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Francis Rouessac and Annick Rouessac

CHeMICAL ANALYSiS

Modern Instrumentation Methods and Techniques

 WILEY

Chemical Analysis

Modern Instrumentation Methods and Techniques

Second Edition

Francis Rouessac and Annick Rouessac

University of Le Mans, France

Translated by

Francis and Annick Rouessac and Steve Brooks



John Wiley & Sons, Ltd

Chemical Analysis

Second Edition

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Modern Instrumentation Methods and Techniques

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West Sussex PO19 8SQ, England
Telephone (+44) 1243 779777

Email (for orders and customer service enquiries): cs-books@wiley.co.uk
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Translated into English by Francis and Annick Rouessac and Steve Brooks

First Published in French © 1992 Masson
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3rd Edition © 1997 Masson
4th Edition © 1998 Dunod
5th Edition © 2000 Dunod
6th Edition © 2004 Dunod

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Wiley-VCH Verlag GmbH, Boschstr. 12, D-69469 Weinheim, Germany

John Wiley & Sons Australia Ltd, 33 Park Road, Milton, Queensland 4064, Australia

John Wiley & Sons (Asia) Pte Ltd, 2 Clementi Loop #02-01, Jin Xing Distripark, Singapore 129809

John Wiley & Sons Canada Ltd, 6045 Freemont Blvd, Mississauga, Ontario, L5R 4J6 Canada

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Anniversary Logo Design: Richard J. Pacifico

Library of Congress Cataloging in Publication Data

Rouessac, Francis.

[Analyse chimique. English]

Chemical analysis: modern instrumentation and methods and techniques / Francis Rouessac and Annick Rouessac; translated by Steve Brooks and Francis and Annick Rouessac. — 2nd ed.

p. cm.

Includes bibliographical references and index.

ISBN 978-0-470-85902-5 (cloth: alk. paper) — ISBN 978-0-470-85903-2 (pbk.: alk. paper)

1. Instrumental analysis. I. Rouessac, Annick. II. Title.

QD79.I5R6813 2007

543—dc22

2006036196

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN 978-0-470-85902-5 (HB)

ISBN 978-0-470-85903-2 (PB)

Typeset in 10¹/₂/12¹/₂pt Times by Integra Software Services Pvt. Ltd, Pondicherry, India

Printed and bound in Great Britain by Antony Rowe Ltd, Chippenham, Wiltshire

This book is printed on acid-free paper responsibly manufactured from sustainable forestry in which at least two trees are planted for each one used for paper production.

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Foreword to the first English edition

In Dutch, *scheikunde* (German: *Scheidekunst* = the art of separation) is synonymous with 'Chemistry'. No chemistry whatsoever without separation, without analysis! And no analysis without 'Instrumentation Methods and Techniques', i.e. without the sub-title of the present book!

Instrumentation was, for a long time, rather crude by today's standards: the furnace, the alembic, the separatory funnel, the filter, the balance . . . Crude, and cheap. Today, no modern analytical laboratory is without M\$ investments in optical, mass and NMR spectrometers, in high performance chromatographs, in electro-analytical equipment.

But also, how heavy the responsibility resting on the shoulders of the analytical chemist! He is the one who in the first place is responsible for the forced closing of a dioxin-delinquent waste incineration plant, for the approval of a new non-persistent pesticide, for the demotion of an athlete from his Olympic title for having used illegal drugs, for the identification of a criminal by the traces of gunpowder on his hands, for the quantification of environmental contaminants, for the detection of diabetes or of poisoning, for the establishment and the enforcement of standards used in World trade . . . The analyst, with his power to say 'yes' or 'no', is one of the most influential of our contemporaries!

There are many books on analytical chemistry, but there were very few, and rather old ones, in French, until F. and A. Rouessac published the first French edition of the present book, eight years ago: they had a niche to fill. Four successive editions have confirmed that they had filled it well: their book was simple, highly informative, and it was kept up-to-date. Through its successive improvements, it has become mature for translation. I am sure the present English version, for which I see no equivalent, will now be useful world-wide to students, as well as to professionals. Fare well, Rouessac & Rouessac!

14 February 2000

Guy Ourisson

President of the French Academy of Sciences

Preface to the first English edition

The book entitled *Analyse Chimique. Methodes et techniques instrumentales modernes*, written originally in French by Professor F. Rouessac and his wife A. Rouessac, has been revised several times and is now in its 4th edition. It is an ongoing project that provides updated versions and increases the usefulness of the manuscript.

The purpose of the work has been to provide basic information on methods of chemical analysis and new instrumentation techniques that have been developed and improved in recent years. Its objective is to provide the analyst with a reference manual while providing students with a teaching tool that covers the basics of most instrumental techniques presently used in chemical analysis. It incorporates basic principles, describes commonly used instruments and discusses the main application for most of the analytical techniques.

The book classifies methods of analysis according to three categories: separation techniques, spectroscopy techniques and other methods. It was written for undergraduate students in chemistry but with the view that it may be of interest for students in other disciplines (physics, biology, etc.) where chemical methods of analysis and instrumental techniques are used. Thus, it provides sufficient information to understand the techniques and their application and allows students to find additional information in more advanced works that discuss specialised instrumental techniques in more detail.

Professor Rouessac gathered the material presented in this book during his teaching career at the University of Le Mans and he has made an effort to integrate theory and practice in a remarkable way. The chapters contain detailed descriptions of instruments and techniques with a few applied examples that are useful to appreciate the scope of the techniques as well as their strengths and limitations in the applied world. The philosophy behind the manuscript is to show that although analytical chemistry and chemical analysis are sometimes considered as different topics, they are inherently intertwined.

Over the years, we have seen a tremendous evolution in chemical analysis. Because of developments in electronics and computer sciences, many new approaches have been developed based on physical measurements and these approaches are now widely used. Nowadays, there is a legion of instrumentation techniques that are more sensitive, more selective and can be applied to analytical problems in many areas of science where the structure determination and quantisation of chemical species is needed. For example, physical methods of chemical

analysis are being used overwhelmingly in the biological sciences. Moreover, the combination of two or more instrumentation analysis techniques had led to the introduction of hyphenated methods that are extremely powerful and require the basic knowledge of the underlying principles. This manuscript provides the essential knowledge for the understanding of these techniques and opens the door to their areas of applications. It also treats some older techniques that maintain their important place in industrial processes. It has been a pleasure for us to translate Professor Rouessac's work. Although we have been able to translate the technical material relatively precisely, there is a flavour of expression used by the authors in their native language that cannot be transposed, as is usually the case with translations. In spite of this limitation, we believe that the content of this book will be extremely useful to readers that are seeking knowledge and information on chemical analysis and analytical instrumentation.

Michel J. Bertrand, Professor
Karen C. Waldron, Assistant Professor
Department of Chemistry
Université de Montréal
September 1999

Preface to second edition

This textbook presents an explanatory and exploratory review of the basic concepts behind the methodologies most frequently encountered in the qualitative, quantitative and structural chemical analysis employed in sectors as diverse as the chemical, pharmaceutical, food and agricultural industries as well as those areas of the environment subjected to stringent controls.

The techniques under review have been classed into one of the following three categories: Separative Methods, Spectral Methods and Other Methods, each of which is the object of an investigative study of the fundamental ideas, their extension, development and application to the corresponding principal, instrumental techniques. Each chapter is illustrated by photographs, numerous diagrams and schemes of chemical reactions or technical principles many of which have been inspired by real instruments and documents obtained from the constructors. However, in order that the book be contained within a reasonable volume, those methods rarely used, or currently in regression are not discussed.

Written and presented as comprehensively as possible, the text addresses a broad spectrum of techniques relevant to a wide range of subjects in chemistry, physics and applied biology and will prove appropriate for students of pre-university, undergraduate and postgraduate levels. Specialist technicians in university support, research and industrial training services will equally find this book useful. The current needs of certain professional sectors in chemical analysis, some of which may appear to have been neglected, linked with the increasing choice of techniques and instruments available, equally justify this compendium of information which updates and unifies several previously available texts.

Though aimed at an otherwise broad readership, this book has been principally designed to engage the interest of students of chemical analysis and to harness their appreciation of the subject as a particular tool employed in a great many experimental sciences and a variety of associated domains.

The authors have included reminders of fundamental principles and have taken account of the evolution of knowledge and the developments in approaches to physical and mathematical phenomena. The text contains only a minimum of theory in order not to lose the attention of the broader readership whose interest is preferential for the technical content. Those readers so wishing may undertake the reading of the more specialised *œuvres* without major difficulty, having acquired from this book a suitable introduction to both the current methods and practical aspects.

The content also reflects the profound changes in analytical techniques currently employed in laboratories. The inherent changes resulting from the growth in demand, the now quite necessary volume of data to be treated, from computerisation and the new requirements most notably in trace analysis.

Comprising a discussion of more than twenty methods, which bearing in mind the large number of applications for each capable of being the subject of a lengthy review, along with the addition of individual exercises, (problems and solutions) and the whole delivered in less than 580 pages, the authors have responded to a considerable challenge. They have preferred a format limited to the presentation of the tools themselves rather than to descriptions of all that their use permits them to do. The choice of applications has therefore been simplified to those which express an illustrative value.

The origin of this book has been the coursework, accumulated over a number of years, which has been presented to the students of the Institute of Technology at the University of Le Mans, France. Colleagues and friends have given graciously of their time for both the re-reading of the text and for critical suggestions. This version of *Chemical Analysis* is the 2nd International Edition and has been updated and completed with respect to its predecessor. The fifth edition of this book has equally been translated into Spanish. The title is *Anàlisis Quimico. Methodos y Técnicas Instrumentales Modernas*, McGraw-Hill Interamerica de Espana, S.A.U.

F. & A. Rouessac
Le Mans, December 2005

Acknowledgments

We are very pleased that the 2nd English edition of *Analyse Chimique* has been published. The authors wish to thank professors Karen Waldron and Michel Bertrand of the University of Montreal for the very hard work they did in translating the manuscript that was used for the first English edition (John Wiley, 2000). The present text leans on this translation, a large part of it being kept.

We also acknowledge Dr Steve Brooks of the Institut de Génétique et de Biologie Moléculaire et Cellulaire in Strasbourg, who translated the text of the French 5th edition. Finally, we have reworked all the chapters and included improved drawings into the text.

The authors also wish to express their gratitude to Professor Guy Ourisson, former President of the French Académie des Sciences, who in agreeing to write a forward for previous editions of this book, bestowed upon it a great honour. Sadly Professor Ourisson passed away in November 2006.

Our special thanks also to the staff of John Wiley & Sons, Ltd (Chichester). We are especially indebted to the following for their kind assistance: Andy Slade, Robert Hambrook, Elizabeth Kingston, Alison Woodhouse (freelance copy-editor) and also Sunita Jayachandran of Integra Ltd (India) for producing a book of pleasing appearance. Those we have inadvertently missed have our sincere apologies.

Below is a list of the companies that have graciously agreed to provide us with both information and documents, certain of which have been reproduced within the text. Their assistance has been particularly precious in view of the speed of technological progress in the sector of analytical instrumentation.

Agilent Technologies, Alltech Associates Inc, American gas & Chemical Co., American Stress Technologies, Amptek Inc, Analytical Instruments, Anotec, Antek, Arelco, ARL, Asoma, ATI Unicam, ATMI Sensoric Div., ATS, Aurora, Beckman Coulter Inc, Berger Mettler Toledo, Bio-Rad, Bosch, Bruker, BW Technologies, Camag, Carbone-Lorraine, Chrompack, Ciba, CTTM, Daiiso Company, Desaga, Dionex, DuPont, Edinburgh sensors, EG&G-ORTEC, ETP Scientific, Eurolabo, Finnigan, Fisons-Instruments, Foxboro, Galileo, Genesis Lab Syst. Inc., Gilson, Grasby-Electronics, Hamamatsu, Hamilton, Imaging Sensing Technology, Jeol, Jenway, Jobin-Yvon, Jordan Valley, Labsystems, LaMotte Co., Leeman

Labs., Leybolds, Merck, Mercury Instr. USA, Metorex, Metrohm, Mettler-Toledo, Microsaic System, Microsensor Technology, Nicolet, Niton, Ocean-optics, Oriel, Ortec, Oxford Instruments, Perkin-Elmer, PESciex, Pharmacia-Biotech, Philips, Photovac, Polymer Lab., PS Analytical, PSS, Rheodyne, RTI, Scientec, Scientific Glass Company, SensIR, Servomex, SGE Europe Ltd, Shimadzu, Siemens, Skalar, SMIS, Spectra Tech, Supelco, Tekmar, Teledyne, Thermo Electron Corp., Thermo-Optek, Thermo-Quest, Thermo Jarrell Ash, Tosohaas, Varian Inc, VG Instruments, Vydac, Waters Corp, Wilmad, Wyatt Technology.

Annick and Francis Rouessac

Introduction

Analytical chemistry is a science close to physical chemistry, which is a branch of pure chemistry. The objective of analytical chemistry is essentially to develop and apply new methodology and instrumentation with the goal of providing information on the *nature* and *composition* of matter. Analytical chemistry also allows the determination of a compound's structure, either partially or totally, in samples of differing complexity. Finally, part of the role of analytical chemistry is to provide an interpretation of the results obtained.

From a more applied point of view, analytical chemistry is the basis of *chemical analysis*, which corresponds to the study of the methods and their diverse techniques designed to solve the concrete problems of analysis. The term *chemistry* is a reminder that analytical chemistry involves the analysis of *chemical elements* and the *defined compounds* derived from these.

The vast discipline of analytical chemistry has implications in all experimental sciences. Its study requires knowledge of many different areas. As a multidisciplinary science, also sometimes referred to as transferable, analytical chemistry calls upon many phenomena, certain of which quite distant from chemistry in the usual sense of the term, in order to provide results. Thus, modern chemical analysis is based on physico-chemical measurements obtained through the use of a variety of instruments, which have greatly benefited from the appearance of microcomputers.

Gradually a tremendous arsenal of processes has been developed, allowing the analyst to respond to an increasing number of various demands. Furthermore, the study of modern chemical analysis techniques is far removed from traditional descriptive chemistry. Many analyses are conducted in non-specialized environments, either on site or at simple workbenches. The determination of compounds is currently quite remote from the use of chemical reactions, which are often avoided for many reasons.

Former wet chemistry methods, at the origin of the term *analytical chemistry*, have become less important because they are labour intensive, require large samples due to their lack of sensitivity, are lengthy and their precision can too easily be altered by the use of insufficiently pure reagents. Nonetheless, wet chemistry methods are still interesting to study.

Analytical chemistry has become indispensable in a number of areas beyond those considered traditional such as chemistry or parachemistry, being increasingly present in activities closely associated with mankind such as applications in medical sciences (diagnostics), biochemistry, food sciences, environmental sciences

(pollution), security in a world often dangerous and in numerous industrial sectors. Analytical chemistry is no longer confined to chemistry. For example, development of regulations in countries regarding the free circulation of products and the agency in charge of environmental protection both require a great number of analyses; evidence that analytical chemistry will assuredly have a privileged position in the future.

More than ever, analytical chemistry is a lively discipline with applied research, specialized journals and many international events (Pittsburgh Conference, Salon du Laboratoire, Analytica, etc.) attended by internationally recognized manufacturers.

One way of determining the importance of a technique is to examine the economic statistics related to the sale of corresponding instruments. The diffusion of a technique increases the probability that an analyst will encounter it during his professional career. For example, chromatographic techniques alone represent more than half of the sales of instruments for molecular analysis, as opposed to elemental analysis which is half of this. However, the latter domain is far from static, other emerging sectors of analysis or novel, alternative methods are being developed and will no doubt come to the prominence. Elsewhere, economic indicators are not the only factor taken into account when assigning the importance of a method. For certain analyses, even a rarely used method can be the most important if it represents the only available means of solving a problem.

Chemical analysis is the proof of many innovations: the evolution of technologies has led to the development of high-performance instruments bringing newer and broader possibilities, notably hyphenated methods and non-destructive methods. *Non-destructive tests* can be conducted on very small samples that do not necessitate extensive sample preparation prior to the measurement. Users can finally acquire instruments that meet the quality and precision requirements necessary for attaining certification. This latter requirement an important step in the official recognition of the quality of the laboratory. Certification procedures are enforced by a number of test compounds all over the world.

Being an analyst requires scientific competence, austerity and honesty. To undertake these kinds of study the analyst must be well trained in different techniques. She/He must be expert and aware of the basic concepts of chemistry realising that a compound might be analysed by alternate methods. Selecting the appropriate method and if possible the best requires knowledge of many parameters. A whole range of questions come up, not necessarily in the following order:

- The sample is of what kind (steel, soil, water...)?
- Does it require a partial or complete analysis of the sample?
- Is the analyte a major component (1 to 100 per cent), minor component (0.01 to 1 per cent) or trace level component (less than 0.01 per cent) of the sample?
- Are qualified personnel available to conduct the analysis?

- Must the analysis be repetitive?
- What is the precision needed?
- What is the cost of analysis?
- Must the sample be recovered after measurement?
- What are the consequences of a possible error in measurement?
- How long will the analysis take?
- What will the reliability of the results be for the method chosen?

As demonstrated by the questions above, when a new analytical objective has been defined, the problem must be approached methodically. The science called chemometrics is aimed at helping to find the best method required for solving an analytical problem as a function of imposed constraints and according to three different directions: methodology, data treatment and interpretation of results.

Taking into account the nature of the analyte to be determined, the starting point consists of *choosing a method* of analysis: the spectroscopic method, electrochemical method, separation method, etc.

The second step concerns that of *choosing a technique*. For example, if chromatography has been chosen as the method, will gas-phase or liquid-phase chromatography be better?

The third decision to make concerns the *choice of sample procedure* and the sample preparation method that will be required to obtain a good result.

As far as experimental protocol is concerned, this will correspond to the mode of operation chosen. This is effectively the 'recipe of the measurement', generally a procedure working within existing norms (AFNOR). This normalization is based upon the standardization of the steps at each stage of analysis from sample preparation to experimental measurement.

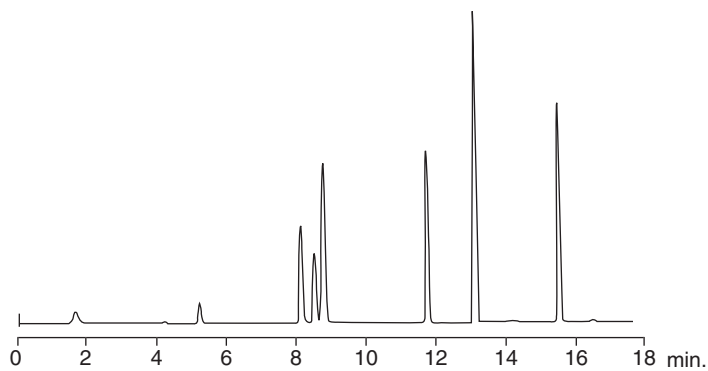
Finally, the results are presented and the raw data from analysis is archived, if possible, as non-rewritable computer files. All of these important aspects of an analysis can be found in official texts under the heading Good Laboratory Practice or GLP.

In order that the most appropriate method is selected for an analysis there exists a science called *chemiometrics* which has the aim of assisting and advising the analyst, as a function of the imperatives required and according to several orientations: appropriate methodology, minimum sampling plan, treatment of the data and interpretation of the results. By use of computerized instruments a correct response can be proposed through exploitation of the results by means of statistical methods in order to reduce the number of lengthy or costly analytical trials.

In conclusion, analytical chemistry includes both chemical analysis and chemiometry. Chemical analysis has the aim of producing results, generally quantitative (concentrations) while chemiometrics applies mathematical methods based on formal logic to extract chemically information from chemical data.

PART 1

Separation methods

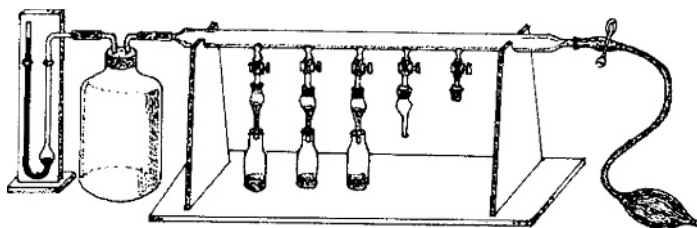


The invention of chromatography

Who invented chromatography, one of the most widely used laboratory techniques? This question leads to controversies. In the 1850s, Schönbein used filter paper to partially separate substances in solution. He found that not all solutions reach the same height when set to rise in filter paper. Goppelsröder (in Switzerland) found relations between the height to which a solution climbs in paper and its chemical composition. In 1861 he wrote 'I am convinced that this method will prove to be very practical for the rapid determination of the nature of a mixture of dyes, especially if appropriately chosen and characterised reagents are used'.

Even if both of them did valuable work towards the progress of paper chromatography, it is traditional to assign the invention of modern chromatography to Michael S. Tswett, shortly after 1900. Through his successive publications, one can indeed reconstitute his thought processes, which makes of him a pioneer, even if not the inventor, of this significant separative method. His field of research was involved with the biochemistry of plants. At that time one could extract chlorophyll and other pigments from house plants, usually from the leaves, easily with ethanol. By evaporating this solvent, there remained a blackish extract which could be redissolved in many other solvents and in particular in petroleum ether (now one would say polar or non-polar solvents). However, it was not well understood why this last solvent was unable to directly extract chlorophyll from the leaves. Tswett put forth the assumption that in plants chlorophyll was retained by some molecular forces binding on the leaf substrate, thus preventing extraction by petroleum ether. He foresaw the principle of adsorption here. After drawing this conclusion, and to test this assumption he had the idea to dissolve the pigment extract in petroleum ether and to add filter paper (cellulose), as a substitute for leaf tissue. He realized that paper collected the colour and that by adding ethanol to the mixture one could re-extract these same pigments.

As a continuation of his work, he decided to carry out systematic tests with all kinds of powders (organic or inorganic), which he could spread out. To save time he had carried out an assembly which enabled him to do several assays simultaneously. He placed the packed powders to be tested in the narrow tubes and he added to each one of them a solution of the pigments in petroleum ether. That enabled him to observe that in certain tubes the powders produced superimposed rings of different colours, which testified that the force of retention varied with the nature of the pigments present. By rinsing the columns with a selection of suitable solvents he could collect some of these components separately. Modern chromatography had been born. A little later, in 1906, then he wrote the publication (appeared in *Berichte des Deutschen Botanische Gesellschaft*, 24, 384), in which he wrote the paragraph generally quoted: 'Like light rays in the spectrum, the different components of a pigment mixture, obeying a law, are resolved on the calcium carbonate column and then can be measured qualitatively and quantitatively. I call such a preparation a chromatogram and the corresponding method the chromatographic method.'



1

General aspects of chromatography

Chromatography, the process by which the components of a mixture can be separated, has become one of the primary analytical methods for the identification and quantification of compounds in the gaseous or liquid state. The basic principle is based on the concentration equilibrium of the components of interest, between two immiscible phases. One is called the stationary phase, because it is immobilized within a column or fixed upon a support, while the second, called the mobile phase, is forced through the first. The phases are chosen such that components of the sample have differing solubilities in each phase. The differential migration of compounds lead to their separation. Of all the instrumental analytical techniques this hydrodynamic procedure is the one with the broadest application. Chromatography occupies a dominant position that all laboratories involved in molecular analysis can confirm.

1.1 General concepts of analytical chromatography

Chromatography is a physico-chemical method of separation of components within mixtures, liquid or gaseous, in the same vein as distillation, crystallization, or the fractionated extraction. The applications of this procedure are therefore numerous since many of heterogeneous mixtures, or those in solid form, can be dissolved by a suitable solvent (which becomes, of course, a supplementary component of the mixture).

A basic chromatographic process may be described as follows (Figure 1.1):

1. A vertical hollow glass tube (the *column*) is filled with a suitable finely powdered solid, the *stationary phase*.
2. At the top of this column is placed a small volume of the sample mixture to be separated into individual components.

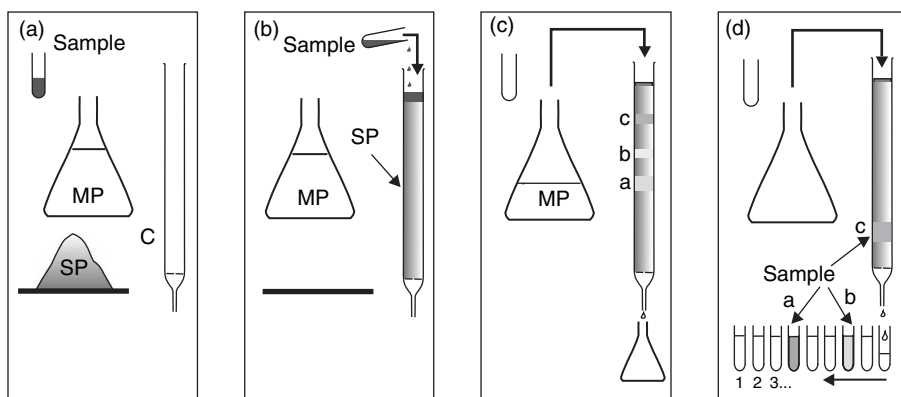


Figure 1.1 A basic experiment in chromatography. (a) The necessary ingredients (C, column; SP, stationary phase; MP, mobile phase; and S, sample); (b) introduction of the sample; (c) start of elution; (d) recovery of the products following separation.

3. The sample is then taken up by continuous addition of the *mobile phase*, which goes through the column by gravity, carrying the various constituents of the mixture along with it. This process is called *elution*. If the components migrate at different velocities, they will become separated from each other and can be recovered, mixed with the mobile phase.

This basic procedure, carried out in a column, has been used since its discovery on a large scale for the separation or purification of numerous compounds (*preparative column chromatography*), but it has also progressed into a stand-alone *analytical technique*, particularly once the idea of measuring the migration times of the different compounds as a mean to identify them had been conceived, without the need for their collection. To do that, an optical device was placed at the column exit, which indicated the variation of the composition of the eluting phase with time. This form of chromatography, whose goal is not simply to recover the components but to control their migration, first appeared around 1940 though its development since has been relatively slow.

The identification of a compound by chromatography is achieved by comparison: To identify a compound which may be A or B, a solution of this unknown is run on a column. Next, its *retention time* is compared with those for the two reference compounds A and B previously recorded using the same apparatus and the same experimental conditions. The choice between A and B for the unknown is done by comparison of the retention times.

In this experiment a true separation had not been effected (A and B were pure products) but only a comparison of their times of migration was performed. In such an experiment there are, however, three unfavourable points to note: the procedure is fairly slow; absolute identification is unattainable; and the physical contact between the sample and the stationary phase could modify its properties, therefore its retention times and finally the conclusion.

This method of separation, using two immiscible phases in contact with each other, was first undertaken at the beginning of the 20th century and is credited to botanist Michaël Tswett to whom is equally attributed the invention of the terms *chromatography* and *chromatogram*.

The technique has improved considerably since its beginnings. Nowadays chromatographic techniques are piloted by computer software, which operate highly efficient miniature columns able to separate nano-quantities of sample. These instruments comprise a complete range of accessories designed to assure reproducibility of successive experiments by the perfect control of the different parameters of separation. Thus it is possible to obtain, during successive analyses of the same sample conducted within a few hours, recordings that are reproducible to within a second (Figure 1.2).

The essential recording that is obtained for each separation is called a *chromatogram*. It corresponds to a two-dimensional diagram traced on a chart paper or a screen that reveals the variations of composition of the eluting mobile phase as it exits the column. To obtain this document, a sensor, of which there exists a great variety, needs to be placed at the outlet of the column. The detector signal appears as the ordinate of the chromatogram while time or alternatively elution volume appears on the abscissa.

- The identification of a molecular compound only by its retention time is somewhat arbitrary. A better method consists of associating two different complementary methods, for example, a chromatograph and a second instrument on-line, such as a

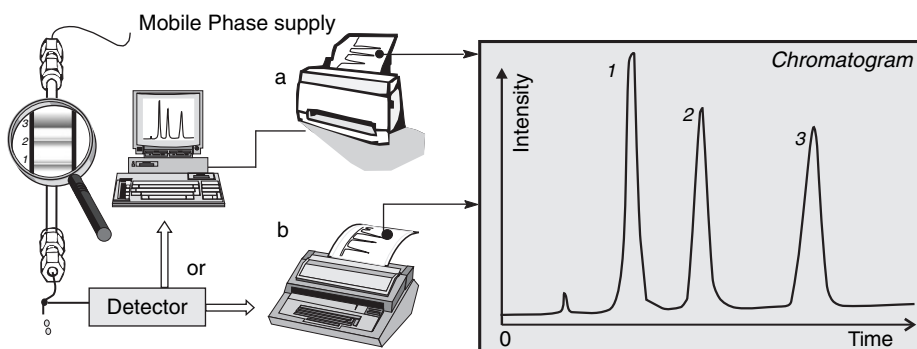


Figure 1.2 *The principle of analysis by chromatography.* The chromatogram, the essential graph of every chromatographic analysis, describes the passage of components. It is obtained from variations, as a function of time, of an electrical signal emitted by the detector. It is often reconstructed from values that are digitized and stored to a microcomputer for reproduction in a suitable format for the printer. (a). For a long time the chromatogram was obtained by a simple chart recorder or an integrator (b). Right, a chromatogram illustrating the separation of a mixture of at least three principal components. Note that the order of appearance of the compounds corresponds to the relative position of each constituent on the column.

mass spectrometer or an infrared spectrometer. These hyphenated techniques enable the independent collating of two different types of information that are independent (time of migration and 'the spectrum'). Therefore, it is possible to determine without ambiguity the composition and concentration of complex mixtures in which the concentration of compounds can be of the order of nanograms.

1.2 The chromatogram

The *chromatogram* is the representation of the variation, with time (rarely volume), of the amount of the analyte in the mobile phase exiting the chromatographic column. It is a curve that has a baseline which corresponds to the trace obtained in the absence of a compound being eluted. The separation is complete when the chromatogram shows as many *chromatographic peaks* as there are components in the mixture to be analysed (Figure 1.3).

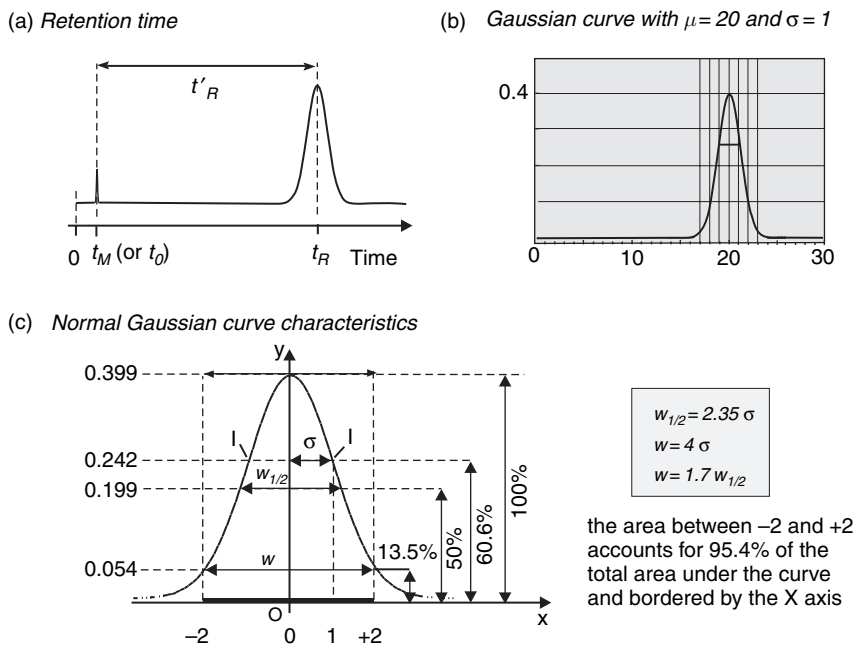


Figure 1.3 *Chromatographic peaks.* (a) The concept of retention time. The hold-up time t_M is the retention time of an unretained compound in the column (the time it took to make the trip through the column); (b) Anatomy of an ideal peak; (c) Significance of the three basic parameters and a summary of the features of a Gaussian curve; (d) An example of a real chromatogram showing that while travelling along the column, each analyte is assumed to present a Gaussian distribution of concentration.

(d) Comparison between a true chromatogram and normal Gaussian-shaped peaks

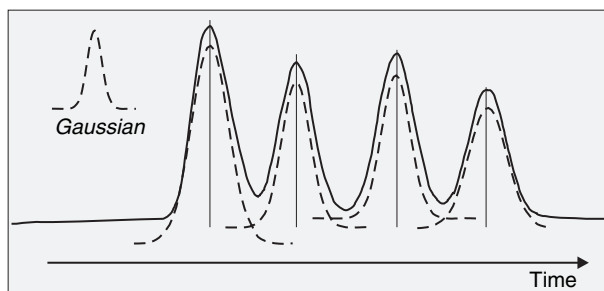


Figure 1.3 (Continued)

A constituent is characterized by its *retention time* t_R , which represents the time elapsed from the sample introduction to the detection of the peak maximum on the chromatogram. In an ideal case, t_R is independent of the quantity injected.

A constituent which is not retained will elute out of the column at time t_M , called the *hold-up time* or *dead time* (formerly designated t_0). It is the time required for the mobile phase to pass through the column.

The difference between the retention time and the hold-up time is designated by the *adjusted retention time* of the compound, t'_R .

If the signal sent by the sensor varies linearly with the concentration of a compound, then the same variation will occur for the area under the corresponding peak on the chromatogram. This is a basic condition to perform quantitative analysis from a chromatogram.

1.3 Gaussian-shaped elution peaks

On a chromatogram the perfect elution peak would have the same form as the graphical representation of the law of Normal distribution of random errors (Gaussian curve 1.1, cf. Section 22.3). In keeping with the classic notation, μ would correspond to the retention time of the eluting peak while σ to the standard deviation of the peak (σ^2 represents the *variance*). y represents the signal as a function of time x , from the detector located at the outlet of the column (Figure 1.3).

This is why ideal elution peaks are usually described by the probability density function (1.2).

$$y = \frac{1}{\sigma\sqrt{2\pi}} \cdot \exp\left[-\frac{(x - \mu)^2}{2\sigma^2}\right] \quad (1.1)$$

$$y = \frac{1}{\sqrt{2\pi}} \cdot \exp\left[-\frac{x^2}{2}\right] \quad (1.2)$$

This function is characterized by a symmetrical curve (maximum for $x = 0$, $y = 0.3999$) possessing two inflection points at $x = \pm 1$ (Figure 1.3), for which the ordinate value is 0.242 (being 60.6 per cent of the maximum value). The width of the curve at the inflection points is equal to 2σ , ($\sigma = 1$).

In chromatography, $w_{1/2}$ represents the width of the peak at half-height ($w_{1/2} = 2.35\sigma$) and σ^2 the variance of the peak. The width of the peak 'at the base' is labelled w and is measured at 13.5 per cent of the height. At this position, for the Gaussian curve, $w = 4\sigma$ by definition.

Real chromatographic peaks often deviate significantly from the Gaussian ideal aspect. There are several reasons for this. In particular, there are irregularities of concentration in the injection zone, at the head of the column. Moreover, the speed of the mobile phase is zero at the wall of the column and maximum in the centre of the column.

The observed asymmetry of a peak is measured by two parameters, the *skewing factor* a measured at 10 per cent of its height and the *tailing factor* TF measured at 5 per cent (for the definition of these terms, see Figure 1.4):

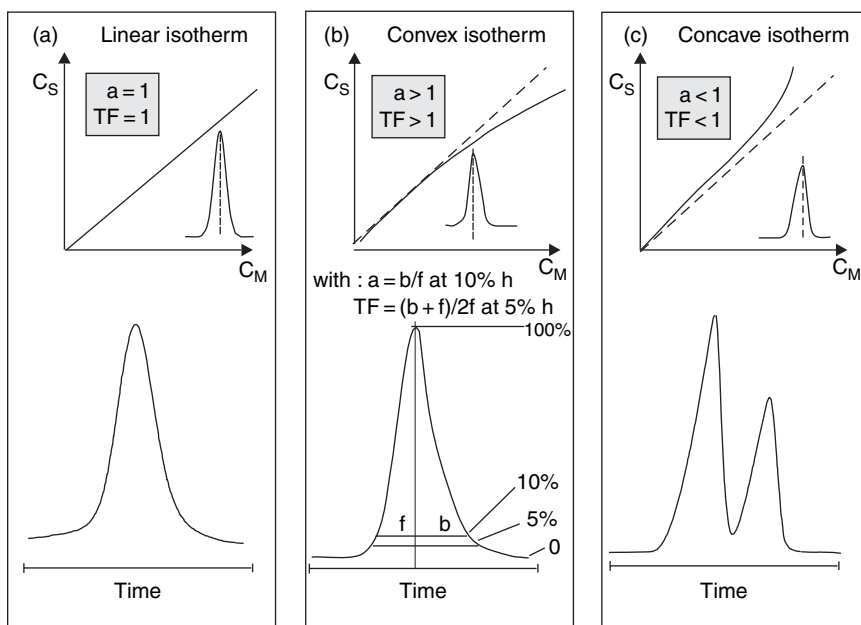


Figure 1.4 *Distribution isotherms.* (a) The ideal situation corresponding to the invariance of the concentration isotherm. (b) Situation in which the stationary phase is saturated – as a result of which the ascent of the peak is faster than the descent (skewing factor greater than 1); (c) The inverse situation : the constituent is retained too long by the stationary phase, the retention time is therefore extended and the ascent of the peak is slower than the descent apparently normal. For each type of column, the manufacturers indicate the capacity limit expressed in ng/compound, prior to a potential deformation of the corresponding peak. The situations (a), (b) and (c) are illustrated by authentic chromatograms taken out from liquid chromatography technique.

$$a = \frac{b}{f} \quad (1.3)$$

$$TF = \frac{b+f}{2f} \quad (1.4)$$

1.4 The plate theory

For half a century different theories have been and continue to be proposed to model chromatography and to explain the migration and separation of analytes in the column. The best known are those employing a statistical approach (stochastic theory), the theoretical plate model or a molecular dynamics approach.

To explain the mechanism of migration and separation of compounds on the column, the oldest model, known as Craig's *theoretical plate model* is a static approach now judged to be obsolete, but which once offered a simple description of the separation of constituents.

Although chromatography is a dynamic phenomenon, Craig's model considered that each solute moves progressively along a sequence of distinct static steps. In liquid–solid chromatography this elementary process is represented by a cycle of adsorption/desorption. The continuity of these steps reproduces the migration of the compounds on the column, in a similar fashion to that achieved by a cartoon which gives the illusion of movement through a sequence of fixed images. Each step corresponds to a new state of equilibrium for the *entire* column.

These successive equilibria provide the basis of *plate theory* according to which a column of length L is sliced horizontally into N fictitious, small plate-like discs of same height H and numbered from 1 to n . For each of them, the concentration of the solute in the mobile phase is in equilibrium with the concentration of this solute in the stationary phase. At each new equilibrium, the solute has progressed through the column by a distance of one disc (or plate), hence the name *theoretical plate theory*.

The *height equivalent to a theoretical plate* (HETP or H) will be given by equation (1.5):

$$H = \frac{L}{N} \quad (1.5)$$

This employs the polynomial approach to calculate, for a given plate, the mass distributed between the two phases present. At instant I , plate J contains a total mass of analyte m_T which is composed of the quantity m_M of the analyte that has just arrived from plate $J - 1$ carried by the mobile phase formerly in equilibrium at instant $I - 1$, to which is added the quantity m_S already present in the stationary phase of plate J at time $I - 1$ (Figure 1.5).

$$m_T(I, J) = m_M(I - 1, J - 1) + m_S(I - 1, J)$$

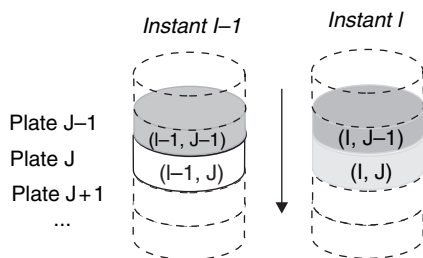


Figure 1.5 Schematic of a column cross-section.

If it is assumed for each theoretical plate that: $m_S = Km_M$ and $m_T = m_M + m_S$, then by a recursive formula, m_T (as well as m_M and m_S), can be calculated. Given that for each plate the analyte is in a concentration equilibrium between the two phases, the total mass of analyte in solution in the volume of the mobile phase V_M of the column remains constant, so long as the analyte has not reached the column outlet. So, the chromatogram corresponds to the mass in transit carried by the mobile phase at the $(N + 1)$ th plate (Figure 1.6) during successive equilibria. This theory has a major fault in that it does not take into account the dispersion in the column due to the diffusion of the compounds.

■ The plate theory comes from an early approach by Martin and Syngé (Nobel laureates in Chemistry, 1952), to describe chromatography by analogy with distillation

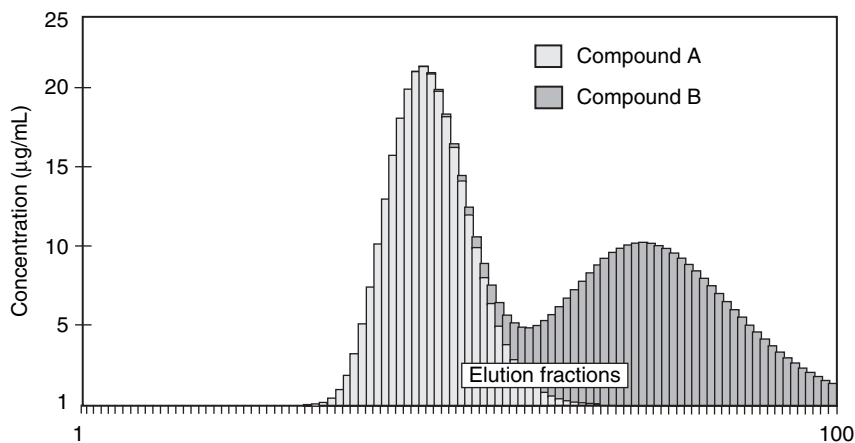


Figure 1.6 Theoretical plate model. Computer simulation, aided by a spreadsheet, of the elution of two compounds A and B, chromatographed on a column of 30 theoretical plates ($K_A = 0.6$; $K_B = 1.6$. $M_A = 300 \mu\text{g}$; $M_B = 300 \mu\text{g}$). The diagram represents the composition of the mixture at the outlet of the column after the first 100 equilibria. The graph shows that application of the model gives rise to a non-symmetrical peak (Poisson summation). However, taking account of compound diffusion and with a larger number of equilibria, the peaks look more and more like a Gaussian distribution.

and counter current extraction as models. This term, used for historical reasons, has no physical significance, in contrast to its homonym which serves to measure the performances of a distillation column.

The *retention time* t_R , of the solute on the column can be sub-divided into two terms: t_M (hold-up time), which cumulates the times during which it is dissolved in the mobile phase and travels at the same speed as this phase, and t_S the cumulative times spent in the stationary phase, during which it is immobile. Between two successive transfers from one phase to the other, it is accepted that the concentrations have the time to re-equilibrate.

■ In a chromatographic phase system, there are at least three sets of equilibria: solute/mobile phase, solute/stationary phase and mobile phase/stationary phase. In a more recent theory of chromatography, no consideration is given to the idea of molecules immobilized by the stationary phase but rather that were simply slowed down when passing in close proximity.

1.5 Nernst partition coefficient (K)

The fundamental physico-chemical parameter of chromatography is the equilibrium constant K , termed the *partition coefficient*, quantifying the ratio of the concentrations of each compound within the two phases.

$$K = \frac{C_S}{C_M} = \frac{\text{Molar concentration of the solute in the stationary phase}}{\text{Molar concentration of the solute in the mobile phase}} \quad (1.6)$$

Values of K are very variable since they can be large (e.g. 1000), when the mobile phase is a gas or small (e.g. 2) when the two phases are in the condensed state. Each compound occupies only a limited space on the column, with a variable concentration in each place, therefore the true values of C_M and C_S vary in the column, but their ratio is constant.

Chromatography and thermodynamics. Thermodynamic relationships can be applied to the distribution equilibria defined above. K , (C_S/C_M), the equilibrium constant relative to the concentrations C of the compound in the mobile phase (M) and stationary phase (S) can be calculated from chromatography experiments. Thus, knowing the temperature of the experiment, the variation of the standard free energy ΔG° for this transformation can be deduced:

$$C_M \rightleftharpoons C_S \quad \Delta G^\circ = -RT \ln K$$

In gas chromatography, where K can be easily determined at two different temperatures, it is possible to obtain the variations in standard enthalpy ΔH° and entropy ΔS° (if it is accepted that the entropy and the enthalpy have not changed):

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

The values of these three parameters are all negative, indicating a spontaneous transformation. It is to be expected that the entropy is decreased when the compound moves from the mobile phase to the stationary phase where it is fixed. In the same way the Van't Hoff equation can be used in a fairly rigorous way to predict the effect of temperature on the retention time of a compound. From this it is clear that for detailed studies in chromatography, classic thermodynamics are applicable.

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2}$$

1.6 Column efficiency

1.6.1 Theoretical efficiency (number of theoretical plates)

As the analyte migrates through column, it occupies a continually expanding zone (Figure 1.6). This linear dispersion σ_1 measured by the variance σ_1^2 increases with the distance of migration. When this distance becomes L , the total column length, the variance will be:

$$\sigma_L^2 = H \cdot L \quad (1.7)$$

Reminding the plate theory model this approach also leads to the value of the height equivalent to one theoretical plate H and to the number N , of theoretical plates ($N = L/H$).

Therefore (Figure 1.7), any chromatogram that shows an elution peak with the temporal variance σ^2 permits the determination of the *theoretical efficiency* N for

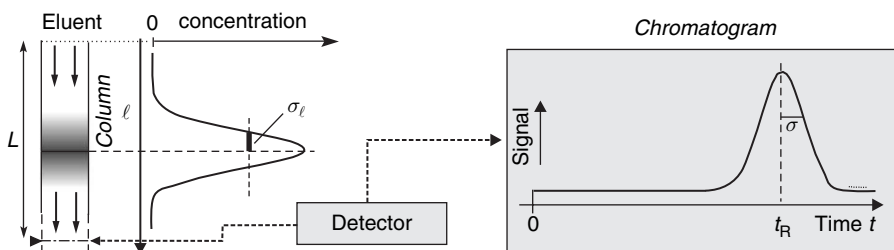


Figure 1.7 Dispersion of a solute in a column and its translation on a chromatogram. Left, graph corresponding to the isochronic image of the concentration of an eluted compound at a particular instant. Right, chromatogram revealing the variation of the concentration at the outlet of the column, as a function of time. t_R and σ are in the same ratio as L and σ_L . In the early days the efficiency N was calculated from the chromatogram by using a graduated ruler.

the compound under investigation (1.8), and by deduction, of the value of H knowing that $H = L/N$;

$$N = \frac{L^2}{\sigma_L^2} \quad \text{Or} \quad N = \frac{t_R^2}{\sigma^2} \quad (1.8)$$

If these two parameters are accessible from the elution peak of the compound, just because t_R and σ are in the same ratio as that of L to σ_L .

On the chromatogram, σ represents the half-width of the peak at 60.6 per cent of its height and t_R the retention time of the compound. t_R and σ should be measured in the same units (time, distances or eluted volumes if the flow is constant). If σ is expressed in units of volume (using the flow), then 4σ corresponds to the 'volume of the peak', that contains around 95 per cent of the injected compound. By consequence of the properties of the Gaussian curve ($w = 4\sigma$ and $w_{1/2} = 2.35\sigma$), Equation 1.9 results. However, because of the distortion of most peaks at their base, expression 1.9 is rarely used and finally Equation 1.10 is preferred.

N is a relative parameter, since it depends upon both the solute chosen and the operational conditions adopted. Generally a constituent is selected which appears towards the end of the chromatogram in order to get a reference value, for lack of advance knowledge of whether the column will successfully achieve a given separation.

$$N = 16 \frac{t_R^2}{w^2} \quad (1.9)$$

$$N = 5.54 \frac{t_R^2}{w_{1/2}^2} \quad (1.10)$$

1.6.2 Effective plates number (real efficiency)

In order to compare the performances of columns of different design for a given compound – or to compare, in gas chromatography, the performances between a capillary column and a packed column – more realistic values are obtained by replacing the *total retention time* t_R , which appears in expressions 1.8–1.10, by the *adjusted retention time* t'_R which does not take into account the *hold-up time* t_M spent by any compound in the mobile phase ($t'_R = t_R - t_M$) The three preceding expressions become:

$$N_{\text{eff}} = \frac{t_R'^2}{\sigma^2} \quad (1.11)$$

$$N_{\text{eff}} = 16 \frac{t_R'^2}{w^2} \quad (1.12)$$

$$N_{\text{eff}} = 5.54 \frac{t_R^2}{w_{1/2}^2} \quad (1.13)$$

Currently it is considered that these three expressions are not very useful.

1.6.3 Height equivalent to a theoretical plate (HETP)

The *equivalent height of a theoretical plate* H , as already defined (expression 1.5), is calculated for reference compounds to permit a comparison of columns of different lengths. H does not behave as a constant, its value depends upon the compound chosen and upon the experimental conditions.

For a long time in gas chromatography an adjustment value called the effective height of a theoretical plate H_{eff} was calculated using the true efficiency.

This corresponds to the Equation 1.14;

$$H_{\text{eff}} = \frac{L}{N_{\text{eff}}} \quad (1.14)$$

In chromatography, in which the mobile phase is a liquid and the column is filled with spherical particles, the adjusted height of the plate h , is often encountered. This parameter takes into account the average diameter d_m of the particles. This eliminates the effect of the particle size. Columns presenting the same ratio (length of the column)/(diameter of the particles) will yield similar performances.

$$h = \frac{H}{d_m} = \frac{L}{Nd_m} \quad (1.15)$$

1.7 Retention parameters

Hold-up times or volumes are used in chromatography for various purposes, particularly to access to retention factor k . and thermodynamic parameters. Only basic expressions are given below.

1.7.1 Retention times

The definition of retention times, *hold-up time*, t_M , *retention time*, t_R and *adjusted retention time*, t'_R , have been given previously (paragraph 1.2).

1.7.2 Retention volume (or elution volume) V_R

The *retention volume* V_R of an analyte represents the volume of mobile phase necessary to enable its migration throughout the column from the moment of entrance to the moment in which it leaves. To estimate this volume, different methods (direct or indirect) may be used, that depend of the physical state of the mobile phase. On a standard chromatogram with time in abscissa, V_R is calculated from expression 1.16, if the flow rate F is constant,

$$V_R = t_R \cdot F \quad (1.16)$$

The volume of a peak, V_{peak} corresponds to that volume of the mobile phase in which the compound is diluted when leaving the column. It is defined by:

$$V_{\text{peak}} = w \cdot F \quad (1.17)$$

1.7.3 Hold-up volume (or dead volume) V_M

The volume of the mobile phase in the column (known as the dead volume), V_M , corresponds to the accessible interstitial volume. It is often calculated from a chromatogram, provided a solute not retained by the stationary phase is present. The dead volume is deduced from t_M and the flow rate F :

$$V_M = t_M \cdot F \quad (1.18)$$

Sometimes, in the simplest cases, the volume of the stationary phase designated by V_S can be calculated by subtracting the dead volume V_M from the total internal volume of the empty column.

1.7.4 Retention (or capacity) factor k

When a compound of total mass m_T is introduced onto the column, it separates into two quantities: m_M , the mass in the mobile phase and m_S , the mass in the stationary phase. During the solute's migration down the column, these two quantities remain constant. Their ratio, called the *retention factor* k , is constant and independent of m_T :

$$k = \frac{m_S}{m_M} = \frac{C_S}{C_M} \cdot \frac{V_S}{V_M} = K \frac{V_S}{V_M} \quad (1.19)$$

The retention factor, also known as the *capacity factor* k , is a very important parameter in chromatography for defining column performances. Though it does

not vary with the flow rate or the column length, k is not a constant as it depends upon the experimental conditions. For this reason it is sometimes designated by k' rather than k alone.

This parameter takes into account the ability, great or small, of the column to retain each compound. Ideally, k should be superior to one but less than five, otherwise the time of analysis is unduly elongated.

An experimental approach of k can be as follows:

Suppose the migration of a compound in the column. Recalling Craig's model, each molecule is considered as passing alternately from the mobile phase (in which it progresses down the column), to the stationary phase (in which it is immobilized). The average speed of the progression down the column is slowed if the time periods spent in the stationary phase are long. Extrapolate now to a case which supposes n molecules of this same compound (a sample of mass m_T). If we accept that at each instant, the ratio of the n_S molecules fixed upon the stationary phase (mass m_S) and of the n_M molecules present in the mobile phase (mass m_M), is the same as that of the times (t_S and t_M) spent in each phase for a single molecule, the three ratios will therefore have the same value:

$$\frac{n_S}{n_M} = \frac{m_S}{m_M} = \frac{t_S}{t_M} = k$$

■ Take the case of a molecule which spends 75 per cent of its time in the stationary phase. Its average speed will be four times slower than if it rested permanently in the mobile phase. As a consequence, if 4 μg of such a compound has been introduced onto the column, there will be an average of 1 μg permanently in the mobile phase and 3 μg in the stationary phase.

Knowing that the retention time of a compound t_R is such that $t_R = t_M + t_S$, the value of k is therefore accessible from the chromatogram ($t_S = t'_R$); see Figure 1.7:

$$k = \frac{t'_R}{t_M} = \frac{t_R - t_M}{t_M} \quad (1.20)$$

This important relation can also be written:

$$t_R = t_M(1 + k) \quad (1.21)$$

Bearing in mind the relations (1.16) and (1.18), the retention volume V_R of a solute can be written :

$$V_R = V_M(1 + k) \quad (1.22)$$

or

$$V_R = V_M + KV_S \quad (1.23)$$

This final expression linking the experimental parameters to the thermodynamic coefficient of distribution K , is valid for the ideal chromatography.

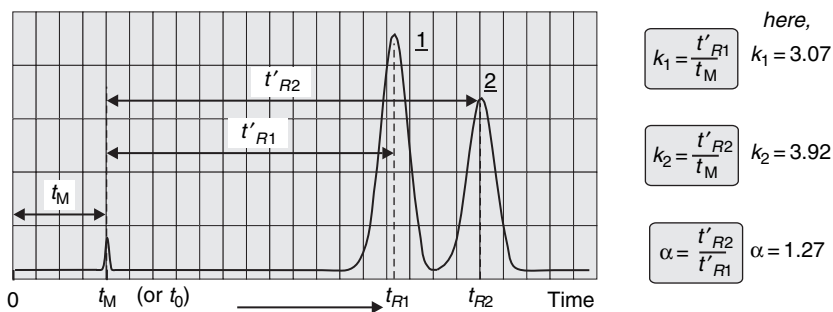


Figure 1.8 Retention factors and separation factor between two compounds. Each compound has its own retention factor. On this figure, the separation factor is around 1.3. The separation factor is also equal to the ratio of the two retention factors. α alone is not enough to determine whether the separation is really possible.

1.8 Separation (or selectivity) factor between two solutes

The separation factor α , (1.24) enables the comparison of two adjacent peaks 1 and 2 present in the same chromatogram (Figure 1.8). Using Equations 1.20 and 1.19, it can be concluded that the separation factor can be expressed by Equation 1.25.

By definition α is greater than unity (species 1 elutes faster than species 2):

$$\alpha = \frac{t'_{R(2)}}{t'_{R(1)}} \quad (1.24)$$

or

$$\alpha = \frac{k_2}{k_1} = \frac{K_2}{K_1} \quad (1.25)$$

For non-adjacent peaks the *relative retention factor* r , is applied, which is calculated in a similar manner to α .

1.9 Resolution factor between two peaks

To quantify the separation between two compounds, another measure is provided by the *resolution factor* R . Contrary to the selectivity factor which does not take into account peak widths, the following expression is used to calculate R between two compounds 1 and 2 (Figure 1.9):

$$R = 2 \frac{t_{R(2)} - t_{R(1)}}{w_1 + w_2} \quad (1.26)$$

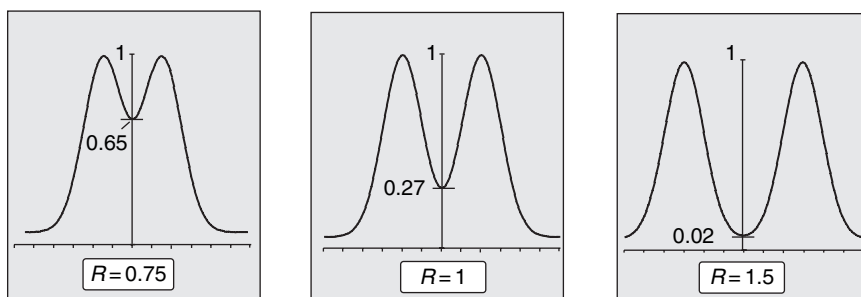


Figure 1.9 Resolution factor. A simulation of chromatographic peaks using two identical Gaussian curves, slowly separating. The visual aspects corresponding to the values of R are indicated on the diagrams. From a value of $R = 1.5$ the peaks can be considered to be baseline resolved, the valley between them being around 2 per cent.

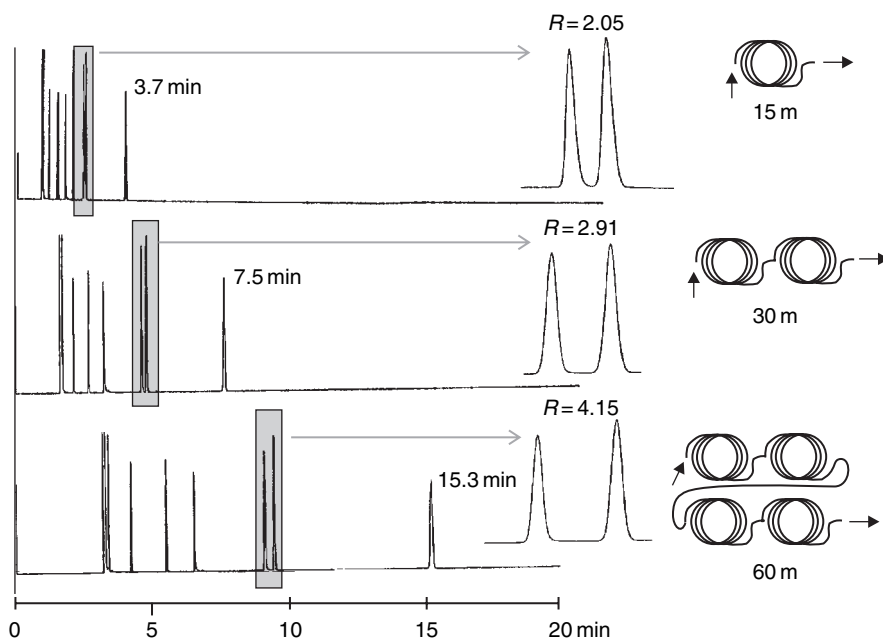


Figure 1.10 Effect of column length on the resolution. Chromatograms obtained with a GC instrument illustrating that by doubling the length of the capillary column, the resolution is multiplied by 1.41 or $\sqrt{2}$ (adapted from a document of SGE Int. Ltd).

Other expressions derived from the preceding ones and established with a view to replacing one parameter by another or to accommodate simplifications may also be employed to express the resolution. Therefore expression 1.27 is used in this way.

It is also useful to relate the resolution to the efficiency, the retention factor and the separation factors of the two solutes (expression 1.28, obtained from 1.26 when $w_1 = w_2$). The chromatograms on Figure 1.10 present an experimental verification.

$$R = 1.177 \frac{t_{R(2)} - t_{R(1)}}{\delta_1 + \delta_2} \quad (1.27)$$

$$R = \frac{1}{4} \sqrt{N_2} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1 + k_2} \quad (1.28)$$

$$R = \frac{\sqrt{N}}{2} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2 - k_1}{k_1 + k_2 + 2} \quad (1.29)$$

1.10 The rate theory of chromatography

In all of the previous discussion and particularly in the plate theory, the velocity of the mobile phase in the column and solute diffusion are, perhaps surprisingly, never taken into account. Of all things, the speed should have an influence upon the progression of the analytes down the column, hence their dispersion and by consequence, upon the quality of the analysis undertaken.

Rate theory is a more realistic description of the processes at work inside a column which takes account of the time taken for the solute to equilibrate between the two phases. It is the dynamics of the separation process which is concerned. The first kinetic equation for *packed columns in gas phase chromatography* was proposed by Van Deemter.

1.10.1 Van Deemter's equation

This equation is based on a Gaussian distribution, similar to that of plate theory. Its simplified form, proposed by Van Deemter in 1956, is well known (expression 1.30). The expression links the plate high H to the average linear velocity of the mobile phase \bar{u} in the column (Figure 1.11).

$$H = A + \frac{B}{\bar{u}} + C\bar{u} \quad (1.30)$$

The three experimental basic coefficients A , B and C are related to diverse physico-chemical parameters of the column and to the experimental conditions. If H is expressed in cm, A will also be in cm, B in cm²/s and C in s (where velocity is measured in cm/s).

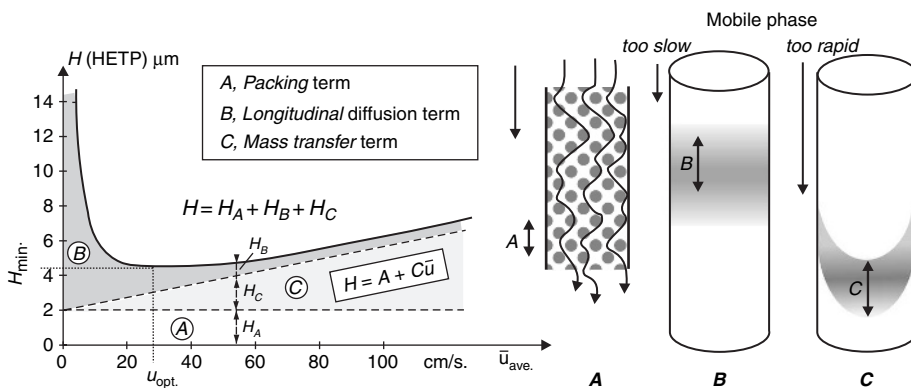


Figure 1.11 Van Deemter's curve in gas chromatography with the domains of parameters A , B and C indicated. There exists an equation similar to that of Van Deemter that considers temperature: $H = A + B/T + CT$.

This equation reveals that there exists an *optimal flow rate* for each column, corresponding to the minimum of H , which predicts the curve described by Equation 1.30.

The loss in efficiency as the flow rate increases is obvious, and represents what occurs when an attempt is made to rush the chromatographic separation by increasing the pressure upon the mobile phase.

However, intuition can hardly predict the loss in efficiency that occurs when the flow rate is too slow. To explain this phenomenon, the origins of the terms A , B and C must be recalled. Each of these parameters represents a domain of influence which can be perceived on the graph (Figure 1.11).

The curve that represents the Van Deemter equation is a hyperbola which goes through a minimum (H_{\min}) when:

$$\bar{u}_{\text{opt}} = \sqrt{\frac{B}{C}} \quad (1.31)$$

Packing related term $A = 2\lambda \cdot d_p$

Term A is related to the flow profile of the mobile phase passing through the stationary phase. The size of the particles (diameter d_p), their dimensional distribution and the uniformity of the packing (factor characteristic of packing λ) can all be the origin of flow paths of different length which cause broadening of the solute band and improper exchanges between the two phases. This results in turbulent or *Eddy diffusion*, considered to have little importance in liquid chromatography and absent for WCOT capillary columns in GC (Golay's equation without term A , cf. paragraph 1.10.2). For a given column, nothing can be done to reduce the A term.

Gas (mobile phase) term $B = 2\gamma D_G$

Term B , which can be expressed from D_G , the diffusion coefficient of the analyte in the gas phase and λ , the above packing factor, is related to the *longitudinal molecular diffusion* in the column. It is especially important when the mobile phase is a gas.

This term is a consequence of the entropy which reminds us that a system will tend spontaneously towards the maximum degrees of freedom, chaos, just as a drop of ink diffuses into a glass of water into which it has fallen. Consequently, if the flow rate is too slow, the compounds undergoing separation will mix faster than they will migrate. This is why one never must interrupt, even temporarily, a chromatography once underway, as this puts at risk the level of efficiency of the experiment.

Liquid (stationary phase) term $C = C_G + C_L$

Term C , which is related to the *resistance to mass transfer* of the solute between the two phases, becomes dominant when the flow rate is too high for an equilibrium to be attained. Local turbulence within the mobile phase and concentration gradients slow the equilibrium process ($C_S \rightleftharpoons C_M$). The diffusion of solute between the two phases is not instantaneous, so that it will be carried along out of equilibrium. The higher the velocity of mobile phase, the worse the broadening becomes. No simple formula exists which takes into account the different factors integrated in term C . The parameter C_G is dependent upon the diffusion coefficient of the solute in a gaseous mobile phase, while the term C_L depends upon the diffusion coefficient in a liquid stationary phase. Viscous stationary phases have larger C terms.

■ In practice, the values for the coefficients of A , B and C in Figure 1.11 can be accessed by making several measurements of efficiency for the same compound undergoing chromatography at different flow rates, since flow and average linear speed are related. Next the hyperbolic function that best satisfies the experimental values can be calculated using, by preference, the method of multiple linear regression.

1.10.2 Golay's equation

A few years after Van Deemter, Golay proposed a modified relationship reserved to capillary columns used in gas phase chromatography. There is no A term because there is no packing in a capillary column (see paragraph 2.5.2).

$$H = \frac{B}{\bar{u}} + C_L \bar{u} + C_G \bar{u} \quad (1.32)$$

Expression 1.33 leads to the minimum value for the HETP for a column of radius r , if the retention factor of the particular compound under examination is known.

The *coating efficiency* can then be calculated being equal to 100 times the ratio between the value found using expression 1.33 and that deduced from the efficiency ($H = L/N$) obtained from the chromatogram.

$$H_{\text{theo.min}} = r \sqrt{\frac{1 + 6k + 11k^2}{3(1+k)^2}} \quad (1.33)$$

1.10.3 Knox's equation

Another, more recent equation, the Knox equation, is applicable to various types of liquid chromatography and includes the adjusted height h :

$$h = A\bar{u}^{1/3} + \frac{B}{\bar{u}} + C\bar{u} \quad (1.34)$$

1.11 Optimization of a chromatographic analysis

Analytical chromatography is used essentially in quantitative analysis. In order to achieve this effectively, the areas under the peaks must be determined with precision, which in turn necessitates well-separated analytes to be analysed. A certain experience in chromatography is required when the analysis has to be optimized, employing all available resources in terms of apparatus and software that can simulate the results of temperature modifications, phases and other physical parameters.

■ In gas phase chromatography, the separations can be so complex that it can be difficult to determine in advance whether the temperature should be increased or decreased. The choice of column, its length, its diameter, the stationary phase composition and the phase ratio (V_M/V_S) as well as the parameters of separation (temperature and flow rate), are amongst the factors which interact with each other.

The *resolution* and the *elution time* are the two most important dependent variables to consider. In all optimizations, the goal is to achieve a sufficiently complete separation of the compounds of interest in the minimum time, though it should not be forgotten that time will be required to readjust the column to the initial conditions to be ready for the next analysis. Chromatography corresponds, in fact, to a slow type of analysis. If the resolution is very good then optimization consists to save time in the analysis. This can be done by the choice of a shorter column – recalling that the resolution varies with the square root of the column length (cf. the parameter N of formula 1.28 and Figure 1.10).

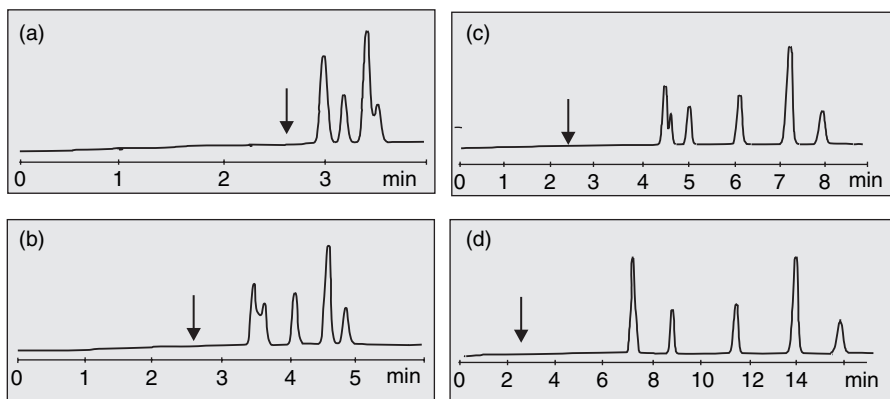


Figure 1.12 *Chromatograms of a separation.* The mobile phase in each trace is a binary mixture water/acetonitrile: (a) 50/50; (b) 55/45; (c) 60/40; (d) 65/35. The arrow indicates the dead time t_M (min) (*J.W. Dolan, LC-GC Int., 1994 7(6), 333*).

Figure 1.12 shows the optimization of a separation, by liquid chromatography, of a mixture of aromatic hydrocarbons. In this case, optimization of the separation has been carried out by successive modifications of the composition of the mobile phase. Note that by optimizing the sequence in this manner, the cycle time of analysis increases.

If only certain compounds present in a mixture are of interest, then a selective detector can be used which would detect only the desired components. Alternately, at the other extreme, attempts might be made to separate the largest number of compounds possible within the mixture.

Depending upon the different forms of chromatography, optimization can be more or less rapid. In gas phase chromatography optimization is easier to achieve than in liquid chromatography in which the composition of mobile phase must

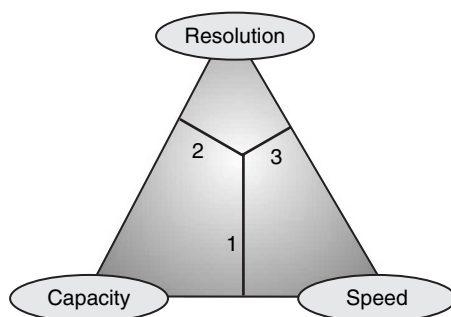


Figure 1.13 *The chromatographer's triangle.* The shaded areas indicate the domain corresponding to analytical chromatography based principally upon the five parameters K , N , k , α and R .

be considered: software now exists that can help in the choice of mobile phase composition. Based upon certain hypotheses (Gaussian peaks), the areas of poorly defined peaks can be found.

The chromatographer must work within the limits bound by a triangle whose vertices correspond to three parameters which are in opposition: the *resolution*, the *speed* and the *capacity* (Figure 1.13). An optimized analytical separation uses the full potential of the selectivity which is the most efficient parameter. In the chromatographer's triangle shown, the optimized conditions are close to the vertex of resolution.

1.12 Classification of chromatographic techniques

Chromatographic techniques can be classified according to various criteria: as a function of the *physical nature* of the phases; of the *process* used; or by the *physico-chemical phenomena* giving rise to the Nernst distribution coefficient K . The following classification has been established by consideration of the physical nature of the two phases involved (Figure 1.14).

1.12.1 Liquid phase chromatography (LC)

This type of chromatography, in which the mobile phase is a liquid belongs to the oldest known form of the preparative methods of separation. This very broad category can be sub-divided depending on the retention phenomenon.

Liquid/solid chromatography (or adsorption chromatography)

The stationary phase is a solid medium to which the species adhere through the dual effect of physisorption and chemisorption. The physico-chemical parameter involved here is the *adsorption coefficient*. Stationary phases have made much progress since the time of Tswett, who used calcium carbonate or inulin (a very finely powdered polymer of ordinary sugar).

Ion chromatography (IC)

In this technique the mobile phase is a buffered solution while the solid stationary phase has a surface composed of ionic sites. These phases allow the exchange of their mobile counter ion with ions of the same charge present in the sample. This type of separation relies on *ionic distribution coefficients*.

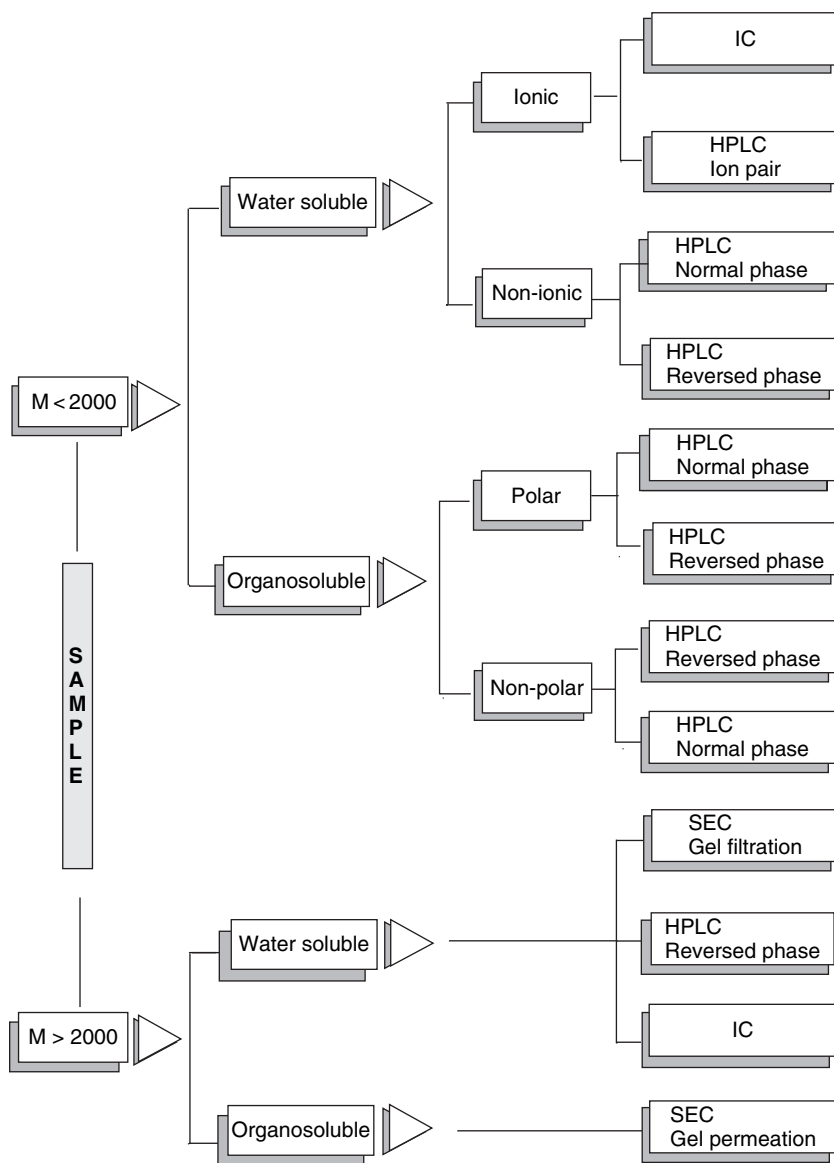


Figure 1.14 Selection guide for all of the different chromatographic techniques with liquid mobile phases. The choice of technique is chosen as a function of the molar mass, solubility and the polarity of the compounds to be separated.

Size exclusion chromatography (SEC)

The stationary phase here is a material containing pores whose dimensions are selected as a function of the size of the species to be separated. This method

therefore uses a form of selective permeability at the molecular level leading to its name, *gel filtration* or *gel permeation* depending on the nature of the mobile phase, which is either aqueous or organic. For this technique, the distribution coefficient is called the *diffusion coefficient*.

Liquid/liquid chromatography (or partition chromatography, LLC)

The stationary phase is an *immobilized* liquid upon an inert and porous material, which has only a mechanical role of support. Impregnation, the oldest procedure for immobilizing a liquid on a porous material, is a method now abandoned because of the elevated risk of washing out the column, which is called *bleeding*.

Liquid/bound phase chromatography

In order to immobilize the stationary phase (generally a liquid polymer), it is preferable to fix it by covalent bonding to a mechanical support. The quality of separation depends upon the *partition coefficient* K of the solute between the two phases, a phenomenon comparable to a liquid-liquid extraction between an aqueous and organic phase in a separating funnel.

1.12.2 Gas phase chromatography (GC)

The mobile phase is an inert gas and as above this form of chromatography can be sub-divided according to the nature of the phase components:

Gas/liquid/ chromatography (GLC)

As indicated above the mobile phase here is a gas and the stationary phase is an immobilized liquid, either by impregnation or by bonding to an inert support which could be, quite simply, the inner surface of the column. This is the technique commonly called *gas phase chromatography (GC)*. The gaseous sample must be brought to its vapour state. It was Martin and Synge who, in 1941, suggested the replacement of the liquid mobile phase by a gas in order to improve the separations. From this era comes the true beginnings of the development of analytical chromatography. Here once again it is the partition coefficient K that is involved.

Gas/solid chromatography (GSC)

The stationary phase is a porous solid (such as graphite, silica gel or alumina) while the mobile phase is a gas. This type of gas chromatography is very effective

for analyses of gas mixtures or of compounds that have a low boiling point. The parameter concerned is the *adsorption coefficient*.

1.12.3 Supercritical fluid chromatography (SFC)

Here the mobile phase is a fluid in its supercritical state, such as carbon dioxide at about 50°C and at more than 150 bar (15 MPa). The stationary phase can be a liquid or a solid. This technique combines the advantages of those discussed above: liquid/liquid and gas/liquid chromatography.

Problems

- 1.1 A mixture placed in an Erlenmeyer flask comprises 6 mL of silica gel and 40 mL of a solvent containing, in solution, 100 mg of a non-volatile compound. After stirring, the mixture was left to stand before a 10 mL aliquot of the solution was extracted and evaporated to dryness. The residue weighed 12 mg.

Calculate the adsorption coefficient, $K = C_S/C_M$, of the compound in this experiment.

- 1.2 The retention factor (or capacity factor), k of a compound is defined as $k = m_S/m_M$, that is by the ratio of the masses of the compound in equilibrium in the two phases. Show, from the information given in the corresponding chromatogram, that the expression used – $k = (t_R - t_M)/t_M$ – is equivalent to this. Remember that for a given compound the relation between the retention time t_R , the time spent in the mobile phase t_M (hold-up or dead time) and the time spent in the stationary phase t_S , is as follows:

$$t_R = t_M + t_S$$

- 1.3 Calculate the separation factor (or selectivity factor), between two compounds, 1 and 2, whose retention volumes are 6 mL and 7 mL, respectively. The dead volume of the column used is 1 mL. Show that this factor is equal to the ratio of the distribution coefficients K_2/K_1 of these compounds ($t_{R(1)} < t_{R(2)}$).

- 1.4 For a given solute show that the time of analysis – which can be compared with the retention time of the compound held longest on the column – depends, amongst other things, upon the length of the column, the average

linear velocity of the mobile phase and upon the volumes V_S and V_M which indicate respectively the volume of the stationary and mobile phases.

- 1.5 Equation (2) is sometimes employed to calculate N_{eff} . Show that this relation is equivalent to the more classical equation (1):

$$N_{\text{eff}} = 5.54 \frac{(t_R - t_M)^2}{w_{1/2}^2} \quad (1)$$

$$N_{\text{eff}} = N \frac{k^2}{(k + 1)^2} \quad (2)$$

- 1.6 The resolution factor R for two solutes 1 and 2, whose elution peaks are adjacent, is sometimes expressed by equation (1):

$$R = \frac{t_{R(2)} - t_{R(1)}}{w_{1/2(1)} + w_{1/2(2)}} \quad (1)$$

$$R = \frac{1}{4} \sqrt{N_2} \frac{\alpha - 1}{\alpha} \frac{k_2}{1 + k_2} \quad (2)$$

1. Show that this relation is different from the basic one by finding the expression corresponding to the classic equation which uses w_b , the peak width at the baseline.
 2. If it is revealed that the two adjacent peaks have the same width at the baseline ($w_1 = w_2$), then show that relation (2) is equivalent to relation (1) for the resolution.
- 1.7 1. Show that if the number of theoretical plates N is the same for two neighbouring compounds 1 and 2, then the classic expression yielding the resolution, equation (1) below, can be transformed into (2).

$$R = \frac{\sqrt{N}}{2} \frac{k_2 - k_1}{k_1 + k_2 + 2} \quad (1)$$

$$R = \frac{1}{2} \sqrt{N} \frac{\alpha - 1}{\alpha + 1} \frac{\bar{k}}{1 + \bar{k}} \quad (2)$$

2. Show that if $\bar{k} = (k_1 + k_2)/2$ then expressions (1) and (2) are equivalent.

- 1.8 The effective plate number N_{eff} may be calculated as a function of the separation factor α for a given value of the resolution, R . Derive this relationship.

$$N_{\text{eff}} = 16R^2 \frac{\alpha^2}{(\alpha - 1)^2}.$$

- 1.9 Consider two compounds for which $t_M = 1$ min, $t_1 = 11.30$ min and $t_2 = 12$ min. The peak widths at half-height are 10 s and 12 s, respectively. Calculate the values of the respective resolutions using the relationships from the preceding exercise.
- 1.10 Certain gas phase chromatography apparatus allows a constant gas flow in the column when operating under programmed temperature conditions.
1. What is the importance of such a device?
 2. Deduce from the simplified version of Van Deemter's equation for a full column the expression which calculates the optimum speed and which conveys a value for the HETP.
- 1.11 Explain how the resolution can be expressed in terms of the retention volumes in the following equation:

$$R = \frac{\sqrt{N} V_{R(2)} - V_{R(1)}}{2 V_{R(1)} + V_{R(2)}}$$

- 1.12 Which parameters contribute an effect to the widths of the peaks on a chromatogram? Is it true that all of the compounds present in a sample and which can be identified upon the chromatogram have spent the same amount of time in the mobile phase of the column? Can it be said that a reduction in the retention factor k of a compound enhances its elution from the column?

2

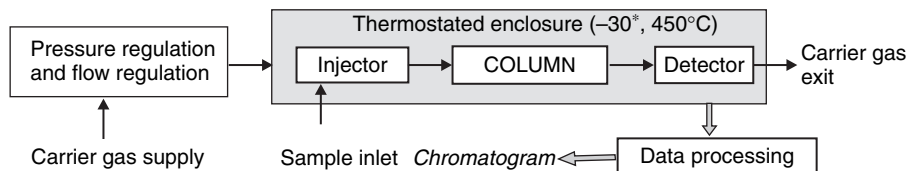
Gas chromatography

Gas chromatography (GC) is a widely used technique whose first applications date back more than 60 years. Since then, development has continued making the best use of the extreme sensitivity, versatility, the possibilities of automation and the ease with which new analyses can be developed. Because separation of compound mixtures on the column occurs while they are in the gaseous state, solid and liquid samples must first be vaporized. This represents, without hesitation, the greatest constraint of gas phase chromatography and weighs against it, since its use is limited to the study of thermostable and sufficiently volatile compounds. However, the applications are numerous in all domains and the development of high speed or multidimensional gas chromatography make this technique even more attractive. Its very great sensitivity permits detection of quantities of the order of picograms for certain compounds.

2.1 Components of a GC installation

A gas chromatograph is composed of several components within a special frame. These components include the injector, the column and the detector, associated with a thermostatically controlled oven that enables the column to attain high temperatures (Figure 2.1). The mobile phase that transports the analytes through the column is a gas referred to as the *carrier gas*. The carrier gas flow, which is precisely controlled, enables reproducibility of the retention times.

Analysis starts when a small quantity of sample is introduced as either liquid or gas into the injector, which has the dual function of vaporizing the sample and mixing it with the gaseous flow at the head of the column. The column is usually a narrow-bore tube which coils around itself with a length that can vary from 1 to over 100 m, depending upon the type and the contents of the stationary phase. The column, which can serve for thousands of successive injections, is housed in a thermostatically controlled oven. At the end of the column, the mobile phase (carrier gas), passes through a detector before it exits to the atmosphere. Some gas chromatographs models of reduced size have their own electrical supply, enabling them to operate in the field (see Figure 2.19).



*or with cryogenic device from -80°C

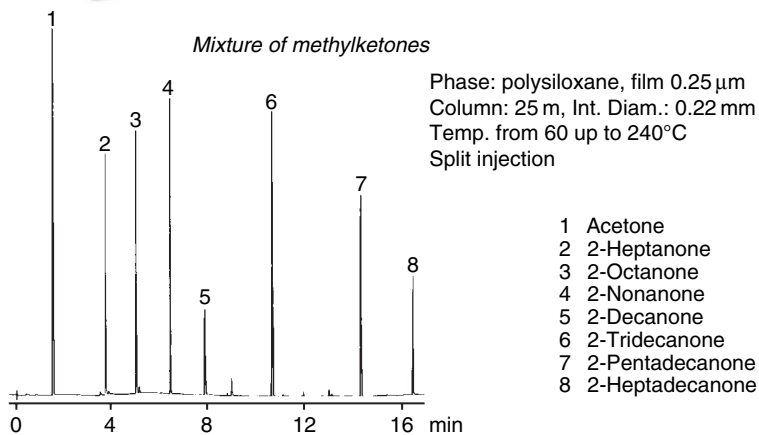


Figure 2.1 A GC installation. Schematic of a gas chromatograph. A commercial gas chromatograph with a mass spectrometry system for detection (Model GCMS 5973 manufactured by Agilent Technologies). The instrument shown here is equipped with an auto-sampler. Below, the chromatogram for a mixture of ketones is displayed.

■ In GC there are four operational parameters for a given stationary phase: L , length of the column, u , velocity of the mobile phase (which affects the theoretical efficiency N , see Section 1.6.1), T , temperature of the column and β , phase ratio (see Section 2.6.4), which affects the retention factor k (Section 1.7.4). The operating conditions of the chromatograph allows modifications in terms of T and u and therefore affects both the efficiency of the column and the retention factors.

2.2 Carrier gas and flow regulation

The mobile phase is a gas (helium, hydrogen or nitrogen), either drawn from a commercially available gas cylinder or obtained, in the case of hydrogen or nitrogen, from an on-site generator, which provides gas of very high purity. The carrier gas must be free of all traces of hydrocarbons, water vapour and oxygen, because all of these may deteriorate polar stationary phases or reduce the sensitivity of detectors. For these reasons the carrier gas system includes filters containing a molecular sieve to remove water and a reducing agent for other impurities. The nature of the carrier gas has no significant influence upon the values of the partition coefficients K of the compounds between the stationary and mobile phases, owing to an absence of interaction between the gas and solutes. By contrast, the viscosity of the carrier gas and its flow rate have an effect on the analytes' dispersion in the stationary phase and on their diffusion in the mobile phase (cf. Van Deemter's equation), and by consequence upon the efficiency N and the sensitivity of detection (Figure 2.2). Hydrogen is the carrier gas of choice. If this gas cannot be used for safety reasons, helium may be substituted.

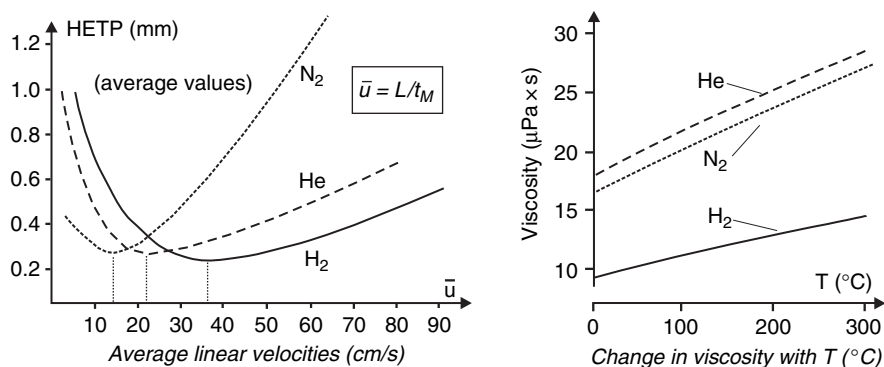


Figure 2.2 Efficiency as a function of the linear velocity of the carrier gas – viscosities of carrier gases. These are typical Van Deemter curves showing that hydrogen, of the three gases studied under the same conditions, allows a faster separation, conveying a greater flexibility in terms of the flow rate, which is very useful for temperature programming. Note the increase in the viscosity of these gases with the temperature and that helium is more viscous than nitrogen at the same temperature.

The pressure at the head of the column (several tens to hundreds of kPa) is stabilized either mechanically or through an electronic pressure control (*EPC*) in order that the flow rate remains constant at its optimal value. This device is valuable because if the analysis is performed with temperature programming, the viscosity of the stationary phase and by consequence the loss of charge in the column, increase with temperature. Therefore to maintain the carrier gas flow constant, the pressure must be finely tuned to compensate this effect. The result is a faster analysis and a longer life for the column.

The injector and the detector have dead volumes (hold-up volumes) which are counted in the total retention volume. In GC, since the mobile phase is a gas, the flow rate measured at the outlet of the column should be corrected by a compression factor J , which compensates for the higher pressure at the head of the column (cf. expression 2.1).

If a chromatogram contains a peak for a compound that is not retained on the stationary phase, it is possible to calculate the average linear velocity of the progression, \bar{u} , of the carrier gas. Elsewhere by installing a flow meter at the outlet of the instrument (atmospheric pressure P_0) and knowing the diameter of the column the velocity \bar{u}_0 of the carrier gas, at the end of the column, can be deduced. The ratio between these two velocities is equal to J , the compression factor, which is linked to the relative pressure P/P_0 (P , the pressure at the head of the column):

$$J = \frac{\bar{u}}{\bar{u}_0} = \frac{3}{2} \cdot \frac{(P/P_0)^2 - 1}{(P/P_0)^3 - 1} \quad (2.1)$$

2.3 Sample introduction and the injection chamber

2.3.1 Sample introduction

The most common injection method is where a microsyringe is used (Figure 2.3) to inject a very small quantity of sample in solution (e.g. $0.5 \mu\text{L}$), through a rubber septum into a flash vaporizer port at the head of the column. For gaseous samples,

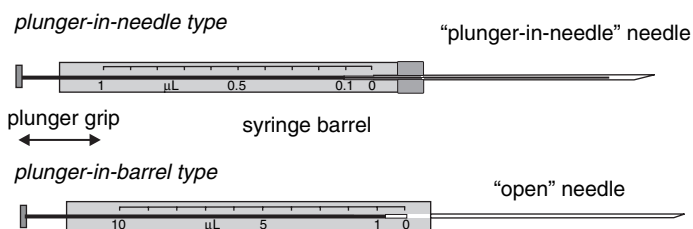


Figure 2.3 Typical syringes used in GC. Several models exist adapted to different types of injectors and columns. For some of them, (0.5 to $1 \mu\text{L}$), a plunger, pushed by the piston, enters the needle to deliver all of the sample.

loop injectors are used similar to those described in liquid chromatography (cf. paragraph 3.4). To better control the reproducibility of the injections – simply changing the user can lead substantial deviations, when in manual mode – most instruments are provided with *autosamplers* in which the syringe movements are automated (Figure 2.1). The assembly operates in a cyclic fashion, taking the sample, injecting it rapidly (0.2 s) and rinsing the syringe. The latter is important to avoid cross-contamination of successive samples that have similar composition.

■ A sampling technique known as 'headspace', of which there are two modes, static and dynamic, is very widespread in GC for the qualitative and even quantitative analyses of volatile constituents present in some samples (cf. Chapter 21).

2.3.2 Injectors

The injector, which is the sample's entrance to the chromatograph, has different functions. Besides its role as an *inlet for the sample*, it must *vaporize*, *mix* with the carrier gas and *bring about* the sample at the head of the column. The characteristics of the injectors, as well as the modes of injection, differ according to column type. The use of an automatic injection system can significantly enhance measurement precision.

Direct vaporization injector

For packed and megabore columns (see paragraph 2.5), which typically use a flow rate of about 10 mL/min, direct vaporization is a simple way to introduce the sample. Any model of this type comprises a metal tube with a glass sleeve (called the *insert*). It is heated to the average boiling temperature of the compounds being chromatographed. The needle of the micro-syringe containing the sample pierces the *septum*, made of silicone rubber, which closes the end of the injector. The other end, also heated, is connected directly to the column (Figure 2.4). Once all of the liquid plug has been introduced with a syringe it is immediately volatilized and enters the column entirely within a few seconds, swept along by the carrier gas.

Split/splitless injector

For *capillary columns* able to handle only a small capacity of sample, even the smallest volume that it is possible to inject with a micro-syringe (0.1 μ L), can saturate the column. Special injectors are used which can operate in two modes, *with* or *without* flow splitting (also called *split* or *splitless*).

In the *split mode* a high flow rate of carrier gas arrives in the vaporization chamber where it mixes with the vapours of injected sample (Figure 2.5). A vent

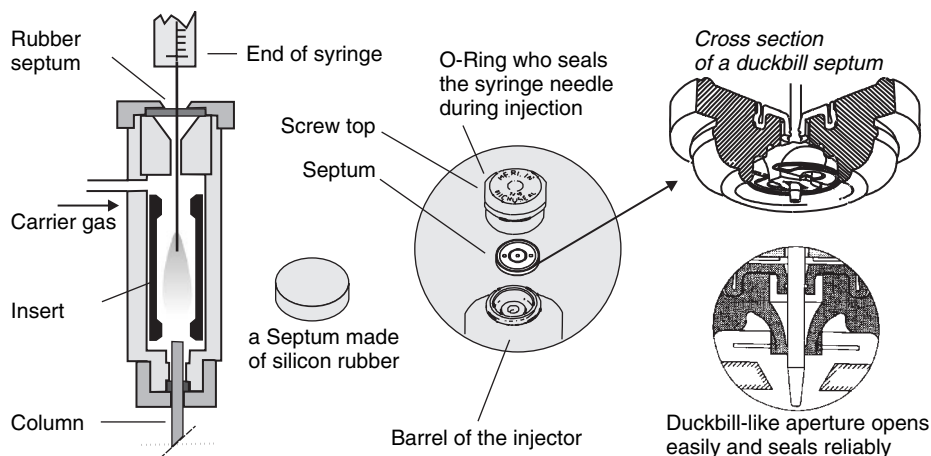


Figure 2.4 Direct vaporization injector used for packed columns. Typical schematic of a model employing a septum. Depending upon the function required a wide range of inserts exist. Right, a variety of self-sealing septum (the ‘Microseal’ Merlin), which can be used thousands of times (reproduced courtesy of Agilent Technologies).

valve, regulated at between 50–100 mL/min, separates this flow into two fractions, of which the largest portion is vented from the injector, taking with it the majority of the sample introduced. The split ratio typically varies between 1 : 20 and 1 : 500. Only the smallest fraction, containing an amount of sample equal to the ratio of division, will penetrate into the column.

$$\text{Split ratio} = \frac{(\text{split outlet flow rate} + \text{column outlet flow rate})}{\text{column outlet flow rate}}$$

When working with capillary columns, this type of injector is also used for very dilute samples in the *splitless* mode. In this mode a smaller volume of solution is injected very slowly from the micro-syringe during which bleeding valve 2 (Figure 2.5) is maintained in a closed position for 0.5 to 1 minute in order that the vaporized mixture of compounds and carrier solvent are concentrated in the first decimetre of the column. The proper use of this mode of injection, which demands some experience, requires a program that starts with a colder temperature in order that the solvent precedes the compounds onto the column. The re-opening of valve 2 provides an outlet for an excess of solvent-diluted sample. Some less volatile compounds are eliminated and that can interfere with the results of the analyses.

■ In quantitative analysis, the use of the split/splitless injector can result in concentration errors owing to a strong discrimination between compounds that are of different volatility: the composition of the fraction entering the column is different to that which is eliminated from the injector. This mode of operation should be avoided when

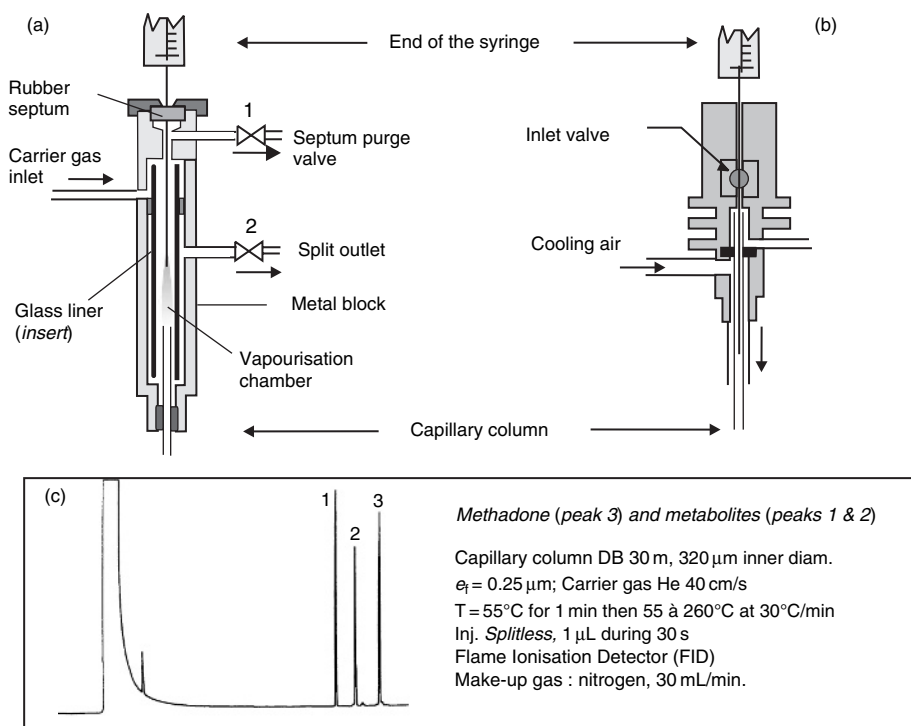


Figure 2.5 *Injectors.* (a) Above left, injection chamber. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). A proportion of carrier gas (1) flows upward and purges the septum, another (2) exits through the split outlet (a needle valve regulates the split) and finally a proportion passes onto the column. (b) Above right, cold injection onto the column. (c) Below, a typical chromatogram obtained in splitless mode. For solvent peaks which are superimposed upon those of the compounds, a selective detector which does not 'see' the solvent is recommended.

using an external standard (cf. paragraph 4.8). However this problem can be partially corrected by a good choice of the glass insert.

Cold on-column injection

This is not a vaporization technique. The sample is deposited directly into the capillary column ('cold on-column' or COC). A special micro-syringe, whose needle (steel or silica) is of 0.15 mm diameter, is necessary for penetrating the column which is cooled to 40 $^\circ\text{C}$ before being allowed to return to its normal operating temperature. This procedure, useful for thermally labile compounds or high boiling compounds, is difficult to master without the aid of an autosampler. It is known not to discriminate between compounds of different volatilities (Figure 2.5).

Programmed temperature vaporization injector

This injector, named PTV (*programmed temperature vaporizer*), is conceptually similar to the split/splitless model. The temperature of the injection chamber can be programmed to effect a gradient, e.g. from 20 up to 300 °C, in a few tens of seconds (Figure 2.6). So, the advantages of the split/splitless injection are combined with those of the cold injection onto the column.

It becomes possible to inject greater volumes with standard syringes avoiding needle-induced discrimination. Furthermore, compounds having low boiling points (particularly solvents) can be eliminated.

The three principal modes of operation are named split cold injection, splitless cold injection and injection with elimination of solvent.

- *Split cold injection*: the sample is introduced into the vaporization chamber and immediately the vent valve is opened and the injector is heated. As the sample is not instantaneously vaporized, the solvent and the different compounds penetrate onto the column in the order of their boiling points. In this way the column is never overcharged.

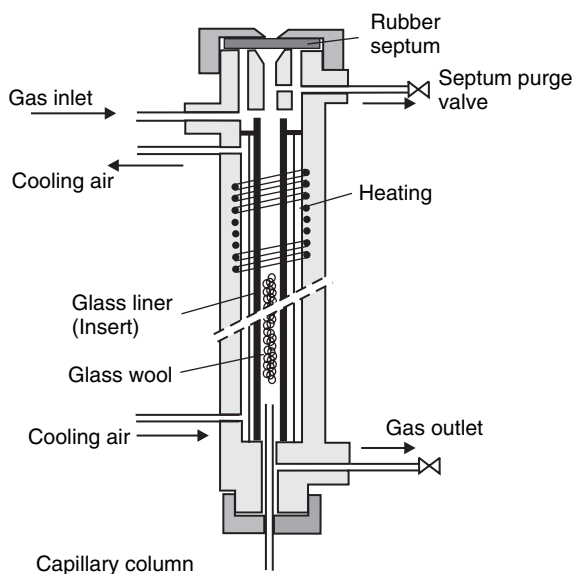


Figure 2.6 PTV injector. To modify very rapidly temperatures, the chamber of the injector is surrounded by an heating element or cooled by the circulation of a cold gas

- *Splitless cold injection*: this mode is employed for trace analysis. The vent valve is closed during the injection. The injection chamber is then heated in order to transfer the sample to the column, which is maintained cold.
- *Injection with elimination of solvent*: the sample is introduced into the cold injector after which the vent valve is opened. Vent flow rate is very high and can attain 1000 mL/min to eliminate all of the solvent. The injector is then heated to permit transfer of the less volatiles compounds onto the column, the bleed valve now being closed (splitless mode). In this way it is possible to inject up to 50 μL in a single injection or up to 500 μL of sample solution, over several injections. This method eliminates the step of the preliminary concentration of sample prior to injection.

2.4 Thermostatically controlled oven

The gas chromatograph comprises an oven with sufficient volume to hold one or two columns easily and which can heat up to more than 400 °C. A weak thermal inertia permits a rapid but controlled temperature climb (gradient able to attain 100 °C/min). The temperature must be controlled to within 0.1 °C in order to get reproducible separations in isothermal or temperature programmed modes. By installation of a cryogenic valve fed with N₂ or CO₂ in the liquid state, the oven can be regulated at low temperature.

2.5 Columns

There are two column types, which differ in their performance: *packed* columns and *capillary* columns (Figure 2.7). For packed columns the stationary phase is deposited or bonded by chemical reaction onto a porous support. For capillary columns a thin layer of stationary phase is deposited onto, or bound to the inner surface of the column.

2.5.1 Packed columns

These columns, less commonly used today, have diameters of 1/8 or 1/4 inch (3.18 and 6.35 mm) and a length of between 1–3 m (Figures 2.7 and 2.8). Manufactured from steel or glass, the internal wall of the tube is treated to avoid catalytic effects with the sample. They can withstand a carrier gas flow rate within the range 10–40 mL/min. They contain an inert and stable porous support on which the stationary phase can be impregnated or bounded (between 3 and 20 per cent). This solid support, having a specific surface area of 2–8 m²/g, is made of spherical

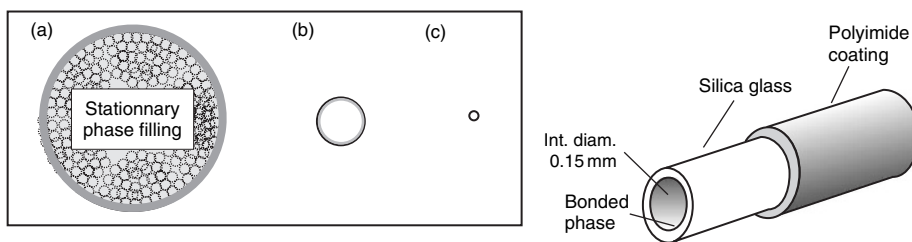


Figure 2.7 Equal scale representation of main types of GC columns. (a) Steel column 3.18 mm; (b) '530' column of 0.53 mm diameter; (c) capillary column of 0.2 mm diameter; (d) detail of a capillary column. At this scale, the thickness of the stationary phase will scarcely be visible. The most common length is of 30 m.

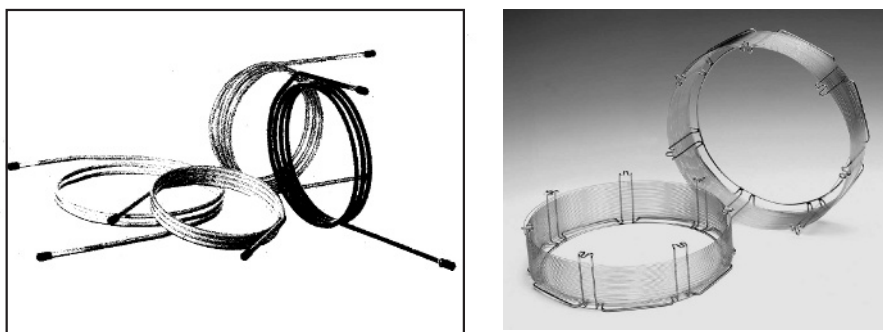


Figure 2.8 Examples of packed (left) and capillary (right) columns (reproduced courtesy of Alltech)

particles of around 0.2 mm diameter, which are obtained from diatomites, silicate fossils (kieselguhr, tripoli) whose skeleton is chemically comparable to that of amorphous silica. A linker assures the cohesion of the grains. After calcination these diatomaceous earths are often designated by the name of Chromosorb®. Other synthetic materials have been developed such as Spherosil®, composed of tiny silica beads. All of these supports have a chemical reactivity comparable to silica gel because of the presence of silanol groups.

Although the performance of packed columns is more modest than capillary columns, they are still usually employed for many routine analyses. Easy to manufacture and with a large choice of stationary phases available, they are not however, well adapted to trace analyses.

2.5.2 Capillary columns (open tubular)

They are usually made of the highest purity *fused silica* obtained by the combustion of tetrachlorosilane (SiCl_4) in an oxygen-rich atmosphere. The internal diameter of

the tube used for these columns varies from 100 to 530 μm , its thickness is 50 μm and the length is of 12 to 100 m. These columns are rendered flexible by the application of a polyimide outer coating, a thermally stable polymer ($T_{\text{max}} = 370^\circ\text{C}$) or a thin aluminium film. They have the advantages of physical strength and can be wound into coils around a lightweight metallic circular support (Figures 2.7 and 2.8). Some manufacturers offer columns made from a metal capillary (aluminium, nickel or steel) which tolerates high operating temperatures of the order of 450°C , providing that the stationary phase is stable enough. The internal surface of the column is usually treated to favour a regular bonding for the stationary phase. This could be a chemical treatment (HCl at 350°C), or the deposit of a thin layer of alumina or silica gel depending on the technique used to bond the stationary phase. The thickness can vary between 0.05 and 5 μm . These are WCOT (*wall-coated open tubular*) or PLOT (*porous layer open tubular*) columns depending upon the nature of the stationary phase employed. Covalent bonding via Si–O–Si–C allows organic compounds to be bound to the silica surface. Columns are particularly stable and can be rinsed periodically with solvents which enable them to recover their initial performance quality.

'530 μm ' columns

Constituted from a capillary of 0.53 mm internal diameter with length varying from 5 to 50 m, these columns are designated, depending on the supplier, by their principal characteristic 'Widebore[®]', 'Megabore[®]', or 'Macrobore[®]'. They require a carrier gas flow rate of at least 5 mL/min and can be as high to 15 mL/min, close to that used in packed columns. However, the resolution and performance of these columns are lower than that of capillary columns of smaller diameter. It is possible to replace a packed column with a '530' column on the older chromatographs, while retaining the same injectors, detectors and flow rates. Their main advantage over packed columns is their lack of bleeding, i.e. the progressive loss of the stationary phase with time. These columns are rarely packed columns.

■ In order to deposit a film of known thickness, a method consists to fill the column with a solution of stationary phase of known concentration (e.g. 0.2 per cent in ether) so that the desired thickness is obtained after evaporation of solvent. This layer can then be reticulated by a peroxide or by γ irradiation. The procedure is similar to the application of a dye on a surface that has been treated beforehand to obtain a firm attachment.

2.6 Stationary phases

For packed columns, for which impregnation techniques are very simple, over 100 stationary phases of various types have been proposed in the literature. On the

other hand, for bonded phase capillary columns the choice of stationary phase is limited because the generation of the film at the surface of the column requires a different principle than impregnation. The current phases correspond in principle to two families: the *polysiloxanes* and the *polyethylene glycols*. Each category can be the object of minor structural modifications. For the study of optically active compounds (enantiomeric separations), particular phases containing cyclodextrins are used.

Each of these phases can be used between a minimum temperature beneath which concentration equilibria are too slow to occur, and a maximum temperature above which degradation of the polymer occurs. This high limit depends on the film thickness and the nature of the polymer.

The stationary phases described below are the more classical types, while in the catalogues, phases especially adapted to particular applications can be found. Among them are phases for the separation of sulfur products, chlorinated pesticides, permanent gases, aldehydes, polycyclic aromatic hydrocarbons (PAH), etc.

2.6.1 Polysiloxanes

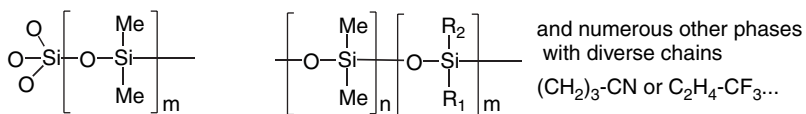
Polysiloxanes (also known as silicone oils and gums) are based upon a repetitive backbone that consists of two hydrocarbon chains per silicon atom (Figure 2.9). There are about 20 different compositions of alkyl or aryl chains (methyl or phenyl) to which can be incorporated further functional groups (e.g. cyanopropyl, trifluoropropyl). Monomers combined in variable proportions also convey changes in the properties of stationary phases (polarity, extended stability from -50 to $300/325$ °C, for the dimethylpolysiloxanes, depending on the column). Owing to their very broad temperature range these phases are the most widely used.

A well-known phase which is used as a reference, since it is the only one that is perfectly defined, is squalane, which on the McReynolds scale has a polarity of zero (cf. Section 2.10.3). This saturated hydrocarbon ($C_{30}H_{62}$) is derived from squalene, a terpenoid natural extract from shark's liver (also present in the sebum of the skin). On this stationary phase, which can be used between 20 and 120 °C (following either deposition or impregnation), the compounds are eluted in increasing order of their boiling temperatures, the retention time being inversely proportional to the analyte vapour pressure. Diverse bonded phases based upon polyalkylsiloxanes, almost apolar, can be used as a replacement for squalane.

2.6.2 Polyethyleneglycols (PEG)

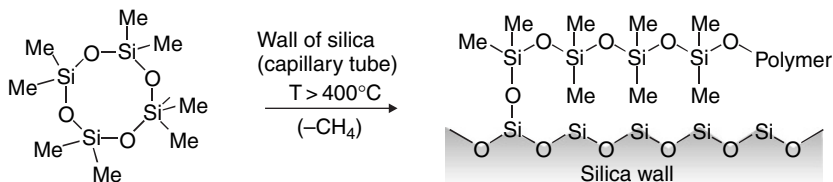
The best known representative of this family is Carbowax® (Figure 2.8). These polar polymers ($M_r = 1500$ to $20\,000$ – for the Carbowax 20M) can be used for

Bonded polysiloxanes (examples):



ex. R₁ and R₂ = Ph m = 95% and n = 5%

Method of formation of a bonded phase



Polyethyleneglycols

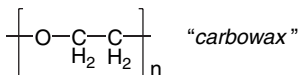


Figure 2.9 Structure of polysiloxanes (silicones), and polyethylene glycols. An inventory of all the compositions of this type of phase, used either for impregnation or bonding, would be lengthy. Treatment of the internal wall of a silica column with tetradimethylsiloxane will obtain a stationary phase bounded, polymerized and later reticulated. (The bonding resembles the fixing of indelible colours in order to create a brightly tinted fabric: the colour contains an active site with which is able to attach itself, for example, to the alcohol functionality of cellulose on cotton fibres).

deposition, impregnation or as bonded phases ($40 < T < 240/260^\circ\text{C}$, depending on column diameter and film thickness).

2.6.3 Chiral stationary phases

These are generally polysiloxane basic phases mixed with 10 to 20 per cent by weight of β -cyclodextrin (polysaccharide) (Figure 2.10). This type of column is suitable for purifying racemic mixtures. If an organic compound, for example, comprises an asymmetric carbon the R and S enantiomers will not have the same affinity for the charged stationary phase in cyclodextrin, which also is expressed as two characteristic peaks. Therefore a chemical compound as a pure racemate will yield two peaks equal in size, each corresponding to an enantiomer (cf. paragraph 3.7).

Some columns accept high temperatures up to 450°C (e.g. DEXSIL 400 or PETROCOL). Amongst the applications here is the analysis of the triglycerides of fatty tissues and simulated distillation as in the petrol industry. The latter

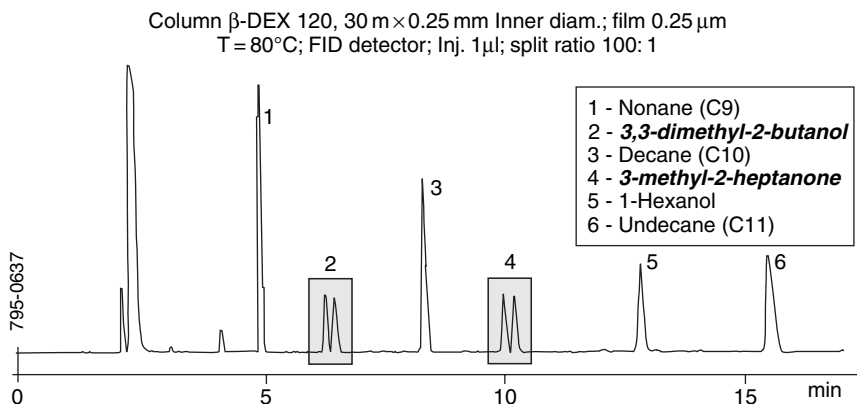


Figure 2.10 Example of a separation with a chiral phase which contains cyclodextrins. The use of a chiral column to separate a racemic mixture of compounds leads to a splitting of the chromatogram signals as can be seen clearly for alcohols, 2 and 4. This chromatogram in isothermal mode, allows the calculation of retention indexes for the separated compounds (adapted from a Supelco illustration).

replaces the conventional distillation, which can take up to 100 hours per analysis (Figure 2.11).

2.6.4 Solid stationary phases

These phases are constituted from a variety of adsorbent materials: silica or alumina deactivated by mineral salts, molecular sieves, porous glass, graphite (e.g. Chromosorb® 100, Poropak®). Capillary columns made by deposition of these materials in the form of a fine porous layer are called PLOT. They are employed to separate gaseous or highly volatile samples. Columns containing graphitized carbon black have been developed for the separation of N₂, CO, CO₂ and very light hydrocarbons. The efficiency of these columns are very high (Figure 2.12).

Historically, silica gel, a thermostable material and insensitive to oxygen, was one of the first compounds to serve as a solid stationary phase for GC columns (Figure 2.9). Today solid phases have become much more elaborate.

To compare or predict the behaviour of capillary columns it is useful to calculate the phase ratio $\beta = V_M/V_S$ (Figure 2.13). Calling d_C the internal diameter of the column and d_f the film thickness deposited on its inner surface, an approximate calculation leads to:

$$\beta = \frac{V_M}{V_S} = \frac{d_C}{4d_f} \quad (2.2)$$

If the compounds to be separated are volatile, a column with a small phase ratio should be chosen ($\beta < 100$) and vice versa. A column of 320 μ m with a stationary

Column HT-5, 6 m × 0.53 mm; film thickness: 0.1 μm
 Column temp. 50 to 480°C (at 10°/min) *Simulated distillation curve of an oil*

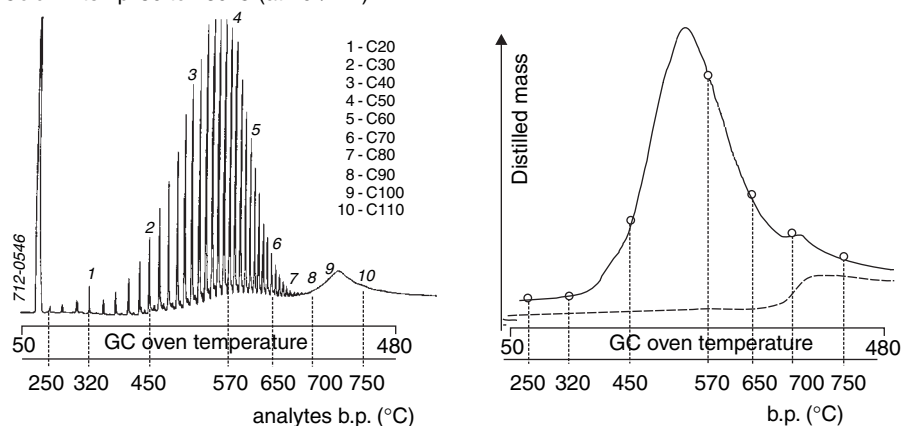


Figure 2.11 *Lubricating oil simulated distillation.* The standard practice calls for the use of a column that can operate at high temperatures and of a series of known oligomers as boiling point references, to calibrate the retention times vs. boiling point curve. Next the sample “to be distilled”, is chromatographed under the same programming conditions. A software package models actual physical distillation, according to the data of the chromatogram. The distribution curve is identical to that which would be obtained from the mixture if it was distilled, a labour intensive and much longer test (adapted from SGE 712-0546 and -0547 documents).

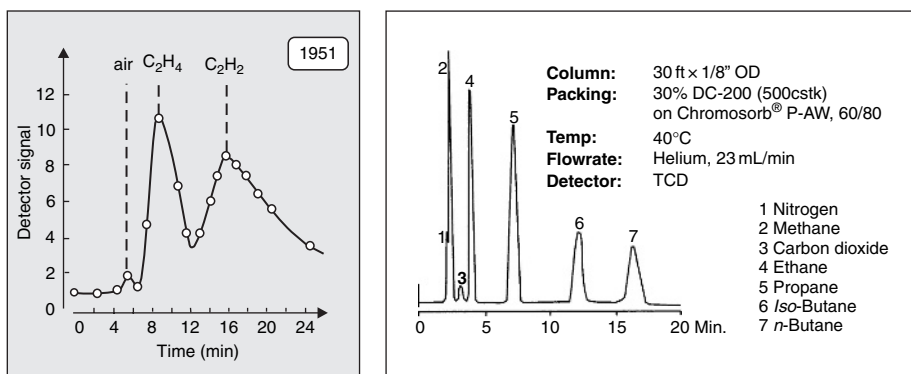


Figure 2.12 *Gas analyses:* Left; one of the earliest ever chromatograms, obtained point by point and representing a mixture of air, ethylene and acetylene separated on silica gel (E. Cremer and F. Prior, *Z. Elektrochem.* 1951, 55, 66). Right; an analysis of gas on a modern packed column (reproduced courtesy of the company Alltech).

phase of 1 μm film thickness leads to a β ratio of 80 while for a column of 250 μm with a film thickness of 0.2 μm, $\beta = 310$. Recalling that $K = k\beta$, it transpires that k , for a given compound and a given stationary phase, will increase if β decreases. The β parameter, which is accessible from the physical characteristics of the

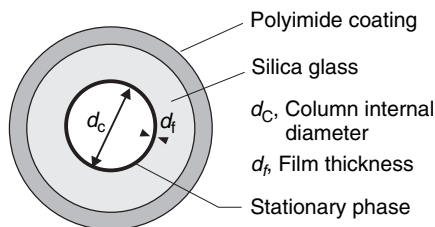


Figure 2.13 Capillary column section

column, allows the calculation of K , the distribution factor. Values are generally very large (e.g. 1000) owing to the nature of the mobile phase which in this case is a gas.

2.7 Principal gas chromatographic detectors

Some detectors are universal; that is they are sensitive to practically every compound that elutes from the column. On the other hand, there are discriminating (selective) detectors that are sensitive only to specific compounds, yielding a very uncomplicated chromatogram. The ideal situation to quantify an analyte, would be to have a detector which sees only this analyte. They can be also categorized as *destructive* or *non-destructive* of the analytes.

Detectors are classified into two groups depending on whether they lead only to a single information such as the retention time and those which yield, besides retention time, structural information of the analyte concerned. For this reason, some gas chromatographs are equipped with two or three detectors linked in series (see Figure 2.17). Nonetheless, the response of all detectors is dependent on the molar concentration or on the mass of analyte in the carrier gas.

2.7.1 Thermal conductivity detector (TCD)

This general purpose and non-destructive detector, in use since the early days of GC, has for a long time remained a mainstay of the technique. Miniaturization has led to it being used as much for packed columns as for capillary columns. Of moderate sensitivity (400 pg/mL carrier gas) when compared with other detectors, it possesses nevertheless a very large linear range (six orders of magnitude). Its operating principle relies on the thermal conductivity of gas mixtures as a function of their composition. The detector incorporates two identical thermistors, resembling minuscule filaments, placed in two tiny cavities of a metal block thermostatically maintained at a temperature above that of the column (Figure 2.14).

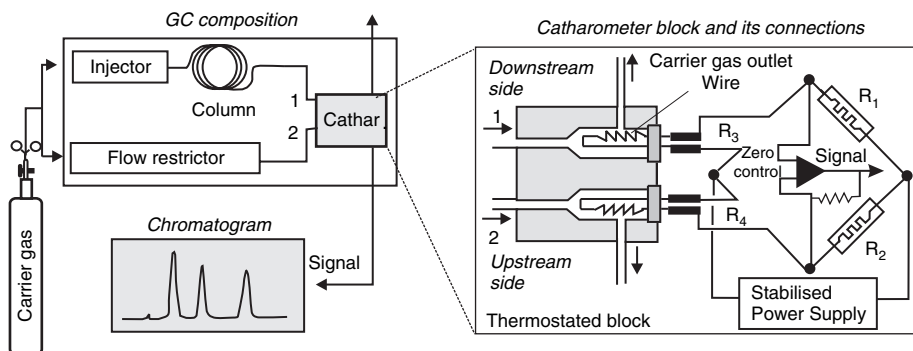


Figure 2.14 Thermal conductivity detector. Left, schematic showing the carrier gas passage. Right, the cross-sectional scheme of the metal block with its operating principle, based on an electrical Wheatstone bridge assembly (equilibrated when $R_1/R_2 = R_3/R_4$).

Both thermistors are located within the path of the carrier gas. One is flushed by the carrier gas evolving the column, while the other is flushed by a part of the carrier gas entering the injector.

In the steady state a temperature equilibrium is established between thermal conductivity of the carrier gas and electrical current through the filament. When a solute elutes there is a change in the mobile phase composition, which in turn modifies its thermal conductivity. The thermal equilibrium being disrupted, this results in a variation of the resistance of one of the filaments which is proportional to the concentration of the compound in the carrier gas.

2.7.2 Flame ionization detector (FID)

Considered as almost universal for organic compounds this is effectively the detector *par excellence*, of GC. The gas flow issuing from the column passes through the flame of a small burner fed by a mixture of hydrogen and air. The detector destroys the organic compound present whose combustion results in the release of ions and charged particles responsible for the passage of a very weak current (10^{-12} A) between two electrodes (pd of 100 to 300 V). One end of the burner, held at ground potential, acts as a polarization electrode while the second electrode, called the collector, surrounds the flame rather like a collar. An electrometer amplifies the signal to a measurable voltage (Figure 2.15).

For organic compounds the intensity of the signal is considered to be proportional to the *mass flow* of carbon, excepted in the presence of heteroelements, such as the halogens. Thus the area under the peak reflects the mass of the compound eluted (dm/dt integrated between the beginning and end of the peak will give the total mass m). The sensitivity is expressed in Coulombs/g of carbon. The detection limit is in the order of 2 or 3 pg/s and the linear dynamic range attains 10^8 . An

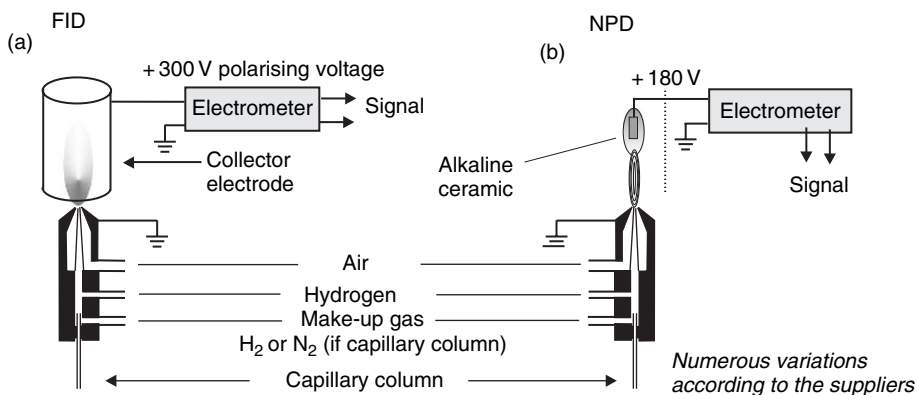


Figure 2.15 FID detector (a) and NPD detector (b). The electrometers used with detectors allow the measurement of very small intensities. The response of these mass flow dependant detectors are unaffected by make-up gas.

FID detector is not affected by variations in flow rate which can lead to errors with TCD.

In order to evaluate the presence of a volatile organic compound in polluted air (the VOCs), there exist portable instruments essentially housing a flame ionization detector that allows the measurement of the carbon content of the atmosphere examined, without chromatographic separation.

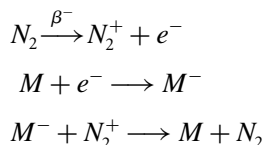
2.7.3 Nitrogen phosphorus detector (NPD)

Compared with the FID, this thermoionic detector has a smaller flame in which the catalytic decomposition of compounds containing nitrogen (N), or phosphorus (P) yields, fairly specifically, negative ions which are received by a collector electrode. As it appears on the representation (Figure 2.15), it comprises a small ceramic cylinder doped with an alkaline salt (e.g. rubidium sulfate). A voltage is applied to maintain a small plasma (800 °C) through the combustion of an air/hydrogen mixture. In these conditions the nitrogen present in air does not yield ions. Detector sensitivity is typically between 0.1 and 0.4 pg/s for nitrogen- or phosphorus-containing analytes, with a linear range of five orders of magnitude.

2.7.4 Electron capture detector (ECD)

This selective detector is considered to be excellent for trace analysis when analytes contain halogen atoms or nitro groups. A flow of nitrogen gas which has been ionized by electrons generated from a low energy β^- radioactive source (a few

mCi of ^{63}Ni) passes between two electrodes maintained at a voltage differential of around 100 V (Figure 2.16). At equilibrium, a base current I_0 is generated, mainly due to free and very mobile electrons. If molecules (M), containing an electrophore such as a halogen (F, Cl, Br), cross the zone between the two electrodes, they capture thermally excited electrons to form heavy negative ions, which by consequence are much less mobile, leading to a decrease in the signal.



The measured intensity decreases exponentially by following a law of type $I = I_0 \exp[-kc]$. The linear range is of about four decades with nitrogen as the carrier gas. The presence of a radioactive source in this detector means that it must be licensed (maintenance visits and regular area testing). This non-destructive detector, well suited for compounds with high electron affinity, is mainly used for analyses of chlorinated pesticides.

■ *Make-up gas.* To provide maximum performances, the three detectors described above should be served with a gas flow of at least 20 mL/min, which is far superior to that within capillary columns. This flow rate is attained by mixing, at the outlet of the column, a make-up gas either identical or different (such as nitrogen), from the carrier gas.

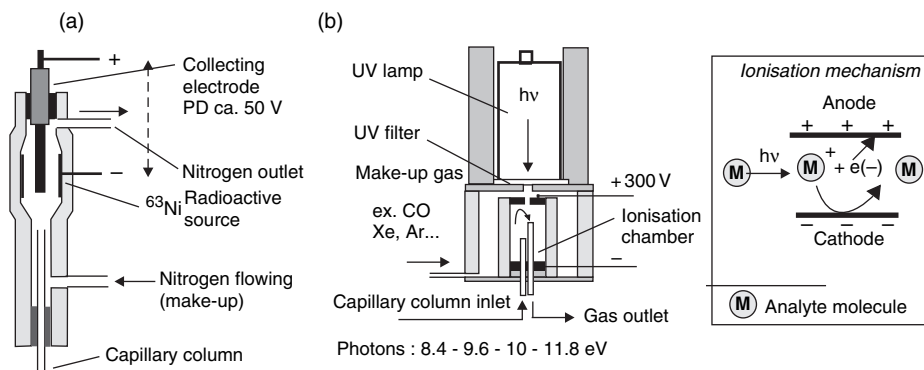


Figure 2.16 *Electron capture detector (ECD) (a) and photo-ionization detector (PID) (b).* The ECD must be installed in a well ventilated position owing to it containing a radioactive source. The PID contains a UV source from which the photons are emitted, having a pre-selected energy, using a filter which prevents undesired carrier gas ionization ($M + h\nu \rightarrow M^+ + e^-$). Examples of filters: LiF at 11.8 eV, MgF_2 at 9.6–10 eV, sapphire at 8.4 eV. On contact with the electrodes the molecules return to uncharged state, ionization being therefore reversible. The use of the make-up gas provides an optimal flow.

2.7.5 Photo-ionization detector (PID)

This detector is fairly selective but it has only a narrow range of application, convenient for hydrocarbons as well as for S or P derivatives. The operating principle consists to provoke ionization of the analytes by irradiation with a UV lamp emitting photons of high energy (of 8.4 to 11.8 eV). The photo-ionization occurs when the energy of the photon is greater to that of the first ionization of the compound (Figure 2.16). A photon of 9.6 eV can, for example, ionize benzene ($PI_1 = 9.2$ eV) but not isopropanol ($PI_1 = 10.2$ eV). Electrons are collected by on an electrode linked to an electrometer.

This detector can function at more than 400 °C and is not destructive since the ionization is reversible and affects only a small fraction of the molecules of each compound passing through. ECD and PID detectors are examples of class-specific detectors often used in trace environmental analysis.

2.8 Detectors providing structural data

None of the detectors previously described yield any information as to the nature of the compound eluted. At most they are selective. Compounds identification proceeds with the use of an internal calibration based on retention times or requires the knowledge of retention indexes (cf. paragraph 2.10). When the chromatogram is very complex, a confusion of identity could occur. To counteract this, several complementary detectors could be associated (Figure 2.17), or a detector able to convey structural information based on spectroscopic data, or elemental composition of the analytes. The retention time and specific characteristics for each compound could then be known. These detectors lead to stand-alone analysis techniques for which the results depend only on the ability of the column to separate properly the constituents of the sample mixture.

2.8.1 Atomic emission detector

The compounds coming out from the column enter into a microwave plasma whose temperature is sufficient to create excited atoms (cf. Chapter 14). Thus, each element present in the solute eluted emits a characteristic spectrum, which permits its identification (see Figure 14.8).

2.8.2 Other detectors

A *mass spectrometry detector* (MSD) which consists of a low resolution mass spectrometer, cf. Chapter 16) can be placed to the outlet of the column. A fragmentation spectrum of each eluted compound is obtained. From the *total ionic current*

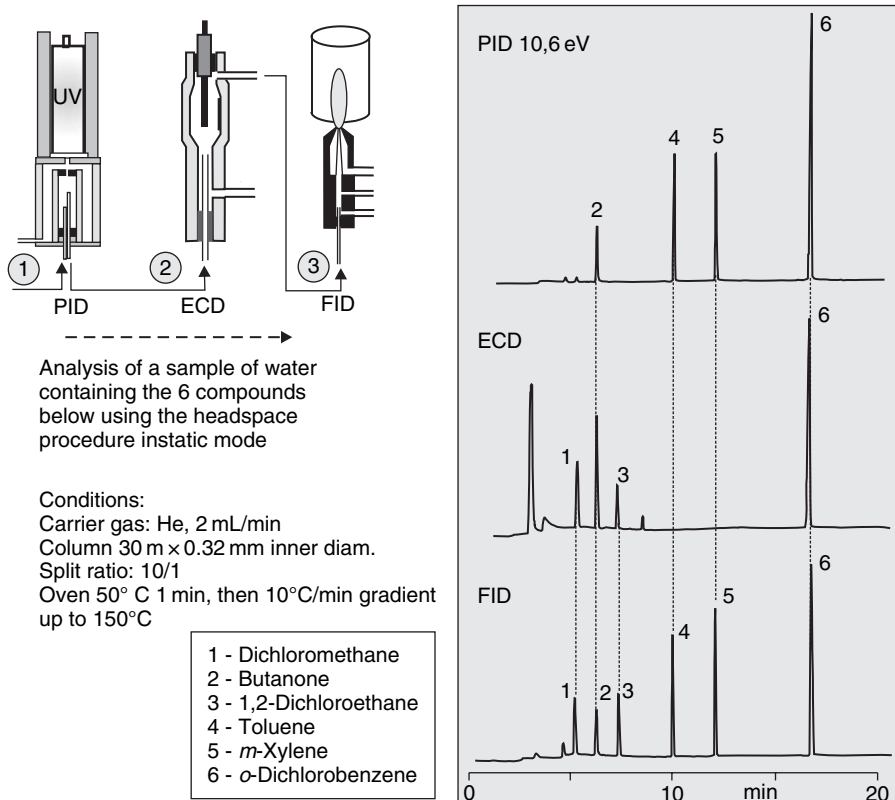


Figure 2.17 Three detectors connected in series. At the outlet of a capillary column, either in series or in parallel and depending upon whether any of them destroy the sample, several detectors can be installed. Here, three chromatograms of an injected mixture are obtained from each detector. Note that the sensitivity varies significantly from one detector to another.

(TIC), a chromatogram can be traced which represents all of the compounds eluted. By choosing a particular ion (*selective ion monitoring*, or *SIM*), a selective chromatogram can be produced. Although this method in some cases leads to a more modest sensitivity than with classic detectors, for many types of analyses it has become essential, notably in the context of environmental studies. Nevertheless it requires the use of high performance columns ($ID = 0.1\text{--}0.2\text{ mm}$) with very low bleeding. Similarly with an *infrared detector*, the IR spectrum (cf. Ch. 10) can be graphically collated while with an *ultraviolet detector* (cf. Ch. 9), the corresponding UV spectrum of each compound eluted can be drawn. This is the domain of coupled 'hyphenated' methods, widely used for trace analyses. The modes of detection mentioned above can be used jointly in the same installation equipped with a capillary column.

2.9 Fast chromatography

Conventional chromatography is a slow method of analysis. The retention times are often longer than an hour when separating components of a complex mixture. To reduce these times, influence can be brought to bear upon several parameters. The most obvious requires the use of a shorter column and so as not to lose efficiency, the diameter of the capillary column should be reduced (cf. expression 1.33). The stationary phase should be a thin film ($0.1\ \mu\text{m}$) and the column must operate with a steep programmed temperature gradient (e.g. $100\ ^\circ\text{C}/\text{min}$), now possible with modern GC instruments. Detector-response time also plays a significant role in achieving the best peak fidelity.

If all the compounds in an existing GC run are too much separated, presenting large vacant spaces between peaks on the chromatogram, then the strategies described above should produce faster analysis times. This leads us to the domain of *fast chromatography* of volatile compounds which uses columns of very different design, for example incorporating resistant outer coverings so that the temperature ($200\ ^\circ\text{C}/20\ \text{s}$) might be increased more sharply. This also avoids to the oven a long time to cool down and get ready for the next run. As a result the retention times are reduced significantly (Figure 2.18). This type of *fast chromatography* sometimes called *high-speed GC* finds its principal use in control analyses.

The detector must be able to store almost immediately the rapid variations in concentration at the moment of each analyte's elution. For detection by mass spectrometry there is good reason to be attentive to the speed of the sweep of the m/z ratio; a slow sequential sweep may lead to a situation in which the concentration in the ionization chamber is not the same from one end of the recording to the other. The TOF-MS (cf. paragraph 16.5) does not suffer from this inconvenience.

There are now portable micro-chromatographs available weighing only a few kg for the rapid analysis of gases and volatile products. Although containing a reservoir of the carrier gas to assure their autonomy they are not bulky (Figure 2.19).

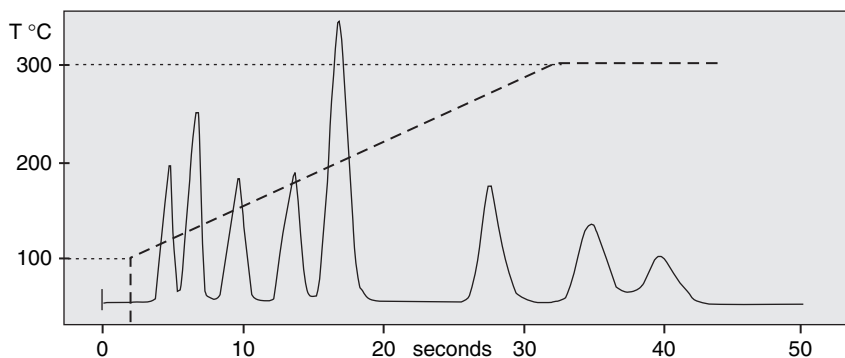


Figure 2.18 *Fast chromatography.* The graph records the programmed temperature of the column

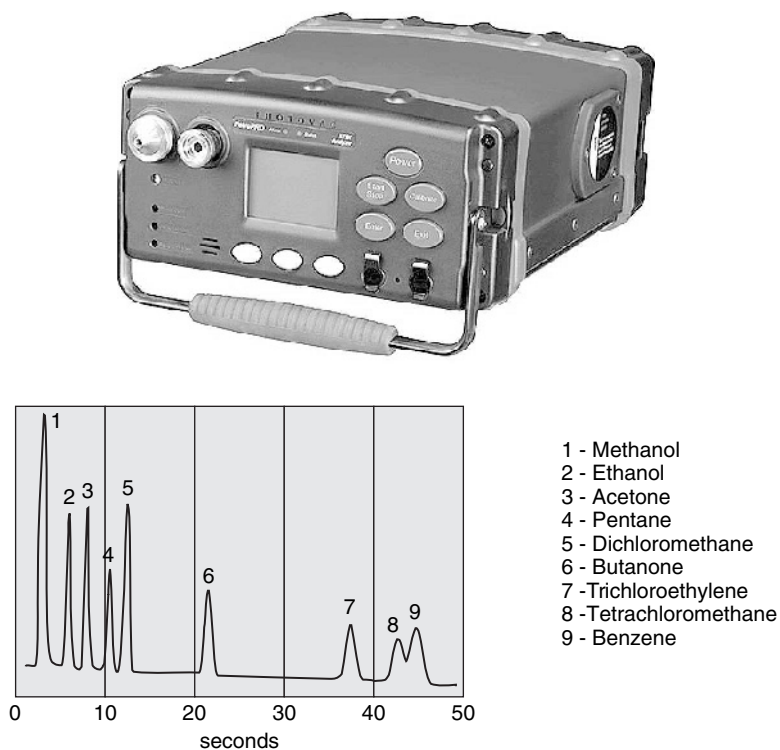


Figure 2.19 *Portable gas chromatograph.* A lightweight (6.6 kg), battery operated, isothermal GC. This miniature analytical engine using a capillary column and a photoionization detector is conceived for the analysis of gas and other volatile compounds (VOCs) (reproduced courtesy of Photovac). Below is an example of chromatogram obtained with such an instrument.

Certain components are obtained by micro-machining on silicon-chips. A short capillary column (5 m) is inserted into the outer metal sleeve, which is able to tolerate a rapid increase in temperature such as a gradient of $20^{\circ}\text{C}/\text{s}$. The efficiency (N) remains fairly poor though the temperature gradient allows an optimization of the selectivity between the compounds.

2.10 Multi-dimensional chromatography

In this technique, that has been used for a long time, the whole (or a fraction) first column effluent is analysed through a second column having different selectivities. This allows the resolution of analytes, which are not separated at the end of the first column. Called GC \times GC or 2D-GC, the installation must comprise two detectors and an injection valve between the two columns positioned in series (Figure 2.20).

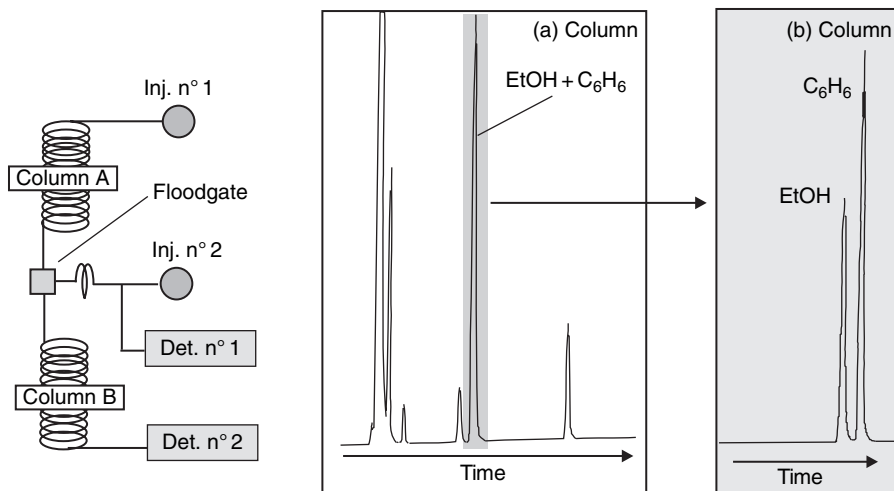


Figure 2.20 *GC × GC setup or two-dimensional chromatography.* The arrangement of two columns each associated with a detector and an injection valve introduced between them. In the example shown in (A), a polar column cannot separate ethanol and benzene. The corresponding fraction is therefore re-injected onto (b), a non-polar column, in which the separation is effected (figure courtesy of Thermoquest).

2.11 Retention indexes and stationary phase constants

These parameters have been developed to pursue at least three objectives:

- To identify a compound by a more general characteristic than its retention time under pre-defined controlled conditions. As a result, a *system of retention indexes* has been developed which is an efficient and cheap means by which to avoid certain identification errors.
- To follow the evolution over time of a column's performance.
- To classify all stationary phases in order to simplify the choice of the column best adapted to a particular kind of separation problem. The chemical nature of the phases and polarities do not allow prediction of which column will be optimal for a given separation. For this, the behaviour of stationary phases with respect to several reference compounds should be examined, preparing the way toward *stationary phase constants*.

2.11.1 Kovats' straight line relationship

The retention index related to a given stationary phase is determined as follows. When a mixture containing compounds belonging to a homologous series of

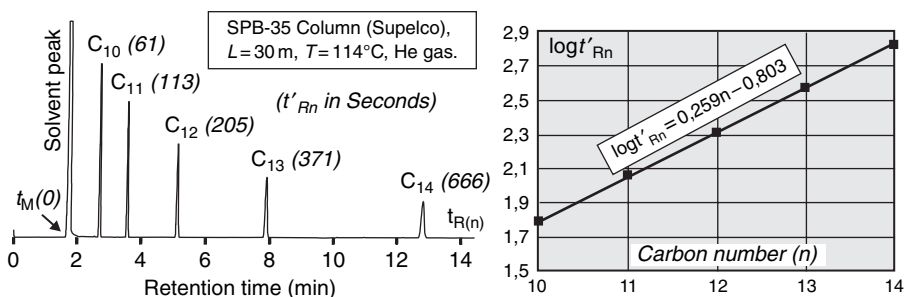


Figure 2.21 Kovats' straight line graph. Left, isothermal chromatogram for a series of five n -alkanes ($C_{10} - C_{14}$). Right, the corresponding plot of the Kovats' relationship for the chosen stationary phase and for the pre-determined analytical conditions.

n -alkanes is injected onto a column maintained under isothermal mode, the resulting chromatogram is such that the logarithm of the adjusted retention times $t'_{R(n)}$ increases linearly with the number n of carbon atoms present in the corresponding n -alkane (Figure 2.21).

On a graphical representation, the carbon number n versus $\log t'_{R(n)}$ usually yields a series of well lined up points according to the following semi-empirical mathematical relationship:

$$\log t'_{R(n)} = an + b \quad (2.3)$$

The adjusted retention time $t'_{R(n)}$ corresponds to the retention time t_R of an alkane having n atoms of carbon, minus the dead time t_M ; a and b are numerical coefficients. The slope of the graph obtained depends on the overall performance of the column and the operating conditions of the chromatograph.

■ This expression (2.3) follows on from another linear relation seen in thermodynamics linking the variation in free energy and the equilibrium constant K , ($\Delta G = -RT \ln K$), for a homologous family of compounds in which each term differs from the preceding one by a supplementary CH_2 unit. Since $K = k\beta$ therefore $t'_R = Kt_M/\beta$, then $\log t'_R$ will increase as $\ln K$ for the homologous family: $\ln t'_R = \ln K + (\ln t_M/\beta)$.

2.11.2 Kovats' retention index (or indice)

A compound (X) is now injected onto the column *without changing the tuning of the instrument*. The resulting chromatogram will enable I_x , the Kovats' retention index, to be calculated for X and the specific column employed: this is equal to 100 times the equivalent number of carbon atoms n_x of the 'theoretical alkane' having the same adjusted retention time than X. Two methods can be used to find n_x :

- The first is based on the Kovats' relationship previously obtained (Figure 2.21). This leads to a calculation of n_x (therefore I_x).
- The second gives a good estimation of I_x from the adjusted retention time of the two n -alkanes (n and $n + 1$ C) that bracket compound X on the chromatogram:

$$I_x = 100n + 100 \frac{\log t'_{R(X)} - \log t'_{R(n)}}{\log t'_{R(n+1)} - \log t'_{R(n)}} [0, 1] \quad (2.4)$$

In contrast to the Kovats' regression line, the retention index depends only on the stationary phase and not on the column dimensions or the flow rate of the carrier gas. Due to this the *retention time* is converted into a *relative retention time* independent of experimental conditions, but normalized to a series of paraffins.

In practice, to ensure of the experimental conditions for the two injections are uniform, compound X and the the n -alkanes mixture are co-injected (Figure 2.22).

The chromatogram that gives the Kovats' relationship for a given stationary phase, can also serve to evaluate the performances of a column. For this, the separation number also known as the *trennzahl number* (TZ) is calculated from expressions 2.5 or 2.6. The two retention times occurring in these relationships relate to two successive alkanes differing by one carbon number (n and $n + 1$ atoms) or to two compounds of a similar type. The separation number indicates how many compounds could be baseline separated reasonably well by the column in the interval of retention time of these two reference compounds. The alkanes

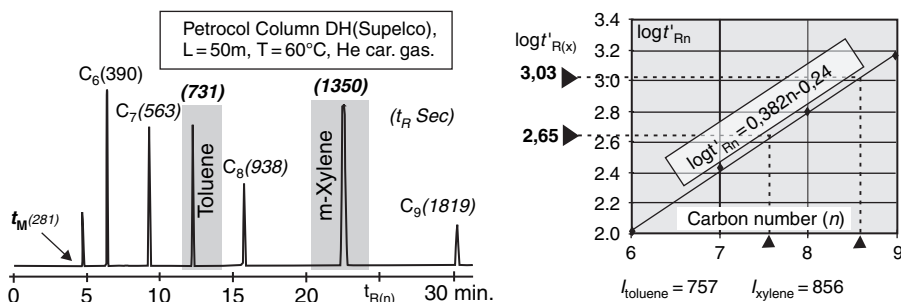


Figure 2.22 Graphical measurement of Kovats' retention index ($I = 100n_x$) on a column in the isothermal mode. The number of equivalent carbons n_x , is found from the logarithm of the adjusted retention time t'_R of X. The chromatogram corresponds to the injection of a mixture of 4 n -alkanes and two aromatic hydrocarbons. The values in italics match the retention times given in seconds. By injecting periodically this mixture the modifications to the Kovats' indexes of these hydrocarbons permits the following of the column's performance. The calculations for retention indexes imply that the measurements were effected under isothermal conditions. With temperature programming they yield good results to the condition to adopt an adjusted formula, though this entails a reduction in precision.

whose elution times are either side of that of the compound being analysed are chosen. For the chromatogram of Figure 2.22, the TZ is around 30.

$$\text{TZ} = \frac{t_{R2} - t_{R1}}{(w_{1/2})_1 + (w_{1/2})_2} - 1 \quad (2.5)$$

or

$$\text{TZ} = \frac{R}{1.18} - 1 \quad (2.6)$$

There are tables of retention indexes of compounds currently in general use on the most common stationary phases. If several retention indexes of the same compound obtained on different stationary phases are available, then this unique collection of values could then characterize the compound more precisely. Identification by retention index is not as reliable as using more popular hyphenated techniques as GC/MS (cf. Section 2.8.2), but it requires a not inconsiderable material investment.

■ *Retention time locking.* The identification of compounds for which the retention times are very close and whose mass spectra are almost identical (certain forms of isomers) is obviously difficult. A current method consists of selecting an internal standard or a compound known to be present in all of the samples to be analysed and through the use of computer software the value of its retention time is locked (unchangeable) for different analyses, even if undertaken on a different apparatus (but on the same stationary phase). The effect of this is to conserve equally the retention times of the other compounds of the mixture, facilitating their identification. This approach, which avoids referring to the retention index, is possible with modern GC instruments and is known as retention time locking (RTL).

2.11.3 McReynolds' constants for stationary phases

To evaluate the behaviour of a stationary phase, a comparison of the Kovats' indexes for five reference compounds belonging to different structural classes is made on the studied phase as well as on squalane, chosen as the reference standard phase for this calculation. The five indexes on a column using squalane, the only reproducible apolar phase since it is formed from a pure material, have been established once and for all (Table 2.1).

The five McReynolds' constants for a given stationary phase are obtained by calculating the differences observed for each of the substances tested between their Kovats' indexes on squalane (I_{Squalane}) and that corresponding to the stationary phase being studied (I_{Phase}):

$$\text{McReynolds constant} = \Delta I = (I_{\text{Phase}} - I_{\text{Squalane}}) \quad (2.7)$$

The sum of the five calculated values, using expression 2.7, has been used to define the overall polarity of the phase under test study.

Table 2.1 McReynolds' constants (ΔI) for several stationary phases normalized to squalane

Stationary phase (symbol)	Benzene X'	1-butanol Y'	2-pentanone Z'	Nitropropane U'	Pyridine S'
Squalane	0	0	0	0	0
SPB-Octyl	3	14	11	12	11
SE-30 (OV-1)	16	55	44	65	42
Carbowax 20M	322	536	368	572	510
OV-210	146	238	358	468	310
Kovats' index for the five reference compounds above (X', Y', Z', U', S') on squalane					
I _{squalane}	653	590	627	652	699

■ Finally this method can tell if two phases should give comparable performances or if a phase is better for an analyte with a specific functional group. Each of the test compounds yields particular information regarding the stationary phase: benzene for the inductive effect, pyridine for proton accepting, butanol for hydrogen bonding, nitropropane for dipolar interactions...

These constants, which are related to molecular structures, allow an appreciation of the interactive forces between stationary phase and solute as a function of compound class (Table 2.1).

An index whose value is high, suggests that the stationary phase strongly retains the compounds that contain the corresponding organic functions. This leads to an improved selectivity for this type of compound. In the same way, to separate an aromatic hydrocarbon from a mixture of ketones, a stationary phase would be selected for which the McReynolds' constant for benzene is rather different to that of pentanone. These differences in retention indexes are provided by suppliers.

The McReynolds' constants have replaced Rohrschneider constants, which were based upon the same principle though using partly different reference compounds.

Problems

2.1 Consider a GC capillary column where the length, diameter and thickness of the film of the stationary phase could all be modified (one factor at a time and without adjustment of the apparatus' physical characteristics, such as temperature and pressure, yet maintaining a flow such that the linear velocity of the gas remains the same).

The three parameters to be modified are displayed in the first column of the table below. Complete the different cases by indicating, using the symbols provided, the observations anticipated: + symbolises an increase, 0 shows a weak variation in either direction and – for a decrease.

Speed of separation	Column capacity	Retention factor, k	Selectivity factor α	Effective plate number, N
<i>increase in column length</i>				
<i>increase in column diameter</i>				
<i>increase in film thickness</i>				

- 2.2 The best-known method for estimating the hold-up time, t_M , consists of measuring the retention time of a compound not retained upon the column. Described here is another method for calculating the hold-up time, which recalls the relation used in establishing factors of retention. Knowing that for a homologous series of organic compounds and where the temperature of the column is constant, it can be written:

$$\log(t_R - t_M) = a \cdot n + b$$

(in which t_R represents the total retention time of a compound having n atoms of carbon, while a and b are constants which depend upon the type of solute and the stationary phase chosen).

1. Recall the chromatographic parameters for which it is essential to know the hold-up time t_M .
 2. Give examples of compounds that might be used to determine t_M in GC.
 3. Calculate t_M from the following experiment, employing the method above: A mixture of linear alkanes, possessing six, seven and eight atoms of carbon, is injected into the chromatograph. The total retention times for these compounds were respectively, 271 s, 311 s, and 399 s, under a constant temperature of 80 °C. (Length of column 25 m, $ID = 0.2$ mm, $d_f = 0.2$ μ m and the stationary phase is made up of polysiloxanes).
 4. If the retention index for pyridine on squalane is 695, what is the McReynolds constant of this compound on the column studied, if it is known that under the conditions of the experiment, the retention time is 346 s?
- 2.3 Find an expression to calculate the thickness of film d_f of a capillary column from K , k and ID , (the column's internal diameter).

A sample of hexane is injected onto a column having an internal diameter of $200\ \mu\text{m}$ for which K is equal to 250. The retention time for the hexane is 200 s, while an unretained compound has a retention time of only 40 s. Calculate the film thickness d_f from this information.

Compound	b.p. ($^{\circ}\text{C}$)	Temperature of the column ($^{\circ}\text{C}$)		
		-35	25	40
ethene	-104	0.249	0.102	0.0833
ethane	-89	0.408	0.148	0.117
propene	-47	1.899	0.432	0.324
propane	-42	2.123	0.481	0.352

- 2.4 Show that for a capillary column, the average flow can be calculated from the following formula:

$$D_{\text{mL/min}} = \bar{u}_{\text{cm/s}} \times 0.47 d_1^2 \text{ mm}$$

(where \bar{u} represents the average linear velocity in a column of internal diameter ID).

- 2.5 The table below contains values of the retention factor k for four refinery gases, studied at three different temperatures upon the same capillary column (length $L = 30\ \text{cm}$, internal diameter = $250\ \mu\text{m}$), whose stationary phase is of type SE-30. The chromatograph is supplied with a cryogenic accessory.

1. Can the polarity or non-polarity of the phase SE-30 be deduced from the elution order of the compounds?
2. Calculate the selectivity factor α for the couple propene–propane at the three temperatures indicated.
3. Why for the same compound does k decrease in response to an increase in temperature?
4. What is the number of theoretical plates of the column for the propane at $40\ ^{\circ}\text{C}$, if it is known that at this temperature the resolution factor for the couple propene–propane = 2?
5. What would be the minimum theoretical value of HETP for the propane at $40\ ^{\circ}\text{C}$?

6. Supposing that the HETP resulting from the Van Deemter curve is the same as that resulting from the calculation which might be performed when considering Question 4, calculate the coating efficiency.
 7. If for a given compound, k is linked to the absolute temperature of the column by the expression: $\ln k = (a/T) + b$, find, for the case of ethane, numerical values for both a and b .
- 2.6 In a GC experiment a mixture of n -alkanes (up to n carbon atoms, where n represents a variable number) and butanol ($\text{CH}_3 \text{CH}_2 \text{CH}_2 \text{CH}_2 \text{OH}$) were injected onto a column maintained at a constant temperature and whose stationary phase was of silicone-type material. The equation of the Kovats' straight line derived from the chromatogram is: $\log t'_R = 0.39n - 0.29$ (where t'_R the adjusted retention time is in seconds). The adjusted retention time of butanol is 168 s. If it is known that the retention index for butanol on a column of squalane is 590 s then deduce its corresponding McReynolds constant upon this column.
- 2.7 A chromatogram reveals a resolution factor of 1.5 between two neighbouring peaks, 1 and 2. If $k_2 = 5$ and $\alpha = 1.05$, and knowing that the retention time of the compound 2 is 5 min, then:
1. Calculate the hold-up time of this chromatogram.
 2. What is the width of the second peak at half-height, if the scale of the chromatogram is such that 1 min corresponds to 1 cm?
- Use the relation 1.28 (cf. paragraph 1.9)

3

High-performance liquid chromatography

Of all the chromatographic techniques whose mobile phase is a liquid, high-performance liquid chromatography (HPLC) is perhaps the best known. Its field of applications overlaps a large section of gas phase chromatography, to which can be added the analyses of many compounds that are thermolabile, or very polar or of high molecular weight. Its success is also due to the possibility, for the chromatographer, to act in a very precise manner upon the selectivity between compounds through an appropriate choice of columns and eluent composition by exploiting the solute/mobile phase/stationary phase interactions. Although the efficiency of the HPLC columns is less than those used for GC, new stationary phases that can operate in several modes such as ion pairing or increasing hydrophobic interactions, reveal further possibilities of HPLC. Finally, miniaturization of the technique (nanochromatography) has facilitated its working association with mass spectrometry.

3.1 The beginnings of HPLC

High-performance liquid chromatography, often called simply by its abbreviation, HPLC constitutes a general purpose analytical technique derived from the most ancient form of preparative liquid chromatography. The modern day technique is greatly enhanced in terms of selectivity, resolution, through miniaturization and the use of very elaborate stationary phases. These phases comprise spherical micro-particles with diameters of 2–5 μm , or porous monolithic material that leads to a significant pressure drop on the column. A large pressure needs to be exerted upon the mobile phase to obtain a continuous flow. In spotting this particularity, the letter *P* of the abbreviation corresponded for a long time to the word *pressure*.

■ The forced migration of a liquid phase in continuous contact with a stationary phase is encountered in several chromatographic techniques. One of the aspects particular to HPLC is that of the partition mechanisms between analyte, mobile phase and stationary phase. They are based on coefficients of adsorption or partition.

3.2 General concept of an HPLC system

An HPLC installation is composed of several specialized units which can be found as separate entities or be integrated within a common framework, usually for reasons of hindrance (Figure 3.1). A tubing system of very small internal diameter (0.1 mm) assures the circulation of the mobile phase between the modules. These

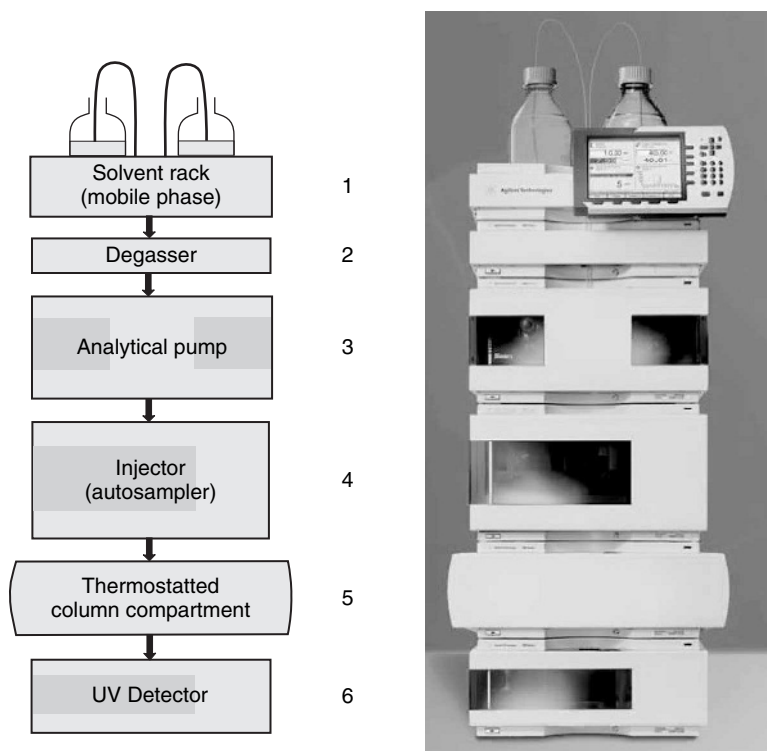


Figure 3.1 Schematic of a modular HPLC instrument. A modular system allows users to adapt the installation according to the applications to be carried out. The vertical assembly of the different modules affords an economy of space. Here the chromatograph, model HP 1200, comprises an auto-sampler that allows continuous operation and a thermostatically-controlled column to improve the reproducibility of the separations. (Reproduced courtesy of Agilent Technologies).

transfer tubes are made of stainless steel or of PEEK® (polyether-etherketone), a coloured and flexible polymer, able to resist the common solvents under high pressure (up to 350 bars).

- The low flow rates obey Poiseuille's law. The maximum velocity of the mobile phase is found at the centre of the tubing while in contact with the wall it is zero. This produces an inevitable dispersion of the compounds. To improve the separations the volume of the mobile phase outside the column is maintained as low as possible (10 per cent dead volume of the column).

3.3 Pumps and gradient elution

3.3.1 Pumps

All HPLC systems include at least one pump to force the mobile phase through the column whose packing is fairly compact. The result of this is a pressure increase at the injector which can attain 20 000 kPa (200 bars) depending upon the flow rate imposed upon the mobile phase, its viscosity, and the size of the particles of the stationary phase.

Pumps are designed in order to maintain a stable flow rate, avoiding pulsations even when the composition of the mobile phase varies. These flow rate metered pumps contain, in general, two pistons in series, working in opposition, to avoid interruptions to the flow rate (Figure 3.2).

- The presence of a non-negligible quantity of ambient gases (N_2 , O_2 , CO_2), dissolved in the solvents, can perturb the separations by modification of the compressibility of the mobile phases which leads to the eventual formation of bubbles. In particular, oxygen can interfere on the lifetime of the column while hindering the working of both electrochemical and photometric UV detectors. The solvents are therefore degassed either by ultrasound, rapid helium bubbling, or by diffusion for which they pass along a polymeric tube, of small diameter, permeable to gas, working like a membrane.

It is obvious that these pumps deliver a series of 'pulses' of the mobile phase. The perfecting of flow rate regulation is achieved by the insertion of a pressure (or pulse) damper, between the pump(s) and the injector. It operates on the mechanical principle of ballasting. Several techniques have been developed to accomplish this. The simplest mechanical ballasting consists of inserting between the pump and the injector a large coil of narrow-bore tubing, several metres long. Under the influence of a wave of solvent the tubing unwinds slightly increasing its internal volume and counteracting the variation in pressure. The most usual damper type is a membrane one (Figure 3.2), usually having a volume of around 0.5 mL. The closed part is filled with heptane. Compressibility of this liquid is enough to compensate for the pulsations of the dual piston pump with piston volume around 100 μ L.

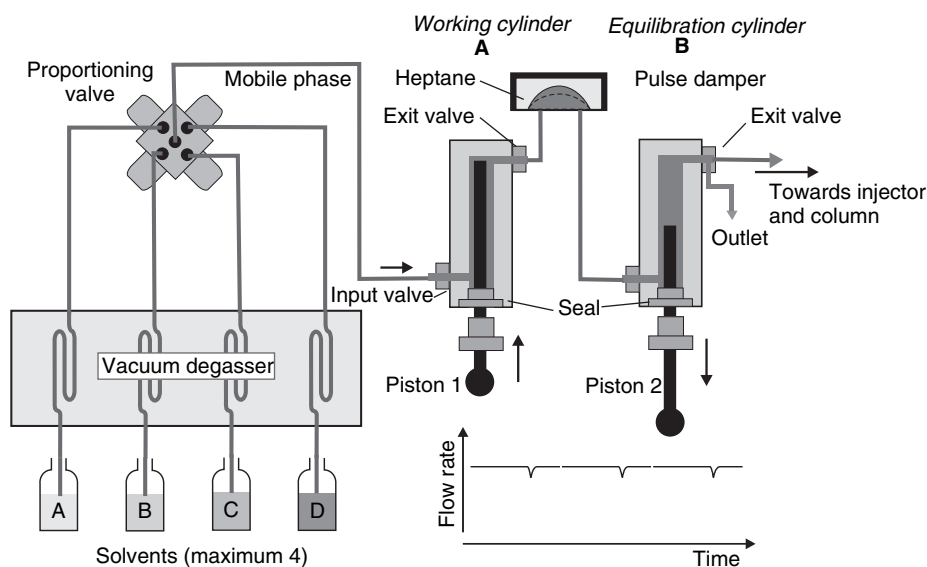


Figure 3.2 Schematic of the dual-headed reciprocating pump. The most simplified way of explaining the cycle of operation, without taking into account the compressibility of the solvents, is as follows. From the moment when the outlet valve of cylinder A closes and its entrance valve opens, the piston in A, moving backwards, sucks the eluent through the inlet check valve and the chamber fills. Meanwhile cylinder B is open and its piston moves forward to force the mobile phase towards the injector and the column. The volume displaced by piston B is half of that available in the chamber of piston A. With chamber A full, the entrance valve of A closes and the corresponding outlet valve opens. Piston A now advances and pushes out the contents of the chamber. Half of this volume is expelled directly towards the column, the other half serves to fill cylinder B as piston B retracts. A pulse absorber is located between the two cylinders (diagram courtesy of Agilent Technologies). Below, graph showing variations in the flow rate as a function of movement of the pistons with time.

■ To obtain micro flow rates (for example, $1 \mu\text{L}/\text{min}$), necessary for a packed capillary column, the same pumps are used, though with the addition at the outlet of a bypass which divides the flow into two fractions of which only the smaller is directed towards the column. To resist the low pHs of many elution mixtures, which can be more corrosive when pressure is high, the components and surfaces that come into contact with the mobile phase need to be inert. The pistons and valves of the pumps are made of sapphire, agate, Teflon or special alloys.

Depending upon the design, an HPLC instrument contains generally two pistons driven by the same motor through a common eccentric cam; this allows one piston to pump while the other is refilling (Figure 3.2). These pumps are associated with a mixing chamber and are located either just before or immediately following. They are capable of delivering an eluent of fixed (*isocratic mode*), or

of variable composition to create an *elution gradient*. For the second case the system must compensate for differences in solvent compressibility in order that the imposed composition be respected.

3.3.2 Low and high pressure gradients

If the composition of the mobile phase has to vary with time, as in a *gradient elution*, then several solvents are required, and in order to attain the desired composition at a given pressure, the instrument must compensate for differences in solvent compressibility. When the system contains a single pump, it should be preceded by a *low-pressure* mixing chamber into which electronically activated valves permit to feed the solvents at a programmed composition. Alternately, in the *high-pressure* arrangement, installations contain more than one pump, depending the number of solvents. The final composition is obtained with a tee located after the pump but before the column (Figure 3.3).

When several analyses are to be done successively, one must avoid the use of gradient elution, by seeking a practical compromise by means of a single eluent of fixed composition. This reduces post-analytical time after each analysis because the re-equilibration of the two phases to their initial composition, after a gradient elution, requires the passage of a volume of at least ten times the dead volume of the mobile phase.

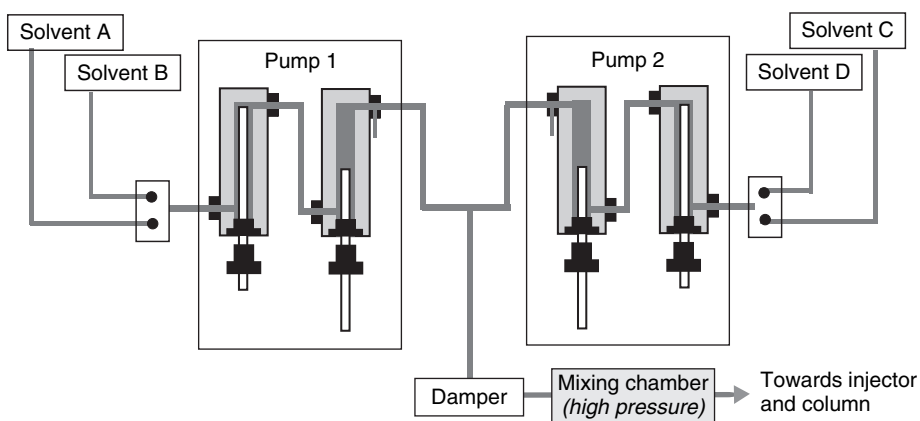


Figure 3.3 A schematic of the core of a high-pressure mixing (two-pump) system. The pumps are called binary, ternary or quaternary depending upon the number of solvents that can be mixed together (here binary). The mixing chamber, which controls the mobile phase composition, is at the output of the two high-pressure pumps on the downstream side of the pumps.

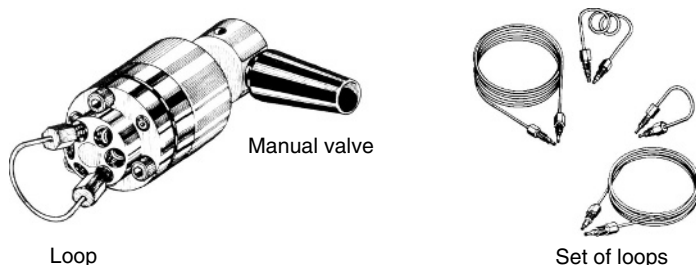


Figure 3.4 Injection valve for HPLC and an assortment of loops. Left, rear view of an injection valve (valve presenting 6 entries/outlets with a loop attached) ; Right, an assortment of loops of different volumes. The volume removed with the syringe must be of the order of 5 to 10 times that of the loop (reproduced courtesy of Rheodyne Inc.).

3.4 Injectors

In HPLC, the injection of a precise volume of sample onto the head of the column must be made as fast as possible in order to cause the minimum disturbance to the dynamic regime of the mobile phase whose flow must be stable from column to detector. This is done by a special high pressure valve, either manual or motorized, possessing several flow paths, which is situated just prior to the column (Figure 3.4). This must be a component of precision able to resist pressures greater than 30 000 kPa. The valve functions in two positions:

- In the *load* position only communication between the pump and the column is assured (Figure 3.5). The sample, contained in a solution, is introduced at atmospheric pressure with the aid of a syringe into a small tubular curved section named a *loop*. Each loop has a small defined volume. They are either integrated into the rotor of the valve or are connected to the outside of the valve's casing.
- In the *inject* position, the sample (which is in the loop) is inserted into the flow of the mobile phase by the 60° rotation of a part of the valve, thus connecting the sample loop to the mobile phase circulation. Highly reproducible injections are attained only if the loop has been completely filled with the sample.

3.5 Columns

The column is a straight stainless steel calibrated tube which measures between 3 and 15 cm in length and whose the inside wall is sometimes coated with an inert material such as glass or PEEK®. Stationary phase is held in the column

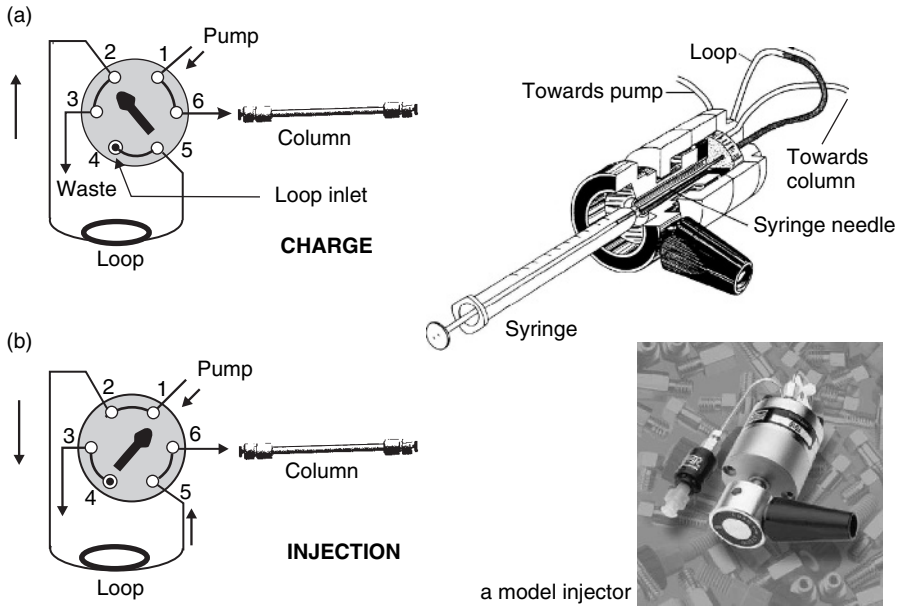


Figure 3.5 The two steps of an injection incorporating a loop. (a) Loading of the loop; (b) Injection onto the column. Schematic of the model 7125 valve from Rheodyne Inc. Most of the injectors are now automated.

between two porous discs situated at each of the extremities (Figure 3.6). The internal diameter of the column, for a long time standardized at 4.6 mm (requiring a mobile phase flow rate of between 0.5 to 2 mL/min) is now often narrower. A wide choice of column now exists with names such as *narrow-bore* (2–4 mm ID), *microbore* (1–2 mm), *packed capillaries* (< 1mm), for which the flow rate

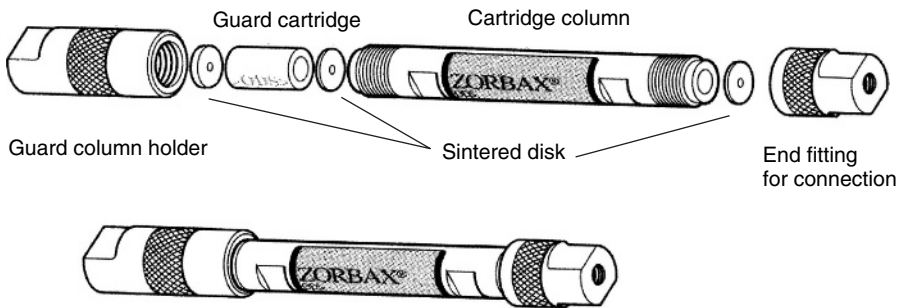


Figure 3.6 Standard column and guard column for HPLC. View of the ZORBAX®column assembly and its exploded view. The replaceable precolumn prevents clogging of the analytical column while extending its lifetime and preserving its performance (Reproduced courtesy of RTI).

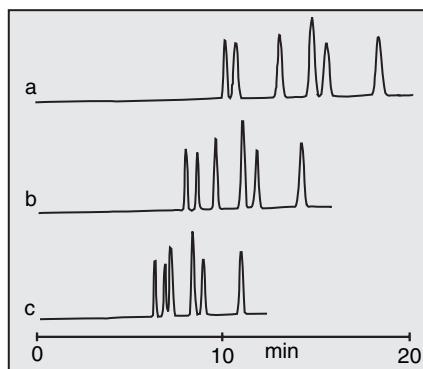


Figure 3.7 The effect of column temperature upon compound separation. Analysis of a compound mixture using the same mobile phase flow rate at three different temperatures (a) 25°C, (b) 35°C, (c) 45°C.

descends to a few $\mu\text{L}/\text{min}$ and therefore requires special pumps and detectors. These narrow columns not only substantially reduce the amount of mobile phase – just a few drops being sufficient to elute all of the compounds – , but also improve resolution by diminishing the diffusion inside the column. They equally have a greater sensitivity and allow HPLC-MS combination (*cf.* Chapter 16).

In order to extend the lifetime of the column, it is often preceded by a precolumn or guard column, short (0.4 to 1 cm), and packed with the same stationary phase as the analytical column (Figure 3.6). This precolumn, which retains the compounds of $R_f = 0$, is periodically changed. It is also recommended, prior to analysis, to pass the samples solutions through a filter of pore size less than $0.5\ \mu\text{m}$.

The influence of temperature upon the quality of separation was for a long time neglected. Now, HPLC instruments are equipped with columns (and therefore eluents) whose temperature is thermostatically controlled. This enhances the reproducibility of analyses and offers a further parameter of separation to be considered (Figure 3.7).

3.6 Stationary phases

The stationary phase (SP) in contact with the mobile phase (MP) is the second medium with which the compounds initially dissolved in the mobile phase will interact. On the column there will be as many particular associations of the three constituents [MP/compound/SP] as there are analytes in the sample.

Many organic and inorganic materials have been tested for use as packing for columns.

3.6.1 Silica gel, the major material for current phases

For the majority of applications *silica gel* still represents the basic material used to pack HPLC columns. It is a rigid, amorphous solid having a composition formula $\text{SiO}_2(\text{H}_2\text{O})_n$ (where n is very close to 0), quite different from natural crystalline silica (SiO_2), which is only a distant precursor of it. Silica gel is in the form of spherical particles, sometimes porous, with a diameter of between 2 to 5 μm . This assures a compact and homogeneous packing of the column which allows a regular circulation of the mobile phase without the formation of preferential routes in the column (Figure 3.8).

It is prepared under conditions of controlled hydrolysis, by a *sol-gel* polymerization of an alkoxysilicate (e.g. tetraethoxysilane) in the form of an emulsion, under the effect of base-catalysed hydrolysis. Initially, tiny particles are formed (0.2 μm) which grow in a regular manner, by various methods, to form spheres that attain a few micrometres in diameter.

■ Recently a new type of column has appeared. Named monolithic it is packed with a porous silica gel which unites to form a single entity (Figure 3.8). This material, more permeable to solvents than traditional bead type stationary phases, conserves a high efficiency even for rapid flow rates of the mobile phase. These columns, now fully reproducible, have equivalent performances to packed columns.

Silica gel is a very polar material corresponding to a three-dimensional network. Though it does not possess the well-ordered structure of crystalline silica, it does maintain the tetrahedral arrangement of four bonds around the silicon atom. This is a reticulated inorganic polymer (Figure 3.8) containing a variable number of *silanol groups*, which have resisted the final calcination. These silanol groups are responsible for the acidic catalytic effect of this material because Si-OH has a $\text{p}K_a$ of 10, comparable with that of phenol. To measure the concentration of silanol groups, solid state ^{29}Si nuclear magnetic resonance (NMR) can be used, or alternatively, in an indirect manner, by quantifying the carbon content for the covalently bound phases.

The characteristics of a silica gel depend upon several parameters, amongst which are the *internal structure*, the *size* of the particles, the *porosity* (dimension and distribution of the pores) the *specific area*, the *resistance to crushing* and the *polarity*. Common silica gels used in HPLC contain around five silanol groups per μm^2 . The specific area is of the order of 350 m^2/g . They can be non-porous or porous (pore size of around 10 nm). The volume of the mobile phase on the column varies from 30 to 70 per cent of the column's total volume. This has come far from the phases used at the beginning of the last century by Tswett, which were of chalk dust or powdered sugar. The treatment to which the silica is submitted makes it something rather like magic sand!

■ The preparation of silica gel in the form of irregular grains, used in preparative chromatography, proceeds in a different fashion. First, orthosilicic acid is formed which

comprises the unstable $[\text{Si}(\text{OH})_4]$, by acidification using sodium silicate $[\text{Na}_2\text{SiO}_3]$ (old name 'pebble liquor' or water-glass) obtained from definite purified sands by alkaline fusion. This unstable orthosilicic acid initially dimerizes then poly-condenses progressively to colloidal particles having a hydroxylated surface. Through aggregation a hydrogel of gelatinous silica is obtained, the calcination of which yields dense grains of silica gel (xerogel). Some of the reactions involved here are similar to those which are encountered for the preparation of the microspheric solid phases used for analytical chromatography.

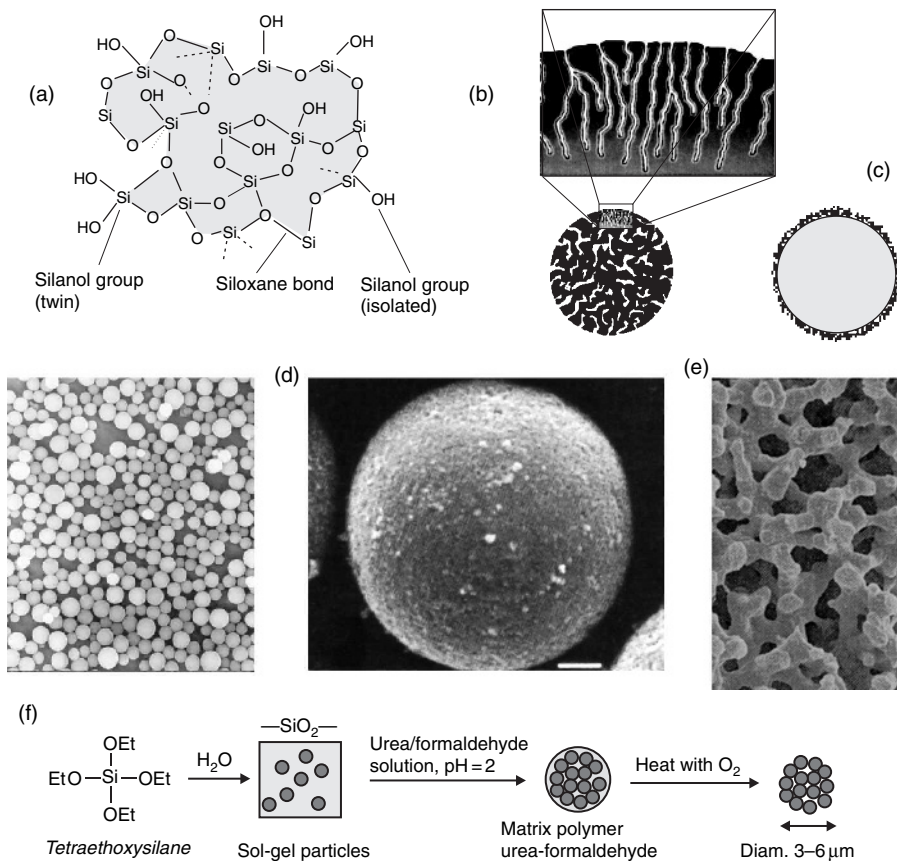


Figure 3.8 *Silica gel for chromatography.* (a) Crude molecular diagram presenting the 3D-structure of a silica gel containing silanol groups. This material must be very pure, traces of metallic elements can increase the acidity of the silanol groups. (b) Porous particle (c) Non-porous particle used for rapid separations. (d) Electron microscopical images of silica gel particles. (e) Monolithic silica gel (reproduced courtesy of Merck). (f) Preparation of spherical grains of silica gel via a sol-gel. The dispersive medium, called sol, is made of spherical particles that are only a few nanometres in diameter. The small spheres agglutinate in the presence of a urea/formaldehyde binding agent until the requisite size ($3\text{--}7\ \mu\text{m}$) is attained. The final treatment is a pyrolysis to eliminate the organic matrix.

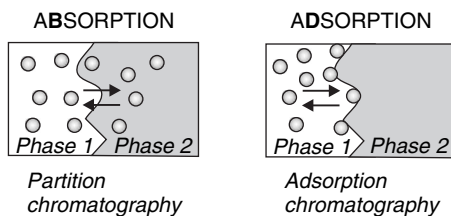
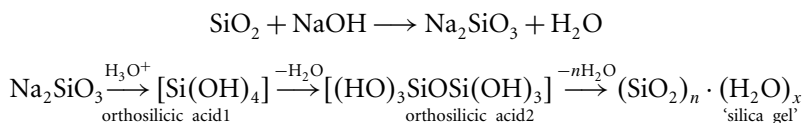


Figure 3.9 The phenomena of adsorption and partition. Contrary to absorption, adsorption is a surface phenomenon. Separation is due to a series of adsorption/desorption steps. Absorption is due to solute partitioning between the two phases.



The mode of action of silica gel is founded upon adsorption (Figure 3.9), a phenomenon that leads to the accumulation of a compound at the interface between the stationary and mobile phases. In simpler cases, a monolayer is formed (known as a Langmuir isotherm), though often some attraction and interaction occurs between the molecules already adsorbed and those still in solution. This contributes to the asymmetry of the elution peaks. Other theories explain the separation phenomenon by a simple slowing down (continuously), at the interface MP/SP which is different for each analyte.

3.6.2 Bonded silica

Though possessing a large capacity for adsorption, silica gel in its simplest state, as described previously, is used less and less for analysis since its qualities change with time, resulting in a lack of reproducibility of separations. For many applications it must be, at least, rehydrated (3–8 per cent water) in order to be deactivated.

To remedy this situation and reduce the often excessive gel's polarity, the silanol groups are exploited in order to provide sites of covalent bonding for organic molecules. Bonded silica gel, modified in this way, behave as a liquid in that the separation mechanism now depends on the *partition coefficient* instead of *adsorption coefficient*. These covalently bonded phases, whose polarity can be easily adjusted, constitute the bases of the *reversed phase polarity partition chromatography* or RP-HPLC, used in the majority of HPLC separations. Two types of syntheses lead to monomeric or polymeric bonded surfaces:

- *Monomeric phases* (10–15 μm thickness): They are obtained by reaction of an alkyl-monochlorosilane in the presence of an alkaline agent with

surface silanol groups (Figure 3.10). The RP-8 (dimethyloctylsilane) or RP-18 (dimethyloctadecylsilane groups, ODS) are prepared in this way. However, a part of the Si-OH groups remain intact and can be the origin of interfering polar interactions. A more complete reaction is obtained with other silyl reactants such as chlorotrimethylsilane (ClSiMe_3) or hexamethyldisilazane ($\text{Me}_3\text{SiNHSiMe}_3$). The sites that remain non-transformed are usually as inaccessible to the reactants as they are to the analytes.

- *Polymeric phases* (25 μm or greater thickness): Here a di- or trichlorosilane is used in the presence of water vapour which provokes a polymerization of the reactant in solution prior to deposit and bonding with the silica. A reticulated polymer layer is obtained. At the molecular scale the final framework of the coating is difficult to imagine: it is mono- or multilayer.

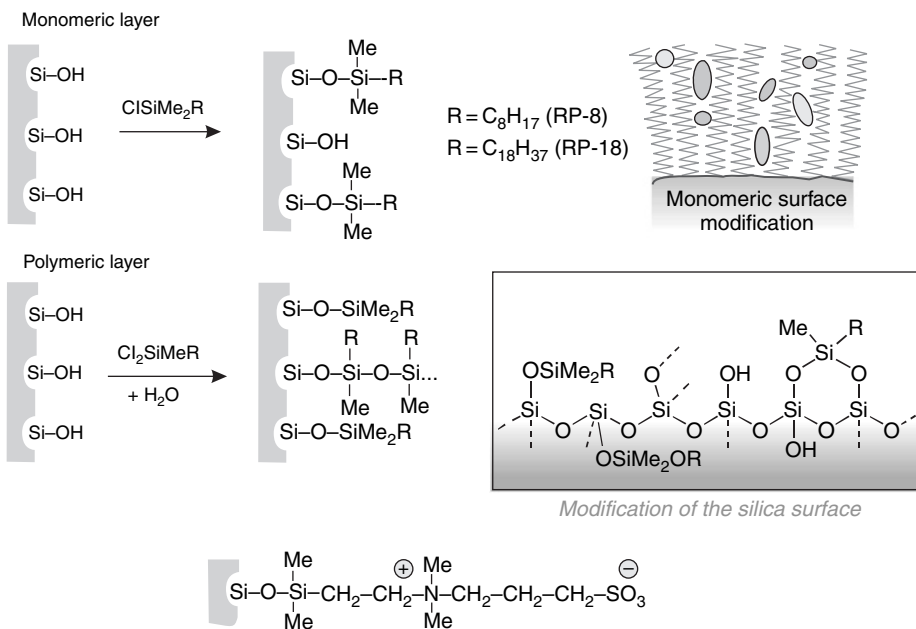


Figure 3.10 Formation of bonded organosilanes at the interface of silica gel. Diagram of organic monomers and polymers at the surface of silica gel. Close-up of a ‘long-haired’ particle with alkyl ligands. When hydrocarbons are bonded onto the silica, they orient themselves at the interface in a particular manner according to their lipophilic and hydrophilic character against the mobile phase. The arrangement Si-O-Si-C is more stable than Si-O-C. For polymeric coatings, a carbon content exceeding 15 per cent can be attained on bonding to accessible silanol groups. Below, an example of a phase containing a zwitterion. Other reactions are also used (hydroxylation in particular).

3.6.3 Other stationary phases of varying polarity

The silica gels discussed previously contain bonded alkyl chains from 8 to 18 carbon atoms. They are versatile and therefore very useful. Yet, for improving the separation of certain classes of compound the tendency is now to use one of the growing number of specific stationary phases.

To the silica are bound linear chains bearing aminopropyl, cyanopropyl, benzyl groups or dipolar ligands (zwitterions) which confer an intermediate polarity to the stationary phase (Figure 3.10). An improvement is noted in the separation of very small polar molecules which require mobile phases rich in water (cf. paragraph 3.8). For example, sugars, peptides and other hydrophilic compounds become separable under these conditions (see, e.g. Figure 3.14). For these modified stationary phases, silica gel acts as a support.

Aluminium oxide (Al_2O_3) or zirconium oxide (ZrO_2) are also used as supports of reticulated deposits based upon polymers of butadiene or styrene-divinylbenzene or hydroxymethylstyrene. Porous graphite, in the form of spheres whose surface is 100 per cent carbon and therefore completely hydrophobic, has been used in applications with compounds possessing atoms carrying lone pairs of electrons thus having high retention factors.

These stationary phases show a greater stability in both acidic and basic media, allowing certain columns to be rinsed with sodium hydroxide 1 M, that Si–O–C bonds would not normally resist.

3.7 Chiral chromatography

A molecular organic compound whose structural formula reveals the presence of an asymmetric centre leads generally to a mixture of two possible enantiomers R and S in variable quantities. If such a mixture is chromatographed on a column packed with a chiral stationary phase (a phase that contains active sites corresponding to only one enantiomer, R for example), two peaks will be observed on the chromatogram (Figure 3.11). These peaks result from the reversible interactions R(compound)/R(stationary phase) and S(compound)/R(stationary phase) of which the stabilities are slightly different. The areas under the peaks are proportional to the abundance of each of the forms, R and S.

Among the general types of chiral stationary phase in current use in LC, are silica matrix bonded to cyclodextrins *via* a small hydrocarbon chain linker (Figure 3.11). These cylindrically shaped ligands, which are oligosaccharides made of five to seven molecules of glucose, possess a hydrophobic internal cavity while the external part is hydrophilic. This gives them a selective permeability to a broad variety of compounds leading ultimately to the formation of reversible diastereoisomer complexes at the surface.

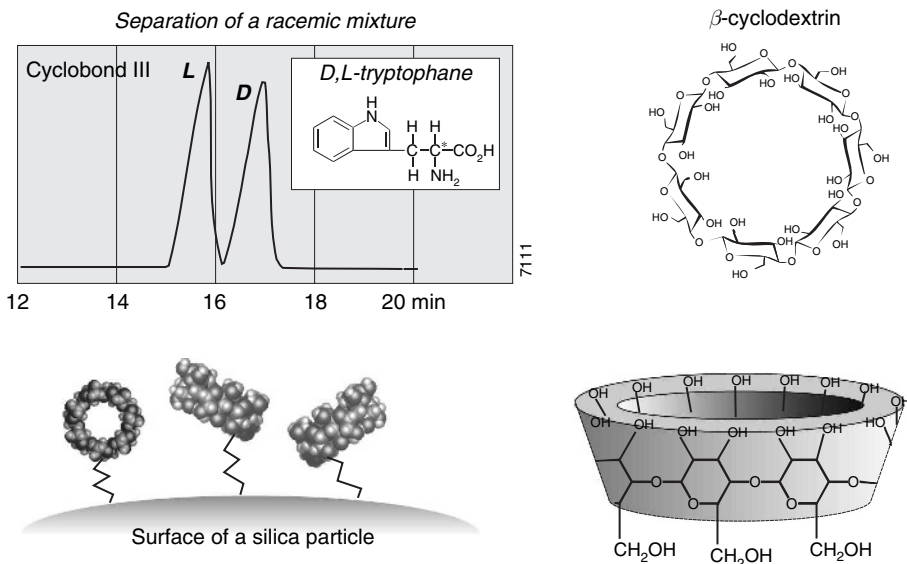


Figure 3.11 Separation on a cyclodextrin-bound stationary phase. Above left, the chromatogram of a racemic mixture of a compound (adopted from an Alltech document). Above right, structural formula of a β -cyclodextrin. These non-reducing cyclic oligosaccharides take the form of a toroid (diameter, 1.5 nm; hydrophobic cavity, 0.8 nm; height, 0.8 nm). The inside of the toroid is hydrophobic as a result of the electron-rich environment provided in large part by the glycosidic oxygen atoms. Below, partial representation of a cyclodextrin bonded through a linker to a silica matrix. These stationary phases are prepared using similar techniques to those for making reverse phases.

The *optical purity* of an analyte defined in terms of its *enantiomeric excess* (*e.e.*), is calculated from the following expression where A_R and A_S represent the areas under the two peaks corresponding to the two enantiomers.

$$\text{optical purity (e.e.\%)} = 100 \frac{|A_R - A_S|}{A_R + A_S}$$

3.8 Mobile phases

The degree of interaction between the mobile phase and the stationary phase whether *normal* or *reversed*, affects the retention time of the analytes. In principle, the polarity of the stationary phase can lead to the following situations:

- If the stationary phase is polar, then the technique is said to be *normal phase chromatography* and a less polar mobile phase is used.
- If the stationary phase is non-polar, or only weakly polar then the technique is called *reversed phase chromatography* (RP-HPLC) – or chromatography on

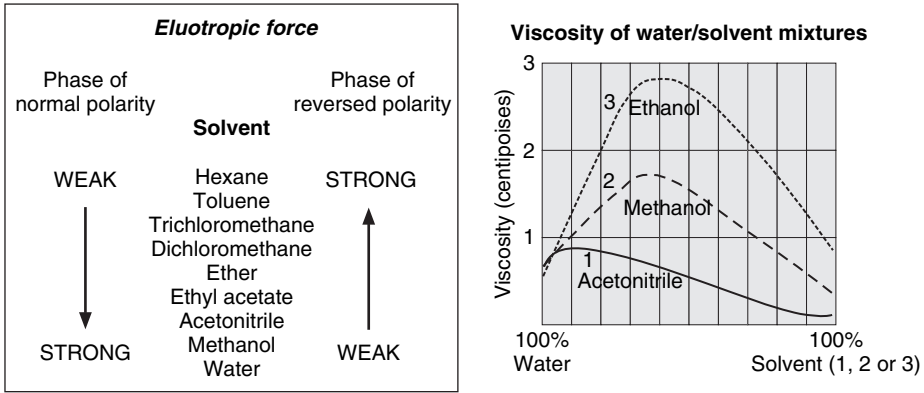


Figure 3.12 *Elutropic force and viscosity of solvents used as mobile phases.* By mixing several solvents the elutropic strength of the mobile phase can be adjusted. Noting that the viscosity and by consequence the pressure at the head of the column varies according to the composition of the mobile phase.

a hydrophobic phase. A polar mobile phase is selected (most commonly water with a modifying solvent such as methanol or acetonitrile). By changing the composition of the mobile phase and therefore its polarity, this affects the distribution coefficients K (C_S/C_M) therefore the retention factors k of the analytes (Figure 3.12). The main difficulty for the chromatographer is, as a function of the compounds to be separated, to make the best choice of mobile phase (Figure 3.13).

Bonded silica phases lead in general to a large loss in polarity. With a phase of this type, of which the surface functionality resembles a paraffin layer the order of elution will be the opposite of that encountered with normal phases. With a polar eluent, a polar compound migrates faster than an apolar compound. Under these conditions hydrocarbons are strongly retained. However, the polar compounds themselves are fairly difficult to separate. To solve this an elution gradient must be used by reducing progressively the water concentration (polar) thus benefitting the modifying solvent chosen (less polar). For example, the elution begins with an 80/20 per cent mixture water/acetonitrile and ends with the composition, 40/60 per cent for water/acetonitrile. This is the domain of hydrophilic interaction chromatography (HIC).

Four different types of interaction are possible between solvent molecules and the analyte:

- *Dipolar* interactions when analyte and solvent both possess dipole moments
- *Dispersion* due to the attraction between molecules in proximity
- *Dielectrics*, which favour the solubility of ionic species in polar solvents

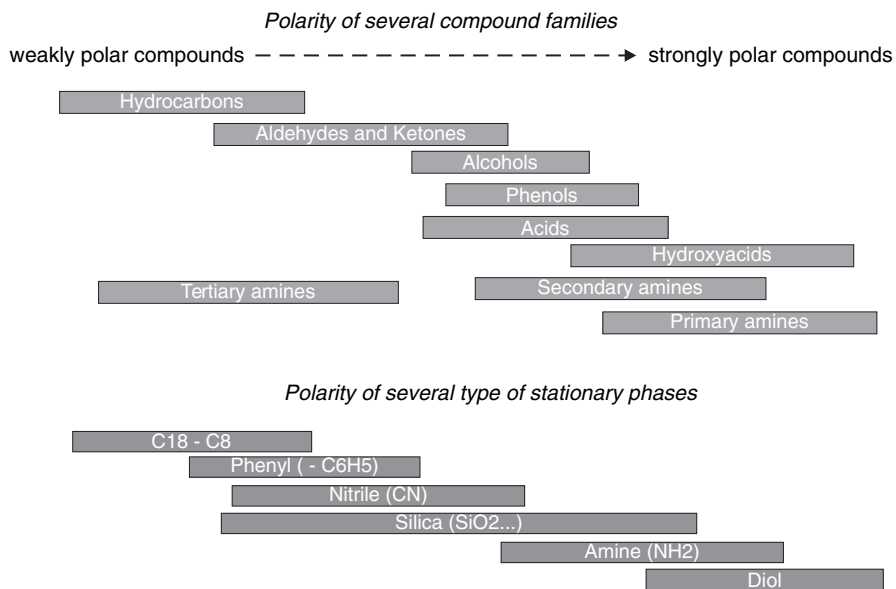


Figure 3.13 Relative polarities of several organic compound families along with some principal types of stationary phase with ligands in current use.

- *Hydrogen bonding* between solvent and analyte when one corresponds to that of a proton donor and the other a proton acceptor.

The separation of very polar compounds on stationary phases of type RP-18 requires the use of a mobile phase rich in water. Under these conditions the stationary phase, which is hydrophobic becomes suddenly waterproof and the capacity for separation weakens. For this reason phases presenting a residual polarity are preferred in order to maintain the interaction between the analytes and the water of the mobile phase (Figure 3.14).

3.9 Paired-ion chromatography

Paired-ion chromatography (PIC) is a technique related to RP-HPLC that affords an improvement in the separation of some highly polar compounds such as amino acids and organic acids that do not adhere at all to the stationary phase if it is apolar (Figure 3.15). Thus this polar character must be reduced to increase retention. To modify the ionic charge, the pH of the mobile phase can be changed but if this is not sufficient then a strongly ionic reagent, called a *ion pairing reagent*, is added to the mobile phase. This is usually a compound with a carbon chain (weakly polar) possessing a functionality whose charge is opposite to the analyte to be separated (e.g. heptane sulfonic acid if the ionic solute is a base). This forms

a 'neutralized' ion pair, a less polar species, fairly stable and lipophilic, which will initially stick to non polar stationary phase but can still be eluted, apparently by a type of ion-exchange mechanism. This principle permits the separation of inorganic cations or anions upon an RP-phase.

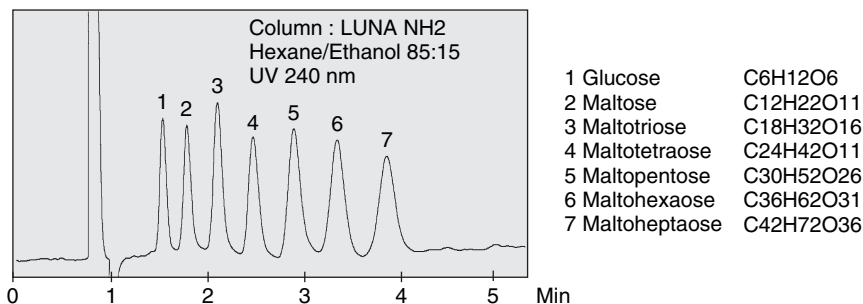


Figure 3.14 The separation of sugars upon an 'amino' phase. The order of elution of 7 homologous sugars indicates that the stationary phase is polar and that the elutropic force of the mobile phase diminishes as the molecular mass increases.

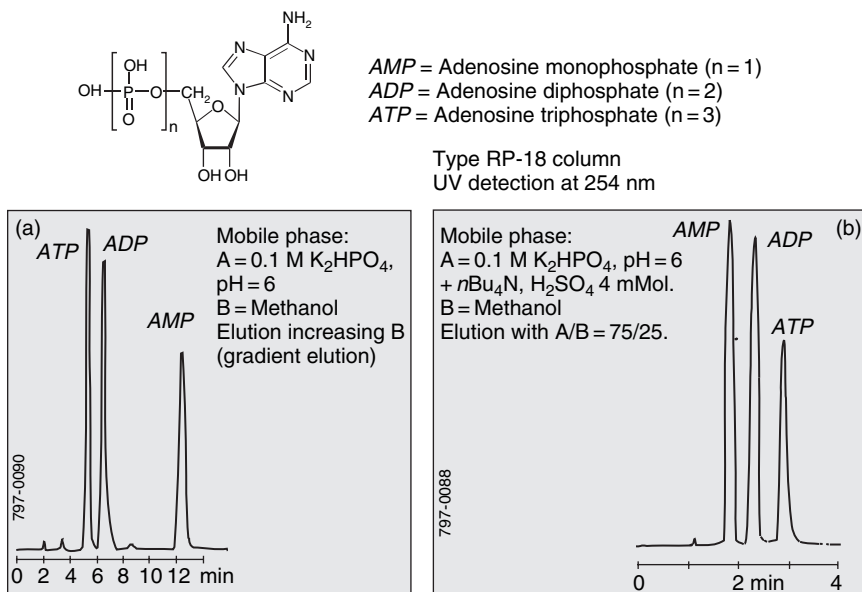


Figure 3.15 The effect upon separation of ion pairing on a column of reversed polarity. (a) The chromatogram of a mixture of three nucleotides displaying the 'normal' situation of an elution in order of reducing polarity; (b) Alternately, in the reverse phase situation: adenosine triphosphate associated with the ion tetrabutylammonium has become more lipophilic and is therefore retained longer (reproduced courtesy of Alltech).

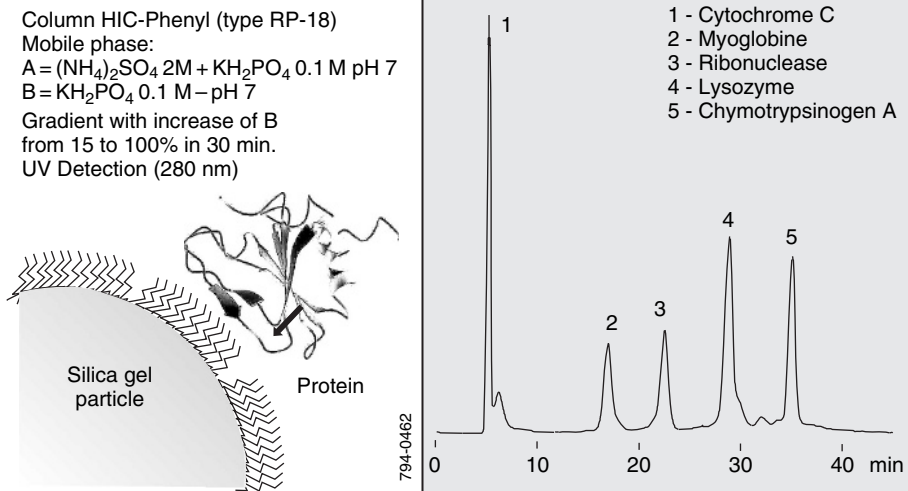


Figure 3.16 Separation improvement due to the principle of hydrophobic interaction. The chromatogram of a mixture of proteins of decreasing hydrophilicity obtained by gradually reducing the saline concentration (reproduced courtesy of Supelco).

3.10 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC), is principally employed for the separation of bio-organic compounds such as water soluble proteins. This separation takes advantage of the hydrophobic differences among the compounds present in the sample. Using an apolar stationary phase the elution is begun under conditions of a high salt concentration (e.g. 2M ammonium sulfate and 0.1 m monosodium phosphate, at pH 7). Under these conditions the proteins attach themselves by their hydrophobic domains to the stationary phase. Next, a reverse salt gradient is started to gradually reduce the saline concentration in order that the proteins return to the mobile phase (Figure 3.16). They are eluted in decreasing order of their hydrophilicity.

■ This operation is similar to a technique employed in organic chemistry: in a separating funnel, in order to extract a compound diluted in an aqueous medium with ether, brine is introduced to reduce the solubility of the compound in water and thereby to facilitate its extraction.

3.11 Principal detectors

The object of chromatography is rarely to determine the global composition of a sample, but rather to measure the concentration of a compound that is present, for which a particularly well-adapted detector must be chosen. The universal

detector is not, therefore, totally indispensable for quantitative analysis. On the contrary it can even be a handicap leading to crowded chromatograms which are indecipherable.

More than in GC, the relative areas of the peaks of a chromatogram often do not have anything to do with the molar or mass composition of the mixture analysed. However, the detector, irrespective of its nature, is required to unite a number of fundamental properties. It should give, for each compound of interest, a response that is proportional to the instantaneous mass flow (indicated by its linear dynamic range), be sensitive, have a small inertia, filter most background noise and be stable over time.

The most widely used detection methods are based upon the optical properties of the analytes: absorption, fluorescence and refractive index (see also ELSD detector, Section 7.4).

3.11.1 Spectrophotometric detectors

Detection is based upon the Lambert–Beer Law ($A = \epsilon_{\lambda} l C$): The absorbance A of the mobile phase is measured at the outlet of the column, at one or several wavelengths λ in the UV or visible spectrum (cf. Chapter 9). The intensity of the absorption depends upon the molar absorption coefficient ϵ_{λ} of the species detected. It is essential that the mobile phase be transparent or possess only a very little absorption (Figure 3.17).

The area of a peak, without taking into account this specific parameter, renders the direct calculation of concentration unfeasible by a simple check of the chromatogram. Spectrophotometric detectors are examples of selective detection. For compounds that do not possess a significant absorption spectrum it is possible to perform derivatization of the analytes prior to detection.

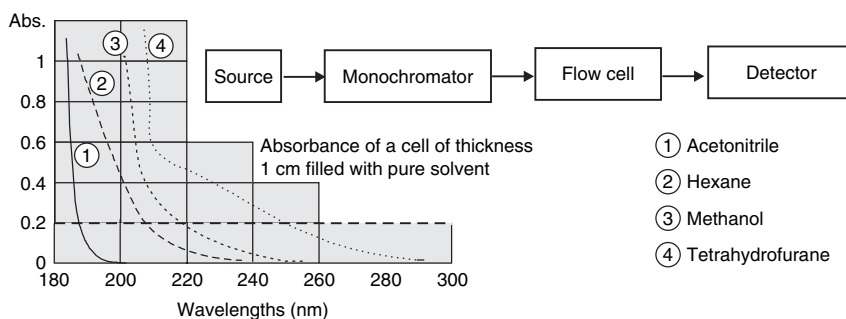


Figure 3.17 *Photometric detection at a single wavelength.* Principle of a photometric detector along with the absorption spectra of several solvents used in liquid chromatography. Here the transparency limit of a solvent corresponds to an absorbance of 0.2 for 1 cm of optical path in the cell.

- *Monochromatic detection.* The basic model comprises a deuterium or mercury vapour light source, a monochromator for isolating a narrow bandwidth (10 nm) or a characteristic spectral line (e.g. 254 nm if the source is a mercury lamp), a flow cell with a volume of a few μL (optical path of 0.1 to 1 cm) and a means of optical detection.
- *Polychromatic detection.* More advanced detectors are able either to change wavelength during the course of an analysis, to record the absorbance at several wavelengths quasi-simultaneously, or even to capture in a fraction of a second, a whole range of wavelengths without interrupting the circulation in the column (Figures 3.18 to 3.20). The diode array detector (DAD) leads not only to a chromatogram but also provides spectral information which can be used to identify the separated compounds (Figure 3.19). This is called *specific detection* (cf. Chapter 9).

The successive spectra of the compounds eluted with the mobile phase are recorded continuously and stored in the memory of the instrument, to be treated later using appropriate software. Often spectacular chromatograms can be obtained (Figure 3.20). The ability to record thousands of spectra during a single analysis increases the potential of these detectors systems. A topographic representation of the separation can be conducted, $A = f(\lambda t)$ (iso-absorption diagrams).

■ The rapid development of biotechnologies, as in biochemistry, requires the analysis of amino acids (proteins hydrolysates); photometric detectors can be used with the condition that prior to passage in the measuring cell a post-column reaction with ninhydrin is carried out (cf. Chapter 8).

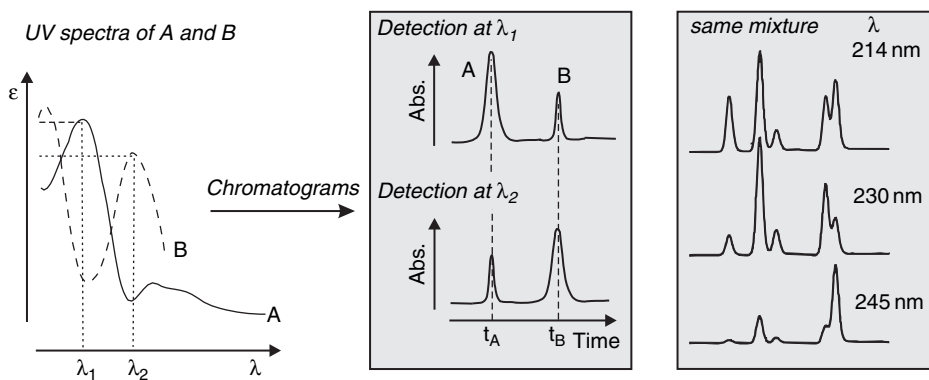


Figure 3.18 Chromatograms of a sample containing two compounds A and B, for which the UV spectra are different. According to the choice of detection wavelength, the chromatogram will not have the same aspect. On the right the chromatograms represent a mixture of several pesticides recorded at three different wavelengths which illustrates this phenomenon. In quantitative analysis therefore the response factors of each of the compounds must be determined prior to the analysis (cf. Quantitative analysis, Chapter 4).

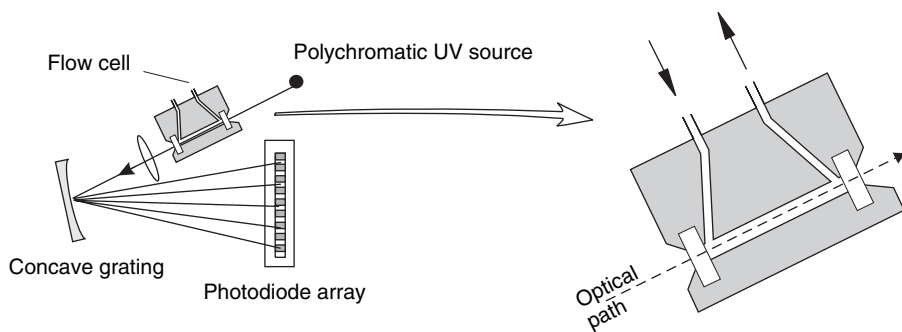


Figure 3.19 *Optical schematic of the diode array detector.* The flow cell is irradiated with a polychromatic UV/Vis light source. The light transmitted by the sample is dispersed by a concave grating towards a detector which comprises a diode array. The number of diodes can attain several hundred, each one monitoring the mean absorption of a very narrow interval of wavelengths (e.g. 1 nm).

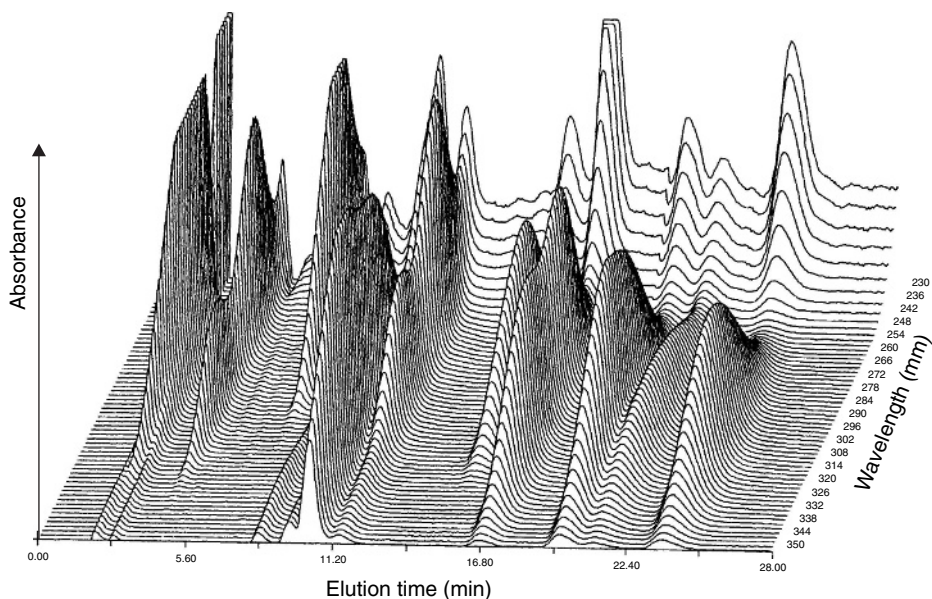


Figure 3.20 *Three-dimensional representation, $I = f(\lambda, t)$, of a chromatographic separation obtained by a rapid recording method (reproduced courtesy of TSP instruments).*

3.11.2 Fluorescence detector

About 10 per cent of organic compounds are fluorescent, in that they have the ability to re-emit part of the light absorbed from the excitation source

(cf. Chapter 11). The intensity of this fluorescence is proportional to the concentration of the analyte, as long as this concentration is kept low. Application to LC chromatography gave rise to fluorescence detectors, very sensitive and for this reason often used for trace analysis. Unfortunately, the response is only linear over a relatively limited concentration range of two orders of magnitude. The domain of this selective detector (Figure 3.21), can be extended by the application of a derivatization procedure pre- or post-column to make the

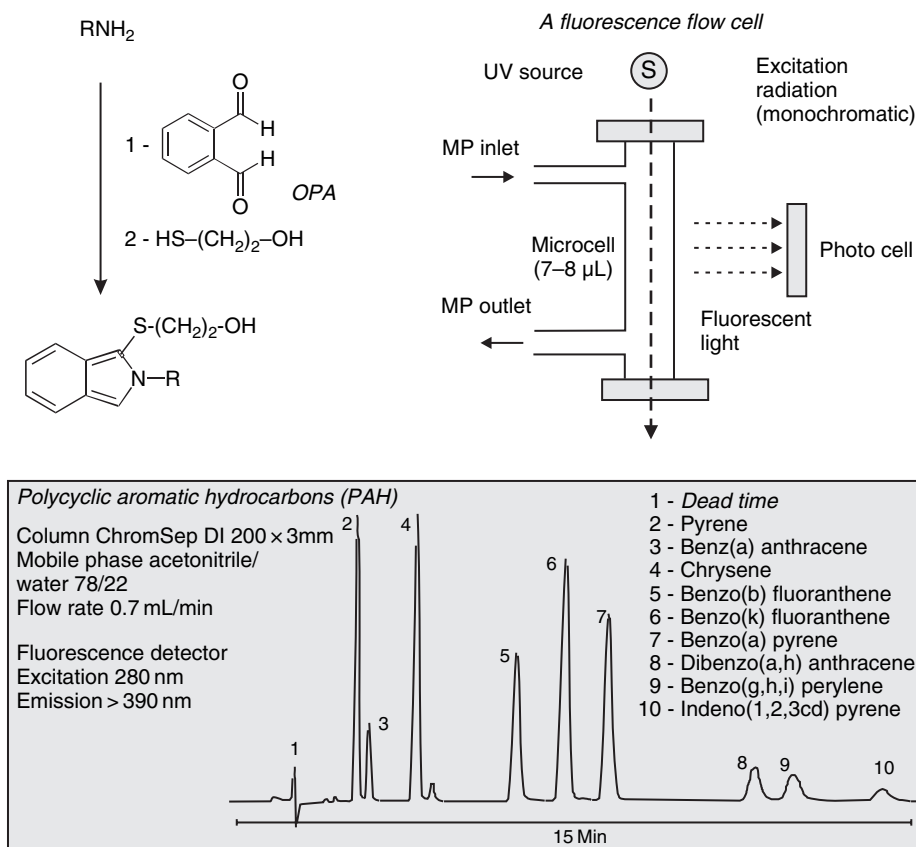


Figure 3.21 Flow-cell of a fluorometric detector. Above left, examples of reagents used to render fluorescent compounds containing primary amines through the action o- phthalic acid (OPA) in the presence of monothio glycol. Above right, a flow cell made of Pyrex glass is used as the sensor through which the excitation light (as UV at 254 nm produced by the mercury lamp) passes axially. A photocell is situated at the side of the cell to receive radially emitted light (perpendicular to the direction of the primary light). Below, chromatogram of a mixture of several polycyclic aromatic hydrocarbons (PAH). The intensity of the fluorescence varies from one compound to another because there are variations in fluorescent quantum yields. To remedy this, the excitation wavelength should be adjusted for each compound. This is done with programmable detectors, able to select the optimal technical conditions for each species.

substances of interest detectable. There are a number of reagents that have been developed specifically to synthesize fluorescent derivatives. For this methodology an automatic reagent dispenser is placed either prior to the column or between the column and the detector to effect one, or several reactions, upon the sample analytes in order to render them fluorescent before or after injection.

■ By following this principle, traces of carbamates (pesticides) in the environment can be measured by saponification with sodium hydroxide and then reaction with *o*-phthalic aldehyde to transform the methylamine into a fluorescing derivative (Figure 3.21).

3.11.3 Refractive index detector (RI detector)

This type of detector relies on the Fresnel principle of light transmission through a transparent medium of refractive index n . It is designed to measure continuously the difference in the refractive index between the mobile phase ahead of and following the column. So a differential refractometer is used. Schematically, a beam of light travels through a cell that has two compartments: one is filled with the pure mobile phase while the other is filled with the mobile phase eluting the column (Figure 3.22). In practice, the optical dispersions of the media are likely to differ, and consequently the refractive index will only match at one particular wavelength. As a result the fully transmitted light will be largely monochromatic. The change in refractive index between the two liquids, which appears when a compound is eluting the column, is visualized as an angular displacement of the refracted beam. In practice, the signal corresponds to a continuous measurement of the retroaction that must be provided to the optical element in order to compensate the deviation of the refracted beam.

The refractive index detector is one of the least sensitive LC detectors. It is very affected by changes in ambient temperature, in pressure and in flow-rate. The temperature of the detector must be regulated with precision (to 0.001°C) and the column thermostatically controlled. This detector leads to both positive and negative peaks, which requires that the baseline is fixed at the mid-height of the graph (Figure 3.22). Also, this detector can only be used in the isocratic mode because in gradient elution, the composition of the mobile phase evolves with time, as does its refractive index. The compensation, easily obtained in the case of a mobile phase of constant composition, is no longer attainable when the composition of the eluent at the outlet of the column differs from that at the inlet. Consequently, it is often arranged in series with other detectors, in the isocratic mode, to give a supplementary chromatogram.

Despite these disadvantages, this detector, considered to be almost universal, is extremely useful for detecting compounds that are non-ionic, do not adsorb in the UV, and do not fluoresce. It finds use in the recognition of those substances (fatty acids, alcohols, sugars, etc.) that are not easily detected by other means.

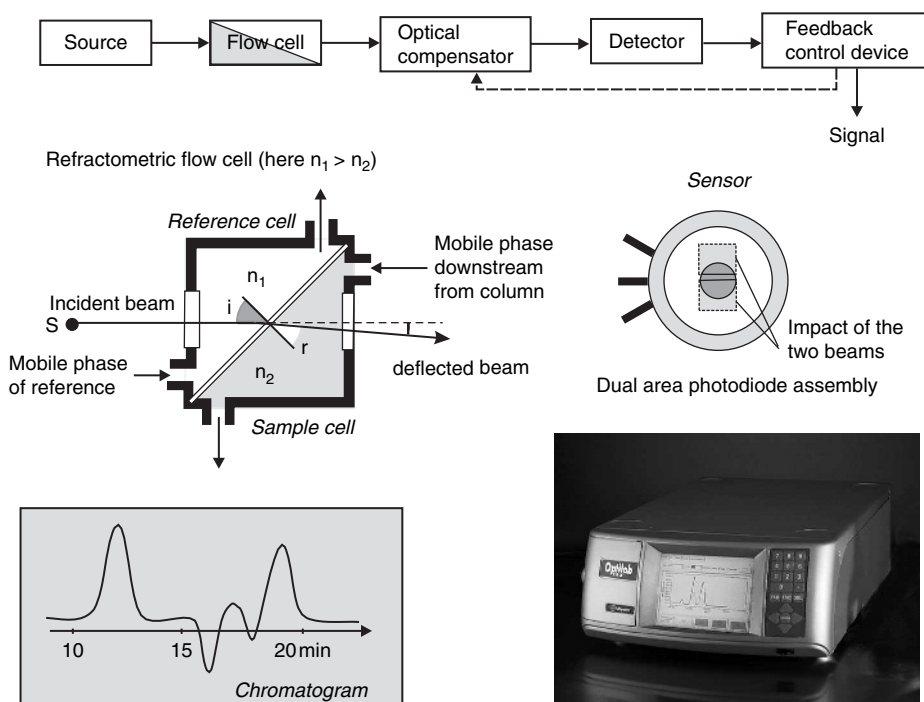


Figure 3.22 Schematics of a differential refractive index detector. The measurement of refractive index in liquid chromatography detection systems can be done in a number of ways. On this schematic, as sample elutes through one side, the changing angle of refraction moves the beam. This results in a change in the photon current falling on the dual stage photodiode which unbalances it. The responses of the two areas are maintained equal by the optical assembly (not displayed). A chromatogram of a mixture of sugars obtained with this type of detector. A commercial instrument, the model Optilab rEX (reproduced courtesy of Wyatt Technology).

3.11.4 Other detectors

As has already been explained for GC, the identification of a compound from its retention time alone can be uncertain. A more useful detector installed at the outlet of the column would be able to give complementary information about the compound being eluted.

When the detector itself is providing a second analytical method of analysis, qualifying it as bi-dimensional, identification becomes more certain. These can be a spectrophotometer used simultaneously as a classic detector (obtaining the chromatogram) and as an identification tool (obtaining the spectrum) for the separated species (cf. section 16.5). Coupling an LC instrument with a mass

spectrometer (tandem HPLC-MS) is equally very useful for identifying mixtures of known substances when a database of compounds under investigation is available. Coupling with a proton-NMR spectrometer (tandem HPLC-NMR ^1H), now possible owing to the increasing sensitivities of modern instruments, facilitates the study of mixtures comprising unknown compounds (cf. Chapters 15 and 16).

3.12 Evolution and applications of HPLC

The success of the tandem HPLC-MS analytical method has enabled liquid chromatography to progress toward miniaturization (Figure 3.23). In fact the principal problem for a mass spectrometer installed at the outlet of a liquid chromatograph is the elimination of the mobile phase. To deal with this, either *capillary liquid chromatography*, or *nano-chromatography*, two improvements of the technique, simplify the interfacing from one apparatus to the other, and allows to solve this problem.

The suitability of micro-columns to HPLC has been revealed by the improved performance of separation. Yet the passage of the technique into the kingdom of Lilliput was not carried out without difficulty. The micro-environment requires understanding, from the micro-flow rates of the mobile phase to the smallest possible void volumes, from the mixing chamber for gradients to the volume of the detection cell. The micro-leaks from conventional pumps are roughly of the order of the flow rates attained in miniature. A customized chromatograph must be used or a conventional system must be adapted because the reproducibility of flow rate gradients is difficult at this scale. A split containing a second packed column or a long capillary is generally used, which acts as a restrictor.

Apart from the reduction in the scale of the quantities separated, two other areas of progression should be noted:

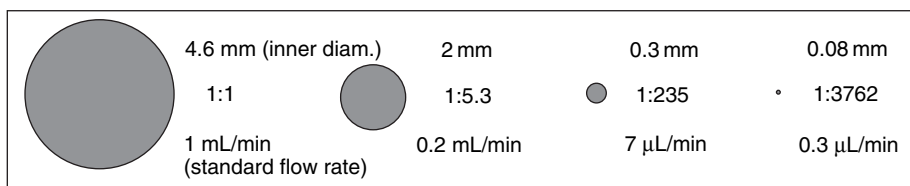


Figure 3.23 Comparison of flow rates in columns of different internal diameters. If the packing in these four columns is of a similar nature, there are advantages by passing from the classic HPLC (left) to the capillary or to the nano-HPLC (right). The narrower the column the greater the decrease in mobile phase consumption.

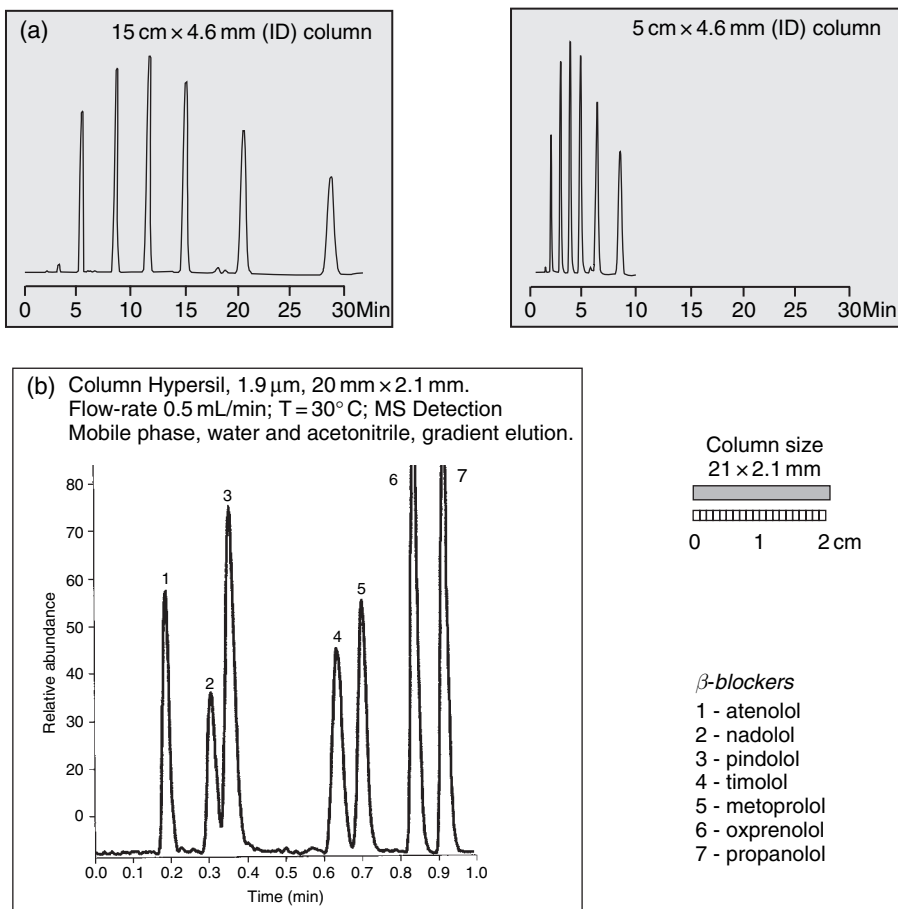


Figure 3.24 *Fast chromatography.* (A) Comparison of two chromatograms obtained from the same compound mixture. The shorter column packed with smaller particles enables to save an appreciable lot of time for comparable performances. (B) Separation of seven β -blockers using a short column and small particles. The seven compounds are separated in under one minute (reproduced courtesy of Thermo Electron Corporation).

1. The use of stationary phases adapted to particular separations: sugars, PAH, amines, nucleotides...
2. Studies to reduce the time of analysis while maintaining the quality of resolution. To achieve this, short columns are used with stationary phases formed either from non-porous particles of small diameter ($3\mu\text{m}$ or less), or of porous networks of silica gel (monolithic column). This allows faster flow rates, then more rapid separations (Figure 3.24). This technique is most frequently used in chemical industries, and areas as food processing, environment, pharmacy and biochemistry.

Problems

3.1 Current HPLC apparatus can use columns of internal diameter (written as ID in the text), of $300\ \mu\text{m}$, and for which the optimum flow rate advised is $4\ \mu\text{L}/\text{min}$.

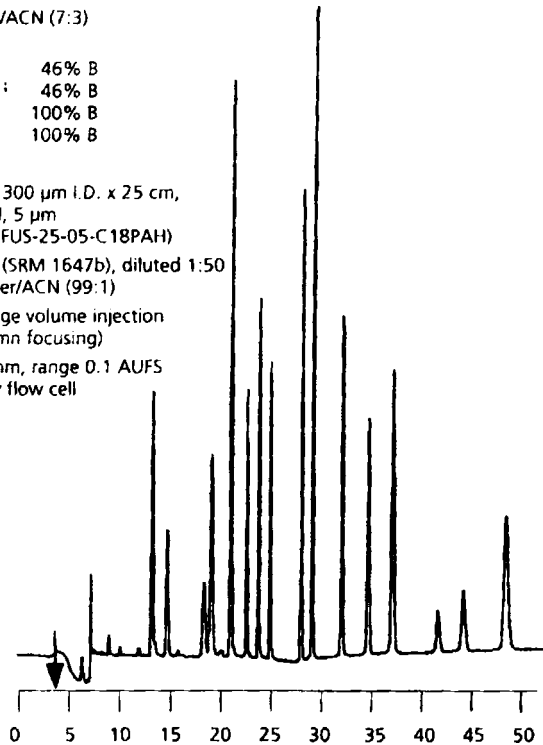
1. Show by simple calculation that this flow rate will conduct the mobile phase at practically the same linear speed as in a column of the same type but possessing a standard diameter of $4.6\ \text{mm}$ for which the advised flow rate is $1\ \text{mL}/\text{min}$.

The chromatogram below corresponds to an example of separation obtained with a narrow column of $300\ \mu\text{m} \times 25\ \text{cm}$.

2. What is the dead volume of this column? (The arrow marked on the chromatogram indicates the hold-up time.)

Separation of PAHs (SRM 1647)

pump	conventional HPLC pump with Acurate
mobile phase	A) water/ACN (7:3) B) ACN
gradient	0 min 46% B 12 min 46% B 20 min 100% B 50 min 100% B
flow rate	$4\ \mu\text{L}/\text{min}$
column	Fusica II, $300\ \mu\text{m}$ I.D. \times 25 cm, C18 PAH, $5\ \mu\text{m}$ (cat. no. FUS-25-05-C18PAH)
sample	16 PAHs (SRM 1647b), diluted 1:50 with water/ACN (99:1)
injection	$10\ \mu\text{l}$ large volume injection (on-column focusing)
detection	UV 254 nm, range 0.1 AUFS U-Z View flow cell



3. What is the retention volume, expressed in ml, of the compound which has the greatest affinity for the stationary phase?
4. Calculate the efficiency of the column for this compound.
5. Calculate the retention factor of this compound on the column.
6. What is the likely nature of the stationary phase? State precisely the superficial structure of such a phase. (On the figure ACN represents acetonitrile.)

Two columns selected for an experiment are filled with the same stationary phase. One column has an internal diameter of 4.6 mm, the other 300 μm , while both have the same length and an equal rate of filling (V_S/V_M). It is decided to use them in succession with the same chromatograph and under identical conditions with the flow rates as advised above. An equal amount of the same compound is injected into each of them in turn.

7. When passing from one column to the other, would a difference in the retention volumes (or elution volume) of the compound be expected?
 8. If the sensitivity of the detector has not been adjusted between the two experiments, will the intensity of the corresponding elution peaks be different?
- 3.2 What is the order of elution of the following acids from an HPLC column whose stationary phase is of type C18 while the mobile phase is a formate buffer $C = 200 \text{ mM}$ of pH 9.

Mixture:

- | | |
|-------------------|--|
| 1. Linoleic acid | $\text{CH}_3(\text{CH}_2)_4\text{CH} = \text{CHCH}_2\text{CH} = \text{CH}(\text{CH})_7\text{CO}_2\text{H}$ |
| 2. Arachidic acid | $\text{CH}_3(\text{CH}_2)_{18}\text{CO}_2\text{H}$ |
| 3. Oleic acid | $\text{CH}_3(\text{CH}_2)_7\text{CH} = \text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$ |

- 3.3 Indicate for each of the chromatographic techniques below the term which best expresses the analyte interaction with the stationary phase

1. Reverse phase
 2. Gel permeation
 3. Ionic chromatography
 4. Normal phase
- a. Molecular mass
 - b. Hydrophilicity
 - c. Hydrophobicity
 - d. Protonation/ionisation

- 3.4 Consider a study, by HPLC, of the separation of three nucleotides (AMP, ADP and ATP), with a column of type RP-18. The mobile phase is a binary mixture of $\text{H}_2\text{O} - \text{KH}_2\text{PO}_4$, 0.1 M (pH 6)/methanol (90/10). The compounds appear in the order ATP, ADP, AMP. If a solution of 4 mM tetrabutylammonium hydrogen sulphate is added to the mobile phase, the order of elution of these compounds is reversed (see Figure 3.15).

Explain the reasons for this phenomenon.

- 3.5 The separation of two compounds A and B is studied by HPLC on a column of type RP-18. The mobile phase is a binary mixture of water and acetonitrile. A linear relationship exists between the logarithm of the retention factor and the % of acetonitrile within the binary mixture ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$).

Two chromatograms were obtained, one in which the mixture of water and acetonitrile was 70/30 v/v, respectively and the other where the same two components were mixed 30/70 v/v. The graph below was constructed from the results. The equations of the straight lines are,

for compound A:

$$\log k_A = -6.075 \times 10^{-3}(\% \text{CH}_3\text{CN}) + 1.3283$$

and for compound B:

$$\log k_B = -0.0107(\% \text{CH}_3\text{CN}) + 1.5235$$

1. Find the composition of the binary phase which leads to the selectivity factor = 1.
2. Suppose that for each compound the width at half height of the corresponding peak in the chromatogram is the same and the efficiency of the column is not modified following the composition of the mobile phase. For which combination of the binary mixtures mentioned above is the resolution between the two peaks the best? Discuss the practicalities of your choice.

4

Ion chromatography

Ion chromatography (IC) is a separation technique which shares numerous common features with HPLC, yet possesses sufficient novel aspects such as its principle of separation or modes of detection, to make it the object of a separate study. IC is adapted to the separation of ions and polar compounds. The mobile phase is composed of an aqueous ionic medium and the stationary phase is an ion-exchange resin. Besides the detection methods based on absorbance or fluorescence, ion chromatography also uses electrochemical methods based on the ionic nature of the species to be separated. Its greatest utility is for analysis of anions for which there are no other rapid analytical methods. Yet current applications of IC are far broader than the analysis of simple ions by which the technique first gained renown. The operating domain, comparable with that of capillary electrophoresis, concerns the separation of many kinds of inorganic or organic species such as amino acids, carbohydrates, nucleotides, proteins and peptides in complex matrices.

This chapter will also review the main methods of quantitative analysis from chromatographic data.

4.1 Basics of ion chromatography

This chromatographic technique is concerned with the separation of ions and polar compounds. Stationary phases contain ionic sites that create dipolar interactions with the analytes present in the sample. If a compound has a high charge density, it will be retained a longer time by the stationary phase. This exchange process is much slower when compared with those found in other types of chromatography. This mechanism may be associated, for molecular compounds, with those already dealt with by HPLC when equipped with RP-columns.

For HPLC, some columns contain ion exchange packings but they are used in significantly different ways. They are not considered to be IC columns. They require concentrated buffers that cannot be suppressed and so are not compatible with conductivity detection. Applications with these columns use more traditional HPLC detection methods (such as UV or fluorescence).

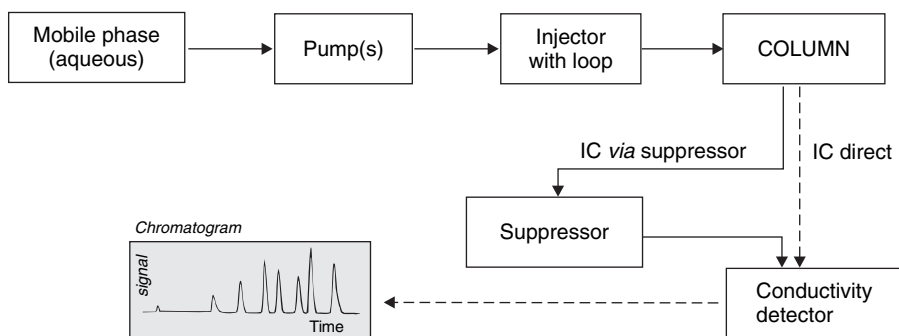


Figure 4.1 Schematic of an ion chromatograph instrument. The classic modular building design of liquid chromatography is seen here again, yet with the difference that the separation is generally performed isocratically. The configuration shows a ‘suppressor’ device installed after the column and in series with a conductivity detector. The suppressor serves to eliminate the ions arising from the eluent to improve sensitivity.

Ion chromatography instruments have the same modules as those found in HPLC (Figure 4.1). They can exist as individual components or as in an integrated model. The pieces into contact with the mobile phase should be made of inert materials capable of withstanding the corrosiveness of acid or alkaline entities, which serve as eluents. The detection of ionic species present in the sample is difficult because these analytes are in low concentrations in a mobile phase that contains high quantities of ions.

The separation of compounds within the sample is founded upon the occurrence of ion exchange, for which two classic examples are given below:

- if cationic species (type M^+) are to be separated, a *cationic* column with a stationary phase capable of exchanging cations will be employed. Such a phase is constituted, for example, of a polymer containing sulfonate ($-\text{SO}_3^-$) groups. Consequently the stationary phase is the equivalent of a polyanion.
- alternately, if anionic species (type A^-) are to be separated, an *anionic* column is selected capable of exchanging anions. This is achieved, for example, by employing a polymer containing quaternary ammonium groups.

To understand the mechanism of a separation, take for example an anionic column containing quaternary ammonium groups, in equilibrium with a mobile phase composed of a solution of hydrogenated carbonate anions (e.g. sodium counter ions). All of the cationic sites of the stationary phase find themselves paired with anions of the mobile phase (Figure 4.2).

When an anion A^- within the sample is taken up by the mobile phase, a series of reversible equilibria are produced which are directed by an exchange equation giving the ion’s distribution between the mobile phase (MP) and the stationary phase (SP).

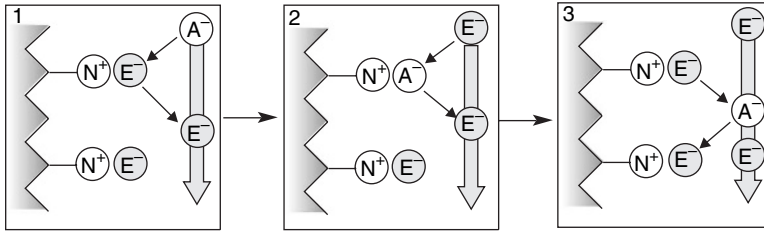
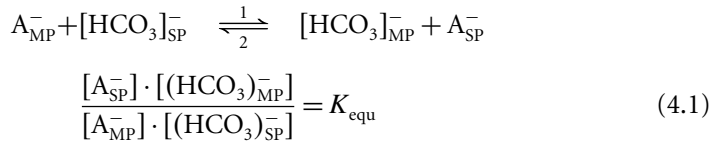


Figure 4.2 Scheme showing the progression of an anion A^- by successive exchanges with the counter anion E^- in contact with an ammonium stationary phase. (1) Initially the counterion E^- fixed to the stationary phase is exchanged with the anionic species A^- present in the mobile phase. (2) Next, the elution inverts the phenomenon by regenerating the stationary phase with the anion E^- which, (3) substitutes again for A^- on the stationary phase. The ion OH^- would be the simplest choice for E^- but mixtures of carbonate and of hydrogenocarbonate (CO_3^{2-} and HCO_3^- at 0.003 M) are preferred since they are more efficient to displace the anions to be separated.

Arrow 1 corresponds to the attachment of the anion A^- to the SP and arrow 2 to its return to the mobile phase and therefore to its progression down the column.



K_{equ} represents the *selectivity* between the two anions with respect to the cation of the stationary phase. As different anions have different K_{equ} they are therefore retained on the column during different times. The time at which a given ion elutes from the column can be controlled by adjusting the pH. Most of instruments use two mobile phase reservoirs containing buffers of different pH, and

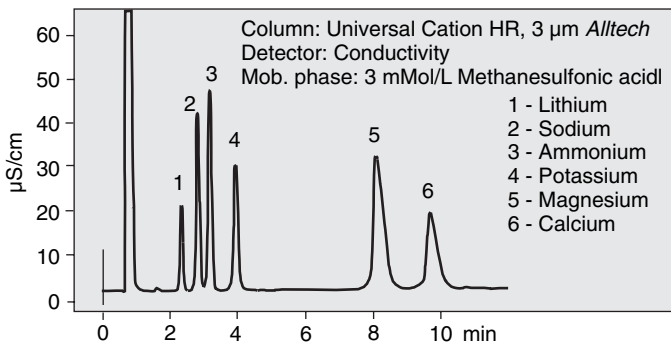
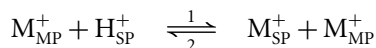


Figure 4.3 Separation of several cations, mono and divalents with a cationic column (Courtesy of Alltech)

a programmable pump that can change the pH of the mobile phase during the separation.

Using a cation exchange resin, a similar situation can be described (the stationary phase SP corresponds, for example, to Polym-SO₃H, strongly acidic):



This exchange phenomenon, which allows polar species to be retained on the resin, is known as *solid phase extraction* (Figure 4.3). If the sample contains two ions X and Y and if $K_Y > K_X$, Y will be retained more than X on the column.

4.2 Stationary phases

Ion chromatography can be subdivided into cation exchange chromatography, in which positively charged ions bind to a negatively charged stationary phase and anion exchange chromatography, in which the negatively charged ions bind to a positively charged stationary phase. The column packings consist of a reactive layer bonded to inert polymeric particles. Stationary phases must satisfy implicitly a number of requirements as narrow granulometric distribution (mono-disperse), large specific surface area, mechanical resistance, stability under acid and basic pHs and rapid ion transfer.

4.2.1 Polymer-based materials

The best known stationary phases are issued from copolymers of styrene and divinylbenzene, in order to obtain packings hard enough to resist pressure in the column. They are made of spherical particles with diameters of 5 to 15 μm (Figure 4.4) that are modified on the surface in order to introduce functional groups with acidic or basic properties.

For cation separation the cation-exchange resin is usually a sulfonic or carboxylic acid. Thus, concentrated sulfuric acid is used to attack the accessible aromatic rings of the copolymer surface to link SO₃H functional groups. A strongly acidic phase is obtained – for cation exchange – on which the anion is fixed to the macromolecule while the cation can be reversibly exchanged with other cationic species present in the mobile phase. These materials are stable over a wide range of pHs and have an exchange capacity of a few mmol/g.

■ Another approach for obtaining these stationary phases is based on the copolymerization of a mixture of two acrylic monomers. One is anionic (or cationic), according to the nature of the phase desired, and the other is polyhydroxylated (Figure 4.5), in order to ensure the hydrophilic character of the stationary phase. There is, however, an inconvenience with these resins as their rate of swelling depends upon the

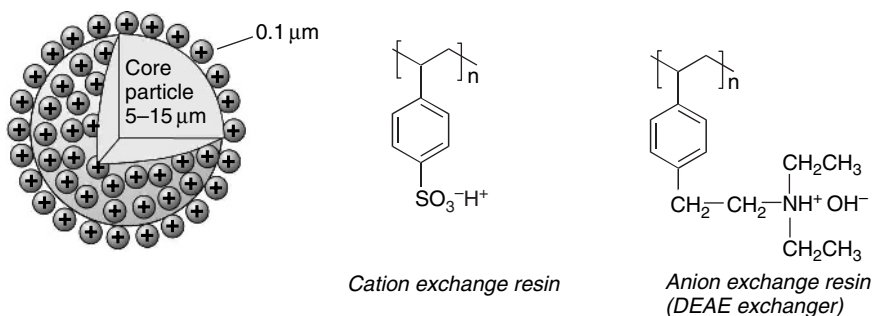


Figure 4.4 *Stationary phases in IC.* Cross-section of a spherical particle of polystyrene used as a cation exchanger. The polystyrene matrix is transformed either to a cation (ex. DOWEX® 4) or to an anion (ex. DOWEX® MSA-1) exchange resin. For anion separation the resin is usually a quaternary ammonium group.

composition of the mobile phase. They are normally reserved for medium pressure chromatography and some biochemical applications.

Starting from the same copolymer, an anion exchange resin can be synthesized, first by chloromethylation, which binds $-\text{CH}_2\text{Cl}$ (Merryfield's resin), followed by reaction with a secondary or tertiary amine depending on the basicity required for the stationary phase.

On contact with water a mildly basic stationary phase such as Polym-NMe_2 yields a weakly ionized phase $(\text{Polym-NMe}_2\text{H})^+\text{OH}^-$ especially when the medium is basic. Alternately in an acidic medium it will appear as a strongly basic phase whose active surface will be strongly ionized: $(\text{Polym-NMe}_2\text{H})^+\text{Cl}^-$. The exchange capacity of these resins varies with the pH.

4.2.2 Silica-based materials

Porous silica particles can serve to support, through covalent bonding, alkylphenyl chains carrying sulfonated groups or quaternary ammonium groups. This fixation step is similar to that used to obtain bonded silica phases developed in HPLC.

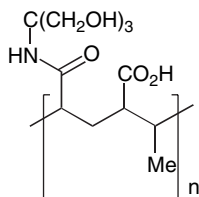


Figure 4.5 *Copolymerization of two monoethylenic monomers (an acid and a trihydroxyamide).* Example of the structure obtained (CM-TRISACRYL M® of IBF-France). Arising from a weak acid the resultant phase will be unusable at acid pH, as it will no longer be in its ionized form.

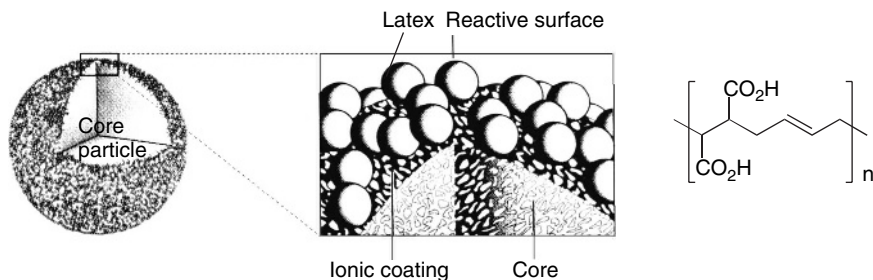


Figure 4.6 *Film resins.* Example of a resin made from a hard core onto which has been deposited a copolymer, derived from the reaction of maleic acid on 1,3-butadiene (Reproduced courtesy of the Dionex Company).

Some of these phases associate the properties of ion chromatography with those of HPLC. Separations depend simultaneously on both ionic coefficients and partition coefficients. Silica packings usually display greater efficiency than their polymeric equivalents.

4.2.3 Resin films

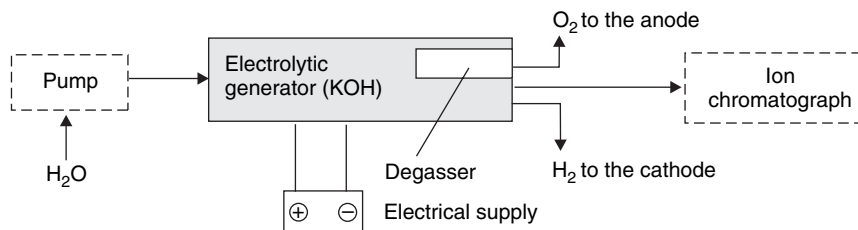
A polymer called 'latex', prepared from a monomer that contains organic groups, is deposited as an array of tiny beads (0.1–0.2 μm in diameter) on an waterproof support to form a continuous film-like layer about 1–2 μm thickness. The support is made of micro-spheres of silica or glass or polystyrene of about 25 μm diameter (Figure 4.6) This gives rapid equilibriums between stationary and mobile phases.

Latex polymer results from the reaction of two unsaturated monomers such as 1,3-butadiene with maleic acid or 2-hydroxyethyl methacrylate.

4.3 Mobile phases

IC mobile phases are usually 100 per cent aqueous with organic or inorganic buffers to control selectivity and when necessary a small content of methanol or acetone used to dissolve certain samples having a low degree of ionization. Depending upon the type of stationary phase, the counter ions present in the mobile phase derived from acids (perchloric, benzoic, phthalic, methane sulfonic), or bases (the most popular for anion analyses are variants of sodium hydroxide and sodium carbonate/bicarbonate).

The pH is adjusted according to the separation to be achieved. The eluents can be prepared in advance remembering that basic solutions have a tendency to absorb atmospheric carbon dioxide, with for consequence a modification in the retention times.



Detail of the electrolytic OH⁻ generating cell

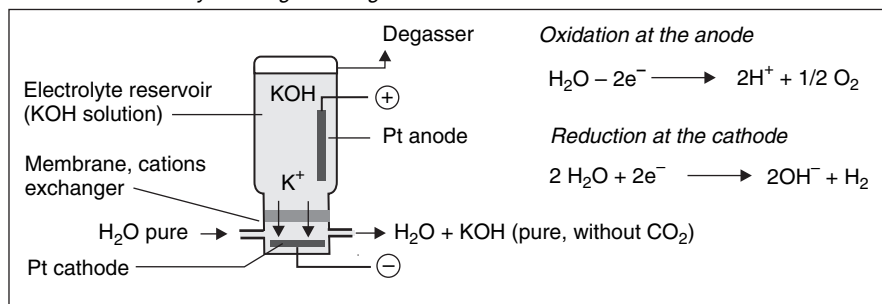
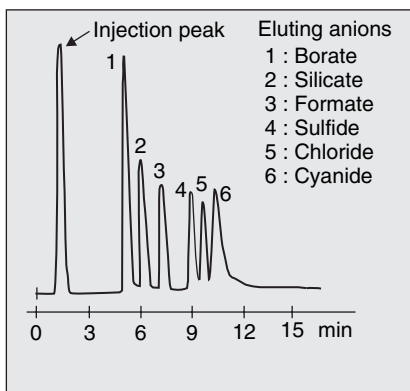


Figure 4.7 Ion chromatograph containing a high-purity OH⁻ generator. Schematic showing the position of the generator between pump and chromatograph. The degasser facilitates the elimination of gas which forms around the electrode located in the eluent stream. A K⁺ ion is formed for every OH⁻ generated. Isocratic or gradient elution is provided on demand (diagram based on a document from Dionex).

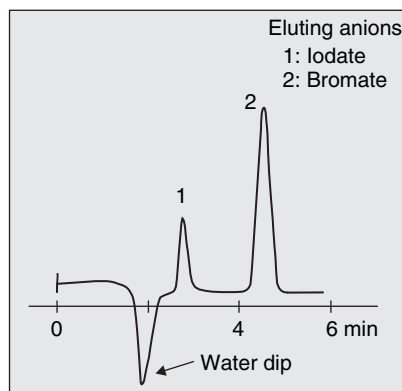
To avoid these inconveniences, an eluent generator can be used (either acidic or basic) which is inserted, as a supplementary module, between the pump and the injector of the ion chromatograph (Figure 4.7). If the flow rate of water and the electrolytic current are known then the concentration of the eluent can be determined with precision and concentration gradients can be effected, a procedure seldom used in ion chromatography.

Injection peak. The first peak in a chromatogram for anions results from the ionic strength of the injected sample being different than that of the eluent. The anions in the sample displace the anions (e.g. carbonate/bicarbonate or hydroxide) that are adsorbed onto the column packing. These displaced anions move forwards with the mobile phase and when passed through the detector appear as a positive peak (Figure 4.8). If a suppressor (cf. section 4.5) is installed at the column outlet and if carbonates make the mobile phase, a negative peak, called the 'water dip' is often present. This peak is the result of carbon dioxide which is formed in the suppressed mobile phase (in the form of carbonic acid).

If the ionic strength of the sample is greater than that of the eluent, there will be a positive peak. These peaks indicate the hold-up time of the chromatogram underway.



Column: Anion/R
 Detector: **Conductivity**
 Mob. phase: 5 mM p-HO-benzoic acid, pH 8.5



Column: Allsep Anion
 Detector: **Suppressed Conductivity**
 Mob. phase: 0.85 mM NaHCO₃

Figure 4.8 Chromatograms displaying injection peak. The injection peak is the unretained peak that allows access to retention factors. This is normally the first peak on the chromatogram. It can interfere with other early-eluting anions such as fluoride (Chromatograms from Alltech).

4.4 Conductivity detectors

Besides the spectrophotometric detectors based on absorbance or fluorescence of UV/visible radiation, and used when the mobile phase does not absorb appreciably, another mode of detection exists based upon electrolyte conductivity. Thus, at the outlet of the column, the conductance (the inverse of the resistance) of the mobile phase is measured between two microelectrodes. The measuring cell should be of a very small volume (approx. 2 μ L). The difficulty is to recognize in the total signal the part due to ions or ionic substances present in the sample. In order to do direct measurements, the ionic charge of the mobile phase has to be as low as possible and the measuring cell requires strict temperature control to within 0.01 $^{\circ}$ C because of the high dependence of conductance on temperature ($\sim 5\%/^{\circ}$ C).

The sensitivity of the detector to an ion X (valency z and molecular concentration C) can be predicted if its equivalent conductance (Λ_X) and that of the eluent ion E (Λ_E) are known. This depends from the difference ΔK between the equivalent conductances of ion X and that of E. ΔK can be calculated according to expression 4.2, knowing that the peak will be either positive or negative.

$$\Delta K = C(\Lambda_X - \Lambda_E) \quad (4.2)$$

■ The *conductance* $G = 1/R$ that corresponds to the reciprocal of the resistance R , is measured between two electrodes which are plunged into the conducting solution and across which is maintained a potential difference. G is expressed in Siemens (S). For a given ion, the conductance of the solution varies with the concentration of the

electrolyte. This relationship is linear for very dilute solutions. The *specific conductance* (in S/mol) or *conductivity* k , permits the measure to be independent of the detection cell parameters:

$$k = GK_{\text{cell}} \quad (4.3)$$

$K_{\text{cell}} = d/A$ represents the *cell constant* (area A and spacing d). Its value cannot be obtained by direct measurement, but is determined from a standard solution for which the conductivity k is known.

Finally the *equivalent ionic conductance* ($\text{S}\cdot\text{m}^2/\text{mol}$) represents the conductivity of an ion with a valence z , in an aqueous solution at 25°C , when the molar concentration C (mol/L) tends towards zero in water (Table 4.1).

$$\Lambda_0 = 1000k/Cz \quad (4.4)$$

Table 4.1 Equivalent ionic conductivities of ions in infinite dilution in water at 25°C

Cations	Λ_0^+ ($\text{S}\cdot\text{m}^2/\text{mol}$) $\times 10^{-4}$	Anions	Λ_0^- ($\text{S}\cdot\text{m}^2/\text{mol}$) $\times 10^{-4}$
H^+	350*	OH^-	198
Na^+	50	F^-	54
K^+	74	Cl^-	76
NH_4^+	73	HCO_3^-	45
$1/2\text{Ca}^{2+}$	60	H_2PO_4^-	33

* $3\,500\,000\ \text{S}\cdot\text{m}^2/\text{mol}$ or 350 in $\text{S}\cdot\text{cm}^2/\text{mol}$.

4.5 Ion suppressors

The mobile phase contains ions that create a background conductivity, making it difficult to measure the conductivity due only to the analyte ions as they exit the column. To improve the signal to noise ratio, when using a conductivity detector, a device called a *suppressor*, designed to selectively remove the mobile phase ions is placed after the analytical column and before the detector. The principle consists to convert the mobile phase ions to a neutral form or replacing them by others of higher conductivity. Suppressor-based detection is more useful for anion analysis than for cation analysis.

The simplest model of a suppressor can be considered as a column which contains a stationary phase having functional groups of opposing charge to those of the separating column. Such a *chemical suppressor*, which contains an anionic resin is associated to a cationic separation column. The mechanism of action can be described using the following example.

Suppose that a mixture containing the cations Na^+ and K^+ has been separated using a cationic column whose mobile phase contains dilute hydrochloric acid. In this acidic medium, at the outlet of the column, the Na^+ and the K^+ ions

are accompanied by H^+ ions coming from the acid and Cl^- anions in order to assure the electroneutrality of the medium. After the separation column, the mobile phase flows through a second column which contains an *anionic exchange resin* whose mobile ion is OH^- . The Cl^- anions will be affixed on this column, thus displacing the OH^- ions that will react with H^+ ions in solution to give water. At the outlet of the suppressor, only (Na^+OH^-) and (K^+OH^-) species are found in water. The ions H^+ and Cl^- have effectively disappeared. As OH^- has a higher conductivity than Cl^- , detection of the Na^+ and K^+ ions is easier, because amplified (Figure 4.9).

In summary, for anion analyses (using a conductivity detector), ion suppressors neutralize the mobile phase, reducing its conductivity, while simultaneously increasing the sample's conductivity.

The limitation of this type of suppressor lies in its very large dead volume that reduces the separation efficiency, due to a remixing of the ions prior to their detection. The ions of the suppressor should be regenerated periodically and it should be used exclusively in the isocratic mode.

Other types of suppressors, having a high ionic capacity, have subsequently been developed. They are made of porous fibres or of micromembranes and possess very small dead volumes in the order of 30–50 μL . That allows gradient elution with a negligible baseline drift. Figure 4.10(a) shows the passage of an anion A^- , in solution in a typical electrolyte used for anionic columns, through a suppressor with a cationic membrane.

Nowadays, continuous regenerated suppressors which make use of electrolytic reactions have been introduced for traces determinations. They behave either

Elution of the cation M^+

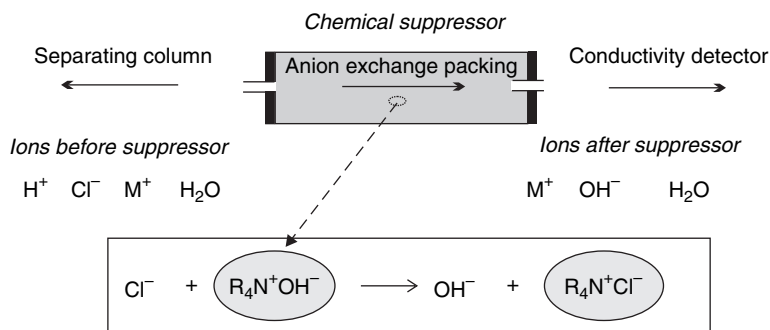
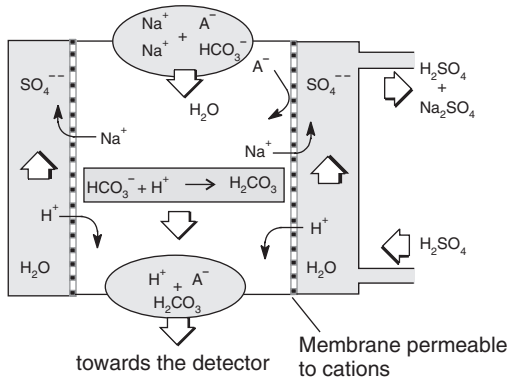


Figure 4.9 Chemical suppressor for an exchange cation column. For cation analysis, the mobile phase is often dilute HCl or HNO_3 solutions, which can be neutralized by an eluent suppressor that supplies OH^- . In this example the anionic suppressor purges the mobile phase of H^+ ions and of almost all of the Cl^- ions, facilitating the detection of the cation M^+ . The same principle holds for anion analysis. In this case, the mobile phase is often dilute NaOH or NaHCO_3 , and the eluent suppressor supplies H^+ to neutralize the anion and retain or remove the Na^+ .

(a) Porous membrane suppressor installed at the outlet of an anionic separating column

Elution of anion A⁻



(c) Separation of anions

Column IonPac (Dionex DX)
Conductivity detection
Eluent: 1.8 mM Na₂CO₃
/ 1.7 mM NaHCO₃
flow: 1 mL/min

1 - fluoride	5 - nitrate
2 - chloride	6 - phosphate
3 - nitrite	7 - sulfate
4 - bromide	8 - oxalate

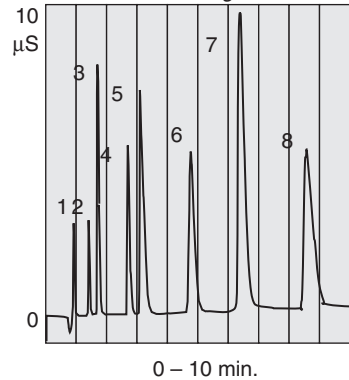
Separation column



Suppressor



Chromatogram



(b) Suppressor with auto-regenerating membrane installed at the outlet of a cationic column

Elution of cation M⁺

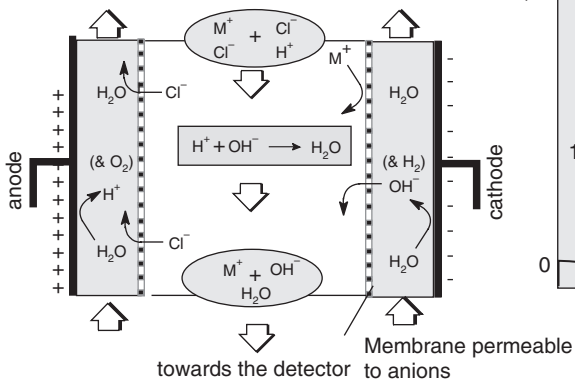


Figure 4.10 Membrane and electrochemically regenerated suppressors. There are two types of membranes, one type permeable to cations (H^+ and in this example Na^+), the other permeable to anions (OH^- and here Cl^-). (a) The microporous cationic membrane is adapted to the elution of anions. Only cations can cross the membrane (corresponding to a polyanionic wall which keeps away the anions in the solution). (b) An anionic membrane suppressor placed, contrary to the preceding model, at the outlet of a cationic column. Ions are regenerated by the electrolysis of water. Note in both cases the counter flow circulation between the eluted phase and the solution of the post-column suppressor. (c) An example of a separation of inorganic cations (concentrations of the order of ppm) using a suppressor of this type.

like a special column containing a resin which regenerates by electrolysis or like a membrane suppressor where the regenerating ions are produced *in situ* by electrolysis of water. Figure 4.10b illustrates the second procedure: it represents the passage of a cation, in a dilute hydrochloric acid solution, through a suppressor whose membrane is permeable to anions.

Alternately, if the problem consists in the separation of a mixture of anions on an anionic column (cationic material), with an eluent containing dilute sodium hydroxide, a membrane allowing the diffusion of cations will be chosen. At the cathode, the passage of hydronium ions towards the main flow of electrolyte will neutralize OH^- ions. At the anode, Na^+ ions will migrate out of the mobile phase and will react with OH^- ions.

Quantitative analysis by chromatography

The significant development of chromatography in quantitative analysis is essentially due to its reliability and its use in standardized analyses. Trace and ultratrace analyses by chromatography are used, particularly the EPA methods for environmental analysis, although their costs are rather high. This type of analysis relies mainly on reproducibility of the separation and on the linear relationship between the injected mass of a compound onto the column and the area of the corresponding peak on the resultant chromatogram. This is an excellent comparative method used in many protocols, which, allied with software used for data treatment allow automation of all the calculations associated with these analyses.

The three most widely used methods are described below accompanied in their simplest formats.

4.6 Principle and basic relationship

In order to calculate the mass concentration of a compound appearing as a peak on a chromatogram, two basic conditions must be met. First, an *authentic sample* of the compound to be measured should be available, as a reference, to determine the detector sensitivity to this compound. Second, a software giving the *heights* or *areas* of the different eluting peaks of interest is also required. All of the quantitative methods in chromatography rely on these two principles. They are comparative but not absolute methods.

For a given tuning of the instrument, it is assumed that a linear relation exists for each peak of the chromatogram, over the entire concentration range, between its area and the quantity of the compound responsible for this peak in the injected sample. This applies for a given concentration range depending on the detector employed. This hypothesis is translated into the following equation:

$$m_i = K_i \cdot A_i \quad (4.5)$$

where m_i is mass of the compound i injected on the column, K_i is the *absolute response factor* for compound i and A_i is the area of the eluting peak for compound i .

The absolute response factor K_i (not to be confused with the partition coefficient), is not an intrinsic parameter of the compound since it depends upon the tuning of the chromatograph. To calculate the response factor K_i , according to expression 4.5, it is essential that both the area A_i and the mass m_i , of compound i injected on the column, are known. However, this mass is difficult to determine with precision since it relies simultaneously upon the syringe, upon the injector type (in GC), or upon the injection loop (in HPLC). This is why most chromatographic methods utilized for quantitative analyses, whether pre-programmed into an integrated recorder or in the multiplicity of available software, do not make use of the absolute response factors, K_i .

4.7 Areas of the peaks and data treatment software

To determine the areas of the peaks appropriate chromatographic software is used which also ensures not only the control and working of the chromatograph but also the data treatment to furnish a report corresponding to one of the pre-programmed methods of quantitative analysis.

The signal recovered by the detector is sampled by the analogue–digital converter (ADC) with a frequency of a few hundreds hertz in order to yield an accurate reproduction of the narrowest peaks in chromatograms obtained from GC with capillary columns. Each software package allows baseline correction, treatment of negative signals and all incorporate different methods to calculate peak areas (Figure 4.11).

- The manual triangulation method and the ‘cut and weight’ method (weight is considered proportional to area) are, of course, no longer employed. However it is useful to remember that for a gaussian eluting peak, the product of its width at half height by its full height, corresponds to approximately 94 per cent of the total area of the peak. In the same way, recorders with an integration system for measuring the peak areas are no longer used.

4.8 External standard method

This method allows the measurement of the concentration (or percentage in mass) of one or more components that appear as resolved peaks on the chromatogram, even in the presence of other compounds yielding unresolved peaks. Easy to use, this method corresponds to the application of a principle common to many quantitative analysis techniques.

The *modus operandi* is based upon the comparison of two chromatograms obtained successively *without changing the control settings of the chromatograph*

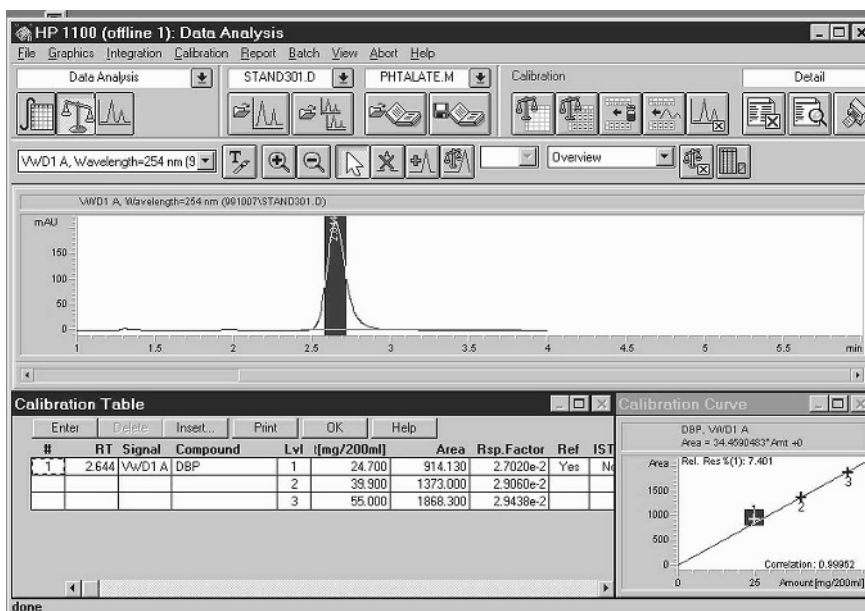


Figure 4.11 Quantitative analysis software for chromatography. Since the signal from the detector situated at the outlet of the column is analogical, an analogue–digital converter (ADC) is necessary. The stored chromatogram, digitized, serves as a basis for its exploitation by the software. Different zones of the screen can display the calibration curve, methodology, etc. (software Chemstation from Agilent Technologies).

(Figure 4.12). The first chromatogram is acquired from a standard solution (reference solution) of known concentration C_{ref} in a solvent. The standard and sample matrix should be as similar as possible. A volume V of this solution is injected. Analysis conditions must be identical. On the resulting chromatogram the area A_{ref} of the corresponding peak is measured. The second chromatogram results from the injection of the *same volume* V of the sample in solution, containing an unknown concentration of the compound to be measured (conc. C_{unk}). The area of the corresponding peak is A_{unk} . Since an identical volume of both samples has been injected, the *ratio of the areas* is proportional to the *ratio of concentrations* which depend upon the masses injected ($m_i = C_i \cdot V$). Applied to the two chromatograms, expression 4.5 leads to relation 4.6, which characterizes this method:

$$m_{\text{ref}} = C_{\text{ref}} \cdot V = K \cdot A_{\text{ref}} \quad \text{and} \quad m_{\text{sam}} = C_{\text{unk}} \cdot V = K \cdot A_{\text{unk}}$$

$$C_{\text{unk}} = C_{\text{ref}} \frac{A_{\text{unk}}}{A_{\text{ref}}} \quad (4.6)$$

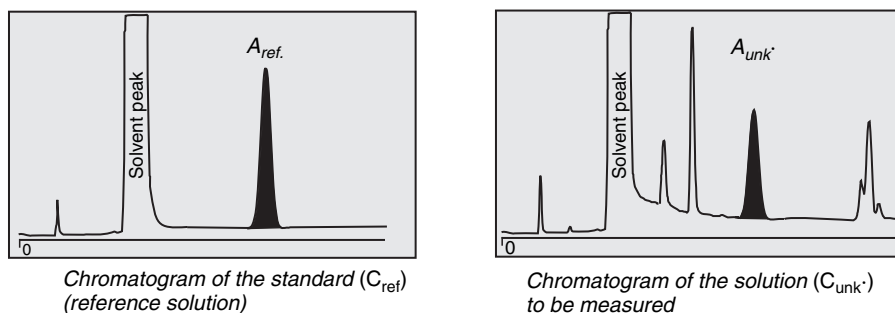


Figure 4.12 Analysis by the external standard method. The precision of this basic method is improved when several solutions of varying concentrations are used in order to create a calibration curve. For trace analyses by liquid chromatography it is sometimes advisable to replace the areas of the peaks by their heights as they are less sensitive to variations in the mobile phase flow rate.

The single point calibration method, as depicted in Figure 4.12, assumes that the calibration line goes through the origin. Precision will be improved if the concentrations of the reference solution and of the sample solution are similar.

This technique, employing the absolute response factors, yields very reliable results with chromatographs equipped with an auto-sampler: a combination of a carousel sample holder and an automatic injector. This permits numerous measurements to be made without interruption, to the condition that no change in the apparatus tuning is made between injections.

The reference solution periodically injected affords a control that can be used to compensate an eventual baseline drift during a sequence of programmed injections.

Precision can be also improved if several injections of the sample and the reference solutions are made, always using equal volumes. In a multilevel calibration, equal volumes of a series of standard solutions are injected. This allows to get a calibration curve of $A = f(C)$, obtained by a regression method (linear least-square or quadratic least-square). This leads to a more precise value for C_{unk} (Figure 4.11).

This method, the only one adapted to gas samples, has the added advantage that nothing needs to be added to the sample solution, unlike the method described below.

4.9 Internal standard method

For trace analysis it is preferable to use a method that relies on the *relative response factor* of each compound to be measured against a marker introduced as a reference. This means that any imprecision concerning the injected volumes, the principal constraint of the previous method, is compensated. As above, this

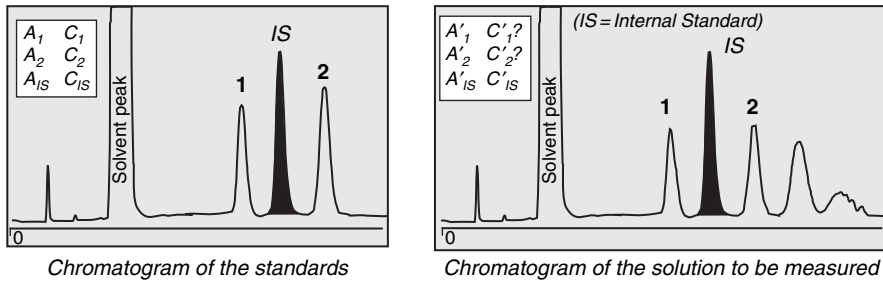


Figure 4.13 Simple example illustrating the internal standard method

more reliable method requires two chromatograms, one to calculate the relative response factors of the compounds of interest, and the other to analyse the sample.

The areas of the peaks to be quantified are compared with that of an *internal standard* (designated by IS), introduced at a known concentration within the sample solution.

Supposing that a sample contains two compounds 1 and 2 to be measured and that compound (IS) represents the additional compound for use as an internal standard (Figure 4.13).

4.9.1 Calculation of the relative response factors

A solution containing compound 1 at known concentration C_1 , compound 2 at known concentration C_2 and the internal standard IS at known concentration C_{IS} is prepared then injected onto the chromatograph. A_1 , A_2 , A_{IS} will be the areas of the elution peaks in the chromatogram due to the three compounds. If m_1 , m_2 and m_{IS} represent the real quantities introduced onto the column, of these three substances, then three relations of type 4.5 can be derived:

$$m_1 = K_1 \cdot A_1$$

$$m_2 = K_2 \cdot A_2$$

$$m_{IS} = K_{IS} \cdot A_{IS}$$

$$\frac{m_1}{m_{IS}} = \frac{K_1 \cdot A_1}{K_{IS} \cdot A_{IS}} \quad \text{and} \quad \frac{m_2}{m_{IS}} = \frac{K_2 \cdot A_2}{K_{IS} \cdot A_{IS}}$$

These ratios enable the calculation of the *relative response factors* of 1 and 2, against IS and designated by $K_{1/IS}$ and $K_{2/IS}$:

$$K_{1/IS} = \frac{K_1}{K_{IS}} = \frac{m_1 \cdot A_{IS}}{m_{IS} \cdot A_1} \quad \text{and} \quad K_{2/IS} = \frac{K_2}{K_{IS}} = \frac{m_2 \cdot A_{IS}}{m_{IS} \cdot A_2}$$

Since the injected masses m_i are proportional to the corresponding mass concentrations $C_i(m_i = C_i V)$, the above equations can be rewritten as follows:

$$K_{1/IS} = \frac{C_1 \cdot A_{IS}}{C_{IS} \cdot A_1} \quad \text{and} \quad K_{2/IS} = \frac{C_2 \cdot A_{IS}}{C_{IS} \cdot A_2}$$

4.9.2 Chromatogram of the sample – calculation of the concentrations

The second step of the analysis is to obtain a chromatogram for a given volume of a solution containing the sample to quantify and to which has been added a known quantity of internal standard IS. This will yield A'_1 , A'_2 and A'_{IS} the areas of this new chromatogram being obtained under the same operating conditions as previously. If m'_1 , m'_2 and m'_{IS} represent the quantities of 1, 2 and IS introduced into the column, then:

$$\frac{m'_1}{m'_{IS}} = K_{1/IS} \cdot \frac{A'_1}{A'_{IS}} \quad \text{and} \quad \frac{m'_2}{m'_{IS}} = K_{2/IS} \cdot \frac{A'_2}{A'_{IS}}$$

From the relative response factors calculated in the first experiment as well as from the known concentration of the internal standard within the sample, C'_{IS} , this leads to :

$$C'_1 = C'_{IS} K_{1/IS} \cdot \frac{A'_1}{A'_{IS}} \quad \text{and} \quad C'_2 = C'_{IS} K_{2/IS} \cdot \frac{A'_2}{A'_{IS}}$$

Expanding to n components it is easy to calculate the mass concentration of the solute i using equation 4.7:

$$C'_i = C'_{IS} K_{i/IS} \cdot \frac{A'_i}{A'_{IS}} \quad (4.7)$$

equally the percentage concentration of i can be expressed using equation 4.8:

$$X_i\% = (C'_i / \text{Mass of sample taken}) \times 100 \quad (4.8)$$

This method becomes even more precise if several injections of the solution and of the sample are carried out. Often a same volume of an IS stock solution is spiked with all standards and samples.

In conclusion, this general and reproducible method demands nevertheless a good choice of internal standard, which should have the following characteristics:

- it must be stable, pure and not exist in the initial sample

- it must be measurable, giving an elution peak well resolved on the chromatogram
- its retention time must be close to that (or those) of the solute(s) to be quantified
- its concentration must be close to, or above that of the analytes to quantify to gain a linear response from the detector
- it must not interfere and co-elute with a sample component.

4.10 Internal normalization method

This method, also called ‘normalized to 100 per cent’ is used for mixtures for which each component is producing a peak on the chromatogram, in order to be able to make a complete assessment of the sample concerned. The solvent, if any, is typically ignored.

Supposing that it is required to find the mass concentrations of three compounds 1, 2, 3 in a mixture (Figure 4.14). The analysis is again carried out in two steps.

4.10.1 Calculation of the relative response factors

A standard solution containing the three compounds 1, 2, and 3 at known concentrations C_1 , C_2 and C_3 is prepared. The chromatogram corresponding to the injection of a volume V of this standard solution shows three peaks of area A_1 , A_2 and A_3 . These areas will be related to the masses m_1 , m_2 and m_3 of the compounds in volume V , by three expressions of type 4.5.

One of the compounds, 3 for example, is chosen as the substance for internal normalization. This compound 3 will serve to calculate the relative response factors $K_{1/3}$ and $K_{2/3}$ for compounds 1 and 2 with respect to 3. As previously deduced:

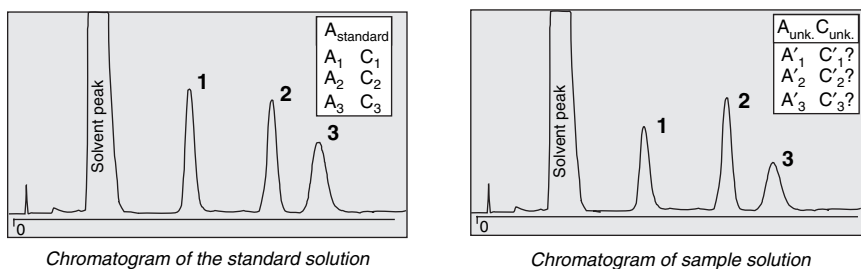


Figure 4.14 Analysis by internal normalization method. This method is commonly reported as the default for early integrators

$$K_{1/3} = \frac{K_1}{K_3} = \frac{m_1 \cdot A_3}{m_3 \cdot A_1} \quad \text{and} \quad K_{2/3} = \frac{K_2}{K_3} = \frac{m_2 \cdot A_3}{m_3 \cdot A_2}$$

Given that $m_i = C_i \cdot V$, then the following expressions for $K_{1/3}$ and $K_{2/3}$ are obtained:

$$K_{1/3} = \frac{C_1 \cdot A_3}{C_3 \cdot A_1} \quad \text{and} \quad K_{2/3} = \frac{C_2 \cdot A_3}{C_3 \cdot A_2}$$

4.10.2 Chromatogram of the sample – calculation of the concentrations

The next step consists to inject a sample of the mixture to be measured containing 1, 2 and 3. Labelling the elution peaks as A'_1 , A'_2 and A'_3 will gain direct access to the percentage mass composition of the mixture represented by x_1 , x_2 and x_3 via three expressions of the following form:

$$x_i\% = \frac{K_{i/3} \cdot A'_i}{K_{1/3} \cdot A'_1 + K_{2/3} \cdot A'_2 + A'_3} \times 100 \quad \text{with } i = 1 \text{ or } 2 \text{ or } 3. \quad (4.9)$$

The condition of normalization being that: $x_1 + x_2 + x_3 = 100$

If the procedure is extrapolated to n components normalized to the component j , a general expression for the response factor of a given compound i can be obtained (equation 4.10).

$$K_{i/j} = \frac{C_i \cdot A_j}{C_j \cdot A_i} \quad (4.10)$$

It is also possible to determine $K_{i/j}$ by plotting a concentration-response curve for each of the solutes.

In a mixture containing n components, if A'_i designates the area of the elution peak of compound i , and if the internal reference is j , then the content of compound i will obey the following equation 4.11:

$$x_i\% = \frac{K_{i/j} \cdot A'_i}{\sum_{i=1}^n K_{i/j} \cdot A'_i} \times 100 \quad (4.11)$$

Supposing that detector gives responses that are independent from the substance (i.e. relative response factors identical as for a TCD detector in GC), this method serves to give an estimation of relative concentrations. This was commonly reported as the default for early integrators (equation 4.12).

$$x_i\% = \frac{A'_i}{\sum_{i=1}^n A'_i} \times 100 \quad (4.12)$$

Problems

4.1 0.604 g of an undiluted stationary phase, comprising SO_3H groups is introduced to an erlenmeyer. 100 mL of distilled water, is added and approximately 2 g of NaCl. The liberated acidity is measured by sodium hydroxide 0.105 M in the presence of helianthin (methyl orange), as an indicator to identify the equivalence point (towards pH 4). If it is known that 25.4 mL of the sodium hydroxide solution must be added to neutralise the acid liberated by the stationary phase, calculate its molar capacity in grams.

4.2 A mixture of proteins is separated on a column with a stationary phase of carboxymethylated cellulose. The internal diameter of the column is 0.75 cm and its length is 20 cm. The dead volume is 3 mL. The flow rate of the mobile phase is 1 mL/min. The pH of the mobile phase is adjusted to 4.8. Three peaks appear upon the chromatogram corresponding to the elution volumes V_1 , V_2 and V_3 at 12 mL, 18 mL and 34 mL respectively.

1. Does this arise from an anionic or cationic phase? Give reasons for your answer.

2. Why, when increasing the pH of the mobile phase, are the times of elution of the three compounds subject to modification? Predict whether these times are increased or decreased.

4.3 In measuring cyclosporin A (a treatment for skin and organ transplant rejection) by HPLC, according to a method derived from that of internal standard, the following procedure is employed.

Preparation of the samples: An extraction of 1 mL of blood plasma is made, to which is added 2 mL of a mixture of water, and acetonitrile (80/20), containing 250 ng of cyclosporin D as internal standard, which has a similar structure to cyclosporin A.

The 3 mL of new mixture is now passed on a disposable solid phase extraction column in order to separate the cyclosporins retained upon the sorbent. Following the rinsing and drying of the column, the cyclosporins are eluted with 1.5 mL of acetonitrile and are then concentrated to 200 μL following evaporation of solvent. A fraction of this final solution is injected into the chromatograph.

1. What is the concentration factor of the original plasma following this treatment?

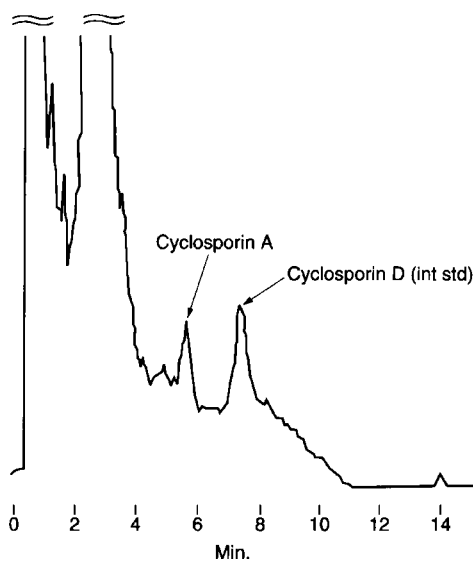
2. Can the rate of recuperation following the extraction step on the solid phase be deduced?

Sample: The standards are created with a plasma originally possessing no cyclosporin. Six different solutions are prepared by adding the necessary quantities of cyclosporin A to each in order to create solutions of 50 ng/mL, 100 ng/mL, 200 ng/mL, 400 ng/mL, 800 ng/mL, and 1000 ng/mL. 1 mL from each of these solutions is subjected to the same extraction sequence following the addition of 250 ng of cyclosporin D to each, as above.

ng/mL en cyclo. A	50	100	200	400	800	1000
Ratio of peaks heights cyclo.A/cyclo.D (R_h)	0.25	0.5	1.02	2.04	4.05	5.1

Column Supelco 75 × 4.6 mm. Silica gel 3 μm, phase RP-8.

3. Determine the concentration in units of ng/mL of cyclosporin A in the blood plasma giving rise to the chromatogram reproduced below. This question should be attempted in two ways:
- By choosing a single point from a standard.
 - By using the gradient which it is possible to draw from the data in the table above (in both cases it will be based upon the heights of the peaks).

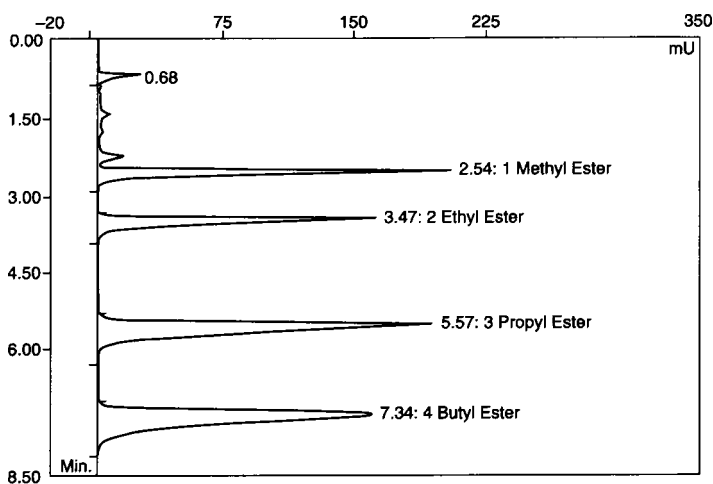


- 4.4 The method of internal normalisation was chosen to determine the mass composition of a sample comprising a mixture of four esters of butanoic acid. To this end, a reference solution containing known % masses of these esters led to the following relative values of the response coefficients of the butanoates of methyl (ME), of ethyl (EE), and of propyl (PE), all three in ratio with butyl-butanoate (BE).

$$K_{ME/BE} = 0.919 \quad K_{EE/BE} = 0.913 \quad K_{PE/BE} = 1.06$$

From the chromatogram of the sample under analysis, reproduced below, and the information given in the table, find the mass composition of this mixture (ignore the first peak at 0.68 min.)

Peak no.	t_R	compound	Area (mV. min)
1	0.68	–	0.1900
2	2.54	methyl ester (ME)	2.3401
2	3.47	ethyl ester (EE)	2.3590
3	5.57	propyl ester (PE)	4.0773
5	7.34	butyl ester (BE)	4.3207



- 4.5 To measure serotonin (5-hydroxytryptamine), by the internal standard method, a 1 mL aliquot of the unknown solution is added to 1 mL of a solution containing 30 ng of N-methyl-serotonin. This mixture is then treated to remove all other compounds which could interfere with the experiment.

The operation performed was an extraction in the solid phase to isolate the serotonin and its methyl derivative, diluted in a suitable medium.

1. Why is the compound forming the internal standard added before the extraction step?
2. Calculate the response factor of the serotonin compared to that of N-methyl-serotonin if it is known that the chromatogram yielded by the standards gave the following results.

<i>Name</i>	<i>Area ($\mu V.s$)</i>	<i>Quantity injected (ng)</i>
Serotonin	30 885 982	5
N-methyl-serotonin	30 956 727	5

3. From the chromatogram of the sample solution, find the concentration of serotonin in the original sample, if it is known that:

— area serotonin	2573 832 $\mu V/s$
— area N-methyl-serotonin	1719 818 $\mu V/s$

5

Thin layer chromatography

Thin layer chromatography (TLC), also known as planar chromatography, is an invaluable method used in chemistry and biochemistry, complementary to HPLC while having its own specificity. Although these two methods are applied differently, the principle of separation and the nature of the phases remain the same. Cheap and sensitive, this technique that is simple to use, can be automated. It has become essential principally since it is possible to undertake several separations in parallel. The development of automatic applicators and densitometers have led to nano-TLC, also called HPTLC, a highly sensitive technique which can be hyphenated with mass spectrometry.

5.1 Principle of TLC

The principle of the separation between phases of the sample components is similar to that of HPLC, though the migration of the constituents through the stationary phase is different. Separation is conducted on a thin layer (100–200 μm) of stationary phase, usually based upon silica gel and deposited on a rectangular plate made out of glass, plastic or aluminium of a few centimetres in dimensions. To maintain the stationary phase on the support and to assure the cohesion of the particles, an inert binder like gypsum (or organic linker) is mixed into the stationary phase during the manufacture of the plate. The constituents can be identified by simultaneously running standards with the unknown.

There are three steps to conduct a separation with this technique:

5.1.1 Deposition of the sample

A small volume of sample (between a few nanolitres to a few microlitres), dissolved in a volatile solvent, is deposited close to the bottom of the plate as a small

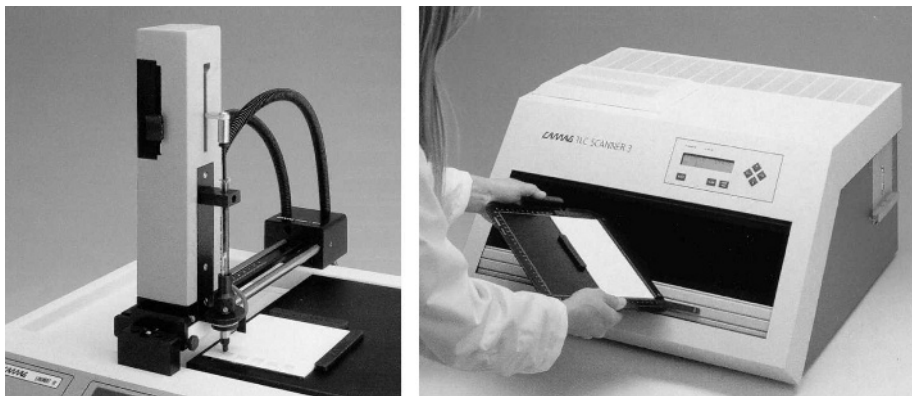


Figure 5.1 Automatic sample deposition device for TLC and a system to 'read' the plate. Left, programmable applicator Linomat IV. Right, densitometer measuring the light either reflected or transmitted by the plate. The optical set up is similar to that of a UV/visible spectrometer (model Scanner 3, reproduced courtesy of Camag).

spot of about 1–2 mm in diameter. This deposit is made either manually, or automatically, with a flat ended capillary (Figure 5.1). The spot can also have the form of a horizontal band of a few millimetres which is obtained by automatic spraying of the sample. This last method has the advantage of having a high reproducibility, indispensable of course, for quantitative analysis. The prepared plate is then placed in a glass developing chamber that contains a small amount of the appropriate developing solvent. The chamber is then covered (Figure 5.2). The position at which the sample has been deposited must be above the level of the solvent.

5.1.2 Developing the plate

The mobile phase rises up the stationary phase by capillarity, moving the components of the sample at various rates because of their different degrees of interaction with the matrix and solubility in the solvent. Their separation may be complete in a few minutes. When the solvent front has travelled a sufficient distance (several centimetres), the plate is withdrawn from the chamber, the position attained by the mobile phase is immediately noted then it is evaporated.

When using a plate of reversed polarity ('RP-TLC'), the mobile phase will generally contain water. In this case it can be useful to add a salt such as lithium chloride which limits diffusion phenomena and thereby increases the resolution.

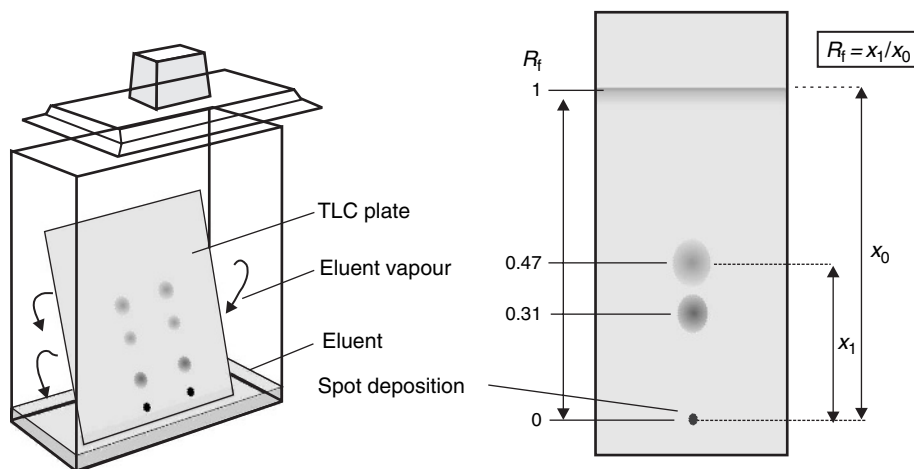


Figure 5.2 *Developing chamber and TLC plate.* Left, available in a variety of dimensions according to the size of the plates (of 5×5 to 20×20 cm), the chambers are made of glass and equipped with a tight fitting cover. Right, typical appearance after the TLC plate is partially dry. A faint line can be observed at the location of the solvent upper limit. This line is called the solvent front. Calculation of R_f (cf. paragraph 5.4). A substance that does not migrate from the sample origin has a $R_f = 0$.

5.1.3 Identifying the spots

The localization of each component on the plate, which has now lost all of the eluent, consists to measure their migration distance from the original deposition. To locate colourless compounds the plate must be developed (Figure 5.2). In order to facilitate the visualization of the spots, manufacturers sell plates that contain a fluorescent salt of zinc which emits a bright green fluorescence when the plate is irradiated with a UV mercury vapour lamp ($\lambda = 254$ nm) and observed in a viewing cabinet. All compounds absorbing at this wavelength appear as a dark spot (or sometimes coloured) against a bright green blue background.

Another method to make compounds visible, almost universally used, consists of heating the plate after spraying it with sulfuric acid which leaves charred blots behind. This approach is, however, not adapted to quantitative TLC; in this case, development is effected by immersion of the plate, using a general (phosphomolybdic acid, vanillin), or specific (e.g. ninhydrin in alcoholic solution for amino acids) reagent. Hundreds of reagents have been described that serve to introduce chromophores or fluorophores groups into the analytes molecules after separation.

The use of square TLC plates allows two-dimensional chromatography to be carried out using two successive elutions with two different mobile phases (Figure 5.3). A high degree of separation can be achieved.

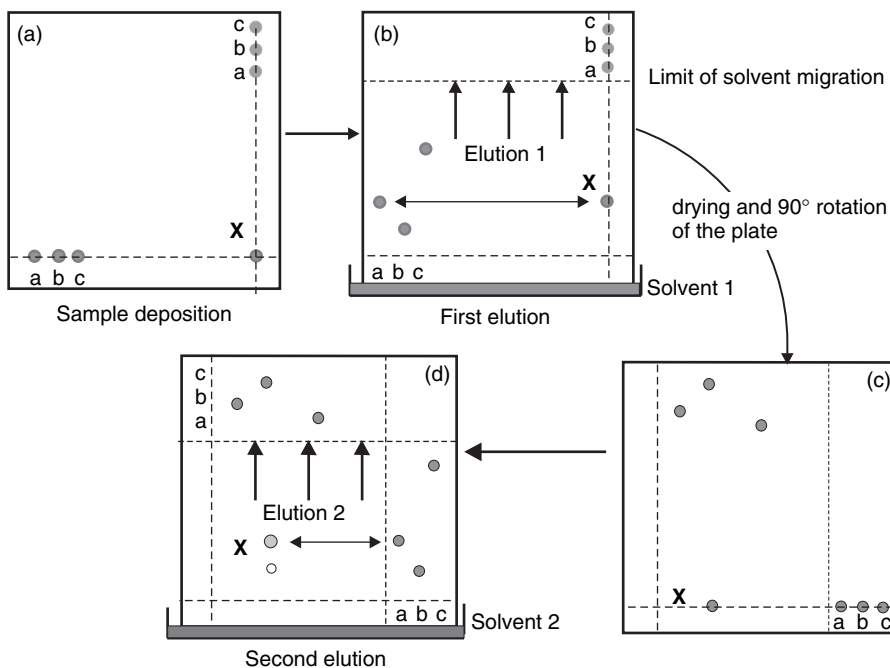


Figure 5.3 *Two-dimensional TLC.* An experiment with separation processes using two different solvents performed in two perpendicular directions. (a) Deposition of an unknown X and three standards; (b) migration in the first direction with the first solvent; (c) drying and rotation of the plate; (d) migration in the second direction with the second solvent. In conclusion, unknown compound X is a mixture of at least two compounds amongst which is the reference compound a (the same R_f in the two solvents) and one other compound which is not b. A typical application of this approach is the separation of amino acids.

5.2 Characteristics of TLC

TLC applies physico-chemical phenomena more complex than HPLC:

- TLC corresponds to a three-phase system between which equilibria are established: solid (stationary), liquid (mobile) and vapour phases.
- The stationary phase is only partially equilibrated with the liquid phase before the migration of the compounds. Depending upon the manner in which the separation is obtained, the mobile phase may or may not be in equilibrium with the vapour phase.
- The adsorption phenomenon of the stationary phase is substantially reduced once a large part of the adsorption sites are occupied. This creates an effect of elongation of the spots. As a result, the R_f (retardation factor) of a compound in the pure state is slightly different from the R_f of the same compound present in a mixture.

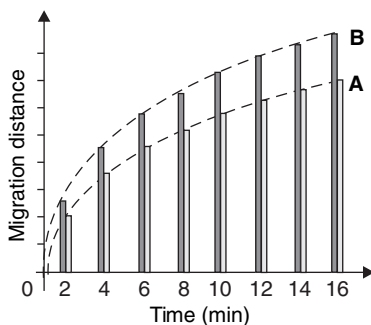


Figure 5.4 Migration distances of the mobile phase on a TLC plate. Over a sequence of regular time intervals, the quadratic progression of the eluant front can be seen. Curve A was obtained in a chamber unsaturated with eluant vapour. Curve B was obtained by saturating the chamber with eluant vapour.

- The flow rate of the mobile phase cannot be modified in order to improve the efficiency of a separation. A remedy to this problem is the multi-development technique, by drying the plate before each new cycle of migration.
- The speed of migration of the solvent front is not constant. It follows a complex function in which the size of the particles of the stationary phase play a part. The migration velocity can be described by a quadratic law : $x^2 = kt$ where x represents the distance of the migration front, t the time and k is a constant (Figure 5.4). As a result the resolution between two spots depends greatly on the R_f values of the compounds. Resolution attains a maximum for an R_f value generally around 0.3.

To sum up, the efficiency N of a TLC plate is very variable. The height equivalent of a theoretical plate has, as in HPLC, an optimal value.

5.3 Stationary phases

Many physico-chemical parameters and several factors must be taken into account when choosing a good stationary phase. The size of the particles, their specific surface area, the volume of the pores and the distribution of particle diameters are all factors that define the properties of the stationary phase. For nano-TLC the size of the particles is of the order of $4\ \mu\text{m}$ and the pores are $6\ \text{nm}$.

The ratio between the silanols and siloxane groups determines the more or less pronounced hydrophilic character of the phase. As with HPLC, it is possible to use bonded silica in which various chains are bound by covalent bonds to silanol groups on the surface. Some phases incorporate alkyl chains (RP-2, RP-8, RP-18), while others contain organic functional groups (nitrile, amine, or alcohol),

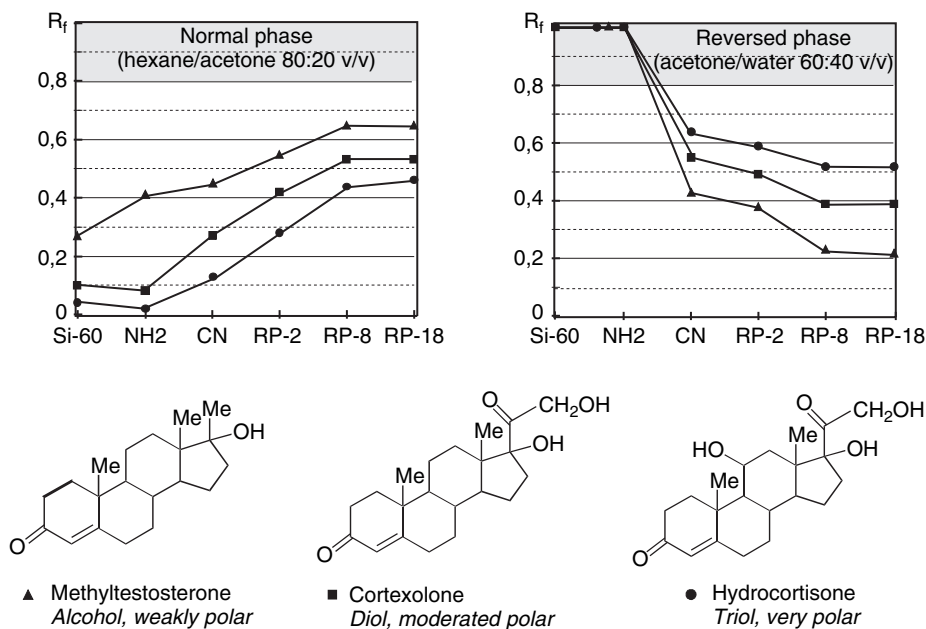


Figure 5.5 Study of the separation of three steroids of different polarities upon six stationary phases with two binary solvent systems. Evolution of the R_f values and inversed migration caused by the change of the eluting mobile phase, one polar and the other non-polar (reproduced courtesy of Merck).

allowing these phases to be used with numerous mobile phases having the appropriate pH and salt concentrations (Figure 5.5). TLC plates can also be prepared to contain chemical groups with net positive or negative charges on the surface. This type of plate is used for ion-exchange TLC.

Modified cellulose supports are also used in TLC, either as fibres or chemically modified micro-crystalline powders. The most widely known is DEAE-cellulose, a phase fairly basic containing diethylaminoethyl groups. Other polar phases, with ion exchange properties can be employed for the separation of ampholytes.

- Mineral binders such as gypsum make the stationary phase fragile, yet serve to advantage to recover the compounds after their separation. This can be done by scraping the zones of interest from the support and extracting the present compounds with a solvent.

5.4 Separation and retention parameters

Each compound is defined by its *retardation factor* R_f (unitless) that corresponds to its relative migration compared to the solvent. R_f values lie between 0 and 1.

$$R_f = \frac{\text{Distance run by the solute}}{\text{Distance run by the solvent front}} = \frac{x}{x_0} \quad (5.1)$$

The efficiency N of the plate for a compound whose migration distance is x and spot diameter w is given by Equation 5.2 and H (HETP) through Equation 5.3:

$$N = 16 \frac{x^2}{w^2} \quad (5.2)$$

and

$$H = \frac{x}{N} \quad (5.3)$$

In order to calculate the retention factor k of a compound or the selectivity coefficient between two compounds, the distances migrated along the plate are compared, for matching, with the migration times read on the chromatogram. Assuming that the *ratio* of the migration velocities u/u_0 is the same on the plate as on the column (which is really only an approximation), then R_f and k can be linked:

$$R_f = \frac{x}{x_0} = \frac{\bar{u}}{\bar{u}_0} = \frac{t_0}{t} = \frac{1}{k+1}$$

such that

$$k = \frac{1}{R_f} - 1 \quad (5.4)$$

R_f values often depend on the solvent use in the TLC experiment and temperature. Thus, the most effective way to identify a compound is to spot known substances on the same plate, next to the unknown.

Finally, by comparison with expression 1.27, the resolution can be given by the relation:

$$R = 2 \frac{x_2 - x_1}{w_1 + w_2} \quad (5.5)$$

5.5 Quantitative TLC

In order to use TLC as a quantitative method of analysis, it is essential to quantify the spots (Figures 5.1 and 5.6), along with definitions for all of the usual parameters (specificity, range of the domain of linearity, precision, etc.). This is done by placing the plate under the lens of a densitometer (or scanner) that can measure either absorption or fluorescence at one or several wavelengths. This instrument produces a pseudo-chromatogram that contains peaks whose areas can be measured. In fact it is actually an isochronic image of the separation at the final instant. In TLC a spot is usually detectable if it corresponds at least to a few ng of a compound UV absorbent.

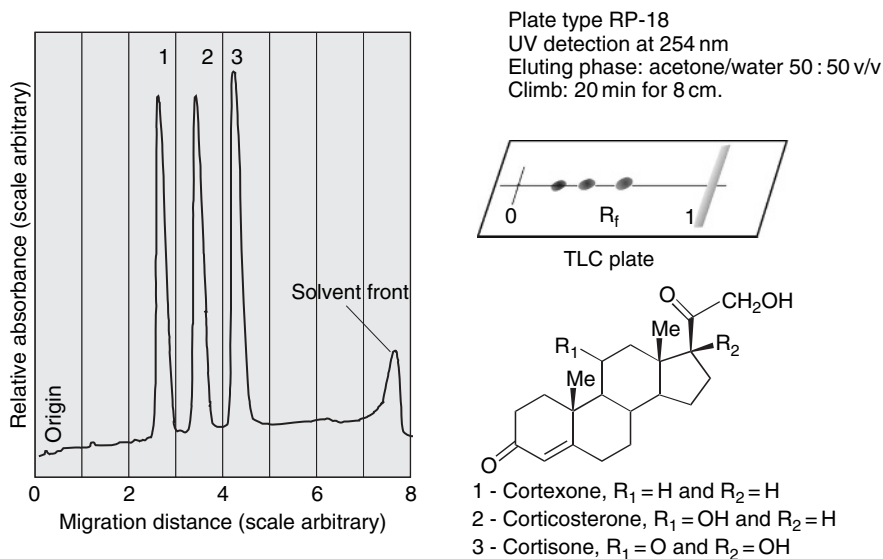


Figure 5.6 Separation of three steroids by TLC on a phase of reversed polarity. The migration distance increases with the polarity of the compound. This pseudo-chromatogram has been obtained by scanning the TLC plate. The same separation effected by HPLC would lead to a chromatogram in which the order of the peaks would be reversed, a compound strongly retained having the longest elution time.

■ In order to reveal radioactively labelled compounds (β^- emission), there exist densitometers that are equipped with a video camera giving an image of the radioactive distribution on the plate. The former procedure of autoradiography, obtained by putting a photoplate in contact with the TLC plate is rather insensitive (exposures could take up to 48 hours). The new densitometers, known as Charpak machines, which are also used in gel electrophoresis, have sufficient sensitivity for detecting activities in the order of a few Becquerels per mm^2 .

For many applications, TLC can replace HPLC (Figure 5.7). Although this technique requires more manual manipulations than HPLC, new improved tools for spotting, migrating, gradient elution, development and recording, confer the necessary reproducibility. Compared with HPLC, TLC is able to treat more samples in the same time period by the setting up of analyses in parallel on the same plate. The plate, only used once and disposable, allows for rapid sample preparation with less risk of loss or contamination. It is very useful for biological samples.

The TLC plate on which the products have been separated is also a means, provisionally, to preserve very small samples after extraction, which can serve for other analyses (mass spectrometry for example).

A recent technological advance allows the eluent to migrate at a constant speed through the application of positive gas pressure in the migration chamber, an effect especially developed which gains in both time and quality of separation.

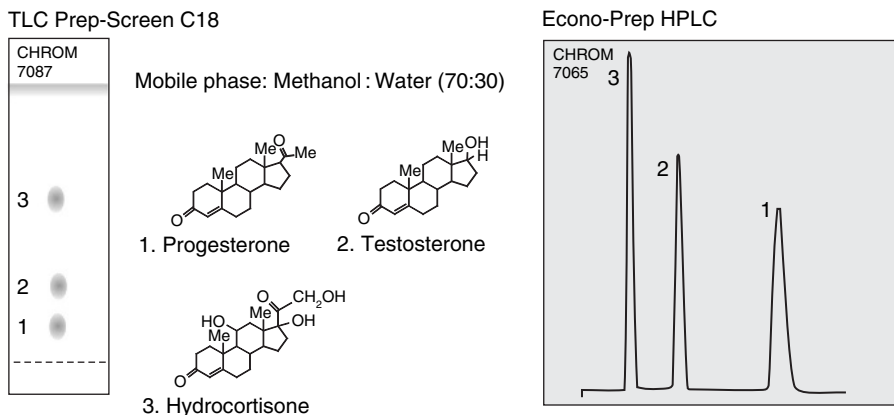


Figure 5.7 Comparison of TLC and HPLC. Separation of a mixture of three steroids on a TLC plate and on a HPLC column. This shows that it is possible to quickly perfect a separation by HPLC, using TLC at first, with the same stationary phase and the same mobile phase for development or elution (reproduced courtesy of Alltech).

High-performance thin layer chromatography HPTLC is an improvement of the technique where the sorbent material (e.g. silica gel 60) has a finer particle size and a narrower particle size distribution than conventional TLC. HPTLC plates have an improved surface homogeneity and are thinner. The resolution is improved, analysis times are shorter and it is sufficient to apply nanolitres or nanograms of sample (Nano-TLC).

Problems

5.1 A mixture of two compounds A and B migrates from the origin to leave two spots with the following characteristics (migration distance x and spot diameter w).

$$x_A = 27 \text{ mm} \quad w_A = 2.0 \text{ mm}$$

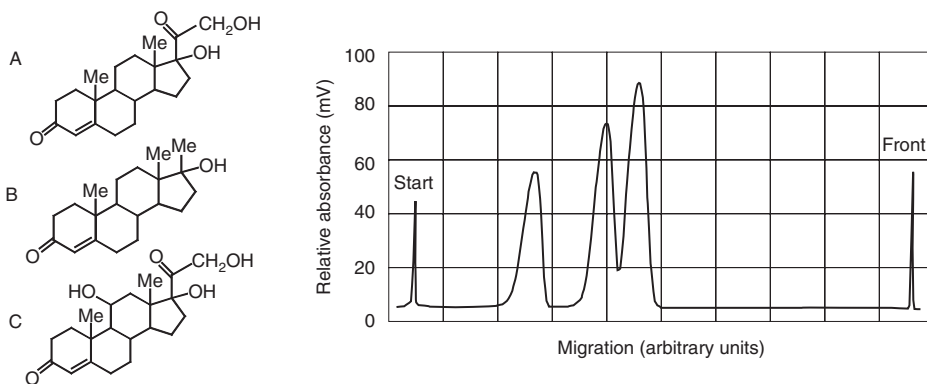
$$x_B = 33 \text{ mm} \quad w_B = 2.5 \text{ mm}$$

The mobile phase front was 60 mm from the starting line.

1. Calculate the retardation factor R_f , the efficiency N and the HETP H for each compound.
2. Calculate the resolution factor between the two compounds A and B.

- Establish the relation between the selectivity factor and the R_f of the two compounds. Calculate its numerical value.

5.2 The following figure represents the results of scanning a TLC plate in normal phase (mobile phase: hexane/acetone 80/20). The three compounds have the structures A, B and C



- Indicate compounds A, B and C, from the identification of the three principal peaks of the recording.
- What would have been the order of elution of these compounds if examined by an HPLC column containing the same types of stationary and mobile phases?
- What would have been the order of elution of these compounds if examined by an HPLC column containing a phase of type RP-18 with a binary mixture of acetonitrile/methanol (80/20) as eluent?
- Calculate the R_f and the HETP for the compound which migrates fastest upon the plate (use the transposed formulae of column chromatography, in particular that giving efficiency, with x as distance of migration).

6

Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) employs, as its name suggests, a fluid in the supercritical state as its mobile phase. This leads to improvements in the separations of thermolabile compounds and more generally for compounds of high molecular weight. The instrument is conceptually a hybrid of gas chromatograph and liquid chromatograph, with either GC capillary columns or HPLC columns, the latter being preferred. The late arrival of this technique on the instrumental market (about 1982), has been a handicap to its development, partly due to the fact that normalized methods have already been developed using other classic chromatography techniques. In addition to this argument the fact that the apparatus is more complex and expensive has meant that unfortunately few instrument manufacturers have been interested in the realization of corresponding analytical instruments. This presentation will aim to highlight the benefits of SFC.

6.1 Supercritical fluids: a reminder

The transformation of a pure compound from a liquid to a gaseous state and vice versa corresponds to a phase change that can be induced over a limited domain by pressure or temperature. For example, a pure substance in the gaseous state cannot be liquefied above a given temperature, called the critical temperature T_C , irrespective of the pressure applied to it. The minimum pressure required to liquefy a gas at its critical temperature is called the critical pressure P_C (Figure 6.1). These points are the defining boundaries on a phase diagram for a pure substance. The curve, which limits the gas and liquid domains, stops at the critical point C. Under these conditions, gas and liquid states have the same density. Above these temperatures and pressures, the compound becomes a supercritical fluid. In particular the viscosity of a supercritical fluid is almost that of a gas (Table 6.1).

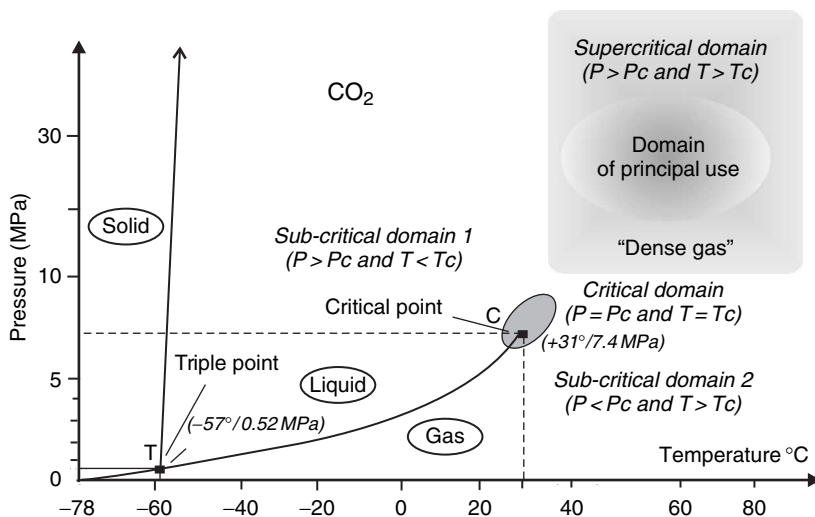


Figure 6.1 Phase diagram temperature/pressure of carbon dioxide. There exists for each pure substance a relation between three variables: temperature T , pressure P and volume V , known as the equation of state. The diagram above is the projection (P/T) for CO_2 . The critical point is located at 31°C and 7.4 MPa ($1 \text{ MPa} = 10^6 \text{ Pa}$, or 10 bar). Getting round the critical point renders it possible to go from the liquid state to the gaseous state without a discontinuity of phase.

Table 6.1 Comparison of densities, viscosities and diffusivities for liquid, supercritical fluid and gas

State	Density (g/mL)	Viscosity (poise $\times 10^3$)	Diffusivity ($\text{cm}^2/\text{s} \times 10^3$)
Liquid	0.8–1	3–24	0.005–0.02
Sup. fluid	0.2–0.9	0.2–1	0.01–0.3
Gas	0.001	0.05–0.35	10–1000

Furthermore its solvation properties (governed by the distribution coefficient K) are similar to those of a slightly polar organic solvent. In this way carbon dioxide, under 300 bars and at 40°C , is comparable to benzene for chromatographic applications.

Depending upon the choice of temperature and pressure, the behaviour of a supercritical fluid can sometimes look like a dense gas and sometimes like a liquid. For these reasons, use of supercritical fluids as mobile phases in chromatography presents certain advantages.

■ Carbon dioxide in its supercritical state can be used for the extraction, on a laboratory scale, of unstable compounds from the matrix. Many industrial applications use also this methodology to extract food products (e.g. decaffeination, recovery of aromas and spices, elimination of fats) or simply as a dry cleaning agent for

clothing. This non-flammable fluid has the advantage that it can be eliminated at low temperature without leaving any toxic residue. For these applications it is considered as a green chemistry partner. However, the use of high pressures linked with large volumes introduce safety concerns and create potential hazards in industrial installations.

6.2 Supercritical fluids as mobile phases

Carbon dioxide is the standard in SFC because its supercritical state is relatively easy to reach ($T_C = 31^\circ\text{C}$ and $P_C = 7400\text{ kPa}$) (Figure 6.1). Beyond these values, the supercritical domain is attained. This low cost compound is not very toxic, non-flammable, non-corrosive. The main disadvantage is its inability to elute very polar or ionic compounds.

More rarely used are nitrous oxide (N_2O , $T_C = 36^\circ\text{C}$, $P_C = 7100\text{ kPa}$) or ammonia NH_3 , $T_C = 132^\circ\text{C}$, $P_C = 11\,500\text{ kPa}$).

The density and therefore the solvating power of supercritical fluids varies according to the pressures to which they are submitted. As a consequence, a pressure gradient in SFC is equivalent to an elution gradient in HPLC, or a temperature gradient in GC.

If the chromatograph can accommodate a double gradient of temperature-pressure, combined with an organic modifier (to displace the coordinates of the critical point), it becomes possible to finely tune the retention of analytes and therefore to modify their selectivities α (Figure 6.2).

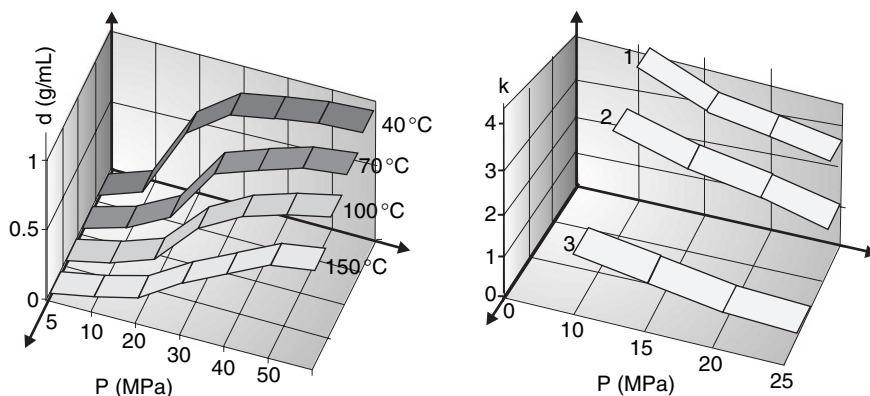


Figure 6.2 Density for carbon dioxide as a function of pressure at four different temperatures. At the critical point, the density of CO_2 is of 0.46 g/cm^3 . Right, the figure represents the variation of the retention factor k for three alkaloids analysed under identical conditions and pressure, fixed by a restrictor at the outlet of the column ($T = 40^\circ\text{C}$, modifier 5% water and 15% methanol; 1: codeine, 2: thebaine and 3: papaverine). The greater the increase in pressure the further the retention factor is reduced.

At 16 000 kPa and 60 °C carbon dioxide has a density of 0.7 g/mL (Figure 6.2). This is why the expression ‘dense gas’, is used in order to indicate that it is not a classic gas. To compensate its low polarity, which under these conditions is similar to that of toluene, an organic modifier (or enhancing agent) such as methanol, formic acid or acetonitrile is frequently added.

6.3 Instrumentation in SFC

The instrumentation for SFC represents hybrid assemblies of GC and HPLC instruments (Figures 6.3 and 6.4). To assure the flow rate of the supercritical fluid a syringe pump or a reciprocal pump is used, which is maintained below the critical temperature by means of a cryostat regulated at around 0 °C. In instances where an organic modifier is added, either a second pump or a tandem pump is utilized which has two chambers, one for the supercritical fluid and the other for the modifier. The liquid then passes through a coil maintained above the critical temperature in order to convert it to a supercritical fluid.

A main difference between SFC and HPLC instrumentation is the need for a back-pressure regulator in SFC, whose function is to restrict outlet flow in such a way to maintain the mobile phase in a supercritical state from the pump through to the end of the column, or even as far as the outlet of the detector depending upon the type chosen (Figure 6.3). The pressure regulator (also called

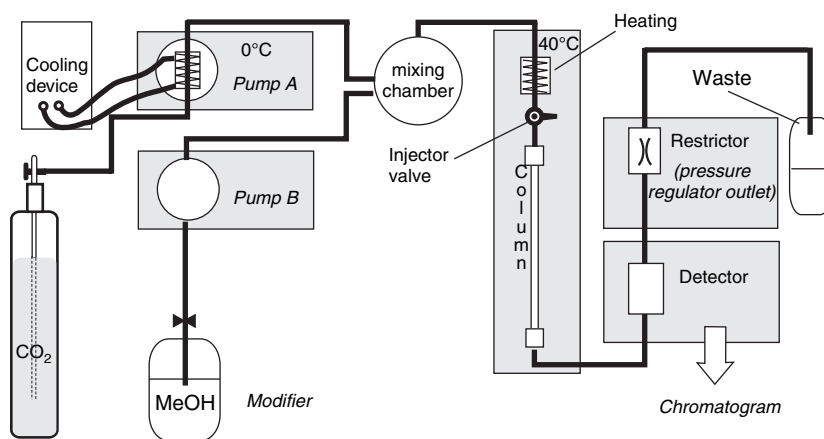
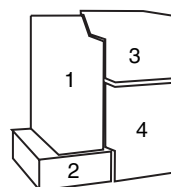


Figure 6.3 Schematic of a SFC installation using a HPLC packed column. Carbon dioxide reaches a supercritical state between the pump and the injector. A pressure regulator (‘restrictor’), is located after the column and either before or after the detector, depending upon its type. It maintains the mobile phase under supercritical conditions until the outlet of the column; A modifier added in small quantity (less than 10 per cent) enables to control the selectivity of the analytes (diagram based upon a document from Vydac).



- 1 - Oven for column
- 2 - UV detector
- 3 - Carousel type autosampler/injector
- 4 - Refrigerated pump

Figure 6.4 A commercial SFC installation. The oven where the column is located also controls the mobile phase pre-heating. The pump is cooled to 0°C (reproduced courtesy of Berger)

the *restrictor*) must correctly manage the important cooling procedure and the consequent volume expansion when the supercritical phase returns to the gas state at atmospheric pressure. Note that the presence of this restrictor avoids the large pressure drop across the column which is considered as a drawback of HPLC.

With a FID (a *flame ionization detector*, which functions at atmospheric pressure) the restrictor is situated before the detector, while it is located after for detectors based upon *UV-absorption, fluorescence or light diffusion*.

A large variety of normal phase HPLC columns (packed type) or GC columns (capillaries) are used. These two types of columns are complementary. For a packed column of HPLC type, the high flow rate of the supercritical mobile phase will render detection by FID or coupling with a mass spectrometer more difficult. Adding a modifier to the supercritical fluid would have the same effect.

6.4 Comparison of SFC with HPLC and GC

SFC is complementary to the other classic techniques of GC or normal phase HPLC. The migration of the solute results from a distribution mechanism between the apolar stationary phase and a slightly polar eluting mobile phase. The solvation capacity of the mobile phase is governed by both temperature and pressure of the supercritical fluid. Therefore as the density of the supercritical fluid mobile phase is increased, components retained in the column can be made to elute. The resistance to mass transfer between the stationary and the mobile phases is less than in HPLC because diffusion is about ten times greater than in liquids. The *C* factor in Van Deemter's equation being smaller, the velocity of the mobile phase can therefore be increased without an appreciable loss of efficiency (Figure 6.5). Moreover, as the viscosity of the mobile phase is close to that of a gas, GC

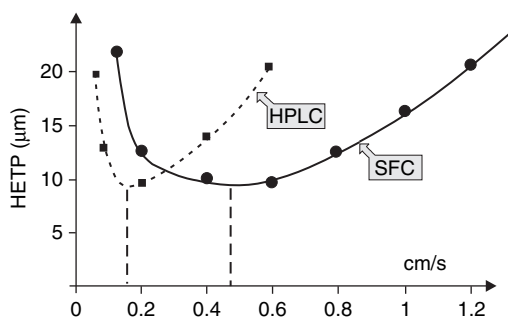


Figure 6.5 Comparison between HPLC and SFC. Both experimental curves have been obtained using the same compound on the same column, but, in one, a classic liquid phase and in the other, carbon dioxide in a supercritical state. The HETP are comparable – but the separation can be up to three times faster by SFC, thus saving analysis time.

capillary columns can be used. However, as a consequence of the position of the restrictor, the pressure drop across the column modifies the distribution coefficients of the compounds between the beginning and the end of their migration. This causes peak broadening. For this reason SFC can provide separations at low temperatures but the efficiencies met in capillary GC are not to be realized with SFC.

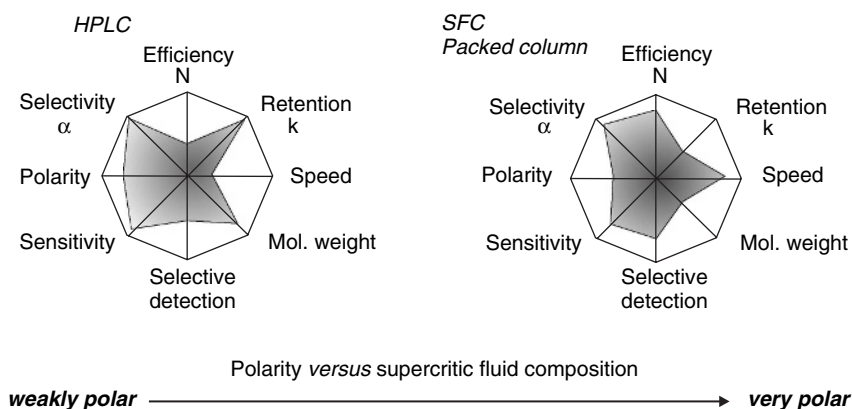


Figure 6.6 Comparison of SFC with HPLC packed columns. When mixed with methanol and additives, supercritical carbon dioxide allows the rebuilding of the whole range of polarities required by the principal types of analyte.

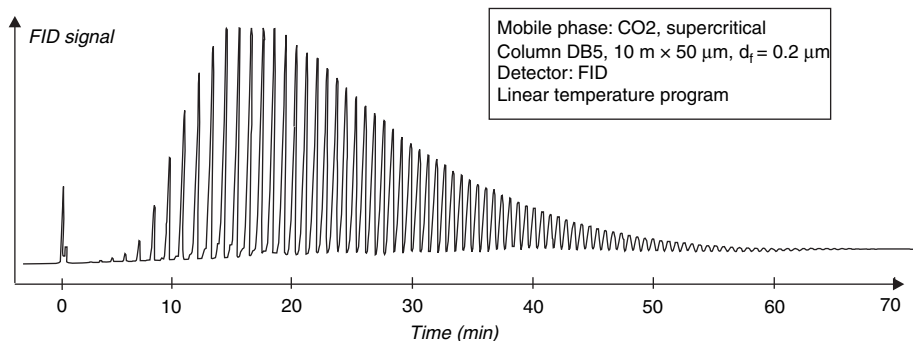


Figure 6.7 Spectacular SFC chromatogram of a mixture of polysiloxane oligomers (reproduced courtesy of Fisons Instruments Inc.). Each compound leads to a unique peak on the chromatogram, thus allowing the determination of the distribution of molecular forms in a polymerization reaction.

6.5 SFC in chromatographic techniques

SFC is potentially useful for various applications (Figures 6.6 and 6.7). The ability to vary selectivity by programming the parameters P (pressure) and T (temperature) rather than by modifying the chemical composition of the eluent represents the technique's major difference. The low viscosity of the mobile phase permits an arrangement of several HPLC-type columns in series. The range of compounds analysed by SFC includes lipids and oils, emulsifiers, oligomers and polymers (Figure 6.7), compounds of molecular mass greater than 1000 which cannot be studied in GC. SFC offers superior speed and efficiency compared to HPLC. Finally, use of supercritical carbon dioxide as a mobile phase facilitates coupling with a mass spectrometer or an infrared spectrophotometer and even with an NMR spectrometer.

7

Size exclusion chromatography

Size exclusion chromatography (SEC) is a method by which molecules can be separated according to their size in solution, thus relating indirectly to their molecular masses. To achieve this, stationary phases contain pores through which compounds are able to diffuse to a certain extent. Although the efficiency of separation can never attain that observed with HPLC, SEC has become an irreplaceable tool to separate natural macromolecules in order to study the distribution of synthetic polymer masses. Though the separation of compounds according to their sizes is not the most efficient process for small and medium molecules, this approach remains very useful in industry where the products are most often mixtures of compounds of very different masses. The instrumentation is comparable to that used in HPLC.

7.1 Principle of SEC

Size exclusion chromatography (SEC) is based upon the ability of the sample molecules to penetrate into the highly porous 'bead'-like structure of the stationary phase (Figure 7.1). Separation arises only as a result of the different degrees of penetration. Molecules of comparatively smaller weight are slowed in their progression in the column because they can enter into the stagnant mobile phase within the pores of the packing. This method is referred to as *gel filtration chromatography* (GFC) when the stationary phase is hydrophilic (the mobile phase being aqueous) and as *gel permeation chromatography* (GPC) when the stationary phase is hydrophobic (the mobile phase being a non-aqueous system).

The total volume V_M of the mobile phase in the column can be considered as the sum of two values $V_M = V_I + V_p$: the interstitial volume V_I (external to the pores) and the volume of the pores V_p .

V_I , called the *void volume*, represents the volume of mobile phase necessary to transport a large molecule assumed to be excluded from the pores and V_M is the volume accessible to a small molecule that can enter all the pores of the packing (volume V_S).

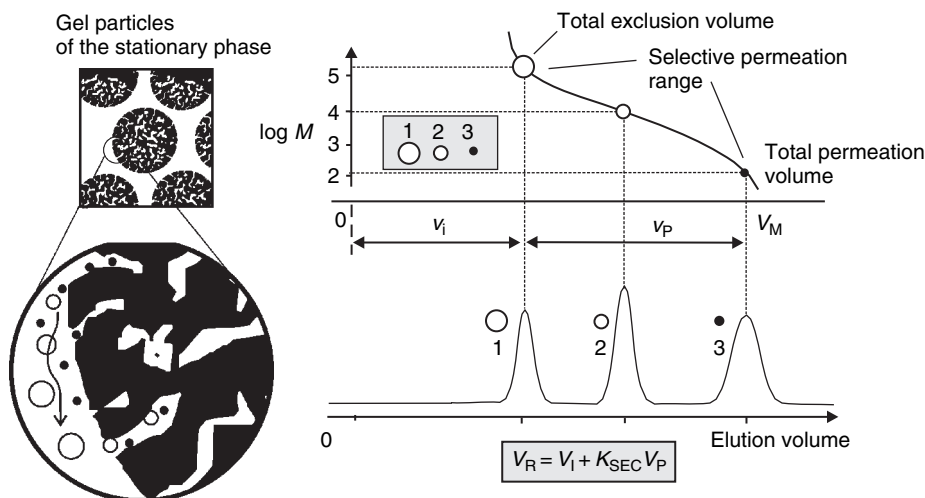


Figure 7.1 Migration across a stationary phase packing. Left, illustrative description of separation in SEC by a porous packing according to the size of the pores. The non porous part of the bead, called the backbone, is inaccessible to the sample molecules. Right, a chromatogram displaying the separation of three species (1, 2, 3) of different sizes. The large molecules (excluded) 1 are the first to arrive followed by medium sized molecules (partial access) 2, and finally by the smallest (full access) 3. The elution volumes V_R are located between V_I for $K_{SEC} = 0$ and V_M for $K_{SEC} = 1$.

The general expression giving the retention (or elution) volumes V_R are therefore (see Section 1.5)

$$V_R = (V_I + K_{SEC} V_P) + K V_S \quad (7.1)$$

K_{SEC} , the diffusion coefficient, represents the degree of penetration of a species dissolved in the volume V_P ($0 < K_{SEC} < 1$). Ideal SFC retention is only governed by the continuous exchange of solute molecules between the void volume and the stagnant mobile phase within the pores. If these conditions are attained, Nernst coefficient K is equal to zero and retention volumes V_R are comprised between V_I and V_M , giving:

$$V_R = (V_I + K_{SEC} V_P) \quad (7.2)$$

For the majority of modern packing materials, V_I and V_P are both of the order of 40 per cent of the volume of the empty column.

When V_R/V_M is greater than 1, the behaviour of the compound on the column no longer follows rigidly the mechanism of size exclusion but as in HPLC it undertakes physico-chemical interactions with the support ($K > 0$).

Each stationary phase is adapted to a separation range expressed in terms of two masses; a higher mass and a lower mass, above and below which there is no obtainable separation. Molecules whose diameter is larger than those of the greatest pores are excluded from the stationary phase (K_{SEC}). This explains the expression

size exclusion for this situation. They flow through the column without being retained or separated. They appear as a single peak in the chromatogram at the position V_1 (Figure 7.1). In contrast, the retention volume of the very small molecules is represented by $V_R = V_M$ (in this case, $K_{\text{SEC}} = 1$). The larger the part of sample molecules in the pores, the larger the retardation. To increase this range of separation, which is fixed by the difference between these two volumes, two or three columns can be put in a series, solvents of low viscosity are used and column are occasionally warmed. The diffusion coefficients K_{SEC} are independent of the temperature.

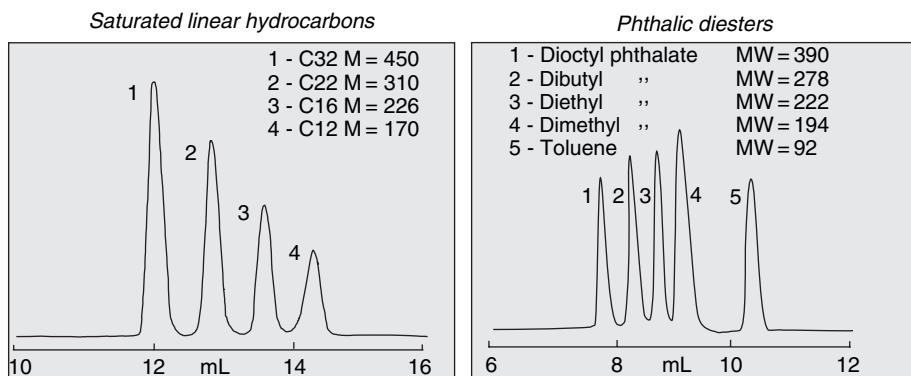
7.2 Stationary and mobile phases

SEC stationary phases are constituted of reticulated organic polymers or minerals that are used as porous rigid or semi-rigid beads (3 to 20 μm). Pores diameters are within the 4–200 nm range. These packings, usually called *gels*, must withstand the pressure at the head of the column and the temperature until about 100 °C in order to allow their utilization for various applications.

A reduction in the diameter of the particles – a gauge for efficiency – reduces the interstitial passages, rendering migration more difficult for the large, excluded molecules. For this reason, it is preferable to increase the size of the particles and to compensate by using a longer column. Standard columns have a length of 30 cm, (with an internal diameter of 7.5 mm). Their efficiency N can attain 10^5 plates/m.

SEC is sub-divided into two techniques:

- *gel permeation chromatography* (GPC). The material packing most often used is a copolymer *styrene-divinylbenzene* (PS-DVB) with an organic mobile phase such as tetrahydrofuran, a good solvent for most polymers. Trichloromethane as well as hot trichlorobenzene are also employed to dissolve synthetic polymers that are not soluble in other solvents (Figure 7.2). GPC is mainly used in chemical analysis.
- *gel filtration chromatography* (GFC). For aqueous SEC, stationary phases must be hydrophilic. Some are based upon a PS-DVB base particle that is made biocompatible with a hydrophilic coating containing hydroxyl or sulfonic groups. Others are based upon polyvinyl alcohols either pure or copolymerized with polyglyceromethacrylates or vinyl polyacetates (Figure 7.3). These packings are called *gels* since they swell on contact with aqueous mobile phases. Porous silica gels containing hydrophilic surfaces are equally employed (presence of glyceropropyl groups) [$\equiv \text{Si}(\text{CH}_2)_3\text{--O--CH}_2\text{CH}(\text{OH})\text{CH}_2\text{OH}$]]. Adsorption phenomena ($K > 0$) are weak, even for the small molecules. GFC is mainly used to separate bio-polymers (e.g. polysaccharides) or other water-soluble biological macromolecules (e.g. proteins).



Conditions: Column, PLgel 5 m, Pores, 5 nm, Dimensions, 300 × 7.5 mm.
Eluent: tetrahydrofurane. Flow-rate: 1 mL/min.

Figure 7.2 GPC. When the stationary phase has small pores, organic compounds of small to medium molecular weight can be separated. In the chromatogram in the right, toluene serves to measure V_M (reproduced courtesy of Polymer Laboratories).

- The term gel filtration should not be confused with the current procedure of filtration which would create the reverse effect. The larger molecules have a greater difficulty than the smaller ones in crossing the filter.

7.3 Calibration curves

For a given solvent, each stationary phase is described by a calibration curve made with isomolecular standards of known masses, M : polystyrenes in THF, polyoxyethylenes, pullulanes or polyethyleneglycols, etc. (Figure 7.3 and Table 7.1). The curves representing $\log M$ as a function of the elution volume have a sigmoidal shape. However, by combining stationary phases of differing porosities, manufacturers can provide mixed columns for which the calibration curve are linear over a broader range of masses. These curves are fairly indicative since size and mass are not mutually dependent parameters when passing from one polymer to another.

Table 7.1 Permeation range (Da) of three gels for various standard compounds

Standard	G2000: 12.5 nm pores	G3000: 25 nm pores	G4000: 45 nm pores
Globular protein	5000–100 000	10 000–500 000	20 000–7 000 000
Dextran	1000–30 000	2000–70 000	4000–500 000
Polyethylene glycol	500–15 000	1000–35 000	2000–250 000

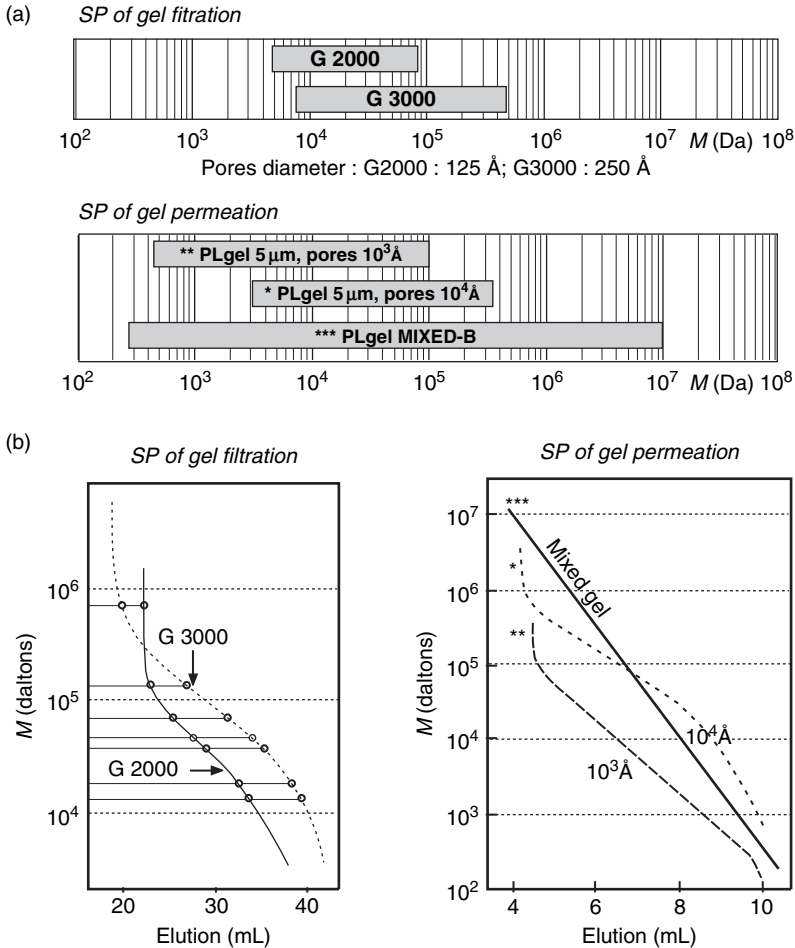


Figure 7.3 Characteristics of stationary phases used for gel filtration and GPC columns. (a) Graphs indicating the mass ranges for two phases in gel filtration and for three phases in gel permeation; (b) Calibration curves ($\log M = f(V)$) for these different phases obtained with proteins for gel filtration and with polystyrene standards for the others (known molecular weights). A weak slope from the linear section reveals a better resolution between neighbouring masses. This is the case when the pores are of regular dimension. The curves $\log M = f(K)$, more rarely studied, reveal the same aspect (reproduced courtesy of Tosohaas and Polymer Lab.). To avoid protein aggregate formation, denaturing compounds are sometimes introduced to the aqueous mobile phase.

7.4 Instrumentation

Instrumentation is similar to that employed in HPLC excepting the columns which are bigger. To increase the resolution it is customary to use two or three columns, with different porosities, in series.

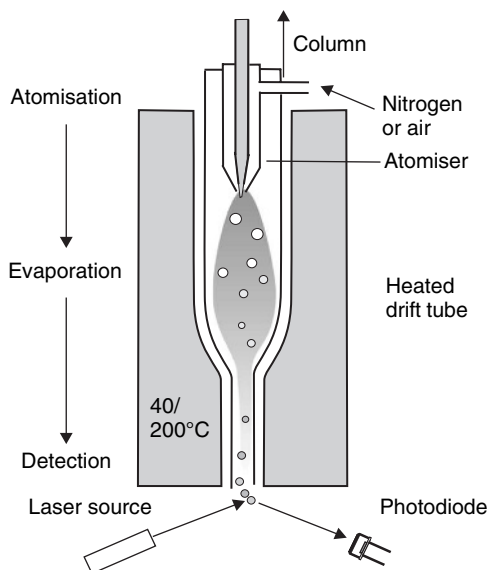


Figure 7.4 *Evaporative light scattering detector.* At the outlet of the column the mobile phase is nebulized under a stream of nitrogen, by a specially atomizing device. When a compound elutes from the column, the droplets under evaporation give a suspension of fine particles. Illuminated by a laser source they scatter the light from a lamp via the Tyndall effect (what happens is comparable to the diffusion by fog through a car headlight beam). The signal detected by a photo-diode is proportional to the concentration of the compound illuminated. Irrespective of the substance, the response factors are very close. This detector is only useful for sample components that cannot be vaporized in the heated section of this detector.

The most commonly used detector is the refractive index detector (cf. Section 3.11.3), considered as universal since for polymers a variation of the refractive index is at first approximation independent of the molecular mass. As this detector is not very sensitive, other detectors are sometimes added to it. They are based upon the light absorption (UV detector) or fluorescence or light scattering (Figure 7.4). This last detector provides a more uniform response to structurally similar analytes than do light absorption detectors. Users can create a universal calibration set from a single analyte to quantify all analytes of the same class.

7.5 Applications of SEC

The main applications are found in the analysis of synthetic polymers or biopolymers because this method permits the separation of nominal masses ranging from 200 to more than 10^7 Da. The absence of a chemical interaction with the stationary phase, associated with a rapid elution time and the possibility to recover all of the analytes, convey many advantages.

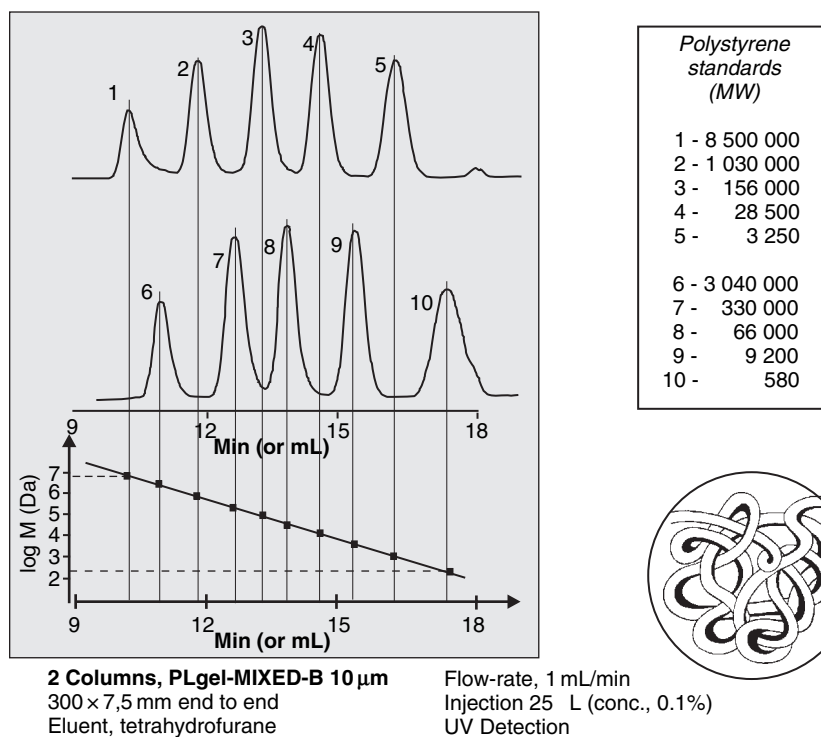


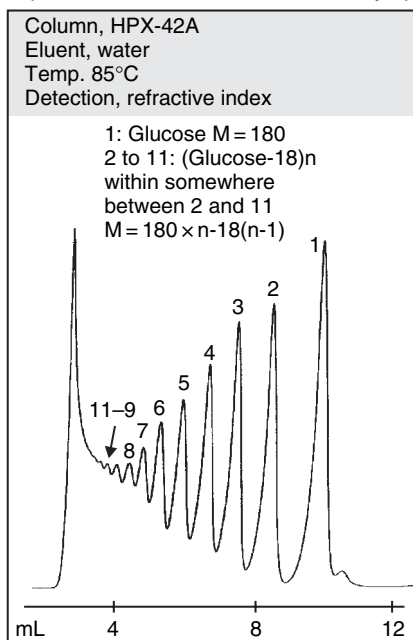
Figure 7.5 Determination of molecular weight in a SEC experiment. The column can be calibrated by using two complementary mixtures of polystyrene standards. Log molecular weight versus retention volume plot. This curve is linear over a wide range of masses due to the use of a mixed stationary phase. Bottom right, conformation supposed for a lipophilic rod or random coil polymer in an aqueous solution. The resulting volume is called *hydrodynamic volume* of the macromolecule.

The choice of stationary phase best adapted to a given separation is made by consultation of the calibration curve of various columns. The column of choice is that which presents a linear range over the masses of the compounds formed in the sample (Figure 7.5). The calibration must be carried out with the same type of standards, because macromolecules can have a variety of forms, from pellet like to thread like (Table 7.1).

7.5.1 Molecular weight distribution analysis

Synthetic polymers differ from small molecules in that they cannot be characterized by a single molecular weight. Even in its pure state, a polymer corresponds to a distribution of macromolecules of different weights. SEC chromatograms and integrated peak areas allow the determination of the molecular weight distribution

Separation of the constituents in corn-syrup



Separation of a mixture of peptides

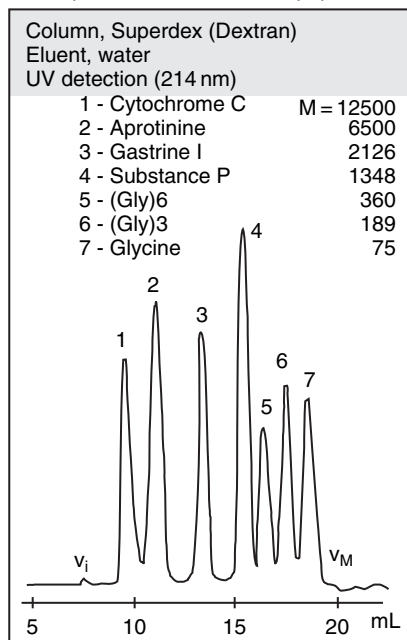


Figure 7.6 Gel filtration chromatogram. Left, separation of a mixture of glucose oligomers ranging from a single unit (glucose $M = 180$), up to 20 units (M around 3000) (reproduced courtesy of Polymer Lab). Right, separation of a mixture of diverse proteins and glycine oligomers (reproduced courtesy of Pharmacia-Biotech).

as well as the most probable mass and the mean mass. This assumes that the mass and the molecular volumes are directly related. Furthermore, the calibration of the column must be done with standards of the same family and with the same mobile phase flow rate.

7.5.2 Other analyses

SEC, which allows high-speed separations, is used for control analysis of samples with unknown compositions. These samples usually contain polymers and small molecules, which is often the case for numerous commercial or industrial products, such as in the biodegradation of polymers.

For typical organic compounds, which can readily be analysed by HPLC or GC, there are fewer applications unless they are sugars or polysaccharides (Figure 7.6) such as starch, paper pulp, beverages and certain pharmaceuticals. SEC with gel columns are widely used for aqueous separations of bio-molecules.

Problems

- 7.1 Occasionally in SEC values of $K > 1$ are observed. How might this phenomenon be accounted for?
- 7.2 Gel permeation chromatography is to be used to separate a mixture of four polystyrene standards of molecular mass: 9 200, 76 000, 1.1×10^6 and 3×10^6 daltons. Three columns are available for this exercise. They are prepacked with gel with the following fractionation ranges for molecular weights:

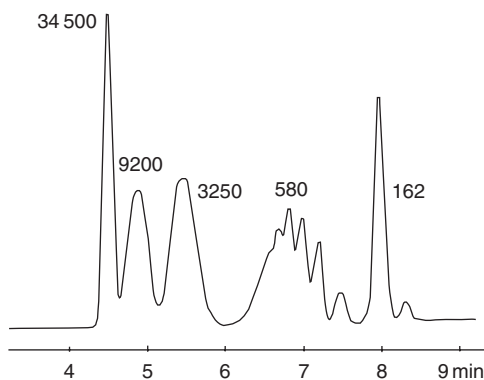
A: 70 000 to 4×10^5 daltons

B: 10^5 to 1.2×10^6 daltons

C: 10^6 to 4×10^6 daltons

How might these four polymers be separated in a single operation if it is permitted to use two of the above columns end to end?

- 7.3 A solution in THF of a set of polystyrene standards of known molecular mass was injected onto a column whose stationary phase is effective for the range 400–3 000 daltons. The flow rate of the mobile phase (THF) is 1 mL/min. The chromatogram below was obtained.



1. Plot the log of relative molecular mass *vs.* retention volume.

2. What is the total exclusion volume for the column (i.e. the interstitial volume), and what is the volume of the pores (the intraparticle volume)?
3. Calculate the diffusion coefficient K_{SEC} for the polystyrene standard whose relative molecular mass is 3 250 daltons.

8

Capillary electrophoresis and electrochromatography

Capillary electrophoresis (CE) is a very sensitive separation method which has been developed largely through the experience acquired from high performance liquid chromatography, thin layer chromatography and from electrophoretic methods. Bio-molecules and compounds of lower molecular mass, difficult to study by HPLC or classical methods of electromigration on slab gel, become separable by CE. From now on, high performance capillary electrophoresis (HPCE), suited to automation, has replaced the traditional electrophoretic techniques on gels. CE, officially recognized by several regulatory agencies, the FDA among them, included in the pharmacopoeia, can be used for quantitative analysis. Capillary electrochromatography (CEC) is a relatively new hybrid separation method that couples the high separation efficiency of CE with HPLC.

8.1 From zone electrophoresis to capillary electrophoresis

Capillary electrophoresis corresponds to an adaptation of the more general electrophoresis methodology. This separative technique is based upon the differential migration of the species, whether or not they carry an overall electric charge, present in the sample solution, under the effect of an electric field and when supported by an appropriate medium.

In the classic semi-manual electrophoretic technique used mainly in bio-analysis, a small slab or strip of plastic material covered by a porous substance (a gel) is impregnated with an electrolyte buffer. The two extremities of the covered gel system are dipped into two independent reservoirs containing the same electrolyte and linked to the electrodes of a continuous voltage supply (Figure 8.1). The sample is deposited in the form of a transverse band, which is cooled and then bedded between two isolating plates. Under the effect of several parameters that

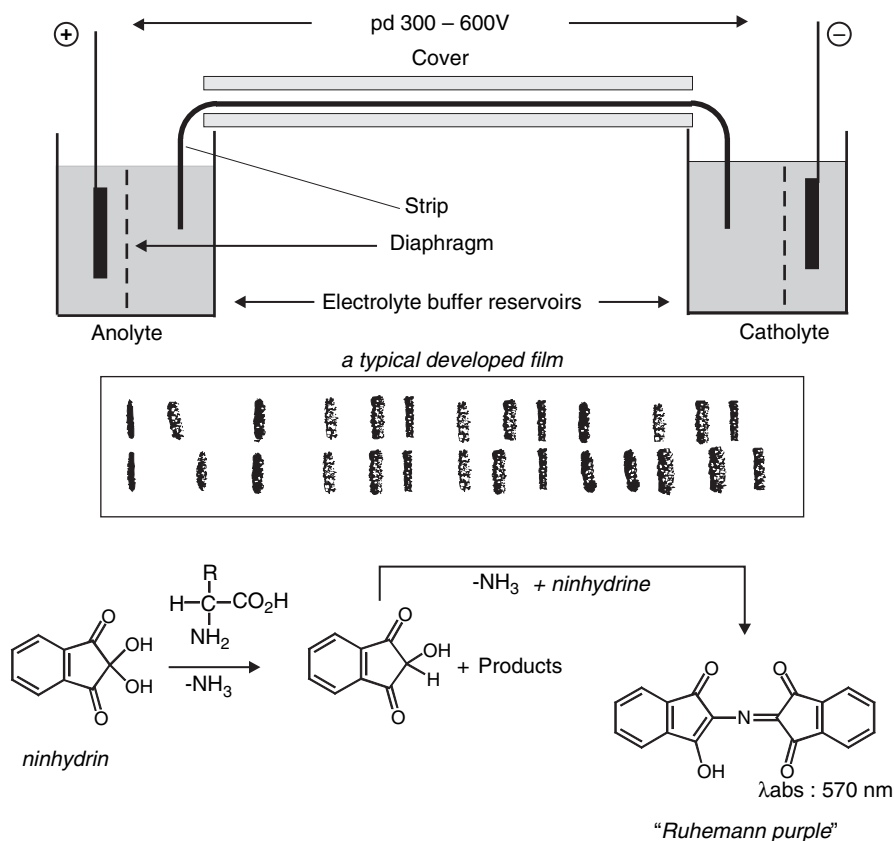


Figure 8.1 Schematic of a horizontal electrophoresis apparatus. Top, each compartment is separated by a diaphragm in order to avoid contamination of the electrolyte by secondary products which are formed during contact with the electrodes. This technique is operated either at constant current or at constant voltage or at constant power. The flat gel is between two plates of glass. Middle, aspect of a standard strip support after revelation. Bottom, ninhydrin is often used to reveal proteins or amino acids. This reagent is transformed on contact with an amino acid that it degrades, to an unstable compound intermediate which reacts in turn with a second molecule of ninhydrin yielding 'Ruhemann purple'. This reagent is one of several compounds existing (e.g. Fluorescamine) to determine position of particular species following migration on the strip. Horizontal gels offer many advantages for nucleic acid separation and remain a widely-used tool for the molecular biologist.

act jointly – voltage (500 V or more in the case of small molecules), charge, size, shape, temperature and viscosity – the hydrated species migrate from one end to the other, generally towards the electrode or pole opposite sign on a time scale that can vary from a few minutes to over an hour. Each compound is differentiated by its mobility but the absence of a measurable solvent front, compared to TLC, requires that the distance of migration is estimated in relation to that of a substance

used as an internal marker. The detection of species, following migration, through a contact process, are transferred onto a membrane where they are derivatized with specific reagents as silver salts, or Coomassie Blue® (Figure 8.1). Markers containing radioactive isotopes (^{32}P or ^3H) can also be used to give traces that can be exploited in the same manner as in TLC.

In capillary electrophoresis, the covered gel slab of the classic technique is replaced by a narrow-bore open-ended fused silica capillary (15–100 μm diameter). The capillary of length L , varying between 20 and 80 cm, is filled with an electrolyte aqueous buffer solution as the two reservoirs (Figure 8.2). For a better control of the migration time, it is advisable to place the capillary in a thermostatic oven. A small inner diameter of the capillary reduces Joule heating to negligible levels and allows the use of high electric fields for very rapid separations (the voltage applied to the electrodes can attain 30 kV).

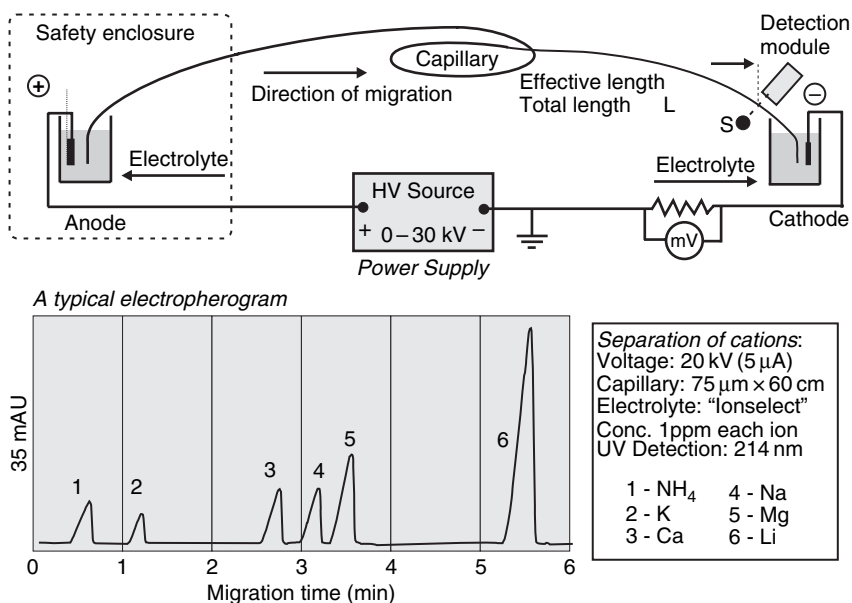


Figure 8.2 Schematic of a capillary electrophoresis instrument. The electrolyte is an aqueous ionic solution, filtered, degassed and containing a variety of additives. A small volume of the sample is injected at the positive end of the capillary (cf. 8.3). For a voltage greater than 5–600 V/cm (30 kV if $L = 50$ cm) special insulation becomes necessary to avoid shorts and arcs of current in a humid atmosphere. For safety reasons, one electrode is usually at ground. The effective distance of migration l is around 10 cm shorter than the length L of the capillary. Below is a typical electropherogram corresponding to the separation of several cations (ordinate given in units of milli-absorbance). The distortion of the peaks is due to electrodispersion. This phenomenon is caused by the differences in conductivity, and hence field, in each zone. The peaks would be symmetrical if their speeds were closer together (reproduced courtesy of Waters).

A detector is placed near the cathode compartment, at a distance l from the head of the capillary. The signal obtained provides the baseline for the electropherogram (Figure 8.2) which yields information about the composition of the sample. The only species detected are those which are directed towards the cathode.

8.2 Electrophoretic mobility and electro-osmotic flow

Particles suspended in a liquid, like solvated molecules, can carry electric charges. The sign and size of the charge depend upon the nature of the species, that of the electrolytic medium as well as on the pH (Figure 8.3). This net charge originates from the fixation to the particle surface, of ions contained in the buffer electrolyte.

Under the effects of various phenomena or simultaneous actions such as temperature, viscosity, voltage, these particles will have migration velocities that will be faster, the smaller they are and the greater their charge. The separation depends upon the charge-to-size ratio of the hydrated analyte ions.

For each ion the *limiting migration velocity* v_{EP} results from the equilibrium between the electric force F , which is exerted in the electric field E acting on the particle of charge q , and the forces resulting from the solution viscosity η . Neutral

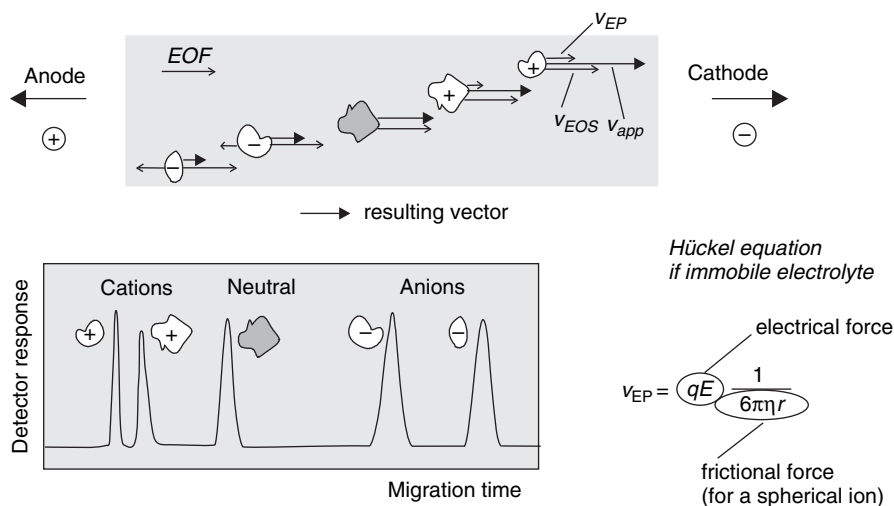


Figure 8.3 *Hückel equation and typical electropherogram.* Influence of the net charge, electric field, volume of the ion and viscosity of the solution upon the migration velocity in an electrolyte animated by an electro-osmotic flow (cf. Section 8.2.2). Higher charge and smaller size confer greater mobility, whereas lower charge and larger size confer lower mobility. The separation depends approximately upon the charge-to-size ratio of each species. Uncharged species move at the same velocity as the electroosmotic flow (see later). The smaller anions arrive last since they would normally go towards the anode. At low pH, electro-osmotic flow is weak and anions may never reach the detector.

species are poorly separated except if an ionic agent is added to the electrolyte in order to associate with them.

Hückel proposed an equation taking account of the influence of these factors upon the electrophoretic velocity v_{EP} of an ion considered as a sphere of radius r .

The charged species present in the sample are submitted to two principal effects, which are their individual *electrophoretic mobility* and the more global *electro-osmotic flow*.

8.2.1 Electrophoretic mobility – electromigration

A compound bearing an electric charge move within the electrolyte *assumed to be immobile* at a velocity v_{EP} (m/s) which depends upon the conditions of the experiment and of its own electrophoretic mobility μ_{EP} . For a given ion and medium, μ_{EP} is a constant which is characteristic of that ion. This parameter is defined from the electrophoretic migration velocity of the compound and the exerted electric field E , using expression 8.1:

$$\mu_{EP} = \frac{v_{EP}}{E} = v_{EP} \frac{L}{V} \quad (8.1)$$

In the above equation, L designates the total length of the capillary (m) and V the voltage applied across the extremities of the capillary. A positive or negative sign is assigned to the electrophoretic mobility, according to the cationic or anionic nature of the species; μ_{EP} ($\text{m}^2/\text{V}/\text{s}$) is equal to zero for a globally neutral species. μ_{EP} can be obtained from an electropherogram by calculating v_{EP} of the compound in a known electric field E (V/m), taking into account of the velocity of the electrolyte (cf. Section 8.2.2). This parameter depends not only on the charge carried by the species, but also on its diameter and on the viscosity of the electrolyte.

8.2.2 Electro-osmotic mobility – electro-osmotic flow (EOF)

The second factor that controls the migration of the solute is the *electro-osmotic flow*. This flow corresponds to the bulk migration of the electrolyte through the capillary. In gel electrophoresis, this flow is small, but in capillary electrophoresis it becomes more important because of the internal wall of the capillary. It is characterized by the *electro-osmotic mobility* μ_{EOS} , as defined by a relation similar to 8.1. v_{EOS} represents the velocity of the electro-osmotic flow.

$$\mu_{EOS} = \frac{v_{EOS}}{E} = v_{EOS} \frac{L}{V} \quad (8.2)$$

In order to calculate μ_{EOS} , the electro-osmotic flow velocity v_{EOS} must first be determined. This corresponds to the velocity in the electrolyte of a species without

any global charge. This is done by measuring the migration time t_{nm} for a *neutral marker* to migrate over the distance l of the capillary ($v_{EOS} = l/t_{nm}$).

As a neutral marker, an organic molecule that is non-polar at the pH of the electrolyte used and easily detected by absorption in the near UV, is selected (e.g. acetone, mesityl oxide or benzyl alcohol).

The electro-osmotic flow has several origins in relation with the internal wall of the capillary. A capillary made of fused silica not having been subjected to any particular treatment bears on its inner surface numerous silanol groups (Si-OH) which become ionized to silanoates (Si-O⁻) when the pH of the electrolyte rises above 3. Under these conditions, a fixed polyanionic layer is formed that attracts cations present in the electrolyte. These cations arrange themselves into two layers of which one is attached to the internal wall while the other remains slightly mobile (Figure 8.4). Between these two layers a potential difference (Zeta potential) develops, the value of which depends upon the concentration of the electrolyte and the pH. When the electric field is applied, H₃O⁺ and ions being solvated by water molecules migrate towards the cathode. In capillary electrophoresis instruments, the net effect of the electro-osmotic flow is to impose for all charged species present in the sample the direction oriented from the anode towards the cathode. The anions progress in an anti-electro-osmotic fashion.

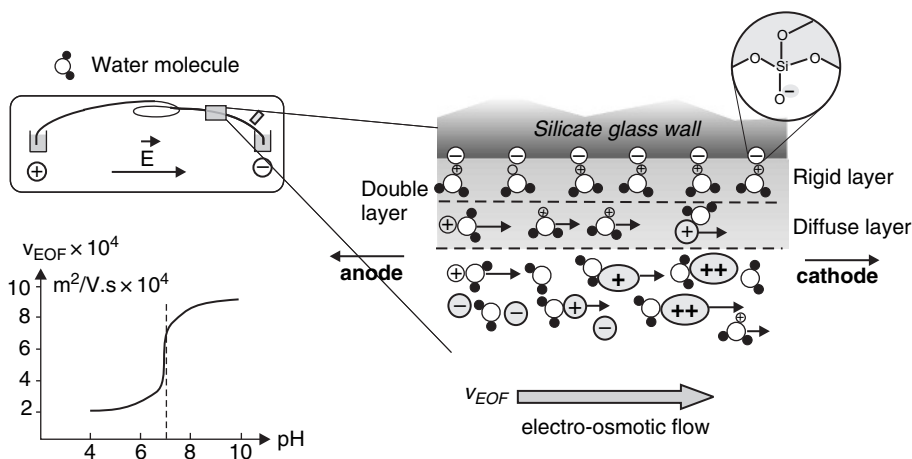


Figure 8.4 Origin of the electro-osmotic flow in a capillary filled with an electrolyte. Model of the double layer. If the inner wall has not been treated (polyanionic layer of a silica or glass capillary) then a pumping effect arises from the anodic to the cathodic compartment: this is the electro-osmotic flow which is reliant upon the potential which exists on the inner surface of the wall. If the wall is coated with a non polar film (e.g. octadecyl) then this flow no longer exists. The electro-osmotic flow is proportional to the thickness of the double cationic layer attached to the wall. It is reduced if the concentration of the buffer electrolyte increases. v_{EOS} is pH dependant: between pH 7 and 8 v_{EOS} can increase by as much as 35 per cent.

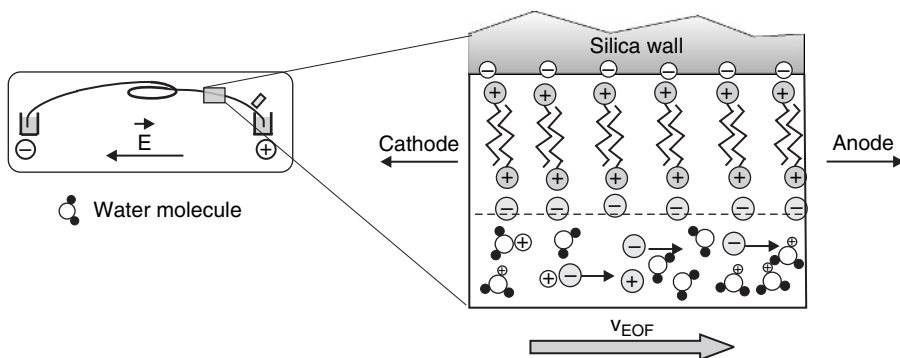


Figure 8.5 Effect of a cationic surfactant upon the silica inner wall. As the migrations must always be made in the direction where the detector is, the voltage polarity of the instrument must therefore be reversed in order for anionic species to progress naturally in the same direction as the electrolyte, that is, toward the detector.

■ Usually, a capillary with a negative inner surface generates a linear displacement of the electrolyte (an electro-osmotic flow) directed towards the cathode. In contrast, if a surfactant is added, such as tetra-alkyl ammonium, capable of reversing the polarity of the inner wall, then the electro-osmotic flow is directed towards the anode (Figure 8.5). By treating the wall with an alkylsilane to make it hydrophobic, proteins become separable, otherwise they tend to adsorb at the surface of bare fused silica.

8.2.3 Apparent mobility

According to the previous arguments, each ion has an apparent migration velocity v_{app} which depends on both the electrophoretic velocity and the electro-osmotic flow (relation 8.3):

$$v_{app} = v_{EP} + v_{EOS} \quad (8.3)$$

v_{app} is easily calculated from the electropherogram. Using l the effective length of the capillary, and t_m , the migration time, v_{app} is given by the expression:

$$v_{app} = l/t_m \quad (8.4)$$

The apparent electrophoretic mobility μ_{app} is defined by an expression analogous to 8.1 or 8.2, such that:

$$\mu_{app} = \frac{v_{app}}{E} = v_{app} \frac{L}{V} \quad (8.5)$$

and, by consequence

$$\mu_{app} = \frac{l \cdot L}{t_m \cdot V} \quad (8.6)$$

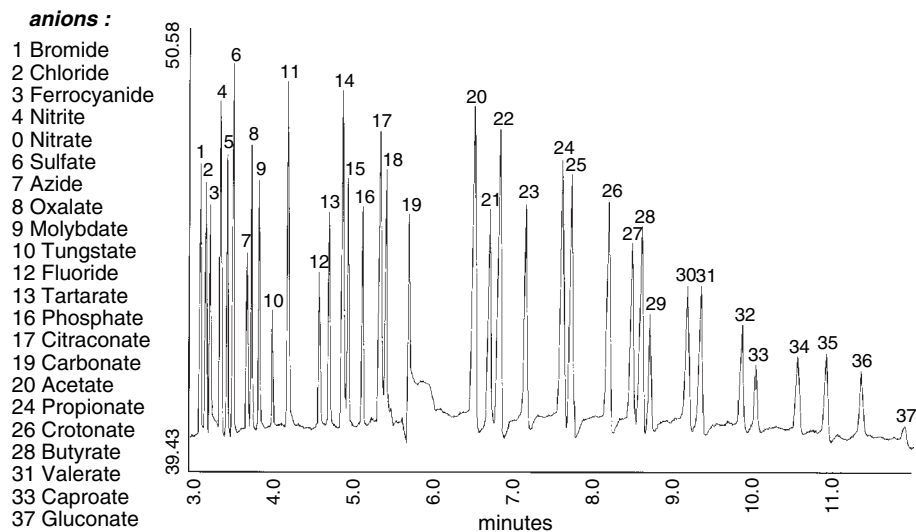


Figure 8.6 An electropherogram. Separation of a complex mixture of anions. The lack of peak symmetry (see also Figure 8.2), is due to variations in the electric field along the whole length of the capillary caused by the dynamic ionic composition (image courtesy of ATI Unicam).

By combining the electro-osmotic flow and the apparent mobility it is possible to calculate the true electrophoretic mobility of the species carrying charges. From relation 8.3 it can be written:

$$\mu_{EP} = \mu_{app} - \mu_{EOS}$$

or alternatively

$$\mu_{EP} = \frac{L \cdot l}{V} \cdot \left(\frac{1}{t_m} - \frac{1}{t_{mn}} \right) \quad (8.7)$$

■ HPCE is commonly used for the separation of anionic species. Generally the polarity of the instrument is reversed in order to change the location of the detector from the cathode end to the anode end of the capillary. As a result the electro-osmotic flow direction is also reversed. Only anions whose μ_{EP} values are greater than those of μ_{EOS} will be detected (Figure 8.6).

8.3 Instrumentation

8.3.1 Different modes of injection

To introduce a micro-volume of sample, which must not exceed 1 per cent of the effective length of the capillary in order to protect the resolution, two procedures are used:

- *Hydrostatic injection.* This action is achieved by dipping the end of the capillary into the solution (also an electrolyte) containing the sample while creating a slight vacuum at the opposite end. The procedure can be improved by exerting a pressure of around 50 mbars upon the sample solution.
- *Electro-migration injection.* This technique, used in gel electrophoresis, is achieved by putting the sample at a potential of appropriate polarity compared to the other extremity and briefly dipping the capillary into it. In contrast to the previous procedure this mode of injection induces a discriminatory action upon the compounds present, which leads to a non-representative composition of the sample.

The hydrostatic injection method is less precise than in HPLC because injection loops do not exist for volumes between 5–50 nL. The quantity entering the capillary is dependent upon many of the parameters that appear in the well-known Poiseuille expression which gives the flow rate F in a tube (radius r , length L) for a liquid having a dynamic viscosity η (expression 8.8). The application of this formula results in an approximate value for what might be termed the ‘entering flow rate’ in the capillary.

$$F = \Delta P \frac{\pi r^4}{8\eta L} \quad (8.8)$$

This relationship reveals that if the radius of a capillary is doubled, then the volume entering will be 16 times greater. This volume is also proportional to the difference in pressure ΔP . An increase in the length of the capillary L produces

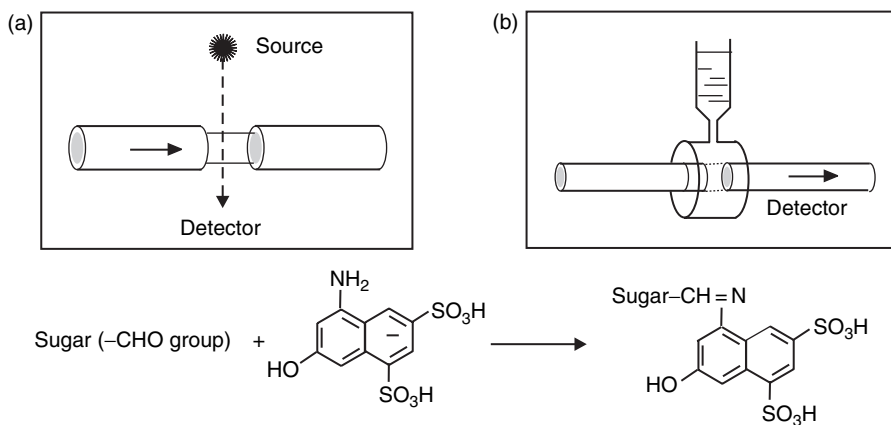


Figure 8.7 *UV detection and post-separation derivatization.* Analytes passing the light source absorb UV radiation resulting in a decrease in light reaching the detector. (a) The measuring cell comprising the capillary itself. (b) Post-derivatization can be undertaken by cutting the capillary just prior to the detector and by introducing an appropriate reagent. Below, example of a reaction to transform a sugar into a fluorescing derivative.

the reverse effect. An internal standard should be used to improve the quantitative performances of HPCE.

8.3.2 Detection methods

Many of the methods of detecting analytes in CE are similar to those used for liquid chromatography. Only three important methods are given here.

- *Direct UV/Vis detection* (cf. Chapter 9). Detection is usually done by UV-visible absorbance with a diode array detector. A very small window is created by burning the polyimide protecting the capillary in such a way that UV light crossing the capillary can be measured. This design is very efficient as the detector cell is basically part of the capillary, which avoids a dead volume (Figure 8.7). UV is most sensitive when used at low wavelengths.
- *Fluorescence detection*. This detection implies a derivatization of the analytes, which are chemically labelled with a fluorphore. Then separation is performed as normal. A light source is used as a source of radiation, and as the analytes move past the detection window the fluorphores emit radiation at a different wavelength. The sensitivity of this procedure is enhanced if a very intense laser source is employed (Figure 8.7).
- *Mass spectrometry detection*. A mass spectrometer linked to a CE instrument allows information on solutes molecular masses and provides structural information helping with the identification of unknowns. However, interfacing of the two instruments is not easy (cf. Chapter 16). Thus an extra flow of liquid must be added to the CE eluent to obtain gas phase ions of the solutes. MS detection is used especially in biochemistry analysis.

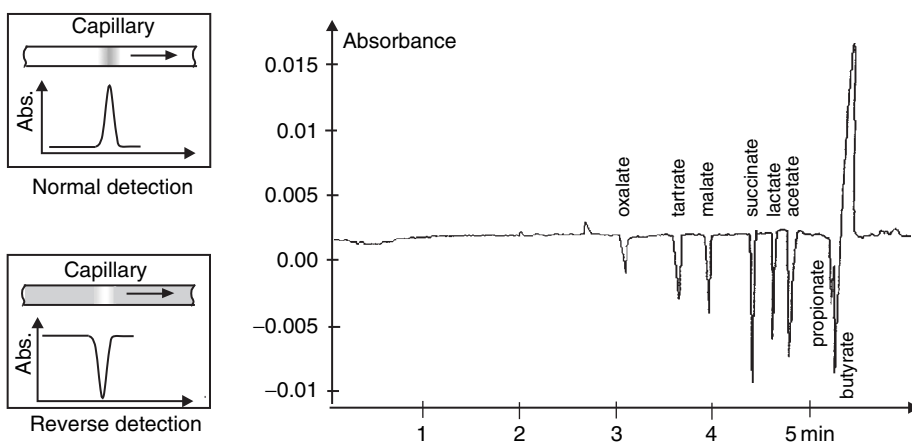


Figure 8.8 Separation of the principal organic acids in white wine by indirect UV detection. Malic acid and lactic acid are indicators for the malo-lactic fermentation (image courtesy of TSP)

■ *Indirect UV detection.* This is used for analytes – as inorganic ions – which do not absorb UV radiation. It involves using a buffer in the capillary such as a chromate or a phthalate which have high absorption coefficients under these experimental conditions. As analytes move past the detector the amount of light passing through the capillary increases as UV absorbing buffer is excluded, leading to negative peaks (Figure 8.8).

8.4 Electrophoretic techniques

8.4.1 Capillary zone electrophoresis (CZE)

This mode of electrophoresis, in which the electrolyte migrates through the capillary, is the most widely used. In this mode, samples are applied as a narrow band that is surrounded by the electrolyte buffer. This electrolyte can be, depending upon the application, acidic (phosphate or citrate) or basic (borate) or an amphoteric substance (a molecule possessing both an acidic and an alkaline function). The electro-osmotic flow increases with the pH of the liquid phase or can be rendered in-existent. This procedure is also called, in contrast to CGE (cf. Section 8.4.3), *free solution electrophoresis*.

8.4.2 Micellar electrokinetic capillary chromatography (MEKC)

In this variant of CZE, adapted to the separation of neutral or polar molecules, a cationic or anionic surfactant, e.g. sodium dodecylsulfate, is added in excess to the background electrolyte to form charged micelles. These small spherical species, immiscible in the solution, form a pseudo-stationary phase analogous to the stationary phase in HPLC. They trap neutral compounds efficiently through hydrophilic/hydrophobic affinity interactions (Figure 8.9). Neutral molecules as well as ionic species can then be conveniently separated as a direct result of their solubilization within the micelles.

MEKC is useful to perform chiral analyses. This involves the addition of a cyclodextrin as a chiral selector to the running buffer. Optical purity analysis can be determined due to the formation of inclusion complexes between a hydrophobic portion of the solute and the cavity of the cyclodextrin, having different stabilities (cf. Section 3.7 and Figure 8.9).

8.4.3 Capillary gel electrophoresis (CGE)

This technique represents the transposition of the classic agarose or polyacrylamide (PAGE) gel electrophoresis into a capillary. The capillary is filled with an electrolyte impregnated into a gel. This produces a filtration effect, which decreases the

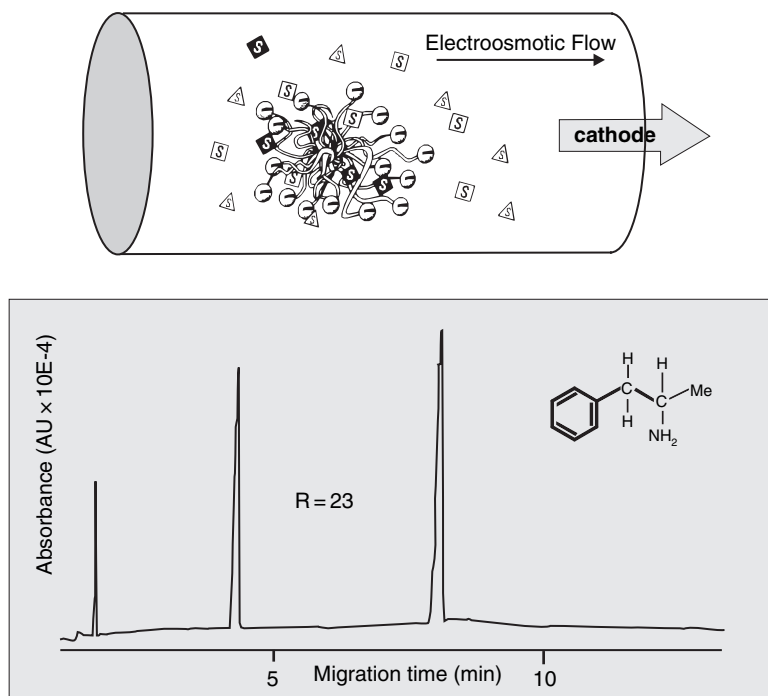


Figure 8.9 Separation of neutral compounds using surfactants (MEKC technique). Above, the lipophilic part of a surfactant, such as an alkylsulfonate, can bind moderately to certain molecules of the substrate S. The negatively charged micelles are directed towards the cathode by the electro-osmotic flow (adapted from Agilent technologies). Below, electropherogram of a racemic sample of an amphetamine in the presence of a cyclodextrin. The electrolyte, NaH_2PO_4 25 mmol (pH 2.5), contains 5 per cent of gamma-cyclodextrin polysulfate. At this pH the electro-osmotic flow is negligible. The hydrogenosulphate ions of the cyclodextrins being directed towards the anode collect the amphetamine molecules retained in their hydrophobic cavities. The two enantiomers, in equal quantity, form two diastereomer complexes which migrate at different velocities. For this experiment the anode is on the detector side. (Reproduced courtesy of Beckman Coulter Inc. USA).

electro-osmotic flow and minimizes convection and diffusion phenomena. Fragile oligonucleotides can be separated in this way.

CGE has been adapted to DNA sequencers. Special instruments fully automated (from sample loading to data analysis) have been designed with multiple capillaries that can simultaneously analyze many samples through a fluorescent-based detection.

The modified technique is known as capillary array electrophoresis for the separation of the DNA or RNA fragments *based on their size*.

■ In classic zone electrophoresis, the support on which the migration takes place, can contain a polyacrylamide gel impregnated with the electrolyte. If the latter

contains sodium dodecylsulfate, then the electrophoresis is named SDS-PAGE, after its acronym. Separations are improved by a filtering phenomenon that superimposes to the electric forces.

8.4.4 Capillary isoelectric focusing (CIEF)

This technique consists of creating a stable and linear pH gradient in a surface-treated capillary, which has been filled with a solution that contains ampholytes.

Ampholyte or zwitterions. An ampholyte is a molecule that contains both acidic and basic groups. At a particular pH, known as the isoelectric point, the charge on the groups is balanced and the molecule is neutral. If an ampholyte is placed in a pH gradient and electrophoresed it will migrate to the point at which it is uncharged and then stops moving.

At the anode side, the capillary is plunged into H_3PO_4 solution while on the cathode side, it is dipped into a NaOH solution. When an electric field is applied to the extremities of a capillary filled with a mixture of solutes and ampholytes, a pH gradient appears. Each of the charged analytes (generally, proteins) migrates through the medium until it reaches the region where the pH has the same value as its isoelectric point (at its pI , the component net charge is zero). Then, by maintaining the electric field and using a hydrostatic pressure, these separated species migrate towards the detector. In order to ensure the high performance of analysis, standards of pI (pI markers) are needed. The high resolutions obtained with this procedure allow especially the separation of peptides whose pI s may differ by only 0.02 pH units.

8.5 Performance of CE

The performance of CE, for the separation of biopolymers is comparable to that of HPLC. The success of a separation relies on the choice of an appropriate buffer medium adapted to the analysis. Only a very small quantity of sample is required and the reagent consumption or solvent is negligible (Figure 8.10). The sensitivity is very high: by using laser induced fluorescence (LIF) detection a few thousands of molecules can be observed.

However, the reproducibility of the analyses is less certain than in HPLC because there are many subtle factors that can affect the precision of the injected volume. For hydrostatic introduction, relative standard deviation is of the order of 2 per cent, if an internal standard is used. When the efficiency N of the separations is sufficiently large, it becomes possible to separate isotopes of an element (Figure 8.11).

HPCE in its 'lab-on-a-chip' version is at the frontier of classic chemistry and is particularly interesting to molecular biologists for the analysis of degradation products of proteins.

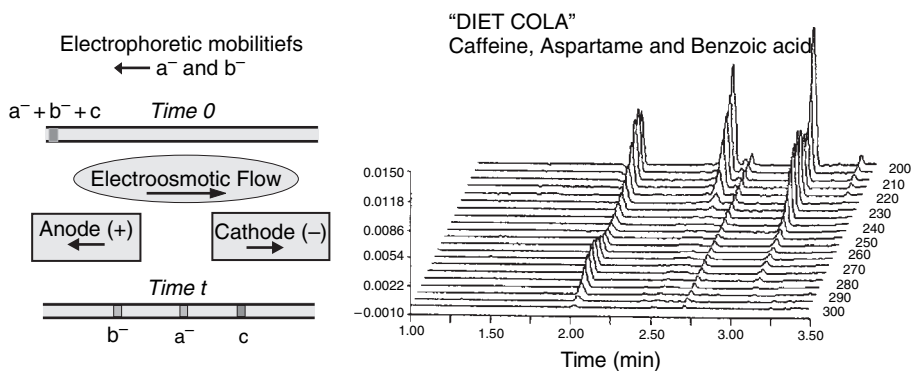


Figure 8.10 *Electro-kinetic separation of three species a^- , b^- and c .* Left, The electro-osmotic flow carrying all of the charged or neutral species along with it, is directed towards the cathode. The negative species though attracted by the positive pole cannot overcome the electro-osmotic flow and are therefore displaced towards the cathode. Right, separation of caffeine c and of the anions of aspartame a^- and of benzoate b^- from a sample of 'DIET COLA.' Presentation in the form of a 3D electroferogram (reproduced courtesy of TSP).

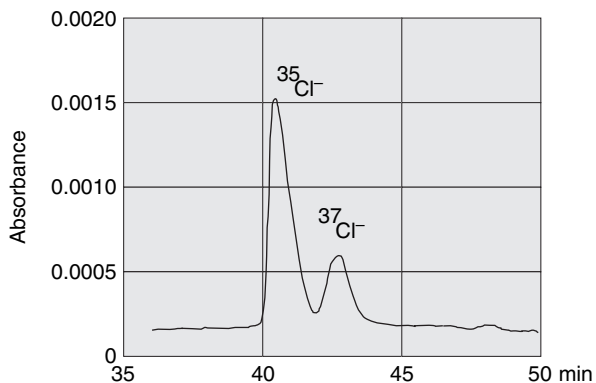


Figure 8.11 *Separation of chlorine isotopes.* When the efficiency is very high isotopes of the same element can be separated which as in this example leads to two clearly defined peaks. Conditions: capillary of $75\ \mu\text{m}/47\ \text{cm}$. $V = 20\ \text{kV}$, $T = 25^\circ\text{C}$, electrolyte: chromate/borate pH 9.2. McDonald LT. *Anal. Chem.* 1995, **67**, 1074.

The separation parameters (efficiency, retention factor, selectivity, resolution) are accessible from electroferograms by using either the same formulae as in chromatography, or those specific to HPCE.

The theoretical efficiency of a separation N – as high as one million plates/metre in a column of length L – can be calculated from its effective length l and from the diffusion coefficient D (cm^2/s). This latest parameter is linked to the dispersion σ and to the migration time t_m via Einstein's law ($\sigma^2 = 2D \cdot t_m$). Expression 8.9

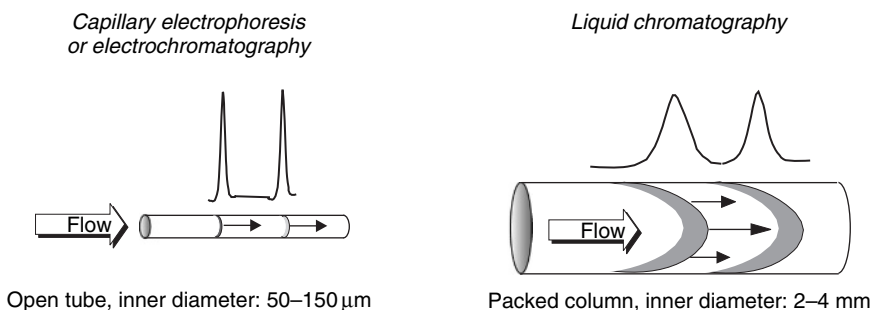


Figure 8.12 Comparison of the conjugated effects of diffusion and of the mode of progression of the mobile phase in HPCE and in HPLC. Diffusion which increases as the square of the diameter of the tube is smaller in electrophoresis than in liquid chromatography, especially if the sample mixture comprises compounds of greater molecular mass. Elsewhere in CE, the electrolyte is drawn along by the internal wall, while it is pushed in HPCE and in CE. Finally the profile of the hydrodynamic flow is almost perfectly flat and not parabolic as in HPLC.

is attained (or 8.10 if t_m is eliminated by using expression 8.6). These expressions may be use also to calculate D if N is experimentally available.

$$N = \frac{l^2}{2Dt_m} \quad (8.9)$$

$$N = \frac{\mu_{app}}{2D} \cdot V \cdot \frac{l}{L} \quad (8.10)$$

Relation 8.10 reveals that the efficiency grows up with the voltage applied. The macromolecules, for which the diffusion factors are slighter than those of small molecules, inevitably lead to better separations (Figure 8.12).

Finally in HPCE, the resolution R between two peaks can be calculated by using the efficiency N , the difference in the speed of migration of the two solutes (Δv) and their average speed \bar{v} (8.9):

$$R = \frac{\sqrt{N}}{4} \cdot \frac{\Delta v}{\bar{v}} \quad (8.11)$$

8.6 Capillary electrochromatography

Capillary electrochromatography (CEC) is a separation method that borrows from CZE and HPLC, by associating the electromigration of ions with their partitioning between the phases, typical to chromatography. Instead of a hydraulic pump, it is the high voltage power supply of the CE instrument that generates a flow through the packed bed capillary. In electrochromatography there is no loss of

charge: each H_3O^+ ion migrates under the effect of an electric field, while in liquid chromatography, one is required working under a pressure close to 1000 bars to push the mobile phase through such a capillary column. The benefit, compared to HPLC, is the fact that in an electrically driven system the flow profile is plug-like and therefore much more efficient than in a pressure driven system where it is parabolic (Figure 8.12).

The method requires a high-purity fused capillary filled with standard HPLC packing materials constituted of particles that can be of very small diameters ($1\text{--}3\ \mu\text{m}$) or of continuous bed (monoliths) since there is no back pressure.

■ Since the surface areas of microparticulate silica based packing materials are much greater than that of the capillary walls, a significant part of the electro-osmotic flow is generated by the remaining surface silanol groups of the stationary phase (Figure 8.13). The packing behaves in a fashion analogous to an untreated internal capillary wall. This does not apply in the case of totally base deactivated materials, such as RP-18 silica which have minimal levels of residual silanol groups.

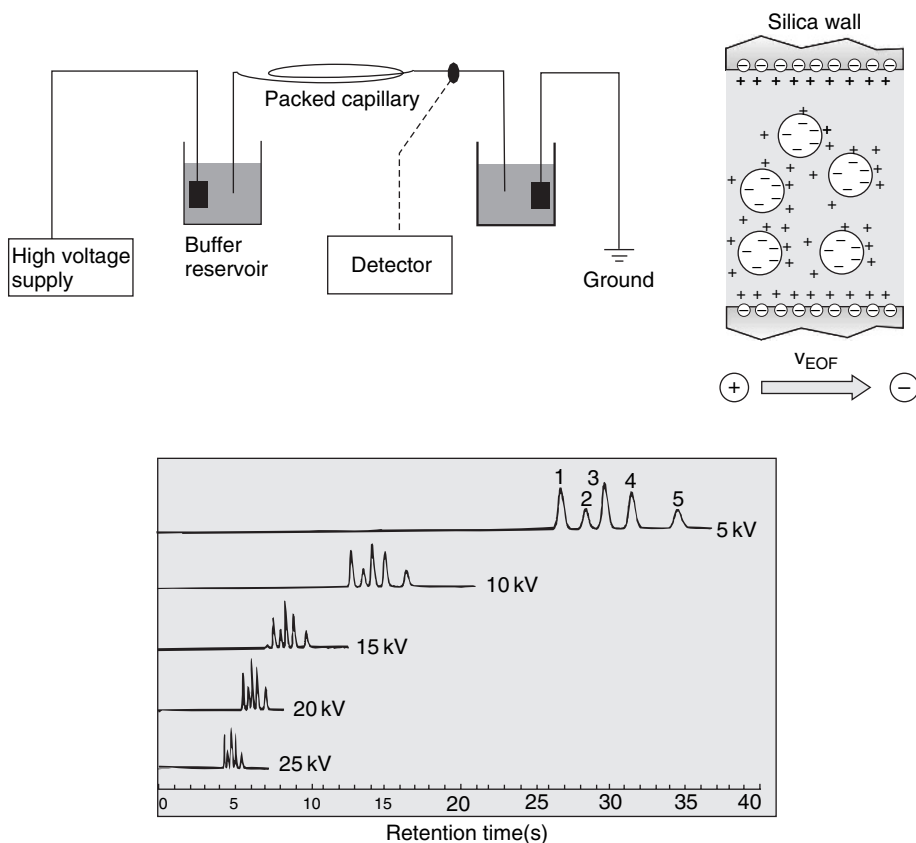


Figure 8.13 Schematic of an electrochromatographic installation and the representation of a small part of the packed capillary. The different attempts to separate a mixture of neutral compounds by varying the voltage, verifies to a first approximation, the relation existing between voltage and velocity of electro-osmotic flow.

Column L = 10 cm
 ϕ = 6.5 cm
 Inner Diam. 100 μ m
 Silica 1.5 μ m ODS
 ddp: see figure
 Fluorometric detector
Mixture:
 1 - Naphthalene
 2 - Fluoranthene
 3 - Benz[a]anthracene
 4 - Benzo[k]fluoranthene
 5 - Benzo[ghi]perylene

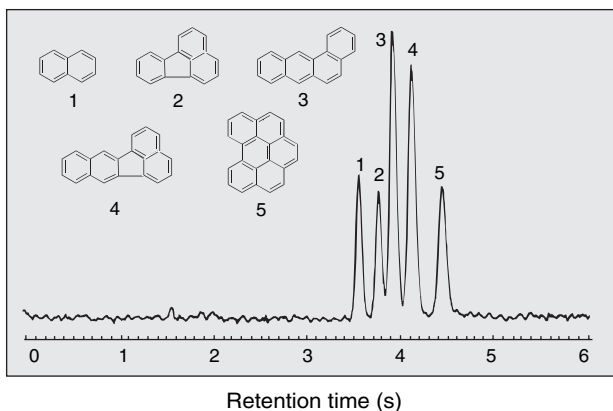


Figure 8.14 Separation of aromatic hydrocarbons by electrochromatography. In this example the use of a very short column and a high voltage enable the separation to be made in few seconds (for $V = 28$ kV of a mixture of non-ionized compounds), with a good resolution. An appreciable efficiency is attained with this procedure (Amox DS *et al.* *Anal Chem* 1998, 70, 4787–92).

The stationary phase acts also with varying success as a retaining material. Non-aqueous mobile phases (for hydrophilic analytes) and untreated silica packings may also be used. The separation factor is very high but a change of composition of the mobile phase or of the organic modifiers will affect the electro-osmotic flow and then the selectivity (Figure 8.14).

As in conventional liquid chromatography the plate height can be expressed using a modified Van Deemter plate height equation.

Some problems remain, such as:

- the speed of separation is limited by the upper limit of the electro-osmotic flow velocity
- the precise control of the volume of sample introduced into the capillary
- the heat generated in the packed capillary
- the band broadening, because electrochromatography involves the use of a stationary phase.

Problems

8.1 An electrophoresis analysis in free solution (CZE), calls for the use of a capillary of 32 cm and with effective length of separation 24.5 cm. The applied voltage is 30 kV. Under the conditions of the experiment the peak of a neutral marker appeared upon the electropherogram at 3 min.

1. Calculate the electrophoretic mobility, μ_{EP} , of a compound whose migration time is 2.5 min. Give the answer in precise units.
2. Calculate the diffusion coefficient under these conditions for this compound, remembering that the calculated plate number, $N = 80\,000$.

8.2 The apparatus for an experiment using a fused silica capillary is set up. The total capillary length $L = 1$ m while the effective capillary length $l = 90$ cm. The applied voltage is 30 kV. The detector is located towards the cathode and the electrolyte is a buffer of pH 5.

In a standard solution a compound has a migration time of $t_m = 10$ min.

1. Sketch a diagram of the apparatus described.
2. From the information given, can it be deduced whether the net charge carried by the compound is positive or negative?
3. Calculate the apparent electrophoretic mobility μ_{app} of the compound.
4. If a small molecule not carrying a charge has a migration time $t_m = 5$ min, deduce the value of the electro-osmotic flux μ_{EOS} .
5. Calculate the electrophoretic mobility of the compound μ_{EP} .
6. What is the sign of the net charge carried by the compound?
7. What would happen if the fused silica capillary was coated by trimethyl-chlorosilane?
8. Supposing that the pI of the compound was 4, what would be the sign of its net charge if the pH of the electrolyte was lowered to 3?
9. Calculate the number of theoretical plates if the diffusion coefficient of the solute is $D = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$.
10. From the relationship between the efficiency N and the diffusion D , explain why small molecules have poorer separations than larger molecules, and why for the smaller variant these separations are much better when the capillary is narrower.

- 8.3 During a capillary electrophoresis experiment it is observed that if the capillary contains an acrylamide gel as well as an electrolyte then the speed of migration is slowed by the mechanical effects of filtration through the gel. This is particularly significant for larger molecules. The following relationship can be considered as proven:

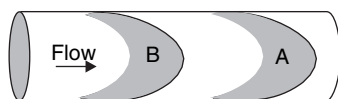
$$\log M = a \cdot v + b$$

where a and b are two constants and M represents the molecular mass (Da) of a molecule migrating with a velocity v .

In an experiment designed to employ this relationship and in order to calculate the molecular mass of an unknown protein, two known standards were used; ovalbumin ($M = 45\,000$ Da) and myoglobin ($M = 17\,200$ Da), whose migration velocities are 1.5 cm min^{-1} and 5.5 cm min^{-1} respectively. In the same experiment, the unknown protein migrates at a velocity of 3.25 cm min^{-1} . Calculate its molecular mass.

- 8.4 Imagine that, following a capillary isoelectric focusing experiment, two polypeptides A and B are immobilised in one section of the capillary as indicated in the figure. The isoelectric point of B is superior to that of A.

Complete the figure indicating how the pH would increase at the positive end of the capillary while showing the orientation of the corresponding electric field.

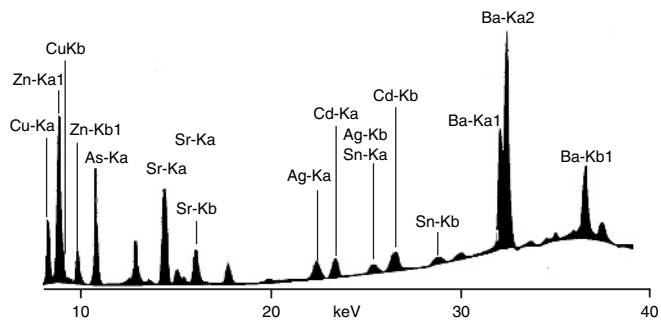


- 8.5 The table below lists a series of proteins along with their respective isoelectric points (pI). By completing the table with positive (+) or negative (−) signs or zeros (0) indicate for each protein whether the net charge will be positive, zero or negative at the three values of pH specified.

Protein	pI	pH = 3	pH = 7.4	pH = 10
insulin	5.4			
pepsin	1			
cytochrome C	10			
haemoglobin	7.1			
albumin (serum)	4.8			

PART 2

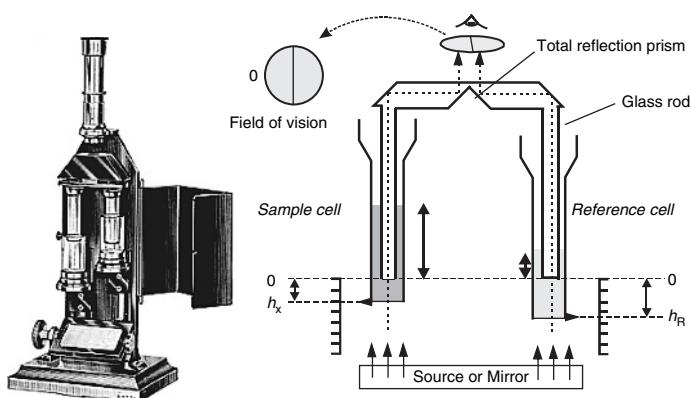
Spectroscopic methods



The creators of modern colorimetry

Visual colorimetry, one of the most ancient of analytical methods was already being used in the times of the Greeks and Romans although it began to take on a more modern scientific character when, in 1729, Pierre Bouguer postulated that 'if a given width of coloured glass absorbs half of the light issuing from a source, then a double width will reduce the light to a quarter of its initial value'.

Some 30 years later, Jean-Henri Lambert (1728–1777) proposed the first mathematical relationship 'the logarithm of the decrease in light intensity (today we would say the inverse of the transmittance) is equal to the product of the opacity of the medium times its thickness'. Finally in 1850, Auguste Beer established a relation between *concentration* and *optical density* (now called *absorbance*), which led to the current form of the Beer–Lambert law (also called Lambert–Beer law or even Lambert–Beer–Bouguer law).



Dubosc's comparator. The observer adjusts the transmitted intensities, according to the two pathways. He can compare the quality of the colours with great precision. The bottoms of the tubes are illuminated by a reflecting surface itself lit by an annexed light source.

Amongst the different devices imagined for visual colorimetry measurements, one of the most original was described by Jules Dubosc in 1868. This instrument which remained in use until the 1960s permits, due to a system of total reflecting prisms, a juxtaposition in a small circular field, of the light intensities that have travelled through two identical cells, one of which contains the sample (concentration C_x) and the other contains a known standard (concentration C_R). The observer sees both fields with one eye, and adjusts the depths of the columns of liquid, h_x of the solution to be measured and h_R of the standard, until the two halves of the field are identical in intensity. When this condition holds, the absorbances are equal. The concentrations of the two solutions are inversely proportional to their depths, which may then be read on the instrument.

If $A_R = A_x$, then $\varepsilon h_R C_R = \varepsilon h_x C_x$

Thus, the concentration C_x can be deduced.

This half-light comparator leads to the required concentration by taking advantage of the Lambert–Beer law through comparison of the thicknesses crossed.

9

Ultraviolet and visible absorption spectroscopy

The absorption by matter of electromagnetic radiation in the domain ranging from the near ultraviolet to the very near infrared, between 180 and 1100 nm, has been studied extensively. This portion of the electromagnetic spectrum, designated as the 'UV/Visible' since it includes radiation perceptible to the human eye, generally yields little structural information but is very useful for quantitative measurements. The concentration of an analyte in solution can be determined by measuring the absorbance at some wavelength and applying the Lambert–Beer Law. The method is known as colorimetry, considered as the workhorse of many laboratories. Colorimetry applies not only to compounds possessing an absorption spectrum in that spectral region but equally to all those compounds which, following modification by specific reagents, lead to derivatives which permit absorption measurements.

This can be achieved by a whole range of instruments, from colour comparators and other basic colorimetric devices to automated spectrophotometers that can carry out multi-component analyses. Liquid chromatography and capillary electrophoresis have favoured the development of improved UV/Vis detectors which reflects the current trend for acquiring chromatograms giving at the same time information concerning the nature and quantification of compounds.

9.1 The UV/Vis spectral region and the origin of the absorptions

This region of the spectrum is conventionally divided into three sub-domains termed *near UV* (185–400 nm), *visible* (400–700 nm) and *very near infrared* (700–1100 nm). Most commercial spectrophotometers cover the spectral range of 185 to 900 nm. The lower limit of the instrument depends upon the nature of the optical components used and of the presence, or not, along the optical pathway of air,

knowing that oxygen and water vapour absorb intensely below 190 nm. Some instruments, on condition that they are operating in a vacuum, can attain 150 nm with samples in the gaseous state. This is the domain of *vacuum* or *far ultraviolet*. The long-wavelength limit is usually determined by the wavelength response of the detector in the spectrometer. High-end commercial UV/Vis spectrophotometers extend the measurable spectral range into the near infrared region as far as 3300 nm.

The origin of absorption in this domain is the interaction of photons from a source with ions or molecules of the sample. When a molecule absorbs a photon from the UV/Vis region, the corresponding energy is captured by one (or several) of its outermost electrons. As a consequence there occurs a modification

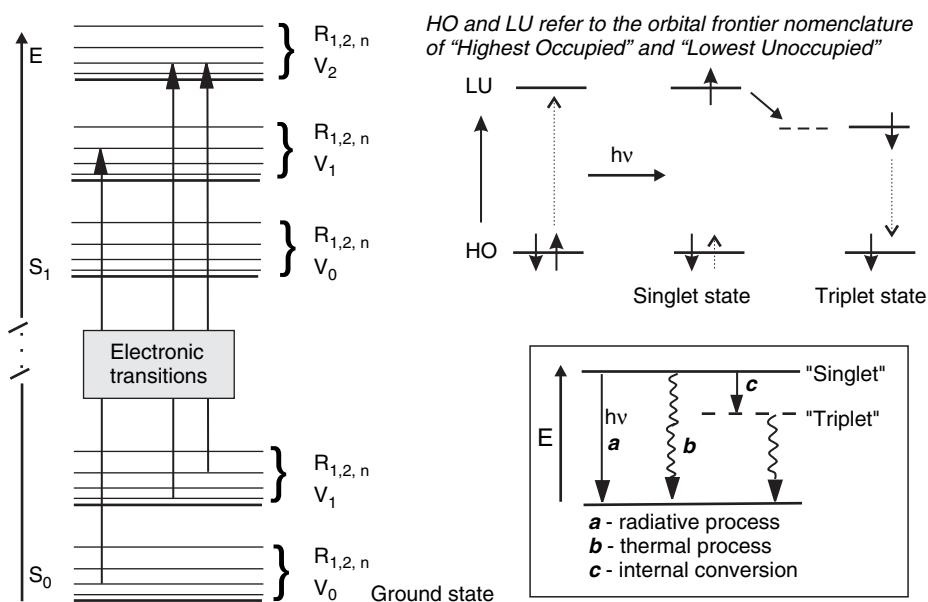


Figure 9.1 Energy quantification of a molecule. Left, diagram showing different energy states of rotation, vibration and electronic of a molecule. Each horizontal line corresponds to a distinct energy level. The hierarchic structure is the following: between each electronic state S there lies several vibrational levels V , themselves also sub-divided into a collection of rotational levels R . The scale of the diagram is, of course, far from being accurate as the vibrational and rotational levels should be closer together. On the figure, the relative distances between the three energy forms should be as their relative values, that is in the order of 1000 : 50 : 1. In condensed phases, as a result of the closeness of molecules, no instrument, regardless of its resolution can record a spectrum upon which are displayed the individual transitions. Right, Conventional representation of the absorption of a photon. Promotion of an electron from one occupied orbital (HO) to an unoccupied orbital (LU) with the apparition of a singlet state giving rise rapidly to a more stable triplet state. This process corresponds to a return of an excited species to the ground state. The transitions being quasi immediate, the distance between the atoms has not had enough time to change (Franck–Condon principle).

of its electronic energy (E_{elec}), a component of the total mechanical energy of the molecule along with its energy of rotation (E_{rot}) and its energy of vibration (E_{vib}) (expression 9.1).

A modification of E_{elec} will result in alterations for both E_{rot} and E_{vib} resulting in a vast collection of possible transitions obtained in all three cases (Figure 9.1), and since the polarities of the bonds within the molecules will be disturbed their spectra are given the generic name of *charge transfer spectra*.

$$\Delta E_{\text{tot}} = \Delta E_{\text{rot}} + \Delta E_{\text{vib}} + \Delta E_{\text{elec}} \quad (9.1)$$

with $\Delta E_{\text{elec}} > \Delta E_{\text{vib}} > \Delta E_{\text{rot}}$.

■ All observed spectra are obtained from a sample containing an huge number of molecules or other individual species which are not all in the same energy state. The resulting graph corresponds to a band type curve that envelops the individual transitions of the present species.

The energy captured during the course of photon absorption can be released through a variety of processes that occur concurrently with emission of a photon (Figure 9.1c). Phosphorescence and fluorescence are transformations of this kind, which are exploited in other analytical methods (cf. Chapter 11).

9.2 The UV/Vis spectrum

UV/Vis spectrometers collate the data over the required range and generate the spectrum of the compound under analysis as a graph representing the *transmittance* (or the *absorbance*) as a function of wavelength along the abscissa, given in nanometres, the recommended unit in this region, rather than in cm^{-1} (Figure 9.2).

In spectroscopy, the *transmittance* T is a measure of the attenuation of a beam of monochromatic light based upon the comparison between the intensities of the transmitted light (I) and the incident light (I_0) according to whether the sample is placed, or not, in the optical pathway between the source and the detector. T is expressed as a fraction or a percentage:

$$T = \frac{I}{I_0} \quad (9.2)$$

or

$$\%T = \frac{I}{I_0} \times 100$$

The *absorbance* (old name *optical density*) is defined by:

$$A = -\log T \quad (9.3)$$

The recorded spectra of compounds in the condensed phase, whether pure or in solution, generally present absorption bands that are both few and broad, while

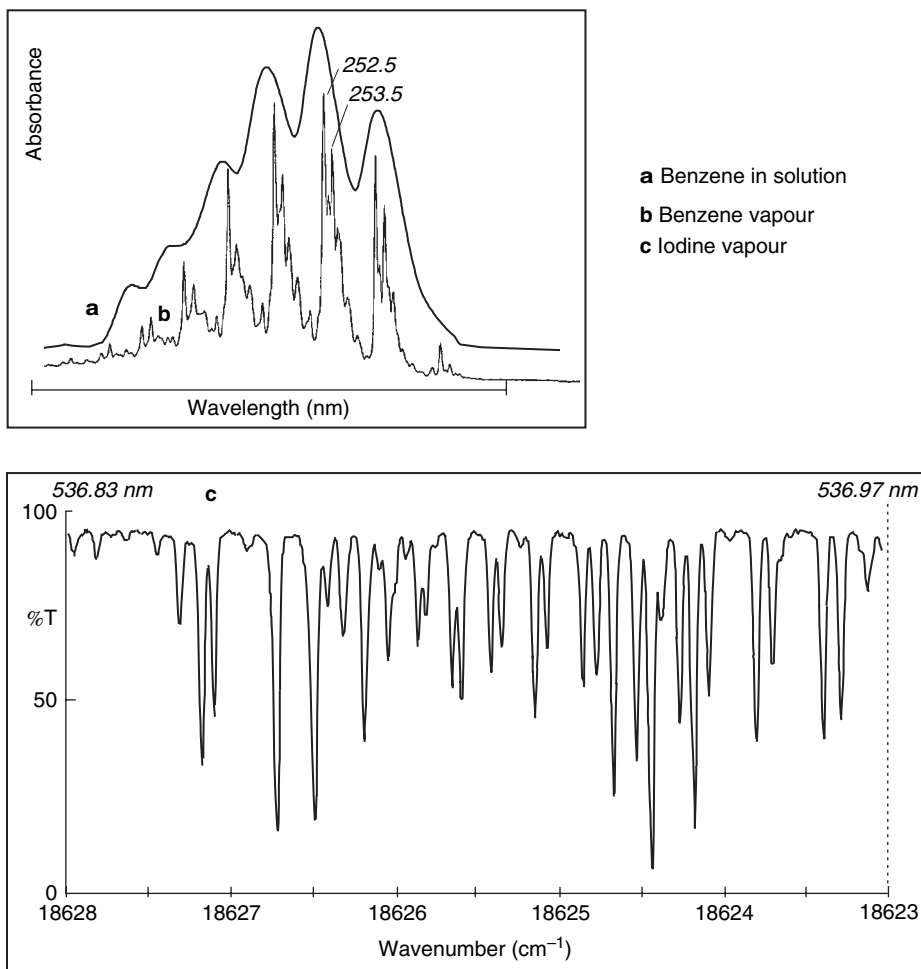


Figure 9.2 *Three different aspects of UV/Vis spectra.* Spectra of benzene (a) in solution (a band spectrum); (b) in the vapour state (spectrum presenting a fine structure); (c) an expansion of a section from the high resolution (0.14 nm total interval) line spectrum of iodine vapour. The spectrum of benzene vapour, obtained from a drop of this compound, deposited in a silica glass cuvette of 1 cm optical path, provides an excellent test to evaluate the resolution of an UV-spectrophotometer.

those spectra obtained from samples in the gas state and maintained under a weak pressure yield spectra of detailed ‘fine structure’ (Figure 9.2). For compounds of simple atomic composition, providing the spectrometer possesses a high enough resolution, the fundamental transitions appear as if isolated. In extreme situations such as these, the positions of the absorptions are recorded in cm^{-1} , a unit better adapted for a precise pointing than the nm (the calculation reveals that there are 111 cm^{-1} between 300 and 301 nm).

9.3 Electronic transitions of organic compounds

Organic compounds represent the majority of the studies made in the UV/Vis. The observed transitions involve electrons engaged in σ or π or non-bonding n electron orbitals of light atoms such as H, C, N, O (Figure 9.3). Where possible the character of each absorption band will be indicated in relation to the molecular orbitals (MO) concerned and the molar absorption coefficient ϵ ($\text{Lmol}^{-1}\text{cm}^{-1}$) calculated at the wavelength corresponding to the maximum of the absorption band.

9.3.1 $\sigma \rightarrow \sigma^*$ transition

This transition appears in the far UV since the promotion of an electron from a σ bonding MO to a σ^* anti-bonding MO requires a significant energy. This is the reason for saturated hydrocarbons that only contain this type of bonding being transparent in the near UV.

Example: *hexane (gas state)*: $\lambda_{\text{max}} = 135 \text{ nm}$ ($\epsilon = 10\,000$).

Cyclohexane and heptane are used as solvents in the near UV. At 200 nm the absorbance of a 1 cm thickness of heptane is equal to 1. Unfortunately, the solvation power of these solvents is insufficient to dissolve many polar compounds.

Similarly, the transparency of water in the near UV ($A = 0.01$ for $l = 1 \text{ cm}$, at $\lambda = 190 \text{ nm}$) is due to the fact there can only be transitions $\sigma \rightarrow \sigma^*$ and $n \rightarrow \sigma^*$ in this compound.

9.3.2 $n \rightarrow \sigma^*$ transition

The promotion of an n electron from an atom of O, N, S, Cl to an MO σ^* leads to a transition of moderate intensity located around 180 nm for alcohols, near

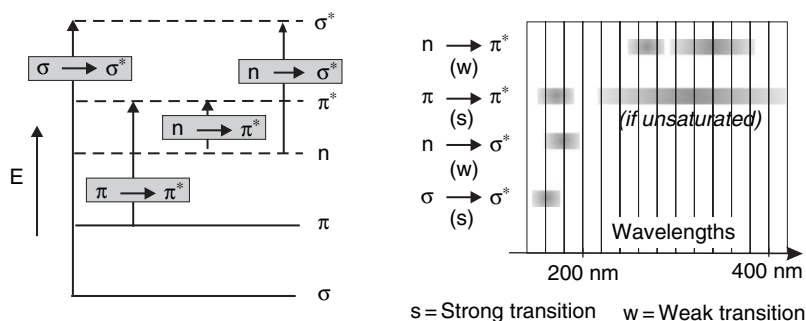


Figure 9.3 Comparison of the transitions met most frequently with simple organic compounds. The four types of transition are united on a single energy diagram in order to situate them with respect to each other and to correlate them with the corresponding spectral ranges concerned.

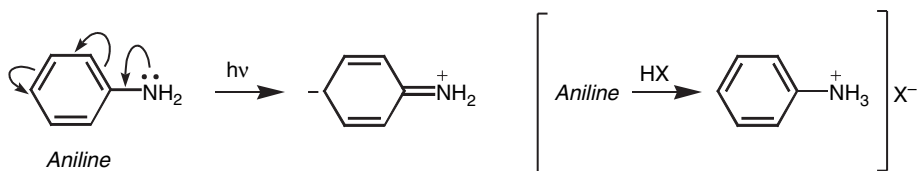


Figure 9.4 Transition $n \rightarrow \sigma^*$ of aniline (a primary amine). This transition corresponds to an increase in the ‘weight’ of the mesomeric form. The absorption peak of aniline representing this transition would disappear if one equivalent of a protonic acid of type HX is added, since the formation of an ammonium salt would immobilize the lone pair of the nitrogen atom necessary for this transition (see the intermediate between the brackets).

190 nm for ethers or halogen derivatives and in the region of 220 nm for amines (Figure 9.4).

Examples: methanol: $\lambda_{\text{max}} = 183 \text{ nm} (\epsilon = 50)$ ether: $\lambda_{\text{max}} = 190 \text{ nm} (\epsilon = 2000)$ ethylamine: $\lambda_{\text{max}} = 210 \text{ nm} (\epsilon = 800)$; 1-chlorobutane: $\lambda_{\text{max}} = 179 \text{ nm}$.

9.3.3 $n \rightarrow \pi^*$ Transition

This transition of low intensity results from the passage of an n electron (engaged in a non-bonding MO) to an anti-bonding π^* orbital. This transition is usually observed in molecules containing a hetero atom carrying lone electron pairs as part of an unsaturated system. The best known is that corresponding to the carbonyl band, easily observed at around 270 to 295 nm. The molar absorption coefficient for this band is weak.

Example: ethanal: $\lambda = 293 \text{ nm} (\epsilon = 12, \text{ with ethanol as solvent})$.

9.3.4 $\pi \rightarrow \pi^*$ Transition

Compounds possessing an isolated ethylenic double bond reveal a strong absorption band around 170 nm. The precise position depends upon the presence of heteroatom substituents.

Example: ethylene: $\lambda_{\text{max}} = 165 \text{ nm} (\epsilon = 16000)$.

A compound that is transparent in a given spectral range of the near UV when it is isolated, can become absorbing if it interact with a species through a mechanism of type donor–acceptor (D-A). This phenomenon is linked to the passage of an electron from a bonding orbital of the donor (which becomes a radical cation) towards a vacant orbital of the acceptor (which becomes a radical anion) of an attainable energy level (Figure 9.5). The position of the absorption band in the visible part of the spectrum is a function of the ionization potential of the donor and of the electron affinity of the acceptor; the value of ϵ is, in general, very large.

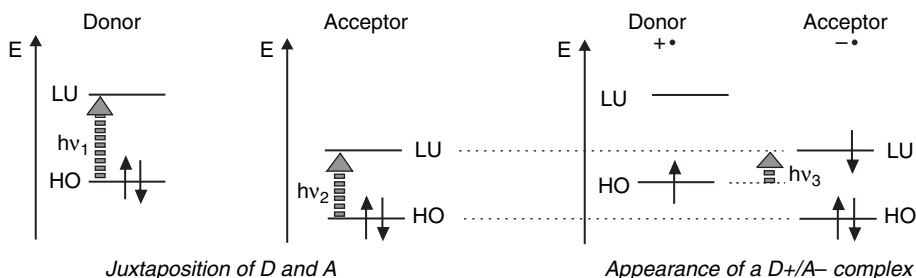


Figure 9.5 Energy diagram for a donor/acceptor interaction. The excited state is supposed to be essentially in the ionic form

9.3.5 $d \rightarrow d$ transition.

Numerous inorganic salts containing electrons engaged in d orbitals are responsible for transitions of weak absorption located in the visible region. These transitions are generally responsible for their colours. That is why the solutions of metallic salts of titanium $[\text{Ti}(\text{H}_2\text{O})_6]^{3+}$ or of copper $[\text{Cu}(\text{H}_2\text{O})_6]^{2+}$ are blue, while potassium permanganate yields violet solutions, and so on.

9.4 Chromophore groups

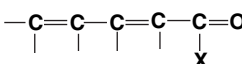
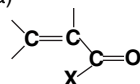
The functional groups of organic compounds (ketones, amines, nitrogen derivatives, etc.), responsible for absorption in UV/Vis are called *chromophores* (Table 9.1). A species formed from a carbon skeleton transparent in the near UV on which are attached one or several chromophores constitutes a *chromogene*.

- *Isolated chromophores*: for a series of molecules having the same chromophore, the position and the intensity of the absorption bands remain constant. When a molecule possesses several isolated chromophores, that is, which do not

Table 9.1 Characteristic chromophores of some nitrogen-containing groups

Name	Chromophore	λ_{max} (nm)	ϵ_{max} ($\text{Lmol}^{-1}\text{cm}^{-1}$)
Amine	$-\text{NH}_2$	195	3000
Oxime	$=\text{NOH}$	190	5000
Nitro	$-\text{NO}_2$	210	3000
Nitrite	$-\text{ONO}$	230	1500
Nitrate	$-\text{ONO}_2$	270	12
Nitroso	$-\text{N}=\text{O}$	300	100

Table 9.2 *Correlation table in UV/Visible spectroscopy.* Fieser–Woodward–Scott rules for the calculation of the position of the absorption maxima for enones and dienones (margin 3 nm).

Type of structure concerned: (chemical formula)	Basic structure (chemical formula)																																								
 <i>solvent: methanol or ethanol</i>	 <i>open chain or ring of 6 C (ring of 5 C: 202 nm)</i>																																								
Increments: - each additional conjugated double bond: 30 nm - exocyclic character of C=C double bond: 5 nm - dienic homoannular character: 39 nm - for each substituent, add (in nm):	<table border="1"> <thead> <tr> <th>positions</th> <th>α</th> <th>β</th> <th>γ</th> <th>δ</th> </tr> </thead> <tbody> <tr> <td>alkyl</td> <td>10</td> <td>12</td> <td>18</td> <td>18</td> </tr> <tr> <td>-Cl</td> <td>15</td> <td>12</td> <td></td> <td></td> </tr> <tr> <td>-Br</td> <td>25</td> <td>30</td> <td></td> <td></td> </tr> <tr> <td>-OH</td> <td>35</td> <td>30</td> <td></td> <td>50</td> </tr> <tr> <td>-O-alkyl</td> <td>35</td> <td>30</td> <td>17</td> <td>31</td> </tr> <tr> <td>-O-acyl</td> <td>6</td> <td>6</td> <td>6</td> <td>6</td> </tr> <tr> <td>-N(R)2</td> <td></td> <td>95</td> <td></td> <td></td> </tr> </tbody> </table>	positions	α	β	γ	δ	alkyl	10	12	18	18	-Cl	15	12			-Br	25	30			-OH	35	30		50	-O-alkyl	35	30	17	31	-O-acyl	6	6	6	6	-N(R)2		95		
positions	α	β	γ	δ																																					
alkyl	10	12	18	18																																					
-Cl	15	12																																							
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-OH	35	30		50																																					
-O-alkyl	35	30	17	31																																					
-O-acyl	6	6	6	6																																					
-N(R)2		95																																							
Solvent increments: - water 30 nm +8 nm - chloroform -1 nm - ether -7 nm - cyclohexane -11 nm																																									

interact with each other because they are separated by at least two single bonds in the skeleton, then the overlapping of the effects of each individual chromophore is observed.

- *Conjugated chromophore systems*: when the chromophores interact with each other the absorption spectrum is displaced towards longer wavelengths (*bathochromic effect*) with an increase in the absorption intensity (*hyperchromic effect*). A particular case is that of molecules with conjugated systems, that is, organic structures containing several adjacent to one another unsaturated chromophores (i.e. separated by a single bond). The spectrum is then strongly disrupted with respect to the overlapping effects of isolated chromophores. The greater the number of carbon atoms upon which the conjugated system is extended, the greater the reduction in the difference between energy levels (Table 9.2). This results in a very large bathochromic effect (Figure 9.6).

9.5 Solvent effects: solvatochromism

Each solvent has its own characteristic polarity. Since it is known that all electronic transitions modify the charge distribution of the compound in solution, it is obvious that the position and intensity of the absorption bands will vary a little with the nature of the solvent used. The nature of the solvent/solute interactions are a greater indication of the type of transition. Two contrasting effects can be distinguished.

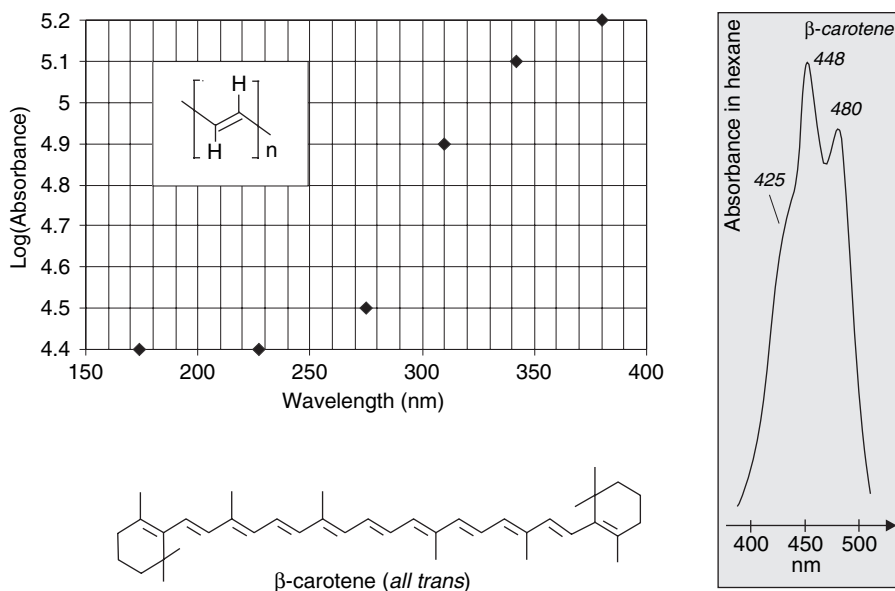


Figure 9.6 The effect of several conjugated double bonds upon the position of the absorption maxima of the transition $\pi \rightarrow \pi^*$ for several conjugated polyenes. Values of λ_{\max} for a family of *E*-disubstituted conjugated polyenes which differ between them by the number of conjugated double bonds. This effect is at the origin of the colour in numerous natural compounds whose structural formulae present extended conjugated chromophores. An example is the orange colour of 'all-trans' β -carotene above which arises from the presence of eleven conjugated double bonds ($\lambda_{\max} = 425, 448$ and 475 nm in hexane). The broader the delocalization of the electrons the greater will be the bathochromic effect. Aromatic compounds lead to spectra more complex than those of ethylenics, the transitions $\pi \rightarrow \pi^*$ being at the origin of 'fine structure'.

9.5.1 Hypsochromic effect (the 'blue shift')

If the chromophore responsible for the observed transition is more polar in its fundamental state than when it is excited, then a polar solvent will stabilize, by solvation, the form prior to absorption of the photon. Molecules of solvent will be clustered around the solute because of electrostatic effects. More energy would therefore be required to excite the electronic transition concerned, causing a displacement of the absorption maxima to shorter wavelengths than would occur in a non-polar solvent. This is the *hypsochromic effect* (Figure 9.7).

This is rather like the $n \rightarrow \pi^*$ transition of the ketone carbonyl in solution. The $C^+ - O^-$ form (characterized by its dipolar moment μ) will be more and more stabilized as the solvent becomes more polar. The excited state being rapidly attained, the solvent shell surrounding the carbonyl has no time to reorient itself and bring about stabilization following absorption of the photon. This same effect is observed for the $n \rightarrow \sigma^*$ transition and is accompanied by a variation of the coefficient ϵ .

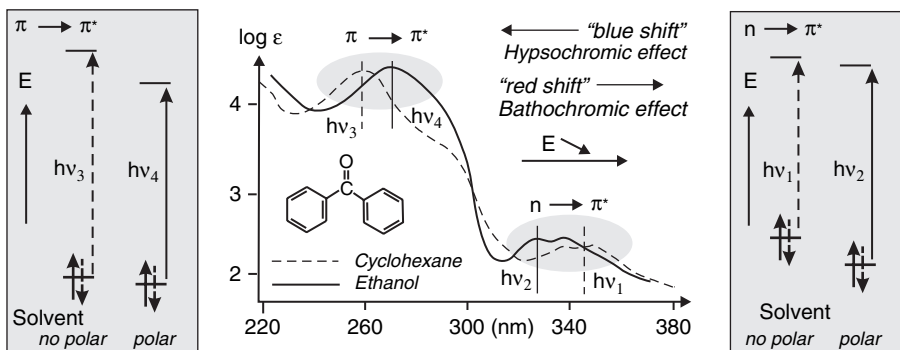


Figure 9.7 Spectra of benzophenone in cyclohexane (1) and in ethanol (2). Observed here with two different solvents and upon two types of transitions are the bathochromic and hypsochromic effects.

9.5.2 Bathochromic effect (the 'red shift')

For less polar compounds the solvent effect is weak. However, if the dipole moment of the chromophore increases during the transition, the final state will be more solvated. A polar solvent can thus stabilise the excited form, which favours the transition: a displacement is observed towards longer wavelengths when compared with the spectra obtained with a non-polar solvent. This is the *bathochromic effect* (Figure 9.7). For example, this is the case for the $\pi \rightarrow \pi^*$ transition of ethylenic hydrocarbons of which the double bond is only weakly polar.

9.5.3 Effect of pH

The pH of the solvent in which the solute is dissolved can have an important effect on the spectrum. Amongst the compounds that present this effect in a spectacular fashion are chemical indicator strips, whose change in colour is used during acidimetric measurements (Figure 9.8). Equivalent points can be located by this methodology.

9.6 Fieser–Woodward rules

Structural analysis from electronic spectra is a fairly vague exercise, in the sense that the relative simplicity of the spectrum has for corollary a poor yield of information. In the 1940s, however, before the development of more powerful techniques of identification, now taken for granted, UV/Vis spectroscopy was employed for this purpose. Studies of spectra for a large number of molecules led to the establishment of a correlation between structures and positions of

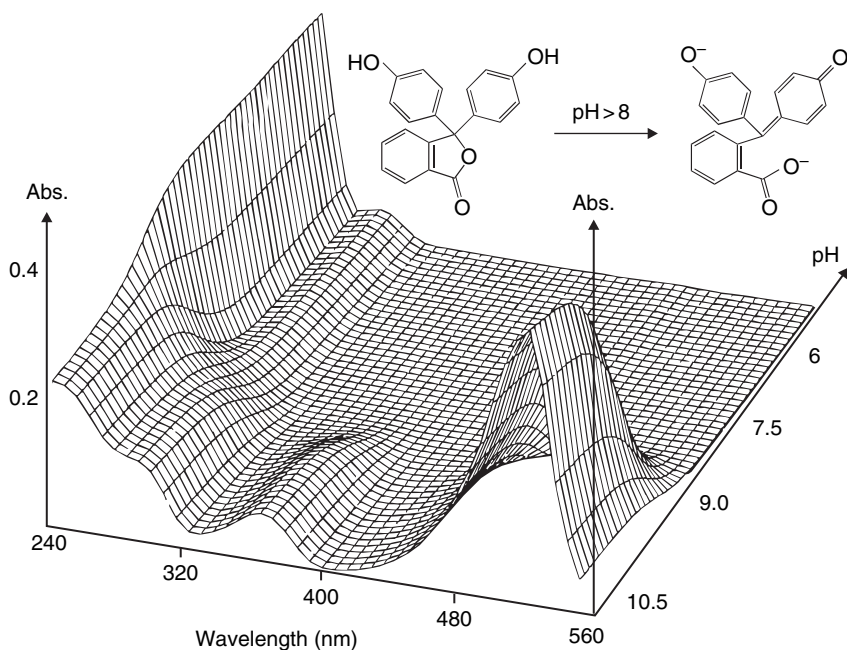


Figure 9.8 *The effect of pH upon a solution of phenolphthalein.* This compound is not coloured at pH values less than 8 though it is bright pink for those greater than 9.5. The graph presented here in 3D perspective reveals that for acid pH there is no absorption in the visible region of the spectrum. In contrast, absorptions towards 500 nm appear when the pH becomes alkaline, which are responsible for the well-known colour of the compound. Notable in this example is the modification in the chemical bonding, which is pH dependent.

the absorption maxima. The best known of these are the empirical rules due to Fieser, Woodward and Scott, which concern unsaturated carbonyl, dienes or steroids compounds. The table presents in the form of increments to λ_{\max} a variety of factors and structural features affecting the position of the absorption band $\pi \rightarrow \pi^*$ for these conjugated systems (Table 9.2).

There is good agreement between the calculated values and those derived from experiment for the four examples displayed in Figure 9.9.

λ_{\max}	Meas. 231	234	217	386 nm
	Calc. 229	234	215	385 nm

Figure 9.9 *Comparison between calculated and experimental values for some unsaturated organic compounds.* The calculations were made from values given in Table 9.2

9.7 Instrumentation in the UV/Visible

A spectrophotometer is designed around three fundamental modules: the *source*, the *dispersive system* (combined in a monochromator), which constitute the optical section and the *detection system* (Figure 9.10). These components are typically integrated in a unique framework to make spectrometers for chemical analysis. A sample compartment is inserted into the optical path either before or after the dispersive system depending upon the design of the instrument.

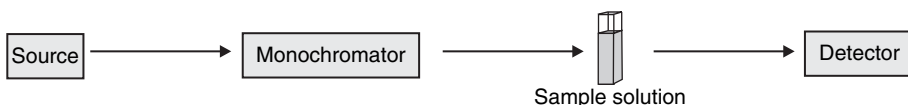
Certain instruments are reserved for routine analyses for which a high resolution is not required. Numerous compounds in solution lead to spectra lacking in fine bands. However it is essential that these instruments are able to give precise quantitative results to within several units of absorbance.

9.7.1 Light sources

All spectrometers require a light source. More than one type of source can be used in the same instrument which automatically swap lamps when scanning between the UV and visible regions:

- for the visible region of the spectrum, an *incandescent lamp* fitted with a tungsten filament housed in a silica glass;
- for the UV region a *deuterium arc lamp* working under a slight pressure to maintain an emission continuum (<350 nm);
- alternatively, for the entire region 200 to 1100 nm, a *xenon arc lamp* can be used for routine apparatuses.

Single or double beam, normal optic sequential spectrometer



Single beam, reverse optic simultaneous spectrometer

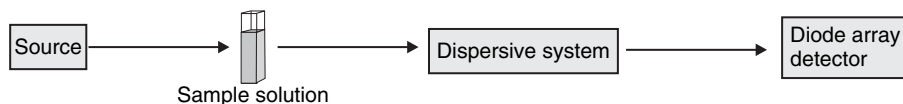


Figure 9.10 *Instrumentation in the UV/Vis.* Two optical schemes are displayed which generate a spectrum by following two different procedures. In the first design on which the majority of instruments are based, the spectrum is obtained in a sequential manner as a function of time (one wavelength after another). In the second, the detector ‘sees’ all of the wavelengths simultaneously.

■ The deuterium discharge lamp contains two electrodes bathed in an atmosphere of deuterium (D_2 is preferred to hydrogen H_2 , for technical reasons). Between the electrodes a metallic screen is placed which is pierced with a circular hole of approximately 1 mm diameter (Figure 9.11). The migration of the electrons toward the anode creates a discharge of current generating an intense arc at this hole, which is in front of the anode. Subjected to this electron bombardment the molecules of deuterium D_2 dissociate, emitting a continuum of photons over the wavelengths range between 160 and 500 nm.

9.7.2 Dispersive systems and monochromators

Sequential instruments

The light emitted by the source is dispersed through either a planar or concave grating which forms part of a monochromator assembly. This device permits the extraction of a narrow interval of the emission spectrum. The wavelength or more precisely the width of the spectral band, which is a function of the slit width, can be varied gradually by pivoting the grating (Figure 9.12). Optical paths with long focal lengths (0.2 to 0.5 m) yield the best resolution.

Simultaneous instruments

This category of instrument functions according to the spectrograph principle. The light beam is diffracted after travelling through the measuring cell (Figure 9.13).

9.7.3 Detectors

The detector converts the intensity of the light reaching it to an electrical signal. It is by nature a single channel device. Two types of detector are used, either a photomultiplier tube or a semiconductor (charge transfer devices or silicon photodiodes). For both of which the sensitivity depends upon the wavelength.

For 'simultaneous' instruments which do not possess a monochromator but a dispersive system, the light intensity at all wavelengths is measured practically at the same instant by aligning a large number of detectors in the form of a diode array (Figure 9.13). The photoelectric threshold, in the order of 1 eV, extends the range of detection up to 1.1 μm .

The efficiency of the photomultiplier tube – a very sensitive device whose linearity of response reaches seven decades – depends upon the yield at the

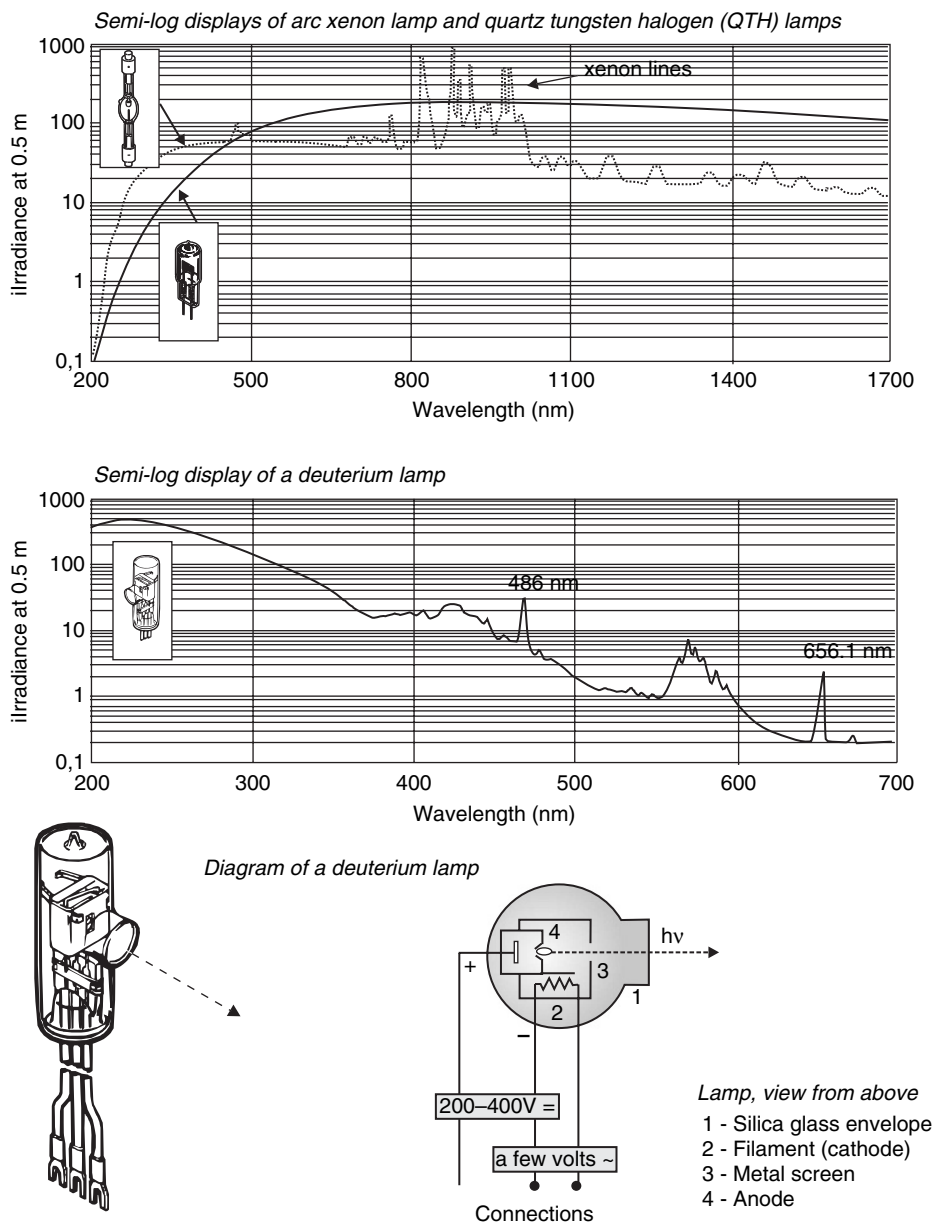


Figure 9.11 Emission spectra of the different types of sources in UV/Vis. A logarithmic scale accounts for the big differences of light intensity according to the wavelengths, notably for filamentless lamps. Below left and middle, general view of a lamp and that seen from above (reproduced courtesy of Oriel). Schematic presenting the circuit details for the lamp. The lamp is booted with a voltage of between 3 to 400 V. The anode is a molybdenum plate while the cathode is a filament of metallic oxide able of emitting electrons and connected to an electrical supply. The emission peaks of deuterium at 486 and 656.1 nm are often used to calibrate the spectrometer wavelength scale.

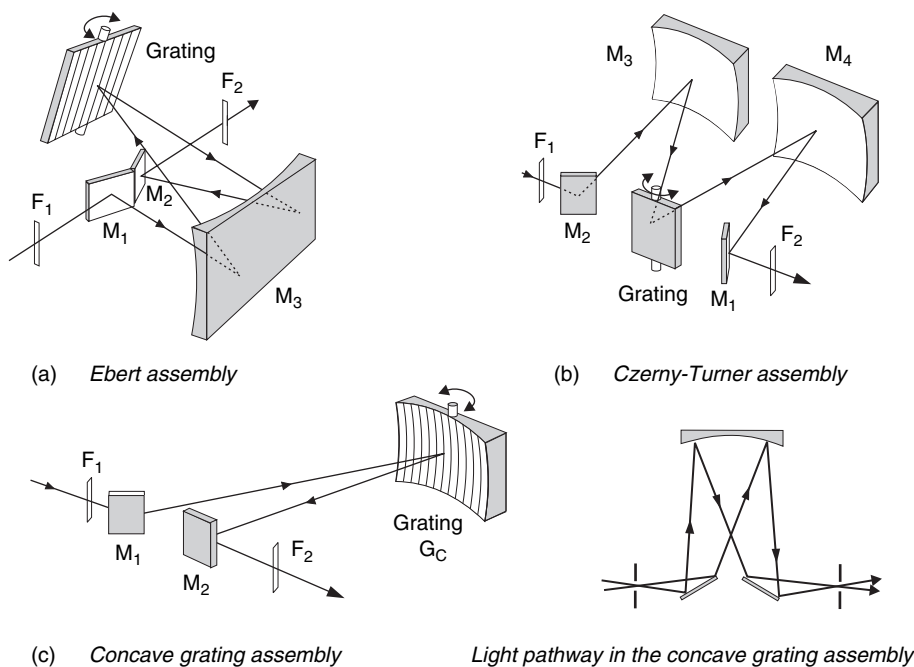


Figure 9.12 Monochromator gratings. (a) Ebert assembly incorporating a single concave spherical mirror M_3 . Able to compensate for aberrations this monochromator gives excellent image quality. (b) Of similar design to Ebert the Czerny–Turner assembly contains two spherical concave mirrors M_3 and M_4 . (c) The concave grating R_c in this design permits both dispersion and focussing of the radiation. The spectral bandwidth of these monochromators depends upon the width of the entrance F_1 and the exit F_2 slits, respectively.

photocathode, which varies with the wavelength (e.g. $0.1 e^-/\text{photon}$ at 750 nm), and on the signal gain provided by the cascade of dynodes (e.g. a gain of 6×10^5). With such values the impact of 10 000 photons/s produces a current of 0.1 nA. For a photomultiplier it is difficult, just as it would be for the eye to compare two light intensities with precision, one arising from the reference beam and the other from the sample, when they are quite different. This is why it is desirable that the absorbance of the solutions does not exceed 1 (see equally Section 9.14). On the other hand, for an instrument whose stray light is of 0.01 per cent (measured in percentage transmittance), an increase in solution concentration produces no significant variation in the signal beyond than 4 units of absorbance.

9.8 UV/Vis spectrophotometers

UV/Vis spectrometers are classified into three optical designs: a fixed spectrometer with a single light beam and sample holder; a scanning spectrometer with double

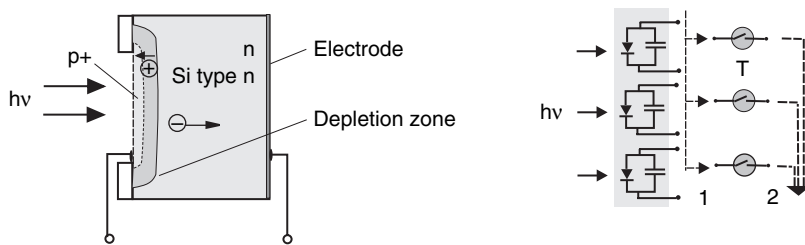


Figure 9.13 Silicon photodiode (PIN) and diode array (CCD detectors – charged coupled device). Each diode, of rectangular shape ($15 \times 25 \mu\text{m}$), is associated with a charged capacitor. Under the impact of photons the diode becomes a conductor and progressively discharges the capacitor. This diode (PIN type) has a large intrinsic region sandwiched between P-doped and N-doped semiconducting regions. Photons absorbed in this region create electron-hole pairs that are then separated by an electric field, thus generating an electric current in a load circuit. The value of the residual charge of each of the capacitors depends upon the number of the photons received. This is measured in a sequential fashion by the electronic circuits (1 and 2). The capacitors are recharged periodically. This is in contrast to the photomultiplier tube which gives an instantaneous measurement of intensity in watts, while the diode leads to an indication of the energy emitted in joules over a time interval. The sensitivity, linearity and the dynamic range of response of the photomultiplier tube are excellent.

light beams and two sample holders for automatic measurement of absorbance; and a non-scanning spectrometer with an array detector for simultaneous measurement of multiple wavelengths.

9.8.1 Single-beam monochannel optical spectrometers

Many routine measurements are conducted at fixed wavelengths by basic photometers fitted with interchangeable interference filters or simple grating monochromators. In single-beam instruments, obtaining a spectrum requires measuring the transmittance of the sample and of the solvent at each wavelength. A control corresponding to the solvent alone or a solution containing the reagents of the measurement (but without the compound to be measured, an *analytical blank*), is first placed in the optical path, then is replaced by the solution prepared from the sample of unknown concentration. These instruments sometimes have a built-in electronic compensation arrangement for variations in light source intensity (Figure 9.14) known as a *splitbeam*. A part of the light beam is diverted before it reaches the sample permitting the stabilization of the source intensity (this is not a true reference beam). These instruments yield absorbance and led to the analyte concentration.

9.8.2 Array-detector spectrophotometers

This type of instrument resembles a spectrograph closely since it allows the simultaneous recording of all wavelengths of the spectrum by using an array of up to

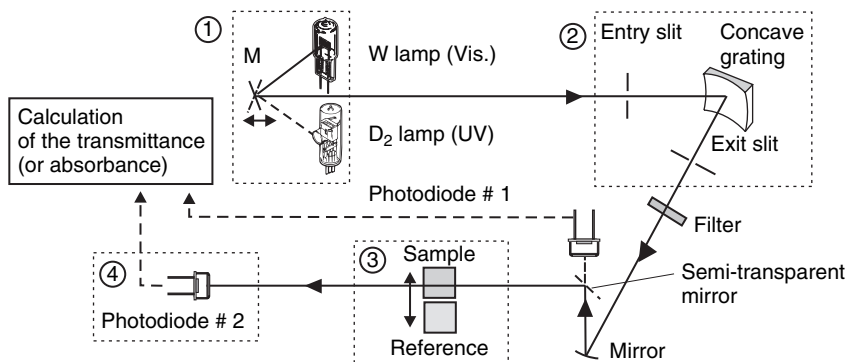


Figure 9.14 Simplified scheme of the optical path of a simple beam, sequential mode spectrophotometer. 1. Two co-existing sources, though only one is selected for the measurement. 2. The monochromator selects the measurement wavelength. 3. The measuring cell containing either sample or control blank is placed in the optical path. 4 and 5. Diode detector and control diode.

a few thousands of miniaturized photodiodes (Figure 9.15). Array-detector spectrophotometers allow rapid recording of absorption spectra, each diode measuring the light intensity over a small interval of wavelength. The resolution power of these diode-array instruments, without a monochromator, is limited by the size of the diodes. These instruments use only a single light beam, so a reference spectrum is recorded and stored in memory to produce transmittance or absorbance spectra after recording the sample spectrum.

9.8.3 Double-beam scanning spectrometer

The double-beam design greatly simplifies the process of the single-beam instrument by measuring the transmittance of the sample and solvent almost simultaneously. One beam passes through the sample while the other passes through the reference solution. Most spectrometers use one (or two) mirrored rotating chopper wheel to alternately direct the light beam through the sample and reference cells. This permits the detector to compare the two intensities transmitted by reference or sample solutions for the same wavelength (Figure 9.16). The amplification of the modulated signal allows the elimination, in large part, of the stray light. The electronic circuit adjusts the sensitivity of the photomultiplier tube as the inverse with respect to the light intensity it receives. A simpler set-up is based on the use of semi-transparent mirror and two connected photodiodes. The signal corresponds to the potential difference required to maintain a constant detector response (principle of a *feedback system*). These instruments are characterized by a fast scanning speed (30 nm/s) and the capability of measuring absorbances to within just a small number of units.

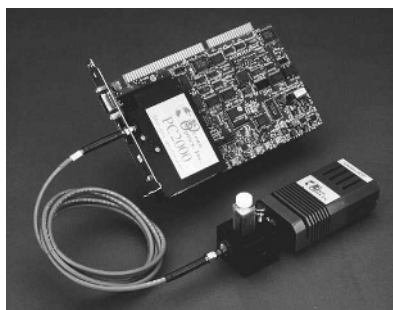
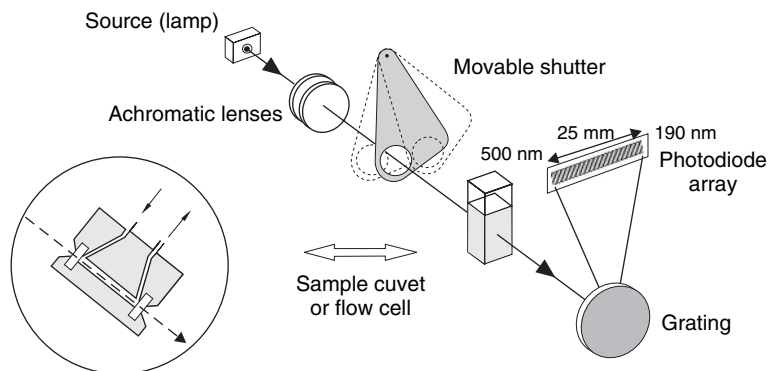


Figure 9.15 Schematics of a single beam spectrophotometer illustrating the simultaneous mode (spectrometer with diode array) and miniature UV/Vis spectrophotometers. Above: the shutter, the only mobile piece in this assembly serves in the reduction of the background noise or dark current produced when no light reaches the photodiodes. All wavelengths pass through the sample. These spectrometers use photodiode arrays (PDAs) or charge-coupled devices (CCDs) as the detector. The spectral range of these array detectors is typically 200 to 1000 nm. With this inverted optical design the sample compartment can be exposed to the external light. These instruments are used mainly as detectors in liquid chromatography (cf. Section 3.7). Below: examples of miniature spectrometers whose dispersive system and detector are integrated into a circuit board, which can be inserted into a personal computer. An optical fibre transmits the light from the source/sample cell situated at a distance (reproduced courtesy of Ocean Optics Europe).

■ **Wavelength calibration in UV/Vis.** To provide accurate measurements, UV/Vis. spectrometers must be accurately calibrated – with regard to both their intensity (transmittance) and location (wavelength) axes. To calibrate the wavelength scale of the spectrometer a solution of a few percent of holmium oxide (Ho_2O_3) in dilute perchloric acid is used. This solution has numerous narrow absorption bands well distributed over the wavelengths for which the different maxima are known. The values depend little upon the spectral bandwidth. In fact, the absorption bands of the spectrum are not symmetrical so that the spectral bandwidth selected for visualization has an influence upon the energy reaching the detector which has in turn repercussions on the intensity of the signal and finally upon the recorded spectrum, e.g. in passing from a slit size 0.1 nm to 3 nm, the maximum at 536.47 nm is displaced to 537.50 nm.

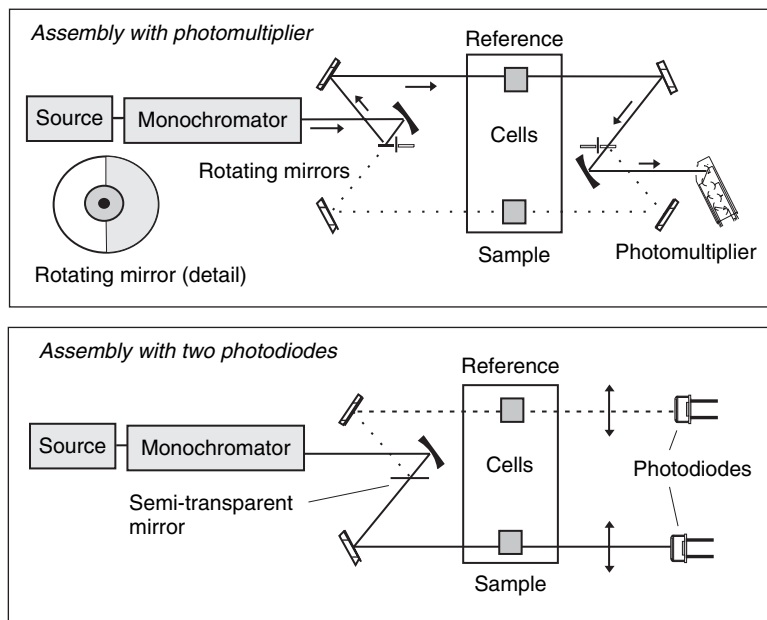


Figure 9.16 Optical path from the exit of the monochromator to the detector for two double beam instruments, (a model with two rotating mirrors and a model with a semi-transparent mirror). The arrangement of the apparatus with rotating mirrors is similar to that of IR spectrophotometers apart from the fact that the light beam issuing from the source passes first through the monochromator before it hits the sample. In this way the photolytic reactions which could occur owing to an overexposure to the total radiation issued from the source are minimized. A more compact and simple optical assembly with a single beam associated with two detectors. A semi-transparent and fixed mirror replaces the delicate mechanism of synchronized, rotating mirrors.

Double beam spectrophotometers allow differential measurements to be made between the sample and the analytical blank. They are preferable to the single beam instruments for cloudy solutions. The bandwidth of high performance instruments can be as small as 0.01 nm. For routine measurements such as monitoring a compound on a production line, an immersion probe is employed. Placed in the sample compartment of the apparatus this accessory contains two fibre-optics, one to conduct the light to the sample and another to recover it after absorption in the media studied. Two types exist: by transmission for clear solutions and by attenuated total reflection (ATR) for very absorbent solutions (Figure 9.17).

■ **Fluorescent compounds.** When the compound studied is fluorescent, the absorption spectrum is less intense because the re-emitted light gives, as a consequence, an inaccurate value for the true absorbed light. Observed using an assembly of reversed optics, the fluorescence of the sample (exposed to the total

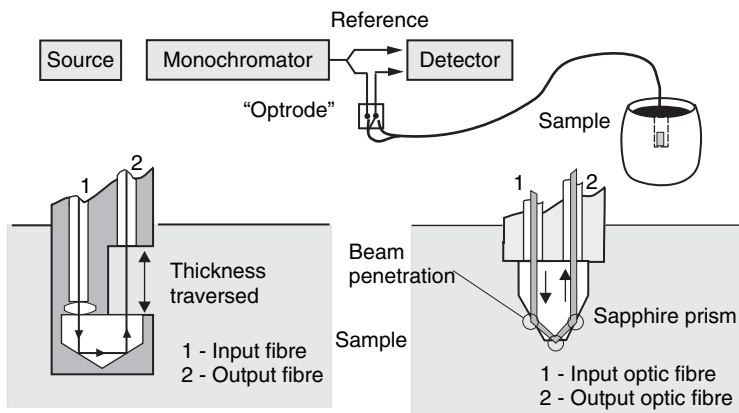


Figure 9.17 The principle of a spectrophotometer fitted with an immersion probe. Monochromatic light issuing from a spectrophotometer is guided towards an immersion cell and then returned to the detector. The route is confined by a fibre optic. Left: transmission probe. Right: ATR probe; the sapphire prism has a refraction index greater than that of the solution. The schematic shows three reflections of the beam and its penetration into the solution (see explanation in Chapter 10, Section 10.9.3).

radiation of the source) reduces the absorbance in the region of the spectrum where the emission is situated. Alternatively, with a traditional optical assembly, the fluorescence appears when the monochromator selects the region corresponding to the excitation: the absorbance will be less intense in the region where compound fluoresces. However, the problem should not be exaggerated as the fluorescence is emitted in all directions and the photons taking the optical path for the absorption experiment constitute a very small part of the emitted light.

9.9 Quantitative analysis: laws of molecular absorption

9.9.1 Lambert–Beer law

The UV/Vis domain has been widely exploited in quantitative analysis and the visible region for a particularly long time. The measurements are based upon the Lambert–Beer law which, under certain conditions, links the absorption of the light to the concentration of a compound in solution.

The origins of this law are found in the work of the French mathematician Lambert who laid down the basis for photometry in the eighteenth century. Later Beer, a German physicist of the following century, proposed a law, which led to the calculation of the quantity of light transmitted through a given thickness of a compound in solution in a non-absorbing matrix.

The result was the *Lambert–Beer law* which is presented here in its current form:

$$A = \varepsilon_{\lambda} l C \quad (9.4)$$

A represents the absorbance, an optical parameter without dimension accessible with a spectrophotometer, l is the thickness (in cm) of the solution through which the incident light is passed, C the molar concentration and ε_{λ} the molar absorption coefficient ($\text{L mol}^{-1} \text{cm}^{-1}$) at *wavelength* λ , at which the measurement is made. This parameter, also called the molar absorptivity, is characteristic of the compound being analysed and depends among other things upon the temperature and the nature of the solvent. Generally its value is given at the wavelength of the maximum absorption. This can vary over a wide range going from zero to over 200 000.

According to Lambert's hypothesis, the intensity I of *monochromatic radiation* is decreased by dI (negative value) after passing through a thickness dx of a material whose *absorption coefficient* is k for the wavelength x chosen (Figure 9.18), being:

$$-\frac{dI}{dx} = kI_x \quad (9.5)$$

In calling I_0 the light intensity of the incident radiation before it passes through the medium of thickness l , that has an absorption coefficient k , expression 9.5 can be written in integrated form giving the transmitted intensity I (Equations 9.6 to 9.8):

$$[\ln I_x]_{I_0}^I = -k [x]_0^l \quad (9.6)$$

$$\ln \frac{I}{I_0} = -k \cdot l \quad (9.7)$$

$$I = I_0 e^{-kl} \quad (9.8)$$

In 1850, Beer proposed the general expression 9.9 for the case of a solution of low concentration dissolved in a transparent medium (non-absorbing), by writing that k is proportional to the molar concentration C of this compound (Figure 9.19):

$$k = k' \cdot C \quad (9.9)$$

By replacing k with $k' C$ in expression 9.7 a new relation is obtained which is better known in the form of expression 9.4, in which the *absorbance* A is represented by one of the comparable expressions:

$$A = \log \frac{I_0}{I} \quad \text{or} \quad A = \log \frac{1}{T} \quad \text{or} \quad A = \log \frac{100}{T\%} \quad \text{or} \quad A = 2 - \log T\% \quad (9.10)$$

The Lambert–Beer law, considered in this approach as a postulate, has been the subject of different interpretations and demonstrations of statistical hypotheses,

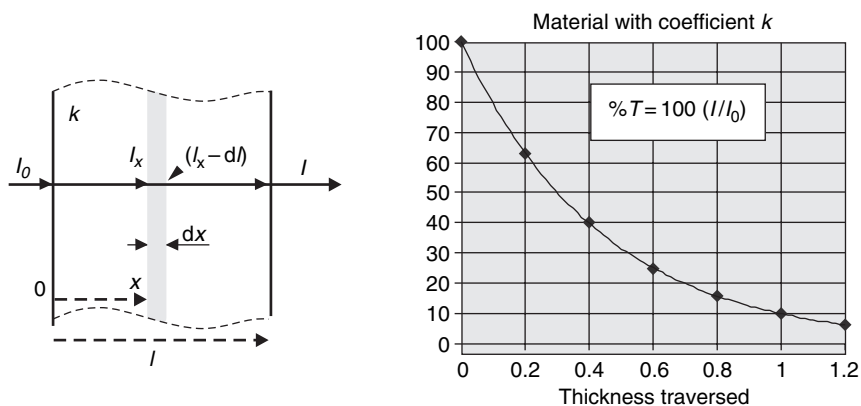


Figure 9.18 Absorption of the light by a homogeneous material and representation of percentage transmittance as a function of the material's thickness. The light reaching the sample can be reflected, diffused, transmitted or absorbed. Here only this last fraction is taken into account.

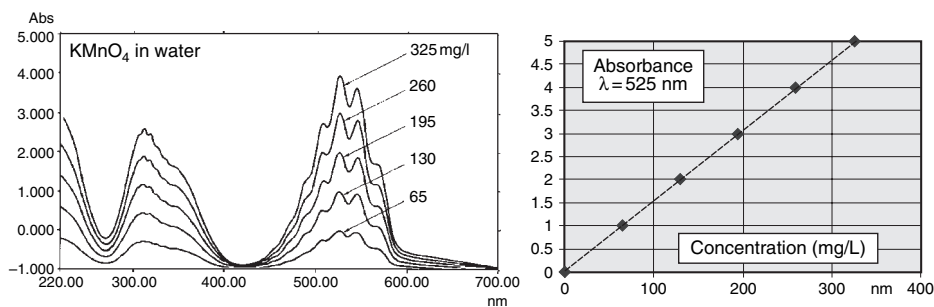


Figure 9.19 Illustration of the Lambert-Beer law. Spectra of aqueous solutions of increasing concentration in potassium permanganate. Graph of the corresponding absorbances measured at 525 nm showing the linear growth of this parameter.

kinetics or simple logic. k may be related to σ , absorption cross-section ($\text{cm}^2/\text{molecule}$) and n , number density (molecules/mL): $k = \sigma.n$.

This law, which concerns only that fraction of the light absorbed can be verified by application of the following conditions:

- the light used must be monochromatic
- the concentrations must be low
- the solution must be neither fluorescent or heterogeneous
- the solute must not undergo to photochemical transformations
- the solute must not undertake variable associations with the solvent.

In general, when the absorbance is to be measured at a single wavelength, the absorption maximum is chosen. In order that the absorbance reflects the concentration, it is required that the spectral band $\Delta\lambda$ reaching the detector and pre-selected by the instrument parameter called bandwidth, will be much narrower (10 times) than the width of the absorption band measured at mid-height (see wavelength calibration in Section 9.8.3).

9.9.2 Additivity of absorbances

The Lambert–Beer law is additive (Figure 9.20). This means that if the absorbance A is measured for a mixture of two compounds, 1 and 2 in a solvent and placed in a cuvette of thickness l the same total absorbance (expression 9.11) will be obtained if the light had successively passed through two cuvettes of thickness l , placed one after the other, one containing compound 1 (Abs. A_1) and the other compound 2 (Abs. A_2). Of course the concentrations and the solvent must remain the same as for the initial mixture (compound 1 is labelled 1 and compound 2 is labelled 2):

Experiment 1:
$$A = l(\varepsilon_1 C_1 + \varepsilon_2 C_2)$$

Experiment 2:
$$A_1 = l\varepsilon_1 C_1 \quad \text{and} \quad A_2 = l\varepsilon_2 C_2$$

$$A = A_1 + A_2 \quad (9.11)$$

This principle is illustrated by the study of isobestic points. Consider a compound A, which is transformed by a reaction of first order to compound B. Assume that the absorption spectra of A and B recorded separately in the same solvent and at the same concentration, cross over at a point I when one is superimposed upon the other (Figure 9.21). Consequently, *for the wavelength of point I*, the absorbances of the two solutions are the same and by corollary the coefficients ε_A and ε_B are equal ($\varepsilon_A = \varepsilon_B = \varepsilon$). In this transformation, compound A is initially

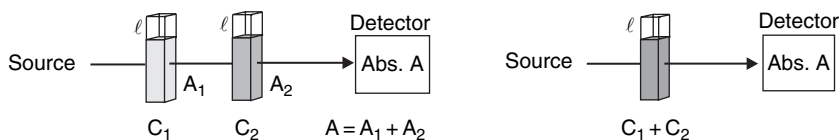


Figure 9.20 *Additive nature of absorbances.* For all wavelengths, the absorbance of a mixture is equal to the sum of the absorbances of each component within the mixture (assuming the same molar concentrations in the two experiments).

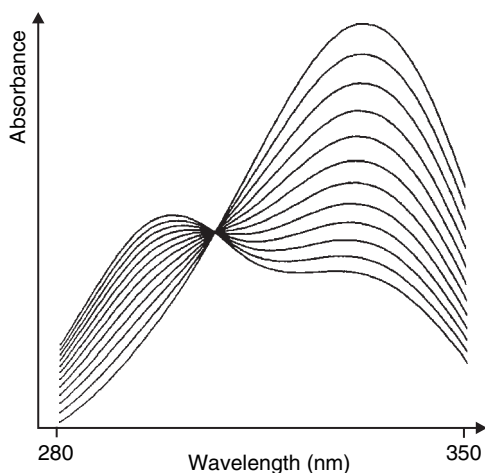


Figure 9.21 *Isobestic point.* Alkaline hydrolysis of methyl salicylate at 25°C. Superimposition of the successive spectra recorded between 280 and 350 nm at 10 min intervals. At isobestic wavelength, the absorbance is invariant.

alone and ultimately compound B is also pure. For all intermediate solutions, the global concentration for mixtures of A and B does not change ($C_A + C_B = C_{\text{ste}}$), which can be given by the following expression:

$$A_I = \varepsilon_A l C_A + \varepsilon_B l C_B = \varepsilon l (C_A + C_B) \quad (9.12)$$

All of the spectra of the various mixtures of A + B formed over time will pass by the point I, called the isobestic point, where the absorbance A will always be of the same value.

This network of concurrent curves is observed when studying coloured indicators as a function of pH, or kinetic studies of particular reactions (Figure 9.21). The isobestic point is useful to measure the total concentration of two species in equilibrium, i.e. an isomerization reaction.

9.10 Methods in quantitative analysis

When a compound does not absorb light, it can nevertheless become the subject of a photometric measurement if it can be transformed beforehand to a derivative that has an exploitable chromophore. Using this method it becomes possible to quantify all kinds of chemical species that have no significant absorption because it is very weak, or because it lies in a region of the spectrum where co-exists other absorptions which interfere. To counteract this, the measurement of absorbance is preceded by a chemical transformation which must be specific, total, rapid,

reproducible and lead to a stable derivative in solution, that is UV/Vis absorbing. This is the principle of colorimetric tests.

The term colorimetry arose from the statement that initial measurements in this field, well before the invention of the spectrophotometer, were carried out with natural light (white light) and by direct visual comparison of the coloration of the sample with that of a control of known concentration. Apart from the eye, no other particular instrumentation was used.

The two situations that can be encountered most frequently are:

- *The analyte to be measured is present in a matrix whose other constituents absorb also in the same spectral range:* the direct measurement of the absorption due solely to this compound is therefore impossible (Figure 9.22, curve a). To overcome this difficulty, the compound is transformed specifically into a derivative whose absorption curve is situated in a region free from interference by the matrix (Figure 9.22, curve b).
- *The analyte to be measured has no exploitable chromophore:* once again this problem is overcome by the creation of an absorbing derivative of the initial species following the same principle (Figure 9.22, curves c and d).

In colorimetry, it is preferable that the measurement is made on a chromophore whose absorbance is situated toward longer wavelengths as this reduces the risk of superimposition of the individual absorptions of the different other compounds. Elsewhere, when measurement is preceded by a chemical reaction, the exact structure of the coloured derivative, whose absorbance is measured, is rarely known: nevertheless, if it is assumed that the reaction implicated is stoichiometric, its molar absorption coefficient is accessible from the molar concentration of the compound that has been derivatized.

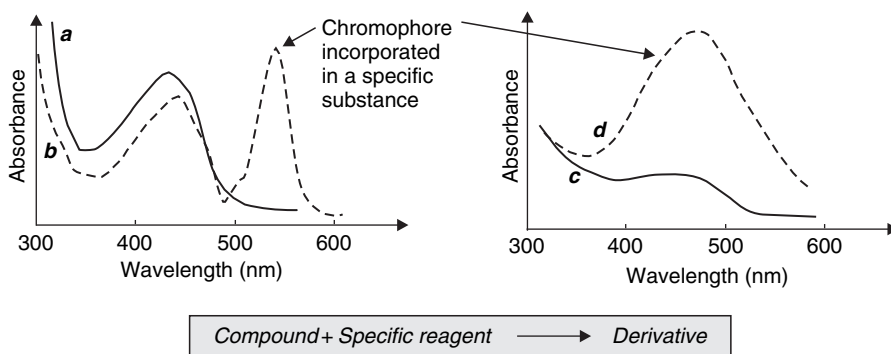


Figure 9.22 Illustration of two situations frequently met. A compound whose absorption is masked by the components of the mixture can otherwise be measured using colorimetry by employing a chemical modification which transforms the molecule into a derivative suffering no interference.

■ Nephelometry, a further technique of colorimetry is also related to the Lambert–Beer law. This method consists of forming a precipitate and from the light absorbed at a given wavelength, to determine the concentration of the precursor. For example, to measure a sulphate ion, a soluble barium salt is added. A sulfate precipitate of barium is formed of which the absorbance is measured following stabilization with a hydrosoluble polymer such as Tween®.

9.11 Analysis of a single analyte and purity control

In practice, the first step is the construction of a calibration curve $A = f(c)$ from solutions of known concentration of the compound to be measured, submitted to the same treatment as the sample. This curve, which is most often a straight line for dilute solutions, allows determination of the concentration C_x of the unknown sample.

Sometimes only a single standard solution is prepared. The concentration is chosen such that the concentration C_R is such that its absorbance A_R is slightly greater than that estimated for the unknown solution A_x (Figure 9.23).

The following formula can then be applied to calculate C_x :

$$C_x = C_R \frac{A_x}{A_R} \quad (9.13)$$

Calculation of the concentration of an analyte by expression 9.13 leads to an erroneous result if the sample contains an impurity (absent in the reference solution) which also absorbs at the wavelength of measurement. Therefore a method commonly named *confirmatory analysis* is used.

For a given pure compound the ratio of the absorption coefficients determined at two wavelengths is constant and a characteristic of this compound (Figure 9.24).

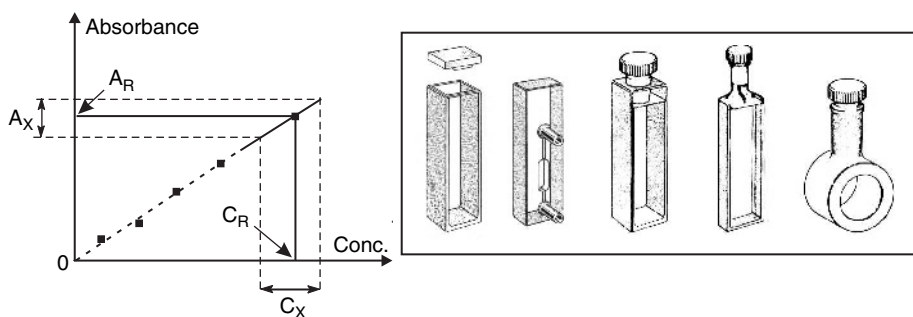


Figure 9.23 Calibration curve and cells of optical glass or silica glass (quartz). If a single reference solution is prepared, the graph will be a straight line that passes through the origin. The accuracy of the result will be precise when the unknown concentration is close to the reference concentration (the result is determined by interpolation, not by extrapolation).

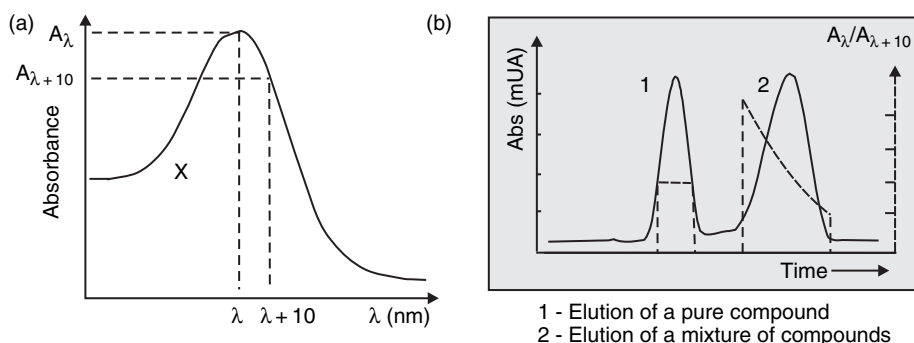


Figure 9.24 Confirmation analysis. (a) UV spectrum of a pure compound; (b) diagram illustrating a chromatogram which presents two peaks of which the first is due to a single compound and the second to the elution of two compounds slightly displaced in time. The evolution of the ratio of absorbances during elution permits a control of the purity of the eluted compounds. These variations are usually revealed by software as coloured areas.

Thus, if one or two extra measurements are performed, displaced by several nanometres with respect to the original measurement, the ratios of absorbance of the sample solution can be calculated and compared with those which have been established in the same way using a pure reference solution. If these ratios are different, it will be presumed that an impurity is present in the sample. The calculation of the concentration would not be reliable in this case.

Starting from this assumption, it is possible to control the homogeneity of the elution peaks in liquid chromatography on the condition that a UV detector undertaking simultaneous measurements of the absorbance at several wavelengths is present (such a diode array detector). Any variation during the course of the elution of a component from the ratio of the absorbances at two wavelengths, suggests that it is a mixture that is eluting from the column instead of a pure compound (Figure 9.24). This method will fail however in the case of the perfect co-elution of two compounds (the retention times being identical).

9.12 Multicomponent analysis (MCA)

When a mixture of compounds whose absorption spectra are known is analysed, then the mixture's composition can be determined. The method is based on absorption spectra of pure individual components and calibrating mixtures of well-defined fraction components. According to the law of additivity (expression 9.11), the spectrum of the mixture to be measured corresponds to the weighted sum of the spectra of each of the individual constituents. The classical method of calculation is reviewed here for revision purposes since it is no longer effected long hand because it is incorporated into computer software.

9.12.1 Basic algebraic method

Given a mixture of three components a, b and c in solution (concentrations C_a, C_b, C_c). The absorbances of this mixture are measured at three wavelengths λ_1, λ_2 and λ_3 giving A_1, A_2 and A_3 . Knowing the values of the specific absorbances for each of the three compounds taken in isolation for the three wavelengths (nine values in total from ε_a^1 to ε_c^3) through application of the additivity law, the following system of three simultaneous equations can be written (it is assumed that the optical path of the cells used is of 1 cm):

$$\begin{array}{ll} \text{at } \lambda_1 & A_1 = \varepsilon_a^1 C_a + \varepsilon_b^1 C_b + \varepsilon_c^1 C_c \\ \text{at } \lambda_2 & A_2 = \varepsilon_a^2 C_a + \varepsilon_b^2 C_b + \varepsilon_c^2 C_c \\ \text{at } \lambda_3 & A_3 = \varepsilon_a^3 C_a + \varepsilon_b^3 C_b + \varepsilon_c^3 C_c \end{array}$$

The resolution of this mathematical system, which corresponds to a $[3 \times 3]$ matrix, leads to the three concentrations required C_a, C_b , and C_c .

$$\begin{bmatrix} C_1 \\ C_2 \\ C_3 \end{bmatrix} = \begin{bmatrix} A_1 \\ A_2 \\ A_3 \end{bmatrix} \cdot \begin{bmatrix} \varepsilon_a^1 & \varepsilon_b^1 & \varepsilon_c^1 \\ \varepsilon_a^2 & \varepsilon_b^2 & \varepsilon_c^2 \\ \varepsilon_a^3 & \varepsilon_b^3 & \varepsilon_c^3 \end{bmatrix}^{-1} \quad (9.14)$$

This approach yields good results when the compounds lead to spectra, which are significantly different, otherwise it loses precision when the spectra are in close proximity as a small measurement error can lead to a large variation in the result. To avoid this risk the instruments housing diode arrays use many tens of data points. Although the system to be resolved (expression 9.14) becomes over-determined but this leads to better results.

9.12.2 Multiwavelength linear regression analysis (MLRA)

The analysis of mixtures has given rise to the development of a variety of methods, made possible with spectrometers that can record data. A computer dedicated to the spectrometer optical platform includes quantitative analysis software that can treat a large number of data points extracted from sample spectra under study and for standard solutions.

Described below is a method of linear regression which leads to the neutralization of the background noise and therefore to an improvement in the results. This method is applied for the analysis of a two-component sample (Figure 9.25).

The instrument uses three recordings: a spectrum of the sample (which contains the two compounds whose concentrations are presently unknown) and a spectrum of the same spectral region for each of the pure compound (reference solutions of known concentration).

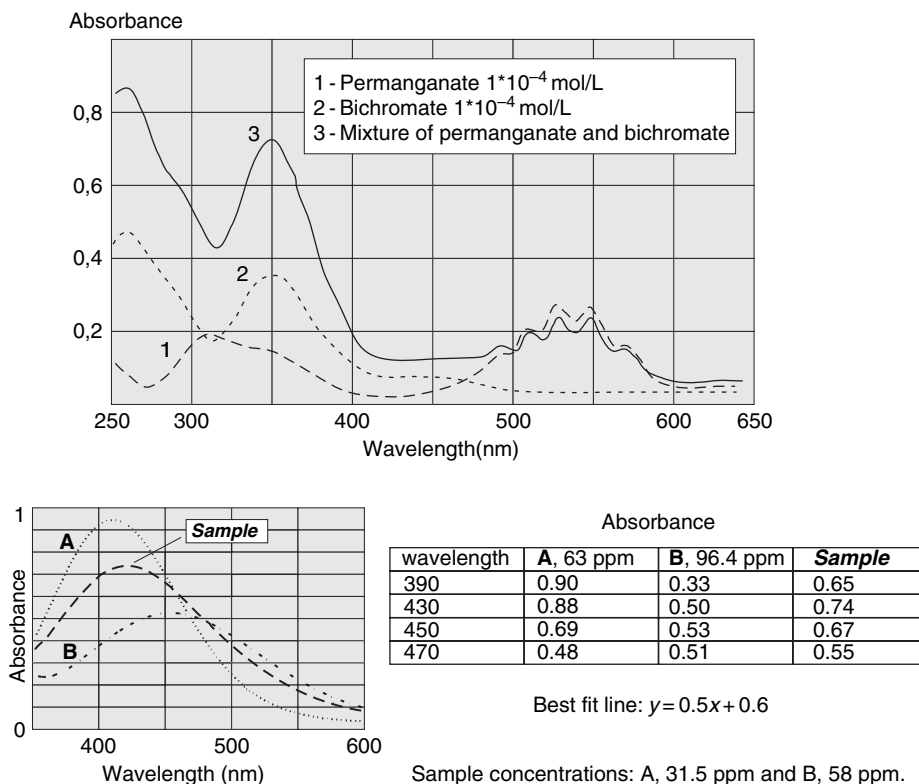


Figure 9.25 Multicomponent analysis. Spectra of a solution 1×10^{-4} M of potassium permanganate, a solution 1×10^{-4} M of potassium dichromate and a solution containing 0.8×10^{-4} M of dichromate and 0.8×10^{-4} M of permanganate (Bianco M. *et al. J Chem Educ* 1989, 66(2), 178). Below is an example of MLRA from experimental data.

For any wavelength, the absorbance of the mixture of the two species a and b (which do not interact with each other) being analysed, is given by the expression (absorbance addivities):

$$A = \varepsilon_a l C_a + \varepsilon_b l C_b \quad (9.15)$$

For each of the two reference spectra, supposing that the thickness of the measuring cell is $l = 1$ cm, the following equations can be written :

$$\text{For compound a} \quad A_{\text{ref.a}} = \varepsilon_a \cdot C_{\text{ref.a}} \quad (9.16)$$

$$\text{For compound b} \quad A_{\text{ref.b}} = \varepsilon_b \cdot C_{\text{ref.b}} \quad (9.17)$$

These two expressions lead to the calculation of the molar absorption coefficients ε of each pure constituent, at each wavelength considered. Therefore expression 9.15 can be re-written as:

$$A = \frac{A_{\text{ref.a}}}{C_{\text{ref.a}}} C_a + \frac{A_{\text{ref.b}}}{C_{\text{ref.b}}} C_b \quad (9.18)$$

By dividing the first element by $A_{\text{ref.a}}$, then for each wavelength:

$$\left(\frac{A}{A_{\text{ref.a}}} \right) = \frac{C_a}{C_{\text{ref.a}}} + \frac{C_b}{C_{\text{ref.b}}} \cdot \left(\frac{A_{\text{ref.b}}}{A_{\text{ref.a}}} \right) \quad (9.19)$$

The first member of relation 9.19 is therefore an affinity function of the ratio of absorbances figuring in the second member. The calculated values are on a straight line whose slope and intercept enable calculations to be made for C_a and C_b .

The precision of the results increases with the number of data points used. The method needs a computerized spectrophotometer.

9.12.3 Deconvolution

Numerous software data treatments authorize the elucidation of mixture composition from spectra. One of the best-known methods is the Kalman's least squares filter algorithm, which operates through successive approximations based upon calculations using weighted coefficients (additivity law of absorbances) of the individual spectra of each components contained in the spectral library. Other software for determining the concentration of two or more components within a mixture uses vector quantification mathematics. These are automated methods better known by their initials: PLS (*partial least square*), PCR (*principal component regression*), or MLS (*multiple least squares*) (Figure 9.26).

9.13 Methods of baseline correction

Some samples, such as biological fluids, contain micelles or particles in suspension, which by light scattering (Tyndall effect) induce a supplementary absorption. This one varies in a regular manner with the wavelength (Figure 9.27).

There are several methods that seek to correct this phenomenon by extracting from the absorbance measured what is not due to the analyte. These are in fact corrections made to the baseline from measurements undertaken at different wavelengths.

9.13.1 Modelling by polynomial function fitting

Supposing that variations in the baseline could be represented by a function $A = k\lambda^n$. To find k and n , a region of the spectrum is located in which the analyte

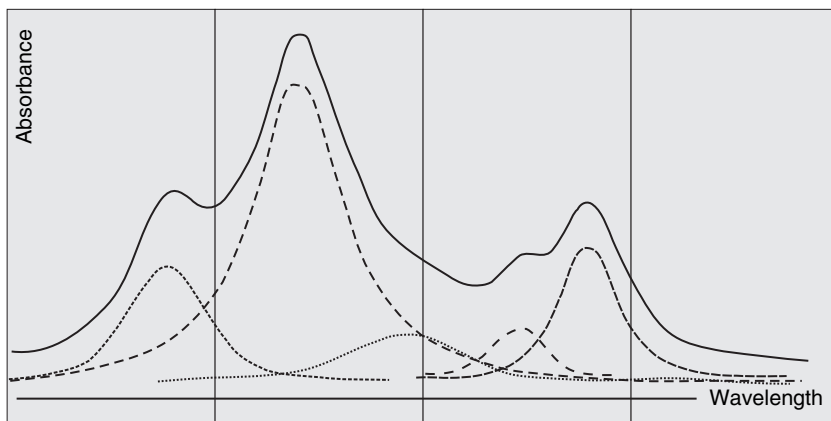


Figure 9.26 *Deconvolution of a spectral curve.* From the experimental spectrum of a mixture of five compounds (the external trace on the figure), the software is able to find the proportion of each one (it is assumed that the individual spectra of the individual components are known).

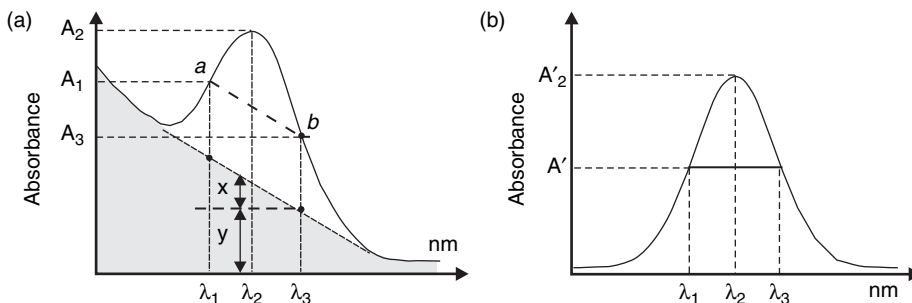


Figure 9.27 *Illustration of the concepts behind Morton and Stubbs' calculation.*

does not absorb in order that the software might use this zone to calculate these coefficients by the method of least squares (knowing that $\log a = \log k + n \log \lambda$). By consequence, whatever the wavelength of measurement it will be possible to remove the absorbance corresponding to the baseline.

9.13.2 Morton–Stubbs three-point correction

The method of Morton and Stubbs (as well as the method using derivative curves (cf. Section 9.15), leads to an efficient correction of the baseline on condition that the underlying absorption varies linearly in the zone of measurement. In a situation such as that represented by Figure 9.27a, in order to quantify the

compound, the absorbance A_2 read at the maximum λ_2 must be corrected by removing the absorbance corresponding to the values noted as x and y .

To bring this about, a reference spectrum of the pure compound must be made in order to choose two wavelengths λ_1 and λ_3 whose absorbance coefficients are the same (Figure 9.27b). As on the figure at, λ_1 and λ_3 this compound has the same absorbance A' and an absorbance maxima A'_2 at the wavelength λ_2 . The ratio R between these two values is calculated ($R = A'_2/A'$). Next on the sample spectrum, the ratio of the absorbances is calculated at these same two wavelengths. On the condition that the baseline is a line of the same slope as the segment ab , the value of x can then be calculated (see figure):

$$x = (A_1 - A_3) \frac{(\lambda_3 - \lambda_2)}{(\lambda_3 - \lambda_1)} \quad (9.20)$$

From R and the ratio A_2/A_3 of the sample, y can be deduced, knowing that the ratio of the absorbances, after the correction of x and y , has the value R (Figure 9.26):

$$R = \frac{A_2 - (x + y)}{A_3 - y}$$

thus,

$$y = \frac{RA_3 - A_2 + x}{R - 1} \quad (9.21)$$

Subtracting x and y from A_2 leads to the calculation of the corrected absorbance, according to

$$A = A_2 - x - y$$

This last equation, which is independent of the concentration of the reference solution yields satisfactory results. This method requires only to have at one's disposal a spectrum of the pure compound.

9.14 Relative error distribution due to instruments

For many contemporary spectrophotometers it is possible to measure absorbance up to within 4 or 5 units. These high values correspond to transmitted intensities that are extremely weak ($I/I_0 = 10^{-5}$ for $A = 5$), giving results that are considered as less reliable.

For transmittance measurements three types of error are recognized as being due to the instrument. These are of independent origin and are potentially accumulative (Figure 9.28):

- *The background noise of the light source.* Represented by the term ΔT_1 is considered to be constant and independent of T : $\Delta T_1 = k$ (curve 1).

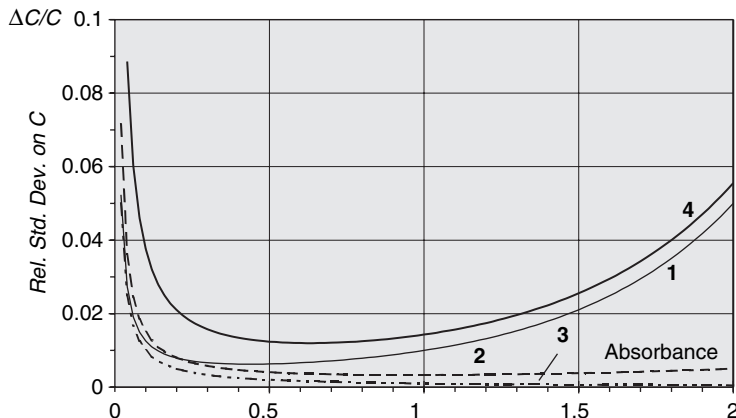


Figure 9.28 Relative errors in quantitative UV spectrometry. Curves representing the average of each of these errors (1 to 3) in absorbance measurements, as well as the relative standard deviation (RSD) of concentration determination as a function of absorbance resulting from their sum (4).

- *The background noise of the photomultiplier tube (or photodiode).* Represented by the term ΔT_2 varies as a function of T following a complex relationship and is represented by the following equation (curve 2):

$$\Delta T_2 = k_2 \sqrt{T^2 + T} \quad (9.22)$$

- *Stray light.* Corresponds to the light which does not pass through the sample but reaches the detector. It decreases the apparent concentration in the sample cell. This term is proportional to T : $\Delta T_3 = k_3 T$ (curve 3).

As these sources of error are additive, the total error in the concentration corresponds – in accepting the three expressions above – to a curve whose ordinate of each point is the sum of the ordinate of the three individual errors discussed above. This curve passes through a minimum generally located around $A = 0.7$. For quantitative measurements it is therefore advisable to adjust the dilutions in order that the absorbances are situated in this favourable domain.

From the Lambert–Beer law it is also possible to link relative standard deviation (see Section 22.2) on the measurement of concentration C to the relative standard deviation made on the transmittance T (curve 4).

$$\frac{\Delta C}{C} = \frac{1}{\ln T} \cdot \frac{\Delta T}{T} \quad (9.23)$$

9.15 Derivative spectrometry

The principle of derivative spectrometry consists of calculating, by a mathematical procedure, derivative graphs of the spectra to improve the precision of certain measurements. This procedure is applied when the analyte spectrum does not appear clearly within the spectrum representing the whole mixture in which it is present. This can result when compounds with very similar spectra are mixed together.

The traces of the successive derived spectral curves are much more uneven than the one of the original spectrum (called *zeroth order spectrum*). These derivative plots amplify the weak slope variations of the absorbance curve (Figure 9.29). The procedure of obtaining the *first derivative* graph, $dA/d\lambda = (d\varepsilon/d\lambda)lC$, can be extended to successive derivatives (*n*th derivatives).

The curve of the *second derivative* matches the points of inflection in the zeroth order spectrum by regions of zero slope which correspond to maxima or minima (Figure 9.30). The fourth derivative reveals even further the weak absorbance variations in the initial spectrum. Numerous analyses would be improved by adopting this principle, knowing that to make a measurement from the derivative spectra is no more difficult than from the absorbance spectrum. The amplitude between the maxima and the minima of the *n*th derivative graph is proportional to the absorbance value of the solution. The calibration graph is established from a few standard solutions of different concentrations to which the same mathematical treatment has been applied, as to the sample solution.

Interest in this procedure manifests itself in three situations in which the absorbance is altered:

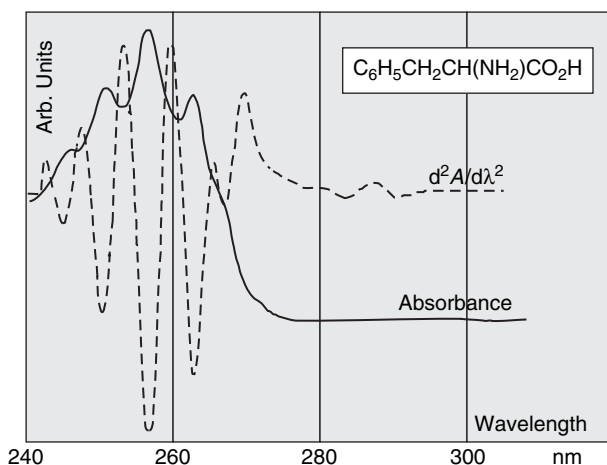


Figure 9.29 Derivative curve. UV spectra of phenylalanine and the curve of its second derivative.

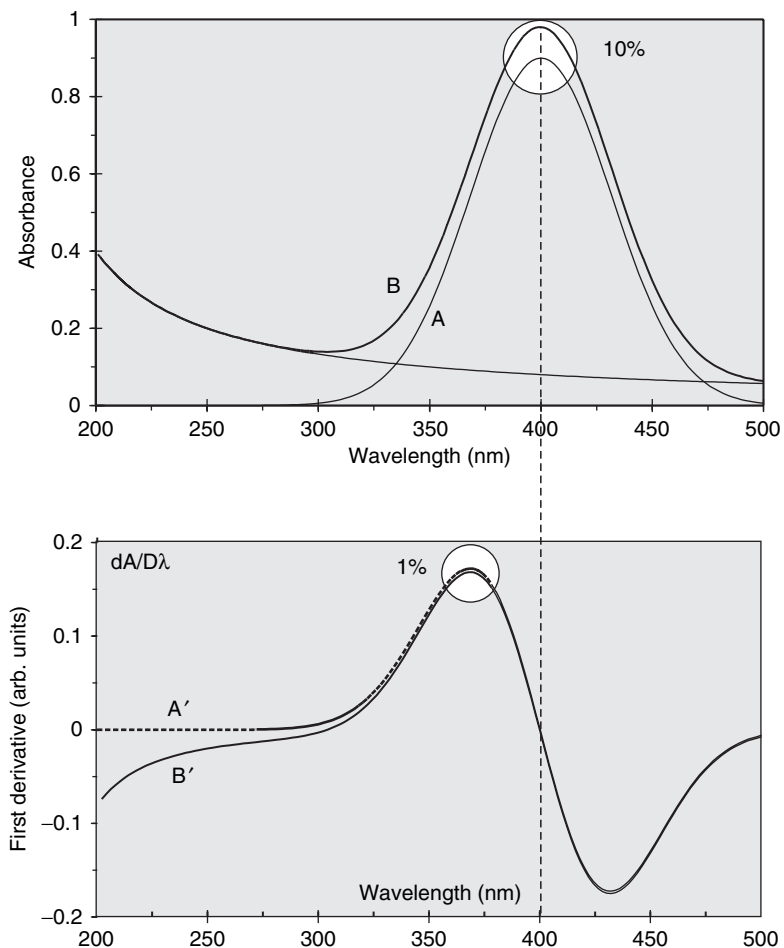


Figure 9.30 Effect of light scattering on the absorbance spectrum and on its first derivative curve. Comparison of the derivative graphs corresponding to A the spectrum of a compound in solution without light scattering and to B, the same spectrum, in the presence of scattering. The phenomenon can be seen to disrupt up to 10 per cent of the absorbance on the regular graph yet only around 1 per cent of the value of the amplitude of the derivative curve (spectra modelled from functions corresponding to Gaussian curves).

- If the spectrum of the sample in solution suffers from an increasing absorbance towards higher energy by consequence of a uniform baseline absorption, the derivative curve, which is sensitive only to variations of slope of the zeroth order curve, will not be affected.
- If light scattering occurs in the heart of the sample solution (which produces an absorption baseline growing moderately at shorter wavelengths), again, the derived graphs will be little affected (Figure 9.30).

- If the absorbance due to the analyte, almost invisible on the original spectrum, is overshadowed by the main absorbance arising from the matrix.

9.16 Visual colorimetry by transmission or reflection

Visual colorimetry, used for more than four centuries, is a simplified form of instrumental absorption spectrometry. Given its modest cost and its frequently surprising precision it is therefore widely practised.

The simplest colorimeters, used for routine measurements, were visual comparators (Figure 9.31). Nessler tubes, used for many years, consisted of graduated flat-bottomed tubes of glass filled with different concentrations of standard solutions mixed with a derivatising agent. The solution to be analysed was placed with the same agent in an identical tube and colour was compared with that of the standards ('same colour, same concentration').

For this purpose, these tubes were placed over a source of white light, to be observed in transmission. These selective tests, ready to use and requiring no instrumentation, complement established methods and by their rapidity help to extend the range of semi-quantitative analyses.

- The innumerable tests, presented in the form of strips, which colour more or less intensely when soaked in the appropriate medium, are among many current applications of colorimetry. However, a result, derived from a visual examination of the reflected light should rather be associated with reflectometry rather than transmission colorimetry.

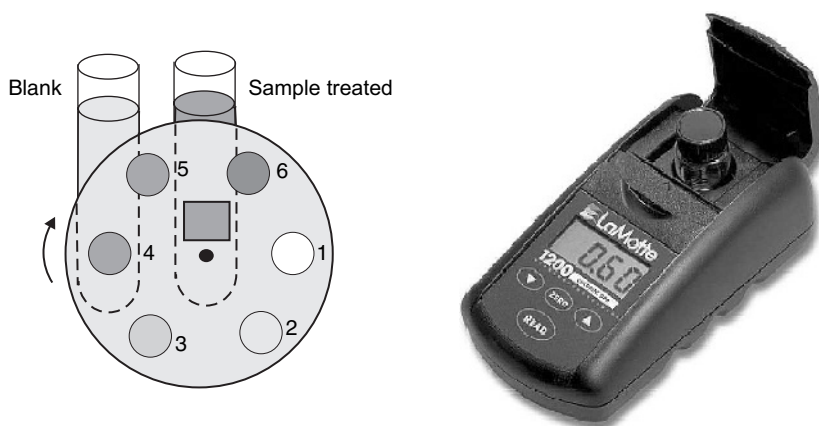
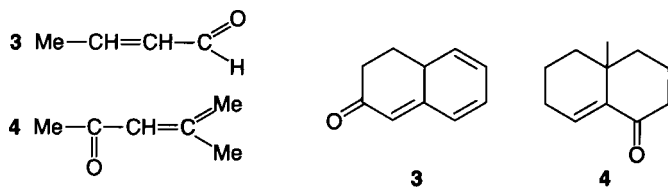


Figure 9.31 *Visual colorimetry.* Left, a disc comparator. The use of this apparatus consists of choosing, by rotation of the filter holder, the filter which permits when it is superimposed upon the analytical blank, a match of the colour of the tube. The observation is made by transmission of white light. The central tube contains the sample after treatment. The disc is calibrated for a particular measurement. Right, a portable colorimeter which allows comparisons without evaluation by the human eye (Model 1200, reproduced courtesy of LaMotte Company).

Problems

- 9.1 Calculate the energy of a mole of photons corresponding to a wavelength of 300 nm.
- 9.2 Calculate the absorbance of an organic dye ($C = 7 \times 10^{-4} \text{ mol L}^{-1}$), knowing that the molar absorptivity $\epsilon = 650 \text{ mol L}^{-1} \text{ cm}^{-1}$ and that the length of the optical path of the cell used is $2 \times 10^{-2} \text{ m}$. What would happen to the absorbance if the cell used was of double its present thickness?
- 9.3 A $1.28 \times 10^{-4} \text{ M}$ solution of potassium permanganate has a transmittance of 0.5 when measured in a 1 cm cell at 525 nm.
1. Calculate the molar absorptivity coefficient for the permanganate at this wavelength.
 2. If the concentration is doubled what would be the absorbance and the percentage transmittance of the new solution?
- 9.4 A polluted water sample contains approximately 0.1 ppm of chromium, ($M = 52 \text{ g mol}^{-1}$). The determination for Cr(VI) based upon absorption by its diphenylcarbazide complex ($\lambda_{\text{max}} = 540 \text{ nm}$, $\epsilon_{\text{max}} = 41\,700 \text{ L mol}^{-1} \text{ cm}^{-1}$) was selected for measuring the presence of the metal. What optimum path-length of cell would be required for a recorded absorbance of the order of 0.4?
- 9.5 Paints and varnishes for use on exteriors of buildings must be protected from the effects of solar radiation which accelerate their degradation (photolysis and photochemical reactions). Given that: $M = 500 \text{ g mol}^{-1}$; $\epsilon_{\text{max}} = 15\,000 \text{ L mol}^{-1} \text{ cm}^{-1}$ for $\lambda_{\text{max}} = 350 \text{ nm}$, what must be the concentration (expressed in g L^{-1}) of a UV additive M such that 90% of the radiation is absorbed by a coating of thickness 0.3 mm?
- 9.6 By employing the empirical rules of Woodward–Fieser, predict the position of the maximum absorption of the compounds whose structures are seen below.



- 9.7 To determine the concentrations (mol/L) of $\text{Co}(\text{NO}_3)_2$ (A) and $\text{Cr}(\text{NO}_3)_3$ (B) in an unknown sample, the following representative absorbance data were obtained.

A (mol/L)	B (mol/L)	510 nm	575 nm
1.5×10^{-1}	0	0.714	0.097
0	6×10^{-2}	0.298	0.757
Unknown	Unknown	0.671	0.330

Measurements were made in 1.0 cm glass cells.

1. Calculate the four molar absorptivities: $\epsilon_{\text{A}(510)}$, $\epsilon_{\text{A}(575)}$, $\epsilon_{\text{B}(510)}$ and $\epsilon_{\text{B}(575)}$.
 2. Calculate the molarities of the two salts A and B in the unknown.
- 9.8 The determination of phosphate concentration in a washing powder requires first the hydrolysis of the tripolyphosphate components into the phosphate ion or its protonated forms. Then a quantitative colorimetric method based upon the absorption of the yellow complex of phosphate and ammonium vanadomolybdate can be used. A series of standard solutions was prepared. The complex was then developed in 10 mL aliquots of these solutions by adding a 5.0 mL aliquot of an ammonium vanadomolybdate solution.

Measurements were made in a glass cell of 1.0 cm pathlength at 415 nm. The following absorbance data were obtained.

1. Produce a calibration curve from these data.
2. By the method of least squares, derive an equation relating absorbance to phosphate concentration.

Solutions	Concentration (mmol/L)	Absorbance
S_0	0.0	0
S_1	0.1	0.15
S_2	0.2	0.28
S_3	0.3	0.4
S_4	0.4	0.55
S_5	0.5	0.7

3. An unknown solution obtained by hydrolysis of 1 g of detergent treated in an identical way gave an absorbance of 0.45. Determine the phosphate concentration of this solution.

9.9 The concentration of the sulphate ion in a mineral water can be determined by the turbidity which results from the addition of excess BaCl_2 , to a quantity of measured sample. A turbidometer used for this analysis has been standardised with a series of standard solutions of NaSO_4 . The following results were obtained:

Standard solution	Conc. SO_4^{2-} (mg/L)	Reading of turbidometer
S_0	0.00	0.06
S_1	5.00	1.48
S_2	10.00	2.28
S_3	15.00	3.98
S_4	20.00	4.61

- In supposing that a linear relationship exists between the readings taken from the apparatus and the sulphur ion concentration, derive an equation relating readings of the turbidometer and sulphate concentration (method of least squares).
- Calculate the concentration of sulphate in a sample of mineral water for which the turbidometer gives a reading of 3.67.

10

Infrared spectroscopy

Analytical infrared studies are based on the absorption (or reflection) of the electromagnetic radiation that lies between 1 and 1000 μm . This is one of the most common spectroscopic techniques used for compound identification and measuring of concentrations in many samples. This spectral range is sub-divided into three smaller areas, the near infrared (near-IR, 1–2.5 μm), the mid infrared (mid-IR, 2.5–50 μm) and the far infrared (beyond 25 μm). Although the near-IR is poor in specific absorptions, it is considered as an important method by quality control laboratories for quantitative applications. By contrast the mid-IR region provides more information upon the structures of compounds and consequently it is much used as a procedure for identifying organic compounds for which it remains a form of functional group fingerprinting. The far IR requires the use of specialized optical materials and sources. To conduct these analyses there is a full range of instruments from Fourier transform spectrometers to a multiplicity of analysers of dispersive or non-dispersive types, specialized in the measurement of pre-defined compounds (e.g. analysis of gases and vapours) or which allow continuous analyses on production lines. The Fourier transform infrared spectrometry offers numerous possibilities for the treatment of spectra and has applications for the analysis of structured microsamples (infrared microanalysis).

10.1 The origin of light absorption in the infrared

In the near and the mid infrared, the absorption of light by matter originates from the interaction between the radiation from a light source and the chemical bonds of the sample. More precisely, if the atoms situated at the two extremes of a bond are different, they form an electric dipole that oscillates with a specific frequency. If such a non-symmetrical bond is irradiated by a monochromatic light source whose frequency is the same as the dipole, then an interaction will occur with the bond. Thus, the electrical component of the wave can transfer its energy to the bond on condition that the mechanical frequency of the bond and the electromagnetic frequency of the radiation are the same (Figure 10.1). This simplified approach can be used to rationalize that in the absence of a permanent

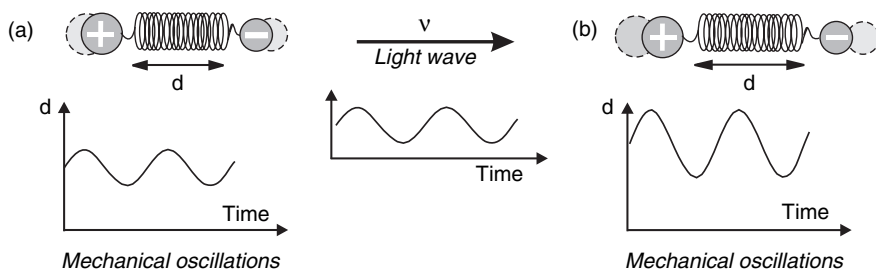


Figure 10.1 Mechanical interpretation of the interaction between a light wave and a polar bond. The mechanical frequency of the wave is not altered by the absorption of a photon, only its amplitude of oscillation increases.

dipole, which is the case with O_2 , N_2 and Cl_2 having non-polar bonds, there will be no coupling with the electromagnetic wave and therefore no absorption of energy will take place. In the mid-IR these bonds are labelled ‘transparent’.

10.2 Absorptions in the infrared

Infrared absorption information, which varies with the radiation wavelengths selected, is generally presented in the form of a *spectrum*, which is the basic document issued from the spectrometer. The ordinate of the graph records the ratio of the transmitted intensities, that is with and without sample, calculated for each wavelength marked on the abscissa. This ratio is called the *transmittance* (T). On the graph it is often replaced by the percent transmittance (per cent T) or by *absorbance* (A), the logarithm to the base 10 of the reciprocal of the transmittance: $A = \log(1/T)$. If the study has been conducted using reflected or diffused light then units of *pseudo-absorbance* will have been used (cf. Section 10.9.2). Finally, it has become appropriate to substitute the values of wavelengths by their equivalent expressed in *wavenumbers* $\bar{\nu}$ whose units are in cm^{-1} or kaysers, (formula 10.1). Figure 10.2 corresponds to a spectrum recorded in the mid-IR, between 2.5 and 25 μm .

$$\bar{\nu}_{cm^{-1}} = \frac{1}{\lambda_{cm}} \quad (10.1)$$

10.3 Rotational–vibrational bands in the mid-IR

Excepting when the temperature is 0 K, atoms within molecules are in perpetual motion and each one of them possesses three *degrees of freedom*, which refer to the three classic Cartesian coordinates. All of these movements confer upon each isolated molecule a combined mechanical energy. The theory is postulated

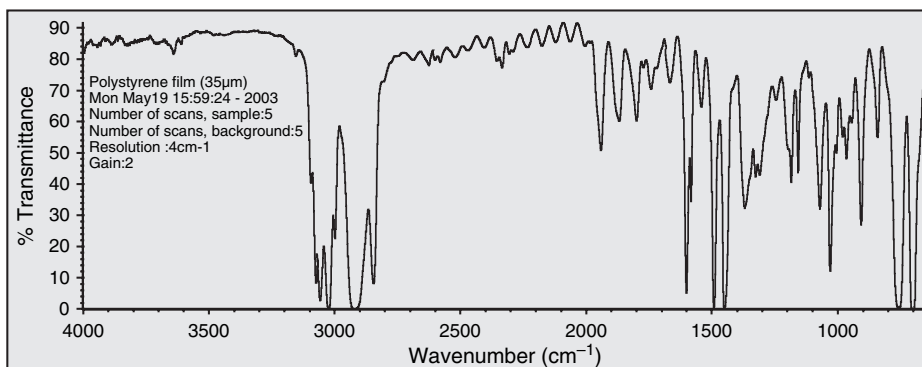


Figure 10.2 *Mid-IR spectrum of a polystyrene film.* The typical representation of an infrared spectrum with a linear scale abscissa in cm^{-1} (see formula 10.1) for an easier observation of the right-hand section and with percentage transmittance along the ordinate. The transmittance is sometimes replaced by the absorbance $A (A = -\log T)$. The scale, in cm^{-1} , (or kaysers) is linear in energy ($E = hc/\lambda$) and will decrease from left to right (i.e. from high to low energy).

that the total molecular energy arises from the sum of the independent though quantified terms named energy of rotation E_{rot} , energy of vibration E_{vib} and electronic molecular energy E_{elec} :

$$E_{\text{tot}} = E_{\text{rot}} + E_{\text{vib}} + E_{\text{elec}} \quad (10.2)$$

The values of these energies are very different and according to the Born–Oppenheimer principle they can vary independently of each other.

In the mid-IR, a source emitting a radiation of 1000cm^{-1} corresponds to a photon energy $E = hc\bar{\nu} = 0.125\text{eV}$. If such a photon is absorbed by a molecule its total energy will be increased by this energy. Theoretically the term E_{vib} will be modified but the term E_{elec} will not change because this energy is too weak to cause transition between two different electronic levels.

In practice, as samples are usually in the form of condensed (liquid) or solid phases, pure or in solution and not in the form of isolated species, numerous dipole-dipole interactions are produced between the species present, which perturb both the energy levels and the absorption wavelengths. Thus a spectrum is always in the form of broadened signals called bands which extend over tens of cm^{-1} , which common apparatus are unable to separate into individual transitions.

It is noteworthy that the width of an absorption signal is inversely proportional to the lifetime of the excited state (Heisenberg’s uncertainty principle). Hence, for gases, the lifetime is long and the absorption lines are sharp. This is why small di- or triatomic molecules sampled in the gas state and at low pressures can lead to spectra containing narrow regular bands whose positions enable the elucidation of the various energetic states predicted by the theory.

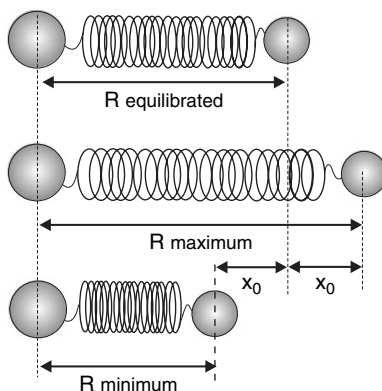


Figure 10.3 A diatomic molecule represented in the form of an harmonic oscillator. The term harmonic oscillator comes from the idea that the elongation is proportional to the force exerted, from which the frequency ν_{vib} is independent.

10.4 Simplified model for vibrational interactions

For modelling the vibrations of bonds, a harmonic oscillator should be imagined, which comprises two masses linked by a spring and able to slide in a plane without friction (Figure 10.3). If the two masses are displaced by a value x_0 with respect to the equilibrium distance R_e and then the system is released, it will begin to oscillate with a period that is a function of the constant relating to the stiffness of the spring, named *force constant* k (N/m) and of the size of the masses involved.

The frequency, which is independent of the elongation, is given by Hooke's law (expression 10.3) in which μ (kg) represents, as indicated, the reduced mass of the system.

$$\nu_{vib} = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \quad (10.3)$$

with

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \quad (10.4)$$

The vibrational energy of this simple model can vary in a continuous fashion. Following a short elongation Δx_0 with respect to the distance from equilibrium R_e , E_{vib} becomes:

$$E_{vib} = \frac{1}{2} k \Delta x_0^2 \quad (10.5)$$

For a bond

$$\bar{\nu} = \frac{1}{2\pi c} \sqrt{\frac{k}{\mu}} \quad (10.6)$$

with

$$\bar{\nu} = \frac{1}{\lambda} = \frac{\nu}{c} \quad (10.7)$$

The model above can be approximated to the chemical bond linking two atoms, on condition that the quantum theory governing species of atomic dimensions is employed. Because a bond whose frequency of vibration is ν can absorb light radiation of an identical frequency, its energy will increase from the quantum $\Delta E = h\nu$. According to this theory, the simplified expression 10.8 will yield possible values for E_{vib} :

$$E_{\text{vib}} = h\nu(V + 1/2) \quad (10.8)$$

$V = 0, 1, 2, \dots$ is called the vibrational quantum number of vibration and can only vary by one unit ($\Delta V = +1$, the 'single quantum transition'). The different values of expression 10.5 are separated by the same interval $\Delta E_{\text{vib}} = h\nu$ (Figure 10.4).

At room temperature, the molecules are still in the non-excited state $V=0$, that is, $E_{(\text{vib})0} = 1/2h\nu$ (energy at 0 K). Besides the standard transition $\Delta V = +1$, that corresponding to $\Delta V = +2$ and 'forbidden' by this theory, appears weakly when the fundamental absorption band is particularly strong (for example, in the case of elongation vibrations in organic compounds containing a carbonyl bond $\text{C}=\text{O}$ (e.g. ketones or aldehydes).

■ No mode of valence vibration is situated higher than 3960 cm^{-1} (H-F). This is the wavenumber which determines the frontier between the mid and the near infrared. However, for reasons of usefulness, many current spectrometers accommodate both near and mid-IR permitting a coverage from 400 up to 8000 cm^{-1} .

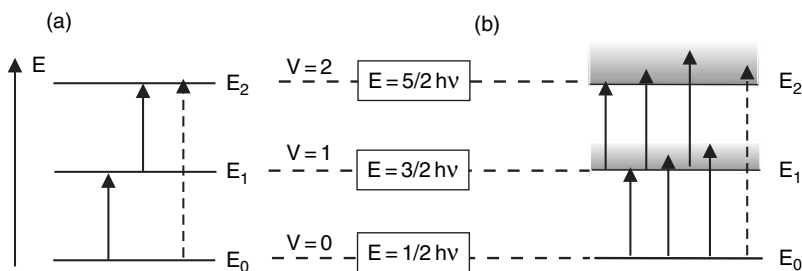


Figure 10.4 Diagram of the vibrational energies levels of a bond. (a) For isolated molecules; (b) for molecules in the condensed phase. The transition $V=0$ to $V=2$ corresponds to a weak harmonic band. Bearing in mind the energies of photons in the mid-IR it can be calculated that the first excited state ($V=1$) is 10^6 less occupied than the ground (fundamental) state. The harmonic transitions are exploited in the near-IR.

10.5 Real compounds

The harmonic oscillator model does not take into account the real nature of chemical bonds, which are far from being perfect springs. The force constant k diminishes if the distance between the atoms is stretched, yet in contrast, increases strongly if the atoms are pushed close together. More precise models employ correction terms related to the anharmonicity of experimentally observed oscillations.

Elsewhere, in the mid-IR, photon energy is sufficient to modify the quantized terms E_{vib} and E_{rot} in expression 10.2. This is therefore a vibration–rotation spectrum, that is, several tens of rotational transitions accompany each vibrational transition. For the simplest molecules it is possible to interpret particular aspects of the absorption bands. Experience and theory have enabled rules of the permitted transitions to be drawn up. Small molecules as carbon monoxide and hydrogen chloride (Figure 10.5) have been intensely studied from this point of view.

Knowing that E_{elec} doesn't vary in the mid-IR, expression 10.2 leads to the energy values corresponding to the bands of absorption (Figure 10.5):

$$\Delta E_{\text{VR}} = (E_{\text{VR}})_2 - (E_{\text{VR}})_1 = \Delta E_{\text{vib}} + \Delta E_{\text{rot}} \quad (10.9)$$

Each atom has three degrees of freedom, corresponding to motions along any of the three Cartesian coordinate axes (x, y, z). Thus a polyatomic molecule of n interacting atoms can be defined by $3n$ degrees of freedom (see Section 10.3). Since it requires 3 coordinates to define its centre of gravity (translational movement of the molecule), the remaining $3n - 3$ are internal degrees of freedom corresponding to vibrations. In general 3 are required to define the rotation of the entire molecule. All of the others correspond to the fundamental vibrations (i.e. $3n - 6$). A molecule containing 30 atoms will have 84 normal modes of vibration, happily not all active, since there must first be variation in the dipole moment to get an absorption. Such a high number of energy levels, to which are added combinations of levels and harmonics, can only lead to complex and unique spectra for each compound.

10.6 Characteristic bands for organic compounds

The application of the above rules to all kinds of compounds has been made in order to exploit the use of infrared spectroscopy as a method of structural analysis. Empirically, it has been observed a correlation between position of certain band maxima and the presence within a molecule of organic functional groups or of particular structural features within the skeleton of molecules (see Table 10.1). This property comes from the fact that each organic functional group corresponds to a collective-type of several atoms. For a given bond, the force constant k (expression 10.3) does not vary significantly from one molecule to another.

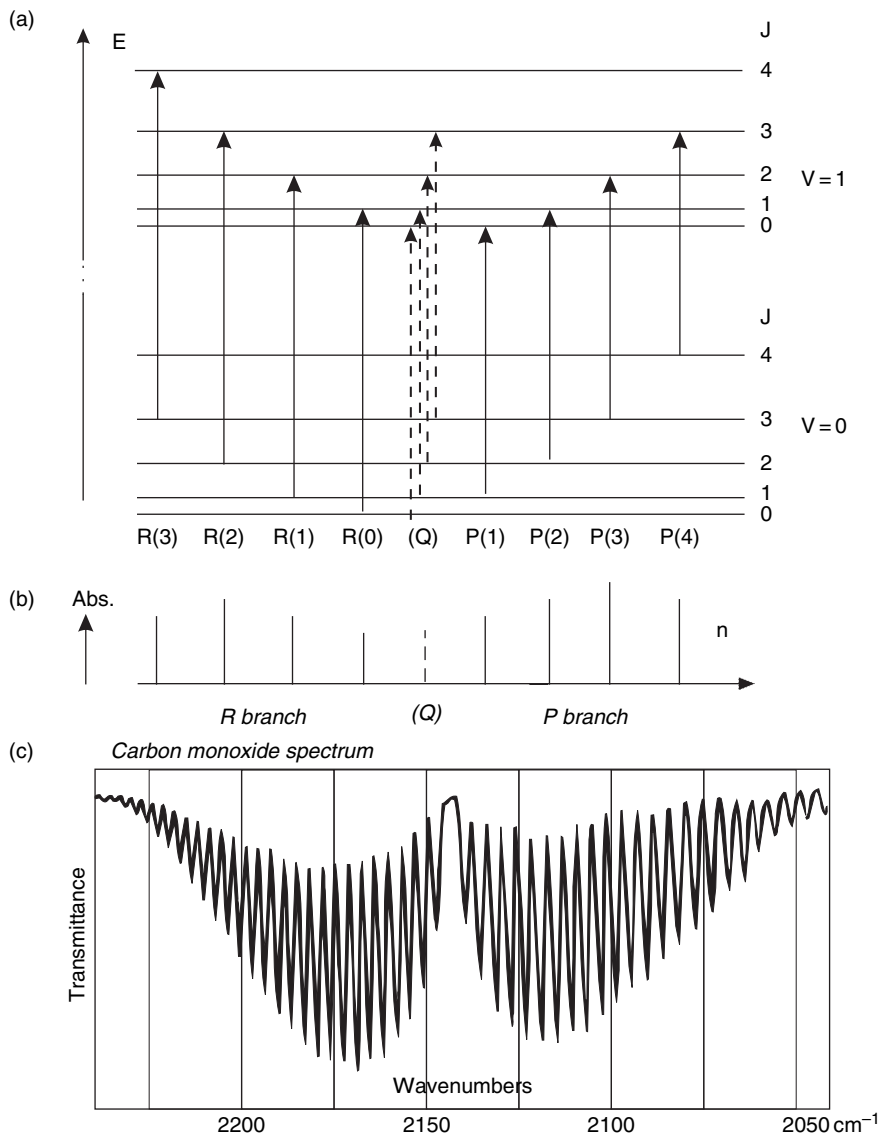
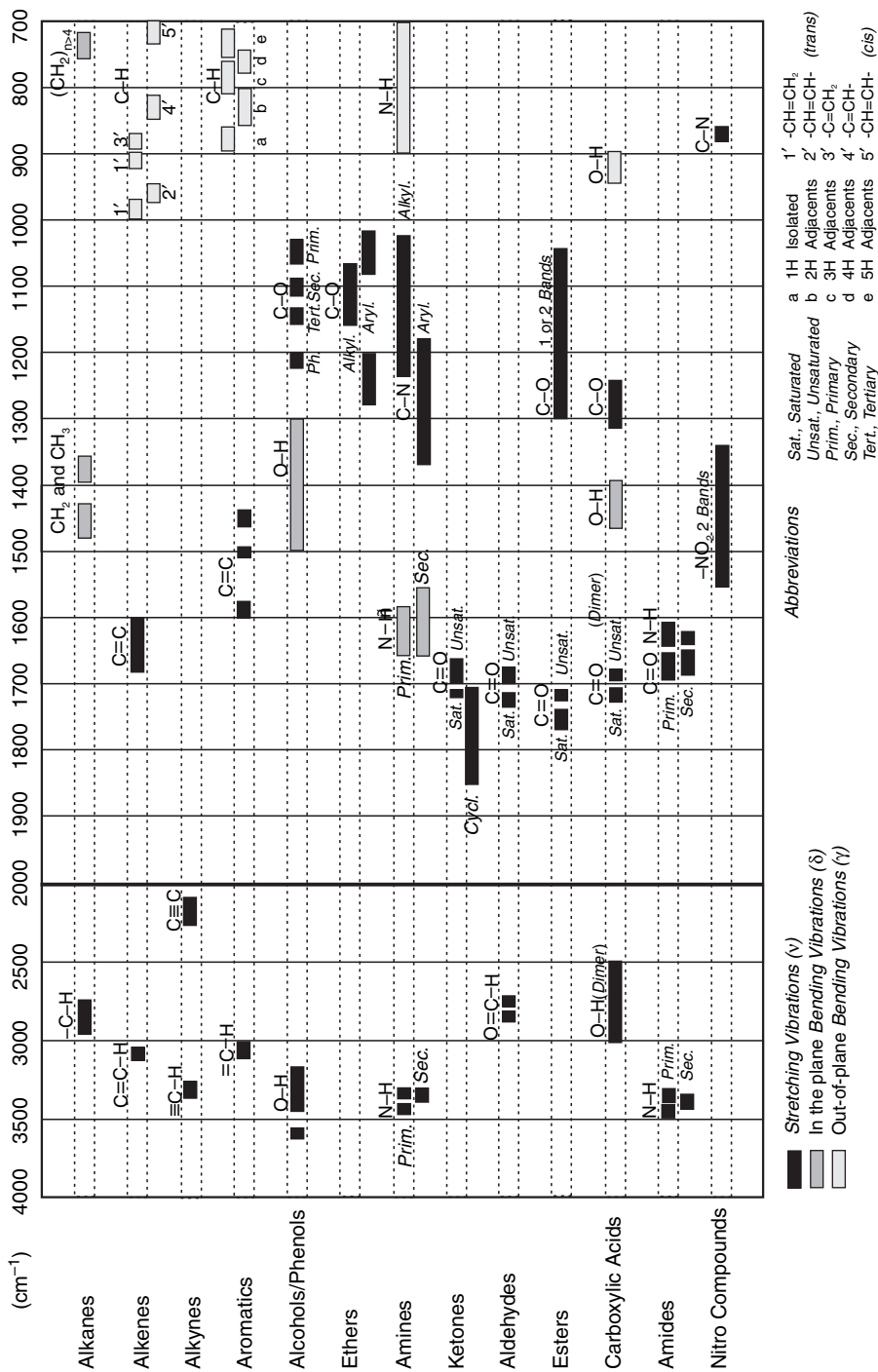


Figure 10.5 *Rotation/vibration levels of carbon monoxide.* V and J are the quantum numbers of vibration and rotation. The fundamental vibration corresponds to $V = +1$ and $J = +1$. (a) A rotation-vibration band corresponds to all of the allowed quantum transitions. If the scale of the diagram is in cm^{-1} , the arrows correspond to the wavenumbers of the absorptions; (b) branch R corresponds to $\Delta J = +1$ and the band P to $\Delta J = -1$. They are situated either side of band Q , absent from the spectrum (here it can be supposed that $\Delta J = 0$ corresponding to a forbidden transition); (c) below, vibration-rotation absorption band of carbon monoxide (pressure of 1000 Pa). The various lines illustrate the principle of the selection rules. The difference (wavenumbers) between successive rotational peaks are not constant due to anharmonicity factors.

Table 10.1 Correlation Chart in the mid-Infrared between functional groups and absorption bands



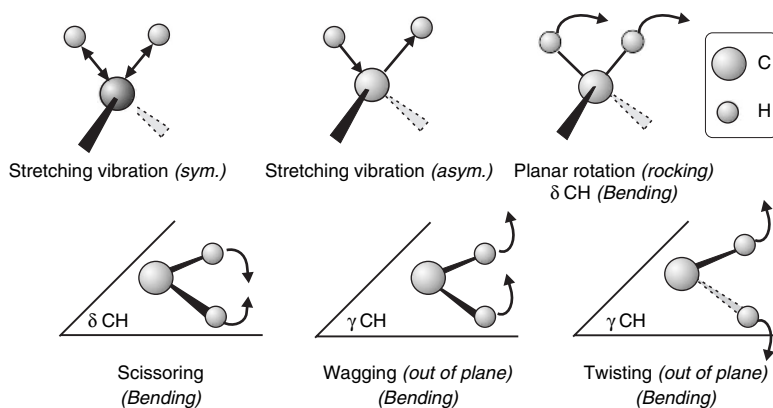


Figure 10.6 Major vibrational modes for a non-linear group: the CH_2 . Characteristic stretching and bending vibrations in and out of the plane. In IR spectroscopy, the position and the intensity of the bands are modified by associations between molecules, solvent polarity, etc.

Moreover, calculation reveals that the reduced mass of a given group of atoms tends toward a limiting value as the molecular mass increases (see expression 10.4). This can explain that absorption of each organic functional group corresponds to a particular value.

The most widely known molecular movements are stretching vibrations (symmetrical and asymmetrical) and bending vibrations of angular deformation (Figure 10.6). Alternately, in the region of the spectrum below 1500 cm^{-1} , the absorption bands are numerous and differ for each compound. These are deformation vibrations of both the bonds and the skeleton and are difficult to assign with accuracy.

For instrumental reasons, the spectral range, which extends from 1 to $2.5\ \mu\text{m}$ (though it has no formal limits) has for a long time been ignored. However the tandem progress of both detectors and transparent fibre optics in the near-IR linked with advances in chemometric methods has permitted the development of the region. The spectra are often presented as curves without clearly defined peaks. Nevertheless, each recording is representative of the sample and therefore can be used as a fingerprint. For organic compounds, the absorption bands in the near-IR originate from either harmonic bands or a combination of the fundamental vibrations of the C–H, O–H and N–H bonds (which are situated close to $3000\text{--}3600\text{ cm}^{-1}$).

As for carbonyl compounds, the first two harmonics of the fundamental vibration of C=O (1700 cm^{-1}) appear towards 3400 and 5100 cm^{-1} respectively. The combination bands result from the interaction of two or more modes of vibration for the same functional group and are superimposed upon the preceding band. The energy of the corresponding transition is approximately the sum of those which have given rise to it.

■ Chloroform, which absorbs at 3020 cm^{-1} and 1215 cm^{-1} will also absorb at 4220 cm^{-1} , which approximately represents the sum of the two preceding values.

10.7 Infrared spectrometers and analysers

These instruments (Figure 10.7) can be divided into two categories: the *Fourier transform spectrometers*, which undertake a simultaneous analysis of the whole spectral region from interferometric measurements, and numerous *specialized analysers* for the second category. *Dispersive-type spectrometers* are also used for the near-IR.

The first category uses a Michelson-type interferometer or similar device, coupled to a specialized microprocessor for calculating the spectrum, while the second category uses filters or a monochromator with a motorized grating that can scan the range of frequencies studied (Figure 10.7).

Dispersive spectrometers. For a long time, mid-IR spectrometers were constructed on the principle of the double beam design. This fairly complex optical assembly, which remains in use for the UV/Vis, yields progressively over real time the

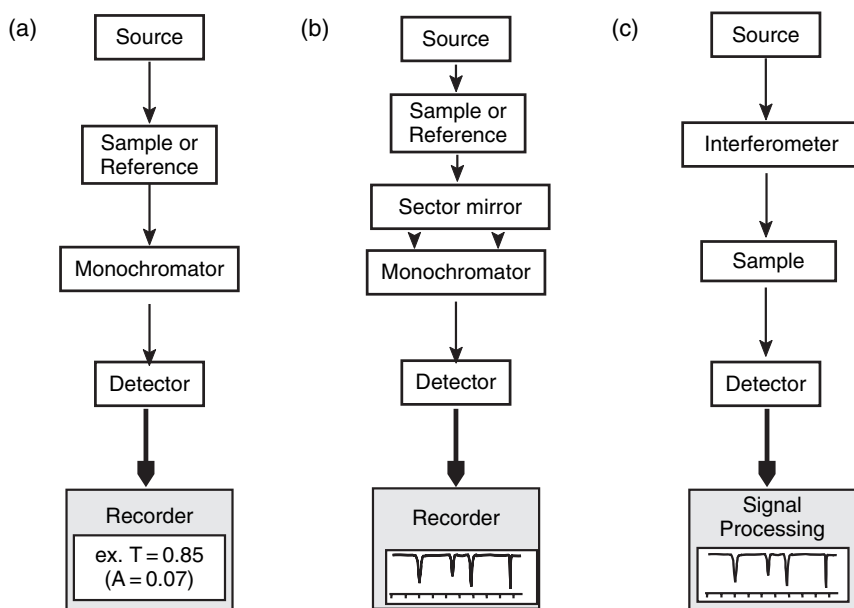


Figure 10.7 Schematic diagram of spectrometers and analysers in the infrared. (a) Single beam analyser containing a fixed monochromator or a filter used when a measurement at a single wavelength will suffice; (b) dispersive spectrometer, double beam system. In contrast to spectrophotometers in the UV/Vis, the sample, located prior to the monochromator is permanently exposed to the full radiation of the source, knowing that the energy of the photons in this region is insufficient to break the chemical bonds and to degrade the sample; (c) Fourier transform single beam model.

spectrum of the sample through the measurement of the transmittance, calculated for each wavelength by comparing the intensity of light arising from two separate pathways, one serving as reference and the other passing through the sample. To this end the light issuing from the source is separated by a series of mirrors into two beams one of which passes through the sample compartment and the other through the reference chamber. The light is directed to a diffraction grating. For each small wavelength interval, defined by the monochromator, the light following each of the two pathways focuses on the detector alternatively. This arrangement is effected by an optical chopper, such a sector mirror which rotates about ten times per second. The ratio of the signals obtained, at practically the same instant, corresponds to the transmittance for the wavelength considered. Each wavelength is measured one at a time. These dispersive instruments are sometimes called grating or scanning spectrometers.

10.7.1 Fourier transform infrared spectrometer (FTIR)

An FTIR spectrometer contains a single beam optical assembly with, as an essential component, an interferometer – often of the Michelson type – located between the source and the sample (Figure 10.7c). It consists of three active components: a moving mirror, a fixed mirror and a *beam-splitter*.

The radiation issuing from the polychromatic source impacts on the beam-splitter acting as a *separator*, which is made of a semi-transparent film of germanium deposited on a KBr support. This device divides the original beam into two halves, one of which is directed towards a fixed mirror and the other towards a moving mirror whose distance from the beam-splitter varies. Recombined, these two beams follow the same optical path passing through the sample before reaching the detector that measures the global light intensity received. This is a multiplexing procedure applied here to the domain of optical signals. The heart of the Michelson interferometer is a moving mirror, the sole mobile component that oscillates between two extreme positions. When its position is such that the pathways travelled by the two beams have the same length until the detector (*zero optical path difference*), then the light composition of the beam leaving the interferometer is identical to that which enters. In contrast, when the mobile mirror changes of this particular position, the light leaving has a spectral composition which depends upon the phase difference of the two beams: the signal transmitted over time by the detector is recorded in the form of an *interferogram*:

$$(\text{Total Int.}) = f(\delta)$$

with δ representing the path difference between both beams (Figure 10.8).

The management of the optical bench and the acquisition of the data is controlled by a specific electronic interface. During the displacement of the mirror, an ADC converter, linked to the detector, samples the interferogram in the form of

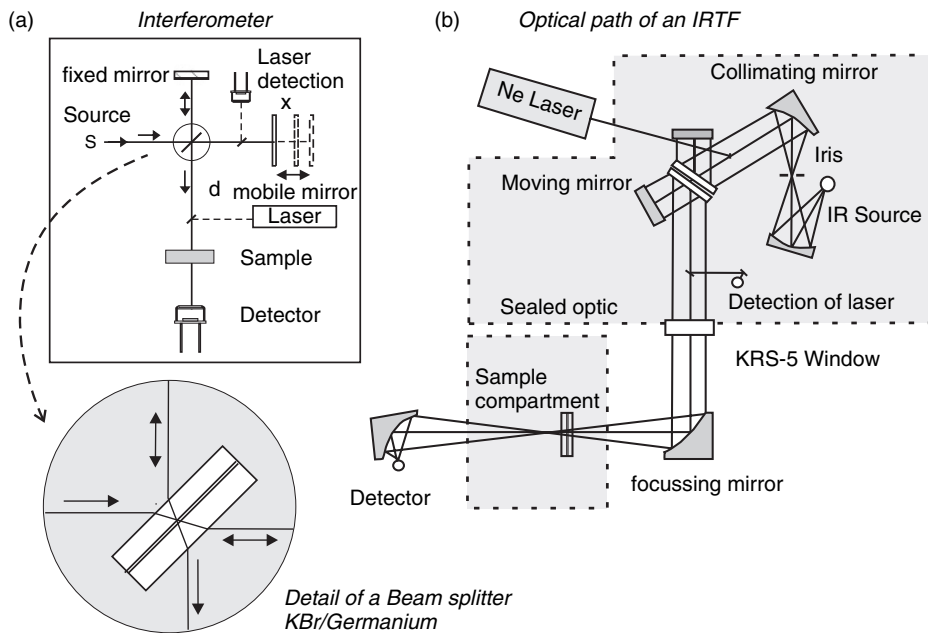
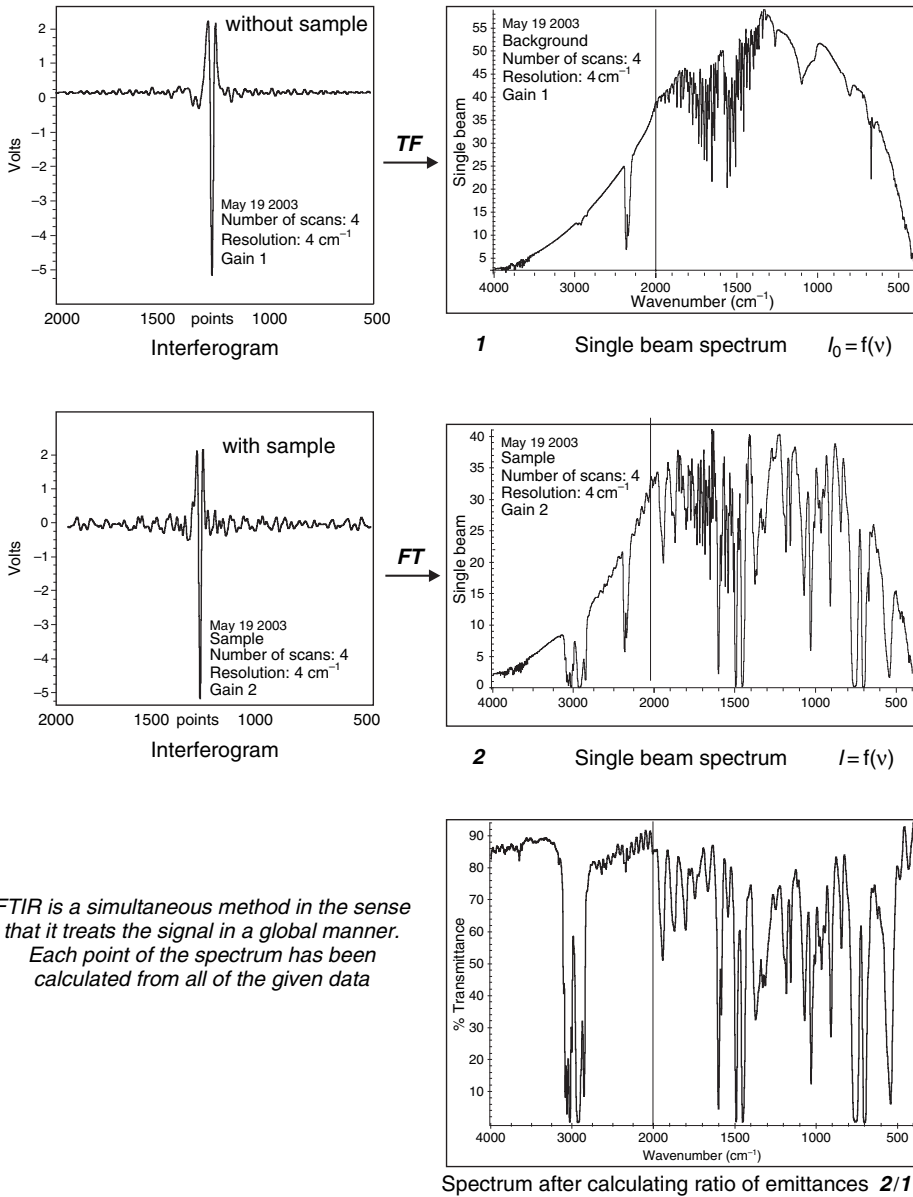


Figure 10.8 The optical assembly of a Fourier transform apparatus. (a) 90° Michelson interferometer with below, some details of the beam-splitter; (b) the optical diagram of a single beam spectrophotometer (picture of Shimadzu model 8300). A low power He/Ne laser is used as an internal standard (632.8 nm) in order to locate with precision the position of the mobile mirror by an interference method (this second sinusoidal interferogram which follows the same optical pathway, is used by the software to determine the optical path difference).

thousands of data points. Each of these values corresponds to a position and represents the global intensity that passes through the sample. This is the second term of a linear equation in which the unknowns correspond to the amplitudes of the n different wavelengths (assumed to be a finite number) considered for the mirror and after absorption by the sample. From these thousands of values a specialized microprocessor will execute, in less time than it would take to describe, using the Cooley algorithm the calculation of a giant matrix leading to the amplitude of each wavelength of the spectral band studied.

Bearing in mind a resolution factor imposed by the method of calculation, the classical representation of the spectrum is obtained, $I = f(\lambda)$ or $I = f(\nu)$. According to Nyquist's theory, at least two points per period are required in order to find, by calculation, a given wavelength of the spectrum. To obtain a sample spectrum equivalent to the one obtained with a double beam spectrometer, two spectra of transmitted intensities are recorded: the first, *without sample* (absorption background) and the second *with sample*. The conventional spectrum, in percentage T , is obtained from these two spectra (Figure 10.9).



FTIR is a simultaneous method in the sense that it treats the signal in a global manner. Each point of the spectrum has been calculated from all of the given data

Figure 10.9 Sequence for obtaining a pseudo-double beam spectrum with a Fourier transform infrared spectrometer. The apparatus records and memorizes two spectra, which represent the variations of I_0 (the blank) and I (sample) as a function of the wavenumber (these are emission spectra 1 and 2). Next the conventional spectrum is calculated, identical to that of an instrument of the double beam type, by calculating the ratio $T = I/I_0 = f(\lambda)$ for each wavenumber. Strong atmospheric absorption (CO_2 and H_2O), which is present along the optical path is eliminated in this way. The illustrations correspond to the spectrum of a polystyrene film.

■ The French mathematician J.-B. Fourier (1768–1830) could never have imagined how famous he would become with the advent of the computer. The principle of his calculations, published in a treatise concerning the propagation of heat in 1808, is applied in many scientific software packages for the treatment of spectra (acoustical, optical or mass-spectrometric) and images. Legend has it that he was elaborating these calculations when Napoleon's army requested him to improve the dimensions of their cannons. In general terms, a transform is a mathematical operation that permits data to be changed from one type of measurement to another (for example, from time, to wavelength).

This FTIR technique of obtaining spectra, also adapted to the near-IR, has significantly modified traditional procedures of obtaining IR spectra. Adopted by almost all manufacturers of spectrometers (e.g. see Figure 10.10) this method has several advantages:

- the entrance slit is replaced by an iris furnishing a better signal to the detector which receives *more energy* (multiplexing advantage);
- the *signal-to-noise ratio is much higher* to that of the sequential method since it can be improved by the accumulation of successive scans (Fellgett advantage);

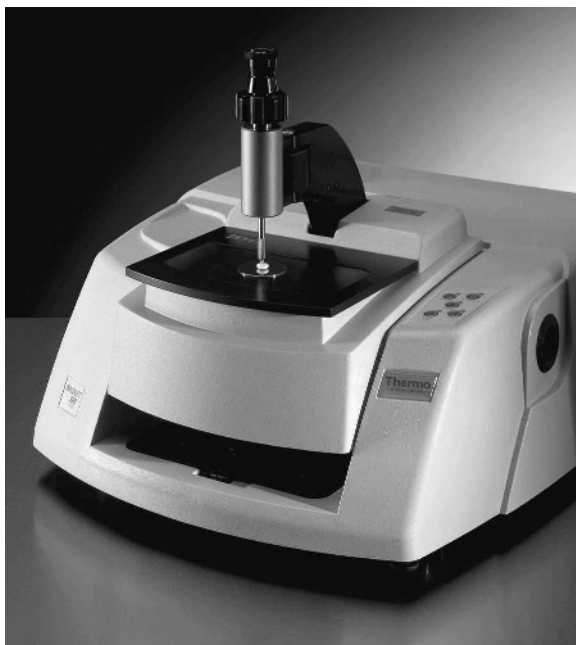


Figure 10.10 *Fourier transform infrared spectrophotometer.* The optical bench of the 380 FTIR, equipped with a diamond ATR accessory (reproduced courtesy of Nicolet).

- the wavelengths are calculated with *higher precision* which facilitate the comparison of spectra;
- the *resolution is better* and constant across the entire region under study.

There exists another way, chosen by a few manufacturers, to incorporate a delay into one of the optical paths when obtaining an interferogram. The principle relies upon the light passing through a crystal having two refractive indices, which constitutes a polarization interferometer.

10.7.2 Infrared analysers

Numerous autonomous small-sized instruments, used for analyses relating to personal protection and other specific targeted applications, are based upon absorbance measurements in the mid-IR or in the near-IR. Sensitivity, ruggedness and easy-to-use are important features for process installations. Designed to quantify one or many compounds from very weak absorbances (1×10^{-5}), they are able to measure reliably concentrations of the order of ppm for gases such as carbon monoxide and dioxide, ammonia, methanal, ethanol, methane, etc. (in total around hundred gases or volatile compounds). The source can be a laser diode, which emits at a precise wavelength or a simple filament with a silica covering (case of the breathalysers). While the principle is simple, the chemistry is sensitive. Measurements of the levels of carbon mono- and dioxide in motor car gas exhausts are carried out by this method. CO is measured by recording the absorbance at 2160 cm^{-1} (cf. Figure 10.5) while CO_2 is measured at 2350 cm^{-1} (Figures 10.11 and 10.12). For ethanol the characteristic absorbance of primary alcohols at 1050 cm^{-1} is considered. These *infrared colorimeters* are competitive with instruments based upon other inventive solutions (e.g. amperometric detection cf. Chapter 20), which are not derived from common laboratory spectrometer technology.

10.8 Sources and detectors used in the mid-IR

10.8.1 Light sources

In the mid-IR several type of sources are used. They are either a lamp filament (Figure 10.13), or a hollow rod, 1–3 mm in diameter and 2 to 4 cm long, made of fused mixtures of zirconium oxide or rare earth oxides (Nernst source) heated by Joule effect by the means of an internal resistor (for example Globar™). These sources are heated to 1500°C , without a protective shield. They dissipate power of the order of a hundred watts by emitting radiation over a large domain ranging from visible to thermal IR. A maximum is observed for $\lambda = 3000/T$ (λ in

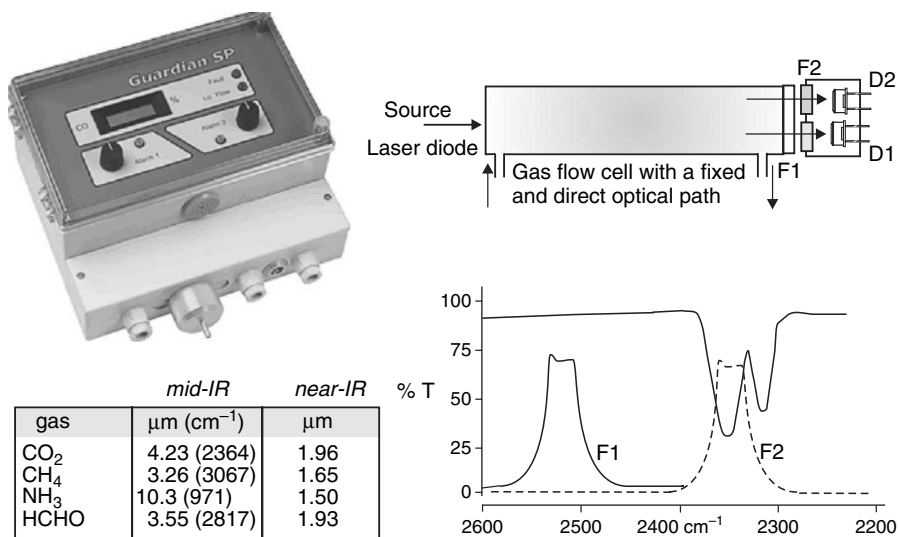


Figure 10.11 Non-dispersive analyser for measuring CO₂ in gaseous media. This assembly is representative of many portable detectors. The selectivity is assured by an adapted optical filter (F2) and by a membrane at the entrance to the cell which only permits gas to pass through to the detector. The assembly contains a second filter (F1) chosen in a non-absorption zone which authorizes to a computation of the transmittance. On the diagram are shown the bandwidths of the two filters and the spectrum as function of transmittance of a gas, here CO₂. The detectors are thermistors. Left, a chart which presents a choice of wavelengths in the mid-IR and the near-IR for several gases (reproduced courtesy of Edinburgh Sensors, GB).

micrometres and T in Kelvin – adapted from Stefan's law). The intensity of the radiation varies enormously as a function of the temperature of the source.

10.8.2 Detectors

The detection of photons in the infrared region was difficult to achieve for a long time and a major cause of this was the mediocre sensitivity of the first spectrophotometers.

The principle relies upon the thermal effect of IR radiation. Sensors that measure radiation by means of the change of temperature of an absorbing material are classified as thermal detectors. They respond to any wavelength radiation that is absorbed and can be made to cover a wide range of wavelengths. Numerous devices have been used, thermistors, thermocouples, thermopiles and other sensors rather imaginative (Figure 10.12b). For FTIR instruments, one of the most common thermal sensors is the *pyroelectric detector*. It has a rapid time response to follow the variations of light intensity, a weak thermal inertia and a linear response. Other detectors are *photo-diodes* (Figure 10.14) and *diode arrays* that

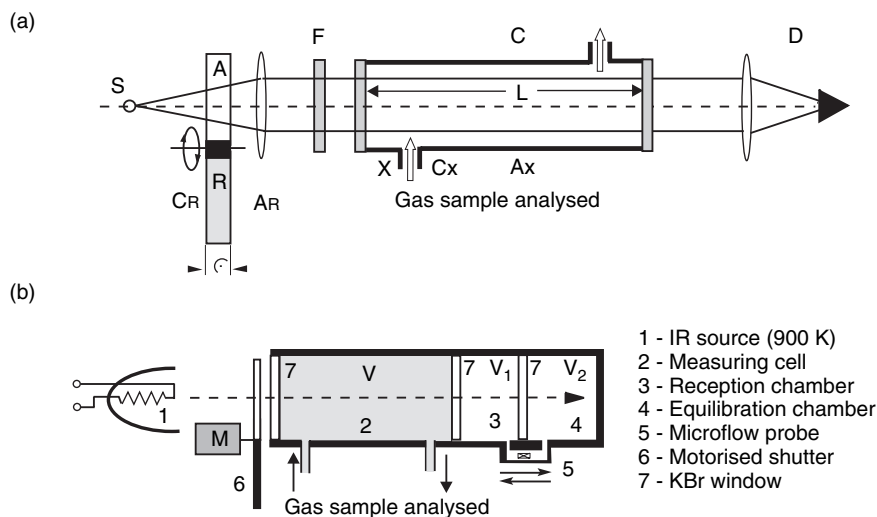


Figure 10.12 Two models of single beam gas analysers. (a) The light issuing from the source S, crosses first a cylindrical assembly serving as a modulator which contains two capsules one R, filled with the gas to be detected and the second A, with a neutral gas (nitrogen). Next, the light passes through an interferential filter F chosen as a function of the wavelength at which the measurement will be made, then cell C containing the sample. When the cell R is in the pathway, a known supplementary absorbance is obtained which allows a deduction of the absorbance due to the gas under study (and the interfering compounds) in the measuring cell (adapted from an instrument of Servomex, GB); (b) the light from the source, after having passed through the measuring cell reaches the cell V_1 containing the reference gas (for measuring CO for example, V_1 and V_2 contain this gas). The measuring of the gaseous flow, by a microflow rate, analyser, circulating between V_1 and V_2 gives an indication of the variations of pressure between these two chambers and therefore of the infrared radiation absorbed by the gas present in V_1 : The radiation reaching V_1 will be all the more attenuated when the sample in the flow cell V will contain this same gas. A beam-chopper serves to break up the light flux to create a pulsed signal (adapted from an instrument of Siemens).

are capable of delivering a full infrared spectrum over a pre-defined wavelength range.

The pyroelectric detector contains a mono-crystal of deuterated triglycine sulfate (DTGS) or lithium tantalate (LiTaO_3), sandwiched between two electrodes, one of which is semi-transparent to radiation and receives the impact of the optical beam. It generates electric charges with small temperature changes. The crystal is polarized proportionally to the radiation received and it acts as a capacitor.

The photodiode detector contains a *semiconductor*, which is used either in photovoltaic mode (no voltage ramp) or in photoconductor mode (with voltage ramp) and contains a P-N junction, which, under the effect of radiation, liberates electron/hole pairs which create a PD measurable in an open circuit.

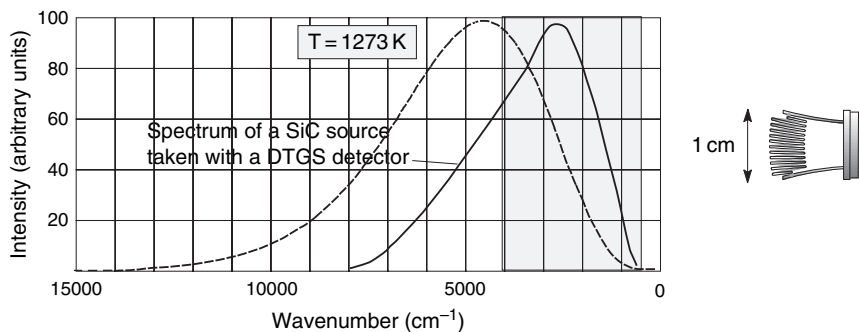


Figure 10.13 Typical emission spectrum of a mid-IR source along with a basic design of a source. In dotted lines is represented the theoretical emission curve of a perfect source ('black body' radiation at 1000°C). According to the model of source, of the beam-splitter and of the detector being used, an experimental curve is registered which appreciably differs from the theoretical curve. It is noticed in particular that between 4000 and 400 cm^{-1} , the signal intensity is divided by a factor ten approximately. Current sources are robust and have a many years' life expectancy.

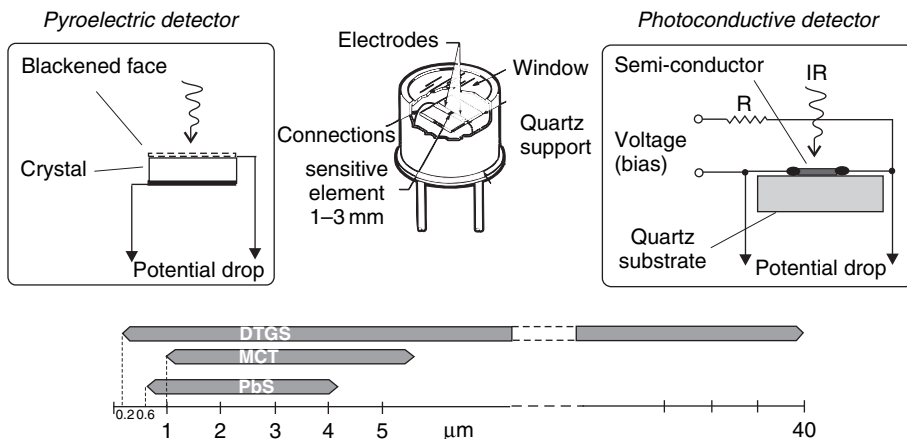


Figure 10.14 Detectors in the infrared. The functioning principles of pyroelectric and semiconductor detectors. Centre, general view of a detector and its container. Below, range of uses for the principal detectors.

This type of detector is constituted, for the mid-IR region, of a ternary alloy of mercury–cadmium telluride (MCT) or indium antimonide (InSb) deposited upon an inert support and for the near-IR of lead sulfide (PbS) or an other ternary alloy of indium/gallium/arsenic (InGaAs). Sensitivity is improved when these detectors are cooled down to liquid nitrogen temperature of (77 K).

10.9 Sample analysis techniques

Spectra are acquired from samples either by transmission or reflection. This second procedure, now common in the infrared, is the fundamental way to meet the needs of qualitative and quantitative techniques for the analysis of all kinds of solid or gaseous samples and aqueous solutions.

10.9.1 Optical materials

Traditional optical material used in the visible or the near-IR becomes opaque in the mid-IR. Other crystalline or amorphous materials, each one having its particular spectral range, must be used for the detectors windows and IR cells. There are more than a dozen. The most common are sodium chloride (NaCl) and potassium bromide (KBr), already mentioned. To replace these fragile and water soluble materials, caesium iodide (CsI is transparent up to 200 cm^{-1}), silver chloride (AgCl), the KRS-5 (thallium bromoiodide) and even diamond can be used. These last materials are hard and insoluble, but unfortunately more expensive. AMTIR (amorphous material transmitting infrared radiation), a glass composed of germanium, arsenic and selenium (e.g. $\text{Ge}_{33}\text{As}_{12}\text{Se}_{55}$) is finally a good choice.

10.9.2 Transmission procedures

- *Gases*, for which the absorbances are weak, require cells for which the optical pathway can be very long, at least of several centimetres (Figure 10.15) but sometimes reaching several hundreds of metres through a complex arrangement of mirrors giving multiple reflections of the infrared beam inside the cell. The cell volume then becomes important (0.2 L).
- In contrast, for the hyphenated techniques such as GC/IR threadlike gas cells (*light pipes*) whose volume does not exceed a few tens of microlitres ($l = 10\text{ cm}$ and diameter $< 1\text{ mm}$) are used. These cells use gilded side walls to provoke multiple reflections.
- *Liquids* are usually analysed with cells, which have two dismountable IR windows. For qualitative analysis, a droplet of the sample is compressed between two NaCl or KBr discs without spacers to create a film. For quantitative analysis, depending upon the wavelength of the measurement, either quartz *Infrasil*TM cells (with optical pathway of 1 to 5 cm), or sealed cells that have a variable or fixed path length (Figure 10.15), can be used. The optical path length must be calibrated and periodically controlled.
- *Solids* are more difficult to study. Some approaches are given below.

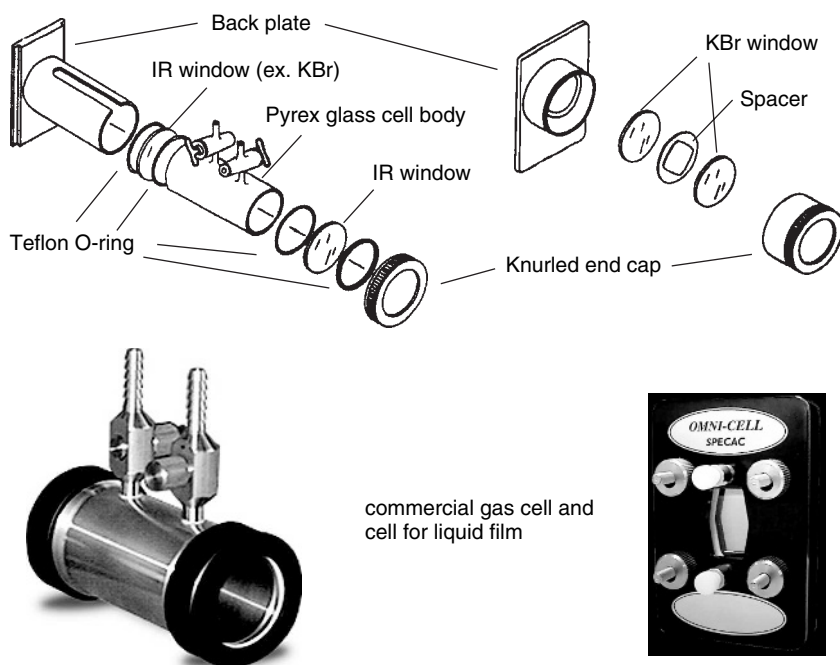


Figure 10.15 Cells in the mid-IR. View of the direct optical pathway for a gas cell and of a simple cell for liquid films with a fixed path length (reproduced courtesy of Spectra Tech). The cell design for the mid-IR allows them to be dismantled, due to the nature of the material chosen for their windows which requires replacement. By contrast, in the near-IR the cells are of quartz as in the UV/Vis.

If the solid can be dissolved in a suitable solvent, then the same method as for a liquid is followed although no solvent exists which is transparent across the whole range of the mid-IR. This procedure permits of quantitative measurements to be performed at all wavelengths except those absorbed by the solvent.

Pellets are used for solid samples that are difficult to melt or dissolve in any suitable IR-transmitting solvents. A few milligrams of the solid grinded to a fine powder (ideally to particles of less than $1\ \mu\text{m}$), is dispersed in a medium which will play the role of matrix, such as a paraffin oil (called Nujol™), or dry potassium bromide (KBr). One obtains a mull or a pellet. The matrix leads to a reduction of the strong optical dispersion caused by the particle/air interface while maintaining the solid in the optical path of the instrument. The finer the powder is ground then the less light will be lost through scattering or diffraction.

Nujol presents three major absorption bands beyond which the spectrum of the sample is exploitable. This spectrum can be supplemented by another carried out in hexachlorobutadiene, transparent in the regions where paraffin absorbs. For the KBr technique, the sample is crushed with KBr in an agate mortar. The mixture is then pressed using an hydraulic press (pressure of 5 to $8\ \text{t}/\text{cm}^2$) or manually into a transparent disc.

10.9.3 Techniques using reflection for solid samples

Obtaining spectra by reflection is an alternative to the procedures described above (see Figure 10.17). The corresponding devices are based upon *attenuated total reflection*, *specular reflection* or *diffused reflection* and are only usable with FTIR as the spectra obtained by reflected light must be corrected by computer software to render them comparable with transmission spectra.

When a light beam impacts at the surface of a medium for which the refractive index is different it can undergo, depending upon the angle of incidence and the variation of the refractive index, either a total reflection as in a mirror, or an attenuated reflection after having in part penetrated into the second medium (just a few micrometres for the mid-IR). The spectral composition of the beam reflected depends of the compound studied and upon the way in which the refractive index of the material varies with the wavelength.

Attenuated total reflection (ATR)

ATR consists of imposing on an optical beam one or several reflections at the interface between the sample and a material upon which the sample has been deposited. This material must be transparent in the region of the wavelength chosen and to possess a high refractive index n such as germanium ($n = 4$), AMTIR ($n = 2.5$), diamond ($n = 2.4$) or KRS-5 ($n = 2.4$) (Figures 10.16 and 10.17a).

If the angle of incidence is greater than the critical angle, the light penetrates the sample that corresponds to half a wavelength at each reflection. The penetration is thus dependent upon the wavelength, the refractive indices of both the crystal and the sample and the angle of incidence. This is sometimes referred to as an evanescent wave. The succession of several total but *attenuated* reflections of this type leads to an effective optical pathway comparable with that which would have been obtained through conventional transmission. The spectrum is nevertheless corrected to take into account the depth of penetration which increases with the wavelength.

ATR requires little or no sample preparation for most samples. This procedure has become indispensable for studying thick or highly absorbing solid and liquid materials, including films, coatings, powders, aqueous liquids and even gases. In some devices the crystal is immersed into the solution being analysed. It is the most versatile sampling technique.

■ **Optical fibre probe.** ATR is used for the study of samples at a distance from the optical bank of the apparatus through the use of suitable optical fibres, that is,

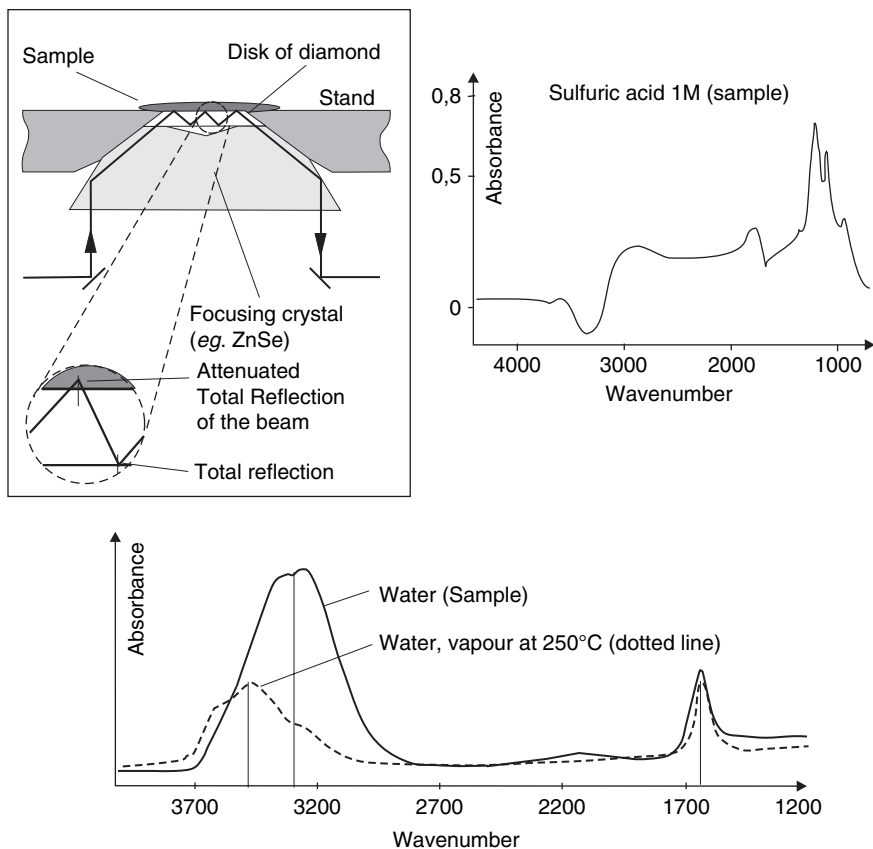


Figure 10.16 ATR three reflection device and examples of spectra. The small diamond disc (diameter 0.75 mm) enables the isolation of the sample examined from the ZnSe crystal. Chemically inert and resistant, diamond is appropriate for examination of all sorts of hard samples which are pressed against its surface or contain water. Above right, are reproduced two spectra obtained with such a device. Spectra obtained from a drop of sulfuric acid (H_2SO_4 1 M) deposited as a sample (reproduced courtesy of SensIR). Below, spectrum of water at 25°C and of heated water vapour (250°C) (*Intl Lab 32,(2), 2002*).

transparent in the infrared over a distance of several metres. They can be used as immersible probes plunged into the product to be analysed enabling rapid control analyses in difficult or aggressive environments.

Specular reflection

This accessory, which can only be used with FTIR spectrometers, is a non-destructive method reserved for samples which reflect at least a minimum of

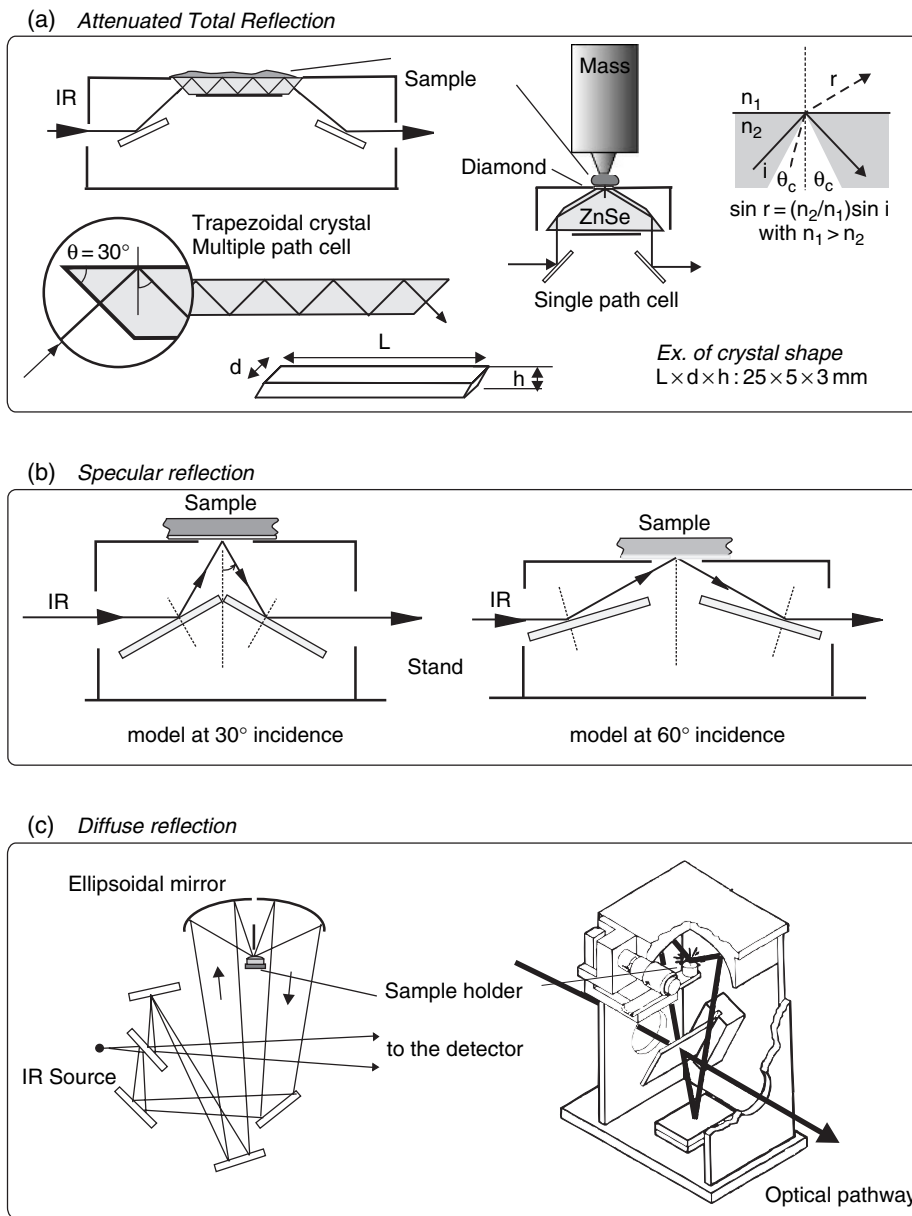


Figure 10.17 Devices allowing the study of samples by reflection. (a) Schematic representation of two ATR devices (attenuated total reflection): a model with a trapezium crystal for multiple reflections and a model for single reflection with solid microsamples (the application of a weight improves the contact of the sample with the crystal's rounded form). Basic formula and notion of critical angle; (b) Specular reflection device. Optical pathway of the apparatus at a fixed angle of 30° for highly reflecting samples and of 60° for the contrary; (c) Optical scheme for a diffuse reflection device and diagram of a Spectra Tech model.

light (polymer films, varnish and some coatings) and measures the *reflectance*, that is the light reflected by the sample in a direction parallel to that of the incident light (Figure 10.17b). The reflected light, which corresponds to only a small part of the incident radiation, is modulated by the variations in the refractive index of the compound as a function of wavelength. By comparing, for each wavelength, the specular reflection I of the sample with the total reflection I_0 obtained by replacing the sample with an aluminium mirror, the instrument can calculate the reflectance spectrum $R = I/I_0 = f(\lambda)$. Finally, by a mathematical transformation known as the Kramers–Krönig (K-K) theory, a pseudo-absorption spectrum is calculated, equivalent to one obtained by transmission (Figure 10.17b).

Diffuse reflection

This is a device containing a series of flat and elliptical mirrors arranged to collect a sufficient part of the light diffused by the sample finely dispersed as a powder in KBr (Figure 10.17c). By comparing the diffused reflection with that of neat KBr, a result resembling the classic transmission spectrum is obtained. A final correction due to Kubelka–Munk is generally added to get a better spectrum. Very time consuming, this technique has been all but abandoned.

Figure 10.18 compares these three approaches using two organic compounds.

10.10 Chemical imaging spectroscopy in the infrared

The high sensitivity of detectors allows the study by transmission or reflection of very small samples such as those that can be examined under an optical microscope. The focusing of the beam upon a zone of only a few micrometres enables to obtain a chemical map which is linked to the composition of the sample if it presents a microstructure. The instrumentation includes a spectrometer coupled with an observation microscope (Figure 10.19).

■ Many industrial or natural compounds are heterogeneous solid mixtures. Traditional methods giving averaged values for the atomic composition are sometimes insufficient. Chemical imaging spectroscopy is a new analytical advance that answers asked questions as what chemical species are present and where are they located? For example, it is possible to study the dispersion of the active agent of a medication within its excipient, or to evaluate the impurities in a foodstuff for cattle. Statistical methods and chemometric approaches as partial least square (PLS) or principal component analysis (PCA) are then very useful in this context. Mid-IR and near-IR are also applied to observation of terrestrial giant samples using satellite-based sensing systems and detectors as plane arrays.

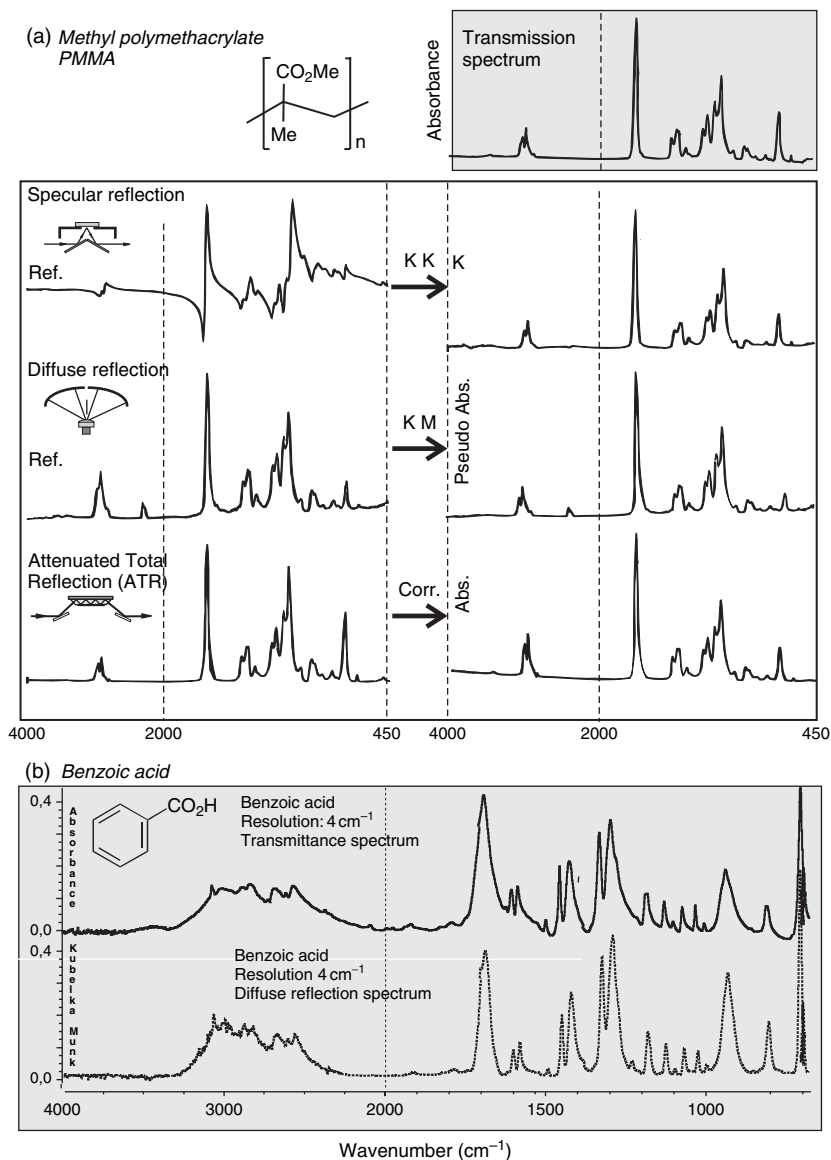


Figure 10.18 Spectra by reflection. (a) From a sample of plexiglass, three types of reflection are displayed. Left, crude spectra and right, spectra after correction. Above, crude signal of specular reflection and the result in units of K following application of the Kramers–Kronig (transformation of the reflectance) calculation; middle, spectrum obtained by diffused light: comparison of the crude spectrum with the Kubelka–Munk correction; below, spectrum obtained by ATR, the latter requiring a fine correction to reduce the absorbance at higher wavenumbers which would be overestimated; (b) comparison of two spectra of benzoic acid, one obtained through transmission, the other by diffused reflection and subsequent K - M correction.

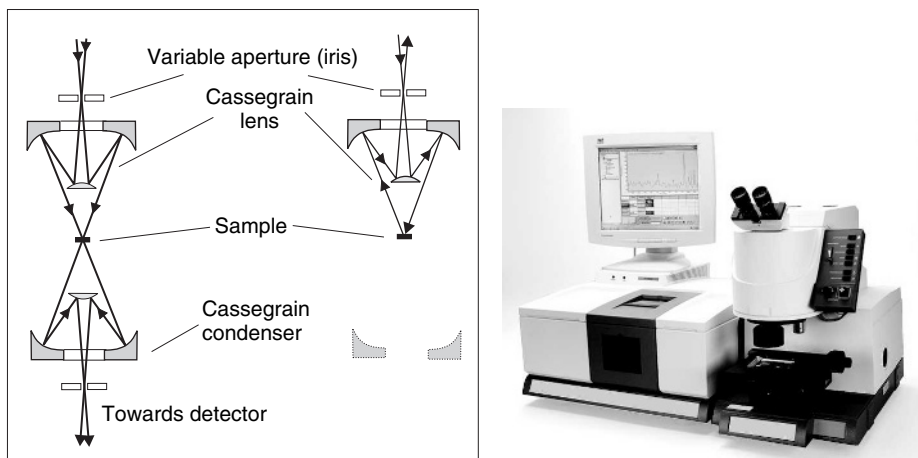


Figure 10.19 *FTIR imaging system.* Left, an optical pathway in an IR microscope associated with a spectrometer. The sample can be examined in transmission (left), or in specular reflection modes (right). This accessory is installed in a spectrometer whose beam is deflected. The Cassegrain optic has the advantage that the light is reflected at the surface of mirrors rather than having to pass through optical lenses. Right, model 400 UMA (reproduced courtesy of Varian Inc. USA).

10.11 Archiving spectra

Different methods are used to store spectra with less memory space than the original files. The experimental data points are mathematically treated by a deresolution procedure in order to replace them by a smaller number of calculated values (e.g. a point calculated each 4 or 2 cm^{-1} reduces a spectrum to less than 1 Ko). Another standard format is the JCAMP-DX (Joint Committee on Atomic and Molecular Physical Data) for exchange spectra in computer readable form. It preserves all of the numerical values of the original spectrum, as well as all of the related information regarding the spectrum in an ASCII file. Most FTIR spectrometers have JCAMP.DX import/export utilities. This format is compatible with all of the comparison algorithms to enable further identification (Figure 10.20).

Coupled techniques such as GC/FTIR and HPLC/FTIR require that chromatograms are displayed in real time on the screen. Consequently, the mobile phase exiting the column is sampled at very short time intervals throughout the elution. IR spectra must be calculated rapidly. They serve to construct a 'pseudochromatograms' called Gram-Schmidt according to the mathematicians whose method is used to treat the data. The individual IR spectra are obtained in differed time (Figure 10.21).

```

##TITLE= CAFFEINE ##JCAMP-DX= 4.24 ##DATA TYPE= INFRARED SPECTRUM ##ORIGIN= EPA1100.SPC
$converted Galactic SPC file
##OWNER= EPA FT-IR Vapor Phase Library ##DATE= 05/05/05 ##TIME= 09:47:00
##MOLFORM= C 8 H 10 N 4 O 2 ##CAS REGISTRY NO= 58-08-2
##NPOINTS= 1842 ##XUNITS= 1/CM ##YUNITS= TRANSMITTANCE ##RESOLUTION= 1.93055
##FIRSTX= 449.413
##LASTX= 4003.553
##XFACTOR= 1.0
##YFACTOR= 5.9604E-8
##FIRSTY= 99.311
##MAXY= 100.92
##MINY= 7.9067
##XYDATA= (X++(Y..Y))
449.41 1666172247 1662340156 1658516880 1643311506 1635761175 1628245537
460.99 1617036772 1605905171 1598526705 1580228522 1562139796 1547818013
472.57 1537162907 1540706434 1537162907 1540706434 1558546974 1572968030

.....(missing values)....
3959.1 1681589144 1681589144 1685465608 1685465608 1685465608 1689351008
3970.7 1689351008 1689351008 1689351008 1689351008 1689351008 1689351008
3982.3 1689351008 1689351008 1689351008 1689351008 1685465608 1685465608
3993.8 1685465608 1685465608 1685465608 1681589144 1681589144 1681589144

```

Figure 10.20 Example of a JCAMP-DX file read by a word processor. This type of file enables the reproduction of the original spectrum by treatment with a suitable software. Experimental points are arranged in sequences of six data points. Only the beginning and the end of the file have been retained.

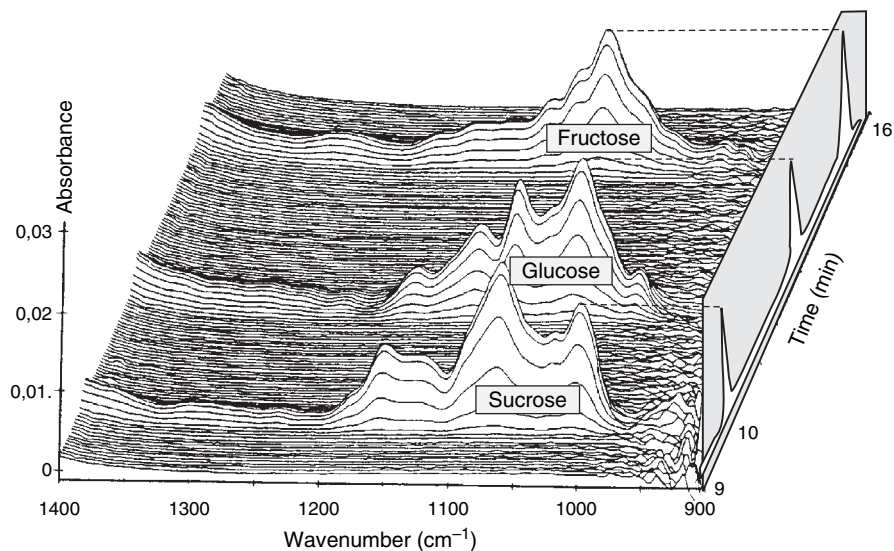


Figure 10.21 Gram-Schmidt process. Three-dimensional pre-recorded reconstruction between 1400 and 900 cm^{-1} of a solution containing 10 mg/mL of three sugars, saccharose, glucose and fructose, by HPLC/FTIR (volume injected = 50 μL) from Keller R. *et al.* 1997 *Anal Chem* 69, 4288.

10.12 Comparison of spectra

One of the most positive identification methods of an organic compound is to find a reference IR spectrum that matches that of the unknown compound. This approach is facilitated using *spectral libraries* as compiled by various societies or

editors (e.g. Aldrich, Sigma, Sadtler, Hummel), whether generalized or specialized (polymers, solvents, adhesives, organic molecules classed by functionality). In order that the comparisons are reliable, these data collections should have been obtained from compounds in the same physical state as the compound to be identified ('EPA Vapor-Phase IR' for example, when the method is GC/FTIR). The archived spectra are normalized by attributing to their most intense band an absorbance value equal to one.

The spectrum to be compared must first be adjusted to conform (absorbance and wavelengths) with the model of the spectra in the library. Then the procedure consists of a mathematical comparison of the unknown compound's spectrum with all those included in the spectral library in order to establish, by the end of the research session, a list of spectra that most resemble that of the unknown compound, each having an index of reliability. The analyst must examine these results with attention recalling that the objective of this exercise is to aid and not to substitute for, because if the algorithm for comparison is changed, the final listing will be probably different. The best methods of identification are associated with interactive programs in which the analyst contributes information in order to narrow the field of investigation, by defining filters.

The algorithm using the absolute difference is the simplest: for each of the j spectra in the library the sum S_j , for the n defined points along the abscissa, is calculated as the absolute difference between the corresponding absorbance of the unknown and that of spectrum j in the library (expression 10.10). Then the j summations S_j are classified in increasing order. The results are presented along with an index of correlation.

$$S_j = \sum_{i=1}^n \left| y_i^{\text{ref}(j)} - y_i^{\text{inc.}} \right| \quad (10.10)$$

Other expressions can be chosen: replacing the differences above by their squares or by the differences of these squares or taking the incremental difference between two consecutive points. Each algorithm may minimize or increase the value of particular factors (differences of intensity, signal-to-background improvement, difference in gradient . . .).

10.13 Quantitative analysis

The precision of absorbance measurements and the possibilities of storage and re-treatment of the spectra have favoured quantitative analysis by infrared. The method is widely used for the facility with which the absorption bands of a particular compound can be located even in a mixture and because efficient methods of statistical analysis are available for the near IR. However, the development of reliable FTIR instrumentation and strong computerized data-processing capabilities have greatly improved the performance of quantitative IR work. Thus,

modern infrared spectroscopy has gained acceptance as a reliable tool for quantitative analysis.

In the early ages of FTIR, some analysts considered that double beam dispersive instruments were preferable for quantitative analysis as they are the only ones to compare the intensities transmitted by the two pathways (of reference and sample) at the same instant.

10.13.1 Quantitative analysis in the mid-infrared

For solid samples dispersed in a KBr disc the thickness of which cannot be measured with precision, a compound is introduced as an internal standard (calcium carbonate, naphthalene, sodium nitrite), in an equal quantity to all of the standards as well as to the sample.

For liquid and solutions, the absorbance measurements are carried out in cells of short optical path l to minimize absorption by the solvent, of which none are really transparent in this spectral domain. The uncertainty in the value of l is linked to the softness of the material used for the construction of cell windows. This means the periodic calibration of their optical path.

The exact measurement of the optical path in cells of low thickness is made by interferometry (interference pattern method). The transmittance of the empty cell is measured for an interval between two wavenumbers $\bar{\nu}_1$ and $\bar{\nu}_2$ (in cm^{-1}). Figure 10.22 shows that the beam S_2 has undergone a double reflection from the internal walls of the cell, thus for a normal incidence, there would be, if $2l = k\lambda$, addition of both light intensities (the two beams S_1 and S_2 are in phase). As a function of the wavelength a modulation of the main beam S_1 of a few percent is observed. After calculation, if N represents the number of interference fringes counted between $\bar{\nu}_1$ and $\bar{\nu}_2$ (in cm^{-1}), then:

$$l_{(\text{cm})} = \frac{N}{2(\bar{\nu}_1 - \bar{\nu}_2)} \quad (10.11)$$

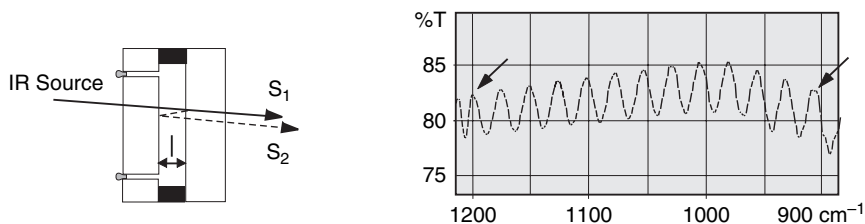


Figure 10.22 Measurement of cell thickness by the method of interference fringes. Left, reflections on the inner wall of a cell (for further clarity, the angle of incidence of the beam on the cell is shifted from normal incidence by a small angle). Right, part of the recording obtained from an empty cell. Twelve fringes are counted between the two arrows. The calculation (expression 10.11) using this data leads to a cell path-length of $l = 204 \mu\text{m}$.

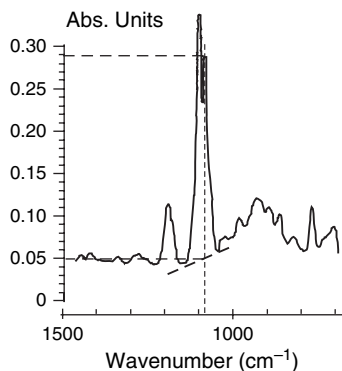


Figure 10.23 *Correction of the absorption baseline.* In supposing, for the given example, that the measurement of the concentration is based upon the absorbance of the band at 970 cm^{-1} , then it will be necessary to deduct from the total absorbance (0.29 AU), the baseline absorption of 0.05 AU which leads to 0.24 AU.

Also to be taken into account is the background absorption which is often visible for a substantial part of the spectrum. In particular it depends upon the manner in which the sample has been prepared. As absorbances are additive, that not due to the compound under study must be subtracted from the total absorbance. This is evaluated by the tangent method (Figure 10.23).

10.13.2 Quantitative analysis in the near infrared

The absorption bands in the near-IR originate from harmonics or combinations of the fundamental vibrations present in the mid-IR.

■ From this point of view the water molecule has been particularly well studied. To a first approximation the band which appears around 5154 cm^{-1} (1940 nm , see Figure 10.24) results from the combination of the asymmetrical vibration situated at 3500 cm^{-1} and the deformation vibration (scissoring, see Section 10.6) at 1645 cm^{-1} (Figure 10.16). These simple calculations ($3500 + 1645 = 5145$) are an imperfect approach, which conceals a certain number of factors of the physical state of the compounds to which infrared is sensitive. Among the approximations are those for the combination bands of the bonds C–H, N–H and C–O in the near-IR.

In this region, essentially reserved for control analyses and where the crude spectra are fairly often disconcerting due to the absence of clear absorption bands, a method employing standards and based upon a single wavelength as in UV/Vis spectrometry can rarely be used (cf. Chapter 9). For many samples, as in the complex matrices of the agricultural and food industries, calibration and validation methods require a great number of representative reference samples, themselves measured by a proven method (Kjeldhal, for example, for the protein nitrogen).

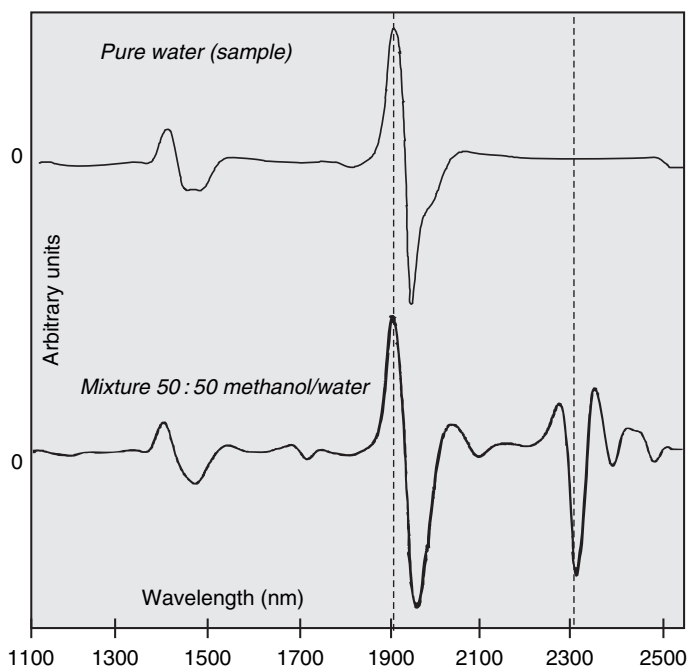


Figure 10.24 *Derivative plots.* Comparison of graphs of the second derivative of pure water and of a mixture of half water/half methanol in the near-IR. Note that it is possible to measure out the methanol from the peak situated around $2.3\ \mu\text{m}$ without interference from the water signal located at $1.94\ \mu\text{m}$. In this spectral region, considered as an extension of the visible range, the wavelengths are rather expressed in nm or μm .

In this way, correlations with around 50 reference samples, very similar to those to be measured, is not unusual. Many products (proteins, oils in cereals, meat), can routinely be analysed by this method and often without preparation beforehand. In general, a relation is sought between certain absorptions and the presence of constituents in the mixtures to be analysed. This is the domain of *correlation analysis*.

As the absorption bands are not very intense, the measurements are undertaken with cells of 1 cm optical path-length and on undiluted sample. They are accomplished either in transmission or reflection (for example, proteins, fats, cellulose or starch are measured by reflectance). By using derivative plots, precision is improved (Figure 10.24).

Some application software programs use methods known as MLR (*multiple linear regression*) that permits the statistical treatment of a large number of data points in order to establish a calibration equation. These chemometric methods take advantage of all the absorptions measured at different wavelengths, irrespective of their origin: analyte to be measured, matrix or artefacts of the instrument.

PCA and PLS methods are commonly used. In conclusion, although certain measurements in the near-IR are not always very sensitive, they are reputed to be very reliable.

Problems

- 10.1
1. What is the energy possessed by a radiation of wavenumber 1000 cm^{-1} ?
 2. Transform $\lambda = 15\text{ }\mu\text{m}$ into units of cm^{-1} and then into m^{-1} . What is the wavelength corresponding to a wavenumber of 1700 cm^{-1} ?
 3. At the maximum of an absorption band, the transmittance is only 5%. What is the corresponding absorbance?
- 10.2
- If chloroform (trichloromethane) exhibits an infrared peak at 3018 cm^{-1} due to the C–H stretching vibration, calculate the wavenumber of the absorption band corresponding to the C–D stretching vibration in deuteriochloroform (experimental value 2253 cm^{-1}).
- 10.3
- A ketone possesses an absorption band with a peak centred around 1710 cm^{-1} . From this information deduce a value for the force constant of the C=O double bond.
- 10.4
- The bond between the two atoms of a diatomic molecule is characterised by a force constant of 1000 N/m . This bond is responsible for a vibrational absorption at 2000 cm^{-1} . Accepting that the energy of radiation is transformed into vibrational energy, estimate a value for the length of the bond at the maximum separation of the two atoms.
- 10.5
- The examination of an absorption band located around 2900 cm^{-1} , expressed by a sample of HCl in the gaseous state, reveals that the band is the result of a superimposition of two forms of vibration, one of which is clearly more intense than the other. These two series are separated by an approximate distance of 2 cm^{-1} . How might this phenomenon be interpreted? Use calculations to illustrate your answer.

- 10.6 Knowing that the fundamental frequency of carbon monoxide is 2135 cm^{-1} when the spectrum is recorded with tetrachloromethane as the solvent, calculate the force constant of this molecule under these conditions.
- 10.7 The following experiment is used to determine the vinyl acetate (VA) level in an ethylene vinyl acetate (EVA) commercial packaging film.

Infrared spectra of packaging films with known vinyl acetate contents are recorded. The absorbance peak at 1030 cm^{-1} used to determine the content of the vinyl acetate was measured by the baseline method ($A = \log(I_0/I)$). The following results were obtained.

(EVA)	%VA	A_{1030}	A_{720}	$d(\mu\text{m})$
1	0	0.01	1.18	56
2	2	0.16	1.55	80
3	7.5	0.61	1.49	82
4	15	0.36	0.45	27

- Taking into account the film thickness, determine, from the data in the table, the best line $A_{1030} = f(\% \text{ VA})$, using linear regression, for a film thickness of $1\ \mu\text{m}$.
 - Explain why the polyethylene peak at 720 cm^{-1} may be chosen as an internal standard, then calculate the ratio A_{1030}/A_{720} for the four films.
 - Using both above methods calculate the vinyl acetate content (%) of an unknown EVA film (giving $d = 90\ \mu\text{m}$, $A_{1030} = 0.7$ and $A_{720} = 1.54$).
- 10.8
- In supposing that the signal to noise ratio obtained for an FTIR is 10 for a signal corresponding to a single scan, what would be the expected value following 16 scans?
 - The resolution (in cm^{-1}) is given by the expression $R = 1/\Delta$, where Δ represents the difference of the maximum optical path between the two beams (in cm) when the mirror glass is moving. What must be the displacement of the mirror relative to the central position to obtain a resolution of 1 cm^{-1} ? (The central position of the mirror is such that δ , the difference between the optical paths, is zero.)

3. To locate the position of the moving mirror with great precision, we superimpose in the apparatus a laser source of monochromatic radiation ($\bar{\nu} = 15\,800\text{ cm}^{-1}$), which permits a computer to pick up a point of the interferogram each time the laser light is extinguished.

What, in μm , is the value of the displacement dx of the mirror between two successive extinctions of the laser beam's light?

11

Fluorimetry and chemiluminescence

Some organic or inorganic compounds, liquids or solids (molecular or ionic crystals), whether pure or in solution, emit light when they are excited by photons from the visible or the near ultra-violet regions. Among the analytical applications of this phenomenon, known as photoluminescence, is fluorimetry, a selective and highly sensitive method for which a wide range of measurements are accessible. The intensity of the fluorescence is proportional to the concentration of the analyte and the measurements are made with the aid of fluorimeters or spectrofluorimeters. The extremely rapid extinction of the light intensity when excitation ceases is the object of analysis. By contrast, phosphorescence is characterized by a more gradual diminution during time. Fluorescence is equally employed as the basis of detectors used in liquid chromatography. Although of different origin, chemiluminescence which comprises the emission of light during certain chemical reactions, has also received several applications in analytical chemistry.

11.1 Fluorescence and phosphorescence

Many compounds, when excited by a light source in the visible or near ultra-violet regions, absorb energy which is nearly instantaneously re-emitted in the form of radiation. According to Stokes' law, the maximum of the spectral emission band is located at a longer wavelength than that of the original excitation light. These are *fluorescent compounds* (Figures 11.1 and 11.2). After excitation, the light intensity decays extremely rapidly according to an exponential law. Expression 11.1 links the intensity of fluorescence I_t and the time passed t since the excitation:

$$I_t = I_0 \cdot \exp[-kt] \quad (11.1)$$

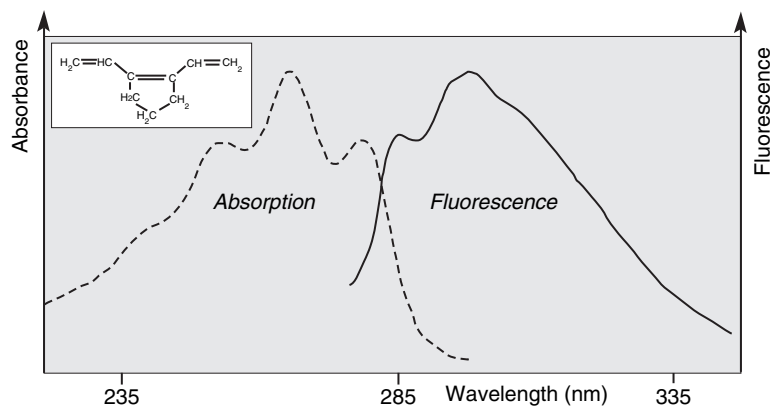


Figure 11.1 Representation on the same graph of the absorbance and fluorescence spectra of an ethylenic compound. The fluorescence spectrum that resembles the mirror image of the absorbance spectrum, as well as the ‘Stokes shift’ can be interpreted by considering the energy diagrams (Figure 11.2). In UV/Vis absorption and fluorescence spectra, bandwidths of 25 nm or more are common. This representation is obtained by uniting on the same graph, with a double scale, the spectrum of absorbance with that of emission. Example extracted from Jacobs H. *et al.*, Tetrahedron 1993, 6045.

This emission can be classified as *fluorescence*, which has a very rapid decrease in intensity or *phosphorescence*, where emission decay is much slower. The difference between the two is characterized by the value of the constant k , which for *fluorescence* is much greater than for *phosphorescence*. The *lifetime* of fluorescence τ_0 is defined, using the rate constant k , by $\tau_0 = 1/k$. At the instant τ_0 , the intensity I_t will become, according to expression 11.1, 36.8 per cent of the initial intensity I_0 . In other words a fluorescent compound corresponds, on the microscopic scale, to a population of individual species of which 63.2 per cent have relaxed to a non-emissive state after this brief period of time.

Fluorescence lifetimes τ_0 are only a few nanoseconds. To facilitate the measurements of fluorescence, current instruments operate in a stationary regime, that is the source of excitation is maintained illuminated, though with the obligatory discrimination between the light from the source and that due to the fluorescence. Figure 11.1 illustrates for example, the apparent mirror symmetry of the absorbance and fluorescence spectra that are common for numerous compounds.

■ Fluorescence can be resolved over time. The use of very short pulsed light sources (picosecond lasers and laser diodes) has rendered accessible graphs of fluorescence decay as a function of the time. New applications based upon a greater knowledge of the lifetime are under development, although they are still not used much in chemical analysis.

11.2 The origin of fluorescence

Subjected to excitation by light, a molecule (the solute, initially in the ground state S_0), is instantaneously promoted to its first electronic excited state S_1 (Figure 11.2). The electrons and those of the neighbouring solvent molecules re-equilibrate very quickly; however the position of the atomic nuclei remains identical to how they were in the fundamental state (this is the Franck–Condon principle). The solute/solvent cage system being out of equilibrium, will adopt a more stable conformation relative to the electronic excited state S_1 . Then, by the process of *internal conversion*, the molecule will rejoin (10^{-12} s), without emitting photons, the state V_0 of level S_1 . If this level is compatible with the fundamental level the system can relax through a fluorescent step (10^{-11} to 10^{-8} s) during which the molecule returns to one of the vibrational states of the ground electronic state S_0 with emission of photons as fluorescence.

Over the course of fluorescence that accompanies the return to the initial state, the molecule can retain a part of the energy absorbed in the form of vibrational

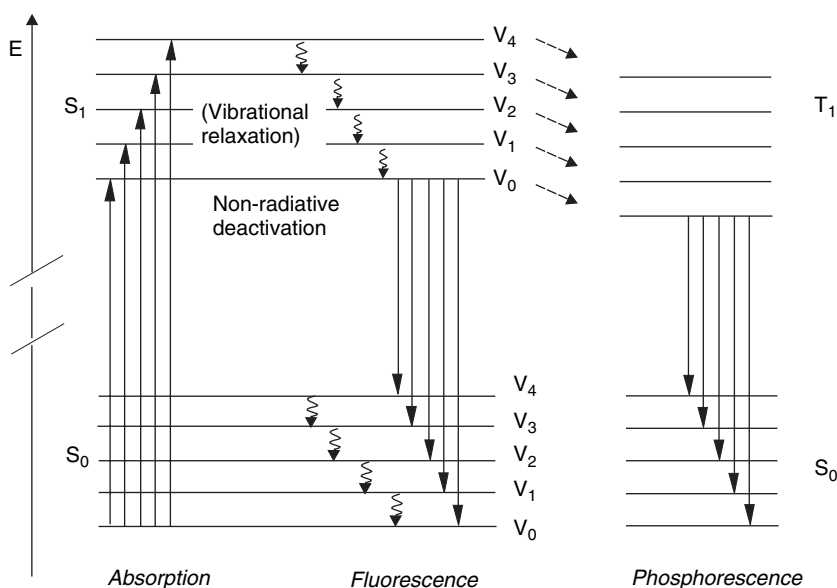


Figure 11.2 Energy diagram comparing fluorescence and phosphorescence. The short arrows correspond to mechanisms of internal conversion without emission of photons. The fluorescence results from transfers between states of the same multiplicity (same spin state) while the phosphorescence results from transfers between states of different multiplicity. The state T_1 produces a delay in the return to the fundamental state, which can last several hours. The ‘Stokes shift’ corresponds to the energy dissipated in the form of heat (vibrational relaxation) during the lifetime of the excited state, prior to photon emission. The real situation is more complex than this simplified Jablonski diagram suggests. To our scale, a compound can be both fluorescent and phosphorescent for, at the molecular scale individual species do not all exhibit the same behaviour.

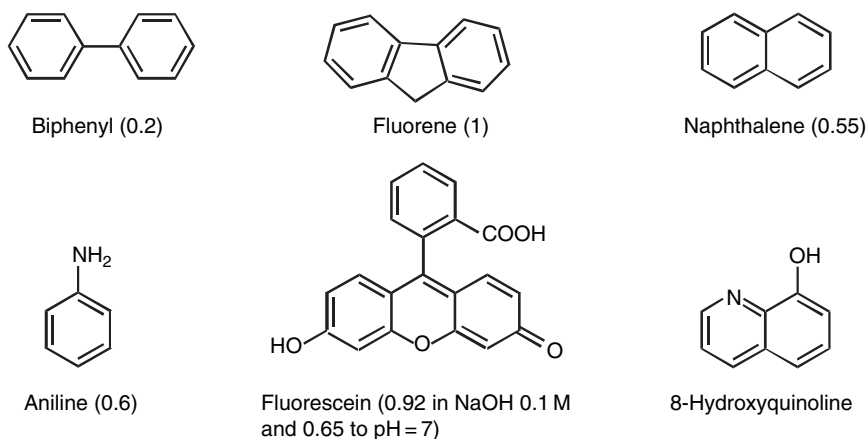


Figure 11.3 *Fluorescing aromatic compounds.* The names are followed by their fluorescence quantum yield Φ_f (cf. Section 11.3), for which the values are obtained by comparison with compounds of known fluorescence. The measurements are made at 77 K. 8-Hydroxyquinoline is representative of various molecules forming fluorescent chelates with certain metal ions.

energy. This excess of vibrational energy is dissipated through collisions or other non-radiative processes known collectively as mechanisms of *vibrational relaxation*. The emission of lower energy photons can equally be produced and be the origin of a fluorescence in the mid-infrared.

Phosphorescence corresponds to one another relaxation process. After the absorption phase, corresponding to the transfer of an electron into the S_1 level (singlet state), an electron spin inversion can occur if the vibrational relaxation is slow enough, which leads to a slightly stable T_1 (triplet state). From here return to the ground electronic state is slowed as it requires a new spin inversion for this electron. These radiative lifetimes can be up to several minutes duration (Figure 11.2).

Sensitivity in fluorimetry is often 1000 times greater to that of the UV/Vis absorption. However, the correct use of these techniques requires a sound knowledge of the phenomenon in order to avoid a number of errors.

Fluorescence, which is often encountered with rigid cyclic molecules possessing π -bonds, is enhanced by the presence of electron-donating groups while being reduced by electron-withdrawing groups (Figure 11.3). Equally, there is a dependency upon the pH of the solvent. In contrast, non-rigid molecules readily lose their entire absorbed energy through degradation and vibrational relaxation.

■ By analogy, this phenomenon can be compared with the effect produced when a hammer hits a block of rubber for example, or by opposition on something hard such an anvil. With the rubber, the energy is dissipated into the mass (as heat) and sound will not be emitted. Alternately on the anvil, a part of the mechanical energy is transmitted towards the outside (as sound waves) and this can be compared to the phenomenon of fluorescence of non-rigid vs. rigid molecules.

11.3 Relationship between fluorescence and concentration

At each point of the solution the intensity of fluorescence is different because a part of the excitation radiation is absorbed before reaching the point being considered and because a part of the emitting radiation light finds itself trapped before it can exit the cell. Globally the fluorescence received by the detector corresponds to the sum of the fluorescence emerging from each of the individual small volumes constituting the space delimited by the entrance and exit slits (Figure 11.4). This is why the calculation of the absolute fluorescence intensity (emission I_f) for the sample is difficult. The phenomenon of radiation damping, called *internal quenching* is due to the partial overlap of the absorption and emission spectra (*colour quenching*), and is increased by transfers of energy from excited species to other molecules or ions through collisions or complexes formation (*chemical quenching*). That is why the presence of oxygen can cause an underestimation of fluorescence.

For solutions, the quantum yield of fluorescence Φ_f (between 0 and 1 inclusive), which is independent of the intensity emitted by the light source, is defined by the ratio of the number of photons emitted to the number of photons absorbed, this latter being equivalent to the ratio of fluorescence intensity I_f over that absorbed I_a (expression 11.2).

$$\Phi_f = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}} = \frac{I_f}{I_a} \quad (11.2)$$

Accepting that $I_a = I_0 - I_t$ (I_t representing the transmitted light intensity), the following reasoning allows to relate I_f to the concentration C , of the compound:

$$I_f = \Phi_f(I_0 - I_t) \quad \text{that is } I_f = \Phi_f \cdot I_0 \cdot \left(1 - \frac{I_t}{I_0}\right) \quad (11.3)$$

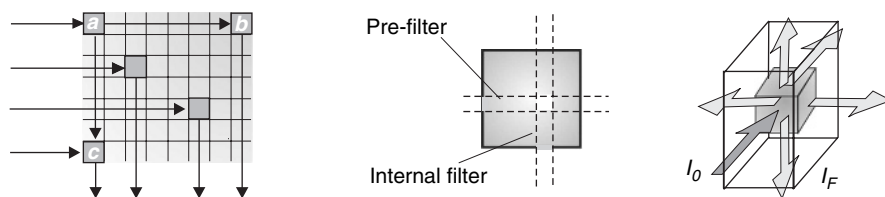


Figure 11.4 *Intensity of fluorescence and radiation damping.* Depending upon the site in the solution where the fluorescence is emitted, a variable intensity of light reaches the detector. By specific positioning of the entrance and exit slits, it is possible to evaluate the re-absorption of the light of fluorescence (comparison between a and c) and the absorption of the incident light (comparison between a and b). In practice, the presence of an iris means that only the light arriving from the central region of the cell is collected.

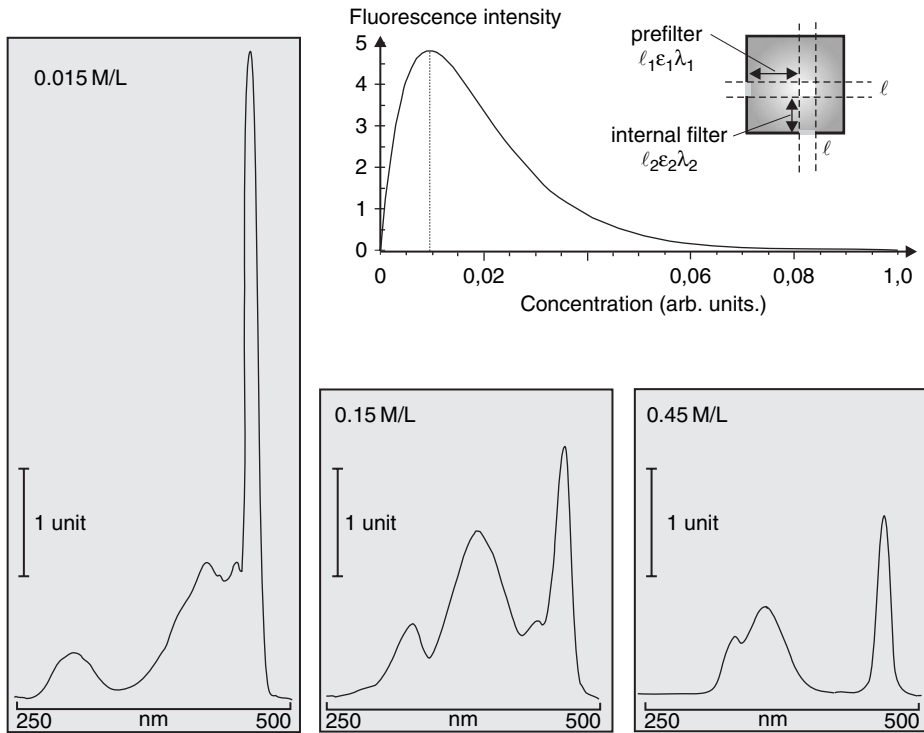


Figure 11.5 Intensity of fluorescence and concentration. Modelling of expression 11.7 reveals the effect of the concentration upon fluorescence intensity. A maximum of fluorescence is observed beyond which, with a continuing increase in concentration, it diminishes. After the maximum the more concentrated the solution then the weaker is the fluorescence – a kind of roll-over or self-quenching. The illustrations correspond to three recordings, at the same scale of biacetyl tetrachloromethane. The curve records the light collected from a small volume situated at the centre of the solution as indicated on Figure 11.4 (parameters ϵ and l chosen arbitrarily).

Knowing that the absorbance A is equal to $\log I_0/I$, expression 11.3 becomes:

$$I_f = \Phi_f \cdot I_0 \cdot (1 - 10^{-A}) \quad (11.4)$$

with

$$10^{-A} = 1 - 2.303A + \frac{(2.303A)^2}{2!} - \dots + \dots$$

If the solution is diluted, the term A is close to 0 and the term 10^{-A} is therefore close to $1 - 2.3A$. The expression (11.4) can then be simplified, becoming:

$$I_f = 2.3 \cdot \Phi_f \cdot I_0 \cdot A \quad \text{thus} \quad I_f = 2.3 \cdot \Phi_f \cdot I_0 \cdot \epsilon \cdot l \cdot C \quad (11.5)$$

with I_0 the intensity of the excitation radiation, C the molar concentration of the compound, ϵ its molar absorption coefficient, l the cell thickness and Φ_f the fluorescence quantum yield.

This last expression reveals that the intensity of fluorescence depends upon the concentration (C), the experimental conditions (I, I_0) and of the compound (ε, Φ_f). If all the parameters due to the instrument and most due the compound are factored into a global constant K , then the following equation can be used for weak concentrations ($A < 0.01$):

$$I_f = K \cdot I_0 \cdot C \quad (11.6)$$

Measurements by fluorimetry make use of several classic methods employing one or more standards (single point calibration or calibration curve, or methods of addition), but to obtain the best results, the solutions must be very dilute.

Above a given limit, fluorescence is no longer directly proportional to concentration. This arises because of the non-linearity of the Lambert–Beer law. The excitation is proportionally weaker and association complexes appear between excited molecules and those in ground state. This leads to the apparently paradoxical result that the fluorescence can diminish even though analyte concentration increases. The curve in Figure 11.5 is a graphic illustration of expression 11.7 following the introduction of reasonable values for these different parameters.

$$I_f = \Phi \cdot k \cdot I_0 \cdot 10^{-(\varepsilon_1 \ell_1 + \varepsilon_2 \ell_2)C} \cdot (1 - 10^{-\varepsilon I C}) \quad (11.7)$$

11.4 Rayleigh scattering and Raman bands

When the wavelengths of excitation and of emission are close together then a confusion becomes possible between the fluorescence of the sample and two artefacts due to the solvent: Rayleigh scattering and Raman diffusion. The limits of detection in fluorescence are often governed by the ability to distinguish the analyte fluorescence from these additives interferences.

11.4.1 Rayleigh scattering

Rayleigh scattering is the re-emission, by the solvent in which the compound is dissolved, of a small fraction of the absorbed excitation light in all directions at the *same wavelength* (Figure 11.6). The Rayleigh scattering intensity depends upon the polarizability of the solvent molecules.

11.4.2 Raman diffusion

Raman scattering, which is 100 to 1000 times weaker than that of Rayleigh scattering, is produced by the transfer of a part of the excitation radiation energy to

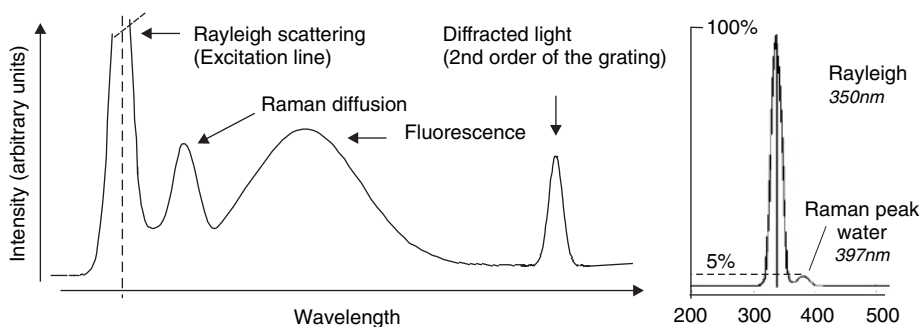


Figure 11.6 The diverse components of a fluorescence spectrum. The position of the Raman peak depends upon the wavelength of the excitation beam and of the solvent. Right, a sensitivity test for a fluorometer.

the solvent molecules in the form of vibrational energy. The solvent molecules re-emit photons of less energy than those having served to excite them. Compared to Rayleigh scattering, the Raman emission band is shifted towards longer wavelengths. For each solvent the difference in energy between the absorbed photons and the re-emitted photons is constant. Therefore it is possible, by modifying the excitation wavelength, to displace the *shift in nanometres* between the position of the Raman and Rayleigh bands (Table 11.1 and Figure 11.6).

The Raman scattering of water serves as a sensitivity test for fluorometers. This consists of measuring the signal/noise ratio of the Raman peak with a cell filled with water, for example at 397 nm if the excitation wavelength is fixed at 350 nm, as a result of the specific shift of 3380 cm^{-1} for this solvent, and to compare with the background signal.

Table 11.1 Position of the Raman scattering band, calculated for four commonly used solvents and five excitation wavelengths from a mercury lamp

Excitation (nm)	254	313	365	405	436	Shift (cm^{-1})
Water	278	350	416	469	511	3380
Ethanol	274	344	405	459	500	2920
Cyclohexane	274	344	408	458	499	2880
Chloroform	275	346	410	461	502	3020

■ The phenomenon of fluorescence is not only used for chemical analyses but is equally applied to substances such as washing powders where a fluorescing agent that sticks to the textile fibres is added. This type of compound absorbs solar radiation in the non-visible part of the spectrum and re-emits at longer wavelength, in the blue spectral region of the spectrum, visible to the eye, which makes clothing appears 'whiter'.

11.5 Instrumentation

A fluorescing compound behaves like a source which emits light in all directions. This emitted light is usually monitored in a direction perpendicular to the beam coming from the primary excitation source. For strongly absorbing solutions, fluorescence is viewed from the face of the sample on which the exciting radiation impinges, while for opaque or semi-opaque samples a frontal measurement is preferable (Figure 11.7).

The excitation source is often a xenon arc lamp with a power of 150 to 800 watts. The measurement of the light intensity is carried out using a photomultiplier tube or a photodiode. The solvent, the temperature, the pH and the concentration are the principal parameters which affect the intensity of fluorescence. For solution samples, rectangular 1 cm glass or fused silica cuvettes are used.

■ Among the numerous applications of LIDARS (light detection and ranging), they are used for gas monitoring, only present as traces in the Earth's atmosphere. Gases are quantified by their retro-scattering fluorescence which is induced following excitation by a very short pulse ($1 \mu\text{s}$) from a strong monochromatic laser. The excitation can be tuned to a specific wavelength adapted to the compound under study.

Two broad categories of commercial instruments are currently proposed by manufacturers: fluorescence ratio fluorometers and spectrofluorometers.

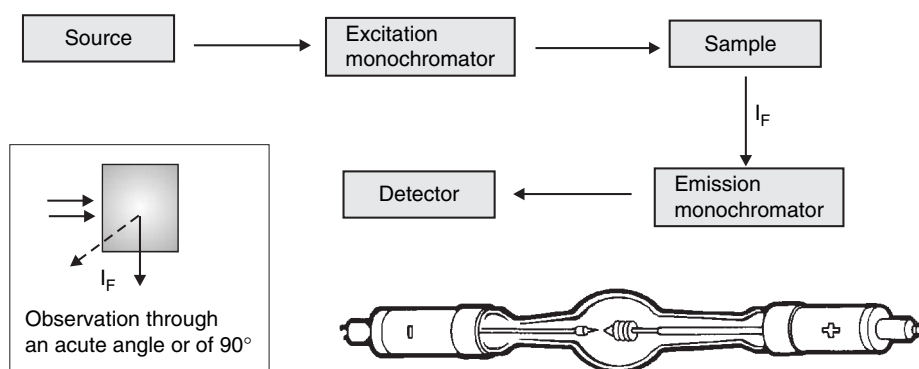


Figure 11.7 A block diagram of a spectrofluorometer with a xenon arc lamp. The fluorescence is measured, in contrast to studies on fluorescence dynamics, under 'steady state' conditions by maintaining the primary excitation source. Below right, a schematic of a xenon arc lamp. The pressure of xenon in the lamp is around 1 MPa. These arc lamps made from a quartz envelope and without a filament, are sources of 'white light'. The cathode is the finer of the two electrodes (reproduced courtesy of Oriol).

11.5.1 Fluorescence ratio fluorimeters

The light emitted by the primary source initially passes through the excitation monochromator which allows a narrow band of wavelengths to be selected (15 nm) to induce fluorescence of the molecules of interest in the sample solution. A part of the fluorescence emitted by the compound is collected in a direction either perpendicular or parallel (depending upon the model) to the direction of the incident beam. Then before the light reaches the detector it passes the emission monochromator allowing the selection of a narrow band of wavelength for measurement. Many manufacturers have commercialized models fitted with filters (Figure 11.8). These basic instruments contain a turret compartment for the standard solutions, the sample and a compound to be used as fluorescing standard. By alternating in the same optical pathway (single beam models) the standard solutions, the sample solution and a fluorescent standard reference (quinine sulfate, rhodamine B or 2-aminopyridine) the fluorescence ratios can be measured allowing to obtain, versus a calibration curve, the expected results. In this manner possible fluctuations from the source and a large number of regulatory parameters of the instrument are eradicated.

The use of a xenon flash lamp as the source enables the study of fluorescence after the source has been switched off. This method, used in immunology, in which fluorescing lanthanide complexes are obtained having a fluorescing lifetime of around 1 ms, is sufficient for very sensitive measurements (in the picomolar concentration range).

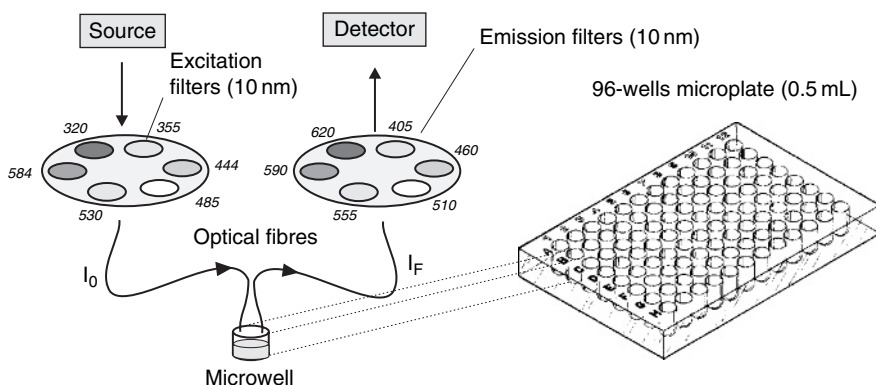


Figure 11.8 Schematic of the optical pathway of a fluorometer microwell-plate reader. A fibre-optic carries the excitation radiation to the wells chosen for analysis and a second fibre recovers the fluorescence *via* a forward geometry. These fluorimeters can read plates containing from 6 up to 384 microwells, that is useful for routine control in combinatorial chemistry and other immunology/enzymology methods of screening.

11.5.2 Bench-top spectrofluorometers

Spectrofluorometers allow a more complete study of fluorescent compounds, notably by recording both excitation and emission spectra (Figures 11.9 and 11.10). They comprise two grating monochromators, both of which are able to scan a spectral band. It is possible to obtain the entire emission spectrum while maintaining a fixed excitation wavelength or to record the entire excitation spectrum while maintaining a constant emission wavelength.

Spectrofluorometers have software which can determine automatically the excitation–emission pair to get the best results (Figure 11.10). Knowing the UV/Vis spectrum of the compound, the procedure can be decomposed in the following steps:

1. The excitation monochromator is set to the value corresponding to the maximum of the UV absorption spectrum.
2. The fluorescence spectrum is recorded.
3. The emission monochromator is set to the wavelength of maximum fluorescence and the excitation wavelength is varied. The excitation spectrum is obtained allowing the best final choice of the excitation radiation (which can be different from the UV absorption maximum).

It may be also useful to scan the excitation and emission monochromators simultaneously (synchronous fluorimetry). Often, this is carried out by scanning the

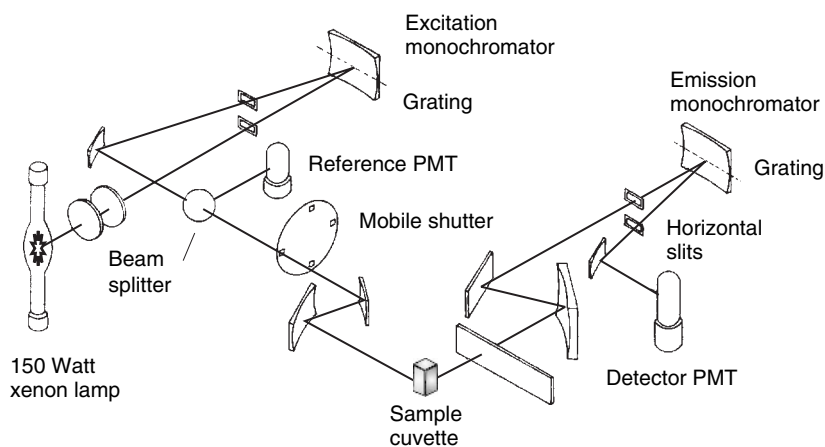
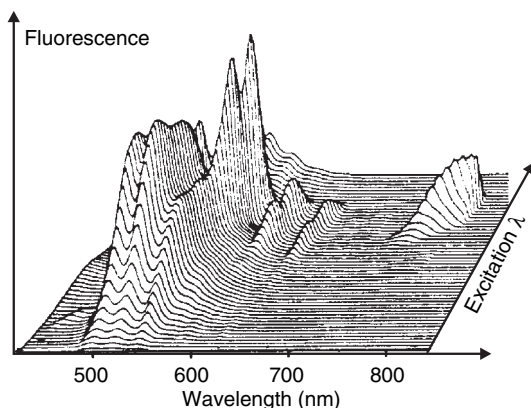


Figure 11.9 Schematic of a Shimadzu F-4500 spectrofluorometer. A fraction of the incident beam, reflected by a semi-transparent mirror, reaches a reference PMT. A comparison of the signals from the two PMTs leads to the elimination of any drifting of the source. This procedure, for single beam instruments, gives approximately the same stability as with a double beam spectrometer. However, the spectrum of a given solution will often present minor differences when recorded upon different instruments (reproduced courtesy of Shimadzu).



o-diaminobenzene bis-hydrochloride $C = 10$ ppm in aqueous solution

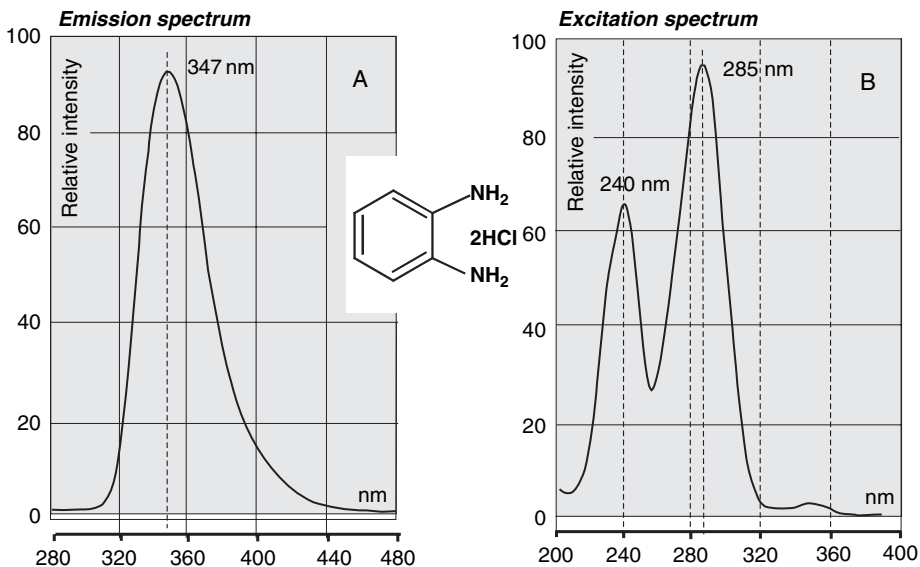


Figure 11.10 Fluorescence spectra. Above, emission–excitation matrix as a topographic representation in pseudo-3D of a mixture of two salts of uranium and terbium by varying the wavelength of excitation. This type of recording leads to the optimum conditions of measurement for such a mixture. Below, emission–excitation spectra. (A) Emission spectrum of fluorescence obtained by maintaining the wavelength of excitation at 285 nm. (B) Excitation spectrum obtained by maintaining the emission monochromator at 347 nm during the recording.

excitation and emission monochromators at the same rate while keeping the wavelength difference (or offset) between them constant.

A fluorescence decay time is a measurement, at fixed wavelength, of fluorescence signal as a function of time. Despite the fact that this time is very short, certain instruments can measure the lifetime of fluorescence. There exist several methods based, either upon the recording of the decrease curve of the light intensity

emitted when the excitation has ceased or, upon the comparison of fluorescence modulation as a function of a rapid modulation of the excitation source.

11.6 Applications

Apart from about 10 per cent of all existing molecules that possess natural fluorescence, many can be made fluoresce by chemical modification or by association with a fluorescent molecule. For example, a *fluorophore reagent* can be bonded to an analyte by chemical reaction (the 7-hydroxycoumarins can be used to this effect). This is called *fluorescence derivatization*, reminiscent of the procedure employed in colorimetry.

For the determination of metal cations, chelates are created with oxine (8-hydroxyquinoline), alizarine or benzoin, then extracted by organic solvents. In biochemistry, fluorescence has numerous applications for the quantification of proteins or nucleic acids by means of reagents which can affix with specificity to these compounds. This approach, sometimes very elaborate, in association with electrophoresis constitutes a more sensitive and less restricting method than detection by radioactive substrates.

■ Chemifluorescence (not to be confused with chemiluminescence) is equally a particularly sensitive method for the detection of proteins. A specific antibody of this protein is required for this which is bound to a conjugated enzyme, such as a phosphatase (Figure 11.11). If mixed with a substrate such as a phosphate derivative of fluorescein, the latter is released then revealed by its fluorescence which is provoked by a suitable source of excitation.

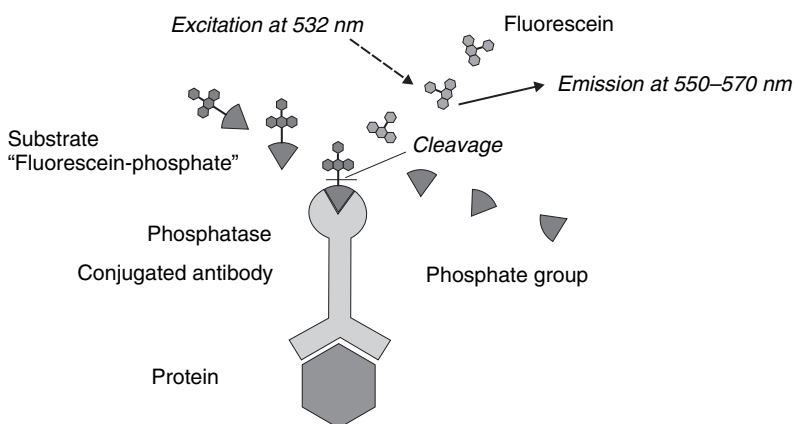


Figure 11.11 Procedure of protein measurement by chemifluorescence in biochemistry. The diagram gathers together the reaction sequence concerned, knowing that to undertake such a measurement a protocol, in which the different reactions are considered as distinct steps, must be followed.

To improve analyses by HPLC, amines can be fluorescently labelled rendering very low thresholds of concentration detectable, in the order of attomoles (10^{-18} M).

Amongst the classic current applications of fluorescence are measurements of polycyclic aromatic hydrocarbons (PAH) in drinking water by HPLC. In this case the detector is equipped with a fluorescence flow cell installed at the outlet of the column. This mode of detection is particularly well adapted for attaining the threshold norms imposed by legislation. The same process can be adopted for measuring the aflatoxins (Figure 11.12), as well as numerous other organic compounds such as adrenalin, quinine, steroids and vitamins.

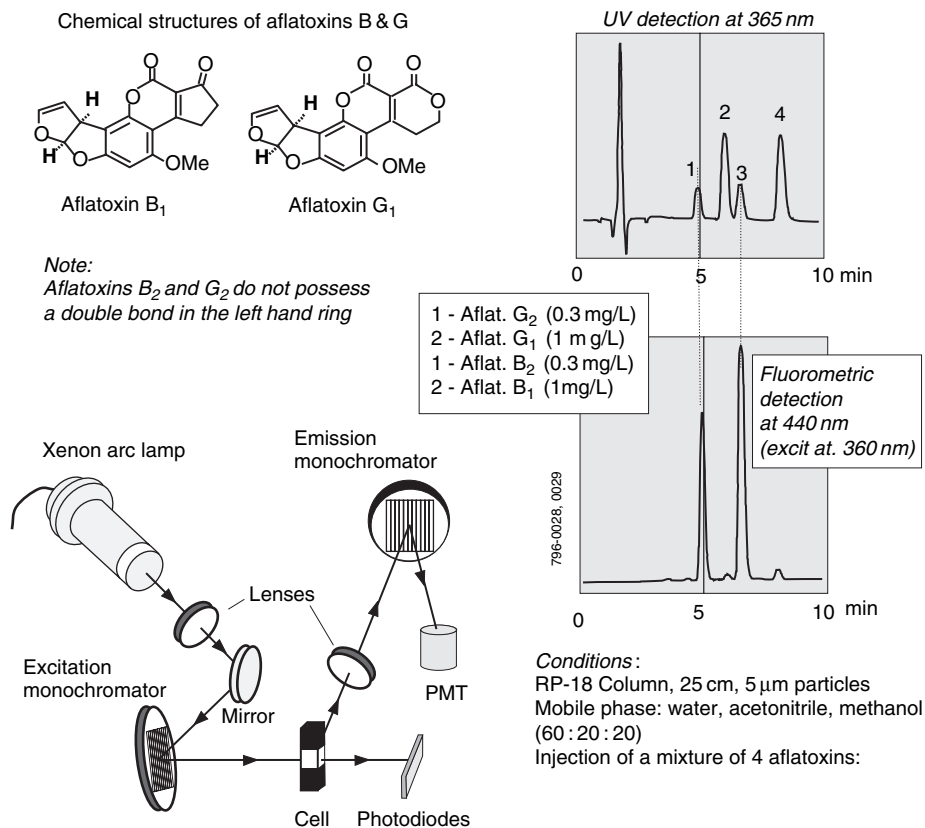


Figure 11.12 Comparison, following a chromatographic separation, of UV and fluorescence detection. Aflatoxins, which are carcinogenic contaminants present in certain batches of grain cereals, are the subject of analysis by HPLC. Detection by fluorescence is much more sensitive to G₂ and B₂ than with UV detection (reproduced courtesy of SUPELCO). Below left, schematic of the different components of a LC-detector based upon fluorescence. This detector is able to find rapidly, for each compound eluted, the best coupling of excitation/emission without interrupting the chromatography underway (reproduced courtesy of a document from Agilent Technologies).

11.7 Time-resolved fluorimetry

This time-resolved fluorescence technique allows a measure of the time dependence of fluorescence intensity after a short excitation pulse. It consists of obtaining a spectrum measured within a narrow time window during the decay of the fluorescence of interest. The usefulness of this technique is now well proven for biochemical assays and immunoassays. Lanthanide chelates have luminescence decay times over $600\ \mu\text{s}$, which allows time-gated fluorescence detection, with a complete rejection of other fluorescence signals. For these quantitative applications, the primary source is generally a quartz lamp associated with a splitter.

There are several ways to perform time-resolved fluorescence measurements. Since the time dependence of fluorescence emission is typically on a picosecond to nanosecond time scale it is very difficult to achieve. To overcome this difficulty either a frequency domain method or the *single photon counting* approach is used.

For studying fluorescent compounds *per se*, *lifetime spectrometers* are used. A highly diluted solution of the compound to be studied is submitted to a pulsed source (laser diode for example) repeatedly for several picoseconds, at frequencies of several megahertz. During these successive cycles, when the source is switched off, the detector measures the time until a photon of fluorescence reaches it. Then this event is stocked in the memory channel reserved for the corresponding time. After having accumulated a large number of photons, the graph of the decay is

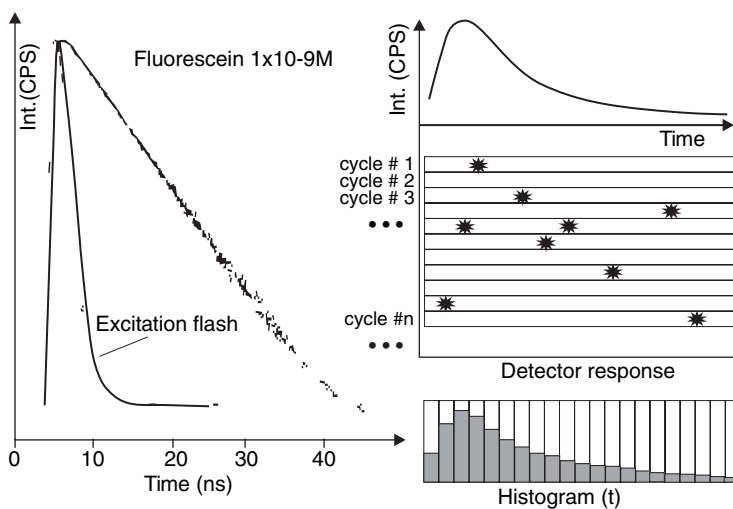


Figure 11.13 Representation of fluorescence decay and of the principle of the measurement. On the graph, each point represents the contents of a memory channel (coupling time/number of photons). The exponential curve of decay appears here in the form of a straight line. This is due to the choice of a logarithmic scale for the ordinate. On the right, a representation of the TCSPC (time-correlated single-photon counting).

finally constructed by creating a histogram of the contents of all of the memory channels (Figure 11.13).

11.8 Chemiluminescence

Chemiluminescence is a particular form of phosphorescence. The excited molecules, which leads to phosphorescence, are produced during a chemical reaction. In the example of Figure 11.14, fluorine acts as a strong oxidizing agent when transforming the dimethyl sulfide. A portion of the energy liberated by this reaction is emitted in the form of light.

■ Luminol and diphenyl oxalate are two compounds very frequently used in a variety of classic applications from non-electric emergency lighting to the hoops, necklaces and light batons sold at fairgrounds. The reactions that causes emission are all oxidations by hydrogen peroxide.

These reactions make available numerous measurements of great sensitivity. If the use of luminol to measure ferrous iron or hydrogen peroxide is anecdotal,

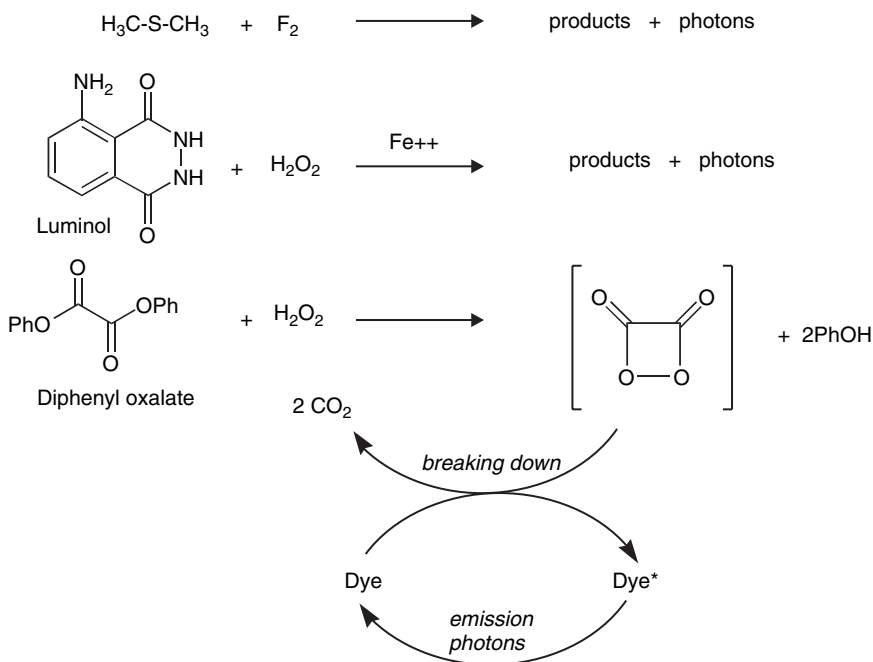


Figure 11.14 Examples of chemiluminescence reactions. The nature of the reaction products is sometimes not well known. Luminol emits an intense ‘electric blue’ light while the diphenyl oxalate, depending upon the colouring agent used, leads to the emission of a great variety of colours.

there exists a certain number of significant applications for chemiluminescence in chemical analysis, such as those which make use of ozone as an oxidizing agent.

This strong oxidizing agent enables the measurement of nitrogen monoxide (oxidized to nitrogen dioxide) with the aid of an automatic instrument that includes an ozone generator (Figure 11.15). The field of application for this measurement is broader than it might seem, ranging from studies of polluted atmospheres to the measurement of the total quantity of nitrogen in an organic sample – on condition that the nitrogen has undergone a previous transformation by combustion to nitrogen dioxide – as the quantitative transformation of nitrogen dioxide to nitrogen monoxide provides the basis for the measurement.

Inversely, ozone can in its turn be measured by chemiluminescence in the presence of ethylene (Figure 11.15).

The same principle can be applied to measuring sulfur first transformed to sulfur dioxide (SO_2) by combustion in the presence of oxygen (Figure 11.16), then reduced to hydrogen sulfide (H_2S) before being finally re-oxidized by ozone (with chemiluminescence).

In gas chromatography some detectors, selective for the elements S and N, use this sequence of reactions (Figure 11.17).

Luminol, whose chemiluminescence in the presence of hydrogen peroxide, can readily be catalysed by horseradish peroxidase (HRP), has found an application in the calibration tests for enzyme-linked immunosorbent assay (cf. Section 17.7). The intensity of the emission, is stable during a few minutes, what makes this method extremely sensitive over an extended range of concentrations, which is unusual for this kind of test.

Chemiluminescence is used in enzymology. With a specific enzyme, some specially prepared reagents liberate unstable intermediates whose decomposition

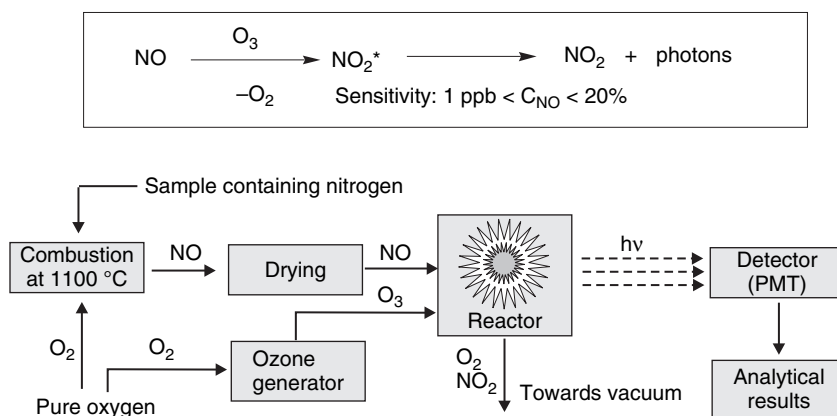


Figure 11.15 *Functioning principle of an analyser based upon nitrogen monoxide luminescence.* This apparatus is able to find all compounds containing nitrogen by a specific emission of light. Installed at the outlet of a GC column, the instrument becomes a selective detector.

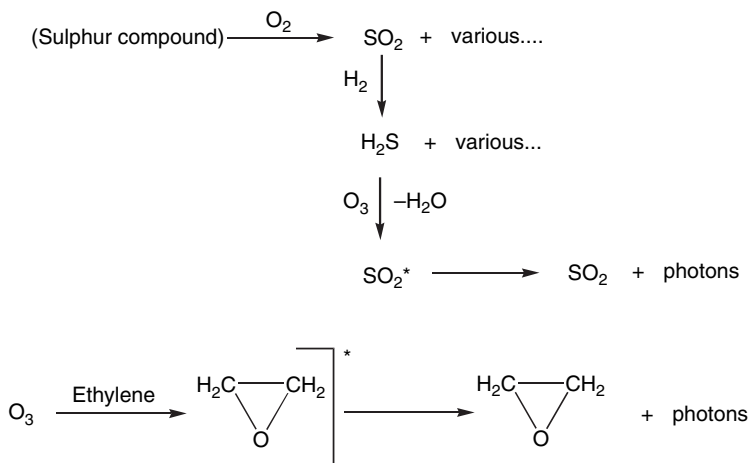


Figure 11.16 *Reactions of chemiluminescence using ozone.* The scheme above summarizes the principle of transformation of a sulfur compound oxidized to sulfur dioxide in the excited state by ozone. Below, the final reaction can be applied to the measurement of ozone just as readily as for ethylene.

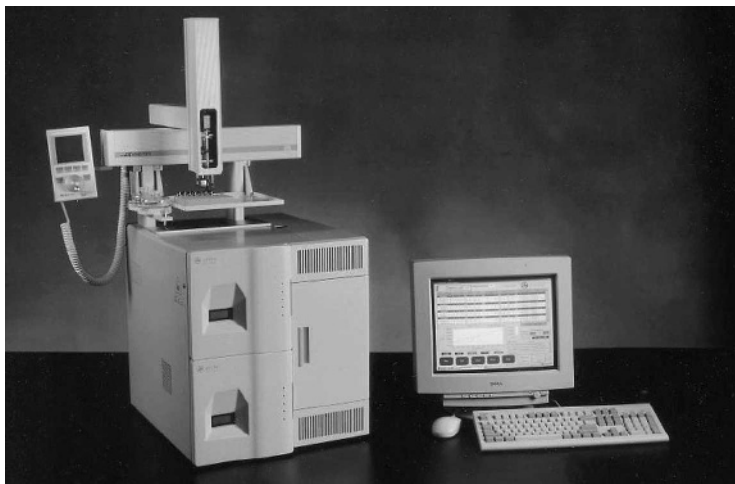


Figure 11.17 *Analyser of sulfur and nitrogen.* Model 9000NS reproduced courtesy of Antek. This instrument combines two luminescence techniques: nitrogen is measured by chemiluminescence of NO (see Figure 11.15) while the sulfur is measured by fluorescence of SO_2 (UV excitation).

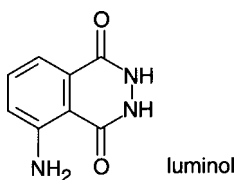
is accompanied by the emission of light. The alkaline phosphatases or the beta-galactosidases are quantified with precision by this procedure.

Problems

- 11.1 Calculate the position of the radiation of Raman diffusion for a monochromator of excitation regulated to 400 nm; the solvent used is cyclohexane. (Recall that for cyclohexane, the Raman diffusion corresponds to a radiation displaced by 2880 cm^{-1} with respect to the wavelength of excitation.)
- 11.2 A test for the sensitivity of a fluorometer is to measure the intensity of fluorescence of the Raman diffusion peak of a cell filled with water and with an optical pathlength of 1 cm. If the wavelength of excitation is regulated to 250 nm, at which wavelength must the measurement be made (the Raman displacement of water is 3380 cm^{-1})?
- 11.3 An unknown aqueous solution of 9-aminoacridine in water leads to an intensity of fluorescence which, measured at 456 nm, is of 60% with respect to an external reference. A standard solution of this compound, the concentration of which is 0.1 ppm in the same solvent, leads to a fluorescence of 40%, under the same conditions. Water alone, equally under the conditions of the experiment, presents a negligible fluorescence.
1. Calculate the ppb concentration of 9-aminoacridine in the unknown sample.
 2. For quantitative analysis, how can one avoid confusing the Raman diffusion and the fluorescence of the compound?
- 11.4 3,4-benzopyrene is a dangerous aromatic hydrocarbon frequently present in polluted air, measurable in a solution of dilute sulphuric acid, by fluorimetry. The excitation wavelength is 520 nm and the measurement taken at 548 nm. 10L of contaminated air is bubbled in 10 mL of sulphuric acid. The fluorescence of 1 mL of this solution measured at 548 nm is 33.33 (arbitrary units). Two standard solutions, one containing 0.75 mg and the other 1.25 mg of 3,4-benzopyrene per mL of the same diluted sulphuric acid, led for extractions of 1 mL to values of 24.5 and 38.6 (same unit scale). A sample not containing the 3,4-benzopyrene was recorded as 3.5 (same unit scale) by the fluorometer under the same conditions.

1. Explain why benzopyrene is fluorescent.
 2. Calculate the mass of 3,4-benzopyrene per litre of air.
 3. Express the result of this measurement in ppm.
- 11.5 Ferrous iron catalyses the oxidation of luminol (see below) by hydrogen peroxide. The intensity of the chemiluminescence which follows increases linearly with the concentration of Fe(II) as it rises from 10^{-10} M to 10^{-8} M. To measure a solution of unknown content in Fe(II), 2 mL of the solution was extracted and then introduced to 1 mL of water, followed by the addition of 2 mL of hydrogen peroxide and finally 1 mL of an alkali solution of luminol. The luminescence signal is emitted and integrated over a period of 10 seconds giving a value of 16.1 (arbitrary units). In a second attempt, 2 mL of the unknown Fe(II) solution is again extracted and on this occasion it was added to 1 mL of a solution 5.15×10^{-5} M in Fe(II) and the same quantities of hydrogen peroxide and luminol were introduced as before. The signal, integrated over 10 seconds, was measured as 29.6.

Calculate the molar concentration of Fe(II) in the original solution.



- 11.6 For the determination of the zinc concentration in an unknown solution A, by the method of standard addition, a standard solution B is prepared whose pure zinc chloride concentration is 0.1 mmol/L ($M = 136.2$ g/mol, $Zn = 65.38$). 5.0 mL aliquots of the unknown solution A were introduced into four separatory funnels. 4.0 mL, 8.0 mL and 12.0 mL portions of the standard solution B were added to three funnels. After stirring and extracting (three times), with 5.0 mL of tetrachloromethane containing an excess of 8-hydroxyquinoline, the extracts were diluted to 25 mL. The following fluorescence readings were obtained:

mL of the solution B added	Fluorescence reading
0	7.65
4	13.95
8	19.6
12	25.8

1. Explain why the extraction with CCl_4 is performed in the presence of hydroxyquinoline.
2. From the data in the table and by the method of least squares derive an equation for the calibration curve.
3. Calculate the ppm concentration of zinc in the unknown solution A.

11.7 Fluorimetry is the method selected to measure the concentration of quinine in a commercially available drink. The excitation wavelength is 350 nm and the wavelength for taking the measurements is 450 nm. From solution A, of quinine, at 0.1 mg/L, a series of standards is prepared to establish a calibration curve.

Solution	Volume of A	Fluorescence (arb. units)	H_2SO_4 0.05 M
Standard 1	20	182	0
Standard 2	16	138.8	4
Standard 3	12	109.2	8
Standard 4	8	75.8	12
Standard 5	4	39.5	16
Blank	0	0	20

An extraction of 0.1 mL is taken from the original solution which is then diluted to 100 mL with H_2SO_4 , 0.05 M. The value for the signal of this solution is 113.

1. Using least squares, equation from these data calculate the calibration.
2. What is the concentration weight/volume (w/v) of quinine (g/L), and in ppm?
3. Why in quantitative analysis is it preferred to take measurements using a fluorometer rather than with a spectrofluorometer?

12

X-ray fluorescence spectrometry

X-ray fluorescence is an atomic spectral property, universally recognized as a very accurate method currently exploited under this same name to provide qualitative and quantitative information on the elemental composition of all types of samples. The principle of operation involves irradiating the sample either with an X-ray beam or by bombardment with particles, such as electrons having sufficient energy, in order to observe the resulting X-ray fluorescence (XRF) emitted by the sample. The universal character of this phenomenon as well as the possibility to rapidly examine samples, most often without preparation, explains largely the success of this non-destructive analytical method. The technique encompasses a wide variety of instruments, ranging in size from portable analysers for instant analyses to high-resolution spectrometers, including X-ray probes fitted to scanning electron microscopes to make specified analyses or a mapping of the elements present.

12.1 Basic principles

When a sample, used as the target, is irradiated with a source of photons or bombarded with particles of high energy (between 5 and 100 keV), a X-ray fluorescence is most often observed (Figure 12.1). The spectrum of this photoluminescence is made up of radiations with wavelengths and intensities that are characteristic to the atoms present in the sample.

The modes of excitation which can provoke X-ray fluorescence are various: photons or particles such fast electrons, protons, α radiation. Whatever the method chosen, the X-ray emission yields nevertheless an identical spectrum.

The emission spectrum depends very slightly on the chemical combination or chemical state of the elements in the sample. For this reason, this non-destructive mode of analysis can be used for all elements starting from boron ($Z = 5$) to uranium ($Z = 92$) in solid or liquid homogeneous samples, without, theoretically, any fastidious preparation.

Although semi-quantitative analysis does not present any major difficulty, quantitative analysis is not quite simple, because a significant part of the emitted radiation from the outer surface of the sample is re-absorbed before being able

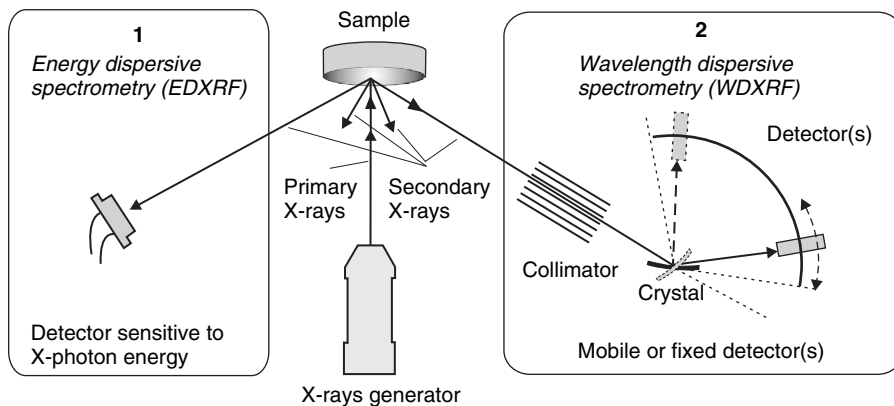


Figure 12.1 *X-ray fluorescence.* The sample when excited by a primary source of X-rays emits a fluorescence radiation which can lead to two representation of spectra depending on the detector: (1) Energy dispersive spectrum (ED-XRF) obtained by means of a cooled diode that gives a signal according to the energy of each incident photon: (2) Wavelength dispersive spectrum (WD-XRF) obtained through use of a rotating crystal functioning as a grating. This goniometric assembly contains one or several mobile detector(s). However, since energy and wavelength are linked (expression 12.2), the spectra are presented in units of energy (eV), whatever the mode of detection.

to escape from the sample. So far the influence of the matrix is clearly taken into account, especially when the sample is not homogeneous, the results can be as precise as those obtained by atomic absorption or emission.

In general, X-ray fluorescence spectrometers consist of a source of excitation radiation, a radiation detector to detect the stimulated radiation from the sample and a display of the spectral output. Since each element has a different and identifiable X-ray signature, the presence and concentration of the element(s) within the sample can be determined.

12.2 The X-ray fluorescence spectrum

X-ray fluorescence of an isolated atom results from a two-step process.

The first step is the *photo-ionization of the atom*. The energy of the incident photon is transferred to an innermost electron (a K electron) which results in the ejection of this electron of the atom, named a *photoelectron*, assuming that the photon has sufficient energy. This *photoelectric effect* leads to an ionized atom as a consequence of the missing electron (Figure 12.2).

- The excitation energy produced is sometimes transferred to one of the outer electrons, causing it to be ejected from the atom. This is an 'Auger electron'. The energy of each photoelectron is equal to the difference between the energy of the incident X-ray photon and that of the energy level initially occupied by the ejected

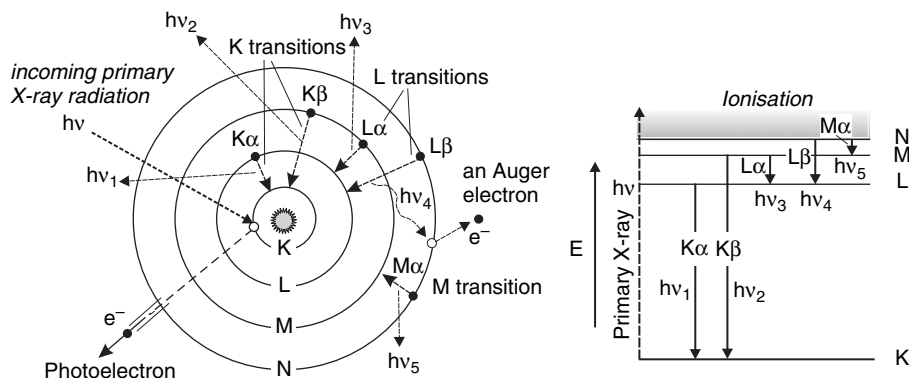


Figure 12.2 Schematic of the X-ray fluorescence process. For this classic image of a medium-size atom are displayed different possible electronic reorganizations when an electron in the K shell is ejected from the atom by an external primary excitation X-ray, creating a vacancy. The different K, L and M transitions are not significantly affected by the nature of the chemical combination in which the atom is found. In this way the sulphur line $K\alpha_1$ changes from 0.5348 nm for S^{6+} to 0.5350 nm for S^0 . This difference of around 1 eV is comparable to the natural linewidth for X-ray radiation.

electron. The photoelectron energy spectrum is the basis of a method called ESCA (electron spectroscopy for chemical analysis).

The second step is the *stabilization of the ionized atom*. It corresponds to the re-emission of all, or part, of the energy acquired during excitation. Almost instantaneous (in 10^{-16} s), an electron from an outer orbit of the atom ‘jumps in’ to occupy the vacancy. Since outer shell electrons are more energetic than inner shell electrons, the relocated electron has an excess of energy that is expended as an X-ray fluorescence photon. In this way, the atom returns to its ground state very quickly.

Cascade electronic rearrangements are observed for heavy atoms, in contrast to lighter elements whose electrons are distributed over a smaller number of ground states.

This reorganization produces photons of fluorescence. If E_1 is the energy of the initial electron and E_2 the energy of the electron which comes to fill the created hole, a radiative transition (‘a *fluorescent photon*’) will occur, with a probability between 0 and 1, and characterized by a frequency ν , such that:

$$h\nu = |E_2 - E_1| \quad (12.1)$$

The interpretation given above is simplified since fluorescence is not the only process by which the atom can lose its excess energy. When a primary X-ray strikes a sample, it can be absorbed or scattered through the material. Other phenomena occur such as Rayleigh scattering (elastic scattering) and the Compton effect (inelastic scattering with release of Compton electrons).

Each atom, from $Z = 3$, leads to a collection of specific radiation, obeying the quantum selection rules of its electrons ($\Delta n > 0$, $\Delta l = \pm 1$). Hydrogen and helium do not have X-ray fluorescence spectra since they do not possess an electron at level L. From beryllium to fluorine a single transition of type $K\alpha$ is noted. Then as the atoms become heavier the number of possible transitions increases (75 for mercury) though the probability of some of them are very small. Fortunately, only a few intense transitions are required to characterize an element (five or six at most).

For all elements, fluorescence appears in the broad energy range from 40 eV to more than 100 keV (wavelengths from 31 to 0.012 nm, as calculated from expression 12.2).

The precise designation of the possible electronic transitions depends upon the orbital energy levels of the atom under study, for which a simplified nomenclature due to Siegbahn is used. For example, for the element iron, $FeK\beta_2$ refers to a transition corresponding to the transfer of an electron to the level K, coming from the neighbouring level M; β indicates the transfer M-K and 2 the intensity relative to the strongest transition in the series (1 being more intense than 2). The $K\beta$ transitions are approximately six times less likely to occur (thus less intense) than the corresponding $K\alpha$ transitions (α marking the shortest 'distance'). For the levels multiple L and M, the notation according to Siegbahn is not always sufficiently explicit.

Taking account of the expression $E = hc/\lambda$ linking the energy of a photon to its wavelength λ , the radiation emitted can be equally characterized by either its wavelength λ (nm or angströms) or by its energy E (eV or keV). The two most commonly used expressions of conversion are:

$$\lambda_{(\text{nm})} = \frac{1240}{E_{(\text{eV})}} \quad \text{or} \quad \lambda_{(\text{nm})} = \frac{1.24}{E_{(\text{keV})}} \quad \text{or} \quad \lambda_{\text{\AA}} = \frac{12.4}{E_{(\text{keV})}} \quad (12.2)$$

12.3 Excitation modes of elements in X-ray fluorescence

To induce X-ray fluorescence of elements in a sample, a source of photons or particles of sufficient energy is required. Generally these sources are produced by X-ray tubes of variable power or, for portable instruments, by radioisotopic sources. If the term of X-ray fluorescence is considered in the broadest sense of X-ray emission, other excitation processes employing particles (e^- , α) can be used.

12.3.1 X-ray generators

In an enclosed space maintained under vacuum a beam of electrons, accelerated through a PD of up to 100 kV, hits a target serving as the anode (called *anticathode*),

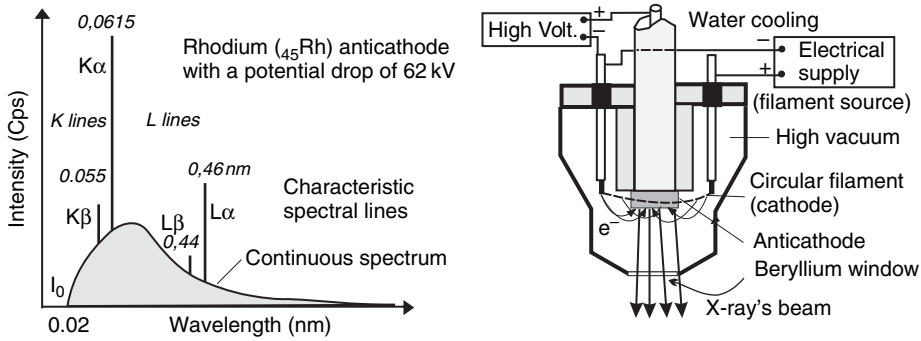


Figure 12.3 X-ray emission spectrum produced by impact of electrons on a target anode and details of an X-ray tube. The line spectrum from the anode can be observed as a superposition on the continuous part of the radiation, which depends upon the potential difference applied. A continuum spectrum is needed for applications requiring high penetration power, such as radiology. These lines after being isolated by means of filters, are used as monochromatic sources. Water cooling is compulsory if the X-ray tube operates at high power (1–4 kW), while not necessary for tubes of routine apparatus generating a few watts. To make monochromatic sources (without continuum spectrum), an adapted monochromator or a tube of this type is used to excite a second target of which the fluorescence relays the first, this time without *bremstrahlung* radiation.

which is made of a metal with an atomic number between 25 and 75. This becomes the source of *primary* X-rays. The emission corresponds to a continuum spectrum (white *bremstrahlung* radiation), due to the slowing down of electrons in the target, and of the very intense fluorescence lines whose wavelengths are characteristic of the metal that constitutes the anode (Figure 12.3). The wavelength end point λ_0 of this spectrum, which depends upon the PD, can be calculated by expression 12.2. In order to have high-energy spectral lines, useful for certain applications, metals with a high atomic number are chosen for the X-ray tube target: rhodium ($K\alpha$ line at $\lambda = 0.061$ nm; $E = 20.3$ keV), tungsten ($K\alpha$ line at $\lambda = 0.021$ nm; $E = 59$ keV), gadolinium, etc. Nowadays, X-ray detectors are extremely sensitive and consequently the power demanded from the generators tends to be reduced.

■ An X-ray generator itself constitutes an application of X-ray fluorescence and it is not surprising therefore that in the past spectrometers were conceived using the sample as the anticathode, on condition that it conducts electric current. The power required to induce fluorescence through a mechanism involving electrons is lower than that required by the impact of photons.

For portable instruments (Figure 12.4), miniature X-ray generators of low power (<500 mW) have been developed. The required electrons that produce X-rays in a target material (Cu) are obtained either by impact laser or from a pyroelectric crystal.



Figure 12.4 *Miniature size X-ray generator and detector.* These probes are used with portable apparatus though their control modules or power units are nevertheless more bulky (reproduced courtesy of Amptek Inc.).

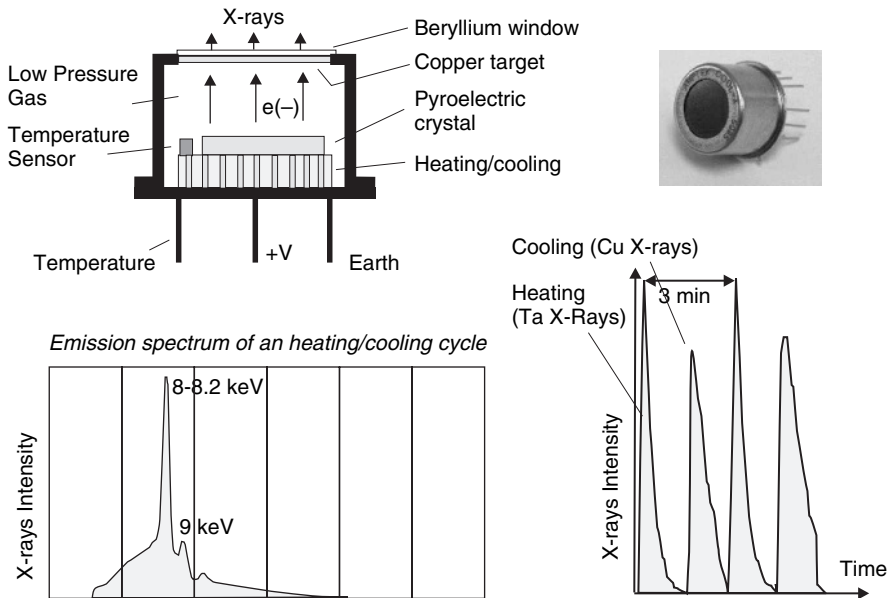


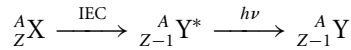
Figure 12.5 *Low-power X-ray generator.* Reserved principally for portable instruments because of their smaller volume (diam. 15 mm). The production of Cu X-rays on the diagram corresponds to the cooling time. The heating/cooling cycle is about 3 min. When the crystal temperature reaches its low point, the heating phase starts again. The above are characteristics of the model Amptek COOL-X.

The generator taken as an example (Figure 12.5) uses a pyroelectric crystal (tantalum) oriented in a fashion so that its top surface depolarizes reversibly when heated. In this way, when the temperature increases the uppermost face gets positively charged and attracts electrons from the ionized gas in the surroundings. White

radiation appears (*bremsstrahlung* X-rays) as well as fluorescent lines of tantalum (L α of ^{73}La). On cooling, the upper face of the crystal becomes negative. The electrons then are accelerated towards a copper target, which is at the ground potential. At this part of the cycle, the radiation contains the spectral lines of this element as well as *bremsstrahlung* X-rays. The cycle time can be varied from 2 to 5 min.

12.3.2 Radio-isotope sources of X-rays

Besides the generators described above there are X-ray sources based on radioactive materials to provide the excitation of the sample. The advantage of using these materials is that an isotope can be selected to provide a mono-energetic beam of radiation that is optimized for the specific application. One method consists to select a radionuclide that is transformed by internal electron capture (IEC). This mode of decomposition corresponds to the transition of one level-K electron into the nucleus of the atom. For a nuclide X, the phenomenon is summarized as follows:



The missing electron is quickly replaced by an other electron coming from an outer shell accompanied with emission of a photon of X-ray fluorescence from the resulting nucleus (Figure 12.6).

There are several known radionuclides of this type, including ^{109}Cd , ^{57}Co , ^{55}Fe , which have sufficiently long periods, that they can be used as different energy sources (Table 12.1). The activity of these isotopic sources is generally of several mCi, generating a flux of 10^6 to 10^8 photons/s/steradian. Of small size they are usually reserved for portable instruments. The isotope or combination of isotopes used for any application is selected based on the ability of the selected isotope(s) to

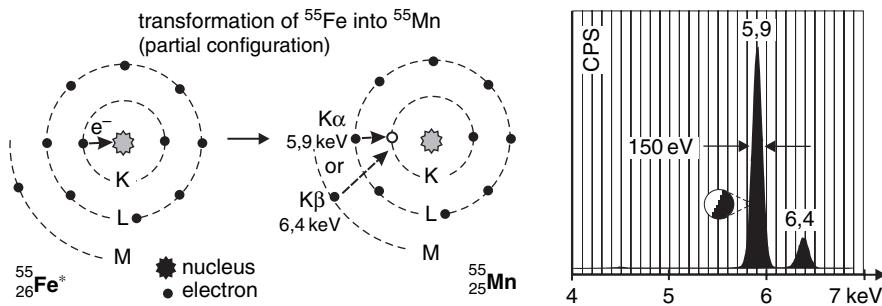


Figure 12.6 Radioactive source ^{55}Fe . Emission spectrum obtained by placing a ^{55}Fe source in the sample compartment of an energy dispersive spectrometer. The signal corresponds to the X-ray fluorescence of ^{55}Mn , that is the nucleus arising from ^{55}Fe decomposition. On this spectrum, the resolution at 5.9 keV, measured at mid-height (FWHM) is around 150 eV.

induce fluorescence of the element being measured. However, these sources require a permit for using, and can pose problems of transport, storage, or maintenance as they emit continuously unlike more classic generators.

A β^- emitting radionuclide may also be associated with a second element to be used as a target which functions as the anode of a conventional X-rays tube. For example, the association $^{147}\text{Pm}/\text{Al}$ ($\tau = 2.6$ years) emits a continuous X-ray decelerating radiation between 12 keV to 45 keV. The chief drawback of the method is the need for periodic source replacements to compensate for source decay.

Table 12.1 Several radio-isotopic X-ray sources

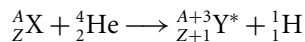
Transition	Half-life (years)	X-ray	λ (nm)	E (keV)
$^{55}\text{Fe} \rightarrow ^{55}\text{Mn}$	2.7	MnK α	0.21	5.9
$^{57}\text{Co} \rightarrow ^{57}\text{Fe}$	0.7	FeK α	0.19	6.4
$^{109}\text{Cd} \rightarrow ^{109}\text{Ag}$	1.3	AgK α	0.056	22.0

12.3.3 Other sources of excitation

α Emitters

To generate X-rays, α radionuclide such as americium ^{244}Am ($\tau = 430$ years) or curium ^{244}Cm can also be used. During the α collisions, internal electrons are ejected, in turn exciting the X-ray fluorescence of the target. The intensity is of several tens of mCi. This source also produces γ radiation of 60 keV.

■ The Martian probes launched in 1996 and during the summer of 2003 by the Jet Propulsion Laboratory were carrying small robotized vehicles, each one containing a APXS (alpha particle X-ray spectrometer) whose source contains ^{244}Cm (30 mCi). The modules are mounted upon a telescopic arm in order to gain a sufficient proximity to the rocks to be able to analyse. This radioisotope provokes a nuclear reaction of type ($\alpha\rho$) with light elements, causing the appearance of protons of energy characteristic to the element, while for heavier elements the X-ray fluorescence yields information upon the identity of the elements present. To improve the resolution of the detectors, which are semi-conductors (cf. section 12.4), the X-ray fluorescence spectra are recorded during the night, which provides the apparatus with a cooler and stable ambient temperature.



Fast electrons

X-ray fluorescence emission can be induced by electrons and therefore it is not surprising that chemical analysis can be performed using electron microscopes

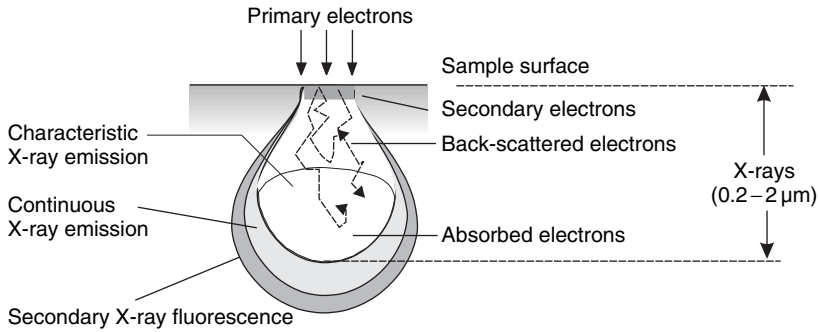


Figure 12.7 ‘Pear’-shaped interaction of the electron beam with the material. Different phenomena are produced in a small volume beneath the surface of the material. A complex emission results in which the origins of a collection of different radiation can be determined. If the energy is appreciably high, then the X-rays are formed at a greater depth within the material and consequently it will be more difficult to detect them.

(SEM). When the object is ‘illuminated’ by highly energetic electrons, which are necessary to generate SEM images, diverse interactions are produced within a small pear-shaped volume surrounding the impact zone (Figure 12.7). An X-ray emission characteristic of the atom composition of that zone being subjected to the electron bombardment is observed. In this case, the sample plays the role of the anode in an X-ray tube. This is why many electron microscopes are equipped with an accessory that can collect a fraction of the X-ray fluorescence to do the analysis of the observed sample. The energy of the electron beam is adjusted to between 20 to 30 keV, for which there is a compromise to allow the appearance of K or L spectral lines, leading to characterization of most elements. This quasi-instantaneous analysis, called *X-ray microanalysis*, relates to a sample volume of approximately $1 \mu\text{m}^3$.

12.4 Detection of X-rays

X-ray detectors are transducers that count individual photons. In a photoelectric interaction, the entire incident energy of the interacting photon is stored up in the detector (while in Compton scattering, only a portion of the incident energy is deposited). The detector works with greater accuracy, as the photon flux is weaker. The two most current types are:

- The *gas transducer* working as a *proportional counter*. Each X-ray photon provokes an ionization in a gas mixture (e.g. argon/methane) which gives a pulse proportional to its energy (Figure 12.8).

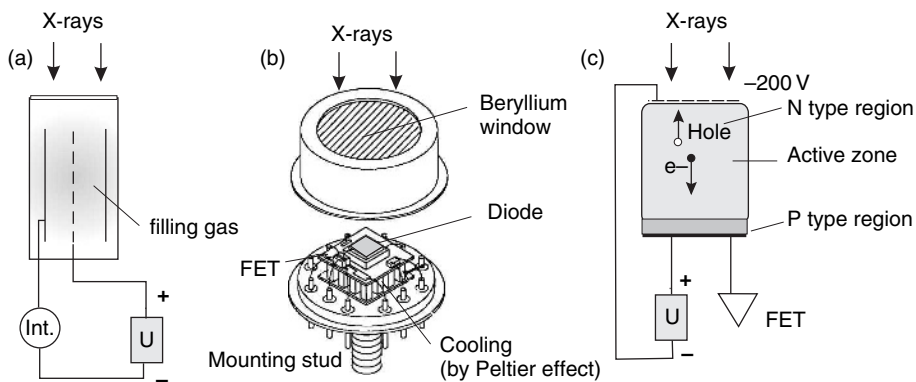


Figure 12.8 The two categories of detectors used for energy dispersive X-ray fluorescence spectrometry. (a) Proportional counter used in pulse mode; (b) Cooled Si/Li diode detector using Peltier effect (XR detector by Amptek Inc.); (c) Functioning principle of a scintillation detector containing a large size reverse polarized semi-conductor crystal. Each incident photon generates a variable number of electron-hole pairs. The very high quantum yield enables the use of low power primary sources of X-rays (a few watts or radio-isotopic sources).

- The *semi-conductor transducer (scintillation counter)*. Each X-ray photon increases the conductivity of the active zone (the junction) of a lithium-doped silicon diode (one electron for around 3.6 eV). The background noise is reduced if the sensor is maintained at low temperature (cooled by liquid nitrogen or a Peltier device). The entry surface is protected by a beryllium film of a few μm (transparent for $Z > 11$) (Figure 12.8). In one or other cases the impulse furnished by the detector allows to go back to the energy of the incident photon.

To classify the photons with better precision their amount is limited to approximately 10 000/s by reducing, if necessary, the source intensity. The instrument's data system should accurately identify the energy of the photons, which arrive as a pack. The resolution of these instruments is given by the width at half-height of the $K\alpha$ line of manganese, emitted by a ^{55}Fe radioactive source (Figure 12.6). Energy dispersive instruments have a resolution around 100 eV, a much greater value than the natural width of the spectral lines.

After having defined a range of acquisition from between 10 to 20 eV, the multi-channel analyser (with, for example, a few thousands channels) will count the pulses caused by photons received during the several minutes of the measurement. Each photon will be classified in an energy channel corresponding to an interval of several electron volts. The resulting spectrum is a histogram (Figure 12.6). These detectors undertake simultaneous analyses over the whole spectral range, which will be better if the acquisition of signals is made during a longer period of time t (the sensitivity increases as \sqrt{t}). Energy calibration is often performed using an external reference such as cobalt $K\alpha$ transition.

12.5 Different types of instruments

X-ray fluorescence spectrometers are classified into two categories depending how the spectrum is obtained: the classic procedure of analysing wavelengths ('WD-XRF') or the alternative methodology of analysing the energy of the photons emitted by the sample ('ED-XRF') (Figure 12.9). To the first category belongs a particular group of instruments fitted with filters (monochannel type) and designated for highly specific measurements.

12.5.1 Energy dispersive X-ray fluorescence instruments (EDXRF)

Energy dispersive instruments of small dimensions are reserved for both qualitative analysis and routine measurements (Figures 12.10 and 12.11). The spectrum is obtained by use of a detector installed quite close to the sample, which distinguishes the energy of each of the fluorescence photons captured. These instruments are equipped with either a small size and low power X-ray tube (around 10 W) or a radioactive source for field instruments.

12.5.2 Wavelength dispersive X-ray fluorescence (WDXRF) spectrometers

In this second class of spectrometers, known for their excellent spectral resolution, the X-ray radiation emitted by the sample passes through a collimator made of long

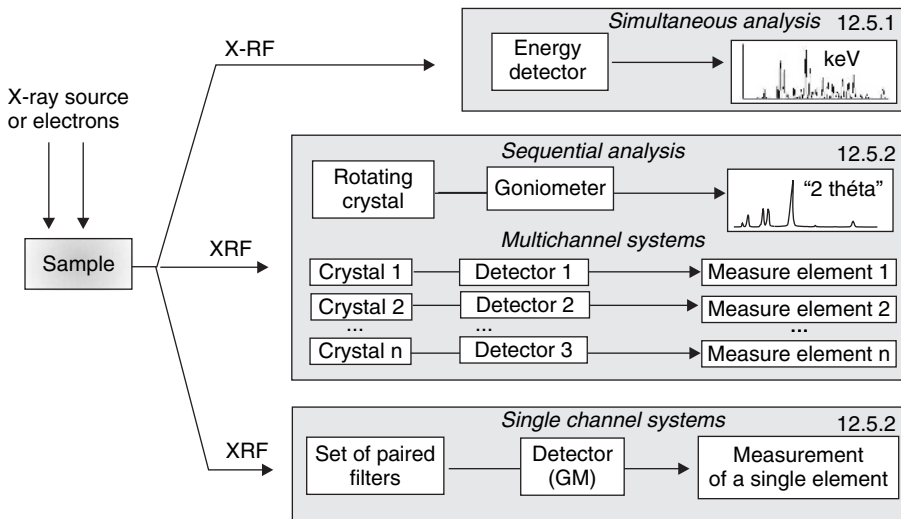


Figure 12.9 Several approaches for obtaining spectra or X-ray fluorescence. On the figure are indicated the paragraph numbers in the text.

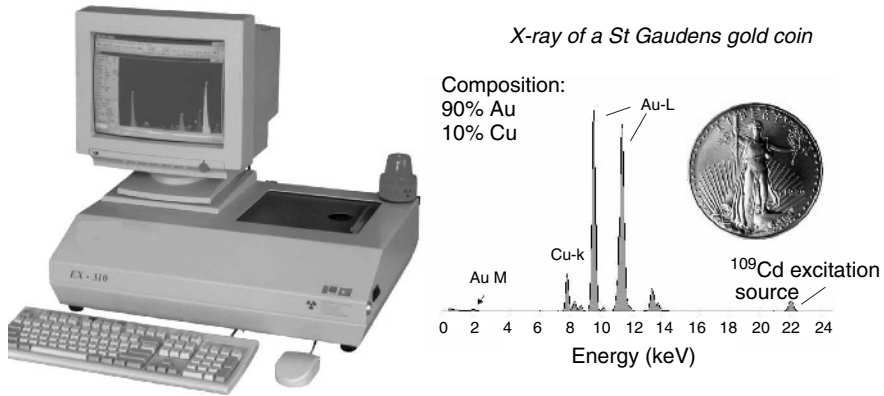


Figure 12.10 EDXRF. Spectrometer containing a low power X-ray tube, as an example of numerous models of this type (model EX-310S reproduced courtesy of Jordan Valley). A typical spectrum obtained with such an instrument (reproduced courtesy of Amptek Inc. USA).

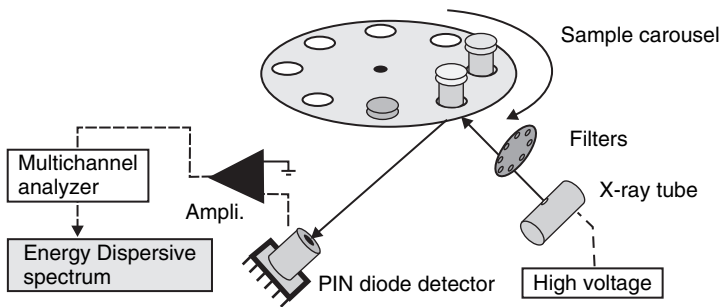


Figure 12.11 Organization of the different sections of an X-ray fluorescence energy dispersive instrument, taking as example the MiniPal by Philips Analytical.

metal wafers (Soller slits), before hitting a crystal which is cut in such a way that its constitutive atoms form planes parallel to the surface (Figures 12.1 and 12.13). These planes, separated between them by a distance d , behave like a stack of parallel mirrors when the angle of incidence θ is the same as the angle of observation of the reflected light (Figure 12.12). The combination of these mirrors has the same effect as a grating: the only radiation observed will be that for which the wavelength satisfies the Bragg relationship (n being a whole number called the order of diffraction).

$$n\lambda = 2d \sin \theta \tag{12.3}$$

For construction reasons, θ can vary from 5 to about 80°. A greater value of d leads to greater wavelengths, however the resolution of the instrument is linked to

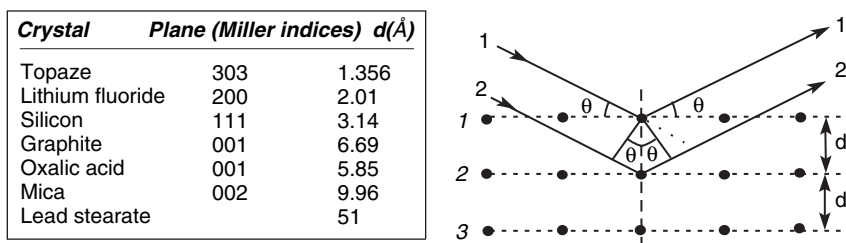


Figure 12.12 Reflecting crystals used in goniometers of wavelength dispersive spectrometers and the Bragg relationship. For the two beams 1 and 2 to be in phase, the difference in their optical path must be a multiple of λ . When this condition is met for the planes 1 and 2, it will be simultaneously for all of the other planes and the global effect will therefore be reinforced. This is the principle of constructive interference. Each crystal may be suitable for a range of wavelengths. The broader this range is searched, the greater the interreticular distance must be for the crystal chosen, though the smaller will be its angular dispersion.

the power of dispersion $d\theta/d\lambda$, which is calculated by differentiating expression 12.3 and which is inversely proportional to d :

$$\frac{d\theta}{d\lambda} = \frac{n}{2d \cos \theta} \quad (12.4)$$

These instruments can be classified as:

- *Sequential analysers.* These instruments are reserved for non-routine elemental measurements. The detector and the crystal undergo simultaneous and synchronized rotations to ensure the detection of θ and 2θ , to a precision of 1/1000 degree of an angle (Figure 12.13).
- *Fixed channel apparatus.* These instruments are able to undertake simultaneous measurements of several elements (Figure 12.13). The Bragg relationship shows that by choosing a crystal (therefore d) and by fixing the angle of detection (and so the value of θ), the radiation of wavelength λ , which will satisfy the Bragg conditions, can be isolated. Starting from this principle several tens of crystal-detector arrangements can be positioned around the same crystal, each coupling (d, θ) , defining a unique wavelength that is specific of a pre-defined element.

Low energy transitions for elements up to phosphorus ($Z = 15$), require operating conditions in a vacuum. The resolution can be as low as a few electronvolts. There exists models of this type which are adapted for scanning electron microscopes.

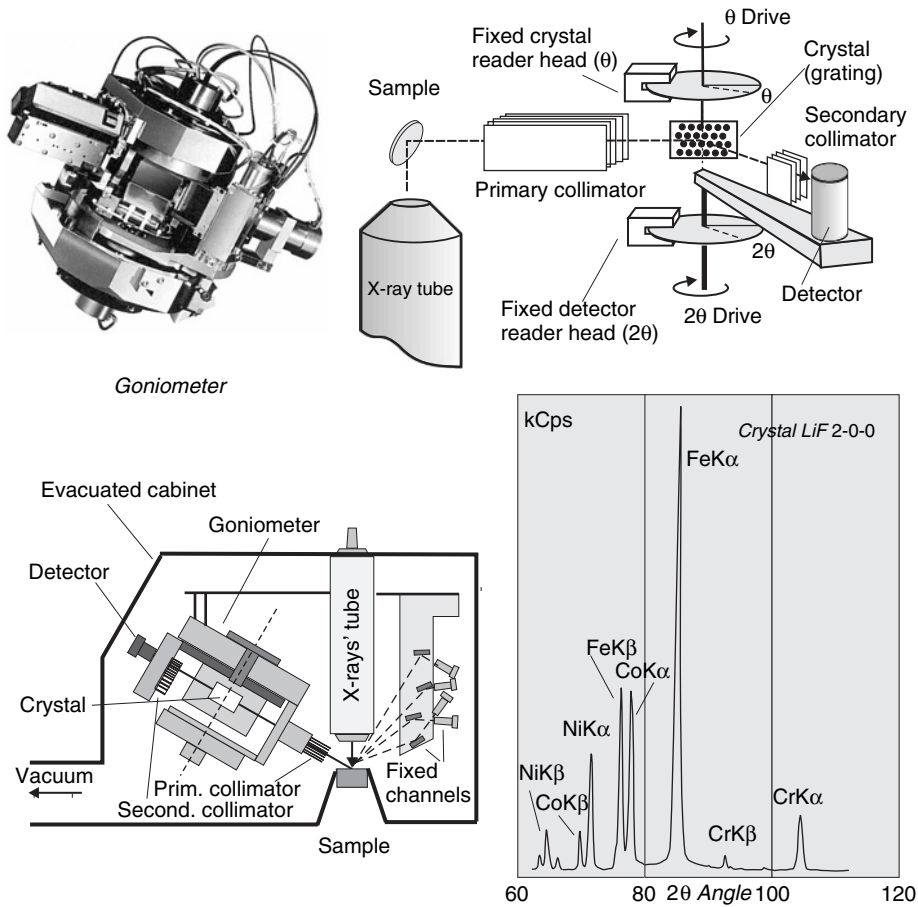


Figure 12.13 Schematics of two models of crystal-based sequential spectrometers. Above, a diagram inspired from a Siemens's SR300 instrument. The primary parallel metallic sheets collimates the X-ray beam emitted by a high power source. The secondary sheets collimator serves to eliminate all of the diffracted light not parallel with the direction 2θ in which the detector is found. Below is a diagram inspired by the model ARL 9800, uniting a goniometric assembly associated with fixed channels from a crystal/detector coupling, for measuring pre-defined elements; right, a typical spectrum of an alloy, recorded as a function of the angle 2θ in degrees as the abscissa and intensity in Cps as the ordinate (document Philips Analytical). The detectors are of the Geiger–Müller type.

12.5.3 Filter instruments – single channel system

These are robust instruments often installed on site for the continuous monitoring of industrial production sequence. In most of these cases, the object is to measure a single element present in a fabrication process, from a particular and typical transition, provided that line can be isolated from the rest of the spectrum.

One method used to isolate a X-ray line from unwanted background and noise, employs equilibrated filters. It consists of linking the concentration of interest to the difference between two measurements. The first is obtained by installing a *transmission filter* between the sample and the detector to isolate the characteristic radiation of the element wanted and the second by fitting an *absorption filter* which is opaque to this same radiation. This will enable, for example, to quantify the copper from its main spectral line by using two filters, one made of nickel and the other made of cobalt. The fluorescence originating from the filters themselves is a limiting factor in this method, which is reserved for routine measurements.

12.6 Sample preparation

Measurements will be repeatable based on the presumption that the sample is inherently uniform in analyte concentration. It is also important to think about the possibility of re-absorption of the emitted fluorescence (the optical *quenching* by the elements present).

Absorption by the matrix can provoke either an underestimation of the result by optical quenching, or an overestimation of the result when some of the X-ray fluorescence causes a secondary excitation of other elements present in the sample. For example, the presence of iron in aluminium provokes an increase in the fluorescence because radiation emanating from iron excites in its turn the fluorescence of aluminium.

Liquid samples do not require particular preparation prior to the analysis. A small volume of sample is placed in a type of vial whose bottom is replaced by a film of polypropylene or mylar (polyester) transparent to X-rays. The depth of absorption can be as much as 1 cm for hydrocarbons.

Solid samples and especially if the matrix is not known, need transformation prior to the measurement. In fact, the measured fluorescence of these materials only concerns a thickness of several micrometres beneath the surface. This thickness depends upon its composition and of the angle of incidence of the primary X-rays: this can be from between several angstroms (if the incident angle is acute), up to half a millimetre. All superficial heterogeneity can have important consequences and cause variations in the result. This is the principal reason for surface treatment prior to analysis.

The two techniques used for sample preparation of a solid are fusion and pellet formation. Fusion involves mixing a little of the sample, reduced to powder, with lithium tetraborate ($\text{Li}_2\text{B}_4\text{O}_7$), and different additives. The glass obtained by fusion in an electric oven by induction heating, called a pearl, constitutes a matrix of light elements and is therefore transparent to X-rays. Pellet formation, using a hydraulic press, is the alternative to the fusion technique. To ensure the cohesion of the pellet, a wax is added (an organic polymer formed from light elements).

12.7 X-ray absorption – X-ray densitometry

When a beam of X-rays passes through a material the result is an exponential attenuation of the primary beam. The different possible interaction processes, as fluorescence, are characterized by a probability of occurrence per unit path length in the absorber. The flux of transmitted primary photons I after having passed through an attenuator with a thickness x (cm) and a *linear attenuation coefficient* called $\mu(\text{cm}^{-1})$ for the wavelength considered, can be calculated with respect to its initial value I_0 and for an angle of penetration of 90° as follows:

$$I = I_0 \cdot \exp[-\mu x] \quad (12.5)$$

This relation which corresponds to the integrated form of the expression $dI = -\mu I dx$, is comparable to that discussed in colorimetry (Lambert–Beer law, see Chapter 9). The linear attenuation coefficient μ can be calculated for all material for which ρ , the density of the medium (g/cm^3) and the composition are known.

Individual attenuation coefficients are available from tables for each element. Next, the weighted mass attenuation coefficient $\mu_M(\text{cm}^2/\text{g})$ must be calculated for the material studied from its elemental composition. Finally, $\mu = \mu_M \rho$.

Figure 12.14 shows that a film of beryllium, such as those serving as windows for X-ray detectors, is opaque to the radiation emitted by the lighter elements ($E < 1 \text{ keV}$ for atomic numbers below 10). The linear attenuation coefficient of a material decreases as the wavelength is decreased. For lighter elements, measurements are made under an atmosphere of helium while for solids a vacuum is imposed.

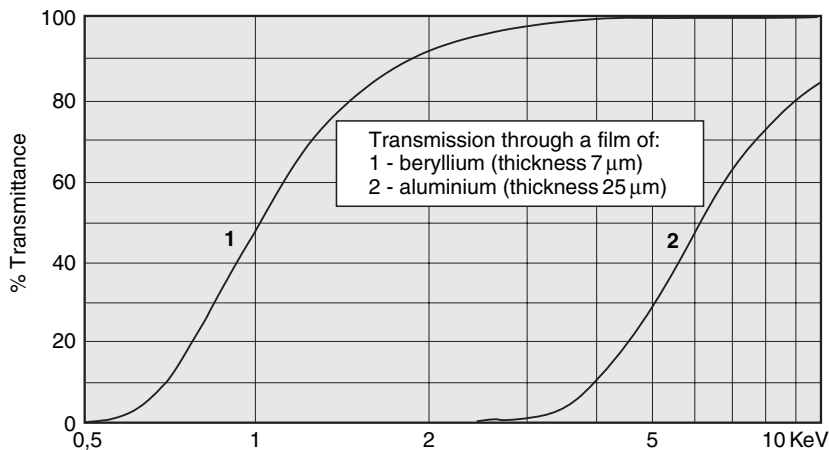


Figure 12.14 X-ray densitometry – examples of percentage transmittance for two films made of light elements. A $7\ \mu\text{m}$ film of beryllium is often used for making windows of energy detectors. For a radiation of 1 keV, (e.g. Na $K\alpha$) it is noteworthy that the attenuation achieved by this film is still of 50 per cent.

■ Expression 12.5 estimates that a household foil of aluminium film of thickness $12\ \mu\text{m}$ ($\rho = 2.7\text{g/cm}^3$) absorbs 57 per cent of the intensity of the transition $\text{TiK}\alpha$ ($\mu_{\text{M}} = 264\ \text{cm}^2/\text{g}$) but only 1 per cent for the transition $\text{AgK}\alpha$ ($\mu_{\text{M}} = 2.54\ \text{cm}^2/\text{g}$).

12.8 Quantitative analysis by X-ray fluorescence

The relationship between the weight concentration of the element to be analysed and the intensity measured from one of its characteristic spectral lines is a complex one. For trace analysis several mathematical models have been developed to correlate fluorescence to the atomic concentration. A series of corrections must be introduced to account for inter-element interactions, preferential excitation, self-absorption and the fluorescence yield (the heavier atoms relax by internal conversion without photon emission). All of these factors require the reference samples to be practically the same structure and atomic composition than the sample under investigation, for all of the elements present. It is mostly because of these reasons that quantitative analysis by X-ray fluorescence is difficult to obtain. When operating upon a solid sample, a perfectly clean surface is important, preferably polished, since the analysis concerns the composition immediately close to the surface.

This method is well adapted to quantitative analysis, where automatic identification of the spectral lines can be made by very sophisticated visual displays. Principal corrections (called ZAF) relate to atomic number (Z), nature of the isotope (A) and fluorescence (F). In semi-quantitative analysis, the software can give an approximate composition of the sample without the need for reference standards.

12.9 Applications of X-ray fluorescence

Initially, X-ray fluorescence was used in industries treating metals or alloys and in more general in the heavy industry (iron and steel industry, cements, ceramics, glassware). The method is non-destructive, requires little sample preparation beforehand and has a large dynamic range. For example, it is possible to measure two elements present in the same sample in which the concentrations are 50 per cent for one and a few ppm for the other. Progress in the detection of photons of high energy has improved the precision for analyses of heavy elements, which can be obtained from their K, and not from their L spectral lines which are situated in the region of the spectrum where background noise levels are too high.



Figure 12.15 *Portable field apparatus.* This instrument represents a category of energy dispersive spectrometers equipped with an X-ray generator but no radioactive source (reproduced courtesy of Niton, USA). Type of information that can be obtained.

With the convenience of current apparatus (Figure 12.15), the scope of application of this technique is considerably broad, either in research and development or as quality control in production. It extends from routine qualitative analyses to measurements that can attain the high level of precision of classic methods. Semi-quantitative analyses are possible, using highly efficient computer software.

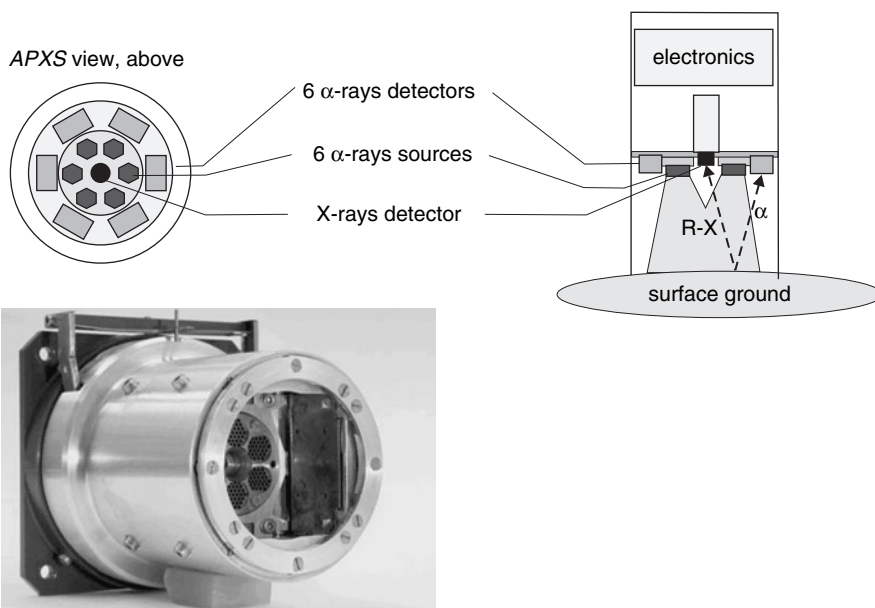


Figure 12.16 *Alpha particle X-ray spectrometer (APXS).* To determine the elemental composition of rocks and soils of Martian surface, the rovers, which landed in 2004 on Mars, carry a deployment device that could be in contact with the rocks. The sensor head contains six ^{244}Cm radioactive sources, six alpha detectors and one X-ray detector in the centre. The accumulation time is a few hours per sample analysed.

X-ray microanalysis, for which samples must ideally be electrically conductors, allows element mapping in heterogeneous samples, if observed with a scanning electron microscope (cf. Section 12.3.3).

The list would be very long if all of the analytical applications of X-ray fluorescence were recorded, photographic industry, paper, semi-conductors (impurities of silicon), petrochemicals (S, P, Cl). In geology, toxicology, environment (dust, combustion fumes, pollution), refuse and rejects (heavy elements such as As, Cr, Cd, or Pb), analysis of ultra light elements (nitrogen) and even space exploration (Figure 12.16). Finally, a current application is the use of X-ray fluorescence to diagnose concentrations of lead in the home (paints and wall coverings).

Problems

12.1 A solution is prepared with 8 g of potassium iodide and 92 g of water.

1. Calculate the mass absorption coefficient (μ_m) of this solution measured with the MoK α radiation of 17.4 keV. Consider that the solution (volume $\rho = 1.05$ kg/L) is an equilibrated mixture of these four elements (K, I, H and O).
2. What fraction of the MoK α line remains after having crossed 1 cm of this solution?

Element	μ_M (cm ² /g)	Atomic mass (g/mol)
K	16.2	39
I	36.3	127
O	1.2	16
H	0.4	1

12.2 Find the following classical conversion formulae:

$$\lambda_{\text{nm}} = \frac{1240}{E_{(\text{ev})}} \quad \lambda_{\text{\AA}} = \frac{12.4}{E_{(\text{kev})}}$$

Recall: $h = 6.6260 \times 10^{-34}$ J s; $c = 2.998 \times 10^8$ m/s; $1 \text{\AA} = 10^{-10}$ m; $1 \text{ eV} = 1.602 \times 10^{-19}$ J.

12.3 An X-ray tube with a tungsten target and which serves as the source in an X-ray wavelength dispersive fluorescence spectrometer is equipped

with a crystal of ethylenediamine tartrate. The plane of reflection in use corresponds to an inter-reticular distance of $d = 4.404 \text{ \AA}$.

1. Calculate the angle of deviation measured with respect to the direction of the incident radiation which collects the emission line $L\beta$ of bromine ($\lambda = 8.126 \text{ \AA}$) of a sample of sodium bromide (it may be considered that the observations recorded are of first order reflection).
2. Knowing that the wavelength of the tungsten line $K\alpha$ occurs at 0.209 \AA , calculate the minimum voltage necessary across the X-ray tube, to excite this line.

12.4 Consider a wavelength dispersive instrument provided with a goniometer comprising a rotating topaz crystal of an appropriate size.

1. Knowing that the crystal spacing of the monochromator (303) has an interplanar distance of 0.1356 nm , calculate the limits of the wavelengths within which it is possible to record the spectrum if the angle of incidence varies through 10° to 75° .
2. Convert these two values into keV.

12.5 Sulphur presents two lines: $K\alpha_1 = 5.37216 \text{ \AA}$ and $K\alpha_2 = 5.37496 \text{ \AA}$

1. What is the energy difference, expressed in eV, between $K\alpha_1$ and $K\alpha_2$?
2. If it is known that the width at the mid-height of these lines is 5 eV , what conclusion can be drawn?
3. If the position of the $K\alpha_2$ line increases by 0.002 \AA when passing from S^{6+} to S^0 , show that this has no impact upon the location of this photoelectron in energy terms.

12.6 Is it possible to protect oneself from X-rays by being wrapped in aluminium foil of thickness $12 \mu\text{m}$?

First calculate for the % transmission for the Ti $K\alpha$ line (4.51 keV , $\mu_m \text{ Al} = 264 \text{ cm}^2/\text{g}$), then for the Ag $K\alpha$ line (22 keV , $\mu_m \text{ Al} = 2.54 \text{ cm}^2/\text{g}$). The volumic mass of the aluminium is 2.66 g/cm^3 .

Consider the same question but, in place of the aluminium, a film of latex is employed $(C_5H_8)_n$ of the same thickness (volumic mass = 1). The final calculation will be made with the transition $K\alpha$ line of titanium ($\mu_m C = 25.6 \text{ cm}^2/\text{g}$, $\mu_m H = 0.43 \text{ cm}^2/\text{g}$).

- 12.7 1. Why does the table of X-ray lines only begin with the third element of the periodic table?
2. Why, when measuring the elements whose energy of emitted radiation is inferior to 3 keV, is it necessary to replace the air in the apparatus by helium?
- 12.8 When establishing the Bragg equation the difference is calculated between the optical path of two rays incident and reflected, making the same angle, α . Why is this?
- 12.9 For determining the % mass of Mn present in a rock, the element Ba is used as an internal reference. Two solid standard solutions in the form of pearls of borax yielded the following results:

No of solution	% Mn by mass	Ratio Mn/Ba
1	0.250	0.811
2	0.350	0.963
unknown sol.	?	0.886

Evaluate the % mass for Mn in the original solution

- 12.10 If a piece of nickel is suspended at a depth of 1 cm in an aqueous solution, what fraction of the intensity of the transition $K\alpha$, emitted when metal atoms are excited, will reach the surface? Mass attenuation coefficients for hydrogen and oxygen are H: $0.4 \text{ cm}^2/\text{g}$; O: $13.8 \text{ cm}^2/\text{g}$.
- 12.11 If the distance between the sample and the detector is 5 cm, calculate the % attenuation of a radiation possessing an energy of 2 keV produced by the presence of air over this distance. Take 80% nitrogen and 20% oxygen for the mass composition of air. The specific weight of air is 1.3 g/L. The mass attenuation coefficients of elemental nitrogen and oxygen are, for a radiation whose energy is 2 keV: $\mu_m = 494 \text{ cm}^2/\text{g}$ for N and $706 \text{ cm}^2/\text{g}$ for O.

13

Atomic absorption and flame emission spectroscopy

Atomic absorption spectroscopy (AAS) and flame emission spectroscopy (FES), also called flame photometry, are two analytical measurement methods relying on the spectroscopic processes of excitation and emission. Methods of quantitative analysis only, they are used to measure of around seventy elements (metal or non-metal). Many models of these instruments allow measurements to be conducted by these two techniques although their functioning principles are different. There exists a broad range of applications, as concentrations to the $\mu\text{g/L}$ (ppb) level can be accessed for certain elements.

13.1 The effect of temperature upon an element

The principle of atomic absorption and flame emission is best understood through an experiment performed by Kirchhoff more than a century ago in which he demonstrated that incandescent gases absorb at the same wavelengths as they are able to emit.

When the light of an electric arc (serving as a source of white light), is dispersed by a prism, a continuous spectrum is obtained (Figure 13.1, top). If this radiation source is replaced by a Bunsen burner onto which a few crystals of sodium chloride is sprinkled, then an emission spectrum of sodium is obtained which possesses the well-known yellow doublet located at 589 nm, among other lines (images of the entrance slit, Figure 13.1(middle) and Figure 13.2). This part of the experiment illustrates *flame emission*. Finally, if the two previous sources, electric arc and the flame of the Bunsen burner are placed in series along the same optical path a spectrum will be obtained which, in contrast to Figure 13.1(top), contains *dark lines* in place of the emission lines of sodium (Figure 13.1, bottom). This is the result of the presence in the flame of a large proportion of ground state sodium atoms that can absorb the same frequencies as the excited sodium atoms emit. This is a manifestation of *atomic absorption*.

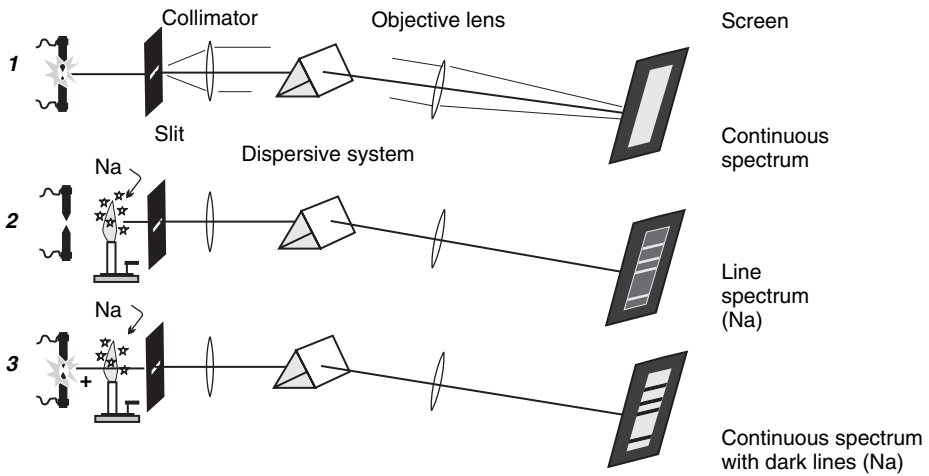


Figure 13.1 Kirchhoff's 'line reversal' experiment. The schematics for the optical set-up (collimator, objective), have been simplified for reasons of clarity. See text for explanations.

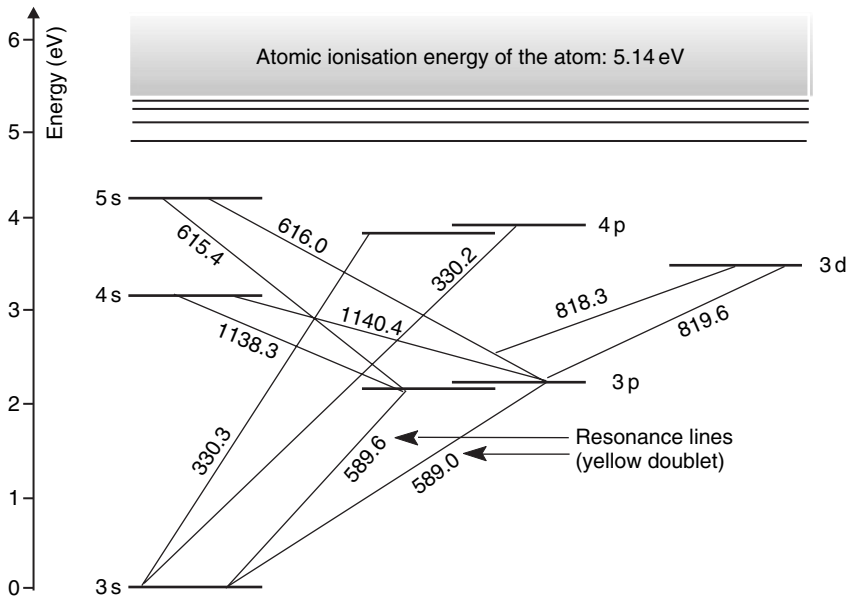


Figure 13.2 Several energy levels of the sodium atom. Simplified representation of the excited states in the sodium atom. Origins of the different emission lines, according the selection rules. The values are indicated in nanometres.

■ Diverse instruments exploiting the principle of this phenomenon are used to measure traces of mercury in a polluted atmosphere. A device has been designed that corresponds to a sort of colorimeter dedicated to this single element. The source is a mercury vapour lamp and the measuring cell is a transparent tube filled with the atmosphere to be monitored. If mercury vapours are present in the optical path, there will be absorption of the radiation emitted by the lamp, a consequence being a diminution of the light intensity in proportion to the mercury concentration.

This experiment illustrates the concept of potential energy states defined for all atoms by their electronic configuration. When an atom in the free state is brought to high temperature or is irradiated by a light source of the near UV/Vis region, then the probability of one of its outer shell electrons to be promoted from the ground state to an excited state is very high. This electron transfer corresponds to an absorption of energy. Conversely, when the atom returns spontaneously to its ground state, it can re-emit this excess of energy in the form of one or many photons. Thus, in the preceding experiments, the flame induces the most probable transitions in the sodium atom (Figure 13.2).

The Maxwell–Boltzmann distribution function permits a calculation of the effect of temperature on each electronic transition. By designating N_0 as the number of atoms in the fundamental state and N_e as those in the excited state, one obtains:

$$\frac{N_e}{N_0} = g \cdot \exp \left[-\frac{\Delta E}{kT} \right] \quad (13.1)$$

where T is absolute temperature in Kelvins, N_e/N_0 is the ratio of the statistical weights of the ground (0) and excited (e) populations of the element considered (whole number), g is a small integer number which depends of the quantum number of each element, ΔE is the energy difference (joules) between the ground (0) and excited (e) state populations concerned and k is the Boltzmann constant ($k = R/N = 1.38 \times 10^{-23}$ J/K).

If ΔE is expressed in eV instead of joules, expression 13.1 becomes:

$$\frac{N_e}{N_0} = g \cdot \exp \left[-11600 \frac{\Delta E}{T} \right] \quad (13.2)$$

Each atomic transition leads to an emission or an absorption of energy, which corresponds on the spectrum to a very narrow interval of wavelength. The uncertainty around the calculated value constitutes the *natural bandwidth of the spectral line*. The width is temperature dependent and can pass from 10^{-5} nm under ideal conditions, to about 0.002 nm at 3000 K. In reality, the line widths as observed through the monochromator of an instrument are much greater owing to the current technical limits of spectrometers.

13.2 Applications to modern instruments

To measure an element by one or other of these two methods, it must be in the form of free atoms. To this end, the sample is heated to a temperature of at least 2000 °C, in order to dissociate all chemical combinations in which the element under study is engaged, including the rest of the sample. This pyrolysis leads to the total concentration of the element without distinguishing the different chemical structures in which the element was possibly bound in the original sample (this is therefore opposite to a *speciation analysis*).

Two thermal devices co-exist: one consists of a burner fed by a combustible gaseous mixture, the other is a type of small tubular electric oven. In the first assembly, used for the majority of elements, an aqueous solution of the sample is nebulized and then introduced into the flame at a constant rate. In the second, the sample is deposited in a small graphite hollow rod open at both ends, where it is volatilized. This more expensive assembly has a greater sensitivity toward refractory elements (V, Mo, Zr). In both methods the optical path source/detector pass through the region containing a tiny cloud of atoms gas in the free state.

In AAS, as in FES, the measurement of light intensity is carried out at a wavelength specific to each element being analysed.

- In AAS, the concentration can be deduced from the measurement of light absorption by the atoms remaining in the ground state when they are irradiated by an appropriate source of excitation.
- In FES, conversely, the concentration can be deduced from the intensity of the radiation emitted by the fraction of atoms that have passed into excited states.

■ Flame emission spectroscopy is based upon the emission of photons by some elements when they are subjected to a temperature of the order of 2000 to 3000 °C. The technique, which is used solely for quantification, is distinguished from atomic emission. This more general term concerns another method of spectral analysis, both qualitative and quantitative, using thermal plasma sources to obtain much higher temperatures and holding a far more higher performance optical arrangement.

13.3 Atomic absorption versus flame emission

Current values of the different parameters of expressions 13.1 and 13.2 are shown in Table 13.1 for some elements. Examination of the data reveals that practically all of the atoms remain in their ground state when the energy difference ΔE is large and the temperature is low.

Therefore it would seem to be preferable, in all circumstances, to base the measurements upon atomic absorption instead of flame emission, as the absorption spectra are simpler than those of emission. However, the matrix in which the

Table 13.1 Ratio of N_e/N_0 for several elements at various temperatures

Element	λ (nm)	E (eV)	g	2000 K	3000 K	4000 K
Na	589	2.1	2	1.0×10^{-5}	6.0×10^{-4}	4.5×10^{-3}
Ca	423	2.93	3	1.2×10^{-7}	3.6×10^{-5}	6.1×10^{-4}
Cu	325	3.82	2	4.8×10^{-10}	7.3×10^{-7}	3.1×10^{-5}
Zn	214	5.79	3	7.3×10^{-15}	5.7×10^{-10}	1.5×10^{-7}

element is found can be the cause of interferences, chemical interactions, instability to the excited states and other phenomena that occur at high temperatures (Figure 13.3) and which render absorbance measurements difficult.

Yet, with the most modern detectors containing a photomultiplier, reliable measurements can be obtained as long as the ratio N_e/N_0 is greater than 10^{-7} . The experience reveals that flame emission is in fact preferable only for five or six elements. This is why the alkaline earths, elements giving coloured flames, are easily measured by emission (Table 13.1).

The value of the ratio N_e/N_0 does not imply that all of the excited atoms N_e return to their initial state as they emit photons. When they fall back down, they can lose their excess energy by other means. Otherwise, the more the temperature increases, the more complex the emission spectrum becomes, mainly due to the emergence of lines originating from ionized atoms (Figure 13.3). To study these complex spectra instruments that possess optics of very high quality are required (cf. Chapter 14).

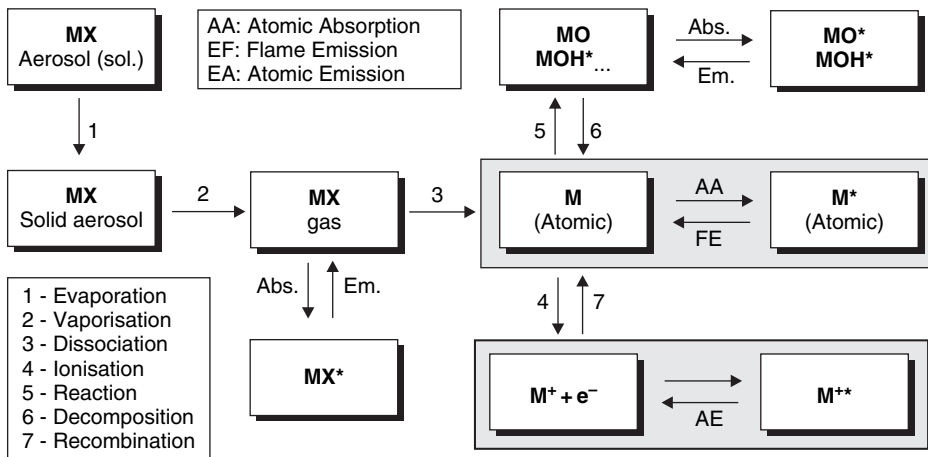


Figure 13.3 Summary of the possible evolution of an aerosol in a flame. Atomic absorption and emission are represented by the shaded areas of the diagram.

13.4 Measurements by AAS or by FES

The quantification of elements by these two methods implies that a relation exists between the concentration and the intensity of the corresponding light absorption or emission. They make use of protocols which comprise a calibration curve from standard solutions of the analyte.

13.4.1 Measurements by atomic absorption

The absorbance of the element in the flame depends upon the number of ground state atoms N_0 remaining on the optical path. Measurements are made by comparing the unknown to the standard solutions.

$$A = k \cdot C \quad (13.3)$$

where A is absorbance, C is concentration of element and k is a specific coefficient to each element at the given wavelength.

The method relies on the Lambert–Beer relationship, but the molar coefficient of absorption ε is not calculated in this case. The instrument yields the absorbance by ratioing the transmitted intensities in the absence and then in the presence of sample. Linearity is only observed for weak concentrations (typically below 3 ppm), or for solutions in which the matrix effect is negligible. The methods, comparables to those used in molecular absorption spectrometry, involve classical protocols *via* a calibration curve. If the matrix is complex, methods using standard additions are used to improve the calibration curve (Figure 13.4).

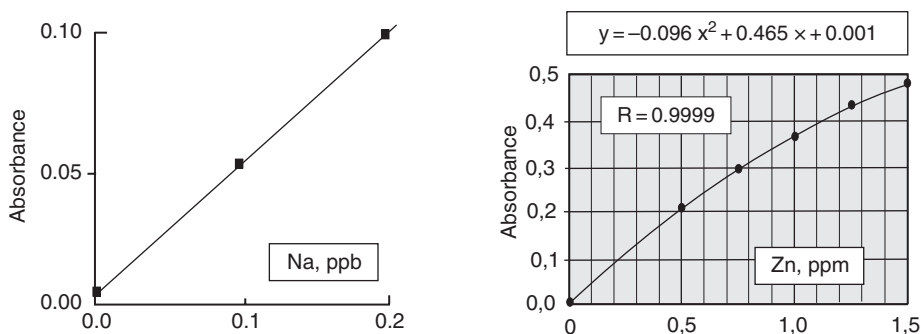


Figure 13.4 Examples of calibration graphs in AAS. Left, a straight calibration line at sub-ppb concentrations obtained with an instrument equipped with a Zeeman effect device (see section 13.7) for the quantification of sodium. Right, a quadratic curve for the measurement for zinc at concentrations in the ppm range with a burner type instrument. This second graph reveals that when concentrations increase, the absorbance is no longer linear. The quantitative analysis software for AAS provides several types of calibration curves.

13.4.2 Measurements by FES

For a population of n excited atoms, the emitted light intensity I_e depends upon the number of atoms dn that return to the ground state during the interval of time dt : ($dn/dt = kn$). Since n is proportional to the concentration of the element in the hot zone of the instrument, the emitted light intensity I_e which varies as dn/dt , is itself proportional to the concentration:

$$I_e = k \cdot C \quad (13.4)$$

This expression is only valid for low concentrations in the absence of self-absorption or of ionization. As previously suggested, to conduct an analysis by flame emission, the response of the instrument requires a calibration with a series of standards.

13.5 Basic instrumentation for AAS

In its simplest form, an AAS resembles a single beam spectrophotometer. The optical scheme is illustrated in Figure 13.5 which shows a basic model. It contains four principal components: the light beam issuing from the *source* (1) passes through the *burner* (2) in which the element is brought to its atomic state before being focused upon the entrance slit of the *monochromator* (3) which selects a very narrow wavelength interval. The optical path ends at the entrance slit of the *detector* (4).

If no sample is present in the flame, the detector will receive all of the light intensity I_0 emitted by the source within this spectral interval selected by the

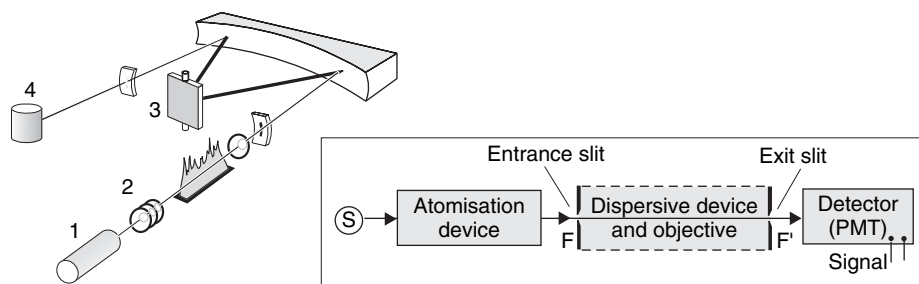


Figure 13.5 The diverse components of a single beam atomic absorption apparatus. Model IL 157(Thermo Jarrell Ash) constructed during the 1980s. 1, source (spectral lamp); 2, flame burner which provides the atomic aerosol; 3, monochromator grating; and 4, detector (photomultiplier). The source illuminates a slit situated at the entrance the dispersive system. The exit slit, is close to the detector window. It determines a narrow bandwidth of the spectrum, ($\Delta\lambda$ of 0.2 to 1 nm), which must not be confused with either the width of the exit slit or with the image of the entrance slit.

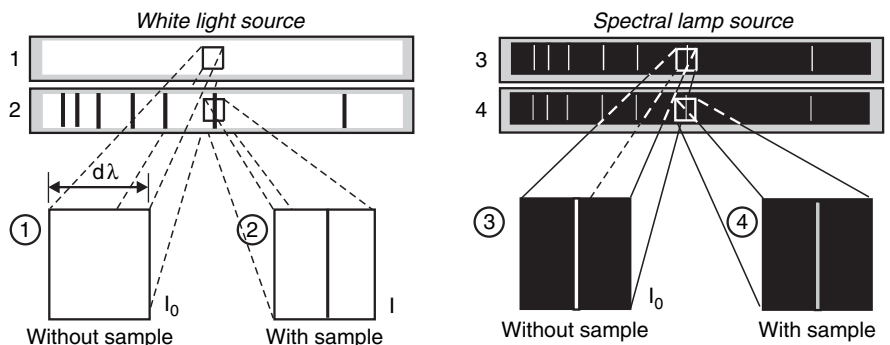


Figure 13.6 Comparison of transmitted intensities in AAS with a continuum light source (1 and 2), and with a lamp that emits spectral lines (3 and 4). The square region describes the wavelength interval seen by the PMT. The PMT signal is proportional to the white parts in the squares. In this way 'the resolution is in the source,' as expressed by Walsh, who is considered to be one of the pioneers of atomic absorption.

entrance slit of the dispersive system. Alternately, if the element is present, the detector receives a reduced intensity I (Figure 13.6).

If the source emits a *continuum of light*, the ratio I_0/I will always be close to 1 because the absorption lines are very fine (1×10^{-3} nm). Such a source would swamp the signal. The very narrow absorption band would appear as only a minute change against the broad incident radiation. However, if a source is chosen which emits only the wavelengths that the element to measure is able to absorb, the ratio I_0/I would become much smaller than 1. The second situation is preferable, for it is more efficient to measure a small change in light intensity against a dark background, since currently used photomultipliers are extremely sensitive.

13.5.1 Hollow cathode lamps (HCL) and electrodeless discharge lamps (EDL)

For the above reasons, atomic absorption instruments use two basic types of lamps. *Hollow cathode lamps* (HCL) are discharge lamps that contain a fill gas (argon or neon). Depending upon the element which constitutes the cathode, the emission spectrum of these sources are different. Hence, to measure an element such as lead, the cathode must contain lead. Each cathode contains the element of choice in a very pure form (99.99 per cent) where possible. This should suggest a major drawback: different lamps are required for each element to be measured. This is why there exist close to a hundred different lamps made from pure elements as well as alloys or fritted powders for multi-element lamps (Figure 13.7). The anode is made of zirconium or tungsten and the window of the lamp is made of either borosilicate or UV transparent glass for wavelengths shorter than 400 nm.

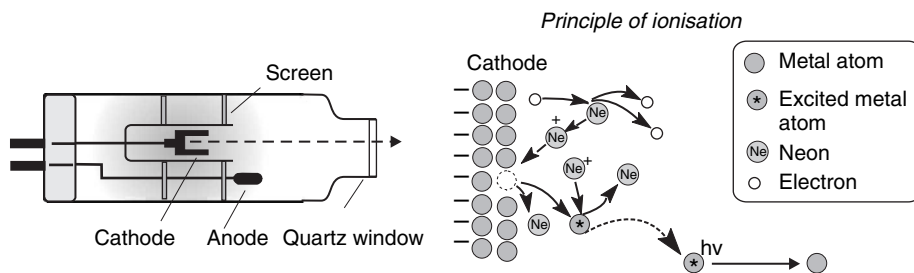
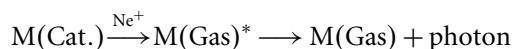


Figure 13.7 *Typical model of a hollow cathode lamp.* The cathode is a hollow cylinder whose central axis corresponds to that of the optical axis of the lamp. The fill gas (normally neon) is always chosen so that the spectral output of the cathode is free from interference. Right, in the box, pictorial representation of atoms of the cathode being excited by the impact of neon ions (Ne⁺). Hollow Cathode lamps are available as either single element or multi-element depending on the application. This particularity of AAS renders it impractical to perform qualitative work.

The assembly shown in Figure 13.7 cannot be used for sodium (melting point too low), or mercury (liquid state). For these elements, glow discharge in metallic vapour lamps are used (GDL).

When a potential difference of around 300 V is applied between the electrodes, the electrons provoke the ionization of the gaseous atmosphere within the lamp. These ions (Ar⁺ or Ne⁺), acquire enough kinetic energy to strip atoms from the cathode which becomes equivalent, at the surface, of an atomic gas. In calling M(Cat.) the element M in its metallic state (cathode) and M(Gas) when it is in its atomic state, emission corresponds to the following sequence :



The spectrum emitted by the lamp corresponds to the superimposition of radiation emitted by the cathode and by the gaseous atmosphere within the lamp. The width of the emission lines, which depends upon different effects (Doppler, Stark (ionization) and Lorentz (pressure)), is narrower than the corresponding absorption band. The monochromator enables the elimination of a large part of the stray light due to the filling gas, and the selection of the most intense spectral line in order to obtain a better sensitivity (Figure 13.8), except for cases of interference caused by other elements.

An alternative to HCL are *electrodeless discharge lamps* (EDL) whose light intensity is about 10–100 times greater but are not as stable as HCL. They are made of a sealed quartz tube that contains a salt of the element of interest along with an inert gas. An RF fields is used to excite the gas which in turn causes the metal to be ionized. These lamps are in general reserved for elements such as As, Hg, Sb, Bi and P.

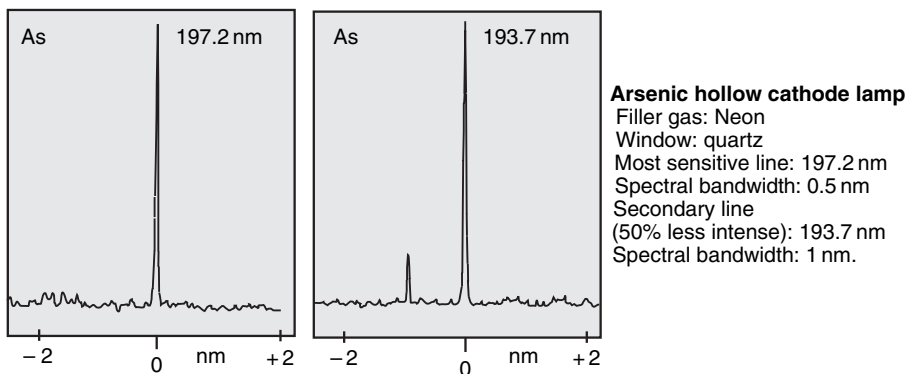


Figure 13.8 Characteristic of the arsenic hollow cathode lamp.

■ Generally, for each HCL there are several interesting emission lines. They do not have the same intensity. The choice of a particular line for an element depends, amongst other things, upon the concentration of the solution being nebulized, knowing that in AAS the precision of the measurement diminishes fairly quickly with concentration (dynamic range no more than 1 to 100). The choice of a second wavelength of weaker absorption avoids the need to dilute a solution when the element is present in high concentration.

13.5.2 Thermal devices for obtaining atomic aerosols

Atomization using a flame – burner and nebulizer

The atomic aerosol for the instrument is provided by a combination of a nebulizer and a burner. The sample in aqueous solution is sucked up by the Venturi effect. Pressurized air is passed through a tube causing the sample solution to be drawn into the burner as a fine mist where it is mixed with a combustible gaseous mixture to produce a flame, which finally contains the atomic aerosol. Naturally, the sample must be in solution form in order to employ this process. This robust mechanical assembly, called the burner, has a rectangular base of about 10 cm in length by 1 mm in width. The optical axis of the instrument is aligned with the longest dimension of the flame (Figure 13.9).

The flame is characterized principally by its chemical reactivity for a given maximum temperature (Table 13.2) and its spectrum. This is a complex medium in dynamic equilibrium, containing free radicals, at the origin of a spectrum in the near UV which results from the superimposition of emission and absorption lines. This can interfere with the measurement of some elements. That is why it is not just any flame which may be used for any element. The chemical reactivity of the flame not being homogeneous, it is important to find a good position for the optical path of the instrument.

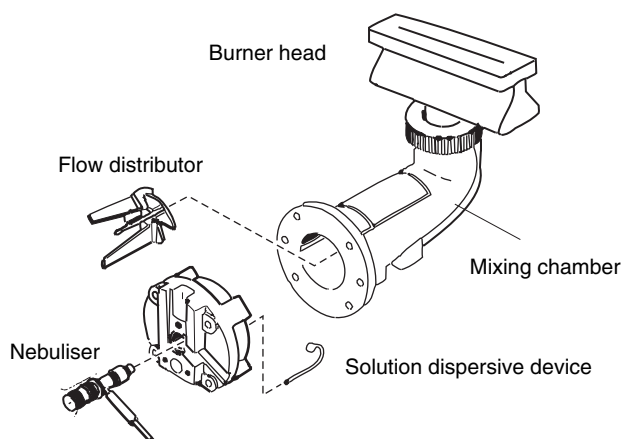


Figure 13.9 The burner of an atomic absorption instrument. This type of burner was used in the Model 3100-3 from Perkin-Elmer (reproduced with permission).

Table 13.2 Upper temperature limits for some gas mixtures

Combustible mixture	Max. temperature (K)
Butane/air	2200
Acetylene/air	2600
Acetylene/nitrous oxide (N_2O)	3100
Acetylene/oxygen	3400

Often an air/acetylene flame is chosen. To attain higher temperatures the air is replaced by nitrous oxide, N_2O .

Thermoelectric atomization

The previous device with flame and nebulizer may be replaced by a *graphite furnace* comprising a tube of graphite with a small cavity that can hold a precise quantity of sample (a few mg or μL using an automatic syringe) (Figure 13.10). This carbon rod, whose central axis coincides with the optical axis of the spectrophotometer behaves as a ohmic resistor that can attain 3000 K by virtue of the Joule effect. The heating cycle generally comprises four steps (Figure 13.10). To avoid loss through projections the temperature is gradually increased, firstly to dry then decompose and finally atomize the sample. In this latter step the temperature gradient can attain $2000^\circ C/s$ because the sample is brought to the atomic gas state in three or four seconds.

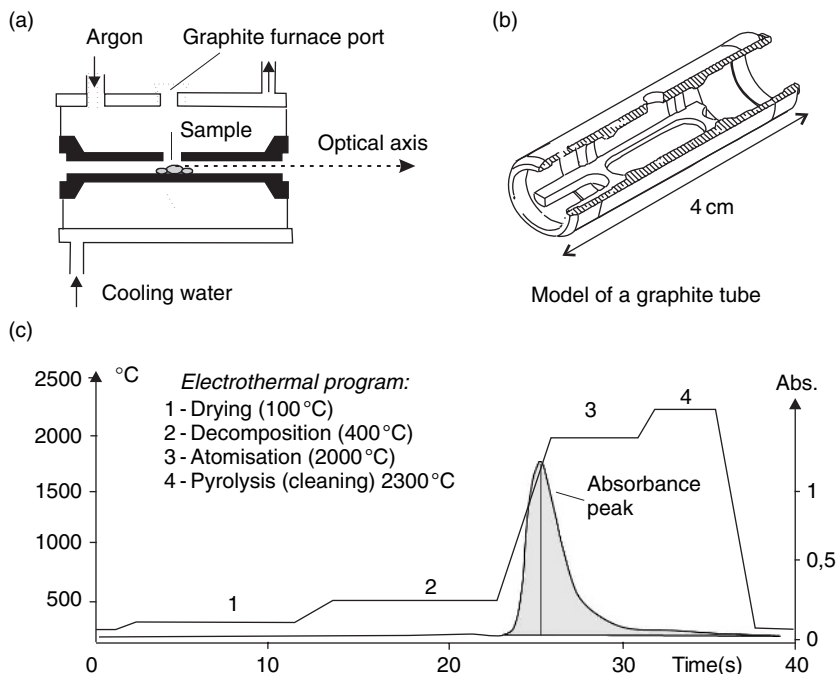


Figure 13.10 *The electrothermal atomization device.* (a) Graphite furnace heated by the joule effect; (b) Example of a graphite rod; (c) Graph displaying temperature programming as a function of time showing the absorption signal. Two types of measurements can be chosen, the peak height absorbance or the integrated absorbance (in grey on the figure). The first two steps of the temperature program are conducted under an inert atmosphere. Solid samples can be assayed.

The graphite tube is surrounded by a double sleeve. One contains an inert gas, such as argon, that circulates to protect the elements from oxidation while the other cools the entire device, using water.

In comparison with the burner, this flameless atomization produces a very high atom density and a longer confinement period which multiplies the overall sensitivity by a factor of 1000.

Chemical vaporization

Some elements such as arsenic (As), bismuth (Bi), tin (Sn) or selenium (Se) are difficult to reduce to atoms in a flame when in higher oxidation states. In order to measure these elements the sample is reacted with a reducing agent constituted by sodium borohydride or tin chloride in an acidic medium, just prior to analysis (Figure 13.11). A volatile hydride of the element is formed which is swept up by a make-up gas into a quartz cell placed in the flame of the burner.

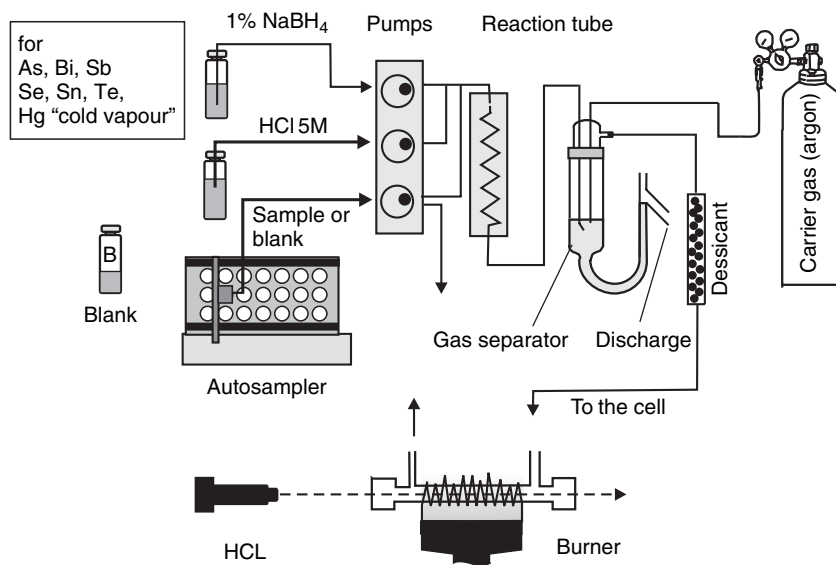
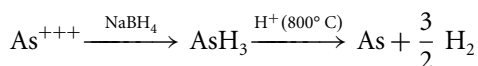


Figure 13.11 Schematic of an hydride reactor used for particular elements. This automatic sampling device houses a mixing tube where the hydride of the metal (or non-metal) is formed during reaction with sodium borohydride. An argon flow extracts the metal hydride formed (gas separator) carrying it to a silica glass tube heated to between 800 and 1000°C in the flame.

Example of the reduction of an arsenic salt by sodium borohydride:



The metallic hydrides, which are easily thermolysed at around 1000 K, liberate the corresponding elements in the atomic state. An electrodeless lamp is preferably used as a light source.

As for mercury, it is not transformed to a hydride but rather remains in the metallic state (Hg^0). Consequently, a special cell that does not require to be put into the flame is used. This is called the 'cold vapour' method, and requires specialized instruments (reduction by SnCl_2).

13.6 Flame photometers

Measurements by flame photometry are carried out either using atomic absorption spectrometers with a burner (but without the light source), or flame photometers. The latter are less sophisticated instruments whose price is ten times less than atomic absorption spectrometers. These photometers are designed to make measurements of only five or six elements. They include interchangeable coloured

filters or a basic monochromator that can isolate a spectral band embracing the selected emission line. They are very useful instruments for certain applications in quality control such as measurements of alkali metals or alkaline earth metals in substances (e.g. calcium in beer or milk, potassium in cements, minerals, etc.) More improved models, possess two measuring cells permitting a comparison of light intensities between the sample and that of a standard, allowing concentration determination. The response linearity is reached rapidly, requiring the use of low concentration solutions (10 to 100 ppm).

■ The principle of flame photometry is employed in a specific GC detector very sensitive to the element sulfur and also in other specialized analysers. So, in GC, when an organo-sulfur compound is pyrolysed in the burner of the detector, the air-hydrogen flame reduces the compound to elemental sulphur which emits radiation of wavelength 394 nm.

13.7 Correction of interfering absorptions

Atomic absorption allows the measurement of around 70 elements (see Figure 13.18). It is widely used because the method can accept samples of various forms at very low concentrations. The scope of applications is therefore considerable. As in visible or infrared spectrophotometry, it is necessary to carry out baseline corrections to eliminate fluctuations coming from the lamp and interfering absorptions.

Instruments with a burner generally have only low background noise in the signal.

Alternately, with graphite furnaces, an incomplete atomization of the solid or liquid sample, due to the matrix at elevated temperatures, can produce interfering absorptions. This is notably the case with samples containing particles in suspension or difficult to reduce, ions or organic molecules remaining unburned owing to a lack of oxygen. This gives a baseline of constant absorption (smokes) within the interval defined by the monochromator. To correct this effect, the manufacturers propose improved instruments that allow to apply various methods (Figure 13.12). However, for this type of sample it is not possible to have the matrix alone without the analyte. Particular double beam instruments have thus been designated.

13.7.1 Background correction using a deuterium lamp

The model taken as example (Figure 13.13) is a pseudo dual beam optical assembly to overcome the fluctuations in light intensity of the source and which contains a second polychromatic source used to determine the absorption due to the matrix alone. This is the most commonly used method.



Figure 13.12 An AA spectrometer, Model AA 280 equipped with a graphite furnace and Zeeman device. A rotating turret holds height HCL (reproduced courtesy of Varian Inc.)

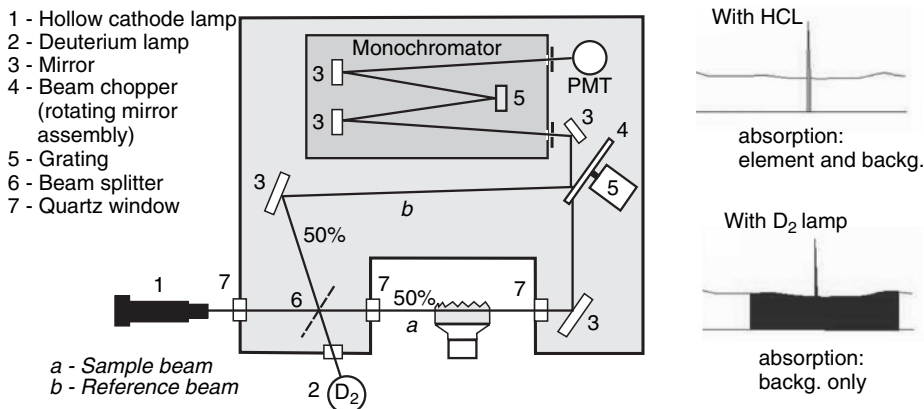


Figure 13.13 Scheme of a AA spectrometer showing deuterium lamp background correction. This 'double beam' assembly includes a deuterium lamp whose broad emission is superimposed, using a semi-transparent mirror, upon the spectral lines emitted by the HCL. Beam path *a* passes through the flame while beam path *b* is a reference path. The instrument measures the ratio of the intensities transmitted by the two beams and for the two sources. The domain of correction is limited to the spectral range of the deuterium lamp, being 200–350 nm (reproduced from the optical scheme of model Spectra AA-10/20, Varian).

For the selected wavelength, using the monochromator, the flame is swept alternately by light issuing, either from the hollow cathode lamp or from the deuterium lamp, which constitutes a continuous source. A rotating mirror is used. When the deuterium lamp is selected, bearing in mind that the sample is nebulized

in the flame, only the background absorption is measured since the bandwidth range is a hundreds of times larger than the absorption line chosen.

When the hollow cathode lamp is selected, the total absorption (background and absorption due to the spectral line of the element) is measured. Absorbances being additive, the difference between the two measurements will yield the absorption due to the background corrected sample, whatever the intensities of the two lamps.

13.7.2 Correction using the Zeeman effect

An alternate approach consists in making use of the Zeeman effect. When a free atom is exposed to a strong magnetic field (10 kG), there is a disturbance of its electron energy states. This phenomenon, called the *Zeeman effect*, modifies the corresponding emission or absorption spectrum of the element. However, all elements do not respond in the same way. Most frequently, it is observed that each absorption line is split into three new polarized lines. One of these lines, called component π , retains in the initial position, while the two others, named component σ , are symmetrically shifted on both sides of the π component (a few picometres in a 1-tesla field). The directions of polarization of the π and σ lines are perpendicular. If a polarizer is installed on the optical path, and oriented in the direction parallel to the field, only component π will absorb the light from the source. Contrary to the atoms of the element, particles and smoke in suspension during the measurement are not affected by the Zeeman effect.

The application of the Zeeman effect in AAS requires the application of an electromagnet at the graphite furnace (or at the flame). Two assemblies exist for correcting the background absorption.

The first set-up is equivalent to a double beam possessing a common optical path. It consists of making two comparative measurements *with* and *without* the magnetic field. In the absence of magnetic field, the detector receives the light from the source minus both the background absorption and the fraction absorbed by the element being measured : these two absorptions are additive. Alternately, when the magnetic field is applied, the absorption band selected separates into several new lines. Those whose position has changed can no longer overlap with the line emitted by the source and they no longer play a role. The line which remains at the original wavelength (as when there is no magnetic field), is polarized along the field's axis and absorbs the radiation of the source exclusively in this direction. Since the polarizer is installed 'perpendicular' to the direction of the magnetic field it will not detect this absorption: the element will have become transparent to the detector. The detector perceives only the continuous background absorption (Figure 13.14).

The second set-up utilizes a fixed magnetic field and a rotating polarizer ; the signal oscillates between two extreme values which correspond either to the background absorption alone or to the latter plus the absorption by the π component of the element (Figure 13.15).

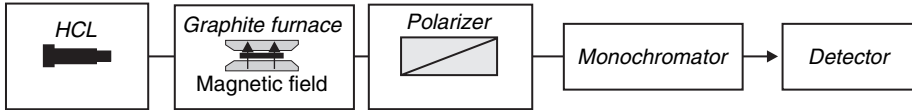


Figure 13.14 Correction through Zeeman effect. Modular scheme of an apparatus using the correction of absorbance by Zeeman effect. Two solutions are applicable: (1) with or without magnetic field and a fixed polarizer; (2) with fixed magnetic field and rotating polarizer.

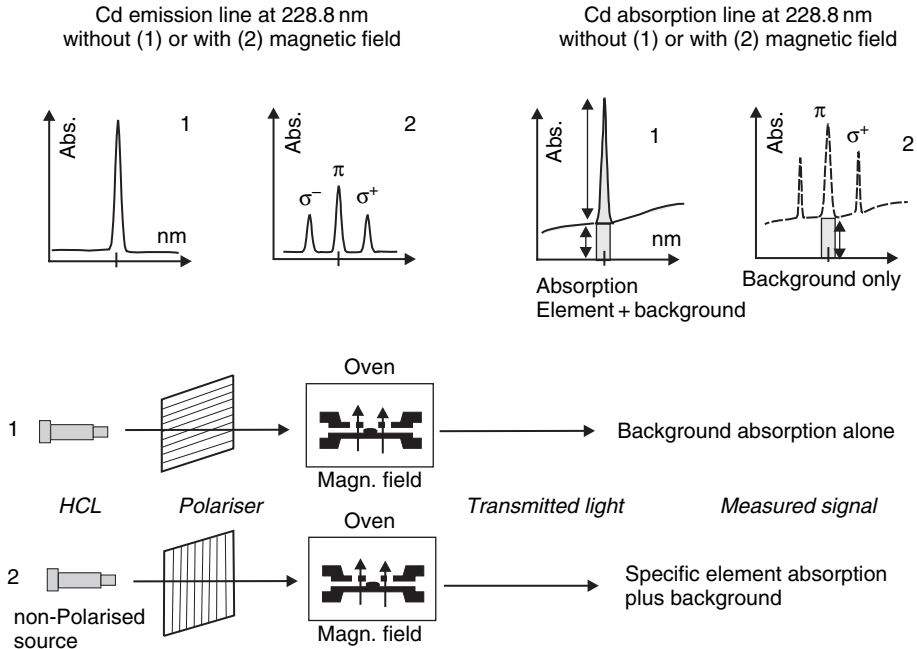


Figure 13.15 Normal Zeeman effect. Pictorial explanation of the rotating polarizer method.

13.7.3 Background correction using a pulsed HCL (Smith-Hieftje method)

When the intensity of a HCL is pulsed with high current by reducing the shunt resistance in its electrical circuit, the profile of the emission lines changes. For each of them the profile broadens, a general consequence of the temperature increase of the cathode. Moreover, the raised temperature of the cathode produces an evaporation of atoms at the centre of the lamp. This cloud of atoms emitted by the cathode re-absorbs in a colder part of the lamp as a very fine spectral line. The net result is that the emission curve dips in the middle at the same wavelength as that emitted by the cathode (Figure 13.16).

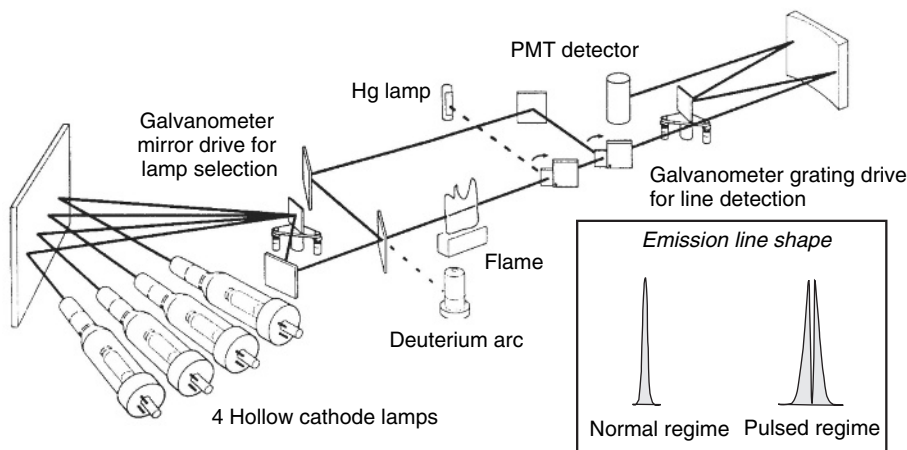


Figure 13.16 *Pulsed lamp for background correction.* The model shown uses the principle of the Smith–Hieftje mercury source background correction. The mercury source as well as the retractable mirrors are used to calibrate the monochromator (reproduced courtesy of Thermo Jarrell Ash). Appearance of an emission line of a HC lamp as a function of its voltage.

This self-absorption is the basis of the pulsed lamp technique for correction of the background absorption. Known as the Smith–Hieftje (S-H) method, this application uses a pulsed lamp which enables a comparison of the two measurements. In normal conditions (e.g. 10 mA) and with the sample into the flame, a global measurement representing the sum of the background absorption and the absorption of the element is observed, while under strained lamp conditions (500 mA) only the background absorption is present as the lamp will no longer emit at the wavelength chosen. The comparison of these two absorbance measurements leads, after correction, to the calculation of the absorption due to the sole analyte.

These three methods of correction present advantages and inconveniences: the deuterium lamp method uses a more complex optical assembly including a second source – the Zeeman method is expensive – the S-H method requires special lamps. The dynamic range is reduced. The choice of the correcting device must be made as a function of the desired applications.

13.8 Physical and chemical interferences

Whenever possible, an element is measured using an intense emission line of the corresponding lamp. Generally the most intense corresponds to the resonance line. However, various factors engendered by the matrix can lead to erroneous analytical results.

13.8.1 Spectral interference

In AAS, the graphite furnace can be the cause of interfering emissions from the walls of the graphite rod. The compounds from the matrix can lead equally to unwanted absorptions.

There is never total impossibility of the superimposition of two absorption lines: such as that chosen for the measurement and that coming from a secondary line belonging to another element. Confusions are rare but it is sometimes advisable to undertake a second measurement at another wavelength. In atomic emission this problem is frequent, not least as the spectrum is more complex (cf. Chapter 14).

13.8.2 Superimposition of absorption and emission of the same element

A non-negligible fraction of the atoms of certain elements pass to the excited state thermally. These atoms emit photons of the same energy that those remaining in the fundamental state are able to absorb. In order to correct the measurement (the emitted intensity has to be subtracted) the HCL voltage is pulsed, which permits differentiation between the emission signal, which is constant, and the absorption signal, which is pulsed. It is also an easy way to account for instrumental variations and 'flame flicker'.

13.8.3 Chemical interactions

When using atomic absorption to search for traces of elements it is important to bear in mind the matrix in which they are found. Well established protocols must be followed to suppress ionic or chemical interference. Mineral salts or organic reagents serving as 'liberating agents' R are often introduced into the solutions to be nebulized for corrective reasons. This treatment will effectively liberate element M from its combination MX, if compound RX is more stable than MX,



■ Thus when calcium is to be measured in a matrix rich in phosphate ions or in refractory combinations containing aluminium, then strontium or lanthanum chloride are added. The effect sought is to liberate calcium and to increase the volatility of the medium to ensure a more efficient elimination during the decomposition step. In the same way to measure sodium or potassium it is usual to add a little of Schinkel's solution (CsCl/LaCl₃).

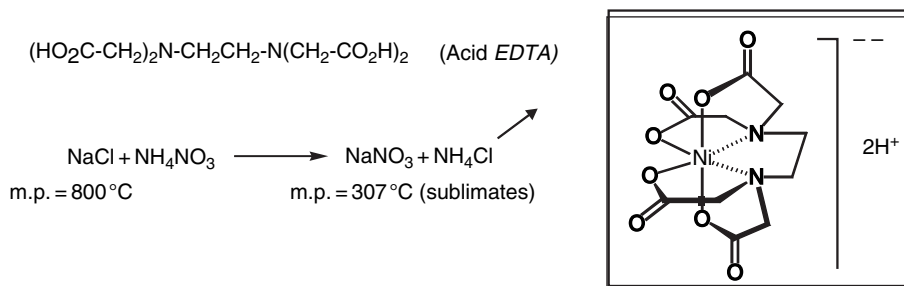


Figure 13.17 *Matrix modification.* Ammonium nitrate or EDTA increases the volatility of certain elements. A volatile complex of type 1 : 1 between a molecule of EDTA and the Ni^{2+} ion.

With apparatus containing a graphite furnace, ethylenediaminetetraacetic acid (EDTA) is introduced to form 1 : 1 complexes with bivalent ions, or ammonium nitrate if the matrix contains a high concentration of sodium (Figure 13.17).

Finally, the use of very hot flames can provoke partial ionization of certain elements which decreases the concentration of free atoms in the flame. This phenomenon is corrected by addition of an *ionization suppressor* in the form of a cation whose ionization potential is lower than that of the analyte. A potassium salt of about 2 g/L is often employed for this purpose.

This variation of the ionization can occur more or less spontaneously when the matrix contains one or more alkaline elements. To avoid these random errors, an ionization buffer based upon a potassium or sodium salt is added systematically to the solutions. An alternative consists to prepare a series of standards in a medium close to that of sample.

13.9 Sensitivity and detection limits in AAS

More than 70 elements can be measured by AAS (Figure 13.18). The lowest concentration that can be quantified in a sample depends upon many factors. In spectrometry the *sensitivity* for an element is defined as being the concentration expressed in $\mu\text{g/L}$ which, in aqueous solution, leads to a 1 per cent decrease ($A = 0.0044$) in the transmitted light intensity. So, for manganese (Mn), this value is 4 pg with an aqueous matrix. When possible, a calibration curve should be established with concentrations in the range of 20 to 200 times this value.

The *detection limit* corresponds to the concentration of the element giving a signal whose intensity is equal three times the standard deviation of a series of measurements made upon the analytical blank or on a very dilute solution (degree of confidence 95 per cent). In practice, the concentrations must be at least ten times higher than the detection limit to give reliable measurements.

IA		Legend										VIII A						VIIIA	
H 1	He 2	M	Air/acetylene flame		xx		Atomic number		M		N ₂ O/acetylene flame		B 5	C 6	N 7	O 8	F 9	Ne 10	
Li 3	Be 4											Al 13	Si 14	P 15	S 16	Cl 17	Ar 18		
Na 11	Mg 12	III B	IV B	V B	V I B	V II B	VIII				IB	II B							
K 19	Ca 20	Sc 21	Ti 22	V 23	Cr 24	Mn 25	Fe 26	Co 27	Ni 28	Cu 29	Zn 30	Ga 31	Ge 32	As 33	Se 34	Br 35	Kr 36		
Rb 37	Sr 38	Y 39	Zr 40	Nb 41	Mo 42	Tc 43	Ru 44	Rh 45	Pd 46	Ag 47	Cd 48	In 49	Sn 50	Sb 51	Te 52	I 53	Xe 54		
Cs 55	Ba 56	La 57	Hf 72	Ta 73	W 74	Re 75	Os 76	Ir 77	Pt 78	Au 79	Hg 80	Tl 81	Pb 82	Bi 83	Po 84	At 85	Rn 86		
Fr	Ra	Ac																	
			Ce 58	Pr 59	Nd 60	Pm	Sm 62	Eu 63	Gd 64	Tb 65	Dy 66	Ho 67	Er 68	Tm 69	Yb 70	Lu 71			
			Th 90	Pa	U 92	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Md	No	Lw			

Figure 13.18 Elements measured by AAS and FES. Most elements can be measured by atomic absorption or flame emission by using one of the available modes of atomization (burner, graphite furnace or device for hydride formation). The sensitivity varies from several ppb (Cu, Cd, Cr) to several ppm (the lanthanides). The elements of the table (in white) for which the atomic number is not shown are not measurable by atomic absorption. However, the hybrid apparatus AAS/OES containing plasmas as a thermal source, has more recently pushed back the limits of this method of elemental analysis.

Problems

- 13.1 If the response of the detector of a flame photometer is proportional to the concentration of the element heated to a state of excitation, by what factor is the signal multiplied when the temperature passes from 2000 K to 2500 K?
(Establish the general expression and then apply it to the case of elemental sodium, whose resonance is promoted by a radiation of 589 nm wavelength.)
- 13.2 Why is it advisable to add ethylenediaminetetracetic acid (EDTA) to measure calcium when it is in the form of a phosphate?
- 13.3 Why is a potassium salt (such as KCl) frequently added when one wants to measure elemental sodium by flame photometer? Remember that the potential for the first ionisation of potassium is 419 KJ mol⁻¹ while that for sodium is 496 KJ mol⁻¹.

- 13.4 The potassium concentration in a blood serum is to be analysed using a method of addition and flame emission. Two extractions of 0.5 mL of serum were taken to create two identical solutions and then both were diluted further with distilled water to a final volume of 5 mL. 10 μ L of 0.2 M KCl is introduced to one of these. The values obtained from the apparatus were 32.1 and 58.6 arbitrary units. What is the potassium concentration of the serum?
- 13.5 An AAS method is employed for the determination of lead (Pb) in a sample of adulterated paprika by the introduction of lead oxide (of the same colour). An electrothermal atomic absorption instrument that provides a background correction based upon the Zeeman effect is used.
0.01 g of the paprika powder is placed in the tube of the graphite furnace. The determination of the area peak absorbance was made at $\lambda = 283.3$ nm first in the absence and later in the presence of a magnetic field. The value of the peak absorption following background correction was 1220 (arbitrary units). Under the same conditions, 0.01 mL of a solution of 10 g/L Pb led to a value of 1000 in the same units.
Calculate the % mass of lead in the sample of paprika under study.
- 13.6 A stock solution of calcium ions was prepared by dissolving 0.1834 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 mL of distilled water and then further diluting by a factor of 10. From this new solution, three standard solutions were prepared by further dilutions of five, 10 and 20 times, respectively. The unknown sample is itself diluted 25 times. Sufficient strontium chloride was then introduced to eliminate any interference due to phosphate ions. An analytical blank containing the same concentration of strontium was the first solution to be examined by the air/acetylene flame. The results were as follows:

Blank	1.5	$\text{Ca} = 40.1 \text{ g mol}^{-1}$
Standard 1:20	10.6	$\text{Cl} = 35.5 \text{ g mol}^{-1}$
Standard 1:10	20.1	
Standard 1:5	38.5	
Sample	29.6	

What is the concentration (in ppm) of the calcium in the sample?

- 13.7 Among the numerous commercial derivatives of ethylenediaminetetracetic acid (EDTA), a mixed salt of Zn/Na containing one atom of zinc and two atoms of sodium per molecule of EDTA is found. This mixed salt presents itself in the form of a crystallized hydrate. An attempt is described to deduce

the number of water molecules of this hydrate by measuring the presence of zinc by atomic absorption. The experimental approach was as follows:

A solution A, is prepared by dissolving 35.7 mg of the hydrate in 100 mL of water. 2 mL were then pipetted and diluted again with H₂O to a final volume of 100 mL. This constitutes the sample solution B. The standardisation is then performed with five solutions of known zinc concentration. (see table below).

1. To effect this measurement the slit bandwidth of the atomic absorption apparatus is 1 nm. What does this parameter represent?
2. Knowing that the calibration curve computed by the apparatus indicates that $A = 0.3692$, $C = 0.99$ mg/L, calculate the number of molecules of water in the mixed salt hydrate of Zn/Na.
3. From the data and by the method of least squares derive an equation for the calibration curve. Calculate the zinc concentration in solution A. Recall: H = 1.01; C = 12.01; N = 14.01; O = 16.0; Na = 22.99 and Zn = 65.39 g/mol respectively.
4. Is the use of the standard calibration curve justified for atomic absorption?

Solution	Conc. Zn mg/L	Absorbance
Blank	0.00	0.0006
Standard 1	0.50	0.2094
Standard 2	0.75	0.2961
Standard 3	1.0	0.3674
Standard 4	1.25	0.4333
Standard 5	1.50	0.4817
Sample	C?	0.3692

14

Atomic emission spectroscopy

Analysis by atomic emission spectroscopy constitutes a general method for measuring elements on the study of the radiation emitted by their atoms present in an excited state, usually following ionization. Several procedures are used to break down the samples into their constitutive elements, most notably by the effect of high temperatures plasmas, spark sources or glow discharges. The emission being far more complex than those of flame emission, instruments required very high quality optics to resolve interferences from both spectral lines and matrix effects. Since this method measures optical emissions with optical spectrometers, the abbreviation OES is better than AES and will be adopted in this chapter.

Employed since its beginning in metallurgical industries, OES, whose analytical domain covers an extended dynamic range, has become an indispensable tool of chemical analysis. The spectrometers are capable of routinely analysing several elements either simultaneously or sequentially. A single instrument can perform the functions of several analytical techniques including X-ray fluorescence, atomic absorption and combustion analyzers. The technique coexists with AAS with which it is more complementary than competitive. This spectroscopy is less expensive than mass spectrometry when applied to elements (ICP-MS) though it is not advisable for the analysis of the lighter elements.

14.1 Optical emission spectroscopy (OES)

When a chemical element in the atomic state is exposed to appropriate conditions of excitation, it emits a characteristic radiation. On this observation is based a general form of elemental analysis, both qualitative and quantitative. This *optical emission spectroscopy* issued from sample is very complex and leads to spectra with thousands of spectral lines accompanied by a continuum background.

The instruments designed for these analyses comprise several parts: the device responsible for bringing the sample in the form of excited or/and ionized atoms (based on gas plasmas, sparks or lasers), an high quality optical bench which conditions the analytical performances, a detector with a sensor (PMT or diode



Figure 14.1 *Atomic emission spectrometer.* Basic concept and an example of a bench model of spectrometer, the Vista-Pro, CCD simultaneous ICP-OES (courtesy of Varian Inc, USA)

array) and finally a computerized section essential to control the entire instrument (Figure 14.1).

The characteristic that most distinguishes these spectrometers from AA or FE instruments is their optical bench and sometimes their impressive size. They are reserved to analytical laboratories that have to treat a great deal of samples.

14.2 Principle of atomic emission analysis

As in FES, the light source of the spectrometer is nothing other than the sample of which all the atoms have been excited. Qualitative analyses of unknowns can therefore be made. This marks an important difference from atomic absorption in which measurements can only be made for elements for which the instrument has been customized (choice of the hollow cathode lamp). From a single run, a multi-element analysis can be obtained in strict contrast to AAS. However only elements for which a calibration has been carefully undertaken can really be measured.

Each atom, once in the excited state can lose its excess energy by emission of one or several photons of which the energies can have many different values (Figure 14.2). For a sample, the dispersive system discloses radiation of a multitude of different wavelengths and intensities. For a given sample, the matrix (all

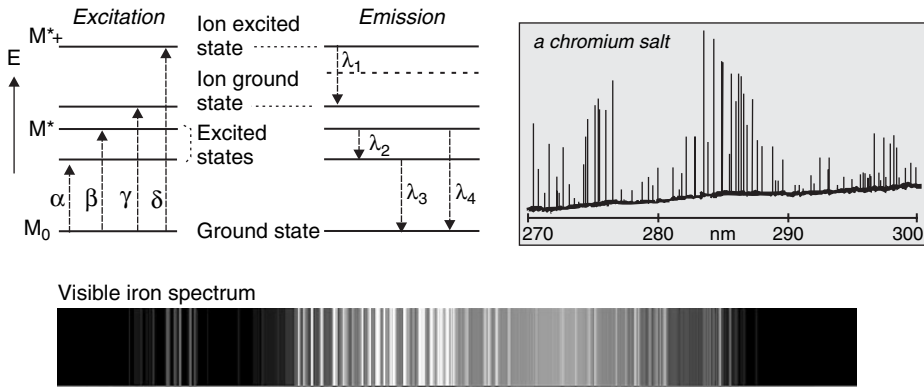


Figure 14.2 Energy transitions in OES. For every atom there exists hundreds of possibilities by which it may return to the ground state, each of these having its own probability. Even the simplest sample is containing an enormous number of emission lines. An example (right) is a partial spectrum of an aqueous solution containing a few ppm of a chromium salt and a visible spectrum of iron.

elements present in the solution) will also emit, therefore the identification of a particular element that is present only as an ultra-trace, will be obtainable to the condition that measurements use multiwavelengths to construct the sample fingerprint. More than 75 elements can be determined in as little as one minute.

OES requires high performance instruments capable of locating weak intensity lines in close proximity to other much more intense. This is why preference is given to spectrometers having a broader dynamic range.

■ FES, which is used for measuring a small number of elements, is of much simpler design. For example, when sodium is analysed with a flame photometer whose flame attains 2000 °C, the sodium atoms are practically the only ones emitting radiation. To measure the emitted light, a simple optical filter put between the flame and the detector, in the absence of a monochromator, isolates a rather broad spectral band including the yellow radiation emitted by the element.

14.3 Dissociation of the sample into atoms or ions

To convert a sample to free atoms, excited or ionized, various procedures are employed based upon gas plasmas, sparks, lasers or glow discharges.

14.3.1 Gas plasma excitation

The most common instruments for OES contain a plasma torch (*inductively coupled plasma* – ICP) that can reach temperatures of up to 8000 K. This device is well adapted to samples in aqueous solution.

A plasma is a highly energized ‘cloud’ of gaseous ions and their electrons. Considered to be the fourth state of matter, it is formed by isolated atoms in equilibrium between their neutral and their ionized states (more than 2 per cent) and with the associated electrons ($10^{18}/\text{cm}^3$) which assure an overall neutrality of the medium.

To initiate the plasma, a flow of argon, weakly ionized by sparks (Tesla discharge), passes through an open quartz tube situated on the axis of several turns of a water-cooled copper tubing (Figure 14.3). This conductive coil is connected to a radiofrequency generator (typically 40.68 MHz and with a power of 1–2 kW).

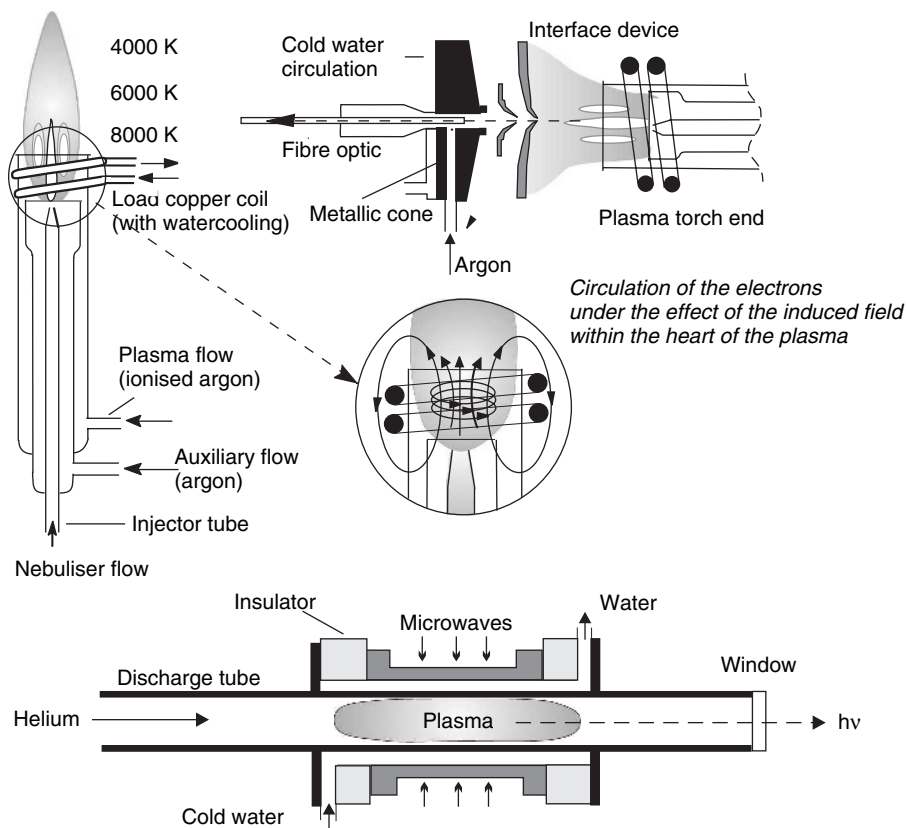


Figure 14.3 Plasmas obtained by inductively coupling or by microwaves. (a) Above left, (radial viewing to collect radial light). A radiofrequency current (between 27 and 50 MHz) that induces circulation of electrons in the inert gas drives the torch. The inner tube is used to inject the sample into the plasma. Right (for axial viewing), cooled device using fibre optic. The torch may consume up to 10 to 15 L/min of argon, which serves simultaneously as the ionization gas, nebulization gas and cooling gas (to avoid the torch melting!). (b) Below is a microwave plasma used at the outlet of a gas chromatograph for a specialized detector. These plasma sources must be stabilized for better reproducibility of the analyses.

The variable magnetic field that is created confines the ions and the electrons to an annular path. As the medium becomes more and more conducting, by appearance of an Eddy current, temperature increases considerably by the Joule effect. The device behaves similar to the secondary coil of a short-circuited transformer. The plasma is isolated from the torch wall by a gaseous sheath of non-ionized argon, which is injected by an external tube concentric to the previous one and is used to keep quartz wall cool.

The temperature of the plasma varies from 2000 to 9000 K, depending on the position. By comparison, the flames used in FES are relatively cold sources. Micro-volumes of sample solutions are introduced at a constant flow with a pneumatic nebulizer *via* a third tube of small diameter directly into the inductively coupled plasma. The position in the plasma chosen for the measurement of light emission (either *radial* or *axial*), depends upon the element and whether an ionic or atomic spectral line is selected for the measurement.

The ICP torch exerts a double action on the atoms: it excites them, which leads to photon emission and ionization. This is why the device is found in elemental analytical methods such as inorganic mass spectrometry, ICP-MS (cf. Sections 16.9 and 16.11). In all cases the sample must arrive in the form of droplets which do not exceed a few microns in diameter. To this end a nebulizer containing two inlets is used, one for the sample solution, the other for a gas serving to generate the aerosol. The choice depends upon the flow rate required and the ionic concentration of the solution (Figure 14.4).

14.3.2 Excitation by sparks or laser

Besides plasmas, which occupy the front rank of atomization and thermal excitation devices, other procedures are used. They gather electrical arcs or sparks (between 3000 and 6000 K) for conducting samples, or pulsed lasers. These are *abrasion methods* which have much progressed and which are used in industrial analysis (foundries, cement works). They can be adapted to different optical assemblies in current apparatuses (Figures 14.5 and 14.6). The UV laser ablation, which leads equally to the production of sparks (even a plasma) at the surface of the studied material is regarded as a 'general purpose workhorse'. The delivered energy densities are sufficient for all sorts of samples geological, biological and environmental specimens.

- Removal by a repetitive sparking device can be used as a means of producing an aerosol from the surface of the sample. Matter volatilized at the point of impact of the sparks (around 1 mg/min) is then directed by an argon flow to an ICP torch operating under argon having an applied voltage of between 20 to 50 kV. Otherwise, the light emission collected as a consequence of the sparks leads to a spectrum of ionic lines while with continuous arcs, neutral atomic lines are provoked.

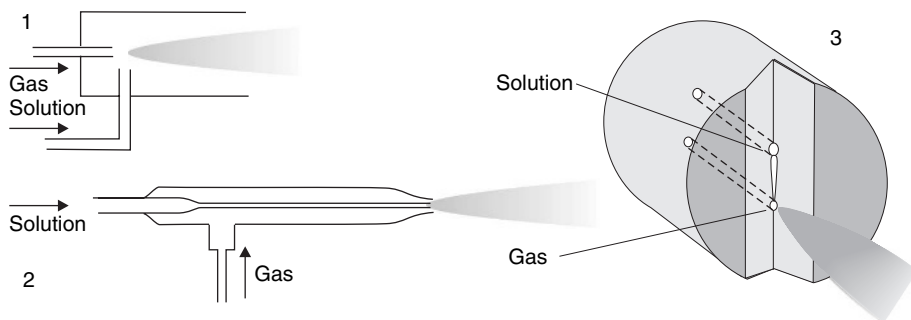


Figure 14.4 Nebulizers. Models: cross flow (1), concentric (2) and parallel (3). In contrast to models 1 and 2, model 3 is not subject to particulate obstruction by impurities in the solution. They leave through a large capillary that drains by gravity along a V-groove design to the outlet where the aerosol is produced (Babington nebulizer). For the solution, a pump regulates the flow of the aerosol. The chamber at the outlet of the nebulizer is used as a drain to eliminate the larger droplets by gravity.

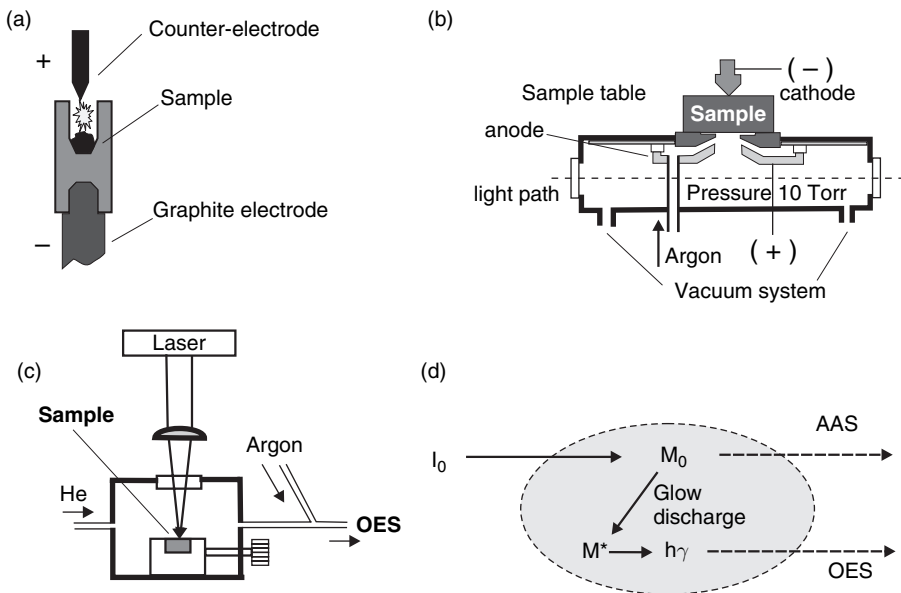


Figure 14.5 Ionization by sparks and glow discharge. (a) Continuous current arc ('globular' technique). The electrodes are submitted to a continuous voltage of several tens of volts ($I = 10\text{--}20\text{ A}$). The use of graphite electrodes is at the origin of emission lines due to radicals and other 'primitive' organic molecules (nitrile (CN) bands between 320 and 400 nm) as well as a continuous background. (b) Cell for glow discharge. The sample is placed on the front surface of the lamp, creating an enclosed region that is filled by a low argon pressure. The plasma confined near the anode, leads to the excitation of the atoms ejected from the sample surface. (c) Schematic of an excitation by UV laser. (d) Glow discharge and laser ablation can also be used for AAS, as a great majority of atoms (M) subsist in the fundamental state M_0 .

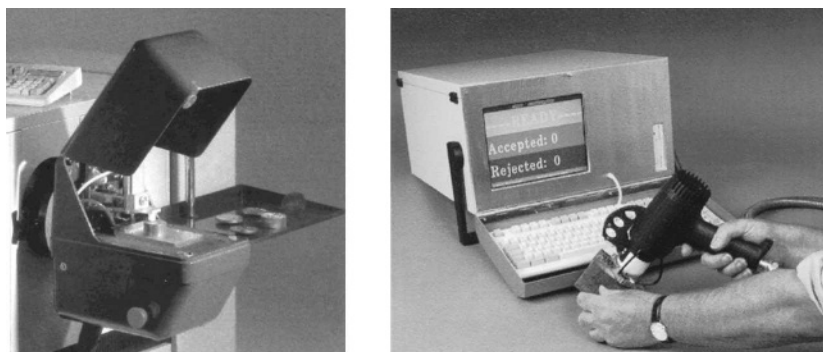


Figure 14.6 Atomic emission instrument with spark ionization. Left, Jobin-Yvon model JY-50. Details of the spark chamber in the open position. Right, mobile post of Metorex ARC Met-900 industrial analysis. The spark is produced by a gun, linked by an optical fibre to the spectrophotometer situated in the console (reproduced courtesy of Jobin-Yvon and American Stress Technologies).

14.3.3 Excitation by glow discharge (GD)

If the sample is a bulk solid sample and is an electrical conductor, it is possible to use it as the cathode of a kind of spectral lamp whose functioning principle is identical to that described for a hollow cathode lamp (cf. Section 13.5.1 and Figure 14.5). The atoms sputtered and removed from the surface of the sample are excited by the plasma. This GD-OES technique provides a rapid and accurate surface analysis, less susceptible to matrix effects and sample homogeneity. It has the advantage of yielding spectra with low background levels whose emission lines are narrow since atomization takes place at lower temperatures than that of the previous techniques.

14.4 Dispersive systems and spectral lines

The active part of the optical set-up begins with the *entrance slit* onto which is focused the light produced by the sample (the light source). The width of this slit is regulated by a mechanical system and cannot be less than a few micrometers because a minimum of light is required to enter, but its length can attain several centimetres.

The source is thus transformed into an *luminous object* of linear profile of which the optical system of the instrument gives as many defined images – the lines – as there are different wavelengths in the source. Each line corresponds to an almost monochromatic image of the entrance slit of the spectrophotometer. A single element can generate more than 2000 lines.

The lines are distributed in the focal plane as a succession of parallel and very narrow luminous segments. The width of these lines depends on several factors.

There are technical causes characteristic to the instrument and collectively known as ‘instrumental parameters’: width of entrance slit, quality of the optics, focal distance, diffraction phenomena through narrow orifices. However, there are also causes due to quantum mechanics, which ensure that the spectral transitions have a ‘natural width’. The radiations emitted by the atoms are not quite monochromatic. In particular with the plasmas, a medium in which the collision frequency is high (this reduces enormously the lifetimes of the excited states), Heisenberg’s uncertainty principle plays a large role (Figure 14.7). Moreover, the elevated temperatures increase the speed of the atoms, enlarging line widths by the Doppler effect. Finally for all of these reasons, the width of the lines at 6000 K reaches several picometres.

To locate the specific radiations of an element to be quantified (the different analytical lines) an optical bench of very high quality is required. These apparatuses can be divided into *spectrographs* and *spectrometers* (Figure 14.8 and see also Figure 9.12). As above pointed out, the source illuminates a slit which becomes a luminous object, which will be analysed by a dispersive system containing a grating (planar, concave or *echelle*).

At the beginning, the qualitative study of these spectra was made by visual comparison using spectrographs. Nowadays these have been replaced by spectrometers capable of resolving line interferences and problems linked to matrix effects. Each element, whether in neutral or ionized form, is responsible for a range of lines of variable intensities. The instrument is therefore programmed to both identify and to quantify the elements in the sample to be measured. Instruments are classified into two major groups.

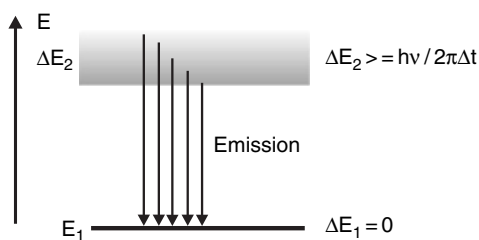


Figure 14.7 *Line width.* Representation of a transition between a stable ground state, whose lifetime is infinite and an excited state, which has a very short lifetime. The uncertainty ΔE_2 leads to an imprecision of the corresponding wavelength.

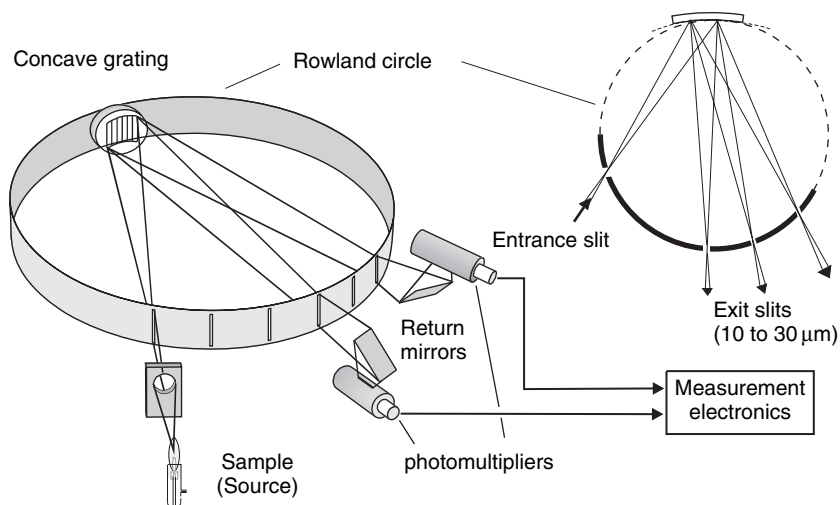


Figure 14.8 Optical schematics of an instrument with a concave grating and a polychromator with long focal distance. Reflecting mirrors can be used when lines to be detected are too close to one another. Several polychromators are sometimes associated. To the right, the principle of Rowland's circle. The optical part of these instruments must be assembled on a very stable metallic support.

14.5 Simultaneous and sequential instruments

Two large families of instruments can be distinguished of which one to measure several elements simultaneously, while the other to measure elements sequentially. Present trends favour systems of the first category with fixed optics to undertake pre-defined applications. The disadvantage of these turnkey solutions is their inability to make an exhaustive analysis of an unknown sample. The second category corresponds to sequential spectrometers in which the detector is fixed and receives only the radiation selected by the monochromator (comprising a motorized optical grating). By consequence this system only permits the measurement of the intensity of one line at a time. Measurements of light intensity by photomultipliers are possible over a very extended dynamic range. Elements can therefore be quantified for which the concentrations, or alternately, the light intensities of the lines are very different.

14.5.1 Fixed optic apparatus containing a polychromator

For each predefined element, the intensities of one or several spectral lines are measured, which constitute the *analytical lines* of measurement. Each line corresponds to a channel which impinges on its own photomultiplier. If the instrument

contains a concave grating, the entrance and the exit slits must be situated on the *Rowland's circle*, at a tangent to the grating and of which the diameter is equal to the radius of the curvature of the grating (Figure 14.8). In this kind of instrument, which has been abandoned, it was not easy to place several detectors, so the preferred type of construction has become that described below.

In a single analysis, close to 20 elements can be measured rapidly. Correction of background noise is also possible by the simultaneous determination of several lines belonging to the same element. The linearity of the measurements extends over a broader range than in atomic absorption.

14.5.2 Fixed optic, *echelle* grating apparatus

Another type of assembly comprises the combination of a particular class of grating, called *echelle grating* with a prism focusing device. This leads to a double dispersion of the light both horizontally, due to the grating and vertically by a CaF_2 prism. This arrangement of high optical resolution, whose focal distance can attain several metres, is an order-separating device. It permits the simultaneous observation of an extended spectral range, for example 200–800 nm (Figure 14.9).

■ For an echelle grating the incident light arrives perpendicular to the sides of the grooves which are fewer than for a conventional grating (e.g. 100 per millimetre) (Figure 14.9). The observation of the diffracted light is made almost at the same angle, in auto collimation, which maximizes the transmission of light (Littrow condition for a blazed grating when the input and output rays propagate along the same axis). Calculation reveals that for a given observation angle a succession of monochromatic wavelengths are found to superimpose as a result of the large interval separating two successive grooves. This multiplexing phenomenon (superimposition of the different orders n of the grating – n is around 100) is resolved by a prism that uses the second dimension of the focal plane to separate the different monochromatic images of the entrance slit. The spectrum remains stationary with respect to the optical assembly.

At the end of the optical path, dispersion of the 'lines' is detected on a matrix of sensors for which the response furnishes quasi-simultaneously information concerning thousands of spectral stains (Figure 14.10). So, analyses are undertaken more rapidly. By a series of improvements, the dynamic range of these sensors has become equivalent to that of photomultipliers.

14.5.3 Wavelengths scanning instruments (monochromator type)

In contrast to the preceding instruments, the dispersive system is movable in order to focus the different wavelengths on the fixed exit slit. A motor allows the pre-programmed selection of the wanted wavelengths and the sequential examination

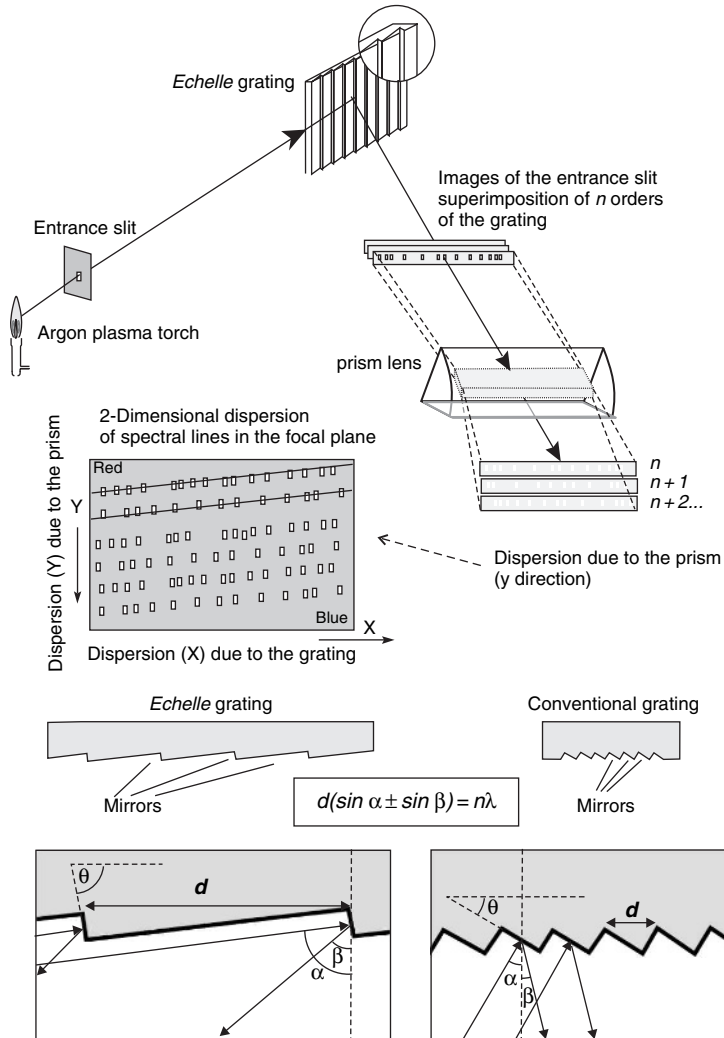


Figure 14.9 Principle of dispersion in the focal plane of an assembly associating an echelle grating and a prism. For clarity, the associated optics (collimating and focusing lenses) have not been represented. Comparison of an echelle grating with a conventional grating. The angle reflecting most of the light of the grating is referred to as the ‘blaze’ angle θ . The basic formula indicates which are the wavelengths diffracted by the grating at an angle of incidence α , with reference to a grating perpendicular and at an observation angle β (the sign is + if the observation is from the same side as the incident beam with respect to the perpendicular and sign – in the contrary case). The resolution can attain that of an apparatus which would elongate the spectrum over 2 m focal length.

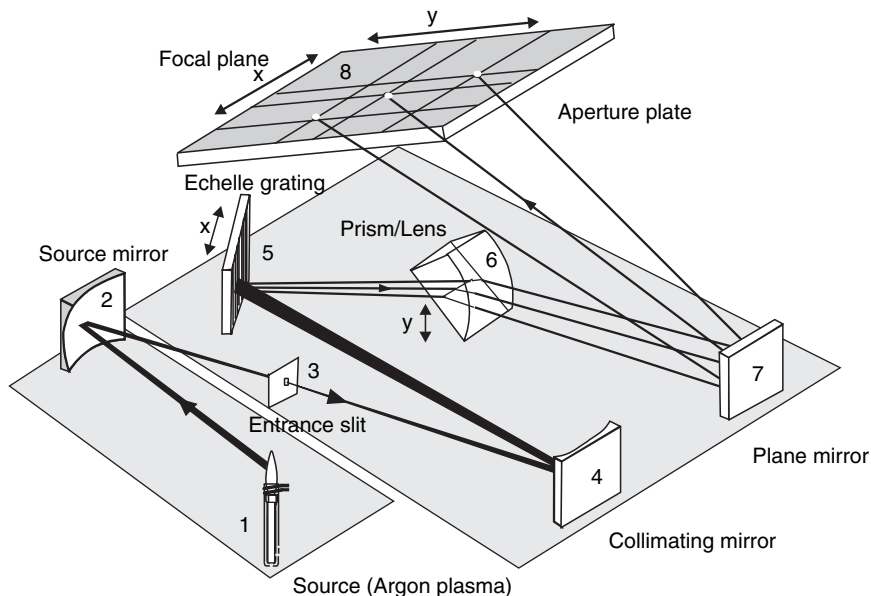


Figure 14.10 *Optical scheme for a spectrophotometer with an echelle grating.* For clarity only the central section of the beam issuing from source 1 is represented (this beam should cover the whole mirror 2). The echelle grating 5, separates the radiations arriving from the source in the horizontal plane (in x). The prism then deviates the radiation in the vertical plane (in y). The path of three different spectral lines is shown. The images of the entrance aperture 2 are in the focal plane 8. In the past, to detect these radiations, photomultiplier tubes (PMT) of reduced size were installed in specific places, but now charge transfer devices (charge coupled or charge injection devices, CCD/CID) are used, as an electronic equivalent of photographic plates. This allows a continuous spectral cover from 190 to 800 nm with excellent resolution. Sensors of 500×2000 pixels (each $12 \times 12 \mu\text{m}$) are now used.

of the spectral lines. The light issuing from the source is analysed either by an Ebert assembly (cf. Section 9.2), or by a Czerny–Turner dual monochromator in order to obtain very high spectral resolution (a few nm), comparable to the emission linewidth at this temperature, over a broad spectral region (160–850 nm). Several assemblies of this type can be placed in series to constitute dual or triple monochromators of very high performance without rendering the assembly too demanding in volume.

These instruments are notable for their flexibility. A mercury vapour light source serves as a means of internally calibrating the wavelengths in order to rapidly determine the position of the grating with the greatest precision. The optical path is maintained in an enclosure maintained under vacuum. Here again the proximity of certain spectral lines necessitates a rigid assembly of a great mechanical precision.

14.6 Performances

Rapidity and detection limits below 1 ppb (10^{-9}) for many elements, make OES one of the best methods of elemental analysis. With instruments containing one array detector it is possible to record in a few seconds the whole spectrum in high resolution (e.g. several thousands emission spectral lines).

Some spectrometers accept more readily dominating matrices as mud or soils for which the elements Si, Fe and Al are in very high concentrations. Each application should be considered as an individual case.

14.6.1 Sensitivity thresholds and detection limits of the spectrometer

The threshold of sensitivity varies according to the instrument and the element being considered. Numerous comparative tables on detection limit values exist which are continually being updated as a result of the progress made in instrumentation. The sensitivity threshold corresponds to the minimum concentration of element in solution that will yield an analytical signal for which the amplitude is equal to three times the standard deviation calculated for an analytical blank. This classic definition leads to rather optimistic values and very variable with respect to the element. The detection limit of the instrument represents the concentration of an element, which allows detection with a confidence of 95 per cent (cf. Chapter 22).

- The detection limit for a methodology is of more importance than the detection limit above. Generally measurements are done in a domain of concentration corresponding to 50 times the detection limit of an instrument. When measuring ultra-traces quantities, it is obvious that sample preparation requires reagents and water of very high purity.

14.6.2 Resolving power

Spectral resolution can be determined directly through an experimental measurement from a recorded spectrum. This corresponds to the chosen emission peak width $\Delta\lambda$, measured at the half-height using the units of the spectrum. Its value depends upon both the dispersive system, the wavelength and the instrument tuning.

The *resolving power* of a spectrometer is its ability to separate adjacent spectral lines. This theoretical concept is given by the dimensionless parameter R such that:

$$R = \lambda / \Delta\lambda \quad (14.1)$$

$\Delta\lambda$ is the difference in wavelength between two spectral lines of equal intensity. Two peaks are considered resolved if the distance between them is such that the maximum of one falls on the first minimum of the other. Atomic emission apparatus have resolving powers in the order of 30 000 rising to 100 000. The use of a too wide exit slit (bandwidth arriving on the photo-multiplier tube) reduces the resolving power of the spectrometer.

14.6.3 Linear dispersion and bandwidth

Linear dispersion defines the extent to which a spectral interval is spread out across the focal field and is expressed in nanometre per millimetre (nm/mm) – (or its inverse, the *reciprocal dispersion* in mm/nm). Linear dispersion is associated with an instrument's ability to resolve fine spectral detail. It depends on several parameters such as the focal distances and the widths of entrance and exit slits of the instrument. In general the better the dispersion the greater is the physical separation distance between two given wavelengths (Figure 14.11).

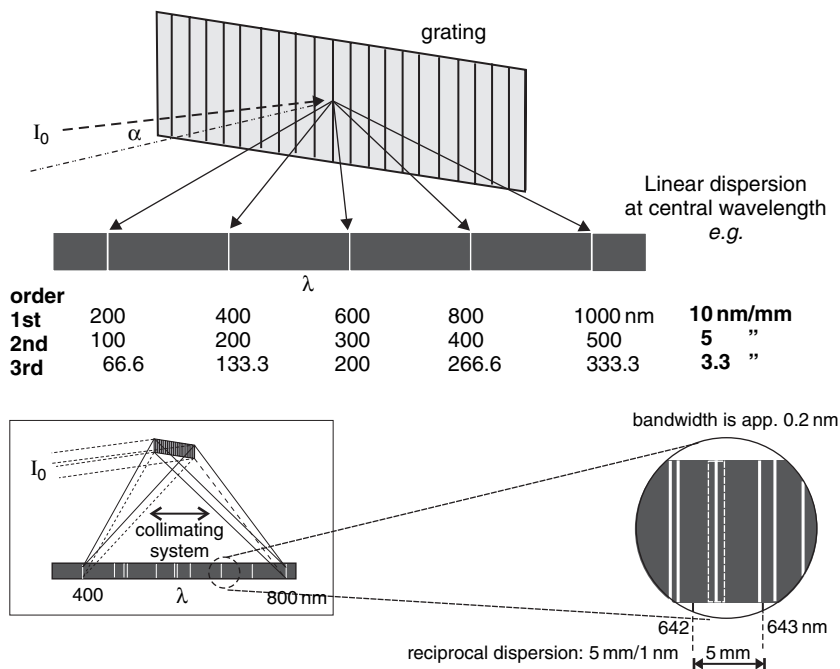


Figure 14.11 Linear dispersion and order of diffraction. Simplified schematics

For example if one instrument disperses a 0.1 nm spectral segment over 1 mm while the other takes a 10 nm spectral segment and spreads it over 1 mm, fine spectral details would be more easily identified with the first instrument than with the second.

The *bandwidth*, which must not be confused with the width of the slit, is the interval of the spectrum, in picometres, which corresponds to the width exiting from the slit. This interval is normally broader than the width of the line on which it is centred.

Thus, if the exit slit is 20 μm and if the reciprocal dispersion is of 5 mm/nm, the bandwidth will be equal to $1 \times (20 \times 10^{-3})/5 = 4 \times 10^{-3}$ nm, being 4 pm.

14.7 Applications of OES

The large number of elements measurable by OES renders this method of analysis indispensable since it permits the measurement of elements chosen in advance or the identification of elements present in any sample. Beyond industrial analytical applications – for example, monitoring the wear of car and jet engine motors, without disassembling them, by measuring the metals present in the oil – it is in the environmental area, without doubt, that analyses are the most widespread.

Applications to the analyses of vegetal or animal production (meat, milk), water, air (dust and ash discharges by incinerators), or of soils in which elements are present over a wide range of concentrations (spreading of industrial sludges on agricultural land) are also common. This methodology has equally a variety of applications in the forensic sciences or in clinical medicine (analysis of tissues or biological fluids). The advantage of OES is the linearity of response over a very large range of concentrations, which enables the treatment of complex matrices with a minimum of preparation. It is frequent that a single solution allows to pass from the measurement of one highly concentrated element to another, present only as a trace.

Detection by flame photometry has been used for a long time in GC for determination into compounds containing phosphorus or sulfur. Starting from this principle, high temperatures generated by gas plasmas can be employed to destroy the compounds eluting from the column while information concerning their elemental composition is collected by hyphenating an atomic emission spectrometer with the chromatograph (Figure 14.12). This is the GC/ICP-OES technique.

By choosing a specific line of an element, a selective chromatogram can be obtained of all of the compounds eluted that contain this element. This is the domain of speciation analysis, the search for classes of compounds corresponding to an association of several elements. This approach avoids interferences due to the sample matrices. It is disappointing that this method of identification of organic

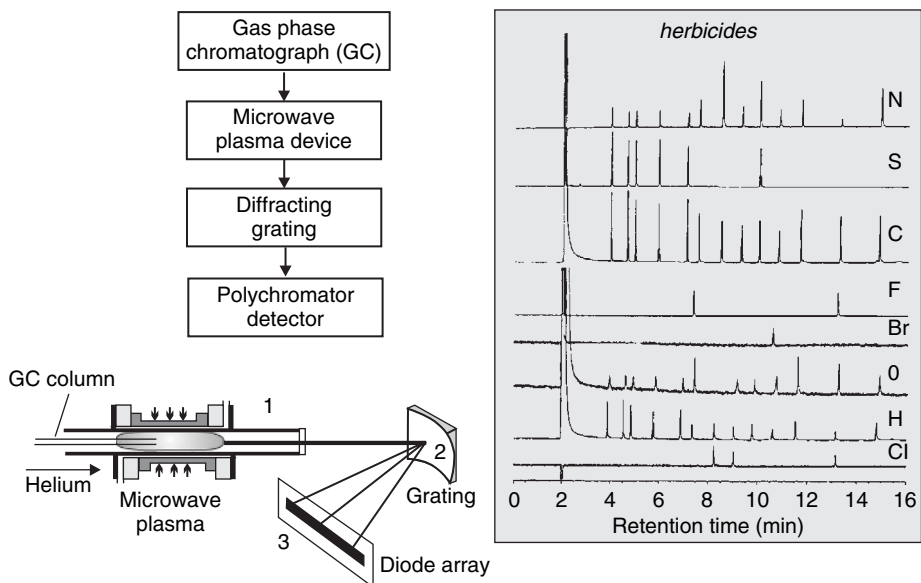


Figure 14.12 Coupling of a gas chromatograph with an atomic emission spectrophotometer. The effluent from the capillary column is injected into a plasma, which effects the decomposition into the corresponding elements. Each chromatographic signal corresponds to a compound containing the element being studied. This is the principle of speciation analysis (chromatograms taken from a Hewlett-Packard document).

molecules implies sample destruction, leaving only information concerning its elemental composition: during this ‘chemical butchering’ the structural information is lost.

Problems

- 14.1
1. Why do the elements which are measured lead to atomic emissions of relatively low intensity?
 2. Why does one generally observe ionic emissions?
- 14.2
- When an atom emits radiation, the frequency of the corresponding photon is linked to the energy of the levels between which the transition occurs. According to the Heisenberg principle, if the excited state has a lifetime of Δt , then this results in a minimum uncertainty for the energy ΔE , such that: $\Delta E \Delta t \geq h/2\pi$.

From this equation calculate the natural width of the 589 nm emission line for sodium if $\Delta t = 1$ ns.

- 14.3 Five standard solutions were prepared for measuring the lead concentration in two solutions, A and B. The two solutions A and B contain the same concentration of magnesium used as an internal standard. The following data were obtained:

Conc. (mg/L)	Emission of signal (arbit. units)	Signal of Mg
0.10	13.86	11.88
0.20	23.49	11.76
0.30	33.81	12.24
0.40	44.50	12.00
0.50	53.63	12.12
sol A	15.50	11.80
sol B	42.60	12.40

From the data, calculate the lead concentration (mg/L) in the two sample solutions, A and B.

- 14.4 The resonance level of the sodium atom is sometimes said to be $16\,960\text{ cm}^{-1}$ from the fundamental level. From this value calculate the corresponding wavelength and the energy of the transition associated with it.
- 14.5 Explain why it is easier to measure traces of radioactive, α -emitting isotopes of long half-life by ICP/MS rather than by radioactivity counting.
- 14.6 If the linear dispersion of a spectrograph used in OES is 2 mm for a difference in wavelength of 1 nm and the size of the exit aperture is $20\ \mu\text{m}$, calculate the spectral bandwidth reaching the detector.

15

Nuclear magnetic resonance spectroscopy

Following on from the pioneering work of physicists Bloch and Purcell around 1945 nuclear magnetic resonance (NMR) very quickly became a flexible and irreplaceable method of spectroscopy for a variety of areas in chemistry. NMR is a powerful and theoretically complex analytical tool that allows the study of compounds in either solution or in the solid state and serves equally in quantitative as in structural analysis, though it is especially in the latter where it is very efficient in gathering structural information concerning molecular compounds. It is therefore of particular practical importance to organic chemistry and biochemistry. Used as a complementary technique to methods of optical spectroscopy and mass spectrometry, it leads to precise information concerning the structural formula, stereochemistry and in some cases the preferred conformation of molecules and even to the identity of the compounds studied. For all of these reasons, NMR has become one of the principal study techniques for inorganic crystals as well as molecular structures. Although NMR has for a long time been considered as not sensitive enough to be adaptable to environmental analysis, this situation has changed, particularly in view of the linking of liquid chromatography with NMR spectroscopy.

15.1 General introduction

Nuclear magnetic resonance has given its name to a very remarkable method of solving the problems of structural determination for organic compounds and for certain types of inorganic material. NMR spectrometers are therefore often located in research laboratories although more trouble-free instruments exist exploiting the same principles for routine applications (see Figures 15.32 and 15.33, at the end of this chapter).

This method for studying matter can be described by choosing solely relevant examples from organic chemistry, the elucidation of molecular structures having

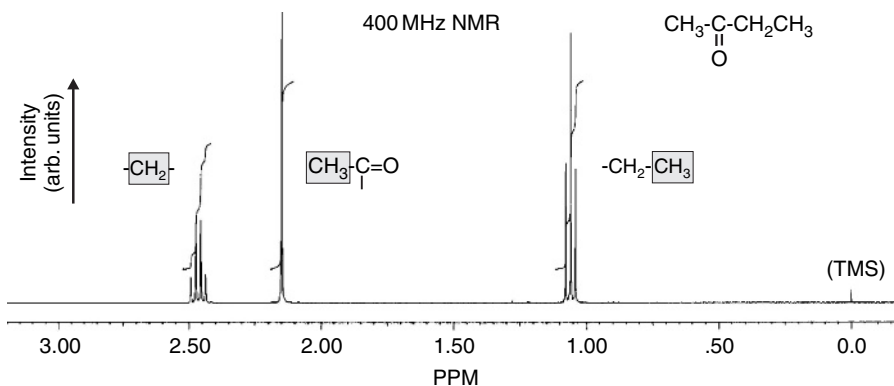


Figure 15.1 Conventional representation of the ^1H NMR spectrum of butanone [$\text{CH}_3(\text{C}=\text{O})\text{CH}_2\text{CH}_3$]. The corresponding integration superimposed over the peaks permits an evaluation of the relative areas of the signals. The meaning of the abscissa will be explained later on.

always served as a driving force for NMR development and to the numerous technical improvements effected since the origin of the technique.

NMR collects information concerning interactions between the nuclei of certain atoms present in the sample when they are submitted to a static magnetic field which has a very high and constant intensity and exposed to a second oscillating magnetic field. The second magnetic field, around 10 000 times weaker than the first is produced by a source of electromagnetic radiation in the radiofrequency domain.

The basic document delivered by these instruments is the *NMR spectrum* (Figure 15.1), a graph representing signals of resonance. It results from the absorption by the sample of definite frequencies sent by this electromagnetic source. The origin of these spectra, which are quite different from optical spectra, can be best understood by consideration of nuclear spins.

- The spectrum of Figure 15.1 is an example of one-dimensional NMR (the intensities of the signals not being counted as a second dimension). More sophisticated studies, in two, three and four dimensions enable a more precise localization of the position of all of the atoms in the molecule studied. This chapter only describes one-dimensional NMR (1D-NMR), for samples in solution.

15.2 Spin/magnetic field interaction for a nucleus

All atomic nuclei – as well as each particle – are characterized by a number of intrinsic parameters, spin \vec{I} being one of them. This vector quantity introduced by quantum mechanics is used to explain the behaviour of atoms in media where there is a preferential direction. A magnetic field creates just such an orientation

for all atoms within this field. The spin of the nucleus has the same dimensions (J s) as the kinetic moment \vec{L} of classic mechanics. The norm of the spin varies from one nucleus to another because it is defined from the spin quantum number I , a characteristic of each nucleus considered, whose value can be zero or a positive multiple of $1/2$.

An isolated nucleus with a non-zero spin number behaves like a small magnet with a magnetic moment $\vec{\mu}$ (JT^{-1}) such that:

$$\vec{\mu} = \gamma \cdot \vec{I} \quad (15.1)$$

This *nuclear magnetic moment* $\vec{\mu}$ is represented by a vector co-linear to \vec{I} (Figure 15.2), and has the same or the opposite direction, according to the sign of γ the *gyromagnetic ratio* (also called the *gyromagnetic constant*).

If an isolated nucleus with a non-zero spin number, which can be compared to a kind of microscopic magnetic needle, is submitted to a magnetic field \vec{B}_0 which makes an angle θ with the spin vector, an interactive coupling occurs between \vec{B}_0 and $\vec{\mu}$; this modifies the potential energy E of the nucleus.

If $\vec{\mu}_z$ represents the projection of $\vec{\mu}$ on the z -axis, which, by convention, is oriented as \vec{B}_0 , then the energy of the interaction (also called the Hamiltonian) is:

$$E = -\vec{\mu} \cdot \vec{B}_0 \quad \text{or} \quad E = -\mu \cos(\theta) B_0 \quad \text{or, finally} \quad E = -\mu_z \cdot B_0 \quad (15.2)$$

According to quantum mechanics, μ_z , for a nucleus, can only have $2I + 1$ different values. This means that in a magnetic field \vec{B}_0 , the potential energy E can also only take $2I + 1$ values. The same goes for angle θ . The quantification

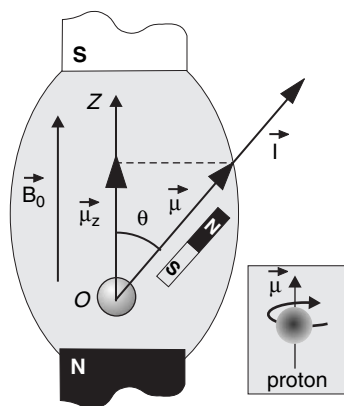


Figure 15.2 Basic NMR vectors. When a spinning nucleus is placed in a magnetic field, the nuclear magnet experiences a torque which tends to align it with the external field. In the conventional NMR coordinate system, the external magnetic field is along the z -axis.

of μ_z are given by the magnetic spin number m which can take the values (in $h/2\pi$ units) expressed by the following series:

$$m = -I, -I + 1, \dots, I - 1, I$$

By using relations 15.1 and 15.2, the $2I + 1$ allowed energy values are given by the following relations:

$$E = -\gamma \cdot m \cdot B_0 \quad (15.3)$$

For $I = 1/2$, the two possible values of E (in joules, and subscripted as α and β) correspond to $m = +1/2$ and $m = -1/2$. They are given as follows:

$$E_1(\text{or } E_\alpha) = -\gamma \frac{1}{2} \frac{h}{2\pi} B_0 \quad \text{and} \quad E_2(\text{or } E_\beta) = +\gamma \frac{1}{2} \frac{h}{2\pi} B_0$$

This splitting in several energy levels recalls the Zeeman effect (cf. Chapter 14) which concerns the separation of electronic states in a magnetic field. It is sometimes referred to in NMR as the Zeeman nuclear separation.

The quantification of the spin vector projection along the z -axis has for effect that \vec{I} traces the surface of a cone of revolution with an angle between the axis and atom that can be calculated if $\vec{\mu}$ and $\vec{\mu}_z$ are known. This *precession movement*, around an axis parallel to that of the magnetic field (Figures 15.3 and 15.5a) is characterized by a frequency which increases with the field intensity.

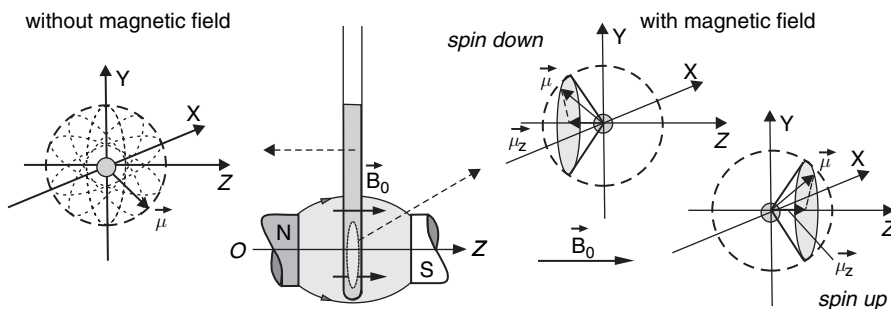


Figure 15.3 Effect of a magnetic field upon a nucleus of spin number $1/2$ for an atom in a compound in solution. If the nucleus is in the upper part of the sample tube, i.e. not submitted to the external magnetic field, $\vec{\mu}$ has no preferential orientation with time. Alternatively, when the nucleus is in the strong magnetic field of the central section, it lines up, precessing in the direction of the applied field. The projection of $\vec{\mu}$ is in the same or the opposite direction to \vec{B}_0 . Unfortunately, here and next figures the dynamic concepts of NMR spectroscopy are difficult to understand with static diagrams.

■ To give a concrete idea of nuclear spin, allusion is often made to the movement of a spinning top rotating around its axis, which makes at a given instant, an angle θ with respect to the direction of the Earth's gravitational field. The spinning top describes a gyroscopic movement about this direction which arises from the combination of its movement of rotation about its own axis and coupling with the gravitational vector. As friction slows down the rotation, the angle θ will increase continually. In contrast to the principle of the spinning top, for a nucleus in a vacuum, the angle is maintained with time, whatever the value of the applied field, because the values of the magnetic moment and of its projection are quantized. The rotational axis of the spinning nucleus cannot be orientated parallel, or anti-parallel (although these terms are used for nuclei) with the direction of the applied field \vec{B}_0 . Therefore, for a nucleus with a spin number $I = 1/2$, calculation leads to the only angles permitted for \vec{I} with respect to z-axis which are approximately 55° and 125° . This takes into account the value of the spin total angular momentum ($\sqrt{I(I+1)}$), and that of the intrinsic angular momentum (I). The angle of 54.74° , used in certain NMR experiments is called the 'magic angle'.

15.3 Nuclei that can be studied by NMR

In a nuclide represented by A_ZX , the nucleus will have a non-zero spin number I , if the number of protons Z and the number of neutrons A , are not both even numbers. ${}^1_1\text{H}$, ${}^{13}_6\text{C}$, ${}^{19}_9\text{F}$, ${}^{31}_{15}\text{P}$ have, for example, a spin number $I = 1/2$ while $I = 1$ for ${}^2_1\text{H}$ (deuterium D) or ${}^{14}_7\text{N}$. All these nuclei will give an NMR signal. Alternatively, the nuclei ${}^{12}_6\text{C}$, ${}^4_2\text{He}$, ${}^{16}_8\text{O}$, ${}^{28}_{14}\text{Si}$, ${}^{32}_{16}\text{S}$ which have a spin number of zero, cannot be studied by NMR. In fact, more than half of the stable nuclei known (at least one isotope per element) leads to an NMR signal although the sensitivity varies enormously depending upon the nucleus.

Hence, the protons (the common name for ${}^1\text{H}$), or ${}^{19}\text{F}$, are easier to detect than ${}^{13}\text{C}$, which is much less sensitive and also for the reason that it represents only 1 per cent of elemental carbon.

15.4 Bloch theory for a nucleus of spin number $I = 1/2$

At a macroscopic scale, even the smallest quantity of a compound is composed of a gigantic number of individual molecules. The number of nuclei being so high, the NMR signal reflect their statistical behaviour, as in optical spectroscopy.

Let us consider a collection of identical nuclei whose spin number is $I = 1/2$. In the absence of an external magnetic field, the individual spin vectors will be randomly oriented and vary constantly with time. From an energy point of view, these nuclei form a population that is considered a *degenerated* state (Figure 15.3).

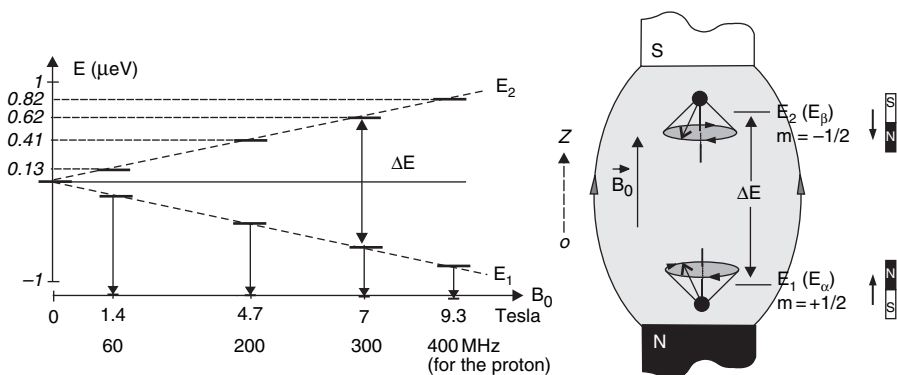


Figure 15.4 Representation of the energy split for a nucleus with a spin number $I = 1/2$ placed into a magnetic field. The parallel alignment (E_1) is slightly lower in energy than the antiparallel one (E_2). The four values chosen for the field \vec{B}_0 correspond, for the proton, as it will be seen later, to commercial instruments of 60, 200, 300 and 400 MHz respectively. \vec{B}_0 represents the magnetic field strength, measured in Tesla (T). The common values are above 1 T. For comparison, the Earth's magnetic field is approximately 5×10^{-5} T.

When these nuclei are placed in a strong external magnetic field \vec{B}_0 (OZ orientation) an interaction will occur between each individual nuclear magnetic vector and this field.

There appears therefore two groups of nuclei according to the direction of their spin vector along the z-axis, whose energies correspond to low energy state E_1 and to high energy state E_2 , as previously defined (Figure 15.4). These orientations are called 'spin up' (magnet image N-S-N-S) and 'spin down' (N-N-S-S).

The difference in energy ΔE between the two states is:

$$\Delta E = E_2 - E_1 = \gamma \frac{h}{2\pi} B_0 \quad (15.4)$$

ΔE is proportional to the field \vec{B}_0 (Figure 15.4). Thus, for the proton (^1H), if $B_0 = 1.4$ T, the difference in energy is very small: 3.95×10^{-26} J or 2.47×10^{-7} eV. The ratio $(E_2 - E_1)/B_0$, depends only upon the value for γ , for the nucleus being studied (Table 15.1).

Table 15.1 Values of γ for the most commonly studied nuclei in NMR

Nuclei N	γ (rad · s ⁻¹ · T ⁻¹)	Sensitivity
^1H	2.6752×10^8	1
^{19}F	2.5181×10^8	0.83
^{31}P	1.084×10^8	6.6×10^{-2}
^{13}C	0.67283×10^8	1.8×10^{-4}

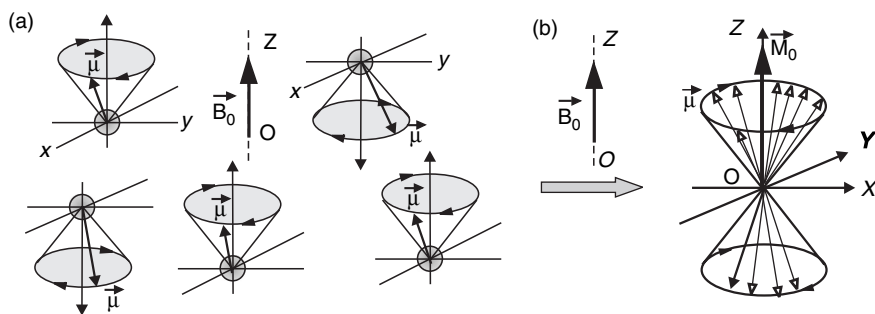


Figure 15.5 *Precession and magnetization.* (a) A snapshot illustrating the precession movement of the spin vector of five independent nuclei in a magnetic field; (b) The macroscopic magnetization vector which results from the individual orientations of a large number of nuclei. If $B_0 = 1.5\text{ T}$, there are less than 10 spin-up more than spin-down per million ^1H nuclei. The values of this very weak net magnetization depends on the excess of population E_1 versus E_2 . By convention the external magnetic field and the net magnetization vector at equilibrium are both along the z-axis.

The population of nuclei located in energy state E_2 , is slightly less numerous than that in the more stable state E_1 . Expression 15.5 calculates the ratio of these two populations (Boltzmann distribution equilibrium).

$$R = \frac{N_2}{N_1} = \exp \left[-\frac{\Delta E}{kT} \right] \quad (15.5)$$

(for ^1H , $R = 0.999964$ if $T = 300\text{ K}$ and $B_0 = 5.3\text{ T}$ where $k = 1.38 \times 10^{-23}\text{ J K}^{-1}\text{ at}^{-1}$).

As a result, it is only the *slight excess* in population E_1 which is responsible for the NMR signal. By increasing the value of the magnetic strength of the magnet, the difference ΔE will be greater and thus sensitivity will increase since the signal is proportional to the populations difference.

On a graphic representation, the weak magnetization of the sample solution is accounted for by using the vector \vec{M}_0 , which is the vector sum of all of the individual vectors $\vec{\mu}$ (Figure 15.5). At equilibrium, this vector lies along the direction of the applied magnetic field \vec{B}_0 and is called the equilibrium magnetization or *net magnetization*. \vec{M}_0 is used to describe pulsed NMR, and to explain the appearance of signals when nuclei enter resonance. \vec{M}_0 is an entity to which the laws of electromagnetism can be applied.

15.5 Larmor frequency

From an analytical point of view, a nuclide can be identified if ΔE , in a field \vec{B}_0 , could be measured (assuming $I = 1/2$), by knowledge of the gyromagnetic

constant γ . As in optical spectroscopy, this difference in energy can be determined under conditions where species can pass between one state and the other, by absorption or emission of a photon.

A basic experiment consist of irradiating the nuclei present in a magnetic field with a source of electromagnetic radiation of variable frequency ν with a propagation direction perpendicular to the external field. Absorption will occur if:

$$h\nu = \Delta E = E_2 - E_1 \quad (15.6)$$

The energy of this photon must exactly match the energy difference between the two states. Expression 15.4 leads to the fundamental relation of resonance (15.7):

$$\nu = \frac{\gamma}{2\pi} B_0 \quad (15.7)$$

This important and general expression, irrespective of the value of I , gives the frequency *Larmor frequency*. It links the magnetic field in which the nuclei being studied are located and the frequency of electromagnetic radiation that induces resonance, hence a signal in the spectrum when the lower energy nuclei spin-flip to the higher state (Table 15.1).

The radiofrequency which induces the exchange between the two levels is equal to the Larmor frequency at which the spin vector rotates about the average direction of the z -axis. Larmor, an Irish physicist whose work preceded that of NMR has shown, by an independent reasoning that ω , the angular rotation frequency of the spin vector about the z -axis, has a value of:

$$\omega = \gamma \vec{B}_0$$

This is the Larmor equation. Since $\omega = 2\pi\nu$, this expression is equivalent to Equation 15.7. The two approaches are different: one leads to the frequency of the quanta which separates both energy states and the other to the mechanical precession frequency. These two frequencies have the same value.

The Larmor frequency of a given nucleus increases with \vec{B}_0 . It is situated in the microwave region of the electromagnetic spectrum and varies, for a field of 1 T, from 42.5774 MHz for the hydrogen ^1H nucleus (proton) to 0.7292 MHz for gold $^{197}_{79}\text{Au}$ (Table 15.2). The instruments are specifically designed for the frequency at which protons resonate, even if they are able to study other nuclei.

Table 15.2 Values in MHz of some resonance frequencies for $\vec{B}_0 = 1 \text{ T}$

Nuclei	^1H	^{19}F	^{31}P	^{13}C	^{15}N
Frequency ν	42.58	40.06	17.24	9.71	4.32

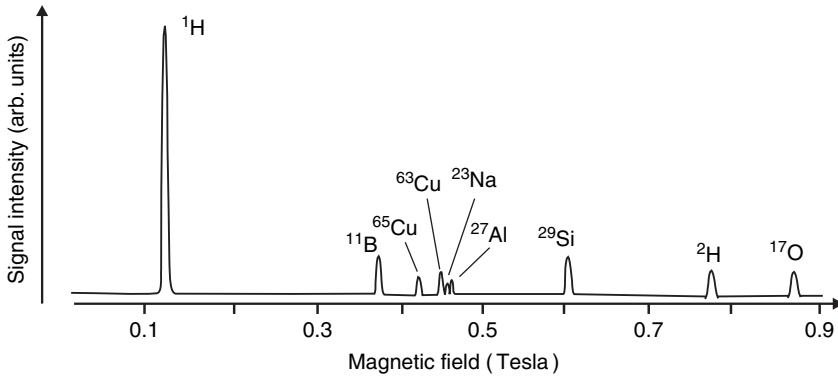


Figure 15.6 NMR spectrum of a water sample placed in a borosilicate glass container, observed at a frequency of 5 MHz (the field is expressed in gauss; Varian document). There are no commercial instruments of this type because NMR is not sensitive enough to solve many of the problems encountered in elemental analysis.

The construction of experimental multi-nuclei instruments was initiated from these principles. These instruments can sweep over a wide range of the field while maintaining a fixed radiofrequency. This leads to a qualitative screening of the easiest elements to detect within the sample through their characteristic resonance (Table 15.2 and Figure 15.6).

15.6 Pulsed NMR

\vec{M}_0 that is shrouded in the strong magnetic field \vec{B}_0 is too weak to be measured. So, to get a signal, resonance of the nuclei is obtained by superimposing upon \vec{B}_0 , in the probe area, a weak oscillating field \vec{B}_1 which originates from a coil that is fed with alternating current radiofrequency.

To understand the interaction of the nucleus with the field \vec{B}_1 in the laboratory frame of reference, it is considered as resulting from the composition of two vectors rotating in opposite directions (clockwise and counter clockwise) in the xy -plane with the same angular velocity (Figure 15.7). If the frame of reference rotates with the precession frequency, the vector component that also rotates clockwise in the laboratory frame of reference will appear stationary. The second vector component that rotates counter clockwise is moving around the z -axis far from the resonance frequency of the spins. It can be ignored. Only the vector turning in the same direction as the precession movement can interact with the nucleus. As transverse magnetization rotates about the z -axis, it will induce a current in a coil of wire located in the xy -plane.

For \vec{B}_1 to be suitably aligned, the propagation axis of the radiofrequency needs to be perpendicular to the z -axis (Figure 15.8). The transfer of energy from the source

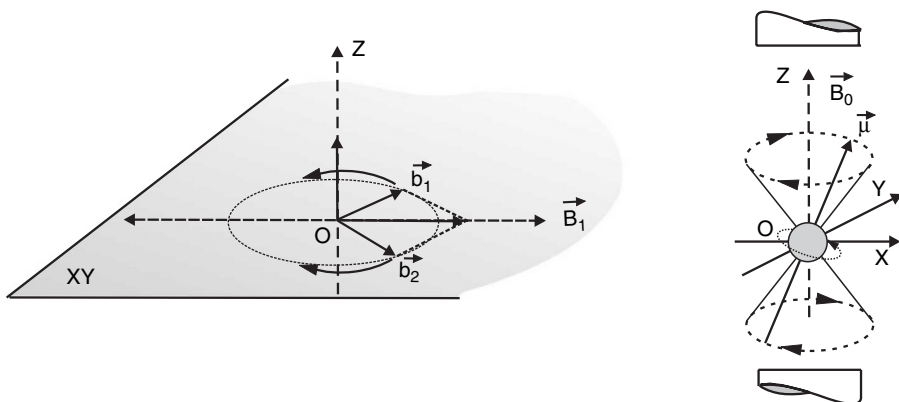


Figure 15.7 Representation of magnetic field \vec{B}_1 . In all points of the plane xy the magnetic component of the wave can be dissociated into two half vectors \vec{b}_1 and \vec{b}_2 rotating in phase with opposite velocities. Here, only \vec{b}_2 can interact with the nuclei of population E_2 .

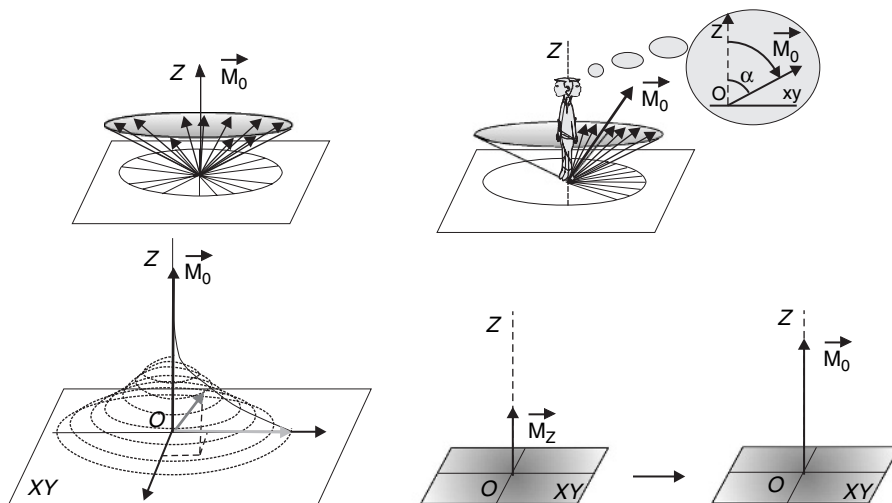


Figure 15.8 Deviation of \vec{M}_0 with irradiation and relaxation of the transverse and longitudinal relaxations of the system following resonance. Schematic representation in which the individual vectors form a spin packet resulting from the non-equilibrium of the populations are shown (laboratory frame of reference). \vec{B}_1 has a double action, flipping the nuclei and packing them in phase. An observer situated in a rotating frame at the precession frequency, would see the magnetization vector tilted by an angle α . Relaxation of \vec{M}_0 to its original position. If $\vec{M}_z = 0$, the spin system is saturated. As transverse magnetization rotates about the z -axis, it will induce a current in a coil of wire located around the x -axis. Plotting current as a function of time gives a sine wave.

to the nuclei occurs when the radiofrequency of the source and the frequency of precession are identical. Under these conditions the spin of some nuclei adopts the second alignment allowed (in the case where $I = 1/2$). Nuclei in energy state E_1 pass to energy state E_2 : in this way, the populations are modified. Saturation is attained when they become equal.

Before irradiation, the individual vectors $\vec{\mu}$ are out of phase with respect to each other, which is represented by the vector \vec{M}_0 , aligned in the direction OZ (Figure 15.5). As the resonance condition is reached, all the individual vectors *pack together and rotate in phase* with \vec{B}_1 . Thus, the direction of \vec{M}_0 changes by an angle α (relative to z -axis), which can be controlled from 0 to π , by adjusting the time and the intensity of the applied radiation. Figure 15.8 illustrates this phenomenon with several individual vectors which join together. It is for this reason that \vec{M}_0 acquires a \vec{M}_{XY} component in the horizontal plane which is maximum when $\alpha = \pi/2$ and conserves a variable component \vec{M}_Z in the z -axis direction (except when $\alpha = \pi/2$). The frequency of the rotation of the magnetization vector is always equal to that of the precession movement. When the irradiation ceases the system returns progressively to the initial state of equilibrium. A coil detects the component in the y -direction (Figure 15.8).

What happens in the probe of the apparatus? A common interpretation suggests imagining an external observer linked to a rotating frame about the z -axis at the frequency of precession of the concerned nuclei (Figure 15.8). For example, let us assume the case of a single precession frequency. When the radiofrequency has this particular value, the nuclei seem, to the observer, to feel only the effects of the rotating component of the field (i.e. perpendicular to z -axis). The magnetization vector deviates until an angle α . After the pulse the individual magnetic moments lose their phase coherence through interaction with the spins of the neighbouring nuclei, faster than they can re-align themselves according to the Boltzmann distribution function which described the populations before the pulse. Thus, \vec{M}_0 loses its xy -plane component, without having returned to its initial value in the OZ direction, which can take a longer period of time.

■ The actual process for obtaining the whole range of frequencies by computation did not exist when the first generation of NMR machines were built. In the earliest NMR instruments, the former approach consisted of obtaining resonance by slightly modifying \vec{B}_0 using a coil wrapped around the pole piece of the magnet (Figure 15.9). Because these instruments used to sweep the magnetic field, they operated at a single frequency (CW-NMR). The spectrum was nevertheless recorded in Hz since the Larmor equation correlates field with frequency. This form of absorption NMR can also be performed with a constant magnetic field and a frequency which is varied. This process for finding the signal is similar to the sequential method of recording optical spectra, or even in daily life, when seeking an FM radio station on an analogue receiver transistor.

In this mode of acquisition, the quality of the spectrum depends on a small fraction of the total recording time. Fourier transform spectrometers make much better use of the recording time.

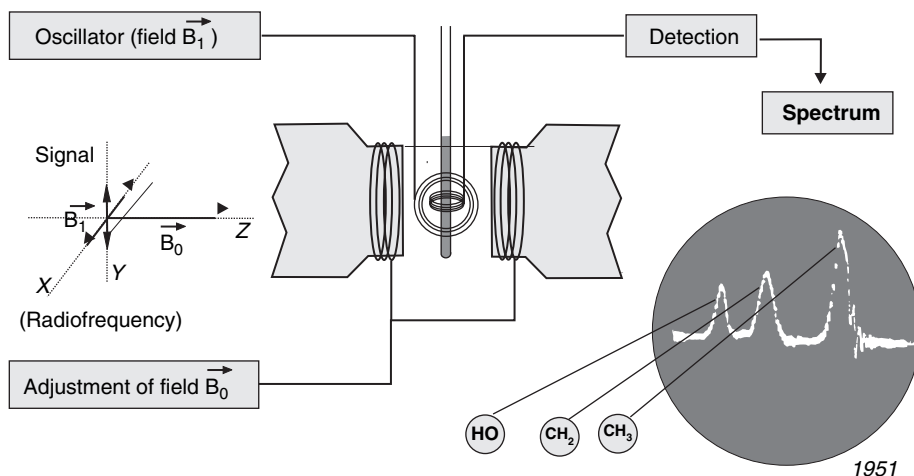


Figure 15.9 CW-NMR. Arrangement of the different coils around the probe between the poles of the magnet. Signal is detected along x or y axes. Right, an historic recording (1951) of the proton spectrum of ethanol. The three signals correspond to the six hydrogen atoms of CH₃, CH₂ and OH. This will be better understood after reading Section 15.8.

Technically speaking, the sample is irradiated for a few microseconds with an intense pulse, which is a means of exposing the sample to all frequencies included in the domain to be sampled. This can be compared to a flash of white light (comparing a polychromatic light with respect to a monochromatic source). For example, when working at 300 MHz, in order to irradiate all of the protons whatever their environment, a frequency range covering at least 6000 Hz is required. Under these conditions a small fraction of each type of proton (but not all the nuclei), will absorb the resonance frequency.

To find a schematic representation for a compound absorbing tens of frequencies is obviously impossible. In such cases, \vec{M}_0 can be dissociated into many vectors, each of which precesses around the field with its own frequency (Figure 15.8, for a simplified situation). During the return to equilibrium, which can take several seconds, the instrument records a complex signal due to the combination of the different precession frequencies present, of which the intensity decreases exponentially with time (Figure 15.10).

This damped interferogram is called *free induction decay* or FID. The signal corresponds at each instant to an overall numerical value which informs on the frequencies of the nuclei that have attained resonance at that particular instant. These values can be transformed by Fourier calculations that converts the signal from the *time domain* into a classically spectrum of the *frequency domain*.

This *pulsed wave* technique, is a simultaneous method in the sense that the apparatus acquires, at each instant, information concerning all of the frequencies present. The major developments of current NMR result from the generalization of this procedure which permits the study of less sensitive nuclei, such as ¹³C.

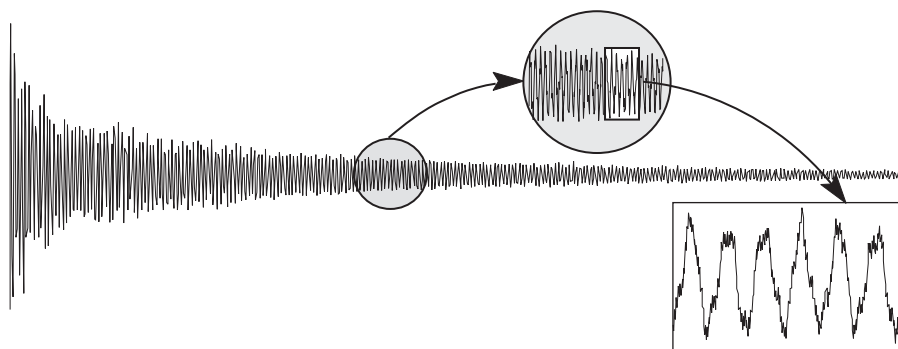


Figure 15.10 Interferogram of fluoroacetone obtained with a pulsed wave NMR instrument. The signal $I=f(t)$, after conversion to a classic spectrum $I=f(\nu)$ by Fourier transform, is that of the spectrum shown in Figure 15.18. The interferogram, recorded over of a few seconds can be accumulated many times to improve the signal/background noise ratio. The computer does not record a continuous FID, but a FID that is sampled at a constant interval.

15.7 The processes of nuclear relaxation

After the pulse of radiofrequency, \vec{M}_0 will regain its equilibrium value after a relaxation time which is depending upon the element and the medium (Figure 15.11). This relaxation period depends on both the reconstitution of the populations to their initial state (time constant T_1), named *longitudinal relaxation* (spin lattice relaxation) and on the loss of phase coherence (time constant T_2), called *transverse relaxation* (spin–spin interactions). Each of the spin packets is experiencing a slightly different magnetic field and rotates at its own Larmor frequency. In solution, T_1 is never longer than a few seconds (for ^1H), while it

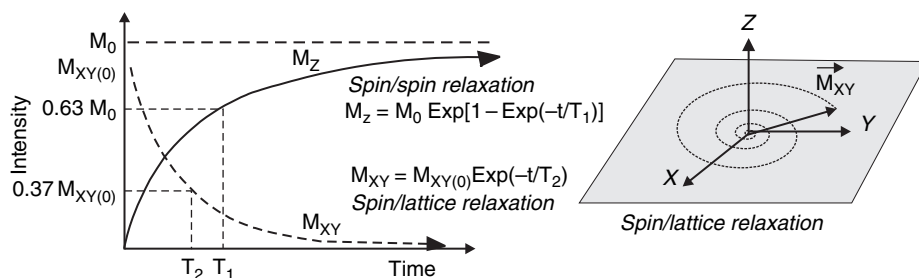


Figure 15.11 The two processes of nuclear relaxation. Evolution over time of spin–spin and spin/lattice relaxation. T_1 is defined as the time required to change the z-component of magnetization by a factor of e . The greater the rigidity of the medium, the smaller T_2 will be. T_2 is always less than or equal to T_1 . The net magnetization in the xy-plane goes to zero and then the longitudinal magnetization grows in until to get \vec{M}_0 along the z-axis.

can be as long as several hours with solids. These two components of \vec{M}_0 must therefore be dissociated. Knowledge of the lifetimes of T_1 and T_2 yields very useful information on the structure of the samples analysed. T_1 decreases when the viscosity of the medium increases, which causes signal broadening. Changes in T_2 also has an effect upon the width of signals. A neat liquid compound leads to broader signals than they would be if the compound was in a dilute solution.

15.8 Chemical shift

So far as NMR is concerned, molecules present in dilute solutions form independent entities that do not interact noticeably between themselves. Alternatively, within a given molecule, the electronic and steric environment of each nucleus creates a very weak local magnetic field which shields it more or less from the action of the external field \vec{B}_0 . Each atom has a particular environment – at least if there are no specific elements of symmetry in the molecule. According to the Larmor equation, these very weak local variations in the intensity of the field will affect the frequency of resonance as compared to what would be seen in a vacuum. This effect is called *shielding* or *deshielding* of the nuclei.

This observation is the basis of the principal exploitation of NMR: rather than observing all of the nuclei present, spread over a wide range of frequencies (several tens of megahertz), the study focuses on a single type of nucleus at a time. In other words the technique zooms over a very narrow range of frequencies (for example 1000 Hz) in order to record the different signals which result from specificities of each compound (Figure 15.1). This screening effect is quantified by the *shielding constant* σ which appears in equation 15.8 linking the effective field B_{eff} which reaches the nucleus with the external field B_0 :

$$B_{\text{eff}} = B_0(1 - \sigma) \quad (15.8)$$

All variations in σ affect the resonant frequency of the corresponding nucleus. This phenomenon is called a *chemical shift*. As many chemical shifts are observed as there are molecules containing different shielding constants σ . Consequently large molecules lead to complex spectra as a result of their numerous σ values. For a nucleus i for which $I = 1/2$, the Larmor equation becomes, on the introduction of the effective field B_{eff} :

$$\nu_i = \frac{\gamma}{2\pi} B_0(1 - \sigma_i) \quad (15.9)$$

Concerning the proton ^1H , expression 15.9 leads to a difference of 1 Hz for the resonance frequency when the surrounding field varies of 2.3×10^{-8} T, while a variation of 10^{-4} T (1 gauss) induces a shift of 4258 Hz. For this reason the setting

of the magnet must be perfectly controlled in order to maintain the stability of the field B_0 in the NMR instrument. Thus, the temperature has to be controlled to within a 1/100th of a degree. Furthermore, the sample is rotated in a tube with very thin walls to reduce the heterogeneity of the field. Considering these constraints, the construction of mobile NMR instruments is a real achievement (Figure 15.33).

15.9 Measuring the chemical shift

According to the Larmor equation, the slightest variation in the field has repercussions on the resonance frequencies. Thus, it would be inadvisable to try to compare spectra or identify compounds using the absolute signal frequencies obtained with different instruments. For this reason the chemical shift is referenced by a relative scale $\Delta\nu/\nu$, which is independent of the instrument. That is why a compound acting as an internal standard is used and can therefore serve as a fixed reference, as well as the frequency ν_{app} of the instrument. For the nucleus considered, the different chemical shifts can then be obtained by dividing the frequency difference $\Delta\nu$ between each signal of the compound studied and that of the standard, by the characteristic frequency ν_{app} of the instrument (expression 15.10).

The values obtained are expressed in parts per million (ppm). In order to calculate the chemical shift δ_i (ppm) corresponding to a signal of frequency ν_i with respect to an internal standard (ν_{ref}), there is no need to know the absolute frequencies, only $\Delta\nu$ is necessary.

$$\delta_i = \frac{\nu_i - \nu_{\text{ref}}}{\nu_{\text{app}}} \cdot 10^6 = \frac{\Delta\nu}{\nu_{\text{app}}} \cdot 10^6 \quad (15.10)$$

The internal standard used for both ^1H and ^{13}C NMR is tetramethylsilane (TMS). This inert and volatile compound (boiling point = 27°C), yields a single signal in both ^1H NMR (for the 12 equivalent protons) and in ^{13}C NMR (for the four equivalent carbons). TMS signal is positioned at origin of the δ scale (Figure 15.11). Almost all organic compounds have positive values for δ , ranging for ^1H from 0 to 15, to 250 for ^{13}C .

- The TMS signal is located on the right-hand of the spectrum, respecting the convention according to which, in spectroscopy, the energy parameter, positioned along the abscissa, decreases when moving towards the right of the spectrum.

The δ scale has enabled the generation of empirical correlation tables based on the chemical shifts as a function of chemical structure, which can be used irrespective of the NMR instrument (Tables 15.5 and 15.6 at the end of this chapter). Expression 15.10 shows that if the spectra of the same compound,

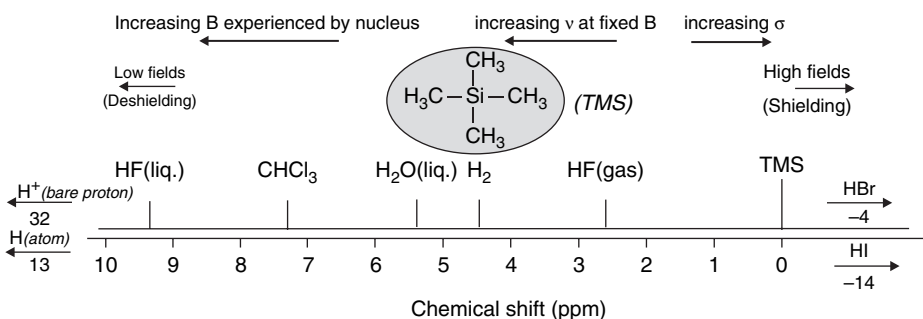


Figure 15.12 Chemical shifts of certain compounds in proton NMR. Shielding effects recorded with a fixed frequency instrument.

recorded with two instruments whose fields B_0 make a ratio between them of k are compared, then the frequencies of the homologous signals for the two spectra will be in this same ratio k , the values of δ remaining the same. However, these tables are not always sufficient to make a correct attribution of the signals. Software programs have been developed to help in this regard.

15.10 Shielding and deshielding of the nuclei

The more marked the *screening* effect, the more the nuclei are said to be *shielded*: in order to obtain resonance the value of the field \vec{B}_0 must be increased, at least when referring to a continuous wave instrument operating at fixed frequency. Signals situated to the right of the spectrum are said to be resonant at *high field* and by contrast signals observed to the left of the spectrum correspond to deshielded nuclei and are said to be resonant at *low field* (Figure 15.12).

15.11 Factors influencing chemical shifts

Examination of a large number of NMR spectra allows to highlight the factors that are responsible for predictable and cumulative effects on the chemical shifts.

15.11.1 Effects of substitution and hybridization

The simple replacement of a hydrogen atom by a carbon-containing group R produces a deshielding of the remaining protons. This substituent chemical shift reaches 0.6 ppm when comparing RCH_3 to CHR_3 . This effect can attain 40 ppm for ^{13}C NMR. The state of hybridization of the carbon atoms greatly influences also the position of the signals.

These *anisotropic* variations are due to the chemical bonds, that is, to the non-homogeneous electronic distribution around bonded atoms, to which can be added the effect of unimportant magnetic fields induced by electron circulation. Ethylenic protons are therefore deshielded as they are positioned in an electron-poor plane. Inversely, acetylenic protons are shielded, because they are located in the axis of the C–C bond and so, plunged into an electron-rich environment. Aromatic protons are displaced towards lower fields because, as well as the anisotropic effect, a local field produced by the movement of the aromatic electrons or the ‘ring current,’ is superimposed to the main magnetic field (Figure 15.13).

15.11.2 Resonance and inductive effects

The chemical shifts of organic compounds are sensitive to the delocalization of electrons bonding. Particularly, the existence of mesomeric forms provokes large shifts of the signals (Figure 15.14).

Thus, it is comprehensible that the electronic effects which modify the polarity of the bonds have an influence upon the chemical shifts. Table 15.3 illustrates the effect of electronegativity upon the position of the methyl signal in the halomethane series $\text{CH}_3\text{-X}$ where the chemical shift of the carbon atom signal increases with the electronegativity of the halogen X.

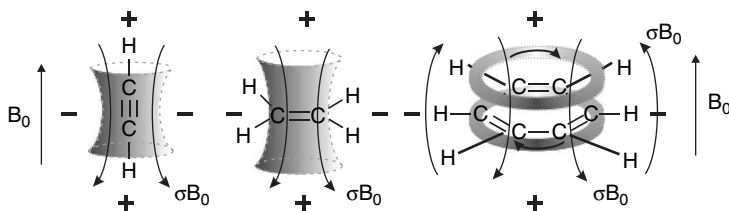


Figure 15.13 *Anisotropic effects and induced local fields.* The presence of π -bonding can be translated as zones of either a (+) shielding or a (-) deshielding effect. The ethylenic protons are at the outside of a kind of double cone of protection while the aromatic protons undergo the effect of the electrons moving in two parallel circular clouds.

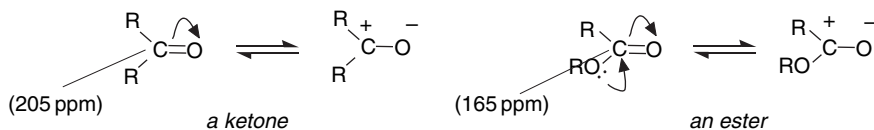


Figure 15.14 *Effects on NMR signals of the resonance effect in mesomeric forms of a carbonyl.* If the carbonyl of a ketone is compared with that of an ester, then it should be noted that for the ketone, the more electropositive of the two, the carbon has less protection than the ester. In ^{13}C NMR the carbonyl signal for a ketone is around 205 ppm while for an ester it is around 165 ppm.

Table 15.3 Influence of the electronegativity χ^* of a halogen on δ (TMS ref.)

	CH ₃ F($\chi = 4$)	CH ₃ Cl($\chi = 3.2$)	CH ₃ Br($\chi = 3$)	CH ₃ I($\chi = 2.6$)
δ_{H} (ppm)	4.5	3	2.7	1.3
δ_{C} (ppm)	75	30	10	-30

* electronegativity λ in eV according to the pauling scale

15.11.3 Other effects (solvents, hydrogen bonding and matrix)

¹H NMR spectra of organic compounds are usually obtained in solvents not containing hydrogen atoms. The solvent, far more abundant than the solute of which the concentration is only of several percent, leads to associations of which the stability depends upon the respective polarities. Consequently, the sample concentration and solvent used must be provided with the correlation tables.

The most widely used solvent is deuterated chloroform (CDCl₃) which is sufficiently polar to dissolve the majority of organic compounds. Also used are acetone-*d*6(C₃D₆O), methanol-*d*4(CD₃OD, pyridine-*d*5(C₅D₅N) or heavy water (D₂O).

■ In ¹H NMR, the position of the chloroform signal in mixtures chloroform/toluene varies from 7.23 ppm (90 per cent chloroform/10 per cent toluene v/v.) to 5.86 ppm (10 per cent chloroform/90 per cent toluene). This shift towards a higher field, for toluene-rich mixtures rich is explained by the presence of complexes causing the proton of chloroform to be located in the protecting zone of toluene's aromatic nuclei.

When the studied compounds have labile hydrogen atoms, D ↔ H exchanges can occur in certain solvents. These exchanges will cause alterations to the intensity and position of the relevant signals. Likewise, hydrogen bonding is able to modify the electronic environment of some protons making their chemical shift sometimes difficult to predict.

Finally, interactions between neighbouring molecules and also the effect of viscosity will alter the resolution of the spectrum through the spin/lattice relaxation time.

15.12 Hyperfine structure – spin–spin coupling

As a general rule, NMR spectra contain more signals than there are nuclei with different chemical shifts. This phenomenon is due to the external magnetic field which influences all of the atoms of the sample compound, causing an alignment of all nuclear spin and that the orientation taken in the magnetic field by one

nucleus affects the neighbouring nuclei, through valence electrons. If the distance between non-equivalent nuclei is less than or equal to three bond lengths, this effect called spin–spin coupling or J coupling is observable. *Homonuclear coupling* (between nuclei of the same type) or *heteronuclear coupling* (between nuclei of different types, as $^1\text{H}/^{13}\text{C}$) gives rise to small shifts in the signal. This hyperfine structure of the spectrum brings additional information about the compound which is studied. Homonuclear coupling between protons is quite frequent. The presence of ^{13}C , ^{31}P and ^{19}F leads also to heteronuclear couplings with the protons.

■ This phenomenon must not be confused with the interaction through distance between two nuclei which exchange their magnetization because the structure of the molecule is such that they are in close proximity in space, although a great number of bonds separate them. This is the Nuclear Overhauser effect which manifests itself by modifications in signal intensities.

15.13 Heteronuclear coupling

15.13.1 Hydrogen fluoride: a typical heteronuclear coupling

Hydrogen fluoride (HF) is a simple molecule that allows the observation of a spin–spin heteronuclear coupling between the two atoms that are linked by a covalent bond. When the ^1H spectrum of this compound is examined, two signals of equal intensity are observed though the molecule itself contains only one hydrogen atom (Figure 15.15). The reason for this is the following: when a sample of this compound is placed in the probe of the instrument, that is equivalent to insert a very large number of individual hydrogen fluoride molecules into the magnetic field B_0 . Knowing that both H and F have the spin number $I = 1/2$, the molecules are distributed at thermal equilibrium into four populations E_1 to E_4 , that correspond to the following spin combinations given on Figure 15.15.

If there was no interaction between the H and F nuclei, the populations E_1 and E_2 on one side and E_3 and E_4 on the other, would have the same energy and consequently in ^1H NMR, only a single signal would be seen (Figure 15.16). However the reality is different as an interaction exists between H and F. With

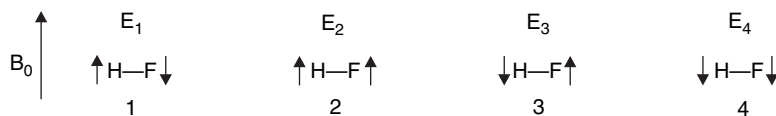


Figure 15.15 HF sample in ^1H NMR – the four populations in the magnetic field \vec{B}_0 .

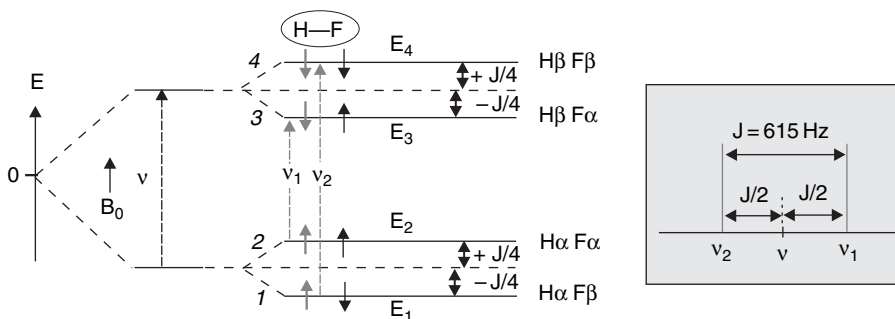


Figure 15.16 Coupling diagram for the HF molecule in ^1H NMR. Hypothetical comparison of the situation in which there is no coupling with the fluorine atom and the real situation. The values for ν_1 and ν_2 differ in length by J Hz.

respect to the situation in which no coupling arises, a theoretical development leads to the conclusion that the two populations E_1 and E_2 correspond now to two different energy states: it follows that there will be a splitting-up of the peak as supposed previously. The energy required to go from E_2 to E_3 is not the same as from E_1 to E_4 . The spin orientation taken by the fluorine atom has an effect on the transition energy observed in ^1H NMR. The spectrum of this compound will therefore contain two peaks corresponding to the transitions situated on Figure 15.16.

The *coupling constants*, expressed in hertz, are available from the NMR spectrum. They are designated by the letter J with an index giving the name and the number of bonds which separate these atoms. If we consider the case of HF, the coupling constant can be written:

$$^1J_{\text{FH}} = 615 \text{ Hz}$$

The spin orientation of the fluorine atom, which has a resonance frequency very far from that of the proton, will not be disturbed by the resonance occurring in protons: in a field of 2.35 T ^1H resonates at 100 MHz while ^{19}F resonates at 94 MHz. The two peaks of the ^1H signal are separated by 615 Hz, irrespective of the intensity of B_0 . The ^{19}F NMR spectrum for this molecule would lead to a similar spectrum. Two peaks would be observed separated by 615 Hz, due this time, to the coupling with the proton following one or other of the orientation chosen.

When the number of bonds separating the atoms concerned increases, the spin-spin couplings weaken very quickly, assuming that there are no multiple bonds (double or triple) which could propagate the spin effect through the π -bond electrons.

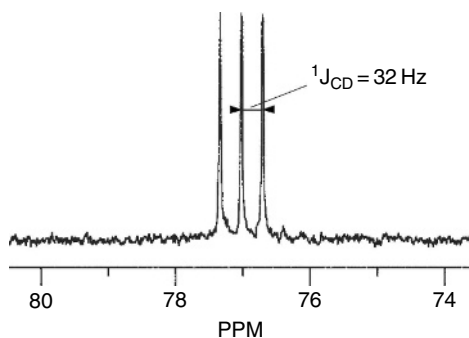


Figure 15.17 ^{13}C Spectrum in CDCl_3 showing the coupling $^1J_{\text{CD}}$. The three signals of this spectrum are caused by the heteronuclear coupling between the ^{13}C and the atom of deuterium ^2H (or D), (coupling $^1J_{\text{CD}} = 32 \text{ Hz}$). Since the deuterium has a spin number $I = 1$, there are three possibilities for m : $-1, 0, +1$, which leads to the triplet. The three components are of equal intensity as there is only a single atom of deuterium. This triplet is present on almost all ^{13}C spectra, in superimposition, when this solvent is used.

15.13.2 Heteronuclear coupling in organic chemistry

The presence in a molecule of atoms such as ^{13}C , ^1H or ^{19}F which possess a spin number of $1/2$, is at the origin of numerous heteronuclear couplings, exploited for their structural information (Figures 15.17 and 15.18).

15.14 Homonuclear coupling

Signal splitting, as studied in the case of hydrogen fluoride, is often encountered in organic compounds with adjacent hydrogen atoms if their chemical shifts are different.

15.14.1 Weakly coupled systems

Nuclei are said to be weakly coupled when the coupling constants are much smaller than the differences in chemical shifts of the nuclei concerned (after conversion in Hz). The ethyl group of butanone (Figure 15.1) illustrates this situation. These five protons – 3 and 2, weakly coupled give rise to three peaks (a *triplet*) towards 1.1 ppm and to four peaks (a *quadruplet*) towards 2.5 ppm.

The origin of these peaks in the spectrum can be ascribed by their chemical shifts (Table 15.5). The triplet is due to the CH_3 and the quadruplet to the neighbouring

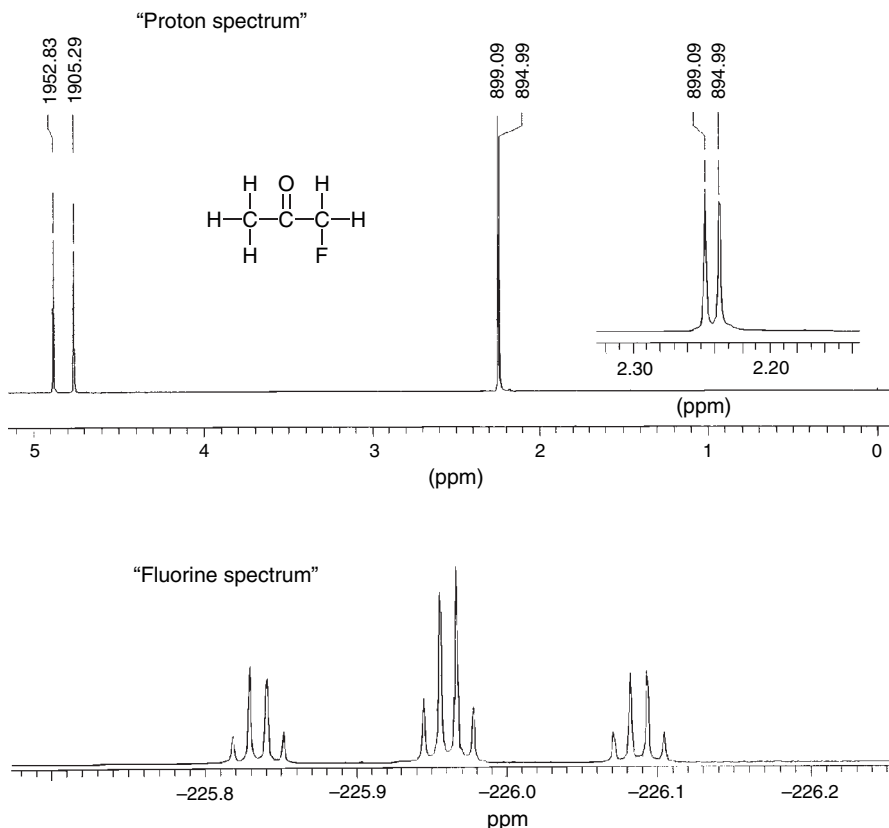


Figure 15.18 NMR spectra of monofluoroacetone. An example of heteronuclear coupling. Above, the ^1H NMR spectrum. The presence of the fluorine atom leads to a doublet for the methyl group ($^3J = 4.1$ Hz) as well as for the CH_2 ($^2J = 47.5$ Hz). Below, spectrum of ^{19}F . The single fluorine atom of this molecule leads to a triplet with the CH_2 group and a quadruplet with the methyl (explanation in next section). The resulting signal is therefore a triplet of quadruplets. With the aid of table 15.4 the coupling constants can be calculated and compared for the two spectra (the scale for the chemical shifts is positioned with respect to FSiCl_3).

CH_2 . The relative intensities of the components, within each of these multiplets can be deduced by the laws of statistics (Figure 15.19 and Table 15.4).

As a general rule for weak couplings, if n nuclei (spin number I), are placed in the same magnetic environment, influencing in an identical manner one or several adjacent nuclei, then the signals observed for the latter will be formed of $2nI + 1$ peaks regularly spaced – i.e. $(n + 1)$ signals in the case where $I = 1/2$. The T_1 and T_2 values of all the spins must be equal. The peak intensities in a multiplet will follow the same pattern found in each row of Pascal's triangle (Table 15.4). However, if a group of protons is submitted to the effect of neighbouring nuclei for which the chemical shifts and the coupling constants are different, the previous

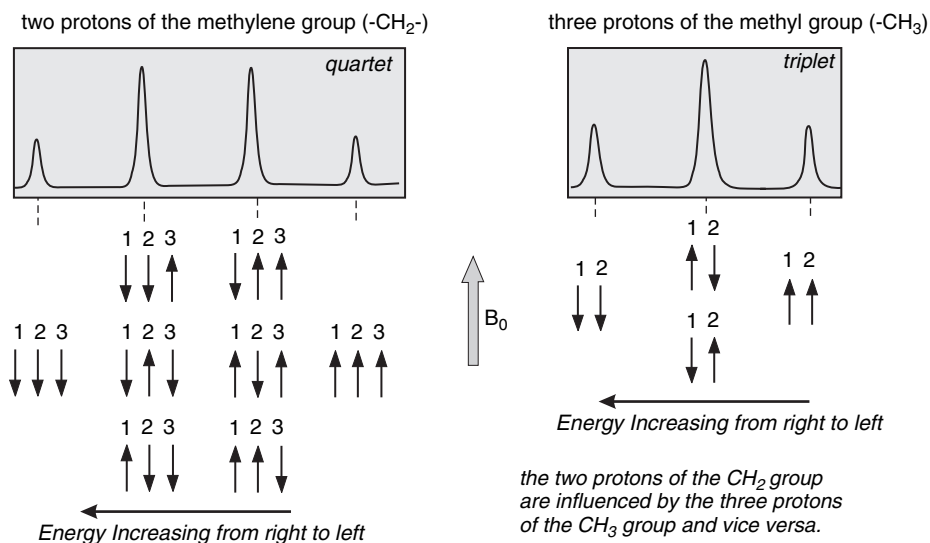


Figure 15.19 Representation of the different spin states for the five protons of an ethyl group. In the same row are found the spin states which produce the same effect on neighbouring nuclei. The sample contains a large number of identical molecules, these are distributed across several populations giving each one a statistically weighted signal like the number of states per row of this scheme.

Table 15.4 Pascal's triangle and its application to NMR for $I = 1/2$

Neighbouring hydrogens	Multiplicity	Intensity							
0	singlet	1							
1	doublet	1	1						
2	triplet	1	2	1					
3	quadruplet	1	3	3	1				
4	quintuplet	1	4	6	4	1			
5	sextuplet	1	5	10	10	5	1		
6	septuplet	1	6	15	20	15	6	1	

approximation is no longer applicable: the multiplicity of the signals and the intensity of the peaks cannot be deduced so easily.

■ **Nomenclature for coupled systems in ^1H NMR.** Spectral interpretation for a molecule with many hydrogen atoms is easier when sub-groups of signals correspond to classic situations. These particular cases are classified by means of a nomenclature using the letters of the alphabet, selected with respect to the chemical shift (Figure 15.20). Protons with the same chemical environment or chemical shift are called equivalent. If the chemical shifts are too little different, the protons are

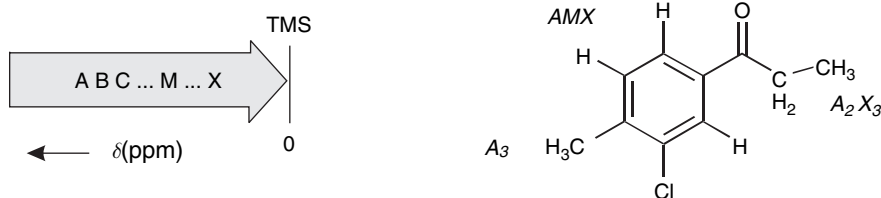


Figure 15.20 *NMR nomenclature of spectra.* The ^1H spectrum of 3-chloro-4-methylpropiophenone is the complex result of the superimposition of the signals of several independent yet easily recognized sub-groups.

designated by the first letters of the alphabet (AB, ABC, A_2B_2 , A_3), while protons with very different chemical shifts are designated by letters such as A, M, and X (i.e. AX, AMX, AX_3 , etc.). Like this, the ethyl group (as ethoxyl), constitutes a system A_2X_3 ; the protons of ethanal represent an AX_3 system while a vinyl group will be ABC, AMX or ABX depending upon the example studied.

In ^{13}C NMR, as the coupling constants $^1J_{\text{C-H}}$ being of the order of 125 to 200 Hz, there are frequent signal overlappings, which makes the spectrum more difficult to interpret. Technically it is possible to eliminate the entire set of couplings for all of the protons (see Figure 15.21).

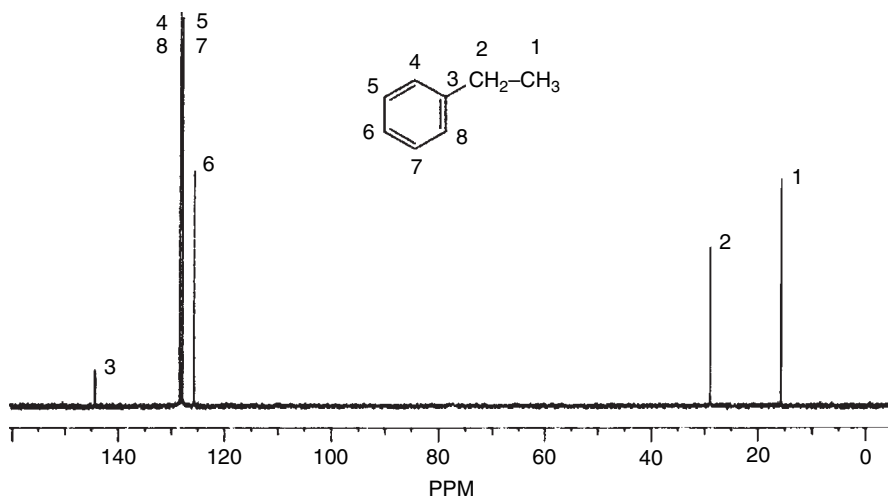


Figure 15.21 *Proton decoupled ^{13}C spectrum of ethylbenzene.* Each carbon atom gives a single signal consisting of a singlet. These simpler 'broad band,' decoupled spectra yield less information.

15.14.2 Strongly coupled systems

When the ratio $\Delta\nu/J$ is small the protons become strongly coupled. The intensities of the multiplets are then very disturbed. New peaks arise from combinations of frequencies hindering the interpretation of spectra. This is one of the reasons for the development of instruments operating at higher frequencies (300 \rightarrow 750 MHz) with supraconducting magnets to obtain the required magnetic fields. Given that $\Delta\nu$ is proportional to the operating frequency, while J remains constant, the ratio $\Delta\nu/J$ increases and again nuclei appear as weakly coupled (Figure 15.22). However there are other phenomena that complicate the spectra when the frequency exceeds 600 MHz.

AB system. When the difference in resonance frequencies between two protons is comparable with their coupling constant J , then this system gives rise to a total of four peaks. This is the simplest strongly coupled system. The signal for each proton contains two peaks separated by J Hz. However the intensities are no longer equal and the chemical shifts, which can no longer be read from the spectrum, must be calculated from the expressions indicated on Figure 15.23.

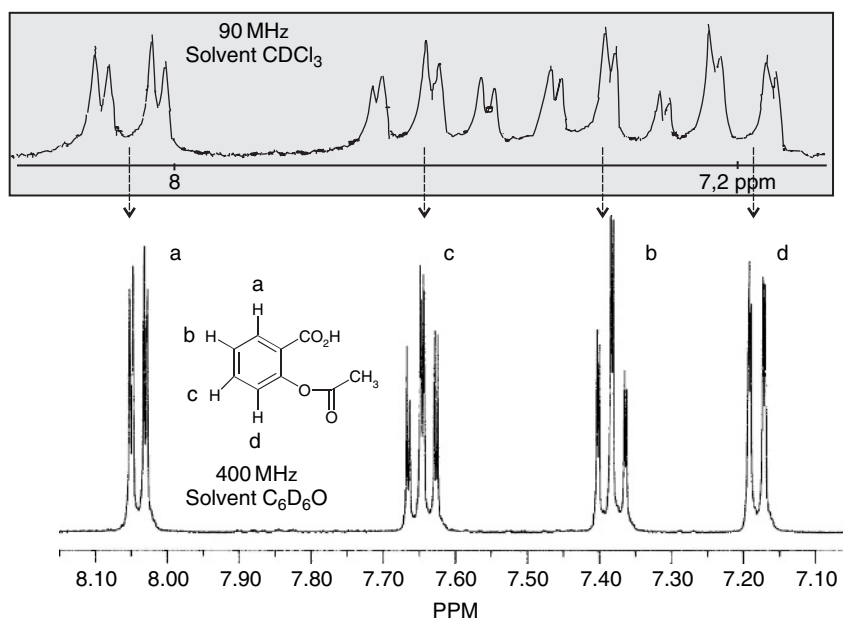


Figure 15.22 Spectrum of the four aromatic protons of aspirin. The figure above reproduces, with a similar scale, aspirin spectrum obtained on two different instruments, one running at 90 MHz (solvent CDCl₃) and the other at 400 MHz (solvent C₆D₆O). The sensitivity of NMR grows as $\vec{B}_0^{3/2}$.

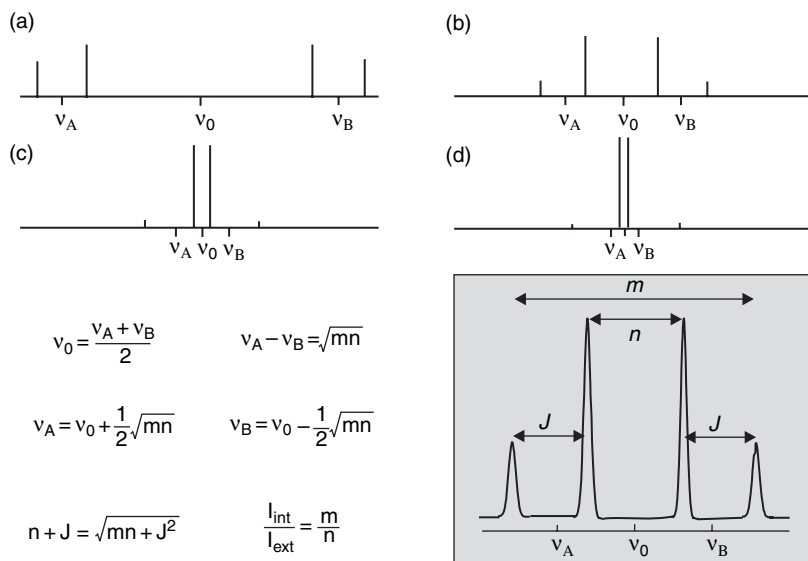


Figure 15.23 Characteristics defining an AB system. (a–d) Gradual modification of a two-proton coupled system as the ratio $\Delta\nu/J$ decreases. Typical appearance and formulae used to analyse an AB system.

15.15 Spin decoupling and particular pulse sequences

NMR instruments have accommodated numerous advances to facilitate spectral interpretation. Thus it is possible to cancel the effect of an existing spin-coupling between neighbouring nuclei. This *spin decoupling* technique is based on the statement that a nucleus in resonance does not always conserve the same spin state with time. This uninterrupted and fast transfer between different states induces a global effect upon the neighbouring nuclei.

In practice, a spin decoupling experiment begins with a record of the spectrum under normal conditions. Next, decoupling is achieved with the aid of a saturation pulse: a second spectrum is recorded while irradiating at the resonance frequency of the nucleus (or nuclei) that is (are) to be decoupled (Figure 15.24). This double resonance technique is used to identify nuclei which are coupled and which cause interpretation difficulties in the spectrum, in particular for superimposed signals.

Fourier transform NMR at high fields ($B_0 = 17.6\text{ T}$ for the study of ^1H at 750 MHz) has considerably extended the potential of this method. This is due to an increase in acquisition speed and rapid computation of spectra.

A variety of experiments based upon particular pulse sequences can be applied to a sample to produce a specific form of NMR signals. For example, the 90-FID pulse sequence rotates the net magnetization down into the xy -plane, through which the relaxation times of the nuclei can be determined. Amongst the more

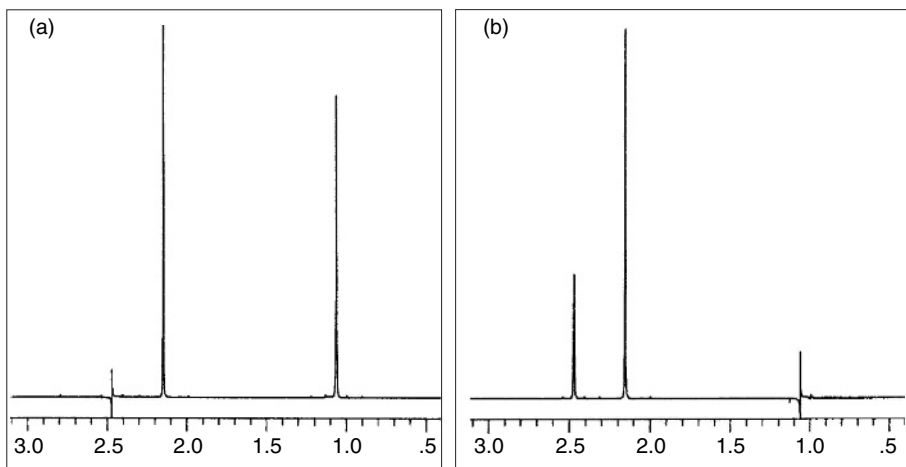


Figure 15.24 *Spin decoupling experiment on butanone.* Modification of the ^1H spectrum by (a) Irradiation of the CH_2 group at 2.47 ppm; (b) Irradiation of the CH_3 group (of ethyl) at 1.07 ppm, both for comparing with the spectrum of butanone, Figure 15.1. For such a simple compound such as this, the experiment is only of illustrative interest. On the other hand, a double resonance experiment would allow the precise determination of the different couplings in aspirin (Figure 15.22).

classical methods, multidimensional 2-D or 3-D NMR allow the deduction of very useful structural information. When analysing bio-polymers such techniques lead to results comparable with those obtained by radiocrystallography, permitting, amongst other things, the representation of molecular conformations in their natural medium.

■ **Magnetic resonance imaging (MRI)** Proton NMR can be exploited to obtain images of all objects containing hydrogen atoms, from living organisms to geological strata. This important development of NMR has been successfully applied to medical analysis under the name of MRI, a form of non-invasive physical observation, without penetration, adapted to soft tissues. The volume to be examined must be placed in a magnetic field, which requires the manufacture of very large supraconducting magnets for total body instruments. Proton signals are the easiest to observe since biological tissues contain around 90 per cent water (an individual of 70 kg comprises 50 kg of water compared to 13 kg of carbon). In other cases the signal of the CH_2 groups, present in lipids, are monitored. The final image is a cartographic presentation of the intensity distribution of the same type of proton signal. The contrast in the image is due to the variations in relaxation times for the protons of the selected plane. Amongst the specific technical complexities is the focusing, or spatial selectivity, needed to obtain a spectrum of a quasi-punctual volume within an object. 'Slices' of the patient are imaged as a matrix of small volumes of tissue (voxels) producing an individual signal. These 'slices' can then be reconstructed to provide images in any plane (Figure 15.25).

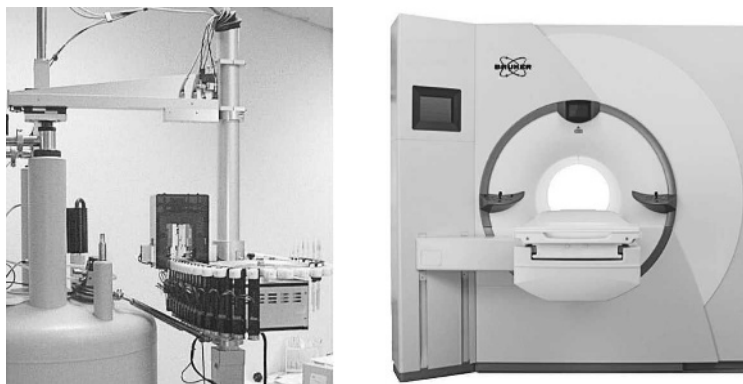


Figure 15.25 Sample introduction for NMR spectrometers. Left, automatic introduction of a sample placed in an ‘NMR tube’ within the magnetic field produced by a superconducting coil maintained at liquid helium temperature (reproduced courtesy of Varian). Right, a large magnet employed for the introduction of a particular kind of sample – the human body (part of a MRI instrument from SMIS).

15.16 HPLC-NMR coupling

The hyphenation of liquid chromatography with NMR spectroscopy is one of the most powerful and time-saving methods for the separation and structural elucidation of unknown compounds and mixtures (Figure 15.26). Unfortunately, HPLC-NMR spectroscopy is a relatively insensitive method requiring sufficient sample concentration in the NMR flow cell. Bearing in mind the tendency toward the miniaturization of HPLC and the consequent reduction of the quantities being chromatographed, on-line coupling of HPLC with NMR offered a real challenge. The use of deuterated organic solvents for mobile phase is generally expensive. The principle consists of passing the mobile phase from the exit of the detector of the chromatograph to a microcell placed in the NMR apparatus. The quantity of a compound in the probe depends about the detection volume.

There exist in principle two general methods for carrying out HPLC-NMR: *on-flow* and *stopped-flow* experiments.

With the on-flow mode of operation the output of the chromatographic separation is recorded simultaneous with ^1H NMR spectra. In most cases the flow of the mobile phase is decreased to allow a large number of NMR spectra to be recorded of one chromatographic peak.

However if the quantity of compound is very small then it may be necessary to interrupt the flow of the mobile phase as soon as the maximum of the peak reaches the flow cell (indicated by UV detector). This stopped-flow mode is used in order to accumulate hundreds of scans to improve the signal/noise ratio of the resultant spectrum.

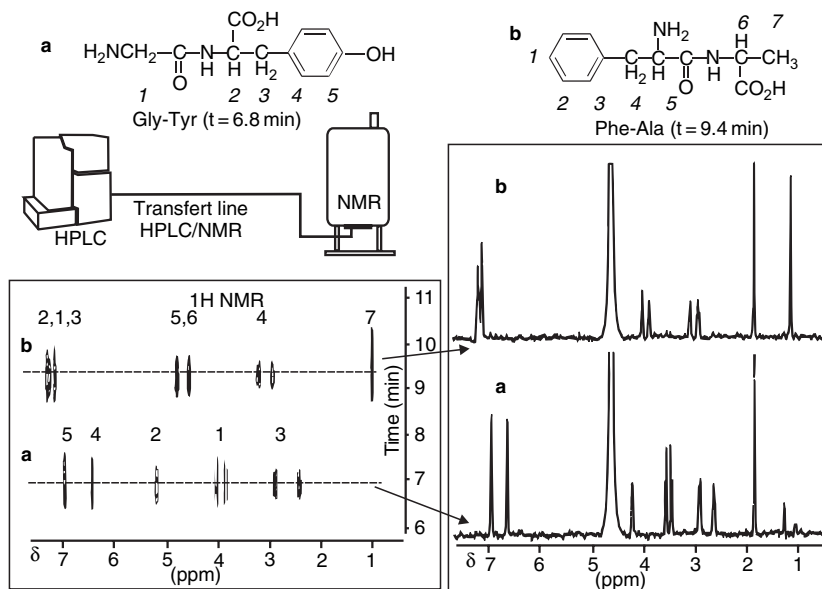


Figure 15.26 An experimental result by HPLC-NMR coupling. Separation of two dipeptides ($5\mu\text{g}$ each) with identification of the signals. For more clarity the peaks of the solvents around 2 and 5 ppm (acetonitrile and water) have been eliminated (Sweeder R.D. *et al. Anal. Chem.* 1999, 71(23), 5335).

15.17 Fluorine and phosphorus NMR

Fluorine and phosphorus are the two heteroatoms in organic chemistry most frequently studied by NMR after hydrogen and carbon.

Fluorine element, consisting of 100 per cent ^{19}F ($I = 1/2$), can be compared with ^1H for its sensitivity. Its electronegativity being greater than hydrogen (4 rather than 2.1), chemical shifts are distributed on a much greater range (Figure 15.27). As a consequence, in ^{19}F NMR, it becomes possible to distinguish between compounds that are chemically very similar. In particular, differences due to stereochemistry are significant with the coupling constants $J_{\text{F-H}}$ extended over a greater range compared to $J_{\text{H-H}}$ (Figure 15.17). On the other hand, relatively few molecules contain atoms of fluorine. Therefore, the fluorine NMR spectra are generally obtained from compounds to which a ^{19}F (or a group CF_3) has been intentionally introduced in a known position by chemical modification, in order to answer questions of structure from the comparison of spectra. The fluorine atom induces a chemical shift comparable to that of an hydroxyl (OH) group, but with minor modification in the stereochemistry of the molecule. The Van der Waals radius of fluorine is 1.35 Angstrom instead of 1.1 for the hydrogen atom.

Phosphorus (^{31}P , $I = 1/2$) another common monoisotopic element, has been studied since the beginnings of NMR. It has a great sensitivity and it is an important element in the composition of numerous biological compounds.

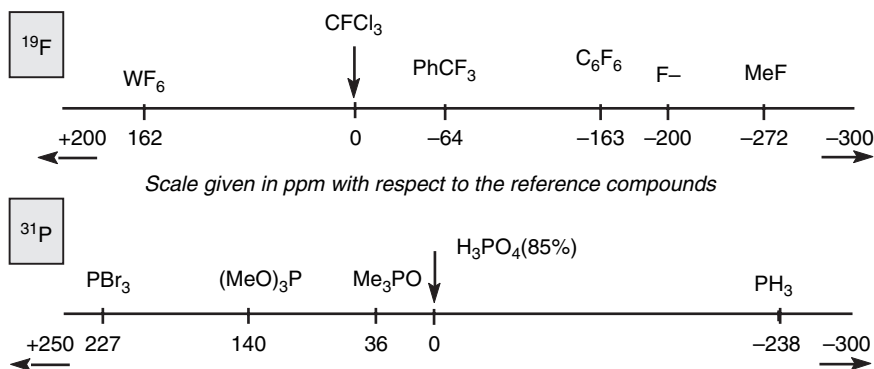


Figure 15.27 Positions of some NMR signals due to fluorine and phosphorus.

15.18 Quantitative NMR

Although NMR is a method essentially reserved for structural analysis, due to the quality of the information that it can yield, it can be used also to give the composition of mixtures. This application is possible if the signals chosen for locating each constituent have areas which can be measured independently. Although the sensitivity and the precision vary with the type of nucleus, this method presents interesting features such as the possibility to carry out analyses without complicated sample preparation and without destruction of the sample. There is no risk of polluting the instrument and in contrast to many other methods, such as chromatography, there is no need of a prior standardization step. From the molar ratios to which spectrum examination yields access, it is possible to deduce mass concentrations.

■ In organic synthesis, ^1H NMR permits to calculate the yields of a reaction. Finally, industry uses ‘low resolution’ analysers based upon ^1H NMR to quantitate water and fats in food industry, like in many other fields.

15.18.1 Measuring areas – application to simple analysis

Areas of peaks on spectra can be given in the form of numerical values, or for older apparatus, can be taken from the integration plots that are superimposed upon the spectrum (Figure 15.1). Yet it is difficult to measure signals to better than 2 per cent accuracy for various technical and physical reasons. NMR-lines are theoretically Lorentzian curves, which are quite broad at their bases, and bear substantial areas even under 1–2 per cent of peak intensity (usually in the noise region). In ^{13}C NMR, it is preferable to add a relaxation agent in order to avoid saturation, linked to the relaxation times, which alters the intensities of the signals. The delay between successive scans must be long enough to allow the magnetization to achieve equilibrium along the z -axis (approximately $5 \times T_1$ for the proton with the longest T_1).

Consider, for example, a sample comprising a mixture of acetone **A** and of benzene **B** diluted in CDCl_3 as solvent. On the ^1H spectrum of this mixture two signals are observed at $\delta = 2.1$ ppm (acetone) and at $\delta = 7.3$ ppm (benzene), with respect to TMS. This spectrum corresponds to the weighted superimposition of the two individual spectra (Figure 15.28).

In this particular case where the molecules of the two compounds each have six protons, the ratio of the areas of the two peaks is representative of the ratio n_A/n_B of the respective numbers of molecules of A and B (given that the response factors are equal for A and B). Assuming that the mixture only contains these two components, and designating S_A as the area of the acetone signal and S_B that of benzene, it follows that:

$$\frac{n_A}{n_B} = \frac{S_A}{S_B} \quad (15.11)$$

If C_A and C_B are the concentrations expressed in percentage mass of A and B, of which the molar masses are M_A and M_B respectively, then:

$$\begin{aligned} C_A + C_B &= 100 \\ \frac{C_A}{C_B} &= \frac{n_A \cdot M_A}{n_B \cdot M_B} \end{aligned} \quad (15.12)$$

Or, by substituting into expression 15.11:

$$\frac{C_A}{C_B} = \frac{S_A \cdot M_A}{S_B \cdot M_B} \quad (15.13)$$

Therefore,

$$C_A = 100 \cdot \frac{S_A M_A}{S_A M_A + S_B M_B} \quad \text{and} \quad C_B = 100 \cdot \frac{S_B M_B}{S_A M_A + S_B M_B} \quad (15.14)$$

15.18.2 Samples containing identifiable compounds

A more general case is that in which the signal (or signals) selected for each compound to be measured do not correspond globally to the same number of

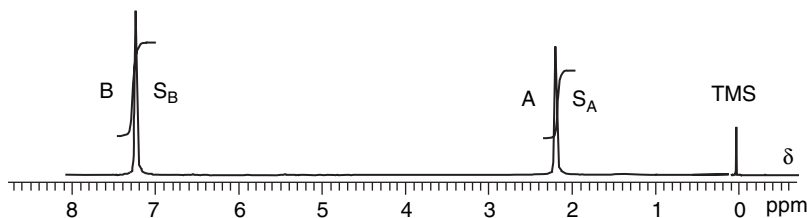


Figure 15.28 ^1H spectrum of a mixture of acetone (**A**) and benzene (**B**). If $S_A = 111$ and $S_B = 153$ (arbitrary units), then knowing that $M_A = 58$ and $M_B = 78$ g/mol, $C_A = 35\%$ and $C_B = 65\%$ total mass.

protons, either because the molecules do not have the same total number of protons, or alternatively, if only a portion of the spectrum of each compound has been chosen in order to identify it.

Supposing for example, that the signal selected for compound A corresponds to a protons and the signal chosen for compound B corresponds to b protons (A and B do not represent acetone and benzene as in above section). When the spectrum of the mixture of A and B is recorded each molecule of B leads to a signal whose intensity is different to that of a molecule of A. The expressions in 15.15 will remain valid provided corrections are inserted to take into account these differences. Each area must be divided by the number of protons that are at the origin of the selected peak, in order to normalize the relative area to one proton of either A or B. Then substituting S_A and S_B by the corrected areas S_A/a and S_B/b , the two preceding expressions become:

$$C_A = 100 \cdot \frac{\frac{S_A}{a} M_A}{\frac{S_A}{a} M_A + \frac{S_B}{b} M_B} \quad \text{and} \quad C_B = 100 \cdot \frac{\frac{S_B}{b} M_B}{\frac{S_A}{a} M_A + \frac{S_B}{b} M_B} \quad (15.15)$$

Expression 15.16 can be transposed to the case where n constituents are visible on the spectrum. By using labels such as A, B, ..., Z and in locating each constituent by a specific area, ca. S_i for i protons for the component I, the general equation 15.16 giving the mass per cent of each one can be formulated :

$$C_i = 100 \cdot \frac{(S_i/i) \cdot M_i}{(S_A/a) \cdot M_A + (S_B/b) \cdot M_B + \dots + (S_i/i) \cdot M_i + \dots + (S_Z/z) \cdot M_Z} \quad (15.16)$$

With this type of calculation, ^1H NMR is frequently used in organic chemistry as a method to find the yield of a reaction $A \rightarrow B$. This is done by recording the spectrum of the coarse material after reaction and by identifying a signal belonging to the product formed (B) and a signal belonging to the remaining starting material (A). The yield of B with respect to A can be written using the previous notations :

$$R = 100 \cdot \left(\frac{S_B}{b} \right) / \left(\frac{S_A}{a} + \frac{S_B}{b} \right) \quad (15.17)$$

15.18.3 The internal standard method

A more general situation corresponds to the measure of a single compound in a sample. To do that, it is not indispensable to know the nature of all the other components that are present, or to identify *all* of the signals in the NMR spectrum. In fact, it is sufficient to find a signal belonging to the compound of interest.

In order to quantify compound X ($M = M_X$) in a mixture called E, a quantity p_R mg of reference compound R (with mass M_R) for use as internal standard, is

added to p_E mg of the sample E containing compound X, before recording the spectrum. The standard R is chosen such that the signal serving as indicator does not interfere with the signal chosen to quantify compound X (Figure 15.29). Next, on the NMR spectrum of the mixture containing the internal reference, a signal belonging to compound X (area S_X for x protons) is chosen as well as a signal belonging to standard R (area S_R for r protons).

By designating n_X/n_R the molar ratio of X and of R, as before, expression 15.19 can be obtained:

$$\frac{C_X}{C_R} = \frac{n_X \cdot M_X}{n_R \cdot M_R} \quad (15.18)$$

knowing that

$$\frac{n_X}{n_R} = \frac{S_X/x}{S_R/r} \quad (15.19)$$

The concentration C_R , expressed as a percentage mass of R, being equal to:

$$C_R = 100 \cdot \frac{p_R}{p_E + p_R} \quad (15.20)$$

the mass concentration (per cent) C_X of compound X can be deduced :

$$C_X = 100 \cdot \frac{p_R}{p_E + p_R} \cdot \frac{(S_X/x) \cdot M_X}{(S_R/r) \cdot M_R} \quad (15.21)$$

15.18.4 The standard addition method

The previous method can be improved by preparing a number of standard solutions that contain both the sample and increasing quantities of the same compound to be measured. From the corresponding NMR spectra of these solutions, a plot can be drawn representing the area of the signal selected for the measurement as a function of the quantity added. The intercept of the graph with the concentration axis will give the unknown concentration (Figure 15.30).

The difficulty of this method is in maintaining the stability of the NMR apparatus long enough to be able to undertake successive measurements of the standard solutions.

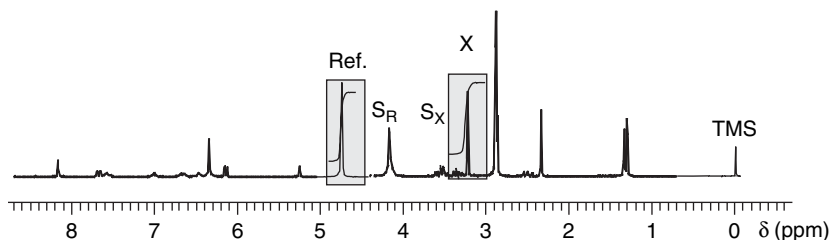


Figure 15.29 Scheme displaying a spectrum of a sample into which a reference compound R has been added. The signal X belongs to the compound to be measured and the signal S to the internal standard.

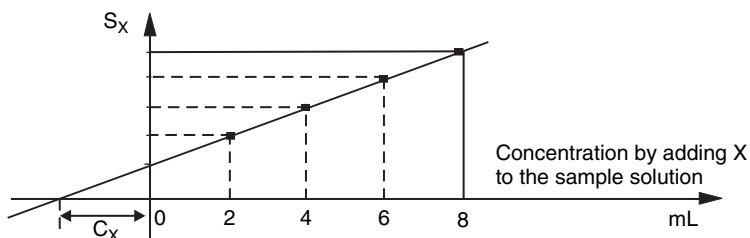


Figure 15.30 Graph obtained for the standard addition method. The unknown concentration corresponds to the abscissa segment between the origin of the axis and the intersection with the calibration curve.

15.19 Analysers using pulsed NMR

Pulsed wave Fourier transform NMR permits another form of routine measurement though rather yet underestimated by quality control analysts. Water and certain other organic compounds such as fats can be quantified by this technique from the hydrogen concentration in the sample. The corresponding instruments do not plot the usual NMR spectrum but give the global intensity of the FID immediately following the period of irradiation, as well as its time decay (Figures 15.31, 15.32 and 15.33). The rate with which the protons relax yields information about the environment of the hydrogen atoms. It thus becomes possible to distinguish the protons that are present in a solid compound and those that are part of a liquid. In food area, following standardization, this give access to the concentration of water and fats in a variety of samples.

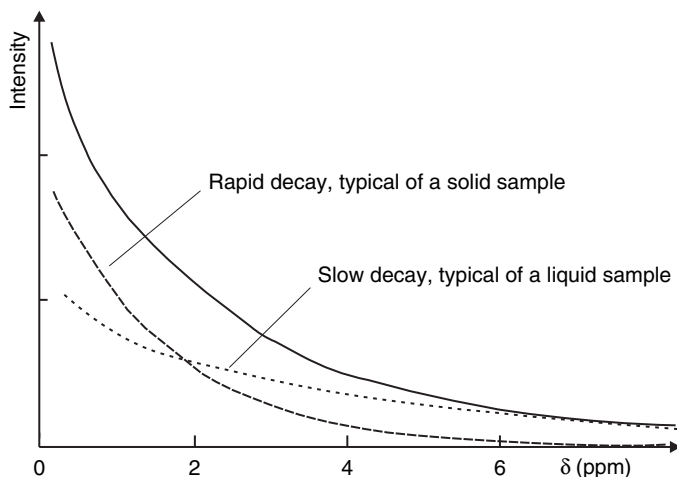


Figure 15.31 FID for a solid/liquid mixture. The amplitude of the FID which relates the quantity of protons as a percentage of the total mass, can be distinguished from relative measures based on T_2 , which permits the percentage of solid to be determined in the sample through deconvolution of the curve envelope and its constituents, as displayed on the graph.

Other applications follow the same principle and are based upon the detection of phosphorus or fluorine elements.

- To determine the water resources of the subsoil (free water quantification and depth discrimination of water-tables), geologists can base their investigations upon ^1H NMR signals submitted to the low ambient Earth's field by using a suitably adapted equipment (the Larmor frequency is a few hundreds Hz).



Figure 15.32 *Low resolution ^1H NMR instrument for routine analyses.* The analyser operates on the principle of pulsed NMR and is used for quantifying water and fat in numerous food products (model minispec, reproduced courtesy of Bruker).



Figure 15.33 *NMR research instrument.* The typical appearance of these instruments corresponds, as here, to the combination of a superconducting magnet (at the bottom), of an electronic cabinet and a workstation for the analysis of results. Model Avance 400 (reproduced courtesy of Bruker).

Table 15.6 ^{13}C NMR chemical shifts of principal types of carbons in organic molecules

		220	200	180	160	140	120	100	80	60	40	20	0
>C=O	Ketones	[Bar from 210 to 200 ppm]											
>C=O	Aldehydes	[Bar from 190 to 170 ppm]											
>C=O	Acids	[Bar from 180 to 165 ppm]											
>C=O	Esters	[Bar from 175 to 155 ppm]											
>C=N	Heteroatomics	[Bar from 165 to 150 ppm]											
>C=C	Alkenes	[Bar from 140 to 110 ppm]											
>C=C	Aromatics	[Bar from 140 to 110 ppm]											
>C=C	Heteroaromatics	[Bar from 140 to 120 ppm]											
$\text{-C}\equiv\text{N}$	Nitriles	[Bar from 125 to 120 ppm]											
$\text{-C}\equiv\text{C}$	Alkynes	[Bar from 80 to 60 ppm]											
>C-O	Quatern. C	[Bar from 75 to 55 ppm]											
>C-N		[Bar from 65 to 45 ppm]											
>C-X		[Bar from 100 to 40 ppm]											
>C-C		[Bar from 55 to 35 ppm]											
>CH-O	Tert. C	[Bar from 75 to 55 ppm]											
>CH-N		[Bar from 65 to 45 ppm]											
>CH-X		[Bar from 100 to 40 ppm]											
>CH-C		[Bar from 55 to 35 ppm]											
$\text{-CH}_2\text{-O}$	Second. C	[Bar from 75 to 55 ppm]											
$\text{-CH}_2\text{-N}$		[Bar from 65 to 45 ppm]											
-CH-X		[Bar from 100 to 40 ppm]											
$\text{-CH}_2\text{-C}$		[Bar from 55 to 35 ppm]											
$\text{CH}_3\text{-O}$	Prim. C	[Bar from 65 to 45 ppm]											
$\text{CH}_3\text{-N}$		[Bar from 55 to 35 ppm]											
$\text{CH}_3\text{-X}$		[Bar from 100 to 40 ppm]											
$\text{CH}_3\text{-C}$		[Bar from 15 to 0 ppm]											

Scale in ppm relative to TMS

Problems

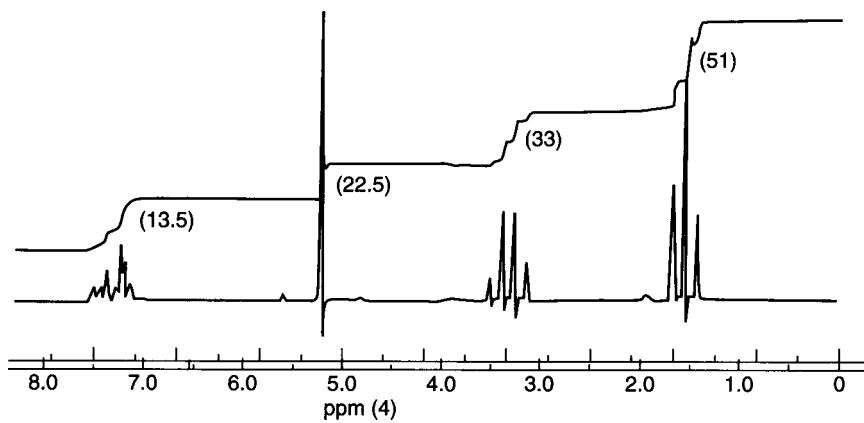
- 15.1 Often in tables the values describing a nucleus' magnetic moment are represented as lying upon an axis parallel to \vec{B} , the applied magnetic field. Therefore, if for a proton, $\mu_z = 1.41 \times 10^{-26} \text{ J} \cdot \text{T}^{-1}$, calculate the constant γ for the proton.
- 15.2 If $T = 300 \text{ K}$, calculate the ratio of the populations N_{E_1}/N_{E_2} for a proton in an NMR spectrometer where the applied magnetic field $B = 1.4 \text{ T}$. Make the same calculation for the case where the field $B = 7 \text{ T}$, given that $\gamma = 2.6752 \times 10^8 \text{ rad T}^{-1} \text{ s}^{-1}$.
- 15.3 In the ^1H NMR spectrum from a 200 MHz spectrometer the scale along the abscissa is represented by $1 \text{ ppm} = \text{cm}$.
1. What is the distance between two signals with a separation of 7 Hz?
 2. If it is known that $\gamma_{\text{H}}/\gamma_{\text{C}} = 3.98$, what would happen to the distance calculated above if a ^{13}C spectrum were to be recorded on the same apparatus?
- 15.4
1. Calculate the chemical shift, δ , in ppm of a proton (^1H), whose NMR signal is displaced by 220 Hz with respect to TMS (the field of the spectrometer is 1.879 T).
 2. The resonance signal for a proton is displaced by 90 Hz with respect to TMS when measured on a 60 MHz spectrometer. What would happen to this displacement if an apparatus of 200 MHz was employed?
 3. What would be the corresponding chemical shift of the same proton with both of these spectrometers?
- 15.5 Two isomers A and B share the same molecular formula, $\text{C}_2\text{HCl}_3\text{F}_2$. What is the structure of each of these isomers when their proton NMR spectra, as recorded upon a 60 MHz apparatus, are the following:

The spectrum of isomer A comprises two doublets at 5.8 ppm and 6.6 ppm respectively ($J = 7$ Hz), while that of isomer B contains a triplet at 5.9 ppm ($J = 7$ Hz).

Consider now that a third isomer is present. Under the existing conditions, describe what its spectrum must be.

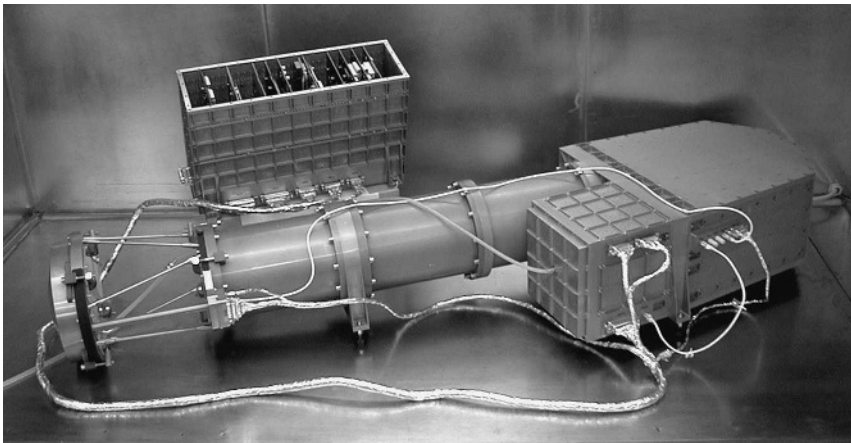
- 15.6 Numerous NMR experiments make use of the 'Magic Angle' technique which corresponds to the angle made by the spin vector and an axis parallel to the direction of the magnetic field in which it is held. What is the value of this angle in degrees?
- 15.7 25 mg of vanillin ($C_8H_8O_3$) is added to 100 mg of an unknown organic compound. The 1H integration curve displayed upon the spectrum of the new mixture can be described as follows: one proton of the original compound corresponds to a value of 20 mm while a single proton of vanillin corresponds to 10 mm. What is the molecular weight of the unknown compound? Remember: H = 1, C = 12, O = 16 g/mole.
- 15.8 If the ratio of the magnetogyric constants γ_F/γ_H is 0.4913, calculate the distance which would separate the TMS signal from that originating from an atom of fluorine, given that the scale upon the spectrum is represented by 1 ppm = 2 cm. (The apparatus is a 200 MHz spectrometer.)
- 15.9 Following the reduction of acetone in isopropanol by means of a hydride the 1H NMR spectrum of the crude, isolated product reveals that the reaction has not completed. The integration curve of the remaining acetone corresponds to a value of 24 (arbitrary units), while that of isopropanol is equivalent to 8. Calculate the molar yield of this transformation.
- 15.10 From the 1H NMR spectrum below, which corresponds to a mixture of bromobenzene, dichloromethane and iodoethane, calculate the percentage composition of the three components. The values (without units) given in brackets on the integration curve are proportional to the areas of the corresponding signals.

Given: H = 1, C = 12, O = 16, Cl = 35.5, Br = 80 and I = 127 g/mol.



PART 3

Other methods



Comet and Interstellar Dust Analyser (CIDA) is a time-of-flight mass spectrometer aboard NASA's Stardust spacecraft launched in February 1999 to explore comet Wild 2 (mission 1999-2006). CIDA has been fabricated by Hoerner & Sulger, under contract by the German Space Agency.

Trace analysis

Modern chemical analysis uses instruments of increasing sensitivity. This is not without logic since more than half of the analyses performed today concern analytes of very low concentration, described as *traces* or *ultra-traces*.

The notion of trace is related to the corresponding mass concentration in the sample. By convention, a compound is considered as being present in trace amount when its concentration in the environment is less than 1000 ppm. Beneath 1 ppm ($1\ \mu\text{L/L}$ or $1\ \text{mg/kg}$), an analyte is considered as being at the ultra-trace level.

Obviously, these limits are not always so clear. They depend upon the analyte, the matrix and of the use of the matter analysed. Even these terms have a subjective character. Thus, if a sample of acetone, in use as a solvent, contains 0.1 per cent methanol, then it can be said that the latter is a trace (1000 ppm) but if the sample constitutes drinking water, then it would be considered an enormous quantity.

To carry out these analyses, the detection limit of the instrument must be imperatively known. This is defined as the concentration of an analyte that allows to obtain a detectable signal with certainty – for example, three times the standard deviation of the background noise signal or of the blank. The detection limit is expressed in parts per million (defined by the mass/mass or mass/volume ratio, depending upon the matrix). If the mass or the volume of solution needed by the instrument to obtain this result is well defined, the preceding value can be transformed to the absolute quantities of analyte or mole fractions (picomole, femtomole, etc.) for obtaining this signal. In general these values are excessively small because current instruments only require minute volumes of sample.

By way of example, if $1\ \mu\text{L}$ of a 1 ppb solution (being about 1 pg), is injected into a chromatograph, and if a mole of the analyte weighs 100 g, this will be 10 femtomol! From this point the analyst must become familiarized with prefixes such as femto- (10^{-15}), atto- (10^{-18}), zepto- (10^{-21}). A zeptomole contains only 602 molecules of a given compound. These quantities tend towards single atoms or individual molecules. The ultimate trace is almost attained.

The detection of individual species is possible with certain methods. Fluorescence methods are used for the detection of molecules and Accelerator mass spectrometry (AMS) for detection of long-lived nuclides. The conventional method of counting the ^{14}C beta-decays is now abandoned, still it is a method that can record the signal of a single atom. This paradox is explained by taking into account that to locate a radioactive atom by counting, it is necessary that decomposition occurs *during the time of the measurement*. If the lifetime of the radionuclide is long, the chance of observing this event will be small.

AMS can measure the ratio of a rare radioisotope relative to a more abundant stable isotope of the same element as for examples $^{14}\text{C}/^{13}\text{C}$ or $^3\text{H}/^1\text{H}$. This provides a new way to measure attomoles of radiocarbon and some other radionuclides.

If the majority of the methods used for trace analyses are the same as those normally employed to measure more abundant compounds, much more care is needed. Many difficulties appear. At this level, containers, flasks and reagents become polluted and the work must be carried out in a clean room, otherwise in the absence of much precautions, it will be possible to discover literally anything, anywhere – it will be just to choose the species to hit upon.

16

Mass spectrometry

Mass spectrometry (MS) is an analytical method of characterizing matter, based on the determination of atomic or molecular masses of individual species present in a sample. The instruments employed for carrying out mass spectrometry can be classified into different categories according to the mass separation technique used. Some are derived from assemblies developed at the beginning of the 20th century for the study of particles or ionized atoms submitted to a magnetic field, while others rely upon different principles. Continuous improvements have made mass spectrometry the most versatile, sensitive and widely used analytical method available today. Miniaturization and the emergence of new ionization techniques allow to this method to be present in a variety of sectors: organic and inorganic chemistry, biochemistry, clinical and environmental chemistry, and geochemistry. This technique is found in many industrial control processes as well as for regulation compliance.

16.1 Basic principles

A minute quantity of sample, transformed in the gas phase or in a suitable form, is ionized and the resulting charged species are then submitted, in an enclosed space maintained under high vacuum, to the action of an electric and/or magnetic field, depending on the type of instrument. The study of the forces exerted upon the ions allows the determination of their *mass-to-charge ratio* and, eventually their nature. This method destroys the compound sample, but owing to the great sensitivity of the technique, only a tiny quantity is required.

■ By way of example, the ions resulting from a sample of methanol (CH_3OH) transformed to the gaseous state and then ionized by bombardment with electrons, will include amongst the charge-carrying species a small amount of positive ions CH_3OH^+ . These ions, formed in an excited state, possess a surplus of internal energy that will induce for many of them an almost immediate fragmentation. They do not, however, all dissociate by the same pathways, thus leading to different ion fragments whose mass is less than that of the original methanol molecule. Generally

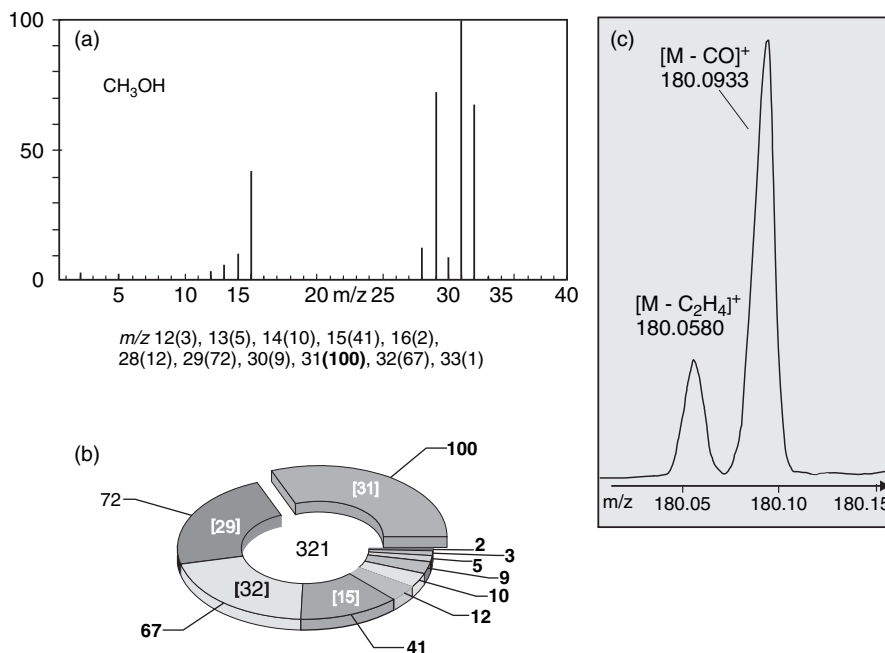


Figure 16.1 Fragmentation spectrum and mass spectrum presented in graphical or tabular form. (a) Fragmentation spectrum of methanol; (b) Unconventional representation of the same spectrum in the form of a circular diagram (hollow pie chart): statistically, for 321 ions formed, there are 100 of mass 31 u (Da), 72 of mass 29, etc. The various ions constitute many different populations; (c) Section of a high-resolution recording of a compound M presenting two ions with very close m/z ratios (one due to loss of CO and the other due to loss of C_2H_4).

these fragments, formed by simple bond cleavage and from subsequent rearrangement reactions are carriers of information on the structure of the original molecule (Figure 16.1a).

The results are displayed as a graph called *mass spectrum* and presented in the form of the abundances of the ions formed and classed in increasing order of their mass/charge ratio (Figure 16.1). Operating under identical conditions, the fragmentation is reproducible and therefore is a means of characterizing the studied compound.

Since mass spectrometers create and manipulate gas-phase ions, they operate in a high-vacuum system. They consist of three essential parts: an ionization source, a mass-selective analyzer, and an ion detector. The sample is submitted to a succession of steps:

1. **Ionization:** the sample in the form of gas or vapour is ionized in the *source* of the instrument. At this stage the mass spectrometer generates gas-phase ions by one of the numerous procedures existing. All molecules lead to a statistical distribution of fragment ions.

2. *Acceleration*: once formed the ions are immediately extracted from this part of the instrument to be *focussed and accelerated* by a series of electronic lenses, to increase their kinetic energy.
3. *Separation*: the ions are then 'filtered' by the *analyser* according to their mass-to-charge ratio, a number of instruments combining several mass analysers in series.
4. *Detection*: after separation, ions terminate their flight by striking the sensor of the *detector* which measures electrical charge and amplifies the weak ionic current.
5. *Display of the mass spectrum* arising from treatment of the signal sent by the detector.

■ This method only yields the mass-to-charge ratio of the ions, m/q . Logically to calculate m , q must be known. Since these ions carry a net charge of type $q = ze$ (where e represents the elementary charge of the electron and z is a small integer), m can be determined to the closest factor of z . This is why the scale of the spectrum is indicated as m/z (m being in atomic mass units). For molecules of small or medium size ($M < 1000$), which generate ions, generally all of them carrying a single charge ($z = 1$), the increasing order by mass is therefore the same as that of the ratio m/z . This assumes that the spectra are graduated in unified atomic mass units (u) or daltons (Da). m/z is normally expressed in Thomsons (Th) but this unit has never been widely accepted.

Ion abundance can be recorded in two very different ways:

- The *normal-mass spectrum* of the mass interval selected. The graph corresponds to a collection of signals in the form of peaks of different width, depending upon the resolution of the instrument (Figure 16.1c). These peaks lead to the determination of the ion masses and can attain a precision better than 10 parts in a million (10^{-5} Da). The upper mass limit, which is constantly being improved, can exceed 10^6 Da.
- The *fragmentation spectrum* (bar spectrum or stick plot). This corresponds to the distribution of all the ions formed, grouped by their *nominal mass* (cf. Table 16.1) as close to their exact masses and presented in the form of vertical lines. The most abundant type of ion leads to the most intense peak, called the *base peak*, which is given 100 per cent intensity. The intensities of the other peaks are then expressed as percentages of the base peak. This graphical representation of masses distributed by population, whose heights are proportional to their abundances, is essentially a histogram (Figure 16.1a, b). Bar spectra such as these are easy to archive and can be used to further comparison and identification. This normalized display, which is

incidentally the most commonly used graph in MS, has however the inconvenience that two or more ions of different atomic composition can occur at the same nominal mass.

The expression ‘mass spectrum’ must not lead to a confusion: the apparatus does not lead to a spectrum in the most usual sense of the term, that met in methods based upon the interaction between a sample and light radiation. The origin of this expression arises most probably from the similarity between the recordings obtained from the first instruments using photographic plates with those of optical spectrographs giving a spatially presentation of resolved spectra. Mass spectrometry does not belong to optical spectroscopy.

Mass spectrometry has progressively become an irreplaceable means of investigation of compound structures in organic chemistry and biochemistry (notably in proteomics). The technique can be applied to analysis of the elemental composition of inorganic samples (ICP/MS) whilst also allowing to study mixtures of molecular species, when combined with chromatography. The *on-line* couplings, such as GC/MS or HPLC/MS are amongst the best methods of analysis for mixtures and samples containing trace amounts of analyte.

The identification of molecular compounds by mass spectrometry can be achieved from either of the two types of spectral diagram:

- Attempting to reconstitute the structure of the compound in the manner of a puzzle. This is an exercise, which becomes more difficult as the mass of the original molecule increases. Successive fragmentations of the ions make identification more complex. However this task becomes less arduous if several spectra recorded under different conditions are available with appropriate software to help in their interpretation.
- Using a spectral library comprising a large number of fragmentation spectra amongst which, in a favourable case, might be found the mass spectrum of the molecule in question.

16.2 The magnetic-sector design

Early mass spectrometry started with the first experiments by J.J. Thomson (1900) and the use of a magnetic field to determine the m/z ratio to study isotopes was developed by Aston or Bainbridge in ca. 1930 (Figure 16.2). The classical equations that describe the movement of ions in magnetic or electric fields are the same on which are based a number of currently mass spectrometers.

In this assembly, the positive ions generated are first accelerated through a voltage difference U . They take up a velocity v which depends upon their mass m (cf. Section 16.3.1) and are then submitted to a transverse magnetic field \vec{B}

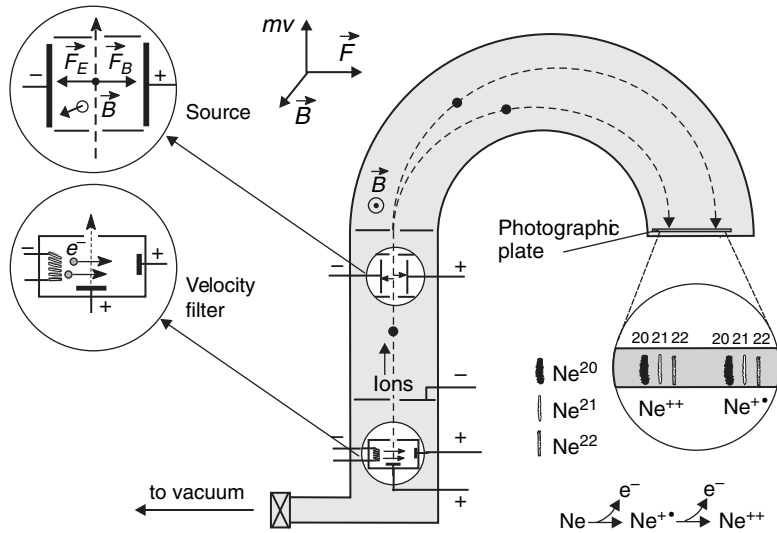


Figure 16.2 A 180° magnetic deflection spectrograph with a velocity filter. The filter eliminates the problem due to the quasi impossibility of having homogeneous and mono-kinetic beams. The diagram represents a sort of photographic recording of the spectrum of neon. The two series of broken lines represent the removal of one or two of the electrons from the different isotopes of elemental neon. The symbol $+ \cdot$ signifies that the ion is both a radical (i.e. has an unpaired number of electrons) and a cation. J.J. Thomson, who around 1913 proved the existence of the isotopes of neon, is considered as the father of this kind of assembly. Later, work performed by F.W. Aston showed the existence of the isotopes of sulphur and chlorine.

responsible for a magnetic flux of density (or field intensity) B . The orientation of field does not modify the ions' velocity but rather forces them on a circular trajectory of which the radius is a function of their m/z ratio.

The fundamental relationship of dynamics, $\vec{F} = m \cdot \vec{a}$ (\vec{a} designates the acceleration), applied to ions of mass m on which is exerted the Lorentz force $\vec{F} = q \cdot \vec{v} \wedge \vec{B}$ leads, after arrangement, to the following relationship:

$$\vec{a} = \frac{q}{m} \vec{v} \wedge \vec{B} \tag{16.1}$$

The orientation of \vec{H} is such that only the centripetal component of the acceleration vector \vec{a} gets involved. The ion trajectory lies in a plane perpendicular to \vec{B} and containing \vec{v} . Since $a = v^2/R$, by substituting a by its form in equation 16.1 (where $q = ze$ is in coulomb, v is in m/s, B is in tesla and m in kg), expression 16.2 is attained which shows that separation occurs according to the *moment* of the ions:

$$R = \frac{mv}{zeB} \tag{16.2}$$

Therefore, the m/z ratio of the ions is only obtained if their speed is known (Equation 16.3):

$$\frac{m}{z} = \frac{RBe}{v} \quad (16.3)$$

For this, in older instruments, a device situated prior to the magnetic sector, called a *velocity filter* was employed. This was done by submitting the ions to two opposing forces through the action of both an electric \vec{E} and a magnetic field \vec{B} . Only the ions remaining on the central trajectory were able to exit the filter. Because the net resulting force must be zero, then $qE = qvB$ and therefore $v = E/B$.

■ **Coulombic force (EMF) and Lorentz force law:** When a potential difference of \vec{V} volts is applied between two parallel plates separated by a distance d , an electric field \vec{E} (V/m) appears, which is uniform and oriented towards the lower potential, if the environment is homogeneous: thus $\vec{E} = \vec{V}/d$. The field \vec{E} determines the Coulomb electrical force \vec{F} acting upon an ion carrying a charge q , whatever its mass and position in the field. $F = qE$ (if the ion is carrying a single elementary charge, $q = e = 1.6 \times 10^{-19}$ C). The coulombic force exerted is independent of the velocity of the ion.

The force that is exerted on a charged particle carrying a charge q , possessing an instantaneous velocity \vec{v} and submitted to the action of a magnetic induction B , is given by the Lorentz force equation:

$$\vec{F} = q\vec{v} \wedge \vec{B}. \quad (16.4)$$

In the general case, when there is an electric field, a positively charged ion will be accelerated in the orientation of \vec{E} , but will spiral when moving through \vec{B} , due to the orientation of the cross-product operator. When there is no electric field, it holds in any reference frame. This equation 16.4 is deduced from Laplace's force law which states the force experienced by a conductor of length dl , through which passes a current I , in a magnetic field \vec{B} . The orientation of this force ($d\vec{F} = I \cdot d\vec{l} \wedge \vec{B}$) can be found from different approaches such as the right-hand three-finger rule or using the orientation of a direct trihedron.

16.3 'EB' or 'BE' geometry mass analysers

Present-day spectrometers with in-built magnetic sectors are the result of the technological evolution of previously double focusing magnetic mass analysers used by Mattauch and Herzog or Bainbridge by the end of the 1930s. They lead to very accurate m/z ratios, though they are limited in the study of high molecular masses (this is the difficulty of producing magnetic sectors of large volume). These instruments also include an electrostatic sector that can be located between the ion accelerator and the magnetic sector (forward geometry, 'EB') or after the magnetic

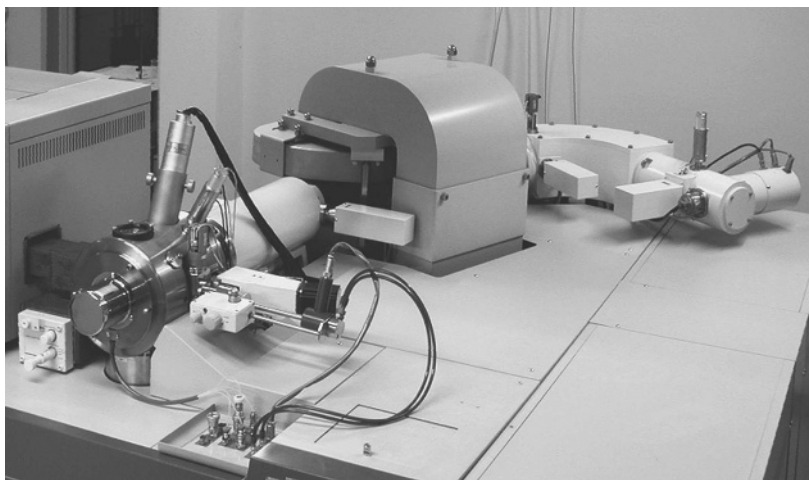
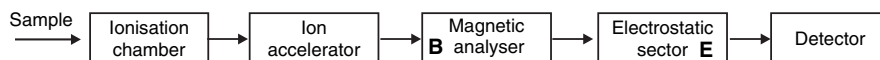


Figure 16.3 A double focusing magnetic sector instrument showing its 'BE' geometry. Model JMS 700. On this photograph, the characteristic shapes of the electromagnet (magnetic sector) and of the electrostatic sector can be seen. The detector lies at the right the ion source is on the left (reproduced courtesy of Jeol, Japan).

sector, as on Figure 16.3 (reversed geometry, 'BE'). The function of the electric sector is to focus the energy of ions produced in the source. Energy focusing leads to a significant improvement in resolution, as we will see later.

■ With a technique known as electromagnetic separation, applications of mass spectrometry began to spread away from the previous academic work into more practical fields like nuclear isotope enrichment. Mass spectrometers have been engaged on a preparative scale (calutrons), notably in the United States where in 1943 several kg of ^{235}U destined for the manufacture of the first atomic bombs were isolated (the Manhattan Project). This, now dated, procedure, which has a low flow rate of under 10^{-3} Pa, is still occasionally in use by other countries.

16.3.1 Ion accelerator

Approximately 5 per cent of the positive ions formed either before or in the ionization chamber or elsewhere in the spectrometer, depending upon the type of instrument, will finally reach the detector. The ions must first be accelerated by means of a series of plates of increasingly negative potential, the total accelerating potential U can be as high as 2 to 10 kV (Figure 16.4). The process must be conducted under a good vacuum ($P < 10^{-4}$ Pa) in order to avoid the formation of electric arcs and to minimize collisions between ions. Under these conditions

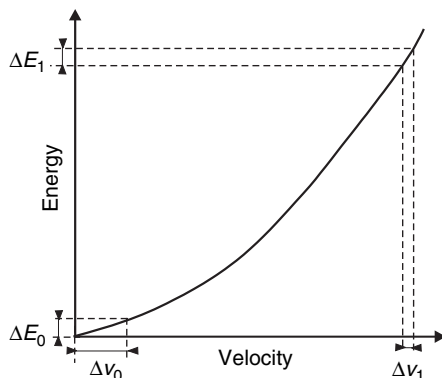


Figure 16.4 Influence of the accelerating voltage upon the range of velocities for ions with the same mass. Assuming the range of velocities prior to acceleration is represented by Δv_0 , it can be observed that the range of velocities after acceleration, Δv_1 , is much smaller. ($\Delta E_1 = \Delta E_0$ but $\Delta v_1 \ll \Delta v_0$).

all the ions carrying the charge $q = ze$, acquire the same kinetic energy $E_1 = zeU$ (relation 16.5). Their velocity after acceleration is therefore inversely proportional to the square root of their mass m_i (Equation 16.6).

$$E_1 = zeU = \frac{1}{2}m_i v_i^2 \quad (16.5)$$

$$v_i = \frac{\sqrt{2zeU}}{m_i} \quad (16.6)$$

However, since the velocities of ions before acceleration are non-zero, their total kinetic energy must take account of their initial kinetic energy, E_0 , which is small and unknown:

$$E_{(\text{total})} = E_1 + E_0 \quad (16.7)$$

The mass accuracy will depend of the velocity of the individual ions with the same mass. For this reason, a high accelerating potential U will restrain the range of velocities for ions of the same mass (Figure 16.4).

16.3.2 Electric sector

An electric sector made of two concentric and cylindrical electrodes is used to further improve the precision of the mass measurements (Figure 16.5).

The force exerted upon the ions, forced to travel tangentially in the electric sector, does not modify their kinetic energy, as the force is perpendicular to the imposed central trajectory that has a radius of curvature R' (Figure 16.5). Its

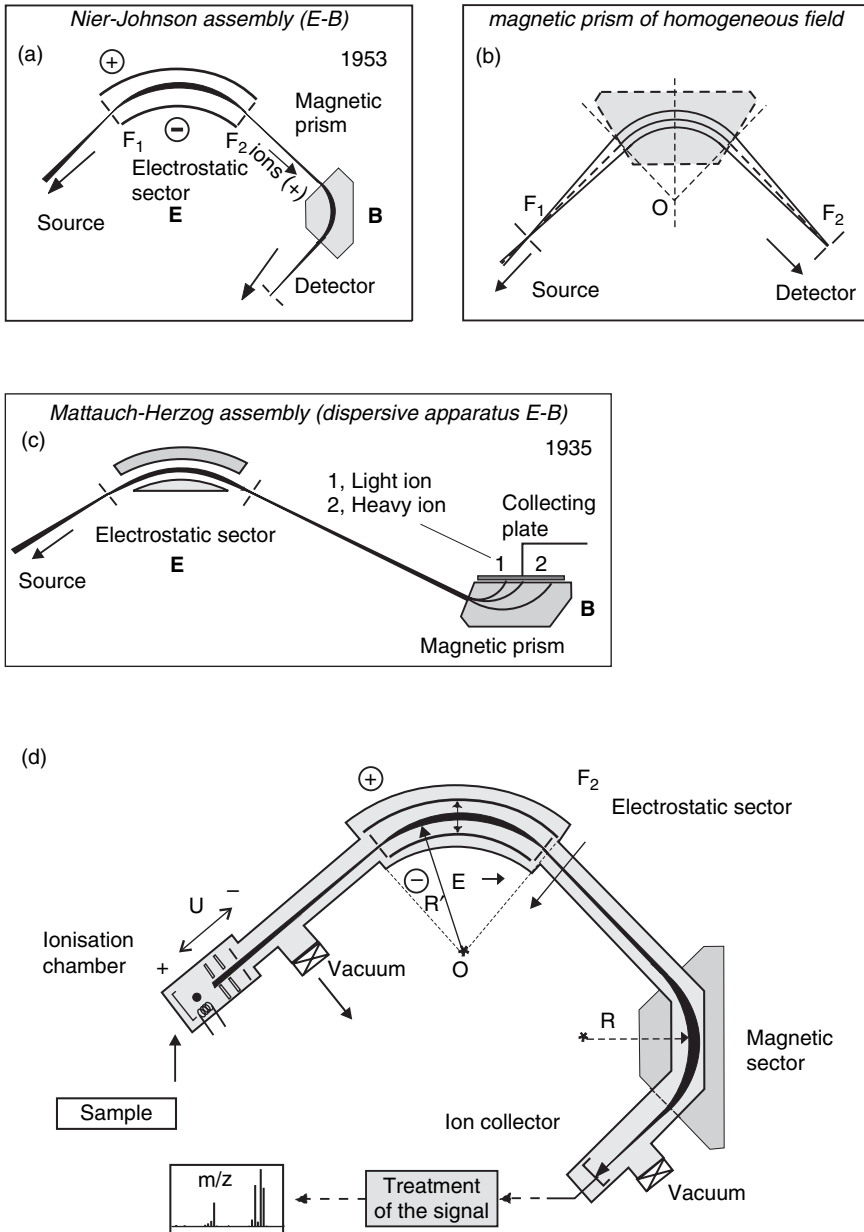


Figure 16.5 Mass spectrometer with magnetic analyser. (a) Nier–Johnson assembly; (b) View of the directional focusing by the magnetic sector (the entrance and exit planes are oblique with respect to the angle of incidence of the beam, ensuring focusing); (c) Mattauch–Herzog assembly; (d) Arrangement of a double focusing spectrometer (e.g. $R' = 40$ cm and $R = 60$ cm).

intensity is equal to $F = mv^2/R'$. Replacement of the term mv^2 by its equivalent in terms of acceleration voltage ($mv^2 = 2zeU$), leads to expression 16.8, which fixes the transmission conditions of the ions through this radial filter. When E and U satisfy this expression, only the ions having acquired the proper energy under acceleration due to the potential drop V , will follow the trajectory of the radius R' and be able to leave the filter. This device acts as an *energy filter*. Moreover, the electric sector has *direction-focusing* properties. It can bring into focus ions, restoring the trajectories which are somewhat deviating before entering the sector. Knowing the potential drop V between the plates of the sector, d distance apart, E can be determined and also the correlation between U and V :

$$E = \frac{2U}{R'} = \frac{V}{d} \quad (16.8)$$

16.3.3 Magnetic analyser

In the EB configuration, a magnetic analyser (or sector) is located after the electric sector. The instruments of this type can be divided into two main categories: the Nier–Johnson geometry in which the radius of curvature imposed upon the ions by the magnetic field is in the same direction as that in the electric field, and the Mattauch–Herzog geometry where these two curvatures are in opposite directions (Figure 16.5). In the latter assemblage, if the magnetic analyser has a proper geometry, all masses are simultaneously focused in the beam, which is a necessary condition for spectrometers housing several detectors that measure isotope ratios. By eliminating the velocity v by combining the expressions 16.2 and 16.5, the *deflection* (or *focusing*) *equation* (16.9) is attained for a magnetic sector:

$$\frac{m}{z} = \frac{R^2 B^2 e}{2U} \quad (16.9)$$

Only the ions which have followed the trajectory with radius R within the flight tube imposed by the instrument will be detected. Hence, to obtain a spectrum within a given mass range, the intensity of the field \vec{B} has to be progressively modified. All ions, whatever the m/z value, will successively follow, one after the other, the unique trajectory leading to the detector. Such a scan requires around 0.1 second.

■ The interdependence of m and U is the origin of a comparative method for a precise determination of masses, called *peak matching*. For this, along with compound X, the precursor of the ion whose mass is to be determined exactly, a mass standard is injected (usually a perfluoro compound whose fragment ions masses are known with high precision). An ion whose mass is close to that of the mass to be determined is chosen as a reference (for example, if the unknown mass is in the order of 200 u, the C_4F_8 ion which has the mass 199.9872 u can be taken and for masses close

to 12, the peak of carbon 12 can be used, which is 12 u by definition). This task is performed automatically on modern instruments.

Both the unknown and the reference peak are forced to overlap. At this point expression 16.10 is verified, and m_x can be calculated:

$$m_x/m_{\text{ref}} = U_{\text{ref}}/U_x \quad (16.10)$$

The magnetic sector is, like the electric sector, focusing in direction: a beam of ions of the same mass and kinetic energy reaching the magnetic sector by F_1 with a small angular dispersion α , is re-focused in F_2 , image of F_1 . At any instant during the scan only ions with a defined mass can follow the radius of curvature R (Figure 16.5). The combination of magnetic and electrostatic deflection of the ions allows focusing on the basis of both momentum and kinetic energy, thus allowing high-resolution mass spectrometric analysis (*directional and energy focusing*). In magnetic sector instruments, the dimensions and the weight of the magnet limit the mass range.

16.4 Time of flight analysers (TOF)

The principle underlying *time of flight* mass spectrometers is based on the relationship that exists between mass and velocity of the ions at a given kinetic energy. The instrument which use a pulsed ionization, measures the time necessary for the masses to travel a distance L in a field free tube, under high vacuum (Figure 16.6). The basic relationship of linear TOF analysers is obtained by eliminating the velocity v from expression 16.5, knowing that $L = vt$:

$$\frac{m}{z} = \left(\frac{2eU}{L^2} \right) t^2 \quad (16.11)$$

In practice, TOF spectrometers enable the analysis of very high masses (300 kDa). To evaluate the masses with precision requires overcoming the initial energy and spatial distributions of the ions. Resolution is related to the duration of the ionization pulse. It can be increased using almost instantaneous ionization methods (a few nanoseconds with a pulsed laser), repeated several hundred times per second, in order that the ions are ejected at the same time. Many instruments contain a *reflectron* to compensate for kinetic energy distribution of ions of the same mass (Figure 16.6). With this kind of electrostatic mirror, the most rapid ions (i.e. higher energies), will penetrate deeper into the decelerating zone than low energy ions, less strongly repelled. The same electrical fields that slowed the ions down as they entered the reflector will now accelerate them back out of the reflector so that they leave with the same velocity that they entered it. In this way the ions of the same mass are time focused, then will arrive on the microchannel detector at the same time.

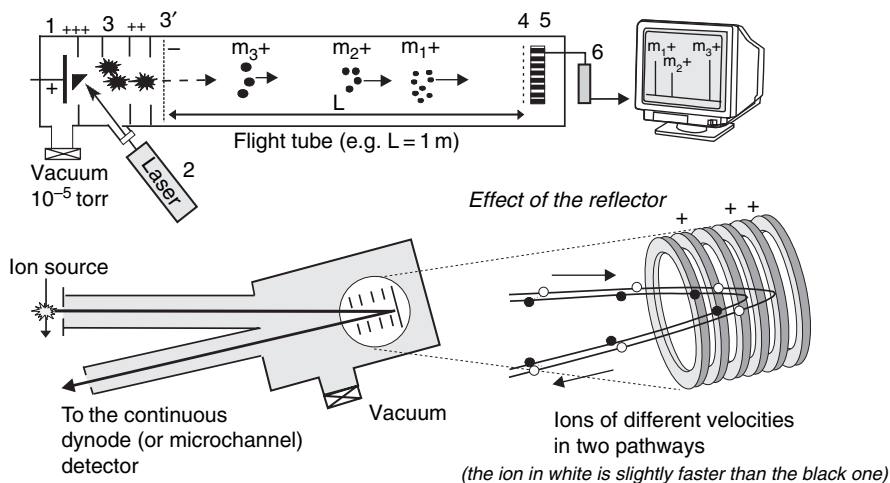


Figure 16.6 A simplified schematic of a time of flight spectrometer and the principle of the ion reflector (reflector). (1) sample and sample holder; (2) MALDI ionization device by pulsed laser bombardment; (3 and 3') ions are formed between a repeller plate and an extraction grid (PD 5000V) then accelerated by another grid; (4) control grid; (5) microchannel collector plate; (6) signal output. Below, a reflectron, which is essentially an electrostatic mirror that is used to time-focus ions of the same mass but which have initially different energies. The widths of the peaks are of the order of 10^{-9} s and the resolution ranges between 15 to 20 000.

■ *Ion mobility spectrometers (IMS)*. This category of portable and fieldable analytical instrument is adapted for research into known and targeted compounds that can be vaporized. Ion mobility is the technique applied most frequently in explosives scanners (airports, terrorist attacks, chemical weapons). They are based upon the time taken by the ions to flight a short drift channel (a few cm) at ambient pressure (Figure 16.7), from ionization chamber to the detector. This technique, which differs from the TOF, to which regrettably it is sometimes compared, is closed to a gas-phase electrophoresis. In the environment of the ion source which contains a radioactive beta particle emitter (^{63}Ni), the presence of air leads to ionic aggregates (clusters) $[(\text{N}_2)_x (\text{H}_2\text{O})_y \text{H}]^+$ and $[(\text{N}_2)_x (\text{H}_2\text{O})_y \text{O}_2]^+$ that will react with the molecules of the sample compound present, depending on their electron affinity. This chemical ionization process forms an aerosol, which migrates in a few milliseconds under the effect of a strong and uniform electric field gradient of about 15 kV/m, which accelerates them towards the detector. The velocity of the ions depends not only on the mass, the charge and the size or shape of the ion, but also on the temperature and pressure of the air. The threshold of detection can reach a few ppb. On calling the speed v , L the distance travelled during the time t_M in the field E , ($E = V/L$) expression 16.12 is obtained:

$$K_{\text{ion}} = \frac{v}{E} = \frac{L}{t_M} \cdot \frac{L}{V} \quad (16.12)$$

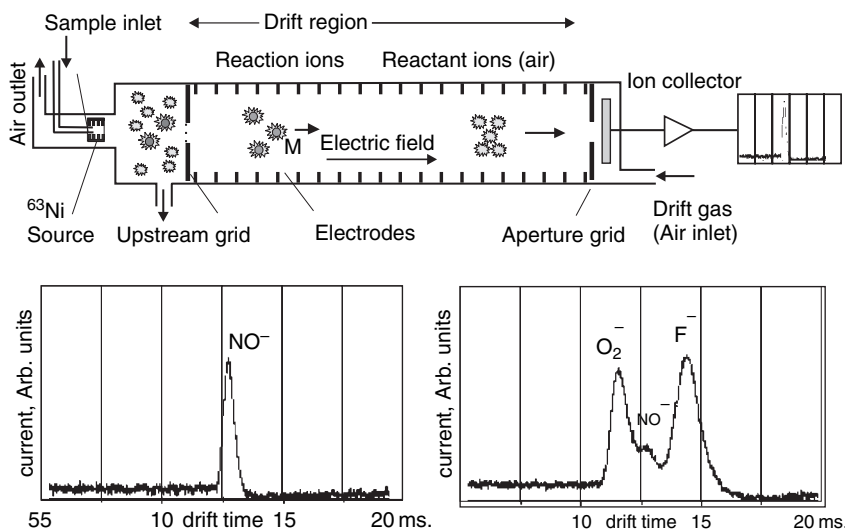


Figure 16.7 *Ion mobility spectrometer.* The ions are admitted into the analyser tube by controlling the polarity of the entry grid. Below, an example of a recording obtained from gaseous compounds. Armies in many countries use this technique for the detection of chemical warfare agents.

The proportional constant (K_{ion}) is called the ion mobility and is usually computed in units of $\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$. K_{ion} is defined by a relation borrowed from capillary electrophoresis.

16.5 Quadrupole analysers

Besides the preceding mass spectrometers, other instruments less voluminous, of lower performance and less expensive have been developed for use in applications that do not require high-resolution spectra. In this category are situated instruments including electric fields only, based upon the use of quadrupole mass filters. These spectrometers, which form the largest category, are very useful as mass detectors (GC/MS or HPLC/MS or ICP/MS installations) as well as in a variety of industrial applications for gas and residual gas in vacuum industry.

16.5.1 Potential and electric fields in a quadrupole

A classic quadrupole is formed of four parallel metallic rods ($L = 5\text{--}20 \text{ cm}$) of hyperbolic cross-section in the interior of the assembly (Figure 16.8). These four

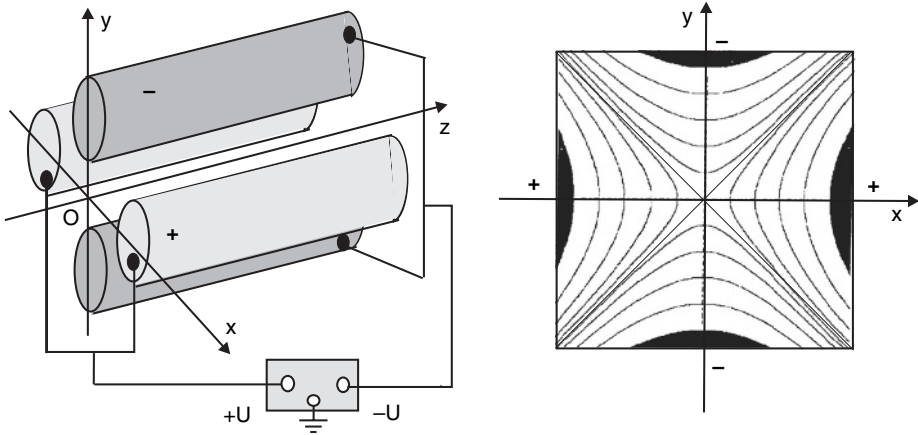


Figure 16.8 Diagram of a quadrupole. Notice the pairing of oppositely charged electrodes. This mechanically simple device requires high-precision manufacture of the rods machined to the hyperbolic shape. In many models the rods have the size of a ball-point pen. Right, graph of a series of equipotential hyperbolic lines in the central part of the filter.

rods are electrodes that are coupled in pairs. The separation between two diametrically opposed rods is designated as $2r_0$. If a potential difference U is applied across the pairs, the potential Φ_E for all points within the xy -plane, irrespective of z , will be given by:

$$\Phi_E = U \cdot \frac{x^2 - y^2}{r_0^2} \quad (16.13)$$

Φ_E remains between $+U$ and $-U$. For a homogeneous medium, the electric potential Φ_E is therefore zero all along the z -axis.

Expression 16.13 implies that in all xy -planes the points having the same potential are situated on the branches of an equilateral hyperbola of which the asymptotes are the straight lines $y = \pm x$. The field lines are orthogonal to the equipotential curves for each point M_{xyz} of the space inside the quadrupole. This field has the value:

$$\vec{E} = -\text{grad}\Phi_E$$

The voltage at the surface of each electrode inside the quadrupole is given by:

$$\begin{aligned} \text{'x electrode'} \quad \Phi_E = +U &\Rightarrow y^2 = x^2 - r_0^2 \\ \text{'y electrode'} \quad \Phi_E = -U &\Rightarrow y^2 = x^2 - r_0^2 \end{aligned}$$

When a positive ion penetrates via the origin O into the filter maintained under vacuum, the components of its velocity vector in xyz -space, represented by (x, y, z) , will determine its trajectory. The central zone behaves like a tunnel along the z -axis, whose walls can attract or repel an ion depending upon the ion's position.

The two positively charged electrodes focus it along the z -axis, which corresponds to the bottom of a potential valley (stability zone), while the two negatively charged bars, in contrast, have a defocusing effect (appearance of a mound of potential in the yz -plane).

■ The image of a marble made to roll along the bottom of a horizontally oriented guttering pipe bears a close similarity to a potential valley. To imagine the effect of a mound of potential, the marble should be imagined as trying to roll along a length of pipe on the outside, a very unstable pathway.

16.5.2 Use of a linear quadrupole as an ion filter

An a.c. voltage of radiofrequency ν and of maximum amplitude V_M is superimposed to the DC potential U (ν is in the order of 2 to 6 MHz and V_M/U of 6 MHz). The two sets of electrodes are submitted to this alternating voltage, but out of phase by π (Figure 16.9).

$$V_{RF} = V_M \cos(2\pi\nu t) \quad (16.14)$$

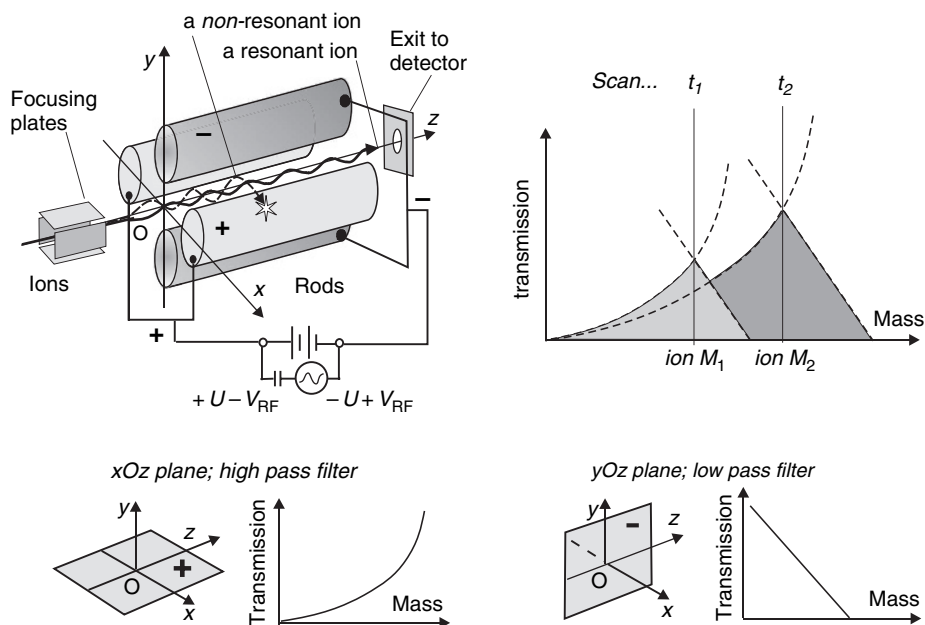


Figure 16.9 *Quadrupolar filter.* Depending upon their mass, the ions respond more or less to the demands of the variable field. Their travel time must be superior to the period of the radiofrequency. For this reason they enter the filter with a weak kinetic energy of several tens of electron volts.

Over time, for each point inside the filter, the potential now corresponds to the algebraic sum of the expressions 16.13 and 16.14:

$$\Phi_{EXY} = [U + V_M \cos(2\pi\nu t)] \frac{x^2 - y^2}{r_0^2} \quad (16.15)$$

The resulting field within the quadrupole is made up partially of a fixed voltage and partially of a variable voltage. Under these conditions the ions which penetrate at O into the quadrupole are submitted to a force that is variable in both intensity and direction. They will follow complex three-dimensional and generally unstable trajectories in the form of a helix or corkscrew. These pathways will generally terminate on contact with one of the electrodes.

To obtain the equations of the movement of an ion the forces F_X , F_Y and F_Z exerted upon it must be known (*Mathieu equations*). As this operation involves complex computation, only two particular cases will be considered here.

1. Assume an ion has a zero velocity component along the y -axis: its trajectory will remain in the xz -plane. The walls being alternately positive and negative ($V_{RF} \gg U$), the heavy ions, due to their high inertia, will not substantially respond to the variations in field orientation: they will only feel the continuous positive voltage U and so will not be perturbed very much. They remain focused on the central axis. However, the lighter ions will be able to oscillate more and more until they discharge, that is be lost, on one of the rods. The two positive rods constitute what is known as a *high-pass filter*.
2. Consider now the case of an ion that does not have a velocity component on the x -axis: its pathway will remain in the yz -plane. The heavy ions will continue to be inexorably attracted towards the negative bars. Only the light ions that can rapidly react to the influence of the radiofrequency will be transmitted. The yz -plane constitutes a *low-pass or highcut filter*.

The calculations related to this technique expose that there are two ways of using this *quadrupolar filter*: one method in which the two voltages, U and V_{RF} , are maintained constant while ν , the radiofrequency, is scanned, or a second method in which the frequency ν is maintained at a given value and the amplitude of the voltages U and V_{RF} are increased regularly in such a way that their ratio remains at a invariable value.

In this way a band-pass is defined, for example 1 u width, unit of resolution, which permits the mass spectrum of the compound to be obtained in a sequential manner.

The resolution R of a quadrupole filter (for details see section 16.8.3) is related to numerous parameters. The instrument can be used either in a mode where Δm is constant – in which case the resolution increases with the mass (Figure 16.10) – or in a mode in which the resolution is constant, close to 1 u.

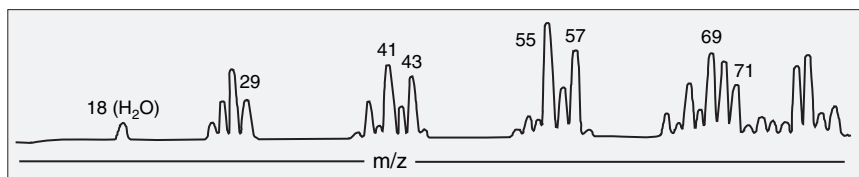


Figure 16.10 Partial spectrum of a hydrocarbon obtained with a gas analyser operating under the quadrupole filter principle. The recording reveals that the instrument has been used in the mode for which Δm is constant for the whole range of masses.

The maximum resolution is given by expression 16.16 for an ion carrying a unit charge e , accelerated under a PD of V_z volts at the entrance to a filter of length L and of radius r_0 by using a radiofrequency whose maximum voltage is V_{\max} .

$$R = Cste \cdot \frac{L^2 \cdot V_{\max}}{r_0 \cdot e \cdot V_z} \quad (16.16)$$

16.6 Quadrupole ion trap analysers

Other mass spectrometers are equipped with three-dimensional ion traps of which the geometry is much different to the quadrupoles previously described. In an ion-trap, the ions are confined between three electrodes (one toroidal and two end-caps), whose particular shape appears to result from a sort of anamorphosis of the four-bar set-up of a classic quadrupole. As in the previous category they operate under the effect of a variable electric field (with or without a superimposed fixed field). Although they are, in appearance, physically simple devices, the fundamental principle of ion trap is complex. These ion trap detectors are sensitive, less costly than quadrupoles and compatible with different ionization techniques. The volume defined by the electrodes, named superior, inferior and annular, is simultaneously the ion source and the *mass filter* (Figure 16.11). These analysers are almost exclusively linked with a separative technique (GC/MS).

The functioning principles can be discussed as follows: the compound is submitted to a very brief electron bombardment yielding ions which subsequently penetrate the central part of the filter. A radiofrequency voltage is then applied to the annular electrode that confines these ions to the central space, by forcing them to follow complex trajectories in the presence of a low pressure of helium, of about 0.01 Pa, introduced to the trap as a form of shock absorber.

To analyse the ions present, the radiofrequency amplitude is increased gradually to destabilize the ions. One by one, in the rising order of m/z ratio,

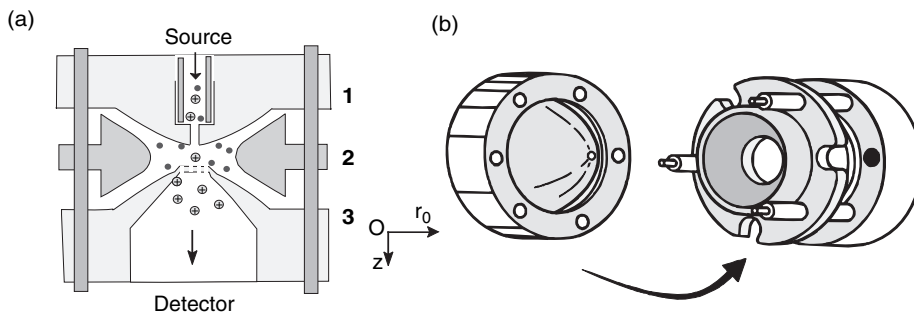


Figure 16.11 *Ion-trap spectrometer.* (a) Design of the electrodes in an ion trap; (b) Diagram giving a perspective of the end-cap and annular electrodes (from *Current Sep.* 1997, 16 : 3, p 85).

the amplitude of oscillation of the ions grows in the axial direction until they are eventually ejected through a series of small holes pierced in one of the end-cap electrodes behind which stands an electron multiplier as detector (Figure 16.12). If a reagent gas is introduced concurrently into the trap to react with the sample then a chemical ionization can also be obtained (section 16.10.2).

These small volume and high sensitivity instruments have, however, the inconvenience of operating over a narrow dynamic range and with an average reproducibility of spectra from the same sample, which represents a serious disadvantage in quantitative analysis.

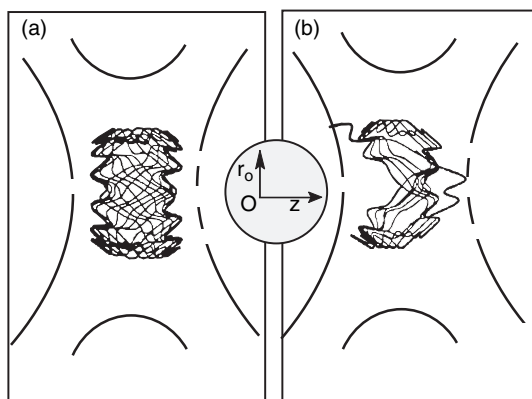


Figure 16.12 *Modelling of the ion pathway in a trap.* (a) Trajectories of a stabilized ion; (b) Pathway of an unstable ion terminating in contact with one of the electrodes (the layout of the trap is the same as in Figure 16.11).

16.7 Ion cyclotron resonance analysers (ICRMS)

These mass spectrometers, not widely used, are rather rare compared to the previous ones. Basically a magnetic ion-trap, a ICRMS is able to ascertain the mass of ions with great precision. The mode of operation can be described in the following way:

The ions, obtained through one of the numerous existing procedures, are directed towards a small cavity situated in the centre of a very intense magnetic field \vec{B} (4 to 9 T) produced by a superconducting coil. In this chamber, the ions are slowed by collisions with residual molecules (the vacuum not being perfect) and trapped through the effects of the repulsion of internal walls carrying positive potentials. Their trajectories are complex but their projections in the xy -plane, orthogonal to the field \vec{B} , correspond to a circular movement (Figure 16.13). The radius of the circular movement depends upon the kinetic energy of the ion and is given by equation 16.2. If the velocity v is small and the magnetic field \vec{B} is intense, the circumference will be very small (less than 1 mm). From the classic expressions $v = \omega R$ and $\omega = 2\pi\nu$, relation 16.17 can be obtained. This shows that to each mass there is a particular frequency, *independent of the velocity of the ion*.

Hence, at any given moment, there will be as many different frequencies of rotation as there are ions of different m/z ratio in the trap.

$$\frac{m}{z} = \frac{eB}{2\pi\nu} \quad (16.17)$$

or

$$\nu = 15350 \frac{Bz}{m} \quad (16.18)$$

The practical expression 16.18 gives the frequency ν (in kHz) of an ion of mass m (Da) carrying a charge z in a field \vec{B} (tesla). Mass analysis is achieved by measuring precisely the different frequencies of the ions.

To establish the frequencies and consequently the m/z ratios of the ions, a brief pulse of RF sweep (e.g. 1 ms), commonly called a *chirp*, is sent, that covers a range of frequencies corresponding to the m/z values to observe. In this way all of the ions will raise their energy. Their frequencies do not change during the time of the pulse, but during the irradiation pulse the radii of their trajectories will increase since their velocities are growing up. They describe spiral orbits. Moreover, following the impulse, the ions of the same m/z ratio come together, phased, which consequently induces a signal (cyclotronic resonance) of the same frequency, in the detector plates (Figure 16.13). Overall the ions lead to a complex signal due to the superimposition of induced frequencies by many different m/z sub-populations. This interferogram, of which the intensity $[I = f(t)]$ decreases when excitation stops, contains information on each of all frequencies which it represents. A calculation on the data allows to extract the frequency and amplitude for each component of the composite signal [Fourier Transform of

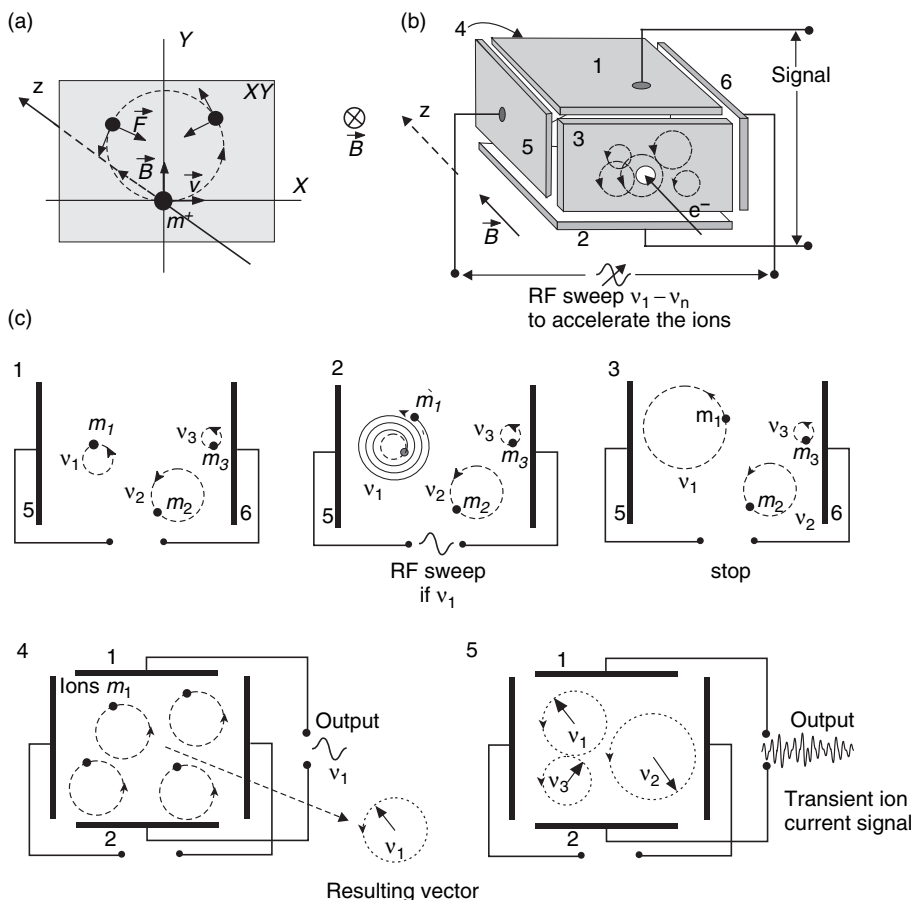


Figure 16.13 The principle of ICRMS. (a) Basic ion trajectory in a magnetic field taking account of the orientation chosen. (b) Simplified view of an ion-trapping cell (plates 5 or 6 are excitatory, plates 3 and 4 trap the ions and plates 1 and 2 are used for detection). The ions are formed either inside or outside the cell. (c) Principle of detection: 1, three ions waiting; 2, pulse of frequency. At ν_1 only ion m_1 is excited; 3, following excitation, ion m_1 adopts a wider trajectory; 4, for a real sample containing a large number of m_1 , these ions will arrange themselves to orbit in phase. On detecting plates 1 and 2 they produce the effect of an electric field vector turning at the same frequency ν_1 whose intensity is a function of its concentration; 5, If the three ions $m_1 - m_3$ have been simultaneously excited, the signal at the exit will be an interferogram. A Fourier calculation would lead to the usual spectrum.

$I = f(t)$ to $I = f(\nu)$ or spectrum $I = f(m/z)$. The main characteristics of ICRMS include very high mass resolution, attomole sensitivity and better than 1 ppm mass accuracy.

■ All categories of mass spectrometers adopt the principle of comparative measurements. The instrument is calibrated with ions of known mass. Although

there are simple relations, some of which are included in this chapter and which lead to the calculation of mass from a variety of parameters, they remain explanatory formulae not used by the computer programs of spectrometers. These basic equations do not take into account secondary factors characteristic to each assembly.

16.8 Mass spectrometer performances

16.8.1 Upper mass limit

All mass spectrometers can determine the m/z ratio up to a maximum value. The greatest mass, in Daltons, will depend on the number of charges z that the ion carries, e.g. if the spectrometer is able to measure m/z up to 2000, it will be possible to detect an ion of mass 80 kDa carrying 40 elementary charges ($q = 40e$).

16.8.2 Sensitivity

The sensitivity of a mass spectrometer is given by the weight of sample consumed per second (e.g. a few pg/s or femtomole/s) in order to obtain a signal of normalized intensity. Since the ion beam persists only a very short time, there is a permanent and dynamic renewal of the ions: spectra are obtained by repetitive scanning.

16.8.3 Mass resolution or resolving power

On spectra with gaussian shaped peaks (obtained with an instrument housing a magnetic sector, a time of flight or cyclotron), masses become more easily distinguished as the corresponding neighbouring peaks become narrower. The width of every mass peak is then a gauge of the performance of the instrument. This important property is at the origin of a parameter called the *resolving power* R (dimensionless), for which several definitions co-exist.

To calculate R , the value m/z of the peak selected is divided by its width $\Delta(m/z)$ either at the mid-height (full width at half maximum, FWHM) for an isolated peak, or at 5 per cent of the height when two neighbouring peaks are being considered (Figures 16.14 and 16.15). To be more precise, the *resolution*, as in most disciplines, is understood to be the smallest observable change in a quantity, here a mass interval (Δm).

$$R = (m/z) / \Delta(m/z) \quad \text{or} \quad R = M / \Delta m \quad (16.19)$$

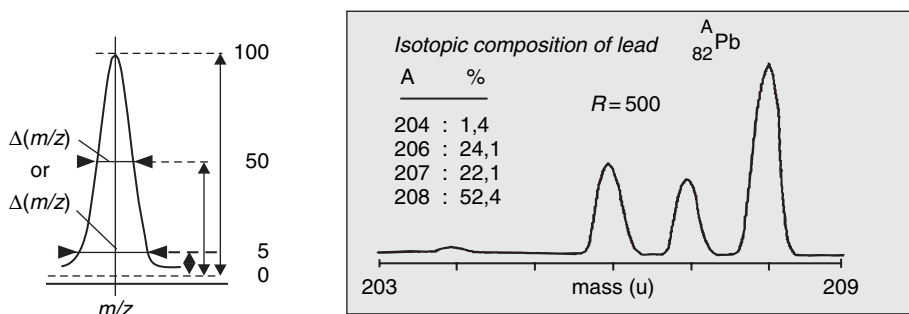


Figure 16.14 Resolving power. Left, definition of this parameter in the case of an isolated peak. Depending upon the manufacturer the width of the peak is measured either at 50 per cent or at 5 per cent of its height. Right, example of a low-resolution spectrum of a sample of lead. The value of R found depends very much on the compound and the mass chosen and of the slit width of the instrument.

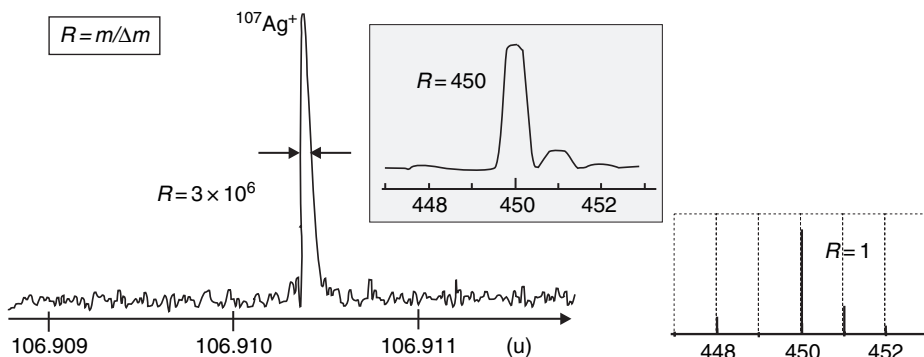


Figure 16.15 Resolving power established using experimental spectra. Left, an example of a recording corresponding to a very high resolving power (ion cyclotron resonance apparatus). With this technology, which requires Fourier Transform, the theoretical maximum for resolution will be: $R = \nu T/2$, (ν being the frequency (Hz) of the ion concerned and T (s), the acquisition time for the interferogram). Centre, a section of a crude signal for a quadrupole mass filter ($R = 450$). Right, a more conventional presentation of the same recording (resolution 1 u).

Fragmentation spectra presented in the form of lines do not permit the calculation above. Furthermore it can be said that for such a spectrum the resolution (and not the resolving power) is exactly 1 u (for example distinguishing peak 28 from peak 29, or peak 499 from peak 500...). In fact, with quadrupoles or ion trap instruments, this parameter is of less interest and the values of the resolving power are modest. An observation of the crude signal from such an instrument shows broad peaks with flat rounded maxima (Figure 16.15). If Δm is constant and close to 1 u, the resolving power will have the approximate value of the mass of interest.

16.9 Sample introduction

Given the variety of forms and the nature of possible samples, there are numerous methods of introducing samples, each with its own particular requirements, into mass spectrometers. The sample can be ionized either before or in the ion source of the spectrometer.

16.9.1 Direct introduction

For gases or volatile liquids a very small quantity of sample is introduced with a micro-syringe to a kind of reservoir linked to the ionization chamber *via* a very narrow channel. Under the effect of a high vacuum that is maintained in the reservoir, the compound is sucked up and vaporized. This procedure is known as a *molecular leak or molecular pumping*. For continuous monitoring of gases and volatile compounds in the vapour state or dissolved in a liquid such as water, the sample can be diffused through a porous membrane.

Solid samples, able to have a sufficient vapour pressure at about 300°C, are deposited on the tip of a metal support, which is inserted in the source of the instrument through a vacuum lock, then heated. For some other modes of ionization the solid sample is mixed into a matrix (e.g. glycerol or benzoic acid).

16.9.2 Sample introduction in hyphenated techniques

Complex mixtures of compounds are often analysed by hyphenated techniques in which a separative method, as chromatography, is interfaced with a mass spectrometer. Thus, the various constituents of the sample can be separately analysed. Coupling of a gas chromatograph to a mass spectrometer (GC/MS) has become widespread. The outlet of the GC capillary column is directly inserted in the ionization chamber of the MS. In this way, compounds eluting from the column, mixed with the carrier gas, are introduced in the ionization chamber and then analysed in the order in which they exit the GC. As the flow rate never exceeds 1–2 mL/min, the pumping capacity of the spectrometer is sufficient for maintaining the high vacuum required for the analysis.

Nowadays, interfacing of a liquid chromatograph or a capillary electrophoresis instrument with a mass spectrometer is used too, although technically more complex. The presence of water in elution solvents is an undesirable compound for the mass spectrometer. With HPLC, the use of micro-columns is desirable, to have very low flow rates. They are also well-suited with different ionization techniques for the analysis of high molecular-weight compounds. A rather old device, whose sensitivity is now judged to be very poor, is the particle beam interface

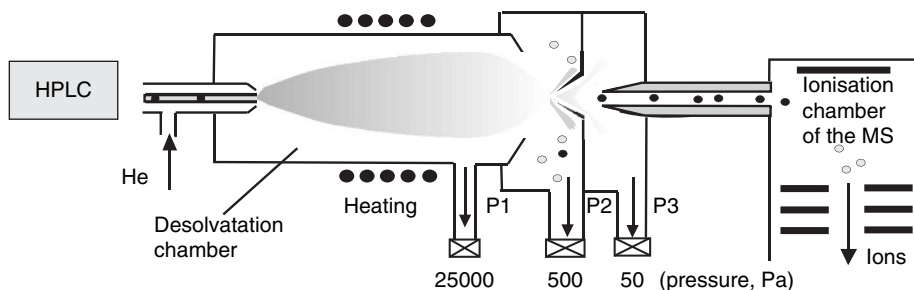


Figure 16.16 *HPLC/MS interface using a particle beam interface.* Solvent molecules are lighter than analytes, so their angular dispersion is wider. Heavier molecules, less deflected by the effect of the vacuum, have a greater probability of remaining on the axis of the system and being transported to the MS. This ballistic approach was much used formerly in other devices, though now is all but abandoned.

(Figure 16.16) in which the liquid phase is vaporized in the form of a spray in the desolvation chamber before entering the area where the sample is concentrated through evaporation.

16.10 Major vacuum ionization techniques

Analysis by mass spectrometry begins with the ionization of a compound into individual, entire or fragmented species. The choice of the ionization method depends upon the planned study and the nature of the subject compound, which could be a small organic molecule, a metal, a biological macromolecule..., and upon its state (gas, liquid, solid). The corresponding devices are complex and often installed during assembling of the spectrometer, giving specialized instrumentation.

16.10.1 Electron ionization (EI)

Electron ionization is the most commonly method used for the analysis of volatile compounds. It is the case for organic molecules. This is a ionization in the gas phase that occurs in the ion source by the collision of the neutral molecules of the sample and electrons emitted from a filament by a thermoionic process (Figure 16.17). The ejection of the most weakly held electron leads to positive ions. This reproducible procedure facilitates the identification of a compound by comparing its spectrum with those collected in a spectral library, assuming the compound is registered within.

Negative ions are also formed though in smaller quantity and by different mechanisms. Thus the technique is usually used in the positive ion mode, but the negative ions can be also studied by inverting the polarity of the accelerating

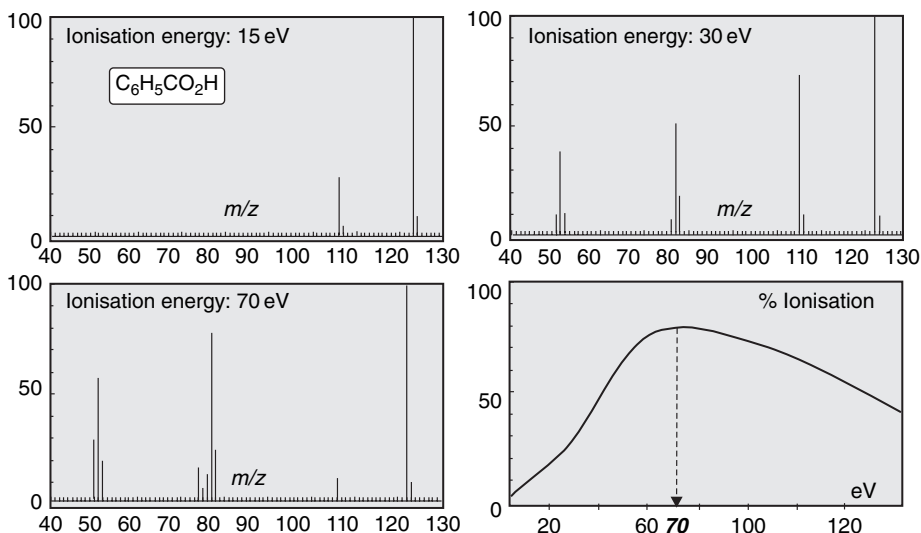


Figure 16.18 Influence of electron energy on fragmentation. An example with benzoic acid is shown here. A graph of the ionization efficiency as a function of electron energy is presented. Note that the spectra are normalized, that is that the most intense peak takes a value of 100. Some claim that this is an application of Procruste's method (according to the Greek legend, the brigand Procruste forced his prisoners to lie on a bed and then, depending of their size, he would either cut their feet or stretch their bodies so they would fit the dimensions of the bed).

the instrument. The relatively high pressure that exists in this section of the MS, reduces the mean free path of the molecules favouring collisions.

This ionization by reaction with gaseous reagents is a mild ionization procedure that produces both positive and negative species. In particular the formation of the pseudomolecular MH^+ ion (which is not a radical cation) is observed, though its concentration depends upon the affinity of M for H^+ , the latter having a lesser tendency to fragment than the ion M^+ obtained by electron impact. This is useful to get the value of the molecular weight of M (Figure 16.19).

However, for compounds of type RH, the ion R^+ is also observed though the chemical ionization always leaves a doubt concerning the value of the molecular mass of the compound studied.

In ammonia chemical ionization, NH_4^+ behaves as CH_5^+ . With isobutane, the main reactive ion is $(CH_3)_3C^+$, which leads to the formation of the stable ion $(CH_3)_3C M^+$, which leads to the ion $(M + 1)^+$, and to isobutene.

16.10.3 Fast atom bombardment (FAB)

This technique of ionization and those described in the following sections are reserved for polar or non-volatile compounds under vacuum conditions.

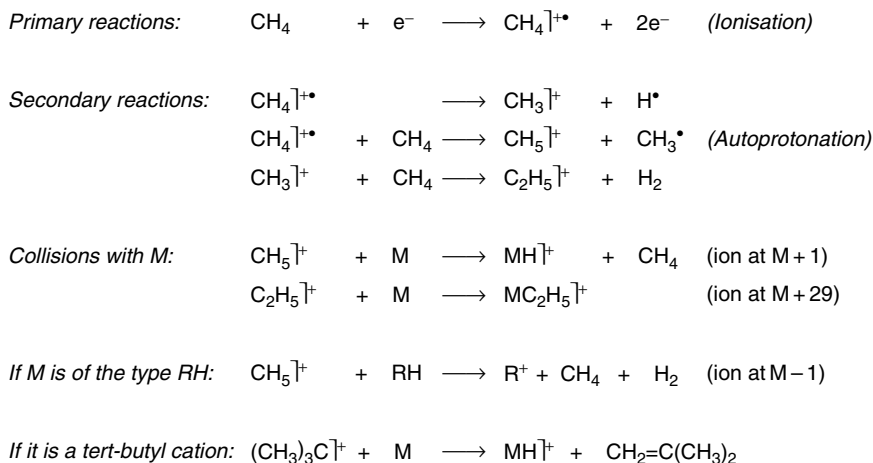


Figure 16.19 *Chemical ionization.* Reactions occurring with methane as reagent gas, and collisions with the molecules M, of the compound. The last equation corresponds to that occurs when isobutene is used. The presence of relatively heavy ions coming from the reagent gas removes all interest in studying the spectrum below 45 Da.

Ionization is achieved through the impact of fast and non-ionized heavy atoms (Ar and Xe) targeting the sample being previously dispersed in a non-volatile liquid matrix (glycerol or diethanolamine) and deposited into the source at the end of a sample probe.

To produce this fast atom bombardment a argon or xenon gas is first ionized by electrons. These primary ions are then accelerated and directed towards a collision chamber where they come into contact with slow-moving neutrals atoms of the same gas. During those collisions, the charge of the fast moving ion is transferred to the slow-moving neutral species resulting in a fast neutral atom, which is directed at the sample (Figure 16.20). Residual ions are eliminated from the principal beam by deflection with an electric field. The sample, on the probe, is ionized when bombarded by the fast atom beam. This technique also causes ionization of the matrix, leading to fairly large background noise, which renders the study of small mass ions not possible.

Instead of fast-moving atoms, fast-moving ions such as Cs^+ or Ar^+ can be used. This is the base of a technique called SIMS (for *secondary ion mass spectrometry*). These ions, which can be accelerated, enhance the number of energetic fragmentations.

16.10.4 Matrix-assisted laser desorption ionization (MALDI)

This mode of ionization–desorption leads to weak fragmentation. The analyte to be studied is first incorporated into a solid organic matrix (dihydroxybenzoic acid

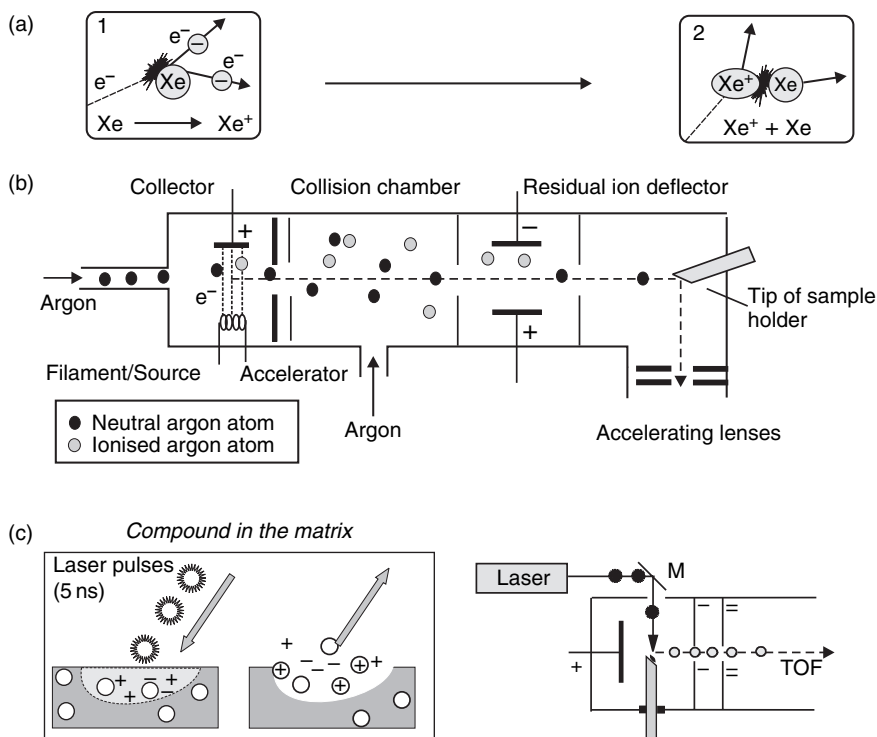


Figure 16.20 FAB and MALDI techniques. (a) The principle of fast-atom beam formation with xenon; (b) The formation of fast atoms of argon in a collision chamber and subsequent bombardment of the sample by this atom beam, usually of 5–10 kV kinetic energy; (c) MALDI or ionization by effect of illumination with a beam of laser generated light onto a matrix containing a small proportion of analyte. The impact of the photon is comparable with that of a heavy atom. Through a mechanism, as yet not fully elucidated, desorption and photoionization of the molecules is produced. These modes of ionization by laser firing are particularly useful for the study of high molecular weight compounds, especially in biochemistry, though not for routine measurements.

for example) and next deposited upon a sample holder that is irradiated with UV laser pulses (e.g. N₂ laser, $\lambda = 337$ nm, 5 ns pulse width). The laser energy strikes the crystalline matrix to cause its excitation and the subsequent ejection of analyte species, which are desorbed and ionized in the gaseous state (Figure 16.20c). Although a substantial amount of energy is involved, this is considered as a soft ionization technique.

Pulsed ionization is particularly well suited to time-of-flight instruments for bio-macromolecules, knowing that it gives ions carrying only a single charge in contrast to atmospheric pressure techniques (see following paragraph).

The analysis of small molecules ($M < 500$ Da) is limited because the matrix decomposes upon absorption of the laser radiation. However, other supports

based upon porous silica can be used as the matrix to overcome this disadvantage.

16.11 Atmospheric pressure ionization (API)

Many new applications in mass spectrometry concern either the measurement of elements or the study of unstable and polar biomolecules ($M > 2000$ Da). These applications have become possible with the advent of atmospheric pressure ionization (API) techniques applicable to compounds in solution following nebulization.

16.11.1 Thermal ionization (TI)

Argon plasmas are used in optical emission spectrometry (cf. section 14.3.1) to atomize and ionize elements in order to provoke the emission of characteristic spectral lines. Hence, it is not surprising that the same plasma torches are employed to ionize inorganic samples in mass spectrometry. Thermal ionization is induced at high temperatures in a gaseous sample with microwave or an inductively coupled plasma.

The sample in solution is nebulized in the plasma generated close to a small aperture (called a skimmer), situated axially, at the entrance to the analyser. A proportion of the ions is sucked up due to the pressure differential (Figure 16.21). A plasma can equally be placed at the exit of a GC capillary column. Since all of the chemical bonds are destroyed in the plasma, only information concerning the elements concentrations becomes available (for example a *speciation analysis* of organometallic compounds).

■ Argon plasmas also contain polyatomic ions at very low concentrations, which arise from the air, from argon or from the sample matrix. These species can be isobaric with the analytes ions and then could be a source of possible confusion when MS have a low resolving power. Thus if we consider N_2 (28 u), O_2 (32 u), ArH (78 u), ClO (51 u), ArO (56 u), CaO (56 u), the corresponding molecular ions have the same m/z ratio as elements such as Si, S, V and Fe, which could be the species under investigation. In this case, two methods for clarification are necessary: selection of another isotope of the element considered (^{54}Fe rather than ^{56}Fe) or choice of another spectrometer with a collision chamber (section 16.12) to dissociate the polyatomic ions.

16.11.2 Spray ionization

Hyphenated techniques as micro-HPLC/SM or HPCE/SM have been the driving force in the development of different highly sensitive devices, which have in common the nebulization of the mobile liquid phase, issued from the separative

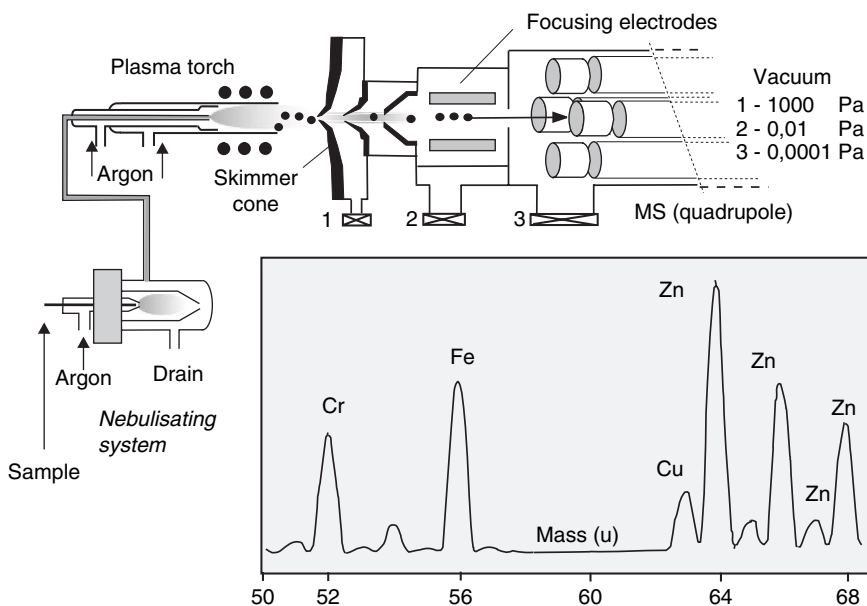


Figure 16.21 Ionization in a plasma torch and recording made by the ICP/MS coupling. A proportion of the ions generated by the horizontal plasma reaches the mass spectrometer. Below, a spectrum of a mixture of several metallic elements with masses recorded between 50 and 68 Da is shown. Note the presence of different isotopes of the elements concerned. The resolving power of this instrument is insufficient to distinguish isobaric ions, that is resulting from different elements yet having practically the same mass and whose signals are superimposed upon each other in the spectrum.

instrument. Depending on the pH this mobile phase can have a variable concentration of H^+ ions and can also contain cations such as NH_4^+ , Na^+ , K^+ (in the case of an electrolyte).

Several techniques are available.

Electrospray or ‘ionspray’ (ESI) (Figure 16.22)

Very small droplets of solvent-containing analyte are formed at the end of a fine silica capillary whose surface has been metallized and which is held at a high positive potential (assuming a study of positive ions). This electric field confers a large charge density (z/m) on these droplets. Solvent is removed through complex mechanisms, by heat or by energetic collisions with a dry gas. As the droplets evaporate, the charge density at their surface increases leading to coulombic explosion (Rayleigh limit). This produces smaller droplets that can repeat the process, finally liberating non-fragmented, protonated or cationic multicharged ions of the analyte (on average 1 elementary charge for each 1000 Da of mass). This is a soft

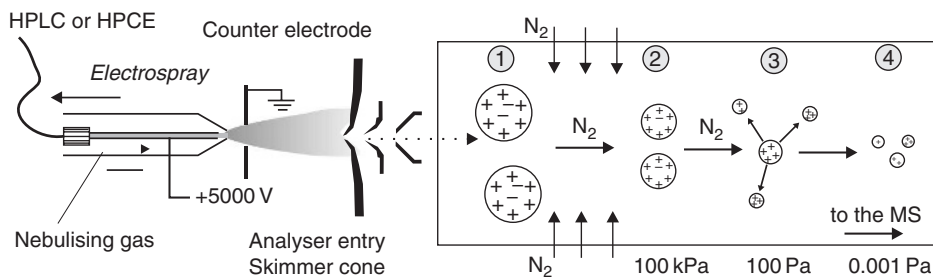


Figure 16.22 A schematic of the mechanism of ion formation in atmospheric pressure ionization by electrospray. The analyte solution flow passes through the electrospray needle that has a potential in the range from 2.5 to 4 kV with respect to the counter electrode. The charged droplets have a surface charge of the same polarity to the charge on the needle. The droplets (1) shrink until they reach the point that the surface tension can no longer sustain the charge ('Coulombic explosion') (2) and the droplet is ripped apart, expelling analyte molecules carrying several charges (3). The presence of nitrogen improves the concentration process (4).

method of ionization because very little fragmentation is produced. Therefore, ESI-MS is an important technique in biological studies.

Photoionization

This mode of ionization is associated with photon induced ionization at around 10 eV. In place of the corona assembly, a UV lamp is used as for the photoionization detection (cf. section 2.7.5). This process, which yields few fragments, is only interesting for molecules of low polarity.

Atmospheric pressure chemical ionization (APCI)

Different from the EI source, the APCI source contains a heated vaporizer which facilitates rapid desolvation/vaporization of the droplets by a very brief heating period up to 500 °C. Reagent ions obtained from the solvent vapour by a corona discharge ionize the vaporized sample molecules through an ion–molecule reaction. Chemical ionization of sample molecules is very efficient due to the numerous ion–molecule collisions (Figure 16.23). This technique leads to multicharged ions of type $(M + nH)^{n+}$ by proton transfer (in the positive mode). Unfortunately this technique is difficult to miniaturize as the required flow rate is higher than that of the electrospray and as such is convenient only for volatile and thermally stable molecules of mass less than 1000 Da.

The advantage of these soft methods of ionization is to obtain pseudomolecular and multicharged ions (z can be greater than 30), formed prior to entry into the spectrometer. With the exception of APCI they are able to extend the range

Atmospheric pressure
chemical ionisation (APCI)

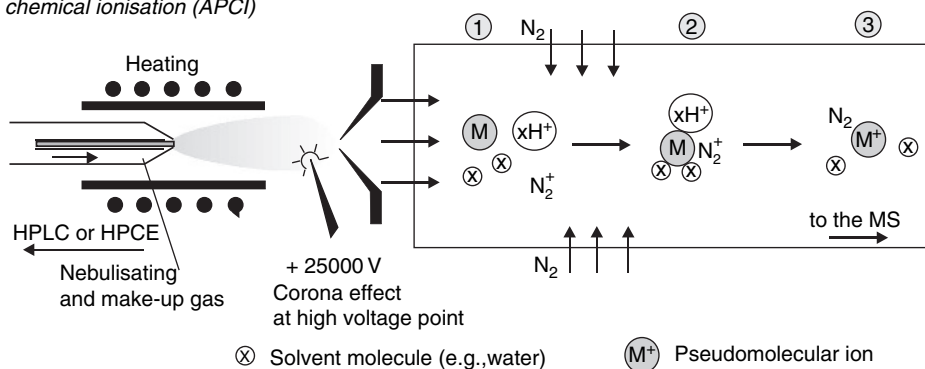


Figure 16.23 Atmospheric pressure chemical ionization. (1) The sample solution is sprayed and mixed with the ions issuing from a reagent gas such as nitrogen, ionized by a polarized needle. Application of a high potential (typically ± 3 to ± 5 kV) produces reagent ion plasma through a corona discharge, mainly from the solvent vapour. These reagent ions associate in their turn with molecules of the analyte, leading to clusters. These are destroyed by a current of nitrogen, which mediates charge transfer to the analyte.

of masses of these instruments up to 10^5 Da (ex. Proteins, polysaccharides and other polymers). These procedures lead to spectra presenting the phenomenon of an ‘ionic envelope’, which leads to the calculation of the molecular mass (Figure 16.24).

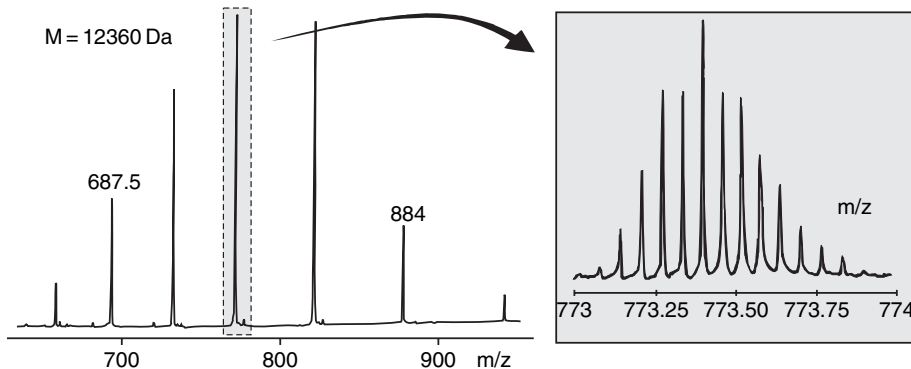


Figure 16.24 Multicharged molecular ions. Left, a spectrum obtained from cytochrome C (horse heart), a protein of molecular weight 12360 Da, by the electrospray method. Between two consecutive peaks in the molecular ion cluster, the difference in the charge on the ion is one unit. The second spectrum, on the right, corresponds to the high-resolution spectrum in the region 773–774 m/z range. From these spectra, it is possible to calculate a very close value of the molecular weight, as well as the number of charges carried by the ions, by two independent methods (courtesy of McLafferty F.W. *et al. Anal. Chem.* 1995, 67, 3802–3805).

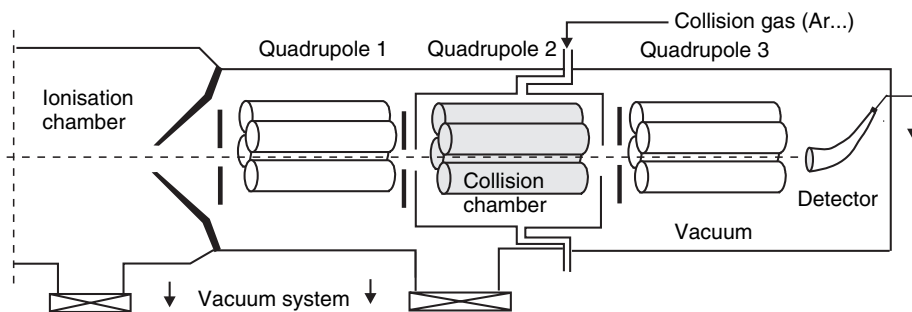


Figure 16.25 A triple quadrupole (QQQ-MS-MS). The middle quadrupole is used as a collision chamber. It is operated in the radiofrequency voltage mode only, where it will transmit all masses. A gas is introduced in this middle quadrupole for collision activation. These instrument can conduct all three types of MS-MS analysis described below.

16.12 Tandem mass spectrometry (MS/MS)

In methods of ionization like ESI or MALDI, spectra often only contain the ionized molecule with very little fragmentation data, contrary to earliest ionization methods like EI and CI. Consequently the spectra are of little use for structural characterization. In these cases, induced fragmentation is required to complement the measurements of mass.

Mass spectrometers have been developed with two or three mass analysers in a series to study ions fragmentation (Figure 16.25). They can be assembled in different configurations but all of them include a collision chamber where ions collide with a target gas such as argon or nitrogen. The kinetic energy of ions is converted to vibrational energy and the ions fragment (*CID, collision induced dissociation*). Many types of tandem mass spectrometers exist. This can be the combination of a magnetic sector followed by a quadrupole, or two quadrupoles, or a TOF with a quadrupole (or the reverse). On the basis of data collected in a first and normal-mode measurement, tandem mass spectrometers have different scanning modes.

- The first method consists of choosing a precursor ion, which corresponds to a pre-selected m/z ratio with the first mass analyser, playing the role of a filter. This ion crosses the collision chamber where it collides with a target gas as argon or nitrogen. The resulting daughter ions continue their course towards the second mass analyser, switched to normal mode. The m/z values of fragment ions are then determined by scanning the second mass analyser. It results a better knowledge of the initially selected ion, to the exclusion of the other ions formed in the ion source (*daughter ion scan*).

- In an alternative method the second mass analyser is set to a selected m/z ratio allowing passage only to those ions, while the first mass analyser is scanned over the mass range. In this way all parent ions leading to that particular product ion can be identified (*search for precursors*).
- Finally, a double synchronized scanning of both mass analysers is made with a pre-programmed shift between them, corresponding to a given neutral mass (e.g. a neutral fragment such as CO or C₂H₂). Thus, all precursor ions present in the ion source, that undergo the same neutral loss, are detected (*search for neutrals*).

16.13 Ion detection

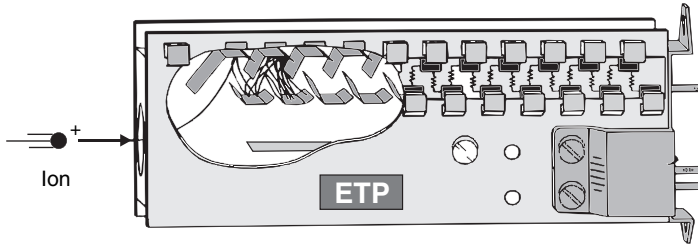
Detection in mass spectrometry is based upon the measurement of the electric charges carried by ions. Normally the number of individual ions of the same species is very large and gives an analogue signal output though certain models have such a power of amplification that they are able to detect the impact of a single ion.

In order to exploit a spectrum quantitatively it is essential that the number of ions detected reflects the number of ions produced, irrespective of their mass. If the mass spectra are obtained by scanning the magnetic field, the deflection formula reveals that the m/z ratio varies with the square of the magnetic field.

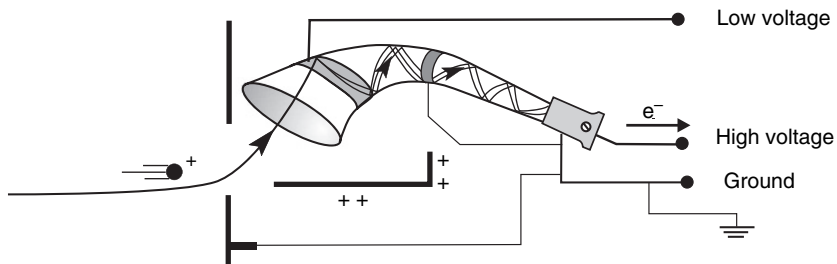
Major types of detection systems:

- *Electron multipliers with discrete dynodes*. In this device, positive ions strike a conversion cathode-liberating electrons which are then accelerated and ‘multiplied’ via a series of up to twenty dynodes (Figure 16.26). This type of detector is extremely sensitive, having a gain of up to 10^8 . Aluminium-based dynodes have improved performances of the traditional materials (Cu/Be alloys) which age rather badly in the residual atmosphere of the spectrometers, or during non-working periods (returning to atmospheric pressure).
- *Continuous dynode multipliers with a channeltron*[®]. The ions are directed towards a collector whose entrance, in the form of a horn, is made of a lead doped glass with which acts as the conversion cathode. The ejected electrons are attracted towards a positive electrode (Figure 16.26) and their collisions against the internal walls give rise to multiplication, as with the separated dynodes. The assembly is usually mounted off-axis to avoid the impact of neutral species as well as photons emitted by the filament, equally susceptible to the removal of the electrons.
- *Microchannel plate detectors (MCP)*. They consist of the union of a large number microchanneltrons arranged like honeycombs. This resembles an electronic version of a photographic plate. Each individual detector is formed

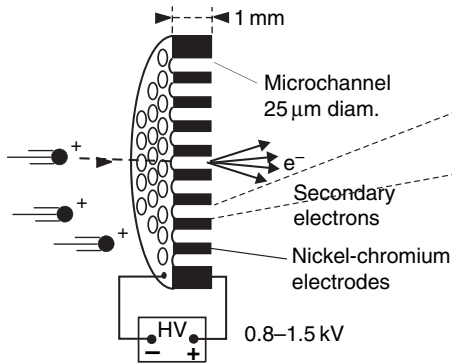
- (a) Multiple stage dynode detector
single-channel electron multiplier



- (b) Continuous dynode detector
single-channel electron multiplier



- (c) Microchannel plate (MCP) detector
(20–100 mm diam.)



- (d) Electron avalanche
within a microchannel

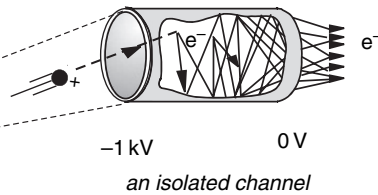


Figure 16.26 *MS detectors.* (a) Discrete dynode model with active film (reproduced courtesy of ETP Scientific Inc.); (b) Continuous dynode model. Diagram of a channeltron[®]: the funnel shaped cathode permits the recovery of ions issuing from different trajectories. The curvature has the effect of preventing the positive ions which appear by the impact of electrons on the residual molecules and restricting therefore the production of further electrons; (c) Microchannel plate. Each plate consists of an array of tiny glass tubes. Each channel becomes a continuous dynode electron multiplier; (d) Details of the conversion cathode. Multiplication of the electrons in a microtube[®] (from illustration by Galileo USA).

from a portion of microtube (25 μm diameter) whose interior is coated by a semi-conductor material acting as a continuous dynode (Figure 16.26). This system preserves the spatial resolution of the input charged ions.

■ The photographic plates used in early mass spectrometers have now been abandoned because the method was considered too slow, the emulsion having both a random reproducibility and a non-linear response to ion current (low dynamics range and contrast). However the principle of simultaneous detection remains attractive. The Mattauch–Herzog type spectrographs employing ionization by sparks used this detection system for a long time.

Amongst the questions to be asked following this brief review of the five categories of mass analysers is if analyses of the same sample compound, leads to an identical mass spectrum. In reality there is no universal analyser. Each one of the above has its own domain. Some of these instruments analyse the ions as soon as they are formed while others begin by stocking them and absorbing the impact of the collisions which can result in the abnormal growth of the $M + 1$ peak. For example, magnetic sector mass analysers and TOF analysers have very fast sampling time (nano- to microseconds) while ion traps can have sampling times as high as one second. For this reason mass spectrometry is held apart when compared with optical spectral methods which are more passive procedures. Therefore, in order to make the spectra comparable it is crucial to standardize the operating conditions.

Applications in mass spectrometry

Mass spectrometry is one of the most dynamic sectors of the analytical instrumentation having innumerable applications. The technique is even now becoming essential in the fields of genomics and proteomics. The following pages show a series of applications of mass spectrometry used in three areas : organic analysis, elemental analysis and isotopic analysis.

16.14 Identification by means of a spectral library

Species identification through comparison of spectra is employed in numerous analytical fields. Several software packages are used to conduct searches in spectral libraries in which the main peaks of known compounds are encoded. The compounds offering the best matches are retained as potential candidates, ranked in order of decreasing similarity with an index of 0 to 1000.

The National Institute of Standards and Technology (NIST) Mass Spectral Library is the most widely used mass spectral reference library, which presently contains a collection of about 200 000 electron impact (EI) mass spectra verified

by experienced mass spectrometrists plus a Kovats retention index library and a MS/MS spectral library.

Yet, when one considers the number of chemicals which is perhaps in excess of 10 million, whereas the largest of the databases of mass spectra contain some 200 000 chemicals, the need to employ other techniques is obvious. In this way, and coexisting with the development of databases some software packages use the concept of artificial intelligence methods to determine chemical structures from their mass spectra. In a number of cases these techniques based upon programmable criteria, chemical classification of the unknown, rarity of mass etc. appear to work well, but are limited to particular classes of compounds. The problem lies in the fact that unknowns are more often totally unknown (such as in environmental samples).

Tandem mass spectrometry has become a tool of proteomics because it leads, in association with degradative methods, to the identification of the amino acid sequences of peptides and proteins. This is obtained by coupling a two-dimensional electrophoresis instrument, as a means of separation, with a MALDI-TOF mass spectrometer, which produces a predominance of singly charged ions and which fragment the peptides by cleavage of the CO-NH bond linking the different amino acids. Although chemical or enzymatic (trypsin) protein digestion remains an important technique for mass spectrometry, it is possible to obtain direct mass measurements of proteins by ESI-MS.

16.15 Analysis of the elementary composition of ions

16.15.1 Methods based upon high-resolution spectra

With a mass spectrometer whose resolving power is at least 10 000, which corresponds to accurate masses to four or five decimal places, it is possible to find the molecular formula of parent or daughters ions. For this, the MS system presents for a given experimental mass, all the probable molecular formulae which could correspond, taking account both of a pre-defined error window and the list of the elements supposed to be present in it (see for example, Table 16.1).

After selection of a molecular formula, the data-processing program gives the value of the corresponding *Index of hydrogen deficiency* (I). This expresses the number of multiple bonds and rings in the corresponding ion (expression 16.20). If the index I is a whole number, then it is a radical cation. If I is a number plus a half, it is a cation. Generally, an ion with an *odd number* of electrons fragments with loss of a radical becoming by consequence a cation with an *even number* of electrons. Though comparatively rare these fragmentations can occur through a process of rearrangement and lead to a radical cation.

By associating several other considerations, such as the nitrogen rule (if the compound contains an atom of nitrogen, its molecular mass is an odd number) or

Table 16.1 Atomic mass of the isotopes of elements commonly encountered in organic compounds

Element	Nominal mass*	Atomic mass (g/mol)	Nucleide (%)	Mass (u)
Hydrogen	1	1.00794	¹ H(99.985)	1.007825
			² H(0.015)	2.014102
Carbon		12.01112	¹² C(98.90)	12.000000
			¹³ C(1.10)	13.003355
Nitrogen	14	14.00674	¹⁴ N(99.63)	14.003074
			¹⁵ N(0.37)	15.000108
Oxygen	16	15.99940	¹⁶ O(99.76)	15.994915
			¹⁷ O(0.04)	16.999131
			¹⁸ O(0.20)	17.999160
Fluor	19	18.99840	¹⁹ F(100)	18.998403
Sulfur	32	32.066	³² S(95.02)	31.972071
			³³ S(0.75)	32.971458
			³⁴ S(4.21)	33.967867
Chlorine	35	35.45274	³⁵ Cl(75.77)	34.968853
			³⁷ Cl(24.23)	36.965903

(*) nominal mass: the integral sum of the nucleons in an atom

the isotopic composition of the elements, the nature of the principal ions present on the spectrum can be identified.

$$I = 1 + \left[0.5 \cdot \sum_1^i N_i (V_i - 2) \right] \quad (16.20)$$

N_i represents the number of atoms of the element i , whose valence is V_i .

16.15.2 Method based upon isotopic abundances

This is another method to find the molecular formula of the analyte. A low-resolution mass spectrometer will suffice. As explained below, the principle is based upon the isotopic composition of elements.

A molecular compound (which consists of an immense population of individual molecules) gives a mass spectrum on which appears several molecular peaks (isotopic cluster at M , $M + 1$, $M + 2$, etc.), whose intensities reflect elemental and isotopic composition of the compound.

If it is known, for example, that this is an organic compound, the list of possible elements is not too great, so, a comparison of this signal pattern with all other patterns of different molecular formulae, limited to those consistent with the information concerning the compound, allows its identification.

Consider a sample of benzene C_6H_6 ($M = 78$ u). Knowing that for 100 atoms of carbon there is approximately 1 atom of ^{13}C , it can be estimated that about 6

per cent of the benzene molecules will include a ^{13}C atom in their skeleton and will therefore have a mass of 79 u (the contribution of ^2H , or D, 0.01 per cent is negligible). This explains the presence of a small peak observed at $m/z = 79$ in the mass spectrum of benzene. Similarly, a peak will be observed at $m/z = 80$, due to the presence of molecules that include either two ^{13}C atoms or one ^{13}C and one ^2H .

Equations 16.21 and 16.22 give the relative intensities in percent of the $M + 1$ and $M + 2$ peaks for organic molecules containing only the elements C, H, N, O, F, P (where $n\text{C}$, $n\text{N}$, etc. symbolize the number of atoms of carbon, nitrogen, etc.):

$$(M + 1)\% = 1.11n\text{C} + 0.36n\text{N} \quad (16.21)$$

$$(M + 2)\% = 1.11 \frac{n\text{C}(n\text{C} - 1)}{200} + 0.2n\text{O} \quad (16.22)$$

16.16 Determination of molecular masses from multicharged ions

Multicharged ions are obtained from macromolecules when they are ionized in the form of a spray (ESI, APCI). Their molecular masses can be accessed through a method alternative to that shown on Figure 16.24. A basic spectrum is required to undertake this calculation, which presents a succession of molecular peaks differing by the number of charges carried.

On the spectrum of the compound whose mass is designated by M , two successive peaks M_1 and M_2 should be located whose charges, z_1 and z_2 , differ by one unit, i.e. $z_2 = z_1 - 1$ (Figure 16.27). The expressions 16.23 and 16.24 then follow:

$$M_1 = \frac{M}{z_1} \quad (16.23)$$

$$M_2 = \frac{M}{z_2} \quad (16.24)$$

Substitution and transposition leads for z_1 to:

$$z_1 = \frac{M_2}{M_2 - M_1} \quad (16.25)$$

Next, knowing z_1 (rounded to the next whole number) M can be calculated (Da) from 16.23.

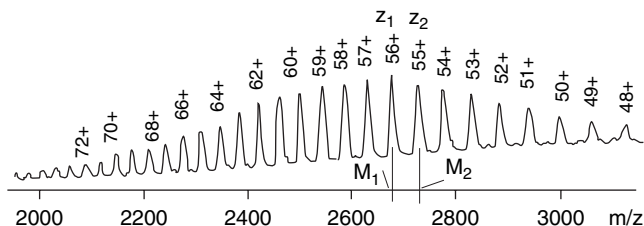


Figure 16.27 Spectral profile of an antibody of high molecular mass. Multicharged ions make accurate mass measurements easier. This spectrum has been obtained with a ESI-MS. Not knowing the values of the charges reported above each signal, the calculation described in the text leads from $M_1 = 2680$ and of $M_2 = 2729$ to $M = 150\,080$ Da.

16.17 Determination of isotope ratios for an element

Determination of isotopic abundance by mass spectrometry can give the geographic source of plant-based organic compounds or mineral compounds. Using the ‘isotopic signature’ of a species leads to better precise results as the different dating methodologies based upon radioactivity.

Isotope fractionation occurs during processes like evaporation or condensation and during chemical reactions. In the living world, isotopes of the same element can be differentiated by the biosynthetic pathways they follow. Furthermore, the rate at which a molecule crosses cellular membranes will depend of the molecule’s isotopic distribution. For example, the isotopic ratio $^{13}\text{C}/^{12}\text{C}$ of natural vanillin is lower than that of the synthetic vanillin; the same effect is observed for glucose, for which the isotopic distribution of carbon and oxygen varies, depending upon the biological cycle being followed by the plant.

The detection of adulterated table oils, flavourings, fruit juices as well as studies of plants metabolism and of numerous medical applications use isotopic abundance as an indicator.

To evaluate variations in the ratio $^{13}\text{C}/^{12}\text{C}$, it is compared with an adopted universal standard. This reference standard is calcium carbonate from Pee Dee (PDB for Pee Dee Belemnite, USA) whose ^{13}C abundance is very high (isotopic ratio $^{13}\text{C}/^{12}\text{C} = 1.12372 \times 10^{-2}$). Experimentally the determination is made by measuring the peak intensities of $^{13}\text{CO}_2$ (45) and $^{12}\text{CO}_2$ (44) obtained from the combustion of the sample. The relative deviation δ , (per mL, or parts per thousand) of the compound is calculated in this way (16.26). This value of δ is generally negative.

$$\delta_{0/00} = 1000 \left[\frac{\left[\frac{^{13}\text{CO}_2}{^{12}\text{CO}_2} \right]_{\text{sam}}}{\left[\frac{^{13}\text{CO}_2}{^{12}\text{CO}_2} \right]_{\text{ref}}} - 1 \right] \quad (16.26)$$

In the presence of a mixture of two compounds A and B whose isotopic ratio distributions are respectively δ_A and δ_B , the experimental value for the total isotopic variation δ_M in the sample, results from the weighted combination of δ_A and δ_B . If x represents the fraction of B and $(1 - x)$ the fraction of A, the following expression allows to calculate the percentages for A and B (16.27):

$$\delta_M = (1 - x)\delta_A + x.\delta_B$$

$$x = \frac{\delta_M - \delta_A}{\delta_B - \delta_A} \quad (16.27)$$

As this measurement involves combustion, the isotopic distribution at the different sites within the compound analysed is not available. For this reason, and when this information is required, it is preferred to compare the D/H ratio by ^2H NMR since this facilitates a comparison at each site of the molecule studied.

The determination of differences in isotopic ratios requires very precise measurements. The combustion step for the sample preparation is usually carried out immediately prior to the injection into the MS. There exist instruments which associate in line a gas chromatograph, a tubular combustion oven, containing copper oxide heated to 800°C , and a low-resolution MS equipped with several Faraday detectors, each collecting the signal corresponding to a specific mass. A calibration compound is co-injected with the product to be studied.

■ Accelerator mass spectrometry (AMS) – precise measurement of the isotopic ratios of the long-lived radionuclides that occur naturally in our environment are beyond the ability of conventional mass spectrometers. For isotopes that exist as infinitesimal traces (a single atom in the presence of 1×10^{15} stable atoms) there exist worldwide, a network of around 50 mass spectrometers, each derived from Van de Graaff accelerators, which are used for these analyses (Figure 16.28).

A few milligrams of a sample, which must be a solid, are first bombarded by caesium ions. The first mass analyser extracts the negative ions corresponding to the

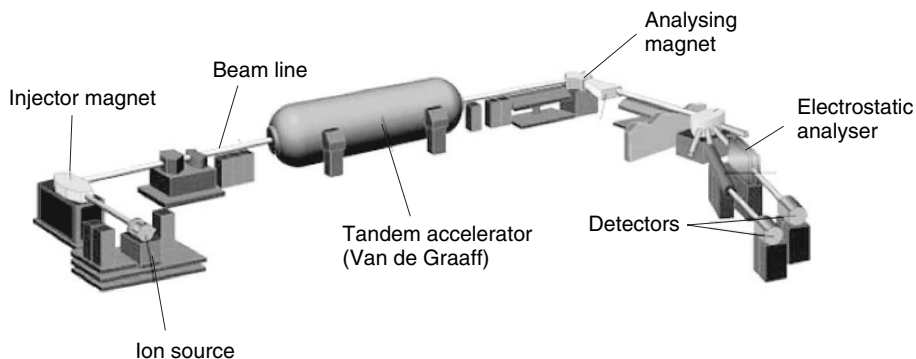


Figure 16.28 Accelerator mass spectrometer. The ANTARES project (Reproduced courtesy of the Australian Nuclear Science and Technology Organization)

pre-selected masses. Next, to eliminate interfering isobars, ions are accelerated by a potential difference of several megavolts towards the positive end of the accelerator. Along the ion beam path they collide with a target (a solid or a gas) in order that they are stripped of electrons. Only isolated atoms, carriers of several positive charges, subsist beyond this point. These ions are reaccelerated. The gas ionization detectors count ions one at a time as they come down the beam line. Abundance measurements are then made by alternately recording isotopic signals arising either from the sample, the standards and the analytical blanks.

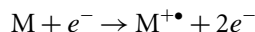
It is the chosen technique for trace level analysis as dating antique objects through their ^{14}C concentration, much more precise and sensitive than Libby's classic method based upon measurements of radiocarbon activity. AMS can detect $1\ ^{14}\text{C}/10^{15}$ total ions.

16.18 Fragmentation of organic ions

The manual interpretation of a mass spectrum – nature and origin of the fragmentation peaks – is a difficult but interesting exercise. Organic chemists are usually familiar with these methods of interpretation since they retrieve different types of transient ions considered to explain reaction mechanisms in condensed phases. The difference here is that these ions move in a vacuum and do not collide. The very short period of time between ion formation and ion detection (a few μs), allows to observe the existence of very unstable species that are unstable under normal conditions.

16.18.1 Basic rules

Fragmentation by electron bombardment, well adapted to the study of organic molecules, results from the instability of the molecular ion formed initially. The molecule M of which the mass is always an even number (excepting when there are an odd number of nitrogen atoms), is ionized and becomes a *radical cation* with an odd number of electrons represented by the symbol $M^{\bullet+}$. For compounds incorporating heteroatoms, ionization will preferentially occur at the heteroatom on a lone pair of electrons (Figure 16.18). At this stage, the molecule M is not yet fragmented:



In a second step, this resulting radical cation generally fragments. Since a mass spectrum results from the fragmentation of a sample that contains billions of molecules, it will show all possible fragmentations. As the relative probabilities of fragmentation are not the same, the fragmentation peaks will have different intensities.

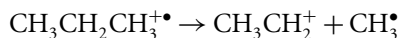
Several factors control the fragmentation procedure of $M^{\bullet+}$ into daughter ions and neutrals (radicals or molecules). Two basic situations are observed:

- a cation with an odd mass number is formed (but with an even number of electrons), accompanied by a neutral fragment in the form of a radical
- a new radical cation appears accompanied by the loss of a neutral from $M^{\cdot+}$.

There is a greater tendency to form stable fragments (ion or neutral) and the fragmentations with rearrangements are favoured when they involve a six-membered ring transition state leading to a hydrogen transfer at the radical cation site.

16.18.2 Fragmentation of an ionized σ bond

In a *hydrocarbon* such as propane, the ionization of a C—C σ bond will lead to the formation of the ethyl cation and a methyl radical rather than the reverse:



Similarly, the ionization of isobutene leads principally, by elimination of a methyl radical, to the formation of an isopropyl cation which is thermodynamically more stable than the methyl cation.

16.18.3 α Fragmentation

In ketones (RCOR'), ionization of the keto group by ejection of an electron from one of the lone pairs of the oxygen atom (Figure 16.29) leads predominantly to the homolytic cleavage of the C—C σ bond that is in position α to the site of ionization. Elimination of the larger alkyl chain as a radical is favoured. However, in the general case of a ketone RCOR' , the four fragment ions $\text{RCO}^{\cdot+}$, $\text{R}'\text{CO}^{\cdot+}$, $\text{R}^{\cdot+}$ and $\text{R}'^{\cdot+}$ (Figure 16.29) will be observed.

In ethers (ROR') the cleavage of the C—C bond adjacent to the oxygen leads to resonance stabilized oxonium ions (Figure 16.30). In primary amines, a similar situation is met with formation of the iminium ion at mass 30: $\text{CH}_2=\text{NH}_2^{\cdot+}$. Elsewhere, split of the ether C—O bond, leads to cations $\text{R}^{\cdot+}$ and $\text{R}'^{\cdot+}$.

16.18.4 Fragmentations with rearrangements

Besides these examples of fragmentation that occur by simple bond cleavage, ions may rearrange, sometimes by very complex pathways. Fragmentation *via* the McLafferty rearrangement is a well-known transformation which occurs in molecular ions containing a carbonyl group $\text{C}=\text{O}$ or a double bond $\text{C}=\text{C}$ (Figure 16.31). The reaction involves a hydrogen transfer to the initially ionization

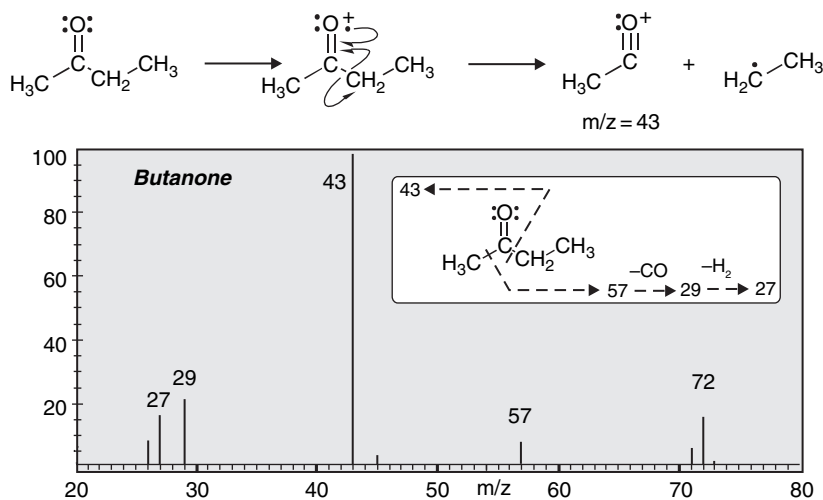
α Fragmentation

Figure 16.29 Electron ionization mass spectrum of butanone. The scheme explains the formation of the principal ion fragments. The molecular ion leads to the acetyl ion CH_3CO^+ ($m/z = 43$), which is ten times more intense than ion 57, $\text{CH}_3\text{CH}_2\text{CO}^+$.

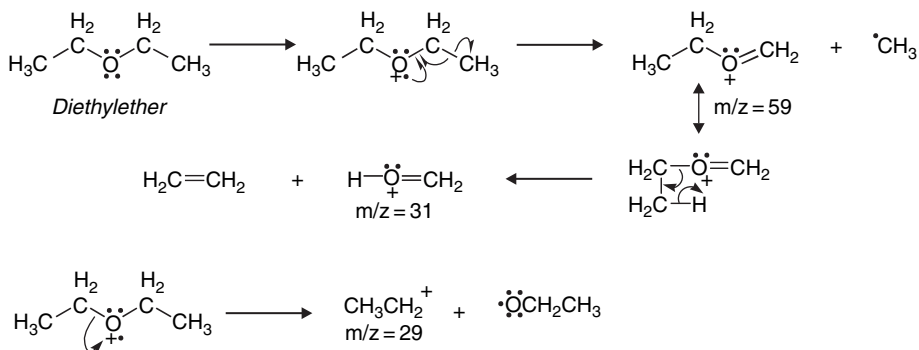


Figure 16.30 Fragmentation modes of an ether, in the case of diethylether

site (oxygen atom) which can be explained by a six-membered intermediate ring. This mechanism goes on by the elimination of a neutral ethylenic residue and formation of a new radical cation of even-numbered mass if the compound does not contain nitrogen.

All these transformations have led to semi-empirical rules which are used in the study of unknown compounds and which also are used to corroborate the results obtained by the automatic exploitation of spectral libraries.

McLafferty rearrangement

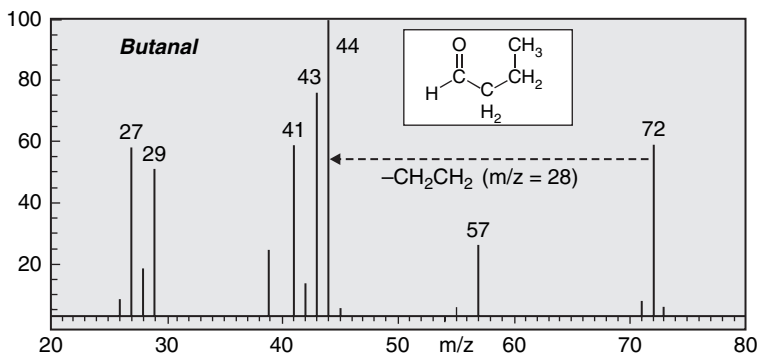
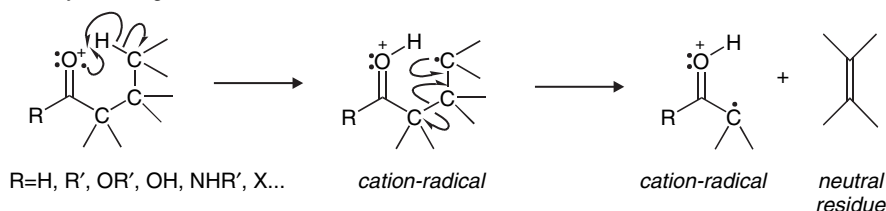
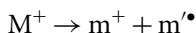


Figure 16.31 McLafferty's rearrangement. Situation for butanal C_4H_8O

16.18.5 Metastable ion peak

Depending on the lifetime of the excited state, an ion that is formed with sufficient excitation may dissociate in the source giving rise to a stable daughter ion or during its flight from the ion source to the detector, giving *metastable ions* (Figure 16.32).



Each individual ion has a random lifetime, which usually is less than a few μs , depending upon its internal energy. This unimolecular decomposition is described by first order kinetics. With a double focusing apparatus, a daughter ion m^+ that is formed before the exit of the electric analyser, will become lost on the internal wall of the instrument because its kinetic energy is insufficient. In contrast, if the decomposition occurs in the field-free region between the exit of the electric accelerator but before the magnetic deflection, the ion that have a momentum mv will be transmitted by the magnetic sector. Because its energy is less than that of a normal ion (its velocity is less than the correct one for an ion of the same mass), the ion m^+ will be observed on the normal mass spectrum as a diffuse peak situated at apparent mass m^* , which does not correspond to its real mass. The formula 16.28 relates the positions of the three signals, parent, daughter and diffuse, on the spectrum when a metastable ion peak is present:

$$m^* = \frac{m^2}{M} \quad (16.28)$$

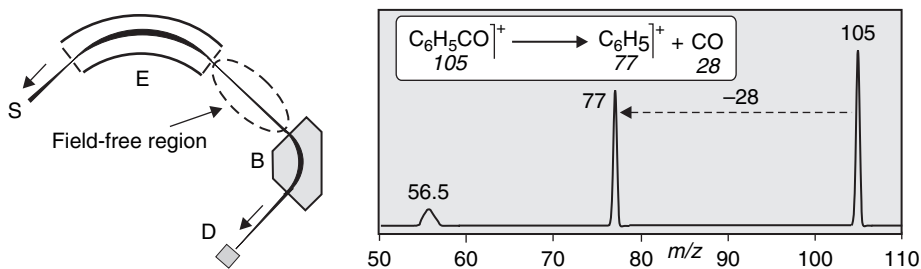


Figure 16.32 *Metastable ion peak.* Theoretical aspect of the three peaks constituting the metastable transition given as an example. For the fragmentation $m/z = 105 \rightarrow m/z = 77$ a metastable ion peak at $m/z = 56.7$ is observed (calcd. 56.5).

The shape of the diffuse peaks are less defined and their positions, because of the mathematical relationship involved, will seldom be near by or at an entire mass (Figure 16.33). Because these peaks are wide and of low intensity, they are generally unsuitable for analytical use except by employing special scanning modes.

Therefore through ‘*peak-matching*’ and metastable peak analysis, it is possible to determine with great confidence the structure of the ions. However the latter procedure has become a little out-dated when compared with the new and more efficient techniques (e.g. MIKE or reversed Nier–Johnson geometry), described in specialized monographs.

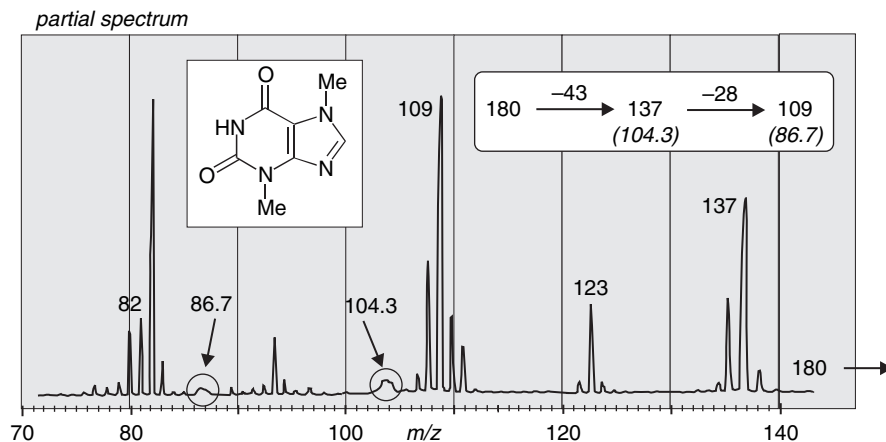


Figure 16.33 *Metastable ions peaks observed with theobromine.* The molecular ion of theobromine (180 Da) gives rise to an ion of mass 137 Da through loss of a CONH^{\bullet} radical (43 Da). This fragmentation is accompanied by a diffuse peak at 104.3 Da. Furthermore, loss of CO (28) from the daughter ion $m/z = 137$ yields a second metastable ion peak which appears at mass 86.7 (reproduced courtesy of Kratos).

Problems

16.1 The isotopic factor δ of carbon, measured from the carbon dioxide resulting from the combustion of a natural vanillin, is $\delta = -20$. A value arising from a synthetic vanillin is $\delta = -30$. Calculate the percentage of these two species within a sample of mixed vanillin, knowing that a previously measured value $\delta = -23.5$ has been obtained.

16.2 To evaluate the volume of a large tank of complex shape the method of isotopic dilution is to be used, in association with a soluble salt of lutetium. This element, which has mass $M = 174.97 \text{ g/mol}^{-1}$, has two stable isotopes ^{175}Lu (97.4%) and ^{176}Lu (2.6%). The procedure is as follows:

After filling the tank with water, 2 g of the trichloride of lutetium hexahydrate $\text{LuCl}_3 \cdot 6\text{H}_2\text{O}$, mass $389.42 \text{ g/mol}^{-1}$, is added.

1. What is the mass X of lutetium, introduced into the tank?

After stirring until the lutetium salt is well mixed, the extraction of a sample of 1 litre of solution is taken from the tank. To this volume of solution is added $20 \mu\text{g}$ of lutetium hexahydrate trichloride prepared from the pure isotope ^{176}Lu ($^{176}\text{LuCl}_3 \cdot 6\text{H}_2\text{O}$ has for mass 390.4 g mol^{-1} ; $^{176}\text{Lu} = 175.94 \text{ g mol}^{-1}$) is added to this solution.

2. Calculate the mass of ^{176}Lu , expressed in μg , added to the sample.

3. By a classical method it is found that the ratio of the two isotopes is now $^{175}\text{Lu} = 90.0\%$ and $^{176}\text{Lu} = 10.0\%$. What type of apparatus would seem to be the best adapted to measure the ratios of isotopes?

4. Discuss the choice of lutetium for use in the experiment.

5. Finally, calculate the volume of the tank.

16.3 A magnetic sector mass spectrometer-type apparatus houses a circular trajectory of radius 25 cm which is imposed upon the ions. The accelerator voltage is raised to 5000 V. A mass spectrum is recorded between 20–200 Da.

If it is assumed that each ion carries a single charge ($e = 1.6 \times 10^{-19} \text{ C}$, $1 \text{ Da} = 1.66 \times 10^{-27} \text{ kg}$):

1. What range of magnetic fields would be required if the accelerating voltage is held constant?

2. Why, in general, are recordings not made by a sweeping variation of voltage?
- 16.4 Calculate the ratio of the $(M + 1)^+$ to M^+ peak heights for footballene (formula: C_{60}), knowing that carbon has two isotopes ^{12}C : 12 amu (98.9%) and ^{13}C : 13 amu (1.1%).
- 16.5 Vanadium (symbol V) has two isotopes whose relative abundances are $^{51}\text{V} = 99.75\%$ and $^{50}\text{V} = 0.25\%$.
- To determine the vanadium concentration in a sample of steel, 2 g of the steel is dissolved in an acid medium and $1\ \mu\text{g}$ of ^{50}V is added to the resulting solution.
- After stirring, an analysis by ICP – MS is performed which obtained a mass spectrum with two peaks centred upon the masses 50 and 51, possessing the same area size.
1. What is the % content of each isotope of vanadium in the unknown steel if the ratio of the areas of the two peaks is the same as that of the masses of the two isotopes?
 2. Give a more thorough answer knowing that $^{50}\text{V} = 49.947\ \text{g/mol}$ and $^{51}\text{V} = 50.944\ \text{g/mol}$.
 3. Explain why this problem would become more complicated if the steel contained either titanium or chrome.
- 16.6 The method of isotopic labelling is employed to determine the presence of traces of copper with precision. An ICP/MS installation was employed. The results of the experiment rely upon the ratio of the intensities of the peaks corresponding to the masses 63 and 65 of the two isotopes ^{63}Cu and ^{65}Cu of elemental copper. The experiment is described below. First, a small quantity of the unknown solution was injected. The intensities of ^{63}Cu and ^{65}Cu are 82 908 and 37 092 respectively (arbitrary units).
1. Calculate the percentage of each copper isotope in this sample. Recall that $^{63}\text{Cu} = 62.9296\ \text{g mol}^{-1}$, $^{65}\text{Cu} = 64.9278\ \text{g mol}^{-1}$.

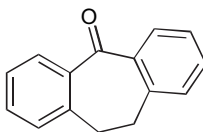
- The above experiment was repeated with a solution of ^{65}Cu which was used as an isotopic marker. For measurements a $250\ \mu\text{L}$ aliquot of the solution containing ^{65}Cu whose concentration is $16\ \text{mg/L}$, is added to an aliquot of $250\ \mu\text{L}$ (approximately $250\ \text{mg}$), of the unknown sample solution. After stirring well, the intensities of the ^{63}Cu and ^{65}Cu peaks were determined and were $31\ 775$ and $79\ 325$ respectively (arbitrary units).

From the above data, calculate the mass concentration of elemental copper in the $250\ \mu\text{L}$ aliquot of the sample solution.

Deduce the ppm copper concentration in the unknown sample solution.

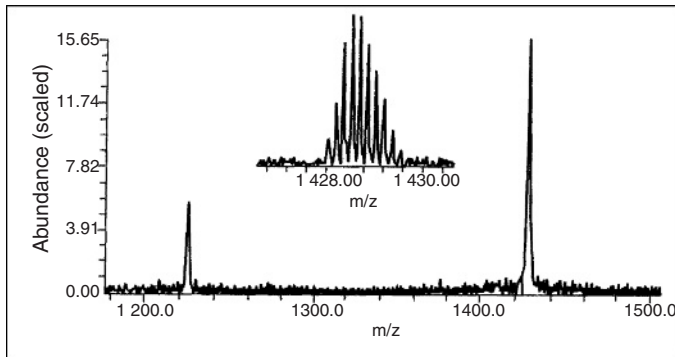
- 16.7 The dibenzosuberone 1 is a ketone whose structure is given below and the spectrum in Figure 16.1.

- Calculate the exact mass from the largest molecular peak, and write the isotopic compositions of the different species constituting peak $M + 1$.
- In the mass spectrum of this compound, among other fragments, one can see two with the same nominal mass which could represent the loss of either CO or of C_2H_4 .
Explain the loss of CO from the parent ion and indicate if, by loss of C_2H_4 , a positive ion, a radical or a cation-radical must result.
- Indicate for the two peaks the corresponding molecular formula and precise molecular weight.
- Knowing that the resolution factor of the mass spectrometer is $15\ 000$, is it possible to distinguish the different species constituting the peak $M + 1$?



- 16.8 The study of macromolecules by mass spectrometry presents differences with respect to the studies of molecules of medium or more modest size.

1. What are these principal differences?
2. Calculate, using two different methods and the information available from the recording below, an approximating molecular mass of ubiquitin (a protein found in baker's yeast).



17

Labelling methods

Several labelling methods of analysis consist of spiking the sample with a known quantity of a labelled (or marked) form of the analyte. After mixing the two forms – labelled and not – a small aliquot of this new solution is withdrawn and by appropriate means it becomes possible, from the ratio of labelled to unlabelled analyte, to calculate its initial quantity in the primary sample. This procedure is well adapted to measure trace analytes in complex mixtures when the extraction step is not total. Two types of labelling or marking are used, either isotopic (stable or radioactive) or enzymatic. In the latter, the compound can be isolated using an immunochemical reaction. This type of approach led to the development of radio-immunological assays (RIA, soon to be abandoned) and immunoenzymological assays (EIA), of which the ELISA tests are part. Although until recently the latter were reserved for studies of biological compounds, their application to chemistry is on the increase.

Neutron activation has been included in this chapter, despite its not being considered as a labelling method. This is one of the most accurate techniques for identifying elemental abundances. This needs irradiation techniques that are not accessible everywhere and this method has the disadvantage of causing the sample to be slightly radioactive.

17.1 The principle of labelling methodologies

The most obvious and ideal method for the determination of the mass concentration of a compound in a mixture would be to totally separate it from all components and weigh it. Unfortunately this can seldom be undertaken, extraction being neither completely selective nor quantitative, indeed impossible if it is in a very low concentration. In this perspective, analytical chromatography of the sample, a separative method *par excellence*, is an indirect way to achieve extraction and to accomplish this goal. The group of methods described below stem from an entirely different principle. These other approaches are gathered under the

general term of labelling. To measure a particular analyte in a sample, a known quantity of the same compound is introduced to the sample in a tagged form (e.g. labelled). This ‘tag’ allows it to be distinguished from the unlabelled form and other compounds present in the initial mixture. After allowing time for mixing and even distribution, a fraction of the sample is collected and the ratio of both forms of the compound is used to obtain the amount initially present in the sample. The labelling of the compound must not alter its behaviour during the recovery step. This technique should not be confused with the classic methods of standard additions described elsewhere.

■ Labelling methods are not only used in chemical analysis. To estimate, for example, the number of salmon in a pond, a definite number of fish are caught, labelled and then released again into the pond. A few days later, a number of fish (the sample) is removed and from the proportion that are labelled, an estimation of the total population can be deduced. For example, if after having labelled and released 500 salmon and if 10 of them are found in a group of 200 fish removed a few days later, then the total number of salmon (x) can be estimated at $10/200 = 500/x$, thus $x = 10\,000$.

Several analytical methods have been developed from this principle. An element or a molecule can be labelled as follows:

- By modifying the isotopic ratio of this element or one of the atoms of the molecule, either with a radio-element (whose activity is measured in becquerels) or with a stable isotope (detection by mass spectrometry or NMR). These are called *isotopic analyses*.
- By using an enzyme or a fluorescent derivative. These are in particular, *immunoenzymatic* and *immunochemical* analyses.

17.2 Direct isotope dilution analysis with a radioactive label

In these types of isotopic analyses, the same compound as that to be measured is used where one of the atoms in it has been replaced by a radionuclide.

To measure a molecular compound X present in a sample, a quantity m_S^* of the labelled compound X (the reference tracer) is added to a precise quantity of the sample. The specific activity (see Section 17.5) of this tracer is represented by A_S (activity in Bq per unit of mass) while A_X is the specific activity after dilution and calculated from a very small aliquot of the spike sample isolated by a fractionation technique as recrystallization or chromatography

A_X is smaller than A_S because the quantity of m_S^* (the tracer implemented) is mixed with the quantity m_X of non-labelled compound. On the other hand, the overall activity is conserved, which is translated by the following expression:

$$A_S m_S^* = A_X (m_S^* + m_X) \quad (17.1)$$

The quantity m_X of analyte in the sample is obtained by rearranging equation 17.1:

$$m_X = m_S^* \left(\frac{A_S}{A_X} - 1 \right) \quad (17.2)$$

If A_X is much smaller than A_S then 17.2 can be approximated by the following equation:

$$m_X \cong m_S^* \frac{A_S}{A_X} \quad (17.3)$$

Having calculated m_X in this way and knowing the mass of sample used, it becomes easy to deduce the concentration of X in the initial sample.

In such a procedure, the labelled tracer must be uniformly mixed in the sample and the aliquot taken must be of a quantity sufficient to avoid weighing errors. If the concentration of the compound to be measured is too low, then recovery as a pure compound, even partially, could be difficult. The adaptation of this method to very small quantities is discussed in the following paragraphs.

The preceding method is known as *reversed isotope dilution analysis* when the compound to be measured is already radioactive. The principle remains the same: the activity of the subject compound (measured from a fraction), is carried out before and after dilution with the same compound, non-labelled. The calculations are identical. This analysis is used for the determination of the isotopic carrier in a solution of a radionuclide using one of its stable isotopes.

17.3 Substoichiometric isotope dilution analysis

Expressions 17.2 and 17.3 suggest that in order to calculate m_X it is not necessary to know A_X and A_S , if their ratio is known. This observation is exploited when the analyte is a very small quantity and impossible to recover in its initial form by employing a traditional procedure.

The method consists in making an insoluble derivative of the analyzed compound to isolate a part from it. To achieve this, a reproducible reaction can be conducted on the labelled standard solution (analytical blank) and, in an identical manner, on the sample solution in order to obtain the same recovery quantity of derivatized compound. This method, named *substoichiometric*, pre-dated the immunochemical methods for trace measurement.

- For example, let us imagine measuring the quantity of sulfate ion present at only a few per cent in an aqueous solution. After having added a known quantity of $^{35}\text{SO}_4^{2-}$ as the label, a solution of a barium salt (to form insoluble BaSO_4) is introduced in a quantity insufficient to precipitate all of the sulfate ions. By comparing the activity of the recovered precipitate with that of the precipitate obtained from the same quantity of labelled sulfate alone, under identical

experimental conditions, the ratio of the activities can be deduced and consequently the quantity of sulfate in the original sample (this is a reverse isotopic dilution).

17.4 Radio immuno-assays (RIA)

The previous methodology, illustrated by the sulfate example, has been employed since the 1960s for the measurement of insulin in serum. The technique, named *radio-immunology*, is a transposition into immuno-chemistry of the preceding experiment.

The basic idea is to separate, after mixing, the analyte by a means of an ultraspecific reagent (an antibody), which renders possible the study of a single type molecule amongst thousands of other substances. This approach has led to immuno-measurements that have benefitted enormously from the development of genetic engineering and biochemistry.

As in the previous method, to the sample containing the analyte, a known quantity of this same analyte in a labelled form with a radioisotope (Figure 17.1) is introduced as a tracer and mixed. Next, to isolate a fraction of the mixture of the two forms, labelled and non-labelled, a specific antibody of this analyte is added in a substoichiometric quantity in order to have a little of the two forms. They will be in the same molar ratio as they are in solution. This mixture of complexes will then be separated by precipitation before assessing its activity.

Radio-immunology has remained the method of choice for certain studies of clinical medicine and has few applications for the measurement of molecules of low molecular mass, because of the use of radioactive substances. Alternatively, the

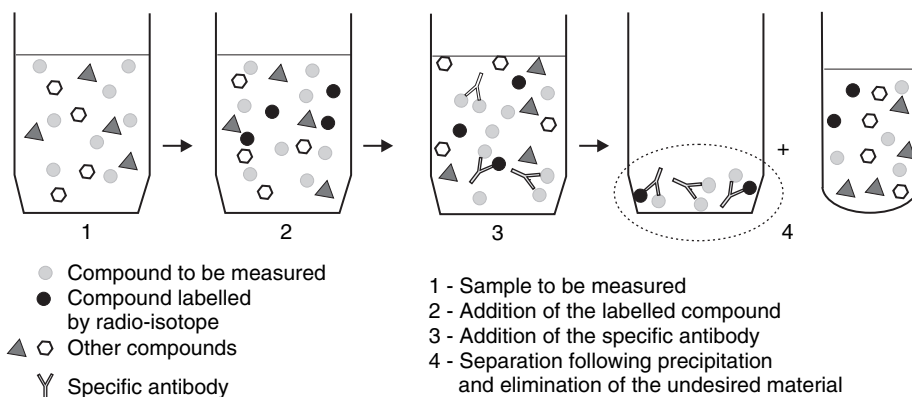


Figure 17.1 Radio-immunology testing – the different steps

method is currently being applied to small compounds labelled with an enzyme (cf. Section 17.7), notably for applications relating to the environment.

- The methods described above, which include measurements of radioactivity, are sources of possible contamination. As such they require an appropriate environment, with the proper authorization to manipulate and handling radioactive substances.

17.5 Measuring radioisotope activity

Any radionuclide, whatever the type of radiation emitted, is characterized by its half-life τ (Table 17.1), which is the time taken for half of corresponding atom population in the sample to decompose (from initial time, $t = 0$). Calling λ the radioactive decay constant, the law of radioactivity decay allows calculation of the number of atoms N present after time t for a population containing N_0 atoms initially. The integrated form of this law is written as equation 17.4:

$$N = N_0 \cdot \exp[-\lambda t] \quad (17.4)$$

with

$$\tau = \ln 2 / \lambda \quad (17.5)$$

Table 17.1 Some characteristics of commonly used radioisotopes

Isotope	Half-life	Type of emission	Energy (MeV)
^3H	12.26 years	β^-	0.02
^{14}C	5 730 years	β^-	0.156
^{32}P	14.3 days	β^-	1.7
^{35}S	88 days	β^-	0.167
^{125}I	60 days	IEC*	0.149

*Internal electron capture, Section 12.3.2.

λ is in units of time⁻¹. In practice, it is not N (the number of atoms remaining) that is known but the activity A ($A = -dN/dt$), expressed in becquerels Bq (1 Bq = 1 nuclear decay per second and 1 curie (Ci), a former unit of activity, is equal to 3.7×10^{10} Bq). The activity, accessible through use of an appropriate detector, is directly related to the concentration of the radionuclide (17.6):

$$A = \lambda N \quad (17.6)$$

A is related to A_0 , the initial activity by an equation analogous to the law of radioactive decay:

$$A = A_0 \cdot \exp[-\lambda t] \quad (17.7)$$

17.5.1 Radioactive labelling of molecules

Only compounds for which at least one of the elements is available in a labelled form can be measured by this method. There are suppliers that specialize in the manufacture of single-atom labelling (C, H, S ...) within a relatively large number of organic molecules (Figure 17.2). Activity can attain 60 mCi/mmol, if each individual molecule contains a ^{14}C atom at a given site. Where possible, ^{14}C is preferred to ^3H (tritium) since the latter is easily exchanged and for which autoradiolysis creates problems as a result of nuclear reactions between that atom and the remainder of the molecule of which it is originally a part (Szilárd–Chalmers effect).

■ The isotope ^{14}C used to prepare labelled compounds is obtained by irradiation in a nuclear reactor, of solid targets containing atoms of nitrogen (aluminium or beryllium nitride), by neutrons of low energy, known as thermal neutrons, themselves the product of the controlled atomic fission of ^{235}U . The radiocarbon formed is next isolated from the target sample by oxidation to $\text{Ba } ^{14}\text{CO}_3$, the variety in which it is delivered to chemists. From $^{14}\text{CO}_2$, it is possible to use a plethora of organic chemical reactions to synthesize different compounds in which the radio-isotope can be introduced to a specific position.

17.5.2 Scintillation counting

The radiation energies of the above radioisotopes are often insufficient to penetrate the window of a Geiger–Müller counter. For this reason, the compound whose activity is to be measured is mixed in solution with a scintillating material called a *scintillator* or a *fluor*, which transforms the β rays into a luminescence proportional to the number of β particles emitted. The sample is diluted in a solvent (toluene, xylene, or dioxane – the latter for water-soluble compounds) that acts as a relay in the transfer of energy to the scintillation mixture comprising for example, PPO (2,5-diphenyloxazole – which emits in the UV) and POPOP emitting in the visible domain (Figure 17.3). The quantum yield of emission depends on the energy of the emitted particles.

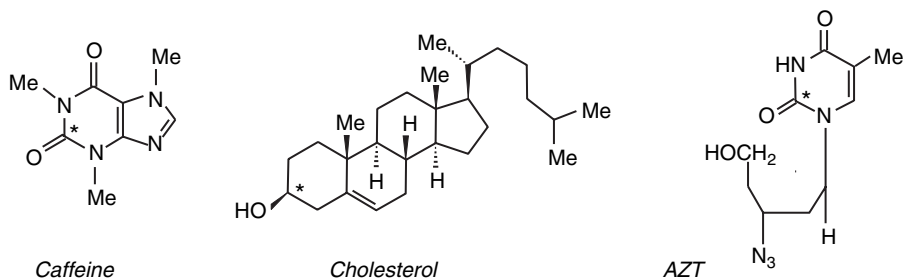


Figure 17.2 Three molecules labelled at a single site with ^{14}C . This radioisotope has a sufficiently long half-life that labelled compounds can easily be stored while avoiding corrections to take into account for natural decay during the measurement.

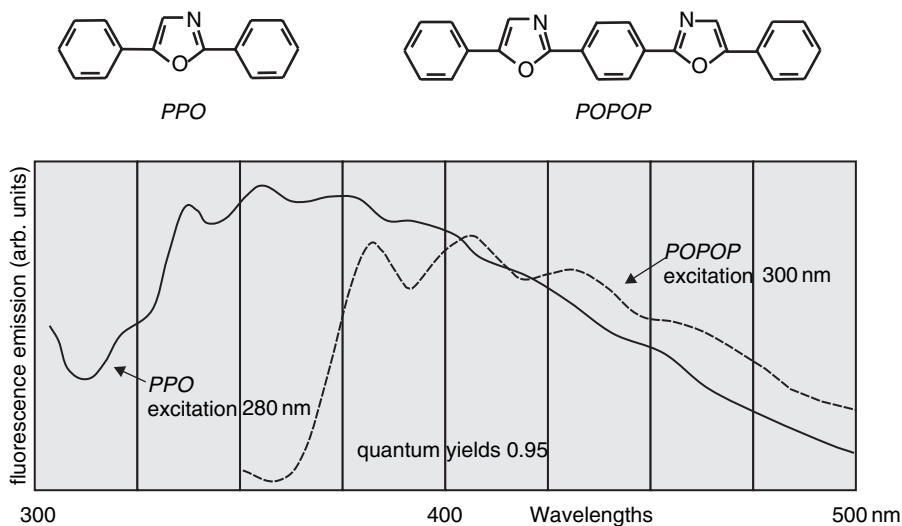


Figure 17.3 Two current examples of scintillators: PPO and POPOP. Fluorescence emission spectra of solutions in cyclohexane of these two compounds well adapted to detection with photomultiplier tubes.

■ Radioactive measurements have, of course, associated problems such as the exposure of people working with them and of the treatment of waste materials which means specific risks to staff and environment. The use of tracers is therefore tightly controlled and possession is by licence granted following an examination of the premises with respect to authorized 'norms' where such material will be stored. The usual precautions must be taken such as working with gloves and behind a protective screen, using material of low activity and if possible, low energy emitters, sufficient to avoid all danger of external radiation.

From amongst the most frequently used radioisotopes only ^{35}S emits γ radiation of high energy. A transparent screen of lead doped plexiglass of 1 cm thickness offers sufficient protection from low activities (less than 1 MBq). However, the operator must be attentive to the risk of internal contamination; a radioisotope, even of weak energy, is very dangerous once it is fixed in the organism. There is no simple and direct relationship between the intensity of radiation (Bq) and the irradiation dose (Sievert). This is a function of the quantity of energy released per mass unit in the irradiated substance.

17.6 Antigens and antibodies

Radio-immunological tests as well as the enzyme-linked immunosorbent assay (ELISA) which are described in the next paragraph require a brief explanation of antibodies because chemistry and immunology are separate disciplines.

The introduction into the organism of an alien substance (*antigen*) of sufficient molecular weight ($M > 5000$ Da), provokes the production of bio-molecules called

antibodies which are glycoproteins referred as to immunoglobulins (principally IgGs).

Antigens and antibodies, in the presence of each other, agglutinate. The forces that bind them are principally due to hydrogen bonding. The hydrophobic interaction exerted by neighbouring water molecules provokes a hydrostatic pressure that ensures the cohesion.

The antigens *induce* not only an immune response but equally *react* with the antibodies, which are caused to appear by the living organism. However, these two properties are different. Small organic molecules such as pesticides are not able of inducing a production of antibodies. In the other hand, the antigens will react if an adapted antibody is already present: these small molecules are called the *haptens*. To generate the antibodies characteristic to a hapten, the latter must be attached by a covalent bond to transporter proteins (BSA or HSA *bovine* or *human serum albumin*), or to a polysaccharide. Therefore, the hapten must have reactive functionality and this structural modification must not influence the specificity of the antibodies that will be produced.

Polyclonal antibodies. The development of antibodies is a rather difficult work. If only a single hapten molecule was linked to a carrier protein such BSA ($M = 66\,000$ Da), the antibody formed would be directed towards several sites on the protein, called the epitotes, corresponding to sections of the protein which trigger the production of the antibodies. Thus several tens of haptens should be bonded. Under these conditions, the antibodies obtained become specific to the hapten and not to the protein.

For this, a subcutaneous or intradermic injection is made, at different points to five or six mice, rats or rabbits of the hapten-bound protein (immunogen) with certain additives. This multi-point primary injection corresponds to around $100\ \mu\text{g}$ of pesticide. Animals having the same genetic background do not necessarily react in the same way. Two weeks later the same process is repeated with one fifth of the immunogen concentration. Two weeks after that, blood samples are taken from the animals to verify if the antibodies have appeared. The injections are repeated two or three times in order to create hyper-immunization. The serum of the animals is now a source of the desired antibody, which is of the polyclonal type. By contrast, monoclonal antibodies are derived from a single cell line.

17.7 Enzymatic-immunoassay (EIA)

Linked with the previous basic description on antibodies, there is an other form of labelling, which employs an enzyme rather than a radioisotope. In this case the tag (the enzyme) is enormous with respect to the subject molecule. Since the concentrations are very low, recovery is undertaken using an antibody. The best known methodology of this type is the enzyme-linked immunosorbent assay

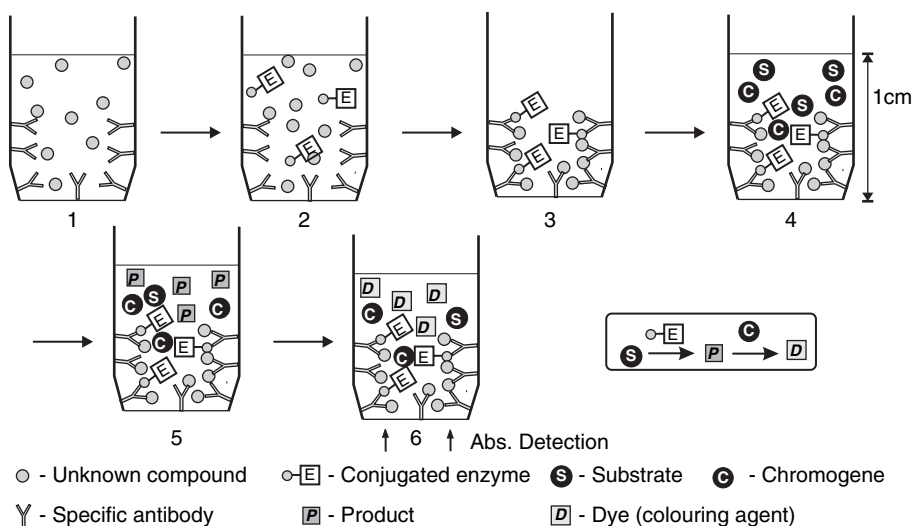


Figure 17.4 The different steps of an enzymatic-immuno test of ELISA type with competition. In clinical analysis, there exist numerous measurements of this type. Kits are available for both organic and inorganic contaminants.

(ELISA) that can be done in several different ways called 'direct', 'indirect' and 'competition' ELISA.

Over the past years, immunoassay field test kits have become a prominent field analytical method for environmental analysis. Below is a brief outline of the method (Figure 17.4).

17.7.1 A 'competition' ELISA test

1. A solution of the test sample (or of the standard or blank) is introduced in the respective plastic tube, generally a well of a microtitre plate with have a transparent bottom for detection purposes. The walls have been sensitized by coating them with the adapted antibodies fixed to the surface through adsorption.
2. A known volume of a solution of the same compound, which is covalently linked ('conjugated') to an enzyme, such horseradish peroxidase, is added. During an incubation period, the two forms (labelled and not labelled) of the compound will enter into competition for binding with the antibodies present on the container walls. Finally they are bound in the proportion of their mixture.
3. Following the incubation period (e.g. 30 min), the wells are rinsed several times with water. Only bound molecules will remain in the tube. The quantity of the enzyme-linked compound fixed on the walls (to the antibody) will

be all the greater, as the amount of free unlabelled compound is low in the sample.

4. The substrate S, specific for the enzyme, as well as chromogen C designed to change colour when acted upon by the reaction product, is now introduced.
5. The fixed enzyme will transform a large number of molecules S into species P, which will react with C to give a coloured product (see above). The amplification factor imparted by the enzyme makes this assay very sensitive. The less there is of the analyte in the sample, the more there will be of the fixed enzyme and therefore the colour intensity will be stronger.
6. Finally the reaction is quenched after a short period of time by addition of a strong acid which destroys the enzyme. The absorbance of the resulting colour, read with a colorimeter, is inversely proportional to the amount of analyte in the sample.

■ Horseradish peroxidase ($M = 44\,000$ Da) is stable and reactive. The linking is obtained with four lysine residues that do not otherwise participate in its activity. According to its name, the substrate S of this enzyme is hydrogen peroxide whose decomposition generates oxygen, which reacts with the chromogen (TMB, tetramethyl benzidine). In the presence of luminol, a chemoluminescence stable for several minutes appears which is at the origin of extremely sensitive measurements.

This classic biological methodology is widely used in research, diagnosis, and testing because it is affordable and sensitive to tiny amounts of material. It is applied to domains such as the environment and agriculture (pesticides, anabolic steroids, aflatoxins, HPA) for which numerous assay kits exist. However only compounds for which there exist adapted antibodies and the conjugated enzymes can be measured.

17.7.2 The relationship absorbance/concentration

The number of bonding sites on the tube wall is very small compared with the number of molecules in solution. Calling N the total number of anchoring sites inside the well, n^* the number of labelled molecules adsorbed, C^* the concentration of the labelled species in the solution and C the concentration of non-labelled unknown, we can write an expression which links the ratio R of the two concentrations with the number of sites concerned:

$$\frac{n^*}{N - n^*} = \frac{C^*}{C} = R \quad (17.8)$$

The absorbance A being proportional to n^* , leads to $A = kn^*$, thus:

$$A = kN \left(\frac{C^*}{C + C^*} \right) = kN \left(\frac{R}{1 + R} \right) \quad (17.9)$$

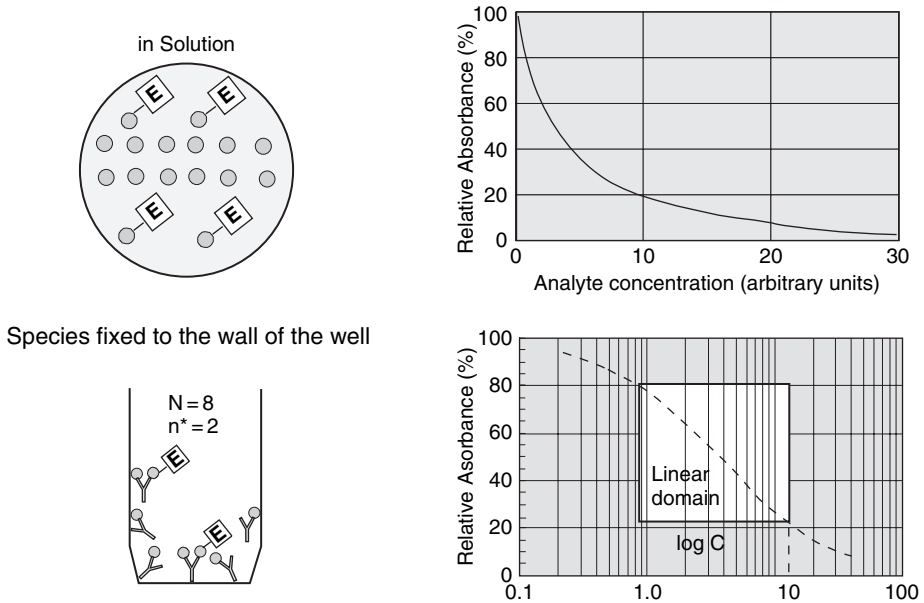


Figure 17.5 Relationship between concentration and absorbance of ELISA assays. The ratio of the two species in solution or fixed to the wells is the same (3, on the figure). Linearity, plotted on a semi-logarithmic scale, is only attained over a narrow range of concentrations.

A typical consequence of this relation 17.9 is the non-linearity of the experimental curve when the concentrations are plotted along the abscissa and at the ordinate the percent absorbance of the solution relative to the reference obtained with the blank ($C=0$). This reference is obtained with the blank (all of the antibody sites are, in this case, occupied by the conjugated enzyme).

If the concentrations are plotted on a logarithmic scale, part of the graph will be linear to a first approximation (Figure 17.5).

The final absorbance measurement can be replaced by fluorescence detection (this is a fluorescence ELISA test). In this case the conjugated enzyme chosen is an alkaline phosphatase which converts the specific substrate into a fluorescent compound.

17.8 Other immunoenzymatic techniques

There are several ways to conduct ELISA techniques with competition in a heterogeneous phase medium. In one of them, the antibodies are covalently bound to magnetic particles of $1\ \mu\text{m}$ diameter instead of to the container wall (Figure 17.6). This approach has the following characteristics:

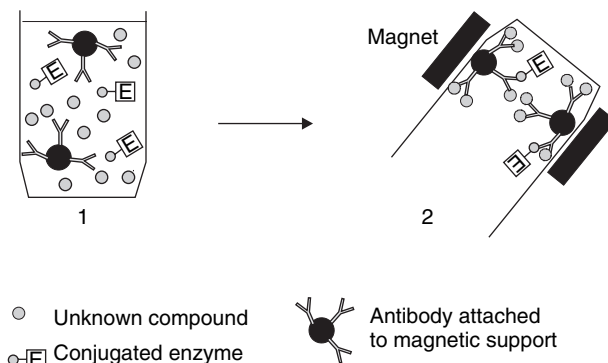


Figure 17.6 *A variant of the basic ELISA assay*

- the quantity of antibodies is unlimited, in contrast to using antibody-coated walls
- the antibody/antigen reaction is favoured because the two partners can be better mixed, the medium being half-way between a homogeneous and a heterogeneous phase.

This assay is essentially the same as previously described, except that since the antibody is fixed to the magnetic particles, a magnetic field is applied after incubation and before rinsing in order to maintain the antibodies against the container walls.

17.9 Advantages and limitations of the ELISA test in chemistry

The various ELISA assays give both semi-quantitative and reliable results more rapidly than chromatography. They are particularly practised to eliminate all of the non-useful negative samples, which need not be submitted to the extraction steps and to a subsequent chromatographic analysis. There are, however, several disadvantages:

1. The eventual recognition of several molecules with different sensitivities can cause a dispersion in the results. There is therefore a risk of crossed-reaction (a false positive).
2. The range of measurement is relatively narrow. As the concentration increases, the measurement becomes less precise (an effect of using a logarithmic scale for the concentrations). A correct dilution for the sample must be found.

3. The wells in microtitre plates have to contain the same quantity of antibody with the same reactivity. This is in fact technically difficult to achieve. The immunization of a laboratory animal is a singular event, which explains that results can differ from one ELISA kit to another.
4. These kits must be stored at low temperature. They have a limited lifetime, especially for field analyses.
5. The costs of these assays with the duplicate tests and the necessary standards must be born in mind. They are economical only for a certain number of analyses.

17.10 Immunofluorescence analysis (IFA)

Labelled antibodies with a fluorescent derivative obtained from fluorescein, rhodamine or luminarin, are well suited for the measurement of a number of human and animal immunoglobulins. However, the transposition of this principle to immunochemical testing for simple organic molecules remains, as yet, non-existent. There may be discrimination between the labelled and non-labelled species at the isolation stage for small organic molecules. This explains that only a few examples of analysis followed by fluorescence measurement.

Several diagnostic techniques are grouped under the term of immunofluorescence which do not correspond to measurements but rather to locate, for example by microscopic examination under ultraviolet light, of certain parts of the preparation derived from a reaction with fluorescein isocyanate or other fluorescent derivatives.

17.11 Stable isotope labelling

The field of application for the isotope dilution method with radioactive tags, extends to measurements using stable isotope. Mass spectrometry or nuclear magnetic resonance are used to determine the variations in the isotopic concentrations. Chemical labelling using externally introduced tags consists of the addition to a sample of the same analyte but containing a stable isotope (e.g. ^2H , ^{13}C , ^{15}N) as an internal standard. This method is as much used for molecular species as for atoms (around 60 have stable isotopes).

The reference and unknown samples differ only by the incorporation of the stable isotope. They have the same properties and behave with essentially identical characteristics under any isolation or separation step. Thus the ratio between intensities of the pair provides an accurate measurement of the unknown concentration. *Isotopic mass spectrometers* are especially adapted for these measurements (Figure 17.7).

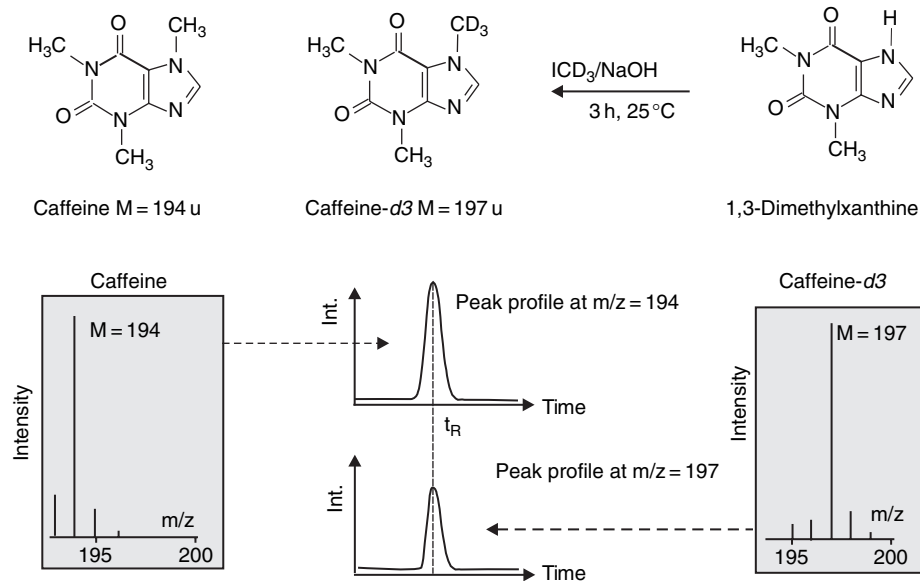


Figure 17.7 HPLC/MS measurement of caffeine by isotopic dilution. The stable isotope used as a tracer is deuterium D. Caffeine-d³ is a product of the methylation of 1,3-dimethylxanthine by ICD₃.

Ultra-trace quantities can also be measured because, in contrast to radioactive labelling, for which the measurement is only based upon the detection of atoms which decompose during the time of the measurement itself; Here, all of the labelled atoms are taken into account.

■ *Measuring traces of caffeine by stable isotope labelling.* First a solution of the sample is made to which a known quantity of labelled caffeine-d³ is introduced. By HPLC these 2 caffeine species have the same retention time. During their co-elution, the detector measures alternately the intensity of the peaks $m/z = 194$ and $m/z = 197$. Two pseudochromatograms are thus obtained (Figure 17.7), one of peak 197 the other of 194, whose areas allow the determination of the caffeine concentration.

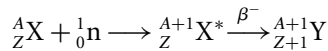
Through synthesis it is possible to replace, in the caffeine ($m = 194$ u), the ³H of the group N-CH₃ of the five-membered ring by three atoms of deuterium (D). This produces labelled caffeine of mass 197 u.

17.12 Neutron activation analysis (NAA)

Activation analysis is a process useful for performing both qualitative and quantitative multi-element analysis from almost every field of scientific or technical interest. The elemental concentrations in the sample are calculated following an irradiation step of the unknown sample and a comparator standard containing

a known amount of the element of interest. Neutron activation is a technique comparatively easier to access than those employing charged particles. Around 60 elements can be identified and measured following their transformation to radioisotopes. This particular process is part of the domain of multi-elemental nuclear activation analysis whose sensitivity varies with the element (from 1000 ppm to 1 ppb).

The nuclear reaction used for NAA is the neutron capture or (n, γ) reaction, as shown below. When a neutron interacts with the target nucleus *via* a non-elastic collision, an isotope of greater mass forms in an excited state (Figure 17.8). By reiteration of this process, one obtains an isotope that becomes unstable and decomposes generally by β^- emission. The total radioactivity resulting from a sample containing several elements – each consisting of an isotopic family – will lead of course to a complex emission spectrum.



The total activity generated in the sample will diminish with time following a decreasing curve corresponding to an envelope of the superimposed activities of the individual radionuclides present. The β^- spectrum being continuous, the composition of the elements cannot be deduced by a simple analysis of the radiation.

The key for identification comes from the γ emission which accompanies the β^- emission and is characteristic to each radionuclide. This emission spectrum occurs in the same spectral range as X-ray fluorescence.

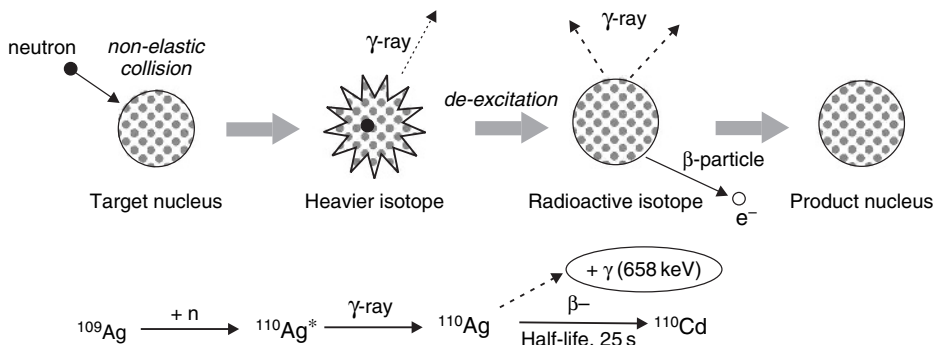
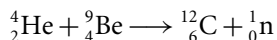


Figure 17.8 Schematic of neutron activation. When a neutron interacts with the target nucleus an isotope forms that can be unstable. If so, it will almost instantaneously de-excite into a more stable configuration through emission of γ -rays. Then, this new radioactive nucleus decays by emission of an electron and one or more characteristic γ -rays, at a rate according to the half-life of this nucleus. Illustration with Ag atom.

17.12.1 Sources of thermal neutrons

There are several types of neutron sources one can use for NAA, but megawatt nuclear reactors with their intense flux of 10^{14} to 10^{16} neutrons $\text{m}^{-2}\text{s}^{-1}$ from uranium fission, offer the highest available sensitivities for most elements. Neutron energy distribution is quite broad, but for conventional NAA, low-energy neutrons (energies below 0.5 eV) are chosen that represent 90–95 per cent of the neutron flux. Sufficient levels of activation are reached in a few minutes, even if the isotope formed has a long half-life. The procedure imposes that the sample to be treated must be thermally stable. It is enclosed in a tube with a comparator standard of known concentration, before being introduced in a reactor beam port.

Small sources of neutrons – containing an α -emitter (several $\mu\text{g}^{241}\text{Am}$ or ^{124}Sb) encapsulated in a beryllium envelope – have been developed to avoid these restrictions. The nuclear reaction generating the neutron is the following:



A variant consists of using a few μg of ^{252}Cf (α -emitter, $\tau = 2.6$ years) as a source of fast neutrons (2 MeV) that are slowed by collision with hydrogen atoms. These low intensity radioisotopic neutron emitters (sealed devices) liberate approximately a few hundred million neutrons per second allowing the measurement of around twenty elements.

17.12.2 Detection – principle of measurement

The choice of the detection procedure depends upon the nature of the emitter and of the complexity of the emitted spectrum. The most effective solution consists of recording the γ spectrum which accompanies the majority of the β^- emissions of the radionuclides present in the sample following activation (Figure 17.9).

The instrumentation used to measure γ -rays is generally a semiconductor detector (NaI(Tl) crystal) associated to a multi-channel analyser to cover energies over the range from about 60 keV to 3.0 MeV. The functioning is similar to that of liquid scintillators.

Introducing a time gap between the end of the irradiation and the counting step can reduce all interferences due to short-lived emitters.

Delayed or prompt NAA. As explained above, γ -rays are measured at some time after the end of the irradiation (delayed-NAA). This is the useful method for the vast majority of elements that produce radioactive nuclides. Yet, in some cases, for elements which decay too rapidly during neutron irradiation or for elements that produce only stable isotopes, γ -rays are measured during neutron irradiation. This is called the prompt-NAA.

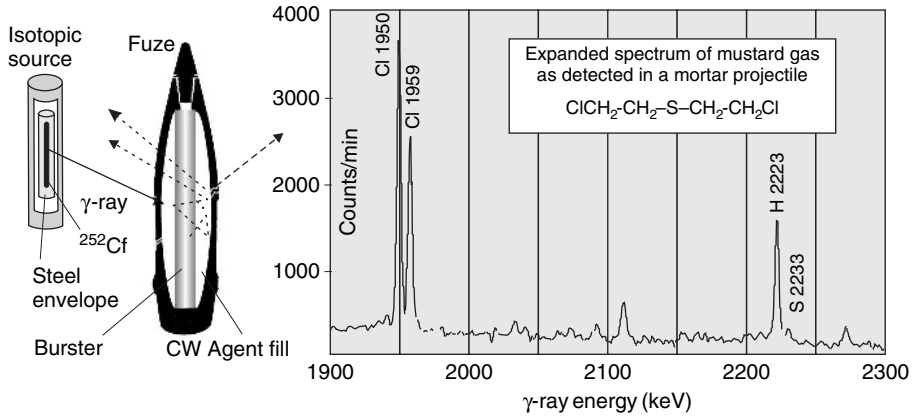


Figure 17.9 A non-conventional application of neutron activation. When it is necessary to destroy a warhead containing chemical warfare agents as mustard gas, NAA is used to identify the contents non-destructively. This diagram, reproduced courtesy of Ametek Inc./ORTEC, illustrates the method. Right, part of the γ -spectrum of bis(2-chloroethyl)sulfide, that shows the S, H and Cl signals.

17.12.3 Induced activity – irradiation time

The probability of inducing a nuclear transformation depends upon the nuclide and of the energy of the neutrons. These two factors are united in a parameter called the *effective cross-section*.

The number of radioactive atoms N^* (the $A + 1$, Z element) which accumulate in the sample during irradiation leads towards an upper limit: at each instant, the increase in the number of nuclei N^* is equal to the difference between the rate of formation, considered as constant, the number of target nuclei N (the A , Z element) being very large, and that of the rate of decay (of $A + 1$, Z element):

$$\frac{dN^*}{dt} = (\varphi\sigma N) - (\lambda \cdot N^*) \quad (17.10)$$

where φ represents the neutron flux, λ the radioactive decay-constant and σ the effective cross-section of each target atom (A , Z). This last number is itself related to the mass m of the isotopic fraction f of the element (A , Z) of atomic mass M . If N_A represents Avogadro's number then:

$$N = (m/M)N_A f \quad (17.11)$$

The integrated form 17.12 of the elementary formula 17.10 allows to evaluate the number of atoms N^* present after a given irradiation time t_i :

$$N^* = \frac{\varphi\sigma N}{\lambda} (1 - \exp[-\lambda t_i]) \quad (17.12)$$

If we consider that the induced activity measured at time t corresponds to $A_t = \varepsilon I_\gamma \lambda N^*$, with ε , the detector efficiency and I_γ the γ -ray abundance, then:

$$A_t = \varepsilon I_\gamma \varphi \sigma N (1 - \exp[-\lambda t_i]) \quad (17.13)$$

The term in brackets, called the *saturation factor*, tends rapidly to 1 as t_i increases. In this way, for $t_i = 6\tau$ the activity reaches 98 per cent of the limiting value. Experimentally, the time of irradiation never exceeds 4 or 5 half-lives of the radioisotope. This method cannot be used for long-lived radioisotopes. When irradiation stops, A_t begins to decrease ($A = A_t \exp[-\lambda t]$)

To calculate the concentration of element X in the unknown sample (C_X), a comparator standard containing a known amount m_{std} of the element of interest is together irradiated in the reactor. Then the unknown sample is measured with the same detector as the standard. Consequently, under the same conditions, the specific activity of the unknown element X will be the same in the sample as in the standard.

When the irradiation, decay and counting times are the same for all samples and standards, the count rate is proportional to the mass of X in the sample (m_X), it follows that the mass of the element in the sample relative to the comparator standard is:

$$\frac{\text{Count rate}_{\text{sample}}}{\text{Count rate}_{\text{std}}} = \frac{m_X}{m_{\text{std}}} \quad C_X = C_{\text{std}} \cdot \frac{W_{\text{std}} \cdot \text{Count rate}_{\text{sample}}}{W_{\text{sample}} \cdot \text{Count rate}_{\text{std}}} \quad (17.14)$$

with $C_i = m_i/W_i$ where C represent the concentrations and W the masses of sample or standard.

The relationship 17.14 is helpful if the radiation induced is simple, or when the counting instrument is fitted with a filter permitting the isolation of the radiation from the element to be measured. However, often the matrix itself becomes active and emits radiation, which comes to be superimposed upon that to be evaluated. By introducing a time gap between the end of irradiation and start of the measurement, the interferences caused by short-lived emitters are eliminated.

■ Suppose that traces of iron are to be measured in a sample of aluminium. The ^{59}Fe ($\tau = 46$ days) is characterized by a γ -ray at 1.29 MeV, but during the irradiation time, the aluminium yields ^{24}Na , responsible for a γ -ray situated at 1.37 MeV, through the reaction $^{27}\text{Al}(n, \alpha)^{24}\text{Na}$ ($\tau = 15$ h). In order to give the short-lived aluminium to decompose, the measurements are postponed by several days.

17.12.4 NAA applications

NAA measures the total amount of an element, even in extremely low concentrations (parts per trillion or lower), in a material without regard to chemical or

physical form. Samples analysed can be liquids or solids and do not have to be put in a solution.

Theoretically, every element can be neutron activated. Yet, some conditions are required. First, the element must have an isotope with a high cross-section that is able to fix the incoming neutrons. Second, if delayed-NAA is used, the half-life of the isotope has to be long enough so the amount of activity is measurable. Third, the isotope itself must be relatively easy to get in sizeable quantities. Lastly, γ -rays must be produced that are reasonably intense and in a limited energy range.

NAA is widely used in many different fields of sciences. Applications include: environmental studies to characterize pollutants, semiconductor materials analysis to measure ultra trace-element impurities, archaeological studies of the distribution of the chemical elements and fossil materials, forensic studies as a non-destructive method (suspect chemical agents, see Figure 17.9), pharmaceutical materials analysis to measure ultra-trace element impurities, etc. Unfortunately, facilities for using this method do not exist everywhere. Otherwise, the sample becomes slightly radioactive, requiring the sample to be quarantined until its activity reaches a state similar to which it was before the NAA.

Problems

- 17.1 To determine the concentration (% mass) of penicillin present in a commercial preparation, an isotopically labelled reference of penicillin is used, whose specific activity is 75 000 Bq/g. 10 mg of labelled penicillin is added to 500 mg of sample. After mixing and equilibration, 1.5 mg of penicillin was recovered whose measured activity was 10 Bq. Calculate the concentration (% mass) of penicillin in the commercial preparation.
- 17.2 In order to measure, by isotopic dilution the mass concentration of the orthophosphate ion PO_4^{3-} in an aqueous solution, 3 mg of labelled phosphate ion $^{32}\text{PO}_4^{3-}$ (^{32}P being a β -emitter whose half-life is 14 days), is added to 1 g of the sample solution.
The specific activity of the added $^{32}\text{PO}_4^{3-}$ is 3100 dps/mg.
After mixing, 30 mg of orthophosphate was isolated whose overall activity was 3000 dps. Find an expression from which the initial quantity of phosphate ion PO_4^{3-} may be calculated, and calculate the % mass of this ion in the aqueous solution.
- 17.3 Patulin ($\text{C}_6\text{H}_7\text{O}_4$), is a compound, dangerous to health in humans which is found in the juice of damaged apples or grapefruits. The current method of measuring this compound resembles that of an immunoenzymatic ELISA test.

A standard solution of pure patulin was prepared in water at a concentration of 1.54 g/L just prior to use.

In the test described, four identical test tubes are used whose inner wall is covered with a suitably adapted antibody. All of the tubes follow the same treatment, differing only in the solution which is introduced in the initial step. The contents are as follows:

- tube 1: a blank, 2 mL of pure water.
- tube 2: 1 mL of pure water plus 1 mL of the standard solution non-diluted.
- tube 3: 1 mL of pure water plus 1 mL of the standard solution diluted twice.
- tube 4: the sample, 2 mL of fruit juice, filtered and diluted twice with water.

(In to each tube the same quantity of conjugated enzyme is introduced, plus all of the other necessary reactants combined).

Following the reaction, the measured absorbance A of the tubes was as follows: tube 1: $A = 1.03$, tube 2: $A = 0.47$, tube 3: $A = 0.58$, tube 4: $A = 0.5$.

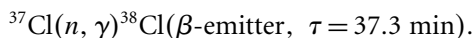
1. Calculate the concentration in ppm of the patulin in the standard solution.
 2. Calculate the % absorbance (also known as the % inhibition) of the solutions in tubes 2, 3 and 4 with respect to tube 1.
 3. Explain why the absorbance of tube 1 is greater than that of the other tubes.
 4. Calculate the concentration of patulin in tubes 2 and 3 in $\mu\text{g/L}$.
 5. Plot the calibration curve, $\% A = f(\log C)$ (C = concentrations of tubes 2 and 3 in $\mu\text{g/L}$)
 6. Calculate the concentration in mg/L of patulin in the fruit juice also giving the result in ppb.
- 17.4 To measure elemental chlorine present in a very weak concentration (of the order of a few ppm) in a sample of steel, by the method of neutron

activation analysis (NAA), a reactor producing a neutron flux of thermal character $2 \times 10^{16} \text{ n/m}^2/\text{s}$ is required.

1. Write clearly, using the symbols provided, the reactions taking place:



and



2. Explain why the γ emissions of the isotope ^{38}Cl are preferred for the experiment rather than those of ^{36}Cl .
3. Indicate two other methods which could be used to measure the chlorine in the steel. Give a precise explanation including reasons, advantages and disadvantages of the methods chosen when they are compared to NAA.

To eliminate the interference afforded by manganese (^{55}Mn) present in steel which leads to ^{56}Mn whose half-life is 2.58 hours and whose radiation is intense, the chlorine is separated by precipitation in the form of silver chloride.

Simplified operating procedure: For five minutes and under the same conditions in the reactor, a) a sample of the steel to be quantified in Cl, and b) a disc of filter paper onto which $100 \mu\text{L}$ of a solution of 0.1 g/L of chloride ion had been previously adsorbed, (previously it had been verified that the filter paper contained no Cl), is irradiated. Then the steel sample is dissolved into solution by boiling with 40 mL of 2M HNO_3 to which is added 2.00 g of dry KCl. This resulting solution is then introduced to 50 mL of an aqueous solution of 15% (w/v) AgNO_3 . The AgCl precipitate is recovered, washed and then dried. The result of the count is displayed in the following table.

	Mass	Mass AgCl	Count γ (1.64 MeV) of ^{38}Cl
Sample	0.51 g of steel	3.726 g	11 203
Standard	$10 \mu\text{g}$ of Cl		48 600

4. Show that the silver nitrate is added in sufficient quantity.
5. Calculate the concentration of elemental chlorine in the steel sample. Recall that $N = 14.007$, $O = 16$, $\text{Cl} = 35.453$, $K = 39.098$, and $\text{Ag} = 107.863 \text{ g/mol}$.

18

Elemental analysis

Certain elements or compounds are more frequently measured than others. Answering to these particular needs, spectrometers and chromatographs, despite their obvious flexibility, are not always the best-adapted instruments for various reasons as operation facility, rapidity of analysis, or even cost. There now exists, alongside classic instrumentation, a whole range of 'customized' apparatus, possessing either modified software or material based upon the particular properties of the elements or species concerned, some of them unique. The target analytes are diverse: elements, ions, molecules (sulfur, carbon, oxygen, ozone, oxides of nitrogen, benzene, polyaromatic hydrocarbons).

These specific instrumental analyses are found in a variety of domains such as the petrochemical industry, metallurgy, agriculture and the sectors of atmospheric pollution, soils and water sectors, the detection of chemical weapons, the fight against terrorism and other areas. These instrumentation of specific analysers should not be neglected. This chapter reviews several original methods limited to single elements only.

18.1 Particular analyses

When hundreds of analyses per day must be undertaken, traditional laboratory methods and instruments can be less effective (e.g. data throughput too slow or too much data to be treated). Therefore there are other instruments for these singular applications which correspond to integrated systems for obtaining more quickly information concerning the concentration of particular elements or compounds. For example, finding out the carbon, hydrogen, nitrogen, oxygen content of unknowns is one need in organic chemistry research. One other area for which there is an increasing requirement is that of continuous systems analysis, which has for objective automation and control in real time, of numerous industrial manufacturing procedures. Amongst the species imposing the most attention are

atoms and molecules. To illustrate these aspects, some methods are described in this chapter.

18.2 Elemental organic microanalysis

The very great number of molecular compounds containing carbon (C) and some other light elements such as hydrogen (H), nitrogen (N) and oxygen (O) have provided the need to measure these elements for a wide range of research, industrial and academic applications. The biosphere uses three or four element combinations as varied as and perhaps more varied than those that form the mineral kingdom. A multiplicity of sectors (petrochemicals, pharmaceuticals, or agrochemicals) in organic chemistry are concerned.

When molecules are synthesized by multi-step sequences or when new compounds are extracted from natural sources, their structure and purity must be elucidated. For this, a quantitative elemental analysis must be performed. This particular type of analysis allows us to find the percent elemental composition, in the pure state, of the molecule under study. The measurement of a single element, indeed two (C and H are the most frequent) will verify the accuracy of the molecular formula proposed for a molecule not as yet fully defined but for which a structure has been deduced from spectral studies. Elsewhere, purity of a compound for which the composition and the molecular weight are known, can be determined by comparison of the experimental results obtained from a sample with the theoretical ones (Figure 18.1).

For such light elements, atomic absorption spectroscopy (unusable due to a lack of suitable sources) or of X-ray fluorescence spectroscopy (lack of sensitivity due to radiation of low energy) are inappropriate.

The principle applied for measuring these elements is to burn a small sample (a few milligrams) of the compound by rising it to a high temperature in the presence of oxygen, with customized analysers. The elements H, C, N, O, S are recovered in the form of gaseous oxidation products. This also has the advantage of separating them physically from the matrix.

■ Elemental microanalysis gives essential information, though insufficient to deduce the molecular formula for a totally unknown compound. Even if all of the elements present had been measured, which is never the case as instruments are designed to determine only a few ones, the molecular formula is known, but not the molecular weight. Taking as an example the results shown on Figure 18.1, the molecular formula $C_{54}H_{54}N_6O_{12}S_2$ would have the same percentage

$C_{27}H_{27}N_3O_6S$	requires % C	62.18	H 5.22	N 8.06
M = 521.5 g/mol	found %	C 62.02	H 5.29	N 8.15

Figure 18.1 Conventional presentation of an elemental analysis for an organic compound

composition in elements than $C_{27}H_{27}N_3O_6S$. In the majority of cases mass spectrometry, or NMR, is called upon to remove this ambiguity and determine the correct formula. Finally, it should be noted that for all compounds the percentage analysis, despite its inherent experimental error, will, when combined to a mass determination method, find the exact molecular weight and formula, the number of atoms being always an integer.

18.2.1 Traditional methods of Dumas and Pregl

Current elemental analytical methods are based on the combustion of a known amount of the sample using a modified Pregl–Dumas technique. To find the atomic composition of an organic compound, a sample is weighed in a consumable tin capsule, then injected into a high temperature furnace and combusted in an excess of pure oxygen. If the elements C, H and N are present, the resulting combustion products, carbon dioxide (CO_2), water (H_2O) and a mixture of nitrogen and nitrogen oxides ($N_2 + NO_x$) will be formed. The amounts of these gas lead by calculation to the percentage of the elements in the initial sample. Elemental oxygen is determined separately by combustion in the presence of carbon to form carbon monoxide.

■ Historically, Dumas founded a new analysis method when he developed in the 1830s, a procedure for estimating the amount of nitrogen in an organic compound. But it is Pregl, Nobel Prize laureate in Chemistry 'for his invention of the method of microanalysis of organic substances' who was the precursor of today's instrumentation. Pregl was able to make measurements of carbon, hydrogen, nitrogen, sulfur, and halogen, using only 3–5 mg of starting materials with results as accurate as those obtained by macroanalysis.

In the early microanalysis apparatus developed by Pregl for carbon and hydrogen, the combustion was made at $750^\circ C$ in a current of oxygen, the transformation of CO into CO_2 being completed by passage over a mixture of copper oxide and lead chromate (Figure 18.2). The masses of elements C and H were calculated from the increase in weight of two tubes (previously weighed), one containing magnesium perchlorate (for H_2O) and the other quicklime (for CO_2): precision reached 0.1 per cent providing the balance could measure in mg.

Later a second generation of analysers were developed (Figure 18.2), which incorporated specialized reagents to remove interferences including halogens, sulfur and phosphorus. The gases are then passed over copper powder to eliminate excess oxygen and reduce oxides of nitrogen to elemental nitrogen. In these CHN analysers, to simplify the operation, the double weighing is replaced by measurements of thermal conductivity differences in gas mixtures before and after passage through two selective traps, the first of which is a water trap (H determination) and the second one for carbon dioxide (C determination). Finally, nitrogen is measured against a helium reference.

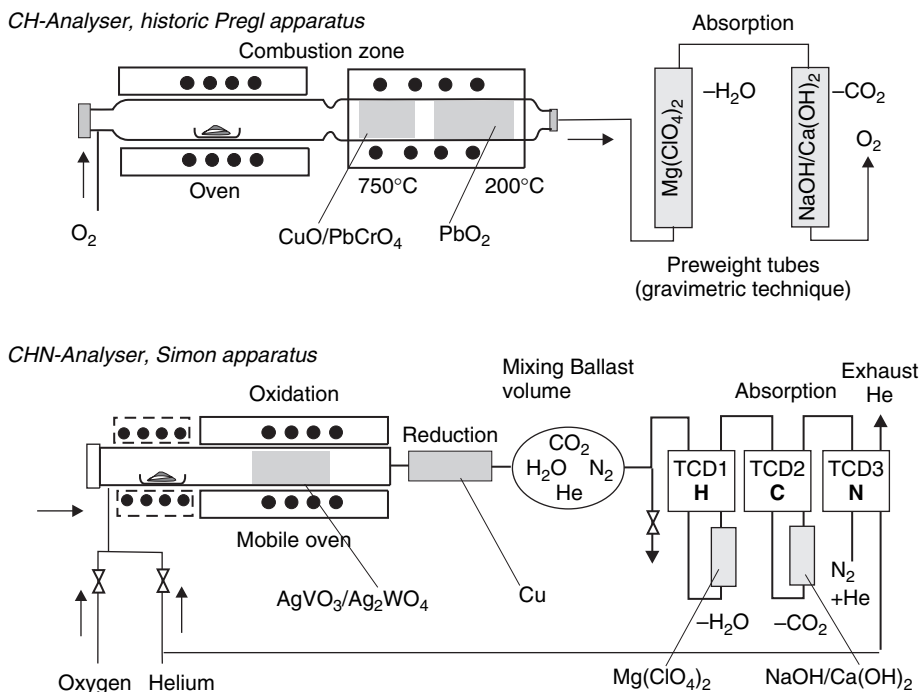


Figure 18.2 Pregl method of microanalysis. Generations of students will recall having calculated, with anxiety, molecular formulae from the masses of carbon dioxide and water trapped in the removable absorbers. In a more recent version, the reaction gases pass over copper powder to reduce the nitrogen oxides to nitrogen. Three thermal conductivity detectors (TCD) signal the presence of H₂O, CO₂ and N₂. The measurement of the three requires elements require around 10 minutes.

18.2.2 Current elemental organic analysers CHNS-O

Other modern analysers keep the sample combustion principle, but the formed gases are measured by a chromatographic method (Figure 18.3). About 1 mg of the sample is pyrolyzed at 900°C in the reactor chamber in the presence of an oxygen excess. The complete oxidation is reached on a tungsten trioxide catalyst. The leaving gas stream includes carbon dioxide, water, nitrogen oxides and an excess of oxygen. The product gas mixture flows through a silica tube packed with copper granules where excess oxygen is fixed and nitrogen oxides reduced to gaseous nitrogen. Finally, the gas mixture is brought to a defined pressure/volume state and is passed to a gas chromatographic system. The elements N, C, H are at the origin of three compounds in the gas state, N₂, CO₂ and H₂O, separable by a packed GC column associated with a thermal conductivity detector. Oxygen in the sample is measured separately, as carbon dioxide, obtained by pyrolysis in the presence of platinumized carbon, and of course, in the absence of oxygen.

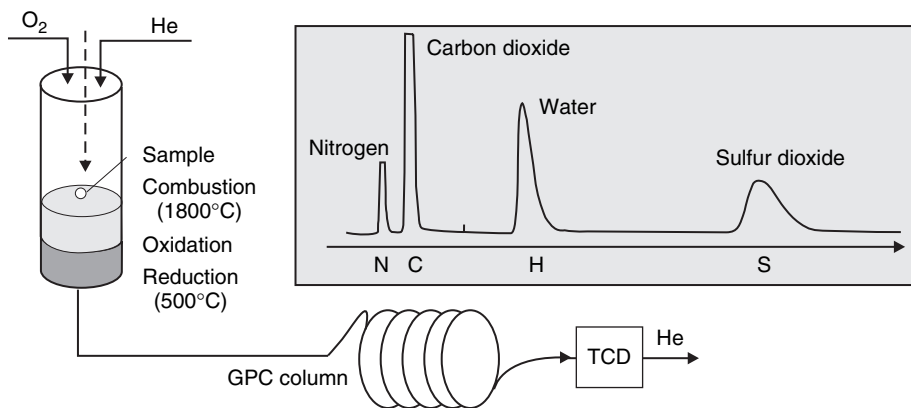


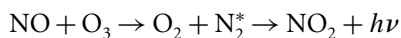
Figure 18.3 *Microanalysis apparatus with chromatographic detection.* The Porapak filled column separates the four gaseous components carried away by the helium carrier gas. Through a prior calibration with standards, the concentration of each of the 4 elements in the sample can be deduced from the areas of the corresponding peaks on the chromatogram.

18.3 Total nitrogen analysers (TN)

The total nitrogen content is used to characterize numerous products and samples in the petrol, paper and carbon industries. These measurements can be carried out with CHN analysers as described previously, but to simplify the procedure, customized analysers have been developed for this single element. They are an alternative for cumbersome reference methods, such as *total Kjeldahl nitrogen* (TKN – corresponds to the sum of organic nitrogen and ammonia).

In the Kjeldahl method the compound containing the nitrogen is first digested in sulfuric acid in the presence of a catalyst before being taken up by a mixture of sodium hydroxide, sodium oxide and calcium hydroxide in order to transform the nitrogen within the substance to a single ammonia-based solution. This sequence ends by taking up this solution in water vapour to remove the ammonia formed, which is finally measured by acidimetry. This method can be rendered semi-automatic but it suffers the inconvenience of using inherent corrosive reagents and titrated solutions as well as being rather time consuming.

After pyrolysis and reduction, the gas mixture containing nitrogen passes through a trap to remove water and the combustion gases (Figure 18.4). The general detection procedure, based on the thermal conductivity of the gas, is replaced by specialized detectors based on the chemiluminescence of nitric oxide (NO) when combined with ozone (O₃) (cf. Section 11.8).



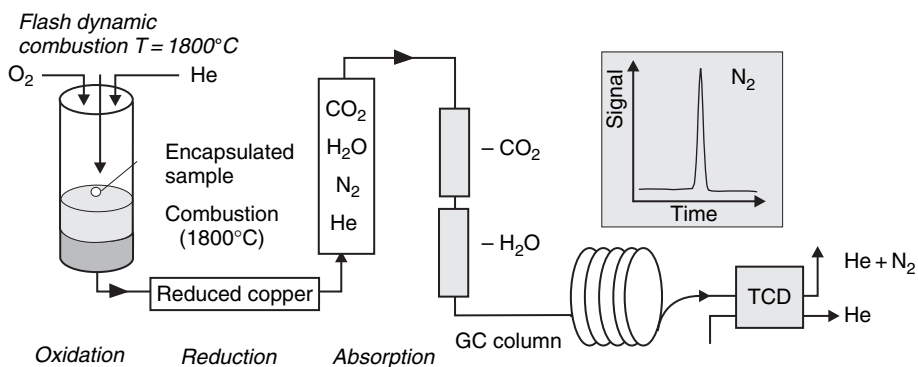


Figure 18.4 *Instrument for nitrogen analysis.* This instrument is a modern adaptation of the Dumas' method, with the conventional detection by means of a catharometer (He is the carrier gas).

or upon the oxidation of NO in contact with an electrochemical sensor (cf. Chapter 20):



■ In Dumas' original method (1840) the substance was heated with copper oxide in an atmosphere of carbon dioxide. Elemental nitrogen was partially oxidized to nitrogen oxides NO_x , which were subsequently reduced over copper. The nitrogen percentage was calculated from the volume of the gas released, measured with a graduated test tube.



Figure 18.5 *A total nitrogen analyser – model TKN, reproduced courtesy of Skalar.* Detection by chemifluorescence. The small amount of light is quantified and is proportional to the nitrogen content of the sample. This type of instrument can be used for measuring proteins.

An example of application can be seen in food-processing industry where the measurement of the total nitrogen content has been adopted by numerous countries as the reference for calculating the protein concentrations, generally accepted as corresponding to 6.5 times the nitrogen value (Figure 18.5).

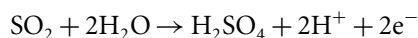
18.4 Total sulfur analysers

Sulfur content is often measured for industrial controls by X-ray fluorescence because the samples usually form part of a series, which may be compared with very similar standards. Yet, for organically bound sulfur compounds that can be destroyed by combustion in a current of oxygen, the measure is based upon the volume of sulfur dioxide (SO₂) formed in an instrument similar to that described previously. Three main procedures of quantification co-exist with which the other gases of combustion do not interfere:

- Detection of sulfur dioxide by fluorescence, a process that can be summarized as follows:



- Specific action of iodine on SO₂ in the presence of water, a reaction exploited elsewhere for the measurement of water by the Karl Fischer method (Chapter 20). The instrument gives the minimum quantity of current required to be generated in a coulometric cell, by an electrolyte containing an iodide, in order to oxidize SO₂. If it is known that 2 electrons are required to oxidize a molecule of SO₂, one sulfur atom will correspond to $2 \times 1.6 \times 10^{-19}$ C.
- Oxidation reaction of sulfur dioxide in nitric acid, in contact with the working electrode of an amperometric cell (cf. Chapter 20):



18.5 Total carbon analysers (TC, TIC and TOC)

With the previous combustion method, analysis is performed when carbon compounds are combusted in an oxygen-rich environment, resulting in the complete conversion of carbon to carbon dioxide. This procedure is not applicable to water samples with a very low concentration in elemental carbon as used for semiconductor manufacturing processes or required in pharmaceutical industries. For these cases the organic contaminants are measured by the *total carbon*

content in water, expressed as the sum of the carbon contained in different organic fractions defined as follows:

- *total inorganic carbon* (TIC) – the sum of inorganic carbon species in a solution (carbon dioxide, carbonic acid, hydrogenocarbonate anion, and carbonate anion) dissolved in the aqueous sample
- *total organic carbon* (TOC) – the amount of carbon covalently bonded in organic molecules
- *total carbon* (TC) – represents the amount of elemental carbon in the sample.

Appropriate analytical methods must be used to measure these three fractions of the total carbon content.

The TIC is available through a coulometric detector. To determine the TOC, all recent analysers employ the same basic technique. The first stage of the method is the removal of the inorganic carbon with an acid such as H_3PO_4 (the TIC being essentially in the form of carbonates) introduced in the liquid sample. Thus the inorganic carbon is converted into carbon dioxide gas that is stripped out of the liquid by a carrier gas. The remaining organic carbon is then oxidized by UV radiation and carbon dioxide generated from the oxidation process is subjected to a conductimetric detection in a conductivity cell (Figure 18.6).

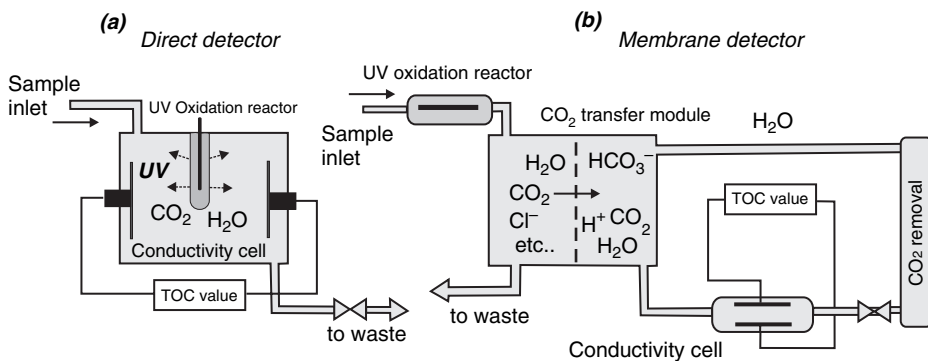
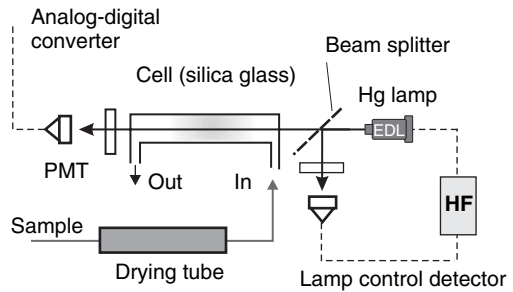
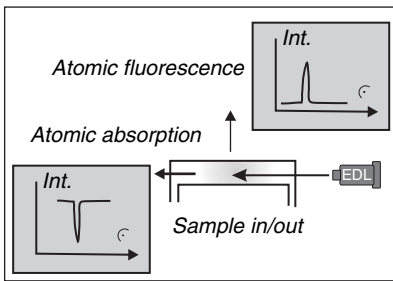


Figure 18.6 Direct and membrane conductimetric detection methods for measuring TOC. Radiation of a high output UV lamp (185 and 254 nm) produce, in water, by different pathways, hydroxyl free radicals (OH), which oxidize the organic compounds to carbon dioxide and water from oxygen dissolved in the water. The difference between types (a) and (b) detectors is that the direct detector is subjected to interference from ionic contamination, acids, bases, and halogenated organics. In the membrane based conductimetric method, the membrane is a protective barrier to interfering ions, enabling the analysis of CO_2 only (reproduced courtesy of GE Analytical Instruments for illustrations).



Cold-vapour atomic absorption spectroscopy



Cold-vapour atomic fluorescence spectroscopy

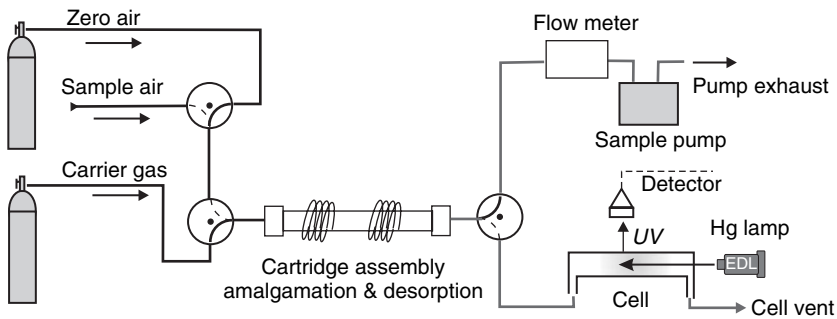


Figure 18.7 *Mercury analysis.* Two models of analyzers (Reproduced courtesy of Mercury Instr. USA and Genesis Laboratory Systems Inc.). The mercury concentration is measured in an optical cell made of fused silica on the path of a mercury discharge lamp (253.7 nm resonance line). Mercury is measured either by AAS or FAS.

18.6 Mercury analysers

Mercury, a heavy metal, is a dangerous pollutant because of the ease with which it may give highly toxic organic derivatives such as dimethylmercury. It is present in a wide variety of sample matrices including water, wastewater or soils and in many sites from chlorine-alkali plants to waste incinerators. The analytical method must be highly sensitive because the gaseous or aqueous samples contain mercury concentrations that may be many orders of magnitude less than possible interfering compounds.

■ The power plants burning coal and municipal waste incinerators are sources of mercury pollution. During thermal processes, all mercury is first converted into elemental form, but during cooling different derivatives can be formed, according to the matrix composition of the flue gas.

To be detectable, mercury must be present in elemental form. Thus for gaseous samples a thermal conditioner unit converts, in the presence of a catalyst, all mercury species present in the sample into elemental form. For liquid samples as polluted water, a first treatment by an acidic oxidizing mixture exchanges mercury compounds into Hg(II) ions that are then reduced to elemental mercury with a tin salt.

When atmospheric mercury is to be measured, the mercury is preconcentrated by passing the sample through a trap containing gold-coated sand. This gold amalgamation enhances detection limits. The amalgam is next heated to desorb the mercury that is detected and measured either by AAS for concentrations within

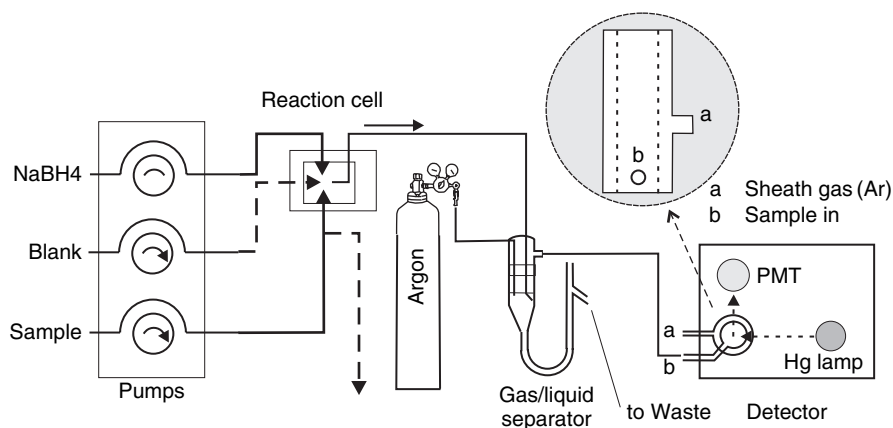


Figure 18.8 Mercury analysis by cold vapour atomic fluorescence spectrophotometry (CVAFS). Measuring principle. (Model reproduced courtesy of P.S. Analytical). The fluorescence is measured at 90° with respect to the incident direction by a photomultiplier tube.

10 ppt to 10 ppm (Figure 18.7) or by atomic fluorescence spectrometry (CVAFS) at ppt or sub-ppt level (Figure 18.8). The last method is less vulnerable to false positive responses and has a better linearity.

Problems

- 18.1 Analysis of an organic compound has revealed that it contains only carbon, hydrogen and oxygen. From the combustion, in a Pregl apparatus, of 5.28 mg of this compound, 10.56 mg of carbon dioxide and 4.32 mg of water are obtained.

What is the molecular formula of the compound knowing that mass spectrometry reveals no significant peaks above $m/z = 89$?

Given: $M(C) = 12 \text{ g/mol}$; $M(O) = 16 \text{ g/mol}$; $M(H) = 1 \text{ g/mol}$

- 18.2 An analyzer based upon AAS and containing a tin chloride elemental reduction device is employed to measure the mercury in an industrial waste water sample. The *cold vapour* mode is used. Three standard solutions and the solution of the sample to be measured give the following results:

Solutions	conc.(ppb)	Signal ($\mu\text{A.s}$)*
Standard 1	0.02	16.66
Standard 2	0.05	42.52
Standard 3	0.2	171.2
Sample	?	48.25

(*) integration of the absorbance signal as a function of time.

1. Indicate the principal advantage and the principal disadvantage of this method.
2. Calculate the concentration (ppb) of mercury in the sample solution.

19

Potentiometric methods

A great deal of quantitative measurements in chemical analyses are based on electrochemistry. Called electrochemical methods, they can be separated into two categories: those based upon measurements of potentials (potentiometry) and those which exploit measurements of current (voltammetry).

The first group, which is developed in this chapter, use ion selective electrodes (ISE). The principle of these chemical sensors is to create an electric cell in which the analyte behaves in such a way that the potential difference obtained relates to its concentration. Measurement of pH, probably the most common and best known electroanalytical method, is part of this group. Most of the measurements concern the determination of ions in aqueous solution, though particular electrodes with selective membranes also allow the determination of molecules. The sensitivity of these methods is very great for certain ions but matrix is sometime responsible for lack of reliability in these measurements. In such cases, complexometric or titrimetric methods must replace direct potentiometry. It remains however for potentiometry multiple applications in which the instruments range from low-cost pH meters to automatic titrators.

19.1 General principles

Potentiometric measurements are based upon the determination of the voltage difference, at zero current, i.e. under equilibrium conditions, between two electrodes which are plunged into a sample solution (Figure 19.1). Each of these electrodes constitutes a half-cell. The following concerning these two electrodes should be noted:

- The *external reference electrode* (ERE), is the electrochemical reference half-cell for which the potential is constant with respect to that of the sample solution.
- The *ion selective electrode* (ISE), also known as the *working electrode*, is composed of an *internal reference electrode* (IRE) bathed in a reference solution of the analyte i subject of the measurement. The electrode, which is

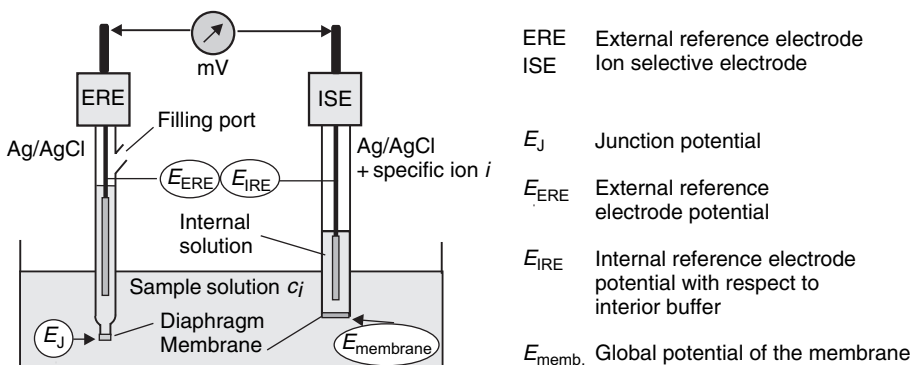
used for measurement, is separated from the sample solution by a *membrane*, selectively permeable to the analyte being studied. The measuring sequence is then as follows:

IRE/internal solution/ion-selective membrane/sample solution/liquid junction/ERE

For studies in aqueous solutions, the external reference electrode (ERE) is often an Ag/AgCl/KCl electrode, made of a silver thread covered with silver chloride bathed in a solution of potassium chloride. Electrical contact with the solution under study is achieved through a finely porous fritted glass. Since the ions have a tendency to migrate across the membrane, there is a resulting low potential junction E_J that can be minimized by a salt bridge, usually a saturated solution of potassium chloride.

The potential difference (PD) between the IRE and the internal membrane surface is constant, as fixed by its design (e.g. the nature of the reference electrode and the activity $a_{i,\text{reference}}$ of the reference solution). Alternatively, the PD, which appears between the external surface of the membrane and the sample solution (E_{memb}), depends upon the activity of the target ion ($a_{i,\text{solution}}$). The potential difference across the membrane is described by the Nernst equation:

$$E_{\text{memb}} = 2.303 \frac{RT}{zF} \log \frac{a_{i,\text{solution}}}{a_{i,\text{reference}}}$$



The membrane is permeable to the selective ion

Figure 19.1 Measurement set-up of an ion selective electrode (ISE). The potential of the membrane permeable to the ion varies with the concentration of the specific ion in the solution sample. The measured signal is the sum of different potentials generated at all interfaces. Measurements are typically conducted by an ionometer. They are made at zero current. Under the equilibrium conditions the transfer of ions from the membrane into solution is equal to the transfer from the solution to the membrane. Manufacturers also supply more robust electrodes called combined electrodes, that include both external and ion selective electrodes in the same device. pH electrodes are of this type. Unfortunately the schematics are less clear due to the compact arrangement.

The cell potential is measured as $E_{\text{cell}} = E_{\text{ISE}} - E_{\text{ERE}} + E_j$

Such as $E_{\text{cell}} = E_{\text{IRE}} + E_{\text{memb}} - E_{\text{ERE}} + E_j$

The terms E_{IRE} and E_{ERE} are independent of the concentration C_i of analyte i . They have no reason to vary during the measurement. Therefore the potential of the cell depends upon that of the membrane (E_j is minimized as explained above).

The activity of the target ion being kept constant inside the electrode ($a_{i,\text{reference}}$) and provided the membrane is only permeable to this single type of ion, the PD, i.e. the electromotive force, is linked to the activity a_i of the ionic species i in the sample solution, by the equation:

$$E_{\text{cell}} = E' + 2.303 \frac{RT}{zF} \log a_{i,\text{solution}} \quad (19.1)$$

In this Nernst-type equation, E' is the standard potential of the measuring sequence used. It takes account of all other potentials. R is the ideal gas constant, F represents Faraday's constant (96 485 C), T is the temperature, z is the charge carried by the target ion i to be measured (*indicating whether + or -*) whose activity is a_i and 2.303 is the logarithmic conversion factor.

The activity a_i of an ion i is linked to its concentration C_i by the relationship $a_i = \gamma_i C_i$ where γ_i is the activity coefficient which depends on the total ionic strength I , that is a measure of the quantity of charge of all ions present in the medium ($I = 0.5 \sum C_i z_i^2$). For example, for an ion i , of charge z , expression 19.1 becomes:

$$E_{\text{cell}} = E' + 2.303 \frac{RT}{zF} \log \gamma_i C_i \quad (19.2)$$

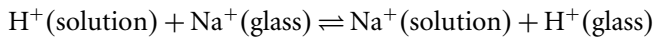
For diluted solutions, the Debye-Hückel law $-\log \gamma_i = -0.5 z_i^2 \sqrt{I}$ indicates that for a given value of I , γ_i is constant. This is why the same quantity of an inert electrolyte, called 'support electrolyte is added to the range of standard and sample solutions', in order to have a large excess of indifferent ions, which stabilize the ionic strength at a constant value. This ISAB (ionic strength adjustment buffer) or TISAB (for total ISAB), limits variations in γ_i . Under these conditions, the measured potential difference depends on the concentration of the ions to be analysed and is given by equation 19.3, which results from equation 19.2:

$$E_{\text{cell}} = E'' + 2.303 \frac{RT}{zF} \log C_i \quad (19.3)$$

19.2 A particular ISE: the pH electrode

This electrode, also called the *glass electrode*, is specific to H^+ ions and highly selective. Glass in this case does not refer to the material of the electrode (which could equally be of plastic), but refers to the membrane that ensures contact

with the solution. This membrane is composed of a thin wall of special glass that has a high sodium concentration (25 per cent). Its surface becomes hydrated in the presence of water and is comparable to a gel, while its inside corresponds to a solid electrolyte. On a microscopic scale this glass is an orthosilicate network with anionic fixed sites and cationic vacancies. This salt of orthosilicic acid $\text{Si}(\text{OH})_4$ has an open structure with sodium cations that allow the transfer of charges from one side of the membrane to the other (Figure 19.2). The outside of the membrane is in contact with the sample solution while the inside is in contact with the internal electrolyte which has a constant acidity (*ca.* pH 7). In short, the membrane is the seat of exchange between cations Na^+ and H^+ :



When the H^+ concentration is different on either side of the membrane, a potential difference PD will appear between them, which is related to the activity of H^+ ions

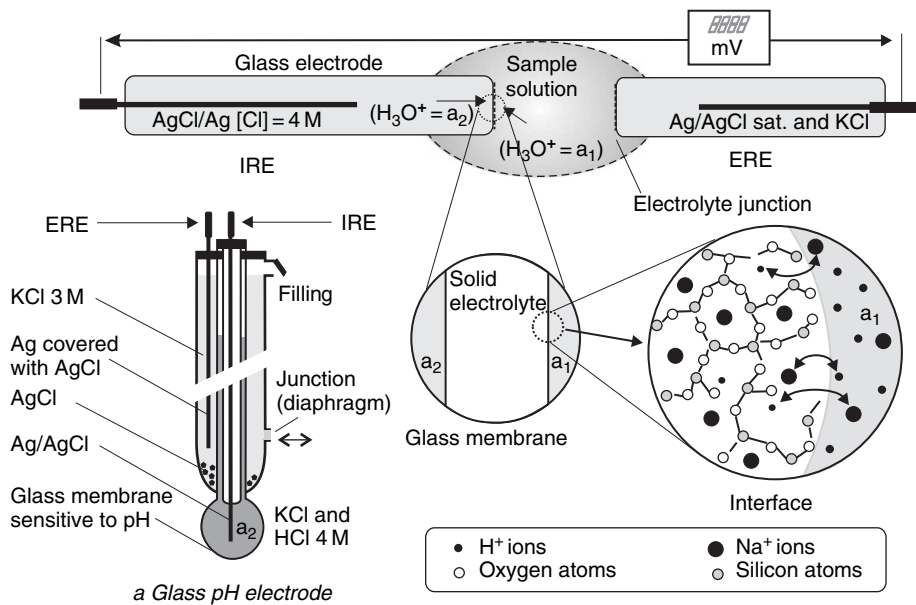


Figure 19.2 Glass electrode for measuring pH . The concentration of H^+ ions is accessible through the potential difference which appears between the glass electrode and the external reference electrode (here an Ag/AgCl electrode). Above, a cross-section of the membrane permeable to H^+ ions. When an H^+ ion forms a silanol bond, a sodium ion moves into solution to preserve electroneutrality of the membrane. Two processes occur: ion-exchange and diffusion. Below left, a cross-section of a combined electrode in a concentric arrangement, where the reference electrode surrounds the glass electrode. The junction permits the migration of ions since the liquids from each side should not mix. Prior to its use, the pH -meter is calibrated with a buffer solution of known pH .

in solution, i.e. its pH. This later is determined by an electronic millivoltmeter, the pH-meter, which monitors the PD between the glass electrode and an external reference electrode ERE. The instrument, after calibration, calculates directly the pH of the solution. For environmental reasons Ag/AgCl electrode is preferred over that of calomel (mercurous chloride/Hg).

19.3 Other ion selective electrodes

During the early days of potentiometry, it was noted that interferences could occur and affect pH value, depending upon the composition of the glass used for the membrane, principally if other alkaline ions were present besides sodium ion. This is known as the *alkaline error*, which occurs for pH above 13. This led to the development of glasses that could respond selectively to monovalent ions such as Na^+ , K^+ , Li^+ and Ag^+ . Selectivity depends on the material that serves to ensure contact between the solution to be analysed and the inside of the electrode, but a membrane truly selective for a single type of an ion does not exist. In all cases an ionic material is required that will permit the establishment of concentration equilibrium for a specific ion. A PD will appear if the activity (or the concentration) is different between the two faces of the membrane. Measurement involves only the free ions in the solution. Standard reference half-cells contain KCl as an electrolyte, which normally oozes through a diaphragm to ensure the junction with the analyte solution. To avoid perturbations when measuring K^+ or Cl^- , an electrolyte gel or a second non-interfering electrolyte (Figure 19.3) is employed.

About 20 ISEs are used and classified according to the nature of the membrane. They serve either in direct ionometry, or as indicator electrodes for measurements involving both titrimetry and complexometry performed with the aid of automatic titrators.

19.3.1 Single crystal and solid-state membrane electrodes

The fluoride electrode is the best known of the ISEs. Its sensitive membrane is a mono-crystal of lanthanum trifluoride doped with a europium salt, which allows the fluorine atoms to migrate within the crystalline network (Figure 19.3). The OH^- ion can cause interference with the F^- ions at alkaline pH.

Crystalline powders agglomerated under pressure or with a suitable inert binder (measurements of Cl^- , Br^- , I^- , Pb^{2+} , Ag^+ and CN^-) provide other mineral membranes. The internal electrolyte can be eliminated (by dry contact). However it is preferable to insert a polymer layer with a mixed-type conductivity to ensure the passage of electrons from the ionic conductivity membrane towards the electronic conductivity electrode (Figure 19.3).

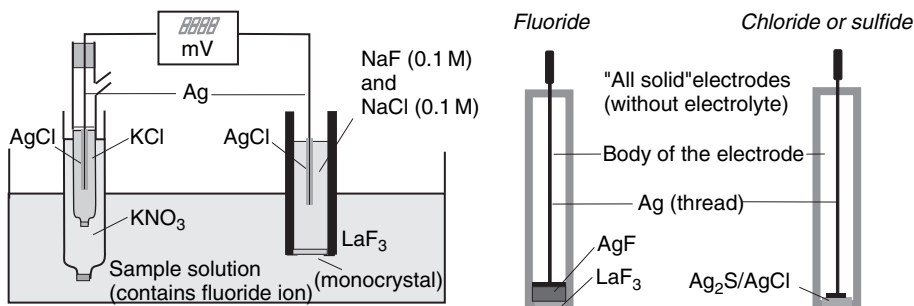


Figure 19.3 *Measurement set-up of ion-specific electrodes.* Schematic of a measuring cell with a ISE targeted for the fluoride ion using a double junction reference electrode. From the two sides of the membrane two equilibria are produced, $\text{LaF}_3 \rightarrow \text{LaF}_2^+ + \text{F}^-$. To avoid osmosis of KCl into the solution, the reference electrode is surrounded by a separate chamber which contains an auxiliary non-interfering electrolyte. KNO_3 1M difficult to oxidize or to reduce, is often used for F^- , Cl^- , I^- , CN^- , S^{2-} or Ag^+ . The measurement requires a high impedance millivoltmeter (type pH-meter). Right, two examples of simple to construct 'coated wire electrodes'. A wire forms an ohmic contact with the membrane (ISE for fluorine with a LaF_3 crystal and ISE for chloride with frit).

19.3.2 Liquid membrane electrode

The separation between the internal and external solutions of the specific electrode is achieved using a porous and hydrophobic disc (of 3 mm diameter). This disc is saturated with an organic solvent that is immiscible with water on either side and contains also an ion-carrier called an *ionophore* (Figure 19.4). Hence, the membrane behaves as a lipophile *immobilized liquid*. The counter ion is a molecule

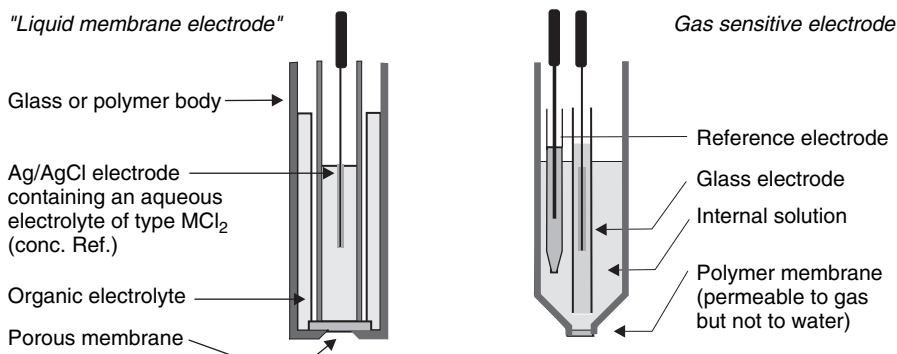


Figure 19.4 *Liquid membrane electrode and gas diffusion electrode.* The ionophore is able to complex ions reversibly and to transfer them through the membrane. The gas diffusion electrode is constructed in part from a pH electrode, which is plunged into an internal solution.

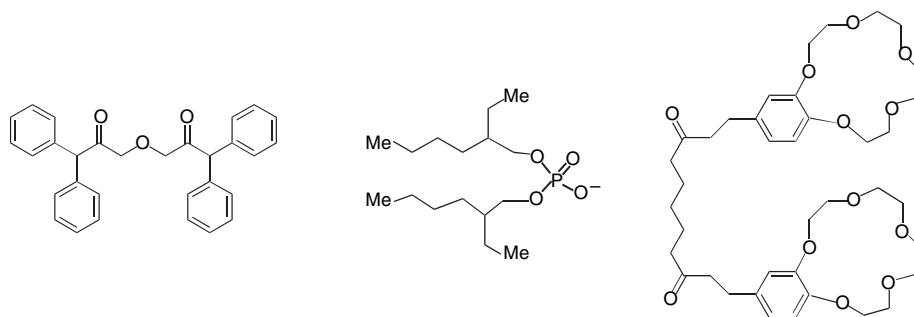
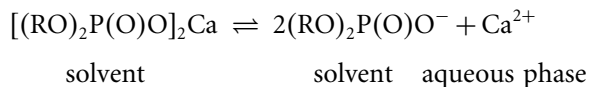


Figure 19.5 *Organic compounds used as ionophores.* From left to right, three commercial chelating ionophores: ETH 129 and the 'dioctyl' phosphate, selective for Ca^{2+} ions and right, the K2 ionophore which is selective for the ion K^+ . Crown ethers of particular sub-structure are frequently used. Some are charged (liquid exchangers), other are neutral.

soluble in the organic phase. The primary interaction between the ion in water and the lipophilic membrane is an extraction process.

For example, to measure calcium ions, an aliphatic diester of phosphonic acid $(\text{RO})_2\text{P}(\text{O})\text{O}^-$ (Figure 19.5) can be used. On each side of the wall a concentration equilibrium is established:



A measurable PD will appear if the concentrations of the calcium ions are different on the two sides of the membrane.

19.3.3 Polymeric membrane electrodes

Many commercial selective electrodes include a membrane made of a plasticized polyvinylchloride (PVC) or polysiloxane film in which are disseminated various ion-carriers, ionic or neutral (Figure 19.5). Around 40 chelate molecules are used for around 10 common ions. Electrodes for ClO_4^- , BF_4^- , NH_4^- , Ca^{2+} are of this type.

19.3.4 Gas-sensing electrodes

Combined electrodes have been developed to measure dissolved gases such as carbon dioxide (CO_2), ammonia (NH_3) and sulfur dioxide (SO_2). These chemical sensors contain an internal solution that is isolated from the sample solution by an hydrophobic membrane which is permeable to dissolved gas molecules

(Figure 19.4). For the three gases given above, the selective electrodes include a glass electrode in contact with a bicarbonate solution of low concentration (0.01 M). The bicarbonate solution is separated from the sample solution by a polymer membrane that allows diffusion of the gas analyte towards the inner electrode compartment.

This diffusion in the bicarbonate brings about a change in pH in the proximity of the membrane. For other gases, such as hydrogen cyanide (HCN), fluorhydric acid (HF) or hydrogensulfide (H₂S), the signal is generated by the modification of the concentration of anions close to the internal wall of the membrane.

■ The selectivity of an electrode is one of the most important characteristics. Among different methods that can be found in the literature the selectivity coefficient of an interfering ion may be defined by $K_{\text{specific ion/interfering ion}}$.

For example, $K_{\text{Br/Cl}} = 2.5 \times 10^{-3}$ indicates that the selectivity of the electrode for the bromide ion will be $1/2.5 \times 10^{-3}$, that is 400 times greater than for the chloride ion.

One other important factor is the response time, defined as the time between the instant at which the selective electrode is dipped in the sample solution and the first instant at which the potential has reached 90 per cent of the final value.

19.4 Slope and calculations

Several methods leading to the determination of the sample concentration C_i of an ionic species i exist. In the presence of a TISAB solution (see section 19.1), that can fix the pH, these methods are based on application of relation 19.3.

For a monovalent ion at 298 K, the *slope factor* S , that represents the term $2.303 RT/F$ is equal to 0.0591 V (the theoretical slope). This is also the value of the gradient factor per pH unit for a glass electrode.

$$E_{\text{cell}} = E'' + \frac{0.0591}{z} \log C_i \quad (19.4)$$

In practice the calibration curve $E_{\text{cell}} = f(C_i)$ using a set of standard solutions, leads to the real slope (i.e. the experimental value of the slope factor), which is an indicator of the performance of the electrode system used.

Table 19.1 Values of the ideal slopes at 298 K for a few ions

Valence	Ion	Slope S (mV/decade)
-2	S ⁻	-29.58
-1	Cl ⁻ , F ⁻	-59.16
+1	Na ⁺ , K ⁺	+59.16
+2	Mg ²⁺ , Ca ²⁺	+29.58

19.4.1 Direct ionometry

A series of standard solutions is prepared by successive dilutions of a stock solution. To each each solution, an excess but constant volume of the recommended ionic buffer (ISAB or TISAB) is added. Sample solutions are prepared in the same way. Next, for each of the standards, the PD across the electrodes is measured and a semi-logarithmic curve $E = f(C_i)$ is plotted (Figure 19.6). Finally, from this curve and the measured PD values for each of the sample solutions, obtained in the same manner, the concentration of the species i is obtained. The precision of measurements can be deduced from equation 19.5:

$$dE = \frac{RT}{zF} \cdot \frac{dC_i}{C_i} \quad \text{that is} \quad \frac{\Delta C_i}{C_i} = \Delta E \cdot \frac{zF}{RT} \quad (19.5)$$

19.4.2 Standard addition method

The following technique may be useful to eliminate the effects of the matrix. It requires only one standard solution and two measurements of potential. However, the volumes must be known with a great precision. First, the PD E_1 is measured

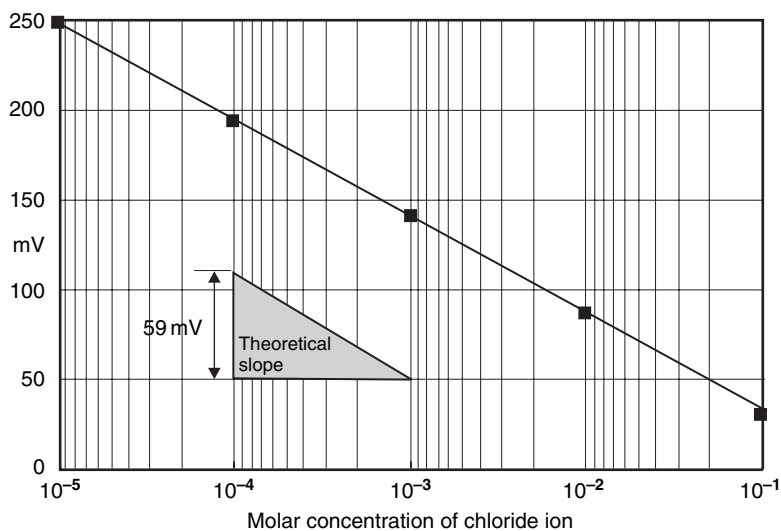


Figure 19.6 Typical calibration curve of an ISE by direct potentiometry. The calibration curve of the specific electrode for the chloride ion has almost an ideal slope value. The range of linear response for the different ISE extends over 4 to 6 orders of magnitude depending on the ion. Expression 19.5 leads to the estimation that an uncertainty of 0.2 mV on E leads to an inexactness of 0.8 per cent in the concentration (for a monovalent ion). Here, the TISAB consists of NaCl 1 M for adjusting the ionic force, a complexing agent for metals and a buffer mixture of acetic acid/sodium acetate.

while the two electrodes (ESI and ERE) are immersed in a known volume V_X of the sample solution of concentration C_X in analyte. After adding to the above solution a small volume V_R of a reference solution of concentration C_R in analyte, the measurement of E is redone (new value E_2).

The calculation, starting from expression 19.4, leads to a new expression giving C_X :

$$C_X = C_R \frac{V_R}{V_R + V_X} \cdot \frac{1}{\left(10^{\Delta E/S} - \frac{V_X}{V_R + V_X}\right)} \quad (19.6)$$

where S represents the actual slope for the electrode used ($0.0591/z$ at 298 K) and $\Delta E = E_2 - E_1$, the potential difference between the two measurements E_1 and E_2 .

■ Instead of introducing the reference solution, a volume V_X of the sample solution at concentration C_X can be added to a volume V_R of the reference solution at concentration C_R . This alternative is preferable when sample solutions are relatively concentrated. Using ΔE as above, it follows that:

$$C_X = C_R \frac{V_X + V_R}{V_X} \cdot \left(10^{\Delta E/S} - \frac{V_R}{V_X + V_R}\right) \quad (19.7)$$

19.4.3 Potentiometric titration

Selective electrodes have a very variable specificity and a limited scope. However, precision can be increased when they are used as indicating electrodes in potentiometric measurements.

The concentration of ions present in solution and the ionic strength vary slightly during the measurement, relative to the concentration of the ion to be measured. This property can be taken into account when two ionic species undergo a stoichiometric reaction. In such cases, the end point in the measurement is characterized by either the complete disappearance of one of the species, or by the appearance of an excess of one of the species. The appearance or the disappearance of a secondary species can also be useful to find the end point.

For example, to calculate the concentration of aluminium in solution, an ion for which no selective electrode exists, this analyte is transformed into insoluble aluminium fluoride AlF_3 by adding sodium fluoride. The end point of this reaction is determined by the presence of an excess of sodium fluoride ions in solution, easily detected by a fluoride selective electrode.

A great number of commercially available automatic titrators perform similar determinations.

■ *The lambda sensor*, which is found in all motor cars with catalytic converters, is an oxygen probe based on the principle of selective electrodes. This sensor, which looks like a spark plug, has a zirconium oxide (ZrO_2) membrane that behaves as a solid electrolyte. The external wall is in contact with the exhaust fumes while the internal wall (the reference) is in contact with air. Between these two walls a PD appears, collected by two electrodes, which is indicative of the difference in oxygen concentrations.

19.5 Applications

Potentiometric measurements are often used in routine analyses because they are simple to achieve and the detection limit of the electrodes are on the order of 10^{-5} – 10^{-6} M. However, the observed detection limit is governed by the presence of interfering ions or impurities.

Some routine applications of the method are:

- in agriculture, the analysis of nitrates in soil samples
- in foodstuffs, the analysis of ions such as NO_3^- , F^- , Br^- , Ca^{2+} , etc. in drinks, milk, meat, or fruit juices
- in industry, the analyses of chlorides in paper paste, cyanides in electrolysis baths, chlorides and fluorides in galvanic processes
- in clinical chemistry, the analysis of certain ions in serum and other biological fluids.

Problems

19.1 Consider an aqueous solution of ammonia, 9×10^{-3} M, to which is introduced a quantity of ammonium chloride in order to attain a pH of 9. At this precise moment, what is the concentration of the salt?

$$(K_B \text{ NH}_3 = 1.8 \times 10^{-5})$$

19.2 Explain how the electrode's glass membrane registers the pH of an aqueous solution.

19.3 What is the degree of dissociation and the pH of an aqueous solution of 0.85 M ethanoic acid? ($K_A \text{ CH}_3\text{CO}_2\text{H} = 1.8 \times 10^{-5}$ at 20°C .)

- 19.4 30 mL of an 10^{-3} M aqueous solution of an Fe^{3+} salt is mixed with 20 mL of a 10^{-3} M solution of a salt of Ti^{3+} . What will be the molar concentration of these salts following reaction in this modified solution? ($\text{Ti}^{3+}/\text{Ti}^{4+} = 0.2$ V vs. SHE; $\text{Fe}^{2+}/\text{Fe}^{3+} = 0.77$ V vs. SHE and $\text{SCE}/\text{SHE} = +0.25$ V).
- 19.5 Knowing that the standard potential of an electrode Cd/Cd^{2+} vs. SHE, has the value 0.404 V, what happens to this potential if the electrode is plunged into an aqueous solution of 0.01 M CdSO_4 ?
- 19.6 Find the expression 19.6 of Section 19.4.2 used in the standard addition method.

20

Voltammetric and coulometric methods

In contrast to potentiometry that operates under zero current conditions, other electroanalytical methods impose an external source of electricity to the sample solution, to induce an electrochemical reaction that would not otherwise spontaneously occur. It is thus possible to measure all sorts of ions or organic compounds that can either be reduced or oxidized electrochemically.

Polarography, the best known of all voltammetric methods, is still nowadays a competitive technique for the measurement of trace elements, ions or organic compounds, although its implementation is rather delicate.

Coulometry is the name given to a group of other techniques that determine an analyte by measuring the amount of electricity consumed in a redox reaction. There are two categories referred to as potentiostatic coulometry and amperostatic coulometry. The development of amperometric sensors, of which some are specific for chromatographic detection, open new areas of application for this battery of techniques. Combining coulometry with the well known Karl Fischer titration provides a reliable technique for the determination of low concentrations of water.

This chapter is an overview of the most important methods in this domain.

20.1 General principles

Voltammetric methods consist of applying a variable potential difference between a *reference electrode* (e.g. Ag/AgCl) and an indicator electrode, called the *working electrode*. On its surface a reaction of type $\text{Ox} + ne \rightarrow \text{Red}$ (or its reverse) is induced. When the potential at the working electrode reaches a value such that a species (the *depolarizer*) present in the solution being studied, is either oxidized or reduced, the current through the circuit outside to the cell increases sharply. In practice, in order that no current passes through the reference electrode, a third electrode called the *auxiliary electrode* (or *counter electrode*), made of an inert metal or carbon employed along with a support electrolyte is used to make the medium electrically conductive (Figure 20.1). The microelectrolysis that occurs

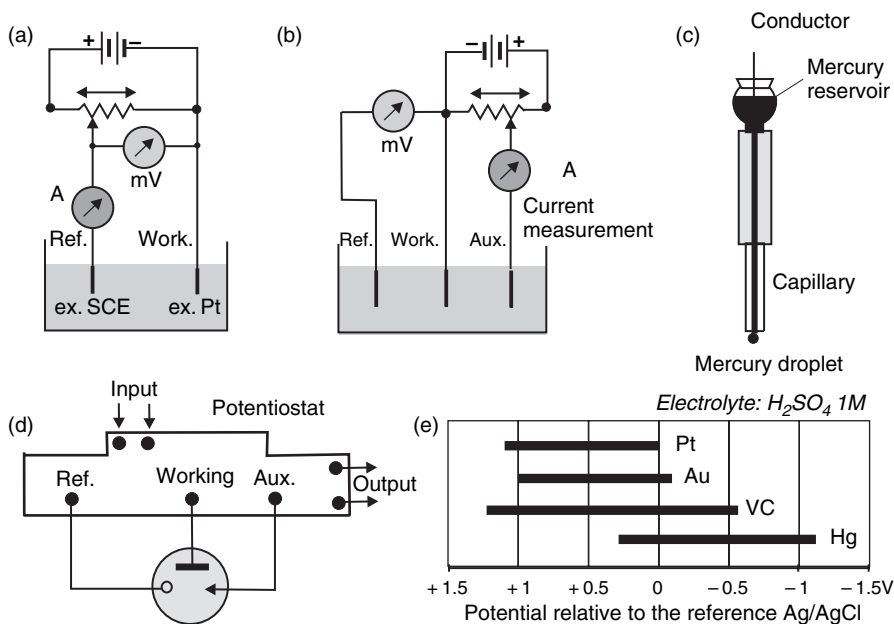


Figure 20.1 Schematics showing 2- and 3-electrode cells. (a) Circuit diagram with two electrodes that is not used in practice as this assembly has the major inconvenience that the current passes through the reference electrode which should be avoided because its potential will be modified; (b) DC set-up in which no current passes through the reference electrode (a consequence of its high impedance); (c) A model indicator electrode (hanging-drop mercury electrode). Many metals can be reduced at the surface of the mercury before H^+ is reduced in its turn (production of hydrogen gas); (d) Representation of a measuring cell controlled by a potentiostat; (e) Ranges of use for the four principal working electrodes in sulfuric acid 1 M as support electrolyte (VC stands for vitreous carbon). The mercury electrode can be used over a wide cathodic range with electrolytes such as KCl or NaOH (-2 V). Although overlaps are observed with different electrodes, their sensitivities can be quite different for a given species.

over a short period during measurements does not notably alter the concentration of analytes in the sample solution.

Briefly, a voltage scan over a range where oxidation or reduction of the analyte is expected, gives a *voltammogram* that is an S shaped curve displaying current vs. voltage, $I = f(E)$. This plot permits the identification and measurement of the concentrations of each species of interest.

The most widely used working electrodes are made of vitreous carbon, platinum, gold or mercury (Figure 20.1). They are flexible in the sense that they can be used between two potential values that depend on the support electrolyte, the pH and the reference electrode. These limits, for example, for a platinum electrode are $+0.65\text{ V}$ relative to the standard calomel reference electrode (SCE) (to avoid oxidation of water: $H_2O \rightarrow 1/2O_2 + 2H^+ + 2e^-$) and -0.45 V (to avoid reduction of water: $H_2O + 2e^- \rightarrow H_2 + 2OH^-$).

■ An electroanalytical voltammetric experiment necessitates a potentiostat. When used in the usual three electrode mode, this device controls the voltage across the working electrode/auxiliary electrode pair. The voltage is adjusted to maintain the potential difference between the working and reference electrodes. So the working electrode potential is maintained, even if a current flows. The cell output provides a measure of the current that flows between the working and auxiliary electrodes. When coupled with a suitable signal generator, it can be used to a variety of voltammetric methods.

20.2 The dropping-mercury electrode

The most original working electrode contains an active mercury micro-dropper. The voltammetric technique making use of this electrode is called *polarography*, invented by Heyrovsky in the 1920s.

This electrode is constituted of a glass tube, maintained in a vertical position, of which the central passageway (10–70 μm in diameter), allows the transfer of mercury between a reservoir and the capillary tip, where very small droplets are formed. This dropping-mercury electrode is immersed in an *unstirred* solution containing the target analyte mixed with a support electrolyte. The surface of the droplet of mercury increases until it falls (around 4–5 s). The fall is most often provoked by a device producing a small impact on the electrode. Instantaneously, a new droplet, identical to the previous one, is formed presenting a fresh uncontaminated surface.

This electrode, rather out of the ordinary, has some inconveniences. The mercury must be very pure (six-time distilled and conserved under nitrogen). Its vapours are poisonous, and after use it must be at least recovered and re-cycled.

20.3 Direct current polarography (DCP)

In the basic experiment – now rarely used – a linear changing time-dependant potential is applied to the mercury droplet, in the order of $v = 1\text{--}2 \text{ mV/s}$ from the initial potential E_i selected. So, it can be written:

$$E_{\text{working}} - E_{\text{ref}} = E_i \pm vt \quad (20.1)$$

The sign \pm of the equation 20.1 signifies that the sweeping can be performed either on the anodic ($V > 0$) or cathodic ($V < 0$) side. The resulting current voltage curve $I = f(E)$ is a *polarogram*, which can show one or several waves (Figure 20.2). The height of each step, corresponds to the *limiting diffusion current* i_D (cf. section 20.4) and is a function of the concentration of the depolarizer (the analyte, which is reduced on contact with mercury), which thus allows its

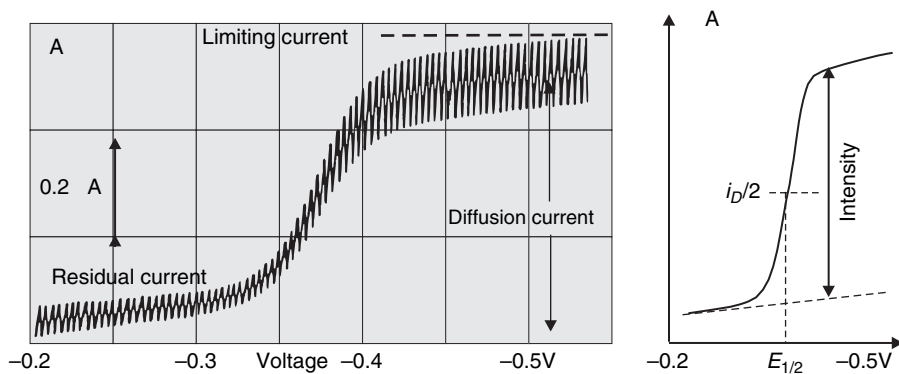


Figure 20.2 A polarographic wave. Polarogram of a solution containing 10 ppm of Pb^{2+} in KNO_3 0.1 M, obtained with a dropping-mercury electrode. The median position of the wave (about -0.35 V) is characteristic of lead while the height of the step, of its concentration. For a better presentation of the graph the oscillations have been damped. Right, a graph shows the measure of i_D . The residual current is due in part to impurities in the support electrolyte and to traces of oxygen.

quantitative determination. Each analyte is characterized by a *half-wave potential*, determined at the mid-height $i_D/2$, of its wave. As a result, several species present in a solution can be studied during the same analysis, on condition that their half-wave potentials are sufficiently different. The polarogram shows also a saw-toothed fine structure, which is produced from the continuous renewal of the mercury droplets.

The mercury is quickly limited at positive potentials ($+0.25\text{ V}$ with respect to the SCE) beyond which it is oxidized in its turn. Instead, for negative potentials it is usable up to -1.8 or -2.3 V depending upon whether the supporting electrolyte is acid or alkaline. This range offers many possibilities in analysis, particularly for the determination of heavy metals.

The elimination of all traces of dissolved oxygen in the solution by nitrogen bubbling is essential otherwise the polarogram would be inexploitable by effect of the reduction of oxygen in water according to the two steps detailed in Figure 20.6.

An advantage of voltammetry over AAS is the ability to distinguish an element in different valence states. For example, iron Fe^{2+} can be quantified apart from Fe^{3+} (cf. Figure 20.4). Some organic molecules are equally electroactive and can be analysed by this method.

20.4 Diffusion current

The particular features appearance of the polarographic wave can be explained as follows. Suppose that a sample solution contains a very much diluted lead salt (Pb^{2+}) in a support electrolyte whose concentration is much greater than that

of the Pb^{2+} . To the working electrode a negative linearly increasing voltage is applied from a starting value lower than required to reduce Pb^{2+} . At -0.35 V (vs. Ag/AgCl), the Pb^{2+} ions are reduced upon contact with the mercury droplet. K^+ ions, non-reduced at this potential, will form a shield around the drop preventing the normal migration of the other Pb^{2+} ions in the solution. However, under the effect of a concentration gradient, these Pb^{2+} ions will reach the surface of the electrode – where they will be reduced – only by diffusion across the layer of K^+ ions. This phenomenon is the basis of the *diffusion current*. The *average limiting value* of the diffusion current \bar{i}_D , is essentially due to the ion flux of the analyte considered at the instant that precedes the falling of the drop. It depends upon several parameters one of which is the *capillary constant*, $(m^{2/3} \cdot t^{1/6})$

$$\bar{i}_D = 607 \cdot n \cdot D^{1/2} \cdot m^{2/3} \cdot t^{1/6} \cdot C \quad (20.2)$$

\bar{i}_D is expressed in μA with the following units: D , diffusion coefficient of the analyte ion (cm^2/s); m , mass flow of mercury (mg/s); t , lifetime of the droplet (s); C , concentration of analyte (mmol/cm^3); n the number of electrons transferred during the electrolysis of the ion. The coefficient 607 is established for 25°C .

This expression – known as the Ilkovic equation – which takes into account several factors, is replaced by the simplified formula 20.3 where K includes parameters related to the method and instrument used. K is a constant for a given analysis.

$$\bar{i}_D = KC \quad (20.3)$$

Experimentally the current passing through the droplet is the result of several effects, amongst which are:

- *The capacitive current* which makes droplet/electrolyte interface analogous to a capacitor by fixation of electrons that face the ions of support electrolyte (e.g. K^+). This capacitive current diminishes very quickly with time because it depends upon the variation of the droplet surface area, which decreases as the droplet expands.
- *The diffusion current* (Faraday-type current) which decreases as $t^{1/2}$ if the surface of the drop does not vary, but increases taking account of the growing potential applied to the drop and its growing surface. This expansion of the droplet's surface more than compensates for the depletion of electroactive substance in close proximity to the electrode. The graph displaying the intensity has at last the appearance shown on Figure 20.3.

■ The *tast polarography* consists of measuring the current only for a very short period, late in the lifetime of the drop. This current sampling avoids the saw-toothed spikes on the current curve. Yet it leads to a reduction in sensitivity since the diffusion current decreases as the analyte flux moves from the bulk of the solution to the surface of the droplet (Figure 20.3).

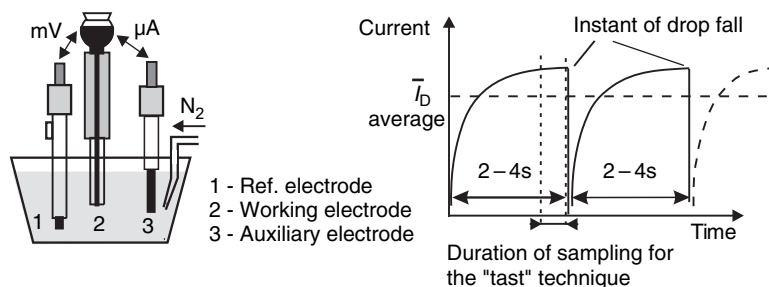


Figure 20.3 *Polarographic cell and diffusion current.* The solution must be free from dissolved oxygen which otherwise causes an interfering double wave (see Figure 20.6). On the right, graph of the diffusion current, growing over time for each drop of mercury in a static solution (unstirred solution). Direct polarography is a slow method of analysis. The recording of a voltammogram requires at least one hundred droplets. A calculation of i_D max is possible with the Ilkovic equation if the coefficient 607 is replaced by 706 in equation 20.2.

20.5 Pulsed polarography

To improve the sensitivity and to be able to distinguish the analytes for which the half-wave potentials differ by only a few tens of mV, the mercury drop is no longer submitted to a gradually increasing voltage but rather to a pulsed voltage. Two major modes of operation coexist.

20.5.1 Normal pulsed polarography (NPP)

To eliminate the jagged appearance of the classic polarogram the standard voltage ramp described earlier is replaced by a series of brief pulses (between 50 to 100 ms), of increasing potential. The pulses are delivered at the same frequency as the mercury drop renewal (between 2 and 4 s). Each pulse begins at the same voltage low enough to avoid the reduction reaction of the analyte (Figure 20.4). Electrolysis is thus blocked between each pulse. Current measurements are made just prior to the fall of the droplet. At this instant the Faraday diffusion current is stabilized while the capacitive current has become negligible. The sensitivity of the basic method here gains 2–3 orders of magnitude.

20.5.2 Differential pulsed polarography (DPP)

A pulse of 50 mV is applied during 50 ms to the working electrode for each successive droplet of mercury. The first measurement is made just before the pulse and the second just before the droplet falls (Figure 20.4). In this way, the

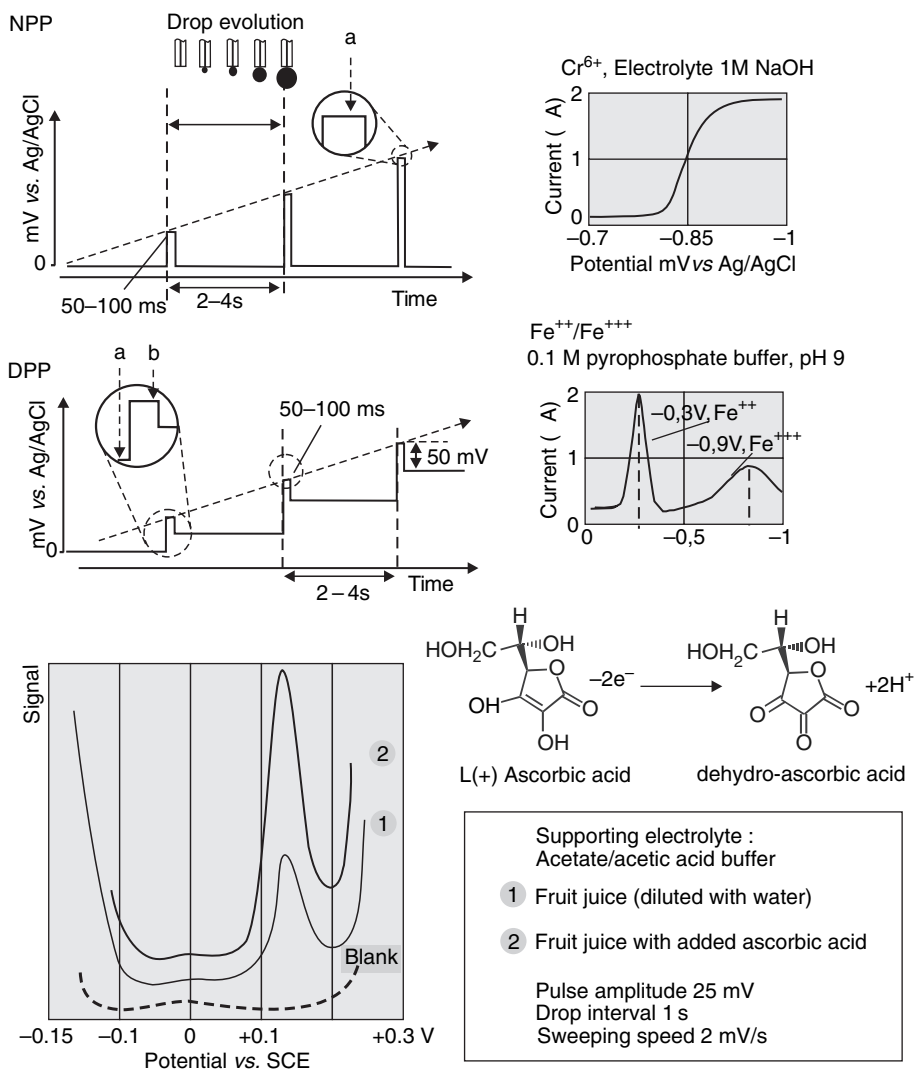


Figure 20.4 Pulsed polarography. NPP and DPP techniques. The diagram shows at which instants the measurements are made (see the arrows a and b). Examples of measurements. The measurement of ascorbic acid (vitamin C) in a fruit juice (see conditions) corresponds to an oxidation on the working electrode.

two currents are measured on the same drop but with a difference of 50 mV voltage. The graph of the difference of these currents, as a function of the voltage, exhibits a peak for each analyte, whose height is proportional to its concentration.

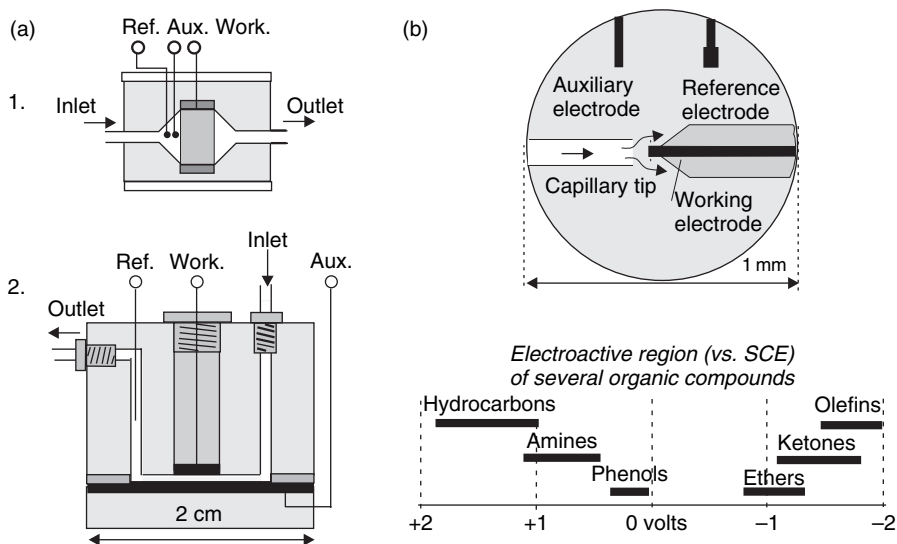


Figure 20.5 Amperometric detection in HPLC and HPCE. (a) Two models of detector cells. The porous graphite working electrode have a large surface and operates under coulometric conditions. The flow of the mobile phase at the working electrode ensures renewal of the electroactive species; (b) Detail of the end of a capillary in HPCE. The working electrode, placed on the cathodic side of the apparatus, is bathed by ions exiting the capillary. Apart from phenols, aromatic amines and thiols, few analytically important molecules are electroactive.

20.6 Amperometric detection in HPLC and HPCE

Voltammetry has been applied to a highly sensitive mode of detection for electroactive compounds in liquid chromatography (providing the mobile phase is conducting) and capillary electrophoresis. The voltammetric cell has to be miniaturized in order not to dilute the analytes after separation. The metal or carbon working microelectrode has a defined voltage with respect to the reference electrode (depending on the substance to be detected) and the detection cell is swept by the mobile phase as it leaves the column (Figure 20.5).

20.7 Amperometric sensors

Voltammetric measurements are not simply restricted to analytical laboratories. The applications of these methods are more numerous than is at first obvious. A large number of analytical instruments, whether portable or not, intended to make precise measurements of substrates present in gas mixtures, vapours or solutions are equipped with electrochemical sensors. These devices operate on the principle of the 2- or 3- electrode cell enclosed in the sensor housing.

20.7.1 Clark oxygen probe

The electrochemical reduction of oxygen in contact with an electrode provides the basis for several models of sensors, which differ only by the reactions used. The best known of these is without doubt Clark's oxygen sensor, which contains a silver anode and a platinum cathode (working electrode), which are both in contact with a KCl solution (Figure 20.6). This electrolyte is separated from the sample by a Teflon membrane that is porous to oxygen but impermeable to the electrolyte. After crossing this membrane the gas comes into contact with the cathode, about $10\ \mu\text{m}$ distant from the inner face of the membrane. A potential difference PD of around 1.5 V is applied between the two electrodes, the cathode being brought to a potential sufficiently negative to reduce all of the oxygen (reactions 1 and 2). The current in the circuit is proportional to the quantity of gas migrating across the membrane and consequently to its concentration in solution (Faraday's law).

The manufacture of electrochemical sensors has evolved considerably and their production has diversified. The three electrodes of the sensor shown in the example of Figure 20.7 result from a manufacturing technique characteristic to microelectronic circuits. Completed by a membrane and an electrolyte, this sensor is used to measure chloride dissolved in water. Based upon the oxidation of the ClO^- (hypochlorous acid) ion its detection limit is 1 ppb.

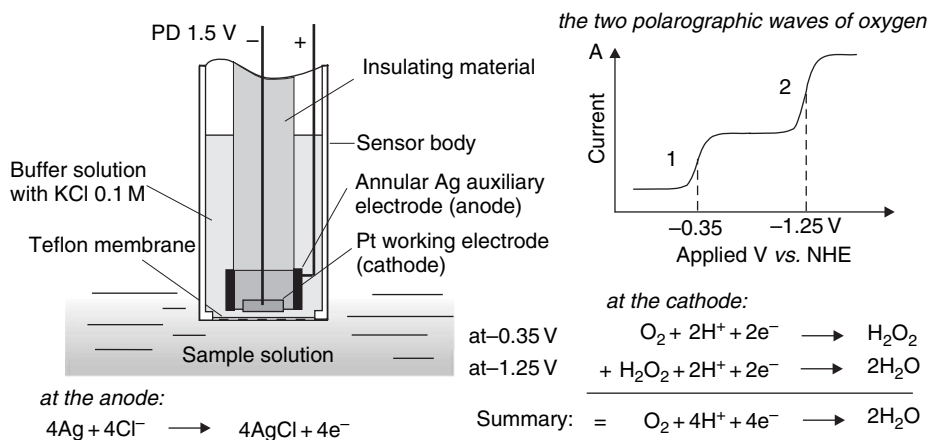


Figure 20.6 A Clark sensor for oxygen determination. Cell housing two concentric electrodes. The Teflon membrane, permeable to oxygen, must be very close to the cathode so that the double diffusion process through the membrane and subsequently the liquid film will lead to a stable signal after a few seconds.

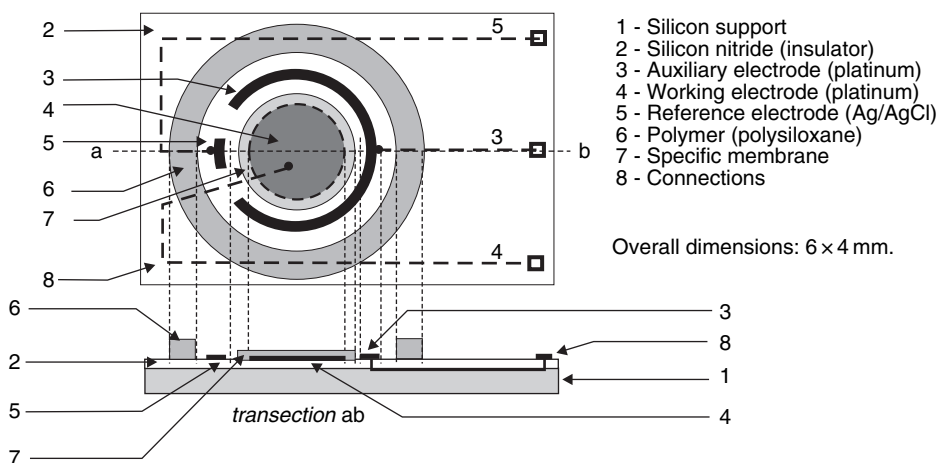


Figure 20.7 Sensor issued from a microlithography technique. Sensor for measuring chloride in aqueous media. Diagram representing a document from Microsens (CH). Sensor MAES-2402. In this diagram, the thicknesses of the circuits are not to scale. This assembly is not complete since it lacks certain elements of the sensor (electrolyte, connections).

20.7.2 Other amperometric gas sensors (AGS)

Many toxic contaminants (SO_2 , NH_3 , PH_3 , $\text{CO} \dots$) can be measured by means of substance-specific electrochemical sensors designed from the classic system of two or three electrodes already discussed. The corresponding instruments are mostly used for a variety of hazardous and confined space applications (Figure 20.8). Being sensitive and having a rapid response time, when the working electrode is covered by a catalyst, they can be used over a wide range of concentrations (4 orders of magnitude). In contrast they function over a restricted temperature range ($-10/+40^\circ\text{C}$), requiring re-standardizing. Their lifetime is normally of a few years, depending of stocking conditions.

When the AGS is exposed to a gaseous mixture, the electroactive species for which it was designed reaches the working electrode where a redox reaction occurs. For example, an oxidation reaction results in flow of electrons from the working electrode to the auxiliary electrode through the external circuit. This flow of electrons constitutes an electric current, which is proportional to the gas concentration. The potentiostat can modify the electrode potentials, allowing different analytes to be measured on condition that the detector is changed.

The H_2S AGS described below corresponds to a typical arrangement (Figure 20.9). The main parts of this sensor consist of three porous noble metal

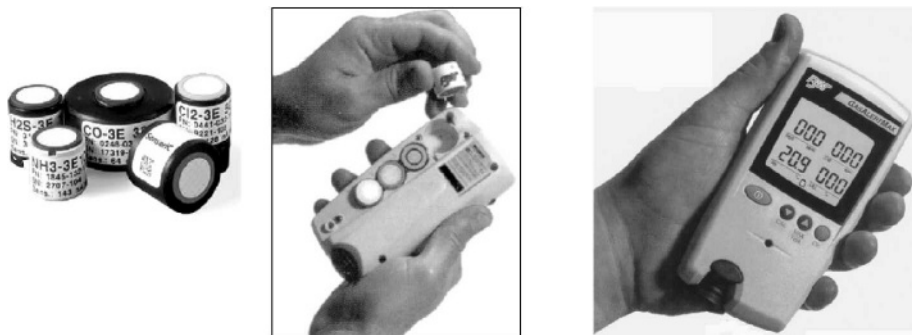
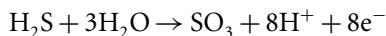


Figure 20.8 *Amperometric gas sensors.* Assortment of sensors from ATMI Sensoric Div. (Ger.). Model GasAlertMax3-DL from BW Technologies (Can); a view shows a model including four specific detectors. Flowing across a specific diffusion barrier, the gases are oxidized (CO, H₂S, NO, H₂, HCN) or reduced (Cl₂, NO₂) in the presence of an electrolyte. This electrolyte is progressively contaminated with by-products, and like non-rechargeable batteries, their life is limited.

electrodes and an acidic aqueous electrolyte, housed within a plastic enclosure. The reference electrode is totally immersed in the electrolyte. It remains at the same potential, and is used to regulate the potential of the working electrode even if a current is generated during operation. Gas enters the cell via a capillary followed by a charcoal filter that removes unwanted interfering gases. Any hydrogen sulfide present undergoes the following oxidation reaction:



The SO₃ generated either escapes from the capillary or dissolves in electrolyte solution to form sulfuric acid. Hydrogen ions migrate within the cell, and finally give rise to water using oxygen from the surrounding atmosphere. The electrons consumed in this reaction are supplied by the external circuit. By connecting the working and the auxiliary electrodes, the current (a few nA) generated between them is measured as directly proportional to the concentration of H₂S in the air. This very weak current gives explanation of the rather long life of many AGS devices: 10 nA for 1 year corresponds to only 0.3 C, a insignificant quantity of electricity for an important electrolysis.

Two other examples on the figure relate the reactions which occur in AGS for carbon monoxide (CO) and for oxygen (O₂). The electrolytes are, for CO, a strong mineral acid such as sulfuric acid, while for O₂ it consists in a weakly alkaline solution with potassium acetate. These liquid electrolytes are immobilized by absorbent materials.

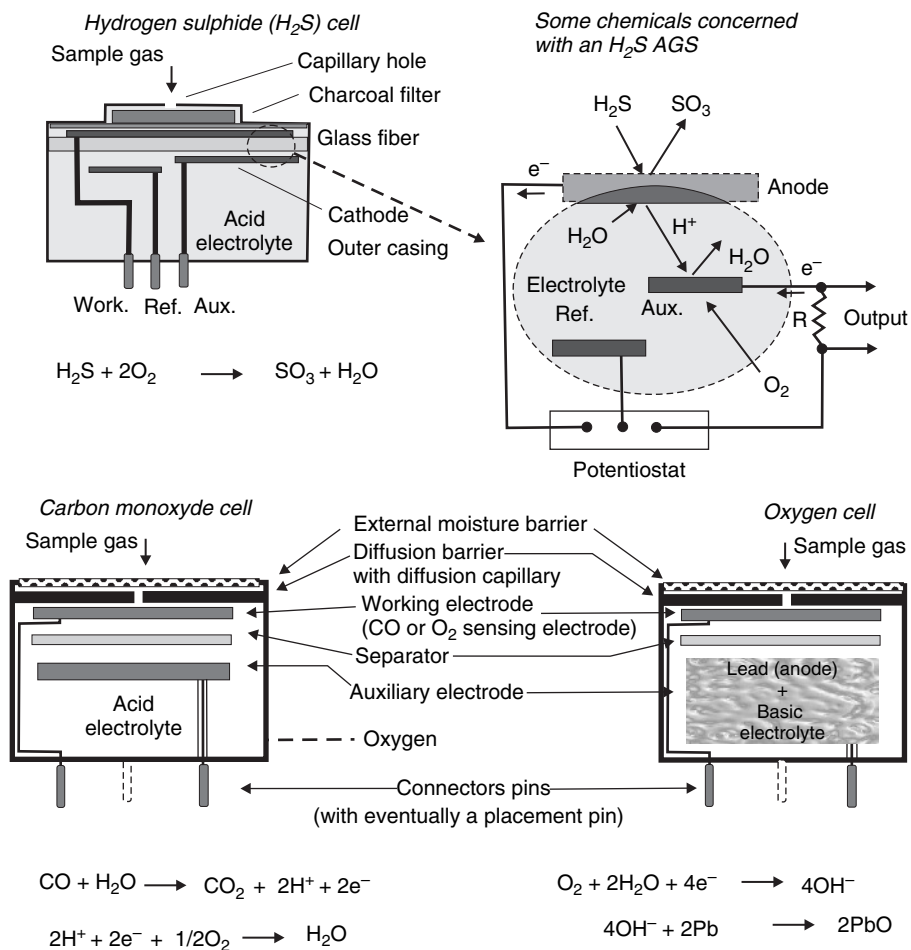


Figure 20.9 AGS, principles of operation. Cells for hydrogen sulfide, for carbon monoxide and for oxygen. For hydrogen sulfide sensor, the reference electrode helps to extend the working range of the sensor and improves the linearity of response. The electrochemical reactions consume the species present in the cell. This can be the anode itself (cell for oxygen), the electrolyte or even a reagent which should be present (oxygen is necessary for the cell detecting carbon monoxide).

20.7.3 Biosensors employing amperometric detection

A *biosensor* contains a compound of biological origin (enzyme, antibody) included in a device holding the electrodes in order to convert a biological signal (e.g. fixation of the antigen to the antibody) to an electric signal.

To make the biosensor specific for an analyte, a membrane is generally used that contains a recognition layer trapping the specific enzyme that catalyses the

transformation of the analyte. This could be a sandwich system in which the enzyme is immobilized between an external cellulose acetate membrane to prevent the passage of heavy molecules and an internal polycarbonate membrane that allows passage of the transformed product to the electrode.

In this area, for insulin-dependent diabetes, there is a miniaturized device for measurement of glucose in blood which uses to assure electron transport *glucose oxydase* (GOX) and either oxygen or a mediator such as quinone or a conducting polymer (Figure 20.10).

For this measurement successive generations of biosensors have adopted the same principle; The mechanism of operation is as follows : glucose is oxidized to gluconolactone, the key reaction, by the oxidized form of glucose oxidase (GOX) an enzyme produced by *Aspergillus niger*. This enzyme contains a flavin-based prosthetic site, which serves as a provisional trap for electrons, by passing from its oxidized form (flavin adenine dinucleotide, FAD) to its reduced form (FADH₂). Two protons and two electrons are transferred to the enzyme as it passes to its reduced form. To return to its initial oxidized state FADH₂ reacts with either oxygen with formation of a molecule of hydrogen peroxide (first generation), or with an oxidant (called a mediator, e.g. quinone), that is easy to mix with the enzyme of known concentration (second generation). This second procedure improves the measurement selectivity.

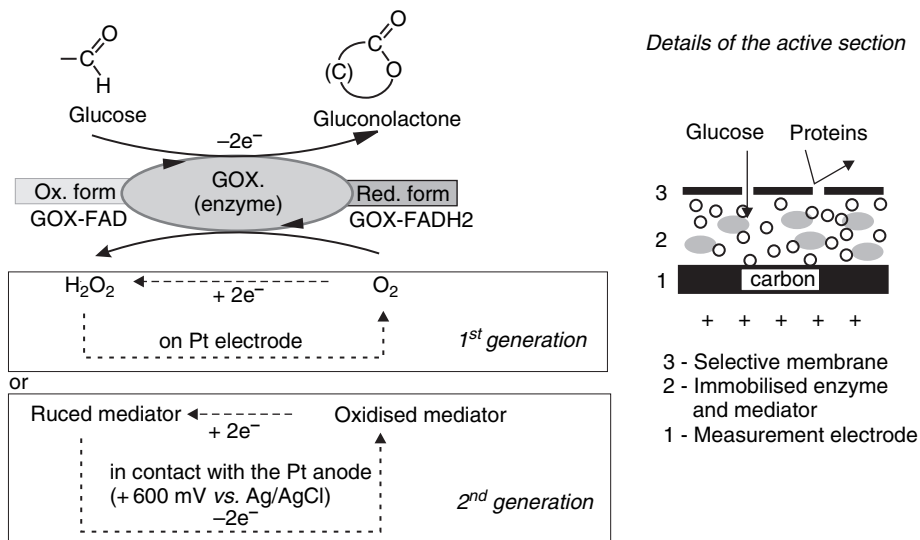


Figure 20.10 Amperometric measurement of glucose. Left, reactions cycle in first and second generation devices. Right, a sandwich-type biosensor involving glucose oxidase. O₂, or a mediator, are necessary because they are small molecules that may reach the prosthetic group of the enzyme, liberating hydrogen peroxide that diffuses to the surface of the platinum electrode. The mediator could equally be a redox system based upon osmium immobilized in a polymer. The reaction can be followed by monitoring oxygen consumption or H₂O₂ formation.

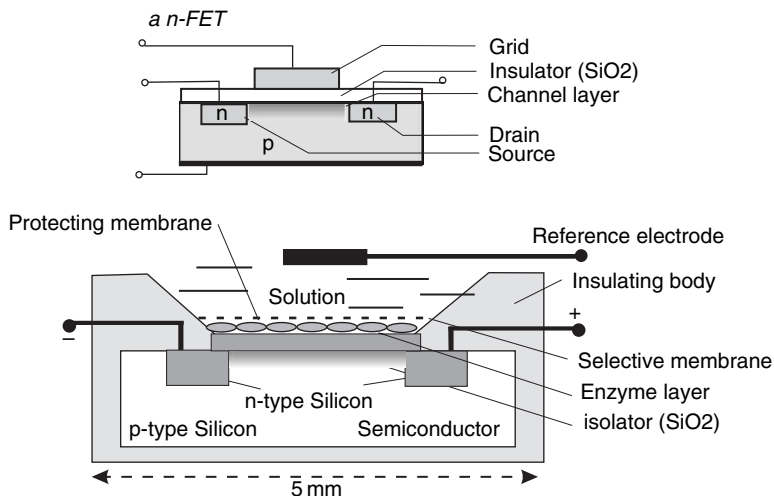


Figure 20.11 A selective electrode based on the principle of the field effect transistor. By placing the enzyme in contact with the electrode, it is possible to follow a specific reaction. The surface of silicon (sites of silanol functionality) is particularly sensitive to H^+ ions.

The amperometric sensors utilize reactions occurring on contact with the working electrode. This category of electrochemical sensor is large. Other forms of detection are met : a urease may be used to transform urea into ammonium ions which will be detected by a selective electrode for those ions, or a penicillinase which will destroy penicillin releasing H^+ detected by a pH electrode.

■ A particular type of biosensor is based upon the principle of the field effect transistor (FET) in which the semi-conducting layer is in contact with a membrane incorporating an enzyme adapted to transform a particular analyte (Figure 20.11). The chemical reaction in contact with the enzyme will modify the polarity of the insulating layer. This in turn will modify the conduction between the source and the collector (drain) of this transistor. The current between these two electrodes serves as the signal.

20.8 Stripping voltammetry (SV)

Two very sensitive methods, called anodic and cathodic stripping voltammetry (SV), are used to measure trace of metal. They are carried out in two stages:

- *Preconcentration.* A fraction of the target electroactive analyte is deposited, in a few minutes, on an electrode held at a constant potential and immersed in a stirred solution of the sample. This electrode can be a hanging mercury

droplet (non-renewed) or a cylinder of vitreous carbon covered by a mercury film or a rotating carbon disc with addition of a mercury salt in the solution. In all cases the surface of the electrode gives an amalgam $M(Hg)$ with metallic analytes M^{n+} .

- *Re-dissolving*. Following electro-deposition, mixing of the solution is stopped and the potential difference is progressively reduced between the working and reference electrodes. Due to reversibility of redox reactions, it appears an electrochemical oxidation of the analyte which is redissolved. The elements are identified by their oxidation potential.

The example given on Figure 20.12 corresponds to anodic voltammetric analysis. When combined with the technique of DPP (Section 20.5.2), stripping voltammetry constitutes the most universally applied voltammetric method. Current instruments for which control of all the parameters is established, allow reproducible conditions and offer an alternative to spectroscopic methods such as atomic emission.

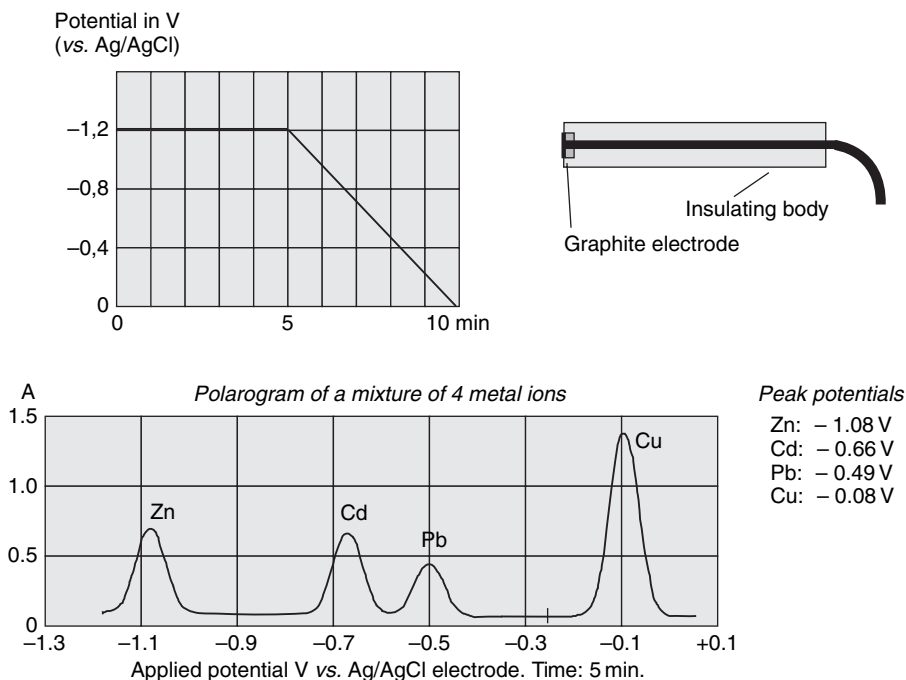


Figure 20.12 *Stripping voltammetry*. Linear voltage programming of the working electrode. Example of an electrode. Analysis of four metals present in a sample of sea water by DPP.

20.9 Potentiostatic coulometry and amperometric coulometry

The methods described previously correspond to the partial electrolysis of the analytes in contact with the working microelectrode. By contrast coulometric methods are based upon stoichiometrical relationships (quantitative conversion of the analytes). The concentration of an analyte is calculated from the quantity of electricity, using the equation $Q = it$. Standardization or calibration curves are not required. This is not therefore a comparative method.

Two types of coulometric methods co-exist:

- The *potentiostatic coulometry*, involves holding the electric potential (the voltage) of the working electrode constant during the reaction. This avoids parasitic reactions. The current decreases as fast as the analyte disappears from the solution. A galvanostat (or amperostat) is used to compute the quantity of current used.
- The *coulometric titration* or *amperostatic coulometry* method in which the current is maintained constant during the measurement by an amperostat until the signal which marks the end of the reaction with the analyte. This is the most simple form of coulometric measurement.

Both techniques take place within an electrochemical cell. A coulometric titrator equipped with a working electrode of large surface area and an auxiliary electrode separated from the reaction compartment by a diaphragm. This protection of the second electrode provides a barrier between the species formed in contact with it and those formed at the working electrode thus avoiding an eventual reaction (see Figure 20.14).

Often, direct and total transformation of the analyte proves difficult because the product formed accumulates in contact with the electrode, causing a polarization. This problem is countered by addition of a precursor in large excess, which is employed for the liberation of an intermediate reagent that will react with all the analyte. In this way the compound being analysed does not participate directly in the electron transfer procedure. This is the case in the coulometric adaptation of the Karl Fischer determination of water, described below.

■ Thus, the measurement of Cl^- ions will be possible from Ag^+ ions generated from the surface of a silver electrode. The quantity of current used, in coulombs, leads to a precise calculation of the quantity of analyte transformed on condition that the current serves only for the formation of ions Ag^+ . Standard solutions are not required for these measurements. The absolute quantity of ions formed is determined from the current consumed. The quantity of current $Q(C) = i(A) \cdot t(s)$ corresponds to a single analyte.

20.10 Coulometric titration of water by the Karl Fischer reaction

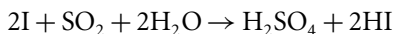
Many manufactured products, as well as solvents and raw materials are analyzed for their water content (percent humidity). Of all the available methods, the Karl Fischer titration is perhaps the most widely used, accounting more than 500 000 determinations performed daily world-wide.

The Karl Fischer method is applied in a multitude of substances from finished products (butter, cheese, dried milk sugar, etc.) to solvents and other industrial products (paper, gas, petroleum, plastic films, etc.). Solids and not soluble samples must, prior to the measurement, either be ground into powders, extracted with anhydrous solvents, eliminated as azeotropes or heated to evaporate the water in special accessories. The only difficult cases are encountered with strongly acidic or basic media since they denature reactants as well as ketones and aldehydes which perturb the titration through formation of acetals (special reagents must be used for these instances).

This method of titration may be sub-divided into two main techniques: volumetric titration making use of a titrator (a potentiograph) with end point detection, and coulometric titration making use of a coulometer, more sensitive for measurement of very low levels of water (concentrations in the order or below 1 mg/L).

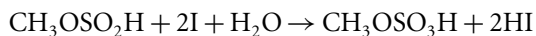
20.10.1 Reactions involved

In the presence of water, iodine reacts with sulfur dioxide through a redox reaction that is specific to these three compounds:



This quantitative reaction can be used to determine water and can be achieved if, for example, a base is added to neutralize the acids formed – for this reason, Karl Fischer used pyridine.

Since iodine is a solid and sulfur dioxide is a gas, an auxiliary polar solvent is used which serves as both a diluent and as a reaction medium. Generally, methanol is used, though in rare cases the methylmonoether glycol, or diethylene glycol, are also used. Under these conditions sulfur dioxide is not simply dissolved in the solvent but interacts with it, leading with methanol to methylhydrogenosulfite. This latter becomes the species that reacts with the iodine in the presence of water.



In contrast to the first reaction, in the presence of methanol, a *single* molecule of water reacts to convert *two* atoms of iodine. Methylhydrogenosulfite is oxidized to

hydrogenosulfate, converted in the presence of a base of type RN into an ammonium salt. The Karl Fischer reaction can therefore be reformulated as follows:



With pyridine as a base, a pyridinium salt $\text{C}_5\text{H}_5\text{NH}^+(\text{CH}_3\text{OSO}_3)^-$ is formed. This base, with its highly disagreeable odour, is now replaced by imidazole or diethanolamine, both odourless and leading to more stable commercial reagents.

Because atmospheric humidity must be avoided, the reaction cell is isolated from the atmosphere by drying tubes. Moreover, since the diluting solvent is rarely anhydrous, owing to its hygroscopic nature, its water content must be determined prior to the measurement. The end point of the reaction is detected by the variation of an alternate current intensity that passes between two small polarized platinum electrodes inserted in the reaction medium (Figure 20.13).

When using a titrator, the *Karl Fischer reagent* (KF reagent) is a mixture of sulfur dioxide, iodine and the base, characterized by the *number of mg of water* that can be neutralized by 1 mL of this reagent. This is referred to as the equivalent mass concentration of water, or the Titer *T* of the reagent.

In the classical method, two steps are involved in the analysis. First, the water equivalence of the KF reagent (Titer *T*) is determined. Then, the water contained in the sample is measured. As for many volumetric measurements it is possible to follow either a method of direct titration or back titration. In the direct titration the end point is reached by gradual addition of the KF reagent. By back titration, an excess of this reagent is introduced, then the excess is measured using a non-anhydrous solvent for which the water equivalence has been determined by pre-titration and which has subsequently been placed in the burette.

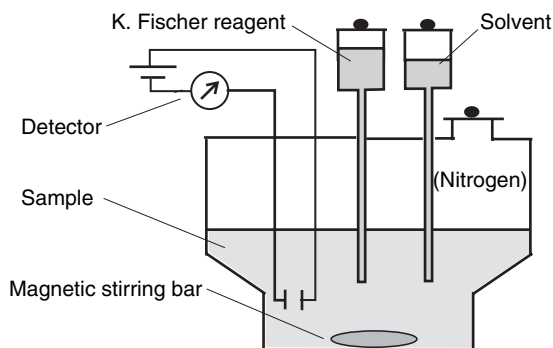


Figure 20.13 *Measurement of water by the Karl Fischer volumetric technique.* The titration cell contains volumetric burettes (which may be automated) and two small platinum electrodes. As long as no iodide is present in solution the electrodes remain polarized and only a weak current circulates between them. Once the equivalence point is reached, an excess of iodine will cause the depolarization of the electrodes and a significant current is registered.

■ Water standards generally consist of a precision-weighed mass of a solid hydrate of known composition: oxalic acid at $2\text{H}_2\text{O}$ (28.57 per cent in water), or tartrate of sodium at $2\text{H}_2\text{O}$ (15.66 per cent in water).

20.10.2 Coulometric adaptation of the Karl Fischer reaction

To obtain results of acceptable precision from the volumetric technique, at least 10 mg of water must be found in the sample, given the magnitude of the equivalent water Titer T of the commercially available KF reagent (several mg/mL). When the amount of water is lower (as low as $10\ \mu\text{g}$ of water) the coulometric version offers many advantages.

The coulometric technique is based on the same chemistry but its fundamental difference is the way by which the iodine is introduced into the titration cell (Figure 20.14).

The iodine necessary for reaction is generated from a precursor by applying electrical pulses to the electrode. Thus, the modified KF reagent contains an iodide (the precursor) which is oxidized to iodine in contact with the working anode ($2\text{I}^- \rightarrow 2\text{I} + 2\text{e}^-$). According to the stoichiometry of the reaction, and combining this with the faraday value, 1 mg of water corresponds to 10.72 coulombs. It is therefore possible to directly determine the amount of water present in a sample by measuring the electrode current in coulombs instead to measure the volume of KF reagent as in the volumetric technique.

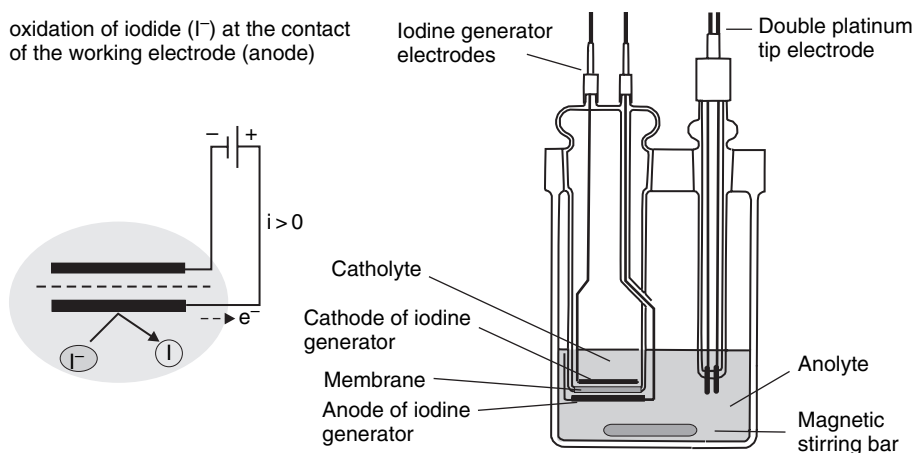


Figure 20.14 Karl Fischer two-component coulometric cell. Model DL-37 reproduced courtesy of Mettler Toledo. The instrument generates iodine by an electrolysis current applied in pulses. The duration of these pulses is reduced as the titration end point is approached. The diaphragm is used to avoid oxidation of the reduced ions appearing at the surface of the cathode. Some models exist without a diaphragm. They are easier to clean but they cannot solve all applications.

Commercial coulometers display the titration results as micrograms of water or directly in ppm or percentage water.

Similar to all other KF reagents, coulometric reagents deteriorate with temperature and light. This is why this reagent is commercially available from the manufacturers in two distinct bottles: one containing a mixture of sulfur dioxide, methanol and base, the other containing an iodide in solution. The contents are mixed prior to use.

Problems

- 20.1
1. Polarography is considered, so far as the sample is concerned, as a non-destructive method of analysis. Is this true?
 2. Why is stirring of the solution avoided in polarography?
 3. The method of standard additions is considered as yielding more reliable results than that of standard solutions. Why?
 4. Why, for the dropping mercury electrode, does the height of the column of Hg have an influence upon the value of the current of diffusion? Upon what is this effect based?
- 20.2
- In a polarography experiment, an aqueous solution containing a concentration of 10^{-3} M $\text{Zn}(\text{NO}_3)_2$ employs 0.1 M KCl as an electrolyte medium. Calculate the ratio of the intensities of migration and diffusion for the Zn ion in the proximity of the cathode.
- Ionic mobilities $\text{Zn}^{++} = 5.5 \times 10^{-8}$, $\text{NO}_3^- = 7.4 \times 10^{-8}$, $\text{K}^+ = 7.6 \times 10^{-8}$ and $\text{Cl}^- = 7.9 \times 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$.
- 20.3
- A dropping mercury electrode is regulated such that one drop falls every 4 seconds ($t = 4\text{s}$). The mass of the Hg corresponding to 20 drops is of 0.16 g. Calculate the average mass flow of Hg in mg/s.
- If the flow is proportional to the height of mercury in the reservoir, what would happen to the lifetime of the drops if the height of Hg was increased three-fold?

- 20.4 Calculate the average current of diffusion \bar{i}_D for the ion Pb^{2+} present as a concentration of 10^{-3} M in 0.1 M KCl aq. at 0°C .
Given: $m = 2.10^{-3}$ g/s, $t = 4$ s, $D = 8.67 \times 10^{-6}$ $\text{cm}^2 \text{s}^{-1}$.
- 20.5 A solution of 1 M KCl, employed as an electrolyte support, is ready to use and contains 0.0001% of Zn. Is this solution richer in zinc than one of 1 M which is prepared by using crystallised KCl for which the content in Zn is of 0.0005%?
- 20.6 A sample of 25 mL prepared for an electrolysis experiment has a zinc concentration of approximately 2×10^{-8} M which leads to the passage of a current of 1.5 nA. Calculate the time necessary to deposit 3% of the Zn present.
Show that the technique of stripping is more sensitive.
- 20.7 In order to measure the quantity of water in powdered milk by the method of Karl Fischer method, the reactant is first standardised as follows:
15 mL of a solvent comprising methanol and formamide is introduced into a cell. 3 mL of KF reactant is added to attain the equivalence point.
Then, 205 mg of oxalic acid dihydrate is introduced. A further aliquot (13 mol) of KF reagent must next be added to attain a new equivalence point. Finally 10 mL of the same solvent containing 1.05 g of dissolved powdered milk is introduced. Again KF reagent (12 mL) is added to find a final equivalence point.
Calculate the % mass of water in the powdered milk.
- 20.8 Calculate the % mass of water in a portion of anhydrous ether knowing that 1 coulomb is required to attain the equivalence point from 1 mL of ether during Karl Fischer measurements (coulometric version). Recall that two atoms of iodide per molecule of H_2O are needed and that the density of ether = 0.78 g/mL.
- 20.9 Determine the % impoverishment of a solution of 20 mL, 1×10^{-3} M zinc nitrate during a measurement of five minutes by polarography. The strength of the current in the circuit is $15 \mu\text{A}$.

21

Sample preparation

For all chemical analyses, the analyte, which refers to the species to be measured, must be in a sufficient quantity and suitable form for the instrument used. The majority of samples require a specific pretreatment. This preliminary stage, which conditions instrument calibration and follows the so-called sampling procedure, has long been the 'bottleneck' in the analytical process. It is often a critical step in a chemical analysis, because it has an influence upon the end result. Sample preparation is therefore an essential step in analysis, as important as the measurement itself, greatly influencing its reliability, its accuracy and its cost. Since trace analyses (less than 1 ppm of analyte in a crude sample), now represent at least half of all analyses being performed, then powerful methods of extraction are required. The particular mode of enrichment is determined by the instrument or the methodology used. This domain is currently very actively studied and benefits from recent knowledge in robotics to shorten the time of preparation. Moreover, the large number of analyses of very small samples has stimulated the development of state-of-the-art equipment and up-to-date procedures, some of which are described in this chapter, dedicated to samples intended for chromatographic analysis.

21.1 The need for sample pretreatment

In order to start an analysis, a sample representative of the batch under study is required. This contains the species being studied called the *analyte*, mixed, in general, with a variable number of other compounds which constitute together what is called the *matrix*. When measuring traces of an analyte within other compounds thousands of times more abundant, there is a fear that interferences between analyte and other compounds may occur. Even the best analytical techniques cannot remedy problems generated by poor sample pretreatment. This makes essential a meticulous sample preparation. Since this supplementary work is often tedious and time consuming (Figure 21.1) methodologies involving

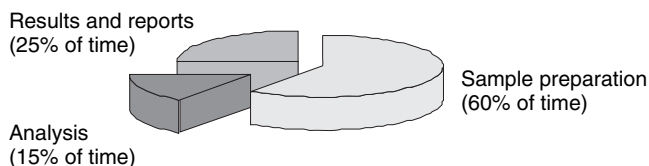


Figure 21.1 Statistics displaying the proportion of the time spent in each stage of a chromatographic analysis. Sample preparation generally represents a large fraction of the total time dedicated to the complete analysis (LC-GC Intl.1991, 4(2)).

adsorption, extraction or precipitation methods have been developed to make it easier and when possible automatic.

■ Measurement by atomic absorption at the mg/L level of elements in drinking water or the scanning of a mid-infrared spectrum of a pure organic compound, as often performed in a teaching laboratory, corresponds to simple situations for which the sample does not need a pretreatment. These conditions are not representative of the difficulties encountered when working on real samples to be analysed. A pretreatment becomes essential.

21.2 Solid phase extraction (SPE)

SPE is currently used for the purification or concentration of a crude extract prior to the measurement of one or several of its constituents. SPE is a clean analytical procedure which has drastically changed the classical approaches of solvent extraction as liquid/liquid extraction in a separating funnel, comparatively slower and that use relatively large amounts of liquid organic solvents.

SPE operates in a small open plastic cartridge, similar to the barrel of a syringe, which contains an appropriate solid sorbent (e.g. a cartridge of 100 to 300 mg of a RP-18 phase type) upon which the process consists of passing a known volume of the liquid sample (Figure 21.2).

Two methods can be used:

- The currently most used method comprises an initial step in which the compound of interest is retained by the sorbent-containing column, followed by the elimination, through rinsing, of the greatest possible number of undesired substances for the subsequent measurement. The analyte is then recovered in a small volume of an appropriate solvent as a solution strongly enriched. This extraction procedure allows not only *isolation* of the analyte but also its *pre-concentration*, which is particularly useful in traces analysis.
- The second method, less frequently used, though very simple, consists of passing the sample through the column in order that the undesired

compounds are retained while the analytes pass freely. This approach is attractive because it eliminates the elution (desorption step). On the other hand, the analyte solution is purified, but not concentrated (enrichment factor of 1).

The solid sorbents closely resemble to that of the solid stationary phases of liquid chromatography. Bonded silica gels (of reverse phase polarity) with a particle size of between 4 and 100 μm , allow a percolation of faster flow rate. Other adsorbents, containing graphite or co-polymers such as styrene-divinylbenzene of large functional group bonded surfaces, are more stable in acidic solutions.

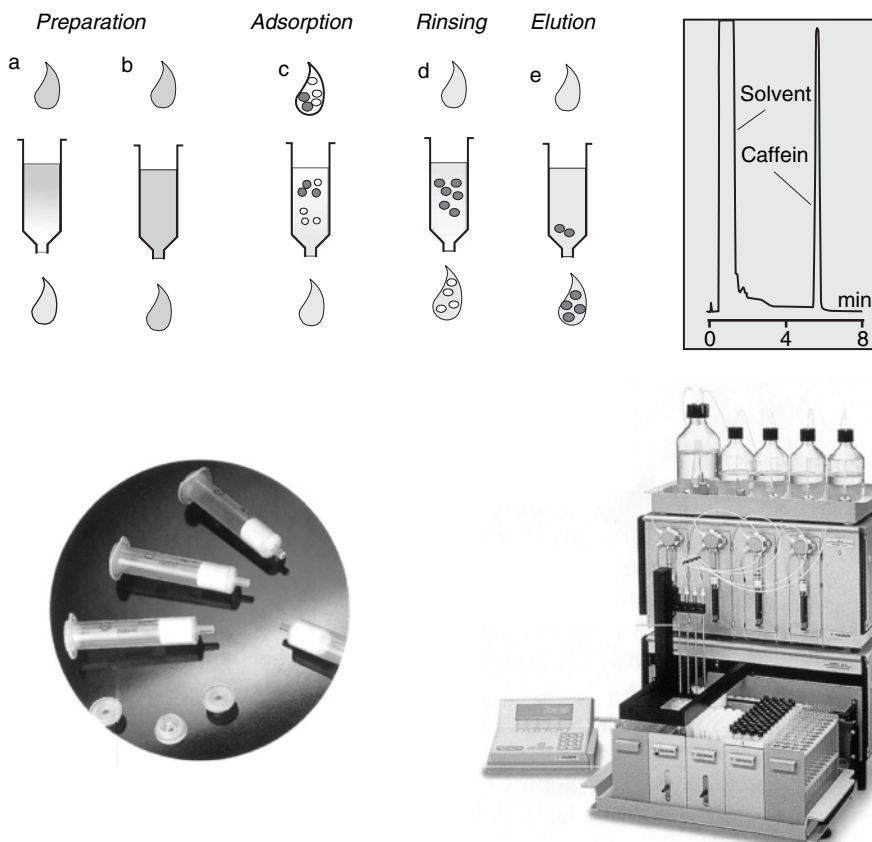


Figure 21.2 *Solid phase extraction.* The separation of an analyte from the matrix. In this example the analyte is the only compound retained on the cartridge. Note the following steps: (a), (b) activation and rinsing of the sorbent prior to use; (c) introduction of a known volume of the sample; (d) interference elimination by rinsing; (e) desorption of the analyte by percolation. On the right, chromatogram (GC) obtained following treatment by this sequence of 1 mL of coffee. Below, on the left, SPE cartridges with reservoirs of 1 to 3 mL; on the right, automated sample preparation (Aspec model XL4 reproduced courtesy of Gilson).

There are also extraction discs (0.5 mm thickness and 25 to 90 mm in diameter) used as a kind of *chemical filters* in the same way as filter paper fit in the funnel of a vacuum flask. The discs are made of bonded phase silica particles trapped in a porous Teflon or glass fibre matrix. They retain traces of organic compounds present in large volumes of aqueous solutions, something that is not possible with the cartridges described above. The analyte is recovered by percolating a solvent through the filter. Trace analysis is made easier because the enrichment factor can be as high as 100.

Non-polar compounds are isolated from a polar matrix with a cartridge or a disc of C-18 derivatized silica gel. Polar compounds are isolated from a non-polar matrix with a cartridge filled with a normal phase. Charged species can be isolated on an ion exchange phase. These three situations are well adapted to analysis by HPLC.

Two particular points require attention. During the extraction step the breakthrough volume must not be reached since beyond this amount the compound to be extracted will no longer be trapped by the cartridge. Elsewhere, during the percolation step, the recovery of the compound absorbed is rarely complete. If the measuring technique calls upon detection by mass spectrometry this quantification problem can be resolved by introducing, prior to extraction, of a given volume of the sample, a known quantity of this same compound labelled with a stable isotope. The behaviour during this sequence of the two forms of the compound being the same, the comparison of their respective signals will permit the establishment of their ratios and consequently the concentration of the unknown. This is the equivalent of the method of internal standards using a standard for which the relative response coefficient will be 1 (cf. paragraph 4.9).

A current research theme concerns the preparation of adsorbents having a marked affinity for a particular type of compound. For this, polymers are synthesized which possess recognition sites in the form of surface deformations, appropriate for trapping molecular targets. The principle resembles that of immuno-extraction, which is even more selective, as explained in the next paragraph.

■ In conventional liquid/liquid extraction, the analyte is diluted in a large volume of solvent. Consequently, when the extracted solution is concentrated, so too are the non-volatile impurities contained in the solvent. This frequently makes the method unacceptable unless ultra-pure solvents are used. For example, if 1 mg of analyte is mixed with 1 mL of 99.9 per cent pure solvent, this latter brings a mass of added contaminants equal to that of the analyte.

21.3 Immunoaffinity extraction

Retention of the analytes with classic SPE cartridges is based upon hydrophobic interactions. This method can lack selectivity if the matrix in which the target analyte is, contains interferent compounds of which greater concentration is

much that are likely to interfere. A lot of progress have been made in developing new types of cartridges based on immunoadsorbents, able of an efficient enrichment of a single analyte from complex environmental matrices. This molecular recognition is based upon the interactions of antigen/antibody type (cf. Chapter 17).

By fixing through covalent bonding an antibody on an adapted solid support, it will result a sorbent that will fix the corresponding antigen, as a molecularly imprinted polymer (Figure 21.3). Immunoaffinity techniques, now used extensively in pharmaceutical and biotechnology applications, can be adapted to extract small organic molecules of environmental samples. There exist extraction cartridges containing antibodies of herbicides or polycyclic aromatic hydrocarbons (HPA) adapted to the extraction of these compounds by a rapid percolation.

As already reported, extraction yield does not attain 100 per cent. For immunoextraction there can be an insufficient number of active sites. It is therefore indispensable to introduce a known amount of a tracer.

21.4 Microextraction procedures

21.4.1 Solid-phase microextraction (SPME)

SPME integrates sampling, extraction, concentration and sample introduction into a single solvent-free step. This technique uses a 1 to 2 cm long segment of porous fused silica fibre coated with an appropriate adsorbent material. This fibre is held in a syringe-like device, protected by the steel needle (Figure 21.4). The device's plunger is depressed to expose the fibre to the sample then retracted at the end of the sampling time (the fibre may be introduced into the aqueous solution or in the space situated above it surface ('headspace', cf. Section 21.6). This process does

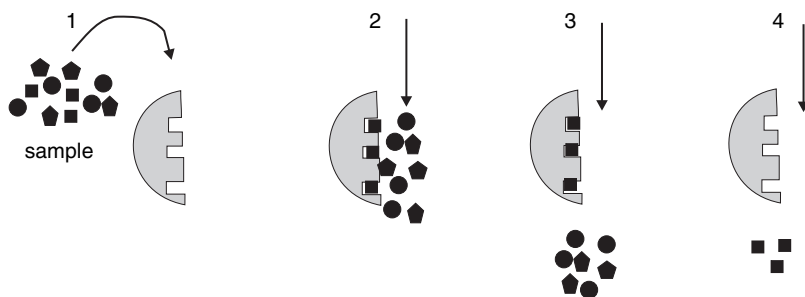


Figure 21.3 Principle of immunoaffinity extraction. The different steps of a classic procedure on a cartridge containing the immobilized antibodies adapted, on this illustration, to the square molecules. After the fixing step (2), rinsing (3), then elution (4) of the analyte is done with an organic solvent.

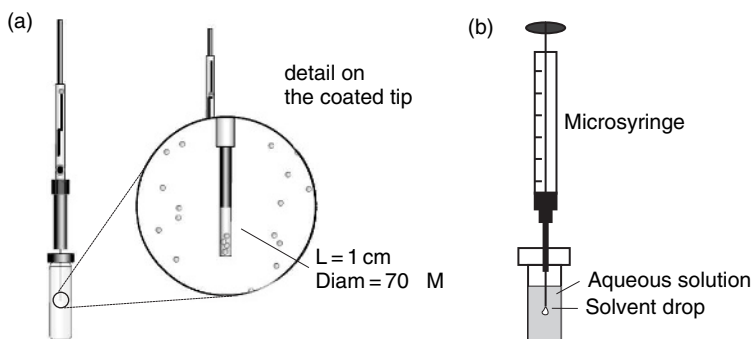


Figure 21.4 *Micro-extraction procedures.* (a) Micro-extraction using an adsorption fibre; (b) Micro-extraction with a single drop of organic solvent. Advantages of this technique are: ultra small volume of sample, low chemical consumption and fast analysis (reproduced courtesy of Supelco).

not need solvents. The sample analytes are directly extracted and concentrated on the extraction fibre. Then the fibre is introduced into a particular injector of a GC, where, through the effect of the temperature, the compounds of interest are desorbed and then separated. The SPME technique can also be routinely used in combination with HPLC, HPCE and MS. Sensitivity is usually low-to-mid per trillion, but this procedure is rather expensive despite the same fibre can be used around 50 times.

21.4.2 Liquid-phase microextraction (LPME)

This technique also called *single-drop microextraction*, operates in a two-phase mode. Analytes are extracted from 1 to 4 mL aqueous samples into 25–50 μL of an organic very pure solvent, as hexane. It uses a microdrop of solvent at the tip of a conventional microsyringe, rather a fragile silica fibre as above. The microdrop, formed at the point of the syringe needle, is immersed over several minutes into the aqueous sample solution containing the products to be extracted (Figure 21.4b). LPME combines extraction and concentration in one step. Subsequent analysis is performed by GC. Quantification is possible, provided operating conditions are held constant through all of the calibration procedure. The method has been applied to the analysis of chemical solvents in foods and pharmaceuticals, natural plant components, environmental volatile and semi-volatile contaminants in water.

The purification of small quantities of aqueous solutions traditionally performed using a separating funnel (liquid-liquid extraction) can be carried out advantageously by a small column containing a porous chemically inert material which is strongly water adsorbent. The aqueous sample is first absorbed into the column (the size must be such that all of the solution should be absorbed). At this stage

the water diffuses into the inert material and behaves as an immobile film of aqueous phase that permits the neutralization and washing the aqueous phase with a non-miscible solvent.

21.5 Gas extraction on a cartridge or a disc

In order to measure low concentrations of molecular compounds present in gaseous samples, for example a polluted atmosphere, the best method consists of trapping them by passing a known volume of the sample through a short tube of absorbing material or through a disposable SPE cartridge (Figure 21.5).

The composition of the trap-column is variable: molecular sieves, graphite-based carbon black, organic polymers. A trap can be composed of a series of several adsorbents. A pump rate is used to deliver a pre-programmed volume of gas, in relation to the capacity of the trap (flow rate of 0.1 to 1 L/min).

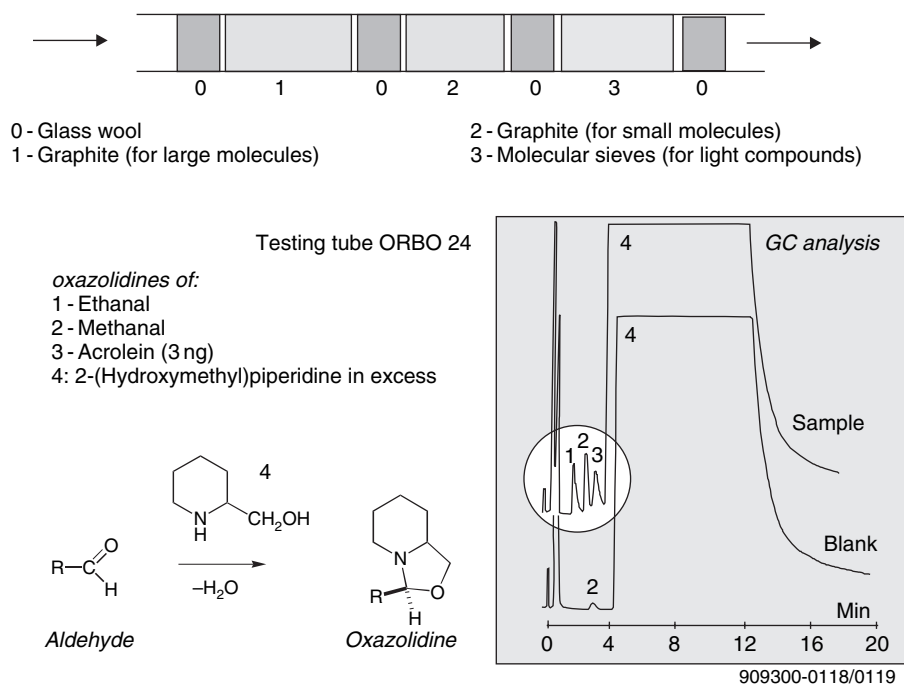


Figure 21.5 Gas extraction. Also called the dynamic purge-and-trap method. Principle of a gas/solid extraction column. A chemical transformation used to detect an aldehyde by derivatization (testing polluted atmosphere, Supelco Inc.).

The compounds absorbed are recovered either through extraction by means of solvents (often using carbon disulfide), or by thermal desorption in a carrier gas which avoids analyte dilution and the introduction of artefacts.

This technique can be adapted to gas phase chromatography. The trap is inserted into a special oven and heated with a temperature gradient able to attain 350 °C in a few seconds. The desorbed compounds are directly introduced into the GC injector. As an alternative to these special ovens there exists extraction tubes of a diameter sufficiently small that can be directly introduced into a modified GC injector.

The recovery of the compounds is considered to be satisfactory when it attains 60 per cent, but it is often almost quantitative with this device.

■ *Purge and trap.* The same extraction tube filled with several layers of adsorbents enables the retention of a broad range of molecules with different polarities. Each layer protects the next, which is more active. The first layer serves to purge the sample of heavy compounds. Lighter compounds and gases are adsorbed by the subsequent layers. To regenerate the trap, the sweeping gas circuit is reversed, so that the high molecular weight compounds retained at the entrance of the tube, are eliminated first (cf. Section 21.5).

A new range of problems appear, affecting more than 50 per cent of the samples treated for trace analyses: absorption by the walls of the containers, action of atmospheric oxygen, evaporation of the analytes, etc. As a consequence those methods are favoured which lead to an enhanced enrichment with a short extraction time.

The same principles are applied for developing *detector tubes*, which contain, along with the adsorbent, a reagent that causes a specific derivatization of the analyte. This can be a change in colour of the tube contents, the analyte being not extracted. Manual breath alcohol tests are found in this category. There now exist many dosimeter badges used as personnel protection indicators in which the circulation of air in contact with the badge is effected naturally and not with a pump. The colour intensity is correlated to the concentration of gas and the time of exposure. This is used for pollution control at places of work or in the environment. The flexibility of this method is due to the wide choice of adsorbent phases that permit selectivity of extraction. When molecules are unstable on the adsorbent and therefore would require stabilization, they can be transformed to a specific derivative by a reagent mixed with the adsorbent.

21.6 Headspace

This relatively simple technique can provide sensitivity similar to the above dynamic purge-and-trap analysis. *Headspace* is a sampling device used in tandem with a GC installation (Figure 21.6). It is reserved for the analysis of volatile compounds present in a matrix, which cannot be itself directly analysed by

chromatography. This is generally the case as most samples are composed of a wide variety of compounds that differ in molecular weight, polarity, and volatility. Basic principles of headspace are illustrated by the two following variations:

- *Static mode*: the sample (solid or liquid matrix) is placed in a glass vial capped with a septum such that the sample occupies only a part of the vial's volume. After thermodynamic equilibrium between the phases present (1/2 to 1 h), a sample of the vapour is taken. Under these conditions, the quantity of each volatile compound present in the headspace (volume above the liquid) will be proportional to its concentration in the matrix. After calibration (using methods of internal or external standards), it is possible to match the real concentrations in the sample with those of the vapours injected in the gas chromatograph (Figure 21.6).
- *Dynamic mode*: instead of working in a closed environment, a carrier gas such as helium is either passed over the surface of the sample or bubbled through it in order to carry the volatile components toward a trap where they are adsorbed and concentrated (Figure 21.7). The procedure continues with a thermal desorption of the trap using reversed gas flow in respect of the injection in the chromatograph. This 'purge-and-trap' principle is semi-quantitative and delivers a sample without residue.

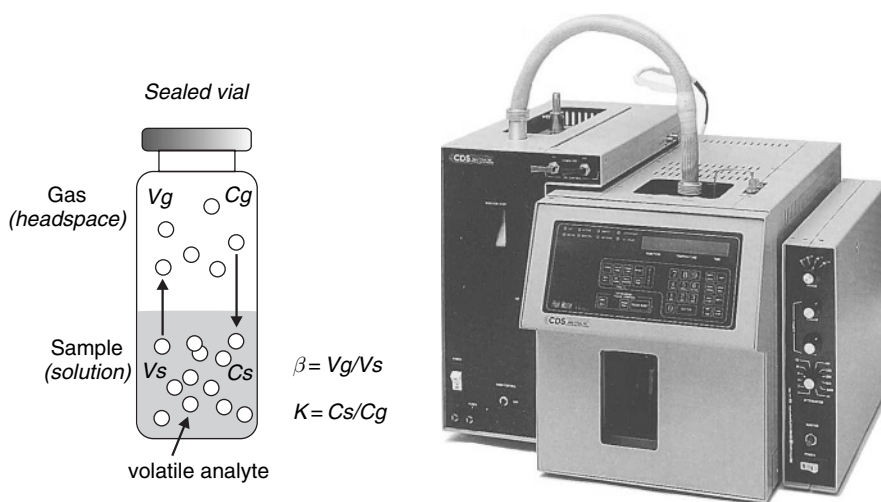


Figure 21.6 *Static headspace*. Sample vial in a state of equilibrium. The state of equilibrium is under the control of two factors: $K = C_s/C_g$ and $\beta = V_g/V_s$. In addition to routine single extraction (one sampling per vial), modes of multiple headspace extraction are incorporated in this model G1888 (reproduced courtesy of Agilent Technologies).

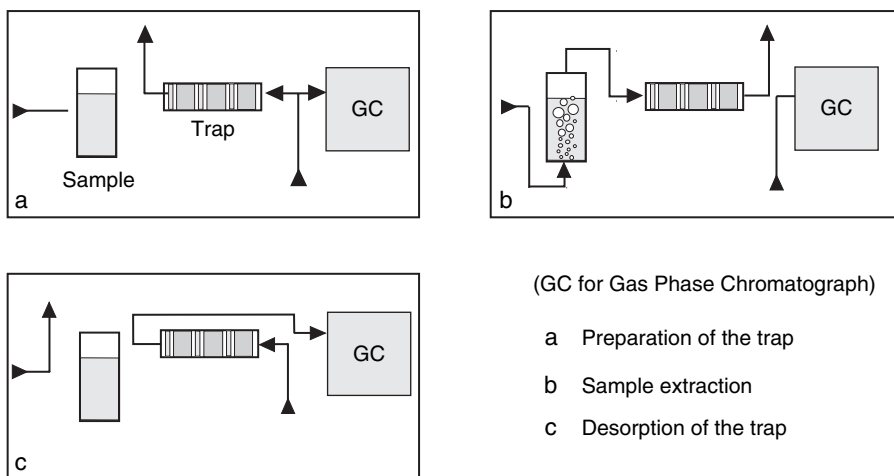


Figure 21.7 *Headspace, dynamic mode.* The sample is recovered by thermal desorption (stripping) of a purge-and-trap cartridge, chosen as a function of the compound to be extracted.

This method is quite reliable for repetitive analyses involving stable matrices. However, if the matrix composition fluctuates, then this disturbs the equilibrium factors and reduces the precision of the result, by consequence of an irregular calibration. In this case cartridge-base extraction would be preferred.

21.7 Supercritical phase extraction (SPE)

Extraction using a fluid in the supercritical state is a well-known procedure employed in the food industry. Analytical extractors operate on the same principle. They incorporate a very resistant tubular container in which is placed the sample (in solid or semi-solid form) with the fluid (CO_2 , N_2O or CHClF_2 – Freon22) in the supercritical state. There are two modes of operation:

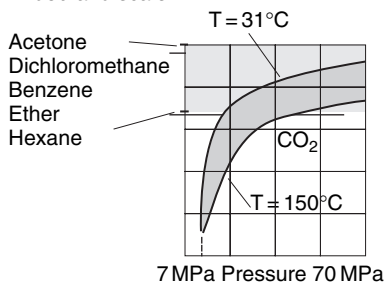
- *Off-line* mode consists of depressurising the supercritical fluid following extraction. By returning to the gaseous state, it leaves the analytes in a concentrated form with the undesirable impurities on the internal wall of the extraction vessel. The concentrate is dissolved using some classical solvent and submitted to a selective extraction on a solid-phase cartridge.
- *On-line* mode consists of performing the direct analysis from the extract, while it is still under pressure, by passing the supercritical fluid through a

chromatographic installation (SFC or HPLC). This procedure is applied only to those compounds for which interferences due to matrix are not likely to occur.

In summary, SFE acts on four parameters that can be modified to obtain a good selectivity: pressure, temperature, extraction period and choice of modifier. It is also possible to change the solvation character of the fluid by the introduction of an organic modifier (e.g. methanol, acetone). Nonetheless, to separate the analyte from the matrix requires a knowledge of the solubility and transfer rate of the solute in the solvent as well as of the physical and chemical interactions between solvent and matrix (Figure 21.8).

The use of microwaves combined with extraction by solvent in a pressurized container is another very efficient process for the rapid treatment of samples.

Hildebrand scale



Supercritical Fluid Properties

Fluid	Critical Point (°C)	Dipole moment (Debye)
CO ₂	32	0
N ₂ O	37	0.2
CHClF ₂	96	1.4

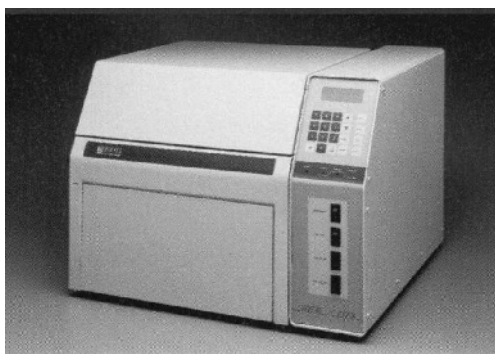


Figure 21.8 *Supercritical fluid extraction.* Comparison of the solvation strength of the CO₂ with respect to the usual solvents (Hildebrand scale) as a function of the temperature and pressure. The polarity of carbon dioxide in the supercritical state is comparable with that of hexane (for 100 atm and 35°C). SPE is a method for which automation becomes a justified investment when the sample throughput is large. Above, sample extractor by supercritical fluids (Model SFE-703 reproduced courtesy of Dionex).

21.8 Microwave reactors

The transformation to elements of samples containing organic material, known as *mineralization*, has motivated the development of many different approaches: dry procedures such as oven heating, combustion, while others by wet treatment such as with mineral acids, fluxes for fusion, etc. In the absence of a universal method applicable to all inorganic elements, the mineralization must be adapted to the sample. This indispensable step of the preparation of a large number of samples, in particular for those analysed by AAS and OES, can be made easier by using of a ‘microwave digester.’

■ Mineralization by strong acids conducted in open containers, in a fume-hood, with the ever present risk of cross contamination and ‘bumping’, is a long and fastidious operation which fundamentally has changed little over a century. Beyond the practical problems involved, some sample matrices are quite impossible to treat under these conditions (refractory or volatile materials, certain minerals, charcoals and heavy oils).

Microwave digesters consists of a Teflon ‘bomb’ of a few mL volume in which the crude sample is placed along with the mineralization solution (necessary for carbonization or oxidation). The heating, induced by the microwaves, occurs *via* molecular agitation due to the dipoles of the water molecules. The closed calorimetric-type vessel avoids losses through droplets or aerosols, or evaporation of volatile components and splashing. The temperatures reached by the acidic solutions under pressure quickly exceed the boiling temperatures under normal conditions (the same effect is seen in a pressure-cooker). This gain in time has been known, in certain cases, to reach more than 90 per cent. In such conditions, digestions by sulfuric or perchloric acids, hydrolyses or mineralizations of Kjeldhal in oxidizing media can be performed. This system can be automated quite safely.

21.9 On-line analysers

All of the laboratory instrumental methods have been practically adapted for on-site analytical procedures, from pH measurement to NMR analyses. The instruments are generally less versatile than their laboratory counterparts. Dedicated to particular measurements and suitable for harsh environments or hazardous areas, they must be robust, so their design is different. They are configured for industrial process control. Many of them are process analysers for concentration measurements. A continuous sample is introduced via a diaphragm pump into a small tank. At intervals the sample or calibration standards are pumped into the ‘chemistry stream’ where they are treated depending of the method of analysis (UV, IR).

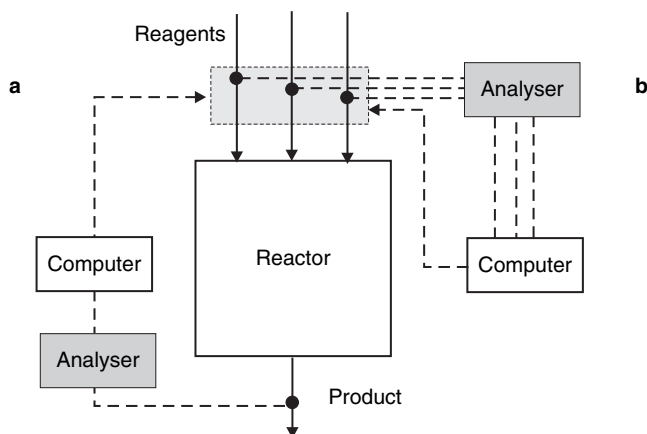


Figure 21.9 *The two control procedures for on-line analysis.* Taking a chemical reaction as an example, procedure (a) corresponds to an analyser situated after synthesis (closed loop feedback through comparison with a reference value). Alternatively, procedure (b) corresponds to an up-stream analyser (in an open loop with continuous adjustment of the reagents' composition).

In this type of analysis, the result must be obtained rapidly as a strategy of feedback is used to optimize or to ensure the stability of the operation. The on-line analyser can control the procedure either up or down stream of the reactor (Figure 21.9).

The use of a selective sensor plunged into the centre of a product to be analysed is not always possible. The preparation of the analyte prior to measurement leads to the removal of a small fraction in order to ensure that the applied treatment is adequate.

22

Basic statistical parameters

In chemical analysis, as in many other sciences, statistical methodologies are unavoidable. The calibration curve constitutes an everyday application, just as an analytical result can only be ascertained if an estimation of the possible error has been considered. Once a measurement has been repeated, a statistical exploitation becomes possible. However, the laws of sampling and tests based upon hypotheses must be understood to avoid non-value conclusions, or to ensure the meaningful quality tests. Systematic errors (user-based, instrumental) or gross errors which lead to results beyond reasonable limits do not enter into this chapter. For the tests most frequently met in chemistry only indeterminate errors are considered here.

22.1 Mean value, accuracy of a collection of measurements

When n measurements are repeated upon the same sample (in the chemical sense of the term) and particularly to a high degree of precision it is frequently found that individual values differ slightly within the group. In these cases a preference for an estimation of the final result based upon the arithmetic mean \bar{x} is expressed. This *mean* \bar{x} of the n measurements is obtained from expression 22.1. This *mean* is accepted as being better than any individual measurement taken at random:

$$\bar{x} = \frac{x_1 + x_2 + \dots + x_n}{n} = \frac{\sum x_i}{n} \quad (22.1)$$

An alternative to the mean or average value, though more rarely used in chemical analysis, consists of taking the median: after having classed the values in increasing order, this is the result situated in the middle of the series, except when the number of the series is even in which the average of the two middle numbers is taken. The median has the advantage of not giving any particular weight to any abnormal value. For example if the values are 12.01, 12.03, 12.05 and 12.68, the arithmetic mean is 12.19 and the median 12.04, a value certainly better than

the mean as it does not take into account the last value in the example given, which appears to be abnormal (dispersion 0.67). The median is a non-parametric statistical method (Section 22.8).

The mean value is impossible to know in advance as the measurements are randomly variable. However, it is not by repeating the same measurement a great many times and finding values with little difference between them that the central value is rendered close to the *exact value*, equally called the *true value* x_0 , (at this stage unknown) because of the possible existence of systematic errors.

Each measurement x must be considered as the sum of the true value x_0 and an *absolute experimental error* value ε . The absolute error ε_i of measurement i is thus expressed as:

$$\varepsilon_i = x_i - x_0 \quad (22.2)$$

If n becomes very large, as in the case of a statistical population, the mean \bar{x} becomes the *true mean* μ and be confused with the *true value* x_0 in the absence of systematic errors. This assumes that the measurements follow the Normal or gaussian distribution (see Figure 22.2).

If the *true value* x_0 is known (e.g. analysis of a standard of perfectly known composition), the *accuracy* of the result or of the method used is characterized by the *total systematic error* ε calculated from the true mean as follows (cf. Table 22.1 and Figure 22.1).

$$\varepsilon = \mu - x_0 \quad (22.3)$$

Table 22.1 Examples of results from an analysis grouping the various parameters of expressions 22.1 and 22.2 (the true value is $x_0 = 20$)

Chemist	1	2	3	4
Measurement 1	20.16	19.76	20.38	20.08
Measurement 2	20.22	20.28	19.58	19.96
Measurement 3	20.18	20.04	19.38	20.04
Measurement 4	20.2	19.6	20.1	19.94
Measurement 5	20.24	20.42	19.56	20.08
Mean \bar{x}	20.2	20.02	19.8	20.02
Median	20.18	20.04	19.38	20.04
Systematic error ε	0.2	0.02	0.2	0.02
Relative error E_R (expression 22.4)	0.01 or 1% or 10^4 ppm	1×10^{-3} or 0.1% or 1000 ppm	0.01 or 1% or 10^4 ppm	1×10^{-3} ou 0.1% or 1000 ppm
Variance (s^2)	7.84×10^{-4}	0.1183	0.1772	3.6×10^{-5}
Standard deviation (s)	0.028	0.344	0.421	0.006
Comments on the results	Precise not accurate	Not precise accurate	Not precise not accurate	Precise accurate

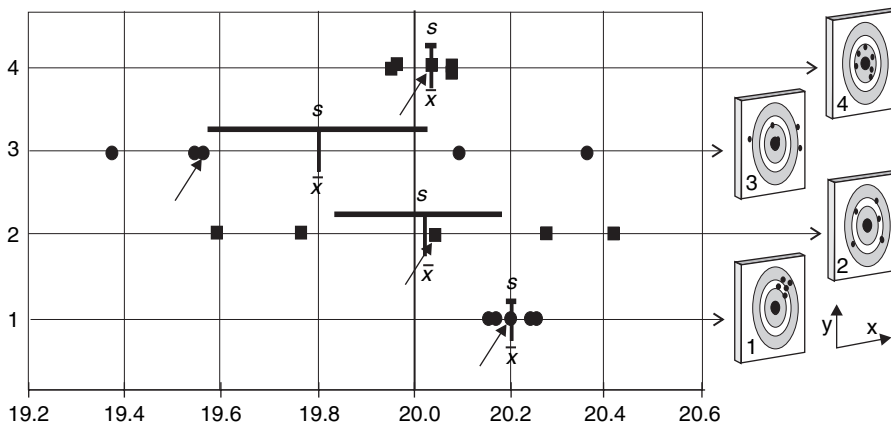


Figure 22.1 Graphical illustration of the data from Table 22.1. To illustrate accuracy and precision the standard deviations have been calculated using formula 22.8. Note the differences between the arithmetic means indicated on the graph by a short vertical bar and the corresponding median values, which are arrowed. For the results from chemists 3 and 4, the difference is fairly large. Chemist 1 has committed very probably a systematic error. On the right, a classic illustration of the precision and accuracy depicted with the aid of a target. This image is less simple than it would appear because there remains an uncertainty in both x and y .

The relative error E_R of a measurement (or on the mean value) corresponds to the ratio of the absolute value of the deviation corresponding to ε_i (or ε), over the true value. E_R can be expressed as a percentage or in ppm.

$$E_R = \frac{|x - x_0|}{x_0} \quad (x \text{ for } x_i \text{ or } \bar{x}) \quad (22.4)$$

If the true value x_0 is not known, which is usually the case in chemical analysis, then the experimental error of the measurement i being e_i , is calculated by replacing x_0 by the mean \bar{x} in 22.2:

$$e_i = x_i - \bar{x} \quad (22.5)$$

e_i represents the algebraic difference between the mean and the i th measurement. The mean deviation \bar{d} or mean of the differences, calculated over n measurements allows an appreciation of the precision:

$$\bar{d} = \frac{\sum |x_i - \bar{x}|}{n} \quad (22.6)$$

In the last expression absolute values of the experimental error are taken since with algebraic values, this parameter \bar{d} would rapidly tend towards 0 as n increases.

22.2 Variance and standard deviation

The above precision (*mean deviation*) has however the inconvenience that it cannot be interpreted statistically since both large and small individual deviations, though not being equally probable, have the same weight. The summing of the squares of the differences is preferred which leads to the most largely used definition for the *precision* (reproducibility) in statistics, known as the *variance* s^2 , which is estimated for a set of n measurements by:

$$s^2 = \frac{\sum (x_i - \bar{x})^2}{n - 1} \quad (22.7)$$

The square root of the *variance* is the *standard deviation*, represented either, when the number n of measurements is small, by s or by σ when a large statistical population of values is available (expression 22.8). The standard deviation is ‘a dispersion index’ expressed in the same units as x .

Calculators and related programmable software possess the corresponding functions:

$$s = \sqrt{s^2} = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}} \quad (22.8)$$

For an infinite number of measurements, s is replaced by σ , n is replaced by $n - 1$ and μ is used instead of the arithmetic mean \bar{x} .

To compare results or to express the uncertainty of a method, s is often expressed in a relative manner. Calculations are made therefore of the *relative standard deviation* (or *RSD*), also called *coefficient of variation* (*CV*) and most often expressed as a percentage:

$$CV = 100 \times \frac{s}{\bar{x}} \quad (22.9)$$

■ When the result of an analysis arises from a calculation involving many experimental values, each having its own standard deviation, there will be, by consequence, a propagation of errors. The precision of the result is calculated using simple equations that are found in most introductory textbooks concerned with statistics.

22.3 Random or indeterminate errors

In the absence of systematic errors, accidental errors ‘due to hazards’, exist that cannot be controlled because they are indeterminate. Their direction and amplitude varies in a non-reproducible manner from one measurement to another. A mathematical analysis of the error curve leads to the conclusion that the arithmetic mean \bar{x} of the individual values is the best estimation of the *true mean* μ (Figure 22.2).

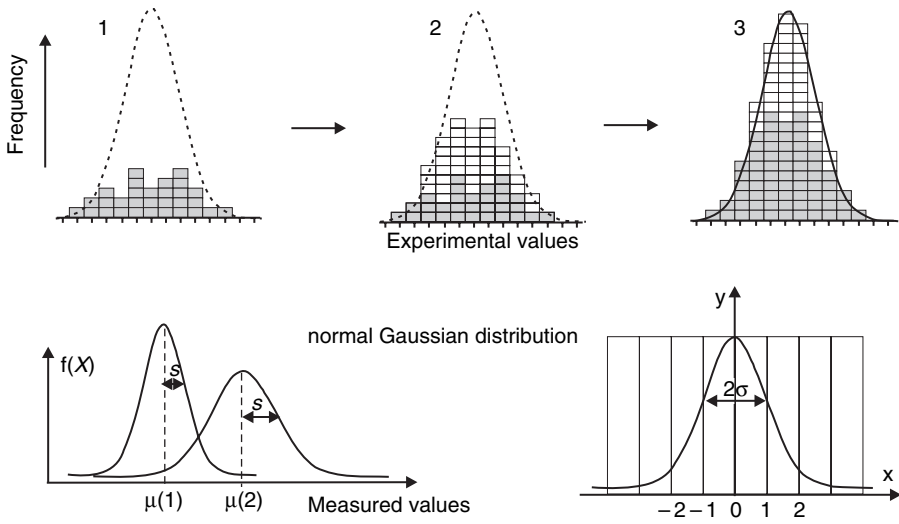


Figure 22.2 *Gaussian curves.* When the number of measurements increases and if the interval determining the group is narrow, then the graphical shape will take (measurement/frequency) the form of a Gaussian curve (Normal distribution law). Below, two series of results centred on two different true means μ . If the number of measurements is very small, it is not possible to estimate the average distribution, thus the true mean. At the bottom right, a reduced form of the Gaussian distribution is shown. The reliability of the mean is given by the 95 per cent confidence interval.

The symmetry and appearance of this curve shows that:

- There are an equal number of errors, positive and negative, with respect to the true mean value
- Small errors are more frequent than large errors
- The most encountered value is the true mean μ (without error).

The normal distribution law (bell-shaped gaussian curve) is the mathematical model which best represents the distribution of random or indeterminate errors due to hazards (Equation 22.10):

$$f(x) = \frac{1}{\sigma\sqrt{2\pi}} \exp \left[-\frac{(x - \mu)^2}{2\sigma^2} \right] \quad (22.10)$$

In order to make this expression universal, the true mean of a population μ , is used for values of x and its standard deviation σ for the unit of measure (Figure 22.2).

With $X = \frac{x - \mu}{\sigma}$ expression 22.13 becomes $f(X) = \frac{1}{\sqrt{2\pi}} \exp \left[-\frac{X^2}{2} \right]$ (22.11)

If the number of measurements is not very large it is important to know their distribution. The curve obtained yields information about the reliability Re of the results. The reliability of the mean, as an estimation of the true value, increases with the square root of the number of measurements n .

$$Re = K\sqrt{n} \quad (22.12)$$

Thus, when the number of measurements n_1 increases to a larger number n_2 , the reliability Re is improved by a factor k such that:

$$k = \sqrt{\frac{n_2}{n_1}} \quad (22.13)$$

The distribution function is the integral to the function $f(X)$. This function is such that 95.4 per cent of the area lies within an interval of width $\pm 2\sigma$ from the value of the true mean. Therefore the chances are 95.4 per cent that the error associated with a given measurement will be contained within the interval $\pm 2\sigma$. A high value for the standard deviation σ signifies a broadened distribution curve. If the number of values is small (a few units), s must be taken instead to estimate σ (Figure 22.2). The greater n is, the better will be the match.

Thus, s and \bar{x} are estimations of σ and of the true mean μ which relates to the total population.

■ In practice, it is not possible to know either the true mean μ or the standard deviation σ since there will be a limited number of values of n . Thus s must be calculated. Elsewhere, when a large number of repetitive measurements concerning the same sample is available, the Chi-square test for the standard deviation (χ^2 test) is used to ascertain if the frequency distribution (that is the number of times where a value is recorded) differs in a significant manner from that of a population, which follows the normal distribution law. Without using proper software this test of normality is a lengthy calculation process.

22.4 Confidence interval of the mean

When the number of the measurements n is small (for example, if n is between 4 and 15), and if there are no systematic errors, the true mean μ can be quite different from the arithmetic mean \bar{x} . An estimation of the true mean must then be made by calculating a confidence interval within which a probability is given (for example 95 per cent), that the real value x_0 will be included.

The *confidence interval*, around the mean \bar{x} , in order that the true mean μ is within (or x_0 in the absence of all systematic errors), is given by the following formula:

$$\bar{x} - \frac{t \cdot s}{\sqrt{n}} \leq \mu \leq \bar{x} + \frac{t \cdot s}{\sqrt{n}} \quad (22.14)$$

In equation 22.14, Student's coefficient t is a statistical factor, which depends upon n and of the level of confidence chosen. These values are displayed in Table 22.2. The greater n is, then the narrower will be this interval, s being the standard deviation of the series of measurements.

Table 22.2 Values of the Student's bilateral confidence coefficient t (calculation of Student's distribution)

n	Level of confidence 90%	Level of confidence 95%	Level of confidence 99%
2	6.31	12.71	63.66
3	2.92	4.30	9.93
4	2.35	3.18	5.84
5	2.13	2.78	4.60
6	2.02	2.57	4.03
7	1.94	2.45	3.71
8	1.90	2.36	3.50
9	1.86	2.31	3.36
10	1.83	2.26	3.25
11	1.81	2.23	3.17
12	1.80	2.20	3.11
15	1.76	2.14	2.98
20	1.73	2.09	2.86
30	1.70	2.05	2.76
60	1.67	2.00	2.66
120	1.66	1.98	2.62

This confidence interval is calculated when a method of measurement is tested with a sample for which the corresponding true value x_0 is known, though it remains to be seen if the latter is situated in the confidence interval calculated.

This test of Student's variable t can equally lead to an adjustment of the number of measurements to be conducted in order to attain a result with the pre-selected level of confidence.

If, as well as the mean \bar{x} , the true value x_0 (or the true mean μ) is also known, expression 22.14 will permit the calculation of the value of t , according to the degree of confidence chosen (expression 22.15 if $n > 20$, else 22.16). A value of t larger than that indicated in Table 22.2, on the line corresponding to the value of n , will be due to a systematic error.

$$t = \frac{|\bar{x} - \mu|}{s} \sqrt{n} \quad (22.15)$$

or

$$t = \frac{|\bar{x} - x_0|}{\sigma} \sqrt{N} \quad (22.16)$$

In the formula, which leads to the calculation of confidence intervals, the standard deviation of the mean $s_{\bar{x}}$, or the uncertainty of the standard deviation may appear, using the following relation that gives the standard deviation of the mean (22.17):

$$s_{\bar{x}} = \pm \frac{s}{\sqrt{n}} \quad (22.17)$$

22.5 Comparison of results – parametric tests

When it is required to compare the results of two methods, or of two instruments applying the same analysis method, or of two laboratories upon the same sample, it is essential to refer to statistical tests. Two main families exist: *parametric* and *non-parametric* tests. The first supposes that data are distributed according to a Normal law (on which the values in Student's table are based), while *non-parametric tests* are based on so-called *robust statistics*, less sensitive to abnormal values. In analytical chemistry it is not often that large quantities of data are collected. Therefore statistical tests must be applied with judgment. They are associated with an acceptable margin of precision, for example 10 or 5 or 1 percent.

22.5.1 Comparison of two variances, Fisher–Snedecor Law

The comparison of the standard deviations s_1 and s_2 of two different sets of results is known as a test of the *variance equality*. In this test, the F factor, which is the ratio of the two variances is calculated in order that $F > 1$:

$$F = \frac{s_1^2}{s_2^2} \quad (22.18)$$

The *null hypothesis* (statistical terminology), states that if there are no significant differences in the variances, then the ratio must be close to 1. Reference should therefore be made to the Fisher–Snedecor values of F , established for a variable number of observations (Table 22.3). If the calculated value for F exceeds that found in the table, the means are considered to be significantly different. Since the variability s_1^2 is greater than s_2^2 , then the second series of measurements is therefore the more precise one.

The adaptation of this test for robust statistics (non-parametric tests) requires simply to calculate the ratio $F = R_1/R_2$ from the two distributions (the difference between the two extreme measurements) of the two series under comparison.

Table 22.3 Summary of the F threshold values established for a confidence level of 95 per cent

Number of measures (denominator)	Number of measures (numerator of the fraction F)						
	3	4	5	6	7	10	100
3	19.00	19.16	19.25	19.30	19.33	19.38	19.50
4	9.55	9.28	9.12	9.01	8.94	8.81	8.53
5	6.94	6.59	6.39	6.26	6.16	6.00	5.63
6	5.79	5.41	5.19	5.05	4.95	4.78	4.36
7	5.14	4.76	4.53	4.39	4.28	4.10	3.67
10	4.26	3.86	3.63	3.48	3.37	3.18	2.71
100	2.99	2.60	2.37	2.21	2.09	1.88	1.00

■ Amongst the examples in Table 22.1 there is no significant difference between the results of chemists 2 and 3 for a degree of confidence of 95 per cent. An experimental value of $F = 1.5$ is obtained using equation 22.18, which is less than the tabulated value of $F = 6.39$ for five measurements made by each chemist. In fact, $F = 1.5$ while from the data in Table 22.1 $F = 6.39$. This verifies that their standard deviations are not significantly different.

22.5.2 Comparison of two experimental means, \bar{x}_1 and \bar{x}_2

Sometimes it is desirable to compare the means obtained from two series of measurements to consider if they are significantly different, bearing in mind that the true value is unknown. Initially it must be determined whether there is any significant difference with respect to the precision of the two means (cf. Section 22.5.1). Next, a calculation using expression 22.19 for the global or *pooled* standard deviation s_p is performed. Then the corresponding value for t is calculated using equation 22.20. It is compared to tabulated value for $n = n_1 + n_2 - 2$ measurements and for the degree of confidence chosen. If the value of t in the table is greater than the value calculated, then it can be concluded that these two means are not significantly different.

$$s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}} \quad (22.19)$$

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{s_p} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \quad (22.20)$$

22.5.3 Estimation of the detection limit of an analyte

The two expressions 22.19 and 22.20 are useful for estimating the smallest concentration of an analyte that can be detected, but not quantified with a pre-selected level of confidence. One of the two means (\bar{x}_2 for example) is considered to be the result of a set of measurements made on the analytical blank. It will be noted as \bar{x}_b with a standard deviation of s_b . If $n_1 = 1$, then expression 22.19 simplifies to $s_p = s_b$, leading to expression 22.21 (using a t value extracted from Table 22.2 for $n_1 + n_b - 2$):

$$\Delta x = \bar{x}_1 - \bar{x}_b = \pm t \cdot s_b \sqrt{\frac{n_1 + n_b}{n_1 \cdot n_b}} \quad (22.21)$$

■ If six measurements are undertaken upon an analytical blank leading to a standard deviation of $0.3 \mu\text{g}$ for the measurement considered, then – with expression 22.21 and for a degree of confidence of 99 per cent, – a value of $1.31 \mu\text{g}$ will be calculated as the detection limit if only a single measurement is made and a value of $0.59 \mu\text{g}$ if five measurements are made ($\bar{x}_b = 0$). Empirically it can be admitted that the instrumental detection limit corresponds to the concentration which leads to a signal whose relative intensity (central value \bar{x}) is twice the standard deviation calculated for 10 measurements of the analytical blank (degree of confidence 95 per cent). The limit of quantification is always higher.

22.6 Rejection criteria Q -test (or Dixon test)

Sometimes, a value within a set might appear aberrant. Although it might be tempting to reject this data point, it must be remembered that it is only abnormal in respect of a given law of probability. There exists a simple statistical criterion for conservation or rejection of this outlier value. This is Dixon's test, which consists of calculating the following ratio (on condition that there are at least seven measurements):

$$Q = \frac{|\text{value in question} - \text{its closest neighbour}|}{\text{largest value} - \text{smallest value}} \quad (22.22)$$

Q calculated in this manner is compared with a table of critical values of Q as a function of the number of data (Table 22.4). If $Q_{\text{calculated}}$ is greater than Q_{critical} the value in question can be rejected.

■ Note: The ASTM standard (American Society for Testing Materials) uses a different test for rejection of an outlier, called the reduced central value $z_j = (x_j - \bar{x})/s$, which has its own table of characteristic critical values.

Henry's curve, the principle of which is available in more advanced statistical texts, also constitutes a good method for detecting outliers using a visual approach.

Table 22.4 Tabular summary of critical values for Q (Dixon's test)

Number of measurements, n	Confidence level 95%	99%
3	0.94	0.99
4	0.77	0.89
5	0.64	0.78
6	0.56	0.70
7	0.51	0.64
8	0.47	0.59
9	0.44	0.58
10	0.41	0.53

22.7 Calibration curve and regression analysis

Instrumental quantitative analysis is commonly based upon comparative methods. For example the sample that contains the analyte and a standard which contains the same concentration of this analyte will yield identical signals, using an instrument with the same settings. In the majority of cases, a series of standards of known concentrations are measured, rather than a single one. Then a calibration or working curve is drawn from the values of the analytical signal as a function of analyte concentration. This *calibration curve* is then used to determine the concentration of an unknown sample or to calibrate the response of the used instrument. Traditionally, on the graph, abscissa corresponds to the concentration and ordinate of the signal value.

The purpose of *regression analysis* as used in quantitative analysis is to generate an equation that relates signals to concentrations, such that if we are given a particular value of a signal we can get the unknown concentration. Therefore, regression analysis takes a step beyond correlation, allowing creation of a predictive model between two (or more) variables. In other words the signals yielded by the instrument (y -axis) are modeled as a function of concentration (x -axis). A model is adopted $Y = f(X)$ allowing an evaluation of y since x and the function are known. Therefore the uncertainty of the results is an accumulation of the uncertainty linked to the measurement and also to the model chosen for the function. Quantitative analysis software uses numerous calculation models. Here only the principal results are given concerning the *simple linear regression*, the statistical approach most frequently met in quantitative analysis (Figure 22.3).

22.7.1 Simple linear regression

As the response of many detectors is linear as a function of the measured variable, taking account of the differences due to experimental conditions as well as of the instrument, the goal is to find the parameters of the straight line that best fits.

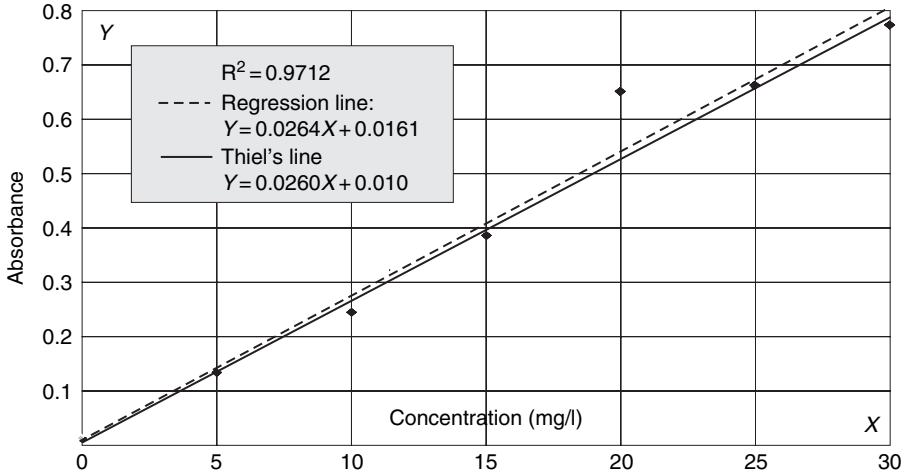


Figure 22.3 Linear regression and the Thiel's line (Section 22.8) corresponding to the data of Table 22.5. Though in close proximity the two lines lead to different results. For example, for $y = 0.5$, leads to $x = 18.85$ (Thiel) and $x = 18.33$ (linear regression), a difference close to 3 per cent. Spreadsheets will often have built-in regression functions to find the best line for a set of data.

The *least squares regression line* is the line which minimizes the sum of the square or the error of the data points. It is represented by the linear equation $y = ax + b$. The variable x is assigned the independent variable, and variable y is assigned the dependent variable. The term b is the y -intercept or regression constant (the value of when $x = 0$), and the term a is the slope or regression coefficient.

Coefficients a and b , as well as the standard deviation on a , may be given by several formulae and specialized software.

Moreover, the dimensionless Pearson *correlation coefficient* R gives a measure of the reliability of the linear relationship between the x and y values. If $R = 1$ it exists an exact linear relationship between x and y . Values of R close to 1 indicate excellent linear reliability. If the correlation coefficient is relatively far away from 1, the predictions based on the first order relationship, $y = ax + b$ will be less reliable.

$$a = \frac{n \sum x_i y_i - \sum x_i \sum y_i}{n \sum x_i^2 - (\sum x_i)^2} \quad (22.23)$$

$$b = \frac{\sum y_i - a \sum x_i}{n} \quad (22.24)$$

$$R = \frac{n \sum x_i y_i - \sum x_i \sum y_i}{\sqrt{[n \sum x_i^2 - (\sum x_i)^2] \cdot [n \sum y_i^2 - (\sum y_i)^2]}} \quad (22.25)$$

A straight line supposes that the errors in y follow the law of Normal distribution. R^2 is the *determination coefficient* that conveys information about how the variations of x overlap variations in y .

In classic calculations the experimental error is considered to affect the y value exclusively and not the concentration recorded in x . If this is not the case, the data points will not have the same quality for the regression line hence comes the idea of according less value to data more distant from the line. Through iterative calculations the equation of a straight line is reached which takes account of the weighting of each point.

In order to compare whether two methods of analysis are highly correlated, a series of n standards of different concentrations are analysed by two pathways. Each standard is next represented by a point on a graph where x bears the results of method 1 and y the corresponding results from the second method. The correlation coefficient R is determined and formula 22.26 is applied. If the t value obtained is greater than the value found in the table for $n - 2$ degrees of freedom, there is a strong correlation between the two analytical methods.

$$t = R \sqrt{\frac{n-2}{1-R^2}} \quad (22.26)$$

22.7.2 Multiple linear regression

Analysis by multiple linear regression leads to the prediction of a result (the *dependent variable*) when it depends upon a certain number of factors (the *independent variables*). Software allows this type of calculation in which the contribution of each factor is determined using standards. The prediction of the result is calculated by a general formula, such as 22.27 and as a composite of three factors in x :

$$y = a + bx_1 + cx_2 + dx_3 \quad (22.27)$$

■ For example, the protein content in cheese, related to the nitrogen percentage (result), can be established from the IR absorbance measured at different wavelengths. This method is equally useful for calculating the parameters A, B and C of the van Deemter equation (Section 1.10), corresponding to experimental data.

22.8 Robust methods or non-parametric tests

The statistical tests developed above assume that the data follow the Normal distribution. However, there are analytical methods for which the results reveal

different distributions. Otherwise they are asymmetric, or symmetric but ‘non-normal’. On some approaches, these distributions are considered as resulting from the superimposition of a normal distribution and abnormal points. The use of the median (cf. Section 22.1), rather than the arithmetic mean, is part of this alternative approach. The standard deviation is replaced by the mean deviation *MD*.

$$MD = \sqrt{\frac{\pi}{2}} \cdot \frac{\sum |x_i - \bar{x}|}{n} \tag{22.28}$$

Under such circumstances, the calibration curve can be reconsidered, as seen in the following example, which illustrates Thiel’s method (Table 22.5 and Figure 22.3).

In order to estimate the best straight line for a series of seven data points (*x*, *y*) in a colorimetric titration, the method consists first to rank the *x* values in increasing order. Since the Thiel’s method requires an even number of data points the median value must be rejected in this example. Then the calculation is the following:

At first, the slopes of three straight lines are calculated, which pass respectively through the first point and that following immediately the median and so on (Table 22.5). The median of the three calculated slope values is taken as the term *a* of the linear equation. Then, the six *b* values are calculated from $b_i = y_i - ax_i$ and also ranked in increasing order. The median will become the term *b* of the line.

Table 22.5 An example of determination of the linear equation coefficients using Thiel’s method from a set of seven data points

Entry #	Conc. (x)	Abs. (y)	slopes a_{ij}	Term b_i	Classification
1	0	0.02	←	0.02	-0.01
2	5	0.14	←	0.01	-0.01
3	10	0.25	←	-0.01	0.01
4	15	0.39	←		
5	20	0.65	←	0.13	0.01
6	25	0.66	←	0.01	0.02
7	30	0.77	←	-0.01	0.13

Equation of line $Y = 0.0260X + 0.010$

In chemistry this method should be more widely used when the number of measurements is too small to be sure of the normality of the distribution.

Three advantages of Thiel's method merit to be highlighted:

- it is not assumed that all of the errors are in y
- it is not assumed that the errors follow the Normal distribution law
- it is to be noted that an aberrant data does not affect the final parameters of the linear equation.

22.9 Optimization through the one-factor-at-a-time (OFAT) experimentation

When a measurement depends upon a signal (absorbance or intensity of fluorescence) which is itself influenced by several factors, then in general, it is customary to seek overall conditions which will lead to the maximum signal.

If the factors involved in an analysis are independent (which is rarely the situation), a common practice is to experiment with *one factor at a time* while holding all others fixed, then the influence of each one can be studied on the result by using a simple repetitive method.

However, the results are often misleading and fail to reproduce conclusions drawn from such an exercise. This method needs some understanding of basic *design of experiments* (DOE) principles.

Imagine that a value given by a sensor depends upon two independent factors x and y . After having fixed the factor x to the value x_1 the influence of factor y on the signal is studied (the signal is measured in the third dimension). For the value $y = Y_1$ it is observed that the signal passes through a maximum. This Y_1 value is then selected and then x is varied in order to optimise its value, which is $x = X_2$ (Figure 22.4). Generally by repeating this procedure a new couple (X, Y) is found which gives slightly improved values each time.

This repetitive method is attractive, as it is simple and supported by common sense, but it does not always constitute the best approach to the problem. If the iso-response curves form a complex envelope, showing notably a ridge – the mathematical illustration of the interaction between the two factors – the preceding method can lead to a false optimum depending upon the parameters selected at the beginning. A more effective method for these situations is to study their effect simultaneously by setting the DOE statistical technique. This different approach has the aim of discovering the optimal conditions using the minimum number of attempts. This is illustrated by the sequential simplex method of optimization and by different designs described in specialized textbooks.

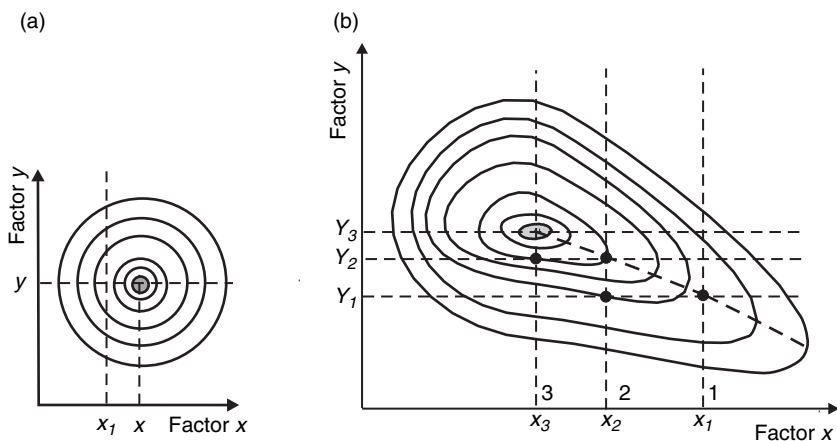


Figure 22.4 The ‘one factor at a time’ method. If there is a surface of continuous response, the iso-response curves have will lead to a variety of optimum situations.

Problems

- 22.1 The true value of a particular measurement is $131.9 \mu\text{g/L}$. Four chemists (A, B, C and D) each repeat the same procedure five times. The individual values obtained are displayed in the table below. Comment upon these results in terms of accuracy and precision (use an indicator of dispersion or standard deviation, s).

Chemist A	130.7	131.6	133.5	132.3	132.6	129.1
Chemist B	125.0	132.3	136.9	137.9	125.9	131.6
Chemist C	136.7	134.5	134.1	135.4	136.0	137.6
Chemist D	130.7	109.9	131.9	115.6	131.3	132.6

- 22.2 With a column whose stationary phase is of squalane and under differing conditions of use, the following values for the Kovats factor of benzene (recognised average value = 653) were determined: 650, 652, 648, 651 and 649. For the value of 653 given, is this significantly different from the other values calculated? Consider a confidence level of 95%.
- 22.3 Supposing that two chemists A and B, whose results figured in question 21.1 above, used two different apparatus. In order to determine if the precision of these apparatus is significantly different apply a test of variance, F .

$$\text{where: } F = s_A^2 / s_B^2$$

- 22.4 Once again return to the results of 21.1, and chemists A and C in particular. If the true value is $131.9 \mu\text{g/L}$ calculate and compare the respective values of parameter t for these chemists. Can the presence of a systematic error be concluded for the data of either A or C?
- 22.5 The results of experimental measurements repeated four times were the following: 24.24, 24.36, 24.87, 24.20, 24.10. Verify whether the third value, which seems to be high compared to the others, should be considered as a value out of acceptable range.
Consider now two complementary measurements: 24.12 and 24.25. Consider again the question posed above and conclude by applying the calculation of the standard deviation, s
- 22.6 The measurements were recorded and then repeated six times for two different methods, for each giving: average 42 ($s = 0.3$), and average 45 ($s = 0.2$).
— Do these two methods yield results that are significantly different?
- 22.7 A compound is accompanied by a certificate indicating its purity as being 99% with $s = 0.08$, established over five analyses. Four control measurements performed upon the same compound led to the following values: 98.58, 98.62, 98.80 and 98.91. Should the original value be rejected?
- 22.8 A series of standards used in atomic absorption led to the following results:

Concentration $\mu\text{g/L}$	1	2.5	5	10	20	30
Absorbance	0.06	0.19	0.36	0.68	1.21	1.58

- Might the method of linear calibration graph be used with these results and, if so, over which range of concentrations?
- 22.9 A series of measurements must be repeated five times. Eight successive values for the blank used in the analysis yielded the following: 0.3, -0.75 , -0.3 , 1.5, -0.9 , 1.8, 0.6 and 1.2.
— Calculate the limit of detection by applying a 99% confidence level.

Solutions

1 Chromatography, general aspects

1.1 At equilibrium the 40 mL of eluant contains $12 \times 40/10 = 48$ mg of compound. There are therefore $100 - 48 = 52$ mg in the stationary phase. If K represents the ratio of the masses present in 1 mL of each phase in equilibrium, then $K = (52/6)/(48/40) = 7.2$.

1.2 When a molecule of the compound migrates along the column it passes alternately from the mobile phase, where it progresses at the speed of this phase, to the stationary phase where it becomes immobilised. The average velocity for the passage is then reduced in relation to the amount of time spent by the molecule in the stationary phase, increasing by consequence the value for t_s .

Now consider the total mass, m_T , of all of the identical molecules in the sample. Statistically at each moment, there will be a certain number of these molecules within the stationary phase while the rest remain in the mobile phase. The ratio of these molecules fixed in the stationary phase (m_S), and of those present in the mobile phase (m_M), will be the same as the ratio representing the sum of the intervals spent in each phase for a single isolated molecule; that is: $m_S/m_M = t_s/t_M$.

Thus k will correspond to the ratio t_s/t_M and since $t_s = t_R - t_M$ then we can refine the classic expression for the retention factor (or capacity factor), as calculated from the chromatogram:

$$k = (t_R - t_M)/t_M$$

1.3 The separation factor (or selectivity factor), is generally calculated from the retention time t_R , yet in the question it is the retention volumes V_R which are given for the two compounds. However, V_R and t_R are linked by an expression which considers the flow of the mobile phase, D :

$$V_R = D \cdot t_R$$

Therefore if for the expression in α below, each t_R (or t_M) is replaced by V_R (or V_M), we will have:

$$\alpha = (t_{R(2)} - t_M)/(t_{R(1)} - t_M) = (V_{R(2)} - V_M)/(V_{R(1)} - V_M).$$

which leads to $\alpha = 1.2$.

Note: The expression in α is defined from the retention volumes and remains useful with a flow gradient but it is very rare that it is employed since the apparatus does not measure the instantaneous rate of the column. Therefore the calculation of the retention volumes (other than by substituting in the relation, $V_R = D \cdot t_R$) is not permissible.

- 1.4 The expression giving the retention factor can be written, $t_R = t_M(1 + k)$, where t_M corresponds to the ratio between the length L of a column and the average velocity u of its mobile phase. Equally, $k = KV_S/V_M$, leading upon substitution to

$$t_R = (L/u)(1 + KV_S/V_M).$$

- 1.5 In order to incorporate k into expression 1, the relation $t_M = t_R/(1 + k)$, is used. Expression 1 then becomes:

$$N_{\text{eff}} = 5.54 \frac{\left(t_R - \frac{t_R}{1+k}\right)^2}{w_{1/2}^2}$$

simplifying to

$$N_{\text{eff}} = 5.54 \times t_R^2 \frac{\left(1 - \frac{1}{1+k}\right)^2}{w_{1/2}^2} \quad \text{and then} \quad N_{\text{eff}} = N \frac{k^2}{(1+k)^2}$$

- 1.6 The classic formula giving the resolution implies that the peaks are Gaussian. The known relationship between w , $w_{1/2}$ and σ permits the replacement of w by $4 \cdot w_{1/2}/2.35$.

1. The basic formula then becomes,

$$R = 2 \frac{t_{R(2)} - t_{R(1)}}{4(w_{1/2})_1/2.35 + 4(w_{1/2})_2/2.35} = 1.18 \frac{t_{R(2)} - t_{R(1)}}{(w_{1/2})_1 + (w_{1/2})_2}$$

The formula, proposed in the question, leads to an error of approximately 20%.

2. If these peaks are adjacent then the widths of their bases will generally be comparable. Allowing therefore $w = w_1 = w_2$ and expressing w as a function of N_2 we will have:

$$R = \frac{t_{R(2)} - t_{R(1)}}{w_2} \quad \text{with} \quad \frac{1}{w_2} = \frac{1}{4} \sqrt{N_2} \frac{1}{t_{R(2)}}$$

leading, on combination, to:

$$R = \frac{1}{4} \sqrt{N_2} \frac{t_{R(2)} - t_{R(1)}}{t_{R(2)}}$$

k can then be introduced by incorporating the general expression, $t_R = t_M(1 + k)$,

$$R = \frac{1}{4} \sqrt{N_2} \frac{k_2 - k_1}{1 + k_2}$$

then, finally by introducing $\alpha = k_2/k_1$:

$$R = \frac{1}{4} \sqrt{N_2} \frac{k_2 - k_2/\alpha}{1 + k_2} \quad \text{leading to} \quad \frac{1}{4} \sqrt{N_2} \frac{k_2}{1 + k_2} \frac{\alpha - 1}{\alpha}$$

1.7 1. In the basic relationship

$$R = 2 \frac{t_{R(2)} - t_{R(1)}}{w_1 + w_2}$$

we can replace w by expressing it as a function of N , $w = 4 \frac{t_R}{\sqrt{N}}$ which will give

$$w_1 + w_2 = \frac{4}{\sqrt{N}} (t_{R(1)} + t_{R(2)})$$

and then,

$$R = \frac{1}{2} \sqrt{N} \frac{t_{R(2)} - t_{R(1)}}{t_{R(2)} + t_{R(1)}}$$

since $t_R = t_M(1 + k)$, substitution of this relation leads to,

$$R = \frac{1}{2} \sqrt{N} \frac{k_2 - k_1}{2 + k_1 + k_2}$$

2. In multiplying the second term in the equation above by $k_2 + k_1/k_2 + k_1$ we can write the following intermediate relation:

$$R = \frac{1}{2} \sqrt{N} \frac{k_2 - k_1}{k_2 + k_1} \frac{\bar{k}}{\bar{k} + 1}$$

Then by introducing α and substituting, we arrive at expression 2.

- 1.8 In supposing that the following equation is true:

$$R = \frac{1}{4} \sqrt{N} \frac{k}{1+k} \frac{\alpha - 1}{\alpha}$$

then,

$$N = 16R^2 \left(\frac{\alpha}{\alpha - 1} \right)^2 \left(\frac{k + 1}{k} \right)^2 \quad \text{but} \quad N_{\text{eff}} = N \left(\frac{k}{1+k} \right)^2$$

which on substitution will lead to the expression given in the question.

- 1.9 $R_1 = (0.5 \times 60)/(10 + 12) = 1.36$; $R_2 = 1.47$, ($k_2 = 9.5$, $\alpha = 1.056$ and $N_2 = 15\,270$) $R_3 = 1.5(k_1 = 9)$; $R_4 = 1.5$.
- 1.10 1. The viscosity of the stationary phase increases with temperature as experienced when a pre-programmed rise in temperature induces a loss of charge across the column. As a result the gas flow vector measured, before the analysis is begun, will be reduced. The average velocity of the gas vector is likely to be different from its optimal value, with an increase in pressure necessary which requires that the chromatograph possesses a flow control.
2. The Van Deemter equation of type $H = f(u)$, has the derivative $d(H)/d(u) = -B/u^2 + C$. This expression cancels to zero when $u = (B/C)^{1/2}$. The value reported in the Van Deemter expression leads to $H = A + 2(BC)^{1/2}$. This calculation becomes important if several experimental points (H_i, u_i) are employed to find the coefficients A, B and C of the equation.
- 1.11 The solution of this problem returns us to the initial three lines of exercise 1.7, where we considered employing the retention volumes in place of the retention times.

- 1.12 1. Flow of the mobile phase, k , column temperature.
 2. Yes.
 3. Yes.

2 Gas chromatography

2.1

	Speed of separation	Injection capacity	Retention factor k	Selectivity factor α	Effectiveness
Increase in column length	—	0	0	0	+
Increase in column diam.	0	+	—	0	0
Increase in film thickness	—	+	+	0	+

2.2 1. N_{eff} , k , α .

2. GC: methane. HPLC: uracil, D_2O . . .

3. Following the experiment three equations can be proposed for the three unknowns: t_M , a and b .

$$\log(271 - t_M) = 6a + b \quad (1)$$

$$\log(311 - t_M) = 7a + b \quad (2)$$

$$\log(399 - t_M) = 8a + b \quad (3)$$

— in multiplying (2) by -1 and adding to (1):

$$\log[(271 - t_M)/(311 - t_M)] = -a \quad (4)$$

— in subtracting (2) from (3):

$$\log[(399 - t_M)/(311 - t_M)] = a \quad (5)$$

The resolution of these simultaneous equations yields, $t_M = 237.7$ s.

4. The Kovats graph passes through the points C7 and C8.

$$\log(399 - 237.7) = 2.207 \quad \text{and} \quad \log(311 - 237.7) = 1.863$$

$$\log(246 - 237.7) = 2.033$$

thus $(2.033 - 1.863)/x = (2.207 - 1.863)/1$; so, $x = 0.494$, therefore $I_k = 700 + 49 = 749$. The McReynolds constant of the pyridine on this stationary phase is $749 - 695 = 54$.

2.3 $K = k\beta$ and $\beta = D_i/4e_f$ therefore $e_f = k \cdot D_i/4 \cdot K$. Since $D_i = 200\mu\text{m}$, $k = (200 - 40)/40 = 4$, and $K = 250$, leading to $e_f = 0.8\mu\text{m}$.

2.4 The volume V leaving a capillary column per second is equal to the internal volume of the column multiplied by the length u . The internal section of the column is given by: $\pi D_i^2/4$,

$$V = u\pi D_i^2/4$$

The flow in mL/min (if u and D_i are in cm) is given by $D = 60V$.

If it is chosen to express D_i in mm, this being a value 10 times larger than that given in cm, we must introduce a factor of 0.1 and the expression would then become: $D = 60u\pi D_i^2 \cdot 0.01/4$, leading to $D = 0.47uD_i^2$.

- 2.5
1. We note that the elution order follows accurately the points of the graph. Since the alkenes are more polar than the corresponding alkanes they migrate faster, as would be expected upon a non-polar column.
 2. The selectivity factor is known, $\alpha = k_2/k_1$ and therefore the following values are found for the temperatures indicated: 1.12 (at -35°C), 1.11 (at 25°C), and 1.09 (at 40°C).
 3. Although the column remains the same, the retention times will be decreased as a result of the parallel reduction in the coefficient $K = C_s/C_M$ with the temperature.
 4. 138 489 theoretical plates are indicated from the application of the formula recalled from exercise 2.7.
 5. $H_{\min} = 0.113$ mm.
 6. Coating efficiency: 52%.
 7. From these three expressions:

$$\ln 0.408 = a/238 + b$$

$$\ln 0.148 = a/298 + b$$

$$\ln 0.117 = a/313 + b$$

we find that $a = 0.00814$ and $b = 0.00493$.

- 2.6 Knowing that the retention time for butanol is reduced we can calculate the number of equivalent carbons in an alkane which would have the same retention time. We find 6.45. The Kovats factor of the butanol is therefore 645. The corresponding McReynold's constant will be

$$645 - 590 = 55$$

- 2.7 1. Knowing the retention time and the capacity factor of the second compound we can use the relation, $t_R = t_M(1 + k)$ to calculate the hold-up time, finding: $t_M = 50$ s.
2. The formula proposed enables the effectiveness of the column N_2 to be calculated for the second compound, which is: $N_2 = 22\ 861$. Subsequently, knowing equally that:

$$N_2 = 5.54 t_R^2 / w_{1/2}^2$$

it is easy to isolate $w_{1/2}$, and to calculate its value: 4.67 s. Bearing in mind the scale of the chromatogram, this time interval corresponds to:

$$(4.67/60) \times 10 = 0.77 \text{ mm.}$$

3 High performance liquid chromatography

- 3.1 1. The flow of the mobile phase in a column is proportional to its cross-section and to the linear velocity of progression for *identical contents* ($D = s \cdot v$). The cross-section equally varies with the square of the diameter ($s = k \cdot d^2$). Thus,

$$v_2/v_1 = k d_1^2 D_2 / k d_2^2 D_1$$

$$v_2/v_1 = (1/0.004) \times (0.3/4.6)^2 = 1.06$$

2. The hold-up volume is $V_M = t_M D = 4 \times 4 = 16 \mu\text{L}$.
3. For the final peak: $t_R = 48$ min, thus $V_R = 48 \times 4 = 192 \mu\text{L}$ or 0.192 mL (around 4 drops!). The first advantage of a narrow column is its economy in the use of solvent.
4. The efficiency of the column, as calculated by the classical formula, gives 67034 plates.
5. $k = (48 - 4)/4 = 11$.
6. The stationary phase is of a functionalised silica-type, as indicated by different annotations on the base of the chromatogram.

7. The hold-up volumes are in the same ratio as the two cross-sections of the columns

$$v_{0.3}/v_{4.6} = (0.3/4.6)^2 = 0.0043$$

Elsewhere the coefficient K (or the values of k) of the compounds does not change from one column to another, therefore the retention volumes, $V_R = V_M(1 + k)$, remain the same as the hold-up volumes. The retention volume of the narrow column (0.3 mm) is therefore equal to that of the broader column (4.6 mm) $\times 0.0043$ (a factor of 235 times less).

8. Since the volume injected was the same, it will be 235 times more concentrated in the mobile phase of the narrow column and therefore in theory the signal should be 235 times more intense at the maximum of the peak. The second advantage of narrow columns is that, for detection, they are more sensitive.

- 3.2 At pH 9, the acids are in the form of their corresponding carboxylate ions. These ions have the tendency to be strongly attracted by the polar aqueous phase. The hydrophobic part (the carbon chain), guards their contact with the stationary phase. The polarity of this chain will decrease in the compound order 1, 3 and 2, which is the order of elution for the compounds.

- 3.3 1. c – Reverse phase

2. a – Gel permeation

3. d – Ionic chromatography

4. b – Normal phase

- 3.4 These compounds are very polar and are therefore little retained by a phase of type RP-18. At pH 6, the ATP is the first to be eluted from the column since it is the most polar. ADP will follow and then finally AMP. If, however, an ammonium salt of type phase transfer is added to the mobile phase then dipole–dipole interactions will be created with the three nucleotides. The complex made with the ATP is the strongest and is therefore retained the longest, hence the order of elution is reversed.

- 3.5 1. If the selectivity factor $\alpha = 1$, it must be that the peaks are superimposed and that the retention times are the same which implies that the respective

retention factors k , and therefore $\log k$, also have the same value. The % MeCN is then verified by solving the following expression:

$$-0.0107[\% \text{MeCN}] + 1.5235 = -6.075 = -6.075 \times 10^{-3}[\% \text{MeCN}] + 1.3283$$

leading to MeCN = 42.2%.

2. The values of $\log k$ can be found from the equations given in the text:

$$\text{Compound A: } k_{30} = 14; k_{70} = 8$$

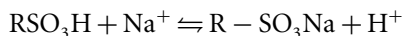
$$\text{Compound B: } k_{30} = 16; k_{70} = 6$$

It is now possible to calculate values of α for the two compositions. When using 30% of MeCN: $\alpha = 16/14 = 1.1428$ while for 70% of MeCN: $\alpha = 8/6 = 1.333$.

In applying the formula yielding the resolution as a function of k and α : $R = 1/4N^{1/2}(\alpha - 1)/\alpha \cdot k/(k + 1)$, we find that substituting the terms in α and k will yield 0.222 for 70% and 0.1176 for 30%. Therefore it is preferable to utilize the MeCN in a proportion of 70%, which also conveys the advantage of leading to a more rapid separation.

4 Ion chromatography

4.1 The stationary phase is of a cationic type. In the dry state it remains in a non-dissociated form but when water is added and in the presence of a high concentration of sodium ions, the following equilibrium is attained in which there is a liberation of H^+ :



The calculation of the molar capacity of the stationary phase is as follows:

$$25.5 \times 0.105/1000 = 2.6775 \times 10^{-3} \text{ mol/g}$$

The equilibrium above is long in establishing itself such that the addition of the sodium hydroxide solution removes all of the protons liberated. A certain time is required for them to reappear in solution.

Note: The *helianthin* $(\text{Me})_2\text{N}(\text{C}_6\text{H}_4)\text{N} = \text{N}(\text{C}_6\text{H}_4) - \text{SO}_3\text{Na}$ is another name for *methyl orange*, which colours red for $\text{pH} < 3.2$ and yellow for $\text{pH} > 4.4$ ($\lambda_{\text{max}} = 505 \text{ nm}$).

- 4.2 1. Such a phase corresponds to a polymer ($250\,000 < M < 900\,000$) comprising acid groups, $-\text{CH}_2\text{COOH}$, and is therefore weakly cationic. The pH of the separation is inferior to the pI of proteins, thus their amino functionality will be fully protonated ($-\text{NH}_3^+$).
2. If the pH is increased then the ionic character of the column is reduced leading to a faster elution of the proteins. Here $pI_1 < pI_2 < pI_3$.
- 4.3 1. The concentration factor is 5 at the beginning since we have 1 mL of plasma and, following treatment of this extract, there will be $200\ \mu\text{L}$ remaining of the solution containing the same amount of cyclosporin as the 1 mL at the beginning.

2. No, because we cannot weigh the collected cyclosporins A or D.

3. a) *Method of internal standard.*

Following convention we will give the letter A to cyclosporin A and D to cyclosporine D as used in the internal standard:

$$k_{A/D} = m_A A_D / m_D A_A = C_A A_D / C_D A_A$$

To calculate the relative response coefficient we will take for example the solution containing 400 ng/mL of cyclosporin A:

$$k_{A/D} = (400/250) \times (1/2.04) = 0.784$$

$$C'_A = C'_D k_{A/D} A'_A / A'_D$$

The chromatogram of the sample for which $A'_A/A'_D = 9/14$ (heights of the peaks measured from the original chromatogram), leads to

$$C'_A = 250 \times 0.784 \times 9/14 = 126\ \text{ng/mL}$$

b) *Method of calibration curve.* Plot the graph of the ratios of the peak heights against concentration C of cyclosporine A.

By the method of least squares we find:

$$R_h = 0.005092C - 0.00411$$

For $R_h = 9/14$, $C = 127.1\ \text{ng/mL}$.

Note: If we evaluate R_h incorrectly then we commit an error of some importance which is of course translated into the result. However, frequently, for very weak concentrations, we can estimate with respect to a limit below

which the error holds less importance, e.g. in taking $R_h = 8/15$ rather than $9/14$, the difference will be 20% and we will find that $C = 104 \text{ ng/mL}$.

- 4.4 Through the application of the general expression we will have a total of four formulae giving the % mass of the various esters. Below is an example for the methyl butanoate ester:

$$\%_{\text{ME}} = 100 \frac{A_{\text{ME}} \times 0.919}{A_{\text{ME}} \times 0.919 + A_{\text{EE}} \times 0.913 + A_{\text{PE}} \times 1.06 + A_{\text{BE}}}$$

We will solve this by taking and substituting the areas given in the question:

$$\%_{\text{ME}} = 16.6$$

$$\%_{\text{EE}} = 16.6$$

$$\%_{\text{PE}} = 33.4$$

$$\%_{\text{BE}} = 33.4$$

Note: The scale in mV corresponds to UV detection.

- 4.5 1. The addition of N-methylserotonin before the extraction is made removes the necessity of counting any eventual loss of product due to the different intermediate manipulations. We can suppose that the yield of the extraction is the same for the two compounds, which are structurally very similar.
2. To determine the relative response factor $k_{\text{S/NMS}}$ of the serotonin (S), with respect to its N-methyl derivative (NMS), the following relation is used:

$$k_{\text{S/NMS}} = \frac{m_{\text{S}}}{m_{\text{NMS}}} \times \frac{A_{\text{NMS}}}{A_{\text{S}}} = \frac{5}{5} \times \frac{30956727}{30885982} = 1.002$$

3. Measuring the sample:

$$m_{\text{S}} = m_{\text{NMS}} k_{\text{S/NMS}} \frac{A'_{\text{NMS}}}{A'_S} = 30 \times 1.002 \frac{2573822}{1719818} = 45 \text{ ng/mL}$$

which gives a concentration of approximately 45 ppb, for an aqueous solution.

5 Thin layer chromatography

5.1 1. $R_{F(A)} = 27/60 = 0.45$; $R_{F(B)} = 33/60 = 0.55$

$$N_A = 16 \times 27^2 / 2^2 = 2916; N_B = 16 \times 33^2 / 2.5^2 = 2788$$

$$H_A = x_A / N_A = 9.26 \times 10^{-4} \text{ cm}; H_B = x_B / N_B = 1.18 \times 10^{-3} \text{ cm}$$

2. $R = 2(33 - 27) / (2 + 2.5) = 2.67$

3. From the definition for the selectivity factor and from the relation linking R_F and k , we arrive at:

$$\alpha = (R_{F(B)} / R_{F(A)}) \cdot (1 - R_{F(A)}) / (1 - R_{F(B)}) = 1.49$$

5.2 1. Since it arises from a normal phase, the more polar compound will be retained the longest. In order of increasing migration distances we will have C, then B and finally A.

2. The order of elution will be A, B then C, reflecting the migration times.

3. Reverse order.

4. Approximate value for $R_{F(A)} = 22.5/50 = 0.45$. HETP = L/N with $N = 5.54x^2/d^2 = 5.54 \times 22.5^2/2^2 = 701$, leading to HETP = $50/701 = 0.07$ mm.

7 Size exclusion chromatography

7.1 In size exclusion chromatography $K < \text{or} = 1$, excepting cases where interactions develop between the solute and stationary phase since this creates a partition phenomenon which superimposes itself upon the diffusion in the pores.

7.2 By placing the following two columns end to end, first column C will separate the masses of 3×10^6 and 1.1×10^6 Da from the remaining two. These two masses will subsequently be separated by the column A. On the corresponding chromatogram we will obtain four distinct peaks.

7.3 1. See graph.

2. The total exclusion or interstitial volume is approximately 4.2 mL. The intraparticle volume of the pore is close to: $7.9 - 4.4 = 3.5$ ml.

3. For the mass of 3 250 Da, $K = (5.4 - 4.4) / (7.9 - 4.4) = 0.29$

8 Capillary electrophoresis and electrochromatography

- 8.1 1. Since it is known that $\mu_{\text{app}} = \mu_{\text{EP}} + \mu_{\text{EOS}}$ therefore $\mu_{\text{EP}} = \mu_{\text{app}} - \mu_{\text{EOS}}$ and since the experiment allows access to the velocities: v_{app} and v_{EOS} . Then by substitution

$$v_{\text{app}} = 24.5 / (60 \times 2.5) = 0.163 \text{ cm/s}$$

and

$$v_{\text{EOS}} = 24.5 / (60 \times 3) = 0.136 \text{ cm/s.}$$

As a result: $v_{\text{EP}} = 0.163 - 0.136 = 0.027 \text{ cm/s.}$

$$\mu_{\text{EP}} = v_{\text{EP}} / E = 0.027 \times 32 / 30\,000 = 2.88 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \text{ V}^{-1}.$$

$$2. D = l^2 / (2Nt_m) = 24.5^2 / (2 \times 80\,000 \times 150) = 2.5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}.$$

- 8.2 1. See the scheme in the book.

2. No, since it arises from a non-treated internal surface, which at the pH considered acts as a polyanion. This results in the creation of an electroosmotic flow. The fact that the compound migrates towards the cathode does not necessarily mean that it is carrying a net positive charge. The possibility remains that it is being trained in that direction, even if carrying a negative charge. Following convention:

3. $l = v_{\text{app}} t$ and $\mu_{\text{app}} = v_{\text{app}} / E = v_{\text{app}} L / V$ therefore $\mu_{\text{app}} = lL / Vt$.

$$\mu_{\text{app}} = 7.5 \times 10^{-4} \text{ cm}^2 \text{ s}^{-1} \text{ V}^{-1}$$

4. Following the same reasoning, this leads to $\mu_{\text{EOS}} = lL / Vt_m$.

$$\mu_{\text{EOS}} = 1.5 \times 10^{-3} \text{ cm}^2 \text{ s}^{-1} \text{ V}^{-1}$$

5. $\mu_{\text{EP}} = \mu_{\text{app}} - \mu_{\text{EOS}}$ thus $\mu_{\text{EP}} = -7.5 \times 10^{-4} \text{ cm}^2 \text{ s}^{-1} \text{ V}^{-1}$

6. The negative character of μ_{app} implies that it originates from a species carrying a net negative charge. The compound will migrate more slowly than a neutral marker.

7. If the internal lining is rendered neutral there would be no more electroosmotic flux and as a result the compound will no longer migrate towards the cathode but will reappear in the anode compartment.

8. If the pI is 4 for all pH less than 4, then the compound will be in the form of a cation. In this case, the migration time will normally be shorter than for a neutral marker.
9. In using the formula recalled in the question, we will find 337 500 theoretical plates.
10. A small molecule diffuses faster than a larger one. Therefore the effectiveness is greater for molecules of greater mass.

8.3 The two control compounds enable the following simultaneous equations to be written:

$$\log 45\,000 = 1.5a + b \quad (1)$$

$$\log 17\,200 = 5.5a + b \quad (2)$$

The resolution of these two equations lead to $a = -0.1$ and $b = 4.8$. Therefore, $\log M = -0.1v + 4.8$ and by substituting $v = 3.25$ we find

$$\log M = 4.48 \quad \text{and so} \quad M = 29\,854 \text{ Da.}$$

Note that the stationary phase does not behave like an SEC gel since it creates obstacles for the larger molecules which are forced to migrate more slowly than their smaller counterparts.

8.4 If the isoelectric pH of B is superior to that of A then this is because the pH at B oversteps with respect to the position of A. Therefore the pH increases from the right to the left of the capillary.

If B is displaced further towards the right it will become positively charged and will therefore migrate towards the left and its original position which is stable. The positive pole is therefore to the right and the negative one to the left. The electric field therefore applies itself from right to left on the corresponding diagrams.

8.5

Protein	pI	pH = 3	pH = 7.4	pH = 10
Insulin	5.4	+	-	-
Pepsin	1	-	-	-
Cytochrome C	10	+	+	0
Haemoglobin	7.1	+	-	-
Albumin serum	4.8	+	-	-

9 Ultraviolet and visible absorption spectroscopy

- 9.1 The application of the expression $E = h\nu$ for one mole is written $E = N.h\nu = N.h.c/\lambda$ leading to:

$$E = 6.022 \times 10^{23} \times 6.6262 \times 10^{-34} \times 3 \times 10^8 / (300 \times 10^{-9})$$

$$= 399\,030 \text{ J}, 399 \text{ kJ or, } 399/4.18 = 95.5 \text{ kcal.}$$

- 9.2 $A = \epsilon.l.C = 650 \times 7 \times 10^{-4} \times 2 = 0.91$. If the optical path length is doubled the absorbance will equally be doubled: $A = 2 \times 0.91 = 1.82$.

- 9.3 1. If $T = 0.5$, $A = \log 1/0.5 = 0.3$.

$$\text{Since, } A = \epsilon.l.C, \epsilon = 0.3/1.28 \times 10^{-4} = 2\,344 \text{ l mol}^{-1} \text{ cm}^{-1}.$$

2. If the concentration is doubled, $A = 0.6$, therefore $\log 1/T = 0.6$ thus $T = 0.25$ and the percentage transmittance is 25%.

- 9.4 The concentration of a solution of 0.1 ppm is $0.1 \times 10^{-3} \text{ g/L}$. The molar concentration is therefore $0.1 \times 10^{-3}/52 = 1.92 \times 10^{-6} \text{ mol/L}$. We can thus calculate:

$$l = A/(\epsilon.C) = 0.4/(41\,700 \times 1.92 \times 10^{-6}) = 4.98 \text{ cm}$$

Therefore a cell of 5 cm pathlength is well adapted.

- 9.5 If 90% of the radiation is absorbed, $T = 0.1$. Thus $A = 1$ and $C = 1/(0.03 \times 15\,000) = 2.22 \times 10^{-3} \text{ mol/L}$. For $M = 500 \text{ g/mol}$, we find, that $m = 1.11 \text{ g/L}$.

- 9.6 1. $207 + 12 = 219 \text{ nm}$ (exp $\lambda_{\text{max}} = 218$ in EtOH);

2. $215 + 12 + 12 = 239 \text{ nm}$.

3. $215 + 60 + 5 + 12 + 18 + 39 = 349 \text{ nm}$ (exp $\lambda_{\text{max}} = 348$ in EtOH);

4. $215 + 5 + 10 + 12 = 242 \text{ nm}$.

9.7 To resolve this problem we use the fact that absorbances are additive:

$$A_T = C_A \cdot l \cdot \epsilon_A + C_B \cdot l \cdot \epsilon_B$$

From these two reference solutions we can calculate ϵ_A and ϵ_B at 510 nm:

$$\epsilon_A = A_A / (C_A \cdot l) = 0.714 / (1.5 \times 10^{-1}) = 4.760$$

$$\epsilon_B = A_B / (C_B \cdot l) = 0.298 / (6 \times 10^{-2}) = 4.967$$

and calculate the same for ϵ'_A and ϵ'_B at 575 nm:

$$\epsilon'_A = A'_A / (C_A \cdot l) = 0.097 / (1.5 \times 10^{-1}) = 0.647$$

$$\epsilon'_B = A'_B / (C_B \cdot l) = 0.757 / (6 \times 10^{-2}) = 12.617$$

By virtue of the addition of absorbances we can now write for the mixture:

$$0.671 = 4.76 C_A + 4.967 C_B$$

$$0.330 = 0.647 C_A + 12.617 C_B$$

The resolution of this two equation system leads to:

$$C_A = 1.2 \times 10^{-1} \text{ mol/L} \quad \text{and} \quad C_B = 2.0 \times 10^{-2} \text{ mol/L.}$$

9.8 1. See graph.

2. The equation of the graph (absorbance against concentration) is:

$$A = 1.3771 C + 0.0024 \quad (R^2 = 0.9987).$$

3. For an absorbance of 0.45, $C = 0.325 \text{ mol/L}$.

9.9 1. The equation of the calibration graph is: $Y = 0.232C + 0.162$ ($R^2 = 0.9834$).

2. For a reading of $Y = 3.67$, $C = 15 \text{ mg/L}$ in the sulphate ion.

10 Infrared spectroscopy

10.1 1. A wavenumber of 1000 cm^{-1} corresponds to a wavelength of $10 \mu\text{m}$ as $E = h\nu = hc/\lambda$. $E = 6.62 \times 10^{-34} \times 3 \times 10^8 / (10 \times 10^{-6}) = 1.99 \times 10^{-20} \text{ J}$. For one mole we would have $12\,000 \text{ J}$ (that is 2.87 kcal/mol).

$$2. \quad \bar{\nu} = \frac{1}{15 \times 10^{-4}} = 666.67 \text{ cm}^{-1}$$

3. The absorbance A is such that: $A = \log 1/T$. If $T = 0.05$, $A = \log 1/0.05 = 1.3$.

10.2 We can accept that the force constant k remains the same for the two species since they are isotopes of the same element. Thus $\mu_{\text{H}} = (12 \times 1)/(12 + 1)$ amu and $\mu_{\text{D}} = (12 \times 2)/(12 + 2)$ amu.

$$\frac{\bar{\nu}_{\text{H}}}{\bar{\nu}_{\text{D}}} = \sqrt{\frac{\mu_{\text{D}}}{\mu_{\text{H}}}}$$

We will find $\bar{\nu}_{\text{D}} = 2215 \text{ cm}^{-1}$ which represents a difference of less than 2% from the experimental value.

10.3 The approximate mass of the carbonyl group will be:

$$\mu_{\text{CO}} = \frac{12 \times 16}{12 + 16} 1.66 \times 10^{-27} = 1.138 \times 10^{-26} \text{ kg.}$$

then substituting,

$$k = 4\pi^2 \bar{\nu}^2 c^2 \mu_{\text{CO}} = 4\pi^2 \times 1710^2 \times (3 \times 10^{10})^2 \times 1.138 \times 10^{-26} = 1183 \text{ N/m}$$

(Note the speed of light must be expressed in cm/s here).

10.4 A photon corresponding to 2000 cm^{-1} will transport an energy of

$$E = hc/\lambda = 3.972 \times 10^{-20} \text{ J.}$$

This energy is transformed into mechanical energy: $E_{\text{TOT}} = E_{\text{KIN}} + E_{\text{POT}}$. At the maximum of the elongation Δx , $E_{\text{KIN}} = 0$ because the velocity is zero. The energy of the photon E is therefore entirely in the form of potential energy: $\Delta E_{\text{POT}} = 1/2k(\Delta x)^2$. $\Delta E_{\text{POT}} = E$ carried by the photon.

$$\Delta x = \sqrt{\frac{2\Delta E}{k}} = \sqrt{\frac{2 \times 3.972 \times 10^{-20}}{1000}} = 8.91 \times 10^{-12} \text{ m}$$

which is about 6% of a bond whose length is 0.15 nm.

10.5 The two sets of vibrational frequencies have for their origin the two isotopes of chlorine in the sample of HCl (75% of ^{35}Cl and 25% of ^{37}Cl). When the

same transition is considered for the two molecules, reunited in the sample, only the reduced mass differs, leading to the separation. The calculation leads to: $\mu_{(35)\text{Cl}}/\mu_{(37)\text{Cl}} = 0.9985$.

$$\frac{\bar{\nu}_{37}}{\bar{\nu}_{35}} = \sqrt{0.9985} = 0.9992$$

Thus towards $3\,000\text{ cm}^{-1}$ the difference is 2.3 cm^{-1} .

10.6 A similar calculation as exercise 10.3 leads to $k = 1\,846\text{ N/m}$.

10.7 1. By linear regression we find: $A/d = 0.0009x + 0.0003$ (1), where x is the VA% in film.

2. By the method of least squares we find: $A_{1030}/A_{720} = 0.0531x + 0.0047$ (2).

3. From equation (1), for the unknown film VA% = 8.44; from equation (2) VA% = 8.46.

10.8 1. From the application of the Fellgett advantage: in calling S/N the signal to noise ratio:

$$S/N = 10 \times (1/16)^{1/2} = 2.5$$

2. $\Delta = 2x$ therefore $R = 1/2x$ and so $x = 1/2R = 0.5\text{ cm}$.

3. If $\nu = 15\,800\text{ cm}^{-1}$, $\lambda = 0.633\text{ }\mu\text{m}$. In passing from one minimum to the next the mirror must be displaced by a distance corresponding to a half-wave-length which is $0.316\text{ }\mu\text{m}$.

11 Fluorimetry and chemiluminescence

11.1 The wavenumber corresponding to a wavelength of 400 nm is $25\,000\text{ cm}^{-1}$ ($1/400 \times 10^{-7} = 25\,000$). The Raman peak is displaced towards longer wavelengths and will be located at $25\,000 - 2880 = 22\,120\text{ cm}^{-1}$. This value corresponds to a wavelength of 452 nm ($10^7/22\,120$).

11.2 A wavelength of 250 nm corresponds to a wavenumber of $40\,000\text{ cm}^{-1}$. The Raman peak of water will therefore be situated at $40\,000 - 3380 = 36\,620\text{ cm}^{-1}$, corresponding to a wavelength of 273.1 nm .

- 11.3 1. The use of the same control source of fluorescence to compare the measurements made under the same conditions leads to the following simple calculations: the concentration of the unknown sample solution is $(60/40) \times 0.1 = 0.15$ ppm or 150 ppb.
2. We can calculate the difference in cm^{-1} between the light of the source and 456 nm (measurement), to see if the displacement is $3\,380\text{ cm}^{-1}$. By the introduction of a fluorospectrometer we could equally judge, by studying the spectra, the effects upon the fluorescence of a displacement of the wavelength of excitation of several nanometers.

- 11.4 1. The benzopyrene has a rigid polycondensed aromatic structure, typical of fluorescent compounds. Forming part of the PAH, it is currently measured by HPLC in drinking water by using a method of detection by fluorescence.
2. The fluorescences being additive, the analysis begins with the correction of the values read for the three solutions by subtracting the value read for the blank. These new values become 21, 35.1 and 29.8. To calculate the concentration of the sample solution one can write: $(1.25 - 0.75)/(35.1 - 21) = (x - 0.75)/(29.8 - 21)$, finding $x = 1.062\text{ }\mu\text{g/mL}$.
3. This value corresponds to the mass of benzopyrene which is found in 1 litre of air ($\rho = 1.3\text{ g/L}$). The mass concentration is therefore

$$1.06 \times 10^{-6}/1.3 = 0.815\text{ ppm} \quad \text{or} \quad 815\text{ ppb.}$$

- 11.5 The method followed is that of addition by measurement: we will resolve this problem easily. In 1 mL of the solution of iron $5.15 \times 10^{-5}\text{ M}$ there are 5.15×10^{-8} moles of Fe. We will therefore have

$$29.6/(5.15 \times 10^{-8} + x) = 16.1/x,$$

which leads to $x = 6.142 \times 10^{-8}$ moles for 2 mL. From this the concentration of the solution of Fe II is $3.07 \times 10^{-5}\text{ M}$.

- 11.6 1. Hydroxyquinoline and zinc form a chelate (one atom of the metal is inserted between two molecules of the complexant), which can be extracted with tetrachloromethane and which yields fluorescence. The four solutions treated being each in the same manner suggests that we might suppose the yields of the extraction to be identical.

2. By application of the least squares calculation the following equation can be derived:

$$I_f = 1.5V + 7.74 \quad (\text{VmL})$$

3. For an intensity of fluorescence $I_f = 0$, we will find $V = 5.16$ mL. In 1 litre of the solution B there is 0.0136 g of zinc chloride, which is $6.538 \mu\text{g/mL}$ of Zn. Therefore 5 mL of unknown solution contains $6.538 \times 5.16 = 33.736 \mu\text{g}$ of Zn. Per litre there will be 200 times more, i.e. $6747.2 \mu\text{g}$, representing a concentration of 6.75 ppm.

- 11.7 1. The five standards (1 to 5) correspond to the following concentrations C (in g/L), (1) = 0.0001; (2) = 0.00008; (3) = 0.00006; (4) = 0.00004; (5) = 0.00002. With these values derived from the method of least squares the equation of the graph is:

$$I_f = 1773285C + 2.22$$

2. The sample of drink whose fluorescence is 113 following a dilution by a factor of 1000 corresponds to a calculated concentration of 6.25×10^{-5} g/L. The drink, non-diluted, yields a concentration of 6.25×10^{-2} g/L which is approximately 62.5 ppm.
3. When we follow a protocol we make measurements of fluorescence at a fixed wavelength which renders the trace of the spectrum redundant.

12 X-ray fluorescence spectrometry

- 12.1 1. The solution is an equilibrated mixture of the elements constituting the sample. In 8 g of KI (166 g/mole), there are:

$$8 \times 39/166 = 1.98 \text{ g of potassium and } 8 \times 127/166 = 6.12 \text{ g of iodine.}$$

In 92 g water (18 g/mole), there are:

$$92 \times 2/18 = 10.22 \text{ g of hydrogen and } 92 \times 16/18 = 81.78 \text{ g of oxygen.}$$

The balance of the mixture by gram is:

$$\begin{aligned} \mu_m &= (1.98 \times 16.2 + 6.12 \times 36.3 + 1.2 \times 81.78 + 10.22 \times 0.4)/100 \\ &= 3.56 \text{ cm}^2/\text{g}. \end{aligned}$$

2. $\mu = 3.56 \times 1.05 = 3.738 \text{ cm}^{-1}$ since $P = P_0 \exp[-\mu x]$, thus finding $P/P_0 = 0.024$ which is 2.4%.

12.2 $E = h.c/\lambda$ therefore $\lambda = h.c/E$

$$\begin{aligned}\lambda_{\text{nm}} &= 10^9 \times (6.626 \times 10^{-34} \times 2.998 \times 10^8) / (E_{\text{eV}} \times 1.602 \times 10^{-19}) \\ &= 1240 / E_{\text{eV}} \\ \lambda_{\text{\AA}} &= 1240 \times 10 / (E_{\text{keV}} \times 1000) = 12.4 / E_{\text{keV}}\end{aligned}$$

12.3 1. $2d \sin \theta = k.\lambda$ (here $k = 1$)

$$\sin \theta = 8.126 / (2 \times 4.404) = 0.9226 \text{ therefore } \theta = 67.31^\circ$$

The deviation $2\theta = 134.62^\circ$.

2. $E = h.c/\lambda$ substituting $(6.626 \times 10^{-34} \times 3 \times 10^8) / (0.209 \times 10^{-10}) = 9.517 \times 10^{-15} \text{ J}$ or 59 480 eV.

- 12.4 1. The crystal sweeps through the angle θ between two extreme values. By the application of $2d \sin \theta = k.\lambda$, if $\theta = 10^\circ$, $\lambda = 0.047 \text{ nm}$ and if $\theta = 75^\circ$, $\lambda = 0.262 \text{ nm}$.

2. The formula for the conversion $\lambda_{\text{\AA}} = 12.4 / E_{\text{keV}}$ leads, for the two wavelengths above, to $E = 26.38 \text{ keV}$ and $E = 4.73 \text{ keV}$, respectively.

12.5 1. $\Delta E/E = \Delta\lambda/\lambda$, therefore $\Delta E = E\Delta\lambda/\lambda = (h.c/\lambda).(\Delta\lambda/\lambda) = (h.c/\lambda^2).\Delta\lambda$ $\Delta E = 1.9284 \times 10^{-19} \text{ J}$ or $1.9284 \times 10^{-19} / (1.6 \times 10^{-19}) = 1.2 \text{ eV}$.

2. We will not be able to distinguish these two transitions arising from the sulphur atom.

3. A variation of $2 \times 10^{-4} \text{ nm}$ corresponds to a difference in energy of less than 1 eV, which will be invisible on the spectrum.

- 12.6 The table of mass attenuation coefficients indicates that for aluminium and the $K\alpha$ of copper, $\mu_{\text{m}} = 264 \text{ cm}^2/\text{g}$. Knowing the density of this metal leads us to the linear coefficient:

$$\mu = 264 \times 2.66 = 702 \text{ cm}^{-1}.$$

The fraction transmitted: $P/P_0 = e^{-702 \times 12.10^{-4}} = 0.43$. By consequence 57% of the radiation is absorbed by the aluminium film.

For the emission $K\alpha$ of silver, $\mu_m = 2.54 \text{ cm}^2/\text{g}$. By application of the same calculations we will find that $P/P_0 = 0.99$. In this case only 1% of this more energetic radiation will be absorbed.

In initial calculation of the coefficient for the latex ($M = 68 \text{ g/mole}$), we are aided by the values given in the question:

$$\mu_m = (60 \times 25.6 + 8 \times 0.43)/68 = 22.64 \text{ cm}^2/\text{g}.$$

The same sequence of calculations will lead to $P/P_0 = 0.89$.

- 12.7 1. At least one electron is required in the shell 'L' e.g. $n = 2$.
2. This is because the constituents of air absorb weakly energetic radiation of fluorescence X. The helium is almost completely transparent.
- 12.8 For the X-ray beam to reach the detector with sufficient intensity the incident radiation will be required to hit the crystal over a surface large enough such that thousands of atoms reflect the light in an identical fashion. If the angle of refraction is different from the angle of incidence then successive atoms from the same horizontal reticular plane will return the radiation with an optical displacement which will appear as interference.
- To avoid this we observe the reflection through an angle equal to the angle of incidence. The differences of pathway only cause interference due to successive horizontal reticular planes.
- 12.9 We begin by establishing the ratio Mn/Ba for the two solid standard solutions. Therefore sol 1 = 0.813 and sol 2 = 0.9625 we can write:

$$(x - 0.25)/(0.886 - 0.811) = (0.35 - 0.25)/(0.9625 - 0.811)$$

leading to: $x = 0.30\%$.

- 12.10 The coefficient of mass attenuation for water for the emission $k\alpha$ of Ni is:

$$\mu_m = (16 \times 13.8 + 2 \times 0.43)/18 = 12.31 \text{ cm}^2/\text{g}.$$

Since $\rho = 1$, $\mu = 12.31 \text{ cm}^{-1}$. We will find that $P/P_0 = e^{-12.31} = 4.5 \times 10^{-6}$. Therefore 1 cm of water is sufficient to stop almost all radiation of this particular strength.

- 12.11 The mass attenuation coefficient of air for a radiation whose energy is 2keV is $\mu_m = (0.8 \times 494 + 0.2 \times 706) = 536.4 \text{ cm}^2/\text{g}$. Since $\rho = 0.0013 \text{ g/mL}$, $\mu = 0.697 \text{ cm}^{-1}$ and $P/P_0 = 0.03$. Attenuation attains 97%.

13 Atomic absorption and flame emission spectroscopy

- 13.1 For a given temperature, $R = N_e/N_o = \gamma \cdot \exp[-\Delta E/kT]$. By combining this equation for the tube values of R such that R_2/R_1 the ratio of the two values of R to 2 000 K and 2 500 K leads to

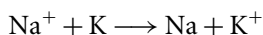
$$\frac{R_2}{R_1} = \exp \left[\frac{\Delta E}{K} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \right]$$

For the resonance emission of sodium the value of $\Delta E = h.c/\lambda$ is

$$\Delta E = 3.37 \times 10^{-19} \text{ J (or } 2.1 \text{ eV)}.$$

Knowing that $k = 1.38 \times 10^{-23} \text{ JK}^{-1}$, $R_2/R_1 = 11.5$. The measurement will therefore be almost 12 times more sensitive at 2500 than at 2000 K.

- 13.2 EDTA yields complexes with a ratio of 1:1 with many metals. Better known among these complexes are those of bivalent cations which lead to hexacoordinated ligands bound through the four acid functions and the two nitrogen atoms. The chelated ions pass more easily to the atomic state in the flame since their volatility is increased.
- 13.3 Only a small proportion of sodium atoms are excited to the atomic state in the flame, which leads to an underestimate of the quantity of this element in a sample. If one adds potassium salt in a sufficient quantity with respect to the atoms of sodium, then a large number of potassium atoms will be present in the flame. These atoms lead to an oxydo-reduction reaction,



In fact, according to the two ionisation potentials given, less energy is required to remove the least held electron within the potassium atom than for the corresponding electron of sodium.

- 13.4 The quantity of potassium ions introduced and expressed in moles is:

$$0.2 \times 10 \times 10^{-6} = 2 \times 10^{-6} \text{ mole}$$

If the total volume of the solution has not changed and we call x the number of moles of potassium in the sample: $32.1/x = 58.6/(x + 2 \times 10^{-6})$ from where it can be deduced that $x = 2.42 \times 10^{-6}$ mole. This quantity is present in 0.5 mL of serum, which is:

$$2.42 \times 10^{-6} \times 1000/0.5 = 4.84 \times 10^{-3} \text{ mol/L.}$$

- 13.5 If we call the signal of absorbance of the sample extracted from paprika A_X and the sample solution A_R , m_X and m_R are the corresponding masses of lead and we will have:

$$A_X/A_R = m_X/m_R \quad \text{therefore} \quad m_X = m_R A_X/A_R$$

$$A_X = 1220, A_R = 1000; \quad m_R = 10 \times 0.01/1000 = 1 \times 10^{-4} \text{ g}$$

The sample introduced into the graphite furnace contains:

$$m_X = 1 \times 10^{-4} \times 1220/1000 = 1.22 \times 10^{-4} \text{ g.}$$

The % mass is therefore $1.22 \times 10^{-4}/10^{-2} = 1.22\%$.

- 13.6 Calcium chloride dihydrate has a molar mass of 147.1 g. The concentration of the parent solution is $1.834/147.1 = 0.01247$ mol/L. Solution diluted 10: 1.247×10^{-3} mol/L. The different solutions made up as standards have the following molar concentrations in Ca^{++} :

Standard 1/20:	0.623×10^{-4} mol/L	signal 10.6
Standard 1/10:	1.247×10^{-4} mol/L	20.1
Standard 1/5:	2.494×10^{-4} mol/L	38.5
Analytical blank:	0 mol/L	1.5

The equation of the calibration curve is: $[\text{signal}] = 148900C + 1.407$. We will find that the unknown solution is such that $C = 1.89 \times 10^{-4}$. The parent solution has a concentration 25 times greater, being 4.73×10^{-3} mol/L, or 0.19 g/L in Ca^{++} .

- 13.7 1. This parameter arises from the spectral band which is selected by the exit slit and will reach the detector. This is not the physical width of the exit slit which cannot be less than several micrometres.
2. EDTA has the molecular formula $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$ while the mixed salt of zinc and sodium is $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_8\text{Na}_2\text{Zn}$.

The mass concentration of the parent solution is $35.7 \times 10 = 357 \text{ mg/L}$. The diluted solution has the concentration $C = 2 \times 357/100 = 7.14 \text{ mg/L}$. The apparatus indicates that this solution corresponds to a concentration of 0.99 mg/L in zinc. As a result the molar mass of the salt is $7.14 \times 65.39/0.99 = 471.6 \text{ g/mol}$.

The salt in the anhydrous state has a molar mass of 399.6 g/mol . We can therefore deduce that the difference $(471.6 - 399.6) = 72 \text{ g}$ represents the mass of water, per mole, of this salt, which is $72/18 = 4$ moles. We conclude therefore that the hydrate comprises four molecules of water.

3. $A = 0.321C + 0.03$, for $A = 0.369$, $C = 1.14 \text{ mg/L}$, a value too high, which shows that for this measurement the calculation of least squares is not well adapted.
4. The general answer to this question is no, since the experimental points are not aligned such that the range of concentrations extends more than 1 mg/L . The apparatus proposes curves of preference.

14 Atomic emission spectroscopy

- 14.1 The elements which are measured are often in much smaller concentrations than all those constituting the matrix. Often it is necessary to identify characteristic emissions of trace elements (in concentrations of the order of ppm or less), mixed with elements which equally will yield emissions but whose concentrations can only attain 10 or 20%.

We observe ionic emissions because the temperatures are very high and the plasma is a medium rich in argon ions and in free electrons which provoke ionisation due to collisions with non-ionised atoms.

- 14.2 From $E = h\nu$, and if $\Delta E \cdot \Delta t > h/2\pi$ then $\Delta\nu > 1/2\pi \Delta t$. Then $\nu = c/\lambda$, so $\Delta\nu = (c/\lambda^2)\Delta\lambda$.

Therefore,

$$\Delta\lambda \geq \frac{1}{2\pi\Delta t} \frac{\lambda^2}{c}$$

If $\lambda = 589 \text{ nm}$ and $\Delta t = 10^{-9} \text{ s}$, we will find $\Delta\lambda > 1.84 \times 10^{-13} \text{ m}$ ($1.84 \times 10^{-4} \text{ nm}$).

Note: The imperfections of spectrometers and the Doppler and Stark effects contribute to an important enlargement of the image of the entrance slit of the apparatus.

- 14.3 From the data below we can construct the calibration graph of the ratio of the signal emission Pb/Mg against concentration.

$$\text{Equation of the graph: } [\text{ratio Pb/Mg}] = 8.219 C + 0.345$$

For the two solutions: A = 0.118 mg/L and B = 0.376 mg/L.

- 14.4 The expression declares that the transition in energy which corresponds to the emission of resonance is $16\,960\text{ cm}^{-1}$. This unit is employed to measure the energies ($E = h.c/\lambda$) with $\lambda = 1/\nu$ and $\lambda = 589.62\text{ nm}$. It is the first component of the doublet called the resonance line ($E = 2.102\text{ eV}$). The second is not a resonance emission.

- 14.5 Measuring radioactivity using a Geiger counter requires a sufficient number of disintegrations to be accumulated in order to obtain a reliable result. If the half-life of the radionucleus is very long and the element not very abundant then the rhythm of the decompositions will not be significant enough to be distinguished from the background noise. An isolated counting can differ enormously from the average since radioactivity results from a series of random events which do not follow Gaussian behaviour.

Alternatively, the method of atomic emission works upon the total atomic population of the isotope.

Example: 1 pCi/1 of ^{237}Np (half-life 2.2×10^6 years) corresponds to a population of $N = A/\lambda = 3.7 \times 10^{12}$ atoms, which can be represented as $1.5 \times 10^{-9}\text{ g/L}$ (or 1.5 ppt).

Such a concentration is at the limit of the method based upon counting yet nonetheless is reliable for atomic emission spectroscopy (AES).

- 14.6 The linear dispersion represents the distance which separates, in mm, two wavelengths which differ by 1 nm. If the exit slit is $20\text{ }\mu\text{m}$ ($2 \times 10^{-2}\text{ mm}$), the bandwidth reaching the detector will be:

$$1 \times (2 \times 10^{-2})/2 = 1 \times 10^{-2}\text{ nm}(10\text{ pm}).$$

15 Nuclear magnetic resonance spectroscopy

- 15.1 $\gamma = \mu_z/m$ with $m = h/4\pi$, thus $\gamma = 4\pi\mu_z/h = 1.41 \cdot 10^{-26} \times 4 \times \pi / (6.6262 \times 10^{-34})$. $\gamma = 2.674 \times 10^8\text{ rad T}^{-1}\text{ s}^{-1}$

15.2 The ratio of the populations is: $N_{E(1)}/N_{E(2)} = e^{\Delta E/KT}$ with $\Delta E = \gamma h B_0 / 2\pi$ from the calculation 1.0000095. If $B_0 = 7\text{ T}$, this ratio becomes 1.0000477.

15.3 1. If on the scale 4 cm represents 200 Hz (then 40 mm = 200 Hz), 7 H will correspond to

$$7/200 \times 40 = 1.4 \text{ mm.}$$

2. If the apparatus operate at 200 MHz for ^1H , it is $n_C = n_H \cdot \gamma_C / \gamma_H$ for ^{13}C . $\gamma_C = 200 \times 1/3.98 = 50 \text{ MHz}$ and 7 Hz will now correspond to $(7/50) \times 40 = 5.6 \text{ mm}$.

15.4 1. The frequency of the apparatus is

$$2.6752 \times 10^8 \times 1.879 / (2 \times 3.1416) = 80 \text{ MHz}$$

The chemical shift is therefore: $220/80 = 2.75 \text{ ppm}$.

2. The shift in Hz will be: $90 \times 200/60 = 300 \text{ Hz}$.

3. The chemical shifts do not change when they are expressed in ppm.

15.5 The proton spectrum of A presents a doublet of doublets. Two different values for the coupling constants can be noted; one large, the other small ($A = 2$).

The proton spectrum of B presents a triplet, therefore the proton is located at the same distance from two atoms of fluorine since the value of the coupling constant is weak and can be considered to originate from compound 3 ($B = 3$).

The third isomer gives a triplet whose coupling constant will be of the order of the distance separating the doublets of B, $1.2 \times 60 = 72 \text{ Hz}$. The structure corresponding to this spectral description is isomer 1.



15.6 When the nucleus of spin $I = 1/2$ is introduced into the magnetic field B_0 , it self-projects into an orientation which follows the rule, $m = (\frac{1}{2}) / (\frac{h}{2}\pi)$. Knowing the value for the spin vector we can write:

$$(\sqrt{3}/2)(h/2\pi) \cos \theta = 1/2(h/2\pi)$$

which is

$$\cos \theta = 1/\sqrt{3} \quad \text{or} \quad \theta = 54.7^\circ$$

- 15.7 In NMR the intensity of the signals reflects the molar concentrations. Vanillin has a molar mass of 152 g, thus for 25 mg of this compound:

$$25 \times 10^{-3} / 152 = 1.645 \times 10^{-4} \text{ mole}$$

Since 1 proton of the vanillin corresponds to a value of integration which is half as strong as that of the proton of the unknown compound, we can deduce that the number of moles of the latter is twice as great, being $1.645 \times 10^{-4} \times 2 = 3.29 \times 10^{-4}$ mole in 0.1 g of sample. The molar mass of the compound is therefore $0.1 / (3.29 \times 10^{-4}) = 304$ g/mol.

- 15.8 From the gyromagnetic ratio we are able to calculate that the resonance frequency of the chlorine is 188.255 MHz. Therefore between the two nuclei there are $200 - 188.255 = 11.745$ MHz.

As $20 \text{ cm} = 10 \times 200 \text{ Hz} = 2000 \text{ Hz}$ and 1 cm corresponds to 100 Hz, then the distance between the signals will be: $11.745 \times 10^6 / 100 = 117450 \text{ cm}$ or 1174.5 m!

- 15.9 Due to the reduction not going to completion the reaction mixture comprises both acetone and isopropanol. 1 proton of acetone corresponds to 24/6 while a proton of isopropanol is 8/1 = 8. The yield will be $R = 100 \times 8 / (4 \times 8) = 66.7\%$.

- 15.10 All of the compounds present in the mixture are submitted to the same conditions. The spectrum recorded corresponds to a superimposition of the spectra of the individual constituents (the dilution of the sample in a solvent does not modify its initial composition). Each constituent leads to either a single or several signals: at 1.6 and 3.3 ppm for $\text{C}_2\text{H}_5\text{I}$, at 5.2 ppm for CH_2Cl_2 and at 7–7.5 ppm for $\text{C}_6\text{H}_5\text{Br}$. To find the composition of the mixture we will follow a reasoning close to that used in internal normalisation. An important difference concerns the relative response factors which can be established without undertaking an analysis of a standard reference. A molecule of dichloromethane (2H) will give a response which will be 2/5 that of a molecule of bromobenzene (5H), or iodoethane (5H). If we divide the respective areas indicated for each (84, 22.5, 31.5) by the corresponding number of protons (5, 2, 5), we will have the value (16.8, 11.25, 6.3) which will be proportional to the molar concentrations. Then, knowing the molar masses of the three constituents (156, 84 and 157 g/mol), we can establish the % mass with the aid of three similar formulas, as follows: take for example CH_2Cl_2 :

$$\%CH_2Cl = 100 [(area CH_2Cl_2/2) \times 84] / [(area CH_2Cl_2/2) \times 84 + (area C_2H_5I/5) \times 156 + (area C_6H_5Br/5) \times 157]$$

$$\%C_2H_5I = 100 \times [16.8 \times 156] / [(16.8 \times 156) + (11.25 \times 84) + (6.3 \times 157)] = 51\%$$

$$\%CH_2Cl_2 = 100 \times [11.25 \times 84] / [(16.8 \times 156) + (11.25 \times 84) + (6.3 \times 157)] = 18.4\%$$

$$\%C_6H_5Br = 100 \times [6.3 \times 157] / [(16.8 \times 156) + (11.25 \times 84) + (6.3 \times 157)] = 30.6\%$$

16 Mass spectrometry

- 16.1 If x_n and x_s are fractions of natural and synthetic vanillin, and $\delta_n = -20$; $\delta_s = -30$; $\delta_m = -23.5\%$ the ratio of the isotopes of these two in natural, synthetic and a mixture of these variants, then:

$$x_n + x_s = 1$$

$$\delta_n x_n + \delta_s x_s = \delta_m$$

then

$$x_n = (\delta_m - \delta_s) / (\delta_n - \delta_s)$$

Substituting, $x_n = 6.5/10 = 0.65$. The composition found is therefore 65% natural vanillin and 35% of the synthetic variant.

- 16.2
1. $X = 2 \times (174.97) / (389.42) = 0.899$ g of the element lutetium.
 2. The extracted volume of 1 litre contains: $(175.94) / (390.4) \times 20 = 9.013 \mu\text{g}$ of ^{176}Lu .
 3. Coupled method ICP/MS.
 4. The ^{175}Lu is the only isotope whose mass spectrum displays a peak at 175 mass units, while a peak at 176 could be due to either Yb or Hf. These elements constitute isotopic families. We could verify beforehand the absence of peaks for the masses for Yb at 171, 172, 173 and 174, and for Hf at 177, 178 and 179 mass units.
 5. Let x be the mass (in μg) of Lu in the extraction of 11. This mass, before addition of ^{176}Lu , comprises $0.974x$ of ^{175}Lu and $0.026x$ of ^{176}Lu .

Following the addition of ^{176}Lu the mass of ^{176}Lu (in μg) is now $0.026x + 9.013$. Since the analysis indicates that the ratio of the intensities of the peaks is such that $^{175}\text{Lu}/^{176}\text{Lu} = 90/10$, then:

$$(0.974x)/(0.026x + 9.013) = 9 \times 174.97/175.90 = 8.95.$$

Thus $x = 108.85 \mu\text{g}$, which leads to a volume of $0.899/108.85 \times 10^{-6} = 82601$.

16.3 1. $m/e = R^2 B^2 / 2U$, therefore $B = (2U)^{1/2} (m/e)^{1/2} / R$. For $M = 20$ mass units, $B_{20} = 0.182 \text{ T}$. For $M = 200$ mass units, $B_{200} = 0.566 \text{ T}$. The ratio between these two values limiting the field is $B_{200}/B_{20} = 10 = 3.16$.

2. If we sweep by a variation in the electric field then there will be, for higher masses, a voltage ten times weaker resulting in a ten-fold decrease in kinetic energy, lowering the resolving power.

16.4 Intensity of the peak: $720 : I_{720} = 0.989^{60} = 0.515$.

Intensity of the peak: $721 : I_{721} = 0.989^{59} \times 0.011 \times 60 = 0.344$.

The ratio of the two intensities: $I_{721}/I_{720} = 100 \times 0.344/0.515 = 66.7\%$.

16.5 1. Approximate answer: If, following mixing, the peaks 50 and 51 have the same area and if we accept that the intensity of the signal is proportional to the mass of the element (and that the intensity reflects the number of ions formed), we would be able to accept that there is $1 \mu\text{g}$ of ^{51}V in 2 g of steel, namely $0.5 \mu\text{g}/1 \text{ g}$. The mass concentration is therefore 0.5 ppm.

2. More accurate answer:

$$(^{51}\text{V}/^{50}\text{V})_{\text{mass}} = (50.944/49.947)(^{51}\text{V}/^{50}\text{V})_{\text{int}} = 1.02(1/1) = 1.02$$

If we call x the quantity of V to be found in 2 g of steel:

$$(^{51}\text{V}/^{50}\text{V})_{\text{mass}} = 1.02 = (0.9975x)/(0.0025x + 1)$$

we find $x = 1.025 \mu\text{g}$, or 0.513 ppm.

3. If Ti or Cr were present in the steel, there would be a peak of nominal mass 51 due to Cr while the intensity indicating a mass of 50 would be disturbed by the presence of the Ti.

- 16.6 1. Calculation of the % mass of each of the two isotopes of copper:

$$\% ^{63}\text{Cu} = \frac{82\,908 \times 62.9296}{82\,908 \times 62.9296 + 37\,092 \times 64.9278} \times 100 = 68.42$$

$$\% ^{65}\text{Cu} = \frac{37\,092 \times 64.9278}{82\,908 \times 62.9296 + 37\,092 \times 64.9278} \times 100 = 31.58$$

Note: On the recording of the mass spectra the areas of the peaks are proportional to the population of the corresponding ions. For the isotopes of an element which do not have the same mass, the % masses will be a little different.

2. We have added 250 μL of a solution of ^{65}Cu at 16 mg/l, therefore

$$0.25 \times 16/1\,000 = 4 \times 10^{-3} \text{ mg or } 4 \text{ mg of } ^{65}\text{Cu}.$$

Following the addition we must calculate, as above, the new % masses of the two isotopes of copper:

$$\% ^{63}\text{Cu} = \frac{31\,775 \times 62.9296}{31\,775 \times 62.9296 + 79\,325 \times 64.9278} \times 100 = 27.97$$

$$\% ^{65}\text{Cu} = \frac{79\,325 \times 64.9278}{31\,775 \times 62.9296 + 79\,325 \times 64.9278} \times 100 = 72.03$$

If we call x the mass of the copper in the sample (x comprises ^{63}Cu and ^{65}Cu), we have (in μg):

$$72.03/27.97 = (0.3158x + 4)/0.6842x$$

We find $x = 2.7658 \mu\text{g}$ (in the 250 mg of the original unknown solution to measure). Thus for 1 g, there will be $4 \times 2.7658 = 11.06 \mu\text{g}$. This solution contains 11.06 ppm of copper.

Note: If in place of the two areas we considered the ratio $^{63}\text{Cu}/^{65}\text{Cu} = 2.2352$ before adding and 0.4006 following the addition, the calculations would lead us to the values above.

For example: If y is the % mass in ^{63}Cu :

$$y/(100 - y) = 2.2352 (62.9296/64.9278) = 2.16641$$

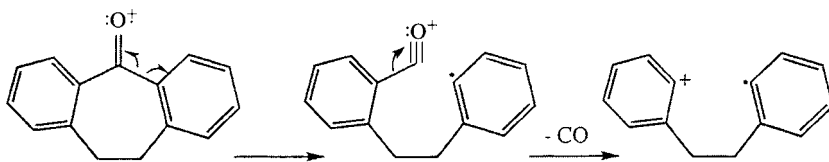
leading to $y = 68.42\%$ and 31.58% of ^{65}Cu .

- 16.7 1. The precise mass corresponding to the molecular formula $C_{15}H_{12}O$ calculated with the more abundant isotopic masses present is as follows:

$$15 \times 12.0000 + 12 \times 1.007825 + 1 \times 15.994915 = 208.088815 \text{ u.}$$

The peak $M + 1$ comprises the sum of the three least abundant isotopic species:

- $^{12}C_{14} \ ^{13}C \ ^1H_{12} \ ^{16}O$
 - $^{12}C_{15} \ ^1H_{11} \ ^2H \ ^{16}O$
 - $^{12}C_{15} \ ^1H_{12} \ ^{17}O$
2. The two modes of decomposition correspond to a loss of 28 u. The ions formed ($m/z = 180$), follow the general rule for radical cations of type CHO.



3. By the loss of CO: $m/z = 208.08822 - (12.0000 + 15.99492) = 180.0939$.
 By the loss of C_2H_4 , $m/z = 208.08822 - (24.0000 + 4.0313) = 180.05732$.
 Following examination of the position of the peaks we can conclude that the more upright peak corresponds to the ions formed when the parent ion loses CO. The molecular formula of this ions is therefore $C_{14}H_{12}$, while the second peak has the molecular formula $C_{13}H_8O$.

4. The resolving power is defined by $R = M/\Delta M$. In considering the scale of the spectrum, ΔM at the mid-height of the largest peak (FWHM) corresponds to approximately 0.012 u, leading to $R = 15\,000$.

In calculating the exact masses of the three isotopomers of the peak $M + 1$ (molecular formulae given in Part One), $a = 209.09217$; $b = 209.095054$ and $c = 209.09321$ u. The differences between these values are very much smaller than the value of ΔM calculated from the spectrum. Under the conditions of recording the spectrum these three types of molecules would appear superimposed.

- 16.8 1. For macromolecules which cannot be vaporised, particular modes of ionisation are used which are similar to methods of impact (FAB or MALDI). Equally ionisation is attained in solution at atmospheric pressure before the sample penetrates the MS (processes of electrospray, thermospray or ionspray). The number of elementary charges carried vary from one

macromolecule to another. Since the apparatus records the ratio m/z it will appear as a series of molecular peaks for the same compound. Following the resolution of the MS we will be able to determine M by calculation from the two peaks (in species carrying different charges), or from the isotopic distribution molecular weight (in species of the same charge state). The recording allows both types of calculation to be made.

2. a) The ratios m/z of the two principal peaks have values close to 1 224 and 1 429. We can express two relations:

$$m/z_1 = 1\,224 \quad \text{and} \quad m/z_2 = 1\,429.$$

Furthermore, we might suppose that the number of charges, greater for the peak to the left, differ by one unit from the peak on the right. Therefore, $z_1 = z_2 + 1$. This will lead to three equations for the three unknowns. We begin by calculating z_1 , finding $z_2 = 5.97$. Since z must be a whole number we will choose $z_2 = 6$. Following on, $m = 6 \times 1\,429 = 8\,574$. The ubiquitin has therefore a molecular mass of about 8 574 Da.

- b) In this method we will consider an enlargement of the isotopic mass $1\,429.2 < m/z < 1\,430$. Between two successive peaks, m varies by a single unit of mass. We have for example $m/z = 1\,428.4$ and $(m + 5)/z = 1\,428.6$. So, $5/z = 0.8$ and $z = 6.25$. If we take the most intense peak located at $m/z = 1\,428.55$, we find $M = 8\,571$ Da.

17 Labelling methods

- 17.1 Calling the specific activity of the marker per g A_S , the mass of this marker used in g, m_S , the activity per g after recuperation A_X and the unknown mass (in g) of penicillin in the sample extracted m_X .

$$m_S = 1 \times 10^{-2} \text{ g}, \quad A_S = 75\,000 \text{ Bq/g} \quad \text{and} \quad A_X = 10 \times 1\,000/1.5 = 6\,666.7 \text{ Bq/g.}$$

$$m_X = 1 \times 10^{-2} \times [(75\,000/6\,666.7 - 1)] = 0.102 \text{ g}$$

In 1 g there is twice as much penicillin, e.g. 0.204 g or 20.4%.

- 17.2 Following the convention of the previous exercise:

$$m_S = 3 \text{ mg}, \quad A_S = 3\,100 \text{ Bq/mg},$$

$$A_X = 3\,000/30 = 100 \text{ Bq/mg}$$

where

$$m_x = 3 \times (3\,100 - 100)/100 = 90 \text{ mg or } 9\%.$$

- 17.3
- The aqueous sample solution of patulin ($M = 154 \text{ g/mol}$), contains $1.54 \times 10^{-3} \text{ g/L}$ of this compound. The concentration is therefore $154 \times 10^{-3}/154 = 1 \times 10^{-5} \text{ M}$ (or 1.54 ppm).
 - The % absorbance (or inhibition) with respect to tube 1:
 - tube 2: $0.47/1.03 = 45.63\%$
 - tube 3: $0.58/1.03 = 56/63\%$
 - tube 4 (sample): $0.50/1.03 = 48.54\%$
 - The absorbance of tube 1 is greater because there is no analyte introduced into the tube. All of the antibody sites are occupied by the conjugated enzyme and the absorbance is, as a result, more intense.
 - The quantity of patulin in tube 2 (2 ml) is $1 \times 10^{-5}/1000 = 1 \times 10^{-8}$ mole, equally $1.54 \mu\text{g}$ or $770 \mu\text{g/L}$ (with $\log C = 2.8865$). In tube 3 the molar quantity of patulin is only half, being 0.5×10^{-8} mole, which is $0.77 \mu\text{g}$ or again $385 \mu\text{g/L}$ (with $\log C = 2.5854$).
 - Along the x-axis, $x = \log C$ (C being in $\mu\text{g/L}$), and along the y-axis, the % of inhibition, which will lead to the following expression:

$$(48.54 - 45.63)/(56.63 - 45.63) = (x - 2.5854)/(2.8865 - 2.5854)$$

leading to $x = 2.6651$. The value of C is thus $462.4 \mu\text{g/L}$. The initial solution is twice the concentration and contains 0.93 mg/L (930 ppb) patulin.

- 17.4
- $$^{35}\text{Cl} + ^1_0\text{n} \rightarrow ^{36}\text{Cl}^* \rightarrow ^{36}\text{Ar}^* \rightarrow ^{36}\text{Ar} \quad (\tau_{\beta^-} = 3.1 \times 10^5 \text{ years})$$

$$^{37}\text{Cl} + ^1_0\text{n} \rightarrow ^{38}\text{Cl}^* \rightarrow ^{38}\text{Ar}^* \rightarrow ^{38}\text{Ar} \quad (\tau_{\beta^-} = 37.3 \text{ min})$$
 - The γ emissions of ^{38}Cl are preferred since their half-life is short. Counting over a period of time (several hours) and with the aid of software we can identify the fraction of this isotope with respect to the radiation constant of the emitter of long half-life (less intense).
 - Atomic absorption, but possible loss of volatile elements during the treatment of the sample. Fluorescence X would be, essentially, analysis of the surface only.

4. KCl and AgCl have 74.551 g and 143.321 g for their respective molar masses. 2 g of KCl corresponds to $2/74.551 = 0.0268$ mole. If AgCl was recovered totally, its mass would be $143.321 \times 0.0268 = 3.845$ g. However, we recover 3.726 g, representing a mass yield of 97%.

50 mL of a solution of AgNO_3 ($M = 203.868$ g/mol, at 15% corresponds to a mass of 7.5 g of this salt, being $7.5/203.868 = 0.037$ mole. We calculate that the quantity of the silver ion is $0.037/0.0268 = 1.38$ times the stoichiometric quantity.

5. The numerical value of the γ count for a total recovery of the isotope ^{35}Cl is $11\,203/0.97 = 11\,549$. The quantity of chlorine in the sample is $11\,203/48\,600 \times 10 = 2.38$ μg , which is $2.38/0.51 = 4.66$ $\mu\text{g/g}$ (or 4.7 ppm).

18 Elemental analysis

- 18.1 $\%C = [10.56 \times (12/44)/5.28] \times 100 = 54.55$; $\%H = [4.32 \times (2/18)/5.28] \times 100 = 9.09$; $\%O = 36.36\%$

If the compound has the molecular formula $\text{C}_x\text{H}_y\text{O}_z$ and taking $M = 88$ then since there are no significant MS peaks above 89 then it can be deduced that: $(12x/\%C) = (y/\%H) = (16z/\%O) = M/100$; Then $x = 4$; $y = 8$; $z = 2$.

- 18.2 1. Advantage: the cold vapour device is specific for mercury; Disadvantage: only inorganic mercury is measured.
2. Equation of the line of least squares : $[\text{signal}] = 858.33 \times [\text{conc}] - 0.46$;
 $C(\text{ppb}) = 0.06$.

19 Potentiometric methods

- 19.1 If we call x the molar concentration of the added NH_4Cl , at $\text{pH} = 9$, $[\text{H}^+] = 10^{-9}$ and $[\text{OH}^-] = 10^{-5}$
 As a result: $K_B = [\text{NH}_4][\text{OH}]/[\text{NH}_3] = (x + 10^{-5})(10^{-5})/(9 \times 10^{-3} - 10^{-5})$.
 Then $x = 1.6 \times 10^{-5}$ M

- 19.2 Two solutions whose concentrations in H^+ ions are different, being C_{int} and C_{ext} , are found in part of the special glass membrane which constitutes the inner wall.

— Potential across the internal face: $E_{\text{int}} = RT/F \ln(\gamma C_{\text{int}})$

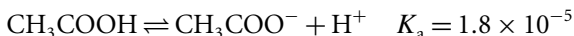
— Potential across the external face: $E_{\text{ext}} = RT/F \ln(\gamma C_{\text{ext}})$

Thus in the chain of potentials between the two limits the contribution of the membrane:

$$\Delta E_{\text{memb}} = 0.059 \log(\gamma C_{\text{ext}}) - 0.059 \log(\gamma C_{\text{int}})$$

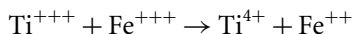
Since C_{int} is constant, and if there are no modifications of other characteristics of the electrodes, then γC_{ext} alone will interfere with the potential of the external face of the membrane. The signal depends upon the pH: 0.059 pH in mV.

19.3 If x is the concentration in H^+ ions:



$K_a = x^2/(0.85 - x)$ therefore $x = 0.0039$ and so $\text{H}^+ = 0.0039 \text{ M}$ with the $\text{pH} = 2.4$. The degree of dissociation will be $(0.0039/0.85) \times 100 = 0.46\%$.

19.4 The reaction is the following:



Since we have added 1.5 times the stoichiometric quantity of Fe^{+++} there will be no more Ti^{+++} . Thus $[\text{Ti}^{+++}] = 0$.

$$[\text{Ti}^{4+}] = (20/1000) \times 10^{-3} \times 1000/50 = (20/50) \times 10^{-3} = 4 \times 10^{-4} \text{ M}$$

$$[\text{Fe}^{++}] = 4 \times 10^{-4} \text{ M}$$

$$[\text{Fe}^{+++}] = 2 \times 10^{-4} \text{ M (half of the preceding value)}$$

19.5 $[\text{Cd}^{++}] = 0.01$. $E = E_0 - RT/nF \ln[\text{Red}]/[\text{Ox}]$ or $E = E_0 - 0.059/n \cdot \log[\text{Red}]/[\text{Ox}]$

$$E = -0.403 - (0.059/2) \times \log(1/0.01) = -0.462 \text{ V.}$$

19.6 The first measurement made with the sample solution was of the following type:

$$E_1 = E'' + S \log C_x$$

The second was of the following type:

$$E_2 = E'' + S \log(C_x V_x + C_R V_R)/(V_x + V_R)$$

The term $(C_x V_x + C_R V_R)/(V_x + V_R)$ represents the new concentration of the compound measured when we add the volume V_R of concentration C_R to the volume V_x of concentration C_x . Therefore:

$$\Delta E = E_2 - E_1 = S \log(C_x V_x + C_R V_R)/[(V_x + V_R)C_x]$$

being:

$$10^{\Delta E/S} = (C_x V_x + C_R V_R)/[(V_x + V_R)C_x]$$

If we isolate C_x from the second member of the expression above then we refine the formula proposed in the question.

20 Voltametric and coulometric methods

- 20.1
1. Not quite since approximately 0.5% of the sample is electrolysed.
 2. The reproducibility of the phenomenon measured to the level of the drops requires that the diffusion establishes itself in the solution at rest. Stirring renders the system unstable. However, an electrode which turns regularly can be used.
 3. Often the sample is complex and the method of standards is not representative of the effect of the matrix for the metallic species or the organic molecules which can react with the electrodes (reductions for example), producing interference.
- 20.2 We calculate the ratio of the number of transport of zinc with respect to the number of migrating or diffusing ions present in the solution:

$$\begin{aligned} t_{\text{Zn}^{++}} &= (2 \times 5.5 \times 10^{-8} \times 10^{-3})/DC \\ DC &= (2 \times 5.5 \times 10^{-8} \times 10^{-3}) + (1 \times 7.4 \times 10^{-8} \times 2 \times 10^{-3}) \\ &\quad + (1 \times 7.9 \times 10^{-8} \times 0.1) + (1 \times 7.6 \times 10^{-8} \times 0.1) \\ t_{\text{Zn}^{++}} &= 6.98 \times 10^{-3} \end{aligned}$$

Thus for each coulomb exchanged, the Zn^{++} ion will transport 6.98×10^{-3} C and the rest through the solution $(1 - 6.98 \times 10^{-3}) = 0.993$ C. As a result $i_m/i_D = 7.03 \times 10^{-3}$ or 0.007. The transport of zinc towards the electrode is therefore controlled by the diffusion.

- 20.3 20 drops (approximately 0.16 g) fall in 80 s. The flow of mercury is therefore:

$$0.16/80 = 2 \times 10^{-3} \text{ g/s.}$$

For a distance three times as high, the flow will be three times as much, i.e. 6×10^{-3} g/s. The drops always have the same mass and follow a rhythm of:

$$(0.16/20)/(2 \times 10^{-3}) = 1.33 \text{ s.}$$

- 20.4 By application of the equation of Ilkovic (i in μA):

$$i_D = 607 \times 2 \times (8.67 \times 10^{-6})^{1/2} \times 2^{2/3} \times 4^{1/6} \times 1 = 7.15 \mu\text{A.}$$

Note: D is in cm^2/s ; m in mg/s and C in mmol/L .

Different units can be used, such as D in m^2/s , m in kg/s and C in mol/m^3 , leading to i_D expressed in amperes.

- 20.5 The 1 M solution of KCl ready to use is such that $[\text{Zn}] = 1 \text{ ppm}$. If we prepare a 1 M solution with crystallised KCl, then we will have $74.6 \times 5/10^6 = 3.73 \times 10^{-4} \text{ g}$ of zinc in 74.6 g of this salt found in 1 litre (which is approximately 1 kg), of solution 0.37 ppm of zinc. The ready-to-use solution is therefore three times more concentrated in zinc than that prepared with crystallised KCl.

- 20.6 The number of moles of Zn in the experiment: $25/1000 \times 2 \times 10^{-8} = 5 \times 10^{-10}$ mole. We would like to deposit 3% i.e. $0.03 \times 5 \times 10^{-10} = 1.5 \times 10^{-11}$. It requires therefore 3×10^{-11} moles of electrons, representing a charge of:

$$Q = 96500 \times 3 \times 10^{-11} = 2.895 \times 10^{-6} \text{ C}$$

Since $Q = it$, $t = 2.895 \times 10^{-6}/1.5 \times 10^{-9} = 1.93 \times 10^3 \text{ s}$ which is 32.16 min.

Note: A current of 1.5 nA is practically undetectable. However, if we employ stripping voltametry, we will redissolve this quantity of zinc in approximately 1 s, which will then produce a much easier signal to detect:

$$i = 2.895 \times 10^{-6}/1 = 2.895 \mu\text{A.}$$

- 20.7 Standardising the reactant: following the question, 1 mL of this solvent has a water content of 3/15 ml of KF reagent. In oxalic acid dehydrate ($M = 126 \text{ g/mol}$), the mass concentration of water is $36/126 = 28.57\%$.

The information given permits the following calculation to find the titre of the reactant:

$$T = 0.2857 \times 205 \times 1/13 = 4.51 \text{ mg/mL}$$

Measurement: 10 mL of this solvent neutralizes $10 \times 3/15 = 2$ mL of reactant, thus 1.05 g of powdered milk will react with $12 - 2 = 10$ mL of this same reagent. There are therefore $4.51 \times 10 = 45.1$ mg of water in the sample, which is a concentration of

$$(45.1/1050) \times 100 = 4.3\% \text{ mass.}$$

- 20.8 In the coulometric version of KF titration 1 molecule of water requires 2 atoms of iodine and 2 electrons. Therefore $2 \times 96\,500$ coulombs (C), are required for one mole, which is 1.8×10^4 mg of water. 1 C corresponds to $1.8 \times 10^4 / (2 \times 96\,500) = 0.0933$ mg of water. This then corresponds to the quantity of water of 1 mL of ether. There are therefore 93 mg of water per litre. Considering the density of ether (0.78 g/ml), the mass concentration will be:

$$93 \times 1/0.78 = 120 \text{ mg/kg (120 ppm)}$$

- 20.9 Two electrons are required to reduce a zinc ion. The quantity of current used in the experiment is $15 \times 10^{-6} \times 5 \times 60 = 4.5 \times 10^{-3}$ C, which will effectively reduce:

$4.5 \times 10^{-3} / (2 \times 96\,500) = 2.33 \times 10^{-8}$ mole of zinc
knowing that at the beginning of the experiment there were $(20/1000) \times 1 \times 10^{-3} = 2 \times 10^{-5}$ mole of zinc. The impoverishment is therefore:

$$(2.33 \times 10^{-8}) / (2 \times 10^{-5}) \times 100 = 0.12\%$$

22 Basic statistical parameters

22.1

Chemist	Average value (\bar{x})	Standard deviation (s)	ϵ	RSD%	Conclusion
Chemist A	131.6	1.56	0.3	1.18	Correct and precise
Chemist B	131.6	5.37	0.3	4.08	Correct yet imprecise
Chemist C	135.7	1.33	3.8	0.98	Incorrect yet precise
Chemist D	125.3	9.93	6.6	7.93	Incorrect and imprecise

- 22.2 The average value is 650 with $s = 1.581$. For the level of confidence indicated, the value of $t = 2.776$. We can then calculate:

$$t \cdot s / (n)^{1/2} = 1.963$$

The results determine a range of 650 ± 1.963 in which we have a 95% chance of finding the true average. There is probably a systematic error in these experiments. However, if we fixed a level of confidence of 99% ($t = 4.6$), we would have $s / (n)^{1/2} = 3.25$ and therefore a range of 650 ± 3.25 . The value of 653 would be included in this interval and would thus be considered as a viable result.

- 22.3 $F = (s_1/s_2)^2 = 11.88$. For two series of measurements, the frontier value is 5.05. Therefore the precision of the two apparatus is significantly different.

- 22.4 The value of t calculated (with $n = 6$) for the chemist A ($s = 1.559$) is 0.471. This value is smaller than those presented in the table of t values: 2.57 (for 95%), and 4.03 (for 99%). There is probably not a systematic error. However, for chemist C ($s = 1.325$), we will find $t = 4.05$, a value which indicates a high likelihood of a systematic error.

- 22.5 The value of $Q = (24.8 - 24.36) / (24.8 - 24.10) = 0.63$ is inferior to that which is found in the table for five measurements and a level of confidence of 95% (value 0.64). We should therefore not reject the value 24.8. However, if we recalculate following the addition of the two new values indicated, Q remains unchanged but in the table of seven values we have 0.51. The value 24.8 is now rejected.

Note: The values of s and of the average in including ($s = 0.239$ and average 24.29), or by rejecting ($s = 0.095$ and average 24.21) these measurements are very different. If we take the middle values we have 24.24 (with), and 24.22 (without). In this case the middle seems preferable to the average.

- 22.6 The pooled standard deviation s_p should be calculated, as follows:

$$s_p^2 = \frac{6 \times 0.3^2 + 6 \times 0.2^2}{14 - 2} = 0.2549^2 \quad t = \frac{3}{0.2549} \sqrt{\frac{7^2}{14}} = 22$$

In the table $t = 2.2$, therefore the two methods do not produce at the same result.

- 22.7 The problem is centred around the comparison of two averages. That for the first percentage purity is 99%, while the average value for the four analyses reported is 98.73 with $s_{n-1} = 0.155$. The 'pooled standard deviation' of the two series of values is:

$$s_p = \{(4 \times 0.08^2 + 3 \times 0.155^2)/7\}^{1/2} = 0.118$$

The value of t based upon $(5 + 4 - 2)$ degrees of freedom leads, according to the table, to 2.365 for a level of confidence of 95%.

We must next calculate $2.365 \times 0.118 \times \{(4 + 5)/(4 \times 5)\}^{1/2} = 0.187$ and compare this value with the difference in the averages of the two series, $99 - 98.73 = 0.23$. The result of this comparison appears rather large and therefore leads to the conclusion that the two averages can be considered to be incompatible. Thus the original value will not be retained.

- 22.8 If we consider that the law of variation of the absorbance with the concentration is a straight line then this will have the equation: $A = 0.05 [\text{conc}] + 0.08$. The difference are relatively large. A quadratic adjustment would be preferred and across a narrower range of concentrations.
- 22.9 For the blank, $s_{n-1} = 0.82$. The value of t calculated for $5 + 8$ measurements is 3.17. Therefore $\Delta x = 3.17 \times 0.82 \times [(5 + 8)/(5 \times 8)]^{1/2} = 1.48$. The limit of the detection is around 1.5 mg.

Appendix

List of acronyms

AAS	Atomic absorption spectroscopy
AC	Alternative current
ADC	Analogue–digital converter
AED	Atomic emission detector
AES	Atomic emission spectroscopy
AGS	Amperometric gas sensor
AMS	Accelerator mass spectrometry
AMTIR	Amorphous material transmitting infrared radiation
APCI	Atmospheric pressure chemical ionisation
APXS	Alpha particle X-ray spectrometer
ASTM	American Society for Testing Materials
ATR	Attenuated total reflectance
BSA	Bovine serum albumin
CCD	Charge-coupled device
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CGE	Capillary gel electrophoresis
CI	Chemical ionization
CID	Collision-induced dissociation
CIEF	Capillary isoelectric focusing
CV	Coefficient of variation
CVAFS	Cold vapour atomic fluorescence spectroscopy
CZE	Capillary zone electrophoresis
CW	Continuous wave
DAD	Diode array detector (or detection)
DCP	Direct current polarography
DPP	Differential pulsed polarography
DTGS	Deuterated triglycine sulfate
ECD	Electron capture detector
EDL	Electrodeless discharge lamp

EDTA	Ethylenediamine-tetracetic acid
EDXRF	Energy dispersive X-ray fluorescence
EI	Electron impact (or ionization)
EIA	Enzymatic immuno-assay
ELISA	Enzyme linked immunosorbent assay
EOF	Electro-osmotic flow
ESCA	Electron spectroscopy for chemical analysis
ESI	Electrospray ionization
FAB	Fast atom bombardment
FES	Flame emission spectroscopy
FID	Flame ionization detector
FID	Free induction decay
FPD	Flame photometry detector
FT	Fourier transform
FTIR	Fourier transform infrared
FTMS	Fourier transform mass spectrometry
FWHM	Full width at half maximum
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GC-ICP	Gas chromatography-inductively coupled plasma
GDL	Glow discharge lamp
GFC	Gel filtration chromatography
GLC	Gas liquid chromatography
GLP	Good laboratory practice
HPCE	High performance capillary electrophoresis
HCL	Hollow cathode lamp
HETP	Height equivalent to a theoretical plate
HIC	Hydrophobic interaction chromatography
HOMO	Highest occupied molecular orbital
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HRP	Horseradish peroxidase
IC	Ion chromatography
ICP	Inductively coupled plasma
ICPMS	Inductively coupled plasma mass spectrometry
ICRMS	Ion cyclotron resonance mass spectrometry
IEA	Immuno-enzymological assay
IEC	Internal electron capture
IFA	Immunofluorescence assay
IMS	Ion mobility spectrometer
IR	Infrared
ISE	Ionic selective electrode
KK	Kramers Krönig
LIDAR	Light detection and ranging

LOMO	Lowest occupied molecular orbital
LPME	Liquid phase micro-extraction
MALDI	Matrix-assisted laser desorption ionization
MCA	Multicomponent analysis
MCT	Mercury cadmium telluride
MEKC	Micellar electrokinetic capillary chromatography
MIKE	Mass ion kinetic energy
MLRA	Multiwavelength linear regression analysis
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MSD	Mass spectrometer detector
NAA	Neutron activation analysis
NADH	Nicotinamide adenine dinucleotide reduced form
NIR	Near infrared
NMR	Nuclear magnetic resonance
NPD	Nitrogen phosphorous detector
NPP	Normal pulsed polarography
OES	Optical emission spectrometry
OFAT	One-factor-at-a-time
PAGE	Polyacrylamide gel electrophoresis
PAH	Polynuclear aromatic hydrocarbons
PCA	Principal component analysis
PDA	Photodiode array
PID	Photo ionization detector
PLS	Partial least square
PMT	Photo multiplier tube
POPOP	1,4-Bis(5-phenyloxazol-2-yl)benzene
ppb	parts per billion
ppm	parts per million
PPO	2,5-diphenyloxazole
ppt	parts per trillion
PT	Peak tailing
PTV	Programmed temperature vaporization
RIA	Radio-immunoassay
RP	Reversed phase
RSD	Relative standard deviation
SCE	Standard calomel electrode
SEC	Size exclusion chromatography
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SEM	Scanning electron microscope
SPE	Solid-phase extraction
SPME	Solid-phase micro-extraction

SV	Stripping polarography
TCD	Thermal conductivity detector
TI	Thermal ionization
TIC	Total ion chromatogram
TISAB	Total ionic strength adjustment buffer
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TOF	Time of flight
TZ	Trennzahl number
UV	Ultraviolet
VOC	Volatile organic compound
WCOT	Wall coated open tubular
WXRF	Wavelength dispersive X-ray fluorescence

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Table of some useful constants

Physical constants

Quantity	Symbol	Value	Units
Speed of light in vacuum	c	2.9979×10^8	m/s
Vacuum permittivity	ϵ_0	8.8542×10^{-12}	F/m
Vacuum permeability	μ_0	$4\pi \times 10^{-7}$	H/m
Elementary charge	e	1.6022×10^{-19}	C
Electron rest mass	m_e	9.1094×10^{-31}	kg
Proton rest mass	m_p	1.6726×10^{-27}	kg
Neutron rest mass	m_n	1.6750×10^{-27}	kg
Atomic mass constant	u	1.6605×10^{-27}	kg
Planck's constant	h	6.6261×10^{-34}	J s
Dirac constant	$\hbar = h/2\pi$	1.0546×10^{-34}	J s
Avogadro number	N_A	6.0221×10^{23}	/mol
Faraday constant	$F = N_A e$	9.6485×10^4	C/mol
Boltzmann constant	$k = R/N_A$	1.3807×10^{-23}	J/K
Molar gas constant	$R = N_A k_B$	8.3145	J/K mol
Gravitational constant	G	6.6720×10^{-11}	N m ² /kg ²

Unit conversion

$$1 \text{ \AA} = 10^{-10} \text{ m} \quad 1 \text{ cm}^{-1} = 2.9979 \times 10^{10} \text{ Hz}$$

$$1 \text{ eV} = 1.6022 \times 10^{-19} \text{ J} \quad 1 \text{ atm} = 1.0133 \times 10^5 \text{ Pa}; \quad 1 \text{ bar} = 10^5 \text{ Pa}$$

Prefixes

<i>Symbol</i>	f	p	n	μ	m	c	d	k	M
<i>Name</i>	femto	pico	nano	micro	milli	centi	deci	kilo	mega
<i>Factor</i>	10^{-15}	10^{-12}	10^{-9}	10^{-6}	10^{-3}	10^{-2}	10^{-1}	10^3	10^6

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