VOL. 705 NO. 2 30 JUNE 1995
THIS ISSUE COMPLETES VOL. 705

JOURNAL OF CHROMATOGRAPHY A

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

ELSEVIER

JOURNAL OF CHROMATOGRAPHY A

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

Scope. The *Journal of Chromatography A* publishes papers on all aspects of **chromatography, electrophoresis** and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental developments and their applications. In the *Symposium volumes*, which are under separate editorship, proceedings of symposia on chromatography, electrophoresis and related methods are published. *Journal of Chromatography B: Biomedical Applications* —This journal, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis or alterations during medical treatment; screening and profiling of body fluids or tissues related to the analysis of active substances and to metabolic disorders; drug level monitoring and pharmacokinetic studies; clinical toxicology; forensic medicine; veterinary medicine; occupational medicine; results from basic medical research with direct consequences in clinical practice.

Submission of Papers. The preferred medium of submission is on disk with accompanying manuscript (see *Electronic manuscripts* in the Instructions to Authors, which can be obtained from the publisher, Elsevier Science B.V., P.O. Box 330, 1000 AH Amsterdam, Netherlands). Manuscripts (in English; *four* copies are required) should be submitted to: Editorial Office of *Journal of Chromatography A*, P.O. Box 681, 1000 AR Amsterdam, Netherlands, Telefax (+31-20) 485 2304, or to: The Editor of *Journal of Chromatography B: Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, Netherlands. Review articles are invited or proposed in writing to the Editors who welcome suggestions for subjects. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Publication information. *Journal of Chromatography A* (ISSN 0021-9673): for 1995 Vols. 683–714 are scheduled for publication. *Journal of Chromatography B: Biomedical Applications* (ISSN 0378-4347): for 1995 Vols. 663–674 are scheduled for publication. Subscription prices for *Journal of Chromatography A*, *Journal of Chromatography B: Biomedical Applications* or a combined subscription are available upon request from the publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where air delivery via SAL is ensured: Argentina, Australia, Brazil, Canada, China, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues must be made within six months of our publication (mailing) date. Please address all your requests regarding orders and subscription queries to: Elsevier Science B.V., Journal Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands. Tel.: (+31-20) 485 3642; Fax: (+31-20) 485 3598. Customers in the USA and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Inc., 655 Avenue of the Americas, New York, NY 10010, USA, Tel. (+1-212) 633 3750, Telefax (+1-212) 633 3764.

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

US Mailing Notice. *Journal of Chromatography A* (ISSN 0021-9673) is published weekly (total 52 issues) by Elsevier Science B.V., (Sara Burgerhartstraat 25, P.O. Box 211, 1000 AE Amsterdam, Netherlands). Annual subscription price in the USA US$ 5389.00 (US$ price valid in North, Central and South America only) including air speed delivery. Second class postage paid at Jamaica, NY 11431. **USA POSTMASTERS**: Send address changes to *Journal of Chromatography A*, Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003. Airfreight and mailing in the USA by Publications Expediting.

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

0021-9673/95/$09.50

Printed in the Netherlands

CONTENTS

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

REGULAR PAPERS

Column Liquid Chromatography

Gas Chromatography

(Continued overleaf)

Contents (continued)

ELSEVIER

Journal of Chromatography A, 705 (1995) 171–184

JOURNAL OF CHROMATOGRAPHY A

Optimization of a modified electrode for the sensitive and selective detection of α-dipeptides

Jian-Ge Chen, Edward Vinski, Kevin Colizza, Stephen G. Weber*

Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA

First received 18 July 1994; revised manuscript received 15 February 1995; accepted 28 February 1995

Abstract

Sensitive and selective detection of dipeptides is important in neurochemistry. We have developed a flexible detection scheme for dipeptides based on a modified carbon electrode. The modification arises from the anodic treatment of the carbon electrode in alkaline solution. The flexibility of the detection scheme arises from the different conditions used in both the modification and the detection. It is shown that the modification step requires the presence of cupric ion, while the detection step does not. On the other hand, it is shown that the presence of copper in the detection eluent, as well as the pH of the environment, can be used in controlling the selectivity of the modified electrode. For example, the modified electrode is more selective for α-dipeptides over β- and γ-dipeptides as well as amino acids at pH 9.8, whereas it is selective for all dipeptides over amino acids at pH 8.0. Detection limits of dipeptides on the order of 10 n*M* were achieved at pH 8.0 by flow-injection analysis with a knotted Teflon tubing connecting the injector and the detector that gave a typical peak volume of about 0.50 ml at 1.0 ml/min. From surface analysis it is shown that the oxygenation of the glassy carbon electrode gives rise to the selectivity. The oxidation of dipeptides at the modified electrode is completely inhibited by 10 m*M* Mg^{2+} in the eluent.

1. Introduction

Due to the increased understanding of the role of L-aspartate (Asp) and L-glutamate (Glu) as excitatory amino acids (EAA), research in the area has turned to the search for other endogenous ligands that act on EAA receptors. In particular, some acidic dipeptides such as γ-Glu-Gln have been observed to interact with EAA receptors in vitro [1–4] and in vivo [4,5]. Numerous β-aspartyl dipeptides have been found in human urine [6] but their functions in the central nervous system (CNS) are not clear. The release of glutathione, a γ-glutamyl tripeptide, has been observed during ischemia [7]. Despite all these findings with β- and γ-dipeptides, little analytical work has been done with the most common dipeptides, the α-dipeptides, because of the lack of sensitive and selective detection techniques.

Traditional analytical methods for the detection of peptides are based on UV absorbance of the amide bond, which is too general and has poor sensitivity [8–11], or fluorescence detection following derivatization with reagents such as *o*-phthalaldehyde which are useful in amino acid analysis [8,12–15]. Although quite effective for amino acids, and for β- and γ-dipeptides, the OPA detection technique suffers a severe loss in

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00286-3

sensitivity for α-dipeptides. Because of its amine specificity, OPA detection of N-acylated compounds such as N-acetyl-Asp-Glu (NAAG), which has no primary amine, is not possible.

Besides sensitivity, an analytical procedure intended for eventual application by neurochemists must have good selectivity for the compounds studied. An example of the need for the selectivity is cysteine sulfinate in the brain. Over time, as analytical methods have improved, the reported concentration of cysteine sulfinate has gone down by four orders of magnitude [16–19].

Previous work on the electrochemical detection of peptides that do not have electroactive functional groups has been done with the biuret reaction [19–21]. In this procedure, the peptide reacts with Cu(II) to form a Cu(II)–peptide complex. This converts previously electroinactive peptides into electroactive species. The oxidation of the Cu(II)–peptide to the Cu(III)–peptide at the upstream anode and the corresponding reduction of Cu(III)–peptide to Cu(II)–peptide at the downstream cathode in a dual series amperometric detector provide for the detection [8]. The reversible Cu(II)/Cu(III) electrochemistry enables the use of the sensitive and selective dual glassy carbon electrode [8,22]. While this detection scheme is useful for peptides containing three or more amino acid residues, it is unfortunately not ideal for dipeptides because the oxidation of the Cu(II)–dipeptide complex requires a high oxidation potential (>0.9 V) [12].

We recently discovered that dipeptides can be detected without the biuret reagent [12]. This involves the use of a modified glassy carbon electrode. The upstream anode becomes modified upon exposure to a high applied potential, ≥ 1.2 V vs. Ag/AgCl. The sensitivity for dipeptides with the modified electrode at a useful analytical anodic potential and pH, i.e. 0.90 V and pH 8.0, is dramatically increased as compared to an unmodified electrode [12]. Both the anodic (modified electrode) and the cathodic (unmodified electrode) signals are useful at pH 8.0 and in the absence of the biuret reagent in the detection eluent. However, it is generally the cathodic signal that is useful at pH >9.5 and in the presence of the biuret reagent in the detection eluent because of the high anodic background. The lowest detection limits are obtained at pH 8.0 and in the absence of the biuret reagent.

There are three objectives of this research. First, the parameters of the modification protocol must be understood in order to optimize conditions with respect to both sensitivity and selectivity. Second, the primary surface chemistry of the modified electrode must be determined to allow intelligent discussion of the surface electrochemistry. Last, we want to demonstrate the applicability of the modified electrode coupled with anion-exchange chromatography for the detection of acidic dipeptides. The biological applications of this detection scheme are underway and will be presented in due course.

2. Experimental

2.1. Chromatographic and detection equipment

A Waters (Milford, MA, USA) 600-MS pump and an ISCO (Lincoln, NE, USA) Model 100DM syringe pump were used to pump the mobile and the post-column phases, respectively. The anodic and cathodic potentials were applied from a BAS (W. Lafayette, IN, USA) LC-4C potentiostat to a BAS dual glassy carbon electrode with a 0.002 inch spacer thickness. All samples were injected into a 20-μl loop using a 100-μl syringe. A Nucleosil 5 SB column (25 × 4.6 cm, Keystone Scientific, Bellefonte, PA, USA) with guard column was used to separate dipeptides. A knotted Teflon tube (0.95 ml, 90 cm × 0.58 mm I.D.) in place of the column was used for flow-injection analysis (FIA) to separate the injection spike from the peak. All data were recorded on a chart recorder and through EZChrom software (Scientific Software, San Ramon, CA, USA) installed on a DTK IBM compatible computer. Peak areas were determined after manually specifying the limits of integration for each peak.

All data presented are obtained from a single dual glassy carbon electrode in a serial configura-

tion. The downstream cathode is always set at 0.0 V vs. Ag/AgCl. All applied potentials given below are referenced to Ag/AgCl, 3 *M* NaCl reference electrode (BAS).

2.2. Reagents

ACS certified monobasic and dibasic sodium phosphate (Fisher, Pittsburgh, PA, USA), sodium bicarbonate (Mallinckrodt, Paris, KY, USA), sodium carbonate (J.T. Baker, Phillipsburgh, NJ, USA), magnesium sulfate (Fisher), glacial acetic acid (J.T. Baker), lithium acetate and lithium sulfate (Aldrich, St. Louis, MO, USA), and analytical grade copper sulfate pentahydrate (Fisher) were used without further purification. Sodium potassium tartrate (Fisher) was recrystallized from water before use.

All amino acids, amino acid derivatives, and peptides were purchased from Sigma (St. Louis, MO, USA) except Asp-Asp, Asp-Glu, Glu-Asp, and Glu-Glu (all from Bachem, King of Prussia, PA, USA). β-Asp-Tau was synthesized after Ienaga et al. [23].

One-letter shorthand for amino acids is used in figure legends and three-letter ones are used in the text. The one-letter abbreviations are: A = Ala, C = Cys, D = Asp, E = Glu, G = Gly, and V = Val.

2.3. Mobile phases and post-column phases

Several buffers were used. The pH 8.0 phosphate buffer (0.1 *M* for FIA and 0.5 *M* for HPLC) consisted of dibasic and monobasic sodium phosphate in a 95:5 molar ratio with sodium bicarbonate at a concentration of 10% of the total phosphate concentration. The 0.2 *M* pH 9.8 carbonate buffer was made from equimolar amounts of bicarbonate and carbonate as sodium salts. The 5.0 m*M* pH 4.6 acetate buffer used as mobile phase for HPLC was made from equimolar amounts of lithium acetate and glacial acetic acid with varying amounts of lithium sulfate. Biuret reagents were prepared in each of the basic buffers. The stock solutions of tartrate (0.3 *M*) and copper sulfate (0.1 *M*) are mixed first, then this solution is mixed with the buffer solution before diluting to the appropriate volume. In this way, copper(II) will not precipitate. The concentrations of copper sulfate and tartrate are 0.1 m*M* and 0.3 m*M*, respectively, in all of the reagent formulations, unless otherwise indicated.

All mobile and post-column phases were dissolved in or diluted with twice-deionized water and vacuum filtered through 0.45-μm type HA filters (Millipore Corp.). The solutions were then purged with helium gas to remove oxygen.

2.4. Electrode modification protocol

The following steps comprise the protocol used for glassy carbon anode modification:

(1) Polish the electrode with 0.05 μm γ-alumina (LECO, St. Joseph, MI, USA) on polishing cloth (BAS).

(2) Vigorously rinse the electrode surface with twice-deionized water.

(3) Ultrasonicate the electrode with 50 ml twice-deionized water twice for 5 min each time.

(4) Assemble the detector cell and place it in the flow stream.

(5) Modify the anode with an applied potential of 1.2 V or 1.4 V vs. Ag/AgCl at a buffer flow-rate of 1.0 ml/min for a given time.

Such a modified electrode can be used for dipeptide detection up to weeks without retreating the electrode surface, and there is no loss of sensitivity observed for up to weeks. During overnight periods the electrode is left open to ambient air without any special storage. The cathode was not modified.

3. Results and discussion

3.1. Optimization of the modified electrode

The parameters that control the modified electrode's performance include the presence of the biuret reagent in solution, the modification and detection pH, and the modification time and potential [12]. The effect of these parameters will be discussed below.

Biuret reagent

In this study, we intend to investigate the effect of copper and tartrate in the modification protocol. We have modified the glassy carbon anode in the presence and absence of copper and tartrate and used both the modified anode and the unmodified cathode (downstream) for dipeptide detection. Table 1 gives the results obtained from flow-injection analysis (FIA) using the modified and the unmodified electrodes for a series of α-, β-, and γ-dipeptides. The sensitivities are corrected for the blank injection. Each number is the mean value from two or three replicate injections. The pooled standard deviation of the areas obtained from each electrode modification is given in the first row of the table. Sensitivities that are less than twice the pooled standard deviation are in parentheses. Negative sensitivities are data in which the peak area is larger in the blank than in the presence of an analyte. The magnitudes of the standard deviations indicate that most of these negative signals are real. We speculate that the negative signals are from a suppression of the ambient background current. The background current is suppressed in the presence of certain peptides because they adsorb and alter the electroxidation of solvent/impurities. The adsorption is reversible.

The data clearly show that the modification requires the biuret reagent. In the absence of the biuret reagent in the detection eluent, the sensitivities of the anode modified in the presence of Cu^{2+} and tartrate are increased as compared to sensitivities obtained from an unmodified and a modified-without-Cu^{2+} and tartrate electrode. Note that experiments cannot be done with Cu^{2+} alone.

Once we established that the modification needed the biuret reagent, we went on to test the detection in the presence and in the absence of the biuret reagent. The results, also obtained

Table 1
Effect of Cu^{2+} on the modification of the anode at pH 9.8

	Cathodic sensitivity (nC/pmol), anode 0.90 V, cathode 0.0 V[a]					
	Unmodified electrode		Modified without Cu^{2+}		Modified with 0.1 m*M* Cu^{2+}	
	Anode	Cathode	Anode	Cathode	Anode	Cathode
Pool S.D., σ_p	0.036	0.001	0.068	0.004	0.120	0.004
α-Asp-Gly	0.202	0.037	0.163	0.034	0.787	0.130
α-Asp-Val	(−0.062)[b]	0.028	−2.98	−0.048	0.476	0.094
α-Ala-Gly	0.406	0.119	−1.93	0.035	1.35	0.260
α-Ala-Ala	0.696	0.173	−1.92	0.078	1.54	0.340
Gly-Gly	0.210	0.048	−2.46	−0.035	(0.139)[b]	0.058
α-Asp-Asp	0.137	0.003	−2.67	−0.072	1.39	0.012
α-Glu-Glu	(−0.235)[b]	0.013	−2.66	−0.052	0.250	0.050
α-Asp-Glu	−0.199	0.016	−2.80	−0.052	(0.026)[b]	0.067
α-Glu-Asp	(−0.067)[b]	(0.001)	−2.37	−0.073	0.790	−0.066
β-Asp-Gly	0.074	0.044	1.98	0.064	2.021	0.093
β-Asp-Val	−0.077	0.014	−2.79	−0.053	0.636	0.036
β-Ala-Gly	0.345	0.020	−1.44	−0.055	1.103	0.039
β-Ala-Ala	0.094	0.012	−2.69	−0.054	0.560	0.021
γ-Glu-Gly	−0.273	0.005	−2.98	−0.059	(−0.007)[b]	(0.007)[b]
γ-Glu-Val	−0.267	0.004	−3.20	−0.067	(−0.182)[b]	0.005

[a] Modification: 0.2 *M* pH 9.8 carbonate buffer with and without the biuret reagent, 1.4 V for 4 h at 1.00 ml/min. Detection: 0.2 *M* pH 9.8 carbonate buffer without the biuret reagent.

[b] Signal magnitude $< 2 \times$ pooled standard deviation, σ_p.

Table 2
Effect of Cu^{2+} on the detection at pH 9.8

Compound	Cathodic sensitivity (nC/pmol), anode at 0.85 V[a]	
	Detected without Cu^{2+}	Detected with Cu^{2+}
Asp	0.009	0.000
α-Asp-Gly	0.100	0.042
β-Asp-Gly	0.075	0.028
γ-Glu-Gly	0.001	0.033
Ala-Ala-Ala	0.570	0.590

[a] Modification: 0.2 *M* pH 9.8 carbonate buffer with the biuret reagent. Detection: 0.2 *M* pH 9.8 carbonate buffer with and without the biuret reagent.

from FIA, for a representative amino acid, α-, β-, and γ-dipeptides, and a tripeptide are shown in Table 2. Note that the tripeptide Ala-Ala-Ala exists as the reversible electroactive biuret complex in Cu(II) containing solution. There are two conclusions to be drawn from the data. First, there is no need for copper in the detection phase once the electrode is modified in the presence of copper. Second, it is more selective to detect α- and β-dipeptides over γ-dipeptides without copper but more selective to detect all dipeptides over amino acids in the presence of copper. This point will become more convincing when results from a larger pool of samples are presented below. Then, we conclude that the biuret reagent is important for the modification and optional during operation for the control of selectivity.

Eluent pH

Four different FIA experiments were performed to determine the effect of pH on the modification and detection. Electrodes were modified at pH 8.0 and pH 9.8, with the biuret reagent at 1.2 V for 4 h at a flow-rate of 1.0 ml/min. These modified electrodes were then tested individually with both pH 8.0 and pH 9.8 eluents which also contained the biuret reagent.

From the results (not shown) from the two experiments in which the electrode is modified in one pH and used for detection at the other pH, we concluded that it is only the detection pH that makes a difference, not the modification pH. Figs. 1 (pH 9.8) and 2 (pH 8.0) show the cathodic sensitivity (cathode at 0.0 V) obtained when the anode is set at 0.8 V, 0.9 V and 1.0 V. The mobile phases are 0.1 *M* pH 9.8 carbonate buffer and 0.2 *M* pH 8.0 phosphate buffer, both with 0.1 m*M* $CuSO_4$ and 0.3 m*M* tartrate. Note that each cluster of three sensitivities is a coarse voltammogram. The negative sensitivities, in this case, are attributed to two different factors; one is the adsorption of amino acids or peptides onto

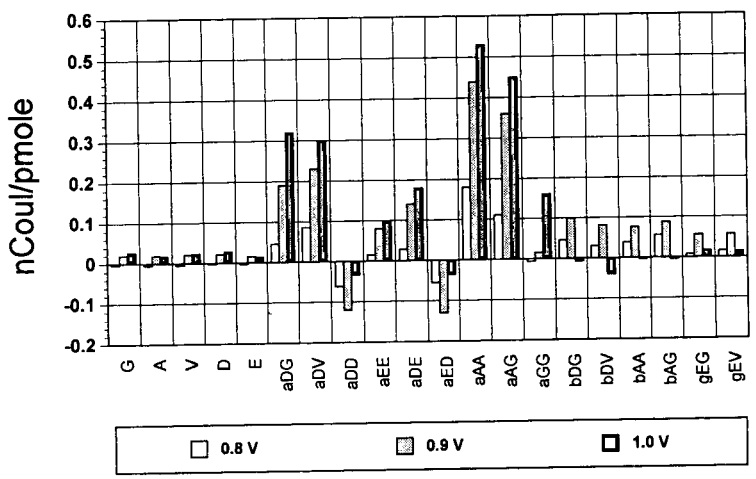


Fig. 1. Cathodic signals with the anode at 1.0, 0.9, and 0.8 V for pH 9.8. Both the modification and the detection are with the same buffer and with the biuret reagent. Modification was done at 1.4 V for 4 h at 1.0 ml/min (a = α, b = β, g = γ).

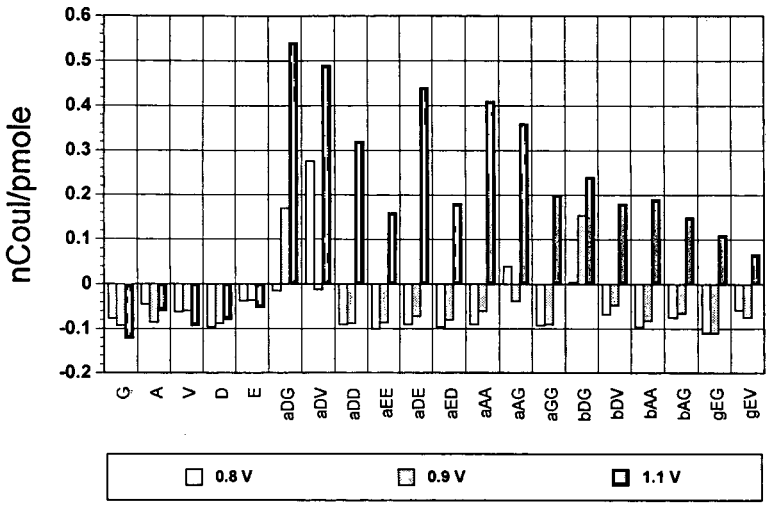


Fig. 2. Cathodic signals with the anode at 1.0, 0.9, and 0.8 V for pH 8.0. Both the modification and the detection are with the same buffer with the biuret reagent. Modification was done at 1.4 V for 4 h at 1.0 ml/min (a = α, b = β, g = γ).

the modified electrode surface as discussed before, and the other is the uptake of copper by the solutes. The onset potentials for significant background current from the Cu(II)–tartrate complex are 0.75 and 0.65 V at pH 8.0 and 10.0, respectively. If the analyte forms the Cu(II)–peptide complex and the oxidation of that complex has a higher onset potential than the Cu(II)–tartrate complex, a negative sensitivity results because of the lowering of the background signal. However, the full mechanistic aspect of this issue is still awaiting to be uncovered. To return to the figures, by comparing the two graphs with the last column of Table 1 (data obtained with an anodic potential of 0.9 V), a few conclusions are clear. In the presence of copper in the detection eluent, the modified electrode is selective for all dipeptides over amino acids at pH 8.0 with an anodic applied potential of ≥ 1.1 V (detecting at the cathode), but it is more selective for α-dipeptides over other dipeptides and amino acids at pH 9.8 with an applied anodic potential of ≥ 1.0 V. Furthermore, the modified anode becomes useful without the copper in the detection eluent, as concluded from comparison of the 0.9 V data of Table 1 and Fig. 1. We conclude in this section that the detection eluent pH and the biuret reagent are important and flexible factors in controlling the selectivity of dipeptide detection.

Modification potential and time

Table 3 gives the anodic signal at 0.85 V for different modification potentials and time at pH 8.0. The electrode is modified with a 0.1 m*M* copper and 0.3 m*M* tartrate in 0.2 *M* pH 8.0 phosphate buffer. The detection is carried out with the same solution. The data show that the increase in signal reaches a saturation point at 1.2 V for 4 h.

We conclude, in this section, that the best modification is done with a pH 8.0 buffer in the presence of copper at 1.2 V for 4 h. The detection buffer pH and the presence and absence of the biuret reagent are factors for controlling the selectivity of the system.

3.2. Hydrodynamic voltammogram by FIA

Figs. 3–5 show the hydrodynamic voltammograms (HDV) obtained by FIA with and without the modified electrode at pH 8.0 and pH 9.8 in the presence of 0.1 m*M* copper. Data plotted at potentials less than 0.4 V are the cathodic signal

Table 3
Effect of modification time and potential; anodic sensitivity (nC/pmol) at 0.85 V[a]

Compound	1.2 V			1.4 V		
	0 h	4 h	11 h	0 h	4 h	11 h
Asp	−0.003	−0.027	−0.074	−0.0005	0.000	−0.050
α-Asp-Gly	0.008	0.160	0.180	0.005	0.170	0.180
β-Asp-Gly	0.010	0.310	0.300	0.004	0.330	0.310
γ-Glu-Gly	−0.005	0.006	−0.021	−0.002	0.004	−0.019
Ala-Ala-Ala	0.710	0.910	0.730	0.690	0.920	0.840

[a] Modification: 0.1 *M* pH 8.0 phosphate buffer with biuret reagents at 1.0 ml/min. Detection: same eluent.

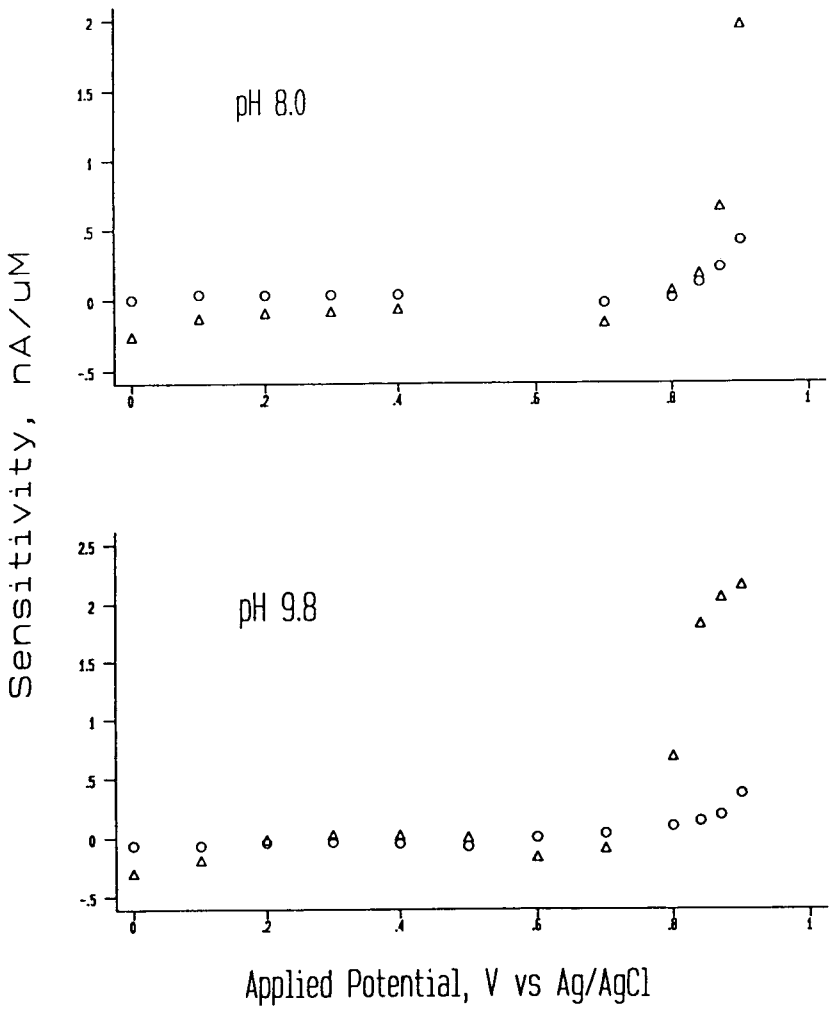


Fig. 3. Hydrodynamic voltammogram of α-Asp-Gly at both pH 8.0 and 9.8. Electrodes were modified in their respective buffers with the biuret reagent and detected with the same eluent; △ = modified electrode, and ○ = unmodified electrode.

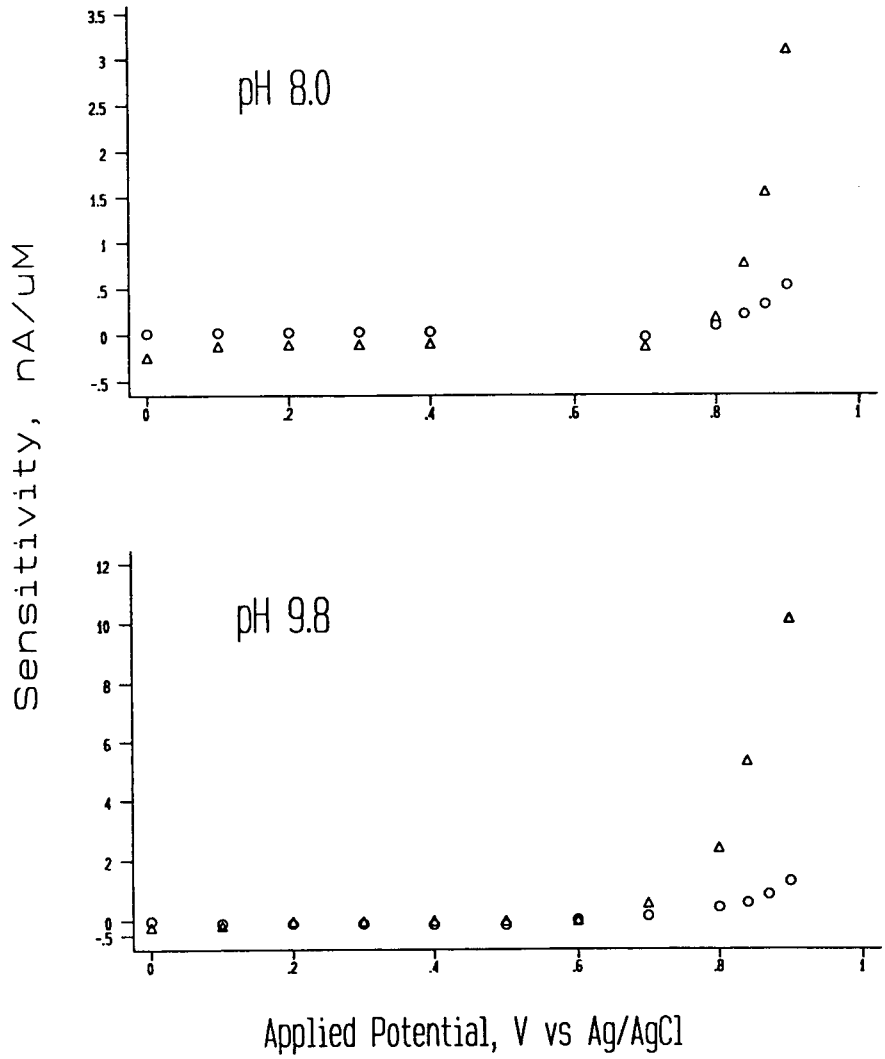


Fig. 4. Hydrodynamic voltammogram of β-Asp-Gly at both pH 8.0 and 9.8. Electrodes were modified in their respective buffers with the biuret reagent and detected with the same eluent; △ = modified electrode, and ○ = unmodified electrode.

(0 to 0.4 V) with the anode at 0.90 V. The remaining data are anode sensitivity. Comparing the modified with the unmodified electrode results reveals a dramatic shift in the onset potential of the signal-generating species upon modification. The control compound, Ala-Ala-Ala, is not affected as much by the modification as the dipeptides.

3.3. Detection limit by FIA

Fig. 6 shows the graph of the inverse of the detection limit in nM^{-1} obtained by FIA for both pH 8.0 and pH 9.8 using the cathodic signals with the anodes at 1.1 and 1.0 V, respectively. The detection limit is defined as the concentration that would give 3/5 of the peak-to-

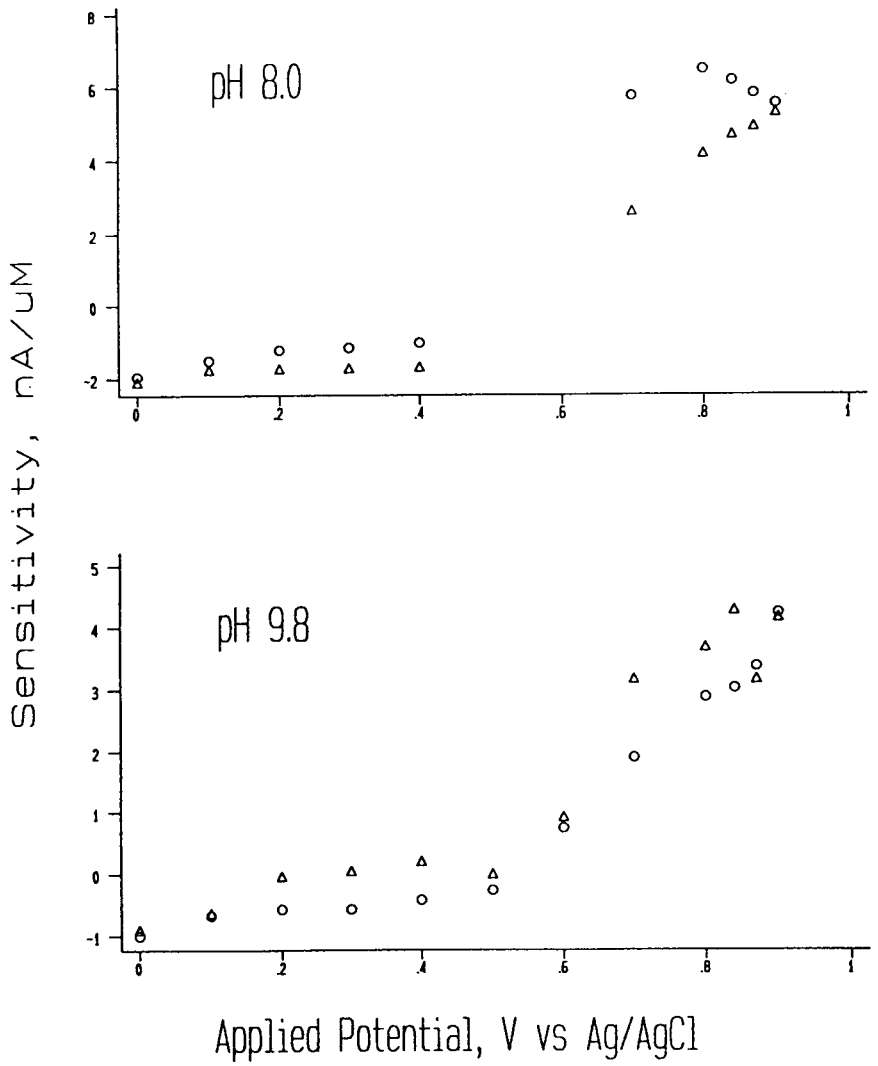


Fig. 5. Hydrodynamic voltammogram of Ala-Ala-Ala at both pH 8.0 and 9.8. Electrodes were modified in their respective buffers with the biuret reagent and detected with the same eluent; △ = modified electrode, and ○ = unmodified electrode.

peak noise which we take to be approximately five times the standard deviation of the baseline. As evidenced by the graph and the discussion above, both the detection limit and the selectivity of dipeptides over amino acids are by far superior with the pH 8.0 buffer. However, selective detection of α-dipeptides over other dipeptides is better with the pH 9.8 buffer. Lastly, we should point out that n*M* detection limits are possible for the dipeptides.

3.4. Surface chemistry

We pointed out in the last few paragraphs that the modification protocol requires copper but it is not necessarily needed for the detection. One

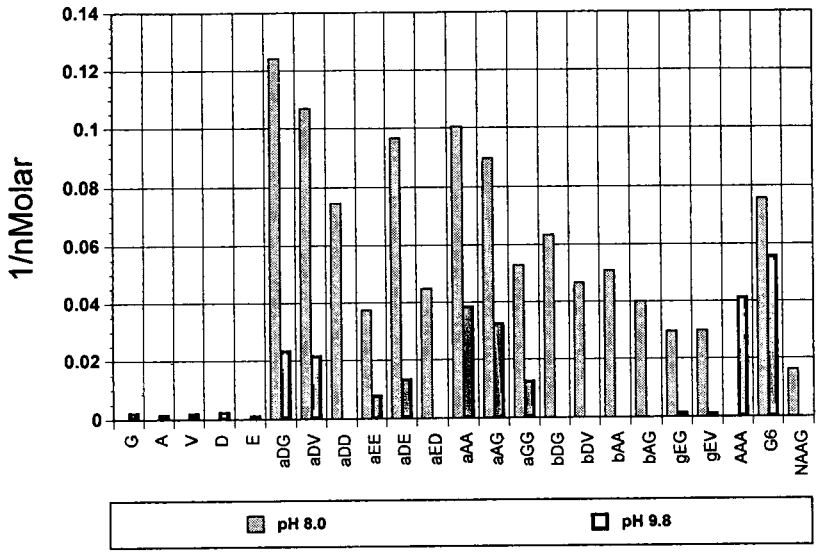


Fig. 6. Inverse of the detection limit plot, obtained at both pH 8.0 and 9.8. Both modification and detection were done in the presence of the biuret reagent (a = α, b = β, g = γ).

might expect some kind of copper salt to have been deposited on the electrode surface during modification that provides the amplification of the signal. After all, a copper electrode has been used to detect amino acids [24]. In answering this question, we turned to spectroscopy for surface analysis. Results of the ESCA spectra of the modified (bottom) and the unmodified (top) glassy carbon electrode surfaces are shown in Fig. 7. The modification was carried out in a polyethylene container with constant stirring with a pH 9.8 carbonate buffer at 1.4 V for 4 h. The control electrode was put in the same solution but without the applied potential. Modification was observed visually by the colored appearance of the surface when observed at an angle. The surprising result is that the expected copper band is not present, rather there is only a change in the O/C ratio and a silicate band that probably originated from the glass reference electrode used. The O/C ratio changes from the expected 0.31 [25] to 0.53. Oxygenation of the carbon surface has been carried out before with much higher potentials, i.e. 1.8 V [25]. The same spectroscopic results were observed with a pH 8.0 modification.

Armstrong et al. [25], studying cytochrome c voltammetry with a pyrolytic carbon electrode at pH 8.0, discovered that the addition of magnesium ion to the electrolyte solution decreased the cytochrome c signal. They attributed this to the Mg^{2+} binding to surface oxygen.

To test the importance of oxygen on the modified electrode surface, we used $MgSO_4$ in the eluent with an electrode that had been modified with a pH 8.0 buffer and biuret reagents. The results obtained with a pH 8.0 detection eluent with various amounts of Mg^{2+} added are shown in Fig. 8 for the analyte β-Asp-Gly. The data clearly demonstrate that the surface oxygen is involved in the oxidation of dipeptides.

3.5. Linearity of the modified electrode response

Fig. 9 shows the calibration curves of β-Asp-Gly obtained with a pH 8.0 modification and detected with the same eluent. Fig. 9A gives the cathodic sensitivity when the anode is at 0.85 V and 1.10 V. Good linearity was observed with the cathode (both with $r^2 > 0.99$). With the anodic signal at 0.80 and an extended concentration

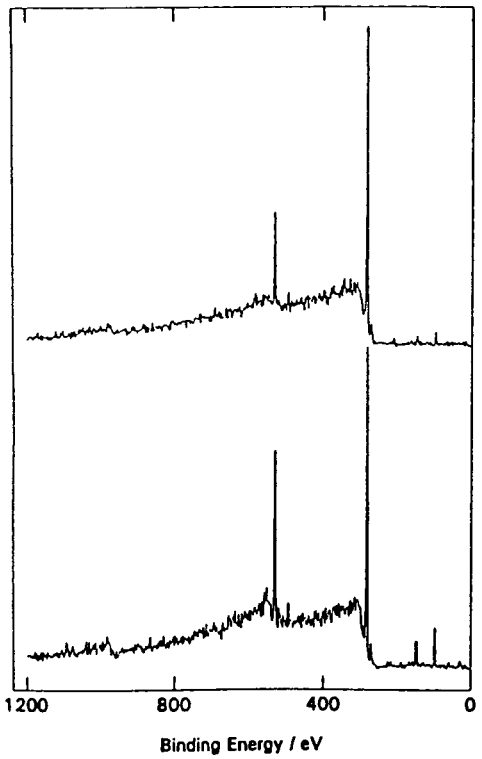


Fig. 7. ESCA spectra of the modified and the unmodified glassy carbon surface. (Top) Unmodified, and (bottom) modified, at pH 9.8 with the biuret reagent for 4 h at 1.4 V with constant stirring of the modification buffer.

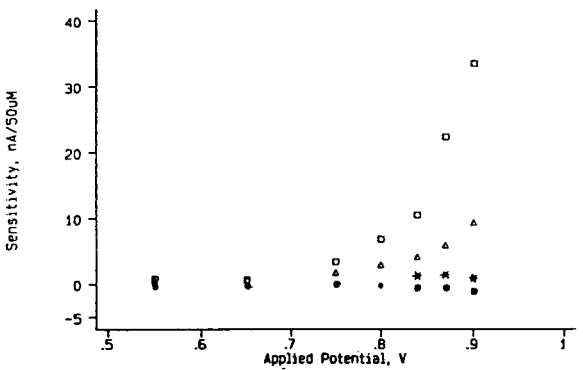


Fig. 8. Effect of Mg^{2+} on the modified GCE surface: □ = without $MgSO_4$, △ = with 3.0 m*M* $MgSO_4$, * = with 10 m*M* $MgSO_4$, and ● = no modification and without $MgSO_4$.

range, however, a curve suggestive of adsorption of peptide onto the modified electrode becomes more clear, as shown in Fig. 9B. These observations support our speculation mentioned previously that these peptides are adsorbed onto the modified electrode surface.

3.6. Example of chromatograms

Fig. 10 shows the chromatogram obtained with an electrode modified under the optimum conditions described above for dipeptides, pH 8.0 buffer with 0.1 m*M* Cu^{2+}, at 1.2 V for 4 h at a flow-rate of 1.0 ml/min. The separation was done with a Nucleosil SB anion-exchange column eluted with a 5 m*M* pH 4.6 acetate buffer and a step gradient from 1.5 m*M* to 15 m*M* Li_2SO_4 after the first 20 min. The post-column phase used is a 0.5 *M* pH 8.25 phosphate buffer with a mixing ratio of 2:1, mobile phase to post-column phase. Thus, there is no Cu^{2+} in the flow stream. An amount of 1 nmole of each of eight dipeptides and glutathione was injected, four α-, two β-, and two γ-dipeptides. In general, the detector under these conditions is much more sensitive for α- than β- than for γ-dipeptides. For example, note that there is no peak for glutathione, a γ-glutamyl tripeptide that is expected to be present in the highest concentration in biological samples.

As discussed before, the best signal-to-noise (*S*/*N*) ratio is obtained in the absence of the biuret reagent in the detection eluent, but the most selective detection for α-dipeptides over β-dipeptides, γ-dipeptides, and amino acids is obtained in the presence of the biuret reagent. The anodic signal is useful only in the absence of biuret reagent. The chromatogram shown clearly demonstrates these points. The cathodic signal in this case, however, exhibits negatively tailing peaks and would not be as useful as the anodic signal. On the other hand, with the presence of the biuret reagent, the cathodic peaks are symmetrical, have the best *S*/*N* ratio, and will be the choice for the detection. Since the surface chemistry of the modified anode is not fully understood, an explanation for the negative tailing of the downstream cathodic signals in the

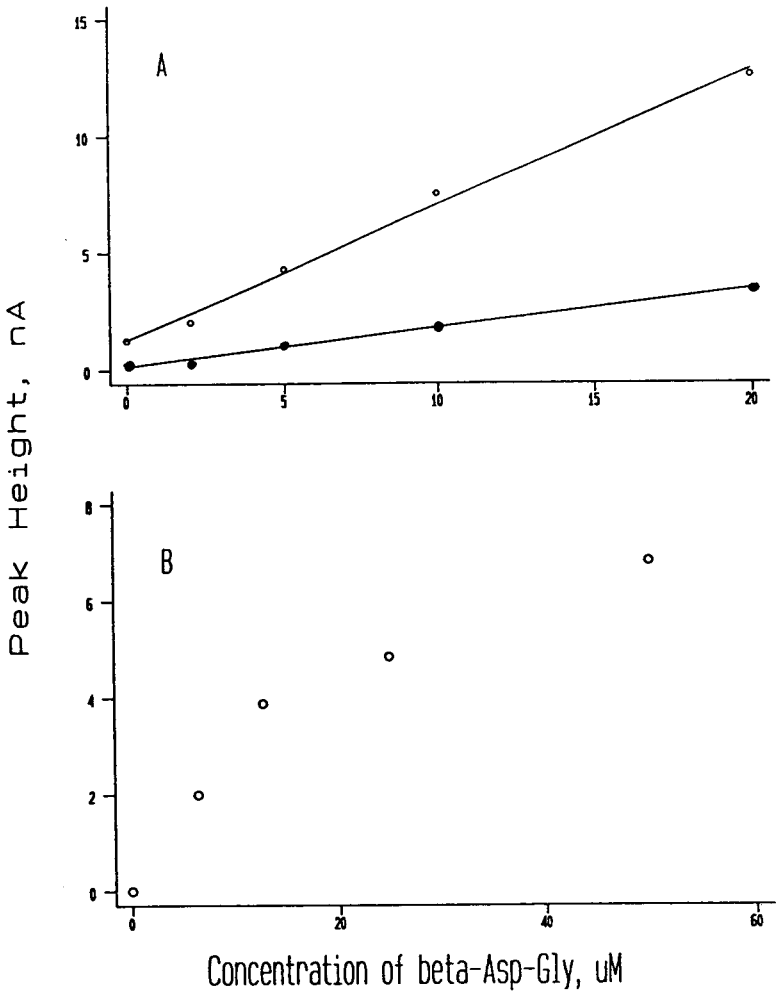


Fig. 9. Calibration curves of β-Asp-Gly with a pH 8.0 modification, cathodic signal. (A) Anode at 1.10 V (○) and at 0.85 V (●). (B) Anodic signal at 0.8 V.

absence of the biuret reagent is not attempted. However, it will be addressed when the modified electrode surface is probed in full detail by microscopy in the near future.

4. Conclusions

We have demonstrated that the modified electrode works for the sensitive and selective detection of dipeptides. The best modification is carried out at pH 8.0 eluent in the presence of the biuret reagent with an applied anodic potential of 1.2 V for 4 h at a flow-rate of 1.0 ml/min. Selectivity can be altered by means of controlling both the pH of the detection eluent and the presence and the absence of the biuret reagent. Surface analysis shows that it is the oxygenation, in the presence of the copper ion during the modification, that is responsible for the selective detection. The detection system exhibits n*M* detection limits. Future work will be

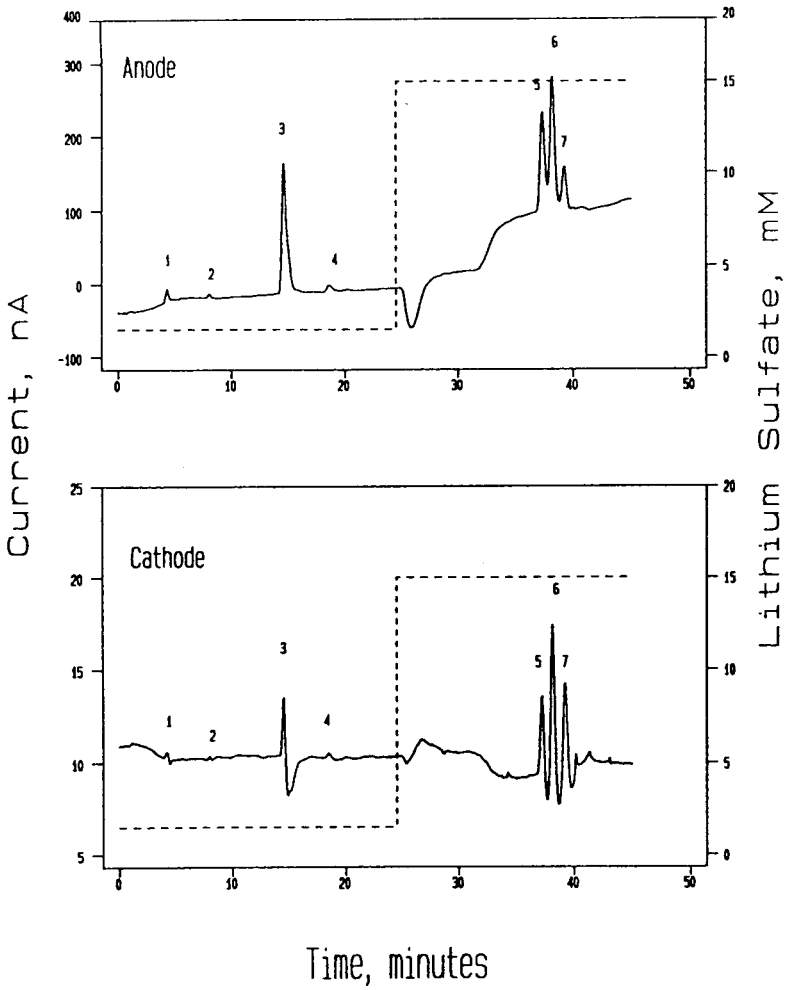


Fig. 10. Chromatograms of 1.0 nmole of eight acidic dipeptides and glutathione obtained with a pH 8.0 modified electrode. Mobile phase: 5.0 m*M* pH 4.6 acetate buffer with a step gradient of Li_2SO_4 as shown by the dotted line at 0.8 ml/min. Post-column phase: 0.5 *M* phosphate–acetic acid pH 8.25 buffer at 0.4 ml/min. Applied potentials are 1.1 V and 0.0 V for the anode and the cathode, respectively. Peaks: 1 = excess acetate, 2 = β-Asp-Tau, 3 = β-Asp-Gly, 4 = γ-Glu-Cys, 5 = α-Asp-Asp, 6 = α-Glu-Asp and α-Asp-Glu, 7 = α-Glu-Glu. No peak was observed for glutathione. See text for more details.

focused on the biological applications of the system.

Acknowledgements

We are grateful to the NIH for financial support through Grant GM44842, to Dr. Andy Proctor for taking the ESCA spectra, and to professor R.L. McCreery for pointing out the Mg^{2+} experiment of Armstrong et al. [Ref. 25].

References

[1] A.A. Francis, A.W. Jones and J.C. Watkins, J. Neurochem., 35 (1980) 1458.

[2] V. Varga, R. Janaky, R.M. Marnela, J. Gulyas, P. Kontro and S.S. Oja, Neurochem. Res., 14 (1989) 1223.
[3] V. Varga, R. Janaky and S.S. Oja, Neurosci. Lett., 138 (1992) 270.
[4] X. Li, O. Orwar, J. Persson, M. Sandberg and I. Jacobsson, Neurosci. Lett., 155 (1993) 42.
[5] C. Mathis, J. De Barry and A. Ungerer, Eur. J. Pharmacol., 185 (1990) 53.
[6] D.L. Buchanan, E.E. Haley and R.T. Markiw, Biochem., 1 (1962) 612.
[7] P. Andiné, O. Orwar, I. Jacobson, M. Sandberg and H. Hagberg, J. Neurochem., 57 (1991) 230.
[8] A.M. Warner and S.G. Weber, Anal. Chem., 61 (1989) 2664.
[9] A.F. Spatola and D.E. Benovitz, J. Chromatogr., 327 (1985) 165.
[10] W.S. III Richardson and L. Burns, J. Chem. Educ., 65 (1988) 162.
[11] K. Tellerova, P. Spacek and M. Adam, J. Chromatogr., 273 (1983) 197.
[12] S.G. Weber, H. Tsai and M. Sandberg, J. Chromatogr., 638 (1993) 1.
[13] R.R. Venn, J. Chromatogr., 423 (1987) 93.
[14] V. Clement-Jones, P.J. Lowry, L.H. Ree and G.M. Besser, Nature, 283 (1980) 295.
[15] O. Orwar, S. Folestad, S. Einarsson, P. Andiné and M. Sandberg, J. Chromatogr., 566 (1991) 39.
[16] J.M. Klancnik, M. Cuenod, B.H. Gahwiler, Z.P. Jiang and K.Q. Do, Neuroscience, 49 (1992) 557.
[17] A. Baba, S. Yamagami, H. Mizuo and H. Iwata, Anal. Biochem., 101 (1980) 288.
[18] I.C. Kilpatrick, S.J., Waller and R.H. Evans, TIPS, 12 (1991) 95.
[19] S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele, Science, 178 (1972) 871.
[20] H. Tsai and S.G. Weber, J. Chromatogr., 515 (1990) 451.
[21] J.W. Mehl, E. Pacovska and R.J. Winzler, J. Biol. Chem., 15 (1949) 13.
[22] D.A. Roston, R.E. Shoup and P.T. Kissinger, Anal. Chem., 54 (1982) 1417A.
[23] K. Ienaga, K. Nakamura, K. Higashiura, Y. Toyomaki and H. Kimura, Chem. Pharm. Bull., 36 (1988) 2796.
[24] P. Luo, F. Zhang and R.P. Baldwin, Anal. Chem., 63 (1991) 1702.
[25] I.-F. Hu, D.H. Karwek and T. Kuwana, J. Eletroanal. Chem., 188 (1985) 59.
[26] F.A. Armstrong, P.A. Cox, H.O.A. Hill, V.J. Lowe and B.N. Oliver, J. Electroanal. Chem., 217 (1987) 331.

ELSEVIER

Journal of Chromatography A, 705 (1995) 185–194

JOURNAL OF CHROMATOGRAPHY A

Broadly applicable polysiloxane-based chiral stationary phase for high-performance liquid chromatography and supercritical fluid chromatography

Gerald J. Terfloth[a,1], William H. Pirkle[a,*], Kenneth G. Lynam[b], Edgar C. Nicolas[b]

[a]*School of Chemical Sciences, Department of Chemistry, University of Illinois at Urbana-Champaign, 1209 West California Street, Urbana, IL 61801-3731, USA*

[b]*DuPont Merck, Deepwater, NJ 08023, USA*

First received 7 September 1994; revised manuscript received 14 February 1995; accepted 21 February 1995

Abstract

A recently reported broad-spectrum chiral selector and two of its homologs were incorporated into polymethylhydrosiloxane and each of the polymers was immobilized on silica gel to afford chiral stationary phases (CSPs) useful for the separation of the enantiomers of a wide variety of compounds by HPLC and/or supercritical fluid chromatography (SFC). These CSPs are stable toward normal-phase, reversed-phase, and sub/supercritical fluid conditions and are not adversely affected by addition of carboxylic acids or amines as mobile-phase modifiers. These polymeric CSPs show higher efficiencies and shorter analysis times when used with sub/supercritical carbon dioxide then when used in the HPLC mode. Additionally, some separations are achievable using sub/supercritical carbon dioxide but not when the same column is used in the HPLC mode.

1. Introduction

Guidelines for the development of new drugs require the development of efficient methods for the analysis and preparation of enantiomerically pure compounds [1]. In this regard, chiral stationary phases (CSPs) are proving to be extremely useful. CSPs are used to monitor asymmetric syntheses, enzymatic transformations, and classical resolutions. They are used to follow the stereochemical preferences of metabolism and to monitor the concentrations of individual enantiomers in patients undergoing treatment. CSPs not only provide a means for determining enantiomeric purity and absolute configuration, they also provide the means to preparatively resolve racemates. Increasingly, they are being used for the preparation of stereochemically pure compounds, an application which will become commonplace with the adoption of simulated moving bed chromatography, a technique especially well suited to the separation of enantiomers.

Most workers would prefer not to have to derivatize analytes prior to chromatography. As improved CSPs are developed, the need for

* Corresponding author.

[1] Present address: SmithKline and Beecham Pharmaceuticals, King of Prussia, PA 19409, USA.

0021-9673/95/$09.50
SSDI 0021-9673(95)00246-4

prederivatization is being diminished. For example, a chiral selector developed recently in these laboratories has been used for the resolution of a wide variety of underivatized enantiomers including (but not limited to) α-aryl propionic acids, epoxides, aziridines, sulfoxides, α-aryloxypropionic acids, diols, dihydropyridines, atropisomeric compounds, heterocycles, and a wide variety of esters and amides [2–5]. Simple prederivatization with inexpensive achiral reagents containing chromophores to simplify detection extends the scope of this CSP even further. Recently, the commercial version of this brush-type CSP, the WHELKO-1 [6], has been used with sub/supercritical carbon dioxide to separate the enantiomers of several profens and of several other compounds of medicinal interest [7,8]. In this paper, we report the incorporation of this chiral selector and two of its homologs into polymethylhydrosiloxanes by a well-precedented procedure [9–24]. The resulting chiral polymers have been coated on silica gel, immobilized by heating, packed into HPLC columns by conventional means and used in both the LC and SFC modes.

The use of sub/supercritical carbon dioxide as a mobile phase offers several significant and previously recognized advantages as described in recent reviews [25–28]. Solvent strength can be adjusted by controlling temperature and pressure. Owing to the low viscosity of carbon dioxide, diffusion rates are increased and column efficiencies are increased considerably [6,29]. The low viscosity allows one to use higher flowrates than might be used for liquid chromatography with the same column without giving rise to undue back pressures or to appreciable efficiency loss. This results in shorter analysis times. The low viscosity also allows one to couple several identical columns in series so as to increase the number of theoretical plates available in the case of difficult separations [30]. Coupling dissimilar chiral columns together for the SFC separation of enantiomers has also been demonstrated [31], a subject worthy of discussion in a later paper. Finally, the use of carbon dioxide reduces or avoids the use of mobile-phase components which may be flammable or environmentally harmful. While some investigators have been aware of these advantages for some time, what may not yet be widely appreciated by chromatographers in general is the potential for heretofore unknown scope, selectivity, convenience, and speed when stereochemical analyses are conducted by sub/supercritical chromatography with carbon dioxide on robust, well-designed chiral selectors. It is evident that these selectors can be incorporated into polysiloxanes in such a way as to equal or exceed the levels of chromatographic efficiency typically encountered with brush-type stationary phases.

2. Experimental

Chromatography was carried out using a Hewlett-Packard supercritical fluid chromatograph equipped with an HP 7673 autosampler (5-μl sample loop), an HP 1050 diode-array detector, and an HP Vectra 486/66u personal computer running the HP ChemStation software. The columns were kept at constant temperature in the instrument's column oven. Tri-*tert.*-butylbenzene was used as a void volume marker. Samples (available from prior studies) were dissolved in methanol at a concentration of 0.5 mg/ml. Carbon dioxide was of SFC grade (Scott Specialty Gases, Plumsteadville, PA, USA). The organic modifiers were HPLC grade solvents (EM Science, Gibbstown, NJ, USA).

2.1. Preparation of the csps

The chiral selectors were prepared in the manner described [2,3] and incorporated into the polysiloxane by the method used by Röder et al. [10]. The chiral polymers were coated onto silica gel (5 μm, 300 Å pore size) and immobilized by heating to 100°C for 24 h under reduced pressure. The CSPs were packed into stainless-steel columns (250 × 4.6 mm I.D.) as methanol slurries. Residual silanol groups were end-capped by passing a solution of 2 ml of hexamethyldisilazane in 50 ml of dichloromethane

through a dichloromethane equilibrated column at a flow-rate of 1.0 ml/min.

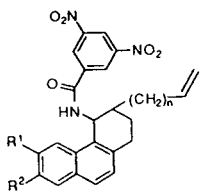
3. Results and discussion

The chiral selector and its two homologs were prepared as the racemates and resolved in the manner described [2]. Incorporation of these selectors into the polymers was done by hydrosilylation of the terminal double bonds with the polymethylhydrosiloxane (Fig. 1). Coating of the chiral polymers onto silica gel of 300 Å pore size, 5 μm particle size, with subsequent immobilization afforded CSPs 1–3. The structural features of the columns are summarized in Table 1. Selectors with different structures were used to study how methyl substituents on the naphthyl of the selector and how the length of the tether influence chromatographic performance. Similar studies have been conducted for brush-type CSPs prepared from these selectors [2–4]. In the reciprocal situation, chromatography of these selectors on an (*S*)-naproxen diallyl amide-derived brush-type CSP has shown that the methyl substituents increase the separation factor of the enantiomers, presumably by increasing their π-basicity [5].

Previous work has shown that incorporation of the chiral selector into a polysiloxane has led to CSPs having decreased retention times relative to those obtained by bonding the same selector to the same support in a brush-type fashion [11–13].

Polymeric CSPs 1–3 are stable under normal-phase and sub/supercritical fluid conditions and do not exhibit any loss of efficiency or selectivity after several months of hard usage. A series of

Table 1
Polysiloxane-based chiral stationary phases 1–3

CSP	Selector[a]			Loading (mmol selector/g CSP)
				
	R^1	R^2	n	
1	H	H	3	0.18
2	H	CH_3	3	0.14
3	CH_3	CH_3	9	0.18

[a] The enantiomers having the (*R*,*R*)-configurations were used for the preparation of CSPs 1–3.

α-arylpropionic acids and various other chiral compounds were used to evaluate the performance of these polysiloxane-derived CSPs by HPLC and SFC. The chromatographic results obtained on CSPs 1–3 (Table 1) using a mobile phase of 10% 2-propanol containing 0.2% acetic acid in carbon dioxide at a pressure of 200 bar and a temperature of 25°C are summarized in Table 2. It should be noted that the mobile phase is subcritical under these conditions. Rapid separations of the enantiomers of α-arylpropionic acids on CSP 1 are achieved under these conditions (Table 2). These enantiomers can be separated without an acidic modifier in the mobile phase (Table 3), although addition of such a modifier may improve band shapes somewhat. Using the corresponding brush-type CSP

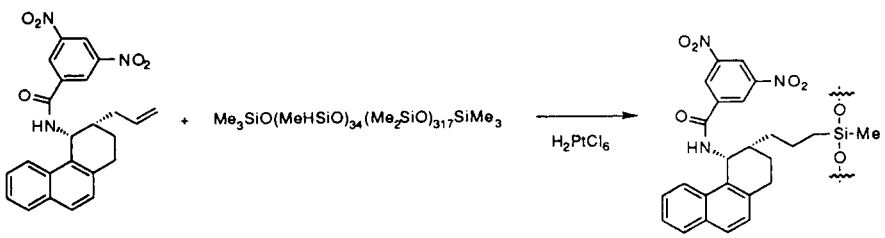


Fig. 1. Formation of the sidechain-modified polysiloxane by hydrosilylation.

Table 2
SFC separations of the enantiomers of arylpropionic acids on CSPs 1–3 using an acidic modifier

Sample		CSP 1	CSP 2	CSP 3
Naproxen	k_1'	1.25	0.84	0.69
	α	2.28	2.21	1.61
Ibuprofen	k_1'	0.09	0.07	0.11
	α	1.44	1.00	1.00
Fenoprofen	k_1'	0.14	0.14	0.21
	α	1.71	1.50	1.00
Flurbiprofen	k_1'	0.18	0.24	0.29
	α	1.39	1.00	1.00
Cicloprofen	k_1'	1.29	0.96	0.81
	α	1.75	1.70	1.37
Pirprofen	k_1'	0.83	0.69	0.73
	α	1.40	1.38	1.00
Carprofen	k_1'	3.09	2.72	3.50
	α	1.79	1.55	1.00
Etodolac	k_1'	0.49	0.51	0.44
	α	1.20	1.14	1.00

Conditions: 10% (0.2% acetic acid) 2-propanol in carbon dioxide, 200 bar, 25°C, 2.0 ml/min, UV detection at 220 nm.

in the HPLC mode, the enantiomers of the α-arylpropionic acids can also be separated without an acidic modifier, but band shapes are poorer than those afforded by the polysiloxane-based CSP. This result either indicates that carbon dioxide suppresses superfluous interactions of the polar analytes with the silica surface or that most of these sites are buried beneath the polymer and are no longer accessible to the analytes.

The separation of several pairs of enantiomers on CSPs 1 and 2 by HPLC using 5% (0.5% acetic acid) ethanol in *n*-hexane are documented in Table 4. Comparison of the chromatographic results obtained on CSP 1 by HPLC (Table 4) and by SFC (Table 2) shows that retention in SFC was greater even though a higher concentration of the polar modifier was used. Furthermore, the enantiomers of all analytes are observed to be separated by SFC, whereas three analytes fail to resolve by HPLC under the indicated conditions. Surprisingly, methyl substituents on the naphthyl system of the chiral selector (CSP 2) do not improve enantioselectivity for these analytes in SFC as they do in HPLC (Table 2). Increasing the tether length is detrimental, for CSP 3 affords separations of just two of these analytes under these same conditions (Table 2). Use of 5% (0.5% acetic acid) ethanol in *n*-hexane as a mobile phase essentially eliminates all retention on CSP 3 (Table 4). The

Table 3
SFC separations of the enantiomers of arylpropionic acids on CSP 1 without use of an acidic modifier

Sample	k_1'	α
Naproxen	2.35	2.00
Ibuprofen	0.25	1.24
Fenoprofen	0.33	1.42
Flurbiprofen	0.46	1.22
Cicloprofen	2.91	1.58
Pirprofen	1.95	1.31
Carprofen	not eluted after 25 min	
Etodolac	1.64	1.14

Conditions: 5% 2-propanol in carbon dioxide, 200 bar, 40°C, 1.0 ml/min, UV detection at 220 nm.

Table 4
HPLC separation of the enantiomers of arylpropionic acids on CSPs 1 and 2

Sample		CSP 1	CSP 2
Naproxen	k_1'	0.26	0.25
	α	2.27	2.32
Ibuprofen	k_1'	0.14	0.23
	α	2.00	1.00
Fenoprofen	k_1'	0.04	0.06
	α	1.00	1.00
Flurbiprofen	k_1'	0.03	0.21
	α	1.00	1.00
Cicloprofen	k_1'	0.18	0.18
	α	1.83	1.00
Pirprofen	k_1'	0.12	0.21
	α	1.50	1.00
Carprofen	k_1'	0.10	0.49
	α	2.80	1.39
Etodolac	k_1'	0.01	0.06
	α	1.00	1.00

Conditions: 5% (0.5% acetic acid) ethanol in *n*-hexane, 25°C, 2.0 ml/min, UV detection at 220 nm.

values given in Table 4 are those determined, However, one should be aware that, when k_1 is quite small, slight errors in determining the void volume of the column significantly affect the apparent values of k_1 and α. Moreover, when the sample is introduced in a solvent other than the mobile phase (as in this study), the k_1 values of weakly retained substances can be significantly affected [32]. Reducing the concentration of the polar modifier concentration increases retention and one now begins to separate the enantiomers of naproxen, cicloprofen, and carprofen (Table 5).

Comparison of the chromatographic data obtained for the α-arylpropionic acids in SFC and HPLC clearly shows that the enantiomers of more of the analytes can be separated by SFC with a single mobile phase than is the case for LC. This suggests that enantiomer separation is more likely to be encountered on the first try if SFC is used. Increasing the length of the tether or adding methyl substituents on the naphthyl group of the selector reduces or destroys enantioselectivity for these analytes. In the corresponding brush-type CSPs increased tether length decreases selectivity, whereas the added methyls improve selectivity. The present observation that the ring methyls are detrimental is surprising and no explanation is offered at present. This behavior may prove to be analyte specific for, on this CSP, the chiral recognition process is more complex for the profens than for most other analytes [33] and separation factors

Table 5
HPLC separation of the enantiomers of arylpropionic acids on CSPs 2 and 3 using a lower modifier concentration

Sample		CSP 2	CSP 3
Naproxen	k_1'	0.99	0.67
	α	2.36	1.72
Ibuprofen	k_1'	0.58	0.33
	α	1.00	1.00
Fenoprofen	k_1'	0.57	0.38
	α	1.00	1.00
Flurbiprofen	k_1'	0.01	0.49
	α	7.00[a]	1.00
Cicloprofen	k_1'	0.56	0.94
	α	1.93	1.78
Pirprofen	k_1'	0.40	0.90
	α	1.70	1.00
Carprofen	k_1'	5.32	4.40
	α	1.74	1.37
Etodolac	k_1'	0.33	0.73
	α	1.76	1.00

[a] This separation factor is artificially high due to the very rapid elution of the less retained enantiomer.
Conditions: 1% (0.5% acetic acid) ethanol in *n*-hexane, 25°C, 2.0 ml/min, UV detection at 220 nm.

Table 6
SFC separation of the enantiomers of several test compounds on CSPs 1-3 at a flow-rate of 2.0 ml/min

Sample		CSP 1	CSP 2	CSP 3
trans-Stilbene oxide	k_1'	0.28	0.18	0.09
	α	3.57	3.35	3.5
Styrene oxide	k_1'	0.07	0.05	0.0
	α	1.7	1.00	1.
Phenyl methyl sulfoxide	k_1'	0.92	0.95	0.45
	α	1.41	1.25	1.20
Warfarin	k_1'	3.29	2.41	1.07
	α	2.04	1.85	1.84
Benzoin	k_1'	0.29	0.21	0.09
	α	3.04	2.74	3.0
Abscissic acid	k_1'	0.40	0.37	0.32
	α	1.45	1.30	1.00

Conditions: 10% 2-propanol in carbon dioxide, 200 bar, 25°C, 2.0 ml/min.

Table 7
SFC separation of the enantiomers of several compounds on CSP 1 using a flow-rate of 4.0 ml/min

Sample	k_1'	α
trans-Stilbene oxide	0.26	3.65
Styrene oxide	0.06	1.8
Phenyl methyl sulfoxide	1.43	1.24
Warfarin	3.05	2.05
Benzoin	0.25	3.12
Abscissic acid	0.36	1.45

Conditions: 10% 2-propanol in carbon dioxide, 200 bar, 25°C, 4.0 ml/min.

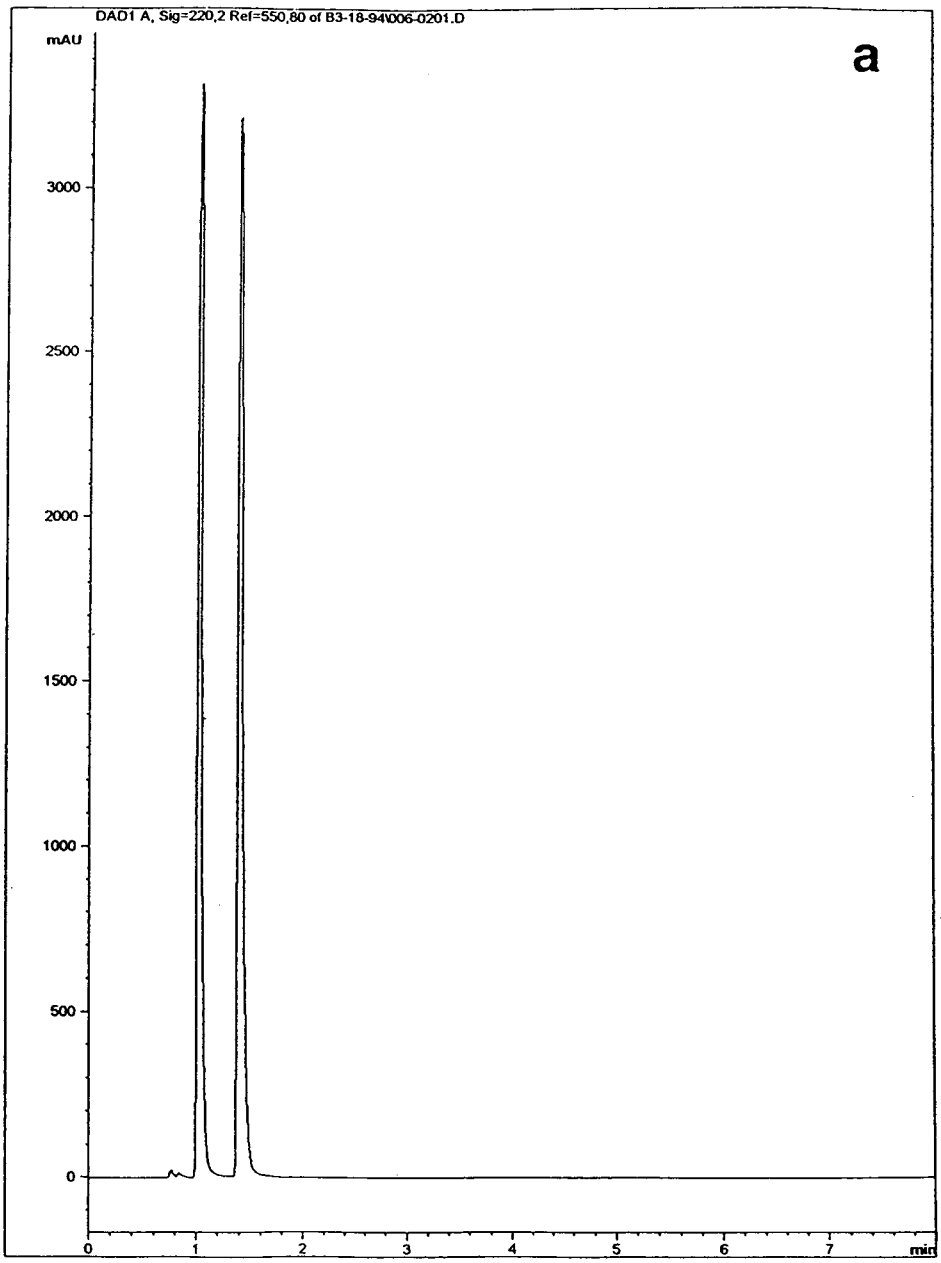


Fig. 2. Chromatograms of (a) *trans*-stilbene oxide, (b) warfarin, and (c) benzoin, obtained using CSP 1 with subcritical carbon dioxide as described in Table 6.

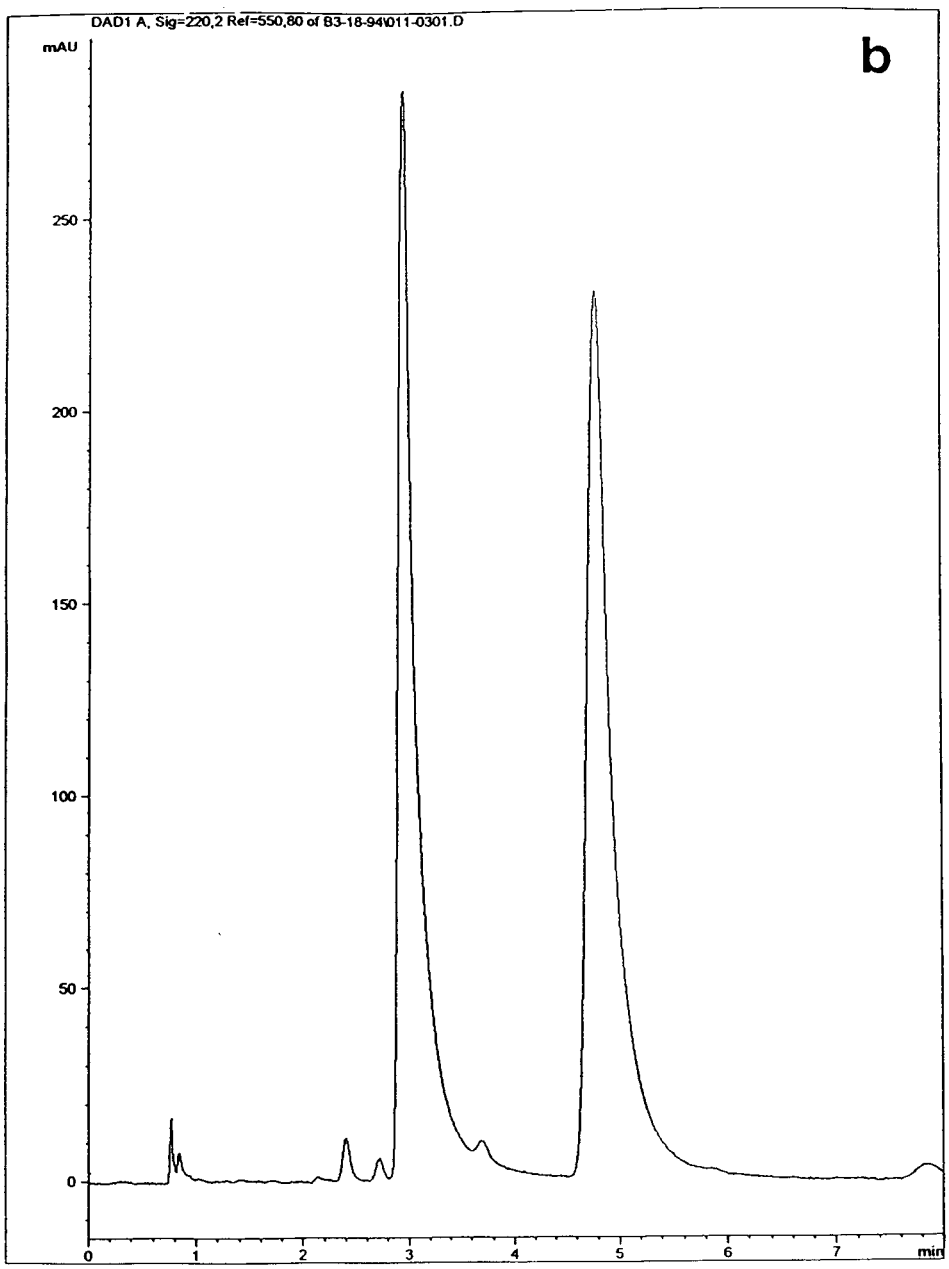


Fig. 2 (*continued*).

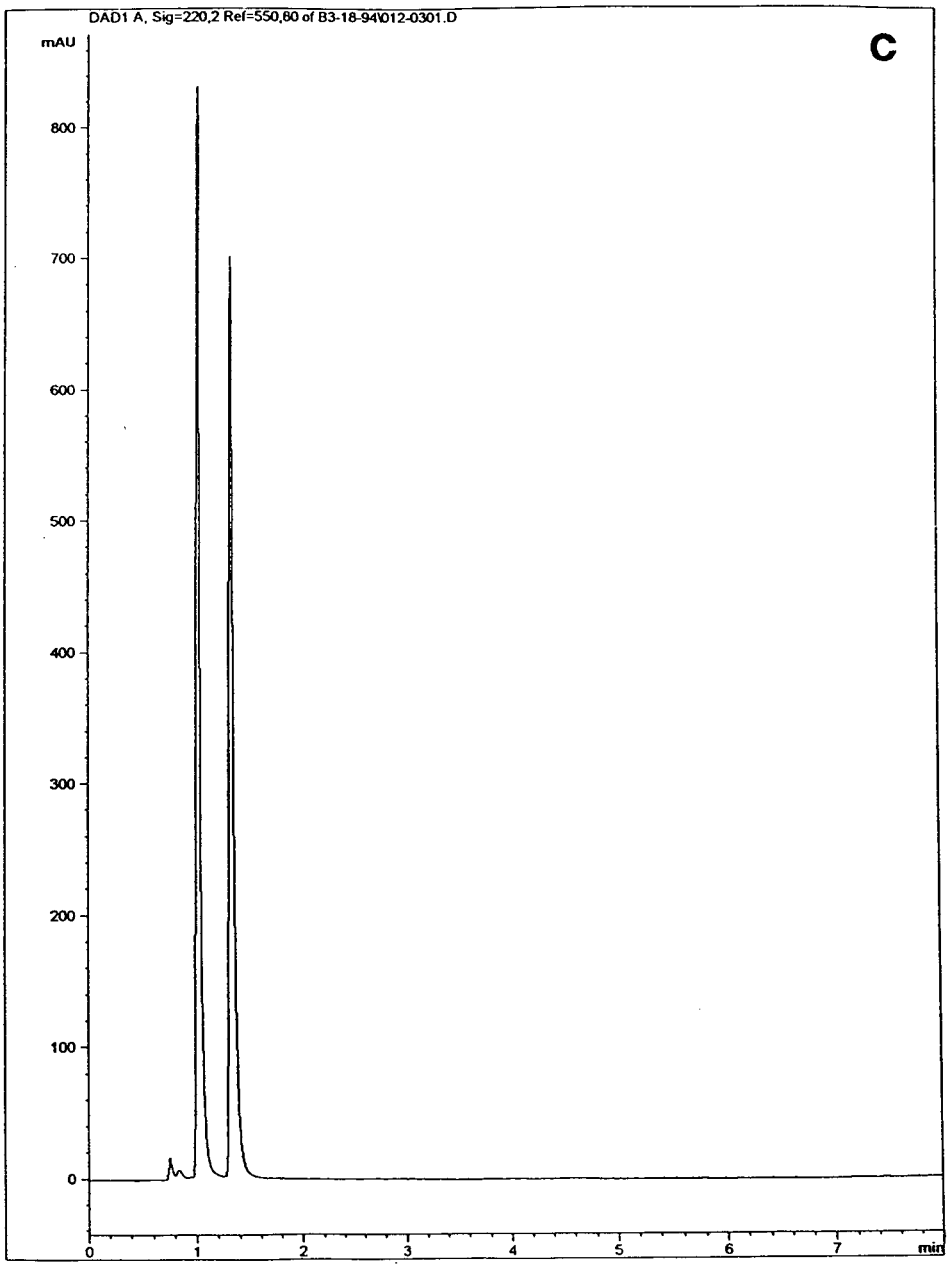


Fig. 2 (*continued*).

are unusually dependent on mobile-phase composition.

The enantiomers of several other racemic analytes were separated on CSPs 1–3 using 10% 2-propanol in carbon dioxide at a pressure of 200 bar and a temperature of 25°C (Table 6). Owing to the low viscosity of the mobile phase, the flow-rate could be increased to 4.0 ml/min to further reduce the time needed for separation (Table 7). Typical chromatograms obtained on CSP 1 are given in Fig. 2.

The use of these polymeric CSPs is not limited to analytical separations. In a preliminary study, a baseline separation of the enantiomers of 5 mg of racemic warfarin was obtained on an analytical column containing CSP 1.

5. Conclusion

Chiral selectors which have proven to be of great scope when used in brush-type CSPs have been incorporated into polysiloxanes and immobilized on silica gel. Columns packed with these materials were used for both the HPLC and SFC separation of the enantiomers of a series of compounds. The use of carbon dioxide as the mobile phase improves column efficiency and reduces analysis times, often to the 2–3 min range. In several instances (to be addressed in a later paper), resolutions were achieved by SFC that could not be achieved by HPLC.

Acknowledgements

The authors gratefully acknowledge the support provided by EM Science, Regis Chemical Company, the National Science Foundation, and the Deutsche Forschungsgemeinschaft (Postdoctoral Fellowship to GJT).

References

[1] Guidelines For Submitting Supporting Documentation in Drug Applications for the Manufacture of Drug Substances. Office of Drug Evaluation and Research (HFD-100), Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, p. 6.
[2] W.H. Pirkle, C.J. Welch and B. Lamm, J. Org. Chem., 57 (1992) 3854.
[3] W.H. Pirkle and C.J. Welch, J. Liq. Chromatogr., 15 (1992) 1947.
[4] W.H. Pirkle and C.J. Welch, Tetrahedron Asymm., 5 (5) (1994) 777.
[5] W.H. Pirkle and G.J. Terfloth, J. Chromatogr. A, in press.
[6] Regis Technologies Inc., 8210 Austin Ave., Morton Grove, IL 60053.
[7] A.M. Blum, K.G. Lynam and E.C. Nicholas, Chirality, 6 (1994) 302.
[8] W.H. Wilson, Chirality, 6 (1994) 216.
[9] M. Schleimer, V. Schurig and W.H. Pirkle, J. Chromatogr. A, 679 (1994) 23.
[10] W. Röder, F.-J. Ruffing, G. Schomburg and W.H. Pirkle, J. High Resolut. Chromatogr., Chromatogr. Commun., 10 (1987) 665.
[11] F.-J. Ruffing, J.A. Lux, W. Röder and G. Schomburg, Chromatographia, 26 (1988) 19.
[12] V. Schurig and H.-P. Nowotny, Angew. Chem., Int. Ed. Engl., 29 (1990) 939.
[13] D.F. Johnson, J.S. Bradshaw, M. Eguchi, B.E. Rossiter, M.L. Lee, P. Petersson and K.E. Markides, J. Chromatogr., 594 (1992) 283.
[14] J.S. Bradshaw, G.-L. Yi, B.E. Rossiter, S.L. Reese, P. Petersson, K.E. Markides and M.L. Lee, Tetrahedon Lett., 34 (1993) 79.
[15] G. Yi, J.S. Bradshaw, B.E. Rossiter, A. Malik, W. Li and M.L. Lee, J. Org. Chem., 58 (1993) 4844.
[16] M. Jung and V. Schurig, J. High Resolut. Chromatogr., 16 (1993) 289.
[17] D. Schmalzing, M. Jung, S. Mayer, J. Rickert and V. Schurig, J. High Resolut. Chromatogr., 15 (1992) 723.
[18] C. Bicchi, G. Artuffo, A. D'Amato, V. Manzin, A. Galli and M. Galli, J. High Resolut. Chromatogr., 16 (1993) 209.
[19] Y. Dobashi, K. Nakamura, T. Saeki, M. Matsuo, S. Hara and A. Dobashi, J. Org. Chem., 56 (1991) 3299.
[20] V. Schurig, D. Schmalzing, U. Muehleck, M. Jung, M. Schleimer, P. Mussche, C. Duvekot and J.C. Buyten, J. High Resolut. Chromatogr., 13 (1990) 713.
[21] P. Fischer, R. Aichholz, U. Boelz, M. Juza and S. Krimmer, Angew. Chem., 102 (1990) 439.
[22] M.B. Saporovskaya, M.M. Volkova and V.A. Pavlov, Zh. Anal. Khim., 44 (1989) 525.
[23] K. Nakamura, S. Hara and Y. Dobashi, Anal. Chem., 61 (1989) 2121.
[24] K. Ernst-Cabrera and W.A. Koenig, React. Polym. Ion Exch. Sorbents, 6 (1987) 267.
[25] C.F. Poole, J.W. Oudsema, T.A. Dean and S.K. Poole, in B. Wenclawiak (Editor), Analysis with Supercritical Fluids: Extraction and Chromatography, Springer-Verlag, Berlin, 1992, p. 116.
[26] M. Schleimer and V. Schurig, in B. Wenclawiak (Editor), Analysis with Supercritical Fluids: Extraction and Chromatography, Springer-Verlag, Berlin, 1992, p. 134.

[27] P. Petersson and K.E. Markides, J. Chromatogr. A, 666 (1994) 381.
[28] K. Anton, J. Eppinger, L. Frederiksen, E. Francotte, T.A. Berger and W.H. Wilson, J. Chromatogr. A, 666 (1994) 395.
[29] R.W. Stringham, K.G. Lynam and C.C. Grasso, Anal. Chem., 66 (1994) 1949.
[30] T.A. Berger and W.H. Wilson, Anal. Chem., 65 (1993) 1451.
[31] P. Sandra, A. Kot and F. David, Chimica Oggi, 12 (1994) 33–36.
[32] M. Zeif, in M. Zeif and L.J. Crane (Editors), Chromatographic Chiral Separations, Marcel Dekker, New York, NY, 1988, p. 323.
[33] W.H. Pirkle and C.J. Welch, J. Chromatogr. A, 683 (1994) 347.

ELSEVIER

Journal of Chromatography A, 705 (1995) 195–203

JOURNAL OF CHROMATOGRAPHY A

On-line dialysis with high-performance liquid chromatography for the automated preparation and analysis of sugars and organic acids in foods and beverages☆

Eric Vérette*, François Qian, Fabrice Mangani

Gilson Medical Electronics (France) S.A., 72 rue Gambetta, BP 45, 95400 Villiers-le-Bel, France

First received 12 May 1994; revised manuscript received 16 February 1995; accepted 24 February 1995

Abstract

A quick, simple and robust technique is described for on-line clean-up and analysis of raw liquid food samples containing complex matrices such as dairy products, soft drinks, and fermented beverages. A completely automated sample preparation system (ASTED XL) provides an efficient way of removing macromolecular and microparticulate interferents by high-performance dialysis, prior to HPLC analysis of the sugars, organic acids and related compounds. Processing samples on-line in the concurrent mode permitted both high reproducibility and optimal throughput.

1. Introduction

Determination of sugars and organic acids is of great importance in the food industry. The goals of such analytical work are various, but are mainly oriented towards quality control at all stages of production, from receipt of raw materials, throughout processing, to the final products.

Several HPLC techniques have been proposed to analyze, in most cases separately, sugars and organic acids. Sugars are generally analyzed using an NH_2-bonded silica column [1,2]. Organic acids are analyzed using ion-exchange or C_{18} columns [3–5], and recently more specific columns [6]. Detection is mainly by UV or refractive index (RI), when very high sensitivities are not required.

The limiting factor of this type of analysis is sample preparation. Efficient sample clean-up is essential for eliminating high-molecular-mass matrix interferents (e.g. proteins, fats, polysaccharides, very condensed phenolic compounds like tannins), in order not to deteriorate the chromatographic results. However, technological advances in sample preparation have been relatively few, when compared with those accomplished for HPLC. Until recently, the clean-up techniques employed prior to the HPLC determination of sugars and organic acids in liquid food samples have been essentially manual and off-line. These techniques vary from simple filtration to more complex clean-up procedures such as liquid–liquid and solid-phase extraction, precipitation and centrifugation [2,4].

This paper presents a more efficient approach

* Corresponding author.

☆ Presented at the 18th International Symposium on Column Liquid Chromatography, Minneapolis, MN, 8–13 May 1994.

0021-9673/95/$09.50
SSDI 0021-9673(95)00274-X

to sample clean-up using dialysis to remove the matrix macromolecules, followed by column switching for injection onto the HPLC system. The power of the technique may be further extended, according to the application, by performing a selective enrichment of the dialysate using a small trace enrichment cartridge before injection. The combination of on-line dialysis and trace enrichment provides an efficient and highly reproducible clean-up of raw samples [7–9]. The technique which is completely automated is performed on the ASTED XL (automated sequential trace enrichment of dialysates).

ASTED technology has already been successfully applied in clinical, pharmaceutical, food and environmental fields [8–10]. The on-line clean-up of a variety of raw foodstuffs permitted the determination of colourants in desserts and confectionery, nitrofurans in eggs and meat homogenates, aflatoxins in milk [11–13]. The application describes the use of on-line ASTED clean-up for the development of a simple and robust HPLC procedure for determining sugars, organic acids, and related compounds directly from raw liquid food samples. Such samples include dairy products, soft drinks, and fermented beverages. These samples were chosen for the complexity of their various matrices composed of high-molecular-mass interferents such as proteins, polysaccharides or condensed phenolic compounds.

2. Experimental

2.1. Chemicals and samples

All chemicals were of analytical grade and the solvents were of HPLC grade. The standards (sugars, organic acids and glycerol) were obtained from Sigma (St. Quentin Fallavier, France). Sulphuric acid was obtained from Merck-Clevenot (Nogent-sur-Marne, France). Water and acetonitrile were obtained from J.T. Baker (Paris, France) and ethanol from Carlo Erba (Rueil-Malmaison, France). All HPLC eluents were filtered before use and degassed with helium. Commercially available food samples were analyzed.

2.2. Instrumentation

ASTED XL

The sample preparation system was a Gilson ASTED XL unit (Gilson Medical Electronics, Villiers-le-Bel, France), consisting of a large XYZ autosampler, two Model 401C dilutors equipped with 1-ml syringes, one flat-bed dialyser with a donor channel volume of 100 μl and a recipient channel volume of 175 μl, fitted with a Cuprophan membrane with a molecular mass cut-off of 15 000, and two automated six-port Model 7010 valves (Rheodyne, Berkeley, CA, USA), one of which was fitted with a 20-μl injection loop. The system control was performed from a keypad using 722 (version 1.03) software.

HPLC

The ASTED XL was coupled to an HPLC system (Gilson Medical Electronics), consisting of a Model 306 pump fitted with a 5SC pump head, a Model 805 manometric module, a Model 831 temperature regulator, a Model 132 refractive index detector, and 715 (V1.2) HPLC system controller software.

Liquid food samples were cleaned-up and analyzed according to the following two methods described in Fig. 1:

Method (a) was used to determine only sugars from soft drinks and dairy products (orange syrup, cola drink, reconstituted baby milk and liquid chocolate yoghurt). In this method the NH_2-Zorbax silica column came from Du Pont Co (Paris, France).

In method (b), fruit juices and fermented beverages (grape juice, red wine, white wine, apple juice and cider) were analyzed. The Ion-300 column (Interchim, Montluçon, France) used for simultaneous determination of sugars, organic acids and some related compounds such as glycerol and ethanol, contained a polymeric cation exchanger in the hydrogen form.

1. LOAD DIALYSER

Sample (*Method a)* 2 x 100 µl; or *Method b)* 4 x 50 µl) loaded into donor channel of dialyser (pulsed mode) at 0.18 ml/min. Wait time of 2 min.

↓

2. ON-LINE CLEAN-UP

Recipient channel of dialyser containing water (static mode).

↓

3. HPLC

Method a) Injection vol: 20 µl

Column:	NH2-Zorbax, 5 µm (250 x 4.6 mm)
Mobile phase:	water-acetonitrile (25:75) at 1.0 ml/min
Temperature:	25°C
Detection:	Refractive index detection (0.06 x 10^{-4} RIUFS, 100 mV)

Method b) Injection vol: 20 µl

Column:	ION-300 (300 x 7.8 mm)
Mobile phase:	0.0085 N sulphuric acid at 0.4 ml/min
Temperature:	65°C
Detection:	Refractive index detection (0.03 x 10^{-4} RIUFS, 100 mV)

↓

4. REGENERATION

Dialyser donor and recipient cells regenerated with 5 ml of water at 3 ml/min

Fig. 1. ASTED XL and HPLC conditions.

3. Results and discussion

3.1. Optimization of the conditions used in HPLC and ASTED XL, and analytical data

Chromatographic and sample preparation conditions were first optimized using analytical standards diluted in water.

HPLC

For both systems, i.e. method (a) and method (b), the main parameters requiring optimization were mobile phase, column temperature and injection volume. In each case, the best compromise had to be established between resolution, analysis time, column lifetime and solvent consumption.

In method (a), the objective was to separate five sugars (i.e. fructose, glucose, sucrose, maltose and lactose). The critical point of the technique described in Fig. 1 was to obtain an acceptable resolution between fructose and glucose within a reasonable analysis time (Fig. 2).

Increasing the percentage of water reduced the retention times of the analytes significantly; a mobile phase of water–acetonitrile (30:70) eluted the five compounds within 10 min, but gave unsatisfactory separation of fructose and glucose ($R_s = 0.4$). On the other hand, a 20:80 composition of this mobile phase provided complete resolution of both analytes, but greatly increased the analysis time, to over 30 min.

The flow-rate was set at 1 ml/min. Higher flow-rates could be used, but with the risks of reducing the resolution, and of decreasing the mechanical resistance of the column, thereby shortening its lifetime.

In this method, the oven temperature was set at 25°C to improve reproducibility of the retention times. Increasing the temperature up to 35°C decreased the analysis time and particularly the retention time of the last peak (lactose) by 9%. Heating the column (to 35°C) was not used, to reduce any stripping of amino groups from the

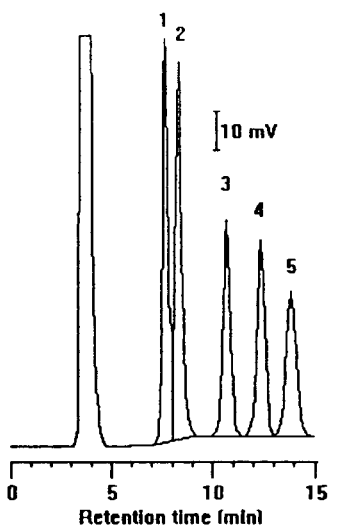


Fig. 2. Chromatogram of standards after processing with ASTED XL. Peaks: 1 = fructose, 2 = glucose, 3 = sucrose, 4 = maltose, 5 = lactose. All concentrations were 10 g/l.

silica surface, to avoid deterioration of the silica itself, and to avoid the formation of Schiff bases between the bonded groups and the reducing sugars [2].

Injection volumes larger than 20 μl resulted in peak broadening, with a loss of resolution between fructose and glucose.

In method (b), the objective was to separate simultaneously six organic acids (i.e. citric, tartaric, malic, succinic, lactic and acetic acids), two sugars (glucose and fructose), glycerol and ethanol. For this technique, we used ion chromatography with a specific column (Fig. 1), where the primary mechanism for the separation of acids is ion exclusion. Steric exclusion and partitioning are involved in separating other molecules.

Recently, work has been done to optimize the chromatographic conditions using this column for the determination of organic acids, sugars, glycerol and ethanol [6]. The variables to be optimized were the temperature of the column, the concentration of sulphuric acid in the mobile phase, and the flow-rate. In that work, the maximum analysis time was established as 25 min, and the optimum conditions (i.e. temperature 71°C, flow-rate 0.572 ml/min, and concentration of sulphuric acid in the mobile phase 0.005 *M*) determined from theoretical models, gave a satisfactory separation except for glucose, malic acid and fructose, which still had very poor resolution.

Therefore, we modified the above conditions to improve significantly the separation of the three compounds. We decreased the flow-rate, the column temperature, and the sulphuric acid concentration. The variation of the retention times observed were greater for organic acids than for other compounds (the best effects were produced by temperature changes). The chromatogram (Fig. 3) showed a much better separation of the three critical analytes (peaks 3, 4 and 5), but the analysis time was increased to 35 min.

Also with this column, injection volumes greater than 20 μl resulted in peak broadening and a loss of resolution.

Optimization of the conditions by this method had two objectives: (1) getting the best separation; and (2) preserving the column lifetime by following the manufacturer's recommendations.

ASTED XL (sample preparation)

A description of the basic operation of the ASTED XL system is given in Fig. 4. Detection problems do not occur, because in the analysis of food, sugars and organic acids are generally

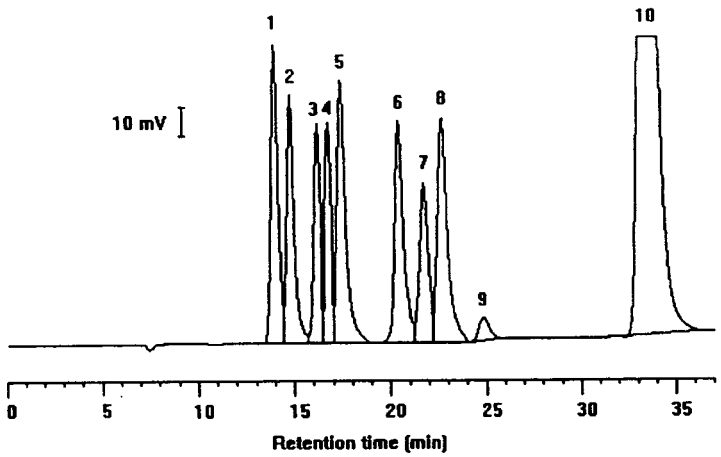


Fig. 3. Chromatogram of standards after processing with ASTED XL. Peaks: 1 = citric acid (6 g/l), 2 = tartaric acid (5 g/l), 3 = glucose (4 g/l), 4 = malic acid (5 g/l), 5 = fructose (5 g/l), 6 = succinic acid (5 g/l), 7 = lactic acid (6 g/l), 8 = glycerol (5 g/l), 9 = acetic acid (1 g/l), 10 = ethanol (121 g/l, i.e. Alc. 15% vol.).

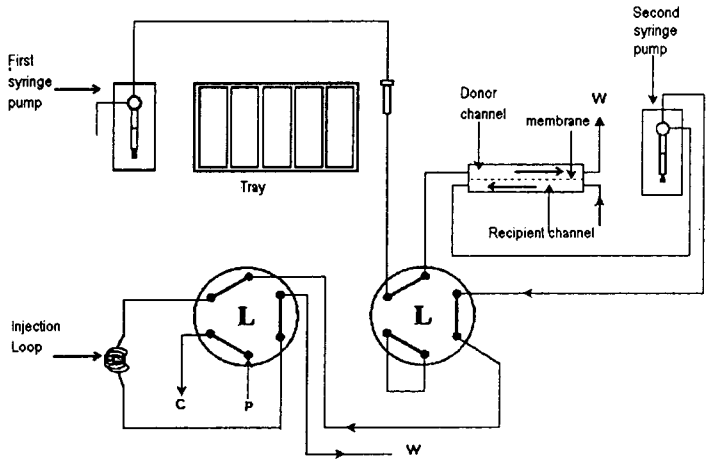


Fig. 4. ASTED XL sample preparation (dialysis and injection process). L = load position; P = HPLC pump; C = chromatography column; W = waste.

present in sufficient quantities. Therefore, on-line dialysis was performed without the need for a trace enrichment step. The sample was loaded into the donor channel of the dialyser and the analytes diffused across the membrane. Then, the dialysate in the recipient channel was transferred into the injection loop. On completion of dialysis, the left injection valve was switched, and the analyte solution was transported by the HPLC mobile phase onto the chromatographic column.

To ensure a favourable concentration gradient across the dialyser membrane, and to optimize the speed and recovery of the dialysis, the liquid flow through both the donor and recipient channels of the dialyser was regulated. Selection of appropriate dialysis models (static, pulsed or continuous) for each channel required consideration of the following points: (1) the absence of a trace enrichment step, which meant that it was not possible to obtain and inject all of the analytes crossing the membrane; (2) the limited size of the injection loop (imposed by chromatographic conditions), which meant that only 20 μl of the dialysate could be analysed.

Consequently, the pulsed dialysis mode was chosen for the donor channel and the static mode for the recipient channel, at the lowest possible flow-rate (0.18 ml/min) available with the 722 (version 1.03) software, which controlled the instrument.

Analytical data

Limits of detection (LOD), recovery rates and linearity were determined for both methods.

In method (a) (Table 1), the LOD was 0.16 g/l for fructose and glucose, and varied from 0.28 g/l to 0.41 g/l for dissacharides. Because of their steric hindrance, recoveries of sucrose, maltose and lactose (4.5–4.9%) were lower than those of the hexoses (6.3–7.1%). Over the linear range the R.S.D.s varied from 2.0% to 9.5%.

In method (b) (Table 2), the LOD varied from 0.04 g/l to 0.11 g/l; the better results observed in this method for glucose and fructose could be explained by a higher sensitivity obtained by using a lower flow-rate inside the flow cell of the detector. Recoveries varied from 6.7% to 9.5% and over the linear range the R.S.D.s varied between 0.6% and 7.9%.

3.2. Liquid food sample analysis

In this part of the study, we used internal standards to minimize any possible fluctuations during dialysis caused by the composition of the matrix.

Table 1
Analytical data on the automated determination of sugars using on-line dialysis and amino-bonded silica column HPLC

Analyte	Limit of detection[a] (g/l)	Recovery[b] (%)	Linearity[c]			Range of linearity (g/l)	Precision[d] (R.S.D.) (%)
			Slope	Intercept	r		
Fructose	0.16	6.3–7.0	137 475	138 907	0.999	1–100	2.4–9.5
Glucose	0.16	6.3–7.1	150 194	92 921	0.999	1–100	3.0–7.2
Sucrose	0.35	4.5–4.8	94 446	121 854	0.997	3–100	2.0–7.6
Maltose	0.28	4.5–4.7	77 896	140 456	0.994	3–100	3.1–9.1
Lactose	0.41	4.6–4.9	66 103	140 145	0.994	3–100	2.8–6.3

[a] LOD at a signal-to-noise ratio of 3.
[b] Recoveries obtained with two concentrations for each analyte (5 and 10 g/l).
[c] Calibration data (based on areas) obtained from 715 HPLC system controller software; r = correlation coefficient; 5 data points for fructose and glucose, 4 data points for sucrose, maltose and lactose, 5 replicates for each analyte.
[d] Minimum and maximum R.S.D. over the linear range ($n = 5$).

Table 2
Analytical data on the automated determination of organic acids, sugars and related compounds using on-line dialysis and polymeric ion 300 column HPLC

Analyte	Limit of detection[a] (g/l)	Recovery[b] (%)	Linearity[c]			Range of linearity (g/l)	Precision[d] (R.S.D.) (%)
			Slope	Intercept	r		
Citric acid	0.07	6.7–7.8	106 181	−15 799	0.999	0.5–10	1.8–4.6
Tartaric acid	0.07	7.6–8.5	114 009	−15 789	0.999	0.5–10	1.7–4.7
Malic acid	0.07	7.6–8.4	104 302	−15 448	0.999	0.5–10	1.7–7.0
Succinic acid	0.07	7.7–9.0	101 606	−14 438	0.999	0.5–10	2.0–4.8
Lactic acid	0.11	6.8–8.8	78 198	−15 015	0.999	0.5–10	2.5–7.1
Acetic acid	0.11	8.5–9.5	71 700	−9457	0.999	0.5–10	1.1–3.5
Glucose	0.05	6.7–7.5	79 137	214 803	0.995	0.5–100	0.6–4.6
Fructose	0.04	7.0–7.4	81 014	220 514	0.995	0.5–100	0.7–5.0
Glycerol	0.04	7.9–9.2	80 587	101 728	0.998	0.4–60	1.1–5.0
Ethanol	0.10	8.1–9.1	45 626	36 144	0.999	8.1–121	1.5–7.9

[a] LOD at a signal-to-noise ratio of 3.
[b] Min.–max. recoveries obtained with 3 concentrations for organic acids (0.5, 1 and 2.5 g/l), 3 for ethanol (0.81, 8.1 and 40.5 g/l), 2 for glucose and fructose (1 and 10 g/l) and 2 for glycerol (0.7 and 7 g/l).
[c] Calibration data (based on areas) obtained from 715 HPLC system controller software, r = correlation coefficient, 5 data points except for ethanol (4 data points) in 5 replicates.
[d] Min.–max. R.S.D. over the linear range ($n = 5$).

Method (a)–Determination of sugars only

Internal standards selected according to the analysis were automatically added to samples by the ASTED XL before dialysis. Orange syrup was automatically diluted six-fold with water, and the cola drink was automatically degassed by slowly bubbling air through it, both prior to dialysis. Table 3 summarises the results obtained from four real foodstuffs. The efficiency of sample clean-up is particularly well illustrated in the cases of the highly proteinaceous samples, reconstituted baby milk and liquid chocolate yoghurt. Samples were processed concurrently. This means that each sample was prepared whilst the previous one was being analyzed. The analytical time was 15 min per sample (when lactose

Table 3
Carbohydrate content of 4 foodstuffs, with corresponding R.S.D.

Sample	Baby milk		Chocolate yoghurt		Orange syrup		Cola drink	
	g/l	R.S.D.%	g/l	R.S.D.%	g/l	R.S.D.%	g/l	R.S.D.%
Fructose	–	–	–	–	46.4	3.4	30.1	2.5
Glucose	–	–	I.S.	–	60.2	3.8	31.5	4.4
Sucrose	I.S.	–	80.0	3.4	7.8	7.6	63	2.3
Maltose	–	–	–	–	7.0	7.0	I.S.	–
Lactose	20.9	3.5	42.6	4.7	I.S.	–	–	–

$n = 10$.

was present in the dialysate). Therefore (apart from the first sample), the sample preparation time of 9 min did not add to the time required to process each sample. The concentrations of the sugars in Table 3 ranged from 7.0 to 80.0 g/l, and the R.S.D. obtained from 10 analyses ranged from 2.3 to 7.6%.

Method (b)–Simultaneous determination of sugars, organic acids and related compounds

This type of determination concerns mainly the analysis of fruit juices, and fermented beverages during their ripening and fermentation processes. Propionic acid has been selected as an internal standard (Fig. 5). Again, concurrent sample processing permitted sample preparation (18 min) to be performed within the analytical run-time (35 min when ethanol is present in the sample). The matrix interferents of these samples, mainly polysaccharides or condensed phenolic compounds like tannins, were removed by the dialysis clean-up. Table 4 summarises the results obtained from five beverages.

The natural organic acids analyzed in the two fruit juices, were citric acid, tartaric acid, malic acid for grape juice, and citric acid, malic acid for apple juice. Malic acid could not be determined in the analysis of the grape juice, because of its low level compared to the high levels of the neighbouring sugars. This problem could be solved (if required) by adjusting the HPLC conditions, in particular by lowering the column temperature, which resulted in a displacement of the malic acid out from the pair of

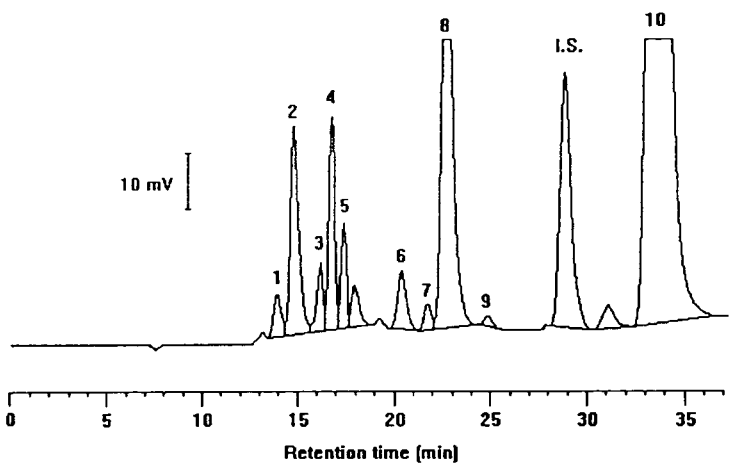


Fig. 5. Chromatogram of a dry white wine after clean-up using ASTED XL. Peaks: 1 = citric acid, 2 = tartaric acid, 3 = glucose, 4 = malic acid, 5 = fructose, 6 = succinic acid, 7 = lactic acid, 8 = glycerol, 9 = acetic acid, I.S. = propionic acid, 10 = ethanol.

Table 4
Organic acid, sugar, glycerol and ethanol content of five beverages, with corresponding R.S.D.

Sample	Grape juice		Red wine		White wine		Apple juice		Cider	
	g/l	R.S.D.%	g/l	R.S.D.%	g/l	R.S.D.%	g/l	R.S.D.%	g/l	R.S.D.%
Citric acid	0.44	2.9	0.45	4.0	0.73	7.2	0.76	3.9	0.29	7.8
Tartaric acid	2.22	2.9	1.99	3.4	2.64	2.5	–	–	0.29	3.6
Malic acid	n.d.	–	0.46	8.0	2.82	2.4	8.99	0.9	n.d.	–
Succinic acid	–	–	0.70	1.6	0.71	2.0	–	–	0.31	5.4
Lactic acid	–	–	1.91	1.4	0.34	1.0	–	–	2.81	1.1
Acetic acid	–	–	0.51	3.2	0.22	8.0	–	–	–	–
Glucose	44.1	1.4	0.15	4.6	0.7	7.0	30.7	1.3	3.4	1.7
Fructose	39.1	1.2	<LOD	3.0	1.0	1.1	39.0	1.6	11.5	1.3
Glycerol	0.9	3.0	6.6	1.0	7.4	1.4	–	–	2.5	1.6
Ethanol	2.2	7.8	122.0	2.2	123.0	2.0	–	–	57.6	3.4

$n = 5$; n.d. = not determined.

sugars, but with an increase of the HPLC runtime. Another solution could be to use a UV detector with the wavelength set at 210 nm, which would give a much lower signal for sugars.

During alcoholic fermentation, the sugars present in fruit juices are transformed into ethanol by yeasts with production of secondary constituents like succinic acid and glycerol. After alcoholic fermentation, white wines (Fig. 5) do not generally undergo any further changes. However, red wines usually undergo a secondary fermentation process, by bacteria, called "malolactic fermentation", in which the malic acid is transformed into lactic acid. A slight oxidation of the wine may also result in the formation of acetic acid (peak 9). The small peak occurring between the internal standard and peak 10 in Fig. 5 has not been determined. Considering its retention time, it may be methanol, and could come from an enzymatic demethoxylation of some polysaccharides, during the crushing of the fruit and in fermentation. The concentrations of organic acids in Table 4 range from 0.22 to 8.99 g/l, the concentrations of glucose and fructose from below LOD to 44.1 g/l. All R.S.D. values range from 0.9 to 8.0%.

The robustness of the method was studied with a real sample, which was a red wine particularly rich in tannins. We observed a loss of resolution between malic acid and fructose ($R_s = 0.8$) only after 120 injections. After a regeneration of the chromatographic column, according to the manufacturer's recommendations (0.025 *M* sulphuric acid), the initial separation was recovered. We repeated this experiment three times without any other noticeable degradation. Recoveries were unchanged and the dialysis membrane remained efficient.

4. Conclusion

This work demonstrates the advantages of on-line sample preparation in the HPLC analysis of liquid food samples. Raw food samples were cleaned up by dialysis and injected directly onto an HPLC system with outstanding reproducibility. The technique was perfectly suited for the removal of macromolecular matrix interferents such as proteins, polysaccharides and condensed phenolic compounds, replacing conventional off-line sample preparation procedures, resulting in more robust HPLC analyses, and widely meeting the criteria required by the food industry for routine analysis. In addition, economical and environmental considerations make this technique very attractive because of the low-cost of consumables (only one membrane for hundreds of analyses) and non-toxic solvent was used (water).

Acknowledgement

The authors thank Sandrine Jacquet, Genevieve Luck and David Hart for helpful services during the production of this manuscript.

References

[1] D.J. Folkes and P.W. Taylor, in R. Macrae (Editor), HPLC in Food Analysis, Academic Press, London, 1982, Ch. 6, p. 159.
[2] F.W. Scott, in Leo M.L. Nollet (Editor), Food Analysis by HPLC, Marcel Dekker, New York, NY, 1992, Ch. 8, p. 259.
[3] K. Saag, in R. Macrae (Editor), HPLC in Food Analysis, Academic Press, London, 1982, Ch. 9, p. 228.
[4] D. Blanco Gomis, in Leo M.L. Nollet (Editor), Food Analysis by HPLC, Marcel Dekker, New York, NY, 1992, Ch. 11, p. 371.
[5] J.P. Goiffon, A. Blanchere and C. Reminiac, Analusis, 13 (1985) 218.
[6] M. Calull, E. López, R.M. Marcé, J.C. Olucha and F. Borrull, J. Chromatogr., 589 (1992) 151.
[7] J.D.H. Cooper, D.C. Turnell, B. Green and F. Vérillon, J. Chromatogr., 456 (1988) 53.
[8] F. Vérillon and F. Qian, Analusis, 19 (1991) 271.
[9] N.C. van de Merbel, J.J. Hageman and U.A.Th. Brinkman, J. Chromatogr., 634 (1993) 1.
[10] N.C. van de Merbel, F. Lagerwerf, H. Lingeman and U.A.Th. Brinkman, Int. J. Environ. Anal. Chem., 54 (1994) 105.
[11] G.M. Greenway, N. Kometa and R. Macrae, Food Chem., 43 (1992) 137.
[12] M.M.L. Aerts, W.M.J. Beek and U.A.Th. Brinkman, J. Chromatogr., 500 (1990) 453.
[13] L.G.M.Th. Tuinstra, P.G.M. Kienhuis, W.A. Traag, M.M. Aerts and W.M.J. Beek, J. High Resolut. Chromatogr., 12 (1989) 709.

ELSEVIER

Journal of Chromatography A, 705 (1995) 205–217

JOURNAL OF CHROMATOGRAPHY A

Development of an "in-source" thermospray-type interface for on-line capillary liquid chromatography–mass spectrometry

Alain Carrier, Louis Varfalvy[1], Michel J. Bertrand*

Regional Center for Mass Spectrometry, Department of Chemistry, University of Montreal, P.O. Box 6128, H3C 3J7 Montréal, Canada

First received 28 December 1994; revised manuscript received 23 February 1995; accepted 3 March 1995

Abstract

The design and the construction of an "in-source" thermospray-type capillary interface for LC–MS experiments on a bench-top quadrupole mass spectrometer is described. The interface consists of a small diameter fused capillary tube which is introduced directly into the CI source through the solid probe inlet and is oriented in the beam axis. In order to produce average linear velocities comparable to those used in conventional thermospray and compatible with the optimum operating conditions of capillary LC–MS systems, a fused-silica transfer capillary of 10 μm is used. Heating of the interface is provided by the ion source and ions are generated using external mode ionization (filament-on). The effect on performance of several operating parameters, such as source temperature, mobile phase composition, sampling distance in the source and flow-rate have been investigated. The results obtained in experiments conducted on the effect of interface temperature and flow-rate demonstrate that the system is very stable under optimal operating conditions which are compatible with the optimum performance of capillary liquid chromatography systems. The interface developed can be used with a series of mobile phases (normal and reversed) and the ionization features in terms of sensitivity are in all cases essentially comparable. The system yields reproducible results and allows picogram range sensitivity to be achieved in flow injection mass spectrometric or liquid chromatographic experiments involving low-molecular-mass polar organic compounds.

1. Introduction

The development of techniques allowing the introduction of liquid samples into the ion source of a mass spectrometer has considerably increased the range of compounds that can be analyzed by mass spectrometry. Alternative ionization techniques for liquid introduction such as thermospray (TSP) [1–5], continuous-flow fast atom bombardment (CF-FAB) [6,7] and more recently the atmospheric pressure ionization (API) techniques such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) [8–10] are most widely used. From the various LC–MS interfaces available, those that allow the introduction of the entire eluent into the ion source are the most appealing for the analysis of low-molecular-mass polar organic compounds. These types of interface use flow-rates that are compatible with the optimum range of operation of miniaturized liquid chromatographic systems and within the pumping capacity of most commercial mass spectrometers

* Corresponding author.
[1] Present address: Hydro-Quebec, Montreal, Canada.

0021-9673/95/$09.50
SSDI 0021-9673(95)00315-0

that have CI capabilities [11]. The use of systems introducing liquids directly into the ion source can lead to three types of operating conditions that are interesting for mass spectrometric analysis. These systems can produce a stable liquid surface under vacuum (continuous-flow FAB), a stable stream of liquid droplets that can be further desolvated, as produced by pneumatic nebulizers with or without electrical assistance (electrospray), or alternatively a steady stream of gas and droplets as obtained with capillary thermal nebulizers (direct liquid introduction, thermospray).

Of the different liquid introduction systems, thermospray has proved to be a useful method. The technique has been widely accepted in the different areas of organic compound analysis such as food and agriculture [12–14], biomedical [15–17] and environmental [18–21] sciences, and is available as an accessory on many mass spectrometers. It is applicable to a wide range of analytes including volatile and nonvolatile, polar and labile molecules, analyzed under a variety of chromatographic conditions [22]. In particular, thermospray ionization can usually provide parent molecular ions from polar and moderate molecular mass compounds ($M_r < 1000$) that are not amenable to analysis using more conventional ionization techniques [23]. In addition, ionization is achieved with the use of filament-on or filament-off, and the abundance of the different fragment ions in the spectra is strongly dependent on the type and concentration of the analyte and the composition of the mobile phase. In this coupling method for liquid chromatography–mass spectrometry, which involves controlled partial vaporization of the LC effluent before the ion source of the mass spectrometer, it is vital that the heat input be properly controlled so that complete vaporization does not occur inside the capillary. As a result of heating, the liquid is nebulized and partially vaporized and the analytes are carried into the ion source as microdroplets or particles in a supersonic jet of vapor. Thermospray can be used successfully for flow-rates in the range of 0.5–2 ml/min. However, the standard thermospray interface cannot be used with lower flow-rates such as those used in capillary liquid chromatography (1–10 μl/min) because the applied heat and the flow-rate become unbalanced which leads to severe instability. Furthermore the use of a large internal diameter vaporizer does not allow the proper average linear velocities leading to thermospray conditions to be achieved.

Over the course of the last decade, liquid chromatography has been subject to a rapid evolution. The application of integrated electronics has lead to the automation and miniaturization of chromatography [24] and this trend has also been reflected in the miniaturization of liquid chromatography. A number of reports have appeared describing the use and advantages of miniaturized liquid chromatographic systems [25]. The low flow-rates used in such systems (1–10 μl/min) allow the introduction of the total liquid chromatographic eluent into the mass spectrometer, thus reducing the total quantity of sample injected necessary to achieve the same signal with respect to systems that require splitting of the eluent.

Previous work in our laboratory has lead to the development of a simple and inexpensive direct liquid introduction (DLI) system that uses a conventional GC–MS interface and that can be used to couple capillary liquid chromatography to mass spectrometry [26]. Experiments conducted with this system have shown that it can be stable under appropriate conditions, that it is reproducible and that it allows picogram range sensitivity to be routinely achieved for low-molecular-mass organic compounds. However, this interface presents limitations in the analysis of more polar compounds.

In order to allow the analysis of thermally labile or involatile compounds and to ensure the direct coupling of capillary liquid chromatography, the design and construction of an in-source capillary thermospray-type interface was undertaken. The interface consists of a small fused capillary tube that allows the direct introduction of liquid samples through a continuous flow loop injection system. Arpino and Beaugrand [27] have demonstrated that it is feasible to use capillary tubes with narrow inside diameters (10 μm) as flow restrictors to nebulize the liquid

solution in order to obtain thermospray conditions with flow-rates compatible with the use of micro-columns. In order to reproduce conditions comparable to those obtained in conventional thermospray, vacuum nebulization of the chromatographic eluents through a fused capillary tube should meet the following requirements: (i) an input liquid flow-rate small enough to allow the normal operation of the mass spectrometer using the available pumping equipment, (ii) an input liquid flow-rate greater than the rate of evaporation of the solvent at the nebulizer orifices, and finally (iii) an operating pressure within the limits fixed by the HPLC pump. The aim of this work was, therefore, to design and construct a thermospray-type interface that meets all these requirements and that would allow the analysis of polar substances.

The scope of applications of the interface has been determined and the influence on performance of operating parameters such as source temperature, flow-rate and vaporizing distance studied. The stability and reproducibility of the system have been investigated along with the effect of type and mobile phase composition on the ionization efficiency.

2. Experimental

2.1. Mass spectrometer

Analyses were performed on a VG TRIO-1 mass spectrometer (VG MassLab, Manchester, UK) equipped with differential pumping (analyzer 50 l/s, source 240 l/s). Data handling capability and control of the instrument were provided by the LAB BASE data system. The ion source pressure (as read on the vacuum gage) was maintained in the range of $2 \cdot 10^{-4}$ to $8 \cdot 10^{-4}$ Torr (1 Torr = 133.322 Pa) and unless otherwise specified the source temperature was maintained at 150°C.

2.2. LC–MS experiments

The capillary system used consisted of a Carlo-Erba (Milano, Italy) Phoenix-20 pump connected to a Valco Model C14W injector with a 60-nl sample loop. The capillary columns used were laboratory made (220 × 0.25 mm I.D.) and packed with 5-μm particles (Spherisorb ODS-2). Typical flow-rates used were between 2.5 and 5 μl/min. In the experiments the sample was introduced by a Valco C14W valve (Valco Instruments Co., Houston, TX, USA). This valve permits direct connection between capillary tubing or LC columns with a minimum dead volume. Detection at 254 nm for the LC–UV experiments was achieved with an ISCO μLC-10 variable-wavelength detector equipped with a 60-nl flow-cell.

2.3. Interface

The capillary thermospray-type interface developed for the mass spectrometer is shown in Fig. 1. It consists of a fused-silica capillary tubing which is introduced directly into the CI source through the solid probe inlet and is oriented in the beam axis. In order to produce average linear velocities comparable to those obtained in conventional thermospray, a fused-silica capillary of 18 mm × 10 μm I.D. is used. Heating of the interface is provided by the ion source and ionization is achieved using external-mode ionization (filament-on mode).

2.4. Chemicals

The compounds used in this study such as gramine, picolinic acid, geraniol, cineole, umbel-

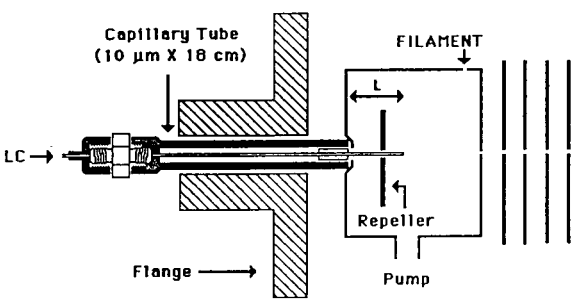


Fig. 1. Schematic of the thermospray interface. L is the sampling distance.

liferone, caffeine, theophylline, theobromine, piperidine, benzonitrile, chloroaniline, chloramphenicol, fluoranthene, anthracene, coumaric acid, ferrulic acid, caffeic acid, *trans*-cinnamic acid, phloroglucinol, salicyl alcohol, adenosine, toluene, ethylbenzene, butylbenzene and amylbenzene were purchased from Aldrich (Milwaukee, WI, USA). All compounds were used without further purification, and the mobile phases were prepared using HPLC-grade (Aldrich) acetonitrile, ethyl acetate, hexane and distilled, deionized water (Milli-Q, Millipore, Bedford, MA, USA).

2.5. Preparation of mobiles phases

The eluents were carefully prepared by mixing appropriate volumes of distilled, deionized water and organic modifiers. In all instances, the solvents were filtered (0.45-μm filter) and degassed in an ultrasonic bath for at least 30 min prior to their use.

3. Results and discussion

In general, most thermospray/plasmaspray ion sources consist of a cylindrical tube with the vaporizer probe at one end and the pumping exit at the other, the vaporizer probe being the heart of the thermospray system. In conventional commercial interfaces, and in most laboratory-built probes as well, vaporizer capillaries of 100–150 μm are usually used. These interfaces are used in conjunction with conventional liquid chromatographic columns and can accommodate flow-rates in the range 0.5–2 ml/min. However, these interfaces, because of their large internal diameter, cannot be used for lower flow-rates, such as those used in capillary liquid chromatography systems (1–10 μl/min). Hirter et al. [28] and Arpino and Beaugrand [29] reported the use of vaporizer capillaries of 10, 25 and 50 μm I.D. in order to introduce typical amounts of liquid between 40 and 80 μl/min. However, these flow-rates are still much higher than the average flow-rates used in capillary liquid chromatographic systems.

The capillary thermospray interface used in this work was designed to fit a VG TRIO-1 mass spectrometer and is shown schematically in Fig. 1. It has the same outside dimensions (7.9 mm O.D.) as the solid probe, and so can be introduced into the mass spectrometer through the solid probe inlet. Basically the interface consists of a stainless-steel tube of 12.5 cm $\times$ 7.9 mm O.D. which allows the introduction of the fused-silica capillary. A teflon disk of 1 cm $\times$ 7.9 mm fitted at the end (near the source) of the stainless steel tube serves as a holder for the capillary tube. The chromatographic column is fitted to the capillary tube with a Valco union. Through this capillary the total effluent of the capillary column is introduced directly into the ion source of the mass spectrometer without a desolvation chamber. In order to develop a thermospray-type interface that can be used for work with packed capillary columns (flow-rates 2–5 μl/min) and still produce average linear velocities comparable to those obtained in conventional thermospray ($\approx$ 900 mm/s), a fused-silica tubing of 18 mm $\times$ 10 μm I.D. was used as vaporizing capillary. This vaporizer capillary is connected to an injector and a pumping system. The characteristics of the in-source capillary thermospray-type interface system are (i) the absence of heating element for the vaporizer capillary, heating being provided by the ion source, and (ii) the absence of a desolvation chamber in contrast to the systems proposed by Hirter and Arpino which use an electrically heated vaporizer capillary and a desolvation chamber. The main appeal of the interface developed is its simplicity and the fact that it requires, except for the probe, no modification to the mass spectrometer such as the addition of a desolvation chamber or additional pumping device.

3.1. Operating parameters

Several parameters that are known to have an effect on the overall performance were studied in order to evaluate the interface and to determine its optimum operating conditions. The main factors affecting the performance of the interface are the temperature settings of the source, the

mobile phase composition and the flow-rate into the ion source of the mass spectrometer. The temperature of the interface governs the rate of evaporation of the mobile phase whereas the flow-rate affects both the evaporation rate and the pressure within the ion source and the composition of the plasma that affects the ionization efficiency. In this instance, another parameter that was found to have an influence on the performance of the system was the sampling distance (L in Fig. 1) which is defined as the length of capillary introduced into the ion source. The conditions (average linear velocities) used in the study were chosen so as to be similar to those involved in conventional thermospray. Optimization of the different parameters was done using the solvent ion intensities [30].

In order to reproduce the average linear velocity obtained with the conventional systems, flow-rates in the order 2.0–7.0 μl/min had to be used. However, a constraint on the flow-rate range that could be used was imposed by the pumping capacity of the mass spectrometer (flow-rates <6.0 μl/min) and the optimum range of operation of miniaturized liquid chromatography (flow-rates >2.0 μl/min). Thus, flow-rates used in this study ranged from 2.0 μl/min to 6.0 μl/min. Under those restrictions, the average linear velocities vary from 530 to 1500 mm/s. The optimization of operating parameters was initially done with a flow of 4.0 μl/min (linear velocity = 900 mm/s) which is similar to the operating conditions in conventional thermospray at a flow rate of 1 ml/min.

The effect of the interface temperature, that is provided by the ion source, on the stability of the plasma was studied. Temperature values have to be such that just enough heat is supplied to almost completely vaporize the liquid as it passes through the vaporizer and the total heat transferred must be equal to the heat required to convert the liquid to vapor at the exit temperature and pressure. If the temperature of the heater is too high then vaporization will tend to occur inside the capillary. On the other hand, if insufficient heat is supplied, superheated liquid will emerge and begin to vaporize in the ion source, thus, creating instability. Both these

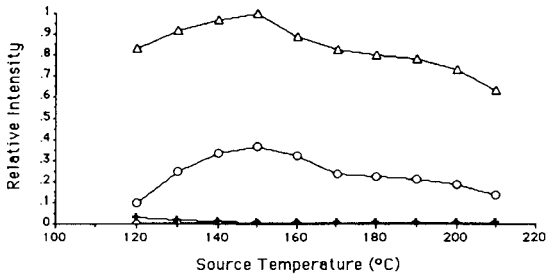


Fig. 2. Relative intensity of solvent ions (acetonitrile–water, 50:50, v/v) as a function of the source temperature. (△) 83, (○) 42, (+) 19, (◇) 18.

conditions will lead to unsatisfactory performance. As can be seen from Fig. 2, that shows the intensities of the solvent ions versus the source temperature, the results obtained when the temperature is varied at constant flow-rate (ca. 4.0 μl/min) of the mobile phase indicate that all ions reached a maximum intensity in the same temperature region of 140–160°C. This temperature region was found to provide extremely stable operating conditions. Partial to severe instability was encountered at both ends of this temperature range for reasons discussed previously. However, the optimum temperature for thermally labile compounds appeared to maximize at lower source temperature (135–145°C), higher temperature increasing the extent of fragmentation as can be predicted.

Another factor that can affect the stability and the operating conditions is the flow-rate. Its value has to be such that the input liquid flow-rate is greater than the rate of vaporization while the pressure, within the chemical ionization source, is adequate to maintain the ionization efficiency and sensitivity. The flow-rate in the system has an effect on the ion source pressure which affects the composition of the plasma and therefore the ionization characteristics of the system. If LC–MS experiments are to be conducted, additional constraints will be imposed on the flow-rate, since it will have to be such that the chromatographic conditions are also optimized in order to maintain chromatographic performance. Hence, the effect of the flow-rate was investigated at the optimum temperature of

the source (150°C). For flow-rate values below 2.0 μl/min, the system is quite unstable, resulting in important variation in the signal. As the flow-rate approaches values of 3.0 μl/min the overall operating conditions tend to stabilize. The range of flow-rates corresponding to stable operating conditions was found to be between 2.5 and 5.0 μl/min corresponding, depending on the solvent used, to indicated source pressures in the range of $2 \cdot 10^{-4}$ to $8 \cdot 10^{-4}$ Torr. This range of flow-rates is directly compatible with the use of 250 μm I.D. packed capillary columns.

As mentioned earlier, the flow-rate affects the ion source pressure and consequently the composition of the plasma. In order to assess the influence of the flow on ionization efficiency the composition of the plasma was monitored over the flow-rate or the pressure range that corresponds to the optimum performance of the system. Several mobile phase compositions were studied in these experiments for both normal and reversed modes, and typical results obtained with a binary mixture of acetonitrile–water (50:50) are shown in Fig. 3. The figure, which gives the ion profiles generated by an ACN–H_2O (50:50) mixture as a function of the flow-rate, indicates that the major ions corresponding to the protonated dimer and monomer of acetonitrile at m/z 83 and m/z 42 decrease in intensity as the flow-rate is increased well above the value of 3 μl/min. Thus, the prefered flow-rate for LC–MS experiments should be chosen in the range of 2–3.5 μl/min and this range approximately corresponds to the optimum linear velocity of the 250 μm capillary columns.

The composition of the mobile phase also has an effect on the quality of the mass spectra obtained since it will determine the reacting species and, thus, the features of the spectra in the filament-on mode. In order to investigate this effect and characterize the background signal (chemical noise) generated by the interface in the filament-assisted mode with varying mobile phase compositions, studies were performed with different solvent compositions. The systems studied were binary mixtures of acetonitrile–water (reversed-phase) and ethyl acetate–hexane (normal-phase) with different solvent ratios (25%, 50%, 75% 100%). Fig. 4A indicates that the major ion for the reversed-phase system (acetonitrile–water) corresponds to the protonated dimer of acetonitrile (m/z 83). Ions at m/z 18 and 19 that correspond to H_2O^+ and H_3O^+, respectively, have much weaker intensities than those generated by acetonitrile. These results are

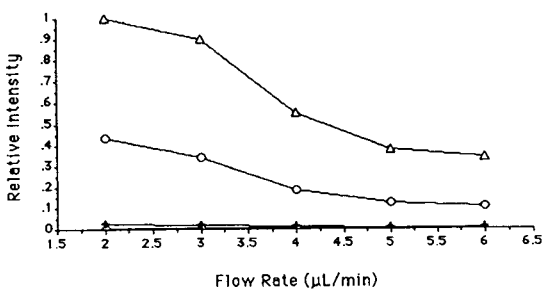


Fig. 3. Relative intensity of the solvent ions (acetonitrile–water, 50:50, v/v) as a function of the flow-rate. (△) 83, (○) 42, (+) 19, (◇) 18.

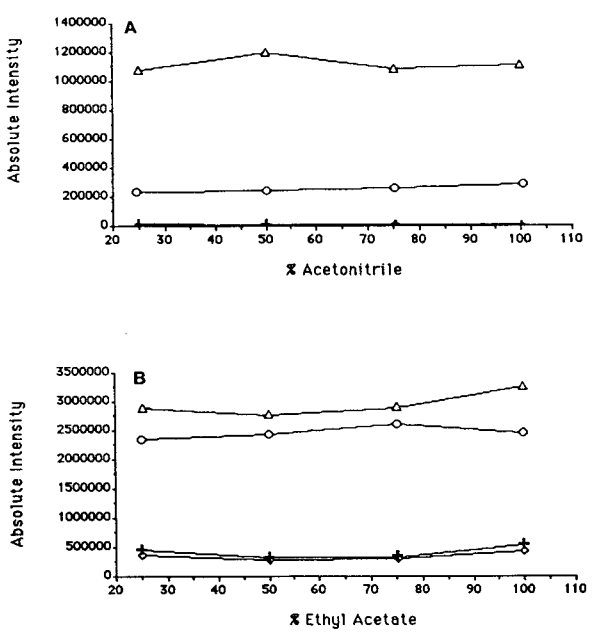


Fig. 4. Variation of the plasma obtained as a function of the mobile phase composition. (A) Acetonitrile–water: (△) 83, (○) 42, (+) 19, (◇) 18. (B) Hexane–ethyl acetate: (△) 177, (○) 89, (+) 85, (◇) 57.

explained by the higher proton affinity of acetonitrile as compared to that of water (approximately 70 kJ/mol higher). Experiments with filament-on thermospray have demonstrated that this mode of operation ionizes analytes by conventional chemical ionization reactions with solvent-derived ions [31] which are also observed in direct liquid introduction LC–MS. The plasma compositions obtained with the capillary in-source thermospray-type interface in the filament-assisted mode differ from those previously observed for ACN–H_2O in direct liquid introduction LC–MS [26] in that the major ion present is the protonated dimer of acetonitrile at m/z 83 instead of the protonated monomer at m/z 42 that is observed in DLI [26]. This reflects the differences in the vaporization conditions that exist between the two types of interfaces.

The results obtained in the filament-assisted mode with the normal-phase systems used (ethyl acetate–hexane) are similar to those obtain for acetonitrile–water mixtures. Fig. 4B indicates that the major ion corresponds to the protonated dimer of ethyl acetate (m/z 177). Ions at m/z 85 corresponding to hydride ion abstraction from hexane have much weaker intensities than those generated from ethyl acetate. From Fig. 4A,B, it can also be observed that the absolute intensities of the major ions of both systems are relatively stable for solvent ratios going from 25% to 100%. Thus, it can be expected that the ionization conditions in the source will be similar over that range of mobile phase compositions.

The efficiency of ionization of several types of compounds was investigated in the filament-assisted mode with various mobile phase compositions using mixture ratios within the determined range. In these experiments, several mobile phase compositions for the two different chromatographic modes, acetonitrile–water (reversed-phase) and ethyl acetate–hexane (normal-phase), were used. The effect of mobile phase composition on ionization was determined by injecting a constant quantity of model compounds and monitoring the relative intensities of the major ions as the composition of the binary mixtures varied. Caffeine (200 ng) was used for the reversed-phase system while chloroaniline (200 ng) and benzonitrile (200 ng) were used for the normal-phase mode. Fig. 5A (reversed-phase) and 5B (normal-phase) show the results obtained for both chromatographic systems. The relative intensities of ions for the model compounds seem to be stable over the range of compositions studied in both systems. These results appear interesting since they indicate that the system can maintain ionization performance in both modes over the composition range 25–100%. In the buffer ionization mode, the efficiency of ionization is greatly reduced which does not, as a general rule, make this mode appealing.

The sampling distance (L in Fig. 1) is a parameter, particular to this interface, that has an effect on the overall performance. This parameter was found to be extremely important since its value determines the heated length of the capillary interface and the vaporizing zone. Furthermore, the sampling distance can directly

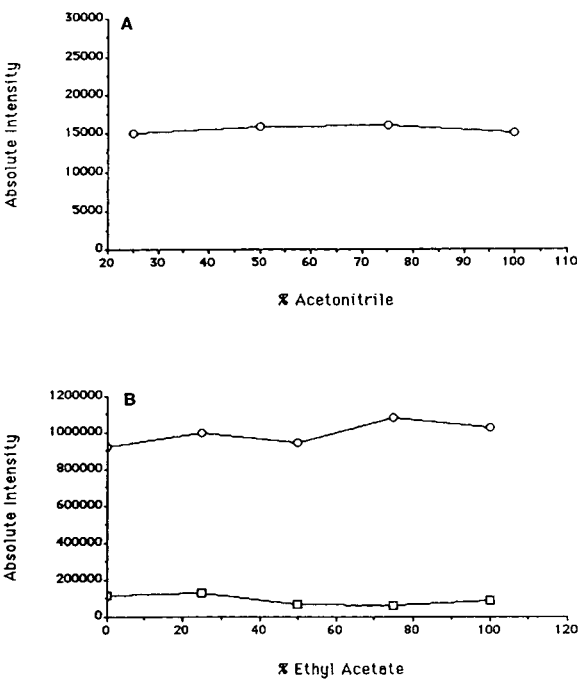


Fig. 5. Variation of the response of model compounds as a function of the mobile phase composition. (A) (○) Caffeine (acetonitrile–water); (B) (○) benzonitrile, (□) chloroaniline (hexane–ethyl acetate).

affect the sensitivity of the system by allowing the path travelled by the ions between the ion slit and the orifice of the interface to be varied. As can be seen from Fig. 6, which gives the relative intensity of solvent ions as a function of the sampling distance, the ion intensities increase with the distance up to a certain maximum, 12.5 mm, at which a drop in intensities occurs. This implies that the heated length of the capillary has to be equal to 12.5 mm in order to meet the proper vaporization conditions. This capillary length of 12.5 mm corresponds to a distance of 3 mm between the ion slit exit and the orifice of the interface. Partial to severe instability of the signal was encountered for shorter heated lengths.

3.2. Spectral and analytical features

The evaluation of the spectral and analytical features achieved with the in-source capillary interface was based on the quality of the mass spectra and on the sensitivity obtained for several types of organic compounds under typical operating conditions. Table 1 shows the structures of the different compounds that were used for the evaluation. In all cases, the protonated species were observed as the major ion and fragment ions were present only to a minor extent. Generally, for full scan spectra the injection of 1–50 ng was needed in order to produce an interpretable spectrum. Typical data obtained are presented in Fig. 7A,B which shows the mass spectra of adenosine (Fig. 7A) and chloramphenicol (Fig. 7B) obtained using acetonitrile as mobile phase. The mass spectra obtained for these compounds were found to be in all points comparable to those obtain under typical thermospray filament-on conditions [32].

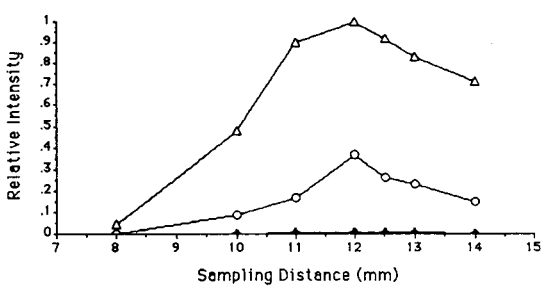
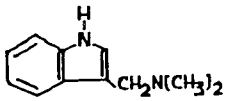
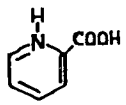
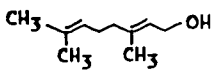
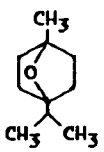
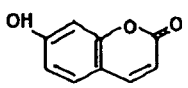
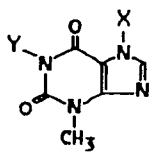
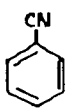
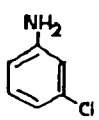
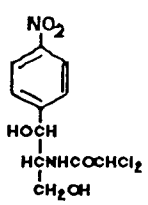


Fig. 6. Relative intensity of solvent ions (acetonitrile–water, 50:50, v/v) as a function of the sampling distance. (△) 83, (○) 42, (+) 19, (◇) 18.

A series of experiments were conducted to evaluate the sensitivity of the interface developed and to determine the detection limits that can be obtained in the scanning mode and in single-ion monitoring (SIM). The data of Table 2, which gives the detection limits measured in both modes (signal-to-noise ratio >3), indicate that limits of detection ranging from 2 to 23 ng were obtained for the different analytes in the scanning mode and ranging from 5 to 30 pg in the single-ion monitoring mode of the parent-molecular ion $[M+H]^+$. The sensitivity observed in both modes with the capillary interface developed is comparable to that obtained with conventional thermospray/plasmaspray (filament-on) interfaces and the detection limits in the order of low nanogrammes in the scanning mode and low pg in the single-ion monitoring mode are comparable. Another important analytical feature is the linearity of the source signal. The results shown in Fig. 8 indicate that the signal is found to be linear over at least 3 orders of magnitude for compounds such as benzonitrile and fluoranthene in the single-ion mode. The system proved to be linear from 2 to 1100 pg in the scanning mode and from 2 to 1000 ng in the single-ion monitoring mode for the two compounds respectively.

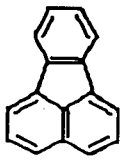
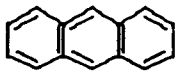
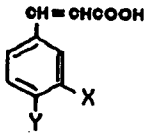
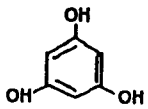
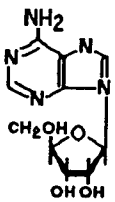
An important factor, when interfacing chromatographic methods to mass spectrometry, is the ability of the interface to preserve chromatographic performance. As mentioned previously, in the type of interface under discussion the flow-rate used has to be compatible with the optimum linear velocity of the mobile phase while allowing the pressure in the ion source to be in the range required for efficient ionization. The situation is similar for the chromatographic separation where the interface must not act as a dilution volume that can reduce the separation efficiency. The latter situation has been demonstrated to occur in LC–CF–FAB-MS systems where the droplet can be considered as a dilution

Table 1
Typical applications of the capillary thermospray interface

Compound	Structure	Ion intensity [a]
Gramine		175 (100)
Piconilic acid		124 (100)
Geraniol		155 (100), 137 (10)
Cineole		155 (100), 137 (35)
Umbelliferone		163 (100), 145 (20)
Caffeine (X = CH_3, Y = CH_3) Theophylline (X = H, Y = CH_3) Theobromine (X = CH_3, Y = H)		195 (100) 181 (100) 181 (100)
Benzonitrile		104 (100)
Chloroaniline		128 (100)
Chloramphenicol		323 (100), 305 (7)

(Table continued on p. 214)

(Table 1 contd.)

Fluoranthene		203 (100)
Anthracene		179 (100)
p-Coumaric acid (X = H, Y = OH) Ferulic acid (X = OCH_3, Y = OH) Caffeic acid (X = OH, Y = OH) *trans*-Cinnamic acid (X = H, Y = H)		165 (100), 147 (40) 195 (100), 177 (25) 181 (100), 163 (30) 149 (100), 131 (30)
Phloroglucinol		127 (100), 108 (20)
Adenosine		268 (100), 136 (18)

[a] Ion intensity (%) (base peak = 100), in parentheses.

Table 2
Typical limits of detection obtained with the capillary thermospray interface

Compound	Detection limit	
	Repetitive scanning[a] (ng)	Selected-ion monitoring (pg)
Chloramphenicol	10	12
Adenosine	23	30
Ranitidine	6	9
Caffeine	3	5
Butylbenzene	7	10
Chloroaniline	2	4
Fluoranthene	3	3
Benzonitrile	10	10
Phenylhexane	2	5

[a] Scan speed of 1 s over a mass range 10–550.

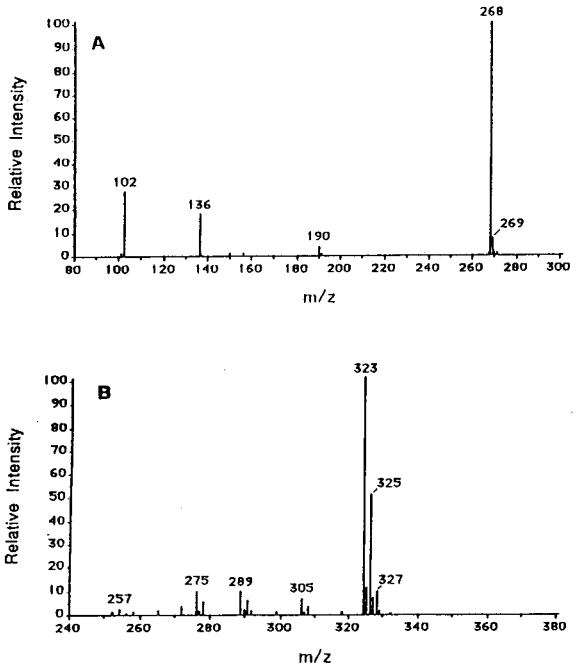


Fig. 7. Mass spectrum of (A) adenosine and (B) chloramphenicol obtained with the capillary in-source thermospray interface.

volume [33]. In order to investigate the possible band broadening that can occur in the interface, a series of experiments were conducted in which the chromatographic peak widths obtained in the μ-TSP interface were compared to those obtained in a conventional LC–UV capillary system. The results of these experiments are depicted in Fig. 9. The figure shows the chromatographic separation of a mixture of alkylbenzenes (toluene, ethylbenzene and propylbenzene) monitored with a UV detector (Fig. 9A) and that observed with the in-source capillary thermospray-type interface (Fig. 9B). Examination of

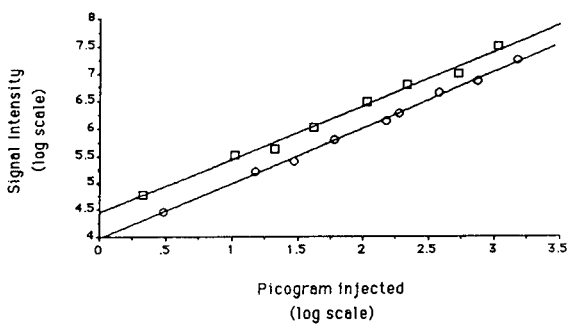


Fig. 8. Calibration curve for parent molecules ions of (○) fluoranthene and (□) benzonitrile.

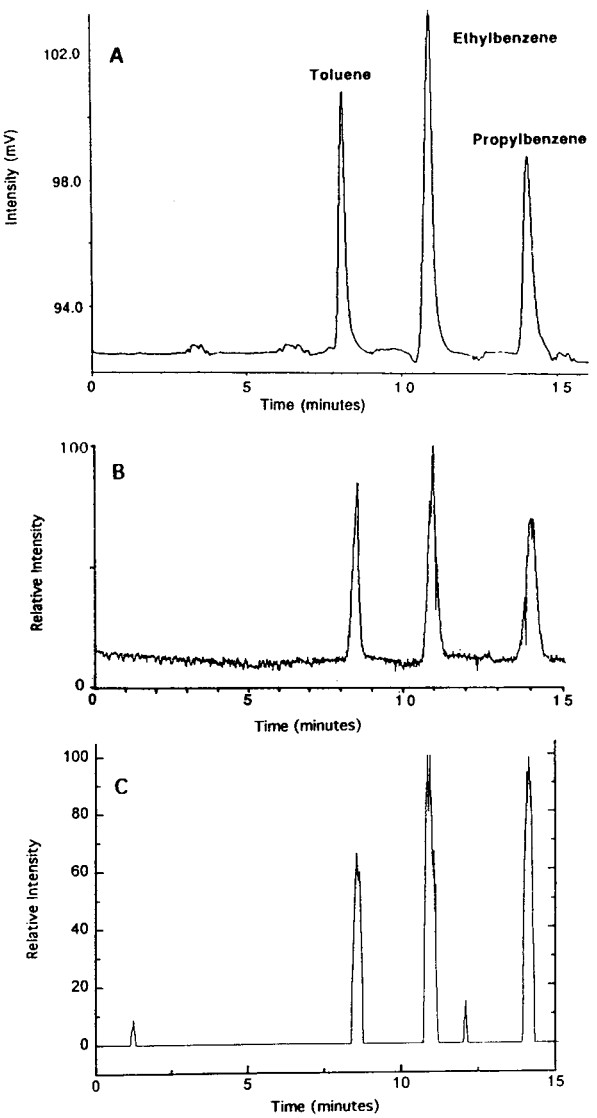


Fig. 9. Separation of a mixture of alkylbenzenes on a 20 cm × 0.25 mm I.D. fused—silica column packed with ODS-2 (5 μm); mobile phase acetonitrile–water (72:25), obtained with (A) UV detector, (B) capillary thermospray interface, (C) treated with the algorithm TICFilt.

the data reveals that the interface developed does not significantly alter the chromatographic profile and that it is not acting as a mixing chamber. Thus, this interface can be advantageously used for LC–MS analysis and also for real-time monitoring of reactions in solution or as a liquid introduction system for mass spectrometry or tandem mass spectrometry (MS–MS) analysis.

The in-source thermospray-type interface, albeit interesting in terms of its performance in the LC–MS analysis of polar molecules of low-molecular-mass, can present limitations when analyzing unknown substances. In the selected-ion monitoring mode the heavy background originating from the mobile phase can usually be avoided by the judicious choice of the m/z ratios that are monitored. However, when analyzing mixtures containing unknown compounds in the repetitive scanning mode, the background contributes a very significant signal that can totally mask signals due to analytes present in small concentrations. Thus, it is preferable to use background treatment algorithms that can eliminate most of the undesirable signal in the TIC as well as in the mass spectra. Fig. 9C shows the TIC that results from the treatment of the LC–MS data by TICFilt, a computer program that we have developed for that purpose [34]. The program allows the detection of small eluting peaks in the TIC generated by direct coupling LC–MS or DLI techniques and provides spectra from which interfering ions have been removed. Hence, the use of a background treatment algorithm minimizes the adverse effect of the important signal generated by the ionization of the mobile phase.

Another difficulty that can be encountered when using direct introduction systems such as the one described is partial or complete clogging of the capillary interface due to the presence of involatile materials. Partial or complete clogging of the vaporizer capillary has been experienced only when the system was used with more concentrated nonvolatile samples with injected quantities above 1 μg or occasionally due to the slow deposition over a long period of time of nonvolatile solvent impurities. The partial clogging can lead to the deviation of the spray resulting in a decrease of the sensitivity. However, this situation is easily detected and remedied.

4. Conclusions

The simple capillary thermospray-type interface that has been described in this work can be very useful to interface capillary liquid chromatography to mass spectrometry and also as a stand-alone system that can be use for flow injection analysis by mass spectrometry or tandem mass spectrometry. The performance of the capillary thermospray-type interface is satisfactory for the analysis of low-molecular-mass polar compounds that cannot always be analyzed by other techniques. It is a reliable system that is easy to operate and offers good stability when operated under optimum conditions. The interface developed has been used to analyze a wide range of compounds and good average sensitivity was obtained both in the repetitive scanning and selected-ion monitoring modes. Detections limits in the low nanogram and low picogram range are obtained in these respective modes of operation. Qualitatively, the mass spectra obtained for a series of compounds with different structures and polarity were found to be in all points comparable to those obtained under typical thermospray conditions. The analytical features of this type of interface can further be improved by the use of appropriate data treatment software that extend the applicability of the technique in the area of analysis of unknown materials.

Acknowledgment

The authors acknowledge the financial support of the Natural Sciences and Engineering Council of Canada (NSERC) and Hydro-Quebec, which permitted this study.

References

[1] C.R. Blakley, J.J. Carmody and M.L. Vestal, J. Am. Chem. Soc., 102 (1980) 5931.
[2] C.R. Blakley, J.J. Carmody and M.L. Vestal, Anal. Chem., 52 (1980) 1636.
[3] C.R. Blakley and M.L. Vestal, Anal. Chem., 55 (1983) 750.
[4] P. Arpino, Mass Spectrom. Rev., 9 (1990) 631.
[5] A.L. Yergey, C.G. Edmonds, I.A.S. Lewis and M.L. Vestal, Liquid Chromatography and Mass Spectrometry, Plenum Press, New York, NY, 1990, pp. 31–85.
[6] Y. Ito, T. Takeuchi, D. Ishii and M. Goto, J. Chromatogr., 346 (1985) 161.
[7] R.M. Caprioli, T. Fan and S.J. Cottrel, Anal. Chem., 58 (1986) 2946.
[8] C.G. Whitehouse, R.N. Dreyer, M. Yamashita and J.B. Fenn, Anal. Chem., 57 (1985) 675.
[9] M. Yamashita and J.B. Fenn, J. Phys. Chem., 88 (1984) 4451.
[10] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong and C.G. Withehouse, Mass Spectrom. Rev., 9 (1990) 37.
[11] V.L. Tal'roze, G.V. Karpov, I.G. Gorodetskii and V.E. Skurat, Russ. J. Phys. Chem., 42 (1968) 1658.
[12] P. Arpino, Mass Spectrom. Rev., 11 (1992) 3.
[13] F.A. Mellon, J.R. Chapman and J.A.E. Pratt, J. Chromatogr., 394 (1987) 209.
[14] J.A. Page, Pestic. Sci., 18 (1987) 291.
[15] R.D. Voyksner, K.L. Tyckzowska and A.L. Aronson, J. Chromatogr., 567 (1991) 389.
[16] K.T. McManos, J.D. deBethizy, D.A. Garteiz, G.A. Kyerematen and E.S. Vesell, J. Chromatogr. Sci., 28 (1990) 510.
[17] G.W. Taylor and D. Watson, J. Chromatogr., 394 (1987) 135.
[18] J.X. De Vries and K.A. Kymber, J. Chromatogr., 562 (1991) 31.
[19] A. Bruchet, M.F. Legrand, P. Arpino and D.J. Dilettato, J. Chromatogr., 562 (1991) 469.
[20] D.F. Gurka, L.D. Betowski, T.L. Jones, S.M. Pyle, R. Titus, J.M. Ballard, Y. Tondeur and W.J. Niederhut, J. Chromatogr. Sci., 26 (1988) 301.
[21] D.F. Gurka, L.D. Betowski, T.A. Hinners, E.M. Heithmar, R. Titus and J.M. Hensshaw, Anal. Chem., 60 (1988) 454A.
[22] D.A. Garteiz and M.L. Vestal, LC Magazine, 3 (1985) 334.
[23] M. Saito, K. Hibi, D. Ishii and T. Takeuchi, in D. Ishii (Editor), Introduction to Microscale High-Performance Liquid Chromatography, VCH Publishers, New York, NY, 1988, pp. 7–31.
[24] W.M.A. Niessen and J. van der Greef, in J. Cazes (Editor), Liquid Chromatography–Mass Spectrometry, Marcel Dekker, New York, NY, 1992.
[25] P. Krien, P. Devant and M. Hardy, J. Chromatogr., 251 (1982) 129.
[26] J.P. Gagné, S.G. Roussis and M.J. Bertrand, J. Chromatogr., 554 (1991) 293.
[27] P.A. Arpino and C. Beaugrand, Int. J. Mass Spectrom. Ion Proc., 64 (1985) 275.
[28] P. Hirter, H.J. Walther and P. Dätwyler, J. Chromatogr., 323 (1985) 89.
[29] P.J. Arpino and C. Beaugrand, Int. J. Mass. Spectrom. Ion Proc., 64 (1985) 275.
[30] C. Lindberg and J. Paulson, J. Chromatogr., 394 (1987) 117.
[31] P.R. Das, B.N. Pramanik, R.D. Malchow, K.J. Ng, Biomed. Environ. Mass Spectrom., 15 (1988) 253.
[32] J.R. Chapman, J. Chromatogr., 323 (1985) 153.
[33] J.P. Gagné, A. Carrier, L. Varfalvy and M.J. Bertrand, J. Chromatogr., 647 (1993) 13.
[34] A. Carrier, D. Zidarov and M.J. Bertrand, Proc. 42nd ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL, 1994, p. 490.

ELSEVIER

Journal of Chromatography A, 705 (1995) 219–227

JOURNAL OF CHROMATOGRAPHY A

Investigations into the epimerisation of tipredane ethylsulphoxide diastereoisomers during chromatographic analysis on reversed-phase silica
I. Investigations into the reaction mechanism

Melvin R. Euerby[a,*], Christopher M. Johnson[a], Ian D. Rushin[a], D.A.S. Sakunthala Tennekoon[b]

[a] *Analytical Chemistry Department, Research and Development Laboratories, Fisons Pharmaceuticals PLC, Bakewell Road, Loughborough, Leicestershire LE11 0RH, UK*

[b] *Department of Pharmaceutical Sciences, University of Strathclyde, Royal College, 204 George Street, Glasgow G11 XW, Scotland, UK*

First received 27 September 1994; revised manuscript received 24 February 1995; accepted 24 February 1995

Abstract

The epimeric α-ethylsulphoxides of tipredane have been shown to undergo sulphoxide epimerisation and elimination of ethylsulphenic acid during reversed-phase (RP) HPLC, prior to the chromatographic separation. This has been shown to be promoted by increased oven temperature, acidic mobile phase, extended analysis time, column chemistry (i.e. silanol population and distribution) and increased metal content (i.e. iron(II) and titanium) of the RP stationary material. Their propensity to undergo such reactions is related to the facile C-17α C-S(O)Et bond breakage. Epimerisation and elimination have been postulated to arise via a common intermediate: an immobilised ethylsulphoxide–metal–geminal silanol complex.

1. Introduction

It has previously been shown that tipredane (I) can undergone S-oxidation in a site- and stereo-selective manner to yield both the methyl (II) and ethylsulphoxide (III) diastereoisomers (β- and α-plane oxidations, respectively) [1,2]. The ethylsulphoxide diastereoisomers of tipredane (III, the steroidal sulphoxide diastereoisomers will be referred to as epimers) are extremely labile compounds undergoing elimination of ethylsulphenic acid at neutral pH at temperatures above 0°C to yield the corresponding C-17 methylthiovinyl derivative (IV).

During chromatographic analysis of the epimers of the ethylsulphoxide (III), elimination of ethylsulphenic acid and epimerisation of the sulphoxide moiety was observed. The degree of elimination and epimerisation was found to be related to the actual chromatographic conditions and the type of octadecylsilyl stationary phase

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00272-3

employed. This paper presents a detailed investigation into the mechanism of the unusual phenomena of epimerisation and elimination arising from a common intermediate. The prerequisites which predispose the ethylsulphoxide (III) to epimerisation and elimination are the labile carbon–sulphur bond of the C-17α substituent, metal ions (present in the native silica or introduced as contaminants from stainless-steel frits) and geminal silanol groups.

The structures of the compounds mentioned in this paper are shown in Fig. 1.

2. Experimental

2.1. Chemicals

All buffer chemicals and solvents used were of HPLC grade, other chemicals were of AR grade (Fisons Scientific Apparatus, Loughborough, UK). Water was purified by means of an Elgastat Spectrum RO and ion-exchange/carbon filter system (High Wycombe, UK). The tipredane epimeric sulphoxides (II, III) were prepared according to the method of Euerby and co-workers [1,2].

The epimeric ethylsulphoxides (III) were stored at −18°C for up to 48 h. The analogous α- and β-sulphoxides of the C-17 epimer of tipredane (V, VI) were prepared and separated in a similar manner and characterised by LC–MS.

2.2. Chromatography

HPLC was performed using a Hewlett-Packard 1090M HPLC system equipped with a Model 1040 linear photodiode-array UV detector. Data acquisition and integration was controlled by a Hewlett-Packard 79994A Chem Station (Hewlett-Packard, Stockport, UK). Detection was at 240 nm, based on the λ_{max} of tipredane.

The purity of the separated fractions (75 μl) was established by analysis using HPLC conditions 1 and 2.

2.3. HPLC analysis of the tipredane sulphoxides

HPLC conditions 1 [2].

Chromatography was performed on a Hypersil ODS "Excel" column (5 μm, 100 × 4.6 mm I.D., Hichrom, Reading, UK). The eluent consisted of mobile phases A and B which were 0.025 *M* NH_4OAc (pH 7.2) and 0.025 *M* NH_4OAc (pH 7.2) in acetonitrile–water (65:35, v/v), respectively. The flow-rate was 1.5 ml/min and the oven temperature was thermostatically held at 26°C. A linear gradient was run during 10 min from 45 to 50% and then from 50 to 95% mobile phase B during 10 min; the final eluent composition was then held for a further 5 min.

HPLC conditions 2

The conditions as described in HPLC conditions 1 were followed with the exception that a Pellicular ODS guard column (37–53 μm, 75 × 2 mm, Whatman, NJ, USA) was placed in front of the Hypersil ODS column (5 μm, 100 × 4.6 mm I.D.), that the oven temperature was thermostatically held at 40°C and that an initial 20 min isocratic mobile-phase composition of 10% B followed by a linear gradient run for 5 min to 45% B were employed prior to that described in HPLC conditions 1. Reaction products were identified by comparison of retention times and spiking experiments with those of authentic materials, and UV diode-array spectroscopy.

All calculations were based on the assumption that the relative response factor for each compound was 1. This assumption is based on the fact that the absorbance at 240 nm is due to the A ring of the steroid and is present in all of the compounds investigated.

2.4. Investigations of the effect of metal ions on epimerisation and elimination

Solutions of the chloride salts of iron(II) and iron(III) in methanol were prepared. To study the effect of these metals in solution, 1 μl (5 μmol) was added to a solution of the tipredane *S*-ethylsulphoxide (III, 90 μl, 90 μmol) in a plastic vial. The contents of the vial were mixed and then incubated at 40°C for 40 min. The

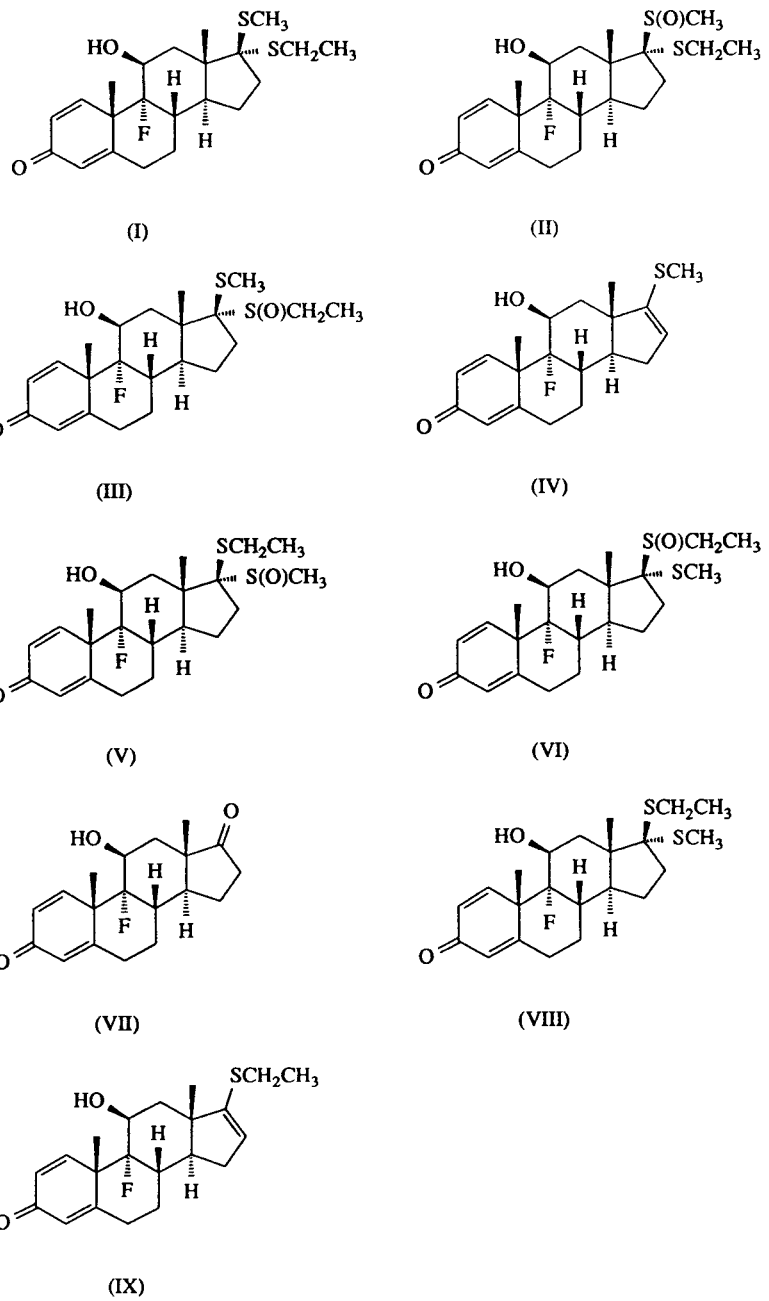


Fig. 1. Structure of compounds mentioned in the present paper. Sulphoxides are chiral because of the stable pyramidal configuration about sulphur, bringing about the formation of steroidal epimers. For simplicity the stereochemistry of the sulphoxide moiety has not been shown.

resulting solution was then analysed using HPLC conditions 1.

3. Results and discussion

3.1. Chromatography of the ethylsulphoxide epimers of tipredane (III)

It has previously been shown that the thermally labile ethylsulphoxide epimers of tipredane (III) can be successfully chromatographed without elimination of ethylsulphenic acid provided that the column temperature is maintained at 26°C or lower, the residency time on the column is minimal and the mobile phase pH is near neutral (see HPLC conditions 1) [2].

In the course of our research into the reaction kinetics of the disulphoxidation of tipredane it was necessary to chromatograph the ethylsulphoxide epimers (III) using a modification of HPLC conditions 1, i.e. the oven temperature was increased from 26 to 40°C and an initial isocratic eluent composition of 10% mobile B for 20 min was substituted prior to commencement of the gradient (see HPLC conditions 2). In addition, a Pellicular ODS guard column was employed.

As expected, due to the elevated column temperature, significant elimination of ethylsulphenic acid occurred yielding the C-17 methylthiovinyl derivative (IV) due to the thermal instability of the ethylsulphoxides (III) (see Table 1). Additionally, a percentage of the C-17 methylthio derivative (IV) underwent hydrolysis to the C-17 keto derivative (VII) (see Fig. 2).

In addition to elimination which occurred, both ethylsulphoxide epimers (III) exhibited epimerisation (see Fig. 2). The epimerisation could be deduced as occurring prior to separation as the peak shape was sharp with no "saddle or reaction zone" between peaks (typically observed if interconversion occurs during the chromatographic process). The phenomenon of "reaction zones" between peaks was first described by Keller and Giddings [3] and a theoretical basis developed by Melander et al. [4]. This phenomenon has subsequently been observed for the *cis–trans* interconversion of peptides [5,6] and various reactions occurring during the chromatographic separation process [7–10]. The *S*-sulphoxide epimer showed a greater tendency towards epimerisation than its corresponding *R*-epimer. This may suggest that in an equilibrium mixture one of the epimers is favoured. This was not verified experimentally as other competing elimination reactions were concomitantly in operation.

Table 1
Effect of chromatographic temperature on the epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (III) using HPLC conditions 2 with a Hypersil ODS column and Pellicular ODS guard column

Temperature (°C)	Epimerisation (%)	Elimination[a] (%)
26	5.8	1.9
40	17.0	8.3
50	36.6	26.0
60	25.9	57.6

[a] Summation of elimination products (IV, VII); the C-17 keto derivative (VII) is classed as an elimination product as it can only arise from subsequent hydrolysis of the elimination product (IV).

The epimerisation observed is quite remarkable, as previously sulphoxides have only been shown to undergo chiral inversion when exposed to harsh conditions such as photolysis or ther-

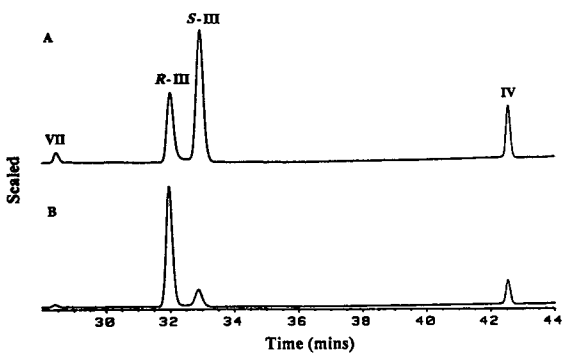


Fig. 2. HPLC analysis of ethylsulphoxide epimers (III) on a Pellicular ODS guard column and a Hypersil ODS column. Chromatographic conditions 2 as in Section 2. (A) *S*-epimer, (B) *R*-epimer.

molysis [11]. The sulphoxides under investigation are known to be extremely labile undergoing elimination at temperatures far below those necessary for thermolytic epimerisation [2].

In contrast to the ethylsulphoxide epimers (III), both methylsulphoxide epimers (II) failed to undergo elimination or epimerisation reactions during chromatography using HPLC conditions 2 with a Pellicular ODS guard column.

3.2. Structural and stereochemical requirements for the epimerisation reaction

Using the same experimental approach as that described previously [2], the C-17 epimer of tipredane (VIII) (in which the position of the ethyl and methyl groups have been interchanged) was oxidised with monomagnesium peroxyphthalic acid to yield the corresponding α-methylsulphoxide (V) and β-ethylsulphoxide (VI) epimers of tipredane. These were subsequently separated using an analytical over-load technique (using HPLC conditions 1, in purities in an excess of 96% and concentration of approximately 50–100 μg/ml) and chromatographed using the epimerisation conditions (HPLC conditions 2). In an analogous manner to the α-ethylsulphoxides of tipredane (III), the α-methylsulphoxides of the C-17 epimer of tipredane (V) showed the same tendency to epimerise and eliminate. The β-methylsulphoxides of tipredane (II) and the β-ethylsulphoxides of the C-17 epimer of tipredane (VI) remained resistant to epimerisation and elimination under the conditions investigated (see Table 2 for results arising from the C-17 epimer of tipredane).

This indicated that it is not the sulphoxide substituent that predisposes it to these anomalous reactions but instead it is the site of the sulphoxide substituent, i.e. epimerisation and the extreme thermal instability are associated with the α-sulphoxides (III, V). This raises the possibility that these processes proceed by a common mechanism. Supporting evidence for this comes from the fact that increasing the column temperature resulted in increased levels of both epimerisation and elimination of the

Table 2
Effect of sulphoxide substituent position on the degree of elimination and epimerisation using HPLC conditions 2 with a Hypersil ODS column and Pellicular ODS guard column

Epimer	Epimerisation (%)	Elimination (%)[a]
α-MeS(O)EtS more polar[b] (V)	3.9	2.3
α-MeS(O)EtS less polar[b] (V)	16.6	5.0
β-EtS(O)MeS more polar[b] (VI)	0.9	0.0
β-EtS(O)MeS less polar[b] (VI)	0.0	0.0

[a] Summation of elimination products (VII and the corresponding vinyl thioether).
[b] Expressed in terms of retention behaviour only.

S-ethylsulphoxide epimer (III) up to a temperature of 50°C, whereas at higher column temperatures, elimination of ethylsulphenic acid became more favoured (see Table 1).

3.3. Chromatographic parameters effecting the degree of epimerisation

The residence time on the column per se was shown not to be crucial to the epimerisation reaction. It was shown that use of a 25-cm column in place of the 10-cm Hypersil ODS column under HPLC conditions 1 resulted in a longer residence time on the column; however, there was only a slight increase in the elimination of ethylsulphenic acid and no epimerisation was noted. This is further evidence to support the conclusion that the reaction does not occur during the separation process.

The duration of the initial isocratic phase, in which the *R*-ethylsulphoxide epimer (III) was strongly adsorbed onto the top of the C_{18} stationary phase, was found to dictate the amount of epimerisation and elimination observed. Exposure of the *R*-ethylsulphoxide epimer (III) to 10% B mobile phase for 40 min rather than 20 min resulted in retention times 20 min longer, and concomitantly more epimerisation and elimination (see Table 3).

The effect of mobile phase pH on the degree

Table 3
Effect of duration of exposure to the initial chromatographic conditions on the elimination and epimerisation of the *R*-epimer of the ethylsulphoxide (III) using HPLC conditions 2 with a Hypersil ODS column and Pellicular ODS guard column

Duration at 10% B (min)	Epimerisation (%)	Elimination[a] (%)
0	0.0	1.0
20	6.3	6.5
40	8.7	12.5

[a] Summation of the products of elimination (IV, VII).

of epimerisation and elimination produced by exposure to chromatography conditions 2 can be seen in Table 4. The results suggest that the *S*-ethylsulphoxide epimer (III) is extremely prone to acid-promoted elimination of ethylsulphenic acid yielding the C-17 methylthiovinyl derivative (IV) which can subsequently undergo hydrolysis to the C-17 keto derivative (VII). In addition, the epimerisation reaction appears to be promoted by acidic conditions; however, at pH 5.3 the elimination reaction is more favoured than epimerisation.

The nature of the stationary phase in these reactions was shown to be important in that when the *S*-ethylsulphoxide epimer (III) was chromatographed using HPLC conditions 2 (reaction on the Pellicular ODS guard column and separation on the Hypersil ODS column), 26.2 and 9.2% epimerisation and elimination, respectively, were observed, whereas without the Pellicular ODS guard column only 9.4 and 3.7% epimerisation and elimination, respectively, were observed. The involvement of the stationary-phase chemistry in these reactions is described in a subsequent paper [12].

Table 4
Effect of pH of mobile phases defined in HPLC conditions 2 on the epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (III), using a Hypersil ODS column with a Pellicular ODS guard column

pH of mobile phase A	Epimerisation (%)	Elimination[a] (%)
7.51	21.9	7.6
6.73	27.8	9.5
6.11	34.6	17.1
5.30	35.0	44.2

[a] Summation of the products of elimination (IV, VII).

3.4. Solution reactions of the ethylsulphoxide epimers of tipredane (III)

Storage of the *R*-ethylsulphoxide epimer (III) in mobile phase (A–B, 45:55, v/v) at 45°C for 32 min and subsequent analysis using HPLC conditions 1 (which do not cause elimination or epimerisation) indicated that only 0.6% and 27% epimerisation and elimination, respectively, had occurred. In comparison, chromatography using HPLC conditions 2 indicated that a significant level of epimerisation had occurred. This confirmed the involvement of the stationary-phase chemistry in the epimerisation reaction.

In the subsequent paper [12] the involvement of the stationary-phase chemistry in the epimerisation and elimination reactions is investigated. It has been shown that the prerequisites for these reactions are the presence of a high proportion of geminal silanols and metals, either present in the native silica or inadvertently added via contamination of the stationary phase with metals leached from the stainless-steel frits when exposed to neat organic solvents.

Only negligible amounts of epimerisations were observed with either iron(II) or (III) in solution; however, elimination was accelerated with both oxidative states of iron. In the case of iron(III) this presumably reflected the acidic pH of the reaction mixture (see Table 5). Whereas for iron(II) the degree of elimination increased with the concentration of iron(II) in the solution, while the pH of the solution did not change significantly, suggesting that iron(II) was directly facilitating the elimination. Hung et al. [10] have also observed that *p*-hydroquinones were oxidised faster during chromatography than in free solution and postulated a heavy metal catalysed reaction at the stationary-phase surface. In this case the metal responsible was iron(III).

Table 5
Effect of iron(II) and (III) concentrations on the epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (III) separated fraction at 40°C for 30 min; HPLC conditions 1 using a 75-μl injection volume (equivalent to approximately 75 μg loaded on-column)

Iron (nmol)		pH*	Epimerisation (%)	Elimination[a]
Fe(II)	Fe(III)	(%)		
50		6.9	0.4	40.6
100		6.8	0.1	52.9
250		6.7	0.0	78.7
0	0	6.9	0.5	13.3
	0	5.0[b]	0.0	99.2
	50	5.9	0.2	41.3
	100	5.6	0.1	67.3
	250	5.0	0.0	94.4

[a] Summation of elimination products (IV, VII).
[b] pH adjusted manually with dilute HCl.
pH* Apparent pH.

3.5. Postulated mechanism of the epimerisation/elimination reaction

The reaction of tipredane *S*-ethylsulphoxide (III) with a bound metal can be rationalised by the formation of an immobilised sulphoxide–metal–geminal silanol complex (using the sulphur of the methylthio group and the oxygen of the ethylsulphoxide group to complex the metal in a five-membered ring (see Fig. 3).

The formation of a similar complex in solution with iron(II) may explain the enhanced elimination of ethylsulphenic acid observed. In this case the iron(II) increases the capacity of the ethylsulphoxide moiety to act as a leaving group and, as the complex is not immobilised once the C-17α C-S(O)Et bond breaks, the metal is free to form the ethylsulphenic acid salt and hence epimerisation does not occur. In comparison, when the complex is immobilised, after the facile breaking of the C-17α C-S(O)Et bond has occurred, the liberated ethylsulphenic acid is held firmly by the metal complex in close proximity to the C-17 position. However, sufficient rotation of the carbon–sulphur bond of the ethylsulphoxide moiety is possible to allow either of the lone pairs of electrons of the sulphur to reform the five-membered ring complex resulting in epimerisation, statistically producing a 1:1 epimeric mixture. This in practice may not be the case as steric effects and the stability of the metal complex may favour formation of one of the epimers or preferential elimination of ethylsulphenic acid.

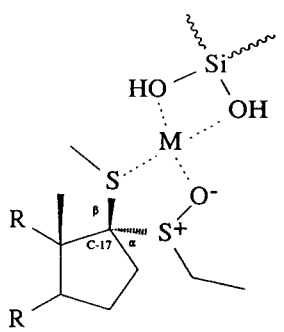


Fig. 3. Schematic representation of the interaction of tipredane ethylsulphoxide epimers (III) and bound metal to geminal silanols. M corresponds to cationic species such as iron(II) and titanium.

The ease with which this reaction occurs depends on the relative instability of the C-17α carbon–sulphur bond of the ethylsulphoxide epimers (III). The increased stability of the *R*-epimer towards epimerisation may be attributed to the small but possibly significant greater thermal stability of the *R*-epimer over the *S*-epimer [2].

3.6. Epimerisation of the methylsulphoxide epimers of tipredane (II) at elevated chromatographic temperatures

The methylsulphoxide epimers (II) failed to undergo any significant epimerisation and/or elimination with either the Pellicular ODS and Kromasil ODS stationary-phase material, using HPLC conditions 2. However, when the column temperature was increased to 80°C, using the Kromasil ODS column, the energy barrier to the C-17β C-S(O)Me bond breakage was overcome, hence, facilitating epimerisation and elimination (see Table 6 and Fig. 4). This implied that the methylsulphoxide epimers (II) possess the ability to complex with immobilised iron(II) under

Table 6
Effect of column temperature on the degree of epimerisation and elimination of the *S*- and *R*-epimers of the methylsulphoxide (II) on a metal-contaminated Kromasil ODS column using HPLC conditions 2

Configuration of methylsulphoxide epimer (II)	Epimerisation (%)		Elimination (%)[a]	
	40°C	80°C	40°C	80°C
S	0.3	10.8	0.4	13.0
R	0.0	4.5	0.0	2.9

[a] Summation of elimination products (VII, IX).

these conditions, in a similar manner to that depicted in Fig. 3 for the corresponding ethylsulphoxide epimers (III). However, at lower temperatures (40°C), the lack of epimerisation observed with the methylsulphoxide epimers may be attributed to either lack of complex formation or, more likely, the increased stability of the carbon–sulphur bond of the C-17β C-S(O)Me moiety. The *R*-epimer has previously been shown to be more stable to thermolysis than its corresponding *S*-epimer [1] and this is reflected in the *R*-epimer's reduced tendency to undergo epimerisation and elimination reactions (see Fig. 4).

4. Conclusions

In conclusion, the tipredane sulphoxides have been shown to undergo sulphoxide epimerisation and concomitant elimination of alkylsulphenic acid prior to chromatographic separation. These reactions are directly related to the stability of the C-17 carbon–sulphoxide bond. The extent of these reactions is related to certain chromatographic parameters (such as mobile-phase pH, column temperature, stationary-phase chemistry and metal content and time bound to the stationary phase). These results can be explained by the formation of an immobilised sulphoxide–metal–geminal silanol 5-membered ring complex, prior to the chromatographic separation process.

The subsequent paper [12] describes and examines the use of the ethylsulphoxide epimerisation reaction to probe the metal content and silanol surface chemistry of a wide range of commercially available RP stationary phases.

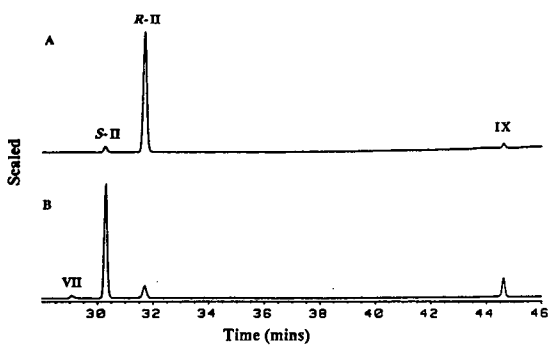


Fig. 4. HPLC analysis of methylsulphoxide epimers (II) on a Kromasil C_{18} column (inadvertently contaminated with metals), no guard column employed. Chromatographic conditions 2 as in Section 2 with the exception that the analysis was performed at 80°C. (A) *R*-epimer, (B) *S*-epimer.

Acknowledgements

We are grateful to Dr. D. Mallern, Messrs. R. Shaw, D. Stanley (Hichrom, Reading, UK), J. Plant and Dr. H. Ritchie (Shandon HPLC, Runcorn, UK) for support and helpful advice, to Dr. J. Crew for critical appraisal of the manuscript, Miss A. Stockley for typographical advice and the British Council for financial support (DASST).

References

[1] M.R. Euerby, J. Hare and S.C. Nichols, J. Pharm. Biomed. Anal., 10 (1992) 269–277.
[2] M.R. Euerby, C.M. Johnson, R.J. Lewis and S.C. Nichols, J. Pharm. Biomed. Anal., 12 (1994) 761–769.

[3] R.A. Keller and J.C. Giddings, J. Chromatogr., 3 (1960) 205–220.
[4] W.R. Melander, H.-J. Lin, J. Jacobson and Cs. Horvàth, J. Phys. Chem., 88 (1984) 4527–4536.
[5] M. Lebel, S. Fang and V.J. Hruby, J. Chromatogr., 586 (1991) 145–148.
[6] J. Jacobson, W. Melander, G. Vaisnys and Cs. Horvàth, J. Phys. Chem., 88 (1984) 4536–4542.
[7] C.-Y. Jeng and S.H. Langer, J. Chromatogr. Sci., 27 (1989) 549–552.
[8] Z. Deng, J.Z. Zhang, A.B. Ellis and S.H. Langer, J. Liq. Chromatogr., 16 (1993) 1083–1103.
[9] B.S. Ludolph, C.-Y. Jeng, A.H.T. Chu and S.H. Langer, J. Chromatogr. A, 660 (1994) 3–15.
[10] J.-X. Hung, J.D. Stuart, W.R. Melander and Cs. Horvàth, J. Chromatogr., 316 (1984) 151–161.
[11] G. Solladie, Synthesis, (1981) 185–196.
[12] M.R. Euerby, C.M. Johnson, I.D. Rushin and D.A.S.S. Tennekoon, J. Chromatogr. A, 705 (1995) 229.

ELSEVIER

Journal of Chromatography A, 705 (1995) 229–245

JOURNAL OF CHROMATOGRAPHY A

Investigations into the epimerisation of tipredane ethylsulphoxide diastereoisomers during chromatographic analysis on reversed-phase silica
II. The involvement of metals in commercially available C_{18} silicas

Melvin R. Euerby[a,*], Christopher M. Johnson[a], Ian D. Rushin[a], D.A.S. Sakunthala Tennekoon[b]

[a] *Analytical Chemistry Department, Research and Development Laboratories, Fisons Pharmaceuticals PLC, Bakewell Road, Loughborough, Leicestershire LE11 0RH, UK*

[b] *Department of Pharmaceutical Sciences, University of Strathclyde, Royal College, 204 George Street, Glasgow G11 XW, UK*

First received 27 September 1994; revised manuscript received 24 February 1995; accepted 24 February 1995

Abstract

The degree of sulphoxide epimerisation (and concomitant ethylsulphenic acid elimination) of the α-ethylsulphoxide epimers of tipredane showed no direct correlation with measured chromatographic parameters for a wide range of commercially available octadecylsilyl stationary phases. Correlation was however established with the metal content (i.e. iron(II) and titanium) of these phases. Low metal content stationary phases have been shown to cause epimerisation (in addition to poor chromatography for analytes with chelating properties) via contamination of the top of the column with metal ions extracted from the stainless-steel or titanium frits when the column had been shipped in neat acetonitrile or methanol. The total metal content of packed columns could be conveniently assessed using the highly sensitive 2,3- and 2,7-dihydroxynaphthalene efficiency ratio test.

1. Introduction

It has previously been shown that the ethylsulphoxide diastereoisomers of tipredane (I, the steroidal sulphoxide diastereoisomers will be referred to as epimers) can undergo epimerisation of the sulphoxide moiety when exposed to certain chromatographic conditions (see preceding paper [1]). The epimerisation (and concomitant elimination of ethylsulphenic acid) occurs when the ethylsulphoxides are adsorbed onto the top of the octadecylsilyl stationary phase prior to the chromatographic separation. The degree of epimerisation was shown to be related to the oven temperature, the pH of the mobile phase, stationary-phase chemistry and the duration that the ethylsulphoxides were adsorbed to the stationary phase prior to separation.

This paper presents a detailed investigation into the involvement of a wide range of commercially available octadecylsilyl stationary-phase

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00273-1

packing materials in the epimerisation and elimination reactions and attempts to correlate numerous chromatographic parameters of the columns (such as hydrophobicity, steric selectivity, hydrogen bonding capacity, ion-exchange capacity at pH > 7 and < 3 and the quantity of alkyl chains) to the degree of epimerisation that the stationary phase promotes. These investigations have additionally shown a direct involvement of metal ions (present in the native silica or introduced as contaminants from stainless-steel frits) in the epimerisation and elimination reactions.

Stainless-steel and titanium frits in the presence of organic shipping solvents have been shown to leach metals including iron(II) and titanium which are then bound to the geminal silanols of the high-purity deactivated silicas. These metals promote the epimerisation and elimination reactions in addition to being deleterious to the chromatography of analytes possessing the capacity for chelation.

The ethylsulphoxides and the 2,3- and 2,7-dihydroxynaphthalenes (IV, V) have been used subsequently to evaluate the metal content/contamination of a range of new octadecylsilyl stationary-phase chemistries and to evaluate the efficiency of procedures used to remove unwanted metals from such stationary phases. The implications of these findings in relation to the chromatography of compounds possessing chelating properties will be discussed.

The structures of the compounds mentioned in this paper are shown in Fig. 1.

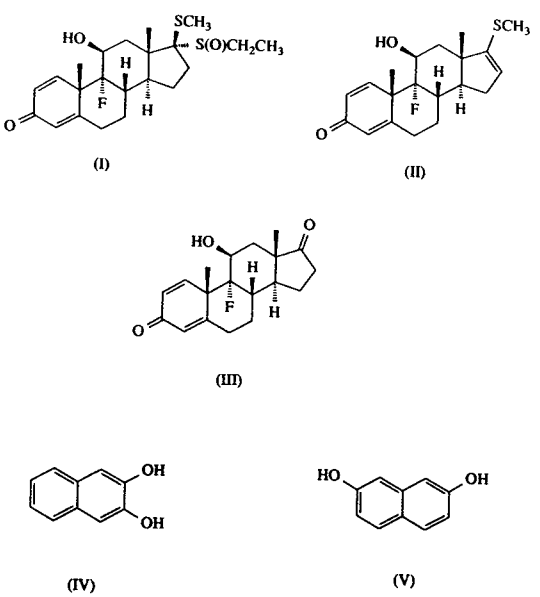


Fig. 1. Structure of compounds mentioned in the present paper. Sulphoxides are chiral because of the stable pyramidal configuration about sulphur, bringing about the formation of steroidal epimers. For simplicity the stereochemistry of the sulphoxide moiety has not been shown.

2. Experimental

2.1. Chemicals

All buffer chemicals and solvents used were of HPLC grade, other chemicals were of AR grade (Fisons Scientific Apparatus, Loughborough, UK). Water was purified by means of an Elgastat Spectrum RO and ion-exchange/carbon filter system (High Wycombe, UK). The ethylsulphoxide epimers of tipredane (I) were prepared according to the method of Euerby and co-workers [2,3] and were stored at −18°C for up to 48 h. 2,3- and 2,7-Dihydroxynaphthalene (IV, V) were obtained from Sigma (Dorset, UK)

2.2. Chromatography

HPLC was performed using a Hewlett-Packard 1090M HPLC system equipped with a Model 1040 linear photodiode-array UV detector. Data acquisition and integration was controlled by a Hewlett-Packard 79994A Chem Station (Hewlett-Packard, Stockport, UK). Chromatography was performed on a variety of columns shown in Table 1. Detection was at 240 nm, based on the λ_{max} of tipredane.

The dead time (t_o) was determined from the first deviation of the baseline after injection.

2.3. Investigations into the effect of commercially available octadecylsilyl stationary phases on the epimerisation/elimination reactions (HPLC conditions 1)

Chromatography was performed on a wide range of commercially available ODS columns

Table 1
Dimensions, particle size and storage solvents of columns investigated

Stationary phase/column	Dimensions (mm)	Particle size (μm)	Storage solvent (v/v)
Hypersil ODS	100 × 4.9	5	MeOH
	150 × 4.9	3	
RP-18 Lichrospher guard column[a]	4 × 4	5	na
RP-18 Lichrospher guard column endcapped[a]	4 × 4	5	na
RP-8 Lichrospher guard column[a]	4 × 4	5	na
Pellicular ODS guard column[a]	75 × 2	37–53	na
Resolve C_{18}	150 × 3.9	5	MeCN/H_2O 60:40
Nova-pak C_{18}	150 × 3.9	4	MeCN/H_2O 50:50
YMC Basic	250 × 4.6	5	MeCN/H_2O 60:40[b]
Lichrosphere 60 RP Select B	125 × 4.0	5	MeCN/H_2O 75:25[b]
SynChropak SCD-100	250 × 4.6	5	MeOH/H_2O 75:25
Spherisorb ODS1	150 × 4.6	5	MeOH/H_2O 65:35
Spherisorb ODS2	150 × 4.6	5	MeOH/H_2O 70:30
Astec C_{18} polymer	150 × 4.6	5	MeCN/H_2O 65:35
Ultrasphere ODS	150 × 4.6	5	MeOH/H_2O 70:30
μBondapak C_{18}	300 × 3.9	10	MeCN/H_2O 60:40
Zorbax R_x C_{18}	150 × 4.6	5	MeOH/H_2O 80:20[b]
Hypersil BDS C_{18}	150 × 4.6	5	MeOH/H_2O 70:30
Suplex pKb-100 C_{18}	250 × 4.6	5	MeCN
Supelcosil LC-ABZ C_{18}	150 × 4.6	5	MeCN
Kromasil C_{18}	150 × 4.6	5	MeOH
Hichrom RPB C_{18}	150 × 4.6	5	MeOH
Spherisorb ODSB	150 × 4.6	5	MeOH/H_2O 70:30
Purospher C_{18}	250 × 4.6	5	MeCN
Symmetry C_{18}	150 × 3.9	5	MeCN
Zorbax SB C_{18}	150 × 4.6	5	MeCN/H_2O 90:10

[a] Guard column material used in conjunction with Hypersil ODS Excel column.
[b] Test evaluation mobile-phase composition.
na = Not applicable.

shown in Table 1. The eluent consisted of mobile phases A and B which were 0.025 *M* NH_4OAc (pH 7.2) and 0.025 *M* NH_4OAc (pH 7.2) in acetonitrile–water (65:35, v/v), respectively. The flow-rate was 1.5 ml/min and the oven temperature was thermostatically held at 40°C. An initial 20-min isocratic mobile phase composition of 10% B followed by a linear gradient run over 5 min to 45% B, 10 min from 45 to 50% and then 50 to 95% mobile phase B over 10 min was employed; the final eluent composition was then held for a further 5 min.

Reaction products were identified by comparison of retention times and spiking experiments with those of authentic materials and UV diode-array spectroscopy.

All calculations were based on the relative response factor for each compound being 1. This assumption relied on the fact that the absorbance at 240 nm was due to the A ring of the steroid and is present in all of the compounds investigated.

2.4. 2,3- and 2,7-Dihydroxynaphthalene efficiency ratio test (DERT)

Chromatography was performed on a range of octadecylsilyl stationary phases. The eluent consisted of 0.025 *M* NH_4OAc (pH 7.2) in acetonitrile–water (25:75, v/v). The flow-rate was 1.5 ml/min and the oven temperature was thermostatically held at 40°C. Detection at 230 nm and a run time of 60 min were employed. A 10-μl amount of the test mixture in acetonitrile was injected (on-column loading of 334 and 157 ng of 2,3- and 2,7-dihydroxynaphthalene IV and V, respectively). The base peak efficiency for each analyte was calculated by Multichrom software (VG Data Systems, Altrincham, Cheshire, UK).

The dihydroxynaphthalene efficiency ratio test (DERT) value was calculated as follows:

$$\text{DERT} = \frac{\text{Base peak efficiency 2,7-dihydroxynaphthalene}}{\text{Base peak efficiency 2,3-dihydroxynaphthalene}}$$

2.5. Investigations into the effect of metal ions on epimerisation/elimination reactions

The effect of loading methanolic solutions of metal ions onto the column was investigated by first equilibrating the column at 40°C in neat methanol at 1.5 ml/min for 20 min and then injecting the methanolic metal solution (100 μl, 500 nmol). The column was then rinsed with pure water to remove the methanol before re-equilibration with the HPLC conditions. The column was then evaluated chromatographically for metal content by the epimerisation/elimination and DERT determinations.

2.6. Atomic absorption spectroscopy

Total iron content was determined using a Perkin Elmer 703 atomic absorption spectrometer (Beaconsfield, UK). Methanolic samples were analysed against external methanolic standards at 248.3 nm using an air/acetylene flame.

2.7. Inductive coupled plasma spectroscopy (ICP)

Elemental analysis was performed using an ARL 3580 optical emission ICP argon plasma spectrometer (Fisons Instruments, Crawley, UK) by external calibration against aqueous standards. Methanolic extracts of frits were evaporated to dryness and then re-dissolved in dilute hydrochloric acid prior to analysis.

2.8. Column characterisation HPLC conditions [4]

The flow-rate was 1.5 ml/min and the oven temperature was thermostatically held at 40°C. Detection at 254 nm and a run time of 20 min were employed.

Amount of alkyl chains (k'_{AB})

The eluent consisted of methanol–water (80:20, v/v); 10 μl of the test solution which contained amylbenzene dissolved in mobile phase, was injected (on-column loading of 6 μg). The capacity factor and the column efficiency at 50% peak height were calculated.

Hydrophobicity [$\alpha(CH_2)$]

The eluent consisted of methanol–water (80:20, v/v); 10 μl of the test solutions which contained amylbenzene dissolved in mobile phase and butylbenzene dissolved in methanol, were injected (on-column loading of 6 and 4 μg, respectively). The quotient of the capacity factor of amylbenzene to that of butylbenzene was calculated.

Steric selectivity ($\alpha_{T/O}$)

The eluent consisted of methanol–water (80:20, v/v); 10 μl of the test solution which contained triphenylene and *o*-terphenyl dissolved in methanol, was injected (on-column loading of 5 μg of each). The quotient of the capacity factor of triphenylene to that of *o*-terphenyl was calculated.

Hydrogen bonding capacity ($\alpha_{C/P}$)

The eluent consisted of methanol–water (30:70, v/v); 10 μl of the test solutions which contained caffeine and phenol dissolved in methanol, were injected (on-column loading of 5 and 10 μg, respectively). The quotient of the capacity factor of caffeine to that of phenol was calculated.

Ion-exchange capacity at pH > 7 ($\alpha_{A/P}$)

The eluent consisted of 0.02 *M* KH_2PO_4 (pH 7.6) in methanol–water (30:70, v/v), 20 μl of the test solutions which contained benzylamine and phenol dissolved in mobile phase, were injected (on-column loading of 10 μg of each). The quotient of the capacity factor of benzylamine to that of phenol was calculated.

Ion-exchange capacity at pH < 3 ($\alpha_{A/P}$)

The eluent consisted of 0.02 *M* KH_2PO_4 (pH 2.7) in methanol–water (30:70, v/v), 20 μl of the test solutions which contained benzylamine and phenol dissolved in mobile phase, were injected (on-column loading of 10 μg of each). The quotient of the capacity factor of benzylamine to that of phenol was calculated.

3. Results and discussion

The preceding paper (Part I, [1]) described the sulphoxide epimerisation and concomitant ethylsulphenic acid elimination from the ethylsulphoxide diastereoisomers of tipredane (I) adsorbed onto a Pellicular ODS guard column prior to the chromatographic separation (see Fig. 2).

The sulphoxide epimerisation and elimination are thought to arise via a common intermediate—an immobilised ethylsulphoxide–metal–geminal silanol complex (see preceding paper, Part I [1]). Therefore, for the sake of simplicity, this paper will initially focus on the epimerisation process and subsequently discuss the elimination process in terms of what has been shown for the former.

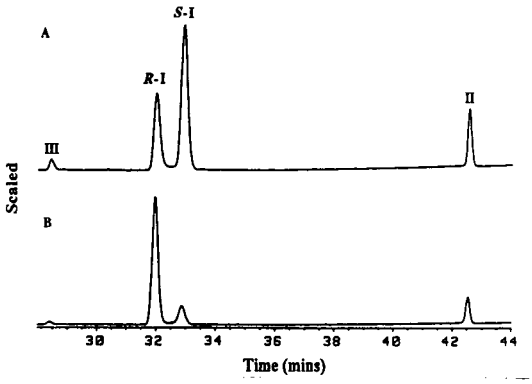


Fig. 2. HPLC analysis of ethylsulphoxide epimers (I) on a Pellicular ODS guard column and a Hypersil ODS column. Chromatographic conditions 1 as in Section 2. (A) *S*-epimer, (B) *R*-epimer.

3.1. Effect of stationary-phase chemistry on the epimerisation reaction

The type of octadecylsilyl (ODS, C_{18}) stationary-phase material appeared to influence the degree of epimerisation observed. For example, the Pellicular ODS guard column resulted in a significantly greater amount of epimerisation compared with the effect of the Hypersil column alone (see Table 2).

Therefore, it was decided to evaluate a wide range of commercially available C_{18} stationary-phase materials (see Table 1) with special emphasis on whether the column had been end-capped or not and whether the column had been deactivated. Preliminary investigations indicated that the previous history of the column could have a pronounced effect on the degree of epimerisation, therefore, for consistency, where possible a new 150 × 4.6 mm I.D., 5-μm column was investigated (where a longer column had to be employed the flow-rate was adjusted accordingly to elute the epimers at approximately the

Table 2
Effect of stationary phases on the epimerisation and elimination of ethylsulphoxide epimers (I) and their resolution using HPLC conditions 1

Stationary phase column	*R*-epimer (I)		*S*-epimer (I)		Resolution *R*- and *S*-epimer (I)
	% epi	% eli[b]	% epi	% eli[b]	
Hypersil ODS	5.4	7.19	9.4	3.7	2.8
Pellicular ODS[a]	12.5	8.7	26.2	9.2	nd
RP-18 Lichrospher[a]	nd	nd	4.8	3.8	nd
RP-18 Lichrospher[a] end-capped	nd	nd	2.8	2.8	nd
RP-8 Lichrospher[a]	nd	nd	4.5	6.9	nd
Resolve C_{18}	13.2	14.3	29.2	20.9	1.3
Nova-pak C_{18}	1.8	4.5	3.5	0.6	2.7
YMC Basic Lichrosphere 60	3.5	5.2	6.6	4.3	3.8
RP Select B	0.8	13.5	2.6	8.1	2.3
SynChropak SCD-100	2.9	12.5	5.8	13.4	2.6
Astec C-18 Polymer	0.0	11.6	0.0	6.6	1.1
Spherisorb ODS1	14.2	33.7	44.7	41.3	0.9
Spherisorb ODS2	16.8	9.7	33.7	13.6	2.5
Ultrasphere ODS	1.5	6.3	3.2	4.4	2.6
μBondapak C_{18}	3.3	7.4	7.5	6.6	nd
Zorbax R_x C_{18}	8.0	8.2	17.0	9.3	2.6
Hypersil BDS C_{18}	0.4	6.5	0.2	2.5	3.2
Suplex pKb-100 C_{18}	0.0	5.3	0.8	3.8	1.6
Supelcosil LC-ABZ C_{18}	nd	7.4	nd	1.1	co-elution
Kromasil C_{18}	24.8	8.3	30.6	45.3	2.6
Hichrom RPB C_{18}	8.7	8.4	24.2	8.7	3.2

[a] Guard column material used in conjunction with Hypersil ODS Excel column.
[b] Summation of elimination products (II and III).
nd = Not determined.

same retention times). Each column was washed with 15 column volumes of mobile phase B and 15 column volumes of initial mobile-phase composition, from HPLC conditions 1, prior to starting the experiment.

Most of the stationary phases showed excellent separation of ethylsulphoxide epimers (I) (see Table 2) in addition to separating the products arising from the elimination process, i.e. the C-17 keto (III) and methylthio (II) derivatives (see Fig. 2).

The Resolve and the Spherisorb materials produced a pronounced "saddle" between the peaks corresponding to the ethylsulphoxides epimers, which was indicative of epimerisation occurring during the chromatographic process (see Fig. 4) [5–8]. This was in contrast to all the other column materials examined which resulted in baseline resolution with no evidence of any true "on-column" epimerisation (see Fig. 3). It was therefore deduced that the epimerisation reaction on the majority of column materials occurred when the ethylsulphoxides were adsorbed onto the stationary phase prior to the gradient commencing and hence prior to the separation process.

3.2. Evidence to implicate the silanol moieties in the epimerisation reactions

Epimerisation of the ethylsulphoxide epimers (I) was not observed with the C_{18} polymer-based column (see Table 2). However, thermal elimi-

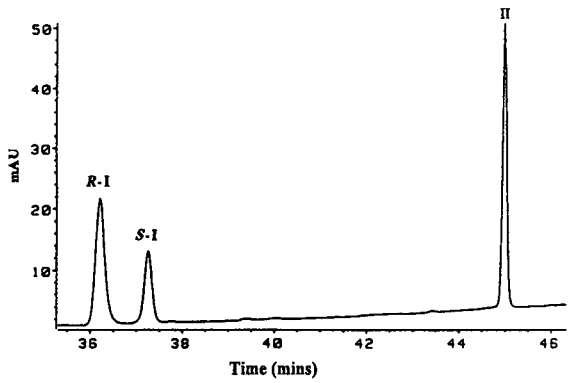


Fig. 3. HPLC analysis of the *S*-epimer of the ethylsulphoxide (I) on a Kromasil C_{18} column (column-packing company A). Chromatographic conditions 1 as in Section 2.

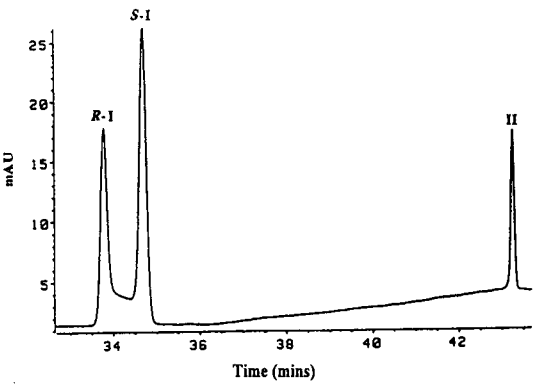


Fig. 4. HPLC analysis of the *S*-epimer of the ethylsulphoxide (I) on a Resolve C_{18} column. Chromatographic conditions 1 as in Section 2.

nation of ethylsulphenic acid still occurred (for results of the *R*-epimer see Table 3).

This observation implicated the involvement of the silanol groups present on the silica-based ODS stationary phase in the epimerisation reaction. Further evidence for this hypothesis came from the fact that the end-capped column, Novapak C_{18}, resulted in lower total epimerisation and elimination than the Resolve C_{18} column which had been prepared from the same base silica but was not end-capped (see Table 2). In addition, the new generation of columns for use with bases, which use nucleophilic shielding groups to protect silanol groups from interactions with basic compounds, e.g. the Suplex pKb-100, showed very little epimerisation.

The deactivated columns, i.e. Lichrosphere 60 RP Select B and Hypersil BDS C_{18}, in general gave lower amounts of epimerisation than traditional columns; however, there appeared to be many anomalies (i.e. the Kromasil C_{18}, Hichrom RPB and Zorbax R_x C_{18} columns).

Table 3
Elimination of ethylsulphenic acid from the *R*-epimer of the ethylsulphoxide (I) as a function of temperature on the Polymer C_{18} column using HPLC conditions 1

Temperature (°C)	Elimination[a] (%)
26	1.5
40	11.6
44	17.2

[a] Summation of the elimination products (II and III).

3.3. Correlation of epimerisation with chromatographic parameters of the various stationary phases

In an attempt to correlate the epimerisation and elimination with specific stationary-phase properties, the columns shown in Table 4 were characterised in accordance with the methodology described by Kimata et al. [4]. This approach provided information regarding the amount of alkyl chains (k'_{AB}), hydrophobicity [$\alpha(CH_2)$], steric selectivity ($\alpha_{T/O}$), hydrogen bonding capacity ($\alpha_{C/P}$) and ion-exchange capacity ($\alpha_{A/P}$) at pH > 7 and pH < 3 for the stationary phase. The efficiency of each column was additionally calculated for amylbenzene (see Table 4).

After extensive examination of the data no direct correlation could be established between the extent of epimerisation and/or elimination with any of the parameters studied. However, the evidence still strongly indicated the involvement of silanol groups in the epimerisation reaction. Although no correlation with total silanol content could be established, silanol groups have been characterised into five subclasses, all with differing acidities and therefore,

Table 4
Characterisation of RP HPLC columns

Columns	k'_{AB}	$\alpha(CH_2)$	$\alpha_{T/O}$	$\alpha_{C/P}$	$\alpha_{A/P}$		N	N/m
					pH 7.6	pH 2.7		
Zorbax R_x C_{18}	5.68	1.57	1.61	0.54	0.55	0.11	13210	88065
Spherisorb ODS	1.78	1.47	1.64	1.57	2.84	2.55	12871	85805
Kromasil C_{18}	7.01	1.48	1.53	0.40	0.31	0.11	12739	84925
Spherisorb ODS2	3.00	1.51	1.56	0.59	0.76	0.23	12390	82601
Hypersil ODS	4.44	1.45	1.28	0.48	1.04	0.64	11412	76078
Hypersil BDS C_{18}	4.50	1.47	1.49	0.39	0.19	0.17	11186	74576
Hichrom RPB C_{18}	4.56	1.40	1.21	0.36	0.18	0.11	10787	71916
Nova-pak C_{18}	4.49	1.49	1.44	0.48	0.27	0.14	10532	70216
Supelcosil LC-ABZ C_{18}	3.14	1.37	2.23	0.24	0.20	0.03	10120	67464
Ultrasphere ODS	6.41	1.52	1.42	0.48	0.31	0.16	9946	66305
YMC Basic	2.33	1.26	0.98	0.57	0.51	0.27	13034	52136
Resolve C_{18}	2.40	1.46	1.59	1.29	4.06	1.23	7161	47741
Suplex pKb 100 C_{18}	2.07	1.35	2.84	0.34	0.29	0.00	10289	41158
Astec C_{18} Polymer	4.92	1.35	4.09	0.15	0.04	0.01	4695	31302

Stationary phase properties and HPLC experimental conditions are as described in Section 2; k'_{AB} number of alkyl chains; $\alpha(CH_2)$ surface density of alkyl groups or hydrophobicity; $\alpha_{T/O}$ steric selectivity; $\alpha_{C/P}$ hydrogen-bonding capacity; $\alpha_{A/P}$ pH 7.6 ion-exchange capacity at pH 7.6; $\alpha_{A/P}$ pH 2.7 ion-exchange capacity at pH 2.7, N (number of theoretical plates) and N/m of amylbenzene.

reactivities [9,10]. It was, therefore, thought that the epimerisation may be attributed to a particular class or classes of silanol groups.

3.4. Evidence for the involvement of a specific class of silanol in the epimerisation reaction

The degree of epimerisation resulting from the use of the Kromasil C_{18} column was of particular interest because of the manufacturer's claims for this column. These include an extremely low metal content in the silica support material and a uniform silanol surface rather than the heterogeneous silanol population of more traditional columns (theoretically producing a stationary-phase material with very low numbers of acidic silanol groups). This was borne out in practice as the column was shown to have low hydrogen bonding and ion-exchange capacities both at pH > 7 and pH < 3, suggesting a low number of acidic silanol sites through the column (see Table 4). However, this column resulted in an extremely high degree of epimerisation for the ethylsulphoxide epimers (I); see Table 2 and Fig. 3 for the chromatography of the *S*-epimer. This further suggested that epimerisation may be facilitated by an active site which was not at present being quantified.

Various manufacturers have made claims that their deactivation processes result in stationary phases with increased numbers of less acidic types of silanols, e.g. geminal silanols. However, due to the highly secret nature of the deactivation procedure there has been an absence of published research into this field. (A ^{29}Si NMR spectroscopic survey of commercially available stationary phases is planned to address this lack of detailed knowledge.) Iron(III) has been reported to selectively block geminal silanols [11], hence it was suggested that if the increased number of geminal silanol groups on the Kromasil material were involved in the epimerisation reaction then loading the column with iron(III) should retard the reaction. However, investigations proved that no reduction in the degree of epimerisation occurred on addition of iron(III) to a Kromasil C_{18} column; in fact a

slight increase in both elimination (Δ2%)[1] and epimerisation (Δ5%) was observed on chromatography of the *S*-epimer of the ethylsulphoxide (I). It was subsequently found that after an EDTA or 0.1% v/v phosphoric acid wash was used to remove the iron(III) from the column, levels of both epimerisation (Δ11 and Δ19% reductions for EDTA and acid washes, respectively) and elimination (Δ9 and Δ12% reductions for EDTA and acid washes, respectively) were reduced, implicating the involvement of metal ions in these reactions.

3.5. Evidence of metal ion involvement in the epimerisation reaction

Investigations of four different batches of new Kromasil C_{18} and a C_8 column, from the same highly reputable column packing company (A), all produced epimerisations; however, the degree of epimerisation and elimination did vary (ranges for epimerisation 30.6–37.3% and elimination 18.7–48.0%, $n = 5$). Examination of the stationary-phase material at the inlet and outlet ends of these columns showed a wide variation in colour ranging from white to dark orange. The dark orange coloration seen with some columns was attributed to hydrated iron(III) oxide. This strongly suggested the presence of metal contamination arising from the stainless-steel frits. It has previously been reported that stainless-steel frits are responsible for poor chromatographic performance [12–15] and on-column reactions [7,16,17].

Atomic absorption spectroscopy of silica from the top few mm of a coloured column revealed levels of approximately 250 ppm total iron, whereas the Kromasil packing material manufacturers "Eka Nobel" claimed < 10 ppm for the native silica.

3.6. Identification of metal ion(s) responsible for the epimerisation reaction

The most probable causative metals which could have been leached from the "316 stainless-steel" frits and were present in any abundance, were nickel (12%), chromium (17%) and iron (69%) [18]. However, the latter in its most stable oxidation state of iron(III) had previously been shown not to cause significant epimerisation. Further investigations of loading methanolic solutions of chromium(III) and nickel(II) chlorides onto the Kromasil C_{18} guard column packing material via injection or stirring overnight at ambient temperature failed to cause any significant increase in epimerisation. However, the adsorption of these metal species onto the silica surface was in doubt as the dihydroxynaphthalene test (see later) suggested that little, if any, metal had been adsorbed presumably due to the slow and/or weak complexation of chromium(III) and nickel(II).

In comparison, when a methanolic solution of the less stable oxidative state of iron [iron(II) chloride] was loaded, significant epimerisation (34.0%) and elimination (15.8%) was observed. It is important to note that although an injection of 5 μmol iron(II) was made, the majority of this passed straight through the column without chelation, as detected by the large UV absorption at the void volume, suggesting that the epimerisation was caused by the much smaller fraction of iron(II) held by the geminal silanols. The dihydroxynaphthalene efficiency ratio test (DERT, see below) indicated that iron(II) had been successfully adsorbed onto the column, which gave a DERT value of 16.7.

3.7. Dihydroxynaphthalene efficiency ratio test (DERT)

The relative concentration of metal ions throughout the column was estimated by an independent method, the DERT value, which compares the peak efficiency, measured at the base, of the two regioisomers 2,3- (IV) and 2,7-dihydroxynaphthalene (V). The former possesses the ability to chelate with metals while the latter does not. Therefore, the nearer the ratio of peak efficiency at base for the 2,7/2,3-dihydroxynaphthalene (DERT value) to unity, the lower the metal content; conversely a value of infinity means that the 2,3-dihydroxynaphthalene

[1] Δ Refers to absolute change (%).

(IV) analyte elutes very slowly over a long time, effectively losing the peak in the baseline noise or that the analyte binds strongly to the metal and fails to be eluted [19]. This test has been shown to be a much better diagnostic probe for metal content than more traditional tests such as salicylaldehyde and the phenol/benzylamine ratio [20]. It has subsequently been shown that the metal probe acetylacetone failed to detect metal contamination on the Waters Symmetry and Merck Purospher columns, whereas the DERT value did.

3.8. Identification of the source of metal contamination

Investigation of an identical batch of Kromasil C_{18} material packed as guard columns "in-house" and also packed as an analytical column by another packing company (B) resulted in only trace levels of epimerisation and minimal elimination. For example, only 0.7 and 3.1% epimerisation and elimination, respectively, were seen with the analytical column for the *S*-epimer (see Table 5, entry 8).

The source of the metal contamination on the Kromasil column batches was suspected to be related to the stainless-steel inlet and outlet frits used by the column packing company (A).

There appeared to be little difference between columns packed with passivated and non-passivated stainless-steel frits (see Table 5, entries 1 and 2); however, the duration of time for which the columns were left prior to testing was directly related to the degree of epimerisation observed (see Table 5, entries 2, 4 and 5).

The epimerisation of the ethylsulphoxides (I) observed with the Kromasil columns under investigation resulted in baseline separation of the epimers indicating that the epimerisation occurred only at the top of the column (see Fig. 3). However, after loading a methanolic solution of iron(II) chloride onto the Kromasil column a saddle was seen between the two epimer peaks illustrating that iron(II) was bound throughout the column resulting in true "on-column" epimerisation (see Fig. 4 for a chromatogram typifying this phenomenon).

The fact that the PEEK alloyed with Teflon (PAT) frits failed to generate significant epimerisation (see Table 5, entries 6 and 7) showed that metals [e.g. iron(II)] were being leached out of the stainless-steel frit. Contrary to our expectations, titanium frits still gave a considerable degree of epimerisation (see Table 5, entry 3), indicating that a unspecified oxidation state of titanium was also able to facilitate the epimerisation.

Substitution of acetonitrile in place of methanol as the storage solvent failed to reduce the extent of epimerisation (compare entries 5 and 12, Table 5). In contrast, the Kromasil C_{18} column supplied by company (B), which caused negligible epimerisation, had been stored in 6:4 v/v acetonitrile–water prior to testing (see Table 5, entry 8). To test the hypothesis that it was the percentage of organic in the shipping solvent which leached metals from the stainless-steel frits, the column packing company (A) stored a newly packed Kromasil C_{18} column in the same shipping solvent as company (B). As anticipated, this column failed to cause epimerisation (see Table 5, entry 13).

(Another potential source of metal contamination lay in the fact that company (A) supplied columns with stainless-steel end stops (epimerisation noted) whereas company (B) used plastic (no epimerisation). This potential source was ruled out as storage of the column with PAT frits supplied with stainless-steel end stops, from company (A), failed to cause epimerisation.)

The most probable explanation was that the leaching of metal ions from the stainless-steel frits was exacerbated by the use of pure organic solvents. Support for this hypothesis comes from the fact that it has previously been shown that various 316 stainless-steel HPLC components are extremely susceptible to corrosion in the presence of "neat" acetonitrile or methanol, iron(II) being generated by the anodic reaction of the corrosion process [21]. "Neat" acetonitrile and methanol are used by certain column manufacturers for packed column storage. It was subsequently shown by ICP that the amount of iron extracted from the passivated stainless-steel frits (1 frit per ml of methanol) at ambient tempera-

Table 5
Effect of column storage solvents and time on the degree of epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (I) on Kromasil C_{18} columns using HPLC conditions 1

Entry number	Frit composition (column storage conditions)	Epimerisation (%)	Elimination (%)[a]	DERT	Batch number of packing material
1	Non-passivated stainless steel (4 months MeOH)	32.4	33.4	∞	0078
2	Passivated stainless steel (4 months MeOH)	39.4	26.2	∞	0078
3	Titanium (4 months MeOH)	25.0	18.0	∞	0078
4	Passivated stainless steel (1 day MeOH)	3.4	4.2	16.9	0078
5	Passivated stainless steel (6 days MeOH)	16.3	11.9	30.9	0078
6	PEEK and Teflon (PAT) (1 day MeOH)	0.3	3.7	16.8	0078
7	PEEK and Teflon (PAT) (12 days MeCN)	0.5	5.5	nd	0033
8	Passivated stainless steel (company B, 7 days MeCN–H_2O, 6:4)	0.7	3.1	0.8	0033
9	Passivated stainless steel (company A, MeOH)	35.4	48.0	nd	0033
10	Passivated stainless steel (company A, MeOH)	37.3	35.2	nd	0077
11	Passivated stainless steel (company A, MeOH)	36.1	44.0	nd	0065
12	Passivated stainless steel (company A, 12 days MeCN)	10.7	10.8	5.1	0078
13	Passivated stainless steel (7 days MeCN–H_2O, 6:4, company A)	0.3	3.9	nd	0078

nd = Not determined.
[a] Summation of elimination products (II and III).

ture was related to the length of exposure of the frits to methanol (see Table 6, entries 1 and 2, and Figs. 5 and 6).

In addition, the incorporation of water into the extraction solvent mixture resulted in lower quantities of iron being extracted (see Table 6, entries 1, 3 and 4).

ICP spectroscopy showed that methanol in contact with 1 passivated frit for 35 days at ambient temperature can extract in the order of

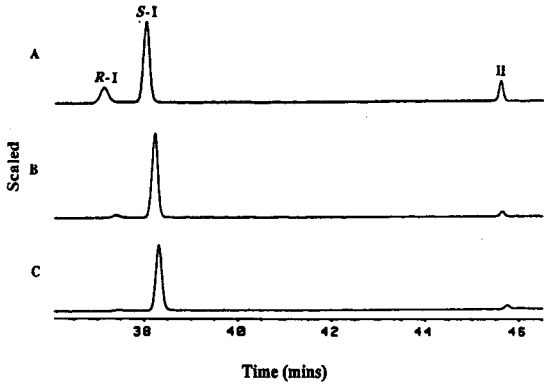


Fig. 5. HPLC analysis of the *S*-epimer of the ethylsulphoxide (I) on a Kromasil C_{18} column (column-packing company A). Chromatographic conditions 1 as in Section 2. Storage of columns in (A) methanol for 6 days, (B) methanol for 1 day, and (C) acetonitrile–water (60:40, v/v) for 7 days at ambient temperatures.

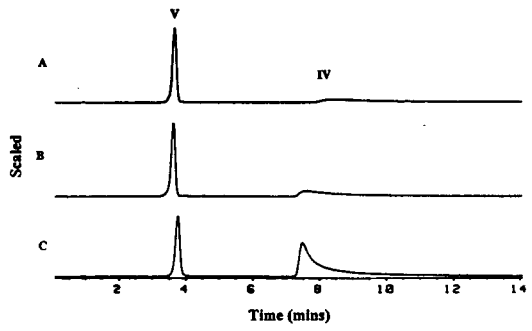


Fig. 6. HPLC analysis of 2,3- and 2,7-dihydroxynaphthalene (IV, V) on a Kromasil C_{18} column (column packing company A). Chromatographic conditions as in Section 2. Storage of columns in (A) methanol for 6 days, (B) methanol for 1 day, and (C) acetonitrile–water (60:40, v/v) for 7 days at ambient temperatures (see Table 5 for DERT values obtained).

1 μg of total iron (see Table 6, entry 1). Typically, $1.8 \cdot 10^{-9}$ mol of the ethylsulphoxide of tipredane was loaded onto the column. Assuming that the total amount of iron was adsorbed onto the top of the column (and available to react), the iron would have been in a molar excess of 10:1 over the ethylsulphoxide (I) and therefore could have a profound effect.

Table 6
Quantity of metals extracted from frits as a function of extraction solvent composition and time as assessed by ICP

Entry number	Frit composition and storage conditions	Quantity extracted (ppm)			
		Iron	Nickel	Chromium	Titanium
1	Passivated stainless steel, 35 days, MeOH	1.0	1.2	0.8	0.0
2	Passivated stainless steel, 4 days, MeOH	0.1	0.8	0.1	0.0
3	Passivated stainless steel, 23 days, 80:20 MeOH–H_2O	0.3	1.0	0.2	0.0
4	Passivated stainless steel, 23 days, 60:40 MeOH–H_2O	0.2	0.9	0.2	0.0
5	Titanium, 35 days, MeOH	0.1	0.0	0.0	1.8

The equivalent of one frit was exposed to 1 ml of extraction solvent at ambient temperature.

The facile nature of extraction of titanium from the titanium frits in methanol was exemplified by entry 5 (see Table 6). The complexed titanium (oxidation state undefined), in addition to iron(II), can promote the epimerisation reaction as titanium frits in contact with methanol have been shown to cause significant epimerisation (see Table 5, entry 3).

Nickel was additionally extracted in reasonable quantities from the passivated stainless-steel frits irrespective of the extraction composition. However, as can be seen from Table 6 (entries 3 and 4), nickel can be ruled out as the causative agent since high levels are extracted with solvent combinations not associated with epimerisation. High levels of chromium were extracted from the passivated stainless-steel frit in the presence of methanol and may be partly responsible for the epimerisation reaction; however, chromium(III) has been shown not to be loaded onto the C_{18} material, therefore, further work is planned to examine the effect of chromium(II).

Neat methanol has been favoured as the storage solvent for reversed-phase columns as it has been previously reported that aqueous methanol storage solvents result in poor stationary-phase stability [22]; no such data is currently available in the open literature for corresponding acetonitrile–aqueous storage solvents.

3.9. The involvement of metal contamination of high-purity deactivated silicas on the degree of epimerisation

Metal ions, extracted from the frits by the use of neat organic shipping solvents, were apparently complexed near the top of the column (in the first few mm) by geminal silanols on the surface of the silica support material. This effect was particularly noticeable for high-purity silica-based C_{18} columns where the intrinsic metal contents are low (<10 ppm iron) and the number of geminal silanols are believed to be high; for example, Kromasil and RPB columns (packed by the same column-packing company) and Zorbax R_x (direct from the manufacturer) produce high levels of epimerisation compared to the Hypersil ODS material (column also stored in neat methanol) which is known to contain a high level of metal in the native silica (i.e. iron >200 ppm, manufacturer's literature).

Evidence to support this comes from the [$\alpha_{A/P}$ pH 7.6] and [$\alpha_{A/P}$ pH 2.7] results of the high-purity silica stationary phases shown above, which indicated a significant difference between the amount of relatively high-acidic silanols (i.e. value at $\alpha_{A/P}$ pH 2.7) and the low-acidic silanols (i.e. value at $\alpha_{A/P}$ pH 7.6) (see Table 4). A large difference between the two values represents a large proportion of low-acidic silanols, such as geminal ones, over that of high-acidity types. It is of interest to note that the difference between the silanols at pH 2.7 and 7.6 for the Hypersil BDS column is very small, suggesting that the few silanols which are present are high-acidity ones and not geminals, hence a low epimerisation result was obtained. In addition, this column was stored in 7:3 methanol–water which causes less iron(II) extraction, and Hypersil materials are known to contain a higher metal content in the native silica than materials such as Kromasil C_{18}. In this case other metals, e.g. iron(III), may already be bound to the geminals, hence preventing access of metal ions which can promote epimerisation to the geminal silanols.

The smaller and more variable degree of epimerisation observed when iron(III) was loaded onto the Kromasil C_{18} material may be caused by a small percentage of the iron(II) species being present in methanolic iron(III) solution or the acidic methanolic injection solution leaching off iron(II) from the stainless-steel frit.

3.10. Implications

The ability of certain stationary phases to enhance the rate of epimerisation of individual tipredane ethylsulphoxide epimers (I) may be attributed to their iron(II) (or titanium) content.

The Zorbax R_x C_{18} column was also shown to cause significant epimerisation (see Table 2), far more than would have been expected given its low metal content (manufacturers claim). Given the fact that the degree of epimerisation and

elimination of the ethylsulphoxide epimer (I) dramatically reduced (epimerisation 0.9 and elimination 5.1%) after the column had been washed with 0.1% v/v phosphoric acid and then an EDTA overnight at 60°C, it was postulated that in common with the Kromasil column and presumably the RPB column, from the same source, inadvertent metal ion contamination from the stainless-steel frits had occurred. This could have been due to the high methanol content used to store the columns (see Table 1) and that the columns appeared to contain a large proportion of non-acidic silanols, e.g. geminals (see Table 4). This result was quite significant as it illustrated that this problem was not confined to only one supplier/manufacturer (see later for further examples).

Columns such as Resolve and Spherisorb ODS1 and 2 (obtained from sources which were not suspected as being involved with inadvertent contamination, i.e. the three columns had not been stored in high-organic solvents) are manufactured from native silica which contains a high metal content and, after the octadecylsilane has been bound, a large proportion of free silanols remain, many will be geminal in nature and possess the ability to complex with metal ions such as iron(II) and titanium. Therefore, the epimerisation can occur throughout the column; this is substantiated by the saddle which is seen between the two ethylsulphoxide peaks (see Fig. 4).

Organic/aqueous based column storage packing solvents such as methanol or acetonitrile–water may, in fact, extract iron(II) from the frits, but the iron(II) may be rapidly converted to iron(III) in the aqueous environment. Alternatively, iron(II) may only be extracted in very high-percentage organic–water mixtures; further investigations in this area are at present being pursued. Our preliminary results indicate that the total iron extracted in aqueous methanol solutions is substantially lower than that extracted in pure methanol (0.3 and 0.2 μg/frit extracted after 23 days exposure to 80:20 and 60:40 methanol–water, respectively, compared to 1 μg/frit with neat methanol; see Table 6, entries 1, 3 and 4).

There may be other metal ions which can facilitate this reaction; e.g. titanium frits have been shown to promote the epimerisation/elimination reaction.

Iron (and possibly other cations) may be present in the initial silica or may be introduced via metal components of the column when there are sufficient geminal silanols to allow significant complexation of iron(II).

3.11. Investigations of further commercially available stationary phases

Subsequent to these findings we have evaluated a range of recently introduced C_{18} stationary-phase materials (see Table 7). It was anticipated that the Symmetry C_{18} and Purospher C_{18} columns may promote the epimerisation reaction owing to the fact that both are formed via polymerisation of silyl ethers which produces a "low acidity" silanol surface (presumably increased geminal population, manufac-

Table 7
Comparison of the epimerisation/elimination reactions and that of the DERT value before and after an EDTA wash

Column material	*S*-epimer (I)		Resolution (I) epimers	DERT value	
	Epimerisation (%)	% Elimination[a] (%)		Before	After EDTA wash
Spherisorb ODSB	3.3	5.8	1.5	∞	nd
Waters Symmetry C_{18}	15.2	11.2	3.3	5.6	0.5
Merck Purospher C_{18}	1.2	0.0	2.1	41.6	0.6
Zorbax SB C_{18}	4.8	15.9	2.4	0.8	nd

nd = Not determined.
[a] Summation of elimination products (II and III).

turer's claim) and are stored in neat organic solvent (acetonitrile) (see Table 1). The latter column's chemistry also possessed a nucleophilic shielding moiety. Due to the cartridge design of the Purospher column, the packing material probably dried out during storage, therefore the cartridge system was assembled and the packing material left in neat acetonitrile for 7 days at ambient temperature.

Contrary to expectation, the Purospher material produced a low level of epimerisation, whereas the DERT value indicated surface metal contamination (see Table 7 and Fig. 7). These results can be rationalised as follows. After the cartridge had been shipped out in pure acetonitrile, the cartridge design allowed the column bed to dry out. Hence, the extracted iron(II) would have been converted to its more stable oxidation state iron(III) which does not promote the epimerisation reaction but its presence was detected in the DERT determination. The refilling and storage of the column in acetonitrile which we performed, did not influence the metal content of the column as most of the "extractable metal" had already been deposited onto the column from the frit.

An EDTA wash successfully removed the surface metal as assessed by the DERT determination (see Table 7 and Fig. 7). The success of this procedure is indicative of surface-bound metal derived from contamination via the frit rather than in the native silica.

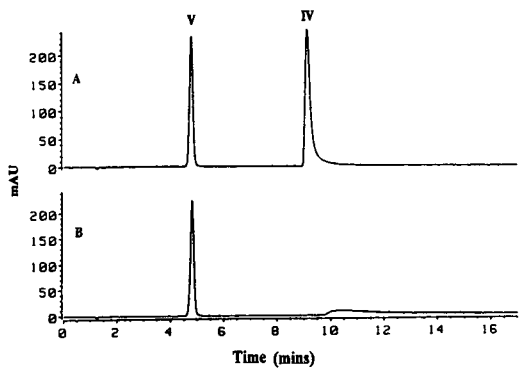


Fig. 7. HPLC analysis of 2,3- and 2,7-dihydroxynaphthalene (IV, V) on a Purospher C_{18} column. Chromatographic conditions as in Section 2. (A) After a subsequent 0.05 *M* Na_2 EDTA wash (1 h at 1.5 ml/min, 40°C), and (B) initial conditions of the column after storage for 1 week at ambient temperature in acetonitrile.

The Spherisorb ODSB column gave a low epimerisation result, indicating low iron(II) content on the column which is in keeping as the column was stored in 7:3 methanol–water; however, the DERT value indicated a high metal content of the silica which was expected (see Table 7).

In contrast to the Zorbax R_x C_{18} column (non-end-capped), the corresponding Stable Bond version in which the silanols are sterically protected by the bulky di-isopropyl silyl side chains gave a low epimerisation result and DERT value, indicative of a low metal content silica (see Table 7). A possible explanation for this may be that metal ions or the probes are prevented access to the surface geminal silanols via the steric protecting groups.

The Symmetry C_{18} column gave a high epimerisation result (see Table 7), indicating the presence of iron(II) adsorbed onto the top of the column (sharp peaks and baseline resolution observed), leached after storage of the column in pure acetonitrile (Coulometric Karl Fischer determination indicated the presence of only 0.1% water content in the storage solvent). This was substantiated by the DERT value which indicated the presence of surface metal on the material which could be easily removed by washing the column for 1 h with 0.05 *M* Na_2 EDTA solution.

3.12. Elimination process

The same overall trends have been observed for the elimination process as for the epimerisation. However, the degree of elimination appears to be more variable than the epimerisation process. This may have been expected as elimination products can arise via another mechanism: that of thermal elimination of ethylsulphenic acid. The wide differences in the degree of elimination (especially seen with different Kromasil columns and batches) may additionally be attributed to the effect of additional metals [other than iron(II)] on the column and/or

metals bound to additional "active sites" on the stationary phase.

4. Summary

The use of the ethylsulphoxide epimers of tipredane and the DERT determination has highlighted vast chromatographic differences between identical packing materials from different packing companies. The source of these differences has been attributed to contamination of the otherwise "metal-free" high-purity stationary-phase material, with metals such as iron(II), by storage of these types of columns with stainless-steel frits in high-organic content solvents. This additionally highlights the possibility of further interactions of analytes possessing chelating potential with metals bound to the surface of these types of stationary phases [7,14,19,23–26].

This research has also indicated the possible need for additional quality control procedures to be introduced by packing companies to monitor for metal content not only in the base silica but at each stage of manufacture through to the final packed column.

Research is presently in progress to assess the feasibility of using the ethylsulphoxide epimers (I) and 2,3- (IV) and 2,7-dihydroxynaphthalene (V) regioisomers before and after various EDTA pre-treatments as probes to chromatographically characterise the proportion of various metal ion(s) bound to differing classes of silanol groups. This may also allow one to determine the proportion of differing classes of silanols such as geminal to non-geminal silanols present on various stationary phases. Preliminary investigations suggest that the two probes exhibit different selectivities (see Table 5, entries 4, 6 and 8). In these three examples the degree of epimerisation is quite similar, whereas with the dihydroxynaphthalene test only entries 4 and 6 gave results suggestive of high metal contamination. One can, therefore, assume that the two tests are measuring two distinct parameters and at present insufficient data are available to allow one to correlate them to one another.

The epimerisation of tipredane ethylsulphoxide (I) has been shown to be an extremely sensitive probe for iron(II) speciation with detection limits below 5 μmol. Further work is presently in progress to assess the feasibility of using this approach to determine trace levels of iron(II) in the presence of iron(III), and to assess the specificity of the epimerisation/elimination reactions to other metals of varying oxidation states.

Acknowledgements

We are grateful to Dr. D. Mallern, Messrs. R. Shaw, D. Stanley (Hichrom, Reading, UK), J. Plant, Dr. H. Ritchie (Shandon HPLC, Runcorn, UK), D. Collis, A. Holmes (Waters Chromatography Division, Watford, UK), M. Clemance (Merck, Poole, UK) and A. Dams (Rockland Technologies, Nuenen, Netherlands) for support and helpful advice. Mr. U. Misty (Fisons Scientific Apparatus, Loughborough, UK) for performing the ICP determinations, Dr. J. Crew for critical appraisal of the manuscript, Miss A. Stockley for typographical advice and the British Council for financial support (DASST).

References

[1] M.R. Euerby, C.M. Johnson, I.D. Rushin and D.A.S.S. Tennekoon, J. Chromatogr. A, XXX (1995) XXX.
[2] M.R. Euerby, J. Hare and S.C. Nichols, J. Pharm. Biomed. Anal., 10 (1992) 269–277.
[3] M.R. Euerby, C.M. Johnson, R.J. Lewis and S.C. Nichols, J. Pharm. Biomed. Anal., 12 (1994) 761–769.
[4] K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosoya, M. Araki and N. Tanaka, J. Chromatogr. Sci., 27 (1989) 721–728.
[5] R.A. Keller and J.C. Giddings, J. Chromatogr., 3 (1960) 205–220.
[6] M. Van Swaay, in R.A. Keller and J.C. Giddings (Editors), Advances in Chromatography, Dekker, New York, NY, Vol. 8, 1969, pp. 363–385.
[7] J.-X. Huang, J.D. Stuart, W.R. Melander and Cs. Horvàth, J. Chromatogr., 316 (1984) 151–161.
[8] W.R. Melander, H.-J. Lin, J. Jacobson and Cs. Horvàth, J. Phys. Chem., 88 (1984) 4527–4536.

[9] M.A. Stadalius, J.S. Berus and L.R. Snyder, LC · GC, 6 (1988) 494–500.
[10] A. Berthod, J. Chromatogr., 549 (1991) 1–28.
[11] B. Pfleiderer and E. Bayer, J. Chromatogr., 468 (1989) 67–71.
[12] S.M. Cramer, B. Nathanael and Cs. Horvàth, J. Chromatogr., 295 (1984) 405–411.
[13] C.-Y. Jeng and S.H. Langer, J. Chromatogr. Sci., 27 (1989) 549–552.
[14] P.C. Sadek, C.J. Koester and L.D. Bowers, J. Chromatogr. Sci., 25 (1987) 489–493.
[15] P.C. Sadek, P.W. Carr, L.D. Bowers and L.C. Haddad, Anal. Biochem., 144 (1985) 128–131.
[16] Z. Deng, J.Z. Zhang, A.B. Ellis and S.H. Langer, J. Liq. Chromatogr., 16 (1993) 1083–1103.
[17] B.S. Ludolph, C.-Y. Jeng, A.H.T. Chu and S.H. Langer, J. Chromatogr. A, 660 (1994) 3–15.
[18] R.C. Weast (Editor), CRC Handbook of Chemistry and Physics, 64th ed., 1983–1984, p. F-112.
[19] R. Vespalec and J. Neca, J. Chromatogr., 281 (1983) 35–47.
[20] M.R. Euerby, C.M. Johnson and D.A.S.S. Tennekoon, Paper presented at the 20th International Symposium on Chromatography, Bournemouth, 19–24 June 1994.
[21] R.A. Mowery Jr., J. Chromatogr. Sci., 23 (1985) 22–29.
[22] H. Engelhardt and M. Jungheim, Chromatographia, 29 (1990) 59–68.
[23] J. Nawrocki, D.L. Moir and W. Szczepanik, Chromatographia, 28 (1989) 143–147.
[24] B. Law and P.F. Chan, J. Chromatogr., 552 (1991) 429–438.
[25] J.W. Dolan, Liq. Chromatogr. HPLC Mag., 4 (1986) 222–226.
[26] G.B. Cox, J. Chromatogr. A, 656 (1993) 353–367.

ELSEVIER

Journal of Chromatography A, 705 (1995) 247–256

JOURNAL OF CHROMATOGRAPHY A

Extraction and measurement of prominent flavonoids in orange and grapefruit juice concentrates

W.E. Bronner*, G.R. Beecher

Food Composition Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, USA

First received 7 September 1994; revised manuscript received 7 February 1995; accepted 1 March 1995

Abstract

A procedure has been developed for determining levels of the major flavonoids in orange and grapefruit juice concentrates. Following addition of an internal standard, commercial juice concentrates were extracted multiple times with methanol, centrifuged, then filtered. Filtrates were analyzed by high-performance liquid chromatography employing UV–Vis detection. Flavonoids present in the extracts were separated on a C_{18} column with an isocratic mobile phase consisting of water–acetonitrile–2-propanol–formic acid (158:23:19:0.2, v/v). A second separation of juice extracts on the same HPLC column with a water–tetrahydrofuran mobile phase (18:7, v/v) was used to confirm the identity of the flavonoids present.

By comparing UV–Vis spectra and retention times with commercial standards chromatographed under identical conditions, three flavanone glycosides were identified and quantitated. The two brands of orange juice concentrate examined were found to contain 120 and 150 mg hg^{-1} hesperidin (where hg corresponds to 100 g), along with 24 and 30 mg hg^{-1} narirutin. The two brands of grapefruit juice concentrate examined contained 62 and 68 mg hg^{-1} narirutin, and identical levels of naringin (200 mg hg^{-1}). Consistent with several previously published reports, these high levels indicate citrus can be a major source of flavonoids in the diet.

1. Introduction

Flavonoids are a class of naturally occurring and structurally related compounds found widely distributed in plants and plant foods. They have been assigned such diverse biological properties as antioxidant, anti-inflammatory, anti-allergic and anti-carcinogenic activity, as well as vitamin C sparing [1]. Recently, dietary flavonoids have been associated with reduced risk of coronary heart disease in an epidemiological study which places a new perspective on these food components [2]. Significant quantities have been detected in many foods common to our diets, although estimates of daily consumption differ considerably [1–3].

Orange juice and grapefruit juice (as well as the fruits, themselves) are highly consumed and, as such, are potentially major contributors to total dietary flavonoids. A number of publications have reported high levels of flavanone glycosides in these juices, although quantitative values showed some disparity [4–14]. While this may be primarily due to sample variability, it may also, to some degree, reflect differences in analytical methodologies. In one comparative study, the manner of juice pretreatment was shown to affect some quantitative results by as much as an order of magnitude, whereas in other instances nearly identical values were obtained [14].

* Corresponding author.

SSDI 0021-9673(95)00304-5

Solvent extraction, solid-phase extraction, and simple filtration have all been used as preparative techniques for analyzing components in foods. Solid-phase cartridges are a convenient means of obtaining purified and concentrated extracts, often directly from the food product, while filtration has the advantages of speed and simplicity. In many applications, however, these extraction methods result in less than quantitative extraction. Consequently, the actual levels in foods may be substantially underestimated. In the case of orange and grapefruit juices, filtration or solid-phase extraction may be ineffective in removing flavonoids located in suspended juice solids, even though these may represent a large fraction of the total flavonoids present. In this kind of food matrix, solvent extraction seems a preferable alternative.

This report describes a method for the extraction and quantitation of the major flavonoids in commercial orange and grapefruit juice concentrates combining solvent extraction with liquid chromatography. Although several HPLC methods for the determination of flavonoid levels in citrus juices have been developed previously [6–9,12–14], the effect of sample preparation, itself, on the quantitative results has been little studied. Consequently, the effectiveness of various sample pretreatments, such as filtration, solid-phase extraction, and solvent extraction with water, aqueous alkali or organic solvents, is largely unknown. In contrast, by measuring the extent of extraction, the present study demonstrated effective extraction of flavonoids from juice concentrates.

2. Experimental[1]

2.1. Materials and instrumentation

Minute Maid and Giant brands of orange juice concentrate and grapefruit juice concentrate were purchased at local food stores. After thawing, concentrates were subdivided into 50-ml polypropylene tubes and stored in a freezer at −20°C. Naringin (naringenin-7-neohesperidoside) hydrate, hesperidin (hesperetin-7-rutinoside), and formic acid were purchased from Aldrich (Milwaukee, WI, USA). Naringin hydrate contained 6.02% H_2O by weight and was greater than 97% pure by TLC; hesperidin contained 3.81% H_2O by weight and was greater than 98% pure by TLC (as analyzed by Aldrich). Narirutin (naringenin-7-rutinoside) and rhoifolin (apigenin-7-neohesperidoside) were purchased from Indofine Chemical Company (Belle Meade, NJ, USA). Narirutin contained 3.45% H_2O by weight and was greater than 97% pure by HPLC (as analyzed by Indofine). HPLC grade acetonitrile and 2-propanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade tetrahydrofuran was purchased from Mallinckrodt Specialty Chemicals Co. (Chesterfield, MO, USA). Anotop 0.2-μm porosity syringe filters (25 mm diameter) and Alltech 1-μm porosity glass fiber syringe filters (25 mm diameter) were purchased from Alltech (Deerfield, IL, USA).

The HPLC system consisted of a Perkin-Elmer Series 4 liquid chromatograph (Norwalk, CT, USA), an Altex manual injection valve (Beckman, San Ramon, CA, USA) equipped with a 20-μl injection loop, a 25 cm × 4.6 mm I.D. Alltima column (Alltech Associates) packed with 5 μm C_{18} modified silica, a Hewlett-Packard 1040M series II diode-array detector, a Hewlett-Packard HPLC ChemStation and a Hewlett-Packard 3630A PaintJet printer (Palo Alto, CA, USA).

The centrifuge used was a Beckman Model J2-21 (Fullerton, CA, USA).

2.2. Standard solutions

Working solutions of hesperidin (0.47 mg ml^{-1}), naringin (2.1 mg ml^{-1}), narirutin (0.065 mg ml^{-1}), and rhoifolin (1.8 mg ml^{-1}) were prepared by dissolving commercial flavonoid standards in methanol. The rhoifolin solution was filtered through a 0.2-μm porosity Anotop filter to remove insoluble material. Stan-

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

dards were stored in dark amber glass bottles at room temperature following preparation and appeared stable during the period of this study. Structures of the flavonoid compounds are shown in Fig. 1.

2.3. Extraction efficiencies

Factors which contribute to the efficiency of solvent extraction include choice of solvent, temperature, and number of extraction steps. In this study, the influence of these variables on the extraction of flavonoids from juice concentrates was examined. In a comparison of solubilities, small quantities each of naringin, hesperidin, and rhoifolin were added separately to methanol, 2-propanol, acetonitrile, and tetrahydrofuran. In all cases, these flavonoids were more soluble in methanol than in the other solvents examined (as indicated by viewing the amounts remaining undissolved), and subsequent juice samples were extracted with methanol. Completeness of extraction was monitored by measuring flavonoid levels in successive solvent fractions of juice concentrates extracted multiple times. The following procedures were employed.

A 0.5-ml volume of 1.8 mg ml^{-1} rhoifolin internal standard and 0.5 ml of methanol were added to approximately 1 g of thawed grapefruit juice concentrate. This mixture was vortex-mixed for 1 min, centrifuged at 25 000 *g* for 15 min, and the supernatant collected. An additional 0.5 ml of rhoifolin solution and 0.5 ml of methanol were added to the remaining solid. The solid material was broken apart with a spatula, vortex-mixed for 1 min, and centrifuged. A second supernatant was collected and the process repeated until a total of 5 supernatants were collected. A 1-ml volume of water was added to each extract 2 through 5. Each extract was vortex-mixed, filtered through a 1-μm porosity glass fiber filter, followed by a 0.2-μm porosity Anotop filter. Following analysis by HPLC, the naringin/rhoifolin and narirutin/rhoifolin absorbance ratios were used to calculate the fraction of flavanone recovered for each individual extraction step (Table 1).

The above extraction procedure was found to be unsuitable for application to orange juice concentrate. Even five extractions resulted in incomplete recovery of hesperidin (see Table 1). Extraction efficiency was improved, however, by extracting at an elevated temperature. A 0.5-ml amount of rhoifolin solution and 3 ml of methanol were added to approximately 1 g of thawed orange juice concentrate. This was vortex-mixed for 30 s, warmed for 15 min in a 55°C water bath, and mixed an additional 30 s. The mixture was centrifuged at 25 000 *g* for 15 min and 3 ml of water were added to the supernatant collected. To the remaining solid, 0.5 ml of rhoifolin, 2 ml of methanol, and 1 ml of water were added. This was vortex-mixed for 30 s, warmed 15 min in the water bath, and vortex-mixed for another 30 s. Following centrifugation, 2 ml of water were added to the supernatant collected. To the remaining solid, 0.5 ml of rhoifolin solution, 1.5 ml of methanol, and 1 ml of water were added. This was vortex-mixed, warmed in the water bath, and mixed again. The mixture was centrifuged, and 3 ml of water were added to the supernatant collected. The three individual extracts were each vortex-mixed, then filtered through 1-μm porosity glass fiber filters, followed by 0.2-μm porosity Anotop filters. Following analysis by HPLC, the hesperidin/rhoifolin and narirutin/rhoifolin absorbance ratios were used to calculate the fraction of flavanone recovered for each individual extraction step.

2.4. Juice extraction

Frozen grapefruit juice concentrate was thawed briefly, and approximately 1-g aliquots weighed into centrifuge tubes. To each aliquot, 1.0 ml of 1.8 mg ml^{-1} rhoifolin was added as an internal standard. Each aliquot was vortex-mixed for 1 min and then centrifuged at 25 000 *g* for 15 min at room temperature. The supernatant was collected, and an additional 1 ml of methanol was added to the remaining solid. The solid material was broken apart with a spatula, vortex-mixed for 1 min, and again centrifuged. The second supernatant was added to the first, and the remaining solid re-extracted with another 1

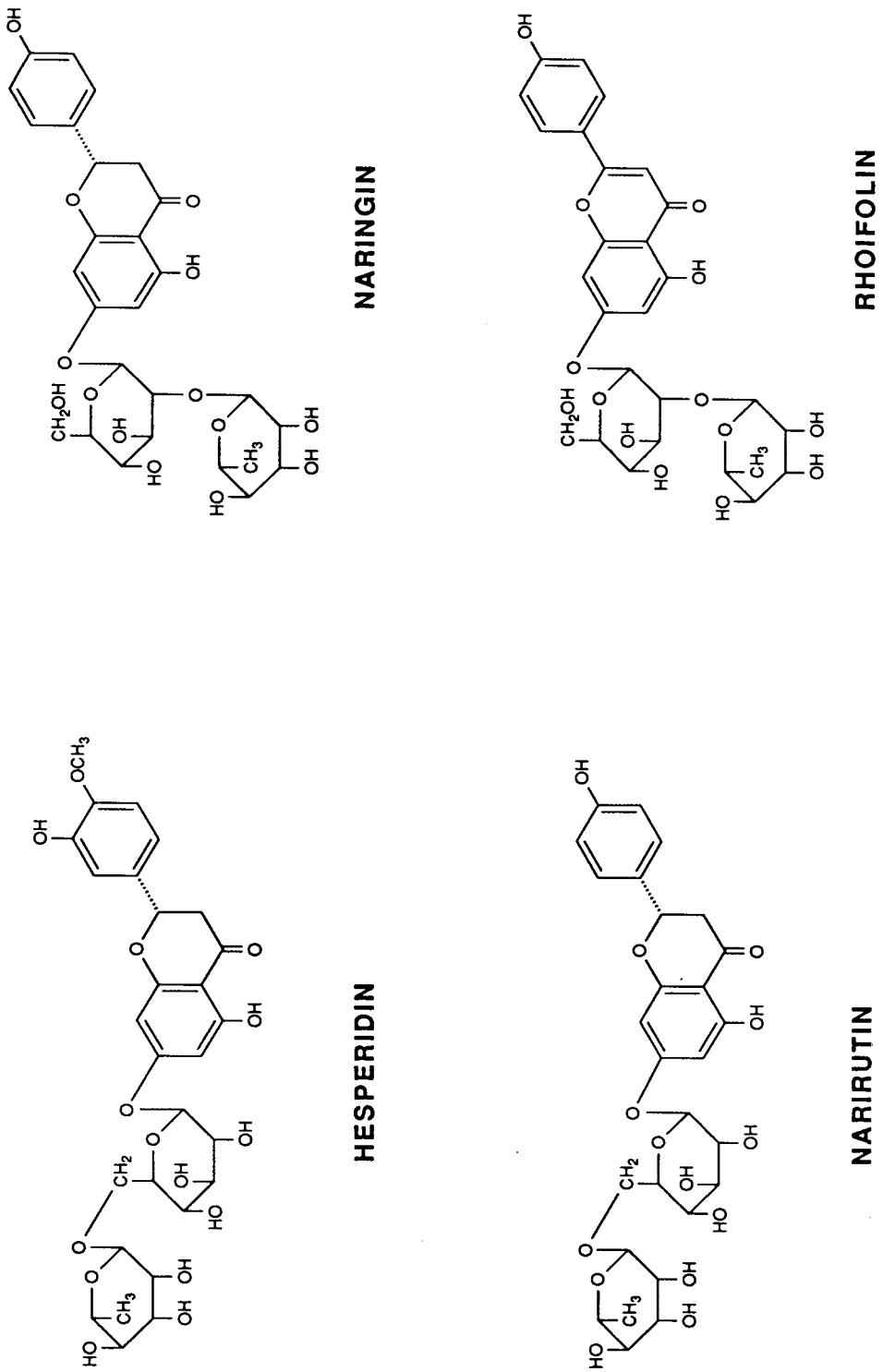


Fig. 1. Structures of hesperidin [15], naringin [16], narirutin [16], and rhoifolin [17].

Table 1
Percentages of prominent flavonoids in juice concentrate extracts[a]

Juice concentrate Exact number[b]	Flavonoid		
	Hesperidin	Naringin	Narirutin
Grapefruit (room temperature)			
extract 1		75	73
extract 2		23	22
extract 3		2	2
extract 4		3	
extract 5		1	
Orange (room temperature/55°C)			
extract 1	32/91		64/94
extract 2	19/8		17/6
extract 3	18/1		8
extract 4	17		7
extract 5	10		3
extract 6	4		

[a] Percentages are amounts extracted relative to the total quantity extracted from the individual juice concentrate aliquot. Values were calculated from ratios of analyte/internal standard chromatographic peak areas for single juice samples.
[b] Extract numbers indicate the particular extract in successive extractions of the same juice concentrate aliquot.
Methanol used as extraction solvent. Flavanone absorbances measured at 283 nm; rhoifolin at 335 nm.

ml of methanol following the same procedure. A 2-ml volume of water was added to the 3 combined methanolic extracts. The resulting mixture was vortex-mixed, filtered through a 1-μm porosity glass fiber filter, followed by a 0.2-μm porosity Anotop filter.

Frozen orange juice concentrate was thawed briefly, and approximately 1-g aliquots weighed into centrifuge tubes. To each aliquot, 0.4 ml of 1.8 mg ml^{-1} rhoifolin and 3 ml of methanol were added. After vortex-mixing for 30 s, each aliquot was warmed for 15 min in a 55°C water bath. Following additional mixing for 30 s, the mixture was centrifuged at 25 000 *g* for 15 min. The supernatant was collected, and 2 ml of methanol plus 1 ml of water were added to the remaining solid. This was vortex-mixed for 30 s, warmed 15 min in the water bath, vortex-mixed another 30 s, and then centrifuged. The supernatant was combined with the first juice extract and 4 ml of water were added. This was vortex-mixed, filtered through a 1-μm porosity glass fiber filter, followed by a 0.2-μm porosity Anotop filter.

A second set of grapefruit and orange juice concentrate samples having no internal standard was prepared for qualitative analysis, only. These samples were processed using the procedure described above, except no rhoifolin was added.

2.5. Recoveries from spiked samples

Recoveries of hesperidin, naringin, narirutin and rhoifolin added to orange and grapefruit juice concentrates were measured by comparing three sets of chromatograms: flavonoid standards, juice concentrates, and spiked juice concentrates. Standards (a single level for each flavonoid) were prepared by diluting measured volumes of working standards with water–methanol to a known final volume. The juice concentrate samples were weighed, extracted as previously described, and brought to the same final volume. Spiked juice concentrates were prepared by adding measured volumes of working standards to weighed amounts of juice concentrates. In each case the quantity of flavonoid added was approximately equal to that present in the juice, itself. The spiked juice concentrates were then extracted as previously described, and

diluted to the same final volume as the other samples.

Quantitative levels in the three sample sets were determined by fully loading the injection loop for each injection. The increased response due to addition of analyte into the juice concentrate (the difference in peak area between a spiked and non-spiked sample) was ratioed against the theoretical increase (the peak area expected from added standard alone) to calculate the recovery.

2.6. Chromatography

All separations were performed at ambient temperature by reversed-phase HPLC on a 25 cm C_{18} column using isocratic runs and premixed eluents. Initial examination of orange and grapefruit juice extracts with varied mobile phase composition indicated only two predominant flavonoids present in each juice. The extracts prepared just for qualitative analysis were analyzed using a water–tetrahydrofuran mobile phase (18:7) at a flow-rate of 0.4 ml min^{-1}. All other separations employed a water–acetonitrile–2-propanol–formic acid (158:23:19:0.2) mobile phase having a flow-rate of 0.6 ml min^{-1}.

Spectral scans were continuously collected from 210 to 450 nm during each run. Peak areas were measured from absorbance/time plots obtained at λ_{max}: naringin, hesperidin, and narirutin plots were integrated at 283 nm; rhoifolin at 335 nm.

3. Results and discussion

3.1. Extraction efficiencies and recoveries

The average recoveries of flavonoids spiked into orange juice concentrate were 106% hesperidin, 84% narirutin and 90% rhoifolin. In grapefruit juice concentrate recoveries were 100% naringin, 84% narirutin and 91% rhoifolin. These values represent duplicate analyses of two spiked samples for each flavonoid examined.

Measured quantities of hesperidin, naringin, and narirutin extracted from juice concentrates are listed in Table 1. Results indicate that in the first two methanolic fractions nearly all extractable material was removed from grapefruit juice concentrate at room temperature, and from orange juice concentrate at 55°C. In both cases, most material was removed in the initial extract. A comparison of orange juice extraction at room temperature and 55°C shows that hesperidin, in particular, was difficult to extract from this matrix without warming. In no case were any of the flavonoid glycosides fully extracted in a single step.

These findings suggest that a significant fraction of total flavonoids were initially present in the juice solids, and these were only partially removed in the first methanolic fraction. Complete extraction of remaining flavonoids required additional extraction stages. The improved extraction of hesperidin in orange juice at an elevated temperature may be indicative of flavonoid glycosides, in general, even though higher temperatures were unnecessary for effective extraction of grapefruit juice.

A minor problem associated with methanolic extraction of juice concentrates, even following centrifugation, was formation of cloudy mixtures, presumably due to suspended particulate matter. This occurred only after multiple extractions, however, and could be corrected by addition of a small percentage of water to the extraction solvent, if necessary. In the absence of water, solids tended to carry over into the supernatant and more readily clog filter units.

In previous work involving analysis of flavonoids in citrus, limited quantitative information regarding the extraction process, itself, has been reported. Except for recovery studies involving fortified samples [6–8,10,12,18], completeness of extraction from the juice matrix has been largely neglected. Trifirò et al. [14], however, in evaluating different methods of extraction, showed that simple filtration resulted in artificially low values for hesperidin in orange and lemon juices. The fact that even added methanol will not totally extract flavonoids from juice solids in a single step (Table 1) is consistent with this finding. Consequently, measured levels of flavo-

noids in citrus juices using only filtration for sample preparation [6,7,9,19] may well underestimate true values. No similar comparative study has been conducted for solid-phase extraction, although 92.5% recovery of hesperidin from orange juice [8] and quantitative recovery of four flavonoids added to citrus juice supernatants [18] have been reported. The effectiveness of solid-phase extraction of citrus juices in two other studies [5,20] is not known.

Methanol [21,22], methanol–2-propanol [4,10], acetone–hexane [4], dimethylformamide [12,13], hot water [14], and aqueous base [14] have all been used as solvents for extraction of flavonoids from citrus juices. Results of the present study suggest that some of these methods, particularly those employing organic solvents at elevated temperature [10,12,13,22] and/or using multiple extraction steps [4,10,21], may be quite effective. Additionally, the increased recovery of hesperidin from orange juice at a higher temperature supports earlier studies of flavonoid glycoside solubilities in aqueous media. Hesperidin, normally sparingly soluble at room temperature, is relatively soluble in boiling water [14]. Similarly, naringin solubility in water is strongly temperature dependent, increasing dramatically at higher temperatures [23].

3.2. Identification and quantitation

The major flavonoids in orange and grapefruit juice concentrates were identified by comparison with commercial standards. The closely matched spectra and retention times confirmed hesperidin and narirutin as major components in orange juice, and naringin and narirutin as major components in grapefruit juice. In addition, application of the peak purity software to the diode-array data indicated no impurities present in any of the chromatographic peaks of interest.

Representative chromatograms of orange and grapefruit juice extracts separated with the water–acetonitrile–2-propanol–formic acid mobile phase are shown in Fig. 2. The associated UV–Vis spectra (Fig. 3) are quite similar and are typical of flavanones, in general [24]. Only at the shorter wavelengths are some minor differences

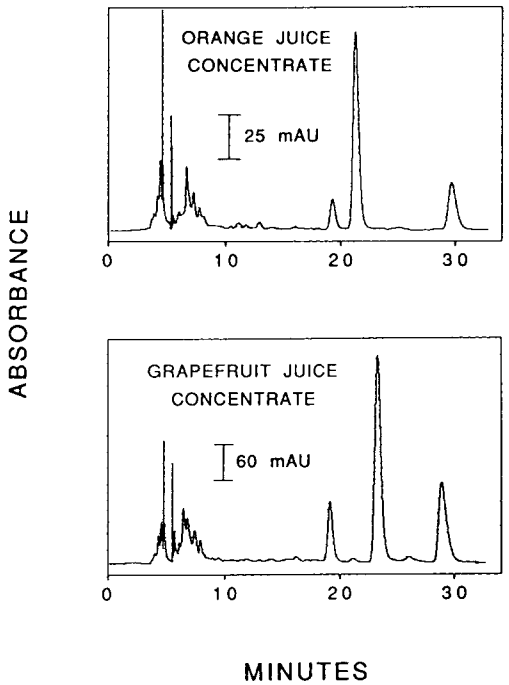


Fig. 2. Chromatograms of orange juice concentrate and grapefruit juice concentrate extracts. All chromatograms are shown with absorbances at 283 nm, whereas peak areas used for quantitative analysis were integrated at 283 nm for flavanone analytes and 335 nm for rhoifolin. Chromatograms show (in order of increasing retention) narirutin, hesperidin, naringin, and rhoifolin (internal standard). C_{18} column; mobile phase of water–acetonitrile–2-propanol–formic acid (158:23:19:0.2); flow-rate = 0.6 ml min^{-1}.

notable. Retention times (average of three runs) of the flavanones separated using the water–tetrahydrofuran mobile phase were 17.6, 22.5 and 26.6 min for hesperidin, narirutin and naringin standards, respectively (not shown). Under these run conditions, the spectra of the three citrus flavanones were, again, quite like one another and, also, similar to those obtained using the other mobile phase.

Rhoifolin, the internal standard added to the juice concentrates, is known to occur in grapefruit peel/pulp [25] and sour orange peel [17], but none was observed in chromatograms of orange or grapefruit juices unless added to the samples. In addition, no components identifiable as flavonoids were found among the group of

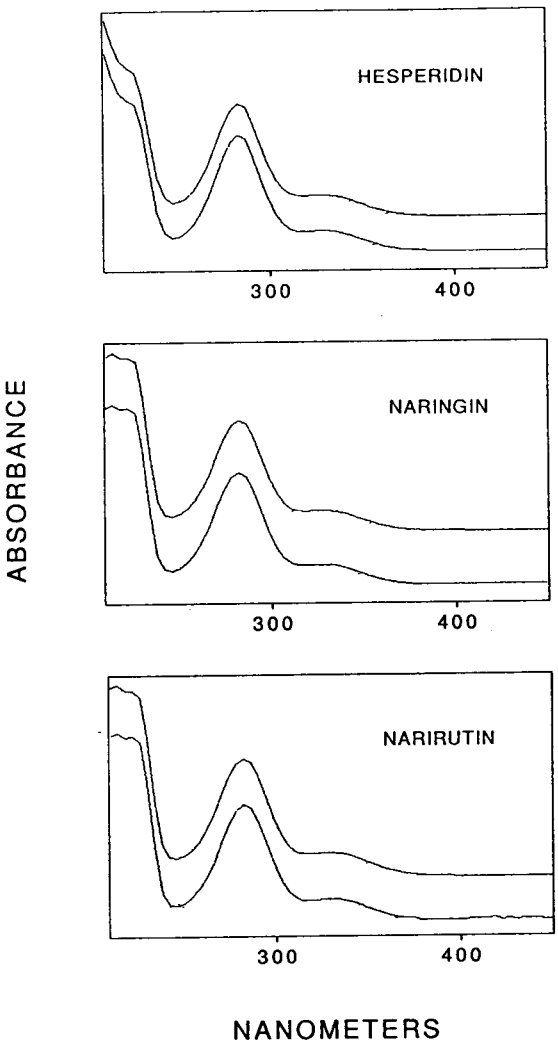


Fig. 3. Absorbance–wavelength spectra of hesperidin, naringin, and narirutin standards (lower tracings), and of corresponding chromatographic peaks from orange juice concentrate or grapefruit juice concentrate extracts (upper tracings) obtained from chromatograms in Fig. 2. Spectra were normalized and offset for purpose of comparison.

early eluting peaks seen in both juice chromatograms (Fig. 2).

Calibration plots of peak-area ratios vs. mass ratios for hesperidin/rhoifolin, naringin/rhoifolin, and narirutin/rhoifolin were obtained for solutions of the pure compounds. Each flavanone standard was prepared at four different concentrations (relative to rhoifolin) in aqueous methanol and analyzed in duplicate. Levels were chosen to bracket those found in the juice concentrates. A least squares linear fit was applied to each data set, the results of which are presented in Table 2. Weights of standards were corrected for percent water (as reported by the suppliers).

All plots were found to be linear across the ranges studied, with intercepts near the origin. Values for the standard errors and correlation coefficients listed in Table 2 indicate good precision was obtained using the internal standard chosen. In addition, results obtained for duplicate runs of the same extract (not listed) were highly reproducible. In contrast to the work of Mouly et al. [13], and Nogata et al. [18], who observed nearly identical response factors for hesperidin, naringin, and narirutin in water–acetonitrile–tetrahydrofuran–acetic acid and water–methanol–phosphoric acid mobile phases, respectively, noticeably different slopes for these three flavonoids were found in the present study.

Quantitative values for juice flavonoids were determined from chromatographic peak areas obtained from absorbance–time plots. Flavanone/rhoifolin absorbance area ratios were converted to flavanone/rhoifolin mass ratios using calibration plots (Table 2). Final concentrations were determined by factoring into calculations the quantity of juice initially aliquoted and the amount of internal standard added.

In orange juice concentrates 120/150 mg hg^{-1} hesperidin and 24/30 mg hg^{-1} narirutin were measured for Giant/Minute Maid brands, respectively. (Calculated values for prepared orange juices, based on diluting 1 part concentrate with 3 parts water, were 31/38 mg hg^{-1} hesperidin and 6.0/7.4 mg hg^{-1} narirutin.) In grapefruit juice concentrates 200/200 mg hg^{-1} naringin and 62/68 mg hg^{-1} narirutin were measured for Giant/Minute Maid brands, respectively. (Calculated values for prepared grapefruit juices, based on diluting 1 part concentrate with 3 parts water, were 49/51 mg hg^{-1} naringin and 16/17 mg hg^{-1} narirutin.) These flavonoid levels are averages of 6 measurements (duplicate analyses of 3 extracts from the same batch of juice

Table 2
Statistical data for calibration plots[a]

	Hesperidin	Naringin	Narirutin
Number of data points	8	8	8
Range[b]	0.49–4.9	0.21–2.1	0.069–0.69
Slope	0.96	0.86	0.61
Intercept	−0.056	−0.016	0.004
Standard error of estimate	0.054	0.014	0.017
(Correlation coefficient)^2	0.999	1.000	0.990

[a] Results derived from linear regression analyses of analyte/internal standard chromatographic peak-area ratios (ordinates) plotted vs. analyte/internal standard mass ratios (abscissae).
[b] Ranges given are for analyte/internal standard mass ratios.
Flavanone absorbances measured at 283 nm; rhoifolin at 335 nm.

concentrate) expressed to 2 significant figures; 1 hg equals 100 g.

The flavonoid levels reported here are in the same general range as the values reported in most previous publications on the subject. Hesperidin in orange juice has been quantitated at 11 [5], 9.8–12.0 (Valencia) and 5.4–10.0 (Hamlin) [7], 31.5–58.3 [8], 7.64–21.9 [9], 29.3–91.5 and 23.5–40.7 [12], and 20–163 and 19.5–350 mg hg^{-1} [14]. Narirutin levels in orange juice have been reported at 1.7–15.3 [8], 2.63–5.42 [9], and 3.69 (Valencia), 8.51 (navel), 4.33 (blood), 8.03 (Thompson), 3.97 (Malta), 5.1–12.8 and 3.0–8.4 mg hg^{-1} [12]. Naringin in grapefruit juice has been quantitated at 23.2–59.9 [4], 21 [6], 30.6 [10], 35.5–46.7 [11], 33.1 (white), 15.91 (pink), 27.54 (red), 25.16 (green), 20.5–20.6, and 11.3–48.1 [12], 13.8–22.7 and 33.6–67.5 [13], and 29.6 and 27.8 mg hg^{-1} [14]. Narirutin levels in grapefruit juice have been reported at 9.6–13.4 [4], 12.4 [10], 10.57 (white), 5.61 (pink), 7.60 (red), 17.86 (green) and 3.3–16.1 [12], and 5.9–7.3 and 10.3–12.2 mg hg^{-1} [13]. Obviously, the large variability in food sample composition precludes direct comparison of results and makes method accuracies difficult to assess. Nonetheless, the high flavonoid content of orange and grapefruit juices, in general, indicates that comparatively large quantities could be consumed in a typical diet.

Based on the levels of hesperidin, naringin, and narirutin found in the Giant/Minute Maid brand juices studied here, a single 8 fluid ounce serving (the standard serving size for fruit juices designated by the Food and Drug Administration [26]) of orange juice would contain 73/90 mg hesperidin (36/44 mg hesperetin) and 14/18 mg narirutin (6.6/8.3 mg naringenin), while 8 fluid ounces of grapefruit juice would contain 120/120 mg naringin (54/57 mg naringenin) and 38/40 mg narirutin (17/19 mg naringenin). These quantities are a substantial fraction of Kühnau's estimate of 1 g daily intake of flavonoid glycosides [1] and exceed the 23–25 mg flavonoid aglycones per day reported by Hertog and co-workers [2,3] who did not include flavanones in their totals. Daily intake of these citrus flavanones could easily exceed that of both β-carotene and vitamin E.

In recent surveys of the American diet, average individual daily consumption of orange and grapefruit juices was estimated as 48 and 4.3 g, respectively [27]. This corresponds to an average daily consumption of 15/18 mg hesperidin, 2.1/2.2 mg naringin and 3.6/4.3 mg narirutin, based on the values obtained for the Giant/Minute Maid juices, respectively. This is higher than the flavanone level reported in the Dutch diet, and up to 50% of the combined total for flavonols and flavones [2,3]. Nonetheless, these quantities are a small fraction of Kühnau's estimate of total flavonoid intake, and do not fully account for the discrepancy in dietary flavonol and flavone levels reported by Hertog and co-workers [2,3] and the levels of all 4-oxo-flavonoids (flavonols, flavones and flavanones) reported by Kühnau [1].

4. Conclusions

The presence of high levels of hesperidin, naringin, and narirutin in orange and grapefruit juice concentrates allows direct HPLC analysis of juice extracts. In the present study, essentially complete extraction of these flavanone glycosides was achieved by extracting multiple times with methanol. For orange juice concentrate, extraction at an elevated temperature resulted in improved recoveries. Flavonoid glycosides were easily separated and detected by reversed-phase HPLC and UV–Vis detection without the need for hydrolysis or derivatization.

Accurate quantitative data are essential to assessing the health benefits of flavonoids in the diet. In addition, the ability to differentiate authentic fruit juices from adulterated products based on flavonoid levels is valuable from a commercial standpoint. The methods presented enable accurate determination of the principal flavonoids in orange and grapefruit juices, and may well be applicable to other citrus not examined in this study. Although the effects of flavanones in humans have not yet been established, these compounds are potentially important dietary components. The increasing evidence that flavonoids may be valuable constituents of the diet underscores the importance of determining their levels in food products.

Acknowledgements

The authors would like to thank Dr. Aldo Ferretti and Dr. Edward Lee for their assistance in translating foreign publications.

References

[1] J. Kühnau, World Rev. Nutr. Diet, 24 (1976) 117.
[2] M.G.L. Hertog, E.J.M. Feskens, P.C.H. Hollman, M.B. Katan and D. Kromhout, The Lancet, 342 (1993) 1007.
[3] M.G.L. Hertog, P.C.H. Hollman, M.B. Katan and D. Kromhout, Nutr. Cancer, 20 (1993) 21.
[4] R.F. Albach and H.K. Wutscher, J. Rio Grande Valley Hort. Soc., 41 (1988) 89.
[5] F. Drawert, G. Leupold and H. Pivernetz, Chem. Mikrobiol. Technol. Lebensm., 6 (1980) 189.
[6] J.F. Fisher and T.A. Wheaton, J. Agric. Food Chem., 24 (1976) 898.
[7] J.F. Fisher, J. Agric. Food Chem., 26 (1978) 1459.
[8] R. Galensa and K. Herrmann, Dtsch. Lebensm. Rdsch., 76 (1980) 270.
[9] P. Gamache, E. Ryan and I.N. Acworth, J. Chromatogr., 635 (1993) 143.
[10] R.E. Hagen, W.J. Dunlap, J.W. Mizelle, S.H. Wender, B.J. Lime, R.F. Albach and F.P. Griffiths, Anal. Biochem., 12 (1965) 472.
[11] R.L. Mansell, C.A. McIntosh and S.E. Vest, J. Agric. Food Chem., 31 (1983) 156.
[12] P.P. Mouly, C.R. Arzouyan, E.M. Gaydou and J.M. Estienne, J. Agric. Food Chem., 42 (1994) 70.
[13] P. Mouly, E.M. Gaydou and J. Estienne, J. Chromatogr., 634 (1993) 129.
[14] A. Trifirò, D. Bigliardi, S. Gherardi and R. Bazzarini, Ind. Conserve, 55 (1980) 194.
[15] C.T. Seitz and R.E. Wingard Jr., J. Agric. Food Chem., 26 (1978) 278.
[16] R.L. Rouseff, Flavonoids and Citrus Quality, ACS Symp. Ser., Am. Chem. Soc., Washington. D.C., The Society, 143 (1980) 91.
[17] T. Nakabayashi, Nippon Nogei Kagaku Kaishi, 35 (1961) 945.
[18] Y. Nogata, H. Ohta, K. Yoza, M. Berhow and S. Hasegawa, J. Chromatogr. A, 667 (1994) 59.
[19] P.F. Cancalon, LC·GC, 11 (1993) 749.
[20] M. Krause and R. Galensa, J. Chromatogr., 588 (1991) 41.
[21] R. Galensa and K. Herrmann, J. Chromatogr., 189 (1980) 217.
[22] J.W. Mizelle, W.J. Dunlap, R.E. Hagen, S.H. Wender, B.J. Lime, R.F. Albach and F.P. Griffiths, Anal. Biochem., 12 (1965) 316.
[23] G.N. Pulley, Ind. Eng. Chem., 8 (1936) 360.
[24] K.R. Markham, Techniques of Flavonoid Identification, Academic Press, New York, NY, 1982, p. 38.
[25] W.J. Dunlap and S.H. Wender, Anal. Biochem., 4 (1962) 110.
[26] Federal Register, Superintendent of Documents, United States Government Printing Office, Washington, D.C., 58 (1993) 2259.
[27] Compilation of 1989 Continuing Survey of Food Intakes by Individuals and 1989 Diet and Health Knowledge Survey (1992), 1990 Continuing Survey of Food Intakes by Individuals and 1990 Diet and Health Knowledge Survey (1993), and 1991 Continuing Survey of Food Intakes by Individuals and 1991 Diet and Health Knowledge Survey (1994), U.S. Department of Agriculture, Human Nutrition Information Service. Computer tape accession numbers PB93-500411, PB93-504803, and PB94-500063.

ELSEVIER

Journal of Chromatography A, 705 (1995) 257–265

JOURNAL OF CHROMATOGRAPHY A

Monitoring of optical isomers of some conformationally constrained amino acids with tetrahydroisoquinoline or tetraline ring structures. Part II

Antal Péter[a,*], Géza Tóth[b], Edit Olajos[a], Ferenc Fülöp[c], Dirk Tourwé[d]

[a]*Department of Inorganic and Analytical Chemistry, Attila József University, P.O. Box 440, H-6701 Szeged, Hungary*
[b]*Biological Research Centre, Hungarian Academy of Sciences, Isotope Laboratory "B" Level, P.O. Box 521, H-6701 Szeged, Hungary*
[c]*Institute of Pharmaceutical Chemistry, Albert Szent-Györgyi Medical University, P.O. Box 121, H-6701 Szeged, Hungary*
[d]*Eenheid Organische Chemie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium*

First received 27 December 1994; revised manuscript received 2 March 1995; accepted 3 March 1995

Abstract

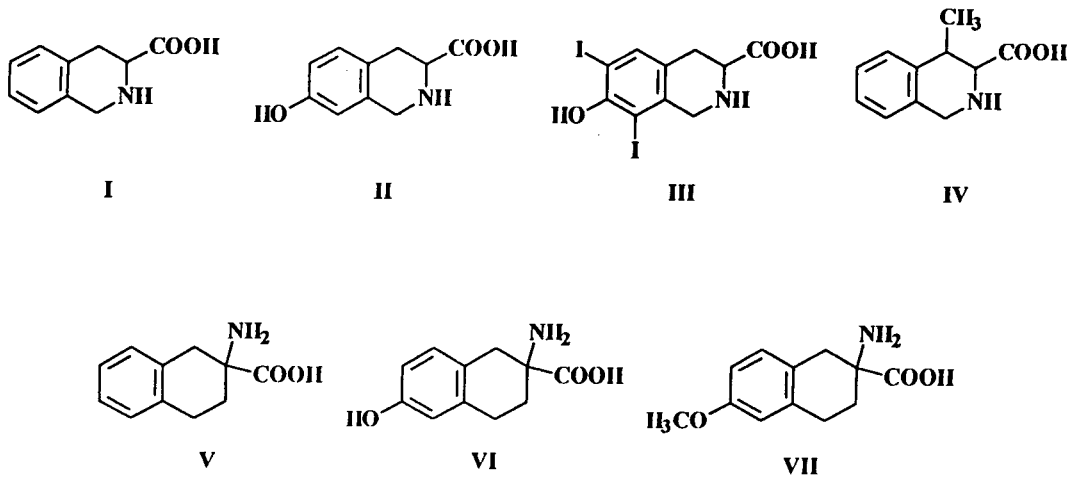
Conformationally constrained amino acids were synthesized in chiral or racemic forms: D- and L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), the *erythro*-D,L-4-methyl analogue, D- and L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, D- and L-7-hydroxy-6,8-diiodo-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, D,L-2-aminotetraline-2-carboxylic acid, D,L-6-hydroxy-2-aminotetraline-2-carboxylic acid and D,L-6-methoxy-2-aminotetraline-2-carboxylic acid. The optical isomers were characterized and identified by applying precolumn derivatization with chiral reagents (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate) and enzymatic digestion with L-amino acid oxidase, carboxypeptidase A and α-chymotrypsin. The HPLC conditions (pH, eluent composition and different buffers) were varied to obtain optimum separations.

1. Introduction

Conformationally constrained or topographically biased amino acids have been shown to provide an important new approach in the design of selectively acting hormone analogues [1,2]. The synthesis of these sterically constrained or biased unusual amino acids either leads to a racemic form, while the asymmetric synthesis may lead to a product which contains only a minor amount of the enantiomeric form. Furthermore, the originally pure amino acids can racemize to some extent in the course of the N^{α}-Boc-protection or during the synthesis of biologically active peptides, resulting in the diastereomers of the peptides. The difficulties in obtaining many of the uncommon amino acids in homochiral form underline the importance of having at hand effective chromatographic methods for the characterization and identification of their enantiomers.

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00316-9



I II III IV

V VI VII

Fig. 1. Structures of the compounds used. I = D- and L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); II = D- and L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (HOTic); III = D- and L-7-hydroxy-6,8-diiodo-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (I_2HOTic); IV = [*erythro*, (*SS*, *RR*)]-D,L-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (β-MeTic); V = D,L-2-aminotetraline-2-carboxylic acid (Atc); VI = D,L-6-hydroxy-2-aminotetraline-2-carboxylic acid (Hat); VII = D,L-6-methoxy-2-aminotetraline-2-carboxylic acid (Matc).

Many attempts have been made to resolve amino acid enantiomers by liquid chromatographic techniques. The enantioselective separations performed by high-performance liquid chromatographic (HPLC) methods can be divided into three main groups: direct separation on chiral stationary phases [3–5], separation on achiral columns with chiral eluents [5–7] and separation of the diastereomers formed by precolumn derivatization with chiral reagents [5,8–19].

For the synthesis of receptor-selective opioid peptides, a number of conformationally constrained aromatic amino acids have been prepared (Fig. 1). The separation of some of these isomers was described earlier [20]. The present paper deals with the separation of enantiomers of these amino acids by using precolumn derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). The eluent system contains methanol as organic modifier instead of the earlier-used acetonitrile. This change in eluent composition proved to be very effective: the analysis time is shortened and the separation is improved.

2. Experimental

2.1. Chemicals and reagents

The amino acids shown in Fig. 1 were synthesized in our laboratory by our own or literature methods. The syntheses of I [21], II and III [22] lead to one enantiomer of the substrate, depending on the configuration of the starting material, whereas IV [23], V [24], VI and VII [25] were obtained in racemic form. The identity of the compounds was checked by means of melting point determination, FAB mass spectrometry, ^{1}H NMR spectroscopy and chiral TLC [26].

GITC was purchased from Aldrich (Steinheim, Germany), FDAA from Pierce Chemical Company (Rockford, IL, USA), L-amino acid oxidase, carboxypeptidase A and α-chymotrypsin Type II from Sigma (St. Louis, MO, USA) and trifluoroacetic acid, sodium

acetate, potassium dihydrogenphosphate of analytical reagent grade, acetonitrile and methanol of HPLC grade and other reagents of analytical reagent grade from Merck (Darmstadt, Germany). Buffers were prepared with twice distilled water and further purified by filtration on a 0.45-μm filter Type HV (Millipore, Molsheim, France). The pH was adjusted with acetic acid (acetate buffer), phosphoric acid (phosphate buffer) and sodium hydroxide.

Chiral plates (Macherey Nagel, Düren, Germany) were used for chiral TLC, and spots were detected with ninhydrin reagent.

2.2. Apparatus

The HPLC system consisted of an M-600 low-pressure gradient pump equipped with an M-996 photodiode array detector, a Millennium 2010 Chromatography Manager data system (Waters Chromatography, Division of Millipore, Milford, MA, USA), an L-6000 liquid chromatographic pump (Merck Hitachi, Tokyo, Japan) equipped with a UV 308 detector (Labor MIM, Budapest, Hungary) and an HP 3395 integrator (Hewlett-Packard, Waldbronn, Germany).

The columns used were Vydac 218TP104 C_{18} and Vydac 218TP54 C_{18} (250 × 4.6 mm I.D.) with 10 and 5 μm particle size, respectively (The Separations Group, Hesperia, CA, USA).

^{1}H NMR spectroscopy was performed on an AM 400 spectrometer (Brucker, Zug, Switzerland).

FAB mass spectra were obtained on a double focussing MS 902 S spectrometer with xenon at 8 kV as bombarding gas (A.E.I. Scientific Apparatus Division, Manchester, UK).

2.3. Derivatization of amino acids

Amino acids (0.5–1 mg) were derivatized with FDAA or GITC by the method of Marfey [14] or Nimura et al. [11].

2.4. Enzymatic digestion of D,L-amino acids

The elution sequence of *erythro*-D- and L-β-MeTic (see Fig. 1 for full name and structure) was determined either by enzymatic degradation of the amino acid with L-amino acid oxidase by the method described in Ref. [20] or with a standard of *erythro*-D-β-MeTic made from *erythro*-D-β-methylphenylalanine by method [21].

The configurations of amino acids containing a tetraline ring were determined by means of enzymatic digestion with carboxypeptidase and α-chymotrypsin. For enzymatic degradation of Atc and Hat (see Fig. 1 for full names and structures), with carboxypeptidase A first the N-trifluoroacetyl derivative of the amino acid was prepared by the method described in Ref. [27]. A 1-mg amount of N-trifluoroacetyl derivative of the amino acid was dissolved in 200 μl 0.1 mol/l Tris buffer (pH 7.2) in a test-tube and 10 μl carboxypeptidase A was added. The test-tube was tightly capped and incubated for 1–10 h at 37°C. The carboxypeptidase A was inefficient in the enzymatic digestion of Matc. For the identification of configuration of Matc the α-chymotrypsin catalysed hydrolysis of D,L-6-methoxy-2-aminotetraline-2-carboxylic acid methyl ester was applied [28]. A 1-mg amount of D,L-amino acid methyl ester was dissolved in 200 μl 0.1 mol/l Tris buffer (pH 7.2) in a test-tube and 1 mg α-chymotrypsin was added. The incubation time was 48 h at 37°C and the pH was checked from time to time. The reaction mixtures were used for derivatization with GITC or FDAA.

3. Results and discussion

The HPLC separations of the derivatized amino acids were carried out in the three different aqueous buffer systems containing methanol as organic modifier: 0.1% trifluoroacetic acid (0.1% TFA), 0.01 mol/l potassium dihydrogenphosphate (pH 3) (phosphate buffer) and 0.01 mol/l sodium acetate (pH 3) (acetate buffer). Comparison with earlier results obtained with acetonitrile [20] reveals that the change of the organic modifier from acetonitrile to methanol improves the separation and the peak shapes.

The results of the separation of Tic derivatives are listed in Table 1. Decrease of the methanol

Table 1
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Tic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
50:50	3.33	4.85	1.45	3.23
55:45	7.25	11.81	1.63	4.42
KH_2PO_4–CH_3OH				
50:50	3.08	4.36	1.42	2.34
55:45	6.66	9.72	1.46	3.86
NaOAc–CH_3OH				
50:50	2.28	2.95	1.29	1.32
52.5:47.5	3.82	5.38	1.40	2.29
55:45	5.49	8.00	1.46	3.07
FDAA derivatives				
TFA–CH_3OH				
35:65	1.62	2.96	1.82	3.84
40:60	2.73	5.25	1.92	4.41
KH_2PO_4–CH_3OH				
30:70	0.86	1.34	1.56	1.66
35:65	1.30	2.15	1.65	2.78
40:60	2.33	4.17	1.79	3.20
NaOAc–CH_3OH				
40:60	1.86	3.26	1.75	4.11
45:55	3.09	5.72	1.85	5.00
50:50	5.92	11.87	2.01	5.47

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

Table 2
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of HOTic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
50:50	1.45	2.35	1.60	1.58
KH_2PO_4–CH_3OH				
50:50	1.66	2.51	1.51	1.92
52.5:47.5	2.05	3.31	1.61	2.37
NaOAc–CH_3OH				
50:50	1.45	2.08	1.43	3.12
52.5:47.5	1.98	3.07	1.55	3.71
FDAA derivatives				
TFA–CH_3OH				
35:65	0.76	1.46	1.92	2.73
45:55	1.46	2.61	1.79	3.50
50:50	2.18	5.34	2.45	8.90
KH_2PO_4–CH_3OH				
50:50	1.34	2.58	1.93	4.00
52.5:47.5	1.48	3.06	2.07	6.06
NaOAc–CH_3OH				
40:60	0.96	1.03	1.08	0.50
50:50	1.82	3.02	1.66	4.00
60:40	2.60	10.33	3.97	13.30

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

content increases the retention times, and the values of α and R_s. Comparison of the three buffer systems demonstrates that sodium acetate is a slightly superior, with relatively low k' values. The GITC derivatives have lower k' values than the FDAA derivatives at the same eluent composition.

The HOTic derivatives exhibit very good separation, with short retention times (Table 2). There is little difference between the three buffer systems, and all appear useful. The GITC derivatives have shorter retention times than the FDAA compounds.

The I_2HOTic derivatives separate very well within a short time in a methanol-containing system (Table 3). At a given percentage of organic modifier, the k' values are 5–10 times lower with methanol as organic modifier than with acetonitrile, while the R_s values improve [20]. To achieve the same R_s values for the separation of GITC and FDAA derivatives, the GITC derivatives should be analyzed at a lower methanol content and thus they require a longer analysis time.

The separation of *erythro*-β-MeTic derivatives can be carried out with good resolution in a

Table 3
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of I_2HOTic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
45:55	3.35	5.44	1.62	3.53
50:50	7.09	13.01	1.83	4.92
KH_2PO_4–CH_3OH				
45:55	1.59	1.89	1.13	1.16
47.5:52.5	2.30	2.92	1.27	1.92
50:50	4.60	8.82	1.92	6.66
NaOAc–CH_3OH				
47.5:52.5	1.99	2.55	1.28	1.33
50:50	3.67	5.98	1.63	4.15
FDAA derivatives				
TFA–CH_3OH				
35:65	1.42	3.10	2.18	5.60
40:60	1.45	3.91	2.70	6.44
KH_2PO_4–CH_3OH				
35:65	1.05	2.21	2.10	4.65
40:60	1.83	5.02	2.74	6.72
NaOAc–CH_3OH				
35:65	1.28	2.36	1.84	3.21
40:60	1.85	3.60	1.95	4.66

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

Table 4
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of *erythro*-β-MeTic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
40:60	1.48	1.92	1.30	1.26
45:55	2.96	4.15	1.39	2.26
KH_2PO_4–CH_3OH				
45:55	2.21	2.82	1.28	1.16
47.5:52.5	3.54	4.67	1.32	1.56
NaOAc–CH_3OH				
50:50	5.25	7.23	1.38	3.83
FDAA derivatives				
TFA–CH_3OH				
40:60	3.73	4.30	1.15	1.38
42.5:57.5	5.49	6.42	1.17	2.11
KH_2PO_4–CH_3OH				
30:70	1.14	1.41	1.25	1.60
35:65	1.69	2.16	1.28	3.00
40:60	2.77	3.73	1.35	3.75
45:55	5.39	7.41	1.37	4.55
NaOAc–CH_3OH				
35:65	1.50	1.95	1.30	2.43
40:60	1.87	2.63	1.40	3.00
45:55	3.83	5.43	1.42	4.36
50:50	6.78	9.94	1.47	6.33

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihidrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

relatively short time in all three buffer systems (Table 4). The peak shapes are best in sodium acetate, the band broadening being larger in trifluoroacetate and phosphate. The GITC derivatives have lower k' values than the FDAA derivatives at the same eluent composition, but the shorter analysis time means a worse resolution.

The four amino acids containing a tetrahydroisoquinoline ring which are investigated can be separated from each other conveniently as GITC derivatives in the acetate buffer–methanol (50:50) system. The elution sequence is HOTic < Tic < I_2HOTic < *erythro*-β-MeTic. The GITC derivatives in all cases have shorter retention times than the corresponding FDAA derivatives at the same eluent composition, but not better R_s values.

The elution sequence of the D- and L-forms of the amino acids I–IV was checked by enzymatic digestion of the L-forms with L-amino acid oxidase, followed by derivatization of the reaction mixture with GITC or FDAA. The results show that, for the GITC and FDAA derivatives of tetrahydroisoquinoline ring-containing unusual

amino acids, the first peak corresponded to the L-form and the second to the D-form.

The uncommon amino acids containing a tetraline ring (Atc, Hat and Matc) were investigated in the same buffer systems. The results of the separation of the Atc derivatives are listed in Table 5. Comparison of the three buffer systems shows that the acetate system is the best in the series of GITC derivatives, while for the FDAA derivatives all three systems are suitable. Otherwise, the FDAA derivatives give better peak shapes and R_s values than the GITC analogues.

The Hat derivatives exhibit good chromatographic results in the acetate buffer system (Table 6). The analyses in the trifluoroacetic acid and phosphate buffer systems take a longer time, but the R_s values do not improve and peaks are broad.

The Matc derivatives can be separated well in all buffer systems, but the chromatograms in sodium acetate display the best peak shapes (Table 7).

Table 5
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Atc derivatives on eluent composition

Eluent composition	k'_D	k'_L	α	R_s
GITC derivatives				
TFA–CH_3OH				
55:45	10.22	11.92	1.17	1.64
KH_2PO_4–CH_3OH				
55:45	9.14	10.64	1.16	1.42
NaOAc–CH_3OH				
50:50	5.25	5.99	1.14	2.22
FDAA derivatives				
TFA–CH_3OH				
45:55	8.62	9.64	1.12	1.46
KH_2PO_4–CH_3OH				
50:50	9.28	11.30	1.22	2.86
NaOAc–CH_3OH				
55:45	14.42	17.36	1.20	3.14

Column: Vydac 218TP54 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

Table 6
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Hat derivatives on eluent composition

Eluent composition	k'_D	k'_L	α	R_s
GITC derivatives				
TFA–CH_3OH				
65:35	15.33	16.94	1.10	1.12
KH_2PO_4–CH_3OH				
65:35	15.83	18.43	1.16	1.49
NaOAc–CH_3OH				
60:40	4.72	5.88	1.25	1.78
FDAA derivatives[a]				
TFA–CH_3OH				
60:40	8.93	7.81	1.14	1.18
65:35	16.44	14.22	1.16	1.64
KH_2PO_4–CH_3OH				
65:35	11.12	9.79	1.14	1.53
NaOAc–CH_3OH				
50:50	2.15	1.96	1.10	1.20
60:40	5.64	5.04	1.12	1.27
65:35	9.41	8.29	1.14	1.43

Column: Vydac 218TP104 C_{18} and Vydac 218TP54 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).
[a] FDAA derivatives were analysed on Vydac 218TP54 C_{18}.

The configurations of the D,L-amino acids containing a tetraline ring were checked by enzymatic digestion of the N-trifluoroacetyl amino acid with carboxypeptidase A and the amino acid methyl ester with α-chymotrypsin. The elution sequence of the isomers was D followed by L, except for Hat, where an interesting reversal of the elution sequence between the GITC and FDAA derivatives was observed.

In Fig. 2 selected chromatograms are shown of the separation of amino acids investigated in this work.

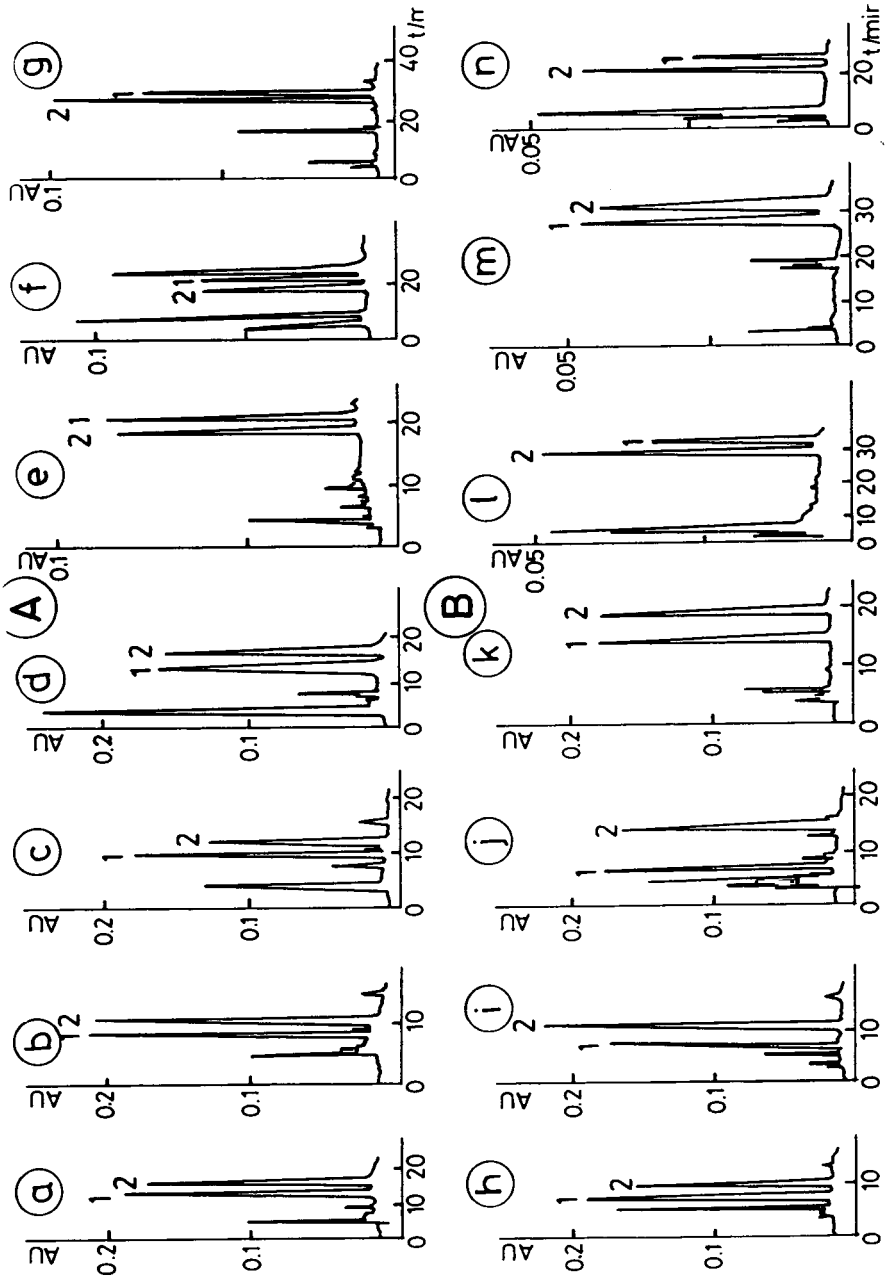


Fig. 2. Selected chromatograms of GITC (A) and FDAA (B) derivatives of amino acids. Amino acids: (a,h) Tic; (b,i) HOTic; (c,j) I_2HOTic; (d,k) *erythro*-β-MeTic; (e,l) Atc; (f,m) Hat; (g,n) Matc. Column, Vydac 218TP104 C_{18}; flow-rate, 0.8 ml/min; detection, (A) at 250 nm, (B) at 340 nm; mobile phase: (a,b) 0.01 *M* phosphate buffer–methanol (50:50), (c,d) 0.01 *M* phosphate buffer–methanol (47.5:52.5), (e,n) 0.01 *M* acetate buffer–methanol (50:50), (f,g) 0.01 *M* acetate buffer–methanol (60:40), (h) 0.01 *M* phosphate buffer–methanol (35:65), (i,l) 0.1% TFA–methanol (45:55), (j) 0.1% TFA–methanol (40:60), (k) 0.01 *M* acetate buffer–methanol (45:55), (m) 0.01 *M* acetate buffer–methanol (65:35); Peaks: 1 = L-isomer, 2 = D-isomer, unlabelled peaks originated from GITC or FDAA reagents.

Table 7
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Matc derivatives on eluent composition

Eluent composition	k'_D	k'_L	α	R_s
GITC derivatives				
TFA–CH_3OH 55:45	9.69	11.94	1.23	2.42
KH_2PO_4–CH_3OH 60:40	4.83	5.78	1.20	2.12
NaOAc–CH_3OH 50:50	7.76	8.50	1.10	2.08
FDAA derivatives				
TFA–CH_3OH 45:55	6.91	8.16	1.18	1.82
KH_2PO_4–CH_3OH 50:50	8.87	11.43	1.29	3.57
NaOAc–CH_3OH 50:50	6.45	8.14	1.26	2.23

Column: Vydac 218TP54 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogen phosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

4. Conclusions

The described procedures can be applied for the separation and identification of conformationally constrained unusual aromatic amino acids. The method permits to check the configuration of amino acids after synthesis and their incorporation into peptides, and hence optimization of the conditions of synthesis of amino acids and peptides. Methanol-containing mobile phase systems seem to be more efficient than acetonitrile-containing ones. The sodium acetate–methanol system is generally more efficient than the TFA or phosphate system. In general the GITC derivatives have lower k' values than the FDAA derivatives. As general rule relating elution sequence to configuration of the amino acid does not seem to be warranted since reversal of elution sequence may occur.

Acknowledgement

This work was supported by OTKA grants 71/91 and 2993/91.

References

[1] W. Kazmierski and V.J. Hruby, Tetrahedron, 44 (1988) 697.
[2] V.J. Hruby, F. Al-Obeidi, W. Kazmierski, Biochem. J., 44 (1990) 697.
[3] W.H. Pirkle and J. Finn, in J. Morrison (Editor), Asymmetric Synthesis, Vol. I, Analytical Methods, Academic Press, New York, 1983, Ch. 6.
[4] W.F. Lindner and C. Petterson, in I. Wainer (Editor), Liquid Chromatography in Pharmaceutical Development: an Introduction, Part 1, Aster, Springfield, VA, 1985, p. 63.
[5] D.W. Armstrong and S.M. Han, CRC Crit. Rev. Anal. Chem., 19 (1988) 175.
[6] V.A. Davankov, A.A. Kurganov and A.S. Bochkov, Adv. Chromatogr., 22 (1983) 71.
[7] C. Petterson, Trends Anal. Chem., 7 (1988) 209.
[8] W.F. Lindner, in L. Crane and B. Zief (Editors), Chromatographic Chiral Separations, Marcel Dekker, New York, 1987, p. 91.
[9] W.F. Lindner, in J.F. Lawrence and R.W. Frei (Editors), Chemical Derivatization in Analytical Chemistry, Vol. 2, Plenum Press, New York, 1982, p. 145.
[10] T. Nambara, in W.S. Hancock (Editor), CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins, Vol. I, CRC Press, Boca Raton, FL, 1984, p. 383.
[11] N. Nimura, H. Ogura and T. Kinoshita, J. Chromatogr., 202 (1980) 375.
[12] T. Kinoshita, Y. Kasahara and N. Nimura, J. Chromatogr., 210 (1981) 77.
[13] N. Nimura, A. Toyama and T. Kinoshita, J. Chromatogr., 316 (1984) 547.
[14] P. Marfey, Carlsberg Res. Commun., 49 (1984) 591.
[15] S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, Anal. Chem., 59 (1987) 1191.
[16] H. Brückner and C. Gah, J. Chromatogr., 555 (1991) 81.
[17] H. Brückner, R. Wittner and H. Godel, Chromatographia, 32 (1991) 383.
[18] H. Brückner and B. Strecker, Chromatographia, 33 (1992) 586.
[19] S. Einarsson ang G. Hansson, in C.T. Mant and R.S. Hodges (Editors), High Performance Liquid Chromatography of Peptides and Proteins, CRC Press, Boca Raton, FL, 1991, p. 369.
[20] A. Peter, G. Tóth, D. Tourwé, J. Chromatogr. A, 668 (1994) 331.

[21] A. Pictet and T. Spengler, Chem. Ber., 44 (1911) 2030.
[22] K. Verschueren, G. Tóth, D. Tourwé, M. Lebl, G. Van Binst and V.J. Hruby, Synthesis, (1992) 458.
[23] M. Lebl. G. Tóth, I. Slaninova and V.J. Hruby, Int. J. Pep. Protein Res., 40 (1992) 148.
[24] R.M. Render, B.H. Butcher, D.H. Buxton and D.I. Howell, J. Med. Chem., 14 (1972) 892.
[25] S.N. Rastogi, J.S. Bendra, N. Anand, Indian J. Chem., 9 (1971) 1175.
[26] G. Tóth, M. Lebl and V.J. Hruby, J. Chromatogr., 504 (1991) 450.
[27] A. Peter, G. Laus, D. Tourwé, E. Gerlo and G. Van Binst, Pept. Res., 6 (1993) 48.
[28] H.I. Abrash, C. Niemann, Biochemistry, 2 (1963) 947.

ELSEVIER

Journal of Chromatography A, 705 (1995) 267–273

JOURNAL OF CHROMATOGRAPHY A

Chromatographic behaviour of opioid peptides containing β-methylphenylalanine isomers

Antal Péter[a,*], Géza Tóth[b], Edit Olajos[a], Dirk Tourwé[c]

[a]*Department of Inorganic and Analytical Chemistry, József Attila University, P.O. Box 440, H-6701 Szeged, Hungary*

[b]*Biological Research Centre, Hungarian Academy of Sciences, Isotope Laboratory, "B" Level, P.O. Box 521, H-6701 Szeged, Hungary*

[c]*Eenheid Organische Chemie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium*

First received 20 December 1994; revised manuscript received 2 March 1995; accepted 6 March 1995

Abstract

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed to obtain pure *erythro*[2*S*3*S*, 2*R*3*R*]- and *threo*[2*S*3*R*, 2*R*3*S*]-β-methylphenylalanine. These amino acids were incorporated into an enkephalin, H-Tyr-D-Ala-Gly-β-MePhe-Val-Val-Gly-NH_2, and into a deltorphin C, H-Tyr-D-Ala-β-MePhe-Asp-Val-Val-Gly-NH_2, analogue, which yielded four diastereoisomers of the peptides. The diastereoisomers were separated on different columns and with different eluent systems. The sequence of elution of the peptide diastereoisomers was determined after hydrolysis of the peptides. For identification of the β-methylphenylalanine enantiomers, enzymatic degradation and an RP-HPLC method were used, with application of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide as derivatizing reagent.

1. Introduction

In the synthesis of receptor selective peptides, unusual amino acids are often used. These unnatural amino acids can either be prepared in racemic form, which is usually the fastest method, or by asymmetric synthesis. Even when using the latter strategy, the chiral purity of the amino acid is often not complete, and small amounts of the stereoisomers can be present.

After incorporation into the peptides, the resulting diastereoisomers may have similar or different physico-chemical or biological properties. The crude peptides are fragmentally purified by reversed-phase high-performance liquid chromatographic (RP-HPLC) methods. In many cases, these purifications are difficult, chromatography of the diastereoisomeric peptides leading to overlapping peaks. Nevertheless, because of the large differences in biological potencies and agonist or antagonist activities of peptide stereoisomers, it is very important to ensure complete separation on both analytical and preparative scales. Another problem is the determination of the configurations of the unusual amino acids in the diastereoisomeric peptides following purification. We described earlier a method for the determination of the configurations of certain unnatural aromatic amino acids in peptide diastereoisomers [1].

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00317-7

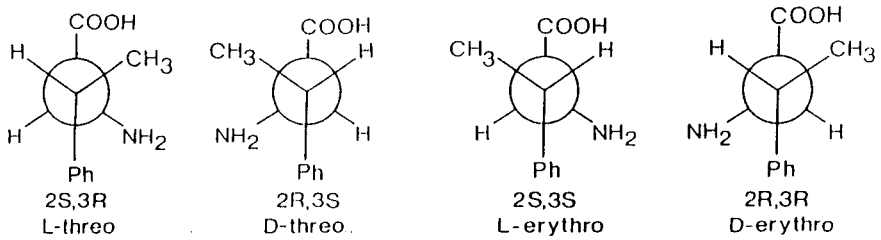


Fig. 1. Structures of four optical isomers of β-methylphenylalanine.

This paper describes a new RP-HPLC method for the preparation of pure *erythro*[(2*S*,3*S*) and (2*R*,3*R*)] and pure *threo*[(2*S*,3*R*) and 2*R*,3*S*)]-β-methylphenlyalanine (β-MePhe). β-MePhe contains two chiral centres, and four stereoisomers are possible (Fig. 1); when incorporated into a peptide, these give rise to different diastereoisomers. We synthesized all four isomers of [β-MePhe[3]]deltorphin C (H-Tyr-D-Ala-β-MePhe-Asp-Val-Val-Gly-NH_2) and of the [β-MePhe[4]] enkephalin analogue (H-Tyr-D-Ala-Gly-β-MePhe-Val-Val-Gly-NH_2) and investigated the effects of the side-chain conformations of the four β-MePhe isomers on the opioid receptor affinity and selectivity [2]. A method was developed for separation of the four diastereoisomers of β-MePhe-containing peptides and for establishment of the configuration of the β-MePhe in these diastereoisomers.

2. Experimental

2.1. Chemicals and reagents

β-MePhe was synthesized by a slight modification of the method of Kataoka et al. [3]. Pure *erythro*[(2*S*,3*S*)- and (2*R*,3*R*)]-β-MePhe and *threo*[(2*S*,3*R*) and (2*R*,3*S*)]-β-MePhe were obtained by chromatographic separation of crystallized β-MePhe. Standard (2*S*,3*S*)-, (2*R*,3*R*)-, (2*S*,3*R*)- and (2*R*,3*S*)-β-MePhe for chromatographic identification were prepared by the method of Kataoka et al. [3]. Their purity and structures were confirmed by fast atom bombardment (FAB) MS and by NMR spectroscopy. The chemical shifts and coupling constants are in agreement with the results of Tsuchihashi et al. [4] and Hruby et al. [5].

Deltorphin C and enkephalin analogues were prepared by solid phase peptide synthesis, using Boc chemistry, starting from 4-methylbenzhydrylamine resin [2,6]. Two syntheses were carried out for both peptides, with Boc-D,L-*erythro*-β-MePhe or Boc-D,L-*threo*-β-MePhe. Both syntheses gave crude diastereomeric peptides.

The mixtures of diastereomeric peptides were separated by means of HPLC, with one of the chromatographic systems described below, and the pure peptides were isolated as white powders. The configuration of β-MePhe in each peptide was determined after hydrolysis to the amino acids, by application of L-amino acid oxidase [7] in combination with the chromatographic methods described previously [8].

L-Amino acid oxidase and 1-fluoro-2,4-dinitrophenyl-5-L-alanin amide (FDAA) were purchased from Sigma (St. Louis, MO, USA).

4-Methylbenzhydrylamine resin was purchased from Bachem Feinchemikalien (Bubsendorf, Switzerland). Amino acids, HPLC-grade solvents (methanol and acetonitrile) and other reagents of analytical-reagent grade were obtained from Merck (Darmstadt, Germany).

Buffers were prepared with doubly distilled water and further purified by pumping through a Type HV 0.45-μm filter (Millipore, Molsheim, France).

2.2. Apparatus

HPLC measurements were performed with three chromatographic systems: (A) the Waters system consisted of an M-600 low-pressure gra-

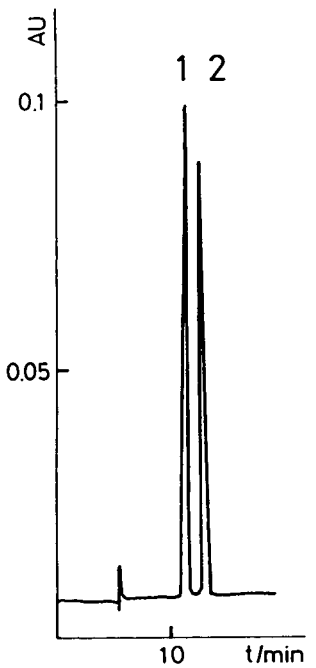


Fig. 2. Chromatogram of D,L-*threo*- and D.L-*erythro*-β-methylphenylalanine. Column, Vydac 218TP54 C_{18}; flow-rate, 1.5 ml/min; mobile phase, 0.2% acetonitrile in water; detection at 210 nm. Peaks, 1 = D,L-*threo*-; 2 = D.L-*erythro*-β-MePhe.

dient pump, an M-996 photodiode-array detector and a Millenium 2010 Chromatography Manager data system (Waters Chromatography, Division of Millipore, Milford, MA, USA); (B) the HPLC system consisted of an L-6000 liquid chromatographic pump (Merck–Hitachi, Tokyo, Japan), a UV 308 spectrophotometric detector (Labor MIM, Budapest, Hungary) and an HP 3395 integrator (Hewlett-Packard, Waldbronn, Germany); and (C) the Gilson Autoprep system contained Gilson Model 302 and 303 pumps, a Gilson Model 115 detector and a Gilson Model 712 system controller (Gilson Medical Electronics, Villiers le Bel, France).

The columns used for analytical separations were as follows: (I) Nucleosil 10 C_{18} (250 × 4.6 mm I.D.), 10-μm particle size (Macherey–Nagel, Düren, Germany); (II) Nova-Pak C_{18} (150 × 3.9 mm I.D.), 4-μm particle size (Waters Chromatography); (III) LiChrospher RP-18 C_{18} (125 × 4 mm I.D.), 5-μm particle size (Merck); and (IV) Vydac 218TP54 C_{18} (250 × 4.6 mm I.D.), 5-μm particle size (Separations Group, Hesperia, CA, USA). For semi-preparative separation, Vydac 218TP1010 C_{18} (250 × 10 mm I.D.), 10-μm particle size, and Vydac 218TP101522 C_{18} (250 × 22 mm I.D.), 10–15-μm particle size, columns (Separations Group) were used.

Quantitative amino acid analysis was performed on an HP 1090 Amino Quant amino acid analyser (Hewlett-Packard). The column used was Hypersyl ODS C_{18} (200 × 2 mm I.D.), 5-μm particle size (Shandon Scientific, Runcorn, Cheshire, UK).

^{1}H NMR spectra were measured on a Bruker (Zug, Switzerland) AM 400 spectrometer.

FAB mass spectra were recorded on a MS 902S double-focussing spectrometer (AEI Scientific Apparatus Division, Manchester, UK) with xenon at 8 kV as bombarding gas.

2.3. Peptide hydrolysis

The deltorphin C and enkephalin analogues containing different stereoisomers of β-MePhe in positions 3 and 4 were hydrolysed under argon pressure in 6 *M* HCl in PTFE bombs in a microwave oven [9]. The solvent was removed by flushing with argon. The dry samples were used for different types of derivatization.

2.4. Enzymatic assay of β-MePhe

A 1-mg amount of *erythro*-D,L- or *threo*-D,L-β-MePhe was dissolved in 0.1 *M* Tris buffer (pH 7.2) in a test-tube and 10 μl of L-amino acid oxidase were added. The test-tube was filled with oxygen, tightly capped and incubated for 24 h at 37°C. The reaction product was used for derivatization reactions.

2.5. Derivatization of β-MePhe

A 0.5–1-mg amount of *erythro*-D,L- or *threo*-D,L-β-MePhe was derivatized with FDAA by the method of Marfey [10].

3. Results and discussion

It was reported previously [8] that D,L-*erythro*- and D,L-*threo*-β-MePhe are not separated on an RP-HPLC column. Only the derivatized *erythro* and *threo* forms could be separated in a conventional buffer–organic modifier system. We now find that with pure water as eluent, the *erythro* and *threo* forms can be separated very well. A better peak shape (more symmetrical peaks) can be achieved if the mobile phase contains 0.1–0.5% of acetonitrile as organic modifier in water (Fig. 2). This finding leads to the separation of D,L-*erythro*- and D,L-*threo*-β-MePhe within a short time on a preparative scale, instead of the very time-consuming crystallization procedure. These pure amino acids were incorporated into [β-MePhe^4]enkephalin and [β-MePhe^3]deltorphin C. The crude peptides were purified by gel-filtration chromatography (Sephadex G-10) with 30% aqueous acetic acid as eluent. Final separation of the diastereoisomers and purification of the lyophilized solid were achieved by semi-preparative RP-HPLC based on one of the analytical methods mentioned below. The analytical separations were carried out on four different reversed-phase columns, in two different buffer systems: a 0.1% aqueous solution of trifluoroacetic acid (TFA) or a 0.01 *M* aqueous

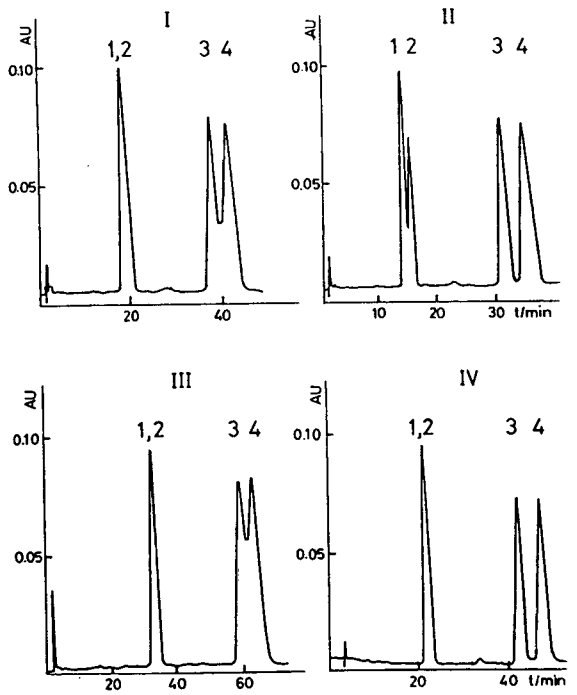


Fig. 3. Chromatograms of four diastereoisomers of [β-MePhe^3]deltorphin C. Column, (I) Nucelosil 10 C_{18}, (II) Nova Pak C_{18}, (III) LiChrospher RP C_{18} and (IV) Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; mobile phase, 0.1% TFA–acetonitrile (80:20); detection at 210 nm. Peaks, 1 = D-*threo*; 2 = D-*erythro*; 3 = L-*threo*; 4 = L-*erythro*.

Table 1
Retention factors (k') and resolutions (R_s) of four diastereoisomers of [β-MePhe^3]deltorphin on different columns

Column	Eluent composition (TFA–CH_3CN)	k'				R_s		
		D-*threo*	D-*erythro*	L-*threo*	L-*erythro*	D–D	D–L	L–L
I	80:20	5.15	7.31	19.08	21.65	3.11	9.56	1.39
II	80:20	4.21	6.14	16.29	18.07	3.00	9.26	1.13
III	80:20	4.28	6.14	16.29	18.08	3.06	9.16	1.18
IV	80:20	3.72	5.04	12.78	14.64	3.20	12.67	2.05

Column: (I) Nucleosil 10 C_{18}; (II) Nova Pak C_{18}; (III) LiChrospher RP C_{18}; (IV) Vydac 218TP54 C_{18}. TFA = 0.1% aqueous solution of trifluoroacetic acid; Flow-rate, 0.8 ml/min; detection at 210 nm. $R_{s,D-D}$ represents the separation of D-*threo*- and D-*erythro*-[β-MePhe^3]deltorphin C isomers; $R_{s,D-L}$ represents the separation of D-*erythro*- and L-*threo*-[β-MePhe^3]deltorphin C isomers; $R_{s,L-L}$ represents the separation of L-*threo*- and L-*erythro*-[β-MePhe^3]deltorphin C isomers.

solution of potassium dihydrogenphosphate (pH 3). The organic modifier was acetonitrile or methanol.

3.1. Separation of diasteroisomers of [β-MePhe^3]deltorphin C

The chromatographic behaviour of the four pure diastereoisomers of [β-MePhe^3]deltorphin C was studied by utilizing four different columns with different mobile phases. The results are given in Table 1 and Fig. 3.

On variation of the organic content of the mobile phase in the range 80–20% at 80% organic modifier content, only two peaks were observed and, on decreasing of the acetonitrile content to 20%, the four diastereoisomers could be separated well. With respect to the four columns used, there was no difference in separation capability, but the same separation on columns II and III could be achieved within a shorter time. With respect to the effects of the buffers and organic modifiers, the 0.1% TFA–acetonitrile system seemed most useful; application of phosphate buffer or methanol did not improve the separation. After peaks 1, 2, 3 and 4 had been collected, lyophilized and hydrolysed, the composition of the amino acids in the samples was determined by amino acid analysis. The configuration of the β-MePhe in the four peptides was determined by enzymatic assay with L-amino acid oxidase. For this reason, D,L-*erythro*-, D,L-*threo*-β-MePhe and the four hydrolysed peptides were digested with L-amino acid oxidase. The reaction products were derivatized with FDAA and analysed by RP-HPLC [8].

This procedure revealed that the first peak in Fig. 3 corresponds to [*threo*-D-MePhe^3]deltorphin C, the second to [*erythro*-D-β-MePhe^3]deltorphin C, the third to [*threo*-L-β-MePhe^3]deltorphin C and the fourth to [*erythro*-L-β-MePhe^3]deltorphin C. From the chromatograms, it is clear that the peptide diastereoisomers containing the D-form of β-MePhe can be separated very well from the L-forms, but that the separation of the *erythro* and *threo* forms is more critical.

3.2. Separation of diastereoisomers of the [β-MePhe^4]enkephalin analogue

The optimum mobile phase compositions for the separation of the four isomers of [β-

Table 2
Retention factors (k') and resolutions (R_s) of four diastereoisomers of [β-MePhe^4]enkephalin with different eluent systems and columns

Column	Eluent composition	k'				R_s		
		L-*threo*	L-*erythro*	D-*threo*	D-*erythro*	L–L	L–D	D–D
	TFA–CH_3CN							
I	80:20	6.70	6.83	18.62	19.62	<0.4	10.02	0.53
II	80:20	4.79	4.79	15.21	16.00	–	8.59	<0.40
III	80:20	9.64	10.07	19.00	20.00	0.55	9.62	0.93
IV	85:15	8.30	8.74	22.20	22.70	0.98	17.95	<0.40
	TFA–CH_3OH							
I	60:40	10.04	10.25	21.36	23.42	<0.4	6.23	0.87
II	60:40	8.72	9.58	20.08	22.50	1.10	10.24	1.12
III	65:35	13.09	13.09	24.46	26.27	–	5.68	0.59
IV	65:35	9.95	9.95	19.95	22.43	–	8.69	1.55

Column: (I) Nucleosil 10 C_{18}; (II) Nova Pak C_{18}; (III) LiChrospher RP C_{18}; (IV) Vydac 218TP54 C_{18}. TFA = 0.1% aqueous solution of trifluoroacetic acid; Flow-rate, 0.8 ml/min; detection at 210 nm. $R_{s,L-L}$ represents the separation of L-*threo*- and L-*erythro*-[β-MePhe^4]enkephalin isomers; $R_{s,L-D}$ represents the separation of L-*erythro*- and D-*threo*-[β-MePhe^4]enkephalin isomers; $R_{s,D-D}$ represents the separation of D-*threo*- and D-*erythro*-[β-MePhe^4]enkephalin isomers.

MePhe[4]]enkephalin are given in Table 2 and Fig. 4. The complete separation of all four isomers in one chromatogram poses considerable difficulties. The procedure for the identification of the isomers is the same as described earlier for [β-MePhe[3]]deltorphin C. The elution sequence for the four diastereomers was L-*threo*, L-*erythro*, D-*threo*, D-*erythro*, i.e. opposite to that observed for [β-MePhe[3]]deltorphin C. The 0.1% TFA–acetonitrile system is suitable for separation of the L- and D-forms, while the separation of the L-*threo* from L-*erythro* and D-*threo* from D-*erythro* forms is completely or partially unsuccessful. Column III seems suitable for the separation of the D-*threo* and D-*erythro* forms while column IV gives the best separation of the L-*threo* and L-*erythro* forms. A better separation can be achieved if the mobile phase contains methanol instead of acetonitrile. On column II, all four isomers are detectable although the resolution of the L-*threo* from the L-*erythro* form is not complete. On changing the buffer system from TFA to phosphate, the results for the separation were worse for any mobile phase composition, except 0.01 *M* phosphate buffer (pH 3)–methanol (60:40), in which the D-*threo* and D-*erythro* forms could be separated with $R_s > 1.3$ within 20 min. It should be mentioned that many variations of gradient elution were tested, but without any considerable improvement in resolution.

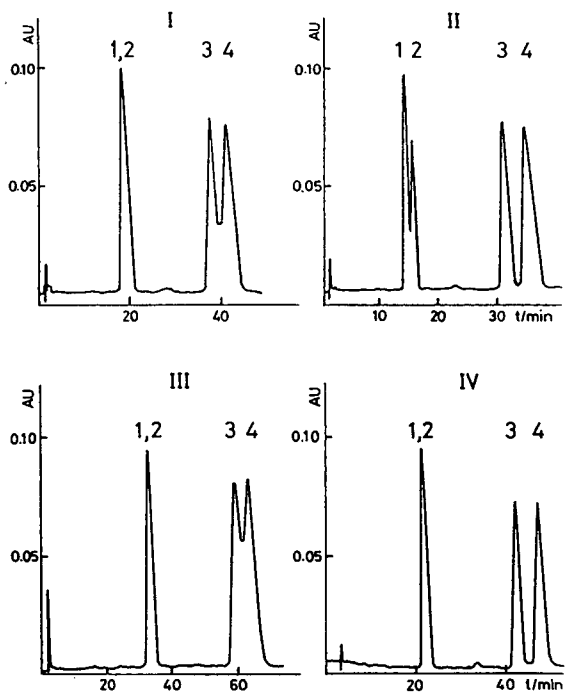


Fig. 4. Chromatogram of four diastereoisomers of [β-MePhe[4]]enkephalin. Column and mobile phase, (I) Nucleosil 10 C_{18}, 0.1% TFA–methanol (60:40), (II) Nova Pak C_{18}, 0.1 TFA–methanol (60:40), (III) LiChrospher RP C_{18}, 0.1% TFA–methanol (65:35) and (IV) Vydac 218TP54 C_{18}, 0.1% TFA–methanol (65:35); flow-rate, 0.8 ml/min; detection at 210 nm. Peaks, 1 = L-*threo*; 2 = L-*erythro*; 3 = D-*threo*; 4 = D-*erythro*.

An important feature of the interaction of peptides and receptors is the three-dimensional structure of the peptide ligand [11]. The structure depends on the amino acid composition, their sequence, their configuration, their side-chain conformation in the peptide and the backbone conformation of the peptide. In the opioid peptides, the distance between tyrosine and phenylalanine is an important factor. The incorporation of one of the four isomers of β-MePhe instead of phenylalanine may result in optimization of this distance, thereby affecting the biological properties, and also the chromatographic behaviour. In the [β-MePhe[4]]enkephalin analogues, the tyrosine and β-MePhe are in the 1,4-positions and the elution sequence is L before D. A similar elution sequence can be observed for H-Tyr-D-Pen-Gly-β-MePhe-D-Pen-OH (Pen = penicillamine) in the TFA–acetonitrile system [1,5]. In [β-MePhe[3]]deltorphin C, the 1,3-positions of tyrosine and β-MePhe leads to a different elution sequences; the first pair of peaks correspond to the D-forms and the L-forms are eluted as the third and fourth peaks. A general prediction of the elution sequence is therefore not possible.

4. Conclusions

Our results demonstrate that the RP-HPLC separation of L- from D-β-MePhe-containing peptides is especially good. However, separation of the *threo* from the *erythro* isomers (epimers at the β-carbon) may be difficult. Therefore, a

synthetic strategy which uses racemic *threo-* or *erythro-β*-MePhe for incorporation in peptides is acceptable in view of the excellent separation of the L- and D-stereoisomers. However, particular attention should be paid to the diastereomeric purity of the *threo* or *erythro* amino acids, as a small amount of the *β*-carbon epimers (e.g., L-*threo* in L-*erythro*) in the final peptide may be difficult to remove.

Acknowledgement

This work was supported by grants 71/91 and 2993/91 from the Hungarian Research Foundation (OTKA).

References

[1] V.J. Hruby, A. Kawasaki and G. Tóth, in CRC Handbook of HPLC of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991, p. 379.
[2] G. Tóth, A. Péter, D. Tourwé, H. Jaspers, P.M.F. Verheyden and G. Tóth, in Peptides 1994, Proceedings of the 23rd European Peptide Symposium, in press.
[3] Y.I. Kataoka, A. Seto, M. Yamamoto, T. Yamads, S. Kuwata and H. Watanabe, *Bull Chem. Soc. Jpn.*, 49 (1976) 1081.
[4] G. Tsuchihashi, S. Mitamura and K. Ogura, *Bull. Chem. Soc. Jpn.*, 52 (1979) 2167.
[5] V.J. Hruby, G. Tóth, C.A. Gehrig, L.-F. Kao, R. Knapp, G.K. Lui, H.I. Yamamura, T.H. Kramer, P. Davis and T.F. Burks, *J. Med. Chem.* 34 (1991) 1823.
[6] A. Misicka, S. Cavagnero, R. Horváth, P. Davis, T.H. Kramer, H.I. Yamamura and V.J. Hruby, Pept. Chem., 1992, Proc. 2nd Jpn. Symp., 1992, p. 378.
[7] J.P. Greenstein and M. Winitz, The Chemistry of Amino Acids, Wiley, New York, 1961, p. 2065.
[8] A. Péter, G. Tóth, E. Cserpan and D. Tourwé, J. Chromatogr. A, 660 (1994) 283.
[9] A. Péter, G. Laus, D. Tourwé, E. Gerlo and G. Van Binst, Pept. Res., 6 (1993) 48.
[10] P. Marfey, Carlsberg Res. Commun., 49 (1984) 591.
[11] V.J. Hruby, F. Al-Obeidi, W. Kazmierski, Biochem. J., 268 (1990) 249.

ELSEVIER

Journal of Chromatography A, 705 (1995) 275–287

JOURNAL OF CHROMATOGRAPHY A

Enantiomeric separation of basic drugs using N-benzyloxycarbonylglyclyl-L-proline as counter ion in methanol

Ngoc-Hang Huynh[a,*], Anders Karlsson[b], Curt Pettersson[a]

[a]*Analytical Pharmaceutical Chemistry, Uppsala University, Biomedical Centre, Box 574, S-751 23 Uppsala, Sweden*

[b]*Product Analysis, Astra Hässle AB, S-431 83 Mölndal, Sweden*

First received 1 December 1994; revised manuscript received 28 February 1995; accepted 3 March 1995

Abstract

Direct separation of enantiomeric amines using mainly N-benzyloxycarbonylglycyl-L-proline (L-ZGP) but also N-benzyloxycarbonylglyclglycl-L-proline (L-ZGGP) as the chiral counter ion in methanol is described. The solid phase was Hypercarb porous graphitic carbon. Several amines of pharmacological interest (e.g., alprenolol, sotalol, terbutaline, promethazine and trimipramine) were separated with high enantioselectivity ($\alpha = 1.16–1.98$) using L-ZGP and L-ZGGP as chiral selectors. In accordance with ion-pair chromatography, the retention of the enantiomeric amines was found to increase with increasing concentration of the anionic form of L-ZGP. Addition of a base (sodium hydroxide or an alkylamine) in excess of L-ZGP gave rise to a decrease in retention and enantioselectivity. The enantioselective retention was also affected by adding 2-propanol or acetonitrile to the mobile phase.

1. Introduction

Chiral ion-pair chromatography was first reported in the late 1970s. Yoneda [1] used chiral counter ions in aqueous mobile phases for the resolution of optically active metal complexes. A chiral zwitterion (L-leucyl-L-leucyl-L-leucine) in a phosphate buffer was used as eluent by Knox and Jurand [2] for the separation of racemic tryptophan and glycylphenylalanine. A chiral counter ion present in an organic mobile phase promotes a high degree of ion-pair formation and has been used successfully for the chiral separation of amines and acids [3]. (+)-10-Camphorsulphonic acid and N-benzyloxycarbonylglycyl-L-proline (L-ZGP) as counter ions in dichloromethane permitted chiral separations of, e.g., amino alcohols [4–6]. Enantioselective retention of carboxylic acids and "N-blocked amino acids" has been obtained by addition of quinine and analogues as chiral counter ions to dichloromethane [7]. Modified silica supports were used as the achiral solid phase. Introduction of porous graphitic carbon as the solid phase made it possible to apply the chiral counter ions L-ZGP [8,9] and (−)-2, 3:4,6-di-O-isopropylidene-2-keto-L-gulonic acid [10] in polar mobile phases (e.g., methanol) for the separation of enantiomeric amines. The possibility of excluding hazardous organic solvents has great

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00302-9

importance in routine analyses. Further, exchanging dichloromethane for methanol or methanol–water mixtures allows the direct injection of biological fluids and facilitates integration of the chiral separation system in coupled-column chromatography for bioanalysis.

The aim of this study was to extend the earlier investigations on L-ZGP in methanol for chiral separations of amines. The influence of the mobile phase composition (total concentration of L-ZGP, concentration of anionic form of L-ZGP, addition of alkylamines and organic modifiers) on the retention and enantioselectivity was studied in detail. A model for the retention of the enantiomers based on the distribution of diastereomeric ion pairs to the solid phase is proposed.

The chiral separation of several amines of pharmacological interest, e.g., β-blocking agents and α-adrenoreceptor stimulating agents, is presented in order to illustrate the potential of L-ZGP as a chiral selector in methanol.

2. Experimental

2.1. Apparatus

The pumps used were a Model 114 M (Beckman Instruments, Fullerton, CA, USA) and a ConstaMetric III (LDC, Riviera Beach, FL, USA). A SpectroMonitor 3100 detector (Milton Roy, Riviera Beach, FL, USA) was set at 270 nm. A Rheodyne (Berkeley, CA USA) Model 7120 injector with a 20-μl loop was used. The column and the solvent reservoir were kept at 17.00 ± 0.1°C using a type 02 PT 923 TC water-bath thermostat (HETO, Birkerød, Denmark).

2.2. Chemicals

All chemicals were of analytical-reagent grade unless indicated otherwise, and were used without further purification. Methanol, 2-propanol and acetonitrile were obtained from Merck (Darmstadt, Germany), sodium hydroxide pellets from Eka Nobel (Surta, Sweden), triethylamine (TEA) and dimethyloctylamine (DMOA) (95% purity) from Janssen (Beerse, Belgium), tributylamine (TBA) from Fluka (Buchs, Switzerland), tripentylamine (TPA) and trihexylamine (THA) (golden label) from Eastman Kodak (New York, USA), N-benzyloxycarbonylglycyl-L-proline from Nova Biochem (Leufärlfingen, Switzerland) and N-benzyloxycarbonylglycylglycyl-L-proline from Sigma (St. Louis, MO, USA). The solutes used are listed in Table 1.

The Hypercarb column (100×4.7 mm I.D., 7 μm) was purchased form Shandon (Astmoor, UK).

2.3. Chromatographic technique

The mobile phase was prepared by dissolving the counter ion, L-ZGP or L-ZGGP (Fig. 1), in methanol. The base, sodium hydroxide or an alkylamine, was added to the mobile phase using a 50 m*M* stock standard solution in methanol.

The column was washed with 250 ml of pure methanol before introducing a new mobile phase. After the breakthrough of the counter ion, as recorded by the UV trace, the mobile phase was recirculated. Less than $2 \cdot 10^{-5}$ mol of L-ZGP was adsorbed on ca. 1 g of the solid phase, Hypercarb.

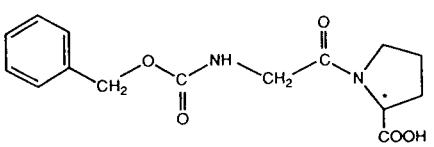
Stock standard solutions were prepared by dissolving the solutes in methanol. Before injection, these solutions were diluted at least tenfold with the mobile phase, giving a solute concentration of 0.2 mmol/l.

3. Results and discussion

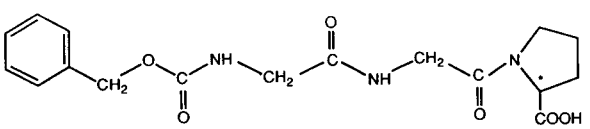
Previous studies of the separation of enantiomeric amines and acids by the ion-pair technique have been based on the addition of a chiral acid or an amine to an organic mobile phase of low polarity [3]. The acid–base interaction (Eq. 1) in inert solvents of low polarity gives rise to the formation of ion pairs or "hydrogen bonded ion pairs" [11]. The chromatographic resolution of the enantiomers is due to differences in ion-pair formation in the organic phase or distribution of the diastereomeric ion pairs between the organic

Table 1
Solutes studied

Entry No.	Compound	Source
1	(*R*,*S*)-Alprenolol · HCl	Astra Hässle
2	(*R*,*S*)-1-(2-Allyl-4-hydroxyphenoxy)-3-isopropyl-amino-2-propanol(*p*-hydroxy alprenolol)	Astra Hässle
3	(*R*,*S*)-Oxprenolol	Astra Hässle
4	(*R*,*S*)-1-(4-Allyloxyphenoxy)-3-isopropylamino-2-propanol (*p*-Oxprenolol)	Astra Hässle
5	(*R*,*S*)-Metoprolol	Astra Hässle
6	(*R*,*S*)-Atenolol	Astra Hässle
7	Pafenolol	Astra Hässle
8	(*R*,*S*)-Sotalol	Bristol Meyers Squibb
9	(*R*,*S*)-Isoprenaline	Apotek Bolaget
10	(*R*,*S*)-Terbutaline	Astra Draco
11	(*R*,*S*)-Salbutamol	Glaxo
12	(*R*,*S*)-Mepivacaine	Astra Pain Control
13	(*R*,*S*)-Ropivacaine	Astra Pain Control
14	(*R*,*S*)-Bupivacaine	Astra Pain Control
15	(*R*,*S*)-Promethazine	Kabi Pharmacia
16	(*R*,*S*)-N-Hydroxyethylpromethazine	Kabi Pharmacia
17	(*R*,*S*)-Trimipramine	Rhône-Poulenc Rorer
18	(*R*)-Alprenolol · HCl	Astra Hässle
19	(*S*)-Alprenolol · HCl	Astra Hässle
20	(*R*)-Sotalol	Bristol Meyers Squibb
21	(*S*)-Sotalol	Bristol Meyers Squibb
22	(*R*)-Terbutaline	Astra Draco
23	(*S*)-Terbutaline	Astra Draco



N-Benzyloxycarbonyl-glycyl-*L*-proline (*L*-ZGP)



N-Benzyloxycarbonyl-glycyl-glycyl-*L*-proline (*L*-ZGGP)

Fig. 1. Structures of counter ions.

mobile phase (org) and the adsorbing stationary phase (stat):

$$(R)\text{-HA}_{org} + (S,R)\text{-B}_{org} \Leftrightarrow (R)\text{-A}^{-}\text{:}(S)\text{–HB}^{+}_{org},$$
$$(R)\text{-A}^{-}\text{:}(R)\text{–HB}^{+}_{org} \Leftrightarrow (R)\text{-A}^{-}\text{:}(S)\text{–HB}^{+}_{stat},$$
$$(R)\text{-A}^{-}\text{:}(R)\text{–HB}^{+}_{stat} \quad (1)$$

where (*R*)-HA is a chiral acid, (*S*, *R*)-B is the enantiomeric pair of an amine and (*R*)-A^{-}:(*S*)-HB^{+} and (*R*)-A^{-}:(*R*)-HB^{+} are the corresponding diastereomeric ion pairs between the chiral acid and the (*S*)- and (*R*)-enantiomers of the amine, respectively.

Organic mobile phases of low polarity promote a high degree of ion-pair formation, but may give rise to disturbing side-reactions (association of the ion pairs). Thus, achiral and also several competing enantioselective equilibria

may decrease or even ruin the enantioselective separation [3]. Chiral ion-pair chromatography in polar mobile phases with medium or high dielectric constants, e.g., methanol, has a great advantage as unfavourable association processes of the diastereomeric ion pairs and their components are less pronounced in these solvent. Mayer discussed, in a review [12], the solvent effects on ion-pair equilibria. Obviously, the ion association behaviour of ionic species in many solvent systems cannot be adequately explained by the electrostatic model (electrostatic interaction, solvent's dielectric constant). Methanol, which has strong electrophilic properties, can solvate the nucleophilic ions by hydrogen bonding. The free energy of solvation depends also on the ion size and influences the ion-pair association constant.

In methanol, acids and bases are partly dissociated and the amount of charged protolytes depends on the degree of protolysis. Coulombic attraction between the charged enantiomer of the amine and the anionic counter ion give rise to the formation of a neutral complex (ion pair) that can be selectively distributed to the solid phase:

$$(R)\text{-}A^-_{mob} + (S,R)\text{-}HB^+_{mob} \Leftrightarrow (R)\text{-}A^-\text{:}(S)\text{–}HB^+_{stat},\ (R)\text{-}A^-\text{:}(R)\text{–}HB^+_{stat} \quad (2)$$

3.1. Retention model

The ion pair and its components can be adsorbed on the surface of the solid support. The adsorption is reversible and the assumption is made that the surface has a limited capacity (K^0) of one type of adsorption sites (A_S) [13]:

$$K^0 = [A_S] + [\text{L-ZGPA}_S] + [(R)\text{-BA}_S] + [(S) - \text{BA}_S] + [\text{L-ZGP:}(R)\text{-HBA}_S] + [\text{L-ZGP:}(S)\text{-HBA}_S] \quad (3)$$

where $[A_S]$ are the available free adsorption sites, $[\text{L-ZGPA}_S]$ are sites occupied by free L-ZGP, $[(R)\text{-BA}_S]$ and $[(S)\text{-BA}_S]$ are sites occupied by the enantiomers of the solute and $[\text{L-ZGP:}(R)\text{-BA}_S]$ and $[\text{L-ZGP:}(S)\text{-BA}_S]$ are those occupied by their ion pairs with L-ZGP. The ion pair of L-ZGP$^-$ and sodium ion is not included in the K^0 expression in order to simplify the retention model. Since an increase in sodium hydroxide concentration gave an increase retention time, it was reasonable to believe that the ion-pair formation between sodium ion and L-ZGP$^-$ was small and did not affect the retention of the enantiomeric amines to a great extent.

The adsorption equilibria for the retention of the ion pairs and the uncharged form of the acid and amine is given by

$$\text{L-ZGP}_{mob} + A_S \overset{K_{\text{L-ZGP}}}{\Longleftrightarrow} \text{L-ZGPA}_S \quad (4)$$

$$(R)\text{-B}_{mob} + A_s \overset{K_{(R)\text{-B}}}{\Longleftrightarrow} (R)\text{-BA}_S \quad (5)$$

$$\text{L-ZGP}^-_{mob} + (R)\text{-HB}^+_{mob} \overset{K_{\text{L-ZGP:}(R)\text{-HB}}}{\Longleftrightarrow} \text{L-ZGP}^-\text{:}(R)\text{-HB}^+A_S \quad (6)$$

The chiral selector, L-ZGP, is added in uncharged form. The pK_a value of L-ZGP in methanol was estimated to be 7–9 [14], which should result in negligible protolysis of L-ZGP. Ionization of L-ZGP was obtained by neutralization with sodium hydroxide. Thus, the concentration of the charged form of the selector, i.e., the counter ion (L-ZGP$^-$), is equal to the concentration of sodium hydroxide added to the mobile phase. The free uncharged L-ZGP is consequently the total concentration of L-ZGP in the mobile phase minus the added concentration of sodium hydroxide.

When the solute concentration is so low that $[(R)\text{-BA}_S] + [(S)\text{-BA}_S] + [\text{L-ZGP:}(R)\text{-HBA}_S] + [\text{L-ZGP:}(S)\text{-HBA}_S] \ll [A_S] + [\text{L-ZGPA}_S]$, the adsorption isotherm is linear and the capacity factor for the enantiomers is given by

$$k' = \frac{qK_{\text{L-ZGP:}(R)\text{-HB}}K^0[\text{L-ZGP}^-]_{mob}}{1 + K_{\text{L-ZGP}}[\text{L-ZGP}]_{mob}} \quad (7)$$

The retention can be controlled by the counter ion (L-ZGP$^-$) concentration and the concentration of uncharged acid (L-ZGP).

The enantiomers of alprenolol showed low retention times ($k' = 0.5$) and no enantioselectivity when using an acidic mobile phase pre-

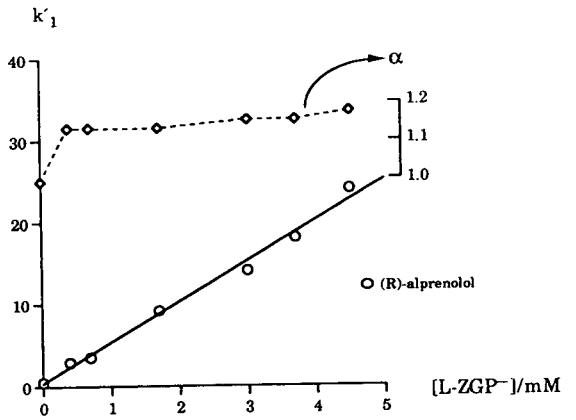


Fig. 2. Enantioretention of (*R*)- and (*S*)-alprenolol related to the anionic form of counter-ion concentration. Solid phase: Hypercarb. Mobile phase: 5 m*M* L-ZGP and *X* m*M* sodium hydroxide in methanol ($0.4 \leq X \leq 4.5$).

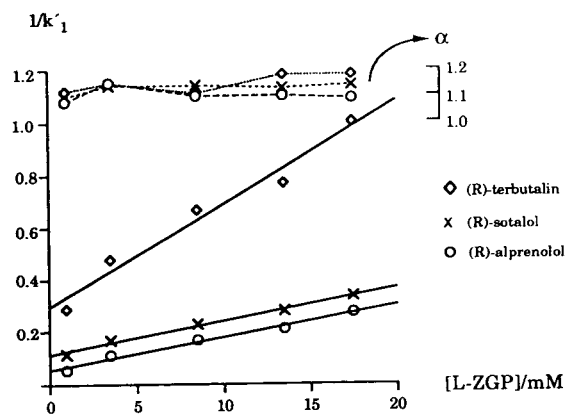


Fig. 3. Influences of the uncharged concentration of counter ion on the enantioretention of amines. Solid phase: Hypercarb. Mobile phase: *X* m*M* L-ZGP and 1.5 m*M* sodium hydroxide in methanol ($2.5 \leq X \leq 19$).

pared by dissolving L-ZGP in methanol (Fig. 2). Clearly, the counter ion concentration generated by the protolysis of L-ZGP was too low to promote enantioselective ion-pair retention. The linear increase in capacity factors (Fig. 2) with increase in the amount of sodium hydroxide in the mobile phase, i.e., increasing concentration of the charged form of the selector, L-ZGP$^-$, is in accordance with ion-pair chromatographic principle (Eq. 7).

A competition for the limited adsorption capacity of the stationary phase by the diastereomeric ion pairs and the uncharged selector, L-ZGP, would give rise to reduced retention for the enantiomeric amines. Inversion of Eq. 7 gave a linear relationship on plotting the inverse of the capacity factor ($1/k'$) versus the concentration of the uncharged form of L-ZGP (Fig. 3). Values for the adsorption constant of the uncharged L-ZGP can be obtained, since the ratio between the slope and intercept is equal to $K_{\text{L-ZGP}}$. The adsorption constants for L-ZGP obtained from the plots for terbutaline and sotalol were in a good agreement, 7.5 and 8.7, respectively. The lower value of $K_{\text{L-ZGP}}$, 4.5, estimated from the plot for alprenolol is probably due to a large uncertainty in the low intercept.

Adjustment of the retention times for the enantiomers by the concentration of either the counter ion (L-ZGP$^-$) or the uncharged acid (L-ZGP) is uncomplicated as it does not affect the enantioselectivity to a significant extent (Figs. 2 and 3).

3.2. Mobile phase amine

An amine is often added to the mobile phase in order to reduce retention times and improve the peak shapes when chromatographing amines on silica-based solid phases [15]. It is generally believed that the improved peak symmetry is due to deactivation of unfavourable adsorption sites on the solid phase by the mobile phase amine [15]. A beneficial effect on the peak symmetry in the presence of a mobile phase amine has also been observed when using porous graphitic carbon, Hypercarb, as the adsorbing phase [8]. Hence, it was of interest to exchange sodium hydroxide for an alkylamine in the mobile phase in order to improve the peak shape of enantiomeric amines. The acid–base reaction between L-ZGP and the alkylamine will promote the necessary counter ion, L-ZGP$^-$. However, the presence of the alkylamine will shorten the retention time and the denominator of the k' expression will be expanded to $1 + K_{\text{L-ZGP}}\text{-}[\text{L-ZGP}^-] + K_{\text{Am}^+\text{L-ZGP}}\text{-}[\text{Am}^+][\text{L-ZGP}^-]$.

Table 2
Influence of alkylamines on enantioselective retention and peak symmetry

Solute	NaOH			Alkylamine													
				TEA			TBA			TPA			THA			DMOA		
	k'_1	a	*asf*	k'_1	a	*asf*	k'_1	a	*asf*	k'_1	a	*asf*	k'_1	a	*asf*	k'_1	a	*asf*
Alprenolol	17	1.10	6.5	14	1.11	5.0	13	1.12	5.5	11	1.14	3.6	7.2	1.14	3.1	9.7	1.16	2.7
Sotalol	6.8	1.10	5.0	7.5	1.11	3.2	7.0	1.12	3.8	6.6	1.14	3.5	4.5	1.14	3.2	5.6	1.11	2.9
Terbutaline	2.4	1.15	2.8	2.7	1.14	1.6	2.7	1.14	3.3	2.5	1.14	2.5	1.6	1.16	1.7	2.0	1.15	1.8

Solid phase: Hypercarb. Mobile phase: 2.5 m*M* L-ZGP and 1.5 m*M* sodium hydroxide or alkylamine in methanol. TEA = triethylamine; TBA = tributylamine; TPA = tripentylamine; THA = trihexylamine; DMOA = dimethyloctylamine; asf = asymmetry factor of the first-eluted enantiomer measured on the baseline.

$K_{Am^+L\text{-}ZGP^-}$ represents the formation constant of the ion pair between the alkylammonium ion (Am^+) and L-ZGP$^-$. The lowest retention of the enantiomeric amines was obtained when trihexylamine (THA) or dimethyloctylamine (DMOA) was added to the mobile phase (Table 2). It is reasonable to assume that the more hydrophobic amines have a higher affinity for the porous graphitic carbon phase and are more effective as competing agents for the limited adsorption capacity of the solid phase. Unfortunately, the improvement in peak shape was modest even for the hydrophobic mobile phase amines THA and DMOA.

The effect of the concentration of the mobile phase amine on the chiral separation was investigated using triethylamine in a solution of 2.5 m*M* L-ZGP in methanol [Table 3(a)]. The retention of the enantiomeric amines was highest when using a triethylamine concentration that was about the same as that of the chiral selector. As discussed above, a low concentration of the mobile phase amine will not neutralize the selector to a sufficient extent, and accordingly the counter ion concentration will be too low. However, a large excess of the mobile phase amine should be avoided as it will result in an alkaline solution where the enantiomeric amines are mainly present as uncharged amines. A decrease in capacity factor was observed at 6.0 m*M* or higher of triethylamine in the mobile phase, indicating that the free amine is less retained (Eq. 5) than the diastereomeric ion pairs (Eq. 6). The enantioselectivity decreased with increasing amounts of mobile phase amines as a result of the retention of the free enantiomeric amines being achiral [Table 3(b)].

3.3. Organic solvent

The elution power of the mobile phase, 5.0 m*M* L-ZGP and 3.0 m*M* sodium hydroxide in methanol, was modified by addition of a second organic solvent to the mobile phase. 2-Propanol and acetonitrile had a profound effect on the retention and enantioselectivity (Tables 4 and 5).

Table 3
Influence of triethylamine on enantioselective retention

Conditions	Solute	[TEA] (m*M*)									
		0.5		1.0		1.5		2.0		2.3	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α
(a) [TEA] < [L-ZGP]	Alprenolol	5.5	1.13	10	1.10	14	1.11	17	1.12	23	1.13
	Sotalol	3.4	1.12	5.5	1.12	7.5	1.11	9.2	1.11	10	1.10
	Trimipramine	11	1.18	18	1.19	24	1.20	29	1.20	33	1.19
	Terbutaline	1.3	1.11	2.0	1.13	2.7	1.14	3.3	1.16	3.8	1.15
	Ropivacaine	2.0	1.03	3.0	1.02	3.17	1.12	–	–	5.1	1.03
		[TEA] (m*M*)									
		3.0		6.0		9.0		18			
		k'_1	α	k'_1	α	k'_1	α	k'_1	α		
(b) [TEA] > [L-ZGP]	Alprenolol	23	1.09	14	1.08	12	1.07	8.8	1.05		
	Sotalol	8.3	1.10	5.6	1.06	4.5	1.07	3.4	1.05		
	Trimipramine	15	1.09	8.6	1.03	7.7	1.0	7.0	1.0		
	Terbutaline	3.7	1.15	2.6	1.16	2.1	1.11	1.6	1.09		
	Ropivacaine	4.9	1.0	4.8	1.0	4.7	1.0	4.5	1.0		

Solid phase: Hypercarb. Mobile phase: 2.5 m*M* L-ZGP and *X* m*M* TEA in methanol.

Table 4
Influence of 2-propanol on enantioselective retention

2-Propanol (%, v/v)	Solute							
	Alprenolol		Terbutaline		Trimipramine		Promethazine	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
0	14	1.15	3.3	1.19	22	1.21	70	1.28
20	–	–	2.4	1.13	17	1.29	–	–
40	11	1.13	2.6	1.18	14	1.38	45	1.44
60	12	1.11	3.8	1.18	11	1.58	37	1.58
80	33	1.02	12	1.13	9.4	1.76	33	1.78
94	–	–	–	–	6.0	1.98	21	2.02

Solid phase: Hypercarb. Mobile phase: 5.0 m*M* L-ZGP and 3.0 m*M* sodium hydroxide and *X*% (v/v) 2-propanol in methanol.

Table 5
Influence of acetonitrile on enantioselective retention

MeOH:AcN (v/v)	Solute							
	Alprenolol		Sotalol		Trimipramine		Terbutaline	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
100:0	17	1.10	6.8	1.10	28	1.16	2.4	1.15
50:50	13	1.07	5.1	1.14	9.9	1.21	3.4	1.21
20:80	33	1.0	16	1.09	15	1.29	16	1.21
6:94	19	1.14	17	1.10	4.1	1.27	64	1.03

Solid phase: Hypercarb. Mobile phase: 2.5 m*M* L-ZGP and 1.5 m*M* sodium hydroxide in methanol–acetonitrile.

The capacity factors for the hydrophobic tricyclic amines (trimipramine and promethazine) decreased whereas the enantioselectivity improved with increasing concentration of 2-propanol in the mobile phase. A different effect with the addition of 2-propanol was observed for the enantiomers of alprenolol and terbutaline. The capacity factor decreased with 20–40% 2-propanol in the mobile phase but increased at higher concentrations of the alcohol. The enantioselectivity for alprenolol and terbutaline deteriorated at 80% 2-propanol in the mobile phase.

There was no clear trend in the changes in retention and enantioselectivity on addition acetonitrile to the mobile phase (Table 5). Clearly, a high concentration of acetonitrile in the mobile phase is favourable for the enantioselectivity of alprenolol and trimipramine but not for the separation of (*R*,*S*)-terbutaline. The best separation of trimipramine and promethazine was observed at 94% acetonitrile in the mobile phase (Fig. 4), whereas the separation factor for sotalol seems less affected by the content of acetonitrile.

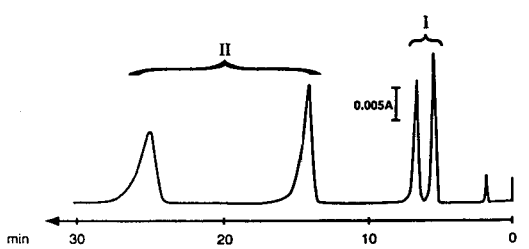


Fig. 4. Separation of racemic trimipramine (I) and promethazine (II). Solid phase: Hypercarb. Mobile phase: 2.5 m*M* L-ZGP and 1.5 m*M* sodium hydroxide in methanol–acetonitrile (6:94, v/v).

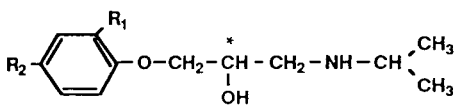
Further studies on this kind of separation system might improve the understanding of the resolution of enantiomeric ions by a chiral counter ion in mixed organic solvents as the mobile phase.

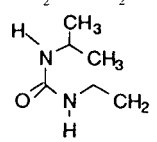
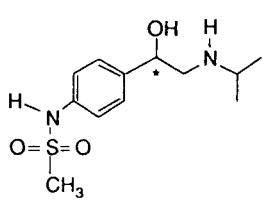
3.4. Structure and stereoselectivity

Previous studies using L-ZGP as chiral counter ion in dichloromethane have shown that intermolecular interactions (e.g., hydrogen bonding) between the counter ion and the enantiomeric solutes are of great importance for the enantioselectivity [8,16]. This study showed that the enantioselectivity for amino alcohols was significantly lower when using methanol ($\alpha = 1.08$–1.17) as mobile phase solvent than with dichloromethane ($\alpha = 1.20$–1.90] [8,16]. A detailed discussion of the effect of the organic solvent and the solute structure on the enantioselectivity is not possible at present. It would require a knowledge of the ion-pair formation constants and also the adsorption constants of the ion pair and the free amine of all solutes in both solvent systems. However, several enantiomeric amines with different hydrogen bonding properties were studied in order to establish the influence of molecular structure on the enantioselectivity when using L-ZGP as chiral counter ion in methanol (Tables 6–9).

Alprenolol and *p*-hydroxyalprenolol (**1** and **2**), lacking an O or an N atom in the *ortho* or *para* substituent in the aromatic ring, gave high enantioselectivies ($\alpha = 1.15$ and 1.17) in methanol as compared with the other β-blockers (Table 6). The retention of *p*-hydroxyalprenolol (**2**) as an ion pair with L-ZGP was significantly higher than that for alprenolol (**1**). The same retention order between alprenolol and *p*-hydroxyalprenolol was observed using alkaline methanol without L-ZGP as the mobile phase ($k'_{alp} = 8.2$, $k'_{p\text{-OH-alp}} = 11.0$), i.e. retention of the β-blockers as free amines. However, almost the same enantioselectivity was observed for alprenolol and *p*-hydroxyalprenolol

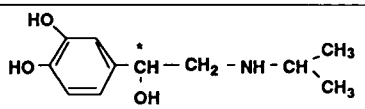
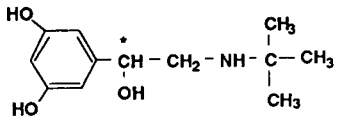
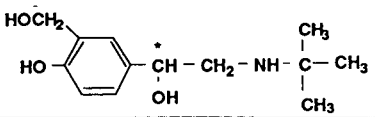
Table 6
Solute structures and enantioselective retention for β-receptor blocking agents



Entry no.	Solute	R_1	R_2	k'_1	α
1	Alprenolol	CH_2=$CHCH_2$	H	22	1.17
2	*p*-Hydroxyalprenolol	CH_2=$CHCH_2$	OH	38	1.15
3	Oxprenolol	CH_2=$CHCH_2O$	H	34	1.09
4	H 74/16	H	CH_2=$CHCH_2O$	50	1.10
5	Metoprolol	H	$CH_2OCH_2CH_2$	18	1.09
6	Atenolol	H	NH_2COCH_2	13	1.09
7	Pafenolol[a]	H		21	1.09
8	Sotalol		9.8	9.8	1.13

Solid phase: Hypercarb. Mobile phase: 5 m*M* L-ZGP and 4.5 m*M* NaOH in methanol.
[a] Mobile phase: 5 m*M* L-ZGP and 3 m*M* NaOH in methanol.

Table 7
Solute structures and enantioselective retention for bronchodilators

Entry No.	Solute	Structure	k'_1	α
9	Isoprenaline		3.9	1.0
10	Terbutaline		3.6	1.17
11	Salbutamol		3.2	1.09

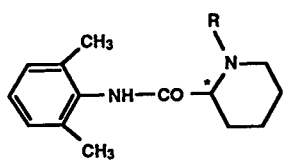
Solid phase: Hypercarb. Mobile phase: 5 m*M* L-ZGP and 4.5 m*M* NaOH in methanol.

(Table 6). Hence it is reasonable to assume that the *p*-hydroxyl group only affects the distribution properties of the diastereomeric ion pairs and is not involved in the interaction with the counter ion. This is also supported by the fact that the same enantioselectivity was obtained for compounds **4–7** with different hydrogen-bonding substituents in the *para* position to the alkanol amine chain. However, the structure of the substituent in the *ortho* position, i.e., closer to the chiral centre, had a significant effect on the enantioselectivity (compounds **1** and **3**).

Despite the low enantioselectivity obtained using L-ZGP in methanol as the mobile phase, it permitted the stereoselective determination of hydrophilic amino alcohols, e.g., sotalol (Table 6), terbutaline (Fig. 5) and salbutamol (Table 7). No enantioselective retention was found for the structurally related α-adrenoreceptor stimulating agent isoprenaline (Table 7).

The retention for enantiomeric local anaesthetics (**9–11**) (Table 8) could be correlated with the hydrophobicity (number of methylene groups) of the alkyl chain attached to the amine function in accordance with ion-pair retention theory [17], whereas the length of the alkyl chain did not affect the enantioselectivity. The slight decrease in separation factor for ropivacaine and

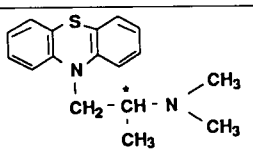
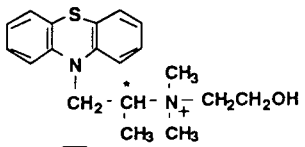
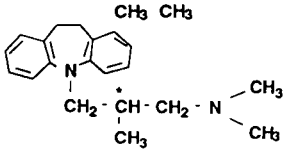
Table 8
Solute structures and enantioselective retention for local anaesthetics



Entry No.	Solute	R	k'_1	α
12	Mepivacaine	CH_3	2.32	1.11
13	Ropivacaine	$CH_2CH_2CH_3$	2.78	1.08
14	Bupivacaine	$CH_2CH_2CH_2CH_3$	3.09	1.08

Solid phase: Hypercarb. Mobile phase: 15 m*M* L-ZGP and 9 m*M* NaOH in methanol–2-propranol (90:10, v/v).

Table 9
Solute structures and enantioselective retention for other amines

Entry No.	Solute	Structure	k'_1	α
15	Promethazine		58	1.23
16	N-Hydroxyethyl promethazine		47	1.0
17	Trimipramine		21	1.19

Solid phase: Hypercarb. Mobile phase: 5 m*M* L-ZGP and 4.5 m*M* NaOH in methanol.

bupivacaine compared with mepivacaine is probably due to steric effects.

Polyaromatic tertiary amines were enantioselectively retained by L-ZGP (Table 9). The chiral separation of trimipramine was as good as that of promethazine; the former has one more methylene group between the asymmetrical carbon and the nitrogen. However, the retention time of promethazine, containing a sulphur atom with free electron pairs, was higher. Previous studies have shown that the retention on porous graphitic carbon with hexane in the mobile phase is sensitive to changes in the solute electron density caused by the electron-donating or -withdrawing ability of substituents and the number and position of the electrondense bonds in the solute [18].

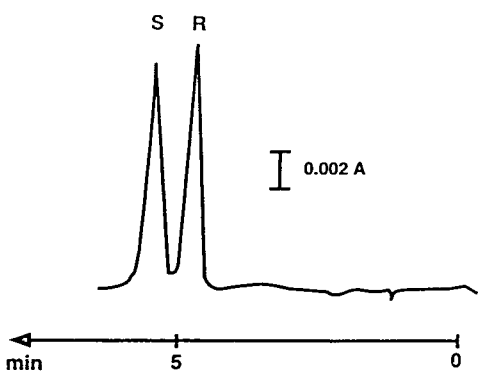


Fig. 5. Separation of racemic terbutaline. Solid phase: Hypercarb. Mobile phase: 5.0 m*M* L-ZGP and 3.0 m*M* sodium hydroxide in methanol.

The influence of the counter-ion structure on the stereoselective retention of β-receptor blocking agents and an α-adrenergic stimulation agent, terbutaline, is presented in Table 10. Exchange of L-ZGP for L-ZGGP resulted in additional hydrogen bonding functions in the counter ion (Fig. 1). Lower retention was observed for the enantiomeric amines when L-ZGGP was the chiral counter ion, probably owing to the lower distribution of the ion pair caused by stronger competition of the uncharged form of L-ZGGP. The effect on enantioselectivity of introducing a glycyl moiety into the counter ion is highly dependent on the solute structure. The use of L-ZGGP instead of L-ZGP gave improved enantioselectivity for metoprolol and pafenolol, whereas no change was found for alprenolol, atenolol and oxprenolol. Several β-blocking agents could be separated in one chromatographic system using L-ZGGP as counter ion, as shown in Fig. 6. The same counter ion was applied to the determination of enantiomeric impurity in (*S*)-alprenolol (Fig. 7). For sotalol

Table 10
Counter-ion structures and enantioselective retention

Entry No.	Solute	Counter ion			
		L-ZGP		L-ZGGP	
		k'_1	α	k'_1	α
5	Metoprolol	13	1.08	9.2	1.16
1	Alprenolol	14	1.15	8.8	1.16
6	Atenolol	9.1	1.08	5.8	1.10
2	Oxprenolol	23	1.08	14	1.09
7	Pafenolol	21	1.09	12	1.13
8	Sotalol	9.9	1.13	9.4	1.06
10	Terbutaline	2.8	1.21	2.6	1.07

Solid phase: Hypercarb. Mobile phase: 5 m*M* of the counter ion and 4.5 m*M* NaOH in methanol.

and terbutaline, with the aromatic ring bound directly to the asymmetric carbon atom, L-ZGP is the more suitable counter ion (Table 10).

4. Conclusion

N-Benzyloxycarbonylglycyl-L-proline and N-benzyloxycarbonylglycylglycyl-L-proline can be used as chiral counter ions in the reversed-phase mode with methanol-containing bases as mobile phase. A retention model based on ion-pair adsorption and competition for adsorption sites according to Langmuir isotherms is shown to be valid. The retention and enantioselectivity can mainly be controlled by the ratio of charged and uncharged chiral selector and the presence of a second organic solvent such as acetonitrile of 2-propanol. Many different chiral amine drugs belonging to several pharmacological groups, i.e., β-blocking agents, local anaesthetics, bronchodilators, antihistamines and antidepressants,

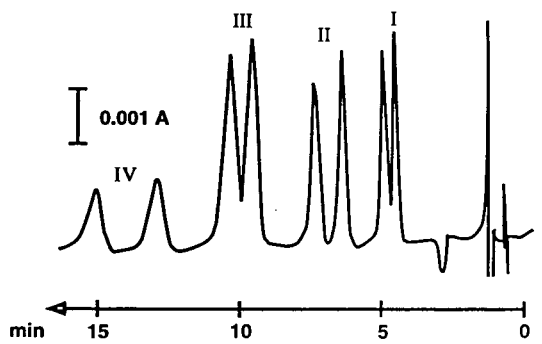


Fig. 6. Separation of β-blocking agents: I = (*R*)- and (*S*)-atenolol; II = (*R*)- and (*S*)-alprenolol; III = (*R*)- and (*S*)-oxprenolol; IV = (*R*)- and (*S*)-*p*-hydroxyalprenolol. Solid phase: Hypercarb. Mobile phase: 5.0 m*M* L-ZGGP and 3.0 m*M* sodium hydroxide in methanol.

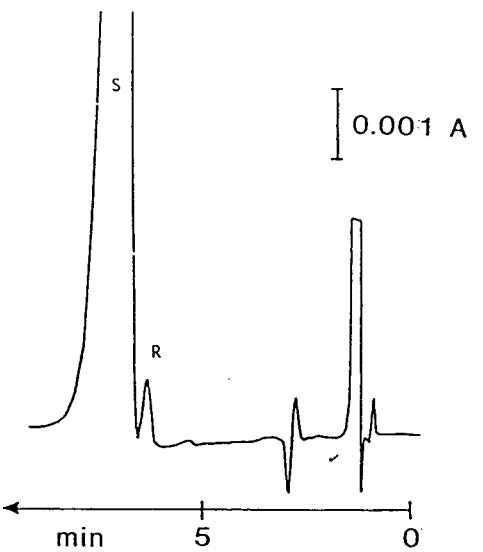


Fig. 7. Determination of enantiomeric impurity in (*S*)-alprenolol using L-ZGGP in methanol as mobile phase. Solid phase: Hypercarb. Mobile phase: 5.0 m*M* L-ZGGP and 3.0 m*M* sodium hydroxide in methanol. Solute: (*S*) and (*R*)-alprenolol (99:1).

can be separated. The relationship between chemical structure and enantioselectivity is complex and cannot easily be predicted.

Acknowledgements

We thank Professor Douglas Westerlund for valuable comments on the manuscript and Dr. Eva Heldin for stimulating discussions. This project was supported by the Swedish National Science Research Council. Research grants from the Swedish Academic of Pharmaceutical Science, the I,F. Foundation for Pharmaceutical Research and the Fredrika Bremer Foundation are gratefully acknowledged.

References

[1] H. Yoneda, J. Liq. Chromatogr., 2 (1979) 1157–1178.
[2] J.H. Knox and J. Jurand, J. Chromatogr., 234 (1982) 222.
[3] C. Pettersson and E. Heldin, in G. Subramanian (Editor), Ion-pair Chromatography in Enantiomer Separations, VCH, Weinheim, 1994, pp. 279–310.
[4] C. Pettersson and G. Schill, J. Chromatogr., 204 (1981) 179–183.
[5] C. Pettersson and M. Josefsson, Chromatographia, 21 (1986) 321–326.
[6] C. Pettersson, A. Karlsson and C. Gioeli, J. Chromatogr., 407 (1987) 217.
[7] C. Pettersson and K. No, J. Chromatogr., 282 (1983) 671.
[8] A. Karlsson and C. Pettersson, J. Chromatogr., 543 (1991) 287.
[9] Y. Bergqvist, J. Kabbani, C. Pettersson and N.H. Huynh, J. Chromatogr., 620 (1993) 217–224.
[10] C. Pettersson and C. Gioeli, Chirality, 5 (1993) 241–245.
[11] M. Davis, Natl. Bur. Stand. (U.S.) Monogr., No. 105 (1968) 19–113.
[12] U. Mayer, Coord. Chem. Rev., 21 (1976) 159–179, and references cited therein.
[13] A Tilly-Melin, Y. Askemark, K.-G. Wahlund and G. Schill, Anal. Chem., 51 (1979) 976–983.
[14] T. Hirokawa, T. Tsuyoshi and Y. Kiso, J. Chromatogr., 408 (1987) 27–41.
[15] A. Sokolowski and K.-G. Wahlund, J. Chromatogr., 189 (1980) 299–316.
[16] A. Karlsson and C. Pettersson, Chirality, 4 (1992) 323–332.
[17] G. Schill, H. Ehrsson, J. Vessman and D. Westlerlund, Separation Methods for Drugs and Related Organic Compounds, Swedish Pharmaceutical Press, Stockholm, 2nd ed., 1984.
[18] R. Kaliszan, K. Osmialowski, B.J. Bassler and R.A. Hartwick, J. Chromatogr., 499 (1990) 333–344.

ELSEVIER

Journal of Chromatography A, 705 (1995) 289–304

JOURNAL OF CHROMATOGRAPHY A

Comparison of three methods for the determination of oxysterols in spray-dried egg

Francesc Guardiola, Rafael Codony*, Magda Rafecas, Josep Boatella

Nutrition and Food Science Unit, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain

First received 24 April 1994; revised manuscript received 5 January 1995; accepted 9 January 1995

Abstract

Three methods for the GC determination of oxysterols (OSs) in spray-dried egg, which combine different steps of purification, are compared. In addition, the efficiency of silica cartridges in the purification of OSs using four different systems of elution with increasing polarities is studied. The absence of cholesterol oxidation during the application of the analytical procedures is checked, and the linearity of the response and the chromatographic limits of detection and quantification are established. The methods are characterized by the calculation of precision and recovery for the different OSs. The method based on saponification alone is rejected, since it shows much lower precision. The method that includes saponification and silica cartridge purification offers higher reliability than the method based on cartridge purification alone, because it shows a higher precision and larger samples can be processed, which improves the limits of detection and quantification.

1. Introduction

The analysis of oxysterols (OSs) is quite a new field and several aspects of the methodology are still controversial. As a consequence, many systems have been proposed, and many of these still need improvement and standardization. The purification of the OS fraction, which involves saponification and/or chromatographic techniques, is the most critical step of the analytical procedure, since the high structural similarity of the various OSs and the complexity of the lipid fraction of many foods demand high selectivity. These initial steps depend on the technique finally used in the OS determination, which is usually GC or HPLC. Moreover, the possibility of cholesterol oxidation during the analytical procedure is another factor that must be borne in mind, but which can be avoided through the optimization of the operating conditions.

Various solvent mixtures have been used for fat extraction in egg samples, but the mixture chloroform–methanol (2:1, v/v) provides the best extraction of the total fat and polar compounds, such as cholesterol and OSs [1,2].

Purification is a critical step in the method, since it improves the selectivity of the determination, and it involves one or two of the following steps: saponification of lipids, and column or TLC fractionation. Saponification is usually applied to the lipid fraction, although some authors attempt to saponify the whole food sample directly [1,3]. However, most authors report better results with the previous extraction of

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00034-8

lipids. Saponification temperature is also controversial. Cold saponification with methanolic KOH has been widely accepted in recent years, instead of hot saponification [1], since the latter increases the possibility of cholesterol oxidation [3,4]. It also increases the instability of some OSs, such as 7-ketocholesterol (7-KC) and cholesterol-5α,6α-epoxide (α-CE). The instability of 7-KC in hot alkaline media has been known since the 1940s, when Bergström and Wintersteiner [5] suggested that cholesta-3,5-dien-7-one could be one of its major decomposition products. Later, several authors supported this observation [4,6–8]. Other authors have reported that hot saponification induced the decomposition by hydrolysis of α-CE to cholestantriol and Tsai et al. [9] reported only 25% recovery of α-CE from hot saponification. The stability of 7-KC and α-CE is well preserved by cold saponification [10–12] and the oxidation of cholesterol is not produced [12,13]. However, contrasting results were reported by Van de Bovenkamp et al. [14] and Bascoul et al. [15], who suggested, respectively, that 7-KC is not stable during cold saponification and that cholesterol is not oxidized in hot alkaline media. In spite of the results reported by Van de Bovenkamp [14] for cold saponification, the methods of OS determination in foods that follow cold saponification give a good recovery of 7-KC and its main decomposition product, cholesta-3,5-dien-7-one, is not detected. This decomposition product is usually detected when hot saponification is used for OS determination in foods [15–17].

Some authors proposed a column fractionation step to purify OSs. This purification can be applied to the total lipids extracted from foods or to the non-saponifiable fraction. Silica is usually the adsorption stationary phase and increasing polarity solvent mixtures are applied to the column first to remove the interference compounds and then to isolate the OS fraction. In recent years, most studies [1,9,18–23] have used short columns or cartridges to purify the OS fraction. Other techniques have found less application, such as gel filtration chromatography [1,21,22,24], argentation chromatography (Florisil-$AgNO_3$ columns) [1,8] or preparative TLC applied to the total lipid or non-saponifiable extract [1,12,13,25].

The lack of comparative studies dealing with analytical methods for the determination of cholesterol oxides led the authors to design the present study, with the aim of comparing three alternative methods. Two of these were chosen from among the most usually reported in recent references. The third was proposed by the authors and resulted from the fusion of the other two methods. In addition, an attempt was made to improve and characterize the three methods and finally select the one that gives the best reliability.

In order to improve these methods, the efficiency of silica cartridges in the separation of a purified OS fraction, in the presence of the main interference attributable to a much higher cholesterol concentration in some samples (e.g. egg products), was studied. Four different systems of elution, chosen from those reported in the reference list and differing in the polarity of the final solvent used for the elution of OSs, were compared. The respective recoveries of each system for OSs and cholesterol, as well as the precision of these recoveries were used to characterize the system of elution, and finally to select the most efficient one. The recovery of phytosterols was studied using the system finally selected in order to apply it in the determination of OSs in samples of plant or mixed origin.

2. Experimental

2.1. Samples

All experiments were performed using the same dried egg sample, obtained by spray drying.

2.2. Reagents and standards

The solvents used were of the following origin and quality: chloroform, methanol, diethyl ether and acetone (all ACS grade) and hexane (for analysis) were from Panreac (Barcelona, Spain), and the ethyl acetate (ACS grade) and the dried

pyridine (max. 0.01% water, for analysis) were from Merck (Darmstadt, Germany).

Other reagents used were sodium hydroxide and anhydrous sodium sulphate (both for analysis) supplied by Panreac and the Sylon BTZ [N,O-bis(trimethylsilyl)acetamide–trimethylchlorosilane–N-trimethylsilylimidazole, 3:2:3, for research], in 0.1-ml glass ampoules, from Supelco, Bellefonte, PA, USA. Silica Sep-Pak cartridges were supplied by Waters, Millipore Division (Milford, MA, USA).

The cholesterol standard was supplied by Merck (>99%, by GC) and 5α-cholestane (99%, by GC) by Supelco. All other standards were from Sigma (St. Louis, MO, USA): cholesterol-5α,6α-epoxide (99%, by TLC), 7β-hydroxycholesterol (7β-HC) (99%, by TLC), cholestanetriol (CT) (>97%, by GC), 7-ketocholesterol (>99%, by HPLC), 25-hydroxycholesterol (25-HC) (>98%, by TLC), β-sitosterol (95%, by GC), stigmasterol (96%, by GC), 11α-hydroxyprogesterone (>98%, by GC), 11β,17α-dihydroxyprogesterone (99%, by HPLC), 19-hydroxycholesterol (19-HC) (>99%, by TLC) and 6-ketocholestanol (>99%, by GC). All these standards were weighed with an accuracy of 0.01 mg, and were made up as ethyl acetate solutions. The purity of these standards was checked by GC.

The choice of a suitable internal standard (I.S.) was made according to the following criteria: (1) the internal standard 5α-cholestane was not recovered through the silica cartridge and had to be added to the final OS extract; (2) recently several authors [12,13,19–21,25,26] used one of several I.S.s (6-ketocholestanol, 7-ketopregnenolone, 5α-androstan-3β-ol-17-one acetate and 19-HC), which were well recovered together with OSs through silica columns and plates; (3) four standards were assayed, 11α-hydroxyprogesterone, 11β,17α-dihydroxyprogesterone, 6-ketocholestanol and 19-HC, in order to find an I.S. well recovered through silica cartridge, and the first two standards were rejected, since they showed unknown impurities when they were injected in the chromatograph; (4) the 6-ketocholestanol was rejected since the chromatographic retention time was too close to that corresponding to the cholestantriol, working under the conditions of temperature programme II. So, 19-HC was chosen as the most suitable I.S., after checking that it was well recovered through the silica cartridges.

2.3. Silica cartridge fractionation

Elution systems compared

Four different solvent elution sequences were compared, as shown in Fig. 1. Elution sequence I is similar to that used by Tsai and Hudson [23] and Tsai et al. [9] for the determination of epoxycholesterols in egg products. Sequence II was proposed by Morgan and Armstrong [18] for the determination of the same OSs in dried egg yolk. Sequence III is similar to that used by Morgan and Armstrong [19,20] for the determination of several OSs in dried egg yolk. Sequence IV was a modification of sequence III, which aimed to increase the polarity of the last solvent used for the OS elution. Thus, four sequences of increasing polarity from I to IV were compared. Solvents were applied to the cartridge at 7 ml/min using a syringe.

Method

In all cases, the cartridge was equilibrated with 5 ml of hexane and then the solution (in 5 ml of hexane) containing the OSs and cholesterol or phytosterols standards was applied to the cartridge. The elution was then carried out applying, first, a further 5 ml of hexane and then solvent mixtures of increasing polarity. The four sequences assayed differed mainly in the composition of the last solvent, by which the OSs were eluted from the cartridge. This OS solution was recovered in a round-bottomed flask and concentrated to approx. 1 ml, by using a vacuum rotatory evaporator. This concentrate was quantitatively transferred with diethyl ether to a glass microtube (75 × 10 mm), containing 25 μg of 5α-cholestane as I.S. The evaporation of solvent was then completed by a slight nitrogen stream, at 25°C, and by keeping the tube in a vacuum desiccator at 10 mmHg (1 mmHg = 133.322 Pa) for 1 h. The residue of OSs was silanized and determined by GC in a Perkin-Elmer chromato-

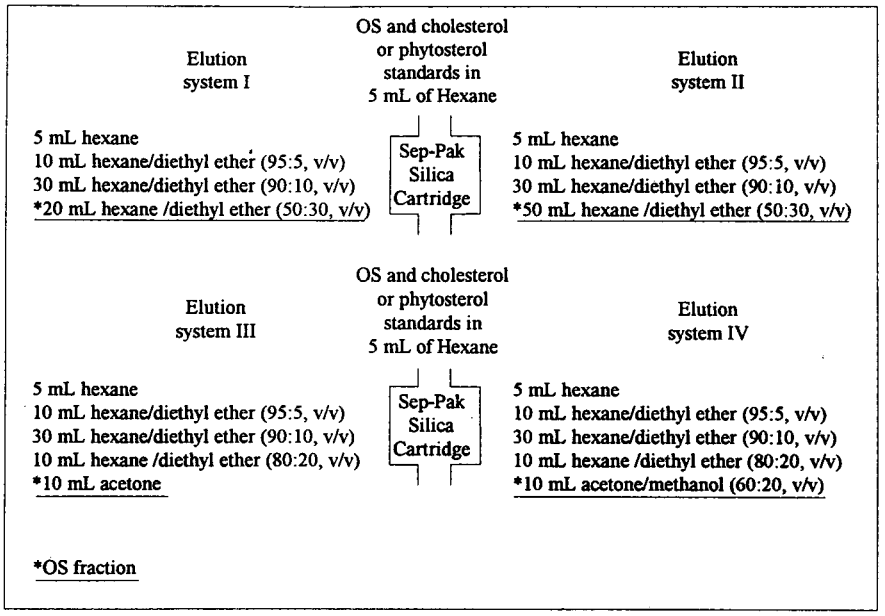


Fig. 1. Diagram of the elution systems compared.

graph, Model Sigma 2000, as described below in the section on GC conditions.

Amounts of standards fractionated; experiment I

The fractionation of three different solutions containing a constant amount of cholesterol (30 μg) and three increasing amounts of five OSs (2.5, 5 and 10 μg of α-CE, CT, 7-KC and 25-HC; and 5, 10 and 20 μg of 7β-HC) was assayed. This concentration range was chosen considering that samples for the analysis were dried egg and that one of the analytical problems was to separate the OS fraction from a high concentration of cholesterol.

Fifteen replicates of the four different elutions were performed, five corresponding to each of the three concentration levels. For the 60 OS extracts obtained, the recoveries of cholesterol, α-CE, 7β-HC, CT, 7-KC and 25-HC were calculated.

Experiment II

The phytosterols were a clear interference, when the determination of OSs was carried out in samples of plant or mixed origin. This led to the study of the recovery of these compounds using the elution system finally chosen. The fractionation of three different solutions containing the amounts of the five OSs, described above; and 2.5, 5 and 10 μg of stigmasterol and β-sitosterol was assayed. Five replicates of each of the three concentration levels were performed and the recoveries for the different standards were calculated.

2.4. Linearity of response

The linearity of the response of the cholesterol and the OS standards up to 55 μg was checked by calculation of calibration curves. These curves were determined on the two Perkin-Elmer gas chromatographs (Sigma 2000 and Autosystem), using two internal standards, 5α-cholestane and 19-HC. The chromatographic conditions were those described below. The formula of these calibrate curves resulted from the regression of the variable y ($A_{xs}/A_{I.S.}$) on the variable x ($W_{xs}/W_{I.S.}$) and its correlation coefficient (r^2) expresses the linearity of the response. From the curve, the relative response factors (RRFs) were also calculated, according to the formula: RRF =

$(W_{xs}A_{I.S.})/(A_{xs}W_{I.S.})$ (where W_{xs} = standard mass; $W_{I.S.}$ = internal standard mass; A_{xs} = standard area; $A_{I.S.}$ = internal standard area).

Three of these curves were calculated. The first two with 5α-cholestane as I.S., using the Sigma 2000 and Autosystem. The third curve was developed using the Autosystem, with 19-HC as I.S. The amounts of standards used were: 0.5, 1, 2.5, 5, 10, 20 and 30 μg, for the first curve; 0.6, 1.2, 2.5, 5, 10 and 20 μg, for the second; and 2.5, 5, 10, and 20 μg for the third. All masses of standards were dissolved in 50 μl of pyridine and silanized with 50 μl of Sylon BTZ, and then injected in duplicate. Five replicates of each level of concentration were performed for the first two curves and four replicates for the third.

2.5. Chromatographic limits of detection and quantification

These limits in relation only to the chromatographic method were determined using the definition and calculation procedure proposed by Knoll [27]. For the calculation of these limits 10 blank solutions and 15 standard solutions (three different concentrations, 2.5, 5 and 10 μg/μl) were injected into each chromatograph described below.

2.6. Procedure of the three methods for the determination of OSs compared

Two of the three methods for the determination of OSs that were compared were chosen from among the most reliable methods proposed in the reference list (methods I and II). Method III was a combination of the other two.

Method I

This method consists of a lipid extraction by agitation with chloroform–methanol (2:1, v/v), a further 1 *M* KOH in methanol cold saponification, the extraction of the unsaponifiable material with diethyl ether, and silanization before GC determination. This method was first introduced by Park and Addis [11] to determine OSs in heated fats and it has been applied by several authors to different food and biological samples [28–34]. Some authors complete the method by TLC fractionation of the non-saponifiable components [12,13,25].

Method II

This method consists of the same lipid extraction as in method I; further purification by adsorption on a silica cartridge and selective elution of the OS fraction and silanization before the GC determination. This method has been used for the determination of OSs in egg yolk powder by Morgan and Armstrong [19,20].

Method III

This is a result of the fusion of methods I and II and consists of the same lipid extraction; the cold saponification and the extraction of unsaponifiable material with diethyl ether; the purification of the OS fraction by selective elution from a silica cartridge and silanization before the GC determination.

Fig. 2 shows a diagram of the three methods compared. All these methods have in common the conditions of attenuated light during all the procedure.

2.7. Lipid extraction (common to the three methods)

Lipids were extracted according to the method proposed by Folch et al. [35], with the following modifications. A 0.65-g amount of powder egg was accurately weighed in a 25-ml conical flask, and 25 μg of 19-HC[1] and 15 ml of chloroform–methanol (2:1, v/v) were added. The mixture was stirred magnetically for 30 min. Then the liquid phase obtained was decanted through a paper filter and recovered in a tube (100 × 26 mm) with a screw cap. The solid residue in the conical flask was reextracted for 10 min with 10 ml of chloroform–methanol (2:1, v/v) and rinsed in 5 ml of the same mixture. The liquid phases obtained were filtered and recovered in the same tube. A 5-ml volume of distilled water was added to the tube, which was agitated and then cen-

[1] When 19-HC is used as I.S., it is added to the sample, and when 5α-cholestane is used, it is added to the final extract.

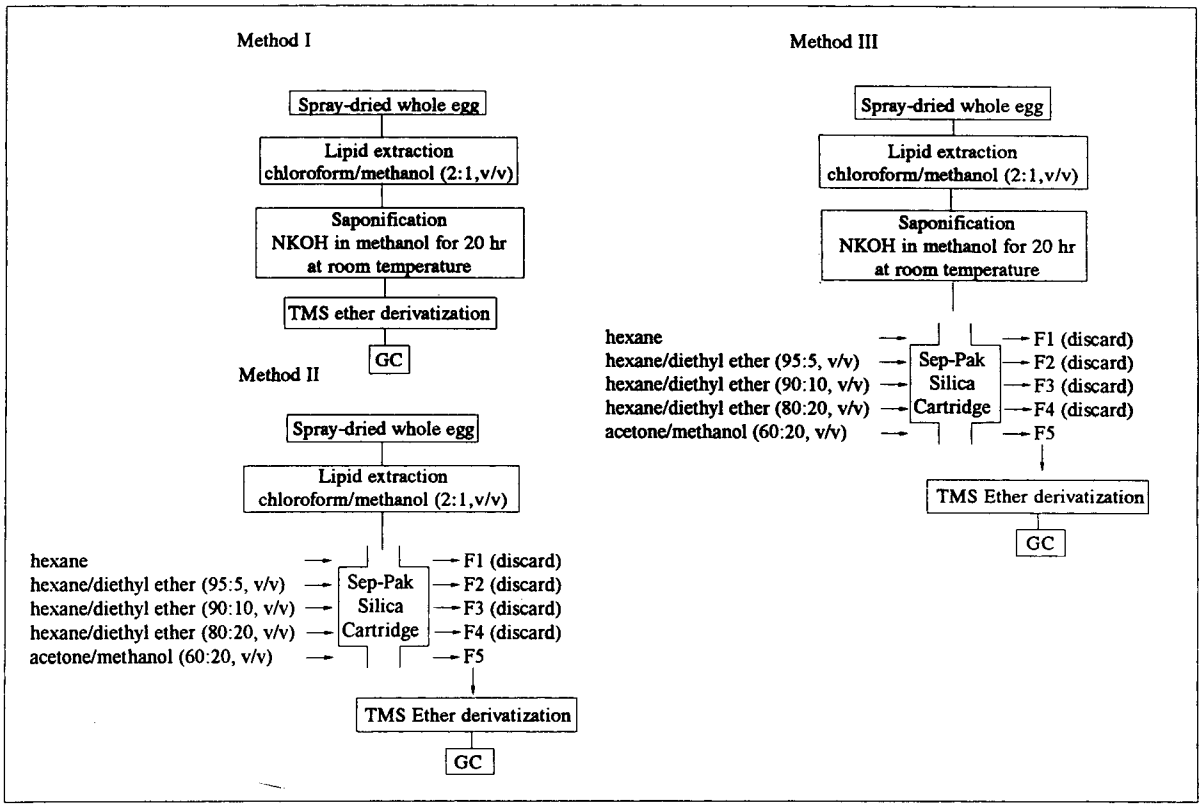


Fig. 2. Diagram of the three methods compared.

trifuged at 2200 rpm for 20 min. The chloroformic extract was transferred to a round-bottomed flask (in method II this extract was previously filtered through anhydrous sodium sulphate), and was concentrated to 1 ml in a vacuum rotatory evaporator, at 35°C. Removal of solvent was completed in a slight nitrogen stream and by keeping the flask in a vacuum desiccator at 10 mmHg for 30 min (in methods I and III) or 1 h (in method II).

2.8. Cold saponification (only in method I and III)

A 10-ml volume of 1 *M* KOH in methanol was added to the flask containing the lipid extract, with gentle agitation to obtain a homogeneous phase. Then the mixture was kept at room temperature for 20 h to complete the saponification. The blend was then quantitatively transferred to a tube (100 × 26 mm) using 10 ml of diethyl ether and 10 ml of distilled water. The sealed tube was then shaken and the organic phase was transferred to a separating funnel. The aqueous phase in the tube was again re-extracted twice with two portions of 10 ml of diethyl ether and the new organic phases obtained were also transferred to the same funnel. The whole organic extract was then washed in the funnel, first in 5 ml of 0.5 *M* aqueous KOH solution, and then in two portions of 5 ml of distilled water. The washed organic extract was filtered through anhydrous sodium sulphate and recovered in a round-bottomed flask, from which the solvent was evaporated to 1 ml, using a rotatory vacuum evaporator at 30°C. From this point, different procedures for methods I and III were followed. In method I, the concentrated

extract was transferred quantitatively with diethyl ether to a glass tube (75 × 10 mm), containing 25 μg of 5α-cholestane (see footnote 1). Glass tubes were used to avoid variability of results, since we have found that plastic materials showed different adsorption of OSs and 5α-cholestane [36]. The non-saponifiable residue was obtained by removal of solvent, using a slight nitrogen stream, at 25°C, and finally in a vacuum desiccator at 10 mmHg for 1 h. Afterwards, the silanization and GC determination were carried out. For method III, the non-saponifiable residue was obtained by removing the remaining solvent by a slight nitrogen stream, at 25°C, and by keeping the flask in a vacuum desiccator at 10 mmHg for 30 min.

2.9. Silica cartridge purification (only in methods II and III)

The lipid extract (method II) or the non-saponifiable extract (method III) was redissolved in 5 ml of hexane and applied to a silica cartridge, previously equilibrated with 5 ml of hexane. The cartridge was then eluted with solvent sequence IV, as described above. The obtained OS fraction plus 25 μg of 5α-cholestane (see footnote 1) was silanized and determined by GC, as described below.

2.10. GC conditions (common to the three methods)

The final residue obtained in any of the three procedures described was redissolved in 50 μl of anhydrous pyridine and 50 μl of Sylon BTZ were then added, and the mixture was kept at room temperature for 20 min to complete the silanization reaction, before injection to the column. The silyl derivatives are stable for several days at −20°C [37]. All injections were performed in duplicate.

GC was performed using two Perkin-Elmer chromatographs, Models Sigma 2000 and Autosystem, equipped with a flame ionization detector and fused-silica capillary column (25 m × 0.25 mm I.D.), with a film thickness of 0.13 μm stationary phase of 100% methylsilicone (Chrompack, Middelburg, Netherlands). Helium was used as carrier gas and the chromatographic conditions were as follows. Oven temperature programmes: (I) from 210 to 240°C at 6°C/min, from 240 to 270°C at 4°C/min, from 270 to 290°C at 2°C/min and 5 or 82 min at 290°C (used in the Sigma 2000); (II) from 210 to 264°C at 2°C/min, from 264 to 290°C at 5°C/min and 2 or 80 min at 290°C (used in the Autosystem). Injector temperature 290°C. Detector temperature 350°C. Split ratio 1:40 for the Sigma 2000 and 1:30 for the Autosystem. Inlet pressure 15 p.s.i. (1 p.s.i. = 6894.76 Pa). Sample volume injected is 2 μl.

2.11. Identification of OSs

Co-chromatographic identification

Firstly, the identification was accomplished by comparison of the relative retention times of the silanized standards with the times corresponding to the peaks obtained for a silanized purified extract from a dried egg sample. After that, the identification was confirmed by addition of silanized OS standards to the same extract (co-chromatography). For major peaks in the sample, like those corresponding to 7β-HC and 7-KC, a previous dilution with pyridine was carried out to obtain a similar size to that obtained with the injection of the standard solution (1.2 μg/100 μl). The dilution was made in order to give lower peaks, which facilitated asymmetry observation. In all OS peaks, the amount of standard added was calculated to increase the area between 50 and 100% in order to facilitate the detection of asymmetrical peaks. All standard-sample mixtures were injected several times and using the two former temperature programmes, as well as the following: (III) from 210 to 290°C at 6°C/min and 10 or 87 min at 290°C; (IV) from 210 to 240°C at 6°C/min, from 240 to 290°C at 4°C/min and 7 or 84 min at 290°C; (V) from 210 to 240°C at 6°C/min, from 240 to 290°C at 2°C/min and 2 or 80 min at 290°C. The last temperature programme did not separate α-CE from 7β-CE and was not used for their identification. Identification was only confirmed when the re-

sulting peak in the spiked sample was symmetrical at all chromatographic temperatures assayed.

Mass spectrometric identification

A further confirmation of the peak identified in egg samples was provided by GC–MS. The system used was a Hewlett-Packard 5988A mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph, equipped with a capillary fused-silica column (25 m × 0.32 mm I.D.), with a film thickness of 0.52 μm stationary phase of 100% methylsilicone (Hewlett-Packard, Geneva, Switzerland). Helium was used as carrier gas and chromatographic conditions were as follows. Oven temperature programme: from 210 to 264°C at 2°C/min, from 264 to 290°C at 5°C/min and 2 or 80 min at 290°C. Injector temperature: 290°C. Split ratio: 1:50. Inlet pressure: 15 p.s.i. Sample volume injected: 5 μl. MS conditions were: interphase temperature 280°C; ion source temperature 200°C; electron energy 70 eV. Cholesterol oxides were identified in the mass range (m/z) from 100 to 650. The SIM (selected ion monitoring) technique was also applied, in which the ions of m/z 120, 129, 131, 321, 367, 382, 384, 403, 456, 472, 474 and 546 were selected as the most characteristic of the OSs determined.

2.12. Absence of cholesterol oxidation during the procedures

A 15-mg amount of cholesterol standard (amount present in approximately 0.95 g of dried egg or in 3.8 g of fresh egg), in which absence of OSs was checked by GC, was subjected to the whole procedure of method III and then injected to the column. This test was carried out in quadruplicate.

3. Results and discussion

3.1. Identification of OSs in the egg sample

Fig. 3 shows the peaks identified according to the procedure described above in the co-chromatographic identification section, which correspond to 7β-HC, α-CE, CT, 7-KC and 25-HC.

Comparing the mass spectra of peaks from the egg sample analyzed with those corresponding to standards injected in the same conditions and with spectra reported by several authors [11,22,24,33,37–40] the identity of the five OSs previously pre-identified was confirmed.

3.2. Comparison of the elution systems

The results of experiment I are given in Table 1. Elution systems I and II did not recover the CT. The recoveries obtained can be explained by the polarity of the compounds analyzed, which increased in the following order: cholesterol < cholesterol-5,6-epoxides < 7-KC < 7-hydroxy-cholesterols < CT [1,24,41,42]. So, systems I and II, having lower polarities, recovered lower amounts of highly polar compounds and did not recover CT. However, 7-KC was more recovered than α-CE in systems I and II, in spite of the polarity scale defined above.

Analysis of variance (ANOVA) was applied in order to ascertain whether the recovery percentage for every elution system and every OS depended on the concentration level. Results of this analysis showed that recoveries did not differ significantly, for any system of elution, according to the concentration. So, a global recovery value was considered for every OS. Then, ANOVA was applied in order to discover whether there were significant differences in the recoveries of cholesterol, α-CE, 7β-HC, 7-KC and 25-HC, according to the elution system. Results of this analysis show statistically significant differences for the five compounds ($P < 0.0001$). For the compounds that showed significant differences the Scheffé's test for "a posteriori" contrasts ($\alpha = 0.05$) was applied, in order to determine which elution systems differ in recovery values. For the four OSs, mean recoveries of elution system III and system IV were significantly higher than II and I, while significantly lower values were found for cholesterol in systems III and IV. In contrast, there were no differences between mean recoveries of systems III and IV. Lower values of cholesterol recovery are inter-

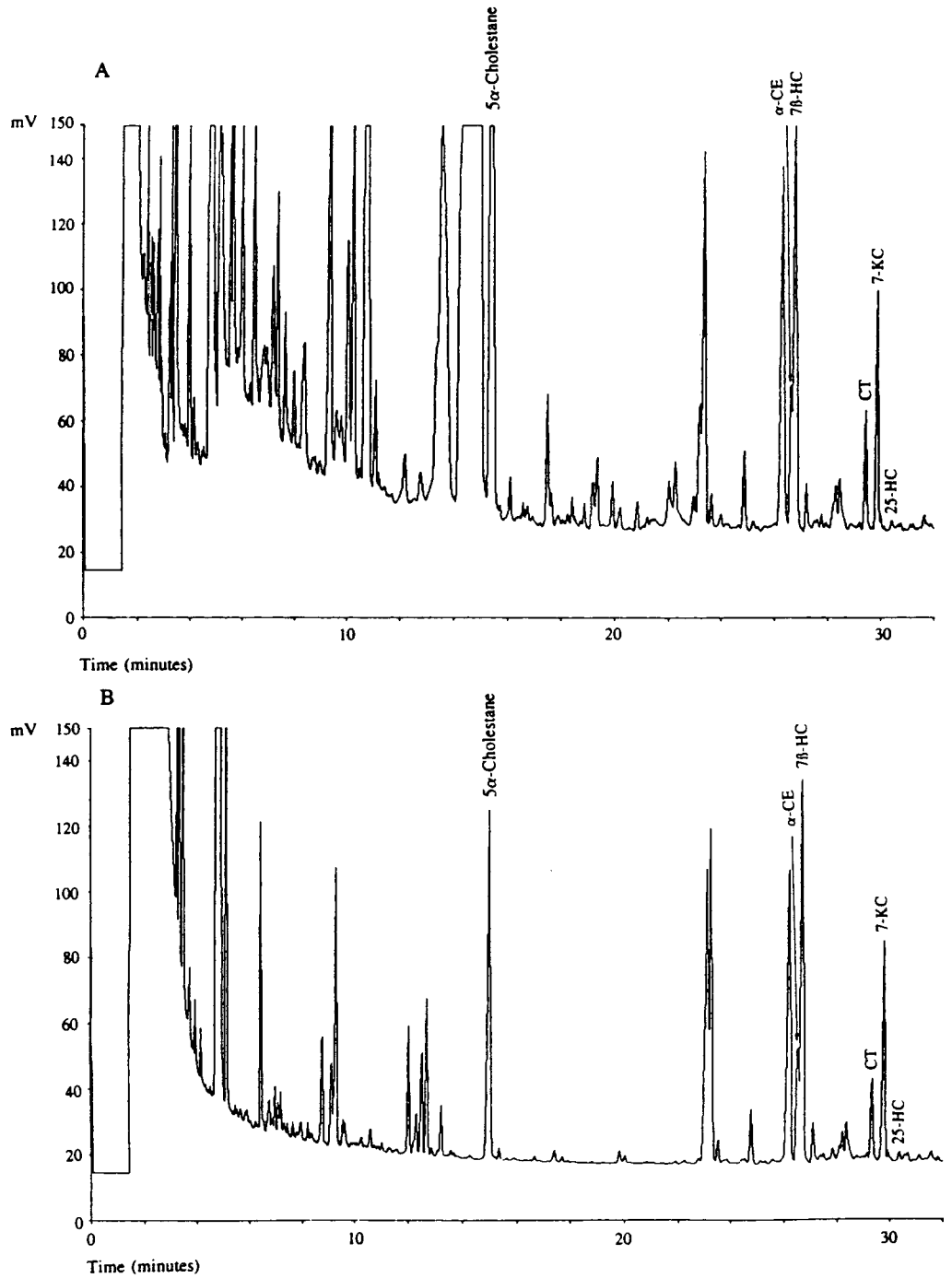


Fig. 3. Chromatograms of silanized extracts obtained from a dried egg sample (temperature programme II). (A) Method II. (B) Method III.

Table 1
Standard recoveries for the different systems of elution

Standard	Amount (μg)	Recovery (%)			
		System I	System II	System III	System IV
Cholesterol	30	7.4[a] (39.6)[b]	9.5 (47.9)	3.4 (26.7)	3.0 (24.8)
α-CE	2.5	33.2 (16.5)	65.5 (14.9)	97.6 (4.0)	98.9 (1.9)
	5	33.8 (12.3)	65.9 (10.3)	98.4 (3.8)	98.9 (1.0)
	10	34.6 (7.6)	65.4 (11.7)	98.2 (3.3)	98.8 (0.9)
	Global recovery	33.9 (11.7)	65.6 (11.5)	98.1 (3.5)	98.9 (1.2)
7β-HC	5	2.7 (117.3)	6.4 (89.2)	96.7 (2.9)	98.6 (1.9)
	10	2.6 (91.8)	6.2 (70.8)	97.3 (2.0)	98.0 (1.3)
	20	2.8 (28.9)	6.5 (39.9)	97.0 (1.3)	98.9 (1.1)
	Global recovery	2.8 (80.1)	6.4 (64.3)	97.0 (2.0)	98.5 (1.4)
CT	2.5	NR	NR	98.7 (3.9)	100.0 (2.1)
	5	NR	NR	98.6 (2.1)	101.2 (1.4)
	10	NR	NR	98.8 (1.6)	101.3 (1.3)
	Global recovery			98.7 (2.5)	100.9 (1.6)
7-KC	2.5	65.4 (6.7)	90.6 (5.2)	100.5 (2.8)	101.3 (2.2)
	5	65.1 (5.6)	90.3 (4.5)	100.5 (2.4)	101.9 (2.0)
	10	65.3 (4.3)	90.5 (3.9)	100.7 (2.0)	102.0 (1.6)
	Global recovery	65.6 (5.2)	90.5 (4.2)	100.5 (2.2)	101.7 (1.9)
25-HC	2.5	48.1 (7.3)	76.5 (5.3)	98.9 (1.5)	100.8 (0.8)
	5	48.2 (7.1)	77.2 (4.6)	98.8 (1.4)	100.4 (0.5)
	10	47.5 (6.5)	76.6 (3.3)	99.0 (1.0)	100.7 (0.3)
	Global recovery	47.9 (6.5)	76.8 (4.2)	98.9 (1.2)	100.7 (0.6)

NR = Not recovered.
[a]Mean value ($n = 5$ for amounts 2.5, 5, 10 and 20, and $n = 15$ for amount 30 and global recovery).
[b]R.S.D. (%).

esting because this compound can interfere in the determination of 7α-hydroxycholesterol since this OS elutes close to cholesterol in the gas chromatogram when a 100% methylsilicone capillary column is used [21,26,37]. In addition, for the four OSs but not for cholesterol, elution system II showed significantly higher recoveries than system I (Table 2). For CT, only the recoveries for systems III and IV could be compared, and the application of the Student–Fisher's t test showed that a significantly higher CT recovery was found using system IV ($P < 0.0009$). According to these results, it can be concluded that elution systems III and IV give significant advantages, due to their lower cholesterol recovery and better recoveries of the different OSs. The significantly higher recovery of CT and the better precision obtained ($n = 15$) in all OS recoveries, using elution system IV, led to the choice of this method for future assays.

For elution system IV similar recovery results were obtained in experiment II for the five OSs. Stigmasterol and β-sitosterol presented low recoveries, a finding of interest, given the desirability of avoiding the interfering effect of these compounds. ANOVA was applied in order to determine whether the recovery for every standard depended on the concentration level. Results of this analysis showed that recoveries do not vary significantly with concentration. There-

Table 2
Significance level and confidence interval of the "a posteriori" contrasts for cholesterol, α-CE, 7β-HC, 7-KC and 25-HC (Scheffé's test, $\alpha = 0.05$)

Standard	S[a]	System II	System III	System IV
Cholesterol	I	NS[b]	$P = 0.0026$ (1.1–7.0)[c]	$P = 0.0007$ (1.6–7.4)
	II		$P < 0.0001$ (3.2–9.1)	$P < 0.0001$ (3.7–9.5)
	III			NS
α-CE	I	$P < 0.0001$ (26.8–36.6)	$P < 0.0001$ (59.3–69.1)	$P < 0.0001$ (60.1–69.9)
	II		$P < 0.0001$ (27.6–37.3)	$P < 0.0001$ (28.4–38.1)
	III			NS
7β-HC	I	$P = 0.0053$ (0.8–6.4)	$P < 0.0001$ (91.5–97.0)	$P < 0.0001$ (92.9–98.5)
	II		$P < 0.0001$ (87.9–93.4)	$P < 0.0001$ (89.3–94.9)
	III			NS
7-KC	I	$P < 0.0001$ (22.1–28.3)	$P < 0.0001$ (32.2–38.3)	$P < 0.0001$ (33.3–39.5)
	II		$P < 0.0001$ (6.9–13.1)	$P < 0.0001$ (8.1–14.3)
	III			NS
25-HC	I	$P < 0.0001$ (26.4–31.3)	$P < 0.0001$ (48.5–53.5)	$P < 0.0001$ (50.3–55.2)
	II		$P < 0.0001$(19.7–24.6)	$P < 0.0001$ (21.4–26.4)
	III			NS

[a]S = System of elution.
[b]For this standard the difference between the mean recoveries (%) using these two systems of elution is not statistically significant (NS = not statistically significant).
[c]P = Significance level of the contrast; the confidence interval is stated in parentheses.

fore, a global recovery value (mean value, $n = 15$) was considered for every standard [relative standard deviation (R.S.D., %) in parentheses]: α-CE, 100.6 (2.1); 7β-HC, 100.2 (2.1); CT, 99.5 (1.6); 7-KC, 101.4 (1.8); 25-HC, 98.5 (1.5); stigmasterol, 3.2 (24.9); and β-sitosterol, 4.1 (22.2).

Finally, for the five OSs, these results were grouped with the former for the system IV and the mean value ($n = 30$) was calculated. Mean recoveries [R.S.D. (%) in parentheses] were: α-CE, 99.7 (1.9); 7β-HC, 99.4 (1.9); CT, 100.2 (2.1); 7-KC, 101.5 (1.8); and 25-HC, 99.6 (1.5).

3.3. Linearity of response

Table 3 gives values obtained with the three curves for the linearity (r^2) and the RRF of each compound analyzed. As can be seen in the table, the linearity of response was good for all standards. RRFs show some differences according to the chromatograph used, which could be related to split discrimination. Values from curve I agree with those reported by different authors [1,12,14,37] suggesting that the RRFs of the more common OSs are close to 1.0 except those of 7-KC and α-CE.

3.4. Limits of detection (DLs) and quantification (QLs)

The DLs and QLs in relation only to the chromatographic method are given in Table 4. To make an approximation to the DL and QL values relative to the whole method, the values corresponding to the chromatographic determination were referred to the mass of sample (0.65 g of dried egg sample was extracted, the extract purified and the final residue was completely silanized). The limits calculated with respect to the sample are given in Table 5.

Table 3
Linearity of response for OSs

Curve	Standard	n	a	b	r^2	RRF (R.S.D.)[a]
C_I	Cholesterol	35	0.022	1.022	0.9968	0.876 (6.2)
	α-CE	35	0.008	0.943	0.9973	1.030 (4.1)
	7β-HC	35	0.046	1.007	0.9985	0.979 (3.3)
	CT	35	0.010	0.984	0.9977	0.985 (6.0)
	7-KC	35	0.001	0.801	0.9929	1.242 (6.1)
	25-HC	35	0.015	0.994	0.9974	0.941 (5.5)
C_{II}	Cholesterol	24	0.012	1.021	0.9988	0.883 (6.1)
	α-CE	24	0.003	1.065	0.9990	0.899 (6.0)
	7β-HC	24	0.003	0.811	0.9999	1.192 (3.0)
	CT	24	−0.006	1.457	0.9978	0.690 (6.1)
	7-KC	24	−0.015	1.387	0.9950	0.762 (6.0)
	25-HC	24	−0.009	1.338	0.9957	0.764 (5.9)
C_{III}	Cholesterol	20	0.061	0.997	0.9915	0.984 (5.5)
	α-CE	20	−0.059	0.871	0.9935	1.181 (5.7)
	7β-HC	20	0.051	0.753	0.9968	1.299 (6.7)
	CT	20	−0.129	1.268	0.9915	0.822 (6.1)
	7-KC	20	−0.108	1.122	0.9950	0.993 (6.3)
	25-HC	20	−0.031	1.274	0.9961	0.801 (6.2)

The regression of y (Area standard/Area internal standard) on x (Weight standard/Weight internal standard) presents the equation $y = a + bx$, where a is the intercept and b the slope; r^2 = Determination coefficient.
[a]Relative response factors, their R.S.D.s (%) are given in parentheses.

Table 4
Detection (DL) and quantification (QL) limits relative to the chromatographic method for each chromatograph

Chromatograph	Parameter	α-CE	7β-HC	CT	7-KC	25-HC
Sigma 2000	DL	1.65	2.34	2.28	2.94	2.16
	QL	4.80	6.82	6.66	8.59	6.31
Autosystem	DL	1.78	1.61	1.40	1.47	1.16
	QL	5.18	4.68	4.09	4.30	3.38

Results expressed as ppm in silanized extract.

Table 5
Detection (DL) and quantification (QL) limits relative to the whole method

Chromatograph	Parameter	α-CE	7β-HC	CT	7-KC	25-HC
Sigma 2000	DL	0.25	0.36	0.35	0.45	0.33
	QL	0.74	1.05	1.03	1.32	0.97
Autosystem	DL	0.27	0.25	0.22	0.23	0.18
	QL	0.80	0.72	0.63	0.66	0.52

Results expressed as ppm in sample.

3.5. Absence of cholesterol oxidation during the procedures

No trace peak corresponding to OSs was detected in the assay described above. Method III is the fusion of methods I and II, and for this reason, it can be concluded that no OS formation should be expected following any of the three methods, paying attention to the following operation conditions: attenuated light; evaporation of solvents until 1 ml, and completing to dryness by using a slight nitrogen stream and by keeping in a vacuum desiccator at 10 mmHg. Moreover, temperature must be lower than 35°C, since traces of some OS (7β-HC and 7-KC) were found when the evaporation was carried to dryness at 45°C.

3.6. Comparison of the three methods for the determination of OSs

Comparison of the precision

In the application of the three methods, the 5α-cholestane was added as I.S. and the same mass of sample (0.65 g of dried egg) and the same chromatograph (Perkin-Elmer Sigma 2000 plus HP 3396A) were used. Ten determinations of the five OSs were performed on the same sample, on successive days, and using the same materials and reagents. The results obtained using method I corresponding to the mean contents of OSs in ppm ($n = 10$), with their R.S.D.s (%) in parentheses, are: α-CE, 8.6 (10.3); 7β-HC, 78.3 (9.2); CT, 12.5 (9.6); 7-KC, 20.2 (9.1); and 25-HC, 1.6 (21.0). For method II: α-CE, 10.8 (9.5); 7β-HC, 91.5 (6.0); CT, 12.3 (7.7); 7-KC, 27.0 (8.2); and 25-HC, 1.6 (19.7). For method III: α-CE, 9.0 (8.0); 7β-HC, 74.6 (3.4); CT, 11.7 (7.5); 7-KC, 21.6 (7.2); and 25-HC, 1.6 (16.6).

As the R.S.D. shows the precision increased from method I to method III. This could be explained by the increasing degree of purification, which improves the selectivity, as the comparative chromatograms show (Fig. 3).

Comparison of the recovery

The recovery was also determined in the three cases on the same chromatograph (Perkin-Elmer Sigma 2000 plus HP 3396A) and using the same I.S. (5α-cholestane). Three levels of the OS standards (5, 10 and 20 μg) were added to 0.65-g aliquots of the same egg dried sample. Five replicas of each level were performed. Table 6 shows the results of the mean recoveries obtained for each OS, at the three levels of addition ($\bar{X}_5$, $\bar{X}_{10}$ and $\bar{X}_{20}$). The application of ANOVA to the recovery values indicates that the differences in the recovery of each method between the three levels of addition were not statistically significant for any OS. For this reason, the global mean values of the recovery ($\bar{X}_G$) are also given in the table.

This table shows that the variability of the recovery increases when the level of addition decreases. Furthermore, the mean recovery values are higher for method II than for method I and method III, which shows the lowest values. The variability of recovery increases from method III to method I. The recoveries of CT were clearly lower for methods I and III than for method II, which could be due to the poor recovery induced by the saponification step (common to methods I and III). Moreover, α-CE recovery was higher than 100% for all three methods, which may be explained by a defect in the integration of the peak, due to its overlap with the 7β-HC peak. Another interesting conclusion of this study is the high recovery obtained for the α-CE and the 7-KC for the methods (I and III) using a saponification step. This demonstrates that these two OSs are stable during cold saponification, which agrees with the data reported by many authors [10–12], although Van de Bovenkamp et al. [14] reported low recovery for the 7-KC with this method.

From all these results of precision and recovery, method III is the most reliable, since it gives a higher precision in the determination and recovery. The other two methods, although they show higher recoveries, have lower precision and in consequence give less reliable results. However, method III should be improved in some aspects, especially the variability. For this reason, improvement was attempted using a new chromatograph, a Perkin-Elmer Autosystem,

Table 6
Mean recoveries of OSs at different levels of addition for the three methods

Method		Oxysterols				
		α-CE	7β-HC	CT	7-KC	25-HC
I	$\bar{X}_5$ [a]	103.6	88.3	61.0	85.4	81.3
	R.S.D. (%)	12.0	14.6	11.8	12.3	8.9
	$\bar{X}_{10}$ [b]	99.5	86.4	60.5	83.8	77.3
	R.S.D. (%)	9.2	9.3	7.9	9.7	9.2
	$\bar{X}_{20}$ [c]	100.6	86.9	61.7	86.5	79.5
	R.S.D. (%)	6.9	5.8	5.2	5.5	5.5
	$\bar{X}_G$ [d]	101.2	87.2	61.1	85.2	79.4
	R.S.D. (%)	9.1	9.9	8.1	9.0	7.8
II	$\bar{X}_5$	111.2	95.1	83.6	86.8	94.9
	R.S.D. (%)	9.8	12.4	10.5	12.1	8.4
	$\bar{X}_{10}$	110.4	97.5	84.7	87.5	95.8
	R.S.D. (%)	7.4	9.9	7.4	8.6	6.5
	$\bar{X}_{20}$	110.4	95.6	86.8	89.3	97.3
	R.S.D. (%)	6.1	8.8	5.6	6.0	3.6
	$\bar{X}_G$	110.7	97.0	85.0	87.9	96.0
	R.S.D. (%)	7.4	9.8	7.6	8.6	6.0
III	$\bar{X}_5$	105.9	80.4	56.2	83.5	70.0
	R.S.D. (%)	5.7	12.7	8.8	8.6	5.4
	$\bar{X}_{10}$	109.3	78.6	51.5	80.3	74.3
	R.S.D. (%)	4.5	8.0	4.7	8.9	4.5
	$\bar{X}_{20}$	107.6	76.2	54.0	77.1	72.1
	R.S.D. (%)	2.7	4.2	5.3	7.4	4.1
	$\bar{X}_G$	107.6	78.4	53.9	80.3	72.1
	R.S.D. (%)	4.3	8.8	7.2	8.4	5.0

[a]Mean recovery at level of addition of 5 μg ($n = 5$).
[b]Mean recovery at level of addition of 10 μg ($n = 5$).
[c]Mean recovery at level of addition of 20 μg ($n = 5$).
[d]Global mean value of recovery ($n = 15$).

equipped with a personal integrator PE Nelson 1020, more reliable than the HP 3396A. The temperature programme of the oven was also modified to give better resolution. The conditions finally used are those described in the Experimental section.

Thus, the following study was carried out to select the most reliable method. Method I was ruled out, since it does not offer any significant advantage, and the comparative study was only focused on methods II and III. In order to improve the precision, 19-HC was used as the I.S., because it could be recovered through the silica cartridge and so could be added to the sample at the beginning of the analytical procedure. These modifications, in addition to the use of a new chromatographic system (PE Autosystem plus PE Nelson 1020 integrator) were applied to methods II and III. The results of precision for method II corresponding to the mean contents of OSs in ppm ($n = 10$), with their R.S.D. (%) in parentheses, are: α-CE, 9.4 (5.1); 7β-HC, 81.1 (5.0); CT, 13.1 (5.8); 7-KC, 25.9 (4.6); and 25-HC, 1.8 (10.5). For method III: α-CE, 9.3 (3.9); 7β-HC, 83.4 (3.8); CT, 14.1 (3.9); 7-KC, 22.2 (3.6); and 25-HC, 1.7

Table 7
Mean recoveries of OSs at different levels of addition for the methods II and III

Method		Oxysterols				
		α-CE	7β-HC	CT	7-KC	25-HC
II	$\bar{X}_5$ [a]	98.6	95.9	92.5	91.2	100.4
	R.S.D. (%)	6.6	14.2	7.7	12.3	7.7
	$\bar{X}_{10}$ [b]	99.3	94.4	91.8	90.5	98.1
	R.S.D. (%)	5.6	7.7	4.7	6.9	6.1
	$\bar{X}_{20}$ [c]	97.4	95.3	93.0	92.5	98.0
	R.S.D. (%)	4.2	4.8	3.5	3.4	2.5
	$\bar{X}_G$ [d]	98.4	95.2	92.4	91.4	98.8
	R.S.D. (%)	5.2	9.1	5.2	7.8	5.6
III	$\bar{X}_5$	96.2	100.3	93.3	93.5	98.1
	R.S.D. (%)	6.4	10.6	6.2	8.3	6.3
	$\bar{X}_{10}$	96.4	99.9	95.0	93.6	97.3
	R.S.D. (%)	4.3	5.8	5.1	5.9	6.7
	$\bar{X}_{20}$	96.0	98.8	95.4	95.0	97.3
	R.S.D. (%)	3.6	3.8	3.0	3.2	2.0
	$\bar{X}_G$	96.2	99.7	94.6	94.0	97.5
	R.S.D. (%)	4.5	6.8	4.7	5.7	5.1

[a]Mean recovery at level of addition of 5 μg ($n = 5$).
[b]Mean recovery at level of addition of 10 μg ($n = 5$).
[c]Mean recovery at level of addition of 20 μg ($n = 5$).
[d]Global mean value of recovery ($n = 15$).

(6.2). The R.S.D. showed that method III gives better precision.

Results of the determination of the recoveries of OSs at three levels of addition (5, 10 and 20 μg) are given in Table 7. The recovery of the I.S. (19-HC), which was 80.96% for method II and 72.98% for method III, was taken into account for the calculations of precision and recovery of the methods. The 19-HC recovery was calculated using 5α-cholestane as the I.S. Applying ANOVA to the results of Table 7, no significant difference in the recovery was found between the three levels of addition. For this reason, the global mean values of these recoveries were calculated, which are also given in the table.

The mean global recoveries of 7β-HC, CT, 7-KC and 25-HC were higher for method III than for II, and the recovery of α-CE was higher for method II. The application of the Student–Fisher's t test showed that these differences are not statistically significant.

From all these data it can be concluded that method III is the most reliable for the determination of OSs in the dried egg, since it shows a higher precision. In addition, this method allows the analysis of a greater mass of sample, since it supplies better purification, and consequently the limit of quantification decreases.

Acknowledgements

This work was supported in part by a research grant from "Comissió Interdepartamental de Recerca i Innovació Tecnològica" (CIRIT). C. Sala-Villaplana provided technical assistance.

References

[1] F. Guardiola, R. Codony, M. Rafecas and J. Boatella, *Grasas Aceites*, 45 (1994) 164.

[2] W.D. Hubbard, A.J. Sheppard, D.R. Newkirk, A.R. Prosec and T. Osgood, *J. Am. Oil Chem. Soc.*, 54 (1977) 81.
[3] E.C. Naber and M.D. Biggert, *Poult. Sci.*, 64 (1985) 341.
[4] G. Maerker and J. Unruh, *J. Am. Oil Chem. Soc.*, 63 (1986) 767.
[5] S. Bergström and O. Wintersteiner, *J. Biol. Chem.*, 141 (1941) 597.
[6] E. Chicoye, W.D. Powrie and O. Fennema, *Lipids*, 3 (1968) 335.
[7] E. Chicoye, W.D. Powrie and O. Fennema, *Lipids*, 3 (1968) 551.
[8] N.A. Higley, S.L. Taylor, A.M. Herian and K. Lee, *Meat Sci.*, 16 (1986) 175.
[9] L.S. Tsai, K. Ijichi, C.A. Hudson and J.J. Meehan, *Lipids*, 15 (1980) 124.
[10] E. Chicoye, W.D. Powrie and O. Fennema, *J. Food Sci.*, 3 (1968) 581.
[11] S.W. Park and P.B. Addis, *J. Agric. Food Chem.*, 34 (1986) 653.
[12] J.E. Pie, K. Spahis and C. Seillan, *J. Agric. Food Chem.*, 38 (1990) 973.
[13] J.E. Pie and C. Seillan, *Lipids*, 27 (1992) 270.
[14] P. van de Bovenkamp, T.G. Kosmeijer-schuil and M.B. Katan, *Lipids*, 23 (1988) 1079.
[15] J. Bascoul, N. Domergue, M. Olle and A. Crastes de Paulet, *Lipids*, 21 (1986) 383.
[16] H.F. Pennock, G. Neiss and H.R. Mahler, *Biochem. J.*, 85 (1962) 530.
[17] T.C. Ryan, J.I. Gray and I.D. Morton, *J. Sci. Food Agric.*, 32 (1981) 305.
[18] J.N. Morgan and D.J. Armstrong, *J. Food Sci.*, 52 (1987) 1224.
[19] J.N. Morgan and D.J. Armstrong, *J. Food Sci.*, 54 (1989) 427.
[20] J.N. Morgan and D.J. Armstrong, *J. Food Sci.*, 57 (1992) 43.
[21] W.W. Nawar, S.K. Kim, Y.J. Li and M. Vadji, *J. Am. Oil Chem. Soc.*, 68 (1991) 496.
[22] J. Nourooz-Zadeh and L.-Å. Appelqvist, *J. Am. Oil Chem. Soc.*, 66 (1989) 586.
[23] L.S. Tsai and C.A. Hudson, *J. Food Sci.*, 50 (1985) 229.
[24] J. Nourooz-Zadeh and L.-Å. Appelqvist, *J. Food Sci.*, 52 (1987) 57.
[25] J.E. Pie, K. Spahis and C. Seillan, *J. Agric. Food Chem.*, 39 (1991) 250.
[26] S.R. Missler, B.A. Wasilchuk and C. Merritt, *J. Food Sci.*, 50 (1985) 595.
[27] J.E. Knoll, *J. Chromatogr. Sci.*, 23 (1985) 422.
[28] H.A. Emanuel, C.A. Hassel, P.B. Addis, S.D. Bergmann and J.H. Zavoral, *J. Food Sci.*, 56 (1991) 843.
[29] S.W. Park and P.B. Addis, *J. Food Sci.*, 51 (1986) 1380.
[30] B.D. Sander, D.E. Smith and P.B. Addis, *J. Dairy Sci.*, 71 (1988) 3173.
[31] B.D. Sander, D.E. Smith, P.B. Addis and S.W. Park, *J. Food Sci.*, 54 (1989) 874.
[32] W.B. Zhang and P.B. Addis, *J. Food Sci.*, 55 (1990) 1673.
[33] W.B. Zhang, P.B. Addis and T.P. Krick, *J. Food Sci.*, 56 (1991) 716.
[34] P.S. Yan and P.J. White, *J. Am. Oil Chem. Soc.*, 67 (1990) 927.
[35] J. Folch, M. Lees and G.H. Sloane Stanley, *J. Biol. Chem.*, 226 (1957) 497.
[36] F. Guardiola, R. Codony, M. Rafecas and J. Boatella, *J. Chromatogr. A*, 705 (1995) 396.
[37] S.W. Park and P.B. Addis, *Anal. Biochem.*, 149 (1985) 275.
[38] S.W. Park and P.B. Addis, *J. Food Sci.*, 52 (1987) 1500.
[39] S.W. Park and P.B. Addis, *J. Am. Oil Chem. Soc.*, 66 (1989) 1632.
[40] S.W. Park and P.B. Addis, in S.K. Peng and R.J. Morin (Editors), *Biological Effects of Cholesterol Oxides* CRC Press, Boca Raton, FL, 1992, p. 33.
[41] A.S. Csallany, S.E. Kindom, P.B. Addis and J.-H. Lee, *Lipids*, 24 (1989) 645.
[42] I. Csiky, *J. Chromatogr.*, 241 (1982) 381.

ELSEVIER

Journal of Chromatography A, 705 (1995) 305–312

JOURNAL OF CHROMATOGRAPHY A

Automated determination of s-triazine herbicides using solid-phase microextraction

I.J. Barnabas[a], J.R. Dean[b,*], I.A. Fowlis[b], S.P. Owen[a]

[a]*Analytical and Environmental Services, Northumberland Dock Road, Wallsend, Tyne and Wear, NE28 0QD, UK*

[b]*Department of Chemical and Life Sciences, University of Northumbria at Newcastle, Ellison Building, Newcastle-upon-Tyne, NE1 8ST, UK*

First received 14 October 1994; revised manuscript received 23 February 1995; accepted 27 February 1995

Abstract

Solid-phase microextraction (SPME) allows the determination of pollutants in aqueous solution by the adsorption of analytes onto stationary-phase coated fused-silica fibres followed by thermal desorption in the injection system of a capillary column gas chromatograph. This technique has been fully automated using a Varian 8100 autosampler and 3400 gas chromatograph fitted with a nitrogen–phosphorus flame thermionic detector. Fibres coated with 7-μm and 100-μm film thicknesses were used to evaluate the adsorption and desorption of four s-triazines. The resulting gas chromatographic peaks desorbed from the fibres were shown to be comparable to those obtained with direct manual injection. The 7-μm fibre, designed for the analysis of semi-volatile analytes was used to investigate the effect of desorption temperature and on-column focusing temperatures on peak response. The desorption temperature was found to be non-critical and an optimum focusing temperature of 40°C was used throughout the analysis. Evaluation of the 100-μm film fibre demonstrated its potential to adsorb greater quantities of analyte from solution and this study established that an adsorption time of 15 min gave an equilibrium distribution of the solutes between the stationary and liquid phases. With the thicker film fibre it was noted that the effectiveness of the desorption process was reduced at temperatures below 140°C. The linear dynamic range of the technique was evaluated over three orders of magnitude. To enhance method sensitivity, the fibre was used to extract a 0.1 ppb solution of herbicide by repeatedly adsorbing and desorbing from the same solution and focusing the combined solutes at the front of the analytical column prior to elution and analysis.

1. Introduction

Modern methods for the analysis of organic pollutants in water are frequently incompatible with the requirements of laboratories to provide a high turnaround of inexpensive analysis without creating additional environmental problems. Semi-volatile compounds are traditionally analysed by liquid–liquid extraction (LLE) procedures using an organic solvent [1]. These methods can be time-consuming, use large volumes of organic solvent and are difficult to automate. In addition, final preconcentration prior to analysis is frequently necessary. The more recent technique of solid-phase extraction (SPE) is now replacing LLE in many situations [2]. However, SPE can be expensive with the cartridges usually being disposed of after one extraction. Also,

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00279-0

SPE can suffer from high blank results and the entire analysis can be lengthy with intermediate washing and drying stages slowing the process. In spite of these difficulties SPE has been accepted for use both on- and off-line for preconcentration and clean-up of environmental analytes [3–5]. Supercritical fluid extraction (SFE) has attracted much attention for its rapid, non-organic solvent extractions, but low recoveries and long extraction times make it unsuitable for direct extraction from water samples [6]. However, the combination of SPE and SFE has been shown to be useful for extraction of pesticides [6–8] and surfactants [9,10] from water.

Solid-phase microextraction (SPME) offers solutions to many of the above problems. The main advantages of SPME include, little modification of existing hardware, faster sample preparation followed by solvent-free analysis of both volatile and semi-volatile compounds with full automation possible [11]. SPME is commonly used for the analysis of volatiles, such as substituted benzenes [12–16], which are routinely analysed by purge-and-trap methods [17], since they are easily desorbed at low temperatures. However, some analyses of semi-volatile analytes have been reported including PAHs [18] and PCBs [19]. Recent fibre developments have included a new polyacrylate fibre which is capable of extracting more polar compounds and has been used to extract phenols direct from water and headspace samples [20]. Much of the work reported so far has used "home-made" SPME fibres and holders [12–16,18–20] which require specialist knowledge and which inevitably suffer from fibre-to-fibre inconsistency. This paper discusses the automated analysis of semi-volatile s-triazine herbicides using a commercially available SPME fibre and holder. The s-triazine group of herbicides is one of the most widely used soil-applied herbicides in Europe [21]. Two fibres with the same coating but of different film thickness are compared with the aim of establishing an analytical method for extraction and detection at sub-ppb levels.

2. Experimental

2.1. SPME apparatus

The fibres used were coated with poly(dimethylsiloxane) at both 100 μm and 7-μm thickness and designed for automated analysis only. The holder used to protect the delicate fibres during adsorption and desorption was also used specifically for automated analysis (both supplied by Supelco, Poole, Dorset, UK). A Varian 8100 autosampler was used to automate the SPME. The standard syringe assembly was removed from the autosampler and replaced with the SPME holder containing the fibre. The autosampler was used together with a Varian 3400 gas chromatograph (GC) fitted with conventional split/splitless injector and a thermionic specific detector (TSD), both supplied by Varian (Walton on Thames, Surrey, UK). The GC was fitted with a 30 m $\times$ 0.25 mm I.D. 0.25 μm DB-5 capillary column (J&W Scientific, supplied by Phase Separations, Clwyd, Wales, UK). Pre-drilled Thermogreen injection septa (Supelco) were used throughout the analysis to reduce septa coring and bleed. Various injection and initial column temperatures were investigated during the study, however, the detector settings, gas flow-rates, pressures and temperature programme remained constant. The detector temperature of 310°C with a TSD bead current of 3.100 A was kept constant. Also the inlet pressure of 15 psi (helium) and the make-up gas velocity of 175 ml/min nitrogen with 55 ml/min hydrogen, remained constant. The temperature programme used to elute the analytes focused at the front of the GC column after desorption was as follows: variable initial column temperature, hold time 1 min, then 15°C/min to 150°C (hold 0.5 min), finally 4°C/min to 210°C.

The SPME autosampler was controlled by Labview software through a PC which allowed adsorption and desorption times to be set, whether headspace or liquid sampling was required, and the number of vials to be automatically analysed. Data collection and handling was also PC controlled via a Varian GC Star

workstation. Only minor modification of the entire autosampler/GC system was required including exchange of the syringe assembly for the SPME holder, installation of the Labview software to control the autosampler and connection of the PC to the autosampler.

2.2. Reagents

All herbicides were purchased from Promochem (Herts, UK) and used to prepare stock standards in methanol (Rathburns, Walkerburn, UK). The stock standards were then diluted to the required concentration, in volumetric flasks, using methanol to produce standard solutions. HPLC grade water (J.T. Baker, Berks, UK) was used throughout the analysis. Aliquots (1.2 ml) of standard solutions were injected into 2-ml autosampler vials (Chromocol, Herts, UK) using a micropipette to ensure the fibre was fully emersed in the solution and to prevent sample-to-sample variation. The vials were then sealed with septa and placed in the autosampler carousel to await analysis.

2.3. Automated SPME procedure

The main advantage of the commercial SPME assembly over a home-made version is its ability to perform fully automated analysis of multiple samples. This was achieved using a standard GC autosampler which was controlled using specialist software from a PC. Once the adsorption and desorption times, sampling mode (liquid or headspace) and the option for multiple or single vial analysis had been put into the software the autosampler routine was as follows. The carousel containing the samples was moved forward, rotated under an optical sensor to count the number of vials and retreated. The fibre on the SPME assembly was then fully protruded and quickly returned to ensure no faults were present. The carousel was once again moved under the SPME assembly and the sheathed fibre allowed to pierce the sample vial septa. Once inside the vial the fibre was extended to a pre-set amount, depending on whether liquid or headspace sampling was selected, where it remained for the set adsorption time. Upon completion of the adsorption period the fibre was again sheathed inside the protective needle and the whole syringe carriage moved to retract the fibre from the sample vial. The carousel was then retreated to expose the injection port and the needle placed in the hot split/splitless injector where once again the fibre was protruded from its protective sheath where it remained for the set desorption time. At the end of the desorption time the GC temperature programme was automatically started together with the Star workstation integrator. When multiple samples were extracted, a pre-adsorb delay could be included into the SPME software which allowed the fibre to begin adsorption in the next sample before the GC temperature programme had finished from the current sample. This allowed considerable time-savings when long adsorptions were being used.

2.4. SPME fibre blanks

Before any extractions can be performed using a new SPME fibre it must first be conditioned in an injector usually at a temperature above that which is to be used for routine desorption, but below the maximum operating temperature of the fibre. For the experiments involving the 100-μm fibre a blank desorption temperature of 220°C was chosen as this was the highest temperature which could be used without possible removal of the poly(dimethylsiloxane) phase. The fibre was conditioned in the injector for a minimum of 3 h at this temperature, with the split vent open, to fully remove any contaminants which might cause very high baseline noise and large ghost peaks. After this the fibre was repeatedly injected into the GC until the resulting chromatogram was clear from any contamination. A blank desorption was also carried out each morning prior to extraction to ensure any airborne interferences adsorbed when the

fibre was left unused overnight in the laboratory atmosphere, were removed.

3. Results and discussion

All experiments were initially done at the 1 ppm level to establish the appropriate protocol before determination of the s-triazines at a more realistic environmental level.

3.1. Comparison between a manual injection and a SPME extraction

Initially a manual injection of 1 ppm of all four s-triazines studied was compared with a 1 ppm aqueous sample containing the same concentration, using the 100-μm fibre. An acetone solution containing simazine, atrazine, propazine and trietazine at 1 ppm was manually injected into the injector at 250°C with the split vent closed. The split vent was re-opened after 0.75 min. This was compared with a SPME extraction using the freshly blanked 100-μm fibre which was absorbed for 5 min in the 1 ppm aqueous solution. After the adsorption was completed the fibre was desorbed for 15 min in the GC injector at 220°C. The injector was operated in split mode throughout all extractions. During the desorption stage the column temperature was maintained at 40°C which allowed the desorbed analytes to be refocused at the front end of the analytical column. No cryogenic cooling was required because of the semi-volatile nature of the herbicides. The two chromatograms resulting from the manual injection and the initial SPME extraction are shown in Fig. 1.

It is obvious from the chromatograms that there is no degradation of peak shape during a SPME extraction and that peak width does remain constant with no tailing observed. This indicates that a column temperature of 40°C during desorption is sufficiently low to successfully focus all of the herbicides. Also the retention times of the four peaks in the extraction exactly match those in the manual injection proving that the peaks shown in the second chromatogram are actually the s-triazine herbicides of interest.

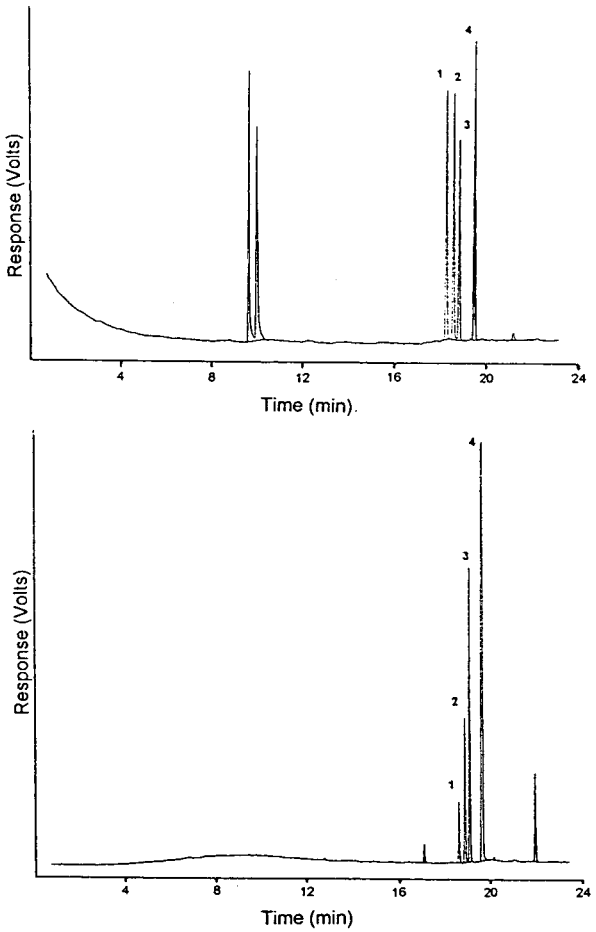


Fig. 1. Comparison between a manual injection (upper) and a SPME extraction using a 100-μm fibre (lower). Peaks: 1 = simazine, 2 = atrazine, 3 = propazine, 4 = trietazine; 1 ppm solution injected in acetone; SPME extraction; 5 min adsorption followed by a 15-min desorption at 220°C.

3.2. Effect of adsorption time

The 100-μm fibre was used to investigate the effect of adsorption time on the peak areas of the four herbicides. A 1 ppm solution of the four analytes was used as a test mixture and the fibre was adsorbed over a range of times from 30 s to 15 min. All extractions were carried out at ambient temperature (~23°C) and were unstir-

red. After each adsorption the fibre was desorbed at 220°C for 15 min. The results are shown graphically in Fig. 2.

Subsequent blank desorption at 220°C after each extraction indicated no carry over of analyte was occurring between extractions. It can be seen from the graph that, as expected, after an initial steep rise as adsorption time is increased the peak area rises less dramatically and is approaching a plateau. The adsorption was not extended beyond 15 min as the peaks obtained, especially for trietazine, were so large that accurate integration became impossible.

3.3. Effect of desorption temperature on the 100-μm fibre

The desorption temperature was determined by maintaining a constant adsorption time of 5 min and using a 0.3 ppm solution so as to reduce the size of the peak areas obtained. This ensured that accurate integration was always possible. After the adsorption period the fibre was inserted into the injector, which was kept at various temperatures ranging from 220°C to 120°C, for 15 min. This is the temperature range recommended to be used with the 100-μm fibre. After each extraction of a 0.3 ppm solution the injector temperature was increased again to 220°C and a blank desorption undertaken to ensure no carry over occurred. Altering the desorption temperature, within the range 220–140°C, has no real effect on peak area. Below 140°C peak areas begin to decrease. Following 140°C desorptions, blank desorptions at 220°C revealed that a residual 5% of analytes were being retained on the fibre coating at the lower desorption temperature. Subsequently the fibre desorptions were run at the maximum temperature of 220°C since this appeared to have no adverse effect on fibre performance.

3.4. Dynamic range of the 100-μm fibre

A short adsorption time of 5 min and desorption time of 15 min at 220°C were then used to study the dynamic range of the 100-μm fibre. A series of aqueous working solutions containing the compounds at various concentrations were made from the stock standards in ranges between 1.0 and 0.1 ppm, 0.1 and 0.01 ppm and 0.01 and 0.001 ppm. Due to operational time constraints the sets of working standards were run on consecutive days. The linear correlation coefficient, for the three separate experiments, was on average 0.978. Thus indicating that the fibre is linear and may be used over three orders of magnitude. Below 0.001 ppm the peaks obtained were very small and it was not possible to integrate them with any certainty. Increasing the bead current to the TSD detector would have increased its sensitivity and may have allowed a lower concentration in solution to be detected but this would have been at the expense of the bead lifetime which is severely reduced when the detector is operated at elevated currents.

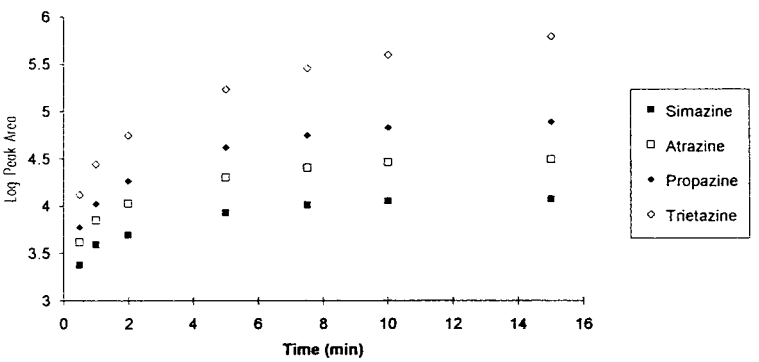


Fig. 2. Effect of adsorption time on peak area using a 100-μm fibre; 1 ppm solution, desorption time 15 min at 220°C.

3.5. Comparison between the 100-μm and the 7-μm fibre

The 100-μm fibre, designed for analysis of volatile compounds, was then replaced with a 7-μm fibre which was specifically designed for extraction of semi-volatile analytes. The much thinner coating on this fibre allowed the phase to be strongly chemically bound to the silica support thus enabling higher desorption temperatures to be used without risk of phase degradation. The fibre was actually capable of being used over the temperature range 220–320°C which should allow higher boiling point semi-volatile compounds to be successfully desorbed. The obvious disadvantage of the thinner film fibre coating was the poor detection limit. Once again the fibre required several hours blank desorption at an elevated temperature before use.

The difference in the amount adsorbed between the 100-μm and 7-μm fibres is illustrated in Fig. 3 where a 1 ppm solution is extracted for 5 min using a 100-μm fibre and for 15 min using the 7-μm fibre. Both fibres were desorbed for 15 min at 220°C and 250°C, respectively. Although the peak shape in both chromatograms is almost identical, it is obvious that the 7-μm fibre is only capable of adsorbing a fraction of the amount adsorbed by the thicker film of the 100-μm fibre.

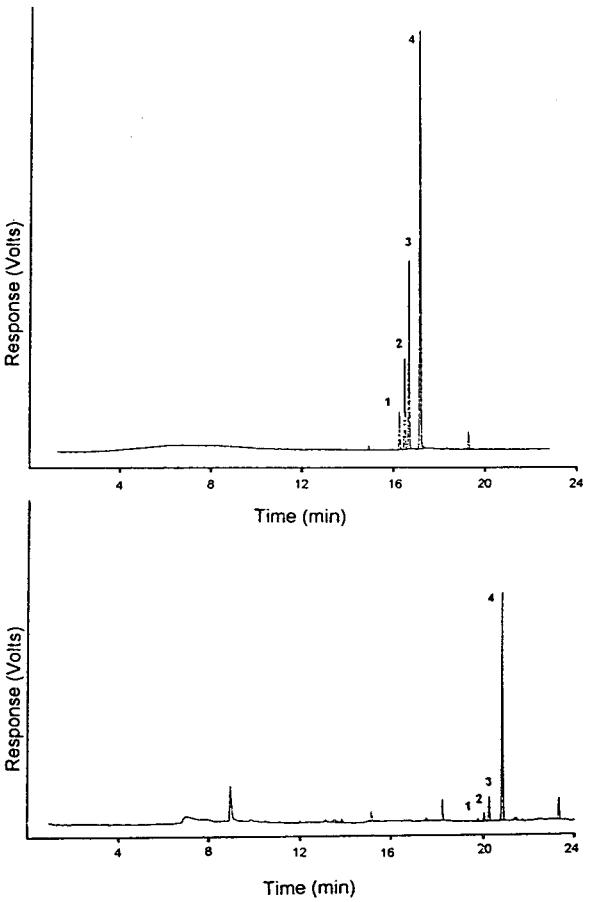


Fig. 3. Comparison between a 1 ppm extraction using a 100-μm fibre (upper) and a 7-μm fibre (lower). Peaks: 1 = simazine, 2 = atrazine, 3 = propazine, 4 = trietazine; 100-μm fibre adsorbed for 5 min, 7-μm fibre adsorbed for 15 min; both desorbed for 15 min at 220°C and 250°C, respectively.

3.6. Effect of desorption temperature on the 7-μm fibre

The effect of desorption temperature on the 7-μm fibre was determined over the working range of the fibre. The 7-μm fibre was used to adsorb a 1 ppm solution for 10 min before a 15 min desorption at the temperature studied. As before, at the end of each extraction a blank desorption was carried out at the maximum operating temperature of 320°C to show any carry over at lower desorption temperatures. No difference in peak area over the temperature range studied was noted. It was observed that regardless of desorption temperature no carry over of any herbicide occurred. Therefore, it was concluded that all analytes could be removed at a desorption temperature of 220°C, irrespective of fibre coating thickness.

3.7. Effect of column focusing temperature

The effect of column focusing temperature was also investigated to determine whether 40°C was the optimum temperature. A range of temperatures between 40°C and 100°C were chosen, at 10°C intervals, since it was not possible to cool the column, in a short period of time, below 40°C without cryogenic cooling. The effect on both peak shape and area was noted for a 10-min adsorption of a 1 ppm solution using the 7-μm

fibre. The fibre was then desorbed for 15 min at 270°C with the column kept at a constant temperature within the range chosen. The usual temperature ramp was used to elute the compounds from the column regardless of the initial column temperature. The focusing temperature ($n = 7$) has no significant effect on peak area (herbicide: mean peak area counts, %R.S.D.; simazine: 850, 39%; atrazine: 1900, 11%; propazine: 5200, 12%; trietazine: 42100, 6%) or peak shape. The %R.S.D. difference for simazine was attributed to its significantly lower response. It was decided to keep the initial column temperature of 40°C since the majority of work had been done at 40°C and there appeared to be no real benefit in using elevated focusing temperatures.

Increasing the desorption temperature above 220°C did not effect the peak. This indicates that the 100-μm fibre may be of use in extracting not only volatile but also semi-volatile compounds. It was therefore decided, from these results, to use the thicker fibre as it was obvious that the 7-μm coating would be incapable of extracting sufficient analyte in a short space of time to be of any use in the analysis of low concentration solutions.

3.8. Multiple extractions and analysis of low concentration solutions

Within Europe the EEC has set limits for individual pesticides and herbicides in drinking water of 0.1 ppb [22]. It is therefore essential for any screening method to be able to detect at this level. From the previous study using the 100-μm fibre it was obvious that it would be impossible to detect a 0.1 ppb solution of s-triazine herbicides in a single extraction. Multiple adsorptions were therefore carried out on the same sample vial and desorbed in the injector. However, instead of following each desorption with a temperature programme to elute the analytes, the herbicides were stacked at the front of the column which was maintained at the focusing temperature. Multiple extractions obviously take considerable time and to reduce this the optimum desorption time was briefly investigated. A 0.5 ppm solution of the four herbicides was placed in the carousel and adsorbed for 3 min using the 100-μm fibre. The fibre was then desorbed at 220°C for the usual 15 min. This was compared with an identical adsorption but with a desorption time of only 5 min. Following the shorter desorption time a blank was run to determine any carry over of analyte. No significant difference in the peak areas after the two different desorptions were noted. The blank carried out after the 5-min desorption indicated a small amount of carry over (<1%) for propazine with the shorter desorption time. Although this is not desirable in single extractions, it is irrelevant when performing multiple adsorptions since all of the analyte is trapped at the front of the column. The 5-min desorption time at 220°C was subsequently used in the multiple adsorption experiments.

The multiple extraction technique was used to extract a 0.1 ppb solution of the herbicides. A 10-min adsorption followed by a 5-min desorption was repeated ten times from a single solution and stacked at the front on the GC column. The whole procedure (15 min per cycle × 10 repeats) in total was repeated three times. The precision of the results ($n = 3$) ranged from 6% R.S.D. for propazine to 20% R.S.D. for atrazine thus indicating the feasibility of the technique to detect low concentration solutions by SPME which would normally be well below the limit of detection of the detector used.

4. Conclusion

SPME has been shown to be a simple and elegant technique which does not require any solvent and only minor modification of existing laboratory hardware. SPME cannot only be used to extract volatile analytes commonly analysed by headspace or purge-and-trap analysis but is also capable of extracting semi-volatile analytes, often extracted using SPE, at a fraction of the cost (the fibres used in this study have been reused approximately 100–150 times). An initial investigation of the principle operating parameters using a 1 ppm mixture of the s-triazines was done. This was followed by the use of a multiple

adsorption technique for the extraction of s-triazine herbicides at the sub-ppb level.

SPME, although still in its infancy as an environmental extraction technique, has indicated sufficient promise to ensure its place for routine analysis of aqueous matrices. At present the amount of data on quantification using SPME is still limited and requires further investigation. Also, the advent of new commercially available fibres with a range of specific analyte coatings will help to promote its use in environmental laboratories of the future.

Acknowledgements

We gratefully acknowledge the financial assistance of Analytical and Environmental Services (AES). The SPME assembly and software was generously supplied by Varian Associates.

References

[1] Determination of nitrogen- and phosphorus-containing pesticides in water by GC–NPD, EPA Method 507, US Government Printing Office, Washington, DC, 1988.
[2] A. Balinova, J. Chromatogr., 643 (1993) 207.
[3] U.A. Th. Brinkman, J. Chromatogr. A, 665 (1994) 217.
[4] V. Pichon and M.C. Hennion, J. Chromatogr. A, 665 (1994) 269.
[5] J.C. Molto, Y. Pico, G. Font and J. Manes, J. Chromatogr., 555 (1991) 137.
[6] I.J. Barnabas, J.R. Dean, S.M. Hitchen and S.P. Owen, J. Chromatogr. A, 665 (1994) 307.
[7] I.J. Barnabas, J.R. Dean, S.M. Hitchen and S.P. Owen, Anal. Chim. Acta, 291 (1994) 261.
[8] P.H. Tang, J.S. Ho and J.W. Eichelbergwer, J. Assoc. Off. Anal. Chem., 76 (1993) 72.
[9] M. Kane, J.R. Dean, S.M. Hitchen, C.J. Dowle and R.L. Tranter, Anal. Proc., 30 (1993) 399.
[10] M. Kane, J.R. Dean, S.M. Hitchen, C.J. Dowle and R.L. Tranter, Analyst, 120 (1995) 355.
[11] C.L. Arthur, L.M. Killam, K.D. Buchholz, J. Pawliszyn and J.R. Berg, Anal. Chem., 64 (1992) 1960.
[12] D. Louch, S. Motlagh and J. Pawliszyn, Anal. Chem., 64 (1992) 1187.
[13] S. Motlagh and J. Pawliszyn, Anal. Chim. Acta., 284 (1993) 265.
[14] B. MacGillivray, J. Pawliszyn, P. Fowlie and C. Sagara, J. Chromatogr. Sci., 32 (1994) 317.
[15] C.L. Arthur, L.M. Killam, S. Motlagh, M. Lim, D.W. Potter and J. Pawliszyn, Environ. Sci. Technol., 26 (1992) 979.
[16] D.W. Potter and J. Pawliszyn, J. Chromatogr., 625 (1992) 247.
[17] Measurement of Purgable Organic Compounds in Water by Capillary Column GC-MS, EPA Method 524.2, US Government Printing Office, Washington, DC, 1988.
[18] M.E. Cisper, W.L. Earl, N.S. Nogar and P.H. Hemberger, Anal. Chem., 66 (1994) 1897.
[19] C.L. Arthur and J. Pawliszyn, Anal. Chem., 62 (1990) 2145.
[20] K.D. Buchholz and J. Pawliszyn, Anal. Chem., 66 (1994) 160.
[21] K.A. Hassall, The Biochemistry and Uses of Pesticides, MacMillan, London, 1990.
[22] D. Barcelo, J. Chromatogr., 643 (1993) 117.

ELSEVIER

Journal of Chromatography A, 705 (1995) 313–317

JOURNAL OF CHROMATOGRAPHY A

Gas chromatographic determination of 5-chloro-2-(2,4-dichlorophenoxy)-phenol in the waste water of a slaughterhouse

Milica Graovac, Miša Todorović, Milan I. Trtanj, Miroslav M. Kopečni, Jožef J. Čomor*

Vinča Institute of Nuclear Sciences, Chemical Dynamics Laboratory, P.O. Box 522, YU-11001 Belgrade, Yugoslavia

First received 22 November 1994; revised manuscript received 7 February 1995; accepted 3 March 1995

Abstract

A straightforward analytical method for the determination of 5-chloro-2-(2,4-dichlorophenoxy)-phenol (commercially known as Irgasan DP 300) in the waste water of a slaughterhouse was developed. The waste water, first cleaned from fats and oils by petroleum ether (b.p. 40–80°C) at pH 11, was extracted by benzene at pH 1 and this Irgasan-containing extract, was further purified by sodium sulfate/silica gel adsorption. After preconcentration and diazomethane derivatization of the extract, capillary GC–ECD determination was applied. The procedure yields a routine recovery of 88.1% regardless of the sample concentration. The lower detection limit for Irgasan in the waste water was found to be 0.2 ng l^{-1}.

1. Introduction

Due to its excellent bactericide properties and very high chemical stability, 5-chloro-2-(2,4-dichlorophenoxy)-phenol (commercially known as Irgasan DP 300) has been widely used in various cosmetic products and as an active component in many commercial disinfectants. One of such disinfectants, coded Albin® DP (product of Albus, Novi Sad, Yugoslavia) has been extensively used in numerous Yugoslav slaughterhouses for the disinfection of working tools.

As pointed out, Irgasan is very stable; it was detected in an industrial waste-water stream following waste water treatment [1], in river water and sediments [2,3], and even in fish tissue [4]. Further, it was confirmed that Irgasan reacts with chlorine in water, producing dichloro- and trichloro-2-(2,4-dichlorophenoxy)-phenols, followed by the decomposition of these intermediates to chlorophenols [5]. As a consequence, the commonly used chlorination of waste water, in order to deodorize and disinfect effluents prior to discharge, will convert Irgasan to no less harmful chlorophenols. Next, incineration of the municipal sewage sludge contaminated with Irgasan represents a potential environmentally hazardous process, because of the ability of chlorinated 2-phenoxyphenols (named predioxines) to undergo ring closure at elevated temperatures, producing extremely toxic polychlorinated dibenzo-*p*-dioxins [6–8].

Since Irgasan DP 300 is a recognized environmental pollutant, we decided to develop a pro-

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00313-4

cedure for its determination in a very special waste-water stream of a slaughterhouse, being a known source of contamination. Previously reported methods were found to be inadequate for precise quantitative determinations of Irgasan in the slaughterhouse waste waters. A reported GC–MS method [1] for Irgasan identification in waste water was stated to be valuable only for qualitative analysis [1]. Renberg [4] published three methods for Irgasan determination in (a) water, (b) fish tissue and (c) soil, based on anion-exchange preconcentration. Method (a), developed for river water, applied to the more complex slaughterhouse waste water gave far lower values than expected. We believe that the reason is the very high Cl^- content in this waste water (several orders of magnitude higher than the Irgasan concentration) and that the ion exchanger is saturated by Cl^- anions instead of by Irgasan. Methods (b) and (c), assuming a rather complex matrix, could have been adopted to our waste water, but the resulting method would be too complicated and time consuming. Therefore, our strategy for the determination of Irgasan was based on simple and fast extraction procedures.

2. Experimental

2.1. Reagents and reference compounds

All solvents used throughout this work were Merck products, of pro analysi grade. The reference sample of Irgasan DP 300 was granted from Albus (Novi Sad, Yugoslavia). Silica gel, particle size of 0.2–0.5 mm, was a product of Kemika (Zagreb, Croatia) and was dried overnight at 105°C. Diazomethane for derivatization was freshly prepared every week.

2.2. Instrumentation and operating conditions

Gas chromatographic measurements were carried out on a Spectra Physics Model SP7100 equipped with an electron-capture detector operating at 350°C. The chromatographic column was a SPB™-5 (Supelco) fused-silica capillary column: 30 m × 0.32 mm I.D., 1.0 μm film thickness. Nitrogen was used as a carrier gas, the injections were made in the splitless mode and the injector was operated at 250°C. The temperature program was started at 200°C and immediately ramped to 250°C at 20°C/min; then without hold-time ramped further to 300°C at 2.5°C/min and held for 5 min.

2.3. Sample preparation and GC–ECD analysis

The pure organic samples (20–250 ml reference organic solutions or organic extracts after clean-up procedure) were preconcentrated in a Kuderna–Danisch apparatus, then evaporated to dryness applying a slow stream of purified dry air to the liquid surface. The residue was redissolved in 0.1 ml of dry diethyl ether containing 10 ng of hexachlorobenzene as an internal standard, and finally 0.1 ml of diethyl ether saturated with diazomethane was added. Injections were made 5 min after the derivatization and the samples prepared in this way were stable during several days.

3. Results and discussion

3.1. Extraction procedure

Selection of the organic extractants for trace analysis in waste-water streams is critical and, therefore it must be performed carefully. As our goal was to develop a method for determination of Irgasan at a concentration as low as possible, the extractants were not tested for selectivity.

The best conditions for extraction were selected on the basis of the distribution coefficient of Irgasan between water and *n*-hexane, carbon tetrachloride, benzene, cyclohexane and dichloromethane. The distribution coefficients were determined as follows: 25 ml of organic solutions containing 5 $\mu g\ ml^{-1}$ of Irgasan were vigorously mixed for 15 min with the same volume of distilled water the pH of which was previously adjusted to a certain value. The lower pH values were adjusted by adding an 8 *M* H_3PO_3 solution to water, while the higher pH

values were adjusted with 1 *M* NaOH. The use of phosphoric acid for pH adjustments during extraction is recommended in the case of phenols, in order to avoid possible oxidation processes which can be initiated when using other, oxidising, acids [9]. After phase separation, an aliquot of 20 ml of organic phase was analysed as described earlier.

The obtained distribution coefficients are shown in Fig. 1. As expected, the distribution coefficient strongly depends on the pH of the aqueous phase: the higher the acidity, the higher the distribution coefficient (a result of the suppression of the phenolic group dissociation). Among the tested extractants, benzene shows the highest distribution coefficient, allowing the extraction of 90.9% of Irgasan in a single step when the water-phase pH is adjusted to 1.0 and the volume ratio used for extraction is 1:4 (benzene–water). This volume ratio was selected as an optimal one, taking into account the Irgasan recovery and benzene consumption. On the other hand, the low distribution coefficient at high pH values of the water phase allows us to remove efficiently fats, oils and tissue residues by extraction with suitable extractants, thereby reducing the amount of unwanted matrix, without significant loss of Irgasan.

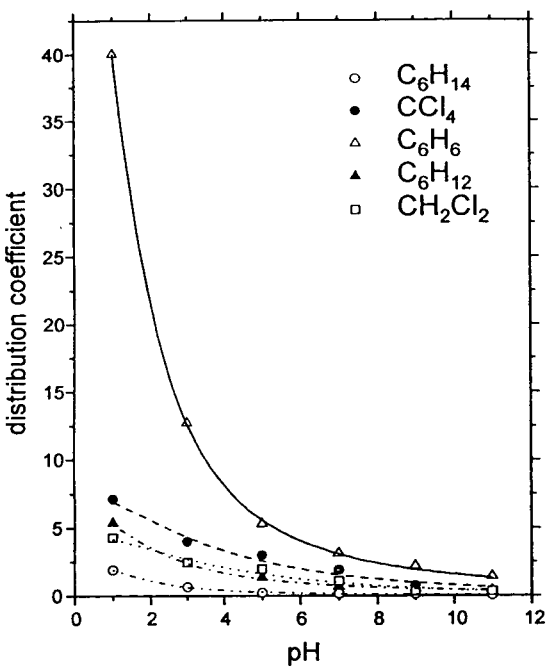


Fig. 1. Experimentally obtained distribution coefficients of Irgasan DP 300 between various organic extractants and water of different pH values.

Petroleum ether is the solvent of choice for cleaning water and solid samples from oils and being a loosely defined mixture of low boiling hydrocarbons its extraction capabilities are well aproximated by hexane. The negligible losses of Irgasan by petroleum ether extraction at pH 11 are corrected by the recovery factor.

3.2. Sample clean-up

Though the concentration of fats and oils can be significantly reduced in the samples by extraction (with petroleum ether at pH 11), this is usually not sufficient, since the fat content in the slaughterhouse waste water very often exceeds several g l^{-1}. In order to extend the life of the chromatographic columns, another sample clean-up procedure is necessary. A short chromatographic column (1 cm I.D. made from glass), filled with 2 g of anhydrous Na_2SO_4 and 4 g of freshly dried silica gel (0.2–0.5 mm particle size) was used for this purpose. After all the organic extract had passed the column, it was rinsed with 25 ml of benzene and all the eluates were joined. The columns were freshly filled for every sample without recycling the adsorbents to avoid possible sample contamination.

3.3. The optimised procedure and recovery

Based upon the dependence of the distribution coefficient on pH, the following procedure was selected for determination of Irgasan in the waste water: Take 1000 ml of the waste water, adjust its pH to 11.0 by adding 1 *M* NaOH, transfer the sample to a mixing funnel, add 250 ml of petroleum ether and shake vigorously for 15 min. After the phases have been separated, adjust the water-phase pH to 1.0 by adding 8 *M* H_3PO_4, transfer the sample to a mixing funnel, add 250 ml of benzene and shake vigorously for 30 min. Separate the phases and allow the

organic phase to pass through the clean-up column, wash the column with 25 ml of benzene and join the eluates. Transfer the cleaned benzene extract to the Kuderna–Danish apparatus and concentrate it to less than 1 ml, rinse the apparatus with 1 ml of benzene, join the concentrate and the benzene rinse in a 2-ml receiving vessel and evaporate the sample to dryness applying a slow stream of cleaned dry air to the liquid surface. Add 0.1 ml of diethyl ether solution with the internal standard (100 ng ml^{-1} of hexachlorobenzene) and 0.1 ml of diazomethane in ether. After 5 min or more inject 5 μl of the sample into the GC injector.

The recovery of Irgasan was determined by analysing the spiked waste-water samples taken from slaughterhouses not using disinfectants based on Irgasan DP 300. Six groups of spiked samples (three samples each) were prepared resulting in an initial Irgasan concentration of 1, 10, 25, 50, 75 and 100 ng l^{-1}. The concentration of Irgasan in water was adjusted by addition of an adequate amount of acetone containing 50 ng ml^{-1} of Irgasan. The spiked samples were homogenised for 1 h by stirring and then analysed according to the described procedure. Based on the known initial concentration, the recovery of all the samples was calculated and the results are shown in Fig. 2. It is obvious that the Irgasan concentration has no significant effect on the recovery (the calculated slope of the data is 0.003% ng^{-1} l) and an average value of 88.1% (with an S.D. of 1.007) was used for all measurements.

The detection limit of the method described above was determined as the standard deviation of three consecutive analyses of the same spiked sample containing 0.5 ng l^{-1} of Irgasan. It was found that the detection limit of the described procedure for determination of Irgasan DP 300 in the slaughterhouse waste water amounts to 0.2 ng l^{-1}.

Fig. 3 shows two typical chromatograms of a spiked (a) and a real (b) sample from two different slaughterhouses. The chromatographic conditions were optimized to give the best resolution around the Irgasan peak to avoid any possible misinterpretation, other parts of the chromatogram were of no interest to our investigation. A small peak next to the Irgasan peak was observed in each chromatogram of spiked samples and of samples where Irgasan was detected. Identification of this compound would require a GC–MS analysis, which we could not afford. However, we believe that this

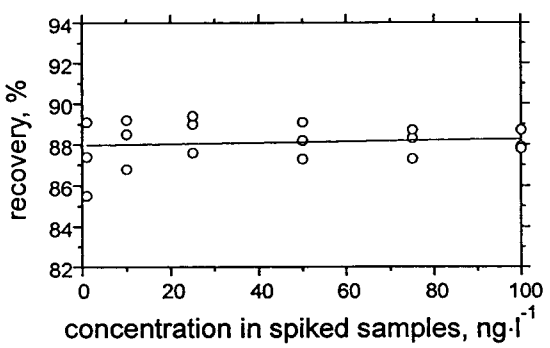


Fig. 2. Recovery of Irgasan DP 300 as a function of its total concentration in spiked samples.

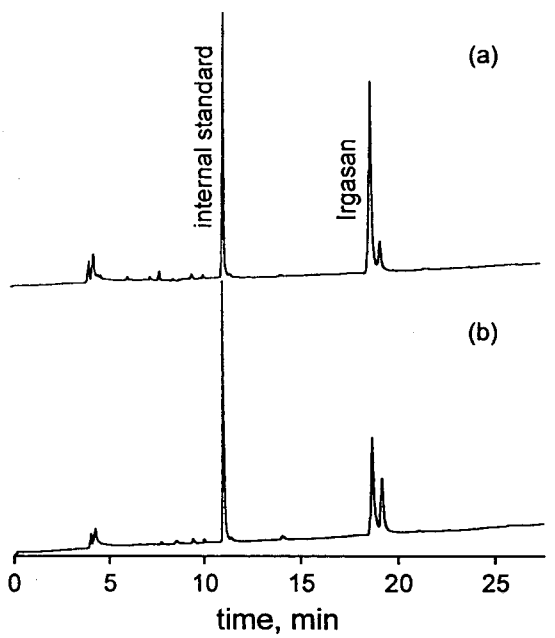


Fig. 3. Typical chromatograms of a spiked (a) and a real sample (b) from different slaughterhouses.

peak comes from an isomer of Irgasan, which is present as an impurity right from its synthesis.

Since the reproducibility of the method (tested by analysing 25 different samples three times) was better than 5%, it was decided to use the developed method in a large scale monitoring study of the waste waters of slaughterhouses in Belgrade and surroundings (these results will be available upon request till the end of 1995).

4. Conclusions

The procedure for the determination of Irgasan DP 300 in the slaughterhouse waste-water stream presented in this paper is carefully developed and optimized in order to get an Irgasan recovery as high as possible, giving very low detection limits, yet being fast and consuming chemicals as little as possible. As has been shown, the Irgasan recovery practically does not depend on the sample concentration in the range from 1 to 100 $ng\,l^{-1}$, offering us a reliable method for determination of Irgasan DP 300.

Acknowledgement

The authors are indebted to the Ministry of Science and Technology of the Republic of Serbia for partial financial support.

References

[1] R.A. Hites, V. Lopez-Avila, Anal. Chem., 51 (1979) 1452A.
[2] G.A. Jungclaus, V. Lopez-Avila and R.A. Hites, Environ. Sci. Technol., 12 (1978) 88.
[3] V. Lopez-Avila, R.A. Hites, Environ. Sci. Technol., 14 (1980) 1382.
[4] L. Renberg, Anal Chem., 46 (1974) 459.
[5] S. Onodera, M. Ogawa, S. Suzuki, J. Chromatogr., 392 (1987) 267.
[6] H.G. Langer, T.P. Brady, P.R. Briggs, Environ. Health Perspect., 5 (1973) 3.
[7] C.-A. Nilsson, L. Renberg, J. Chromatogr., 89 (1974) 325.
[8] C.-A. Nilsson, K. Andersson, C. Rappe, S.-O. Westermark, J. Chromatogr., 96 (1974) 137.
[9] M.M. Kopečni, M.V. Tarana, S.D. Čupić and J.J. Čomor, J. Chromatogr., 462 (1989) 392.

ELSEVIER

Journal of Chromatography A, 705 (1995) 319–324

JOURNAL OF CHROMATOGRAPHY A

Control of selectivity in micellar electrokinetic chromatography by modification of sodium dodecyl sulfate micelles with organic hydroxy compounds

Shoichi Katsuta*, Tohru Tsumura, Koichi Saitoh, Norio Teramae

Department of Chemistry, Faculty of Science, Tohoku University, Sendai 980-77, Japan

First received 12 December 1994; revised manuscript received 21 February 1995; accepted 1 March 1995

Abstract

Modification of micellar phases with solubilized solutes was examined to control the separation in micellar electrokinetic chromatography (MEKC). The addition of organic hydroxy compounds (1-hexanol, cyclohexanol and phenol) as modifiers to a micellar solution of sodium dodecyl sulfate specifically decreased the capacity factor of some aromatic analytes possessing hydrophilic functional groups. The effect of phenol was different in selectivity to that of 1-hexanol and cyclohexanol. The effects of these modifiers were mainly explained in terms of the saturation of the micellar palisade layer with the modifiers and the hydrogen-bond interaction between the modifier and analyte molecules in the micellar phase. Such micellar modification were applied to improve the MEKC separation.

1. Introduction

Micellar electrokinetic chromatography (MEKC) is a useful separation method which allows excellent separations of neutral compounds utilizing capillary electrophoretic techniques [1]. The separation of neutral compounds in MEKC is performed on the basis of their differential partitioning between an electroosmotically pumped aqueous phase and a slower moving, electrophoretically retarded, ionic micellar phase. Therefore, it is difficult in principle to separate compounds whose partition coefficients between the micellar and aqueous phases are close to each other. In such a case, selective control of their partition coefficients is necessary to improve the separation.

Several ionic surfactant micelles [2,3] and mixed micelles [4,5] have been used to optimize the selectivity of MEKC separation. The types of surfactants commercially available and efficient for MEKC [1] are, however, not numerous. The use of additives such as hydrophilic organic solvents (methanol [6], dimethylformamide [7], etc.) and hydrophilic solutes (urea [8], cyclodextrin [9], etc.) in the micellar solution has also been studied. These additives, except for cyclodextrin, are mainly used to enhance the solubility of hydrophobic analytes in the aqueous phase and usually decrease the partition coefficient of the analytes unselectively.

On the other hand, our preliminary study

* Corresponding author. Present address: Department of Chemistry, Faculty of Science, Science University of Tokyo, Kagurazaka, Shinjuku-ku, Tokyo 162, Japan

0021-9673/95/$09.50
SSDI 0021-9673(95)00291-X

indicated that the selectivity of MEKC separation is changed considerably by the addition of a small amount of cyclohexanol to the micellar solution [10]. This effect can probably be attributed to the changes in micellar properties with solubilized cyclohexanol. This concept, "modification of micellar phases with solubilized solutes," may widen the scope and the application range of MEKC because of the diversity of the solutes to be used as modifiers.

In this study, organic hydroxy compounds, 1-hexanol, cyclohexanol and phenol, were used as modifiers for sodium dodecyl sulfate micelles, and their effects on the MEKC separation of various neutral aromatic compounds were compared. These modifiers contain the same number of carbon atoms, but the structures of their hydrocarbon parts and the acidities of their hydroxyl groups are different. Cyclohexane was also examined as a modifier for comparison. The variation of the capacity factors of the aromatic analytes was evaluated, and the features and mechanism of the effect of micellar modification are discussed.

2. Experimental

2.1. Apparatus

The apparatus used was a Jasco (Tokyo, Japan) Model CE-800 capillary electrophoresis system with a 700 mm × 0.05 mm I.D. fused-silica capillary tube. Detection was performed by on-column measurements of UV absorption at a position 500 mm from the injection end of the capillary. A Shimadzu (Kyoto, Japan) Chromatopak CR-1A data processor was used for recording chromatograms.

2.2. Reagents

All reagents were of analytical-reagent grade and used as received. Sodium dodecyl sulfate (SDS) was obtained from Wako (Osaka, Japan). The modifiers, 1-hexanol, cyclohexanol, phenol and cyclohexane, were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The aromatic analytes used were benzene, chlorobenzene, *o*-cresol, *p*-cresol, 2-chlorophenol, 4-chlorophenol, 2-naphthol, nitrobenzene and acetophenone, which were purchased from Wako or Tokyo Kasei Kogyo. Water was doubly distilled and further purified using a Milli-Q Labo system (Millipore, Bedford, MA, USA).

2.3. Procedure

The fused-silica capillary was filled with pH 6.8 buffer solution (0.010 *M* sodium phosphate–0.0020 *M* sodium tetraborate) containing 0.075 *M* SDS and 0.050–0.10 *M* additive compound. Sample solutions containing $5 \cdot 10^{-4}$–$5 \cdot 10^{-3}$ *M* aromatic analytes, 10% (v/v) methanol and $3 \cdot 10^{-5}$ *M* Sudan III or Oil Yellow OB were introduced from the positive end of the capillary by siphoning. Separations were performed by applying 20 kV at 25 ± 1°C. The detector was operated at 250 or 260 nm. Before each run, the capillary was rinsed successively with acetone (5 min), 0.1 *M* sodium hydroxide (5 min), water (5 min) and running buffer (10 min).

3. Results and discussion

3.1. Characteristics of the effect of micellar modification with organic hydroxy compounds

Fig. 1 shows examples of chromatograms in the absence and presence of 0.10 *M* 1-hexanol in the micellar solution of 0.075 *M* SDS. The presence of 1-hexanol (only 1.4%, v/v) in the micellar solution has a considerable effect on the retention order of the analytes. The partition coefficient of 1-hexanol at a low concentration was reported to be $2.25 \cdot 10^3$ on the mole fraction concentration scale [11]. Therefore, it is estimated that about 70% of 1-hexanol in the micellar solution is distributed into the micellar phase at a surfactant concentration of 0.075 *M*; the number of moles of 1-hexanol in the micellar phase is comparable to that of the surfactant and the properties of the micelles should be modified with the solubilized hexanol. Substantial changes in the selectivity of separation were also ob-

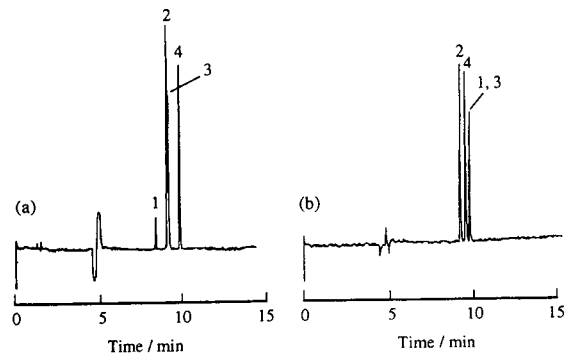


Fig. 1. Effect of the addition of 1-hexanol on the micellar electrokinetic chromatogram of aromatic compounds. Micellar solution: (a) 0.075 *M* SDS in phosphate–borate buffer (pH 6.8); (b) 0.075 *M* SDS and 0.092 *M* 1-hexanol in phosphate–borate buffer (pH 6.8). Peaks: 1 = benzene; 2 = nitrobenzene; 3 = *p*-cresol; 4 = acetophenone.

served in the presence of cyclohexanol and phenol.

In order to evaluate quantitatively the micellar modification effect on the partitioning of analytes, the capacity factor (k'), defined as the mole ratio of the analytes in the micellar phase to that in the aqueous phase, were calculated from the following equation [1]:

$$k' = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})} \quad (1)$$

where t_0, t_R and t_{mc} denote the migration times of the aqueous solution, analyte and micelle, respectively. Here, t_0 and t_{mc} were determined from the measurement of the migration time of methanol as the solute insolubilized in the micellar phase and that of Sudan III or Oil Yellow OB as the solute completely solubilized in it, respectively. Sudan III and Oil Yellow OB, which differ considerably in molecular size and structure, always gave an identical migration time, and this result supports the contention that both dyes are completely solubilized in the micellar phase.

The capacity factors of various aromatic compounds in the absence and presence of each modifier in 0.075 *M* SDS solution are summarized in Table 1. Here the modifier concentration was 0.1 *M*, except for cyclohexane, the concentration of which was 0.05 *M* because of its limited solubility. In Fig. 2, the dependence of the log k' values of some analytes on the concentration of 1-hexanol, cyclohexanol and phenol in the micellar solution is shown.

The presence of 1-hexanol and cyclohexanol decreases considerably the capacity factors for acetophenone and nitrobenzene but not for *p*-cresol. In contrast, the presence of phenol, whose hydroxyl group is much more acidic than that of alcohols, decreases the capacity factor of *p*-cresol but does not change those of acetophenone and nitrobenzene. All the hydroxy modifiers, especially 1-hexanol, increase the capacity factors of analytes that possess no hydrophilic groups (benzene, toluene and chlorobenzene).

Table 1
Variation of capacity factors by the use of organic modifiers

Solute	Log k' without modifier	Increment of log k' with modifier			
		Cyclohexane	1-Hexanol	Cyclohexanol	Phenol
Benzene	0.22	+0.04	+0.14	+0.06	+0.01
Nitrobenzene	0.33	0.00	−0.04	−0.05	−0.04
p-Cresol	0.34	+0.01	+0.02	−0.01	−0.16
Acetophenone	0.44	0.00	−0.11	−0.14	−0.03
Toluene	0.65	+0.04	+0.15	+0.06	
Chlorobenzene	0.76	+0.04	+0.16	+0.05	
2-Naphthol	1.05	+0.01	−0.03	−0.05	

SDS concentration, 0.075 *M*; modifier concentration, 0.10 *M*, except for cyclohexane (0.050 *M*).

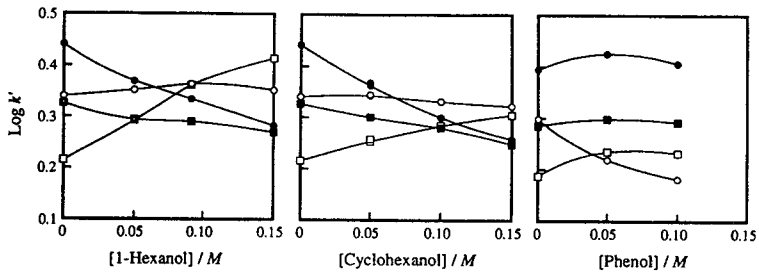


Fig. 2. Dependence of log k' on the concentration of modifiers in the micellar solution (0.075 M SDS, pH 6.8): □ = benzene; ■ = nitrobenzene; ○ = p-cresol; ● = acetophenone.

On the other hand, cyclohexane as the modifier has no effect on the capacity factors of most of the analytes, although those of benzene, toluene and chlorobenzene are slightly enhanced. It is apparent that the presence of a hydroxyl group in the modifier molecules is important.

The solubilization of long-chain alcohols generally lowers the critical micelle concentration (CMC) [11,12]. The CMC value of SDS in the present buffer without the modifiers was determined to be $4.6 \cdot 10^{-3}$ M by the electrical conductivity method. Therefore, in this study where the total concentration of SDS was 0.075 M, the increment of the surfactant forming micelles with the decrease in CMC should be negligibly small (less than 6%). According to a study employing fluorescence quenching measurements [13], the aggregation number of SDS is decreased by the addition of alcohols but the micelle size is nearly invariant. On the other hand, recent work using dynamic light scattering [14] showed that the size of SDS micelles increases with increasing concentration of 1-octanol. Anyway, it is difficult to explain the selective effect of the modifiers in MEKC in terms of the changes in CMC, aggregation number and micelle size.

In general, solutes that possess hydrophilic functional groups are mainly solubilized in the surface palisade layer of the micelle, as shown in Fig. 3a, whereas solutes that possess no hydrophilic groups are incorporated in the hydrocarbon core of the micelle [15]. As shown in Fig. 3b, the organic hydroxy compounds as modifiers should be solubilized in the surface palisade layer, and it is probable that the saturation of the palisade layer with the modifiers causes a decrease in the partition coefficient of analytes that possess hydrophilic groups. On the other hand, if there is an attractive interaction between the modifier and analyte molecules in the micelle as shown in Fig. 3c, the partition coefficient of the analyte should be increased. In the less polar field inside of the micelle, stronger interaction via hydrogen bonding is expected between acidic (phenols) and basic (alcohols, acetophenone and nitrobenzene [16,17]) solutes than in the aqueous

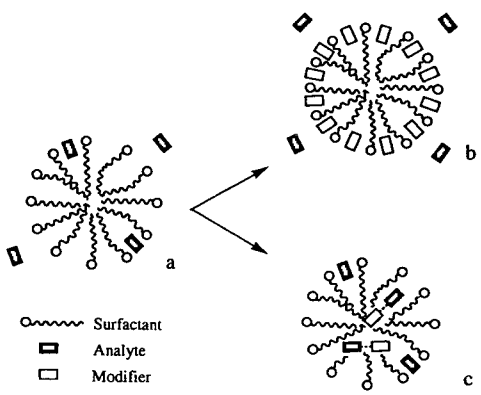


Fig. 3. Schematic illustrations explaining the effect of organic hydroxy modifiers on the capacity factors of analytes that possess hydrophilic functional groups. (a) Partitioning of the analyte between micellar palisade layer and bulk water; (b) decrease in the partition coefficient of the analyte caused by saturation of the palisade layer with the modifier; (c) increase in the partition coefficient of the analyte caused by interaction between the analyte and the modifier in the micelle.

phase. When the combination of modifier and analyte is such an acid–base pair, the effect shown in Fig. 3c should compensate for the effect in Fig. 3b, and apparently the effect of the modifers should become small. Consequently, the effects of the modifiers on the capacity factors of analytes that possess hydrophilic groups, shown in Table 1 and Fig. 2, are explained in terms of the saturation of the micellar palisade layer and the intermolecular hydrogen bonding in the micelles.

The effect of increasing the capacity factors of solutes that possess no hydrophilic groups can be attributed to the expansion of the micellar core with the hydrocarbon part of the solubilized modifiers. The greatest effect of 1-hexanol in increasing the capacity factor corresponds to the deeper permeation of its straight hydrocarbon chain into the micellar core than the cyclic chains of cyclohexanol and phenol.

3.2. Applicability to improvement of MEKC separation

In Fig. 4, the micellar electrokinetic chromatograms of *o*-cresol, *p*-cresol and nitrobenzene with 0.075 *M* SDS solution in the absence and presence of 0.10 *M* 1-hexanol are shown. The partition coefficients of these analytes between the micellar and aqueous phases are similar and complete separation with the pure micelles is difficult. However, in the presence of 1-hexanol, baseline separation is achieved accompanying a change in retention order. This improvement in separation is primarily due to the selective decrease in the capacity factor of nitrobenzene.

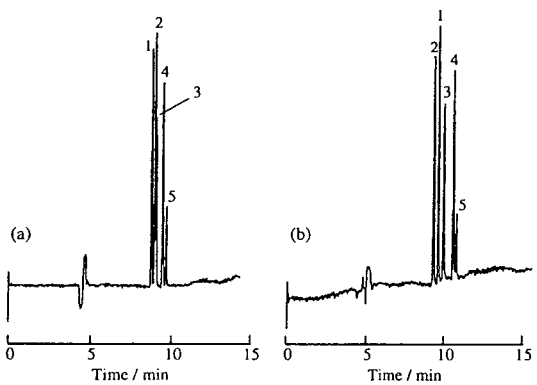


Fig. 4. Improvement in the MEKC separation of (1) *o*-cresol, (2) nitrobenzene, (3) *p*-cresol, and (4) 2-chlorophenol by the addition of 1-hexanol. Micellar solution: (a) 0.075 *M* SDS in phosphate–borate buffer (pH 6.8); (b) 0.075 *M* SDS and 0.10 *M* 1-hexanol in phosphate–borate buffer (pH 6.8).

Generally, resolution in MEKC becomes higher when the value of t_{mc}/t_0 and the number of theoretical plates (N) increase [1]. The addition of 1-hexanol, cyclohexanol and phenol did not influence the t_0 value but increased the t_{mc} value: t_{mc}/t_0 was 3.63 with 0.075 *M* SDS and rose to 4.47, 4.23 and 3.99 in the presence of 0.1 *M* 1-hexanol, cyclohexanol and phenol, respectively. Further, the N value was increased by the addition of the alcohols, e.g., N for acetophenone rose from $1.7 \cdot 10^5$ to $2.2 \cdot 10^5$ and that for 2-naphthol from $2.6 \cdot 10^5$ to $3.2 \cdot 10^5$ when 0.10 *M* cyclohexanol was added. Although the reasons for the increases in t_{mc}/t_0 and N are still unclear, the micellar modification with the hydroxy compounds is obviously effective for the improvement of resolution in MEKC.

4. Conclusions

This work clearly shows that the solubilization selectivity of SDS micelles is modified by the addition of a small amount of organic hydroxy compounds to the micellar solution. Such micellar modification with organic solutes offers a simple and useful method for controlling the MEKC separation and makes it possible to separate analytes that are hardly separated with pure micelles. In addition, this method improves the resolution by increasing the migration time of micelles and the number of theoretical plates.

When using UV adsorption detectors in MEKC, aliphatic alcohols are favourable as modifiers because they show little absorbance in the UV range. Of course, various hydrophobic compounds other than hydroxy compounds can be used, and further investigations are in progress.

Acknowledgement

This work was supported in part by a Grant-in-Aid for JSPS Fellows from the Ministry of Education, Science and Culture, Japan.

References

[1] R. Kuhn and S. Hoffstetter-Kuhn, Capillary Electrophoresis: Principle and Practice, Springer, Berlin, 1993, Ch. 5, p. 191.
[2] D.E. Burton, M.J. Sepaniak and M.P. Maskarinec, J. Chromatogr. Sci., 25 (1987) 514.
[3] S. Terabe, M. Shibata and Y. Miyashita, J. Chromatogr., 480 (1989) 403.
[4] E.L. Little and J.P. Foley, J. Microcol. Sep., 4 (1992) 145.
[5] E.S. Ahuja, B.P. Preston and J.P. Foley, J. Chromatogr. B, 657 (1994) 271.
[6] S. Takeda, S. Wakida, M. Yamane, A, Kawahara and K. Higashi, Anal. Chem., 65 (1993) 2489.
[7] C. Kiyohara, K. Saitoh and N. Suzuki, J. Chromatogr., 646 (1993) 397.
[8] S. Terabe, Y. Ishihama, H. Nishi, T. Fukuyama and K. Otsuka, J. Chromatogr., 545 (1991) 359.
[9] S. Terabe, Y. Miyashita, O. Shibata, E.R. Barnhart, L.R. Alexander, D.G. Patterson, B.L. Karger, K. Hosoya and N. Tanaka, J. Chromatogr., 516 (1990) 23.
[10] S. Katsuta and K. Saitoh, Anal. Lett., 27 (1994) 743.
[11] K. Hayase and S. Hayano, Bull. Chem. Soc. Jpn., 50 (1977) 83.
[12] I.V. Rao and E. Ruckenstein, J. Colloid Interface Sci., 113 (1986) 375.
[13] M. Almgren and S. Swarup, J. Colloid Interface Sci., 91 (1983) 256.
[14] M. Abe, Y. Tokuoka, H. Uchiyama, K. Ogino, J.F. Scamehorn and S.D. Christian, Colloids Surf., 67 (1992) 37.
[15] M.J. Rosen, Surfactants and Interfacial Phenomena, Wiley, New York, 2nd ed., 1989, Ch. 4, p. 170.
[16] R.W. Taft, D. Gurka, L. Joris, P. von R. Schleyer and J.W. Rakshys, J. Am. Chem. Soc., 91 (1969) 4801.
[17] M.J. Kamlet and R.W. Taft, J. Am. Chem. Soc., 98 (1976) 377.

ELSEVIER

Journal of Chromatography A, 705 (1995) 325–333

JOURNAL OF CHROMATOGRAPHY A

Migration behaviour and optimization of selectivity of dichlorophenols in capillary zone electrophoresis

Ching-erh Lin*, Wei-chen Lin, Wen-cheng Chiou

Department of Chemistry, National Taiwan University, Taipei, Taiwan

First received 26 October 1994; revised manuscript received 28 February 1995; accepted 3 March 1995

Abstract

The migration behaviour and selectivity of six isomeric dichlorophenols were systematically investigated using capillary zone electrophoresis. The pH, concentration of background electrolyte and applied voltage were optimized to give the best separation of these isomeric compounds. The migration behaviour and selectivity of each compound were affected strongly by pH, but much less by the composition and concentration of the electrolyte. The dependence on pH of differences in electrophoretic mobilities between two consecutively migrating solutes was used to determine the optimum buffer pH for the separation. The results confirmed that this optimum pH agrees satisfactorily with that predicted from the mean value of the solute pK_a. The pK_a values of these six dichlorophenols are reported.

1. Introduction

Capillary electrophoresis (CE) is a popular and powerful separation technique that possesses many advantageous features, such as high resolution, high efficiency of separation and rapid analysis [1–9]. It has been successfully applied to diverse analytical samples. In CE, the migration times, resolution and separation efficiency are governed by the applied voltage, electrophoretic mobility (μ_{ep}) of a charged species of interest and the electroosmotic flow (μ_{eo}). In practice, among several separation parameters that affect mobility, the pH generally plays an important role in CE separation as it determines the extent of ionization of each individual analyte. Therefore, manipulation of the buffer pH becomes a key strategy in optimizing separation. However, the combined effects of other separation parameters, such as composition and concentration of the background electrolyte and of the applied voltage, must also be considered.

Chlorinated phenols are of great environmental concern because of their high toxicity. The separation of chlorinated phenols by CE has been investigated in several laboratories [10–16]. Terabe et al. [10] successfully resolved a mixture of nineteen chlorinated phenols by means of micellar electrokinetic capillary chromatography (MECC) using a fused-silica capillary with sodium dodecyl sulfate (SDS) in phosphate–borate solution at pH 7.0. Ong et al. [13] separated eleven priority phenols in a similar manner at pH 6.6. The separation of eight chlorinated phenols in industrial waste water was achieved by Gaitonde and Pathak [14] using capillary zone electrophoresis (CZE) with a phosphate–borate electrolyte at pH 8.0. The pH for separating

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00301-0

eight substituted phenols with pK_a in the range 7.04–10.28 was optimized by Smith and Khaledi [15]; they proposed a mathematical model to predict the mobility of each solute as a function of pH, and suggested that the optimum pH can be predicted with a window diagram. However, based on the resolution of the worst resolved peak pair, the optimum pH was about 10.5, which is even greater than the corresponding pK_a. The optimized CZE separation of thirteen chlorinated phenols as a function of pH, concentration of the background electrolyte and applied voltage was investigated by Gonnord and Collet [16]; they obtained the best resolution at pH 6.9. As the pH is varied for effective separation of diverse chlorinated or substituted phenols in an empirical manner, questions about the optimum pH for obtaining the best resolution of chlorinated phenols and about a baseline separation arise.

In order to address these issues, one has to achieve a profound understanding of not only the migration behaviour of these solutes but also the exact role of important parameters that have significant effects on the migration and selectivity of the solutes considered. We therefore investigated systematically the effect of pH and other parameters on migration behaviour and resolution in the CE of chlorinated phenols, even though the migration and selectivity depend strongly only on pH. we chose six isomeric dichlorophenols as test solutes and determined their pK_a values. We describe here an approach to determine the optimum buffer pH for the separation of these dichlorophenols.

2. Experimental

2.1. Chemicals and reagents

All six isomeric dichlorphenols (DCP) were purchased from Aldrich, sodium dihydrogenphosphate dihydrate from Showa Chemicals, anhydrous disodium tetraborate from Merck and methanol (HPLC grade) from Mallickrodt. All other chemicals were of analytical-reagent grade and used as received.

Sample solutions were prepared at a concentration about 20 ppm in methanolic solution. Phosphate–borate buffer solution was prepared by mixing disodium tetraborate and sodium dihydrogenphosphate solution in an appropriate ratio. The pH of the buffer solution was then adjusted with sodium hydroxide (0.1 *M*) or hydrochloric acid (0.1 *M*) to the desired value. All solutions were degassed ultrasonically and filtered through a membrane filter (0.22 μm) before use.

2.2. Apparatus

Capillary electrophoretic experiments were carried out on a Model 1000 capillary electrophoresis system (Spectra-Physics, Fremont, CA, USA), equipped with a UV detector, a thermostated fused-silica capillary cartridge (either 44 cm × 75 μm I.D. or 44 cm × 50 μm I.D.) and an autosampler. The length of capillary between injection and detection was 37 cm. The CE system was interfaced with a miocrocomputer and printer with CE 1000 1.05A software. A Model SP-701 pH meter (Suntex, Taipei, Taiwan) was used to measure pH with an accuracy of ±0.01 unit.

2.3. Procedure

All experiments were performed with phosphate–borate buffer systems suitable for the desired pH at 25°C and the measurements were run at least in triplicate to ensure reproducibility. An applied voltage of 10 kV was selected to keep the total current less than 100 μA in order to avoid experimental complications resulting for Joule heating when using a 75 μm I.D. capillary. Sample injections were made in the hydrodynamic mode. The sample solution was typically injected for 1 s. All measurements were monitored at 215 nm.

When a new capillary was used, the capillary was washed for 10 min with sodium hydroxide solution (1.0 *M*) at 60°C, followed by 10 min with sodium hydroxide solution (0.1 *M*) at 60°C and 5 min with deionized and purified water at

60°C. The capillary was prewashed for 5 min with the running buffer before each injection.

2.4. Calculations

Electrophoretic mobilities of solutes were calculated with the equation

$$\mu_{ep} = \mu - \mu_{eo} = \frac{L_t L_d}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right) \quad (1)$$

where μ_{ep} is the electrophoretic mobility of the solute tested, μ is the apparent mobility, μ_{eo} is the electroosmotic mobility, t_m is the migration time measured directly from the electropherogram, t_{eo} is the migration time for an uncharged solute (methanol as neutral marker), L_t is the total length of capillary, L_d is the length of capillary between injection and detection and V is the applied voltage.

3. Results and discussion

3.1. Optimization of separation

Optimization of the separation of dichlorophenols in CZE is achieved by controlling either the difference in the electrophoretic mobilities of solutes (μ_{ep}) or the difference between the mobility and electroosmotic flow (μ_{eo}). In practice, both μ_{ep} and μ_{eo} can be modified by varying the separation parameters. We considered the combined effects of pH, composition and concentration of background electrolyte and applied voltage on the mobilities of dichlorophenols to optimize the separation, which involves a compromise among resolution, speed and sensitivity. We discuss each parameter in turn.

Applied voltage

The experiments were conducted below the maximum operating voltage for the conditions of pH, composition and concentration of the buffer electrolyte; otherwise the temperature of the buffer increases, resulting in peak broadening and irreproducible data. Fig. 1 shows the current generated as a function of applied voltage for a chosen concentration of as phosphate–borate buffer system and a fused-silica capillary (length 44 cm, I.D. either 75 or 50 μm) to separate isomeric dichlorophenols at pH 7.72. In order to ensure the absence of complications due to Joule heating, we conducted all experiments at or below 10 kV when using a 75 μm I.D. capillary or below 17 kV when using a 50 μm I.D. capillary, so that the current generated was kept below 100 μA. For this reason and because the number of theoretical plates obtained at 10 kV for a 75 μm I.D. capillary has the largest value, we used an applied voltage 10 kV with a 75 μm I.D. capillary.

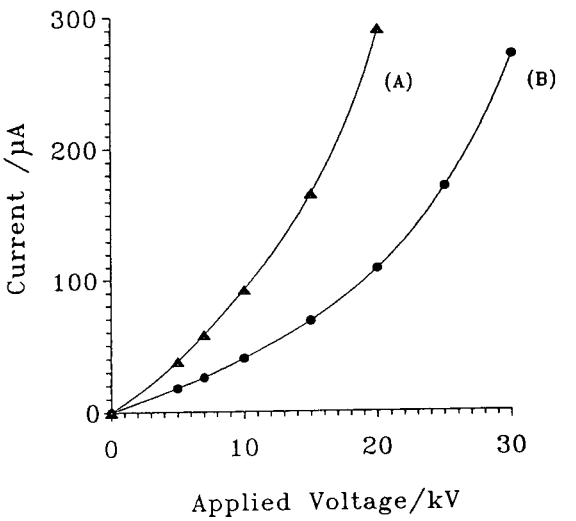


Fig. 1. Current generated as a function of applied voltage for 50 m*M* phosphate–100 m*M* borate buffer at pH 7.72 at 25°C. Fused-silica capillary: length, 44 cm; I.D. (A) 75 and (B) 50 μm.

Buffer composition

As the pK_a of dichlorophenols (DCPs) reported in the literature are in the range 6.5–8.5, a phosphate–borate buffer system was used as background electrolyte. Electropherograms of dichlorophenols obtained at various moral ratios of phosphate to borate are shown in Fig. 2. The migration order of the dichlorophenols is 3,4-DCP < 3, 5-DCP < 2, 4-DCP < 2, 3-DCP < 2, 5-DCP < 2,6-DCP. This order is consistent with that reported previously [16].

The effects of buffer composition on the

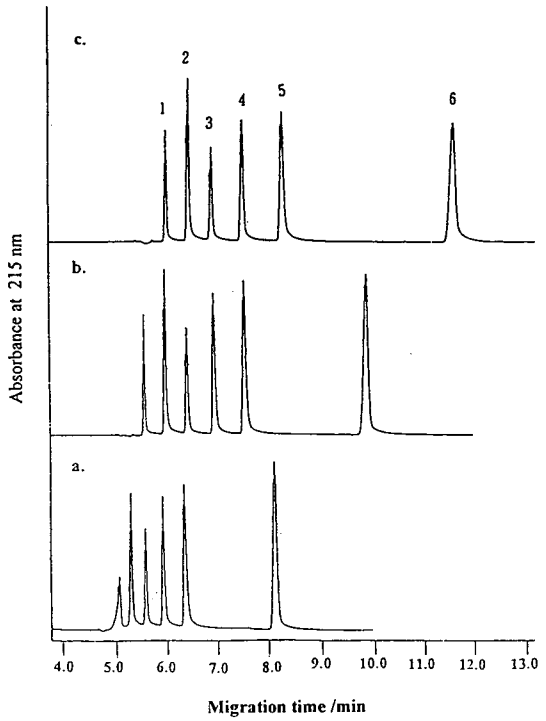


Fig. 2. Electropherograms of isomeric dichlorophenols obtained for various molar ratios of phosphate–borate of buffer composition: (a) 20:80; (b) 50:50; (c) 80:20. Total buffer concentration, 100 mM; buffer pH, 7.72 at 25°C; applied voltage 10 kV; fused-silica capillary, 44 cm × 75 μm I.D. Peaks: 1 = 3,4-DCP; 2 = 3,5-DCP; 3 = 2,4-DCP; 4 = 2,3-DCP; 5 = 2,5-DCP; 6 = 2,6-DCP.

electroosmotic mobility and electrophoretic mobilities of each dichlorophenol are illustrated in Fig. 3. Both the electrophoretic and electroosmotic mobilities decrease when the ratio of phosphate to borate at a total buffer concentration of 100 mM varies from 20:80 to 80:20. As all dichlorophenols exhibit similar migration behaviour, variation of the electrolyte composition affects the relative selectivity only slightly. The difference between the electrophoretic mobility of each solute and the electroosmotic mobility decreased with increasing concentration of phosphate in the buffer solution. The conductivity increased linearly from $5.10 \cdot 10^{-3}$ to $6.64 \cdot 10^{-3}$ mho cm^{-1} before adjustment of the pH and from $5.55 \cdot 10^{-3}$ to $11.13 \cdot 10^{-3}$ mho cm^{-1} after adjustment of the pH to 7.72 when the molar ratio of

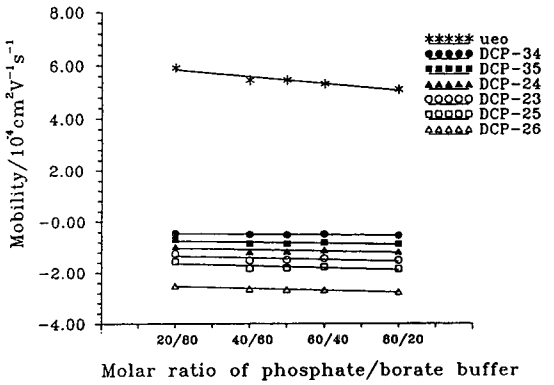


Fig. 3. Effect of buffer composition on electrophoretic mobility of dichlorophenols. Other conditions as in Fig. 2.

phosphate–borate buffer was varied from 20:80 to 80:20. Consequently, the migration times of dichlorophenols increased when the fraction of phosphate in the buffer solution was increased.

Buffer concentration

The variation of the electrophoretic mobility of dichlorophenols as a function of ionic strength of the buffer solution is shown in Fig. 4. The magnitudes of both the electroosmotic and electrophoretic mobilities of each isomeric dichloro-

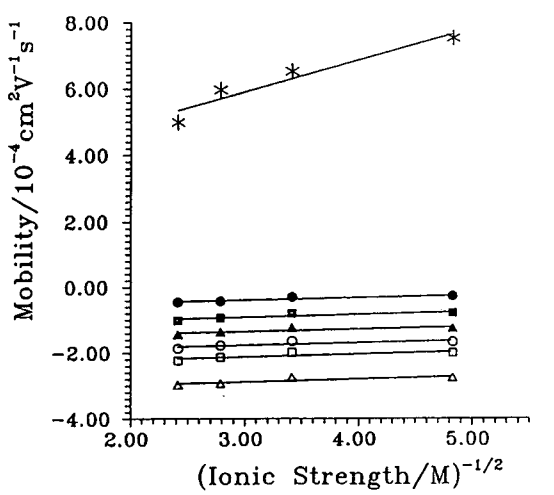


Fig. 4. Electrophoretic mobility as a function of ionic strength. Molar ratio of phosphate to borate, 1:2; buffer pH, 7.72; operating conditions, 10 kV, 25°C; fused-silica capillary, 44 cm × 50 μm I.D. Symbols as in Fig. 3.

phenol decreased with increasing ionic strength. As the variation of mobility of each isomer was about the same, six straight lines nearly parallel to each other were observed. The migration times of dichlorophenols increased with increasing total concentration of phosphate and borate (Fig. 5).

Buffer pH

The electropherograms in Fig. 6 clearly illustrate that the selectivity of dichlorophenols was greatly affected by buffer pH in the range 6.29–9.63. Because at pH 6.29 we see a single feature for 3,4-DCP, 3,5-DCP and 2,4-DCP, these isomers co-migrate almost together, whereas 2,3-DCP, 2,5-DCP and 2,6-DCP isomers are barely separated. The resolution of the former three isomers improved with increasing buffer pH. Baseline separation of all six isomers was achieved when the buffer pH was in the range 6.7–9.6. At higher pH, the migration times of dichlorophenols were longer and electrophoretic peaks appeared broadened. This is mainly due to the longitudinal diffusion. Table 1 lists the mobility data for the six dichlorophenols; the relative standard deviation of the mobility was found to be less than 2%.

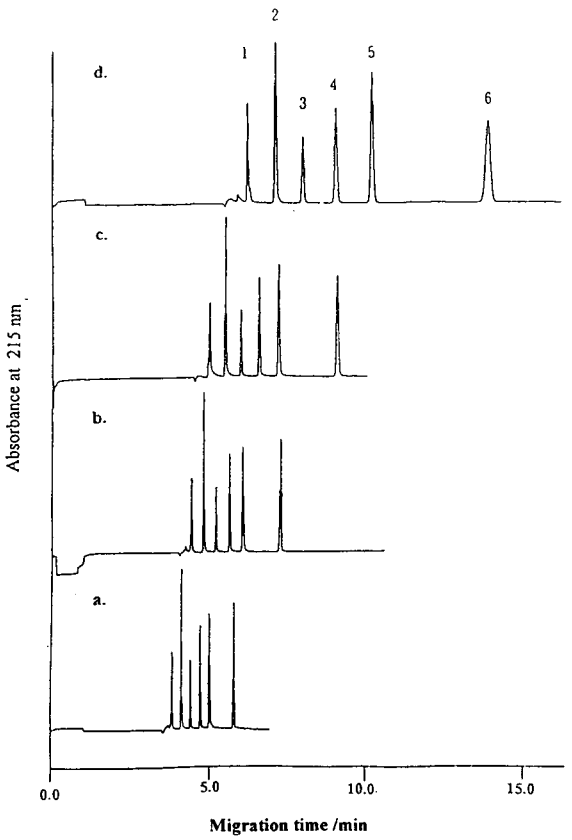


Fig. 5. Electropherograms of isomeric dichlorophenols obtained for various ionic strengths. Concentrations of phosphate/borate in phosphate–borate buffer (m*M*), (a) 12.5/25; (b) 25/50; (c) 37.5/75; (d) 50/100; other operating conditions as in Fig. 4. Peaks as in Fig. 2.

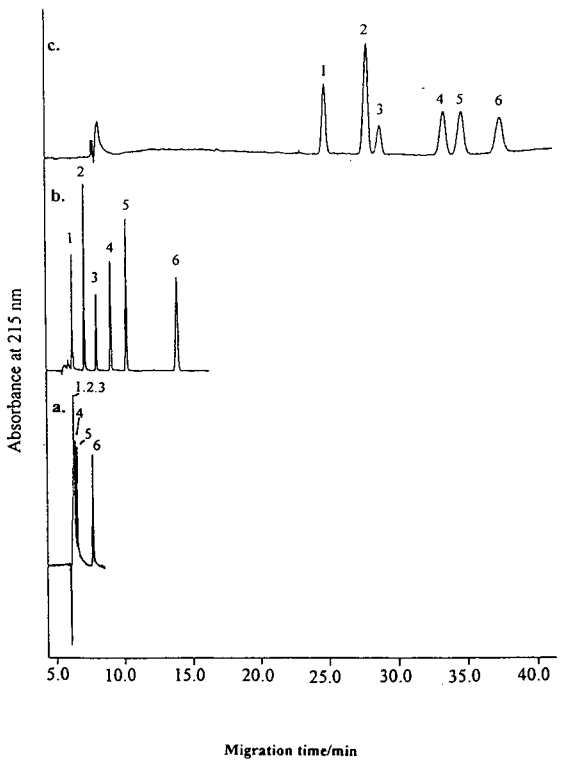


Fig. 6. Electropherograms of isomeric dichlorophenols obtained for various buffer pH values: (a) 6.29; (b) 7.72; (c) 9.63. Buffer composition, 50 m*M* phosphate–100 m*M* borate; fused-silica capillary, 44 cm × 50 μm I.D.; other operating conditions and peak identification as in Fig. 2.

The migration behaviour of each dichlorophenol is described by the equation [17]

$$\mu_{ep} = \mu_A - \left(\frac{K_a}{[H^+] + K_a}\right) \quad (2)$$

Table 1
Mobilities (10^{-4} cm^2 V^{-1} s^{-1}) of dichlorophenols at various buffer pH values

pH	μ_{eo}	μ_{ep}					
		3,4-DCP	3,5-DCP	2,4-DCP	2,3-DCP	2,5-DCP	2,6-DCP
5.30	4.10	0.00	0.00	0.00	0.00	0.00	−0.14
5.80	4.31	0.00	0.00	0.00	0.00	−0.05	−0.34
6.14	4.32	0.00	0.00	0.00	−0.07	−0.13	−0.63
6.29	4.40	0.00	−0.02	−0.05	−0.11	−0.19	−0.81
6.70	4.49	−0.01	−0.08	−0.16	−0.29	−0.46	−1.39
6.99	4.56	−0.09	−0.22	−0.37	−0.56	−0.80	−1.82
7.35	4.60	−0.16	−0.39	−0.65	−0.95	−1.26	−2.20
7.72	4.52	−0.36	−0.77	−1.13	−0.15	−1.78	−2.47
8.14	4.34	−0.67	−1.22	−1.61	−1.94	−2.17	−2.58
8.53	4.24	−1.24	−1.83	−2.12	−2.37	−2.50	−2.71
9.11	4.05	−1.98	−2.24	−2.37	−2.54	−2.60	−2.70
9.63	3.69	−2.31	−2.45	−2.48	−2.62	−2.65	−2.71
10.10	3.37	−2.48	−2.53	−2.53	−2.64	−2.66	−2.70

where μ_{ep} is the electrophoretic mobility of an isomeric dichlorophenol at a given pH, μ_{A^-} is the mobility of the anionic form of the corresponding isomeric dichlorophenol and pK_a is the acid dissociation constant. Accordingly, a sigmoidal curved for the migration behaviour of each isomeric dichlorophenol is predictable when electrophoretic mobilities are plotted against buffer pH. Fig. 7 depicts the electrophoretic mobilities of six dichlorophenols as a function of buffer pH. At low pH (<pK_a − 2), the electrophoretic mobilities of dichlorophenols asymptotically approach zero, whereas at high pH (>pK_a + 2), where the deprotonated form of the isomeric dichlorophenol becomes dominant, the mobilities approach their corresponding μ_{A^-} values. This electromigration behaviour is consistent with that observed for 2,5-DCP and 3,5-DCP by Smith and Khaledi [15].

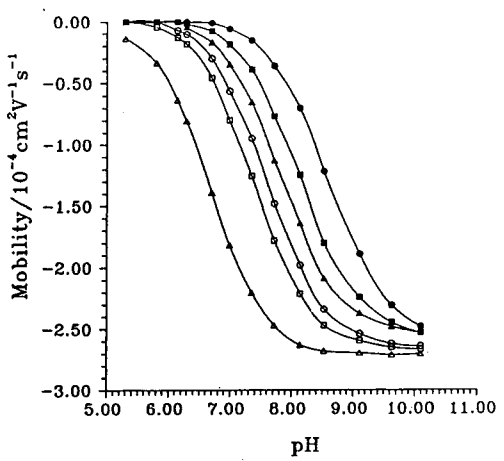


Fig. 7. Plots of electrophoretic mobility of dichlorophenols as a function of pH. Buffer, 50 m*M* phosphate–100 m*M* borate; fused-silica capillary, 44 cm × 50 μm I.D.; other operating conditions as in Fig. 2. Symbols as in Fig. 3.

Capillary electrophoresis is useful for determining the dissociation constants of acids [15] and bases [18]. With the aid of the plots in Fig. 7, one can easily determine the pK_a of each isomer of dichlorophenol, by estimating the best value of μ_{A^-} from measurement of the mobility of the anionic form of each isomeric dichlorophenol at high pH (>pK_a + 2), and then measuring the pH corresponding to $\mu_{ep} = \frac{1}{2}\mu_{A^-}$ from the plot of μ_{ep} versus pH. Hence, the pK_a of each isomeric dichlorophenol is equal to the buffer pH at $\mu_{ep} = \frac{1}{2}\mu_{A^-}$. Table 2 gives the values of pK_a and μ_{A^-} determined for each isomeric species with values from the literature. The accuracy of the pK_a value is ±0.05 unit.

Agreement between these two sets of data is satisfactory. We predicted the mobility of each isomeric dichlorophenol over the pH range 5.30–10.10 with Eq. 2 and calculated the pK_a and μ_{A^-} values. The predicted and actual mobilities are plotted in Fig. 8. The slope (1.0007) and the

Table 2
pK_a and μ_{A^-} values for dichlorophenol isomers

Solute	pK_a		μ_{A^-}-(10^{-4} cm^2 V^{-1} s^{-1})	
	This work	Others	This work	Others[b]
3,4-DCP	8.50	8.62[a]	−2.48	–
3,5-DCP	8.10	8.25[a], 7.98[b]	−2.53	−2.83
2,4-DCP	7.90	7.90[a]	−2.53	–
2,3-DCP	7.60	7.71[a]	−2.64	–
2,5-DCP	7.30	7.51[a], 7.32[b]	−2.66	−3.06
2,6-DCP	6.70	6.78[a]	−2.70	–

[a] From Ref. [15].
[b] From Ref. [13].

correlation coefficient ($r = 0.9995$) indicate an excellent correlation between predicted and actual mobilities. Hence the values of pK_a and μ_{A^-} are demonstrated to be reliable.

3.2. Optimization of selectivity

Even if the six isomeric dichlorophenols are easily separated in a suitable range of buffer pH, it is still of interest to determine the optimum pH in an alternative way, based on optimizing the overall difference in the effective mobilities of these dichlorophenols.

An explicit equation for optimum pH of the background electrolyte to separate two weak acids is [3]

$$pH_{opt} = \frac{1}{2}(pK_{a_1} + pK_{a_2}) - \log \frac{(\sqrt{\mu_{A_2^-}/\mu_{A_1^-}} - \sqrt{K_{a_2}/K_{a_1}})}{1 - \sqrt{(\mu_{A_2^-} K_{a_2})/(\mu_{A_1} - K_{a_1})}} \quad (3)$$

where subscripts 1 and 2 refer to two solutes to be separated. Hence, if the pK_a and ionic mobilities are known, the optimum buffer pH can be calculated. For substances with equal ionic mobilities, such as positional isomers, the equation becomes simplified to

$$pH_{opt} = \frac{1}{2}(pK_{a_1} + pK_{a_2}) \quad (4)$$

Based on the pK_a values in Table 2, the optimum buffer pH values for the five pairs of consecutively migrating isomeric dichlorophenols are 8.35, 8.00, 7.74, 7.52 and 7.04, respectively, in the order of increasing migration time. For instance, the optimum pH for the pair 2,5-DCP and 2,6-DCP is predicted to be 7.04. Similarly, the optimum pH predicted for the six isomeric dichlorophenols is 7.61, which is the mean of the pK_a values of 3,4-DCP and 2,6-DCP.

Fig. 9 shows plots of differences in electrophoretic mobilities between each two consecutively migrating isomeric dichlorophenols ($\Delta\mu_{ep}$) against buffer pH. The curves exhibit maxima at pH ≈7.0, 7.5, 7.8, 8.0, and 8.4 for the five pairs of consecutively migrating isomers of dichloro-

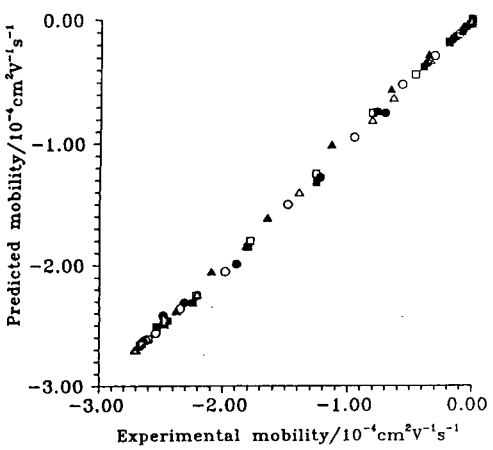


Fig. 8. Predicted and experimental mobilities for six isomeric dichlorophenols over the pH range 6.7–9.1. Symbols as in Fig. 3.

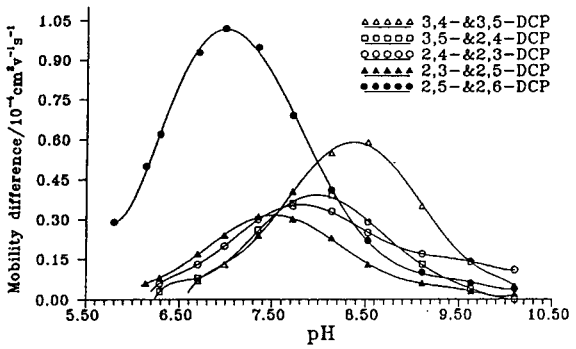


Fig. 9. Plots of differences in electrophoretic mobilities between two consecutively migrating isomeric dichlorophenols against buffer pH. Other conditions as in Fig. 7.

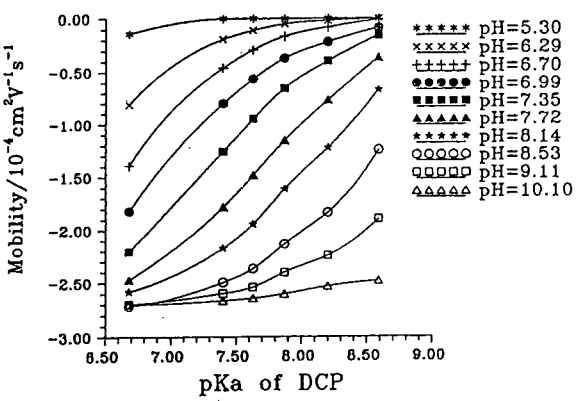


Fig. 10. Plots of electrophoretic mobility of dichlorophenols at a given buffer pH versus pK_a.

phenol. Thus, the optimum pH for resolving all six isomeric dichlorophenols is about 7.6–7.7, because the overall difference of effective mobilities of the six isomers is maximized. Table 3 summarizes the predicted and observed optimum pH values for each pair of consecutively migrating isomers and for all six isomeric dichlorophenols. The experimental results agree satisfactorily with the predicted values.

The resolution evaluated on the basis of the observed electropherograms at various buffer pH clearly indicates that, if the difference in electrophoretic mobilities between any two consecutively migrating isomers is not less than $6 \cdot 10^{-6}$ cm^2 V^{-1} s^{-1}, baseline separation of all isomeric dichlorophenols is achieved. This condition occurs at a buffer pH in the range 6.7–9.6. The minimum electrophoretic mobility difference for baseline resolution was evaluated on the basis of the equation $R_S = 2\Delta w/(w_1 + w_2)$, where $R_s = 1.5$ is the resolution for baseline separation, w_1 and w_2 are the two peak widths at the base and Δw is the minimum difference in migration times for the pair of consecutively migrating isomers. Δw was then converted into $\Delta\mu_{ep}$ to obtain the minimum difference in electrophoretic mobility for baseline separation.

Table 3
Predicted and experimental optimum pH values of dichlorophenols

Migrating pair	Predicted value[a]	Experimental value[b]
3,4- and 3,5-DCP	8.35	8.40
3,5- and 2,4-DCP	8.00	8.00
2,4- and 2,3-DCP	7.74	7.80
2,3- and 2,5-DCP	7.52	7.50
2,5- and 2,6-DCP	7.04	7.00
3,4- and 2,6-DCP	7.61	7.6–7.7

[a] Based on the equation $pK_{a_{opt}} = \frac{1}{2}(pK_{a_1} + pK_{a_2})$.
[b] Determined from Fig. 9.

To confirm that the present approach is suitable for determining the optimum buffer pH, the mobility data for six isomeric dichlorophenols at a given buffer pH were plotted against their corresponding pK_a values. Fig. 10 shows such plots obtained by treating the buffer pH as a parameter. The curves change from a concave to a convex shape as the pH is increased from 5.3 to 10.10. At pH 7.72, the curve is nearly a straight line when the overall difference in effective mobilities of the six isomers approaches its maximum value. As this pH falls in the predicted range of optimum buffer pH, this nearly straight line between the concave and convex conditions indicates the optimum condition.

4. Conclusion

The electromigration behaviour and selectivity of six isomeric dichlorophenols were greatly affected by the buffer pH in capillary zone electrophoresis. In addition to the investigation

of the buffer pH, the effects of composition and concentration of the buffer electrolyte and of applied voltage on the electrophoretic mobility of these solutes were studied to optimize the separation. Baseline separation of these dichlorophenols was achieved at 10 kV with a phosphate–borate buffer with pH in the range 6.7–9.6. The optimum buffer pH determined for the best resolution of the dichlorophenols is about 7.7, in agreement with the prediction from the mean of pK_a values of 2,6-DCP and 3,4-DCP.

Acknowledgement

We thank the National Science Council of the Republic of China for financial support.

References

[1] S.F.Y. Li, Capillary Electrophoresis: Principles, Practice and Applications, Elsevier, Amsterdam, 1992.
[2] P. Jandik and G. Bonn, Capillary Electrophoresis of Small Molecules and Ions, VCH, New York, 1993.
[3] Foret, L. Křivánková and P. Boček, Capillary Zone Electrophoresis, VCH, Weinheim, 1993.
[4] A.G. Ewing, R.A. Wallingford and T.M. Olefirowicz, Anal. Chem., 61 (1989) 292A.
[5] B.L. Karger, A.S. Cohen and A. Guttman, J. Chromatogr., 492 (1989) 585.
[6] H.H. Lauer and J.B. Ooms., Anal. Chim. Acta, 250 (1991) 45.
[7] O. Vesterberg, Electrophoresis, 14 (1993) 1243.
[8] M.W.F. Nielen, Trends Anal. Chem., 12 (1993) 345.
[9] P.E. Jackson and P.R. Haddad, Trends Anal. Chem., 12 (1993) 231.
[10] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando., Anal. Chem., 56 (1984) 113.
[11] S. Terabe, K. Otsuka and T. Ando., Anal. Chem., 57 (1985) 834.
[12] K. Otsuka, S. Terabe and T. Ando., J. Chromatogr., 348 (1985) 39.
[13] C.P. Ong, C.L. Ng, N.C. Chong, H.K. Lee and S.F.Y. Li, J. Chromatogr., 516 (1990) 63.
[14] C.D. Gaitonde and P.V. Pathak, J. Chromatogr., 514 (1990) 389.
[15] S.C. Smith and M.G. Khaledi, Anal. Chem., 65 (1993) 193.
[16] M.F. Gonnord and J. Collet, J. Chromatogr., 645 (1993) 327.
[17] C.J.O.R. Morris and P. Morris, Separation Methods in Biochemistry, Pitman, London, 2nd ed., 1974, p. 714.
[18] J. Cai, J.T. Smith and Z.E. Rassi, J. High Resolut. Chromatogr., 15 (1992) 30.

ELSEVIER

Journal of Chromatography A, 705 (1995) 335–341

JOURNAL OF CHROMATOGRAPHY A

High-performance capillary electrophoretic separation of human serum albumin using a neutral coated capillary

K.A. Denton*, R. Harris[1]

Department of Protein Chemistry, Delta Biotechnology Ltd., Castle Court, 59, Castle Boulevard, Nottingham NG7 1FD, UK

Received 17 January 1995; accepted 27 February 1995

Abstract

A rapid, simple and highly resolving capillary electrophoresis technique is described for the separation of sub-species of clinical grade human serum albumin (HSA) using a neutral coated capillary. As the buffer pH approached the p*I* of HSA (5.2), eight peaks representing HSA sub-populations were resolved. On a non-coated capillary, no such resolution was obtained and buffers several pH units away from HSA's p*I* were required to prevent protein adsorption to the capillary wall. Some sub-species could be identified by mass spectrometric analysis as representing intact and amino-terminally damaged HSA with and without a blocked thiol at cysteine 34.

1. Introduction

Capillary electrophoresis (CE) is recognised as a powerful separation technique for the analysis of a diverse range of molecules [1–4]. High resolution, short analysis times and ease of use make it an ideal choice for monitoring therapeutic proteins and biopolymers. Separation efficiencies for CE are typically high (>100 000 theoretical plates). In both quality control (QC) and research environments, CE now stands alongside the more established high-resolution separation techniques such as HPLC and polyacrylamide gel electrophoresis (PAGE) [5,6].

An advantage of CE is the ability to separate molecules in free solution. Large proteins can be separated, but may suffer from a lack of resolution between native and modified forms (e.g., post translational modifications, single residue mutations). This lack of resolving power is often due to protein interaction with the walls of untreated fused-silica capillaries which act as cation exchangers towards negatively charged samples. This leads to band broadening, loss of sensitivity and/or loss of sample [7,8]. Normally, this can only be counteracted effectively by using either extremes of pH away from the protein's p*I*, high salt concentrations to compete with available binding sites or by addition of solvent modifiers such as detergents or ion-pairing reagents to reduce wall effects. However, these actions tend to reduce the separation capabilities of CE and the ability to differentiate between closely related sub-populations of the protein.

Approaches have been made to overcome these problems. A wide range of coatings have been linked covalently to the fused silica in order to reduce adsorption of biomolecules such as

* Corresponding author.

[1] Present address: Andaris, Unit 21–22, Whitemoor Court, Nuthall Road, Nottingham NG8 5BY, UK.

0021-9673/95/$09.50
SSDI 0021-9673(95)00285-5

proteins to the capillary wall [7–9]. These coatings can confer further advantages such as an increase in the stability of the capillary, often at extremes of pH, and a potential increase in the capillary lifetime. Neutral coated capillaries can offer many of these benefits allowing sharp peaks to be resolved whilst using buffers near the p*I* of the protein [10]. Thus, short analysis times can be maintained and adsorption to the capillary wall is reduced significantly.

HSA exists in more than one form, as shown by the broad p*I* range quoted for this protein [11] and the band profile observed on an isoelectrofocusing (IEF) gel [12,13]. The ability to separate these different species in free solution by CE offers many advantages over alternative methods such as PAGE and IEF. CE is generally less time-consuming, less labour-intensive and allows rapid, quantitative separation of biomolecules such as proteins. The high-resolution separation of HSA is described using a new neutral coated capillary. The silica is bonded with polyacrylamide to give a neutral coating which reduces electroosmotic flow (EOF) and analyte/sample interactions with the capillary wall. Buffer conditions ensure that samples are negatively charged (just above the p*I* of the protein). As the EOF is negligible, electrophoretic migration is achieved through charge attraction of the negative protein to the anode. The separation of HSA sub-species using this neutral coated capillary is compared with that of a non-coated fused-silica capillary and the identification of the sub-species is discussed.

2. Experimental

2.1. Materials

Clinical grade HSA was obtained from a commercial supplier. Glycated HSA was purchased from Sigma (Poole, Dorset, UK). The neutral coated capillary and citrate 2-N-morpholino-ethanesulfonic acid (MES) buffers (kit part No. 477445) and non-coated capillaries (part No. 338451) were obtained from Beckman (High Wycombe, Buckinghamshire, UK). All other chemicals used were purchased from Sigma (analytical grade). Milli-Q grade water (Millipore, Watford, Hertfordshire) was used throughout.

2.2. Instrumentation

Capillary electrophoresis was performed on a P/ACE 2210 unit controlled by System Gold software (Beckman). Fixed-wavelength detection was set at 214 nm for the neutral capillary separations, as stipulated in the manufacturer's instructions, and 200 nm for non-coated fused-silica capillary separations for maximum sensitivity. In both cases, the data collection rate was 5 Hz and the capillary internal diameter (I.D.) was 50 μm; the length of the neutral coated capillary was 37 cm, that of the non-coated capillary was 43 cm. All separations were performed at 25°C.

2.3. Preparation of samples

HSA, glycated HSA and other albumin samples were diluted to 1 mg/ml in water prior to separation. Dilution into one tenth concentration of appropriate buffer did not affect the separation achieved and the use of water greatly facilitated method development. Reduction of HSA was performed by adding 2m*M* dithiotrietol (DTT) to the sample vial and incubating in the autosampler for 1 h prior to injection. Amino-terminal degradation of HSA was increased by incubating the protein at 37°C for 8 weeks [14]. The reference marker Orange-G was included in samples separated on the neutral coated capillary.

2.4. Separation conditions

The neutral coated capillary was used with separation buffers of 20 m*M* citrate MES at pH 5.2, pH 5.5 and pH 6.0. To achieve separation of HSA at these pH values the instrument polarity was reversed (with the anode at the capillary outlet). Prior to separation the capillary was rinsed for 2.5 min with the appropriate buffer. Sample was introduced using a 3-s pressure

injection. Electrophoresis was performed at a constant voltage of 18.5 kV (500 V cm^{-1}). The non-coated capillary was used with separation buffers of 50 m*M* phosphate at pH 1.6, pH 5.2, pH 6.5 and pH 8.0 and the instrument polarity was normal (with the cathode at the capillary outlet). Prior to separation the capillary was rinsed firstly with 0.1 *M* NaOH for 1 min then with the appropriate buffer for 2 min. A 3-s pressure injection of the sample was followed by constant voltage separation at 20 kV (465 V cm^{-1}).

2.5. Electrospray mass spectrometry (ESMS)

HSA was prepared for ESMS by reverse-phase HPLC and subjected to mass analysis on a VG Quattro mass spectrometer which had been calibrated over the *m/z* range 950–1750 Da/e using horse heart myoglobin.

3. Results

3.1. A comparison of neutral coated with non-coated silica capillaries for the separation of HSA

The separation of HSA sub-populations using a neutral coated capillary is demonstrated (Fig. 1) and shown to improve as the pH approached the p*I* of HSA. Six poorly separated peaks identified using a buffer at pH 6 (Fig. 1c) were resolved into eight well defined peaks at pH 5.2 (Fig. 1a). The migration time increased as the pH neared the p*I* of HSA but there was no evidence of loss of protein at any of the pH values tested. Using buffers further away from the p*I* of HSA (pH 2 or pH 7) to achieve separation offered no benefits using the neutral coated capillary. A comparison of the separation of HSA using fused-silica capillaries can be seen in Fig. 2. Here, in contrast to the neutral coated capillary separation, buffers with pH extremes were required to prevent adsorption of the protein to the capillary wall. The best separation of HSA was found using buffers at pH <3 or >7. Only single major peaks were obtained at

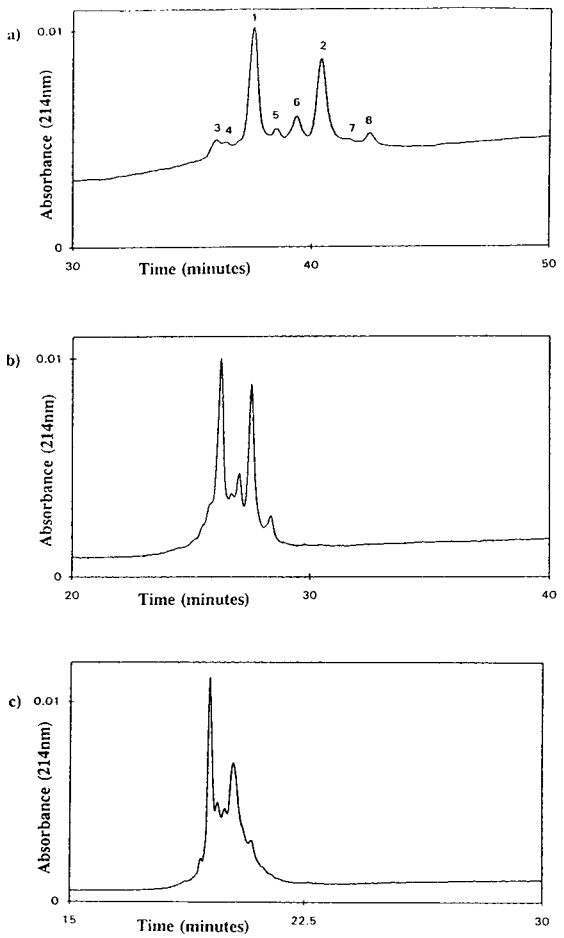


Fig. 1. Separation of HSA on a neutral coated capillary. HSA was separated at pH 5.2 (a), pH 5.5 (b) and pH 6.0 (c). Eight peaks (1–8) were resolved at pH 5.2.

these pH values with no significant resolution of the sub-populations. As the pH neared the p*I* of HSA, rather than observing an increase in resolution, the response decreased, the peaks broadened and migration times varied due to excessive interaction between the protein and the capillary wall (Fig. 2b). The inclusion of additives such as ion-pairing reagents (1,3-diaminopropane) or detergents (sodium dodecyl sulfate) failed to improve resolution (results not shown). These additives decreased the protein–wall interactions but did not allow high peak resolution.

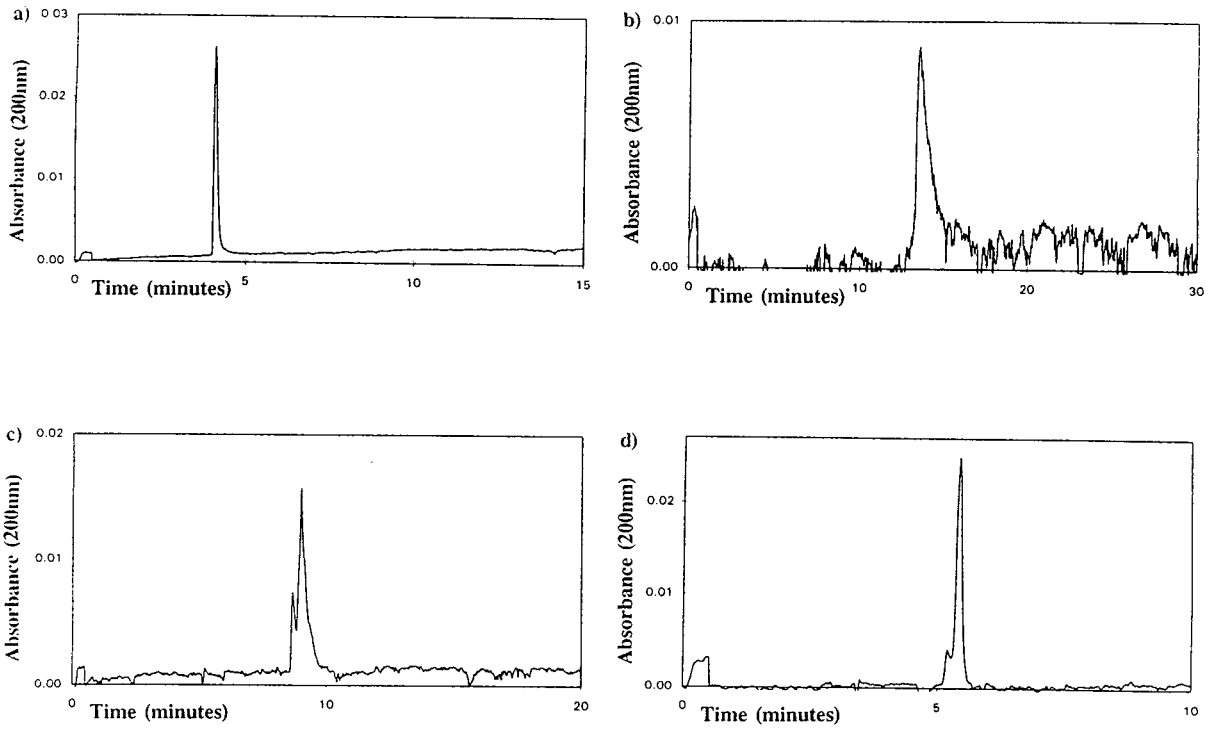


Fig. 2. Separation of HSA on a non-coated capillary. HSA was separated at pH 1.6 (a), pH 5.2 (b), pH 6.5 (c) and pH 8.0 (d). At the two pH extremes one peak was resolved whilst closer to HSA's p*I* poor/variable resolution was achieved.

3.2. Identification of HSA sub-species separated on the neutral coated capillary

HSA is regarded as a heterogeneous protein [15] which can be confirmed by ESMS (Fig. 3). The main species were identified from their relative masses to be HSA monomer with a free thiol at cysteine 34 (Fig. 3, peak A), HSA monomer with the thiol at cysteine 34 blocked with cysteine (Fig. 3, peak B) and amino-terminally degraded HSA (Fig. 3, peak C) [14]. The first major peak on the CE electropherogram (Fig. 1a, peak 1) represents HSA with a free thiol group and the second major peak (Fig. 1a, peak 2) represents HSA with a blocked thiol group. This was verified after reduction of the sample with DTT which resulted in the disappearance of peak 2 with a concomitant increase in peak 1 (Fig. 4).

The assignment of the other peaks was more difficult. The mass difference between the degraded HSA (Fig. 3, peak C) observed in the mass spectrum and HSA represents the loss of the amino-terminal residues aspartic acid and alanine. After reduction, the electropherogram shows a drop in the height of peak 8 with a corresponding increase in the height of peak 6 (Fig. 4). These two peaks could represent amino-terminally damaged HSA with free (peak 6) and blocked (peak 8) thiol groups. Furthermore, it has been shown that there is an increase in amino-terminal degradation of HSA when incubated at elevated temperatures (e.g. >30°C) [14] which can be monitored by mass spectrometric analysis (data not shown). The separation of HSA after incubation at 37°C shows increased levels of peaks 6 and 8, with corresponding drops in the heights of the main peaks (1 and 2) when compared to non-incubated HSA (Fig. 5), providing further evidence of the nature of these species. Peak 3, which was not affected by reduction with DTT, also increased after incubation at elevated temperatures (Fig. 5). This could represent a further degradation prod-

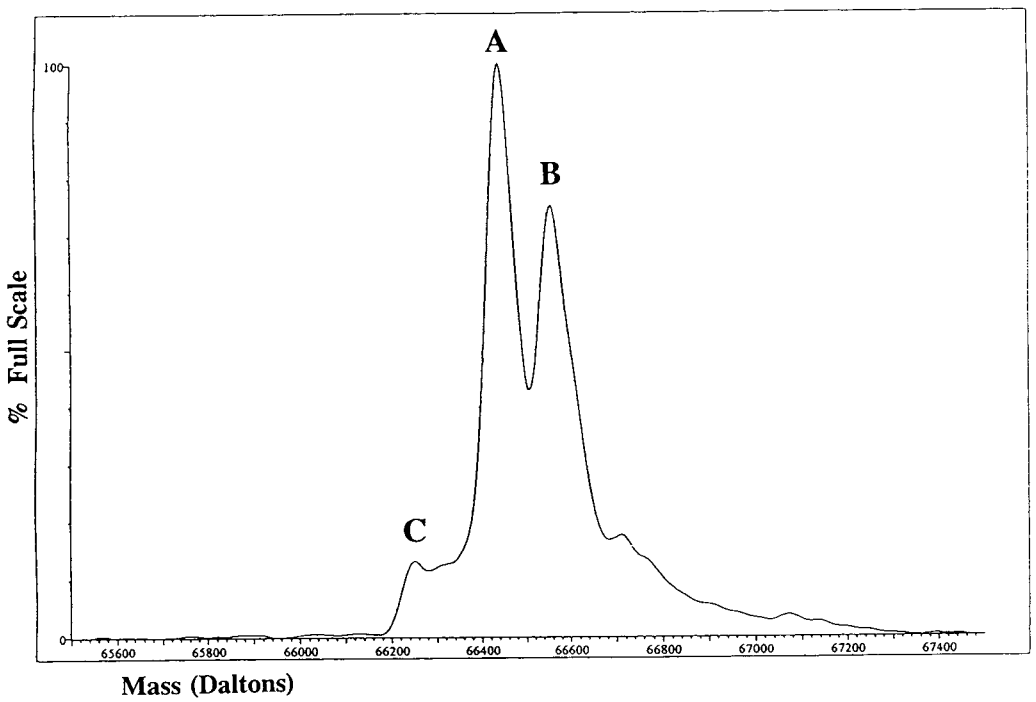


Fig. 3. Electrospray mass spectrometry of HSA. Three masses for HSA were observed; monomer, free thiol group (A, M_r 66 437); monomer, blocked thiol group (B, M_r 66 555); and amino-terminally degraded HSA (C, M_r 66250).

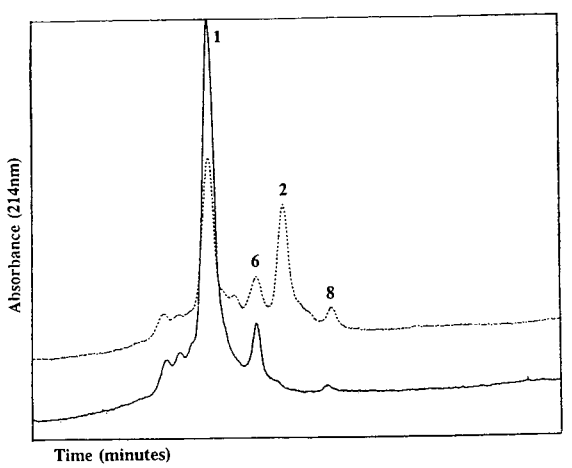


Fig. 4. Separation of reduced and non-reduced HSA on a neutral coated capillary. The reduced sample (——) showed an increase in peaks 1 and 6 (intact and amino-terminally degraded HSA with a free thiol group respectively) with corresponding decreases in peaks 2 and 8 (intact and amino-terminally degraded HSA with a blocked thiol group respectively) when compared to the non-reduced state (– – –).

uct of HSA, deamidation or dimerisation/polymerisation of the protein. Further analysis is required to identify this species.

HSA is glycated [16,17] and it would seem probable that one of the resolved peaks could be due to a glycated form. Analysis of glycated HSA on the neutral coated capillary demonstrated that the neutral species bound to the protein did affect the resolution but not the profile of the separation, as the same peaks were obtained as for HSA, but significantly broader (data not shown). As previously described, separation at pH values above the p*I* of the protein is dependent on negative charges. Sugars such as glucose and mannose bind to lysine residues (lysine 525 of HSA is a major site for glycation) which would not affect the neutral overall charge of the protein under these conditions and thus not the separation. Therefore, it would be expected that separation at pH values value below the p*I* of HSA may differentiate between glycated and non-glycated species. However, the

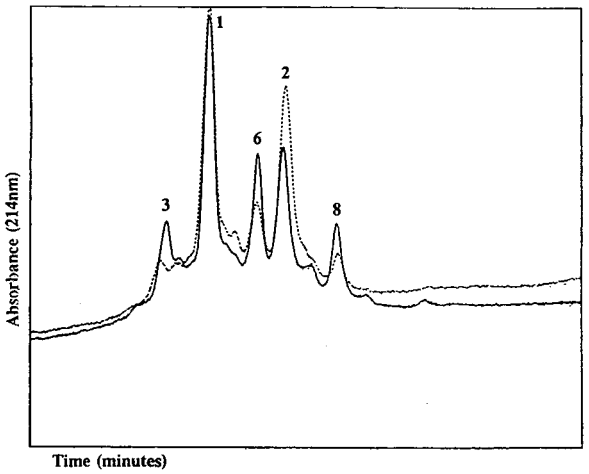


Fig. 5. Separation of HSA and HSA after incubation at 37°C on a neutral coated capillary. The 37°C incubated sample (—) demonstrated accelerated amino-terminal degradation by increases in peaks 6 and 8 (amino-terminally degraded HSA with free and blocked thiol groups respectively) with corresponding decreases in peaks 1 and 2 (intact HSA with free and blocked thiol groups respectively) compared to the non-incubated sample (– – –). The unidentified peak 3 also increased on incubation.

separation was found to be similar to that above the p*I* of HSA (the profile for glycated HSA constituted the same peaks as for HSA but broader and less well resolved). Therefore, we have been unable to demonstrate that a glycated form of albumin is separated by the neutral coated capillary. Further analyses are required to verify the identity of the remaining species resolved by this technique.

4. Discussion

High-resolution separation of clinical grade HSA and many other proteins is difficult using convential non-coated capillaries, especially with buffers near to the p*I* of the protein. This is demonstrated in this study where adequate HSA separation and limited resolution was only achieved at extremes of pH. However, the utilisation of a neutral coated capillary reversed this situation. Separation of HSA can be performed near to its p*I* and, because wall effects are greatly reduced, with high resolution. The resolving power of this technique could possibly be achieved or improved upon using IEF–PAGE, a method involving the casting of gels and staining of the proteins for identification and quantitation. However, this method requires the use of highly pure and expensive reagents to guarantee accuracy and reproducibility. To date, we have been unable to achieve a comparable separation using CE–IEF. The use of a neutral coated capillary, therefore, offers several advantages for the researcher or QC analyst for monitoring protein quality, even with large proteins such as HSA. The method is simple, requires no buffer additives, and allows for further method development depending on the individual protein being analysed. The method also has the capability to be micro-preparative if instrumentation allowing fraction collection is used. Resolved peaks can then be collected and subjected to further analysis. Potentially, the combination of this technique with electrospray mass spectrometry is an ideal method for further characterisation of sub-populations of proteins (as is the case with clinical HSA), the monitoring of minor specific protein modifications and for use in protein stability studies.

Acknowledgements

We thank Beckman Instruments (UK) for their help and support with this project and Neil Dodsworth for HSA analysis by electrospray mass spectrometry.

References

[1] Z. Deyl and R. Struzinsky, J. Chromatogr., 569 (1991) 63.
[2] Z.K. Shihabi, Annals Clin. Lab. Sci., 22 (1992) 398.
[3] W. Thormann, S. Molteni, J. Caslavska and A. Schmutz, Electrophoresis, 15 (1994) 3.
[4] Yan Xu, Anal. Chem., 65 (1993) 425.
[5] K.D. Altria and M.M. Rogan, Chromatogr. and Analysis, 32 (1994) 5.
[6] K.D. Altria, LC-GC Int., 6 (1993) 616.

[7] Z.E. Rassi and W. Nashabeh, in N.A. Guzman (Editor), Capillary Electrophoresis Technology: Chromatographic Science Series, Vol. 64, Marcel Dekker, New York, 1993, p. 383.
[8] T. Wehr, LC-GC Int., 6 (1993) 70.
[9] F.E. Regnier and D. Wu, in N.A. Guzman (Editor), Capillary Electrophoresis Technology: Chromatographic Science Series, Vol. 64, Marcel Dekker, New York, 1993, p. 287.
[10] D. Schmalzing, C.A. Piggee, F. Forest, E. Carrilho and B.L. Karger, J. Chromatogr. A, 652 (1993) 149.
[11] L. Anderson, in B. Blomback and L.A. Hanson (Editors), Plasma Proteins, Wiley, New York, 1979, p. 43.
[12] B.M. Van Liedekerke, H.J. Nelis, J.A. Kint, F.W. Vanneste and A.P. De Leenheer, J. Pharmaceut. Sci., 60 (1991) 11.
[13] T. Jr. Peters, in F.W. Putman (Editor), The Plasma Proteins, Academic Press, New York, 1975, p. 133.
[14] B. Chan et al., Eur. J. Biochem., 227 (1995) 524.
[15] J. Janatova, J. Med., 5 (1974) 149.
[16] N. Shaklai, R.L. Garlick and H.F. Bunn, J. Biol. Chem., 259 (1984) 3812.
[17] J.P. Bohney and R.C. Feldhoff, Biochem. Pharmacol., 43 (1992) 1829.

ELSEVIER

Journal of Chromatography A, 705 (1995) 343–349

JOURNAL OF CHROMATOGRAPHY A

Separation of stereoisomers of aminoglutethimide using three capillary electrophoretic techniques

Vincent C. Anigbogu[a], Christine L. Copper[b], Michael J. Sepaniak[b,*]

[a]*Department of Chemistry, Agnes Scott College, Decatur, GA 30030, USA*

[b]*Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600, USA*

First received 25 November 1994; revised manuscript received 25 January 1995; accepted 1 March 1995

Abstract

A novel approach to capillary electrokinetic chromatography involving differential distribution between neutral and charged cyclodextrins is described for the separation of the enantiomeric pair of the drug aminoglutethimide. For comparison purposes, micellar electrokinetic capillary chromatography and capillary electrophoresis with neutral cyclodextrin additives are also evaluated for this separation. The three techniques are compared in terms of their ability to resolve the two enantiomers. At pH 9, where aminoglutethimide is neutral, the enantiomers were resolved using a running buffer containing 5 m*M* carboxymethyl-β-cyclodextrin, 1 m*M* β-cyclodextrin, and 50% (v/v) methanol. At the same pH, micellar electrokinetic capillary chromatography, using a running buffer containing 50 m*M* sodium dodecyl sulfate and 17.5 m*M* β-cyclodextrin, only partially resolved the enantiomers. However, at pH 3, where aminoglutethimide is ionized, the enantiomeric pair was separated using capillary electrophoresis with either 10 m*M* α-cyclodextrin or 5 m*M* γ-cyclodextrin added to the running buffer. Enantiomeric separations, by way of the three electrophoretic capillary chromatographic techniques mentioned above, are presented and mechanisms of chiral recognition of aminoglutethimide by cyclodextrins under various experimental conditions are also discussed.

1. Introduction

The difference in the pharmacodynamic activities of enantiomers has created a need to study the pharmacological and toxicological properties of optically active compounds including drugs, agrochemicals, pesticides, herbicides, halogenated hydrocarbons and/or their stereoisomeric metabolic products [1–3]. Unfortunately, such studies are hampered by difficulties in obtaining both enantiomers of the target compound in optically pure forms either through stereospecific synthesis or chromatographic separation. Nevertheless, it is widely believed that in the near future, the pharmaceutical and agrochemical industries will be required by regulatory agencies, such as The Food and Drug Administration, to provide detailed information regarding the enantiomeric purity of drugs and therapeutic or toxic effects of individual enantiomers [4,5]. Hence, the development of rapid and accurate methods for stereochemical resolution of drugs is expected to remain an important issue for some time.

A racemic mixture of aminoglutethimide (AGT) (see Fig. 1 for structures), also known as

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00292-8

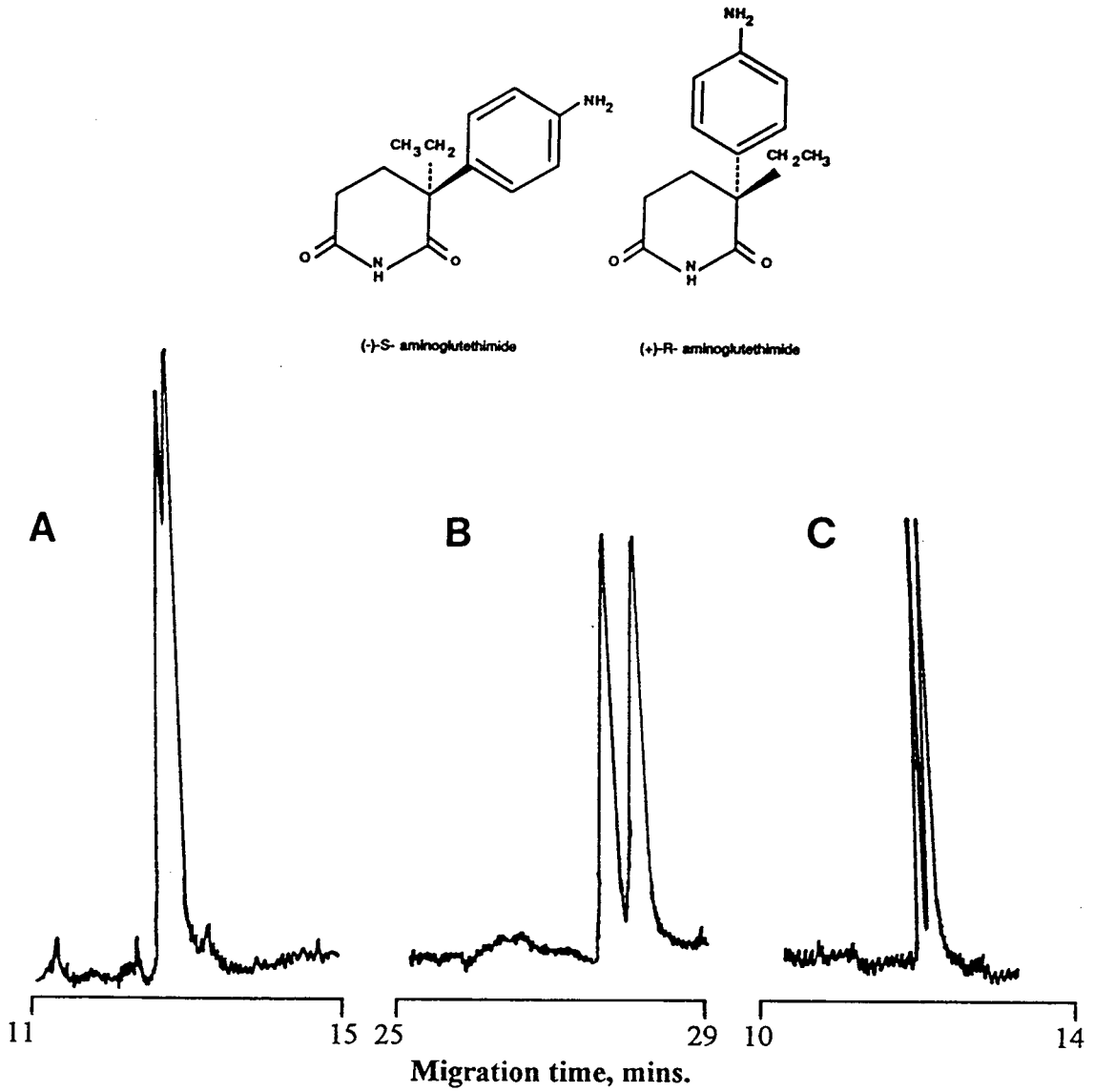


Fig. 1. Dual CD-phase CE (dual-CD-CE) separations of the enantiomers of AGT. Running buffer: NaH_2PO_4–$Na_2B_4O_7$ (pH 9) with 5 m*M* CM-β-CD and 1 m*M* β-CD; (A) 0% methanol and 10 kV applied voltage; (B) 50% (v/v) methanol at 10 kV; and (C) 50% (v/v) methanol at 20 kV.

(±)-3-(aminophenyl)-3-ethyl-2,6-piperidinedione, is clinically used for the treatment of adrenocortical tumors, metastatic breast cancer, and Cushing's syndrome [6,7]. HPLC has been used to resolve the enantiomers of AGT and its acetylated metabolite using a 10-cm long α_1-acid glycoprotein column [8]. However, the method was found to be time-consuming and very sensitive to mobile-phase conditions. Enantiomers of AGT have also been resolved using Chiralcel OD (cellulose tris-3,5-dimethylphenyl carbamate) and Chiralcel OJ (cellulose tris-(4-methylphenyl benzoate) ester) columns with some success [9]. More recently, the use of

Chiralcel OD and OJ columns in series was reported for the resolution of AGT stereoisomers and its acetylated metabolite in urine [10]. Besides suffering from the typical disadvantages of HPLC, the use of these specialty columns is very expensive. To circumvent these problems, various capillary electrophoretic techniques can be employed.

Capillary electrophoresis (CE) has been used extensively to obtain highly efficient separations (up to 10^6 plates/m) of charged solutes. However, neutral compounds cannot be separated using CE unless a charged "secondary phase" is employed to produce a capillary electrokinetic chromatographic mode of separation. Surfactants, above their critical micelle concentration (cmc), were first utilized in electrophoretic separations of neutral compounds by Terabe et al. in 1984 [11]. This technique, often dubbed micellar electrokinetic capillary chromatography (MECC), is instrumentally and operationally similar to CE [12]. In MECC, solutes are separated based on a differential distribution between the running buffer and the electrophoretically retarded micellar phase. MECC exhibits a finite "elution window", bordered by the column void time (t_o) and the effective elution time of the micelle (t_m). The existence of this elution window can limit peak capacity and hinder the separation of hydrophobic compounds, since they tend to completely associate with the micellar phase and co-elute near t_m [12].

Enantiomeric resolution by MECC can be achieved by using chiral surfactants, such as bile salts, or by adding chiral selectors to the running buffer. In fact, the combination of cyclodextrins (CDs) and micelles, referred to herein as CD-MECC, is widely reported for successful separation of enantiomers [13–15]. Native CDs are neutral, cylindrically shaped molecules consisting of a hydrophillic exterior and a hydrophobic cavity. Cavity diameters vary depending on the number of glucose units present (6, 7, or 8 units for α-, β-, and γ-CDs, respectively) [16]. Various CD derivatives (neutral and ionizable), such as hydroxypropyl (HP) and carboxymethyl (CM) variations, are also available and have been utilized in electrophoretic separations.

Terabe et al. first used carboxylated CDs as a charged secondary phase, which functioned in a manner similar to negatively charged micelles in MECC. They separated non-optically active, water-soluble compounds using this capillary electrokinetic chromatography format [17]. More recently, reports of charged CDs as running buffer additives in CE to separate enantiomers have appeared in the literature [18–20]. For example, Schmitt and Engelhardt [20] employed carboxylated CDs in different modes (charged or uncharged according to the pH of the buffer system) for the separation of neutral, cationic or anionic enantiomeric drugs.

It is likely that a comparable, but more predictable, alternative to the above-mentioned techniques would prove advantageous for some enantiomeric separations. In this report, we describe a dual (neutral and charged) CD-phase mode of capillary electrokinetic chromatography, referred to herein as dual-CD-CE, for the separation of the enantiomeric pair of AGT. Enantiomeric separations of AGT via CD-MECC and neutral CD-modified CE (CD-CE) are also presented for comparative purposes.

2. Experimental

2.1. Apparatus

An in-house constructed system, which consisted of a Hipotronics (Brewster, NY, USA) Model 840A high-voltage power supply and a Linear (Reno, NV, USA) Model 204 UV–Vis absorbance detector operated at 205 nm and 0.001 AUFS, was used in this work. Separations were performed using unmodified, fused-silica capillaries that were 50 cm × 50 μm I.D. (40 cm to the detector) and purchased from Polymicro Technologies (Phoenix, AZ, USA). Samples were hydrostatically injected for 10 s by raising the anodic end of the capillary 10 cm above the cathodic reservoir. The applied voltage ranged from 10 to 20 kV and the observed current ranged from 5 to 40 μA. All separations were carried out in a 10 m*M* NaH_2PO_4–6 m*M* $Na_2B_4O_7$ running buffer at various pHs (which

were reached by addition of phosphoric acid). Electropherograms were recorded with a Kipp and Zonen (Delft, Netherlands) strip-chart recorder.

2.2. Reagents

The *d,l*-aminoglutethimide (98% purity) sample was purchased from Sigma (St. Louis, MO, USA) and was used as received. The bile salt, sodium cholate, was purchased from Aldrich (Milwaukee, WI, USA). Buffer salts and other conventional chemicals were purchased from Baxter Scientific (McGaw, IL, USA). All cyclodextrin samples used were gifts from American Maize Products (Hammond, IN, USA) or CTD (Gainesville, FL, USA).

2.3. Procedure

A 1.0 m*M* stock solution of AGT was prepared in the appropriate buffer solution (1.4 mg AGT into 5.0 ml of buffer). The mixture was sonicated for about 30 min to insure complete dissolution. Working solutions containing 0.1 m*M* AGT were found to be stable for about two days.

At the beginning of each day, the column was rinsed with 100 m*M* NaOH and water. The column was then filled with the appropriate running buffer and allowed to equilibrate by applying 10 kV for several minutes before making the first injection. The column was also rinsed with 10 m*M* NaOH, followed by water, at regular intervals to insure consistent electroosmotic flow.

3. Results and discussion

Charged CDs alone, including carboxymethyl β-CD (CM-β-CD), have been employed to separate enantiomers of some optically active species. However, when charged CDs by themselves do not provide enantioselectivity, adding another chiral selector to the running buffer is a logical decision. The addition of a second optically active phase, such as a neutral CD, can result in enantiomeric resolution. This creates a capillary electrokinetic chromatographic mode of separation involving a charged CD secondary phase and a primary phase composed of running buffer with neutral CD. In separations of neutral, water-soluble compounds, interactions with the running buffer are significant. Conversely, water-insoluble compounds interact with the primary phase based solely on association with the neutral CD [21].

In a dual phase system containing negatively charged and neutral CDs (such as CM-β-CD and β-CD), the mobility of each enantiomer is altered depending upon its interaction with each of the two chiral phases. The magnitude of the difference between the mobilities of the enantiomers strongly influences the resolution observed. This difference can be amplified, thus improving resolution, by altering the types and concentrations of the enantioselective charged and/or neutral CD phases, or by extending the elution window. By analogy with MECC, the elution window is bordered by t_o and the effective migration time of the charged CD phase, t_{ch}. The elution window can be extended by adding certain organic solvents to the running buffer [12]. It should be noted that the polydispersity of the charged CD phase renders the value of t_{ch} poorly defined. It also can be a source of band broadening if the solute–charged CD association–dissociation kinetics are not rapid [21].

Since the enantiomers of AGT have no intrinsic mobility under neutral or basic pH conditions, negatively charged CM-β-CD was employed to impart an effective mobility to the enantiomers upon complexation. Despite an obvious complexation, indicated by an increase in migration time, the presence of CM-β-CD alone provided no enantiomeric resolution. Therefore, combinations of CM-β-CD and several neutral CDs (α, β, γ, HPβ, and HPγ) were tested. In these cases, complexation with the neutral CD inhibited complexation with the charged CD and reduced migration time. It was discovered that only the CM-β-CD/β-CD system imparted some enantioselectivity on AGT. The best resolution was observed at concentrations of 5 m*M* CM-β-CD and 1 m*M* β-CD (Fig. 1A).

Increasing the β-CD concentration above 1 m*M* further reduced migration time (i.e., further reduced the effective capacity factor of AGT), but degraded resolution. The dual-CD-CE technique is expected to mimic MECC in that the dependency of resolution on capacity factor exhibits an optimum that is influenced by the size of the elution window [17]. Because of a strong inclusion with β-CD, concentrations greater than 1 m*M* result in capacity factors less than the optimum. Further improvement in resolution was achieved through extension of the elution window by adding 50% (v/v) methanol (Fig. 1B). The ability to add such a large amount of methanol is unique to dual-CD-CE as compared to MECC, which can only tolerate up to 25% (v/v) organic modifier before micellization is disrupted [22]. The addition of methanol also resulted in a sharp drop in current (11 μA to 5 μA) which allowed a higher voltage (up to 20 kV) to be applied across the capillary. This led to a two-fold decrease in analysis time with no degradation of efficiency (Fig. 1C).

As stated above, the presence of an elution window in the dual-CD-CE system makes it comparable to the familiar MECC technique. For this reason, optimization of enantiomeric separation of AGT using a sodium dodecyl sulfate (SDS) micellar system was attempted in order to directly compare these similar techniques. Since SDS micelles alone cannot impart chiral recognition, neutral CDs were investigated as additives to the SDS mobile phase. It was found that β-CD, at a concentration of 17.5 m*M*, exhibited maximum, yet poor, resolution of the AGT enantiomers (Fig. 2A). In an attempt to improve resolution via an extension of the elution window, methanol was added to the running buffer. Unlike the dual-CD-CE case described above, the addition of methanol decreased selectivity (see Fig. 2B), even at concentrations as low as 5% (v/v). Other means of extending the elution window include using coated capillaries or employing surfactants other than SDS. However, these options were not explored in this work.

Bile salt surfactants, unlike SDS, are chiral in nature and have been used alone in MECC to provide enantiomeric resolution [15]. However, an attempt to resolve the neutral AGT enantiomers using 10, 20, and 50 m*M* concentrations of sodium cholate, with and without methanol, proved unsuccessful.

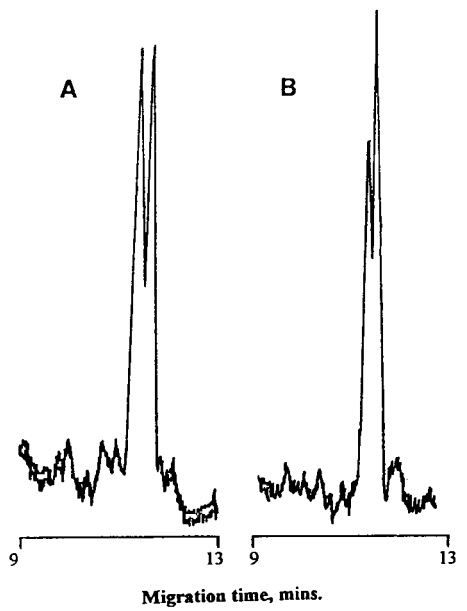


Fig. 2. Neutral CD-modified MECC (CD-MECC) separations of the enantiomers of AGT. Running buffer: NaH_2PO_4–$Na_2B_4O_7$ (pH 9) with 50 m*M* SDS at 15 kV applied voltage; (A) 17.5 m*M* β-CD; (B) conditions same as (A) with 5% (v/v) methanol.

A comparison of the dual-CD-CE system to CD-MECC for the enantiomeric separation of AGT enantiomers gives rise to several general advantages of the former technique. Firstly, the dual-CD-phase system is more resilient to the addition of organic solvents. This is a result of CDs being stable molecules while micelles are dynamic aggregates which are continually exchanging with free surfactant. This equilibrium has been shown to be affected by the addition of organic solvents. Therefore, larger quantities of organic solvents can be used in dual-CD-CE to extend the elution range or to increase solute solubility in the primary phase. Secondly, solute–CD interactions are generally more selective than solute–micelle interactions. Solutes form inclusion complexes with CDs based on their

size, geometry, and physiochemical properties, while interactions with micelles are largely based on solute hydrophobicity [12,16]. Therefore, with the specificity and wide variety of CDs available, the possibilities of unique selectivities in dual-CD-CE are expected to outnumber those of CD-MECC.

While dual-CD-CE and CD-MECC are both potentially applicable to the separation of neutral and charged stereoisomers, CD-CE can result in enantiomeric resolution of charged solutes only. In a pH 3 running buffer, AGT is positively charged and its enantiomers can be separated by addition of various neutral CD additives, as illustrated in Fig. 3. As expected, no enantiomeric resolution was observed in the absence of CD (Fig. 3A). The long migration time of the AGT cation is a consequence of the very slow electroosmotic flow at pH 3. Further increases in migration time result from a reduction in the positive mobility of the AGT cation upon complexation with the neutral CD. Near baseline enantiomeric resolution resulted from the addition of 10 m*M* α-CD while 5 m*M* γ-CD provided complete resolution (see Figs. 3B and 3C, respectively). However, any concentration of β-CD resulted in an increased migration time for AGT with no enantiomeric resolution observed.

While the CD-CE separation performance of AGT enantiomers is comparable to that of dual-CD-CE, this may not be the case in separations of other enantiomeric compounds. Resolution in CD-CE requires solutes to be ionized giving rise to several problems. First, at very low pHs, electroosmotic flow is reduced leading to increased analysis time. Second, the compound of interest may be unstable at extreme pH.

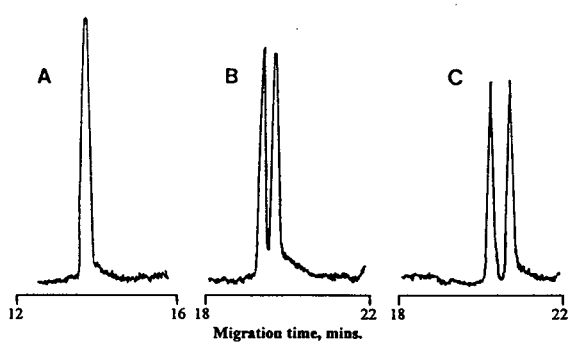


Fig. 3. Neutral CD-modified CE (CD-CE) separations of the enantiomers of AGT. Running buffer: NaH_2PO_4–$Na_2B_4O_7$ (pH 3) at 15 kV applied voltage (A) no CDs added; (B) conditions same as (A) with 10 m*M* α-CD; and (C) conditions same as (A) with 5 m*M* γ-CD.

It seems that pH plays an important role in each of the separation methods described above. Specifically, AGT enantiomers interact differently with native cyclodextrins depending upon the pH of the running buffer. In dual-CD-CE and CD-MECC at pH 9, only β-CD exhibited enantioselectivity. However, at pH 3 in the CD-CE mode, both α- and γ-CDs exhibited stereoselectivity towards AGT while β-CD did not. It is likely that these behavioral differences of AGT, in the presence of the various native CDs, are reflections of its conformational changes at different pHs. However, they may also stem from more complex occurrences. For, example, in dual-CD-CE, the AGT enantiomers may form complexes of equal energies with the neutral β-CD and these diastereomers are resolved by differentially interacting with the charged CM-β-CD. However, detailed confirmation of such behavior has yet to be determined experimentally.

It is quite obvious that for ionizable stereoisomers, the more straight forward technique is CD-CE, as evidenced by the ease of separation of the AGT enantiomers illustrated above. However, for neutral molecules, CD-MECC or dual-CD-CE is the method of choice. A comparison of these two techniques for the separation of AGT enantiomers revealed that the latter is easier to optimize than the former. But this is not without a price. The prerequisite to enantiomeric separation of neutral compounds using dual-CD-CE is that the enantiomers must form inclusion complexes with the charged CD phase. The degree to which a solute molecule is complexed by a charged CD depends on the size of the CD cavity, steric hindrances at the entrance of the cavity, and the types and charges of substituents on the solute and CD molecules [23]. Since a wide variety of charged CDs, with varying degrees of substitution, are available, it

is important to be able to predict the encapsulating ability of specific CD derivatives with molecules to be separated. Previously, we have used computer-aided molecular modeling to study CD–solute interactions [14,24]. We expect that similar studies involving derivatized CDs will provide insight into their usefulness in capillary electrophoretic separations.

Acknowledgements

This work was supported by the Division of Chemical Sciences, Office of Basic Sciences, United States Department of Energy, under Grant DE-FGO5-86ER13613, and the National Science Foundation, under Grant CHE-9320461, with The University of Tennessee, Knoxville. Support was also contributed by The Procter and Gamble Co. and by Merck and Co., Inc. The authors are grateful to G.A. Reed of American Maize Products and S. Herschleb of CTD Inc. for providing the cyclodextrin samples used in this study.

References

[1] M. Novotony, H. Soini and M. Stefansson, Anal. Chem., 66 (1994) 646A.
[2] T.J. Ward, Anal. Chem., 66 (1994) 633A.
[3] S.C. Stinson, Chem. Eng. News, Sept. 27 (1993) 38.
[4] P. Camilleri, Capillary Electrophoresis: Theory and Practice, CRC Press, Ann Arbor, MI, 1993.
[5] W.H. DeCamp, Chirality, 1 (1989) 2.
[6] G.A. Nagel and R.J. Stanten, Aminoglutethimide as an Aromatase Inhibitor in the Treatment of Cancer, Hans Huber Publishers, Berne, 1984.
[7] P.E. Lonning and S. Kvinnsland, Drugs, 35 (1988) 685.
[8] H.Y. Aboul-Enein and M.R. Islam, J. Chromatogr. Sci., 26 (1988) 616.
[9] H.Y. Aboul-Enein and M.R. Islam, Chromatographia, 30 (1990) 223.
[10] H.Y. Aboul-Enein and M.R. Islam, in S. Ahuja (Editor), Chiral Separations by Liquid Chromatography, American Chemical Society, Washington, DC, 1991, Ch. 12, p. 203.
[11] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem., 56 (1984) 111.
[12] M.J. Sepaniak, D.F. Swaile, A.C. Powell and R.O. Cole, in P. Grossman and J. Colburn (Editors), Capillary Electrophoresis, Academic Press, New York, NY, 1992, Ch. 6, p. 159.
[13] H. Nishi, T. Fukuyama and S. Terabe, J. Chromatogr., 553 (1991) 503.
[14] C.L. Copper and M.J. Sepaniak, Anal. Chem., 66 (1994) 147.
[15] R.O. Cole, M.J. Sepaniak and W.L. Hinze, J. High Resolut. Chromatogr., 13 (1990) 579.
[16] C. Strattan, BioPharm, 4 (1991) 44.
[17] S. Terabe, H. Ozaki, K. Otsuka and T. Ando, J. Chromatogr., 332 (1985) 211.
[18] A. Nardi and A. Eliseev, J. Chromatogr., 638 (1993) 247.
[19] N.W. Smith, J. Chromatogr. A, 652 (1993) 259.
[20] T. Schmitt and H. Engelhardt, Chromatographia, 37 (1993) 475.
[21] M.J. Sepaniak, C.L. Copper, K.W. Whitaker and V.C. Anigbogu, J. Chromatogr. B., in press.
[22] J. Gorse, A.T. Balchunas, D.F. Swaile and M.J. Sepaniak, J. High Resolut. Chromatogr., 11 (1988) 554.
[23] J. Szejtli, Cyclodextrins and Their Inclusion Complexes, Akademiai Kiado, Budapest, 1982.
[24] C.L. Copper, J.B. Davis, R.O. Cole and M.J. Sepaniak, Electrophoresis, 15 (1994) 785.

ELSEVIER

Journal of Chromatography A, 705 (1995) 351–361

JOURNAL OF CHROMATOGRAPHY A

Application of modified cyclodextrins in capillary electrophoresis for enantiomeric resolution of propranolol and analogues

M.W. Matchett, S.K. Branch, T.M. Jefferies*

School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK

First received 21 July 1994; revised manuscript received 20 February 1995; accepted 1 March 1995

Abstract

The enantio-resolution of propranolol and four derivatives was examined by free solution capillary electrophoresis (FSCE) using various cyclodextrin molecules. Of the three modified cyclodextrins investigated, hydroxyethyl-β-cyclodextrin provided the largest chiral resolution values for all five analytes. This may be linked to its extended hydrogen bonding chains on the cyclodextrin rim. Methyl-β-cyclodextrin and *heptakis*(2,3-di-O-acetyl) β-cyclodextrin gave lower maximum analyte resolutions, probably due to differences in their macrocyclic structure and hydrogen bonding ability. The presence of a bulky, non-polar alkyl group on the analytes was found to enhance chiral recognition. Methanol was found to have a varied effect on chiral resolutions, dependent on the type of cyclodextrin and structure of the analyte.

1. Introduction

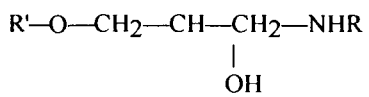
Propranolol [1-(isopropylamino)-3-(1-naphthyloxy)-2-propranol] is a widely prescribed optically active β-blocker used in the treatment of various cardiovascular disorders. There is considerable interest in developing methods to separate its enantiomers [1–3], which show different pharmacological effects in vivo [4]. The effects of various modified cyclodextrins on the enantiomeric resolution of propranolol and four analogues by free solution capillary electrophoresis (FSCE) are reported here.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of between 6 and 12 α-(1,4) linked glucopyranose units, with α- (6units), β- (7 units) and γ-CD (8 units) being the only commercially available forms of the parent macrocycles. The molecules have nonpolar inner cavities and are hydrophilic in nature on their external rims due to the presence of either primary or secondary hydroxyl groups [5]. With these characteristics, CD "hosts" are able to form diastereomeric inclusion complexes with a wide range of optically active compounds, allowing the "guest" molecules to be resolved into their enantiomers. CDs have thus been used for chiral discrimination in a variety of analytical techniques [6].

FSCE involves the application of a high voltage across a narrow capillary filled with buffer in order to separate components based on their relative electrophoretic mobilities, often in the presence of electroendosmotic flow (EOF) [7].

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00294-4



1 = R' = naphthyl, R = *i*-pro
2 = R' = naphthyl, R = *t*-butyl
3 = R' = naphthyl, R = ethyl
4 = R' = phenyl, R = *i*-pro
5 = R' = phenyl, R = *t*-butyl

Fig. 1. Structures of propranolol (1) and four analogues used in this study.

When CDs are added to a buffer containing cationic analytes, compounds which have higher association constants with the CD are expected to display increased migration times in FSCE (anode destination electrode). The CD is electrically neutral and thus possesses no electrophoretic mobility. Consequently, enantiomers which are charged may be chirally resolved if their CD stability constants, which are strongly dependent upon the structures of both CD and analyte, are sufficiently different to provide for separate migration times.

In this work propranolol and four closely related analogues (1–5 in Fig. 1) were used to study how changes in molecular structure can affect the complexation process with available cyclodextrins, selected on the basis of their different hydrophobicities and hydrogen-bonding abilities: methyl-β-cyclodextrin (Me-β-CD), hydroxyethyl-β-cyclodextrin (HE-β-CD) and *heptakis*(2,3-di-O-acetyl)β-cyclodextrin (Ac-β-CD). Possible host–guest interactions are proposed by relating the analyte and CD structures to the resulting migration times and enantio-resolutions.

A method previously applied to propranolol by Fanali [8] was also employed to rapidly assess the ability of urea solubilized β-CD to resolve the structurally related analogues. In addition, the effect of methanol on enantio-resolutions and migration times was briefly examined.

2. Experimental

Me-β-CD (average degree of substitution 1.8) and HE-β-CD (molar substitution 0.6) were supplied by Wacker (Munich, Germany). *Heptakis*(2,3-di-O-acetyl)β-cyclodextrin was generously donated by H. Mallwitz and U. Holzgrabe of the Pharmazeutisches Institut der Universität Bonn (Germany). Propranolol and its derivatives (1–5) were kindly provided by Dr. G. Bedford of Zeneca, Macclesfield, UK.

A Biorad HPE CE system was used with a BioRad 20 cm × 25 μm I.D. coated capillary. Samples were loaded by electromigration and separated at room temperature at a constant current of 5 μA. Data were recorded at the analyte λ_{max} values (λ_{288} for compounds 1–3 and λ_{254} for compounds 4 and 5) with the Biorad 800 HRLC detector (0.005 = AUFS), version 2.30. Samples of compounds 1–5 were prepared by dissolution in methanol–50 m*M* potassium dihydrogen phosphate pH 3.0 (30:70, v/v) at about 0.5 mg/ml. Buffers were prepared from potassium dihydrogen phosphate (50 m*M*) using freshly distilled and filtered water and then adjusted to the appropriate pH with orthophosphoric acid before the addition of methanol. The required amount of CD was then added to the buffers, which, along with the sample solutions, was then filtered through a 0.2-μm filter (Whatman, Maidstone, UK) and centrifuged at 11 400 *g* for 5 min before use.

Enantiomeric resolution values determined by FSCE have been calculated according to Eq. 1; [9]

$$R_s = 1.177(t_2 - t_1)/(Wa_{1/2} + Wb_{1/2}) \quad (1)$$

where t_1 and t_2 are the migration times for peaks 1 and 2, whilst $Wa_{1/2}$ and $Wb_{1/2}$ are the widths of peaks 1 and 2 respectively, at half peak height. It is appreciated that the experimentally determined resolution values include a factor for column efficiency as well as selectivity, however all the data reported here has been obtained using the same column and all the analytes were examined under a range of identical operating conditions under which changes in resolution values are mainly due to changes in selectivity values.

Coated capillary columns, such as used in this work, are able to minimize EOF, which can prove detrimental to resolution [10]. Apparent

electrophoretic mobilities (μ_{ep}) can be calculated according to Eq. 2; [2]

$$\mu_{ep} = lL/Vt_m \quad (2)$$

where l is the length to the detector, L is the total capillary length, V is the operating voltage, and t_m is the migration time of a neutral marker.

Electroendosmotic flow (EOF) measurements were attempted according to recommendations from the manufacturers Biorad. A neutral marker, in our case acetone, was loaded at the outlet reservoir in a running buffer to acetone ratio of 20:1. The polarity was set from negative to positive and the time taken for the neutral marker to pass from the outlet reservoir to the detector window was measured (a distance of 4.6 cm). The λ_{max} was 264 nm, the maximum for acetone in an aqueous acid [11].

3. Results and discussion

The aqueous/buffer mixture was chosen to ensure a stable pH (3.0) at which analytes 1–5 possessed a positive charge (pK_a's of 1–5 are around 9 ± 0.5) and thus migrated towards the cathode. Attempts to measure the EOF under these conditions were successful only in that they gave a maximum possible value of $8 \cdot 10^{-6}$ cm^2 V^{-1} s^{-1}. This is very low and compares favourably with Wren and Rowe [2], who ignored EOF at the higher value of $0.04 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}.

3.1. Effect of heptakis(2.3-di-O-Acetyl)β-cyclodextrin (Ac-β-CD)

With Ac-β-CD only 1 and 2 were enantiomerically resolved under these experimental conditions (Table 1). Acetylation increases the hydrophobicity of the CD and changes the hydrogen bonding ability of the CD which often results in low enantioselectivity [12]. However Yamashoji et al. [13] have shown that Ac-β-CD can be used to give improved chiral resolution of DL-alanine β-naphthylamide in CE when compared to β-cyclodextrin.

With only the two most hydrophobic compounds (1 and 2) showing any enantio-recognition (Fig. 2) and possessing the longest migration times, it would appear that analyte hydrophobicity is a major factor in the determination of resolution values and complex formation constants when using this CD derivative in FSCE. There are few published incidents were Ac-β-CD has provided enhanced separation over the underivatized β-CD [14]. Previous work in this laboratory has in fact demonstrated the ability of Ac-β-CD to provide enhanced enantio-selectivity for a series of phenethylamines in FSCE, when compared to results achieved using β-CD [15]. All five analytes however showed longer migration times as the concentration of Ac-β-CD was increased, indicating that they underwent

Table 1
Migration times (t_m, min) for compounds 1–5 in the presence of increasing amounts (mM) of *heptakis*(2,3-di-O-acetyl)β-cyclodextrin (Ac-β-CD). Resolution values (R_s) in parentheses. FSCE conditions: methanol–50 mM KH_2PO_4, 30:70, pH 3. Constant current of 5 μA.

Analyte	0.0 mM	4 mM	7.4 mM	15 mM	25 mM	35 mM[a]
1	6.84	10.82	10.88	11.56, 11.65	11.78, 11.89	8.48
	(0)	(0)	(0)	(<0.1)	(0.26)	(0)
2	6.94	11.07	11.35, 11.39	12.27, 12.41	12.39, 12.56	8.98
	(0)	(0)	(<0.1)	(0.63)	(0.81)	(0)
3	6.32	10.58	10.67	11.29	11.37	8.24
	(0)	(0)	(0)	(0)	(0)	(0)
4	4.30	9.82	10.02	10.34	10.39	6.53
	(0)	(0)	(0)	(0)	(0)	(0)
5	5.59	10.27	10.43	10.86	11.05	7.51
	(0)	(0)	(0)	(0)	(0)	(0)

[a] 4 M urea was necessary to enable dissolution of this amount of cyclodextrin.

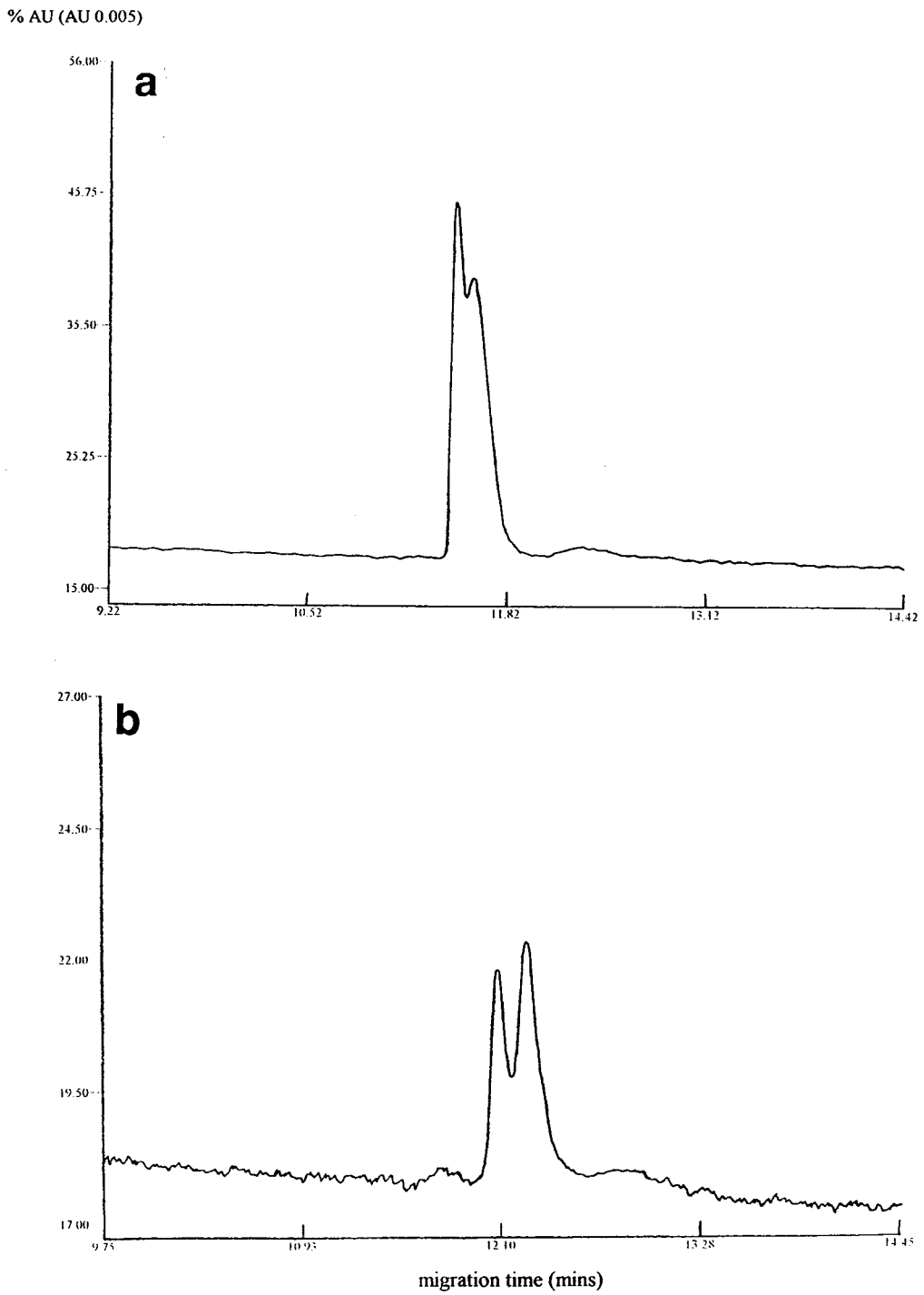


Fig. 2. Electropherograms for compounds 1 (a) and 2 (b). FSCE conditions: methanol–50 m*M* KH_2PO_4, 30:70, pH 3, containing 25 m*M* Ac-β-CD. Constant current of 5 μA.

complexation to some extent. These migration times were longer in the presence of Ac-β-CD than Me-β-CD up to the 15 m*M* level (suggesting higher formation constants), but shorter than the migration times seen with HE-β-CD (suggesting lower formation constants). Yet enantio-resolution was much higher for all the analytes with HE-β-CD and also Me-β-CD, except in the case of 2. Therefore the ability of a molecule to form an inclusion complex with a CD does not cause enantio-discrimination per se [16], although it is deemed to be a necessary pre-requisite in most instances [17].

Due to the insoluble nature of Ac-β-CD it was necessary to use 4 *M* urea to solubilise 35 m*M* of this CD in the buffer. This resulted in lower migration times than would have been expected if urea was absent, and also in the removal of all signs of enantio-recognition for 1 and 2. The strongly polar urea molecules may have significantly reduced any possible hydrogen-bonding of the CD with 1 and 2, thus negating resolution and shortening the migration times by increasing the amount of time 1 and 2 spent as the faster moving, free analytes.

3.2. Effect of methyl-β-cyclodextrin (Me-β-CD)

Methylated β-cyclodextrins have been extensively employed in CE chiral separations, often demonstrating enhanced resolutions over the parent macrocycle [18–22]. Wren and Rowe stated that in the presence of Me-β-CD, hydrophobicity would be the major force driving complexation in a series of β-blockers [2]. More hydrophobic solutes would require the presence of less CD to achieve their individual optimum resolution values. This hypothesis is partly supported by our results.

Compounds 1 and 2 each gave maximum resolution values at 25 m*M* Me-β-CD, with the more hydrophobic 2 having a higher resolution value of 0.73, compared with compound 1 which is less non-polar and gave a lower resolution of 0.54 (see Table 2). Compound 3 is less hydrophobic than either 1 or 2 and gave a lower maximum resolution of 0.32 at the highest Me-β-CD concentration of 35 m*M* (see Fig. 3). Similar in behaviour was compound 5 which is less hydrophobic than 1, 2 or 3 and also gave its maximum resolution at 35 m*M*. However the resolution of 5 (1.54) was higher than observed with the other more non-polar analytes. Also, the least hydrophobic of the compounds, 4, unexpectedly showed its maximum resolution of 0.68 at only 25 m*M* Me-β-CD, when it would have been thought to require a higher concentration than the other compounds, based on the simple model advanced by Wren and Rowe [2].

Different structural features of the five analytes therefore seem to be important not only in the actual complexation process but also in the

Table 2
Migration times (t_m, min) for compounds 1–5 in the presence of increasing amounts (m*M*) of methyl-β-cyclodextrin (Me-β-CD). Resolution values (R_s) in parentheses. FSCE conditions: methanol–50 m*M* KH_2PO_4 30:70, pH 3. Constant current of 5 μA.

Analyte	0.0 m*M*	4 m*M*	7.4 m*M*	15 m*M*	25 m*M*	35 m*M*
1	6.84	7.84	8.39, 8.48	9.37, 9.51	11.07, 11.23	11.87, 12.05
	(0)	(0)	(0.22)	(0.26)	(0.54)	(0.48)
2	6.94	8.12	9.37, 9.42	10.12, 12.23	14.20, 14.39	14.34, 14.45
	(0)	(0)	(0.17)	(0.27)	(0.73)	(0.31)
3	6.32	7.50	7.82, 7.88	9.57, 9.68	13.44, 13.62	14.07, 14.18
	(0)	(0)	(0.16)	(0.20)	(0.30)	(0.32)
4	4.30	6.50	6.57, 6.63	8.39, 8.53	10.90, 11.14	11.58, 11.82
	(0)	(0)	(<0.1)	(0.21)	(0.68)	(0.53)
5	5.59	7.00	7.90, 7.99	9.08, 9.26	11.51, 11.78	13.87, 14.23
	(0)	(0)	(0.63)	(0.73)	(1.32)	(1.54)

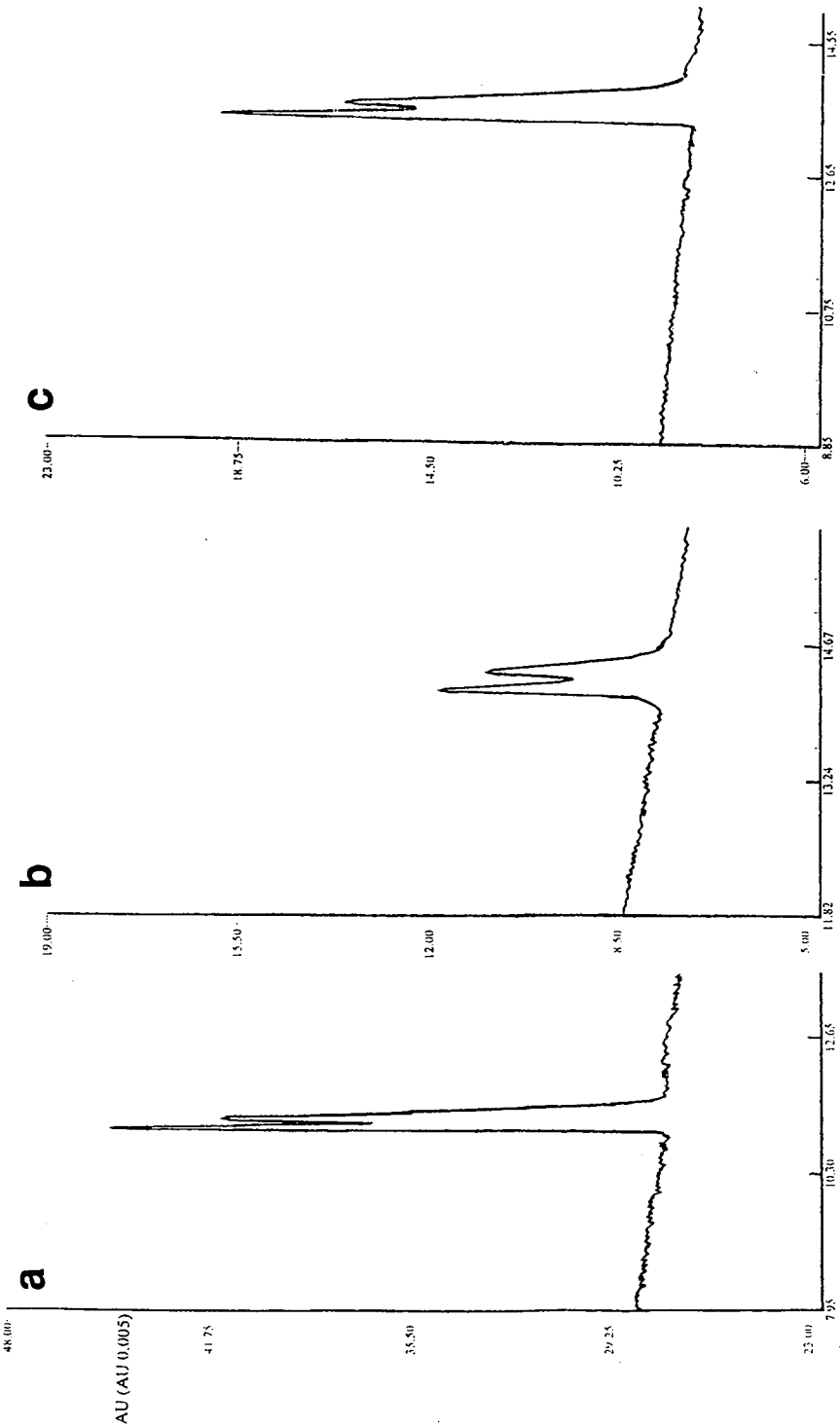


Fig. 3. Electropherograms for compounds 1 (a), 2 (b) and 3 (c). FSCE conditions: methanol–50 m*M* KH_2PO_4, 30:70, pH 3, containing Me-β-CD at 25 m*M*, 25 m*M* and 35 m*M*, respectively. Constant current of 5 μA.

overall enantio-recognition mechanism. Compounds 4 and 5 gave higher maximum resolution values than 1 and 2 respectively, suggesting that the presence of the naphthyl moiety proved detrimental to resolution by perhaps holding the stereogenic centre too far from the CD rim to permit optimum hydrogen-bonding with the CD hydroxyl groups.

The bulky *tert.*-butyl R group promoted enantio-recognition in comparison to the *iso*-propyl R group with maximum resolution values of $2 > 1$ and $5 > 4$. This may be related to the proposed hydrophobicity effect, with the more non-polar compounds having a stronger association with the Me-β-CD and hence also displaying longer migration times. Chang et al. [23] found that *tert.*-butyl derivatives of amino acids were well resolved on a bonded hydroxypropyl-β-CD column in HPLC, clearly supporting the idea that this group can be beneficial for chiral discrimination. However, by virtue of the insignificant chemical shifts demonstrated by the *iso*-propyl protons of propranolol, NMR spectroscopy has demonstrated that this R group (see compound 1, Fig. 1) is not incorporated into the β-CD cavity [24]. Therefore the R groups must interact with the modified CD rim to influence enantio-recognition, perhaps via some steric and/or hydrophobic effect, which alters the analyte's position in the CD cavity and by doing so, holds the analyte hydroxyl and amine groups in a more favourable orientation to encourage chiral recognition.

Methylation is known to not only make the parent β-CD more hydrophobic [25] but also to increase the cavity depth and enhance the flexibility of the macrocycle due to a reduction in the number of possible hydrogen bonds between the O(2) and O(3) hydroxyl groups [26]. These changes may explain the improved analyte enantio-recognition seen with Me-β-CD by allowing a "better fit" of the analyte (in this case those compounds with R = *tert.*-butyl) with the more hydrophobic and conformationally flexible Me-β-CD molecule.

3.3. Effect of hydroxyethyl-β-cyclodextrin (HE-β-CD)

The enantiomers of propranolol have been resolved using HE-β-CD in CE [27], although in the same work the authors were unable to achieve a similar degree of separation with Me-β-CD. Other work has indicated that HE-β-CD generally gave poorer chiral separations than both methylated β-cyclodextrins [20] and even β-cyclodextrin itself [21]. In this work HE-β-CD gave longer migration times for all five compounds than did Me-β-CD or Ac-β-CD, suggesting that the analytes formed stronger inclusion complexes with this CD derivative under the experimental conditions used here. Strongly related to this is the fact that the highest resolution values for all the analytes occured with this CD (Table 3). The extended hydrogen-bonding sites

Table 3
Migration times (t_m, min) for compounds 1–5 in the presence of increasing amounts (m*M*) of hydroxyethyl-β-cyclodextrin (HE-β-CD). Resolution values (R_s) in parentheses. FSCE conditions: methanol–50 m*M* KH_2PO_4 30:70, pH 3. Constant current of 5 μA.

Analyte	0.0 m*M*	4 m*M*	7.4 m*M*	15 m*M*	25 m*M*	35 m*M*
1	6.84	12.48, 12.56	12.64, 12.78	14.39, 14.63	15.84, 16.12	16.06, 16.36
	(0)	(<0.1)	(0.44)	(0.74)	(0.83)	(0.74)
2	6.94	12.42, 12.54	12.53, 12.71	13.85, 14.14	15.34, 15.68	16.00, 16.39
	(0)	(0.66)	(1.08)	(1.31)	(1.51)	(1.67)
3	6.32	11.19	11.99, 12.07	13.86, 14.01	15.61, 15.80	16.63, 16.84
	(0)	(0)	(<0.1)	(0.62)	(0.69)	(0.77)
4	4.30	10.10	10.64, 10.72	12.19, 12.41	13.57, 13.84	13.78, 14.04
	(0)	(0)	(<0.1)	(0.94)	(1.03)	(0.29)
5	5.59	10.52	10.90, 11.01	13.08, 13.32	14.45, 14.77	15.53, 15.90
	(0)	(0)	(<0.1)	(1.12)	(1.30)	(1.54)

on the HE-β-CD chains may be responsible for these observations, by allowing a closer spatial interaction with the hydrogen donor-acceptor sites near the asymmetric centre on the lengthy analyte alkyl chain. HE-β-CD was also the only CD to provide any enantio-recognition at the 4 m*M* level, where it partially resolved 1 and 2, with the more non-polar 2 having a higher resolution value of 0.66.

However, HE-β-CD caused a reversal in the migration order of 1 and 2 when compared to the results obtained with Me-β-CD and Ac-β-CD. Thus 2 had faster migration times than 1 over the entire CD concentration range, yet still maintained higher enantio-resolution values than 1. Evidently structural changes to the rim of the CD cavity are responsible for the behaviour seen here, although the exact mechanism is not as yet understood. NMR studies are in progress to help to clarify the situation.

A second difference noted with HE-β-CD was that the presence of the analyte naphthyl moiety had a more varied effect on enantio-resolution. Although the maximum resolution value of 4 was still greater than that of 1 (as with Me-β-CD), 5 had a lower maximum resolution than 2 which was not the case with Me-β-CD. The most hydrophobic of the solutes, i.e. 2, therefore gave the highest individual maximum resolution with HE-β-CD (see Fig. 4), whereas the less non-polar 5 displayed the largest resolution with Me-β-CD. In both these cases neither 2 nor 5 had the longest migration times with HE-β-CD and Me-β-CD, respectively.

^{1}H NMR investigations have indicated that the naphthyl moiety of propranolol (1) penetrates into the β-CD cavity, forming complexes with a 1:1 stochiometry, whilst its alkyl chain lies directly over the rim of the CD cavity [24]. This allows the hydroxyl and amine groups of 1 to hydrogen-bond with the secondary hydroxyl groups of the β-CD molecule [28]. It is therefore not surprising that both pairs 1/4 and 2/5 should have portrayed different enantio-resolution values as respectively, they differ only in their R′ groups, i.e. that part of the structure which is responsible for inclusion into the CD cavity and which thus plays a crucial role by holding the molecule in the correct orientation for optimum enantio-recognition.

As with Me-β-CD, the existence of a *tert.*-butyl group on the analytes promoted enantio-recognition in the presence of HE-β-CD, with resolution values of $2 > 1$ and $5 > 4$ at all CD concentrations.

HE-β-CD has additional hydrogen-bonding sites on its modified rim, accounting perhaps for it displaying the highest maximum resolution values observed in this work. The ability of many modified CDs to improve on enantio-recognition values seen with the parent CDs has been described before in many other FSCE applications [8,16,25,29].

3.4. Effect of β-cyclodextrin (β-CD)

It was not possible to fully investigate the effects of β-CD under the buffer conditions used with the other CDs due to its very low solubility in 30% methanol (0.64 g/100 ml) [30]. Therefore only a 4 m*M* β-CD buffer was prepared (methanol–50 m*M* KH_2PO_4, 30:70, v/v, pH 3). No enantio-resolution was observed for any of the analytes at this CD concentration (unlike HE-β-CD which resolved 1 and 2) and the migration times were faster here than with the other three CD derivatives, suggesting the possibility of lower CD analyte formation constants.

A method developed by Fanali [8] was briefly tested to observe how the structural differences of the five analytes affected their ability to be resolved using a urea solubilized β-CD (40 m*M*) methanol–phosphate (30:70) buffer (Table 4). Optical resolution values were of the order $2 > 1 > 5 > 3 > 4$. As seen with the CD derivatives, the presence of a *tert.*-butyl group helped promote resolution with $2 > 1$ and $5 > 4$. When R′ = naphthyl, enantio-resolutions increased with $1 > 4$ and $2 > 5$, which is directly opposite to the results obtained with Me-β-CD and also in contrast to the resolution order seen for 1 and 4 with HE-β-CD. It would seem therefore that under these conditions β-CD favours the recognition of compounds 1 and 2, the bulkiest and most hydrophobic of the five analytes.

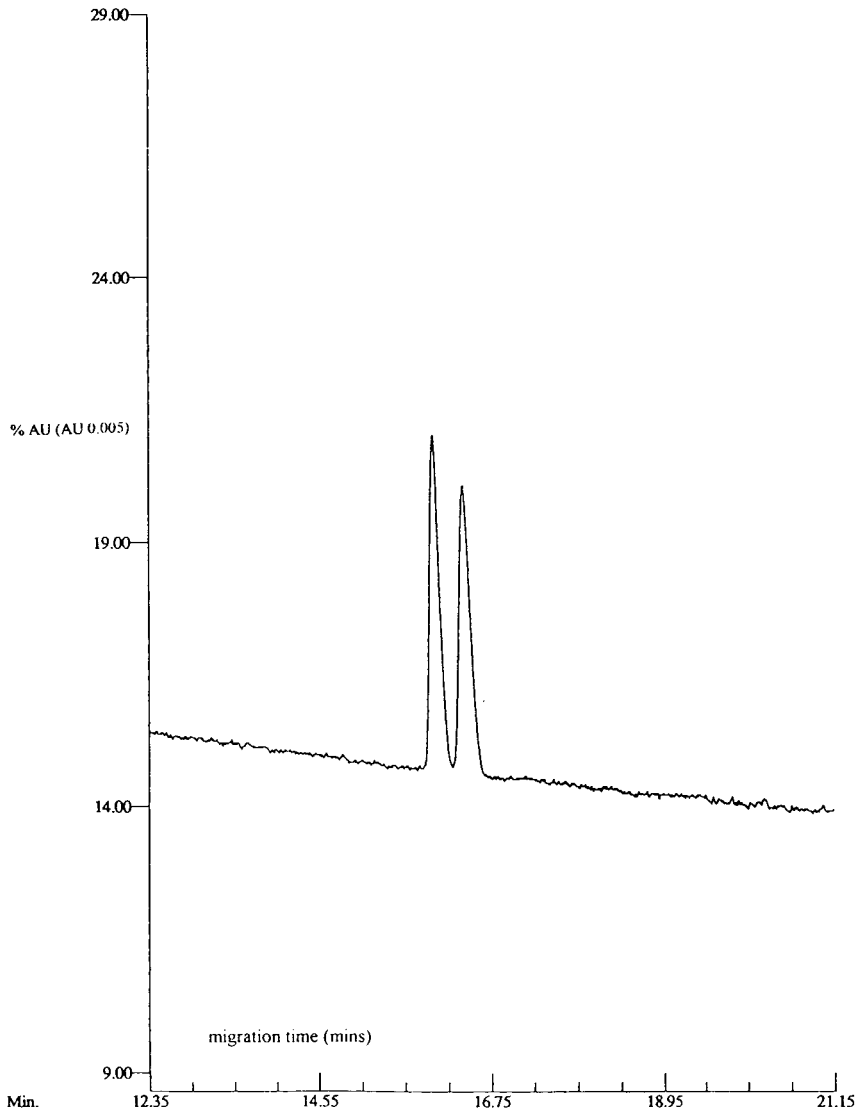


Fig. 4. Electropherogram for compound 2 FSCE conditions: methanol–50 m*M* KH_2PO_4, 30:70, pH 3, containing 35 m*M* HE-β-CD. Constant current of 5 μA.

3.5. Effect of methanol on resolution and migration times

It was decided to examine what effect the removal of methanol would have on the analyte resolutions and migration times achieved in the presence of the CD derivatives. Limited by the relatively insoluble Ac-β-CD, the 7.4 m*M* level was chosen as the maximum CD concentration in the absence of organic modifier. Me-β-CD and HE-β-CD concentrations were thus kept equal to this level to allow for a direct comparison of results.

With both HE-β-CD and Ac-β-CD the removal of methanol had a detrimental effect on resolution. None was observed for any of the

Table 4
Resolution values (R_s) and migration times (t_m, min) for compounds 1–5 in the presence of β-cyclodextrin. FSCE conditions: methanol–50 m*M* KH_2PO_4 30:70, pH 2.5, 4 *M* urea. Constant current of 5 μA.

1	1.05	12.06, 12.23
2	1.31	10.80, 11.03
3	0.28	10.43, 10.54
4	<0.1	8.76, 8.87
5	0.63	9.86, 10.04

analytes with HE-β-CD in the absence of methanol, whereas they all formally displayed some resolution when methanol was present. Ac-β-CD (at 7.4 m*M*) had only previously resolved 2 when methanol was present. This slight enantio-resolution was removed when methanol was absent. With Me-β-CD the effect on resolutions seemed more variable. Enantio-resolutions of 1, 2 and 3 increased when no methanol was present, whilst those of 4 and 5 decreased. Migration times increased in every case when methanol was removed.

As stated by Wren and Rowe [9], organic solvents such as methanol are believed to change the apparent mobility difference between the analyte enantiomers in FSCE, which in turn can increase or decrease the observed enantioselectivity depending on the CD type and concentration. This finding is mirrored in these results where 30% methanol was shown to have a beneficial effect on resolution with HE-β-CD and Ac-β-CD, yet it produced both higher and lower resolutions using Me-β-CD, dependent on the analyte structure (and hence its CD complex formation constant). Solvation can radically alter both the geometry and relative stabilities of various cyclodextrin–analyte conformations [17], so it is not unexpected that enantio-resolution values and migration times should be affected when methanol is added to the buffer.

4. Conclusions

Of the three CD derivatives used, HE-β-CD gave the largest resolutions for all five analytes and was the only CD to show any enantio-selectivity at the 4 m*M* level. Increased flexibility of the Me-β-CD macrocycle and additional hydrogen bonding sites on the HE-β-CD rim may have been responsible for the resolution values seen with these CD derivatives. Ac-β-CD, the most hydrophobic of the CDs, showed the poorest enantio-selectivities, resolving only 1 and 2 to a small extent. This was related to a change in the hydrogen-bonding ability of Ac-β-CD [13]. In the presence of urea, β-CD (as used by Fanali [8]) showed some enantio-selectivity to all five analytes and even gave the largest resolution value seen for 1 (1.05).

The presence of a *tert.*-butyl group on the analyte alkyl chain (which is not believed to enter the CD cavity) enhanced resolutions with all the CDs used here (except in the case of 4 and 5 with Ac-β-CD) viz. maximum resolution values were of the order $2 > 1$ and $5 > 4$. The analyte positioning in the CD cavity and around the CD rim may have been altered by the *tert.*-butyl group due to some steric and/or hydrophobic effect, which in turn led to an increase in the observed enantio-selectivity.

When only the R′ group differed (i.e., for 1 and 4, and 2 and 5), resolution values either increased or decreased depending on the CD used. This R′ group is believed to enter the CD cavity [24]. If the cavity dimensions are altered by changes to the CD rim [22,26] then it would not be unexpected that enantio-resolution values should also change, perhaps as the analyte experiences a better or poorer "fit" with the CD cavity.

The presence of methanol was seen to have a varied effect on enantio-resolutions which was linked to the findings of Wren and Rowe [9] who stated that organic solvents altered the apparent mobility difference between enantiomers. Such a change could then alter the resolution values depending on the type and concentration of CD being used.

References

[1] S.A.C. Wren and R.C. Rowe, J. Chromatogr., 603 (1992) 235.

[2] S.A.C. Wren and R.C. Rowe, J. Chromatogr., 635 (1993) 113.
[3] J. Haginaka, C. Seyama, H. Yasuda and K. Takahasi, J. Chromatogr., 598 (1992) 67.
[4] A.M. Krustulovic (Editor), Chiral Separation by HPLC: Application to Pharmaceutical Compounds, Ellis Horwood, Chichester, 1989, Ch. 2, pp. 31–68.
[5] A.M. Krustulovic (Editor), Chiral Separation by HPLC: Application to Pharmaceutical Compounds, Ellis Horwood, Chichester, 1989, Ch. 7, pp. 147–172.
[6] D.T. Burns, J. Pharm. Biomed. Anal., 12 (1994) 1.
[7] J. Snopek, I. Jelinek and E. Smolkovia-Keulemansová, J. Chromatogr., 609 (1992) 1.
[8] S. Fanali, J. Chromatogr., 545 (1991) 437.
[9] S.A.C. Wren and A.C. Rowe, J. Chromatogr., 609 (1992) 363.
[10] S.A.C. Wren, J. Chromatogr., 636 (1993) 57.
[11] A.C. Moffat (Editor), Clarke's Isolation and Identification of Drugs, The Pharmaceutical Press, London, 1986, p. 315.
[12] A.M. Krustulovic (Editor), Chiral Separation by HPLC: Application to Pharmaceutical Compounds, Ellis Horwood, Chichester, 1989, Ch. 10, pp. 208–231.
[13] Y. Yamashoji, T. Ariga, S. Asano and M. Tanaka, Anal. Chim. Acta, 268 (1992) 39.
[14] R.J. Barnaby and A.M. MacLeod, J. Liq. Chromatogr., 14 (1991) 287.
[15] S.K. Branch, U. Holzgrabe, T.M. Jefferies, H. Mallwitz and M.W. Matchett, J. Pharm. Biomed. Anal, in press.
[16] S. Fanali, J. Chromatogr., 474 (1989) 441.
[17] J.E.D. Davies (Editor), Spectroscopic and Computational Studies of Supramolecular Systems, Kluwer Academic Publishers, Dordrecht 1992, Ch. 3, pp. 187–205.
[18] M.W.F. Nielen, Anal. Chem., 65 (1993) 885.
[19] H. Soini, M.-L. Riekkola and M.V. Novotny, J. Chromatogr., 608 (1992) 265.
[20] M.M. Rogan, K.D. Altria and D.M. Goodall, Electrophoresis, 15 (1994) 808.
[21] M. Heuermann and G. Blaschke, J. Chromatogr., 648 (1993) 267.
[22] P. Gareil, J.P. Gramond and F. Guyon, J. Chromatogr., 615 (1993) 317.
[23] S.C. Chang, L.R. Wang and D.W. Armstrong, J. Liq. Chromatogr., 15 (1992) 1411.
[24] D. Greatbanks and R. Pickford, Mag. Res. Chem., 25 (1987) 208.
[25] M. Tanaka, T. Miki and T. Shono, J. Chromatogr., 330 (1985) 253.
[26] K. Harta, K. Uekama, M. Otagiri and F. Hirayama, J. Incl. Phen., 2 (1984) 583.
[27] T.E. Peterson, J. Chromatogr., 630 (1993) 353.
[28] D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, Science, 232 (1986) 1132.
[29] A. Nardi, A. Eliseen, P. Bocek and S. Fanali, J. Chromatogr., 638 (1993) 247.
[30] M. Taghvaei and G.H. Stewart, Anal. Chem., 63 (1991) 1902.

ELSEVIER

Journal of Chromatography A, 705 (1995) 363–368

JOURNAL OF CHROMATOGRAPHY A

Short communication

Purification of preparative quantities of group B *Streptococcus* type III oligosaccharides

Lawrence C. Paoletti*, Kenneth D. Johnson

Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, 180 Longwood Avenue, Boston, MA 02115, USA

First received 23 December 1994; revised manuscript received 20 February 1995; accepted 1 March 1995

Abstract

Many bacterial capsular polysaccharides are regularly repeating units of oligosaccharides. Bacterial oligosaccharides have been used in neoglycoconjugate vaccines and as reagents in the study of specific antibody binding. Unfortunately, separation methods have not been adequate for the purification of preparative quantities of bacterial oligosaccharides. Here we describe a size-exclusion procedure that resulted in the resolution of group B *Streptococcus* type III oligosaccharides composed of 4–25 sugars.

1. Introduction

Oligosaccharides are composed of more than 2 but less than 10 monosaccharides [1]; however, in the vaccine field more common usage refers to an oligosaccharide as a polymer smaller in molecular size than that of the native polysaccharide [2–5]. Many bacterial capsular polysaccharides, composed of several repeating units of oligosaccharide, are well characterized virulence factors. Some bacterial polysaccharides serve to evade the host immune mechanisms by impeding antibody binding and/or complement activation. Oligosaccharides, including those of *Haemophilis influenzae* [2,6,7] and group B *Streptococcus* (GBS) type III [8,9], have been used in the design of neoglycoconjugate vaccines.

GBS type III capsular polysaccharide (CPS) is composed of pentasaccharide repeating units of galactose, glucose, N-acetylglucosamine and N-acetylneuraminic (sialic) acid (Fig. 1). The sialic acid moiety is critical in maintaining the conformation of the antigenic epitope of GBS type III cps [10]. High-affinity binding of specific antibody to the type III CPS is dependent on the presence of the negatively charged sialic acid moiety positioned as the terminal saccharide of the disaccharide side chain of GBS type III CPS [5]. Protective antibody to GBS recognizes the conformationally determined epitope on the type III CPS with negligible binding to a single type III pentasaccharide. However, increasingly higher affinity binding is found as the chain length of the oligosaccharide increases beyond two pentasaccharide repeating units [4]. Desialylation of GBS type III CPS results in a structure identical to that of the CPS of *S. pneumoniae* type 14 (Fig. 1) and a diminution of binding by type III-specific antibody [11]. Therefore, desialylated GBS type III CPS has been a useful reagent in

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00295-2

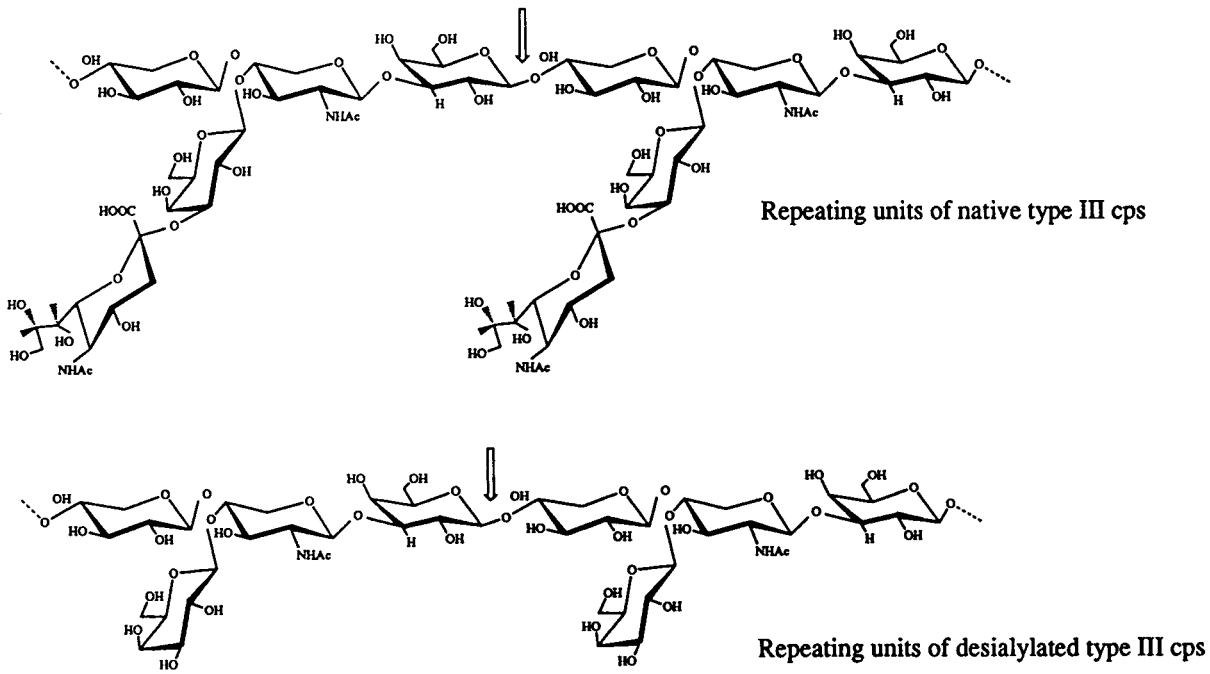


Fig. 1. Two repeating unit oligosaccharides structure of native, sialylated GBS type III [5] and desialylated GBS type III [11]. The arrow indicates site of depolymerization by endo-β-galactosidase.

experiments designed to study the role of sialic acid in binding of type III-specific antibody.

The need to study further the effect of GBS type III oligosaccharide chain length on the binding of specific antibody prompted an investigation into ways of generating preparative amounts of pure GBS type III oligosaccharides. Depolymerization of GBS type III CPS can be accomplished by digestion of the native CPS with crude preparations of endo-β-galactosidase prepared from spent culture fluids of *Citrobacter freundii* [8]. Endo-β-galactosidase cleaves internal β-D-galactose(1→4) linkages [12] and digestion of type III CPS with this enzyme results in the generation of oligosaccharides (Fig. 1) of one or more pentasaccharide repeating units [8]. Moreover, this method of depolymerization does not affect the acid-labile, α(2→3) ketosidic linkage between the side chain galactose and sialic acid of GBS type III CPS. Endo-β-galactosidase also cleaves desialylated GBS type III CPS, thus allowing for generation of type III oligosaccharides that lack sialic acid.

In general, chromatographic techniques have been used to analyse analytically component saccharides of oligosaccharides found on mammalian glycoproteins. Guile et al. [13] have recently reported the use of ammonium formate buffers in preparative anion-exchange high-performance liquid chromatographic (HPLC) separation of anionic sugars. Although excellent separation of anionic sugars was achieved by these investigators, the amount of oligosaccharides purified by this method was not reported. Previous attempts for the purification of GBS type III oligosaccharides using anion-exchange HPLC was labor-intensive and resulted in low (≤5 mg) yields [5]. Here we describe a procedure for the separation and purification of GBS oligosaccharides by size-exclusion chromatography with use of a Superdex 30 preparation grade column and an fast protein liquid chromatography (FPLC) system. Superdex 30 is a new size-exclusion medium composed of dextran covalently coupled to highly crosslinked porous agarose beads (Pharmacia LKB Biotechnology

product sheet). This matrix has a molecular mass separation range of $\leq 10\,000$ useful for the separation of proteins, peptides and nucleic acids. This is the first report of the application of this chromatographic matrix for the separation of bacterial oligosaccharides.

2. Experimental

2.1. Chemicals

Native, sialylated GBS type III CPS was isolated and purified from GBS strain M781 as described previously [14]. Desialylated type III CPS was generated with an acid extraction procedure as described by Lancefield [15]. Endo-β-galactosidase was partially purified from spent culture fluids of *C. freundii* and was used to depolymerize GBS type III as described previously [8]. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Equipment

An FPLC system consisting of an LCC-501 plus controller, P-500 pumps, MV-7 mixing valve, a Sephadex G-75 column (31 cm × 25 mm I.D.), a HiLoad 16/60 Superdex 30 prep grade column (60 cm × 16 mm I.D.) and a Superfrac fraction collector were purchased from Pharmacia (Uppsala, Sweden). A differential refractometer Model 401 (Waters, Division of Millipore, Marlborough, MA, USA) was used as the detector.

2.3. Chromatographic conditions

Sialylated type III oligosaccharides

Sialylated type III oligosaccharides were purified in a two-step procedure. Depolymerized type III CPS was placed on a G-75 column equilibrated with 10 m*M* Tris, pH 7.0 and fractions that corresponded to large (eluting close to the void volume of the column), medium, and small (eluting close to the bed volume of the column) were pooled and lyophilized to dryness. This procedure was repeated with several depolymerized batches of type III CPS. Each of three size pools was suspended in 0.7 ml of water and 0.5 ml was placed onto a Superdex 30 column equilibrated with 330 μM phosphate, 5 m*M* NaCl buffer, pH 7.3. The flow-rate was 1.0 ml/min. Oligosaccharide peaks were detected with a refractometer and fractions corresponding to each peak were individually collected and dried by lyophilization.

Desialylated type III oligosaccharides

Desialylated type III oligosaccharides were purified by loading enzyme-depolymerized, desialylated type III material directly onto the Superdex column. Fractions containing different-size oligosaccharides were collected as described above.

2.4. Thin layer chromatography and NMR analysis

Type III oligosaccharides composed of 10 sugars or less were visualized by TLC by methods described previously [5]. The number of repeating units in the oligosaccharides obtained from each of the peaks in the fractionation was determined by NMR spectroscopic analysis of coded samples in the laboratory of Dr. Harold J. Jennings, National Research Council of Canada, Ottawa. The intensity of the reducing-end galactose anomeric (α plus β) signals (doublets at 5.234 and 4.571 ppm, respectively) were compared with the coincident methyl signals (singlet at 2.03 ppm) of the N-acetylglucosamine and sialic acid residues. NMR spectra were run in ^2H_2O on a Bruker 600-Mz spectrometer at 290 K. For this analysis, acetone was used as an internal standard.

3. Results and discussion

3.1. Separation of sialylated GBS type III oligosaccharides

The elution profiles of the three sialylated type III oligosaccharide pools are shown in Fig. 2. The smallest-size pool recovered from the

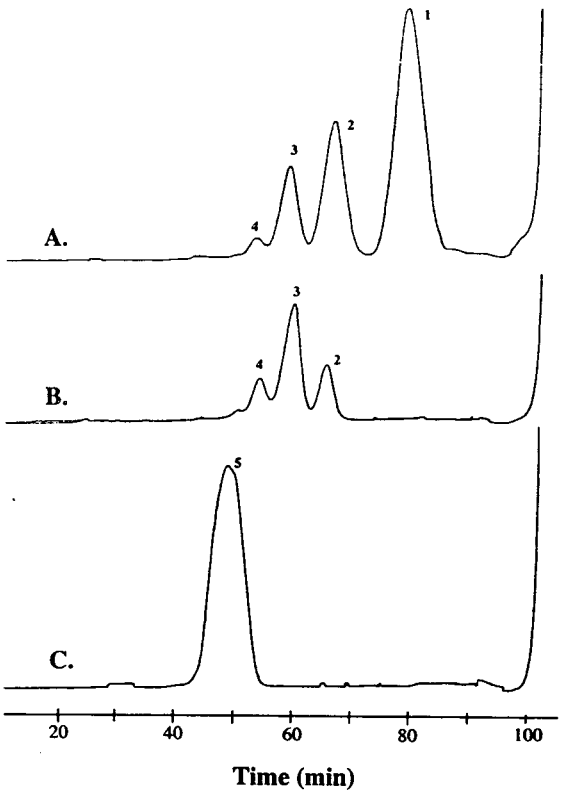


Fig. 2. Elution profiles of sialylated type III oligosaccharides. The numbers of type III repeating units are indicated above each peak. The attenuation setting on the refractometer was 16×, 16× and 8× for profiles A, B and C, respectively.

Sephadex G-75 column resolved into four fractions on the Superdex 30 column with peak volumes of 81 ml (peak 1), 68 ml (peak 2), 61 ml (peak 3), and 55 ml (peak 4) as shown in Fig. 2A. The medium-size pool recovered from the Sephadex G-75 column resolved into three fractions on the Superdex 30 column with peak volumes of 68 ml (peak 2), 61 ml (peak 3), and 55 ml (peak 4) as shown in Fig. 2B. The largest-size pool from the G-75 column resolved into a single fraction with a peak volume of 48 ml (peak 5) as shown in Fig. 2C. Elution volumes and K_{av} of these oligosaccharides are shown in Table 1. These data indicate baseline separation of sialylated type III oligosaccharides in the range of 5 to 25 sugars.

3.2. Separation of desialylated GBS type III oligosaccharides

Desialylated type III polysaccharide possessed 1% (w/w) sialic acid as measured by the thiobarbituric acid assay [16] with commercially available sialic acid as the standard. Desialylated type III (dIII) oligosaccharides were separated into distinct fractions of 6 repeating units (24 sugars) to 1 repeating unit (4 sugars). The elution volumes and K_{av} of these dIII oligosaccharides are shown in Table 1. During these experiments,

Table 1
Separation of GBS type III sialylated or desialylated oligosaccharides with use of a Superdex 30 size-exclusion column

Type III oligosaccharides	Repeating units (No. sugars)	FM ($\log_{10}$)	Elution volume (V_e) (ml)	K^a_{av}
Sialylated	5 (25)	3.702	48	0.067
	4 (20)	3.606	55	0.186
	3 (15)	3.481	61	0.288
	2 (10)	3.307	68	0.407
	1 (5)	3.009	81	0.627
Desialylated	6 (24)	3.633	56	0.203
	5 (20)	3.554	61	0.288
	4 (16)	3.458	66	0.373
	3 (12)	3.333	71	0.458
	2 (8)	3.159	79	0.593
	1 (4)	2.864	92	0.814

[a] V_o (44 ml) and V_t (103 ml) were determined using native type III polysaccharide and acetone, respectively. FM = formula mass.

we learned that digested CPS can be placed directly onto the Superdex 30 column eliminating the need for the Sephacel G-75 size-exclusion step and without loss of resolution. Furthermore, void volume material that contained both enzyme and native polysaccharide was collected and again incubated at 37°C for further digestion and processing of native polysaccharide.

Recovery of pure type III oligosaccharides typically ranged from 5 to 17 mg with greater amounts obtained with the smaller-size oligosaccharides.

3.3. TLC analysis of type III oligosaccharides

Type III oligosaccharides consisting of 4, 5, 8, and 10 saccharides were resolved by TLC (Fig. 3). The resolution limit of type III repeating units by TLC is 2 pentasaccharide repeating units or 10 sugars. These results are in complete agreement with published reports on the purification of these oligosaccharides obtained using more labor-intensive methods of purification [5,17]. In addition to TLC analysis, purity of all type III oligosaccharides was independently confirmed by NMR (not shown) as described above.

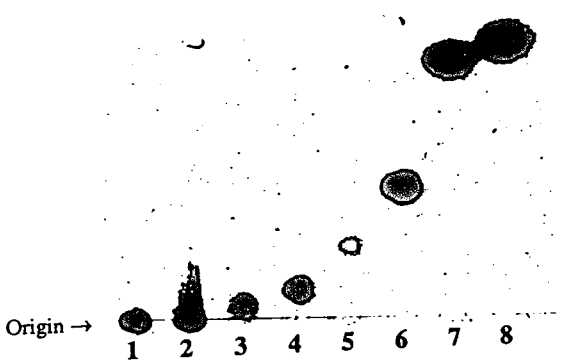


Fig. 3. TLC analysis of GBS type III oligosaccharides. Lane 1 = native type III polysaccharide; lane 2 = 2 pentasaccharide repeating units; lane 3 = 2 tetrasaccharide repeating units; lane 4 = 1 pentasaccharide repeating unit; lane 5 = 1 tetrasaccharide repeating unit; lane 6 = sialic acid; lane 7 = galactose; lane 8 = glucose.

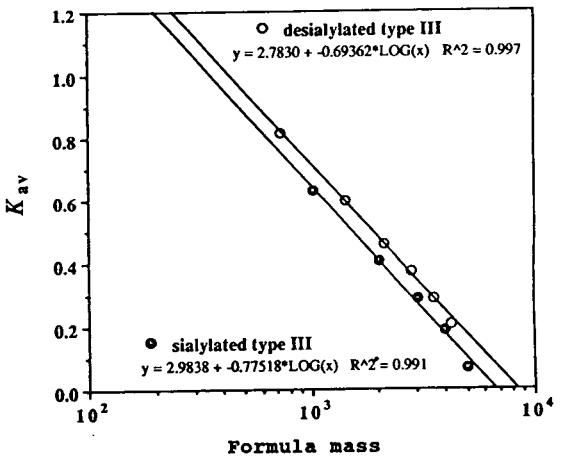


Fig. 4. Relationship between K_{av} and the formula mass of purified sialylated (●) and desialylated (○) type III oligosaccharides.

3.4. Relationship between type III oligosaccharide molecular mass and K_{av}

The formula mass and K_{av} of purified, native and desialylated type III oligosaccharide is shown in Table 1. There was a direct, positive relationship between the log-transformed formula mass and determined K_{av} of sialylated ($r^2 = +0.991$) and desialylated ($r^2 = +0.997$) GBS type III oligosaccharides as shown in Fig. 4. The Superdex 30 column matrix also resolved a four (desialylated single repeating unit) and a five (sialylated single repeating unit) sugar structure as indicated by the different K_{av} values for these oligosaccharides (Table 1 and Fig. 4).

4. Conclusions

Preparation of pure sialylated and desialylated GBS type III oligosaccharides consisting of 25 sugars or less was accomplished by size-exclusion chromatography using a Superdex 30 column. The procedure for generating oligosaccharides described here is efficient and rapid and results in baseline separation of preparative quantities of oligosaccharides of different chain length. A

direct linear correlation existed between the K_{av} measured and the log formula mass of type III oligosaccharides. These oligosaccharides will be valuable reagents for the study of the influence of polysaccharide conformation to the binding of GBS type III-specific antibody.

Acknowledgements

The authors thank April Blodgett and Julieanne Pinel for polysaccharide production and purification, and Dr. H.J. Jennings at the NRCC for NMR analysis. This work was supported by NIH grant AI-23339 and NIH contract AI-25125.

The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

[1] Joint Commission on Biological Nomenclature, Abbreviated terminology of oligosaccharide chains, Recommendations 1980, Eur. J. Biochem., 126 (1982) 433.
[2] P. Anderson, Infect. Immun., 39 (1983) 233.
[3] I. Seppala and O. Mäkelä, J. Immunol., 143 (1989) 1259.
[4] M.R. Wessels, A. Munoz and D.L. Kasper, Proc. Natl. Acad. Sci. USA, 84 (1987) 9170.
[5] M.R. Wessels, V. Pozsgay, D.L. Kasper and H.J. Jennings, J. Biol. Chem., 262 (1987) 8262.
[6] P.W. Anderson, M.E. Pichichero, R.A. Insel, R. Betts, R. Eby and D.H. Smith, J. Immunol., 137 (1986) 1181.
[7] P.W. Anderson, M.E. Pichichero, E.C. Stein, S. Porcelli, R.F. Betts, D.M. Connuck, D. Korones, R.A. Insel, J.M. Zahradnik and R. Eby, J. Immunol., 142 (1989) 2464.
[8] L.C. Paoletti, D.L. Kasper, F. Michon, J. DiFabio, K. Holme, H.J. Jennings and M.R. Wessels, J. Biol. Chem., 265 (1990) 18278.
[9] L.C. Paoletti, D.L. Kasper, F. Michon, J. DiFabio, H.J. Jennings, T.D. Tosteson and M.R. Wessels, J. Clin. Invest., 89 (1992) 203.
[10] H.J. Jennings, C. Lugowski and D.L. Kasper, Biochemistry, 20 (1981) 4511.
[11] H.J. Jennings, K.G. Rosell and D.L. Kasper, Can. J. Biochem., 58 (1980) 112.
[12] M.N. Fukuda, J. Biol. Chem., 256 (1981) 3900.
[13] G.R. Guile, S.Y.C. Wong and R.A. Dwek, Anal. Biochem., 222 (1994) 231.
[14] M.R. Wessels, L.C. Paoletti, D.L. Kasper, J.L. DiFabio, F. Michon, K. Holme and H.J. Jennings, J. Clin. Invest., 86 (1990) 1428.
[15] R.C. Lancefield, J. Exp. Med., 59 (1934) 441.
[16] L. Warren, J. Biol. Chem., 234 (1959) 1971.
[17] M.R. Wessels and D.L. Kasper, J. Exp. Med., 169 (1989) 2121.

ELSEVIER

Journal of Chromatography A, 705 (1995) 369–373

JOURNAL OF CHROMATOGRAPHY A

Short communication

Separation of hydroxyl protected heparin derived disaccharides using reversed-phase high-performance liquid chromatography

R.J. Kerns, R.J. Linhardt*

Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242, USA

Received 5 January 1995; accepted 2 March 1995

Abstract

A simple and efficient method for the separation of hydrophobic derivatives of glycosaminoglycan-derived disaccharides is described. Hydroxyl-protected derivatives of a trisulfated disaccharide, prepared from heparin using heparin lyase, were separated by reversed-phase high-performance liquid chromatography. These disaccharide derivatives differed by the number, position, and stereochemistry of acetyl and pivaloyl groups. Separation was achieved on a C_{18} column using a reversed gradient of ammonium sulfate in water. This method has application in the purification of disaccharide derivatives being used as chiral synthons in the preparation of higher oligosaccharides.

1. Introduction

High-performance liquid chromatography (HPLC) is commonly used in the analysis of oligosaccharides that are chemically and enzymatically derived from glycosaminoglycans (GAGs); including heparin, heparan sulfate, chondroitin sulfates, and dermatan sulfate. Amino columns [1–5], reversed-phase (RP) columns [6–11], strong anion-exchange (SAX) columns [12–15], and others [16–19] have been used for separating these highly polar acidic oligosaccharides. While a number of RP-HPLC methods have been developed for the analysis of GAG-derived oligosaccharides, pre-column derivatization and/or ion-pairing reagents are usually required for separation of these highly polar compounds. Pre-column derivatization has included lipophilic dansylhydrazone [16] and pyridylamino [10] groups attached through the sugars reducing-end. These derivatives can be detected by ultraviolet absorbance, fluorescence, and chemiluminescence. Ion-pairing reversed-phase (IPRP)-HPLC has utilized quaternary ammonium groups (tetrapropylammonium, tetrabutylammonium) to cap the negatively charged groups of GAG-derived oligosaccharides, providing hydrophobic interaction sites [6–9,11]. Most derivatization reactions are irreversible and the complete removal of the ion-pairing agents is often difficult. Thus, after a separation has been accomplished there is no practical way to recover the starting acidic oligosaccharides. This has limited RP- and RPIP-HPLC to the analysis of acidic oligosaccharides, precluding its use for preparative chromatography.

SAX-HPLC has been widely used for both the analysis and preparation of oligosaccharides en-

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00293-6

zymatically-derived from GAGs [12]. This is the optimal method for the fractionation of underivatized oligosaccharides containing unsaturated uronic acid residues allowing detection based on UV absorbance. Pre-column derivatization, with agents, such as 7-amino-1,3-naphthalene disulfonic acid (AGA), facilitates sensitive fluorescence detection [20]. These fluorescent tags are often charged to minimize hydrophobic interactions that are deleterious to fractionation on SAX-HPLC.

Enzymatic depolymerization of heparin with heparin lyases (as well as heparan sulfate, chondroitin sulfates, dermatan sulfate, and hyaluronic acid with their corresponding polysaccharide lyases) provides a reproducible distribution of unsaturated uronic acid containing oligosaccharide products [21]. The trisufated disaccharide 1 (Fig. 1) is the most abundant disaccharide obtained from heparin. In our laboratory, we have recently synthesized a number of hydroxyl-protected derivatives (2–8) of disaccharide 1 (Fig. 1) [22]. Purification of reaction mixtures utilizing SAX-HPLC afforded poor resolution. The pivaloyl derivatives (7 and 8) underwent hydrophobic collapse, precipitating on the column, while the various acetylated derivatives were poorly resolved, possibly the result of the minimal effect of acetate groups on their elution position. These results suggested that RP-HPLC might be useful for the separation of these highly charged disaccharide derivatives, which differ only in position, number, and stereochemistry of relatively small hydrophobic groups. The results presented describe a simple RP-HPLC method for the separation of these disaccharide derivatives. Derivatization and the use of ion-pairing reagents are not required, making this method potentially useful for preparative applications.

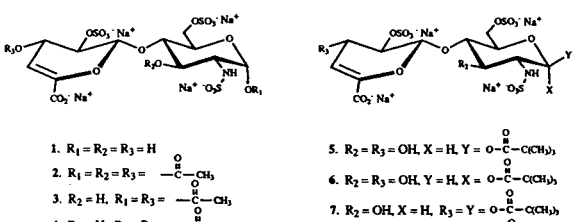


Fig. 1. Structures of heparin disaccharide derivatives.

2. Experimental

Trisulfated disaccharide (1) and derivatized disaccharides (2, 3 and 5–8) were prepared and characterized in our laboratory as previously reported [22]. Compound 4 was prepared from 2 by selective removal of the anomeric acetate using standard conditions [23]. All high-resolution NMR and FAB-MS data were consistent with structure. Composition of mixtures and purity of the disaccharide derivatives were determined by capillary electrophoresis as previously reported [22].

RP-HPLC used dual, face programmable, Shimadzu (Kyoto, Japan) LC-7A titanium-based pumps. The system was equipped with a Rheodyne (Cotati, CA, USA) No. 7125 titanium injector and a Pharmacia LKB (Piscataway, NJ, USA) 2141 variable-wavelength UV detector with both Shimadzu Chromatopac C-R2A integrating recorder and Rainin (Woburn, MA, USA) analog converter connected to a Macintosh system utilizing Dynamax chromatography software. Analytical separations were performed using a reversed-phase C_{18} column (25 cm × 4.6 mm I.D.), 5 μm particle size from Vydac (Hesperia, CA, USA). UV detection of these compounds was at 232 nm based on the chromophore of the unsaturated uronic acid residues [21].

All chemicals were of analytical or reagent grade. Ammonium sulfate (200 m*M*) was adjusted to pH 6 using 5% ammonium hydroxide. De-ionized, distilled water was adjusted to pH 6.0 with NaOH. All mobile phase solutions were filtered through a 0.45-μm membrane filter and degassed prior to use. Specific conditions for all separations are given below.

3. Results and discussion

The aim of this investigation was to develop a rapid and efficient method for the separation of

highly charged, structurally complex, heparin-derived disaccharide derivatives containing hydrophobic hydroxyl protecting groups. Both SAX-HPLC and RPIP-HPLC, used to separate enzymatically prepared disaccharide 1 [11,12], require conditions that create problems with the fractionation of derivatives 2–8.

SAX-HPLC was first used to purify these disaccharide derivatives on a semi-preparative scale [22]. This separation method has limited utility for purification of compounds 2–8 due to low resolution and long separation times. The acetate derivatives (2–4) are poorly resolved by SAX-HPLC, but this separation was satisfactory for the preparation of small quantities of these compounds. The more hydrophobic monopivaloylated derivatives, 5 and 6, are not resolvable by SAX-HPLC. Strong hydrophobic interactions between the dipivoylated derivatives 7 and 8 and the stationary phase of the SAX column occur at the high salt concentrations (>2 *M*) required for their elution. These hydrophobic interactions combined with the strong ionic interactions from the disaccharide's four acidic groups, give long retention times and broad peaks. The addition of up to 10% methanol to the aqueous salt eluent provided little improvement in resolution.

RP-HPLC separation of acidic oligosaccharides generally requires the use of quaternary ammonium ion-pairing reagents in the mobile phase [6–9,11]. Removal of the ion-pairing reagent following separation poses a serious purification problem. Additionally, when a fully hydroxyl-protected disaccharide derivative, such as 2, is converted to a tetrabutylammonium salt (prepared by neutralizing the acid form, obtained by passing 2 through H^+ Dowex, with tetrabutylammonium hydroxide) water solubility is lost and the regeneration of the sodium salt form becomes very difficult.

Pyridylamino derivatization of dermatan sulfate and chondroitin sulfate disaccharides through reductive amination has been reported [10]. This derivatization facilitates RP-HPLC separation in sodium phosphate buffer mobile phase containing methanol without the use of an ion-pair [10]. This observation suggested that compounds 2–8, also containing hydrophobic functionality, might be separated effectively using RP-HPLC without further derivatization and in the absence of ion-pairing reagents.

Compounds 1, 2, 7, and 8 were first examined using a C_{18} column with water (at pH 6.0) as the mobile phase. While 1 and 2 eluted with the void volume, 7 and 8 were slightly retained, giving broad overlapping peaks (chromatograms not shown). These preliminary results suggested that loading the samples in salt, followed by elution at decreasing salt concentrations might afford an improved separation. Ammonium sulfate was chosen to be added to the mobile phase since it had been used similarly in the analysis of phosphorylated peptides [24]. Ammonium sulfate can be easily removed after fractionation using a desalting column or by evaporating to dryness and recovering and disaccharide derivative from salt by dissolving it in methanol. Initially, this separation was performed isocratically with 200 m*M* ammonium sulfate. Trisulfated disaccharide 1 again eluted with the void, but disaccharide derivatives 2 and 7 were retained until the column was washed with water.

The optimum system for the separation of all of the positional and stereochemical isomers of the seven derivatized disaccharides (2–8) consisted of loading the sample in pH 6, 200 m*M* ammonium sulfate followed by a linearly decreasing ammonium sulfate gradient (200 m*M* to 0 m*M*) over 10 min. Analytical RP-HPLC chromatograms of disaccharride 1 (Fig. 2a) and acetylated disaccharides 2–4 (Figure 2b,c,e) show all components are well resolved. Resolution is based on the number of acetylated hydroxyl groups as well as the position of these groups (i.e., 3 and 4 are positional isomers). Although compound 4 was analyzed as >95 percent pure by capillary electrophoresis, a double peak was observed in Fig. 2e. A previous report [25] suggests that, under certain RP-HPLC conditions, the α- and β-anomers of a free anomeric center can result in a double peak.

Analytical RP-HPLC chromatography of the pivaloylated derivatives (5–8) are shown in Fig. 3. Differing degrees of pivaloylation results in dramatic differences in elution position. However, the α- and β-isomers of the monopivaloylated derivatives 5 and 6 (Fig. 3b) and the

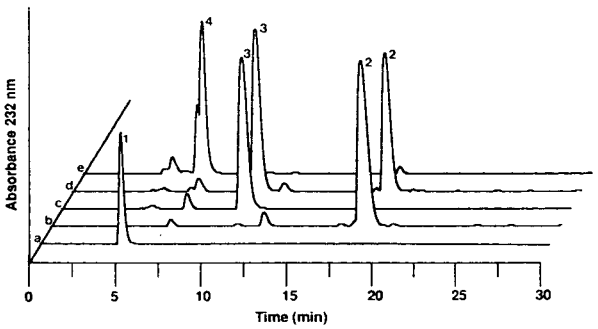


Fig. 2. Analytical RP-HPLC chromatograms of disaccharides 1–4 (peak labels correspond to the structures in Fig. 1). The stationary phase is a Vydac RP C-18 (5 μm), 250 × 4.6 mm I.D. column. The mobile phase is composed of solution A (200 m*M* ammonium sulfate, pH 6.0) and solution B (water, pH 6.0). A linear gradient elution of $t = 0.0$ min [solution A (0.6 ml/min), solution B (0.0 ml/min)] to $t = 10$ min [solution A (0.0 ml/min), solution B (0.6 ml/min)] and $t = 10$–50 min [solution B, 0.6 ml/min] was used. The injection volume was 30 μl and 40–60 μg of sample was analyzed per injection. Detection relied on UV absorbance at 232 nm.

dipivaloylated derivatives 7 and 8 (Fig. 3e) also gave well-resolved peaks. Compounds 5 and 6 had previously coeluted when analyzed by SAX-HPLC.

In conclusion, these data show that RP-HPLC provides a rapid and efficient separation of highly acidic heparin-derived disaccharide derivatives and that this separation is based on degree, position, and stereochemistry of both acetyl and pivaloyl hydroxyl protecting groups. The mobile phase used contains only water and ammonium sulfate making this method generally applicable for preparative separations and avoids the problems associated with using ion-pairing reagents and mixed solvent systems. These methods should facilitate the purification of GAG-derived disaccharides containing limited hydrophobicity or differing slightly in the hydrophobic moieties that are present. Current efforts include applying these separation techniques in the preparation of protected chiral synthons useful for the chemical synthesis of larger, biologically important GAG-like oligosaccharides.

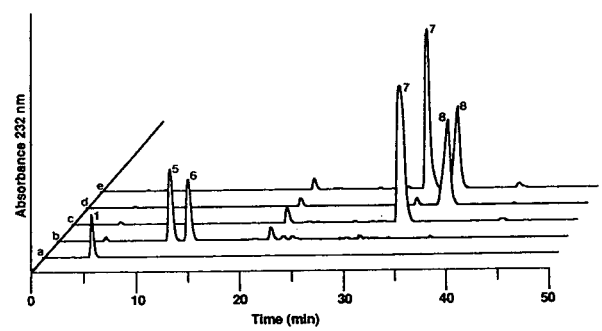


Fig. 3. Analytical RP-HPLC chromatograms of disaccharides 1, 5–8 (peak labels correspond to the structures in Fig. 1). Analysis was conducted as described in the legend of Fig. 2.

Acknowledgements

This work was supported by a research grant form The Mizutani Foundation for Glycoscience, Tokyo, Japan and a NIH pre-doctoral fellowship (NIH GM08365) to RK.

References

[1] D.C. Seldin, N. Seno, K.F. Austen and R.L. Stevens, Anal. Biochem., 141 (1984) 291.
[2] K. Shinomiya, I. Koshishi, T. Imanari, M. Takeda, M. Maeda and A. Tsuji, Chem. Pharm. Bull., 34 (1986) 4887.
[3] D.P. Norby, V.M. Goldberg, R.W. Moskowitz and C.J. Malemud, Anal. Biochem., 131 (1983) 324.
[4] K. Yoshida, S. Miyauchi, H. Kikuchi, A. Tawada and K. Tokuyasu, Anal. Biochem., 177 (1989) 327.
[5] H. Akiyama, T. Toida and T. Imanari, Anal. Sci., 7 (1991) 807.
[6] N. Ototani, M. Kikuchi and Z. Yosizawa, J. Biochem., 94 (1983) 233.
[7] Y. Guo and H.E. Conrad, Anal. Biochem., 168 (1988) 54.
[8] L. Silvestro, I. Viano, A. Naggi, G. Torri, R. Da Col and C. Baiocchi, J. Chromatogr., 591 (1992) 225.
[9] L.E. Chun, T.J. Koob and D.R. Eyre, Anal. Biochem., 171 (1988) 197.
[10] C. Kodama, N. Ototani, M. Isemura and Z. Yosizawa, J. Biochem., 96 (1984) 1283.
[11] R.J. Linhardt, K.N. Gu, D. Loganathan and S.R. Carter, Anal. Biochem., 181 (1989) 288.
[12] K.G. Rice, Y.S. Kim, A.C. Grant, Z.M. Merchant and R.J. Linhardt, Anal. Biochem., 150 (1985) 325.
[13] R.J. Linhardt, K.G. Rice, Y.S. Kim, D.L. Lohse, H.M. Wang and D. Loganathan, Biochem. J., 254 (1988) 781.

[14] S.R. Delaney and H.E. Conrad, Biochem. J., 209 (1983) 315.
[15] R.N. Shaklee and H.E. Conrad, Biochem. J., 235 (1986) 225.
[16] H. Akiyama, S. Shidawara, A. Mada, H. Toyoda, T. Toida and T.J. Imanari, J. Chromatogr., 579 (1992) 203.
[17] M.E. Zebrower, F.J. Kieras and W.T. Brown, Anal. Biochem., 157 (1986) 93.
[18] Y. Takazono and Y. Tanaka, J. Chromatogr., 288 (1984) 167.
[19] A. Hjerpe, C.A. Antonopoulos and B. Engfeldt, J. Chromatogr., 254 (1982) 365.
[20] K.-B. Lee, A. Al-Hakim, D. Loganathan and R.J. Linhardt, Carbohydr. Res., 214 (1991) 155.
[21] R.J. Linhardt, in A. Varki (Editor), Current Protocols in Molecular Biology, Vol. 2, Wiley-Interscience, New York, 1994, ch. 17.
[22] R.J. Kerns, I.R. Vlahov and R.J. Linhardt, Carbohydr. Res., 267 (1995) 143.
[23] C.A.A. Van Boekel, T. Beetz, J.N. Vos, A.J.M. de Jong, S.F. van Aelst, R.H. van den Bosh, J.M.R. Mertens and F.A. van der Vlught, J. Carbohydr. Chem., 4 (1985) 293.
[24] Z. Zhao, D.A. Malencik and S.R. Anderson, Biochem. Biophys. Res. Comm., 176 (1991) 1454.
[25] N. Ototani, N. Sato and Z. Yosizawa, J. Biochem., 85 (1979) 1383.

ELSEVIER

Journal of Chromatography A, 705 (1995) 374–379

JOURNAL OF CHROMATOGRAPHY A

Short communication

Determination of azadirachtin by reversed-phase high-performance liquid chromatography using anisole as internal standard

R. Thejavathi, Shirish R. Yakkundi, B. Ravindranath*

Vittal Mallya Scientific Research Foundation, P.O. Box 406, K.R. Road, Bangalore 560 004, India

First received 18 October 1994; revised manuscript received 22 February 1995; accepted 6 March 1995

Abstract

An accurate and convenient method of quantitation of the well-known biopesticide azadirachtin by reversed-phase high-performance liquid chromatography has been developed, using anisole as the internal standard. The method is precise and accurate within limits of ± 1.0% in the concentration range of 0.00002% (w/v) to 0.004% (w/v) of azadirachtin in solution (0.2–40 ppm) or 2–400 ng by weight of azadirachtin (10 μl injection; 20 ng of internal standard). Application of the method to stability studies of azadirachtin showed that the compound is reasonably stable (95–99%) in a variety of common solvents at room temperature (7 days) and to ultrasound (30 min). An efficient sample preparation protocol has been developed giving a recovery of 99.5 ± 4%.

1. Introduction

Azadirachtin (also known as azadirachtin A; Fig. 1) is a tetranortriterpenoid (limonoid) present in neem seeds (*Azadirachta indica* A. Juss) to amounts of 0.2 to 0.6% [1]. The neem tree, which yields about 30–40 kg/year of the seeds, is widely distributed in South Asian and several other tropical countries. Azadirachtin has gained world-wide attention for its insect antifeedant and ecdysis inhibiting properties [2,3]. This compound is highly potent at low concentrations against more than 200 agricultural pests and it is ecofriendly [4]. Thus, it has the potential to be a safe alternative to the toxic synthetic pesticides and a number of commercial formulations are being introduced world-wide. However, wide application of azadirachtin as a pest control agent requires a sensitive and reliable method for its quantitation in neem extracts, commercial formulations and foods, for monitoring its efficacy, stability and toxicity.

Azadirachtin occurs in neem together with nearly 100 other limonoids including azadirachtins B–K with closely related structures and its separation and quantitation is a challenging task. Reversed-phase high-performance liquid chromatography has been conveniently used for separation and quantitation of azadirachtin. The known methods of quantitation of azadirachtin generally use an external standardization method [4,5]. The main drawback of these methods is the inaccuracy in azadirachtin determination due to the possible loss of the compound during sample preparation. Such problems in quantita-

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00314-2

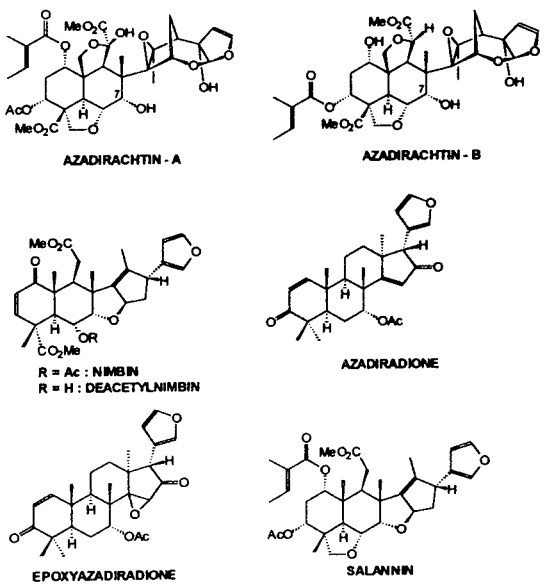


Fig. 1. Structures of major limonoids from *Azadirachta indica* A. Juss.

tion can generally be overcome by using an internal standard.

Quantitation by the internal standard method is sensitive and reproducible as a known amount of internal standard is added to the sample early in the sample preparation protocol and the compound is analysed as a ratio with respect to the internal standard. In the most recent paper on the HPLC analysis of azadirachtin by Hull et al. [7], benzyl alcohol, phenylethanol and phenylpropanol have been suggested as possible internal standards but without supporting data. None of these compounds are satisfactory because of the large differences in the chromatographic and spectral properties between the analyte and the standards. Also, the accuracy is only ± 10% in the range of 0.005 to 0.75% which is not sufficient for monitoring azadirachtin in the concentration ranges in which it is effective. Thirdly, the HPLC system used (3 μm Supelcosil RP-8 column, 150 × 4.6 mm I.D.) requires a 40-min run time and an elaborate column washing protocol with tetrahydrofuran. Therefore, the method is not very convenient for routine use.

In this paper, we report a fast, sensitive and accurate method for the quantitation of azadirachtin by using anisole as the internal standard. The efficacy of the method is supported by recovery studies and the method has been used to determine the stability of azadirachtin in different solvents.

2. Experimental

2.1. Chemicals and materials

Anisole from SD Fine Chemicals Pvt. (Bombay, India) was used without further purification. Solvents were obtained from E. Merck (India) (Bombay, India), and were distilled before use. Water used was deionised and purified using a Milli-Q water purifier from Millipore (Bedford, MA, USA). Azadirachtin was isolated from neem seeds by the method described by Schroeder and Nakanishi [8]. A 50-mg amount of azadirachtin (90% pure) thus obtained was subjected to preparative HPLC to yield 99% pure compound which was used to prepare calibration curves. Neem seeds were collected from Nagallur, a village in Andhra Pradesh, India.

2.2. Equipment

A Model 2100 UV spectrophotometer from Shimadzu Corporation (Kyoto, Japan) was used for spectroscopic studies. The HPLC system used consisted of two Model 510 reciprocating pumps, a Model U6K loop injector, a Model 484 tunable wavelength absorbance detector at 217 nm, a Maxima 820 FC system controller and data processor, a Novapak C_{18} (4 μm, 150 × 3.9 mm I.D., for analytical chromatography) and μBondapak C_{18} (10 μm, 300 × 10 mm I.D., for preparative purpose), all from Waters Associates (Milford, MA, USA); the sonicator (VC 600 Watt model dual output) was obtained from Sonics and Materials (Danbury, CT, USA), and the Remi centrifuge (Model R23) from Remi Sales (Bombay, India). The Whatman 3-piece filter funnel with glass microfibre filters (2.5-cm)

was from Whatman International (Maidstone, UK).

2.3. Chromatographic procedure

The solvent program used for complete analysis of neem extract was an isocratic elution with acetonitrile–water (40:60, v/v) for 5 min followed by a linear gradient to 100% acetonitrile in 3 min (20% increase per min) with a total run time of 12 min including a preacquisition delay of 2 min to eliminate the solvent peak in quantitation experiments. For preparative HPLC, 40% aqueous acetonitrile was used as the mobile phase with a flow-rate of 4.4 ml/min.

2.4. Standard solutions

Standard solution of azadirachtin (0.1%) was prepared by dissolving 50 mg of the compound in 50 ml of methanol. An anisole stock solution of 2 mg/ml was made and later diluted 100-fold to obtain a concentration of 20 μg/ml. A 1-ml volume of this solution was mixed with a calculated volume of azadirachtin solution and made up to 10 ml to cover the concentration range 0.2–100 μg/ml, with an anisole concentration of 2 μg/ml. Three 10-μl aliquots of each mixture were injected onto the HPLC system and the area and height responses determined using the data processor. For stability studies, the anisole stock solution (2 mg/ml) was diluted 10-fold to yield a concentration of 200 μg/ml.

2.5. Sample preparation

A 5-g amount of the powdered solid sample (e.g. neem seed powder) was sonicated (18 mm horn; 50% duty cycle; amplitude 5) for 30 min with methanol (12.5 ml) containing 250 μg of anisole. The mixture was centrifuged and the supernatant decanted. The solid was mixed with 8 ml of methanol and centrifuged again. The supernatants were pooled in a 25-ml volumetric flask and made up to the mark with 50% aqueous methanol to bring the total concentration of methanol in the test solution to 90% aqueous methanol. The solution was passed through a Whatmann three-piece filter funnel. A 1-ml volume of the filtrate was passed through a C_{18} Sep-Pak cartridge ("Classic", volume = 1 ml, 400 mg of ODS) and eluted with 3 ml of 90% aqueous methanol. The eluants were pooled and made up to 5 ml in a volumetric flask with 90% aqueous methanol and a 10-μl aliquots was injected onto the HPLC column (Fig. 2).

2.6. Recovery studies

De-oiled neem seed powder was obtained by extracting 100 g of neem seed powder with chloroform (500 ml) in a percolator. A 5-g amount of the powder was analysed for azadirachtin content as described above (0.005%). A 50-g amount of the neem seed powder (containing 2.5 mg of azadirachtin) was added to 100 ml of dichloromethane containing 10 mg of azadirachtin. Dichloromethane was evaporated and the dry powder was used for recovery studies the following day. A 5-g amount of the neem seed powder was subjected to the sample preparation protocol given above.

2.7. Stability of azadirachtin

To determine the stability of azadirachtin in different solvents, approximately 2.5 mg of azadirachtin was dissolved in the organic solvent (chloroform, acetone, ethyl acetate, acetonitrile, methanol and ethanol) and made up to the mark in a volumetric flask (10 ml) with the same solvent. A 1-ml volume of this solution was immediately mixed with 1 ml of anisole solution (200 μg/ml) in a 10-ml volumetric flask and made up with methanol, and a 10-μl aliquot was analysed on the HPLC system. A 1-ml volume of each of the solutions was refluxed for 8 h and taken in a 10-ml volumetric flask containing 1 ml of anisole stock solution and made up with methanol as before. The remainder of the solution was kept at room temperature for 7 days and analysed as before. Stability of azadirachtin to the sample preparation protocol was checked by dissolving 1.0 mg azadirachtin in methanol (10 ml) containing anisole (1 mg) and determin-

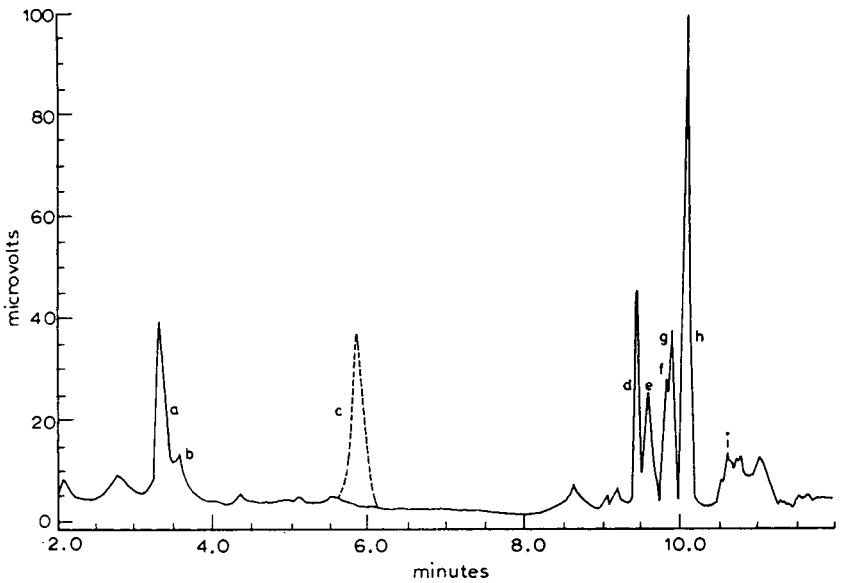


Fig. 2. Chromatogram of the neem seed extract. For sample preparation and chromatographic conditions, see text. Peaks: a = azadirachtin A; b = azadirachtin B; c = anisole (internal standard); d = deacetylnimbin; e = unidentified; f = azadiradione; g = nimbin; h = salannin; i = epoxyazadiradione.

ing the ratio of azadirachtin to internal standard before and after 30 min sonication in methanol.

3. Results and discussion

A variety of reversed-phase columns based on octadecylsilyl (C_{18}) and octylsilyl (C_8) stationary phases have been used for separation of neem limonoids. These columns show a wide range of selectivities and efficiencies. In the present study, we have used a Waters Novapak C_{18} column (150 × 3.9 mm I.D.) which, we have found, is particularly efficient for the separation of azadirachtin A and azadirachtin B ($\alpha = 1.1$), the main active constituents of neem, as well as the other major limonoids. Chromatographic analysis of a neem seed extract using a water–acetonitrile gradient required less than 12 min (Fig. 2).

Choice of an internal standard for azadirachtin was indeed difficult. An ideal internal standard should have similar chromatographic and spectral properties as the compounds to be analysed but should not normally co-occur with them nor co-elute with any of the peaks in the analyte. Though structurally very different from azadirachtin, we have found that anisole is a very good candidate as internal standard for azadirachtin, the reasons being: (a) the $A_{1\%}$ of anisole at 217 nm is 233, which is comparable to that of azadirachtin (173); (b) the separation factor of anisole to azadirachtin is comfortably close (around 1.8); (c) anisole is stable and neutral in nature and does not react with azadirachtin or other components of the sample; (d) the ratio of azadirachtin to anisole remained constant through the sample processing protocol; (e) anisole does not interfere with other components of the sample as shown by the chromatograms of neem extract with anisole in Fig. 2; and (f) the suitability of anisole as internal standard is further proved by recovery studies. It may also be mentioned that because of the different ranges of UV absorption (λ_{max} at 205 nm and 280 nm, respectively) and its appearance in the middle of the chromatogram (Fig. 2), well separated from both polar and nonpolar limonoids, anisole could also be the internal standard for several of the other important limonoids of

neem, the structures of which are shown in Fig. 1.

3.1. Calibration curve for azadirachtin with anisole as internal standard

The minimum detectable amount of azadirachtin (defined at a signal-to-noise level of 3) was 2 ng (10-μl injection volume). The response ratio of azadirachtin to anisole, taking either area or height, was found to be linear within the concentration ratio 0.1–20. Thus, the calibration curves for the response of azadirachtin with respect to anisole were obtained with the concentration range of azadirachtin being 0.2–40 μg/ml with anisole at 2 μg/ml (10-μl injection volume). Data was processed on Maxima 820 FC system controller and data processor. The linear regression analysis ($n = 33$) of the calibration curves yielded the following equations (a) for areas, $y = 0.413x - 0.053$ (y = area response ratio, x = mass ratio) with the standard error for slope and intercept being 0.002 and 0.015 (correlation coefficient $r = 0.9996$), respectively; (b) for heights, $y = 0.52x - 0.041$ (y = height response ratio, x = mass ratio) with the standard error for slope and intercept being 0.002 and 0.015, respectively, with correlation coefficient $r = 0.9998$. Beyond the linear range, the slope of the plot deviated appreciably from the above values. Thus, at an anisole-to-azadirachtin ratio of 1:25 the deviation from the slope was ± 2.5% and at 1:30, it was ± 5%. For concentrations beyond the upper limit, i.e. 40 μg/ml, and up to 1 mg/ml, it was found more accurate to use the internal standard at a concentration of 20 μg/ml, the equations being $y = 0.32x + 0.015$ and $y = 0.43x + 0.024$ for area and height ratios, respectively. The standard errors are 0.004 and 0.011 (height ratio) and 0.003 and 0.010 (area ratio) for slope and intercept, respectively.

3.2. Precision and accuracy

For the determination of precision and accuracy of the method, a sample of known concentration of azadirachtin (0.0016%) was analysed using slopes of the calibration curves (both by area and height ratio). The precision (relative standard deviation = 100 × standard deviation/mean, $n = 3$) was ± 0.95% and ± 1.8% considering height and area ratios, respectively. Accuracy (measured concentration × 100/actual concentration) was 100.1% when the height ratio was considered and 98.9% when the area ratio was used. Thus, use of the height ratio gives more accurate results than the area ratio for lower concentrations of azadirachtin.

3.3. Stability of azadirachtin

Azadirachtin is known to be susceptible to light and variation in pH. It is also said to be unstable in solution. Knowledge of the stability of azadirachtin would help in developing methods for its extraction, separation and formulations for use as a pesticide as well as for developing reliable analytical protocols. The stability of azadirachtin was therefore examined in six common organic solvents.

Azadirachtin (2.5 mg) was dissolved in different solvents, i.e. chloroform, acetone, ethyl acetate, acetonitrile, methanol and ethanol (10 ml each; azadirachtin concentration, 0.025% w/v). A 1-ml volume of each of these solutions was mixed with 1 ml of anisole solution (200 μg/ml) and the detector response ratio of azadirachtin to anisole at zero time was determined. The azadirachtin concentration in the different solvents was determined in a similar manner after 7 days at room temperature and indicated that azadirachtin was reasonably stable in all solvents tested (95–99%). The azadirachtin concentration after 8 h of reflux in the same solvents indicated that azadirachtin was less stable to higher temperatures (90–95%). For sample preparation purposes (see below), the stability of the azadirachtin to anisole ratio was also determined before and after sonication for 30 min in methanol (100%; $n = 5$).

3.4. Sample preparation and recovery

As noted earlier, the types of samples in which azadirachtin may need to be determined is quite varied. They may be solids (example; neem

seeds) or solutions with a variety of diluents, requiring different methods of sample preparation. For liquid samples Hull et al. [7] have suggested dilution with 90% aqueous methanol followed by solid-phase extraction using appropriate reversed-phase columns to remove the lipids prior to injection onto the HPLC columns.

For solid samples, rapid extraction by sonication in methanol was found to be efficient. The suitability of using anisole as internal standard has been demonstrated by recovery studies. Five samples of de-oiled neem seed powder, spiked with a known amount of azadirachtin (1 mg/5 g), were subjected to the sample preparation protocol and analysis as given above. Azadirachtin was determined using the calibration curves. The recovery (mean ± S.D.) was 99.5 ± 4% considering height ratios. It may be mentioned that since the neem seed powder used in the experiment was de-oiled by extraction with chloroform, it contained much less of azadirachtin (5 mg% compared to 0.25% w/w in the original seeds) and other limonoids than those reflected in Fig. 2.

Acknowledgements

We acknowledge the financial support from International Development Research Centre, Canada. We also thank Dr. P.R. Krishnaswamy, Scientific Director V.M.S.R.F., and Prof. P.V. Subba Rao, Indian Institute of Science, Bangalore, for encouragement and support.

References

[1] T.R. Govindachari, G. Sandhya and S.P.G. Raj, J. Nat Prod., 55 (1992) 596.
[2] J.H. Butterworth and E.D. Morgan, Chem. Comm., (1968) 23.
[3] J.D. Warthen, Jr., *Azadirachta indica*: a source of insect feeding inhibitors and growth regulators, USDA, Sci. and Educ. Adm. Agric. Reviews and Manuals, Northeastern Ser. 4, Beltsville, MD, 1979, p. 21.
[4] A.J. Mordue and A. Blackwell, J. Insect Physiol., 39 (1993) 903.
[5] J.D. Warthen, Jr., J.B. Stokes, M. Jacobson and M.F. Kozempel, J. Liq. Chromatoqr., 7 (1984) 591.
[6] K.M.S. Sundaram and J. Curry, J. Liq. Chromatogr., 16 (1993) 3275.
[7] C.J. Hull, Jr., B.S. Switzer and W.R. Dutton, J. Chromatogr., 633 (1993) 300.
[8] D.R. Schroeder and K. Nakanishi, J. Nat. Prod., 50 (1987) 241.

ELSEVIER

Journal of Chromatography A, 705 (1995) 380–384

JOURNAL OF CHROMATOGRAPHY A

Short communication

High-performance liquid chromatographic separation and determination of small amounts of process impurities of ciprofloxacin in bulk drugs and formulations☆

Sajid Husain*, Sara Khalid, V. Nagaraju, R. Nageswara Rao

Analytical Chemistry Division, Indian Institute of Chemical Technology, Hyderabad 500 007, India

First received 2 January 1995; revised manuscript received 24 February 1995; accepted 27 February 1995

Abstract

A simple and rapid high-performance liquid chromatographic method was developed for the separation and determination of small amounts of process impurities such as chlorofluoroaniline, dichlorofluoroacetophenone, cyclopropylacrylate and quinolinic acid in ciprofloxacin. The separation was achieved on a reversed-phase C_{18} column using water–methanol–acetic acid (84:15.9:0.1, v/v/v) as eluent. The method was used not only for quality assurance but also for process development of ciprofloxacin. The mean recovery of ciprofloxacin from authentic samples was $99.57 \pm 1.95\%$ and the limit of detection was $5 \cdot 10^{-9}$ g.

1. Introduction

Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)quinolone-3-carboxylic acid] is a broad-spectrum antibiotic used in the treatment of urinary and respiratory tract infections and also gastrointestinal and sexually transmitted diseases [1,2]. It has been used effectively against Gram-positive and Gram-negative bacteria that are resistent to penicillins, cephalosporins, aminoglycosides, β-lactums and tetracyclines. It is produced synthetically in large amounts by cycloarylation of dichlorofluoroacetophenone followed by condensation with piperazine [3,4]. In this process, intermediates, viz., cyclopropyl acrylate and quinolinic acid, are formed at different stages of the reactions and are ultimately converted into ciprofloxacin. It is likely that the unreacted intermediates may be present in small amounts as impurities in ciprofloxacin and reduce its quality. Therefore, the separation and determination of ciprofloxacin and its process components is of great importance not only for quality assurance but also for process development.

Several high-performance liquid chromatographic (HPLC) methods using fluoroscence or ultraviolet detection have been reported [5–7], but these are mainly applicable to the determination of ciprofloxacin and its metabolites in biological fluids. Microbiological assays have been used extensively but suffer from the interference from active metabolites that are generally present in sample matrices [8]. Flow-injection spectrophotometry and differential-pulse polarography have been tried but are not reproducible [9,10]. A thorough literature search

* Corresponding author.

☆ IICT Communication No. 3459.

0021-9673/95/$09.50
SSDI 0021-9673(95)00278-2

revealed that no single analytical method for the separation and determination of potential process impurities in ciprofloxacin has been reported. In this paper, we describe a simple and rapid HPLC method for the separation and determination of small amounts of the principal process components of ciprofloxacin using a reversed-phase C_{18} column and methanol–water–acetic acid (84:15.9:0.1, v/v/v) as the eluent at ambient temperature.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical-reagent grade unless stated otherwise. Glass distilled water, HPLC-grade methanol (Spectrochem, Bombay, India) and acetic acid (Qualigens, Bombay, India) were used. Samples of ciprofloxacin and its intermediates were prepared in our laboratory.

2.2. Apparatus

A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) with a 20-μl loop injector having a six-way high-pressure valve was used. A Shimadzu SPD 6AV variable-wavelength UV–Vis spectrophotometric detector was connected after the column. A reversed-phase C_{18} (Flexit, Pune, India) column (300 mm × 3.5 mm I.D.; particle size 10 μm) was used for separation. The chromatographic and integrated data were recorded with a Chromatopac C-R3A processing system.

2.3. Chromatographic conditions

The mobile phase was methanol–water–acetic acid (84:15.9:0.1, v/v/v). Samples were dissolved in the mobile phase. The analysis was carried out under isocratic conditions at a flow-rate of 1 ml/min and a chart speed of 5 mm/min at room temperature (27°C). Chromatograms were recorded at 254 nm using a UV detector.

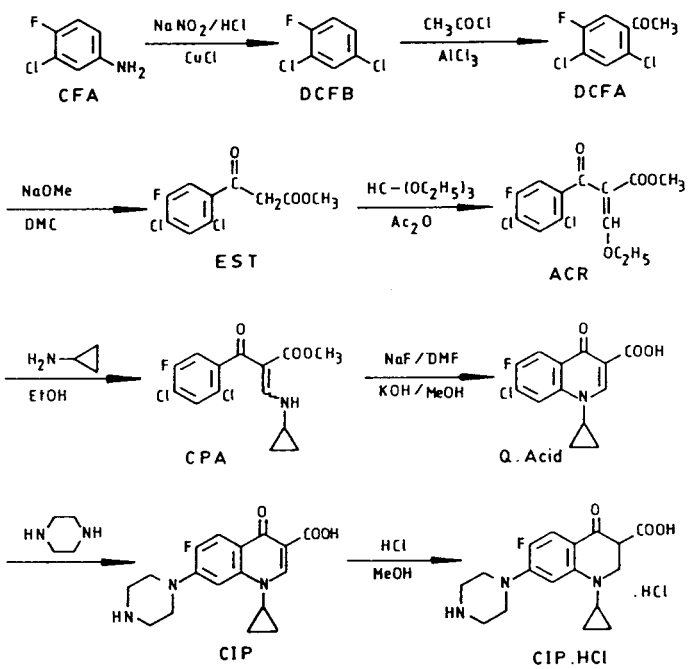


Fig. 1. Reactions involved in the preparation of ciprofloxacin hydrochloride.

2.4. Analytical procedure

Samples (10 mg) were dissolved in the mobile phase (100 ml) and a 20-μl volume of each sample was injected and chromatographed under the above conditions. Synthetic mixtures, bulk drugs and formulations were analysed under identical conditions. The amounts of impurities were calculated from their respective peak areas.

3. Results and discussion

Fig. 1 shows the molecular structures of potential impurities of ciprofloxacin (CIP) produced industrially. The impurities and CIP were subjected to separation by HPLC (Fig. 2). The peaks were identified by injecting the individual compounds. It can be seen that CIP is well separated from the reactants and intermediates. Aqueous acetic acid (0.1%) was found to improve the separation significantly. Earlier attempts using different columns, i.e. μBondapak C_{18} and Bondclone C_{18}, did not yield the desired separation.

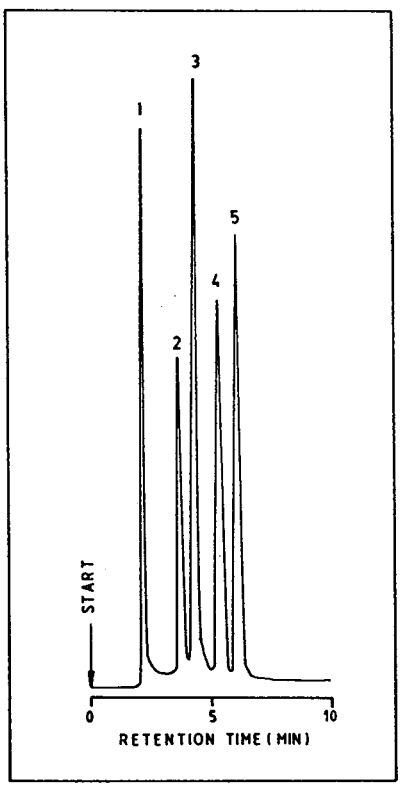


Fig. 2. Typical chromatogram of a mixture containing (1) CIP (21 μg), (2) DCFA (18 μg), (3) Q-acid (21 μg), (4) CPA (20 μg) and (5) CFA (16 μg).

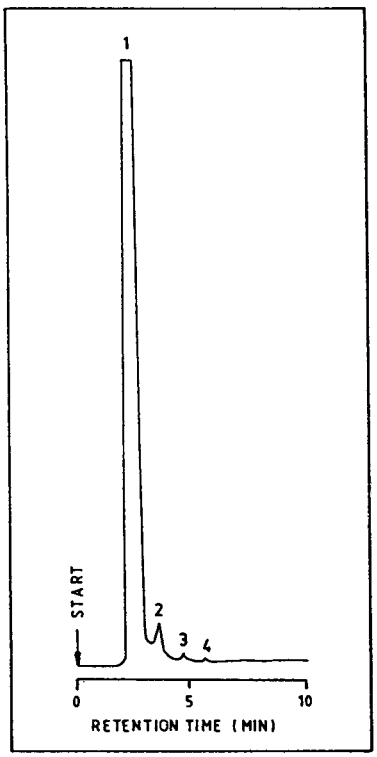


Fig. 3. Chromatogram of a commercial formulation of ciprofloxacin. For identification of peaks, see Fig. 2.

The retention times (t_R) and wavelengths of maximum absorption (λ_{max}) were determined and are given in Table 1. It can be seen that the early elution of CIP ($k' \approx 0.2$) indicates the possibility of overlap of some of the impurities of the process with the CIP band. Therefore, the retention times of all the process impurities were measured systematically and it was confirmed that they do not overlap with the band of CIP. However, the impurity ACR could not be chromatographed as it is unstable and not isolated from the process. The solubility of another impurity, EST, is low in the mobile phase and on

Table 1
Retention data for CIP and potential impurities

Compound	Abbreviation	t_R (min)	λ_{max} (nm)
Ciprofloxacin	CIP	2.25	278
Dichlorofluoro acetophenone	DCFA	3.83	242
Quinolinic acid	Q-acid	4.52	259
Cyclopropyl acrylate	CPA	5.60	308
Chlorofluoroaniline	CFA	6.37	235

examination it was found that at low concentrations it does not interfere with any of the other compounds during the analysis ($t_R = 7.58$ min). The raw material DCFB was also subjected to analysis by HPLC and it was found that it does not overlap with CIP, but interferes with one of the impurities of the process (t_R 3.90 min). DCFB is generally not present in the final product of CIP as it is highly volatile.

The UV detector was set at 254 nm and used for both detection and quantification. Good linearity was found between the mass and integral response for each compound under examination. At 0.001 AUFS the limit of detection for CIP was $5.0 \cdot 10^{-9}$ g with a signal-to-noise ratio of 4.0. The relative response factors for all the compounds are given in Table 2.

Standards containing known amounts of CIP, CPA, CFA, DCFA and Q-acid were prepared and analysed by HPLC. The accuracy of the method was checked by the standard addition technique. Subsequent additions of small amounts were accurately reflected in their peak areas. The measured amounts agreed well (within 1.95%) with the actual values. The results are given in Table 3.

Table 2
Response data

Compound	Concentration range (μg/ml)	Relative response factor	R.S.D. (%)[a]
CIP	950–990	2.33	1.45
DCFA	1–10	1.00	1.97
Q-acid	5–15	7.83	1.08
CPA	3–15	2.34	1.63
CFA	1–10	2.93	1.57

[a] $n = 3$.

Table 3
Analytical data for standard mixtures

Compound	Taken (%)	Found (%)[a]	Error (%)
CIP	95.96	96.09	0.14
DCFA	0.82	0.79	3.65
Q-acid	1.27	1.23	3.14
CPA	1.02	0.99	2.94
CFA	0.93	0.90	3.22

[a] Average of three determinations.

The quality of CIP in several batches of samples obtained commercially was thoroughly checked. The concentrations of various impurities were determined by HPLC and the purity of CIP was calculated. The results are given in Table 4. From these results, it is clear that the method is precise and accurate for the separation and determination of small amounts of process impurities that are present in CIP. The method is suitable not only for process development but also for quality assurance of CIP and related products.

References

[1] K. Grohe, Chem. Br., 28 (1992) 34.
[2] H.C. Neu, A. Percival and H. Lode, Am. J. Med., 87 (1989) 1.
[3] G. Lukacs and M. Ohno (Editors), Recent Progress in the Chemical Synthesis of Antibiotics, Springer, Berlin, 1990, p. 256.

Table 4
Determination of process impurities in a typical sample of ciprofloxacin

Compound	Concentration (%)	S.D. (%)[a]
DCFA	0.41	1.75
Q-acid	0.26	1.89
CPA	0.03	2.30
CFA	0.02	2.78

[a] $n = 3$.

[4] L.M. Isidro, P.C. Alberto and D.C. Antonio, Span. Pat., ES 2 009 072 (1989); Chem. Abstr., 114 (1991) 164281r.
[5] G.J. Krol, A.J. Noe and D. Deermann, J. Liq. Chromatogr., 8 (1986) 340.
[6] F. Vallee, M.L. Bel and M.G. Bergeron, Ther. Drug. Monit., 8 (1986) 340.
[7] W.M. Awni, J. Clarkson and D.R.P. Guay, J. Chromatogr., 5 (1986) 414.
[8] K. Borner, H. Lode and G. Hoffken, Eur. J. Clin. Microbiol., 5 (1986) 476.
[9] S.M. Sultan and F.O. Suliman, Analyst, 117 (1992) 1523.
[10] P.O. Dea, A.C. Garcia, A.J.M. Ordiers and P.T. Blanco, Electroanalysis, 2 (1990) 637.

ELSEVIER

Journal of Chromatography A, 705 (1995) 385–389

JOURNAL OF CHROMATOGRAPHY A

Short communication

High-performance liquid chromatographic determination of ionic compounds in cosmetic emulsions: application to magnesium ascorbyl phosphate

A. Semenzato*, R. Austria, C. Dall'Aglio, A. Bettero

Centro di Cosmetologia Chimica dell'Università di Padova, Dipartimento di Scienze Farmaceutiche, Via Marzolo 5, 35131 Padova, Italy

First received 18 October 1994; revised manuscript received 31 January 1995; accepted 31 January 1995

Abstract

A rapid, reliable method based on reversed-phase high performance liquid chromatography for the qualitative and quantitative determination of magnesium ascorbyl phosphate (VC-PMG) in both standard solutions and cosmetic products is reported. The procedure, based on an aminic stationary phase and acetonitrile–0.3 *M* phosphate buffer (pH 4) (40:60) as the mobile phase, allows the determination of VC-PMG in cosmetic samples after dilution with a tetrahydrofuran–0.3 *M* phosphate buffer (pH 4) (3:7) solvent mixture.

1. Introduction

The analysis of untreated heterophase systems may easily be performed after dilution with aqueous–organic solvent mixtures [e.g., water–tetrahydrofuran (1:9)]. This dilution step avoids any pretreatment of the sample with some practical advantages, such as rapidity, flexibility and decrease of the analytical error [1]. This procedure, combined with RP-HPLC, allows the qualitative and quantitative determination of each ingredient, so that its stability in the finished product and compatibility with other formula components may be studied [2]. Although this method has been successfully applied to the determination of non-polar and polar molecules in multi-component systems [3,4], it cannot be considered as optimum for the determination of ionic compounds.

In this paper, we report a new approach to the direct determination of ionic molecules in cosmetic products; we investigate the chromatographic behaviour of the magnesium salt of ascorbyl phosphoric acid (VC-PMG), a new derivative recently introduced commercially and used in cosmetic emulsions as a skin whitener and radical scavenger [5–7].

It is well known that ascorbic acid has important physiological effects on the skin, including the inhibition of melanogenesis, promotion of collagen formation and prevention of free radical formation [8–10]; these effects are closely related to the well known antioxidant properties of this molecule [11–13].

The use of vitamin C in finished products is limited by its chemical properties: this molecule, readily soluble in water, is extremely unstable in

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00149-2

aqueous solution [14,15] and is poorly absorbed through the skin [14,15]. The reactivity of ascorbic acid and its solubility properties may be modified by esterification with organic or inorganic acids [14,15]. Of the various derivatives available on the market, magnesium ascorbyl phosphate (VC-PMG) (Fig. 1) seems to be the most stable molecule in aqueous solution [8,14].

Previous publications have only described spectroscopic procedures for the determination of magnesium ascorbyl phosphate [8,14], and no chromatographic study for a reproducible, objective evaluation of its behaviour in finished products has yet been performed. The aim of this study was to develop a rapid, reliable method based on RP-HPLC for the qualitative and quantitative determination of magnesium ascorbyl phosphate in cosmetic products [16,17]. As VC-PMG is a polar molecule, its chromatographic separation may be carried out using an anion-exchange column. However, direct analysis of heterophase systems on such a column is not easy to perform, because they need a high percentage of organic modifiers, often incompatible with high-molarity buffer solutions. For these reasons, we investigated the chromatographic behaviour of VC-PMG on reversed and bonded phases with increasing polarity.

The determination of VC-PMG in untreated cosmetic samples may be performed using an aminic stationary phase and acetonitrile–phosphate buffer as the mobile phase after sample dilution with a tetrahydrofuran–0.3 *M* phosphate buffer (pH 4) (3:7) solvent mixture.

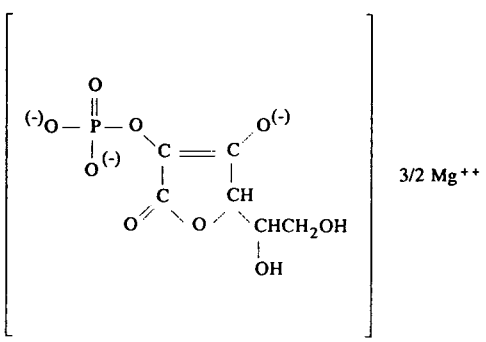


Fig. 1. Structure of magnesium ascorbyl phosphate (VC-PMG).

2. Experimental

2.1. Materials and reagents

Magnesium ascorbyl phosphate (VC-PMG) was obtained from Nikkol (Tokyo, Japan). LiChrosorb RP-18 (Li-RP18), LiChrosorb CN (Li-CN) and LiChrosorb NH_2 (Li-NH_2) columns (7 μm, 250 mm × 4 mm I.D.) and analytical-reagent grade reagents and solvents were obtained from Merck (Darmstadt, Germany).

2.2. Apparatus

A Gilson liquid chromatograph (Biolabo Instruments, Milan, Italy) equipped with two pumps (Models 305 and 306), a Gilson Model 805 manometric module, a Gilson Model 811B dynamic mixer, a Rheodyne Model 9010 valve, a Perkin-Elmer LC 95 UV–Vis detector and a Shimadzu C-R5A data station was used.

2.3. Chromatographic conditions

The stationary phase was Li-NH_2 (7 μm), the eluent was acetonitrile–0.3 *M* phosphate buffer (pH 4) (40:60) at a flow-rate of 1 ml/min, UV detection at 255 nm was applied and the injection volume was 20 μl.

2.4. Standard solutions

VC-PMG standard solution (1%, w/v) was prepared in 0.3 *M* phosphate buffer (KP) (pH 4) and stored in the dark at 4°C. Further dilutions were performed, using the same solvent, in the range 5–30 μg/ml for obtaining calibration graphs.

2.5. Samples

About 1 g of each cosmetic sample, accurately weighted, was diluted 1:20 with tetrahydrofuran–0.3 *M* phosphate buffer (pH 4) (3:7) in a screw-

capped tube and stirred in a vortex mixer until completely homogeneous. Further dilutions were performed only with 0.3 *M* phosphate buffer (pH 4) until a final dilution of 1:200–4000, depending on the VC-PMG concentration in the cosmetic sample considered. The solutions obtained were injected directly into the chromatographic system.

3. Results and discussion

3.1. VC-PMG analysis

The chromatographic behaviour of magnesium ascorbyl phosphate in aqueous solution was investigated using three stationary phases of increasing polarity and acetonitrile to acid phosphate buffer mobile phase ratios.. As expected, no interaction between VC-PMG and cyanopropylic or octadecyclic column was detectable, as the molecule eluted with the solvent.

In contrast, a chromatographic separation occurred on the aminopropylic column (Fig. 2), which can be considered a weak anionic exchanger under the conditions employed. Moreover, when the amount of organic modifier in the mobile phase was increased, no significant change in the retention process could be noted, confirming the ionic nature of the interaction.

The capacity factor (k') of magnesium ascorbyl phosphate on the aminic column closely depended on the ionic strength of the buffer. As reported in Fig. 2, it gradually decreased with increasing molarity, and was not affected by acetonitrile at concentrations up to 40%.

The chromatographic process also was significantly influenced by the pH of the mobile phase: acidity of the eluent is essential for the anion-exchange mechanism, as it provides salt dissociation and protonation of aminic groups. This chromatographic process did not occur if a neutral mobile phase was used (Fig. 3).

In conclusion, the chromatographic elution of VC-PMG on an aminic stationary phase closely depends on the molarity and acidity of the buffer, although in order to determine this molecule directly in a cosmetic sample, avoiding pretreatment steps (i.e., extraction of the oily phase), a significant percentage of organic modifier had to be used.

Considering the time of analysis, peak res-

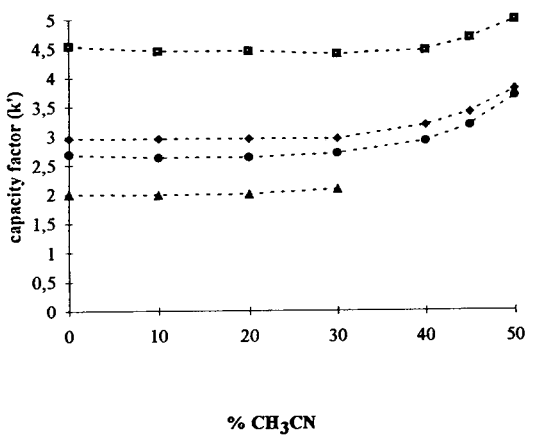


Fig. 2. Influence of ionic strength on capacity factor (k') of VC-PMG. Column, Li-NH_2; eluent, acetonitrile–phosphate buffer (pH 4); flow-rate, 1 ml/min; detection at 255 nm. ■ = 0.15; ◆ = 0.25; ○ = 0.3; ▲ = 0.5 *M*. An acetonitrile concentration over 30% is not compatible with 0.5 *M* phosphate buffer.

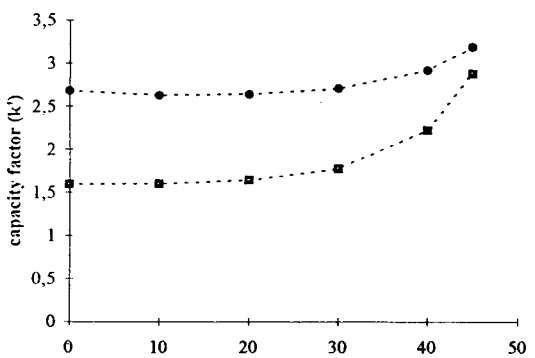


Fig. 3. Influence of pH on capacity factor (k') of VC-PMG. Column, Li-NH_2; eluent, acetonitrile–0.3 *M* phosphate buffer; flow-rate, 1 ml/min; detection at 255 nm. □ = pH 6.5; ○ = pH 4.

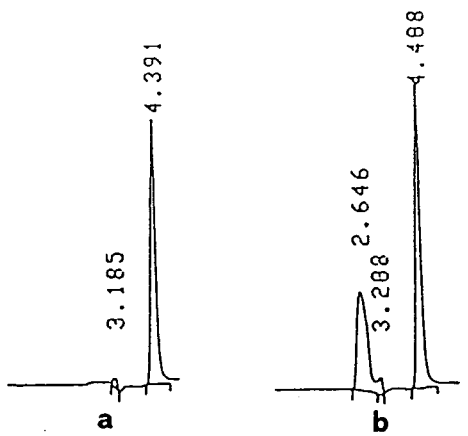


Fig. 4. Chromatographic patterns of VC-PMG (30 μg/ml) in (a) standard solution and (b) a cosmetic emulsion.

olution and compatibility between acetonitrile and phosphate buffer, the optimum chromatographic parameters were as given in Section 2.3.

Fig. 4a shows the chromatographic pattern of a standard solution of VC-PMG in 0.3 *M* phosphate buffer (pH 4) (30 μg/ml); linear calibration graphs [y = peak area; x = concentration (μg/ml)] were obtained in the range 5–30 μg/ml with good regression coefficients ($y = 2.15x - 0.05$; $r = 1.00$). Repeatability of analysis was verified at various concentrations, and revealed good precision and accuracy (recovery 99.10–101.00%; R.S.D. 0.17–0.48%).

The stability of VC-PMG standard solutions in 0.3 *M* phosphate buffer (pH 4) was studied in order to verify its compatibility with the mobile phase employed; 1% standard solutions, stored at room temperature in the dark and periodically analysed for 5 months, did not show any significant concentration loss (Table 1).

Table 1
Recovery of VC-PMG standard solutions after storage for 5 months at room temperature

Standard solution (1%)	Recovery (%) ($n = 5$)
H_2O	91.27 ± 0.22
KP (pH 4)	99.12 ± 0.28
Dil. HCl (pH 4)	73.58 ± 0.24

3.2. Application to cosmetic samples

Analysis of cosmetic formulations can easily be performed, avoiding any extraction step, by dilution of samples with suitable aqueous–organic mixtures [12,13]. The procedure normally employed for heterophase systems is based on sample dilution with a tetrahydrofuran–water (9:1) solvent mixture, followed by direct RP-HPLC. This mixture is not suitable for the determination of magnesium ascorbyl phosphate, as the complete solubilization of this molecule can be achieved only using a highly polar medium. For this reason, we investigated the solubilization power of various mixtures prepared with tetrahydrofuran and phosphate buffer; good results were obtained using tetrahydrofuran–0.3 *M* phosphate buffer (pH 4) (3:7).

The determination of VC-PMG in cosmetic samples can be performed by first applying a dilution step (1:20) of about 1 g of the sample, accurately weighed, with tetrahydrofuran–0.3 *M* phosphate buffer (pH 4) (3:7) solvent mixture. Under these conditions, we obtained a homogeneous suspension of the ingredients, which could be directly analysed in the chromatographic system. Depending on the VC-PMG concentration in the sample, further dilutions can be performed using only phosphate buffer.

Fig. 4 compares the chromatographic patterns of VC-PMG (a) in standard solution and (b) in a cosmetic emulsion, and shows a good peak resolution. The matrix effect was evaluated using the standard addition method (standard solution, $y = 2.15x - 0.05$ ($r = 1.00$); standard solution + sample, $y = 2.20x + 13.90$ ($r = 1.00$)).

In order to evaluate the applicability of this method to cosmetic emulsions, several oil–water systems containing known concentrations of VC-PMG were prepared. Recovery trials and relative standard deviations for each sample confirmed the reproducibility and flexibility of the method, which appears to be suitable for the rapid, sensitive determination of VC-PMG (Table 2).

VC-PMG was then determined in six "skin whitener" emulsions marketed in Italy; no inter-

Table 2
Assay results for VC-PMG in various oil–water emulsions

Concentration found (%, w/w) ($n = 5$)	Recovery (%)	R.S.D. (%)
1.04 ± 0.01	104.00	0.96
1.07 ± 0.01	97.27	0.93
1.07 ± 0.03	97.27	2.80
1.06 ± 0.02	96.36	1.89
1.53 ± 0.01	102.00	0.65

ferences with other formula components were detected, confirming the applicability of the methodology.

In conclusion, chromatographic determination of this highly polar molecule can easily be directly performed in cosmetic emulsions using a tetrahydrofuran–buffer solvent mixture and a weak anion exchanger as the stationary phase, and can be used to perform stability studies of this molecule in cosmetic products.

In order to overcome the problems due to the short lifetime of the silica aminic column (hydrolysis of aminic groups), the use of a polymeric aminic stationary phase can be considered. This kind of column, more expensive than the former, provides the same chromatographic results with stable performance.

Acknowledgements

Financial support from MURST (40%), CNR Special Project on Fine Chemicals, and L. Crosetto is gratefully acknowledged.

References

[1] A. Bettero, B. Casetta, F. Galiano, E. Ragazzi and C.A. Benassi, *Fresenius' Z. Anal. Chem.*, 318 (1984) 525.
[2] A. Bettero, A. Semenzato and C.A. Benassi, *J. Chromatogr.*, 507 (1990) 403.
[3] C.A. Benassi, A. Semenzato and A. Bettero, *J. Chromatogr.*, 464 (1989) 387.
[4] A. Semenzato, M. Secchieri, A. Baù, A. Nicolato and A. Bettero, *Farmaco*, 47 (1992) 1407.
[5] M. Tagawa, T. Murata and T. Onuma, *Preprints of the 17th IFSCC Yokohama*, 2 (1992) 896.
[6] R. Crippa, V. Horak, G. Prota, P. Svoronos and L. Wolfram, in A. Brossi (Editor), *The Alkaloids. Chemistry and Pharmacology*, Academic Press, New York, 1989, Ch. 36, p. 253.
[7] J.M. Wilmott, M.C. Duggan and A.P. Znaiden, in N.J. Lowe and N.A. Shaath (Editors), *Sunscreens. Developments, Evaluation and Regulatory Aspects*, Marcel Dekker, New York, 1990, Ch. 10, p. 279.
[8] *NIKKOL VC-PMG: Stable Vitamin C Derivative*, Nikko Chemicals, 1989, Tokyo.
[9] G. Karg, J. Wilmott and A. Znaiden, *Cosmet. Toilet.*, 102 (1987) 37.
[10] S. Murad, D. Grove, K. Lindberg, G. Reynolds, A. Siverajah and S.R. Pinnell, *Proc. Natl. Acad. Sci. USA*, 78 (1981) 2879.
[11] K. Furuse, *Cosmet. Toilet.*, 102 (1987) 99.
[12] P.T. Pugliese and C.B. Lampley, *J. Appl. Cosmetol.*, 3 (1985) 129.
[13] A. Sparavigna, G. Viscardi, G. Galbiati and U. Citernesi, *Cosmet. Toilet. Ital. Ed.*, 5 (1993) 29.
[14] M. Galesso, M. Gatta and F. Galiano, *Cosmet. Toilet. Ital. Ed.*, 2 (1993) 58.
[15] H. Takashima, H. Nomura, Y. Imai and H. Mima, *Am. Perfum. Cosmet.*, 86 (1971) 29.

ELSEVIER

Journal of Chromatography A, 705 (1995) 390–395

JOURNAL OF CHROMATOGRAPHY A

Short communication

Phosphorus speciation in nickel plating baths by ion chromatography

Magdalena Biesaga, Marek Trojanowicz*

Department of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland

First received 1 December 1994; revised manuscript received 28 February 1995; accepted 3 March 1995

Abstract

A non-supressed ion chromatographic method with conductivity detection for the simultaneous determination of hypophosphite, phosphite and orthophosphate was developed. Separation of phosphorus oxyanions from lactate present in the plating bath samples was achieved by modification of the succinate eluent with acetonitrile.

1. Introduction

Despite significant progress in recent years in the instrumentation and applications of high-performance ion chromatography, especially in environmental analysis, relatively few applications have been reported to the speciation of phosphorus. Because of the wide use of simple and condensed oxyanions of phosphorus in various industrial process, in various detergent preparations and fertilizers, there is a demand for this determination and high-performance liquid chromatography seems to be especially suitable for this purpose.

Several examples of such an application in single- and dual-column ion chromatographic systems have been reported. In a single-column system a mixture of monovalent oxyanions (hypophosphite, phosphite and orthophosphate) was analysed with succinic acid as eluent and conductivity detection [1] and with 4-amino-2-hydroxybenzoic acid as eluent and indirect UV absorptive detection [2]. These studies were carried out only with standard mixtures of anions. In the determination of pyrophosphate and tripolyphosphate, also in a single-column system, a better sensitivity with indirect UV than conductivity detection was found [3]. Series of carboxylic acids of various structures were recently tested as eluents in the single-column ion chromatography of phosphorus oxyanions with refractive index detection [4]. The best eluents for the determination of non-condensed anions were *p*-hydroxybenzoic acid and *p*-aminobenzoic acid, although complete baseline resolution of oxyanions was not achieved. The determination of the same anions was carried out also in a more complex system, where single-column chromatography was combined with a flow-injection postcolumn reaction detection system [5]. The separated hypophosphite and phosphite anions were oxidized to orthophosphate and then reacted with a chromogenic molybdenum reagent. Postcolumn reaction with Fe(III) with UV

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00303-7

absorption detection was also developed for the determination of phosphorus anions in a dual-column system with a sodium carbonate–sodium hydroxide eluent [6].

A technological process in which control of the speciation of phosphorus anions is needed for optimization of the process run is electroless nickel plating from hypophosphite baths. This process is based on the reaction [7]

$$Ni^{2+} + H_2PO_2^- + H_2O \rightarrow Ni + 2H^+ + H_2PO_3^-$$

However, the details of how this reaction occurs have not been finalised [8]. An important feature of this reaction is that nickel provides the necessary catalytic effect for this electroless process. The nickel plating process is improved by additions of small amounts of organic compounds, which results in increased stability of the bath, speed of deposition and brightness of the deposit.

Nickel coatings formed in the electroless process have high hardness and good mechanical resistivity. They are primarily employed as a corrosion-protective coatings on functional articles of complex shape. They are formed at 85–95°C in acidic solutions of pH 3.9 (fresh bath) containing 30 g/l nickel sulphate or chloride, lactic acid, 20 g/l sodium hypophosphite and some brightening components. Nickel salt is added to the bath during its use as long as the phosphite ($H_2PO_3^-$) concentration is lower than 1 *M*.

The aim of this study was to develop a method for the determination of hypophosphite, phosphite and orthophosphate in such a bath using a single-column ion chromatographic system with conductivity detection. The application of this technique for this purpose has not previously been reported.

2. Experimental

2.1. Apparatus

Ion chromatographic measurements were carried out using a Model 6200 ion chromatograph with a conductivity detector from Tecator (Hoganas, Sweden) and an HPLC system from Perkin-Elmer consisting of a Series 100 pump and a Bacharach Type 3D conductivity detector. As analytical columns, a 302 IC column from Vydac (Hesperia, CA, USA) and an OmniPac PAX 500 column from Dionex (Sunnyvale, CA, USA) were used.

2.2. Reagents

Sodium hypophosphite was purchased from Aldrich, sodium phosphite from BDH and succinic acid from Merck. Acetonitrile of HPLC grade was obtained from Baker. All other reagents were of analytical-reagent grade from POCh (Gliwice, Poland).

Stock standard solutions (1000 mg/l) of sodium hypophosphite, sodium phosphite and sodium orthophosphate were prepared once a week by dissolving the appropriate amount of solid reagent in deionized water and kept in a refrigerator. Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions with deionized water.

A 0.1 *M* eluent solution was prepared by dissolving succinic acid in deionized water and was stored in refrigerator. More dilute solutions for measurements were prepared daily by appropriate dilution with water and pH adjustment prior to the addition of organic modifier. Then, after filtration with a 0.45-μm nylon 66 membrane filter (Supelco), it was ultrasonicated for 15 min. All solutions were prepared using deionized water obtained from a Waters Milli-Q water-purification system.

Samples of nickel plating baths were kindly provided by Ms. Danuta Przybylska of the Institute of Precision Mechanics, Warsaw, Poland.

2.3. Procedure

Natural bath samples were diluted 10^4-fold with deionized water prior to injection. Injected samples and standard solutions were filtered through 0.22-μm nylon single-use syringe filters of 25 mm diameter (Supelco). Chromatography was performed using a 100-μl injection volume

and an eluent flow-rate of 1.0 ml/min. An optimized eluent solution of pH 3.6 contained 0.2 m*M* succinic acid and 5% acetonitrile. Measurements were carried out in room temperature without thermostating. An overnight constant flow of 0.3 ml/min was maintained.

3. Results and discussion

3.1. Optimization of separation of hypophosphite, phosphite and orthophosphate

Owing to the better detectability obtained for simple phosphorus oxyanions with conductivity detection in comparison with indirect UV measurements, the former was employed. The optimization of the ion chromatographic separation of hypophosphite, phosphite and orthophosphate with a Vydac 302 column and succinic acid solution as eluent confirmed the results obtained earlier by Ryder [1]. The best results were obtained with 20 m*M* succinic acid of pH 3.0, but a serious drawback to separation under such conditions was partial overlapping of the hypophosphite peak with the broad peak of chloride (Fig. 1); the resolution of the peaks for chloride ($t_R = 12.3$ min) and hypophosphite ($t_R = 13.6$ min) was only 0.7. Hence, in spite of the satisfactory separation of oxyanions of phosphorus, this method cannot be applied to real samples containing an excess of chloride as matrix component.

The application of a Dionex OmniPac PAX 500 anion column in the chromatography of oxyanions of phosphorus has not been reported previously. For this column succinic acid was also examined as the eluent in the concentration range 0.2– to 20 m*M* (Fig. 2). In this case the separation of simple phosphorus oxyanions together with lactate was carried out, as lactic acid is usually present in nickel plating baths. At a very low concentration of the eluent, when satisfactory separation for all the oxyanions considered was obtained, lactate exhibits the same retention time as hypophosphite. An increase in succinic acid concentration improves the separation of lactate and hypophosphite, but significantly deteriorates the separation of phosphite and orthophosphate.

An increase in pH above 4.0 for 0.2 m*M* succinic acid solution also results in the simultaneous elution of phosphite and orthophosphate and a worse separation of hypophosphite from the solvent peak than at pH 3.6.

For a given column and a particular eluting anion, a certain differentiation of the retention of separated anions can be expected from the change in dielectric properties of eluent. In high-performance ion chromatography, the addition of a non-aqueous solvent to the eluent has been employed, for instance, to change the extent of the hydrophobic adsorption of the eluent on the

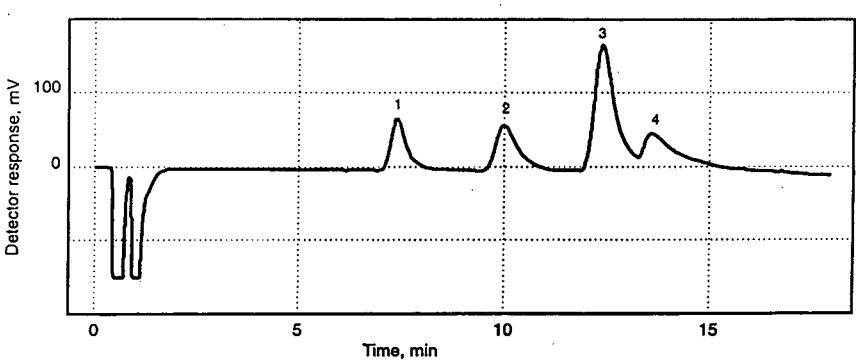


Fig. 1. Chromatogram obtained for a mixture of (1) 5 mg/l orthophosphate, (2) 5 mg/l phosphite and (4) 5 mg/l hypophosphite using 20 m*M* succinic acid (pH 3.0) as eluent with a Vydac 302 IC column. Sample volume, 500 μl; eluent flow-rate, 3.9 ml/min. Peak 3 corresponds to 5 mg/l chloride.

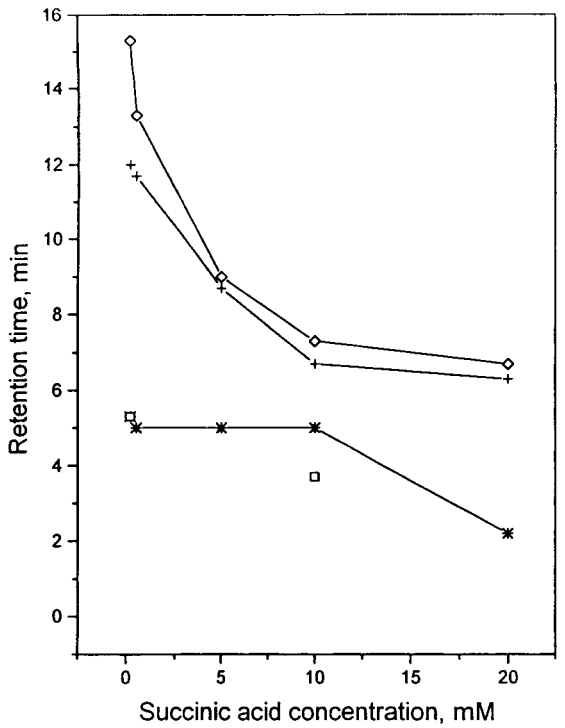


Fig. 2. Effect of concentration of succinic acid solution of pH 3.6 used as eluent on retention times of (*) hypophosphite, (◇) phosphite, (+) orthophosphate and (□) lactate at a flow-rate of 1 ml/min. Injection of 100 μl of solutions containing 0.1 m*M* lactate and 10 mg/l hypophosphite, orthophosphate and phosphite. Column, OmniPac PAX-500.

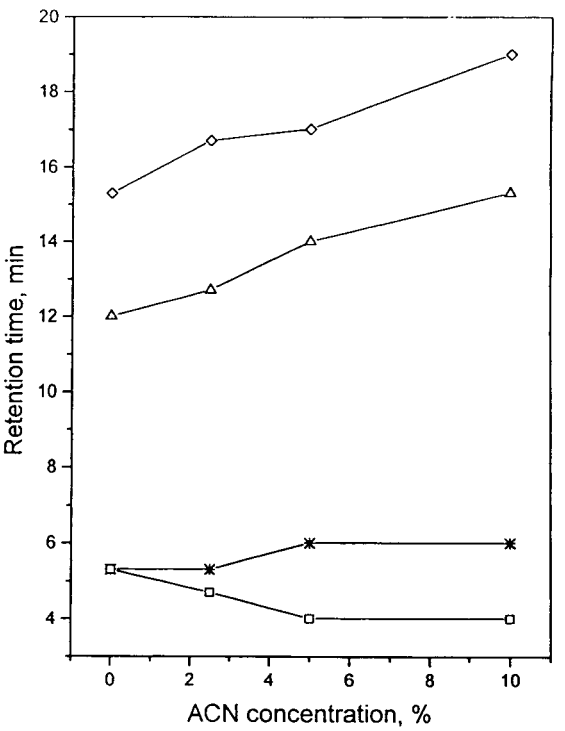


Fig. 3. Effect of addition of acetonitrile to 0.2 m*M* succinic acid (pH 3.6) used as eluent on retention times of (*) hypophosphite, (◇) phosphite, (△) orthophosphate and (□) lactate at a flow-rate of 1 ml/min. Injection of 100 μl of solutions containing 0.1 m*M* lactate and 10 mg/l hypophosphite, orthophosphate and phosphite. Column, OmniPac PAX-500.

stationary phase [9] and for selectivity mediation [10]. In this study it was found that an increase in the acetonitrile content in the succinic acid eluent up to 10% does not affect significantly the retention of phosphite and orthophosphate, but essentially improves separation of hypophosphite and lactate by lowering the retention time of lactate (Fig. 3). For a 0.2 m*M* succinate eluent of pH 3.6 with 5% acetonitrile added, the retention time for chloride was 20.6 min and no signal was observed for 20 mg/l nitrate and sulfate under such conditions. Under the optimized conditions it was also found that the presence of nickel(II) in the injected solutions at concentrations up to 2 mg/l, corresponding to the Ni content in fresh baths, does not influence of phosphorus species. The chromatogram of a synthetic mixture of phophorus oxyanions is shown in Fig. 4A and the calibration data are given in Table 1. The limit of detection under optimized conditions for a 100-μl sample volume was estimated to be 0.1 mg/l for hypophosphite and 0.5 mg/l for phosphite and orthophosphate.

An advantage of the use of an OmniPac PAX-500 column instead of a Vydac 302 IC is the use of a 100-fold less concentrated eluent at a four-fold lower flow-rate.

3.2. Determination of phosphorus oxyanions in plating baths

Owing to high concentration of species to be determined in samples of nickel plating baths, the analyte solutions were diluted 10^4-fold with

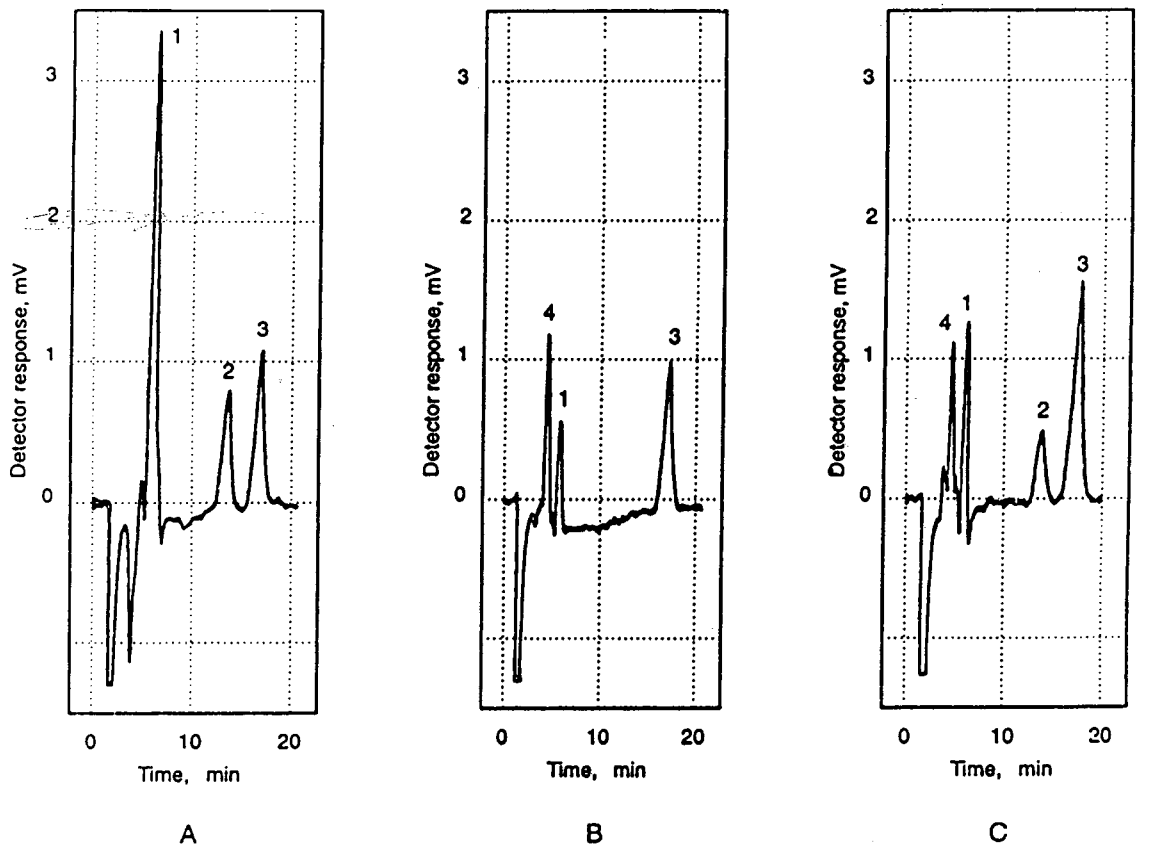


Fig. 4. Chromatograms obtained for (A) a standard mixture containing 5 mg/l each of (1) hypophosphite, (2) orthophosphate and (3) phosphite, (B) a 10^4-fold diluted plating bath sample and (C) a 10^4-fold diluted bath sample spiked with 2 mg/l of orthophosphate, phosphite and hypophosphite, using as eluent 0.2 m*M* succinic acid (pH 3.6) containing 5% acetonitrile with an OmniPac PAX-500 column. Flow-rate, 1 ml/min; sample injection volume, 100 μl. Peak 4 corresponds to lactate.

distilled water prior to chromatographic analysis. Examples of chromatograms obtained for fresh and spiked bath solutions are shown in Fig. 4B and C, respectively. The quantitative results are given in Table 2. The R.S.D. ($n = 9$) obtained for a 5 mg/l concentration of each analyte was

Table 1
Characteristics of calibration graphs (peak height vs. concentration) obtained for ion chromatography of phosphorus oxyanions with an OminPac PAX 500 column using as eluent 0.2 m*M* succinic acid (pH 3.6) with 5% acetonitrile at a flow-rate of 1 ml/min and an injection volume of 100 μl

Analyte	Retention time (min)	Parameters of calibration plot		
		Upper limit of linear response (m*M*)	Slope (mV/m*M*)	Intercept (mV)
Hypophosphite	5.3	0.08	43.5	0.13
Phosphite	12.0	0.13	13.3	0
Orthophosphate	15.3	0.10	13.2	0.02

Table 2
Determination of oxyanions of phosphorus in $1:10^4$ diluted solution of nickel plating bath samples under optimized conditions with an OmniPac PAX 500 column using as eluent 0.2 m*M* succinic acid (pH 3.6) with 5% acetonitrile at a flow-rate of 1 ml/min and an injection volume of 100 μl

Sample	Analyte	Concentration found (mg/l)	Recovery (%)	
			2 mg/l added	4 mg/l added
Fresh bath	Hypophosphite	1.0	110	94
	Phosphite	<0.5	104	101
	Orthophosphate	<0.5	104	99
Spent bath	Hypophosphite	1.3	91	84
	Phosphite	3.5	127	120
	Orthophosphate	0.5	104	99

3.4, 1.0 and 4.0% for hypophosphite, phosphite and orthophosphate, respectively.

Acknowledgement

This work was financed by the University of Warsaw, Grant No. BST-472/9/94.

References

[1] D.S. Ryder, J. Chromatogr., 354 (1986) 438.
[2] M.C. Mehra and C. Pelletier, Chromatographia, 30 (1990) 337.
[3] W.D. MacMillan, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 102.
[4] D. Hatton and W.F. Pickerin, Talanta, 3 (1993) 307.
[5] Y. Hirai, N. Yoza and S. Ohaski, J. Chromatogr., 206 (1981) 501.
[6] P. Hoffman, I. Schmidtke and I.H. Lieser, Fresenius Z. Anal. Chem., 335 (1989) 402.
[7] A. Brenner and G. Riddel, Proc. Am. Electroplaters Soc., 33 (1946) 23; 34 (1947) 156.
[8] J.K. Dennis and T.E. Such, Nickel and Chromium Plating, Newnes-Butterworths, London, 1972.
[9] Y.E. Pazukhina, O.A. Shpigun, I.N. Voloshchik and M.L. Litvina, Zh. Anal. Khim., 43 (1988) 117.
[10] S. Rabin and J. Stillian, J. Chromatogr. A, 671 (1994) 63.

ELSEVIER

Journal of Chromatography A, 705 (1995) 396–399

JOURNAL OF CHROMATOGRAPHY A

Short communication

Adsorption of oxysterols on different microtube materials during silanyzation prior to gas chromatographic determination

Francesc Guardiola, Rafael Codony*, Magda Rafecas, Josep Boatella

University of Barcelona, Nutrition and Food Science Unit, Faculty of Pharmacy, Av. Joan XXIII s/n, 08028-Barcelona, Spain

First received 29 April 1994; revised manuscript received 8 September 1994; accepted 19 October 1994

Abstract

The observation of increasing variability in the GC determination of oxysterols when the material of the silanization microtubes was changed led to a comparative study of three kinds of microtubes (Pyrex glass, polypropylene homopolymer and polypropylene copolymer). The characteristics of the adsorption of each material on different oxysterols and their influence on the reliability of the determination are reported. The variability of the adsorption in the different materials is high for 5α-cholestane, one of the most widely used internal standards in the GC determination of oxysterols. This will affect the precision and accuracy of the relative response factors. Significant differences were also found for cholesterol and the five oxysterols analysed, especially between the polypropylene copolymer and the other two materials.

1. Introduction

There have been few reports on the adsorption of the sterols and oxysterols (OS) on the surface of the materials used during the analytical procedures involved in their determination [1,2]. However, this is not a trivial phenomenon, as variable results are obtained if changes in the material used are introduced without strict control. This variability can lead to large errors in the determination of some OS, owing to their low concentration in foods.

The need for this study was suggested by the observation of an unexpected variability of results in the GC determination of OS [3] when the nature of the plastic silanization microtubes was changed. We designed a study with the aim of establishing the degree of adsorption on glass and two different plastic materials for cholesterol, the main OS, and 5α-cholestane, the internal standard (I.S.) used in the determination.

2. Experimental

2.1. Reagents and standards

Ethyl acetate (ACS grade) and dried pyridine (maximum 0.01% water, for analysis) were obtained from Merck (Darmstadt, Germany) and Sylon BTZ [N,O-bis(trimethylisilyl)acetamide–trimethylchlorosilane–N-trimethylsilylimidazole (3:2:3), for research], in 0.1-ml glass ampoules, from Supelco (Bellefonte, PA, USA). Standard cholesterol (>99%, by GC) was supplied by Merck and 5α-cholestane (99%, by GC) by Supelco. All other standards were obtained from

* Corresponding author.

0021-9673/95/$9.50
SSDI 0021-9673(94)01018-8

Sigma (St. Louis, MO, USA): cholesterol-5α,6α-epoxide (α-CE) (99%, by TLC), 7β-hydroxycholesterol (7β-HC) (99%, by TLC), cholestanetriol (CT) (>97%, by GC), 7-ketocholesterol (7-KC) (>99%, by HPLC) and 25-hydroxycholesterol (25-HC) (>98%, by TLC).

All these standards were weighed in a Sartorius 2004 MP microbalance with an accuracy of 0.01 mg. The standards were handled as ethyl acetate solutions (2.5 mg per 10 ml for internal standards and 2.0 mg per 10 ml for the others). When dilutions of these solutions were needed, they were prepared with ethyl acetate immediately prior to use.

2.2. Gas chromatographic conditions

GC was performed on a Perkin-Elmer Sigma 2000 chromatograph equipped with a flame ionization detector and fused-silica capillary column (25 m × 0.25 mm I.D.), with a stationary phase film thickness of 0.13 μm methylsilicone (CP-Sil 5 CB) from Chrompack (Middelburg, Netherlands). Helium was used as the carrier gas and the chromatographic conditions were as follows: oven temperature, programmed from 210 to 240°C at 6°C/min, from 240 to 270°C at 4°C/min and from 270 to 290°C at 2°C/min with a 5-min hold at 290°C; injector temperature, 290°C; detector temperature, 350°C; splitting ratio, 1:40; inlet pressure, 15 psi; and sample volume injected, 2 μl.

2.3. Design of the experiments

We designed two experiments to study the use of microtubes made of three common materials: Pyrex glass, polypropylene (PP) homopolymer and polypropylene copolymer.

In experiment I, 5 μg of each OS and 5 μg of cholesterol were added to six microtubes of each type of material. Then 50 μl of pyridine were added as solvent and 50 μl of Sylon BTZ as silanization reagent, and the blend was kept for 20 min to complete the reaction. Then, 50 μl of the blend were transferred into a glass microtube and 37.5 μg of 5α-cholestane (I.S.) were added. After 20 min, 2 μl of this blend were injected into the chromatograph. In order to check the adsorption of 5α-cholestane, 50 μl of Sylon BTZ were added to six microtubes of each type containing 75 μg of 5α-cholestane dissolved in 50 μl of pyrine. After 20 min, 50 μl of the blend were transferred into a glass microtube containing 37.5 μg of cholesterol, which was used as the I.S. After 20 min of reaction, 2 μl were injected into the chromatograph. In all instances, as can be observed, the I.S. was added directly to the final glass microtube, so that its adsorption should not affect the results.

In experiment II, four microtubes of each material were filled with 75 μg of 5α-cholestane (I.S.), 5 μg of each OS standard, 5 μg of cholesterol and 50 μl of pyridine as solvent. The blend was silanized with 50 μl of Sylon BTZ and, after 20 min, 2 μl of the reaction mixture were injected into the chromatograph.

3. Results and discussion

3.1. Results of experiment I

From the chromatograms obtained, the ratio between the areas of the standard and of the internal standard (A_{xs}/A_{is}) was calculated for each compound. In all instances, mean values of these ratios were higher in glass microtubes, owing to the higher degree of adsorption of standards in the plastic materials than in the glass (Table 1).

An analysis of variance (ANOVA) applied to these results showed that there are significant differences in the area ratio (A_{xs}/A_{is}) for cholesterol ($P = 0.0035$), 5α-cholestane ($P < 0.0001$) and α-CE ($P < 0.0001$), depending on the microtube used. For these three compounds, in order to know which mean ratios differ, we applied the Scheffé's test for a posteriori contrasts ($\alpha = 0.05$). The results obtained are given in Table 2.

Table 2 shows that 5α-cholestane was adsorbed in PP homopolymer more than in glass because, on average, A_{xs}/A_{is} in glass was between 0.0392 and 0.1217 higher than in PP homopolymer. In addition, this compound in PP

Table 1
Mean values for the ratio A_{xs}/A_{is} of 5α-cholestane, cholesterol and the five OS in the different microtube materials assayed ($n = 6$)

Material	5α-Cholestane	Cholesterol	α-CE	7β-HC	CT	7-KC	25-HC
Pyrex glass	0.8743	0.0776	0.0681	0.0698	0.0693	0.0569	0.0559
PP homopolymer	0.7939	0.0711	0.0604	0.0586	0.0667	0.0505	0.0538
PP copolymer	0.7047	0.0686	0.0628	0.0624	0.0692	0.0519	0.0551

Table 2
Significance level and confidence interval of the a posteriori contrasts (Scheffé's test, $\alpha = 0.05$)

Standard	Material	PP homopolymer	PP copolymer
5α-Cholestane	Pyrex glass	$P < 0.0001$ (0.0392–0.1217)[a]	$P < 0.001$ (0.1283–0.2109)
	PP homopolymer		$P < 0.0001$ (0.0435–0.1184)
Cholesterol	Pyrex glass	NS[b]	$P = 0.0035$ (0.0275–0.1756)
	PP homopolymer		NS
α-CE	Pyrex glass	$P < 0.0001$ (0.0567–0.2250)	$P = 0.0311$ (0.0094–0.1759)
	PP homopolymer		NS

[a] P = Significance level of the contrast; the confidence interval is given in parentheses.
[b] For this standard the difference between the mean ratios (A_{xs}/A_{is}) using these two kinds of microtube is not statistically significant.

copolymer was highly adsorbed and presented a value for A_{xs}/A_{is} between 0.1283 and 0.2109 lower than in glass and between 0.0435 and 0.1184 lower than in PP homopolymer. Cholesterol and α-CE presented higher adsorption in PP copolymer than in glass and, moreover, α-CE presented higher adsorption in PP homopolymer than in glass.

3.2. Results of experiment II

As the I.S. (5α-cholestane) showed a higher adsorption in the plastic materials, we carried out a second experiment, in which the I.S. was added to the same microtube as the other standards, to simulate the real procedure followed for calibration. From the chromatograms obtained, the ratio A_{xs}/A_{is} was calculated for each standard and the mean values are given in Table 3.

The results in Table 3 indicate that the mean ratio A_{xs}/A_{is} is maximum in the PP copolymer and minimum in the Pyrex glass, for all standards. This is Because the 5α-cholestane is more adsorbed in the PP copolymer than in the PP homopolymer, and much more so than in the glass. The application of the ANOVA to the mean values of A_{xs}/A_{is} for each standard in the

Table 3
Mean values of A_{xs}/A_{is} for each standard in the different microtubes ($n = 4$)

Material	Cholesterol	α-CE	7β-HC	CT	7-KC	25-HC
Pyrex glass	0.0862	0.0582	0.0751	0.0710	0.0556	0.0489
PP homopolymer	0.0918	0.0622	0.0801	0.0755	0.0601	0.0567
PP copolymer	0.1129	0.0843	0.1084	0.1082	0.0795	0.0783

Table 4
Significance level and confidence interval of the a posteriori contrasts (Scheffé's test, $\alpha = 0.05$)

Standard	Material	PP homopolymer	PP copolymer
Cholesterol	Pyrex glass	NS[a]	$P = 0.0005$ (0.014–0.039)[b]
	PP homopolymer		$P = 0.0024$ (0.009–0.033)
α-CE	Pyrex glass	NS[a]	$P < 0.0001$ (0.019–0.034)
	PP homopolymer		$P < 0.0001$ (0.015–0.030)
7β-HC	Pyrex glass	$P = 0.0130$ (0.001–0.010)	$P < 0.0001$ (0.029–0.037)
	PP homopolymer		$P < 0.0001$ (0.024–0.032)
CT	Pyrex glass	NS[a]	$P = 0.0003$ (0.022–0.053)
	PP homopolymer		$P = 0.0007$ (0.017–0.048)
7-KC	Pyrex glass	NS[a]	$P = 0.0004$ (0.013–0.035)
	PP homopolymer		$P = 0.0017$ (0.009–0.030)
25-HC	Pyrex glass	$P = 0.0254$ (0.001–0.015)	$P < 0.0001$ (0.023–0.036)
	PP homopolymer		$P < 0.0001$ (0.015–0.028)

[a] For these standards the difference between the mean ratios (A_{xs}/A_{is}) using these two kinds of microtube is not statistically significant.
[b] P = significance level of the contrast; the confidence interval is given in parentheses.

different materials showed that statistically significant differences exist for cholesterol ($P = 0.0003$), 7β-HC ($P < 0.0001$), α-CE ($P < 0.0001$), CT ($P = 0.0001$), 7-KC ($P = 0.0003$) and 25-HC ($P < 0.0001$). Therefore, applying the Scheffé's test for a posteriori contrasts ($\alpha = 0.05$), we can conclude that, for all standards, the mean value of A_{xs}/A_{is} is significantly higher in the PP copolymer than in glass and than in the PP homopolymer. Moreover, these mean values are significantly higher in the PP homolymer than in glass only for 7β-HC and 25-HC (Table 4).

The main conclusion was that the type of microtubes used in the method can significantly affect the results obtained, mainly in the calculation of the relative response factors of sterols and in the calibration graph because one of the most usual I.S., 5α-cholestane, shows significantly different adsorption rates on each kind of microtube. For this reason, we think that the most reliable results can be obtained using the Pyrex glass microtubes for derivatization, as they offer lower and less variable adsorption of all compounds.

Acknowledgements

This work was supported in part by a research grant from the Comissió Interdepartamental de Recerca i Innovació Tecnològica (CIRIT). C. Sala-Villaplana provided technical assistance.

References

[1] J. Levin, E.H. Friederich and J. Lobotsky, *J. Clin. Endocrinol. Metab.*, 25 (1965) 1519.
[2] *Packaging Materials – Plastics*, Newnes-Butterworth, London, 1975, p. 94.
[3] F. Guardiola, R. Codony, M. Rafecas and J. Boatella, *J. Chromatogr.*, 705 (1995) 289.

ELSEVIER

Journal of Chromatography A, 705 (1995) 400–402

JOURNAL OF CHROMATOGRAPHY A

Short communication

Purification of α-L-arabinofuranosidase using a single-column mini-scale isoelectric focusing unit

Sathivel Chinnathambi[a], Anil Lachke[a,*], S. Radhakrishnan[b]

[a]*Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India*

[b]*Polymer Sciences and Engineering, National Chemical Laboratory, Pune 411 008, India*

First received 15 August 1994; revised manuscript received 27 January 1995; accepted 31 January 1995

Abstract

Fabrication of a single-tube mini-scale isoelectric focusing unit with useful modifications is described. Provision of a side-arm to the unit facilitated easy collection of protein samples without disturbing the density and pH gradients. An ion-conducting polymer containing ether and hydroxy groups was fixed at the anodic end of the unit that separated the anodic solution from other solutions and also provided an easy path for charge transport. Using this unit, α-L-arabinofuranosidase (EC 3.2.1.55) was purified from crude broth of *Sclerotium rolfsii* in three steps with 33-fold purification.

1. Introduction

The overall operations and manoeuvring of the commercial instrumentation for electrofocusing of proteins prohibit their use for routine experimentation, hence Weller et al. [1] described a simple U-shaped mini-scale apparatus in which the p*I* of enzymes could be determined. Despite many advantages, the mini-scale units lack proper facilities for the collection of fractions from the column. This step must be followed meticulously without disturbing the pH and density gradient in order to achieve maximum resolution and recovery of the proteins. We have developed an improved single-tube electrofocusing column in which a special ion-conducting polymer is used. Using this column, α-L-arabinofuranosidase was purified to homogeneity in three simple steps.

2. Experimental

2.1. Strain and medium

Maintenance and cultivation of *Sclerotium rolfsii* were described previously [2]. The activity of α-L-arabinofuranosidase (α-L-AF) in international units (U) was measured using *p*-nitrophenyl α-L-arabinofuranoside (Sigma, N-3641) as the substrate.

2.2. Electrofocusing unit

The unit consists of single main column provided with a side-arm for easy collection of

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00147-6

protein fractions without disturbing the pH and density gradient. This unit is provided with an ion-conducting polymer at the anodic end.

The following solutions were prepared: (A) electrolyte solutions, (i) 0.1 *M* phosphoric acid as anode solution and (ii) 0.1 *M* sodium hydroxide as cathode solution; (B) separation solution, 1.5 ml of glycerol + 1 ml of water; (C) gradient solutions, (i) high-density solution, consisting of 3 ml of glycerol, 2 ml of water, 0.2 ml of carrier electrolyte and the protein sample (10–100 μg) and (ii) low-density solution, consisting of 5 ml of water, carrier 0.2 ml of electrolyte and the protein sample (10–100 μg); and (D) the ion-conducting polymer, which was prepared as follows. A prepolymer was prepared by heating a mixture of medium molecular mass (2000–4000) ethylene glycol (4.0 g) 1,2,6-hexanetriol (0.4 g) and $FeCl_3$ (10 mg) for 4 h at 90°C and precipitating the mass in acetone to obtain a powder. This powder was mixed with poly(vinyl alcohol) (molecular mass 50 000) in the proportion of 30% (w/w) and blended thoroughly. A 2-g amount of the resulting blend was mixed with concentrated phosphoric acid (0.5 ml) and allowed to soak for 1 h to obtain a paste, which was applied to the end of an isoelectric focusing tube [3]. The various layers of the solutions are shown in Fig. 1.

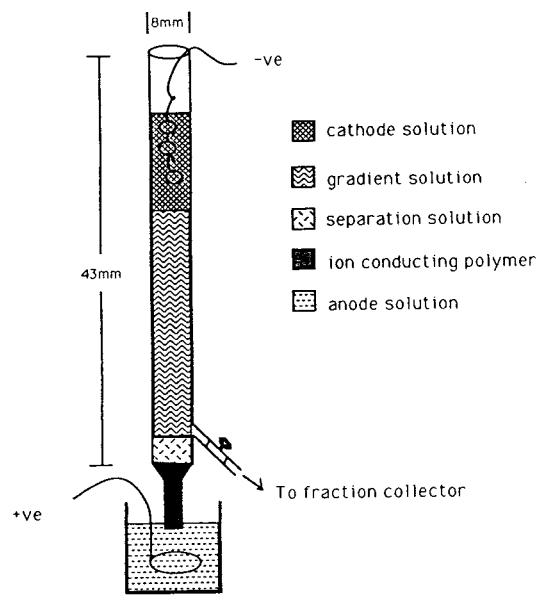


Fig. 1. Schematic diagram of mini-scale electrofocusing unit.

The apparatus assembly was placed in a cold room (5 ± 2°C) and the separation solution was layered above the conducting polymer up to the level of the side-arm (S). Gradient solutions (i) and (ii) were layered on the separation solution using a gradient mixer. Cathode solution was layered gently above the gradient using a 10-ml syringe. The platinum electrodes were dipped into respective solutions. Isoelectric focusing (IEF) was carried out for 8–10 h at 400 V, giving a current of 1.5 mA. Electrofocusing was discontinued and fractions of 0.2 ml each were collected by opening the tap on the side-arm. p*I* was determined by checking the pH of each fraction; protein can be detected either by measuring the absorbance at 280 nm or by carrying out an enzyme assay.

3. Results and discussion

3.1. Single-column IEF unit

It is possible to isolate and purify various protein samples conveniently using the improved apparatus shown in Fig. 1. The use of a side-arm with a tap is convenient for the collection of protein fractions. This design avoids many constraints during experimentation and its fabrication is simple.

3.2. Ion-conducting polymer

The anodic end of the IEF unit contains a layer of ion-conducting polymer, which has two functions: (a) it separates the anodic solution from the rest of the solutions and (b) it provides an easy path for charge transport. Ordinary gels or semipermeable membranes would normally create a large potential drop across them and decrease the net current flowing in the cell, giving rise to low efficiency. Hence a polymer containing ether and hydroxy groups was chosen, which would complex with the anodic solution (0.1 *M* phosphoric acid) and form an ionically conducting medium. Typically, a paste was made from 1 g of polymer powder and 0.7 ml of 1 *M*

phosphoric acid (85%) and fixed at the lower end of the column. It was ensured that the unit is leak-proof. The polymer was soaked in phosphoric acid for 1 h before the experimentation, which increased the conductivity owing to swelling and ionic dissociation. We have observed that the ion-conducting polymer shortens the duration of IEF: most models generally required 24–72 h but this has been shortened to 8–10 h.

3.3. Purification of α-L-AF

Crude broth of *S. rolfsii* was concentrated by ultrafiltration using an Amicon XM-300 membrane. The concentrate was subjected to Sephacryl S-300 gel column chromatography (104 × 1 I.D. cm). The fractions exhibiting high specific activity of α-L-AF were pooled and dialysed in a collodion bag (Sartorius SM 13200) for 8 h against 1% glycine solution. Finally dialysed enzyme was loaded for electrofocusing. The homogeneity of the enzyme was ascertained by observing a single protein band in polyacrylamide gel electrophoresis and also in analytical IEF in polyacrylamide gel. The steps of enzyme purification are summarized in Table 1.

α-L-AF is of increasing importance in biopulping, structural studies of carbohydrates [4] and cancer chemotherapy [5]. Therefore, screening for the microbial α-L-AF having the desired properties needs small amounts of a homogeneous or a highly refined preparation for preliminary characterization. The unit described here was useful for this purpose.

Commercial electrofocusing units need proportionately large amounts of expensive carrier electrolytes and a long period for the formation of a pH gradient, which often results in a loss of enzyme activity. To overcome these constraints, the modified single-column mini-scale IEF unit described here can be used routinely.

Acknowledgement

CSIR (New Delhi, India) is gratefully thanked for a Fellowship to C.S.

References

[1] D.L. Weller, A. Heaney and R.E. Sjorgren, *Biochim. Biophys. Acta*, 168 (1968) 576–579.
[2] A.H. Lachke and M.V. Deshpande, *FEMS Microbiol. Rev.*, 54 (1988) 177–194.
[3] S. Radhakrishnan, S. Chinnathambi and A.H. Lachke, *Indian Pat.*, filed 22 November 1994.
[4] K.K.Y. Wong and J.N. Saddler, in M.P. Coughlan and P.H. Geoffren (Editors), *Hemicellulose and Hemicellulase*, Portland Press, London, 1993, Ch. 6, pp. 127–143.
[5] G. Butschak, W. Foerster and A. Graffi, *Z. Allg. Microbiol.*, 16 (1976) 507–519.

Table 1
Purification of α-L-arabinofuranosidase from *S. rolfsii*

Step	Total activity (U/ml)	Total protein (mg/ml)	Specific activity	Purification (-fold)	Recovery in each step (%)
Crude broth	7.5	115	0.06	–	–
XM-300 concentrate	7.2	104	0.07	1.1	96
Sephacryl S-300	0.4	0.56	0.71	11.8	57
IEF	0.05	0.025	2.0	33.3	83

Author Index Vol. 705

Vol. 705, No. 2

Journal of Chromatography A

NEWS SECTION

ANNOUNCEMENTS

SHORT COURSE ON VALIDATION IN CAPILLARY ELECTROPHORESIS: SCIENTIFIC AND REGULATORY ISSUES FACING PRACTITIONERS OF CAPILLARY ELECTROPHORESIS, YORK, UK, 5–6 OCTOBER 1995

The first ever course in Validation on Capillary Electrophoresis will complement the basic course at which many European scientists have been trained and the biennial Capillary Electrophoresis Symposium which has become a forum where CE users meet the world's experts. Validation is increasingly important in all industries as an essential component in the maintenance of quality. Additionally in the regulated environment of the pharmaceutical and agrochemical industries, the need to satisfy the regulatory authorities has focussed attention on all aspects of validation. The relative newness of CE as an analytical technique and its increasing acceptance throughout industry as a rapid separatory method makes it timely to outline the issues of standards and calibration, robustness, reliability, compliance and transferability of methods.

The course will be of interest to all users of CE, to their managers and to those responsible for compliance and product registration. The exploration of the scientific issues surrounding the development and use of reliable methods will be of value to all practitioners whether subject to regulatory control or not. The questions raised and discussed will however be of particular interest and immediate importance to those seeking to gain regulatory approval.

Lectures

- Introduction and overview
- Validation – instrumental aspects
- Optimisation of precision and sensitivity
- Standards in capillary electrophoresis
- Harmonisation of analytical methods: company and regulatory perspectives
- Validation of capillary electrophoresis in biopharmaceuticals
- Validation of chiral capillary electrophoresis.

There will be three workshops during the course. The first will be based on a critical examination of selected papers from the literature. The second will be a computer workshop using an optimisation programme as an aid to identifying robust conditions. The final workshop will explore stability testing and standard operating procedures. Opportunity for informal discussion with the tutors will be present throughout the course, but in addition there will be formal feedback and discussion sessions.

For further details contact: Dr Terry Threlfall, Industrial Liaison Executive, Department of Chemistry, University of York, Heslington, York YO1 5DD, UK. Tel.: (+44-1904) 432-576 or 434-079; Fax: (+44-1904) 432-516.

6th ANNUAL FREDERICK CONFERENCE ON CAPILLARY ELECTROPHORESIS, FREDERICK, MD, USA, 23–25 OCTOBER 1995

The format of the conference will include oral and poster presentations by individual conference participants, and optional enrollment in a one-day CE course on 22 October. Invited speakers will provide an expert overview of the basic aspects and applications of CE and MECC including instrument and column design, detection, optimization, and factors that influence mobility, selectivity, resolution, and the application of CE in the separation of small ions as well as large biomolecules with emphasis on clinical, pharmaceutical and biomedical applications. Investigators interested in presenting their work for consideration by the Scientific Committee should send a 200-word abstract (accepted as 20-minute talks or poster displays).
For further information contact Margaret L. Fanning, Conference Coordinator, PRI, NCI-FCRDC, P.O. Box B, Frederick, MD 21702-1201, U.S.A. Tel. (+1-301) 846-5865; Fax (+1-301) 846-5866.

12th LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY MONTREUX SYMPOSIUM, HILTON HEAD ISLAND, SC, USA, 1–3 NOVEMBER 1995

The 12th Montreux Symposium on LC–MS, SFC–MS, CE–MS, and MS–MS will deal with all areas of these topics including: technical development with on-line and off-line aspects; theoretical considerations; and applications of the techniques in environmental, clinical, and pharmaceutical analysis as well as analysis in other fields.
Subtopics will be introduced by plenary and invited research lectures, followed by oral and poster presentations. A major emphasis of the symposium will be devoted to panel and group discussions on the state-of-the-art of LC–MS, SFC–MS, CE–MS, and MS–MS.
You are invited to submit an abstract of original research in one of these topics by 1 August 1995.
An introductory course in LC–MS, SFC–MS, and CE–MS will be offered on the two days preceding the symposium. Topics to be covered include:
1. Ionization methods
2. LC–MS Interfaces: principles, techniques, and applications of
   - Continuous flow FAB
   - Thermospray LC–MS
   - Particle beam interfaces
   - Electrospray/ionspray interfaces
   - APCI
   - Novel interfaces
3. LC–MS–MS: principles and applications
4. SFC–MS
5. CE–MS.

Instructors for the Short Course will be Jack Henion from Cornell University, Ithaca, NY, USA, and Dai Games from the University College of Swansea, UK.
For further details contact: Shirley Schlessinger, LC/MS '95, Suite 1015, 400 East Randolph Drive, Chicago, IL 60601, U.S.A. Tel.: (+1-312) 527-2011; Fax (+1-312) 528 3437.

ISPPP '95, 15th INTERNATIONAL SYMPOSIUM ON THE SEPARATION AND ANALYSIS OF PROTEINS, PEPTIDES AND POLYNUCLEOTIDES, BOSTON, MA, USA, 19–22 NOVEMBER 1995

ISPPP '95 will once again bring together researchers from industry and academia working with new developments and application in the separation and analysis of biomolecules.
The three day scientific program consists of oral, poster and discussion sessions. Recognized authorities will review current trends and future perspective in various topics, such as:
- Analytical applications
- Column technology and support materials
- Detection and amplification
- Electroseparation techniques
- Isolation and purification techniques
- MicrochipTech, miniaturized systems
- Molecular design and surfaces
- Oligonucleotides
- On-line analytical techniques
- Protein–surface interactions
- Regulatory issues: validation
- Sensors of the future.

You are invited to submit an abstract describing original research in any of the aforementioned areas.
The Scientific Committee welcomes your suggestions

for additional topics to be covered in the symposium. Completed manuscripts submitted at the time of the symposium will be subject to normal review procedures and, if accepted, be published in a collected proceedings in the *Journal of Chromatography A*.
For further details contact: ISPPP '95, Judy Heine, Department of Chemistry, Brown Hall, Purdue University, West Lafayette, IN 47907-1393, USA. Tel.: (+1-317) 494-1648; Fax: (+1-317) 494-0359.

3rd INTERNATIONAL GLYCOBIOLOGY SYMPOSIUM, SAN DIEGO, CA, USA, 29 NOVEMBER–1 DECEMBER 1995

This meeting will bring experts together to critically discuss established and novel methods for analysis of glycoconjugates. The program will include plenary lectures, poster presentations and discussion sessions. You are invited to submit abstracts describing original research in areas including:

- Established and novel techniques for the analysis of glycoconjugates
- Specific enzymatic release of oligosaccharides from glycoconjugates
- Automated approaches for the chemical release and enzymatic sequencing of carbohydrates
- One- and two-dimensional liquid chromatography analysis of oligosaccharides
- Electrophoresis of oligosaccharides and glycoproteins in gels and in capillaries
- Linkage analysis using GC–MS
- Structural elucidation using chemical fragmentation approaches
- Derivatization schemes for chromatography and mass spectrometry
- Accurate mass measurements of glycoproteins and glycopeptides
- One- and two-dimensional NMR methods for complete structural elucidation of carbohydrates
- Analysis of glycoforms using matrix-assisted laser desorption mass spectrometry
- Glycopeptide analysis using LC–electrospray MS
- Solution conformation of oligosaccharides.

For further details contact: Patty Batchelder, Conference Manager, International Glycobiology Symposium, 7948 Foothill Knolls Drive, Pleasanton, CA 94588, USA. Tel. (+1-510) 426-9601; Fax: (+1-510) 484-3024.

1996 WINTER CONFERENCE ON PLASMA SPECTROCHEMISTRY, FORT LAUDERDALE, FL, USA, 8–13 JANUARY 1996

This symposium features developments in plasma spectrochemical analysis by inductively coupled plasma (ICP), dc plasma (DCP), microwave plasma (MIP), and glow discharge (GDL, HCL) sources.
The topics for the symposium are:

- sample introduction and transport phenomena
- flow injection spectrochemical analysis
- element speciation with plasma/chromatographic techniques
- plasma instrumentation, including chemometrics, expert systems, on-line analysis, software, and remote-system automation
- sample preparation, treatment and automation
- excitation mechanisms and plasma phenomena
- spectroscopic standards and reference materials
- plasma source mass spectrometry
- glow discharge atomic and mass spectrometry
- applications of stable isotope analysis
- laser-assisted plasma spectrometry.

For further information contact: 1996 Winter Conference on Plasma Spectrochemistry, c/o ICP Newsletter, Attn. Dr. R. Barnes, Department of Chemistry, Lederle GRC Towers, University of Massachusetts, Box 34510, Amherst, MA 01003-4510, USA. Tel.: (+1-413) 545-2294; Fax: (+1-413) 545-4490.

PREPTECH '96, EAST RUTHERFORD, NJ, USA, 5–7 FEBRUARY 1996

Topics of interest will include: preparative liquid chromatography, membrane separations, electroseparation techniques, extraction, and centrifugation.
Scientists with a professional interest in these and related areas are invited to submit abstracts for both oral and poster presentations. Abstracts should include the title, the names of the authors and their affiliations (omitting degrees, but indicating the name of the presenting author), company name, complete mailing address, telephone- and fax numbers; a clear statement of the objective of the work, methodology, and results and conclusions.
The deadline for receipt of oral abstracts is July 28, 1995; for poster abstracts, December 1, 1995. Ab-

stracts not exceeding 350 words should be submitted as both hard copy and as PC or Macintosh files (WordPerfect, Word, or ASCII) to Robert Stevenson, Chairman Technical Committee, PrepTech'95, 3338 Carlyle Terrace, Lafayette, CA 94549, USA. Tel.: (+1-510) 283-7619; Fax: (+1-510) 283-5621.
Information about attendance and exhibiting should be directed to: Joan Lantowski, Conference Coordinator, ISC Technical Conferences, Inc., 30 Controls Drive, P.O. Box 559, Shelton, CT 06484, U.S.A. Tel.: (+1-203) 926-9300; Fax: (+1-203) 926-9722.

7th INTERNATIONAL SYMPOSIUM ON SUPERCRITICAL FLUID CHROMATOGRAPHY AND EXTRACTION, INDIANAPOLIS, IN, USA, 31 MARCH–4 APRIL 1996

The purpose of the symposium will be to provide a forum for maximum exchange of information on techniques, theory and applications of supercritical fluid chromatography, extraction and fractionation. The program will emphasize recent accomplishments and research results rather than review. It will consist of lecture presentations featuring leading researchers from academia, government, and industry; posters; intensive and informal discussion sessions; an instrument exhibit; an optional tutorial.
Newcomers and those interested in review are invited to participate in a one-day tutorial preceding the symposium.
The exposition program will be designed to ensure that participants have ample opportunities to meet commercial vendors who will display state-of-the-art SFC, SFE, and SFF instrumentation and supplies.
A limited number of travel scholarships are available to students wishing to present a poster. You may apply by submitting a short letter of nomination from the major professor and a two-page abstract to the Symposium Manager by 15 December 1995.
Our past symposia have emphasized personal conversations and exchange of recent knowledge among researchers. We wish to continue this tradition. The best form has proven to be poster sessions combined with separate, open microphone discussion sessions. Therefore, participants are invited and encouraged to present posters on SFE, SFC, and SFF.
Two-page abstracts are required in advance and will be reproduced in a bound volume for attendees. The deadline for submission is 29 January 1996.
For further details contact: Mrs Janet Cunningham, SFC/SFE '96 Symposium Manager, Barr Enterprises, 10120 Kelly Road, P.O. Box 279, Walkersville, MD 21793, USA. Tel.: (+1-301) 898-3772; Fax (+1-301) 898-5596.

26th INTERNATIONAL SYMPOSIUM ON ENVIRONMENTAL ANALYTICAL CHEMISTRY, VIENNA, AUSTRIA 9–12 APRIL 1996

Major topics to be covered will include:
- development of new analytical techniques, instrumentation and procedures for environmental studies
- application of analytical techniques to environmental problems.

Program highlights covered by invited lectures of leading scientists will include:
- Sampling strategies and sample preparation (e.g. SFE)
- Hyphenated techniques (e.g. LC–GC, GC–MS, GC–AES, HPLC–MS)
- Elemental analysis, inorganic separation and organic trace analysis
- Sensors for environmental monitoring
- Biochemical detection techniques
- Multiresidue (screening) procedures
- Quality assurance in environmental analysis
- Air, water and soil analysis
- Transnational environmental problems in Europe
- Environmental pollution in sensitive ecosystems.

Posters and oral contributions on all topics related to environmental analytical chemistry are welcome. Deadline for submission of abstracts: 15 November 1995. Lectures and poster contributions will be published in the *International Journal of Environmental Analytical Chemistry*. Manuscript should be submitted at the conference.
An exhibition of analytical instruments, reagents and books will be organized.
For further details contact: Dr Erwin Rosenberg, Institut für Analytische Chemie, Technische Universität Wien, Getreidemarkt 9/151, A-1060 Vienna, Austria. Tel. (+43-1) 58801 4824 (or 4940, 4837); Fax: (+43-1-) 586 7813; E-mail: erosen@fbch.tuwien.ac.at

BIO-CHROMATOGRAPHY AND PURIFICATION OF BACTERIAL, VIRAL AND OTHER NATURAL ANTIGENS, PARIS, FRANCE, 13–15 MAY 1996

The Tenth Anniversary Meeting of the European Society for Bio-Chromatography will focus on bio-chromatography essentially used for purifying antigens. All correlated topics will also be considered such as: column and supports, protein structure and chromatographic behaviour, peptides, oligonucleotides, polysaccharides and mucopolysaccharides, molecular recognition and molecular imprinting; electrokinetic techniques will not be forgotten.
Those wishing to contribute are requested to submit abstracts on the official forms. Forms will be mailed on request or as complementary information with the first announcement.
The deadline for submission is 31 December 1995.
The subject matter of the posters and oral communications is not necessarily limited to the areas above.
Manufacturers, distributors and suppliers of products and service to the special fields of bio-chromatography, immunology and microbiology will find this meeting an opportunity to display information on their products or services. They can also present oral communications during special sessions.
For further details contact: Bio-Chromatographie 96, Secrétariat du Congrès, Unité d'Immuno-Allergie, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (+33-1) 4568 8448; Fax: (+33-1) 4061 3160.

20th INTERNATIONAL SYMPOSIUM ON HIGH PERFORMANCE LIQUID PHASE SEPARATIONS, SAN FRANCISCO, CA, USA, 16–21 JUNE, 1996

The traditional areas of chromatography have been enriched by the growth of allied separation methods, such as capillary electrophoresis, mass spectrometry, and the development of hyphenated approaches. The permanent scientific committee has recognized these developments by changing the name of the meeting to the International Symposium on High Performance Liquid Phase Separations.
Scientific highlights:

- Advances in sensor technology;
- Validation of capillary electrophoresis methods;
- Developments in mass spectrometry;
- Manufacture of consistent HPLC columns;
- Micro-separations;
- Environmental applications for fieldable instruments;
- Government regulations in environmental and pharmaceutical industry;
- Development of user friendly software;
- Multi-dimensional analysis for complex biological samples.

For further details contact: Mrs. Janet Cunningham, HPLC '96 Symposium/Exhibit Manager, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA. Tel.: (+1-301) 898-3772; Fax (+1-301) 898-5596.

8th INTERNATIONAL SYMPOSIUM ON CHIRAL DISCRIMINATION, EDINBURGH, UK, 30 JUNE–3 JULY 1996

This Symposium series provides an excellent international forum for the exchange of ideas, information and recent advances on all aspects of molecular chirality. Its interdisciplinary nature encompasses many scientific disciplines including chemistry, biochemistry, pharmacology, biology, pharmacy, medicine and analytical chemistry.
The scientific programme will feature invited lectures, submitted lectures and posters and poster discrimination sessions. Topics that will be presented include the following:

- Fundamental aspects and molecular recognition
- Novel developments in asymmetric synthesis
- Stereoselective catalysis
- Catalytic antibodies
- Enzymatic methods
- Determination of absolute configuration
- Chiroptical systems including circular dichroism and polarimetry chiral NMR shift reagents
- Advances in enantiomeric separations including LC, CE, MEKC, GC and SFC
- Large-scale enantiomeric separations
- Chiral ligand binding
- Chiral pharmacokinetics
- Chiral pharmacodynamics
- Racemisation

- Environmental issues involving stereochemistry
- Food and beverage issues involving stereochemistry
- Validation issues for the regulatory environment
- Regulatory issues of chiral drugs and pesticides
- Applications of chiral analysis for problem-solving in industry.

A book of Abstracts of all scientific contributions will be distributed to each participant at he 8th ISCD. All papers submitted will be considered for publication in a special issue of *Chirality* after refereeing.

A major exhibition of scientific equipment and materials will be held in conjunction with the 8th ISCD. All those interested in taking part in this exhibition are invited to contact the Secretariat for further details.

All scientists involved in any aspect, fundamental or applied, of chiral discrimination are invited to contribute.

For further details contact: ISCD Conference Secretariat, c/o Meeting Makers Ltd., 50 George Street, glasgow G1 1QE, UK. Tel.: (+44-141) 553-1930; Fax: (+44-141) 552 0511.

PBA '96: 7th INTERNATIONAL SYMPOSIUM ON PHARMACEUTICAL AND BIOMEDICAL ANALYSIS, OSAKA, JAPAN, 20–23 AUGUST 1996

The theme of PBA '96 will be the development of new techniques and methods for the analysis of drugs, related materials and endogeneous compounds. Emphasis will be put on "The analytical approach to biofunctions" relating to chemotherapy and diagnosis. All those who are involved in any area of pharmaceutical and biomedical analysis are cordially invited to participate.

The scientific programme will include invited plenary and keynote lectures by internationally recognized scientists, contributed papers (oral and posters) and poster discussion sessions. The topics will include the following:

- Analysis of drug materials and formulations
- Drug analysis in clinical and preclinical phases of drug development
- Analysis of endogeneous substances
- Sample preparation and derivatization
- Advances in separation techniques – HPLC, SFC, GC, CE etc.
- Advances in spectroscopy – NMR, UV–VIS, MS, CD, FT-IR, CL, FL etc.
- Radio- and immuno-assay methods
- Automated analytical instruments
- Assay validation
- Chemometrics
- Therapeutic drug monitoring, clinical and forensic toxicology
- Chemical clinical tests
- Biofunction analysis for diagnosis, mass-screening and therapy
- Analysis of drug enantiomers
- Pharmaceutical analysis for quality control and general tests for the pharmacopoeia.

Participants who wish to present a paper are hereby invited to submit an abstract, the deadline being 1 February 1996. All submitted papers will be considered for publication in the *Journal of Pharmaceutical and Biomedical Analysis.*

For further details contact: Professor Susumu Honda, Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi, Osaka 577, Japan. Fax: (+81-6) 721 2353.

EUROANALYSIS IX, BOLOGNA, ITALY, SEPTEMBER 1–7, 1996

Following Euroanalysis traditions, the 9th conference will aim to cover all branches of analytical chemistry with emphasis on the "problem solving" role of the discipline.

The scientific program will consist of invited lectures and contributed papers (oral and posters). Special topics planned will include:

- Education (where the complete scheme of the "Eurocurriculum" in analytical chemistry will be discussed)
- Validation in analytical chemistry
- Reference materials
- Calibration and traceability.

The official language of the Conference is English. No simultaneous translation will be provided.

For further details contact: Professor L. Sabbatini, EUROANALYSIS IX, Dipartimento di Chimica, Università di Bari, Via Orabona 4, I-70126 Bari, Italy. Tel.: (+39-80) 242-020/16/14; Fax: (+39-80) 242-026.

2nd WORKSHOP ON BIOSENSORS AND BIOLOGICAL TECHNIQUES IN ENVIRONMENTAL ANALYSIS, LUND, SWEDEN, 11–13 SEPTEMBER 1996

This is the follow-up of the successful first workshop held in Paris in September 1994 under the auspices of the International Association of Environmental Chemistry. This workshop intends to cover advances of different techniques based on biological recognition for environmental purposes. The close link between environmental pollution and its impact on xenobiotic human exposure links the environmental area with the toxicological and biomedical fields in several ways. Similar analytical problems must be solved with related techniques and it is therefore the intention of the Lund meeting to stimulate and explore the progress achieved in these parallel research areas.
For further details contact: Mrs M. Frei-Häusler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwil 2, Switzerland. Tel. (+41-61) 481-2789; Fax (+41-61) 482 0805.

21st INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY, STUTTGART, GERMANY, 15–20 SEPTEMBER 1996

This symposium will be organized by the Gesellschaft Deutscher Chemiker, Arbeitskreis Chromatographie der Fachgruppe Analytische Chemie in association with the Chromatographic Society (UK) and Grouppe pour l'Avancement des Sciences Analytique (G.A.M.S.).
Themes: New aspects in applications, instrumentation and the fundamentals of the various chromatographic methods (gas chromatography, liquid chromatography, planar chromatography, supercritical fluid chromatography) and related separation techniques such as capillary electrophoresis. Special emphasis will be on new developments and on the practical application of the different techniques in problem solving for environmental, pharmaceutical, biomedical and industrial analysis,
For further details contact: Gesellschaft Deutscher Chemiker, Abteilung Tagungen, P.O. Box 900440, D-60444 Frankfurt am Main, Germany. Fax: (+49-69) 7917 475.

ITP '96: 10TH INTERNATIONAL SYMPOSIUM ON CAPILLARY ELECTROPHORESIS AND ISOTACHOPHORESIS, PRAGUE, CZECH REPUBLIC, 17–20 SEPTEMBER 1996

Topics covered include
- theoretical aspects
- applications
- instrumentation in
  - –zone electrophoresis
  - –isotachophoresis
  - –electrokinetic chromatography
  - –isoelectric focusing

The symposium language will be English. The program will consist of oral presentations and posters. All papers presented will be considered for publication in a special issue of the *Journal of Chromatography A*. Abstracts of all contributed papers will be distributed to the participants as a Book of Abstracts. The deadline for submission of abstracts is 30 June 1996.
An exhibition of electrophoretic instruments and literature will be held adjacent to the symposium. Companies interested in participating in the exhibition are invited to send their enquiries to the contact address.
For further details contact: Dr Bohuslav Gas, Faculty of Science, Charles University, Albertov 2030, 12840 Prague 2, Czech Republic. Tel.: (+42-2) 2491 5472; Fax: (+42-2) 291 958; Internet: e-mail address (preferred): itp96@prfdec.natur.cuni.cz

CALENDAR OF FORTHCOMING EVENTS

□ 1–5 October 1995
Dallas, TX, USA
IICS '95: International Ion Chromatography Symposium. *Contact:* Century International Inc., P.O. Box 493, 25 Lee Road, Medfield, MA 02052, USA. Tel.: (+1-508) 359-8777; Fax: (+1-508) 359-8778.

□ 5–7 October 1995
Lexington, KY, USA
International Symposium on Clinical Enzymology. *Contact:* Dr Norbert W. Tietz, Department of Pathology and Laboratory Medicine, University of Kentucky Medical Center, Lexington, KY 40536, USA. Tel. (+1-606) 323-5319; Fax: (+1-606) 257-8932.

6–8 October 1995
York, UK
Short Course on Validation in Capillary Electrophoresis. *Contact:* Dr Terry Threlfall, Industrial Liaison Executive, Department of Chemistry, University of York, Heslington, York YO1 5DD, UK. Tel.: (+44-1904) 432-576 or 434-079; Fax: (+44-1904) 432-516.

□ 10–11 October 1995
Atlanta, GA, USA
Silicones for Biomedical/Pharmaceutical Applications. *Contact:* Program Division, Technomic Publishing Company, Inc., 851 New Holland Avenue, Box 3535, Lancaster, PA 17604, USA. Tel.: (+1-717) 291-5609; Fax (+1-717) 295-4538; Toll-free in the US/territories and Canada: 800-233-9936.

□ 11–13 October 1995
Secaucus, NJ, USA
Biosensors. *Contact:* Program Division, Technomic Publishing Company, Inc., 851 New Holland Avenue, Box 3535, Lancaster, PA 17604, UA. Tel.: (+1-717) 291-5609; Fax (+1-717) 295-4538; Toll-free in the US/territories and Canada: 800-233-9936.

15–20 October 1995
Cincinnati, OH, USA
FACSS XXII: 22nd Annual Conference of the Federation of Analytical Chemistry and Spectroscopy Societies. *Contact:* FACSS, 201-B Broadway Street, Frederick, MD 21701-6501, USA. Tel.: (+1-301) 846-4797; Fax: (+1-301) 694-6860.

23–25 October 1995
Frederick, MD, USA
6th Annual Frederick Conference on Capillary Electrophoresis. *Contact:* Margaret L. Fanning, Conference Coordinator, PRI, NCI-FCRDC, P.O. Box B, Frederick, MD 21702-1201. Tel. (+1-301) 846-5865; Fax: (+1-301) 846 5866.

1–3 November 1995
Hilton Head Island, SC, USA
12th LC/MS Montreux Symposium. *Contact:* Shirley E. Schlessinger, LC/MS '95, Suite 1015, 400 East Randolph Drive, Chicago, IL 60601, USA. Tel.: (+1-312) 527-2011.

□ 5–10 November 1995
Cordoba, Spain
1995 First Mediterranean Basin Conference on Analytical Chemistry. *Contact:* Professor Alfredo Sanz-Medel, Department of Physical and Analytical Chemistry, Faculty of Chemistry, Universşity of Oviedo, C/ Julian Claveria, s/n, 33006 Oviedo, Spain. Tel.: (+34-8) 510-3480 or 510-3474; Fax: (+34-8) 510-3125.

□ 14–16 November 1995
Helsinki, Finland
KEMIA 95. *Contact:* Ms Tea Siili, Association of Finnish Chemical Societies, Hietaniemenkatu 2, 00100 Helsinki, Finland. Tel. (+358-0) 408-022; Fax: (+358-0) 408-780.

19–22 November 1995
Boston, MA, USA
ISPPP '95. *Contact:* Judy Heine, Department of Chemistry, Brown Building, Purdue University, Lafayette, IN 47907, USA. Tel.: (+1-317) 494-1648; Fax: (+1-317) 494-0359.

29 November–1 December 1995
San Diego, CA, USA
3rd International Glycobiology Symposium. *Contact:* Paddy Batchelder, Conference Manager, 7948 Foothill Knolls Drive, Pleasanton, CA 94588, USA. Tel.: (+1-510) 426-9601; Fax: (+1-510) 484-3024.

□ 8–9 December 1995
Nürmberg, Germany
2nd Nürmberg Conference of EUFEPS: Interchangeability of Topical Products for Local Action. *Contact:* EUFEPS Secretariat, P.O. Box 1136, S-111 81 Stockholm, Sweden. Fax: (+46-8) 205-511.

5–7 February 1996
East Rutherford, NJ, USA
PrepTech '96. *Contact:* Robert Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA. Tel.: (+1 510) 283-7619; Fax: (+1 510) 283-5621.

6–9 February 1996
Bruges, Belgium
HTC 4: 4th International Symposium on Hyphenated Techniques in Chromatography. *Contact:* Dr R. Smits, BASF Antwerp N.V., Centraal Laboratorium, Haven 725, Scheldelaan 600, B-2040 Antwerp, Belgium. Tel.: (+32-3) 561-2831; Fax: (+32-3) 561-3250; or Dr R. Senten, Municipal Laboratory, Slachthuislaan 68, B-2060 Antwerp, Belgium. Tel.: (+32-3) 217-2905; Fax: (+32-3) 235-3323.

31 March–4 April 1996
Indianapolis, IN, USA
7th International Symposium on Supercritical Fluid Chromatography and Extraction. *Contact:* Janet Cunningham, c/o Barr Enterprises, 10120 Kelly Road, P.O. Box 279, Walkersville, MD 21793, USA. Tel. (+1-301) 898-3772; Fax: (+1-301) 898-5596.

9–12 April 1996
Vienna, Austria
ISEAC 26. 26th International Symposium on Environmental Analytical Chemistry. *Contact:* Dr Erwin Rosenberg, Institut für Analytische Chemie, Technische Universität Wien, Getreidemarkt 9/151, A-1060 Vienna, Austria. Tel. (+43-1) 58801 4824 (or 4940, 4837); Fax: (+43-1-) 586 7813; E-mail: erosen@fbch.tuwien.ac.at

□ 17–19 April 1996
Nice, France
7th International Symposium on Luminescence Spectrometry in Biomedical Analysis – Detection Techniques and Applications in Chromatography and Capillary Electrophoresis. *Contact:* Dr Willy R.G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. Pharmaceutical Analysis, Laboratory of Drug Control, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel. (+32-9) 221-8951; Fax (+32-9) 221-4175.

□ 13–15 May 1996
Paris, France
Bio-Chromatography and Purification of Bacterial, Viral and Other Natural Antigens. *Contact:* Bio-Chromatographie 96, Secrétariat du Congrès, Unité d'Immuno-Allergie, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel. (+33-1) 4568 8448; Fax: (+33-1) 4061 3160.

20–24 May 1996
Riva del Garda, Italy
18th International Symposium on Capillary Chromatography. *Contact:* Prof. P. Sandra, I.O.P.M.S., Kennedypark 20, B-8500 Kortrijk, Belgium. Tel. (+32-56) 204-960; Fax: (+32-56) 204-859.

16–21 June 1996
San Francisco, CA, USA
HPLC '96: 20th International Symposium on High Performance Liquid Phase Separations. *Contact:* Mrs. Janet E. Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA. Tel.: (+1-301) 898-3772; Fax: (+1-301) 898-5596.

30 June–3 July 1996
Edinburgh, UK
8th International Symposium on Chiral Discrimination. *Contact:* ISCD Conference Secretariat, c/o Meeting Makers Ltd., 50 George Street, Glasgow G1 1QE, UK. Tel.: (+44-141) 553-1930; Fax: (+44-141) 552-0511.

20–23 August 1996
Osaka, Japan
PBA '96: 7th International Symposium on Pharmaceutical and Biomedical Analysis. *Contact:* Prof. S. Honda, Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi, Osaka 577, Japan. Fax: (+81-6) 721-2353.

21–23 August 1996
York, UK
4th International Symposium on Capillary Electrophoresis. *Contact:* Dr Terry Threlfall, Industrial Liaison Executive, Department of Chemistry, University of York, Heslington, York YO5 1DD, UK. Tel.: (+44-1904) 432-576 or 434-079; Fax: (+44-1904) 432-516.

1–7 September 1996
Bologna, Italy
Euroanalysis IX. *Contact:* Prof. L. Sabbatini, Euroanalysis IX, Dipartimento di Chimica, Università di Bari, Via Orabona 4, I-70126 Bari, Italy. Tel.: (+39-80) 242-020/16/14; Fax: (+39-80) 242-026.

11–13 September 1996
Lund, Sweden
2nd Workshop on Biosensors and Biological Techniques in Environmental Analysis. *Contact:* Mrs. M. Frei-Häusler, IAEAC Secretariat, Postfach 46, CH-

4123 Allschwil 2, Switzerland. Tel.: (+41-61) 481-2789; Fax: (+41-61) 482-0805.

15–20 September 1996
Stuttgart, Germany
21st International Symposium on Chromatography. *Contact:* Gesellschaft Deutscher Chemiker, Abteilung Tagungen, P.O. Box 900440, D-60444 Frankfurt am Main, Germany. Fax: (+49-69) 7917-475.

17–20 September 1996
Prague, Czech Republic
ITP '96. Tenth International Symposium on Capillary Electrophoresis and Isotachophoresis. *Contact:* Dr Bohuslav Gas, Faculty of Science, Charles University, Albertov 2030, 12840 Prague 2, Czech Republic. Tel. (+42-2-) 2491-5472; Fax: (+42-2) 291-958. Internet e-mail (preferred): itp96@prfdec.natur.cuni.cz.

■ **9–11 July 1997**
Kyoto, Japan
HPCE '97 Kyoto: Tenth International Symposium on High Performance Capillary Electrophoresis. *Contact:* Professor Shigeru Terabe, Faculty of Science, Himeji Institute of Technology, Kamigori, Hyogo 678-12, Japan. Tel.: (+81) 7915-80172; Fax: (+81) 7915-80132.

■ Indicates new or amended entry.

Characterization and Chemical Modification of the Silica Surface

By **E.F. Vansant, P. Van Der Voort** and **K.C. Vrancken**

Studies in Surface Science and Catalysis Volume 93

Oxide surface materials are widely used in many applications, in particular where chemically modified oxide surfaces are involved. Indeed, in disciplines such as separation, catalysis, bioengineering, electronics, ceramics, etc., modified oxide surfaces are very important. In all cases, the knowledge of their chemical and surface characteristics is of great importance for the understanding and eventual improvement of their performances. This book reviews the latest techniques and procedures in the characterization and chemical modification of the silica surface, presenting a unified and state-of-the-art approach to the relevant analysis techniques and modification procedures, covering 1000 references integrated into one clear concept.

Contents:

©1995 572 pages Hardbound
Price: Dfl. 410.00 / US$ 241.25
ISBN 0-444-81928-2

ELSEVIER
An imprint of Elsevier Science

ORDER INFORMATION

ELSEVIER SCIENCE
Customer Service Department
P.O. Box 211
1000 AE Amsterdam
The Netherlands
Fax: +31 (20) 485 3432

For USA and Canada:
ELSEVIER SCIENCE
Customer Service Department
P.O. Box 945
New York
NY 10159-0945
Fax: +1 (212) 633 3764

US$ prices are valid only for the USA & Canada and are subject to exchange rate fluctuations; in all other countries the Dutch guilder price (Dfl.) is definitive. Customers in the European Union should add the appropriate VAT rate applicable in their country to the price(s). Books are sent postfree if prepaid.

Chromatography in the Petroleum Industry

Edited by **E.R. Adlard**

Journal of Chromatography Library, Volume 56

Petroleum mixtures consist primarily of relatively unreactive complex hydrocarbons covering a wide boiling range. Such mixtures are difficult to separate by most analytical techniques. Therefore, the petroleum industry has for many years played a leading role in the development of chromatographic methods of analysis. Since the last book specifically concerned with chromatographic analysis of petroleum appeared 15 years ago, numerous advances have been made including developments in liquid and supercritical fluid chromatography, the advent of silica capillary columns with bonded stationary phases and the commercial availability of new selective detectors.

The current book contains chapters written by experts concerning the analysis of mixtures ranging from low boiling gases to waxes and crude oils.

Although the volume is specifically aimed at the petroleum analyst, there is much information of general interest which should be of benefit to a very wide readership.

Contents:

©1995 452 pages Hardbound
Price: Dfl. 435.00 (US$ 255.75)
ISBN 0-444-89776-3

ELSEVIER
An imprint of Elsevier Science

ORDER INFORMATION

ELSEVIER SCIENCE B.V.
P.O. Box 330
1000 AH Amsterdam
The Netherlands
Fax: +31 (20) 485 2845

For USA and Canada:
P.O. Box 945, New York
NY 10159-0945
Fax: +1 (212) 633 3680

US$ prices are valid only for the USA & Canada and are subject to exchange rate fluctuations; in all other countries the Dutch guilder price (Dfl.) is definitive. Customers in the European Union should add the appropriate VAT rate applicable in their country to the price(s). Books are sent postfree if prepaid.

PUBLICATION SCHEDULE FOR THE 1995 SUBSCRIPTION

Journal of Chromatography A and *Journal of Chromatography B: Biomedical Applications*

MONTH	1994	J–M	A	M[a]	J	J	
Journal of Chromatography A	Vols. 683–688	689–695	696/1 696/2 697/1 + 2 698/1 + 2	699/1 + 2 700/1 + 2 702/1 + 2 703/1 + 2	704/1 704/2 705/1 705/2	706/1 + 2 707/1 707/2 708/1	The publication schedule for further issues will be published later.
Bibliography Section		713/1			713/2		
Journal of Chromatography B: Biomedical Applications		663–665	666/1 666/2	667/1 667/2	668/1 668/2	669/1 669/2	

[a] Vol. 701 (Cumulative Indexes Vols. 652–700) expected in October.

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in *J. Chromatogr. A*, Vol. 657, pp. 463–469. A free reprint can be obtained by application to the publisher, Elsevier Science B.V., P.O. Box 330, 1000 AH Amsterdam, Netherlands.)

Types of Contributions. The following types of papers are published: Regular research papers (full-length papers), Review articles, Short Communications and Discussions. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as full-length papers, but should preferably not exceed five printed pages. Discussions (one or two pages) should explain, amplify, correct or otherwise comment substantively upon an article recently published in the journal. For Review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the *Journal of Chromatography A* or *B*.

Manuscripts. Manuscripts should be typed in **double spacing** on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Abstract, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Abstract. All articles should have an abstract of 50–100 words which clearly and briefly indicates what is new, different and significant. No references should be given.

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.

Experimental conditions should preferably be given on a *separate* sheet, headed "Conditions". These conditions will, if appropriate, be printed in a block, directly following the heading "Experimental".

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 caption, all the *captions* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be provided. 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".

Vols. 1–651 of the *Journal of Chromatography*; *Journal of Chromatography, Biomedical Applications* and *Journal of Chromatography, Symposium Volumes* should be cited as *J. Chromatogr.* From Vol. 652 on, *Journal of Chromatography A* (incl. Symposium Volumes) should be cited as *J. Chromatogr. A* and *Journal of Chromatography B: Biomedical Applications* as *J. Chromatogr. B.*

Dispatch. Before sending the manuscript to the Editor please check that the envelope contains four copies of the paper complete with references, captions 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.

Reprints. Fifty reprints will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

0021-9673(19950630)705:2;1-3