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high-performance liquid chromatography of biopolymers and biooligomers

part A: principles, materials and techniques

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To Irene
my wife, effective helper, and my best friend

PREFACE TO PART A

Originally, about six years ago, the author aimed to put together a medium-sized publication on rapid methods of column chromatography of biopolymers and their medium-molecular-weight fragments, i.e., proteins, enzymes, polypeptides, nucleic acids, polynucleotides, oligonucleotides, polysaccharides, oligosaccharides, glycopeptides and other glycoconjugates (glycolipids and glycoproteins). Since the very beginning low-molecular-weight monomers were not included, in hope of simplifying the book. Moreover, by that time more monographs had been published on the separation of amino acids, simple sugars and low-molecular-weight components of nucleic acids, but books on the high-performance liquid chromatographic separation of high- and medium-molecular-weight biopolymers and biooligomers were rare. They were still to come as this area was just in its infancy.

When setting up the structure of this book I did not anticipate such an explosive development of the new branch of chromatography of biopolymers and biooligomers that resulted, after five years of effort by the writer, in a five-fold expansion of the originally proposed extent to the publication of two large volumes. By agreement with the publisher it was decided to separate general methods, description of packings, instrumentation and experimental techniques (Part A) from descriptions of the chromatography of individual compounds, a Register of chromatographed substances and a full title Bibliography (Part B).

Putting together such large a monograph was time-consuming, requiring great activity and continuous supplementation with up-to-date insertions to already written parts. Parts A and B altogether contain 2891 citations, 1303 of which are summarized alphabetically in the Bibliography. The processing of such a large number of references required various auxiliary tables and to put them together was also laborious. The situation has changed so dramatically since the beginning of this book that, to my mind, it now far exceeds the capabilities of one person to perform such an enormous task; the field is developing so quickly that all the new aspects cannot be processed effectively by a single person. It would be more effective, simpler and quicker to edit volumes written by several authors. This book is an exception to this rule, but I hope a beneficial one for most readers, as a single authorship enables some unifying principles for the whole work to be included.

Work on the whole monograph began with a detailed background literature search in *Chemical Abstracts* and other journals, and looking back to this preparatory work in the library I remember with gratitude the effective help given

by my previous assistant, Ms. Jana Sedláčková. On the basis of the preliminary study I requested reprints of the cited papers, which were sent willingly by their authors. In addition to such reprints, some of the authors also sent other reprints and booklets on the topic concerned, together with unrequested but valuable reprints on other related topics of interest. As a result, the search for original papers in libraries was reduced to only a few missing papers and side citations that emerged subsequently during the writing of individual chapters when there was not enough time to request reprints. I thank cordially all colleagues who made my literature search much easier.

This book was not translated from Czech but written directly in English. I considered it necessary for the manuscript to be read by other persons in order to eliminate possible errors and oversights that might have easily occurred. In this connection special thanks are due to Dr. Ivana Zemanová (Research Institute for Pharmacy and Biochemistry, Prague), who read the whole text, pointed out shortcomings and made preliminary language corrections. I am also grateful to Dr. M. Ryba (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague) for reading and professional comments on the whole of Part A. Individual chapters of Part B were read by other colleagues, who I shall thank in the Preface to that part. With pleasure I also thank all the others whose help in various ways contributed to the publication of this book. Above all I thank my wife Dr. Irene Mikešová, who ensured the transfer of all corrections and insertions from the manuscript to the final copy, ordered and typed all the literature and also participated in the organizational work involved. Next is my daughter, Dr. Eve Zažímalová-Mikešová, who carefully put together the Subject Index. My warm thanks belong also to the staff of the publisher.

Because of my age I am due to retire, and with this monograph I part with active scientific life in the Institute of Organic Chemistry and Biochemistry at the Czechoslovak Academy of Sciences, Prague, where I found an outstanding professional basis for my work. The driving motive for putting this book together was philosophical: I feel that one, especially when beyond the zenith of one's productive life, should strive for a "positive life balance", which means contributing to the development of human society by giving back to it more value than one has received during one's life. During my career as a scientist I acquired much from my colleagues by studying their original papers, reviews, monographs and lectures. This book, and my previous publications, I consider to be a small pay-back for my great debt. It is up to others to estimate its value.

Prague, Czechoslovakia

OTAKAR MIKEŠ

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I am most grateful to the authors cited, to the publishers of the journals and books listed below and to copyright owners for their kind permission to use some of the figures and data from their papers.

Journals and periodicals

American Laboratory (Fairfield, Connecticut); by courtesy of International Scientific Communications, Inc., Fairfield, CT, U.S.A., Figs. 3.6 and 3.7.

Analytical Biochemistry; by courtesy of Academic Press, Inc., Orlando, FL, U.S.A., Figs. 1.2, 4.8, 4.13, 5.17, 5.21 and 5.22.

Analytical Chemistry; by courtesy of the American Chemical Society, Washington, DC, U.S.A., Fig. 2.12 and Table 2.2.

Chemie für Labor und Betrieb; by courtesy of Umschau Verlag, Breidenstein GmbH, Frankfurt am Main, F.R.G., Figs. 5.12 and 5.15.

Chimia; by courtesy of the redaction, Basel, Switzerland, Figs. 5.16 and 5.27.

Clinical Chemistry; by courtesy of the American Association for Clinical Chemistry, Winston-Salem, NC, U.S.A., and by the courtesy of Martin Marietta Energy Systems, Inc., Oak Ridge, TN, U.S.A., Figs. 5.19 and 5.20.

International Journal for Biochemistry; by courtesy of Pergamon Press, Ltd., Oxford, U.K., Fig. 3.10.

Journal of Chromatographic Science; by courtesy of Preston Publications, Inc., Niles, IL, U.S.A., Figs. 3.15 and 3.17.

Journal of Electroanalytical Chemistry; by courtesy of Elsevier Science Publishers, Amsterdam, The Netherlands, Fig. 5.23.

Journal of Chromatography; by courtesy of Elsevier Science Publishers, Amsterdam, The Netherlands, Figs. 2.10, 2.11, 3.3, 3.5, 3.12, 3.13, 3.14, 3.16, 4.3, 4.4, 4.6, 4.7, 4.9, 4.10, 4.11, 4.12, 4.14, 4.15, 5.6, 5.7, 5.18, 5.25, 5.26, 5.29, 5.30, 5.32, 6.3 and 6.7.

Journal of Liquid Chromatography; by courtesy of Marcel Dekker, New York, NY, U.S.A., Fig. 5.9.

Journal of Molecular Biology; by courtesy of Academic Press, Inc., London, U.K., Fig. 4.5.

Monatsschrift für Brauerei; by courtesy of Versuchs- und Lehranstalt für Brauerei in Berlin, Berlin, F.R.G., Fig. 3.17.

Nature (London); by courtesy of Macmillan Journals Limited, London, U.K., Figs. 1.1 and 4.2.

Oncodevelopmental Biology and Medicine; by courtesy of Elsevier Science Publishers (Biomedical Division), Amsterdam, The Netherlands, Figs. 3.8 and 3.9.
Science Tools; by courtesy of LKB Produkter AB, Bromma, Sweden, Fig. 2.6.
Trends in Analytical Chemistry; by courtesy of Elsevier Science Publishers, Amsterdam, The Netherlands, Figs. 3.11 and 6.5.

Books

Laboratory Handbook of Chromatographic Methods (O. Mikeš, Editor), published in 1979 by Ellis Horwood, Ltd., Chichester, U.K., Fig. 2.1 and Table 3.5.
Liquid Column Chromatography (Z. Deyl, K. Macek and J. Janák, Editors), published in 1975 by Elsevier Science Publishers, Amsterdam, The Netherlands, Fig. 5.4.
Nucleic Acid Constituents by High Performance Liquid Chromatography (by T.C. Wehr), published in 1980 by Varian Instruments Group, Walnut Creek, CA, U.S.A., Fig. 6.4.
Perspectives in Peptide Chemistry (A.N. Eberle, R. Geiger and T. Wieland, Editors), published in 1981 by S. Karger, AG, Basel, Switzerland, Fig. 6.6.
Separation Methods. New Comprehensive Biochemistry, Vol. 8 (Z. Deyl, Editor), published in 1984 by Elsevier Science Publishers (Biomedical Division), Amsterdam, The Netherlands, Fig. 5.28.
Úvod do vysokoúčinné kapalinové kolonové chromatografie (Introduction to High Performance Liquid Column Chromatography) (by J. Churáček and P. Jandera), published in 1984 by SNTL, Prague, Czechoslovakia, Figs. 5.13, 5.24 and 6.2.

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Chapter 1

INTRODUCTION

1.1 HISTORICAL

Effective separation processes are very important requirements for advances in chemical, biochemical and biological sciences. Among other separation methods used in these fields, chromatography is the most general. This term was first used at the beginning of the 19th century by M.S. Tswett, a Russian botanist, who described the separation of plant pigments on columns of inert adsorbents. Tswett did not limit the chromatographic separation process only to coloured substances. His findings did not gain widespread recognition until the rediscovery of the technique by Kuhn and Lederer in 1931. Since that time, adsorption chromatography has been widely used in many fields of chemistry, for the separation of both natural substances and synthetic products.

The next important step followed in 1941 when Martin and Synge invented liquid-liquid partition chromatography, which was quickly extended by Consden et al. by the discovery of paper chromatography; in its time this method played an important role in biochemistry.

The ideas behind these chromatographic separations inspired other discoverers. Kirchner et al. opened the way for thin-layer chromatography (capable of replacing column chromatography by flat-bed techniques for analytical and semi-preparative purposes), and Martin and James, and Janák for gas-liquid and gas-solid chromatography. Gas chromatography became a successful separation technique and quickly spread to many branches of chemistry and biochemistry because of its simplicity, efficiency and wide applicability and the possibilities of automation and programming.

The process of ion exchange, discovered by the English soil chemists Way and Thompson in 1850, which later found important industrial applications especially in water softening, was adapted to a chromatographic form, valuable in biochemistry, mainly after 1951, the year in which Moore and Stein worked out their separation of amino acids. The amino acid analyser constructed by Spackman et al. is one of the first excellent examples of automation of relatively complicated liquid chromatographic equipment. In connection with the ion-exchange chromatography of amino acids, Hamilton in 1958 emphasized the importance of particle dimensions and size homogeneity of chromatographic packings. The value of using small particles for adsorption chromatography was appreciated by Snyder.

Another chromatographic principle important for biochemistry was introduced in 1959 by Porath and Flodin, using the term "gel filtration", which was later often termed "gel permeation chromatography" (Moore, 1964). A similar principle was published by Stere and Ackers and termed "restricted diffusion chromatography" and by Pedersen "exclusion chromatography". The principle of gel chromatography or size (steric) exclusion chromatography, based on different diffusion rates into the porous support of molecules differing in size, found numerous applications in the separation of biopolymers.

Adsorption, partition, ion-exchange and steric exclusion chromatography, in spite of the fact that their separation principles are different, became closer and closer with time owing to the development of similar separation equipment in all the modes. Therefore, the general term liquid chromatography became to be mostly used for these separation techniques. It covers all the chromatographic processes that proceed using a liquid mobile phase and is the counterpart of gas chromatography, where the mobile phase is gaseous.

In the subsequent development of liquid chromatography, an important contribution was made by Giddings, who recognized that the theory developed for gas chromatography can be applied (with some minor modifications) to liquid chromatography. His theoretical findings indicated that by using very small particles and the necessary higher pressures, liquid chromatography can match gas chromatography in both speed and resolving power. Further efforts were therefore devoted by chromatographers to the speed and effectiveness of the separation process, which of course depended on particle size and quality. Credit in these endeavours must go to Kirkland (1969) of DuPont, who achieved high-speed separations (completed in a few minutes) with good resolution using specially prepared fine particles and high pressures, thus opening the way to modern high-pressure (high-performance) liquid chromatography (HPLC).

Two main factors contributed to its further evolution: (1) unification of equipment for all modes of liquid chromatography* permitted concentrated development, and (2) research on and the production of very fine and effective non-compressible porous chromatographic packings, with as homogeneous a size as possible. This joint effort led to great successes in liquid chromatographic separations of various substances (which need not be volatilizable as in gas chromatography; most biological substances to be separated are thermally unstable and not volatile) and Done et al. (1972) were certainly accurate in designating these methods as "a revolution in liquid chromatography".

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* The only exception was amino acid analysis, owing to special requirements for detection and the relative slowness of the separation process.

Modern column liquid chromatography is referred to as high-pressure or high-performance and sometimes also high-speed, high-flow or high-efficiency liquid chromatography. For liquid chromatography the abbreviation LC is used, and HPLC was selected for modern versions covering both the terms high-pressure and high-performance. For classical low-pressure methods, the abbreviation LPLC is sometimes used, and MPLC for medium-pressure liquid chromatography.

After the main basis for HPLC had been elaborated and this method had become widely applied, three new chromatographic principles appeared and were incorporated into subsequent trends, viz., hydrophobic, reversed-phase and affinity chromatography. The first two principles are similar. Shaltiel and co-workers and Hofstee studied the chromatographic purification of proteins on alkyl-substituted carbohydrate gels with hydrocarbon arms of various lengths and described the first laws governing the separation. The retardation or retention of proteins depended on the strength of hydrophobic interactions (in lipophilic pockets of protein surfaces) with hydrocarbon arms immobilized on the support. This separation principle was named hydrophobic interaction chromatography. The principle and importance of hydrophobic interactions in natural processes were very well explained by Tanford.

Reversed-phase chromatography is based on a similar interaction principle. The term was first used by Howard and Martin in 1950 to distinguish it form the usual partition chromatography with "normal phases", where an aqueous polar phase was anchored (stationary) and a non-polar phase was mobile. In reversed-chromatography, a hydrophobic support is used and the mobile phase is hydrophilic and polar. The modern development of this type of chromatography is associated with the name of Horváth (see, e.g., the discussion by Horváth and Melander). Porous silicas with hydrocarbon-organosilane bonded phases became the most popular reversed-phase high-performance liquid chromatographic (RP-HPLC) supports. Packings of this type found one of their most important uses in peptide separations. Hearn and Hancock made substantial contributions in this field; the first communication of their voluminous series of papers appeared in 1976 (Hancock et al.). Karger and Giese reviewed the biochemical applications of reversed-phase liquid chromatography.

Although the history of the discovery of affinity chromatography is complicated (see, e.g., Turková), the main steps towards the modern concept of this technique were made by Porath et al. (1967) and Cuatrecasas et al. (1968) (see also Cuatrecasas and Anfinsen). The method is based on biospecific interactions between ligands (affinants) immobilized on suitable supports and biologically active substances, which are reversibly retained on chromatographic columns. This type of separation has been also developed into an HPLC technique [see, e.g., Kato et al. (1977), who chromatographed nucleic acid bases and nucleosides

on a synthetic resin support, coupled with thymine]. The term HPLAC (high-performance liquid affinity chromatography) was proposed by Ohlson et al.

1.2 CONCISE BIBLIOGRAPHY OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Modern high-speed and highly efficient liquid chromatography spread quickly to many branches of chemistry and biochemistry, and this is mirrored in the large number of books and the very many reviews that have appeared. In this short section it is not possible to give a complete survey and therefore only a concise view will be presented, dealing with the general aspects. More specialized books and reviews relating to subsequent sections of this book will be cited later.

A historical overview of the evolution of liquid chromatography, starting from Tswett's papers up to the beginnings of HPLC, was published by Ettre (1980) and a historical dialogue on the occasion of the 75th anniversary of the invention of chromatography (consisting of personal stories of 59 pioneers of various chromatographic techniques) was edited by Ettre and Zlatkis in 1979,

Eight international symposia on column liquid chromatography have been already organized and their Proceedings surveyed progress in this field. One of the latest symposia, "Twenty Years of Modern Liquid Chromatography", took place in New York in 1984 and the proceedings were edited by Horváth and Heftman (1985). Molnar edited the proceedings of the Symposium on Practical Aspects of Modern HPLC, held in West Berlin in 1981, and Kalász those of the 2nd Annual American-Eastern Symposium on Advances in Liquid Chromatography (organized in Szeged, Hungary in 1982). Column liquid chromatography is reviewed very thoroughly at 2-year intervals in *Analytical Chemistry*; references for the last three reviews are Walton (1980) and Majors et al. (1982, 1984). The nomenclature of liquid chromatography including HPLC was dealt with by Ettre (1981). Many basic introductory reviews have been written on rapid and efficient column liquid chromatography by workers from various countries. Only a few of them will be cited here as examples, in chronological order, viz., Yashin and Frolov, Brown, Hennion et al., Meyer, Acquaro and Barretta, Buege and Peinhardt, Freeman, Lim, McNair, Pietrzyk, Ruggeri and Fonseca, Augusto and Perazo, Hatano, Janssen and Kanji, Roth, Ditz, Fink de Cabutti et al., Novotny, Collins et al. and Imai.

Starting with the famous monograph on modern liquid chromatography edited by Kirkland in 1971, number of books on chromatography have appeared that deal, in addition to LC or other separation methods, also with HPLC techniques. Some of them are comprehensive treatises [such as those by Karger et al., Deyl et al. (1975), Mikeš, Deyl (1984) and Poole and Schuette (1985)] whereas others are concise basic introductions to liquid chromatographic techniques, surveys or

practical handbooks (Perry et al., Bristow, Scott, Schram). Another group of books are specialist texts on HPLC, such as the extensive three-volume series by Horváth (1980, 1981, 1983). The second edition of the popular monograph by Snyder and Kirkland is also a voluminous treatise important for every practitioner of modern liquid chromatography. Other books by Rajcsanyi and Rajcsanyi, Simpson, Johnson and Stevenson, Knox, Yost et al., Hamilton and Sewell and Kucera (specializing in microcolumn techniques) are not so extensive and represent concise practical introductions to HPLC methods. Several books on HPLC have been published in languages other than English, such as those by Rosset et al., Engelhardt (1979, 1980), Eppert, Savoia and Churáček and Jandera.

Many books have been devoted to applications of HPLC. Some citations from areas that will not be dealt with in other sections of this book are given here. Books on general applications were published by Done et al. (1974) and by Pryde and Gilbert. Most of the books that conform with the general direction of our book are from the fields of biochemical, biomedical and pharmaceutical applications of HPLC, such as those by Brown (1973), Dixon et al. (1976), Rivier and Burgus, Tsuji and Morozowich, Wessely and Zech, Ando and Ariji, Gerson, Lawson et al., Kabra and Marton, Hawk and Krstulović and Brown. Some reviews in this area were written by Schmid and Beschke, Brown and Krstulović, Courtier and Thomas, Games et al., Hori, Onishi and Itoh, Perrett, Gooding and Regnier, Hartwick and Brown, Kaplan, Riedmann and Wagner, Wong, Keffer, Regnier and Gooding and Erni. Low-temperature HPLC for the separation of thermally labile species is interesting also from the point of view of biochemical applications of HPLC; it was reviewed by Henderson and O'Connor. Henschen et al. (1985) edited a specialized monograph on HPLC in biochemistry.

Some other publications on applications are from the field of food analysis and the foodstuffs industry, such as those by Charalambous, Macrae (1982) and Pearson et al. Reviews in this field were published by Saxby, Macrae (1980, 1981). Tweeten and Euston, Battaglia, Mikeš (1981, 1982), Piesiewicz, Van der Haar and Cornet and Fogy et al. (1980, 1981; comprehensive survey, 587 references).

In addition to the cited books and reviews, many other examples and summaries from various fields of applied biochemistry form parts of the general books on HPLC, cited in the preceding paragraphs. Other specialized books and reviews will be cited later.

1.3 PROBLEMS WITH CLASSICAL LIQUID CHROMATOGRAPHY OF BIOPOLYMERS

The discovery of liquid-liquid partition chromatography and paper chromatography in the early 1940s increased the interest in studies of the composition and structure of proteins and their fragments and led to a search for effective preparative isolation and separation methods for proteins. Column adsorption

and partition chromatography, successful for low-molecular-weight substances, seemed to show possibilities. However, column chromatography requires suitable packings and this was a great problem for many years, not only in the chromatography of proteins, which were treated first, but also with other biopolymers. The inorganic supports available in the early chromatographic experiments often sorbed proteins irreversibly. Column packings based on aromatic polymer matrices (which were available when industrial ion-exchange processes began to be developed) were too hydrophobic and denatured proteins. The only exception was the methacrylate ion exchanger Amberlite IRC 50, well known to protein chemists at that time, and similar resins. Because these packings were microporous, only the functional groups on the surface were active, so the materials had to be finely ground in order to obtain large specific surface areas with correspondingly higher sorption capacities. Bead forms of fine packings were not available at that time.

In the mid-1950s a fundamental step of great importance was made in the chromatography of biopolymers: Sober and Peterson, and Peterson and Sober, introduced ion-exchange derivatives of cellulose, which proved very suitable not only for the separation of proteins but later also for nucleic acids, and were quickly developed nearly to perfection (cf., Peterson, 1970). These materials were macroporous with nearly all functional groups accessible to biopolymers and therefore they showed high sorption capacities. At the end of 1950s another important step contributed to the chromatographic separation of biopolymers: Porath and Flodin applied macroporous derivatives of cross-linked polydextran and achieved the gel chromatography of water-soluble macromolecular substances. Two years later Porath and Lindner prepared ion-exchange derivatives of these materials. Later, cross-linked derivatives of agarose were introduced by Porath et al. (1975). All the column packings were soon commercially available in standard qualities. Thousands of papers have been published describing the successful separation of biopolymer mixtures using these materials. Without exaggeration, it can be said that these chromatographic methods have made major contributions to the present state of development of biochemistry and molecular biology. In the author's opinion the contribution of the above mentioned methods has not yet been fully acknowledged.

Low-pressure liquid chromatography (LPLC) has already performed its task in the separation of biopolymers and, in spite of the fact that it will further effectively serve biochemists, it is not suitable for rapid separations. For this purpose it must step aside and leave the way for more modern methods. General disadvantages of column packings with polysaccharide matrices mentioned above are the following: (1) they are too soft, they do not allow the use of higher pressures (they stop the flow) and the separation requires a long time, which