VOL. 600 NO. 1 MAY 27, 1992

8th Danube Symp. on Chromatography Warsaw, September 2–6, 1991

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SYMPOSIUM VOLUMES

ELSEVIER

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis or alterations during medical treatment; screening and profiling of body fluids or tissues with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; drug level monitoring and pharmacokinetic studies; clinical toxicology; analytical studies in occupational medicine.

Submission of Papers. Manuscripts (in English; *four* copies are required) should be submitted to: Editorial Office of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, Netherlands, Telefax (+31-20) 5862 304, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, Netherlands. Review articles are invited or proposed by letter to the Editors. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications*) has 39 volumes in 1992. The subscription prices for 1992 are:

J. Chromatogr. (incl. *Cum. Indexes, Vols. 551–600*) + *Biomed. Appl.* (Vols. 573–611):
Dfl. 7722.00 plus Dfl. 1209.00 (p.p.h.) (total *ca.* US$ 4880.25)

J. Chromatogr. (incl. *Cum. Indexes, Vols. 551–600*) only (Vols. 585–611):
Dfl. 6210.00 plus Dfl. 837.00 (p.p.h.) (total *ca.* US$ 3850.75)

Biomed. Appl. only (Vols. 573–584):
Dfl. 2760.00 plus Dfl. 372.00 (p.p.h.) (total *ca.* US$ 1711.50).

Subscription Orders. The Dutch guilder price is definitive. The US$ price is subject to exchange-rate fluctuations and is given as a guide. Subscriptions are accepted on a prepaid basis only, unless different terms have been previously agreed upon. Subscriptions orders can be entered only by calender year (Jan.–Dec.) and should be sent to Elsevier Science Publishers, Journal Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands, Tel. (+31-20) 5803 642, Telefax (+31-20) 5803 598, or to your usual subscription agent. Postage and handling charges include surface delivery except to the following countries where air delivery via SAL (Surface Air Lift) mail is ensured: Argentina, Australia, Brazil, Canada, China, Hong Kong, India, Israel, Japan*, Malaysia, Mexico, New Zealand, Pakistan, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. *For Japan air delivery (SAL) requires 25% additional charge of the normal postage and handling charge. For all other countries airmail rates are available upon request. Claims for missing issues must be made within three months of our publication (mailing) date, otherwise such claims cannot be honoured free of charge. Back volumes of the *Journal of Chromatography* (Vols. 1–572) are available at Dfl. 217.00 (plus postage). Customers in the USA and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 655 Avenue of the Americas, New York, NY 10010, USA, Tel. (+1-212) 633 3750, Telefax (+1-212) 633 3990.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Clinical Chemistry Lookout, Current Awareness in Biological Sciences (CABS), Current Contents/Life Sciences, Current Contents/Physical, Chemical & Earth Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Pharmaceutical Abstracts, Referativnyi Zhurnal, Research Alert, Science Citation Index and Trends in Biotechnology.

US Mailing Notice. *Journal of Chromatography* (main section ISSN 0021-9673, *Biomedical Applications* section ISSN 0378-4347) is published (78 issues/year) by Elsevier Science Publishers (Sara Burgerhartstraat 25, P.O. Box 211, 1000 AE Amsterdam, Netherlands). Annual subscription price in the USA US$ 4880.25 (subject to change), including air speed delivery. Application to mail at second class postage rate is pending at Jamaica, NY 11431. **USA POSTMASTERS**: Send address changes to *Journal of Chromatography*, Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003. Airfreight and mailing in the USA by Publication Expediting.

See inside back cover for Publication Schedule, Information for Authors and information on Advertisements.

0021-9673/92/$05.00

Printed in the Netherlands

For Contents, see p. VII.

JOURNAL of CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SYMPOSIUM VOLUMES

ELSEVIER
AMSTERDAM — LONDON — NEW YORK — TOKYO

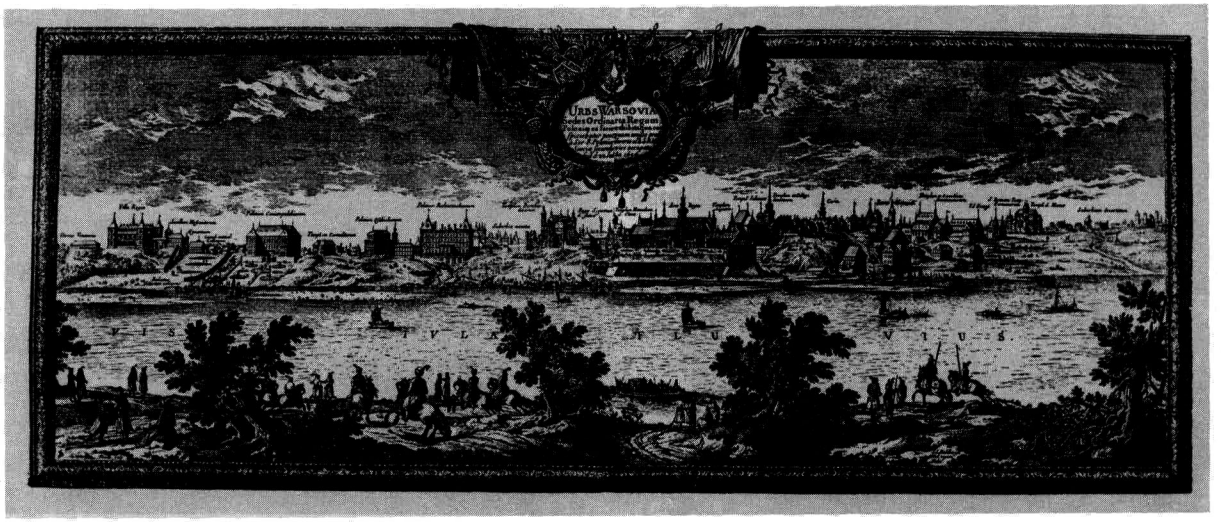
J. Chromatogr., Vol. 600 (1992)

Panorama of Warsaw from the right bank of the Vistula River (by E. J. Dahlberg, 1656)

0021-9673/92/$05.00

Printed in the Netherlands

SYMPOSIUM VOLUME



8TH DANUBE SYMPOSIUM ON CHROMATOGRAPHY

Warsaw (Poland), September 2–6, 1991

Guest Editors

Z. WITKIEWICZ
(Warsaw)

J. LIPKOWSKI
(Warsaw)

CONTENTS

8TH DANUBE SYMPOSIUM ON CHROMATOGRAPHY, WARSAW, SEPTEMBER 2–6, 1991

In articles with more than one author, the name of the author to whom correspondence should be addressed is indicated in the article heading by a 6-pointed asterisk (*).

Stationary Phases in Gas Chromatography

by **H. Rotzsche**, *VEB Chemiewerk, Nünchritz, Radebeul, Germany*

Journal of Chromatography Library Volume 48

The primary aim of this volume is to make the chemist familiar with the numerous stationary phases and column types, with their advantages and disadvantages, to help in the selection of the most suitable phase for the type of analytes under study. The book also provides detailed information on the chemical structure, physicochemical behaviour, experimental applicability, physical data of liquid and solid stationary phases and solid supports. Such data were previously scattered throughout the literature. To understand the processes occurring in the separation column and to offer a manual both to the beginner and to the experienced chromatographer, one chapter is devoted to the basic theoretical aspects. Further, as the effectiveness of the stationary phase can only be considered in relation to the column type, a chapter on different column types and the arrangement of the stationary phase within the column is included.

The secondary aim of this book is to stimulate the development of new and improved standardized stationary phases and columns, in order to improve the reproducibility of separations, as well as the range of applications.

Contents: **1. Introduction. 2. Basic Concepts.** Basic components of a gas chromatographic system. Raw data measured from the chromatogram. Derived basic chromatographic parameters. Flow of gases in a gas chromatographic column and formation of bands. Thermodynamic bases of gas chromatography. The quality of chromatographic separation. The time of analysis. Definition of symbols used and list of essential relationships. **3. The Chromatographic Column.** Packed columns. Micro-packed columns. Open-tubular columns. Properties and comparison of the main column types. **4. Characterization of Stationary Phases.** Intermolecular forces. Quantities for the description of interactions. **5. Solid Stationary Phases.** Classification of adsorbents. Carbon adsorbents. Boron nitride and molybdenum disulphide. Adsorbents with hydroxylated and dehydroxylated surfaces. Porous organic polymers. Substances forming inclusion compounds. Modified adsorbents. **6. Chemically Bonded Stationary Phases.** Adsorbents for bonding reactions. Bonding reactions. Properties and characterization of chemically bonded phases. Outlook and prospects for chemically bonded phases. **7. The Solid Support.** The particle size and shape. The surface area. Activity of the original and of the coated solid support. Diatomite supports. Synthetic silica-based supports (Volaspher and quartz). Silica gel. Micro glass beads and porous layer beads. Fluorocarbon supports. Other support materials. **8. Liquid Stationary Phases.** General properties of liquid stationary phases. Hydrocarbons. Silicones. Alcohols, ethers and carbohydrates. Esters. Nitriles and nitrile ethers. Nitro compounds. Amines. Amides. Heterocyclics. Sulphur compounds. Fluorine compounds. Fatty acids and their salts. Salts. Chiral stationary phases. Liquid crystals. Mixed stationary phases. **9. Selection of Stationary Phases.** General recommendations for choosing a suitable stationary phase. Choosing stationary phases for special separation problems with regard to the desired selectivity. Preferred stationary phases. Approaches to stationary phase selection. **Literature. Indexes.**

1991 xlv + 410 pages
Price: US $ 166.50 / Dfl. 325.00
ISBN 0-444-98733-9
Co-edition with Akademische Verlagsgesellschaft Geest & Portig K.-G., Leipzig, Germany

Elsevier Science Publishers
P.O. Box 211, 1000 AE Amsterdam, The Netherlands
P.O. Box 882, Madison Square Station, New York, NY 10159, USA

New Developments in Ion Exchange

Materials, Fundamentals and Applications

Proceedings of the International Conference on Ion Exchange, ICIE '91, Tokyo, Japan, October 2-4, 1991

edited by **M. Abe**, Tokyo Institute of Technology, Tokyo, Japan,
T. Kataoka, University of Osaka Prefecture, Osaka, Japan and
T. Suzuki, Yamanashi University, Yamanashi, Japan

This volume contains the papers presented at the International Conference on Ion Exchange (ICIE '91). Included are reviews and original papers reflecting recent progress in new branches of science and technology based on novel ion-exchange materials and ion-exchange theory.

Topics covered include: Fundamentals (Ion exchange theory and process), Synthesis of New Materials, Chromatography (Ion and liquid chromatography, and associated techniques), Water Purification, Environmental, Membranes, Hydrometallurgy, Separation Science and Technology (Food, pharmaceutical, biological and others).

Contents:

1991 xlii + 636 pages
Price: US $ 218.00 / Dfl. 425.00
ISBN 0-444-98688-X

Co-edition with and distributed in Japan by Kodansha Scientific Ltd.

Elsevier Science Publishers

P.O. Box 211, 1000 AE Amsterdam, The Netherlands
P.O. Box 882, Madison Square Station, New York, NY 10159, USA

JOURNAL OF CHROMATOGRAPHY

VOL. 600 (1992)

Journal of Chromatography, 600 (1992) 1
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 072

Foreword

The *8th Danube Symposium on Chromatography* was held in Warsaw (Poland) from September 2 to 6, 1991. It was organized by the Institute of Physical Chemistry of the Polish Academy of Sciences and the Institute of Chemistry of the Military Technical Academy. The Committee of Analytical Chemistry of the Polish Academy of Sciences and, in particular, its Commission of Chromatographic Analysis gave their local support to the organization of the symposium.

About 250 participants from 18 countries, including the USA, Canada and Japan, took part in the symposium and numerous world-renowned scientists delivered informative lectures. The inaugural lecture by Professor E. Bayer (Tübingen) was entitled "Multi-coupling of HPLC–capillary electrophoresis and mass spectrometry", followed during the week by 25 plenary lectures and 15 oral and 181 poster presentations. Some of the papers are included in this special issue of the *Journal of Chromatography*. It can be seen that most of the currently important topics in chromatographic science were discussed during the symposium, including problems in electrophoresis and electrochromatography. The theory of chromatography, the development of chromatographic equipment and the application of chromatography to the analysis of different compound types were discussed.

The afternoon session on the first day of the symposium was devoted to the history of chromatographic discovery. A special reason for this was that, as not many young chromatographers may know, Michail Tswett discovered chromatography during his work in Warsaw. L. S. Ettre and K. I. Sakodynskii presented very interesting lectures about Tswett's life and his work. R. Mierzecki, a well known Polish chemist and historian of science, recalled the state of science in Poland at that historical time.

The Conference Centre of the Polish Ministry of National Defence was the location for the symposium. The organizing committee and the participants all agreed that this Centre was an ideal venue ensuring good conditions for the symposium events.

The International Committee of the Danube Symposia discussed, in connection with the new political situation in Europe, the possibilities for organizing future symposia in this series. It was decided that the *9th Danube Symposium on Chromatography* will take place in Budapest in 1993 and it will probably be the last one. Subsequent meetings will be incorporated in the series of *International Symposia on Chromatography*, organized hitherto in Western Europe, the next of which, the 19th, will take place in 1992 in Aix-en-Provence (France).

During the symposium Professor Edward Soczewiński of the Medical Academy in Lublin and Professor Zygfryd Witkiewicz of the Military Technical Academy in Warsaw received Tswett Medals, awarded to them by the Chromatographic Society of the former Soviet Union.

Zygfryd Witkiewicz
Janusz Lipkowski

Journal of Chromatography, 600 (1992) 3–15
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 969

Early evolution of chromatography: the activities of Charles Dhéré*

Veronika R. Meyer

Institut für Organische Chemie der Universität, Freiestrasse 3, CH-3012 Bern (Switzerland)

Leslie S. Ettre*

Department of Chemical Engineering, Yale University, P.O. Box 2159, Yale Station, New Haven, CT 06520 (USA)

ABSTRACT

Charles Dhéré (1876–1955), professor at the University of Fribourg (Switzerland), was the first in Europe to recognize the importance of chromatography. While with his students W. Rogowski and G. Vegezzi he improved the laboratory set-up used for chromatography, he also proved the correctness of Tswett's assumptions on the existence of a multitude of chromophyllic and carotenoidic pigments, demonstrated that chromatography can provide purer substances than any of the then accepted methods and further extended the use of chromatography into animal biochemistry. He also provided the first reliable discussion on the life and activities of Tswett, the inventor of chromatography.

CONTENTS

1. INTRODUCTION

In 1991 we are celebrating the eighty-fifth anniversary of the two classical publications of M. S. Tswett on the chromatographic separation method [1,2]. This development already started in St. Petersburg where he moved in 1896 and was finished in Warsaw**. In the 85 years which have passed

* Parts of this paper were presented at the *15th International Symposium on Column Chromatography, Basel, June 3–7, 1991* and at the *8th Danube Symposium on Chromatography, Warsaw, September 2-6, 1991.*

** Mikhail Semenovich Tswett was born in Asti, Italy, in 1872. Although his father was a Russian, he spent the first 24 years of his life in Switzerland where he studied at the University of Geneva, receiving his *Docteur ès Sciences* degree in botany in 1896. At that time he followed his father to Russia. However, the Russian educational system and science establishment did not recognize foreign degrees: therefore, he had to submit new master's and doctor's theses to Russian universities. He worked on the first in St. Petersburg, in the laboratories of the Imperial Academy of Sciences and submitted it in 1901 to the University of Kazan. He received his Russian doctor degree later, in 1910, in Warsaw, where he had been associated with various universities between December 1901 and the Summer of 1915. For details on the life and activities of Tswett see the publications of Sakodynskii (refs. 3–6), Senchenkova (ref. 7) and Hais (ref. 8), and the paper of Dhéré discussed in the present publication (ref. 9).

0021-9673/92/$05.00

since these publications, chromatography became the most important laboratory method used in chemistry and biochemistry.

Tswett's invention, chromatography, and his results in the field of plant pigments, did not receive the recognition they deserved: they were greeted with skepticism and even by ridiculing them [10,11]. This so-called dormant period finally ended in 1931, when the group of Richard Kuhn, in Heidelberg, demonstrated the superiority of chromatography [12].

In the 25 years which have passed between Tswett's two fundamental papers and the start of the work in Heidelberg, only a very few scientists recognized the importance of chromatography as a separation technique and utilized it in their research. Among these two are particularly important: Charles Dhéré, in Switzerland, and Leroy Sheldon Palmer, in the USA. The purpose of this paper is to discuss in detail the activities of Dhéré and his students with special emphasis on their contribution to the evolution of chromatography. We plan to deal with the activities of Palmer in a separate publication.

2. DHÉRÉ'S LIFE; HIS FIELD OF INTEREST

Charles Dhéré (Fig. 1) was born on March 5, 1876, in Paris, and first studied medicine, receiving his *Docteur en Médecine* degree in 1898 with a thesis dealing with the variation of nerve centers as a function of size [13]. However, he never practiced as a physician. First he became an assistant at the Sorbonne, in the Department of Natural Sciences; then, in 1900, he joined the University of Fribourg, in Switzerland, as an associate professor (*professeur extraordinaire*) of physiology, biological chemistry and microbiology. In 1908, he became a full professor and, in 1909, he received the *Docteur ès Sciences* degree from the Sorbonne, with a thesis on the investigation of albuminoides, proteides and their derivatives using ultraviolet spectroscopy [14]. During his long tenure at the University of Fribourg, Dhéré served twice (in 1916–1917 and 1933–1934) as the dean of the Faculty of Science. He retired in 1938 when the title of a *professeur honoraire* was bestowed on him.

After his retirement Dhéré moved from Fribourg to Geneva where an office and laboratory space was provided for him in the Institute of Zoology of the University. He continued his scientific studies until 1951. He died on January 18, 1955[a].

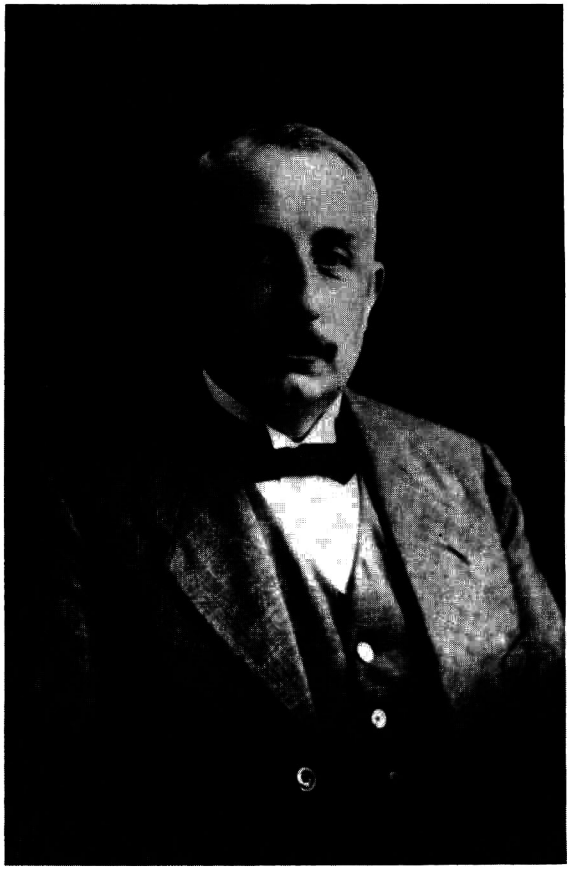


Fig. 1. Charles Dhéré, in the 1930s.

Dhéré's main interest was the investigation of biologically important substances, particularly by ultraviolet and fluorescence spectroscopy. He even coined two special terms to express his field of interest: *optochimie and optochimie biologique*. As a summary of his life's activities Dhéré in 1937 published a major book on fluorescence in biochemistry

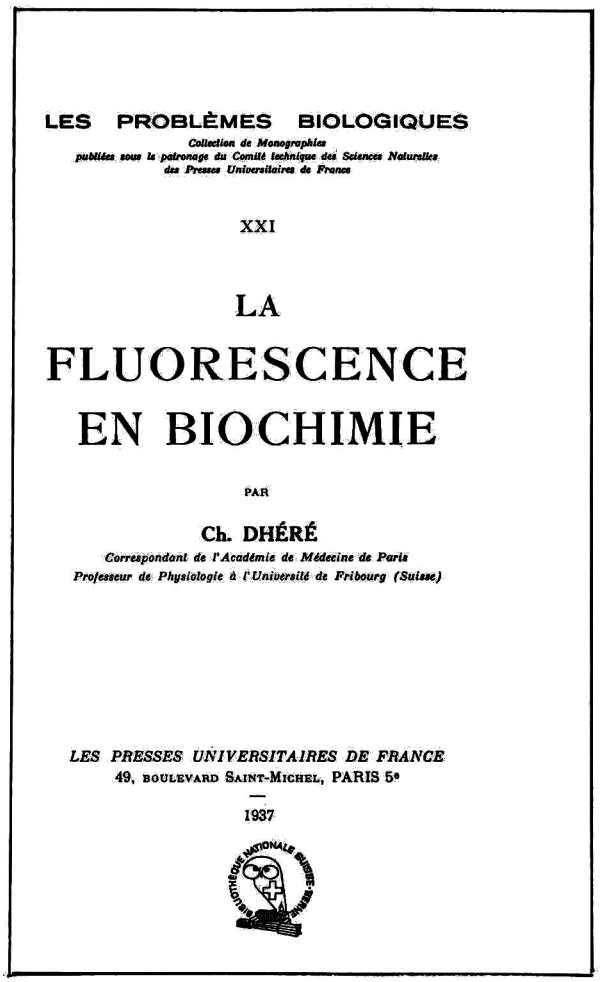
[a] Most data on Dhéré's life were taken from the eulogy by L. Laszt (ref. 15). Note that this Bulletin was always published in one volume during the summer of the following year. This is the reason that although Dhéré died in January 1955, his eulogy was published in the 1954 volume.

[16][a] (Fig. 2), which was considered at that time so important that in 1938, he received the Marcel Benoist Prize for it[b].

Naturally a prerequisite of any spectroscopic investigation is the ability to prepare pure substances. For this reason Dhéré became interested already at the beginning of his professional career in fundamental laboratory techniques which can be utilized for this purpose. One of them was electrodialysis and Dhéré contributed significantly to the advancement of this technique. His interest in methods permitting the separation of biologically important substances and their preparation in pure form also led him to chromatography.

It is interesting that Dhéré never met Tswett, not even during Tswett's frequent visits to his friend Edouard Claparède[c], in Geneva. However, obviously, he had learned about chromatography fairly early and immediately realized its advantages for his own work: after all, he needed pure substances for the spectroscopic measurements and chromatography seemed to be the ideal method for it.

Dhéré started to use chromatography around 1911 and the first work in which this is documented is the doctorate thesis of Wladyslaw de Rogowski, a student from Warsaw. A few years later, the chromatographic technique was further improved in the work of another graduate student, Guglielmo Vegezzi. Let us deal in more detail with these two students and their work.


LES PROBLÈMES BIOLOGIQUES

Collection de Monographies
publiées sous le patronage du Comité technique des Sciences Naturelles
des Presses Universitaires de France

XXI

LA
FLUORESCENCE
EN BIOCHIMIE

PAR

Ch. DHÉRÉ

Correspondant de l'Académie de Médecine de Paris
Professeur de Physiologie à l'Université de Fribourg (Suisse)

LES PRESSES UNIVERSITAIRES DE FRANCE
49, BOULEVARD SAINT-MICHEL, PARIS 5e

1937

Fig. 2. Title page of Dhéré's book on fluorescence in biochemistry, published in 1937.

3. ROGOWSKI AND HIS THESIS WORK

Wladyslaw Franciszek de Rogowski[d] was born on December 3, 1886, in Warsaw, in the Russian-occupied part of Poland. His grandfather was involved in the 1831 Polish uprising against Russia and this rebellious nature was evidently inherited by

[a] It is indicative that for his book (ref. 16) Dhéré selected a motto from the book of Thudichum, *On Chemical Identification of Diseases*, published in 1868, in London: "The phenomena of fluorescence will in future yield important means of diagnosis in animal chemistry."

[b] This prize was established by a Frenchman, Marcel Benoist, to honor annually important scientific inventions which particularly help to improve human life. The winner is always selected by the Swiss Federal Department of the Interior. The prize was presented for the first time in 1919. In 1938, the Prize carried an amount of 30 000 Swiss Francs, a very large sum at that time.

[c] Edouard Claparède (1873–1940) was a physician and professor of psychology at Geneva University. He had been a student at the University when Tswett studied there and they became close friends (see ref. 8).

[d] Rogowski's Christian name is given in different ways in the various sources, as *Wladyslaw, Wladislas* and *Ladislas*. The first is the correct Polish spelling of the name while the two other forms represent an attempt to spell it in the French way. Unless we quote the source verbatim, we shall give it in the Polish form.

his grandson: in 1905, as a senior in high school, he organized a strike, most likely induced by the disturbances in Warsaw in conjunction with the 1905 Russian revolution. Therefore, he had to transfer to a private school (the Jezewski School of Commerce) in order to finish his secondary education. Subsequently he went to Switzerland, to start his university studies. Again, this was probably connected with the political events in Russian-occupied Poland where, for a period, the universities were closed to students.

We have found his police registration in the city of Bern dated October 12, 1906, indicating him as a student at the University. However, he left after one year: an entry at the police dated September 14, 1907, indicates that he departed to Russia which undoubtly means his return to Warsaw. We have information that he also studied at the Jagellonian University of Cracow (in the Austrian-occupied part of Poland) which served not only the Polish people under Habsburg rule: large numbers of Poles from the territories under Russian and German rule also studied there. A Polish biography [17] indicates further studies by Rogowski at the universities of München and Leipzig, but the autobiography added to his doctorate thesis (see below) does not mention any of these.

The next definite information we have about Rogowski is an entry in the student registration book of the University of Fribourg dated October 19, 1911 (Fig. 3). He carried out his thesis work under Professor Dhéré and received his doctorate on December 21, 1912[a].

Toward the end of Rogowski's laboratory investigations a co-authored paper discussing part of his results was submitted by Dhéré to the journal of the French Academy of Sciences (where most of his papers were published): this report was presented at the October 7, 1912, session of the Academy [18]. In this short paper chromatography is indicated in a single sentence only. However, in the thesis of Rogowski [19] the technique and the system used by him is described in more detail.

In Rogowski's chromatography system (Fig. 4) the glass column was not tapered at its lower end (as originally proposed by Tswett [2]) but closed by a cork having multiple perforations; it was standing on a perforated porcelain disk held by a tapered outer glass sleeve. Calcium carbonate which was prebaked at 150°C for 10 h was used as the adsorbent; the height of the chromatographic packing in the column tube was 60–80 mm.

[a] In his later paper on Tswett and on the evolution of chromatography (ref. 9) Dhéré mentions that "my student and collaborator W. de Rogowski left my laboratory (and, most likely, also Switzerland) immediately after finishing his doctorate examination at Fribourg, in 1912." As discussed later, Rogowski's thesis was never printed and is not deposited at the Swiss National Library. We should also mention that his name is not included in the Annual Reports of the University of Fribourg which list all the persons who received a doctorate: he is missing in the Report of 1912 as well as in the Reports for the following years.

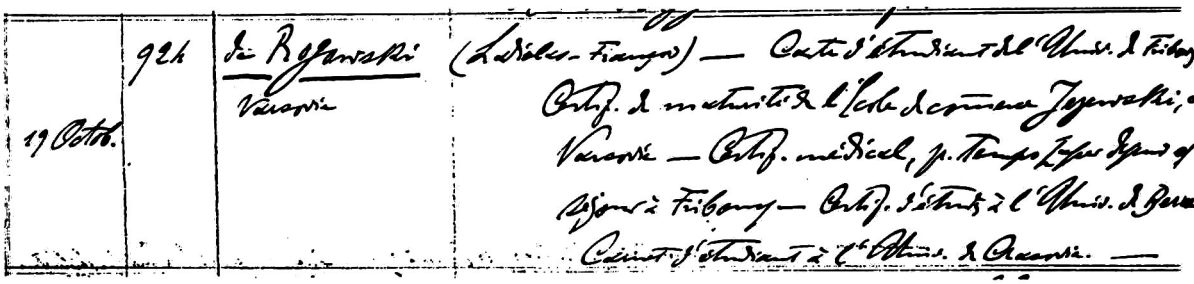


Fig. 3. Entry in the Student Registration Book of the University of Fribourg, for the 1911–1912 Winter Semester. We give the transcript of the hand-written text: de Rogowski, Varsovie, (Ladislas – Franzen) – Carte d'étudiant de l'Univ(ersité) de Fribourg – Certif(icat) de maturité de l'Ecole de commerce Jezewski, à Varsovie – Certif(icat) médical, p. temps.. (?).. séjour à Fribourg – Certif(icat) d'étud(iant) à l'Univ(ersité) de Berne. Carnet d'étudiant à l'Univ(ersité) de Cracovie.

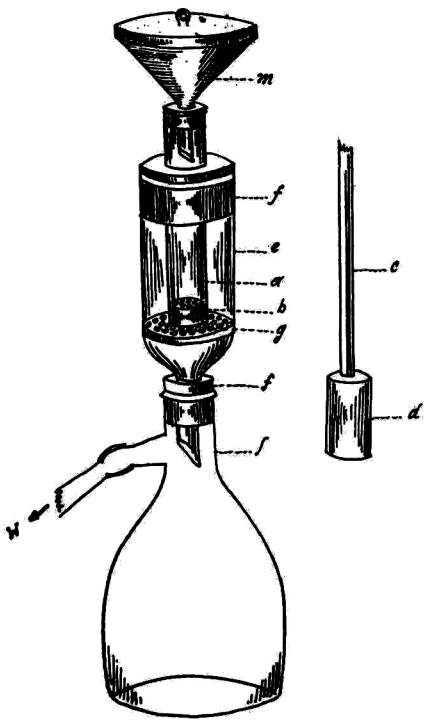


Fig. 4. Rogowski's chromatography system (from ref. 19). *a* = Chromatographic column (250 mm × 20 mm I.D. glass tube); *b* = cork (10 mm high) with multiple perforations; *c*, *d* = wooden pestle; *e* = outer glass sleeve; *f* = rubber stoppers; *g* = perforated porcelain disk; *l* = glass flask; *m* = funnel; *w* = water pump.

After separation and formation of the colored rings, the column packing (the "chromatogram") was slowly pushed out of the tube and the colored rings were carefully separated from each other and from the rest of the packing. This represented a major improvement: for a long time it had always been a problem how to isolate and collect the separated colored rings, without loss and contamination by compounds present in the other rings. Let us not forget that elution of the separated compounds from the column by a continuous solvent flow (obtaining a "liquid chromatogram", an expression used at that time to distinguish it from the column serving as the "chromatogram") started to be used only in the second half of the 1930s [11]. In his later paper on the evolution of chromatography [9] (discussed below) Dhéré emphasized this special feature of Rogowski's system, that it permits one to obtain the individual fractions without rapture of the column packing.

The subject of Rogowski's thesis was the spectroscopic investigation of chlorophyll *a* (α) and *b* (β)[a], of the so-called "crystallizable chlorophyll"[b] and of certain carotenoids.

With regard to the so-called 'crystallizable chlorophyll" Rogowski's investigations proved again that Tswett had been correct: it was not a pure substance but rather a mixture. Concerning the leaf carotenoids investigated by Rogowski, Dhéré emphasized later [9] that this work was the first after Tswett preparing pure substances and demonstrating (independently of Tswett) that pure xanthophyll does not have a red fluorescence in alcoholic solution, as described by Escher – a close collaborator of Willstätter – in his Ph. D. thesis of 1909 [23]. As stated by Dhéré [9], "if Escher had used purification by the chromatographic method, he would not have committed this error."

The main subject of Rogowski's work was the UV adsorption of chlorophyll *a* (α) and *b* (β), and an important point in the investigation was to check the purity of the isolated fractions, comparing data of Tswett *vs.* Willstätter and his collaborators. Twenty-five years later Oscar Biermacher[c] (another student of Dhéré), in his thesis [24] summarized the situation in the following way:

> "By the classical Willstätter and Stoll method of 1913, fractionation between immiscible solvents was employed to separate chlorophyll components *a* and *b* from the yellow pigments and from each other. The spectrograms inserted in the thesis of W. de Rogowski show, however, that the chlorophyll *b* previously prepared by Dhéré and de Rogowski by the chromatographic adsorption method was notably more pure than

[a] Chlorophyll *a* and *b* are identical to chlorophyll α and β; Tswett used Greek letters while later literature adapted the use of Roman characters (*cf.* ref. 10).

[b] "Crystallizable" chlorophyll was an isolated substance which Willstätter believed to be a single, native pigment. Tswett, in a number of publications (refs. 20–22) demonstrated that, in fact, is was an artifact, formed during the long alcoholic extraction of the living tissue and that it was a mixture of two substances.

[c] Oscar Biermacher was born on September 10, 1904, in Cleveland, Ohio (USA). He received the B.Sc. degree from the University of Dayton, Ohio, in 1928. After four years' teaching at high schools in Ohio, he returned to the University of Dayton in 1932 for graduate studies. In 1934 he enrolled at the University of Fribourg and carried out his thesis work under Prof. Dhéré, receiving his doctorate in 1936.

the chlorophyll *b* prepared at the same period by Willstätter and Stoll."

The same fact was emphasized in 1940 by Hans Fischer and Adolf Stern [25] who stated that

"M. Tswett was able to establish exactly the existence of two green components with help of the adsorption analysis introduced by him and to describe in detail the spectra of these pigments. These investigations were, however, later violently contradicted and they were forgotten, although, as we know it today, M. Tswett was the first who actually obtained – be it in solution – really pure chlorophyll. Only Ch. Dhéré and W. de Rogowski (1912) could again prepare pure chlorophyll solutions according to the method of M. Tswett and describe the fluorescent spectra of both components."

Although this is not the place to discuss and value Rogowski's spectra it is interesting to compare his VIS spectra of chlorophyll *a* and *b* with the ones published by Willstätter in those years. Twenty years later Alfred Winterstein [26,27] analyzed in detail Willstätter's spectra and proved that his chlorophyll *b* was not pure but had a 15% impurity of chlorophyll *a*. This is evident from the spectra published by Willstätter and co-workers [28,29] where the 644 nm absorption band of chlorophyll *b* is accompanied by a weak band at 663 nm (being the most prominent but not the only extra band in the spectrum): the 663 nm absorption is caused by chlorophyll *a* present in the solution (see Fig. 5). Today it is funny to read that Rogowski, in his thesis, was worrying about this missing band and that he only obtained *une légère ombre* (a weak shadow) at this wavelength when he increased the optical pathlength to 26 nm [19]. Obviously the chlorophylls prepared by him were really pure (Fig. 6) and indeed he confirmed Tswett's results. However, in those days Willstätter was the big authority in this field and people believed him more than the little-known Russian botanist.

It is noteworthy that F. M. Schertz (one of the translators of the chlorophyll book by Willstätter and Stoll [28]) published a paper [30] as late as 1929 in which he highly criticized Tswett's work and methodology. The following two sentences are quoted from this paper:

"It is evident that Tswett was never at any time dealing with pure pigments for not once were the substances crystallized, nor did he report any attempt made at crystallization."
"... Tswett's methods have been shown to be unreliable in identifying and distinguishing carotin and xanthophyll."

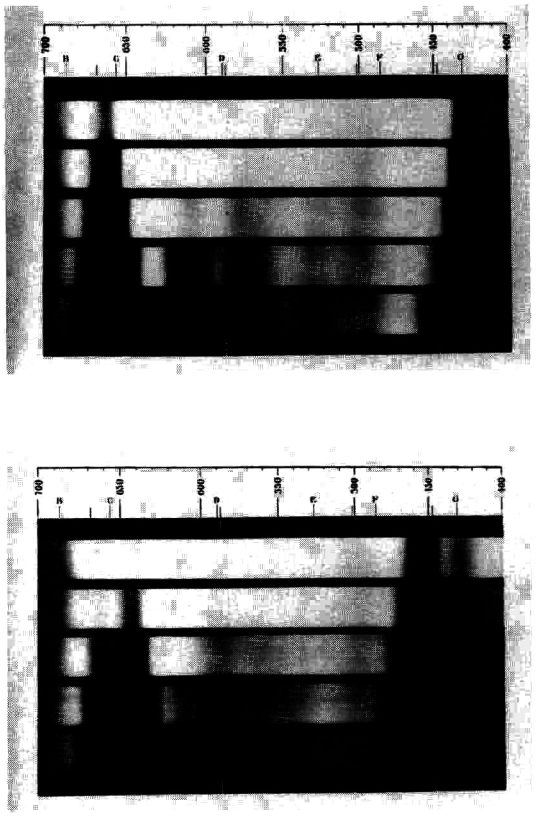


Fig. 5. Willstätter's visible spectra of chlorophylls (from ref. 28). The spectra were obtained by the direct exposure of a cell filled with solution to a photographic plate. Optical pathlengths were, from top down: 5, 20, 40 and 80 mm. Concentration: 31.2 mg of chlorophyll in 1 l of ether. The wavelength scale is in nm. Upper spectrum: chlorophyll *a*; lower spectrum: chlorophyll *b*. The 644 nm absorption band of chlorophyll *b* is accompanied by the 663 nm band of chlorophyll *a*.

It took ten more years until this argument was finally confuted by P. Karrer who stated [31] that "... it would be a mistake to believe that a preparation purified by crystallization should be purer than one obtained from chromatographic analysis. In all recent investigations chromatographic purification widely surpassed that of crystallization."

These facts emphasize even more the farsightedness of Dhéré who realized at such an early stage of the evolution of science that chromatography can give purer compounds than the classical methods.

When we were looking for the thesis of Rogowski, we could not find it at the Swiss National Library where all doctorate theses submitted to Swiss

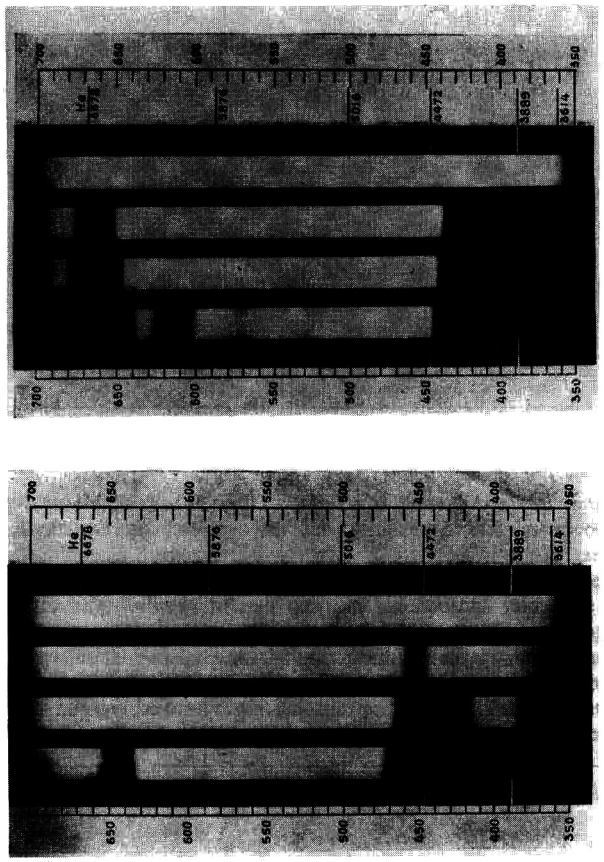


Fig. 6. Rogowski's visible spectra of chlorophylls, (from ref. 19). The uppermost strip is a blank followed by optical pathlengths of 5, 10 and 20 mm. Concentration of the solution was not known. The wavelength scale is in nm. Upper spectrum: chlorophyll *a*; lower spectrum: chlorophyll *b*. These spectra were expanded for this reproduction in order to obtain equal distances in the 400–700 nm region for Figs. 5 and 6.

universities are deposited, and there was no record of it there. Finally, we found a poor copy of the typewritten text at the University of Fribourg [19]; this text is unusual in a number of aspects. Although the text on the title page of the typed thesis specifies that it is presented by Wladislas de Rogowski to the Faculty of Science of Fribourg University in order to obtain the degree of a *Docteur ès Sciences*, the date (year) on this title page is given as 1914 (and not as 1912) and the city as Warsaw (and not as Fribourg). Also, in the brief autobiography of the author included with the thesis the fact that he received the doctor degree is given in past tense: "*A la suite de mes examens à la Faculté des Sciences de Fribourg j'ai obtenu le title de docteur le 21 décembre 1912.*" This would mean that this text is a later typescript and that he probably did not have a formal written thesis at the time of his final examinations[a].

The fact that the text we found at the University of Fribourg was typed later, outside the French-speaking part of Switzerland, is also indicated by our observation that the typewriter used did not have the accented vowels use in French (*e.g.*, *à*, *é*, *è*, etc.) and he had to add the accents by hand.

Zechmeister and Cholnoky, in the bibliography section of their fundamental chromatography book published in 1937 [32] list Rogowski's thesis as submitted to two universities: to Fribourg, in 1912, and to Warsaw, in 1914. Thus, an easy interpretation of the past tense, and of the year 1914, would be that this text was typed later and while a copy was sent to Dhéré, it was actually submitted by Rogowski to the University of Warsaw, for the second doctorate[b]. There is, however, a major problem in this interpretation. On the title page of the thesis, the author is listed as *Wladislas de Rogowski de Varsovie (Pologne)*. There is no possibility that the Universi-

[a] See footnote *a* on p. 6.

[b] In this respect we would like to refer to the well-known problem Tswett had with his Swiss doctorate. When moving to Russia, he found out that foreign scientific degrees are not accepted there; therefore, he had to repeat both his master's and doctorate degrees (*cf.* footnote ** on p. 3). It is quite possible that this "second doctorate" of Rogowski simply meant the same, submitted for official recognition.

If we assume this "second doctorate" theory, there is an intriguing possibility: in connection with submitting his thesis to the University of Warsaw Rogowski may have met Tswett. It is true that from 1908 on, Tswett was affiliated with the Polytechnique Institute and not with the University: however, undoubtedly, he maintained connections with the University where he had been active between 1901 and 1908. In his thesis Rogowski frequently cited Tswett and the thesis discussed a subject only a few could understand; therefore, it would not be unusual if his colleagues gave Tswett this thesis for review or at least, to read it. Furthermore, let us not forget that in Russia, defending a doctorate thesis had always been a well-publicized public affair. It would have been unusual if Tswett did not go to this open session: after all, the candidate used *his* method and the main subject of the thesis was to prove that *his* (Tswett's) results disputed by others were correct. The only reason which could have prevented this was Tswett's illness: we know (*cf.* refs. 5 and 8) that in 1914, he was absent between the end of March and the middle of November.

ty of Warsaw would have permitted to submit a thesis in which the country is indicated as "Poland": let us not forget that officially, "Poland" did not exist and Warsaw was in Russia[a]! On the other hand, it is also possible that this title page was prepared only for the copy mailed to Dhéré. Most likely one can never resolve this mistery.

The most interesting in Rogowski's thesis is that he quoted Tswett's magnum opus, his book published in 1910 [33] by giving its Russian title, written in Cyrillic, while *e.g.*, Vegezzi, in his thesis (see below) was quoting the book by giving its title in French[b]. We can safely assume that Dhéré already knew about Tswett's activities from his papers published in German and French scientific journals[c]. However, most likely, it was through Rogowski that he became exposed to Tswett's Russian book. Obviously Rogowski knew Russian and we would not be surprised if he brought the book with him to Fribourg. Here, we have the direct connection between Tswett's publication and the work in Dhéré's laboratory.

Just a few more words about Rogowski (Fig. 7). After his doctorate he was not engaged any more in original scientific work. He was an educator, both in Poland and Brazil (setting up schools for the children of Polish immigrants); a poet and a writer; a politician and a pioneer in modern agricultural methods; and when needed, he served his country against those who wanted to destroy it. He survived the 1944 Warsaw uprising but, on January 20, 1945, a few days after the liberation of Warsaw, Rogowski was arrested by the Red Army. He died on April 25, 1945, in the Soviet concentration camp Baskoje, in the Ural Mountains.

Fig. 7. Wladyslaw Franciszek de Rogowski, in 1935.

Rogowski's wife, Irena Maczewska, survived her husband and died on January 4, 1973, in Warsaw, at the age of 81. They had two daughters, Barbara Rogowska-Piotrowska (born in 1923, now living in Warsaw), and Kalina Rogowska-Przeslawska (born in 1927 and died in 1972)[d].

4. VEGEZZI AND HIS THESIS WORK

In 1913–1914 Dhéré had seven publications on animal and plant pigments and their spectroscopic investigations. Among these only one, the paper coauthored with Ryncki, dealing with the UV spectroscopic investigations of carotenoid pigments [35], mentioned that pure carotene was prepared according to the method of Tswett, using the technique

[a] We would like to express our appreciation to Prof. Edward Soczewinski (Department of Inorganic and Analytical Chemistry, Medical Academy, Lublin, Poland) who drew our attention to this fact.

[b] It should be noted that on the top of the front page of Tswett's book, the French translation of its title *(Les Chromophylles dans les Mondes Végétal et Animal)* is given. This can clearly be seen in the photo of the title page shown on p. 211 of Senchenkova's book (ref. 7). The identical French title of Tswett's book is cited by Vegezzi, in his thesis (ref. 34) and also in the bibliography of Dhéré's paper on Tswett (ref. 9).

[c] In the comprehensive bibliography section of her book (ref. 7) Senchenkova lists three papers in German from 1906; five in German and one in French from 1907; seven in German and one in French from 1908; none from 1909; one in German from 1910; three in German and two in French from 1911.

[d] We obtained this information and the photograph of Fig. 7 through Dr. H. Lamparczyk (Faculty of Pharmacy, Medical Academy, Gdansk, Poland) from Mrs. Barbara Rogowska-Piotrowska (Warsaw) for which we express our special gratitude.

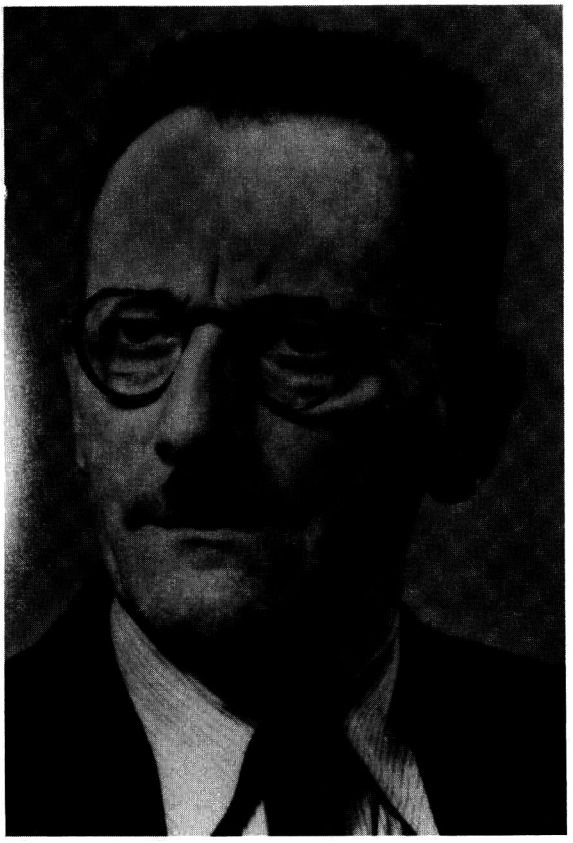


Fig. 8. Guglielmo Vegezzi, in his middle age.

described by Rogowski, and reference is given to the paper by Dhéré and Rogowski [18].

The next major work carried out in Dhéré's laboratory in which the chromatographic technique was further improved was the thesis of Guglielmo Vegezzi, born on August 1, 1890, in Ticino, the Italian-speaking area of Switzerland (Fig. 8).

Vegezzi did his undergraduate studies at the Universities of Zürich and Fribourg; he started his thesis work under Dhéré in 1913 and it was mostly finished by the Summer of 1914. However, his military service in 1914–1915 prevented him from finishing it until the spring of 1916. He received his *Docteur ès Sciences* degree on July 25, 1916. Subsequently he joined the Swiss Federal Administration of Alcohol as a chemist, later advancing to the position of the vice-director of the Agency. He died on September 3, 1955.

The subject of Vegezzi's thesis was the spectroscopic investigation of various pigments present in invertebrates, such as in the bile and liver of the escargot *Helix pomatia*, and in the eggs of the spider crab *Maja squinado*. These investigations were very important because they represented the first application of chromatography in the preparation of such pigments in pure form, permitting the measurement of their UV absorption bands and their fluorescence. In addition to the printed thesis of Vegezzi [34] the results were summarized in six papers co-authored by Dhéré and Vegezzi [36–41].

The chromatographic system of Vegezzi (Fig. 9) differed only slightly of that of Rogowski. Now, the

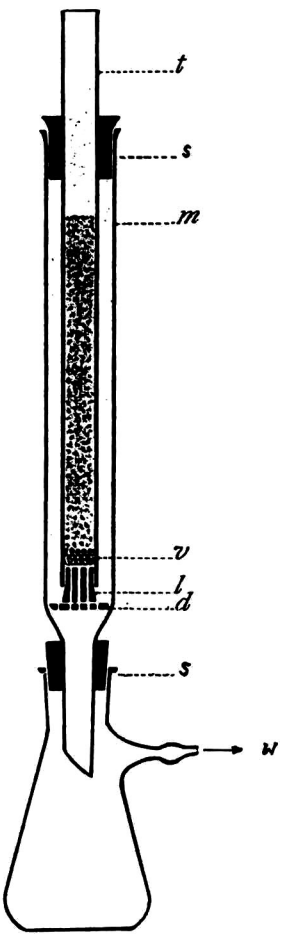


Fig. 9. The chromatographic system of Vegezzi, (from ref. 34). t = Chromatographic column (350 mm × 16 mm I.D. glass tube), m = outer glass sleeve, v = glass wool layer (about 5 mm high), l = cork with multiple perforations, d = perforated porcelain disk, s = rubber stoppers, w = water pump.

outside glass sleeve extended to the whole length of the chromatographic column: in his 1943 paper [9] Dhéré implied that in the Rogowski design (*cf.* Fig. 4), the upper part of the "chromatogram" could not be seen well because the upper rubber stopper blocked the view of it and this was the reason for the change. In Dhéré's later book on fluorescence [16] the Vegezzi design of a chromatographic system is recommended.

It is interesting to note that after finishing the investigations with Vegezzi, Dhéré evidently did not carry out any more chromatography. He also slowed down in having graduate students. In the list of Laszt [15] there is one thesis from 1906, six are from 1910–1912, one each from 1916 (Vegezzi's), 1924, 1927 and 1928, two each from 1932 and 1936 and one from 1941. In other words, there is an eight-year hiatus after Vegezzi, there are a couple of theses from the second part of the twenties and then, there is again an indication of renewed activity toward the end of Dhéré's professional career.

When going through the doctorate theses published from 1924 on, one may wonder why chromatography was no longer used in Dhéré's laboratory. A great number of pigments from various sources were prepared in pure form (or whatever was assumed to be pure) by precipitation, dialysis, crystallization and liquid–liquid partition. We may assume that the know-how for chromatography was lost when Vegezzi left Dhéré's laboratory, just after his exam, to start working in Bern. As mentioned, for some years Dhéré did not have any graduate students and the next thesis was only finished in 1924. The only one who was thinking about chromatography was Oscar Biermacher who finished his thesis in 1936 [24]. In this he discussed the method, but then decided to use liquid–liquid partition for the isolation and purification of chlorophylls, because he found that this technique is not inferior to chromatography. This is even more strange because by then, chromatography started to be widely used in many laboratories, also in Switzerland.

We have discussed the activities of Dhéré only from the point of chromatography. An investigation of his whole professional life was beyond our task.

5. PAPER ON TSWETT AND ON THE EVOLUTION OF CHROMATOGRAPHY

In 1937 Zechmeister and Cholnoky published their bestseller, their book on chromatography [32] and one year later a second, greatly enlarged edition was published. In the preface of the second edition, the authors stated that "it was intended to begin this volume with a biography of Tswett, but reliable data about the life of this pioneer were not available up to this time". Indeed this was true: the first brief biography of Tswett (a total of 28 lines, with a bibliography listing 56 publications) was published only in 1940, in a collection of the biographies of botanists from Geneva [42] written by John Briquet[a].

Evidently this publication induced Dhéré to start collecting data on Tswett. Dhéré was now retired and living in Geneva, with strong connection to the University. Unfortunately Edouard Claparède, Tswett's closest friend in Geneva, with whom he studied at the University and whom he visited almost yearly from Russia[b], was already dead when Dhéré became interested in Tswett's life and activities, but his widow provided a photo of the young Tswett which he presented in 1896 to his friend Claparède. However, Dhéré could still contact P.G. Hochreutiner, professor at Geneva University, who was also a graduate student together with Tswett in the laboratory of professor Marc Thury.

It took about two years for Dhéré to compile his 50-page long paper which he finally submitted on March 23, 1943 to the journal *Candollea* [9]. This paper not only dealt with the life of Tswett but it also discussed in detail his scientific work presenting a critical evaluation of the controversies related to the early years of chromatography and to Tswett's chromatographic investigations of plant pigments. In addition, Dhéré also presented a summary of the evolution of chromatographic analysis up to 1940. The article finished with a brief discussion of the

[a] John Briquet (1870–1931) was assistant at the Botanical Institute of the University of Geneva when Tswett studied there. He later became professor of botany at the university. After Tswett's return to Russia, they remained in touch through frequent correspondence. Briquet finished the compilation of ref. 42 just before his death but it was published only nine years later, on the occasion of the 50th anniversary of the Société Botanique Suisse.

[b] See footnote *c* on p. 5.

influence of Tswett's method on the results of "the princes of contemporary science", the Laureates of the Nobel Prize in Chemistry. Finally a detailed bibliography listing the most important papers related to chromatography, including 36 papers by Tswett, was given[a]. For 30 years, until the start of the publications of Sakodynskii, Senchenkova and others, this was the most comprehensive discussion of Tswett's life and activities.

6. CONCLUSIONS

In the early evolution of chromatography Charles Dhéré occupies a very important place. He was the first who, by independent investigations, proved the correctness of Tswett's assumptions on the existence of multitude of chlorophyllic and carotenoidic pigments. Dhéré extended the use of chromatography into animal biochemistry[b] and demonstrated that indeed, chromatography can provide purer substances than any of the then accepted methods. We might rightly cite Winterstein and Willstaedt in praising his activities: In 1933 Winterstein stated that "the significance of this method (*i.e.*, of chromatography) to biochemical research subsequently (*i.e.*, after Tswett) appeared to be understood only by Charles Dhéré and his co-workers" [26]; and, according to Willstaedt, "one of the first authors to appreciate the advantages of this new procedure (*i.e.*, chromatography) was Dhéré, who made use of it in a number of investigations" [43].

7. ACKNOWLEDGEMENTS

Many people helped us with valuable information and discussion; we cannot list all of them. However, we particularly would like to acknowledge the contributions of Mrs. H. Bürgi (Chancellery of the University of Fribourg, Switzerland), Professor em. E. Giovannini (University of Fribourg), Mrs. Ann Klus (Chief Librarian, Medical Academy, Gdansk, Poland), Professor F. J. A. Kreuzer (Institute of Physiology, University of Nijmegen, Netherlands), Dr. habil. Henryk Lamparczyk (Faculty of Pharmacy, Medical Academy, Gdansk, Poland), Mrs. S. Pinz (Institute of Systematic Geobotany, University of Bern, Switzerland), Mrs. Barbara Rogowska-Piotrowska (Warsaw, Poland), Professor Edward Soczewiński (Department of Inorganic and Analytical Chemistry, Medical Academy, Lublin, Poland) and Dr. G. Vegezzi Jr. (Aarau, Switzerland).

NOTE ADDED IN PROOF

In our paper we often used the term "Russia": by this we simply referred to the Russian Empire of the Tsar prior to 1916 and not to the geographical area of Russia formed after the dissolution of the Soviet Union at the end of 1991. Similarly, the term "Russian" meant a subject of the Russian Empire.

Actually, the true nationality of M. S. Tswett and of his father is not so simple. His father, Semen N. Tswett, was born in Chernigov which is in the Ukraine; thus, according to present-day standards he might be considered a Ukrainian. However, he left that area as a teenager and never returned (except for brief visits). He studied at the University of Tartu (the same school where, 70 years later, his son became a professor) in Estonia (which, at that time, was part of the Russian Empire) and, after graduation, he had been active in Russia proper or abroad (in Italy and Switzerland). In the last few years of his life he was a Councilor of State, the Chairman of the Provincial Finance Department in the Crimean Peninsula which then was part of Russia proper but today, because of a decree of Khrushchev from the mid-1950s, belongs to Ukraine.

Mikhail S. Tswett's mother, who was of Italian origin, was born in Turkey but grew up in Russia. As mentioned (*cf.* footnote *a* on p. 4) M. S. Tswett was born in Italy and grew up in Switzerland. In fact, when he returned to Russia, in 1896, he spoke only fairly poor Russian and we know for instance (see ref. 8) that at home, he and his wife preferred to

---

[a] We have found two apparent errors in Dhéré's paper: (a) the date of Tswett's death is given as May 1920, while he actually died on June 26, 1919. This error can be traced back to the announcement of Tswett's death in the *Berichte der deutschen botanischen Gesellschaft* giving this false date. (b) Dhéré stated that Edouard Claparède died in 1939; however, according to Hais (ref. 8) he died in 1940.

[b] Actually L. S. Palmer at the University of Missouri (Columbia, MO) in the USA, also carried out chromatographic investigations of pigments present in plants and animal tissues, from 1911 on. However, at that time, neither knew about the other's work.

use French in conversation. In his life M. S. Tswett spent 25 years in Switzerland, seven years in Russia proper, fifteen in Poland and less than one in Estonia (both within the Russian Empire). So what nationality was he?

REFERENCES

1 M. Tswett, "Physikalisch-chemische Studien über das Chlorophyll. Die Adsorptionen", *Ber. Dtsch. Botan. Ges.*, 24 (1906) 316–323. An English translation of this article has recently been published in: V. G. Berezkin, Editor, *Chromatographic Adsorption Analysis: Selected Works of M. S. Tswett*, Ellis Horwood, New York, London, 1990, pp. 21–26.

2 M. Tswett, "Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls", *Ber. Dtsch. Botan. Ges.*, 24 (1906) 384–393. An English translation of this article has recently been published in: V. G. Berezkin, Editor, *Chromatographic Adsorption Analysis: Selected Works of M. S. Tswett*, Ellis Horwood, New York, London, 1990, pp. 27–34.

3 K. I. Sakodynskii, "M. S. Tswett – His Life", *J. Chromatogr.*, 49 (1970) 2–17.

4 K. I. Sakodynskii, "The Life and Scientific Works of Michael Tswett", *J. Chromatogr.*, 73 (1972) 303–360.

5 K. I. Sakodynskii, "New Data on M. S. Tswett's Life and Work", *J. Chromatogr.*, 220 (1981) 1–28.

6 K. I. Sakodynskii, *Michel Tswett – Life and Work*, Carlo Erba Strumentazione, Milan, undated (1981).

7 E. M. Senchenkova, *Mikhail Semenovich Tsvet*, Nauka, Moscow, 1973.

8 I. M. Hais, "Tswett's Letters to Claparède", *J. Chromatogr.*, 440 (1988) 509–531.

9 Ch. Dhéré, "Michel Tswett, le Créateur de l'Analyse Chromatographique par Adsorption. Sa Vie, ses Traveaux sur les Pigments Chlorophylliens", *Candollea (Genève)*, 10 (1943) 23–73.

10 T. Robinson, "Michael Tswett", *Chymia*, 6 (1960) 146–161.

11 L. S. Ettre, "Evolution of Liquid Chromatography: A Historical Overview", In Cs. Horváth (Editor), *High-Performance Liquid Chromatography – Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, pp. 1–74.

12 E. Lederer, "La Renaissance de la Méthode Chromatographique de M. Tswett en 1931", *J. Chromatogr.*, 73 (1972) 361–366.

13 Ch. Dhéré, *Sur la Variation des Centres Nerveux en Fonction de la Taille*, Thesis for the *Docteur en Médicine* degree, University of Paris, Paris, 1898.

14 Ch. Dhéré, *Recherches Spectrographiques sur l'Absorption des Rayons Ultraviolets par les Albuminoïdes, les Protéides et leurs Dérivés*, Thesis for the *Docteur ès Sciences* degree, University of Paris, Paris, 1909.

15 L. Laszt, *Bull. Soc. Frib. Sci. Nat.*, 44 (1954) 304–313.

16 Ch. Dhéré, *La Fluorescence en Biochimie*, Presses Universitaires de France, Paris, 1937, 318 pp. + 27 plates.

17 Data on Rogowski's life were mostly taken from the book *Slownik Biograficzny 1988–1989*, Ossolineum Publisher, Warsaw, 1990, pp. 455–457.

18 Ch. Dhéré and W. de Rogowski, "Sur l'Absorption des Rayons Ultraviolets par les Chlorophylles et par la Chlorophylle Crystallisée", *Comp. Rend. Acad. Sci. Paris*, 155 (1912) 653–656 (presented on October 7, 1912).

19 Wladislas de Rogowski, *Recherches sur les Spectres d'Absorption Ultra-Violets et sur les Spectres d'Emission par Fluorescence des Pigments Chlorophylliens*, Thesis for the *Docteur ès Sciences* degree, 79 (typed) pages + 4 plates. The year given in the thesis is 1914.

20 M. Tswett, "Über die Natur des sogenannten 'Krystallisierbaren Chlorophylls' (Meta-chlorophyllins)", *Biochem. Z.*, 44 (1908) 414–425.

21 M. Tswett, "Das sogenannte 'krystallisierte Chlorophyll' – ein Gemisch", *Ber. Dtsch. Chem. Ges.*, 43 (1910) 3139–3141.

22 M. Tswett, "Über die Löslichkeitsverhältnisse der Chlorophylline und eine neue Methode zur Isolierung derselben", *Ber. Dtsch. Chem. Ges.*, 45 (1911) 1124–1127.

23 H. Escher, *Zur Kenntniss des Carotins und des Lycopins*, Thesis, E. T. H., Zürich, 1909.

24 O. Biermacher, *The Visible and Infrared Fluorescence Spectra of Chlorophyll a, Chlorophyll b and Several Porphyrins*, Thesis for the *Docteur ès Sciences* degree, University of Fribourg, Fribourg, 1936.

25 H. Fischer and A. Stern, *Die Chemie des Pyrrols, II., 2. Hälfte: Die Chemie der Chlorophylle*, Akademische Verlagsgesellschaft, Leipzig, 1940.

26 A. Winterstein and G. Stein, "Fraktionierung und Reindarstellung organischer Substanzen nach dem Prinzip der chromatographischen Adsorptionsanalyse. I. Anwendungsbereich; II. Chlorophylle", *Z. Physiol. Chem.*, 220 (1933) 247–263, 263–277.

27 A. Winterstein, "Fraktionierung und Reindarstellung von Pflanzenstoffen nach dem Prinzip der chromatographischen Adsorptionsanalyse", in G. Klein (Editor), *Handbuch der Pflanzenanalyse*, Springer Verlag, Wien; Vol. IV, Part 3 (1933), pp. 1403–1437.

28 R. Willstätter and A. Stoll, *Untersuchungen über Chlorophyll: Methoden und Ergebnisse*, Julius Springer, Berlin, 1913; viii + 424 pp. An English translation of the book was published 15 years later: *Investigations on Chlorophyll: Methods and Results* (translated by F. M. Schertz and A. R. Merz), Science Press, Lancaster, PA, 1928.

29 R. Willstätter, A. Stoll and M. Utzinger, "Absorptionsspektren der Komponenten und ersten Derivate des Chlorophylls", *Ann. Chem.*, 385 (1911) 156–188.

30 F. M. Schertz, "The Pure Pigments Carotin and Xanthophyll, and the Tswett Adsorption Method", *Plant Physiology*, 4 (1929) 337–348.

31 P. Karrer, "Zur Frage der Reinheit und Wirkungsstärke von Vitamin A Präparaten", *Helv. Chim. Acta*, 22 (1939) 1149–1150.

32 L. Zechmeister and L. Cholnoky, *Die Chromatographische Adsorptionsmethode*, Julius Springer, Wien, 1937, 2nd ed., 1938.

33 M. S. Tswett, *Khromofilly v Rastitel'nom i Zhivotnom Mire* ("Chromophylls in the Plant and Animal World"), Izd. Karbasnikov, Warsaw, 1910. A part of this book specifically dealing with the chromatographic method has recently been published in English translation in: V. G. Berezkin, Editor,

Chromatographic Adsorption Analysis: Selected Works of M. S. Tswett, Ellis Horwood, New York, London, 1990, pp. 35–79. (Note that in this publication the Russian title of Tswett's book is given incorrectly, by reversing the order of "rastitel-'nom" and "zhivotnom").

34 G. Vegezzi, *Recherches sur Quelques Pigments des Invertébrés: Hélicorubine, Hépatochlorophylle, Tétronérythrine*, Thesis for the *Docteur ès Sciences* degree, University of Fribourg, Fribourg, 1916, 111 pp. + one plate.

35 Ch. Dhéré and L. Ryncki, "Sur l'Absorption des Rayons Visibles et Ultraviolets par les Pigments Carotinoïdes", *Compt. Rend. Acad. Sci. Paris*, 157 (1913) 501–503.

36 Ch. Dhéré and G. Vegezzi, "Sur l'Hémochromogène Acide", *Compt. Rend. Acad. Sci. Paris*, 163 (1916) 18–20 (presented on July 3, 1916).

37 Ch. Dhéré and G. Vegezzi, "Influence Exercée par le Degré de Réduction des Hémochromogènes sur leurs Propriétés Spectrales", *Compt. Rend. Acad. Sci. Paris*, 163 (1916) 209–211 (presented on August 23, 1916).

38 Ch. Dhéré and G. Vegezzi, "Sur la Composition Pigmentaire de l'Hépatochlorophylle", *Compt. Rend. Acad. Sci. Paris*, 163 (1916) 399–401 (presented on October 16, 1916).

39 Ch. Dhéré and G. Vegezzi, "Sur l'Hélicorubine", *Compt. Rend. Acad. Sci. Paris*, 164 (1917) 869–870 (presented on May 29, 1917).

40 Ch. Dhéré and G. Vegezzi, "Recherches sur l'Hélicorubine, I", *J. Physiol. Pathol. Gén.*, (1917) 44–52.

41 Ch. Dhéré and G. Vegezzi, "Recherches sur l'Hélicorubine, II", *J. Physiol. Pathol. Gén.*, (1917) 53–66).

42 John Briquet, "Biographies des Botanistes à Genève (1500–1931)", *Bull. Soc. Bot. Suisse*, 50a (1940) 1–494; Tswett's biography is on pp. 463–466.

43 H. Willstaedt, *L'Analyse Chromatographique et ses Applications*, Hermann, Paris, 1938.

Journal of Chromatography, 600 (1992) 17–36
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 008

Review

Role of ion-exchange and extraction chromatography in neutron activation analysis

Rajmund Dybczyński

Department of Analytical Chemistry, Institute of Nuclear Chemistry and Technology, Dorodna 16, 03-195 Warsaw (Poland)

ABSTRACT

The role and uses of ion-exchange chromatography and extraction chromatography in neutron activation analysis are surveyed. Examples of post-irradiation group separations and isolation of individual radionuclides and also pre-irradiation separations are presented and their significance for achieving the best detection limits and the highest accuracy are emphasized, including the contribution of column chromatography to the construction of "definitive" methods of analysis. Special emphasis is given to difficult cases such as micro–macro separations of ions with similar properties (*e.g.*, rare earth elements, alkali metals). The importance of the proper choice of the chromatographic system, including such factors as the kind of ion exchanger–solution combination, resin cross-linking and temperature, is pointed out.

CONTENTS

1. INTRODUCTION

Most of the papers presented at this Symposium were devoted to the demonstration of merits of various types of chromatography used as individual and self-dependent method of quantitative analysis. This paper presents a brief survey of selected applications of ion-exchange and extraction chromatography used mainly as a separation tool in conjunction with neutron activation for the deter-

0021-9673/92/$05.00

mination of elements in inorganic trace analysis.

Neutron activation analysis (NAA) is one of the most powerful techniques available for the determination of trace amounts of elements. The principles of the method are such that typically samples and standards are irradiated in a high flux of thermal neutrons in a nuclear reactor, then the characteristic radiation of the radionuclides formed as a result of the nuclear reactions is measured with a suitable detection system, most often with a γ-ray spectrometer [1–5].

The general scheme of analysis is shown in Fig. 1. The purely instrumental (non-destructive) version of the method is indicated with thick arrows, whereas thin arrows show the radiochemical (destructive) variant of the method.

NAA occupies a unique position among other methods of inorganic trace analysis owing to several features. First, it is a nuclear method and so the induced radioactivity does not depend on the chemical form of an element. Hence NAA to a much lesser extent than other methods of analysis is vulnerable to the so-called matrix effects, which is in turn reflected in the generally good accuracy of the method. Second, once the sample had been irradiated, any subsequent contamination with elements from the ambient atmosphere, reagents, container walls, etc., does not influence the signal measured (*i.e.*, the radioactivity of a given radionuclide). In analytical terms, the blank is eliminated or drastically reduced. Finally, the method shows very favourable detection limits with respect to a large number of elements (Table 1).

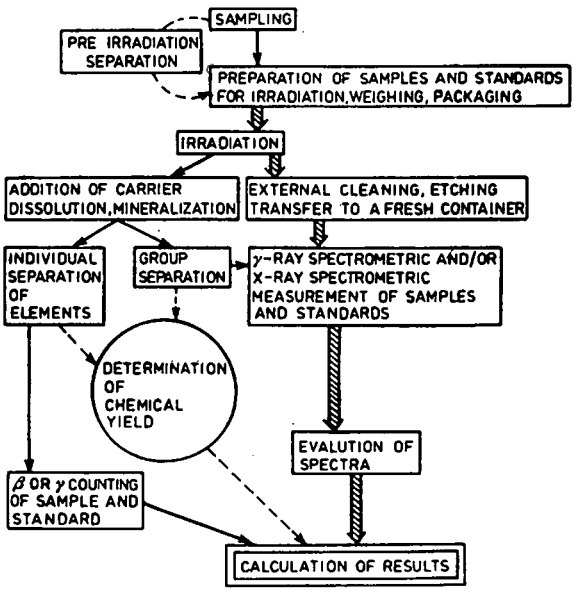


Fig. 1. General scheme of NAA method. Thick arrows, purely instrumental version; thin arrows, radiochemical version. (After Dybczyński [4]).

The detection limits shown in Table 1 are the so-called "interference-free detection limits", *i.e.*, those attainable when the activity in question is measured in the absence of other radionuclides. For real samples the detection limits may be worse by several orders of magnitude, owing to spectral interferences, the necessity for measuring small photopeaks on a high and varying Compton background, interfering nuclear reactions, etc. [1–5]. At the same time, the accuracy of determinations is adversely affected by phenomena such as the count losses due to pile-up and dead-time effects [5,6]. Therefore, although the great progress observed

TABLE 1

CALCULATED BEST DETECTION SENSITIVITIES FOR 68 ELEMENTS IN THE ABSENCE OF INTERFERING ACTIVITIES

For $\Phi_{th} = 10^{13}$ n/cm^2 · s; $t_i = 5h$ maximum; $t_d = 0$; $t_c = 100$ min maximum; 40 cm^3 Ge(Li) detector; 2 cm distance; largest photopeak. (After Guinn and Hoste [2]).

Detection limit (μg)	Elements[a]
1–3 × 10^{-7}	In, Eu, Dy
4–9 × 10^{-7}	Ho
1–3 × 10^{-6}	Mn, Sm, Au
4–9 × 10^{-6}	Rh, Lu, Re, Ir
1–3 × 10^{-5}	Co, Cu, Ga, As, I, Cs, La, Er, W, Hg, U
4–9 × 10^{-5}	Na, V, Br, Ru, Pd, Sb, Yb, Th
1–3 × 10^{-4}	Sc, Ge, Sr, Te, Ba, Nd, Ta
4–9 × 10^{-4}	Cl, Se, Cd, Gd, Tb, Tm, Hf, Pt
1–3 × 10^{-3}	Al, Zn, Mo, Ag, Sn, Ce, Os
4–9 × 10^{-3}	K, Ti, Cr, Ni, Rb, Y, Pr
1–3 × 10^{-2}	Mg
4–9 × 10^{-2}	Zr
1–3 × 10^{-1}	F, Ca, Nb
1–3	Fe
4–9	Si
10–30	S, Pb

[a] In addition to the above 68 elements, three can be determined by β^- counting: P (5 × 10^{-4} μg), Bi (1 × 10^{-2} μg) and Tl (2 × 10^{-2} μg).

over the past two decades in the technology of semiconductor detectors, pulse-height analysers and associated electronics has made possible the use of γ-ray spectrometry for the purely instrumental determination of several elements in a variety of matrices, nevertheless to achieve the best detection limits the use of separation methods is indispensable.

2. THE PLACE OF ION-EXCHANGE CHROMATOGRAPHY AMONG OTHER METHODS USED FOR RADIOCHEMICAL SEPARATIONS

Of the various separation methods used in NAA, such as distillation, precipitation and coprecipitation, solvent extraction, ion exchange and electrochemical deposition, ion exchange shows several advantages. The availability of several types of ion exchangers with different exchange groups provides a wide range of selectivities which can be further augmented by the proper choice of the composition of the mobile phase, including the use of complexing agents.

The broad literature on the subject [7–12], containing many tabulations of distribution coefficients for different elements in a variety of ion exchanger–solution systems, facilitates the planning of ion-exchange separation schemes. In most instances it is possible to find an ion-exchange system with sufficiently large differences in selectivities of the ions in question that quantitative separation can be achieved by stepwise elution from relatively short columns with simple gravity flow.

Good selectivity, quantitativeness and simplicity of operation have made ion-exchange chromatography (IEC) the leading separation technique employed in the radiochemical version of NAA.

IEC is often complemented by extraction chromatography, *i.e.*, the technique in which a porous hydrophobic support such as polytetrafluoroethylene, styrene–divinylbenzene copolymer or silanized silica gel is coated with a suitable extractant and used in columns similarly to ion exchangers [7,13–16]. Many of the extractants employed in this technique, such as tri-*n*-octylamine and di-(2-ethylhexyl)orthophosphoric acid, extract ions by an ion-exchange mechanism. In the following, selected examples of the use of ion-exchange and extraction chromatography in the determination of several elements by radiochemical NAA will be given. This review is not intended to give a comprehensive coverage of the field but rather to demonstrate the salient role of chromatography in cases when the determination of trace elements by the purely instrumental version of NAA is impossible.

3. REMOVAL OF MACROCONSTITUENTS (PREVAILING ACTIVITIES) AND GROUP SEPARATIONS

As was mentioned in section 1, despite the progress in instrumentation for γ-ray spectrometry, the measurement of weak γ lines is often impossible in the presence of high activity originating from activatable macro-constituents of the sample or even from trace elements with especially high activation cross-sections.

3.1. Analysis of biological materials

A common interference in the determination of trace elements in biological materials is the preponderant activity of radiosodium (^{24}Na). Girardi and Sabbioni [17] showed that sodium can be effectively separated from nearly all other elements by retention on a column with hydrated antimony pentoxide (HAP) from concentrated acid solutions, *e.g.*, 8 *M* HCl. This principle, among others, was used by Tijoe *et al.* [19] in a method for the determination of some elements essential for life or toxic in biological materials (see Fig. 2). Here the removal of ^{24}Na matrix activity on an HAP column is combined with further separation of trace elements into groups by stepwise elution from anion-exchange columns, permitting their interference-free determination in ensuing fractions by γ-ray spectrometry. HAP is still widely in use for the elimination of interference from ^{24}Na in NAA [19].

The use of crystalline hydrated antimony pentoxide in columns is often cumbersome because of its poor mechanical stability and tendency to clog the column. Recently, a "composite ion exchanger" containing HAP incorporated into a phenolsulphonic–formaldehyde resin matrix was prepared [20]. Its use in columns eliminates most of the problems mentioned above. Several other separation schemes for multi-element determination of trace components in biological materials by NAA have been devised [21–24].

Some of these methods employ, in addition to

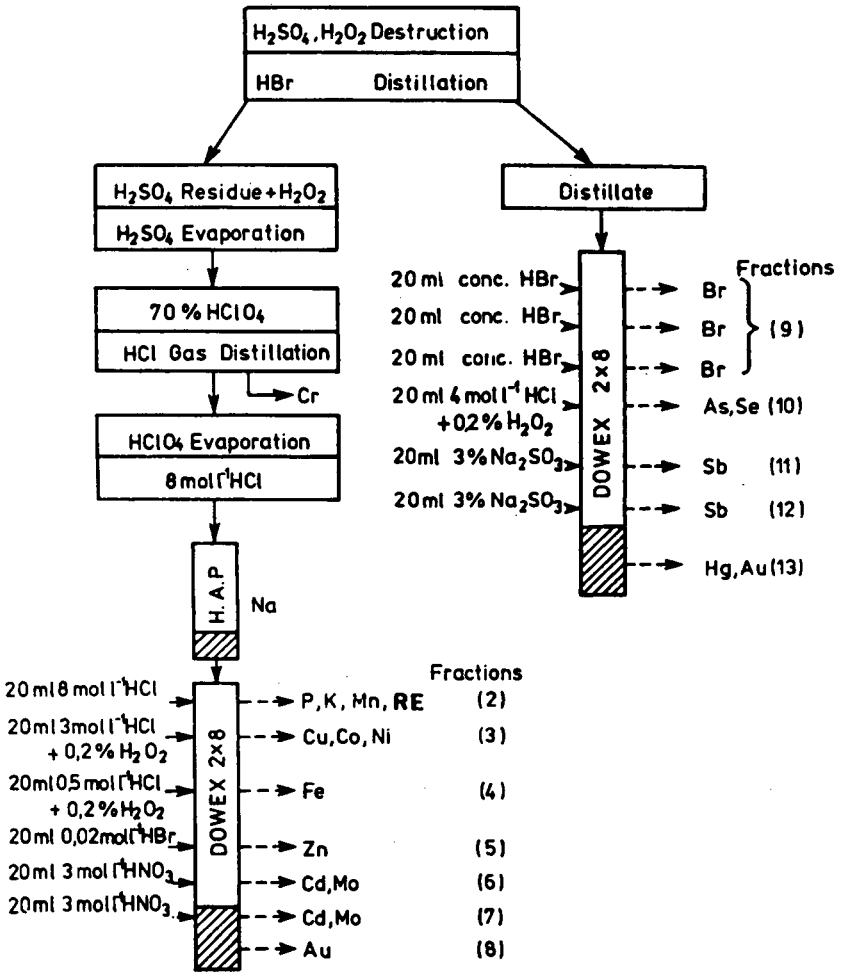


Fig. 2. Separation scheme devised for the determination of some toxic and/or essential trace elements in biological materials by NAA. (After Tijóe *et al.* [18]).

conventional strongly acidic cation exchangers and strongly basic anion exchangers, also the chelating resin Chelex 100 with iminodiacetic exchange groups and extractants such as di(2-ethylhexyl) orthophosphoric acid (HDEHP) adsorbed on a hydrophobic support.

The separation schemes are based either on stepwise elution or on selective fixation of a radionuclide or group of radionuclides with differing γ-ray energies on particular columns.

Other, less extensive, chromatographic procedures have been proposed for the determination of a single element or group of elements that are especially difficult to determine in neutron-irradiated biological materials.

Several rare earth elements (La, Ce, Nd, Eu, Gd, Yb and Lu) were determined, after wet ashing of the irradiated sample, by retention on a Dowex 50-X8 column followed by sequential elution of impurities. Rare earths were finally eluted with 6 *M* HCl and determined by γ-ray spectrometry [25].

The use of inorganic ion exchangers as selective sorbents for some elements in NAA procedures should be noted. For example, zinc hexacyanoferrate(II) was used for the selective retention of cadmium [26].

Tin dioxide (TDO) was used for the isolation of selenium from 1 *M* nitric acid solution, while hydrated manganese dioxide (HMD) served as a sorbent selective for chromium [27]. In another study HMD was employed as an effective sorbent for the whole group of elements of interest, namely Cr, As, Se, Mo, Ag, Sb and Sn [28]. Molybdenum and tungsten were separated from most other elements by extraction chromatography with a stationary phase consisting of α-benzoin oxime supported on Bio-Beads SM-2 [29].

New separation possibilities were revealed when studying the properties of an amphoteric resin, Retardion 11A8, containing both quaternary ammonium and carboxylic acid exchange groups. Selective separation of zinc [30] and cadmium [31] from nearly all other elements can be achieved with a single column of Retardion 11A8 by exploiting both anion and cation functions of the resin.

An interesting example of another kind of chromatography employed in NAA is a recently proposed procedure in which some elements, *e.g.*, Mn(II), Cu(II) and Zn(II) but also Se(IV), Hg(II), As(III) and Sb(III), are first complexed with a chelating agent such as 8-hydroxyquinoline, pyrrolidine dithicarbamate or diethylammonium diethyldithiocarbamate and then retained on columns of C_{19}-bonded silica gel [32,33].

3.2. Analysis of high-purity materials and geological samples

Chromatographic methods have been widely used in radiochemical NAA for the determination of trace elements in a variety of other matrices such as semiconductor materials, high-purity metals and geological materials.

When the technique of γ-ray spectrometry still relied on the use of NaI(Tl) detectors, but sometimes also in a later period very elaborate schemes of ion-exchange separation, *e.g.*, for the determination of trace elements in silica, were devised [34,35]. With the introduction of Ge(Li) and HPGe detectors, many more elements could be determined in Si or SiO_2 matrices non-destructively, but in several instances ion-exchange separations are still necessary. This may be exemplified by the procedure for the determination of indium, in which short-lived ^{116m}In was selectively retained on an inorganic ion exchanger, cerium oxalate, from hydrofluoric acid solution with 92–95% yield [36]. Another example is the determination of uranium and thorium in silica and aluminium where the activation products of both elements, *i.e.*, ^{239}Np and ^{233}Pa, were separated by retention on Dowex 1-X8 (Cl^-), followed by selective elution with 3 *M* HF–9 *M* HCl, coprecipitation with LaF_3 and measurement by γ-ray spectrometry [37].

Tantalum is an example of a material in which the determination of any impurity by instrumental NAA is virtually impossible owing to the high activity of the matrix due to both short-lived (^{182m}Ta) and long-lived (^{182g}Ta and ^{183}Ta) radionuclides. The separation scheme [38] devised for the determination of several trace impurities in tantalum metal (Fig. 3) makes use of the high affinity of tantalum to an anion-exchange resin in HF medium. Elements that do not form stable fluoride complexes (Co, Cr, Cu, K, La, Mn, Ni) are quantitatively eluted from a Dowex 1-X8 (F^-) column with 1–2 *M* HF. Elements taken up by the resin (Hf, Mo, Re, Sc and W) can be selectively eluted from the column with 40–50 *M* HF, leaving macro amounts of tantalum on the column (Fig. 4).

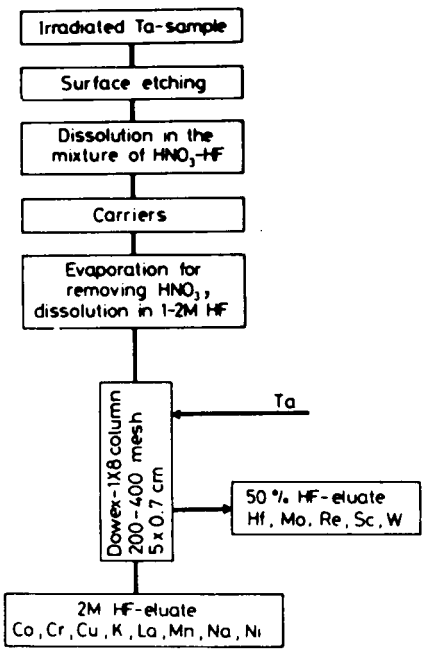


Fig. 3. Flow-scheme of the procedure for the post-irradiation separation of impurities from Ta metal matrix. (After Caletka *et al.* [38]).

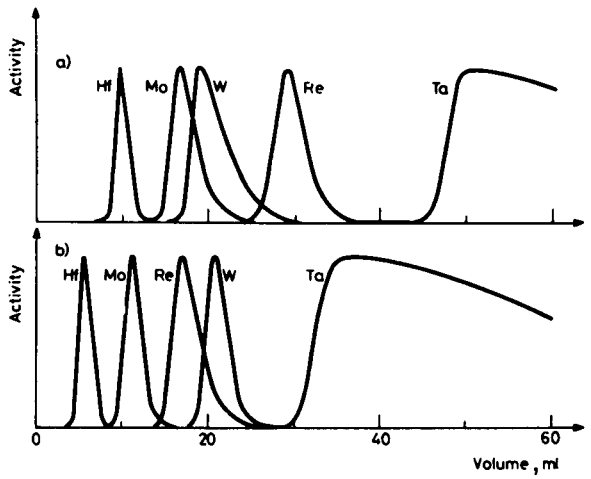


Fig. 4. Separation of Hf, Mo, W and Re from macro amounts of Ta. (a) Column, 12 cm × 0.38 cm^2 Dowex 1-X8 (F^-) (200–400 mesh); adsorption stage, 5 ml of 2 *M* HF containing 500 mg of Ta and respective tracers followed by 10 ml of 2 *M* HF washing solution; elution stage, 40% HF (22.5 mol/l); flow-rate, 0.4–0.5 ml/min. (b) Column, 13 cm × 0.38 cm^2 Dowex 1-X8 (F^-) (200–400 mesh); adsorption stage, as above; elution stage, 50% HF (31.2 mol/l); flow-rate, 0.4–0.5 ml/min. (After Caletka *et al.* [38]).

Similar methods also based on anion exchange in HF were used for the determination of several impurities in tungsten metal [39]. Another separation scheme for the determination of impurities in molybdenum metal employed first a Dowex 50W-X8 (H^+) column, which made it possible to isolate a group of several elements (Sm, Cr, Cs, Rb, Zr, Fe, Zn, Co, La), and then a Dowex 1-X8 (NO_3^-) anion-exchange column to retain ^{239}Np and ^{233}Pa for the determination of uranium and thorium [40].

Extraction chromatography was used for the determination of trace elements in high-purity tellurium [41]. After irradiation, dissolution and distillation of ^{131}I, tellurium was reduced to Te(IV) with hydrazine and retained from 12 *M* HCl on a column with TBP supported on polytetrafluoroethylene. The impurities (Na, K, Cr, Mn, Co, Cu, Zn, As, Ag, Cd and La) were recovered in the eluate and determined by γ-ray spectrometry.

A similar method with the use of an additional anion-exchange column in the bromide form to retain cadmium was used for the determination of impurities in cadmium telluride [42]. A modified version of the method served also for the determination of trace elements in the ternary compound $Pb_xSn_{1-x}Te$ [43].

In the analysis of geological materials, a strongly basic anion-exchange resin in the chloride form [44] or newly synthesized chelating resins [45] were used for the group separation of noble metals. Low levels of iridium in sedimentary rocks were determined by separating cationic interfering elements from the anionic $IrCl_6^-$ complex on a Bio-Rad AG 50W-X8 sulphonic cation exchanger [46].

3.3. Unconventional separations

In ion-exchange separations, sometimes unusual selectivities occurring in concentrated electrolyte solutions are exploited. In Fig. 5, a flow scheme of the separation procedure [47] devised for the determination by NAA of La, Ga, Hf and Sc in chamotte brick and related refractory materials is presented. These elements were studied and employed as "internal isotopic tracers" for the investigation of metallurgical processes. In this method gallium and scandium were separated from most of the other elements by retaining them on a column with a low

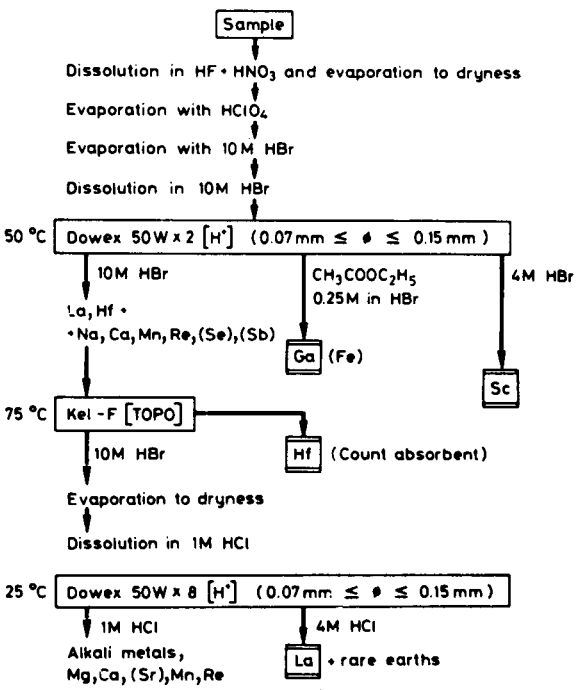


Fig. 5. Radiochemical separation scheme for the quantitative isolation of Ga, Sc, Hf and La from refractory materials. (After Fołdzińska and Dybczyński [47]).

cross-linked strongly acidic sulphonic cation exchanger, following by selective stepwise elution (Fig. 6).

It is interesting that the retention of both elements from 10 *M* HBr seemingly contradicts the mass-action law of a cation-exchange reaction. In reality, gallium, which is known to form a negatively charged bromide complex, is probably taken up by the resin owing to some kind of complex formation between the undissociated $HGaBr_4$ (or ion pair $H^+ GaBr_4^-$) and the benzene rings of the resin network. Consequently, it can be eluted with an organic extractant containing a small amount of HBr.

Scandium, which does not form negative bromide complexes, is apparently at least partially dehydrated in 10 *M* HBr and fulfills its solvation needs via sulphonate groups of the resin. When the concentration of the acid is diminished to 4 *M*, scandium elutes easily, as could be expected on the basis of the mass-action law of an ion-exchange reaction. Lanthanum, hafnium and other elements were eluted with 10 *M* HBr at 50°C because the elevated temperature reduced tailing. Hafnium was selectively retained from the same solution at 75°C on a column with tri-*n*-octylphosphine oxide (TOPO) supported on Kel-F powder and measured on the bed by γ-ray spectrometer. Lanthanum (together with other lanthanides) was finally separated from accompanying elements by stepwise elution from a Dowex 50W-X8 column.

This separation scheme ensured virtually quantitative ($\geq 96\%$) recovery of the elements in question and good radiochemical purity of the respective fractions so that a γ-ray spectrometer with only moderate energy resolution [NaI(Tl) detector] could be used for determination. Amounts of the order of mg/kg (ppm) for all four elements could be conveniently determined by this method in a variety of refractory materials.

3.4. Problems with micro–macro separations of ions with similar chemical properties

The separation procedures and schemes discussed above and based on stepwise elution or selective fixation on the column exploit large differences in the selectivities of individual ions occurring in various ion-exchange and extraction systems. Sometimes, however, it is necessary to separate elements with very similar chemical properties.

A classical example is the group of rare earth elements. While the separation of lanthanide ions present in trace amounts can be relatively easily achieved, the realization of macro–micro separations may create serious problems. This will be

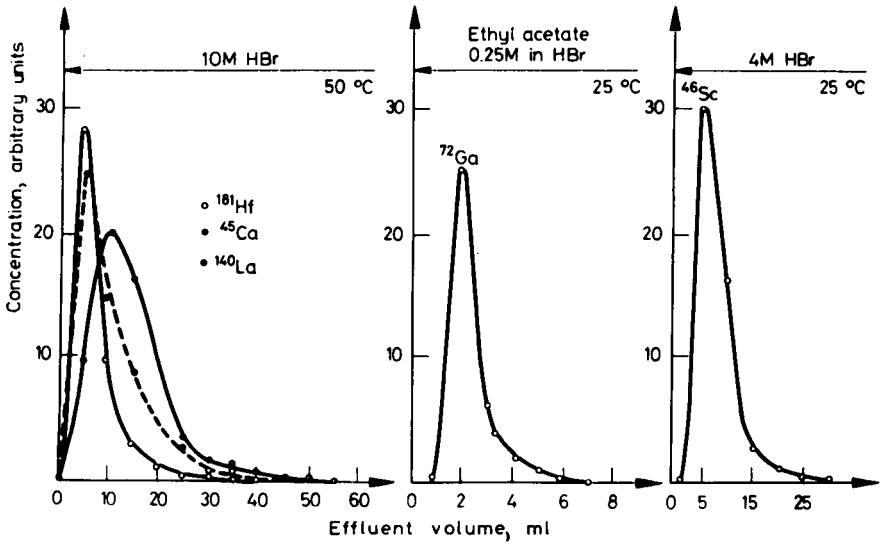


Fig. 6. Isolation of Ga and Sc by ion-exchange chromatography. Column, 10.0 cm × 0.18 cm^2 Dowex 50W-X2 (H^+) (0.07 mm $\leq \phi \leq$ 0.15 mm); flow-rate, 5 cm/min. (After Fołdzińska and Dybczyński [47]).

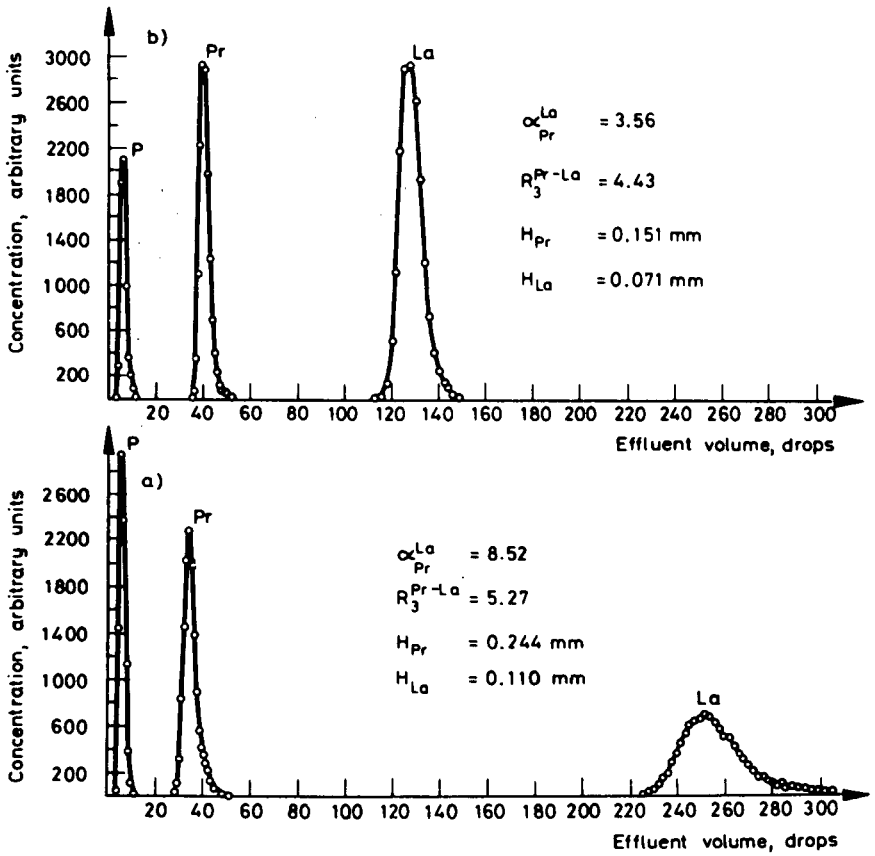


Fig. 7. Comparison of elution curves for trace amounts of La and Pr in various systems. Column, 5.0 cm × 0.0306 cm² Dowex 50W-X8 (11 μm $\leq \phi \leq$ 23 μm). (a) Eluent, 0.2 *M* iminodiacetate (pH 6.3); flow-rate, 0.87 cm/min. (b) Eluent, 0.5 *M* lactate (pH 3.6); flow-rate, 0.76 cm/min. (After Dybczyński [49]).

illustrated by the example of developing a separation method for the NAA determination of lanthanum in Pr_6O_{11} [48,49].

During the search for suitable separation system, elution of rare earth elements (trace amounts) from cation-exchange columns with various complexing agents was studied with the aid of radioactive tracers. Some results are shown in Fig. 7.

Separation factors, α_1^2, theoretical plate heights, H, and resolutions, R_3^{1-2}, were calculated from the following equations:

$$\alpha_1^2 = \frac{\lambda_2}{\lambda_1} = \frac{U_{\max(2)} - (U_0 + V)}{U_{\max(1)} - (U_0 + V)} \tag{1}$$

$$H = \frac{L}{N} = \frac{L\sigma^2}{(U_{\max} - U_0)^2} \tag{2}$$

$$R_3^{1-2} = \frac{U_{\max(2)} - U_{\max(1)}}{3(\sigma_1 + \sigma_2)} = \frac{(\alpha_1^2 - 1)\sqrt{L}}{3(\alpha_1^2 + 1)\sqrt{H}} \tag{3}$$

where:

λ = distribution coefficient;
$U_{\max}$ = retention volume (ml);
U_0 = dead volume of the column;
V = free volume of the resin bed;
L = length of the resin bed;
N = number of theoretical plates;
σ = standard deviation of chromatographic peak.

As can be seen from Fig. 7a, the separation of trace amounts of lanthanum and praseodymium on a micro-column employing 0.2 *M* ammonium iminodiacetate (pH 6.3) as eluent seemed to be

especially promising, better than that obtained with ammonium lactate (Fig. 7b).

For scaling up the separation to amounts of praseodymium up to fractions of milligram, the use of larger columns and a coarser mesh size of the resin to maintain a reasonable flow-rate was necessary. This adversely influenced the column performance but the separation was still fairly good (Fig. 8a). A further increase in the amount of praseodymium in the sample, however, resulted in rapid deterioration of the separation and with 10 mg of praseodymium no separation could be achieved (Fig. 8b).

Hence this system was shown to be very sensitive to overloading. On the other hand, separation of the lanthanides on an anion exchanger, after converting them into anionic complexes with disodium ethylenediaminetetraacetate (Na_2H_2Y), proved to be very insensitive to loading effects. One can see from Fig. 9 that, despite the moderate separation factor ($\alpha_{La}^{Pr} = 3.28$), much smaller than that observed in the cation-exchange system discussed previously, an excellent separation of trace amounts of lanthanum from 1 mg of praseodymium could be easily accomplished.

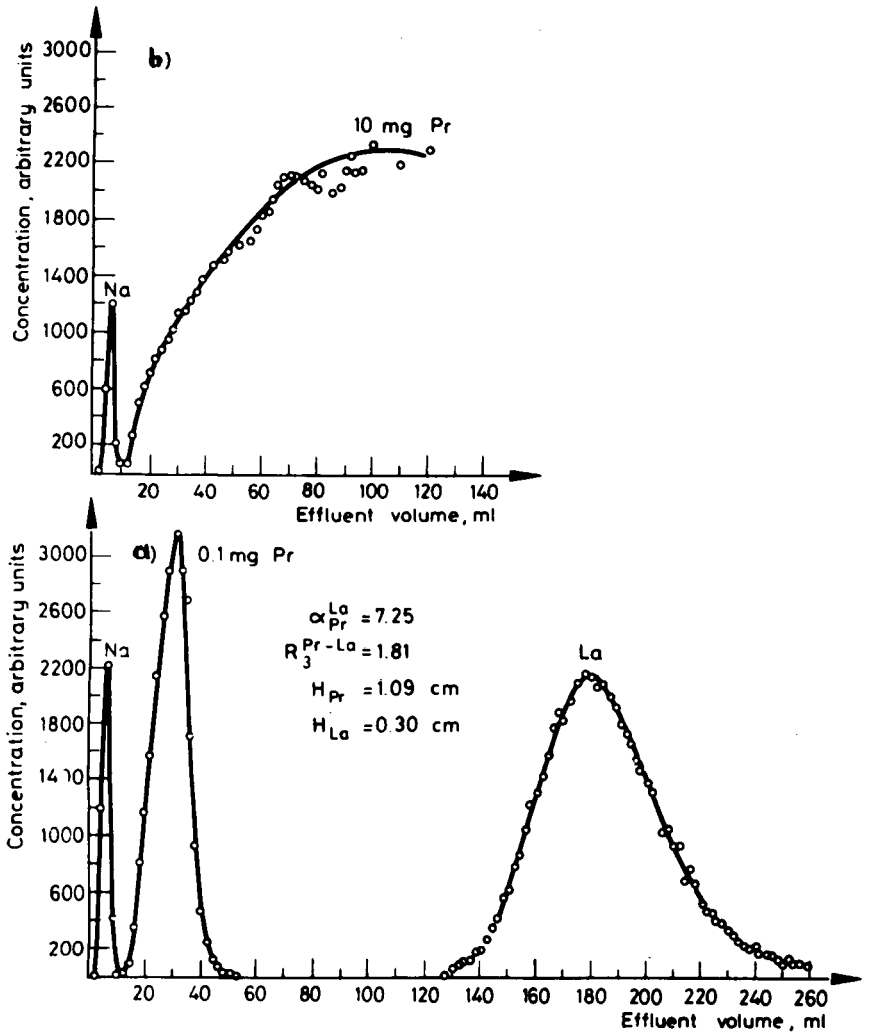


Fig. 8. Effect of loading on the separation of trace amounts of La from Pr by cation-exchange chromatography. Column, 19.6 cm × 0.392 cm^2 Dowex 50W-X8 (0.10 mm $\leq \phi <$ 0.20 mm); eluent, 0.2 *M* iminodiacetate (pH 6.4); room temperature; flow-rate, 2 cm/min. (a) 0.1 mg Pr; (b) 10 mg Pr. (After Dybczyński [49]).

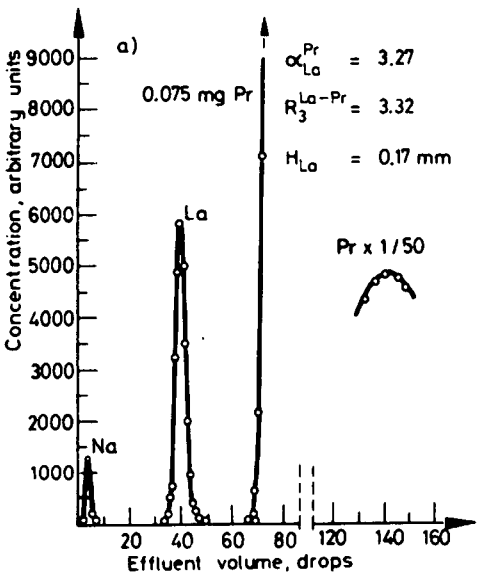


Fig. 9. Effect of loading on the separation of trace amounts of La from Pr by anion-exchange chromatography. (a) Column, 5.8 cm × 0.0306 cm² Dowex 1-X4 (H_2Y^{2-}) (11 μm ≤ ϕ ≤ 42 μm); eluent, 0.05 *M* Na_2H_2Y; temperature, 50°C; flow-rate, 1 cm/min; load, 0.075 mg Pr. (b) Column, 6 cm × 0.116 cm² Dowex 1-X4 (H_2Y^{2-}) (15 μm ≤ ϕ ≤ 48 μm); eluent, 0.03 *M* Na_2H_2Y; temperature, 50°C; flow-rate, 0.91 cm/min; load, 1 mg Pr. (After Dybczyński [49]).

It is interesting that the proper choice of temperature played an important role here in optimization of the separation conditions. In Fig. 10 the temperature dependence of the selectivity coefficients $k_{H_2Y^{2-}}^{LnY^-}$ for the two lanthanides (Ln) in the system Dowex 1-X4 (H_2Y^{2-})–Na_2H_2Y (aq.) is presented. The selectivity coefficient for an ion-exchange reaction:

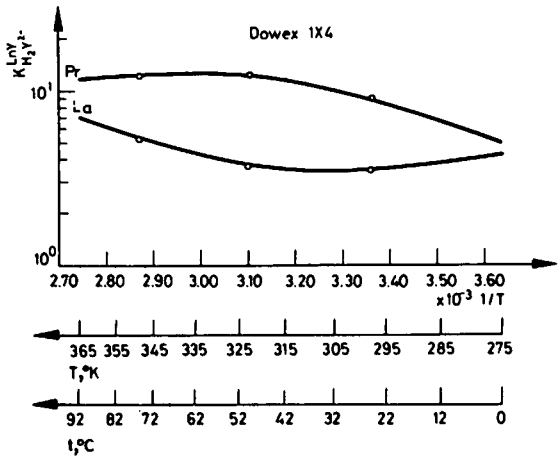


Fig. 10. Temperature dependence of selectivity coefficients for an ion-exchange reaction: $\frac{1}{2}R_2H_2Y + LnY^- = RLnY + \frac{1}{2}H_2Y^{2-}$ (After Dybczyński [49]).

$$\frac{1}{2}R_2H_2Y + LnY^- = RLnY + \frac{1}{2}H_2Y^{2-} \qquad (4)$$

where R denotes a structural unit of the ion exchanger and Y^{4-} is the anion of ethylenediaminetetraacetic acid (H_4Y), may be expressed as [50]

$$k_{H_2Y^{2-}}^{LnY^-} = \frac{\lambda_{LnY^-} m_{H_2Y^{2-}}^{1/2} d}{C_r} \qquad (5)$$

where

λ_{LnY^-} = distribution coefficient of the lanthanide complex;
m = molality of eluent (Na_2H_2Y) solution;
d = density of eluent solution;
C_r = concentration of the resin phase (mmol per g of dry resin [H_2Y^{2-}]),

and so is directly proportional to the distribution coefficient. One can easily note from Fig. 10 that the highest ratio of distribution coefficients, *i.e.*, separation factor, may be expected at 50°C. Both lower and higher temperatures would result in deterioration of the separation conditions.

The significance of the resin cross-linking should

also be emphasized. As was shown in earlier studies [50], the theoretical plate height when separating complex ions of large dimensions may vary considerably. When separating rare earth ethylenediaminetetraacetates on strongly basic anion-exchange resins, Dowex 1 (H_2Y^{2-}), the plate height varies by two orders of magnitude within the cross-linkings X2 and X16, reaching a minimum for Dowex 1-X4 [50]. Hence the proper choice of temperature, resin cross-linking and also the flow-rate is a key factor when aiming at successful separations.

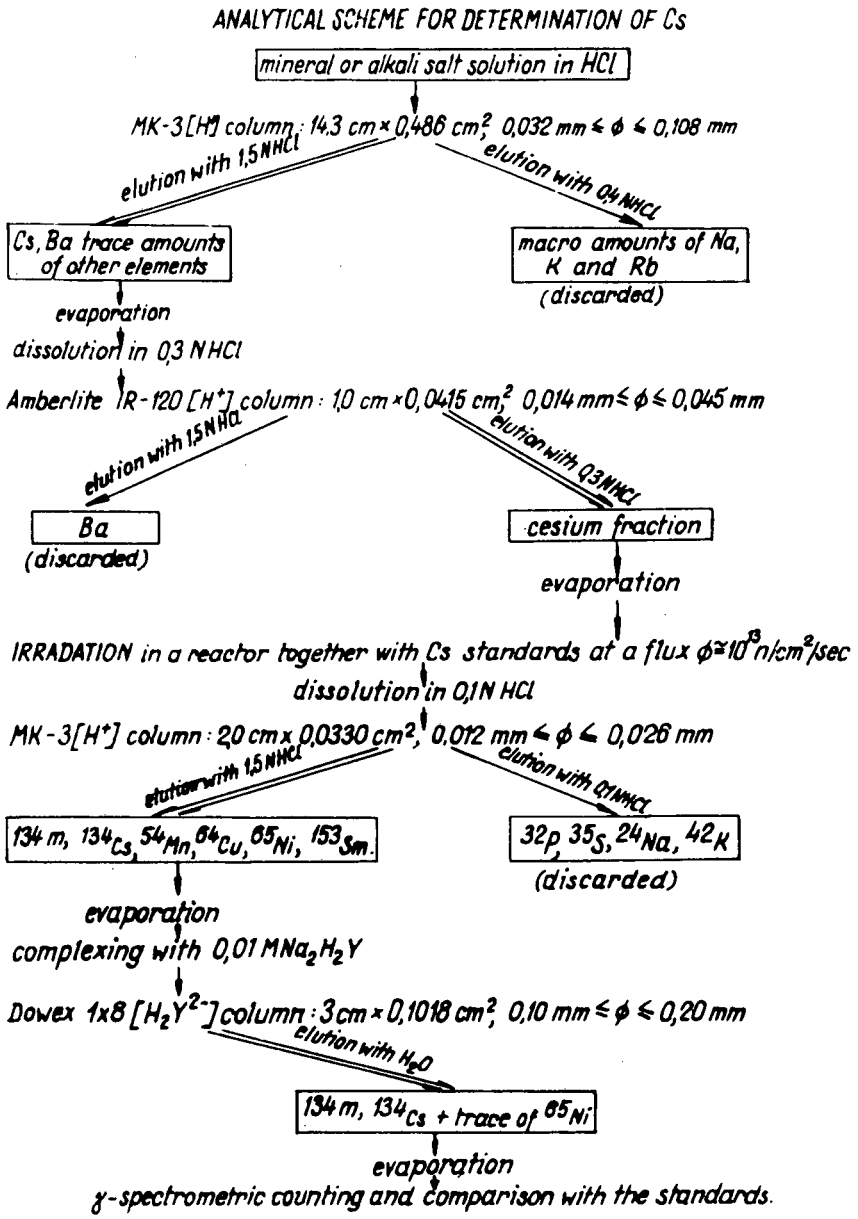


Fig. 11. Analytical scheme for the determination of traces of Cs in mineral salts by NAA, involving pre- and post-irradiation ion-exchange separations. (After Dybczyński and Sterliński [52]).

Somewhat similar to the case of rare earth elements is the problem of micro–macro separations of alkali metals. Some inorganic ion exchangers, such as hexacyanoferrates and salts of heteropoly acids, show high selectivity towards heavier alkali metals, especially caesium [51]. However, the elution of

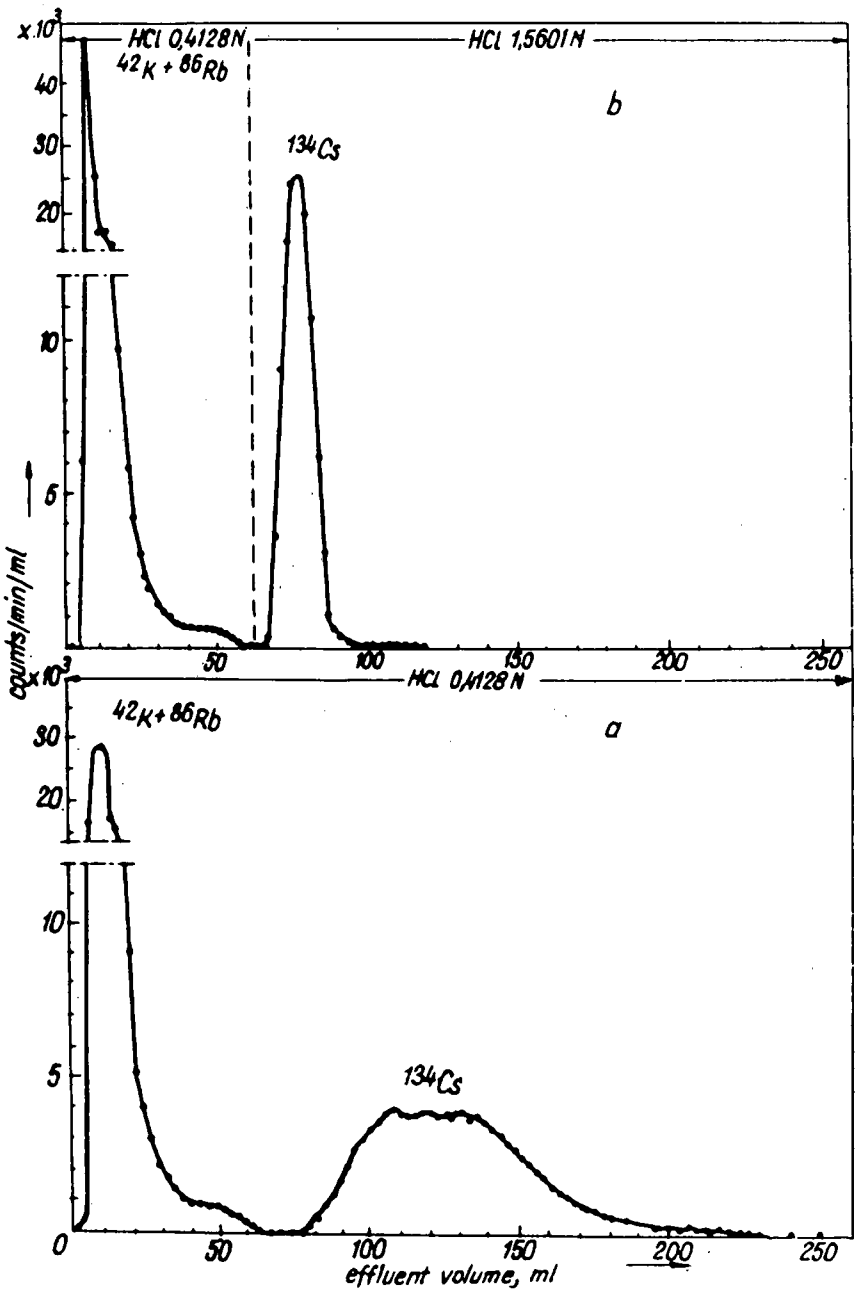


Fig. 12. Typical elution curve illustrating the separation of trace amounts of Cs from macro amounts of potassium (0.5 g KCl) and rubidium impurity. Column, 14.3 cm × 0.486 cm² MK-3 (H^+) (phenolsulphonic cation exchanger) (0.032 mm $\leq \phi \leq$ 0.108 mm); temperature, 25°C. Eluent: (a) 0.4128 *M* HCl; (b) 0.4128 *M* HCl until collection of 61 ml of the eluate, then 1.56 *M* HCl; flow-rate, *ca.* 2 ml/cm² · min. (After Dybczyński and Sterliński [52]).

caesium from these sorbents is difficult and inorganic ion exchangers are not completely insoluble in aqueous solutions, which may cause problems especially when preconcentration of caesium before irradiation is desired. In such instances the use of organic ion-exchange resins for micro–macro separations may be preferred.

A flow scheme of the analytical procedure for the determination of traces of caesium in mineral salts (NaCl, KCl, etc.) is shown in Fig. 11. In this method, a large sample (0.5 g) is taken and caesium is separated from other elements by stepwise elution from two cation-exchange columns [52]. The first is filled with phenolsulphonic resin, MK-3 (H^+), which shows increased selectivity towards caesium owing to the complexing properties of the phenolic groups, which permits the separation of traces of caesium from macro amounts of sodium, potassium and rubidium (Fig. 12). The other column is filled with a conventional sulphonic-type resin [Amberlite IR-120 (H^+)] on which it is relatively easy to achieve separation of univalent metal ions from those of higher valency.

The combination of pre-irradiation isolation of caesium with post-irradiation separation from impurities and measuring the 31-keV gamma line of the short-lived ^{134m}Cs isotope ($t_{1/2}$ = 3.1 h) with an NaI(Tl) wafer coupled to a pulse-height analyser made it possible to achieve a detection limit as low as 0.08 ppb.

4. PRECONCENTRATION OF TRACE ELEMENTS BEFORE NEUTRON IRRADIATION

All the examples quoted above except the last one concerned radiochemical separations, *i.e.*, procedures in which the sample is first irradiated with neutrons and then, after dissolution, IEC and/or extraction chromatography are employed to isolate the nuclide(s) of interest and to permit the interference-free measurement of its (their) characteristic radiation.

Sometimes, however, it may appear necessary to remove matrix elements before irradiation. Certain types of samples activate too strongly to permit their safe handling with moderate shielding, *i.e.*, without the use of hot chambers. Especially difficult is the situation when the radionuclide to be measured is short-lived and emits γ-rays of low energy. A typical example is shown in Fig. 11. Another typical case is the determination of very low concentrations of elements in large volumes of solution (*e.g.*, water, wastes), where it is necessary to perform preconcentration and to bring the element(s) in question into the form compatible with the requirements set for irradiation in a nuclear reactor.

Therefore, although performing the separation before irradiation eliminates one of unique advantages of NAA, *viz.*, the lack of a blank, there are cases where such an approach is the only possible solution.

4.1. Rare earth elements

Several rare earth elements (REE), *e.g.*, Sm, Gd, Ho, Yb and Lu, form in (n,γ) reactions isotopes that emit γ-rays lying exclusively or mostly in the low-energy region of the γ-ray spectrum. Hence their determination in the presence of a high activity of matrix elements is usually impossible. REE are usually preconcentrated as a group, and this procedure may also be combined in case of need with post-irradiation separation.

Trace amounts of REE in briny groundwaters were preconcentrated by coprecipitation with $Fe(OH)_3$ followed by separation of iron on a Dowex 1-X8 (Cl^-) column from 10 *M* HCl. REE were recovered in the eluate, and sent for irradiation in a reactor [53].

In determine REE at ultratrace abundance levels in geological materials, the samples were decomposed with HF–$HClO_4$ and, after evaporation and removal of fluorides, REE were coprecipitated with calcium oxalate. The precipitate was ignited, dissolved in HNO_3 and REE were purified by several adsorption–elution cycles using Dowex 1-X8 (NO_3^-) anion exchanger and nitric acid mixtures with acetic acid, propanol and methanol. The REE were finally eluted with dilute HNO_3 and subjected to irradiation with neutrons [54].

In another study, REE were preconcentrated from 1.8 *M* HCl solution (obtained after dissolution of rocks in HF–$HClO_4$–HNO_3, evaporation and dissolution in HCl) by retention on a Dowex 50W-X8 (H^+) column. Macro constituents were removed with 2 *M* HCl, then REE were eluted with 6 *M* HCl, converted into nitrates and sent for irradiation [55].

REE in environmental samples were preconcen-

trated on a column with HDEHP adsorbed on porous PTFE powder. Other metals were removed by stepwise elution and the fraction was analysed by NAA [56]. Similarly, extraction chromatography with TOPO adsorbed on Wofatit EP-60 as a stationary phase was used for the separation of REE by stepwise elution from virtually all other elements, including scandium [57].

4.2. Noble metals

Preconcentration is often used when determining noble metals, the contents of which in most of materials are very low. Trace amounts [in the ng/g (ppb) range] of gold and iridium in steel were determined by retention of the two metals on an anion exchanger, elution of most of the impurities and ashing the resin before irradiation [58].

A newly synthesized chelating ion exchanger was used for selective retention of the group of noble metals, *viz.*, Ru, Pd, Os, Ir, Pt and Au [59]. The resin was then ashed and irradiated in a nuclear reactor. This method was employed for the determination of some of these elements in an ore and in high-purity copper metal.

The determination of ultratrace amounts of palladium and platinum may be hindered by the presence of even trace amounts of gold in the sample sent for irradiation. A new method for the determination of palladium and platinum in ores, concentrates and other products of the copper industry consisted in removal of most of cations on a column of Dowex 50W-X8 (H^+) from 0.5 *M* HCl solution, selective retention of gold by "anomalous" sorption on a column of Dowex 50W-X4 (H^+) from 3 M HCl–Cl_2 solution, followed by adsorption of anionic complexes of palladium and platinum on a Dowex 1-X8 (Cl^-) column. The resin was irradiated and wet ashed and post-irradiation separation also by ion-exchange chromatography was used to obtain palladium and platinum fractions of high radiochemical purity with almost 100% yield [60].

Gold, palladium and platinum together with uranium and tungsten were determined in natural water samples by complexation with pyrrolidinecarbodithioate and retention on the column with C_{19}-bonded silica gel at pH 2 followed by neutron irradiation and γ-ray spectrometry [61].

4.3. Other elements

Several elements (Al, Ba, Ca, Ce, Cr, Cu, Fe, La, Mg, Mn, Sc, Sm, V and Zn) can be retained as a group on the chelating resin Chelex 100 at pH 5–6 with simultaneous separation from macro amounts of sodium and chlorine. The resin can then be irradiated with neutrons and measured by high-resolution γ-ray spectrometry, thus permitting the determination of many elements from one sample. This approach was used for the analysis of rain water [62], sea water [63], etc. In a similar way, Al, Cu, Mn and V were determined in solutions obtained from the wet-ashing of biological materials [64].

Another method of group separation before irradiation consisted in retaining Ag, Cd, Co, Cr, Cu, Fe, Hg, Mo, Se and Zn from water and waste on Dowex 1-X2 containing 8-hydroxyquinoline-5-sulphonate as a counter ion [65].

Some preconcentration methods were designed for the determination of selected single elements. For example, vanadium was determined in biological tissues by wet-ashing, retention of vanadium on Dowex 1-X8 from ammoniacal solution, elution of vanadium with 1 *M* HNO_3 and irradiating the eluate [66]. Copper in biological materials was determined via short-lived ^{66}Cu using extraction chromatography with LIX 64 (active component 2-hydroxy-5-nonylbenzophenone oxime) supported on Bio-Beads SM-1. After selective retention of copper from the solution obtained by wet-ashing of the sample in HNO_3–$HClO_4$ and neutralization to pH 4.4, the resin was irradiated in a reactor and measured by γ-ray spectrometry [67].

Two variants of ion-exchange preconcentration were proposed for the determination of several trace elements in tantalum metal [38]. In the first, tantalum solution in HF was passed through Dowex 50W-X8 (H^+) cation exchanger. Tantalum, which forms negatively charged fluoride complexes, passed to the eluate and trace elements were retained on the resin, which was subsequently dried and irradiated with neutrons. The second method consisted in retention of tantalum on Dowex 1-X8 (F^-) anion exchanger and elution of groups of trace elements with HF of various concentrations. The eluates were evaporated and irradiated in a reactor.

Other workers preconcentrated trace amounts of uranium and thorium in tantalum metal by reten-

tion on Dowex 50W-X8 (H^+) from HF–H_3BO_3 solution in which the affinity of tantalum to the resin is low, followed by elution of uranium and thorium with 0.5 *M* HF. As the elution curve of tantalum showed considerable tailing, multiple column operation was used to achieve the desired decontamination factor ($\geq 10^6$) (Fig. 13) [68].

It is perhaps worth mentioning that although ion-exchange chromatography is usually used as an auxiliary tool in NAA, sometimes the reverse is also true. For example, NAA has been used for studying the speciation of some elements in biological tissues. In particular, NAA was employed to monitor the separation of protein-bound trace elements (Cu, Mn, Zn, Cd, etc.) as performed by ion-exchange chromatography and other techniques (size-exclusion chromatography, electrofocusing, etc.) [69,70].

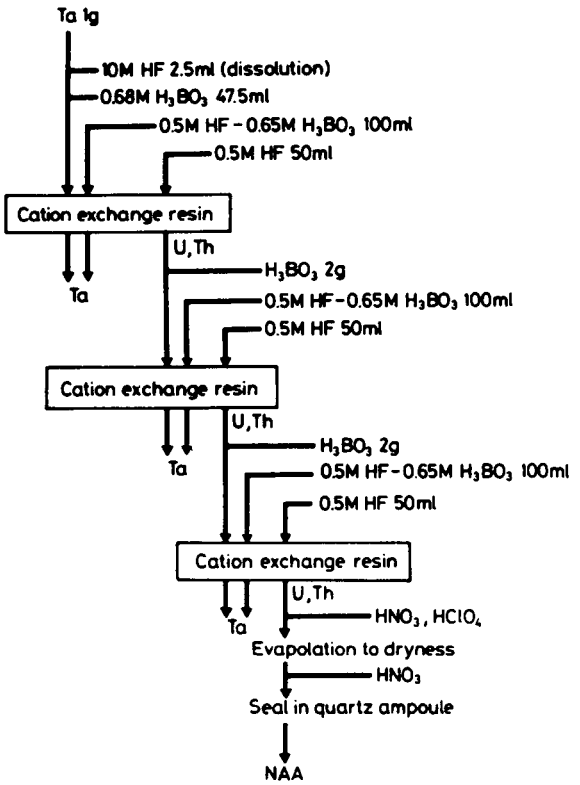


Fig. 13. Scheme for the separation of trace amounts of U and Th from macro amounts of Ta employing multiple column operation. (After Sasaki *et al.* [68]).

5. COLUMN CHROMATOGRAPHY AS A COMPONENT OF "DEFINITIVE" METHODS OF ANALYSIS

In inorganic trace analysis, accuracy is still a problem, as evidenced by the results of numerous interlaboratory comparisons in which results provided by individual laboratories sometimes differed by several orders of magnitude [71–73].

Analytical difficulties tend to increase, often drastically, on going to analyses of materials with very low contents of trace elements [74]. Therefore, there is an obvious need to devise very reliable methods for the determination of individual trace elements, whose status would be close to that of "definitive" methods.

As formulated by Uriano and Gravatt [75], "definitive" methods of chemical analysis are those that have a valid and well described theoretical foundation, have been experimentally evaluated so that reported results have negligible systematic error and have high levels of precision".

Definitive methods reported so far are not numerous. Isotope dilution mass spectrometry has almost exclusively been used to devise definitive methods for the determination of trace amounts of elements [75].

Recently, the first definitive method based on a combination of NAA and column chromatography, intended for the determination of trace amounts of copper in biological materials, was devised in our laboratory [76]. The flow scheme of the method is shown in Fig. 14. The most important step is the highly selective and quantitative separation of radiocopper formed as a result of the $^{63}Cu(n,\gamma)^{64}Cu$ reaction from virtually all other radionuclides present in a neutron-irradiated biological sample. This separation is accomplished with the use of a relatively short column with stationary phase consisting of LIX 70 (active component 2-hydroxy-3-chloro-5-nonylbenzophenone oxime) supported on Bio-Beads SM-1 (styrene–divinylbenzene copolymer) (Fig. 15) [77]. Almost all matrix and accompanying trace elements are eluted with 0.1 *M* glycine buffer (pH 2.8), 0.2 *M* in sodium hypophosphite and 1 *M* in $NaNO_3$, followed by elution of copper with 4 *M* HCl. As can be seen from Fig. 16, the copper fraction is virtually radiochemically pure.

Definitive methods are not intended for routine determinations, but rather for verification of results

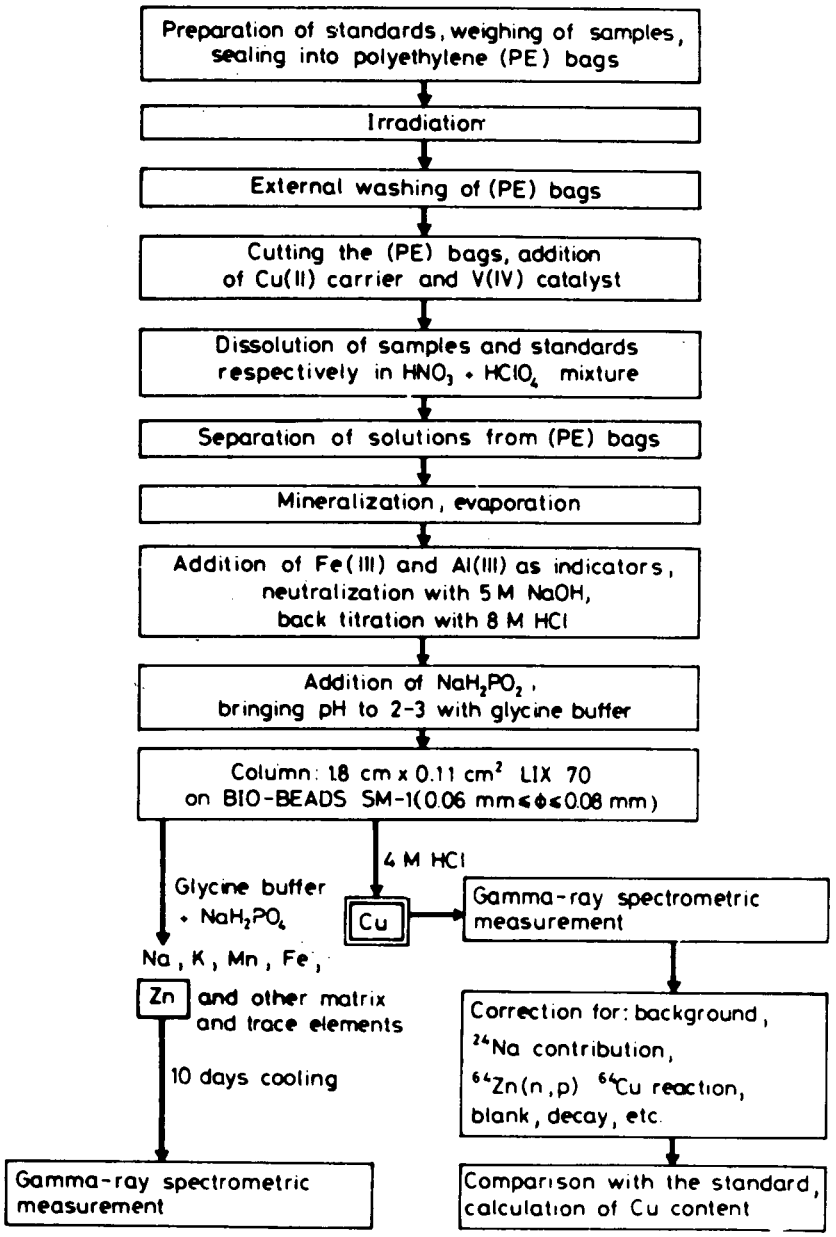


Fig. 14. Flow scheme of the analytical procedure for the determination of Cu in biological materials by NAA, involving selective and quantitative separation of Cu from other elements by extraction chromatography. (After Dybczyński *et al.* [76]).

obtained by other methods of trace analysis and for certification of candidate reference materials. Definitive methods are based on procedures comprehensively elaborated and tested in every detail. However, despite all the precautions taken, theoretically there is always a possibility that something may go wrong without being noticed by the analyst. Therefore, a definitive method should have some warning mechanisms incorporated into the procedure to safeguard against making gross errors.

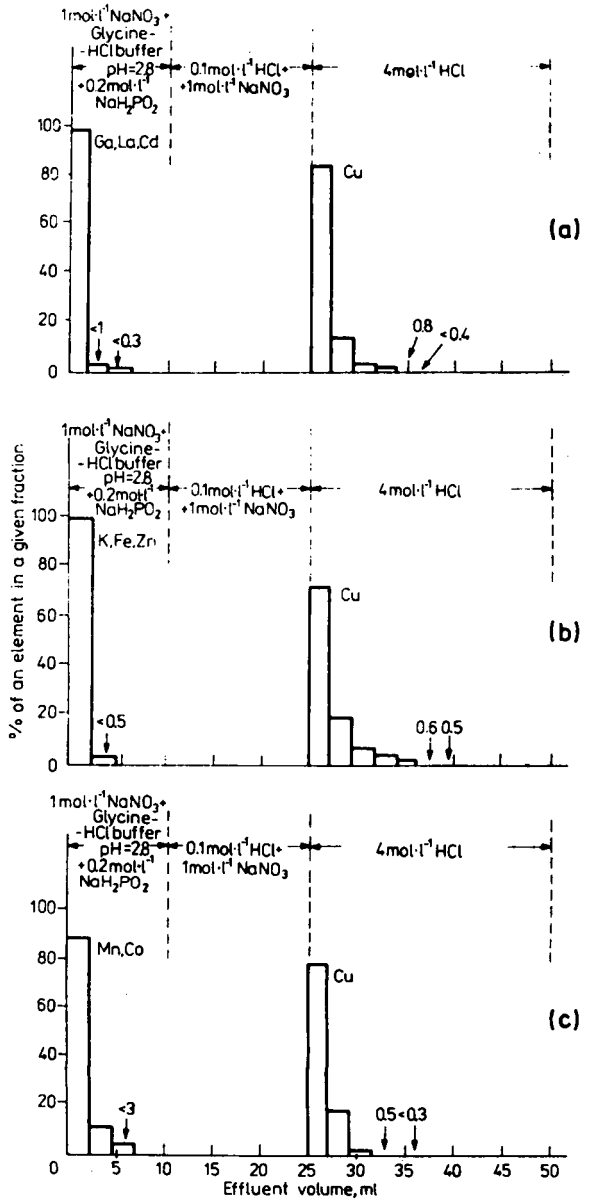


Fig. 15. Typical separations of Cu from various elements by extraction chromatography. Column, 2.3 cm × 0.116 cm^2 LIX 70 on Bio-Beads SM-1 (0.8 g/g); particle size, 0.06 mm $\leqslant \phi \leqslant$ 0.08 mm; flow-rate, 1.6–2.7 cm/min. (After Dybczyński and Maleszewska [77]).

In this method, the criteria for accepting the results of analysis, in addition to agreement of the normalized count rates of at least two standards, one of which has passed through the whole mineralization and separation procedure and the other is measured directly, low value of the residual blank, correct result for the parallel analysed reference material, etc., include also the visual inspection of the separation process.

Owing to the addition of 50 μg of inactive copper carrier to the sample at the beginning of the analysis, the movement of the greenish blue copper band with a sharp front can easily be followed visually. In the correctly performed procedure the band should not have travelled more than one to two thirds of the bed length by the end of the elution run. If this (and the other conditions mentioned above are not simultaneously fulfilled, then the results of this series of determinations are not considered to be obtained by a definitive method.

6. CONCLUSIONS

This review is intended to demonstrate the significance and important role that ion-exchange and extraction chromatography play in neutron activation analysis. Despite the current trend towards purely instrumental analysis, the use of chemical separations and in particular ion-exchange and extraction chromatography is still indispensable in many instances in NAA, especially when achieving the highest accuracy and interference-free detection limits is required.

A similar philosophy could be applied also to other methods of inorganic trace analysis with this reservation, but contrary to NAA, the problem of the blank is here of much greater significance.

7. ABBREVIATIONS AND SYMBOLS

HAP	hydrated antimony pentoxide (ion exchanger)
HDEHP	di(2-ethylhexyl)orthophosphoric acid
HMD	hydrated manganese dioxide (ion exchanger)
IEC	ion-exchange chromatography
NAA	neutron activation analysis
TBP	tributyl phosphate
TDO	tin dioxide (ion exchanger)
TOPO	tri-*n*-octylphosphine oxide
REE	rare earth elements
Na_2H_2Y	disodium ethylenediaminetetraacetate

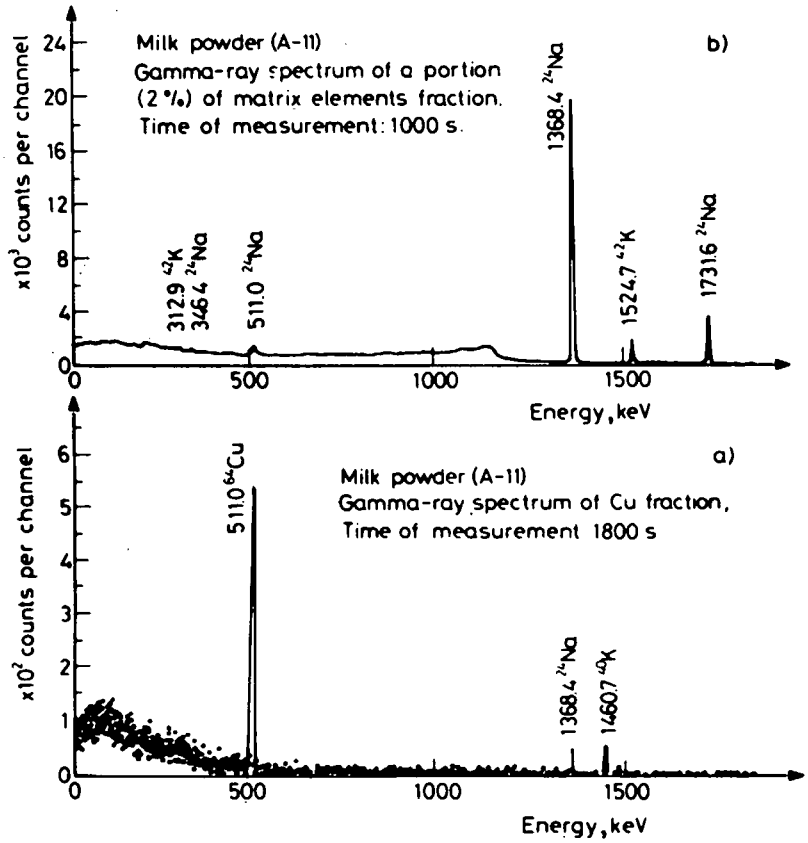


Fig. 16. Typical γ-ray spectra of (a) copper fraction from Milk Powder A-11 [reference material with very low copper content (0.33 mg/kg)] and (b) an aliquot from "matrix elements fraction" from the same material. (After Dybczyński *et al.* [76]).

Ge(Li) detector	lithium-drifted germanium detector
HPGe detector	high-purity germanium detector
NaI(Tl) detector	thallium-activated sodium iodide detector
α_1^2	separation factor
σ	standard deviation of chromatographic peak
λ	distribution coefficient
Φ_{th}	flux of thermal neutrons
C_r	concentration of resin phase
d	density of eluent solution
H	theoretical plate height
k	selectivity coefficient
L	length of the resin bed
m	molality of eluent solution
N	number of theoretical plates
R_3^{1-2}	resolution
$t_{1/2}$	half life
t_i	irradiation time
t_d	decay time
t_c	counting time
U_{max}	retention volume (ml)
U_0	dead volume of the column
V	free volume of the resin bed
ø	particle diameter

REFERENCES

1 D. De Soete, R. Gijbels and J. Hoste, *Neutron Activation Analysis*, Wiley, New York, 1972.
2 V. P. Guinn and J. Hoste, in *Elemental Analysis of Biological Materials (Technical Reports Series*, No. 197), International Atomic Energy Agency, Vienna, 1980.
3 V. Krivan, *Angew. Chem., Int. Ed. Engl.*, 18 (1979) 123.
4 R. Dybczyński, *Chem. Anal. (Warsaw)*, 30 (1985) 749.

5 *Quality Assurance in Biomedical Neutron Activation Analysis, IAEA-TECDOC-323*, International Atomic Energy Agency, Vienna, 1984.
6 S. Sterliński and W. Hammer, *J. Radioanal. Chem.*, 31 (1976) 235.
7 J. Minczewski, J. Chwastowska and R. Dybczyński, *Separation and Preconcentration Methods in Inorganic Trace Analysis*, Ellis Horwood, Chichester, 1982.
8 M. Marhol, *Ion Exchangers in Analytical Chemistry*, Academia, Prague, 1982.
9 E. Egorov and S. B. Makarova, *Ionnyi Obmien v Radiokhimii*, Atomizdat, Moscow, 1971.
10 K. Dorfner (Editor), *Ion Exchangers*, Walter de Gruyter, New York, 1991.
11 F. Girardi, R. Pietra and E. Sabbioni, *J. Radioanal. Chem.*, 5 (1970) 141.
12 F. Girardi and R. Pietra, *At. Energy Rev.*, 14 (1976) 521.
13 E. Cerrai, *Chromatogr.* Rev., 6 (1964) 129.
14 T. Braun and G. Ghersini (Editors), *Extraction Chromatography*, Akadémiai Kiadó, Budapest, 1975.
15 G. S. Katykhin, *Zh. Anal. Khim.*, 20 (1965) 615.
16 T. A. Bolshova and E. N. Shapovalova, *Zh. Anal. Khim.*, 38 (1983) 1489.
17 F. Girardi and E. Sabbioni, *J. Radioanal. Chem.*, 1 (1968) 169.
18 P. S. Tijoe, J. J. M. De Goeij and J. P. W. Houtman, *J. Radioanal. Chem.*, 37 (1977) 511.
19 N. Levi, F. Lux and Z. B. Alfassi, *J. Radioanal. Nucl. Chem.(Articles)*, 129 (1989) 93.
20 A. Bilewicz, B. Bartoś, J. Narbutt and H. Polkowska-Motrenko, *Anal. Chem.*, 59 (1987) 1737.
21 K. Samsahl, *Sci. Total Environ.*, 1 (1972) 65.
22 J. A. Velandia and A. K. Perkons, *J. Radioanal. Chem.*, 20 (1974) 743.
23 L. O. Plantin, *Nuclear Activation Techniques in the Life Sciences, Proceedings of Symposium, Bled, 1972*, International Atomic Energy Agency, Vienna, 1972, p. 73.
24 G. V. Iyengar, *J. Radioanal. Nucl. Chem. (Articles)*, 110 (1987) 503.
25 P. Collecchi, M. Esposito, S. Meloni and M. Oddone, *J. Radioanal. Nucl. Chem. (Articles)*, 112 (1987) 473.
26 A. Brandone, P. A. Borroni and N. Genova, *Radiochem. Radioanal. Lett.*, 57 (1983) 83.
27 M. B. A. Vasconcellos, V. A. Maihara, D. I. T. Fararo, M. J. A. Armelin, E. Cortes-Toro and R. Ogris, *J. Radioanal. Nucl. Chem. (Lett.)*, 153 (1991) 185.
28 R. Zeisler, R. R. Greenberg and S. F. Stone, *J. Radioanal. Nucl. Chem. (Articles)*, 124 (1988) 47.
29 B. Danko, R. Łobiński and R. Dybczyński, *J. Radioanal. Nucl. Chem. (Lett.)*, 137 (1989) 145.
30 R. Dybczyński and S. S. Aldabbagh, *Analyst (London)*, 112 (1987) 449.
31 R. Dybczyński and Z. Samczyński, *J. Radioanal. Nucl. Chem. (Articles)*, 150 (1991) 143.
32 J. J. Fardy, G. D. Meorist and T. M. Florence, *Anal. Chim. Acta*, 159 (1984) 199.
33 J. J. Fardy and M.-G. Tang, *J. Radioanal. Nucl. Chem. (Articles)*, 123 (1988) 573.
34 A. I. Kalinin, R. A. Kuztentsov, V. V. Moiseev and V. E. Tspepurnek, *Radiokhimicheskie Metody Opredeleniya Mikroelementov*, Nauka, Moscow, 1965, p. 161.
35 M. M. Ivanova, I. P. Oglobina, S. A. Genel, V. V. Mitina, A. I. Kalinin and V. G. Lambrev, *Zh. Anal. Khim.*, 32 (1977) 1066.
36 M. Gallorini, M. di Casa and G. F. Cerofolini, *Radiochem. Radioanal. Lett.*, 55 (1982) 77.
37 T. Mitsugashira, Y. Koma, S. Hirai, I. Okada, N. Kurashima and H. Sakurai, *J. Radioanal. Nucl. Chem. (Articles)*, 147 (1991) 69.
38 R. Caletka, R. Hausbeck and V. Krivan, *J. Radioanal. Nucl. Chem. (Articles)*, 120 (1988) 319.
39 R. Caletka, R. Hausbeck and V. Krivan, *J. Radioanal. Nucl. Chem. (Articles)*, 120 (1988) 305.
40 K. S. Park, N. M. Kim, Y. S. Kim, K. Y. Lee, H. W. Choi and Y. Y. Yoon, *J. Radioanal. Nucl. Chem. (Articles)*, 123 (1988) 585.
41 S. S. Grazhulene, V. K. Karandashev and Yu. V. Yakovlev, *Radiochem. Radioanal. Lett.*, 57 (1983) 273.
42 S. S. Grazhulene, V. K. Karandashev and Yu. V. Yakovlev, *Radiochem. Radioanal. Lett.*, 59 (1983) 225.
43 V. K. Karandashev, S. S. Grazhulene and Yu. V. Yakovlev, *J. Radioanal. Nucl. Chem. (Lett.)*, 106 (1986) 223.
44 M. Oddone, S. Meloni and R. Vanucci, *J. Radioanal. Nucl. Chem. (Articles)*, 142 (1990) 489.
45 C. F. Chai, S. L. Ma, X. Y. Mao, K. N. Liao and W. C. Liu, *J. Radioanal. Nucl. Chem. (Articles)*, 114 (1987) 281.
46 M. J. A. Armelin, M. B. A. Vasconcellos, E. B. Pereira and F. Sireilli Neto, *J. Radioanal. Nucl. Chem. (Articles)*, 132 (1989) 261.
47 A. Fołdzińska and R. Dybczyński, *J. Radioanal. Chem.*, 21 (1974) 507.
48 R. Dybczyński, S. Sterliński and C. Golian, *J. Radioanal. Chem.*, 16 (1973) 105.
49 R. Dybczyński, *J. Radioanal. Chem.*, 31 (1976) 115.
50 R. Dybczyński, *J. Chromatogr.*, 50 (1970) 487.
51 K. H. Lieser, in K. Dorfner (Editor), *Ion Exchangers*, Walter de Gruyter, New York, 1991, p. 519.
52 R. Dybczyński and S. Sterliński, in I. Buzas (Editor), *Proceedings of the Analytical Chemical Conference, Budapest, 20–23rd April 1966*, Technical University, Budapest, 1966, p. 398.
53 J. C. Laul, F. A. Lepel and H. R. Smith, *J. Radioanal. Nucl. Chem. (Articles)*, 123 (1988) 349.
54 C. Koeberl, F. Kluger and W. Kiesl, *J. Radioanal. Nucl. Chem. (Articles)*, 112 (1987) 481.
55 Y. Terakado, T. Fujitani and T. Takada, *J. Radioanal. Nucl. Chem. (Articles)*, 129 (1989) 23.
56 L. A. Smakhtin, L. I. Mekhryusheva, N. V. Filippova, N. V. Migalina and T. S. Sinitsyna, *J. Radioanal. Nucl. Chem. (Lett.)*, 154 (1991) 293.
57 B. Gorski, Yu. S. Korotkin and V. N. Kosyakov, *J. Radioanal. Nucl. Chem. (Lett.)*, 135 (1989) 27.
58 X.-L. Wang, Y.-L. Chen, X.-Y. Wang, Y. Sun, Y.-B. Fu and G.-P. Gao, *J. Radioanal. Nucl. Chem. (Articles)*, 147 (1991) 377.
59 K. Huang, G. S. Zhuang and Y. O. Cheng, *J. Radioanal. Nucl. Chem. (Articles)*, 112 (1987) 193.
60 R. Dybczyński, J. Majchrzak, H. Stokowska and H. Szyszko, *Chem. Anal. (Warsaw)*, 35 (1990) 609.

61 N. K. Shah and C. M. Wai, *J. Radioanal. Nucl. Chem. (Articles)*, 130 (1989) 451.
62 J. E. Milley and A. Chatt, *J. Radioanal. Nucl. Chem. (Articles)*, 110 (1987) 345.
63 R. R. Greenberg and H. M. Kingston, *Anal. Chem.*, 71 (1982) 147.
64 R. R. Greenberg, H. M. Kingston, R. Zeisler and J. Woittiez, *Biol. Trace Elem. Res.*, 26 (1990) 17.
65 W.Żmijewska, H. Polkowska-Motrenko and H. Stokowska, *J. Radioanal. Nucl. Chem. (Articles)*, 116 (1987) 243.
66 A. J. Blotcky, F. G. Hamel, A. Stranik, A. Ebrahim, R. B. Sharma, E. R. Rack and S. S. Solomon, *J. Radioanal. Nucl. Chem. (Articles)*, 131 (1989) 319.
67 R. Dybczyński, B. Danko and J. Kaczorowski, *Chem. Anal. (Warsaw)*, 34 (1989) 103.
68 Y. Sasaki, H. Takeishi, T. Adachi and K. Izawa, *J. Radioanal. Nucl. Chem. (Articles)*, 139 (1990) 143.
69 C. K. Jayawickreme and A. Chatt, *J. Radioanal. Nucl. Chem. (Articles)*, 124 (1988) 257.
70 C. K. Jayawickreme and A. Chatt, *Biol. Trace Elem. Res.*, 26 (1990) 503.
71 G. H. Morrison, *Anal. Chem.*, 43 (1971) 22A.
72 D. J. von Lehmden, R. H. Jungers and R. E. Lee, Jr., *Anal. Chem.*, 46 (1974) 239.
73 R. Dybczyński, A. Veglia and O. Suschny, in P. Bratter and P. Schramel (Editors), *Trace Element Analytical Chemistry in Medicine and Biology*, Walter de Gruyter, New York, 1980, p. 657.
74 R. Dybczyński, in *Nuclear Techniques for Analysis of Environmental Samples, IAEA/RL/135*, International Atomic Energy Agency, Vienna, 1986.
75 G. A. Uriano and C. C. Gravatt, *CRC Crit. Rev. Anal. Chem.*, 6 (1977) 361.
76 R. Dybczyński, M. Wasek and H. Maleszewska, *J. Radioanal. Nucl. Chem. (Articles)*, 130 (1989) 365.
77 R. Dybczyński and H. Maleszewska, *Chem. Anal. (Warsaw)*, 32 (1987) 619.

Journal of Chromatography, 600 (1992).37–42
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 934

Contribution of ionically immobilized bovine serum albumin to the retention of enantiomers

Stephen C. Jacobson and Georges Guiochon*

**Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1501 and Division of Analytical Chemistry, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120 (USA)*

ABSTRACT

The retention of the enantiomers of mandelic acid and N-benzoylalanine was studied on columns prepared by immobilizing bovine serum albumin (BSA) on an anion exchanger. The amount of BSA fixed on the column is easy to adjust and measure. The adsorption isotherms were determined. For each enantiomer, the isotherm is well accounted for by a bi-Langmuir equation. One term of the isotherm (which is the same for both enantiomers) corresponds to non-selective interactions and the other term to the chiral selective interactions. The column saturation capacity of this second term is 8% larger for the less strongly retained enantiomer. This saturation capacity corresponds approximately to one enantiomer molecule adsorbed for five BSA molecules immobilized. This result is in agreement with the assumption of the hydrophobic cavity of BSA being the chiral selective site.

INTRODUCTION

The study of the behavior of enantiomers in a chiral selective environment has received much attention over the past decade [1,2]. A wide variety of enantiomeric pairs have been resolved on an ever increasing number of chiral stationary phases (CSPs). One class of CSPs is protein immobilized on a solid support, and bovine serum albumin (BSA) is one of these proteins. Three methods of immobilization include covalent bonding to silica [3,4], adsorption on silica [4,5] and ionic immobilization to an anion exchanger [6].

In order to improve the enantioselectivity of a stationary phase, the two options are to increase the density of the enantiomeric discriminator, which improves the selectivity between the antipodes, and to decrease the contribution of the achiral retention which degrades the resolution of a separation without enhancing the selectivity. Usually, these two complement each other, *i.e.*, as the surface density of the discriminator increases, the non-selective interactions decrease. However, an alternative when using chemically bonded silica is end-capping of the residual silanol groups of the support, which has been shown to decrease the total retention while enhancing the resolution [7].

When using an anion exchanger as a support, a varying degree of adsorbed BSA can be obtained via changes in the buffer composition used. With various densities of BSA on the support surface, the retention behavior and the selectivity are altered, allowing the separation of the different contributions toward retention and their study. The system under consideration is the elution of N-benzoyl-DL-alanine and DL-mandelic acid on ionically immobilized BSA.

THEORY

Adsorption isotherm model

The bi-Langmuir isotherm [8,9] is constructed from two Langmuir terms. It has been used previously to describe the adsorption behavior observed when two independent, non-cooperative types of sites coexist on the surface and the solute adsorbs on them with different energies. This model is particularly valid to describe the adsorption of

0021-9673/92/$05.00

enantiomers on a chiral selective stationary phase [10]. The equation of this model is

$$q_x = \frac{q^x_{s,cs} b_{x,cs} C_x}{1 + b_{x,cs} C_x} + \frac{q_{s,ns} b_{ns} C_x}{1 + b_{ns} C_x} \quad (1)$$

The first term of the isotherm (x = L or D) represents the chiral selective interactions responsible for the separation of the enantiomers. The second term accounts for the non-selective interactions affecting the retention of both enantiomers but, as an achiral medium, without favoring either isomer. The chiral selective site has a higher affinity for the enantiomers, which explains the substantial tailing observed at high concentrations.

The saturation capacity, $q_s = a/b$, of the chiral selective site, $q_{s,cs}$, is expected to be similar for the two enantiomers, although steric restrictions due to the geometric configuration could cause slight differences. On the other hand, the saturation capacity of the non-selective sites, $q_{s,ns}$, has to be the same because enantiomers behave identically in achiral environments.

Isotherm determination

The experimental adsorption data to which eqn. 1 is fit for each enantiomer is determined using the method of elution by characteristic point (ECP) [11]. A high-concentration, narrow-plug injection of each enantiomer is eluted from the column and the diffuse, rear boundary of the band profile is monitored. The profile is then converted from detector response to concentration units by direct calibration of the signal. Knowing the retention volume for each mobile phase concentration on the rear boundary of the band profile, the equilibrium concentration of the adsorbed enantiomer is determined from the following differential mass balance equation [12]:

$$q = \int_0^C \frac{V_R - V_0}{V_S} \cdot dC \quad (2)$$

There is substantial agreement between isotherm data derived from the diverse possible methods, and ECP has the advantages of being fast and of requiring a much smaller amount of sample than frontal analysis [12].

EXPERIMENTAL

Equipment

A modular chromatograph was assembled, consisting of a Waters (Milford, MA, USA) Model 510 pump, a Waters gradient controller, a Scientific Systems (State College, PA, USA) LP-21 pulse damper, a Valco (Houston, TX, USA) electric injector with a 50-μl injection loop, a Spectroflow 757 UV detector (Kratos Analytical, Ramsey, NJ, USA) and a Spectra-Physics (San Jose, CA, USA) integrator with a Labnet data acquisition card connected to an IBM AT computer.

Materials

Column. The columns (150 mm × 4.6 mm I.D.) were packed in the laboratory with a quaternary ammonium anion-exchange stationary phase (Vydac, Hesperia, CA, USA) having an average particle size of 10 μm and an average pore diameter of 30 nm.

Chemicals. N-Benzoyl-D-alanine, N-benzoyl-DL-alanine, N-benzoyl-L-alanine, D-mandelic acid, DL-mandelic acid, L-mandelic acid and bovine serum albumin (BSA) (No. A-7638) were purchased from Sigma (St. Louis, MO, USA) and used as received.

Mobile phase. For the pumping of BSA onto the column, the mobile phase was 50 m*M* aqueous phosphate buffer (pH 6.8) for column A and 100 m*M* aqueous phosphate buffer (pH 6.8) for column B. For the elution profiles, the mobile phase was 50 m*M* aqueous phosphate buffer (pH 6.8).

Procedures

After the columns had been packed, BSA was fixed to the anion exchanger by pumping a 2 mg/ml BSA solution onto the column until breakthrough was detected. The mass balance showed that column A had 153.5 mg, column B had 137.8 mg and column C had 0 mg of BSA. Before the elution profiles were measured, the column was equilibrated with *ca.* 100 column volumes of phosphate buffer (50 m*M*, pH 6.8). For the duration of the experiments, the leakage of BSA was negligible. This was checked by measuring the retention volume and the selectivity of the racemic mixtures of mandelic acid and N-benzoylalanine under linear conditions at the start and end of the experiments reported here. No variations of either were observed.

Two sets of elution profiles were obtained. First, the racemic mixtures were injected under linear conditions to determine the capacity factors at infinite dilution, k', and the selectivity, α. Second, the pure enantiomers were injected under overloaded conditions in order to determine their adsorption isotherms by ECP. The elution profiles of the pure enantiomers were converted by direct calibration from the detector response into concentration profiles, the response being the same for two enantiomers. At the wavelengths used, 240 nm for mandelic acid and 280 nm for N-benzoylalanine, the calibration graphs were linear and the regressions were carried out using a standard procedure. A SAS algorithm was used to obtain the best-fit bi-Langmuir parameters by non-linear regression carried out on the University of Tennessee VAX 8800 computer.

RESULTS AND DISCUSSION

Elution profiles

Under linear conditions (Figs. 1 and 2 and Table I), the selectivity of the column for the enantiomers increases with increasing amounts of BSA immobilized in the column. The retention of the less retained enantiomer of each pair, the L-isomer, decreases with increasing amounts of BSA immobilized to the support, while the retention of the more retained enantiomer, the D-isomer, increases. The molecular interaction energy of either L-isomer with the pure anion-exchanger surface (column C) is greater than that of either L-isomer with the BSA molecule (columns A and B). In effect, the BSA molecules are shielding the L-isomer molecules from access to the anion-exchanger surface, causing its retention time and retention factor to decrease (Table I). On the other hand, the D-isomers experience stronger interactions with the BSA molecules than with the anion-exchanger surface. As a consequence, the capacity factors of the D-isomers increase with increasing BSA load (Table I).

Under overloaded conditions (Figs. 3 and 4), a similar behavior is observed. One phenomenon that is not ealisy explained, however, is the crossing of the elution band profiles of the two enantiomers (insets in Figs. 3 and 4). This should be related to

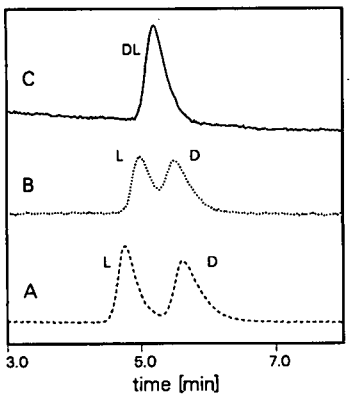


Fig. 1. Chromatograms of DL-mandelic acid resolved under linear conditions for the three columns (A, B and C). For each column, 0.2 nmol of the racemic mixture was injected. Experimental conditions: stationary phase, BSA ionically immobilized on a quaternary ammonium anion exchanger; mobile phase, 50 m*M* phosphate buffer (pH 6.8); flow-rate, 1 ml/min.

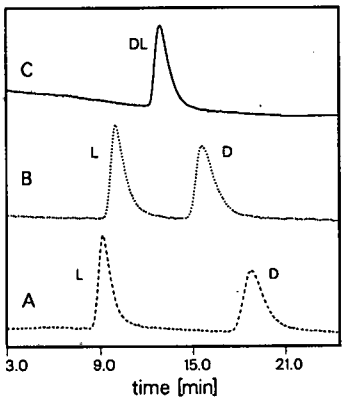


Fig. 2. Chromatograms of N-benzoyl-DL-alanine resolved under linear conditions for the three columns (A, B and C). For each column, 0.2 nmol of the racemic mixture was injected. Experimental conditions as in Fig. 1.

TABLE I

CAPACITY FACTORS AND SELECTIVITIES

Compound	Column	k'_L	k'_D	α
Mandelic acid	A	1.20	1.71	1.43
	B	1.32	1.65	1.25
	C	1.38	1.38	1.00
N-Benzoylalanine	A	3.43	8.08	2.35
	B	3.85	6.53	1.69
	C	4.91	4.91	1.00

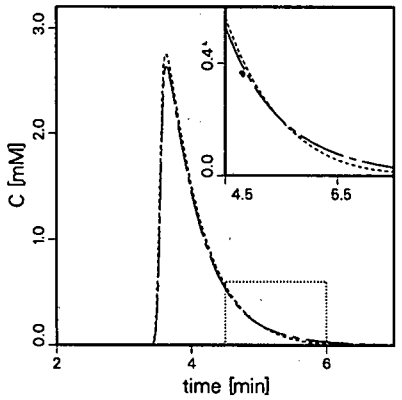


Fig. 3. Overloaded elution profiles of DL-mandelic acid for column B. (– – – –) L-Isomer (amount injected, 1.81 μmol); (–·–·–) D-isomer (amount injected, 1.74 μmol). Experimental conditions as in Fig. 1. Inset: enlargement of dotted region showing the crossing of the band profiles.

the difference observed between the saturation capacities of the selective sites for the two enantiomers and is discussed in the next section.

If non-selective molecular interaction with the surface of the anion exchanger are considered to make the dominant contribution to the enantiomer retention, this contribution should decrease in addition to the column capacity when the density of the BSA immobilized on the surface increases. If we compare the overloaded elution profiles recorded on the three columns (Figs. 5 and 6), it is clear that

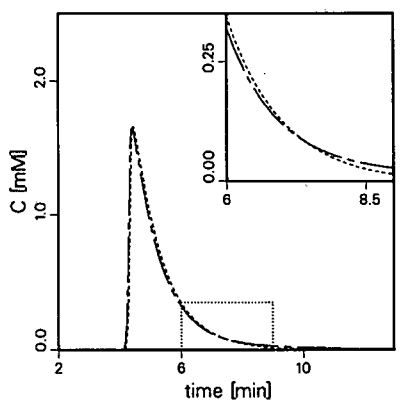


Fig. 4. Overloaded elution profiles of N-benzoyl-DL-alanine for column B. (– – – –) L-Isomer (amount injected, 1.90 μmol); (–·–·–) D-isomer (amount injected, 1.92 μmol). Experimental conditions as in Fig. 1. Inset: enlargement of dotted region showing the crossing of the band profiles.

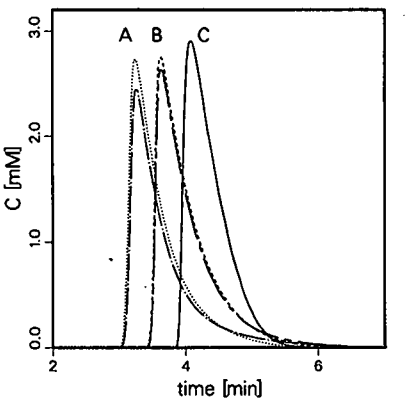


Fig. 5. Overloaded elution profiles of DL-mandelic acid for the three columns. Column A: (· · · ·) L-isomer (amount injected, 1.69 μmol); (– · – · –) D-isomer (amount injected, 1.52 μmol). Column B: (– – – –) L-isomer (amount injected, 1.81 μmol); (–·–·–)D-isomer (amount injected, 1.74 μmol). Column C: (———) (amount injected, 1.79 μmol). Experimental conditions as in Fig. 1.

this is realized as the fronts of the overloaded peaks of comparable amounts injected elute earlier as the amount of BSA increases in the column.

Adsorption isotherms

The adsorption data (Figs. 7 and 8) for both pairs of enantiomers on the three columns were deter-

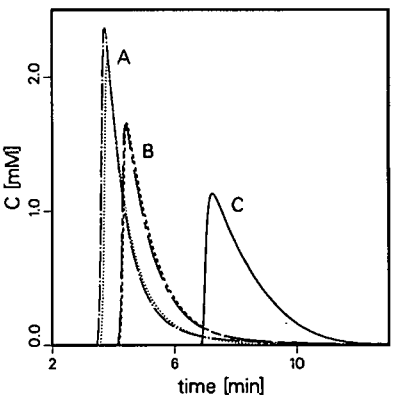


Fig. 6. Overloaded elution profiles of N-benzoyl-DL-alanine for the three columns. Column A: (· · · ·) L-isomer (amount injected, 2.00 μmol); (– · – · –) D-isomer (amount injected, 2.23 μmol). Column B: (– – –) L-isomer (amount injected, 1.90 μmol); (–·–·–) D-isomer (amount injected, 1.92 μmol). Column C: (———) (amount injected, 1.93 μmol). Experimental conditions as in Fig. 1.

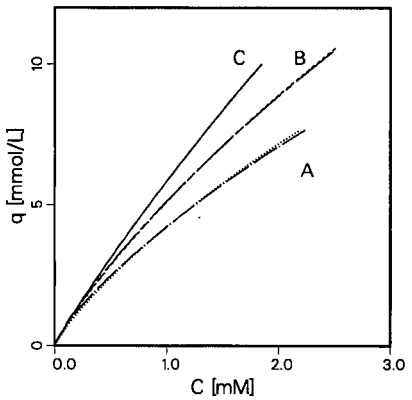


Fig. 7. Adsorption isotherms of DL-mandelic acid determined from the corresponding elution profiles in Fig. 5. Column A: (· · · ·) L-isomer; (– · – · –) D-isomer. Column B: (– – –) L-isomer; (– – – –) D-isomer. Column C: (———). For best-fit bi-Langmuir parameters, see Table II.

mined using the ECP method (eqn. 2) and the band profiles in Figs. 5 and 6. The best-fit bi-Langmuir isotherm parameters were calculated from the data for columns A and B, using this isotherm (eqn. 1) as the model. For column C, only a single Langmuir expression was used for the fit of the adsorption data. The values obtained for the parameters are listed in Tables II (mandelic acid) and III (N-benzoylalanine). As the loading of the BSA increases, the initial slope of the selective term of the bi-Langmuir isotherms increases, showing the effect of the protein on the retention of the enantiomers. On the other hand, the initial slope of the non-selective term of the isotherm decreases, demonstrating the shielding of the anion-exchanger surface by the BSA.

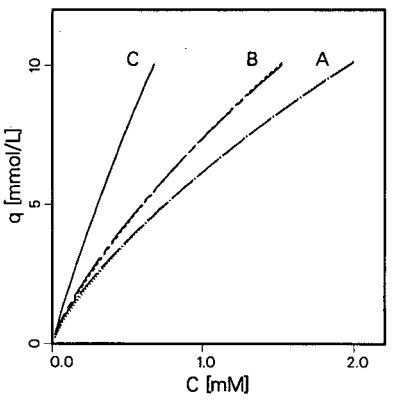


Fig. 8. Adsorption isotherms of N-benzoyl-DL-alanine determined from the corresponding elution profiles in Fig. 6. Lines as in Fig. 7. For best-fit bi-Langmuir parameters, see Table III.

Since we know the amount of BSA that is immobilized on the column (column A = 2.30 μmol; column B = 2.07 μmol) and the volume of the stationary phase (0.52 ml), we can express the saturation capacities of the selective sites (Tables II and II) as the number of molecules of enantiomers adsorbed at saturation per molecule of BSA. For mandelic acid, this ratio is 0.20 for the L-isomer and 0.19 for the D-isomer. For N-benzoylalanine, it is 0.22 for the L-isomer and 0.21 for the D-isomer. These numbers imply that there exists at most one chiral selective site per immobilized BSA molecule. Hence the active site is probably the inside of the hydrophobic cavity of the protein, as suggested previously [13,14]. Unfortunately, with the method of protein immobilization used here, we have no control of the orientation of the protein on the surface of the anion exchanger. It depends largely on the charge distribution on the protein surface. Consequently, it is reasonable to assume that every molecule of protein cannot be accessed by the enantiomers. Only one out of five would be available with our stationary phase. This number should be different, and probably larger, for covalently bonded BSA. As expected from previous work [6,10] and from the numbers just reported, the saturation capacity of the chiral selective sites is low, *e.g.*, *ca.* 40 times smaller than the saturation of the non-selective sites for column A. This explains why the BSA columns are so easily overloaded.

The most intriguing feature of these results is that

TABLE II

BI-LANGMUIR ISOTHERM PARAMETERS FOR MANDELIC ACID

Column	Site	Isomer	a	b (l/mmol)	q_s (mM)
A	Selective	L	2.8	3.2	0.89
		D	5.2	6.6	0.79
	Non-selective		4.1	0.13	31.7
B	Selective	L	2.5	3.1	0.80
		D	3.8	5.1	0.75
	Non-selective		5.2	0.11	46.9
C	Non-selective		6.9	0.13	52.8

TABLE III

B-LANGMUIR ISOTHERM PARAMETERS FOR N-BENZOYLALANINE

Column	Site	Isomer	a	b (l/mmol)	q_s (mM)
A	Selective	L	7.0	7.4	0.95
		D	13.8	15.5	0.89
	Non-selective		6.5	0.19	34.2
B	Selective	L	6.1	6.90	0.89
		D	11.6	14.0	0.83
	Non-selective		7.9	0.21	37.6
C	Non-selective		18.6	0.41	45.3

the isotherms of the two pairs of enantiomers cross for columns A and B where enantioselection occurs. This, of course, is expected following the observation that the elution profiles do cross (inset in Figs. 3 and 4). The assumption made when fitting the adsorption data to the bi-Langmuir isotherm model was that the non-selective site parameters are identical for any pair of enantiomers, but the selective site parameters can vary without constraint (other than when the adsorption behavior is Langmuirian in nature). In all instances, we find that the selective site saturation capacity of the L-isomer is greater than that of the D-isomer by an average of 8% (Tables II and III). This could be explained in one of two different ways.

First, two separate selective sites of adsorption could exist, one for each type of enantiomers. This situation would lead to the profile of the more retained enantiomers passing through the profile of the less retained enantiomers when the column is overloaded. However, under competitive conditions a strong displacement of the less retained enantiomer by the more retained enantiomer is observed [6,10], thus rebutting this possibility. The second possible reason is that the orientation of the enantiomers within the hydrophobic cavity of the protein might impose stronger steric restrictions on the more retained enantiomer, which requires three interactions as opposed to only two for the less retained enantiomer.

ACKNOWLEDGEMENTS

The gift of the anion exchange stationary phase by Vydac is gratefully appreciated. We thank Edwin Murphy for technical assistance. This work was supported in part by grant CHE-8901382 from the National Science Foundation and by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory. We acknowledge the continuous support of our computational effort by the University of Tennessee Computing Center.

SYMBOLS

- a first coefficient of the Langmuir isotherm
- b second coefficient of the Langmuir isotherm
- C mobile phase concentration of the solute
- q equilibrium stationary phase concentration of the solute
- q_s saturation capacity
- V_0 dead volume
- V_R retention volume
- V_S stationary phase volume

Subscripts

- x D or L, corresponding to the D-isomer and the L-isomer, respectively
- cs chiral selective site of adsorption
- ns non-selective site of adsorption

REFERENCES

1 D. W. Armstrong and S. M. Han, *CRC Crit. Rev. Anal. Chem.*, 19 (1988) 175.
2 W. H. Pirkle and T. C. Pochapsky, *Chem. Rev.*, 89 (1989) 347.
3 S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.*, 237 (1982) 473.
4 S. Andersson, S. Allenmark, P. Erlandsson and S. Nilsson, *J. Chromatogr.*, 498 (1990) 81.
5 P. Erlandsson, L. Hansson and R. Isaksson, *J. Chromatogr.*, 370 (1986) 475.
6 S. C. Jacobson and G. Guiochon, *J. Chromatogr.*, 590 (1992) 119.
7 W. H. Pirkle and R. S. Readnour, *Chromatographia*, 31 (1991) 129.
8 D. Graham, *J. Phys. Chem.*, 57 (1953) 665.
9 R. J. Laub, *ACS Symp. Ser.*, 297 (1986) 1.
10 S. Jacobson, S. Golshan-Shirazi and G. Guiochon, *J.Am. Chem. Soc.*, 112 (1990) 6492.
11 E. Cremer and J. R. K. Huber, *Angew. Chem.*, 73 (1961) 461.
12 S. Golshan-Shirazi, S. Ghodbane and G. Guiochon, *Anal. Chem.*, 60 (1988) 2630.
13 S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.*, 316 (1984) 617.
14 R. H. McMenamy in V. M. Rosenow, M. Oratz and M. A. Rotschild (Editors), *Albumin, Structure, Function and Uses*, Pergamon Press, Oxford, 1977, p. 143.

Journal of Chromatography, 600 (1992) 43–49
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 944

High-performance liquid chromatographic retention behaviour of ring-substituted aniline derivatives on a porous graphitized carbon column

Esther Forgács* and Tibor Cserháti

Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest (Hungary)

ABSTRACT

The retentions of sixteen ring-substituted aniline derivatives were determined on a graphitized carbon column using unbuffered acetonitrile–water and methanol–water eluent mixtures at various organic phase concentrations. Principal component analysis calculated from both the correlation and covariance matrices was used to detect the similarities and dissimilarities between the retention behaviours of the aniline derivatives. Each aniline derivative showed narrow and symmetrical peaks in each eluent. The high percentage of variance explained in the first principal component suggests that the eluents have common elution characteristics, but according to the second principal component they showed slightly different selectivities. The retention of aniline derivatives is not governed by their lipopholicity and bulkiness. The data indicate that a mixed type of retention mechanism accounts for the retention.

INTRODUCTION

The poor stability of silica at alkaline pH values [1] and the presence of free silanol groups on the surface of silica-based reversed-phase supports [2,3] limits the application of silica materials in chromatography. In order to suppress the effect of electrostatic interactions between the polar solutes and the acidic silanol groups, the eluent has to be buffered or various eluent additives such as salts and organic amines have to be added to the eluent [4]. To increase the pH range of application, many other supports have been developed, such as alumina [5], octadecyl-coated alumina [6], zirconia [7,8] and various polymer-based supports [9]. A promising new support, porous graphitic carbon (PGC), has been developed in the last decade [10,11]. The characteristics of PGC have recently been discussed [12]. Successful separations of basic solutes have been achieved on PGC columns [13,14]. It was found that the electronic interactions between the solutes and the surface of PGC have a considerable impact on the retention [15], and the structural planarity of solutes exerted a much greater influence on the selectivity on PGC than on C_{18} columns [16].

Principal component analysis (PCA) has frequently been used for the evaluation of retention data matrices [17]. In situations with many variables (chromatographic systems) and observations (solutes), the comparison of the retention behaviours of chromatographic systems taking into consideration simultaneously the retention of each solute is difficult owing to the multi-dimensionality of the data matrix. PCA allows the easier evaluation of the similarities or dissimilarities between solutes and eluent systems.

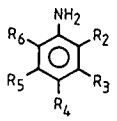
EXPERIMENTAL

A porous graphitic carbon column (Hypercarb, 100 × 4.7 mm I.D. particle diameter 7 μm) was purchased from Shandon Scientific (Runcorn, UK). The high-performance liquid chromatographic system consisted of a Liquopump Model 312 pump (LaborMIM, Budapest, Hungary), a Model CE-212 variable-wavelength UV detector (Cecil In-

0021-9673/92/$05.00

TABLE I

STRUCTURES OF RING-SUBSTITUTED ANILINE DERIVATIVES



No.	R2	R3	R4	R5	R6
1	CH_3	CH_3	H	H	H
2	CH_3	H	H	CH_3	H
3	CH_2CH_3	H	H	H	CH_3
4	H	H	Cl	H	H
5	Cl	H	Cl	H	H
6	H	Cl	H	Cl	H
7	H	Br	H	H	H
8	H	H	Br	H	H
9	Br	H	Br	H	H
10	I	H	H	H	H
11	H	H	I	H	H
12	NO_2	H	H	H	H
13	H	NO_2	H	H	H
14	H	H	NO_2	H	H
15	NO_2	H	H	H	NO_2
16	Cl	H	NO_2	H	H

struments, Cambridge, UK), an injector (Valco, Houston, TX, USA) with a 20-μl sample loop and a Waters Model 740 integrator (Waters–Millipore, Milford, MA, USA). The flow-rate was 1 ml/min and the detection wavelength was set at 254 nm. Mixtures of methanol–water and acetonitrile–water were used as eluents, with methanol and acetonitrile concentrations ranging from 90 to 97.5% (v/v) (in steps of 2.5%, v/v) and 85–92.5% (v/v) (in steps of 2.5%, v/v), respectively. The structures of the ring-substituted aniline derivatives are shown in Table I. The aniline derivatives were dissolved in methanol or acetonitrile at a concentration of 0.05 mg/ml. The retention time of each compound in each eluent was determined with three consecutive determinations.

To compare the performance of PGC with that of a traditional reversed-phase column, the retentions of compounds **4, 9** and **10** were determined on a 150 × 4 mm I.D. Hypersil ODS (5 μm) column with water–methanol (4:6, v/v) and 0.005 M K_2HPO_4–methanol (4:6, v/v) (pH adjusted to 7.80 with orthophosphoric acid) eluents. The asymmetry factor and the plate number for each derivative were calculated as described [18]. The plate number for toluene as a non-polar compound was also determined on both columns. The performance of the column was characterized by the ratio of the plate number for aniline derivative to the plate number for toluene. We assume that this ratio reflects the influence of solute polarity on the column performance.

In the PCA the mean retention time ± 2 standard deviations (S.D.) of aniline derivatives determined with in eight eluents formed the data matrix. The various acetonitrile–water and methanol–water eluents (four of each) were considered as variables, and the mean retenton times ± 2S.D. of aniline derivatives were the observations. The calculation was carried out on both the correlation and covariance matrices. The applicaton of both PCA methods for the same data matrix was motivated by the finding that the normalization (needed for the calculation of the correlation matrix) causes distortion of the data which may lead to inadequate results [19]. The explained variance was set to 99.9% in both instances. To compare the information content of PCA methods, linear correlations were calculated between PCA variables and loadings calculated from the two different matrices. Dependent variables were always those calculated fromt he covariance matrix. Only the PCA variables and loadings with the same serial number were correlated.

$$A_{cov} = a + b\,A_{corr} \quad (1)$$

$$Z_{cov} = a + b\,Z_{corr} \quad (2)$$

where A = PCA loadings, Z = PCA variables, corr = calculated from correlation matrix and cov = calculated from covariance matrix.

RESULTS AND DISCUSSION

Each aniline derivative showed symmetrical peaks in each eluent system (Figs. 1 and 2), that is, the carbon column can be successfully used for the separations of ring-substituted aniline derivatives without buffering the eluent. The retention order of solutes does not follow the retention order expected according to their lipophilicity. The more hydrophilic nitro derivative elutes after the more hydrophobic methoxy, chloro and iodo derivatives (Fig. 1). Substitution isomers could be well separated on

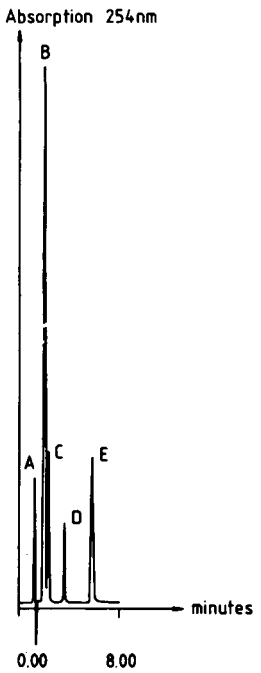


Fig. 1. Separation of aniline derivatives on a porous graphitic carbon column. Eluent, methanol–water (95:5, v/v); flow-rate, 1 ml/min; detection, 254 nm. A = Aniline; B = 2-methoxyaniline; C = 2-chloroaniline; D = 2-iodoaniline; E = 2-nitroaniline.

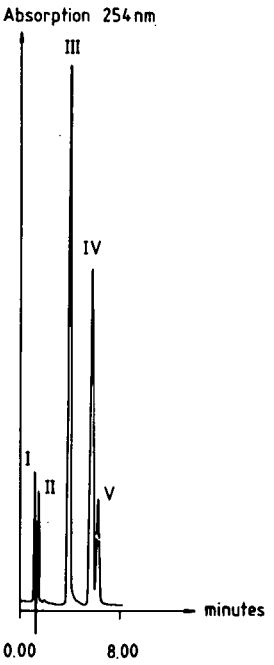


Fig. 2. Separation of aniline derivatives on a porous graphitic carbon column. Eluent, methanol–water (97.5:2.5, v/v); flow-rate, 1 ml/min; detection, 254 nm. I = Dead time; II = aniline; III = 3-nitroaniline; IV = 2-nitroaniline; V = 4-nitroaniline.

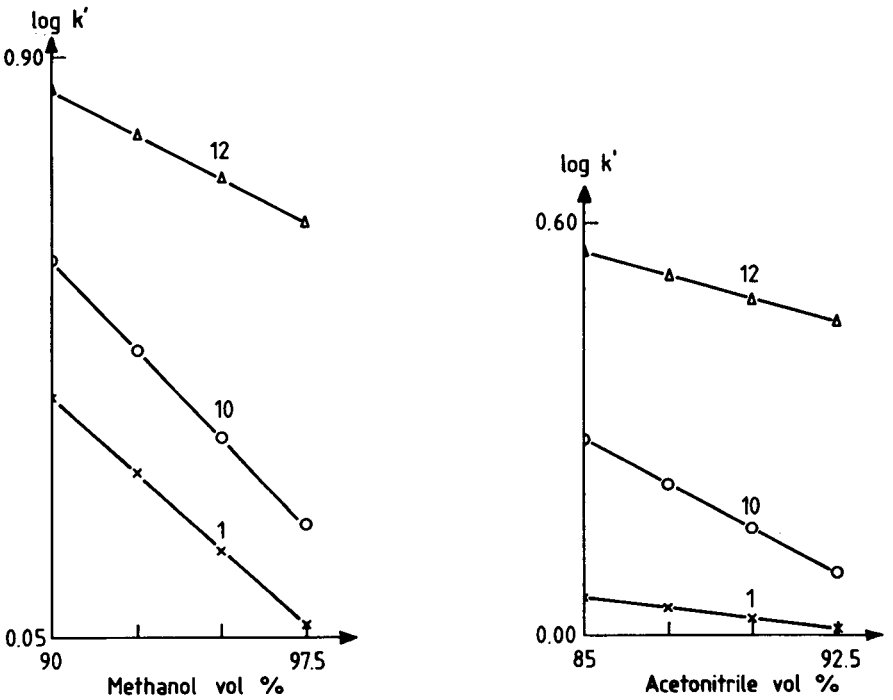


Fig. 3. Dependence of log k' values of ring-substituted aniline derivatives on the concentration of organic modifier in the eluent. Numbers refer to aniline derivatives in Table I.

TABLE II

RETENTION TIMES OF ANILINE DERIVATIVES

Compound	Retention time (min)			
	Methanol (%, v/v)			
	90	92.5	95	97.5
1	$2.92 \pm 1.4 \cdot 10^{-2}$	$2.73 \pm 5.6 \cdot 10^{-2}$	$2.42 \pm 7.0 \cdot 10^{-3}$	$2.25 \pm 7.0 \cdot 10^{-3}$
2	$4.45 \pm 3.5 \cdot 10^{-2}$	$4.90 \pm 7.0 \cdot 10^{-3}$	$2.70 \pm 2.8 \cdot 10^{-2}$	$2.69 \pm 4.3 \cdot 10^{-2}$
3	$5.66 \pm <10^{-3}$	$4.95 \pm <10^{-3}$	$3.78 \pm 1.4 \cdot 10^{-2}$	$3.48 \pm <10^{-3}$
4	$3.46 \pm 2.1 \cdot 10^{-2}$	$3.00 \pm 7.0 \cdot 10^{-3}$	$2.77 \pm 3.5 \cdot 10^{-2}$	$2.02 \pm 1.4 \cdot 10^{-2}$
5	11.30 ± 0.19	$8.92 \pm 7.0 \cdot 10^{-3}$	$6.30 \pm 7.0 \cdot 10^{-3}$	$5.18 \pm <10^{-3}$
6	$10.80 \pm <10^{-3}$	8.70 ± 7.10^{-3}	$5.91 \pm 7.0 \cdot 10^{-3}$	$4.66 \pm 2.8 \cdot 10^{-2}$
7	$2.69 \pm <10^{-3}$	$2.32 \pm <10^{-3}$	$1.93 \pm 7.0 \cdot 10^{-3}$	$1.76 \pm <10^{-3}$
8	$4.13 \pm 4.9 \cdot 10^{-2}$	$3.50 \pm 1.4 \cdot 10^{-2}$	$2.81 \pm 1.4 \cdot 10^{-2}$	$2.41 \pm 1.4 \cdot 10^{-2}$
9	$16.60 \pm <10^{-3}$	$14.04 \pm 3.5 \cdot 10^{-2}$	$10.19 \pm <10^{-3}$	8.47 ± 0.12
10	$5.75 \pm <10^{-3}$	$5.25 \pm <10^{-3}$	$4.05 \pm <10^{-3}$	$3.27 \pm 7.0 \cdot 10^{-3}$
11	$5.82 \pm 6.3 \cdot 10^{-2}$	$4.83 \pm 3.5 \cdot 10^{-2}$	$3.65 \pm <10^{-3}$	$3.13 \pm 1.4 \cdot 10^{-2}$
12	$10.56 \pm 7.0 \cdot 10^{-3}$	$9.13 \pm <10^{-3}$	$6.79 \pm 2.1 \cdot 10^{-2}$	$5.53 \pm 3.5 \cdot 10^{-2}$
13	$8.10 \pm <10^{-3}$	6.53 ± 0.21	$4.84 \pm <10^{-3}$	$3.92 \pm 7.0 \cdot 10^{-3}$
14	$12.55 \pm <10^{-3}$	10.31 ± 0.11	$7.89 \pm 2.1 \cdot 10^{-3}$	$6.42 \pm 3.5 \cdot 10^{-3}$
15	$63.40 \pm <10^{-3}$	$52.30 \pm <10^{-3}$	$41.50 \pm <10^{-3}$	36.0 ± 0.21
16	$48.49 \pm <10^{-3}$	$34.04 \pm <10^{-3}$	$27.50 \pm <10^{-3}$	$21.11 \pm 7.0 \cdot 10^{-3}$

the column, indicating the importance of the steric parameters in the retention (Fig. 2).

The retention of each compound decreases with increasing concentration of organic modifier (Fig. 3) and it depends on the number and position of substituents (Table II), which indicates the involvement of steric parameters in the mechanism of retention. The peak symmetry is similar on PGC with unbuffered eluent and on an ODS column with buffered eluent, but the relative performance of the PGC column is better than that of the ODS column even with a buffered eluent (Table III).

In both PCA methods the first principal component explained most of the variance (Tables IV and V), hence the main retention characteristics of the eight eluent systems applied can be expressed by only one hypothetical eluent system. Comparing the ratio of variance explained by the individual principal components, both calculation methods give similar results. According to eqn. 1, highly significant correlations were found between the PCA variables (Table VI). This result indicates that in our case the information contents of the PCA variables computed from different matrices are similar. However, the r values show that the predictive powers of the two calculation methods are different, which means that they are not entirely interchangeable. The distributions of PCA loadings (eluent systems) calculated according to the covariance and correlation matrices are shown in Fig. 4. The coordinates (F_1 and F_2) have no concrete physical meaning; they only indicate the relative distances between the eluent systems in two dimensions.

TABLE III

COMPARISON OF RETENTION CHARACTERISTICS OF POROUS GRAPHITIZED CARBON AND HYPERSIL ODS COLUMNS

(A) Porous graphitized carbon column; (B) ODS column, non-buffered eluent; (C) ODS column, buffered eluent. $P(\%)$ = (plate number for compound/plate number for toluene) · 100.

Compound	Asymmetry factor			$P(\%)$		
	A	B	C	A	B	C
4	1.23	1.60	1.25	64.02	40.4	47.7
9	1.17	1.67	1.33	65.3	65.6	67.3
10	1.12	1.50	1.05	66.12	39.0	40.1

Acetonitrile (%, v/v)			
85	87.5	90	92.5
$2.96 \pm < 10^{-3}$	$2.50 \pm 7.0 \cdot 10^{-3}$	$2.22 \pm < 10^{-3}$	$1.97 \pm 7.0 \cdot 10^{-3}$
$2.46 \pm < 10^{-3}$	2.33 ± 0.21	$2.14 \pm < 10^{-3}$	$1.75 \pm 7.0 \cdot 10^{-3}$
$3.22 \pm < 10^{-3}$	$3.01 \pm < 10^{-3}$	$2.75 \pm < 10^{-3}$	$2.71 \pm < 10^{-3}$
$2.33 \pm < 10^{-3}$	$2.20 \pm < 10^{-3}$	$2.14 \pm 7.0 \cdot 10^{-3}$	$2.07 \pm < 10^{-3}$
$6.53 \pm < 10^{-3}$	$5.84 \pm 1.4 \cdot 10^{-2}$	$5.20 \pm < 10^{-3}$	$5.08 \pm < 10^{-3}$
$5.92 \pm < 10^{-3}$	$5.61 \pm < 10^{-3}$	$5.19 \pm < 10^{-3}$	$4.65 \pm < 10^{-3}$
$2.60 \pm 7.7 \cdot 10^{-3}$	$2.53 \pm 4.2 \cdot 10^{-3}$	$2.37 \pm < 10^{-3}$	$2.25 \pm < 10^{-3}$
$2.82 \pm 7.0 \cdot 10^{-3}$	$2.52 \pm < 10^{-3}$	$2.34 \pm 7.0 \cdot 10^{-3}$	$2.05 \pm < 10^{-3}$
$10.94 \pm < 10^{-3}$	$9.87 \pm < 10^{-3}$	$9.24 \pm < 10^{-3}$	$8.32 \pm < 10^{-3}$
$3.67 \pm < 10^{-3}$	$3.52 \pm < 10^{-3}$	$3.36 \pm < 10^{-3}$	$3.17 \pm < 10^{-3}$
$2.35 \pm < 10^{-3}$	$2.17 \pm < 10^{-3}$	$1.96 \pm 7.0 \cdot 10^{-3}$	$1.80 \pm 7.0 \cdot 10^{-3}$
$5.76 \pm < 10^{-3}$	$5.24 \pm < 10^{-3}$	$4.95 \pm 7.0 \cdot 10^{-3}$	$4.60 \pm < 10^{-3}$
$4.26 \pm 5.6 \cdot 10^{-3}$	$4.07 \pm 2.8 \cdot 10^{-3}$	$3.88 \pm 7.0 \cdot 10^{-3}$	$3.64 \pm 7.0 \cdot 10^{-3}$
$6.22 \pm 7.0 \cdot 10^{-3}$	$5.89 \pm 1.4 \cdot 10^{-3}$	$5.55 \pm < 10^{-3}$	$5.16 \pm < 10^{-3}$
$39.72 \pm < 10^{-3}$	$26.95 \pm < 10^{-3}$	$11.71 \pm 7.0 \cdot 10^{-3}$	$6.35 \pm < 10^{-3}$
$13.28 \pm < 10^{-3}$	$19.65 \pm < 10^{-3}$	$18.54 \pm < 10^{-3}$	$17.08 \pm < 10^{-3}$

Eluents containing different organic mobile phases (methanol or acetonitrile) form separate clusters. This result indicates that methanol and acetonitrile show different selectivities, but this difference is small (compare the information contents of the first and second principal components in Table IV).

The relationships between the PCA variables are shown in Fig. 5. Also here the coordinates (F_I and F_{II}) have no concrete physical meaning; they only indicate the relative distances between the aniline derivatives in two dimensions. The centre of the circle represents the mean retention time and the radi-

TABLE IV

RESULTS OF PRINCIPAL COMPONENT ANALYSIS CARRIED OUT ON THE COVARIANCE MATRIX

Eluent		PCA loadings[a]	
No.	Composition (v/v)	I[b]	II[c]
1	Methanol–water (90:10)	4.52	−0.24
2	Methanol–water (92.5:7.5)	5.94	−0.19
3	Methanol–water (95:5)	7.37	−0.06
4	Methanol–water (97.5:2.5)	0.62	−0.68
5	Acetonitrile–water (85:15)	2.84	1.22
6	Acetonitrile–water (87.5:12.5)	4.27	0.37
7	Acetonitrile–water (90:10)	4.03	0.31
8	Acetonitrile–water (92.5:7.5)	3.70	0.33

[a] PCA loadings are related to the individual contributions of eluents to the first and second hypothetical eluent systems.
[b] Eigenvalue 278.96, variance explained 98.94%.
[c] Eigenvalue 2.38, variance explained 0.82%.

TABLE V

RESULTS OF PRINCIPAL COMPONENT ANALYSIS CARRIED OUT ON THE CORRELATION MATRIX

Eluent		PCA loadings[a]	
No.	Composition (v/v)	I[b]	II[c]
1	Methanol–water (90:10)	0.99	−0.09
2	Methanol–water (92.5:7.5)	0.99	−0.11
3	Methanol–water (95:5)	0.99	−0.06
4	Methanol–water (97.5:2.5)	0.99	−0.12
5	Acetonitrile–water (85:15)	0.94	0.33
6	Acetonitrile–water (87.5:12.5)	0.99	0.03
7	Acetonitrile–water (90:10)	0.99	0.03
8	Acetonitrile–water (92.5:7.5)	0.99	0.01

[a] PCA loadings are related to the individual contributions of eluents to the first and second hypothetical eluent systems.
[b] Eigenvalue 7.82, variance explained 97.77%.
[c] Eigenvalue 0.15, variance explained 2.12%.

TABLE VI

PARAMETERS OF LINEAR CORRELATION BETWEEN THE PRINCIPAL COMPONENT VARIABLES AND LOADINGS

$y = a + bx$ (y = calculated from covariance matrix; x = calculated from correlation matrix).

Dependent and independent variables[a]	Parameters[b]					
	n	a	b	S_b	r	$r_{tabulated}$
(1) PCA variables	48	18.10	5.95	0.05	0.9975	0.4648 (99.9%)
(2) PCA variables	48	0.55	4.12	0.55	0.7391	0.4648 (99.9%)
(1) PCA loadings	8	37.18	0.43	0.50	0.3262	0.6215 (90.0%)
(2) PCA loadings	8	0.12	3.64	0.48	0.9518	0.9249 (99.9%)

[a] PCA variables = related to the individual contributions of solutes to the first and second hypothetical solutes; PCA loadings = related to the individual contributions of eluents to the first and second hypothetical eluent systems.

[b] n = Sample number; a = regression constant; b = regression coefficient; S_b = standard deviation of regression coefficient; r = correlation coefficient; $r_{tabulated}$ = critical value of correlation coefficient.

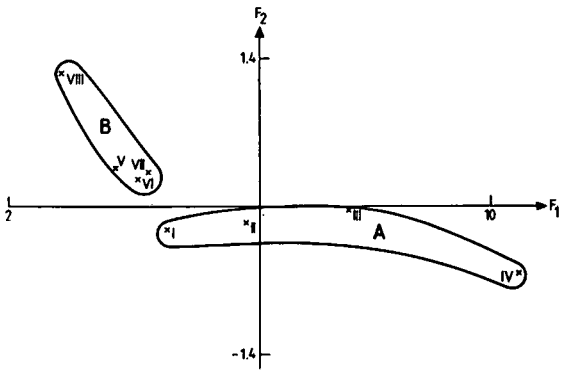


Fig. 4. Distribution of eluent systems according to the covariance matrix. A = Methanol–water eluents; B = acetonitrile–water eluents. The coordinates (F_1 and F_2) have no concrete physical meaning; they only indicate the relative distances between the eluent systems.

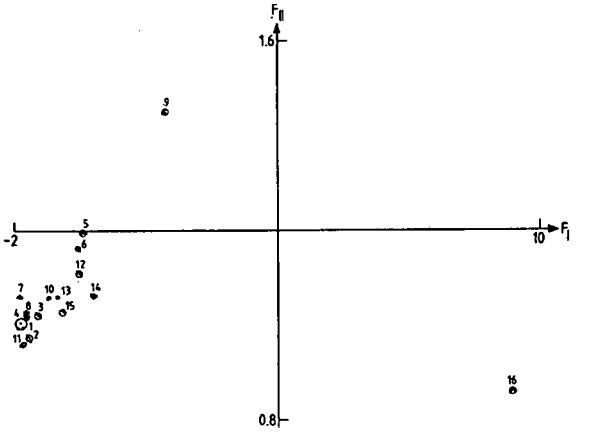


Fig. 5. Two-dimensional map of principal component variables based on the covariance matrix. Numbers refer to aniline derivatives in Table I. The coordinates (F_T and F_{II}) have no concrete physical meaning; they only indicate the relative distances between the aniline derivatives.

us of the circle characterizes the ±2 S.D. value.

We conclude that this procedure may represent a new graphical method including the standard deviation in PCA. The aniline derivatives do not form separate clusters either according to the number of substituents or according to the lipophilicity of the substituents. This result indicates that the number of substituents, the lipophilicity of the derivatives and possibly other molecular parameters have a similar impact on the retention, that is, with the PGC column the aniline derivatives show a mixed retention mechanism.

ACKNOWLEDGEMENT

This work was supported by grant OTKA 2670 from the Hungarian Academy of Sciences.

REFERENCES

1 A. Berthod, *J. Chromatogr.*, 549 (1991) 1.
2 A. Nahum and C. Horvath, *J. Chromatogr.*, 203 (1981) 53.
3 E. B. Klaas, C. Horvath, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
4 H. Tayar, H. Waterbeend and B. Testa, *J. Chromatogr.*, 320 (1985) 305.

5 C. J. Laurent, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 285 (1984) 161.
6 J. J. Sun and J. S. Fritz, *J. Chromatogr.*, 522 (1990) 95.
7 J. A. Blackwell and P. W. Carr, *J. Chromatogr.*, 549 (1991) 43.
8 J. A. Blackwell and P. W. Carr, *J. Chromatogr.*, 549 (1991) 59.
9 T. Takeuchi, W. Hu and H. Haraguchi, *J. Chromatogr.*, 517 (1990) 257.
10 M. T. Gilbert, J. H. Knox and B. Kaur, *Chromatographia*, 16 (1982) 138.
11 J. H. Knox, B. Kauer and G. R. Millward, *J. Chromatogr.*, 352 (1986) 3.
12 B. Kaur, *LC · GC*, 3 (1990) 41.
13 D. Roberts and D. W. Ruane, *J. Pharm. Biomed. Anal.*, 7 (1989) 112.
14 J. C. Berridge, *J. Chromatogr.*, 449 (1989) 56.
15 R. Kaliszan, K. Osmialowski and B. B. Bassler, *J. Chromatogr.*, 499 (1990) 33.
16 N. Tanaka, T. Tanagawa, K. Kimata, K. Hosoya and T. Araki, *J. Chromatogr.*, 549 (1991) 29.
17 T. Cserhati and H. E. Hauck, *J. Chromatogr.*, 514 (1990) 45.
18 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, pp. 28 and 222.
19 T. Cserhati and Z. Illes, *Chromatographia*, 31 (1991) 152.

Journal of Chromatography, 600 (1992) 51–57
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 866

Comparison of adsorption properties of Florisil and silica in high-performance liquid chromatography

I. Retention behaviour of monofunctional model solutes

Monika Waksmundzka-Hajnos*, Teresa Wawrzynowicz and Tadeusz H. Dzido

Department of Inorganic and Analytical Chemistry, Medical Academy, Staszica 6, 20-081 Lublin (Poland)

ABSTRACT

Earlier investigations demonstrated some special properties of Florisil and a greater selectivity than silica for the adsorption of some pairs of organic compounds. Differences in selectivity of the two adsorbents may be characterized by graphical correlations of retention parameters for these adsorbents. Consideration of relationship between $\log k'_{\text{Florisil}}$ and $\log k'_{\text{silica}}$ allows the interpretation of some factors that influence the adsorptive behaviour of organic compounds. The retention behaviour of several monofunctional aromatic compounds (quinoline bases, nitroarenes, primary and secondary aromatic amines and phenols) was therefore investigated by high-performance liquid chromatography in chromatographic systems using Florisil and silica and binary mobile phases consisting of heptane and a polar modifier (isopropanol, 1,4-dioxane or tetrahydrofuran).

INTRODUCTION

Florisil (magnesium silicate), produced mainly for thin-layer chromatography (TLC) or for classic column chromatography is frequently used in practical separations because of its special selectivity [1,2]. However only a single theoretical study of its properties has been published [3,4].

Soczewiński and co-workers [5–7] found that the molecular model of adsorption elaborated for silica can also be used for describing the adsorption behaviour of organic compounds on Florisil. Some information on the mechanism of adsorption from solution in polar solvents can be obtained from relationships between R_m ($\log k'$) and log (eluent composition).

Systematic studies of these relationships have been performed earlier for a number of model compounds belonging to different chemical groups [8,9] using TLC in sandwich tanks and Florisil as an adsorbent. These experiments indicated that steric and mesomeric effects have a significant influence on the chromatographic parameters of organic solutes. Although TLC in sandwich tanks operated in a quasi-column manner (continuous development, samples spotted behind the solvent front) [10] made it possible to obtain results close to those obtained in column chromatography [11], they were less accurate. Therefore, it was of interest to verify those observations using high-performance liquid chromatography (HPLC).

In this work, the retention behaviour of several monofunctional aromatic compounds (quinoline bases, primary and secondary aromatic amines, phenols) was investigated by HPLC in chromatographic systems using Florisil and binary mobile phases consisting of heptane and a polar modifier (tetrahydrofuran, 1,4-dioxane or isopropanol). These chromatographic data were compared with earlier results obtained for silica by HPLC [12,13].

0021-9673/92/$05.00

EXPERIMENTAL

The chromatographic experiments for Florisil were performed at 19 ± 1°C using a Type 302 liquid chromatograph (Institute of Physical Chemistry, Polish Academy of Sciences, Warsaw, Poland) equipped with syringe pump and a UV detector (254 nm) at a flow-rate of 1.2 ml/min. Single solutes were injected in the eluent with the help of a 5-μl injection valve.

HPLC-grade 10-μm Florisil was prepared in the Institute of Inorganic Chemistry (Gliwice, Poland). Florisil (Fluka, Buchs, Switzerland) for TLC was milled in a Model 100 AFG counterflow-fluid mill produced by Alpine (Augsburg, Germany). Particle segregation of ground Florisil was made in several stages in a Model 100 MZR pneumatic separator (Alpine). Characteristics of this separator were obtained by a CILAS 715E laser granulometer (Alcatel, France). Separated material was analysed in a Model A 200 LS pneumatic apparatus (Alpine). The porosity parameters (determined using a Carlo Erba porosimeter) of the fraction used as a column packing were specific surface area (BET) 340 m^2/g and total porosity 46.2%.

The column (150 × 4 mm I.D.) was packed with 10-μm Florisil by the slurry method. It had an efficiency of about 3000 theoretical plates determined by the adsorption of diphenylamine or 1-nitronaphthalene using 2% tetrahydrofuran in *n*-heptane as the eluent. The void volume of the column was determined by injection of pure *n*-heptane. The experimental results represent the averages of three runs.

The chromatographic experiments with silica were performed using a Perkin-Elmer (Norwalk, CT, USA) Model 1210 liquid chromatograph with dual syringe pumps and a UV detector (254 nm). The column (125 × 4 mm I.D.) was packed with 10-μm LiChrosorb Si 60 (E. Merck, Darmstadt, Germany) with a declared surface area (BET) of 500 m^2 g. For further details see, refs. 12 and 13.

As mobile phases, *n*-heptane solutions of isopropanol (for HPLC, E. Merck), 1,4-dioxane (for HPLC, Romil Chemicals, Shepshed, UK) and tetrahydrofuran (for chromatography, redistilled, International Enzymes, Windsor, UK) were used.

RESULTS AND DISCUSSION

The experimental results for the solutes listed in Table I are illustrated graphically as plots of log k' (capacity factor) against log c (concentrations; %, v/v), where c is the concentration of the polar component of the solvent. Fig. 1 shows these relationships for monofunctional solutes (of class AB according to the Pimentel–McClellan classification [14]) in the system with Florisil and tetrahydrofuran–*n*-heptane as eluent. The plots obtained for Florisil are linear, in accordance with the Snyder–Soczewiński model of adsorption [15,16]. The slopes of the lines can be differentiated: for anilines the plots are slightly steeper than those for the phenols. This indicates stronger adsorption of aromatic amines than phenols on the acidic centres of the Florisil surface.

Fig. 2 shows correlation plots of log k' values obtained for monofunctional solutes adsorbed on Florisil against log k' values of these compounds

TABLE I

SOLUTES INVESTIGATED

Solute	Abbreviation
Aniline	An
1-Aminonaphthalene	1AN
2-Aminonaphthalene	2AN
4-Methylaniline	4MAn
2,3-Dimethylaniline	23MAn
2,4-Dimethylaniline	24MAn
2,5-Dimethylaniline	25MAn
N-Benzylaniline	BylAn
Diphenylamine	diϕA
Phenol	P
3-Methylphenol	3MP
4-Methylphenol	4MP
2,3-Dimethylphenol	23MP
2,5-Dimethylphenol	25MP
Thymol	T
2-Napthol	2HN
Quinoline	Q
6-Methylquinoline	6MQ
7-Methylquinoline	7MQ
8-Methylquinoline	8MQ
3-Methylisoquinoline	3MiQ
5,6-Dimethylquinoline	26MQ
Benzophenone	Bf
1-Nitronaphthalene	1NtN

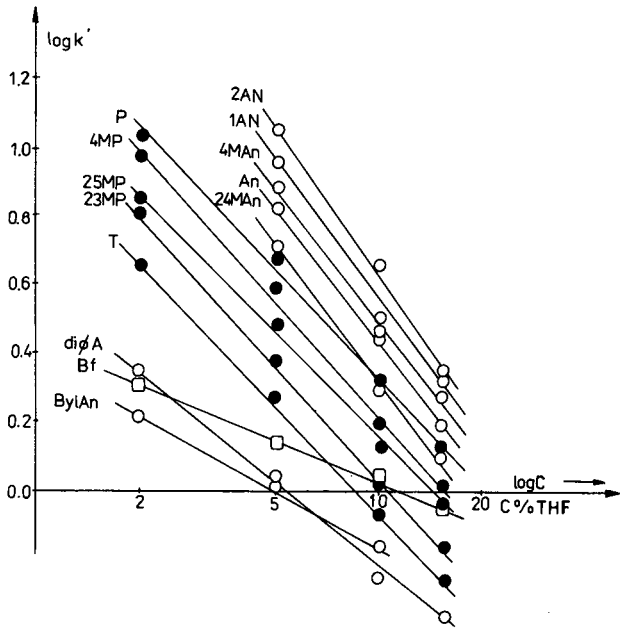


Fig. 1. Plots of log k' *versus* log (concentration of modifier in the mobile phase) (c, %, v/v) for monofunctional aromatic compounds. Active solid Florisil; mobile phase, tetrahydrofuran–*n*-heptane. For identification of solutes, see Table I.

adsorbed on silica in different eluent systems. These correlations illustrate the differences in adsorption on the Florisil and silica surfaces. In all three diagrams for three eluent systems, tetrahydrofuran–*n*-heptane, dioxane–*n*-heptane and isopropanol–*n*-heptane for phenols and for aniline derivatives (primary aromatic amines) respectively two correlation lines can be plotted. These lines can be expressed by the following equations:

(a) for tetrahydrofuran as a modifier:
for phenols (upper line):

$$\log k'_{\text{Florisil}} = 1.29 \log k'_{\text{silica}} + 0.05; \quad r = 0.89 \ (n = 8)$$

for anilines (lower line):

$$\log k'_{\text{Florisil}} = 0.82 \log k'_{\text{silica}} - 0.28; \quad r = 0.84 \ (n = 6)$$

(b) for dioxane as a modifier:

for phenols (upper line);

$$\log k'_{\text{Florisil}} = 1.98 \log k'_{\text{silica}} - 0.19; \quad r = 0.98 \ (n = 7)$$

for anilines (lower line):

$$\log k'_{\text{Florisil}} = 1.33 \log k'_{\text{silica}} - 0.37; \quad r = 0.97 \ (n = 7)$$

(c) for isopropanol as a modifier:
for phenols (upper line):

$$\log k'_{\text{Florisil}} = 1.62 \log k'_{\text{silica}} + 0.08; \quad r = 0.88 \ (n = 7)$$

for anilines (lower line):

$$\log k'_{\text{Florisil}} = 1.50 \log k'_{\text{silica}} - 0.37; \quad r = 0.94 \ (n = 7)$$

For combined phenols and anilines much lower correlation coefficients (r) would be obtained.

The points for secondary amines (diphenylamine, N-benzylamine), nitronaphthalene and benzophenone are dispersed near these two lines. For all compounds, especially in systems with tetrahydrofuran and isopropanol, the correlation coefficients are lower, which is due to differences in selectivity between the two adsorbents. The correlation coefficients for dioxane are high and the lines are close to each other. It seems that dioxane (class B) modifies the Florisil and silica surfaces by ether oxygen. This leads to adsorption of solutes by displacement and solvation of molecules [12,17]. The adsorbed molecules of dioxane on the Florisil or silica surface can interact with the second ether oxygen with the chromatographed solutes. This leads to co-adsorption of class AB solutes on the monolayer of dioxane, which causes the differences in the surface properties of Florisil and silica in this system to decrease and therefore the adsorption of solutes is similar.

It should be noticed that, for example, anilines are separated on Florisil using isopropanol (Fig. 2c) in the range of 0.5 log k' units and on silica only 0.3 log k' units, which means that the corresponding k' values are in the range of about 3.2 units on Florisil and 2 units on silica. A contrary effect can be observed for the same group of compounds using tetrahydrofuran as a modifier (Fig. 2a). In this instance the log k' values are in the range of about 0.25 units for Florisil and 0.35 units for silica and hence the separation of anilines on Florisil is worse. It seems that tetrahydrofuran binds to the Florisil surface, so that its specific polar properties decrease. Probably this results from the formation of a monolayer of tetrahydrofuran molecules. This film of adsorbed modifier molecules shields and deactivates particularly the surface of Florisil.

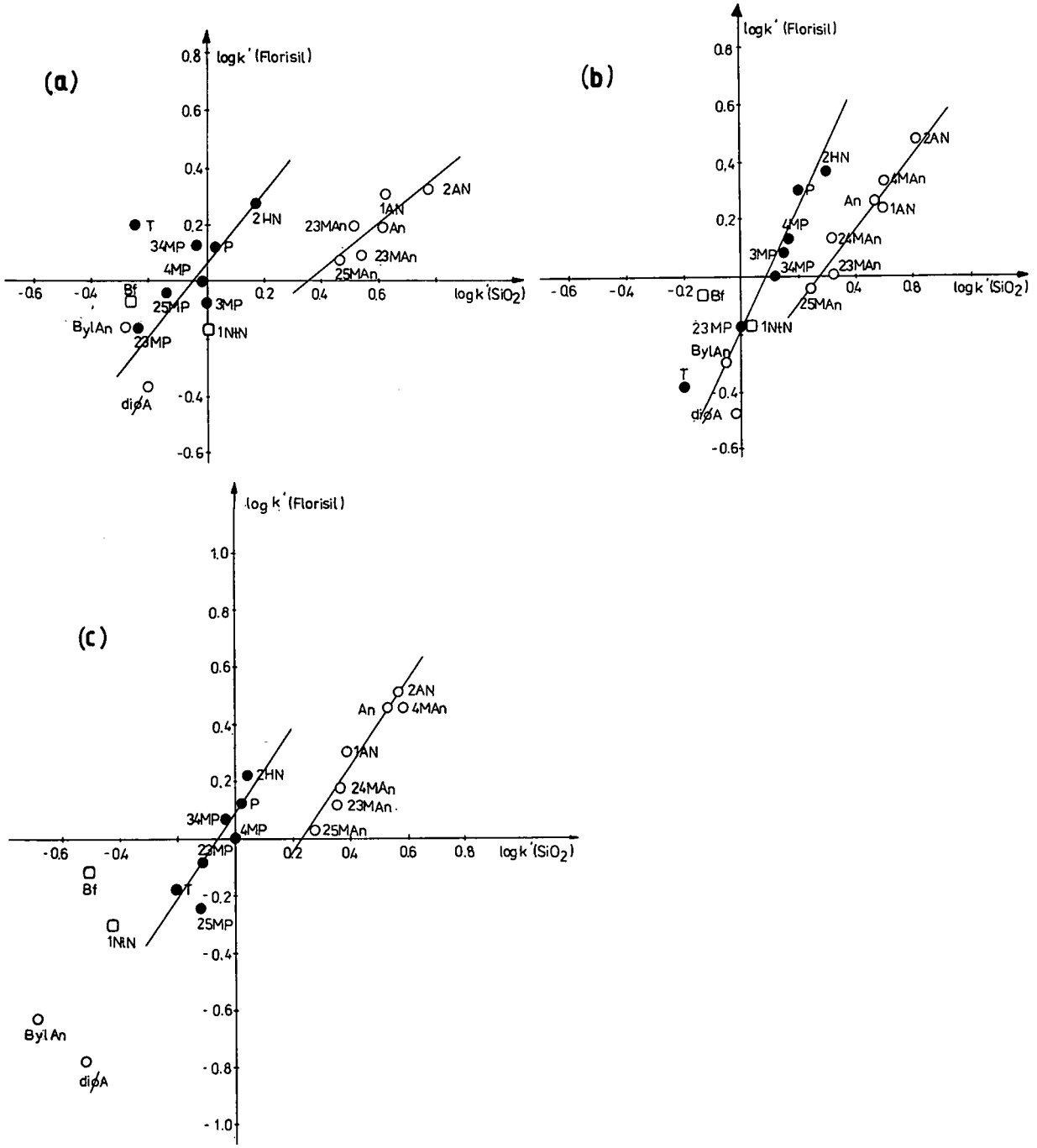


Fig. 2. Correlation between log k' values of monofunctional aromatic compounds on Florisil and silica. Solutes: (●) phenols; (○) anilines; (□) other compounds. Mobile phase: (a) tetrahydrofuran–*n*-heptane (15%, v/v, for Florisil and 20%, v/v, for silica); (b) dioxane–*n*-heptane (20%, v/v, for both adsorbents); (c) isopropanol–*n*-heptane (5%, v/v, for both adsorbents).

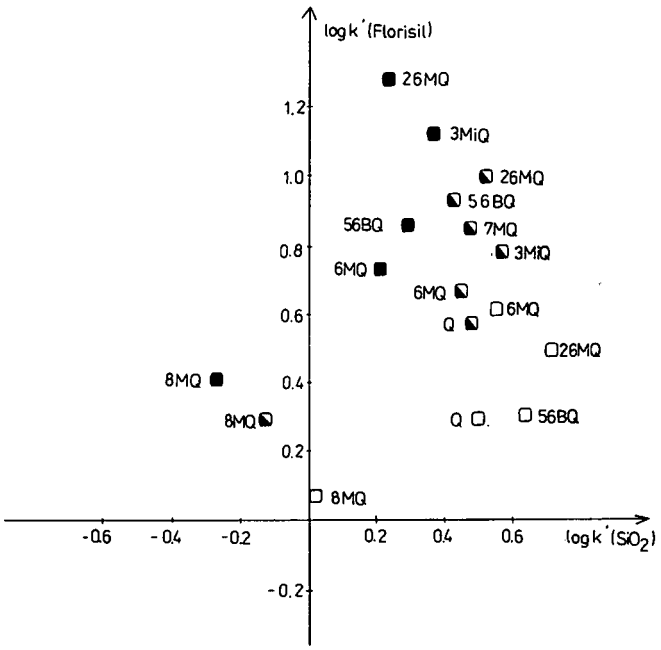


Fig. 3. Correlation between log k' values of monofunctional solutes (quinoline bases) on Florisil and silica using following systems: (□) tetrahydrofuran–*n*-heptane (40%, v/v, for Florisil and 20%, v/v, for silica); (◪) dioxane–*n*-heptane (50%, v/v for Florisil and 30%, v/v, for silica); (■) isopropanol–*n*-heptane (30%, v/v for Florisil and 5% v/v, for silica).

The correlation plots of log k'_{Florisil} against log k'_{silica} (Fig. 2) allow a comparison of the selectivities of the systems. For instance, it can be seen that although anilines are less strongly adsorbed on the Florisil than on the silica surface, in most instances they can be better separated using Florisil than silica, especially when using isopropanol or dioxane as a modifier (see Fig. 2b and c). For example, isomers such as dimethylanilines (23MAn, 24MAn, 25MAn) and aminonaphthalenes (1AN, 2AN) are better separated on Florisil using isopropanol–*n*-heptane or dioxane–*n*-heptane as the eluents. However, the adsorption of phenols on Florisil and on silica is similar, although there are some individual differences in chromatographic behaviour (*e.g.*, dimethylphenols). The isomers of xylenol are better separated on Florisil than on silica with all the eluent systems investigated (see Fig. 2). There are greater differences for Florisil than for silica in the retention of phenol and 2-naphthol and of phenol and 4-cresol with all three eluent systems.

The chromatographic properties of the above systems can also be compared by the retention of quinoline bases (class B). Quinolines are more strongly adsorbed on Florisil than on silica in all the systems investigated. The effect of shielding of the Florisil surface and its deactivation, especially by tetrahydrofuran molecules, can be observed (see Fig. 3). In all instances the compounds can be better separated using Florisil than silica. For example, in all eluent systems methylquinolines (7MQ or 6MQ) can be separated from dimethylquinoline (26MQ), and also from quinoline (Q).

Fig. 4 compares the selectivity of Florisil with different solvent systems as the log k' "spectrum" obtained for a constant concentration of a modifier (comparable solvent strength) for monofunctional solutes of class AB. The different slopes of the straight segments of lines indicate marked changes in selectivity; changes in the sequence of the elution of the compounds are also apparent. The effect of the individual substituents on retention can be

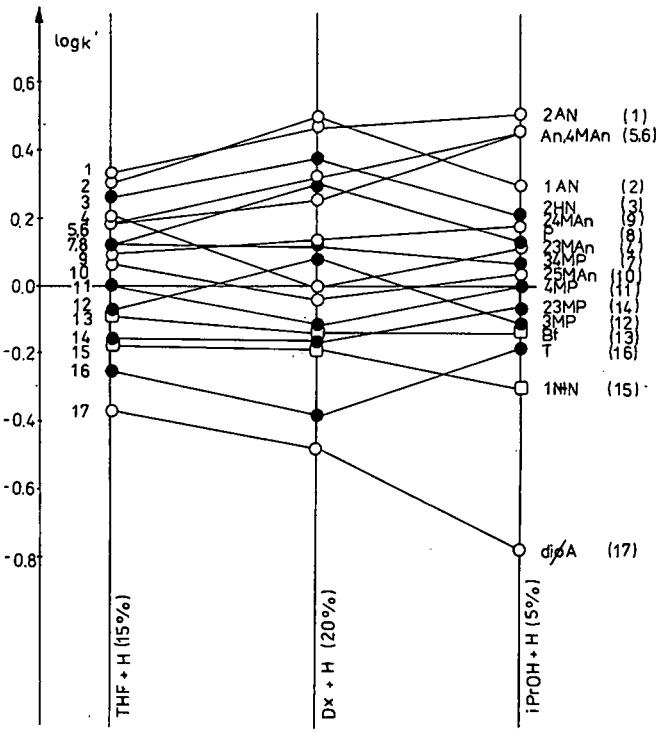


Fig. 4. Graphical comparison of log k' values obtained for Florisil. Mobile phases: tetrahydrofuran (15%), dioxane (20%) and isopropanol (5%) in *n*-heptane (H).

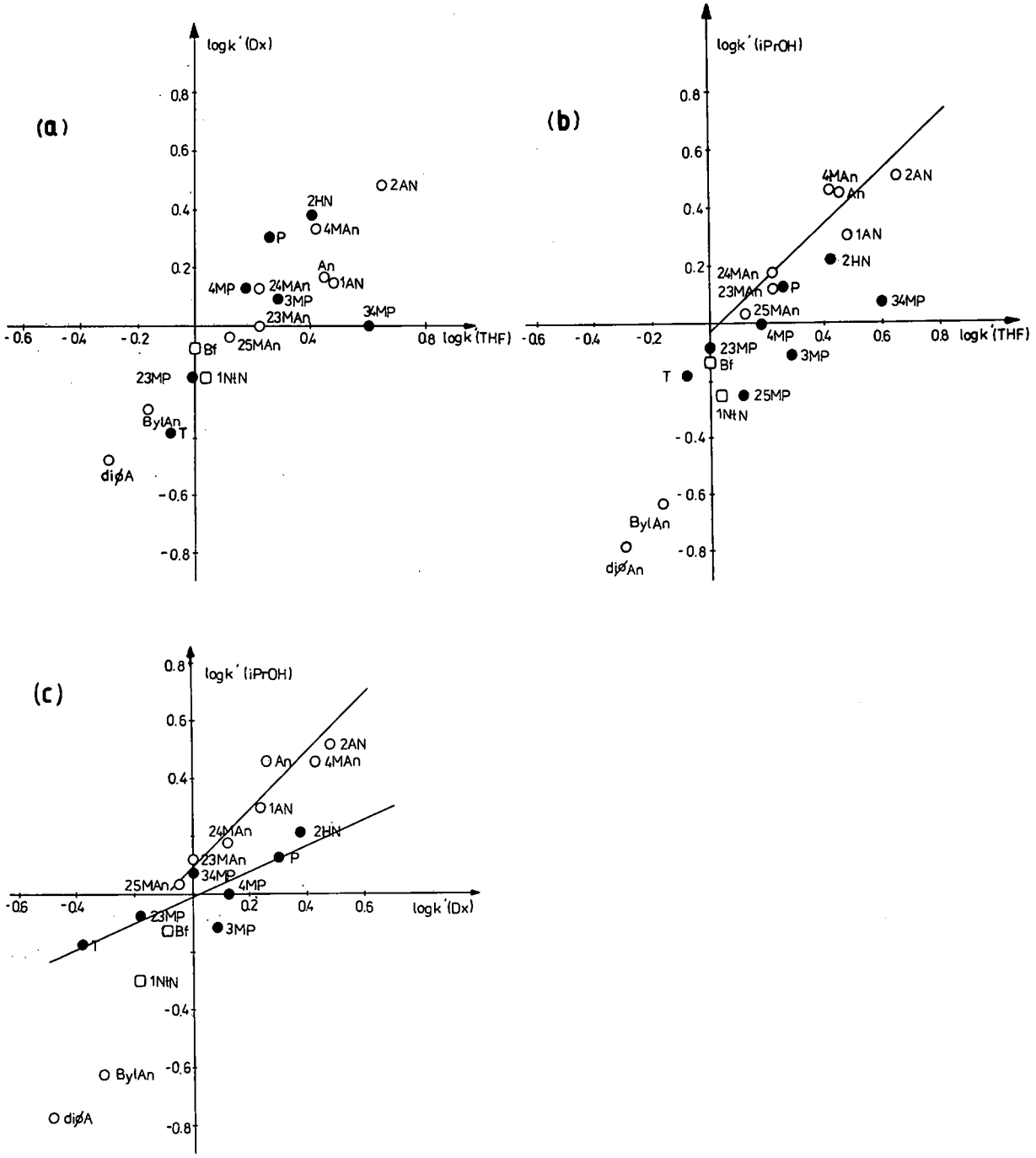


Fig. 5. (a) Correlation of log k' values for 20% (v/v) of dioxane (Dx) in *n*-heptane against log k' values for 10% (v/v) of tetrahydrofuran (THF) in *n*-heptane. Active solid, Florisil. (b) Correlation of log k' values for 5% (v/v) of isopropanol (iPrOH) in *n*-heptane against log k' values for 10% (v/v) of tetrahydrofuran in *n*-heptane. Active solid, Florisil; correlation line plotted for primary anilines. (c) Correlation of log k' values for 5% (v/v) of isopropanol in *n*-heptane against log k' values for 20% (v/v) of dioxane in *n*-heptane. Active solid, Florisil.

quantified by direct estimation from these diagrams.

Another method for comparison of the investigated eluent systems is to plot the log k' *versus* log k' relationships obtained using Florisil as an active solid and different modifiers in the mobile phase for all the investigated monofunctional solutes of class AB.

Comparison of dioxane–*n*-heptane and tetrahydrofuran–*n*-heptane eluent systems shows a common correlation line for all the compounds investigated (see Fig. 5a). However, there is a wide spread of points within each group; moreover, it is advantageous from the viewpoint of differences in selectivity between these modifiers (*i.e.*, there are no separate parallel correlation lines for phenols and anilines as when using silica [12]).

Fig. 5b illustrates the correlations between the eluents isopropanol–*n*-heptane and tetrahydrofuran–*n*-heptane. The points for aromatic primary amines form the upper line, which can be expressed by the following equation:

$$\log k'_{\mathrm{iPrOH}} = 0.93 \log k'_{\mathrm{THF}} - 0.04; \quad r = 0.91 \ (n = 7)$$

The points for phenols are dispersed below this line.

Fig. 5c presents a correlation of isopropanol–*n*-heptane and dioxane–*n*-heptane systems. The points form two crossing lines. The upper line for anilines can be expressed by the following equation:

$$\log k'_{\mathrm{iPrOH}} = 0.98 \log k'_{\mathrm{Dx}} + 0.10; \quad r = 0.95 \ (n = 7)$$

For the lower line for phenols the equation is

$$\log k'_{\mathrm{iPrOH}} = 0.43 \log k'_{\mathrm{Dx}} - 0.02; \quad r = 0.83 \ (n = 7)$$

These relationships show the specific properties of dioxane as a modifier and indicate that phenols are strongly adsorbed on the Florisil surface modified by dioxane molecules forming a film with ether oxygen–electrodonor atoms opposing the bulk phase.

CONCLUSIONS

In spite of a number of analogies between adsorption on Florisil and on silica, especially for AB class compounds, distinct differences in the acidity of the adsorption centres and their distribution were observed (*e.g.*, stronger adsorption of quinolines of class B and capability for separation of isomers). Either Florisil or silica may be used as a complementary adsorbent for some particular separations.

Modifiers such as isopropanol and dioxane are very interesting from the viewpoint of selectivity, especially the dioxane system, where an additional co-adsorption mechanism is observed.

REFERENCES

1 T. Wawrzynowicz, M. Waksmundzka-Hajnos and M. L. Bieganowska, *Chromatographia*, 28 (1989) 161.
2 M. Waksmundzka-Hajnos and T. Wawrzynowicz, *J. Planar Chromatogr.*, 3 (1990) 439.
3 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.
4 L. R. Snyder, *J. Chromatogr.*, 12 (1963) 488.
5 E. Soczewiński, T. Dzido and W. Golkiewicz, *Chromatographia*, 10 (1977) 298.
6 E. Soczewiński, W. Golkiewicz and T. Dzido, *Chromatographia*, 10 (1977) 221.
7 E. Soczewiński, J. Kuczmierczyk and T. Wawrzynowicz, *Chem. Anal. (Warsaw)*, 29 (1964) 527.
8 M. Waksmundzka-Hajnos, *Chem. Anal. (Warsaw)*, in press.
9 M. Waksmundzka-Hajnos, *J. Planar Chromatogr.*, 4 (1991) 127.
10 T. Wawrzynowicz, E. Soczewiński and K. L. Czapińska, *Chromatographia*, 20 (1985) 223.
11 T. Dzido and T. Wawrzynowicz, *Chem. Anal. (Warsaw)*, 24 (1979) 953.
12 T. Dzido and E. Soczewiński, *J. Chromatogr.*, 388 (1987) 99.
13 T. Dzido and E. Soczewiński, *J. Chromatogr.*, 395 (1987) 489.
14 G. C. Pimentel and A. L. McClellan, *The Hydrogen Bond*, Freeman, San Francisco, 1960.
15 E. Soczewiński, *Anal. Chem.*, 41 (1969) 179.
16 L. R. Snyder in Cs. Horváth (Editor), *High Performance Liquid Chromatography*, Vol. 3, Academic Press, New York, 1983.
17 E. Soczewiński, *J. Chromatogr.*, 388 (1987) 91.

Journal of Chromatography, 600 (1992) 59–65
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 909

Chromatographic properties of graphitized thermal carbon black modified with a monolayer of liquid crystal

V. I. Nazarova* and K. D. Shcherbakova

Laboratory of Adsorption and Chromatography, Chemistry Department, Lomonosov State University, 119899 Moscow (USSR)

O. A. Shcherbakova

Institute of Chemistry of Nonaqueous Solutions, Academy of Sciences of the USSR, Ivanovo (USSR)

ABSTRACT

Data obtained by chromatography, adsorption from solution and theoretical calculations show that a monolayer of liquid crystal adsorbed on the surface of graphitized thermal carbon black (GTCB) has an orientational order that is analogous to the mesomorphic state of the substance. A decrease in the adsorption potential and an increase in the general structural selectivity in the separation of polynuclear aromatic and partially hydrogenated compounds were observed for the modified sorbent. This is due to the influence of the liquid crystal monolayer on the adsorption of the studied substance on the one hand, and to the influence of GTCB on the monolayer on the other. The order of elution of isomeric polynuclear hydrocarbons on the modified and unmodified surfaces is the same, which makes it easier to identify the investigated isomeric mixtures.

INTRODUCTION

One of the most interesting possibilities of chromatography is in elucidating the relationship between retention and the molecular structure of the compounds under investigation. In gas chromatography on the non-specific monoatomic adsorbent graphitized thermal carbon black (GTCB) such a relationship has been demonstrated by many workers [1,2]. This forms the basis of the Kiselev and Poshkus method of studying of molecular structure [3,4].

In gas–liquid chromatography, the relationship between retention characteristics and molecular structure is much more complicated [5–9]. Nevertheless, when a liquid crystal material is used as a stationary liquid phase there is still sufficient sensitivity to the form and geometric size of the adsorbed molecules. The structural selectivity in this instance is due to the ordering of the mesophase. According to Witkiewicz [10], long and plane molecules can easily enter the lattice of the liquid crystal while non-linear and non-planar molecules cannot penetrate easily between anisotropic molecules of the mesogen and, hence they pass through the column more quickly.

It has been established that the state of a liquid crystal stationary phase is defined by the nature of the support and the character of its surface, the orienting influence of which on the structure of the immobile layer being greater in this instance than with traditional liquid stationary phases [10–12]. With inert supports, and also with active supports, the sensitivity of the layer to the molecular structure of adsorbates depends on the amount of liquid crystal deposited [13–15].

It has been observed [16] that there is a connection between conformational changes and electronic structures of molecules with different types of ordering in the mesophase. Owing to the short-range surface specific and non-specific interactions in the first adsorption layer, liquid crystal molecules applied as a monolayer on an active support of silica (glass, Silochrome) or on to split graphite form

0021-9673/92/$05.00

highly oriented layers [17]. By means of scanning tunnelling microscopy, some data on the state of the liquid crystal monolayer on split graphite have been obtained [18]. The position and orientation of molecules in the liquid crystal lattice were determined [18]. The overlapping of terminal groups in the monolayer film is weaker than in the bulk. It was also noted [18] that the structure of the layer is more likely to be molecular than of liquid crystalline type. Hence the use of the mesophase notion is inappropriate and there are no mesomorphic transitions in this instance. High structural selectivity of a monolayer of liquid crystal of cholesteric type on hydroxylated and dehydroxylated Silochrome was found in the gas chromatographic separation of xylene isomers [19].

The aim of this work was to determine the structural selectivity of a monolayer of liquid crystal of nematic type applied to GTCB. The adsorption of molecules on a non-porous homogeneous surface of GTCB depends on their geometry and polarizability. The same tendency was observed in the papers cited for the adsorption of molecules on a liquid crystal film. However, GTCB has a high adsorption potential and requires high working temperatures; GTCB pellets can easily be ruined. In addition, the majority of the liquid crystal exists in the nematic state in a narrow temperature interval. Hence, with the known advantages and disadvantages of GTCB and of liquid crystal stationary phases, it was considered of interest to modify GTCB with a liquid crystalline substance. Studies were made of the influence of non-specific adsorption of the support on the anisotropic properties of the monolayer on the one hand, and the influence of the monolayer on the adsorption properties of GTCB on the other.

EXPERIMENTAL

Sterling MT 3100 D4 GTCB (Cabot, Boston, MA, USA) with a specific surface area of 7.6 m^2/g was used as the adsorbent. For application of a monolayer we chose a high-temperature liquid crystal of the nematic type, bis(hexyloxybenzylidene) phenylendiamine (BHOBPDA), trademark H-75 (USSR):

$$p\text{-}C_6H_{13}O\text{–}C_6H_4\text{–}CH=N\text{–}p\text{-}C_6H_4\text{–}N=CH\text{–}C_6H_4\text{–}OC_6H_{13}\text{–}p$$

$$C \xrightarrow{425\,K} S \xrightarrow{453\,K} N \xrightarrow{523\,K} I$$

where C = crystal state, S = smectic state, N = nematic state and I = isotropic liquid. This compound has a low saturated vapour pressure and a wide temperature interval of the mesophase.

The calculation of the amount of liquid crystal which corresponds to a monolayer is based on the Van der Waals size of the molecules and on the following circumstances: (a) liquid crystal molecules have no active terminal groups; (b) the maximum gain in energy is for horizontal packing of the molecules; and (c) there are no pores on the GTCB surface. The area occupied by one liquid crystal molecule on the GTCB surface was found to be 185 Å, and the amount of liquid crystal corresponding to monolayer covering was 0.9 $\mu mol/m^2$ according to calculations. This value was proved experimentally by adsorption from solution. The equilibrium concentrations were determined with a Model SF-4A spectrophotometer (USSR).

Gas chromatographic measurements were carried out on a Chrom-5 analytical chromatograph (Czechoslovakia) equipped with a flame ionization detector and modified for physico-chemical investigations [20] and on an LKhM-8 MD chromatograph (USSR).

The following micropacked columns (MPC) were used: (1) GTCB modified with a monolayer of BHOBPDA, 45 cm × 1 mm I.D., pellet fraction 0.16–0.18 mm, mass of GTCB 0.5482 g and of liquid crystal 0.0018 g; (2) modified Chromaton N AW, pellet fraction 0.16–0.18 mm, 5 wt.% of liquid crystal, columns 25 cm × 1 mm I.D. and 90 cm × 0.75 mm I.D., mass of the packing 0.0841 g and 0.2029 g, respectively. The liquid crystal was precipitated on the GTCB and Chromaton N AW from dichloromethane solution under constant vibration followed by conditioning by heating in a flow of nitrogen.

The chromatographic sorption characteristics of the investigated compounds on the modified GTCB were compared with those obtained on unmodified GTCB [21] and in the bulk of the liquid crystal applied to Chromaton N AW.

Isomeric mixtures of partially or fully hydrogenated aromatic compounds were used as ad-

sorbates. The injection of adsorbates into the column was performed with 10% solutions in ethanol or benzene without splitting of the gas flow.

RESULTS AND DISCUSSION

The obtained adsorption isotherm has a saturation region, which corresponds to a monolayer. The amount of liquid crystal is 0.9 μmol/m^2. The dependences of the logarithms of the retention volumes of phenanthrene and anthracene on reciprocal of temperature in the bulk phase of the liquid crystal on Chromaton N AW are presented in Fig. 1. The character of these curves is in agreement with the common concept of phase transitions in liquid crystals. The three regions of the different phase states of BHOBPDA can easily be distinguished, namely the crystalline, smectic and nematic states. The transition of the liquid crystal from crystal to smectic is accompanied by a decrease and that to the nematic state by an increase in retention. A further increase in temperature leads to a decrease in retention.

The fact that the temperatures of the phase transitions of the pure liquid crystal and liquid crystal applied to Chromaton coincide proves that at the given degree of impregnation the inert support does not influence the properties of liquid crystal phase. The separation of the sorbates is due to the difference in the enthalpy of dissolution of the sorbates in the liquid crystal bulk. The maximum

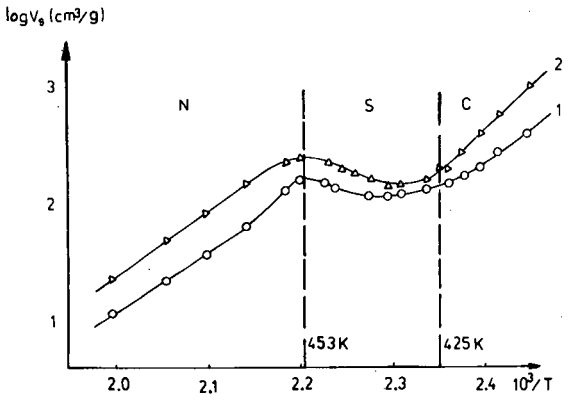


Fig. 1. Temperature dependence of the retention volumes of (1) phenanthrene and (2) anthracene on Chromaton N AW with 5% liquid crystal.

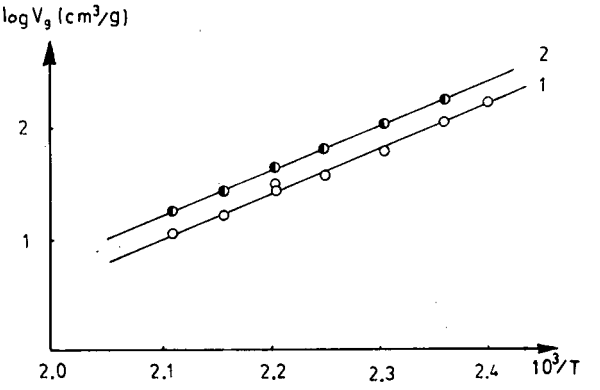


Fig. 2. Temperature dependence of the retention volumes of (1) phenanthrene and (2) anthracene on GTCB modified with a liquid crystal monolayer.

structural selectivity, $\alpha = V_g(\text{anthracene})/V_g(\text{phenanthrene})$ corresponds to the nematic state.

As can be seen from Fig. 2, there are no bends on the curves of the dependences of the logarithms of the retention volumes of phenanthrene and anthracene on the GTCB surface modified with a monolayer of BHOBPDA. Hence no phase transition of the liquid crystal monolayer on the GTCB surface takes place and its physical characteristics change continuously.

In the investigated temperature range, a high selectivity of separation of these components was observed. In addition to anthracene and phenanthrene, acenaphtene, acenaphthylene, fluorene and pyrene were also studied on modified GTCB. Their retention characteristics are given in Table I for different stationary phases for comparison.

It can be seen from Table I that the retention values on modified GTCB are lower than those obtained for adsorption on unmodified GTCB, and also for dissolution in the liquid crystal. The retention values of aromatic compounds on the unmodified GTCB at 473 K were obtained by extrapolation. The decrease in the adsorption potential on modified GTCB can be related to the increase in the distance between the interacting molecular centres of the adsorbate and the support by the thickness of the layer. The retention values also reflect the contribution of the specific interactions between molecules of the adsorbate and those of the liquid crystal. Here the presence of benzene rings in the

TABLE I

RETENTION VOLUMES, V_g^{473} (473 K), INTERNAL ENERGY, $-\Delta U$, AND SELECTIVITY COEFFICIENT, α, MEASURED ON DIFFERENT STATIONARY PHASES

$\alpha = V_g$(anthracene)/V_g(phenanthrene).

Substance	Liquid crystal		GTCB		GTCB + liquid crystal	
	V_g^{473} (cm^3/g)	$-\Delta U$ (kJ/mol)	V_g^{473} (cm^3/g)	$-\Delta U$ (kJ/mol)	V_g^{473} (cm^3/g)	$-\Delta U$ (kJ/mol)
Acenaphthene	822	55	1751	91	16.7	76
Acenaphthylene	836	51	1975	93	6.7	64
Fluorene	384	38	8334	79	15.5	79
Pyrene	5380	43	–	–	83.3	64
Phenanthrene	1194	45	53662	97	19.5	77
Anthracene	820	42	44017	97	11.8	76
α	1.45		1.22		1.65	

liquid crystal was responsible for the high affinity to aromatic molecules of the sorbates.

The increase in selectivity obtained in the separation of phenanthrene and anthracene on modified

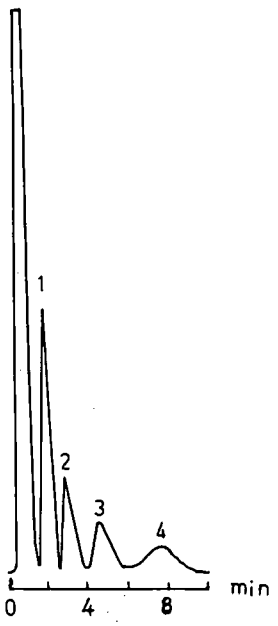


Fig. 3. Chromatogram of a mixture of tetracyclic aromatic hydrocarbons in a micropacked column with 5% liquid crystal on Chromaton N AW, pellet fraction 0.16–1.18 mm, column 25 cm × 1 mm I.D., temperature 443 K, mass of adsorbent 0.841 g, inlet pressure $1.92 \cdot 10^5$ Pa (1.9 atm), carrier gas nitrogen, flow-rate 10 ml/min. Peaks: 1 = pyrene; 2 = triphenylene; 3 = benzanthracene; 4 = chrysene.

GTCB reflects the effect of the action of the adsorption field of GTCB and of the monolayer of BHOBPDA, which is in a structured state similar to the mesomorphic state of a bulk phase. It should be noted that the order of elution on the unmodified GTCB and in the bulk phase of the liquid crystal on Chromaton coincides for nearly all the compounds. The selectivity of the mesomorphic phase to aromatic compounds is high, as can be seen, for example, in the separation of a mixture of tetracyclic aromatic hydrocarbons in the short micropacked column (Fig. 3).

The structural selectivity of the liquid crystal bulk phase to the isomers of perhydro compounds in the absence of specific interactions is not so high. In this instance the separation of the isomers is due only to the difference in their molecular structures. On the 90-cm column with liquid crystal stationary phase applied to Chromaton N AW the mixture of isomers of perhydroanthracene was not separated completely (Fig. 4a), whereas on the unmodified GTCB such a separation is possible (Fig. 4b). The identification of peaks was based on mass composition in the case of their separation on 5 wt.% liquid crystal (Fig. 4a). The separation of the same mixture on modified GTCB in the 45-cm column with temperature programming is shown in Fig. 4c. The order of elution for all the above columns coincides. For the separation of perhydroanthracene isomers on modified GTCB in comparison with unmodified GTCB, the selectivity estimated with only the first two peaks

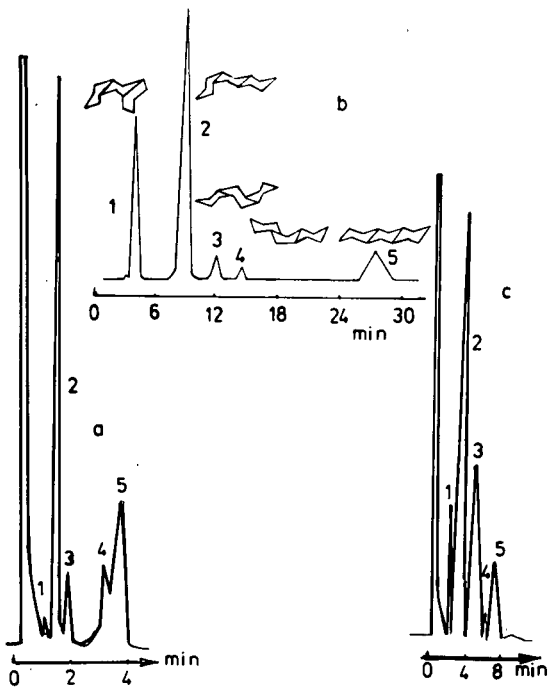


Fig. 4. (a) Chromatogram of the mixture of perhydroanthracene isomers in a micropacked column with 5% liquid crystal on Chromaton N AW; column 90 cm × 1 mm I.D., temperature 458 K, mass of adsorbent 0.2029 g, inlet pressure $1.92 \cdot 10^5$ Pa (1.9 atm), carrier gas nitrogen, flow-rate 3 ml/min. (b) Separation of perhydroanthracene isomers in a micropacked column with unmodified GTCB [21]; column 1.2 m × 1 mm I.D., carrier gas hydrogen, flow-rate 10 ml/min, flame ionization detection, mass of GTCB 0.788 g, inlet pressure $2.12 \cdot 10^5$ Pa (2.1 atm). (c) Separation of perhydroanthracene isomers in a micropacked column with GTCB modified with liquid crystal; column 45 cm × 1 mm I.D., temperature programmed from 433 to 443 K starting at the 4th minute, carrier gas nitrogen, flow-rate 10 ml/min, mass of adsorbent 0.55 g, inlet pressure $1.92 \cdot 10^5$ Pa (1.9 atm), GTCB fraction (as in b) 0.16–0.18 mm. Peaks: 1 = *cis–syn–cis*; 2 = *cis–syn–trans*; 3 = *cis–anti–cis*; 4 = *trans–anti–trans*; 5 = *trans–syn–trans*.

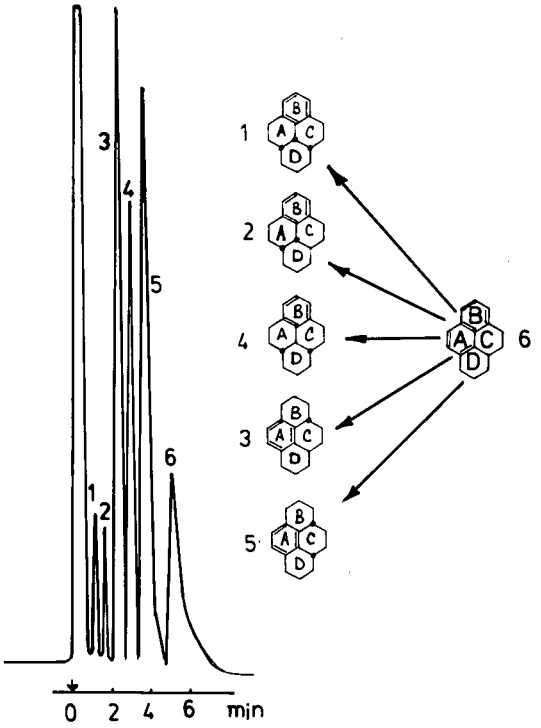


Fig. 5. Separation of a mixture of perhydropyrene isomers in a micropacked column with GTCB modified with a liquid crystal monolayer. Temperature 453 K, other conditions as in Fig. 4c.

being taken into account is slightly lower and the working temperature necessary for the separation decreases (by 70–80 K).

The use of GTCB modified with a liquid crystal monolayer can be successful for the separation of technological mixtures obtained by hydrogenation of high-boiling polyaromatic compounds, the result of which is the formation of isomeric compounds with different degrees of hydrogenation. We obtained a complete separation of a mixture of decahydropyrenes (obtained by hydrogenation of hexahydropyrene at low temperatures), as shown in Fig. 5. In this instance theory predicts the existence of five possible isomers. The identification of the peaks of this mixture was performed as described previously [20] according to the model concepts of their molecular structures and using the sum of the distances of their atoms from the plane surface of the adsorbent ($\sum\tau_i$). According to the decreasing order of this value, the first to elute from the column with GTCB modified with BHOBPDA should be the isomer with the molecule containing an incompletely hydrogenated B-ring and the rings in the row A–D–C have *cis* linkages, *i.e.*, hydrogen atoms at carbon atoms 8, 12 and 16 are situated at one side of the system of rings. This isomer, ADC-*cis*-decahydro-B-pyrene, has the most bent form of the molecule and the maximum sum of distances from the adsorption field plane, $\sum\tau_i$. The other isomers are eluted according to the decrease of this sum. The isomer ADC-*cis*, *trans*-decahydro-B-pyrene is the

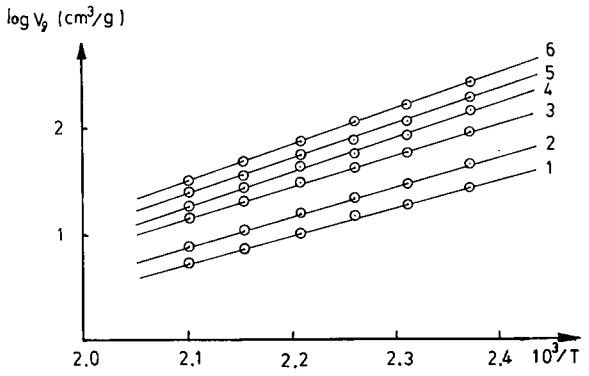


Fig. 6. Temperature dependence of the retention volumes of perhydropyrene isomers in a 45-cm micropacked column with GTCB modified with a liquid crystal monolayer. The curve numbers correspond to the peak numbers in Fig. 5.

second to elute. In its molecule the B-ring is incompletely hydrogenated and the A–D–C rings have *cis* and *trans* linkages. The third peak on the chromatogram belongs to the isomer in which the A-ring is incompletely hydrogenated and the B–C–D rings have *trans*, *cis* linkages, *i.e.*, to BCD-*trans*,*cis*-decahydro-A-pyrene. The fourth peak corresponds to ADC-*trans*,*trans*-decahydro-B-pyrene and the fifth to the isomer with an incompletely hydrogenated A-ring and *cis* linkages of the B–C–D rings. The sixth peak belongs to the initial compound hexahydropyrene, the molecule of which has the most planar structure.

The separation of isomers of this mixture on OV-1 liquid stationary phase has been published [22]. The temperature necessary for the elution of isomers of this mixture on carbon black modified with liquid crystal is lower than that for their elution on unmodified carbon black by 150–180 K. The chromatogram in Fig. 5 was obtained with a 45-cm micropacked column of GTCB modified with BHOBPDA. The high selctivity of the separation on this sorbent can be explained by the overall contribution of non-specific dispersion and specific interactions caused by the presence of the aromatic ring in an A or B position in molecules of decahydropyrenes. The selectivity of separation of this mixture on modified GTCB is higher than that on the liquid crystal phase.

The curves of the dependence of log V_g on the reciprocal of temperature are presented in Fig. 6. As it can be seen, a high selectivity is observed over the whole interval of temperatures studied. This confirms the assumption about high orientational ordering and stability of the liquid crystal monolayer applied to a homogeneous graphite surface. The column with GTCB modified with BHOBPDA has been used for 1 year without changes in its properties.

CONCLUSIONS

Adsorption from solution and theoretical calculations have shown that the amount of liquid crystal adsorbed on a GTCB surface corresponds to a monolayer. The molecular orientational ordering of a two-dimensional BHOBPDA film is similar to the mesomorphic state of a bulk phase. There are no phase transitions in the adsorbed liquid crystal monolayer and the working temperature range is widened. Comparison of the retention characteristics obtained for polynuclear hydrocarbons in chromatographic columns containing unmodified GTCB, bulk phase of BHOBPDA and GTCB modified with a BHOBPDA monolayer shows that the decrease in adsorption potential and working temperature is due to the action of the liquid crystal monolayer. The structural selectivity of GTCB modified with liquid crystal for the separation of aromatic and partially hydrogenated isomeric polynuclear compounds is higher than that with unmodified GTCB or with the liquid crystalline bulk phase.

GTCB modified with a monolayer of BHOBPDA or other liquid crystals with the use of more effective capillary columns appears promising for the separation of complex isomeric mixtures of high-boiling compounds. It has been established that for GTCB modified with a liquid crystal monolayer the relationship between the retention values of hydrocarbons and their molecular structures is of the same character for unmodified GTCB.

REFERENCES

1 A. V. Kiselev and Ya. I. Yashin, *Adsorbtsionnaya Gazovaya i Zhidkostnaya Khromatografiya (Adsorption Gas and Liquid Chromatography)*, Khimiya, Moscow, 1979.
2 A. V. Kiselev, *Molekulyarnye Vzaimodeistviya v Adsorbtsii i Khromatografii (Molecular Interactions in Adsorption and Chromatography)*, Vysshaya Shkola, Moscow, 1986.
3 A. V. Kiselev and D. P. Poshkus, *Faraday Discuss. Chem. Soc.*, 15 (1980) 13.

4 A. V. Kiselev, in Ya. M. Kolotyrkin (Editor), *Sovremenniye Problemy Fisicheskoi Khmii (Modern Problems of Physical Chemistry)*, Khimiya, Moscow, 1982, p. 180.
5 A. V. Ladon, *Chromatographia*, 4 (1971) 171.
6 R. V. Golovnya and D. N. Grigoryeva, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 3 (1987) 596.
7 W. Engewald and L. Wennrich, *Chromatographia*, 9 (1976) 540.
8 V. M. Nabivach and W. E. Vasiliev, *Zh. Fiz. Khim.*, 62 (1988) 1873.
9 A. Voelkel, *J. Chromatogr.*, 464 (1989) 251.
10 Z. Witkiewicz, *J. Chromatogr.*, 251 (1982) 311.
11 Oweimreen Ghassan Andrawes, *Mol. Cryst. Liq. Cryst.*, 68 (1981) 257.
12 K. Shoji, T. Ishiji and M. Takeda, *J. Chem. Soc. Jpn.*, 4 (1980) 557.
13 J. Rayss and F. Waksmundzki, *J. Chromatogr.*, 292 (1984) 207.
14 W. Marciniak and Z. Witkiewicz, *J. Chromatogr.*, 207 (1981) 333.
15 W. Marciniak and Z. Witkiewicz, *J. Chromatogr.*, 324 (1985) 299.
16 A. C. Sarkisyan and S. M. Yayloyan, *Zh. Strukt. Khim.*, 30 (1989) 173.
17 C. S. Mullin, P. Guyot-Sionnest and R. Shen, *Phys. Rev. A*, 39 (1989) 3745.
18 D. P. E. Smith, H. Horber, Ch. Gerber and G. Binnig, *Science*, 245 (1989) 43.
19 S. P. Vetrova, N. P. Karabanov and Ya. I. Yashin, *Dokl. Akad. Nauk SSSR*, 250 (1980) 1165.
20 A. V. Kiselev, V. I. Nazarova and K. D. Shcherbakova, *Chromatographia*, 14 (1981) 148.
21 A. V. Kiselev, V. I. Nazarova and K. D. Shcherbakova, *Chromatographia*, 18 (1984) 183.
22 G. Mann, A. Sicker, R. Herzschuh, W. Engewald and K. Praefcke, *J. Prakt. Chem.*, 331 (1989) 267.

Journal of Chromatography, 600 (1992) 67–77
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 946

Chromatographic determination of the physico-chemical parameters of adsorption on activated carbon fibres

H. Grajek, S. Neffe and Z. Witkiewicz*

Institute of Chemistry, Military Technical Academy, 01489 Warsaw 49 (Poland)

ABSTRACT

Inverse gas chromatography was used to determine the adsorption isotherms of benzene and nitrogen on activated carbon fibres (ACFs) obtained from viscose and polyacrylonitrile cloths. For benzene the results were compared with those obtained by the static method. The entropies and pure heats of adsorption were calculated from adsorption isotherms of benzene and nitrogen determined statically and chromatographically by the method suggested by Bering and Serpinsky. The specific surface areas of these adsorbents were determined by the heat desorption method proposed by Nelsen and Eggertsen and were calculated from the isotherms by the BET method. The results were compared with the surface areas of the walls of micropores calculated assuming the slit-like model of micropores. The surface areas of the walls of micropores are smaller than those obtained using other methods. Inverse gas chromatography was used successfully for determination of the surface properties of carbon adsorbents.

INTRODUCTION

In recent years, there has been increasing interest in carbon adsorbents of the fibre type, the so-called activated carbon fibres (ACFs). They have begun to replace activated carbons, which were commonly used in pollution adsorption installations [1,2]. ACFs are obtained mainly from cellulose, viscose, polyacrylonitrile and poly(vinyl chloride) cloths and others.

In order to explain the processes occurring on the surface of ACFs, and on the surface of activated carbons, the determination of their surface properties through measurement of their porous structure, and a knowledge of the adsorption isotherms and thermodynamic functions is required. For this purpose, gravimetric and volumetric methods are widely used. These methods are accurate but time consuming. In this connection, research is in progress to find new methods that are as precise as static methods but less time consuming. Many investigators have been trying to use adsorption gas chromatography for this purpose [3–8]. The possibility of applying it in physico-chemical investigations originates from the fact that the chromatographic process is related to the properties and physico-chemical interactions of the column filling (*e.g.*, ACFs) with the analyte substances.

Adsorption gas chromatography has already been used to investigate carbon fibres with relatively small surface heterogeneity. Investigations of the adsorption phenomena have been carried out only in the Henry's law region. From the retention data the net retention volumes were calculated. From the net retention volumes at zero coverage of the surface of the adsorbate, physico-chemical parameters of the adsorption, *e.g.*, standard free energy of adsorption, surface energy, entropy of specific interactions, Kováts retention index and isosteric heat of adsorption have been calculated [9–12]. Gray and co-workers [13–16] determined adsorption isotherms using adsorption gas chromatography beyond the Henry's law region. They investigated the adsorption phenomena on the surface of cellulose fibres, darning thread, ramie cloths and Cellophane. They calculated the constants of the BET and Antoine equations and isosteric heats of adsorption from adsorption isotherms [13–16].

0021-9673/92/$05.00

In this work, the adsorption isotherms of benzene and nitrogen on activated carbon fibres obtained from viscose and polyacrylonitrile cloths were determined chromatographically. From these isotherms, the constants of the Dubinin–Radushkevich (DR) and BET equations, pure heat of adsorption and molar differential entropy of adsorption were calculated. The results for benzene obtained chromatographically are compared with those obtained by the static method.

EXPERIMENTAL

Adsorbents

The following fibrous carbon adsorbents were used: W_{w-a}, viscose fibre, carbonized at 1073 K and activated by the steam-gas method for 1 h at 1123 K; W_{w-a+k}, viscose fibre carbonized and activated in the same way as W_{w-a} and impregnated with a catalyst consisting of 6.5% (w/w) Cu(II), 3.1% (w/w) Cr(VI) and 0.035% (w/w) Ag(I); W_{p-a}, fibres obtained from polyacrylonitrile cloths, carbonized at 973 K and activated by the steam-gas method for 1 h at 1073 K; and W_{p-a+k}, polyacrylonitrile fibres carbonized and activated in the same way as W_{p-a} and impregnated with a catalyst consisting of 5.8% (w/w) Cu(II), 2.5% (w/w) Cr(VI) and 0.03% (w/w) Ag(I).

Impregnated carbon fibres (W_{w-a+k}, W_{p-a+k}) were obtained by soaking activated fibres in an aqueous solution of the complex salts of copper, chromium and silver.

Determination of adsorption isotherms by the static method

The adsorption isotherms of benzene vapour on the surfaces of activated and impregnated fibres were determined gravimetrically using a McBain–Bakr adsorption balance [17]. The sensitivity of the quartz spiral was $4.8 \cdot 10^{-3}$ kg m^{-1}. The samples of carbon fibres were degassed in a vacuum drier at 373 K and 20.6 kN m^{-2}. After degassing, the sample was thermostated at 293 K and benzene vapour was introduced.

Determination of adsorption isotherms by the chromatographic method

The chromatographic investigations were carried out by means of a Mera-Elwro 504 (INCO, Poland) gas chromatograph with a thermal conductivity detector. The carbon fibres were placed in glass columns (65 cm × 0.2 cm I.D.). The length of the adsorbent bed in column was 15–18 cm, which corresponded to 0.10–0.15 g depending on the kind of carbon fibre used. The part of the column that was not occupied by the ACF bed was filled with glass beads of mesh size 0.20–0.25 mm [18]. The filled column was mounted in the chromatograph thermostat and heated for 8 h at 453 K in a stream of hydrogen flowing at 40 cm^3 min^{-1}. Benzene and nitrogen were used as adsorbates. Benzene was injected into the column by means of a Hamilton microsyringe. The size of the samples introduced was 45–50 μl. Nitrogen was injected into the column by means of the six-way sampler valve. The size of the sample introduced was 5.732 cm^3. The adsorption isotherms were determined at 293 and 373 K. The temperature of the injection device and of the detector was 473 K. During the measurement at 293 K the columns were placed in a water-bath cooler to thermostat the carbon fibre bed. The fluctuations of the thermostat temperature, as measured by means of Anschütz thermometers, did not exceed 0.2 K. The adsorption isotherms of nitrogen were determined at 77 K. The temperature of the injection device and of the detector was 293 K. In the determination of the isotherms, hydrogen purified and dried over Carbosorbit N active carbon and molecular sieves 5A was used.

The pressures and the corresponding adsorption values were calculated from the following equations:

$$p = \frac{m_a q R T_c h}{S_{peak} v_0} \tag{1}$$

$$a = \frac{m_a S_{ads}}{m S_{peak}} \tag{2}$$

where

m_a = amount of the adsorbate injected (mmol);

m = mass of the adsorbent in the column (g);

v_0 = reduced carrier gas flow-rate (cm^3 min^{-1}):

$$v_0 = \frac{v T_c (p_0 - p_{H_2O})}{T_0 p_0} \cdot \frac{3\left(\frac{p_i}{p_0}\right)^2 - 1}{2\left(\frac{p_i}{p_0}\right)^3 - 1}$$

v = carrier gas flow-rate at the column temperature (cm^3 min^{-1});

q = speed of the recording tape (cm min^{-1});
R = universal gas constant;
T_c = temperature of the column (K);
T_0 = temperature of the environment (K);
p_i = pressure at the column inlet;
p_0 = pressure at the column outlet;
h = height of the peak (cm);
p_{H_2O} = pressure of water vapour at the temperature of the environment;
S_{peak} = total surface area of the peak (cm^2).

When the isotherms were determined from the peak maximum, the S_{ads} value corresponded to the adsorption surface area (in cm^2) of the tested adsorbate sample. When the peak profile method was used, the value S_{ads} was found by dividing the adsorption surface area into n parts.

The peaks obtained were unsymmetrical for all the adsorption systems. This indicates the dominating effect of diffusion on the rate of establishment of adsorption equilibrium. Therefore, when calculating the adsorption isotherms we took into account the effect of diffusion of the adsorbate in the ACF bed on adsorption in accordance with the postulates of Dollimore *et al.* [19].

Determination of specific surface area

The specific surface areas of the investigated ACFs were determined by Nelsen and Eggertsen's method, which is known as the heat desorption method [20]. The column with the carbon fibre bed was immersed in liquid nitrogen. A stream of H_2–N_2 mixture was passed through the column. This mixture, of known composition, was obtained previously by mixing the streams of adsorbate and carrier gas. The amount of nitrogen adsorbed by the adsorbent bed at a given relative pressure was calculated from the desorption peak because it was more symmetrical. Each experiment performed for one concentration of adsorbate in the mixture resulted in one point on the adsorption isotherm.

The partial pressure of the adsorbate was calculated from the equation

$$p = \frac{v_{ads}}{v_p} \cdot p_b \tag{3}$$

where

v_{ads} = volumetric flow-rate of the adsorbate (cm^3 min^{-1});
v_p = volumetric flow-rate of the H_2–N_2 mixture through the carbon fibre bed during measurement (cm^3 min^{-1});
p_b = barometric pressure.

The appropriate values of adsorption for the above pressure were calculated from the expression

$$a = kS_{peak} \cdot \frac{v_p}{v_k} \tag{4}$$

where

k = detector constant determined from a calibration plot [21] [from the function $S_{peak}v_k = \xi(m_a q)$];
S_{peak} = area of the desorption peak (cm^2);
v_k = volumetric flow-rate of the H_2–N_2 mixture during calibration (cm^3 min^{-1}).

Microscope investigation

The investigation of the surface morphology of activated carbon fibres was carried out by scanning electron microscopy (SEM) with a Tesla BS-300 microscope. These were only qualitative measurements.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the typical surface morphology of ACFs obtained from viscose and impregnated with catalysts, respectively. SEM of these ACFs did not reveal any substantial morphological changes of the fibre surface caused by the activation process. This suggests that activation causes changes mainly in the internal structure [22]. In Fig. 2 there are bright and dark spots which depend on the hue of the base. They probably represent the catalyst distributed on the surface of the ACF. They should disappear with a uniform concentration of the catalyst on the surface of the carbon fibre.

In Figs. 3 and 4, the adsorption isotherms for benzene vapour on the surface of the ACFs used, determined at 293 K by the static method, are compared with those found chromatographically at 293 and 373 K. It can be concluded that the chromatographic measurements at 293 K yield lower adsorption values than those obtained by the static method. The adsorption isotherms of nitrogen determined chromatographically at 77 K are shown in Fig. 5.

In order to characterize better the ACFs used, the constants of the two-term Dubinin–Radushkevich

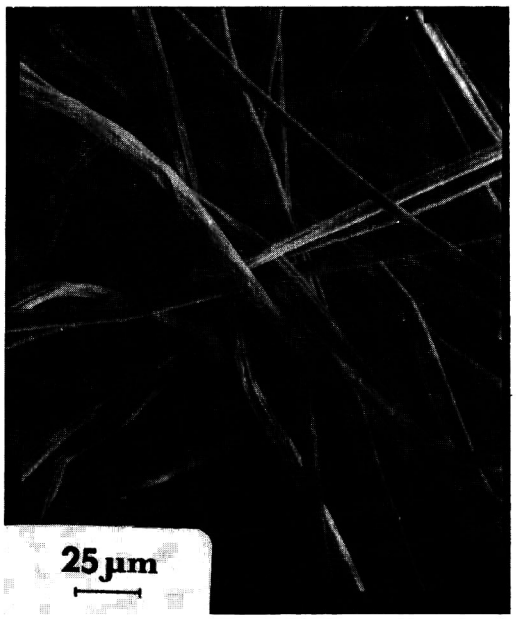


Fig. 1. Microphotograph of activated carbon fibres obtained from viscose.

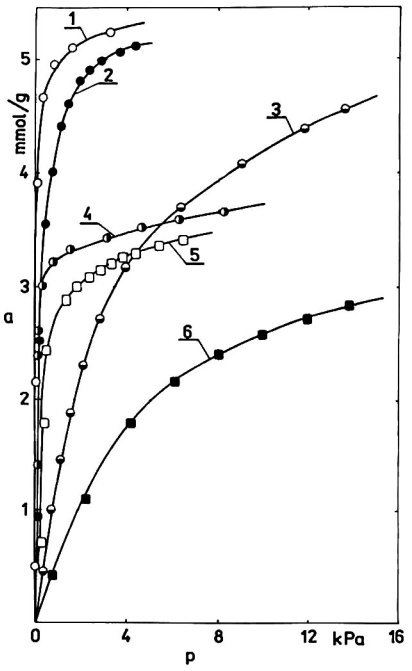


Fig. 3. Adsorption isotherms of benzene determined by the static method at 293 K (1, 4) and by the chromatographic method at 293 K (2, 5) and at 373 K (3, 6) on the ACFs obtained from viscose cloths.

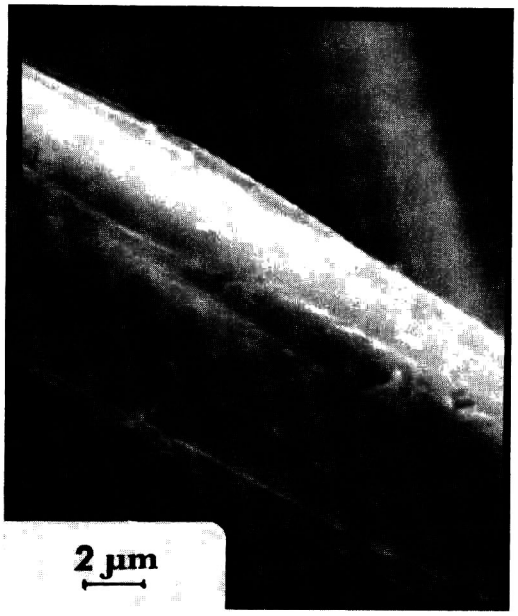


Fig. 2. Microphotograph of carbon fibres obtained from viscose, activated and impregnated with catalysts.

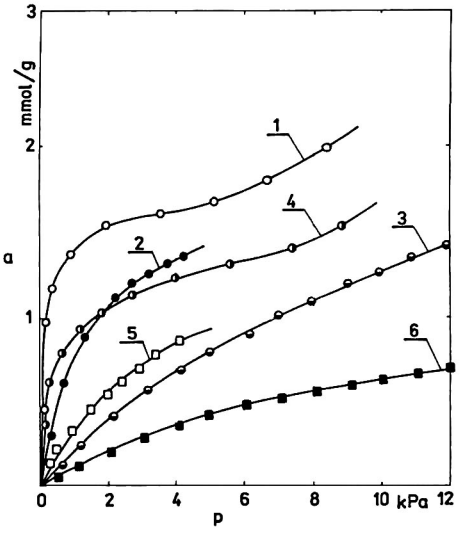


Fig. 4. Adsorption isotherms of benzene determined by the static method at 293 K (1, 4) and by the chromatographic method at 293 K (2, 5) and at 373 K (3, 6) on the ACFs obtained from polyacrylonitrile cloths.

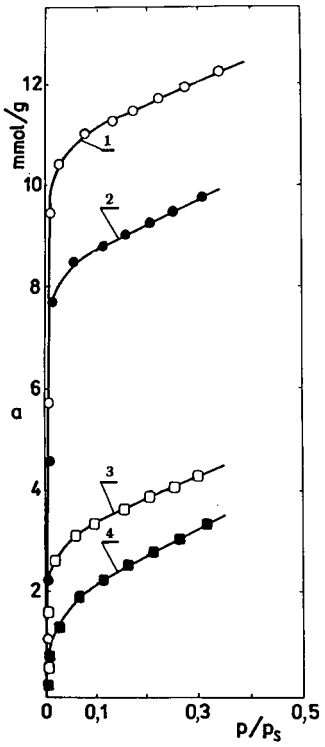


Fig. 5. Adsorption isotherms of nitrogen determined by the chromatographic method at 77 K on the ACFs: (1) W_{w-a}; (2) W_{w-a+k}; (3) W_{p-a}; (4) W_{p-a+k}.

(DR) equation were calculated from the benzene and nitrogen isotherms. The adsorption isotherms which were determined statically and chromatographically at 293 and 373 K are shown using linear coordinates of the DR equation (also called the characteristic adsorption equation [23]):

$$\ln W = \ln W_0 - \left(\frac{1}{\beta E_0}\right)^2 A^2 \qquad (5)$$

where

W = volume of the adsorption sphere of micropores ($cm^3\ g^{-1}$);
W_0 = limiting volume of the adsorption sphere of micropores ($cm^3\ g^{-1}$);
β = affinity coefficient;
A = differential molar work of adsorption (kJ mol^{-1});
E_0 = characteristic adsorption energy (kJ mol^{-1}).

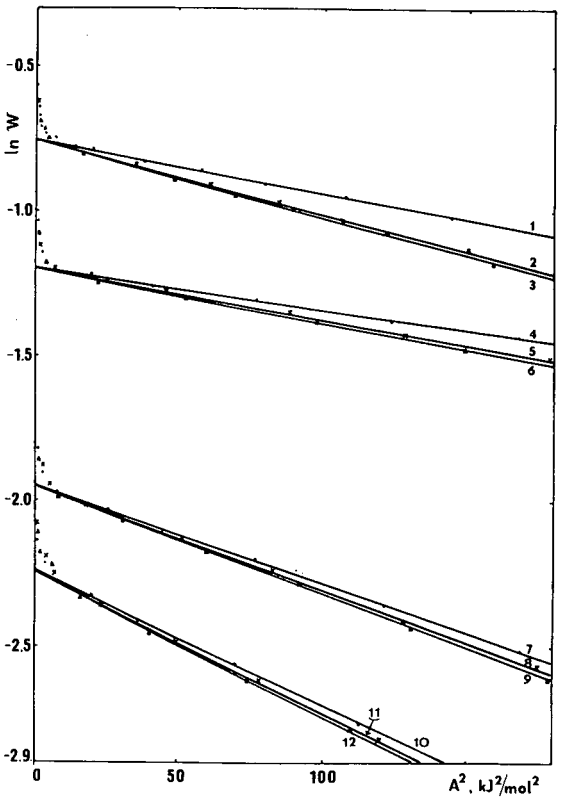


Fig. 6. Changes in the characteristic curves of the DR equation calculated from the adsorption isotherms of benzene determined statically at 293 K (1, 4, 7, 10) and chromatographically at 293 K (3, 6, 9, 12) and at 373 K (2, 5, 8, 11) on the surfaces (1, 2, 3) W_{w-a}, (4, 5, 6) W_{w-a+k}, (7, 8, 9) W_{p-a} and (10, 11, 12) W_{p-a+k} for the first (I) structure of pores.

A graph for the first structure of micropores is shown in Fig. 6 and that for the second structure in Fig. 7. The results are also given in Tables I and II. For every adsorption system the characteristic curves calculated from statically determined adsorption isotherms of benzene are above those calculated from the chromatographically determined adsorption isotherms. The characteristic curves calculated from adsorption isotherm of benzene at 293 K, determined chromatographically, are below those calculated from the adsorption isotherms determined chromatographically at 373 K.

It is possible to explain the temperature dependence of the characteristic curves when it is taken into account that part of the adsorption sphere is inaccessible to the adsorbate molecules. Dubinin *et*

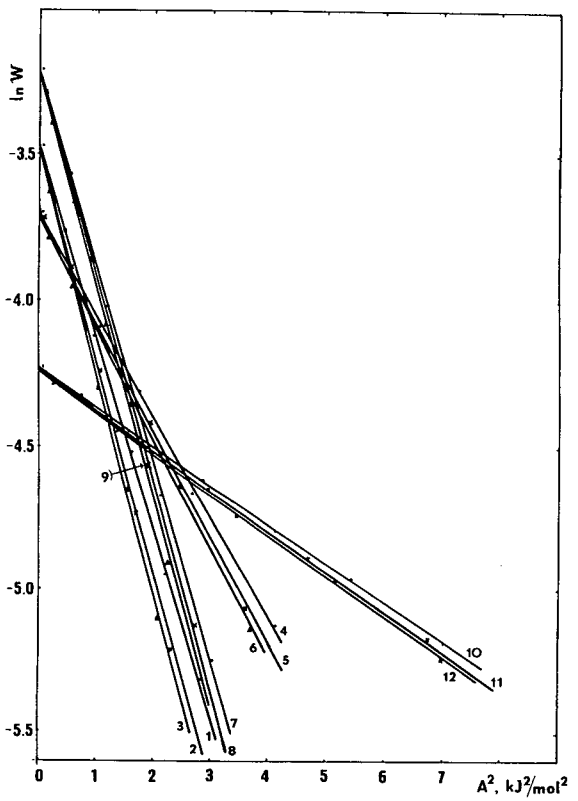


Fig. 7. Changes in the characteristic curves of the DR equation at 293 and 373 K for benzene on the ACFs for the second (II) structure of pores. Notation as in Fig. 6.

al. [24,25] suggested that this effect increases with increase in the linear dimensions of the adsorbate molecules and with decrease in the geometric dimensions of the entrances into the micropores. This effect was observed during static measurements. In adsorption from the stream of the adsorbate–carrier gas mixture flowing through the ACF bed in the column, this effect will probably increase. The diffusion coefficient increases with increase in temperature. Therefore, at higher temperatures the ACF bed in the column is better penetrated by the adsorbate molecules from the stream of the adsorbate–carrier gas mixture and therefore the values of W_0 are higher at higher temperatures.

The limiting volumes of the adsorption sphere for the first (I) and second (II) structures of pores calculated from adsorption isotherms determined statically are higher than the values calculated from chromatographic measurements.

From the results of the measurements presented in Figs. 3–5 and the numerical data in Tables I and II, we can say that the investigated ACFs are characterized by a highly developed structure of micropores, low values of W_{02} and high values of the structure–energy constants B_2. Deposition of the chromium–copper–silver catalyst on the surface of carbon fibres causes a decrease in the physical adsorption capacity of the ACFs.

The coefficients of the linear regression of the DR equation indicate that the linearity of this equation is better fulfilled for chromatographic measurements (for benzene as the adsorbate) at 373 K than 293 K.

The linearity for the second (II) pore structure for every adsorption system is worse than that for the first (I) pore structure. The values of the limiting volume of the adsorption sphere of micropores calculated from the DR equation were compared with the values of the volume of the monolayer W_m calculated from the BET equation [26]:

$$\frac{1}{(1-h)W} = \frac{1}{W_m c} + \frac{c-1}{W_m c} \cdot h \tag{6}$$

where

$W_m = a_m v$ = volume of monolayer ($cm^3\ g^{-1}$);
v = molar volume of adsorbate at the measurement temperature ($cm^3\ mmol^{-1}$);
c = constant of the BET equation;
h = relative pressure of adsorbate ($5 \cdot 10^{-3} < h < 0.1$).

Dubinin [26] suggested that the value of W_m calculated from the BET equation for the range of relative pressure of adsorbate $5 \cdot 10^{-3} < h < 0.1$ more accurately describes the structure of a monolayer compared with the value W_m calculated for the range $0.05 < h < 0.35$ usually used by other workers.

The constants of the BET equation (W_m and c) and the specific surface areas calculated for the investigated ACFs are given in Tables III and IV. In Table IV results of measurements of specific surface areas determined by the heat desorption method are also presented.

The specific surface areas were calculated from the following equation:

$$S\ (m^2\ g^{-1}) = 602.3 \cdot \frac{\omega}{v} \cdot W_m \tag{7}$$

where

TABLE I

PARAMETERS OF THE TWO-TERM DUBININ–RADUSHKEVICH EQUATION CALCULATED FROM ADSORPTION ISOTHERMS OF BENZENE DETERMINED STATICALLY (Stat.) AT 293 K AND CHROMATOGRAPHICALLY (Chrom.) AT 293 AND 373 K

Carbon fibre	T (K)	W_{01} (cm^3 g^{-1})		E_{01} (kJ mol^{-1})		$B_1 \cdot 10^6$ (K^{-2})		r^a	
		Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.
W_{w-a}	293	0.4225	0.3893	21.06	19.06	0.83	1.01	0.9993	0.9924
W_{w-a}	373	–	0.4001	–	19.60	–	0.94	–	0.9932
W_{w-a+k}	293	0.2869	0.2796	24.84	23.46	0.59	0.67	0.9977	0.9912
W_{w-a+k}	373	–	0.2814	–	23.82	–	0.65	–	0.9959
W_{p-a}	293	0.1160	0.0981	16.49	16.13	1.35	1.41	0.9968	0.9913
W_{p-a}	373	–	0.1022	–	16.36	–	1.37	–	0.9992
W_{p-a+k}	293	0.067	0.048	15.48	15.00	1.53	1.63	0.9991	0.9889
W_{p-a+k}	373	–	0.054	–	15.42	–	1.54	–	0.9975
		W_{02} (cm^3 g^{-1})		E_{02} (kJ mol^{-1})		$B_2 \cdot 10^6$ (K^{-2})		r^a	
		Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.
W_{w-a}	293	0.014	0.010	1.25	1.21	235.9	250.5	0.9910	0.9898
W_{w-a}	373	–	0.012	–	1.23	–	241.4	–	0.9910
W_{w-a+k}	293	0.013	0.010	1.88	1.65	104.0	134.7	0.9903	0.9885
W_{w-a+k}	373	–	0.012	–	1.69	–	128.5	–	0.9921
W_{p-a}	293	0.020	0.019	1.23	1.20	245.2	254.7	0.9915	0.9899
W_{p-a}	373	–	0.020	–	1.22	246.4	–	0.9930	
W_{p-a+k}	293	0.008	0.007	2.83	2.66	45.9	51.8	0.9902	0.9894
W_{p-a+k}	373	–	0.008	–	2.69	–	50.6	–	0.9920

[a] r = Linear regression coefficient.

ω = surface area occupied by one adsorbate molecule in the monolayer (nm^2) (ω = 0.162 for nitrogen and 0.410 for benzene);

v = molar volume of adsorbate at the measurement temperature by the method proposed by Nikolajev and Dubinin [27] (cm^3 mmol^{-1}) (for nitrogen at 77 K v = 0.0346 and for benzene at 293 K v = 0.0889 and at 373 K v = 0.1053).

The widening of the theory for the volume filling

TABLE II

PARAMETERS OF THE TWO-TERM DUBININ–RADUSHKEVICH EQUATION CALCULATED FROM ADSORPTION ISOTHERMS OF NITROGEN DETERMINED CHROMATOGRAPHICALLY AT 77 K

Carbon fibre	W_{01} (cm^3 g^{-1})	E_{01} (kJ mol^{-1})	$B_1 \cdot 10^6$ (K^{-2})	r	W_{02} (cm^3 g^{-1})	E_{02} (kJ mol^{-1})	$B_2 \cdot 10^6$ (K^{-2})	r
W_{w-a}	0.3988	19.25	0.99	0.9988	0.1213	2.44	61.7	0.9895
W_{w-a+k}	0.2992	19.14	1.00	0.9953	0.098	2.38	64.6	0.9912
W_{p-a}	0.1185	11.96	2.56	0.9899	0.068	2.44	61.6	0.9907
W_{p-a+k}	0.069	9.94	3.71	0.9912	0.022	2.94	42.3	0.9908

TABLE III

PARAMETERS OF THE BET EQUATION AND THE SPECIFIC SURFACE AREAS OF THE ACFs CALCULATED FROM ADSORPTION ISOTHERMS OF BENZENE AT 293 AND 373 K

Carbon fibre	T (K)	c		W_m (cm^3 g^{-1})		r		S_{BET} (m^2 g^{-1})	
		Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.
W_{w-a}	293	169.4	172.2	0.3586	0.3618	0.9985	0.9914	995.6	1004.5
W_{w-a}	373	–	73.6	–	0.3612	–	0.9986	–	846.7
W_{w-a+k}	293	86.6	71.6	0.2786	0.2862	0.9998	0.9944	773.5	794.6
W_{w-a+k}	373	–	60.2	–	0.2876	–	0.9995	–	674.1
W_{p-a}	293	49.3	10.4	0.1125	0.1114	0.9990	0.9896	312.4	309.3
W_{p-a}	373	–	9.2	–	0.1120	–	0.9996	–	263.3
W_{p-a+k}	293	12.2	6.2	0.0673	0.0648	0.9966	0.9910	186.9	179.9
W_{p-a+k}	373	–	6.6	–	0.0619	–	0.9996	–	147.4

of micropores on adsorbents with a non-homogeneous micropore structure [28] creates new possibilities for describing the porosity of ACFs. Assuming the slit-like model of micropores, it is possible to describe the volume distribution of micropores as a function of half-width, x_0, in the slit-like model:

$$x_0 = k_0/E_0 \tag{8}$$

where k_0 is a parameter depending on E_0, being described by the experimental equation

$$k_0 = 13.028 - 1.53 \cdot 10^{-5} E_0^{3.5}$$

For each increase in the elementary volume of the micropores, dW is responsible for the increase in the elementary geometric area of their walls, dS_g:

$$dS_g = \frac{dW}{x_0} \cdot 10^3 \tag{9}$$

Introducing additional assumptions, Dubinin simplified eqn. 9 to a simple mathematical expression which makes it possible to calculate the geometric surface area of micropore walls. For the two-term DR equation its form is as follows:

$$S_g \text{ (m}^2 \text{ g}^{-1}) = \left(\frac{W_{01}}{x_{01}} + \frac{W_{02}}{x_{02}} \right) \cdot 10^3 \tag{10}$$

The parameters calculated according to eqns. 8 and 10 are given in Tables V and VI. The values of the specific surface area calculated from the adsorption isotherms of benzene and nitrogen determined statically and chromatographically differ from the values of the surface area of the walls of micropores calculated from these isotherms.

Bering *et al.* [29] stated that the concept of the specific surface area in the case of materials with a

TABLE IV

THE PARAMETERS OF THE BET EQUATION AND SPECIFIC SURFACE AREAS OF THE ACFs CALCULATED FROM ADSORPTION ISOTHERMS OF NITROGEN AT 77 K (S_{BET}) AND DETERMINED BY THE HEAT DESORPTION METHOD (S_d)

Carbon fibre	c	W_m (cm^3 g^{-1})	r	S_{BET} (m^2 g^{-1})	S_d (m^2 g^{-1})
W_{w-a}	353.5	0.3557	0.9976	1002.6	998.6
W_{w-a+k}	244.8	0.2810	0.9985	792.0	785.7
W_{p-a}	212.9	0.1087	0.9981	306.4	305.3
W_{p-a+k}	89.1	0.0698	0.9991	196.7	191.8

TABLE V

GEOMETRIC SURFACE AREAS OF THE WALLS OF PORES AND HALF-WIDTHS OF THEIR SLITS CALCULATED FROM ADSORPTION ISOTHERMS OF BENZENE DETERMINED STATICALLY AND CHROMATOGRAPHICALLY AT 293 AND 373 K ON USED ACFs

Carbon fibre	T (K)	x_{01} (nm)		S_{g1} ($m^2\ g^{-1}$)		x_{02} (nm)		S_{g2} ($m^2\ g^{-1}$)		S_g ($m^2\ g^{-1}$)	
		Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.	Stat.	Chrom.
W_{w-a}	293	0.618	0.682	684.5	570.4	10.400	10.743	1.3	0.9	685.8	571.3
	373		0.658		607.9		10.569		1.1		609.0
W_{w-a+k}	293	0.521	0.556	550.9	504.0	6.915	7.879	1.9	1.3	552.8	505.3
	373		0.547		513.7		7.692		1.6		515.3
W_{p-a}	293	0.789	0.806	147.0	121.6	10.656	10.833	1.9	1.8	148.9	123.4
	373		0.795		129.3		10.656		1.9		130.2
W_{p-a+k}	293	0.840	0.867	79.8	55.4	4.594	4.887	1.7	1.4	81.5	56.8
	373		0.842		64.1		4.833		1.7		65.8

highly developed pore structure has no physical meaning, because no independent method for determining this area is known. However, if it is assumed that the BET surface area characterizes well the range of the micropores, mesopores and macropores in the case of carbon materials, this parameter can be used to describe the structure of the ACFs.

In Figs. 8 and 9, the values of the pure heats of adsorption of benzene and nitrogen, calculated by the method suggested by Bering and Serpinsky [30–32], from chromatographic measurements are compared with the pure heats of adsorption calculated from static measurements. For all the adsorption systems used, the pure heats of adsorption calculated from chromatographic measurements are lower than those calculated from static measurements. The greatest difference occurs in the initial range of coverages. An increase in temperature of 90 K causes a decrease in the pure heat of adsorption of *ca.* 12 kJ mol^{-1} for viscose-based ACFs and *ca.* 14 kJ mol^{-1} for polyacrylonitrile-based ACFs. Higher values for the pure heat of adsorption for benzene and nitrogen were obtained for viscose-based than for polyacrylonitrile-based ACFs.

Fig. 10 shows the variation of the molar differential entropy of adsorption of the adsorbates on viscose-based ACFs. Every adsorption system was characterized by a negative entropy.

CONCLUSIONS

The chromatographic method may be used successfully to characterize the adsorption properties of the ACFs. The fundamental advantages of the chromatographic method are speed, high reproduci-

TABLE VI

GEOMETRIC SURFACE AREAS OF THE WALLS OF PORES AND HALF-WIDTHS OF THEIR SLITS CALCULATED FROM ADSORPTION ISOTHERMS OF NITROGEN DETERMINED CHROMATOGRAPHICALLY AT 77 K ON USED ACFs

Carbon fibre	x_{01} (nm)	S_{g1} ($m^2\ g^{-1}$)	x_{02} (nm)	S_{g2} ($m^2\ g^{-1}$)	S_g ($m^2\ g^{-1}$)
W_{w-a}	0.675	591.1	5.328	22.7	613.8
W_{w-a+k}	0.679	440.4	5.462	17.9	458.3
W_{p-a}	1.087	109.5	5.328	12.8	122.3
W_{p-a+k}	1.308	52.8	4.422	5.0	57.8

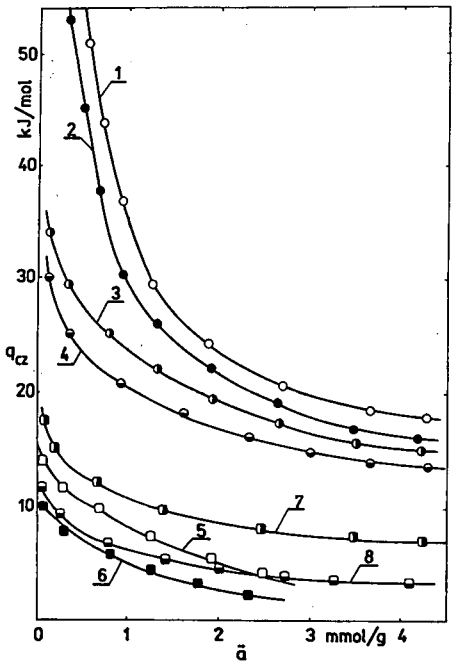


Fig. 8. Changes in the pure heats of adsorption on the activated (1, 3, 5, 7) and impregnated (2, 4, 6, 8) ACFs obtained from viscose cloths calculated by the BS method from adsorption isotherms of benzene (1, 2, 3, 4, 5, 6) and nitrogen (7, 8) determined statically at 293 K (1, 2) and chromatographically at 77 K (7, 8), 293 K (3, 4) and 373 K (5, 6).

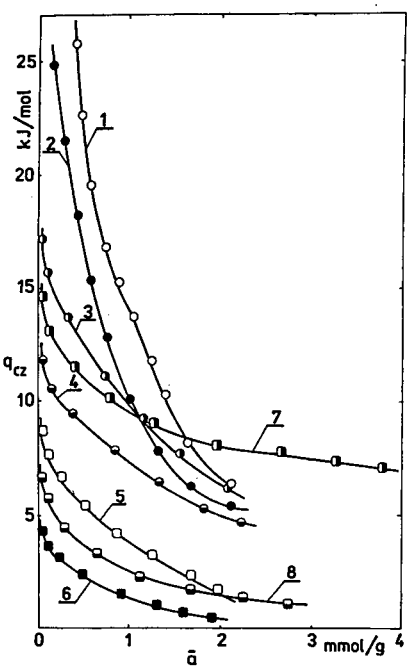


Fig. 9. Changes in the pure heats of adsorption on the activated (1, 3, 5, 7) and impregnated (2, 4, 6, 8) ACFs obtained from polyacrylonitrile cloths calculated by the BS method from adsorption isotherms of benzene (1, 2, 3, 4, 5, 6) and nitrogen (7, 8) determined statically at 293 K (1, 2) and chromatographically at 77 K (7, 8), 293 K (3, 4) and 373 K (5, 6).

bility of the adsorption systems and the possibility of investigating the adsorption phenomena in the initial range of coverges.

The isotherms obtained for all the systems studied are convex with respect to the pressure axis, indicating that the adsorbate–adsorbent interactions have a dominant effect on the initial progress of adsorption of the adsorbates.

The observed differences in the adsorption values and the pure heats of adsorption found from static and dynamic measurements may be due to (i) blocking of the active adsorption sites on the adsorbent surface by the carrier gas molecules and (ii) the so-called molecular sieve effect connected with the similarity of the dimensions of the adsorbate molecules and the entrances to the pores, which results in the whole adsorption volume of the micropores not being filled with the adsorbate in the chromatographic process; W_0 values calculated from the chromatographic measurements are higher at higher temperatures.

The viscose ACFs are characterized by a higher ability to adsorb benzene vapor and nitrogen compared with polyacrylonitrile-based ACFs.

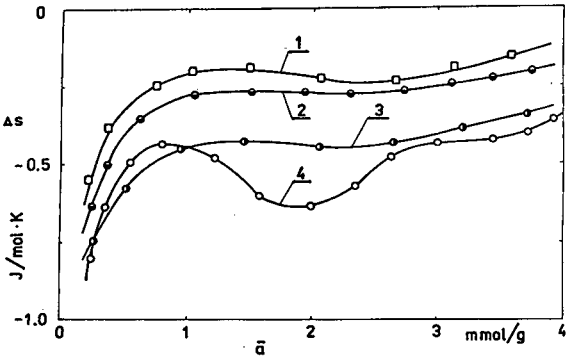


Fig. 10. Changes in the molar differential entropy of adsorption of nitrogen (1) and benzene (2, 3, 4) calculated by the BS method from adsorption isotherms, determined statically at 293 K (4) and chromatographically at 77 K (1), 293 K (3) and 373 K (4) on the surface of W_{w-a}.

The values of the specific surface areas of ACFs calculated by the BET method from the adsorption isotherm of benzene determined statically and chromatographically at 293 K are similar to the results obtained by the heat desorption method.

Taking into account the contribution of micropores to the general porosity of ACFs, it can be concluded that there is a similarity between ACFs and activated carbons of good quality.

ACKNOWLEDGEMENT

This work was kindly supported by the Polish State Committee for Scientific Research, Grant No. 3035 39101.

REFERENCES

1 G. N. Arons, R. N. Macnair, L. G. Coffin and H. D. Hogan, *Text. Res. J.*, 44 (1974) 874.
2 R. N. Macnair and G. N. Gilbert, in P. N. Cheremisinoff and F. Ellerbusch (Editors), *Carbon Adsorption Handbook*, Ann Arbor Sci. Publ., Ann Arbor, MI, 1978, p. 819.
3 A. V. Kiselev and Ya. I. Yashin, *Adsorpcyjna Chromatografia Gazowa*, PWN, Warsaw, 1969.
4 G. Deininger, J. Asshauer and I. Halasz, *Chromatographia*, 8 (1975) 143.
5 M. Domingo-García, F. J. Lopez-Garzon, R. Lopez-Garzon and C. Moreno-Castilla, *J. Chromatogr.*, 324 (1985) 19.
6 M. Rozwadowski, J. Siedlewski and R. Wojsz, *Chem. Stosow.*, 24 (1980) 535.
7 R. Wojsz, *Pol. J. Chem.*, 63 (1989) 223.
8 W. R. Betz and W. R. Supina, *J. Chromatogr.*, 471 (1989) 105.
9 P. J. M. Carrott and K. S. W. Sing, in *Proceedings of the 4th International Carbon Conference—CARBON 86, Baden-Baden*, 1986, p. 283.
10 A. J. Vukov and D. G. Gray, in *Proceedings of the 4th International Carbon Conference—CARBON 86, Baden-Baden*, 1986, p. 394.
11 P. J. C. Chappell and D. R. Williams, *J. Colloid Interface Sci.*, 128 (1989) 450.
12 M. Nardin, H. Balard and E. Papirer, *Carbon*, 28 (1990) 43.
13 U.-B. Mohlin and D. G. Gray, *J. Colloid Interface Sci.*, 47 (1974) 747.
14 P. R. Teremaine and D. G. Gray, *J. Chem. Soc., Faraday Trans. 1*, 71 (1975) 2170.
15 G. M. Dorris and D. G. Gray, *J. Colloid Interface Sci.*, 71 (1979) 93.
16 S. Katz and D. G. Gray, *J. Colloid Interface Sci.*, 82 (1981) 318, 326 and 339.
17 W. J. McBain and A. M. Bakr, *J. Am. Chem. Soc.*, 48 (1926) 690.
18 J. Volf, J. Koubek and J. Paszek, *J. Chromatogr.*, 81 (1973) 9.
19 D. Dollimore, G. R. Heal and D. R. Martin, *J. Chromatogr.*, 50 (1970) 209.
20 F. M. Nelsen and F. T. Eggertsen, *Anal. Chem.*, 30 (1958) 1387.
21 T. Paryjczak, *Gas Chromatography in Adsorption and Catalysis*, Polish Scientific Publishers, Warsaw and Ellis Horwood, Chichester, 1986.
22 J. B. Donnet and R. Ch. Bansal, *Carbon Fibers*, Marcel Dekker, New York, 1984.
23 M. M. Dubinin, *Carbon*, 27 (1989) 457.
24 M. M. Dubinin, K. M. Nikolajev, N. S. Poljakov and N. I. Seregina, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1970) 761.
25 M. M. Dubinin, K. M. Nikolajev, N. S. Poljakov and N. I. Seregina, *Izv. Akad. Nauk. SSSR, Ser. Khim.*, (1971) 1871.
26 M. M. Dubinin, *Carbon*, 21 (1983) 359.
27 K. M. Nikolajev and M. M. Dubinin, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1958) 1165.
28 M. M. Dubinin, *Carbon*, 19 (1981) 321.
29 V. R. Bering, M. M. Dubinin and V. V. Serpinsky, *J. Colloid Interface Sci.*, 38 (1972) 185.
30 V. R. Bering and V. V. Serpinsky, *Dokl. Akad. Nauk SSSR*, 114 (1957) 1244.
31 V. R. Bering and V. V. Serpinsky, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1957) 125.
32 V. R. Bering and V. V. Serpinsky, *Dokl. Akad. Nauk SSSR*, 148 (1963) 1331.

Journal of Chromatography, 600 (1992) 79–81
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 002

Ion-exchange high-performance liquid chromatography of diastereoisomers of some phosphonodipeptides

S. V. Galushko*, M. Y. Belik, V. A. Solodenko, T. N. Kasheva and V. P. Kukhar

Institute of Bioorganic Chemistry and Oil Chemistry, Academy of Sciences of the Ukraine, 253660 Kiev 94 (Ukraine)

ABSTRACT

The chromatographic behaviour of L-L,D-peptides Val–AlaP, Leu–PheP, Ala–AlaP, Phe–LeuP, Leu–PheP(O-iso-C_3H_7)$_2$ and Leu–Phe on aminopropyl and sulphonyl sorbents was studied. An increase in the H_3PO_4 concentration in the eluent resulted in a decrease in the retention but hardly affected the selectivity of the separation of the phosphonopeptides on an aminopropyl sorbent. The retention and selectivity of the separation of the phosphonopeptides on a sulphophenyl sorbent decreased with increasing H_3PO_4 concentration in the eluent. The optimum conditions for the separation of the diastereoisomers were found.

INTRODUCTION

Aminophosphonic acids[a] and phosphonopeptides (PPepts) are a promising class of bioactive compounds [1]. The separation of diastereoisomers of phosphonodipeptides (PDs) that differ in the configuration of the α-carbon atom of the aminophosphonic acid residues by normal and reversed-phase (RP) high-performance liquid chromatography (HPLC) was described in a previous paper [2]. It should be noted that the separation of hydrophilic and poorly retained PDs by RP-HPLC is sometimes difficult. Hence it seemed expedient to use ion-exchange HPLC (HPIEC) for this purpose. Only a few data concerning the separation of PDs by IEC have been reported previously. The separation of diethyl esters of 1-(N-L-alanylamino)benzylphosphonic acid isomers by IEC has been described [3].

The aim of this work was to determine the optimum conditions for the HPIEC of PD on aminopropyl and sulphophenyl sorbents.

[a] Generally accepted abbreviations for α-aminophosphonic acids were used, *e.g.*, L,D-1-(N-L-alanylamino)ethylphosphonic acid = L-Ala-L,D-AlaP.

EXPERIMENTAL

Chromatographic conditions

The experiments were performed on an LKB (Bromma, Sweden) liquid chromatographic system consisting of a Model 2150 HPLC pump, a Model 7410 injector, a Model 2140 rapid spectral detector set at 225 nm and a Model 220 recording intergrator. The column used were (1) Separon SIX NH_2, 5 μm (150 × 3.3 mm I.D.) from Tessek (Prague, Czechoslovakia) and (2) Nucleosil 100 5-SA, 5 μm (200 × 4.0 mm I.D.) from Macherey–Nagel, (Düren, Germany). The mobile phases were methanol (0–5%)–orthophosphoric acid (0.1 m*M*–0.5 *M*) with isocratic elution. The flow-rate was (1) 0.25 or (2) 0.4 ml/min.

Materials

L-L,D-Dipeptides Val–AlaP (**1**), Leu–PheP (**2**), Ala–AlaP (**3**), Phe–LeuP (**4**), Leu–PheP(O-iso-C_3H_7)$_2$ (**5**) and Leu–Phe (**6**) were obtained as described [4,5]. Orthophosphoric acid and methanol were used as received. Water was doubly distilled and filtered for HPLC use.

RESULTS AND DISCUSSION

When PPepts are chromatographed on an aminopropyl sorbent with an eluent containing acids a mode of anion-exchange chromatography is realized. An increase in the phosphoric acid concentration in the mobile phase results in a decrease in the PPept retention but has virtually no effect on the selectivity of separation of diastereoisomers (Fig. 1). The retentions of the PDs (**1**–**4**) increase in the order **3** > **1** > **2** > **4**. Hence we can conclude that under the conditions used hydrophobic interactions between PPepts and alkyl fragments of the sorbent have a moderate effect on their retention. Both changes in the amino acid sequence (compounds **2** and **4** and the volume of the substituent in the α-position in the aminocarboxylic acid residue (compounds **1** and **3** exert a weak effect on the retention and selectivity. The selectivity of PDs separation ranges from 1.2 to 1.6 depending on their structures (Fig. 1). The L–L isomers have lower retentions. Eluents containing 0.1–10 m*M* orthophosphoric acid and 5% methanol are the optimum for the separation of PDs on an aminopropyl sorbent (Fig. 2). In the absence of methanol in the eluent the efficiency of separation of the PDs decreases.

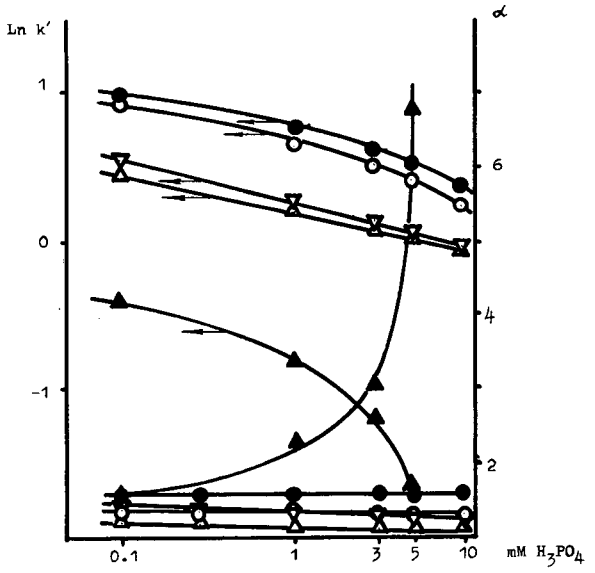


Fig. 1. Effect of orthophosphoric acid concentration on logarithm of the capacity factors (ln *k'*) of L–D isomers and the selectivity (α) of diastereoisomer separation. Column, Separon SIX NH_2, 5 μm (150 × 3.3 mm I.D.); eluent, H_3PO_4–methanol (95:5); flow-rate, 0.25 ml/min. Compounds: ● = L-Phe–D-LeuP (**4**); ○ = L-Leu–D-PheP (**2**); ▽ = L-Val–D-AlaP (**1**); △ = L-Ala–D-AlaP (**3**); ▲ = L-Leu–D-Phe (**6**).

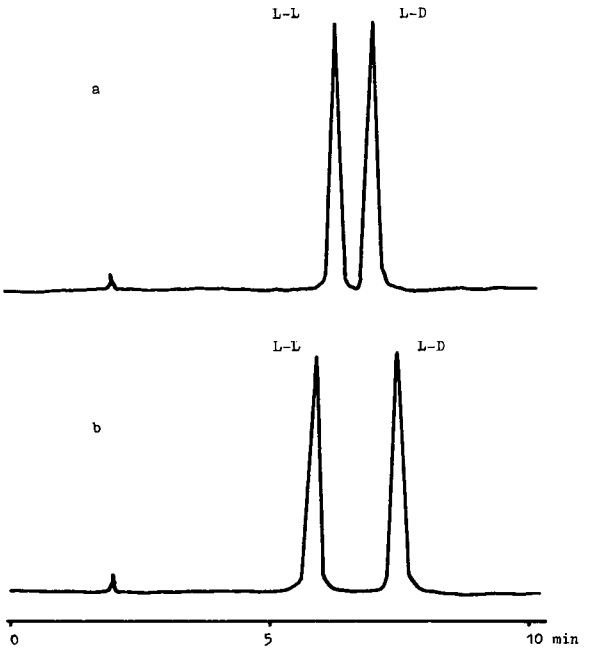


Fig. 2. Chromatograms of the separation of PPepts diastereomers. Column, Separon SIX NH_2, 5 μm (150 × 3.3 mm I.D.); eluent, 0.1 m*M* H_3PO_4–methanol (95:5); flow-rate, 0.25 ml/min. (a) L-Ala–L,D-AlaP (**3**); (b) L-Val–L,D-AlaP (**1**).

It is interesting to determine the effect of replacement of a carboxylic group by a phosphonic group on the chromatographic behaviour of peptides. For an aminopropyl sorbent the effect of the concentration of orthophosphoric acid in the eluent on the retention and selectivity of separation of the diastereoisomers of the carboxylic peptide **6** differs greatly from that for the phosphono analogues **2** (Fig. 1). The sharp increase in the selectivity of separation of diastereoisomer with increase in the acid concentration in the eluent can be explained by the difference in the p*K* values of carboxylic groups of L–L and L–D peptides. It has been shown that under conditions of RP-HPLC the selectivity of separation of diastereoisomers changes appreciably just within the range of pH values of the eluent where the peptides are converted from the ionized to the molecular form [4].

The retention of PPepts **2** and **4** on the phenyl-

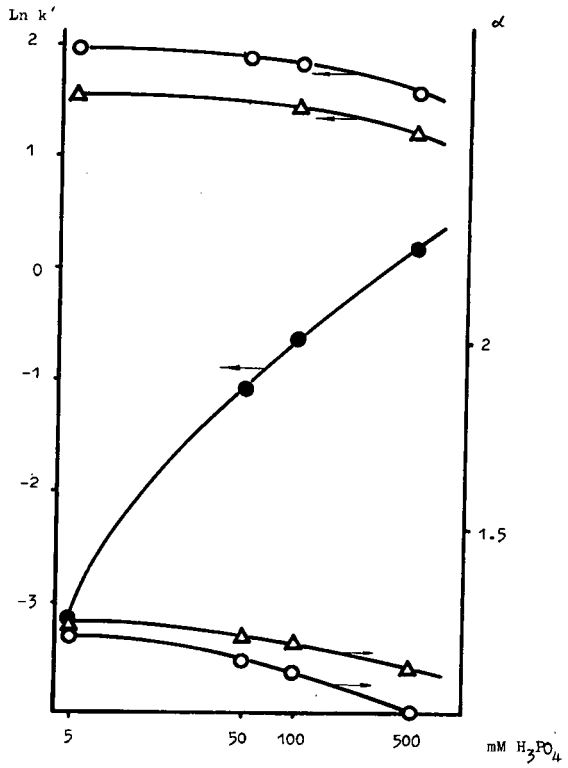


Fig. 3. Effect of orthophosphoric acid concentration on logarithm of capacity factors (ln k') of L–D isomers and the selectivity (α) of diastereoisomer separation. Column, Nucleosil 100 5-SA, 5 μm (200 × 4.0 mm I.D.); flow-rate, 0.40 ml/min. Compounds: ○ = L-Val–D-AlaP (**1**); △ = L-Ala–D-AlaP (**3**); ● = L-Leu–D-PheP(O-iso-C_3H_7)$_2$ (**5**).

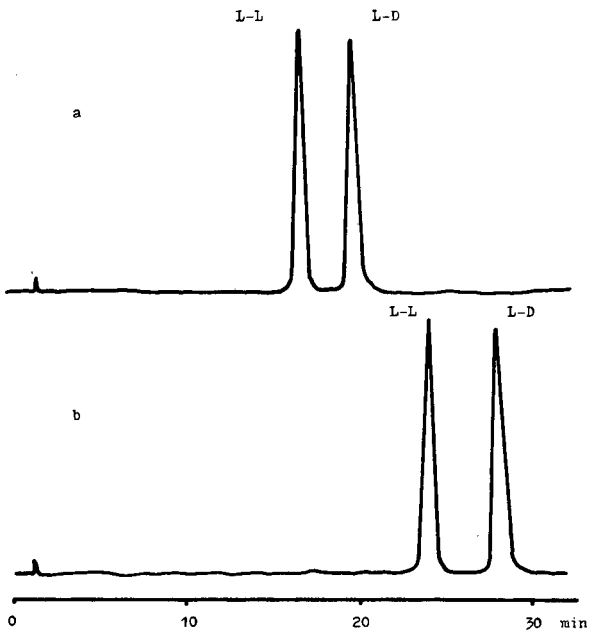


Fig. 4. Chromatograms of the separation of PP diastereomers. Column, Nucleosil 100 5-SA, 5 μm (200 × 4.0 mm I.D.); eluent, 0.1 mM H_3PO_4–methanol (95:5); flow-rate, 0.40 ml/min. (a) L-Ala–L,D-AlaP (**3**); (b) L-Val–L,D-AlaP (**1**).

sulphonate sorbent under conditions of cation-exchange chromatography is higher and the eluents with higher ionic strength should be used in this instance. A mobile phase containing 0.1 M potassium sulphate is the optimum for the separation of these PDs. Neither the retention nor the selectivity of the separation of these PDs is significantly influenced by the concentration of the phosphoric acid over the range 0.1–50 mM.

The selectivity and retention of the more hygrophilic PDs **1** and **3** decrease with increasing orthophosphoric acid content in the mobile phase (Fig. 3). Diastereoisomers of these PPepts were separated on column 2 by using an eluent containing 0.05–0.1 M orthophosphoric acid (Fig. 4).

Our attempts to separate diastereoisomers of the protected PPept **5** under conditions of cation-exchange chromatography on a sulphophenyl sorbent were unsuccesful. It can be assumed that the two hydrophobic isopropyl protecting groups of the PPept interact with aryl fragments of the sorbent. In this instance a fragment of the molecule with an asymmetric carbon atom is located in the mobile phase and it does not interact with the surface of the sorbent or contribute to the selectivity of separation. It is worth noting that the retention of protected peptides, in contrast to that of unprotected peptides, is considerably influenced by the concentration of phosphoric acid in the eluent (Fig. 3).

The results obtained permit optimum conditions and a sorbent for the complete separation of phosphonopeptide diastereoisomers to be chosen.

REFERENCES

1 P. Mastalerz, L. Kupczyk-Subotkowska, Z. S. Nerman and G. Laskawiec, *Naturwissenschaften*, 69 (1982) 46.
2 S. V. Galushko, M. Y. Belik, V. A. Solodenko, T. N. Kasheva and V. P: Kukhar, *J. Chromatogr.*, 553 (1991) 143.
3 Y. P. Belov, V. A. Davankov, V. A. Tsiryapkin and C. V. Rogozhin, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 7 (1975) 1619.
4 F. R. Atherton, C. H. Hassall and R. W. Lambert, *J. Med. Chem.*, 29 (1986) 29.
5 V. A. Solodenko, T. N. Kasheva and V. P. Kukhar, *Zh. Obshch. Khim.*, 59 (1989) 2786.

Journal of Chromatography, 600 (1992) 83–85
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 009

High-performance ligand-exchange liquid chromatography of fluoro derivatives of alanine

S. V. Galushko*, I. P. Shishkina, I. I. Gerus and M. T. Kolycheva

Institute of Bioorganic Chemistry and Oil Chemistry, Academy of Sciences of the Ukraine, 253660 Kiev 94 (Ukraine)

ABSTRACT

The separation of enantiomers of 3-fluoroalanine, 3,3-difluoroalanine and 3,3,3-trifluoroalanine on ChiralProCu, ChiralValCu and Chiral-1 Nucleosil columns was studied. The substitution of hydrogen for fluorine atoms results in a significant increase in the selectivity of separation of the enantiomers. The relationship between the number of fluorine atoms and the retention of enantiomers on different columns was studied. Optimum conditions for the separation of enantiomers were found.

INTRODUCTION

Fluorine analogues of amino acids have high biological activity depending on the absolute configuration of the molecule. High-performance liquid chromatography (HPLC) has been used successfully to separate the enantiomers of fluoroamino acids but no data concerning the effect of replacement of hydrogen atoms in molecules of amino acids by fluorine atoms on the retention and selectivity of the separation of enantiomers on chiral sorbents have been reported. The separation of some polyfluoro derivatives of alanine reversed-phase HPLC with a chiral mobile phase has been developed [1–3]. It was shown that the introduction of fluorine substituents into the molecule of amino acids leads to increased retention and selectivity of enantiomers [1]. The separation of some aromatic fluoroamino acids by ligand-exchange chromatography (LEC) has been described [4] and the chromatographic behaviour of α-trifluoromethyl-α-amino acids on different chiral sorbents has been studied [5]. It was shown that changes in the selectivity of the separation of enantiomers after the introduction of a CF_3 group into the molecule of amino acids depended greatly on the type of sorbent used.

The aims of this work were to separate the enantiomers of 3-fluoro, 3,3-difluoro and 3,3,3-trifluoro derivatives of alanine by LEC and to compare the selectivities of the separation of the enantiomers on different chiral sorbents.

EXPERIMENTAL

Chromatographic conditions

The experiments were performed on an LKB (Bromma, Sweden) liquid chromatographic system consisting of a Model 2150 HPLC pump, a Model 7410 injector, a Model 2140 rapid spectral detector set at 235 nm, a Model 2200 recording integrator and a Model 2155 column oven.

The columns used were (1) ChiralProCu = Si100, (2) ChiralValCu = Si100, both 5-μm (250 × 4.6 mm I.D.) (Serva, Heidelberg, Germany), and (3) Nucleosil Chiral-1, 5 μm (250 × 4.6 mm I.D.) (Macherey–Nagel, Düren, Germany). The mobile phases were 1–2.5 m*M* copper sulphate solutions at a flow-rate of 0.75 ml/min.

Materials

Stereoisomers of fluorine-containing amino acids were synthesized as described [6,7]. Copper sulphate was of analytical reagent grade. Water was doubly distilled and filtered before HPLC use.

0021-9673/92/$05.00

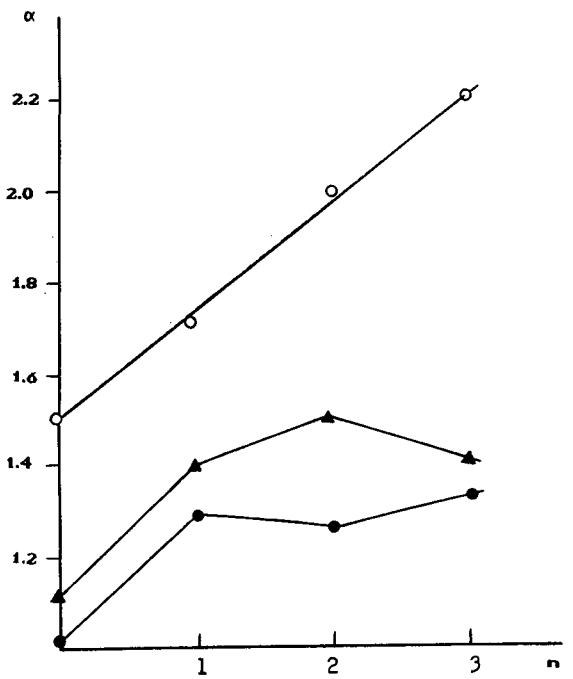


Fig. 1. Effect of the number of F atoms in the molecule of alanine (n) on selectivity of enantiomers separation. Column: ● = ChiralProCu, 5 μm (250 × 4.6 mm I.D.); ▲ = ChiralValCu, 5 μm (250 × 4.6 mm I.D.); ○ = Nucleosil Chiral-1, 5 μm (250 × 4.0 mm I.D.). Eluent, 2.5 mM $CuSO_4$; flow-rate, 0.75 ml/min; temperature, 35°C; wavelength, 235 nm.

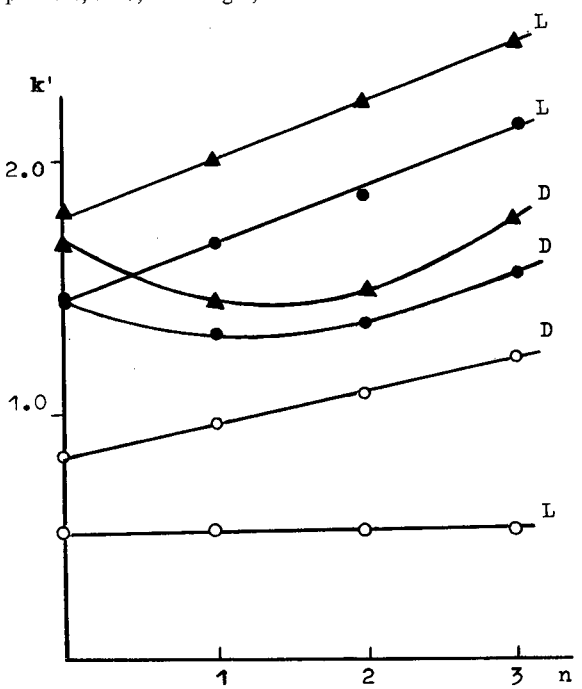


Fig. 2. Effect of the number of F atoms in the molecule of alanine (n) on the retention of enantiomers. Columns, eluent and conditions as in Fig. 1.

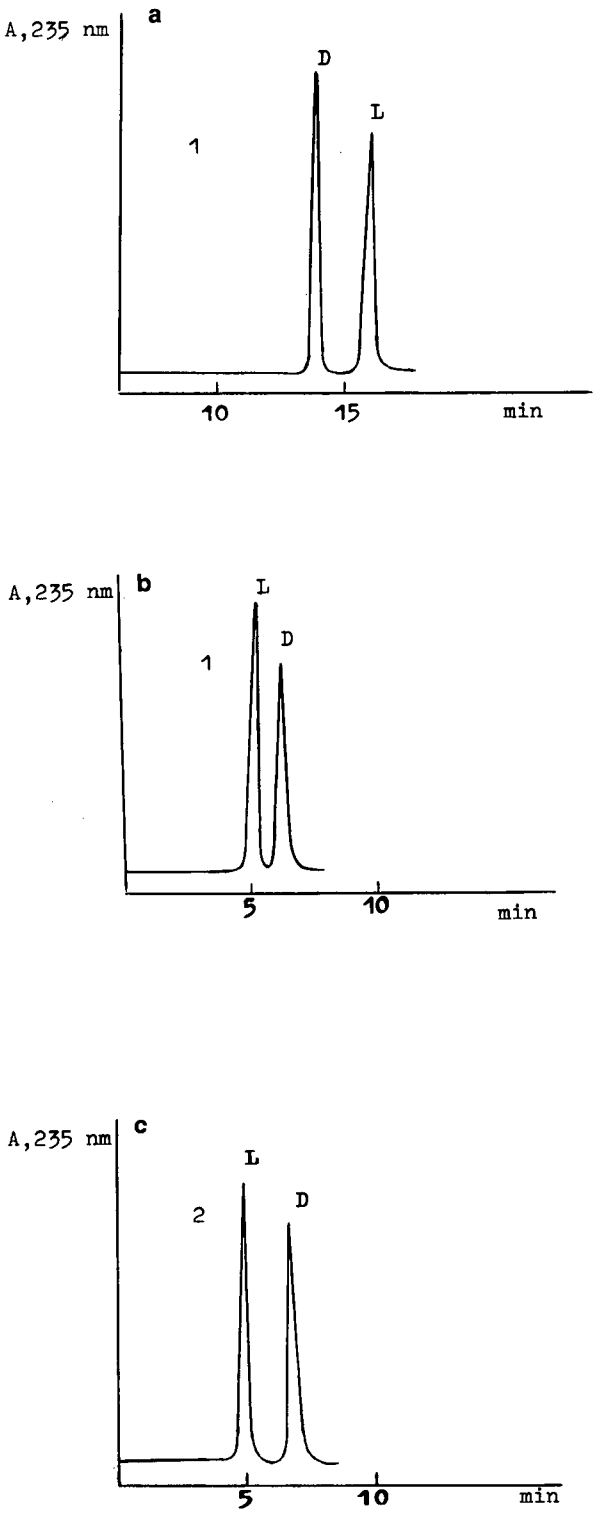


Fig. 3. Separation of enantiomers of (1) 3-fluoroalanine and (2) 3,3-difluoroalanine. Columns: (a) ChiralProCu, 5 μm (250 × 4.6 mm I.D.); (b and c) Nucleosil Chiral-1, 5 μm (250 × 4.0 I.D.). Eluent and conditions as in Fig. 1.

RESULTS AND DISCUSSION

The selectivity of the separation of amino acid enantiomers by LEC on chiral stationary phases depends substantially on the structure of ligand bonded [8]. Fig. 1 shows the dependence of the selectivity (α) of the separation of amino acids enantiomers on the number of atoms of fluorine in the molecule. The results show that for the sorbents studied there are considerable differences in the change in this parameter when H atoms are successively replaced with F atoms. For Nucleosil Chiral-1 the introduction of each F atom into the molecule of alanine leads to an increase in the selectivity of 0.23, whereas the introduction of only the first atom of fluorine significantly affects the selectivity of separation on ChiralProCu and ChiralValCu columns. The introduction of F atoms affects differently the retention of L- and D-isomers. For all the sorbents studied the plot of capacity factor (k') *vs.* number of F atoms (n) is a straight line of slope $0.15–0.25k'/n$ for more retained enantiomers (Fig. 2). The absence of any difference in the retention of L-isomers of the amino acids studied on the Nucleosil Chiral-1 column indicates that only a slight or no interaction occurs between the fluoro-containing part of the molecules and the surface ligand of the sorbent. It is also interesting that on columns 1 and 2 the order of elution of D- and L-isomers is the reverse of that with column 3 (Fig. 3). The order of elution on the Nucleosil Chiral-1 column ($k'_D > k'_L$) is characteristic of sorbents with a polymer covering a silica matrix [6].

Hence these experiments have shown that the enantiomers of all the fluoroamino acids studied can be successfully separated by LEC (Fig. 3). The efficiency of the columns is fairly high and the separation is easily achieved at ambient temperature, but some heating of the column enabled the efficiency of separation to be improved. The optimum concentration of Cu(II) ions in the mobile phase is 1.0–2.5 mM. The method may be useful both for the small-scale resolution of enantiomers and for monitoring the enantiomeric purity of fluoro derivatives of alanine.

REFERENCES

1 J. W. Keller and B. I. Hamilton, *Tetrahedron Lett.*, 27 (1986) 1249.
2 J. W. Keller and K. O. Dick, *J. Chromatogr.*, 367 (1986) 187.
3 J. W. Keller and K. Niwa, *J. Chromatogr.*, 486 (1989) 434.
4 J. R. Gerson and M. I. Adam, *J. Chromatogr.*, 325 (1985) 103.
5 S. V. Galushko, I. P. Shishkina, V. A. Soloshonok and V. P. Kukhar, *J. Chromatogr.*, 511 (1990) 115.
6 T. Taushima and K. Kawada, *Tetrahedron Lett.*, 26 (1985) 2445.
7 I. I. Gerus, Y. L. Yagupolsky, V. P. Kukhar, L. S. Boguslavskaya, N. N. Chuvatin, A. V. Kartashov and Y. V. Mitin, *Zh. Org. Khim.*, 27 (1991) 537.
8 V. A. Davankov, J. D. Navratil and H. F. Walton, *Ligand-Exchange Chromatography*, CRC Press, Boca Raton, FL, 1988.

Journal of Chromatography, 600 (1992) 87–92
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 929

Buffer and pH dependence of the retention of phenylthiocarbamylamino acids in reversed-phase high-performance liquid chromatography

Magdolna Morvai, Valéria Fábián and Ibolya Molnár-Perl*

Institute of Inorganic and Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112 (Hungary)

ABSTRACT

An exhaustive study was made to find the optimum conditions for the determination of phenylthiocarbamyl (PTC)-amino acids. The effects of the type of salt dissolved in the eluent (sodium or ammonium acetate), the pH of the eluent, the presence of additives such as triethylamine and phosphate and the maximum storage time of the dissolved derivatives were investigated. On Hypersil or Nucleosil columns (15 cm × 4.6 mm I.D.; 5 μm) the determination of 21 PTC-amino acids [including cyst(e)ine, tryptophan and ornithine] require 22 min. The reproducibility of the measurments was < 5.0% (relative standard deviation).

INTRODUCTION

The separation of amino acids after precolumn derivatization with phenyl isothiocyanate (PITC) and their subsequent determination by reversed-phase high-performance liquid chromatography (HPLC) has gained wide acceptance (Table I). The determination of the components of protein hydrolysates and those of free amino acids as phenylthiocarbamyl (PTC) derivatives has considerable advantages over other derivatization methods, including the quantitative reaction of all common amino and imino acids with PITC, the high stability of the PTC-amino acids, the relatively good sensitivity and reproducibility for the resulting PTC derivatives and the relative ease and reasonable cost of their preparation and HPLC separation.

After pioneering work [1,2] numerous papers [3–13] have dealt with the improvement of the initial methods. In this work, we made a systematic study in order to optimize the pH, the salt type and the maximum detector responses of 21 amino acids, including cyst(e)ine, tryptophan and ornithine, using the same two linear-increment gradient-type elution systems, to find the differences (if any) between two different stationary phase-containing columns of the same size, to define the stability of the PTC-amino acid derivatives after their dissolution in buffer and to examine the role of the presence of triethylamine and phosphate in the eluents.

EXPERIMENTAL

Materials

Triethylamine (TEA), PITC, amino acids and proteins were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade acetonitrile and methanol were purchased from Reanal (Budapest, Hungary). All other reagents were of the highest purity available. Hydrolysis and derivatization tubes were supplied with the Pico-Tag Workstation (Waters Assoc., Milford, MA, USA).

Derivatization of amino acids with PITC

Standards of free amino acids in a mixture containing 12.5, 25.0 or 37.5 nmol of each amino acid were placed in the 50 × 6 mm I.D. tubes and dried under vacuum. Free amino acids were redried, adding 10 μl of ethanol–water–TEA (2:2:1) to each tube. Thereafter to each redried sample 20 μl of derivatization reagent [ethanol–TEA–water–PITC

0021-9673/92/$05.00

TABLE I

HPLC CONDITIONS SUGGESTED FOR THE DETERMINATION OF PTC-AMINO ACIDS

Column (cm × mm I.D.)	Particle size (μm)	Manufacturer	pH	Temp. (°C).	Eluent[a]		No. of amino acids measured	Analysis time (min)[b]	Ref.
					A	B			
15 × 3.9	3	Waters, Picotag	6.4	38	0.14 *M* Na.ac. + 0.5 ml/l TEA	ACN–H_2O (6:4)	18	12	1,6
25 × 4.6	5	IBM, DuPont, Altex	6.8	52	0.1 *M* Amm. ac.	ACN–Met–H_2O (44:10:46 and others)	16	35	2
25 × 4.6	5	Waters, Picotag and others	6.4	45	0.7 *M* Na.ac. + 2.5 ml/l TEA	H_2O; C = ACN–H_2O (80:20)	23	30	3,4
10 × 4.6	3	Thomson Instrument (Newark, DE, USA)	6.4	36	0.05 *M* Na.ac. + 2.25 ml/l TEA	Solvent A: ACN–Met (5:4:1)	17	20	5
15 × 3.9	3	Waters, Picotag	6.4	?	0.14 *M* Na.ac–ACN (9.4:0.6) + 1 ml/l TEA	ACN–H_2O (6:4)	18	14	7
15 × 3.9	3	Waters, Picotag	5.7	33	0.14 *M* Na.ac. + 0.7 ml/l TEA	ACN–H_2O (6:4)	20	16	8
25 × 4.6	5	Hypersil (?)	6.5	55	0.15 *M* Na.ac.	ACN	16	20	9
30 × 3.9	?	Waters, Picotag	6.4	46	0.14 *M* Na.ac.–ACN (9.4:0.6) + 0.05 ml/ml TEA	ACN–H_2O (6:4)	29	20	10
25 × 4.6	5	Dynamex C_{18}, Rainin (Emeryville, CA, USA)	5.7	45	0.1 *M* Na.ac. + 0.5 ml/l TEA	ACN–H_2O (6:4)	18	45	11
25 × 4.6	5	Hypersil, Jones (Hengoed, UK)	6.4	?	0.01 *M* Na.ac.	0.01 *M* Na.ac–ACN (4:6)	17	60	12
25 × 4.6	5	Vydac C_{18} (?)	5.0-6.8	40-60	0.05 *M* Amm.ac.	0.023 *M* Amm.ac.–ACN–H_2O (44:46:10)	17	50	13
15 × 4.6	5	Hypersil (Shandon) Nucleosil (Macherey–Nagel)	7.2	amb.	0.05 *M* Na.ac. or 0.05 *M* Amm.ac.	0.1 *M* Na.ac (or Amm.ac.)–ACN–Met (46:44:10)	21	22	This work

[a] ACN = Acetonitrile; Met = methanol; TEA = triethylamine; Na.ac. = sodium acetate; Amm.ac. = ammonium acetate.

[b] Time from injection to elution of the last PTC derivative.

TABLE II

DETECTOR RESPONSES AND REPRODUCIBILITY STUDY OF PTC-AMINO ACIDS IN MODEL SOLUTIONS OBTAINED WITH (A) SODIUM ACETATE- AND (B) AMMONIUM ACETATE- CONTAINING ELUENTS OF VARIOUS pH VALUES

Values in parentheses and in quotation marks have been omitted from averages: (i) data in quotation marks indicate that the detector responses of glycine and alanine obtained with different eluents (A and B) are different; (ii) values in parentheses are higher (threonine, proline; pH = 7.6; eluent A) or lower [cyst(e)ine, lysine; pH = 7.0; eluents A and B] than the average.

Eluent	pH	Detector response (arbitrary units/pmol of amino acid)																			
		1 Aspartic acid	2 Glutamic acid	3 Hydroxyproline	4 Serine	5 Glycine	6 Histidine	7 Threonine	8 Alanine	9 Proline	10 Arginine	11 Tyrosine	12 Valine	13 Methionine	14,15 Cyst(e)ine	16 Isoleucine	17 Leucine	18 Phenylalanine	19 Ornithine	20 Tryptophan	21 Lysine
A	7.6	168	182	190	181	180	170	(214)	219	(226)	217	213	207	205	133	208	216	208	310	231	294
	7.2	165	176	181	179	174	162	189	215	208	192	201	192	197	122	208	206	202	304	226	287
	7.0	163	170	178	178	174	159	172	206	207	197	200	201	193	(111)	189	217	198	302	217	(260)
						"176"			"213"												
B	7.6	165	170	177	175	210	175	186	199	208	202	203	200	200	123	192	192	200	309	231	297
	7.2	167	174	178	179	212	169	171	182	195	203	207	201	208	129	195	199	198	309	231	283
	7.0	151	167	179	171	203	161	175	183	197	215	194	180	200	(101)	194	198	199	302	215	(264)
						"208"			"188"												
Average		163	173	181	177		166	179		203	204	203	197	201	127	198	205	201	306	225	290
Standard error		6.2	5.4	4.9	3.6		6.3	8.3		6.4	9.9	6.5	9.5	5.4	5.2	8.3	10.2	3.8	3.7	7.4	6.4
Standard error (%)		3.8	3.1	2.7	2.0		3.8	4.7		3.1	4.8	3.2	4.8	2.7	4.1	4.2	5.0	1.9	1.2	3.3	2.2

pH	Found amounts (% of the quantitative values)																			
7.0	100	100	100	100	100	100	100	100	100	100	100	100	100	84	100	100	100	100	96	90
6.8	91	93	95	96	94	96	98	96	91	95	90	91	92	80	90	94	93	95	95	85
6.4	89	95	96	94	95	96	98	95	92	95	90	92	90	78	91	95	92	96	94	88
6.0	90	92	94	94	91	96	98	91		90[a]	91	89	87	73	87	93	89	96	93	82
5.6	84	89	90	91	86	95	99	88		90[a]	91	90	85	54	87	90	79	95	86	81

[a] Could not be calculated separately.

(7:1:1:1)] were added and vortex mixed. They were prepared according to the Waters Pico-Tag Workstation manual.

The derivatized standards were dissolved in 1.00 ml of 0.05 *M* sodium acetate solution (pH 7.2). Hence 20-μl aliquots of standards contained 250, 500 and 750 pmol of each amino acid.

Chromatography

The system was a Liquochrom Model 2010 liquid chromatograph (Labor MIM, Budapest, Hungary), which consisted of two Liquopump 312/1 solvent-delivery systems and a Type OE-308 UV detector with a wavelength range of 195–440 nm. Samples were injected in 20-μl volumes using an injector supplied by Labor MIM. The columns (BST, Budapest, Hungary) were 15 cm × 4.6 mm I.D. containing Hypersil ODS bonded phase (5 μm) (Shandon) or Nucleosil 5 C_{18} (5 μm) (Macherey–Nagel). Eluents were kept under a blanket of nitrogen.

The two eluent systems each consisted of two components: (A) = 0.05 *M* sodium acetate or 0.05 *M* ammonium acetate (pH 7.2) and (B) = 0.1 *M* sodium acetate or 0.1 *M* ammonium acetate–acetonitrile–methanol (46:44:10) (mixed in volume ratios and titrated with glacial acetic acid or 50% sodium hydroxide to pH 5.6, 6.0, 6.4, 6.8, 7.2 and 7.6), henceforth called sodium acetate- or ammonium acetate-containing eluents). The gradient which was optimized for the separation was from 100% A to 100% B in 22 min; for 5 min a washing step with 100% B was applied, followed by a return to 100% A in 2 min. After an additional 4 min, elution with solvent A was performed. Thereafter the system was equilibrated for the next injection. Thus, the total time required from one injection to the next is 32 min. Both the derivatization and elution procedures were carried out at ambient temperature.

RESULTS AND DISCUSSION

The pH dependence of the elution order of PTC derivatives applying sodium acetate- or ammonium acetate-containing eluents of the same composition with various pH values (5.6, 6.0, 6.4, 6.8, 7.2 and 7.6) was tested. As shown in Figs. 1 and 2 and Table II, the detector responses obtained with eluents of pH 7.2–7.6 are independent of the nature of the salts dissolved. Thus, in reproducibility studies all values with the exception of those for cyst(e)ine and lysine, obtained with eluents of pH 7.0–7.6, could be taken into account [Table II, values of averages, standard deviations (S.D.) and relative standard deviations (R.S.D., %)].

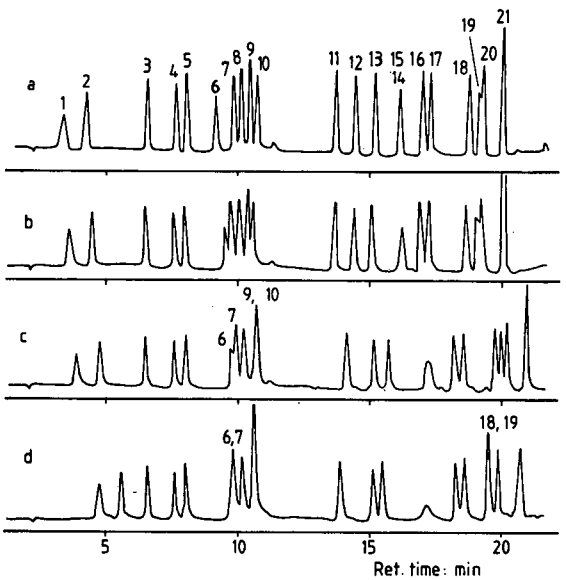


Fig. 1. HPLC with sodium acetate-containing eluents of various pH values: (a) 7.2; (b) 6.4; (c) 6.0; (d) 5.6. For peak identification, see Tables II and III. Column: Hypersil.

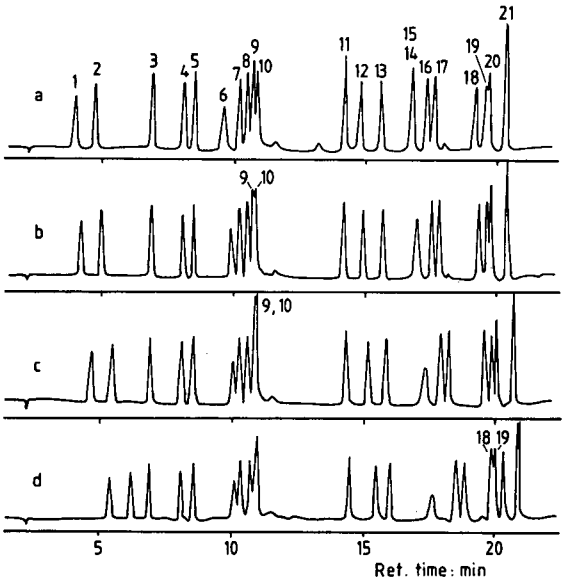


Fig. 2. HPLC with ammonium acetate-containing eluents of various pH values: (a) 7.2; (b) 6.4; (c) 6.0; (d) 5.6. For peak identification, see Tables II and III. Column: Hypersil.

TABLE III

STABILITY STUDY OF PTC-AMINO ACIDS DISSOLVED IN SODIUM ACETATE-CONTAINING ELUENT A AND STORED AT 4°C

The amounts of amino acids injected were all 500 pmol.

Time of storage (h)	Found amounts (%) of the quantitative values)																			
	1 Aspartic acid	2 Glutamic acid	3 Hydroxyproline	4 Serine	5 Glycine	6 Histidine	7 Threonine	8 Alanine	9 Proline	10 Arginine	11 Tyrosine	12 Valine	13 Methionine	14,15 Cyst(e)ine	16 Isoleucine	17 Leucine	18 Phenylalanine	19 Ornithine	20 Tryptophan	21 Lysine
0–7	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
18	100	100	100	96	100	93	100	97	100	91	100	100	100	95	100	100	100	97	100	100
26	100	96	100	96	97	91	100	91	100	89	100	100	100	95	100	100	100	97	100	100
44	100	97	100	95	98	87	92	92	100	85	100	96	100	90	100	100	95	97	94	93
73	93	92	100	92	96	85	89	89	100	85	90	87	95	84	95	95	95	93	95	91
120	91	91	100	92	96	82	80	87	100	85	90	82	95	79	89	89	94	93	95	90
190	80	79	93	87	92	81	72	77	92	76	79	66	80	70	81	81	89	91	91	88

Although between pH 7.0 and 7.6 the sodium acetate- and ammonium acetate-containing eluents resulted in the same detector responses, because of the perfect resolution of peaks 6–10 (Fig. 2a and d), and in order to obtain maximum detector responses even for cyst(e)ine and lysine, the sodium acetate-containing eluents of pH 7.2 are to be preferred [Table II, values for cyst(e)ine at pH 7.0].

The studies with eluents of lower pH 6.8–5.6) revealed following disadvantageous changes. The retention times of the earlier eluting members (peaks 1–9) increase in parallel with the decreasing pH values of eluents. Thus, at pH < 7, the peaks of histidine (peak 6) and threonine (peak 7), and those of alanine (peak 8) and arginine (peak 9) become increasingly closer to each other, then merge (Figs. 1 and 2). The detector responses of all amino acids decrease continuously (Table II, pH 6.8–5.6); considerable losses can be observed with phenylalanine, aspartic acid, glycine and methionine and an extremely high loss with cyst(e)ine. The last result could be the reason for the fact that about the half of the published studies do not mention the problem with cyst(e)ine [5,7,9,11,14–18].

Regarding the type of column, no differences in resolution and sensitivity were observed with the Hypersil and Nucleosil packings when applying strictly the same elution conditions.

Regarding the stability of PTC derivatives after dissolving them in buffers either no [2,5,7–9,11,12] or contradictory [1,3,4,6,10,13] literature data can be found. The statement [1,6] that no loss could be detected after 3 days (in the cold) proved to be incorrect [3,4,10,13]. On keeping the dissolved PTC amino acids at 5–8°C, their stability lasted only 16 h [3,4], whereas others [10,13] reported as *ca.* 5% loss after storage for 48 h at 4°C.

The stability of the PTC-amino acids dissolved in eluent A and stored at 4°C was checked immediately after their preparation and after 30 min and 7, 18, 26, 44, 73, 102 and 190 h (Table III). As can be seen, the most sensitive PTC derivatives, in decreasing order, are histidine, serine and cyst(e)ine. Hence, according to our experience, the maximum time for retaining the dissolved derivatives in their initial amounts was $\leqslant 7$ h.

The effects of the presence of TEA and "phosphate" in eluent A were also investigated, applying our optimum conditions, *i.e.*, sodium acetate-containing eluent of pH 7.2, completed with 5 and 50 μl/l and 1, 2, 3 and 4 ml/l of TEA or with $5 \cdot 10^{-3}$, $1 \cdot 10^{-2}$ and $2 \cdot 10^{-2}$ *M* phosphate. The data obtained showed that the use of both additives is of secondary importance. Although the presence of 50 μl/l and 1–4 ml/l of TEA increased the detector response of cyst(e)ine by 5–10%, at the same time the proline and arginine peaks became inseparable. Also, employing phosphate, increased sharpness of the histidine peak could not be approved confirmed [5] and only an increased aspartic acid response was observed.

In conclusion, it has been shown that in the determination of PTC-amino acids, the use of sodium acetate- instead of ammonium acetate-containing eluents improves the separation of histidine, threonine, alanine, proline and arginine, from the point of view of the maximum detector response the optimum pH of the eluents is 7.2–7.6 and PTC-amino acids dissolved in buffer should not be stored at 4°C for longer than 7 h.

REFERENCES

1 B. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin, *J. Chromatogr.*, 336 (1984) 93.
2 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65.
3 P. S. L. Janssen, J. W. van Nispen, P. A. T. A. Melgers, H. W. M. van den Bogaart, R. L. A. E. Hamelinck and B. C. Goverde, *Chromatographia*, 22 (1986) 345.
4 P. S. L. Janssen, J. W. van Nispen, P. A. T. A. Melgers, H. W. M. van den Bogaart, G. W. M. van Aalst and B. C. Goverde, *Chromatographia*, 22 (1986) 351.
5 R. F. Ebert, *Anal. Biochem.*, 154 (1986) 431.
6 B. A. Bidlingmeyer, T. L. Tarvin and S. A. Cohen, in K. A. Walsh (Editor), *Methods in Protein Sequence Analysis 1986*, Humana Press, Clifton, NJ, 1986.
7 L. Robitaille and L. J. Hoffer, *Can. J. Physiol. Pharmacol.*, 66 (1988) 613.
8 R. G. Elkin and A. M. Wasynczuk, *Cereal Chem.*, 64 (1987) 226.
9 R. Mora, K. D.Berndt, H. Tsai and S. C. Meredith, *Anal. Biochem.*, 172 (1988) 368.
10 S. A. Cohen and D. J. Strydom, *Anal. Biochem.*, 174 (1988) 1.
11 S. Gunawan, N. Y. Walton and D. M. Treiman, *J. Chromatogr.*, 503 (1990) 177.
12 R. A. Sherwood, A. C. Titheradge and D. A. Richards, *J. Chromatogr.*, 528 (1990) 293.
13 A. Guitart, P. H. Orte and J. Cacho, *Analyst (London)*, 116 (1991) 399.
14 T. H. Maugh, *Science*, 225 (1984) 42.
15 H. P. J. Bennett and S. Solomon, *J. Chromatogr.*, 359 (1986) 221.
16 M. M. T. O'Hare, O. Tortora, U. Gether, H.V. Nielsen and T. W. Schwartz, *J. Chromatogr.*, 389 (1987) 379.
17 B. L. Rosenlund, *J. Chromatogr.*, 529 (1990) 258.
18 R. M. Marcé, M. Calull, J. Guasch and F. Borrull, *Am. J. Enol. Vitic.*, 40 (1989) 194.

Journal of Chromatography, 600 (1992) 93–97
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 911

Solid-phase extraction and high-performance liquid chromatographic analysis of a toxic compound from γ-irradiated polyurethane

Hideharu Shintani

National Institute of Hygienic Sciences, Department of Medical Devices, 1–18–1, Kamiyoga, Setagaya, Tokyo 158 (Japan)

ABSTRACT

Polyurethane (PU) is used in medical devices, but it has been reported that a carcinogen, 4,4′-diaminodiphenylmethane (MDA), is produced in PU when subjected to autoclave sterilization. MDA formation has also been reported in PU when sterilized by γ-irradiation. Analysis of serum MDA extracted from sterilized PUs was performed. Determination was by reversed-phase high-performance liquid chromatography using a mixture of an aqueous solution of ammonium acetate and acetonitrile as eluent. An ODS column and electrochemical detection were used. Prior to analysis, serum samples containing MDA were subjected to solid-phase extraction using C_{18}, phenyl or cyclohexyl reversed-phase columns. Elution was performed with alkaline methanol containing 1 *M* ammonia. A satisfactory recovery was attained. The efficiencies of solid-phase extraction and liquid–liquid extraction was compared.

INTRODUCTION

Polyurethane (PU) is widely used in medical devices due to its biocompatibility and biostability [1–4]. Two types are used for this purpose: thermoplastic and thermosetting PU. Fabrication of thermoplastic PU is identical with that of Pellethane 2363 [1–4].

We were interested in the leaching of 4,4′-diaminodiphenylmethane (MDA) from thermosetting PU when sterilized with γ-irradiation. Thermosetting PU is used as a potting material for the connection of blood dialysis fibres to device modules [5]. Thermosetting PU is fabricated by reaction of 4,4′-diphenylmethane diisocyanate (MDI) with partially saponified caster oil [5,6]. As a larger amount of MDI than castor oil is used, there is the potential for an excessive residue of unreacted MDI. MDI is both a toxic and a reactive compound [1–6].

Medical devices require sterilization, therefore sterilization procedures should not be detrimental to the device. In spite of the biocompatibility and biostability of PU [1–6], it has been reported that toxic MDI is residual in PU [1–6]. MDA is reportedly produced by hydrolysis of residual MDI during degradation of PU by heat, by irradiation, by chemical or enzymatic reactions [7–11]. MDA is reportedly toxic [12], mutagenic [13], teratogenic [14] and carcinogenic [15,16].

We considered it important to evaluate the degree of MDA production in sterilized medical devices and the subsequent migration of MDA into human blood. For this purpose, analysis of serum MDA was studied. A liquid–liquid extraction for serum MDA has been reported previously [17–19]. In these cases, the MDA recovery yields were low and complex processes were required [17–19], therefore a solid-phase extraction (SPE) was studied. As MDA is unstable when heated [20], reversed-phase high-performance liquid chromatography (HPLC) was considered to be more appropriate.

EXPERIMENTAL

Materials

Thermoplastic PU was fabricated as described for Pellethane 2363 (Dow Chemical) [1–4]. Thermosetting PU potting material was fabricated by reac-

0021-9673/92/$05.00

ting MDI with partially saponified castor oil [5,6]. An excess of MDI over castor oil was used, thus leaving residual MDI. MDI is readily converted to MDA in an aqueous environment [21]. This sample was identical with sample B in a previous study [22].

Other chemicals were of extra grade supplied by Wako (Tokyo, Japan). Serum was obtained from Kanto Kagaku (Tokyo, Japan).

HPLC analysis

A conventional end-capped ODS column (insufficiently end-capped), ODS 120T-1251, from Toso (Tokyo, Japan) was used with an aqueous eluent consisting of 50 m*M* ammonium acetate–acetonitrile (1:3) at a flow-rate of 1.2 ml/min. Detection was carried out simultaneously using electrochemical detection (ED) (900 mV application) and UV detection. The ED apparatus used was a VMD 501 supplied by Yanagimoto (Kyoto, Japan) with a glassy carbon working electrode and an Ag/AgCl reference electrode. UV detection was carried out at 250 nm, the wavelength of maximum absorption of MDA, with a Model SPD-2A spectrophotometer supplied by Shimadzu (Kyoto, Japan).

Solid-phase extraction procedure

Bond Elut® columns (C_1, C_2, C_8, C_{18}, phenyl, cyclohexyl and silica and a SCX strong cation-exchange column) were supplied by Analytichem (Harbor City, CA, USA).

Reversed-phase columns and the silica column were conditioned using 1 ml of methanol followed by 1 ml of water. Serum (1 ml containing MDA at a concentration of 0.1–10 ppm) was applied to the conditioned column, which was rinsed with 1 ml of water. The column-trapped MDA was eluted with 250 μl of methanol containing 1 *M* ammonia.

The Bond Elut SCX was conditioned with 2 ml of 1 *M* HCl and washed with 2 ml of methanol and thereafter with 2 ml of water. Serum (1 ml containing MDA at a concentration of 0.1–10 ppm) was adjusted to pH 2.5. The serum was deproteinized and centrifuged. The supernatant was applied to the conditioned SCX column. The column-trapped MDA was rinsed with 1 ml of 0.01 *M* HCl and eluted with 1.5 ml of methanol containing 1 *M* ammonia.

Conditioning and elution in SPE were carried out using a Model AP-115 AN vacuum pump supplied by Iwaki (Tokyo, Japan).

RESULTS AND DISCUSSION

HPLC analysis

MDA was detectable by ED due to the presence of two primary aromatic amino groups. When using an insufficiently end-capped ODS column such as ODS 120T-1251, it was necessary to add 50 m*M* ammonium acetate to attain a common ion effect for rapid elution and to prevent MDA peak tailing. For reproducible and sensitive detection by ED, a salt concentration above 50 m*M* is advisable.

MDA was detectable by ED above 500 mV. The response increased (Fig. 1) and the selectivity decreased with increasing voltage. As voltages above 1000 mV application deteriorates the glassy carbon working electrode, 900 mV is more appropriate. The UV absorption spectrum of MDA is presented in Fig. 1. MDA was detected at a maximum wavelength of 250 nm.

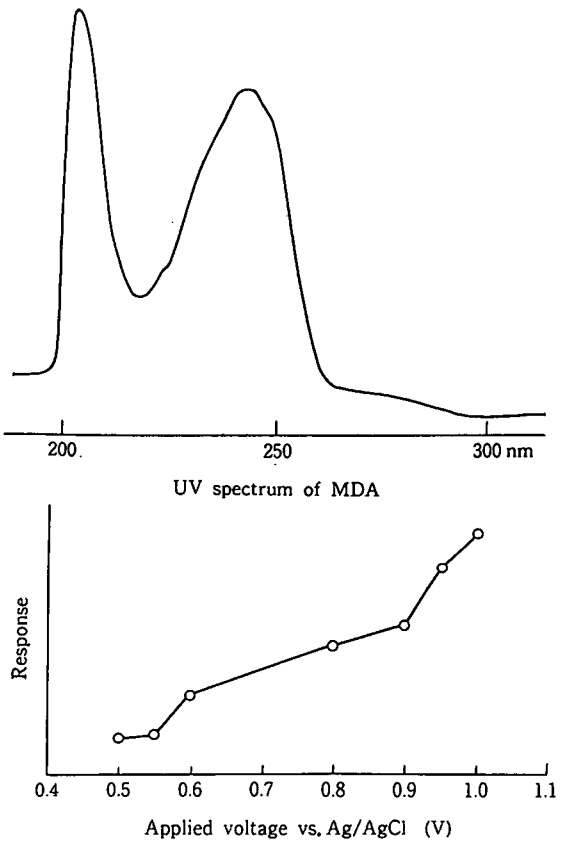


Fig. 1. (Top) UV spectrum of MDA and (bottom) relationship between applied voltage and response of MDA.

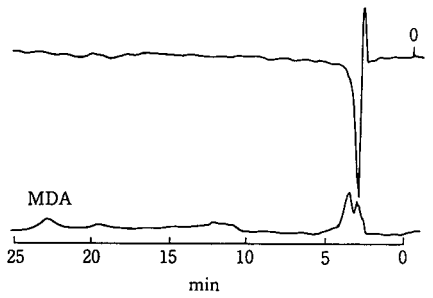


Fig. 2. HPLC of methanol extract of irradiated PU. In the lower chromatogram 0.48 ppm of MDA was detected by ED. No MDA was detected by UV (250 nm) in the upper chromatogram.

As shown in Fig. 2, there is a significant difference between the selectivity and sensitivity of UV and ED in MDA detection. UV detection showed a greater baseline fluctuation due to impurities in the ammonium acetate eluent. ED is clearly superior, being 50 times more sensitive. The detection limits of ED and UV detection are 3 and 150 μg/l, respectively.

Liquid–liquid extraction of serum MDA

A liquid–liquid extraction has been reported [17–19]. In order to suppress ionization of MDA, serum alkalized and extracted repeatedly with hydrophobic organic solvents such as heptane, diethyl ether or benzene [17–19]. These methods are complex procedures, requiring repeated extraction. The recovery yield is reported to be 70–80%, which is unsatisfactory [17–19].

When one part of alkaline serum was extracted twice with seven parts of chloroform–methanol (3:1), satisfactory recovery of serum MDA was achieved [21]. We have recently developed a more efficient method, in which two parts of acetonitrile are added to one part of serum [22]. In both instances, the extracts were vacuum evaporated in a water-bath maintained at 50°C. Temperature is a critical factor; above 50°C MDA degrades and an unsatisfactory recovery is obtained. The acetonitrile procedure requires a single extraction and shows a satisfactory recovery [average recovery yield 98% ($n=5$), coefficient of variation (C.V.) = 1.6%] [22]. As liquid—liquid extraction requires condensation and a large volume of solvent, we elected to use SPE.

Solid-phase extraction of serum MDA

Bond Elut columns (C_1, C_2, C_8, C_{18}, phenyl, cyclohexyl, silica and SCX) were tested. The recovery yields are averages for five specimens. In all instances the C.V. is less than 1.4%.

The recovery yields of serum MDA from the C_1, C_2, C_8 and silica columns are 56%, 75%, 90% and 12%, respectively. Residual MDA was determined in the drain, indicating that MDA was insufficiently retained on the column. The recovery yields from the C_{18}, phenyl and cyclohexyl columns are 100%. When following this procedure, the concentration of recovered MDA from the C_{18}, phenyl and cyclohexyl columns is five times the original concentration without condensation (Fig. 3).

The lower recovery yield for the C_1, C_2, C_8 and silica columns and the higher recovery yields for the C_{18}, phenyl and cyclohexyl columns indicate that the predominant factors for retaining MDA on reversed-phase columns are Van der Waals binding and π-π binding between benzene rings. The binding of MDA to silanol in reversed-phase columns is not significant due to the lower recovery yield of serum MDA from the silica column. This is thought to be due to the interference of serum water with the combination of MDA to silanol. The recovery yield increased with increasing hydrophobicity from the C_1 to C_8 column, indicating that Van der Waals binding is involved in the binding factor.

A satisfactory recovery yield was attained using the SCX column, but conditioning of SCX is complex, requiring deproteinization and centrifugation of serum prior to application.

When eluting MDA from reversed-phase columns, methanol resulted in a 6% MDA recovery (Fig. 3); 10% was recovered by elution using 10 mM ammonium acetate–methanol (1:1) (Fig. 3). MDA was satisfactorily recovered by using strongly alkaline methanol containing 1 M ammonia (Fig. 3). Both methanol and acidified methanol resulted in an unsuccessful recovery of serum MDA. The lower recovery when using an acidified methanol cannot be explained, but acidification is thought to be depressed in alkaline serum.

When the chromatograms of serum obtained using C_8, C_{18}, phenyl and cyclohexyl columns were compared, the fast elution peaks proved to be retained more on the phenyl than on the other columns (Fig. 4). The recovery yield remained satis-

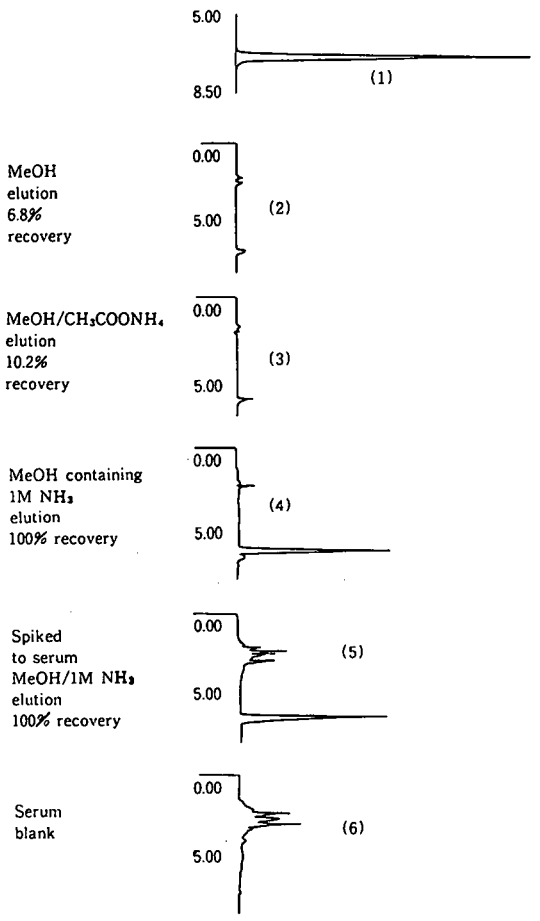


Fig. 3. HPLC of serum containing MDA. Serum MDA was recovered by SPE using a C_{18} column combined with various eluents. HPLC conditions: C_{18} column; eluent, methanol (MeOH)–10 m*M* aqueous ammonium acetate (1:1); flow-rate, 1.2 ml/min; detection, UV (254 nm); 10 μl of sample applied to the column. The MDA peak has a retention time of 6.95 min. (1) 105 ng per 10 μl of MDA standard solution. (2) 100 μl of 105 ng per 10 μl of MDA aqueous solution were applied to C_{18} resin (100 mg of resin and 120 μl void volume), eluted with 250 μl of methanol); 200 μl were collected and applied to HPLC. (3) The same procedure as in (2) except for the eluent: methanol–10 m*M* aqueous ammonium acetate (1:1), identical with HPLC eluent. (4) The same procedure as in (2) except for the eluent: methanol containing 1 *M* NH_3. (5) 50 μl of 21 μg/ml of MDA were added to 1 ml of serum to prepare the serum sample containing MDA at a concentration of 1 μg/ml. This was applied to C_{18} resin, eluted with 250 μl of methanol containing 1 *M* NH_3. 200 μl were collected and applied to HPLC. (6) Serum blank.

factory, confirming the sufficient binding capacity to retain MDA and other serum admixtures.

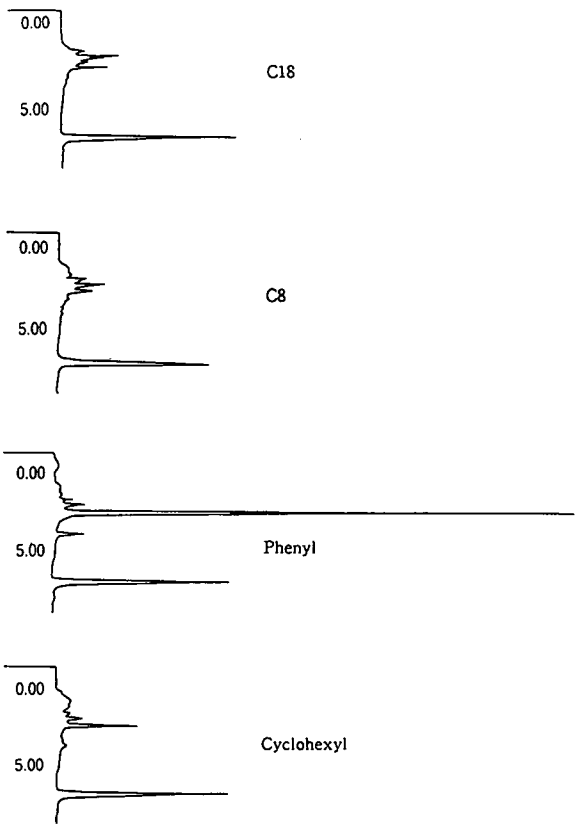


Fig. 4. Solid-phase extraction of serum MDA using C_8, C_{18}, phenyl and cyclohexyl columns. A 50-μl volume of a 21 μg/ml concentration of MDA was added to 1 ml of serum to prepare the serum sample containing MDA at a concentration of 1 μg/ml. This was applied to C_8, C_{18}, phenyl and cyclohexyl columns and eluted with 250 μl of methanol containing 1 *M* NH_3. Conditions are the same as in Fig. 3.

Determination of MDA in potting material sterilized by autoclaving or gamma irradiation

There was no indication of MDA formation in thermosetting PU potting material sterilized by autoclaving at 121°C for 60 min.

Potting material sterilized by 10 Mrad γ-irradiation produced MDA at a level of a few ppm. MDA formation increased with increasing irradiation dose according to a quadratic equation. At 2.5 Mrad, the irradiation level officially approved, MDA was formed at the concentration of less than 1 ppm.

The HPLC chromatogram of serum extract from irradiated potting material indicates that serum ex-

tracts both hydrophilic and hydrophobic compounds including MDA. Detection was by ED. As these extracts indicate mutagenicity, further study is required.

The risk factor of MDA formed at 10 Mrad irradiation (a few ppm) was estimated using ref. 16. In our opinion, the result is "not significant" as the estimated cancer-causing risk factor is 0.29 (29 in 100 persons) when intaking 1 mg/kg body mass of MDA per day. The MDA elution level is far less than the risk factor level.

The elution of compounds other than MDA from irradiated potting material is greater. They are mutagens, therefore other compounds must be evaluated as they are thought to migrate to patients. It is necessary to estimate the risk factor to patients exposed to these toxic compounds. For this purpose, the chemical structures and the biological characteristics of the exact mutagens in the extract should be further clarified.

REFERENCES

1 M. D. Lelah and S. L. Cooper, *Polyurethane in Medicine*, CRC Press, Boca Raton, FL, 1987.
2 S. Gogolowski, *Colloid Polym. Sci.*, 267 (1989) 757.
3 S. Gogolowski, *Trends Polym. Sci.*, 1 (1991) 47.
4 H. J. Griesser, *Polym. Degrad. Stabil.*, 33 (1991) 329.
5 H. Frommelt, *Makromol. Chem., Maromol. Symp.*, 12 (1987) 281.
6 Z. S. Petrovic and D. Fajnik, *J. Appl. Polym. Sci.*, 29 (1984) 1031.
7 J. Autian, A. R. Singh, J. E. Turner, G. W. C. Hung, L. J. Nunez and W. H. Laurence, *Cancer Res.*, 35 (1975) 1591.
8 J. Autian, A. R. Singh, J. E. Turner, G. W. C. Hung, L. J. Nunez and W. H. Laurence, *Cancer Res.*, 36 (1976) 3973.
9 T. D. Darby, H. J. Johnson and S. J. Northup, *Toxicol. Appl. Pharm.*, 46 (1978) 449.
10 W. C. Huepper, *Am. J. Clin. Pathol.*, 34 (1960) 328.
11 W. C. Huepper, *J. Natl. Cancer Inst.*, 33 (1964) 1005.
12 L. J. Casarett, in L. H. Cassarett and J. Daull, (Editors), *Toxicology: the Basic Science of Poisons*, Macmillan, New York, 1976, p. 24.
13 H. Shimizu and N. Takemura, *Sangyo Igaku*, 18 (1976) 138.
14 J. MaLaughlin, Jr., P. P. Marliac, M. J. Verett, N. K. Mutchler and J. G. Fritzhung, *Toxicol. Appl. Pharmacol.*, 5 (1963) 760.
15 D. Steinhoff and E. Grundmann, *Naturwissenschaften*, 58 (1971) 578.
16 *National Toxicology Program Technical Report on the Carcinogenesis Bioassay of 4,4'-Methylenedianiline dihydrochloride, NTP-81-143*, NIH Publication No. 82-2504, National Institute of Health, Bethesda, MD, 1982.
17 M. Tortoreto, P. Catalani, M. Bianchi, C. Blonda, C. Pantarotto and S. Paglialunga, *J. Chromatogr.*, 262 (1983) 367.
18 J. Cocker, L. C. Brown, H. K. Wilson and K. Rollins, *J. Anal. Toxicol.*, 12 (1988) 9.
19 M. C. Bowman, *J. Assoc. Off. Anal. Chem.*, 61 (1978) 1253.
20 E. Dyer and R. J. Hammond, *J. Polym. Sci., Part A*, 2 (1964) 1.
21 H. Shintani in C. P. Sharma and M. Szycher (Editors), *Sterilization Systems*, Technomic, Lancaster, 1992, in press.
22 H. Shintani an A. Nakamura, *J. Biomed. Mater. Res.*, 25 (1991) 1275.

Journal of Chromatography, 600 (1992) 99–108
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 813

Determination of erythromycin ethylsuccinate by liquid chromatography

Th. Cachet☆, P. Lannoo, J. Paesen, G. Janssen and J. Hoogmartens*

Katholieke Universiteit Leuven, Laboratorium voor Farmaceutische Chemie, Instituut voor Farmaceutische Wetenschappen, Van Evenstraat 4, B-3000 Leuven (Belgium)

ABSTRACT

A method is described for the determination of erythromycin ethylsuccinate by liquid chromatography. A C_{18} reversed-phase column (25 cm × 4.6 mm I.D.) was used with acetonitrile–0.2 *M* tetrabutylammonium sulphate (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water [*x*:5:5:(90 − *x*)] as mobile phase. The proportion of acetonitrile (*x*) has to be adapted to the type of stationary phase used. For RSil C_{18} LL, 42.5% was used. The column was heated at 35°C, the flow-rate was 1.5 ml/min and UV detection was performed at 215 nm. The main component, erythromycin A ethylsuccinate, was separated from all other components which were present in commercial samples. The main impurities were erythromycin A and the ethylsuccinate esters of erythromycin B and C. The amide N-ethylsuccinyl-N-demethylerythromycin A was shown to be present in all the samples examined. The method was successfully applied to the analysis of specialties.

INTRODUCTION

Soon after the introduction of the antibiotic erythromycin in 1952, a number of esters were prepared and marketed [1]. Esters of erythromycin are biologically inactive prodrugs and have to be hydrolysed to produce antimicrobial activity. Especially the ethylsuccinate ester of erythromycin (EES) is nowadays used in various pharmaceutical forms.

At present, pharmacopoeias prescribe a microbiological method for the assay of erythromycin ethylsuccinate [2,3]. After hydrolysis of the ester, the total activity is determined against an erythromycin standard. Liquid chromatography (LC) allows the separation of the ester from erythromycin and other related substances and therefore allows the specific and accurate determination of the ester. An LC method for EES has already been described by Tsuji and co-workers [4,5]. The same method was applied to the fluorimetric determination of EES in serum [6]. A method using electrochemical detection to measure EES and erythromycin in plasma, urine and saliva has been reported [7]. Methods for the separation of the ester from biological fluids are less suitable for quality control as the main concern is the separation of the ester from the biological background. The method reported by Tsuji and Kane [5] for the analysis of bulk EES suffers from the use of an elevated column temperature (70°C) and a relatively high pH of the mobile phase (pH 7.4), needed to reduce peak tailing and analysis time. Such conditions severely affect the lifetime of the silica-based reversed-phase stationary phase.

More recently, an LC method for the determination of erythromycin was described in which moderate conditions of temperature (35°C) and pH (6.5) could be used after addition of quaternary ammonium ions to the mobile phase [8]. In this paper, a modification of this LC method is described for the determination of EES in bulk or in pharmaceutical forms.

☆ Present address: Proctor & Gamble European Technical Centre, Temselaan 100, B-1853 Strombeek-Bever, Belgium.

0021-9673/92/$05.00

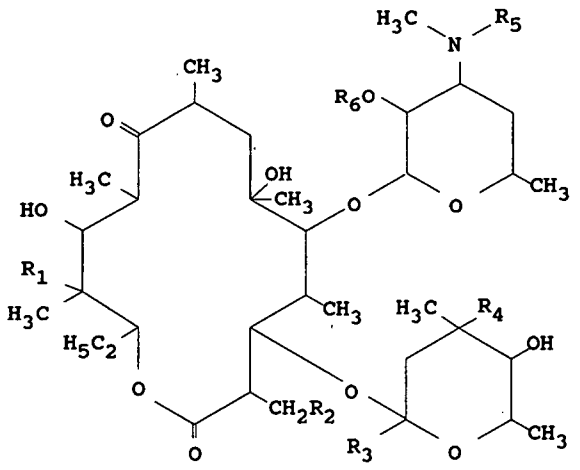
EXPERIMENTAL

Samples and reference substances

Bulk samples of EES were kindly provided by Proter (Milan, Italy) and Upjohn (Kalamazoo, MI, USA) or were a gift from Professor H. Vanderhaeghe (Rega Institute, Katholieke Universiteit Leuven, Belgium). Specialties containing EES were obtained from the Belgian market. Pure erythromycin A (EA) was obtained by crystallization of a commercial sample of erythromycin as described [9]. Anhydroerythromycin A (AEA) [10] and erythromycin A enol ethr (EAEN) [11] were prepared from EA according to previously described methods. Analogous methods were used to prepare anhydroerythromycin A ethylsuccinate (AEAES) and erythromycin A enol ether ethylsuccinate (EAENES) from EAES.

Details of the preparation of EAENES and AEAES and analytical data will be reported elsewhere. Ethylsuccinate esters of erythromycin B (EBES), erythromycin C (ECES), erythromycin E

(a)



	R1	R2	R3	R4	R5	R6
Erythromycin A (EA)	OH	H	H	OCH_3	CH_3	H
Erythromycin B (EB)	H	H	H	OCH_3	CH_3	H
Erythromycin C (EC)	OH	H	H	OH	CH_3	H
Erythromycin F (EF)	OH	OH	H	OCH_3	CH_3	H
Erythromycin E (EE)	OH	- O -		OCH_3	CH_3	H
N-desmethylerythromycin A (dMeEA)	OH	H	H	OCH_3	H	H

The corresponding ethylsuccinate esters (..ES) have R6 = $CO-CH_2-CH_2-COOC_2H_5$. N-ethylsuccinyl, N-demethylerythromycin A (ESdMeEA) corresponds to dMeEA with R5 = $CO-CH_2-CH_2-COOC_2H_5$.

Fig. 1.

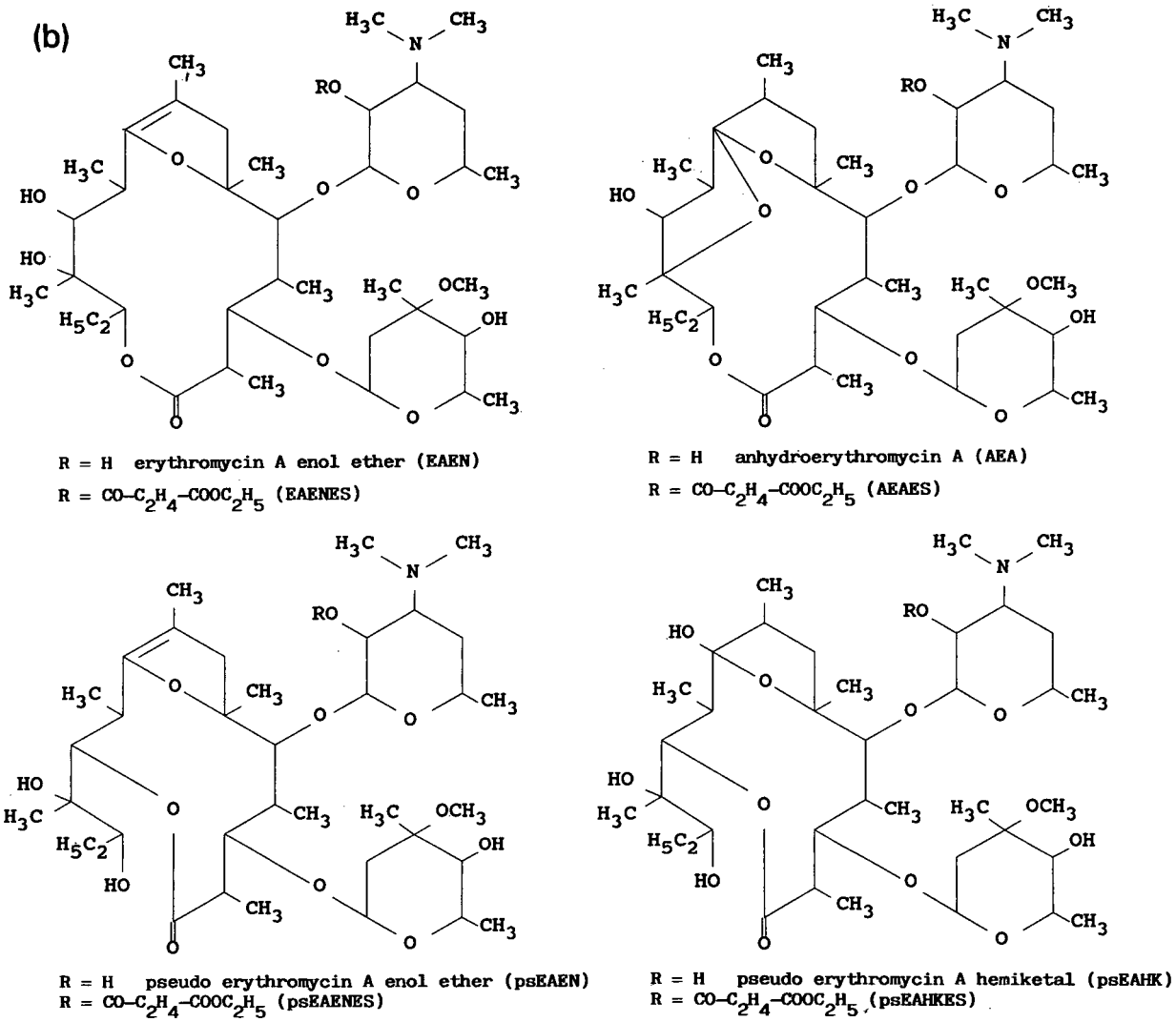


Fig. 1. Structures of the erythromycins examined.

(EEES), erythromycin F (EFES), N-demethylerythromycin A (dMeEAES), pseudo-erythromycin A hemiketal (psEAHKES) and pseudo-erythromycin A enol ether (psEAENES) were prepared by reaction with ethylsuccinyl chloride. The following typical procedure was used: to a mixture of 100 mg of starting material (*ca.* 0.15 mmol) dissolved in 10 ml of dry acetone and 500 mg of dry sodium carbonate, an equivalent amount of ethylsuccinyl chloride (Janssen Chimica, Beerse, Belgium) was added. The mixture was stirred for 2 h, filtered and evaporated under reduced pressure. Smaller amounts of starting material (up to 10 mg) were used to prepare the derivatives of EB, EC, EE and EF. The starting materials EB and EC were obtained by preparative LC of mother liquor residues from erythromycin production [12]. EE [13] and EF [14] were isolated from commercial erythromycin; dMeEA [15], psEAEN and psEAHK [16] were prepared according to described procedures. After analysis, the derivative obtained from dMeEA turned out to be an amide (N-ethylsuccinyl-N-demethylerythromycin A = ESdMeEA) and not a 2'-O-ester (dMeEAES). The determination of the structure is discussed below. Structures are shown in Fig. 1.

Instrumentation

The chromatographic system was composed of a Waters (Milford, MA, USA) M45 pump, a Valco (Houston, TX, USA) Model CV-6-UHPa N60 injection valve equipped with a 20-μl loop, a Merck–Hitachi Model 4200 variable-wavelength detector set at 215 nm (Merck, Darmstadt, Germany) and a Hewlett-Packard (Avondale, PA, USA) Model 3390 A integrator.

Mass spectra were recorded on a Kratos (Ramsey, NJ, USA) Concept 1H mass spectrometer at 8 kV accelerating voltage, 70 eV ionization energy and 150°C ion-source temperature. The sample was introduced by the direct insertion probe. ^{13}C NMR spectra were measured on an FX90Q 90-MHz Fourier transform instrument (Jeol, Tokyo, Japan). The sample was dissolved in C^2HCl_3.

Stationary phases

The stationary phases used are listed in Table I and were laboratory-packed in columns of 25 cm × 4.6 mm I.D. following a classical slurry packing procedure [17]. Spherisorb materials were obtained from Phase Separations (Queensferry, UK), Partisil materials from Whatman (Clifton, NJ, USA), μ-Bondapak from Waters and RSil from RSL–Bio-Rad (Eke, Belgium).

Solvents and mobile phases

Phosphate buffers (0.2 *M*) were prepared by mixing suitable amounts of 0.2 *M* solutions of ammonium dihydrogenphosphate and diammonium hydrogenphosphate (analytical-reagent grade, E. Merck). Tetrabutylammonium hydrogensulphate (TBA) (Janssen Chimica) was used to prepare 0.2 *M* TBA solutions. These solutions were adjusted to the required pH with 40% (w/v) sodium hydroxide solution before the solutions were brought to the final volume. LC-grade acetonitrile was obtained from Rathburn Chemicals (Walkerburn, UK). Water was distilled twice from glass. Mobile phases were degassed by sonication. Acetone was obtained from Janssen Chimica and was purified by distillation after refluxing in the presence of potassium permanganate.

Sample preparation and stability of the solutions

About 150 mg of EES bulk sample were dissolved in 6 ml of acetone and diluted to 10.0 ml with a mixture containing 12.5% (v/v) 0.2 *M* TBA (pH 6.5), 7.5% (v/v) 0.2 *M* phosphate buffer (pH 6.5) and water up to 100% (v/v).

TABLE I

INVESTIGATED STATIONARY PHASES

Stationary phase	Column No.	d_p (μm)	Particle shape[a]	Carbon content (%)
Spherisorb C_8	I	5	S	6
Spherisorb ODS 1	II	10	S	7
Spherisorb ODS 2	III	10	S	12
Partisil CCS/C_8	IV	10	I	9
Partisil ODS	V	10	I	5
Partisil ODS 2	VI	10	I	15
Partisil ODS 3	VII	10	I	10
μBondapak C_{18}	VIII	10	I	9
RSil LL C_{18}	IX	10	I	12

[a] S = spherical; I = irregular.

For dispersible powders and tablets, 10 ml of acetone were added to an amount corresponding to about 250 mg of EES. The suspension was sonicated for 5 min in a glass-stoppered test-tube and then centrifuged at 2500 *g* for 5 min. An aliquot of 6.0 ml of supernatant was diluted to 10.0 ml with the same aqueous mixture as described for bulk EES.

The stability of EAES in this solution was examined at room temperature and at 6°C using the described LC method. After 1 h the concentration of EAES was 96.2% of the initial concentration at room temperature and 98.7% at 6°C, owing to hydrolysis of EAES and formation of EA. Samples for analysis were therefore prepared immediately before use. Rapid hydrolysis of EAES to EA in neutral aqueous solution has been reported previously [18].

Quantitative analysis

Analysis of the samples was finally performed on an RSil LL C_{18} (10 μm) column (25 cm × 4.6 mm I.D.). For the determination of the main component a house standard of EAES was used. The standard was purified by four consecutive crystallizations from acetone–acetonitrile (1:1). By LC this standard was found to contain 2.1% EA, 0.2% ESdMeEA and 0.6% ECES. Titrations with perchloric acid in non-aqueous medium gave a mean value of 97.9% calculated as EAES with a relative standard devia-

tion (R.S.D.) of 0.2% for $n = 11$ determinations. When the titration result was corrected for the amount of EA, which is also titrated and which has a lower molecular mass than EAES, a total base content of 97.5% was obtained. The water content was determined by means of a Karl Fischer titration using a 10% (w/v) solution of imidazole in methanol as the solvent [19]. The result was 2.6% (R.S.D. = 1.5%, $n = 9$). The presence in the house standard of residual organic solvents was examined by gas chromatography under conditions where acetonitrile could be detected at the 0.05% (w/v) level and acetone at the 0.1% (w/v) level. No solvents were detected. The total mass (100%) was thus sufficiently explained by the base titration result (97.5%) and the water content (2.6%). Therefore, the standard was accepted to contain 100% − 2.9% (total impurities by LC) − 2.6% water = 94.5% EAES.

Regression lines for EAES, EA, ESdMeEA and EAENES are given in Table II. The regression line obtained with small amounts of EAES was used for the quantification of EBES or ECES. Regression lines obtained with EAES were corrected for a purity of 94.5%. Other curves were not corrected. Limits of quantification for an injected amount of 300 μg were 1 μg (0.3%) for EA, 1.5 μg (0.5%) for EB, 2 μg (0.6%) for AEA, EAES and AEAES, 0.15 μg (0.05%) for ESdMeEA and 0.5 μg (0.15%) for EAENES.

RESULTS AND DISCUSSION

Structure of N-ethylsuccinyl-N-demethylerythromycin A (ESdMeEA)

The amide structure of ESdMeEA was determined by mass and NMR spectrometry. The mass spectrum exhibited a weak molecular ion at *m/z* 847 (0.1%) and other weak ions at *m/z* 829 ($M-H_2O$, 0.2%), 802 ($M-C_2H_5O$, 0.1%) and 784 (829 − C_2H_5O, 0.5%). Prominent ions occurred at *m/z* 272 (desosamine, 100%), 113 (94%) and 101 (91%). The ^{13}C NMR spectrum in C^2HCl_3 clearly showed the 21 aglycone resonances, the 8 cladinose and the 6 ethylsuccinyl peaks at their expected positions. The remaining 7 desosamine signals (103.9, 71.5, 68.3, 54.1, 35.5, 28.6 and 20.7 ppm) showed a pattern indicative of an N-ethylsuccinyl moiety. Indeed, esterification of the 2′-OH function normally results in a characteristic 2–2.5 ppm upfield shift of C-1′ and C-3′ and a *ca.* 1 ppm downfield shift of C-2′. In this case, however, when compared with the spectrum of dMeEA [20], a 1 ppm downfield shift of C-1′ was observed together with a substantial upfield shift for C-3′ (−5.7 ppm), the N-CH_3 group (−3.4 ppm) and C-4′ (−1.2 ppm). Another strong argument in favour of the 3′-N-amide structure is the appearance of supplementary resonances at a 30% intensity level for almost all desosamine signals. These are probably due to the presence of different rotamers in solution as a result of restricted rotation of the amide bond.

Development of the mobile phase

As erythromycin ethylsuccinate (EES) is prepared by reaction of erythromycin with ethylsuccinyl chloride, the chromatographic conditions were examined in relation to the separation of EA and its decomposition products AEA and EAEN, which are formed in acid, and of the corresponding esters EAES, AEAES and EAENES. First, acetonitrile–

TABLE II

REGRESSION LINES

y = Peak area; x = mass injected (μg); $S_{y,x}$ = standard error of estimate; R = range of injected mass (μg) examined.

Compound	Slope	Intercept	Correlation coefficient	$S_{y,x}$	R
EAES	253 394	−412 491	0.999	635 562	230 –360
	255 940	−41 860	0.998	48 278	5 –60
EA	196 001	44 537	0.999	27 924	2 –16
ESdMeEA	1 242 087	102 394	0.998	99 786	0.4–3.5
EAENES	2 069 873	129 400	0.999	52 827	0.5–3.0

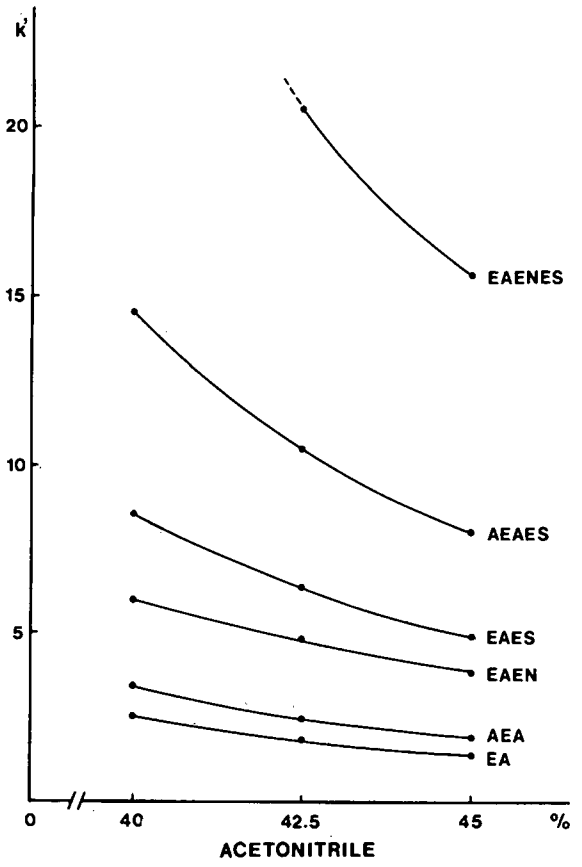


Fig. 2. Influence of the acetonitrile content on the separation. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (*x*:5:5:90 − *x*); column, Partisil ODS 3, 10 μm (250 mm × 4.6 mm I.D.); flow-rate, 1.5 ml/min; temperature, 35°C; detection, UV at 215 nm.

0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (*x*:5:5:90 − *x*) was chosen as the mobile phase. This was derived from that previously used for erythromycin [8,21].

Owing to the stronger retention of the esters as compared with erythromycin, it was necessary to use mobile phases with a higher acetonitrile content.

In Fig. 2 it is demonstrated that on a Partisil ODS 3 column the proportion of acetonitrile should be at least 42.5% for EAENES to be eluted within a reasonable period of time. However, these increased levels of acetonitrile negatively affect the separation of the other components. Variation of the pH of the mobile phase between 6 and 7 did not have much effect on the separation. At pH 7 EAEN was eluted closer to EAES than at pH 6. A pH higher than 7 was not considered because it lowers the stability of the stationary phases. Below pH 6 the peak symmetry of the esters was severely affected and on most of the higher loaded stationary phases fronting was observed. Therefore, pH 6.5 was adopted in subsequent chromatography.

The influence of the TBA content of the mobile phase is shown in Fig. 3. The use of TBA influenced the order of elution and reduced the total analysis time. The effect of TBA on the retention times was less pronounced for the esters. This can be explained by their pK_b values. Esters (pK_b 6.3) are considerably less basic than erythromycin (pK_b 4.5) [18]. Beyond 5%, a further increase in TBA content did not substantially alter the separation. Therefore, this concentration was adopted. The influence of the concentration of the buffer in the mobile phase on

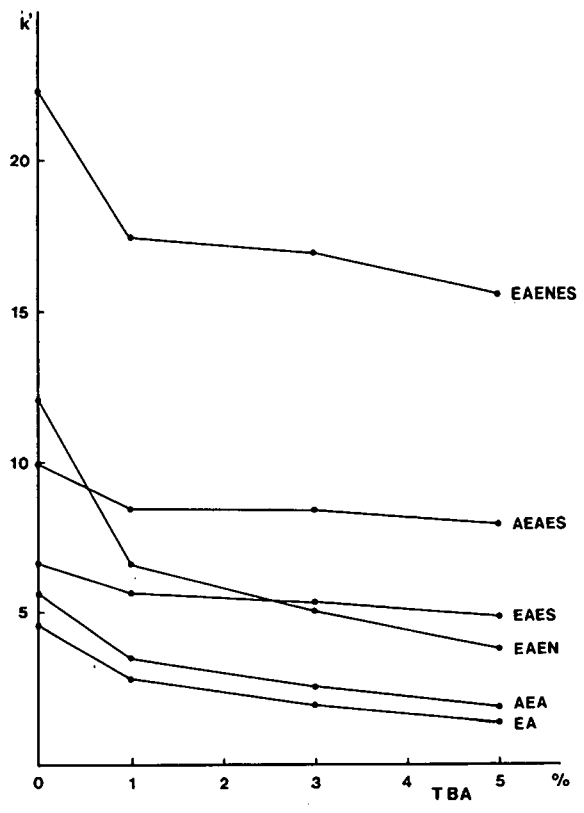


Fig. 3. Influence of the concentration of tetrabutylammonium ion (TBA) on the separation. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (45:*x*:5:50 − *x*); other conditions as in Fig. 2.

the chromatography was small. Only the separation between EAEN and EAES was improved slightly on increasing the buffer concentration from 1% to 10%. An intermediate value of 5% was adopted.

With the mobile phase acetonitrile–0.2 *M* phosphate buffer (pH 6.5)–0.2 *M* TBA (pH 6.5)–water (45:5:5:45), the capacity factors of EA, AEA, EAEN, EAES, AEAES and EAENES were determined on the stationary phases mentioned in Table I. The results are shown in Fig. 4. The same elution order was observed on all the columns. For this reason, the position of the substances is indicated by their elution number. The elution pattern is dependent of the stationary phase. The higher loaded phases show the strongest retention, but the carbon content alone does not explain the total analysis time, as is shown, for example, by columns V and VIII, nor does a higher carbon content guarantee better selectivity towards all the compounds examined. The total analysis time can be adapted by adjusting the acetonitrile content of the mobile phase. The chromatography of these erythromycin esters was not influenced by the age of the column, as was mentioned previously for erythromycins [8,22,23].

Quantitative analysis of bulk erythromycin ethylsuccinate

With the mobile phase acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phisphate buffer (pH 6.5)–water (42.5:5:5:47.5) and an RSil C_{18} LL column, a number of commercial EES samples of various origin were analysed. Small amounts of EA were found in most samples whereas AEA, EAEN and AEAES were not present. EAENES was present in very small amounts. The chromatograms of most samples showed the presence of other impurities.

As EB and EC may be present in the erythromycin used for the preparation of the ester, the corresponding esters EBES and ECES can be present as impurities. The presence of EBES and ECES in commercial samples has already been mentioned by Tsuji and Goetz [4]. Meanwhile, we demonstrated that, in addition to EB and EC, commercial-grade erythromycin may contain appreciable amounts of EE, EF and dMeEA (up to 5%) and small amounts of the decomposition products EAEN and psEAEN (up to 1%). AEA and psEAHK were found in negligible amounts [21]. Therefore, the separation of the esters EBES, ECES, EFES, EEES, psEAENES and psEAHKES and of the amide ESdMeEA was also examined. The retention times of the various components relative to that for EAES are given in Table III. There was no separation between EEES and EAES or between EFES and ESdMeEA. Except for ESdMeEA, the order of elution of the various components was consistent with the order of elution of the parent components [22]. Based on these

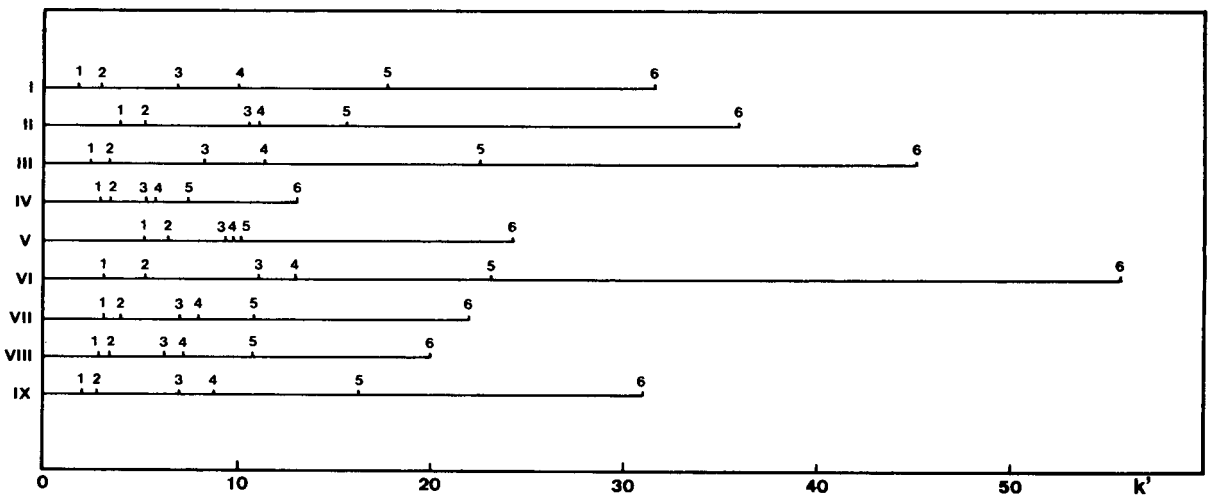


Fig. 4. Influence of the stationary phase on the separation. See Table I for column identification and characteristics. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* ammonium phosphate buffer (pH 6.5)–water (45:5:5:45), other conditions as in Fig. 2. 1 = EA; 2 = AEA; 3 = EAEN; 4 = EAES; 5 = AEAES; 6 = EAENES.

TABLE III

RELATIVE RETENTION TIMES OF THE VARIOUS ETHYLSUCCINATE DERIVATIVES

Column, RSil C_{18} LL, 10 μm (250 mm × 4.6 mm I.D.); mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (42.5:5:5:47.5); temperature, 35°C; flow-rate, 1.5 ml/min; detection, UV at 215 nm.

Compound	Relative retention time
Erythromycin F ethylsuccinate (EFES)	0.41
N-Ethylsuccinyl-N-demethylerythromycin A (ESdMeEA)	0.43
Erythromycin C ethylsuccinate (ECES)	0.65
Erythromycin E ethylsuccinate (EEES)	0.97
Erythromycin A ethylsuccinate (EAES)	1.00
Pseudo-erythromycin A hemiketal ethylsuccinate (psEAHKES)	1.51
Erythromycin B ethylsuccinate (EBES)	1.68
Anhydroerythromycin A ethylsuccinate (AEAES)	1.90
Pseudo-erythromycin A enol ether ethylsuccinate (psEAENES)	2.08
Erythromycin A enol ether ethylsuccinate (EAENES)	3.83

relative retention times we were able to identify EBES, ECES and ESdMeEA in a number of EES samples. Fig. 5 shows a typical chromatogram of a commercial EES sample. As there was no separation between EEES and EAES or between EFES and ESdMeEA, the samples were methanolysed and it was possible to show, by thin-layer chromatography (TLC), the presence of EF, but not of EE. This infers the presence of EFES in commercial EES samples. TLC of erythromycin has been extensively discussed elsewhere [14].

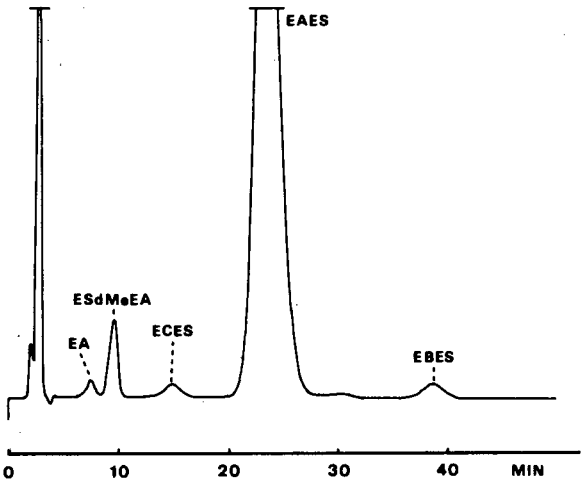


Fig. 5. Typical chromatogram of a bulk sample of erythromycin ethylsuccinate. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (42.5:5:5:47.5); column, RSil C_{18} LL, 10 μm (250 mm × 4.6 mm I.D.); other conditions as in Fig. 2. EA = erythromycin A; ESdMeEA = N-ethylsuccinyl-N-demethylerythromycin A; ECES = erythromycin C ethylsuccinate; EAES = erythromycin A ethylsuccinate; EBES = erythromycin B ethylsuccinate.

Determination of erythromycin ethylsuccinate

Table IV gives the results obtained in the analysis of commercial samples of bulk erythromycin ethylsuccinate. Some samples from manufacturer B contained large amounts of EBES (up to 16.4%). However, recent batches from the same manufacturer showed the highest content of EAES (94.9%). The content of ESdMeEA never exceeded 1.1%. Owing to the five times higher specific absorbance of ESdMeEA at 215 nm compared with EA, ESdMeEA gave relatively high peaks. In faster eluting systems ESdMeEA may be eluted together with EA, leading to overestimation of the EA content. EFES, which was eluted together with ESdMeEA, was determined by means of TLC. Only one sample contained a detectable amount of EAENES. By addition of the different components, figures close to 100% were obtained. The R.S.D. for EAES was never higher than 1.0%. Sample No. 1 was used as a secondary standard. It was analysed 47 times over a period of 20 days with an R.S.D. of 0.9% on the peak area corresponding to EAES.

TABLE IV

COMPOSITION (%, w/w) OF BULK SAMPLES OF ERYTHROMYCIN ETHYLSUCCINATE

Manufacturer	Sample No.	EA	ESdMeEA[a]	EFES[b]	ECES	EB	EAES (*n*, R.S.D., %)	EBES	EAENES	H_2O[c] (*n*, R.S.D., %)	Total
A	2	1.0	1.0	0.5	0.8	<0.5	92.8 (3, 0.1)	0.7	<0.15	1.7 (5, 2.2)	98.5
B	6	0.6	0.5	0.5	0.5	3.0	75.0 (3, 0.7)	16.4	<0.15	1.6 (4, 1.7)	98.1
	13	1.0	0.5	<0.5	0.5	<0.5	94.9 (3, 0.7)	2.3	<0.15	1.5 (3, 3.0)	100.7
C	9	0.7	0.4	<0.5	1.2	<0.5	94.6 (3, 1.0)	0.6	<0.15	1.8 (3, 1.5)	98.7
D	14	1.1	1.1	<0.5	1.7	<0.5	91.8 (3, 1.0)	2.8	0.4	1.4 (3, 4.6)	100.3
E	1	1.0	0.8	0.5	1.2	<0.5	93.1 (3, 0.9)	2.3	<0.15	1.4 (7, 5.3)	100.3

[a] No correction was applied for EFES, eluted together with ESdMeEA. Under the conditions used, the specific absorbance of ESdMeEA is about five times larger than that of EFES.
[b] Evaluated by TLC.
[c] KF titration.

Results obtained by analysis of specialties are reported in Table V. The content of EAES ranged from 90.4 to 112.9% of the label claim, with EA being the most important impurity (up to 6.3%). The higher content of EA in specialties compared with that in bulk samples suggests that some EA is formed during processing. This is consistent with the lability of the ester towards hydrolysis.

The results show that LC is very suitable for the determination of erythromycin ethylsuccinate in bulk samples and in preparations.

TABLE V

COMPOSITION OF SPECIALTIES AS A PERCENTAGE (w/w) OF LABEL CLAIM

Manufacturer	Sample No.	Pharmaceutical form[a]	EA	ESdMeEA	ECES	EAES (*n*, R.S.D.)	EBES	Total
A	19	G	4.0	0.4	0.5	91.6 (3, 0.4)	1.0	97.5
	21	T	1.0	0.8	1.5	95.3 (3, 0.4)	0.6	99.2
E	22	DP	0.4	0.9	1.7	96.6 (3, 0.8)	1.9	101.5
F	15	DP	6.3	0.4	0.5	112.9 (3, 0.6)	0.8	120.9
G	18	DP	3.1	0.3	0.7	90.4 (3, 1.0)	0.6	94.5

[a] DP = Dispersible powder, G = granules, T = tablets.

ACKNOWLEDGEMENTS

The authors thank Mrs. A. Decoux for skilful secretarial assistance. Dr. R. Busson is acknowledged for the NMR spectrometry.

REFERENCES

1 V. C. Stephens and J. W. Conine, *Antibiot. Ann.*, (1959) 346.
2 *European Pharmacopoeia*, Maisonneuve, Sainte Ruffine, 2nd ed., 1984, monograph 274.
3 *United States Pharmacopeia XXII*, United States Pharmacopeial Convention, Rockville, MD, 1988, p. 524.
4 K. Tsuji and J. F. Goetz, *J. Chromatogr.*, 157 (1978) 185.
5 K. Tsuji and M. P. Kane, *J. Pharm. Sci.*, 71 (1982) 1160.
6 K. Tsuji, *J. Chromatogr.*, 158 (1978) 337.
7 D. Croteau, F. Vallée, M. C. Bergeron and M. LeBel, *J. Chromatogr.*, 419 (1987) 205.
8 Th. Cachet, I. O. Kibwage, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 409 (1987) 91.
9 I. O. Kibwage, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 330 (1985) 275.
10 P. F. Wiley, K. Gerzon, E. H. Flynn, M. V. Sigal, O. Weaver, U. C. Quarck, R. R. Chauvette and R. Monahan, *J. Am. Chem. Soc.*, 79 (1957) 6062.
11 P. Kurath, P. H. Jones, R. S. Egan and T. J. Perun, *Experientia*, 27 (1971) 362.
12 I. O. Kibwage, G. Janssen, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 346 (1985) 309.
13 Th. Cachet, C. Haest, R. Busson, G. Janssen and J. Hoogmartens, *J. Chromatogr.*, 445 (1988) 290.
14 Th. Cachet, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 403 (1987) 343.
15 E. H. Flynn, H. W. Murphy and R. E. McMahon, *J. Am. Chem. Soc.*, 77 (1955) 3104.
16 I. O. Kibwage, R. Busson, G. Janssen, J. Hoogmartens, H. Vanderhaeghe and J. Bracke, *J. Org. Chem.*, 52 (1987) 990.
17 J. Hoogmartens, E. Roets, G. Janssen and H. Vanderhaeghe, *J. Chromatogr.*, 244 (1982) 299.
18 B. Steffansen and H. Bundgaard, *Int. J. Pharm.*, 56 (1989) 159.
19 Th. Cachet and J. Hoogmartens, *J. Pharm. Biomed. Anal.*, 6 (1988) 461.
20 J. G. Nourse and J. D. Roberts, *J. Am. Chem. Soc.*, 97 (1975) 4584.
21 Th. Cachet, K. De Turck and J. Hoogmartens, *J. Pharm. Biomed. Anal.*, 9 (1991) 547.
22 Th. Cachet, I. Quintens, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.*, 12 (1989) 2171.
23 Th. Cachet, I. Quintens, J. Paesen, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.*, 14 (1991) 1203.

Journal of Chromatography, 600 (1992) 109–113
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 928

High-performance liquid chromatography of benzodiazepines using sorbents with thermally immobilized Carbowax 20M

Irena Choma*, Andrzej L. Dawidowicz and Roman Lodkowski

Department of Chemical Physics, Marie Curie-Skłodowska University, Marie Curie-Skłodowska Sq. 3, 20-031 Lublin (Poland)

ABSTRACT

The use of siliceous supports (controlled-porosity glass or silica gel) with thermally immobilized Carbowax 20M as sorbents for the separation of benzodiazepines by high-performance liquid chromatography (HPLC) was studied. The physico-chemical and chromatographic properties of these sorbents were compared with those of LiChrosorb DIOL and LiChrosorb RP-18. The results indicate that normal-phase HPLC chromatography of benzodiazepines using sorbents with thermally immobilized Carbowax 20M is a simple and effective method characterized by a simple binary mobile phase, very short column equilibration, high selectivity and a short time of analysis.

INTRODUCTION

Benzodiazepines belong to a group of anti-insomnia and anti-anxiety drugs [1]. In some instances the level of these drugs and their metabolites in body fluids such as blood or urine need to be monitored and controlled [2]. The methods most frequently used for this purpose are spectrophotometry [3,4], electrochemical methods [5,6], radioimmunoassay [7–9], supercritical fluid chromatography [10], gas chromatography (GC) [4,11,12], thin-layer chromatography [13–15] and high-performance liquid chromatography (HPLC) [8,14,16–21]. Chromatographic methods are superior to the others owing to their selectivity. They allow the separation and determination of individual drugs and their metabolites. However, GC analysis is complicated by the need for derivatization and the thermal instability of some drugs, particularly chlordiazepoxide and oxazepam. For this reason, HPLC analysis is prefered to GC.

According to the literature, the separation of benzodiazepines by HPLC is performed mainly using reversed-phase (RP) systems on sorbents composed of a silica support material with chemically bonded alkyl radicals (octyl or octadecyl) [9,14,22]. In these instances multi-component and buffered mobile phases are used [14,19,23,24]. However, RP columns working with buffered phases have a short lifetime. In addition, the analysis requires time-consuming pH adjustment depending on the composition of the sample mixture. Ion-pair chromatography [20,25] involves prolonged column equilibration before analysis.

In order to avoid the above disadvantages, in normal-phase chromatography can be considered [21,26,27]. Our preliminary investigations with silica gel as a column packing showed that unmodified silica materials are unsuitable. However, it was found that the use of less polar sorbents should resolve the problem. Such sorbents can be prepared by chemical bonding or immobilization of medium-polarity stationary phases to a silica support. This paper deals with the determination of benzodiazepines using a normal-phase HPLC system on siliceous supports with a bonded diol phase and a Carbowax 20M chain. The results are compared with those obtained in RP systems.

0021-9673/92/$05.00

EXPERIMENTAL

Preparation of sorbents with immobilized Carbowax 20M

The modified Aue–Daniewski procedure was employed [28,29]: 4-g portions of LiChrosorb Si 100 (Merck, Darmstadt, Germany) or controlled-porosity glass (CPG) (prepared in our laboratory) were placed in a round-bottomed flask that contained 100 ml of hexadecane (International Enzymes, Windsor, UK). A stream of nitrogen with an oxygen content below 2 ppm (Róży-Luxemburg Factory, Warsaw, Poland), additionally dried on molecular sieves 4A and 5A (Fluka, Buchs, Switzerland), was turned on 30 min before the heating began. A 4-g amount of Carbowax 20M (Aldrich, Milwaukee, WI, USA) was placed in a small funnel located between the flask and a reflux condenser. Heating was then started. First, most of the hexadecane liquefied on the wall of the condenser without touching the polymer. Subsequently, the cold finger of the condenser was cooled with air and the walls of the flask and the condenser were insulated, so vapour of hexadecane melted the polymer so that it dropped into the flask. After about 15 min all the polymer was in the flask and the process was continued for 5 h. The temperature of immobilization was controlled by a thermocouple set in the flask and maintained in the range 270–275°C. After allowing it to cool under nitrogen to 25–30°C, the hexadecane was decanted and the sorbent washed a few times with hexane (Reachim, Moscow, USSR) and chloroform (POCH, Gliwice, Poland). The material was then placed in a stainless-steel column (300 × 8 mm I.D.) thermostated at 60°C and washed with 250 ml each of hexane, chloroform and methanol (POCH) at a flow-rate of 1 ml/min. The solvents were pumped by means of a syringe-type pump. The sorbent wat then removed from the column and dried on a Schott funnel. Controlled-porosity glass and silica gel with immobilized Carbowax 20M are referred to as CPG-C and Si100-C, respectively.

Preparation of porous glass

Controlled-porosity glass (CPG) was obtained from Vycor-type alkali–boron–silica glass. This material was converted into the porous form by appropriate thermal and chemical treatment as decribed elsewhere [30]. In order to obtain the packing for the HPLC columns, the porous glass was ground and the *ca.* 8-μm fraction was separated.

Other materials

LiChrosorb DIOL (referred to as SiDiol) and LiChrosorb RP-18, particle size 10 μm (Merck), were used as additional columns packings.

Chromatographic measurements

Chromatographic measurement were made using a Liquochrom 2010 liquid chromatograph (MIM, Budapest, Hungary) equipped with a UV detector. The sorbents were packed into stainless-steel columns (250 × 4 mm I.D.) using the balanced-density slurry method. As the mobile phase in the normal-phase system isopropanol–hexane (30:70) was employed and in the reversed-phase system methanol–acetonitrile–0.005 *M* KH_2PO_4 buffer (pH 6.0) (26.5:16.5:57) was used (isopropanol from POCH; acetonitrile from Fluka). In all instances the flow-rate was 1 ml/min. Analyte substances (see Table I) were obtained from Polfa (Tarchomin, Poland).

Physico-chemical measurements

Specific surface area, pore diameter and pore volume were determined using a Sorptomat 1800 (Carlo Erba, Milan, Italy) on the basis of nitrogen adsorption–desorption hysteresis. The Carbowax content was calculated from CHN analysis data (Type 185 CHN Analyzer; Hewlett-Packard, Avondale, PA, USA).

Particle size fractionation

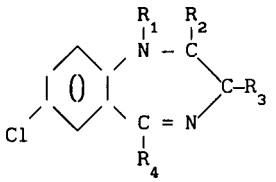
Particle size fractionation was carried out using Multi-Plex Laboratory Classifier 100 MZR (Alpina, Germany).

RESULTS AND DISCUSSION

The physico-chemical properties of the sorbents and siliceous supports of immobilized Carbowax 20M employed are given in Table II. The values of the specific surface area (S), mean pore diameter (D) and pore volume (V_p) of the porous glass (CPG) and silica gel (Si100) are similar. Immobilization of Carbowax 20M leads to some changes in the physico-chemical properties of sorbents obtained in this way (CPG-C and Si100-C), mainly a decrease in S

TABLE I

STRUCTURAL FORMULAE OF BENZODIAZEPINES



Name	R_1	R_2	R_3	R_4
Diazepam (Relanium)	$-CH_3$	=O	H, H	
Oxazepam (Serax)	-H	=O	H, OH	
Medazepam (Norbrium)	$-CH_3$	H, H	H, H	
Nitrazepam (Mogadon)				
Chlorodiazepoxide (Elenium)				Cl^-

and V_p. As can be seen from Table II, the amounts of bonded polymer are almost the same.

Retention data obtained for a benzodiazepine mixture on columns packed with the discussed materials are given in Table III. The capacity factors indicate that unmodified siliceous materials are not useful for analyse for benzodiazepines, at least with the applied chromatographic system. The differences among the capacity factors of the injected compounds are very small and some of the compounds, *e.g.*, chlordiazepoxide and oxazepam, do not appear in the chromatogram. This is connected with

TABLE II

PHYSICO-CHEMICAL PROPERTIES OF THE SORBENTS

S = specific surface area; V_p = pore volume; D_{max} = pore diameter corresponding to the maximum of the pore size distribution function; w/S = amount of Carbowax 20M immobilized on 1 m^2 of sorbent surface..

Sorbent	S (m^2/g)	V_p (cm^3/g)	D_{max} (Å)	$(w/S) \cdot 100$ (g/m^2)
CPG	347	1.16	100	–
Si100	318	1.32	140	–
SiDiol	230	0.90	100	–
CPG-C	92	0.39	100	0.154
Si100-C	119	0.62	130	0.147

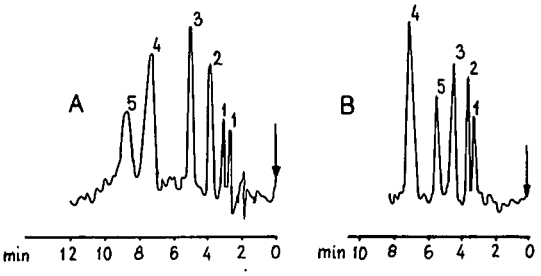


Fig. 1. Chromatograms of benzodiazepines in normal-phase system. Chromatographic conditions as described under Experimental. (A) CPG-C; (B) SiDiol. Peaks: 1 = medazepam; 2 = diazepam; 3 = chlordiazepoxide; 4 = oxazepam; 5 = nitrazepam.

the zone spreading effect caused by adsorption. When sorbents with an immobilized Carbowax 20M layer are used as column packings, the separations are considerably improved, especially with the CPG-C sorbent. This sorbent effects the complete separation of the benzodiazepines.

As was mentioned, SiDiol shows a polarity similar to those of packings with immobilized Carbowax 20M. The capacity factors of benzodiazepines obtained on SiDiol are given in Table III. The main difference between these data and those obtained on CPG-C is the elution sequence of oxazepam and nitrazepam. In addition, the capacity factors of benzodiazepines obtained on the SiDiol column are slightly lower than those obtained on the CPG-C column. The respective chromatograms are presented in Fig. 1. As can be seen from both Fig. 1 and Table III, medazepam is eluted from the CPG-C column as two peaks. Medazepam was obtained from a pharmaceutical manufacturer as a pure clinical preparation. It can be assumed that the two peaks correspond to geometric isomers and are separated owing to the high selectivity of the Carbowax 20M phase (the same is observed with Si100-C). It is also probable that they are products of the decomposition of medazepam (the drug was not capsuled), but Carbowax 20M still seems to be a more selective phase than SiDiol.

Fig. 2. shows a chromatogram of nitrazepam, oxazepam, chlordiazepoxide and diazepam obtained on LiChrosorb RP-18 in an RP system (the corresponding capacity factors are given in the last column of Table III). Medazepam was excluded from the mixture because of its extremely long elution time. Despite this, the analysis of the four drugs

TABLE III

CAPACITY FACTORS (k') OF BENZODIAZEPINES

Mobile phase (flow-rate 1 ml/min): (A) isopropanol–hexane (30:70); (B) methanol–acetonitrile–0.005 M KH_2PO_4 buffer (pH 6.0) (26.5:16.5:57).

Substance	k'					
	CPG (A)	CPG-C (A)	Si100 (A)	Si100-C (A)	SiDiol (A)	RP-18 (B)
Medazepam	0.59	0.51 0.77	0.56	0.44 0.61	0.43	–
Diazepam	0.83	1.22	0.86	1.13	0.65	19.3
Chlorodiazepoxide	–	1.90	–	1.64	1.05	11.0
Oxazepam	1.02	3.26	–	4.49	2.25	7.1
Nitrazepam	0.91	4.09	1.00	4.49	1.48	5.2

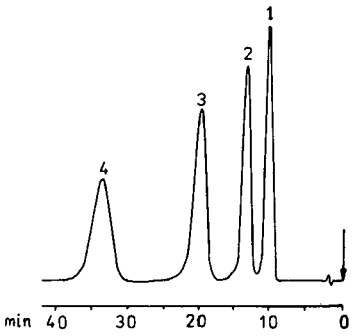


Fig. 2. Chromatograms of benzodiazepines in reversed-phase system. Chromatographic conditions as described under Experimental. Peaks: 1 = nitrazepam; 2 = oxazepam; 3 = chlordiazepoxide; 4 = diazepam.

present requires much more time than that for all five drugs on Carbowax 20M or Diol phase. Probably by using gradient elution and increasing the flow-rate it would be possible to separate all the described drugs (including non-polar medazepam) in an RP system. However, even then, comparing the mobile phase preparation and equipment, the advantages lie with isocratic chromatography in a normal-phase system.

CONCLUSIONS

Normal-phase chromatography of benzodiazepines using sorbents with thermally immobilized Carbowax 20M is a simple and effective method. Its main advantages are a simple binary mobile phase, very short column equilibration before analysis, high selectivity and a short time of analysis.

REFERENCES

1 M. Linnoila, in E. Coster (Editor) *The Benzodiazepines — From Molecular Biology to Clinical Practice*, Raven Press, New York, 1983, p. 267.
2 H. Schütz, *Benzodiazepines — A Handbook*, Springer, Heidelberg, 1982, p. 21, 24, 123.
3 P. Jatlow, *Clin. Chem.*, 18 (1972) 516.
4 I. A. Werner, H. Altorfer and X. Perlia, *Chromatographia*, 30 (1990) 225.
5 M. R. Hackman, M. A. Brooks and J. A. F. de Silva, *Anal. Chem.*, 46 (1974) 1045.
6 M. A. Brooks, *Bioelectrochem. Bioenerg.*, 10 (1983) 37.
7 I. L. Slightom, J. C. Cagle, H. H. McCurdy and F. Castagna, *J. Anal. Toxicol.*, 6 (1982) 22.
8 T. Yamaguchi, R. Konaka, K. Sugeno, K. Hirai and M. Kono, *J. Chromatogr.*, 417 (1987) 216.
9 W. R. Dixon, J. Earley and E. Postma, *J. Pharm. Sci.*, 64 (1976) 231.
10 R. M. Smith, M. Marsin sanagi, *J. Chromatogr.*, 483 (1989) 51.
11 C. Drouet-Coassolo, C. Aubert, P. Coassolo and J. P. Cano, *J. Chromatogr.*, 487 (1989) 295.
12 H. Maurer and K. Pfleger, *J. Chromatogr.*, 422 (1987) 85.
13 T. Inoue and T. Niwaguchi, *J. Chromatogr.*, 339 (1985) 163.
14 K. Valko, S. Olajos, T. Cserhati, *J. Chromatogr.*, 499 (1990) 361.
15 M. Rojowska, M. Przyborowska and H. Szumilo, *Chem. Anal. (Warsaw)*, 33 (1988) 581.
16 P. M. Klockowski and G. Levy, *J. Chromatogr.*, 422 (1987) 334.
17 C. M. Selavka and I. S. Krull, *J. Liq. Chromatogr.*, 10 (1987) 345.
18 T. B. Vree, A. M. Baars, Y. A. Hekster and E. van der Kleijn, *J. Chromatogr.*, 224 (1981) 519.
19 P. Mura, A. Piriou, P. Fraillon, Y. Papet and D. Reiss, *J. Chromatogr.*, 416 (1987) 303.
20 E. I. Minder, R. Schaubhut, Ch. E. Minder and D. J. Vonder-Schmitt, *J. Chromatogr.*, 419 (1987) 135.
21 M. C. Pietrogrande, F. Dondi, G. Blo, P. A. Borca and C. Bighi, *J. Liq. Chromatogr.*, 11 (1988) 1313.
22 *Supelco Rep.*, 5, No. 3 (1986) 3.
23 M. Mazhar, and S. R. Binder, *J. Chromatogr.*, 497 (1989) 201.
24 N. de Giovanni and M. Chiarotti, *J. Chromatogr.*, 428 (1988) 321.
25 C. J. Sohr and A. T. Buechel, *J. Anal. Toxicol.*, 6 (1982) 286.
26 I. Fellegvari, J. Visy, K. Valko, T. Lang and M. Simonyi, *J. Liq. Chromatogr.*, 12 (1989) 2719.
27 H. Stampfli, G. Patil, R. Sato and C. Y. Quon, *J. Liq. Chromatogr.*, 13 (1990) 1285.
28 M. M. Daniewski and W. A. Aue, *J. Chromatogr.*, 147 (1978) 119.
29 W. A. Aue, M. M. Daniewski, J. Müller and J. P. Laba, *Anal. Chem.* 49 (1977) 1465.
30 A. L. Dawidowicz, A. Waksmundzki and A. Derylo, *Chem. Anal. (Warsaw)*, 24 (1979) 811.

Journal of Chromatography, 600 (1992) 115–121
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 848

Retention of basic drugs on porous polymers in high-performance liquid chromatography

Barbara Gawdzik

Faculty of Chemistry, Maria Curie Skłodowska University, Pl. M. Curie-Skłodowskiej 3, 20031 Lublin (Poland)

ABSTRACT

The behaviour of basic drugs on two polymeric high-performance liquid chromatographic columns in the presence of alkaline mobile phases was studied. The packings were PRP-1 and di(methacryloyloxymethyl)naphthalene–divinylbenzene copolymer. Using the alkyl aryl ketone scale, the retention indices for ephedrine, clonidine, lidocaine, benzocaine, procaine, propranolol, diazepam, strychnine, cyproheptadine, amitriptyline and promethazine were calculated. To determine the influence of the pH of the mobile phase on the retention of these compounds, peak asymmetry factors and reduced plate heights were measured.

INTRODUCTION

In recent years there has been growing interest in polymeric materials for use in reversed-phase high-performance liquid chromatography (HPLC), the most popular being macroporous styrene–divinylbenzene copolymers [1–9]. Unfortunately, polymeric sorbents are characterized by some important drawbacks such as swelling in strong organic solvents owing to the presence of micropores in the polymeric matrix and low column efficiencies [8,9]. On the other hand, porous polymers are stable with eluents over the whole pH range [10]. This important feature permits the direct separation of basic compounds at high pH.

Commonly used alkyl-bonded silica cannot be employed for the chromatography of amines in an uncharged from because the pH limit of silica is about 8 and many of these supports possess strongly adsorptive properties. To diminish these effects, deactivating agents such as aliphatic amines were added to the eluent [11] or eluents containing a high proportion of an organic component [12] and different buffers were applied [13–16]. In spite of this, limited selectivity and poor peak shapes were often achieved.

According to Jane [17], the retention of basic compounds is predominantly controlled by the analyte pK_a. To determine them in a non-ionized form, a mobile phase with pH − pK_a > 2 should be used [18]. For this reason, PRP-1 and Amberlite XAD-2 porous copolymers have been applied for the determination of basic drugs in alkaline eluents [18,19].

In this work, a comparison of two polymeric columns in the reversed-phase HPLC separation of basic drugs was made. The retention of basic drugs was studied on a column packed with a copolymer of two cross-linking agents, di(methacryloyloxymethyl)naphthalene and divinylbenzene (DMN–DVB), whose properties were studied previously [20–22], and on a PRP-1 styrene–divinylbenzene column (Hamilton, Reno, NV, USA). Methanol–aqueous sodium hydroxide (90:10) was employed according to De Biasi *et al.* [18] as the optimum mobile phase for reasonably short retention times of the studied basic drugs on the PRP-1 column.

Using mobile phases of different pH (8.9, 10.3, 11.5 and 12.5), the effect of pH on retention data and peak symmetry was determined.

In order to determine the selectivities of the columns, retention indices for the studied basic drugs based on the alkyl aryl ketone scale were calculated.

0021-9673/92/$05.00

EXPERIMENTAL

Chemicals and mobile phases

Methanol of HPLC grade was obtained from Merck (Darmstadt, Germany) and sodium hydroxide of laboratory-reagent grade from POCh (Gliwice, Poland). Mobile phases were made up by volumetric mixing of methanol with 0.001, 0.005, 0.05 and 0.25 *M* sodium hydroxide solution, prepared from sodium hydroxide pellets. The pH of the mobile phases was measured with a PHM-64 pH meter (Radiometer, Copenhagen, Denmark). All mobile phases were filtered through suitable filters and degassed by agitation in an ultrasonic bath and kept under a weak stream of helium. Alkyl aryl ketones were of laboratory-reagent grade from a range of sources.

Basic drugs were kindly provided by the Medical Academy in Lublin, Poland. Except for benzocaine, diazepam and strychnine, they were in a form of hydrochlorides. Sample solutions were prepared at a concentration of *ca.* 0.1 mg/ml by dissolving the bases in methanol–water (90:10, v/v) [18]. Sodium nitrate (30 mg/ml) in the same mobile phase was used as a void volume marker [23].

HPLC equipment

Chromatographic measurements were carried out using an HPLC system consisting of a Techma-Robot (Warsaw, Poland) Model 302 syringe pump fitted with a Rheodyne (Cotati, CA, USA) Model 7125 injection valve equipped with a 10-μl sample loop and a Laboratórni Přístroje (Prague, Czechoslovakia) LCD 2563 UV–visible detector set at 254 nm, [16,23] and 0.04 a.u.f.s.

The columns used were a 150 × 4.1 mm I.D. column packed with PRP-1 (5 μm) and a 100 × 4 mm I.D. column packed with DMN–DVB porous copolymer (3–10 μm). The columns were maintained at 30°C in a circulating water-bath. The measuring cell of the detector was also water thermostated at 30°C.

TABLE I

PEAK ASYMMETRY FACTORS (A_s) AND REDUCED PLATE HEIGHTS (h) FOR BASIC DRUGS MEASURED ON DMN–DVB AND PRP-1 COLUMNS

Column	Drug	pH 8.9		pH 10.3		pH 11.5		pH 12.5	
		A_s	h (μm)	A_s	h (μm)	A_s	h (μm)	A_s	h (μm)
DMN–DVB	Ephedrine	1.2	51	1.0	51	1.0	30	1.0	29
	Clonidine	1.0	32	1.0	35	1.0	31	1.0	23
	Lidocaine	1.2	57	1.0	37	1.0	35	1.0	31
	Benzocaine	1.0	38	1.0	39	1.0	35	1.0	29
	Procaine	1.7	71	1.5	71	1.2	35	1.2	34
	Propranolol	–	–	–	–	1.4	54	1.3	36
	Diazepam	1.8	95	1.8	95	1.6	92	1.3	84
	Strychnine	2.4	178	2.2	178	1.8	109	1.4	69
	Cyproheptadine	2.0	178	2.0	142	1.8	109	1.8	109
	Amitriptyline	–	–	1.7	142	1.0	71	1.0	51
	Promethazine	1.8	95	1.8	102	1.6	95	1.2	84
PRP-1	Ephedrine	2.0	186	2.0	171	1.2	37	1.0	30
	Clonidine	1.5	58	1.3	29	1.2	34	1.2	31
	Lidocaine	1.5	42	1.3	40	1.3	36	1.3	36
	Benzocaine	1.4	42	1.0	27	1.0	30	1.0	29
	Procaine	2.0	44	1.2	31	1.2	31	1.2	30
	Propranolol	–	–	2.5	64	1.6	61	1.5	52
	Diazepam	3.4	166	3.2	166	2.8	150	2.8	153
	Strychnine	3.4	230	3.0	162	2.7	142	2.5	136
	Cyproheptadine	3.4	214	2.8	187	1.8	166	1.7	150
	Amitriptyline	2.0	214	1.5	156	1.5	115	1.2	117
	Promethazine	3.0	187	2.0	142	1.8	100	1.4	109

Peaks were recorded on a Laboratórni Přístroje Model 4001 recorder. The recorded retention distances were the means of three determinations. The flow-rate of the mobile phase was 0.5 ml/min. At the end of each working day the column was flushed with methanol–water (90:10, v/v).

Titration

A 1-g amount of the DMN–DVB copolymer in 20 ml of 1 *M* NaCl and 10 ml of methanol was titrated potentiometrically using an automatic titrator set and PHM-64 digital pH meter (Radiometer); 0.01 *M* sodium hydroxide solution and 0.01 *M* hydrochloric acid were used for titration. For each point on the potentiometric titration curve, the equilibrium state with respect to the display of the pH meter was achieved.

Chromatographic measurements

Capacity factors (k') were calculated as $k' = (l_R - l_0)/l_0$, where l_R and l_0 are the retention distances on the chromatogram of the sample and a non-retained sample (sodium nitrate), respectively. Retention indices (I) derived from the alkyl aryl ketones from butyrophenone to heptanophenone were calculated as described previously [20,22]. Peak asymmetry factors (A_s) were measured at 10% of the peak height [24]. Reduced plate heights, $h = H/d_p$, where H is the theoretical plate height and d_p (μm) is the particle diameter, were calculated to compare column packings of different particle diameters [24].

RESULTS AND DISCUSSION

Accordding to De Biasi *et al.* [18], basic drugs can be successfully separated on a PRP-1 polymeric column using mobile phases of high pH and with a high percentage of the organic component. Following their work, the chromatographic behaviour of a range of basic drugs with methanol–aqueous sodium hydroxide solutions (90:10, v/v) as mobile phases was studied. In order to investigate te influence of pH on retention four mobile phase of different pH were used.

In Table I, peak asymmetry factors (A_s) and reduced plate heights (h) for the bases determined on the DMN–DVB and PRP-1 columns are summarized. At pH 8.9 on both columns significant asymmetry of the peaks is visible. The peak shapes improve with increasing pH of the mobile phase. As the pK_a values of the studied amines do not exceed 10 (Table II), in a mobile phase of pH 12.5 they should be in a non-ionized form and peak asym-

TABLE II

IONIZATION CONSTANTS OF BASIS DRUGS [23,25]

Drug	Ionization constant	Drug	Ionization constant
Ephedrine	9.6	Diazepan	3.3
Clonidine	8.2	Strychnine	2.3; 8.0
Lidocaine	7.9	Cypropheptadine	–[a]
Benzocaine	2.5	Amitryptyline	9.4
Procaine	9.0	Promethazine	9.1
Propranolol	9.5		

[a] Not available.

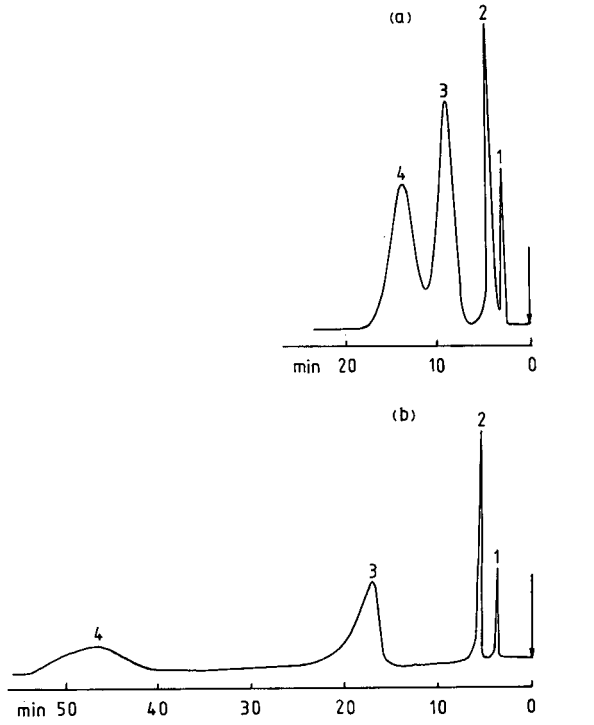


Fig. 1. Separation of basic drugs on polymeric columns: (a) DMN–DVB; (b) PRP-1. Peaks: 1 = ephedrine; 2 = benzocaine; 3 = strychnine; 4 = promethazine. Mobile phase, methanol–0.25 *M* NaOH (90:10, v/v); flow-rate, 0.5 ml/min; column inlet pressure, 17.4 MPa for DMN–DVB and 10.2 MPa for PRP-1; detection, 254 nm.

metry should disappear. Unfortunately, even at this pH significant asymmetry is observed, for cyproheptadine, strychnine and diazepam on the PRP-1 column and for cyproheptadine on the DMN–DVB column.

From a comparison of the chromatograms (Fig. 1), one can conclude that the efficiency of the PRP-1 column is much better but the values of the reduced plate heights are very similar. On both columns the reduced plate heights depend on the pH of the mobile phase, with the lowest values occurring at pH 12.5. Simultaneously, the efficiency of these columns decreases with increasing retention.

Tables III and IV give the capacity factors for the basic drugs and the alkyl aryl ketone standards used for calculation of retention indices. On both copolymers, changes in the pH of the mobile phase cause marked changes in the capacity factors of the basic drugs. Only diazepam and benzocaine behave exceptionally, their capacity factors remaining nearly constant independently of the mobile phase used. This lack of sensitivity to pH changes is due to their low ionization constants (Table II). For the other bases a decrease in the capacity factors is characteristic. On the PRP-1 column it occurs below pH 10.3 and on the DMN–DVB column below pH 11.5.

Changes in the pH of the mobile phase have a smaller influence on the k' values for alkyl aryl ketones. On the DMN–DVB column, an increase in pH causes a regular decrease in the k' values of alkyl aryl ketones but on the PRP-1 column their retentions are fairly constant. Such a behaviour of these copolymers results from their different chemical character. PRP-1 copolymer does not possess any functional groups whereas DMN–DVB copolymer has ester groups in a polymeric network and these groups are responsible for the sensitivity

TABLE III

CAPACITY FACTORS (k') AND RETENTION INDICES (I) FOR BASIC DRUGS OBTAINED ON THE DMN–DVB POLYMERIC COLUMN

Compound	pH 8.9		pH 10.3		pH 11.5		pH 12.5	
	k'	I	k'	I	k'	I	k'	I
Alkyl aryl ketones								
Acetophenone	1.18	841	1.16	856	1.14	854	1.09	854
Propiophenone	1.71	950	1.61	966	1.56	965	1.52	965
Butyrophenone	2.04	1002	1.80	1001	1.74	1001	1.69	1001
Valerophenone	2.82	1098	2.41	1099	2.30	1098	2.25	1098
Hexanophenone	3.98	1198	3.25	1200	3.10	1201	3.09	1203
Heptanophenone	5.67	1302	4.41	1301	4.13	1300	4.10	1299
Basic drugs								
Ephedrine	2.00	997	1.16	856	0.47	500	0.30	410
Clonidine	1.98	993	1.21	869	0.61	638	0.55	621
Lidocaine	0.98	788	0.80	733	0.65	673	0.63	653
Benzocaine	0.94	784	0.94	773	0.86	759	0.79	739
Procaine	6.56	1345	3.57	1230	0.94	792	0.90	784
Propranolol	–	–	–	–	1.45	940	1.25	901
Diazepam	2.43	1062	2.33	1088	2.18	1081	2.18	1086
Strychnine	16.33	1613	9.36	1552	2.82	1169	2.70	1158
Cyproheptadine	8.58	1426	4.86	1333	3.20	1213	3.06	1200
Amitriptyline	–	–	6.57	1434	3.50	1244	3.45	1241
Promethazine	8.24	1412	5.85	1395	3.73	1266	3.69	1263
Void volume for sodium nitrate (ml)	0.93		0.93		0.92		0.92	
Correlation coefficients								
Acetophenone–heptanophenone	0.9963		0.9934		0.9936		0.9937	
Butyrophenone–heptanophenone	0.9998		0.9999		0.9999		0.9998	

TABLE IV

CAPACITY FACTORS (k') AND RETENTION INDICES (I) FOR BASIC DRUGS OBTAINED ON THE PRP-1 POLYMERIC COLUMN

Compound	pH 8.9		pH 10.3		pH 11.5		pH 12.5	
	k'	I	k'	I	k'	I	k'	I
Alkyl aryl ketones								
Acetophenone	2.97	796	2.90	797	2.90	795	2.92	796
Propiophenone	5.00	922	4.85	921	4.83	921	4.90	921
Butyrophenone	7.00	1003	6.77	1002	6.71	1002	6.85	1002
Valerophenone	10.29	1096	10.00	1096	9.87	1097	10.18	1098
Hexanophenone	15.79	1199	15.49	1201	15.07	1201	15.54	1200
Heptanophenone	24.14	1301	23.39	1300	22.57	1300	23.53	1301
Basic drugs								
Ephedrine	2.80	782	1.09	562	0.69	449	0.65	435
Clonidine	2.58	766	1.19	583	1.10	557	1.07	556
Lidocaine	2.21	725	2.06	716	2.14	721	2.10	718
Benzocaine	1.53	636	1.55	619	1.37	615	1.39	619
Procaine	2.17	720	1.68	666	1.66	658	1.66	661
Propranolol	–	–	4.36	896	3.74	858	3.49	841
Diazepam	7.29	1013	7.27	1019	7.50	1023	7.25	1017
Strychnine	9.93	1087	6.29	985	6.36	989	6.11	975
Cyproheptadine	23.70	1297	18.07	1242	18.03	1241	18.20	1237
Amitriptyline	29.43	1356	21.27	1283	21.11	1278	21.37	1276
Promethazine	23.57	1296	18.42	1243	18.19	1242	18.15	1238
Void volume for sodium nitrate (ml)	1.18		1.18		1.18		1.18	
Correlation coefficients								
Acetophenone–heptanophenone	0.9987		0.9989		0.9988		0.9987	
Butyrophenone–heptanophenone	0.9997		0.9998		0.9998		0.9998	

of the copolymer to pH changes. The weakly acidic character of the DMN–DVB copolymer, confirmed by the titration curve (Fig. 2), increases the dissociation of basic drugs. Hence a mobile phase of higher pH than for the PRP-1 copolymer is required for the separation of bases in a non-ionized form. Nevertheless, the decrease in k' for basic drugs observed on the PRP-1 column also suggests the existence of weakly acidic groups in this copolymer.

On comparing the retention indices of the basic drugs (Figs. 3 and 4), differences in the retention mechanism on these two copolymers can be seen. On both copolymers, an increase in the mobile phase pH generally causes a decrease in retention indices. The retention indices of diazepam and benzocaine do not change with increase in pH. The other amines are more or less ionized, so their retention changes in comparison with alkyl aryl ketones. The influence of the mobile phase pH on the retention indices of basic drugs is especially noticeable with the DMN–DVB copolymer (S-shapes curves). In a mobile phase of pH 12.5, in which interactions of ion-exchange character disappear, the differences in copolymer selectivities are more visible. At this pH the retention indices of the basic drugs are greater on the DMN-DVB than on the PRP-1 column. This means that some specific interactions between the copolymer with ester functional groups and the solutes take place.

The results presented here indicate that the retention of basic drugs depends on the pH of the mobile phase but the mechanism is complex and involves the degree of ionization of the analytes and the chemical character of the polymeric phase. In mobile phases of high pH interactions of ion-exchange character disappear but the separation efficiency of basic drugs on both of the copolymers studied is still poor.

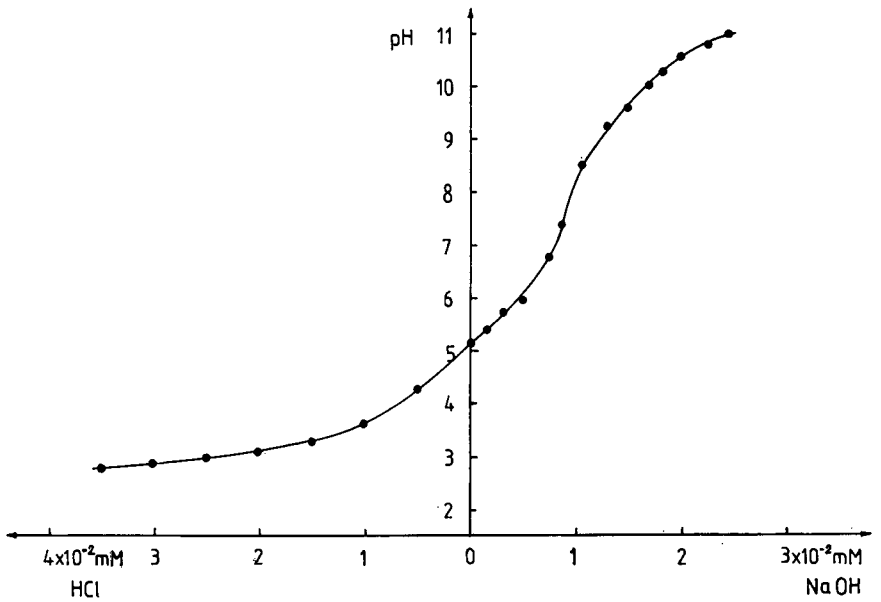


Fig. 2. Potentiometric titration of DMN–DVB porous copolymer.

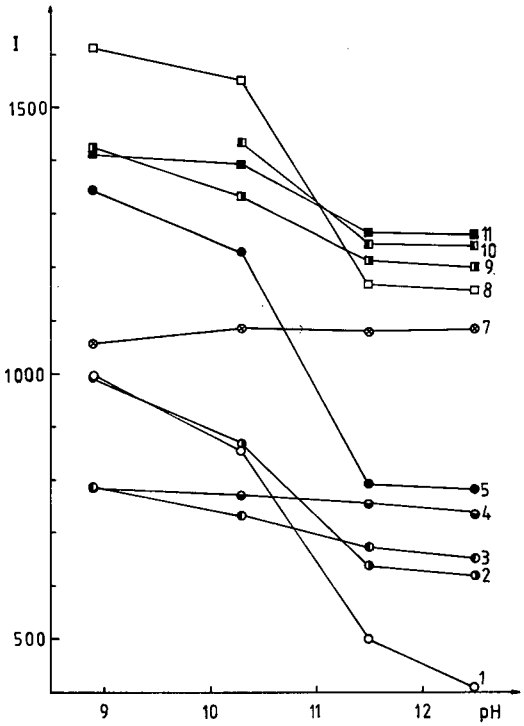


Fig. 3. Effect of mobile phase pH on retention indices of basic drugs obtained on the DMN–DVB column. Drugs: 1 = ephedrine; 2 = clonidine; 3 = lidocaine; 4 = benzocaine; 5 = procaine; 7 = diazepam; 8 = strychnine; 9 = cyproheptadine; 10 = amitriptyline; 11 = promethazine.

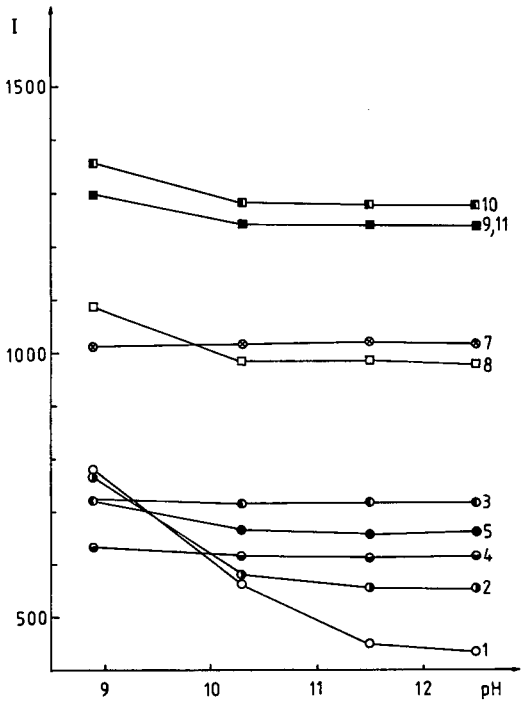


Fig. 4. Effect of mobile phase pH on retention indices of basic drugs obtained on the PRP-1 column. Drugs as in Fig. 3.

REFERENCES

1 Z. Iskandarani and D. J. Pietrzyk, *Anal Chem.*, 53 (1981) 489.
2 Z. Iskandarani and D. J. Pietrzyk, *Anal Chem.*, 54 (1982) 1065.
3 J. W. Benson and D. J. Woo, *J. Chromatogr. Sci.*, 22 (1984) 386.
4 R. M. Smith, *J. Chromatogr.*, 291 (1984) 372.
5 R. M. Smith and D. R. Garside, *J. Chromatogr.*, 407 (1987) 19.
6 L. D. Bowers and S. Pedigo, *J. Chromatogr.*, 371 (1986) 243.
7 L. L. Lloyd and F. P. Warner, *J. Chromatogr.*, 512 (1990) 365.
8 H. W. Stuurman, J. Köhler, S. O. Jansson and A. Litzen, *Chromatographia*, 23 (1987) 341.
9 F. Nevejans and M. Verzele, *J. Chromatogr.*, 406 (1987) 325.
10 D. P. Lee, *J. Chromatogr. Sci.*, 20 (1982) 203.
11 R. Gill, S. P. Alexander and A. C. Moffat, *J. Chromatogr.*, 247 (1982) 39.
12 S. O. Badiru and T. M. Jefferies, *J. Pharm. Biomed. Anal.*, 6 (1988) 859.
13 R. J. Flanagan and I. Jane, *J. Chromatogr.*, 323 (1985) 173.
14 I. Jane, A. McKinnon and R. J. Flanagan, *J. Chromatogr.*, 323 (1985) 191.
15 D. J. Smith, *J. Chromatogr. Sci.*, 19 (1981) 253.
16 R. M. Smith, J. P. Westlake, R. Gill and M. D. Osselton, *J. Chromatogr.*, 514 (1990) 97.
17 I. Jane, *J. Chromatogr.*, 111 (1975) 227.
18 V. De Biasi, W. J. Lough and M. B. Evans, *J. Chromatogr.*, 353 (1986) 279.
19 H. J. E. M. Reeuwijk and U. R. Tjaden, *J. Chromatogr.*, 353 (1986) 339.
20 B. Gawdzik, J. Gawdzik and U. Czerwińska-Bil, *Chromatographia*, 26 (1988) 399.
21 B. Gawdzik, *Chromatographia*, 31 (1991) 21.
22 B. Gawdzik, *J. Chromatogr.*, 549 (1991) 77.
23 R. M. Smith, T. G. Hurdley, R. Gill and M. D. Osselton, *J. Chromatogr.*, 398 (1987) 73.
24 S. Coppi, A. Betti, C. Bighi, G. P. Cartoni and F. Coccioli, *J. Chromatogr.*, 442 (1988) 97.
25 J. E. F. Reynolds (Editor), *Martindale: The Extra Pharmacopoeia*, Pharmaceutical Press, London, 28th ed., 1982.

Journal of Chromatography, 600 (1992) 123–127
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 910

Gas chromatographic separation of *cis–trans* isomers of alkylcyclohexylbenzenes on a capillary column with a liquid crystalline stationary phase

J. Mazur*, Z. Witkiewicz and R. Dąbrowski

Institute of Chemistry, Military Technical Academy, 01 489 Warsaw (Poland)

ABSTRACT

Liquid crystal derivatives of cyclohexylbenzene with an isothiocyanate group have interesting properties with respect to application in displays. However, only *trans* isomers of these compounds are useful for this purpose, hence separation of the isomers during synthesis and the determination of the amount of the waste *cis* isomer are necessary. The *cis–trans* isomers can readily be separated and analysed using short (15 m) glass capillary columns coated with a liquid crystalline stationary phase. A liquid crystal derivative with an isothiocyanate group has been applied. For comparison, some chromatographic separations on Hewlett-Packard Ultra-2 column were performed. Because of the specific retention mechanism of the liquid crystalline phase it was possible to separate isomers with better selectivity and in a shorter time than on the Ultra-2 column. However, as a result of its high efficiency, the Ultra-2 column is better for the chromatographic separation of the other by-products of the synthesis.

INTRODUCTION

Trans isomers of alkylcyclohexylbenzene derivatives with an isothiocyanate group are a family of liquid crystal compounds that are attractive for application in displays. The usefulness of these compounds is greater than that of the analogous cyano compounds because of their superior physicochemical properties, *viz.*, a smaller bulk viscosity, smaller bend elastic constants, smaller dipole moment, lower dielectric anisotropy and higher birefringence [1–3]. Liquid crystal isothiocyanates make it possible to obtain a wide assortment of liquid crystalline mixtures revealing good chemical stability and high display dynamics; the method of their synthesis has been published [4].

Liquid crystalline isothiocyanates and intermediate products of their syntheses exist in two isomeric forms, *cis* and *trans*. The final liquid crystal products of the syntheses should not contain *cis* isomers, hence it is necessary to remove *cis* isomers from all the intermediate products and to determine the contents of waste *cis* isomers in the intermediate products.

The *cis* isomers are removed step by step from successive intermediate products by applying different methods such as crystallization, distillation and column liquid chromatography [4].

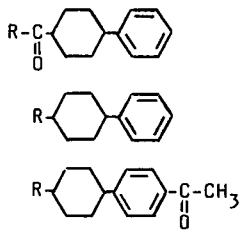
For the determination of the content of *cis* isomers we applied gas chromatography on a column with a liquid crystalline stationary phase. Liquid crystalline stationary phases provide a unique selectivity for the separation of many classes of isomers whose molecules have different length-to-breadth ratios [5–10]. Considering the high boiling points of the analyte isomers, only liquid crystalline stationary phases having high thermal resistance can be used.

EXPERIMENTAL

Compounds

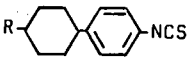
Intermediate products of liquid crystal syntheses whose *cis–trans* isomers were analysed have the following general formulae:

0021-9673/92/$05.00



where R = C_1–C_8 alkyl. These compounds were isolated from the reaction mixtures and 0.1–0.4 μl of 1–2.5% solutions in dichloromethane (POCh, Gliwice, Poland) were injected into the gas chromatograph. Some raw reaction mixtures containing all by-products of the syntheses were also analysed.

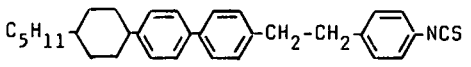
We also attempted to determine the purity and *cis–trans* isomer contents in the final liquid crystal products of the syntheses. Compounds with the following general formula were analysed:



where R = C_2–C_8 alkyl.

Chromatographic procedure

The formula of the 1-[4'-(4-*trans*-pentylcyclohexyl)biphenyl]-2-(4-isothiocyanatophenyl)ethane liquid crystal used as the stationary phase is



and the temperatures of the phase transitions are

$$K \overset{135°C}{\rightleftharpoons} S \overset{163°C}{\rightleftharpoons} N \overset{280°C}{\rightleftharpoons} I$$

the phases being the solid crystal (K), smectic mesophase (S), nematic mesophase (N) and isotropic liquid (I). Its synthesis and some of its chromatographic applications have been reported [11–13].

The liquid crystalline stationary phase was deposited statically in a glass capillary column (12 m × 0.3 mm I.D.) according to the procedure described previously [13]. The column efficiency was about 700 theoretical plates/m (capacity factor = 7.53; pyrene; temperature 250°C). Column preparation was performed at Maria Curie-Skłodowska University, Lublin, Poland.

Some analyses were carried out on a fused-silica capillary column (25 m × 0.2 mm I.D.) with a 0.33-μm film of non-mesomorphic Ultra-2 stationary phase (Hewlett-Packard). The column efficiency was 4900 theoretical plates/m (capacity factor = 6.98).

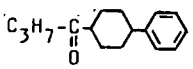
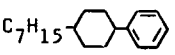
A Pye Unicam (Cambridge, UK) Model GCV gas chromatograph equipped with a split-stream injector and a flame ionization detector was used. The carrier gas was helium and the make-up gas was nitrogen.

RESULTS AND DISCUSSION

The separations of *cis–trans* isomers of alkylcyclohexylbenzenes on a capillary column with a liquid crystalline stationary phase are characterized by high selectivity because of the specific retention

TABLE I

RELATIVE RETENTION TIMES (α) OF *TRANS–CIS* ISOMERS

Compound	Column temperature (°C)	α	
		On column with liquid crystal	On column with Ultra-2
	226	1.80	1.09
	200	2.18	1.13

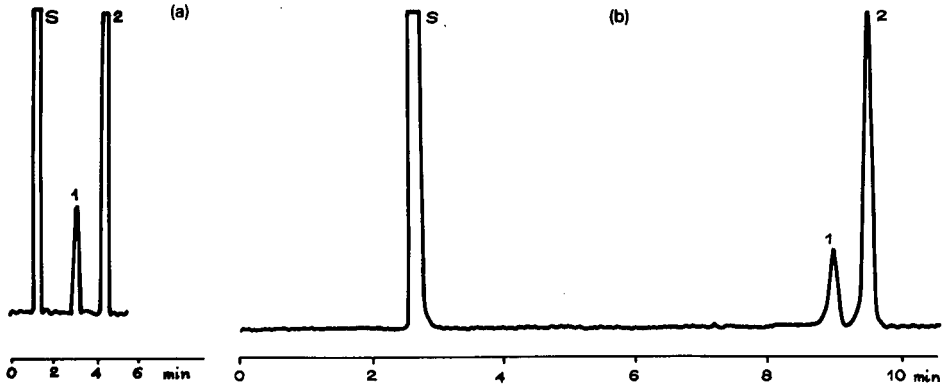


Fig. 1. Chromatograms of *cis-trans* isomers of butanoylcyclohexylbenzene. (a) On capillary column with liquid crystalline stationary phase. Column temperature, 226°C; injector and detector temperatures, 340°C; carrier gas (helium) linear velocity, 20 cm/s; make-up gas (nitrogen) flow-rate, 40 cm^3/min; splitting ratio, 1:100. (b) On capillary column with Ultra-2 stationary phase. Carrier gas (helium) linear velocity, 16 cm/s; other conditions as in (a). Peaks: 1 = *cis* isomer; 2 = *trans* isomer; S = solvent.

mechanism. The *trans* isomers, having a larger length-to-breadth ratio than the *cis* isomers, were eluted with a longer retention time. Comparison of the separation selectivity (expressed in terms of the relative retention time of *trans* isomers with respect to the *cis* isomers obtained on the two columns indicates an advantage with the use of the column with the liquid crystalline phase. The relative retention times of *trans–cis* isomers of two compounds are given in Table I.

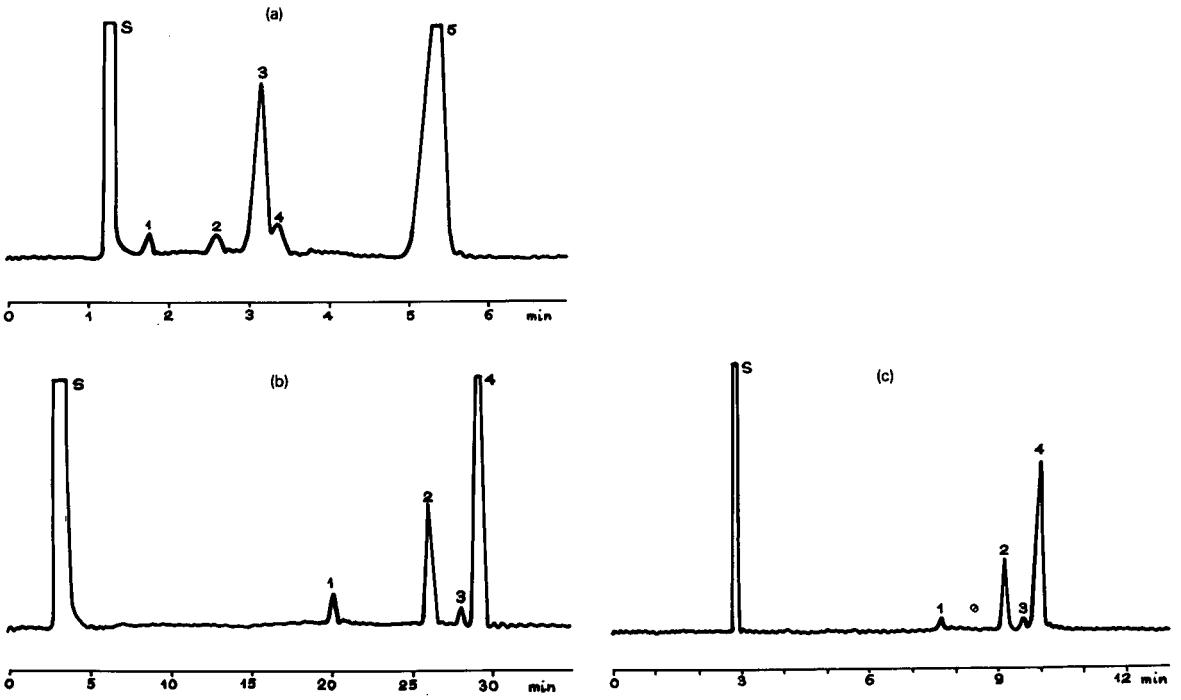


Fig. 2. Chromatograms of *cis–trans* isomers of 4-heptylcyclohexylbenzene. (a) On capillary column with liquid crystalline stationary phase. Column temperature, 200°C; injector and detector temperatures, 320°C; carrier gas (helium) linear velocity 20 cm/s; make-up gas (nitrogen) flow-rate, 40 cm^3/min; splitting ratio, 1:100. Peaks: 1,2,4 = unidentified substances; 3 = *cis* isomer; 5 = *trans* isomer; S = solvent. (b) On capillary column with Ultra-2 stationary phase. Carrier gas (helium) linear velocity, 16 cm/s; other conditions as in (a). Peaks: 1,3 = unidentified substances; 2 = *cis* isomer; 4 = *trans* isomer; S = solvent. (c) chromatogram as in (b) obtained at 240°C.

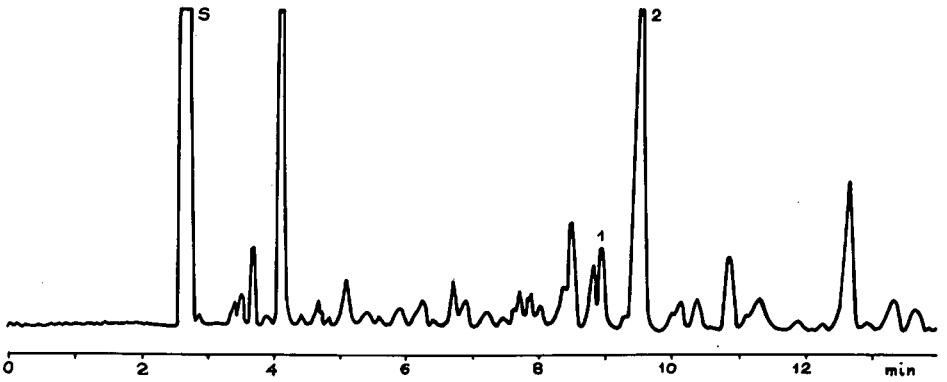


Fig. 3. Chromatogram of reaction mixture after synthesis of butanoylcyclohexylbenzene obtained on a capillary column with Ultra-2 stationary phase. Carrier gas (helium) linear velocity, 16 cm/s; other conditions as in Fig. 1a. Peaks: 1 = *cis* isomer; 2 = *trans* isomer; S = solvent.

The time required to obtain chromatograms on the column with the liquid crystalline phase was considerably shorter than that on the column with Ultra-2 (see Figs. 1 and 2, which show the chromatograms of the *cis–trans* isomers of the substances in the Table I obtained on both columns).

As a result of the much higher efficiency of the Ultra-2 column, the separations of by-products in the raw reaction mixtures after syntheses are better on this column (see Fig. 3).

Examples of the chromatographic separation of *cis–trans* isomers of other compounds are shown in Figs. 4 and 5.

An attempt to perform the chromatographic analysis of liquid crystals on the column with the liquid crystalline stationary phase gave negative results. The similarity of the molecular shapes of the stationary phase and the analyte liquid crystals resulted in very high retentions, some retention times even being over 60 min. Under the same conditions the liquid crystals were eluted from the Ultra-2 column in 10–15 min (Fig. 6).

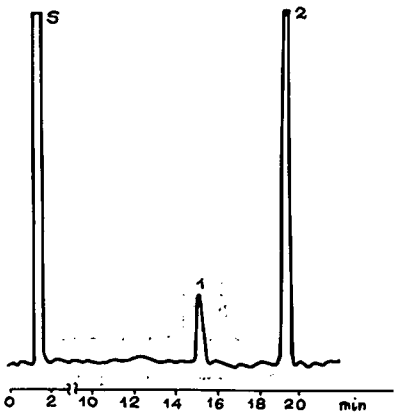


Fig. 4. Chromatogram of *cis–trans* isomers of 4-(4-pentylcyclohexyl)acetophenone on a capillary column with liquid crystalline stationary phase. Conditions as in Fig. 1a. Peaks: 1 = *cis* isomer; 2 = *trans* isomer; S = solvent.

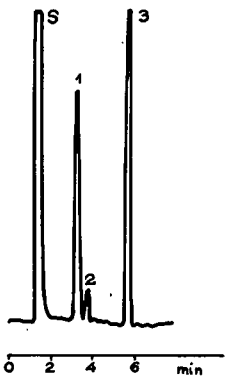


Fig. 5. Chromatogram of *cis–trans* isomers of 4-propylcyclohexylbenzene on a capillary column with liquid crystalline stationary phase. Column temperature, 165°C; other conditions as in Fig. 1a. Peaks: 1 = *cis* isomer; 2 = impurity; 3 = *trans* isomer; S = solvent.

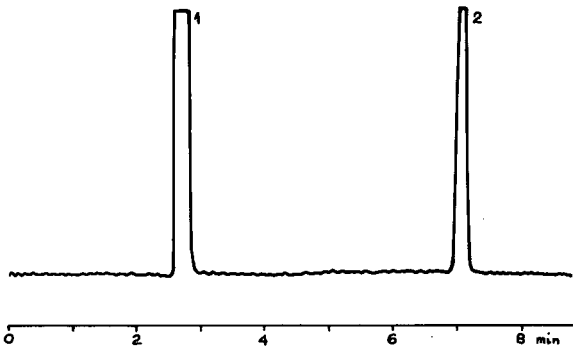


Fig. 6. Chromatographic analysis of the purity of 4-(isothiocyanatophenyl)-1-(*trans*-4-propyl)cyclohexane on a capillary column with Ultra-2 stationary phase. Column temperature, 280°C injector and detector temperatures, 360°C; carrier gas (helium) linear velocity, 16 cm/s; other conditions as in Fig. 1a. Peaks: 1 = dichloromethane (solvent); 2 = *trans* isomer of liquid crystal.

CONCLUSIONS

The application of a liquid crystalline stationary phase to the chromatographic determination of *cis–trans* isomers of alkylcyclohexylbenzenes allows the analysis time to be shortened in comparison with that on the Ultra-2 column.

The chromatographic separation of raw syntheses mixtures which contain many by-products are better on the Ultra-2 column. After purification, for checking isomer contents, it is preferable to apply the column with the liquid crystalline stationary phase.

The use of liquid crystalline stationary phases for analysing liquid crystals is unsatisfactory because of very long retention times.

REFERENCES

1 R. Dąbrowski, *Mol. Cryst. Liq. Cryst.*, 191 (1990) 17.
2 Cz. Puchała, W. Wacławek and R. Dąbrowski, *Biul. Wojsk. Akad. Tech.*, 37 No. 1 (1988) 3.
3 J. W. Baran, Z. Raszewski, R. Dąbrowski, J. Kędzierski and J. Rutkowska, *Mol. Cryst. Liq. Cryst.*, 123 (1985) 237.
4 R. Dąbrowski, J. Dziaduszek, T. Szczuciński, Z. Stolarz, J. Zieliński and K. Kenig, *US Pat.*, 4 528 116 (1985); *Eur. Pat.*, 0126883 (1987).
5 Z. Witkiewicz, *J. Chromatogr.*, 466 (1989) 37.
6 J. Mazur and Z. Witkiewicz, *LC·GC Int.*, 5 (1990) 34.
7 L. Sojak, I. Ostrovsky, R. Kubinec, G. Kraus and A. Kraus, *J. Chromatogr.*, 509 (1990) 93; 520 (1990) 75.
8 T. J. Betts, *J. Chromatogr.*, 513 (1990) 311.
9 I. Nesterova, B. Rekhter, G. Roshka and Z. Witkiewicz, *J. Chromatogr.*, 537 (1991) 482.
10 J. P. Kithinji, M. W. Raynor, B. Egia, I. L. Davies, K. D. Bartle and A. A. Clifford, *J. High Resolut. Chromatogr.*, 13 (1990) 27.
11 J. Mazur, Z. Witkiewicz and R. Dąbrowski, *Pol. Pat. Appli.*, P-268841 (1987).
12 J. Mazur, Z. Witkiewicz and R. Dąbrowski, *Biul. Wojsk. Akad. Tech.*, 37, No. 9 (1988) 33.
13 J. Mazur, Z. Witkiewicz and R. Dąbrowski, *J. Chromatogr.*, 455 (1988) 323.

Journal of Chromatography, 600 (1992) 129–132
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 943

Integral Doppler anemometry in porous membranes for the analysis of liquid mixtures and examination of membrane properties

S. N. Semenov

Institute of Chemical Physics, Russia's Academy of Sciences, Kosygin str. 4, 117977 Moscow (Russia)

ABSTRACT

The possibility of field flow fractionation (FFF) using integral Doppler anemometry (IDA) in the pores of transparent membranes is theoretically examined. The IDA spectra were calculated for cylindrical pores, where a transverse force exists owing to the electrical field of the double electrical layer on the pore walls. Two possibilities were considered: (a) particles are repulsed from the wall and focused near pore axis, and (b) they are attracted to the wall and concentrated in its vicinity. The IDA spectra are essentially different in these cases and this circumstance may be the basis for programming analyses of liquid mixtures containing ampholytic particles. It is shown that FFF in pores is possible if the pore-size distribution is narrow enough. If the pore-size distribution is broad, IDA can be used for the investigation of this distribution. It is also shown that such an investigation is most convenient if particles are focused near the pore axis. In this case IDA spectra averaged over a large number of pores are especially simply linked to the pore-size distribution and do not depend on the interaction of a particle with an electrical field in a pore.

INTRODUCTION

The application of correlation spectroscopy to studies of liquid mixture in a laminar flow and a transverse force field has been proposed [1–3] and a clear dependence of averaged spectra of scattering on the transverse particle distribution has been shown. This distribution is due to the interaction of particles with a transverse force field and to thermal motion. As a result of theoretical examination, the experimental method of analytical field flow fractionation (FFF) called integral Doppler anemometry (IDA) has been developed. The main features of IDA are continuous injection of the sample mixture and a short time of analysis, approximately equal to the time of relaxation in FFF [4–6]. However, a further decrease in analysis time is very desirable, especially in programming processes of analysis. For this purpose channels with a small transverse size are necessary.

THE MATHEMATICAL PROBLEM

Porous membranes containing pores transparent to light may be a suitable system for IDA. For example, nuclear filters may be used; basically they represent polymer films, in which pores are produced by the action of heavy ions in accelerators of electrically charged particles. These membranes may have positive or negative surface electrical charge at different pH values. The transverse electrical field in the pores causes redistribution of the charged particles driven by flow through the pores. The IDA spectra, depending on the transverse distribution of particles, may provide information about the properties of particles and also the properties of the pores and membrane surface. For particle examination it is necessary to use calibrated pores. For example, nuclear filters with an average pore size of 0.5–2.0 μm with a pore size dispersion of about 5% may be used. In this case the main problem is the

0021-9673/92/$05.00

calculation of IDA spectra in a cylindrical capillary, where transverse force and laminar flow exist. As in a flat channel, the spectrum of scattering on a single particle can be considered as

$$S_0(\omega) = \delta(\omega - qu) \tag{1}$$

where ω is frequency, q is the wave vector of scattering (difference between the wave vectors of the initial and scattered light) and u is the velocity of the scattering particle. Eqn. 1 can be used at $u \gg qD$, where D is the diffusion coefficient of the particle. Usually these parameters are $q \approx 10^4\ \mathrm{cm}^{-1}$ and $D \lesssim 10^{-6}\ \mathrm{cm}^2/\mathrm{s}$, tha is, u must be $\gg 10^{-2}$ cm/s. Because all particles scatter laser light independently, the integral spectrum of scattering averaged over the cross-section of the channel can be written as

$$S_1(\omega) = 2\pi \int_0^R r\mathrm{d}r W(r) S_0[\omega - qu(r)] \tag{2}$$

where r is the distance from the axis of the capillary, $W(r)$ is the probability of finding a particle at distance r, $u(r)$ is the flow profile in the capillary and R is the pore radius. If we neglect electroosmotic flow, the flow profile can be considered as

$$u(r) = u_0[1 - (r/R)^2] \tag{3}$$

where

$$u_0 = \Delta p R^2/4\eta h \tag{4}$$

is the maximum flow velocity, Δp is the pressure difference at the membrane applied, η is the viscosity of the liquid and h is the thickness of the membrane. Substituting eqn. 1 in eqn. 2, we find that

$$S_1(\omega) = 2\pi r(qu) W[r(qu)]\ \mathrm{d}r/\mathrm{d}(qu)|_{qu=\omega} \tag{5}$$

where

$$r(qu) = R(1 - qu/qu_0)^{1/2} \tag{6}$$

according to eqn. 3. The probability $W(r)$ is considered as a Boltzmann distribution:

$$W(r) = \mathrm{e}^{-qf(r)/kT} \Big/ \int_0^R 2\pi r\mathrm{d}r \mathrm{e}^{-qf(r)/kT} \tag{7}$$

where q is the effective electrical charge of a particle, $f(r)$ is the potential of the double electrical layer in the pore and kT is the thermal energy. Usually the energy of electrostatic interaction in double electrical layers is about $10\ kT$. Therefore, the distribution in eqn. 7 must be very narrow: particles repulsed from the pore wall are focused near its axis and attracted particles are concentrated in the vicinity of the wall. In the former instance eqn. 7 can be written as

$$W(r) = [E_0 \mathrm{e}^{-E_0(r/R)^2}]/\pi R^2 \tag{8}$$

and in the latter as

$$W(r) = [E_0 \mathrm{e}^{-E_0(R-r)/R}]/2\pi R^2 \tag{9}$$

where $E_0 = \alpha q f_0/kT$, $\alpha \approx 1$ and f_0 is the potential of the double electrical layer. The coefficient α is defined by the distribution of potential, $f(r)$; for example, if this distribution is parabolic, $\alpha = 1$ in eqn. 8 and $\alpha = 2$ in eqn. 9. Substituting eqns. 6 and 8 in eqn. 5, we find that

$$S_1(\omega) = [E_0 \mathrm{e}^{-E_0(1-\omega/qu_0)}]/qu_0 \quad \text{if } \omega \leqslant qu_0$$
$$S_1(\omega) = 0 \quad \text{if } \omega > qu_0 \tag{10}$$

for particles focused near the axis of the pore. The spectrum represenred by eqn. 10 has an asymmetric peak which has a tail of width *ca.* qu_0/E_0 (see Fig. 1). The maximum of this peak is at $\omega = qu_0$. The spectra of particles with different charges q must have different widths and the general spectrum of mixture containing these particles can be a source of information about the particle charges.

The velocities of particles attracted to the wall are much less than u_0 and instead of eqn. 6 we can use the aprpoximate expression

$$r \approx R(1\text{–}2qu/2qu_0) \tag{11}$$

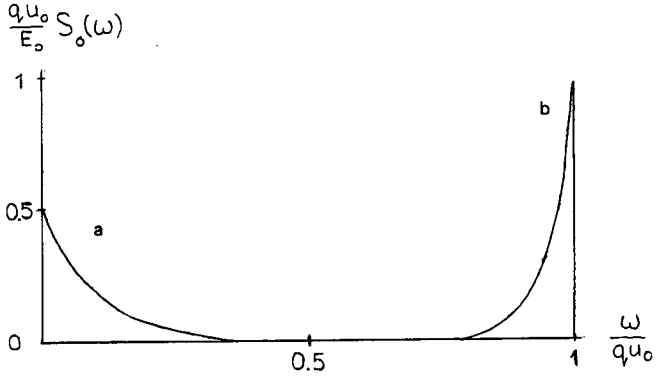


Fig. 1. IDA spectra in a cylindrical capillary: (a) particles attracted to the wall; (b) particles focused near the axis. $E_0 = 20$.

Substituting eqns. 9 and 11 in eqn. 5, we find that

$$S_1(\omega) = (E_0 e^{-E_0\omega/2qu_0})/2qu_0 \quad (12)$$

The IDA spectrum of attracted particles occurs at $\omega \approx 2qu_0/E_0 \ll qu_0$ and has a qualitatively different shape than the spectrum represented by eqn. 10 (see Fig. 1). The difference between the spectra of attracted and repulsed particles can constitute the basis for a programmed analysis of mixtures containing ampholytic particles. This process may be controlled by programmed pH changes with concomitant changes in particle charge. Of course, changes in the potential distribution, $f(r)$, must be small or known at any pH. The IDA spectrum with a uniform transverse particle distribution will be used in further considerations. It has been calculated [7] and has a rectangular shape:

$$S_1(\omega) = qu_0 \quad \text{if } \omega \leqslant (qu_0)^{-1} \quad (13)$$

$$S_1(\omega) = 0 \quad \text{if } \omega > (qu_0)^{-1}$$

Usually a large number of pores are simultaneously illuminated by laser light. If the pore-size distribution is narrow, the observed spectrum is still described by eqns. 10 and 12. However, if the pore-size distribution is broad, the IDA spectrum observed on unit area of the membrane must be written as

$$\langle S_1(\omega)\rangle = N_0 c_0 h \int_0^\infty S_0[\omega, E_0, q\Delta p R^2/4\eta h]\pi R^2 n(R)\mathrm{d}R \quad (14)$$

where $n(R)$ is the pore-size distribution normalized in the following way:

$$\int_0^\infty n(R)\mathrm{d}R = 1$$

c_0 is the concentration of particles scattering laser light, N_0 is the number of pores in unit area of the membrane and h is the thickness of the membrane. Eqn. 4 for the maximum flow velocity u_0 is used in eqn. 14; the spectrum of scattering on a single particle is also normalized per unit area, similarly to eqn. 1. It is taken into account also that the IDA spectrum in any pore is proportional to the number of particles present, which is equal to $c_0\pi R^2 h$. Eqn. 14 can be expressed in another way using the pore distribution according to the cross-sectional area, σ, $n^*(\sigma)$:

$$\langle S(\omega)\rangle = N_0 c_0 h \int_0^\infty S_0[\omega, E_0, \omega_0(\sigma/\sigma_0)]\sigma n^*(\sigma)\mathrm{d}\sigma \quad (15)$$

where $\sigma_0 = \Delta p/4\eta$ and $\omega_0 = \pi q/h$. The averaged IDA spectrum gives the possibility of finding pore distributions $n(R)$ and $n^*(\sigma)$ by solving integral eqns. 14 and 15. In some situations, however, the relationship between $\langle S_0(\omega)\rangle$ and the pore distributions $n(R)$, $n^*(\sigma)$, can be simplified. For example, if particles are focused near the pore axis. The IDA spectrum of this single pore has the shape of a narrow peak, the width of which is more or less equal to the dispersion of pore distribution $n^*(\sigma)$. In this case a "unit" spectrum $S_0(\omega)$ in eqn. 15 can be expressed as

$$S_0(\omega) = \delta[\omega - \omega_0(\sigma/\sigma_0)] \quad (16)$$

Substituting eqn. 16 in eqn. 15, we obtain the following expression for the averaged IDA spectrum:

$$\langle S_0(\omega)\rangle = N_0 c_0 h(\sigma_0/\omega_0)^2 \sigma n^*(\sigma)|_{\sigma=\sigma_0(\omega/\omega_0)} \quad (17)$$

Eqn. 17 shows that focusing of particles near the pore axis simplifies the problem: the registered averaged IDA spectrum permits the pore distribution $n^*(\omega)$ to be reconstructed immediately. The second advantage of these conditions is the weak dependence of the averaged spectra on the details of the particle interaction with the electrical field in the pores: eqn. 17 does not contain the parameter E_0. This indicates that calibrated particles are not necessary for measurement, it is only necessary to use particles focusing near the pore axis. These conditions may be realized if $E_0 \gg 1$ and if the width of the pore distribution $n^*(\sigma)$ is $\gg E_0^{-1}$. A similar situation arises with a uniform transverse distribution of particles in the pores if they are uncharged or there is no electrical field. Substituting eqn. 13 into eqn. 15 and taking derivatives, we find that

$$\mathrm{d}[\langle S(\omega)\rangle]/\mathrm{d}\omega = N_0 c_0 (\sigma_0/\omega_0)^2 n^*(\sigma)\sigma|_{\sigma=\sigma_0(\omega/\omega_0)} \quad (18)$$

Eqn. 18 shows that the distribution $n^*(\sigma)$ can be obtained from averaged IDA spectra relatively

simply if the particles are uniformly distributed across the pores.

Next, it is necessary to determine the limits of approximations used in the calculations and the conditions necessary to carry out informative experiments. The time of transverse relaxation in an electrical field to a Boltzmann distribution, $\tau_\perp$, must be much less than the time for a particle to passing through the pore, $\tau_\parallel$. The time of relaxation, $\tau_\perp$, is *ca.* R^2/E_0D. E_0 can be evaluated as bf_0/D, where b is the electrophoretic mobility. If the particle size is about 10^{-5} cm, usually $b \approx 10^{-4}$ cm^2/V · s and $D \approx 10^{-7}$ cm^2/s. The characteristic value of the surface potential is *ca.* 10^{-1} V. Hence $E_0 \approx 100$ for such particles and the time of transverse relaxation $\tau_\perp \approx 10^{-3}$–$10^{-2}$ s if the pore radius R is about 10^{-4} cm. The time of passing $\tau_\parallel$ is *ca.* h/u_0. Usually h is about 10^{-2} cm and the velocity u_0 satisfying the condition $u_0 \gg qD$ is about 10^{-2} cm/s. Under these conditions $\tau_\parallel \approx 10\tau_\perp$ and a Boltzmann distribution of particles is established along the whole pore length. According to eqn. 4, these flow velocities need a pressure gradient across the membrane of $\Delta p/h \approx 10^4$ g/cm^3. This value is comparable to the pressure gradient across a horizontal membrane due to the force of gravity.

CONCLUSIONS

Registration of averaged IDA spectra of particles driven by flow through a transparent membrane can provide a source of information about the particle charge distribution and their dependence on pH if the pore-size distribution is narrow. The investigation of porosity parameters determining the selectivity of membrane separation processes is possible if the pore-size distribution is broader. In the latter instance the shape of the averaged IDA spectrum is particularly simply related to the pore-size distribution if particles are focused near the pore axis. The data obtained by a pure hydrodynamic process of particles passing through pores can give the most direct information about the parameters determining membrane selectivity.

REFERENCES

1 S. N. Semenov and V. L. Kononenko, *Russ. J. Phys. Chem.*, 60 (1986) 3117.
2 S. N. Semenov and V. L. Kononenko, *Russ. J. Phys. Chem.*, 61 (1987) 1947.
3 V. L. Kononenko and S. N. Semenov, *Russ. J. Phys. Chem.*, 60 (1986) 000.
4 S. N. Semenov, V. L. Kononenko and Ya. K. Shimkus, *J. Chromatogr.*, 446 (1988) 141.
5 V. L. Kononenko and Ya. K. Shimkus, *J. Chromatogr.*, 520 (1990) 270.
6 V. L. Kononenko and Ya. K. Shimkus, *Russ. J. Phys. Chem.*, 64 (1990) 000.
7 V. V. Blazhenkov, V. V. Vlasenko, F. M. Penkov and S. I. Zheglov, *Zh. Prikl. Mech. Tech. Fiz.*, No. 2 (1987) 24.

Journal of Chromatography, 600 (1992) 133–137
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 942

Transverse particle redistribution in a flat channel for SPLITT or integral Doppler anemometry

S. N. Semenov

Institute of Chemical Physics, Russia's Academy of Sciences, Kosygin str. 4, 117977 Moscow (Russia)

ABSTRACT

The solution of the convective diffusion equation for the case of a flat channel with a transverse focusing force is proposed. It is shown that in practically important situations this solution can be expressed as a series containing Hermite polynomials. Using this solution, the transversal distributions of particles at different distances and corresponding to different distributions at the channel input were calculated. The situations in which there are uniform and delta-function-like transverse distributions of particles were examined. First, these curves show that transverse non-equilibrium peaks of concentration may exist if the distance from the channel input is short enough. Second, it is confirmed that there exists a possibility of high resolution under very non-equilibrium conditions. The transversal peaks of concentration may be used for the analysis of fractions that cannot be separated under equilibrium conditions.

INTRODUCTION

Recently, methods of continuous liquid mixture separation and analysis based on field flow fractionation (FFF) principles have been developed. A splitting of the flow in an FFF channel by means of longitudinal splitters was suggested [1,2] for the separation of different fractions in a field of transverse force. This method was called SPLITT (continuous separation in split-flow thin cell) separation. The analytical variant of continuous FFF has been considered [3–5] in which photon-correlation spectroscopy is used for the visualization of transverse particle distribution, *i.e.*, integral Doppler anemometry (IDA). IDA analysis is easiest if a focusing transverse field is applied. The possibility of protein separation in a SPLITT cell with a transverse electrical field has been demonstrated [6]. IDA spectra have been registered in a flat channel where a transverse focusing hydrodynamic force was acting [7,8]. A model suspension containing relatively large (*ca.* 1 μm) particles was used, and it was shown that the transverse particle distribution was essentially non-equilibrium. Because the particle size was relatively large, the trajectories of particles were used [7,8] for the calculation of IDA spectra, neglecting the Brownian motion. The possibilities of SPLITT separation in a non-equilibrium (transfer) regime have been discussed [6]. Most experimental situations do not permit diffusion to be neglected, and the examination of the convective diffusion equation is necessary.

THE MATHEMATICAL PROBLEM

In a flat channel the dimensionless form of the convective diffusion equation is as follows:

$$\partial/\partial x_1(\partial/\partial x_1 + \partial E/\partial x_1)c = Pe(1 - x_1^2)\partial c/\partial z_1 \qquad (1)$$

where $x_1 = x/h$, $z_1 = z/h$; x, z = transverse and longitudinal coordinates ($x = 0$ is placed in the plane of symmetry and $z = 0$ at the inlet of a channel), h = half-width of a channel, c = concentration, $E(x)$ = transverse potential in kT units, kT = thermal energy and Pe = longitudinal Peclet number:

$$Pe = u_0 h/D$$

where u_0 = maximum flow velocity and D = diffusion coefficient of particles. If the transverse potential is of the focusing type, then

0021-9673/92/$05.00

$$E(x_1) = E_0(x_1 - x_0)^2 \quad (2)$$

where E_0 = transverse Peclet number and x_0 = dimensionless coordinate of a focusing point. The boundary conditions for eqn. 1 take into account the inpermeability of the walls and the constancy of concentration at the channel inlet:

$$(\partial/\partial x_1 + \partial E/\partial x_1)c = 0 \qquad \text{at } x_1 = \pm 1 \quad (3)$$

$$c = c_0 \qquad \text{at } z_1 = 0 \quad (4)$$

where c_0 is the initial concentration. These boundary conditions permit a search for the solution of eqn. 1 in the form

$$c = c_1(x_1)e^{-\lambda z_1} \quad (5)$$

and its transformation into an ordinary differential equation:

$$d/dx_1(d/dx_1 + dE/dx_1)c_1 + Pe\lambda(1 - x_1^2)c_1 = 0 \quad (6)$$

with the boundary conditions in eqn. 3 and eigenvalue λ. The substitution

$$c_1 = u(x_1)e^{-E(x_1)/2} \quad (7)$$

transforms eqn. 6 into an equation of the Sturm–Liouville type:

$$d^2u/dx_1^2 + [E_0 - E_0^2(x_1 - x_0)^2 + Pe\lambda(1 - x_1^2)]u = 0 \quad (8)$$

with the boundary conditions

$$du/dx_1 + E_0(x_1 - x_0)u = 0 \qquad \text{at } x_1 = \pm 1 \quad (9)$$

Different solutions of a Sturm–Liouville equation are orthogonal:

$$\int_{-1}^{1}(1 - x_1^2)u_m u_n dx_1 = \delta_{mn}\int_{-1}^{1}(1 - x_1^2)u_n^2 dx_1 \quad (10)$$

where $n, m = 0, 1, 2 \ldots$ and δ_{mn} = Kronecker symbol.

Eqn. 9 permits the solution of eqn. 1 with potential as in eqn. 2 to be written in the following form:

$$c(x_1,z_1) = \sum_{n=0}^{n=\infty} B_n e^{-\lambda z_1 - E_0(x_1 - x_0)^2/2}u_n(x) \quad (11)$$

where

$$B_n = \int_{-1}^{1} e^{E(x_1)/2}(1 - x_1^2)_n dx_1 / \int_{-1}^{1}(1 - x_1^2)u_n^2 dx_1 \quad (12)$$

After introducing a new coordinate:

$$y = (E_0^2 + Pe\lambda)^{1/4}[x_1 - x_0E_0^2/(Pe\lambda + E_0^2)] \quad (13)$$

we obtain the equation

$$d^2u/dy^2 + \{[(E_0 + Pe\lambda)(E_0^2f_0 + Pe\lambda)/(E_0^2 + Pe\lambda)]/(E_0^2 + Pe\lambda)^{1/2} - y^2\}u = 0 \quad (14)$$

where $f_0 = 1 - x_0^2$, and the boundary conditions are

$$du/dy + E_0(E_0^2 + Pe\lambda)yu = 0 \quad (15)$$

at

$$y = (Pe\lambda + E_0^2)^{1/4}[\pm 1 - E_0^2x_0(E_0^2 + Pe\lambda)] \quad (16)$$

According to eqn. 16, the boundary conditions in eqn. 15 must be satisfied at $y \approx E_0^{1/2} \gg 1$. In this situation we may assume that the boundary conditions in eqn. 15 are satisfied at $y = \pm\infty$. In addition to the conditions in eqn. 15, we must assume that $c = 0$ at the walls of channel, because for the focusing potential in eqn. 2 virtually all the particles are rejected from the walls at sufficiently short distances from the inlet. Usually $E_0 \approx$ 10–100 in FFF, but it has been shown [7,8] that $E_0 \approx 1000$. After our simplification of the boundary conditions, only Hermite functions [9] can be the solutions of eqn. 14:

$$u_n(y) = e^{-y^2/2}H_n(y) \quad (17)$$

where

$$H_n(y) = (-1)^n e^{y^2}d^n(e^{-y^2})/dy^n \quad (18)$$

is the Hermite polynomial. For the eigenvalues λ we obtain the following equation:

$$E_0 + Pe\lambda(E_0^2f_0 + Pe\lambda)/(E_0^2 + Pe\lambda) = (2n + 1)(E_0^2 + Pe\lambda)^{1/2} \quad (19)$$

TRANSVERSE PARTICLE DISTRIBUTIONS AT $Pe\lambda \ll E_0^2$

The expression on the left-hand side of eqn. 19 rises monotonically with λ, and for this reason only one λ corresponds to any n. However, eqn. 19 has no analytical solution. Usually the channel length is *ca.* 1 cm and $h \approx 10^{-2}$ cm, so $z_1 \approx 10^2$ and $\lambda \leqslant z_1^{-1} \approx 10^{-2}$. Under usual FFF conditions E_0 = 10–100 and $Pe \approx 10^3$–10^4, so $Pe\lambda \approx (1$–$10^{-2})E_0^2$. As can be shown [7,8], $Pe \approx 10^6$ and $E_0 \approx 10^3$, so $Pe\lambda \approx 10^{-2}E_0^2$. In other words, in most situations we can

assume that $Pe\lambda \ll E_0^2$, and obtain from eqns. 17–19

$$u_n = e^{-E_0(x_1-x_0)^2/2} H_n[E_0^{1/2}(x_1 - x_0)] \quad (20)$$

$$Pef_0\lambda/E_0 = 2n \quad (21)$$

Eqns. 20 and 21 hold true for $n \ll E_0 f_0/2$. Because $H_n(y) \approx (2y)^n$ for $y \gg 1$, any member of the series 10 is *ca.* $(E_0)^{1/2} \exp(-E_0) \ll 1$ near the walls, and the solution in eqns. 20 and 21 should be considered to be true. The conditions of orthogonality of the polynomials 20 are simpler than eqn. 10:

$$\int_{-\infty}^{\infty} e^{-x^2} H_n(x) H_m(x) dx = \delta_{mn} n! (\pi)^{1/2}$$

and for coefficients B_n we obtain instead of eqn. 12

$$B_n = c_0 \int_{-\infty}^{\infty} H_n[(E)^{1/2}(x_1 - x_0)] dx_1/[2^n n! (\pi)^{1/2}] \quad (22)$$

Using the recurrent relationships [9]

$$d[H_n(y)]/dy = 2nH_{n-1}(y)$$

and the asymptotic expression for $H_n(y)$ at $y \gg 1$, we obtain

$$B_n = c_0(E_0)^{(n+1)/2}[(1 - x_0)^{n+1} - (-1 - x_0)^{n+1}]/[2(\pi)^{1/2}(n + 1)!] \quad (23)$$

Expressions 20, 21 and 23 permit us to obtain the function $c(x_1, z_1)$ for $E_0, Pe \gg 1$ and $n \ll E_0 f_0/2$:

$$c(x_1, z_1) = c_0(E_0/\pi)^{1/2}\{[(E_0)^{1/2} e^{-2E_0 z_1/f_0 Pe}]^n[(1 - x_0)^{n+1} - (-1 - x_0)^{n+1}](-1)^n d[e^{-E_0(x_1-x_0)^2}]/dx_1\}/(n + 1)! \quad (24)$$

With the help of eqn. 24 we can explain most of the phenomena that take place in the process of redistribution of particles in a flat laminar flow and transverse focusing potential.

RESULTS AND DISCUSSION

In the non-equilibrium (transfer [6] regime, transverse peaks of concentration appear owing to the decrease in the transverse force and velocities of particles on approaching the focusing point. In this situation the particles, which are released from the walls, are concentrated in the regions where the transverse force is small. The positions of non-equilibrium peaks, as shown [7,8], can be different at the same distance z for particles with different values of E_0, even if they have the same focusing point, for example $x_0 = 0$. In this case,

$$c(x_1, z_1) = c_0(E_0/\pi)^{1/2} \sum_{n=0}^{n=\infty} [a^n d^{2n}(e^{-E_0 x_1^2})/dx_1^{2n}]/[(2n + 1)!(E_0)^n] \quad (25)$$

where $a = E_0 e^{-(4E_0 z_1/Pe)}$.

Eqn. 25 gives the possibility of calculating approximately the maximum distance at which the non-equilibrium peaks still exist. At large distances z_1, where $a \ll 1$, eqn. 25 transforms into the Boltzmann exponent, which has the maximum at $x_1 = 0$. At smaller distances two members of the series 25 should be taken into account:

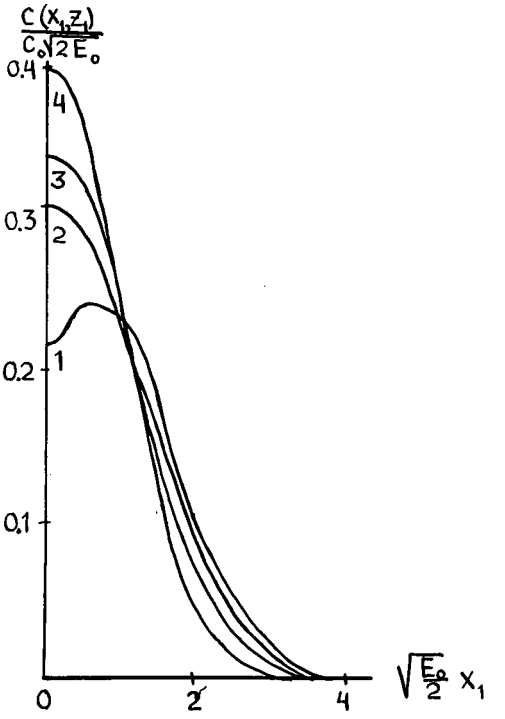


Fig. 1. Transverse particle distributions with a uniform particle distribution at the channel input. $E_0 = 20$; $Pe = 2 \cdot 10^3$. (1) $a = 2.0$; (2) $a = 1.0$; (3) $a = 0.5$; (4) Boltzmann distribution. $a = E_0 e^{-4E_0 z_1/Pe}$.

$$c(x_1, z_1) \approx c_0(E_0/\pi)^{1/2} e^{-E_0 x_1^2}[1 + + (a/6)(4E_0 x_1^2 - 2)] \quad (26)$$

This expression has a maximum at $x = 0$ if $a > 1$; tht is, the non-equilibrium peak is possible at the distances

$z_1 < z_1^* = Pe \ln E_0 / 4E_0$

For $Pe \approx 10^6$ and $E_0 \approx 10^3$, we obtain $z_1^* \approx 1727$. If the half-width of a channel $h \approx 10^{-2}$ cm, then the distance corresponding to this value is equal to *ca.* 17.3 cm. Although the comparison is very conditional, these parameters are in accordance with specified conditions [7,8], where non-equilibrium peaks were observed. The transverse distributions of concentration t different values of a (*i.e.*, at different distances from the channel input) are shown in Fig. 1. These curves are obtained using first four members of series 25 with $n = 0, 1, 2, 3$. It can be shown that the maximum error is less that 5% even at $a = 2$. The curves in Fig. 1 confirm the possibility of the existence of non-equilibrium transverse peaks of concentration at $a > 1$. A special inlet has been proposed [10,11] to make the initial transverse distribution of particles narrower and to increase the resolution of FFF. If the initial transverse distribution of particles is much narrower that $E_0^{-1/2}$, we can write the initial concentration in eqn. 4 as

$$c = 2c_0\delta(x) \text{ at } z_1 = 0 \quad (27)$$

where δ is the Dirac delta function. The distribution 27 is normalized to obtain the same number of particles in any cross-section of the channel that gives eqn. 4. Using eqns. 22 and 27 we can find coefficients B_n:

$$B_n = 2c_0(E_0/\pi)^{1/2} H_{2n}(0)/[2^{2n}(2n!)] \quad (28)$$

Substituting the values of $H_{2n}(0)$ obtained from the recurrence relationship

$$H_{n+1}(x) = 2xH_n(x) - 2nH_{n-1}(x)$$

$$H_{2n}(0) = (-2)^n(2n-1)!! \quad (29)$$

where $(2n-1)!!$ is the product of all uneven numbers $< 2n$, we obtain

$$B_n = 2c_0(E_0/\pi)^{1/2}(-2)^{-n}/[2^n(2n)!!] \quad (30)$$

where $(2n)!!$ is the product of all even numbers from 2 to $2n$. Using eqn. 30, the transverse distribution of particles can be written as

$$c(x_1, z_1) = \quad (31)$$
$$2c_0(E_0/\pi)^{1/2} \sum_{n=0}^{n=\infty} [b^n/(2n!!)] d^{2n}(e^{-E_0 x_1^2})/d(E_0^{1/2} x_1)^{2n}$$

Transverse distributions corresponding to eqn. 31 with different values of $b = (e^{-4E_0 z_1/Pe})/2$ (*i.e.*, at different distances from the channel input) are shown in Fig. 2. A characteristic feature of these curves is the increase in their width with increasing distance from the channel input. Only at large distances, when $b \ll 1$, does the transverse distribution reach the maximum width $E_0^{-1/2}$ and a Boltzmann transverse distribution is established. This is in good agreement with the suggestion [10,11] that a "pinched" inlet must be used to increase the resolution of FFF or SPLITT in the non-equilibrium regime when the transverse particle distribution is narrow.

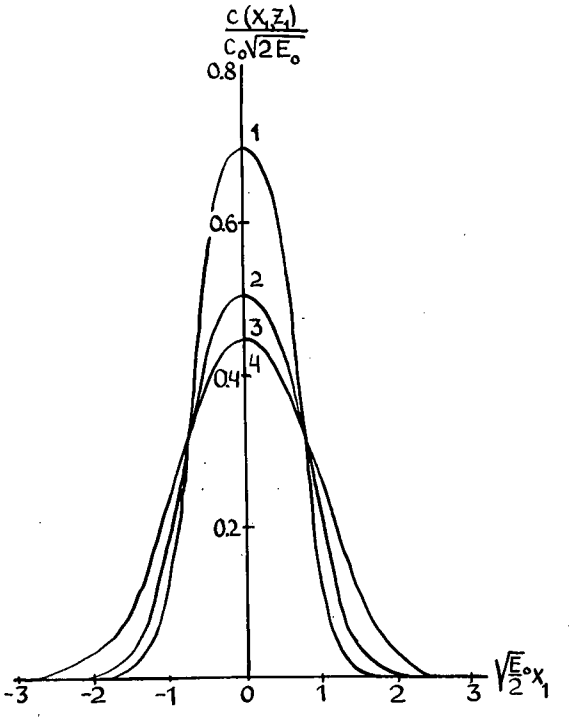


Fig. 2. Transverse particle distributions with a delta-function-like particle distribution at the channel input. $E_0 = 20$; $Pe = 2 \cdot 10^3$. (1) $b = 2.0$; (2) $b = 1.0$; (3) $b = 0.5$; (4) Boltzmann distribution. $b = (1/2)e^{-4E_0 z_1/Pe}$

REFERENCES

1 J. C. Giddings, *Sep. Sci. Technol.*, 19 (1984) 831.
2 J. C. Giddings, *Sep. Sci. Technol.*, 20 (1986) 749.
3 S. N. Semenov and V. L. Kononenko, *Russ. J. Phys. Chem.*, 61 (1987) 1947.
4 S. N. Semenov and V. L. Kononenko, *Russ. J. Phys. Chem.*, 60 (1986) 3117.
5 S. N. Semenov, V. L. Kononenko and Ya. K. Shimkus, *J. Chromatogr.*, 446 (1988) 141.
6 S. Levin, M. N. Myers and J. C. Giddings, *Sep. Sci. Technol.*, 24 (1989) 1245.
7 V. L. Kononenko and Ya. K. Shimkus, *Russ. J. Phys. Chem.*, 64 (1990) 2754.
8 V. L. Kononenko and Y. K. Shimbus, *J. Chromatogr.*, 520 (1990) 270.
9 L. D. Landau and E. M. Lifchitz, *Kvantovaja Mechanika*, GIFML, Moscow, 1963.
10 J. C. Giddings, *Med. Lab. Sci.*, 46 (1990) 39.
11 M. H. Moon, M. N. Myers and J. C. Giddings, *J. Chromatogr.*, 517 (1990) 423.

Journal of Chromatography, 600 (1992) 139–148
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 902

Use of integral Doppler anemometry in field-flow fractionation

V. L. Kononenko* and J. K. Shimkus

Institute of Chemical Physics, Russia's Academy of Sciences, Kosygin str. 4, 117334 Moscow (Russia)

ABSTRACT

Integral Doppler anemometry (IDA) is applied in analytical field-flow fractionation (FFF) of particles by registering the transverse concentration profiles of particle mixtures in FFF channels. Several applications of IDA in FFF are considered. First, its use in FFF systems with a linear focusing force is discussed, for two qualitatively different schemes: (a) a conventional FFF scheme with a single probe injection; and (b) a laterally non-equilibrium, time-independent scheme with a steady-state particle concentration at the channel inlet. In the conventional scheme IDA allows direct and precise measurements of the equilibrium focusing positions of fractions and, if necessary, the registration of the usual elution curve. In the stationary non-equilibrium regime it allows the analysis time and channel length to be decreased considerably compared with the conventional FFF regime. Second, the possibility of IDA measurements of the lateral field geometry and intensity inside FFF channels by registering the characteristic trajectories of test particles is shown theoretically and experimentally. The specially developed kinematic formalism valid in the case of large transverse Peclet numbers, *i.e.*, for strong enough fields and/or large enough particles, is used. It allows the time-dependent concentration distribution of particles in a flat channel flow with a lateral force applied (FFF conditions) to be obtained analytically for arbitrary profiles of velocity and force immediately following the probe injection.

INTRODUCTION

Field-flow fractionation (FFF) has become a well established technique for the analysis and separation of particle mixtures [1,2]. Analytical fractionation is done by registering the particle concentration profiles with time along the channel axis near its outlet. However, long before the axial profiles are formed, the quasi-equilibrium transverse concentration profiles of fractions are established across the channel. These profiles can be measured using integral Doppler anemometry (IDA), recently developed and applied in analytical FFF [3–7]. It has been demonstrated experimentally [6,7] that the use of IDA opens up the possibility of a considerable decrease in the analysis time and the channel length. It also enables a conceptually new approach in analytical FFF, the detection of fractions even during their lateral equilibration (IDA–FFF) [6], to be implemented. The most advantageous approach is the use of IDA in focusing FFF [4]. In previous papers [4,6,7], the basic principles of IDA–FFF were formulated, the necessary theory was developed and feasibility experiments on IDA–FFF were done. These experiments demonstrated the IDA fractionation of particles under stationary, laterally non-equilibrium conditions in FFF channels, using the intrinsic hydrodynamic focusing force alone [6] and in combination with another force (gravity) [7].

In this paper, other promising applications of IDA in FFF are considered theoretically and experimentally: (1) analytical IDA–FFF of particles in a flat channel with a linear focusing force, both for time-dependent and time-independent particle concentration distributions in a flow; (2) the use of the integral Doppler anemometer as a conventional particle detector at the outlet of a channel in a standard scheme of focusing FFF; and (3) the measurements of the spatial characteristics of the lateral field and the calibration of FFF channels by

0021-9673/92/$05.00

means of IDA and the test particles in a flow. The analysis is based on the kinematic formalism, which is developed below for the theoretical description of the FFF process in the case of sufficiently large particles and/or strong enough lateral fields. The main advantages of this formalism are that it gives the analytical expressions for the time-dependent concentration distribution of particles in a flat channel for arbitrary profiles of flow velocity and lateral force starting from the moment in time immediately after the probe injection.

GENERAL RELATIONSHIPS

Previous IDA–FFF experiments [6,7] showed a very promising possibility of analytical fractionation of particles under essentially non-equilibrium lateral conditions, *i.e.*, at the very early stages of the classical FFF process. This raises once again the old problem of the detailed description of particle concentration distributions in a flow during the initial or relaxation stage of FFF [2]. Extensive work has been done on this problem [8–12] and has been reviewed [2], resulting, in particular, in calculation of the appropriate relaxation terms in the expressions for the retention ratio R and the zone-spreading parameter H. These two quantities are among the main parameters of the classical FFF scheme, but for IDA–FFF the transient (non-equilibrium) lateral concentration profiles are of primary interest. This is due to the essence of IDA–FFF, in which the fractions are detected via registering their transverse concentration profiles.

A comprehensive description of the FFF process is possible using the convective diffusion equation, which gives the particle concentration distribution in the channel as a function of time [11,12]. However, the mathematical complexity of this equation leads to tedious numerical calculations [11,12], which greatly impedes its practical use. The situation is simplified in the case of sufficiently strong lateral fields and/or sufficiently large particles (the high value of the transverse Peclet number [2] is essential). Here the transformation of the particle concentration distribution along and across the channel is governed mainly by the kinematic trajectories of particles. The diffusive smearing of these trajectories can be neglected during a considerable period of time, especially in the view of their spread due to the polydispersity of any real particle system. These features allow the use of the kinematic (non-diffusive) approach in the FFF theory. It has been used successfully for the description of stationary non-equilibrium IDA–FFF [6,7].

In this paper a more general formalism is develope, which allows the kinematic analysis of time-dependent situations also. Let us consider the laminar flow of a dilute suspension of spherical particles of radius a in a flat channel of width $2h$ in the presence of some lateral force. We choose the dimensionless coordinate system in units of h, with the z axis along the flow, the x axis perpendicular to the channel walls and the origin in the middle of a channel. In the kinematic approximation the convective diffusion equation is reduced to the time-dependent continuity equation:

$$\frac{\partial C(x,z,t)}{\partial t} + \mathrm{div}[C(x,z,t)\cdot\vec{v}(x,z)] = 0 \qquad (1)$$

where $C(x,z,t)$ is the particle concentration and $\vec{v}(x,z)$ is the particle velocity. We assume the longitudinal component of $\vec{v}$ to coincide with the local flow velocity and the lateral component to be determined by the transverse force according to the Stokes law. Let F_0, $v_{\|}$ be the characteristic values, and $\varphi(x)$, $u(x)$ be the dimensionless lateral profiles of the external force and the flow velocity. Then,

$$v_x(x) = \frac{F_0}{6\pi\eta\alpha}\varphi(x); \quad v_y = 0; \quad v_z(x) = v_{\|}\cdot u(x) \qquad (2)$$

where η is the fluid viscosity. Inserting eqn. 2 into eqn. 1 and solving the partial differential equation arising by the characteristics method [6] with the initial conditions $C = C_0(x_0,z_0)$, $x = x_0$, $z = z_0$ at $t = 0$, we obtain

$$C(x,z,t) = C_0(x_0,z_0)\cdot\frac{\varphi(x_0)}{\varphi(x)}\cdot\Theta(1+x_0)\cdot\Theta(1-x_0) \qquad (3a)$$

$$t = \mu\int_{x_0}^{x}\frac{\mathrm{d}\xi}{\varphi(\xi)}; \quad z = z_0 + \mu\int_{x_0}^{x}\frac{u(\xi)}{\varphi(\xi)}\mathrm{d}\xi;$$

$$\mu = \frac{6\pi\eta a v_{\|}}{F_0} = \frac{Pe_{\|}}{Pe_{\perp}} \qquad (3b)$$

where $Pe_{\|}$ and $Pe_{\perp}$ are the longitudinal and trans-

verse Peclet numbers, respectively [2], and $\Theta(x) = 1$ for $x \geqslant 0$, $\Theta(x) = 0$ for $x < 0$ is the step function. The dimensionless time $t \geqslant 0$ is expressed in the units of $(h/v_{\|})$. The variables x_0 and z_0 in eqn. 3a are connected with x,z,t by eqns. 3b. In the case of the stationary (time-independent) concentration distribution considered previously [6], eqn. 1 lacks the time derivative $\partial C/\partial t$, so the first of eqns. 3b is absent, while the second eqn. 3b interconnects the variables x, x_0 and z.

Eqns. 3 are simple and have great generality, being written for arbitrary profiles of flow velocity and lateral force. It is important that they describe the FFF process starting from the moment immediately after the probe injection. They are suitable both for the quantitative description of specific FFF systems and for the general qualitative analysis of various stages of the FFF process, including the influence of the specific forms of the flow velocity profile and the lateral force profile on this process.

LINEAR FOCUSING FORCE AND PLANE POISEUILLE FLOW

The most advantageous are IDA applications in focusing FFF [4]. In previous papers [6,7] the special case of the intrinsic hydrodynamic focusing force was considered. We now consider a simpler and more general case of the linear profile of a focusing force. This case corresponds closely to the focusing or hyperlayer FFF systems suggested elsewhere [13,14]. For a linear focusing force and plane Poiseuille flow, we have

$$\varphi(x) = (x_f - x); \quad u(x) = (1 - x^2) \tag{4}$$

where $-1 < x_f < 1$ is the focusing point, which can differ for different fractions of particles.

The theoretical description of IDA–FFF requires the calculation of transverse concentration profiles of particles at the given z and t [6,7]. First we consider conventional focusing FFF, *i.e.*, a time-dependent scheme with a single probe injection [2]. We adopt the initial concentration distribution to be the same for all fractions, and to have a Gaussian shape along the z axis, being homogeneous along the x and y axes:

$$C_0(x_0,z_0) = C_0(x_f,\mu) \cdot \exp\left(-\frac{z_0^2 h^2}{2l^2}\right) \cdot \Theta(1 + x_0) \cdot \Theta(1 - x_0) \tag{5}$$

where $C_0(x_f,\mu)$ is the distribution function of particle fractions [6,7] and l is the characteristic axial width of the initial distribution (the case of a slab-like initial distribution can be treated analogously, giving similar results). Using eqns. 3b and 4 to express x_0, z_0 in terms of x, z, t, factorizing the expression for z_0 thus obtained and substituting the results into eqns. 3a and 5, we finally obtain

$$C(x,z,t) = C_0(x_f,\mu) \cdot \exp\left\{-\frac{\mu^2 h^2}{8l^2} \cdot \left[\exp\left(\frac{2t}{\mu}\right) - 1\right]^2 \cdot [x - x_1(z,t)]^2 \cdot [x - x_2(z,t)]^2 + \frac{t}{\mu}\right\} \cdot \Theta[x_l(t) - x] \cdot \Theta[x - x_r(t)] \tag{6a}$$

$$x_{1,2}(z,t) = x_m(t) \mp \left\{\frac{2[z_m(t) - z]}{\mu\left[\exp\left(\frac{2t}{\mu}\right) - 1\right]}\right\}^{1/2} \tag{6b}$$

$$x_m(t) = x_f \cdot \frac{1 - \exp\left(-\frac{t}{\mu}\right)}{1 + \exp\left(-\frac{t}{\mu}\right)};$$

$$z_m(t) = (1 - x_f^2)t + 2\mu x_f \cdot x_m(t)$$

$$x_r(t) = x_f - (1 + x_f) \cdot \exp\left(-\frac{t}{\mu}\right);$$

$$x_l(t) = x_f + (1 - x_f) \cdot \exp\left(-\frac{t}{\mu}\right) \tag{6c}$$

Eqns. 6 give a clear description of the focusing process. The concentration distribution has left, $x_l(t)$, and right, $x_r(t)$, lateral boundaries due to the displacement of the particles away from the channel walls under the action of lateral force. The positions of these boundaries depend only on time, and approach x_f asymptotically for $t \gg \mu$. The total lateral width of the distribution, $x_l(t) - x_r(t) = 2\exp[-(t/\mu)]$, decreases exponentially with time. The instantaneous distribution $C(x,z,t = \text{constant})$ has the shape of a curved crest within the lateral boundaries $x_l(t)$, $x_r(t)$, the maxima line $z_m(x,t)$ being determined by the parabolic equation $[x - x_1(z,t)] \cdot [x - x_2(z,t)] = 0$ (see Fig. 1). At this line, as eqn. 6a shows, the concentration has a maximum value that depends only on time: $C(x_{1,2},z,t) = C_0(x_f,\mu) \cdot \exp(t/\mu)$. The leading maximum point of the concentration crest lies at $x = x_m(t)$, $z = z_m(t)$. It advances along the channel with the maximum

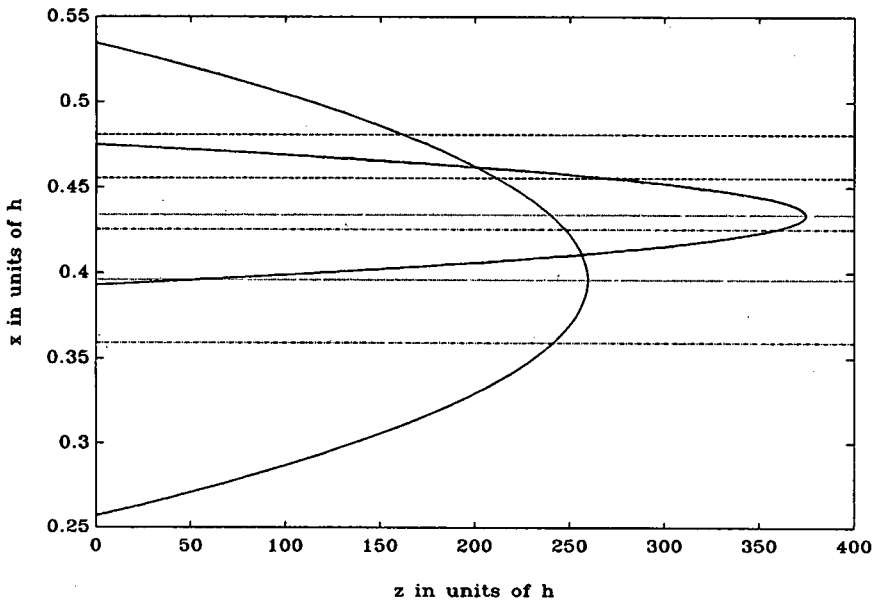


Fig. 1. Maxima lines $z_m(x,t)$ (solid), the minimum–maximum lines $x = x_m(t)$ (dotted), and the left $x_l(t)$ (dashed) and the right $x_r(t)$ (dot–dash) boundary lines of the instantaneous concentration distribution of particles in a plane Poiseuille flow and linear focusing lateral force, computed with eqns. 6 for two moments of time, $t_1 = 280$ and $t_2 = 420$; $x_f = 0.4472$, $\mu = 100$.

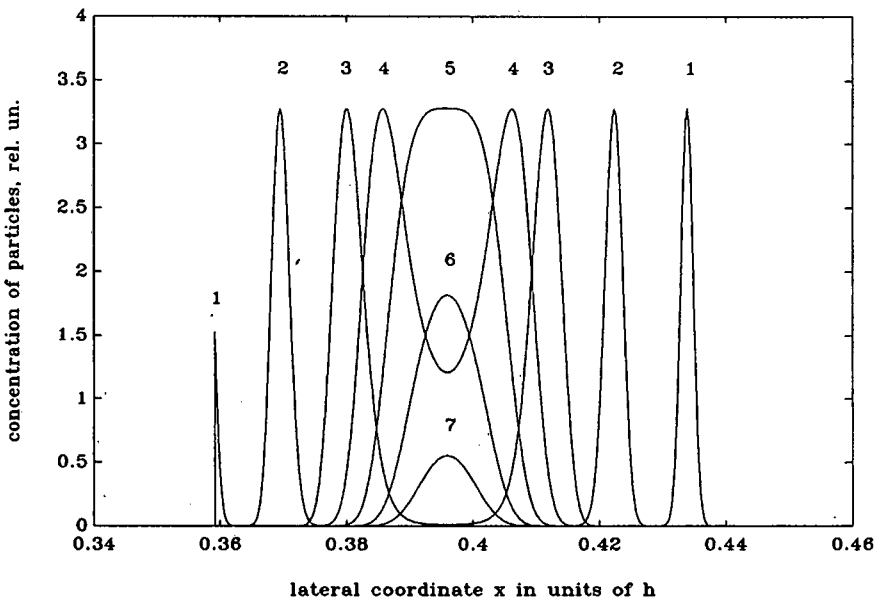


Fig. 2. Series of lateral profiles of instantaneous concentration distribution of particles in a plane Poiseuille flow and linear focusing lateral force, computed with eqns. 6 for $t = 280$, $x_f = 0.4472$, $\mu = 100$, $z =$ (1) 240, (2) 250, (3) 256 and (4) 258, (5) $z_m(t)$, (6) 260.5 and (7) 261.2.

flow velocity $v_{\|}$ when $t \ll \mu$, and with the flow velocity at the focusing point $(1 - x_f^2)v_{\|}$ when $t \gg \mu$. The shape of the transverse concentration profile $C(x) = C(x,z = \text{constant}, t = \text{constant})$, which is relevant for IDA–FFF, depends essentially on the relationship between z and $z_m(t)$. For $z > z_m(t)$ it has a single maximum at $x = x_m(t)$. For $z < z_m(t)$ it has two maxima separated by a minimum at $x = x_m(t)$. These maxima are reached either at maxima lines $x = x_1(z,t)$, $x = x_2(z,t)$, or at the lateral boundaries, depending on the relationship between $x_{1,r}(t)$ and $x_{1,2}(z,t)$ (see Figs. 1 and 2). Eqn. 6a also shows, that the concentration distribution has essentially different axial and lateral widths. All longitudinal profiles $C(z) = C(x = \text{constant}, z,t = \text{constant})$ are characterized by an original effective width l, while the peaks of the lateral profiles $C(x)$ have an effective width $(4l/\mu)\ [\exp(2t/\mu) - 1]^{-1}$, which decreases sharply with time owing to the focusing.

Next we consider the concentration profiles for the stationry scheme of analytical FFF [6], when a steady-state concentration distribution is maintained at the channel entrance instead of a single probe injection. For the stationary case the variables x_0 and z_0 entering eqn. 3a do not depend on time and are related to x and z by the second of eqns. 3b. Setting for definiteness $z_0 = 0$, and substituting eqns. 4 into eqns. 3, we obtain

$$C(x,z) = C_0(x_0,x_f,\mu) \cdot \cdot \exp\left[\frac{-x_f(x - x_0) - \frac{1}{2}(x^2 - x_0^2)}{1 - x_f^2}\right] \cdot \Theta[x_{m2}(z) - x] \cdot \Theta[x - x_{m1}(z)] \quad (7a)$$

$$\frac{z}{\mu} = (1 - x_f^2) \cdot \ln\left(\frac{x_f - x_0}{x_f - x}\right) + x_f(x - x_0) + \frac{1}{2}(x^2 - x_0^2) \quad (7b)$$

The boundary trajectories $x_{m1}(z)$ and $x_{m2}(z)$, which determine the lateral width of the concentration distribution, are given by eqn. 7b for $x_0 = -1$ and $x_0 = 1$, respectively. They approach x_f asymptotically as z tends to infinity. The maxima of the lateral concentration are reached at the boundaries (see Fig. 3), and are given by eqns. 7 for $x_0 = \mp 1$, $x = x_{m1}, x_{m2}$.

USE OF INTEGRAL DOPPLER ANEMOMETER

The integral Doppler anemometer can be used for the detection of particle fractions in both the stationary and non-stationary FFF regimes. The potential advantages of IDA fractionation are revealed most effectively under essentially non-equilibrium lateral conditions and in the stationary regime [6,7]. Under such conditions, as eqns. 7 and Fig. 3 show, the lateral concentration profile of each fraction has two peaks lying at $x_{m1}(z)$ and $x_{m2}(z)$. The positions of these peaks are influenced by the characteristic fraction parameters x_f and μ entering eqns. 7. The concentration peaks create the corresponding peaks in the integral Doppler spectrum, two for each fraction. The necessary equations connecting the shape and the peak positions of the IDA spectrum with the particle concentration profile and its peak positions were given previously [6,7]. They enable one to determine the x_f and μ values for each fraction from the measured IDA spectrum. The parameters x_f and μ are, in turn, connected with the appropriate physico-chemical parameters of particles, specified by the nature of the lateral field.

The doublet structure of the fraction line in the IDA spectrum for $x_f \neq 0$ may partly complicate the identification of fractions. This complication does not arise in the special case of a symmetrical profile of the focusing force, when $x_f = 0$ for all the fractions, and, consequently, $x_{m2}(z) = -x_{m1}(z)$ for each fraction. Here IDA detection enables the μ parameter of the fraction to be determined.

Qualitatively the IDA–FFF system with a linear focusing force is similar to the systems with an intrinsic hydrodynamic focusing force implemented previously [6,7]. The basic theory and the general analytical approach developed in that work, together with eqns. 7, can easily be used to obtain all the necessary characteristics of the present system. The concentration profiles and IDA spectra presented previously [6,7] can be used as qualitative illustrations for the present case also.

Apart from the stationary non-equilibrium separation scheme feasible only with IDA detection (IDA–FFF), the integral Doppler anemometer can be used in conventional FFF systems also, as a particle detector at the outlet of the fractionating channel. In this scheme the anemometer registers the IDA spectra continuously, in an accumulating and

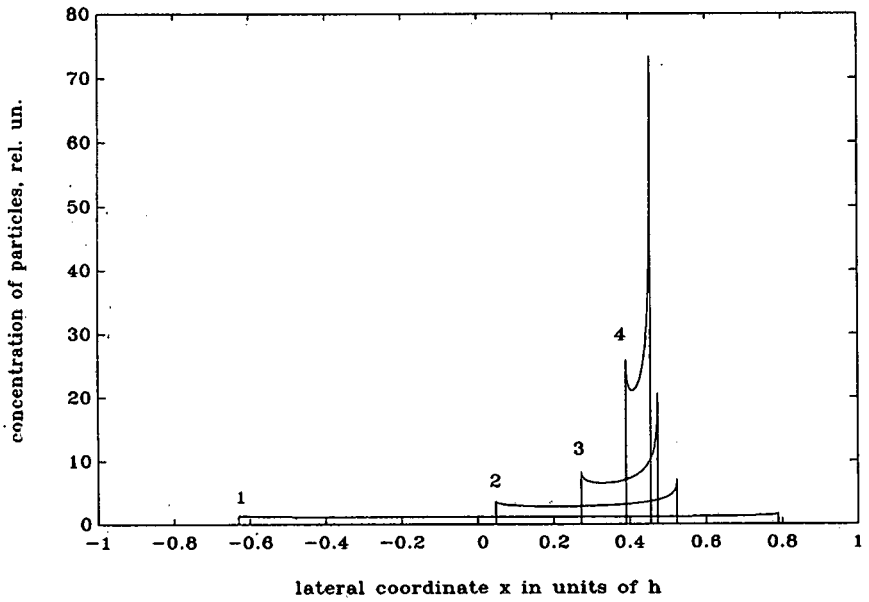


Fig. 3. Series of lateral profiles of a stationary concentration distribution of particles in a plane Poiseuille flow and linear focusing lateral force, computed with eqns. 7 for $x_f = 0.4472$, $\mu = 100$ and $z =$ (1) 10, (2) 100, (3) 180 and (4) 280.

averaging mode. Using the measured IDA spectrum, the time-averaged lateral profile of the particle concentration is obtained as described above. For FFF systems of the focusing type this lateral profile gives directly the equilibrium focusing positions $(x_f)_n$ of various fractions, because the registration is being

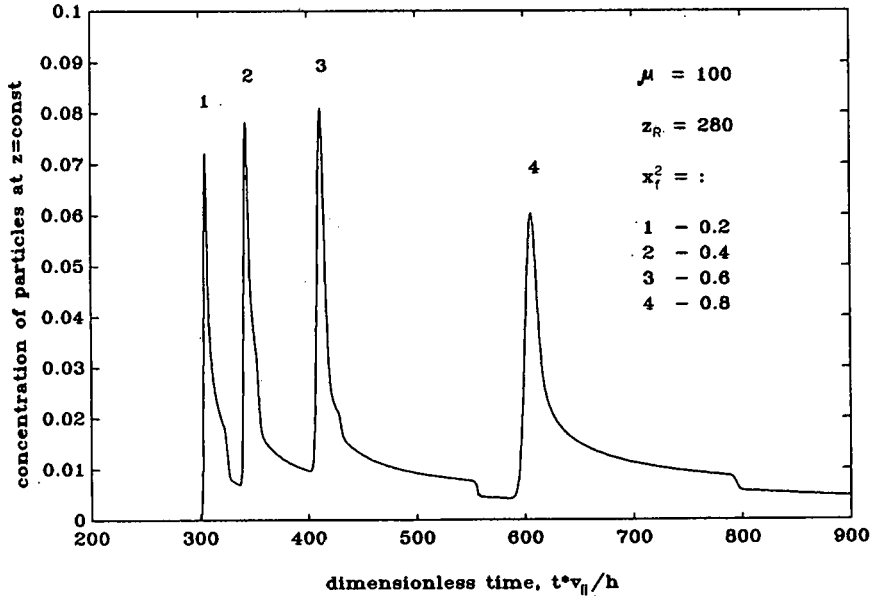


Fig. 4. Elution curve, computed with the kinematic approximation for a model mixture of four particle fractions of equal concentrations, passed through a focusing FFF system with a linear focusing force. Concentration at $z = 0$ is equal to unity.

done under laterally equilibrium conditions (the limiting case $t \gg \mu$ in eqns. 6 of the present model). These positions are connected with the appropriate physico-chemical parameters of the particles, allowing their determination for different fractions. In addition, one of the two laser beams of the differential optical IDA set-up [5] can be directed to another photodetector for the parallel registration of the absorption or low-angle light scattering. This allows one to obtain the usual elution curve, or fractogram [2]. The use of two independent evaluation procedures may be desirable in some instances to increase the accuracy and reliability of fractionation.

We illustrate this scheme by considering a model particle mixture of four fractions that have different focusing positions, $(x_f)_n^2 = 0.2, 0.4, 0.6$ and 0.8, and (for simplicity) the same values of μ. Proceeding from typical experimental situations [2], we assume $\mu = (Pe_{\|}/Pe_{\perp}) = 100$ and $t \geqslant 3\mu$. According to eqns. 6c, at $t = 3\mu = 300$ the most "rapid" fraction (with $x_f = \sqrt{0.2}$) has its concentration maximum at $z_m = 276.2$. This allows us to choose the detector position to be, say, $z_R = 280$ (it should be noted that time is expressed in units of $h/v_{\|}$, and the coordinates x and z in units of h). Fig. 4 presents the usual FFF fractogram computed for this model mixture, using eqns. 6 with $l = h$ and the other parameter values listed above. The aim is to show the complex "convective" shape inherent in the elution curve, and to demonstrate the efficiency of the kinematic approach. The shape is strongly asymmetric, and has a long post-maximum tail due to the kinematic smearing of the initial Gaussian-type concentration distribution by the flow. The parameters of the fractions can be deduced from the measured positions of the elution peaks using eqn. 6c for $z_m(t)$. However, this is not a simple procedure, because two parameters, x_f and μ, are involved, the latter describing the influence of the relaxation effects on the peak position.

Fig. 5 shows the time-averaged IDA spectrum of this model mixture, computed for the averaging interval $t = 300$–1500 and $z_R = 280$. The calculations used the time-averaged lateral concentration profile obtained from eqns. 6, and eqn. 6a from ref. 7, which connects this profile with the IDA spectrum in the case of Poiseuille flow. Fig. 5 indicates that each fraction has the corresponding Doppler line at the frequency $(f/f_{max}) = 1 - (x_f)_n^2$, which gives directly the focusing position $(x_f)_n$. The Doppler lines have the characteristic doublet structure discussed above, which reflects the existence of two peaks of the lateral concentration for $z < z_m(t)$. The line width sharply decreases, and its height increases

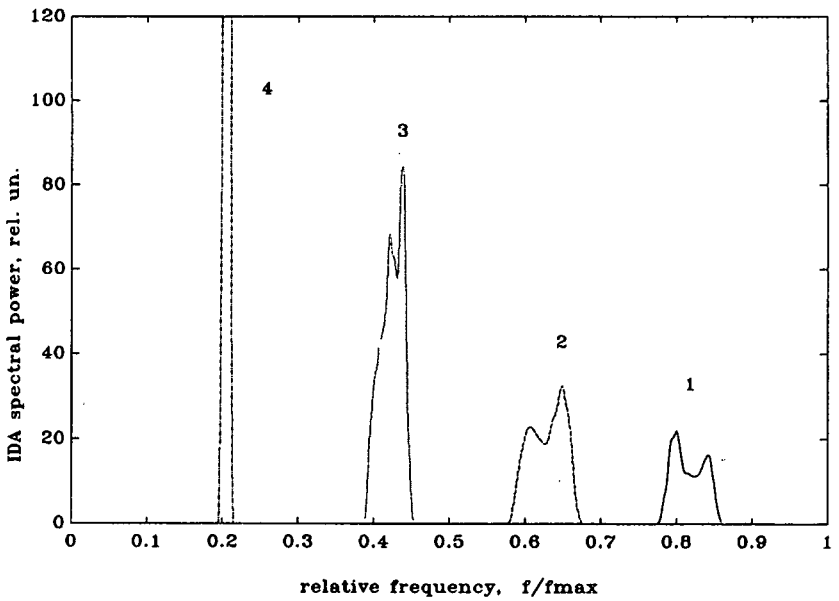


Fig. 5. Time-averaged IDA spectrum, computed for a model suspension probe under the conditions in Fig. 4.

from the "rapid" to the "slow" fractions. This is a consequence of a longer travelling time of the latter, which means their better focusing. In real measurements the narrowing and the growth of the lines are limited by the diffusive broadening and by the instrumental width of the anemometer. However, as the estimates show and the measurements demonstrate [7], these lines remain very narrow for particles with radius $a > 1$ μm, ensuring the high resolution and accuracy of x_f determination.

IDA MEASUREMENTS OF LATERAL FORCE

In the kinematic regime, when diffusion plays a minor role in particle motion, the particle concentration distribution in FFF channels has cut-off type lateral boundaries. In the stationary situation, when the steady-state concentration distribution is maintained at the channel inlet, the positions of the cut-off points along the channel are determined by the boundary lines $x_{m1}(z)$ and $x_{m2}(z)$. These lines are described in the general form by eqn. 6 in ref. 6 or by eqn. 8 below for arbitrary profiles of flow velocity and lateral force, and by eqn. 7b here and eqns. 4 in ref. 7 for some particular profiles. At these lines the lateral concentration of particles changes from nearly zero to some final or even maximum value, depending on the shape of the $F(x)$ profile [6,7]. This concentration step procedures the corresponding peak or shoulder in the IDA spectrum [6,7]. Hence the position of $x_m(z)$ lines can be registered by measuring a series of IDA spectra along the channel. If specially prepared test particles with known specific parameters are used in such measurements, then the lateral field geometry and strength can be measured or calibrated by this procedure. The formal relationship follows from the particle trajectories (eqn. 3b). For the stationary case the boundary lines are the particle trajectories, which begin from the channel walls, $x_0 = 1$ and $x_0 = -1$, at the inlet of a channel, $z_0 = 0$:

$$z = 6\pi\eta v_{\|} \int_{x_0}^{x_m} \frac{u(x)}{F(x)} \cdot \mathrm{d}x \tag{8}$$

Taking the x-derivatives of the left- and right-hand sides of eqn. 8 and replacing the former by the inverse function derivative, we obtain

$$F(x) = 6\pi\eta a v_{\|} u(x) \frac{\mathrm{d}x_m}{\mathrm{d}z}(x) \tag{9}$$

We used this procedure for the measurement of the real profile of the hydrodynamic focusing force in a flat channel. The point is that contrary to the theory [15], the position of the focusing plane of a particle depends on its relative size (a/h) [6,7]. We measured the concentration peak positions along the channel for various widths $2h = 40$–200 μm, using human erythrocytes ($a \approx 3.4$ μm) and latex particles ($a \approx 2$ μm) as test particles. The experimental procedure is described elsewhere [16]. The typical IDA spectra registered and the corresponding lateral concentration profiles obtained, in addition to the measured $x_m(z)$ curves have also been presented previously [16]. The processing of these data according to eqn. 9 gives the hydrodynamic force profile in the range $0.2 < |x| < 0.8$. The resultant profiles show a strong dependence on the flow velocity $v_{\|}$ and the particle size a for $(a/h) > 0.05$. With increase in a the focal positions $\pm x_f$ shift gradually to the centre of the channel, but for $(a/h) < 0.1$ the focusing remains non-central. Then, at $(a/h) \approx 0.1$ and $v_{\|} \approx 1$ cm/s the focal position changes fairly abruptly to become the central plane of a channel.

DISCUSSION AND CONCLUSIONS

The present and the previous papers [3,6,7] show the advantages and limitations, formulate the theoretical and instrumental approaches to and outline the application areas of integral Doppler anemometry [5] in FFF. IDA permits analytical FFF, which can be done in two qualitatively different regimes. The first regime is stationary with time and non-equilibrium relative to the lateral field, and can be called non-equilibrium IDA fractionation or IDA–FFF [6,7]. In this regime the probe circulates in suspension inside a closed circuit, which includes the FFF channel. The detection of fractions is made via IDA registration of their non-equilibrium lateral concentration profiles in a channel. The lateral fields can be both of focusing ([6,7] and this work) and non-focusing [7] type. The flow velocity profiles can be either symmetrical (*e.g.*, Poiseuille flow in a plane or in a circular cross-section channel) or of another type. The linear (Couette) velocity profile is especial-

ly convenient for IDA–FFF owing to the one-to-one correspondence between the axial velocity and the lateral position of a particle in a flow. The evident advantages of the non-equilibrium regime are the short channel length (up to several centimetres) and the short analysis time (up to tens of seconds) [6,7]. The instrumental complications compared with the conventional FFF scheme are the necessity for optical windows in the lateral field section of a channel, a more sofisticated optical set-up and more complex electronics.

The second regime is the use of the integral Doppler anemometer as an ordinary detector in standard FFF scheme, especially in focusing FFF. Here the IDA registration gives immediately the equilibrium focal positions of fractions (see Fig. 5). This is very convenient for the precise determination of the appropriate parameters of particles. Second, it opens up the possibility for efficient calibration and metrology of FFF instruments. A further advantage of the IDA detector is the opportunity for parallel registration of the usual elution curve by measuring some optical characteristic of particles in a flow, such as absorption, low-angle light scattering or dynamic light scattering. Just as in the first IDA regime, these measurements require optical windows. However, in the second regime, such windows can be placed in the usual way, after the lateral field part of a channel (of course, without flow distortion).

The optical set-up of the IDA instrument can be very simple and compact [5,17]. The light can be registered with an ordinary photodetector (photomultiplier, photodiode, etc.). The signal processing unit requires a simple bandpass amplifier and a real-time spectrum analyser [5]. The latter can be successfully (and preferably) replaced by a computer supplied with a plug-in real-time Fourier transformation unit. This computer can control the whole system operation and carry out complete data processing.

For IDA detection, only the light scattered by the particles in a flow is necessary. However, it is hindered by another portion of scattered light, coming from inhomogeneities of the optical windows and from impurities and suspended particles adsorbed on their surfaces. The scattered light intensity depends strongly on the particle or inhomogeneity size [6,17]. In practice, this makes IDA fractionation the most efficient with large particles ($a \gtrsim 0.5$ μm), where the hindering light scattering from the windows can be made comparatively small. This is the case in the analytical fractionation of biological cells. Steric FFF is also known to be efficient for the separation of 1–100-μm particles [2], but it is sensitive to the particle size only. However, in bilogical applications it is often necessary to distinguish between subpopulations of cells with nearly the same size, but with different values of surface electric charge, electric dipole moment or magnetic moment (cell separation using the immunologically attached magnetic markers). IDA–FFF based on an appropriate lateral force (electric, dielectrophoretic, magnetic) can be used for these purposes. The implementation of such systems requires additional research and development.

SYMBOLS

a	radius of a particle
$C(x,z,t)$	concentration of suspended particles in a flow
$C_0(x_f,\mu)$	distribution function of particle fractions
$F_x(x)$ and F_0	lateral force and its magnitude
f	frequency (Hz)
h	half-width of a flat channel
$Pe_\parallel, Pe_\perp$	longitudinal and transverse Peclet numbers
t	dimensionless time (in units of $h/v_\parallel$)
$u(x)$	dimensionless flow velocity profile
$v_\parallel$	maximum flow velocity
x	dimensionless lateral coordinate (in units of h)
x_f	lateral position of the focusing point
$x_l(t), x_r(t)$	lateral boundaries of instantaneous non-stationary particle concentration distribution
$x_m(t)$	minimum–maximum line of a particle concentration distribution
$x_{m1}(z), x_{m2}(z)$	lateral boundaries of a stationary particle concentration distribution
z	dimensionless coordinate along the flow (in units of h)
$z_m(x,t)$	maxima line of non-stationary concentration distribution
$z_m(t)$	leading point of the maxima line
z_R	position of a fraction detector

η	fluid viscosity
$\Theta(x)$	unit step function
μ	characteristic separation parameter of a particle fraction
$\varphi(x)$	dimensionless profile of lateral force

REFERENCES

1 J. C. Giddings, *Sep. Sci.*, 19 (1984–85) 831.
2 J. Janča, *Field-Flow Fractionation*, Marcel Dekker, New York, 1988.
3 V. L. Kononenko and S. N. Semyonov, *Russ. J. Phys. Chem.*, 60 (1986) 1530.
4 S. N. Semenov, V. L. Kononenko and J. K. Shimkus, *J. Chromatogr.*, 446 (1988) 141.
5 V. L. Kononenko and J. K. Shimkus, *Sov. Tech. Phys. Lett.*, 14 (1988) 896.
6 V. L. Kononenko and J. K. Shimkus, *J. Chromatogr.*, 520 (1990) 271.
7 V. L. Kononenko and J. K. Shimkus, *J. Chromatogr.*, 553 (1991) 517.
8 J. C. Giddings, *J. Chem. Phys.*, 49 (1968) 81.
9 M. E. Hovingh, G. H. Thompson and J. C. Giddings, *Anal. Chem.*, 42 (1970) 195.
10 J. C. Giddings, Y. H. Yoon, K. D. Caldwell, M. N. Myers and M. E. Hovingh, *Sep. Sci.*, 10 (1975) 447.
11 S. Krishnamurthy and R. S. Subramanian, *Sep. Sci.*, 12 (1977) 347.
12 K. Jayaraj and R. S. Subramanian, *Sep. Sci. Technol.*, 13 (1978) 791.
13 J. Janča, *Makromol. Chem. Rapid Commun.*, 3 (1982) 887.
14 J. C. Giddings, *Sep. Sci.*, 18 (1983) 765.
15 P. Vasseur and R. G. Cox, *J. Fluid Mech.*, 78 (1976) 385.
16 V. L. Kononenko and J. K. Shimkus, in S. A. Akhmanov, M. Yu. Poroshina, N. I. Koroteev, B. N. Toleutaev (Editors), *Laser Applications in Life Sciences, Proc. SPIE*, 1403 (1991) 381.
17 F. Durst, A. Melling and J. M. Whitelaw, *Principles and Practice of Laser-Doppler Anemometry*, Academic Press, London, 1976.

Journal of Chromatography, 600 (1992) 149–156
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 849

Support matrix effects in the reversed-phase thin-layer chromatography of some peptides

Tibor Cserháti

Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O.B. 17, H-1525 Budapest (Hungary)

ABSTRACT

The retentions of 28 peptides in reversed-phase thin-layer chromatography (RPTLC) were determined on cellulose and on impregnated cellulose and alumina layers with 1-propanol as the organic component of the mobile phase. Each peptide showed a support matrix effect: their R_M values first decreased to a minimum, then increased with increasing 1-propanol concentration. On cellulose layers only the increasing phase was observed. The retention behaviour of peptides was adequately described with a quadratic or linear function, but the slope value of the linear function had a positive value. The results demonstrate that the support matrix effect can be observed on non-silica supports and it may occur in reversed-phase chromatography in the case of polar solutes and supports with free adsorptive centres on their surfaces. Both the intercept and slope values of the function are needed to describe the lipophilicity of peptides, but the correlation is not strong enough for the determination of the lipophilicity of peptides by RPTLC. Principal component analysis showed that the peptides form distinct clusters on the basis of their retention characteristics: peptides containing a basic amino acid, peptides with a ring structure in the amino acid side-chain and peptides containing uncharged amino acids.

INTRODUCTION

Reversed-phase thin-layer chromatography (RPTLC) has been extensively applied to determine the lipophilicity of bioactive molecules [1,2]. To increase the accuracy of the lipophilicity determination, linear correlations have been calculated between the R_M values and the concentration of the organic component of the mobile phase; the R_M value extrapolated to zero organic phase concentration (R_{M0}) was regarded as the most accurate estimate of lipophilicity [3,4]. However, in the case of peptides [5], quaternary amino steroids [6] and crown ether derivatives [7,8], no linear correlation was found between the R_M value and the concentration of the organic component of the mobile phase. The R_M value decreased with increasing organic concentration in the lower concentration range, reached a minimum and then increased with further increase of the organic phase ratio. This phenomenon was tentatively explained in terms of a silanophilic effect: at higher organic concentrations, the solute molecules have an enhanced probability of access to the silanol groups uncovered by the impregnating agent. The interaction with the free silanol groups results in an increased retention and an increased apparent lipophilicity [5]. The adsorptive side-effect of free silanol groups can be eliminated or decreased by the addition of alkylamines [9] or salts [10] to the eluent.

Recent research indicates that in RPTLC the adsorptive character of the support material has a considerable influence on retention, even after impregnation [11–13], because the adsorptive sites not covered with the impregnating agent also affect the binding of solutes. This indicates that the surface pH value and adsorption characteristics of the support may have some impact on the retention of polar compounds even after impregnation. It has recently been established that the surface pH of the silica influences the RPTLC retention of dansylamino acids [14] and of free amino acids [15].

So far as we are aware, the structural characteristics of solutes accounting for the silanophilic effect,

0021-9673/92/$05.00

and the influence of the surface pH of the support on it, have never been studied in detail. The term "silanophilic" effect becomes misleading when supports other than silica are used in the study of the polar (possibly hydrogen bonding) effects mediated by the matrix. We consider that the expression "support matrix effect" better describes the phenomena discussed above. We assumed that owing to their amphipathic character peptides are ideal test solutes to study the effects outlined above.

Reversed-phase chromatography has been extensively applied to separate peptides on both the analytical [16] and preparative scale [17,18]. The retention depended on the type [19] and density of the hydrophobic ligand [20]. Moreover, reversed-phase chromatography has been utilized as a physico-chemical tool for the study of peptide behaviour at hydrophobic liquid–solid interfaces which mimic biological lipid bilayers. This helped to identify and characterize both the hydrophobic interaction sites and the existence of conformational equilibria of peptides such as β-endomorphin [21,22], luteinizing hormone-releasing hormone [23], myosin kinase analogues [24] and human growth hormone related peptides [25,26].

The objectives of this work were to determine the contributions of the physico-chemical parameters of peptides and supports to the support matrix effect in the RPTLC of some peptides.

EXPERIMENTAL

The structures of the peptides are given in Table I. DC Fertigplatten Cellulose, DC Fertigplatten Aluminiumoxid 60 and DC Alufolien Kieselguhr F_{254} (Merck, Darmstadt, Germany) plates were impregnated by overnight predevelopment in *n*-hexane–paraffin oil (95:5, v/v). As it had been demonstrated previously that non-impregnated cellulose may behave as a reversed-phase sorbent under appropriate conditions [4], non-impregnated cellulose plates were also used. The peptides were dissolved in water–1-propanol (2:1, v/v) at a concentration of 2 mg/ml, and 2 μl of each solution was spotted on the plates. 1-Propanol was applied as the organic component of the mobile phase in the concentration range 0–90 vol.% at 5% (cellulose and impregnated cellulose) and 10% intervals (alumina and diatomaceous earth). After development, the peptides were detected with ninhydrin. For each experiment, five independent parallel determinations were carried out.

TABLE I

STRUCTURE OF PEPTIDES

All amino acids had the L-configuration. β-Abu = β-aminobutyric acid; γ-Abu = γ-aminobutyric acid; γ-Ape = γ-aminopentanoic acid; γ-Amh = γ-amino-δ-methylhexanoic acid.

Compound No.	Structure	Compound No.	Structure
1	β-Abu–Ala	15	Pro–Thr–Ile–Pro
2	Gly–Gly	16	Trp–Ser–Tyr–Gly
3	Phe–Ala	17	Trp–Ala–Ile
4	Ala–Ala	18	Phe–Leu–Glu–Glu–Val
5	β-Abu–β-Abu	19	Phe–Gly–Glu–Glu–Leu
6	γ-Amh–γ-Amh	20	Arg–Thr–Asn–Thr–Gly
7	γ-Ape–γ-Abu	21	Leu–Ala–Ala–Ala
8	γ-Abu–γ-Abu	22	Lys(Ala)–Pro–Arg
9	Ala–β-Abu	23	Leu–Lys–Pro–Arg
10	Ala–Thr	24	Lys–Ala
11	Gly–Leu–Gly	25	Ala–Lys–Pro–Lys
12	Gly–β-Abu–Gly	26	Val–His–Asn
13	Glu–Cys–Gly	27	Reduced glutathione
14	Thr–Ile–Pro	28	Oxidized glutathione

MATHEMATICAL METHODS

When in a given RPTLC system the peptide spot remained at the start, or was very near to the front (deformed spot shape), or the relative standard deviation of the five parallel determinations was higher than 8%, the data were omitted from the calculations. Linear correlations between the R_M values of the peptides and the concentration of 1-propanol (C) in the eluent were calculated:

$$R_M = R_{M0} + b_1 C \quad (1)$$

where b_1 is the slope. The calculation was carried out separately for each peptide and for each layer. In some instances eqn. 1 did not give a good fit to the experimental data. Hence a quadratic correlation was calculated for each peptide–1-propanol pair exhibiting an irregular (non-linear) dependence of the retention on the organic phase ratio:

$$R_M = R_{M0} + b_1 C + b_2 C^2 \quad (2)$$

Application of eqn. 2 was motivated by the fact, that the irregular retention behaviour of peptides on impregnated silica layers has been successfully described with a quadratic function [27].

To find the relationships between the retention behaviour of peptides and their lipophilicity, linear correlations were calculated between the parameters of eqn. 2 (independent variables) and the lipophilicity values of the peptides taken from ref. 28 or calculated accordingly. As the inclusion of non-significant independent variables in the equation lessens the information power of the equation, stepwise regression analysis [29] was applied to overcome this difficulty. The number of accepted variables was not limited; they were accepted above 95% significance level. The calculation was carried out separately for both cellulose and alumina supports. As the peptides did not show any retention on the diatomaceous earth support these data were omitted from the calculations.

To elucidate the similarities and dissimilarities between the retention behaviour of the peptides and the parameters of eqns. 1 and 2, principal component analysis (PCA) was applied [30]. The peptides were taken as observations, and the parameters (R_{M0}, b_1 and b_2 values separately for both celluloses and alumina) served as variables. Peptides 6, 16–23, 25–26 and 28 were omitted from the PCA because their retention behaviour cannot be significantly modelled by eqn. 2 on one or both supports. Two-dimensional non-linear mapping of the PC loadings and variables was also carried out [31]. The iteration was carried out to the point when the difference between the two last iterations was less than 10^{-8}.

RESULTS AND DISCUSSION

The peptides did not show any measurable retention on the diatomaceous earth support, the spots being very near to the front both with pure distilled water and water–1-propanol (1:9, v/v) mobile phases. This finding shows again that the hydrophobic impregnating agent does not entirely cover the active adsorption centres on the support surface and the presence of adsorption centres uncovered with the hydrophobic impregnation agent is necessary for retention.

The peptides were not retained on the cellulose surfaces at lower 1-propanol concentrations; at higher concentrations they showed the opposite retention behaviour to that expected (Fig. 1).

The retention of solutes generally decreases with increasing mobile phase ratio, but the retention of peptides increased with increasing concentration of 1-propanol in the mobile phase. This means that the peptides showed typical support matrix effects even

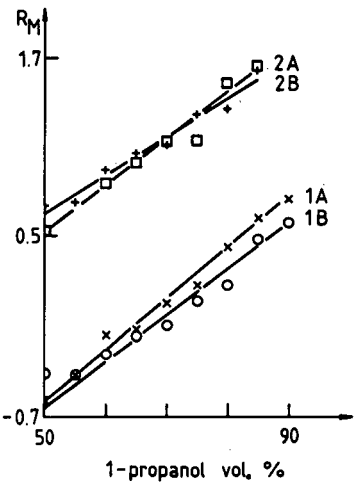


Fig. 1. Dependence of R_M value of (1) Ala–Thr and (2) Lys(Ala)–Pro–Arg on the 1-propanol concentration in the mobile phase. (A) Unimpregnated cellulose; (B) impregnated cellulose.

on cellulose and impregnated cellulose surfaces, where the silanol groups were definitely absent.

Two types of peptide retention behaviour were observed on impregnated alumina (Fig. 2). The retention behaviour of each peptide exhibited typical support matrix effects. In some instances the R_M value decreased with increasing 1-propanol concentration in the lower concentration range, and then increased with further increase in 1-propanol concentration. In other instances the retention of the peptides increased monotonically with increasing 1-propanol concentration. These results also indicate that the support matrix effect can be observed on non-silica layers, that is, the presence of silanol groups is not a prerequisite for the support matrix effect.

The parameters of the equations describing the dependence of R_M values on the 1-propanol concentration on impregnated and non-impregnated cellulose layers are given in Table II. In most instances a significant linear correlation was found between the R_M value of peptides and the 1-propanol concentration in the mobile phase. The r^2 values (ratio of the change in a dependent variable determined by the change in an independent variable or variables) indicate that the change in the 1-propanol concentration explained 92–99% of the change in peptide

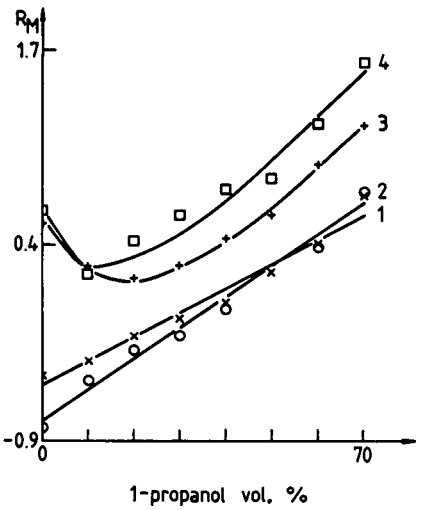


Fig. 2. Dependence of the R_M value of (1) β-Abu–Ala, (2) γ-Ape–γ-Abu, (3) Gly–β-Abu–Gly and (4) Lys–Ala on the 1-propanol concentration in the mobile phase. Impregnated alumina layer.

retention. We should stress that the equations are valid only for the higher concentration range (50–90 vol.%) of 1-propanol; at lower concentrations the peptides were at the front, showing no measurable retention.

The parameters of the equations describing the dependence of R_M values on the 1-propanol concentration on impregnated alumina layers are given in Table III. In most instances a significant correlation was found between the R_M value of the peptides and the 1-propanol concentration in the eluent. In contrast to the cellulose layers, the correlation was not linear in each instance. In some instances a typical support matrix effect was observed, which can be well described with a quadratic function. The equations were similar to those calculated for impregnated silica layers. The r^2 values indicate that the change in the 1-propanol concentration explained 74–98% of the change in peptide retention.

The results suggest that the irregular retention behaviour of peptides (and probably other amphiphilic compounds) cannot be simply ascribed to the presence and availability of free silanol groups. We assume that the phenomenon is the result of at least two effects: (a) the free adsorption centres of the support (silanol groups or not) may influence the retention in eah instance when they are not totally covered (which is momentarily impossible) or masked with appropriate eluent additives; and (b) the dissociation of polar groups is suppressed in mobile phases with lower dielectric constants (higher organic phase ratio). As in reversed-phase chromatography, the retention is governed by the lipophilicity of the solute in the given mobile phase system and the apparent lipophilicity of a solute with dissociable substituents depends strongly on the mobile phase composition, and the variation in the apparent lipophilicity may contribute to the irregular retention behaviour. We believe that with the increasing application of modified alumina [32] and various cellulose derivatives [33–35] as HPTLC sorbents, the problem will increase in importance and its study will help in the elaboration of more efficient separations and a better understanding of the underlying retention mechanisms.

The parameters of the correlations between the chromatographic parameters and the calculated lipophilicity values for various layers are listed in Table IV. The significance level was over 99.9% with

TABLE II

PARAMETERS OF CORRELATIONS BETWEEN THE R_M VALUE OF PEPTIDES AND THE 1-PROPANOL CONCENTRATION IN THE MOBILE PHASE ON CELLULOSE LAYERS

See eqn. 1.

Compound No.[a]	Cellulose support							
	Impregnated				Unimpregnated			
	n^b	R_{M0}	$b_1 \cdot 10^2$	r^2	n^b	R_{M0}	$b_1 \cdot 10^2$	r^2
1	8	−1.95	2.88	0.9493	8	−2.87	4.12	0.9328
2	8	−1.89	3.72	0.9698	8	−2.30	4.08	0.9829
3	9	−2.30	2.91	0.9384	9	−2.19	2.74	0.9700
4	9	−1.78	2.87	0.9440	9	−2.09	3.16	0.9667
5	9	−2.15	2.98	0.9685	8	−2.95	4.28	0.9557
6		n.s.[c]			7	−1.84	1.64	0.9645
7	9	−2.70	4.00	0.9649	8	−2.70	3.77	0.9426
8	9	−2.97	4.37	0.9750	8	−3.18	4.81	0.9673
9	8	−2.07	3.34	0.9520	8	−2.94	4.56	0.9665
10	9	−2.13	2.99	0.9769	8	−2.26	3.33	0.9872
11	9	−2.40	3.35	0.9262	8	−2.79	3.87	0.9791
12	9	−2.26	3.46	0.9663	8	−2.45	3.98	0.9738
13	9	−2.40	4.23	0.9655	7	−2.91	5.04	0.9849
14	9	−1.71	1.78	0.9677	7	−1.71	1.61	0.9671
15	9	−1.75	1.95	0.9347	7	−2.05	2.25	0.9685
16	9	−2.62	3.57	0.9781		n.s.		
17		n.s.			7	−2.00	1.84	0.9299
18	7	−3.62	4.97	0.9696	8	−1.64	1.63	0.9516
19	4	−1.40	1.68	0.9397	6	−2.07	3.22	0.9584
20	7	−1.81	4.01	0.9326	8	−2.19	4.54	0.9746
21		n.s.			8	−1.92	2.13	0.9549
22	8	−0.62	2.56	0.9744	7	−1.06	3.15	0.9612
23	9	−2.57	4.24	0.9604	8	−3.16	4.77	0.9333
24	8	−0.99	2.43	0.9355	9	−1.56	3.17	0.9720
25	8	−4.02	5.28	0.9706	7	−2.83	3.80	0.9736
26	6	−0.69	2.33	0.9724	8	−2.59	4.88	0.9732
27	6	−2.39	4.14	0.9571	8	−2.40	4.10	0.9912
28	6	−2.34	4.94	0.9910	7	−2.82	5.43	0.9775

[a] See Table I.
[b] Number of observations.
[c] Not significant.

cellulose supports and over 95% for impregnated alumina. Eqns. I and II in Table IV support the results of ref. 36 that the intercept (R_{M0}) and slope (b) values of the correlation between the retention value of a compound and the concentration of the organic component in the mobile phase are equally descriptors of the lipophilicity and both are needed for the exact determination of lipophilicity. This finding is supported by the result that both chromatographic parameters have a similar impact on the lipophilicity (see b' values). The r^2 values demonstrate that the predictive power of the equations, although significant, is fairly low. They explain only 50–72% of the lipophilicity change, which from a practical point of view is unacceptable. Our data indicate that the traditional RPTLC method is probably not suitable for the determination of the lipophilicity of peptides and the results obtained by similar methods for similar compounds have to be treated with caution.

The results of PCA are given in Table V. The first and second principal components explain most of

TABLE III

PARAMETERS OF CORRELATIONS BETWEEN THE R_M VALUE OF PEPTIDES AND THE 1-PROPANOL CONCENTRATION IN THE MOBILE PHASE ON AN ALUMINA SUPPORT

See eqn. 2.

Compound No.[a]	n	R_{M0}	$b_1 \cdot 10^2$	$b_2 \cdot 10^3$	r^2
1	8	−0.53	1.62	0	0.9675
2	8	0.04	1.57	0	0.9620
3	7	1.47	−11.14	1.32	0.9374
4	8	−0.26	1.77	0	0.9530
5	6	−0.52	1.54	0	0.9471
6	7	1.18	−8.58	1.02	0.8252
7	7	−0.78	2.07	0	0.9819
8	7	−0.56	2.16	0	0.9767
9	8	−0.26	1.54	0	0.9700
10	8	0.52	−5.15	0.78	0.7403
11	7	1.30	−7.13	0.95	0.8299
12	8	0.48	−2.01	0.45	0.9430
13	5	1.23	1.70	0	0.9249
14	7	1.36	−10.24	1.28	0.8369
15	8	1.33	−10.45	1.32	0.7628
16–19		n.s.			
20	7	−0.72	2.80	0	0.9526
21	8	1.41	−12.40	1.54	0.8172
22, 23		n.s.			
24	8	0.51	−2.01	0.45	0.8808
25, 26		n.s.			
27	7	1.09	1.03	0	0.9600
28		n.s.			

[a] See Table I.

TABLE IV

PARAMETERS OF CORRELATIONS BETWEEN THE LIPOPHILICITY VALUE (y) AND CHROMATOGRAPHIC PARAMETERS (R_{M0}, b_1 AND b_2) OF PEPTIDES ON VARIOUS LAYERS[a]

Results of stepwise regression analysis.

Unimpregnated cellulose: $y = a + b_3R_{M0} + b_4b_1$ (I)

Impregnated cellulose: $y = a + b_3R_{M0} + b_4b_1$ (II)

Impregnated alumina: $y = a + b_4b_1$ (III)

Parameter[a]	No. of equation (layer type)		
	I	II	III
n	23	23	17
a	3.69	1.62	1.58
b_3	−2.03	−2.41	–
s_{b_3}	0.63	0.47	–
b_4	−1.77	−1.45	−0.16
s_{b_4}	0.28	0.34	0.04
b'_3 (%)	34.0	54.9	–
b'_4 (%)	66.0	45.1	–
r^2	0.7203	0.5693	0.5016
F	27.04	13.22	–

[a] b_1–b_4 = Slopes; n = number of observations; a = intercept; b' (%) = normalized slope values (path coefficients); F = calculated value of the F-test.

the total variance, the former explaining about 67% of it. The loadings of each variable are high in the first component. As the slope values (variables 2, 4, 6 and 7) have the highest loadings in the first component and the slope values are related to the hydrophobic surface area of solutes [37], this PC can be regarded as the hydrophobic surface area of the peptides.

Owing to the high intercorrelation between the intercept and slope values, the R_{M0} values also have fairly high loadings in the first component. The two-dimensional non-linear map of PC variables (Fig. 3) shows the clustering of peptides, taking into consideration simultaneously all seven variables. The peptides form distinct clusters according to their structure and polarity. Cluster I contains the peptides with a linear side-chain. This cluster includes

TABLE V

RESULTS OF PRINCIPAL COMPONENT ANALYSIS

Parameter	No. of principal component		
	1	2	3
Eigenvalue	4.72	1.40	0.53
Sum of variance explained (%)	67.45	87.45	94.97
Principal component loadings			
Variable:			
1	0.84	0.36	0.26
2	−0.93	−0.08	0.20
3	0.62	0.72	0.22
4	0.86	−0.34	0.29
5	0.68	−0.51	0.50
6	−0.88	0.44	0.13
7	0.89	−0.42	−0.14

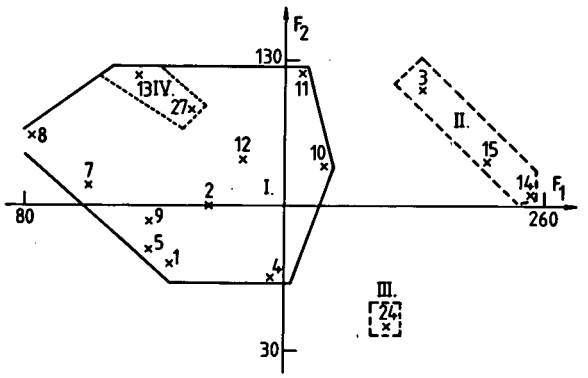


Fig. 3. Two-dimensional non-linear map of peptides. Number of iterations, 59. Error of mapping, $1.04 \cdot 10^{-2}$. Numbers are peptide numbers in Table I.

cluster IV, containing dicarboxylic amino acids. This finding indicates that the acidity of the peptide has a negligible effect on the retention under our experimental conditions. This cluster includes both di- and tripeptides, that is, the number of amino acids in the peptides does not influence the retention characteristics appreciably. The peptides with one or two ring structures are well separated from the others (cluster II). Peptide 24 with a net basic charge forms the third cluster. In contrast to the effect of the net acid charge, the basicity of the peptide markedly influences the retention. This clustering suggests that the polarity and the dimensions of the amino acid side-chain (bulkier ring structure) mainly influence the retention behaviour of peptides in RPTLC even on non-silica supports.

The two-dimensional non-linear map of PC loadings shows the similarities and dissimilarities of the seven variables (Fig. 4). The R_{M0} (points 1, 3 and 5) and linear b (slope) values (points 2, 4 and 6) form distinct clusters independent of the type of support. This finding suggests that the structural characteristics and polarity parameters of the peptides have a greater impact on their retention than the adsorptive character of the supports.

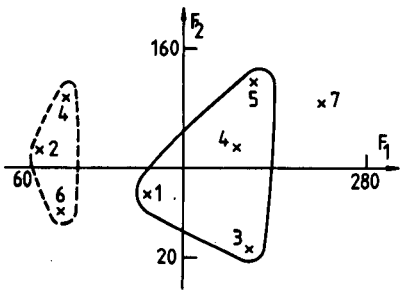


Fig. 4. Two-dimensional non-linear map of parameters of eqn. 2. Number of iterations, 31. Error of mapping, $7.60 \cdot 10^{-3}$. 1 and 2 = R_{M0} and b_1 values of eqn. 2 (unimpregnated cellulose); 3 and 4 = R_{M0} and b_1 values of eqn. 2 (impregnated cellulose); 5, 6 and 7 = R_{M0}, b_1 and b_2 values of eqn. 2 (impregnated alumina).

ACKNOWLEDGEMENT

This work was supported by grant OTKA 2670 from the Hungarian Academy of Sciences.

REFERENCES

1 C. B. C. Boyle and B. V. Milborrow, *Nature (London)*, 208 (1965) 537.
2 G. L. Biagi, A. M. Barbaro and M. C. Guerra, *J. Chromatogr.*, 41 (1969) 371.
3 J. Draffehn, B. Schonecker and K. Ponsold, *J. Chromatogr.*, 205 (1980) 113.
4 T. Cserháti, *Chromatographia*, 18 (1984) 18.
5 K. E. Bij, Cs. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
6 É. János, T. Cserháti and E. Tyihák, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 634.
7 T. Cserháti, M. Szögyi and L. Györfi, *Chromatographia*, 20 (1985) 253.
8 A. Nahum and Cs. Horváth, *J. Chromatogr.*, 203 (1981) 53.
9 S. G. Weber and W. G. Tramposh, *Anal. Chem.*, 55 (1983) 1771.
10 S. G. Weber and J. D. Orr, *J. Chromatogr.*, 322 (1985) 433.
11 M. C. Guerra, A. M. Barbaro, G. Cantelliforti, M. T. Foffani, G. L. Biagi, P. A. Borea and A. Fini, *J. Chromatogr.*, 216 (1981) 93.
12 W. F. Giesen and L. H. M. Janssen, *J. Chromatogr.*, 237 (1982) 199.
13 T. Cserháti, Y. M. Darwish and Gy. Matolcsy, *J. Chromatogr.*, 270 (1983) 97.
14 Z. Illés and T. Cserháti, *J. Planar Chromatogr.*, 1 (1988) 231.
15 Z. Illés and T. Cserháti, *J. Planar Chromatogr.*, 2 (1989) 92.
16 A. J. Albert, *J. Chromatogr.*, 444 (1988) 269.
17 L. R. Snyder, G. B. Cox and P. E. Antle, *J. Chromatogr.*, 444 (1988) 303.
18 G. B. Cox, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 444 (1988) 325.
19 G. Jilge, R. Janzen, H. Giesche, K. K. Unger, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 397 (1987) 71.
20 K. D. Lork, K. K. Unger, H. Brückner and M. T. W. Hearn, *J. Chromatogr.*, 476 (1989) 135.
21 M. I. Aguilar, A. N. Hodder and M. T. W. Hearn, *J. Chromatogr.*, 327 (1985) 115.
22 M. T. W. Hearn and M. I. Aguilar, *J. Chromatogr.*, 352 (1986) 35.
23 M. T. W. Hearn and M. I. Aguilar, *J. Chromatogr.*, 359 (1986) 31.

24 M. T. W. Hearn and M. I. Aguilar, *J. Chromatogr.*, 392 (1987) 33.
25 A. W. Purcell, M. I. Aguilar and M. T. W. Hearn, *J. Chromatogr.*, 476 (1989) 113.
26 A. W. Purcell, M. I. Aguilar and M. T. W. Hearn, *J. Chromatogr.*, 476 (1989) 125.
27 T. Cserháti, Gy. Osapay and M. Szögyi, *J. Chromatogr. Sci.*, 27 (1989) 540.
28 R. F. Rekker, *The Hydrophobic Fragmental Constant, Its Derivation and Application*, Elsevier, New York, 1977, p. 34.
29 H. Mager, *Moderne Regressionsanalyse*, Salle, Sauerlander, Frankfurt am Main, 1982, pp. 135–157.
30 K. V. Mardia, J. T. Kent and J. M. Bibby, *Multivariate Analysis*, Academic Press, London and New York, 1969.
31 J. W. Sammon, Jr., *IEEE Trans. Comput.*, 18 (1969) 401.
32 J. J. Pesek and H. D. Lin, *Chromatographia*, 28 (1989) 565.
33 M. Krause and R. Galensa, *J. Chromatogr.*, 441 (1988) 417.
34 R. Isaksson, P. Erlandson, L. Hansson, A. Holmberg and S. Berner, *J. Chromatogr.*, 498 (1990) 257.
35 T. Nagai and S. Kamiyama, *J. Chromatogr.*, 525 (1990) 203.
36 K. Valkó, *J. Liq. Chromatogr.*, 7 (1984) 1405.
37 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.

END OF SPECIAL ISSUE

Vol. 600, No. 1

Journal of Chromatography

NEWS SECTION

SHORT CONFERENCE REPORT

8th DANUBE SYMPOSIUM ON CHROMATOGRAPHY, WARSAW, POLAND, SEPTEMBER 2–6, 1991

For those readers of this issue who did not have the opportunity to participate at the Warsaw meeting we present here a few pictures to attempt to show the atmosphere of this gathering. The pleasant environment in the lecture hall, around the poster sessions and last but not least at the festive dinner contributed to the success of this symposium. Familiar faces could be seen making this meeting a sort of chromatography family event.

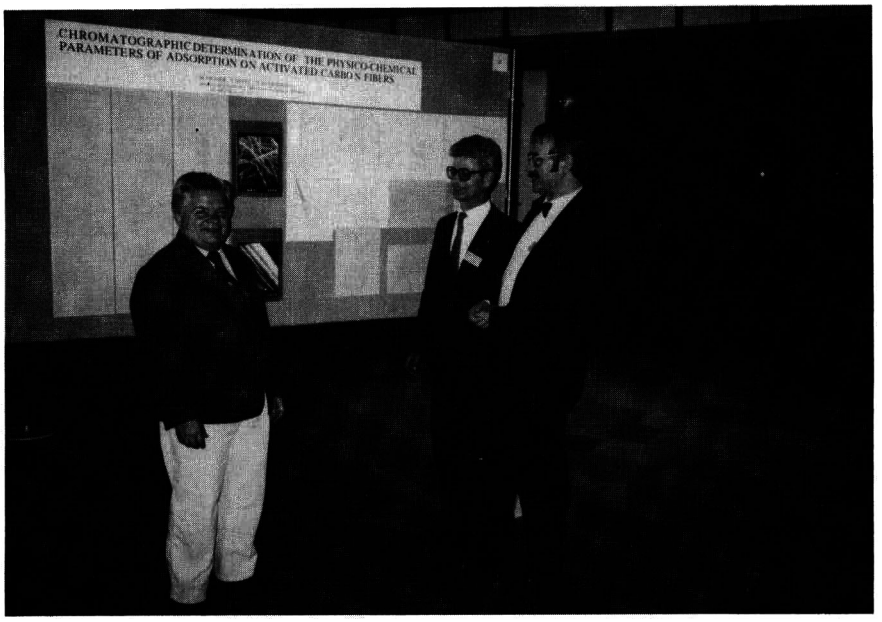


Fig. 1. Organizers of the Warsaw meeting: from the left Professors Soczewinski, Witkiewicz and Lipkowski in front of a picture in the poster section.

As these meetings will cease to continue (see Preface) due to the contemporary attempts for unified Europe, the pictures presented here will be ones of historical value; they will represent a commemoration of Eastern Europe scientific activities of the past.

Fig. 2. Listening to science is a tough job. Tired before the end of the meeting, Professors Bayer (second from the left), Huber, Macek and Sz. Kovacs.

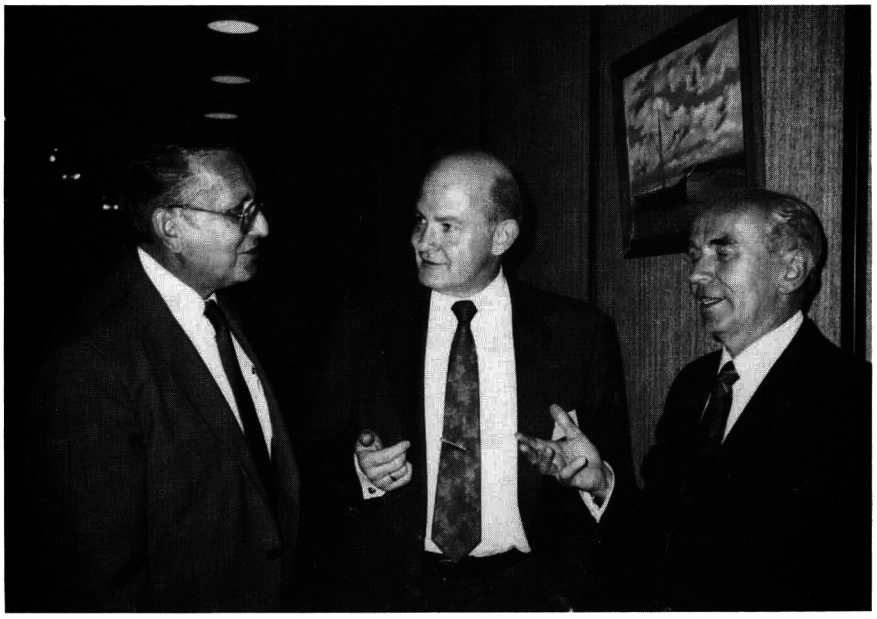


Fig. 3. A friendly chat: from left to right Professors Deyl, York and Macek.

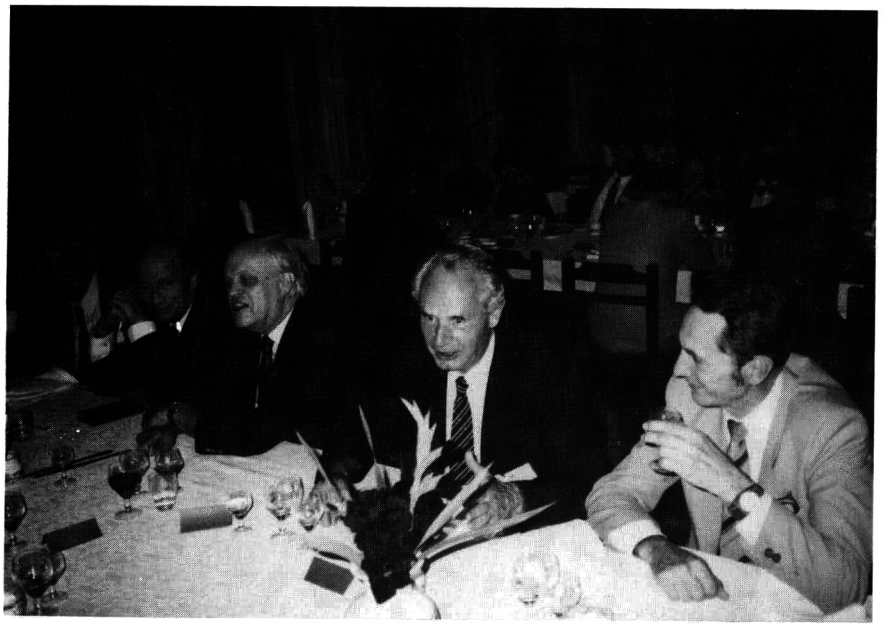


Fig. 4. The best comes at the end: enjoying their dinner (from the left) Professors Sahodinski, Berek, Ettre, Szepesy and Garaj.

PUBLICATION SCHEDULE FOR 1992

Journal of Chromatography and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1991–F 1992	M	A	M	J	
Journal of Chromatography	Vols. 585–593	594/1 + 2 595/1 + 2	596/1 596/2 597/1 + 2	598/1 598/2 599/1 + 2 600/1 600/2	602/1 + 2 603/1 + 2 604/1	The publication schedule for further issues will be published later.
Cumulative Indexes, Vols. 551–600					[a]	
Bibliography Section		610/1			610/2	
Biomedical Applications	Vols. 573 and 574	575/1 575/2	576/1	576/2 577/1	577/2	

[a] Cumulative Indexes will be Vol. 601, to appear early 1993.

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 558, pp. 469–472. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Review articles and Short Communications. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as Full-length papers, but should preferably not exceed five printed pages. For Review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the *Journal of Chromatography*.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Abstract, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Abstract. All articles should have an abstract of 50–100 words which clearly and briefly indicates what is new, different and significant.

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

Dispatch. Before sending the manuscript to the Editor.please check that the envelope contains four copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers and Short Communications will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.