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AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY

edited by **P.L. DUBIN,** *Indiana-Purdue University*

(Journal of Chromatography Library, 40)

The rapid development of new packings for aqueous size-exclusion chromatography has revolutionized this field. High resolution non-adsorptive columns now make possible the efficient separation of proteins and the rapid and precise determination of the molecular weight distribution of synthetic polymers. This technology is also being applied to the separation of small ions, the characterization of associating systems, and the measurement of branching. At the same time, fundamental studies are elucidating the mechanisms of the various chromatographic processes.

These developments in principles and applications are assembled for the first time in this book.

- Fundamental issues are dealt with: the roles of pore structure and macromolecular dimensions, hydrophobic and electrostatic effects, and the determination and control of column efficiency.
- High-performance packings based on derivatized silica are reviewed in detail.
- Special techniques are thoroughly described, including SEC/LALLS, inverse exclusion chromatography, and frontal zone chromatography.
- Attention is focussed on special applications of size-exclusion methods, such as the characterization of micelles, separations of inorganic ions, and Hummel-Dreyer and related methods for equilibrium systems.
- Protein chromatography is dealt with in both dedicated sections and throughout the book as a whole.

This is a particularly comprehensive and authoritative work - all the contributions review broad topics of general significance and the authors are of high repute.

The material will be of special value for the characterization of synthetic water-soluble polymers, especially polyelectrolytes. Biochemists will find fundamental and practical guidance on protein separations. Researchers confronted with solutes that exhibit complex chromatographic behavior, such as humic acids, aggregating proteins, and micelles should find the contents of this volume illuminating.

Contents: Part I. Separation Mechanisms. Part II. Characterization of Stationary Phases. Part III. New Packings. Part IV. Biopolymers. Part V. Associating Systems. Subject Index.

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Analytical Artifacts

GC, MS, HPLC, TLC and PC

by **B.S. MIDDLEDITCH**, *Dept. of Biochemical and Biophysical Sciences, University of Houston, Houston, TX, USA*

(Journal of Chromatography Library, 44)

This encyclopaedic catalogue of the pitfalls and problems that all analysts encounter in their work is destined to spend more time on the analyst's workbench than on a library shelf. The author has dedicated the book to "the innumerable scientists who made mistakes, used impure chemicals and solvents, suffered the consequences of unanticipated side-reactions, and were otherwise exposed to mayhem yet were too embarrassed to publish their findings".

Traditionally, the mass spectroscopist or gas chromatographer learnt his trade by participating in a 4-6 year apprenticeship as graduate student and post-doctoral researcher. Generally, no formal training was provided on the things that go wrong, but this information was accumulated by sharing in the experiences of colleagues. Nowadays, many novice scientists simply purchase a computerized instrument, plug it in, and use it. Much time can be wasted in studying and resolving problems due to artifacts and there is also a strong possibility that artifacts will not be recognized as such. For example, most analysts realize that they should use glass rather than plastic containers; but few of them would anticipate the possibility of plasticizer residues on glassware washed using detergent from a plastic bottle.

This book is an easy-to-use compendium of problems encountered when using various commonly used analytical techniques. Emphasis is on impurities, by-products, contaminants and other artifacts. A separate entry is provided for each artifact. For specific chemicals, this entry provides the common name, mass spectrum, gas chromatographic data, CAS name and registry number, synonyms and a narrative discussion. More than 1100 entries are included. Mass spectral data are indexed in a 6-peak index (molecular ion, base peak, second peak, third peak) and there are also formula, author and subject indexes. An extensive bibliography contains complete literature citations.

The book is designed to be *used*. It will not only allow experienced analysts to profit from the mistakes of others, but it will also be invaluable to other scientists who use analytical instruments in their work.

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Automatic Methods of Analysis

by **M. VALCÁRCEL** *and* **M.D. LUQUE DE CASTRO,**
Department of Analytical Chemistry, University of Córdoba, Córdoba, Spain

(Techniques and Instrumentation in Analytical Chemistry, 9)

This new book gives a comprehensive overview of the state of the art of the automation of laboratory processes in analytical chemistry. The topics have been chosen according to such criteria as the degree of consolidation, scope of application and most promising trends.

The book begins with the basic principles behind the automation of laboratory processes, then describes automatic systems for sampling and sample treatment. In the second part the principal types of analysers are discussed: continuous, batch and robotic. The third part is devoted to the automation of analytical instrumentation: spectroscopic, electroanalytical and chromatographic techniques and titrators. The last part presents examples of the application of automation to clinical chemistry, environmental pollution monitoring and industrial process control.

The text is supplemented by 290 figures and 800 literature references. It is written primarily for those directly involved in laboratory work or responsible for industrial planning and control, research centres, etc. It will also be useful to analytical chemists wishing to update their knowledge in this area, and will be of especial interest to scientists directly related to environmental sciences or clinical chemistry.

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Polymer Thermodynamics by Gas Chromatography

by **R. Vîlcu** *and* **M. Leca**, *Polytechnic Institute, Bucharest, Romania*

(Studies in Polymer Science, 4)

This book presents direct and inverse gas chromatography as a powerful tool for determining a great number of thermodynamic properties and quantities for micro- and especially for macromolecular substances. In order to ensure the continuity and clarity of the presentation, the book first considers some frequently used concepts of chromatography with a mobile gas phase, i.e. the mechanism of separation, retention parameters and the theories of gas chromatography. The employment of this technique as an important method of studying solutions through the most representative statistical models is also discussed. The thermodynamics of direct gas chromatography, as applied to dissolution, adsorption and vaporization underlies the thermodynamic treatment of inverse gas chromatography.

The most extensive chapter of the book is devoted to the thermodynamics of inverse gas chromatography and deals with a number of important topics: phase transitions in crystalline-amorphous polymers and liquid crystals, glass transitions, other second order transitions in polymers, the determination of diffusion coefficients, the segregation of block copolymers and other applications.

This book is intended for those specialists in research and industry who are concerned with the modification and characterization of polymers, with establishing polymer applications, and with the processing of polymers. It will also be useful to students and specialists interested in the physico-chemical basis of the phenomena involved in gas chromatography in general and its inverse variant in particular.

Contents:

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THE STANDARD TEXT ON THE SUBJECT...

Chemometrics: a textbook

D.L. Massart, *Vrije Universiteit Brussel, Belgium,*
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S.N. Deming, *Dept. of Chemistry, University of Houston, TX, USA,*
Y. Michotte *and* **L. Kaufman,** *Vrije Universiteit Brussel, Belgium*

(Data Handling in Science and Technology, 2)

Most chemists, whether they are biochemists, organic, analytical, pharmaceutical or clinical chemists and many pharmacists and biologists need to perform chemical analyses. Consequently, they are not only confronted with carrying out the actual analysis, but also with problems such as method selection, experimental design, optimization, calibration, data acquisition and handling, and statistics in order to obtain maximum relevant chemical information. In other words: they are confronted with chemometrics.

This book, written by some of the leaders in the field, aims to provide a thorough, up-to-date introduction to this subject. The reader is given the opportunity to acquaint himself with the tools used in this discipline and the way in which they are applied. Some practical examples are given and the reader is shown how to select the appropriate tools in a given situation. The book thus provides the means to approach and solve analytical problems strategically and systematically, without the need for the reader to become a fully-fledged chemometrician.

Contents: Chapter 1. Chemometrics and the Analytical Process. 2. Precision and Accuracy. 3. Evaluation of Precision and Accuracy. Comparison of Two Procedures. 4. Evaluation of Sources of Variation in Data. Analysis of Variance. 5. Calibration. 6. Reliability and Drift. 7. Sensitivity and Limit of Detection. 8. Selectivity and Specificity. 9. Information. 10. Costs. 11. The Time Constant. 12. Signals and Data. 13. Regression Methods. 14. Correlation Methods. 15. Signal Processing. 16. Response Surfaces and Models. 17. Exploration of Response Surfaces. 18. Optimization of Analytical Chemical Methods. 19. Optimization of Chromatographic Methods. 20. The Multivariate Approach. 21. Principal Components and Factor Analysis. 22. Clustering Techniques. 23. Supervised Pattern Recognition. 24. Decisions in the Analytical Laboratory. 25. Operations Research. 26. Decision Making. 27. Process Control. Appendix. Subject Index.

"The many examples, the eye-pleasing presentation, and the references to other texts and articles make the book useful as a teaching tool. Beginners and those more familiar with the field will find the book a great benefit because of that breadth, and especially because of the clarity and relative uniformity of presentation... this book will be the standard text on the subject for some time." (Journal of Chemometrics)

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With the *Chemometric Newsletter*, this journal is the official bulletin of the Chemometrics Society. It publishes articles about new developments on laboratory techniques in chemistry and related disciplines which are characterized by the application of statistical and computer methods. Special attention is given to emerging new technologies and techniques for the building of intelligent laboratory systems. The following topics are dealt with:

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Review

Environmental applications of ion chromatography

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(Received December 8th, 1989)

CONTENTS

1. INTRODUCTION

Ion chromatography (IC) is a versatile, selective and sensitive method for the determination of a variety of anions and cations at trace and ultra-trace levels. It has been applied to hundreds of problems in various fields involving ionic analysis in clinical, food, pharmaceutical, industrial, plating solution and environmental samples. Perhaps the major reason for the rapid growth in popularity of IC is its usefulness in the environmental field. Due to the general complexity of environmental samples, IC has become an integral part in environmental analysis. Detection of specific trace elements at the sub-part-per-billion (10^9) level is a major challenge for the analytical chemist. The enhanced separating powers of the method also permits speciation of environmental contaminants. Different species of elements have different orders of toxicities.

Early publications (1970s) of IC work described environmental applications. Ion chromatography was introduced in 1975[1], but soon after two books were published in 1978 and 1979[2,3]. There is a need to update and assemble new application papers in applied environmental sciences.

Several books[4–8] and general review articles[9–40] have been published on IC in recent years. The most recent book by Smith[5] discusses IC applications up to 1986.

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However, much of the work reviewed focuses on company information and lacks many of the published references. Several review articles on environmental applications have also been published[41–56].

Ion chromatography is a rapidly changing field. This review includes the most recent papers published in the literature as well as information available from the major IC instrument and column manufacturers on environmental pollution applications. The literature is reported according to applications in water, soil, sediment, sludge, plants, air (aerosols) and fossil fuels. A survey of the literature indicates that replication of the analysis can be a problem. Often good or unique work on specific elements can get "buried" with the description on separation of multiple ions. The speciation of different oxidation states of related ions and separation of less common ions have been emphasized in this review.

2. WATER ANALYSIS

IC has shown great promise for the sequential determination of ionic species in a wide variety of water samples. In many cases, little sample pretreatment is required. The technique has an intrinsic ability to cope with complex water samples with large quantities of matrix ions in the detection of the target solutes at trace or ultra-trace concentrations within the sample.

There are numerous reports on the use of IC in the determination of common anions such as Cl^-, NO_3^-, PO_4^{3-} and SO_4^{2-} in a wide range of water samples such as wastewater, rain water, drinking water, snow and Antarctic ice (Table 1). The use of high-performance liquid chromatography (HPLC) for the determination of organic anions and inorganic ions in such samples has been comprehensively reviewed by Bombaugh[57]. The determination of organic and inorganic pollutants in different water samples using amperometric and colorimetric detection methods was recently reviewed[58]. Amperometric detection tends to be selective and is very sensitive. The sensitivity and effectiveness of IC has also been compared to classical spectrometric methods[59]. Many IC procedures have been standardized against colorimetric, titrimetric, steam distillation, autoanalyzer, atomic absorption spectrometry (AAS) and inductively coupled argon plasma emission spectrometry (ICAP).

In addition to separation and detection of various common anions, IC has been used for the determination of various uncommon species in water. Bromide has been selectively determined in water samples using amperometric detection[60]. In a recent paper by Urasa and Nam[61], chromium(III) and chromium(VI) in water samples were analyzed directly using cation- and anion-exchange columns employing a direct current plasma-AAS as an element-selective detector. Detectable concentrations of chromium species were in the low ppb range using preconcentration procedures. Cyanide, an extremely toxic ion which is used in electroplating and gold industries, has been determined in wastewater by IC using electrochemical detection[41,107,110,114]. The simultaneous determination of CN^-, Cl^-, Br^- and I^- was recently reported with amperometric detection[115].

Different oxidation states of some elements have different levels of toxicities and hence the separation and determination of these species is very important. While ICAP and AAS only detect the total elemental content, IC can determine different species simultaneously. IC has been useful for the speciation of toxic ions such as chromium[61,124], arsenic[91,158] and selenium[135,136].

TABLE 1

APPLICATIONS OF IC IN THE ANALYSIS OF WATER SAMPLES

R.S.D. = Relative standard deviation; Cond. = conductivity; GFAAS = graphite-furnace atomic absorption spectrometry; LOD = limit of detection; DAP = diaminopropionic acid; PDCA = pyridine dicarboxylic acid.

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Rain water, groundwater	Br^-	Dionex AS3	3.0 m*M* $NaHCO_3$–2.0 m*M* Na_2CO_3	Amperometric	LOD, 10 $\mu g\ l^{-1}$; recovery, 97–110%; Cl^- interferes if Cl^-/Br^- ratio > 1000:1	60
Rain, drinking water, lake and interstitial waters	NO_3^-, SO_4^{2-}, HCO_3^- NO_2^-, $S_2O_3^{2-}$, Cl^- Br^-, $H_2PO_4^-$, $HCOO^-$	Vydac 302IC	4.0 m*M* Sodium hydrogen phthalate	Cond.	Use of several different eluents	62
River water	Cl^-, NO_3^-, SO_4^{2-}	TSKgel IC-anion PW	0.4 m*M* Trimellitate	UV–VIS	LOD, 0.1, 0.2 and 0.1 mg l^{-1} for Cl^-, SO_4^{2-} and NO_3^-, respectively	63
Rain water	CH_3COO^-, $HCOO^-$, Cl^-, NO_2^-, NO_3^-, SO_4^{2-}	Dionex AS3	2.0 m*M* $NaHCO_3$–1.67 m*M* Na_2CO_3	Cond.	Detection of volatile organic acids and inorganic anions, simultaneously	64
Rain, well water, snow	NO_2^-	Shodex IC I-613	1.0 m*M* Phthalic acid–0.35 m*M* $(C_4H_9)_4$ NOH–0.5% tetrahydrofuran	Amperometric	LOD, 3 $\mu g\ ml^{-1}$	65
Rain, wastewater, soil pore water	Cl^-, NO_3^-, SO_4^{2-}, total S	Vydac 302 IC	4.0 m*M* Phthalic acid (pH 5)	Cond.	LOD, 0.6, 0.2 and 0.2 $\mu g\ ml^{-1}$ for Cl^-, NO_3^- and SO_4^{2-}, respectively	66
Rain water, seawater	Dimethylsulfoxide	Bio-Rad HPX-87H	5.0 m*M* H_3PO_4	UV (195 nm)	Ion exclusion; LOD, 10 $\mu g\ l^{-1}$	67
Rain water, Antarctic ice	S(IV)	Dionex AS3	0.9 m*M* Bicarbonate–0.2% formaldehyde	Cond.	LOD, 0.1 $\mu g\ l^{-1}$; formaldehyde protects against oxidation	68

(Continued on p. 214)

TABLE 1 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Rain water	F^-, Cl^-, NO_2^-, SO_4^{2-}, Br^-, NO_3^-, PO_4^{3-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Semi-automatic (analysis of 60 samples per day)	69
River water	Arsenic anions	TSKgel IC-anion PW	$NaNO_2$	Cond.	Recovery, 88–102%; LOD, 0.3–5 $\mu g\ ml^{-1}$	70
Rain water	Na^+, NH_4^+, K^+	Dionex cation exchange	6.0 m*M* HNO_3	Cond.	Significant variation between IC and AAS at lower ion concentrations	71
Simulated rain water	(i) F^-, Cl^-, NO_3^-, SO_4^{2-},	(i) Dionex anion exchange	(i) 3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	(i) Cond.	Required 1–4 ml sample within 0.05–20 mg l^{-1} range	72
	(ii) Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}	(ii) Dionex cation exchange	(ii) 5.0 m*M* HCl	(ii) Cond.		
Rain water	Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}	Dionex	$NaHCO_3$–Na_2CO_3	Cond.	Step-gradient elution; lacks sensitivity for Mg^{2+}	73
River water	HCO_3^-, CO_3^{2-}	TSKgel IC-Anion PW	0.4 m*M* Trimellitate (pH 7.5)	UV (270 nm)	Compared well with Floco injection analysis	74
Rain, lake	(i) SO_4^{2-}, Cl^-, NO_3^-, F^-, PO_4^{3-},	Dionex AS4	(i) 1.8 m*M* Na_2CO_3–2.2 m*M* $NaHCO_3$	(i) Cond.	Cost-effective compared to spectroscopy methods; LOD, 0.005–0.05 mg l^{-1}	75
	(ii) Li^+, Na^+, K^+, NH_4^+ Ca^{2+}, Mg^{2+}	HPIC-CS3	(ii) 4.8 m*M* HCl–4.0 m*M* DAP · HCl–4.0 m*M* histidine	(ii) Cond.		
Rain water	Alkali and alkaline earth cations	Dionex	35 m*M* HCl	Cond.	LOD, 0.05–0.15 μmol l^{-1} for alkali ions and 0.2–0.6 μmol l^{-1} for alkaline earth ions	76
Rain water	Na^+, Cl^-	Bio-Rad S-X2 and Dionex AS4	Lithium hydrogen phthalate–phthalic acid	Cond.	Simultaneous determination of anions and cations; LOD, ng level; R.S.D. $<0.5\%$	77
Natural and wastewater	NO_3^-	C_{18} reversed phase	60 m*M* KH_2PO_4–15 m*M* H_3PO_4	UV (210 nm)	NO_2^- and organic chromophores do not interfere; LOD, 7 $\mu g\ l^{-1}$	78

River and drinking water	Cl^-, NO_3^-	Spherisorb ODS-2 (Phase separations, Queensferry, U.K.)	Sodium hydrogen phthalate	UV	High pH and high ionic strength of eluent not recommended; LOD, 3–4 nmol	79
River water	NO_3^-, NO_2^-	Dionex AS4	4.0 m*M* Na_2CO_3–4.0 m*M* $NaHCO_3$	UV (220 nm)	Time of analysis for NO_3^-, 5 min	80
Lake, sea, well and tap waters	F^-	Shodex IC I-524A	2.5 m*M* phthalic acid (pH 4.0)	Cond.	Microdiffusion of F^- from sample matrix; LOD, 4 $\mu g\ l^{-1}$	81
River, drinking water	Li^+	HPIC-CS2	8.0 m*M* HCl	Cond.	LOD, 1 $\mu g\ l^{-1}$	82
River water	Na^+, K^+	Zorbax SIL	0.01 *M* Lithium acetate	Cond.	Time of analysis, 10 min	83
Pond water	Ca^{2+}, Mg^{2+}, (Sr^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{3+})	Dionex cation exchange	1.0 m*M* $Ba(NO_3)_2$	Cond.	Al^{3+} or Cr^{3+} did not elute under the conditions described	84
River water	Ca^{2+}, Mg^{2+}	Synthesized polystyrene–divinylbenzene	0.12 *M* $HClO_4$	VIS (590 nm)	Post-column reactor	85
Pond water	Ca^{2+}, Mg^{2+}	Bio-Rad AG1-X8	1.0 m*M* $Pb(NO_3)_2$	Cond.	Good agreement with AAS	86
River water	Al^{3+}	Dionex CS2	0.2 *M* $(NH_4)_2SO_4$ (pH 2.8)	UV (310 nm)	Post-column derivatization; LOD, 7 $\mu g\ l^{-1}$	87
Tap water	Al^{3+}	Dionex CS2	0.1 *M* K_2SO_4 (pH 3.0)	Fluorescence	Post-column reaction; LOD, 1 $\mu g\ l^{-1}$	88
River and sewage waters	NH_4^+	Hitachi 2632 (anion-exchange resin)	Water	Coulometric	Results agree with phenate method; LOD, ng levels	89
River water	Quaternary ammonium compounds	Whatman Partisil PAC 10 (cyano–amino bonded phase)	$CHCl_3$–CH_3OH	Cond.	LOD, 0.02 $\mu g\ l^{-1}$ (50 μl sample size)	90
River water	AsO_3^{3-}, AsO_4^{3-}, SeO_3^{2-}, SeO_4^{2-}	C_{18} reversed phase	1.0 m*M* Hexadecyltrimethylammonium bromide	GFAAS	LOD, 15–25 ng	91

(Continued on p. 216)

TABLE 1 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Seawater	F^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}	Dionex anion exchange	2.5 m*M* Na_2CO_3–3.0 m*M* $NaHCO_3$	Cond.	R.S.D. 0.4–4.2%	92
Seawater	Ni^{2+}, Co^{2+}, Cu^{2+}	Waters Nova-Pak C_{18}	10 m*M* CH_3COONa–CH_3OH–H_2O (76:24%) (pH 8.4)	UV (340 nm)	Time of analysis, 20 min	93
Seawater	I^-	TSKgel IC-anion PW (polymethacrylate, anion-exchange capacity, 0.03 meq)	0.1 *M* NaCl + 5 m*M* Sodium phosphate buffer (pH 6.7)	Amperometric	LOD, 5 $\mu g\ l^{-1}$; R.S.D. 3%	94
Seawater	Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}	Waters IC-Pak-C	10 m*M* Phenylethylamine (pH 5.5)	Cond. and UV/VIS	LOD, 0.2–21.0 ng ml^{-1}; aromatic base eluents were assessed	95
Surface water	Cl^-, NO_3^-, SO_4^{2-}	Waters IC-Pak-A	Borate–gluconate	Cond.	System peaks were avoided by treating sample with cation exchanger	96
Surface water	NO_3^-, NO_2^-	Merck LiChrosphere-RP_{18}	*n*-Octylamine (pH 6)	UV	Good agreement with photometric determination	97
Groundwater	Se(IV) and Se(VI)	Dionex AS3	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Preservation of sample techniques discussed	98
Pore water	Cl^-, F^-, NO_3^-, Br^-, SO_4^{2-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.0 m*M* Na_2CO_3	Cond.	Results compared with colorimetric methods; LOD, 0.005–0.1 mg l^{-1}; recovery, 97–103%	99
Natural water	F^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}	Waters IC-Pak-A	Borate–gluconate, KOH and lithium benzoate	UV/VIS or Cond.	Time of analysis, 16 min	100

Antarctic snow and ice	NO_3^-, SO_4^{2-}, Cl^-, Na^+, NH_4^+, K^+	Dionex anion exchange; Dionex cation exchange	2.0 m*M* Na_2CO_3–2.5 m*M* $NaHCO_3$ (anions) 5.0 m*M* HCl (cations)	Cond.	Efforts were made to reduce contamination problems	101
Antarctic ice	(i) Cl^-, $HCOO^-$, methane sulfonate, NO_2^-, NO_3^-, SO_4^{2-}	(i) Dionex 030-985 anion exchange	(i) 0.7 m*M* $NaHCO_3$–2.0 m*M* Na_2CO_3	(i) Cond.	Sample preservation info. Time of analysis, 30 min	102
	(ii) CH_3COO^-, F^-, Na^+, NH_4^+	(ii) Wescan 269-004 cation exchange	(ii) 4.0 m*M* HNO_3	(ii) Cond.	Sample preservation info.	102
Wastewater	Cl^-, NO_3^-	HIKS-1	1.0 m*M* Tyrosine (pH 10.8)	Cond.	LOD, 10 ng ml^{-1}; R.S.D. <2%	103
River water	NO_3^-, Cl^-, SO_4^{2-}, PO_4^{3-}	Dionex AS1	36 m*M* $NaHCO_3$–24 m*M* Na_2CO_3	Cond.	Complex Fe with cyanide to avoid hydroxide interference	104
Wastewater	Cl^-, NO_3^- PO_4^{3-}, SO_4^{2-}	Dionex 30827	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Compared with various other methods	105
Wastewater	Cl^-, SO_4^{2-}, PO_4^{3-}, $S_2O_3^{2-}$, SCN^-, NO_2^-, NO_3^-	Dionex AS3 or AS5	2.55 m*M* $NaHCO_3$–2.05 m*M* Na_2CO_3	Cond.	Time of analysis, 15 min	106
Wastewater	CN^-	Dionex AS4	2.2 m*M* Na_2CO_3	Cond.	Oxidation of CN^- to CNO^- by hypochlorite	107
Wastewater	SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$	Bio-Rad Bio-gel TSK IC-anion PW	Gluconate–borate (pH 7.6)	UV (254 nm)	Compares with conventional conductivity and electrochemical detection	108
Wastewater	F^-, Cl^-, NO_3^-, SO_4^{2-}, PO_4^{3-}	Dionex 30170	2.1 m*M* $NaHCO_3$–1.7 m*M* Na_2CO_3	Cond.	Na_2CO_3 fusion used to decompose sample; LOD, 8–10 μg	109
Wastewater	CN^-	Dionex AS3	14.7 m*M* Ethylene-diamine–10.0 m*M* Na_2HBO_3–1.0 m*M* Na_2CO_3	Amperometric	LOD, 0.02 mg l^{-1}; compares well with colorimetric determination	110
Saline water	NO_3^-, PO_4^{3-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–1.9 m*M* Na_2CO_3	Cond.	LOD, 0.1 mg l^{-1}	111
Natural water	S^{2-}	Dionex-AS3	H_3BO_3–NaOH–ethylene-diamine	Amperometric	LOD, 1.9 ng ml^{-1}; R.S.D. 0.64%	112

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TABLE 1 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Natural water	F^-, Cl^-, NO_3^-, SO_4^{2-}	Anion-exchange resin	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	R.S.D., 1.3%; LOD, 30, 40, 240, 400 pg for F^-, Cl^-, NO_3^- and SO_4^{2-}, respectively	113
Wastewater	$CN^-(Cl^-, ClO_3^-, SO_4^{2-})$	Dionex anion exchange	2.2 m*M* Na_2CO_3	Cond.	Free CN^- and metal CN^- complexes determined	114
Lake water	CN^-, Cl^-, Br^-, I^-	Vydac 302 IC	Potassium hydrogen phthalate (5.0 m*M*, pH 4.3)	Amperometric	Simultaneous determination; LOD, 0.2–12 $\mu g\ l^{-1}$; recovery 95–105%; time of analysis, 10 min	115
Wastewater	PO_4^{3-}	Dionex anion exchange	3.0 m*M* Na_2CO_3–1.0 m*M* NaOH	Cond.	LOD, 1.5 mg l^{-1}; R.S.D. 0.49%	116
Refinery wastewater	NH_4^+, ethanolamines	Dionex MPIC NS-1	5.0 m*M* hexane–sulfonic acid	Cond.	Time of analysis, <11 min; R.S.D. 2%; LOD, 0.05–5 mg l^{-1}	41
Wastewater	(i) SCN^-, $S_2O_3^{2-}$, (ii) $S_4O_6^{2-}$, SO_3^{2-}, SO_4^{2-}	Dionex anion exchange	(i) 2.4 m*M* Na_2CO_3–3.0 m*M* $NaHCO_3$ (ii) 7.2 m*M* Na_2CO_3–9.0 m*M* $NaHCO_3$	Cond.	Long elution time; badly tailing peaks for $S_2O_3^{2-}$; recovery, 97–101%; R.S.D. 3.8%	117
Wastewater	Borate	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	R.S.D. 0.05–2.6%; recovery, 96.4–99.3%; LOD, 0.05 mg l^{-1}	118
Wastewater	F^-, Cl^-, PO_4^{3-}, NO_3^-, SO_4^{2-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Time of analysis, 24 min	51
Wastewater	F^-, Cl^-, NO_3^-, HPO_4^{2-} SO_4^{2-}, I^-, SCN^-, ClO_4^-	Biotronik IC-1000	1.0 m*M* Tyrosine	Cond.	R.S.D. <0.02%; LOD, 0.01 $\mu g\ ml^{-1}$	119
Natural and wastewater	F^-, Cl^-, NO_2^-, PO_4^{3-}, Mg^{2+}, Ca^{2+}, HCO_3^-, CO_3^{2-}	Dionex IC16	Na_2CO_3 or HNO_3	Cond.	Linear response	120

Wastewater	NH_4^+	Dionex CS1	H_3BO_3	Fluorescence	Post-column reaction with *o*-phthaldialdehyde	121
Wastewater	SO_4^{2-} (F^-, Cl^-, NO_2^-, NO_3^-, PO_4^{2-}, and Br^-)	Dionex anion exchange	$NaHCO_3$–Na_2CO_3	Cond.	LOD, 40 $\mu g\ l^{-1}$; R.S.D. <3%	122
Wastewater	Cu^{2+},Pb^{2+},Zn^{2+}, Ni^{2+},Co^{2+},Cd^{2+}, Fe^{2+},Mn^{2+}	Waters μBondapak C_{18}	2.0 m*M* NaOS–30 m*M* tartaric acid–20 m*M* citric acid (pH 3.4)	VIS (520 nm)	Sensitivity, low ng ml^{-1}; post-column derivatization	123
Wastewater	Cr(VI) + Cr(III)	Dionex CS5	2.0 m*M* PDCA, 2.0 m*M* Na_2HPO_4, 10 m*M* NaI, 50 m*M* $CH_3CO_2NH_4$, 2.8 m*M* LiOH	VIS (520 nm)	Post-column reaction; LOD, 5 $\mu g\ l^{-1}$	124
Acid rain	F^-, Cl^-, NO_3^-, SO_4^{2-}	Dionex AS3	$NaHCO_3$–Na_2CO_3	Cond.	LOD, 0.03–0.25 mg l^{-1}	125
Drinking water	NO_3^-, (F^-, Cl^-, PO_4^{3-}, SO_4^{2-}, Br^-, NO_2^-)	Dionex AS1	4.5 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Compares well with potentiometry; R.S.D. 0.5–2%; LOD, 1 mg l^{-1}	126
Natural water	F^-, Cl^-, PO_4^{3-}, NO_2^-, NO_3^-, SO_4^{2-}	Dionex AS1	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Compares to emission spectroscopy; interferences by humic acid removed by ultrafiltration	59
Natural water	F^-, Cl^-, NO_2^-, NO_3^-, $H_xPO_4^{(3-x)}$, SO_4^{2-}	PRPX-100 polystyrenedivinylbenzene	0.8 m*M* Phthalate (pH 6.8)	UV (265 nm)	LOD, low ng; interference by carbonates; time of analysis, 15 min	129
Drinking water	NO_2^-	High capacity ion-exchange resin	4.0 *M* NaCl	UV (210 nm)	Sample concentration (10–15 ml) through ion exchange; LOD, 0.1 $\mu g\ l^{-1}$	128
Drinking water, rain, lake water	NO_2^-	Anion exclusion/HS	5.0 m*M* H_2SO_4	Amperometric	Recovery, 96–104%; R.S.D. 2.9–7.3%; time of analysis, 6 min	127
Drinking water	(i) NO_3^-	Waters IC-Pak	5.0 m*M* LiOH	UV (214 nm)	LOD, 0.26 $\mu g\ l^{-1}$	130
	(ii) NO_3^-	Dionex HPIC AS4	28 m*M* $NaHCO_3$–22 m*M* Na_2CO_3	Cond.	Compared to EPA test method 300.0; LOD, 4 $\mu g\ l^{-1}$	

(Continued on p. 220)

TABLE 1 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Natural water	F^-, Cl^-, NO_3^-, SO_4^{2-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	R.S.D. <2%	131
Drinking and pond water	NO_3^-	Partisil 10 SAX	1.0 m*M* Potassium hydrogen phthalate	UV (265 nm)	LOD, 0.1 μg ml^{-1}	132
Natural water	NO_3^-, SO_4^{2-}	Dionex anion exchange	0.5 m*M* Potassium hydrogen phthalate	Cond.	Time of analysis, 20 min includes cleanup procedure to remove humic substances	133
Tap water	Cl^-, NO_3^-; SO_4^{2-}	Toyo Soda IC anion PW	2.0 m*M* Tiron	Cond. or UV (290 nm)	R.S.D. 2%; time of analysis, 10 min	134
River and drinking water	Se(IV), Se(VI)	Dionex anion exchange	2.5 m*M* Na_2CO_3–2.0 m*M* KOH	Cond.	Also detects TeO_3^{2-}, TeO_4^{2-}, NO_3^-, SO_4^{2-}, PO_4^{3-}; time of analysis, 30 min; R.S.D. <0.7%	135
River and drinking water	Se(IV), Se(VI)	Nucleosil 55B (Gasukuro Kogyo, Tokyo)	75 m*M* Ammonium phosphate	Fluorescence	Post-column derivatization; time of analysis, 10 min; LOD of Se(IV), 0.17 μg l^{-1}	136
Tap water	F^-	Interaction ORH-801	0.001 *M* H_2SO_4	Cond.	Ion exclusion; time of analysis, <15 min	137
Drinking water	HCO_3^-	Interaction Ion-310	Deionized water	Cond.	Anion-exclusion method; time of analysis, <10 min	137
Drinking water	F^-, Cl^-, NO_2^-, NO_3^-, Br^-, PO_4^{3-}, SO_4^{2-}	Dionex anion exchange	2.0 m*M* Na_2CO_3–0.75 m*M* $NaHCO_3$	Cond.	Recovery, 90–108%; R.S.D. 1–6%	138
Drinking water	Br^-, I^-, NO_2^-, SCN^-	Nucleosil 10-CN	Cetyltrimethyl-ammonium bromide	Amperometric	Ion pair C_{18}; LOD for I^- or SCN^-, 1 μg l^{-1}	139
Natural water	MoO_4^{2-}, WO_4^{2-}	Dionex anion exchange	6.0 m*M* Na_2CO_3	Cond.	LOD, 1 μg l^{-1}; R.S.D. 15%; time of analysis, 17 min	140
Tap water	Al^{3+}	Dionex CG2	0.1 *M* K_2SO_4 (pH 3.0)	Fluorescence	Post-column derivatization; LOD, 1 μg l^{-1}; R.S.D. 3.4%; time of analysis, 2 min	141

Drinking water	(i) NH_4^+, Ca^{2+}, Mg^{2+}	(i) Vydac SC cation exchanger	(i) 1.25 m*M* HNO_3	(i) Cond.	(i) Time of analysis, 10 min	142
	(ii) Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}	(ii) Resin BN-X4 blend	(ii) 1.0 m*M* Ethylene diammonium dinitrate (pH 6.1)	(ii) Cond.	(ii) Time of analysis, 5 min; R.S.D. <2.5%	142
Drinking water	Ca^{2+}, Mg^{2+} (Ni^{2+}, Zn^{2+}, Pb^{2+}, Mn^{2+}, Sr^{2+})	Dionex CS-2	2.0 m*M* Ethylenediamine–2.0 m*M* citric acid (pH 4.0)	Cond.	LOD, <20 $\mu g\ l^{-1}$	143
Drinking water	(i) Cl^-, NO_3^-, SO_4^{2-}	(i) Waters IC-pak™ anion	(i) 2.0 m*M* Ethylenediamine–octanesulfonic acid (pH 6)	(i) Cond.	Simultaneous analysis of anions and cations; time of analysis, 16 min	144
	(ii) Ca^{2+}, Mg^{2+}	(ii) Cation columns	(ii) 3.0 m*M* Sodium octanesulfonate	(ii) Cond.		144
Drinking water	(i) Na^+, K^+, NH_4^+	(i) Cation exchanger	(i) HNO_3	(i) Cond.	LOD, 1 $mg\ l^{-1}$	145
	(ii) Mg^{2+}, Ca^{2+}	(ii) Cation exchanger	(ii) Ethylenediamine	(ii) Cond.	LOD, 1 $mg\ l^{-1}$	145
Drinking water	NO_3^-, NO_2^-	Waters μBondapak C_{18}	5.0 m*M* Tetramethyl ammonium phosphate	UV (214 nm)	Time of analysis, 6 min; LOD, 0.1 $mg\ l^{-1}$	146
Natural and industrial process stream samples	Cr(III) and Cr(VI)	Dionex-CS2 and Dionex-AS7	7.5 m*M* Trilithium citrate–10 m*M* oxalic acid	Direct current plasma-AAS	LOD, 1 $\mu g\ l^{-1}$; element specific; time of analysis, <6 min	61
Snow	NO_3^-, SO_4^{2-}	Dionex AS1	2.4 m*M* Na_2CO_3–3.0 m*M* $NaHCO_3$	Cond.	Also detected F^-, Cl^-, Br^-, PO_4^{3-}; R.S.D. 3%	147
Fog samples	(i) Cl^-, NO_2^-, NO_3^-, SO_3^{2-}, SO_4^{2-}	(i) Dionex AS4	0.5 m*M* $NaHCO_3$–1.3 m*M* Na_2CO_3	(i) Cond.	LOD, <20 $\mu g\ l^{-1}$; time of analysis, 12 min	148
	(ii) F^-, CH_3COO^-, $HCOO^-$	(ii) Dionex AS4	1.5 m*M* $Na_2B_4O_7$	(ii) Cond.	Time of analysis, 10 min	148
	(iii) Na^+, NH_4^+, K^+	(iii) Sykam LCA K01	4.5 m*M* HNO_3	(iii) Cond.	Time of analysis, 7 min	148
	(iv) Ca^{2+}, Mg^{2+}	(iv) Sykam LCA K01	1.0 m*M* Histidine–1.0 m*M* diaminopropionic acid–12 m*M* HCl	(iv) Cond.	Time of anlysis, 10 min	148

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TABLE 1 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Antarctic ice	(i) $CH_3SO_3^-$, $HCOO^-$, F^-, Cl^-, NO_3^-, SO_4^{2-}	(i) Dionex AS4	(i) 0.65 m*M* $NaHCO_3$–2.5 m*M* $NaHCO_3$–4.0 m*M* Na_2CO_3	(i) Cond.	Time of analysis, 6 min; requires 5 ml sample to reach 10^{-10} g g^{-1}	149
	(ii) Na^+, NH_4^+, K^+	(ii) Dionex CS2	(ii) 25 m*M* HCl	(ii) Cond.		149
Wastewater	Organic acids	Dionex AS2	0.01 *M* HCl	Cond.	Ion exclusion	149
Demineralized water	Fe^{3+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, alkaline earth cations	Silica-based cation exchanger	0.1 *M* Tartrate (pH 3.05)	VIS	Post-column derivatization; LOD, 2 μg l^{-1}	150
Wastewater	CN^-	Dionex AS3	1.0 m*M* Na_2CO_3– 10 m*M* NaH_2BO_3–15 m*M* ethylenediamine	Amperometric	Application to photographic wastes	151
Wastewater	CN^-, S^{2-}, Br^-	Dionex AS3	1.0 m*M* Na_2CO_3–10 m*M* NaH_2BO_3–15 m*M* ethylenediamine	Amperometric (Ag electrode)	R.S.D. <2%; time of analysis, 8 min	41
Spiked water sampled	Cd^{2+}, Co^{2+}, Mn^{2+}	Dionex CS5	3.0 m*M* Pyridine-2,6-dicarboxylic acid–4.3 m*M* LiOH	UV (520 nm)	LOD, 0.1 μg l^{-1}; recovery, 97–114%; R.S.D. 0.57%	152
Rain water	Organic acids (formic, acetic, propionic, oxalic, malonic, succinic glutaric acid)	Dionex AG3, AS3, AF3	0.1 *M* Octane sulfonic acid	Cond.	R.S.D. 3–4.9%; LOD, 4–10 ng ml^{-1}	153
Rain water	F^-, CH_3COO^-, $HCOO^-$	Dionex AX1	2.0 m*M* $Na_2B_4O_7$	Cond.	Time of analysis, 15 min; R.S.D. 3.7–7.1%; LOD, 0.05–0.91 mg l^{-1}	154
Snow samples (Antarctic)	NO_3^-	Biotronik BT II AN	$2 \cdot 10^{-3}$ *M* Na_2CO_3	UV (215 nm)	R.S.D. 0.1%; LOD, 5 ng g^{-1}	155

Seawater (artificial)	I^-	TSKgel IC anion SW separator	0.5 m*M* Sodium citrate (pH 5.0)	UV/Cond.	R.S.D. 4%; LOD, 3 ng; also detected Cl^-, NO_3^-, ClO_4^-; Cl^-, Br^-, NO_3^- may cause interference if present in large quantities	156
Natural water	I^-	SAX-1	30 m*M* Na_2CO_3	UV (226 nm)/ Cond.	Also separates Cl^-, PO_4^{3-}, Br^-, NO_2^-, NO_3^-, SO_4^{2-}; LOD, 0.005 μg (100 μl); recovery, 95%	157
Natural water	As(III) + As(V)	Bio-Rad organic analysis column (Aminex HPX-87H ion exclusion)	0.01 *M* Orthophosphoric acid	Amperometric/ UV (200 nm)	R.S.D. 1.3%; detection was linear up to 1.0 μM; time of analysis, 20 min	158
River water	Na^+, K^+	Zorbex SIL	0.01 *M* Lithium acetate	Cond.	LOD, 0.005 *M*; time of analysis, 10 min	159
Purified water	F^-, Cl^-, NO_2^-, NO_3^-, Br^-, PO_4^{3-}, SO_4^{2-}	Dionex AS4	2.0 m*M* Na_2CO_3– 0.75 m*M* $NaHCO_3$	Cond.	LOD, 3–10 $\mu g\ l^{-1}$; recovery, 90–107%; R.S.D. 0.4–6.3%	160
Lake water	NO_3^-	Dionex AS2	Water	UV (210 nm)	R.S.D. 0.45%; time of analysis, 10 min; LOD, low ppb	161
Natural water	Cl^-, Br^-, NO_3^-, SO_4^{2-}	Wescan anion exchange (269-001)	Sodium hydrogen phthalate	Cond.	LOD, 1 mg l^{-1}; chromatography on a guard cartridge	162
Wastewater effluent	PO_4^{3-}	Dionex AS4	2.25 m*M* $NaHCO_3$– 2.0 m*M* Na_2CO_3	Cond.	Other anions separated Cl^-, F^-, NO_2^-, Br^-, NO_3^-, SO_4^{2-}	163

Organic acids appear in wastewater as a result of biological decomposition of organic materials. Ion-exclusion chromatography was found to be extremely reliable in the determination of weak organic acids in various water samples[102,153]. McDowell and Stedman[44] reported the interference of carbonic acid in their studies on the determination of formic, acetic, propionic and butyric acids in various water samples. Ethanolamines have also been determined using IC[43].

Another serious pollution problem is acid rain[125]. IC has been applied in the analysis of acid rain to determine the anion and cation composition. There are also several reports on the ionic analysis of Antarctic snow samples[101,102]. Sensitivity is an extremely important parameter since the ion content in these samples can be about 100 times lower than that of comparable samples taken from industrial areas of the northern hemisphere[5].

3. SOIL SEDIMENT, SLUDGE AND PLANT ANALYSIS

IC analysis of the ionic species in complex systems such as soil, sediments, sludges and plants has received little attention in recent years. Soil, sediment and sludge materials are often analyzed with the objective to determine a single constituent. However, the composition of other ions present may be determined by IC. The advantages of IC in the analysis of such complex samples over conventional chemical techniques include the determination of a variety of ions in a single run, speed and speciation and minimal sample preparation. Table 2 shows the environmental applications of IC in soil, sediment, sludge and plant samples.

Dick and Tabatabai[170] were the first to determine NO_3^- and SO_4^{2-} in soil samples using suppressed IC after extraction with LiCl. Inorganic nitrogen and sulfur are of prime importance in soils since they are essential plant nutrients. Frankenberger and co-workers have used IC for the determination of the following ions in soil: Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_3^{2-}, SO_4^{2-}, CN^-, Br^-, I^-, Li^+, Na^+, NH_4^+, K^+, Ca^{2+}, Mg^{2+}, Ba^{2+} (refs. 115, 167, 168, 185) as well as Sr^{2+} (ref. 185), SeO_3^{2-} (refs. 174,175), SeO_4^{2-} (refs. 173, 175) AsO_4^{3-} (ref. 177), WO_4^{2-}, (ref. 183), MoO_4^{2-} (ref. 182), CrO_4^{2-} ref. 180) $H_2BO_3^-$ (ref. 190) and HCO_3^- (ref. 190). Recently, Mehra and Frankenberger[175] developed an IC method to separate and determine SeO_3^{2-} and SeO_4^{2-}, simultaneously in soils. Speciation of Se is important in understanding its potential toxicity and mobility in groundwater and soils. Simultaneous determination of arsenite and arsenate has also been reported in sediments with no interferences from other anions[196].

IC is becoming an attractive alternative to the conventional spectroscopic methods for determination of metal ions in soil. Meaney *et al.*[205] used IC for the determination of Fe^{3+} and Al^{3+} in soil and clay samples. Bertsch and Anderson[188], using a fully automated IC system, determined Al^{3+} extracts using a UV–visible detector. Alkali and alkaline earth cations in soil have been detected using conductometric detection both by single column and suppressed techniques. Reversed-phase columns have been used for determination of Pb, Zn, Co, Fe and Mn in soils[123]. Determination of total Cu, Ni and Zn in soils has recently been reported by (i) digestion of soil with HF, $HClO_4$ and HNO_3 followed by (ii) extraction of metals as dithiozonate in $CHCl_3$, (iii) destruction of the metal dithiozonate complex with HNO_3 and (iv) final determination by IC using a chromogenic reagent for the detection of the metals by visible photometry[187].

TABLE 2

APPLICATIONS OF IC IN THE ANALYSIS OF SOIL, SEDIMENT, SLUDGE AND PLANT SAMPLES

R_s = Resolution. MS = mass spectrometry; DAN = 2,3-diaminonaphthalene.

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Soil	SO_4^{2-} (total S)	Dionex AS4	18 m*M* $NaHCO_3$–1.2 m*M* Na_2CO_3	Cond.	Time of analysis, 10 min; recovery, 84–98%; sample prep., Na_2O_2 fusion	164
Soil	SO_4^{2-}	(i) Waters IC-Pak A (ii) Vydac 302 IC	(i) Borate–gluconate buffer (ii) 4 m*M* Phthalic acid (pH 4.5)	(i) Cond. (ii) Cond.	Compared extractants: R.S.D. 1.9–8.4%; compared to ICAP; recovery, 97–108%	165
Soil	NO_3^-	Dionex AS4	0.75 m*M* $NaHCO_3$–2.2 m*M* Na_2CO_3	Cond.	LOD, 0.1 ng N dm^{-3}; Cl^- interference eliminated with Ag^+ cation-exchange resin; compared well with steam distillation	166
Soil	PO_4^{3-}	Vydac 302 IC	1.5 m*M* Phthalic acid (pH 2.7)	Cond.	LOD, 0.3 $\mu g\ l^{-1}$; time of analysis, 20 min; R.S.D. 1.1% (500 μl); compared well with autoanalyzer	167
Soil	Cl^-, NO_3^-, SO_4^{2-} (NO_2^-, SO_3^{2-})	Vydac 301 IC	4.0 m*M* Phthalic acid	Cond.	Time of analysis, 11 min; LOD, 0.025–1.0 ng l^{-1}; R.S.D. 3.6–8.4%; compared well to conventional methods	168
Soil	Cl^-, NO_3^-, SO_4^{2-}	Wescan Ion-guard anion cartridge (269-003)	15 m*M* Phthalic acid	UV (300 nm)	Time of analysis, 3 min	169
Soil	NO_3^-, SO_4^{2-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–1.8 m*M* Na_2CO_3	Cond.	Time of analysis, 12 min; R.S.D. 0.4–4.3%; compared well with methylene blue	170

(Continued on p. 226)

TABLE 2 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
					(SO_4^{2-}) and steam distillation (NO_3^-); recovery, 98–102%; LOD, 0.2 μg ml^{-1}	
Soil, plants	Cl^-, NO_3^-, PO_4^{3-} SO_4^{2-}	Apex ODS	0.5 m*M* Tetrabutyl ammonium hydroxide, 50% methanol, potassium hydrogen phthalate	UV (255 nm)	Recovery, 84–108%; time analysis, 20 min; interference by aspartic acid	171
Soil, plants	NO_3^- (NO_2^-)	Dionex AS4	16 m*M* Na_2CO_3–16 m*M* $NaHCO_3$	UV (220 nm)	Time of analysis, 5 min	130
Soil	Se (total)	Cosmosil 5SL	Cyclohexane–ethyl acetate (95:5)	Fluorescence	Post-column derivatization (DAN); time of analysis, 6 min., R.S.D. 3.9%; recovery, 93–97%	172
Soil	SeO_4^{2-} (NO_2^-, NO_3^-, SO_4^{2-})	Vydac 302 IC	4.0 m*M* Phthalic acid (pH 4.6)	Cond.	Applicable with high SO_4^{2-} levels; R.S.D. 1.46 (500 μl); LOD, 18 μg l^{-1}; compared well with AAS and ICAP	173
Soil	SeO_3^{2-} (Cl^-, NO_2^-, NO_3^-, PO_4^{3-})	Vydac 302 IC	1.5 m*M* Phthalic acid (pH 2.7)	Cond.	Applicable with high Cl^- levels; R.S.D. 2% (500 μl); LOD, 3 μg l^{-1}; time of analysis, 8 min; comparable to AAS and ICAP	174
Soil	SeO_3^{2-} and SeO_4^{2-} (Cl^-, NO_3^-, SO_4^{2-})	Wescan resin-based (269-029)	4.0 m*M* *p*-Hydroxybenzoic acid	Cond.	Humic materials removed through soil phase extraction; LOD, 60–110 μg l^{-1}; R.S.D. 0.9–1.86% (500 μl); time of analysis, 15 min	175
Soil	F^-, Cl^- (NO_2^-, PO_4^{3-}, NO_3^-, SO_4^{2-})	Dionex AS3	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Various extractants used, 0.01 *M* NaOH produces maximum F^- signal; time of analysis, 15 min	176

Soil	AsO_4^{3-} (Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-})	Wescan resin-based (269-029)	6.0 m*M* *p*-Hydroxybenzoic acid	Cond.	AsO_3^{3-} was not detected; R_s between AsO_4^{2-} and PO_4^{2-} 1.93; LOD, 92 μg l^{-1}; time of analysis, 20 min; compared well with AAS and ICAP	177
Soil	Br^-, SCN^-, I^-	Whatman Partisil 10 SAX	5.0 m*M* KH_2PO_4–10% CH_3CN organic modifier	UV (205 nm)	Interference with NO_2^-; time of analysis, 23 min; LOD, 1–3 ng	178
Soil	CN^-	Waters IC-Pak anion	5.0 m*M* KOH	Amperometric	LOD, 100 ng ml^{-1}	179
Soil	CN^-, Cl^-, Br^-, I^-	Vydac 302 IC	Potassium hydrogen phthalate (5.0 m*M*, pH 4.3)	Amperometric	Simultaneous determination; LOD, 0.2–12 μg l^{-1}; recovery 95–105%; time of analysis, 10 min	115
Soils, sludges	CrO_4^{2-} (NO_3^-, SO_4^{2-})	Wescan resin-based (269-029)	5.0 m*M* *p*-Hydroxybenzoic acid (pH 8.5)	Cond.	Organic impurities removed through solid phase extraction; R.S.D. 1.98% (500 μl); LOD, 92 μg l^{-1}; compared well with ICAP; time of analysis, 16 min	180
Soil	Cl^-, NO_3^-, HCO_3^-, SO_4^{2-}	Vydac anion exchange	3.0 m*M* Potassium hydrogen phthalate	Cond.	Samples included soil, and drinking water	181
Soil	MoO_4^{2-} (Cl^-, NO_3^-, PO_4^{3-} and SO_4^{2-})	Waters TSKgel IC-Pak Anion 1 (26770)	5.0 m*M* *p*-Hydroxybenzoic acid (pH 8.25)	Cond.	LOD, 45 μg l^{-1}; time of analysis, 15 min; R.S.D. 2.7% (500 μl)	182
Soil, sludge	WO_4^{2-} (Cl^-, NO_3^-, SO_4^{2-})	Wescan resin-based (269-029)	5.0 m*M* *p*-Hydroxybenzoic acid (pH 8.5)	Cond.	LOD, 170 μg l^{-1}; time of analysis, 13 min; R.S.D. 1.9% (500 μl); R_s, WO_4^{2-} and SO_4^{2-}, 2.84	183
Soil	Li^+, Na^+, NH_4^+, K^+	DuPont ZIPAX SCX	3.0 m*M* HNO_3	Cond.	Li^+/Na^+ separation poor; time of analysis, 7 min; LOD, 0.1–5.0 mg l^{-1}	184
Soil	Li^+, Na^+, NH_4^+, K^+	Vydac 401 TP cation exchange	1.0 m*M* HNO_3 (pH 2.1)	Cond.	LOD, 0.05–1 mg l^{-1}; time of analysis, 5 min; compared well with conventional methods	185

(Continued on p. 228)

TABLE 2 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Soil	Mg^{2+}, Ca^{2+} (Sr^{2+}, Ba^{2+})	Vydac 401 TP cation exchange	5.0 m*M* Ethylenediammonium dinitrate (pH 6.1)	Cond.	LOD, 0.1–1 mg l^{-1}; time of analysis, 4 min; compared well with conventional methods	186
Soil	Ca^{2+}, Mg^{2+} (Sr^{2+}, Mn^{2+} and Zn^{2+})	Dionex CS2	2.0 m*M* Ethylenediamine–2.0 m*M* citric acid	Cond.	LOD for Ca^{2+} and Mg^{2+} were 20 and 10 μg l^{-1}, respectively	143
Soil	Ca^{2+}, Mg^{2+} (Sr^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+})	Dionex cation exchange	0.3 m*M* $Ba(NO_3)_2$	Cond.	Time of analysis, 10 min; compared well with AAS; LOD, 10^{-6} *M*	84
Soil	Li^+, Na^+, K^+, Rb^+, Cs^+	Dionex cation exchange	5.0 m*M* HCl	Cond.	Time of analysis, 12 min; compared well with AAS and flame photometry; R.S.D. 2.3–6.9%	186
Soil	Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}	Dionex cation exchange	2.5 m*M* HCl–2.5 m*M* *m*-phenylenediamine dihydrochloride	Cond.	Time of analysis, 16 min; compared well with AAS; R.S.D. 2.3–6.9%	186
Soil	Cu^{2+}, Ni^{2+}, Zn^{2+}	Dionex CS5	4.0 m*M* Pyridine 2,6-dicarboxylic acid–50 m*M* CH_3COOH-CH_3COONa (pH 4.8)	UV (520 nm)	Detection as dithiozone complexes; compared well with AAS	187
Soil	Al^{3+}	Dionex CG3	0.4 *M* NH_4Cl (pH 3.1)	UV–VIS	Post-column reaction; LOD, 20 μg l^{-1} (100 μl); time of analysis,3 min; compared well with AAS	188
Soil	Pb, Zn, Co, Fe, Mn	Waters μBondapack C_{18}	2.0 m*M* NaOS–10 m*M* tartaric acid (pH 3.4)	UV (520 nm)	Post-column reaction; time of analysis, 20 min	123
Soil	Alkyl methylphosphonic acid	Dionex MPIC-NS1	2.0 m*M* Tetrabutylammonium hydroxide–1.0 m*M* Na_2CO_3–10–24% CH_3CN	Cond.	Time of analysis, 15 min; LOD, 0.2 mg kg^{-1}	189

Soil and sediment	Borate, bicarbonate	Wescan ion exclusion column	D-Sorbitol	Cond.	Time of analysis, 6 min; R.S.D. 0.68–1.42%; LOD for borate, 0.1 $\mu g\ l^{-1}$	190
Soil	F^-, Cl^-, NO_2^-, PO_4^{3-}, Ca^{2+}, Mg^{2+}	Dionex anion-exchange resin	Na_2CO_3–$NaHCO_3$	Cond.	Linear response was reported	120
Soils, plants	MoO_4^{2-} (WO_4^{3-})	Cosmosil C_{18}	1.5 m*M* Tiron–30 m*M* tetrabutylammonium bromide	UV (315 nm)	Time of analysis, 11 min; recovery, 97–105%; R.S.D. 2.4–4.5%	191
Plants	NO_3^-	Wescan anion exchange	4.0 m*M* Phthalic acid	Cond.	Used for determination of ^{15}N	192
Plants	NO_3^-, SO_4^{2-} (Cl^-, PO_3^{2-}, Br^-)	Hamilton PRP X-100	0.5 m*M* Pyromellitate buffer (pH 3)	UV (295 nm)	LOD, 2–5 ng; time of analysis, 10 min	193
Plants	Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}	Dionex AS4	2.0 m*M* Na_2CO_3–0.7 m*M* $NaHCO_3$ in 2% 2-propanol	Cond.	Time of analysis, 10 min	194
Plants	(i) Li^+, Na^+, NH_4^+, K^+ Rb^+, Cs^+, Ca^{2+}, Mg^{2+}	(i) Dionex CS3	30 m*M* HCl–1.0 m*M* 2,3-diaminopropionic acid–0.5 m*M* $ZnCl_2$	(i) Cond.	Time of analysis, 8 min	194
	(ii) Ca^{2+}, Mg^{2+}	(ii) Dionex CS3	27.5 *M* HCl–2.25 m*M* 2,3-diamino-propionate · HCl–2.25 m*M* L-histidine · HCl	(ii) Cond.	Time of analysis, 12 min	194
Plants	Cl^-, PO_4^{3-}, NO_3^-, SO_4^{2-}, glycolate, malate	Dionex AS3	2.8 m*M* $NaHCO_3$–2.2 m*M* Na_2CO_3	Cond.	Time of analysis, 12 min; R.S.D. 0.1–2.1%; compared well to NO_3^- electrode	195
Plants	Cl^-, HPO_4^{2-}, NO_3^-, SO_4^{2-}, malate, oxalate	Dionex AS4	2.8 m*M* $NaHCO_3$–2.2 m*M* Na_2CO_3	Cond.	R.S.D. <5%; recovery, 95–104%; compared well with conventional methods; time of analysis, 16 min; sample: tobacco	196
Plants	(i) K^+, Na^+	(i) Dionex cation exchange	(i) 5.0 m*M* HCl	(i) Cond.	Time of analysis, 8 min; compared well with AAS and photometry; R.S.D. 0–7.69%	197

(Continued on p. 230)

TABLE 2 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
	(ii) Ca^{2+}, Mg^{2+}	(ii) Dionex cation exchange	(ii) 2.5 m*M* HCl–2.5 m*M* *m*-phenyldiamine dihydrochloride	Cond.	Time of analysis, 8 min; compared well with AAS; R.S.D. 0–7.14%	197
Plants	HCOOH	Alltech Model OA-1000 organic acid column (58°C)	5 m*M* H_2SO_4	UV (214 nm)	Time of analysis, 12 min; similar results with GC–MS	198
Sediments	Cl^-, NO_3^-, SO_4^{2-}	Dionex AS4	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	High Cl^- concentration interferes with NO_3^- and SO_4^{2-} determination; time of analysis, 25 min	199
Sediments	I^-	Dionex AS3	20 m*M* $NaNO_3$–25 m*M* NaOH	Amperometric (Ag^+ electrode)	R.S.D. 3.5%; recovery 90–111%; time of analysis, 10 min	200
Sediments	AsO_3^{3-}, AsO_4^{3-}	Dionex AS1	3.0 m*M* $NaHCO_3$–1.4 m*M* Na_2CO_3	Cond.	No interference from NO_3^-, PO_4^{3-}, SO_4^{2-}; time of analysis, 30 min	201
Sludges	Cl^-, NO_3^-, SO_4^{2-}	Dionex AS4	2.8 m*M* $NaHCO_3$–2.2 m*M* Na_2CO_3	Cond.	Time of analysis,12 min	202
Soil	NO_3^-	Waters C_{18} Radial-Pak	Tetrabutylammonium hydrogen sulfate	UV (220 nm)	Time of analysis, 6 min; compared well with colorimetric method	203
Soil, plants	Total S	Dionex AS3	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Time of analysis, 8 min; compared well with methylene blue method; R.S.D. 0.3–6.5%	204

The chief advantage of IC in plant nutrition studies is its ability for rapid analysis of many anions including intracellular organic acids. Little sample preparation is required for these analyses. Deproteinised plant extracts (obtained routinely by boiling) avoid column contamination. Other advantages of IC in the anion analysis of plant extracts have been described recently by Grunau and Swiader[195]. However, they emphasized the need for precaution such as the use of membrane filters for the removal of interfering extractables since some of these extractables may cause column poisoning. Various digestion methods have been reported including wet and dry procedures[194,195,197].

4. AIRBORNE MATERIALS

IC is a valuable tool for the determination of ions of various airborne samples such as ambient air, aerosols and dust (Table 3). The sample species may be gases including NO_x, SO_x or mists such as H_2SO_4 that have been emitted from anthropogenic sources. Airborne samples also include non-volatile salts and inorganic ions sorbed on particulates such as fly ash or dust.

An airborne sample must first be dissolved in a solvent before it can be analyzed by IC. But this is not always so straightforward. For example, if SO_2 is collected, it must first be converted to an ionic form such as SO_4^{2-} (refs. 208,209) before the IC sample analysis can be performed. Peroxybenzoylnitrate is determined by hydrolysis of the sample into the benzoate anion which is then determined by IC[206]. Sulfuryl fluoride is hydrolyzed to F^- for the IC analysis[207].

Collection of airborne samples is performed with an impinger or sampling train where the gas is sparged through a collection solvent. Frequently, the impinger solvent contains a reagent to convert the sample into the ionic form, *e.g.*, NO_2 to NO_3^-, SO_2 to SO_4^{2-}.

Impingers containing solvents may not be convenient for field investigations or for monitoring worker exposure. Solvent-free systems are often preferred. Much of the recent work describes solid trapping samplers. Gases are passed through the sampler and then desorbed with a solvent. The sampler may be an activated carbon cartridge or flow-through denuder. Although activated charcoal may be used for direct sampling, frequently the support is coated with a chemical to make it more reactive with the target species, i.e., triethanolamine-coated supports to collect SO_2 and NO_2 (refs. 208–210).

Airborne particulates can be dry-filtered out of the air and extracted with a solvent before analysis. Frequently, water or the IC eluent is used as the extraction solvent.

Once the sample is collected, the chromatography becomes fairly straightforward. Tsitouridou *et al.*[235] reported a comprehensive study on eluent and column selection for determination of more than 15 different ions in aerosol and fog water samples. Anion-exchange chromatography is most commonly used for anion samples, but ion-exclusion chromatography is becoming popular for weak inorganic and organic acid samples[217].

Much of the work on airborne samples involves the determination of Cl^-, NO_3^- and SO_4^{2-}. However, as indicated in Table 3, there are considerable studies conducted on other ions of interest. For example, determination of ammonia[211–213], amines[211]

TABLE 3

APPLICATIONS OF IC IN THE ANALYSIS OF AIRBORNE SAMPLES

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Air	Peroxybenzoylnitrate	Dionex anion exchange	2.6 m*M* $NaHCO_3$–3.0 m*M* Na_2CO_3	UV (224 nm)	Potential interference by C_6H_5CHO and C_6H_5COOH; LOD, 0.03 $\mu g\ l^{-1}$	206
Air	Sulfurylfluoride	Dionex AS-1	3.5 m*M* Na_2CO_3–4.0 m*M* NaOH	Cond.	Determination based upon hydrolyis with detection of F^- and SO_4^{2-}: recovery, 95.8%; R.S.D. 11%	207
Air	Sulfur dioxide nitrogen dioxide	Dionex AS-4A	8.0 m*M* Na_2CO_3–3.0 m*M* $NaHCO_3$	Cond.	Trapped on Sep-Pak C impregnated with triethanolamine–potassium hydroxide; R.S.D. 2.4–5.3%; recovery, 85–105%	208
Air	Sulfur dioxide nitrogen dioxide	Dionex AS-1	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Trapped on triethanolamine impregnated with molecular sieve; recovery, 85%	209
Air	Nitrogen dioxide	Dionex AS-4A	2.0 m*M* Na_2CO_3–0.7 m*M* $NaHCO_3$	Cond.	Trapped on Sep-Pack C_{18} impregnated with triethanol-amine; R.S.D. 2.7% (84 ng ml^{-1}); recovery, 87–94%	210
Air	Ammonia and amines	Dionex cation exchange	HCl–HNO_3	Cond.	Trap ammonia/amines by oxalic acid–glycerol treated filter paper; LOD, 8.4 ng ml^{-1} NH_3, 7.9–12.1 ng ml^{-1} amines	211
Air	Ammonia	Dionex cation exchange	5.0 m*M* HCl	Cond.	LOD, 10 $\mu mol\ l^{-1}$; R.S.D. <3%; compares favorably to colorimetric method	212
Auto exhaust	NH_3, alkylamines	Dionex cation exchange	2.5 m*M* HNO_3	Cond.	Agreed well with spectroscopy at >10 $\mu g\ ml^{-1}$ NH_3	213

Air	F^-	Dionex anion exchange	0.3 m*M* $NaHCO_3$	Cond.	Al did not interfere; LOD, 0.1 $\mu g\ ml^{-1}$	214
Air	Cl^-, NO_3^-, SO_4^{2-} acetate, formate, oxalate	Dionex AS-3	2.2 m*M* Na_2CO_3–2.8 m*M* $NaHCO_3$	Cond.	Components of copper patina	215
Air	Formaldehyde	Dionex anion exchange	5.0 m*M* $Na_2B_4O_7$	Cond.	Charcoal adsorption; detected as $HCOO^-$; recovery, 99%; R.S.D. 8%	216
Air	Di- and monomethyl sulfate	Dionex anion exchange	3.0 m*M* Na_2CO_3–2.6 m*M* NaOH	Cond.	Collected on filter pack adsorbent bed; LOD, 2–10 nmol	218
Air	N_3^-	Dionex AS2	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Air bag inflator effluent; R.S.D. 2.5%; linear range, 0.1–10 $\mu g\ ml^{-1}$	219
Air	N_3^-	Dionex AS1	2.0 m*M* NaOH–2. 4 m*M* Na_2CO_3	Cond.	Air bag inflator effluent; Br and adipate cause interference; R.S.D. 2.5%; LOD. 0.5 $\mu g\ ml^{-1}$ (100 μl)	220
Air	HCHO, CH_3CHO	Dionex AS1	1.5 m*M* $NaHCO_3$–5.0 m*M* $Na_2B_4O_7$–6.0 m*M* Na_2CO_3	Cond.	Conversion to formate; R.S.D. 2.6%; LOD, 0.5 ng ml^{-1}	221
Air	AsO_3^{3-}, AsO_4^{3-}, monomethyl arsonate, dimethyl arsinite	Dionex anion exchange	2.4 m*M* $NaHCO_3$–1.9 m*M* Na_2CO_3–1.0 m*M* $Na_2B_4O_7$	AAS	R.S.D. 11%; LOD, <10 ng ml^{-1}; time of analysis, 10 min.	222
Air	SO_2	Dionex anion exchange	3.0 m*M* Na_2CO_3–3.0 m*M* $NaHCO_3$	Cond.	Conversion to SO_4^{2-}; recovery 83–98%; R.S.D. 2.4–7.5%; LOD, 6.0 $\mu g\ ml^{-1}$	223
Air	SO_2	Dionex AS1	$NaHCO_3$–Na_2CO_3	Cond.	Impinger, H_2O_2 solution with conversion to SO_4^{2-}	224
Air	NO_2, NO_x	Dionex AS4	0.20 m*M* Na_2CO_3–0.75 m*M* $NaHCO_3$	Cond.	Collected in alkaline $KMnO_4$ and converted into NO_2^-; LOD, 13 mg NO_x/m^3	225
Air	NO_2	Dionex AS3	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	LOD, 0.25 $\mu g\ m^{-3}$; R.S.D. 5.7%	226

(Continued on p. 234)

TABLE 3 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Air	H_2SO_4 mist	Dionex anion exchange	4.0 m*M* Na_2CO_3–4.0 m*M* $NaHCO_3$	Cond.	Collected on PTFE filter; extracted with benzylaldehyde; interference by benzoic acid; LOD, 0.005 $\mu g\ m^{-3}$	227
Air	Cl_2, ClO_2	Dionex AS3	Na_2CO_3–$NaHCO_3$	Cond.	Time of analysis, 25 min; R.S.D. 1.4–7.3%; LOD, 0.02 $\mu g\ ml^{-1}$ ClO_2 and 0.06 $\mu g\ ml^{-1}$ Cl_2	228
Air	$HCOO^-$, CH_3COO^-	Dionex AS1	5.0 m*M* $Na_2B_4O_7$	Cond.	LOD, 0.2 $\mu g\ m^{-3}$ for formic acid and formaldehyde and 0.4 $\mu g\ m^{-3}$ for acetic acid and acetaldehyde	229
Air	$ClCH_2COOH$	Dionex anion exchange	1.5 m*M* $NaHCO_3$	Cond.	R.S.D. 1.7%; no interference by glycolic acid, acetic acid, dichloroacetic acid, F^- and Cl^- compounds or water vapor	230
Air	Ammonium sulfamate	Dionex anion exchange	3.0 m*M* Na_2CO_3–2.0 m*M* $NaHCO_3$	Cond.	R.S.D. 1.3–3.5%; retention time, 3.4 min; recovery 94–100%; LOD, 0.9 mg m^{-3} (90-l sample)	231
Air	SO_4^{2-}, CO_3^{2-}, NO_3^-	(i) Dionex 35311 (ii) Dionex 30890	(i) 1.5 m*M* Na_2CO_3–1.9 m*M* $NaHCO_3$ (ii) 70 m*M* Na_2CO_3	Cond.	Samples collected in alkaline $KMnO_4$; recovery, 99–102%; R.S.D. 0.4–4.6%	232
Air	$HCOO^-$, CH_3COO^-, $CH_3CH_2COO^-$, Cl^-, NO_2^-, NO_3^-, SO_2	Dionex AS2	1.0 m*M* Tridecafluoro-heptanoic acid, 1% isopropanol	Cond.	Recovery, 95–98%	64
Air	Chloroacetyl chloride	Dionex AS1	1.5 m*M* $NaHCO_3$	Cond.	Recovery, 99%; R.S.D. 7.2–8.8%, LOD, 0.01 $\mu g\ ml^{-1}$	233

Air	Cl^-, NO_2^-, NO_3^-, SO_4^{2-}	Vydac 302 IC	1.6 m*M* Salicylate	UV and fluorometry	LOD, 2.3–3.2 mg l^{-1}; time of analysis, 25 min	234
Aerosol	(i) Cl^-, NO_2^-, NO_3^- SO_4^{2-} (ii) Na^+, NH_4^+, K^+ (iii) F^-, glycolate, lactate, formate, formic acid	Wescan anion/R Wescan cation Wescan ion exclusion	(i) 4.0 m*M* Potassium hydrogenphthalate (ii) HNO_3 (iii) H_2SO_4	(i) Cond. (ii) Cond. (iii) Cond.	Portable IC, LOD, 0.02–0.15 mg l^{-1}	236
Aerosol	NH_4^+	Dionex cation exchange	6.0 m*M* HNO_3	Cond.	R.S.D. 0.9–2.9%; time of analysis, 10 min; compared well with ion selectivity method	236
Paint aerosol	Cr(VI)	Dionex AS5	0.5 m*M* NaOH–7.0 m*M* Na_2CO_3	Cond.	LOD, 0.1 μg ml^{-1}; R.S.D. 4.3%; time of analysis, 8 min	237
Aerosol	SO_4^{2-}, NO_3^-	Dionex AS1	3.0 m*M* $NaHCO_3$–0.9 m*M* Na_2CO_3	Cond.	Br^- and PO_4^{3-} interfered with analysis; time of analysis, 20 min; R.S.D. 1–3%	238
Aerosol	SO_4^{2-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	LOD, 0.5 μg m^{-3}	239
Aerosol	Cl^-, NO_3^-, SO_4^{2-}	Vydac 302	5.0 m*M* Potassium hydrogenphthalate	Cond.	Time of analysis, 7 min	240
Aerosol	Cl^-, NO_3^-, SO_4^{2-}	Dionex AS4	2.8 m*M* $NaHCO_3$–2.2 m*M* Na_2CO_3	Cond.	LOD, 5 ng m^{-3}; time of analysis, 8 min	241
Aerosol	F^-, Cl^- (NO_2^-, NO_3^-, SO_4^{2-})	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Time of analysis, 20 min	242
Flue dust	AsO_4^{3-}, SO_3^{2-}	Dionex anion exchange	3.5 m*M* Na_2CO_3–2.6 m*M* NaOH	Cond.	No detection of AsO_3^{3-}; LOD, 1.8 μg ml^{-1} AsO_4^{2-}; oxidation of SO_3^{2-} to SO_4^{2-} by addition of $FeCl_3$ or $CuCl_2$	243
Flue dust	AsO_4^{3-}, AsO_3^{3-} (Cl^-, NO_3^-, SO_4^{2-})	Dionex AS1	3.5 m*M* Na_2CO_3–2.6 m*M* NaOH	Cond.	Time of analysis, 30 min	244
Flue gas	SO_3^{2-}, SO_4^{2-}, H_2SO_4 mists	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	SO_3^{2-} and SO_4^{2-} are detected simultaneously	245

(Continued on p. 236)

TABLE 3 *(continued)*

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Flue gas	NO_x, SO_x	Toya Soda IC-Anion-PW	0.7 m*M* Gluconate–0.7 m*M* boron–0.1 m*M* KH_2PO_4–EDTA	UV–VIS–Cond.	Ions were determined from flue gas scrubbing	246
Flue gas	SO_3^{2-}, SO_4^{2-}	Dionex anion exchange	9.0 m*M* $NaHCO_3$–7.2 m*M* Na_2CO_3	Cond.	IC method was used to determine sulfoxy ions in flue gas desulfurization solutions	247
Flue gas	Hydrogen halides and halogens	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	No SO_2 or NO_x interference; recovery 99.4%; LOD, low $\mu g\ ml^{-1}$	248
Flue gas	Cl^-, SO_3^{2-}, SO_4^{2-}, Ca^{2+}, Mg^{2+}, Na^+, K^+, NH_4^+	(i) Dionex anion exchange (ii) cation exchange	(i) 3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3 (ii) 5.0 m*M* HNO_3	Cond.	Time of analysis, 30 min	249
Combustion gases	F^-, Cl^-, NO_x, SO_3^{2-}, SO_4^{2-}, Br^-, HPO_4^{2-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–4.0 m*M* Na_2CO_3	Cond.	Column temperature critical to obtain good resolution	250

Dusts, fly ash	F^-, Cl^-, NO_2^-, NO_3^-, I^-, SO_4^{2-}, PO_4^{3-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Time of analysis, 16 min; R.S.D. 0.12–9.99%; compared well with ion selective electrodes, potentiometry, AAS, ICP	251
Coal fly ash	F^-, Cl^-, NO_3^-, SO_4^{2-}	Dionex anion exchange	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	R.S.D. <3%; LOD, 0.05–150 $\mu g\ ml^{-1}$; unable to detect PO_4^{3-}	252
Diesel exhaust	Formic acid	Dionex ICE	5.0 m*M* $Na_2B_4O_7$	Cond.	Time of analysis, 11 min; peak height linear from 0.1–4 $\mu g\ ml^{-1}$; LOD, 0.05 $\mu g\ ml^{-1}$ (100 μl sample)	217
Fuel combustion products	F^-, Cl^-, PO_4^{3-}, NO_2^-, NO_3^-, SO_x, Mg^{2+}, Ca^{2+},Na^+, NH_4^+, K^+	Dionex AS1	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Time of analysis, 20 min; compares to ASTM methods with greater sensitivity	253
Atmospheric pollutants	NO_2^-, NO_3^-, SO_4^{2-}	IC-PAK anion	Potassium hydrogenphthalate *vs.* borate–gluconate	Cond.–UV (284 nm)	IC compared well to colorimetric method for NO_2^-; time of analysis, 16 min; LOD by UV for SO_4^{2-}, 5 ng (50 μl sample)	254

TABLE 4

APPLICATIONS OF IC IN THE ANALYSIS OF FOSSIL FUELS

Sample	*Ion*	*Analytical column*	*Eluent*	*Detector*	*Comments*	*Ref.*
Fuels, coal	F^-, Cl^-, NO_2^-, PO_4^{3-}, Br^-, NO_3^-, SO_4^{2-}	Dionex anion exchange	Na_2CO_3–$NaHCO_3$	Cond.	Compared well to ASTM methods; LOD, 2–10 μg ml^{-1}	255
Fuels	Total S (F^-, Cl^-, NO_2^-, NO_3^-, SO_4^{2-})	Dionex anion exchange	2.4 m*M* $NaCO_3$–3.0 m*M* $NaHCO_3$	Cond.	Compared well to ASTM methods; R.S.D. 0.7%; LOD, 2 μg ml^{-1} SO_4^{2-}	256
Fuel oil	Total S	Dionex AS1	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Compared well with titrimetry	257
Fuel oil	Total S	Dionex AS1	3.0 m*M* $NaHCO_3$–2.4 m*M* Na_2CO_3	Cond.	Compared well with X-ray fluorescence and titrimetry; LOD, 0.007%; R.S.D. 2.7%; time of analysis, 25 min	258
Crude oil	Total S (Cl^-, NO_3^-, SO_4^{2-})	Waters IC-Pak	Borate–gluconate–acetonitrile–glycerol	Cond.	Recovery, 95.7–102.2%; R.S.D., 1.0–2.9%; LOD, 0.5 μg kg^{-1}; time of analysis, 15 min	259
Coal, ash, geological materials	F^-	Dionex AS3	1.5 m*M* $NaHCO_3$	Cond.	LOD, 1 ng; R.S.D. 4.9%; compared well with ion selective electrode	260
Coal	Total Cl^-	Wescan 260-001	Potassium hydrogenphthalate	Cond.	Extraction with dimethyl sulphoxide; converted to Cl^-	261
Coal	Cl^-, NO_3^-, NO_2^-, SO_4^{2-}	SAX 1	4.0 m*M* Na_2CO_3–4.0 m*M* $NaHCO_3$	Cond.	Precision not satisfactory for NO_2^-, NO_3^-; time of analysis, 10 min	262
Coal	Ag, Al, Ba, Cd, Cu, Fe, Mg, Mn, Ni, Pb, Si, Sn, Ti, V, Zn	100Å μStyragel	Pyridine (chloroform, tetrahydrofuran)	ICP-AES	LOD, 3–530 ng ml^{-1}; size-exclusion chromatography; multiple peaks of Fe	263

and alkylamines[213] have been reported. Acetate, formate and oxalate[215], sulfamate[231], monochloroacetic acid[230], chloroacetyl chloride[233], formic acid[64,217,235], dimethyl- and methyl-sulfate[218], azide[219,220], formaldehyde[216] and acetaldehyde[221] have also been monitored.

5. FOSSIL FUELS

The elemental composition of fossil fuels such as coal and various petroleum products is an important environmental consideration in the use of these energy sources. IC can be used to determine the fuel elemental composition for halogens, N, S, P and various metals. The samples may first be combusted in an oxygen bomb or a furnace, the gases are absorbed in water and the ions are determined by IC. In other cases, the sample is simply extracted with a solvent. Dimethyl sulfoxide has been used to leach Cl^- from a number of coal samples[261]. Water extract of coal has been used by Honma *et al.*[262] for the determination of Cl^-, NO_2^-, NO_3^- and SO_4^{2-}. Recently Burns *et al.*[264] found IC to be very attractive for the separation of a number of radionuclides of elements such as plutonium, americium, curium, uranium and thorium in radiochemical wastes. Table 4 lists the applications of IC in the analysis of fossil fuels.

6. CONCLUSIONS

New analytical developments in IC have allowed the simultaneous determination of ions from complex matrices at trace levels. IC can be tailored to be selective for specific target solutes in the presence of other matrix ions, *i.e.*, SeO_4^{2-} *vs.* SO_4^{2-} (ref. 173), SeO_3^{2-} *vs.* Cl^- (ref. 174) and AsO_4^{3-} *vs.* PO_4^{3-} (ref. 177). Much of the IC applications involve suppressed systems marketed by Dionex (Sunnyvale, CA) but recent advances have now become available with non-suppressed systems (single column ion chromatography, SCIC).

Important parameters in optimizing the separation and detection of target solutes include choice of eluent, concentration of the mobile phase, working pH, column selection and choice of detectors. Among the most popular eluents, low ionic strength aromatic organic acids (phthalate, benzoate and citrates) and mineral acids have been used as the mobile phase in SCIC. Stationary phases include macroporous styrenedivinylbenzene resins, quaternized silica and polymethacrylate gels. Conductivity is frequently used as the mode of detection in IC but other detectors include absorbance (UV or visible light), fluorescence, AAS, coulometry and amperometry. Electrochemical detection is popular because of its high selectivity and its ability to detect ultra-trace levels.

New developments in IC do not simply involve "pick and choose" of each component (mobile phase, column and detector), but require extensive knowledge in determining the compatibility of these components in developing a useful analytical system. The pK_a of the target ion of interest must be known as well as the presence of potential interfering substances. Column selection is based on the upper and lower pH limit of the mobile phase to optimize separation.

Among the advantages of IC are: (i) its relatively high sensitivity with detection limits in the low $\mu g\ ml^{-1}$ (conductivity, UV/VIS, AAS), low $ng\ ml^{-1}$ (amperometry)

and pg ml^{-1} range (fluorometric detection); (ii) simultaneous determination of several species and other elements, *e.g.*, separation and determination of SO_3^{2-}, SO_4^{2-} and $S_2O_3^{2-}$ (ref. 108); (iii) IC is non-destructive and fractions can be collected for further confirmation; (iv) the cost of IC is relatively low (the system can be modified easily by combining different components to accommodate changes in analytical requirements); (v) sample preparation is often minimal. IC can be used in routine analysis of environmental samples and operated after minimal instruction.

7. SUMMARY

Ion chromatography (IC) is going through rapid growth and popularity because of its usefulness in the applied environmental field. This review includes recent IC applications in rainwater, groundwater, surface water, wastewater, drinking water, fog samples, ice, snow, soil, sediments, sludge, plants, air, exhaust, aerosols, flue dust, fly ash, fuel oil and coal. A major emphasis of this review is on speciation of ions.

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Two-dimensional field-flow fractionation

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SUMMARY

Multidimensional field-flow fractionation (FFF) is described in two major forms: one in which different separative stages are coupled together and one in which two independent displacements, at least one of them FFF, are carried out in a generally planar channel structure. The latter, the subject of this paper, is relatively promising for implementation in FFF systems because in most cases the geometry of the FFF channel is already planar; the channel structure needs mainly to be broadened along the second dimension and modified with different inlets and outlets for this two-dimensional use.

The large number of potential two-dimensional FFF systems is discussed. These systems are described at greater length in four categories: (1) FFF displacement used in both dimensions, (2) FFF along one axis and chromatography along another, (3) FFF along one axis and a field-induced displacement along the other, and (4) FFF separation combined with bulk or flow displacement at right angles.

Finally, theoretical equations are obtained for the deflection of the trajectories away from the main flow axis z. The sensitivity of deflection to component properties is described in terms of the *deflection selectivity*. Several examples are discussed in which the deflection selectivity is remarkably high.

INTRODUCTION

Multidimensional separation methods are those techniques that utilize, according to well-defined criteria, two or more relatively independent separative stages to achieve the resolution of sample components[1,2]. Well-designed multidimensional systems have a number of advantages over normal one-dimensional or linear separation systems. These advantages include enhanced resolving power, increased flexibility, and in some cases an improved compatibility in the matching of the separation system to the sample[2].

The primary limitation of multidimensional separation systems is their complexity. Perhaps the simplest means for realizing multidimensional capabilities is to couple or link different separative stages to one another. In coupled column chromatography,

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for example, partially resolved fractions taken from the eluent of the first column are shunted one at a time into a subsequent column, usually with a different stationary phase. Such systems have great flexibility and they are relatively easy to operate[3,4]. However, a conceptually simpler multidimensional [in this case, two-dimensional (2D)] approach involves carrying out two different displacement (usually separation) steps along the two axes of a surface, most often a planar bed[5]. This powerful approach has been used extensively in thin-layer chromatography and in electrophoresis[6–8]. While coupled column operation can be usefully applied to field-flow fractionation (FFF)[9], it is the "planar" 2D system, in which a FFF mechanism is used for one or both of two right angle displacement steps, that will be considered in this paper.

For a number of reasons (see below), the dominant geometry for FFF channels has been that of a thin rectangular space enclosed between close-lying parallel plates. In most cases, separation has been realized only along the principal or flow axis of the system. The thin dimension, across which the field is applied, is used for the enrichment process that underlies separation[10,11]. This leaves a final dimension, the "breadth" coordinate, available for further separative manipulations. Thus without a change in basic geometry, this latter coordinate, perhaps physically expanded somewhat, can in theory be used as a second dimension along which separation can be realized. The feasibility of this approach has been demonstrated by the development of continuous steric FFF, a 2D method in which gravitational sedimentation is applied along the breadth dimension in order to convert steric FFF into a continuous separation system[12].

In this paper we develop the conceptual foundations for 2D-FFF in a broader context, outlining a large number of possible experimental configurations. We examine the use of the second (breadth) dimension both for continuous separations and for discrete methods yielding true 2D fractograms. For the convenience of the ensuing discussion, the coordinates of all such systems are defined in Fig. 1.

The reasons for the dominance of thin parallel plate channels in FFF are instructive; some of these considerations carry over to 2D-FFF. First we note that the field in FFF should generally impinge on the channel surface at right angles to the

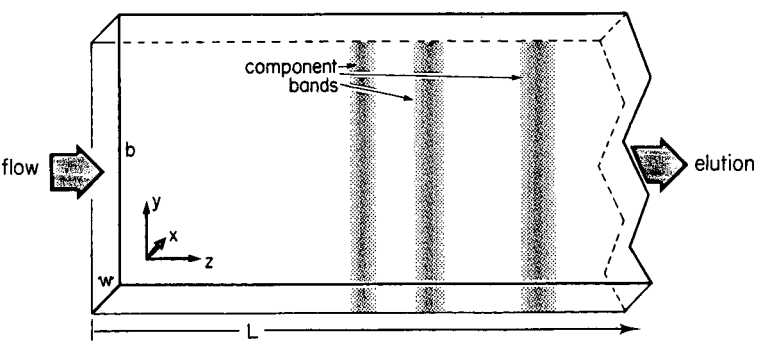

Fig. 1. Illustration of coordinate system of FFF channel: z = flow direction (forming principal separation axis), x = field direction, y = lateral direction (available as a second dimension for separation). Component bands extending from $y = 0$ to $y = b$ and separating along axis z demonstrate normal one-dimensional FFF.

surface plane in order to avoid spurious transport parallel to the plane. Because a majority of fields and gradients are simplest to apply with all field lines more or less parallel in the apparatus, flat channel surfaces are preferable. Furthermore, if one wants to avoid possible convective effects due to temperature gradients in the channel (thermal FFF) or density gradients induced by the sample, then the surface should be flat and normal to the earth's gravity. Finally, the parallel plate configuration is relatively easy to construct with carefully controlled dimensions.

The substantial breadth b (several centimeters) ordinarily found in FFF channels is employed to diminish edge effects, increase sample capacity, and increase channel volumes so that extraneous volumes are negligible by comparison.

If a radial field can be generated, then an annular thin channel configuration is also desirable. (We define an annular channel as one in which the flow direction is parallel to the axis of symmetry of the field. Normal sedimentation FFF, in which the ribbon-like channel is wrapped around the inside of a centrifuge basket, employs a flow direction at right angles to the axis of rotation with all the attributes of a parallel plate channel of finite breadth b.) True annular channels have the advantage of doing away with edge effects entirely. However, in any configuration chosen, an annular channel is subject to possible disturbances by gravity-induced convection. (In at least one case, thermogravitational FFF, convective flow can be used to advantage[13].) Annular channels as well as parallel plate channels are expected to be convenient for 2D separations. An exception is the subtechnique of shear FFF in which the breadth dimension (measured around the circumference) must be employed to generate the shear-induced forces[14]. Consequently, there will be no further reference to shear FFF in this paper.

CATEGORIES OF 2D-FFF

Following the general pattern of enormous variability in 2D separation methods[5], the more specific implementation of 2D-FFF methods can, in principle, assume many forms. We can identify a number of major categories and subcategories; these are summarized in Table I (see explanation of Table I below). These categories vary, as shown, with respect to possible elution axes and regarding the possibility of simultaneous operation, in which both displacements take place at the same time. Operation in the simultaneous mode is a potentially streamlined approach to discrete analytical separations; it is essential for continuous separations[5].

Table II, applicable only to one-dimensional operation, is suggestive of the range of possibilities. The elements of the table's matrix represent different potential kinds of one-dimensional FFF subtechniques, based on the combination of six fundamentally different modes of operation of FFF and various displacement forces originating in some of the different fields and gradients conceivable for FFF. Each subtechnique (element) can, in theory (many are untested), be operated under a wide range of conditions and field strengths. For 2D operation, we can combine each FFF subtechnique with most other FFF subtechniques or with other displacement effects (of a form indicated by Table I) to generate hundreds of possible 2D-FFF approaches. These are clearly too numerous to detail here, but by examining different categories we can better understand the potential characteristics, advantages, and limitations of FFF in various 2D forms.

TABLE I

MAJOR CATEGORIES AND SUB-CATEGORIES OF 2D-FFF SEPARATION

See text.

2D category	*2D sub-categories*	*Possible elution axes*	*Simultaneous/ continuous operation*
(1) FFF × FFF	$F_jFF \times F_kFF$	FFF	No[a]
	$F_jFF(1) \times F_jFF(2)$		
	$A_1FFF \times A_2FFF$		
(2) FFF × chromatography	FFF × open channel	FFF	No[a]
	FFF × packed channel	chromatography	
(3) FFF × field displacement	$F_jFF \times F_j$	FFF	Yes
	$F_jFF \times F_k$		
(4) FFF × bulk/flow displacement	FFF × bulk	FFF	Yes
	FFF × flow	flow	

[a] Subject to occasional exception.

Category 1: FFF × FFF

The two FFF displacements in this category must generally be carried out sequentially. The reason for this, as explained more fully in the original 2D publication[5], is that in most cases of simultaneous operation, all components take the same vector direction, which is the axis of flow. (Exceptions can be developed by using different mechanisms of flow in the two orthogonal directions.) In the absence of differences in the deflection angle, discrete separations are no better (and much more complicated) than their one-dimensional form; continuous separations are impractical[5].

The sequential operation of the FFF × FFF mode allows a great deal of

TABLE II

GREAT VARIETY OF FFF METHODS IS SUGGESTED BY THIS MATRIX, MOST ELEMENTS OF WHICH ARE POTENTIAL FFF SUBTECHNIQUES

The table is not comprehensive as some potential displacement phenomena, *e.g.*, photophoretic, have been omitted.

Operating mode	*Displacement force*						
	Sedimentation (Sd)	*Thermal (Th)*	*Electrical (El)*	*Dielectrical (Dl)*	*Flow (Fl)*	*Shear (Sh)*	*Magnetic (Mg)*
Normal (Nl)	SdNlFFF	ThNlFFF	ElNlFFF	DlNlFFF	FlNlFFF	ShNlFFF	MgNlFFF
Steric (St)	SdStFFF	ThStFFF	ElStFFF	DlStFFF	FlStFFF	ShStFFF	MgStFFF
Hyperlayer (Hy)	SdHyFFF	ThHyFFF	ElHyFFF	DlHyFFF	FlHyFFF	ShHyFFF	MgHyFFF
Cyclical-field (Cy)	SdCyFFF	ThCyFFF	ElCyFFF	DlCyFFF	FlCyFFF	ShCyFFF	MgCyFFF
Secondary equilibrium (Sy)	SdSyFFF	ThSyFFF	ElSyFFF	DlSyFFF	FlSyFFF	ShSyFFF	MgSyFFF
Chromatographic hybrid (Ch)	SdChFFF	ThChFFF	ElChFFF	DlChFFF	FlChFFF	ShChFFF	MgChFFF

flexibility. Different carrier fluids can be applied along the two axes with operation possible at different temperatures, pressures, etc. Even more, the edge strip along which the first FFF displacement occurs can have its own thickness and surface characteristics; the wall along this strip can be designed to transmit a different external field than that applied over the bulk of the channel for the second displacement.

As shown in Table I, there are some distinct sub-categories of FFF × FFF operation. The use of different fields for the two displacements, say fields j and k, is symbolized by $F_jFF \times F_kFF$. The same field applied under two different sets of conditions, (1) and (2), which may involve different field strength, flow velocity, carrier density, pH, etc., is indicated by $F_jFF(1) \times F_jFF(2)$. Separations combining two fundamentally different modes of operation, A_1 and A_2 (see above), are designated by $A_1FFF \times A_2FFF$. In some cases more than one of these characteristics may differ between the two component runs.

Category 2: FFF × chromatography

The combination of FFF and chromatographic mechanisms is subject to many of the same considerations (such as sequential operation) that apply to category 1. With thin enough channels having walls coated with a retentive phase (except along an edge strip reserved for FFF), effective chromatography could be carried out in the second dimension. However, channels of extraordinary thinness are needed for effective chromatographic operation in open channels, particularly for macromolecules[15]. Such extreme thinness is not needed in FFF because the diffusion path is reduced by the field[11]. It is possible that a channel in which the thickness is stepped would help resolve the unlike requirements of FFF and chromatography.

Alternatively, the part of the flat channel used for chromatography could be carefully packed, the remainder left open for FFF. Either mechanism could be used first in sequence but with FFF used first along a channel formed at one edge, flow control might be simpler.

Category 3: FFF × field displacement

By applying an external field at right angles to the FFF displacement (and also at right angles to the primary FFF field), simultaneous operation is possible, yielding either discrete or continuous separation. The previously cited example of continuous steric FFF (better specified as Sd/StFFF × gravitational sedimentation) illustrates simultaneous, continuous separation in this category. In this example, both displacements are selective (separative), but this is no particular advantage for continuous separation[5]. For example, it should be possible to combine electrophoresis, which provides little size selectivity for colloidal particles of fixed surface composition, with sedimentation FFF (in essentially any mode), or with some other size-selective FFF methods to realize continuous size-based separation.

For cases in which electrophoretic displacements are selective, continuous separation can be realized by using (non-selective) flow rather than FFF for the second direction. Continuous flow or deflection electrophoresis is based on this combination[16,17]. However, when carried out in the space between parallel plates, the solute bands are smeared out (forming "crescent zones") because of the nonuniform parabolic flow[18]. If FFF is used in place of non-selective flow, it appears that this resolution loss can be largely avoided and that both discrete and continuous separation could be achieved[19].

The variability of the FFF × field displacement category is also large, reflecting the possible combination of the many FFF methods (along with extensions and variations) spelled out in Table II with a number of field-induced displacements, including sedimentation, electrophoresis, possibly isotachophoresis and dielectrophoresis, thermal diffusion, and so on.

Category 4: FFF × bulk flow displacement

There is a possibility of combining FFF and non-selective bulk or flow displacement to achieve separation. Thus an annular channel subject to slow rotation (bulk displacement) could yield continuous separation by the same general mechanism proposed for annular chromatography[20,21]. Separation on a rotating disc, with different components deflected along different trajectories to their unlike FFF velocities, could also be used.

In order to realize selective deflection using nonselective flow at right angles to FFF displacement, the flow profiles in the two directions would need to be different (*e.g.*, electroosmotic flow along the flow axis).

DEFLECTION AND SELECTIVITY

Some general aspects of 2D separation theory were discussed in the earlier 2D paper[5] and in some of the references noted therein[6,22]. Here we will focus on the deflection of components in 2D-FFF systems and on the resulting selectivity of deflection with respect to component properties.

In many cases[5] the coordinates of the component zones in a 2D separation system are most conveniently described in terms of radial distance R' and deflection angle θ (see Fig. 2). For resolving large numbers of components in the discrete (analytical) mode, separation must occur along both of these coordinates, although any two species can be separated by differences in only one coordinate[1,2]. However, for continuous separation, only differences in θ are required. Because a θ-based

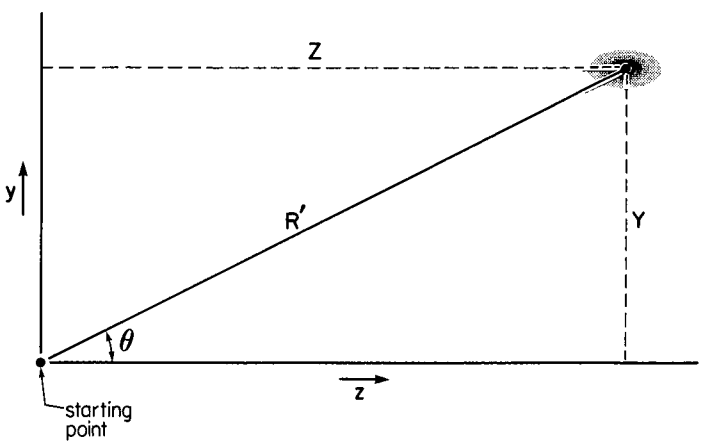

Fig. 2. Specification of component zone location by the polar coordinates R' and θ.

separation is central in both cases, we will describe some aspects of selectivity along this coordinate. (We note that an R'-based separation, governed by the increment $\delta R' = [(\delta X)^2 + (\delta Y)^2]^{1/2}$, depends directly on the individual increments δX and δY responsible for separation in the two contributing displacement steps.)

For theoretical simplicity, deflection will be referenced to $\tan\theta$ rather than θ. We have

$$v = \tan\theta = \frac{Y}{Z} \tag{1}$$

where Y and Z are the respective displacements along the y and z axes. When displacements occur uniformly along these axes for times t_y and t_z at the respective velocities υ_y and υ_z, we have

$$v = \frac{\upsilon_y t_y}{\upsilon_z t_z} \tag{2}$$

For simultaneous operation, for which $t_y = t_z$, v reduces to

$$v = \frac{\upsilon_y}{\upsilon_z} \tag{3}$$

The magnitude of deflection may depend upon several different component properties or upon a single property, depending on the nature of the sample and of the 2D separation system. The deflection selctivity with respect to some single property p (where p may be molecular weight M, particle diameter d, density ρ, etc.) is defined analogously to other selectivity expressions, namely[23]

$$S_p = \frac{\mathrm{d}\ln v}{\mathrm{d}\ln p} \tag{4}$$

For category 1 (FFF × FFF) operation, generally feasible only in the sequential mode, component velocities are expressed in terms of the respective retention ratios R_y and R_z in much the same way as in the one-dimensional case

$$Y = \upsilon_y t_y = R_y \langle\upsilon\rangle_y t_y \tag{5}$$

$$Z = \upsilon_z t_z = R_z \langle\upsilon\rangle_z t_z \tag{6}$$

where $\langle\upsilon\rangle$ is the cross-sectional average velocity. For the normal mode of FFF operation, each R is expressed in terms of its respective retention parameter λ, often according to the limiting equation[24]

$$R \approx 6\lambda \tag{7}$$

The parameter λ, in turn, is formulated in terms of property p $\lambda(p)$. Thus v can be expressed as

$$v = \frac{R_y\langle\upsilon\rangle_y t_y}{R_z\langle\upsilon\rangle_z t_z} \approx \frac{\lambda_y\langle\upsilon\rangle_y t_y}{\lambda_z\langle\upsilon\rangle_z t_z} \tag{8}$$

Retention parameter λ can be written in the general form[24]

$$\lambda = \frac{kT}{F_x w} \tag{9}$$

where kT is thermal energy, w is the channel thickness, and F_x is the x component of force exerted on the particle by the field. If we write $F_x = fU_x = fm_x S_x$, we get

$$\lambda = \frac{kT}{f}\frac{1}{wm_x S_x} = \frac{D}{wm_x S_x} \tag{10}$$

where U_x is the velocity of the particle along axis x due to the FFF field of strength S_x, m_x is the generalized mobility of the particle, f is the friction coefficient, and D the diffusion coefficient. When this λ is substituted back into eqn. 8, we get

$$v = \frac{(m_x)_z(S_x)_z\ \langle\upsilon\rangle_y t_y\ D_y}{(m_x)_y(S_x)_y\ \langle\upsilon\rangle_z t_z\ D_z} \tag{11}$$

where $(m_x)_z$ and $(m_x)_y$ are the mobilities and $(S_x)_z$ and $(S_x)_y$ the x-directed field strengths applicable during displacement along axes z and y, respectively, and D_z and D_y are the respective diffusion coefficients in effect during these two displacements. While D_z and D_y may be equal, they can differ substantially because the carrier liquid, pH, and temperature may not be the same in the two displacement steps.

In the case of category 2 (FFF × chromatography), generally involving sequential operation, v is obtained from eqn. 2 with the corresponding migration velocities (υ) inserted. Since the υ for chromatography is usually a complex function of most continuous properties p, no simple expressions for υ emerge. However, for FFF × size exclusion chromatography, υ can be readily calculated.

For category 3, involving FFF × direct field operation, we have for the field displacement

$$Y = U_y t_y = m_y S_y t_y \tag{12}$$

where U_y is the velocity induced along coordinate y by the field whose strength is S_y. The mobility of the particle due to the y-directed field is m_y.

To get the FFF displacement (operating in the normal mode) along z in comparable terms, we follow eqns. 6 and 7 to get

$$Z = R_z\langle\upsilon\rangle_z t_z \approx 6\lambda\langle\upsilon\rangle_z t_z \tag{13}$$

The substitution of λ from eqn. 10 into eqn. 13 gives

$$Z = \frac{6D\langle v\rangle_z t_z}{w m_x S_x} \tag{14}$$

This expression along with eqn. 12 yields

$$v = \frac{Y}{Z} = \frac{w m_x m_y S_x S_y\ t_y}{6D\langle v\rangle_z\ t_z} \tag{15}$$

Normally, operation would be simultaneous with $t_y = t_z$, giving

$$v = \frac{w m_x m_y S_x S_y}{6D\langle v\rangle_z} \tag{16}$$

When the same kind of field (*e.g.*, electrical or sedimentation) is used in both x and y directions, we have $m_x = m_y = m$ and this equation becomes

$$v = \frac{w m^2 S_x S_y}{6D\langle v\rangle_z} \tag{17}$$

which shows that deflection is very sensitive to mobility m, offering the possibility of high resolution, particularly in continuous operation. The mobility-based selectivity of such operation is found from eqn. 4 to be $S_m = 2$, an extraordinarily high value. This equation shows that charged species (*e.g.*, proteins) could be continuously separated in an ElFFF × electrophoresis system with a selectivity of two in electrophoretic mobility ($m = \mu$). A similar high promise applies to sedimentation, as will be shown in the next section.

If FFF is carried out in the steric rather than the normal mode, we can replace the R in eqn. 10 by

$$R = 6\gamma d/w \tag{18}$$

where γ is the steric correction factor and d is particle diameter. Eqns. 13 and 12 then lead to

$$v = \frac{w m_y S_y}{6\gamma d\langle v\rangle_z} \tag{19}$$

Finally, for FFF × bulk flow displacement (generally occurring simultaneously), the flow velocity v_y is constant and v becomes

$$v = \frac{v_y}{R_z\langle v\rangle_z} \tag{20}$$

All selectivity in this case originates from changes in R_z.

EXAMPLES OF POTENTIAL 2D SYSTEMS

It is interesting to apply these equations to several potential forms of 2D operation. For this purpose we assume that the sample consists of spherical particles of diameter d and density ρ_s. For such particles the sedimentation force is

$$F(\text{sed}) = \frac{\pi}{6} d^3 \, |\Delta\rho| \, G \tag{21}$$

where $\Delta\rho = \rho_s - \rho$, ρ is the carrier density, and G is the field strength expressed as acceleration. The mobility relative to G (the sedimentation coefficient) is

$$m(\text{sed}) = \frac{d^2 \, |\Delta\rho|}{18\eta} \tag{22}$$

For crossflow the force is

$$F(\text{flow}) = 3\pi\eta d U = 3\pi\eta d \dot{V}_c / Lb \tag{23}$$

where U is the velocity of crossflow, $\dot{V}_c$ is the volumetric crossflow rate, η is the carrier velocity, and L and b are the channel length and breadth, respectively. The mobility referenced to $\dot{V}_c$ becomes

$$m(\text{flow}) = \frac{1}{Lb} \tag{24}$$

The deflection for the 2D combination, sedimentation FFF × flow FFF, can be obtained by substituting eqns. 22 and 24 into eqn. 11

$$v(\text{SdN|FFF} \times \text{FlN|FFF}) = \frac{18\eta \dot{V}_c}{Lbd^2 \, |\Delta\rho| \, G} \frac{\langle v \rangle_y t_y}{\langle v \rangle_z t_z} \tag{25}$$

where η is now the viscosity of the flow FFF carrier liquid (instead of the carrier for sedimentation FFF, if different) by virtue of the D_y/D_z ratio in eqn. 11. The selectivity S_d of v with respect to d is two.

If sedimentation FFF at one carrier density is combined with that at another carrier density (thus falling in the $F_jFF(1) \times F_jFF(2)$ category of Table I), a similar analysis shows that the diameter-based selectivity vanishes and is replaced by a particle density selectivity.

When FFF is combined with direct field displacement, a variety of results are obtained. For the case of SdStFFF × gravitational sedimentation mentioned earlier, the substitution of eqn. 22 into eqn. 19 with S_y replaced by g (the acceleration of gravity) × cos θ(θ is the angle between the sedimentation path and a vertical axis) yields

$$v = \frac{wd \, |\Delta\rho| \, g \cos\theta}{108\gamma\eta\langle v \rangle_z} \tag{26}$$

The apparent diameter selectivity of this deflection is unity but the actual selectivity is somewhat higher because γ decreases somewhat with d.

If we now use the pair SdNlFFF × centrifugal sedimentation, v is obtained from eqn. 17 as

$$v = \frac{w \mid \Delta\rho \mid^2 d^4 G_x G_y}{1944\eta^2 D\langle v\rangle_z} \tag{27}$$

Since $D = kT/3\pi\eta d$, we have

$$v = \frac{\pi}{648} \frac{w \mid \Delta\rho \mid^2 d^5 G_x G_y}{kT\eta\langle v\rangle_z} \tag{28}$$

Eqn. 28 shows a remarkably high selectivity both with respect to diameter ($S_d = 5$) and density difference $\Delta\rho$($S_{\Delta\rho} = 2$). This high selectivity can, in theory, be realized using a sedimentation FFF channel "tilted" with respect to the rotating axis. The angle of tilt will specify the relationship between G_x and G_y. Unfortunately, preliminary efforts to utilize such a channel constructed in this laboratory have not proven successful, perhaps because of secondary flow.

The above cases, of course, represent only a few examples from the rich repertoire of potential 2D-FFF techniques. In most cases the calculation of deflection, selctivity and other separation properties is straightforward and can be carried out in much the same manner as illustrated above.

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Capillary column gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry detection of chemical warfare agents in a complex airborne matrix

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SUMMARY

The chemical warfare agents sarin, soman and mustard were detected and confirmed during full-scanning gas chromatography (GC)–mass spectrometry (MS) at the nanogram level in spiked extracts of a diesel exhaust environment sampled onto the charcoal of a Canadian C2 respirator canister. This matrix, typical of what might be expected under battlefield conditions, was used for the development of a GC–MS–MS method for the verification of trace levels of sarin, soman and mustard. Chemical interferences associated with this complex sample were virtually eliminated and low-picogram GC–MS–MS detection limits were estimated for these chemical warfare agents in the presence of numerous interfering diesel exhaust and charcoal bed components.

INTRODUCTION

Chemical weapons use, although prohibited by the 1925 Geneva Protocol, has been documented during several armed conflicts, including the Iran/Iraq war[1–4]. Verification of chemical agent use has often been difficult, due in part to inadequate battlefield sampling and identification procedures. Capillary column gas chromatography (GC)–flame ionization detection (FID) may be used for the routine screening of samples for the presence of chemical warfare agents[5,6]. However, it is generally agreed that confirmation of the chemical warfare agents or their degradation products requires identification by mass spectrometry (MS). Electron impact (EI), the traditional MS method of ionization, has gained wide acceptance for the verification of chemical warfare agents, as the EI mass spectra of many organophosphorus[7–12] and

sulfur vesicant[13–17] chemical warfare agents, their decomposition products and related compounds have been published. Comparison of acquired mass spectra with published data, along with supporting chromatographic and/or other spectroscopic data meets suggested verification requirements[18].

The availability of commercial tandem mass spectrometry (MS–MS) systems with triple quadrupole or hybrid (*e.g.*, sector/quadrupole) design has provided researchers with the opportunity to confirm the presence of "target" compounds in a highly specific manner without the need for extensive sample handling. Tandem mass spectrometers offer a number of specific scan functions including parent ion, daughter ion, constant neutral loss and reaction ion monitoring. During reaction ion monitoring, the method of choice for many trace "target" compound applications, the first mass analyser is tuned to allow a desired mass (*e.g.*, $M^{+\bullet}$) into the collisional activated dissociation (CAD) cell while the second mass analyser allows only characteristic ion(s) derived from fragmentation(s) of the ion selected by the first analyser to be detected. The two degrees of selectivity offered by the MS–MS instrument are further enhanced by the use of gas chromatographic sample introduction.

MS–MS has been reviewed recently[19–22], and methodology has been reported for selected organophosphorus pesticides[23–25] and organophosphorus nerve agent standards[26]. Although MS–MS has been suggested as a possible means of chemical warfare agent verification in complex environmental samples[26], there have been no reports of development and application of GC–MS–MS methodology for this purpose.

A capillary column GC study using FID, EI-MS and MS–MS detection was initiated with the principal objective being the development and evaluation of these methods for the detection and confirmation of sarin (isopropyl methylphosphonofluoridate), soman (pinacolyl methylphosphonofluoridate), and mustard [bis(2-chloroethyl)sulfide] in a complex airborne matrix. The air sampled during this study contained the volatile components of diesel exhaust and was very similar in composition to battlefield air sampled onto charcoal during a recent interlaboratory analytical exercise[27]. Charcoal from exposed C2 Canadian respirator canisters was solvent extracted and spiked at several levels to allow evaluation of these analytical methods for the trace detection of the sarin, soman and mustard.

Capillary column GC–FID was of little utility due to the complexity of the sample extract. Sarin, soman and mustard could be detected and confirmed during full-scanning GC–MS at nanogram levels in spiked extracts of the diesel exhaust environment sampled onto the C2 canister charcoal. This airborne matrix, being the most complex of those sampled, was used in the development of a GC–MS–MS approach for the identification of sarin, soman and mustard. Chemical interferences were virtually eliminated and low picogram GC–MS–MS detection limits were estimated for these chemical warfare agents in the presence of numerous interfering diesel exhaust components.

EXPERIMENTAL

Standards

Sarin (GB), soman (GD) and mustard (H) were provided by our Organic

Chemistry Laboratory. Distilled-in-glass dichloromethane was purchased from BDH (Edmonton, Canada). All samples and standards were stored in PTFE-lined screw-capped vials at 4°C prior to GC analysis.

Sample collection and handling

Air from a diesel exhaust environment was sampled through a Canadian C2 charcoal canister for 4 h at the typical working respiratory rate of 20 l/min. The canister charcoal (108 g) was Soxhlet extracted for 6 h with 250 ml of dichloromethane and concentrated to 10 ml under a gentle stream of nitrogen. Portions of the extract were then spiked at the 50-μg/ml, 5-μg/ml and 500-ng/ml levels with GB, GD and H. Extraction efficiencies were evaluated prior to spiking and found to be in the 10–15% range for GB and GD and 70% for H (based on the extraction of 100 μg of agent spiked onto 10 g of blank charcoal).

Instrumental

GC injection volumes of 0.4 and 1 μl, equivalent to $2 \cdot 10^{-4}$ and $5 \cdot 10^{-4}$ m^3 of air sampled on the charcoal of C2 canisters respectively, were used for all GC analyses of the spiked and unspiked charcoal extracts.

Capillary column GC–FID analyses were performed with a Hewlett-Packard 5890 gas chromatograph equipped with an on-column injector of our own design[5]. A 15 m × 0.32 mm I.D. J&W DB-5 (0.25 μm) capillary column was used for all analyses with the following temperature programme: 40°C (2 min), then 10°C/min to 280°C (5 min).

Capillary column GC–MS analyses were performed at Defence Research Establishment Suffield with a VG 70/70E double-focusing mass spectrometer (VG Analytical, Wythenshawe, U.K.) interfaced to a Varian 3700 gas chromatograph under chromatographic conditions identical to those employed during GC–FID analysis. EI-MS operating conditions were as follows: accelerating voltage, 6 kV; emission, 100 μA; electron energy, 70 eV; source temperature, 200°C; resolution (10% valley definition), 1000; and scan function, 400 to 35 u at 1 s/decade.

Capillary column GC–MS–MS analyses were performed at the Institute of Sedimentary and Petroleum Geology with a VG 70/70SQ hybrid tandem mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph. All injections were on-column at 40 or 50°C using a Hewlett-Packard injector. The 15 m × 0.32 mm I.D. J&W DB-5 capillary column was held at this temperature for 2 min and then programmed at 10°C/min to a maximum of 280°C. EI-MS conditions were identical to those employed during GC–MS analysis with the exception of source temperature (250°C) and accelerating voltage (8 kV). The daughter spectrum of *m/z* 158 for H was obtained under the following conditions: CAD cell, 58 eV (laboratory scale)/air ($5 \cdot 10^{-7}$ Torr) and, quadrupole scan function, 200 to 40 u at 0.5 s/decade. Reaction ion monitoring for H was carried out on the *m/z* 158 to *m/z* 109 and *m/z* 158 to *m/z* 96 transitions with a 80-ms dwell time and a 20-ms delay. GB and GD daughter spectra were obtained for *m/z* 99 under similar conditions: CAD cell, 50 eV (laboratory scale)/air ($5 \cdot 10^{-7}$ Torr) and, a quadrupole scan function, 200 to 40 u at 0.5 s/decade. Reaction ion monitoring for GB and GD was carried out on the *m/z* 99 to *m/z* 79 transition with a 80-ms dwell time and a 20-ms delay.

RESULTS AND DISCUSSION

The diesel exhaust environment sampled onto Canadian C2 charcoal canisters contained primarily hydrocarbon compounds (Table I) and was similar in composition to the volatile battlefield components extracted from a respirator canister circulated as part of a recent interlaboratory analytical exercise. Charcoal extracts used in this study were however further complicated by the presence of silicon-containing compounds adsorbed onto the charcoal bed of the Canadian C2 respiratory canisters. The development of suitable confirmation methods for chemical warfare agents adsorbed onto charcoal under realistic conditions would be valuable in a chemical weapons convention verification role as charcoal mask canisters represent a possible retrospective sampling device.

TABLE I

MAJOR SAMPLE COMPONENTS IDENTIFIED IN CHARCOAL AIRBORNE SAMPLE EXTRACTS

Chromatogram peak No. (Figs. 1 and 2)	*Molecular weight*	*Compound*
1	128	*n*-C_9 alkane
2	142	*n*-C_{10} alkane
3, 5, 8, 11, 13		Charcoal impurity
4	156	*n*-C_{11} alkane
6	170	*n*-C_{12} alkane
7	184	*n*-C_{13} alkane
9	198	*n*-C_{14} alkane
10	212	*n*-C_{15} alkane
12	226	*n*-C_{16} alkane
14	240	*n*-C_{17} alkane
15	254	*n*-C_{18} alkane

Capillary column GC–FID

The chemical warfare agents GB, GD (two chromatographic components due to diastereoisomeric pairs) and H were easily detected in a standard solution at the 20-ng level during GC–FID analysis (Fig. 1a). However, GD and H were not detected by GC–FID in the presence of the charcoal extract components at the 20-ng level. Only GB was detected at this level, since it eluted prior to most of the sample extract components (Fig. 1c). Clearly, a more specific chromatographic detector, such as a flame photometric detector, would be more suitable for sample screening and tentative identification of these chemical warfare agents[28,29] in the presence of this matrix (Fig. 1b).

Capillary column GC–EI-MS

Fig. 2 illustrates capillary column GC–MS chromatograms obtained for the charcoal extract (Fig. 2a) and chemical warfare agent spikes of this extract at the 20-ng (Fig. 2b) and 2-ng (Fig. 2c) levels. Recognizable full scanning EI mass spectra were

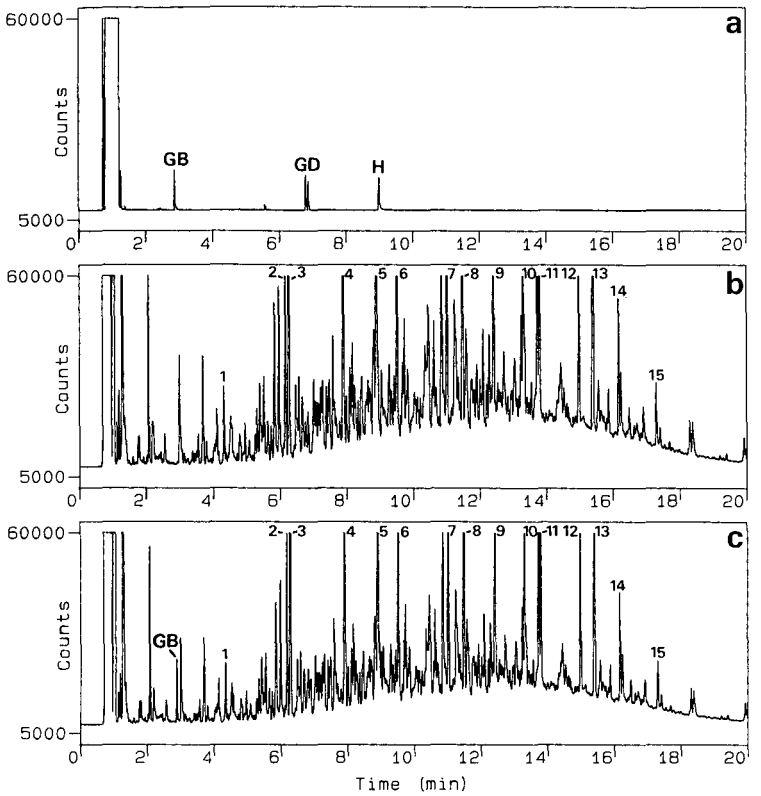

Fig. 1. Capillary column GC–FID chromatograms of (a) 20 ng sarin (GB), soman (GD) and mustard (H), (b) dichloromethane extract of the equivalent of $2 \cdot 10^{-4}$ m^3 of air sampled onto the charcoal of a C2 canister and (c) the previous sample spiked with 20 ng of GB, GD and H. Principal airborne extract sample components are identified in Table I. Column: 15 m × 0.32 mm I.D. J&W DB-5; temperature programme: 40°C (2 min), 10°C/min to 280°C (5 min).

only possible at the 20-ng level for all the spiked chemical warfare agents due to the complexity of this airborne extract.

Amounts of 200–500 pg of chemical warfare agent standard were routinely detected during full scanning operation, but the presence of the diesel exhaust components severely hampered trace confirmation of the chemical warfare agents. Selected ion monitoring under EI conditions typically improves sensitivity by about two orders of magnitude over full scanning so that low-picogram levels may be verified. However, this technique was not applicable at a resolution of 1–2000 due to chemical noise. For this reason no detection limits were estimated. Higher-resolution (*e.g.*, 10 000) selected ion monitoring under capillary column GC–MS conditions, while not readily achieved on our instrument, may reduce the matrix chemical noise so that lower levels of the chemical warfare agents may be confirmed during capillary column GC–EI-MS analysis. This approach to the reduction of chemical noise has been used for the confirmation of compounds such as dioxins[30]. However, recent reports of interferences even at high resolution in some sample matrices suggest that an alternative approach such as MS–MS be considered[30].

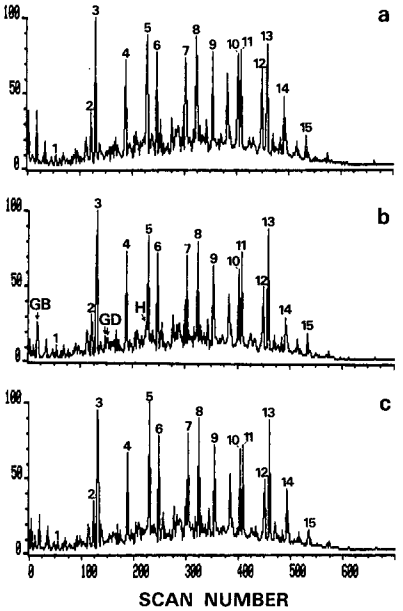

Fig. 2. Capillary column GC–EI-MS chromatograms of (a) dichloromethane extract of the equivalent of $2 \cdot 10^{-4}$ m^3 of air sampled onto the charcoal of a C2 canister, and the previous sample spiked at the (b) 20 ng and (c) 2 ng level with sarin (GB), soman (GD) and mustard (H). Principal airborne extract sample components are identified in Table I. Column: 15 m × 0.32 mm I.D. J&W DB-5; temperature programme: 40°C (2 min), 10°C/min, 280°C (5 min).

Capillary column GC–MS–MS

Hesso and Kostiainen[26] reported the daughter spectra for the pseudo-molecular ions formed during ammonia chemical ionization of GB, GD, tabun and VX. The utility of reaction ion monitoring for the detection of chemical warfare agents in

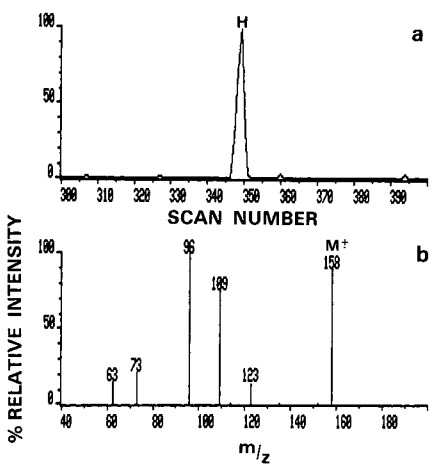

Fig. 3. (a) Collisional activated dissociation chromatogram obtained for the daughters of *m/z* 158 during GC–MS–MS analysis of mustard (H). (b) Daughter spectrum of H. Column: 15 m × 0.32 mm I.D., J&W DB-5; temperature programme: 50°C (2 min), 10°C/min, 280°C (5 min).

a complex or environmental matrix, while mentioned, was not demonstrated. The inability of conventional capillary column GC–EI-MS to confirm trace levels of chemical warfare agents in a real environmental matrix prompted investigation into application of MS–MS instrumentation for the trace detection of chemical warfare agents in a complex matrix.

The daughter spectrum of the molecular ion (*m/z* 158) for H was acquired under CAD conditions, which, while perhaps not optimal, did provide significant lower mass ions for use in a reaction ion monitoring experiment. The use of a higher mass ion, such as the molecular or higher mass fragmentation ion, for the acquisition of daughter spectra is usually preferred to minimize potential interferences. Daughter ions of *m/z* 158 at *m/z* 63, 73, 96, 109 and 123 were observed by scanning the quadrupole after CAD at 58 eV using air (Fig. 3). Both the *m/z* 158 to 109 (loss of CH_2Cl) and *m/z* 158 to 96 (loss of C_2H_3Cl) transitions were considered suitable and monitored during reaction ion monitoring of H in the diesel exhaust extract. Fig. 4 illustrates the reaction ion monitoring chromatograms obtained for the *m/z* 158 to 109 transition for 500 pg of H, the charcoal extract, and the charcoal extract spiked with 500 pg of H. H was easily confirmed without any interference in the presence of more than twice as much sample extract (*i.e.*, $5 \cdot 10^{-4}$ m^3 of diesel exhaust air) as was used during GC–FID and GC–EI-MS evaluation. The signal-to-noise ratio for 500 pg of H (greater than 80:1) was independent of the matrix and virtually identical for both the standard (Fig. 4a) and spiked extract (Fig. 4c). A conservative method detection limit of 30 pg (signal-to-noise ratio 5:1) was estimatd for H based on these findings.

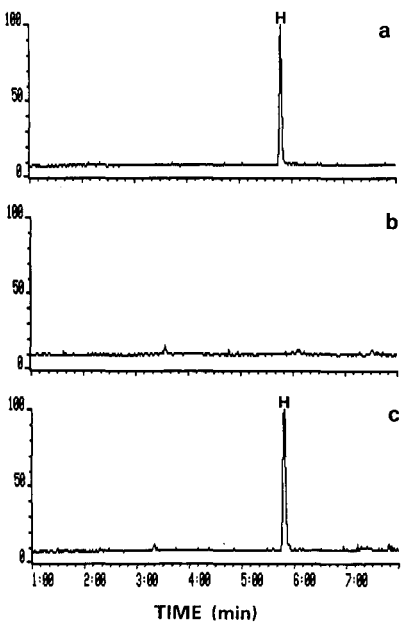

Fig. 4. Reaction ion monitoring chromatogram for *m/z* 158 to *m/z* 109 obtained during GC–MS–MS analysis of (a) 500 pg of mustard (H), (b) dichloromethane extract of the equivalent of $5 \cdot 10^{-4}$ m^3 of air sampled onto the charcoal of a C2 canister and (c) the previous sample spiked with 500 pg of mustard (H). Column: 15 m × 0.32 mm I.D., J&W DB-5; temperature programme: 50°C (2 min), 10°C/min, 280°C (5 min).

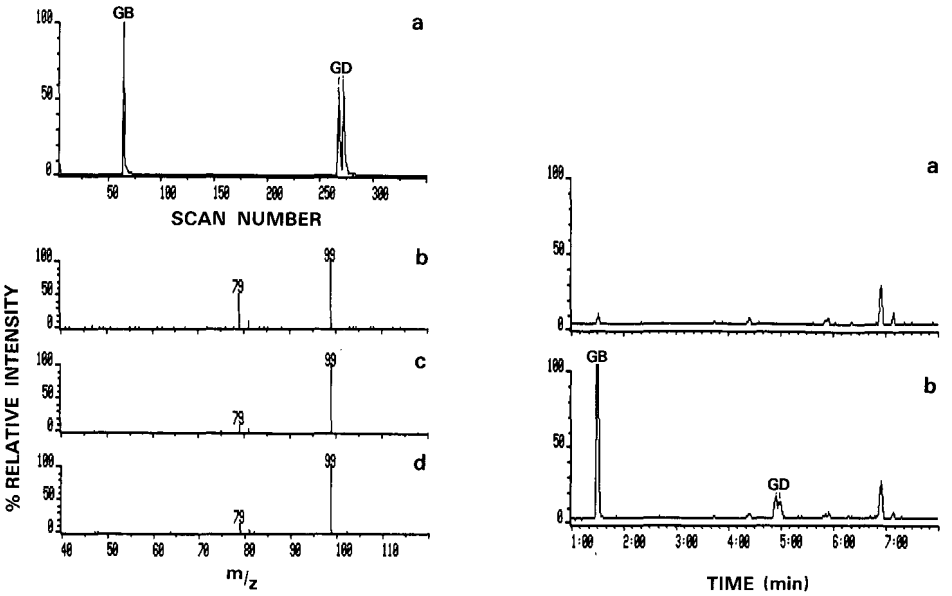

Fig. 5. (a) Collisional activated dissociation chromatogram for the daughters of *m/z* 99 during GC–MS–MS analysis of sarin (GB) and soman (GD). Daughter spectra of GB (b) and chromatographic peaks for GD (c and d). Column: 15 m × 0.32 mm I.D., J&W DB-5; temperature programme: 40°C (2 min), 10°C/min, 280°C (5 min).

Fig. 6. Reaction ion monitoring chromatogram for *m/z* 99 to *m/z* 79 obtained during GC–MS–MS analysis of (a) dichloromethane extract of the equivalent of $5 \cdot 10^{-4}$ m^3 of air sampled onto the charcoal of a C2 canister and (b) the previous sample spiked with 500 pg of sarin (GB) and soman (GD). Column: 15 m × 0.32 mm I.D., J&W DB-5; temperature programme: 40°C (2 min), 10°C/min, 280°C (5 min).

Unlike H, molecular ions are not observed for GD or GB following EI ionization. Both these compounds and other methylphosphonofluoridates do however form a diagnostic EI fragmentation ion at *m/z* 99 due to $[(CH_3)(F)P(OH)_2]^+$. CAD (50 eV in the presence of air) of *m/z* 99 resulted in the detection of a daughter ion at *m/z* 79 for both GB and GD (Fig. 5). Reaction ion monitoring of the *m/z* 99 to 79 transition, due to loss of HF, should be highly specific to methylphosphonofluoridates. Both GB and GD were readily detected at 500 pg in the preseance of $5 \cdot 10^{-4}$ m^3 of diesel exhaust air (Fig. 6). A minor interference (approx. 3% the height of the GB peak) was observed at the retention time of GB, while no interferences were detected at the retention times of the GD peaks. The method detection limit for GB was estimated to be 70 pg in the presence of this interference and 5 pg in matrices that do not contain this interference (signal-to-noise ratio 5:1). A GD method detection limit of 60 pg (signal-to-noise ratio 5:1) was estimated based on these findings.

CONCLUSIONS

The chemical warfare agents sarin, soman and mustard were detected and confirmed during capillary column GC–EI-MS conditions at nanogram levels in spiked extracts of diesel exhaust environment sampled onto the charcoal of Canadian

C2 respiratory canisters. Capillary column GC–FID was of little utility due to the complexity of the sample extract.

Daughter spectra, obtained during capillary column GC–MS–MS of the chemical warfare agents, suggested the use of the *m/z* 158 to 109 or 96 collisional activated dissociation processes for the detection of H and the *m/z* 99 to 79 collisional activated dissociation process for the identification of GB and GD. Reaction ion monitoring of these collisional activated processes during GC–MS–MS proved to be the most sensitive of the methods evaluated for the confirmation of the chemical warfare agents GB, GD and H in the presence of components commonly found in an airborne battlefield environment. GC–MS–MS detection limits in the 30–70-pg range were estimated for each of the chemical warfare agents in the presence of sample component concentrations levels two to three orders of magnitude greater than the spiked agents. Application of MS–MS for the detection of chemical warfare agents, or other compounds of chemical defence interest, appears to be an attractive approach for the verification of "target" compounds in complex environmental matrices such as those that may be encountered during airborne sampling of battlefield emissions.

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Calculation of programmed temperature gas chromatographic characteristics from isothermal data

III. Predicted retention indices and equivalent temperatures

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SUMMARY

Theoretical procedures, described in Part I [*J. Chromatogr.*, 405 (1987) 67], for predicting retention indices in programmed temperature gas chromatography from isothermal data are experimentally tested for a number of compounds under a range of experimental conditions. In general, and taking into full consideration random error predictions, agreement is reasonably satisfactory. Also calculated by procedures of Part I are two distinct kinds of equivalent temperature, these being the temperatures of isothermal chromatographic experiments giving the same calculated retention time (a) or index (b) as a corresponding programmed temperature experiment. These temperatures are compared with various simple functions of initial and calculated final temperatures in such an experiment.

INTRODUCTION

Part I[1] describes theoretical and computational procedures for predicting programmed-temperature gas chromatography (PTGC) characteristics using, as input information, experimental data for the same column and carrier gas pressure differential, but obtained under isothermal conditions (isothermal gas chromatography, IGC). Part II[2] describes the calculation (with due consideration of random errors), and comparison with experiment, of retention times and elution temperatures for a range of organic compounds (*n*-alkanes, monocyclic aromatic hydrocarbons and ketones) on a capillary column under a variety of programmed-temperature conditions; the temperature programme corresponded in each case to a linear increase of temperature with time ("single linear ramp"). The present paper extends the comparison to PTGC retention indices and also calculates two kinds of equivalent

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temperature[1]. These are the temperatures of IGC experiments for which, on the one hand, retention times are the same as calculated PTGC values, and on the other hand, retention indices are the same. The equivalent temperatures are then compared with various averages of initial and elution temperatures and with the Giddings significant temperature[3].

EXPERIMENTAL

The experimental procedure is fully described in Part II[2], which also lists the compounds studied. The list is repeated in the first column of Tables I and II. For the present purpose, it should be noted that it includes a contiguous set of *n*-alkanes, to be used as retention index standards for the remaining compounds.

PREDICTED EQUIVALENT TEMPERATURES (RETENTION TIME TYPE)

Part II[2] presents (a) experimental isothermal retention times for the compounds studied, (b) two simple alternative relationships between column dead time and

TABLE I

PREDICTED EQUIVALENT TEMPERATURES (RETENTION TIME TYPE) AND VARIOUS TRADI

Values in parentheses are estimated standard deviations.

Compound	*Heating rate = 2.00 K min⁻¹*							
	Initial temperature = 333.16 K				*Initial temperature = 393.16 K*			
	Equivalent temperature/K	*Comparison temperatures/K*[a]			*Equivalent temperature/K*	*Comparison temperatures/K*[a]		
		(a)	*(b)*	*(c)*		*(a)*	*(b)*	*(c)*
n-Nonane	338.7(0.2)	338.7	338.6	316.7	395.4(0.0)	395.4	395.4	365.8
n-Decane	342.9(0.0)	342.6	342.3	323.8	396.0(0.1)	396.0	396.0	367.0
n-Undecane	348.6(0.1)	347.7	347.1	333.3	397.0(0.0)	397.0	397.0	368.8
n-Dodecane	356.6(0.1)	354.3	353.0	345.0	398.7(0.1)	398.6	398.6	371.8
n-Tridecane	365.1(0.5)	360.9	358.8	357.6	401.1(0.1)	401.0	400.8	376.1
n-Tetradecane	376.7(0.9)	369.0	365.5	372.5	405.0(0.8)	404.5	404.2	382.5
n-Pentadecane	384.5(0.1)	374.5	370.0	382.7	409.4(0.1)	408.3	407.8	389.6
Nonan-5-one	347.7(0.1)	346.9	346.3	331.7	396.9(0.0)	396.9	396.8	368.6
Propiophenone	353.5(0.2)	351.9	350.9	340.9	398.3(0.1)	398.2	398.2	371.0
Butyrophenone	360.6(0.2)	357.7	356.0	351.6	400.2(0.1)	400.0	399.9	374.4
Valerophenone	370.2(0.2)	364.9	362.1	364.9	403.4(0.1)	403.0	402.8	379.9
Hexanophenone	380.0(0.1)	371.8	367.8	377.6	407.5(0.1)	406.8	406.3	386.8
Isopropyl benzoate	356.8(0.1)	354.6	353.5	345.9	399.0(0.1)	398.9	398.8	372.3
2-Phenylpropane	339.8(0.1)	339.7	339.6	318.5	395.6(0.1)	395.6	395.6	366.2
1-Phenylpropane	340.8(0.1)	340.6	340.4	320.2	395.8(0.1)	395.8	395.8	366.5
1-Phenylbutane	345.8(0.1)	345.3	344.9	328.8	396.7(0.1)	396.7	396.6	368.2
1-Phenylpentane	352.8(0.1)	351.3	350.3	339.8	398.1(0.1)	398.0	398.0	370.7
1-Phenyloctane	380.3(0.1)	372.0	367.9	378.0	407.7(0.1)	407.0	406.5	387.1

[a] Values are given in the order (a) arithmetic mean of initial and calculated elution temperature, (b) corresponding harmonic mean, (c) Giddings significant temperature, *i.e.*, 0.92 × calculated elution temperature.

temperature derived from the values of (a) for *n*-alkanes, (c) least squares linear regression coefficients for plots of ln(capacity factor) against reciprocal absolute temperature, and (d) a selection of predicted PTGC retention times and elution temperatures for a variety of single-linear-ramp programmed conditions using the theoretical/computational procedures described in Part I[1]. From this starting point, one can calculate[1] the temperatures of corresponding IGC experiments predicted to have the same retention times as the PTGC experiments; in Part I, such an "equivalent temperature" has been symbolised $T_{eq(1)}$. Tables I and II give a selection of these, together with estimates of standard deviations derived from a numerical application of error propagation theory[1,2].

Because of the close similarity in predictions using two alternative column dead time formulae[2] (and this is also the case in the predictions of Part II), these and subsequent tables in this paper have been restricted to predictions from one of the formulae, *viz.*,

$$t/\mathrm{s} = -46.4\,(3.6) + 6.54\,(0.179)\sqrt{T/\mathrm{K}};\ \text{covariance} -0.64 \qquad (1)$$

where t is the column dead time at absolute temperature T (in this paper standard

TIONAL EQUIVALENT TEMPERATURES FOR HEATING RATE OF 2.00 AND 7.00 K min^{-1}

Heating rate = 7.00 K min^{-1}

Initial temperature = 333.16 K				*Initial temperature = 393.16 K*			
Equivalent temperature/K	*Comparison temperatures/K*[a]			*Equivalent temperature/K*	*Comparison temperature/K*[a]		
	(a)	*(b)*	*(c)*		*(a)*	*(b)*	*(c)*
348.6(0.5)	348.4	347.8	334.6	400.5(0.1)	400.6	400.5	375.5
356.3(0.0)	355.2	353.9	347.1	402.2(0.1)	402.4	402.2	378.7
365.1(0.1)	362.5	360.1	360.5	404.8(0.0)	404.9	404.5	383.3
375.5(0.1)	370.5	366.7	375.2	408.6(0.0)	408.5	407.9	389.9
385.6(0.5)	377.8	372.5	388.7	413.4(0.2)	412.8	411.9	397.9
397.6(1.6)	385.4	378.3	402.6	419.5(1.6)	417.8	416.4	407.1
406.7(0.1)	391.8	393.0	414.4	426.2(0.1)	423.4	421.2	417.3
363.9(0.1)	361.6	359.3	358.8	404.5(0.0)	404.6	404.3	382.8
372.1(0.2)	368.2	364.9	371.0	407.8(0.1)	407.8	407.3	388.6
381.2(0.2)	374.9	370.3	383.3	411.8(0.1)	411.4	410.6	395.3
392.1(0.2)	382.7	376.3	397.6	417.6(0.1)	416.5	415.2	404.6
402.6(0.0)	389.6	381.4	410.4	424.0(0.1)	421.8	419.9	414.4
376.2(0.1)	371.2	367.3	376.5	409.3(0.0)	409.1	408.5	391.1
350.9(0.2)	350.5	349.6	338.4	401.2(0.1)	401.4	401.2	376.8
352.8(0.2)	352.2	351.2	341.6	401.6(0.1)	401.8	401.6	377.6
361.4(0.1)	359.6	357.7	355.2	404.0(0.2)	404.2	403.9	382.0
371.2(0.1)	367.4	364.2	369.5	407.4(0.1)	407.3	406.8	387.8
403.0(0.0)	389.9	381.6	410.9	424.3(0.0)	422.0	420.1	414.9

TABLE II

PREDICTED EQUIVALENT TEMPERATURES (RETENTION TIME TYPE) AND VARIOUS TRADITIONAL EQUIVALENT TEMPERATURES FOR HEATING RATE OF 15.00 K min^{-1}

Values in parentheses are estimated standard deviations.

Compound	*Initial temperature = 333.16 K*				*Initial temperature = 393.16 K*			
	Equivalent temperature/K	*Comparison temperatures/K*[a]			*Equivalent temperature/K*	*Comparison temperatures/K*[a]		
		(a)	*(b)*	*(c)*		*(a)*	*(b)*	*(c)*
n-Nonane	359.4(0.6)	359.5	357.6	354.9	407.6(0.2)	408.5	407.9	389.9
n-Decane	368.9(0.1)	367.7	364.5	370.1	410.5(0.1)	411.3	410.5	395.1
n-Undecane	379.1(0.1)	375.9	371.0	385.1	414.3(0.1)	415.0	413.9	401.9
n-Dodecane	390.3(0.1)	384.3	377.5	400.5	419.6(0.0)	419.8	418.1	410.8
n-Tridecane	401.0(0.5)	391.9	383.1	414.5	425.7(0.3)	425.2	422.8	420.7
n-Tetradecane	412.5(2.1)	398.9	388.1	427.4	432.3(2.1)	430.5	427.2	430.3
n-Pentadecane	422.5(0.2)	405.9	392.9	440.4	440.2(0.2)	436.9	432.5	442.2
Nonan-5-one	377.8(0.1)	374.9	370.3	383.4	414.0(0.1)	414.7	413.6	401.3
Propiophenone	387.3(0.2)	382.4	376.1	397.1	418.8(0.2)	419.2	417.6	409.6
Butyrophenone	396.9(0.2)	389.4	381.3	410.0	424.0(0.1)	423.8	421.6	418.2
Valerophenone	408.3(0.2)	397.3	386.9	424.5	431.0(0.1)	429.8	426.7	429.1
Hexanophenone	419.0(0.0)	404.2	391.7	437.3	438.3(0.1)	435.6	431.5	439.8
Isopropyl benzoate	391.4(0.1)	385.3	378.3	402.5	420.7(0.0)	420.9	419.0	412.7
2-Phenylpropane	362.5(0.2)	362.0	360.0	360.2	408.9(0.3)	409.7	409.1	392.2
1-Phenylpropane	364.9(0.2)	364.5	361.8	364.1	409.6(0.1)	410.5	409.8	393.6
1-Phenylbutane	375.3(0.0)	373.1	368.8	379.9	413.4(0.1)	414.1	413.1	400.3
1-Phenylpentane	386.1(0.2)	381.5	375.4	395.4	418.1(0.2)	418.6	417.0	408.4
1-Phenyloctane	419.4(0.0)	404.5	391.9	437.8	438.6(0.1)	435.9	431.7	440.4

[a] Values are given in the order (a) arithmetic mean of initial and calculated elution temperature, (b) corresponding harmonic mean, (c) Giddings significant temperature, *i.e.*, 0.92 × calculated elution temperature.

deviations are given in parentheses). A full set of data covering both formulae and 32 combinations (listed in Part II) of initial oven temperature and heating rate is available from the authors on request. Also included in Tables I and II, for comparison purposes, are the arithmetic and harmonic means of initial and *calculated* elution temperatures and the *calculated* Giddings significant temperature (0.92 × calculated elution temperature)[3], all three of which have been used in the past as "equivalent temperatures".

PREDICTED PTGC RETENTION INDICES AND EQUIVALENT TEMPERATURES (RETENTION INDEX TYPE)

For the same selection of experimental conditions, Tables III–V give calculated retention indices by two alternative procedures, (a) by linear interpolation between adjacent points on a plot of programmed temperature retention time against carbon number for *n*-alkanes, and (b) by cubic spline interpolation for the same plot (the two methods are detailed in Part I[1]). Observed values corresponding to the calculated values of method (b) are included for comparison. The tables also present calculated

TABLE III

PREDICTED AND OBSERVED RETENTION INDICES AND EQUIVALENT TEMPERATURES (RETENTION INDEX TYPE) FOR INITIAL TEMPERATURES OF 333.16 AND 393.16 K AND A HEATING RATE OF 2.00 K min^{-1}

For each compound, the first and second lines correspond to initial temperatures of 333.16 and 393.16 K, respectively.

Compound	*Retention index*			*Equivalent temperature/K*[a]	
	Predicted[a]		*Observed*[b]		
	(a)	*(b)*		*(a)*	*(b)*
(1) Nonan-5-one	1084 (2)	1086 (1)	1073.1(0.1)	330(4)	371(17)
	1088 (2)	1091 (1)	1076.2(0.1)	376(6)	405(15)
(2) Propiophenone	1163 (3)	1164 (3)	1165.3(0.1)	351(0)	361 (6)
	1175 (2)	1179 (2)	1180.3(0.1)	388(2)	400(12)
(3) Butyrophenone	1251 (4)	1252 (4)	1252.2(0.1)	363(0)	378 (7)
	1261 (4)	1264 (4)	1265.3(0.0)	390(1)	404(11)
(4) Valerophenone	1349 (6)	1349 (7)	1355.6(0.2)	380(1)	372(11)
	1359(12)	1364(10)	1366.2(0.0)	399(5)	405(12)
(5) Hexanophenone	1450 (8)	1443(10)	1459.9(0.1)	388(1)	365(20)
	1460 (8)	1456(18)	1467.7(0.1)	404(1)	395(24)
(6) Isopropyl benzoate	1204 (2)	1204 (2)	1207.2(0.0)	359(1)	368(10)
	1212 (1)	1213 (1)	1216.7(0.0)	391(2)	399(15)
(7) 2-Phenylpropane	926 (5)	928 (5)	929.2(0.1)	315(8)	319 (6)
	938 (4)	942 (3)	941.6(0.1)	371(6)	385 (4)
(8) 1-Phenylpropane	950 (4)	953 (3)	958.2(0.1)	314(5)	319 (9)
	964 (3)	968 (3)	971.5(0.1)	375(4)	385 (9)
(9) 1-Phenylbutane	1053 (1)	1057 (1)	1056.4(0.1)	334(2)	351(13)
	1067 (1)	1072 (2)	1071.8(0.1)	382(3)	399(16)
(10) 1-Phenylpentane	1154 (1)	1155 (1)	1157.3(0.1)	349(2)	362 (7)
	1163 (3)	1169 (3)	1169.9(0.1)	382(4)	400 (9)
(11) 1-Phenyloctane	1454 (7)	1448(10)	1465.1(0.1)	388(1)	368 (9)
	1464 (7)	1460(17)	1471.7(0.1)	405(1)	396(29)

[a] Values in parentheses are estimated standard deviations. Retention index (a)[1] is obtained by linear interpolation between adjacent points on a plot of programmed temperature retention time against carbon number for *n*-alkanes. Retention index (b)[1] is obtained by cubic spline interpolation for the same plot. Equivalent temperature (a)[1] uses retention index (a) in conjunction with a linear interpolation between adjacent points on a plot of log(isothermal capacity factor) against carbon number for *n*-alkanes. Equivalent temperature (b)[1] uses retention index (b) in conjunction with a least squares linear fit to the points of the same plot.

[b] Values in parentheses are 95% confidence deviations. Experimental values are analogues of the predicted indices (b).

temperatures of IGC experiments for which Kováts retention indices are the same as calculated PTGC indices; in Part I, such an "equivalent temperature" has been symbolised $T_{eq(2)}$. Again two different methods have been used to calculate these: method (a) uses retention index (a) in conjunction with a linear interpolation between adjacent points of a plot of log(isothermal capacity factor) against carbon number for *n*-alkanes; method (b) uses retention index (b) in conjunction with a least squares linear fit to the points of the same plot. Standard deviation estimates (see Part I[1]) are included for all calculated values.

TABLE IV

PREDICTED AND OBSERVED RETENTION INDICES AND EQUIVALENT TEMPERATURES (RETENTION INDEX TYPE) FOR INITIAL TEMPERATURES OF 333.16 AND 339.16 K AND A HEATING RATE OF 7.00 K min^{-1}

For each compound, the first and second lines correspond to initial temperatures of 333.16 and 393.16 K, respectively.

Compound	*Retention index*			*Equivalent temperature/K*[a]	
	Predicted[a]		*Observed*[b]		
	(a)	*(b)*		*(a)*	*(b)*
Nonan-5-one	1087 (1)	1088 (1)	1074.5(0.1)	363(1)	384(23)
	1089 (2)	1091 (1)	1076.9(0.0)	392(4)	410(18)
Propiophenone	1174 (2)	1171 (3)	1172.8(0.1)	378(0)	380 (5)
	1181 (2)	1183 (2)	1183.0(0.0)	403(0)	408 (8)
Butyrophenone	1260 (4)	1260 (5)	1261.0(0.1)	390(1)	396 (8)
	1268 (4)	1269 (4)	1270.2(0.0)	408(1)	414(12)
Valerophenone	1364(12)	1363(13)	1365.7(0.1)	407(1)	403(15)
	1373(19)	1374(18)	1372.6(0.1)	419(1)	423(21)
Hexanophenone	1466 (8)	1463(14)	1470.8(0.1)	415(2)	409(16)
	1471 (7)	1472(20)	1475.7(0.1)	425(2)	426(18)
Isopropyl benzoate	1210 (2)	1209 (2)	1212.7(0.1)	383(2)	387 (5)
	1215 (1)	1216 (2)	1219.7(0.0)	405(1)	408(16)
2-Phenylpropane	931 (5)	931 (4)	931.6(0.0)	340(3)	334(15)
	940 (4)	944 (3)	944.9(0.1)	381(5)	392 (4)
1-Phenylpropane	956 (4)	956 (3)	960.8(0.1)	343(3)	336(12)
	976 (3)	970 (3)	974.4(0.1)	386(3)	392 (8)
1-Phenylbutane	1060 (1)	1062 (1)	1062.2(0.1)	361(1)	368 (8)
	1071 (2)	1075 (2)	1074.5(0.0)	394(4)	406 (7)
1-Phenylpentane	1162 (2)	1161 (2)	1163.5(0.1)	377(1)	380 (8)
	1169 (3)	1172 (3)	1173.5(0.1)	399(2)	408 (8)
1-Phenyloctane	1470 (7)	1467(13)	1474.2(0.1)	416(3)	410(14)
	1476 (6)	1476(18)	1478.3(0.1)	426(1)	426(20)

[a,b] See corresponding footnotes for Table III.

DISCUSSION

Corresponding PTGC retention indices calculated by the two methods are very similar; in the main, bearing in mind the estimated standard deviations, they may indeed be regarded as indistinguishable. Agreement between predicted and observed is generally good, again in the light of the stated uncertainties, although there is certainly a tendency for observed values to be higher. The situation is shown graphically in Fig. 1, which excludes any error considerations; the compound to box number correspondence is given in Table III. It will be seen that for four compounds predictions are less satisfactory than for the others. These four are nonan-5-one (compound 1) where predictions are *consistently* high, and valerophenone, hexanophenone and 1-phenyloctane (compounds 4, 5 and 11) where there is significant spread of predictions around the observations. Possible reasons for imperfect prediction have been presented in Part

TABLE V

PREDICTED AND OBSERVED RETENTION INDICES AND EQUIVALENT TEMPERATURES (RETENTION INDEX TYPE) FOR INITIAL TEMPERATURES OF 333.16 AND 393.16 K AND A HEATING RATE OF 15.00 K min^{-1}

For each compound, the first and second lines correspond to initial temperatures of 333.16 and 393.16 K, respectively.

Compound	*Retention index*			*Equivalent temperature/K*[a]	
	Predicted[a]		*Observed*[b]		
	(a)	*(b)*		*(a)*	*(b)*
Nonan-5-one	1089 (1)	1089 (1)	1075.3(0.0)	382(4)	394(22)
	1091 (2)	1092 (1)	1077.6(0.1)	406(5)	417(16)
Propiophenone	1178 (2)	1177 (3)	1179.0(0.0)	396(1)	396(10)
	1187 (3)	1188 (3)	1188.6(0.1)	417(3)	418 (7)
Butyrophenone	1268 (4)	1267 (5)	1267.9(0.0)	408(1)	409(17)
	1275 (3)	1274 (5)	1275.6(0.1)	423(1)	423(16)
Valerophenone	1377(20)	1377(21)	1373.2(0.1)	424(2)	428(22)
	1387(28)	1388(28)	1378.7(0.1)	434(1)	444(23)
Hexanophenone	1476 (7)	1477(17)	1478.7(0.1)	434(1)	434(10)
	1480 (6)	1484(21)	1482.4(0.1)	441(2)	445(11)
Isopropyl benzoate	1214 (2)	1214 (2)	1217.0(0.1)	401(1)	400 (5)
	1219 (1)	1219 (2)	1223.0(0.1)	419(1)	417 (8)
2-Phenylpropane	935 (5)	934 (4)	934.3(0.1)	358(2)	349 (6)
	944 (4)	946 (3)	946.5(0.1)	393(6)	401 (6)
1-Phenylpropane	960 (4)	960 (3)	963.9(0.1)	362(1)	352(16)
	970 (3)	973 (3)	976.6(0.0)	398(3)	402 (8)
1-Phenylbutane	1066 (1)	1066 (1)	1066.4(0.1)	379(0)	382 (7)
	1076 (2)	1078 (2)	1078.0(0.1)	407(2)	414 (7)
1-Phenylpentane	1167 (3)	1167 (3)	1168.2(0.2)	395(0)	395 (7)
	1174 (4)	1176 (4)	1177.0(0.1)	414(1)	418 (7)
1-Phenyloctane	1481 (6)	1481(14)	1480.3(0.0)	435(1)	433 (4)
	1484 (5)	1488(17)	1484.1(0.1)	442(1)	444 (7)

[a,b] See corresponding footnotes for Table III.

II[2]. Predictions based upon similar theory by Curvers *et al.*[4] (but without any error considerations) are rather closer to their experimental values than those given here.

Because both predicted elution temperatures[2] and predicted retention indices generally compare well with experiment, it may be supposed that comparison of *calculated* equivalent temperatures (both types) and *calculated* "comparison temperatures" (three kinds as given in Tables I and II) corresponds reasonably closely with comparison of *experimental* values. The difference in nature between the two types of equivalent temperature [retention time type $T_{eq(1)}$ (Tables I and II) and retention index type $T_{eq(2)}$ (Tables III–V)] has been emphasised in Part I[1]. Comparison of values for corresponding eluent and PTGC conditions certainly indicate significant differences in many cases. Furthermore, there are some significant differences between values of $T_{eq(2)}$ calculated by the two different methods; incidentally, the generally higher estimated standard deviations by method (b) arise from the involvement of *all* *n*-alkanes [in contrast with only two for method (a)] in the linear regression aspect of

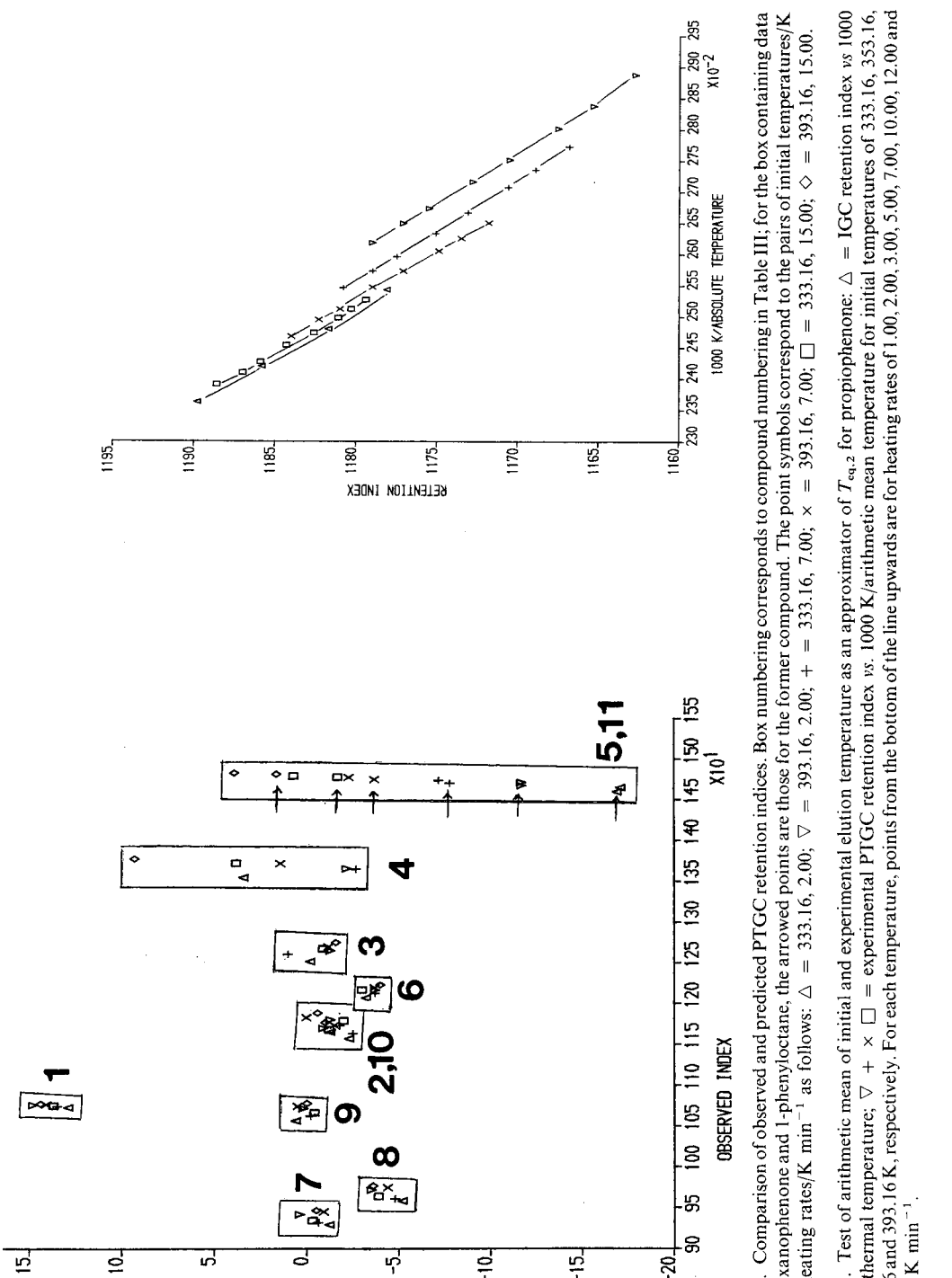

Fig. 1. Comparison of observed and predicted PTGC retention indices. Box numbering corresponds to compound numbering in Table III; for the box containing data for hexanophenone and 1-phenyloctane, the arrowed points are those for the former compound. The point symbols correspond to the pairs of initial temperatures/K and heating rates/K min^{-1} as follows: △ = 333.16, 2.00; ▽ = 393.16, 2.00; + = 333.16, 7.00; × = 393.16, 7.00; □ = 333.16, 15.00; ◇ = 393.16, 15.00.

Fig. 2. Test of arithmetic mean of initial and experimental elution temperature as an approximator of $T_{eq,2}$ for propiophenone: △ = IGC retention index *vs* 1000 K/isothermal temperature; ▽ + × □ = experimental PTGC retention index *vs*. 1000 K/arithmetic mean temperature for initial temperatures of 333.16, 353.16, 373.16 and 393.16 K, respectively. For each temperature, points from the bottom of the line upwards are for heating rates of 1.00, 2.00, 3.00, 5.00, 7.00, 10.00, 12.00 and 15.00 K min^{-1}.

TABLE VI

CALCULATED EQUIVALENT TEMPERATURES (RETENTION INDEX TYPE) FOR HYPOTHETICAL COMPOUND

$\ln(k_1) = -12.2\,(0.02)$, $k_2 = 5000\,(10)$ K, covariance -0.2. Column dead time/temperature dependence as eqn. 1; initial temperature = 333.16 K.

Heating rate/K min^{-1}	*Equivalent temperature/K (and "estimated standard deviation")*	
	(a)	*(b)*
1.00	1140 (1932)	422(126)
2.00	1939 (17 632)	417 (70)
3.00	−9762(4 089 722)	416 (66)
5.00	−154 (4045)	415(116)
7.00	146 (1052)	415 (72)
10.00	283 (321)	414(138)
12.00	326 (186)	414 (64)
15.00	364 (59)	414(117)

the method. There is certainly a possibility of ill-conditioning in the calculation of $T_{eq(2)}$ in situations where the IGC (Kováts) indices are only very slightly dependent on temperature. To illustrate this, a hypothetical example [for which[1] $\ln(k_1) = -12.2$ (0.02) and $k_2 = 5000$ (10) K; covariance $= -0.2$; $\ln(k_1)$ and k_2 are the intercept and slope, respectively, of the least squares linear plot of ln (isothermal capacity factor) against reciprocal absolute temperature] gave the absurd results shown in Table VI; at least in this example, there is more "stability" in $T_{eq(2)}$ values obtained by method (b).

In comparing both $T_{eq(1)}$ (all compounds) and $T_{eq(2)}$ (inapplicable for the *n*-alkanes) with the various comparison temperatures given in Tables I and II, it is apparent that *generally* (and there are exceptions) (i) neither equivalent temperature compares well with the Giddings significant temperature, (ii) $T_{eq(1)}$ compares better with one or other of the means of initial and elution temperatures than does $T_{eq(2)}$. The quality of various comparison temperatures as approximators of $T_{eq(2)}$ has been previously[5] approached in a different way, *viz.*, by plotting experimental IGC retention indices against reciprocal absolute temperature (this being nearly linear, and equivalent to a plot of PTGC index against experimental $T_{eq(2)}^{-1}$), and then examining the proximity of points [(experimental comparison temperature)$^{-1}$, experimental PTGC index] to the plot; the closer the points, the better the comparison temperature as an approximator of $T_{eq(2)}$. This approach is illustrated in Figs. 2–4 for propiophenone; experimental IGC indices at 393.16, 403.16, 413.16 and 423.16 K are 1178.1, 1181.7, 1185.8 and 1189.8, respectively. Generally, and this is particularly true at

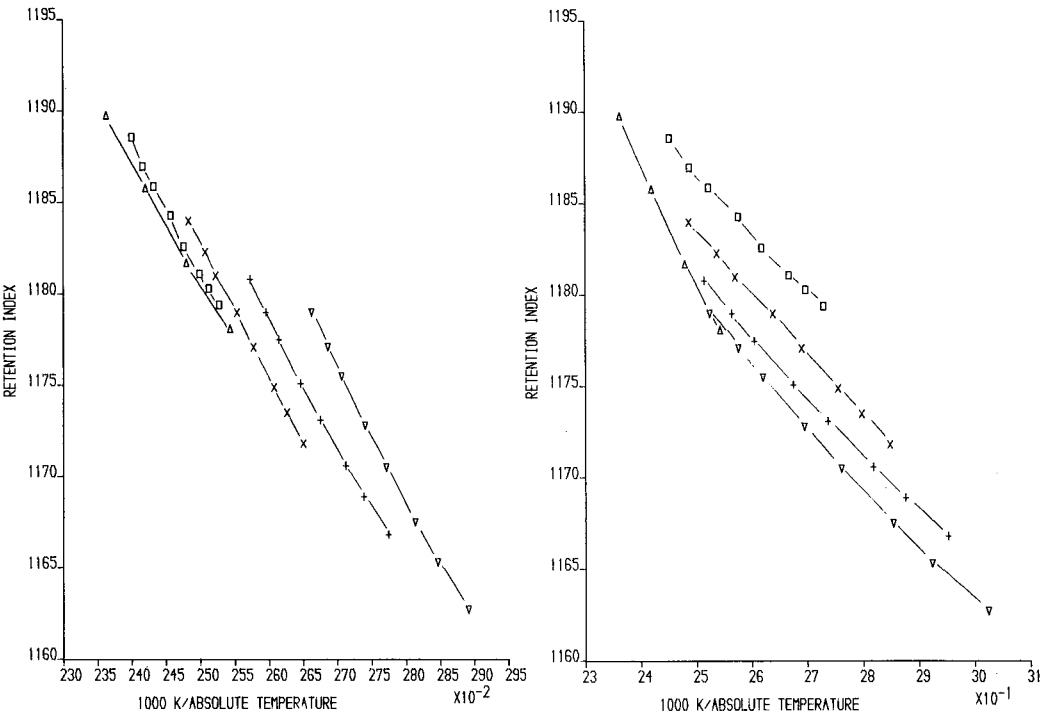

Fig. 3. Test of harmonic mean of initial and experimental elution temperature as an approximator of $T_{eq,2}$ for propiophenone: △ = IGC retention index *vs.* 1000 K/isothermal temperature; ▽ + × □ = experimental PTGC retention index *vs.* 1000 K/harmonic mean temperature for initial temperatures of 333.16, 353.16, 373.16 and 393.16 K, respectively. For each temperature, points from the bottom of the line upwards are for heating rates of 1.00, 2.00, 3.00, 5.00, 7.00, 10.00, 12.00 and 15.00 K min^{-1}.

Fig. 4. Test of Giddings significant temperature as an approximator of $T_{eq,2}$ for propiophenone: △ = IGC retention index *vs* 1000 K/isothermal temperature; ▽ + × □ = experimental PTGC retention index *vs.* 1000 K/Giddings significant temperature for initial temperatures of 333.16, 353.16, 373.16 and 393.16 K, respectively. For each temperature, points from the bottom of the line upwards are for heating rates of 1.00, 2.00, 3.00, 5.00, 7.00, 10.00, 12.00 and 15.00 K min^{-1}.

higher initial column initial temperatures, the arithmetic mean (and to a slightly lesser extent the harmonic mean) is a better $T_{eq(2)}$ approximator than is the Giddings significant temperature. The same tendency applies for other eluents.

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Preparation and gas chromatographic characterization of some immobilized crown ether–polysiloxane stationary phases

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SUMMARY

The cross-linked fused-silica capillary columns, SE-54–30% 18-crown-6, SE-54–50% 18-crown-6 and SE-54–50% 15-crown-5, were prepared and characterized by gas chromatography, including the determination of column bleeding, phase transition temperature, polarity and selectivity. The polarity and selectivity of these phases are comparable to those of SE-54 and OV-1701. The hydrogen bonding contribution of crown ether phases in separating solutes is discussed.

INTRODUCTION

Crown ethers have a polar ring formed by the oxygen atoms and the ring structure confers the ability to form stable complexes with metal cations and electrically neutral organic solutes[1,2] They have received considerable attention especially in analytical chemistry[3–8], and a few gas chromatographic (GC) studies have been reported[9–13]. Crown ethers have great potential in GC because of their unique selectivity. However, small-molecule crown ether stationary phases have low maximum operating temperatures and the column efficiency and thermal stability are poor[13].

Polysiloxane polymers provide the best performance as stationary phases in GC. They have good solute diffusivity, thermal stability and film-forming ability. Most of the phases developed recently for capillary chromatography are based on the polysiloxane backbone, substituted with different organic groups. These polar side-groups on the polysiloxane backbone give specific selectivities for particular components.

In this work, a new method for preparing crown ether–polysiloxane stationary phases by direct cross-linking crown ether with SE-54 stationary phase in different proportions was developed. These polymeric phases were tested for efficiency, polarity, selectivity, phase transition temperature and thermal stability.

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EXPERIMENTAL

Reagents

ω-Undecyleneoxymethyl-18-C-6 and ω-undecyleneoxymethyl-15-C-5 (C = crown), shown in Fig. 1, were obtained from the Department of Chemistry, Wuhan University. All other chemicals used were of analytical-reagent grade.

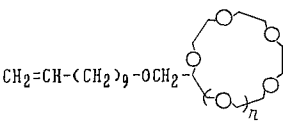

Fig. 1. Structure of alkylene crown ethers. n = 1, Monomer I; n = 2, monomer II.

Preparation of crown ether polysiloxane columns

Fused-silica capillary tubing (Academy of Post and Telecommunication, Wuhan, China) was rinsed with water and methanol and purged with nitrogen at 280°C for 2 h. Columns were then statically coated at room temperature with 0.5% (w/v) mixed stationary phase (SE-54 with monomer I or II in different proportions) and 5% (w/v) dicumyl peroxide (DCUP) in methylene chloride, which was previously filtered through a 0.2-μm Millipore filter. Both ends of the colomn were sealed and the latter was placed in an oven at 150°C for 1 h, then heated to 190°C at 8°C min^{-1} and held at 190°C for 1 h. The non-cross-linked molecules of the stationary phase were removed during the first washing with ten column volumes of methylene chloride and the column capacity was decreased by 10–14%, as indicated by the decrease in the capacity factors of the test substances. Prolonged washing of the column with five column volumes of this solvent did not lead to additional removal of the stationary phase, with an unchanged capacity. Finally, the column was conditioned at 280–300°C for 12 h.

Column evaluation

Column evaluation was performed with a Shimadzu Model GC-7A gas chromatograph equipped with a capillary split injection system and flame ionization detector, using nitrogen as the carrier gas. Grob's test mixture, an isomer mixture and two samples were used to demonstrate the selectivity of the phases. The columns were tested for polarity and efficiency by measuring the retention indices and number of plates per metre for naphthalene at 120°C. The thermal stabilities of the crown ether polysiloxane stationary phases were tested by measuring the column bleeding.

RESULTS AND DISCUSSION

The presence of terminal vinyl groups in the synthetic crown ether monomers I and II facilitates its cross-linking with the SE-54 matrix by treatment with DCUP, forming a crown ether polysiloxane in the inner surface of the capillary column which is non-extractable by solvents. Table I summarizes the chromatographic characteristics of those crown ether columns. The results indicate that the theoretical plate

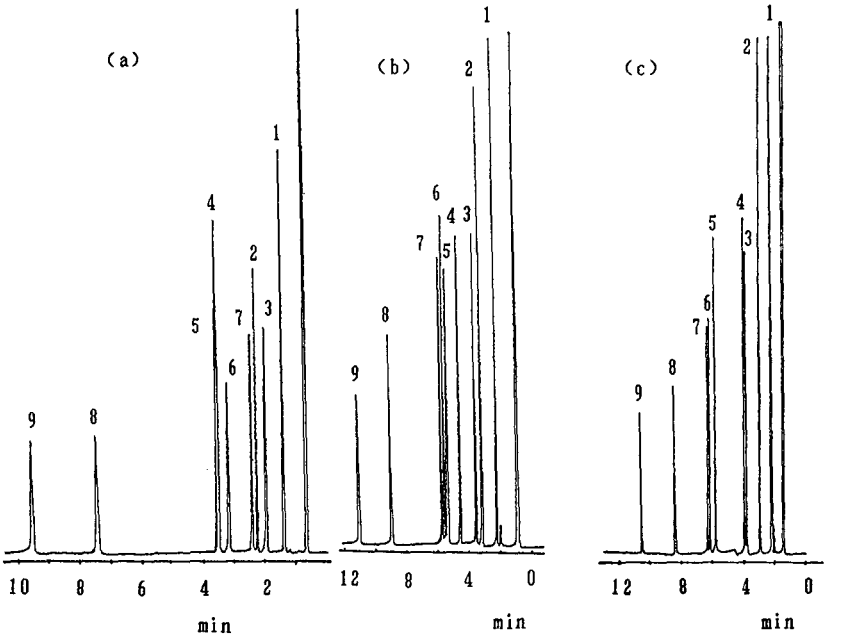

Fig. 2. Chromatogram of the Grob test mixture. (a) On SE-54. Column temperature programmed from 100 to 160°C at 6°C min^{-1}. (b) On SE-54–50% monomer II. Column temperature programmed from 100 to 170°C at 6°C min^{-1}. (c) On SE-54 – 50% monomer I. Columnn temperature programmed from 100 to 150°C at 4°C min^{-1}. Peaks 1 = *n*-decane; 2 = *n*-undecane; 3 = 1-octanol; 4 = *n*-dodecane; 5 = naphthalene; 6 = 2,6-dimethylphenol; 7 = 2,4-dimethylaniline; 8 = methyl undecanoate; 9 = methyl dodecanoate.

number is over 3800 per metre for all of the columns and the column efficiency decreases slightly with increasing crown ether content. The peak–area ratio of acidic P (2,6-dimethylphenol) and basic A (2,4-dimethylaniline) test substances and the peak asymmetry factor of octanol on fused-silica columns coated with SE-54–crown ether and cross-linked with DCUP showed no increase in polarity and activity.

Fig. 2 shows chromatograms of the Grob test mixture obtained on the different columns. For the SE-54–50% 18-C-6 and SE-54–50% 15-C-5 columns, the polar components of the Grob test mixture, 1-octanol, 2,4-dimethylaniline and 2,6-dimethylphenol, elute after undecane and naphthalene, in contrast to the reference SE-54 column. It can be concluded that crown ether–polysiloxane columns have different polarities and selectivities to the SE-54 column. The results also indicate that crown ether monomers were cross-linked to the SE-54 polysiloxane backbone and contributed to the separation mechanism.

The selectivity and average polarity of those crown ether—polysiloxane stationary phases, represented by the McReynolds constants (*I*), are shown in Table II. It can be seen that the crown ether phases have an average polarity between those of SE-54 and OV-1701 and SE-54 – 50% 18-C-6 has a slightly lower polarity than SE-54–50% 15-C-5, owing to the similar D_{3d} comformation of 18-C-6.

As illustrated in Table III, the relative retention values of all the test samples

TABLE I

CHARACTERISTICS OF CROWN ETHER–POLYSILOXANE CAPILLARY COLUMNS

Column No.	*Column dimensions [length (m) × I.D. (mm)]*	*Stationary phase*	*Naphthalene capacity factor (k')*	*Efficiency (n/m)*	*P/A*	*Peak asymmetry*[14]
1	12 × 0.24	SE-54 – 30% monomer II	2.80	4633	1.03	1.00
2	14 × 0.26	SE-54 – 50% monomer II	2.76	3806	1.05	1.00
3	14 × 0.26	SE-54 – 50% monomer I	3.41	4176	1.01	1.00

TABLE II

SELECTIVITY (McREYNOLDS CONSTANTS, *I*) AND POLARITY OF THE CROWN ETHER–POLYSILOXANES

Stationary phase	*Benzene*	*Butanol*	*2-Pentanone*	*Nitropropane*	*Pyridine*	*Average polarity*
SE-54	33	72	66	99	67	60
SE-54 – 30% monomer II	61	129	75	132	128	105
SE-54 – 50% monomer II	63	152	84	151	126	115
SE-54 – 50% monomer I	64	149	86	163	133	119
OV-1701	67	170	153	228	171	158

with the SE-54–crown ether columns are greater than those with the SE-54 column. With the exception of trimethylbenzene, this indicates that the former have much better selectivity than SE-54. In comparison with OV-1701, which has a higher polarity, they have roughly the same selectivity with respect to nitro-substituted benzene positional isomers, but with respect to cresol, chlorophenol and nitrophenol compounds, SE-54 – crown ether columns provide a higher selectivity. This suggests that the crown ether–polysiloxanes are convenient for separating apolar and polar compounds, especially compounds that have the ability to form hydrogen bonds with the oxygen atom in the crown ether ring.

TABLE III

RELATIVE RETENTION VALUES OF TEST COMPOUNDS IN THE VARIOUS COLUMNS

Compound	*Isomer*	*SE-54*	*SE-54 – 50% 18-C-6*	*SE-54 – 50% 15-C-5*	*OV-1701*
Ethylnitrobenzene	*o-*	1.00	1.00	1.00	1.00
	m-	1.32	1.40	1.37	1.36
	p-	1.46	1.61	1.63	1.60
Dinitrobenzene	*o-*	1.00	1.00	1.00	1.00
	m-	1.10	1.10	1.14	1.14
	p-	1.23	1.44	1.43	1.41
Cresol	*o-*	1.00	1.00	1.00	1.00
	p-	1.04	1.26	1.23	1.03
Chlorophenol	*o-*	1.00	1.00	1.00	1.00
	p-	2.23	6.30	5.39	4.49
Nitrophenol	*o-*	1.00	1.00	1.00	1.00
	m-	3.95	39.5	45.00	14.90
	p-	5.17	70.60	78.70	23.70
Trimethylbenzene	1,3,5-	1.00	1.00	1.00	1.00
	1,2,4-	1.15	1.17	1.14	1.19
	1,2,3-	1.36	1.33	1.36	1.47
Naphthalene		1.00	1.00	1.00	1.00
2-Methylnaphthalene		1.62	1.75	1.76	1.74
Biphenyl		1.97	2.27	2.44	2.22
Acenaphthylene		3.16	3.93	4.17	3.81
Dibenzyl		3.43	3.86	4.19	4.01
Fluorene		4.70	4.91	7.00	5.92

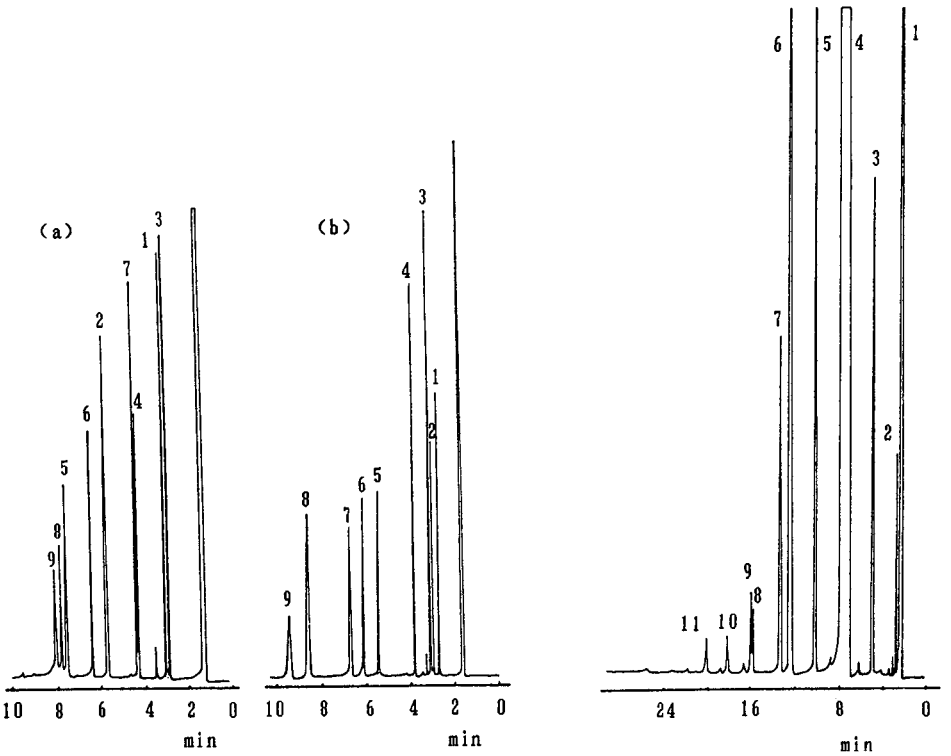

Fig. 3. Chromatogram of phenol compounds. (b) On SE-54 – 30% monomer I. Column 10 m × 0.25 mm I.D., temperature programmed from 105 to 230°C at 12°C min^{-1}. (a) On SE-54. Column, 16 m × 0.25 mm I.D., temperature programmed from 75° to 200°C at 12°C min^{-1}. Peaks: 1 = *o*-chlorophenol; 2 = *o*-nitrophenol; 3 = phenol; 4 = 2,4-dimethylphenol; 5 = 2,4,6-trichlorophenol; 6 = *p*-bromophenol; 7 = 2,4-dinitrophenol; 8 = *m*-nitrophenol; 9 = p-nitrophenol.

Fig. 4. Chromatogram of ethyl-substituted nitrobenzene compounds on SE-54–50% 15-C-5 (column). Column temperature programmed from 100 to 200°C at 4°C min^{-1}. Peaks: 1–3 = solvents; 4 = nitrobenzene; 5 = *o*-ethylnitrobenzene; 6 = *m*-ethylnitrobenzene; 7 = *p*-ethylnitrobenzene; 8 = 2,3-diethylnitrobenzene; 9 = 2,5-diethylnitrobenzene; 10 = 3,5-diethylnitrobenzene; 11 = 3,4-diethylnitrobenzene.

The ring structure of the crown compounds provides selectivity for organic compounds with hydroxyl groups, which is based on accessibility and availability of hydrogen bonding between the hydroxylic hydrogens and the ether oxygen atom. The separation of nitrophenol isomers is a good example, the relative retention of the *para*- and *ortho*-substituted pair on the crown ether–polysiloxane phases being higher than that on other phases. Because the *para* substituted compound is a rod-like molecule fitting into the cavity of the crown ether ring it shows a stronger hydrogen bonding interaction of the ether oxygen than the *ortho*-substituted compound, which forms intramolecular hydrogen bonds between the nitro oxygen atom and the phenolic hydrogen atoms, thus leading to a more pronounced difference in the relative retention times.

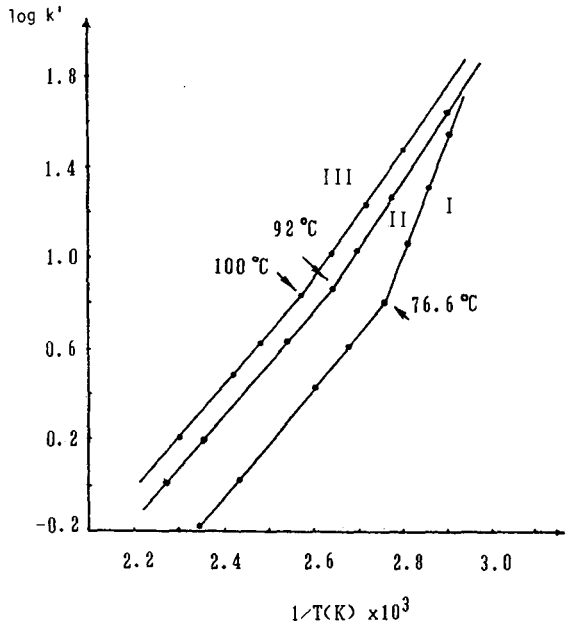

Fig. 5. Plots of log k' (capacity factor) against inverse of absolute temperature for 1-octanol. I, SE-54 – 30% monomer II; II, SE-54 – 50% monomer II; III, SE-54 – 50% monomer I.

The three crown ether–polysiloxane phases also gave excellent separations of various environmental substituted phenol mixtures (Fig. 3) and ethyl-substituted nitrobenzene products (Fig. 4). In comparison with an SE-54 column, SE-54–50% 18-C-6 results in a superior separation without tailing for phenol compounds.

To determine the operating temperature range of the SE-54 – crown ether columns, the phase transition temperature and maximum allowable operating temperature of each column were measured. Fig. 5 shows changes in the retention behavior of 1-octanol on columns 1, 2 and 3 at 76.6, 92 and 100°C respectively; these transitions occur at temperatures which may correspond to the glass transition temperature and a liquid–liquid transition. The maximum allowable operating temperature was determined by identifying the temperature (300°C) at which a shift in the baseline of $7.5 \cdot 10^{-13}$ A was observed. The baseline was not increased when the column temperature was below 245°C.

CONCLUSION

The three SE-54 – crown ether columns discussed exhibit a high column efficiency, a wide operating temperature range and possess unique selectivity for polar positional isomers and compounds with the ability to form hydrogen bonds with the crown ether ring. Their preparation is simple.

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Quantitative gas chromatographic analysis of mandelic acid enantiomers

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SUMMARY

A procedure was developed for the quantitative gas chromatographic (GC) analysis of mixtures of mandelic acid enantiomers. Past methods for the GC resolution of α-hydroxy acid enantiomers on optically active stationary phases are systematically reviewed, with special attention to mandelic acid, which is particularly prone to racemization during derivatization. Modification of an earlier procedure involving the conversions: mandelic acid → mandelyl chloride → N-propylmandelamide → O-trimethylsilyl-N-propylmandelamide finally permitted the preparation of O-trimethylsilyl-N-propylmandelamide on a macro scale with negligible racemization and allowed the precise GC analyses of mandelic acid enantiomers on a stainless-steel capillary column coated with N-docasanoyl-L-valine *tert.*-butylamide.

INTRODUCTION

A recent need to evaluate the precise enantiomeric composition of mixtures of *R* and *S*-mandelic acid has prompted us to explore the options for the gas chromatographic (GC) resolution of α-hydroxy acid enantiomers. The GC resolution of enantiomers has been accomplished by either one of two general techniques: (1) conversion of the enantiomers into volatile diastereomer derivatives by reaction with an appropriate optically active reagent, followed by separation on conventional GC phases[1,2] and (2) conversion of the enantiomers into a suitable volatile derivative, followed by separation on an optically active stationary phase. Both methods were originally developed primarily for the resolution of amino acids, and Gil-Av and co-workers[3,4] were the first to describe the use of optically active stationary phases for such applications. These high-molucular-weight phases, which included N-trifluoroacetyl (TFA)-L-isoleucine lauryl ester[3], N-TFA-L-valyl-L-valine cyclohexyl ester[4] and other L-L-dipeptide ester derivatives[5], as well as N-lauroyl-L-valine *tert.*-butylamide[6] and N-docosanoyl-L- valine *tert.*-butylamide[7], were capable of cleanly resolving racemic amino acids as their N-TFA ester derivatives, and offered a number of practical advantages[8] over previous resolutions of amino acids as diastereomeric ester derivatives using conventional GC phases[1,2]. An evaluation of several of these

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procedures for the precise enantiomeric analysis of a number of known mixtures of leucine enantiomers was subsequently undertaken[9], with the finding that such methods allowed for comparable accuracy (0.03–0.7%) and precision (0.03–0.06% standard deviation) in replicate analyses. The above optically active phases had the drawback, however, of being limited to relatively low operating temperatures, thus jeopardizing their application to higher-molecular-weight amino acids[8,10]. This difficulty was overcome in 1977 with the development of "Chirasil-Val", an optically active polysiloxane phase made by coupling a copolymer of dimethylsiloxane and carboxy-alkylmethylsiloxane with L-VALINE *tert.*-butylamide[10]. Since that time this versatile GC phase has been used for the resolution not only of amino acids, but also of the enantiomers of a wide variety of additional classes of organic compounds[11–13].

The GC resolution of α-hydroxy acid enantiomers has not received the attention hitherto devoted to amino acids. In a study which included the resolution of certain amino acids, amines and amino alcohols, lactic acid was first resolved as its O-pentafluoropropionyl N-cyclohexylamide derivative using glass capillary columns coated with Chirasil-Val[14]. In a later study utilizing Chirasil-Val the resolution of eighteen different α-hydroxy acids as five different alkyl ester derivatives was described[15]. The peak tailing due to the free hydroxyl groups was overcome by the use of fused-silica capillary columns and the separation factor, α, for the 3-pentyl esters was found satisfactory. Frank *et al.*[16] studied the resolution of eight different α-hydroxy acids as a number of different derivatives using Chirasil-Val on glass or fused-silica capillary columns. The derivatives included methyl esters, O-heptafluorobutyryl N-propylamides, O-trimethylsilyl N-propylamides, O-isopropylcarbamoyl methyl esters and O-isopropylcarbamoyl N-isopropylamides. Generally speaking, baseline resolution of each enantiomer pair was achieved, but it was found that mandelic acid was racemized to the extent of 54% in some of the derivitization procedures. The authors speculate broadly on the nature of the intermolecular interactions between various groups on the chiral derivative and the Chirasil-Val phase. Later, Wang *et al.*[17] applied one of the above derivatives, the O-trimethylsilyl N-propylamides to the baseline separation and resolution of a mixture of nine α-hydroxy acids of increasing molecular weight ranging from that of lactic to that of *m*-hydroxymandelic acid on Chirasil-Val. Using the readily racemized mandelic acid as a probe, they also worked out the details for a three-step derivatization procedure that was accompanied with a minimum of racemization. Finally, the most recent application of Chirasil-Val to the separation of mandelic acid enantiomers has been that of Koppenhoefer and Allmendinger[18], who converted mandelic acid into a number of derivatives, each of which was analyzed independently for the extent of racemization during preparation. These included methyl, isopropyl and 3-pentyl esters, N-isopropylamide, N-isopropylurethane methyl and isopropyl esters and N-isopropylurethane N-isopropylamide. The extent of racemization at each step of the derivatization was measured by GC on Chirasil-Val and it was found that only the simple esterification of mandelic acid with an alcohol (*e.g.* 3-pentanol) was free (<0.1%) of significant racemization in these multi-step derivatization procedures.

Since 1980 numerous chiral stationary phases other than Chirasil-Val have been employed in generally successful attempts to resolve α-hydroxy acid enantiomers. These phases include optically active Cu(II) complexes of salicylaldehyde Schiff bases[19] and 1,3,5-triazine derivatives of L-valine di- and tripeptide isopro-

pyl esters[20], each of which has been used to resolve the lower alkyl and/or cycloalkyl esters of lactic acid. König *et al.*[21] used O-benzyloxycarbonyl derivatives of *S*-mandelic acid *tert.*-butylamide and *S*-α-phenylethylamide as optically active phases on glass capillary columns to resolve six different α-hydroxy acids as their O-TFA isopropyl esters. They later extended the study[22] to include a number of additional chiral phases derived from *R*- and *S*-mandelic acid as well. A year later König *et al.*[23,24] employed *S*-α-phenylethylamide or *tert.*-butylamide derivatives of other optically active α-hydroxy or O-benzyloxycarbonyl-α-hydroxy acids as stationary phases to resolve ten different hydroxy acids, again as their O-TFA isopropyl esters. At the same time these investigators introduced three new optically active polymeric phases, hexanoyl-*S*-valine-OV-225 and XE-60–*S*-valine–*S(or R)*-α-phenylethylamide, made by chemically modifying the conventional polysiloxane phases OV-225 and XE-60. These phases proved capable of resolving amino acids, amines, amino alcohols and carbohydrates, as well as α-hydroxy acids. König *et al*[25] later extended the use of the XE-60–*S*-valine–*S*-α-phenylethylamide phase to the resolution of some fourteen different α-hydroxy acid enantiomers as their methyl or isopropyl ester isopropylurethane derivatives, formed by reaction of their hydroxyl groups with isopropyl isocyanate. The latter reagent was subsequently proposed[26,27] as a one-step "universal" reagent to form derivatives for the GC resolution of optically active alcohols, amines, amino and N-methylamino acids, as well as α- and β-hydroxy carboxylic acids. Derivatizations were reported to proceed without racemization. The same XE-60–*S*-valine phase was later reported[28] effective in resolving a number of derivatives formed by the action of phosgene on optically active diols, amino alcohols, N-methylamino acids and α-hydroxycarboxylic acid, the latter as 1,3-dioxolan-2,4-diones. Again, the derivatizations were reported to proceed without racemization.

As opposed to the above optically active stationary phases for resolving α-hydroxy acids and other enantiomers directly, the earlier diastereomer GC resolution technique, originally pioneered for resolving amino acids, has received only scant application to hydroxy acids. König and Benecke[29] partially or completely resolved the O-TFA and O-trimethylsilyl (TMS) derivatives of seventeen different α-hydroxy acids as their (+)-3-methyl-2-butyl ester diastereomers on conventional Carbowax 20M, SE-30 and OV-17 phases and later Slessor *et al.*[30] resolved several higher alcohols as well as methyl 9-hydroxy-2-decenoate as their diastereomeric S(+)-lactyl esters on a methylsilicone DB-1 phase.

Previous resolutions of α-hydroxy acids have thus generally employed the optically active stationary phases indicated above, loaded onto glass or fused-silica capillary columns. Stainless steel columns have not been utilized, nor have the earlier optically active GC phases such as N-docosanoyl-*S*-valine *tert.*-butylamide (or the enantiomeric *R*-valine phase[31]), so successfully utilized for amino acids. In fact, it has been stated[22] that the N-acyl-*S*-valine *tert.*-butylamides, while excellent for amino acids, "have no enantioselectivity for α-hydroxy acid derivatives". In addition, while the above studies frequently extol the use of optically active phases for quantitative enantiomer analyses, they generally report only the separation factors achieved with the phase in question, or display actual GC traces showing the separations and the (usually) baseline resolution of the α-hydroxy acid derivatives involved. Thus an actual quantitative GC analysis, checking on accuracy and reproducibility, of mixtures of an α-hydroxy acid of known enantionmeric composition, similar to that

accomplished for leucine enantiomer mixtures[9], has not previously been undertaken. With the above in mind, the objectives of our present work were to develop a method for the accurate GC analysis of mixtures of *R*- and *S*-mandelic acid enantiomers using, if possible, our previously employed[31] stainless steel capillary columns coated with N-docosanoyl-*S* (or *R*)-valine *tert.*-butylamide phases.

EXPERIMENTAL

Gas chromatography

The GC column employed was a 44 m × 0.1 mm I.D. open tubular stainless-steel capillary column, wall-coated with N-docosanoyl-*S*-valine-*tert.*-butylamide (75 mg). This was installed in a Hewlett-Packard Model 5700A gas chromatograph equipped with a flame ionization detector and a Hewlett-Packard Model 18740B capillary inlet system and attached to a Hewlett-Packard Model 3380A digital electronic integrator-recorder. Each product to be analyzed was dissolved in dichloromethane at the molar concentrations indicated in the experiments described below, and 1 μl of each solution was injected onto the column at the temperatures (isothermal) and carrier gas (nitrogen) pressures specified below.

Methyl R-mandelate

R(−)-Mandelic acid (Aldrich, 99+%; 948.3 mg) was esterified by heating with methanol (25 ml) and concentrated sulfuric acid (0.7 ml). The product, 974.7 mg (94.1%) of clear oil, crystallized on standing, m.p. 55.5–56.5°C; $[\alpha]_D^{25}$ − 182° (*c*, 0.9; C_6H_6). Literature: m.p. 55.5°C[32]; $[\alpha]_D^{25}$ − 173° (*c*, 15; C_6H_6)[33].

Propylaminolysis of methyl R-mandelate

The above methyl *R*-mandelate (103.0 mg; 0.620 mmol) was dissolved in propylamine (Aldrich, 99+%; 1.0 ml; 12.2. mmol). The solution was placed in a 1-dm polarimeter tube and its rotation at 25°C was observed at the time intervals shown in Table I. After 2785 min the reaction mixture was stripped of volatiles at 65°C using a rotary evaporator ("vacuum evaporated") and the residue was "chased" twice by dissolving in dichloromethane (3 ml) and vacuum evaporating. This yielded 113.1 mg (94.2%) of syrupy N-propyl-*R*(−)-mandelamide.

The constancy of the observed rotation between 1275 and 2785 min indicates that, once formed, the N-propyl-*R*(−)-mandelamide product was optically stable at 25°C in propylamine solution. The relative consistency of the first-order rate constants calculated for six observed rotations between 115 and 425 min indicates further that the above propylaminolysis is a simple pseudo first order reaction.

The optical stability of methyl R-mandelate in triethylamine

A solution of 30 mg methyl *R*-mandelate [having $[\alpha]_D^{25}$ − 171.8° (*c*, 1.77; C_6H_6)] in 1.00 ml triethylamine (Aldrich) had an observed rotation of −2.315°/dm at 25°C. The rotation remained essentially constant on standing and after 22.75 h was −2.298°. In another experiment a solution of 81.1 mg of the above ester in 3.00 ml triethylamine was heated on the steam bath for 45 min. After cooling to 25°C the solution had an observed rotation of −2.265°/dm.

TABLE I

PROPYLAMINOLYSIS OF METHYL *R*-MANDELATE

Time (min)	α°/dm	$k(c)\ min^{-1}\ (\times 10^3)^c$
0	−8.00[a]	
10	−7.33	
15	−6.98	
115	−1.89	16.4
155	−1.41	15.8
175	−1.23	16.0
245	−0.94	15.8
365	−0.82	15.0
425	−0.80	15.5
1275	−0.79	
1805	−0.79	
2785	−0.79[b]	
Mean ± S.D.		15.8 ± 0.5

[a] By extrapolation.
[b] Corresponds to $[\alpha]_D^{25} = -6.6^\circ$ (*c*, 12.0; propylamine) for N-propyl-*R*(−)-mandelamide.
[c] Calculated by $k = (2.3/t) \times \log([\alpha_0 - \alpha_\infty]/[\alpha_0 - \alpha_t]$, where t = time (min).

O-Trifluoroacetyl-N-propyl-R-mandelamide (R-I)

The above N-propyl-*R*-mandelamide (113.1 mg) was dissolved in dichloromethane (1 ml) and treated with trifluoroacetic anhydride (TFAA; 1.0 ml). After 30 min the volatiles were removed at 60°C by vacuum evaporation, chasing twice with dichloromethane (3 ml). An amount of 171.1 mg (101%) of crude, crystalline *R*-I was obtained. This was recrystallized by dissolving in acetone (0.5 ml), adding hexane (3 ml) and chilling. The purified *R*-I (62 mg) had m.p. 124.5–125°C and showed one sharp peak at 12.81 min under the GC conditions: 0.01 *M*, T = 140°C, P = 0.45 p.s.i. Pure *R*-I product proved unstable, liquefying on standing for several days. GC of this liquid failed to show the previous 12.81 min peak. GC of the original crude *R*-I showed the presence of a small shoulder at a longer retention time on the main peak, suggesting that crude *R*-I contained a small, undetermined amount of the *S*-enantiomer.

O-Trifluoroacetyl-N-propyl-RS-mandelamide (RS-I)

A mixture of methyl *RS*-mandelate (167.5 mg; 1.01 mmol) and propylamine (1.5 ml; 18.3 mmol) was allowed to stand for 96 h, then was processed as above to yield 202.4 mg (103.9%) of crude crystalline N-propyl-*RS*-mandelamide. An amount of 107.1 mg of this was dissolved in dichloromethane (0.5 ml), treated with TFAA (0.5 ml) and after 30 min was processed as above to yield 154.9 mg (96.6%) of solid *RS*-I. This was recrystallized as above to yield 84.4 mg of fine needles, m.p. 105–105.5°C. Under the GC conditions used above for the *R*-enantiomer, *RS*-I was incompletely resolved and variations in the GC parameters were accordingly investigated. For GC conditions of: 0.005 *M*, P = 0.2 p.s.i., T = 140, 145 and 150°C approximately baseline resolution was achieved with retention times [*R*:*S* (min)], respectively, of 28.70:31.42, 24.26:26.10 and 20.53:21.97. The quantitative GC analyses [*R*:*S* (%)]

under these conditions were: 140°C (53.3:46.7), 145°C (50.3:49.7) and 150°C (48.6:51.4).

O-Trimethylsilyl-N-propyl-RS-mandelamide (RS-II)

A portion of the above crude N-propyl-*RS*-mandelamide (95.3 mg; 0.494 mmol) was dissolved in pyridine (2.5 ml) and treated with bis(trimethylsilyl)trifluoroacetamide (BSTFA; Aldrich; 970 mg; 3.77 mmol). The mixture was heated on the steam bath for 30 min then vacuum evaporated, and the residue was chased twice with benzene (4 ml) and thrice with dichloromethane (4 ml). An amount of 139.6 mg (106%) of thick oily *RS*-II was obtained which was analyzed using GC conditions of: 0.001 *M*, P = 0.35 p.s.i., *T* = 150°C. These led to baseline resolution, with *R*-II eluting at 18.77 and *S*-II at 21.52 min and an enantiomer analysis of *R:S*(%):50:50.

N-Propyl-*RS*-mandelamide has been previously resolved as its O-heptafluorobutyryl[16] and O-trimethylsilyl[16,17] derivatives on a Chirasil-Val glass capillary column, but no quantitative data were given.

O-Trimethylsilyl-N-propyl-R-mandelamide (R-II)

Since the above propylaminolysis of methyl *R*-mandelate appeared to be attended by an undetermined amount of racemization, an alternative preparation of N-propyl-*R*-mandelamide was employed. Finely powdered *R*-mandelic acid (50.0 mg) and thionyl chloride (0.5 ml) were placed in a test tube and sonicated for 15 min at 25°C. Additional thionyl chloride (0.5 ml) was added, sonication was continued for 15 min, and the mixture was transferred to a small flask with the aid of dichloromethane (2 ml). The volatiles were vacuum evaporated at 25°C and the residue was chased at 25°C with dichloromethane (4 ml), benzene (4 ml) and thrice with additional dichloromethane (4 ml), yielding 47.4 mg (84.5%) of oily mandelyl chloride. This was dissolved in dichloromethane (2 ml) and treated with propylamine (1 ml). After 5 min the solution was vacuum evaporated at 25°C and chased thrice with dichloromethane (4 ml), to yield 70.8 mg (112%) of N-propyl-*R*-mandelamide, a sweet-smelling, thick oil. This was dissolved in triethylamine (Aldrich; 1.0 ml), treated with BSTFA (0.5 ml) and heated on the steam bath for 30 min. The amber solution was vacuum evaporated at 50°C, chasing twice with benzene (4 ml) and four times with dichloromethane (4 ml). An amount of 94.0 mg (108%) of *R*-II was obtained, an amber oil which was analyzed immediately using GC conditions: *ca.* 0.01 *M*, *T* = 150°C, *P* = 0.35 p.s.i. A sharp peak at 18.79 min was noted, along with a very small (<0.12%) peak at slightly longer retention time, indicating that insignificant racemization occurred during the preparation. The next day similar GC results were obtained on the original *ca.* 0.01 *M* dichloromethane solution. The neat *R*-II product, however, appeared unstable. The original oil lost 18% of its weight on standing 24 h and the 18.79 min GC peaked had vanished. Similar instability was noted for an oily *RS*-II product prepared as above form *RS*-mandelic acid.

Quantitative analyses of known mixtures of R- and S-mandelic acid enantiomers as their O-trimethylsilyl-N-propylamides (R-II and S-II)

Mixture 1. A mixture (28.5 mg) containing 73.0% *R*(−)- and 27.0% *S*(+)-mandelic acid was converted to 49.7 mg (100%) of a mixture of R and *S*-II exactly as described above for *R*-II. Half of the product was dissolved in dichloromethane for

immediate analysis using the GC conditions employed above for *R*-II. The *R*-II eluted at 18.68 and the *S*-II at 21.46 min, resulting in: %*R*: 72.2, 72.3; %*S*: 27.8, 27.7. Two and six days later, respectively, the same dichloromethane solution showed: %*R*: 73.7, 72.3; %*S*: 26.3, 27.7; overall average: %*R*: 72.6; %*S*: 27.4 ± 0.7. After 3 weeks the same solution showed: %*R*: 70.2; %*S*: 29.8. By contrast, the original oily product showed a 7% weight loss on standing for 2 days and its GC showed a new peak at 15.41 min, with the original 18.68 and 21.46 min peaks absent. Thus *R*-II and *S*-II appear reasonably stable in dichloromethane solution at 25°C, but decompose rapidly when undissolved.

Mixture 2. A mixture (25.5 mg) containing 39.6% *R*(−)- and 60.4% *S*(+)-mandelic acid was converted to 44.5 mg (114%) of a mixture of *R*-II and *S*-II exactly as above and the product was dissolved (0.01 *M*) in dichloromethane. Immediate GC analyses under the above conditions showed: %*R*: 39.1, 39.2; %*S*: 60.9, 60.8. Replicate analyses were conducted on the same dichloromethane solution after 1, 4 and 5 days with comparable results. The overall average of six analyses was: %*R*: 38.8; %*S*: 61.2 ± 0.4.

RESULTS AND DISCUSSION

Since mandelic acid is so readily racemized, our initial goal was to develop a derivatization method proceeding with minimum racemization. While simple esterification of mandelic acid proceeds with negligible inversion (<0.1%)[15,18], subsequent conversions of the ester to the N-isopropylamide (with isopropylamine in dichloromethane, 80°C) and then to the N-isopropylurethane (with isopropylisocyanate in dichloromethane, 100°C) were reported to involve up to 4–14% inversion[18], and the direct formation of N-isopropylmandelamide N-isopropylurethane was accompanied by a host of byproducts[18] as well. The three-step conversion of mandelic acid to its O-heptafluorobutyryl N-propylamide has been reported to proceed with up to 27% inversion[16]. Thus the most promising procedure appeared to be the three-step conversion of Wang *et al.*[17] of mandelic acid to O-trimethylsilyl-N-propylmandelamide (II) using (1) thionyl chloride (25°C), (2) propylamine (25°C) and (3) BSTFA–pyridine (80°C), during which racemization was reported as low as 2%. Variations of this procedure were accordingly explored.

The conversion of methyl *R*-mandelate into N-propyl-*R*-mandelamide was accomplished by dissolving the ester in propylamine at 25°C. The propylaminolysis was complete in *ca.* 7 h (Table I), proceeding with pseudo first order kinetics. The resulting N-propyl-*R*-mandelamide was optically stable once formed (Table I), but there apeared to be a small amount of racemization of the starting ester by action of the propylamine prior to or during its conversion to the amide, as indicated by GC analysis after subsequent conversion of the amide to its O-TFA derivative (*R*-I). Applying the same reaction sequence to methyl *RS*-mandelate gave O-trifluoroacetyl N-propyl-*RS*-mandelamide (*RS*-I), which could be reasonably but not optimally resolved under our GC conditions. Conversion of the above amide to its trimethylsilyl ether using BSTFA, however, gave O-trimethylsilyl-N-propyl-*RS*-mandelamide (*RS*-II), which could be quantitatively resolved.

When the Wang *et al.*[17] conversion of *RS*-mandelic acid to *RS*-II was scaled up from the original μg level to the 50-mg level, GC analysis of the *RS*-II product showed

baseline resolution and reasonable quantitative agreement: %*R*: 49.4; %*S*: 50.6. When the same conversion was applied on this scale to *R*-mandelic acid, however, the anticipated *R*-II product proved to contain 25% of the *S*-II enantiomer. In another experiment where N-propyl-*R*-mandelamide, prepared as above by the propylaminolysis of methyl *R*-mandelate, was converted to its O-trimethylsilyl ether using BSTFA in pyridine at 80°C, the *R*-II product contained 15.4% *S*-II.

Since methyl *R*-mandelate was found to be optically stable in triethylamine solution, we thought that substituting triethylamine for pyridine in the final trimethylsilation step of the Wang *et al.* conversion[17] might lead to less overall racemization. This proved to be the case since when *R*-II was prepared in this way it contained $<0.12\%$ of the *S*-II enantiomer.

Finally, the latter procedure was applied to the quantitative analyses of two known mixtures of mandelic acid enantiomers, the first having *R*: 73.0; *S*: 27.0% and the second having *R*: 39.6; *S*: 60.4%. Replicate analyses of the II product from the first mixture showed *R*: 72.6; *S*: 27.4 ± 0.7% and from the second mixture *R*: 38.8; *S*: 61.2 ± 0.4%. Since the first analysis missed the known composition by only 0.4% and the second by 0.8%, we conclude that the above procedure enables reasonably accurate and reproducible GC analyses of mandelic acid enantiomer mixtures using N-docosanoyl-L–valine *tert.*butylamide phase on a stainless-steel capillary column.

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Comparison of various stationary phases for normal-phase high-performance liquid chromatography of ethoxylated alkylphenols

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SUMMARY

Unmodified silica and chemically bonded diol, nitrile and amino phases were tested as column packings for normal-phase liquid chromatographic separations of ethoxylated alkylphenols in mobile phases consisting of aliphatic alcohol(s) and a non-polar hydrocarbon. The performance of unmodified silica is improved when ethanol is used instead of propanol in binary mobile phases or when ethanol–propanol–aliphatic hydrocarbon ternary mobile phases are applied.

Diol and nitrile bonded phases show regular retention behaviour only in mobile phases with a low content of propanol, where 6–8 ethoxymers can be resolved in a reasonable time. In propanol-rich mobile phases, a mixed retention mechanism causes a non-linear increase in log k' with increasing number of oxyethylene units, which hinders the prediction of retention by calculation and deteriorates the separation of the individual oligomers in propanol–*n*-alkane mobile phases.

The amino-bonded stationary phase shows regular retention behaviour even for higher ethoxymers in mobile phases containing high proportions of propanol in *n*-alkane, so that the capacity factors can be predicted by calculation. The amino-bonded phase offers better separation possibilities for the individual ethoxymers than the other stationary phases tested, in both the isocratic and gradient modes of elution.

INTRODUCTION

Ethoxylated alcohols and alkylphenols are important non-ionic surfactants with widespread industrial and domestic applications. Consequently, there is considerable

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interest not only in the determination of these materials, but also in the characterization of the distribution of the individual ethoxymers in both industrial and environmental samples. Although gas and thin-layer chromatography have been applied for this purpose, the applications of high-performance liquid chromatography (HPLC) have acquired major attention recently because of the simplicity of the analytical procedure and its straightforward applicability to the separation of higher ethoxymers.

Steric exclusion chromatography usually requires recycling and long analysis times[1], so that interaction modes of liquid chromatography are more suitable in practice.

To improve the possibilities of UV detection, ethoxylated alcohols can be separated after derivatization with 3,5-dinitrobenzoyl chloride in either reversed-phase[2] or normal-phase[3] systems, or as anthroyl derivatives in reversed-phase systems[4]. Underivatized condensates of ethylene oxide with alcohols or fatty acids may be separated using refractometric[5] or mass spectrometric[6] detection. Normal-phase gradient-elution chromatography on a bonded amino phase[6] and isocratic partition chromatography on a bonded diol phase using a mobile phase consisting of *n*-hexane, 2-propanol, water and acetic acid[5] have been employed for this purpose.

Ethoxylated alkylphenols can be partially separated in reversed-phase chromatographic systems using octadecyl- or octylsilica columns[7–11], but the quality of the separation depends on the type of alkyl group and on the mobile phase components and the individual ethoxymeres may be eluted in order of either decreasing or increasing number of oxyethylene units[7–9]. In normal-phase systems, silica gel and mobile phases consisting of an *n*-alkane and propanol, ethanol or acetonitrile have been employed for the separation of ethoxylated alkylphenols[10,12,13]. Nitrile-[4,12–14], diol-[13] and amino-bonded phases[6,13,15–21] have been employed more often than unmodified silica, but the mobile phases used usually consisted of water, acetic acid or other very strongly polar compounds in addition to the *n*-alkane and another organic solvent of medium polarity, which makes these systems more difficult to understand; obviously these are dynamically induced partition rather than adsorption systems. Ethoxylated alkylphenols are readily detected by UV detectors but fluorimetric[18] or mass spectrometric[8] detection systems are also occasionally used.

Desbène *et al.*[3] compared the performances of diol-, amino- and nitrile-bonded phases for the separation of 3,5-dinitrobenzoyl esters of ethoxylated long-chain aliphatic alcohols, but no systematic comparison of stationary phases for the separation of underivatized ethoxylated alkylphenols has been reported.

This work is a continuation of previous investigations of selectivity in various oligomeric series in reversed-phase and normal-phase systems[13]. The main aim was to compare the chromatographic behaviour of underivatized ethoxylated nonylphenols on unmodified silica gel and chemically bonded diol-, nitrile- and amino-bonded stationary phases in mobile phases consisting of an *n*-alkane and lower aliphatic alcohols and to find the optimum separation conditions. Further, the suitability of the individual normal-phase systems for the prediction of retention was tested. This aspect is considered to be important for two reasons: (a) this prediction may be useful for identifying the peaks in chromatograms of more complex samples, which may contain not only the pure oligomers but also isomers, adducts, additives, and other compounds; and (b) irregular band spacing in the chromatogram may affect

unfavourably the results of quantification of the oligomer distribution and the prediction of retention is important for optimization of the separation process so as to obtain regularly spaced symmetrical peaks of the individual oligomers in as short a separation time as possible.

Therefore, chromatographic systems that yield regular and predictible spacing of the peaks of the individual oligomers in the chromatogram are to be preferred to other systems. For this reason, we compared the individual chromatographic systems from the point of view of regular band spacing, predictability of retention and reliability of the quantitative results.

As has been shown earlier, the dependence of the capacity factors, k', of the individual oligomers on the number of oligomeric units, n, and on the concentration, φ, of the more polar component in a binary mobile phase can often be described by[22]

$$\log k' = a_0 - m_0 \log \varphi + n (a_1 - m_1 \log \varphi) \quad (1)$$

where a_0, m_0, a_1 and m_1 are constants related to the adsorption energy and adsorbed area of the repeat structural (oligomeric) unit and of the structural residue (end-groups)[13,22].

EXPERIMENTAL

Some experiments were performed using an M6000A pump, a U6K injector and an M440 UV detector operated at 254 nm (all from Waters Assoc., Milford, MA, U.S.A.), connected to a TZ 4241 line recorder and a CI 100 computing integrator (both from Laboratory Instrument Works, Prague, Czechoslovakia). An HP 1090M liquid chromatograph equipped with a UV diode-array detector, operated at 230 nm, an automatic sample injector, a 3DR solvent delivery system, a thermostated column compartment, a Series 79994A workstation and an HP 2225 Think-Jet printer (Hewlett-Packard, Avondale, PA, U.S.A.) was used for other experiments.

Stainless-steel columns (300 × 3.8, 4.2 or 4.4 mm I.D.) were packed in the laboratory with the spherical materials Silasorb 600 SPH (silica gel), 5 μm, Silasorb SPH Amine and Silasorb SPH Nitrile, 7.5 μm (all from Lachema, Brno, Czechoslovakia), using a high-pressure slurry packing technique. A Silasorb Diol (7.5 μm) stainless-steel column (250 × 4 mm I.D.) was obtained pre-packed from Lachema.

Oligomeric ethoxylated nonylphenols with various declared average stoichiometric ratios of oxyethylene units to nonylphenol were obtained from Servo (Delden, The Netherlands) under the commercial names Serdox NNP 1.5, Serdox NNP 4, Serdox NNP 8, Serdox NNP 12 and Serdox NNP 20. The samples were dissolved in the mobile phase in appropriate concentrations to yield good UV detector responses.

1-Propanol, 2-propanol, ethanol and *n*-heptane used as mobile phase components were all of spectroscopic or analytical-reagent grade from Lachema. The solvents were kept dried over molecular sieves S5A (Lachema) and were filtered using a Millipore non-aqueous 0.45-μm filter before use. The mobile phases were either pre-mixed in the required volume ratios and de-gassed by ultrasonication prior to use in the Waters Assoc. equipment, or were prepared directly in the HP 1090M instrument by mixing the pure solvents and de-gassed during operation by continuous stripping with helium.

Column dead volumes, V_M, were determined using refractometric detection with an R401 differential refractometer (Waters Assoc.) and *n*-heptane as the dead volume marker. The retention volumes, V_R, of the individual ethoxymers in the samples tested were measured at different mobile phase compositions. The capacity factors, k', were calculated from the mean V_R value of three repeated experiments under given conditions ($k' = V_R/V_M - 1$). Linear regression analysis was used to calculate the constants a_0, m_0, a_1 and m_1 in eqn. 1, necessary for predictive calculations of k'.

For mass spectrometric identification of the individual ethoxymers in fractions from the liquid chromatograph, a Varian-MAT 44s quadrupole mass spectrometer (Varian, Bremen, F.R.G.) was employed, using chemical ionization with methane. Mass spectrometric measurements were performed at the Institute for Synthetic Resins and Lacquers, Pardubice, Czechoslovakia.

RESULTS AND DISCUSSION

Identification of individual ethoxymers

As pure etoxymer standards were not available and the commercial samples were mixtures of various oligomers the identification of the individual oligomers was based on mass spectrometry of the fractions separated by HPLC. For this purpose, Serdox NNP 1.5 was chosen, as it contains only low oligomers in relatively high concentrations (declared stoichiometric ratio of ethylene oxide to nonylphenol = 1.5:1). The individual ethoxymers in the sample were completely separated on the column packed with Silasorb SPH Amine in 1-propanol–*n*-heptane (2.5:97.5). A 150-μl volume of a 5% solution of Serdox NNP 1.5 was separated into three main fractions (A, B and C, Fig. 1), the individual fractions from two repeated runs were collected and their mass spectra measured. These spectra are shown in Fig. 2. Fractions A and B, corresponding to the first two chromatographic peaks, show peaks of the molecular ion at $m/z = 264$,

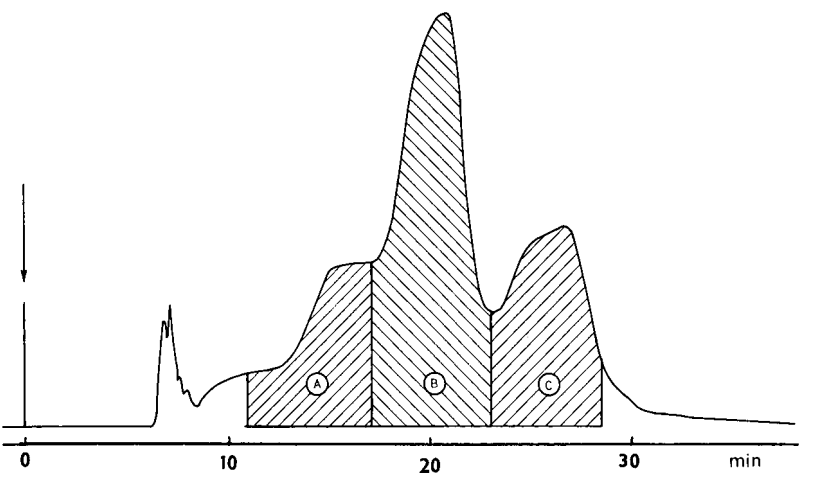

Fig. 1. Semi-preparative separation of 150 μl of a 5% sample of Serdox NNP 1.5 on a Silasorb SPH Amine (7.5 μm) column (300 × 4.2 mm I.D.) with 1-propanol–*n*-heptane (2.5:97.5) mobile phase. Instrument, Waters Assoc.; UV detection at 254 nm. Fractions A, B and C correspond to the three dominant peaks in the chromatogram.

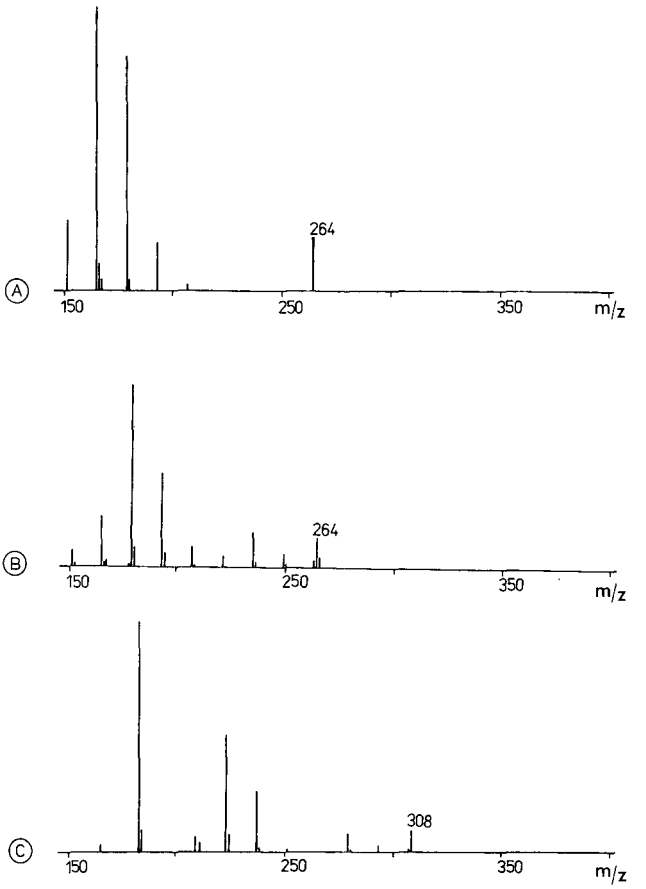

Fig. 2. Mass spectra of fractions A, B, C in Fig. 1. Conditions are given under Experimental.

which corresponds to nonylphenol with one oxyethylene unit, and the third peak (fraction C) shows the peak of the molecular ion at $m/z = 308$, which represents the molecular mass of nonylphenol with two oxyethylene units. In fraction B, the spectrum is dominated by a fragment at $m/z = 179$, which is probably formed by the loss of a hexyl radical ($\Delta m/z = 85$) from the molecular ion. The fragment peak at $m/z = 233$ in the mass spectrum of fraction C corresponds to the same loss. In the mass spectrum of fraction A, the fragment peak at $m/z = 165$ is more intense than at $m/z = 179$. The former peak probably corresponds to the loss of a heptyl radical from the molecular ion.

From this it can be concluded that the first peak in Fig. 1 (fraction A) probably corresponds to $C_7H_{15}CH(CH_3)C_6H_4$-*p*-OCH_2Ch_2OH (I), the second peak (fraction B) to $C_6H_{13}C(CH_3)_2C_6H_4$-*p*-OCH_2CH_2OH (II) and the third peak (fraction C) to $C_6H_{13}C(CH_3)_2C_6H_4$-*p*-$O(CH_2CH_2O)_2H$ (III).

From Fig. 1 it can be seen that isomer II is present in the sample at a *ca.* five times higher concentration than isomer I. In some of the experiments the chromatograms

TABLE I

EXPERIMENTAL RETENTION VOLUMES (V_R, cm^3) AND CAPACITY FACTORS [k'(e)] OF THE INDIVIDUAL OLIGOMERS WITH n OLIGOMERIC UNITS ON SILASORB 600 SPH, 5 μm (COLUMN 300 × 3.8 mm I.D.), IN MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS [φ, % (v/v) · 10^{-2}] OF 1-PROPANOL IN n-HEPTANE

Sample: Serdox NNP 4. V_M = column dead volume (cm^3); k'(c) are the capacity factors predicted by calculation using the equation $\log k' = -1.47 + 0.35n - (1.00 + 0.07n) \log \varphi$.

n	$\varphi = 1$			$\varphi = 0.9$			$\varphi = 0.8$			$\varphi = 0.6$			$\varphi = 0.5$		
	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$
2	3.22	0.15	0.17	3.29	0.19	0.19	3.38	0.21	0.22	3.58	0.32	0.30	3.76	0.35	0.37
3	3.83	0.37	0.38	4.15	0.50	0.43	4.38	0.57	0.50	4.75	0.76	0.71	5.31	0.90	0.89
4	5.06	0.80	0.85	5.56	1.02	0.97	6.28	1.25	1.13	7.09	1.62	1.64	8.34	1.99	2.06
5	7.75	1.77	1.91	8.88	2.22	2.20	10.74	2.86	2.58	12.62	3.67	3.80	16.20	4.81	4.86
6	14.29	4.10	4.26	17.13	5.22	4.95	21.82	6.84	5.86	26.52	8.81	8.81			11.41
V_M		2.80			2.75			2.78			2.70			2.79	

showed a slight indication of the separation of isomeric peaks of higher ethoxymers, possibly of type I, but in most instances the isomeric peaks were not apparent and therefore they are not considered in further discusson.

From a comparison of the elution pattern of the Serdox NNP 1.5 sample with chromatograms for other samples with higher nominal degrees of etoxylation, the peaks of the individual ethoxymers were readily identified.

Comparison of retention behaviour on silica gel in binary and ternary mobile phases

The experimental retention volumes and capacity factors in mobile phases containing various concentrations of 1-propanol in *n*-heptane are given in Table I. The logarithms of the capacity factors increases regularly both with increasing number of oligomeric units, n, and with decreasing logarithm of the concentration of propanol in the mobile phase, φ, so that the eqn. 1 can be used to describe the retention of lower oligomers (up to five oxyethylene units). The capacity factors of the oligomer with six oxyethylene units show significant positive deviations from the values predicted by calculation using eqn. 1 in mobile phases containing 80–90% of propanol. A relatively high value of the constant $a_1 = 0.35$ and low value of the constant $m_1 = 0.07$ in eqn. 1 in propanol–heptane mobile phases mean a very high separation selectivity between neighbouring oligomers, so that it is not possible to separate more than four or five oligomers in a single run within a reasonable separation time under isocratic conditions.

In mobile phases consisting of ethanol and *n*-heptane, eqn. 1 can be used to describe appropriately the retention of the first seven oligomers in the ethoxylated nonylphenol series, higher oligomers showing negative deviations from the capacity factors predicted by calculation (Table II). The constants a_1 and m_1 in eqn. 1 are lower in ethanol–*n*-hexane mobile phases than in mobile phases containing propanol, which has been attributed to the higher polarity (solvent strength) of ethanol with respect to

TABLE II

AS TABLE I, FOR MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS [φ, % (v/v) · 10^{-2}] OF ETHANOL IN *n*-HEPTANE

k'(c) are the capacity factors predicted by calculation using the equation $\log k' = -2.46 + 0.29n - (3.28 - 0.27n) \log \varphi$.

n	$\varphi = 0.3$			$\varphi = 0.4$			$\varphi = 0.5$			$\varphi = 0.6$		
	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$
1	3.22	0.26	0.25	2.89	0.08	0.11	2.78	0.03	0.03			0.02
2	3.38	0.32	0.36	3.10	0.15	0.16	2.90	0.08	0.09	2.88	0.05	0.05
3	3.83	0.50	0.50	3.38	0.26	0.25	3.15	0.17	0.14	3.05	0.12	0.09
4	4.57	0.79	0.71	3.84	0.43	0.38	3.43	0.28	0.23	3.25	0.18	0.15
5	4.94	0.93	1.00	4.17	0.56	0.57	3.76	0.40	0.37	3.54	0.29	0.26
6	5.96	1.33	1.41	4.96	0.85	0.87	4.33	0.61	0.60	4.03	0.47	0.44
7	7.47	1.92	1.98	5.99	1.23	1.33	5.15	0.92	0.98	4.77	0.74	0.76
8	9.31	2.64	2.79	7.60	1.84	2.02	6.26	1.33	1.57	5.76	1.10	1.28
9	12.12	3.75	3.39	9.92	2.70	3.08	7.93	1.96	2.55	7.08	1.59	2.18
V_M		2.55			2.68			2.68			2.73	

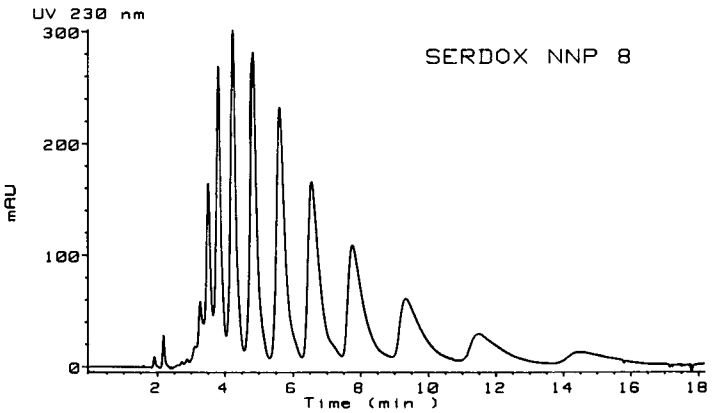

Fig. 3. Isocratic separation of Serdox NNP 8 on a Silasorb 600 SPH, 5 μm (silica gel) column (300 × 3.8 mm I.D.) with ethanol–*n*-heptane (30:70) mobile phase. Flow-rate, 1 cm^3/min; detection, UV (230 nm); instrument, HP 1090M; sample volume, 5 μl.

propanol[13]. Consequently, both the absolute retention and selectivity are lower in ethanol–*n*-heptane mobile phases, which in this instance favours isocratic separation of the individual oligomers in a shorter time than in propanol–*n*-heptane mobile phases. Fig. 3 shows the chromatographic separation of Serdox NNP 8 (with eight nominal oxyethylene units) on Silasorb 600 SPH, using ethanol–*n*-heptane (30:70) as the mobile phase. These conditions allow the first twelve oligomers to be separated in about 16 min.

In ternary mobile phases containing ethanol and 1-propanol in *n*-heptane, the chromatographic behaviour of ethoxylated nonylphenols is approximately between those in ethanol–*n*-heptane and propanol–*n*-heptane binary mobile phases[13]. This means that the selectivity of the separation of neighbouring oligomers is lower than in 1-propanol–*n*-heptane but higher than in ethanol–*n*-heptane mobile phases. Table III

TABLE III

AS TABLE I, FOR MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS OF 1-PROPANOL (φ_P) AND ETHANOL (φ_E) IN *n*-HEPTANE [φ_E, φ_P IN % (v/v) · 10^{-2}; $\varphi_E : \varphi_P$ = 1:1).

k'(c) are the capacity factors predicted by calculation using the equation $\log k' = -1.97 + 0.31n - (1.83 - 0.11n) \log (\varphi_E + \varphi_P)$.

n	$\varphi_E = \varphi_P = 0.5$			$\varphi_E = \varphi_P = 0.166$			$\varphi_E = \varphi_P = 0.125$		
	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$
2			0.04			0.26			0.42
3	3.10	0.09	0.09	4.00	0.50	0.47	4.74	0.71	0.73
4	3.34	0.17	0.19	4.91	0.84	0.86	5.97	1.15	1.28
5	3.96	0.38	0.38	6.50	1.44	1.55	8.42	2.03	2.24
6	5.06	0.77	0.78	9.49	2.56	2.81	13.17	3.74	3.93
7	7.10	1.49	1.58	15.30	4.75	5.08	22.34	7.06	6.89
V_M		2.85			2.66			2.77	

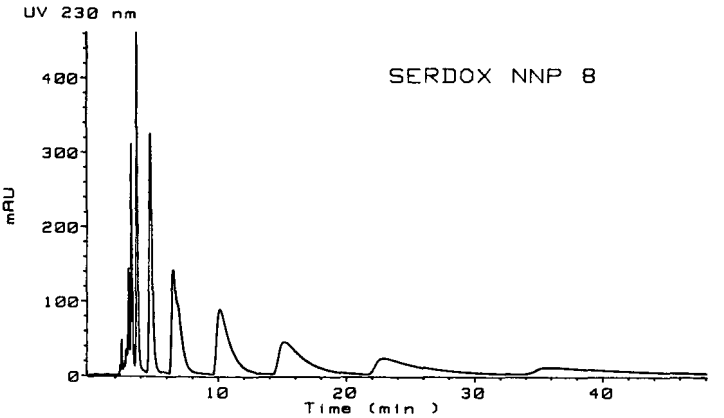

Fig. 4. Isocratic separation of Serdox NNP 8 on a Silasorb 600 SPH column with ethanol–1-propanol–*n*-heptane (1:1:3) mobile phase. Other conditions as in Fig. 3.

shows that eqn. 1 may be adequately used to describe the dependence of the retention ($\log k'$) of the first seven oligomers on the number of oligomeric units and on the sum of the concentrations of ethanol and propanol. Fig. 4 shows the separation of the first ten oligomers in ethanol–1-propanol–*n*-heptane (20:20:60) mobile phase, which takes about 45 min. Using a ternary gradient with simultaneously increasing concentration of ethanol and 1-propanol at a constant ratio of ethanol and 1-propanol concentrations (3:1), it was possible to achieve the separation of fourteen oligomers in 30 min (Fig. 5). The separation achieved under these conditions compares favourably with that using a binary gradient of increasing concentration of ethanol in *n*-heptane (Fig. 6). Hence the ratio of the concentrations of ethanol and 1-propanol in the mobile phase may be used with advantage to control the retention and the separation selectivity.

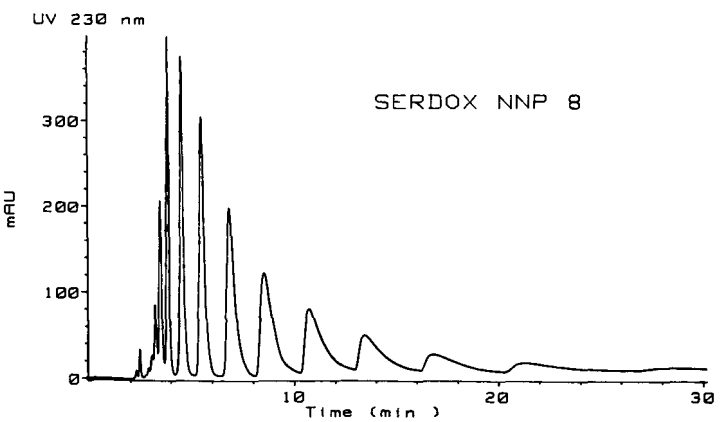

Fig. 5. Gradient elution separation of Serdox NNP 8 on a Silasorb 600 SPH column using a linear ternary gradient from ethanol–1-propanol–*n*-heptane (24:8:68) to ethanol–1-propanol (75:25). Other conditions as in Fig. 3.

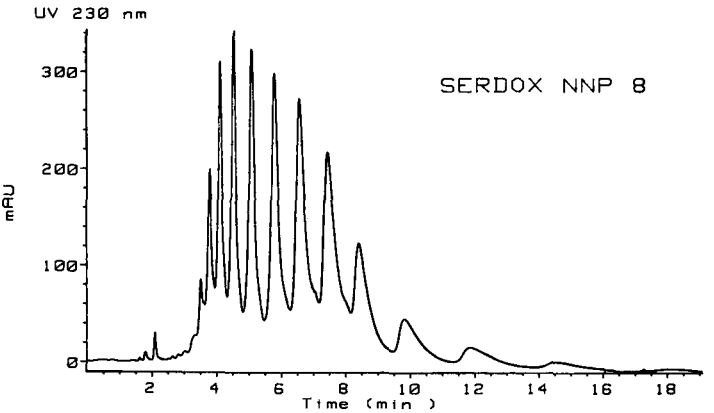

Fig. 6. Gradient elution separation of Serdox NNP 8 on a Silasorb 600 SPH column using a linear gradient from ethanol–*n*-heptane (25:75) to ethanol–*n*-heptane (85:15) in 30 min. Other conditions as in Fig. 3.

Retention behaviour on chemically bonded polar phases in organic mobile phases

The nitrile phase is the least polar of the chemically bonded phases tested. In mobile phases containing less then 25–30% of 2-propanol in *n*-heptane, the retention of the individual ethoxylated nonylphenols on Silasorb SPH Nitrile is adequately described by eqn. 1, *i.e.*, $\log k'$ increases regularly with increasing number of oligomeric units, n, and with decreasing logarithm of propanol concentration, φ (Table IV).

The elution behaviour of ethoxylated nonylphenols in mobile phases with a higher content of propanol is different (Table V). The decrease in $\log k'$ with increasing $\log \varphi$ of 1-propanol is almost linear and negative slopes of these plots increase with increasing number of oligomeric units, as expected according to eqn.

TABLE IV

EXPERIMENTAL RETENTION VOLUMES (V_R, cm^3) AND CAPACITY FACTORS [k'(e)] OF THE INDIVIDUAL OLIGOMERS WITH n OLIGOMERIC UNITS ON SILASORB SPH NITRILE, 7.5 μm (COLUMN 300 × 4.4 mm I.D.), IN MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS [φ, % (v/v) · 10^{-2}] OF 2-PROPANOL IN *n*-HEPTANE

Sample: Serdox NNP 4. V_M = column dead volume (cm^3); k'(c) are the capacity factors predicted by calculation using the equation $\log k' = -1.81 + 0.06n - (0.94 + 0.14n) \log \varphi$.

n	φ = 0.25			φ = 0.20			φ = 0.14		
	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$
1	4.40	0.07	0.08	4.52	0.10	0.10	4.75	0.14	0.15
2	4.53	0.10	0.11	4.71	0.14	0.14	5.10	0.22	0.22
3	4.74	0.15	0.15	4.97	0.21	0.21	5.58	0.34	0.34
4	4.96	0.21	0.21	5.29	0.28	0.30	6.15	0.48	0.51
5	5.26	0.28	0.30	5.75	0.40	0.43	7.14	0.72	0.78
6	5.66	0.37	0.42	6.44	0.57	0.62	8.69	1.09	1.17
7	6.14	0.49	0.58	7.56	0.84	0.89	11.14	1.68	1.78
V_M		4.11			4.11			4.15	

TABLE V

AS TABLE IV, FOR MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS [φ, % (v/v) 10^{-2}] OF 1-PROPANOL IN *n*-HEPTANE

Column dead volume (V_M = 3.45 cm^3).

n	φ=0.5		φ=0.6		φ=0.7		φ=0.8	
	V_R	$k'(e)$	V_R	$k'(e)$	V_R	$k'(e)$	V_R	$k'(e)$
1	4.09	0.19	4.08	0.18	–	–	–	–
2	4.27	0.24	4.22	0.22	4.19	0.21	4.18	0.21
3	5.34	0.55	5.26	0.52	5.05	0.46	4.96	0.44
4	10.12	1.93	9.18	1.66	8.11	1.35	7.54	1.19
5	16.92	3.90	14.32	3.15	11.64	2.37	10.29	1.98
6	25.73	6.46	21.26	5.16	15.87	3.60	13.06	2.78
7	33.57	8.73	25.75	6.46	18.40	4.33	14.66	3.25
8			29.76	7.63	20.53	4.95	15.97	3.63
9			33.22	8.63	22.38	5.49	17.03	3.94
10			37.15	9.77	24.45	6.09	18.22	4.28
11			42.59	11.35	27.40	6.94	20.17	4.85
12					32.00	8.28	23.10	5.70
13					38.74	10.23	27.47	6.96
14					48.35	13.01	33.42	8.69
15							41.21	10.96

1 (Fig. 7), but the plots of log k' *versus* n are non-linear and show a sigmoidal shape (Fig. 8). The increase in log k' per oligomeric unit is most significant in between two and five oxyethylene units; this increase is approximately six times less for the oligomers with more than six oxyethylene units, but for oligomers with more than ten oxyethylene units the increment in log k' per oligomeric unit again increases. The behaviour has not been observed either in mobile phases with a lower content of propanol or in chromatographic systems with unmodified silica Silasorb 600 SPH, and is not easy to explain. The increment in log k' is equivalent to a free energy of transfer from the mobile to the stationary phase per oligomeric unit and the retention behaviour observed with the nitrile stationary phase in propanol-rich mobile phases suggests a change in conformation of the oxyethylene chains depending on the chain length of the oligomer. This conformation change may be connected with solvation of the oxyethylene chains by propanol molecules and with steric possibilities for contact between the oxyethylene units and active sorption centres on the surface of the bonded phase. The different retention behaviour in mobile phases with low and high contents of propanol indicates that a possible mixed partition–adsorption mechanism may play an important role in propanol-rich mobile phases. This may possibly explain the lower capacity factors in 2-propanol–*n*-heptane (25:75) than in 1-propanol–*n*-heptane (50:50) (Tables IV and V). As a practical consequence, isocratic (Fig. 9) or gradient-elution (Fig. 10) separations of ethoxylated nonylphenols in propanol-rich mobile phases yield irregularly spaced peaks with poor separation of the first oligomers.

The retention behaviour of ethoxylated nonylphenols on the bonded diol phase is very similar to that on the nitrile phase. In mobile phases containing less than 25%

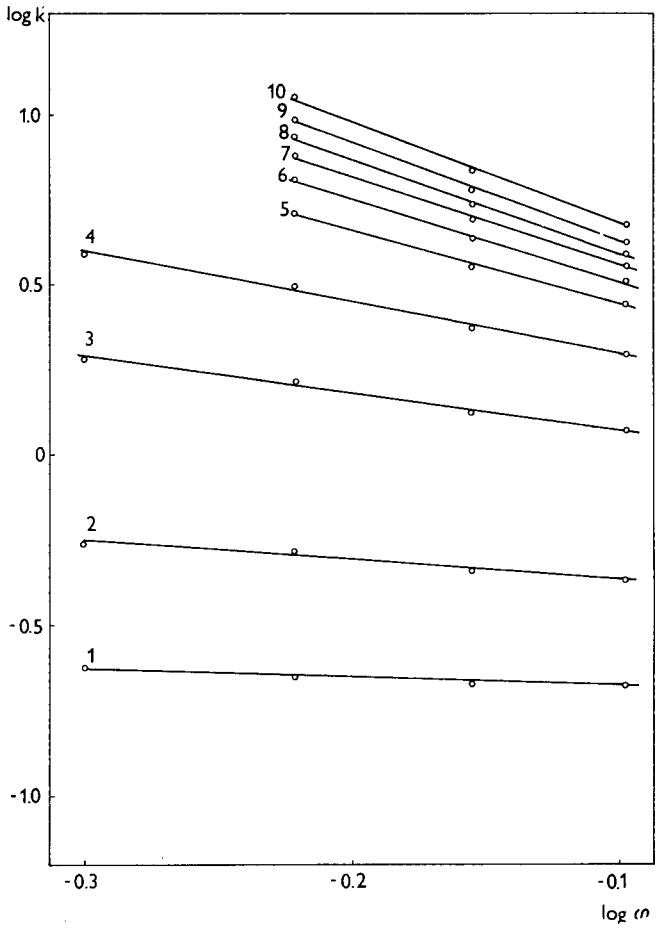

Fig. 7. Dependence of retention (k') of the individual ethoxymers in the sample of Serdox NNP 8 on the concentration [φ, % (v/v) · 10^{-2}] of 2-propanol in *n*-heptane as the mobile phase on a Silasorb SPH Nitrile, 7.5 μm, column (300 × 4.0 mm I.D.). The numbers on the lines are the numbers of oxethylene units in the ethoxymers.

1-propanol in heptane, the retention behaviour of the ethoxylated nonylphenols with up to eight oligomeric units is well described by eqn. 1 (Table VI). However, the capacity factors are greater in the mobile phase containing 40% than in that with only 20% of propanol (Table VI), and the plots of log k' *versus* the number of oligomeric units in propanol-rich mobile phases show an analogous sigmoidal shape to the corresponding dependences for Silasorb Nitrile (Fig. 11). Obviously, the reason for this behaviour is the same with the two stationary phases. Also, the spacing of the peaks of the individual oligomers is similar to that with the nitrile phase (Fig. 12).

The retention behaviour of ethoxylated nonylphenols on the bonded amino phase differs significantly from that on the other bonded phases tested. Eqn. 1 can describe adequately the retention of ethoxylated nonylphenols on Silasorb SPH Amine up to the seventh or eighth oligomer in mobile phases containing 20–75% of

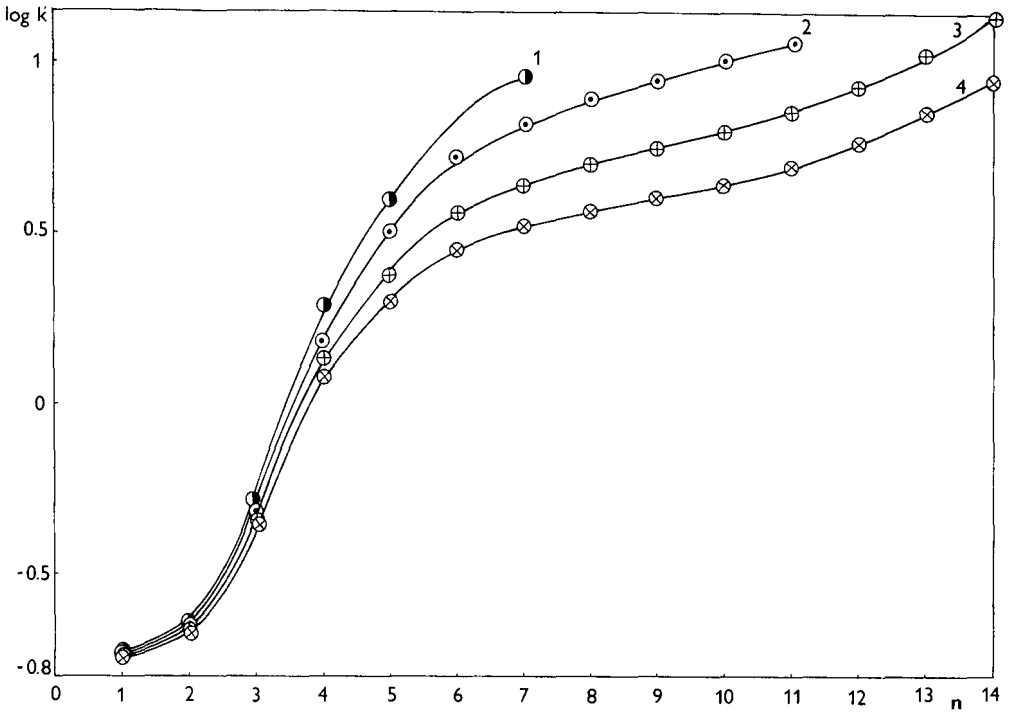

Fig. 8. Dependence of retention (k') of the individual ethoxymers in the sample of Serdox NNP 8 on the number of oxyethylene units, n. Column as in Fig. 7. Mobile phases with different ratios of 2-propanol to n-heptane: (1) 50:50; (2) 60:40; (3) 70:30; (4) 80:20.

2-propanol in n-heptane; positive deviations are observed with 10% propanol for the oligomers with $n \geqslant 5$ (Table VII). This "regular" dependence of k' on the number of oligomeric units and on the mobile phase composition is similar to that observed with unmodified silica Silasorb 600 SPH. Unmodified silica and the bonded amino phase

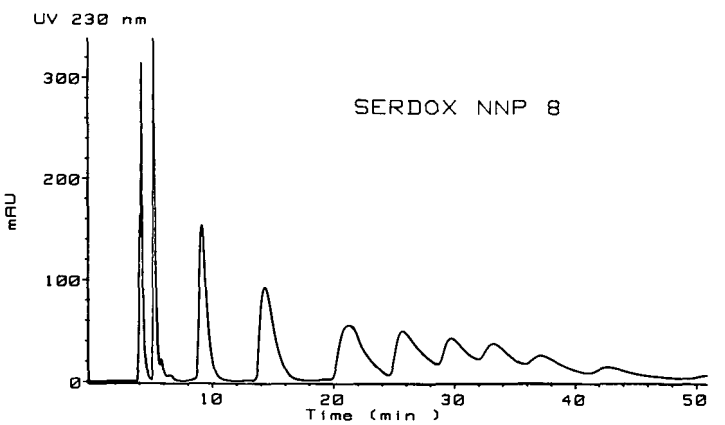

Fig. 9. Isocratic separation of Serdox NNP 8 on a Silasorb SPH Nitrile, 7.5 μm, column (300 × 4.4 mm I.D.) with 1-propanol–n-heptane (60:40) mobile phase. Other conditions as in Fig. 3.

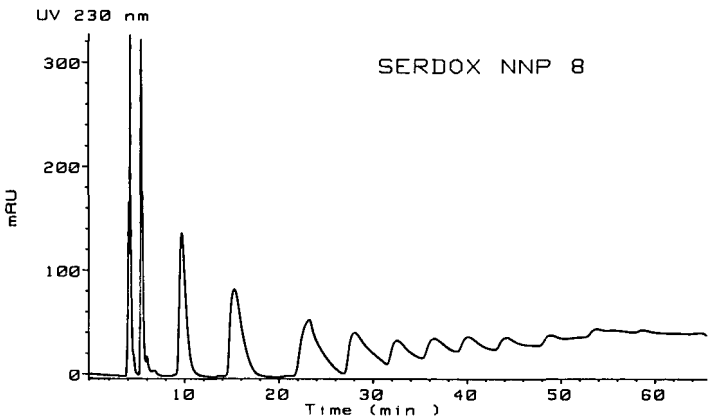

Fig. 10. Gradient elution separation of Serdox NNP 8 on a Silasorb SPH Nitrile column using two-step elution with 1-propanol–*n*-heptane (60:40) for 30 min, followed by a linear gradient to 1-propanol–*n*-heptane (90:10) in the following 30 min. Column as in Fig. 9; other conditions as in Fig. 3.

possess stronger polar adsorption centres than the nitrile and diol phases and the mixed retention mechanism and conformational change of the oxyethylene chain depending on the chain length probably do not occur, or are far less significant on the former two stationary phases. The spacing of the individual oligomers on the chromatograms obtained with the bonded amino phase is more regular than that with the nitrile- and diol-bonded phases and allows a better separation of the first

TABLE VI

EXPERIMENTAL RETENTION VOLUMES (V_R, cm^3) AND CAPACITY FACTORS [k'(e)] OF THE INDIVIDUAL OLIGOMERS WITH n OLIGOMERIC UNITS ON SILASORB DIOL, 7.5 μm (COLUMN, 250 × 4 mm I.D.), IN MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS [φ, % (v/v) · 10^{-2}] OF 1-PROPANOL IN *n*-HEPTANE

Sample: Serdox NNP 4. V_M = column dead volume (cm^3); k'(c) are the capacity factors predicted by calculation using the equation $\log k' = -1.60 + 0.15n - (0.58 + 0.03n) \log \varphi$.

n	$\varphi = 0.2$			$\varphi = 0.1$			$\varphi = 0.05$			$\varphi = 0.01$		
	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$
1												
2	3.02	0.12	0.14	3.29	0.23	0.22	3.50	0.31	0.34	5.41	0.91	0.95
3	3.20	0.16	0.21	3.58	0.34	0.33	4.00	0.49	0.53	7.41	1.61	1.54
4	3.41	0.24	0.31	4.08	0.53	0.50	4.58	0.71	0.81	9.50	2.35	2.51
5	3.95	0.43	0.46	4.62	0.73	0.76	5.50	1.06	1.25	13.91	3.91	4.07
6	4.50	0.63	0.68	5.75	1.15	1.15	7.37	1.76	1.94	21.29	6.51	6.61
7	5.12	0.86	1.00	7.70	1.89	1.74						
8	6.75	1.45	1.49	9.45	2.54	2.63						
9												
10												
11												
12												
V_M		2.73			2.73			2.73			2.73	

oligomers, with relatively short elution times of the ethoxymers up to $n = 14$–18 (Fig. 13). If gradient elution is applied in this chromatographic system, approximately 25 oligomers can be separated in 1 h (Fig. 14). The separation achieved on Silasorb SPH Amine was better than that on the other column packings tested, which can be explained as follows. As the adsorption energy and activity of the adsorbent surface are lower on the amino-bonded phase than on silica gel, so are the partial constants a_1 and m_1 in eqn. 1, which determine the selectivity of separation between neighbouring oligomers. Hence, the rate of increase in the capacity factors with increasing number of oligomeric units is lower on the amino-bonded phase and a greater number of oligomers can be separated in reasonable time than on unmodified silica. The diol- and nitrile-bonded phases are similar to the amino bonded phase as far as the separation of lower oligomers is concerned, but unfortunately the possibilities of separating higher oligomers are impaired by the irregular retention behaviour discussed above. Previous workers using nitrile- or diol-bonded phases usually added water, acetic acid or methanol to the mobile phase to achieve a good separation, but such systems are more difficult to understand and describe because of the mixed separation mechanism (partition–adsorption). Although the separation efficiency in these systems is similar to those in non-aqueous systems employing amino-bonded phases, in our experience the latter chromatographic system increases the number of oligomers that can be separated or suppresses band tailing and makes it possible to achieve more reproducible quantitative results.

$\varphi = 1$		$\varphi = 0.8$		$\varphi = 0.7$		$\varphi = 0.6$		$\varphi = 0.4$	
V_R	$k'(e)$	V_R	$k'(e)$	V_R	$k'(e)$	V_R	$k'(e)$	V_R	$k'(e)$
		2.56	0.08	2.56	0.08	2.55	0.08	2.67	0.13
2.62	0.10	2.67	0.12	2.69	0.14	2.67	0.13	2.83	0.20
3.16	0.33	3.30	0.39	3.38	0.42	3.53	0.49	3.94	0.66
4.65	0.96	5.46	1.30	6.05	1.55	6.85	1.89	8.51	2.59
6.08	1.56	7.75	2.27	9.34	2.94	11.44	3.83	16.65	6.02
7.72	2.26	10.45	3.41	13.66	4.76	18.38	6.76	29.75	11.55
8.67	2.66	11.98	4.06	17.36	6.32	24.55	9.36		
9.50	3.01	13.34	4.63	20.43	7.62	29.77	11.56		
9.99	3.21	14.71	5.10	21.87	8.23	33.84	13.28		
10.51	3.44	15.99	5.75			38.69	15.32		
11.78	3.97	18.64	6.87			46.16	18.48		
13.83	4.84	23.20	8.79						
	2.37		2.37		2.37		2.37		2.37

TABLE VII

EXPERIMENTAL RETENTION VOLUMES (V_R, cm^3) AND CAPACITY FACTORS [k'(e)] OF THE INDIVIDUAL OLIGOMERS WITH n OLIGOMERIC UNITS ON SILASORB SPH AMINE, 7.5 μm (COLUMN, 300 × 4.2 mm I.D.), IN MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS [φ, % (v/v) · 10^{-2}] OF 2-PROPANOL IN n-HEPTANE

Sample: Serdox NNP 4. Column dead volume (V_M) = 3.50 cm^3. k'(c) are the capacity factors predicted by calculation using the equation $\log k' = -1.78 + 0.16n - (1.31 + 0.01n) \log \varphi$.

n	$\varphi = 0.735$			$\varphi = 0.5$			$\varphi = 0.4$			$\varphi = 0.3$			$\varphi = 0.2$			$\varphi = 0.1$		
	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$	V_R	$k'(e)$	$k'(c)$
1										3.91	0.11	0.12	4.24	0.21	0.20	5.33	0.52	0.50
2	3.66	0.04	0.05	3.83	0.09	0.09	3.91	0.11	0.12	4.08	0.16	0.18	4.57	0.30	0.30	6.00	0.71	0.74
3	3.75	0.07	0.08	4.00	0.14	0.13	4.08	0.16	0.18	4.33	0.23	0.26	4.94	0.41	0.44	7.16	1.04	1.09
4	3.91	0.11	0.12	4.16	0.19	0.20	4.33	0.23	0.26	4.79	0.36	0.38	5.65	0.61	0.65	9.12	1.60	1.62
5	4.08	0.16	0.17	4.50	0.28	0.29	4.83	0.38	0.39	5.54	0.58	0.57	6.86	0.96	0.97	13.08	2.73	2.40
6	4.50	0.28	0.26	5.16	0.47	0.43	5.66	0.61	0.58	7.00	1.00	0.84	9.23	1.63	1.43	21.08	5.02	3.55
7	4.75	0.35	0.38	5.83	0.66	0.64	6.50	0.85	0.85	8.45	1.41	1.24	11.80	2.37	2.12	31.50	8.00	5.25
8	5.08	0.45	0.57	6.50	0.85	0.94	7.41	1.11	1.26	10.08	1.88	1.84	14.92	3.26	3.13			
9	5.41	0.54	0.84	7.25	1.07	1.39	8.45	1.41	1.87	12.04	2.44	2.72	18.87	4.39	4.63			

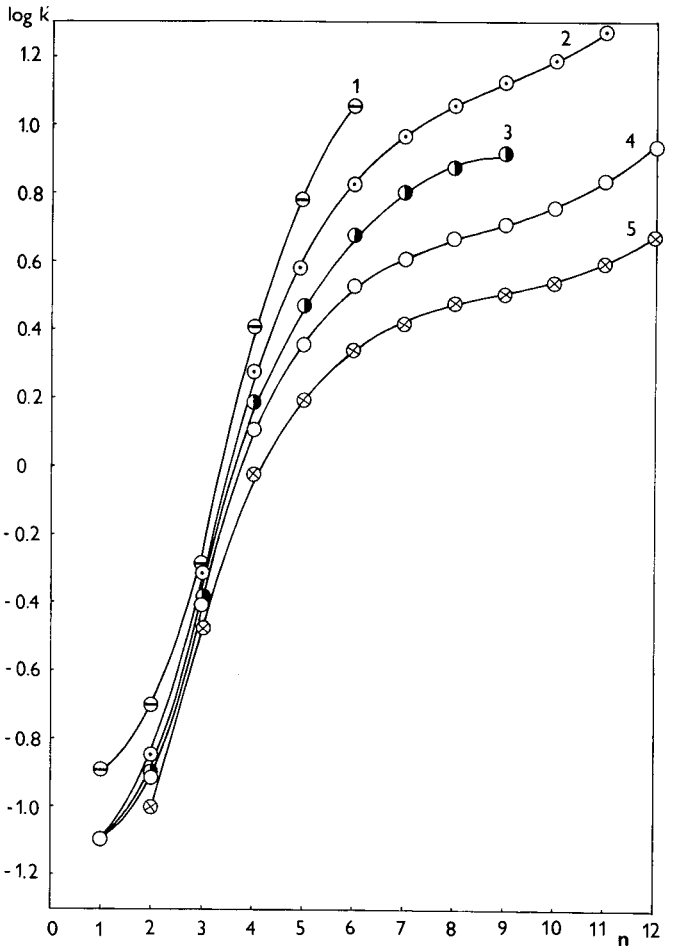

Fig. 11. Dependence of retention (k') of the individual ethoxymers in the sample of Serdox NNP 8 on the number of oxyethylene units, n, on a Silasorb SPH Diol, 7.5 μm, column (250 × 4 mm I.D.). Mobile phase with different ratios of 1-propanol to n-heptane: (1) 40:60; (2) 60:40; (3) 70:30; (4) 80:20; (5) 100:0.

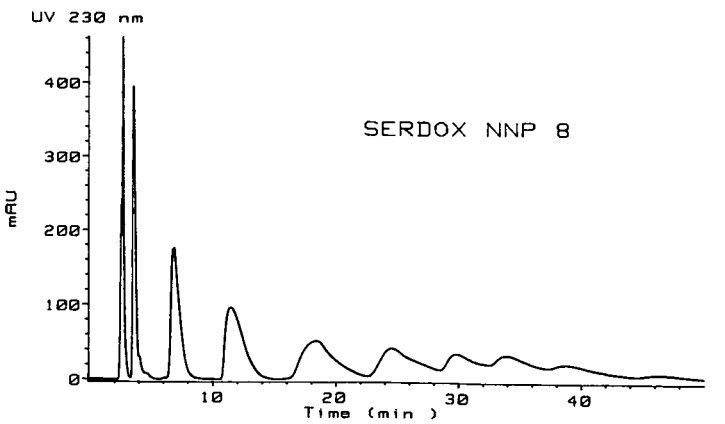

Fig. 12. Isocratic separation of Serdox NNP 8 on a Silasorb SPH Diol, 7.5 μm, column (250 × 4 mm I.D.) with 1-propanol–n-heptane (60:40). Other conditions as in Fig. 3.

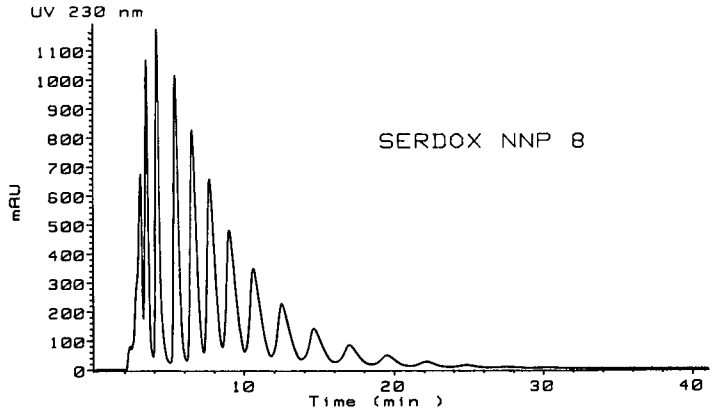

Fig. 13. Isocratic separation of Serdox NNP 8 on a Silasorb SPH Amine, 7.5 μm, column (300 × 4.2 mm I.D.) with 2-propanol–*n*-heptane (25:75). Other conditions as in Fig. 3.

Dependence of results of quantification on separation conditions

The results of the distribution of the individual ethoxymers in a given product determined by the evaluation of chromatographic data can be subject to a systematic error, depending on the separation conditions. Sources of errors in quantitative determinations are imperfect resolution of low ethoxymers and band tailing in some chromatographic systems, which may lead to errors in the integration of the peak areas and to underestimation of the concentrations of higher ethoxymers. The latter errors should increase with decreasing concentration of polar solvent(s) in the organic mobile phase, as the tailing usually increases with increasing elution volume in inadequately selected chromatographic systems.

Chromatographic systems with Silasorb Nitrile and Silasorb Diol as the stationary phases yield irregular spacing of the peaks of ethoxymers and only 8–12 ethoxymers could be resolved in a reasonable time. For this reason, these systems were

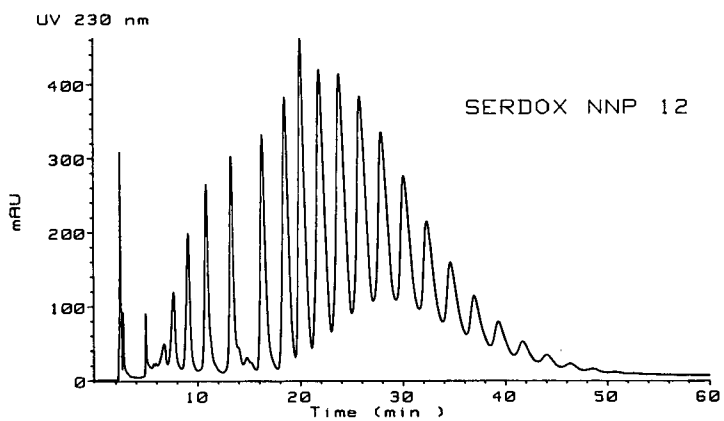

Fig. 14. Gradient elution separation of Serdox NNP 12 on a Silasorb SPH Amine column using linear gradient from 100% *n*-heptane to 2-propanol–*n*-heptane (90:10) in 60 min. Column as in Fig. 13; other conditions as in Fig. 3.

omitted from the quantification experiments. Systems with silica gel and propanol–heptane mobile phases were also omitted, as they were poorer in this respect than systems with ethanol–heptane or propanol–ethanol–heptane mobile phases.

As pure ethoxymer standards were not available, theoretical response factors were considered, based on a constant value of the molar absorption coefficients of ethoxylated nonylphenols, independent of the number of oxyethylene units. Ahel and Giger[20] found previously that such theoretical response factors were very close to the experimental values for ethoxylated alkylphenols. Serdox NNP 8, with eight nominal oxyethylene units, was used as the test sample in quantitation experiments and the response factors were related to the response factor of the oligomer with five oxyethylene units. The results of the quantitative evaluation of the ethoxymer distribution were expressed relative to the oligomer with eight oxyethylene units, rather than to the sum of all the ethoxymers in the sample, as in some experiments a significant proportion of higher ethoxymers could not be eluted. The results are given in Table VIII.

TABLE VIII

RESULTS OF THE DETERMINATION OF DISTRIBUTION OF THE INDIVIDUAL ETHOXYMERS IN SERDOX NNP 8 USING VARIOUS NORMAL-PHASE HPLC SYSTEMS

The results are expressed as mass % calculated from peak areas relative to the oligomer with eight oxyethylene units, considering the theoretical (stoichiometric) response factors, R, related to the oligomer with five oxyethylene units ($R_5 = 1$). Columns: (I) Silasorb 600 SPH, 5 μm, 300 × 3.8 mm I.D.; (II) Silasorb SPH Amine, 7.5 μm, 300 × 4.2 mm I.D. Mobile phases: (1) ethanol–*n*-heptane (70:30); (2) ethanol–*n*-heptane (30:70); (3) ethanol–1-propanol–*n*-heptane (20:20:60); (4) gradient from ethanol–1-propanol–*n*-heptane (8:24:68) to ethanol–1-propanol (75:25) in 30 min; (5) 2-propanol–*n*-heptane (25:75); (6) gradient from 0 to 90% 2-propanol in *n*-heptane in 60 min. n = Number of oxyethylene units in the individual ethoxymers.

n	R	*Mass %*					
		I,1	*I,2*	*I,3*	*I,4*	*II,5*	*II,6*
1	0.6		1.8	1.9	1.6		1.4
2	0.7		6.8	8.8	6.8	2.7	6.8
3	0.8	27.9	22.2	25.2	22.4	38.0	21.2
4	0.9	49.2	44.9	48.5	44.9	47.4	42.7
5	1.0	66.7	64.3	67.3	64.4	68.0	62.3
6	1.1	81.5	81.0	85.9	80.1	80.2	80.1
7	1.2	91.4	94.4	97.9	93.5	93.5	92.5
8	1.3	100.0	100.0	100.0	100.0	100.0	100.0
9	1.4	98.7	95.4	82.9	100.7	98.2	100.4
10	1.5	88.8	81.2		84.0	86.8	90.3
11	1.6	71.9	57.7		57.1	70.2	73.3
12	1.7	52.1	29.8		38.8	52.6	55.6
13	1.8	27.4				36.9	38.5
14	1.9					24.2	24.6
15	2.0					14.8	14.6
16	2.1					8.6	8.1
17	2.2					4.6	4.4
18	2.3					2.0	2.4
19	2.4						1.2
20	2.5						0.7
21	2.6						0.4
22	2.7						0.2

On a silica gel column, the first three ethoxymers are eluted in a common peak with ethanol–*n*-heptane (70:30) as the mobile phase, but the peak areas of ethoxymers with $n = 4$–12 are in approximate agreement with the results achieved on Silasorb SPH Amine. With 30% ethanol, the peak areas of ethoxymers with up to nine oxyethylene units and in the ternary mobile phase ethanol–1-propanol–*n*-heptane (20:20:60) those for the oligomers with up to eight oxyethylene units agree approximately with the results achieved on the amine column, but the peak areas of higher ethoxymers are significantly lower on the silica gel column. Ternary gradient elution on Silasorb 600 SPH yields a distribution in approximate agreement with the results on the amine column for ethoxymers with up to ten oligomeric units. With the Silasorb Amine column, approximately identical results were obtained for the ethoxymers with 5–8

TABLE IX

MASS DISTRIBUTION OF THE INDIVIDUAL ETHOXYMERS IN SERDOX NNP 4, NNP 8, NNP 12 AND NNP 20 WITH VARIOUS STOICHIOMETRIC RATIOS OF ETHYLENE OXIDE TO NONYLPHENOL

Results are given as mass % in sample calculated from peak areas with stoichiometric response factors (Table VIII). Column, Silasorb SPH Amine, 7.5 μm, 300 × 4.2 mm I.D.; linear gradient from 0 to 90% 2-propanol in *n*-heptane in 60 min; flow-rate, 1.0 cm^3/min. n = Number of oxyethylene units in the individual ethoxymers.

n	*Mass %*			
	NNP 4	*NNP 8*	*NNP 12*	*NNP 20*
1	3.12	0.17	0.17	0.07
2	12.86	0.83	0.53	0.10
3	17.11	2.58	1.01	0.14
4	18.49	5.20	1.61	0.19
5	16.16	7.58	2.08	0.18
6	11.89	9.74	3.16	0.10
7	8.17	11.26	4.68	0.25
8	5.16	12.17	6.58	0.37
9	3.19	12.22	8.45	0.60
10	1.81	10.99	10.03	0.96
11	1.09	8.93	11.10	1.54
12	0.52	6.77	11.32	2.37
13	0.28	4.68	10.37	3.49
14	0.14	2.99	8.72	4.93
15		1.78	6.90	6.32
16		0.99	5.14	7.65
17		0.54	3.58	8.84
18		0.29	2.31	9.82
19		0.15	1.35	9.85
20		0.08	0.68	8.61
21		0.04	0.23	7.06
22		0.01		5.74
23				4.77
24				3.98
25				3.21
26				2.86
$\Sigma > 26$				6.00

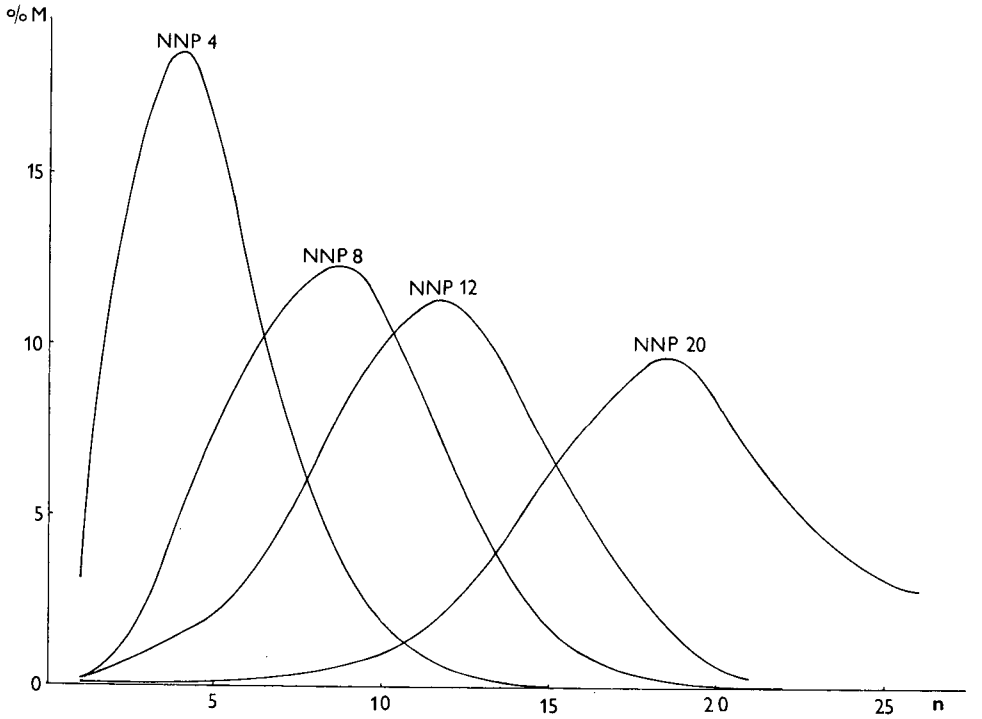

Fig. 15. Percentage mass distribution (%M) of the individual ethoxymers with n oxyethylene units in Serdox NNP 4, NNP 8, NNP 12 and NNP 20 determined with conditions as in Table IX.

oxyethylene units using either isocratic elution with 2-propanol–n-heptane (25:75) or gradient elution from 0 to 90% 2-propanol in n-heptane. The contents of the ethoxymers with $n>9$ were higher than those found with the Silasorb 600 SPH column, probably because of a considerably smaller peak tailing observed with the amino-bonded phase. With 25% propanol, the contents of the first three ethoxymers are not precise because of imperfect resolution of the individual compounds.

In conclusion, silica gel Silasorb 600 SPH can be used reliably only for the determination of the ethoxymer distribution up to n = 10, with an ethanol–propanol–n-heptane ternary gradient to obtain the best results with this stationary phase. If the distribution of the individual ethoxymers with 1 to 20–25 oxyethylene units is to be determined, an amino-bonded phase with a gradient of propanol in n-heptane offers the best results of all the systems tested. Table IX and Fig. 15 show the mass distributions of ethoxymers in samples with various stoichiometric ratios of ethylene oxide to nonylphenol (4, 8, 12 and 20:1).

CONCLUSIONS

In normal-phase HPLC with mobile phases consisting of dried aliphatic alcohols and n-alkanes, silica gel and an amino-bonded phase show regular retention behaviour, which can be predicted by calculation using the approach introduced

recently[22]. On the other hand non-linear log k' *versus* n plots are observed with diol- and nitrile-bonded phases using dried organic mobile phases, which indicates a mixed retention mechanism. Moreover, the peaks of the higher ethoxymers show significant tailing with the latter two bonded phases and with silica gel and only a limited number of ethoxymers can be resolved in a reasonable time. The results of integration depend on the mobile phase composition and, consequently, the diol- and nitrile-bonded phases are not suitable for the reliable determination of ethoxymer distributions using dried organic mobile phases. More or less successful separations of ethoxymers using the latter bonded phases have been reported recently using a strongly polar additive in the organic mobile phase, such as water [5,12], acetic acid[18] or 2-methoxyethanol[14]; however, a mixed retention mechanism controls the chromatographic behaviour in such systems, which makes it difficult to predict the elution volumes of ethoxymers in these systems and to compare different chromatographic systems.

Band tailing on silica gel columns in ethanol-containing mobile phases is less than in mobile phases containing propanol and ethoxymers with up to ten oligomeric units can be separated readily using gradient elution with ternary mobile phases consisting of ethanol, propanol and an *n*-alkane. The most reliable results can be achieved with the amino-bonded phase, where ethoxymers with up to 20–25 oxyethylene units can be separated in 1 h using gradient elution with an increasing concentration of propanol in an *n*-alkane, without the necessity to add water to the mobile phase, as has been suggested earlier[6,15,16,19,20].

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High-performance liquid chromatographic separation of biomolecules using calcium phosphate supported on macroporous silica microparticles

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SUMMARY

The preparation of a chromatographic support showing similar selectivity and chemical inertness to hydroxyapatite (HA) and mechanical resistance to the pressures generally used in high-performance liquid chromatography is described. A mixed matrix was formed by covering macroporous silica microparticles with a thin layer of calcium phosphate (CaP–HA). The porous (500, 1000 and 4000 Å) silica microparticles (5 and 10 μm) had been previously inactivated with glycidoxypropyltrimethoxysilane in acidic medium, to convert the silanol groups into hydrophilic groups, producing a biocompatible support with excellent properties in terms of mechanical resistance to high pressure (5000 p.s.i.), selectivity, chemical inertness and efficiency. Columns packed with small particles (5 μm) were run at high pressure and high flow-rates for prolonged periods. Mixed silica WP-DIOL/CaP–HA matrices were used to separate a standard protein mixture antimelanoma monoclonal antibody and some small molecules such as carnitine derivatives and sugars.

INTRODUCTION

Hydroxyapatite [HA; $Ca_{10}(PO_4)_6(OH)_2$] has long been used for the separation and purification of biomolecules. It is chemically inert, stable over a wide pH range (5.5–10.0), heat resistant and allows for a high recovery of biomolecules with unaltered physico-chemical properties.

Tiselius *et al.*[1] first used columns packed with HA for the liquid chromatography of proteins; the method has also been successfully utilized to separate nucleic acids such as native and denatured DNA, linear and circular DNA and RNA[3]. Studies on the structure, mode of action and applicability of HA by Bernardi and

co-workers[3–5] and others[6–9] have made this adsorbent especially popular. However, it is well known that HA has some serious drawbacks; in particular, its fragility often precludes the use of high flow-rates and high pressures such as are realized in high-performance liquid chromatography (HPLC) systems. Better results in terms of mechanical resistance and permeability have been achieved only recently by several workers[10–17] and new HPLC supports based on irregular microparticles or spherical ceramic aggregates of microcrystals of HA are now commercially available.

In order to rectify the above problems, we studied the possibility of preparing a support showing the chromatographic selectivity and the chemical inertness of HA and, at the same time, satisfactory mechanical resistance to the high pressures generally used in HPLC. For this purpose, we prepared a mixed matrix by covering spherical macroporous (500, 1000 and 4000 Å) silica microparticles (5 and 10 μm) with a thin calcium phosphate (CaP)–HA layer. In order to reduce the chemical reactivity of the support, the siliceous matrix was previously treated with glycidoxypropyltrimethoxysilane to convert the reactive silanol groups into diol groups to produce a biocompatible support[18] (Fig. 1, first step).

The mixed supports we synthesized showed excellent mechanical resistance, selectivity, chemical inertness and efficiency.

In this paper the preparation of these supports is reported. Their physico-chemical properties are described in addition to their chromatographic performance with low-molecular-weight organic molecules and with proteins.

EXPERIMENTAL

Equipment

Fourier transform infrared (FT-IR) spectra were recorded as KBr pellets on a Nicolet 20SX FT-IR spectrometer. Analytical liquid chromatography was performed on a Waters Assoc. (Milford, MA, U.S.A.) chromatograph equipped with a U6K universal injector, two M510 solvent-delivery systems, a temperature control module (TMC) and an M490 programmable multi-wavelength detector. Chromatographic data were collected and processed on a Waters 840 data and chromatography control station. Analytical data (% C, H, Ca, P) were obtained from Mikroanalytisches Laboratorium, Dr. H. Pascher (Bonn, F.R.G.). Thermogravimetric data were obtained by means of a Mettler TA3000 system consisting of a Mettler TG50 thermogravimeter, TC10A microprocessor and GRAPHWARE TA70.1 data station. Micrographs were obtained with ISI-Akashi Model S × 40A electron microscope system.

Reagents

Nucleosil silica gels were obtained from Macherey, Nagel & Co. (Düren, F.R.G.). All chemicals were purchased from Carlo Erba (Monterotondo, Italy), except 3-glycidoxypropyltrimethoxysilane (GOPTMS), from Janssen (Beerse, Belgium). Transferrin (bovine), myoglobin (from horse skeleton muscle), lysozyme (from chicken egg white) and cytochrome *c* (from beef heart) were obtained from Sigma (St. Louis, MO, U.S.A.). Anti-melanoma monoclonal antibody (IgG class) was kindly supplied by Prof. Soldano Ferrone (Department of Microbiology and Immunology, New York Medical College, Valhalla, NY, U.S.A).

Preparation of wide-pore glycidoxypropylsilica gel (WP-GPSG)

Nucleosil 1000-5 silica gel (3 g) was suspended in toluene (65 ml) and the suspension boiled under argon at 110°C for about 2 h to remove traces of water by azeotropic distillation. Glycidoxypropyltrimethoxysilane (1.5 ml, 6.8 mmol) was then added; the mixture was boiled for 3 h under the same conditions until about 10 ml of distillate had been collected. The suspension was cooled, filtered and washed successively with 20 ml each of toluene, methanol, methanol–water (50:50), methanol and acetone. The wide-pore glycidoxypropylsilica gel (WP-GPSG-1000-5) obtained was dried under vacuum (C, 1.10%; H, 0.18%).

Preparation of wide-pore silica gel DIOL (WP-DIOL)

WP-GPSG 1000-5 (1 g) was suspended in water (20 ml) and the pH was adjusted to 3.5 with 1 *M* sulphuric acid. The suspension was heated at 90°C for 2 h, then filtered and washed successively with 20 ml each of water, methanol and dichloromethane. The wide-pore silica gel DIOL (WP-DIOL 1000-5) obtained was dried under vacuum (C, 1.03%; H, 0.17%).

Preparation of supported calcium phosphate–HA (CaP–HA) on WP spherical microparticles of silica gel DIOL (WP-DIOL)

Procedure A (matrices I and II). An amount of $CaCl_2 \cdot 2H_2O$ [87.8 mg (0.5976 mmol) for CaP–HA 10%; 43.9 mg (0.2988 mmol) for CaP–HA 5%; 21.9 mg (0.1494 mmol) for CaP–HA 2.5%] was dissolved in water (30 ml). WP-DIOL (1 g) was suspended in this solution. Water was removed by distillation *in vacuo* (80°C, 14 mmHg). The product was washed with methanol and dried under vacuum (0.1 mmHg). The dried powder was slowly added to a volume of 0.5 *M* sodium phosphate[a] of pH 6.8 (3.42 ml for CaP–HA 10%; 1.71 ml for CaP–HA 5%; 0.85 ml for CaP–HA 2.5%) under sonication at room temperature. The suspension (brushite-WP-DIOL) was filtered, washed with water, resuspended in a saturated solution of $Ca(OH)_2$[b] (100 ml) and boiled for 30 min [always checking the $Ca(OH)_2$ consumption with methanolic phenolphthalein) to convert brushite into CaP–HA[19]. The suspension was cooled, filtered and washed with water until the pH paper was neutral; it was then washed successively with 20 ml each of methanol and dichloromethane and dried (80°C, 0.1 mmHg).

Procedure B_1 (matrices III–VII). WP-DIOL (1 g) was suspended in water (30 ml). Equal volumes of 0.5 *M* sodium phosphate (pH 6.8) and 0.5 *M* $CaCl_2 \cdot 2H_2O$ (1.71 ml for CaP–HA 5%; 0.85 ml for CaP–HA 2.5%) were added dropwise to the suspension, always with stirring at 60°C. The precipitate (brushite-WP-DIOL) was filtered, washed with water and suspended in water (50 ml) together with a few drops of methanolic phenolphthalein; saturated $Ca(OH)_2$ was added dropwise until the suspension turned pink (pH $\geqslant$8.5). The suspension was boiled for 30 min, with

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[a] 0.5 *M* sodium phosphate buffer (pH 6.8) was prepared by mixing 0.5 *M* $NaH_2PO_4 \cdot H_2O$ and 0.5 *M* $Na_2HPO_4 \cdot 2H_2O$ until the desired pH was achieved.

[b] Saturated solutions of $Ca(OH)_2$ were prepared by boiling $Ca(OH)_2$ in deaerated water (helium) and filtering under argon to prevent precipitation of calcium carbonate. Similarly, treatment of brushite with boiling saturated $Ca(OH)_2$ was performed under an argon atmosphere.

TABLE I

KINETIC PERFORMANCES OBTAINED USING DIFFERENT SILICEOUS MATRICES, DIFFERENT Ca PERCENTAGES AND DIFFERENT PREPARATIVE PROCEDURES FOR CaP–HA

Matrix		*Ca(%, w/w) (Ca/P)*[a]	*Preparative procedure*	*Efficiency (plates/m)*[b]	*φ*	*Column dimensions, length × I.D. (mm)*
No.	*Type, particle size, shape*					
–	HPHA (10 μm, irregular)	–	Commercial	8000	3000	30 × 4.6
I	Nucleosil 1000-DIOL (5 μm spherical)	4.50 (1.3)	A	20 000	4000	50 × 4.0
II	Nucleosil 1000-DIOL (5 μm spherical)	2.30 (1.3)	A	26 000	2400	50 × 4.0
III	Nucleosil 500-DIOL (5 μm spherical)	2.33 (1.55)	B_1	25 000	1300	50 × 4.0
IV	Nucleosil 1000-DIOL (5 μm spherical)	2.0 (1.65)	B_1	30 000	2500	50 × 4.0
V	Nucleosil 1000-DIOL (5 μm spherical)	0.98 (1.58)	B_1	50 000	1500	100 × 6.0
		0.98 (1.58)	B_1	55 000	1300	50 × 4.0
VI	Nucleosil 1000-DIOL (10 μm spherical)	0.99 (1.54)	B_1	30 000	2000	100 × 6.0
VII	Nucleosil 4000-DIOL (5 μm spherical)	2.10 (1.55)	B_1	19 700	620	50 × 4.0
VIII	Nucleosil 1000-DIOL (5 μm spherical)	0.99 (1.3)	B_2	24 000	1200	50 × 4.0
IX	Nucleosil 1000-DIOL (5 μm spherical)	2.0 (1.3)	B_2	22 000	2600	50 × 4.0

[a] Calcium/phosphate molar ratio.
[b] See *Chromatographic performance* for the definition of the reported symbols.

dropwise addition of $Ca(OH)_2$ as necessary to maintain the pink coloration. The suspension was then cooled, filtered and washed as described in procedure A.

Procedure B_2 (matrices VIII and IX). WP-DIOL (1 g) was suspended in 0.5 *M* NaCl (15 ml). Equal volumes of 0.5 *M* $Na_2HPO_4 \cdot 2H_2O$ (pH 9.2) and 0.5 *M* $CaCl_2 \cdot 2H_2O$ (0.69 ml for CaP–HA 5%; 0.35 ml for CaP–HA 2.5%) were added dropwise to the suspension, stirring at room temperature. The precipitate was filtered, washed with water and resuspended in water (50 ml) containing a few drops of methanolic phenolphthalein. Saturated $Ca(OH)_2$ was added dropwise until the suspension turned pink (pH 8.5). The suspension was boiled for 30 min, always adding $Ca(OH)_2$ as required to keep the pink coloration; it was then cooled, filtered and washed as described in procedure A.

Chemical and physical characterization of mixed silica WP-DIOL/CaP–HA matrix

Definitions of the components of "mixed silica WP-DIOL/CaP–HA matrix" are functionalized macroporous silica = WP-DIOL and CaP–HA = calcium phosphate as calcium-deficient hydroxyapatite. The Ca/P molar ratio is always less than the theoretical value of pure HA (1.67) (see Table I).

Microanalytical data (Ca, P) of mixed silica WP-DIOL/CaP–HA matrices are reported in Table I; FI-IR spectra (KBr pellets) show bands at 604 and 562 cm^{-1} identical with those found in an authentic sample of HA.

Column packing

Columns (50 × 4.0 mm or 100 × 10 mm I.D.) made of stainless steel with titanium frits were packed using the slurry-packing procedure. Mixed silica WP-DIOL/CaP–HA matrices (0.7 or 4.0 g) were dispersed in 0.35 *M* sodium phosphate buffer (pH 6.8) (30 ml for the small column, 50 ml for the large column) and then sonicated for 5 min. The resulting slurry was packed with a Haskel Model DSTV-122 pump using water as pressurizing agent.

Chromatographic performance

Efficiency test. Mixed silica WP-DIOL/CaP–HA supports were tested using a mixture of benzene, methyl benzoate, nitrobenzene and 1,3-dinitrobenzene [eluent, *n*-hexane–chloroform (90:10, v/v); flow-rate, 1 ml min]. Only the last peak (1,3-dinitrobenzene) of the chromatogram was considered for the calculation of the number of theoretical plates.

The column dead volume (V_0) was determined from the elution time of an unretained marker (benzene; eluent, dichloromethane). Dimensionless parameters such as reduced plate height (h), flow resistance parameter (Φ) and separation impedance (E) were calculated according to Bristow and Knox[20]. Diffusion coefficients of solutes in the mobile phase were determined using the empirical Wilke–Chang equation[20]. The results are reported in Table I.

Applications. Protein solutions (10–50 μl; 1 μg μl/protein) were loaded onto columns and eluted with a linear gradient of sodium phosphate (pH 6.8) (1–350 m*M*). Different flow-rates and different run times were tried in order to optimize the resolution of a standard protein mixture; the effluent was monitored at 280 nm. Separation of some small polar molecules was also attempted (see Figs. 9 and 10) using acetonitrile–water (80:20, v/v) as the eluent; the effluents were monitored at 220 nm.

RESULTS AND DISCUSSION

The utility of HA for the chromatographic separation of biomolecules is dependent on the bioactivities of the biomolecules not being affected by adsorption to HA[3,9,21] Nevertheless, HA has some serious disadvantages: the fragile crystals break easily under mechanical load, especially if high-pressure micropumps are used. In order to improve its mechanical resistance without compromising its chromatographic performance, we produced a mixed matrix by covering functionalized silica microparticles with a thin layer of CaP–HA. The preparative procedure is presented in Fig. 1[22].

The siliceous matrix was inactivated by treatment with 3-glycidoxypropyltrimethoxysilane of the reactive silanolic groups followed by acid hydrolysis of the epoxide rings; the same result was also obtained following a single-step procedure[18] involving derivatization of the siliceous matrix with 3-glycidoxypropyltrimethoxylsilane in acidic medium. The principal difference between these two methods is that in the two-step method, described in this paper, polymerization reactions occurring during derivatization are avoided.

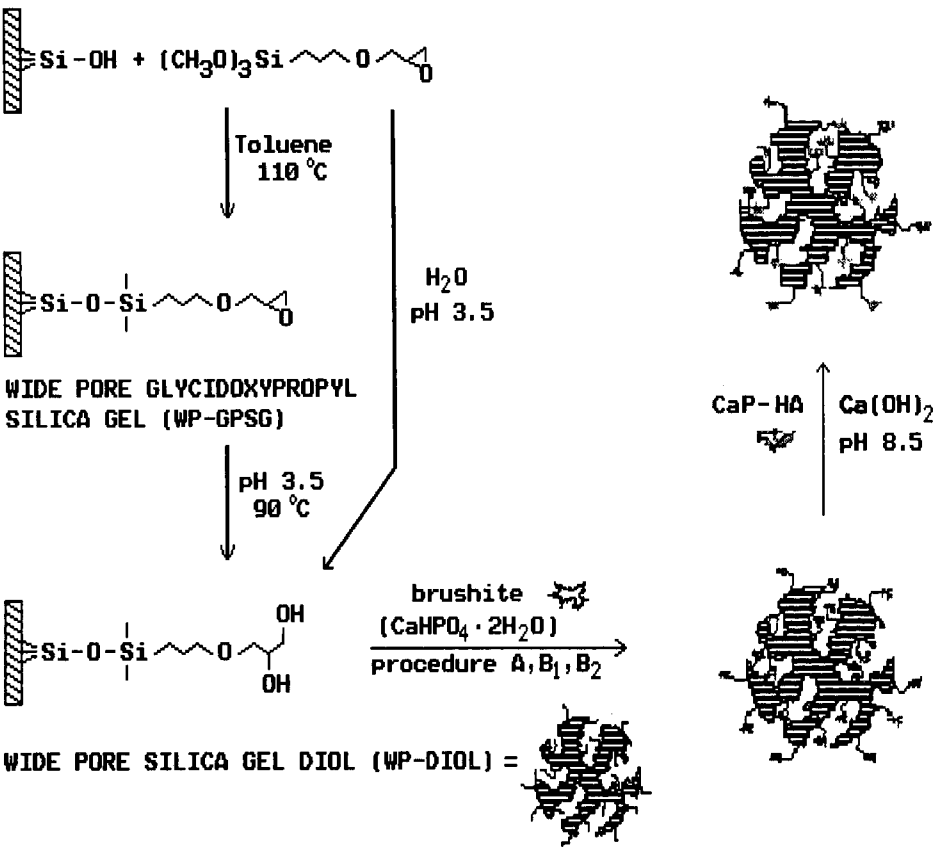

Fig. 1. Preparative procedure for calcium phosphate supported on functionalized macroporous silica microparticles (WP-DIOL/CaP–HA mixed matrices).

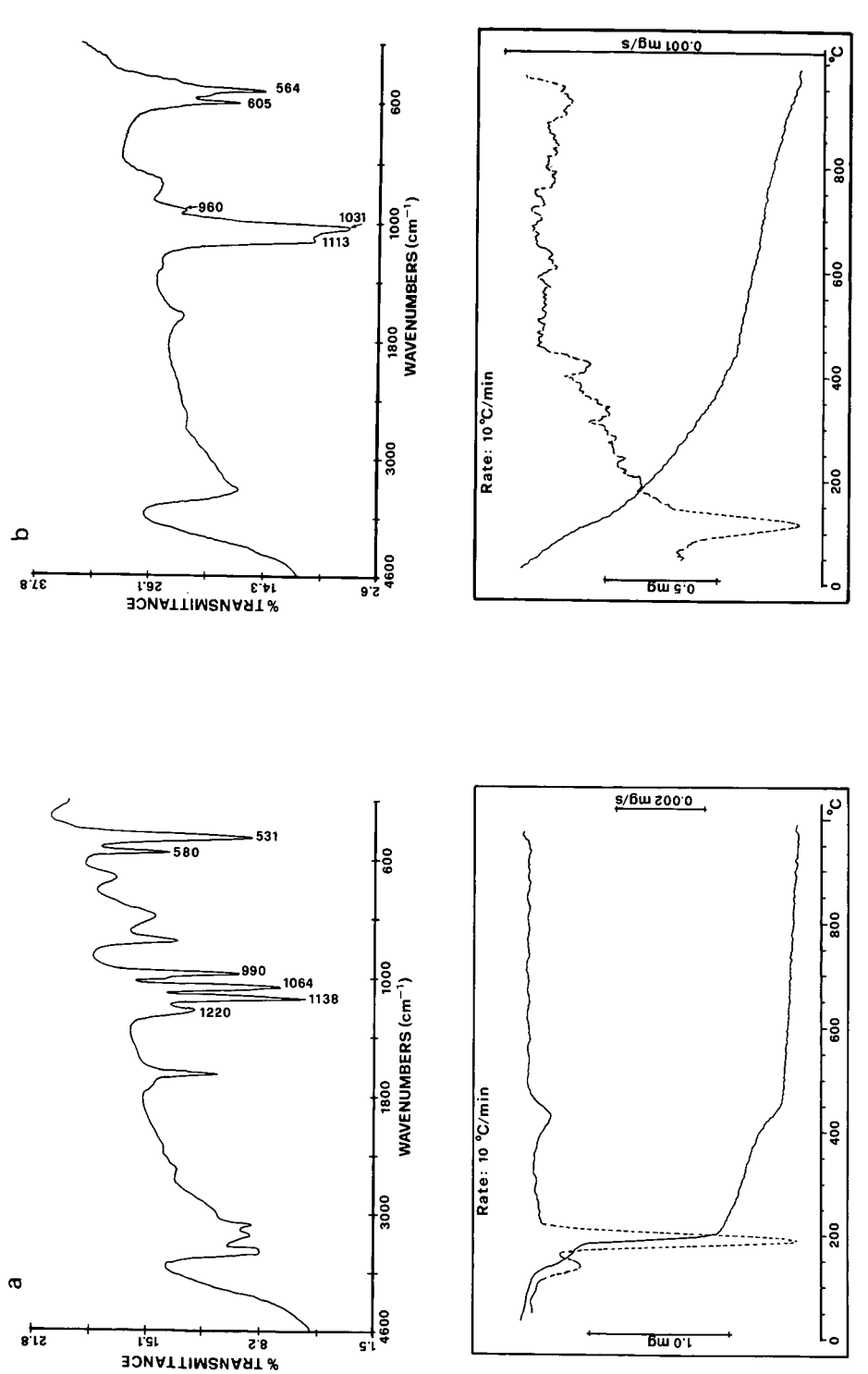

Fig. 2. FT-IR spectra and thermogravimetric analysis of (a) brushite obtained according to procedure B_1 up to 60°C and (b) octacalcium phosphate obtained according to procedure B_2 at 95°C.

For the deposition of CaP–HA two different procedures were adopted; in the first step of procedure A Ca^{2+} ions were adsorbed on the silica surface by evaporation *in vacuo* of a suspension of silica gel and a $CaCl_2$ solution of known concentration; a convenient volume of sodium phosphate solution was then added to convert Ca^{2+} ions into calcium phosphate crystals. In the first step of procedures B_1 and B_2, $CaHPO_4 \cdot 2H_2O$ (brushite) crystals were deposited on silica microparticles by slow addition of $CaCl_2$ and sodium phosphate solutions. Sodium phosphate buffers of different pH were tested (B_1, pH = 6.8; B_2, pH = 9.2).

Preliminary precipitation tests, performed at different temperatures in acidic and basic media (without silica microparticles), showed that in procedure B_1 brushite ($CaHPO_4 \cdot 2H_2O$) is obtained up to 60°C[6,17], whereas in procedure B_2, at temperatures higher than 60°C octacalcium phosphate [$Ca_8H_2(PO_4)_6 \cdot 5H_2O$] is formed[6,17]. These results are supported by FT-IR spectra[23–26] and by the thermogravimetric analyses reported in Fig. 2. These matrices were subsequently treated with $Ca(OH)_2$ to convert the brushite to hydroxyapatite[19].

Fig. 3 compares the FT-IR spectra of a mixed matrix (1000-5 DIOL + CaP–HA, matrix V) obtained according to procedure B_1, the glycolated siliceous matrix (Nucleosil 1000-5 DIOL) and a sample of commercial HA. Spectrum (b) and more clearly (c) (the latter obtained by subtracting (a) from (b) show typical absorption peaks at 604 and 562 cm^{-1} of commercial HA crystals. Hence the total conversion of brushite into CaP-HA follows from lack of IR absorption at 580 and 531 cm^{-1} and it is in agreement with the reported results[19] for a similar conversion of brushite into Ca-deficient HA by $Ca(OH)_2$.

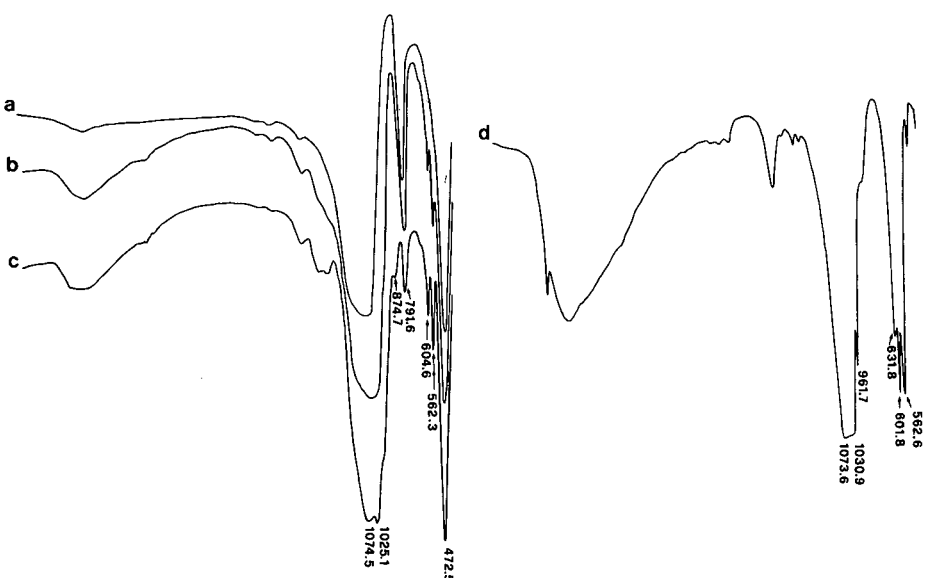

Fig. 3. FT-IR spectra: (a) control siliceous matrix Nucleosil 1000-5 DIOL; (b) Nucleosil 1000-5 DIOL CaP–HA (matrix V) mixed matrix obtained according to procedure B_1 (see Table I); (c) difference spectrum [(b)–(a)]; (d) irregular commercial HA.

Fig. 4 shows some micrographs (2000–5000 × enlargements) of a mixed matrix of 5 μm and 1000 Å porosity, obtained according to procedure B_1 (matrix V; see Table I). The macroporous structure of the support and the uniform distribution of CaP–HA are evident.

Spherical siliceous microparticles of different diameters and porosities were examined. Column kinetic performances were evaluated according to Bristow and Knox[20], and the results are reported in Table I.

It can be seen that CaP–HA supports prepared by procedure B_1 allowed us to adjust and optimize the chromatographic performance of the support in terms of efficiency, permeability and resolution of protein mixtures. The less satisfactory performance of supports prepared by procedure A might be due to the formation of unadsorbed crystals which are subsequently lost, causing a lower column efficiency and permeability.

In order to evaluate kinetic and thermodynamic performances for the separation of biomolecules, we prepared supports of different porosities (500, 1000 and 4000 Å) and particle sizes (5–10 μm); data obtained using different standard protein samples (see examples) showed that the 1000 Å support was the best, because it had a suitable porosity and high mechanical resistance (5000 p.s.i.), thereby ensuring a long column lifetime; moreover, a particle size of 5 μm in conjunction with a porosity of 1000 Å results in a high chromatographic efficiency. However, good efficiency and low column pressure can also be obtained with a particle size of 10 μm.

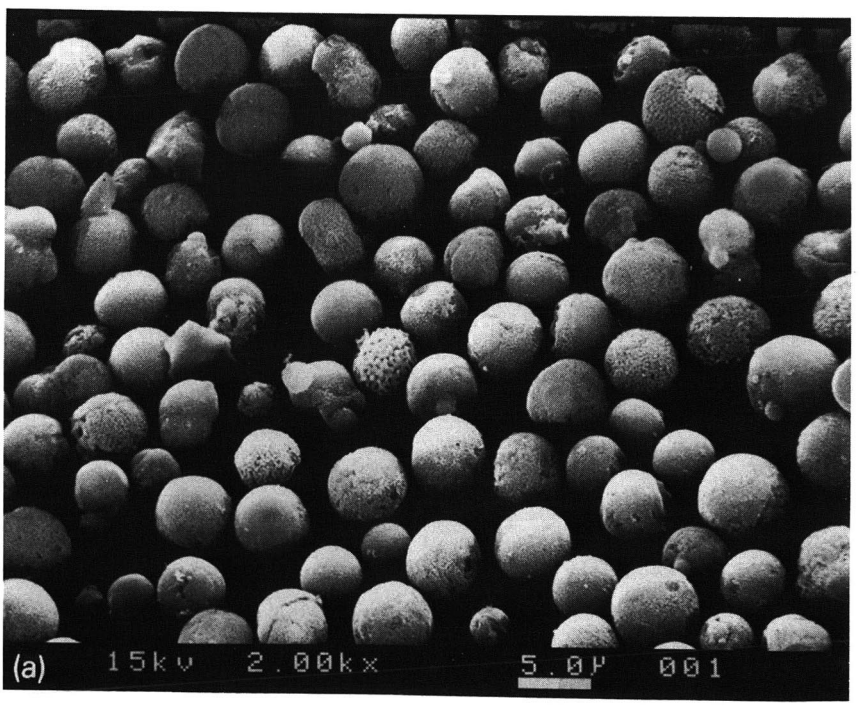

Fig. 4.

(Continued on p. 328)

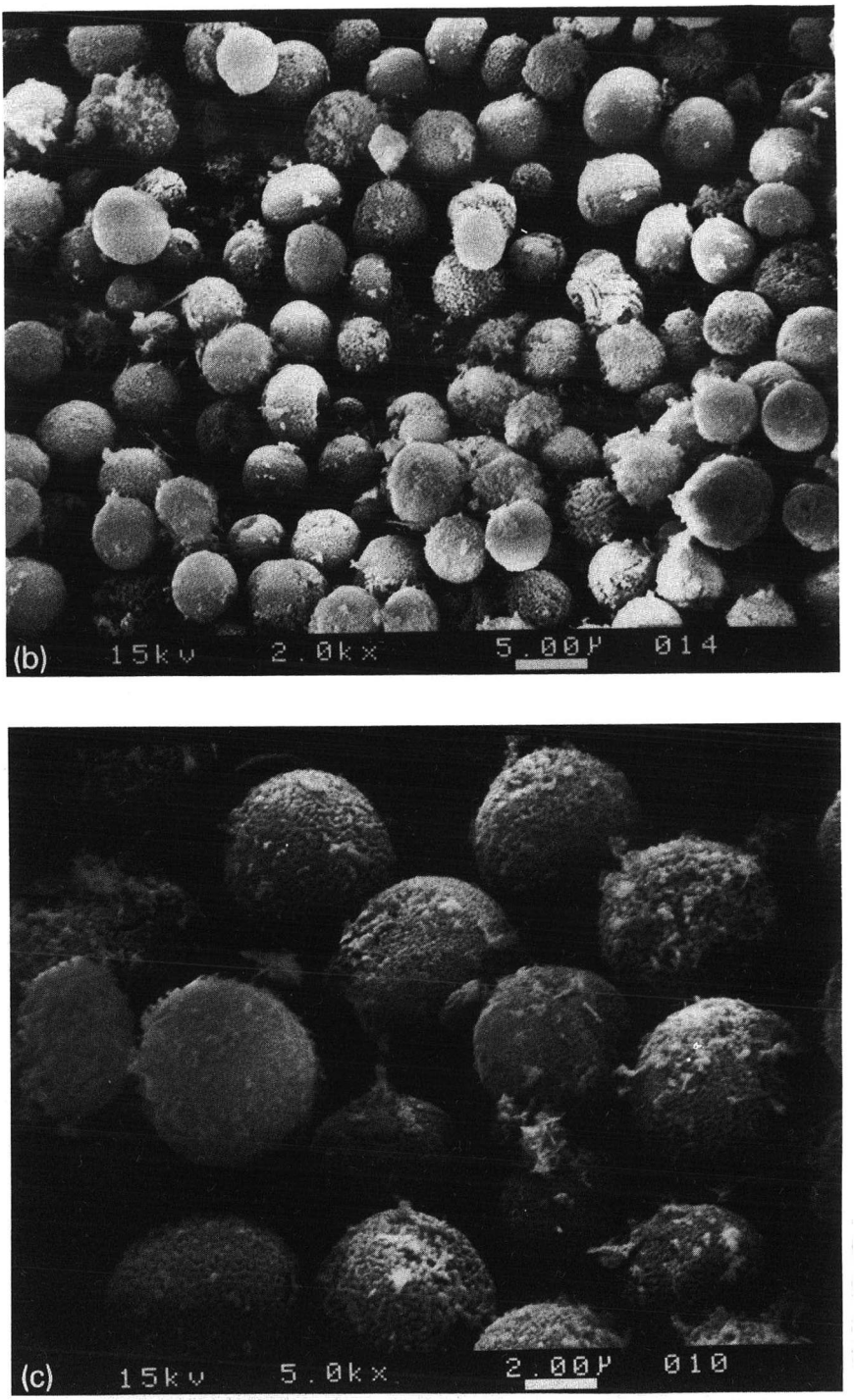

Fig. 4. Micrographs of some supports: (a) Nucleosil 1000-5 DIOL (1:2000); (b) and (c) Nucleosil 1000-5 DIOL CaP–HA (matrix V, see Table I) (1:2000 and 1:5000, respectively).

Applications

In order to investigate the separation properties of the mixed CaP–HA matrix columns, protein samples (single proteins and protein mixtures) were loaded onto the column and eluted with a linear phosphate gradient. The identity of each peak in a protein mixture was established by means of an internal standard, and/or by comparison of the capacity factors (k') of the peaks with those of the individual protein components.

We achieved separations of complex mixtures of proteins using CaP–HA mixed matrices; the small particles gave sharp peaks with minimum tailing at high flow-rates, allowing a fast analysis and more sensitive UV detection. The resolving power of the columns was demonstrated by separating a standard protein mixture containing transferrin, myoglobin, lysozyme and cytochrome *c* (Fig. 5). These chromatograms also illustrate the chromatographic behaviour of acidic and basic proteins: acidic proteins (transferrin, p*I* 5.5–5.9; myoglobin, p*I* = 7.2) are eluted at lower sodium phosphate concentrations than basic proteins (lysozyme, p*I* = 10.7; cytochrome *c*, p*I* 9.8–10.3). The interaction of acidic and basic moieties of proteins with hydroxyapatite crystals has been extensively studied by several groups[8,27–30].

A blank experiment with the above mixture of model proteins on a column packed only with WP-DIOL 1000-5 shows a very low retention of the basic proteins (lysozyme and cytochrome *c*), and no retention of the acidic proteins.

The support macroporosity and the reduced particle size allow very fast kinetics of the chromatographic process. Fig. 6 shows the results obtained in the separation of the same protein mixture using a flow-rate twice that employed for the chromatogram in Fig. 5; the elution is faster with no decrease in resolution.

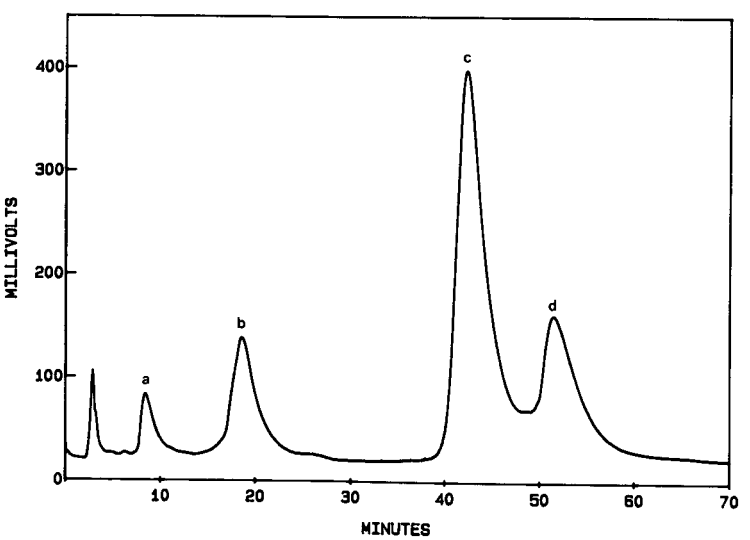

Fig. 5. Separation of a protein mixture containing (a) transferrin, (b) myoglobin, (c) lysozyme and (d) cytochrome *c* (oxidized form). Packing: Nucleosil 1000-5 DIOL CaP–HA 2.5% (matrix V) (100 mm × 6.0 mm I.D.). Linear gradient of sodium phosphate (pH 6.8), 1–350 mM (60 min); flow-rate, 1.0 ml/min; temperature, 25°C; detection, UV (280 nm).

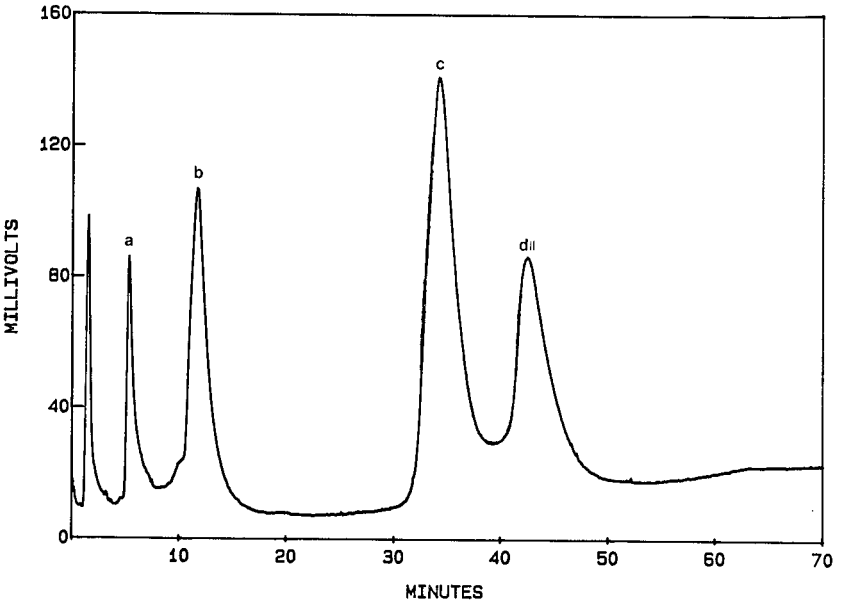

Fig. 6. Separation of a protein mixture containing (a) transferrin, (b) myoglobin, (c) lysozyme and (d) cytochrome *c* (oxidized form). Flow-rate, 2.0 ml/min; other conditions as in Fig. 5.

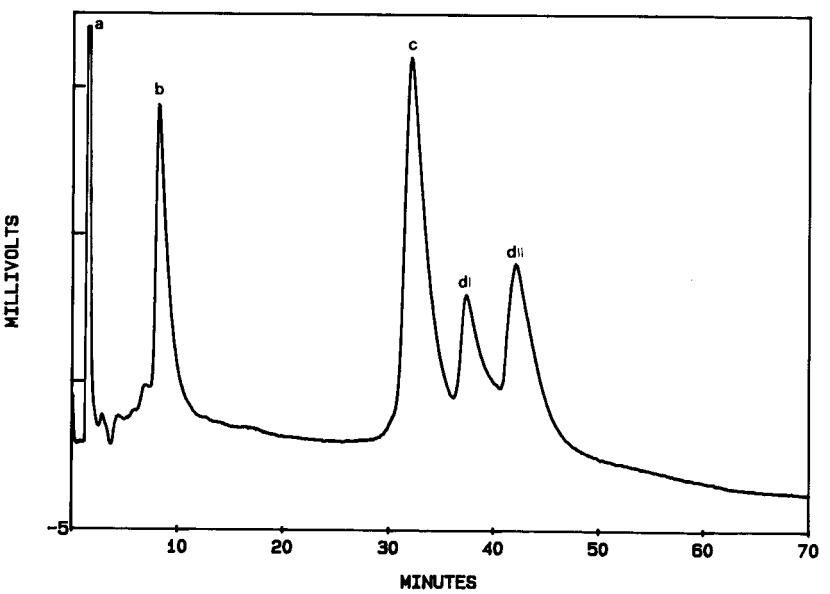

Fig. 7. Separation of a protein mixture containing (a) transferrin, (b) myoglobin, (c) lysozome and (d) cytochrome *c* (I = reduced; II oxidized). Temperature, 40°C; other conditions as in Fig. 5.

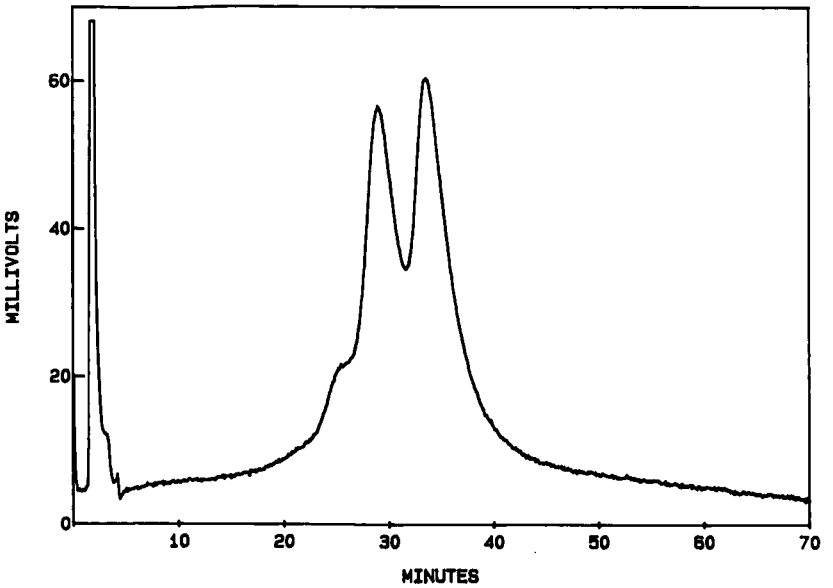

Fig. 8. Anti-melanoma monoclonal antibody (IgG class). Conditions as in Fig. 5.

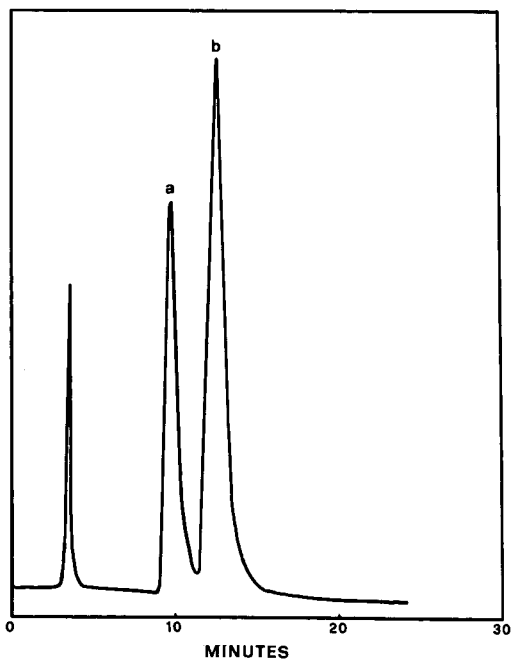

Fig. 9. Separation of (a) D,L-carnitine and (b) acetyl-D,L-carnitine. Packing: Nucleosil 1000-10 DIOL CaP–HA (matrix VI) (100 mm × 6.0 mm I.D.). Eluent, acetonitrile–water (80:20, v/v); flow-rate, 2.0 ml/min; temperature, 25°C; detection, UV (220 nm).

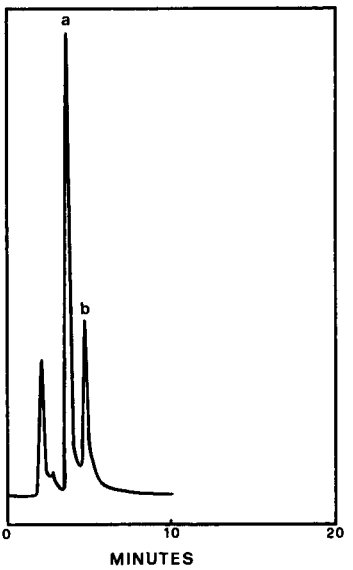

Fig. 10. Separation of (a) fructose and (b) maltose. Packing: Nucleosil 1000-10 DIOL CaP–HA (matrix VI) (100 × 6.0 mm I.D.). Conditions as in Fig. 9.

The column can also be utilized at higher temperatures; Fig. 7 shows the separation of the standard protein mixture performed at 40°C; the transferrin is very little retained under these conditions, whereas a better resolution is obtained for lysozyme and cytochrome *c*, the latter being completely separated in both its oxidized and reduced forms.

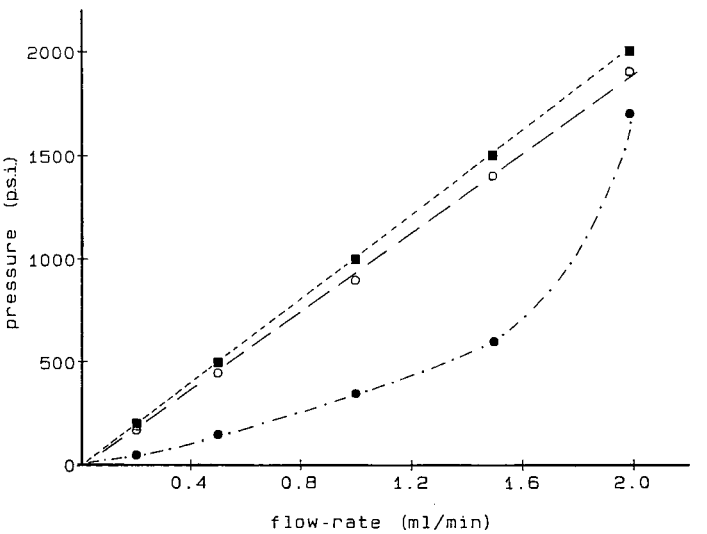

Fig. 11. Flow-rate *versus* pressure. ● = Irregular HA (10 μm, 30 × 4.6 mm I.D.; ○ = matrix III, Nucleosil 500-5 DIOL (5 μm, 50 × 4.0 mm I.D.); ■ = matrix V, Nucleosil 1000-5 DIOL (5 μm, 50 × 4.0 mm) I.D. Mobile phase, 1 m*M* sodium phosphate (pH 6.8); temperature, 25°C.

These supports have proved to be particularly selective in the separation of monoclonal antibodies, as shown in Fig. 8 for an IgG-type monoclonal antibody.

Apart from analytical and semi-preparative separations of proteins, the column can also be used for the separation of small molecules containing quaternary ammonium groups. Fig. 9 shows a separation of some carnitine derivatives; good selectivity, peak symmetry and high efficiency are evident. A good separation of small polar molecules, such as sugars, can also be achieved (Fig. 10)[31].

The columns have been repeatedly used at high flow-rates over a period of 6–12 months with no reduction in column performance (kinetic or thermodynamic). Fig. 11 indicates the relationship between pressure and flow-rate; its linearity confirms the great mechanical resistance of the support. The use of a 0.45-μm prefilter before the analytical column is an advisable precaution. The usable pH range is 4.5–8.5.

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Adsorption behaviour of albumin and conalbumin on TSK-DEAE 5PW anion exchanger

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SUMMARY

The adsorption behavior of chicken albumin and conalbumin on TSK-DEAE 5PW ion exchanger was studied by determining the equilibrium isotherms of these compounds at different temperatures, with different mobile phase ionic strengths and pH, using frontal analysis. The last two parameters have a considerable influence on the isotherms, including the saturation capacity, whereas temperature has a much less important influence. In most instances, and especially with chicken albumin, these isotherms are poorly approximated by a Langmuir isotherm equation, but rather correspond to high-affinity isotherm types. The Scatchard plots exhibit a downward convexity, indicating non-cooperative adsorption on two kinds of sites. The adsorption process tends to be partially irreversible towards dilution in the mobile phase.

INTRODUCTION

The investigation of protein adsorption at liquid–solid interfaces is of great importance for theoretical and practical reasons. The surface area occupied by a protein molecule on the surface of a known adsorbent, the adsorption energy at low surface coverage, the importance of lateral molecular interactions in the sorbed layer, the competition between different protein molecules for adsorption and the displacement of protein molecules by "strong" solvents all contribute to shed light on the ternary structure, its fluctuations and the surface energy of the proteins studied[1]. From a more practical standpoint, an accurate knowledge of the adsorption isotherms of a number of pure proteins and of their competitive isotherms is required for a better understanding of the chromatographic behavior of these compounds, especially at high concentrations, under the experimental conditions favored in the preparative applications of liquid chromatography.

Preparative liquid chromatography has become the essential tool used in

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biochemical laboratories and in the pharmaceutical industry for the extraction, separation and purification of proteins. The literature concerning these applications of chromatography has experienced a very rapid growth in recent years[2–4]. The optimization of the experimental conditions for preparative separations of proteins is still carried out empirically, however, because of a lack of the information required to apply the results of the theory of non-linear chromatography[5,6]. The most pressing need, at present, is for data on the competitive adsorption behavior of proteins on the most conventional stationary phases.

Among the packing materials that are currently used for the chromatographic separation and analysis of peptides and proteins, TSK gels have become very popular[7,8]. However, to our knowledge no systematic investigation of the adsorption behavior of proteins on these semi-rigid, porous polymer beads has yet been published.

This paper reports measurements of the adsorption isotherms of two common, basic proteins, chicken albumin and conalbumin, carried out by frontal analysis on TSK-DEAE 5PW, a weak anion exchanger, under various experimental conditions, and on the effect of the mobile phase flow velocity, temperature, pH and ionic strength of the mobile phase on these isotherms. A miniaturized equipment, similar to that described previously[9], was used for these determinations because of the special requirements of protein adsorption isotherm determinations.

In a further paper, the use of these isotherm data for the prediction of the profiles of the high-concentration bands obtained on analytical-scale columns will be discussed[10].

THEORETICAL

The experimental results derived from frontal analysis are amounts, q, adsorbed at equilibrium at various concentrations, C, of the protein in the mobile phase. These data can be plotted either as classical isotherms, *i.e.*, plots of q *versus* C, or as Scatchard plots of q/C *versus* q. If the equilibrium isotherm is well accounted for by a Langmuir expression:

$$q = \frac{aC}{1 + bC} \tag{1}$$

the Scatchard plot is a straight line of slope $-b$ and ordinate intercept a. This straight line intersects the abscissa at $q = a/b$, which corresponds to the column saturation capacity (and C infinite). The determination of the best estimates for a and b is thus done by fitting a linear relationship to the data on a Scatchard plot[1]. In most instances, however, the data points are not aligned and the equilibrium isotherm is not accounted for by a Langmuir equation.

The simplest model that can account for the type of data obtained in this work assumes that there are two types of non-cooperative independent adsorption sites[1]. In such a case, the amount adsorbed is the sum of the amounts adsorbed on each of the two types of sites:

$$q = \frac{a_1 C}{1 + b_1 C} + \frac{a_2 C}{1 + b_2 C} \tag{2}$$

In this case, the Scatchard plot is curved, with a downward convexity. The coefficients of the isotherm can be derived from the characteristics of the plot.

It should be emphasized here that the theoretical conditions for the validity of eqn. 1 are very retrictive (*e.g.*, ideal solution and adsorbed layer, no adsorbate–adsorbate interactions). We should be more surprised to see that eqn. 1 accounts well for many sets of adsorption data than to observe failures. There are many sources of failure of this equation. A heterogeneous surface covered with two different types of sites, a model leading to the bi-Langmuir isotherm (eqn. 2), is not the only possible reason. Obviously, a heterogeneous surface is covered by patches which exhibit a certain distribution of surface energy. The resulting isotherm is very complex and cannot be expressed simply. The relative success of eqn. 2 reflects more our ability to fit experimental data to a four-parameter equation of this type than its soundness as a model.

The molecular interactions of a charged protein with an ion exchanger exhibit many possibilities of complex retention mechanisms involving combinations of ionic interactions between the protein (which may carry several charges) with one or several ionic groups bonded to the surface of the ion exchanger, and/or hydrophobic interactions between the neutral patches on the protein surface and the organic groups at the surface of the exchanger. The ion-exchange mechanism alone becomes complex when the eluite carries several distant charges. Regnier and co-workers[11–13] have derived relationships valid at low sample concentrations. Eble *et al.*[14] have shown that, at high concentrations, the equilibrium isotherm resembles a two-site distribution. Finally, at high concentrations, protein–protein interactions may further complicate the phenomenon.

Therefore, the successful use of either eqn. 1 or 2 to account for experimental data does not mean that the retention mechanism is simple or that it proceeds after one or two well defined and identifiable steps.

EXPERIMENTAL

Materials

TSK-DEAE 5PW samples (average particle size *ca.* 10 μm) were a gift from Y. Kato (Toyo Soda, Tonda, Japan). Chicken albumin (denoted throughout the paper as albumin) and conalbumin were purchased from Sigma (St. Louis, MO, U.S.A.). All solvents were of high-performance liquid chromatographic (HPLC) grade from American Scientific Products (McGaw Park, IL, U.S.A.). Reagents were of analytical-reagent grade from Mallinckrodt (Paris, KY, U.S.A.). All these chemicals were used as received, without further purification. The purity of the proteins used was not tested. However, no spurious peak or step appeared during any experiment which could have indicated the presence of impurities.

Columns

Microbore columns (50 mm × 1 mm I.D.) were slurry packed with a suspension of TSK-DEAE 5PW in water under a pressure lower than 800 p.s.i. Under higher pressures the polymer bead particles are destroyed and the efficiency of the column obtained and its permeability may become very low. The columns must be also operated under low or moderate pressures. According to the manufacturer's

recommendations for the use of TSK gel columns, the maximum mobile phase flow-rate for a 7.5 mm I.D. column should be 1.20 ml/min, which corresponds to a flow-rate of 21 μl/min through a 1 mm I.D. column, as used in this work. Probably because of the narrower diameter, higher velocities could be used without damage, and columns were operated at flow-rates between 20 and 50 μl/min. Above this flow-rate, the packing material may be crushed and the column performance severely affected.

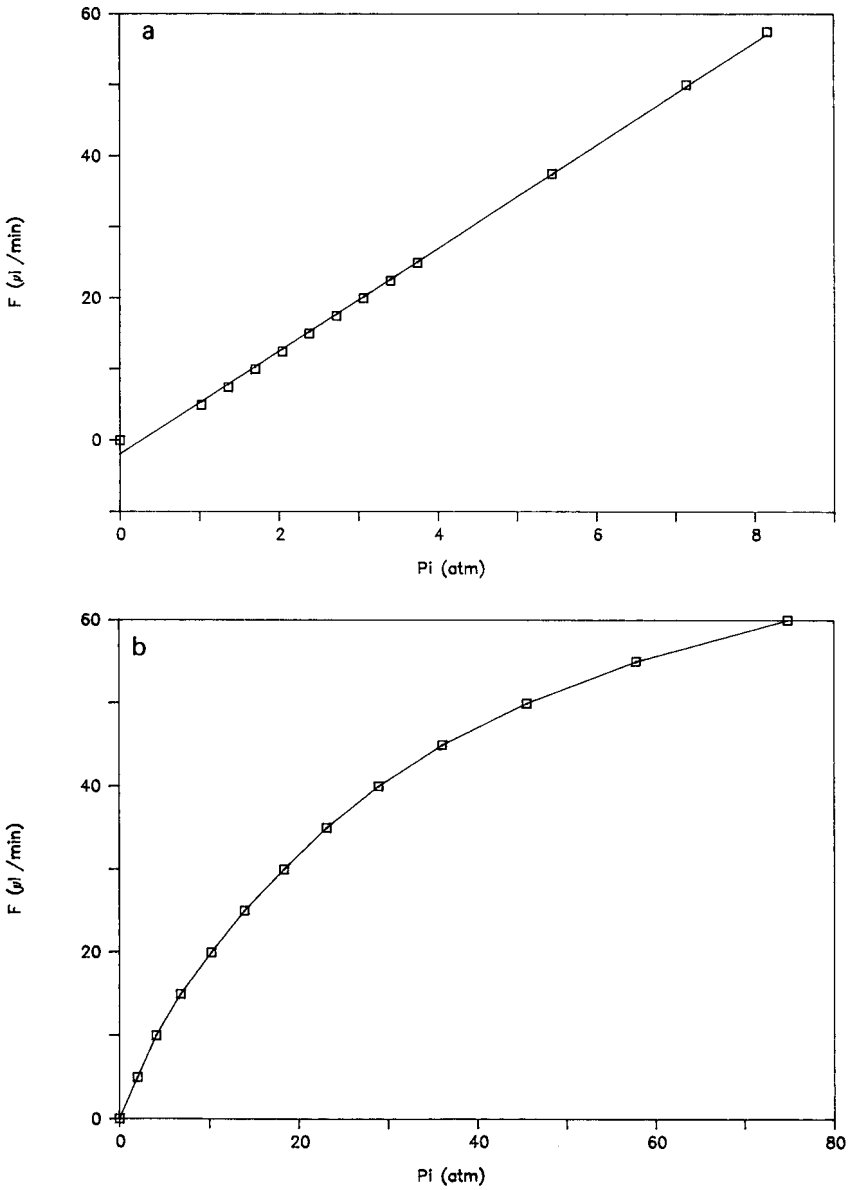

Fig. 1. (a) Plot of mobile phase flow-rate (F) *versus* column inlet pressure (P_i). (b) The same, over a wider pressure range for a poorly packed column.

Fig. 1a shows a plot of flow-rate *versus* inlet pressure for one of our typical columns. The relationship is linear in the pressure range used. Fig. 1b shows that, over a wider pressure range, the flow-rate of a poorly packed column does not increase linearly with increasing pressure, but more slowly. Provided that the pressure remains moderate, the phenomenon is reversible.

Equipment

The isotherm measurements were carried out with a miniaturized liquid chromatograph of conventional design, similar to that described previously[9]. A flow cell of very small volume was used with a Kratos (Ramsey, NJ, U.S.A.) 757 Spectroflow UV detector. All tubings and connections were kept as small as possible.

Procedures

Isotherm measurements were conducted using the classical frontal analysis method[15]. The measurement procedure was identical with that used previously[16]. The aqueous buffer solutions used contained Tris–acetate, adjusted with acetic acid to the required pH. Solutions were stored in a refrigerator and used after filtration on a 0.2-μm nylon 66 membrane filter from Gelman (Ann Arbor, MT, U.S.A.).

When the determination of an isotherm has been carried out under a certain set of experimental conditions, the column is thoroughly washed with a concentrated solution (8 *M*) of urea before any further experiments are carried out. This permits the complete elimination of all traces of strongly adsorbed protein and supplies a clean adsorbent surface for further investigations. Hence all the curves described in this paper are comparable. The need to apply this cleaning procedure carefully demonstrates, however, that a certain, albeit small, amount of protein is quasi-irreversibly adsorbed by the surface, thus pointing to a probable adsorption hysteresis[17].

Calculations

After having determined which Scatchard plots were curved, the values of the coefficients of the bi-Langmuir equations were calculated for those instances. The two asymptotes of these hyperbolic plots were linearly regressed to give the best initial guesses for the four coefficients, a_1, a_2, b_1 and b_2. Those initial values were entered into a simple simplex optimization program, together with the experimental isotherm data, in order to find the best fit. The program iterates until the sum-of-squares error between the experimental and calculated isotherms is minimized.

RESULTS AND DISCUSSION

In all the isotherm determinations, the breakthrough fronts recorded were very steep, showing that the corresponding isotherms are convex upward, *i.e.*, the amount of the studied protein sorbed by the gel at equilibrium increases less rapidly than its concentration in the mobile phase. As the isotherms were determined by frontal analysis, they are adsorption isotherms; no attempt was made to investigate the desorption isotherms, the desorption kinetics or the degree of hysteresis involved in the equilibrium studied[17]. The necessity to clean the column with urea solution after each determination in order to obtain consistent results indicates that such a phenomenon takes place to some extent.

Adsorption isotherms of albumin and conalbumin

Fig. 2a shows the adsorption isotherm determined for albumin and conalbumin with a 50 m*M* Tris–acetate buffer solution at pH 8.6. Both isotherms have a convex upward shape, with a steep initial slope. This isotherm shape is characteristic of macromolecular adsorption. The isotherm for conalbumin is reasonably well fitted by the simple Langmuir isotherm equation, as illustrated by the Scatchard plot[18] shown in Fig. 2b. In contrast, the adsorption isotherm of albumin is poorly fitted by a Langmuir equation. First, the initial slope is extremely steep, much steeper than with conalbumin. At the lowest mobile phase concentration at which a measurement could be carried out, the adsorbed amount is nearly 80% of the column saturation capacity, instead of 25% for conalbumin (Fig. 2a). This steep initial rise is followed by a rapid saturation. A constant amount is adsorbed at mobile phase concentrations above 4.0 mg/ml. The Scatchard plot, shown in Fig. 2c, exhibits the characteristic hyperbolic shape. Such an adsorption behavior cannot be accounted for by a simple mechanism.

Influence of the mobile phase flow-rate on the isotherm

By definition of an equilibrium property, the equilibrium isotherm should be independent of the mobile phase flow-rate during the measurements. Because of the very low molecular diffusion coefficients of proteins in aqueous solutions, however, and because of the possible steric hindrance to their radial mass transfer by diffusion across the porous particles of stationary phase, it is necessary to determine the range of flow-rates within which equilibrium determinations can safely be made.

The influence of the mobile phase flow-rate on the equilibrium isotherm was studied by carrying out isotherm determinations with the same mobile phase under increasing flow-rates. Fig. 3 shows the adsorption isotherm of albumin determined at flow-rates of 25, 50 and 75 μl/min. The data are reproducible, even at the highest

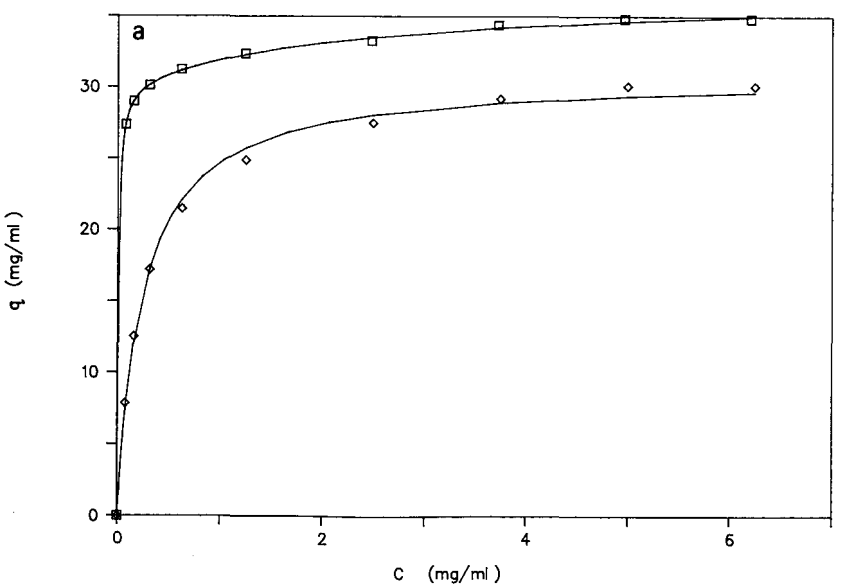

Fig. 2.

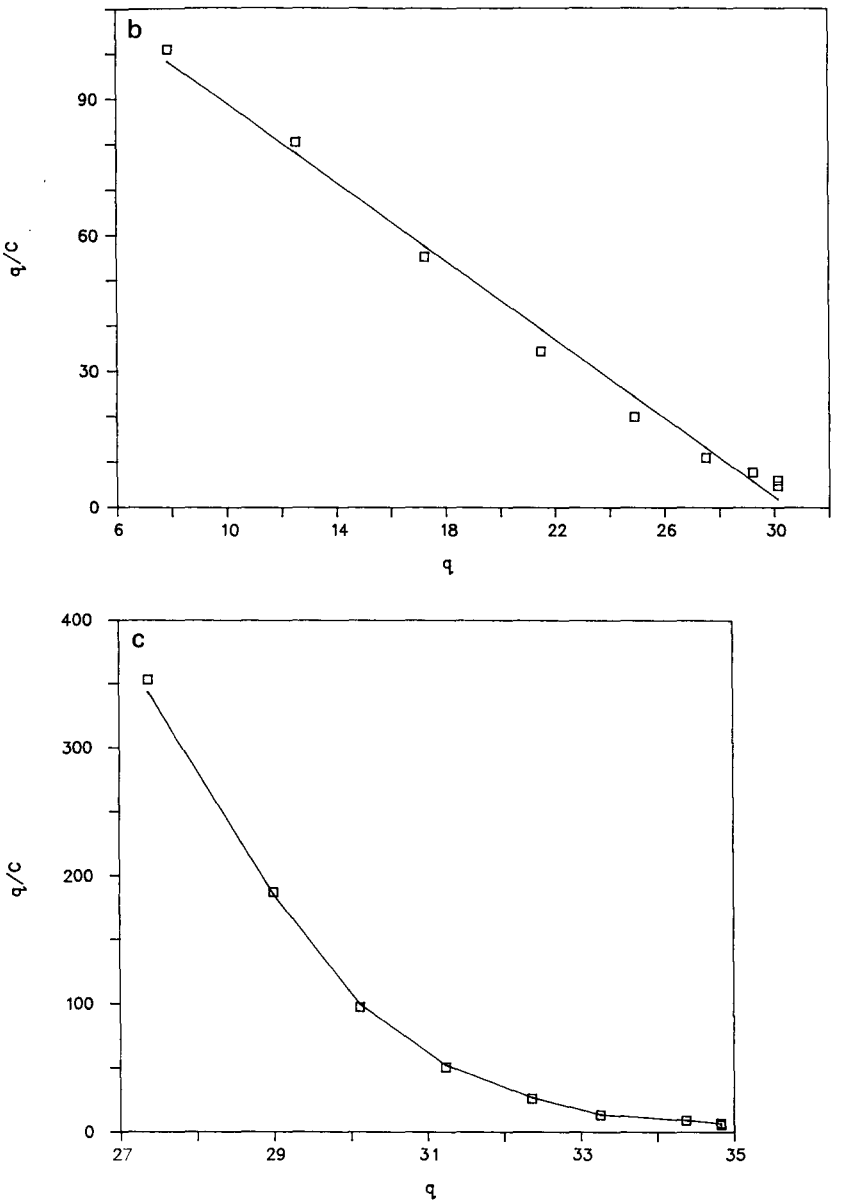

Fig. 2. Experimental data on the distribution equilibrium of chicken albumin and conalbumin between TSK-DEAE 5PW gel and the mobile phase [aqueous solution of Tris–acetate buffer (50 m*M*) at pH 8.6]. (a) Adsorption isotherms of chicken albumin (□) and conalbumin (◇). Plot of concentration in the stationary phase (q) *versus* concentration in the mobile phase at equilibrium (C). Experimental points and best curve obtained by a least-squares fitting of these data points to the Langmuir equation (conalbumin, *cf.*, Fig. 2b) or eqn. 2 (albumin, *cf.*, Fig. 2c). (b) Scatchard plot for the conalbumin data, showing a linear dependence of q/C on q. (c) Scatchard plot for the albumin data, showing a strongly curved plot, with downward convexity (non-cooperative binding).

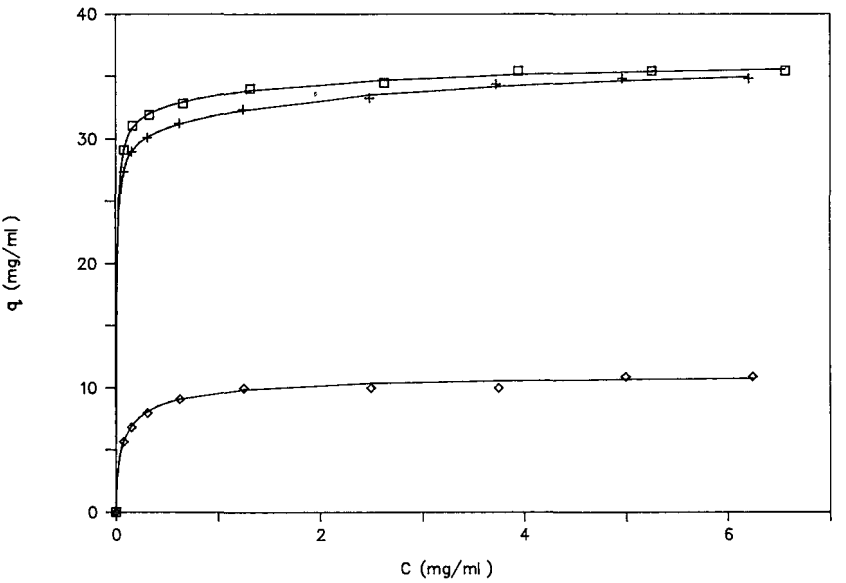

Fig. 3. Influence of mobile phase flow-rate on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot. Flow-rate: □ = 25; + = 50; ◇ = 75 μl/min.

flow-rate used. There is no real significant difference between the results obtained at 25 and 50 μl/min or at lower flow-rates. It is clear, however, that isotherm determinations carried out at flow-rates above 50 μl/min are meaningless, as the system is not equilibrated. The amount of albumin adsorbed decreases dramatically when the flow-rate increases above 50 μl/min. This latter flow-rate corresponds to a linear velocity of 0.13 cm/s and a reduced velocity for albumin of the order of 100–150. Although a 0.13 cm/s velocity may seem unexceptional for conventional HPLC experiments, the reduced velocity is fairly high for this kind of determination, whether or not the packing material suffers from excessive crushing. This illustrates that HPLC of proteins should always be carried out at velocities much lower than that of conventional organics[19].

All further measurements were carried out at a flow-rate of 50 μl/min.

Scatchard plots and representation of the isotherms

The Scatchard plots for Fig. 2 demonstrate that under the corresponding experimental conditions conalbumin exhibits non-cooperative binding to the weak ion exchanger used as the stationary phase, whereas chicken albumin exhibits either negatively cooperative binding or, more probably, adsorption on two different types of interaction sites[17]. Accordingly, Scatchard plots were made for each set of adsorption data collected. In all instances where the plots were not linear, they exhibited the same curvature, with a downward convexity. An iteration program[20] permits the determination of the best values of the slopes and ordinates of the two asymptotes of these plots (Fig. 2c).

TABLE I

ADSORPTION ISOTHERMS OF ALBUMIN

A_1/B_1 and A_2/B_2 are the column saturation capacities for the two kinds of sites.

Variable	*Value*	A_1	B_1	A_1/B_1	A_2	B_2	A_2/B_2	$\Sigma A/B$
Temperature (°C)	15	3248	94.9	36.1	4.14	0.854	4.8	40.9
	25	3472	115	30.2	2.85	0.429	6.6	36.8
	35	5156	215	23.9	7.83	1.168	6.7	30.6
Flow-rate (l/min)	25	3568	111	32.2	2.64	0.609	4.3	36.5
	50	3472	115	30.2	2.85	0.429	6.6	36.8
	75	290	50.00	5.8	16.30	3.19	5.1	10.9
Sodium acetate (m*M*)	0	3472	115	30.2	2.85	0.429	6.6	36.8
	25	331	18.8	17.6	4.101	0.517	7.9	25.5
	50	119	18.0	6.6	6.71	0.868	7.7	14.3
pH	7.6	9.6	9.7	1.0	2.53	0.208	12.1	13.1
	8.6	331	18.8	17.6	4.10	0.517	7.9	25.5
	9.6	105	21.2	5.0	2.14	0.333	6.4	11.4

The data are reported in Tables I (albumin) and II (conalbumin). The values obtained for the coefficients a_1, a_2, b_1 and b_2 are not very accurate, because of the experimental errors and of the compensation between the two terms of eqn. 2. The reproducibility for the column saturation capacity of the first type of sites is not much better than *ca.* 10% (see the data for 25 and 50 μl/min). The reproducibility for the column saturation capacity of the second type of sites is poor, of the order of 40%, mostly because adsorption data cannot be measured accurately at low concentrations. For conalbumin, most isotherms were well accounted for by a simple Langmuir model and in these instances only one set of coefficients is reported. In two instances (*e.g.* pH = 7.6), however, corresponding to very low column saturation capacities, the values of the coefficients calculated by the program had no physical meaning. They are not reported, but the estimated column saturation capacity is given in the last column of the Table II.

In Figs. 4–8, the solid lines represent the isotherms calculated from the coefficients in Tables I and II.

TABLE II

ADSORPTION ISOTHERMS OF CONALBUMIN

Symbols as in Table I.

Variable	*Value*	A_1	B_1	A_1/B_1	A_2	B_2	A_2/B_2	$\Sigma A/B$
Sodium acetate (m*M*)	0	125	4.05	30.8				30.8
	25	36.7	2.77	13.2				13.2
	50	2.0	0.42	4.8				4.8
pH	7.6	–	–	–	–	–	–	2.5
	8.6	36.7	2.77	13.2				13.2
	9.6	353	34.0	10.4	4.93	0.77	6.4	16.8

Influence of the ionic strength of the solution

Figs. 4 and 5 show the equilibrium isotherms of conalbumin and albumin, respectively, obtained with mobile phases of increasing ionic strength. Both the initial slope and the column saturation capacity of these basic proteins decrease dramatically with increasing ionic strength of the bulk solution. The range of sodium acetate concentrations investigated was 0–50 m*M*. In this range, the driving force for protein adsorption on the TSK-DEAE 5PW gel remains the electrostatic interactions between the ionized protein molecules and the charge carriers of the weak anion-exchange resin.

The influence of the mobile phase ionic strength on the equilibrium isotherm of proteins is explained by the increasing competition for adsorption between the negative ions in the mobile phase and the negative protein ions. The hydrophobic interactions of the proteins with the organic groups at the surface of the resin still have a negligible influence. It is most probable, however, that at higher ionic strength an opposite trend would take place and adsorption of the proteins would begin to increase with increasing ionic strength, as reported previously[9].

The equilibrium isotherms of conalbumin at all ionic strengths can be well accounted for by the Langmuir equation, as we have already reported. The same is not true for albumin. The initial slope of the isotherm is too steep and the saturation takes place too fast. The Scatchard plots show the same curvature as the plot in Fig. 2c. The isotherm is well accounted for by the sum of two Langmuir isotherms, corresponding to non-cooperative adsorption on two different kinds of sites. It is remarkable (see Table I) that the saturation capacity for the first sites decreases steadily with increasing

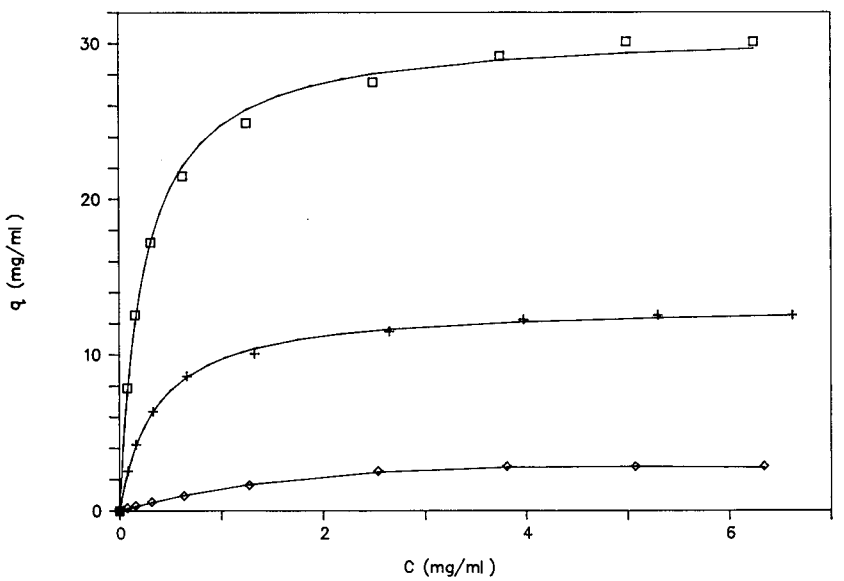

Fig. 4. Influence of mobile phase ionic strength on the equilibrium isotherm of conalbumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot (linear except for 50 m*M* sodium acetate). Additional concentration of sodium acetate buffer: □ = 0; + = 25; ◇ = 50 m*M*.

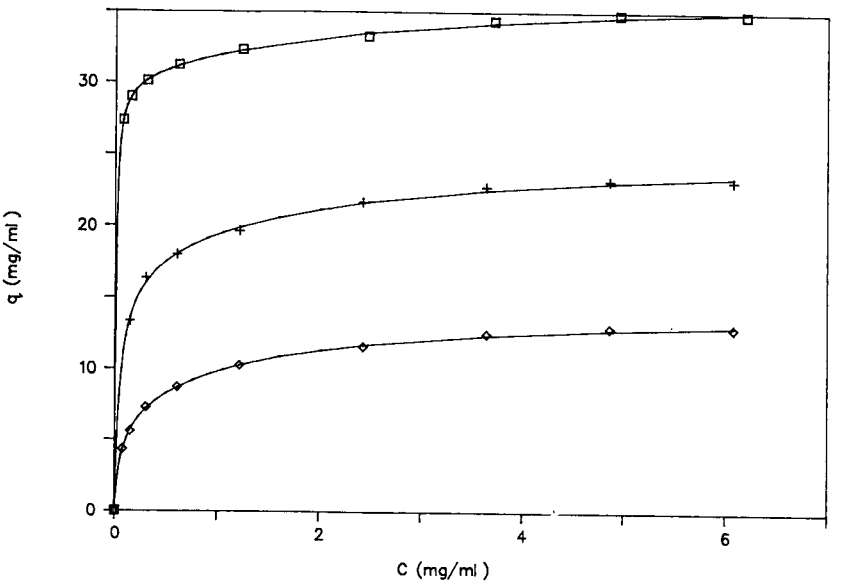

Fig. 5. Influence of mobile phase ionic strength on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot. Additional concentration of sodium acetate buffer: □ = 0; + = 25; ◇ = 50 m*M*.

ionic strength, whereas that of the second sites remain constant, within experimental error. Probably the former sites could be responsible for ionic interactions, whereas the latter would be involved in the hydrophobic interactions.

Influence of the pH of the mobile phase

Figs. 6 and 7 show the equilibrium isotherms of conalbumin and albumin, respectively, at increasing pH of the mobile phase. The ionic strength of the mobile phase was higher in these experiments than in those on the influence of flow-rate and temperature, as 25 m*M* sodium acetate was added to the mobile phase. This explains the difference between the isotherms of the two proteins obtained at pH 8.6 in Figs. 2, 6 and 7.

The amount of conalbumin adsorbed at equilibrium between the two phases increases with increasing pH, from 7.6 to 9.6 (see Fig. 6). The increase in amount sorbed is larger when the pH increases from 7.6 to 8.6 than when it increases from 8.6 to 9.6. The data at pH 7.6 were difficult to measure for conalbumin, partly because the column saturation capacity is small at that pH. The best isotherm obtained by curve fitting has no physical meaning and the coefficients are not reported. The superimposition of several phenomena is probably responsible for the pH dependence of the adsorption behavior of proteins.

The most important of these phenomena are the variation of the relative concentration of the ionized species with increasing pH and the increasing competition for adsorption between the negative protein ions and other negative ions, such as OH^- or AcO^-, whose concentration increases rapidly with increasing solution pH. The first phenomenon dominates in the pH range 7.6–8.6, whereas the concentration of the

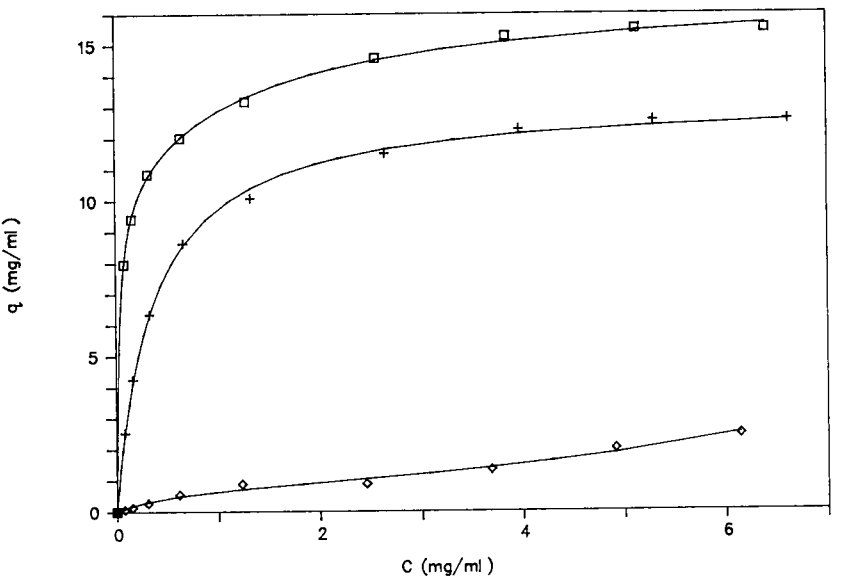

Fig. 6. Influence of mobile phase pH on the equilibrium isotherm of conalbumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot (except pH 8.6, Langmuir isotherm). Mobile phase pH: □ = 9.6; + = 8.6; ◇ = 7.6.

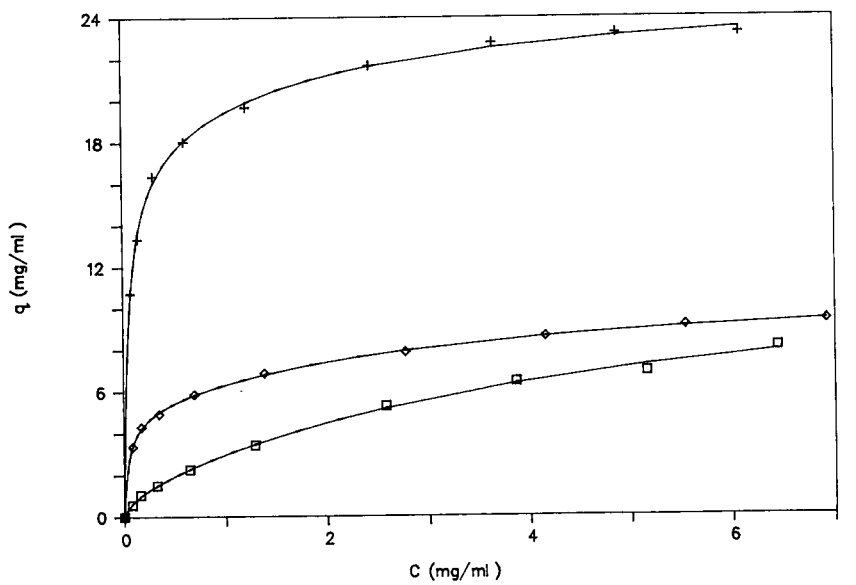

Fig. 7. Influence of mobile phase pH on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2, except the mobile phase also contains 25 m*M* sodium acetate. Solid lines calculated using the best coefficients derived from the Scatchard plot. Mobile phase pH: □ = 7.6; + = 8.6; ◇ = 9.6.

OH^- ions remains low. When the pH is increased from 8.6 to 9.6, however, the concentration of protein ions increases more slowly, and these ions are in increasing competition with the negative ions of the solution, so the amount of protein adsorbed increases only slowly, as observed.

With albumin, the competition between the protein ions and the other negative ions in the solution is strong enough at high pH that the amount of protein adsorbed is actually smaller than at pH 8.6. This decrease is essentially due to the lower column saturation capacity for the sites of the first type, which decreases 3-fold when the pH is raised from 8.6 to 9.6. The column saturation capacity of the sites of the second type decreases sharply when the pH is raised from 7.6 to 8.6, but then remains nearly constant.

Influence of the column temperature

Fig. 8 shows the equilibrium adsorption isotherm determined for albumin at 15, 25 and 35°C. The amount of protein adsorbed at saturation decreases slightly with increasing temperature, by *ca.* 1.5%/°C. The origin slope of the isotherm increases slightly with increasing temperature (see Table I).

The decrease in the column saturation capacity with increasing temperature is essentially due to the decrease in the capacity of the sites of the first type. The effect of a temperature change on the sites of the second type appears to be minimal, within the range of experimental error.

The effect of a change in the column temperature on the amount of protein adsorbed at equilibrium, from a solution at a constant concentration, is much less important than the influence of changes in the mobile phase composition.

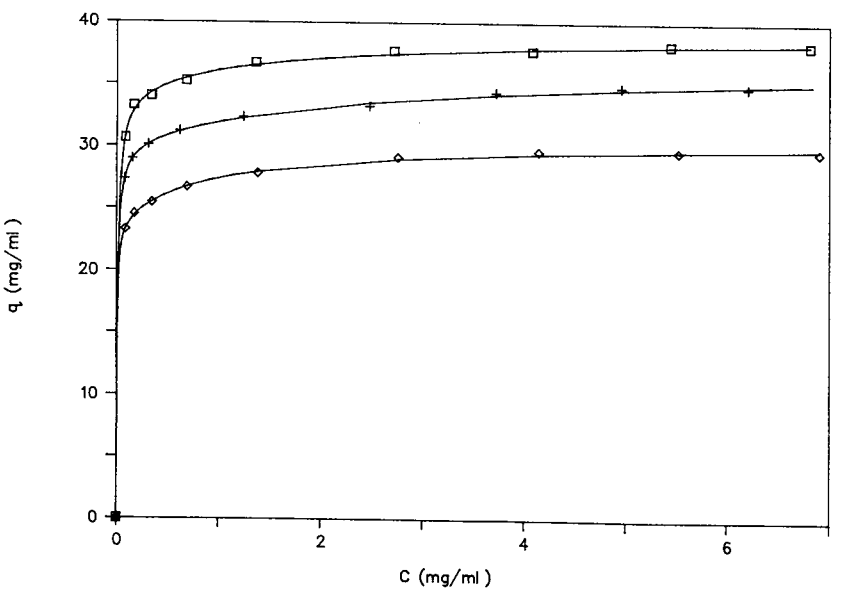

Fig. 8. Influence of the column temperature on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot. Column temperature: □ = 15; + = 25; ◇ = 35°C.

Adsorption capacity of TSK-DEAE gels

The adsorption capacity of the TSK gel investigated depends heavily on the experimental conditions. At most, it is of the order of 35–38 mg/ml for both proteins. This value is smaller than that obtained in a similar study performed on ion-exchange packing materials based on silica[9]. The difference is mostly explained by the larger average pore size of the polymeric packing particles and its smaller specific surface area.

In most instances, the column saturation capacity for the first type of sites is about 20% of the total column saturation capacity. The sites which are responsible for the highest fraction of the column saturation capacity are also those for which the Gibbs free energy of association is the largest. Adsorption on these sites is also the most sensitive to changes in the electrolytic properties of the mobile phase. The lack of precision prevents a more detailed discussion. The data, however, demonstrate that in most instances the surface of the stationary phase is not homogeneous, which, considering the organic structure of these ion exchangers, is not surprising.

The adsorption data presented here have been used for an investigation of the elution profiles of high concentration zones of albumin and conalbumin on TSK-DEAE 5PW gels under isocratic conditions. A comparison between experimental results and the prediction of the semi-equilibrium model[21] confirmed the slow kinetics of mass transfer observed in this work[10].

ACKNOWLEDGEMENTS

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Separation by cation-exchange high-performance liquid chromatography of three forms of Chinese hamster ovary cell-derived recombinant human interleukin-2

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SUMMARY

Purified recombinant (r) interleukin 2 (IL-2) produced by a transformed Chinese hamster ovary cell line shows a single peak when analysed by reversed-phase high-performance liquid chromatography, but it can be resolved into three forms by sodium dodecyl sulphate polyacrylamide gel electrophoresis. These three forms were successfully isolated by narrow-bore ion-exchange chromatography through optimization of the elution conditions. The addition of *n*-propanol as an organic modifier to the mobile phase proved to be essential for the recovery of the protein from the column in a yield of 90% or better based on protein quantification and biological activity determination. This chromatographic method was used for the purification of these three rIL-2 forms which represent variable glycosylation of a single polypeptide chain. A comparison of the biological activities using the murine CTLL-2 cell proliferation assay showed that the specific activities of the three forms are similar.

INTRODUCTION

Interleukin-2 (IL-2), an activated T-lymphocyte lymphokine that plays an important role in the immune response[1,2], is a glycoprotein heterogeneous with respect to charge and size; this heterogeneity is caused by variable glycosylation on a single polypeptide chain[3–5]. Recently, the carbohydrate structures of the two major forms of IL-2 produced by peripheral blood lymphocytes have been determined as the tetrasaccharide NeuAc(α2–3)Gal(β1–3)[NeuAc(α2–6)]GalNAc-ol for the form called N2 and as the trisaccharide NeuAc(α2–3)Gal(β1–3)GalNAc-ol for the N1 form (NeuAc = neuraminic acid; Gal = galactose; GalNAc = N-acetylgalactosamine)[5]. Both structures are O-linked to threonine 3 of the polypeptide chain. Stimulated T-lymphocytes also secrete a nonglycosylated IL-2, the M form[6].

Using partially purified material it was shown that variable glycosylation and sialylation accounted for heterogeneity of size and charge but made no difference to the *in vitro* biological activity of the lymphokine[7]. However, no further comparative

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studies were done to extend these observations probably owing to the difficulty in producing and purifying sufficient material of each form from T-lymphocytes.

We have previously described the isolation of a transformed Chinese hamster ovary (CHO) cell line that secretes large amounts of glycosylated recombinant (r) IL-2[8]. As the CHO-derived rIL-2, like natural IL-2, is heterogeneous with a variable sialic acid content, we searched for a simple procedure to separate the different glycovariants. Methods such as isoelectric focusing or sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) can be used to analyse the forms[7,9,10] but are not suitable for purification. Chromatofocusing has been used for IL-2 purification[3,8,11] but the method is time consuming and not well adapted for low-level protein detection because of the interference of the polyampholytes present in the mobile phase. Recently, a reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the separation of the different natural IL-2 forms was described, but it allowed only a partial separation of these forms[5].

We describe here the development of a rapid narrow-bore cation-exchange HPLC (CEX-HPLC) method that separates the different glycosylated rIL-2 forms and the partial biochemical characterization of these forms.

EXPERIMENTAL

Production of glycosylated recombinant interleukin-2

Glycosylated rIL-2 was produced by a transformed CHO cell line and purified to homogeneity as described previously[8].

High-performance liquid chromatography

All chromatographic separations were carried out at room temperature on a Hewlett-Packard HP1090 HPLC system equipped with a diode-array detector. RP-HPLC was performed on a BU 300 column (100 × 2.1 mm I.D. (Brownlee) and developed with a 15-min linear gradient from 40 to 60% acetonitrile–0.1% trifluoroacetic acid (TFA) at a flow-rate of 0.4 ml/min. Protein monitoring was performed at 220 nm.

CEX-HPLC was performed on a CX 300 column (100 × 2.1 mm I.D.) (Pierce) with a sodium acetate eluting buffer. The pH and organic modifier (*n*-propanol) were adjusted as described under Results and Discussion. A 15-min linear gradient from 20 to 100 m*M* sodium acetate and a flow-rate of 0.4 ml/min were used throughout. Elution was monitored at 280 nm and peaks were quantified according to their areas.

SDS-PAGE

SDS-PAGE was carried out as described by Laemmli[12] on 15% polyacrylamide gel; the acrylamide: bisacrylamide ratio was set at 29:1 to improve the separation in the 12 000–17 000-dalton range. Gels were silver stained (Bio-Rad Labs. kit) and scanned with a 2202 Ultroscan laser densitometer (LKB).

Neuraminidase treatment

Samples of rIL-2 were digested overnight at room temperature with *V. cholerae* neuraminidase (Calbiochem) in 50 m*M* sodium acetate buffer (pH 5.5) containing 154 m*M* sodium chloride and 4 m*M* calcium chloride. One unit of enzyme was used for

100–200 μg of rIL-2. TFA was added (1% final concentration) to stop the reaction, then the samples were stored at −20°C until chromatographic analysis.

Sialic acid determination

Sialic acid was liberated from rIL-2 by mild 1-h acid hydrolysis with 50 m*M* sulphuric acid, then quantified by HPLC on a Aminex HPX-87H column (300 × 7.8 mm I.D.) (Bio-Rad Labs.) with 5 m*M* sulphuric acid as the eluent at a flow-rate of 0.5 ml/min as described elsewhere[13]. Monitoring was done at 195 nm. The sialic acid standard was obtained from Calbiochem.

Protein determination

The concentration of the rIL-2 was determined either by amino acid analysis as previously described[8] or with a Bio-Rad Labs. kit using bovine serum albumin as the standard.

Biological assay

The activity of IL-2 was measured by the spectrophotometric assay described by Mosmann[14]. Units are expressed with reference to the standard provided by the National Cancer Institute Biological Response Modifier Program.

RESULTS AND DISCUSSION

Purified rIL-2 produced by a transformed CHO cell line[8] shows a single peak on RP-HPLC using a C_4 column and an acetonitrile gradient at pH 2.1 (Fig. 1). However, it can be resolved into three bands by SDS-PAGE, two major bands of 16 500 and 16 000 dalton and a minor band of 15 000 dalton (Fig. 2, lines a and h). These three bands have been separated by chromatofocusing and have been proved to be derived from post-translational modifications of a single polypeptide chain[8]. The time-consuming chromatofocusing separations resulted in low and variable recoveries and, for further characterization, an additional purification step was often required to eliminate the polyampholytes. Recently an RP-HPLC method based on a hexyl

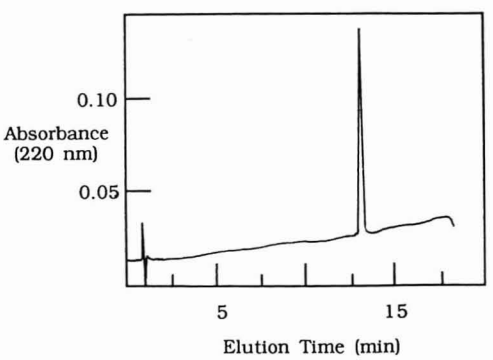

Fig. 1. Reversed-phase HPLC of rIL-2. Approximately 3 μg of purified rIL-2 were injected onto a BU 300 column (100 × 2.1 mm I.D.) and eluted with a linear gradient from 40 to 60% acetonitrile containing 0.1% TFA at a flow-rate of 0.4 ml/min. The single peak eluting at 13 min was collected for SDS-PAGE analysis.

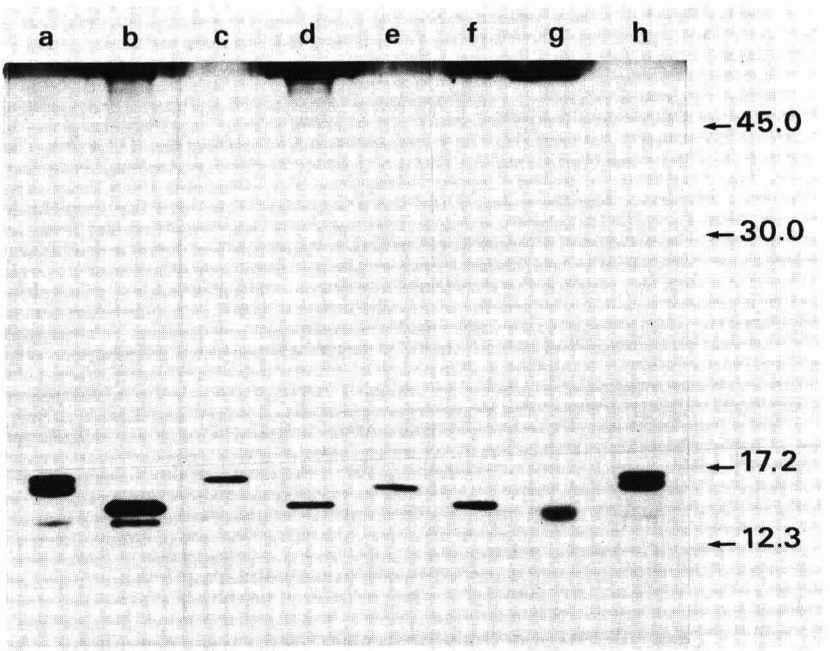

Fig. 2. SDS-PAGE. Lanes: a and h = purified glycosylated rIL-2; b = glycosylated rIL-2 treated with neuraminidase; c and d = rIL-2 eluting in peak I (Fig. 4) before and after neuraminidase treatment; e and f = rIL-2 eluting in peak II (Fig. 4) before and after neuraminidase treatment; g = rIL-2 eluting in peak III (Fig. 4). Values on the right hand side indicate molecular weights in kilodalton.

column using a propanol gradient at pH 4.3 for the separation of the different natural IL-2 forms was described, but it allowed only a partial separation of the different glycosylated forms[5]. Because of these problems and taking into account the rapidity of HPLC, we developed and optimized a CEX-HPLC system for the separation of the different sialylated forms of IL-2.

The first attempts to isolate the IL-2 forms on a silica-based cation-exchange column with an aqueous salt gradient resulted in a very poor recovery; however, better than 90% recovery, based on protein determination and biological activity, was obtained when an organic modifier, *n*-propanol, was used as a mobile phase additive (Table I). Concentrations of *n*-propanol up to 60% were tested, with optimum resolution between 30 and 45% (Fig. 3). Quantitative recovery of the IL-2 was not obtained if *n*-propanol was replaced with acetonitrile, suggesting a strong hydrophobic interaction of this protein with the chromatographic support. This behaviour can be attributed to the hydrophobicity of IL-2, as several other small proteins when chromatographed on the same silica based column were recovered nearly quantitatively without the need for additives (results not shown). The effect of the pH of the mobile phase was also studied; sharper peaks and good resolution were obtained at pH 6.5. At a pH higher than 7.6, the components were barely retained and poorly separated; at a pH lower than 6.5 the resolution was maintained even if the retention times were increased. pH 6.5 was chosen because it proved to be very convenient for

TABLE I

SEPARATION OF rIL-2 FORMS BY CEX-HPLC: TYPICAL RECOVERY IN A PREPARATIVE EXPERIMENT

360 μg of purified rIL-2 (determined by amino acid analysis[7]) were loaded onto the CX 300 column and eluted with a 30-min linear gradient from 20 to 100 m*M* sodium acetate in 40% *n*-propanol. Each indicated fraction was collected and the IL-2 content was determined by amino acid analysis. The values are means of three determinations. Peaks are labelled according to Fig. 4.

Material	*Amount of IL-2 (μg)*	*Recovery (%)*
Starting material	360	–
Eluting material:		
Peak I	155 ± 15	43
Peak II	155 ± 15	43
Peak III	43 ± 5	12
Total	353	98

storing the glycosylated IL-2 forms, as lower pH values favoured a slow desialylation of the molecules[5].

A flow-rate of 0.4 ml/min and a gradient of sodium acetate from 20 to 100 m*M* in 15 min were chosen because they resulted in a good compromise between resolution and separation time.

Neuraminidase treatment of the rIL-2 shifted the electrophoretic mobility of the 16 500- and 16 000-dalton forms to 15 500 dalton, leaving unchanged the 15 000-dalton band (Fig. 2, line b), indicating that sialic acids are covalently linked to the CHO-derived rIL-2 as previously described[8]. In order to identify the eluting material in peaks I, II and III from the CEX-HPLC (Fig. 4A), each peak was collected and analysed by SDS-PAGE; the material in peaks I, II and III gave single bands of 16 500,

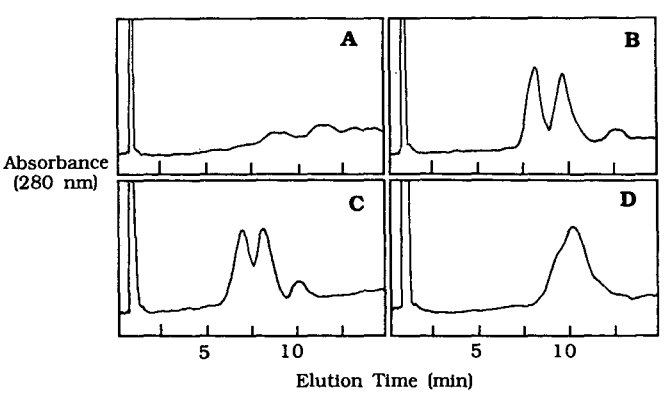

Fig. 3. Effect of *n*-propanol on the CEX-HPLC separation of rIL-2. Approximately 4 μg of purified rIL-2 were injected onto a CX 300 column (100 × 2.1 mm I.D.) and eluted with a 15-min linear gradient from 20 to 100 m*M* sodium acetate buffer (pH 6.5) in the presence of (A) 15%, (B) 30%, (C) 45% and (D) 60% *n*-propanol.

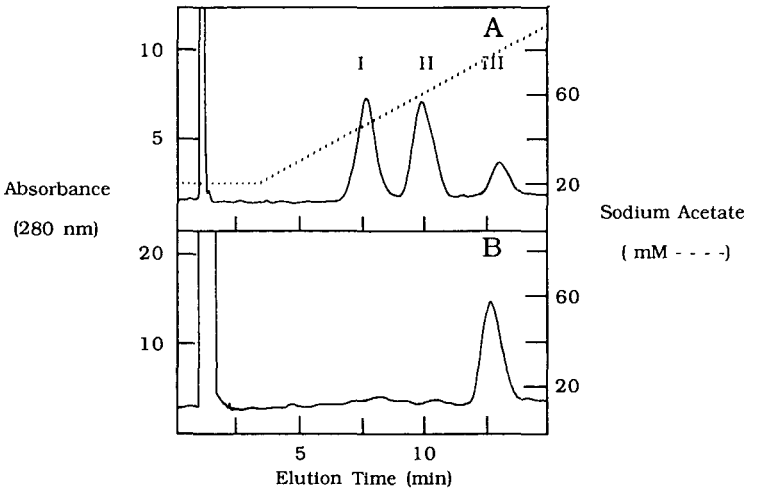

Fig. 4. Neuraminidase treatment of rIL-2. Approximately 4 μg of purified rIL-2 were injected (A) before and (B) after neuraminidase treatment onto a CX 300 column (100 × 2.1 mm I.D.) and eluted with a 15-min linear gradient from 20 to 100 m*M* sodium acetate buffer (pH 6.5) in the presence of 30% *n*-propanol. Individual peaks, labelled I, II, and III, were collected for SDS-PAGE analysis.

16000 and 15000 dalton, respectively (Fig. 2, lines c, e and g). Neuraminidase treatment of each isolated peak confirmed the presence of sialic acid covalently linked to the forms eluting in peaks I and II as the molecular weights, M_r, were shifted, in both cases, to 15500 dalton (Fig. 2, lines d and f) while the M_r of the material in peak III remained unchanged (not shown). The changes in electrophoretic mobility were accompanied by a shift in the eluting positions of peaks I and II into III (Fig. 4B). In addition, when the neuraminidase-treated 15500-dalton molecules of peaks I and II were treated with an O-glycanase specific for the Gal(β1-3)GalNAc conjugates, the M_r values were reduced to 15000 dalton without any changes in the CEX-HPLC behaviour (results not shown). Sequence analysis of the material eluting in peak III showed the expected NH_2-terminal Ala–Pro–Thr–Ser–Ser–Ser–Thr–Lys–Lys; the NH_2-terminal sequences of the material eluting in peaks I and II were identical with that determined for the material eluting in peak III, but no amino acid was identified in the third position, confirming our previous results that this threonine residue is the attachment point of the sugar moiety[8].

These results are compatible with a carbohydrate structure for the rIL-2 eluting in peak I as that of the natural IL-2 form called N2[5] containing the tetrasaccharide NeuAc(α2–3)Gal(β1–3)[NeuAc(α2–6)]GalNAc O-linked to the threonine in position 3 of the polypeptidic chain and for the recombinant material eluting in peak II as that of the natural IL-2 form called N1[5], identical with N2 but missing one sialic acid. The rIL-2 in peak III corresponds to non-glycosylated rIL-2 (M form). Additional information that supports the assignment of these structures was obtained by fast atom bombardment (FAB) mass spectrometric analysis of the purified amino-terminal tryptic peptides of each of the separated forms[15].

We compared CEX-HPLC with SDS-PAGE for IL-2 analysis. Both techniques provide similar resolution and correlate well in the quantification of the different

TABLE II

COMPARISON BETWEEN SILVER-STAINED SDS-PAGE DENSITOMETRY, CEX-HPLC AND SIALIC ACID DETERMINATION FOR THE QUANTIFICATION OF THE DIFFERENT FORMS OF rIL-2

Method	*IL-2*			*Sialic acid content (mol/mol IL-2)*
	N2 (%)	*N1 (%)*	*M (%)*	
SDS-PAGE	44	44	12	1.32[a]
CEX-HPLC	41	44	15	1.26[a]
Sialic acid	–	–	–	1.18[b]

[a] Deduced from the percentage of each form.
[b] Determined after mild acid hydrolysis as described under Experimental.

sialylated and non-sialylated forms with the amount of sialic acid released from the rIL-2 after mild acid hydrolysis (Table II). However, CEX-HPLC is advantageous because it is rapid and offers a simple way to recover the isolated forms. In fact, as the same resolution was observed with loads ranging from 2 to 200 μg (for loads of 200 μg or more, a 30-min gradient was used), we used this chromatographic system to purify each CHO-derived rIL-2 form for further characterization of their specific biological activities.

The biological activities of the purified forms were determined on a murine IL-2-dependent cytotoxic T cell line. In this system, the three forms showed similar specific activities [N2, $(2.1 \pm 0.2) \cdot 10^7$; N1, $(1.9 \pm 0.2) \cdot 10^7$; M, $(1.8 \pm 0.2) \cdot 10^7$ IU/mg (means $\pm$ S.D., $n = 3$)], suggesting that these forms interact similarly with the high-affinity IL-2 receptor present on this cell line and responsible for the cell proliferation as previously described[7]. However, as this proliferation assay may overlook small structural differences, more precise IL-2 receptor binding studies are being undertaken with the purified forms.

In conclusion, we have developed a simple chromatographic method for the separation of the different glycosylated and non-glycosylated rIL-2 forms. The availability of these highly purified forms of IL-2 will allow not only a better understanding of the structural function of these post-translational modifications, but also a more complete comparison in different biological systems *in vitro* and *in vivo*. The latter are of particular interest because, as has been shown for many others glycoproteins[16], the sialic acid residues can be critical for the survival of this molecule in the circulation.

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Precolumn derivatization technique for high-performance liquid chromatographic determination of penicillins with fluorescence detection

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SUMMARY

A precolumn derivatization method was developed for the high-performance liquid chromatographic (HPLC) determination of penicillins using fluorescence detection. Penicillins were derivatized by a two-step reaction, the β-lactam ring being opened by hydrolysis in aqueous sodium carbonate solution in the first step to give a secondary amine functionality, and the secondary amino group being reacted with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole in the second step to give a fluorescent derivative. The resulting reaction mixture was injected directly onto a reversed-phase column and analysed by HPLC. At a penicillin concentration of 10 μg/ml, the precision (relative standard deviation) ranged from 1.49 to 2.20%. In the concentration range 0.2–100 μg/ml, a linear response was observed. The detection limits of this method were 30–85 ng/ml for five different penicillins at a signal-to-noise ratio of 3:1. The proposed method was applied to the determination of penicillins added to serum following pretreatment by deproteinization and removal of compounds containing amino functionalities with a cation-exchange resin.

INTRODUCTION

Numerous pre- and postcolumn derivatization methods have been developed for the high-performance liquid chromatographic (HPLC) determination of penicillins[1–13]. These methods can be classified into two categories: spectrophotometric methods with the use of mercury(II) chloride combined with alkaline, imidazole or triazole[1–7] and fluorimetric methods with the use of labelling agents for amino compounds such as fluorescamine or *o*-phthalaldehyde (OPA)[8–11]. As the former methods allow the derivatization of various penicillins, this type of derivatization method has recently been applied to the analysis of numerous β-lactam compounds. In

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contrast, the latter methods allow the detection of β-lactams at picomole levels, but they are not applicable to penicillins that have no primary or secondary amino residues for the attachment of the label.

It is well known that the β-lactam ring is easily hydrolysed in weakly alkaline solution to give a corresponding penicilloic acid having a secondary amino functional group[14]. This allows the indirect labelling of β-lactam compounds that have no primary or secondary amino residue with labelling agents for amino compounds, and suggests the possibility of their highly selective determination in biological fluids when penicillins are derivatized after all interfering amino compounds in the biological sample have been removed by suitable pretreatment.

The present paper describes the precolumn fluorimetric derivatization of penicillins by using the above-mentioned reactions. Penicillins hydrolysed by aqueous sodium carbonate were labelled with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) and the derivatives were separated by reversed-phase HPLC and detected fluorimetrically. The method was applied successfully to the determination of penicillins added to serum.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and water were purchased from Kanto Chemicals (Tokyo, Japan). Sodium cloxacillin (Cl-PC) and sodium dicloxacillin (diCl-PC) were donated by Meiji Seika Kaisha (Tokyo, Japan). Sodium piperacillin (PI-PC) was purchased from Toyama Chemical Industries (Toyama, Japan) and sodium methicillin (Me-PC) from Sigma (St. Louis, MO, U.S.A.). Hyland Q-pack Chemistry Control Serum I was obtained from Cooper Biomedical (Tokyo, Japan). NBD-F was obtained from Dojindo Labs. (Kumamoto, Japan) and potassium benzylpenicillin (PC-G) and other reagents from Wako (Osaka, Japan). A cation-exchange resin column (55 × 5.0 mm I.D.) packed with AG 50W-X8 (H^+) (100–200 mesh) (Bio-Rad Labs., Richmond, CA, U.S.A.) was washed with 10 ml of 50% acetonitrile solution prior to use.

Apparatus

The HPLC system consisted of an L-6200 delivery system (Hitachi, Tokyo, Japan), a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.), an ODS-80T_M pre-packed column (150 × 4.6 mm I.D.) (Tosoh, Tokyo, Japan) and an L-1200 fluorescence spectrophotometer (Hitachi). The detector excitation and emission wavelengths were set at 470 and 530 nm, respectively. All chromatographic studies were performed at room temperature.

Derivatization procedures

The derivatization reagent was prepared by dissolving NBD-F in acetonitrile at a concentration of 80 m*M*. A 1 mg/ml stock solution of each penicillin was prepared with 50 m*M* phosphate buffer (pH 7.4.). Standard solutions of each penicillin were prepared by diluting the stock solution to appropriate concentrations with 50 m*M* phospate buffer (pH 7.4) or control serum.

For standard sample. To 45 μl of the sample solution were added 45 μl of

acetonitrile and 10 μl of 5% sodium carbonate solution. The reaction mixture was allowed to stand for 60 min at 60°C and then cooled in an ice-bath. To the reaction mixture were added 15 μl of 0.2 *M* phosphate buffer (pH 6.0) and 35 μl of the derivatization reagent and then it was allowed to stand for 10 min at 60°C. After being cooled in an ice-bath, the mixture was mixed with 30 μl of 1 *M* hydrochloric acid solution. An aliquot (5–10 μl) of the resulting mixture was injected directly onto the HPLC column.

For serum. An aliquot of serum (600 μl) was pipetted into a tube for centrifugation. After addition of 600 μl of acetonitrile, the tube was vortex mixed for 1 min. Following centrifugation at 2000 *g* for about 5 min, 1 ml of the supernatant was passed through the cation-exchange resin column. The effluent from column was collected, after the first 200 μl had been discarded. To 450 μl of this solution were added 50 μl of 10% sodium carbonate solution and the mixture was allowed to stand for 60 min at 60°C. After cooling in an ice-bath, 100 μl of the resulting hydrolysis mixture were derivatized as described above.

RESULTS AND DISCUSSION

Fig. 1 shows the reaction course for the hydrolysis of the β-lactam ring and the formation of NBD derivatives. The hydrolysis and derivatization reactions should proceed in a weakly alkaline aqueous medium. The five penicillins were hydrolysed in 5% sodium carbonate solution at 60°C to give the corresponding penicilloic acids (II) and were further reacted with NBD-F at 60°C to give fluorescent NBD derivatives (III). The formation of by-products interfering with the detection of the NBD derivatives was not observed in the hydrolysis reaction. An excess of NBD-F gave NBD-OH and a few other products in the derivatization, but they did not interfere with the detection of the NBD derivatives, as they were eluted earlier than any of the NBD derivatives of penicillins. Consequently, the resulting reaction mixture could be injected directly into the chromatograph without further treatment. The NBD derivatives eluting from column were monitored spectrofluorimetrically as described by Watanabe and Imai[15]. Typical chromatograms of the NBD derivatives of five penicillins are shown in Fig. 2.

The hydrolysis reaction also proceeds in β-lactamase solution, but penicillins having β-lactamase resistance cannot be hydrolysed. Indeed, in this study, this enzyme

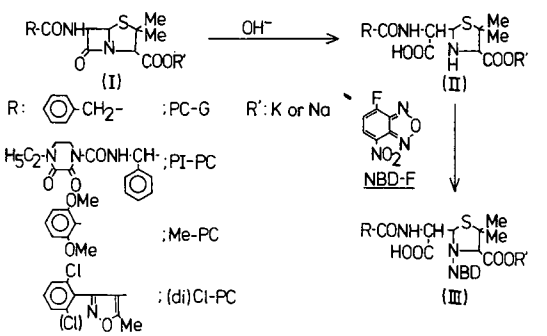

Fig. 1. Reaction scheme for the hydrolysis of β-lactam ring and formation of NBD derivatives.

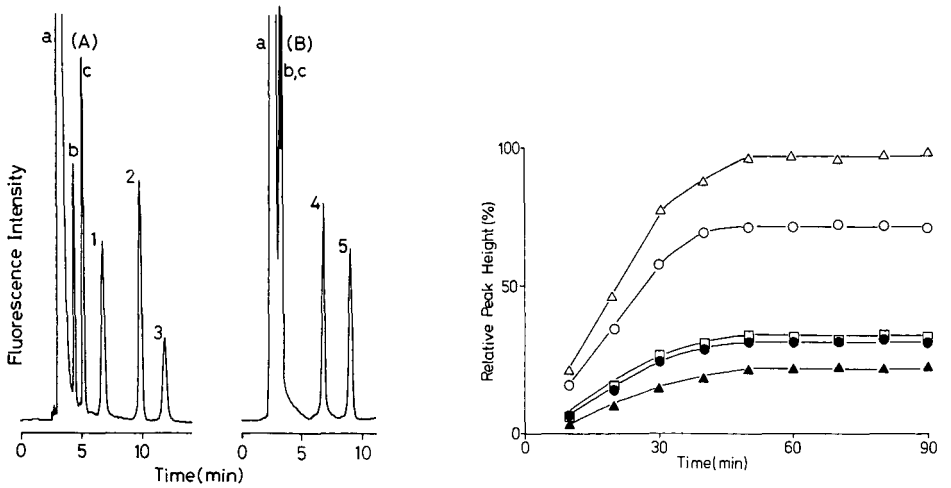

Fig. 2. Chromatographic profiles of the NBD derivatives of five penicillins. 1 = PI-PC; 2 = PC-G; 3 = Me-PC; 4 = Cl-PC; 5 = diCl-PC; a = NBD-OH; b, c = unknowns. Mobile phase, 0.1 *M* phosphate buffer (pH 3.0)–methanol, (A) 60:40 and (B) 45:55; flow-rate, 1.0 ml/min; injection volume, 5 μl; sample concentration, 20 μg/ml for each penicillin; detector sensitivity, 0.5.

Fig. 3. Effect of hydrolysis time on the fluorescence intensity of the NBD derivatives. Reaction temperature, 60°C; sample concentration, 50 μg/ml. △ = PC-G; ○ = PI-PC; □ = Me-PC; ● = Cl-PC; ▲ = diCl-PC.

applied to all five compounds. PC-G and Me-PC were easily hydrolysed, but with the other three compounds this reaction hardly proceeded. Therefore, weakly alkaline conditions were adopted in the procedure.

The effects of the precolumn reaction conditions, time of hydrolysis and derivatization reactions, pH of reagent buffer added to the resulting hydrolysis mixture prior to the derivatization and concentration of NBD-F were investigated by HPLC with spectrofluorimetric detection of the reaction products. The results obtained with five penicillins are illustrated in Figs. 3–6.

The time course for hydrolysis was tested at 60°C. In 5% sodium carbonate solution, hydrolysis of all penicillins was completed in 50 min, and longer periods of hydrolysis at this temperature produced no significant improvement in the fluorescence intensity (Fig. 3).

The reaction conditions of the derivatization were also tested at a 60°C. The maximum fluorescence intensity for PC-G, PI-PC and Me-PC was obtained at NBD-F concentrations of 80 m*M* or above, and that for Cl-PC and diCl-PC at cqncentrations of 60 m*M* or above (Fig. 4). The buffer solutions were investigated in the pH range 5.0–7.0. In this range, the fluorescence intensity for Cl-PC and diCl-PC was almost constant. The fluorescence intensity for PC-G, PI-PC and Me-PC was also constant in the pH range 5.0–6.0, but higher pH decreased the fluorescence intensity (Fig. 5). Fig. 6 shows the effect of time on the yield of the fluorescent derivative. The maximum fluorescence intensity for PC-G and PI-PC was obtained after 10 min and that for Me-PC, Cl-PC and diCl-PC after 5 min. In both instances, longer periods of reaction gave no significant improvement in the peak height.

In order to determine the reproducibility of the present method, several analyses

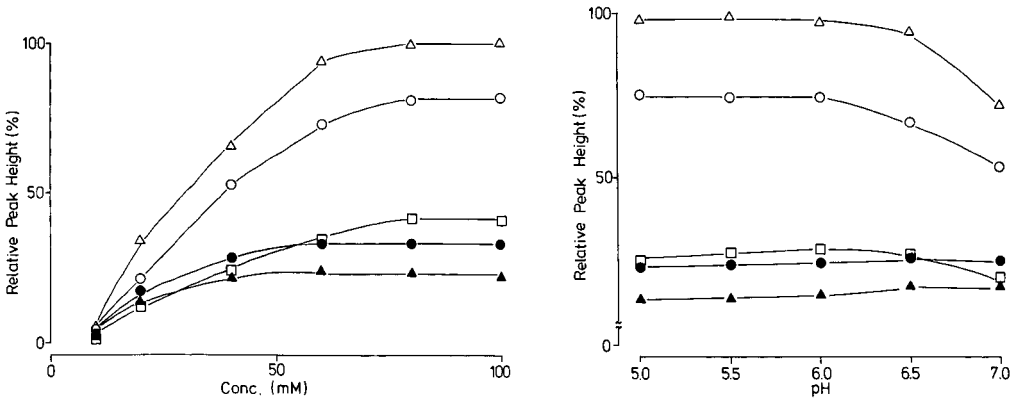

Fig. 4. Effect of NBD-F concentration on the fluorescence intensity of the NBD derivatives. Conditions and symbols as in Fig. 3.

Fig. 5. Effect of pH of reagent buffer on the fluorescence intensity of the NBD derivatives. Conditions and symbols as in Fig. 3.

were performed. Table I lists the relative standard deviations of the peak height for five penicillins at a concentration of 10 μg/ml. Detection limits (signal-to-noise ratio = 3) for all the NBD derivatives are also listed in Table I. A linear response was observed in the range 0.2–100 μg/ml. The regression equations and the correlation coefficients (r^2) of calibration graphs for the penicillins are given in Table II. Highly sensitive detection for all five penicillins was achieved.

Our findings demonstrate that penicillins that have no primary or secondary amino function can be derivatized with NBD-F as a fluorimetric labelling agent after hydrolysis. This suggests that the method may be useful for the highly selective determination of penicillins that have no amino function in a biological fluid or fermentation medium. Thus penicillins can be easily derivatized after all amino

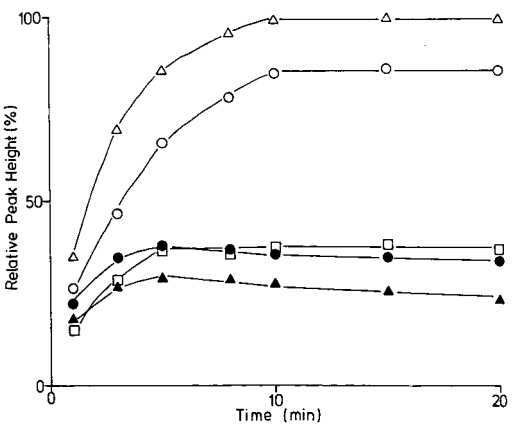

Fig. 6. Effect of reaction time of the derivatization on the fluorescence intensity of the NBD derivatives. Conditions and symbols as in Fig. 3.

TABLE I

REPRODUCIBILITIES OF PEAK HEIGHT AND DETECTION LIMITS FOR PENICILLINS

Sample	*Relative standard deviation (%)*[a]		*Detection limit (signal-to-noise ratio 3) (ng/ml)*
	Intra-assay	*Inter-assay*	
PI-PC	0.99	1.49	50
PC-G	1.32	1.82	30
Me-PC	1.70	2.20	85
Cl-PC	1.01	1.51	30
diCl-PC	1.23	1.73	45

[a] 10 μg/ml, $n = 12$.

TABLE II

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS OF CALIBRATION GRAPHS FOR PENICILLINS

Concentration range of 0.2–100 μg/ml.

Sample	*Regression equation*[a]		*Correlation coefficient* (r^2)
	Slope	*Intercept*	
PI-PC	8.37	2.63	0.998
PC-G	11.60	4.21	0.998
Me-PC	3.90	2.29	0.996
Cl-PC	5.26	1.82	0.998
diCl-PC	4.34	0.54	1.000

[a] Peak height = intercept + slope × concentration (μg/ml).

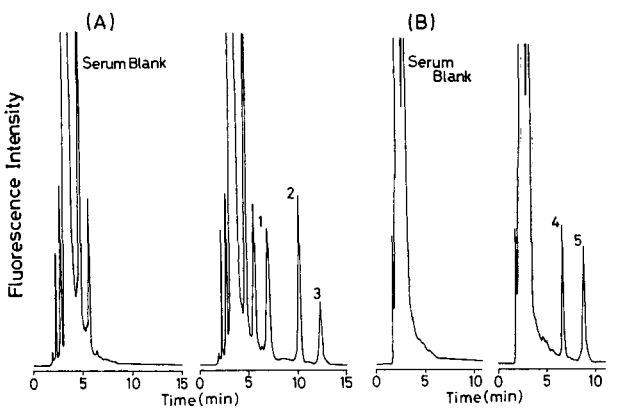

Fig. 7. Chromatographic profiles of serum blank and five penicillins added to serum. Mobile phase, 0.1 *M* phosphate buffer (pH 3.0)–methanol, (A) 60:40 and (B) 45:55. Peak assignments and other conditions as in Fig. 2.

TABLE III

RECOVERY OF PENICILLINS FROM SERUM

Penicillin	*Recovery (%, n = 6)*		
	5 μg/ml	*20 μg/ml*	*50 μg/ml*
PI-PC	95.0	93.7	92.5
PC-G	93.8	91.0	93.9
Me-PC	90.4	91.2	92.8
Cl-PC	95.1	97.0	98.7
diCl-PC	95.8	96.2	100.7

TABLE IV

REPRODUCIBILITIES OF DETERMINATION OF PENICILLINS SPIKED IN SERUM

Penicillin	*Relative standard deviation (%, n = 6)*		
	5 μg/ml	*20 μg/ml*	*50 μg/ml*
PI-PC	1.97	1.90	1.92
PC-G	2.00	1.95	1.80
Me-PC	2.36	2.35	2.01
Cl-PC	3.04	2.40	2.25
diCl-PC	2.85	2.80	2.11

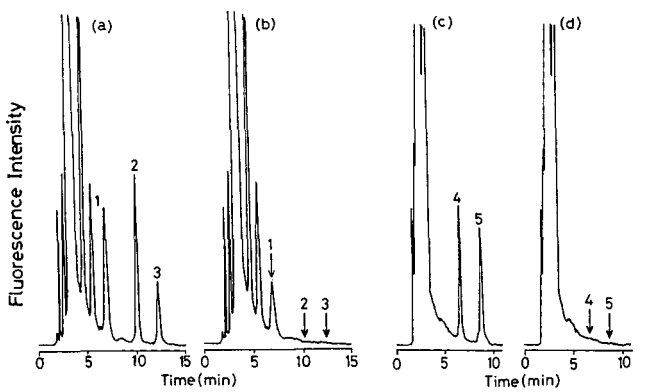

Fig. 8. Chromatographic profiles of five penicilloic acids added to supernatant of deproteinized serum, derivatized (b and d) after pretreatment by the cation-exchange resin and (a and c) without the pretreatment. Sample concentration, 20 μg/ml as corresponding penicillins. Peak assignments and other conditions as in Fig. 7.

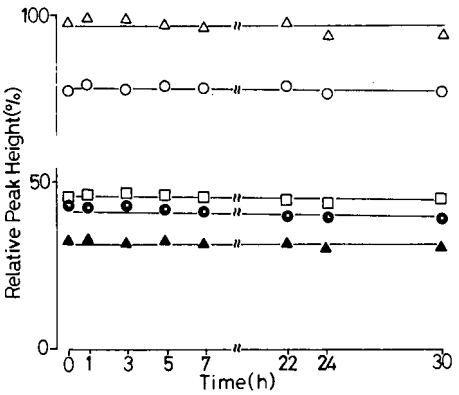

Fig. 9. Stability of the NBD derivatives on the resulting reaction mixture at ambient temperature. Symbols as in Fig. 3.

compounds in the biological sample have been removed by pretreatment with a cation-exchange resin.

Fig. 7 shows chromatograms of serum blank and the five penicillins added to serum, derivatized by the present method following pretreatment by deproteinization and passage through a cation-exchange column. Interference-free chromatograms, sufficient recovery and reproducibilities were obtained (Tables III and IV). In these pretreatment methods, as the pH of the effluent from the cation-exchange column was very low compared with the standard solution, the hydrolysis and derivatization reactions did not proceed. This problem was solved by pH adjustment using 10% instead of 5% sodium carbonate solution.

Penicilloic acid is a major metabolite of penicillins. If it is not completely removed by the pretreatment, it will result in a positive systematic error during the determination of unchanged drug. Fig. 8 shows the chromatograms of the five penicilloic acids (penicillins hydrolysed with 5% sodium carbonate solution) added to the supernatant of deproteinized serum. Apart from that arising from PI-PC, they were completely removed by the present pretreatment. The penicilloic acid from PI-PC was only 60% removed, so the method was unsuitable in this instance. In order to resolve this problem, other pretreatment methods are being studied.

The NBD derivatives of the five penicillins were stable for at least 24 h in the resulting reaction mixture at room temperature (Fig. 9). This stability is sufficient to allow the use of an autosampler, which suggests that the present method may be applied to routine analysis. The results also suggest the possibility of determination by the use of one of the penicillins as an internal standard for other penicillins, except for PI-PC.

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Preparative and analytical separation of oligosaccharides from κ-carrageenan

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SUMMARY

The separation of the enzymic degradation products of κ-carrageenan, as studied by size-exclusion chromatography on Bio-Gel P-6 and reversed-phase high-performance liquid chromatography on μBondapak C_{18}, resulted in a homologous series of oligomers in addition to non-homologues substances. On the former column steric exclusion competed with ion exclusion originating from solute–solute and solute–gel interactions. Competition was reduced with increasing ionic strength of the eluent. Separation on μBondapak C_{18} was influenced by the type of salt, indicating that the reversed-phase separation was not solely based on solute–column interactions.

INTRODUCTION

During the last few years, galactan oligosaccharides have gained considerable interest as they are used as model compounds for ^{13}C NMR and Raman spectral band assignment of native polysaccharides[1–3]. Since the tetramer and longer chain oligomers undergo a conformational transition as a function of temperature[4] and in the presence of certain counter ions[5], they can be used to provide evidence for structural changes[6] occurring in the polymer solution. In contrast to the polymer, the oligomers have a well characterized structure. Initially in the hydrolysate of κ-carrageenan a homologous series of four oligomers was detected using thin-layer chromatography (TLC) on cellulose[7]. These sulphated oligosaccharides were isolated by dry-column cellulose chromatography. Size-exclusion chromatography (SEC) on Sephadex G-25 resulted in the separation of a disaccharide and a tetrasaccharide[8]. However, on analysis with Bio-Gel P-6 the presence of up to ten oligomers in the hydrolysate was reported and the separation was studied as a function of the ionic strength of the eluent[9]. In this paper, the preparative separation parameters involved in the separation of the oligomers from the hydrolysate and the use of reversed-phase high-performance liquid chromatography (HPLC) as a fast and suitable adequate technique for the analysis of this type of sample are considered.

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EXPERIMENTAL

Materials

Standard oligomer samples for HPLC were purchased from Grampian Enzymes (Aberdeen, U.K.). The hydrolysate was a kind gift from C. Rochas (Grenoble, France). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

SEC

SEC was performed with two Pharmacia (Uppsala, Sweden) columns. One (850 × 26 mm I.D.) was filled with a Bio-Gel P-6 (Bio-Rad Labs., Richmond, CA, U.S.A.) packing, particle size 200–400 mesh and the other (700 × 15 mm I.D.) with Sephadex G-50 Super Fine (Pharmacia) gel. Detection was performed with an R410 differential refractive index (RI) detector from Waters Assoc. (Milford, MA, U.S.A.). The fraction collector and pump were also from Pharmacia. The flow-rate of the Bio-Gel P-6 column was maintained at 0.45 ml min^{-1} and 5-ml samples were injected. Samples of 0.5 ml were injected onto the Sephadex G-50 column and the flow-rate was 0.15 ml min^{-1}.

HPLC

The HPLC system from Waters Assoc. was equipped with an R410 detector. All data were treated with a 740 data module. Two Model 510 HPLC pumps were connected in series and a flow-rate of 0.5 ml min^{-1} was usually used. All operations were made with the isocratic operating mode of an automatic gradient controller. Injections were carried out with a U6K injector.

All hydrolysate samples were dissolved in 1 ml of the eluent, centrifuged and filtered (Millipore, 0.45 μm) before injection (10 μl) onto a μBondapak C_{18} column coupled with a C_{18} guard column (Waters Assoc.). The system was run at ambient temperature. The eluent was prepared with ultrapure water (Milli-Q), filtered (0.22 μm) and degassed with a Millipore vacuum filtration apparatus.

TLC

The samples were applied to Kieselgel 60 Fertigplatten (200 × 100 mm) (Merck) with a calibrated glass capillary and were placed in a closed glass tank. Butanol–acetic acid–water (2:1:1) was used as the solvent system and detection was performed with naphthoresorcinol–sulphuric acid reagent[7].

RESULTS AND DISCUSSION

Preparative separation

κ-Carrageenan is an alternating copolymer consisting of mainly → 4)-3,6-anhydro-α-D-galactopyranosyl-(1 → 3)-β-D-galactopyranosyl 4-sulphate-(1 →. The repeating unit is called neocarrabiose 4-sulphate (Fig. 1) and the oligomers will be denoted by their degree of polymerization (DP), characterized by *n*, the number of repeating units. Previously, it was shown that the enzyme κ-carrageenase hydrolyses κ-carrageenan in a random-*endo* manner[10]. It has been reported that a homologous series of κ-carrageenan oligomers can be separated from the hydrolysate on a Bio-Gel P-6 column using sodium nitrate solution as eluent[9].

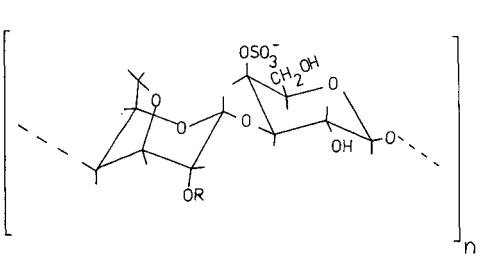

Fig. 1. Idealized repeating structure of κ-carrageenan (R = H).

In Fig. 2 the logarithm of the distribution coefficients (K) for the separation of different oligomers on Bio-Gel P-6 with $5 \cdot 10^{-2}$ and $1 \cdot 10^{-1}$ M sodium nitrate solution at 20 and 35°C are plotted as a function of n. K is calculated using the expression

$$K = \frac{V - V_0}{V_T - V_0} \tag{1}$$

where V, V_0 and V_T are the elution volume of a particular oligomer, the high-molecular-weight enzyme-resistant fraction and the salt, respectively. Because in SEC the

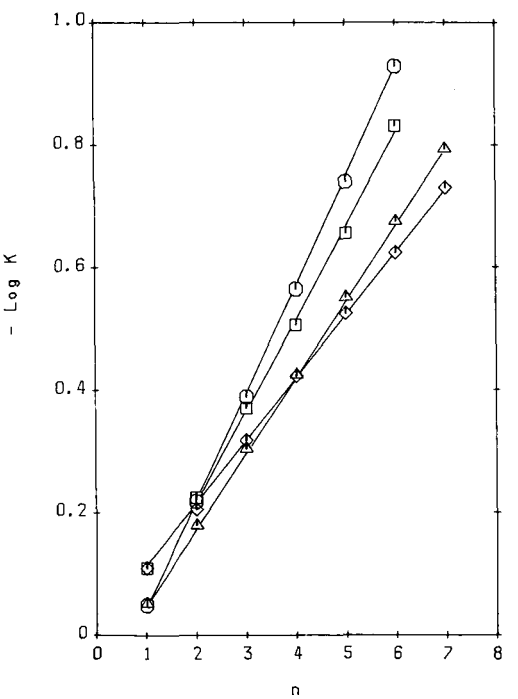

Fig. 2. Negative logarithm of the distribution coefficient (K) of κ-carrageenan oligomers on Bio-Gel P-6 as a function of the degree of polymerization in 10^{-1} M salt solution at (△) 20°C and (◇) 35°C and in $5 \cdot 10^{-2}$ M salt solution at (○) 20°C and (□) 35°C.

separation depends on the hydrodynamic volume of the solutes[11], the straight lines observed in Fig. 2 indicated the presence of homologous substances. In contrast to the literature[9], the curves in Fig. 2 did not overlap at either 20 or 35°C. According to the SEC theory of polyelectrolytes[12,13], an eluent salt concentration of $5 \cdot 10^{-2}$ M is sufficient to screen the electrostatic repulsions between the solutes.

Also for charged oligomers a higher salt content in the eluent does not result in large changes in K. This can be illustrated for the separation of the κ-carrageenan oligomers from the hydrolysate on a Sephadex G-50 column (Fig. 3). At both temperatures the influence of the salt content in the eluent was small on Sephadex compared with Bio-Gel. Therefore, on the latter a more complex separation mechanism seems to occur. The large influence of temperature on K further suggests that the separation on Bio-Gel P-6 is not based merely on the hydrodynamic volume of the solutes. Theoretically, K should be independent of temperature. As the temperature and the eluent salt concentration had different effects on the two SEC packings used, it was concluded that solute–gel interactions were involved in the separation mechanism on Bio-Gel P-6. At 20°C the oligomers eluted faster on Bio-Gel with $5 \cdot 10^{-2}$ M than with 10^{-1} M sodium nitrate solution (Fig. 2), which indicated ion exlusion[14] due to electrostatic repulsions between the charged oligosaccharides and the amide groups of the gel. Addition of more salt suppressed this effect. As a result, the solutes are retained much longer in the gel pores. The salt in the eluent is therefore necessary in order to avoid competition between the steric exclusion mechanism and

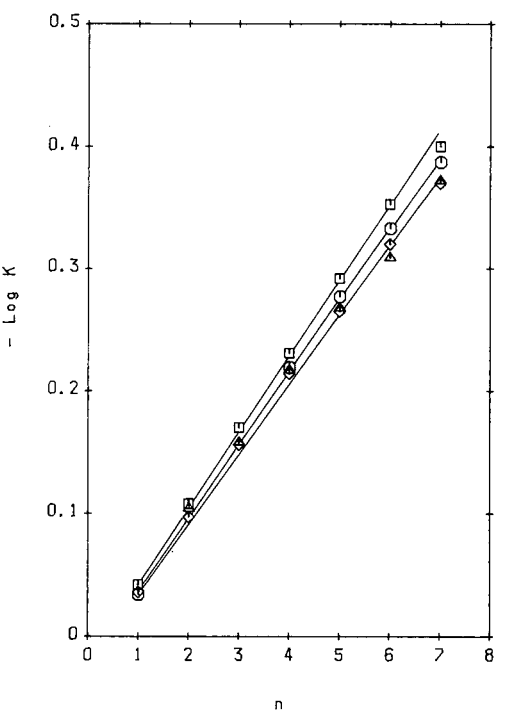

Fig. 3. Negative logarithm of the distribution coefficient (K) of κ-carrageenan oligomers on Sephadex G-50 Super Fine. Symbols as in Fig. 2.

ion exclusion caused by electrostatic repulsions between the solutes and between the solutes and the gel. To overcome both interactions, higher salt concentrations were needed than in the absence of solute–column effects[12,13].

The specific interactions of cabohydrates with polyacrylamide gels are well known and have been reviewed[15]. In agreement with Figs. 2 and 3, the influence of the ionic strength of the eluent on the K values of oligosaccharides separated on Bio-Gel is known to be larger than that on Sephadex[16]. The effect of temperature on the elution volumes of carbohydrates analysed on Bio-Gel has also been shown[17]. It depended on the carbohydrate structure of the solutes[18].

Although K is of considerable importance in the study of the SEC mechanism on a particular column, in preparative SEC mainly the resolution (R) is of interest. The high sample concentrations used in SEC affect the resolution and can give rise to band broadening or peak tailing[19]. On Bio-Gel P-6 increasing concentrations up to 15 mg ml^{-1} resulted only in increased peak heights without a distinct loss of resolution for all oligomers. High sample concentrations for preparative SEC also affect the hydrodynamic size of the solutes and thus K, which may change owing to overloading. The value of K for DP 1 remained unchanged within 1% for concentrations up to 15 mg ml^{-1}. However, with increasing molecular weight the concentration dependence of K increases and the reproducibility of the determined elution volume decreases[19]. Repeated measurements revealed, *e.g.*, that the uncertainty of the determined K for DP 7 is 7% for sample concentrations of 15 mg ml^{-1}, compared with only 1% for DP 1.

Fig. 4 shows the influence of the salt concentration in the eluent and the temperature on the resolution (R) for Bio-Gel P-6. The values of R are plotted against the average molecular weight of two neighbouring compounds (M) in the chromatogram and are calculated according to

$$R = \frac{2(V_2 - V_1)}{W_2 + W_1} \tag{2}$$

where V and W are the elution volume and the peak width at the base, respectively. Better resolution is obtained with 10^{-1} M than $5 \cdot 10^{-2}$ M sodium nitrate solution, especially for the high-molecular-weight oligomers. With 10^{-1} M salt solution oligomers even up to DP 9 were detected. However, with $5 \cdot 10^{-2}$ M salt solution DP 8 and 9 oligomers were never resolved. Therefore, the preparative separation of these oligomers on this column has to be performed with at least 10^{-1} M salt solutions. In a previous study[5] we used ammonium hydrogencarbonate as an electrolyte as it can be partially removed from the oligomer samples during freeze-drying, with ammonia, carbon dioxide and water as decomposition products. The oligomers up to DP 4 are easily separated with both 10^{-1} and $5 \cdot 10^{-2}$ M salt solutions (Fig. 4). Elution with the latter eluent offered the advantage that less salt has to be removed during the very time-consuming desalting step, particularly with these small molecules.

The large influence of temperature on R (Fig. 4) in both eluents is probably a consequence of the non-ideal SEC behaviour. Increasing temperature usually enhances the sample solubility and reduces the solvent viscosity. Both improve the resolution. However, this was not so here. The resolution at 20°C is always higher than at 35°C, for which we have no explanation. Only for the large oligomers in $5 \cdot 10^{-2}$ and 10^{-1} M salt solution did R reach the same values at both temperatures.

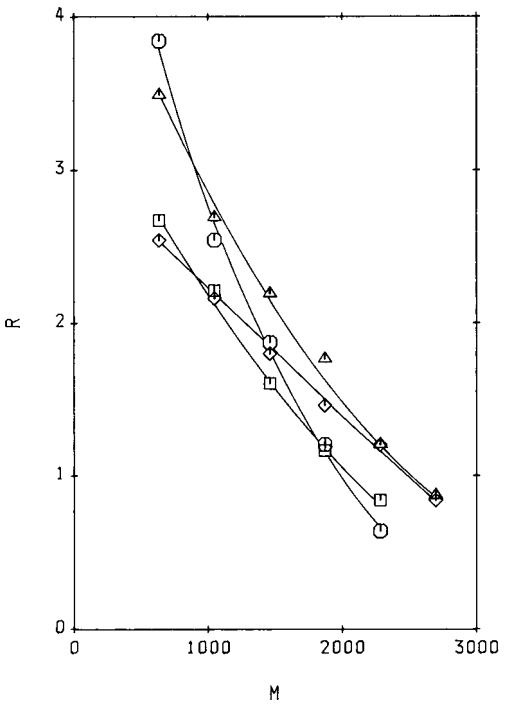

Fig. 4. Resolution (R) of κ-carrageenan oligomers on Bio-Gel P-6 as a function of the average molecular weight of two neighbouring compounds on the chromatogram. Symbols as in Fig. 2.

It is well known that temperature can be used to prevent adsorption of solutes. Small molecules are easily adsorbed on the column, because through permeation they are exposed to a much higher surface area in the packing[20]. In preparative SEC, loss of product due to adsorption should be prevented. However, on Bio-Gel P-6 at 20°C with 10^{-1} M salt solution a peak was recorded after the elution of the salt ($K = 1.14$). Adsorption depended on the ionic strength and disappeared at 35°C.

In order to establish which oligomers were lost at 20°C, the relative peak areas with 10^{-1} M salt solution were compared at both temperatures. Table I shows that no significant difference existed. Therefore, adsorption was probably due to a low-molecular-weight contaminant which at 35°C was eluted with the salt. By ageing and in the presence of traces of acid the oligomers may degrade and the hydrolysis of the 3,6-anhydrogalactose residue results in the formation of galactose and 5-(hydroxymethyl)-2-furaldehyde[9]. The latter compound was responsible for a sometimes slight yellow colour[21] of concentrated oligomer solutions and was adsorbed on Bio-Gel P-2 packing[9]. With $5 \cdot 10^{-2}$ M salt solution at 20°C, owing to the excellent separation of oligomers of DP 1 and 2 (Fig. 4), two other contaminating compounds became apparent at $K = 0.661$ and 0.791. Moreover, detailed examination of the chromatogram revealed the existence of another contaminant between the DP 3 and 4 peaks ($K = 0.330$). These compounds, not previously reported, occur in only minor amounts (<0.5% of the total oligomer fraction) and were not isolated. These non-homologous

TABLE I

INFLUENCE OF THE BIO-GEL P-6 COLUMN TEMPERATURE ON THE RELATIVE FRACTION OF ELUTED κ-CARRAGEENAN OLIGOMERS

Eluent salt concentration = 10^{-1} *M*. *n* = Number of repeating units. ± Values are standard deviations calculated from three replicates.

n	*20°C*	*35°C*
1	15.0 ± 0.3	15.7 ± 0.5
2	29.5 ± 0.5	29.8 ± 0.4
3	22.3 ± 0.2	22.3 ± 0.2
4	15.3 ± 0.2	14.4 ± 0.3
5	8.7 ± 0.6	8.3 ± 0.2
6	4.4 ± 0.1	4.2 ± 0.2
>6	2.4 ± 0.1	2.6 ± 0.1
ERF[a]	2.4 ± 0.1	2.6 ± 0.1

[a] ERF = Enzyme-resistant fraction.

substances probably originate from "kinking residues" in the substrate[22] which are insensitive to enzymic hydrolysis.

Analytical separation

Although the analysis of the mixture could be easily performed on the preparative Bio-Gel P-6 column, analytical studies of processes such as κ-carrageenan digestion would be very time consuming as optimum results required 16–18 h. Therefore, reversed-phase HPLC has been suggested for these types of sugars[23]. All separations were effected within 30 min and oligomers up to DP 13 were observed. As in reversed-phase chromatography the separation depends on the hydrophobic interactions between the sample solute and the column, salt was again added to the eluent to screen the electrostatic repulsions between the oligomers. Figs. 5 and 6 demonstrate that the separation improved with increasing sodium nitrate concentra-

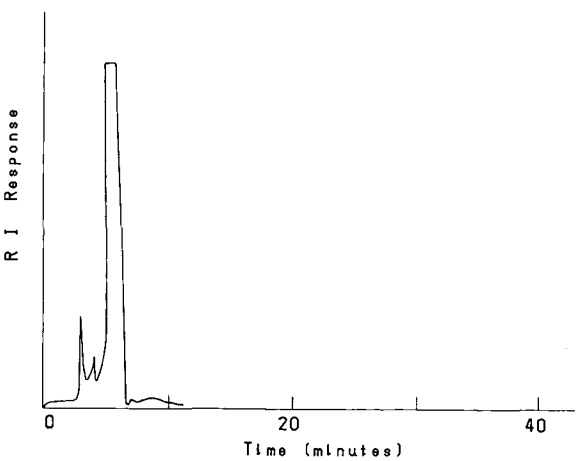

Fig. 5. HPLC on μBondapak C_{18} of the κ-carrageenan oligomers in water.

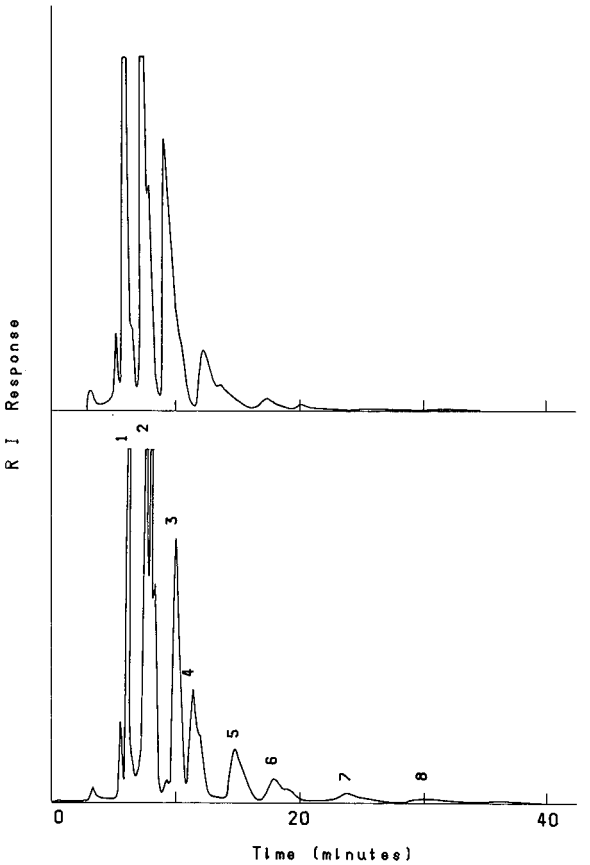

Fig. 6. HPLC on μBondapak C_{18} of the κ-carrageenan oligomers with (top) $5 \cdot 10^{-2}$ *M* and (bottom) $5 \cdot 10^{-1}$ *M* sodium nitrate solution.

tion, which for an optimum separation was distinctly higher than the 10^{-1} *M* used in SEC.

Moreover, the HPLC and SEC traces showed different patterns. In the former instance more peaks were observed and some were broadened owing to the appearance of small shoulders. The peaks in Fig. 6 were assigned using DP 1 and DP 2 standard samples. According to the theory of the retention behaviour of oligomeric series in reversed-phase chromatography[24], the capacity factors of the standards in $5 \cdot 10^{-1}$ *M* sodium nitrate solution were plotted against *n* following the equation

$$\log k = (\log \alpha)n + \log \beta \tag{3}$$

where $\log \alpha$ is the selectivity and β the capacity factor for the hypothetical oligomer with $n = 0$. The capacity factor is defined as

$$k = \frac{V}{V_M} - 1 \tag{4}$$

where V is the retention volume of a particular oligomer and V_M the column dead volume. Based on eqn. 3, the k values of the larger oligomers (n = 3–8) were calculated and compared with the experimental data. The oligomers separated on the HPLC column were also collected and analysed by TLC using the standards as references. Chromatographic movements were observed up to n = 5 and the migration fitted a linear relationship:

$$\log\left(\frac{1}{R_F} - 1\right) = f(n) \tag{5}$$

which indicates the presence of a homologous series.

In contrast with the previously reported HPLC result[23], oligomers of DP 1 and DP 2 were well separated. In addition, we observed a splitting of the DP 2 peak which was due to anomerization. Separation of carbohydrate anomers on HPLC columns is well known[25]. However, in our work it is not yet clear why the anomerization was mostly pronounced for DP 2. When injecting the DP 1 and DP 2 standards only the latter chromatogram showed two peaks, nearly baseline separated with 1 M salt solution. For both the hydrolysate and the pure DP 2 sample the relative peak areas for the first and second peaks were 63.9 ± 0.5% and 36.1 ± 0.5%, in agreement with the β/α anomerization ratio obtained by ^{13}C NMR spectroscopy[4].

Fig. 5 shows that two peaks were eluted before the oligomer of DP 1. The first was due to the presence of salt and corresponded with V_M. The second had a relative peak area of 2.5%, which agreed with the value for the enzyme-resistant fraction eluting at the void volume on Bio-Gel P-6 (Table I). This value was distinctly lower than that of 10.8% reported recently[26]. As it was shown that the enzyme-resistant fraction consisted mainly of irregular galactan polysaccharides, contaminating the carrageenan sample[26], the amount of this fraction depends on the purity of the carrageenan extracted from seaweed.

In all HPLC traces it was observed that the peak width increased with increase in n. Especially for $n > 5$ the peaks became extremely broad, which has also been reported for the separation of other oligomers on this column[27]. This can be explained by the assumption that the larger oligomers gave rise to a decreased diffusion rate through the column packing, which resulted in peak broadening. Also the conformation could have had an effect. We showed that an increased chain length results in a larger conformational freedom. In water the conformation of the oligomer of DP 4 is different from that of DP 1 and the presence of high salt concentrations can induce further conformational changes[5]. The appearance of shoulders on the broad peaks again indicated the presence of the anomers.

The influence of the ionic content of the eluent on the elution of the oligomers on a C_{18} packing was studied with a theory proposed for chromatography with binary mobile phases composed of water and an organic solvent. However, the expressions, such as eqn. 3, remain valid in the extreme case where the concentration of the organic solvent in the mobile phase is zero. In Table II the selectivity of the κ-carrageenan oligomers (log α) and the intercept (log β) are given for two sodium chloride and sodium nitrate eluent concentrations. When log α is positive, the theory predicts that a relatively non-polar repeat structural unit determines the retention. Therefore, it

TABLE II

EXPERIMENTAL VALUES FOR THE SLOPE (LOG α) AND THE INTERCEPT (LOG β) ACCORDING TO THE LINEAR EXPRESSION BETWEEN THE LOGARITHM OF THE CAPACITY FACTOR AND THE DEGREE OF POLYMERIZATION FOR κ-CARRAGEENAN OLIGOMERS WITH VARIOUS SODIUM NITRATE AND SODIUM CHLORIDE ELUENT MOLARITIES

Column: μBondapak C_{18}.

Salt	*Concentration (M)*	*Log α*	*Log β*
$NaNO_3$	$5 \cdot 10^{-2}$	0.138	−0.159
	$5 \cdot 10^{-1}$	0.139	−0.170
NaCl	$5 \cdot 10^{-2}$	0.136	−0.167
	$5 \cdot 10^{-1}$	0.138	−0.112

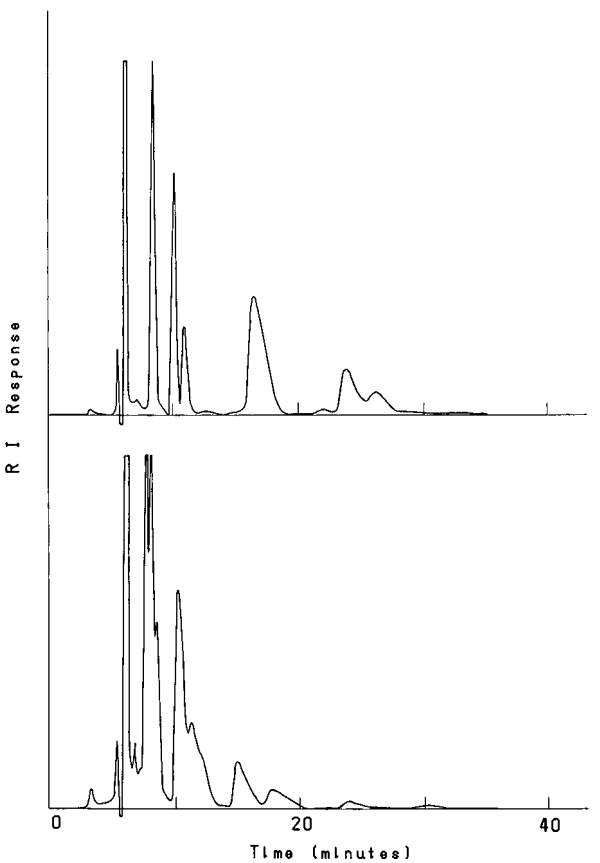

Fig. 7. HPLC on μBondapak C_{18} of the κ-carrageenan oligomers in (top) $5 \cdot 10^{-1}$ M and (bottom) $5 \cdot 10^{-2}$ M sodium chloride solution.

seems reasonable to assume that the separation is based on the hydrophobic interactions between the C_{18} stationary phase and the repeating anhydrogalactopyranosyl unit (Fig. 1).

It is also seen from Table I that the selectivity with sodium chloride and sodium nitrate eluents was constant for both concentrations. This seems normal as the salt was only necessary to screen the electrostatic repulsions. With sodium nitrate, the value of the intercept also remained ralatively constant, which indicated that no large differences in retention volumes were observed with $5 \cdot 10^{-2}$ and $5 \cdot 10^{-1}$ *M* solutions. With sodium chloride, however, we found that a concentration of $5 \cdot 10^{-2}$ *M* was as effective for the separation as $5 \cdot 10^{-1}$ *M* sodium nitrate (Table II and Fig. 7). Indeed, all peaks were well separated, even the anomers of DP 2, but all were detected at larger retention volumes. However, for higher sodium chloride concentrations, only oligomers up to DP 5 were observed and the others probably adsorbed on the (guard) column. With $5 \cdot 10^{-1}$ *M* sodium sulphate, adsorption was even more pronounced (chromatogram not shown) as only oligomers of DP 1 and DP 2 were detected as broad but well separated peaks.

In analogy with the HPLC analysis of other charged compounds[19], the stationary phase of the column plays an essential role in the separation and in some instances gives rise to adsorption. As the chromatographic behaviour of this series oligomers varied with the type of salt in the eluent, we conclude that the separation with eluent salt concentrations above $5 \cdot 10^{-2}$ *M* is not exclusively dependent on the hydrofobic interactions between the anhydropyranosyl units and the C_{18} groups of the stationary phase. The exact influence of the salt on the solute–stationary phase interaction is not yet clear, however.

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Determination of non-steroidal anti-inflammatory analgesics in solid dosage forms by high-performance liquid chromatography on underivatized silica with aqueous mobile phase

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SUMMARY

A high-performance liquid chromatography procedure for the determination of selected non-steroidal anti-inflammatory analgesics (acetylsalicylic acid, fenbufen, fenoprofen, ibuprofen, indomethacin, ketoprofen, naproxen, sulindac and tolmetin) from pharmaceutical dosage forms has been developed. The individual analytes are extracted from the dosage forms with 0 to 10% aqueous acetonitrile and chromatographed on a 22-cm underivatized silica column at ambient temperature (23 ± 1°C). The mobile phases consisted of 5 m*M* aqueous sodium phosphate–phosphoric acid buffer, pH 2.6 containing 0 to 10% acetonitrile. Accuracy and precision of the method were shown to be excellent. This study was performed to extend the applicability of underivatized silica stationary support with aqueous eluents to the analysis of acidic compounds.

INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) on bonded stationary phases has developed into a major analytical tool for separation and quantitation of analytes. Recently, reports have appeared in the scientific literature describing the separation of basic[1–8] and neutral[9] compounds on underivatized silica using typical reversed-phase mobile phases. These systems showed considerable improvement in peak shape, plate numbers and efficiency as compared to conventional bonded phase chromatography. The predominant retention mechanism for basic compounds was determined to be cation exchange with the silica surface[2–8]. Hydrogen bonding or other non-specific forces were cited for the retention of neutral compounds on silica in the reversed-phase mode[9]. When used with aqueous buffered eluents, the silica surface is deactivated by several layers of adsorbed water over a layer of strongly hydrogen bonded water[10–12]. This makes the silica surface capable of interacting with neutral and possibly acidic analytes.

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This study was designed to explore the applicability of underivatized silica to the analysis of acidic drugs from pharmaceutical dosage forms. Non-steroidal anti-inflammatory analgesics were chosen as test compounds due to their wide use and availability. Chromatographic procedures suitable for dosage form assays of these pharmaceutical preparations were developed using underivatized silica with aqueous buffered acetonitrile eluents.

EXPERIMENTAL

Reagents and chemicals

The structural formulae of the compounds studied are shown in Fig. 1. Fenoprofen calcium and naproxen sodium were purchased from United States Pharmacopeial Convention (Rockville, MD, U.S.A.). Acetylsalicylic acid and salicylic acid were obtained from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). Fenbufen, fenoprofen, ibuprofen, indomethacin, ketoprofen, sulindac and tolmetin were purchased from Sigma (St. Louis, MO, U.S.A.). Commercial tablet and capsule dosage forms of the various non-steroidal anti-inflammatory analgesics were obained at a local pharmacy.

Acetonitrile, methanol and water were HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Monobasic and dibasic sodium phosphate and concentrated phosphoric acid were Baker analyzed reagents.

Instrumentation

Chromatography was performed on an HPLC system consisting of two Varian Model 2510 HPLC pumps (Walnut Creek, CA, U.S.A.) connected to a Varian Model 2584 static mixer, a Rheodyne Model 7125 injector equipped with a 10-μl loop (Cotati, CA, U.S.A.) and a Varian Model 2550 variable-wavelength UV detector. The analytical wavelength was set to the absorbance maximum of each particular analyte. Data acquisition and reduction were performed on a Spectra-Physics Model SP4290 recording integrator (San Jose, CA, U.S.A.).

Separation was accomplished on a 5-μm silica column (220 mm $\times$ 4.6 mm I.D., Brownlee Labs., Santa Clara, CA, U.S.A.) fitted with a 7-μm silica precolumn (15 mm $\times$ 4.6 mm I.D., Brownlee). A second precolumn was placed between the static mixer and the injector to saturate the mobile phase with silica. The column was maintained at ambient temperature (23 $\pm$ 1°C).

UV spectra were obtained using a Beckman Model DU-7 scanning spectrophotometer (Fullerton, Ca, U.S.A.).

Mobile phases

Mobile phase buffers of various pH values and molarities were prepared using the Henderson-Hasselbach equation. The actual pH of each mobile phase was measured carefully for subsequent calculation of ionic strength. All mobile phases were filtered through a 0.45-μm nylon-66 filter (MSI, Westborough, MA, U.S.A.) and degassed by sonication. The flow-rate was set at 1–1.1 ml/min.

Preparation of standard solutions

Standard solutions of each drug were prepared by dissolving 0, 2, 4 and 6 mg of

the drug in 100 ml of aq. acetonitrile containing the same concentration of acetonitrile as the appropriate mobile phase. A four-point standard curve was constructed for each analyte.

Analysis of dosage forms

Capsule contents and tablets were weighed and finely ground. An accurately weight portion, equivalent to 4 mg drug substance was transferred to a 100-ml volumetric flask. Approximately 80 ml of aqueous acetonitrile containing the same concentration of acetonitrile as the mobile phase was added and the flask was placed in an ultrasonic bath for 5 min. The mixture was allowed to cool, diluted to volume and mixed in a mechanical shaker for 2–3 min. Any remaining solids in the mixture were allowed to settle. An aliquot of the solution was drawn up and filtered through a 0.2-μm nylon-66 syringe filter (Lida Manufacturing, Bensenville, IL, U.S.A.) prior to injection into the HPLC system. Quantitation was based on linear regression of peak heights.

RESULTS AND DISCUSSION

The goal of this study was to demonstrate the applicability of an underivatized silica stationary support and aqueous buffered eluents to the analysis of drug substances containing the carboxylic acid moiety. Non-steroidal anti-inflammatory analgesics were chosen as test compounds (Fig. 1). Not only are these drugs widely available, but they also exhibit enough variation in structure and functional group chemistry to provide a representative sample of acidic compounds of pharmaceutical interest.

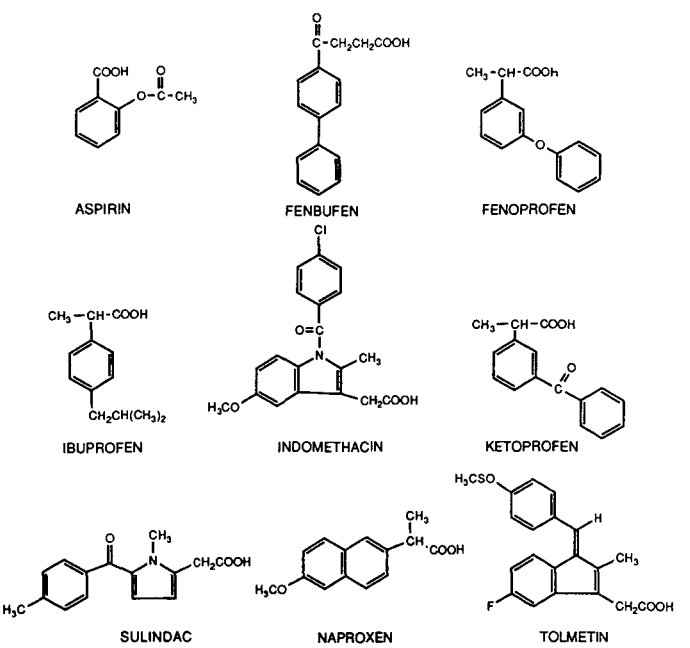

Fig. 1. Structures of non-steroidal anti-inflammatory analgesics.

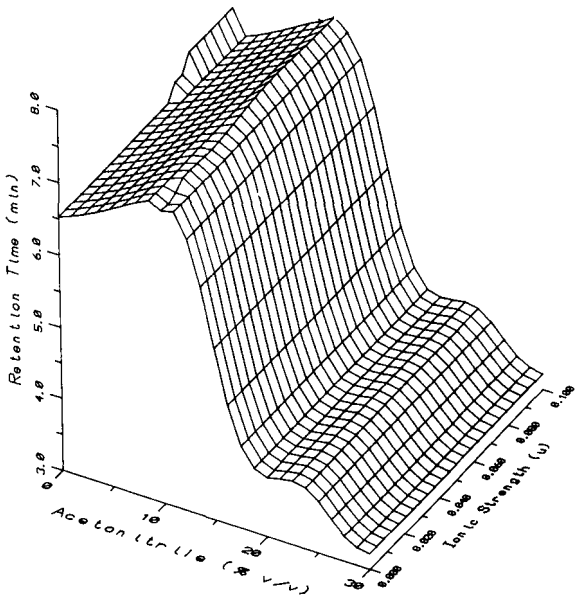

Fig. 2. Response surface of fenbufen. Response surface was generated from retention data, ionic strength and organic modifier concentration of the mobile phase. Mobile phase: sodium phosphate–phosphoric acid buffer, pH 2.5; flow-rate: 1 ml/min.

There are no reports in the scientific literature describing the reversed-phase separation of acidic compounds on silica. Therefore, several mobile phases differing in ionic strength, concentration and type of organic modifier and pH were prepared and tested for the chromatography of these non-steroidal anti-inflammatory analgesics.

It has been reported that not only the ionic strength but also the type of buffer and competing cation influences the retention of basic analytes on underivatized silica[13]. Therefore sodium phosphate buffers were chosen to prepare the mobile phases since phosphate buffer covers a wide pH range. The buffer pH of each mobile phase was not adjusted since the actual amounts of sodium phosphate and phosphoric acid were calculated using the Henderson–Hasselbach equation. The final pH of each mobile phase was then accurately measured and the ionic strength of the buffer was calculated. The pH varied from 2.5 to 7.5 with calculated ionic strengths of 0 to 0.1 U and 0 to 40% organic modifier.

Ionic strength of the mobile phase had a minor effect on retention as shown for fenbufen in Fig. 2. Increases in buffer concentration of the mobile phase only increased retention slightly. Increasing the ionic strength of the mobile phase shifted the equilibrium of the carboxylic acid to the unionized species. In this form, the analyte is able to interact more effectively with the stationary phase. To avoid excessive pump seal wear, the buffer strength was held to 5 m*M*.

As observed previously in this laboratory on the chromatography of neutral compounds[9], the concentration of organic modifier in the mobile phase was the

TABLE I

EFFECT OF ACETONITRILE CONCENTRATION AND MOBILE PHASE pH ON RETENTION OF SELECTED ANALGESICS ON UNDERIVATIZED SILICA

Compound	*Acetonitrile (%)*[a]			*pH*[b]		
	10	*20*	*30*	*2.5*	*5.1*	*7.5*
Tolmetin	6.3[c]	3.7	3.2	–[d]	–	–
Sulindac	–[d]	–	–	15.2[c]	12.1	4.5

[a] Mobile phase: 5 m*M* sodium phosphate/phosphoric acid pH 2.5–acetonitrile; flow-rate was 1 ml/min with detector set at 254 nm.

[b] Mobile phase: 20 m*M* sodium phosphate/phosphoric acid–acetonitrile, (90:10, v/v) flow-rate was 1 ml/min with detector set at 254 nm.

[c] Retention time in minutes.

[d] Not applicable.

predominant parameter affecting retention (*i.e.* fenbufen in Fig. 2, tolmetin in Table I). Furthermore, increases in the organic modifier beyond 30% were shown to move the analyte peak into the solvent front. This identical retention behavior was also observed with all the other analgesic compunds. The type of organic modifier in the mobile phase also affected retention. Substituting an equal concentration of methanol for acetonitrile caused an approximate doubling in retention and deterioration in peak shape. Peak width increased and tailing was evident on late eluting peaks (capacity factor, $k' > 5$). Similar chromatographic behavior was observed in our earlier study of neutral compounds on underivatized silica with reversed-phase eluents. As explained by Scott *et al.*[10–12], the deactivated silica stationary phase has one layer of water strongly hydrogen bonded to the surface silanol groups, 2 to 3 layers of water are more loosely held over the strongly held primary water layer. Being able to act both as a proton donor and acceptor, methanol can interfere with the formation of the secondary and tertiary water layer, replacing water molecules and, hence, altering the stationary phase. These changes result in a more lipophilic stationary phase that will alter the partitioning of the unionized acidic analytes into the stationary phase and behave analogous to non-polar bonded phase chromatography.

The pK_a values of these analgesic drugs range between 3.5 and 4.6. At a mobile phase pH greater than 5, the silanols are ionized and the analytes would be expected to pass unretained through the column due to charge–charge repulsion. As was shown for sulindac in Table I, it was surprising to observe retention of the drug on the silica column at a mobile phase pH of 7.5 where the analyte is totally ionized. Even though retention of all of the analgesics was shown to decrease considerably with increasing pH, there was enough interaction between each analyte and silica to allow retention.

It has been our experience that the silica column has shorter equilibration times, less prominent solvent fronts and is much more stable when operated at a low pH[8,9]. Since low pH was more applicable to these separations, the mobile phase pH was held at 2.5 for the analysis of the dosage forms.

Traditionally, silica has been used in conjunction with less polar mobile phases in normal phase chromatography. The current use of normal phase chromatography

TABLE II

COMPARISON OF RELATIVE RETENTION BEHAVIOR OF SELECTED ANALGESICS ON OCTADECYLSILANE *versus* UNDERIVATIZED SILICA

Octadecylsilane[a]		*Underivatized silica*[b]	
Compound	*k'*	*Compound*	*k'*
Tomletin	3.77	Sulindac	0.7
Sulindac	5.02	Fenoprofen	1.1
Ketoprofen	5.98	Ibuprofen	1.1
Naproxen	6.59	Naproxen	1.3
Fenbufen	7.46	Tolmetin	1.4
Fenoprofen	8.54	Ketoprofen	1.5
Indomethacin	8.86	Fenbufen	2.6
Ibuprofen	10.01	Indomethacin	3.6

[a] Values derived from solvent programming using acetonitrile–0.05 *M* acetate buffer pH 4.5 at a column temperature of 35°C; flow-rate of 0.8 ml/min and detector set at 254 nm (see ref. 17).
[b] See Table IV for chromatographic conditions.

in drug analysis is rather limited when compared to reversed-phase chromatography on bonded phases. The latter technique is employed for the vast majority of all analytical separations of drugs[14,15]. Factors governing analyte retention in an underivatized silica system are different from those in bonded phase chromatography. For the separation of basic compounds such as amines and/or quaternary ammonium ions, cation exchange has been identified as the predominant retention mechanism[1–8]. Neutral compounds have also been separated on underivatized silica with an acetonitrile–sodium phosphate buffer eluent. In this instance, the retention mechanism is most likely hydrogen bonding or other non-specific analyte–silica interactions[9]. In both cases, peak shape of the various analytes was equal or improved compared to conventional reversed-phase chromatography. In addition, exceedingly simple mobile phases were used consisting of small amounts of organic modifier in buffer. Columns are very stable and exhibit high efficiencies (up to 70 000 plates/m)[5].

Whereas the predominant retention of basic compounds on underivatized silica is cited to be cation exchange, a different mechanism must be proposed to explain the retention of these acidic analytes. Based on our results, there is most likely a mixed retention mechanism of hydrogen bonding and quasi reversed-phase retention[9,16].

A marginal disadvantage of this underivatized silica system lies in the limited amount of analyte that can be injected at one time. Once the analyte size exceeds 0.5 to 1 μg on column, the tailing factor increases slightly (*ca.* 0.05 to 0.15) and a decrease in retention time is observed (*ca.* 0.2 min). A comparison of the relative retention behavior of selected analgesic compounds on octadecylsilane *versus* underivatized silica is shown in Table II.

Accuracy and precision of the method were evaluated using spiked samples of selected anti-inflammatory drugs. The results are shown in Table III. The above described chromatographic system was then applied to the analysis of acetylsalicylic acid, fenoprofen, ibuprofen, ketoprofen, naproxen and tolmetin in commercialy available dosage forms. The mobile phase was optimized for each compound to

TABLE III

ACCURACY AND PRECISION FROM SPIKED DRUG SAMPLES

	Concentration		*Accuracy (%)*	*R.S.D. (%)*
	Added (μg/ml)	*Found*[a] *(μg/ml)*		
Aspirin	2.50	2.55 ± 0.05	2.00	1.91
	5.50	5.52 ± 0.04	0.36	0.73
Fenoprofen	2.50	2.45 ± 0.31	2.00	1.24
	5.50	5.53 ± 0.11	0.55	0.81
Ibuprofen	2.50	2.53 ± 0.09	1.20	1.01
	5.50	5.51 ± 0.07	0.18	0.68
Ketoprofen	2.50	2.49 ± 0.12	0.40	0.84
	5.50	5.50 ± 0.06	0.00	0.72
Naproxen	2.50	2.54 ± 0.12	1.60	1.00
	5.50	5.52 ± 0.03	0.36	0.87
Tolmetin	2.50	2.52 ± 0.09	0.80	1.02
	5.50	5.49 ± 0.08	1.64	0.56

[a]Based on $n = 3$.

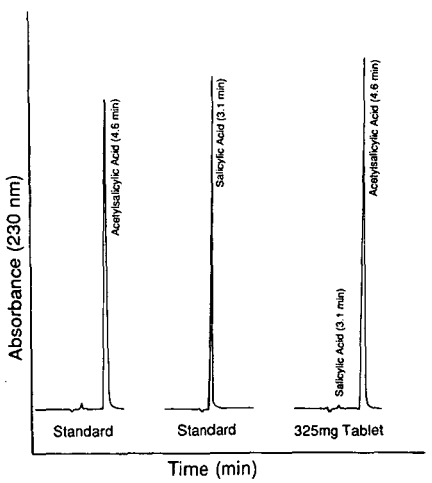

Fig. 3. Typical chromatograms of acetylsalicylic acid and salicylic acid in standard solutions and a solid dosage form (chromatographic conditions in text and Table IV).

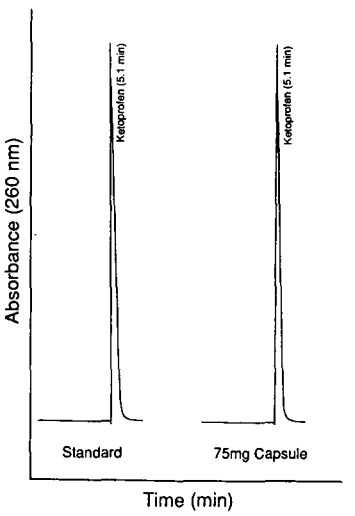

Fig. 4. Typical chromatograms of ketoprofen in standard solution and dosage form (chromatographic conditions in text and Table IV).

TABLE IV

ANALYTICAL FIGURES OF MERIT

	r^{2a}	*System suitability*[b]	*LOD*[c]	k'^{d}	*Theoretical plates*[d]	*Tailing factor*[e]
Aspirin	0.9995	0.95	1 ng at 230 nm	1.4	4957	1.03
Fenbufen	–[f]	1.08	–[f]	2.6	5028	1.07
Fenobrufen	0.9999	1.05	1 ng at 272 nm	1.1	2750	1.04
Ibuprofen	0.9994	1.12	0.5 ng at 225 nm	1.1	2052	1.11
Indomethacin	–[f]	1.10	–[f]	3.6	6352	1.10
Ketoprofen	0.9997	0.99	0.3 ng at 260 nm	1.5	4758	1.02
Naproxen	0.9995	0.99	0.5 ng at 260 nm	1.3	4571	1.01
Salicylic acid	–[f]	–[f]	–[f]	0.6	3462	1.02
Sulindac	–[f]	1.16	0.5 ng at 230 nm	0.7	5963	1.03
Tolmetin	0.9999	1.01	0.4 ng at 260 nm	1.4	3973	1.04

[a] Range examined from 0–60 μg/ml, $n = 4$. Mobile phase consisted of 5 m*M* sodium phosphate buffer, pH 2.6–acetonitrile (95:5, v/v) at 1 ml/min, except for aspirin (100% sodium phosphate buffer).
[b] R.S.D. (%) of 6 replicate injections at analyte concentration of 40 μg/ml.
[c] Limit of detection on column, signal-to-noise ratio of 3.
[d] Determined with mobile phase of 5 m*M* sodium phosphate buffer, pH 2.6–acetonitrile, (95:5 v/v), 1 μg/ml analyte solution in 5% acetonitrile, 220 mm × 4.6 mm I.D., 5 μm Brownlee silica column.
[e] Calculated at 10% peak height.
[f] Not applicable.

obtain a short retention time ($k' < 2.5$) for high sample throughput. For aspirin, the mobile phase consisted of 5 m*M* sodium phosphate–phosphoric acid buffer, pH 2.5 (Fig. 3); for all the other analgesics studied, 50% (v/v) acetonitrile was added to the mobile phase as shown for ketoprofen in Fig. 4. Quantitative recoveries were ob-

TABLE V

RESULTS OF TABLET AND CAPSULE DOSAGE FORM ASSAYS

Compound	*Label strength (mg)*	*Amount found (mg)*[a]	*Percentage of label claim*[a]
Aspirin[b]	325	328.7 ± 2.9	101.1 ± 0.9
Fenoprofen[c]	300	304.8 ± 0.8	101.6 ± 0.3
Ibuprofen[d]	200	201.1 ± 0.5	100.5 ± 0.3
Ketoprofen[e]	75	78.8 ± 0.2	105.0 ± 0.2
Naproxen[f]	250	252.2 ± 0.6	100.9 ± 0.2
Tolmetin[g]	400	398.0 ± 0.7	99.5 ± 0.2

[a] Mean ± S.D. based on $n = 4$.
[b] Enteric coated aspirin, Rugby, Lot No. 010-0488T.
[c] Nalfon, Dista, Lot No. 1FA78A.
[d] Advil, Whitehall Lab., Lot No. 9E09.
[e] Orudis, Wyeth Lab., Lot No. 9880476.
[f] Naprosyn, Syntex, Lot No. 11150.
[g] Tolectin, McNeil Pharmaceuticals, Lot No. DP8613P.

tained for all analytes from the dosage forms. The analytical figures of merit are summarized in Table IV. The results of the dosage form assays are listed in Table V.

CONCLUSION

Underivatized silica with aqueous eluents was shown to be amenable for the separation and quantitation of non-steroidal anti-inflammatory drugs in pharmaceutical dosage forms. This HPLC system has advantages of using simple and inexpensive mobile phases and a comparatively inexpensive and very stable silica column. This study suggests that the use of underivatized silica can be expanded to solve other separation problems where selectivities other than those found in bonded phases are needed.

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Isolation, identification and separation of isomeric truxillines in illicit cocaine

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SUMMARY

Seven out of the eleven possible intact isomeric truxillines present in illicit cocaine have been isolated and identified. These truxilline alkaloids included α-, β-, γ-, δ-, ε-, ω- and *neo*-isomers. The individual truxillines were characterized via high-performance liquid chromatography–diode array detection, capillary gas chromatography–electron ionization mass spectrometry and capillary supercritical fluid chromatography–flame ionization detection. α- and β-truxilline were also identified using nuclear magnetic resonance spectrometry. Good resolution of the intact truxillines was obtained using high-performance liquid chromatography.

INTRODUCTION

The eleven alkaloidal isomeric truxillines, the structures of which are shown in Fig. 1, have been reported recently to be present in illicit cocaine at the <0.01%–1% level[1]. This is not surprising since α- and β-truxilline have been shown to exist in coca leaf[2,3]. The presence of all eleven isomeric truxillines in illicit cocaine was originally based on good indirect evidence provided mainly from the chromatographic and spectrometric analysis of the dimethyl esters of diphenylcyclobutanedicarboxylic acids formed after acid hydrolysis[1]. The truxillic and truxinic acids (isomeric diphenylcyclobutanedicarboxylic acids) are obtained from the acid hydrolysis of the intact truxillines. The detection of these trace constituents in cocaine samples could be important for intelligence purposes, in particular for the comparative analysis of exhibits.

The chromatographic analysis described by Moore *et al.*[1] involved lithium aluminum hydride reduction of the truxillines followed by derivatization with heptafluorobutyric anhydride and analysis via capillary gas chromatography with electron-capture detection (GC–ECD). Sensitivity at the low pg level on-column was obtained. Previous attempts at chromatographing the intact truxillines via capillary GC proved unsuccessful[1]. In contrast, high-performance liquid chromatography (HPLC) and capillary supercritical fluid chromatography (SFC) appeared promising

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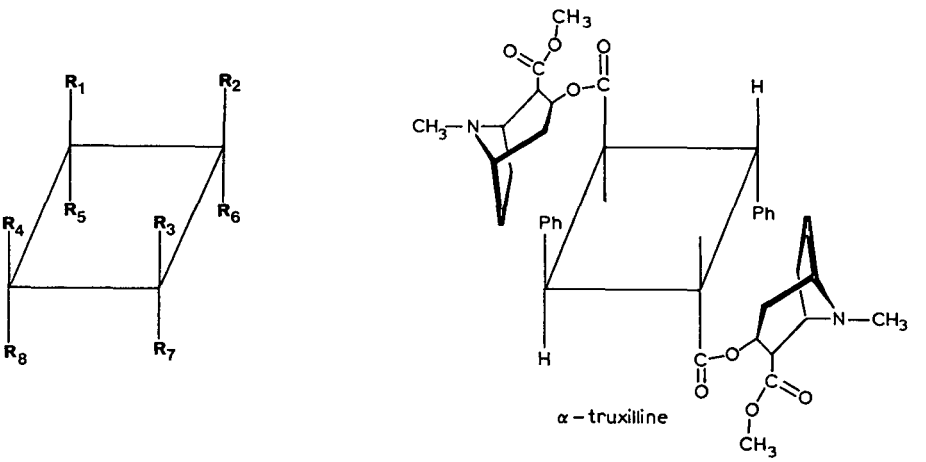

Fig. 1. Structures of the truxillines. (Left) General structure. (1) α-, $R_1 = R_7 =$ methyl ecgonine ester, $R_4 = R_6 =$ phenyl, $R_2 = R_3 = R_5 = R_8 =$ H; (2) β-, $R_5 = R_6 =$ methyl ecgonine ester, $R_3 = R_4 =$ phenyl, $R_1 = R_2 = R_7 = R_8 =$ H; (3) δ-, $R_2 = R_5 =$ methyl ecgonine ester, $R_4 = R_7 =$ phenyl, $R_1 = R_3 = R_6 = R_8 =$ H; (4) ε-, $R_5 = R_7 =$ methyl ecgonine ester, $R_2 = R_4 =$ phenyl, $R_1 = R_3 = R_6 = R_8 =$ H; (5) μ-, $R_1 = R_6 =$ methyl ecgonine ester, $R_4 = R_7 =$ phenyl, $R_2 = R_3 = R_5 = R_8 =$ H; (6) γ-, $R_1 = R_3 =$ methyl ecgonine ester, $R_4 = R_6 =$ phenyl, $R_2 = R_5 = R_7 = R_8 =$ H; (7) *neo*-, $R_2 = R_5 =$ methyl ecgonine ester, $R_3 = R_4 =$ phenyl, $R_1 = R_6 = R_7 = R_8 =$ H; (8) ζ-, $R_5 = R_6 =$ methyl ecgonine ester, $R_4 = R_7 =$ phenyl, $R_1 = R_2 = R_3 = R_8 =$ H; (9) *epi*-, $R_1 = R_7 =$ methyl ecgonine ester, $R_2 = R_4 =$ phenyl, $R_3 = R_5 = R_6 = R_8 =$ H; (10) *peri*-, $R_1 = R_3 =$ methyl ecgonine ester, $R_2 = R_4 =$ phenyl, $R_5 = R_6 = R_7 = R_8 =$ H; (11) ω-, $R_1 = R_2 =$ methyl ecgonine ester, $R_3 = R_4 =$ phenyl, $R_5 = R_6 = R_7 = R_8 =$ H. (Right) Structure of α-truxilline. Ph = phenyl.

for the direct analysis of these compounds[4,5]. Two HPLC systems have been reported for the analysis of the truxillines using reversed-phase chromatography and UV detection[4]. In these works the identities of the individual truxilline peaks were not determined. One system used dodecylsulfate as a counter ion and was good for the simultaneous separation of acids, monoprotic amines and diprotic amines in illicit cocaine. Resolution obtained for seven peaks believed to be truxillines was fair. The second system was used only for the analysis of truxillines and gave good reslution for eight peaks believed to be truxillines. UV detection afforded on-column sensitivity at the low-ng level[6]. Excellent methodology sensitivity was obtained via HPLC because of the ability to use large injection volumes. Capillary SFC analysis of a HPLC size-exclusion extract also believed to contain truxillines has been reported[5]. Sensitivity of these compounds on-column, using UV detection, was at the high-pg level[6]. Capillary SFC was used as a viable alternative to HPLC and GC because of the possibility of a high-resolution separation of the intact isomeric truxillines. In the capillary SFC system the identities of the individual truxillines were not determined.

In this study seven intact truxillines were isolated using previously reported chromatographic methods including alumina column chromatography[1], reversed-phase chromatography[4] and size-exclusion chromatography[5]. Analytical data were obtained for the individual truxillines via HPLC using a diode array detector, by capillary GC using both an electron-capture cell and a low-resolution mass spectrometry (MS) system operated in the electron ionization (EI) modes as detectors, and by

capillary SFC using a flame ionization detector. Two of the truxillines (α and β) were also analyzed via nuclear magnetic resonance (NMR) spectroscopy.

EXPERIMENTAL

Equipment

The equipment used for size-separation chromatography, analytical–preparative HPLC separations, analytical HPLC separations using diode array detection, capillary GC–ECD, capillary SFC with flame ionization detection (FID) and NMR analysis have been described elsewhere[1,4,5]. For capillary SFC analysis various 10 m × 50 μm I.D. fused-silica capillary columns were used (Lee Scientific, Salt Lake City, UT, U.S.A.). The stationary phases included SB-octyl-50 (0.25 μm film thickness), SB-methyl-100 (0.25 μm film thickness), SB-biphenyl-30 (0.25 μm film thickness), SB-cyanopropyl-25 (0.25 μm film thickness) and SB-smectic (0.15 μm film thickness).

For semi-preparative HPLC separations the same equipment used for analytical–preparative analysis was used[4] except the column consisted of a Magnum 9 ODS-3 column (25.0 cm × 9.4 mm I.D.) (Whatman, Clifton, NJ, U.S.A.).

Low-resolution EI-MS spectra were obtained on a Finnigan MAT 4630 (San Jose, CA, U.S.A.) quadrupole mass spectrometer. The column for the capillary GC–MS system consisted of a DB-1 (8 m × 0.25 mm I.D., film thickness 0.25 μm) (J & W Scientific, Rancho Cordova, CA, U.S.A.). Sample injection was accomplished using an on-column injector (J & W Scientific) and helium was used as the carrier gas at a velocity of 40 cm/s. EI data were acquired at an ionization potential of 50 eV and a source temperature of 140°C. Samples were dissolved in chloroform [some solutions contained 25% (v/v) N,O-bis(trimethylsilyl)acetamide (BSA)].

200-MHz proton NMR spectra were obtained as described previously[1]. HPLC fractions were made alkaline with sodium bicarbonate and extracted with deuterated chloroform. The organic layer was backwashed with deuterated water to diminish the presence of hydrophilic artifacts and to reduce the water proton signal. Standards were dissolved in deuterated chloroform and evaporated to dryness in a warm water bath (50°C) under a stream of nitrogen. The solution and evaporation steps were repeated as before in order to remove or otherwise diminish volatile organic solvent impurities. The residue was then taken up with deuterated chloroform and washed successively with sodium bicarbonate–deuterated water and deuterated water.

Materials

Methanol, acetonitrile and tetrahydrofuran (Burdick & Jackson, Muskegon, MI, U.S.A.) were HPLC grade. Other chemicals were reagent grade.

The HPLC mobile phases were internally mixed from solvent reservoirs containing methanol, acetonitrile, tetrahydrofuran, methylene chloride and either phosphate buffer or phosphate buffer containing 0.02 *M* sodium dodecyl sulfate, pH 2.0. The phosphate buffer was a mixture of 3480 ml water, 120 ml 2 *M* sodium hydroxide and 40.0 ml phosphoric acid.

α-Truxilline was prepared from α-truxilloyl chloride and methyl ecgonine in a procedure similar to the one used for the synthesis of *trans*-cinnamoylcocaine[7]. α-Truxilloyl chloride was prepared from α-truxillic acid and thionyl chloride.

α-Truxillic acid was synthesized and characterized as previously described[1]. The synthesized standard was characterized via proton NMR, MS, IR and UV.

β-Truxilline was prepared as above from β-truxilloyl chloride and methyl ecgonine. β-Truxinic acid was synthesized and characterized as previously described[1]. Unlike the α-truxilline synthesis, a mixture of roughly equal amounts of β- and *neo*-truxilline was produced. β-Truxilline was isolated from this mixture using alumina column chromatography, potassium permanganate oxidation and semi-preparative HPLC. The standard was characterized via proton NMR, MS, IR and UV.

Semi-preparative isolation of truxillines

An equivalent of 1.0 g of cocaine–HCl was subjected to extractions and alumina column chromatography[1].

The extract from the column was evaporated to dryness and reconstituted in 5 ml of diethyl ether. Three 1-ml portions were evaporated to dryness. Each fraction was then reconstituted in 200 μl of the HPLC starting mobile phase prior to injection of the sample onto the liquid chromatograph.

Gradient elution for HPLC analysis was used at ambient temperature with a flow-rate of 6.3 ml/min. A 60-min linear gradient from methanol–phosphate buffer (20:80) to (50:50) was used.

Analytical–preparative isolation of truxillines

An illicit cocaine–HCl sample equivalent to 1.0 g cocaine–HCl was dissolved in 20 ml 0.05 *M* sulfuric acid and then extracted with three 20-ml aliquots of methylene chloride. The solution was made basic by the addition of 20.0 ml of a saturated solution of sodium bicarbonate and then extracted with three 40-ml aliquots of methylene chloride. The combined methylene chloride extracts were concentrated to 1 ml on a water bath at 60°C under a stream of nitrogen, prior to nine 100-μl injections into the liquid chromatograph fitted with size-exclusion column.

Size-exclusion chromatography was performed as previously described[5], except the effluent was diverted to a fraction collector.

The pooled fractions of interest from size-exclusion chromatography representing compounds of higher molecular size were evaporated to dryness on a water bath at 60°C under a stream of nitrogen and reconstituted in 500 μl of starting mobile phase prior to injection into the liquid chromatograph.

A previously reported gradient system for the analysis of these compounds[4] was modified as follows. A 30-min linear gradient from methanol–phosphate buffer (20:80) to (50:50) was employed.

RESULTS AND DISCUSSION

α- and β-truxilline were isolated from an illicit cocaine–HCl exhibit using liquid–liquid extractions, alumina column chromatography and semi-preparative HPLC. The proton NMR spectra of the extracted HPLC fractions representing these compounds were consistent with their respective structures in view of spectra previously reported for the dimethyl esters of the corresponding acids (and in contrast with those reported, or otherwise calculated, for the nine other isomers[1,8–10]) and for methyl ecgonine[11] and the relative intensities of selected signals representing the acid portion of each compound to those of the methyl ecgonine moieties.

Most of the major signals observed in the two truxillines exhibited an upfield shift of 0.1 ppm or less as compared with their counterparts in the diphenylcyclobutanedicarboxylic methyl esters and in methyl ecgonine. The phenyl signals, at about 7 ppm, did not shift at all. The tritium absorbance of methyl ecgonine (3.8 ppm) exhibited a dramatic downfield shift in the formation of the truxillines, appearing at 4.5 and 4.6 ppm in the α-isomer and at 5.0 ppm in the β. Deshielding shifts of this magnitude are common for the signals of secondary protons geminal to hydroxyl groups when they undergo esterification (the "acylation shift"[12,13]).

NMR spectra of these two extracts closely matched synthesized α- and β-truxilline, respectively, as shown in Figs. 2 and 3. Minor differences noted between sample and standard, particularly upfield of 1.3 ppm, are ascribed to moisture and other impurities. Interferences of this sort are virtually unavoidable when sub-mg quantities of a compound are extracted from a large chromatographic fraction of a natural substance and spectrally compared with an identical compound synthetically prepared.

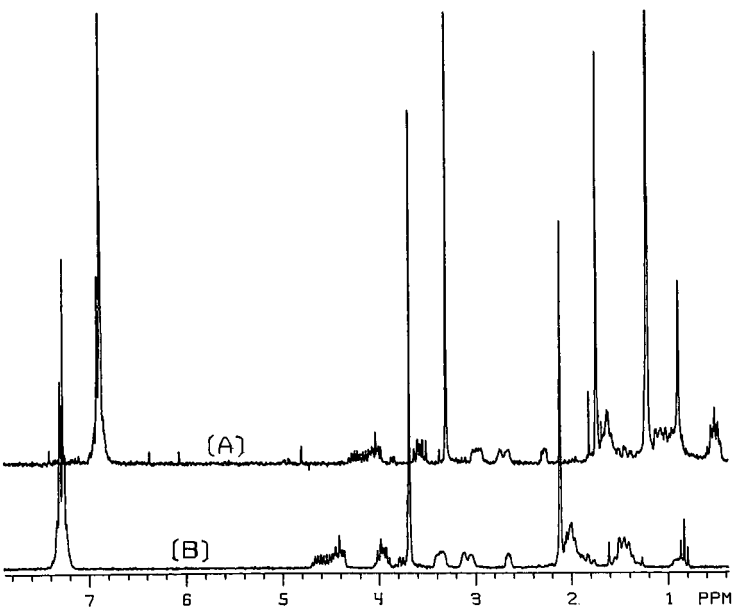

Fig. 2. 200-MHz proton NMR spectra of α-truxilline as obtained from (A) extracted HPLC fraction and (B) synthesized standard.

Seven truxillines, including α- and β-truxilline, were isolated from an illicit cocaine–HCl exhibit using liquid–liquid extractions, size-exclusion chromatography and analytical–preparative HPLC. Size-exclusion chromatography gave a cleaner extract and a more nearly quantitative recovery than alumina column chromatography. HPLC analysis of the truxilline content before and after alumina column chromatography revealed different ratios of the truxilline isomers.

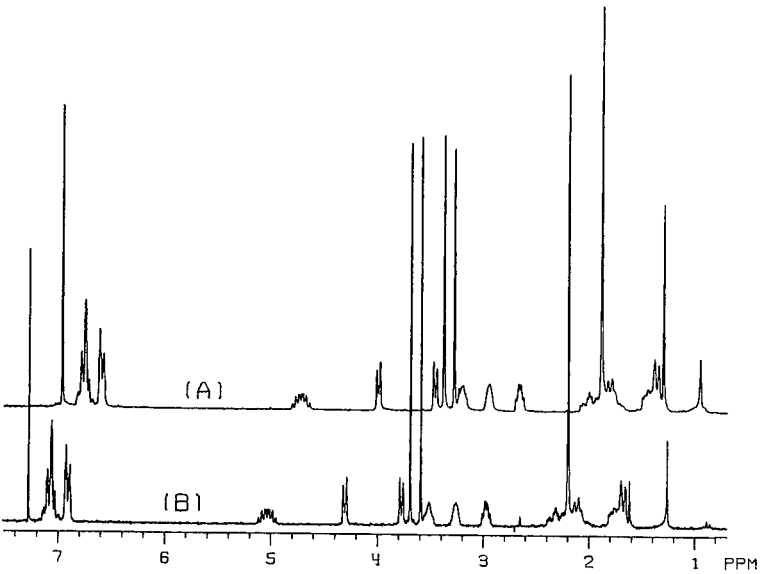

Fig. 3. 200-MHz proton NMR spectra of β-truxilline as obtained from (A) extracted HPLC fraction and (B) synthesized standard.

The seven truxillines isolated via analytical–preparative HPLC dissolved in mobile phase were reanalyzed on the same system with one major peak obtained for each fraction. In addition the seven fractions were also analyzed via HPLC and diode array detection using the previous reported system for the simultaneous analysis of acidic and basic impurities in cocaine[4]; with again only one major peak obtained. UV spectra obtained for the individual peaks using the diode array detector were consistent with compounds containing benzene rings without extended conjugation. An example of a UV spectrum obtained from an isolated fraction is shown in Fig. 4.

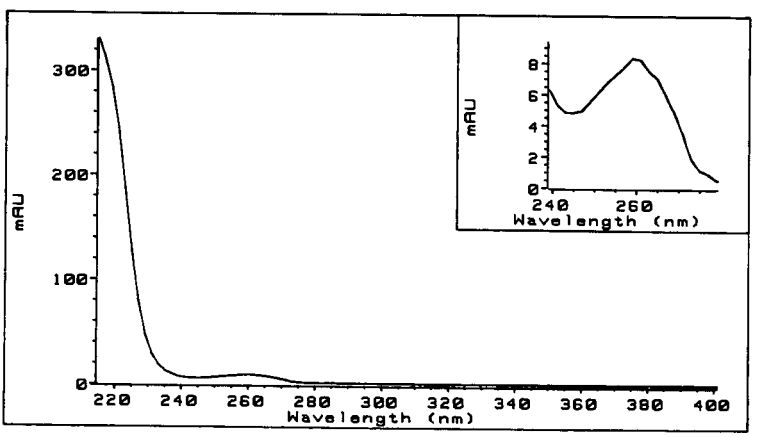

Fig. 4. Apex-UV spectrum for peak obtained from isolated fraction representing α-truxilline. Chromatographic conditions described in ref. 4.

Fractions, each representing a truxilline compound, were then made basic with saturated sodium bicarbonate and extracted with methylene chloride prior to capillary GC–ECD, capillary GC–MS and capillary SFC–FID analysis.

The isolated truxillines were subjected to lithium aluminum hydride reduction[a] followed by derivatization with heptafluorobutyric anhydride (HFBA) and capillary GC–ECD analysis[1]. Each truxilline fraction gave peaks matching ecgonine and, as expected, a truxillic or truxinic acid. The resulting data obtained on the truxilline isolates were correlated to the work of Moore *et al.*[1] which in turn allowed the identification of the respective truxillines as the α-, β-, γ-, δ-, ε-, ω- and *neo*-isomers. GC–ECD analysis also revealed that minor amounts of the *peri*- and/or *epi*- and ζ-isomers were also present in a few of the isolated fractions.

Direct spectrometric proof that the isolated fractions contained truxillines was provided by capillary GC–MS under EI conditions. Previous attempts at chromatographing intact truxillines via capillary GC using either a standard Grob injection port with a DB-1 stationary phase or an on-column injector with a DB-1701 stationary phase proved unsuccessful[1]. However, in this work it was found that a cold (less than 100°C) on-column injection of the truxilline in a solution of methylene chloride–BSA (75:25, v/v) into a fused-silica capillary column (DB-1) resulted in a chromatogram having less than 10% decomposition products of the respective truxilline[b], that is, *ca.* 90% of the truxilline chromatographed without decomposition. Without the addition of BSA, the response obtained for the truxilline was estimated to be *ca.* 25% of expected. The improved performance with BSA is believed to be due to the masking of active sites by the silylating reagent.

EI-MS data for the truxillines examined in this work are shown in Table I. Although all of the truxilline spectra are very similar, they can be distinguished from one another easily as the relative abundances of several ions are sensitive to the intra-molecular stereochemical relationships of these molecules. MS data obtained for the various suspected truxillines correlated very well with those obtained for standard α-truxilline. The capillary GC–MS data for isolated α-truxilline were in excellent agreement with those obtained for standard α-truxilline. The total ion chromatogram for the capillary GC–MS analysis of the cocaine extract before fraction collection is shown in Fig. 5. Based on a comparison of the capillary GC–MS data with the capillary GC–ECD results, small amounts of ζ-, *epi*- and *peri*-truxilline were also believed to be present.

The chromatogram of the various truxilline isomers on the reversed-phase HPLC system used to isolate these compounds is shown in Fig. 6. This system, optimized just for isomeric truxillines, was developed based on the model of Kirkland and Glajch[14]. The gradient optimization scheme was carried out using binary gradients at approximately equal solvent strengths. A phosphate buffer (pH 2.0) was used with the modifiers methanol, acetonitrile and tetrahydrofuran. The retention characteristics of the truxilline isomers on the HPLC system used for the simultaneous analysis of acidic and basic impurities in cocaine[4] are shown in Fig. 7. The latter

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[a] The lithium aluminum hydride reduction of 1 mol of a truxilline results in the production of two moles of a tropanediol and 1 mol of a diphenylcyclobutanediol.

[b] The thermal decomposition products of a truxilline are 2-carbomethoxytropidine and either one or both of the two possible hydrolysis products of the respective truxillic or truxinic acid.

TABLE I

MASS SPECTRUM ABUNDANCES OF TRUXILLINES

Mass	*Relative abundance*						
	Truxilline						
	δ	*Neo*	*β*	*ε*	*ω*	*γ*	*α*
82	6370	6050	5430	5260	5770	5050	5610
83	2390	2590	2730	2100	4370	2870	3070
94	2530	2360	2050	2190	2160	2070	2160
95	430	450	310	380	380	360	340
96	1900	2110	1920	1510	1610	1940	1650
98	120	120	70	80	100	120	70
102	1	70	5	40	1	1	40
103	170	270	290	280	390	290	380
108	320	280	300	290	150	220	280
119	320	390	270	250	270	230	210
120	130	130	90	100	10	90	110
122	540	550	460	480	410	400	480
124	360	230	190	250	100	180	140
131	260	550	440	900	900	600	1220
150	600	690	430	500	60	160	140
152	410	380	190	260	170	210	210
155	160	130	150	180	110	120	170
166	140	160	90	110	10	50	110
168	250	270	300	220	290	280	420
180	350	280	230	120	120	110	170
182	10 000	10 000	10 000	10 000	10 000	10 000	10 000
194	60	60	50	10	1	1	20
198	640	540	460	470	570	480	760
233	40	60	10	70	1	40	90
234	30	50	10	20	1	30	20
238	1	90	180	20	1	1	80
239	1	1	20	1	1	1	6
298	30	1	40	20	1	1	30
313	40	60	1	1	1	50	1
329	160	190	590	140	10	70	160
330	30	80	550	20	90	10	190
432	50	1	10	1	1	1	1260
433	5000	4440	2740	5000	770	2960	1200
476	180	280	90	40	1	1	140
477	10	10	5	140	1	1	130
478	290	360	420	220	1	80	130
515	200	60	60	160	20	1	20
577	40	60	180	40	1	160	60
627	1	1	50	50	210	100	520
630	130	100	70	100	1	1	60
658	1360	1810	870	1440	2400	840	3190

chromatographic system, although not optimum for the analysis of truxillines, gives the "best" overall separation via HPLC of acids, monoprotic amines and diprotic amines present in illicit cocaine. This was accomplished using a similar optimization scheme as above with a phosphate buffer (pH 2.0) containing dodecyl sulfate[4]. Acidic

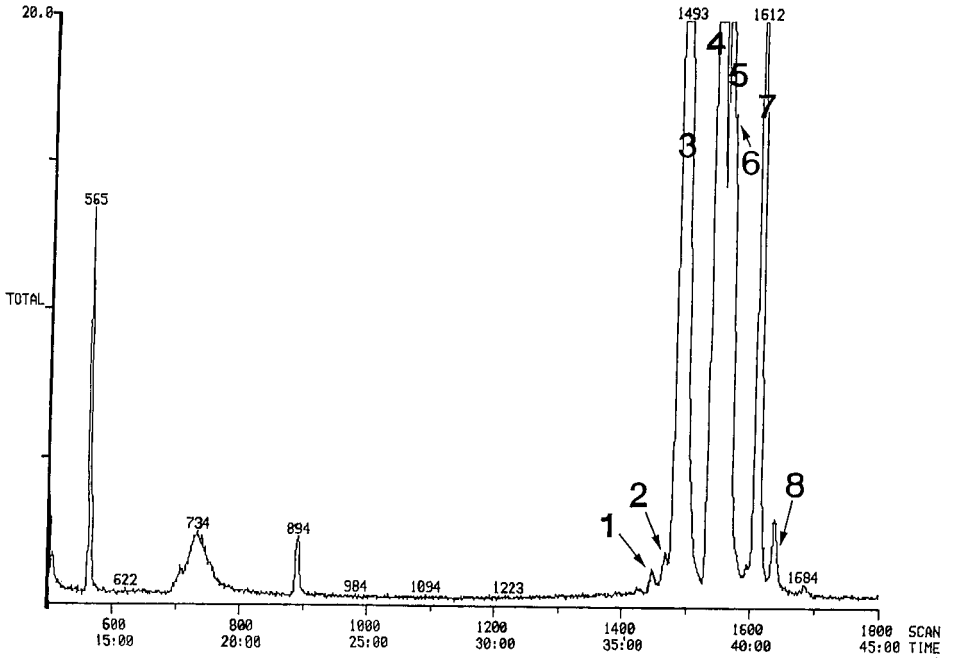

Fig. 5. Total ion chromatogram of illicit cocaine impurities including isomeric truxillines isolated via size-exclusion chromatography. Peaks: 1 = possibly ζ-truxilline; 2 = ω-truxilline; 3 = β-truxilline; 4 = *neo*-, α- and γ-truxilline; 5 = δ-truxilline; 6 = possibly *epi*- or *peri*-truxilline; 7 = ε-truxilline; 8 = *peri*- or *epi*-truxilline. A DB-1 column was used with helium carrier gas at a linear velocity of 55 cm/s. The initial temperature was held at 120°C for 2 min, programmed to 220°C at a rate of 15°C/min and finally programmed to 295°C at a rate of 2°C/min.

compounds are little affected by the addition of dodecyl sulfate while the retention of basic compounds increases, with the increase in retention for diprotic amines double that of monoprotic amines[4]. For the above HPLC systems the possibility exists based on the capillary GC–ECD and capillary GC–MS experiments that additional truxilline isomers may be present under the ε- and β-truxilline peaks.

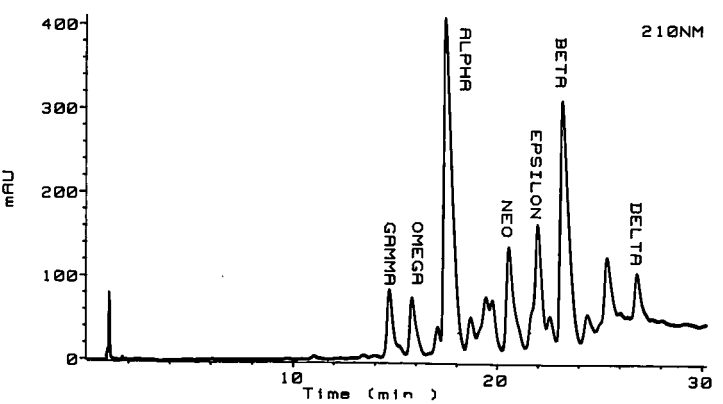

Fig. 6. HPLC of illicit cocaine impurities including isomeric truxillines isolated via size-exclusion chromatography. Chromatographic conditions are described under Experimental pertaining to analytical–preparative isolation of truxillines.

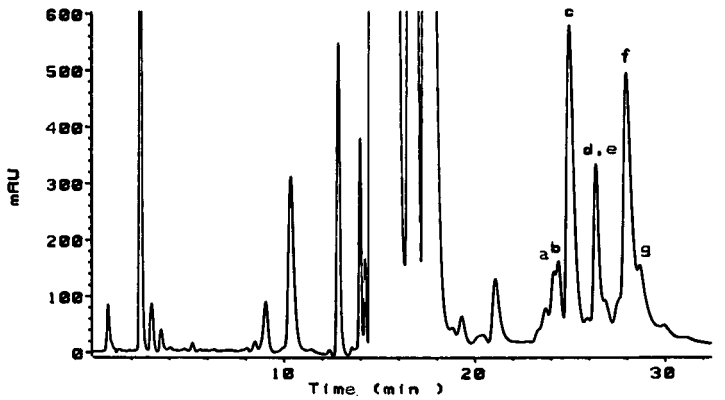

Fig. 7. HPLC at 215 nm (20-nm bandpass) of an illicit cocaine–HCl sample. Peaks: a = γ-truxilline; b = ω-truxilline; c = α-truxilline; d = *neo*-truxilline; e = ε-truxilline; f = β-truxilline; g = δ-truxilline. Chromatographic conditions and sample preparation described in ref. 4.

As shown in Figs. 6 and 7, except for *neo*- and ε-truxilline which coelute in the system containing dodecyl sulfate, identical retention order was obtained for the truxillines on two different reversed-phase systems. The retention properties of α-, β-, ε- and δ-truxillines are vastly different from what has been reported for N,N-dimethyl-

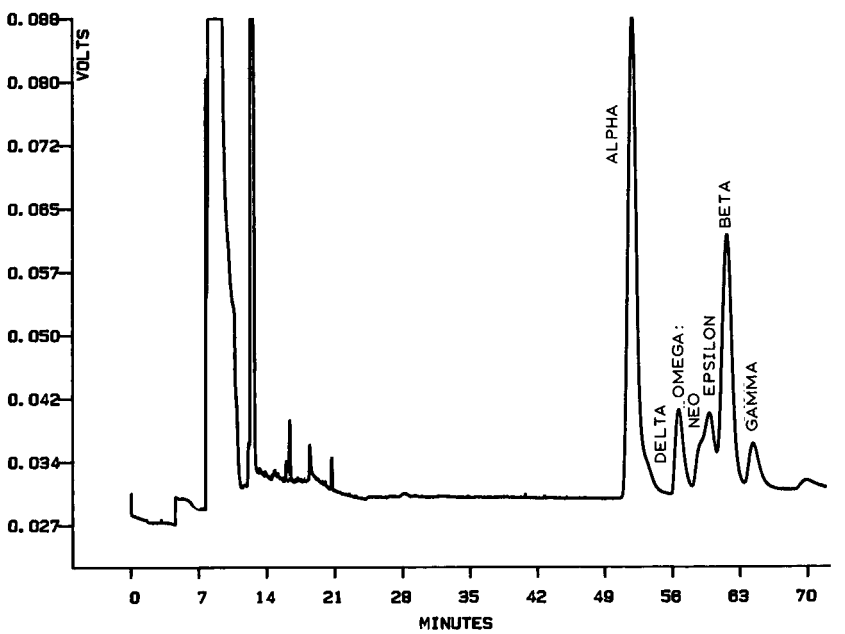

Fig. 8. Capillary SFC of illicit cocaine impurities including isomeric truxillines isolated via size-exclusion chromatography. An SB-biphenyl-30 capillary column was used with density programming for carbon dioxide at 80°C. A density ramp was employed where the initial density was held at 0.25 g/ml for 5 min, then linearly increased to 0.35 g/ml at 6 min and finally linearly increased to 0.65 g/ml at 18.5 min prior to holding the final density.

amides of the same parent truxillic and truxinic acids[15]. The separation of the diamides was explained by differences in the stereochemistry of the solute molecules which allowed various degrees of overlap of the non-polar portions with the alkyl stationary phase. It would appear the polar methyl ecgonine moiety in the truxilline molecule is a major cause of the differences in retention behavior of the truxillines and the truxillic and truxinic diamides. Solute properties such as non-polar surface area, dipole moment and charge have been shown to contribute to retention in reversed-phase chromatography[16].

Despite the use of various stationary phases and experiments at different temperatures only a partial separation of truxilline isomers was obtained via capillary SFC as shown in Fig. 8. The stationary phases investigated included a 100% methyl polysiloxane, a 50% *n*-octyl–50% methyl polysiloxane, a 25% cyanopropyl–25% phenyl–50% methyl polysiloxane, a liquid crystalline polysiloxane and a 30% biphenyl–70% methyl polysiloxane. For the latter stationary phase temperatures of 80–140°C were used.

For the various chromatographic techniques investigated, the best separation for the γ-, ω-, α-, *neo*-, ε-, β- and δ-isomers was obtained via HPLC where baseline resolution was achieved. As shown in Figs. 4, 5 and 8, vastly different retention orders were obtained for the isomeric truxillines using capillary GC, HPLC and capillary SFC. Depending on the technique, π–π, dipole–dipole, dispersion, and hydrogen-bonding interactions as well as sample volatility and sample stereochemistry can play a role in the various separation mechanisms.

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Determination of the charge of ions by partition coefficient measurements in gel permeation chromatography

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SUMMARY

A method is presented for determining the charge of ions from data on their gel permeation chromatographic partition coefficients with two eluents containing different electrolytes over a range of ionic strengths. The method was verified by measurements on Bio-Gel P-4 with simple inorganic ions of known charge ranging from -4 to $+2$.

INTRODUCTION

In a recent paper[1] we proposed a method for the determination of the charge of ions from partition measurements in aqueous polymer two-phase systems, containing two different electrolytes. Briefly, the concept of this method is as follows. As the two phases of such a system have different polarities, the partition constant of any substance is not exactly unity. This is particularly true for (highly) charged ions, leading to partition constants of electrolytes (strongly) dependent on their chemical nature. Therefore, if a trace amount of an ion P^z is added to such a two-phase system containing an electrolyte NX, its partition coefficient can be expected to depend on K_{NX} and on its charge z, as electroneutrality must be maintained in both phases. From the combined results in the presence of two different electrolytes (NX and NY), the charge z can be derived. The method was verified in sodium perchlorate and sodium sulphate containing polyethylene glycol–dextran systems with simple inorganic ions of known charge. It was argued that this method has distinct advantages over charge determination by ion-exchange chromatography, mainly because measurements can be performed at much lower ionic strengths, permitting activity coefficient corrections and extrapolation of the experimental data to zero ionic strength.

However, a few drawbacks of the method should be mentioned. As a result of the high viscosity of the two polymer layers, concentrations must be expressed as molalities (mol kg^{-1}). Further, the calculation of activity coefficients requires assumptions about the dielectric constants of the two polymer layers.

Gel permeation chromatography can be considered as a special case of partition

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in an aqueous polymer two-phase system: the gel particles and the eluent represent two phases of different polymer concentration and therefore different polarity. Hence, also in this case, when using an eluent containing an electrolyte NX, the transfer of an ion P^{z+} from the eluent to the gel phase must be accompanied by a simultaneous transfer of z ions X^- from the eluent to the gel phase (or of z ions N^+ from the gel phase to the eluent). Therefore the partition coefficient of P^z depends on K_{NX} and z.

The analogy between the two methods was described in previous papers[2,3]. This means that gel permeation chromatographic partition experiments using two eluents containing different electrolytes can also be used for charge determination. It also means that the advantage mentioned above for the aqueous polymer two-phase partition method holds for the gel permeation chromatographic method. Moreover, the latter is simpler. First, as the partition coefficients are derived from elution volumes, a single universal detector can be used for all the ions investigated. Further, there is no need for the use of a molality concentration scale. Finally, if eluents of equal ionic strength are used, activity coefficient corrections are simpler and the dielectric constant of water can be used for the solvent in the gel phase.

In this work, the gel permeation chromatographic method was verified by experiments on Bio-Gel P-4, using sodium perchlorate and sodium sulphate as the eluent electrolytes, with simple inorganic ions of known charge ranging from -4 to $+2$.

THEORY

For the partition of a trace amount of an ion with charge z, P^z, in an aqueous polymer two-phase system, containing the electrolytes NX and N_2Y, respectively, an equation was derived (eqn. 7 in ref. 1) showing that $\Delta\log K_{P^z} \equiv \log [K_{P^z}(NX)/K_{P^z}(N_2Y)]$ and the charge z are proportionally related, where K_{P^z} represents the thermodynamic partition constant of the ion P^z.

For gel permeation chromatography, using the eluent electrolytes NX and N_2Y, this equation can be written as

$$\Delta\log K_{P^z} = \Delta\log K'_{P^z} + \log\left[\frac{y_S(NX)\, y_M(N_2Y)}{y_M(NX)\, y_S(N_2Y)}\right] = z\Delta\log K_{N^+} \qquad (1)$$

where the subscripts S and M denote the stationary and mobile phase, respectively, K' is the partition coefficient, *i.e.*, the ratio of the concentrations in the two phases ($K' = c_S/c_M$), y is the activity coefficient of P^z on the molar scale and $\Delta\log K'$ represents $\log [K'(NX)/K'(N_2Y)]$. For NX- and N_2Y-containing eluents of equal ionic strength, the activity coefficients of P^z in the two eluents can be taken as equal, *viz.*, $y_M(NX) = y_M(N_2Y)$, which simplifies eqn. 1 to

$$\Delta\log K_{P^z} = \Delta\log K'_{P^z} + \log\left[\frac{y_S(NX)}{y_S(N_2Y)}\right] = z\Delta\log K_{N^+} \qquad (2)$$

Eqn. 2 predicts that a plot of $\Delta\log K_{P^z}$ for several ions of different charge (data that can be obtained by measuring $\Delta\log K'_{P^z}$ for these ions and correcting for the activity

coefficient term, see below) *versus* z gives a straight line passing through the origin with a slope $\Delta\log K_{N^+}$. In this paper eqn. 2 will be verified.

The way in which corrections of $\Delta\log K'_{P^z}$ for the activity coefficient term were made is outlined below. Clearly, the error of such corrections decreases with decreasing ionic strength of the NX and N_2Y solutions. Therefore, we performed measurements of $\Delta\log K'_{P^z}$ at three low levels of the ionic strength I of the eluent ($I = 1/2 \sum_i z_i^2 c_i$), *viz.*, 0.30, 0.10 and 0.03 mol l^{-1}, and we extrapolated the corrected data to zero ionic strength. As shown in the Appendix, the activity coefficient term occurring in eqn. 2 can be written as

$$\log\left[\frac{y_S(NX)}{y_S(N_2Y)}\right] = -z^2A\sqrt{I}f(I,\mathring{a}_i) - f'(c) + C'I \tag{3}$$

Substitution in eqn. 2 yields

$$\Delta\log K_{P^z} = \Delta\log K'_{P^z} - z^2A\sqrt{I}f(I,\mathring{a}_i) - f'(c) + C'I \tag{4}$$

where f and f' are known functional relationships and C' is an unknown constant. This equation shows that linear extrapolation of $[\Delta\log K'_{P^z} - z^2A\sqrt{I}f(I,\mathring{a}_i) - f'(c)]$ to $I = 0$ yields $\Delta\log K_{P^z}$.

EXPERIMENTAL

Chemicals and apparatus

The following chemicals and materials were used: Bio-Gel P-4 (200–400 mesh) (Bio-Rad Labs., Richmond, CA, U.S.A.), $NaClO_4 \cdot H_2O$ and Na_2SO_4 (Analyzed, Baker, Deventer, The Netherlands), $^{22}NaCl$ (Amersham, Little Chalfont, U.K.), $^{99}Mo/^{99m}Tc$ generator (Mallinckrodt, Petten, The Netherlands), human serum albumin (Sigma, St. Louis, MO, U.S.A.) and $K_4Mo(CN)_8$ (gift from the laboratory of inorganic chemistry, University of Utrecht, The Netherlands). All other chemicals [$MgCl_2 \cdot 6H_2O$, methanol, NaCl, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6 \cdot 3H_2O$] were of analytical-reagent grade.

The chromatographic equipment consisted of a column (C 10/40), flow adapter (AC10), laboratory valve (LV4), gel and eluent reservoir (RC 10), polyethylene tubing (Pharmacia, Uppsala, Sweden), peristaltic pump (Minipuls-2; Gilson, Villiers-le-Bel, France), differential refractometer (R 403; Waters Assoc., Milford, MA, U.S.A.) and a well-type NaI(Tl) scintillation crystal, HV supply and ratemeter (Baird Atomic, Bedford, MA, U.S.A.).

Sample preparation

Sample solutions of $MgCl_2$, methanol, NaCl, Na_2SO_4, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and $K_4Mo(CN)_8$ were prepared by dissolution at a concentration of 3–5 mM in the eluents. Sample solutions of $^{22}NaCl$ in the eluents were prepared by adding 50 μl of a 1 mM solution of NaCl, spiked with $^{22}NaCl$, to 5 ml of eluent. Sample solutions of $Na^{99m}TcO_4$ in the eluents were prepared by addition of 50 μl of the generator eluate in

0.15 *M* NaCl to 5 ml of eluent. For human serum albumin, 0.2‰ solutions in the eluents were used.

Chromatography

The pretreatment of Bio-Gel P-4 and the packing of the column (38 × 1.0 cm I.D.) were performed as recommended by the manufacturer. Aliquots of about 0.15 ml of the samples were applied and eluted at a flow-rate of 8.5–9.5 ml h^{-1}. Flow-rates were accurately determined by weighing the column effluent. The eluents used were $NaClO_4$ and Na_2SO_4 solutions of ionic strength 0.30, 0.10 and 0.03 mol l^{-1}.

Calculation of partition coefficients

Elution volumes were corrected for the extra-column dead space. Partition coefficients, K'_{P^z}, were calculated from

$$K'_{P^z} = \frac{V_e - V_0}{V_1 - V_0}$$

where V_e is the corrected elution volume of P^z, V_0 is the corrected elution volume of human serum albumin and V_1 is the total liquid volume in the column, obtained from $V_1 = V_{bed} - W/\rho$, where V_{bed} is the bed volume, W is the weight of Bio-Gel P-4 used in packing the column and ρ is its density. For ρ an arbitrary value of 1.1 g ml^{-1} was taken; this value results in a mean value of $\Delta \log K'_{CH_3OH} = 0$, averaged over four levels of the ionic strength (I = 0.005 mol l^{-1} was also used).

RESULTS AND DISCUSSION

The experimental K'_{P^z} values are given in Table I. Note, that $K'_{SO_4^{2-}}(Na_2SO_4)$, measured by eluting a small excess of SO_4^{2-} in Na_2SO_4 eluents, is not equal to $K'_{Na^+}(Na_2SO_4)$, measured by eluting a small amount of ^{22}Na in Na_2SO_4 eluents. This may be due to some ion-exchange behaviour of the gel; it is well known that Bio-Gel

TABLE I

PARTITION COEFFICIENTS, K'_{P^z}, OF DIFFERENT SPECIES IN THE PRESENCE OF $NaClO_4$ AND Na_2SO_4 OF IONIC STRENGTH 0.3, 0.1 AND 0.03 mol l^{-1}

Species	*$NaClO_4$*			*Na_2SO_4*		
	0.3	*0.1*	*0.03*	*0.3*	*0.1*	*0.03*
Mg^{2+}	1.962	2.191	2.301	1.044	1.211	1.428
Na^+	1.321	1.382	1.553	1.016	1.069	1.235
MeOH	1.016	0.951	1.014	0.979	1.018	0.998
TcO_4^-	1.903	1.934	2.037	2.712	2.668	2.503
Cl^-	0.942	0.916	0.935	1.194	1.158	1.120
SO_4^{2-}	0.694	0.663	0.657	0.928	1.018	1.029
$Fe(CN)_6^{3-}$	0.861	1.032	1.037	1.889	1.913	1.968
$Fe(CN)_6^{4-}$	0.621	0.724	0.776	1.415	1.529	1.725
$Mo(CN)_8^{4-}$	0.933	1.149	1.351	2.255	2.793	2.933

contains some carboxylate groups that are negatively charged at neutral pH. This leads to a slight increase in $K'_{Na^+}(Na_2SO_4)$ by ion-exchange and to a slight decrease in $K'_{SO_4^{2-}}(Na_2SO_4)$ by ion exclusion (measurements at $I = 0.005$ mol l^{-1} showed these effects to a large extent and are therefore not reported). A second effect that may be responsible for this discrepancy arises if the sorption isotherm of the eluent electrolyte is not linear. In that case, the measurement of the elution volume of a slight concentration disturbance does not yield $K'_{SO_4^{2-}} = (c_{SO_4^{2-}})_S/(c_{SO_4^{2-}})_M = (c_{Na^+})_S/(c_{Na^+})_M$ but the derivative of the sorption isotherm, $K'^*_{SO_4^{2-}} = d(c_{SO_4^{2-}})_S/d(c_{SO_4^{2-}})_M = d(c_{Na^+})_S/d(c_{Na^+})_M$. On the other hand, the measurement of the elution volume of a $^{22}Na^+$ spike does yield $K'_{Na^+} = (c_{Na^+})_S/(c_{Na^+})_M$. Obviously $K'^* \neq K'$ if the sorption isotherm is not linear. This effect has been clearly demonstrated[4] in gel chromatographic results on Sephadex G-10.

From the data in Table I, values of $\Delta\log K'_{P^z} = \log [K'_{P^z}(NaClO_4)/K'_{P^z}(Na_2SO_4)]$ were calculated. For the ions the latter data were corrected for the terms $z^2A\sqrt{I}f(I,\mathring{a}_i)$ and $f'(c)$ occurring in eqn. 4. The correction terms are given in Table II for several values of z at the three levels of I considered. They were calculated using $\mathring{a}_i = 5$ Å, $A = 0.506$ and $\beta = 0.329$ (the values[5] of the Debye–Hückel constants of water at 25°C), values of K'_{NX} and K'_{N_2Y} from Table I [$K'_{NX} = K'_{Na^+}(NaClO_4)$ and $K'_{N_2Y} = K'_{Na^+}(Na_2SO_4)$], $\nu_{NX} = 2$, $\nu_{N_2Y} = 3$ and values of d from ref. 6.

The results obtained after applying these corrections are given in Table III, together with the extrapolated data, representing $\Delta\log K_{P^z}$ values. In Fig. 1 a plot of $\Delta\log K_{P^z}$ *versus* z is shown. It is seen that the linear relationship predicted by eqn. 2 is obtained. Least-squares fitting of the data to the relationship $\Delta\log K_{P^z} = a + bz$ yields $a = -0.010 \pm 0.006$ and $b = 0.106 \pm 0.002$. The line thus passes through the origin within the 90% confidence interval of the intercept ($a = -0.010 \pm 0.011$). The slope, $b = 0.106 \pm 0.002$ (7 degrees of freedom), is not significantly different from the theoretical value, $\Delta\log K_{Na^+} = 0.095 \pm 0.005$ (1 degree of freedom) (see Table III). The standard deviation of the experimental points to the calibration line, $s(\Delta\log K_{P^z}) = 0.0148$, is even smaller than the pooled standard deviation of the data in the last column of Table III, $s_p = 0.0205$. Therefore, the expression $\Delta\log K_{P^z} = 0.106z - 0.010$ can be used as a calibration line for the determination of ionic charge. Of course, as the activity coefficient corrections depend on z, this determination must be done by successive approximations. Table IV (first column) gives the deviations from the real charge, calculated from the data in the last column of Table III and the relationship $\Delta\log K_{P^z} = 0.106z - 0.010$, for the ions investigated. For comparison, deviations from

TABLE II

VALUES OF $z^2A\sqrt{I}f(I,\mathring{a}_i)$ FOR VARIABLE I AND z AT $\mathring{a}_i = 5$ Å AND VALUES OF $f'(c)$ FOR VARIABLE I

I (mol l^{-1})	$z^2A\sqrt{I}f(I,\mathring{a}_i = 5\ \mathring{A})$				$f'(c)$
	$z = \pm 1$	$z = \pm 2$	$z = \pm 3$	$z = \pm 4$	
0.3	0.013	0.052	0.118	0.210	0.024
0.1	0.009	0.037	0.083	0.147	0.008
0.03	0.007	0.027	0.060	0.107	0.003

TABLE III

VALUES OF $[\Delta\log K'_{P^z} - z^2A\sqrt{I}f(I,\mathring{a}_i = 5\ \text{Å}) - f'(c)]$ OF DIFFERENT SPECIES P^z AT VARYING IONIC STRENGTH, VALUES OF $\Delta\log K_{P^z}$, OBTAINED BY EXTRAPOLATION *VERSUS I* AT $I = 0$ AND STANDARD DEVIATIONS OF THE EXTRAPOLATED VALUES

Species	*I (mol l^{-1})*			
	0.3	*0.1*	*0.03*	*→0*
Mg^{2+}	0.198	0.212	0.177	0.189 ± 0.022
Na^+	0.077	0.094	0.090	0.095 ± 0.005
MeOH	0.016	−0.030	0.007	−0.013 ± 0.029
TcO_4^-	−0.191	−0.157	−0.099	−0.106 ± 0.025
Cl^-	−0.140	−0.119	−0.088	−0.091 ± 0.013
SO_4^{2-}	−0.202	−0.231	−0.225	−0.233 ± 0.009
$Fe(CN)_6^{3-}$	−0.483	−0.359	−0.341	−0.316 ± 0.014
$Fe(CN)_6^{4-}$	−0.592	−0.480	−0.457	−0.436 ± 0.009
$Mo(CN)_8^{4-}$	−0.617	−0.541	−0.447	−0.453 ± 0.036

the real charge found for some ions by the aqueous polymer two-phase partition method[1] and by ion-exchange chromatography[1,7] are also given in Table IV.

We conclude from the data in Table IV that the precision of this gel permeation chromatographic method is almost identical with that of the method based on partition in an aqueous polymer two-phase system and is much better than that attainable by ion-exchange procedures. The gel permeation chromatographic method is the method of choice in cases where the charge of individual components of an ionic

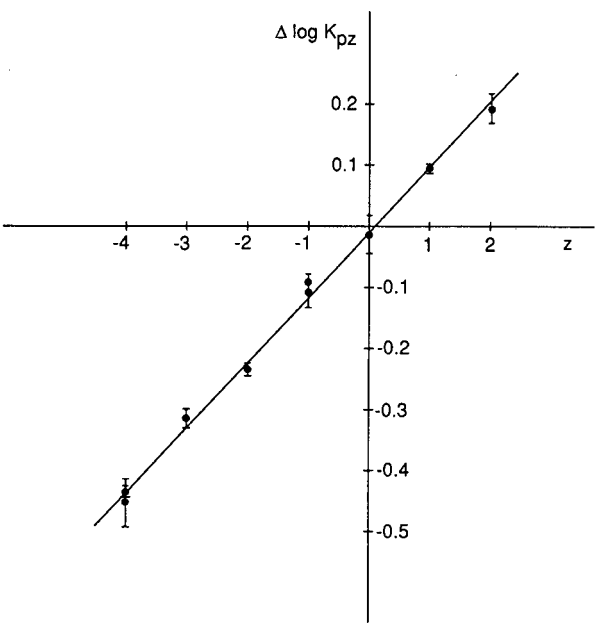

Fig. 1. $\Delta\log K_{P^z}$ *versus* z. Data from Table III. Regression line: $\Delta\log K_{P^z} = 0.106\ z - 0.010$.

TABLE IV

DEVIATIONS FROM THE REAL CHARGE FOR SOME IONS FOUND BY GEL PERMEATION CHROMATOGRAPHY (THIS WORK), BY AQUEOUS POLYMER TWO-PHASE PARTITION[1] AND BY ION-EXCHANGE CHROMATOGRAPHY[1,7]

Ion	*Gel permeation chromatography*	*Aqueous polymer two-phase partition*	*Ion-exchange chromatography*	
			DEAE-Trisacryl	*Aminex 28*
Mg^{2+}	−0.12	0.00		
Na^{+}	−0.01	+0.11		
TcO_4^-	+0.09	−0.08		
Cl^-	+0.24		+0.37	
SO_4^{2-}	−0.10	−0.11	+0.60	−0.20
$Fe(CN)_6^{3-}$	+0.11	−0.12	+1.80	
$Fe(CN)_6^{4-}$	−0.02	+0.18		
$Mo(CN)_8^{4-}$	−0.18			

mixture has to be determined. In fact, by this method the charge of each separable component can be determined, whereas only the mean charge is accessible by the aqueous polymer two-phase partition method.

From the standard deviation of the experimental points to the calibration line, $s(\Delta\log K_{P^z}) = 0.0148$ and the values of the slope b and its standard deviation s_b ($b = 0.106 \pm 0.002$), the standard deviation s_z of a charge z, to be determined by the proposed method, can be estimated using[8]

$$s_z^2 = b^{-2}[(1 + 1/n)s^2(\Delta\log K_{P^z}) + (z - \bar{z})^2 s_b^2]\left(1 + \frac{p}{n - 1 - p}\right)$$

where n is the number of experimental data points and p the number of parameters used in calculating the regression line and $\bar{z}$ is the mean charge of the ions used. The resulting errors (using $n = 9$, $p = 2$ and $\bar{z} = -1.33$) are given in Table V.

TABLE V

STANDARD DEVIATION, s_z, OF AN IONIC CHARGE z, TO BE DETERMINED BY THE PROPOSED METHOD, USING THE CALIBRATION LINE OF FIG. 1

z	−4	−3	−2	−1	0	+1	+2
s_z	0.18	0.17	0.17	0.17	0.17	0.18	0.18

APPENDIX

The activity coefficient y_{P^z} of an ion P^z, present in a trace amount in an electrolyte solution of ionic strength I is given by the extended Debye–Hückel equation[5] (see also ref. 1)

$$-\log y_{P^z} = \frac{z^2 A\sqrt{I}}{1 + \beta\mathring{a}_i\sqrt{I}} + \log\left[\frac{d + 10^{-3}c(\nu M - M_{\text{solv.}})}{d^0}\right] + CI \qquad \text{(A1)}$$

where A, β and C are constants, $\mathring{a}_i$ is the distance of closest approach of the electrolyte ions to the ion P^z, d and d^0 are the densities of the solution and the solvent, respectively, c is the concentration of the electrolyte, v is the number of ions into which one molecule of electrolyte dissociates and M and $M_{solv.}$ are the molecular weights of the electrolyte and the solvent, respectively. The activity coefficient term, occurring in eqn. 2, can then be written as

$$\log\left[\frac{y_S(NX)}{y_S(N_2Y)}\right] = -z^2A\left[\frac{\sqrt{I_S(NX)}}{1+\beta\mathring{a}_i\sqrt{I_S(NX)}} - \frac{\sqrt{I_S(N_2Y)}}{1+\beta\mathring{a}_i\sqrt{I_S(N_2Y)}}\right] - \log$$
$$-\log\left[\frac{[d+10^{-3}c_S(vM-M_{solv.})]_{NX}}{[d+10^{-3}c_S(vM-M_{solv.})]_{N_2Y}}\right] - C_S(NX)I_S(NX) + C_S(N_2Y)I_S(N_2Y) \quad \text{(A2)}$$

The ionic strength of the stationary phase, I_S, and the concentration of the electrolyte in the stationary phase, c_S, are related to those in the mobile phase, I and c, by

$$\frac{I_S(NX)}{I} = \frac{c_S(NX)}{c} = K'_{NX}$$
$$\frac{I_S(N_2Y)}{I} = \frac{c_S(N_2Y)}{c} = K'_{N_2Y} \quad \text{(A3)}$$

Substitution in eqn. A2 gives an expression that can be written in the following simplified form

$$\log\left[\frac{y_S(NX)}{y_S(N_2Y)}\right] = -z^2A\sqrt{I}f(I,\mathring{a}_i) - f'(c) + C'I \quad \text{(A4)}$$

where f and f' are known functional relationships and C' is an unknown constant.

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Centrifugal counter-current chromatography, a promising means of measuring partition coefficients

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SUMMARY

Centrifugal counter-current chromatography (CCCC) was examined as a means of measuring lipophilicity values. Using an aqueous buffer as the stationary phase and 1-octanol as the eluent, seventeen solutes of widely different structure and of log D_{oct} [log (distribution coefficient) at pH 7.4] spanning 2.5 units were examined. A very good correlation was found with literature log P [log (partition coefficient) of neutral species] values measured by the classical shake-flask method. The efficiency and precision of the CCCC method makes it a promising technique for measuring lipophilicity.

INTRODUCTION

Lipophilicity is an important molecular property of drugs and other xenobiotics often correlated with their biological activity. A number of experimental techniques have been developed to simulate partition processes in biological systems and to measure lipophilicity. The best known is the shake-flask (SF) method, which remains the standard system for measuring the lipophilicity of chemical compounds. In this system, two poorly miscible liquid phases (water and an organic solvent) are stirred gently until an equilibrium is reached; 1-octanol is universally accepted as the reference organic solvent[1–3], but other solvents are very useful, for example in comparing the hydrogen-bonding ability of series of solutes[4–6]. However, despite its value, the SF method suffers from a number of practical limitations due to various perturbing factors such as time consumption, solute stability or volatility, solute impurities, formation of microemulsions and concentration and salt effects, etc., as cogently discussed by Dearden and Bresnen[7].

Solid–liquid partition chromatography has been used as an alternative means

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for measuring lipophilicity for many years. In particular, chromatographic retention parameters obtained by reversed-phase high-performance liquid chromatography (RP–HPLC) have become increasingly popular in replacing the 1-octanol–water partition coefficient in quantitative structure–activity relationship (QSAR) studies[8,9]. Unfortunately, the presence of a solid support with a non-negligible proportion of residual silanol groups on the surface of the alkyl-bonded phase dramatically influences the partitioning process of polar basic compounds owing to an additional adsorption mechanism; the use of a masking agent becomes necessary[10–12], but this introduces an additional variable in the experimental conditions.

Recently, a new method[13–15] called centrifugal partition chromatography (CPC), high-speed counter-current chromatography (HS-CCC) or centrifugal counter-current chromatography (CCCC) has found its first applications in determining lipophilicity. This is a liquid–liquid chromatographic method in which two non-miscible solvents are used as the stationary and mobile phase, respectively. A centrifugal force maintains the stationary phase, while the mobile phase is pumped through the system. Using a Sanki CPC model[16], the method has demonstrated its potential for measuring partition coefficients[13,14,17]; limitations are the high cost of the equipment, its restricted availability and a number of technical difficulties. Another apparatus is available, namely the Ito multi-layer coil separator–extractor[15,18–20], which is cheaper and easy to obtain. To the best of our knowledge, this equipment has never been used to determine partition coefficients. Our preliminary investigations showed a narrow range of measurable partition coefficients, a problem that can be partly overcome by varying the relative volumes of the two phases in the apparatus using two distinct pumps to fill the coil.

EXPERIMENTAL

Chemicals

β-Pyridylalkanols were synthetized as described[21]. All other solutes were commercially available; 1-octanol (purum) was purchased from Fluka (Buchs, Switzerland).

Apparatus

Measurements were performed at room temperature using an Ito Multi-layer coil separator–extractor (P.C, Potomac, MD, U.S.A.) equipped either with preparative coil No. 10 (I.D. 2.6 mm, volume 370 ml) or with analytical coil No. 14, which we shortened to about one third of the initial length (I.D. 1.6 mm, volume 107 ml). The speed of rotation was controlled with a d.c. motor speed control/basic speed range ASH-600 (Bodine Electric, Chicago, IL, U.S.A.). For commuting between "head" and "tail" ends of the coil, an SRV-4 four-way valve (Pharmacia, Uppsala, Sweden) was installed. The samples were injected through a Lobar six-port valve injector (Merck, Darmstadt, F.R.G.) with the 2.5-ml loop mounted. The samples were detected at 254 nm (morphine at 287 nm) using a Uvikon 725 UV detector (Kontro, Zurich, Switzerland) equipped with a QS 1.000 80-μl UV cell. The chromatograms were recorded with a Model 3392A integrator (Hewlett-Packard, Meyrin, Switzerland). The coil was filled using two T414 LC pumps (Kontron), which were joined with a metallic T-piece. All connecting tubes were Lobar No. 15455 (Merck). A more detailed description of the apparatus was published by Slacanin *et al.*[22].

Procedures

Preparation of phases. The organic and aqueous phases were mutually saturated by stirring for 1–2 h and then separated in a decantation funnel.

Preparation of samples. Concentrated or saturated solutions of each solute were prepared in the mobile phase (*i.e.*, water-saturated octanol).

Filling of the coil. To allow the volume ratio of the two phases to be chosen at will, the technique of coil filling was modified by using two pumps, one for each phase. While the system was in rotation, the coil was first completely filled with the mobile phase and then a desired volume of the stationary phase was added to the column by displacing the mobile phase. This allowed good control of the volume ratio of the two phases in the coil (as checked repeatedly by emptying the coil), and no exclusion of stationary phase was observed once the mobile phase had been pumped through the coil during measurement of retention volumes. It was found useless to empty and wash the coil between days; the pump and the motor were stopped overnight, and it was sufficient the next day to set the coil in rotation, wait for 15–30 s and then start pumping the mobile phase.

Although the distribution of the stationary phase in the coil may not be totally uniform, this cannot affect the distribution equilibrium and hence the results, provided that the interface area is not decreased[15].

Sample injections. These were done manually without stopping either the eluent flow or rotation. The usual volume injected was 0.5–1.0 ml (loop capacity 2.5 ml), making it possible to inject simultaneously three or four compounds, provided that they were of sufficiently different lipophilicity and did not interfere with each other's retention.

Determination of column dead time. In the normal operating mode used (the apolar solvent being the mobile phase), anthracene (log P = 4.5) was taken as the non-retained compound whose duration of passage was the column dead time, t_0.

Reuse of solvents. The mobile phase was immediately recycled, provided that it did not contain any solute; contaminated mobile phase was distilled and saturated with the aqueous phase before reuse, thus saving significant volumes of solvent.

Washing of the coil. This was performed with methanol; nitrogen was then used to dry the coil.

Operating conditions

The temperature throughout was 21 ± 1°C. The mobile phase was 1-octanol and the aqueous stationary phase was a 0.01 M solution of 3-morpholinopropanesulphonate at pH 7.4; both phases were mutually saturated. Three different sets of conditions were used. When the preparative coil was used with a volume ratio of mobile and stationary phases of *ca.* 15:1, the flow-rate of the eluent was measured as 7.45 ml/min (setting 8 ml/min) and the speed of rotation was 800 rpm. When the preparative coil was used with a volume ratio of mobile and stationary phases of *ca.* 1:2, the flow-rate of the eluent was 4.95 ml/min (setting 5 ml/min) and the speed of rotation was again 800 rpm. When the analytical coil was used, a volume ratio of mobile and stationary phases of *ca.* 3:2 was chosen, the flow-rate of eluent was 3.8 ml/min (setting 4 ml/min) and the speed of rotation was 1000–1200 rpm (no precise scale on the apparatus).

The octanol–water distribution coefficient of a given solute (apparent partition

coefficient at the pH of study, here 7.4) was calculated according to the equation[23].

$$\log D^{ITO} = \log [V_s/(V_r - V_0)] \quad (1)$$

where D^{ITO} is the octanol–water distribution coefficient, V_0 the dead volume (mobile phase volume, calculated as V_0 = flow · t_0), V_s is the volume of the stationary phase (calculated as total volume minus V_0) and V_r the retention volume of the solute (calculated as V_r = flow · t_r from the retention time of the solute, t_r).

As some of the solutes examined were partially or completely ionized at pH 7.4, the distribution coefficient at pH 7.4 (also called the apparent partition coefficient, and designated here as log D^{ITO} or log D^{SF}, respectively) was distinguished from the "true" partition coefficient of the neutral species (log P). Log D and log P are identical for non-ionizable compounds, whereas for ionizable compounds log P was calculated by correcting log D for ionization as described by Rekker[24].

RESULTS AND DISCUSSION

Preparative coil, volume ratio of mobile and stationary phases ca. 15:1

In this system, the mean dead time t_0 was 46.55 ± 0.26 min (anthracene as the non-retained solute; average of eight measurements over four days). The small standard deviation indicates the stability and the reproducibility of the equipment. The dead volume V_0 was calculated to be 346 ml and the volume of the stationary phase V_s was 24 ml, *i.e.*, a volume ratio close to 15:1.

Twelve solutes with published log P values covering a range of almost 2 units were examined in this sytem. The solutes were also chosen for their structural differences (acids, bases, phenols, heterocyclic derivatives, etc.). Their t_r values are reported in Table I; the derived distribution coefficients (as log D^{ITO} values) and calculated log P^{ITO} values are also given. These values are the means of two or three injections, the deviations always being smaller than 0.01 on the log D scale.

Preparative coil, volume ratio of mobile and stationary phases ca. 1:2

In this system, the mean dead time t_0 was 25.50 ± 0.38 min (anthracene as the non-retained solute; average of four measurements over two days). The dead volume V_0 was calculated to be 126 ml and the volume of the stationary phase V_s was 244 ml, *i.e.*, a volume ratio close to 1:2.

Eight solutes were examined, their published log P values ranging from −0.1 to 1.1. Their t_r values are reported in Table I together with log D^{ITO} (deviations <0.01) and log P^{ITO} values. Note that three solutes (Nos. 12, 13 and 16) were examined in both systems, the differences in the log D^{ITO} values being 0.32, 0.08 and 0.11, respectively.

Analytical coil, volume ratio of mobile and stationary phase ca. 3:2

In this system, the mean dead time t_0 was 18.00 ± 0.20 min (anthracene as the non-retained solute; average of six measurements over four days). The dead volume V_0 was calculated to be 68 ml and the volume of the stationary phase V_s was 39 ml, *i.e.*, a volume ratio close to 3:2.

The ten solutes examined had published log P values ranging from −0.8 to 2.7.

TABLE I

OCTANOL–WATER PARTITION COEFFICIENTS MEASURED BY CCCC USING A PREPARATIVE COIL

No.	*Solute*	t_r *(min)*	*Log* D^{ITO} [a]	*Log* D^{SF} [b]	*Log* P^{ITO} [c]	*Log* P^{SF} [d]
1	4-Iodobenzoic acid	53.77[e]	−0.36		3.00	3.02
2	4-Bromobenzoic acid	61.19[e]	−0.66		2.74	2.86
3	4-Chlorobenzoic acid	64.54[e]	−0.75		2.66	2.65
4	4-Hydroxybenzoic acid	105.5[e]	−1.26		1.56	1.58
5	Benzyl alcohol	28.42[f]	1.22	1.10	1.22	1.10
6	Benzylamine	67.50[e]	−0.82		1.15	1.09
7	4-Fluorobenzamide	30.89[f]	0.96	0.91	0.96	0.91
8	2-Bromobenzamide	35.00[f]	0.71	0.73	0.71	0.73
9	Benzamide	36.56[f]	0.65	0.64	0.65	0.64
10	β-Pyridylpropanol	38.00[f]	0.59	0.60	0.59	0.60
11	Sulpiride	98.44[e]	−1.21	−1.15	0.52	0.58
12	2-Aminopyridine	47.04[e]	0.73		0.83	0.49
		44.81[f]	0.41		0.51	0.49
13	3-Aminophenol	48.33[e]	0.23	0.17	0.23	0.17
		60.00[f]	0.15	0.17	0.15	0.17
14	Sulphamerazine	57.32[e]	−0.53	−0.12[g]	0.07	0.14
15	Sulphathiazole	54.82[e]	−0.42	−0.41[g]	0.04	0.05
16	β-Pyridylmethanol	50.97[e]	−0.15	−0.02	−0.15	−0.02
		79.21[f]	−0.04	−0.02	−0.04	−0.02
17	Sulphanilamide	69.35[e]	−0.85	−0.89[g]	−0.85	−0.72

[a] Log (distribution coefficient) (apparent partition coefficient) measured by CCCC at pH 7.4.

[b] Log (distribution coefficient) (apparent partition coefficient) measured by the shake-flask method at pH 7.4 (literature data[25]).

[c] Log (partition coefficient), corrected for ionization when applicable, measured by CCCC.

[d] Log (partition coefficient), corrected for ionization when applicable, measured by the shake-flask method (literature data[25]).

[e] Measured by CCCC at a phase ratio of about 15:1.

[f] Measured by CCCC at a phase ratio of about 1:2.

[g] Experimental value at pH 7.5.

TABLE II

OCTANOL–WATER PARTITION COEFFICIENTS MEASURED BY CCCC USING AN ANALYTICAL COIL

No.	*Solute*	t_r *(min)*	*Log* D^{ITO} [a]	*Log* D^{SF} [b]	*Log* P^{ITO} [c]	*Log* P^{SF} [d]
1	Sulphanilamide	76.50	−0.76	−0.89	−0.76	−0.72
2	Morphine	29.00	−0.03	−0.12	0.84	0.76
3	β-Pyridylmethanol	30.10	−0.08	−0.02	−0.08	−0.02
4	Sulphathiazole	42.66	−0.38	−0.43	0.08	0.05
5	Sulphamerazine	46.00	−0.44	−0.12	0.16	0.14
6	3-Aminophenol	25.59	0.13	0.17	0.13	0.17
7	Sulpiride	120.0	−1.00	−1.15	0.73	0.58
8	β-Pyridylpropanol	20.43	0.61	0.60	0.61	0.60
9	Benzylamine	76.75	−0.76		1.17	1.09
10	4-Chlorobenzoic acid	69.50	−0.70		2.71	2.65

[a–d] See Table I.

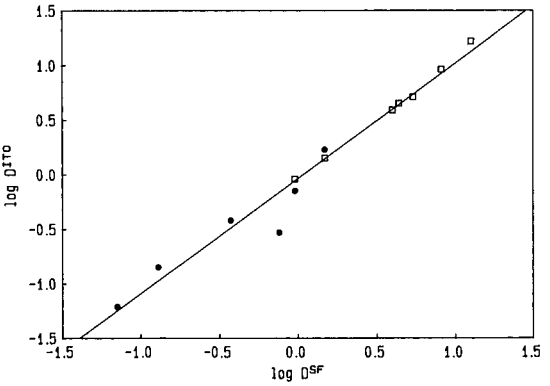

Fig. 1. Correlation of log D values (apparent partition coefficient at pH 7.4) measured by CCCC (Ito apparatus equipped with preparative coil) with literature values obtained by the shake-flask (SF) method. The line corresponds to eqn. 2. The symbols ● and □ correspond to values measured at phase ratios of 15:1 and 1:2, respectively.

Their retention times (t_r), log D^{ITO} (deviations <0.01) and log P^{ITO} values are reported in Table II.

Comparison of distribution and partition coefficients measured by the CCCC method with literature values

Preparative coil. A correlation was first sought between log D^{ITO} values and log D values measured by the shake-flask method and reported in the literature (*i.e.*, log D^{SF} values, see Table I), yielding the equation

$$\log D^{ITO} = 1.055(\pm 0.122) \log D^{SF} - 0.036(\pm 0.081) \quad (2)$$
$$n = 13;\ r^2 = 0.970;\ s = 0.130;\ F = 364$$

where n is the number of observations, r the correlation coefficient, s the standard deviation and F the Fisher's test parameter.

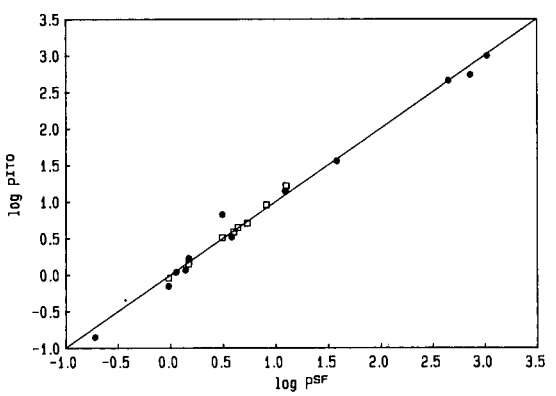

Fig. 2. Correlation of log P values ("true" partition coefficient corrected for ionization) determined by CCCC (Ito apparatus equipped with preparative coil) with literature values obtained by the shake-flask (SF) method. The line corresponds to eqn. 3. Symbols as in Fig. 1.

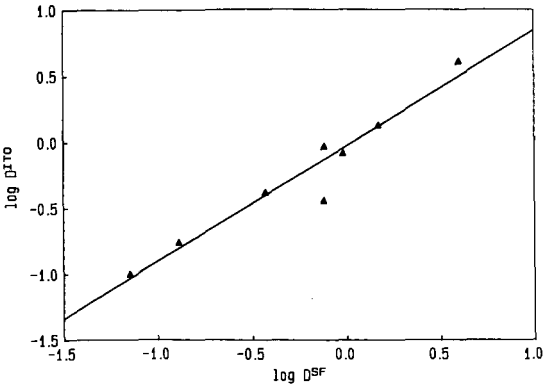

Fig. 3. Correlation of log D values (apparent partition coeffient at pH 7.4) measured by CCCC (Ito apparatus equipped with analytical coil) with literature values obtained by the shake-flask (SF) method. The line corresponds to eqn. 4.

In a second step, the correlation between log P^{ITO} and log P^{SF} (Table I) was calculated, yielding the equation

$$\log P^{ITO} = 1.001(\pm 0.051) \log P^{SF} + 0.001(\pm 0.065) \quad (3)$$
$$n = 20;\ r^2 = 0.990;\ s = 0.105;\ F = 1730$$

Figs. 1 and 2 show these relationships and demonstrate the regular lipophilicity distribution of the seventeen solutes investigated.

Analytical coil. Here, the following equally satisfactory equations were obtained:

$$\log D^{ITO} = 0.875(\pm 0.234) \log D^{SF} - 0.029(\pm 0.136) \quad (4)$$
$$n = 8;\ r^2 = 0.933;\ s = 0.143;\ F = 84$$

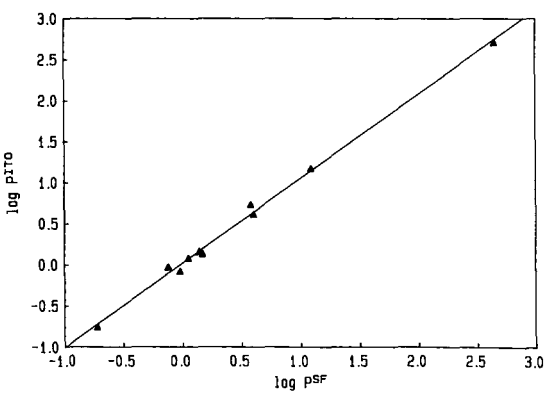

Fig. 4. Correlation of log P values ("true" partition coefficient corrected for ionization) determined by CCCC (Ito apparatus equipped with analytical coil) with literature values obtained by the shake-flask (SF) method. The line corresponds to eqn. 5.

$$\log P^{ITO} = 1.030(\pm 0.054) \log P^{SF} + 0.017(\pm 0.053) \quad (5)$$
$$n = 10; r^2 = 0.996; s = 0.064; F = 1927$$

These relationships are represented in Figs. 3 and 4.

The four equations, but especially eqns. 3 and 5, are characterized by good correlation coefficients, indicating that the CCCC method indeed is an effective means of measuring lipophilicity. Even more interesting is the fact that in the four equations the slope is equal to unity and the intercept is zero, implying that within the lipophilicity range investigated log P values determined by the CCCC method can be equated with those determined by the shake-flask system without any calibration or proportionality factor.

CONCLUSION

This study has established the interest of CCCC as a novel technique for measuring lipophilicity. Using an aqueous buffer as the stationary phase and octanol as the eluent, log P values were found that correlate well with literature values as measured by the shake-flask method. In addition, the very good reproducibility of the CCCC system suggests that its precision must be significantly better than that of the shake-flask method, as shown by subsequent studies in our laboratory (work in progress). Certainly it is less time consuming and thus more efficient, our estimate being that the gain in time is 4–6-fold.

The compounds investigated span a log D range of 2.5 units (Fig. 1) and a log P range of almost 4 units (Fig. 2). This is still too limited a range to render the CCCC method generally applicable, but work is now in progress to alleviate this limitation, one possibility worth exploring being the reversed elution mode (organic solvent as stationary phase). The use of coils with different internal diameters and volumes could yield additional advantages, allowing retention times and amounts injected to be closer to those in high-performance liquid chromatography.

SYMBOLS AND ABBREVIATIONS

$\log D_{oct}$	log (octanol–water distribution coefficient) at a given pH
$\log D^{SF}$, $\log D^{ITO}$	log (distribution coefficient), solvent system not specified, measured by the shake-flask method or with the Ito equipment, respectively
$\log P$	log (partition coefficient) of neutral species, solvent system not specified
$\log P^{SF}$, $\log P^{ITO}$	log (partition coefficient), solvent system not specified, measured by the shake-flask method or with the Ito equipment, respectively
t_0	column dead time
t_r	retention time of the solute
V_0	dead volume of the column (volume of the mobile phase)
V_r	retention volume of the solute
V_s	volume of the stationary phase
CCCC	centrifugal counter-current chromatography
SF	shake-flask method

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Quantification of cyclic 2,3-diphosphoglycerate from methanogenic bacteria by isotachophoresis

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SUMMARY

Cyclic 2,3-diphosphoglycerate (cDPG), a metabolite exclusively present in methanogenic bacteria, was separated from other phosphate-containing compounds in extracts of *Methanobacterium thermoautotrophicum* by means of isotachophoresis. The detection limit was 100 pmol per injected sample. Quantification of intracellular concentrations of cDPG with the isotachophoretic assay and with a spectrophotometric cDPG assay gave comparable results, but the isotachophoretic assay was faster, less laborious and more sensitive. A possible involvement of cDPG in the energy metabolism was indicated by studies of *Mb. thermoautotrophicum* in batch and continuous cultures.

INTRODUCTION

In 1983 a unique cyclic pyrophosphate was isolated from *Methanobacterium thermoautotrophicum*, the structure of which was identified[1–3] as the intramolecularly cyclized pyrophosphate of 2,3-diphospho-D-glycerate (Fig. 1), referred to in the following as cDPG. Under optimum growth conditions, this unusual metabolite is the major soluble carbon- and phosphorous-containing compound in cells of *Mb. ther-*

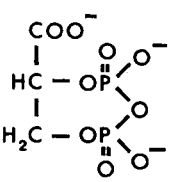

Fig. 1. Structure of cyclic 2,3-diphospho-D-glycerate (cDPG).

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moautotrophicum. Intracellular concentrations have been found to range from 2 to 200 m*M* under different growth conditions[3–6].

Up to now, cDPG has been found in significant concentrations in methanogenic bacteria belonging to the genera *Methanobacterium, Methanobrevibacter, Methanothermus* and *Methanosphaera,* whereas it has not been detected in members of the genera *Methanococcus, Methanosarcina* and *Methanogenium*[4,5,7]. One important difference between these two groups of methanogens is that the former possess a rigid pseudomurein cell wall, whereas the latter, with the exception of *Methanococcus,* do not. This might indicate a possible involvement of cDPG in the carbohydrate metabolism as an intermediate in the synthesis of the pseudomurein component of the cell wall. However, several studies point to a multi-functional role of cDPG in the metabolism of methanogens. For instance, cDPG might be involved in energy storage as it contains a high-energy phosphoryl group which could be utilized in ATP synthesis[1]. In addition, it has been suggested to function as a phosphate storage compound, comparable to polyphosphates in other bacteria, as the intracellular cDPG pool was found to be depleted during growth in the absence of exogenous phosphate[2,6]. cDPG has also been linked to phospholipid synthesis[6] and gluconeogenesis[8]. Finally, the potassium salt of the trianionic cDPG has been found to stabilize the thermolabile enzymes glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase *in vitro*[4].

The spectrophotometric cDPG assay most commonly used, which employs acid hydrolysis of cDPG to 2,3-diphosphoglycerate (2,3-DPG) and subsequent quantification of 2,3-DPG using a multi-enzyme assay[3], is laborious and time consuming. As isotachophoresis has been found to be well suited for accurate quantification of ionic metabolites from methanogens[9], we adopted this fast and simple technique as the basis for a novel cDPG assay. The assay was developed using authentic cDPG obtained by isolation from mass-cultured cells. For reference, cDPG was prepared from 2,3-DPG by organic synthesis. The assay was employed to study the possible role of cDPG in the energy and phosphate metabolism of *Mb. thermoautotrophicum* by investigating the relationship between growth and intracellular levels of cDPG under different physiological conditions.

EXPERIMENTAL

Organism and growth conditions

Mb. thermoautotrophicum strain △H (DSM 1053) was cultured at 65°C under hydrogen–carbon dioxide (80:20, v/v) in a medium composed of (per litre) KH_2PO_4, 6.8 g; $Na_2CO_3 \cdot 10H_2O$, 9.0 g; NH_4Cl, 2.1 g; L-cysteine · HCl, 0.6 g; $Na_2S \cdot 9H_2O$, 0.6 g; and minerals solution, 2 ml. The latter contained (per litre) nitrilotriacetic acid, 96 g; $MgCl_2 \cdot 6H_2O$, 41 g; $MnSO_4 \cdot H_2O$, 5 g; $FeCl_2 \cdot 4H_2O$, 5 g; $NiCl_2 \cdot 6H_2O$, 1.2 g; $ZnSO_4 \cdot 7H_2O$, 1 g; $CoCl_2 \cdot 6H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 1 g; H_2SeO_3, 0.8 g; $NaMoO_4 \cdot 2H_2O$, 0.24 g; $CuSO_4 \cdot 5H_2O$, 0.1 g; $AlK(SO_4)_2 \cdot 2H_2O$, 0.1 g; H_3BO_3, 0.1 g; and $NaWO_4 \cdot 2H_2O$, 3 mg. The pH of the culture medium and of the minerals solution was adjusted to 7.0. Cells were grown in a 0.5-l fermentor operated as a batch or continuous culture with hydrogen–carbon dioxide at a flow-rate of 22.4 l/h. Cell growth was monitored by reading the absorbance at 578 nm with a Hitachi Model 191 spectrophotometer and by measuring methane production by gas chromatogra-

phy[10]. Cells were harvested, suspended (10:1, w/v) in 10 m*M* N-tris(hydroxymethyl) methyl 2-aminoethanesulphonate (TES) buffer at pH 7.0 and, following centrifugation, resuspended in this buffer to a minimum concentration of 4 mg dry weight (dw)/ml. Cell-free extracts were prepared from these suspensions as described previously[9]. Crude cell extracts were obtained by boiling (45 min, 100°C) or sonication (seven times 10 s at 70 W; Branson Sonifier B-12) of 0.5- and 1.5-ml samples of cell suspensions, respectively. The intracellular volume of the cells was assumed to be 1.8 ml/g dw of cells[11].

Isotachophoresis

Isotachophoretic separations were performed at ambient temperature with an LKB 2127 Tachophor carrying a 28-cm Teflon capillary tube of 0.4 mm I.D. The leading electrolyte solution contained chloride (10 m*M*) as the leading anion and 6-amino-*n*-hexanoic acid as the buffering counter ion at pH 4.5. Poly(vinyl alcohol) (0.05%) was included in the leading electrolyte to sharpen the zone boundaries. The terminal electrolyte was acetate (10 m*M*) at pH 4.8. The driving current was 500 μA at 3–4 kV. The total assay time was about 15 min. Detection was effected with an a.c. conductivity detector[12]. Zone lengths of eluting anions were derived from the differential signal of the conductivity meter and expressed in units of time (s). Reference solutions were prepared in Milli-Q deionized water (Millipore). Aliquots of 1–5 μl were subjected to isotachophoresis. Calibration graphs were constructed by plotting the zone lengths against the amounts of anion injected.

Spectrophotometric cDPG assay

The assay was performed essentially as described previously[3] with 200-μl samples of cell extracts, containing 0.5–1.0 mg dw of cells. Briefly, the samples were treated with 1 *M* hydrochloric acid (1:1, v/v) for 15 min at 100°C in order to hydrolyse the pyrophosphate bond in cDPG, resulting in a complete conversion to 2,3-DPG[2]. 2,3-DPG was quantified by use of the Boehringer (Mannheim, F.R.G.) commercial assay, which measures the amount of NADH oxidized during the conversion of 2,3-DPG to glycerol-3-phosphate catalysed by five consecutive enzymatic reactions.

Isolation and purification of cDPG from Mb. thermoautotrophicum

Cells were mass-cultured in a 10-l fermentor under hydrogen–carbon dioxide (80:20, v/v; 100 l/h) and harvested at the end of the logarithmic phase of growth. All of the subsequent manipulations were performed at 4°C. Cells were suspended (1:1, w/v) in buffer consisting of 100 m*M* TES and 10 m*M* EDTA at pH 7.2. The cells were broken by passage through a French press at 138 MPa. Perchloric acid (2.1%, w/v) was added to the suspension. After vigorous shaking for 20 min, the suspension was centrifuged at 12 000 *g* for 10 min. The supernatant obtained was neutralized, whereupon centrifugation was repeated. cDPG was purified from the supernatant fraction using a DEAE-Sephadex A-25 column (20 cm × 3 cm I.D.), eluted with a linear gradient of 0.3–1.5 *M* ammonium acetate at pH 7.5, followed by a QAE-Sephadex A-25 column (45 cm × 1.5 cm I.D.), eluted with a linear gradient of ammonium acetate at pH 5. cDPG was traced and quantified in the eluent fractions by spectrophotometric assay. The fractions containing cDPG were pooled, lyophilized and taken up in deionized water. Judged by isotachophoretic analysis, the product obtained was pure.

Chemical synthesis of cDPG

cDPG was prepared by incubating 500 μmol of 2,3-DPG, dissolved in 10 ml of 0.5 *M* Bis–Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane] buffer containing 0.2 *M* $MgCl_2$ (pH 6.5), in the presence of 1 *M* EDAC [1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride]. cDPG formation was monitored by use of the isotachophoretic assay. After incubation for 2 days at 40°C with constant shaking, 2,3-DPG conversion to cDPG ceased. The reaction mixture was adjusted to pH 7.5 with ammonia and diluted 100-fold with deionized water. cDPG was isolated from this solution by use of the ion-exchange chromatographic procedure outlined above.

Chemicals

EDAC, Bis–Tris and TES were obtained from Sigma (St. Louis, MO, U.S.A.), poly(vinyl alcohol) from Merck (Darmstadt, F.R.G.), 2,3-diphosphoglycerate from Boehringer and 6-amino-*n*-hexanoic acid from Serva (Heidelberg, F.R.G.).

RESULTS

Isolation of authentic cDPG

A mass culture of *Mb. thermoautotrophicum,* about 3.8 g dw of cells, was used for the isolation of cDPG. Judged by the spectrophotometric assay, the intracellular cDPG content was 160 μmol/g dw of cells (89 m*M*), which is comparable to values reported by others[3,5,6]. Isolation of cDPG from perchloric acid-treated cells by ion-exchange chromatography yielded 120 μmol/g dw of cells extracted. Hence 75% of the cDPG contained in the whole cells was recovered.

Chemical synthesis of cDPG

Incubation of 500 μmol of 2,3-DPG with EDAC–$MgCl_2$, which catalyzes the intramolecular cyclization process, resulted in the formation of 50 μmol of cDPG. Although this is a low transformation yield, the conversion proceeds rapidly (48 h), and can be performed with large amounts of 2,3-DPG to obtain any desired amount of pure cDPG.

Isotachophoretic assay of cDPG and related compounds

The separation obtained with the isotachophoretic assay of a mixture of pure compounds, *i.e.,* cDPG and five other phosphate-containing metabolites, is illustrated in Fig. 2A. It can be seen that each of the compounds has a characteristic position in the isotachopherogram, referred to as the relative step height. In addition to the compounds shown, the assay is suitable for the separation of tripolyphosphate, phosphoenolpyruvate and pyruvate, which have relative step heights of 0.16, 0.29 and 0.40, respectively. Fig. 2B shows a typical isotachopherogram of a cell-free extract of *Mb. thermoautotrophicum* cells. cDPG, P_i and 2,3-DPG were present at millimolar concentrations in the cells, whereas other phosphate-containing compounds possibly present were below the limit of detection at the recorder setting employed. Quantification of cDPG in crude cell extracts prepared by boiling or sonication yielded identical results.

Calibration graphs were prepared for each of the phosphate-containing metab-

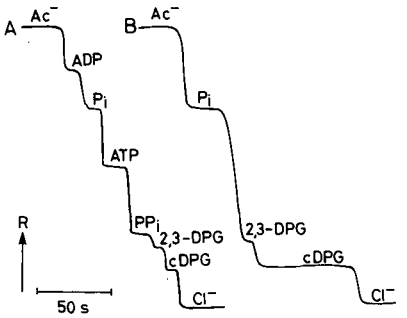

Fig. 2. Isotachopherograms of (A) a reference mixture of phosphate-containing metabolites (1 μl sample volume; cDPG and 2,3-DPG at 1 m*M*, the other ions at 2 m*M*) and (B) a cell-free extract prepared from cells of *Mb. thermoautotrophicum* harvested during logarithmic-phase growth in batch culture (1 μl, containing about 30 μg dw of cell material). Chloride was used as the leading ion and acetate as the terminating ion. 1 = Chloride (0.0); 2 = cDPG (0.13); 3 = 2,3-DPG (0.23); 4 = PP_i (inorganic pyrophosphate) (0.26); 5 = ATP (0.50); 6 = P_i (inorganic orthophosphate) (0.70); 7 = ADP (0.85); 8 = acetate (1.0). Values in parentheses are relative step heigths. R = Resistance.

olites under investigation. A linear relationship was found in all instances between the zone lengths and the amounts of compound injected. In Fig. 3 this is shown for P_i, 2,3-DPG and cDPG. Linearity was good up to about 20 nmol per injection. In all instances the zone lengths were found to be proportional to the amounts of compound injected. Zone lengths recorded for equimolar amounts of the metabolites varied in the proportions P_i:2,3-DPG:cDPG = 1:1.5:2. The limit of detection was in the range 50–200 pmol per injection for the various metabolites, whereas the detecton

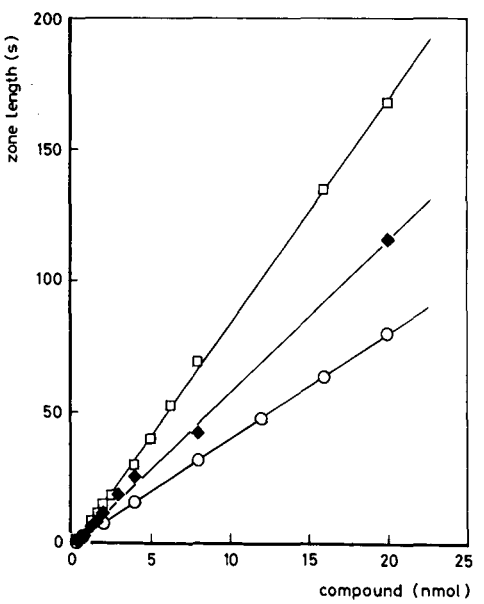

Fig. 3. Calibration graphs indicating the dependence of the zone lengths on the amounts of (○) P_i, (◆) 2,3-DPG and (□) cDPG injected. Solid lines are linear regression curves. All linear regression coefficients were $\geqslant 0.996$.

TABLE I

CELLULAR LEVELS OF cDPG AND 2,3-DPG AT DIFFERENT SPECIFIC GROWTH RATES, ASSESSED BY SPECTROPHOTOMETRIC AND ISOTACHOPHORETIC ASSAYS

Cells of *Mb. thermoautotrophicum* were grown in a hydrogen-limited continuous culture with cysteine (0.35 g/l) and thiosulphate (1 g/l) as the source of sulphur.

Specific growth rate (h^{-1})	*Intracellular content (μmol/g dw)*			
	Spectrophotometric assay		*Isotachophoretic assay*	
	cDPG	*2,3-DPG*	*cDPG*	*2,3-DPG*
0.036	210	23	204	12
0.108	111	31	173	30
0.180	113	37	105	24
0.252	79	12	90	7
0.288	70	3	84	4

limit of the spectrophotometric assay is 2 μmol/g dw of cells[6], which is equivalent to about 20 nmol per assayed sample. Results obtained with the isotachophoretic assay were generally in good agreement with those of the spectrophotometric assay, as is shown in Table I for levels of cDPG and 2,3-DPG measured in samples of *Mb. thermoautotrophicum* cultured at different specific growth rates in a continuous culture.

Growth and cDPG levels

Mb. thermoautotrophicum was cultivated batchwise to assess growth and intracellular levels of cDPG as a function of time (Fig. 4). Growth proceeded exponentially up to cell densities of absorbance 0.8. At absorbance values of 0.8–2.0 growth was linear, probably as a result of hydrogen limitation due to inefficient hydrogen transfer into the culture medium[6]. From Fig. 4 it can be seen that high levels of cDPG

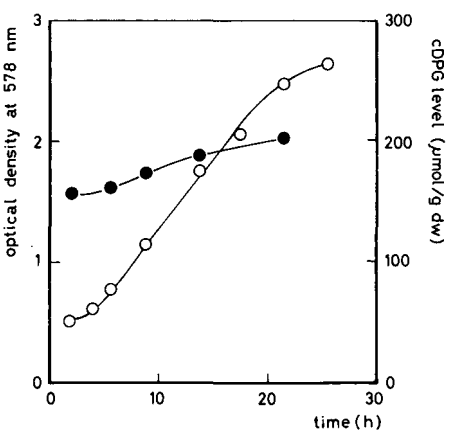

Fig. 4. Relationship between growth and cellular cDPG content of cells of *Mb. thermoautotrophicum* grown in a 0.5-l batch culture. ○ = Absorbance at 578 nm; ● = cDPG concentration. dw = Dry weight.

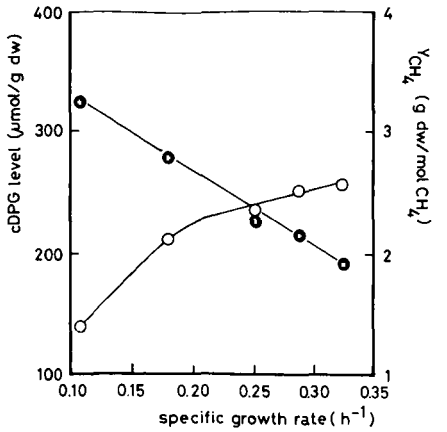

Fig. 5. (●) Intracellular cDPG level and (○) specific growth yield (Y_{CH_4}) in relation to the specific growth rate in a hydrogen-limited chemostat culture of *Mb. thermoautotrophicum*.

are maintained in stationary- and logarithmic-phase cells and also in cells during linear growth. A relatively small increase in the cDPG concentration was recorded during the course of growth.

To determine the cellular level of cDPG in relation to the yield of growth at different specific growth rates, *Mb. thermoautotrophicum* was cultured in a hydrogen-limited chemostat under steady-state conditions at different dilution rates. The values obtained for both parameters are given in Fig. 5. The concentration of cDPG was found to increase with decreasing growth rate, an observation substantiated by the data in Table I, while the yield of cells per mole of methane produced (Y_{CH_4}) decreased with decreasing growth rate.

DISCUSSION

Isotachophoresis can be used for the qualitative and quantitative analysis of anionic metabolites[9,12]. Sample components are introduced at the interface of a discontinuous buffer system, the leading and terminating electrolytes, and separated into discrete, consecutive zones with homogeneous concentrations. Each compound can be identified by its relative step height in the isotachopherogram, and its concentration can be derived from the associated zone length. Isotachophoresis is a simple and rapid technique and was found here to be very well suited to measure the concentration of phosphate-containing metabolites in cell extracts of a methanogenic bacterium. Quantification of cDPG and 2,3-DPG with the novel isotachophoretic assay yielded values that were in good agreement with those obtained with the spectrophotometric assay commonly employed for this purpose. With the isotachophoretic assay, however, the detection limit is two orders of a magnitude lower and the manipulations are less elaborate and less time consuming in comparison with the spectrophotometric assay.

cDPG is one of several unusual metabolites unique to methanogenic bacteria[13]. As cDPG levels as high as 200 μmol/g dw, equivalent to 5% of the total dry weight of the cells, have been found in *Mb. thermoautotrophicum*, an important physiological

function of cDPG is evident. Here we investigated possible functions of cDPG in the phosphate and energy metabolism of this methanogen, using the isotachophoretic assay for quantification.

During batch cultivation, high concentrations of cDPG were maintained both in logarithmic-phase cells and in stationary-phase cells. Apparently, intracellular levels of cDPG are not strongly influenced by the metabolic state of the cell. Although cDPG has been suggested[2,6] to function as a phosphate storage compound, the observation that cDPG levels do not increase significantly during stationary growth, as polyphosphate levels in other microorganisms do, does not support this view.

In hydrogen-limited chemostat cultures, an increase in the cellular content of cDPG was observed with a decrease in specific growth rate. The latter phenomenon might be related to changes in the energy metabolism of the cell and could indicate a function of cDPG as an energy-storage compound in *Mb. thermoautotrophicum*. Assuming that the amount of ATP synthesis is comparable at different growth rates, the higher availability of ATP at a low growth rate could then account for a lower cDPG turnover, and hence the higher cDPG concentrations found in the cells at low rates of growth. A role of cDPG as phosphagen, however, does not find much support in the literature[1], and several groups have reported results to the contrary of such a function[2,5,6,8].

The question of why *Mb. thermoautotrophicum* and a number of other methanogenic bacteria maintain high intracellular levels of cDPG remains unanswered. The relevance of the various functions attributed to this compound needs to be substantiated by future research, which might be facilitated by the isotachophoretic cDPG assay presented here.

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Note

Simple, direct gas chromatography–mass spectrometry interface for the ion trap detector

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Since its introduction in 1983[1,2], the utility of the Finnigan-MAT ion trap detector for gas chromatography–mass spectrometry (GC–MS) has been augmented by the addition of automatic gain control[3] and chemical ionization capability[4]. However, the GC–MS interface has remained unchanged and consists of an approximately 4-ft. long flexible transfer line designed for use as an open-split interface[5]. In an open-split interface, the exit of the capillary GC column is designed to operate at atmospheric pressure. The deactivated fused-silica restrictor housed within the transfer line connects the capillary GC column to the mass spectrometer and provides the pressure drop required by the mass spectrometer's vacuum system. For our work in pesticide residue analysis, we often employ short (3–4 m) capillary GC columns for rapid analysis of labile pesticides and their metabolites[6]. Such analyses can best be accomplished with the exit end of the capillary column directly coupled to the MS vacuum in conjunction with high carrier gas linear velocities[7–9]. For use as a direct GC–MS interface, the existing interface is cumbersome to work with, particularly when changing columns, and results in a significant portion of a short capillary column residing within the transfer line.

In this paper, we report on a simple, direct GC–MS interface of less than 1 ft. in length which provides a line-of-sight path for the capillary column enabling direct insertion of the column outlet into the ion trap. The interface was designed to facilitate insertion and removal of the column from the ion trap and can be readily constructed and retrofitted to existing ion trap detectors. The new interface has made possible the GC–MS confirmatory analysis of terbufos, terbufos sulfoxide and terbufos sulfone at residue (1 ng) levels, an analysis previously performed[6] on a Finnigan-MAT TSQ-70.

EXPERIMENTAL

Apparatus

A drawing of the interface is shown in Fig. 1. The transfer line consists of an empty stainless-steel liquid chromatography column (25 cm × 2.1 mm I.D. × 1/4 in. O.D.; Part No. 5-9127, Supelco, Bellefonte, PA, U.S.A.) and is connected to the ion

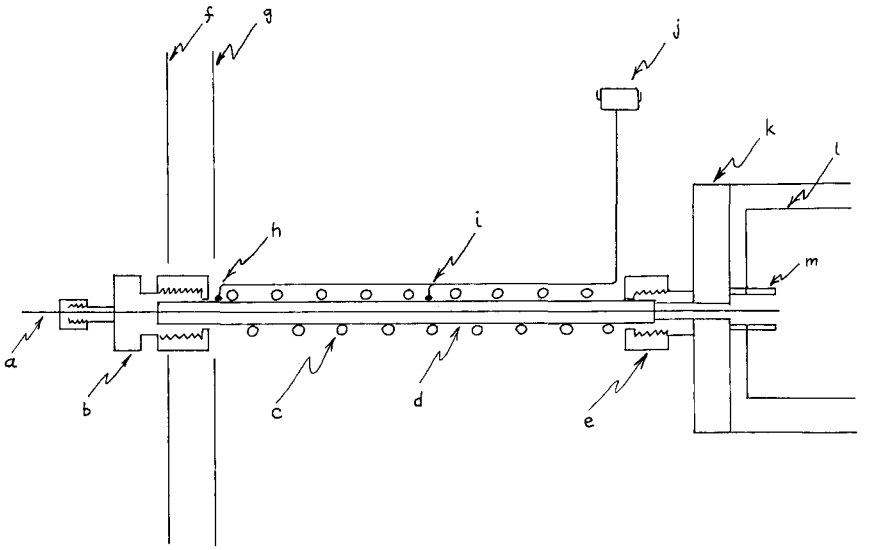

Fig. 1. Drawing of the direct GC–MS interface. a = Capillary GC column; b = 1/4 in.-to-1/16 in. Swagelok reducing union; c = coiled cable heater; d = 1/4 in. O.D. stainless-steel column; e = 9/16 in. Swagelok nut; f = GC oven inner wall; g = GC oven outer wall; h = "open-split" thermocouple; i = "transfer line" thermocouple; j = Burndy connector; k = ion trap manifold; l = ion trap; m = Rulon spacer.

trap manifold by a 9/16-in. Swagelok nut and graphitized vespel ferrule. A 1/4 in.-to-1/16 in. Swagelok reducing union connects the transfer line to the capillary GC column and provides the vacuum seal for the mass spectrometer. Removing the 5/16-in. Swagelok nut on the reducing union is all that is required for changing capillary GC columns interfaced to the mass spectrometer. The overall length of the transfer line from the back of 5/16-in. Swagelok nut to the end of the 1/4 in. O.D. tubing is 270 mm. The distance from the back of the 5/16-in. nut to the inside wall of the ion trap manifold is 283 mm. Combining the latter value with a measured length of 15 mm for the Rulon guide within the ion trap itself yields an overall length of 298 mm of capillary GC column to reach from the back of the 5/16-in. nut to the outlet of the Rulon guide within the ion trap.

Heating of the transfer line is provided by a coiled cable heater (24 in., 120 V, 225 W, 0.25 in. coil I.D., 9.00 in. coil width, standard 2A lead orientation; Part No. 62H24A6X, Watlow Electric, St. Louis, MO, U.S.A.) and uses the same heater power supply as used by the manufacturer's "transfer line" heater. The "transfer line" thermocouple (iron–constantan, Part No. TC-GG-J-20-36-STD, Omega Engineering, Stamford, CT, U.S.A.) is attached with high-temperature cement (Omega Bond 200, Omega Engineering) approximately midway along the transfer line and equally spaced between two turns of the coiled cable heater. The "open-split" thermocouple is attached in the same manner next to the reducing union. The heater and thermocouple leads are attached to a Burndy connector used by the manufacturer for the ion trap detector "transfer line" (Part No. 00004-22050, Finnigan-MAT, San Jose, CA, U.S.A.). The transfer line is insulated with flexible ceramic tape (Part No. 395-41, Cotronics Corp., Brooklyn, NY, U.S.A.) and two layers (1/2 in. I.D. × 1/8 in. wall and

1 in. I.D. × 1/8 in. wall) of braided fiberglass sleeving (Flextex, Ambler, PA, U.S.A.). The "exit nozzle" heater is placed on top of the 9/16-in. Swagelok nut at the ion trap manifold and the "exit nozzle" thermocouple is positioned between the nut and the heater.

The gas chromatograph is a Varian 3500 and is sited with the right side (viewed from the front) facing the rear of the ion trap detector. A 1 3/4 in. diameter hole through the right side panel and a 3/4 in. diameter hole through the GC oven wall are drilled on-line with the GC inlet port on the ion trap manifold. Following attachment of the transfer line to the ion trap manifold, the holes in the GC are slid over the interface until the 9/16-in. nut on the reducing union rests on the inside wall of the column oven. This positions the side of the GC approximately 5 in. from the rear of the ion trap detector and allows adequate room for air intake and exhaust to cool the electronics of the mass spectrometer.

Materials

Terbufos, terbufos sulfoxide and terbufos sulfone were obtained from American Cyanamid Company (Princeton, NJ, U.S.A.). Lauryl laurate was purchased from Pfaltz & Bauer (Stamford, CT, U.S.A.).

RESULTS AND DISCUSSION

The first test of the direct GC–MS interface for a difficult pesticide residue analysis was to determine if chromatography obtained on terbufos and its oxidative metabolites previously performed on a TSQ-70 could be replicated with the ion trap detector. Terbufos sulfoxide is an organophosphorous pesticide metabolite which is notoriously difficult to chromatograph[10,11]. A short capillary GC column from injector to detector, high carrier gas linear velocities, and rapid temperature programming of the column oven (30°C/min) are required for a successful analysis[6]. Using the same chromatographic conditions as employed previously, the chromatography of terbufos and its oxidative metabolites excellently matches that reported in the literature. As shown in Fig. 2, excellent peak symmetry is evident for each analyte

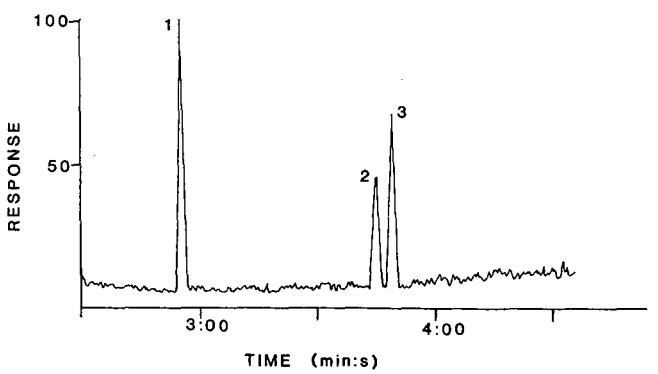

Fig. 2. Total ion current chromatogram from 1 ng each of terbufos (1), terbufos sulfoxide (2), and terbufos sulfone (3) using the direct GC–MS interface.

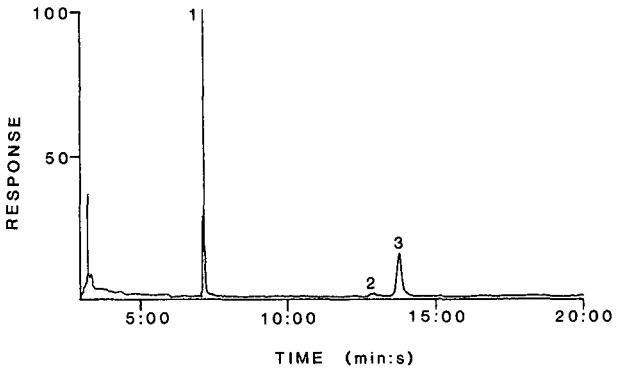

Fig. 3. Total ion current chromatogram from 100 ng each of terbufos (1), terbufos sulfoxide (2), and terbufos sulfone (3) using the manufacturer's open-split interface.

at the 1-ng level. The electron impact mass spectra of the analytes compare favorably with those in the literature[10,11].

Earlier attempts to replicate this analysis on the ion trap detector with the manufacturer's open-split interface gave the results shown in Fig. 3. This chromatogram was generated from 100 ng of each analyte. At the 1-ng level, only terbufos gave a reasonable response.

To check for chromatographic peak tailing of high boiling analytes because of cold spots on the interface, lauryl laurate (dodecyl dodecanoate) was chosen. Lauryl laurate has been reported to be a useful compound for checking GC–chemical ionization MS performance[12]. The analysis used the same chromatographic conditions as those employed for terbufos and it oxidative metabolites with the exceptions of extending the temperature program to 220°C and raising the transfer line temperature to 200°C. The total ion current chromatogram from an 11-ng splitless injection is illustrated in Fig. 4 and shows no evidence of peak tailing. Comparable chromatographic performance was also obtained on the higher boiling *n*-hexatriacontane (*n*-$C_{36}H_{74}$, b.p. = 265°C at 1 Torr) after extending the column oven temperature program to 300°C and raising the transfer line temperature to 250°C. Under these experimental conditions, *n*-$C_{36}H_{74}$ eluted at 285°C.

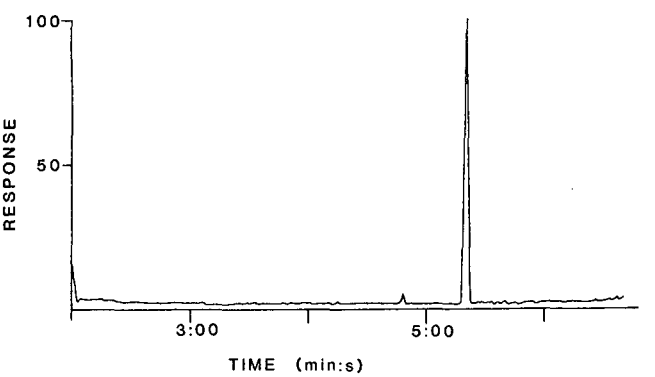

Fig. 4. Total ion current chromatogram from 11 ng of lauryl laurate.

In conclusion, a simple, direct GC–MS interface has been constructed for the Finnigan-MAT ion trap detector. The short, line-of-sight path permits the capillary GC column to be placed within the ion trap thus facilitating the analysis of labile analytes as shown for terbufos sulfoxide. The interface shows no evidence of chromatographic peak tailing due to active sites or cold spots. Capillary GC columns can be easily inserted and removed from the interface, and the interface can be readily constructed and retrofitted to existing ion trap detectors. While used here with short capillary columns for pesticide residue analysis, the interface is just as applicable for capillary GC–MS using much longer columns. Improved chromatographic performance could be achieved by operating at subambient inlet pressures[8,13], but with a resultant increase in the complexity of the GC–MS system.

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CHROM. 22 309

Note

High-performance liquid chromatographic post-column reaction system for the electrochemical detection of ascorbic acid and dehydroascorbic acid

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Recently the simultaneous determination of ascorbic acid (AA) and dehydroascorbic acid (DHAA) in a variety of samples utilizing high-performance liquid chromatography (HPLC) has received considerable attention. This has ocurred to a large extent since Tolbert and Ward's[1] complaint in 1980 about a lack of a "completely satisfactory" assay for DHAA, their suggestions to reduce DHAA to AA, after separation, followed by ultraviolet (UV) detection or electrochemical detection (ED) and their belief in the importance of the DHAA/AA ratio.

Many detection schemes have been used. Some, with ED, determined AA followed by total AA and DHAA after reduction of the DHAA to AA with no separation[2,3]. Also UV detection was employed in a similiar manner[4–6]. Post-column derivatization followed by fluorescence detection has also been used[7–9]. The use of both ED for AA and UV detection for derivatized DHAA has been described[10,11]. Recent reviews have been published[12,13].

Ziegler *et al.*[14] reported a convenient HPLC post-column system in which DHAA is reduced to AA by dithiothrietol (DTT) followed by UV detection. The UV detection of DHAA in its reduced form, AA, is more sensitive and selective than UV detection of DHAA itself.

ED of both AA and DHAA (after reduction to AA) would be advantageous because it is more sensitive than UV detection and often more selective than UV and fluorescence detection. Ziegler *et al.*[14] also reported an unsuccessful attempt to use ED, attributing their difficulties to high background and electrode poisoning that resulted from the excess DTT. We report an extension of the system of Ziegler *et al.*[14] in which the excess DTT is reacted with N-ethylmaleimide (NEM), thereby permitting ED of both AA and DHAA. Okamura[15] did this in a non-chromatographic method.

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EXPERIMENTAL

Fig. 1 is a schematic diagram of the system. Numbers 1–10 refer to this figure. No. 1 is a Waters M-45 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) with a solvent injection valve (Model 7010; Rheodyne, Cotati, CA, U.S.A.) equipped with a 20-μl loop. The analytical column (2) is a Nova-Pak C_{18} reversed-phase column from Waters.

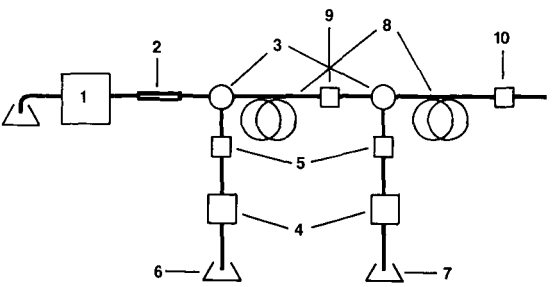

Fig. 1. Schematic diagram of reaction system. 1 = HPLC delivery system and sample injector; 2 = analytical column; 3 = mixing tees; 4 = pumps; 5 = pulse dampeners; 6 = DTT solution; 7 = NEM solution; 8 = reaction coils; 9 = UV detector; 10 = electrochemical detector. See text for details.

The mobile phase is 0.010 *M* $HClO_4$ and a flow-rate of 1.1 ml/min was used. Visco-mixer mixing tees (Lee Co., Westbrook, CT, U.S.A.) (3) are employed to combine the post-column reagents with the mobile phase. Reagent delivery pumps (4) are 350 pumps (SSI, State College, PA, U.S.A.) and pulse dampeners (5) are SSI LP-21.

The first post-column reagent (6) is 0.010 *M* DTT, 0.25 *M* NaH_2PO_4 and 0.25 *M* Na_2HPO_4, added at 0.50 ml/min. The second post-column reagent (7) is 1.0% NEM, 0.25 *M* NaH_2PO_4 and 0.25 *M* Na_2HPO_4, added at 0.60 ml/min. These reagents were stored under refrigeration for no more than a few days. Reaction coils (8) are 0.010 in. (0.254 mm) I.D. tubing of about 10 cm diameter. All tubing is stainles steel. The first reaction coil, in which DTT reduces DHAA to AA, is 24 m long. The second reaction coil, in which NEM reacts with the excess DTT, is 15 m long. The entire system was maintained at room temperature (22 $\pm$ 3°C).

A UV detector (9) (Waters Model 440 at 254 nm) was used for comparison with the electrochemical detector (10), an LC-3A Amperometric Detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with an Re-1 Ag/AgCl reference electrode and a TL-3 glassy carbon working electrode. The glassy carbon electrode was pretreated each day by polishing with BAS polishing alumina (CF-1050, Bioanalytical Systems) followed by about 1 min of ultrasonic cleaning (Model SC-40; Sonicor, Copiague, NY, U.S.A.) and thorough rinsing with water and methanol. The working electrode was set at +0.60 V *vs.* the reference electrode. This potential was chosen because it was in the diffusion current region of hydrodynamic voltammograms obtained on this equipment.

DTT and NEM were obained from Aldrich (Milwaukee, WI, U.S.A.). All other reagents were A.C.S. reagent grade.

Standard AA solutions were made by dissolving the weighed reagent-grade compound in 0.010 *M* $HClO_4$. Standard DHAA solutions were made by oxidation of

AA solutions with saturated bromine water[15]. Excess bromine was removed by sparging with high-purity-grade nitrogen. AA and DHAA solutions of 10^{-6} *M* and higher (in 0.010 *M* $HClO_4$) were stable for at least 6 h. Standard solutions were prepared daily.

The mobile phase and post-column reactor solutions were filtered through 0.45-μm filters (Type HA; Millipore, Bedford, MA, U.S.A.) and vacuum deaerated.

RESULTS AND DISCUSSION

Standard solutions and selection of conditions

Fig. 2 shows examples of chromatograms of DHAA, AA and their mixture using the system described above. For the same concentrations the peak heights of AA and DHAA are essentially the same; this indicates, in accord with Ziegler *et al.*[14], that DHAA is completely reduced in the reactor (see data below). Background current due to oxidation of DTT is suppressed successfully by this system so that the electrochemical detector can be used at its most sensitive setting (1 nA full scale) with only small offset settings. Analyte concentrations as low as 10^{-7} *M* are easily determined (see below).

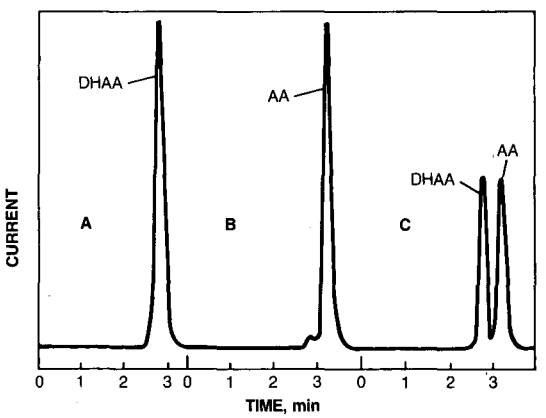

Fig. 2. Chromatograms of AA and DHAA. (A) 10^{-5} *M* DHAA; (B) 10^{-5} *M* AA; (C) $5 \cdot 10^{-6}$ *M* AA and DHAA.

Under he optimized conditions (described in the Experimental section), DTT was 3.1 m*M* in the first reaction coil and 2.3 m*M* in the second coil while NEM was 22 m*M* in the second coil. The pH was approximately 7. When the DTT concentration in the first coil was decreased to less than about 2 m*M*, DHAA was not completely reduced, as illustrated by these data:

DTT (mM)	*DHAA signal/AA signal*
3.9	1.04
2.7	1.04
2.1	0.94
1.5	0.88
1.3	0.85
0.83	0.36

The length of the first reaction coil was chosen as the minimum length yielding a ratio of 1 for the DHAA/AA signals using the optimized DTT concentration and 10^{-5} *M* DHAA samples. The NEM concentration, flow-rate and the second reaction coil length were chosen to give minimum background current on the electrochemical detector, along with acceptable broadening of the chromatographic peaks and an acceptable total pressure. The use of different chromatographic conditions may result in different optimum post-column reactor conditions.

Electrochemical detector drift

Carbon electrodes, in spite of their utility, are notorious for their changing sensitivity, "poisoned surfaces", and their varying behavior resulting from different pretreatments. A recent report[16] discusses this and shows how AA signals can vary with pretreatment of the electrode.

Fig. 3 shows how the sensitivity of the carbon electrode in this system decreases with time. This decrease in sensitiviy was not due to the loss of AA and DHAA in the standard solutions. This is clear because the UV detector (in the system as a check) gave the same signals over the course of the experiment. When the system was run without DTT and NEM, but otherwise the same, the sensitivity for AA changed considerably less for similar times.

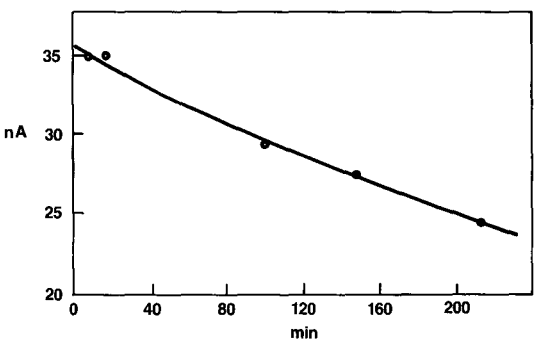

Fig. 3. Decrease in sensitivity with time. Signal obtained using 10^{-5} *M* AA and DHAA solutions.

Even though the residual current is sufficiently suppressed, apparently the DTT NEM system is slowly "poisoning" the electrode surface. The only noticeable change is in sensitivity; peak shapes remain the same. Chromatograms were obtained successfully with an electrode that had been used for about a week without cleaning and had lost approximately 90% of its original sensitivity.

This drifting sensitivity indicates that standards must be used along with each sample and, depending on the sensitivity required, the electrode must be cleaned at appropriate intervals. The length of these time intervals increases if the electrochemical detector is off between samples. The cleaning procedure, described above, can be completed in about 15 min. This pretreatment and the frequent use of standards is a minor disadvantage when the sensitivity and selectivity of the electrochemical detector are important.

Detection limits and linearity

Table I gives representative data which show that, after correction for the changing sensitivity of the detector, the peak height is linear with concentration of AA and DHAA from 10^{-5} to 10^{-7} *M* utilizing the entire sensitivity range of the instrument. The correction was made by multiplying each result by the ratio of the original sensitivity to the sensitivity at the time of measurement, obtained from a plot as in Fig. 3. The signals from repeat injections of 10^{-6} *M* and higher AA and DHAA solutions were reproducible to about 2%. The representative data in Table I have a relative sample standard deviation (coefficient of variation) of 3% ($n=6$) for the signal/concentration ratio of AA and 4% ($n=4$) for DHAA.

TABLE I

SIGNAL *VS.* CONCENTRATION

Representative data corrected for changes in detector sensitivity (see text).

DHAA		*AA*	
Concentration (M)	*Signal[a]/ concentration (mA/M)*	*Concentration (M)*	*Signal[a]/ concentration (mA/M)*
–	–	$1.33 \cdot 10^{-5}$	1.03
–	–	$1.33 \cdot 10^{-5}$	1.01
$1.10 \cdot 10^{-5}$	0.99	$1.10 \cdot 10^{-5}$	0.96
$1.10 \cdot 10^{-5}$	0.94	$1.10 \cdot 10^{-5}$	0.99
$5.50 \cdot 10^{-6}$	1.07	$5.50 \cdot 10^{-6}$	0.98
$1.10 \cdot 10^{-6}$	1.02	$1.10 \cdot 10^{-6}$	1.02
$2.20 \cdot 10^{-7}$	1.03	$2.20 \cdot 10^{-7}$	1.15
$1.10 \cdot 10^{-7}$	1.04	$1.10 \cdot 10^{-7}$	1.03
$1.10 \cdot 10^{-7}$	0.70	$1.10 \cdot 10^{-7}$	0.90

[a] Corrected as described in text.

The detection limit of $1 \cdot 10^{-7}$ *M* (about 3 × noise) was 10 times lower than the limit with the UV detector used for comparison purposes. This limit corresponds to detection of 0.3 ng of analyte; Ziegler *et al.*[14] report a detection threshold of 1.4 ng at a signal-to-noise ratio of about 2 with a UV system.

DHAA contaminant or inadvertent oxidation of AA

A small amount of DHAA (1–2%) was found in every sample of AA (see Fig. 2). The following indicate that the DHAA was a contaminant in the AA and not due to artifactual oxidation: (1) the DHAA peak remains in the same proportion to the AA peak upon serial dilution; (2) the DHAA peak remains constant with time; (3) the same peak is observed whether the AA is diluted with 0.010 *M* $HClO_4$ made from helium-sparged deionized water, air saturated deionized water or ordinary tap water; (4) the peak has the definite retention time of DHAA, unlike the flat, broadened signal obtained by Seki *et al.*[8] which was attributed to AA oxidation in the analytical column.

Disconcerting, however, are the facts that three different sources of AA, including the sodium salt of AA, gave essentially the same DHAA signal, and the most dilute (10^{-7} *M*) solution of AA did not always abide by finding point 1 above.

This problem will be investigated further but it is another illustration of the success of the post-column reaction system.

Examples of real samples

A few "real" samples were analyzed for DHAA and AA as illustrations of the use of this system. Fig. 4 shows chromatograms from a urine sample diluted 1 to 100 with 0.010 *M* $HClO_4$. Under these conditions the electrochemical detector is far superior to the UV detector because of its selectivity. This urine sample contained 0.36 m*M* AA and a trace of DHAA.

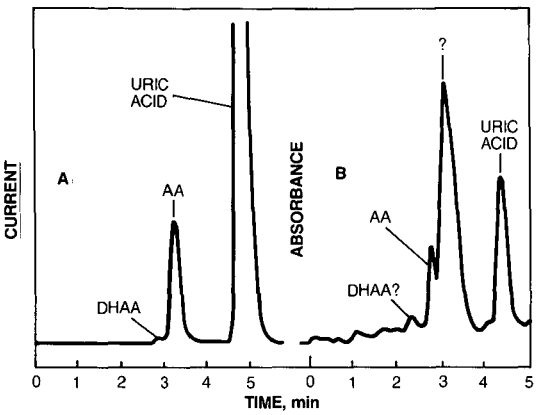

Fig. 4. Chromatograms of a urine sample. Sample diluted 1:100 with 0.010 *M* $HClO_4$. (A) ED; (B) UV detection.

A sample of rose hips tea made from a commercial brand contained 80 mg/l DHAA and 140 mg/l AA.

Saliva from one individual was found to contain 1.1 mg/l DHAA and only a trace amount of AA; a range of 0–3.7 mg/l of total AA in saliva has been reported[17].

CONCLUSION

The post-column reaction system using ED is an improvement over one previously described[14] which uses UV detection. ED affords greater sensitivity and selectivity than UV detection.

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CHROM. 22 227

Note

Determination of tyramine in cheese by reversed-phase high-performance liquid chromatography with amperometric detection

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(First received September 5th, 1989; revised manuscript received December 18th, 1989)

Tyramine, the biogenic amine derivative of tyrosine, is an active pressor amine which occurs naturally in many foods[1,2], especially fermented food products. Tyramine is formed during the cheese-making process by the bacterial degradation of milk protein and subsequent decarboxylation of tyrosine. Tyramine generally does not represent any hazard to individuals unless large amounts are ingested or the normal routes of catabolism are inhibited or are genetically deficient. Usually tyramine is metabolized to *p*-hydroxyacetic acid by monoamine oxidase in the human intestine, liver and kidneys and excreted via the urine. However, tyramine can cause a hypertensive crisis[3–6] in patients treated with monoamine oxidase inhibitor drugs such as antidepressants and antitubercular drugs. Particularly with cheese, it is well known as the so-called cheese effect or cheese reaction.

The tyramine content of cheese is known to be variable not only in different types of cheese but also in the same type of cheese. Therefore, the determination of the tyramine content of cheese is required for pharmaceutical, therapeutic and food hygiene purposes. Tyramine in cheese has been determined by fluorometric methods[7–10] and with amino acid analyzers[11,12]. However, these methods are time-consuming and require relatively large amounts of sample.

In this paper, we describe a method for the detection of tyramine in cheese by reversed-phase high-performance liquid chromatography (HPLC) with amperometric detection (AMD), which provides a sensitive and specific method for compounds that possess intrinsic electrochemical activity[13].

EXPERIMENTAL

Apparatus

The liquid chromatograph was a Trirotor-VI (Jasco, Tokyo, Japan) with a

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LiChrosorb RP-Select B (7 μm) column (250 × 4 mm I.D.) (Cica-Merck, Tokyo, Japan). The detector was an E-558 amperometer (IRICA, Kyoto, Japan) with a glassy carbon electrode. The applied potential was maintained at 0.7 V *versus* an Ag/AgCl reference electrode with a sensitivity setting of 4 nA full-scale. An F-1000 fluorescence spectrometer (Hitachi, Tokyo, Japan) with an excitation wavelength of 225 nm and an emission wavelength of 305 nm was used.

Other equipment consisted of an Ultra-Turrax homogenizer (IKA-Werk, F.R.G.), an H-107 type B centrifuge (Kokusanenshinki, Tokyo, Japan) and a 0.45-μm Chromatodisc 13A membrane filter (Kurashikiboseki, Osaka, Japan).

Reagents

A standard solution was prepared by dissolving 10 mg of tyramine (Sigma, St. Louis, MO, U.S.A.) in 10 ml of mobile phase and diluting to 1 μg/ml with mobile phase.

The mobile phase consisted of 0.05 *M* phosphate buffer (pH 7.5) containing 1 m*M* EDTA. This solution was passed through the 0.45-μm membrane filter and acetonitrile added to make a final concentration 10%. The flow-rate was 1.0 ml/min at ambient temperature.

Perchloric acid (5%) was prepared by diluting 70% perchloric acid (Kanto Chemical, Tokyo, Japan).

Sample preparation

Weigh 5 g of cheese into a 50-ml centrifuge tube. Add 10 ml of 5% perchloric acid and 10 ml of dichloromethane and homogenize for 2 min in the Ultra-Turrax homogenizer. Decant the aqueous phase into another flask and discard the organic phase. Repeat the procedure twice, then collect all the aqueous phases. Neutralize a 1-ml aliquot of aqueous phase with 0.1 *M* sodium hydroxide solution and dilute to 5 ml with distilled water. Filter through the 0.45-μm membrane filter and inject 10 μl of the eluate into the HPLC system.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of tyramine obtained from a cheese extract. The retention time of tyramine was *ca.* 10 min and the detection limit was 0.2 ng at the a signal-to-noise ratio of 3. The detection limit of tyramine in cheese with this method was 0.3 μg/g.

Fig. 2 shows the relationship between the applied potential of the working electrode and the recorder response. The onset potential of tyramine oxidation is about 0.55 V. The response of the detector to tyramine depends on the applied voltage. Higher potentials were impractical because of the background noise generated. Therefore, the detector operating potential was set at 0.7 V. The calibration graph was checked by measuring peak areas. Linearity of the detector response for tyramine was obtained by injecting 1–5 ng.

A recovery study using five tyramine-spiked Camembert cheeses at levels of 1, 10 and 100 μg/g showed recoveries of 92.3–95.3% with a coefficient of variation of 2.4–3.1%.

Fluorimetric detection was used for comparison with the proposed method. A

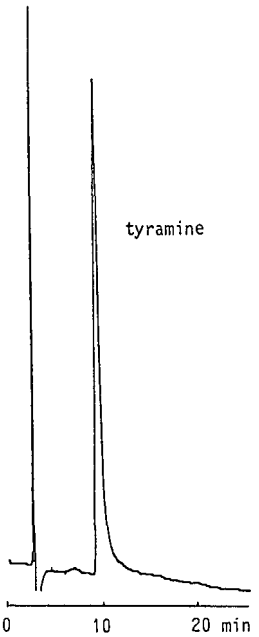
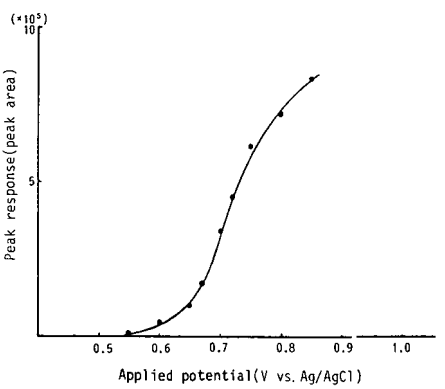


Fig. 1. Typical high-performance liquid chromatogram of tyramine obtained from cheese extract. Chromatographic conditions: stationary phase, LiChrosorb RP-Select B prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column; mobile phase, 0.05 *M* phosphate buffer (pH 7.5) containing 10% acetonitrile; flow-rate, 1 ml/min at ambient temperature; detector applied voltage, 0.7 V *vs.* Ag/AgCl.



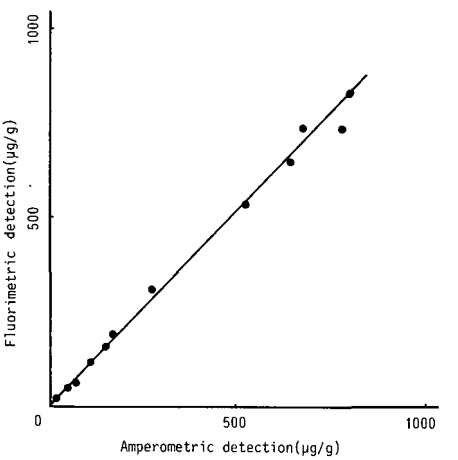


Fig. 2. Effect of applied potential on detector response to tyramine with amperometric detection.

Fig. 3. Correlation of tyramine contents in various cheeses measured with amperometric and fluorimetric detection. $y = 0.999x + 8.393$; $r = 0.995$ ($n = 12$).

good correlation between the two methods was obtained, as shown as in Fig. 3 using twelve different cheeses. The minimum detectable amount with the method is 0.2 ng; this represents a *ca.* 25-fold increase over the fluorimetric detection sensitivity.

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CHROM. 22 217

Note

Direct liquid chromatographic resolution of (*R*)- and (*S*)-abscisic acid using a chiral ovomucoid column

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Abscisic acid (ABA) is a plant hormone with an important role in plant growth and development. It occurs naturally as the (+)-*S*-enantiomer. Most studies on ABA metabolism in plants have used racemic, synthetic material. As differences in the physiological effects and metabolism of enantiomers of ABA have been reported[1,2], the resolution of the enantiomers is essential for the elucidation of ABA metabolism in plants.

The enantiomers have only been separated with difficulty. Resolution has been achieved by repeated crystallization of brucine salts[3], acetylcellulose column chromatography[4], high-performance liquid chromatography (HPLC) of the methyl ester[5] and the diol derivative[6] and immunoaffinity chromatography[7]. Unfortunately, these methods are time consuming and unable to resolve ABA directly. Recently, Nonhebel[8] and Okamoto *et al.*[9] reported the direct optical resolution of ABA by HPLC using a chiral stationary phase, but the mechanisms involved in the resolution of (*RS*)-ABA have not been described. This paper describes the direct and rapid resolution of (−)-(*R*)- and (+)-(*S*)-ABA by HPLC using a chiral ovomucoid column[10,11]. The enantiomeric ratio can also be determined accurately by this method. Finally, the possible mechanisms involved in the resolution of (*RS*)-ABA are discussed.

EXPERIMENTAL

Apparatus

A Shimadzu LC-5A instrument equipped with an SPD-2A variable-wavelength UV monitor was used. A stainless-steel column (150 × 4.6 mm I.D.) was packed with ovomucoid-conjugated aminopropylsilica gel (5 μm), an available as ULTRON ES-OVM from Shinwakako (Kyoto, Japan).

Chemicals

Racemic ABA was purchased from Wako (Osaka, Japan). (+)-(*S*)-ABA was provided by Dr. N. Hirai. The methyl ester of (*RS*)-ABA was obtained by treatment of ABA with ethereal diazomethane. Sodium 1-heptanesulphonate (SHS) and tetra-*n*-

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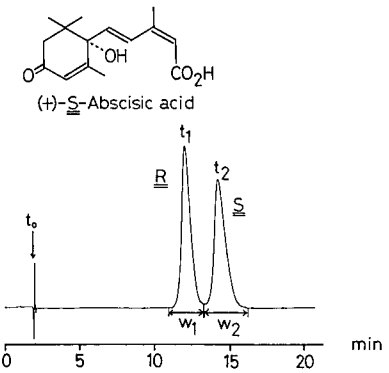

Fig. 1. Chromatogram resulting from an injection of 1 μl of a mixture of (*RS*)-ABA (0.5 μg). Mobile phase, 2% of 2-propanol in 20 m*M* potassium phosphate buffer (pH 3.50) at a flow-rate of 1.0 ml/min and ambient temperature. UV detection (254 nm, 0.04 a.u.f.s.). $k'_1 = (t_1 - t_0)/t_0$; $k'_2 = (t_2 - t_0)/t_0$; $\alpha = k'_2/k'_1$; $R_s = 2(t_2 - t_1)/(w_1 + w_2)$.

pentylammonium bromide (TPAB) were obtained from Tokyo Kasei (Tokyo, Japan). All other chemicals were of analytical-reagent grade.

RESULTS AND DISCUSSION

Fig. 1 shows the optical resolution of (−)-(*R*)- and (+)-(*S*)-ABA on an ovomucoid column. Baseline separation was almost achieved by using 2% 2-propanol in 20 m*M* potassium phosphate buffer (pH 3.50) as the mobile phase. The capacity factors (k') were 13.6 and 16.59, respectively. The separation factor (α) and resolution factor (R_s) were 1.22 and 1.01, respectively. The enantiomer with the longer retention time was identified as the natural *S*-enantiomer by co-chromatography with (+)-(*S*)-ABA standard.

We carried out tests on the recovery of the *R*-enantiomer from (*S*)-ABA to establish whether the enantiomeric ratio could be determined accurately. (*RS*)-ABA was added to (*S*)-ABA to give a concentration of (*R*)-ABA of 0.5 and 1.0%. The added *R*-enantiomer was recovered quantitatively at both concentrations by this procedure (Table I). The detection limit of (*R*)-ABA was 2.5 ng per injection. Hence the method allows the proportion of *R*-enantiomer in a sample to be measured precisely.

TABLE I

RESULTS OF TESTS ON THE RECOVERY OF (−)-(*R*)-ABA FROM (+)-(*S*)-ABA

Calculated (%)	*Found (%)*	*Recovery (%)*
0.99	1.00	101.0
	1.01	102.0
	Av. 1.01	Av. 101.5
0.50	0.50	100.0

TABLE II

INFLUENCE OF MONOVALENT ALCOHOLS AND ACETONITRILE ON THE RESOLUTION OF ABA

Mobile phase: modifier + 20 m*M* phosphate buffer (pH 3.50).

Modifier	k'	α	R_s
None	38.21	1.21	0.51
Methanol (7%)	15.96	1.16	0.61
Ethanol (5%)	10.61	1.00	—
2-Propanol (2%)	13.6	1.22	1.01
Acetonitrile (3%)	13.82	1.20	0.70

The retention and resolution of ABA can be regulated in three ways, either by addition of an organic modifier to the mobile phase, or by varying the ionic strength or the pH of the mobile phase. The content and kind of organic modifier in the mobile phase greatly influenced the retention and selectivity. An increased concentration of the organic uncharged modifier reduces the capacity factor. Table II gives some results obtained using monovalent alcohols and acetonitrile as an uncharged modifier. Ethanol removed the chiral resolution effect of the column, but the reason for this phenomenon is not clear. 2-Propanol was found to be the best modifier for ABA. ABA was strongly retained by the ovomucoid column at pH 4.0–5.0, as shown in Fig. 2. This pH range coincides with the isoelectric point of ovomucoid.

The above results imply that hydrophobic interactions are involved in the retention of the solute. Table II shows that the separation factors were almost unchanged despite about a three-fold reduction in the capacity factor on addition of 2% of 2-propanol. This suggests that hydrophobic interactions play an important role in the retention of ABA, but that they are not essential for the resolution.

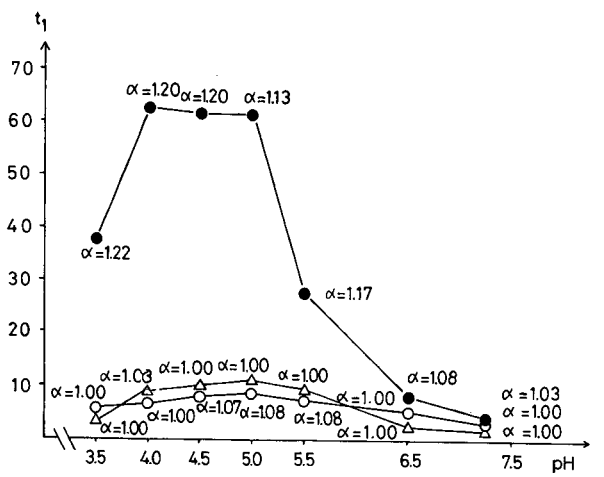

Fig. 2. Influence of pH and the charged modifiers, SHS and TPAB, on retention times (t_1) and separation factors (α). Mobile phase, potassium phosphate. ● = No modifier; ○ = 5 m*M* SHS added; △ = 5 m*M* TPAB added.

In a system with this chiral stationary phase, the addition of both SHS and TPAB charged modifiers to the mobile phase markedly reduced the capacity and separation factors (Fig. 2), as reported by Miwa *et al.*[11]. When SHS is used as anionic ion-pairing agent, the explanation for this phenomenon is as follows. 1-Heptanesulphonate ions may compete with ABA for the cationic cavity of the protein, so that the retention and resolution of ABA become small. With TPAB, the reason for the above observation is not yet clear. In both instances we could not improve the resolution by the use of additives, unlike the results obtained with Enantiopac[12].

The retention was also influenced by the ionic strength of the mobile phase. ABA exhibited a strong retention on this column at lower ionic strength, whereas the separation factors were almost unchanged (Fig. 3). This indicates that coulombic interactions, *i.e.*, an ion-exchange process, are involved in the retention of ABA, but that it is not so important for the chiral recognition and hydrophobic interaction with 2-propanol.

Variation of the pH of the mobile phase between 3.5 and 7.3 influences the capacity and separation factors of ABA, as demonstrated in Fig. 2. The separation factor decreases slightly with increasing pH, whereas the capacity factors are greatly affected. This effect seems to be caused by a change in the properties of the stationary phase with pH. As shown in Fig. 2, the enantiomers of ABA are best resolved at pH 3.50. Ovomucoid has an isoelectric point of 4.1 and has a net negative charge at lower pH. ABA is a weak acid having a pK_a of 4.8[13]. At pH 3.50 the carboxyl group in ABA appears to be almost undissociated. Hence strong hydrogen bonding between the carboxyl group in ABA and ovomucoid may play an important role in the chiral recognition. This also supports the fact that the methyl ester of (*RS*)-ABA shows a very poor resolution (R_s = 0.19) under the conditions used in Fig. 1.

In conclusion, fairly high separation factors can be obtained for the enantiomers of ABA by using ovomucoid as the chiral bonded phase. The optical purity of small amounts of ABA can be also determined directly, rapidly and accurately by this HPLC

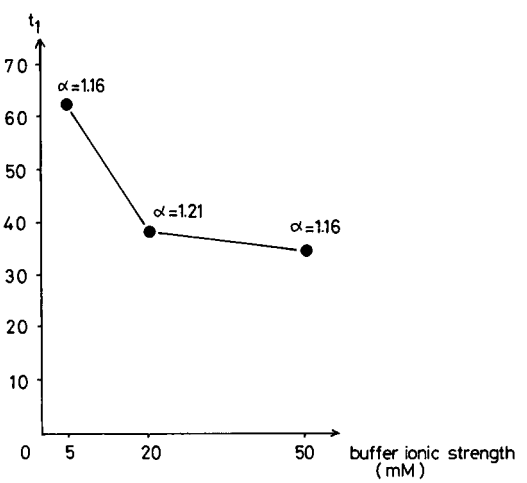

Fig. 3. Influence of buffer ionic strength (m*M*) on retention times (t_1) and separation factors (α). Mobile phase, potassium phosphate.

method. The experiments reported here indicate that strong hydrogen bonding may play an important role in the chiral recognition of ABA. Studies on the resolution of some ABA derivatives are in progress, and will be reported elsewhere.

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CHROM. 22 311

Note

Chiral thin-layer chromatographic separation of phenylalanine and tyrosine derivatives

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(First received August 11th, 1989; revised manuscript received January 26th, 1990)

Ligand-exchange chromatography is extremely useful for the separation of enantiomers of amino acids, peptides and other optically active substances[1]. Recently the commercially available thin-layer chromatography (TLC) plates of Macherey-Nagel, Chiralplate, have become routine equipment for laboratories synthesizing amino acids and peptides[2].

Our interest has focused on the asymmetric synthesis of phenylalanine and tyrosine *derivatives* and *analogues*, with special attention to analogues which can conformationally fix or bias such residues in biologically active peptides. Conformational constraint or topographic bias residues have been shown to provide an important new approach in the design of selectively acting hormone analogues[3–5]. The synthesis of appropriately modified phenylalanine and tyrosine derivatives and their characterization is obviously the first step in this task. We have employed chiral TLC plates in the evaluation of reaction products and in their stereochemical characterization, and we would like to report here the chromatographic behavior of these sterically constrained or biased phenylalanine and tyrosine derivatives.

EXPERIMENTAL

Materials and reagents

The amino acids phenylalanine (I, Fig. 1), tyrosine (IX), and 3′,5′-dibromotyrosine (XIII) were purchased from Sigma. The other unusual aromatic amino acids used in these studies were synthesized in our laboratories at the Department of Chemistry, University of Arizona, Tucson, and the Department of Peptide Chemistry, Institute of Organic Chemistry and Biochemistry, Prague. Their structures were determined by a variety of physico-chemical methods, including high-field NMR, mass spectrometry, elemental analysis and optical rotation. The new synthetic procedures developed

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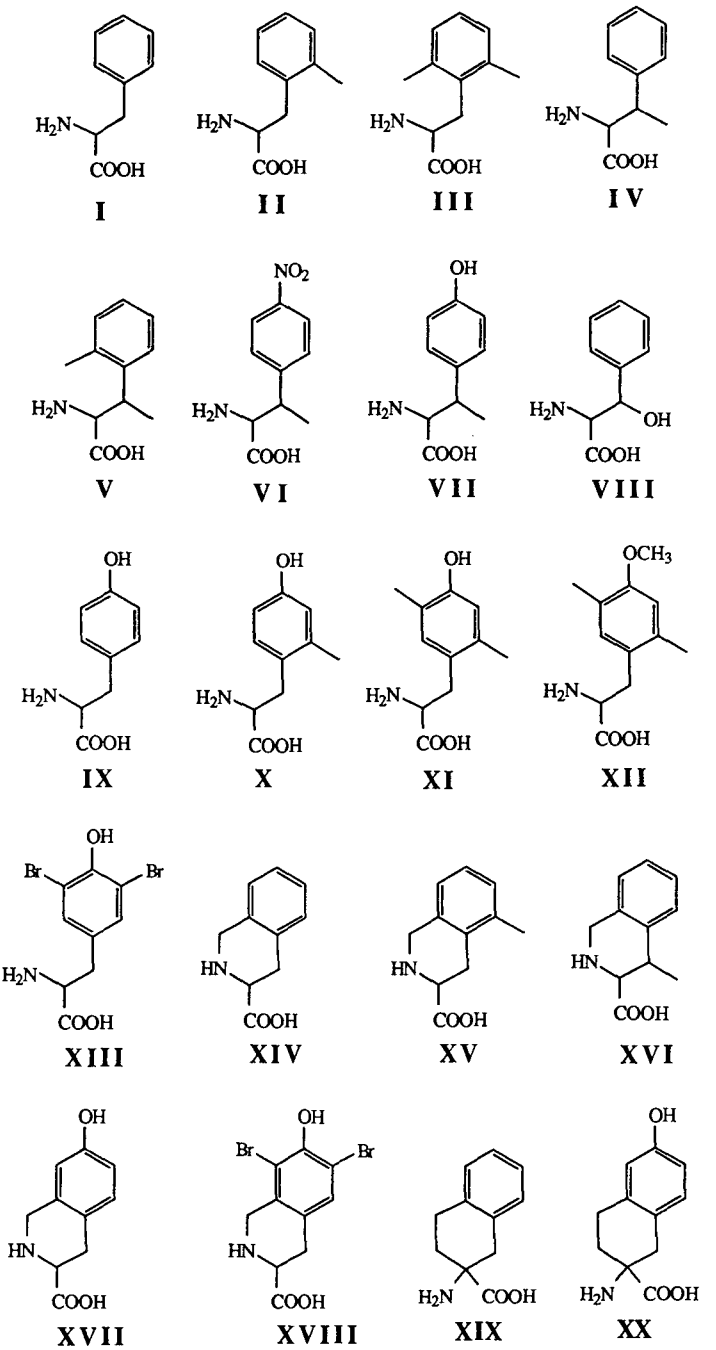

Fig. 1. Structures of the amino acid derivatives examined.

for the synthesis of many other new amino acids will be published elsewhere, as will the peptide analogues described in the Results and Discussion section. The following amino acids were prepared for this study (see superscript references for synthetic methodology, the Roman numbers used refer to Fig. 1): 2′-methylphenylalanine (II)[6]; 2′,6′-dimethylphenylalanine (III)[6]; β-methylphenylalanine (IV)[7]; 2′-methyl-β-methylphenylalanine (V)[8]; β-methyl-*p*-nitrophenylalanine (VI)[9]; β-methyltyrosine (VII)[10]; β-hydroxyphenylalanine (VIII)[11]; 2′-methyltyrosine (X)[12]; 2′,5′-dimethyltyrosine (XI)[12]; 2′,5′-dimethyl-4-methoxyphenylalanine (XII)[12]; tetrahydroisoquinoline carboxylic acid (XIV)[13]; 2′-methyltetrahydroisoquinolinecarboxylic acid (XV)[13,14]; β-methyltetrahydroisoquinolinecaboxylic acid (XVI)[13,14]; 4′-hydroxytetrahyhydroisoquinolinecarboxylic acid (XVII)[14]; 3′,5′-dibromo-4′-hydroxytetrahydroisoquinolinecarboxylic acid (XVIII)[14]; 2-aminotetralincarboxylic acid (XIX)[15]; 2-amino-6-hydroxytetralin carboxylic acid (XX)[15].

L-Amino acid oxidase (*Crotalus adamateus* crude venom) was purchased from Sigma. Chiral TLC plates were from Macherey-Nagel, trifluoroacetic acid was purchased from Chemical Dynamics Corporation, acetonitrile was obtained from American Burdick and Jackson (American Scientific Products).

The peptides Tyr-D-Pen-Gly-X-D-Pen (Pen = penicillamine or β,β-dimethylcysteine, X = β-methylphenylalanine, *p*-NO_2-β-methylphenylalanine) and oxytocin with β-methylphenylalanine in position 2 were synthesized in our laboratory by solid-phase peptide synthesis methods we have developed[16,17]. HPLC experiments were performed on a Perkin-Elmer 3D instrument equipped with an LP 90 UV detector and an LC1-100 Laboratory Computing Integrator. The HPLC columns used were Vydac 218TP1004 (25 cm × 0.46 cm) columns. Fourier transform ^1H NMR spectra were run on a Bruker AM 250 spectrometer.

The R_F values of the individual amino acids on chiral plates were identical to those previously reported by Günther[1]. The solvent system used was acetonitrile–methanol–water (4:1:1).

Enzymatic digestion of aromatic amino acids[18]

Amino acids I–XII (0.3 mg) were dissolved in Tris buffer (0.1 *M*, pH 7.2), the pH was adjusted by the addition of 0.2 *M* sodium hydroxide to 7.2 and 0.3 mg of L-amino acid oxidase (*Crotalus adamanteus* crude venom, Sigma) was added. The test tube was filled with oxygen, tightly closed and incubated for 24 h at 37°C. After 24 h new enzyme was added and the incubation was continued for another 24 h. The reaction mixture was spotted directly on a Chiralplate without any dilution or concentration. Development was performed in the system specified in Table I.

Determination of configuration of aromatic amino acid in peptide (general method)

An oxytocin analogue containing an aromatic amino acid in position 2 (0.2 mg) was hydrolysed in 6 *M* hydrochloric acid at 100°C for 20 h. The solution was evaporated *in vacuo*, and the residue was dissolved in 50 μl of water and injected into a C_{18} Vydac column (25 × 0.46 cm) mentioned above, which had been equilibrated with 0.1% trifluoroacetic acid in water. Gradient elution (0–3 min: 0% acetonitrile, 3–23 min: to 50% acetonitrile) afforded a strong peak at the dead volume of the column (containing Asp, Glu, Gly, Pro, Leu, Ile and Cys, as determined by TLC comparison with standards), and a second major peak eluted at 12–15 min [depending on the

TABLE I

R_F DATA FOR THE PHENYLALANINE AND TYROSINE DERIVATIVES

Chiralplate, acetonitrile–methanol–water (4:1:1), detection by ninhydrin.

Compound	*Configuration*	$R_{F(L)}$	$R_{F(D)}$	$R_{F(L)}/R_{F(D)}$
I		0.56	0.42	1.33
II		0.54	0.43	1.26
III		0.52	0.38	1.37
IV	*S,S+R,R (erythro)*	0.56	0.36	1.56
	R,S+S,R (threo)	0.55	0.47	1.17
V	*S,S+R,R (erythro)*	0.57	0.33	1.73
	R,S+S,R (threo)	0.55	0.48	1.15
VI	*S,S+R,R (erythro)*	0.62	0.43	1.44
	R,S+S,R (threo)	0.60	0.52	1.15
VII	*S,S+R,R (erythro)*	0.67	0.52	1.29
	R,S+S,R (threo)	0.67	0.55	1.22
VIII	*S,S+R,R (erythro))*	0.63	0.49	1.29
IX		0.63	0.51	1.24
X		0.62	0.54	1.15
XI		0.67	0.56	1.20
XII		0.57	0.45	1.27
XIII		0.62	–	–
XIV		0.54	0.50	1.08
XV		0.51	0.49	1.04
XVI	*S,S+R,R (erythro)*	0.51	0.45	1.13
XVII		0.60	–	–
XVIII		0.58	–	–
XIX		0.55	0.47	1.17
XX		0.64	0.59	1.08

structure of the aromatic amino acid(s)]. All peaks were collected, lyophilized, and the residue was dissolved in water (30 μl) and spotted onto a Chiralplate. Comparison with standards revealed the configuration of the aromatic amino acids.

RESULTS AND DISCUSSION

The structures of the amino acid derivatives examined are given in Fig. 1. Table I shows the R_F values of all available optical isomers. We were able to determine the absolute configuration of these derivatives containing the primary amino group (with the exception of XIX and XX) by enzymatic digestion using L-amino acid oxidase[18]. The configuration of XIX was determined with the use of carboxypeptidase[7]. The configuration of compounds containing a secondary amino group was secured by synthesis starting from an optically pure amino acid. Due to the substitution of the side chain moiety of the phenylalanine analogues, the enzymatic digestion was significantly slower in comparison to Phe itself. However, complete digestion of the L-isomer was obtained using a larger excess of enzyme and longer reaction times. Results obtained with Chiralplate (enzymatic conversions of L-enantiomer) were further verified by quantitative amino acid analysis[18].

Only the D-amino acids are then detectable by amino acid analysis. It is in-

teresting to compare the amino acids containing a tetrahydroisoquinoline ring structure with the corresponding amino acids in which the rotation of the aromatic side chain group is not so constrained. The more constrained compounds have displayed much poorer separation of enantiomers. Comparison of β-substituted amino acids shows that the *erythro* compounds have very different R_F values in comparison with compounds of *threo* configuration. Compounds with *threo* configuration of substituents can be compared to the β-unsubstituted compounds. The influence of aromatic ring substitution on separation can be observed in compounds I–III, IV–VII, IX–XII, XIV and XV.

The use of chiral plates was extremely useful in the cases of amino acid derivatives in which a mixture of D-, L-*erythro* or D-, L-*threo* stereoisomers was obtained during the synthesis. The combination of this technique with NMR spectroscopy and enzymatic digestion has enabled us to determine the stereochemistry of all chiral carbons[7,15,19].

We have also used chiral TLC separation for the determination of amino acid configuration of synthetic peptide analogues which were prepared starting from the racemic aromatic amino acid. After separation of the diastereoisomeric peptides on a reversed-phase HPLC column, we hydrolyzed the sample of each isomer and either analysed it directly on a Chiralplate (in cases when no amino acids interfered with the separation), or applied the hydrolysate to a reversed-phase HPLC column and analysed on TLC only fractions containing the aromatic amino acids. We can recommend this method for determination of the configuration of aromatic amino acids in synthetic peptides including oxytocin, enkephalin and cholecystokinin-8 analogues.

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CHROM. 22 313

Note

2,4-Dinitrophenylpyridium chloride, a novel and versatile reagent for the detection of amino acids, primary and secondary amines, thiols, thiolactones and carboxylic acids during planar chromatography

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Hemlock alkaloids[1] and nicotinamide[2] have been detected with 1-chloro-2,4-dinitrobenzene and primary aromatic amines[3] and nitrofurans[4] by a photochemical reaction with pyridine but 2,4-dinitrophenylpyridium chloride (DPPC) has not been previously used as a chromatographic detecting reagent.

DPPC can be considered a charge-transfer complex, in which the electron-withdrawing dinitrophenyl moiety causes a decrease of electron density within the pyridine ring, rendering its α-carbon atoms susceptible to nucleophilic attack[5–7], as shown in Fig. 1. In this paper, we report the first simple synthesis of DPPC and its subsequent use in differentiating between the various classes of nucleophiles, colorimetrically and fluorimetrically.

EXPERIMENTAL

Unless stated otherwise, all procedures were carried out at 20–23°C.

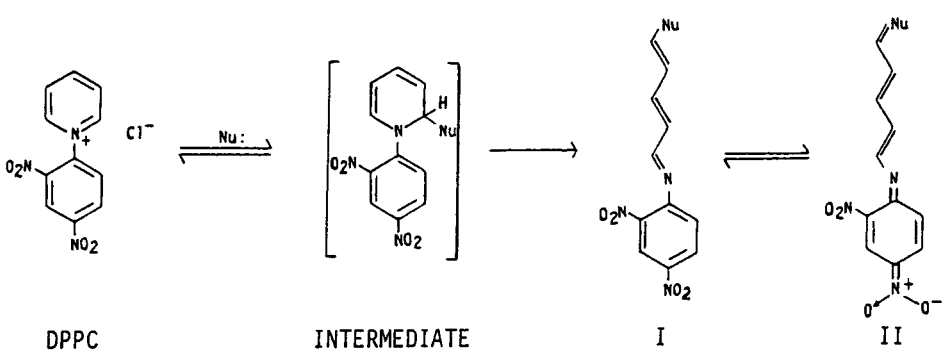

Fig. 1. The general reaction of DPPC with nucleophiles (Nu:) to give the mixed glutaconic aldehyde derivative (I) or the aci-nitro form (II) of I.

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Materials

Chemicals were mainly supplied by BDH (Poole, U.K.). Ammonia solution (sp.gr. 0.88) and most of the solvents used for chromatography were of analytical-reagent grade like the starting materials for DPPC synthesis but butanone, butan-1-ol, 2-methyl-propan-2-ol and propan-2-ol were ordinary-reagent grade. Glass-distilled water was used throughout.

Preparation of DPPC

1-Chloro-2,4-dinitrobenzene (0.2 g) in 2.5 ml acetone was mixed with 0.4 ml pyridine and left to stand for 72 h in a stoppered Pyrex tube in the dark. The solid formed was filtered by suction, washed with 5 ml ice-cooled acetone and air dried. Crystal colour: light beige brown. Yield, 0.135 g (48%): M.p., 187–193°C (Lit. 183–200°C)[18]. Solid DPPC slowly dissociates on standing so the reagent was kept in methanolic solution (200 mg l^{-1}), which is stable for at least 0.5 year at 20°C in darkness.

TLC

All samples chromatographed were made up at 25 m*M* and stored at −20°C. Amino acids and carboxylic acids were dissolved in 10% (v/v) aqueous propan-2-ol; asparagine, tyrosine and cystine were solubilised by the further addition of HCl to a final concentration of 0.05 *M*. Amines were dissolved in 70% (v/v) aqueous methanol containing 0.025 *M* HCl if as free bases.

Aliquots (0.2–0.6 μl) were spotted 1 cm from the edge of Merck cellulose (Art. 5578, DC Plastikrolle, 100 μm thickness) or Merck silica gel (Art. 5553, DC Alufolien, 200 μm thickness, without fluorescent indicator) layers.

Ascending chromatography was in one of the following solvents: (I) 2-methyl-propan-2-ol–butanone–propanone–methanol–water–ammonia (40:20:20:1:14:5, v/v) or (II) butan-1-ol–propanone–acetic acid–water (35:35:10:20, v/v) in a glass chromatographic tank. These were selected as examples of acidic and basic solvents already established for the separation of amino acids[8,9].

After the solvent front had migrated 9 cm, plates were dried in an air current, 15 min for solvent I and 45 min for solvent II. When all the R_F values were initially low, layers were chromatographed a second time in the same direction and re-dried.

Detection procedure

Plates were sprayed with DPPC in methanol (200 mg l^{-1} for cellulose and 100 mg l^{-1} for silica gel) until they just appeared translucent. After drying in an air stream for 10 min, the reaction was initiated by placing them in a sealed glass chromatographic tank (30 × 27.5 × 8.7 cm), which had been thoroughly rinsed with a mixture of 8 ml methanol and 2 ml triethylamine, the excess having been poured away. Methanol was essential for full colour differentiation and development of a yellow background.

When the plates were yellow–yellow brown, after *ca.* 1 min for cellulose and 8 min for silica gel, they were removed and examined. This was designated stage A. The layers were then returned to the alkaline atmosphere for a further 15 min. and either examined immediately or after air drying for 30 min (stage B). They were then kept in a dark cupboard for 24 h before recording the fluorescences that had normally developed (stage C).

TABLE I

AMINO ACIDS

R_F values were determined at a loading of 2.5 nmol. Key: BH = bleached; Bg = beige; Br = brown; Dk = dark; G = green, Lt = light; ND = no absorbance or fluorescence at a loading of 25 nmol; Or = orange; Pk = pink; L = lilac; Pu = purple; Rd = red; W = white; * = colour of *fluorescence* as recorded under 366 nm UV light. (nmol) = Detection limits in nmol at the given stage. Where, as in Tables II and III, colours were identical at stages A and B, sensitivities, except those marked °, were recorded at stage A. When sensitivity was based on fluorescence, irradiation was at 366 nm. Sometimes (Table II) the fluorescence intensity increased when the layers were kept a further 14 days in darkness and this is recorded as an extension of the stage C results.

Compound[a]	*Cellulose, solvent I run* ×2				*Cellulose, solvent II*			
	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*
1 4-Aminobutyric acid	17	Br–Rd (1.0)	Lt Rd (0.75)	Bg–Or* (1.0)	56	Or–Br (1.0)	Lt Br (0.5)	Bg–Or* (0.75)
L-Arginine–HCl	5	Or–Br (7.5)	Lt Or (2.5)	Bg* (0.5)	15	Lt Or-Br (2.5)	BH (2.5)	Bg* (0.75)
L-Aspartic acid	2	Or–Br (15.0)	BH (1.0)	Bg–Or* (0.75)	21	Lt Or (10.0)	BH (2.5)	Bg–Or* (0.75)
L-Asparagine	9	Or–Br (10.0)	BH (1.0)	Bg–Or* (0.75)	15	Or–Br (15.0)	BH (5.0)	Bg–Or* (0.75)
L-Cysteic acid	4	Br Rd (5.0)	Or Br (5.0)	Bg–Or* (1.0)	8	Or–Br (5.0)	Lt Rd (1.0)	Bg–Or* (0.75)
L-Cystine	1	Lt Or (5.0)	BH (2.5)	Bg–Or*(2.5)	4	Or–Br (1.0)	Or (2.5)	Bg–Or* (2.5)
Glycine	11	Rd (0.75)	Rd (0.5)	Bg–Or* (0.25)	26	Rd (0.5)	Rd (0.75)	Bg–Or* (0.75)
L-Histidine	18	Br Rd (5.0)	BH (1.0)	Bg–Or* (0.75)	16	Or–Br (2.5)	BH (1.0)	Bg Or* (0.75)
L-Leucine	54	Rd (0.5)	Lt Br (2.5)	Bg–Or* (0.75)	80	Lt Rd (0.75)	Lt Rd (1.0)	Bg–Or* (0.75)
Isoleucine	54	Rd (0.5)	Lt Br (2.5)	Bg–Or* (0.75)	79	Lt Br (0.75)	Lt Rd (1.0)	Bg–Or* (0.75)
L-Lysine–HCl	11	Lt Rd (1.0)	Rd (0.5)	Bg–Or* (0.75)	14	Or–Br (2.5)	BH (1.0)	Bg–Or* (0.25)
L-Methionine	37	Or–Br (5.0)	Br (1.0)	Bg–Or* (2.5)	57	Or–Br (1.0)	Lt Rd (2.5)	Bg–Or* (5.0)
L-Methionine sulphone	23	Or–Br (5.0)	Lt Or (1.0)	Bg–Or* (0.75)	31	Or–Br (2.5)	Or (2.5)	Bg–Or* (0.75)
5-Methyl-L-Tryptophan	64	Br (1.0)	Y (1.0)	Y* (0.75)	79	Or–Br (1.0)	Y (1.0)	Y* (0.75)
L-Phenylalanine	58	Or–Br (1.0)	Lt Rd (5.0)	Bg–Or* (0.75)	70	Or–Br (1.0)	Lt Rd (7.5)	Bg–Or* (0.75)
L-Serine	22	Rd (1.0)	Lt Rd (5.0)	Bg–Or* (1.0)	22	Or–Br (2.5)	Lt Rd (2.5)	Bg–Or* (0.75)
L-Tyrosine	29	Br (2.5)	BH (0.75)	Bg–Or* (0.75)	58	Or–Br (1.0)	BH (1.0)	Bg–Or* (0.75)
L-Tryptophan	48	Lt Br (2.5)	BH (1.0)	Bg–Or* (0.75)	58	Or–Br (2.5)	Lt Br (7.5)	Bg–Or* (0.75)
2 L-Azetidine-2-carboxylate	17	Pu (0.15)	Dk Br (0.1)	Y–G* (0.25)	36	Pu (0.2)	Dk Br (0.1)	G* (0.25)
4-Hydroxy-L-proline	15	Pu (0.2)	Pu (0.15)	G* (0.25)	23	Pu (0.25)	Dk Br (0.15)	G* (0.5)
4-Hydroxymethyl-L-proline	17	Pu (0.1)	Dk Br (0.05)	G* (0.25)	32	Pu (0.25)	Dk Br (0.1)	Y–G* (0.25)
4-Methyl-L-proline	37	Pu (0.05)	Dk Br (0.025)	G* (0.25)	63	Pu (0.15)	Dk Br (0.05)	G* (0.25)
N-Methyltaurine	39	Rd–Pu (0.5)	Dk Rd (0.25)	Y–G* (0.5)	37	Rd–Pu (0.25)	Dk Rd (0.2)	Y–G* (0.5)
D-Pipecolate–HCl	42	Pu (0.75)	Dk Br (1.0)	Lt G* (1.0)	52	Pu (1.0)	Dk Br (0.75)	Lt G* (0.75)
L-Proline	23	Pu (0.05)	Dk Br (0.025)	G* (0.25)	41	Pu (0.15)	Dk Br (0.05)	G* (0.25)
Sarcosine	21	Pu (0.15)	Br–Pu (0.1)	Y–G* (0.25)	39	Pu (0.25)	Dk Br (0.2)	Y–G* (0.25)

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

TABLE II

AMINES

For details, see Table I.

Compound[a]	*Cellulose, solvent II*			*Silica gel, solvent II*			
	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*	*(nmol)*
1 Putrescine–di-HCl	10	Rd (0.5)^0	Bg–W* (2.5)	6	Or–Br (1.0)	W* (1.0)	
Benzylamine	74	Or–Br (0.5)	Or* (5.0)	62	Or–Br (0.15)	W* (2.5)	
Ethanolamine	34	Or–Br (5.0)	Bg–Or* (2.5)	25	Or–Br (2.5)	W* (5.0)	
3-Methoxy-4-hydroxyphenylethylamine–HCl	63	Or–Br (2.5)	Bg–Or* (5.0)	61	Br (0.2)	W–Pu* (1.0)	→ Pu* (0.1)
m-Tyramine	68	Br (1.0)	W* (1.0)	66	Or–Br (0.2)	W–L* (0.15)	→ L* (0.025)
o-Tyramine–HCl	73	Br (1.0)	Bg–Or* (2.5)	69	Br (0.25)	W* (0.5)	→ Or* (0.2)
p-Tyramine–HCl	69	Or–Br (0.5)	Bg–Or* (2.5)	66	Or–Br (0.5)	W* (0.25)	→ Or* (0.15)
2 Cytisine	38	Dk Rd–Pu (0.15)^0	G* (0.5)	15	Rd–Pu (0.025)	G* (0.5)	→ G* (0.25)
Diethanolamine	39	Pu (0.1)^0	G* (0.5)	29	Dk Rd (0.5)	G* (2.5)	
N-Methylbenzylamine HCL	79	Pu (0.1)	G* (0.75)	59	Rd–Pu (0.075)	G* (1.0)	→ G* (0.75)
N-Methyl-3-methoxy-4-hydroxyphenylethylamine-HCl	66	Pu (0.05)	Y–G* (1.0)	56	Rd–Pu (0.1)	W* (0.5)	→ Or* (0.1)
Morpholine	45	Rd–Pu (0.5)	Y–G* (1.0)	31	Dk Rd (1.0)	Y–G* (2.5)	
Piperazine–di-HCl	6	Pu (0.05)	G* (0.5)	2	Pu (0.2)	G* (1.0)	
Spermine–tri-HCl	2	Rd–Pu (0.2)	Y–G* (0.75)	1	Dk Rd (0.25)	W–G* (0.75)	

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

RESULTS AND DISCUSSION

Tables I–IV show the sequential colours/fluorescences that developed after reaction with DPPC, together with minimum detectable quantities for four classes of compounds.

Table I indicates that all primary amino acids tested initially gave brown spots which had developed bleached centres by stage B if loadings exceeded 5 nmol: they were totally bleached by stage C but could still be detected, normally at enhanced sensitivity, by their beige–orange fluorescence under UV light. Primary amines (Table II) reacted similarly but were better recorded at stage A/B. In contrast, secondary amino acids and secondary amines first gave a purple colour which formed a yellow centre at loadings of > 2.5 nmol (stage B): finally a yellow spot was associated with a green fluorescence. Table II confirms that initial colour and the wavelength of fluorescence do provide a reliable way of distinguishing between amino and imino groups.

The best discrimination between the two types of fluorescence is obtained under 254 nm UV light, while the greatest sensitivity is given by irradiation at 366 nm. Fluorescence intensity often increases with time, at least on silica gel, doubling on average when stage C plates are kept a further 14 days in darkness: the effect is recorded in Table II where it leads to a substantial increase in sensitivity for individual primary and secondary amines. Attempts to stimulate fluorophore production by heat and/or by increased exposure time to triethylamine–methanol were unsuccessful. Replacing triethylamine with ammonia solution actually diminished subsequent fluorescence.

A cellulose chromatogram at stage A, if kept in air, will absorb moisture; its background will darken; purple spots will become dark brown and other colours will similarly degenerate leading to a loss of sensitivity. However, layers at stage A can be stabilised by sandwiching them immediately between pairs of glass plates, pre-washed in acetone.

The pH of the chromatography solvent sometimes has a minor effect on colour

TABLE III

AROMATIC AMINES

For details, see Table I.

Compound[a]	*Cellulose, solvent II*			*Silica gel, solvent II*		
	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*
p-Phenylenediamine–di-HCl	56	Pu (2.5)^0	Or* (1.0)	54	Br-Pu (1.0)	Or* (0.75)
Sulphathiazole	85	ND	Lt Br (25)	84	ND	Lt Rd–Br (20)
2-Aminoimidazole sulphate	48	ND	Lt Br (20)	55	ND	Br (25)
p-Aminobenzoate	91	Br (10.0)	Or* (2.5)	92	Br (2.5)^0	Or* (1.0)
p-Anisidine	77	Br (10.0)	Pk–Or* (2.5)	71	Br (2.5)^0	Or* (1.0)
2 Diphenylamine	98	ND	ND	92	ND	Y (15.0)
Skatole	99	ND	ND	96	ND	Br (25.0)

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

quality (Table I) but none at all on the appearance of the fluorescence at stage C. Table I also implies that sensitivity is unimpaired by residues from either solvent.

No data are given for amines run in solvent I on cellulose as the corresponding chromatograms streak extensively. However, Tables II and III do show results for amines run on both cellulose and silica gel in solvent II. Silica gel may produce minor changes in absorbance/fluorescence characteristics and on average doubles sensitivity towards primaries and halves that for secondary amines.

Tables II and III only contain a selection of the amines tested. *n*-Butylamine, cadaverine, diethylamine, di-*n*-butylamine, octopamine, 2-phenylethylamine, piperidine, pyrrolidine, spermidine and synephrine also react with DPPC giving the colours/fluorescences predicted from their chemical structures: all were easily visible at 5 nmol.

Unlike most equivalent reagents, DPPC will detect amino and imino groups linked directly to aromatic rings (Table III) but sensitivities are often very poor: indeed aniline, N-methylaniline and indole have been excluded from the table because they did not react visibly at 25 nmol. Even those aromatic amines that were detectible often gave no fluorescence at stage C, just a yellow or brown colouration.

Amides and amidines seem not to react: urea and guanidine were certainly negative at 25 nmol.

The DPPC reagent is bleached at all stages by thiol compounds like mercaptoethanol and thioglycolic acid. It will detect the non-volatile thiols, dithiothreitol and cysteine on Whatman 3MM paper at levels down to 0.5 and 2.5 nmol cm^{-2} respectively. However, it has been impossible to assay sensitivities under the standard conditions adopted here because of the chemical changes that occur during chromatography. For example, all preparations of cysteine tested (3 of the free base and 4 of the hydrochloride) showed two spots as recorded in Table IV. The slower is almost certainly cysteine, by comparison with its characteristics in Table I, but relative R_F values suggest that the faster and major component is cystine thiolactone. Certainly

TABLE IV

THIOLS AND CARBOXYLIC ACIDS

For details, see Table I

Compound[a]	*Cellulose, solvent I run* $\times 2$				*Cellulose, solvent II run* $\times 2$			
	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*
L-Cysteine-HCl	33	BH (0.75)	BH (0.75)	BH (2.5)	63	BH (1.0)	BH (1.0)	BH (2.5)
	1	ND	ND	Bg-Or*	4	ND	ND	Bg-Or*
DL-N-Acetylhomocysteine thiolactone	98	BH (20)	BH (10)	BH (10)	95	BH (15)	BH (5.0)	BH (5.0)
DL-Malic acid	3	BH (0.75)	BH (10)	BH (15)	82	BH (7.5)	BH (10)	BH (15)
Glycolic acid	18	BH (1.0)	BH (15)	BH (20)	84	BH (7.5)	BH (15)	BH (20)
L-Pyroglutamic acid	23	BH (2.5)	BH (15)	BH (20)	87	BH (10)	BH (15)	BH (20)
Itaconic acid	6	BH (0.75)	BH (5.0)	BH (10)	98	BH (5.0)	BH (10)	BH (15)
Succinic acid	4	BH (0.75)	BH (5.0)	BH (10)	96	BH (5.0)	BH (10)	BH (15)

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

DPPC will detect the much more stable acetylhomocysteine thiolactone, albeit poorly.

Carboxylic acids also bleach the reagent (Table IV) but this time the visible effect declines rapidly as the pH increases from stage A to B.

DPPC has considerable value as a functional group reagent. Initial colour and fluorescence wavelength at stage C certainly provide a reliable way of distinguishing between primary and secondary nitrogen, while the bluish fluorescence developed by 3-methoxy/hydroxy primary phenylethylamines 14 days after stage C may be a specific test for this class of compound. It is even possible to distinguish between thiols and carboxylic acids by monitoring their bleaching reactions carefully.

However, DPPC often lacks sensitivity. It would not, for example, be the reagent of choice for detecting thiols or carboxylic acids. Even for primary amino compounds it is generally less sensitive than ninhydrin[10], *o*-phthalaldehyde[11] and fluorescamine[12,13] by factors of *ca.* 4, 8, and 2–8 respectively. This probably overstates its disadvantage as we have measured sensitivities under conditions that give good chromatographic separation and it is competitive for individual compounds like *m*-tyramine and 3-methoxy-4-hydroxyphenylethylamine. Nevertheless it is most satisfactory for the detection of aliphatic and alicyclic secondary amino compounds which it can generally do at the 0.2-nmol level. Here competing reagents include vanillin[14] and nitrobenzoyl chloride[15], but these will only detect NH in amino acids, not in amines. Nitroprusside–acetaldehyde[16] detects the same classes of secondary nitrogen as DPPC but its sensitivity towards the secondary amines listed in Tables II and III was at least 10 times less when tested after solvent 2 on silica gel. The fluorescamine–taurine[17] spray has a broader specificity than any reagent so far mentioned as it will also reveal NH linked to an aromatic ring but its sensitivity is sometimes poor, >5 nmol for proline and is impaired by both acidic and alkaline solvent residues.

As for the chemistry of these reactions, R_2NH groups allow greater delocalisation of electrons over the conjugated system than do RNH_2 groups, yielding the red–purple aci-nitro structure (Fig. 1) at a considerably lower pH. The carboxyl groups of amino acids also promote delocalisation to a lesser degree: hence the tendency of amino acids to give reddish colours at stage A. The structure of the fluorophores at stage C is unclear at present.

Thiols react with the dinitrophenyl moiety, dissociating the DPPC and so preventing colour formation at all stages. Pyridine is known to catalyse this type of acylation[6].

Finally, bleaching by carboxylic acids is due to their buffering properties: they resist the increase in pH when the plates are exposed to triethylamine, so temporarily prevent colour development.

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Note

Paper chromatographic studies of metal complexes

II. Comparison of square planar and octahedral complexes

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Despite numerous theories (adsorption, partition, solvent extraction, ion exchange, etc.), the actual mechanism of the chromatographic separation of inorganic compounds on filter paper is not well understood[1–12]. It may be considered as consisting of the following steps: (1) adsorption of metal ions by the cellulose molecules of filter paper[13–15] (Fig. 1a); (2) desorption of metal ions by acids adsorbed as a result of the formation of oxonium ions by the interaction of cellulose with the H^+ ions of an acid[14,16,17] (Fig. 1b); and (3) solvation of the desorbed ions by solvent[18,19] and the washing away of the solvated complex by the solvent stream.

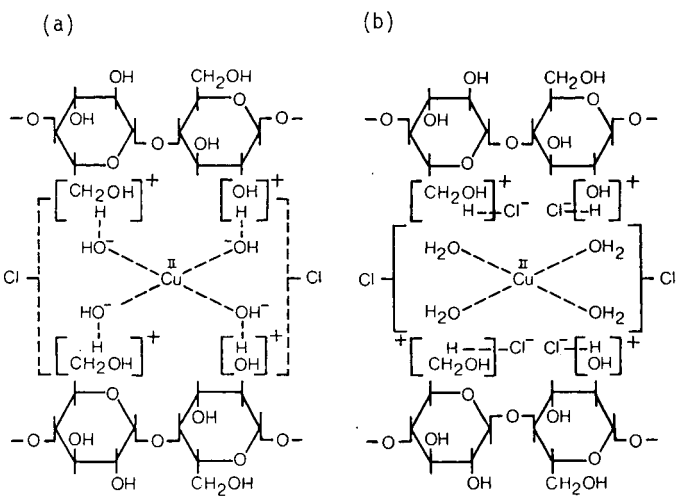

Fig. 1. (a) Mode of adsorption of Cu^{2+} aqua complex on cellulose. (b) Mode of desorption of Cu^{2+} aqua complex on cellulose in the presence of H^+ ions.

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In this paper, the movement of square planar metal(II) complexes on cellulose is compared, under identical conditions, with that of octahedral metal(III) and metal(II) complexes.

EXPERIMENTAL

Materials

The complex compounds were synthesized according to published procedures[20–26]. Their purity was confirmed by elemental analysis and spectral measurements. The complexes are very stable at pH $\approx$ 7, but they begin to disssociate at pH $<$ 7[27].

Procedure

The complex compounds were dissolved in water and subjected to ascending chromatography using Whatman No. 1 paper strips (3 cm $\times$ 52 cm). Aqueous solutions (0.1 *M*) of various salts were used as developers (Table II). The chromatograms were developed for about 3 h at 25°C. The cobalt(III) complexes were detected by spraying with an aqueous solution of sodium sulfide, which colors the spots black. With nickel(II) and copper(II) complexes an ethanolic solution of dithiooxamide (rubeanic acid) gave blue and green spots, and with palladium(II) complexes an acidic solution of dithiooxamide gave yellow spots.

RESULTS AND DISCUSSION

When cobalt(III) complexes of different charges were developed with water, only anionic and neutral complexes easily migrated on paper and yielded high R_F values (*ca.* 0.9). However, the cationic complexes remained at the baseline or diffused a long distance from the point of application[27]. The paper (cellulose) is charged negatively in contact with water, and the cellulose anion strongly attracts the cationic complexes[27]. Such strong binding was reduced considerably by using ionic electrolytes[27]. In general, the R_F values of cationic complexes belonging to a particular charge type ($+3$, $+2$ or $+1$) are the lowest in KI solution, and then gradually increase as the electrolyte changes to KBr, KCl, etc., and then to bi–univalent electrolytes such as K_2SO_4, $Na_2S_2O_3$, etc. These variations in R_F values indicate outer-sphere association between the complex cations and the anion present in the developer[27]. Investigations of the outer-sphere association constants of $[Co(NH_3)_6]^{3+}$, $[Co(bigH)_3]^{3+}$, etc., have shown that these constants follow the order[28,29] $I^- < Br^- < Cl^- \ll SO_4^{2-}$. The larger the association constants, the larger will be the R_F values. The anion of the developer reduces the overall charge of the complex cation and thus allows easy passage of the complex ion along the anionic filter-paper:

$$[Co(bigH)_3]^{3+} + X^- \rightleftharpoons \{[Co(bigH)_3]^{3+}, X^-\}^{2+}$$

($X^- = Cl^-$, Br^- or I^-);

$$[Co(bigH)_3]^{3+} + SO_4^{2-} \rightleftharpoons \{[Co(bigH)_3]^{3+}, SO_4^{2-}\}^{+}$$

TABLE I

R_F VALUES FOR OCTAHEDRAL Co(III) AND Ni(II) COMPLEXES WITH DIFFERENT DEVELOPERS (0.1 *M*)

Complex[a]	*KI*	*KBr*	*KCl*	CH_3COONH_4	K_2SO_4	$Na_2S_2O_3$	$KNaC_4H_4O_6$
$[Co(bigH)_3]Cl_3$	0.50	0.54	0.60	0.48	0.82	0.85	0.84
$[Co(MebigH)_3]Cl_3$	0.52	0.57	0.62	0.49	0.83	0.87	0.90
$[Co(NH_3)_6]Cl_3$	0.40	0.55	0.65	0.40	0.85	0.87	0.90
$[Co(PhbigH)_3]Cl_3$	0.45	0.52	0.59	0.46	0.90	0.90	0.84
$[Co(gly)(bigH)_2]Cl_2$	0.65	0.67	0.70	0.64	0.93	0.91	0.93
$[Co(\alpha\text{-alan})(bigH)_2]I_2$	0.67	0.71	0.78	0.67	0.96	0.95	0.96
$[Co(\beta\text{-alan})(bigH)_2]I_2$	0.62	0.65	0.73	0.63	0.91	0.92	0.94
$[Co(IDA)(bigH)_2]Cl$	0.77	0.81	0.82	0.78	0.95	0.93	0.94
$[Co(en)_2CO_3]Cl$	0.78	0.79	0.83	0.79	0.94	0.94	0.95
$[Co(NH_3)_3(NO_2)_3]$	0.95	0.93	0.96	0.93	1.00	1.00	1.00
$[Co(gly)_3]$	0.96	0.94	0.96	0.95	1.00	1.00	1.00
$[Ni(en)_3]Cl_2$	0.43	0.46	0.52	0.94	0.73	0.72	0.73
$[Ni(gly)_2(H_2O)_2]$	0.94	0.94	0.96	0.96	0.98	0.99	0.99
$[Co(dipy)(bigH)_2]Cl_3$	0.52	0.55	0.61	0.53	0.83	0.84	0.84
$[Co(o\text{-phen})(bigH)_2]Cl_3$	0.49	0.53	0.61	0.50	0.86	0.85	0.85
$K[Co(NO_2)_2(gly)_2]$	1.00	1.00	1.00	1.00	1.00	1.00	1.00
$NH_4[Co(NO_2)_4(NH_3)_2]$	1.00	1.00	1.00	1.00	1.00	1.00	1.00
$Na_3[Co(NO_2)_6]$	1.00	1.00	1.00	1.00	1.00	1.00	1.00
$K_3[Co(NO_2)_6]$	1.00	1.00	1.00	1.00	1.00	1.00	1.00

[a] alanH = Alanine; bigH = biguanide; dipy = α,α′-dipyridyl; en = ethylenediamine; glyH = glycine; $IDAH_2$ = iminodiacetic acid; MebigH = N^1-methylbiguanide; *o*-phen = *o*-phenanthroline; PhbigH = N^1-phenylbiguanide.

Square planar complexes of nickel(II), copper(II) and palladium(II) behaved identically when water, methylamine, ethylamine, pyridine, etc., were used as developing solvents. In order to reduce such strong binding of the cationic complexes, several electrolytes were used (Table I). With water-containing KCl (or KBr or KI) developer, although the complexes moved from the point of application, the complexes soon diffused a long distance along the filter-paper. Other aqueous salt solutions were also used as developers.

However, KCl-containing pyridine developer not only helped the spot to migrate from the point of application but also provided a good-sized spot of about 1.5 cm (Table II). Pyridine substantially increased the solubility of the complexes. The solubilities of $[Cu(bigH)_2]Cl_2$, $[Cu(MebigH)_2]Cl_2$, $[Cu(EtbigH)_2]Cl_2$ and $[Cu(PhbigH)_2]Cl_2$ are 18.92, 7.15, 0.38 and 0.40 g per 100 ml, respectively, in 0.5 *M* KCl and 20.84, 7.96, 0.45 and 0.48 g per 100 ml in 0.5 *M* KCl–pyridine (100:5, v/v) developing solvent.

Table II shows that an increase in the molecular weight of the alkyl group substituted on the N^1 atom of the biguanide molecule increases the R_F values of the corresponding copper(II), nickel(II) and palladium(II) bis(biguanide) complexes. However, phenyl substitution lowers the R_F values (Table II). Because biguanide, alkylbiguanides and arylbiguanides have more or less similar basic characters (pK_1 and pK_2 of bigH = 11.5, 2.9; MebigH = 11.4, 3.0; EtbigH = 11.5, 3.1; and PhbigH = 10.7, 2.2), the attractive influence of the doubly charged cations on the cellulose anion

TABLE II

R_F VALUES OF SQUARE PLANAR METAL(II) COMPLEXES WITH DIFFERENT DEVELOPERS

Complex[a]	*Developer I*[b]	*Developer II*[c]	*Developer III*[d]
$[M(bigH)_2]Cl_2$	0.48	0.75	0.42
$[M(MebigH)_2]Cl_2$	0.54	0.81	0.53
$[M(EtbigH)_2]Cl_2$	0.65	0.85	0.60
$[M(Pr^ibigH)_2]Cl_2$	0.70	0.91	–
$[M(PhbigH)_2]Cl_2$	0.43	0.70	0.31

[a] bigH = Biguanide; EtbigH = N^1-ethylbiguanide; MebigH = N^1-methylbiguanide; PhbigH = N^1-phenylbiguanide; Pr^ibigH = N^1-isopropylbiguanide.

[b] 100 ml 0.5 *M* KCl + 5 ml pyridine; M = Cu(II).

[c] 100 ml 1 *M* KCl + 5 ml pyridine; M = Ni(II).

[d] 70 ml 0.5 *M* KCl + 30 ml pyridine–water (50:50), M = Pd(II).

is also probably similar. However, replacement of the H atom attached to the N^1 atom of the biguanide molecule by hydrophobic groups should reduce considerably the chances of forming hydrogen-bonded species with cellulose anions, and thus increasing substitution should result in higher R_F values.

If solubility alone were responsible for differences in the R_F values, a reverse order with increasing alkyl substitution should be observed. The increase in R_F values from biguanide to higher alkyl-substituted biguanides is also likely to be related to the inductive influence of the electron-releasing alkyl groups, which diminishes the overall charge on the complex cation to some extent. This lowering of charge should increase the R_F values, whereas electron-withdrawing phenyl substitution should lead to lower R_F values.

Ammonium acetate solution of pH *ca.* 6.5 was not a good developer as spectrophotometric studies revealed that for all these square planar complexes the electronic spectra were substantially modified. This developer, therefore, does not provide R_F values of genuine metal(II) bis(biguanide) complexes.

It is noteworthy that nickel(II) complexes are likely to form the very insoluble $[Ni(bigH)_2]SO_4$ in aqueous K_2SO_4 developer[20]. By virtue of its extreme insolubility, the spot remained on the baseline. Similarly, $Na_2S_2O_3$ solution was unsuitable, particularly for copper(II) complexes, because in the presence of $Na_2S_2O_3$, copper(II) species may be reduced to copper(I) species. Therefore, if the overall charge of the complex cation alone were responsible for the retention of complex cations, migration of square planar complex cations on paper would have been equally possible by using only a suitable electrolyte developer. Therefore, it can be safely assumed that the retention of square planar complexes on a cellulose bed is due to direct coordination of the cellulose anion in the metal(II) complex, making the complex a hexacoordinate structure. It sould be noted that in the presence of suitable coordinating ligands a square planar complex of palladium(II) or platinum(II) may add two more ligands and be transformed into an octahedral complex ($D_{4h} \rightarrow O_h$ symmetry)[30].

During thin-layer chromatographic studies, Baba and Yoneda[31] observed that tris(glycinato)cobalt(III) (O_h symmetry) moved almost with the solvent front, whereas bis(glycinato)copper(II), also a non-electrolyte, remained at the baseline. Such

a strong retention of bis(glycinato)copper(II) on silica gel is due to direct coordination of silica to the copper(II) complex, giving it a hexacoordinate structure ($D_{4h} \rightarrow O_h$ symmetry)[31]. It should be noted that bis(glycinato)copper(II) crystallizes as a monohydrate, and its X-ray pattern shows a distorted octahedral structure through coordination of a water molecule along a carboxyl oxygen of the neighboring molecule[32].

ACKNOWLEDGEMENTS

R.K.R. thanks Prof. R. L. Dutta, Department of Chemistry, University of Burdwan, and Prof. N. Saha, University College of Science, Calcutta, for providing some facilities and for helpful discussions. The authors are grateful to the referees for valuable suggestions for improving the paper.

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CHROM. 22 291

Book Review

Advances in Chromatography, Vol. 30, Selectivity and retention in chromatography, edited by J. C. Giddings, E. Grushka and P. R. Brown, Marcel Dekker, New York, Basle, 1989, XIX + 255 pp., price US$125 (U.S.A. and Canada), US$150 (rest of the world), ISBN 0-8247-8155-4.

The series *Advances in Chromatography* consists of some excellent books which are present in many libraries and laboratories. The books contain various reviews on differents aspects of chromatography and also other separation methods. The subjects of the present volume are very diverse and even the wide subtitle *Selectivity and retention in chromatography* does not really cover the whole contents.

The first chapter, written by Schoenmakers and Uunk, is a a very good, critical survey of mobile and stationary phases for supercritical fluid chromatography (SFC). After a clear introduction to retention in SFC, the choice and the possibilities of the mobile phase composition are extensively discussed. The section on stationary phases is more concise because mostly well known liquid and gas chromatographic phases are used in SFC.

Chapter 2 (Tanaka and Araki) shows the potential of polymeric materials for reversed-phase liquid chromatography. Some illustrative tables with stationary phases and applications are presented, but self-evidently these are not up-to-date. Only a few examples of separations are shown and, unfortunately, not much attention is paid to the theoretical aspects of these materials.

Chapter 3 summarizes the work of the author (Jinno) on the behaviour of polycyclic aromatic hydrocarbons in reversed-phase liquid chromatography with different stationary phases and computer-assisted retention prediction. Mixtures of methanol and dichloromethane have been used as mobile phases, which is exceptional for reversed-phase chromatography. Generally, more explanation of the experimental results is needed. The description of the chemometric aspects of the work, *e.g.*, cluster analysis, is short for many chromatographic specialists. However, the possibilities of the combination of chromatography and chemometrics for the identification of similar compounds is well demonstrated.

Chapter 4 (Goto, Takeuchi and Ishii) shows a few interesting systems in miniaturized liquid chromatography (LC), but the title "Miniaturization in high-performance liquid chromatography" is too general and somewhat misleading. The authors present nearly only their own excellent work on precolumn enrichment, electrochemical detection, LC coupled with fast atom bombardment mass spectrometry and so-called unified chromatography. The discussion is often superficial and does not give sufficient details. However, the (few) figures and references demonstrate mthe high potential of micro-LC.

Chapters 5 and 6 (Pollak) are devoted to densitometric analysis. Chapter 5 deals with the sources of error related to non-linearity problems in thin-layer chromatography (and electrophoresis). More or less well known theory, such as the Kubelka–

Munk model, is discussed. For non-specialists in this field this is certainly interesting, but sufficient details for a complete understanding are sometimes missing. The main limitation of this chapter is that examples illustrating the theory are not present. In Chapter 6 attention is focused on electronic scanning for densitometric analysis. Some hardware possibilities such as vidicon tubes and solid-state sensors are presented. Diagrams of the systems are not shown. Generally, the references are not recent and the last two references mentioned in the text have been omitted from the list of references.

The book is well organized and is easily readable. For many libraries it is again a very useful book and a worthwhile contribution to a recognized series. However, for specialists in the above topics the small number of pages devoted to their specialism may not justify the book having an important place on their shelf. Moreover, some chapters are short and fragmentary.

Amsterdam (The Netherlands) G. J. DE JONG

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CHROM. 22 213

Book Review

Computerized multiple input chromatography, by M. Kaljurand and E. Küllik, Ellis Horwood, Chichester and Halsted (Wiley), New York, 1989, 226 pp., price £ 45.00, US$ 88.00, ISBN 0-7458-0120-X (Ellis Horwood), 0-470-21228-4 (Halsted)

This book deals mainly with a technique in which chemometrics is integrated in an analytical (chromatographic) procedure; data obtained in a conventional way are not processed, but rather the analytical method is modified using the capabilities of a computer. However, the expression "multiple input chromatography" is obviously used here with two different meanings: multi-dimensional chromatography (column switching) with only a few chemometric aspects, and multiplex chromatography, better known as correlation chromatography.

The book starts with a brief theoretical introduction to system theory, followed by a chapter dealing with the theoretical basis of correlation chromatography. This method is related but not similar to techniques such as Fourier transform spectroscopy and Hadamard spectroscopy. In correlation chromatography a drastic reduction of the detection limit is achieved without preconcentration. Also, it offers the possibility of (semicontinuous) process monitoring.

The next chapter is devoted mainly to two-dimensional chromatography, comprising both computer-controlled multiple injections with varying concentrations and column switching.

In the last part of the book the required instrumentation is discussed and practical applications are reviewed.

One can conclude that correlation chromatography is obviously still an experimental technique, not very well known, despite the impressive possibilities. A number of diverse problems prevent a general application: the theory is not easy to understand, a visual interpretation of the output signal is impossible and the method requires a special high-quality injection system, not commercially available. In addition, real-time (micro)computer control of the chromatographic system, including an appropriate data acquisition and correlation software package, is required.

It is hoped that this book may contribute to the acceptance of correlation chromatography in modern routine analytical practice. In certainly gives a good reflection of the state of the art at the time of writing (1987).

However, some remarks should be made. The important so-called "correlation noise" is almost always considered to originate from varying input concentrations. It may be an important factor when concentration variations are large, *e.g.*, in pyrolysis measurements, but it is of minor importance in ordinary correlation chromatography. Sometimes the disturbance level of the correlogram, caused by detector noise, is also called correlation noise. It is advisable to make a sharp distinction between noise in the correlogram, originating from (additive) random detector noise, and correlation "noise", typically caused by the correlation procedure as a result of both varying input concentrations and system non-idealities such as non-linearity and injection errors.

Recently, injection errors have proved to be the dominant source of correlation noise with a deterministic nature (ghost peaks). This kind of noise, observed during trace analysis in the presence of high concentrations of the main components and mostly incorrectly attributed to non-linearity, has restricted considerably the practical applicability of correlation chromatography so far. Adding the newest theoretical and practical insights with respect to this aspect is essential, although the book emphasizes the crucial importance of a perfect injection system.

A useful but concise description of the instrumentation, in particular input systems, is given. However, the remark in the chapter on instrumentation that correlation chromatography requires a detector with a high noise level is puzzling. Some attention is paid to the subject of chemical concentration modulation correlation chromatography, a modification of conventional correlation chromatography with interesting and important capabilities.

The review of applications in the last part covers both trace analysis and process monitoring, the latter obviously being the field of interest of the authors. Particularly in thermochromatography (pyrolysis) interesting results are presented. It must be emphasized that only typical experimental results are presented; commercial correlation chromatography instruments are not (yet) available.

In conclusion, this is a book with much information on a not widely known chemometric technique with promising possibilities, supplemented with details on areas such as thermochromatography and column switching.

Amsterdam (The Netherlands) H. C. SMIT

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CHROM. 22 290

Book Review

Chemiluminescence and photochemical reaction detection in chromatography, edited by J. W. Birks, VCH, Weinheim, New York, X + 291 pp., Price DM 138.00; £ 49.45, ISBN 3-527-26782-4.

The groups of J. W. Birks and of R. E. Sievers at the University of Boulder, Boulder, CO, U.S.A., have done a lot of research on chemiluminescence (CL) both in the liquid and in the gas phase. This explains why five chapters of the interesting book considered here have been written by researchers at the above-institution; the other two are from T. A. Nieman (University of Illinois) and R. S. Givens and R. L. Showen (University of Kansas). The book has been dedicated to Roland W. Frei (Free University, Amsterdam), one of the pioneers in the field of reaction detection in high-performance liquid chromatography (HPLC), who died in January 1989.

In general, chemiluminescence reactions (and their counterparts, photochemical reactions) proceed according to complicated and not completely known mechanisms. Further, in practice, gas-phase and liquid-phase CL are almost independent fields of research. Nevertheless in this book a successful attempt has been made to cover the complete field of CL with emphasis on detection in gas chromatography (GC) and LC, while the reader is assumed to be familiar with chromatographic principles. The book will be of interest to chromatographers willing to try something a little out of the ordinary to solve their analytical problems. For these readers, especially the commercially available CL detectors for GC might be of interest and further the involvement of fully developed LC detection procedures, *e.g.*, based on luminol or peroxyoxalate CL. In addition, the book will be very useful to analytical chemists with research interests in CL. Commonly the latter confine their attention to the analytical implications of a single CL reaction; this book will enable them to become involved in alternative reactions in an efficient manner.

The characters of the seven contributions to the book differ. In the first chapter, John W. Birks gives a comprehensive overview of photophysical and photochemical principles. In 37 pages an overwhelming number of subjects are discussed. Although the chapter is clearly written, it will be difficult to assimilate by chemists inexperienced in this field and these readers will have to resort to complete books on this subject. In contrast, for specialists, such a compact treatment is appropriate. Of course, it is the intention of the first chapter to provide a background for the subsequent ones. However, the various chapters seem to have been written simultaneously so that they can be read independently.

Two chapters are devoted to CL detection in GC. Despite the fact that detection is not a general problem in GC, at the moment CL detectors have a place in some specific problems of analysis. Here the reagent gas (usually composed of highly reactive species generated in a gas discharge or a microwave bridge) is mixed with the GC effluent and, if the reaction is sufficiently exothermic, one or more of the products may be formed in electronically or high-vibrationally excited states, subsequently

losing their excess energy by emitting luminescence (without substantially heating the detector cell). Highly reactive species such as O_3, active nitrogen (nitrogen molecules in an electronic excited state and nitrogen atoms), F atoms, F_2, Na atoms and OClO are stable enough to be transported to the detector cell, where the CL reaction takes place. Various CL reactions based on these species and their potential for GC detection are clearly discussed. A complete chapter is devoted to the reaction between NO and O_3 producing NO_2*, which provides a broad CL emission centring around 1200 nm which can be easily distinguished from other CL signals. Three commercial detectors have been developed based on this reaction, *viz.*, the thermal energy analyzer (TEA), the nitrogen-compound-selective detector and the redox CL detector. The first is especially directed to the analysis of nitroso compounds which undergo catalysed pyrolysis producing NO. The second is based on the same principle, but here oxygen is added to the GC column effluent before it passes through the pyrolyser. To be detected by the third type, the analytes do not have to contain a nitrogen moiety; the GC effluent is mixed with nitrogen dioxide or nitric acid and all analytes that react, producing NO, are detected indirectly. Interesting applications to real samples are presented based on the fact that selective CL detection of NO at ppt (v/v) concentration levels up to six orders of magnitude is possible.

Chapter 4 gives a clearly written, comprehensive overview of the existing CL detection systems in liquid solutions, considering the most frequently studied reactions, *i.e.*, luminol, lucigenin, peroxyoxalate and tris(2,2-bipyridine)ruthenium(II), and the possibility of electrochemiluminescence and the bioluminescent luciferin–luciferase reaction. The reader will acquire a good impression of current accomplishments. Of course, the character of this chapter is that of an overview; it would not be difficult to write a complete book devoted to the luminol reaction or to the peroxyoxalate reaction itself.

The contents of Chapter 5 may be unexpected. Here an in-depth analysis of the peroxyoxalate CL reaction is given, not presented in the literature before. Such a physical–organic approach is not only of interest to researchers studying reaction mechanisms. To apply peroxyoxalate CL in HPLC detection at trace levels, the reaction variables have to be carefully defined and evaluated. The peroxyalate reaction basically has a biphasic intensity *versus* time profile, indicating that at least two light-generating intermediates and one storage intermediate play a role (instead of the generally, uncritically assumed dioxetanedione). In the mixed aqueous solvents characteristic of reversed-phase HPLC, a simplified picture is observed: a single, rapid burst of light, decaying exponentially. In a flow dynamic system, this reaction–time profile is not the only parameter: the reaction occurs in a continuous flow reactor which is composed of the mixing chambers, the connecting tubing and the flow cell.

The longest chapter is devoted to photochemical reaction detection in HPLC. Obviously, it is advantageous to utilize photons as a "reagent" compared with chemical derivatization procedures, the most important point being that light can be introduced without additional pumps or mixing devices. Much attention is paid to photochemical reactor design in HPLC, including a comparison of light sources. Further, the photochemical reactions are classified in four basic categories, *i.e.*, photolysis (the analyte is decomposed into smaller frequents), molecular rearrangements (as a result of changes in the molecular structure, the analyte is transformed to a more detectable product), photocatalytic reactions (the analytes function as catalysts for

the photochemical production of detectable surrogates) and finally, sensitized photochemical reactions (in which light-absorbing reagents are used to detect analytes which do not absorb light themselves). Many practical applications are presented, emphasizing both the selectivity and sensitivity of photochemical reaction detection. Nevertheless, in practice conventional detectors are still preferentially used. To become universally accepted, off-the-shelf photoreactors and chemiluminescence detection apparatus should be commercially available.

The final chapter is an extensive treatment of photochemical reaction detection based on singlet oxygen sensitization. The basic idea is that analytes, on being excited, decay at least partly via the electronic triplet state and subsequent reaction with molecular oxygen, thus producing singlet oxygen. Indirect measurement of this reactive and short-living form of molecular oxygen in principle opens up an interesting new detection possibility for UV-absorbing analytes. However, to date, only one application in LC has been reported in the literature. In this particular field a lot of work still has to be done.

Summarizing, this book should be readily available to all chromatographers interested in alternative detection methods.

Amsterdam (The Netherlands) CEES GOOIJER

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CHROM. 22 287

Book Review

Business opportunity report: chromatography —Markets, systems, applications, by R. H. Vermuyten, Business Communication Co., Norwark, CT, 1988, 150 pp., price US$ 1950.00.

The purpose of this report is to supply companies, already actively engaged in the chromatographic market or planning to enter this market, with information such as marketing needs and requirements, research and development and corporate planning activities. The report identifies and discusses some of the major trends that are occurring in the market place and provides forecasts of the market through 1992.

According to the author, an exhaustive research of sources including key trade publications, key trade associations, appropriate business directories, available published research reports, interviews and discussions with authorities in industry and chromatographic manufacturers was carried out.

The report consists of eight sections and an Appendix with a total of 151 pages.

In Section I, the methodology which was used to gather the necessary information is explained. One may always dispute why a certain source of information has been used or not. These days, however, many trends and new developments can be identified during meetings, seminars, international symposia and in the scientific literature. Probably owing to the lack of these sources, important techniques are misrepresented, *e.g.*, electrophoresis is cited as being "not expected to create any impact on the size and growth of the chromatographic market". In 1987, when this report was written, this statement was probably justified. However, today in 1990, it is simply incorrect.

In Section II, the report features an excellent review of the different types of chromatographic techniques, including gas, liquid and supercritical fluid chromatography.

Section III is dedicated to the end-user market and divides the chromatographic market according to its applications into three end-user groups (industrial, research and clinical).

Section IV provides an overview of the "current" market, based on data from 1987. Major suppliers of chromatographic hardware, their market share in different liquid chromatography categories such as high-performance liquid chromatography, ion, thin-layer and affinity chromatography, are listed. The stated expectation that the market in supercritical fluid chromatography is going to triple within the next few years was certainly correct 2 years ago, but today it seems that this market is growing much more slowly than was expected.

Section V provides detailed profiles of the major suppliers of chromatographic products, which are definitely of use for a marketing report; however, the data and information need to be updated.

Section VI is probably the most inspiring chapter in the whole report. It discusses

in detail some of the factors that influence the market place such as trends in technology, applications and research.

Section VII deals with an overview of the future market, and two growth areas in particular are specified, instrumentation and applications.

Section VIII is addressed to business opportunities identified during this research. With liquid chromatography–mass spectrometry, supercritical fluid chromatography, affinity chromatography, large-scale chromatography, *etc.*, certainly some of the most important growth areas for future business opportunities have been briefly addressed.

The Appendix contains a glossary of major terms used in the field of chromatography and will be a useful tool particularly for non-chromatographers, *e.g.*, sales and marketing specialists.

In general, the report is well structured and represents a comprehensive overview of the market situation as it was in the U.S.A. in 1987. In my opinion, however, it is vital today that a marketing report aimed at assisting chromatographic companies in their marketing, research and development and planning decisions should contain also the newer separation techniques such as electrophoresis[a], microseparations and membrane separations.

Amsterdam (The Netherlands) J. P. CHERVET

[a] According to Business Communication Co., this subject will be treated in an upcoming report on electrically driven separations.

Author Index

Errata

J. Chromatogr., 405 (1987) 67–76.
Page 70, 3rd line, "eqn.4" should read 'eqn. 4a".
Page 70, 4th line from bottom, "exp(k_2T)" should read "exp(k_2/T)".
Page 71, last line, "$k_{3(1)}$" should read "$k_{3(1)}t_1$".
Page 73, eqn. 13, "$I_{eq(2)}$" should read "$T_{eq(2)}$".
Page 74, 9th line, "i" should read "I".

J. Chromatogr., 503 (1990) 155–165.
Page 156, last two text lines, sentence "B_1 and B_2 were stainless-steel pipes, 1.1 m in length and of 0.5 mm I.D." should be deleted.

Request for manuscripts

Y. Ito, W.D. Conway, M. Knight and Y.-W. Lee will edit a special, thematic issue of the *Journal of Chromatography*, entitled **"Counter-current Chromatography"**. Both reviews and research articles will be included.

Topics such as the following will be covered:

- theoretical aspects
- instruments
- solvent systems
- applications
- any other topics relating to counter-current chromatography.

Potential authors of reviews should contact Yoichiro Ito, National Institutes of Health, Bldg. 10, Rm. 7N 322, Bethesda, MD 20892, U.S.A. (Tel. 301-496-3237 or -2557, Fax: 301-402-0013).

The deadline for receipt of submissions is **May 1, 1990**. Manuscripts submitted after this date can still be published in the Journal, but then there is no guarantee that an accepted article will appear in this special thematic issue. **Five** copies of the manuscript should be submitted to Y. Ito. All manuscripts will be reviewed and acceptance will be based on the usual criteria for publishing in the *Journal of Chromatography*.

Request for manuscripts

R. Majors, F. Regnier and K. Unger will edit a special, thematic issue of the *Journal of Chromatography* entitled **"LC Column Packings"**. Both reviews and research articles will be included.

Topics such as the following will be covered:

- organic packings
- inorganic packings
- non-porous particles
- macroporous particles
- restricted access media
- functionalized membranes
- solid-phase extraction materials
- commercially available packings
- physical-chemical characterization
- relative performance of packings
- packing procedures and hardware
- column care.

Only minor coverage of topics such as affinity chromatography and chiral separations is planned since these will be the topics of other thematic issues.

Potential authors of reviews should contact Roger Giese, Editor, prior to any submission. Address: 110 Mugar Building, Northeastern University, Boston, MA 02115, U.S.A.; tel.: (617) 437-3227; fax: (617) 437-2855.

The deadline for receipt of submissions is **June 1, 1990**. Manuscripts submitted after this date can still be published in the Journal, but then there is no guarantee that an accepted article will appear in this special, thematic issue. **Five** copies of the manuscript should be submitted to R. Giese. All manuscripts will be reviewed and acceptance will be based on the usual criteria for publishing in the *Journal of Chromatography*.

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journal of chromatography news section

AWARDS

PITTSBURGH ANALYTICAL CHEMISTRY AWARD

Professor George H. Morrison has been named by the Society for Analytical Chemistry of Pittsburgh as recipient of the 1990 Pittsburgh Analytical Chemistry Award. The award was presented by Mary Louise Theodore, 1989–1990 Chairman of the Society on Tuesday morning, March 6, 1990. Professor Morrison received his undergraduate degree in chemistry from Brooklyn College and his Ph.D. in chemistry from Princeton University. His positions in academia and industrial institutions have uniquely equipped him to bridge the diverse needs of analytical chemistry. He served as principal investigator of the Apollo Lunar Analysis Program. Director of the Cornell Materials Science Center Analytical Facility, NIH Senior Fellow at the Harvard Medical School, Head of Inorganic and Analytical Chemistry at the General Telephone and Electronics Laboratories and served on the Manhattan Project at Princeton University. He is presently Professor of Chemistry at Cornell University.

Professor Morrison has served on the editorial boards of numerous journals including: *Analytical Chemistry, Analyst, Analytica Chimica Acta, Microchimica Acta, Separation Science,* and the *Journal of Physical and Chemical Reference Data.* He has served on numerous scientific committees including those of the International Union of Pure and Applied Chemistry, National Research Council, National Science Foundation, National Bureau of Standards, and the American Chemical Society.

Professor Morrison has received numerous awards including the American Chemical Society Award in Analytical Chemistry, Medal of the Society for Applied Spectroscopy, and the Eastern Analytical Symposium Award.

Professor Morrison has documented his scientific, editorial, and research efforts in more than 350 papers. He has shown the applicability of ion microscopy to many new fields, including the medical field. As editor of *Analytical Chemistry,* he has advanced this internationally recognized journal.

KEENE P. DIMICK AWARD

Professor Milos V. Novotny has received the Keene P. Dimick Award on Monday morning, March 5, 1991. This award is administered by the Society for Analytical Chemists of Pittsburgh and sponsored by Keene P. Dimick. Professor Novotny received his B.S. degree from the University of Brno, Czechoslovakia and his Ph.D. in biochemistry in 1965 from that institution. Following postdoctoral assignments at the Czechoslovak Academy of Science, Royal Karolinska Institute of Stockholm, and the University of Houston, he joined the faculty of Indiana University, Bloomington, Indiana. He is presently Rudy Professor of Chemistry at that institution. He has received numerous honors including chairmanship of the Gordon Research Conference on Analytical Chemistry. M.S. Tswett Award and Medal in Chromatography, Eastern Analytical Symposium Award in chromatography and is a Fellow, American Association for the Advancement of Science.

Professor Novotny's research has been focused in the areas of chromatography applications and research. He has published numerous papers on chromatography and served on many editorial boards associated with chromatographic sciences. These include: *Journal of Chromatography/Biomedical Applications, Journal of High Resolution Chromatography* (co-editor), *Chromatography Forum* (guest editor), *Chromatographia* and *Journal of Microcolumn Separations* (associate editor).

DAL NOGARE AWARD

The Dal Nogare Award has been presented to Dr. Robert L. Grob of Villanova University. The presentation of the Award will take place during the Dal Nogare Award Symposium on Tuesday afternoon. Dr. Grob's award is for his achievements in the field of chromatography, particularly for his contributions in the study of theory, instrumentation and applications of gas and liquid chromatography to environmental analysis.

Bob Grob received his M.S. and Ph.D. at the University of Virginia. He worked at Esso Research and Wheeling College before joining the faculty of Villanova in 1963. In 1967 he was promoted to full professor and since that time has directed a very active research group on chromatography. He has authored or coauthored six books and his research has been published in more than 100 papers.

The Dal Nogare Award has been given by the Chromatography Forum of the Delaware Valley since 1972 in recognition of scientists who have contributed significantly to the understanding and practice of chromatography.

ANNOUNCEMENTS OF MEETINGS

CHROMATOGRAPHY — PAST, PRESENT, AND FUTURE; HONORARY SYMPOSIUM ON THE OCCASION OF THE 90TH BIRTHDAY OF PROFESSOR ERIKA CREMER, INNSBRUCK, AUSTRIA, 19–20 JUNE, 1990

The symposium will cover the entire scope of chromatographic and related separation techniques, *e.g.* gas chromatography, HPLC, ion chromatography, SFC, and capillary electrophoresis.

The lectures, given by speakers from universities and from industry, will contrast specialized review presentations with present-day applications and developments. Major new perspectives in this field of science will be discussed in a number of invited contributions.

A social program will be organised for all participants and accompanying persons.

For further information, contact: Dr. G.K. Bonn, Institute of Radiochemistry, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria. Tel.: (512) 507-3213; Fax: (512) 580-519.

AMSTERDAM SUMMERCOURSE ON CAPILLARY ZONE ELECTROPHORESIS, AMSTERDAM, THE NETHERLANDS, 15–17 AUGUST, 1990

The separation technique capillary zone electrophoresis (CZE) receives rapidly growing attention. The analytical interest in CZE arises from the wide spectrum of compounds that can be separated with high efficiency, varying from inorganic ions to very large biopolymers. Among the fields of application we find pharmaceutical analysis, clinical analysis and biotechnology.

The Amsterdam CZE Summercourse is meant to guide newcomers in the basic concepts of CZE. In overview lectures and informal seminar sessions leading experts in the field will show the possibilities of the technique. Participants have the opportunity to subscribe for specialized topics in seminar sessions and to discuss the application of CZE in their own laboratory.

Lecture/seminar topics will include: general introduction to capillary electrophoresis; micellar electrophoresis and electrochromatography; detection in CZE; electrophoresis of biopolymers; capillary gel electrophoresis; mass loadability and peak distortion; coating of the capillaries in CZE; CZE–MS coupling; applications of CZE in the pharmaceutical industry; and applications of CZE in biotechnology. The course organizers are: Dr. W.Th. Kok, Dr. J.C. Kraak and Dr. J. Kragten.

The tuition fee will be approximately Dfl. 1500. The fee includes the coursebook and manual, lunches, refreshments and the course dinner.

For further details, contact: Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV, Amsterdam, The Netherlands. Tel.: (31 20) 525-6515/6539/6546/6567. Fax: (31 20) 525-5802 CCITT3.

INTERNATIONAL SYMPOSIUM ON CAPILLARY ELECTROPHORESIS YORK, U.K., AUGUST 22–24, 1990

This symposium will be the first on capillary electrophoresis (CE) to be held in the U.K. The enormous topical interest in this rapidly expanding technique promises that it will be a lively event. Plenary speakers will include leading American and European investigators.

The symposium will be preceded by a three-day short course in CE (August 19–22 and starts its first session at 8 pm on Wednesday August 22. A commercial Exhibition of the latest CE systems and components will also be featured.

Lecture and poster topics will include: instrumentation, detector developments, CE–mass spectrometry, micellar separations, capillary gel electrophoresis, analytical applications, clinical and pharmaceutical analysis, neurosciences, peptides and proteins, carbohydrates, oligonucleotides.

Abstracts describing original research are invited in the area of CE for inclusion in the oral programme or for presentation as a poster (max. 300 words), by Friday April 27, 1990.

The cost of the meeting including registration, accommodation, meals, admission to the Instrument Exhibition and a copy of the abstracts will be £160 for Chromatographic Society and British Electrophoresis Society members, and £195 for non-members. The reduced fee for students is £140.

For further information, contact: Dr. Carys Calvert (Symposium Manager), Dept. of Chemistry, University of York, Heslington, York, YO1 5DD, U.K. Tel.: 0904 432576 (direct line) or 0904 432511 (general office); Telex: 57933 YORKUL; Fax: 0904 433433; E. Mail: CGGC1 @VAXA.YORK.AC.UK.

6TH INTERNATIONAL SYMPOSIUM ON ENVIRONMENTAL RADIOCHEMICAL ANALYSIS, MANCHESTER, U.K., SEPTEMBER 19–21, 1990

The symposium aims to provide a forum for professional radiochemists who analyse environmental and biological materials, to discuss their latest work and ideas. It will also give an opportunity for environmental scientists who apply radiochemical methods to their studies, to present their results.

The preliminary scientific programme is as follows: nuclear counting techniques applicable to environmental measurements; the determination of radionuclides in environmental materials (solids and aqueous matrices); the determination of radionuclides in biological samples (bioassay); the application of radiochemical methods to environmental studies; and quality assurance.

The Symposium will be held in the Department of Chemistry at the University of Manchester. The closing date for final registrations will be 20th July 1990. The registration fee will be about £75.00 plus Value Added Tax and will include the Symposium Dinner and lunches.

For further details, contact: Mrs F.J. Johnson, Concilia, P.O. Box 18, Ilkley LS29 6RA, U.K.

EURO HPLC TRAINING COURSE, WATERFORD, IRELAND, 25–29 JUNE, 1990, AND MONTPELLIER, FRANCE, 8–12 OCTOBER, 1990

This Euro HPLC training course is organised with the support of EC programme COMETT. The aim of this programme is to disseminate the latest information and research methodology related to HPLC to a wide range of pharmaceutical, chemical, medical, cosmetic and food enterprises.

This course covers: developments in columns and stationary phases; enantiomeric separations; trends in detection techniques; photodiode array detection (evaluation, limits and possibilities optimization); derivatization techniques; sample pre-treatment by solid phase extraction techniques; method development and optimization; expert systems; method validation and equipment qualification; ion chromatography; evaluation of super-critical fluid chromatography relative to HPLC.

Lectures will be given by specialists from seven EC countries. Instrument demonstrations will also be a feature of the course. A simultaneous English–French translation will be provided for the course in Montpellier.

Course fee will be: Waterford, Ir£ 100, contact Mr J. Griffith, Dept. of Chemical and Life Sciences, Waterford Regional College, Waterford, Ireland, Tel.: (05) 175934, Fax: (05) 178292. Montpellier, FF 2500, Contact Prof. H. Fabre, Laboratoire de Chimie Analytique, Faculté de Pharmacie, 34060 Montpellier Cedex, France, Tel.: (67) 635432, Fax: (67) 611622.

1991 SYMPOSIUM ON SUPERCRITICAL FLUID CHROMATOGRAPHY, PARK CITY, UT, U.S.A., JANUARY 15–17, 1991

The 1991 Symposium on Supercritical Fluid Chromatography, sponsored by the State of Utah and Brigham Young University, will be held at Prospector Square, Park City, UT, U.S.A., January 15–17, 1991.

The purpose of the workshop is to provide a forum fox maximum exchange of information on techniques and applications of supercritical fluid chromatography, including analytical supercritical fluid extraction. Scientists, both experienced chromatographers and potential users of SFC, are encouraged to attend.

The workshop will consist of oral presentations, poster sessions, and planned informal discussion sessions. Emphasis will be placed on open discussion of the details of the practice of SFC. Attendees are invited to present results of basic research or practical applications of SFC. Both packed column and capillary column topics are appropriate. Informal discussion sessions on important issues are planned. Prospective attendees are encouraged to suggest topics for discussion to be included in the program.

Registration fee is US$ 400 for regular attendees, and US$ 150 for graduate students.

For more information please contact Dr. Milton L. Lee, Department of Chemistry, Brigham Young University, Provo, UT 84602, U.S.A. Tel: (801) 378-2135; Fax: (801) 378-5474.

HPCE '91, 3rd INTERNATIONAL SYMPOSIUM ON HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS, SAN DIEGO, CA, U.S.A., FEBRUARY 3–6, 1991

The 3rd International Symposium on High Performance Capillary Electrophoresis will be held on February 3–6, 1991 in San Diego, CA, U.S.A. This is the continuation of the series which began in Boston in 1989 and San Francisco in 1990.

The three-day program will include invited and contributed lectures. As in the previous symposia, posters will play a major role in the scientific program, and ample time will be set aside to view them and to speak to their authors. Sufficient space has also been allocated for the poster area so that all posters will remain up during the entire symposium. Lecture and poster topics will include: zone electrophoresis; isoelectric focusing; isotachophoresis; micellar separations; CE–mass spectrometry; gel columns; detector design; instrumentation; and analytical and micropreparative applications for pharmaceuticals, peptides, proteins, carbohydrates, oligonucleotides, sub-cellular structures, and whole cells.

The deadline for submission of abstracts is September 1, 1990.

A three-day exhibition of HPCE systems and components is also planned.

For further details, contact: Shirley E. Schlessinger, Symposium Manager, HPCE '91, Suite 1015, 400 East Randolph Drive, Chicago, IL 60601, U.S.A.

2-D PAGE 1991, LONDON, U.K., JULY 16–18, 1991

The meeting will cover various aspects of methodology and applications of two-dimensional polyacrylamide gel electrophoresis. The meeting will consist of plenary sessions, contributed lectures and poster presentations. There will also be an exhibition of equipment and supplies for 2-D PAGE.

Topics will include: methodology, visualisation, western blotting, densitometry, computer analysis, protein databasing, protein characterisation, microsequencing, and applications.

The meeting will be held in English.

The meeting will take place in the Conference Centre at Kensington Town Hall, Horton Street, London W8. Accommodation at a reduced rate available through the Secretariat has been reserved in the London Tara Hotel; alternatively you may make your own arrangements.

For further details contact: 2-D PAGE 1991 Secretariat, Department of Cardiothoracic Surgery, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY, U.K.

SYMPOSIUM PROGRAMME

HPLC '90, 14th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY, BOSTON, MA, U.S.A., MAY 20–25, 1990

The 14th International Symposium on Column Liquid Chromatography will be held at the Boston Park Plaza Hotel, Boston, MA, U.S.A., from May 20 to 25, 1990. Registration information and full details can be obtained from: Shirley E. Schlessinger, Symposium Manager, HPLC '90, Suite 1015, 400 East Randolph Drive, Chicago, IL 60601, U.S.A., Tel.: (312) 527-2011. The detailed programme of the symposium is given below.

SCIENTIFIC PROGRAM SUMMARY

MONDAY		TUESDAY		WEDNESDAY		THURSDAY		FRIDAY	
8:30	Session 1: Critical Issues in Separation Science (Plenary)		Session 5: FFF/Colloids	8:30	Session 11: Microcolumn Separations	8:30	Session 17: LC/CE	8:30	Session 23: (Plenary)
			Session 6: Detection		Session 12: HPLC of Biopolymers I		Session 18: Novel Packing Materials II	10:00	Coffee
			Session 7: Novel Packing Materials I		Session 13: Clinical Drug Analysis		Session 19: Polymer/ Biopolymer Characterization	10:30	Session 24: (Plenary)
10:45	Coffee/ Posters: (at the 57)	10:45	Coffee/ Exhibition/ Posters: (at the Castle and 57)	10:30	Break	10:45	Coffee/ Posters: (at the Castle)	12:00	Invitation to HPLC '91
12:15	Lunch	12:15	Lunch	11:00	Session 14: CE Fundamentals	12:15	Lunch	12:10	Close of Symposium
1:15	Exhibition/ Posters (at the Castle)	1:15	Exhibition/ Posters: (at the Castle and 57)		Session 15: Sample Handling	1:15	Posters: (at the 57)		
3:00	Session 2: Fundamentals Session 3: Chiral Separations I Session 4: Analytical Biotechnology	3:00	Session 8: Preparative Scale Separations Session 9: Optimization Session 10: Chiral Separations II		Session 16: LC and LC/MS of Polypeptides	3:00	Session 20: Trace Analysis Session 21: HPLC of Biopolymers II Session 22: Novel Applications		
5:15	Discussion Sessions	5:15	Discussion Sessions	1:00	Lunch	5:15	Discussion Sessions		
					Exhibits open to the public				
				6:00	Clambake at New England Aquarium				

NOTE: All registrants are invited to a **Welcoming Reception** on Sunday evening from 6:00 to 7:30 PM.

SCIENTIFIC PROGRAM

Sunday, May 20, 1990

6:00-7:30pm Welcoming Reception

Monday, May 21, 1990

8:15am Opening of Symposium
B. L. Karger, Northeastern University, Boston, MA USA

8:30-10:30 Lecture Session 1 (Plenary): Critical Issues in Separation Science, *B. L. Karger,* Moderator

Participants:

Dieter Hoehn
Hewlett-Packard Co., Palo Alto, CA USA

Jack J. Kirkland
E.I. duPont de Nemours, Wilmington, DE USA

Fred E. Regnier
Purdue University, West Lafayette, IN USA

H. Michael Widmer
Ciba-Geigy, Basel, SWITZERLAND

10:45-12:15 Poster Sessions:

Fundamentals
Microcolumn Liquid Chromatography
Preparative Scale Separations
Optimization
Supercritical Fluid Chromatography

1:15-2:45 Poster Sessions:

Chiral Separations I
HPLC Applications: General
Advances in Detection

3:00-5:00pm, Monday, May 21, 1990

Lecture Session 3: Chiral Separations I, Continued

Chiral Amino Acid Microanalysis by Direct Optical Resolution of Fluorescent Derivatives on Resolvosil® Columns
Stig G. Allenmark and Shalini Andersson, University of Gothenburg, Gothenburg SWEDEN

Coupled Column Chromatography in Chiral Separations
Andreas M. Rizzi, University of Vienna, Vienna, AUSTRIA

Lecture Session 4: Analytical Biotechnology

Analysis of Glycoprotein Carbohydrate Using High-pH Anion Exchange Chromatography*
R. R. Townsend, University of California, San Francisco, CA USA

Carbohydrate Structural Detail Using Supercritical Fluid Chromatography Mass Spectrometry*
Vernon N. Reinhold, Harvard University School of Medicine, Boston, MA USA

Effect of Preferred Binding Domains on Peptide Retention Behavior in Reversed-Phase Chromatography
Robert S. Hodges, University of Alberta, Edmonton, Alberta CANADA

Comparative Oligosaccharide Mapping of Soluble CD4 N-Linked Glycosylation Sites
Kaylan Anumula and James J. L'Italien, SmithKline Beecham Pharmaceuticals, King of Prussia, PA USA

An Assessment of Separation Methods for High Molecular Weight Biological Substances
Gerard P. Rozing, Hewlett-Packard GmbH, Waldbronn, FDR

5:15 - 6:00pm

Discussion Sessions: Chiral Separations
Column and Packing Materials
Analytical Biotechnology

Lecture Session 2: Fundamentals

Evolution of Liquid Chromatography: Explorers, Pioneers and Settlers*
Leslie S. Ettre, The Perkin-Elmer Corporation, Norwalk, CT USA

Recognition of Overlapped Exponentially Modified Gaussians Using Second Derivatives*
Eli Grushka and Dror Israeli, The Hebrew University, Jerusalem ISRAEL

Comprehensive Thermodynamic and Theoretical Investigation of Retention in Capillary Supercritical Fluid Chromatography
Daniel E. Martire, Rebecca L. Riester and Chao Yan, Georgetown University, Washington, DC USA

Optimization of Peak Compressions Induced by Large System Peaks in Ion-Pair RPLC
Torgny Fornstedt and Douglas Westerlund, University of Uppsala, Uppsala, SWEDEN

Using the Electrostatic Theory for Ion Pair Chromatography In Practice
Jan Ståhlberg, Astra Pharmaceutical Production AB, Sodertalje, SWEDEN

Lecture Session 3: Chiral Separations I

A Commentary on the Chromatographic Separation of Enantiomers*
William H. Pirkle, University of Illinois, Urbana, IL USA

HPLC-Enantioseparation Using Immobilized Chiral Acids or Amines as Selectors*
Wolfgang Lindner, Sabine Rauch-Puntigam, Franz Reiter, Herbert Schnedl and Georg Uray, Karl-Franzens-University of Graz, Graz AUSTRIA

Circular Dichroism Detector in HPLC
Carlo Bertucci, Enrico Domenici and Piero Salvadori, University of Pisa, Pisa, ITALY

8:30-10:30am, Tuesday, May 22, 1990

Lecture Session 5: Field-Flow Fractionation and Colloid Separations

Recent Advances in Field-Flow Fractionation*
J. Calvin Giddings, University of Utah, Salt Lake City, UT USA

Size Fractionation of Emulsified Drugs Using Sedimentation FFF*
Jianmin Li and Karin D. Caldwell, University of Utah, Salt Lake City, UT USA

Developments in Asymmetrical Flow Field-Flow Fractionation
Anne Litzén and Karl-Gustav Wahlund, University of Uppsala, Uppsala, SWEDEN

Coriolis-Induced Secondary Flow Effect in Sedimentation FFF
Mark R. Schure, Rohm and Haas Company, Spring House, PA USA

Hydrodynamic Chromatography on Small Sized Porous and Non-Porous Packings
Gerrit Stegeman, Rob Oostervink, Johan. C. Kraak and H. Poppe, University of Amsterdam, Amsterdam, THE NETHERLANDS

Lecture Session 6: Detection

Separation and Detection in Capillary Electrophoresis and Capillary Liquid Chromatography*
Edward S. Yeung, Iowa State University, Ames, IA USA

Post Column Reaction Detection and FIA*
Heinz Engelhardt, Universität des Saarlands, Saarbrucken FDR

Attomole Detection Limits with an Improved Coulometric Electrochemical Detector for Liquid Chromatography
E.W. Zink, W.M. Krebs, P.A. Blanco, R.S. Pollard, T.J. Sapienza, R. Vitukevich and F. Puma, ESA, Bedford, MA USA

* Keynote address

8:30-10:30am, Tuesday, May 22, 1990

Lecture Session 6: Detection, Continued

Protein Aggregation Detection by Modern HPLC On-Line Coupling to Low Angle Laser Light Scattering (LALLS)
Shiaw-Lin Wu, Jerry Cacia and William S. Hancock, Genentech, Inc., South San Francisco, CA USA

Refractive Index Gradient Detection of Biopolymers Separated by High Temperature Liquid Chromatography
Robert E. Synovec and Curtiss N. Renn, Center for Process Analytical Chemistry, Seattle, WA USA

Lecture Session 7: Novel Packing Materials I

Perfusion Chromatography*
Fred E. Regnier, Purdue University, West Lafayette, IN USA

High-Speed Separations of Macromolecules with New Superficially-Porous Column Packings*
Jack J. Kirkland, E.I. duPont de Nemours and Company, Wilmington, DE USA

A Novel Type of Chromatographic Column Comprising a Cylinder with Communicating Micro-Channels*
Stellan Hjertén and Jia-Li Liao, University of Uppsala, Uppsala, SWEDEN

Advanced Packing Materials for Reversed-Phase Liquid Chromatography*
Nobuo Tanaka (1), Kazugiro Kimata (1), Ken Hosoya (1), Takeo Araki (1), Yoshihiro Shiojima (2), Yutaka Ohtsu (2), Riyou Tsuboi and Hajime Tsuchiya (4), (1) Kyoto Institute of Technology, Kyoto, (2) Shiseido, Yokohama, (3) Nacalai Tesque, Mukoh and (4) Nitto Technical Information Center, Ibaraki, JAPAN

10:45am - 12:15pm

Poster Sessions: Chiral Separations II
Pre- and Post-Column Derivatization
Analysis of Oligonucleotides

1:15 - 2:45pm

Poster Sessions: Capillary Electrophoresis Applications
Column and Packing Materials
Environmental Analysis

3:00 - 5:00pm

Lecture Session 8: Preparative Scale Separations

A Comparison of Overloaded Elution Chromatography and Displacement Chromatography*
A.M. Katti and Georges Guiochon, University of Tennessee, Knoxville, TN and Oak Ridge National Laboratory, Oak Ridge, TN USA

Membrane Displacement Chromatography of Biomolecules*
Michael W. Phillips (1), Steven Fraleigh (1), Richard Hamilton (2) and Steven M. Cramer (1), (1) Rensselaer Polytechnic Institute, Troy, NY USA and (2) Millipore Corporation, Bedford, MA USA

Practical Laboratory Applications of the Self-Displacement Effect
Joan Newburger (1), Susan Taylor (1) and Georges Guiochon (2),
(1) Squibb Institute for Medical Research, Princeton, NJ USA and (2) University of Tennessee, Knoxville, TN USA

Perfusion Chromatography: Application to Rapid Process Development and Scaleup
Noubar Afeyan, Scott Fulton, Neal Gordon, István Mazsaroff and Lászlo Várády, PerSeptive Biosystems, Cambridge, MA USA

Systematic Purification Process Development and Scale-Up for Monoclonal Antibodies Using Ion Exchange and Hydrophobic Interaction Chromatography
Peter S. Gagnon (1), Roy Eksteen (2), John J. Maikner (3) and Peter G. Cartier (3), (1) Validate Biosystems Inc., Tucson, AZ, (2) TosoHaas Technical Center, Woburn, MA, (3) Rohm and Haas Research Laboratory, Spring House, PA USA

3:00 - 5:00pm, Tuesday, May 22, 1990

Lecture Session 9: Optimization

Optimization Studies in HPLC*
Lloyd R. Snyder and John W. Dolan, LC Resources, Inc., Lafayette, CA USA

A Generalized Liquid Chromatographic Approach to the Analysis of Drugs from Serum Using Direct Injection Techniques with Surfactant Containing Eluents*
Brian A. Bidlingmeyer (1), F. Vincent Warren, Jr.(1), J. Detlef Bentrop (1), Ralph Grohs (2) and Larry Bowers (3), (1) Waters Division of Millipore Corp., Milford, MA USA, (2) Universität des Saarlandes, Saarbrucken, FDR, (3) University of Minnesota, Minneapolis, MN USA

Fully Automatic HPLC Optimization
Nebojsa M. Djordjevic, Ernst P. Lankmayr, Wolfhard Wegscheider, Ludwig Jaufman, Fritz Erni and Bernhard Schreiber, Sandoz, Basel, SWITZERLAND

Computer-Assisted Retention Prediction System for Biooligomers. Optimizing Gradients in Ion-Exchange, Reversed-Phase, and Mixed-Mode HPLC
Yoshinobu Baba, Oita University, Oita, JAPAN

Multi-Dimensional HPLC for High-Resolution Separation of Complex Biochemical and Physiological Samples
Kazuo Seta and Tsuneo Okuyama, Faculty of Science, Tokyo Metropolitan University, Tokyo, JAPAN

Lecture Session 10: Chiral Separations II

The Application of Enantioselective Chromatographic Techniques to Clinical, Pharmacological and Regulatory Assays*
Irving W. Wainer, Saint Jude Children's Research Hospital, Memphis, TN USA

The Role of New Derivatized Cyclodextrins in Chromatography*
Daniel W. Armstrong, University of Missouri-Rolla, Rolla, MO USA

Recent Developments in the Displacement-Chromatographic Separation of Enantiomers on Cyclodextrin-Silica Columns
G. Quintero, Gy. Farkas, A. Bartha and Gy. Vigh, Texas A&M University, College Station, TX USA

Chiral Ion-Pair Chromatography on Porous Polygraphite Carbon
Anders Karlsson and Curt Pettersson, Uppsala University, Uppsala, SWEDEN

HPLC Resolution of Isomers of Drugs with Mobile Phases Containing Urea-Solubilized β-Cyclodextrin
Joyce Noroski, William Merkl, Donald Mayo and Joel Kirschbaum, The Squibb Institute for Medical Research, New Brunswick, NJ USA

5:15 - 6:00pm

Discussion Sessions: Preparative Scale Separations
Clinical/Pharmaceutical Applications
Capillary Electrophoresis

8:30 - 10:30am, Wednesday, May 23, 1990

Lecture Session 11: Microcolumn Separations

New Fluorogenic Reagents for High-Sensitivity Detection of Biological Compounds by Microcolumn Liquid Chromatography and Capillary Electrophoresis*
Milos Novotny, Donald P. Wiesler, Jinping Liu, Kelly A. Cobb, Stephen C. Beale and Osamu Shirota, Indiana University, Bloomington, IN USA

Microcolumn Separations and the Analysis of Single Neurons*
James W. Jorgenson, Robert T. Kennedy, Mary D. Oates, Bruce R. Cooper, Beverley Nickerson, Curtis A. Monnig and Stephen C. Beale, University of North Carolina, Chapel Hill, NC USA

Concept, Implication, and Features of Universal Microbore Columns for GC, SFC and LC
Frank Yang, Yan Liu, Ilona Davies and Chris Pohl
Dionex Corporation, Sunnyvale, CA USA

Performance of Open Tubular Columns (5-10 μm I.d.) in Reversed-phase LC Using Immobilized Polysiloxanes of Different Selectivity
Anne B. Buskhe, Karin M. Göhlin and Marita Larsson, Chalmers University of Technology and University of Göteborg, Göteborg, SWEDEN

Biological Applications Using Packed Capillary LC
David Demorest and Bruce Black, Applied Biosystems, San Jose, CA USA

Lecture Session 12: HPLC of Biopolymers I

Monitoring Ligand-Induced Alterations in Protein Surface Metal Ion Binding Sites with High-Performance Immobilized Metal Ion Affinity Chromatography*
T. William Hutchens and Tai-Tung Yip, Baylor College of Medicine, Houston, TX USA

Interaction and Characterization of Coulombic Interactive Regions of Proteins by High Performance Anion Exchange Chromatography and Computer Graphics Modelling Analysis*
Anthony N. Hodder, Mibel I. Aguilar, Irena Cosic and Milton T.W. Hearn, Monash University, Clayton, Victoria AUSTRALIA

Isolation of Interferon Receptors Using Biotinylated Interferon Immobilized onto Affinichrom Streptavidin-Coated Glass Beads
Terry M. Phillips and James V. Babashak, George Washington University Medical Center, Washington, DC and Kontes Scientific Glassware, Vineland, NJ USA

Covalent Binding of Dnp Amino Acids to Highly Crosslinked Polystyrene Beads and Application for HPAC of IgE
S. Wongyai, J.M. Varga and G.K. Bonn, University of Innsbruck, Innsbruck AUSTRIA

Boronate High-Performance Affinity Chromatography
Ram P. Singhal, Y. Sarwar and S.S.M. de Silva, Wichita State University, Wichita, KS USA

Lecture Session 13: Clinical and Drug Analysis

Critical Review of Typical and Atypical Antidepressant Monitoring by HPLC*
Steven H.Y. Wong, University of Connecticut School of Medicine, Farmington, CT USA

Chromatographic Techniques in the Synthesis of New Anti-AIDS Dideoxyribonucleoside Chemotherapeutic Agents*
Phyllis R. Brown, Nan-In Jang and Joseph G. Turcotte, University of Rhode Island, Kingston, RI USA

Analysis of Antineoplastic Agents by Liquid Chromatography with Pre-column Fluorescent Derivatization: Multidimensional Approaches for the Enhancement of Detection
Christopher M. Riley, Karsten A. Holm, Carl G. Kindberg, John F. Stobaugh, Joseph M. Ault, Jr., Cheryl D. Schlaegel and Milan Slavik, University of Kansas, Lawrence, KS USA

8:30 - 10:30am, Wednesday, May 23, 1990

Lecture Session 13: Clinical and Drug Analysis, Continued

Advances in the Analysis of New Cardio-Active Drugs by HPLC
David W. Holt, St. George's Hospital Medical School, London, UK

HPLC in Therapeutic Monitoring of Immunosuppressive Drugs
Raman Ventaramanan, Gilbert J. Burckart and Richard J. Ptachcinski, University of Pittsburgh, Pittsburgh, PA USA

10:30 Coffee

11:00am - 1:00pm

Lecture Session 14: Fundamentals of Capillary Electrophoresis

The Influence of Temperature and Field Strength Profile on Resolution in HPCE: Practical and Theoretical Points of View
Stellan Hjerten, Tasanee Srichaiyo and Karin Elenbring, University of Uppsala, Uppsala, SWEDEN

Aqueous-Phase Modification for Selectivity Manipulation in Micellar Electrokinetic Chromatography
Shigeru Terabe (1), Yosuke Miyashita (1), Osamu Shibata (1), Hiroyuki Nishi (2), Tsukasa Fukuyama (2) and Masaaki Matsuo (2), (1) Kyoto University, Kyoto and (2) Tanabe Seiyaku Co. Ltd., Osaka, JAPAN

The Use of Capillary Electrophoresis Coupled to Fluorescence Microscopy for Determination of Brain Constituents
Juan P. Advis (1) and Norberto Guzman (2), (1) Rutgers University, New Brunswick, NJ and (2) Princeton Biochemicals, Inc., Princeton, NJ USA

Thermal Effects in Capillary Zone Electrophoresis
John H. Knox and Kathleen A. McCormack, University of Edinburgh, Edinburgh, Scotland UK

Capillary Zone Electrophoresis of Glycoconjugates
Ziad El Rassi and Wassim Nashabeh, Oklahoma State University, Stillwater, OK USA

Lecture Session 14: Fundamentals of Capillary Electrophoresis, Continued

Practical Aspects of Capillary Electrophoresis of Peptides and Proteins
Dean E. McNulty, Kathryn L. Stone, and Kenneth R. Williams, Yale University School of Medicine, New Haven, CT USA

Lecture Session 15: Sample Handling

Various Aspects of Sample Handling Techniques for HPLC*
Udo A. Th. Brinkman and H. Lingeman, Free University, Amsterdam, THE NETHERLANDS

New Polymeric Activated Reagents for the Derivatization of Nucleophiles in HPLC-UV/FL*
Ira S. Krull, Andre J. Bourque, Dieter Schmalzing, Chun-Xin Gao, Asa Trogen and Mike Szulc, Northeastern University, Boston, MA USA

In Vivo Microdialysis Sampling Coupled to Liquid Chromatography for Pharmokinetic Studies
Dennis O. Scott, Lori R. Sorenson, Karla L. Steele, Deanna L. Puckett, Ada M. Herrara and Craig E. Lunte, University of Kansas, Lawrence, KS USA

Peptide Recognition in RPLC with Post-Column Reaction and Photodiode Array Detection by Second-Derivative UV-Difference Spectroscopy
Anthony F. Fell (1), John B. Castledine (1), Bertil Sellberg (2), Rolf Modin (2) and Robert Weinberger (3), (1) University of Bradford, Bradford, UK, (2) Pharmacia Leo Therapeutics, Uppsala, SWEDEN and (3) ABI, Ramsey, NJ USA

The Use of Pre- and Post-column Techniques in the Development of Automated HPLC Methods for the Quantitative and Sensitive Analysis of Biogenic Amines
Stephan V. Rose and Jim P. Crombeen, Chrompack International, Middelburg, THE NETHERLANDS

11:00am - 1:00pm, Wednesday, May 23, 1990

Lecture Session 16: LC and LC/MS of Polypeptides

The Use of Reversed Phase HPLC to Characterize Norleucine Containing Variants*
W. S. Hancock, E. Canova-Davis, J. Battersby, R. Harris, R. Keck and B. Keyt, Genentech, South San Francisco, CA USA

Desorption and Spray Ionization Interferences for LC/MS: Current Applications and Future Directions*
Richard Caprioli, University of Texas Medical School, Houston, TX USA

Liquid Chromatography/Time-of-Flight Mass Spectrometry and Its Application to Peptide Analyses
Richard C. Simpson (1), W. Bart Emary (2), Ihor Lys (2) Robert J. Cotter (2) and Catherine C. Fenselau (1), (1) University of Maryland, Baltimore MD USA and (2) Johns Hopkins University School of Medicine, Baltimore, MD USA

HPDC-MS: Chromatography for the Masses
John Frenz, James H. Bourell and William S. Hancock, Genentech, Inc., South San Francisco, CA USA

Rapid HPLC of Proteins and Peptides with Coupled Columns
Krishna Kalghati, Tetsuro Ogawa and Csaba Horváth, Yale University, New Haven, CT USA

8:30 - 10:30am, Thursday, May 24, 1990

Lecture Session 17: LC/CE

Recent Developments in Chemically Synthesized Stationary Phases*
G. Schomburg, Max Planck Insitute, Mlheim-Ruhr, FDR

Pressure and Electrically Driven Open Tubular Liquid Chromatography: An Assessment and Comparison*
H. Poppe, J.C. Kraak, P.P.H. Tock and G.J.M. Bruin, University of Amsterdam, Amsterdam, THE NETHERLANDS

Electrophoresis and Electrodialysis as Methods for the Automated Work Up of Biological Samples
K.-P. Hupe (1), A.J. Debets (2) and W.Th. Kok (2), (1) Hewlett-Packard GmbH, Waldbronn, FDR, (2) Free University, Amsterdam, THE NETHERLANDS

Comparison of the Separation of the Components of Fetal Calf Serum by Traditional and Capillary Electrophoresis
Barbara B. Vanorman, Vivian L. Steel, Elaine M. Merisko, Ronald P. Lirette, Gary G. Liverside, and Gregory L. McIntire, Sterling Drug, Great Valley, PA USA

Separation of Ionizable Solutes by Micellar Electrokinetic Capillary Chromatography (MECC) and Micellar Liquid Chromatography
Joost K. Strasters, Andrew H. Rodgers and Morteza G. Khaledi, North Carolina State University, Raleigh, NC USA

Lecture Session 18: Novel Packing Materials II

Polystyrene-Polyoxethylene Craft Copolymers, Chameleon Type Supports for HPLC*
Ernst Bayer and Wollgang Rapp, University of Tübingen, Tübingen, FDR

Porous Titania and Zirconia as Packing Materials in HPLC*
U. Trüdinger, G. Müller and K. Unger, Johannes Gütenberg University, Mainz, FDR

New Ion-Exchangers for the Chromatography of Biopolymers
Werner Müller and Christa Jansen, E. Merck, Darmstadt, FDR

New Inorganic Non-Porous Ion Exchangers for Protein Analysis and Purification
Joseph P. Barone, Donna M. Dion, Edward R. Grover, Raymond B. Hanselman and Dorothy J. Phillips, Waters Division of Millipore Corp., Milford, MA USA

Monosized Stationary Phases for Chromatography
Turid Ellingsen and Oddvar Aune, SINTEF, Trondheim, Norway

8:30 - 10:30am, Thursday, May 24, 1990

Lecture Session 19: Polymer/Biopolymer Characterization

New Polymer Characterization Capabilities in Separation Science*
Howard G. Barth and Wallace W. Yau, E.I. duPont de Nemours & Co., Wilmington, DE USA

Hypercross-Linked Polystyrene-Type Phases for Liquid Chromatography*
Vadim A. Davankov, Maria P. Tsjurupa and Alexander A. Kurganov, USSR Academy of Sciences, Moscow, USSR

Pitfalls in Molecular Weight Determination by SEC
Paul L. Dubin, Indiana University-Purdue University at Indianapolis, Indianapolis, IN USA

Determination of Ligand-Protein Binding Parameters by Means of a Modified Hummel-Dreyer HPLC Method Using Internal Surface Reversed Phase (ISRP) Columns
Thomas C. Pinkerton and Kenneth A. Koeplinger,
The Upjohn Company, Kalamazoo, MI USA

On-line Molecular Weight Detection for Protein Chromatography
Paul Claes (1), Paul Griew (1), Penny Vardy (1), Sue Fowell (1), Michael A. Boss (2) and Andrew Kenney (1),
(1) Oros Instruments, Slough, UK and (2) Oros Instruments, Cambridge, MA USA

10:45am - 12:15pm

Poster Sessions: Clinical Analysis I
HPLC of Biopolymers I
Analysis of Drugs I

1:15 - 2:45pm

Poster Sessions: Clinical Analysis II
HPLC of Biopolymers II
Analysis of Drugs II

3:00 - 5:00pm, Thursday, May 24, 1990

Lecture Session 20: Trace Analysis

Measurement of DNA Adducts by Mass Spectrometry*
Roger W. Giese and Paul Vouros, Northeastern University, Boston, MA USA

Amino Acids, Peptides and Proteins: Practical Pursuits at Picomole Levels*
Daniel Strydom, Brigham and Women's Hospital, Boston, MA USA

Amino Acid Analysis of Proteins at the Femtomole Level
Mary D. Oates and James J. Jorgenson, University of North Carolina, Chapel Hill, NC USA

Electrochemical Detection of Peptides and Their Cyanobenz[f]isoindole Derivatives
Mark A. Nussbaum (1), Christopher M. Riley (2) and Susan M. Lunte (2),
(1) Ripon College, Ripon, WI and (2) University of Kansas, Lawrence, KS USA

Trace Enrichment by Displacement Chromatography
R.S. Ramsey (1), A.M. Katti (2) and G. Guiochon (1),
(1) Oak Ridge National Laboratory, Oak Ridge, TN and (2) University of Tennessee, Knoxville, TN USA

3:00 - 5:00pm, Thursday, May 24, 1990

Lecture Session 21: HPLC of Biopolymers II

HPLC Thiophilic Adsorbents for Purification of Antibodies and Their Application in Immunoaffinity Chromatography*
Meir Wilchek, Bernhard Nopper, Edward A. Bayer and Fortune Kohen, The Weizmann Institute of Science, Rehovot, ISRAEL

Functional Flexibility and Protein Chromatography*
Shmuel Shaltiel, The Weizmann Institute of Science, Rehovot, ISRAEL

Adsorption Behavior of Human Growth Hormone (hGH) and N-Methionine hGH in Reversed Phase Chromatography
Peter Oroszlan (1), Glen Teshima (2), Shiaw-Lin Wu (2), William S. Hancock (2) and Barry L. Karger (1), (1) Northeastern University, Boston, MA and (2) Genentech, South San Francisco, CA USA

Visualization of Proteins on Surfaces of Macroporous Chromatographic Resins
Edward Firouztale (1), Christine Beaty(1), Gerry M. Von Blohn (1) and Jarrett Burton (2), (1) Rohm and Haas Company, Spring House, PA USA, (2) Lehigh University, Bethlehem, PA USA

A Chromatographic Determination of the Relative Free Energies of Amino Acid Sidechain-Sidechain Interactions Using an Aqueous Mobile Phase and Their Applicability to the Protein Unfolding Problem
Thomas C. Pochapsky and Quinton Gopen, Brandeis University, Waltham, MA USA

Lecture Session 22: Novel Applications of HPLC

HPLC of Catechol-Related Compounds in a Clinical Diagnostic Laboratory and in Therapeutic Monitoring
Claudio Lucarelli, Istituto Superiore di Sanità, Rome, ITALY

Trace Analysis of Aliphatic Amines in the Environment Using Derivatization-Sorption Techniques
Jaroslav Churácek, Helena Pechová, Karel Ventura and Pavel Jandera, University of Chemical Technology, Pardubice, CZECHOSLOVAKIA

Reversed Phase HPLC of Erythromycin Using Chemiluminescence and UV-VIS Detection
Neil D. Danielson (1), Li He (1), Letithia Trelli (1) and Dana K. Morgan (2), (1) Miami University, Oxford, OH USA, (2) Abbott Laboratories, North Chicago, IL USA

HPLC Method Development for Pharmacokinetic and Metabolic Studies of GYKI-14766 Using Pre- or Post-Column Derivatization
Gábor Szepesi and István Valkó, Institute for Drug Research, Budapest, HUNGARY

On-Line HPLC-NMR Coupling
Klaus Albert and Ernst Bayer, University of Tübingen, Tübingen, FDR

Multiple Peak Formation and Interconversion in the Reversed-Phase Liquid Chromatography of Triostin A and Its Analogs
Thomas V. Alfredson (1), August H. Maki (1), Marye E. Adaskaveg (2), Jean-Louis Excoffier (2) and Michael J. Waring (3) , (1) University of California, Davis, CA USA, (2) Varian Associates, Walnut Creek, CA USA, (3) Cambridge University, Cambridge, UK

5:15 - 6:00pm

Discussion Session: HPLC of Biopolymers

8:30 - 10:00am, Friday, May 25, 1990

Lecture Session 23 (Plenary)

Rapid HPLC of Biological Macromolecules: Means and Applications
Csaba Horváth, Krishna Kalghatgi, Judit Horváth and Firoz Antia, Yale University, New Haven, CT USA

Applications of HPCE in the Biological Sciences
B. L. Karger, A. S. Cohen, D. N. Heiger, R. S. Rush, J. Y. Chen and D. Najarian, Northeastern University, Boston, MA USA

10:00 Coffee

10:30am - 12:00pm

Lecture Session 24 (Plenary)

Combinations of Chromatography and Electrophoresis with Mass Spectrometry
Richard D. Smith, Harold R. Udseth, Charles G. Edmonds, Charles J. Barinaga and Joseph A. Loo, Pacific Northwest Laboratories, Richland, WA USA

The Role of Temperature in High-Speed HPLC
Fritz Erni, Sandoz, Basel, SWITZERLAND

12:00 - 12:10pm

Invitation to HPLC '91
Fritz Erni, Sandoz, Basel, SWITZERLAND

12:10pm

Close of Symposium
Georges Guiochon, University of Tennessee, Knoxville, TN USA

POSTER SESSIONS Monday, May 21, 10:45 - 12:15

Fundamentals

The Effect of Concentration of Mobile Phase Components on the Retention and Areas of System Peaks and Solutes Peaks
Naomi Ben-Dom and Eli Grushka, The Hebrew University, Jerusalem ISRAEL

On the Origin of 'Eddy-Dispersion' in Packed Beds
Alexander L. Berdichevsky and Uwe D. Neue, Waters Division of Millipore Corp., Milford, MA USA

Effect of Intermolecular Interactions Between Functional Groups on Retention Prediction in RP-HPLC
Roger M. Smith, Christina M. Burr, and Wang Rui, Loughborough University of Technology, Loughborough, Leicestershire UK

Separation of Acidic Compounds Using Controlled Ionization Reversed-Phase HPLC
Gary T. Marshall and David J. Hometchko, Interaction Chemicals Inc., Mountain View, CA USA

Thermodynamic Comparison of Methanol + Water and Acetonitrile + Water Mobile Phases in Reversed-Phase Liquid Chromatography
Daniel E. Martire, Aurelio Alvarez-Zepeda and Bhajendra N. Barman, Georgetown University, Washington, DC USA

Plate Height Theory for Compressible Mobile Phase Fluids and Its Application to Gas Chromatography, Liquid Chromatography, and Supercritical Fluid Chromatography
Daniel P. Poe (1) and Daniel E. Martire (2), (1) University of Minnesota, Duluth, MN and (2) Georgetown University, Washington, DC USA

POSTER SESSIONS Monday, May 21, 10:45 - 12:15

Fundamentals, Continued

Theory for Secondary Chemical Gradient Elution in Liquid Chromatography
Joe P. Foley, Louisiana State University, Baton Rouge, LA USA

Application of Tri-Modal Solvent Gradients in Reversed-Phase HPLC for the Pseudo Two-Dimensional Resolution of Different Functional Classes of Components in Complex Samples
Edward L. Little and Joe P. Foley, Louisiana State University, Baton Rouge, LA USA

The Retention Behavior of Homologous Solutes in Reversed-Phase Liquid Chromatography
Lane C. Sander and Stephen A. Wise, Center for Analytical Chemistry, National Institute of Standards and Technology, Gaithersburg, MD USA

Measurement of Limiting Ionic Equivalent Conductance by Single-Column Ion Chromatography
Shan Zhong Lian (1), Xiao He (2) and Zhu Pengling (2), (1) Gansu Institute of Environmental Protection, Lanzhou, (2) Lanzhou University, Lanzhou, CHINA

Computer Simulation of the Bonded Phase-Solvent-Solute System in HPLC
Mark R. Schure, Rohm and Haas Company, Spring House, PA USA

Liquid-Solid Chromatography and Adsorption from Solutions of Polychloroprenes
Yuri A. Eltekov, USSR Academy of Sciences, Moscow, USSR

Theoretical and Practical Aspects of Multiphase Columns for Liquid and Ion Chromatography
Rosanne W. Slingsby and Maria Rey, Dionex Corporation, Sunnyvale, CA USA

Multiple Species Model for Prediction of Retention Using Amino-Acid Eluents in Ion-Chromatography
Péter Hajós, Miklós Magyari and János Inczédy, University of Veszprém, Veszprém, HUNGARY

POSTER SESSIONS Monday, May 21, 10:45 - 12:15

Modern Theory of the Liquid Chromatography of Polymers
Alexander Skvortsov (1) and Alexey Gorbunov (2), (1) Chemical-Pharmaceutical Institute, Leningrad and (2) All-Union Research Institute for Highly Pure Biopreparations, Leningrad, USSR

Anomalous Bandspreading of Ethlyenediaminetetraacetato-Chromium (III) Ion in Reversed-Phase Liquid Chromatography
John H. Knox and Masami Shibukawa, University of Edinburgh, Edinburgh, UK

System Peaks: An Important Tool for Studying the Solvation Layer in Reversed Phase Liquid Chromatography
David M. Bliesner and Karen B. Sentell, University of Vermont, Burlington, VT USA

Microcolumn Liquid Chromatography

Novel Liquid-Like Ion-Exchange Stationary Phases for Open Tubular Column LC
Anne B. Buskhe, Karin M. Göhlin and Marita Larsson, Chalmers University of Technology and University of Göteborg, Göteborg, SWEDEN

Evaluating the Experimental Performance of Open Tubular and Packed Capillary Columns in Liquid Chromatography
Daniel M. Dohmeier and James J. Jorgenson, University of North Carolina, Chapel Hill, NC USA

Analysis of Single Adrenal Cells by Reversed Phase Micropacked Liquid Chromatography
Bruce R. Cooper, David J. Leszczyszyn, Jeffrey A. Jankowski, R. Mark Wightman and James J. Jorgenson, University of North Carolina, Chapel Hill, NC USA

Gradient Microbore LC Biochromatography
Joe Tehrani and Karen J. Potter, Isco, Inc., Lincoln, NE USA

POSTER SESSIONS Monday, May 21, 10:45 - 12:15

Optimization

Computer Simulations of Reversed-Phase Gradient Elution Separations: an Experimental Study of the Predictive Accuracy of Retention Times and Bandwidths for OPA-Derivatized Amino Acids
Diana D. Lisi (1), James D. Stuart (1) and Lloyd R. Snyder (2), (1) University of Connecticut, Storrs, CT and (2) LC Resources, Orinda, CA USA

The Application of Multivariate Analysis Techniques to Derive Quantitative Relationships in Micellar Liquid Chromatography
Emelita D. Breyer, Joost K. Strasters and Morteza G. Khaledi, North Carolina State University, Raleigh, NC USA

Micellar Liquid Chromatography of Zwitterionic Compounds: Retention Behavior and Optimization Strategies
Andrew H. Rodgers, Joost K. Strasters, Sang Te Kim and Morteza G. Khaledi, North Carolina State University, Raleigh, NC USA

The Use of Computer Aided Optimization in Practical Samples
A.C.J.H. Drouen, Hewlett-Packard GmbH, Waldbronn, FDR

A Flexible Approach to Peak Tracking Based on Relative Retention, Band Areas and Supplemental Information
John W. Dolan and Lloyd R. Snyder, LC Resources, Lafayette CA USA

Characterization of Stationary Phases for HPLC by Means of Chemometric Methods
S.J. Schmitz and H. Engelhardt, Universität des Saarlandes, Saarbrücken, FDR

Ruggedness Testing of Liquid Chromatographic Methods: Using a Linear Retention Model to Predict Retention in Non-Linear Systems
John W. Dolan, Lloyd R. Snyder and Dana C. Lommen, LC Resources, Lafayette, CA USA

Computer-Assisted Internal Standard Selection (CAISS) for Reversed-Phase Liquid Chromatography
Norman E. Skelly, Stephen W. Barr and Anthony P. Zelinko, The Dow Chemical Co., Midland, MI USA

POSTER SESSIONS Monday, May 21, 10:45 - 12:15

Preparative Scale Separations

Large Scale Purification of Haptenated Oligonucleotides Using HPLC
Joseph E. Celebuski and Ronald L. Morgan, Abbott Laboratories, Abbott Park, IL USA

Applications of Preparative HPLC in Fat Chemistry Research
Andreas Bruns, Henkel Research Corporation, Santa Rosa, CA USA

Novel Displacer Compounds for the Displacement Chromatography of Biopolymers
Guhan Jayaraman, Guhan Subramanian and Steven M. Cramer, Rensselaer Polytechnic Institute, Troy, NY USA

Porous Polymeric Packings for the Large Scale Purification of Peptides, Proteins, and Antibiotics
Peter G. Cartier (1), Karl C. Deissler (1), John J. Maikner (1) and Warren G. Schwartz (2), (1) Rohm and Haas Research Laboratory, Spring House, PA and (2) TosoHaas Technical Center, Woburn, MA USA

Application of Biocryl Bioprocessing Aids in the Processing and Purification of Proteins Prior to Chromatography
Kathleen Fletcher, Peter G. Cartier, Sheryl G. Foxall, John J. Maikner and Michael J. Byers, Rohm and Haas Research Laboratories, Spring House, PA USA

A Practical Study of the Influence of Column Efficiency Upon the Width of Overlapping Displacement Zones in Preparative Elution Chromatography
Geoffrey B. Cox, Prochrom, Indianapolis, IN USA

Large Scale High Performance Liquid Chromatography with Polymer-Based Packings in Axially Compressed Column Systems
Geoffrey B. Cox and Harlene I. Marks, Prochrom, Indianapolis, IN USA

Displacement Chromatography Using Non-Ideal Distribution Isotherms
P.K. de Bokx and H.P. Urbach, Philips Research Laboratories, Eindhoven, THE NETHERLANDS

POSTER SESSIONS Monday, May 21, 10:45 - 12:15

Preparative Scale Separations, Continued

Separation of a Synthetic Peptide Fragment Using High Resolution 5 Micron Reversed Phase Packings
Patricia Young (1), Thomas Wheat (1), Jenny Grant (1) and Thomas Kearney (2), (1) Waters Division of Millipore Corp., Milford, MA (2) MilliGen Biosearch, Millipore Corp., Burlington, MA USA

Comparison of Experimental and Theoretical Band Profiles in Overloaded Elution Chromatography
S. Golshan-Shirazi, S. Jacobsen and G. Guiochon, University of Tennessee, Knoxville, TN, and Oak Ridge National Laboratory, Oak Ridge, TN USA

Optimization of Methods Development and Scale-Up for Peptide Purification by Reversed Phase Chromatography
Richard F. Meyer and Robert S. Cooley, YMC Inc., Morris Plains, NJ USA

Displacement HPLC of a Biosynthetic Protein Product
Jana M. Jacobson, BioWest Research, Brisbane, CA USA

Simulation of Nonlinear Chromatographic Techniques Using Different Multicomponent Isotherms
Firoz D. Antia and Csaba Horváth, Yale University, New Haven, CT USA

Preparative Reversed-Phase Sample Displacement Chromatography of Peptides: Effects of Sample Load, Sample Volume, Flow-Rate and Run Time
Colin T. Mant, T.W. Lorne Burke and Robert S. Hodges, University of Alberta, Edmonton, Alberta CANADA

Supercritical Fluid Chromatography

Retention of Cyano-Bonded Silica Columns in SFC and a Comparison with Normal Phase HPLC
Roger M. Smith and Simon Cocks, Loughborough University of Technology, Loughborough, Leicestershire UK

POSTER SESSIONS Monday, May 21, 10:45 - 12:15

Analysis of Pharmaceutical Compounds via Modified Supercritical Fluid Chromatography
Paul Zimmerman (1) and Mehdi Ashraf-Khorassani (2), (1) University of Pittsburgh, Pittsburgh, PA and (2) Suprex Corp., Pittsburgh, PA USA

A Study of Formic Acid Modified Carbon Dioxide Mobile Phase in Capillary Supercritical Fluid Chromatography
Jeffrey A. Crow and Joe P. Foley, Louisiana State University, Baton Rouge, LA USA

Investigation of Explosive Materials Using Liquid Chromatography and Supercritical Fluid Chromatography
Bruce R. McCord (1) and Frederic W. Whitehurst (2), (1) FBI Academy, Forensic Science Research and Training Center, Quantico, VA and (2) FBI, JEH Building, Washington, DC USA

Supercritical Fluid Extraction and Chromatography of Polar Compounds
Sam F.Y. Li, Hian K. Lee and Chye P. Ong, National University of Singapore, Republic of SINGAPORE

POSTER SESSIONS Monday, May 21, 1:15 - 2:45

Chiral Separations I

Displacement-Chromatographic Separation of Alpha-Alkyl-Arylcarboxylic Acid Enantiomers on Cyclodextrin-Silica Columns
Gy. Farkas, G. Quintero, A. Bartha and Gy. Vigh, Texas A&M University, College Station, TX USA

Adsorption Isotherm Studies on Pirkle-Type Chiral Stationary Phases
P. Camacho, E. Geiger, L. Zimmermann, Gy. Farkas, A. Bartha and Gy. Vigh, Texas A&M University, College Station, TX USA

On-Line HPLC System for the Stereoselective Determination of Free Drug Concentration in Protein Binding Equilibrium by Direct Sample Injection
Akimasa Shibukawa, Miwa Nagao and Terumichi Nakagawa, Kyoto University, Kyoto JAPAN

POSTER SESSIONS Monday, May 21, 1:15 - 2:45

Chiral Separations I, Continued

Attempts to Recognize and Determine the Structures of Stereoisomers Using HPLC
Ken Hosoya (1), Kazugiro Kimata (1), Nobuo Tanaka (1), Takeo Araki (1), Riyou Tsuboi (2), Kunihiko Takabe (3) and Hidemi Yoda (3), (1) Kyoto Institute of Technology, Kyoto, (2) Nacalai Tesque, Mucoh, and (3) Sizuoka University, Hamamatsu JAPAN

Separation and Study of Corrinoid Stereoisomers Using HPLC
Susan H. Ford, Alva Nichols, and Jean M. Gallery, Chicago State University, Chicago, IL USA

Development and Performance of Covalently Immobilized Protein Stationary Phases for Enantiomeric Separation
Sunanda R. Narayanan, Sunil V. Kakodkar, and Laura J. Crane, J.T. Baker Inc., Phillipsburg, NJ USA

Synthesis and Evaluation of a Chiral Urea Bonded Phase Based on PEI-Silica
Sunil V. Kadokar and Morris Zief, J.T. Baker Inc., Phillipsburg, NJ USA

Measurement of Norephedrine Enantiomers in Serum Using Coupled Achiral/Chiral HPLC
Irving W. Wainer and Ya-Qin Chu, St. Jude Children's Research Hospital, Memphis, TN USA

Measurement of Leucovorin Diastereomers in Serum Using Coupled Chiral/Achiral HPLC
Irving W. Wainer, Philippe Jadaud, Connie Young and Lin Silan, St. Jude Children's Research Hospital, Memphis, TN USA

Chiral Stationary Phase HPLC Separation and Racemization Kinetics of Enantiomeric 3-Hydroxy-1,4-Benzodiazepines
Xiang-Lin Lu and Shen K. Yang, Uniformed Services University of the Health Sciences, Bethesda, MD USA

The Separation of Drug Enantiomers using Automated Pre-Column Derivatization with New Chiral Reagents
Heinz Goetz, Hewlett-Packard GmbH, Waldbronn, FDR

POSTER SESSIONS Monday, May 21, 1:15 - 2:45

Liquid Chromatographic Separation of Isomeric Phenanthrols on Monomeric and Polymeric C18 Columns
Shen K. Yang and Zi-Ping Bao, Uniformed Services University of the Health Sciences, Bethesda, MD USA

Separation of the Enantiomers of β-Receptor Blocking Agents and Other Cationic Drugs Using the CHIRAL-AGP Column
Märit Enquist (1) and Jörgen Hermansson (2), (1) Apoteksbolaget AB, Stockholm SWEDEN and (2) ChromTech AB, Norsborg SWEDEN

Determination of Saterinone Enantiomers in Plasma Samples by HPLC with an Internal Standard Using a Chiralcel OD Column, Fractionation and Reversed-Phase Chromatography
Martin Rudolph, Beiersdorf AG, Hamburg FDR

Enantiomeric Resolution of Pharmaceutical Amines and Aminoalcohols on HPLC Chiral Stationary Phases, via Facile Formation of Dinitrophenyl Urea Derivatives
Thomas D. Doyle, Charlotte A. Brunner and William M. Adams, Food and Drug Administration, Center for Drug Evaluation and Research, Washington, DC USA

A Chiral Recognition Mechanism for the LC Resolution of N-(3,5-dinitrobenzoyl-aminoacid Methyl Esters Upon a Chiral Phase Derived from (-)-(2-phenylcarbamoyloxy)propionic Acid
C. Rosini, D. Pini, G. Uccello-Barretta and P. Salvadori, CNR-C.S., University of Pisa, Pisa, ITALY

Ring-Substituted Phenyl Isocyanates as Derivatizing Agents for the Resolution of Chiral Amines of Pharmaceutical Interest on the Pirkle Covalent Phenylglycine (DNBPG) Column
Elfriede Nusser and Joseph Gal, Colorado University School of Medicine, Denver, CO USA

HPLC Applications: General

Development and Use of a Stability-Indicating HPLC Assay for Meldrum's Acid
Joseph R. Kaczvinsky and Scott A. Read, The Proctor & Gamble Co., Cincinnati, OH USA

POSTER SESSIONS Monday, May 21, 1:15 - 2:45

HPLC Applications: General, Continued

Determination of Caffeine and Polyphenol Compounds in Coffee Pulp by HPLC
M. Laure Hannibal, Ernesto Favela, and Irina Gerlal, Universidad Autonoma Metropolitano, Iztapalapa, MEXICO, and Institut Francais de Recherche pour le Developpement en Cooperation, Paris, FRANCE

The Analysis of Petroleum Products by HPLC Group Type Analysis and by Multidimensional Chromatography, Combining HPLC with High Resolution Capillary GC
Stephan V. Rose and Jaap de Zeeuw, Chrompack Int. B.V., Middelburg, THE NETHERLANDS

Rapid Semi-Preparative HPLC and TLC Separation and Determination of *Oxytropis ochrocephala (Bunge)*
Li Ping (1), Yu Weile (1), Lu Yinghua (2), and Gao Nu (2), (1) Lanzhou Institute of Chemical Physics, Academia Sinica, Lanzhou, CHINA, (2) East China Normal University, Shanghai, CHINA

High Pressure Liquid Chromatographic Detection of Hydroxyl Radical in Heart
Anna George, Parinum S. Rao, and Dipak K. Das, University of Connecticut School of Medicine, Surgical Research Center, Farmington, CT and Long Island Jewish Medical Center, New Hyde Park, NY USA

Quantification of Rat Myocardial Prostaglandin and Thromboxane by High Performance Liquid Chromatography (HPLC) Using Fluorimetric Detection
Gerald A. Cordis and Dipak K. Das, University of Connecticut School of Medicine, Farmington, CT USA

Qualitative and Quantitative Determination of Carbohydrate Sweeteners and Organic Acids from the Human Oral Cavity Using High Performance Liquid Chromatography
Harold A.B. Linke and Stephen J. Moss, New York University Dental Center, New York, NY USA

Non-Aqueous Ion-Exclusion Column Applications
David W. Togami, Interaction Chemicals Inc., Mountain View, CA USA

POSTER SESSIONS Monday, May 21, 1:15 - 2:45

The On-Line Analysis of Dissolution Samples Using Liquid Chromatography
Kurt Lampart (1) and Menno van den Oever (2), (1) Anapharm Instruments, Thun, SWITZERLAND and (2) Anapharm Instruments, Bound Brook, NJ USA

Evaluation of Chemical Structural Heterogeneity of Cationic Acrylamide Copolymers Using High-Performance Liquid Chromatography
Shyhchang S. Huang, Hercules Research Center, Wilmington, DE USA

Separation of 3-Substituted 2-(1H) Pyridones on Silica Gel Using a Tetramethyl-ammonium Hydroxide Modified Mobile Phase
David A. Nelson and Stephen C. Preece, University of Wyoming, Laramie, WY USA

Separation of Cationic Aluminum Chlorhydrate Polymers by Gel Filtration
Nelson P. Ayala, Westwood Chemical Company, Middletown, NY USA

Comparison of Anion Exchanger Capacity for Low and High Molecular Weight Anions
D. Bentrop and H. Engelhardt, Universität des Saarlandes, Saarbrücken, FDR

Determination of Inorganic Anions by Flow Injection Analysis and Ion Chromatography
G. Schöndorf and H. Engelhardt, Universität des Saarlandes, Saarbrücken, FDR

Determination of Hydroquinone and Some of Its Ethers in Cosmetic Cream by Reversed-Phase HPLC
Ber-Lin Chang, Chun-Sheng Chien, Ching-Yih Shaw and Ming-Hsin Lee, Division of Drug Chemistry, National Laboratories of Foods and Drugs, Department of Health, Taipei, Taiwan ROC

Characterization of Dextran Sulfate by High Performance Aqueous Gel Filtration Chromatography (GFC) and Gel Electrophoresis
Gene F. Ray, Dan R. Soderberg and Evelyn A. Murrill, Midwest Research Institute, Kansas City, MO USA

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HPLC Applications: General, Continued

The GPC Analysis of Starches with Alkaline Eluents
T. Suortti and E. Pessa, Technical Research Centre of Finland, FINLAND

High Performance Liquid Chromatographic Determination of Lactose in Lactose-Reduced Milk
David B. Pautler, Hewlett-Packard Company, Valley Forge, PA USA

Characterization of Some Surfactants Used in Building Materials by Chromatographic Techniques
Jai P. Gupta, Lindsay A. Cupitt and Jeanette Knowles, Fosroc Division of Foseco International Ltd., Nechells, Birmingham UK

High Temperature Liquid Chromatography of Polyethylene
Yuri A. Eltekov and Tatyana A. Romanova, USSR Academy of Sciences, Moscow, USSR

Resolution in GPC of Polymers and Proteins
Alexey Gorbunov (1) and Alexander Skvortsov (2), (1) All-Union Research Institute for Highly Pure Biopreparations, Leningrad, USSR and (2) Chemical-Pharmaceutical Institute, Leningrad, USSR

Retention Behaviour of Metal (II, III) Ethylenediaminetetraacetate Complexes in Reversed-Phase Ion-Pair Chromatography
Masami Shibukawa, University of Edinburgh, Edinburgh, UK and St. Marianna University, Kawasaki, JAPAN

Advances in Detection

Modern HPLC Approaches Coupled to Low Angle Laser Light Scattering Detection for Biopolymer Molecular Weight Determination
Hans H. Stuting and Ira S. Krull, Notheastern University, Boston, MA USA

Comparison of Performance Characteristics of Commercial HPLC Instrumentation for On-Line Micro-LC-Continuous Flow FAB-MS of Biopolymers
John A. Bullock and Mark Olsen, Sterling Research Group, Malvern, PA USA

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Purity Assessment of Recombinant Tissue Plasminogen Activator (rt-PA) Using UV Spectra for the Quantitative Comparison of Tryptic Maps from Reference Material and Production Lots
Hans-Jurgen P. Sievert (1), Shiaw-Lin Wu (2), Rosanne Chloupek (2) and William S. Hancock (2), (1) Hewlett-Packard Company, Avondale, PA USA and (2) Genentech, South San Francisco, CA USA

Use of UV Spectral Libraries to Identify Tryptic Peptides in Different Chromatographic Systems
Cliff Woodward (1), Hans-Jurgen P. Sievert (1), Shiaw-Lin Wu (2), and William S. Hancock (2), (1) Hewlett-Packard Company, Avondale, PA USA and (2) Genentech, South San Francisco, CA USA

Optimization of Photodiode Array Detection Parameters for Qualitative and Quantitative HPLC Analyses
Michael E. Swartz, Thomas Wheat, and Isaac Neitring, Waters Division of Millipore Corp., Milford, MA USA

A New HPLC Separation System for the Detection of Inorganic and Organic Anions
Bernd Glatz, Mingbiao Yang, and Manfred Riedmann, Hewlett-Packard GmbH, Waldbronn, FDR

Indirect Detection Strategies in Free Amino Acid Separations
P. G. Simonson and D. J. Pietrzyk, University of Iowa, Iowa City, IA USA

The Use of LC/MS for the Determination of Guar Gum in Carrageenan Gum Blends
W. Jeffrey Hurst (1), Robert A. Martin, Jr. (1) and Robert Isensee (2), (1) Hershey Foods Corporation Technical Center, Hershey, PA USA, (2) Oneida Research Services, Whitesboro, NY USA

Response of Different UV-Vis Detectors in HPLC Measurements of the Absolute Number of Moles of an Analyte
G. Torsi, G. Chiavari, C. Laghi and A.M. Asmudsdottir, 'G. Ciamician', Bologna, ITALY

Precision of Trace Level Analysis
R.E. Pauls, L.N. Polite and R.W. McCoy, Amoco Research Center, Naperville, Il USA

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Advances in Detection, Continued

Mathematical Function for Peak Shape Characterization and the Detection of Co-Eluting Impurities Using Only Single-Channel Detection
Mark S. Jeansonne and Joe P. Foley, Louisiana State University, Baton Rouge, LA USA

Influence of the Retention Time and the Dead Volume on the Separation and Sensitivity by Multidimensional LC with Conventional and Micropacked Columns
Georgi S. Georgiev and Nicolay M. Nicolov, Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia BULGARIA

HPLC Analyses of Optically Active Compounds Using a New Optical Rotation Detector
Hiroshi Suzuki, Tsunemi Tokieda, Hiroko Watanabe, John D. MacFarlane and Soyao Moriguchi, JM Science Inc., Buffalo, NY USA

Indirect Detection in Reversed-Phase Liquid Chromatography: Retention and Response Studies in Systems with Several Retatined System Peaks
Jacques Crommen (1), Isabelle Bechet (1), Patrick Herne (1), Goran Schill (2) and Douglas Westerlund (2), (1) University of Liege, Liege, BELGIUM and (2) University of Uppsala, Uppsala, SWEDEN

A New Sensitive Multimode Electrochemical Detector for Organic Species with Weak UV Absorbances: Carboxylic, Phosphonic, and Sulfonic Acids, Carbohydrates, Alcohols, Amines and Amino Acids
Roy D. Rocklin, Rosanne W. Slingsby and Maria A. Rey, Dionex Corporation, Sunnyvale, CA USA

Amperometric Detection of Retinoids in Non-Aqueous Solvents by HPLC
P.D. Bryan (1), I.L. Honigberg (1) and N.M. Meltzer (2), (1) University of Georgia, Athens, GA USA and (2) Roche Dermatologicals, Nutley, NJ USA

POSTER SESSIONS Tuesday, May 22, 10:45 - 12:15

Chiral Separations II

Enantiomeric Resolution and Absolute Configuration of Triols and Triol-hydroxyethylthio-ethers Derived from Benzo(a)pyrene Diol-Epoxides by Chiral Stationary Phase HPLC
Henry B. Weems and Shen K. Yang, Uniformed Services University of the Health Sciences, Bethesda, MD USA

Separation of Enantiomers Using Chiral Modified Hypercarb
Eva Heldin and Curt Pettersson, Biomedical Center, Uppsala, SWEDEN

Structural Factors Affecting Chiral Recognition and Separation on Cellulose Based Chiral Stationary Phases
Hassan Y. Aboul-Enein and Rafiqul Islam, King Faisal Specialist Hospital and Research Center, Riyadh, SAUDI ARABIA

A High Performance Liquid Chromatographic Assay of Tocainide Enantiomers
Stewart R. Wirebaugh and Douglas R. Geraets, University of Iowa College of Pharmacy, Iowa City, IA USA

Stereochemical Resolutions on HPLC Chiral Stationary Phases Based Upon Proteins: the Effect of Mobile Phase Composition on Retention and Stereoselectivity
G. Felix (1), I. Leroux (1), C. Netter (2) and C. Dumas (2), (1) Laboratoire de Chimie Organométallique (CNRS URA35), Université de Bordeaux I, Talence Cedex, FRANCE, (2) SFCC, Neuilly-Plaisance, FRANCE

O-(3,5-dinitrobenzoyl)quinine: An Optically Active Selector for the Preparation of a New Chiral Stationary Phase
D. Pini, C. Rosini, P. Altemura and P. Salvadori, CNR-C.S., Pisa, ITALY

Chiral Chromatography in Monitoring the Biotransformation of 3-substituted 1,4-Benzodiazepin-2-ones
Enrico Domenici, Carlo Bertucci, and Piero Salvadori, CNR-C.S. and University of Pisa, Pisa, ITALY

HPLC Resolution of Optical Isomers: Investigation of Solute-CSP Interactions by Variable Temperature Chromatography
F. Gasparrini, D. Misiti and C. Villani, La Sapienza University, Rome, ITALY

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Chiral Separations II, Continued

Elution Orders in Enantiomer Separation by HPLC with Some Chiral Stationary Phases
Naobumi I, Hajimu Kitahara and Reiko Kira, Sumika Chemical Analysis Service, Osaka JAPAN

Preparative Chromatographic Separation of Enantiomers on Polymer/Silica Composites as Chiral Stationary Phases
Joachim N. Kinkel, F. Eisenbeis and K. Reichert, E. Merck, Darmstadt, FDR

Effect of Column Temperature on the Enantioselectivity of Chiral Stationary Phases in HPLC
S. Rauch-Puntigam (1), W. Lindner (1) and F. Erni (2), (1) Karl-Franzen University of Graz, Graz, AUSTRIA and (2) Sandoz, Basel, SWITZERLAND

HPLC Separation of the Three Stereoisomers of Diaminopimelic Acid in Hydrolyzed Bacterial Cells
M. Zanol and L. Gastaldo, Merrell Dow Research Institute, Gerenzano, ITALY

HPLC Separation of Radiopharmaceutical Ligand Enantiomers with a Gamma-Cyclodextrin Bonded Phase Column
J. Mark Green, Rosemarie Jones, D. Scott Edwards and Joseph L. Glajch, E.I. duPont de Nemours and Co., North Billerica, MA USA

Chiral Separation of Amino Acids on Porous Graphite Carbon with an Eluent Containing Copper Complex of L-Phenylalanine
John H. Knox and Qian Hong Wan, University of Edinburgh, Edinburgh, Scotland, UK

Enantioselective and non-Enantioselective Methods Enabling the Assay of the Components of l-Carnitine Family
Gloria Cardace, Giulia Bruno, Stefano Di Donato (2), Antonio Marzo, Edoardo Arrigoni Martelli, Sandra Muck, Camilla Corbelletta and Eleanor Bassani, (1) Sigma Tau SpA, Pomezia/Roma and (2) C. Besta Neurological Inst., Milano, ITALY

POSTER SESSIONS Tuesday, May 22, 10:45 - 12:15

Pre- and Post-Column Derivativization

N-(9-Acridinyl)-bromoacetamide - A Powerful Reagent for Phase-Transfer-Catalyzed Fluorescence Labelling of Carboxylic Acids for Liquid Chromatography
Stig G. Allenmark, Monika Chelminska-Bertilsson, and Richard A. Thompson, University of Gothenburg, Gothenburg, SWEDEN

Sensitive and Selective Fluorescence Detection of Carboxylic Acids by Liquid Chromatography with On-Line Ion-Pair Extraction
C. de Ruiter, W.A. Minnaard, H. Lingeman, E.M. Kirk and U.A.Th. Brinkmann, Free University, Amsterdam, THE NETHERLANDS

Pre-Column Derivatization of Biogenic Carboxylic Acids for Liquid Chromatography with Peroxyoxalate Chemiluminescence Detection
P.J.M. Kwakman, H. Koelewijn, U.A.Th. Brinkmann, and G.J. deJong, Free University, Amsterdam, THE NETHERLANDS

The Fluorogenic Derivatization of Primary and Secondary Amines: Evaluation of 2-Chloro-3-Isothiocyanopyridine as a Precolumn Reagent
Lisa L. McChesney and John F. Stobaugh, University of Kansas, Lawrence, KS USA

An Improved Liquid Chromatographic Method for the Determination of Eflornithine in Plasma Utilizing the Precolumn Fluorogenic Reagent Naphthlene-2,3-Dicarboxaldehyde/Cyanide
Mary F. Kilkenny, Milan Slavik, and John F. Stobaugh, University of Kansas, Lawrence, KS USA

Optimization of the Precolumn Derivatization of Amino Acids and Small Peptides with 2,3-Napthalene Dicarboxaldehyde/Cyanide (NDA/CN)
Martha A. Kral, John F. Smasal, and John F. Stobaugh, University of Kansas, Lawrence, KS USA

Quantitative Comparisons of Established Fluorogenic Primary Amine Reagents and Synthesis of Newer Analogs
Arun Thakur and John F. Stogaugh, University of Kansas, Lawrence, KS USA

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Pre- and Post-Column Derivativization, Continued

Peptide and Amino Acid Determination by Micro HPLC with a Two-Chemistry, Post-Column Reaction Method
Dennis C. Shelly and Yanxia Du, Stevens Institute of Technology, Hoboken, NJ USA

Oxazole-Based Fluorescent Tagging Reagents for the Analysis of Amines and Thiols Using High Performance Liquid Chromatography
Robert G. Carlson, Toshimasa Toyo'oka, Hitesh P. Chodshi, Richard S. Givens and Susan M. Lunte, University of Kansas, Lawrence, KS USA

Post Column Reaction for the Simultaneous Analysis of Chromatic and Leuco Forms of Malachite Green and Crystal Violet by HPLC with Colorimetric Detection
John L. Allen and Jeffery R. Meinertz, National Fisheries Research Center, La Crosse, WI USA

Zone-Electrophoretic Sample Treatment Valve for the Analysis of Biological Samples by High Performance Liquid Chromatography
A.J.J. Debets (1), K.-P. Hupe (1), W.Th. Kok (2) and U.A.Th. Brinkman (1), (1) Free University, Amsterdam, THE NETHERLANDS and (2) University of Amsterdam, Amsterdam, THE NETHERLANDS

Liquid Chromatographic Trace Enrichment and Clean-Up with On-Line Gas Chromatography
G.J. de Jong, J.J. Vreuls, W.J.G.M. Cuppen and U.A.Th. Brinkmann, Free University, Amsterdam, THE NETHERLANDS

Analysis of Derivatized Opioid Peptides Utilizing Coupled-Column Liquid Chromatography with Peroxyoxalate Chemiluminescence Detection
David A. Jencen, John F. Stobaugh, Christopher M. Riley, and Richard S. Givens, University of Kansas, Lawrence, KS USA

A New Polymeric Achiral Reagent Containing the 3,5-Dinitrophenyl Tag for Off-Line Derivatizations of Amines and Alcohols in HPLC-UV/EC
A.J. Bourque, C.-X. Gao, D. Schmalzing and I.S. Krull, Northeastern University, Boston, MA USA

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Determination of Amino Acids and Peptides by Reversed Phase Liquid Chromatography Using On-line, Post-Column Photolysis-Electrochemical Detection (HPLC-hv-EC)
Lin Dou and Ira S. Krull, Northeastern University, Boston, MA USA

Simultaneous Determination of Allopurinol and Oxypurinol Using Immobilized Xanthine Oxidase by HPLC with Electrochemical Detection
Eugene J. Eisenberg, Gary G. Liversidge and Kenneth C. Cundy, Sterling Research Group, Great Valley, PA USA

HPLC Methodology for the Analysis of Prolil and Hydroxyprolil-Peptides in Urine with Pre-Column Derivatization and Fluorometric Detection
Michael Codini (1), Carlo Alberto Palmerini (1), Claudio Lucarelli (2) and Ardesio Floridi (1), (1) Univ. Perugia, Perugia, and (2) Istituto Superiore di Sanità, Rome, ITALY

High-Resolution Analysis of Protein Amino Acids and Hydrolysis By-Products by the Use of HPLC and Automated Pre-Column Derivatization
Bernd Glatz, Herbert Godel and Petra Seitz, Hewlett-Packard GmbH, Waldbronn, FDR

Selective Trace Metal Analysis Using Single Step Chromatographic Preconcentration and ICP-AES
S. Nathakarnkitkool (1), P. Jandik (2) and G.K. Bonn (1), (1) University of Innsbruck, Innsbruck, AUSTRIA and (2) Waters Division of Millipore Corporation, Milford, MA USA

Derivatization Procedure Suitable for HPLC Analysis of Clenbuterol
Pier Antonio Biondi (1), Marco Montana (2), Francesco Manca (2) and Gianfranco Brambilla (3), Istituto Fisiol. Veterinaria e Biochim., Milano, (2) Jockey Club, Milano and (3) Istituto Zooprof. Lombardia ed Emilia, Brescia, ITALY

Automated Solid Phase Extraction and HPLC Injection of Theophylline from Serum
Brian G. Lightbody, Brian D. Holden and Julie Tomlinson, Zymark Corporation, Hopkinton, MA USA

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Pre- and Post-Column Derivativization, Continued

Postcolumn Indirect Fluorescence Detection of Fluoride
Michael J. Lovdahl and Donald J. Pietrzyk, University of Iowa, Iowa City, IA USA

The Determination of Phenylurea Metabolites Using HPLC with Post Column Photolysis and Fluorescence Detection
Ronald G. Luchtfeld, Food and Drug Admin., Kansas City, MO USA

Multielement Analysis by Reversed-Phase HPLC with Benzoylacetone Extraction
Mitsuru Yamazaki and Susumu Ichinoki, Hokuriku University, Kanazawa, JAPAN

Trace Analysis of Oligomeric Carbohydrates by HPLC and Post Column Derivatization
M. Krämer and H. Engelhardt, Universität des Saarlandes, Saarbrücken, FDR

HPLC of Oligonucleotides

Measuring the Effects of UV-B Radiation on DNA: Separations and Detection Methodology
Roswitha S. Ramsey, Chen-Huei Ho, and Robert J. Shine, Oak Ridge National Laboratory, Oak Ridge, TN USA

Application of High Performance Ion-Exchange Chromatography for Separation of Large DNA Restriction Fragments on a Nonporous Resin
Ann M. Stancavage and Terrence S. Reid, Supelco, Bellefonte, PA USA

Rapid High Performance Purification of Nucleic Acids
Bill Warren (1), George Vella (1), Kevin O'Connor (1), Theresa Dunne (2) and Edouard Bouvier (2), (1) Waters Division of Millipore Corp., Milford, MA USA (2) MilliGen Biosearch, Millipore Corp., Burlington, MA USA

High-Resolution Purification of Synthetic Oligonucleotides by Analytical and Macroscale Ion-Exchange Chromatography
J.R. Thayer and C.A. Pohl, Dionex Corporation, Sunnyvale, CA USA

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Capillary Electrophoresis: Applications

Polymer Coated Fused Silica Capillaries for MECC Separations of Organic and Organometallic COmpounds
J. A. Lux, H. Yin and G. Schomburg, Max-Planck-Institut, Mülheim, FDR

Capillary Electrophoretic Separations of Proteins at High pH Using a New Capillary Deactivation Method
Kelly A. Cobb, Vladislav Dolnik, and Milos Novotny, Indiana University, Bloomington, IN USA

The Evaluation of Capillary Zone Electrophoresis as a Potential Tool for the Separation of Closely-Related Peptides
Elizabeth A. Monnot-Chase, John F. Stobaugh, Susan M. Lunte, and Christopher M. Riley, University of Kansas, Lawrence, KS USA

The Reproducibility of Free Solution Capillary Electrophoresis
Bruce Black, Applied Biosystems, San Jose, CA USA

Comparison of the Resolving Power of Capillary Zone Electrophoresis, Micellar Electrokinetic Chromatography, and Micellar Liquid Chromatography: the Role of pH and/or Surfactant Concentration
Joe P. Foley, Louisiana State University, Baton Rouge, LA USA

Buffer and Temperature Gradients in Micellar, Electrokinetic, Capillary Electrophoresis
Scot R. Weinberger and Timothy D. Schlabach, Spectra-Physics Corp., San Jose, CA USA

A Comparison of Selectivity Control in Capillary Electrophoresis and Ion Exchange Chromatography
J.R. Thayer and A. Wainwright, Dionex Corporation, Sunnyvale, CA USA

An Evaluation of Soluble CD4 and Its Peptide Fragments by Capillary Electrophoresis
Sudhir Burman, Kaylan Annumula, I.Y. Huang, Betsy Hughes, Anthony Jurewicz and James J. L'Italien, SmithKline Beecham Pharmaceuticals, King of Prussia, PA USA

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Capillary Electrophoresis: Applications, Continued

Influence of Peptide Structure and Buffer Composition on Peptide Mobility in Capillary Electrophoresis
Richard Palmieri (1), Leslie Holladay (1), Jo-Lynne Boone (2) and Tom Lobl (2), (1) Beckman Instruments, Palo Alto, CA USA and (2) Immunotech Pharmaceuticals, San Diego, CA USA

Comparison of Different Methods of High Performance Capillary Eletrophoresis (HPCE) to Separate Whey Proteins
Alejandro Cifuentes, Mercedes de Frutos and Jose C. Diez-Masa, Institute of Organic Chemistry (C.S.I.C.), Madrid SPAIN

Approaches for Generating Linear and Shaped Gradients of Nanoliter Volumes for Future Micro-LC and Capillary Electrophoresis (CE) Methods
Vern Berry (1), Egkmont Rohwer (2), Victor Pretorius (2), (1) Salem State College, Salem, MA USA, (2) University of Pretoria, Pretoria, Republic of SOUTH AFRICA

Analysis of 6-Mercaptopurine and Nucleotide Metabolites by Liquid Chromatography and Capillary Electrophoresis with Laser-Induced Fluorescence
Shelley R. Rabel, Steve Soper, John F. Stobaugh, Robert Trueworthy and Christopher Riley, University of Kansas, Lawrence, KS USA

A Comparative Study for the Measurement of Solute-Micelle Binding Constants
Ali S. Kord, Joost K. Strasters and Morteza G. Khaledi, North Carolina State University, Raleigh, NC USA

Optical Resolution by Micellar Electrokinetic Chromatography
Koji Otsuka (1) and Shigeru Terabe (2), (1) Osaka Prefectural College of Technology, Osaka, (2) Faculty of Engineering, Kyoto University, Kyoto JAPAN

Quantitation in Gel Filled Capillary Columns
Aran Paulus and Ernst Gassmann, Ciba-Geigy Ltd., Basel, SWITZERLAND

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Comparison of the Separation of the Components of Fetal Calf Serum by Traditional and Capillary Electrophoresis
Barbara B. Vanorman, Vivian L. Steel, Elaine M. Merisko, Ronald P. Lirette, Gary G. Liverside, and Gregory L. McIntire, Sterling Drug, Great Valley, PA USA

Column and Packing Materials

Structure and Separation Characteristics of Polymer-Coated C18 Packing Materials for Reversed-Phase Liquid Chromatography
Osamu Shirota (1), Yutaka Ohtsu (1), Okitsugu Nakata (1), Shoji Hukushima (1) and Milos Novotny (2), (1) Shiseido Toxicological & Analytical Research Center, Yokohama, JAPAN and (2) Indiana University, Bloomington, IN USA

Chemical Synthesis of Internal-Surface Reversed-Phase Silica Support for Liquid Chromatography and Its Application to the Assays of Drugs in Serum
Jun Haginaka, Junko Wakai and Hiroyuki Yasuda, Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya JAPAN

The Preparation and Application of Wide Pore Silica-based Anion Exchangers with a Narrow Pore Size Distribution
Harald J. Ritchie, Paul Ross, Alan Wiltshire, and David R. Woodward, Shandon Scientific Ltd., Runcorn, Cheshire UK

A New Family of High Resolution Ion Exchangers for Protein and Nucleic Acid Purifications from Laboratory to Process Scales
Donna M. Dion, Kevin O'Connor, Dorothy J. Phillips, George J. Vella, and William Warren, Waters Division of Millipore Corporation, Milford, MA USA

Studies and Applications on a Direct Injection Shielded Hydrophobic Phase for Liquid Chromatography
Carmen T. Santasania, Supelco, Inc., Bellefonte, PA USA

Applications and Mechanistic Studies on a Highly Base Deactivated HPLC Column; Analytical Techniques to Assess Silanophilic Interactions
Tracy L. Ascah and Jennifer M. Wilson, Supelco, Inc., Bellefonte, PA USA

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Column and Packing Materials, Continued

A New Preparation Method of Internal Surface Reversed-Phase Packing Materials Starting from Alkylsilylated Silica Gels
Kazuhiro Kimata (1), Riyou Tsuboi (2), Ken Hosoya (1), Takeo Araki (1) and Nobuo Tanaka (1), (1) Kyoto Institute of Technology, Kyoto JAPAN, (2) Nacalai Tesque, Mukoh JAPAN

Silicone Polymer-Coated Silicas for Reversed Phase Liquid Chromatography
Neil T. Miller and Joseph T. Kowalewski, The PQ Corporation, Conshocken, PA USA

Two New Polymeric Ion-Exchange Packings for Solid Phase Extraction of Ionic Compounds
Ragnia M. Patel and David J. Hometchko, Interaction Chemicals Inc., Mountain View, CA USA

Stability-Testing of RP-Phases
W. Eberhardt and H. Engelhardt, Universität des Saarlandes, Saarbrücken, FDR

Systematic Evaluation of Reverse Phase Column Packings for HPLC of Basic Analytes
B. Lay and R.R. Ryall, R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ USA

Reequilibration of Polymer Coated Alumina Columns in Reversed Phase Liquid Chromatography: Effect of Solvent, Temperature, Gradient Step and Flow Rate
Rene V. Arenas and Joe P. Foley, Louisiana State University, Baton Rouge, LA USA

Preparation and Characterization of Aryl/Butadiene Copolymer Coated Alumina and Silica HPLC Packings
Walton B. Caldwell (1), Mark A. Buese (2) and James J. Perkins (2), (1) ES Industries, Marlton, NJ USA, (2) Temple University, Philadelphia, PA USA

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Characterization of a New Polymeric Media for the Rapid Reverse Phase Chromatography of Peptides and Proteins
David J. Lanteigne and A.H. Heckendorf, The Nest Group, Southborough, MA USA

Effect of Component of Polyacrylamide Gels on Exclusion Limits
Kiichi Suzuki and Ken'Ichi Nakazato, Kitasato University, Kanagawa JAPAN

Comparison of Omnipac and C-18 Columns for the Separation of Ionized and Neutral Species
L.N. Polite, R.E. Pauls and R.W. McCoy, Amoco Research Center, Naperville, IL USA

Polybutadiene Coated 1.7 μm Nonporous Silicas in Reversed Phase Chromatography (RPC)
M. Hanson and K. Unger, Institüt für Anorganische Chemie und Analytische Chemie, Johannes Gütenberg-Universität, Mainz, FDR

Improved Silica Matrices for More Reliable Use in Chromatographic Separation Technology
R. Ditz, F. Eisenbeis, W. Müller, J. Kinkel, K. Reichert and M. Wotschokowsky, E. Merck, Darmstadt, FDR

Novel Ceramic Microspherical Beads
Kenji Miyasaka (1), Takashi Ohbayashi (1), Hideyuki Kuwahara (1), Toshihiro Ishikawa (1) and Tsuneo Okuyama (2), (1) Tonen Corporation, Saitama, JAPAN and (2) Tokyo Metropolitan University, Tokyo, JAPAN

Basic Characterization of Fluorapatite (FAP) Column Chromatography
Kenji Miyasaka (1), Takashi Ohbayashi (1), Hideyuki Kuwahara (1), Toshihiro Ishikawa (1) and Tsuneo Okuyama (2), (1) Tonen Corporation, Saitama, JAPAN and (2) Tokyo Metropolitan University, Tokyo, JAPAN

Normal Phase Column Chromatography on Hydroxyapatite
Kenji Miyasaka (1), Hideyuki Kuwahara (1), Toshihiro Ishikawa (1) and Tsuneo Okuyama (2), (1) Tonen Corporation, Saitama, JAPAN and (2) Tokyo Metropolitan University, Tokyo, JAPAN

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Column and Packing Materials, Continued

Perfusion Chromatography: The Next Generation in Real-Time Separation of Biomolecules
Noubar Afeyan (1), Scott Fulton (1), Neal Gordon (1), István Mazsaroff (1), Lászlo Várády (1) and Fred Regnier (2), (1) PerSeptive Biosystems, Inc., Cambridge, MA USA and (2) Purdue University, West Lafayette, IN USA

HPLC of Morphine, Codeine and Other Bases in Porous Graphitized Carbon (PGC) Using Various Mobile Phase Additives
John H. Knox and Qian Hong Wan, University of Edinburgh, Edinburgh, UK

HPLC Applications: Environmental Analysis

The Analysis of Priority Pollutants with High Performance Liquid Chromatography
Stephan V. Rose and Jim P. Crombeen, Chrompack Int. B.V., Middelburg, THE NETHERLANDS

A Simple High-Performance Liquid Chromatographic Method for the Analysis of Tebuthiuron and Hexazinone
John Lydon, Beatriz F. Engelke and Charles S. Helling, US Department of Agriculture, Beltsville, MD USA

Selective Determination of Octachlorodibenzofuran and Perchlorinated Polynuclear Aromatic Hydrocarbons in Complex Mixtures, by Reverse-Phase HPLC with Fluorescence Detection
Ladislav M. Markovec and Neville N. Brown, ICI Australia, Ascot Vale, Victoria AUSTRALIA

HPLC Assay of Deoxyribonucleoside Di- and Triphosphates in Tomato Roots
Indrani Dutta, Probir K. Dutta, Don W. Smith and Gerard A. O'Donovan, University of North Texas, Denton, TX USA

An Evaluation of Microwave Technology for the Hydrolysis of Proteins III. Hydrolysis with Alternative Acids
Cliff Woodward (1) and W. Gary Engelhart (2), (1) Hewlett-Packard Co., Avondale, PA and (2) CEM Corporation, Matthews, NC USA

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Use of Ion-Exclusion Chromatography for Monitoring Fatty Acids Produced by Anaerobic Degradation of Priority Pollutants in Ground Water
N. Chamdasem (1) and G.W. Sewell (2), (1) NSI Technology Services, Ada, OK and (2) R.S. Kerr Environmental Research Laboratory, USEPA, Ada, OK USA

A Rapid Method for the Quantitative Detection of the Mycotoxin Citrinin by HPLC
Robert B. Vail and Michael J. Homann, Schering-Plough, Union, NJ USA

Optimization of an HPLC Method Using Post-Column Derivatization and Fluorescence Detection for the High Sensitivity Analysis of Pesticides
Patrick D. McDonald, Jessie Hansen, Wade Leveille, Arthur E. Sims, William J. Wildman, Valerie Zener and John Morawski, Waters Division of Millipore, Milford, MA USA

Clonal Variation in the Alkaloid Content of *Erythrina Spp.* Leaves
Lori D. Payne and Joe P. Foley, Louisiana State University, Baton Rouge, LA USA

Standardizing HPLC Parameters for Environmental Risk Assessment
Martha J.M. Wells, Tennessee Technological University, Cookeville, TN USA

Comparison Between GP- and RP-HPLC in the Analysis of Lignocellulose Oligomers and Humic Substances
Guido C. Galletti (1), Roberta Piccaglia (1) and Giuseppe Chiavari (2), (1) C.N.R., Bologna and (2) 'G. Ciamician', Bologna, ITALY

New Pyrene Column for Environmental Analysis
Shoji Kubota, Nobuo Tanaka and John MacFarlane, JM Science Inc., Buffalo, NY USA

Determination and Sampling of Aldehyde Compounds in Air by High Pressure Liquid Chromatography
Maurizio Colli (1), Angel Gironi (1), Vittorio Molina (1), Franceso Altilia (1) and Gianvico Melzi D'Eril (2), (1) C. Analisi Monza, Monza, and (2) Univ. Pavia, Pavia, ITALY

POSTER SESSIONS Tuesday, May 22, 1:15 - 2:45

HPLC Applications: Environmental Analysis, Continued

Use of IC-MS for Environmental Applications
Rosanne W. Slingsby and John Stillian, Dionex, Sunnyvale, CA USA

The Effects of Column Temperature, Particle Size, Pore Size and Surface Chemistry of C18 HPLC Columns on the Separation of Polynuclear Aromatic Hydrocarbons
H. Dean Rood and Mark Wareing, J&W Scientific, Folsom, CA USA

Differences in Retention Behavior of Congeners of Chlorinated Dibenzothiophene by Reversed Phase LC with Different Stationary Phases
Elizabeth R. Barnhart, Nobuo Tanaka, Kazuhiro Kimata, Ken Hosoya, Louis R. Alexander, Shigeru Terabe and Donald G. Patterson, Jr., Centers for Disease Control, Atlanta, GA USA

HPLC Applications: Clinical Analysis I

HPLC Separation of the Three Stereoisomers of Diaminopimetic Acid in Hydrolyzed Bacterial Cells
Margherita Zanol and Luigi Gastaldo, Lepetit Research Center, Gerenzano-Milano, ITALY

A Simple Method of Liquid Chromatographic Determination of Cyclosporin (Cy A) in Human Blood
Angela Iacona (1), Domenico Adorno (2) and C.U. Casciani (2), (1) Univ. L'Aquila, and (2) Univ. Torvergata, Roma, ITALY

HPLC Determination of Methyl Ethyl Ketone (MEK) in Urine
Augusta Brega (1), Paolo Prandini (2), Patrizia Villa (1) and Armido Quadri (3), (1) Lab. Biomed, Concesio, (2) SPE Sist. e Prog. Elettronici (Bs), and (3) Osp. P. Richiedei, Gussago, ITALY

Phenol, m-, o- and p-Cresol, p-Aminophenol, p-Nitrophenol Determination in Urine by HPLC
Augusta Brega (1), Paolo Prandini (2), Carlo Amaglio (2) and Emilio Pafumi (1), (1) Lab. Biomed, Concesio and (2) SPE Sist. e Prog. Elettronici, (Bs) ITALY

POSTER SESSIONS Thursday, May 24, 10:45 - 12:15

Analytical and Micropreparative High-Performance Size-Exclusion Chromatography (HPSEC) of Mucins
Andrew S. Feste, Dominique Turck, and Carlos H. Lifschitz, Baylor College of Medicine, Houston, TX USA

Simultaneous Determination of p-Hydroxylated and Dihydrodiol Metabolites of Phenytoin in Urine by HPLC
George K. Szabo, Richard J. Pylilo, Hamid Davoudi and Thomas R. Browne, Boston University School of Medicine, Boston, MA USA and V.A. Medical Center, Boston, MA USA

HPLC Detection of 3-Methylhystidine in Hydrolyzed Proteins
Luciano Dalla Libera, P. Cavallini and M. Fasolo, Univ. Padova, Padova, ITALY

Determination of 2',3'-Dideoxyguanosine in Human Plasma by High Performance Liquid Chromatography
Andrew P. Cheung and Mechelle Carnine, SRI International, Menlo Park, CA USA

A Generic Method for the Assay of Aminotetralin Derivatives in Blood Plasma Using Solid Phase Extraction and HPLC with Modified Reversed Phases
Stephen A. Wood, Susan A. Rees and Roger J. Simmonds, Upjohn Limited, Crawley, West Sussex UK

Simultaneous Measurement of Retinol, α-Tocopherol, Zeaxanthin, β-Cryptoxanthin, Lycopene, α-Carotene, cis- and trans-β-Carotene, and Four Retinyl Esters in Fasting Serum by Reversed Phase HPLC with Multiwavelength Detector
Anne L. Sowell, Daniel L. Huff, Patricia R. Yeager and Elaine W. Gunter, Centers for Disease Control, Atlanta, GA USA

Isolation and HPLC Analysis for 6-Mercaptopurine in Human Plasma
Kun-tsan Lin, G.E. Rivard and J.M. LeClerc, Ste-Justine Hospital, Montreal, Quebec CANADA

A High Performance Liquid Chromatographic Method for the Analysis of Tricyclic Antidepressants in Serum
David B. Pautler, Hewlett-Packard Company, Valley Forge, PA USA

POSTER SESSIONS Thursday, May 24, 10:45 - 12:15

HPLC Applications: Clinical Analysis I, Continued

An Improved, Sensitive Method for Measurement of Plasma Homovanillic Acid by HPLC with Coulometric Detection
Peter J. Knott, Ren-Kui Yang, Henry Cheng, Patricia Warne, Vahram Haroutunian, Michael Davidson and Kenneth L. Davis, Bronx VAMC, Bronx, NY USA

HPLC Analysis of Chlorhexidine in Gingival Fluid from Periodontal Pockets
Maurizio Tonetti, Roberto Corio, Roberto Mangione, Costantino Eftimiadi and Livio Radin, Univ. Genova, Genova, ITALY

HPLC Chemotaxonomy of Oral Bacteroides: Analysis of Metabolic End-Products
Livio Radin, Roberto Corio, Costantino Eftimiadi and Giuseppe A. Botta, Univ. Genova, Genova, ITALY

The Role of Column Switching HPLC in Clinical Pharmacokinetics: Applications to CNS-Active Drugs
Vittorio Ascalone (1) and Gabrio Bianchetti (2), (1) Synthelabo Research, L.E.R.S., Milano, ITALY and Synthelabo Research, L.E.R.S., Paris, FRANCE

Extraction and Quantitation of the Alkaloid, Sanquinarine, from Blood, Urine and Feces
Paul C. Reinhart, Robert E. Wylie II, Randy M. McEvoy and Katherine H. Andersen, Vipont Research Laboratories, Inc., Fort Collins, CO USA

The Determination of 4-Amino-1-Hydroxybutane-1,1-Bisphosphonic Acid in Urine by Automated Pre-Column Derivatization and High Performance Liquid Chromatography with Fluorescence Detection
Walter F. Kline, Bogdan K. Matuszewski and William F. Bayne, Merck Sharp & Dohme Research Laboratories, West Point, PA USA

Quantitative Determination of Terbutaline in Plasma Using Coupling of Conventional and Microbore LC with Electrochemical Detection
Claes Roos, Anders Blomgren, Lars-Erik Edholm, Britt-Marie Kennedy and Ann Svensson, AB Draco, Lund, SWEDEN

POSTER SESSIONS Thursday, May 24, 10:45 - 12:15

HPLC Separation of Hemoglobin Adducts from Trichloroethylene (TCE)
Steven C. Goheen, Berta L. Thomas, Janis E. Hulla, Michael G. Horstman and David L. Springer, Battelle, Pacific Northwest Laboratory, Richland, WA USA

Improved Determination of Ranitidine in Rat Plasma by High-Performance Liquid Chromatography (HPLC)
Alvin B. Segelman, Vidyasagar E. Adusumalli and Florence J. Segelman, Rutgers University, College of Pharmacy, Piscataway, NJ USA

HPLC of Biopolymers I

HPLC for the Assessment of Immobilizing Enzymes on Metallic Membranes
Huoy-Jiun J. Wang and Ronald L. Thomas, Clemson University, Clemson, SC USA

Purification of a Biospecific Monoclonal Antibody by High-Performance Hydroxyapatite Chromatography from Ascitic Fluid
Claudio Poiesl, Giovanna Bugari, and Salvatore Ghielmi, University of Brescia, Brescia, ITALY

A Phosphate Gradient High Performance Liquid Chromatographic Method for the Analysis of Synthetic Salmon Calcitonin
William J. Mayer (1), David A. Long (2), and Dilip K. Parikh (2), (1) Rorer Pharmaceutical Corp., Fort Washington, PA USA and (2) Armour Pharmaceutical Company, Kankakee, IL USA

Further Studies on the Effects of Chromatographic Pore Size, Particle Size, Surface Area and Mobile Phase Conditions on the Binding of Antibodies and Other Large Proteins to Ion Exchange Matrices
David R. Nau, J.T. Baker Inc., Phillipsburg, NJ USA

Scale-Up of Group Specific Affinity Chromatography
Terrence S. Reid, Daryl J. Gisch, and Ann M. Stancavage, Supelco, Bellefonte, PA USA

POSTER SESSIONS Thursday, May 24, 10:45 - 12:15

HPLC of Biopolymers I, Continued

Optimization of Reversed Phase HPLC Separations of Picomole Amounts of Enzymatic Digests of Proteins
Kathryn L. Stone, Dean E. McNulty, J. Myron Crawford, Mary B. Lopresti, and Kenneth R. Williams, Yale University School of Medicine, New Haven, CT USA

A Multi-Mode Chromatographic Approach for the Purification and the Structure Elucidation of N-Acetylglucosamine Oligomers from Hydrolyzed Chitin
Charles H. Phoebe, Jr., George J. Vella, Dale A. Cumming, Taymond T. Camphausen, and Dennis R. Benjamin, Waters Division of Millipore Corp., Milford, MA USA and Genetics Institute, Andover, MA USA

Scalable Purification of Biomolecules by High Performance Affinity Chromatography in Inert Columns
Bonnie Bell-Alden, Mark Cava, W. Harry Mandeville, Dorothy J. Phillips, and George J. Vella, Waters Division of Millipore Corp., Milford, MA USA

Non-Ideal Behaviour of Several Silica-Based Stationary Phases in the TFA/Acetonitrile-Based RP-HPLC Separation of Insulins
Susanne Linde and Benny S. Welinder, Hagedorn Research Laboratory, Gentofte, DENMARK

Characterization and Disulfide Assignment of Tryptic Peptides from Human Relaxin
T. Jeremy Kessler and Eleanor Canova-Davis, Genentech, South San Francisco, CA USA

Isocratic Separation of Angiotensins and Related Peptides and Prediction of Isocratic Solvent Composition Employing Step-Wise Gradient Elution
Hasmukh B. Patel and Christopher M. Riley, University of Kansas, Lawrence, KS USA

A New Systematic Approach for the Development of Protein Separations
Nancy E. Astephen and Thomas E. Wheat, Waters Division of Millipore Corporation, Milford, MA USA

POSTER SESSIONS Thursday, May 24, 10:45 - 12:15

Prediction of Isocratic Conditions for the Reversed Phase Liquid Chromatography of Opioid Peptides and Their Cyanobenz[f]isoindole (CBI) Derivatives from Gradient Elution Data
Kaushik J. Dave, Christopher M. Riley, and John F. Stobaugh, University of Kansas, Lawrence, KS USA

Analysis of Drugs I

Fast LC in Pharmaceutical Analysis
Michael W. Dong and Frank L. Vandemark, The Perkin-Elmer Corporation, Norwalk, CT USA

Identification of System Peaks in a High Performance Liquid Chromatographic Assay for Sodium Lactate
Cheryl L. Martin, James L. Gorski, and Robert H. Jacobus, Baxter Health Care Corporation, Round Lake, IL USA

Determination of Clorsulon in Animal Plasma at Sub-Parts-Per-Million Concentrations by HPLC with Fluorescence Detection Using a Post-Column Photochemical Reactor
James V. Pivnichny, Jung-Sook K. Shim, and Pierre de Montigny, Merck Sharp and Dohme Research Laboratories, Rahway, NJ USA

An Isocratic HPLC-Photodiode Array Detection Method for Determination of Lysine- and Arginine-Vasopressin and Oxytocin in Biological Samples
Parinam S. Rao, Nisa Rujikarn, Gerald S. Weinstein, and Denis H. Tyras, Long Island Jewish Medical Center, New Hyde Park, NY USA

Simple RP-HPLC Method for the Separation of Tocopherols and Tocopherol Esters
Nancy J. Holt and Joseph R. Kaczvinsky, The Proctor & Gamble Co., Cincinnati, OH USA

High Performance Liquid Chromatographic Isolation and Purification of Lecithin for Use in the Synthesis of Anti-AIDS Drugs
Phyllis R. Brown, John V. Amarl and Joseph G. Turcotte, University of Rhode Island, Kingston, RI USA

POSTER SESSIONS Thursday, May 24, 10:45 - 12:15

Analysis of Drugs I, Continued

A Rapid HPLC Method for Determining Octanol/Water Partition Coefficients for Low Molecular Weight Compounds Used in the Treatment of Brain Tumors
William L. Strond, Robert Feldman, and Dennis Groothuis, Evanston Hospital, Evanston, IL USA

Pharmaceutical Applications of Alternative Bonded Phases and Support Materials for Reverse Phase HPLC
Michael E. Swartz, Thomas Wheat, and Isaac Neitring, Waters Division of Millipore Corp., Milford, MA USA

Liquid Chromatographic Analysis of a Potential Polymeric-Pendant Drug Delivery System for Peptides: Application of HPSEC, RP-HPLC, and IC to the Evaluation of Biodegradable Poly(Chloro Tri-Alanine Methyl Ester)Phosphazenes (polyTame)
W. Mark Eickhoff (1), Gary G. Liversidge (2), and R. Mutharasan (1), (1) Drexel University, Philadelphia, PA USA and (2) Sterling Research Group, Great Valley, PA USA

HPLC Purity and Potency Assays: Designing Methods That SPARkLe
Jackson P. Scholl and Steven K. MacLeod, The Upjohn Company, Kalamazoo, MI USA

Determination of Vitamin D in a Protein Hydrolysate Infant Formula Using Column Switching
Bruce E. Molitor, Aaron M. Jones, Sandra L. Yeager and Benjamin D. Travis, Ross Laboratories, Columbus, OH USA

Investigation of Sulfadrug Residues in Pork and Chicken Tissues in Taiwan
Shin-Shou Chou, Chia-Fen Tsai and Chieu-Chen Cheng, National Laboratories of Foods and Drugs, Taipei, Taiwan ROC

Ion Exchange Chromatographic Study of Oral and Injectable Versions of Some ACE and Enkephalinase Inhibitors
David Koharsky, Schering-Plough Research, Bloomfield, NJ USA

POSTER SESSIONS Thursday, May 24, 10:45 - 12:15

Coupled Column LC-Techniques for the Bioanalysis of Drugs: Advantages of Miniaturization
Lars-Erik Edholm, Anders Blomgren and Claes Roos, AB Draco, Lund, SWEDEN

HPLC Analysis of Bleomycins
Hans-Peter Fiedler and Jutta Wachter, Universität Tübingen, Tübingen, FDR

HPLC Applications: Clinical Analysis II

Ion Chromatographic Determination of Serum Phosphate and Sulfate
Michele Petrarulo, Ornella Bianco, Martino Marangella, Sergio Pellegrino, Corrado Vitale and Franco Linari, Osp. Maur. Umberto I, Torino, ITALY

Measurement of Adenine, Inosine and Hypoxanthine in Cell Suspensions Using Small-Bore, Isocratic Column High Performance Liquid Chromatography
Richard H. Gayden, Francis Bodola, Robert E. Beach and Claude R. Benedict, University of Texas Medical Branch, Galveston, TX USA

Direct Injection of Physiological Sample and Simultaneous Analysis of Water-Soluble and Fat-Soluble Components by HPLC Using Column Switching and Tandem Column System
Kazuo Seta and Tsuneo Okuyama, Tokyo Metropolitan University, Tokyo, JAPAN

Multi-Dimensional HPLC for High-Resolution Separation of Complex Biochemical and Physiological Components
Kazuo Seta and Tsuneo Okuyama, Tokyo Metropolitan University, Tokyo, JAPAN

HPLC: A Powerful Tool in Case of Congenital Adrenal Enzymatic Defect
Guido Carpene (1), Giuseppe Opocher (1), Annapaola Vettoretti (1), Francesco Pedini (1), Stefano Rocco (1) and Franco Mantero(2), (1) Univ. Padova, Padova, and (2) Univ. Catania, ITALY

POSTER SESSION Thursday, May 24, 1:15 - 2:45

HPLC Applications: Clinical Analysis II, Continued

Assay of Hydroxyfarrerol (IdB 1031) in Biological Fluids
Antonio Marzo (1), Edoardo Arrigoni Martelli (1), Giulia Bruno (1), Ernesto M. Martinelli (2) and Giorgio Pifferi (2), (1) Sigma Tau SpA, Pomezia/Roma and (2) Inverni della Beffa, Milano, ITALY

Proglucagon and Chromogranin B Synthesized in the Pancreatic Islets
Egon Nielsen, Ingelise Fabrin, Benny S. Welinder and Ole D. Madsen, Hagedorn Research Laboratory, Gentofte, DENMARK

Ion-Pair Reversed-Phase HPLC Determination of Nucleotides, Nucleosides and Nucleobases in Cells of Different Metabolic Complexity
Andreas Werner, Werner Siems and Gerhard Gerber, Institute of Biochemistry, Berlin, DDR

Neonatal and Pediatric Drug Monitoring - Shielded Hydrophobic Phase for Direct-Sample-Analysis of Carbamazepine
Steven H.Y. Wong, Lisa A. Butts and Ken Broder, University of Connecticut School of Medicine, Farmington, CT USA

HPLC Evaluation of Broncoplus and Its Metabolite in Biological Fluids
Antonio Marzo (1), Edoardo Arrigoni Martelli (1), Giulia Bruno (1), Demetrio Nava (1), Alain Mignot (2), Richard Vidal (2) and Marc A. Lefebvre (2), (1) Sigma Tau SpA, Pomezia/Roma, ITALY and (2) Centre D'Etude et de Recherche in Pharmacie Clinique, Saint Benoit, FRANCE

Determination of L-DOPA, Carbidopa and Related Impurities by HPLC with Electrochemical Detector
Peppino Betto, Claudio Lucarelli, Flavio Belliardo, Giuseppe Ricciarello and Massimo Giambenedetti, (1) Istituto Superiore de Sanità, Roma and (2) Univ. Torino, Torino, ITALY

Isolation and Identification of Neurolite Metabolite
Mark W. Watson, Richard C. Walovitch, Michael V. Ganey and Tina M. Nason, E.I. du Pont de Nemours and Co., North Billerica, MA USA

POSTER SESSION Thursday, May 24, 1:15 - 2:45

Detection of Oxidation Products in Neurolite Raw Material
Peta A. Ryan, Bobbie A. Ewels, Michele E. Foster, Lynne S. Manne and Neil E. Williams, E.I. du Pont de Nemours and Co., North Billerica, MA USA

HPLC Methodology for the Determination of 3-Hydroxy-α-Methyl-1-Thyrosine in Plasma
Claudio Lucarelli (1), Peppino Betto (1), Gabriele Grossi (2), and Giuseppe Ricciarello (1), (1) Istituto Superiore di Sanità, Roma and (2) Policl. S. Orsola, Bologna, ITALY

Is Alumina Extraction Always Suitable for Plasma Catecholamine Determination by RP-HPLC?
Claudio Lucarelli (1), Peppino Betto (1), and Michela Codini (2), (1) Istituto Superiore di Sanità, Roma and (2) Univ. Perugia, Perugia, ITALY

Improvements in Catecholamines and Related Metabolites Automated Analysis by Column Switching HPLC
Gabriele Grossi (1), Claudio Lucarelli (2), Alberto M. Bargossi (1) and Giuseppe Sprovieri (1), (1) Lab. Centr. Policl. S. Orsola, Bologna, and (2) Istituto Superiore di Sanità, Rome, ITALY

High Performance Liquid Chromatography Assay and Pharmacokinetic Studies of Doxophylline in Plasma
Aldo Lagana (1), Mariano Bizzarri (2) and Massimo Mancini (2), (1) Univ. Bologna, Bologna, (2) Univ. La Sapienza, Roma, ITALY

Extraction and Isolation of Anabolic Oestrogens from Animal Tissues by a Double Trap
Aldo Lagana (1), Massimo Mancini (2) and Aldo Marino (2), (1) Univ. Bologna, Bologna, (2) Univ. La Sapienza, Roma, ITALY

Measurement of Urinary Tryptophan and Its Metabolites of the Kynurenine Pathway by HPLC
Mariano Bizzari (1), G. Frezza (1), Massimo Mancini (1) and Aldo Lagana (2), (1) Univ. La Sapienza, Roma and (2) Univ. Bologna, Bologna, ITALY

d-Aminolevulinic Acid in Plasma by Free Amino Acid Analysis
Charles J. Hannan, Thomas M. Kettler, Irwin B. Dabe and Timothy S. Clark, Madigan Army Medical Center, Tacoma, WA USA

POSTER SESSION Thursday, May 24, 1:15 - 2:45

HPLC Applications: Clinical Analysis II, Continued

Determination of Neurochemicals in Biological Fluids Using a Coulometric Array Detector and Automated HPLC System
V. Rizzo (1), G. Melzi d'Eril (1), G. Achilli (2) and G. Cellerino (2), (1)University of Pavia, Pavia, ITALY and ESA, Bedford, MA USA

Narrow-Bore HPLC with Post-Column Reduction and Fluorometric Detection for the Simultaneous Determination of Vitamin K1-Epoxide and Vitamin K1 at Femtomole Levels in Biological Samples
Kenneth W. Davidson and James A. Sadowski, USDA Human Nutrition Research Center at Tufts University, Boston, MA USA

Reversed Phase Liquid Chromatographic Determination of 2',3'-Dideoxycytidine and Its Nuceolside Analogue 2',3'-Dideoxyinosine
Gene F. Ray, Tim B. Skinner, Phyllis J. Harrison and Evelyn A. Murrill, Midwest Research Institute, Kansas City, MO USA

Determination of Hydroxylated Pyridinoline in Urine by HPLC
Jan Macek, Alexander Lichy and Milan Adam, Research Institute for Rheumatic Diseases, Prague, CZECHOLSLOVAKIA

High Performance Liquid Chromatographic Determination of Diltiazem and Its Major Metabolites, N-Monodemethyldiltiazem and Desacetyldiltiazem in Plasma
K. J. Swart and S. Viljoen, The University of the Orange Free State, Bloemfontein, Republic of SOUTH AFRICA

Direct-Injection Analysis of Carbovir (CBV) and Its Major Metabolite in Mouse Plasma and Urine by Internal Surface Reverse Phase (ISRP) Liquid Chromatography
L.M. Rose, A.A. Beaty, S.M. el Dareer, K.F. Tillery, and D.L. Hill, Southern Research Institute, Birmingham, AL USA

Simultaneous Separation and Determination (in Serum) of Phenytoin and Carbamazepine and Their Deuterated Anlogues by HPLC/UV for Tracer Studies
G. K. Szabo, R. J. Pylilo and T. R. Browne, Boston University School of Medicine, Boston, MA and V.A. Medical Center, Boston, MA USA

POSTER SESSION Thursday, May 24, 1:15 - 2:45

HPLC of Biopolymers II

A Selective Chromatographic ystem for Carbohydrates by Complex Formation with Metals in Alkaline Solution
Morgan Stefansson, Uppsala University, Uppsala SWEDEN

Polymeric versus Silica-Based Stationary Phases for RP-HPLC of Polypeptides
Benny S. Welinder, Hagedorn Research Laboratory, Gentofte DENMARK

Peptide Analysis and Purification
Linda L. Lloyd and Frank P. Warner, Polymer Laboratories Ltd., Church Stretton, Shropshire UK

Studies on a Unified Retention Mechanism of Proteins in Liquid Chromatography
Bian Liujiao, Feng Wenke and Geng Xindu, Northwest University, Xi'an CHINA

RP-HPLC of Whey Proteins from Different Animal Species
Mercedes de Frutos (1), Alejandro Cifuentes (1), Jose C. Diez-Masa (1), Manuel V. Dabrio (1), Lourdes Amigo (2), Mercedes Ramos (2) and Carmen Polo (2), (1) Instituto de Quimica Orgánica, Madrid SPAIN, (2) Instituto de Fermentaciones Industriales, Madrid, SPAIN

A Cation-Exchange HPLC Method for Synthetic Salmon Calcitonin
Maria E. Munera, Fred R. Borger, Everett Flanigan and Dilip K. Parikh, Armour Pharmaceutical Company, Kankakee, IL USA

Validation of an Analytical Reversed Phase HPLC Method for Synthetic Salmon Calcitonin
Everett Flanigan, Fred R. Borger, Laurence L. Ho, William J. Mayer, Maria E. Munera and Dilip K. Parikh, Armour Pharmaceutical Company, Kankakee, IL USA

A New Method for the Characterization of Packings for the Separation of Proteins with HPLC
R.E. Huisden, J.C. Kraak and H. Poppe, University of Amsterdam, Amsterdam, THE NETHERLANDS

POSTER SESSION Thursday, May 24, 1:15 - 2:45

HPLC of Biopolymers II, Continued

Novel Mobile Phase Combinations and Alternative Stationary Phases for HPLC Separation of Peptides
Patricia M. Young and Thomas E. Wheat, Waters Division of Millipore Corporation, Milford, MA USA

High Performance Liquid Chromatography of SDS-Protein Complex on Hydroxyapatite Column
Kenji Miyasaka (1), Yoshio Yamakawa (2) and Tsuneo Kuyama (3), (1) TONEN Corporation, Saitama, JAPAN, (2) National Institute of Health, JAPAN and (3) Tokyo Metropolitan University, Tokyo, JAPAN

Characterization of Variants of a Recombinant Human IGF-1 Formed During Hydroxylamine Cleavage of a Fusion Protein
Victor T. Ling, Marian Eng and Eleanor Canova-Davis, Genentech, South San Francisco, CA USA

Affinichrom™ Plastic-Coated Glass Beads: A Support for Immunoaffinity Chromatography
Terry M. Phillips and James V. Babashak, George Washington University Medical Center, Washington, DC USA, and Kontes Scientific Glassware, Vineland, NJ

Separation of Inositol Phosphate (InsP) Isomers by Reversed Phase High Performance Liquid Chromatography (HPLC) Employing Positively Charged Surfactants
Clara Brando, Thomas Hoffman and Ezio Bonvini, Laboratory of Cell Biology, DBBP/CBER/US-FDA, Bethesda, MD USA

Comparison of Ion-Exchange Columns for Protein Analysis
H. Engelhardt, E. Schweinheim and E. Wickel, Universität des Saarlandes, Saarbrücken, FDR

Optimization of Separation Conditions for the FMOC-ADAM Approach to Amino Acid Analysis: RP-HPLC of 'basic acids'
W. Götzinger and H. Engelhardt, Universität des Saarlandes, Saarbrücken, FDR

POSTER SESSION Thursday, May 24, 1:15 - 2:45

Two-Stage Enzymatic Digestion of Proteins and Isolation of Peptides from Proline-Endopeptidase Digestion Mixtures by Reversed Phase HPLC
Cynthia P. Quan, John Frenz and William S. Hancock, Genentech, South San Francisco, CA USA

Evaluation of the State of Oligomerization of Soluble CD4 by Size Exclusion HPLC and Analytical Ultracentrifugation
Sudhir Burman, Preston Hensley, Dwight Moore, Shing Mai, Anthony Jurewicz and James J. L'Italien, SmithKline Beecham Pharmaceuticals, King of Prussia, PA USA

Characterization of Some Recombinant Proteins by HPLC: Interpretation and Reliability
Vladas Bumelis, Juozas Daugvila, Vytautas Maktinis and Arvidas Neniskis, Institute of Applied Enzymology, Vilnius, Lithuania USSR

Hysteresis of Protein Adsorption Isotherms with Hydrophobic Interaction Chromatographic Adsorbents
R. Blanco, S. W. Lin and B. L. Karger, Northeastern University, Boston, MA USA

Analysis of Drugs II

Liquid Chromatographic Assay of Dimetridazole, Ronidazole and Ipronidazole in Premixes and Feeds
Roberto Laffi, Silvia Marchetti, Stefano Rossato and Mario Marchetti, Università di Bologna, Bologna, ITALY

HPLC of Liposomes: Monitoring Surfactant-Induced Drug Release
M.C. Bañó, E. Pérez-Payá, L. Braco and C. Abad, Universitat de València, Burjassot, València, SPAIN

High Performance Liquid Chromatography (HPLC) Separation and Isolation of Sassafras Albidum (Nuttall) Nees (Lauraceae) Root Bark Alkaloids
Florence H. Segelman, Alvin B. Segelman and Jerrold Karliner, Rutgers University, College of Pharmacy, Piscataway, NJ USA

POSTER SESSION Thursday, May 24, 1:15 - 2:45

Analysis of Drugs II, Continued

Determination of Phenothiazine Drugs in Kidney and Meat Samples by HPLC
B. Keshavan, University of Mysore, Mysore, INDIA

The Determination of Acrivastine and Its Major Metabolite, BW270C, in Human Plasma by HPLC with UV Detection
Graham S. Land and Neil P. Shortman, Wellcome Research Laboratories, Beckenham, Kent UK

An Investigation Into Alternative Methods of Assessing HPLC Bioanalytical Data to Optimize Accuracy Over a Wide Range of Drug Concentrations
Graham S. Land and William J. Leavens, Wellcome Research Laboratories, Beckenham, Kent UK

Metal Interaction Chromatography of Sulfonamides
Alfred V. Del Grosso, U.S. Food and Drug Administration, Center for Biologics Evaluation and Research, Bethesda, MD USA

Micro-LC Method for the Determination of Bamifylline and Its Metabolites in Human Plasma
Flavio Belliardo (1) and Claudio Lucarelli (2), (1) Univ. Torino, Torino, and (2) Istituto Superiore di Sanità, Rome, ITALY

Analysis of Tricyclic Antidepressant Drugs in Serum
Maria Pia Segatti (1), G. Nisi (1) and Claudio Lucarelli (2), (1) Osp. B. Garofolo, Trieste, and (2) Istituto Superiore di Sanità, Rome, ITALY

Proceedings of the Symposium will be published by the *Journal of Chromatography*

PUBLICATION SCHEDULE FOR 1990

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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INFORMATION FOR AUTHORS

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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), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two 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 is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, 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.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

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 three 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.

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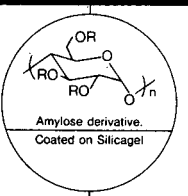
•CHIRALPAK AS

R: $-C(=O)-N(H)-C^{*}(H)(CH_3)-$ (phenyl)

*: S体

for β-Lactam antibiotics



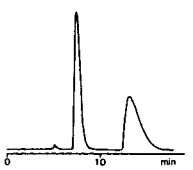
NEW CHIRALPAK AD

•CHIRALPAK AD

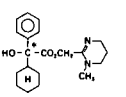
R: $-C(=O)-N(H)-$ (2,5-dimethylphenyl)

4-Acetoxy-2-azetidine

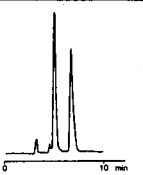
Eluent : Hexane/Ethenol = 8/2
Flow rate : 1.0mℓ/min
Temperature : r.t.
Detection : UV254nm



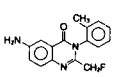
Oxyphencyclimine



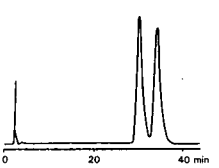
Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0mℓ/min
Temperature : r.t.
Detection : UV254nm



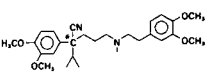
Afloqualone



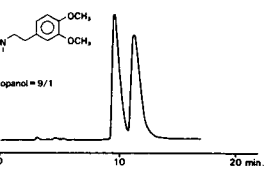
Eluent : Hexane/EtOH = 95/5
Flow rate : 1.3mℓ/min
Temperature : 50°C
Detection : UV254nm



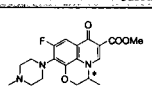
Verapamil



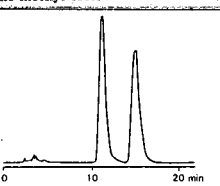
Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0mℓ/min
Temperature : r.t.
Detection : UV254nm



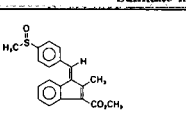
Ofloxacine methyl ester



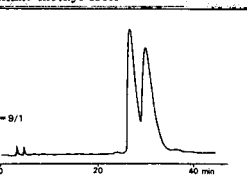
Eluent : Hexane/EtOH = 8/2
Flow rate : 1.2mℓ/min
Temperature : 40°C
Detection : UV254nm



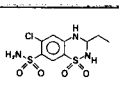
Sulindac methyl ester



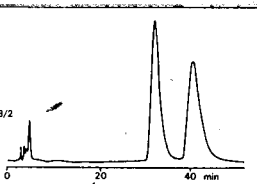
Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0mℓ/min
Temperature : r.t.
Detection : UV254nm



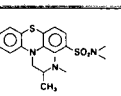
Ethiazide



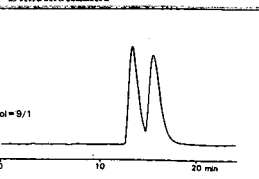
Eluent : Hexane/Ethenol = 8/2
Flow rate : 1.0mℓ/min
Temperature : 40°C
Detection : UV254nm



Dimethotiazine



Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0mℓ/min
Temperature : r.t.
Detection : UV254nm



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