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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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The numbering and title system used for the *Journal of Chromatography*, while allowing maximum flexibility to most users of the journal, has led to some confusion, particularly among librarians.

Starting with the 1994 subscription, a new title and numbering system will, therefore, be used, reflecting the feed-back we have received from our subscribers. Effective from Volume 652, the first volume of the 1994 subscription, the two sections will have independent volume numbers and slightly revised titles. The volume numbers for the respective sections will each continue from the section numbers completed in 1993 for the full set. The modified titles of the two sections will be *Journal of Chromatography A* and *Journal of Chromatography B: Biomedical Applications* (see also the summary below).

Authors citing Volumes 1–651 of the *Journal of Chromatography* and *Journal of Chromatography, Biomedical Applications* in their publications should, as before, refer to *J. Chromatogr.* From Volume 652 onwards, *Journal of Chromatography A* should be cited as *J. Chromatogr. A* and *Journal of Chromatography B: Biomedical Applications* as *J. Chromatogr. B.*

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We trust that these changes will contribute to easy usage of the journal.

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Epoxy- and diol-modified silica: optimization of surface bonding reaction

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ABSTRACT

The 3-glycidyloxypropyltrimethoxysilane–silica bonding reaction was investigated. The carbon and bonded epoxide content after the bonding reaction and the vicinal diol and total hydroxy group content after the subsequent opening reaction were determined. The highest yield of bonded epoxide groups was obtained in water at pH 8.5 and ambient temperature. The corresponding diol phase behaves well in the size-exclusion chromatography of poly(ethylene glycol)s in water. Neither the silane bonding reaction nor the subsequent epoxide opening reaction give rise to diol structures only; formation of glycol ethers cannot be avoided. The bonding reaction in toluene gives mostly surface-bonded glycol–ether structures.

INTRODUCTION

High-performance size-exclusion chromatography (SEC) has become a well accepted technique for the characterization of water-soluble polymers [1]. The column packings used may be organic or silica-based. Although the wider pH stability [2] favours organic packings, silica-based materials are frequently preferred because of their higher efficiency and more robust nature. Among them diol-bonded silica [3,4] is very popular. Its hydrophilic moiety is believed to be a vicinal diol obtained by simultaneous opening of the oxirane ring in the bonding reaction [3] with 3-glycidyloxypropyltrimethoxysilane (GPTMS) in water at pH 3.5 and 90°C. The oxirane intermediate was shown to be useful for coupling a variety of ligands used as ion exchangers [5] and affinity packings [6]. To prevent the oxirane ring from opening, the silane bonding reaction is usually carried out at pH 5–6 [5,7,8].

In fact, GPTMS is an exception among other silanes used to prepare bonded phases in the sense that the oxirane ring is highly reactive [9]. The formation of the desired diol grouping might be accompanied by some by-products and their formation could be highly pH and temperature dependent; all of them may be expected to be less hydrophilic. Thus, methanol formed by hydrolysis of GPTMS may participate in oxirane opening and form the corresponding glycol ethers, the diol grouping formed may react with another oxirane ring and also polyaddition may take place. Moreover, both bonded and unbonded GPTMS may participate in these reactions. At the same time, the starting silane is hydrolysed and forms the corresponding trisilanol during the initial part of the bonding reaction in aqueous solution; silanol polymerization giving cyclic oligosiloxanes [10] could then compete with the surface bonding reaction and these cyclic siloxane structures may be also bonded to the silica surface.

The complexity of the bonding reaction may be responsible for the differences in ionic and hydrophobic interactions among diol phases of a different origin [11]. Hydrophobic interactions arise from hydrocarbon spacer groups and the number of hydroxy groups should play a decisive

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role [12]. Thus, diol-bonded silica adsorbs [13] oligo(ethylene glycol)s and cannot be used for SEC of these polymers if water is used as a mobile phase.

There is a lack of knowledge [8] concerning a detailed description of the chemical species which exist on diol-modified silica. Therefore, the bonding reaction was studied in detail; bonded oxirane, vicinal diol, total hydroxy and CH contents were determined as a function of temperature, pH and time. The aim was to find conditions giving the maximum surface coverage of oxirane groups on silica (epoxy activation) and, after opening oxirane rings, to obtain a diol phase with a high coverage of hydroxy groups.

EXPERIMENTAL

Materials

The silica used (Separon SGX, mean particle diameter $d_p = 10$ μm, specific surface area 480 m^2/g) was obtained from Laboratory Instrument Works, Prague (present manufacturer Tessek, Prague, Czech Republic). The 3-glycidyloxypropyltrimethoxysilane and poly(ethylene glycol)s ($M_r = 200$, 2000 and 20 000) were purchased from Fluka (Buchs, Switzerland).

Analytical-reagent grade reagents used in functional group analysis and organic solvents were supplied by Lachema (Brno, Czech Republic). Doubly distilled water was used throughout.

Silica modification

Silica was activated by boiling (30 min) in dilute hydrochloric acid (1:10, v/v), washed with water until neutral, boiled in water (30 min), washed and dried at 150°C.

Aqueous solutions (5%) of GPTMS were prepared by adding the silane dropwise to water, keeping the pH of stirred solutions at given value with KOH (10^{-3}–10^{-1} *M*) or HCl (10^{-3} *M*) solutions. A PHM-64 pH meter (Radiometer, Copenhagen, Denmark) was used. Silicas were added in the ratio of 1.0 g of silica to 1.5 g of GPTMS and the reactions were performed with periodic swirling in sealed flasks or under reduced pressure (8–9.5 kPa) at 24–26°C. Reactions at higher temperatures were performed in a drying oven. The bonded-phase supports were filtered and washed with water, dioxane and diethyl ether and dried at laboratory temperature and then at 85°C for 3 h. Bonding reactions in toluene were performed under reflux or at laboratory temperature in sealed flasks; again, 5% solutions of the silane reagent and the ratio of 1 g of silica to 1.5 g of GPTMS were used and toluene replaced water in the washing procedure. Oxirane ring opening was achieved during 3 h at 85°C (drying oven, periodic swirling) in 0.1 *M* H_2SO_4; washing and drying were identical to those above after the bonding reaction in water.

Analytical procedures

There is a broad range of analytical techniques available for the determination of oxirane, vicinal diol and hydroxy functions [14]. In the case of surface-bonded groups, a decrease in reactivity [15] had to be expected and, therefore, the most efficient procedures were selected after testing several possibilities found in the literature [14]. The main criterion was the reproducibility of the end-point determination as a function of the speed of the titration (epoxy groups) and of the duration of the oxidation (acetylation) in the case of back-titrations.

Direct titration of epoxy groups of the sample suspended in acetic acid with standard (0.1 *M*) perchloric acid in acetic acid in the presence of an excess of tetraethylammonium bromide (25% solution in acetic acid) to the crystal violet end-point, described by Jay [16], was evaluated as the best alternative.

The periodate oxidation method [14] was found to be the best for determination of vicinal diol groups: the bonded silica sample after reaction (30 min) with periodic acid (0.1 *M*) solution was partially neutralized with saturated sodium hydrogencarbonate solution, then sodium arsenite solution (0.06 *M*) was added and the excess of arsenite was back-titrated with iodine (0.0125 *M*) to the starch end-point. Blank titration was performed in the same way.

Perchloric acid-catalysed acetylation [17] was used to determine the total content of hydroxy groups: a silica sample was treated (60 min) with acetic anhydride (0.25 *M*) in ethyl acetate containing 0.006 *M* $HClO_4$, then pyridine–water

(9:1) was added, the sample was filtered and washed with methanol and the filtrate was titrated with sodium hydroxide (0.15 *M*) in methanol to the cresol red–thymol blue mixed indicator end-point. Again, the same blank titration was performed. Using unmodified silica no silanol interference was observed.

The precision of the epoxide and vicinal diol determinations was estimated as 0.02 μmol/m^2; for total hydroxy group determination *ca.* 0.06 μmol/m^2 was obtained.

Chromatography

The SEC equipment consisted of a VCR 40 HPLC pump (CSAS Development Works, Prague, Czech Republic), a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 100-μl loop, an R401 differential refractometer (Waters, Milford, MA, USA) and a Servogor 2S recorder (Goerz Electro, VIenna, Austria). Stainless-steel columns (250 × 8 mm I.D., supplied by Tessek) were packed by a slurry technique at 30 MPa with methanol–dioxane (1:1, v/v) as the slurry liquid.

RESULTS AND DISCUSSION

To avoid opening of oxirane ring during the bonding reaction, pH 4.9 was selected and the influence of reaction temperature and time was investigated (Table I). The theoretical content of bonded epoxide ligand in μmol/m^2 was calculated from carbon analysis. From the calculated loss of epoxide due to the bonding reaction, it is immediately seen that heating decreases the content of epoxides considerably. A similar decrease is seen if a longer reaction time at ambient temperature is chosen. Both the carbon and measured epoxide content are low in all cases. Primary aminopropyl (C_3 ligand) phases bonded to the same silica matrix [18] exhibit typical values of 5–6% C and 2.4–2.7 μmol/m^2 NH_2 groups; in the present instance *ca.* 10–12% C should be expected for the C_6 ligand. The conversion parameters, X = *vic.*-OH/found epoxide content and Y = *vic.*-OH/epoxide content calculated from %C, allow a comparison of samples with various carbon contents and indicate a complex behaviour. Assuming no loss of epoxide during the bonding reaction and oxirane ring opening exclusively to diol, we obtain $X = Y = 2$. If the oxirane ring is opened during the bonding reaction only to diol, $X > 2$ and $Y = 2$ should be obtained. The values of $Y < 2$ may be interpreted as a result of side-reactions, formation of ether bonds during bonding and/or a ring-opening reaction. Then, if epoxide loss is low (or none) in the bonding reaction and ether formation during sulphuric acid treatment proceeds, $X < 2$ may be found. Experimental X

TABLE I

REACTION OF GPTMS WITH SILICA IN WATER AT pH 4.9

X = *vic.*-OH (μmol/m^2): epoxide (μmol/m^2) content from titration; Y = *vic.*-OH (μmol/m^2): epoxide (μmol/m^2) ligand calculated from carbon content; reaction described under Experimental.

Sample No.	Temperature (°C)	Duration (h)	Elemental analysis		Titration		Epoxide loss (%)	X	Y
			Carbon (%)	Ligand (μmol/m^2)	Epoxide (μmol/m^2)	*vic.*-OH (μmol/m^2)			
1	90	0.5	3.45	1.00	0.12	0.98	86.4	7.17	0.98
2	45	0.5	2.45	0.71	0.19	0.64	72.3	3.26	0.91
3	26[a]	2.0	1.30	0.38	0.27	0.44	46.7	1.62	1.17
4	26[a]	7.5	2.40	0.69	0.50	0.85	28.5	1.74	1.24
5	26[a]	54	5.98	1.73	0.79	2.00	54.6	2.55	1.16
6	Ambient	168	7.06	2.04	0.50	2.58	75.3	5.12	1.26

[a] Under vacuum.

and Y values (Table I) confirm a considerable formation of ether bonds in both reaction steps; Y values indicate that the ether formation increases with increasing temperature. A disappointing result was obtained: neither higher temperature nor time can be used to increase the content of unopened epoxide.

Therefore, the next step was to look in detail into the pH dependence of the bonding reaction of GPTMS at ambient temperature. In this respect, some controversy is found in the literature. Regnier and Noel [3], using pH 3.5, pointed out that basic bonding conditions and extended reaction times increased the amount of bonded phase, but no decrease in protein adsorption was observed. Another report [19] shows that reaction at pH 6 gives a material with inferior chromatographic characteristics and recommends the use of lower pH. Stout and DeStefano [20] used pH 8.8 and 90°C and obtained packings exhibiting good performance. According to Plueddemann [10], using epoxy functional silanes in aqueous solutions, the pH of the solution must be maintained above 4 to prevent hydrolysis of the oxirane ring to a glyceryl ether. It is worth mentioning that the mechanism of oxirane ring opening is different [9] in acidic and base media. Alkyloxiranes form corresponding ether and primary hydroxy groups capable of further addition of another oxirane ring under acid catalysis; basic catalysis gives mainly secondary hydroxy groups of low reactivity towards another epoxide group. Hence the basic catalysis might decrease the formation of higher adducts. When methanol adds to the oxirane ring, the same effect should operate in the first step owing to the formation of a secondary hydroxy group. Hydrolysis of trialkoxysilanes to silanetriols is rapid [10]; a threefold molar excess of methanol to epoxide may be expected to be formed in the initial period of the bonding reaction.

The results of the GPTMS bonding in water at ambient temperature and different pH are summarized in Table II. It is seen that the lowest epoxide loss is achieved at pH 8.5. The decrease in Y at low pH indicates more side-products in agreement with the previous scheme. The small increase in X in this case of high epoxide loss also confirms considerable ether formation during the opening reaction, in agreement with Plueddemann [10].

Consequently, the reaction at pH 8.5 giving the lowest loss of epoxide and highest conversion parameter Y was studied further. The effects of reduced pressure (expected to decrease the methanol content in the reaction mixture) and reaction time and reproducibility of bonding were investigated. To obtain a deeper insight into the bonding reaction, the determination of

TABLE II

pH DEPENDENCE OF GPTMS–SILICA BONDING REACTION IN WATER

X and Y as in Table I. All experiments under vacuum; 24–26°C

pH	Duration (h)	Elemental analysis		Titration		Epoxide loss (%)	X	Y
		Carbon (%)	Ligand (μmol/m^2)	Epoxide (μmol/m^2)	*vic.*-OH (μmol/m^2)			
3.1	2	2.18	0.52	0.27	0.48	58.3	1.81	0.75
4.9	2	1.30	0.38	0.27	0.44	30.0	1.64	1.15
5.5	2	1.39	0.40	0.23	0.40	45.6	1.79	0.97
7.0	2	2.79	0.81	1.00	1.48	16.0	1.49	1.25
7.0	5	4.09	1.19	1.58	2.25	18.8	1.42	1.15
8.5	2	6.78	1.96	1.71	–	13.4	–	–
8.5	1	7.19	2.08	1.90	–	9.0	–	–
8.5	1	7.11	2.06	1.79	2.90	12.5	1.60	1.40
9.0	2	7.59	2.19	1.81	–	17.0	–	–

total hydroxy group content was added and a conversion parameter Z = total OH (μmol/m^2)/epoxide (μmol/m^2) calculated from carbon content was introduced. Table III summarizes the results. It is immediately seen that the reproducibility at equal reaction times is not very good, although the same batch of silica was used. Therefore, great care is needed with respect to conclusions. The complexity of both bonding and opening reactions obviously determines these differences and this effect adds to the well known divergence of properties [21,22] of apparently identical bonded phases based on different silica matrices. The entirely different behaviour of various diol silicas described in the literature [11] is then easily understood. Comparing average values of X, Y and Z in experiments under vacuum and at ordinary pressure a small decrease in these parameters is observed and may be interpreted as a result of enhanced methyl ether formation. No relative increase in the epoxide loss with time is the first satisfactory result; the other is the increase in the total amount of hydroxy groups per ligand with time as expressed by parameter Z. X values again indicate that the opening reaction is accompanied by significant glycol ether formation. Sample 12 therefore represents the best epoxy-modified reactive silica with surface coverage of 2.4 μmol/m^2 of epoxide groups. After the epoxide opening, 4.8 μmol/m^2 of hydroxy groups (75% of them being diol groups) are obtained. In terms of theoretical epoxide content (calculated from carbon content), 57% undergo bonding and opening to form diol groupings and the remainder give only some isolated hydroxy groups. Fig. 1 shows that using this optimized diol packing a correct SEC behaviour of poly(ethylene glycol)s is achieved in water as mobile phase.

To evaluate the effect of cyclic oligosiloxane formation in the bonding reaction, the reactions were performed with GPTMS prehydrolysed for given periods of time before silica was added (Table IV). Indeed, cyclization takes place and the cyclic products are able to be attached to the silica surface, as follows from the increased carbon content with prehydrolysis for 3 h. The epoxide loss compares well with Table III and the same holds for X. A small decrease in Y and

TABLE III

OPTIMIZATION OF GPTMS–SILICA REACTION IN WATER AT pH 8.5

X and Y as in Table I; Z = total OH (μmol/m^2): epoxide (μmol/m^2) ligand calculated from carbon content; reaction described under Experimental; 24–26°C; samples 1–6 under vacuum.

Sample No.	Duration (h)	Elemental analysis		Titration			Epoxide loss (%)	X	Y	Z
		Carbon (%)	Ligand (μmol/m^2)	Epoxide (μmol/m^2)	*vic.*-OH (μmol/m^2)	Total OH (μmol/m^2)				
1	1	7.11	2.06	1.79	2.90	3.15	12.5	1.60	1.40	1.52
2	1	7.16	2.06	1.58	2.65	2.75	23.1	1.66	1.28	1.33
3	1	6.79	1.96	1.56	2.12	2.42	20.3	1.36	1.09	1.23
4	1	7.43	2.15	1.75	2.62	2.94	18.1	1.50	1.22	1.37
5	1	6.56	1.90	1.58	2.17	2.52	16.0	1.36	1.14	1.33
Average 1–5	–	7.01	2.02	1.65	2.50	2.75	18.0	1.50	1.23	1.36
6	0.33	5.87	1.69	1.50	2.10	2.29	11.8	1.41	1.25	1.36
7	1	6.55	1.90	1.58	2.17	2.38	16.0	1.36	1.14	1.25
8	1	7.48	2.17	1.54	2.10	2.38	28.2	1.35	0.97	1.10
9	1	6.11	1.77	1.50	1.87	2.42	14.6	1.23	1.06	1.36
Average 7–9	–	6.71	1.94	1.54	2.04	2.40	19.6	1.31	1.06	1.24
10	20	9.30	2.69	2.17	3.12	4.08	19.8	1.44	1.16	1.52
11	96	9.95	2.87	2.21	2.92	4.15	23.5	1.32	1.02	1.44
12	192	10.81	3.12	2.42	3.58	4.81	22.9	1.48	1.15	1.54

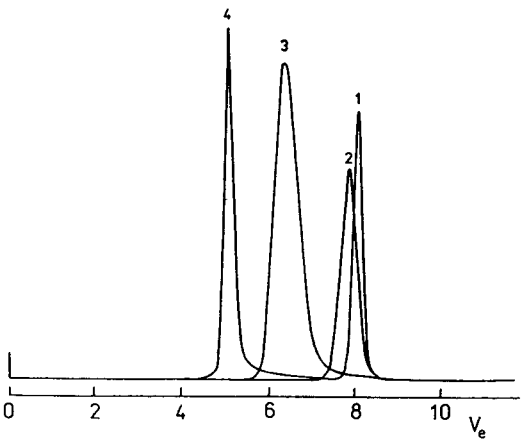

Fig. 1. SEC behaviour of poly(ethylene glycol)s on optimized diol packing with water as mobile phase. Injection of 100 μl of (1) 0.05% ethylene glycol, (2) 0.05% PEG 200, (3) 0.1% PEG 2000, (4) 0.05% PEG 20 000. Packing No. 12 (Table III); flow-rate, 1.92 ml/min; refractive index detection.

Z might indicate slightly higher formation of glycol ethers in solution during prehydrolysis for 3 h.

Bonding reactions of GPTMS in organic solvents are seldom found in the literature [23,24] and, as a rule, the reaction conditions are not well described. To obtain a full picture, the bonding reaction was performed in toluene (common solvent for anhydrous silane bonding [25]) at 110 and 26°C (Table V). An unexpected result was obtained: most of the epoxide groups disappeared even at ambient temperature and very low conversion to hydroxy groups was obtained in comparison with the optimum conditions in water. The total hydroxy conversion (Z) is approximately constant in all instances and only a slightly increased yield of vicinal hydroxyls (Y) is found at 26°C. Alumina acidified with 5% acetic acid is known to be an efficient catalyst in oxirane opening reactions [9]; the acidic silica surface may exhibit the same effect especially under these conditions. The oxirane opening may be initiated by released methanol or hydration water present on the silica surface, and extensive polyaddition may follow that can be terminated by another methanol or hydration water molecule. Diol-bonded silica prepared in CCl_4 at 60°C has been examined by C and Si NMR spectroscopy [23] and no methoxy groups were observed; this might indicate the participation of hydration water. Such a comparison requires caution; the bonding reaction mechanism may depend strongly on the selection of the organic solvent. For instance, we observed no surface bonding if dioxane was used as a reaction solvent. Anyway, the low content of hydroxy groups obtained especially at 110°C indicates rather an "ether"-bonded phase structure described many years ago [26].

CONCLUSIONS

Bonding of 3-glycidyloxypropyltrimethoxysilane to silica in water should be performed at pH 8.5 and ambient temperature to obtain a high yield of epoxide activation. The corresponding diol phase obtained after the oxirane opening

TABLE IV

REACTION OF SILICA WITH PREHYDROLYSED GPTMS IN WATER AT pH 8.5

X, Y, and Z as in Table III; duration 1 h; 24–26°C.

Prehydrolysis time (h)	Elemental analysis		Titration			Epoxide loss (%)	X	Y	Z
	Carbon (%)	Ligand (μmol/m²)	Epoxide (μmol/m²)	*vic.*-OH (μmol/m²)	Total OH (μmol/m²)				
0.25	6.94	2.00	1.60	1.98	2.60	19.7	1.23	0.99	1.30
0.50	5.71	1.65	1.40	1.79	2.67	16.0	1.28	1.09	1.62
0.50	6.30	1.81	1.54	1.92	2.58	15.3	1.24	1.06	1.42
3.00	9.29	2.69	2.06	2.44	3.44	23.5	1.18	0.91	1.28

TABLE V

GPTMS–SILICA BONDING REACTION IN TOLUENE

X, *Y* and *Z* as in Table III.

Sample	Elemental analysis		Titration			Epoxide loss (%)	*X*	*Y*	*Z*
	Carbon (%)	Ligand (μmol/m^2)	Epoxide (μmol/m^2)	*vic.*-OH (μmol/m^2)	Total OH (μmol/m^2)				
1[a]	16.3	4.71	*ca.* 0	0.29	1.96	*ca.* 100	–	0.06	0.41
2[b]	12.7	3.67	0.06	0.38	1.67	98	6.0	0.10	0.45
3[c]	5.4	1.56	0.17	0.46	0.65	89	2.8	0.29	0.41
4[d]	5.9	1.69	1.50	2.10	2.29	12	1.41	1.25	1.36

[a] 110°C, 15 h.
[b] 110°C, 4 h.
[c] 26°C, 1 h.
[d] Comparable sample in water at pH 8.5.

reaction exhibits the correct SEC behaviour of poly(ethylene glycol)s in water. Neither the silane bonding reaction nor the subsequent oxirane opening gives rise to diol structures only; considerable formation of glycol ethers varies with changing reaction conditions. The bonding reaction in toluent performs much worse; an "ether"- rather than a "glycol"-bonded phase is obtained.

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CHROM. 25 329

Chromatographic determination of enantiomeric purity by achiral means

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ABSTRACT

A chromatographic method for determination of the enantiopurity of a chiral analyte using achiral reagents is described. The analyte is first converted to diastereomers utilizing either dimerization reactions of the analyte or a bifunctional (achiral) reagent. The diastereomers thus obtained constitute a *meso* compound and a *dl* pair, *e.g.* two epimeric pairs. The epimeric pairs are separated by HPLC on a C_{18} (achiral) column. A mathematical equation was derived for determination of enantiomeric purity utilising the peak areas of the eluted epimers. This method takes into account differences in both reaction rates of the formation of the epimers and differences in their detector responses. The minimum amount of enantiomeric impurities (in the analytes studied) that could be detected by this method was approximately 0.002%.

INTRODUCTION

Determination of the enantiomeric composition of an analyte is routinely made by either direct or indirect methods. Direct separation of enantiomers based on a chiral stationary phase (CSP) is, of course, the method of choice. CSPs, however, frequently show low efficiencies and/or loading capacities, which restrict their ability to determine very small amounts of optical impurities in the analytes. The enantiomeric purity of the analyte can be determined even when the chiral selector is partially racemized [1]. Chiral additives [2], not necessarily enantiomerically pure, in the mobile phase in combination with an achiral column are also commonly used in evaluation of the enantiomeric composition of analytes.

An indirect method is based on the preparation of diastereomers by reacting the solute with a chiral reagent followed by separation of the epimers on an achiral column. The limitation of this approach is the enantiomeric purity of the chiral reagent. Determination of enantiomeric contaminants less than, say, 0.1% requires a reagent with an enantiomeric purity of at least 99.9%.

Vingeron *et al.* [3] were the first to recognize the possibility of utilizing the intrinsic difference in chirality between a pure enantiomer and an enantiomeric mixture for determination of optical purity by means of bifunctional achiral reagents. By reacting enantiomers with a bifunctional symmetrical achiral reagent it was possible to convert the mixture of enantiomers into a *meso* compound and a *dl* pair. Vingeron *et al.* [3]

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showed that it is possible to determine the optical purity of the initial enantiomer by analysing the diastereomeric mixture [1] by either NMR or gas chromatography. A similar method utilizes phosphorus trichloride (PCl_3) as derivatizing agent and the integrals of ^{31}P NMR signals for determination of enantiomeric purity [4]. A somewhat modified version of this ^{31}P NMR [5] method allows determination of enantiomeric excess within 2%, the limitation being the accuracy of the integration due to the signal-to-noise ratio.

The potential of the method of Vingeron *et al.* [3] has further been demonstrated using ligand exchange chromatography [6] or gas chromatography [7] to separate the *meso* compound and the *dl* pair. We report a method based on the original approach of Vingeron *et al.* [3] in which the diastereomers are prepared by randomly linking the enantiomers of an analyte either directly by a disulphide bridge or by means of an achiral bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene (DFDNB). The diastereomers formed are separated by HPLC on a C_{18} phase using an achiral mobile phase.

This report also includes the derivation of a mathematical equation for the calculation of the enantiomeric purity as the peak areas of the eluted epimers are not directly proportional to the concentrations of the initial enantiomers of the analyte. In the derivation of the mathematical expression differences in reaction due to chiral discrimination in the formation of the diastereomers as well as differences in their detector response factors are considered.

The accuracy of the method was tested by determination of the enantiomeric composition of two different kinds of chiral analytes, one

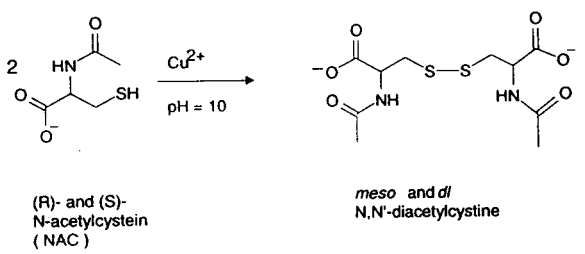

Fig. 1. Synthesis of *meso* and *dl* N,N'-diacetylcysteine.

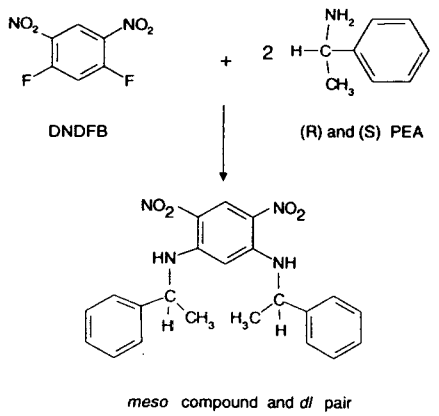

Fig. 2. Coupling of (*R*)- and (*S*)-PEA to bifunctional reagent DNDFB.

containing mixtures of (*R*)- and (*S*)-N-acetylcysteine (NAC) and the other containing mixtures of (*R*)- and (*S*)-1-phenylethylamine (PEA). The enantiomers of both samples were converted to diastereomers, a *meso* compound and a *dl* pair, by achiral means (see Figs. 1 and 2). The epimers were in a subsequent step separated by HPLC using a C_{18} phase (see Figs. 3 and 4). To allow comparisons the enantiomeric composition of PEA was also determined using a chiral reagent (Marfey's reagent [8]). The main

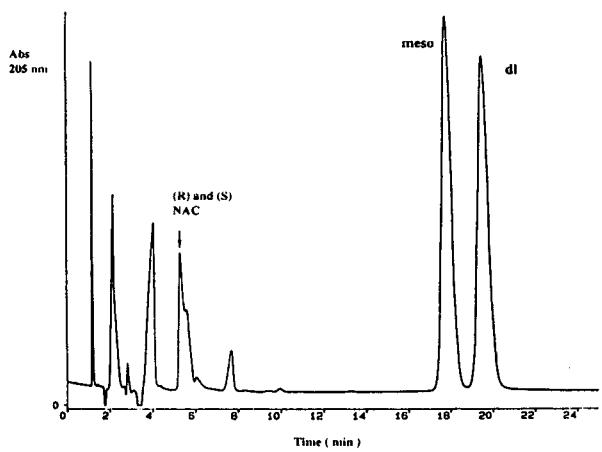

Fig. 3. Separation of *meso* and *dl* N,N'-diacetylcystine epimers by HPLC. Column: Kromasil DR 100-5 C_{18}, 150 × 4.6 mm I.D. Mobile phase: (20 m*M* tetrabutylammonium sulphate + 10 m*M* NaH_2PO_4, pH 7.0)–acetonitrile (95:5, v/v). Detector: UV 205 nm. Flow-rate: 1.0 ml/min. Injection volume: 20 μl.

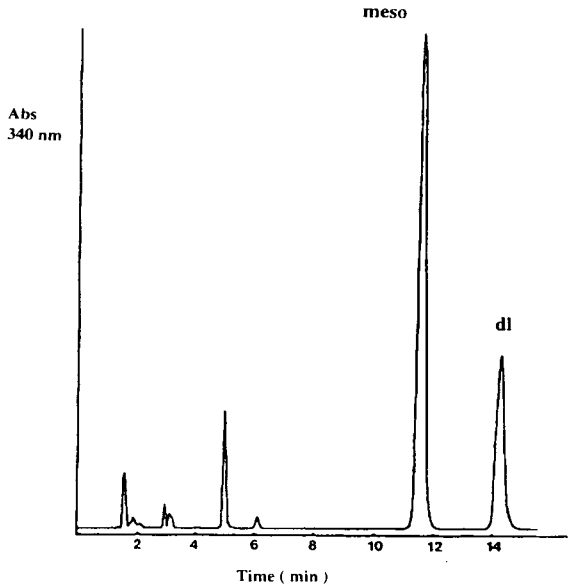

Fig. 4. HPLC separation of *meso* and *dl* PEA. Column: Supelcosil LC-C_{18}, 5 μm, 150 × 4.6 mm I.D. Mobile phase: 10 m*M* NaH_2PO_4, pH 7.0–acetonitrile (30:70, v/v). Detector: UV 340 nm. Flow-rate: 1.0 ml/min. Injection volume: 20 μl.

purpose of this work was to get access to highly purified PEA. The enantiopurity of the material was determined after each recrystallization according to the method presented in this paper.

EXPERIMENTAL

Apparatus and chromatography

A Hewlett-Packard 1050 (Hewlett-Packard, Waldbronn, Germany) was used as solvent-delivery system and a Kratos Spectroflow 783 (ABI Analytical, Ramsey, NJ, USA) as detector. The analytes were introduced onto the column by a Carnegie Medecin CMA/200 autoinjector (Carnegie Medecin, Stockholm, Sweden). The chromatographic conditions are described in the figure legends.

The peak areas were calculated using a chromatographic data system (Nelson Analytical, Cupertino, CA, USA).

Chemicals and reagents

DFDNB was obtained from Jansen (Beerse, Belgium). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) was supplied by Pierce (Rockford, IL, USA). (*R*)- and (*S*)-PEA were purchased from Merck (Darmstadt, Germany) and NAC was prepared (*i.e.* acetylation of cysteine) at Draco (Lund, Sweden).

Purification of (R) or (S)-1-phenylethylamine

To remove possible enantiomeric impurities in the commercial (*R*)- and (*S*)-(enantiomers) PEA, these were recrystallized as diastereomeric salts according to a previously published method [9]. A 4.0-g aliquot of (*R*) or (*S*)-PEA and 5.0 g of (−)- or (+)-tartaric acid were dissolved in 15 ml of boiling water and the solution was allowed to crystallize overnight. The isolation of the enantiomer after each recrystallization was carried out as previously described. All other chemicals were of analytic or chromatographic grade and used as obtained.

Preparation of diastereomeric derivatives

N,N'-Diacetylcystine. NAC is converted to N,N'-diacetylcystine according to a standard procedure (see Fig. 1). To approximately 1.0 g of NAC dissolved in a 0.2 *M* sodium carbonate buffer (pH 10) 50 μl of 8.0 m*M* copper sulphate solution were added. The reaction mixture was allowed to stand for 3–10 h, after which time 20 μl of the crude mixture were injected onto the column.

Preparation of diastereomers of PEA with DFDNB. PEA was allowed to react with DFDNB according to Fig. 2. A 200-μl sample of a 10.0 m*M* solution of PEA (as free base or salt) in water, 400 μl of a 2.5 m*M* solution of DFDNB in acetone and 80 μl of 1.0 *M* sodium carbonate buffer (pH 9.4) were mixed and kept at 40°C. After 1 h the mixture was acidified with 80 μl of 2 *M* HCl. A 20-μl aliquot of this mixture was injected onto the column.

Occasionally a small amount of precipitated material was observed, which could change the diastereomeric ratio of the solution. This could be avoided by exchanging the sodium carbonate buffer for 10 μl of triethylamine.

Preparation of diastereomers of PEA with Marfey's reagent. Marfey's reagent, 2.5 m*M* acetone, was used according to the same procedure as DFDNB.

DERIVATION OF EQUATIONS

Formation of diastereomers from a mixture of enantiomers could be accomplished either by a direct dimerization or by means of a bifunctional reagent (A). In the following derivations the bifunctional reagent is included but the resulting equations are unaffected whether or not a reagent is used.

Reacting a bifunctional symmetrical achiral reagent, A, with enantiomers of an analyte, (*R*) and (*S*), gives the *dl* pair (*S*)-A-(*S*) and (*R*)-A-(*R*) and the *meso* compound (*S*)-A-(*R*) or (*R*)-A-(*S*) (see Fig. 5). The (*S*)-A-(*R*) and (*R*)-A-(*S*) compounds are identical due to the symmetrical reagent, A.

The *meso* and *dl* compounds are epimers and can be separated by achiral means. In the first stage of the reaction the enantiomers, (*R*) and (*S*) In Fig. 5, with molar ratios x and $1-x$, respectively, yield the intermediates (*R*)-A and (*S*)-A with molar ratios x and $1-x$, respectively. In the next stage of the reaction the intermediates (*R*)-A and (*S*)-A can react with either (*R*) or (*S*) to form (*R*)-A-(*R*) or (*R*)-A-(*S*) and (*S*)-A-(*R*) or (*S*)-A-(*S*), respectively. However, it is likely that the epimers (*R*)-A-(*R*) and (*R*)-A-(*S*) are formed at different reaction rates and consequently the amount of (*R*)-A-(*R*) and (*R*)-A-(*S*) that is formed will be proportional to xxk_2 and $(1-x)xk_1$, respectively, where k_2 and k_1 are constants that take into account different reaction rates and/or different detector response factors.

It can be assumed that the enantiomers (*R*)-A-(*R*) and (*S*)-A-(*S*) are formed at the same reaction rate and have the same UV response.

The area of the peak containing the *meso* compound (Q_1) and the area of the peak containing the *dl* pair (Q_2) are related to the original amounts of (*R*)- and (*S*)-enantiomers in the analyte in the following manner.

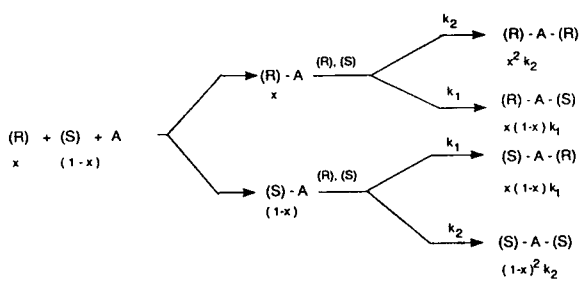

Fig. 5. Symbols used in the derivation of the equations.

$$\frac{Q_1}{Q_1+Q_2} = q_1 = \frac{2k_1x(1-x)}{2k_1x(1-x)+k_2x^2+k_2(1-x)^2}$$

If $K = \frac{k_2}{k_1}$

$$q_1 = \frac{2x(1-x)}{2x(1-x)+Kx^2+K(1-x)^2} \quad (1)$$

Eqn. 1 gives x as a function of K and q_1:

$$x = 0.5 \pm \sqrt{0.25 - K/2(1/q_1 + K - 1)} \quad (2)$$

Each value of q_1 gives two possible solutions of x, which means that there are always two possible compositions (x and $1-x$) of the original enantiomeric mixture (analyte) that will give the same peak area ratio (q_1). Consequently, this method cannot be used to determine which of the enantiomers, (*R*) or (*S*), is predominant in a specific sample, but only their relative amounts.

To solve the equations, K has to be determined from a sample with known composition. The simplest way to do this is with a racemate where $x = 0.5$. Eqn. 1 then takes the simple form:

$$q_1 = \frac{1}{1+K} \text{ or}$$

$$K = \frac{1}{q_1} - 1 = \frac{Q_2}{Q_1} \quad (3)$$

If $K = 1$ then eqn. 2 is simplified to:

$$x = 0.5 \pm \sqrt{0.25 - 0.5q_1} \quad (4)$$

For very small values of x ($1-x = 1$, $x^2 = 0$ and $1-q_1 = 1$) eqn. 1 reduces to:

$$q_1 = \frac{x}{x+0.5K} \text{ or } x = 0.5q_1K$$

RESULTS AND DISCUSSION

In order to check the reliability of the equations above, a series of different enantiomeric mixtures of purified PEA with known compositions (x) were prepared. The mixtures were

analysed and q_1 was determined for each mixture. With an iterative regression program a value of K was calculated that made the best fit to eqn. 2 of all x and corresponding q_1 values. The enantiomeric purity of an analyte is then easily obtained from eqn. 2 using the calculated K value and q_1, which is obtained from the evaluation of the chromatogram. The results are shown in Table I. Each value of q_1 is the mean of at least two runs. The best fit to eqn. 2 was obtained with $K = 0.51927$, which shows that the reaction rate for formation of the *meso* compound is almost twice the reaction rate for the formation of the *dl* pair.

Marfey's reagent is well recognized for its usefulness as a chiral derivatizing reagent, especially for amino acids. It is structurally similar to DFDNB; one of the fluorine atoms is replaced by L-alanine amide. In another set of experiments the enantiomeric purity of PEA was determined with both Marfey's reagent and DFDNB after each recrystallization (see *Chemicals and reagents* section) in the purification of PEA. Each determination is a mean value of at least two chromatographic runs and the results are shown in Table II.

As the content of (*R*) in (*S*) decreases, the determinations performed with Marfey's reach a level of approximately $x = 0.0025$, in contrast to the determinations performed with DFDNB. This discrepancy is probably due to a contamination of Marfey's reagent with approximately 0.25% of the opposite enantiomer.

TABLE I

DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF PEA IN MIXTURES OF KNOWN COMPOSITION

Molar ratio of (*R*) (added x)	Peak area ratio (q_1)	Molar ratio of (*R*) (found x)[a]
0.00016	0.00067	0.00017
0.00067	0.00272	0.00071
0.00304	0.0115	0.00301
0.0123	0.0461	0.0124
0.0355	0.1222	0.0349
0.1222	0.3455	0.1226

[a] Regression analysis: Minimum (the best fit) was found after 23 iterations. The *residual sum of squares* at minimum was $4.111 \cdot 10^{-6}$.

TABLE II

DETERMINATION OF ENANTIOMERIC PURITY USING MARFEY'S REAGENT OR DFDNB

Batch of PEA (number of recrystallizations)	Marfey's reagent [molar ratio of (*R*)]	DFDNB [molar ratio of (*R*)]
0[a]	0.00208	0.0156
1	0.0070	0.0040
2	0.0027	0.00019
3	0.0033	0.00052
4	0.0030	0.00042
5	0.0025	0.000034

[a] Starting material.

The surprisingly pure product obtained after the second recrystallization could be due to a non-representative sampling.

Enantiomeric mixtures of NAC of known compositions were also prepared and analysed, and the results are shown in Table III. In this case the reaction rates for the formation of the *meso* and the racemic forms are very similar ($K \approx 1$). A good correlation is obtained between added and found values.

CONCLUSIONS

The success of the determination is highly dependent on a simple reaction between a

TABLE III

DETERMINATION OF ENANTIOMERIC COMPOSITION OF NAC IN MIXTURES OF KNOWN COMPOSITION

Molar ratio of (*R*) added (x)	Peak-area ratio (q_1)	Molar ratio of (*R*) found (x)
0	0.0003	0.00015
0.0053	0.0099	0.0050
0.0154	0.0285	0.0145
0.0538	0.1022	0.0542
0.1584	0.2591	0.1534
0.3090	0.4209	0.3022
0.5000	0.4991	0.5000

bifunctional reagent and substrate or a dimerization of the substrate.

The detection limit is mostly dependent on the linear dynamic range of the detector and the loading capacity of the column. Detection of enantiomeric impurity of PEA and DFDNB was possible down to approximately 0.002%. DFDNB has a strong UV absorption maximum at 340 nm, which is often distinguishable from the absorption of most organic compounds. This facilitates the identification of the reaction products. However, the method does not permit the identification of each enantiomer; only a determination of their relative concentrations is possible.

Detection of enantiomeric impurity of NAC by dimerization was possible down to at least 0.02%. In this case the native UV absorbance of N,N'-diacetylcystine at 205 nm was utilized for detection.

The great variety of commercially available bifunctional reagents should make it possible to find suitable ones that fulfil the requirements for derivatization and the subsequent chromatographic separation of a number of chiral compounds.

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CHROM. 25 380

High-performance liquid chromatography of fatty acid derivatives in the combined silver ion and reversed-phase modes

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ABSTRACT

In order to learn more of the mechanism of silver ion complexation in chromatographic systems, the retention characteristics of some unsaturated fatty acid esters (phenacyl and phenethyl) were determined using reversed-phase high-performance liquid chromatography with silver ions in the mobile phase. It was possible to calculate equivalent chain length and fractional chain length values, which appeared to indicate that double bonds and silver ions formed simple 1:1 complexes in the mobile phase. The overall lipophilicity of the complex had an effect in determining the resolution of positional isomers. The equilibrium constant for the formation of phenacyl oleate (or "argentation constant") was calculated to be in the range 0.059–0.067, depending on the experimental conditions. The mechanism is compared with that for systems in which silver ions are bound to the stationary phase.

INTRODUCTION

In a previous paper [1], we described the retention characteristics of derivatives of unsaturated fatty acids in silver ion high-performance liquid chromatography (Ag-HPLC). A column was used with silver ions linked via ionic bonds to phenylsulphonic acid moieties, which were in turn bound to a silica matrix [2]. As there were appreciable differences in retention of the phenacyl derivatives of isomeric monoenoic fatty acids, base-line resolution of the natural 6-, 9- and 11-18:1 isomers was possible at ambient temperature. The results enabled some suggestions to be made about the probable complexation mechanism.

In an effort to understand better the nature and strength of the interaction between a double bond in a lipid and silver ions, we have turned to another method of introducing silver ion into an HPLC system, *i.e.* reversed-phase HPLC (RP-HPLC) with a mobile phase containing a silver salt. This modification of silver ion HPLC had been applied to the separation of simple fatty acid derivatives by others [3–6], but has been of rather limited use because of numerous technical problems, as discussed elsewhere [7,8]. However, we considered that this form of silver ion chromatography might be a suitable tool for estimation of the interaction between a double bond in a fatty acid derivative and a silver ion,

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distinct from hydrophobic and other effects. By this means, it is possible to compare the retention characteristics of a solute in the presence and absence of silver ions in the mobile phase, while keeping all other chromatographic conditions constant.

The chromatographic behaviour of derivatives of some positional isomers of monoenoic fatty acids and common polyunsaturated fatty acids were investigated in this study in an attempt to reveal the contribution of the position and number of double bonds in the complexation reaction. The equilibrium constant of the reaction between silver ions and phenacyl oleate was determined quantitatively by using an approach suggested by Horváth *et al.* [9].

EXPERIMENTAL

Materials and reagents

Free fatty acids with 0 to 6 double bonds, including the three naturally occurring *cis*-octadecenoic positional isomers (oleic, petroselinic and *cis*-vaccenic acids) were purchased from Sigma (Poole, UK). The rest of the isomeric *cis*-octadecenoic acids had been prepared earlier by total synthesis [10]. All solvents were analytical or HPLC grade and were supplied by FSA Scientific Apparatus (Loughborough, UK). Silver nitrate (AnalaR), silver perchlorate (Sigma), ammonium nitrate (AnalaR) and potassium nitrate (FSA) were used to modify the mobile phase.

High-performance liquid chromatography

A Spectra-Physics (St. Albans, UK) Model 8770 solvent-delivery system was used, together with a Pye Unicam Model 4025 UV detector and an octadecyl (Spherisorb S5ODS2, Hichrom, Reading, UK) or an octyl (Spherisorb S5C8, Hichrom) column (250 mm × 4.6 mm I.D.). Methanol–water mixtures were used as mobile phase at a flow-rate of 1 ml/min. The proportion of water was varied depending on the column used and the derivative studied. Silver, ammonium and potassium salts (0.0025 to 0.08 *M*) were added to the mobile phase as aqueous solutions in such proportions that their final concentration in the mobile phase was in the range 0.0025 to 0.08 *M*.

The phenacyl and phenethyl derivatives were detected at 257 nm, as this wavelength was less sensitive toward the presence of silver salts in the mobile phase [3].

The dead volume was measured using acetone, which is not retained in the column and absorbs strongly below 330 nm. The appropriate myristic acid (14:0) derivative was used as an internal standard in each run. Experiments were performed at constant temperature, 20°C, by fitting the column into a water jacket, through which ethyleneglycol was pumped from a Chrompack Model RTE-110B temperature control unit (Chrompack UK, London, UK).

Derivatization

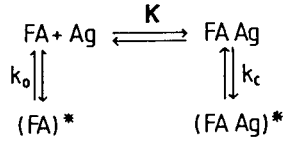
Fatty acids were converted into phenacyl [11] and phenethyl derivatives. To produce the phenethyl derivatives, the free acids (10 mg) were first converted to the corresponding acid chlorides by reaction with oxalyl chloride (0.5 ml) for 36 h at room temperature; the excess reagent was evaporated in a stream of nitrogen and finally by a rotary evaporator. The residue was dissolved in toluene (0.5 ml), and phenethyl alcohol (10 mg) in toluene (1 ml) and pyridine (0.2 ml) were added. The mixture was heated overnight at 50°C, then the excess solvent and pyridine were removed in a rotary evaporator. Hexane (5 ml) was added to the residue and the solution was washed with water (2 × 5 ml). Finally, the product was cleaned by elution through a Florisil column (0.5 g) with hexane–acetone (5 ml; 99:1, v/v). The solvents were evaporated under nitrogen and the required phenethyl derivatives were re-dissolved in methanol for analysis (0.02 to 0.2 mg/ml). Phenacyl and phenethyl derivatives were injected onto the column as methanolic solutions (10 μl).

RESULTS AND DISCUSSION

Retention characteristics of fatty acid derivatives

The interactions that take place in reversed-phase HPLC with a mobile phase containing

silver ions may be presented as:



where FA denotes a fatty acid molecule, FAAg is the complex with a silver ion, and * denotes a molecule retained by the support. If the concentration of the complexing ion in the mobile phase is sufficiently high, the solute (*e.g.* a fatty acid derivative) should be in the form of a complex, and the capacity factor (k'_c) would represent the retention of the complex in the RP-HPLC system. When the mobile phase contains no silver ions, the retention factor (k'_o) would represent the retention of the uncomplexed fatty acid derivative. The difference between the two values corresponds to the effect of complex formation [9].

Phenacyl esters of unsaturated fatty acid were studied, because of the interesting results reported earlier [1] for their retention characteristics on a silver loaded column and because of the convenience of using UV detection. Phenethyl esters were prepared as they are similar to phenacyl esters in molecular weight, shape and properties, but lack a second carbonyl group thought to participate in complexation [1].

Preliminary experiments showed that the most suitable mobile phase was a mixture of methanol and water in proportions that depended on the nature of the fatty acid derivative and the chain-length of the bonded stationary phase. The composition of the mobile phase was considered to be optimum when the best resolution of 6-18:1 and 9-18:1 derivatives, used as test substances, was achieved. Mobile phases based on acetonitrile were not suitable because of their strong interaction with silver ions.

The retention characteristics were measured by the capacity factor k', or, as specified below, by the ratio of the capacity factor of the analyte to that of the same myristic acid (14:0) derivative, and denoted here as k''. k' values were measured with a standard deviation of ± 0.01.

Phenacyl derivatives of fatty acids, including *cis*- and *trans*-isomers, have been resolved with high selectivity by RP-HPLC on octadecyl columns [3,11,12]. A column with an octyl bonded phase was less selective because of the lower lipophilic interaction, and separations of positional isomers of fatty acids could not be achieved with the common mobile phases. On the other hand, the effects of adding silver ions in the mobile phase were expected to be more distinctive.

The temperature was maintained at 20°C. Although preliminary experiments with lower temperatures (10°C and 0°C) improved the resolution, inconvenient increases in the retention time and peak broadening also occurred. When the composition of the mobile phase was adjusted to give comparable retention times and peak shapes, no effect of temperature on resolution was apparent. This confirmed our earlier conclusion [1] that the whole chromatographic system responds to the change in the temperature.

The silver salt concentration was varied between the relatively narrow limits of 0.0025 *M* to 0.08 *M*. Measurements above this range were not possible because of the background UV absorption of the silver ions and the salt anions.

When silver ions were added to the mobile phase, the capacity factors (i.e. k'_{Ag} and k''_{Ag}) decreased gradually with the increasing silver salt concentration. In Fig. 1, the k''_{Ag}/k''_o ratio is

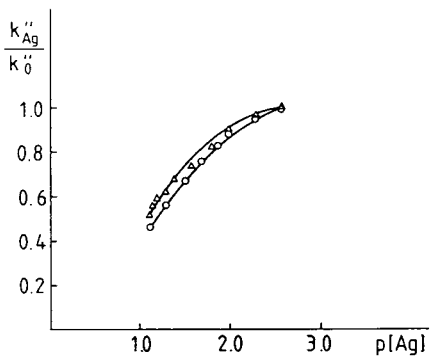


Fig. 1. The dependence of the k''_{Ag}/k''_o of phenacyl oleate on the negative logarithm of the silver ion concentration (molar), p[Ag^+], in the mobile phase. △ = Spherisorb S5C8 column and a mobile phase of methanol–water (87:13, v/v); ○ = Spherisorb S5ODS2 column with mobile phase 92.5:7.5 (v/v).

plotted against the silver ion concentration with the phenacyl ester of 9-18:1 as test compound for both columns. It has been suggested [9] that this ratio is a measure of the complexation effect. Evidently, since the complex is retained less strongly than the non-complexed derivative (*i.e.* $k'_{Ag} < k'_o$), the ratio is less than 1 and its value decreases with an increasing degree of complexation. The effect of complexation was slightly stronger for the octadecyl bonded phase and this was probably connected with the stronger response of the octadecyl moieties toward the changed physical characteristics of the complexed fatty acid derivative.

Although other monoene isomers were studied, the retention behaviour of the three naturally-occurring isomers, 6-, 9- and 11-18:1, was most important from a practical point of view. Under all condition, the 6-18:1 isomer was retained more than was 9-18:1. However, 6-18:1 was also found to be retained more in the silver-loaded column, presumably as a result of the formation of a stronger silver ion complex [1], so it was expected that it would be retained less in a reversed-phase system with silver ions in the mobile phase. In fact, the phenacyl derivatives of the monoenoic fatty acid isomers followed the elution order reported by Wood [12] for a RP-HPLC system (Fig. 2) with two important exceptions. The 4- and 5-18:1 isomers were retained more than their neighbours, and this was ascribed to an interaction between the carbonyl oxygen and the double bond that lowered the overall polarity of the molecule. In the presence of silver ions (0.05 *M*) in the mobile phase, the capacity factors of these isomers fell on their "correct" places on the graph. Fig. 2 reveals that the lipophilic interaction governs the elution order. Thus, 6-18:1 and 9-18:1 were separated completely with an R_s value of 2.5 (with 0.075 *M* silver nitrate in the mobile phase). No conditions were found under which 9- and 11-18:1 were separated. Comparative experiments with the same mobile phase and no silver nitrate showed that 6-18:1 and 9-18:1 were readily resolved (with an R_s value close to 1.0) on an octadecyl but not on an octyl column. Thus, silver ions in the mobile phase enable or improve the resolution but do not affect the elution order.

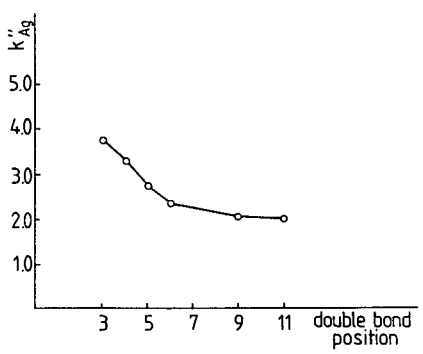


Fig. 2. The relative capacity factors, k'', of some isomeric C_{18} monoenoic phenacyl derivatives with the position of the double bond.

Silver perchlorate was found to be superior to nitrate, with lower concentrations needed to separate 6- and 9-18:1 isomers completely on the octadecyl column; an R_s value of 3.5 was obtained with 0.05 *M* silver perchlorate *versus* 2.1 with 0.05 *M* silver nitrate. The type of salt had no significant effect with the octyl column.

Capacity factors decreased with an increasing number of double bonds in the fatty acid. To demonstrate the degree of retention, the capacity factors were determined relative to those of the corresponding oleate derivatives with the results listed in Table I. Evidently, the nature of the ester moiety had little influence on the relative degree of retention of the polyunsaturated fatty acids, as the two sets of results are almost the same. The C_{18} derivatives with two to four double bonds eluted from 2 to 5 times faster, respectively, than did the monoene. Because of the differences in chain lengths, eicosapentaenoate and docosahexaenoate eluted as quickly as the corresponding 18:4 derivatives.

A more impressive presentation of the effect of the complexation on retention is the plot of the $k''_{Ag} - k''_o$ values (the fraction of the capacity factor that corresponds directly with the effect of complexation) *versus* the concentration of silver ion in the mobile phase. Fig. 3 illustrates this effect for the same fatty acids as in Table I. With a greater degree of unsaturation, higher silver ion concentrations increased the effect of the complexation. The greater the number of double bonds, the smaller was the effect of the silver

TABLE I

RATIO OF THE RELATIVE CAPACITY FACTORS (k''_{Ag}) OF PHENETHYL AND PHENACYL ESTERS OF SOME POLYUNSATURATED FATTY ACIDS TO THAT OF THE OLEATE DERIVATIVE

The Spherisorb S5C8 column was used with a mobile phase of methanol–water (88:12, v/v) for the phenethyl esters and (81:19, v/v) for the phenacyl esters with 0.08 *M* silver perchlorate.

Fatty acid	$k''_{pufa}/k''_{18:1}$	
	Phenethyl ester	Phenacyl ester
9-18:1	1.0	1.0
9,12-18:2	0.6	0.5
9,12,15-18:3	0.3	0.3
6,9,12,15-18:4	0.2	0.2
5,8,11,14-20:4	0.4	0.3
5,8,11,14,17-20:5	0.2	0.2
4,7,10,13,16,19-22:6	0.2	0.2

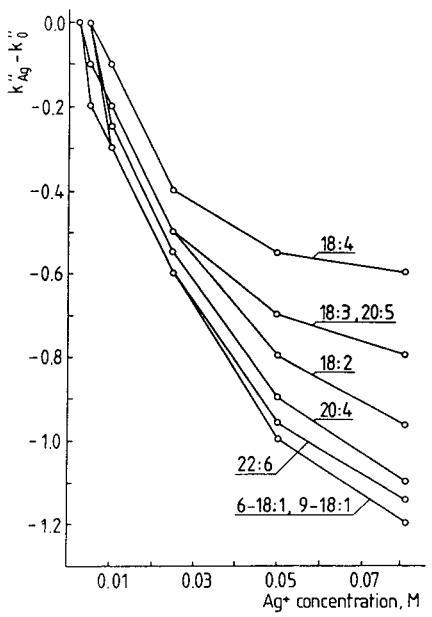


Fig. 3. The $k''_{Ag} - k''_o$ values for phenacyl derivatives of some polyunsaturated fatty acids on a Spherisorb S5C8 column and mobile phase methanol–water (87:13, v/v); k''_o was measured with 0.08 *M* ammonium nitrate in the mobile phase; k''_{Ag} was measured with 0.08 *M* silver nitrate in the mobile phase.

ions on the capacity factor. Finally, the retention of the two monoenoic isomers was affected equally by the presence of silver ions in the mobile phase, suggesting that retention order is not determined by a difference in the strength of the complexes but by the general lipophilicity of a molecule. This property should be greater for 6-18:1 as it possesses a longer lipophilic "tail". It is evident that 18:3 and 20:5 fatty acids have the same $k''_{Ag} - k''_o$ values, while those of 22:6, 20:4 and 18:2 are also similar, over the range of silver ion concentrations.

The contribution of the double bonds and the nature of the ester moiety in the retention characteristics of the fatty acids with different degrees of unsaturation and chain length are better understood by calculating the equivalent chain length (ECL) values [13,14]. This was possible because of the linear relationship between the logarithms of the capacity factors (k') and the chain length of the saturated fatty acid derivatives (both phenethyl and phenacyl esters), as has been shown by others [15,16] (Fig. 4). Fractional chain length (FCL) values, *i.e.* the increment in ECL value of a given fatty acid derivative below that of the saturated derivative of the same chain length, were also calculated. As in GLC [17], the FCL value is influenced by the number and position of the double bonds in the aliphatic chain. Because of their higher lipophilicity, phenethyl esters were retained more than were phenacyl esters.

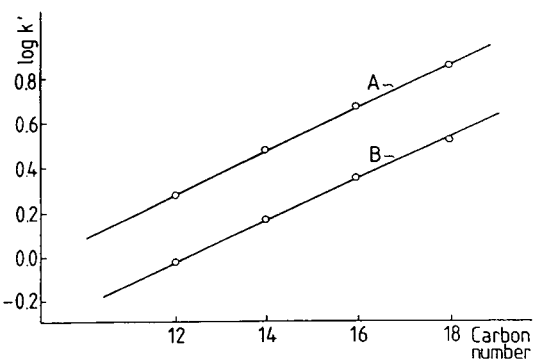


Fig. 4. The capacity factors (k') of saturated fatty acids as phenethyl (A) and phenacyl (B) derivatives in relation to chain length as measured on a Spherisorb S5C8 column with a mobile phase of methanol–water (88:12, v/v).

TABLE II

THE EQUIVALENT CHAIN LENGTH (ECL) AND FRACTIONAL CHAIN LENGTH (FCL) VALUES OF SOME POLYUNSATURATED FATTY ACIDS AS PHENETHYL (PHE) AND PHENACYL (PHA) DERIVATIVES ON A RP-HPLC COLUMN

The Spherisorb S5C8 column was used with a mobile phase of methanol–water (88:12, v/v); the water contained 0.08 *M* ammonium nitrate.

Fatty acid	ECL		FCL	
	PhE	PhA	PhE	PhA
6-18:1	16.87	17.00	−1.13	−1.0
9-18:1	16.71	16.94	−1.26	−1.06
9,12-18:2	15.63	15.72	−2.37	−2.28
9,12,15-18:3	14.22	14.61	−3.78	−3.39
6,9,12,15-18:4	13.60	13.50	−4.68	−4.50
5,8,11,14-20:4	15.21	15.28	−4.79	−4.72
5,8,11,14,17-20:5	14.16	13.94	−5.84	−6.06
4,7,10,13,16,19-22:6	15.00	14.39	−7.00	−7.61

When silver ions were absent from the mobile phase, irrespective of the nature of the ester moiety, the corresponding ECL values were similar, as were the relevant FCL values listed in Table II. For the C_{18} fatty acids, monoene:diene:triene:tetraene, the ratio of the FCL values was close to 1:2:3:4, indicating that the double bonds have an equal contribution to the overall polarity of the fatty acid–silver ion complex. The effect of the ester group on ECL values is small in this system.

The picture was different when silver ions were added to the mobile phase, and the ECL and FCL values are listed in Table III. ECL values of phenethyl derivatives were lower, implying a stronger effect of the silver ions. Also, the ratio of the FCL values of the phenacyl derivatives of the monoenoic to tetraenoic C_{18} fatty acids were the same as in RP-HPLC, *i.e.* 1:2:3:4, but that of the phenethyl esters was 1:1.3:1.9:2.4. More information was obtained by comparing the ΔFCL values, where

$$\Delta FCL = FCL_o - FCL_{Ag}$$

and represents the effect of complexation alone on the chromatographic behaviour of the fatty acid derivative. The ΔFCL values of the C_{18} phenethyl derivatives are higher than those of phenacyl esters derivatives by a value of about the same magnitude. Evidently, a further factor contributed to the complexation and must be connected with the phenethyl moiety. As the ratio of the FCL values for the C_{18} monoene:diene:triene:tetraene was not far from

TABLE III

THE EQUIVALENT CHAIN LENGTH (ECL) AND FRACTIONAL CHAIN LENGTH (FCL) VALUES FOR SOME POLYUNSATURATED FATTY ACIDS AS PHENETHYL (PHE) AND PHENACYL (PHA) DERIVATIVES ON AN RP-HPLC COLUMN IN THE PRESENCE OF SILVER IONS

The Spherisorb S5C8 column was used with a mobile phase of methanol–water (88:12, v/v); the water contained 0.08 *M* silver nitrate.

Fatty acid	ECL		FCL		ΔFCL	
	PhE	PhA	PhE	PhA	PhE	PhA
6-18:1	13.73	15.50	−4.27	−2.50	−3.14	−1.50
9-18:1	13.10	15.06	−4.90	−2.94	−3.64	−1.88
9,12-18:2	10.79	12.39	−7.21	−5.61	−4.84	−3.33
9,12,15-18:3	7.29	9.28	−10.71	−8.72	−6.93	−5.33
6,9,12,15-18:4	4.50	6.28	−13.50	−11.72	−8.82	−7.22
5,8,11,14-20:4	8.58	10.28	−11.42	−9.72	−6.63	−5.00
5,8,11,14,17-20:5	5.63	7.50	−14.37	−12.50	−8.53	−6.44
4,7,10,13,16-22:6	5.63	7.50	−16.37	−14.50	−9.37	−6.89

2:3:4:5, the phenethyl moiety appeared to be roughly equivalent to one double bond in its effect. A confirmation of this assumption is given by the results in Table IV, where the ECL and FCL values of the phenethyl and phenacyl derivatives were calculated using the relative capacity factor, k'' (note that this leads to small differences from analogous values in Tables II and III). As the contribution of the ester moiety was eliminated, ECL, FCL and ΔFCL values of both types of derivative were very close.

Complexation of oleic acid with silver ions

The data presented in Fig. 1 can be utilised to evaluate the equilibrium constant (K) of the silver ion complex of phenacyl oleate by the method described by Horváth *et al.* [9]. The approach has several limitations, the most important being that a correct evaluation can only be made when a 1:1 complex is formed. In the reaction between an olefinic double bond and silver ions, the information is contradictory. It has been reported that silver ions can form a *bona fide* complex with two ethylene molecules [18] or with a diene system [19], but other data show that 1:1 complexes can also be formed [20]. In this system, the simple relationship between the number of double bonds and ECL values suggests the latter. Our calculations assume that phenacyl oleate and a silver ion form a 1:1 complex in the mobile phase that interacts with the hydrocarbon moieties of the bonded phase, and that k'' can be substituted for k' values.

Another requirement was to perform the measurements over as broad a range of silver ion concentrations in the mobile phase as possible, but as discussed above the upper limit was only 0.08 M unfortunately. With these provisos, the results allowed a quantitative evaluation of the equilibrium constant. The equation used was [9]:

$$k''_{Ag} = (k''_o + K[Ag^+])/(1 + K[Ag^+])$$

which can be linearized [9] for graphical evaluation of the constant K in the form

$$Y = A + BX$$

where

$$Y = k''_{Ag} - k_o; X = (k''_{Ag} - k''_o)/[Ag^+] \text{ and}$$

$$K = -1/B$$

The experimental conditions and the results are listed in Table V. The K values are very close despite the different columns, silver salts and phenacyl oleate concentrations. To our knowledge there is only one study, which deals with measurements of the so-called "argentation constant" [21] for a fatty acid derivative, when a value of 8.4 was found for methyl oleate. In this

TABLE IV

THE EQUIVALENT CHAIN LENGTH (ECL) AND FRACTIONAL CHAIN LENGTH (FCL) VALUES OF SOME POLYUNSATURATED FATTY ACIDS AS PHENETHYL (PHE) AND PHENACYL (PHA) DERIVATIVES ON SPHERISORB S5C8 COLUMN CALCULATED BY USING THE RELATIVE CAPACITY FACTORS k'' ($k'_{FA}/k'_{14:0}$)

The subscript "o" denotes measurement with ammonium nitrate and "Ag" with silver nitrate in the mobile phase.

Fatty acid	ECL_o		FCL_o		ΔECL_{Ag}		FCL_{Ag}		ΔFCL	
	PhE	PhA	PhE	PhA	PhE	PhA	PhE	PhA	PhE	PhA
6-18:1	16.58	16.66	−1.42	−1.34	13.79	13.80	−4.21	−4.20	2.79	2.86
9-18:1	16.47	16.56	−1.53	−1.44	13.15	13.38	−4.85	−4.62	3.22	3.18
9,12-18:2	15.29	15.22	−2.71	−2.78	10.80	10.22	−7.20	−7.78	4.49	5.00
9,12,15-18:3	14.00	14.00	−4.00	−4.00	7.40	7.11	−10.60	−10.89	6.60	6.09
6,9,12,15-18:4	12.83	12.89	−5.17	−5.11	4.50	4.11	−13.50	−13.89	8.33	8.78
5,8,11,14-20:4	14.86	14.89	−5.14	−5.11	8.77	8.22	−11.23	−11.78	6.09	6.67
5,8,11,14,17-20:5	13.69	13.78	−6.31	−6.22	5.78	5.78	−14.22	−14.22	7.91	9.00
4,7,10,13,16,19-22:6	14.65	13.78	−7.35	−8.22	5.67	4.89	−16.33	−17.11	8.98	8.89

TABLE V

EQUILIBRIUM CONSTANT OF THE PHENACYL OLEATE COMPLEX WITH SILVER IONS AS MEASURED BY RP-HPLC UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Column	Mobile phase[a]	Silver salt	Sample concentration, M	Equilibrium constant	Regression coefficient
Spherisorb S5ODS2	92.5:7.5	$AgNO_3$	$6.7.10^{-4}$	0.067 ± 0.002	0.988
Spherisorb S5C8	87.0:13.0	$AgNO_3$	$6.7.10^{-5}$	0.059 ± 0.002	0.971
Spherisorb S5C8	81.0:19.0	$AgClO_4$	$6.7.10^{-5}$	0.062 ± 0.002	0.953

[a] Methanol–water.

instance, the distribution of methyl oleate was measured between isooctane and aqueous methanol containing silver nitrate by means of iodine values rather than by modern chromatographic methods. This very high value does not agree with other data on the equilibrium constants of monounsaturated hydrocarbons and other derivatives [20], which did not exceed 1.0. We believe that our value is more accurate than that reported earlier [21].

CONCLUSIONS

In silver ion absorption chromatography (TLC or HPLC), silver ions are held directly by the support [8], and complexation takes place on the surface. In silver ion chromatography in a reversed-phase mode, complexation occurs in the mobile phase and the complex interacts with the bonded phase. Complexation with silver ions is important in both instances, but it seems that the selectivity for the separation of positionally-isomeric monoenes, for example, depends more on the other factors. In adsorption silver ion chromatography, steric factors and the molecular conformation are more important; the ester moiety and one double bond, or two double bonds, in a single molecule appeared to interact simultaneously with one silver ion in a system in which silver ions were attached via ionic bonds to the stationary phase [1]. In RP-HPLC in the reversed-phase mode, it appeared that 1:1 complexes with double bonds and silver ions only were formed. If the ester moiety interacted with silver ions, it did so independently of complexation with double bonds.

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Triacylglycerol analysis of partially hydrogenated fats using high-performance liquid chromatography

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ABSTRACT

This study describes a method that has been developed for the separation and quantification of triacylglycerols in partially hydrogenated fats rich in oleic, elaidic, palmitic and stearic acids. Determination of triacylglycerols is according to carbon number, number of double bonds and geometrical isomers (*i.e. cis* and *trans* configuration). The method described is a two-stage procedure, utilizing RP-HPLC for preliminary fractionation into partition numbers, and following this with subsequent separation into geometrical isomers using RP-HPLC/silver ions. The method has been applied to a number of different model triacylglycerols, and also to partially hydrogenated soya-bean oil and palm oil. The relative standard deviations are 2% for triacylglycerol species on the 15% absolute level, and *ca.* 5% on a 3% absolute level.

INTRODUCTION

The major constituents of natural vegetable fats are triacylglycerols (TGs). Since a TG molecule is built up of three fatty acids, combinations of just a few kinds of fatty acid can give rise to several different TGs. The complexity of TG analysis is therefore obvious.

In a partially hydrogenated fat, the number of fatty acid isomers is dramatically increased by the appearance of geometrical isomers (*i.e. cis* and *trans* configurations) and various positional isomers (*i.e.* different positions of double bonds within the fatty acid chain). Thus the increased number of fatty acid isomers will result in a drastic increase in the number of possible TGs. For example, OOO (three *cis* double bonds) may convert to geometrical isomers OOEl (two *cis*, one *trans*), OElEl (one *cis*, two *trans*) and ElElEl (three *trans* double bonds). Except in the section dealing with positional isomers, in this study all C18:1 *cis* fatty acids have been referred to as oleic acid (O), regardless of the position of the double bond. Similarly, all C18:1 *trans* fatty acids have been referred to as elaidic acid (El), and all C18:2 fatty acids have been referred to as linoleic acid (L).

The importance of being able to determine the geometrical isomers of TGs in partially hydrogenated fats can be ascribed to their effect on the physical behaviour of the fats, such as their polymorphic behaviour and melting properties.

Silver ion chromatography has, since several decades, been utilised for separation of geometrical isomers of lipids, such as fatty acids and TGs [1–7]. This type of separation is possible due to the formation of π-complexes between double bonds and silver ions. *trans*-Olefins form weaker π-complexes with silver ions than do *cis*-olefins; hence *cis–trans* isomers can be separated using silver ion chromatography. Initially silver ions were used in conjunction with TLC, with silver nitrate being incorporated in the silica gel layer. With this approach De Vries and Jurriens [1] have demonstrated resolution of

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just a few geometrical isomers of TGs that include oleic acid (*cis*) and elaidic acid (*trans*). In recent years the technique has been adapted to HPLC. Christie [4] has demonstrated such a separation of some geometrical isomers of TGs into the groups SSEl, SSM, SElM and SMM (S = saturated fatty acid, M = monounsaturated fatty acid) using an ion-exchange column impregnated with silver ions. Hammond and Irwin [5] have demonstrated a similar separation of StElSt and StOSt (St = stearic acid) on a silica column impregnated with silver ions. It is also worth mentioning in conjunction with silver ion chromatography that many workers have shown the separation of symmetric and asymmetric isomers of mixed saturated and unsaturated fatty acids, *e.g.* SOS and SSO [8].

This study deals with separation of the major geometrical isomers of TGs in partially hydrogenated vegetable fats. It includes separation of geometrical isomers, such as all combinations of oleic acid and elaidic acid. The proposed method, which is based on HPLC and includes silver ion chromatography, has been applied to partially hydrogenated soya bean oil and palm oil. To the best of our knowledge, no results of either separation of complete homologous series of geometrical isomers or detailed separation of the complex fats mentioned above have previously been published.

EXPERIMENTAL

Chemicals

Acetone, methanol and 2-propanol, all of analytical-reagent quality and propionitrile of synthesis quality, distilled over Siccapent, were used for the mobile phases. In addition, silver nitrate of analytical-reagent quality was used as a component. For dissolution of PN fractions (PN = partition number), benzene of analytical-reagent quality was used. All these chemicals were produced by Merck (Darmstadt, Germany).

Samples

All model TGs (purity ≥99%) were purchased from Larodan (Malmö, Sweden). The TGs are listed in Table I. The partially hydrogenated fats were hydrogenated and refined by the company Karlshamns (Karlshamn, Sweden). Both soya-bean oil and palm oil were hydrogenated to a melting point of 41°C and referred to as Soya 41 and Palm 41 respectively. For fatty acid composition, see Table II.

Preparative HPLC separation into partition numbers (PNs)

An LDC pump was used for the PN separation, together with an ERC 7512 refractive index detector (ERMA, Tokyo, Japan). The mobile phase was methanol–acetone (3:2; v/v) at a flow-rate of *ca.* 1 ml/min. The separation was performed on a 5-μm particle size Hibar RP C_{18} column (250 mm × 4 mm I.D.) (Merck) at ambient temperature. About 320 mg fat was dissolved in 1.5 ml acetone in tightly sealed vials. The vials were then tempered at *ca.* 40°C, and 20 μl of the solution were injected into the HPLC system. The PN fractions yielded were collected and evaporated to dryness at 60°C under a stream of nitrogen. Finally, the fractions were dissolved in benzene to give a concentration of *ca.* 20 mg/ml.

Ag–HPLC separation into geometrical isomers

The chromatographic system used comprised an Optilab 5931 liquid chromatograph (Tecator, Höganäs, Sweden), with the refractive index detector equipped with a 10-mm measuring cell. The separation was performed on a 5-μm particle size Hibar RP C_{18} LiChrosorb column (250 mm × 4 mm I.D.) from Merck. The mobile phase was methanol–2-propanol (3:1; v/v), with $AgNO_3$ in a concentration of 0.085 *M*, at a flow-rate of *ca.* 1 ml/min. The $AgNO_3$ was dissolved in methanol by stirring, before the 2-propanol was added, to reach dissolution at room temperature. The temperature of the total chromatographic system was kept at 20°C. The major PN fractions, at a concentration of 20 mg/ml benzene, were injected in volumes of 5–10 μl. A computer-based system, CHROMATIC (KEBO Computer Applications, Stockholm, Sweden) was used for integration purposes.

TABLE I

CHARACTERISTICS OF TRIACYLGLYCEROL STANDARDS

Structure refers to the sum of the carbon atoms in the fatty acid moieties in relation to the sum of the number of double bonds. The abbreviations c and t refer to the double bonds in *cis* and *trans* configuration, respectively, in the linoleic acid moieties. In the interests of simplification, all $C_{18:2}$ fatty acid moieties have been designated as linoleic acid (L), regardless of *cis* or *trans* configuration.

Triglyceride	Abbreviation	Structure	PN
Dioleolinolein (c,c)	OOLc,c	$C_{54:4}$	46
Dioleolinolein (t,t)	OOLt,t	$C_{54:4}$	46
Oleoelaidolinolein (c,c)	OElLc,c	$C_{54:4}$	46
Dielaidolinolein (c,c)	ElElLc,c	$C_{54:4}$	46
Palmitooleolinolein (c,c)	POLc,c	$C_{52:3}$	46
Palmitooleolinolein (t,t)	POLt,t	$C_{52:3}$	46
Palmitoelaidolinolein (c,c)	PElLc,c	$C_{52:3}$	46
Dipalmitolinolein (c,c)	PPLc,c	$C_{50:2}$	46
Dipalmitolinolein (t,t)	PPLt,t	$C_{50:2}$	46
Triolein	OOO	$C_{54:3}$	48
Dioleoelaidin	OOEl	$C_{54:3}$	48
Oleodielaidin	OElEl	$C_{54:3}$	48
Trielaidin	ElElEl	$C_{54:3}$	48
Palmitodiolein	POO	$C_{52:2}$	48
Dipalmitoolein	PPO	$C_{50:1}$	48
Dipalmitoelaidin	PPEl	$C_{50:1}$	48
Tripalmitin	PPP	$C_{48:0}$	48
Stearodiolein	StOO	$C_{54:2}$	50
Stearooleoelaidin	StOEl	$C_{54:2}$	50
Stearodielaidin	StElEl	$C_{54:2}$	50
Palmitostearoolein	PStO	$C_{52:1}$	50
Distearoolein	StStO	$C_{54:1}$	52
Distearoelaidin	StStEl	$C_{54:1}$	52

RESULTS AND DISCUSSION

Preparative fractionation into triacylglycerol groups

The aim of the preparative fractionation was to divide the TGs into well-defined groups, each containing a moderate number of TGs. Three procedures were considered for this purpose: separation according to PN, separation according to number of double bonds (NDB) and separation according to carbon number (CN). However, since CN analysis is performed by GC this procedure is not suitable for fractionation. Separation according to NDB can be performed by separation of brominated TGs. However, the bromination reaction produces irreversibly chemically altered compounds that become identical for both *cis* and *trans* configurations, and this procedure was therefore also excluded. The third procedure, separation according to PN [9] accordingly proved to be the best solution. It follows the simple relation that $PN = CN - 2 \cdot NDB$. By way of example, PN separation of Soya 41 is illustrated in Fig. 1A. Since the PN48 fraction from Soya 41 includes the geometrical isomers of at least nine different TGs, the PN peak is broadened. Among others, it includes the four geometrical isomers OOO, OOEl, OElEl and ElElEl, which are partly separated as pure standards (Fig. 1D). For purposes of comparison, Fig. 1C shows the resolution of OOO and ElElEl only. The triunsaturated OOO and the saturated PPP (Fig. 1B) are nearly separated at baseline but, since Soya 41 includes several

TABLE II

FATTY ACID COMPOSITION OF PALM 41 AND SOYA 41

Structure refers to number of carbon atoms in relation to the number of double bonds. The abbreviations c and t refer to the double bonds in *cis* and *trans* configuration, respectively. Fatty acid composition has been determined by GC on a polar fused-silica column.

Fatty acid	Abbreviation	Structure	Palm 41 (%)	Soya 41 (%)
Lauric acid	La	$C_{12:0}$	0.3	0.1
Myristic acid	M	$C_{14:0}$	1.2	0.1
Palmitic acid	P	$C_{16:0}$	42.3	10.7
Palmitoleic acid	Po	$C_{16:1}$	0.3	0.1
Margaric acid	Mg	$C_{17:0}$	0.0	0.1
Stearic acid	St	$C_{18:0}$	7.0	18.9
Oleic acid + elaidic acid	O + El	$C_{18:1c} + C_{18:1t}$	45.1	65.4
Linoleic acid	L	$C_{18:2}$	3.4	3.4
Arachidonic acid	A	$C_{20:0}$	0.4	0.4
Gadoleic acid	G	$C_{20:1}$	0.2	0.3
Behenic acid	B	$C_{22:0}$	0.0	0.4
Unknowns	–	–	0.0	0.3
Total	–	–	100	100

TGs with retention times between those of OOO and PPP, the PN48 peak looks like the peak of a single component. This is also due to the relative proportions of the different TGs within the peak. If the relative proportions are altered, the peak may become asymmetrically shaped, as for the PN50 peak of Palm 41 (Fig. 8). As the retention times of the different TGs within a PN differ, it is important to collect the whole peak at fractionation. One total fractionation, with an elution time of *ca.* 1.5 h, will provide enough material for the subsequent separation procedure.

Subsequent separation into geometrical triacylglycerol isomers

Optimization of analytical system. The subsequent separation procedure was based on argentation HPLC, in order to separate geometrical isomers.

When work on developing the method was first started, some initial experiments were carried out with silver ions bonded to the stationary phase [10]. However, this procedure also separated the positional isomers to a high degree, and the resulting chromatograms were too complex. The procedure made both identification and quantification difficult, due to the broadening of the peaks. Another procedure making use of silver ions in the mobile phase was therefore tested. This gave good separation between geometrical isomers, but separation of positional isomers was less pronounced. Also the separation of symmetric and asymmetric TG isomers (*e.g.* POP and PPO) were studied using silver ions in the mobile phase. No resolution occurred which means that our intended separation into geometrical isomers was not disturbed. Another advantage of utilizing silver ions in the mobile phase was that a RP C_{18} column could be successfully used for the separation.

Several points had to be taken into consideration in choosing the mobile phase. First, the mobile phase had to dissolve the silver nitrate properly while at the same time being sufficiently non-polar for the elution of saturated TGs. Secondly, the mobile phase had to be inert to the silver ions, so that no reaction would take place. Thirdly, the refractive index of the mobile phase had to be different from that of the TGs, since TGs were to be detected using a refractive index detector. For these reasons mobile phases such as propionitrile, acetonitrile, methanol and 2-propanol were tested. The mobile phase that

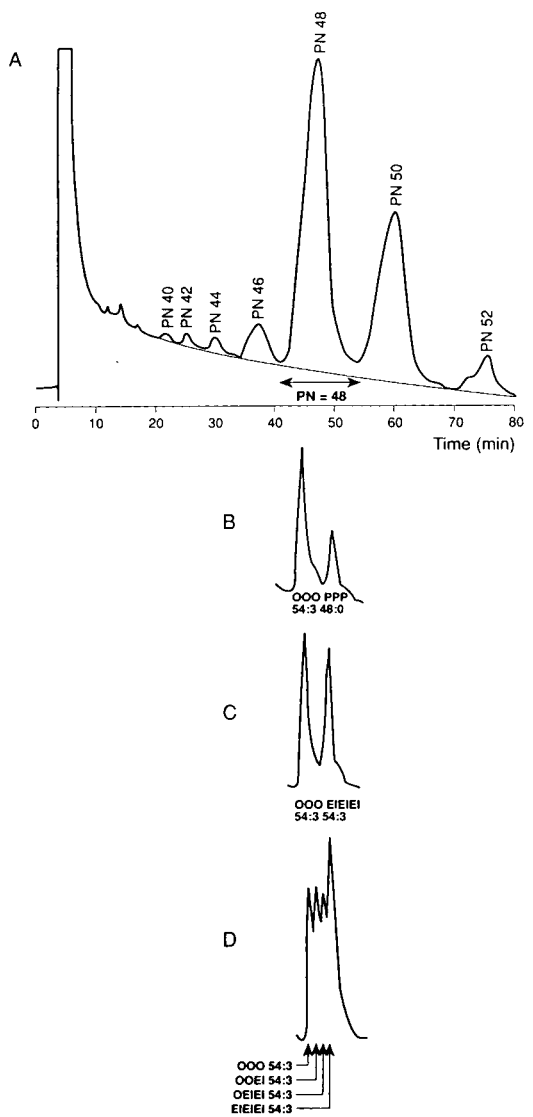

Fig. 1. PN separation of Soya 41 and illustration of the separation of different types of TG isomers included in the PN48 peak. Conditions: column Hibar RP C_{18} (250 mm × 4 mm I.D.), mobile phase methanol–acetone (3:2; v/v). Ambient temperature, flow-rate 1 ml/min. For abbreviations see Table I; for A, B, C and D see text.

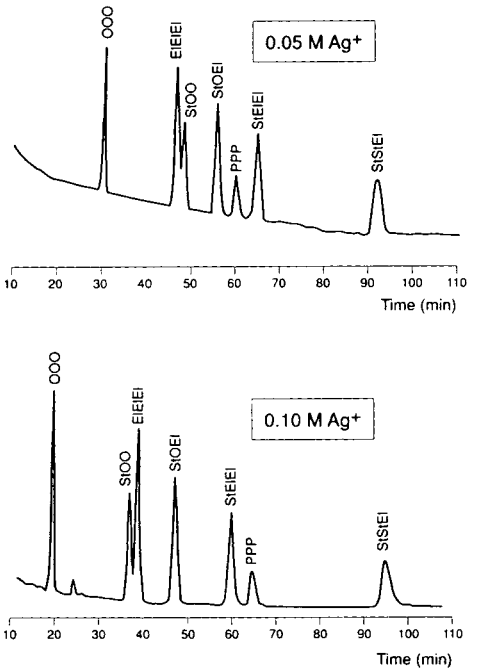

Fig. 2. Influence of silver nitrate concentration on retention times of different TGs, including geometrical isomers. Conditions: Column Hibar RP C_{18} LiChrosorb (250 mm × 4 mm I.D.), mobile phase methanol–2-propanol (3:1; v/v), system temperature 20°C, flow-rate 1 ml/min. For abbreviations see Tables I and II.

gave the best separation results was methanol–2-propanol (3:1; v/v) with dissolved silver nitrate.

The silver ions affect in two ways. They act as complexing agents with double bonds (π-complexes), and they also increase the polarity of the mobile phase. From Fig. 2 it can be seen that a saturated TG (PPP) has a longer retention time when the silver concentration is increased. The silver ions cannot form π-complexes with a saturated compound and this longer retention time must be attributable to the increased polarity of the mobile phase. Unsaturated TGs are of course also affected by the polarity of the mobile phase. The formation of π-complexes is also illustrated in Fig. 2, from which it can be seen that PPP-StElEl and ElElEl-StOO are eluted in inverted order when the silver concentration is increased. When the elution order of PPP and StElEl is inverted in this way, the *trans* double bonds must have formed a complex with silver ions as otherwise, with only a change in the polarity of the mobile phase taking place, no inversion would have resulted. The inversion in the order in which ElElEl and StOO are eluted demonstrates that there is a difference in the

strength of silver complexes with *cis versus trans* double bonds. StOO with only *two cis* double bonds is so strongly affected by the increase in the silver concentration that it elutes before ElElEl, in which there are *three trans* double bonds. Since, due to the increased polarity of the mobile phase, the stearic acid in StOO contributes to an increase in retention time, it is clear that the silver complexes with *cis* double bonds are stronger than those with *trans* double bonds. This is probably due to a sterical hindrance from the *trans* double bonds.

Silver concentrations of 0.01–0.10 *M* $AgNO_3$ were tested on mixtures with standards and on partially hydrogenated fats mainly based on monounsaturated and saturated C_{16} and C_{18} fatty acids. Higher concentrations than 0.10 *M* are not practicable because of the limited solubility of $AgNO_3$. The effect of increased silver concentration on the PN48 of a partially hydrogenated soya-bean oil is shown in Fig. 3. Here a low concentration of 0.02 *M* $AgNO_3$ is compared with a high concentration of 0.10 *M*. As can be seen, the high silver concentration gives much better separation of geometrical isomers. A silver concentration of 0.085 *M* was finally chosen, based on total overall evaluation of separation of all major PN fractions and also bearing in mind the risk of precipitation occurring with higher concentrations.

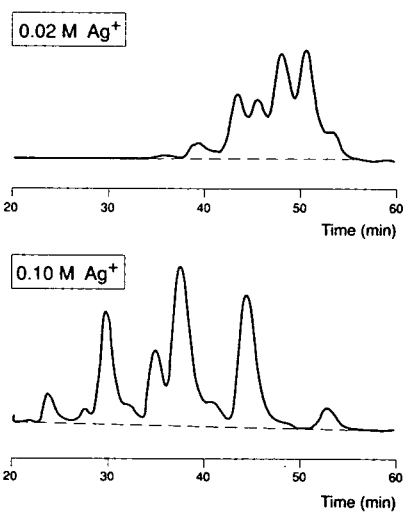

Fig. 3. Influence of silver nitrate concentration on separation of the PN48 fraction of a partially hydrogenated soya-bean oil. Conditions as in Fig. 2.

The use of silver in the mobile phase made special precautions necessary to avoid its reduction, which might have damaged the detector cell. The mobile phase had to be kept in dark bottles to protect it from light. Furthermore, the light source emitting radiation through the refractive index detector had to be in the OFF position whenever samples were not being analyzed. In the event of precipitation occurring, the silver nitrate might have damaged the pump. To avoid damage to either the detector cell or the pump, low flow had to be on even when no analysis was being performed. During longer periods with no analysis, the mobile phase with silver nitrate had to be changed to the mobile phase without. By taking these precautions we have used an HPLC system for several years without encountering any serious problems.

The influence of temperature on separation was tested briefly. Temperatures ranging from 18 to 30°C for the mobile phase and the column were tested. It was noticed that lower temperatures in this range gave better resolution, and a temperature of 20°C was finally chosen. Temperatures lower than 18°C were not considered advantageous, due to the risk of precipitation occurring.

The number of columns, *i.e.* the number of theoretical plates was arrived at from the separation point of view. With two columns, peak broadening and analysis time increased but no appreciable improvement in separation took place. One column was therefore chosen. The number of theoretical plates for the TGs present was 36 000 to 42 000.

Separation capacity. The resolution of unsaturated and saturated TGs with the same PN is strongly increased in the subsequent separation, using silver in the mobile phase, compared to in the initial fractionation. For example the separation of OOO and PPP increased dramatically (see Fig. 4 and Fig. 1B). Consequently, an extended space between the first and the last eluted component, within a PN, becomes avail-

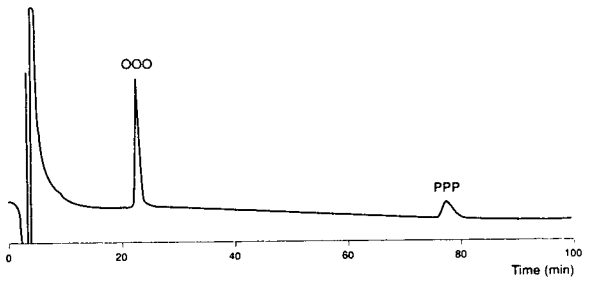

Fig. 4. Separation of two TGs, OOO and PPP, with a substantial difference in saturation but with the same PN. Conditions as in Fig. 2, but with silver concentration of 0.085 *M*. For abbreviations see Table I. PN = CN − 2 · NDB; here: $PN_{OOO} = 54 - 2 \cdot 3 = 48$ and $PN_{PPP} = 48 - 2 \cdot 0 = 48$.

able for the geometrical isomers of that particular PN. Such separations of geometrical isomers of homologous series within PN46, PN48 and PN50 are illustrated in Fig. 5A–C. PN, CN and NDB remain constant within a series and only the geometrical configurations vary. A transition from oleic acid with a *cis* configuration to elaidic acid with a *trans* configuration contributes to a major change in retention time, due to the effect of the silver ions in the mobile phase. As a result, resolution between isomers which differ in their geometrical configuration by just one double bond is excellent. From Fig. 5A–C it can also be seen that it would not have been possible to analyze the different PNs in a single analysis, as this would have resulted in overlapping peaks. Also for TGs containing palmitic acid (P), the resolution between isomers which differ in their geometrical configuration by just one double bond is excellent (Fig. 6A). Fig. 6B illustrates the separation of two isomers that differ only in the geometrical configuration of linoleic acid. In the first peak, both the double bonds in the linoleic acid are in *cis* configuration. In the second, both are in *trans* configuration.

Fig. 7A illustrates the way in which a change from one oleic acid moiety in OOLc,c to one elaidic acid moiety (OElLc,c), affects retention time to the same extent as a corresponding change from linoleic acid with both double bonds in *cis* configuration in OOLc,c to linoleic acid with both double bonds in *trans* configuration (OOLt,t). A similar change is illustrated in Fig.

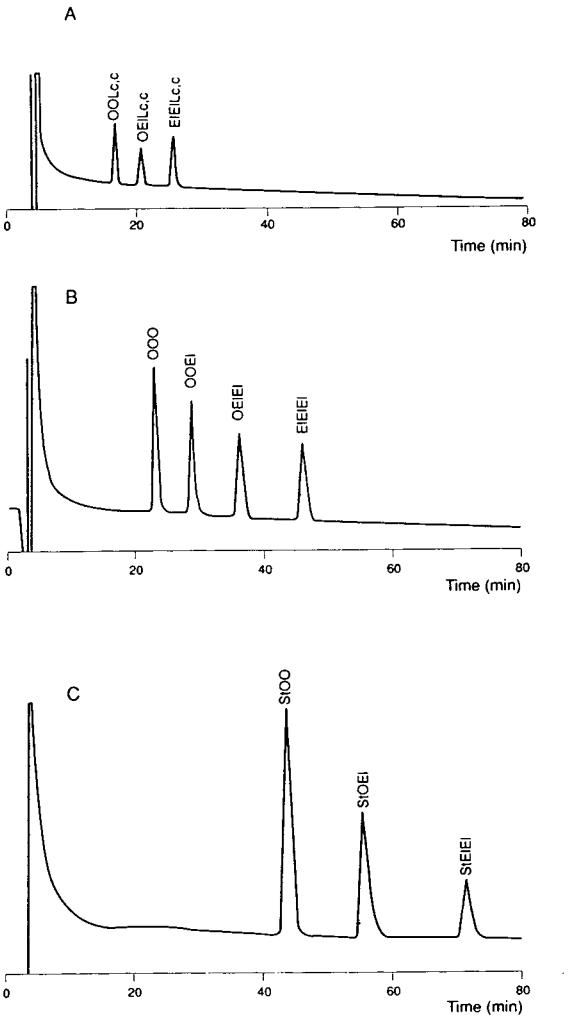

Fig. 5. Separation of homologous series of geometrical TG isomers, with C_{18} fatty acid moieties, for PN46 (A), PN48 (B) and PN50 (C). Within each series the difference between adjacent peaks can be seen to be the geometrical configuration of just one double bond. Conditions as in Fig. 4. For abbreviations see Table I.

7B. From this it would seem that a change in the configuration of the only double bond in a monounsaturated fatty acid in a given TG corresponds to a change in the geometrical configuration of both double bonds in a diunsaturated fatty acid in the same TG.

A strong separation capacity of the system for other types of TG isomers, such as the positional

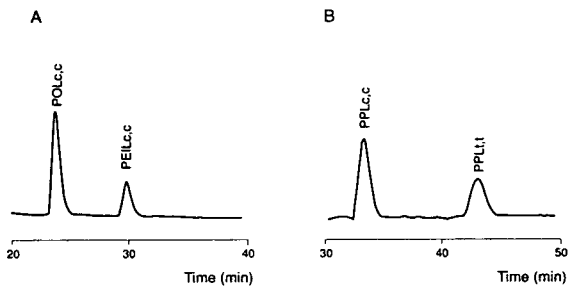

Fig. 6. Separation of homologous series of geometrical TG isomers containing palmitic acid, for PN46. Conditions as in Fig. 2, but with silver concentration of 0.085 *M*. For abbreviations see Table I; for A and B see text.

and symmetric/asymmetric (*e.g.* POP and PPO), was undesirable because this could cause overlapped and broadened peaks as well as identification problems. To check the degree of separation of positional isomers in the silver nitrate system used, two extremes were tested. These extremes, OOO (oleic acids with double bonds in position 9) and PePePe (petroselinic acids with double bonds in position 6) were clearly separated. Separation occurred in spite of the fact that attempts were made to suppress it with a view to obtaining better defined chromatograms. However, it seems unlikely that hydrogenation results in any considerable amounts of such extremes. The broadening of peaks for a partially hydrogenated fat, compared to those for model substances, can be explained by a slight variation in positional isomers (Figs. 8 and 9). The separation of symmetric/asymmetric isomers has also been studied. The pairs POP/PPO and StOSt/StStO showed no resolution.

Applications

With a view to illustrating some applications two fats were chosen, both hydrogenated to melting points of 41°C but with different fatty acid compositions (Table II).

Fig. 8 illustrates the separation and identification of Palm 41, which included a high percentage of palmitic acid (42.3%). With nearly 70% of total TGs, the peak of PN48 dominates. The sizes of the remaining PNs can be seen in Table III. The peaks that elute immediately after the solvent peak are also worthy of note. These are the diglycerides included in the fat.

The PN46, PN48 and PN50 fractions were subsequently separated into geometrical isomers (Fig. 8). The largest fractions, PN48 and PN50,

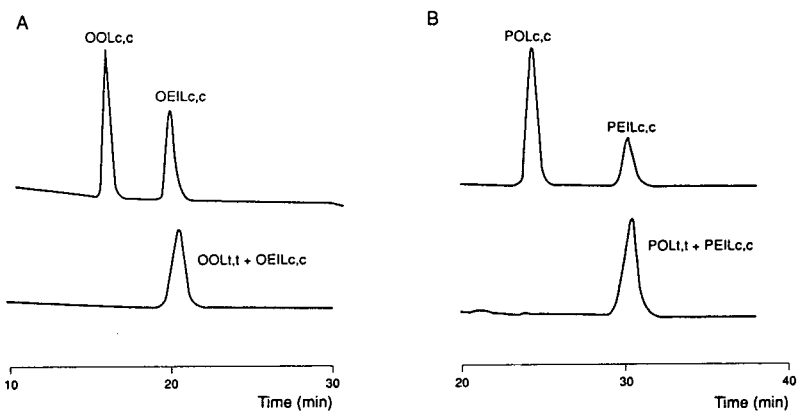

Fig. 7. Influence of a change in the geometrical configuration of a mono-unsaturated fatty acid moiety within a TG compared with that of a di-unsaturated fatty acid moiety. Conditions as in Fig. 2, but with silver concentration of 0.085 *M*. For abbreviations see Table I; for A and B see text.

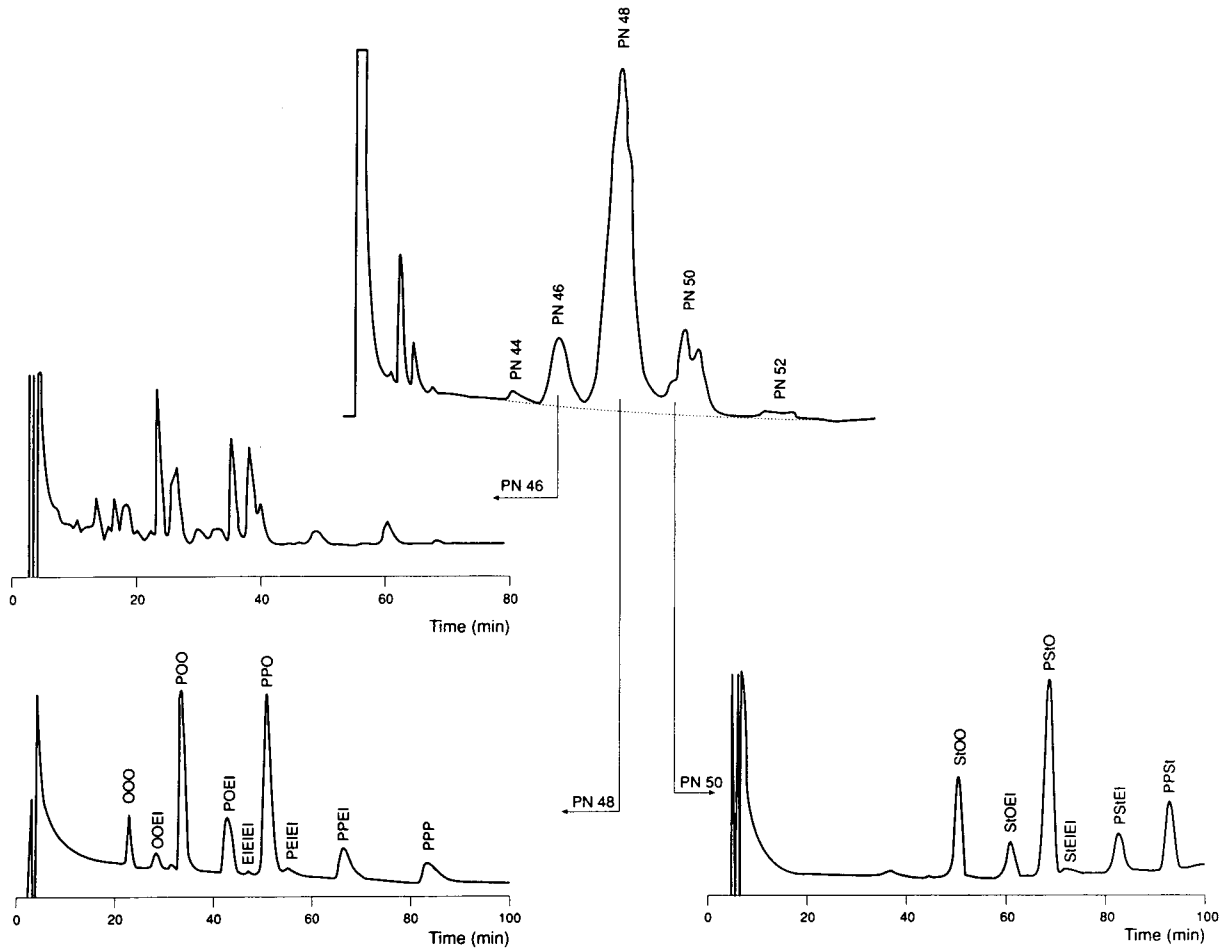

Fig. 8. Application of the proposed method on Palm 41. For conditions see under Experimental. For abbreviations see Table I.

were identified with the help of standard solutions. The PN46 peaks have not been indicated, due to incomplete identification as a result of the wide variety of TGs included. One of the reasons for this variety is that the TGs in this fraction include a large percentage of linoleic acid, which as a fatty acid can appear in four different geometrical isomers. However, since most of the TGs belong to PN48 and PN50, many of them have been identified anyway. The TGs have been separated into geometrical isomers nearly at baseline (Fig. 8).

Fig. 9 illustrates separation and identification in the case of Soya 41. This fat is especially rich in oleic, elaidic and stearic acid. Consequently, PN distribution becomes displaced to higher PNs than in the case of Palm 41 (Table III). The PN52 fraction was therefore analyzed and identified as geometrical isomers, and has been included in Table IV.

In the case of Soya 41 too, the diacylglycerols can be seen to have eluted after the solvent peak. If the chromatograms for Soya 41 and Palm 41 are compared it can be seen, as might

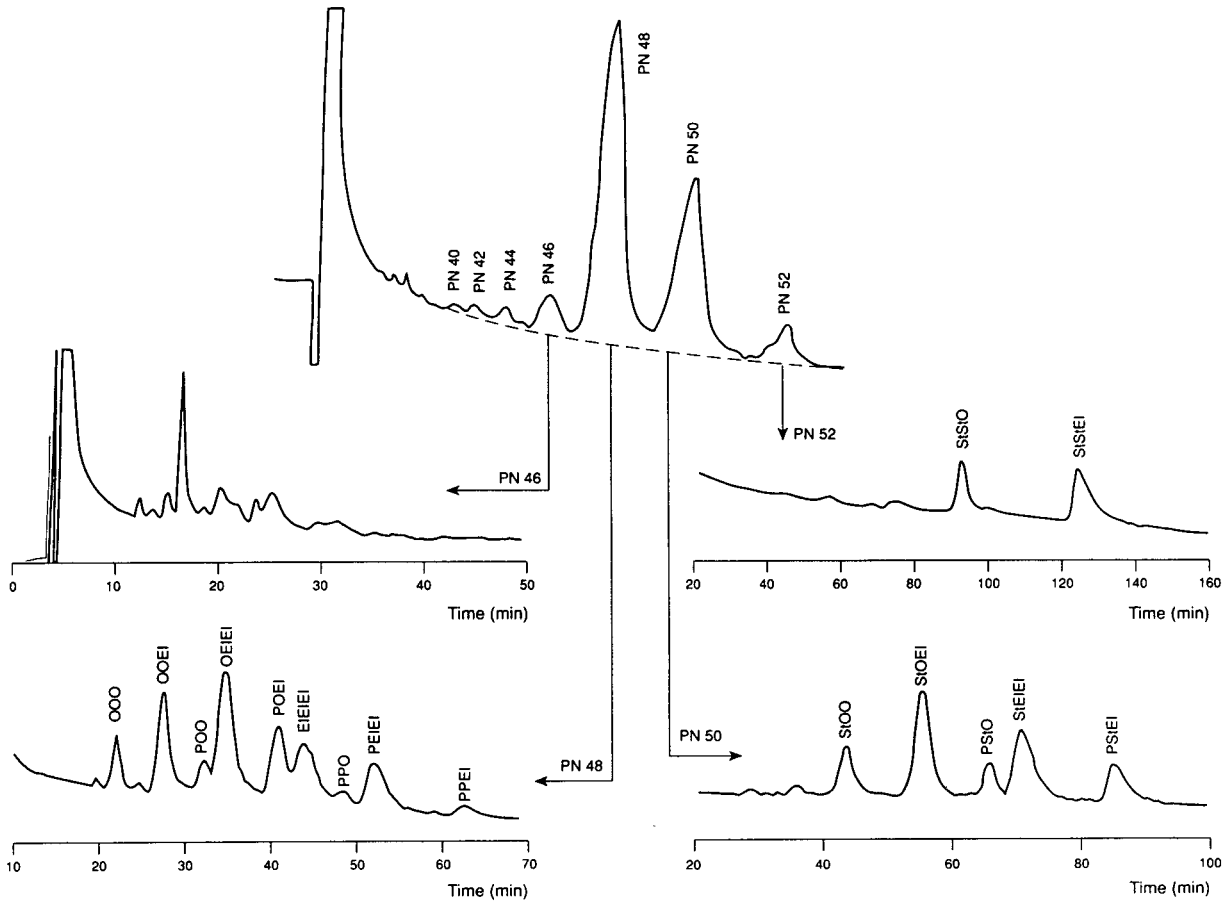

Fig. 9. Application of the proposed method on Soya 41. For conditions see under Experimental. For abbreviations see Table I.

TABLE III

PARTITION NUMBER DISTRIBUTION OF PALM 41 AND SOYA 41

PN	Palm 41 (%, w/w)[a]	Soya 41 (%, w/w)[a]
40	0.0	0.6
42	0.2	0.9
44	1.2	1.4
46	9.9	5.5
48	68.3	54.5
50	18.0	32.8
52	2.5	4.5
Total	100	100

[a] % (w/w) of total amount of TGs.

be expected, that Soya 41 contains a much smaller percentage of diacylglycerols.

Quantification of TGs for the two fats is as shown in Tables III and IV. The amounts have been expressed in % (w/w), which is approximately equal to area% when TGs do not differ much in CN and NDB.

Reproducibility

The relative standard deviation of the total analysis of TG amounts of 15 and 3% has been calculated as 2 and 5%, respectively. The calculations of the standard deviations are based on that the total analysis can be designated as a multiplicative expression [11].

TABLE IV

TRIACYLGLYCEROL COMPOSITION OF PALM 41 AND SOYA 41, INCLUDING GEOMETRICAL ISOMERS

For abbreviations see Table I.

Triglyceride			Palm 41 (%, w/w)[a]	Soya 41 (%,w/w)[a]
Abbreviation	Structure	PN		
OOO	$C_{54:3}$(c,c,c)	48	3.6	2.7
OOEl	$C_{54:3}$(c,c,t)	48	1.7	8.1
POO	$C_{52:2}$(c,c)	48	17.8	2.5
OElEl	$C_{54:3}$(c,t,t)	48	0.1	14.9
POEl	$C_{52:2}$(c,t)	48	7.9	7.3
ElElEl	$C_{54:3}$(t,t,t)	48	0.4	8.7
PPO	$C_{50:1}$(c)	48	24.3	1.5
PElEl	$C_{52:2}$(t,t)	48	1.4	6.3
PPEl	$C_{50:1}$(t)	48	5.9	1.1
PPP	$C_{48:0}$	48	4.8	0.0
Unidentified peaks	–	48	0.4	1.4
StOO	$C_{54:2}$(c,c)	50	3.1	4.0
StOEl	$C_{54:2}$(c,t)	50	1.6	10.8
PStO	$C_{52:1}$(c)	50	8.1	3.0
StElEl	$C_{54:2}$(t,t)	50	0.4	9.1
PStEl	$C_{52:1}$(t)	50	2.1	4.9
PPSt	$C_{50:0}$	50	2.8	0.0
Unidentified peaks	–	50	0.0	1.0
StStO	$C_{54:1}$(c)	52	–	1.4
StStEl	$C_{54:1}$(t)	52	–	2.5
Unidentified peaks	–	52	–	0.6

[a] % (w/w) of total triacylglycerols.

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Determination of sodium 5,6-benzylidene-L-ascorbate and related compounds by high-performance liquid chromatography

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ABSTRACT

A simple high-performance liquid chromatographic process with ultraviolet detection was established to determine ascorbic acid, benzoic acid, benzaldehyde and the two different diastereomers of sodium 5,6-benzylidene-L-ascorbate. The five substances were reproducibly separated on a reversed-phase C_{18} column with gradient elution with an acetate buffer (pH 6.85)–methanol system. This method was applied to the determination of the concentrations of these substances in various biological fluids after their extraction and deproteinization with acetonitrile.

INTRODUCTION

Benzaldehyde, an antitumour substance, can be isolated from the volatile fraction of figs [1]. Oral administration of sodium 5,6-benzylidene-L-ascorbate (SBA) (see Fig. 1 for the structure of its two diastereomers) to patients with advanced, inoperative carcinoma induced remarkable necrotic change of the tumours [2]. We have previously shown that intravenous administration of SBA induced degeneration of 3′-methyl-4-dimethylaminoazobenzene-induced rat hepatocellular carcinoma (vacuolar, eosinophilic degeneration, nuclear debris) [3,4]. In cultured L-929 cells, SBA induced chromatin condensation, DNA fragmentation into nucleosomal oligomers and disappearance of cell surface microvilli, which are characteristic of apoptotic cell death [5]. However, the process of induction of the antitumour action of SBA is still unclear. We report here a method for the determination of SBA and related compounds using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and reagents

The following chemicals and reagents were used: SBA (ChemiScience, Tokyo, Japan); acetonitrile (for HPLC) and methanol (for HPLC) (Kanto Chemical, Tokyo, Japan); RPMI 1640

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medium (Gibco, Grand Island, NY, USA); foetal bovine serum (FBS) (Filtron, Brooklyn, Australia); L-(+)-ascorbic acid (Wako, Osaka, Japan); benzoic acid (Koso Chemical, Tokyo, Japan); benzaldehyde (Kanto Chemical); bovine serum albumin (Fraction V) (Wako).

Preparative separation of SBA diastereomers

One gram of SBA diastereomers was separated by chromatography using a column prepacked with Toyopearl HW-40S (850 × 30 mm I.D.) (Tosoh, Tokyo, Japan), eluted with methanol–water (10:90, v/v) delivered by a peristalic pump. After the fractionated solutions had been checked by analytical HPLC, all solutions including the two diastereomers, compounds A and B, were combined. The methanol in them was evaporated *in vacuo* at 40°C or lower. Lyophilization of the resulting aqueous solutions shielded from light gave 0.2 and 0.6 g of each diastereomeric SBA, both of which were pale-orange amorphous compounds.

The structures of the two SBA diastereomers, separated by preparative HPLC, were confirmed from the NMR chemical shifts, coupling constants and nuclear Overhauser effect (NOE) (Fig. 1). To confirm the configuration of the benzylidene site by NMR, compounds A and B were subjected to permethylation of the hydroxy groups by, respectively, excess of methyl iodide and sodium hydride in dimethylformamide (DMF).

^{1}H NMR spectroscopy in C^2HCl_3 of permethylated compound A (A-Me) showed the presence of a signal at δ 5.78 (s) due to the benzylic proton of the benzylidene group, whereas permethylated compound B (B-Me) showed the presence of a signal at δ 5.86 (s) due to that of the benzylic proton. The configuration at the benzylidene centre was not clear from these data alone. A notable difference between A-Me and B-Me was revealed by an NOE difference of 1.52–2.03% between the benzylic proton of the benzylidene group and C_5–H of A-Me. On the other hand, no NOE was observed between the benzylic proton of benzylidene group and C_5–H

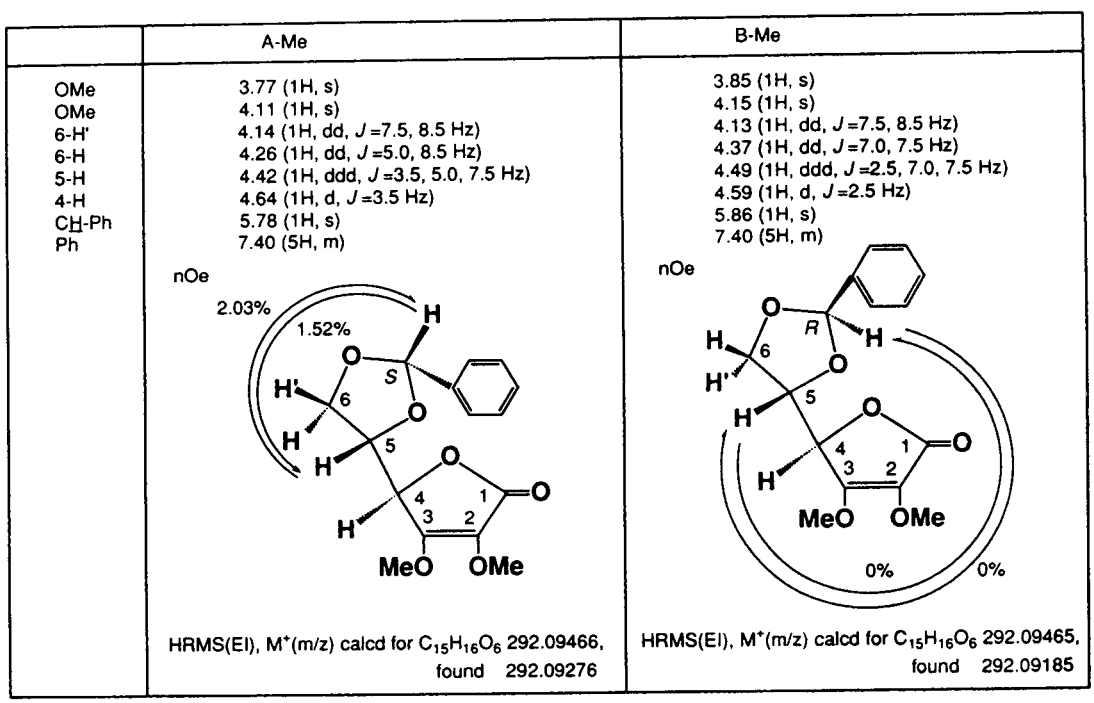

Fig. 1. Structures, chemical shifts and NOE value for the two SBA diastereomers, A-Me (*S* configuration) and B-Me (*R* configuration) (δ ppm, C^2HCl_3, Varian VXR-400, 400 Mz).

of B-Me. Therefore, we concluded that the stereochemistry of the benzylic site of A-Me is *S* and that of B-Me is *R* (Fig. 1).

Extraction and deproteinization of samples by acetonitrile

Cell lysate was prepared from human myeloblastic leukaemic ML-1 cells [6] by sonication (1 min at 0°C) in 50 m*M* acetate buffer (pH 6.85) and centrifugation for 10 min at 13 000 *g*. An extract from the liver of 5-month-old Donryu rats (Sankyo Laboratory Service) was prepared by sonication in the same buffer.

To investigate the efficiency of acetonitrile extraction, a mixture of SBA (786 μg), ascorbic acid (105 μg), benzoic acid (1313 μg) and benzaldehyde (179 μg) was added to 0.45 ml of each of the following: regular culture medium (RPMI 1640 medium supplemented with 10% of FBS), FBS, cell lysate (containing 10.8 mg/ml of protein) or rat liver extract (containing 62.2 mg/ml of protein). After allowing the mixtures to stand for 30 s at room temperature, they were vigorously mixed with 1.05 ml of acetonitrile (final acetonitrile concentration = 70%) and centrifuged for 1 min at 4200 *g*. The supernatant (80 μl) was diluted with 200 μl of 50 m*M* acetate buffer containing 15% of methanol and 20 μl of the diluent was applied to HPLC.

Apparatus

Chromatographic analyses were performed at 25°C on a Hitachi HPLC system (D-2500 chromato-integrator, L-4200 UV–Vis detector, L-6200 intelligent pump) with an ODS-80T$_M$ column (150 × 4.6 mm I.D., 5 μm) (TSK-Gel, Tosoh) and a Guardgel ODS-80T$_M$ column (15 × 3.2 mm I.D.) (Tosoh). The sample was loaded and separated with a linear gradient of 15–40% (v/v) methanol in 50 m*M* sodium acetate buffer (pH 6.85). The flow-rate was 1.0 ml/min and the elution profile, monitored by the absorbance at 255 nm, was recorded at attenuation 6. The gradient elution was run for 30 min.

Protein determination

The amount of protein was determined by the method of Lowry *et al.* [7], using bovine serum albumin as standard.

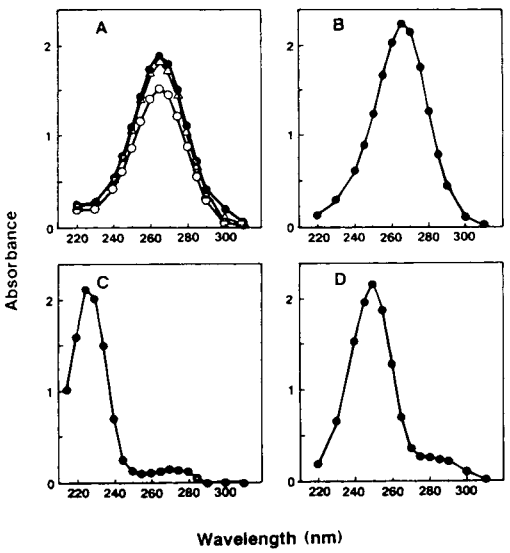

Fig. 2. Absorption spectra of (A) SBA and its two diastereomers, (B) ascorbic acid, (C) benzoic acid and (D) benzaldehyde. SBA or its two diastereomers (△ = *S* configuration; ○ = *R* configuration (50 μg/ml) and ascorbic acid (25 μg/ml) were dissolved in 50 m*M* acetate buffer (pH 6.85). Benzoic acid (25 μg/ml) was dissolved in methanol. Benzaldehyde (15.9 μg/ml) was dissolved in 50 m*M* acetate buffer (pH 6.85) containing 0.086% (v/v) of methanol.

RESULTS AND DISCUSSION

Absorption spectrum

Fig. 2 shows the UV absorption maxima (A) at 265 nm for unseparated SBA and its two diastereomers, (B) at 265 nm for ascorbic acid, (C) at 225 nm for benzoic acid and (D) at 250 nm for benzaldehyde. Therefore, their elution profiles were monitored by HPLC at 255 nm. The molar absorption coefficient (577) of benzoic acid, measured at this wavelength, was *ca.* one order of magnitude lower than those of SBA, ascorbic acid and benzaldehyde (8294, 11 765, 12 533).

Effect of pH on retention time

The optimum pH of the running buffer for HPLC separation was determined. At pH < 3, elution of two SBA diastereomers was relatively slow, as indicated by the longer retention time (Fig. 3A). The elution time was a minimum at pH 6–9. The peak area of SBA diastereomers, determined with a D-2500 chromato-integrator,

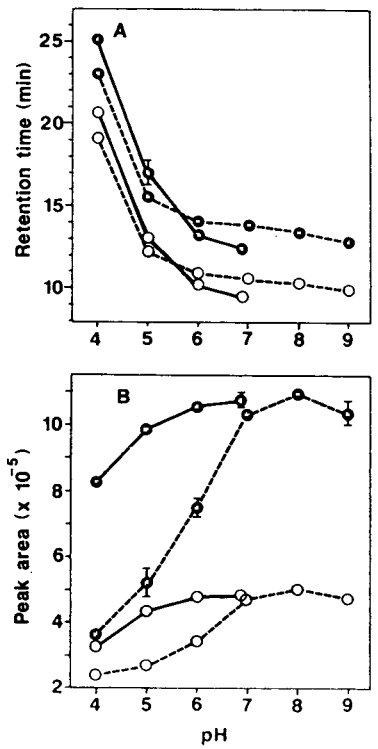

Fig. 3. Effect of pH of running buffer on the retention time and peak area of SBA diastereomers. SBA (3 μg) was equilibrated with 50 m*M* phosphate buffer (broken line) or 50 m*M* acetate buffer (solid line), adjusted to the indicated pH, and (A) the retention time and (B) peak area of two SBA diastereomers (● = *R* configuration; ○ = *S* configuration) were then determined. Each point is the mean from two independent experiments.

decreased with decrease in the pH of the running buffer (Fig. 3B). This was more apparent when the pH was adjusted with phosphate buffer than when it was adjusted with acetate buffer. Therefore, acetate buffer (adjusted to pH 6.85) was used in subsequent HPLC experiments. SBA was found to be extremely unstable at low and high pH: the degradation half-life of SBA in 50 m*M* phosphate buffer at pH 2 and 9 at 37°C was 4 and 5 min, respectively. The major degradation products of SBA at pH 2 were ascorbic acid and benzaldehyde (data not shown).

HPLC separation

When SBA was subjected to HPLC with elution with a 15–40% linear gradient of methanol in 50 m*M* acetate buffer (pH 6.85), it was separated into two diastereomers, one with *S* configuration (retention time 9.24 min) and the other with *R* configuration (retention time 12.18 min) (Fig. 4A). These peaks were confirmed by co-elution with purified diastereomers (Fig. 4D). The ratios of *S* and *R* diastereomers to total SBA, determined with the chromato-integrator, were 31.04 ± 0.27% and 68.96 ± 0.27% ($n = 13$), respectively. Two small peaks that eluted at 1.70 and 27.27 min, representing about 5% of the total peak, were superimposed with the ascorbic acid (peak I) and benzaldehyde (peak III in Fig.

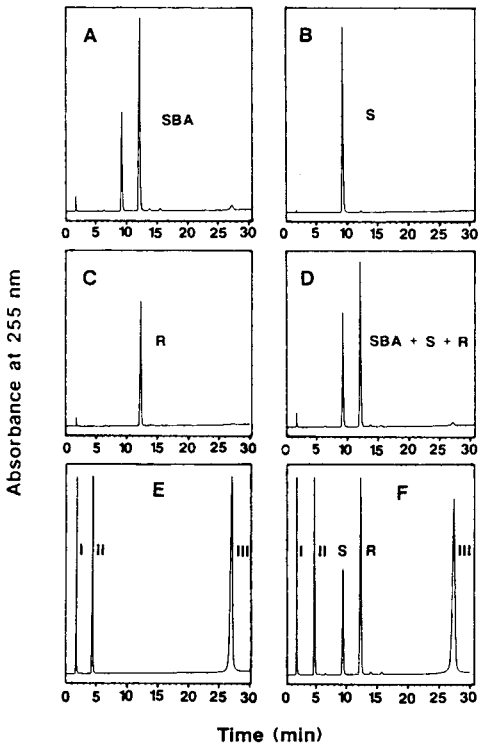

Fig. 4. Separation of the two SBA diastereomers using a linear 15–40% gradient of methanol in 50 m*M* acetate buffer (pH 6.85). Flow-rate, 1.0 ml/min; detection wavelength, 255 nm; attenuation, 6 (100% absorbance on ordinate = 0.16). Applied sample amounts: (A) SBA (3 μg); (B) diastereomer of SBA with *S* configuration (1.5 μg); (C) diastereomer of SBA with *R* configuration (1.5 μg); (D) mixture of SBA (1.5 μg) and its two diastereomers (0.75 μg each); (E) mixture of ascorbic acid (0.4 μg) (peak I), benzoic acid (5 μg) (peak II) and benzaldehyde (0.68 μg) (peak III); (F) mixture of ascorbic acid (0.4 μg), benzoic acid (5 μg), SBA (3 μg) and benzaldehyde (0.68 μg).

4F) peaks, respectively. SBA showed no detectable amount of benzoic acid (peak II in Fig. 4E).

Evaluation of present method

Repeated experiments showed that ascorbic acid (peak I), benzoic acid (peak II), the *S* and *R* diastereomers of SBA and benzaldehyde (peak III) could be reproducibly eluted at retention times of 1.693 ± 0.013 ($n = 24$), 4.497 ± 0.105 ($n = 18$), 9.061 ± 0.212 ($n = 25$), 11.826 ± 0.337 ($n = 25$) and 26.992 ± 0.290 min ($n = 24$), respectively. This indicates the reproducibility of the gradient formation, as judged by the elution profiles and retention times.

Linearity of quantification of the peak areas on the chromatogram was investigated (Fig. 5). When the amounts of ascorbic acid, benzoic acid, SBA and benzaldehyde subjected to HPLC were within the range 0.001–10 μg, their peak areas increased linearly. At a higher level (100 μg), the peak areas were slightly smaller than expected.

Extraction and deproteinization by acetonitrile

The efficiency of acetonitrile as a deproteinizing agent was examined. Acetonitrile precipitated serum proteins, cellular proteins and rat liver proteins in a concentration-dependent manner (Fig. 6). Addition of 70% acetonitrile precipitated 97–99% of these proteins. When the 70% acetonitrile-soluble fraction was subjected to HPLC, several peaks, referred to as back-

TABLE I

EXTRACTION OF SBA AND RELATED COMPOUNDS WITH 70% ACETONITRILE

Compound	Peak area (recovery, %)				
	Added[a]	Recovered from[b]			
		RPMI 1640 +10% FBS	FBS	Cell lysate	Liver extract
Ascorbic acid	626 391 ±17 285	613 625 ±14 133 (98)	500 248 ±98 729 (80)	671 397 ±57 431 (107)	512 253 ±20 054 (82)
Benzoic acid	704 648 ±26 736	768 416 ±1844 (109)	792 467 ±4590 (112)	748 744 ±2846 (106)	781 080 ±25 078 (111)
(*S*)-SBA	494 631 ±10 535	504 941 ±22 602 (102)	475 020 ±35 705 (96)	497 634 ±2950 (101)	480 986 ±15 537 (97)
(*R*)-SBA	1 094 309 ±24 458	1 118 257 ±45 465 (102)	1 052 998 ±78 777 (96)	1 098 017 ±6326 (100)	1 060 961 ±33 368 (97)
Benzaldehyde	1 796 129 ±31 463	1 726 382 ±91 435 (96)	1 911 461 ±57 661 (106)	1 767 409 ±38 912 (98)	1 713 563 ±124 982 (95)

[a] A mixture of ascorbic acid (0.4 μg), benzoic acid (5 μg), SBA (3 μg) and benzaldehyde (0.68 μg) in 20 μl was subjected to HPLC.

[b] The samples were added to culture medium, FBS, cell lysate or liver extract and then extracted with 70% acetonitrile. The acetonitrile-soluble fraction was diluted and amounts equivalent to those in footnote a were subjected to HPLC, as described under Experimental. The peak areas of these samples were determined, after subtraction of the background peaks (indicated by the lower chromatograms in Fig. 7A–D). Each value is the mean ± S.D. from 2–4 independent experiments. Numbers in parentheses are percentage recovery.

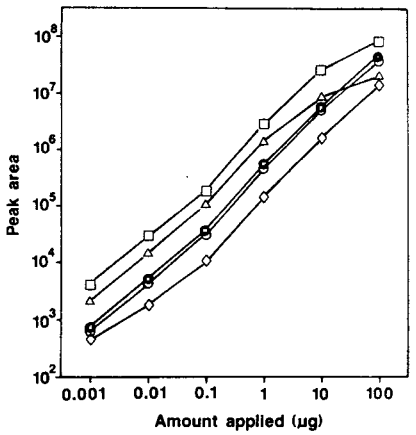

Fig. 5. Relationship between amount applied and peak area. The indicated amount of (○) the *S* diastereomer of SBA, (●) the *R* diastereomer of SBA, (△) ascorbic acid, (◇) benzoic acid or (□) benzaldehyde was subjected to HPLC. Attenuation, 6; detection wavelength, 255 nm. Each point is the mean of two independent experiments.

ground peaks, appeared at retention times from 1.7 to 5.0 min (lower chromatograms in Fig. 7B–D).

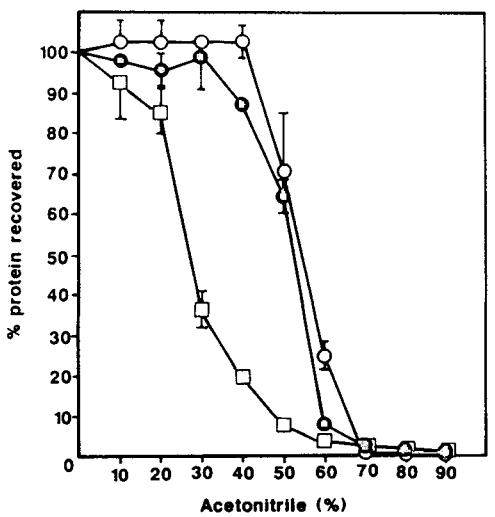

Fig. 6. Deproteinization by acetonitrile. Acetonitrile was added to the (○) FBS, (●) ML-1 cell lysate or (□) rat liver extract to the indicated final concentrations and the protein concentration of the supernatant was determined. Initial concentrations of FBS, cell lysate, and rat liver were 3819 ± 101, 1080 ± 4, and 6219 ± 934 μg/ml, respectively. Each value is the mean ± S.D. from three independent experiments.

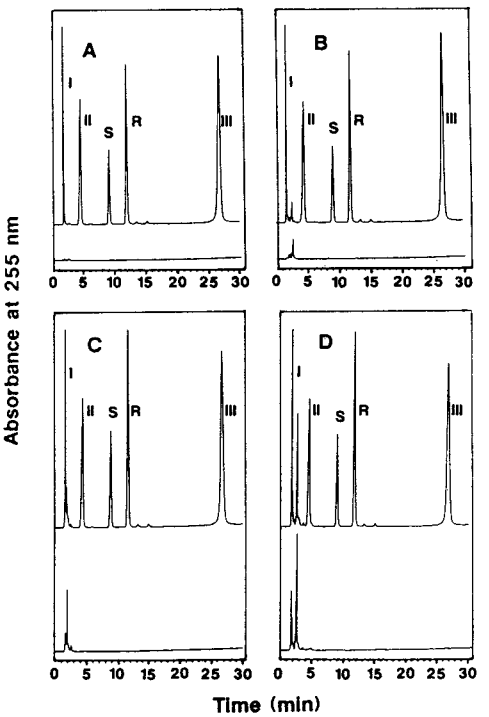

Fig. 7. HPLC separation of SBA and related compounds after extraction from various biological fluids with acetonitrile. Ascorbic acid, benzoic acid, SBA and benzaldehyde was added to (A) RPMI 1640 medium supplemented with 10% of FBS, (B) FBS, (C) ML-1 cell lysate or (D) rat liver extract. Acetonitrile was added at a final concentration of 70%, and then the acetonitrile-extractable fraction, equivalent to 0.4 μg ascorbic acid, 5 μg benzoic acid, 3 μg SBA and 0.68 μg benzaldehyde, was subjected to HPLC. The lower chromatogram in each part is for a control without test compounds. I = Ascorbic acid; II = benzoic acid; III = benzaldehyde; S = *S* diastereomer of SBA; R = *R* diastereomer of SBA.

Ascorbic acid, benzoic acid, two diastereomers of SBA and benzaldehyde were added to the culture medium, FBS, cell lysate or liver extract and then extracted with 70% acetonitrile. When one of the acetonitrile extracts was subjected to HPLC, these compounds were separated as distinct peaks (upper chromatograms in Fig. 7A–D). It was necessary to subtract the absorbance of the background peaks in order to calculate the concentrations of these compounds accurately. These compounds, except for ascorbic acid in FBS and liver extract, were quantitatively recovered in the acetonitrile supernatant (Table I).

CONCLUSIONS

The proposed HPLC technique is useful for the determination of the two SBA diastereomers (compounds A and B), which were separated from the major metabolites, and for ascorbic acid and benzaldehyde. To extract the sample present in culture medium or serum, the use of acetonitrile is recommended (Table I). The use of an acidic solution, such as perchloric acid or trichloroacetic acid, for extraction and deproteinization should be avoided, as SBA is extremely unstable at low pH (Fig. 3B). This study has demonstrated the applicability of the proposed technique to the study of the metabolism of SBA.

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Automated recognition of target compounds at low levels in environmental samples by means of capillary gas chromatography–mass spectrometry with dedicated mass spectral libraries and the macro program AUTARG

I. Description of the macro program AUTARG☆

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ABSTRACT

Automatic pesticide screening was performed using a macro program to compare mass spectra acquired during GC–MS with those in designated mass spectral libraries containing a limited number of target compounds. The automated evaluation procedure enabled fast recognition of pesticides in complex chromatograms. The application of reconstructed ion chromatograms of two characteristic ions and time window programming even leads to the recognition of target compounds overlapped by matrix substances and background noise.

INTRODUCTION

In environmental analysis, capillary gas–liquid chromatography (GLC) plays a major role in the detection of pollutants or pesticide residues in soil or water samples. Many hundreds of such contaminants can be directly analysed by GLC, a great many others after derivatization. These large numbers of environmental pollutants have to be separated from each other and from matrix compounds. Screening analysis has been successfully performed using gas chromatography with capillary columns, and simultaneous detection with two different selective detectors, usually combinations of electron-capture detection (ECD), nitrogen–phosphorus detection and flame photometric detection. Although these detectors are highly selective, matrix compounds that survive the clean-up procedures may also produce signals. As a consequence, the chromatograms generated often appear very complex.

Therefore, in recent years, the mass spec-

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☆ The macro program AUTARG can be obtained from Professor Dr. H.-J. Stan, Institute of Food Chemistry, Technical University of Berlin, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany. A description is included.

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trometer, which has been used in confirmatory analysis for decades in the field of environmental analysis, has also gained popularity in screening analysis. This new application may become a domain of the mass selective detectors. In the course of the investigation of a series of groundwater samples, we found that most of the chromatograms obtained by GLC with ECD could not be evaluated satisfactorily because of the large number of matrix peaks. Screening analysis with the HP 5970 mass-selective detector with cyclic scanning and searching in a designated mass spectral pesticide library showed surprisingly good results. Manual data evaluation still remained very time-consuming, although the search was done only in a designated library. Therefore, the macro program AUTOEVAL was developed, which simulated automatically all the steps usually applied in manual data evaluation [1,2]. The program has been used successfully for about the last 2 years for pesticide residue analysis in food and various kinds of environmental analyses in ground- and surface water as well as soil samples.

In this paper we describe an updated version of this program, now called AUTARG, which stands for automated target analysis.

EXPERIMENTAL

Sample preparation and the experimental conditions of GC–MS are described in Part II of the paper [3].

HARDWARE AND SOFTWARE REQUIREMENTS

After installation the program AUTARG runs as a part of the HP ChemStation software for MS-DOS. Therefore, hardware requirements are the same as for the HP ChemStation software (personal computer with a 386 processor, hard disk and at least 4 MB of RAM). The use of a math coprocessor is recommended to speed up data analysis.

We run the HP ChemStation on a 486 PC with 8 MB of RAM, a 120-MB hard disk and MS-DOS 5.0 and Windows 3.1 as the operating system.

DESIGN OF THE PROGRAM AUTARG

When environmental samples are analysed by GC–MS in full-scan mode, all eluting substances produce signals. The resulting total-ion chromatograms (TICs) are, therefore, often of a very complex nature. One of the merits of mass spectrometric detection is, however, that information about substances is highly specific and can be matched with mass spectral libraries. In order to obtain the best search results, most of the spectra measured require background subtraction to reduce interfering signals. Manipulation and library search are aided by processing functions offered by the operating software.

The aim, which was successfully achieved with the macro program AUTOEVAL, was the automated performance of all the steps necessary for manual data evaluation. The new program AUTARG, taking full advantage of Windows, follows the same line and consists of five program parts that can be integrated into the data analysis window of the HP ChemStation software. They provide the following functions.

AUTARG Level 1 performs an automated library search of every automatically integrated peak against one or two user-defined libraries. It is written in HP ChemStation macro language.

AUTARG Level 2 uses reconstruced ion chromatograms (RICs) to search for compounds at very low concentrations. It is also useful in detecting peaks that are overlapped by matrix compounds. It is written in HP ChemStation macro language.

AUTARG Set-up is used to define user parameters, *e.g.* the names of the mass spectral libraries for automatic searching, thresholds, time windows and so on. It too is written in HP ChemStation macro language.

AUTARG Control-file editor is a program to build up the so-called control-files needed for the AUTARG Level 2 search. It is written in Visual Basic for Windows.

AUTARG Results is a program for viewing, editing and printing the results obtained from Level 1 and Level 2 searches. It is written in Visual basic for Windows.

Fig. 1 shows the data analysis screen with the opened pull-down menu of AUTARG.

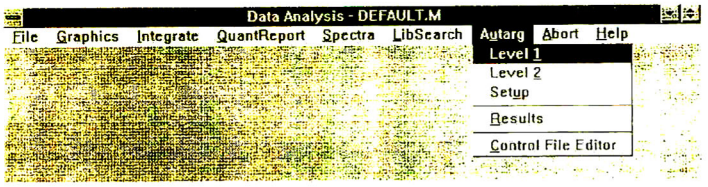

Fig. 1. Data analysis screen with the opened pull-down menu of AUTARG.

Description of Level 1

A click on menu point Level 1 starts the integration of the TICs according to user-defined integration parameters. The user can choose, for example, to use autointegration or a special events file. After integration, every integrated peak is background subtracted by subtracting successively the mass spectra at peak start and peak end and then compared with one or two target compound libraries. It is also possible to specify a hit quality threshold, so that Level 1 only switches into the second library if this threshold is not met. By testing various conditions, we found that library searches on small peaks may give poor results if background subtraction has been performed. Therefore, Level 1 also performs a library search without background subtraction for each peak and checks whether the hit quality is higher with or without background subtraction. The results, compiled in three different result tables together with a picture of the TIC, can easily be accessed by choosing Results from the AUTARG menu, as shown in Fig. 2.

The first results table, called AUTARG Results Level 1, contains the complete analysis report of AUTARG Level 1, including the sample description (sample name, corresponding data file, acquisition date, method file, operator) as well as the names of the target compound libraries. All integrated peaks are listed with their retention times and search results. If a library search is successful, the three best matches of each library search are reported with their substance name, hit quality and reference number in the corresponding library. If the user has also specified a retention time reference standard (*e.g.* aldrin), the expected retention time for each compound found by library search is calculated. Peaks with no search results are reported as such, with only their retention times.

In a second results table, called Important Peak List, only those results of the library search that have met user-defined thresholds for high

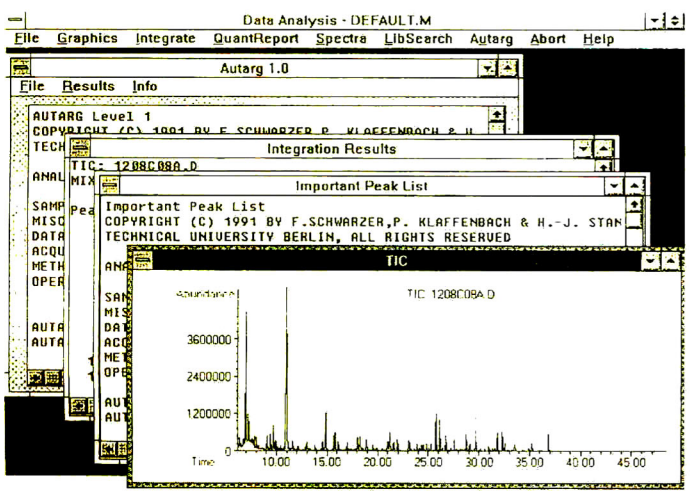

Fig. 2. Results of a AUTARG Level 1 search.

quality and retention time windows are compiled. For example, this list might contain only search results with a hit quality better than 80 and also all peaks with a high quality less than 80 if exhibiting a difference in retention time smaller than 1 min from that laid down in the library for the suggested compound.

This also enables the recognition of target compounds overlapped by matrix components, which produce poor library search results, and emphasizes the retention time as a very important independent piece of information. On the other hand, any similarity between the mass spectrum of the sample peak and the reference spectrum must be considered as purely coincidental if the retention times of the reference target compound and sample peak are significantly different.

The third results table contains the integration results of all peaks with all details such as peak area, peak width, resolution, peak start and peak end. Together with the TIC picture, the integration results table presents an overview of all compounds detected in the sample.

Compared with AUTOEVAL, AUTARG Level 1 offers the following advantages. With AUTOEVAL, all results were directly sent to the printer. This turned out to be very time-consuming when using AUTOEVAL as a part of a method because of the low speed of the printing process. AUTARG Level 1, therefore, prints all results to files, which speeds up the data analysis process considerably. Data processing now consumes less time than needed by the GC–MS system to cool down after a run with temperature programme and to equilibrate before the next start. A second advantage of AUTARG Level 1 is that the analyst can check the results first on the screen and can decide later what to print out. This saves printing costs and reduces paper consumption enormously. Finally, the automatic comparison of search results with and without background subtraction always guarantees the best search results.

Description of Level 2

As mentioned above, the AUTARG Level 1 results table may contain some search results with a poor hit quality but excellent correlation between the retention times for the peak searched and the suggested library compound. In this case, the target compound may be overlapped by a co-eluate from the matrix and manual evaluation must, therefore, be performed. Such a manual verification procedure, performed to confirm the identity of a compound generating a peak overlapped by the peak of a matrix compound, is demonstrated below.

In a groundwater sample AUTARG Level 1 found 2-(4)-chlorophenoxy-2-methyl-propionic acid pentafluorobenzyl (PFB) ester with a hit quality of only 44 but an excellent match in retention time. Fig. 3 shows the TICs of the groundwater sample and a zoomed view of the peak under investigation.

The mass spectrum taken at the apex and the library search result are shown in Fig. 4. The base peak in the spectra of the unknown is ion m/z 149, which commonly belongs to plasticizers of the phthalic ester group. Visual comparison of the spectrum of the unknown and the spectrum of the reference compound from the library shows that the appropriate ions for 2-(4)-chlorophenoxy-2-methyl-propionic acid PFB ester also appear in the spectrum of the unknown.

For further confirmation of the hypothesis of two compounds overlapping in that one peak,

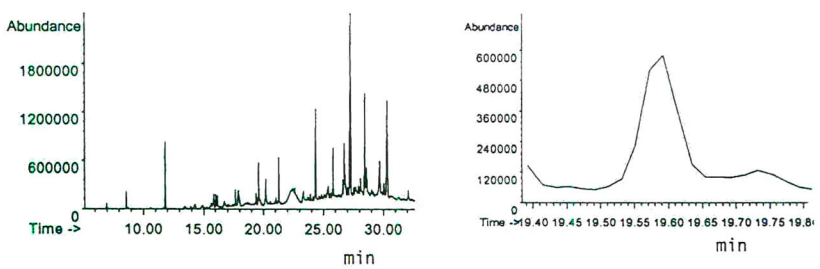

Fig. 3. TIC of a ground water sample. Left: Complete chromatogram. Right: Portion under investigation enlarged.

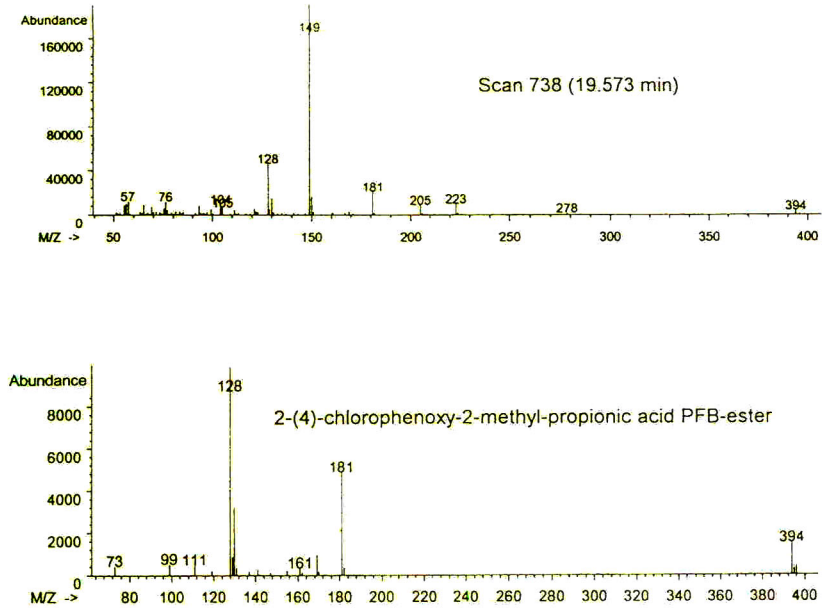

Fig. 4. Mass spectrum of the "unknown" (top). Reference spectrum from the library (bottom).

the use of reconstructed ion chromatograms (RICs) proved to be helpful. The RICs of the two selected specific ions of 2-(4)-chlorophenoxy-2-methyl-propionic acid PFB ester, m/z 128 and m/z 394, and m/z 149, which is suspected to originate from the co-eluting phthalic ester, are shown in Fig. 5.

It is evident that the traces of the target compound ions and that of the ion suspected to belong to the plasticizers show that the two compounds do not elute exactly at the same time. In order to obtain a target compound spectrum of maximum purity, a scan was taken at that time at which the plasticizers (ion m/z 149) have almost completely eluted. After background subtraction, the library search reported the recognition of 2-(4)-chlorophenoxy-2-methyl propionic acid PFB ester with a hit quality of 99.

As shown in the example, manual evaluation of peaks exhibiting poor hit quality to suggested library compounds but a good correlation in retention time can provide excellent results when using RICs of appropriate selected ions. A disadvantage of this technique is that it is very time-consuming when applied to a large number of peaks. Therefore, we developed AUTARG Level 2 to execute automatically all the steps described above. The only task the user has to perform is to create so-called control-files for AUTARG Level 2. This can easily be done with the Control-file editor, of which a screenprint is shown in Fig. 6.

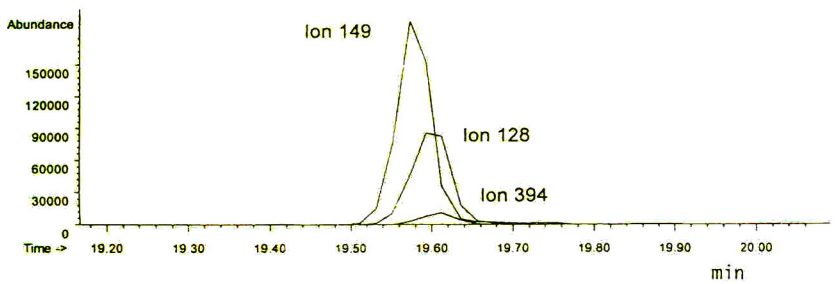

Fig. 5. RIC of the "unknown".

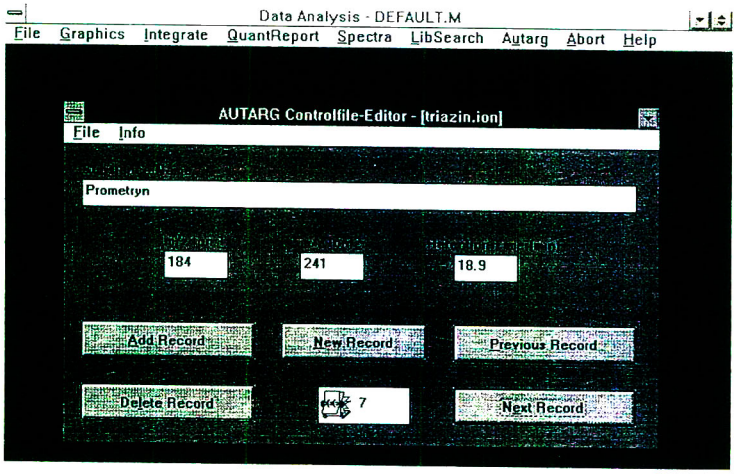

Fig. 6. Control-file editor for AUTARG Level 2.

For each substance, AUTARG Level 2 needs the target compound name, the two selected specific ions and the retention time expected for the target compound. A group of target compounds can be combined in one control file. For example, all triazine herbicides can be compiled in a control file named triazin.ion, which we use in our laboratory, as shown in Fig. 6. One control file can hold up to 100 target compounds.

When starting the Level 2 search from the AUTARG menu, Level 2 asks for the number of control files that should be used for search. Up to five such control files are allowed for one search run. Having started by simply marking the control file names on the corresponding file list, Level 2 begins to check for the first target compound in the first control file. This is done by redrawing the specified ion traces in a user-defined time window, usually 1 min, around the expected retention time of the target compound. Only if Level 2 has found peaks in the traces of both ions will it proceed to check whether peaks appear at the same retention time in both of the ion traces. If their difference in retention is smaller than 0.015 min, Level 2 assumes that the two ions originate from the same target compound and continues by taking a scan at the apex of the peak followed by background subtraction. This is performed by subtracting the scans taken at peak start and peak end. The resulting spectrum is then searched against a target compound library. When looking at the example illustrated in Fig. 5, it can be seen that the apex of the target compound ion traces does not fit exactly to the apex of the matrix compound peak. Therefore, the spectrum of the scan selected by Level 2 shows already a good correspondence to that of the target compound sought. Further background subtraction at peak start and peak end of the target compound eliminates the interfering ion m/z 149 almost completely, so that the library search now results in a hit quality of 99. In other cases, usually when the peak is very small, it may be preferable not to subtract any background from the current spectrum. Therefore library search is also performed without background subtraction. Only the better result is reported.

Level 2 then continues to search for the next target compound listed in the control file. When no more compounds are found in the current control file, Level 2 switches to the next control file. As mentioned above, a maximum number of five control files can be used for one Level 2 run with a total search capacity of up to 500 target compounds.

All positive search results are compiled in the AUTARG results Level 2 table with a complete sample description (sample name, corresponding data file, acquisition date, method file, operator), the names of the control files and the library used. Each peak with a positive search

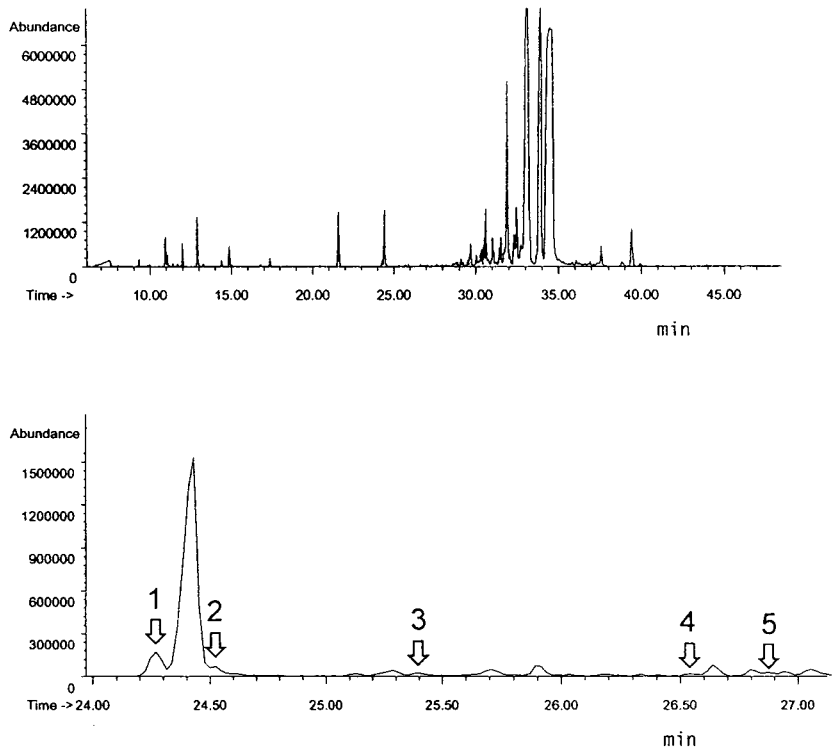

Fig. 7. TIC of groundwater sample. Top: Complete chromatogram. Bottom: Zoomed part with the peaks labeled which were found by AUTARG Level 2.

result is described by the scan number, the retention time at which the spectrum was taken and the three best hits.

A particular feature of Level 2 is also based on the RIC technique. Level 2 can find such peaks that are normally overlooked, being hidden in the noise. These peaks naturally are not integrated by AUTARG Level 1, or by any integration software, and as a consequence are not searched. Since the signal-to-noise ratio is, with most of the ion traces, orders of magnitudes better than with TIC, the presence of compounds can be spotted at very low concentration levels depending, the course, on the overall abundance of the fragment ions selected. Hence, we developed AUTARG Level 2 not only to support the recognition of a target compound overlapped by matrix compounds, but as a general supplement for AUTARG Level 1 in order to reduce the determination limit. This is demonstrated with the example below. Fig. 7 shows the TIC of a groundwater sample for which Level 1 reported no search results. Level 2 was therefore started, with a control file for phenoxy acid herbicides for further search, resulting in the recognition of five target compounds as listed in Table I.

The peaks were labelled with arrows in the zoomed part of the TIC.

Searching for target compounds in parts of a TIC where no analyst would suspect the presence of any target compounds, because no peaks can be seen (*e.g.* peaks 3, 4 and 5), makes Level 2 a very valuable tool. The reason why such peaks, which are easily overlooked in the TIC, can be recognized is the high signal-to-noise ratio in the traces of discrete target compound ions. In Fig. 8, as one example, the RIC traces of two ions of dichlorpropionic acid PFB ester (peak 5) are shown.

With a scan taken at the apex of the peak (Fig. 9, top) a library search was performed, which

TABLE I

TARGET PESTICIDES RECOGNIZED WITH AUTARG LEVEL 2 APPLYING A CONTROL FILE FOR PHENOXY ACID HERBICIDE PFB ESTERS

MCPA = 4-Chloro-*o*-tolyloxyacetic acid.

Peak	Target compound	Hit quality
1	2-(4)-Chlorophenoxy-2-methyl-propionic-acid PFB ester	98
2	2,4-Dichlorobenzoic acid PFB ester	96
3	Mecoprop PFB ester	83
4	MCPA PFB ester	42
5	Dichlorprop PFB ester	52

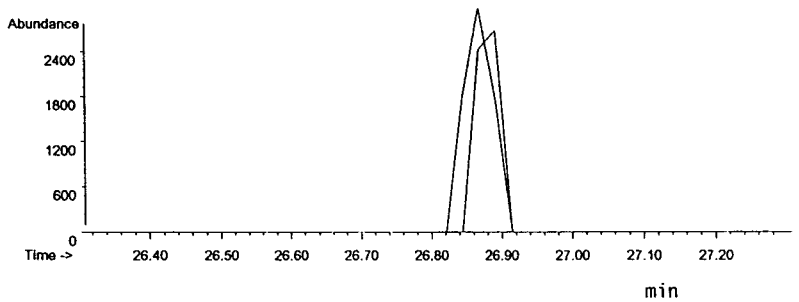

Fig. 8. RIC of two ions of dichlorprop-PFB-ester (*m/z* 414 and 162).

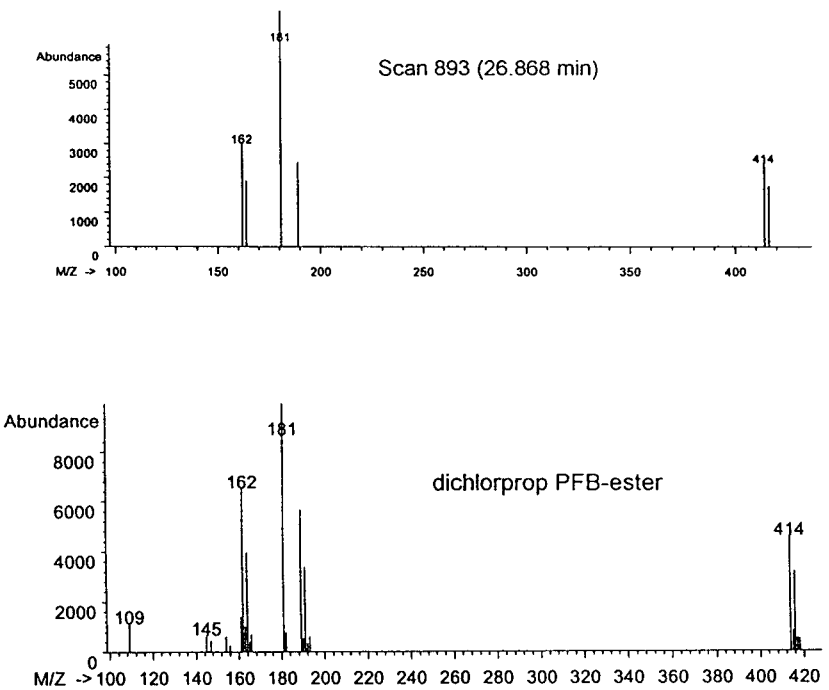

Fig. 9. Scan taken at the apex of the ion traces (top). Result of library search (bottom).

resulted in a hit quality of 53 for dichlorpropionic acid PFB ester (Fig. 9, bottom).

A visual inspection of the two mass spectra confirms their correspondence in the indicative ions.

CONCLUSIONS

Automated evaluation of GC–MS data from complex total ion chromatograms of environmental samples can be performed easily with the AUTARG program. The evaluation is executed in the short time usually necessary to re-equilibrate the GC oven for the next temperature-programmed analysis. The quality of the evaluation is constant throughout the analysis, in contrast to that of manual data evaluation. The use of the macro in combination with designated libraries containing only a limited number of target compounds leads to reliable results that can be verified very quickly by the analyst.

The application of reconstructed ion chromatograms of two characteristic ions with time window programming enables the recognition of target compounds overlapped by co-eluting matrix compounds. The method is also valid for target compounds at very low concentration levels, which produce peaks in the total ion chromatogram not exceeding the noise level.

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Journal of Chromatography A, 653 (1993) 55–62
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CHROMSYMP. 2901

Automated recognition of target compounds at low levels in environmental samples by means of capillary gas chromatography–mass spectrometry with dedicated mass spectral libraries and the macro program AUTARG

II. Application to pesticides in groundwater samples

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ABSTRACT

The use of the macro program AUTARG in the daily routine analysis of pesticides in groundwater was investigated. AUTARG Level 1 proved to be a valuable and reliable tool for the automated evaluation of GC–MS data. It is able to replace time-consuming manual evaluation by providing similar reliable results. AUTARG Level 2 is a powerful addition to Level 1, especially in trace level analysis, when looking for specific compounds using dedicated control files. It has been proved that the use of ion traces by Level 2 makes possible the detection of target compounds hidden in the chromatographic background. In our investigations, using an older GC–MS system, it has been shown that the limits of AUTARG are determined by the detection limits. Today, new GC–MS systems promise much lower detection limits. Using AUTARG for automated evaluation of scan chromatograms to analyse water samples according to the tolerances for drinking water of the European Community should present us with no problems.

INTRODUCTION

In Part I [1] of this paper we described the macro program AUTARG, which is designed to reduce the workload of analysts by automating the evaluation of full scan GC–MS chromatograms.

In this part, we wish to demonstrate the merits of this program in daily routine water analysis. The program was applied to a series of groundwater samples, which were screened for the most relevant pesticides. The study was performed with groundwater samples from the Berlin area, which usually contain a considerable amount of interfering humic substances. Therefore, recoveries were analysed for each individual water sample. The three classes of pesticides used for the recoveries were: (1) chlorinated hydrocarbons, (2) triazines and similar nitrogen-containing pesticides and (3) phenoxycarboxylic acids and other acidic herbicides.

In this paper we present a few examples from these field studies, in order to demonstrate the

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potential and limits of automated chromatogram evaluation using AUTARG Level 1 and AUTARG Level 2.

EXPERIMENTAL

Material

All pesticide standards were of analytical purity, purchased from Promochem, Wesel, Germany, or Pestanal quality from Riedel de Haen, Seelze, Germany. Sample vials, screw caps and septa were purchased from Zinsser, Frankfurt, Germany. Inserts of 200 μl for the sample vials were obtained from CS-Chromatographie Service, Langerwehe, Germany. Stock solutions of all compounds were prepared in toluene or methanol. Standards and samples were finally dissolved in toluene. All solvents were Pestanal products from Riedel de Haen. Pentafluorobenzylbromide was obtained from Aldrich, Steinheim, Germany. Triethylamine was purchased from Merck, Darmstadt, Germany. Solid-phase extraction (SPE) cartridges, 6 ml (polypropylene), and RP-18 material were obtained from Baker, Frankfurt, Germany. Adjustable transferpettors (1–10 μl and 10–100 μl) were from Brand, Wertheim, Germany.

Sample preparation

Chlorinated hydrocarbons and triazines (recoveries). The water samples (1 l) were spiked with the mixture of pesticides to achieve a concentration of 100 ng/l of each substance, and then extracted by liquid–liquid partition with 50 ml of dichloromethane. The neutral extract was evaporated and separated into two fractions by chromatography on small silica gel columns. The extracts were finally dissolved in 100 μl of toluene.

Phenoxycarboxylic acids (recoveries). A water sample of 1 l was spiked with a mixture of pesticides to achieve a concentration of 100 ng/l of each substance. The internal standard 2,4-dichlorobenzoic acid was added at twice the concentration level. The sample was then acidified to pH <2 with HCl. Each SPE cartridge was filled with 2 g of RP-18 adsorbent. Conditioning was performed successively with 5 ml of dichloromethane, 5 ml of methanol and finally 5 ml of distilled, deionized water. The solvents were drawn through the cartridges by means of a gentle vacuum and the cartridge was not permitted to run dry after addition of the water. The water sample spiked with the herbicides was then percolated through the cartridge at a flow-rate of *ca.* 8 ml/min. After drying the cartridge for 2–3 h under a gentle stream of nitrogen, the herbicides were eluted with 3 ml of dichloromethane and 5 ml of methanol. The eluate was dried under a gentle stream of nitrogen.

Derivatization was performed at 90°C using 200 μl of pentafluorobenzylbromide (2% in toluene) and 2 μl of triethylamine as catalyst. The derivatized sample was then dried under nitrogen and finally dissolved in 100 μl of toluene.

GC–MS parameters

All MS measurements were performed with an HP 5970 mass-selective detector combined with an HP 5890 gas chromatograph fitted with a 25 m × 0.2 mm I.D. × 0.33 μm HP-5 capillary column. The oven temperature was maintained at 100°C for 1 min following injection, then programmed at 30°C/min to 150°C, which was held for 1 min, then at 3°C/min to 205°C followed by 10°C/min to 260°C, which was held for 23 min. The injector and transfer line temperatures were 210 and 250°C, respectively, and 2-μl quantities of sample were injected by means of an HP 7673 autosampler using hot splitless injection with the split closed for 0.9 min.

Scan parameters: scanned mass range, 50–510; scan rate, 0.93 scans/s; solvent delay, 6 min.

Hardware and software requirements

These were as described in Part I [1].

RESULTS AND DISCUSSION

Since the tolerance for drinking water was fixed by the European Community Commission (EEC) and since it has been established by the individual European member states, it has become general practice to keep these tolerances in mind when developing methods for residue analysis in either groundwater or surface water. Therefore in our study, standard mixtures of

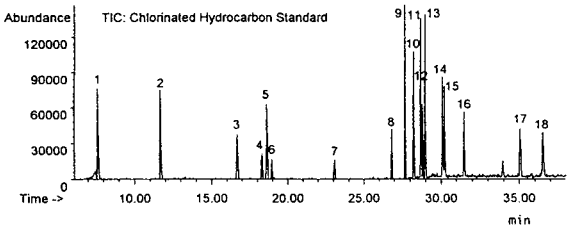

Fig. 1. TIC of the chlorinated hydrocarbon mixture containing eighteen pesticides (each at 1 ng/μl) as listed in Table I.

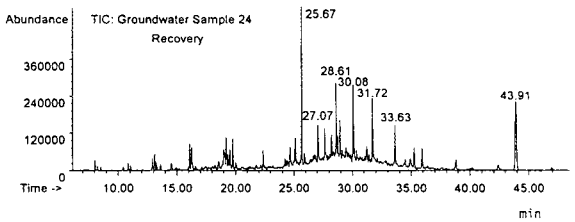

Fig. 2. Recovery of groundwater sample (No. 24) spiked with 100 ng/l of the chlorinated hydrocarbon mix.

pesticides considered relevant in water contamination were applied at a concentration level of 100 ng/l for recovery studies. Because of the detection sensitivity of a GC–MS system applying cyclic scanning, this is a demanding goal, as can be seen from the chromatograms of the standard pesticide mixtures. According to our lengthy experience of pesticide analysis, the recognition of 2 ng of pesticide injected, by means of library search, is a satisfactory result in routine analysis. This cannot, however, be expected to be achieved with all the 400 pesticides documented in the HPPEST Library [2].

Chlorinated pesticides

In Fig. 1, the total-ion current chromatogram (TIC) of a standard mixture of eighteen pesticides (1 ng/μl) is shown. Each standard was recognized, as shown in Table I.

Fig. 2 shows the chromatogram of a groundwater sample spiked with this standard mixture at a concentration of 100 ng/l. An analyst

TABLE I

RESULTS OF THE ANALYSES OF THE CHLORINATED HYDROCARBON STANDARD MIXTURE AND THE RECOVERY OF SAMPLE 24 BY MEANS OF AUTARG LEVELS 1 AND 2

Numbers in columns Level 1 and Level 2 indicate the match quality of the pesticide found by automated library search. HCH = Hexachlorocyclohexane.

Pesticide	Peak No.	Expected t_R (min)	Chlorinated hydrocarbon standard			Recovery (sample 24)			
			Level 1	Level 2	t_R (min)	Level 1	t_R (min)	Level 2	t_R (min)
Dichlobenil	1	7.65	91	91	7.65	Not id.	–	Not id.	–
Pentachlorobenzene	2	11.70	91	91	11.70	Not id.	–	Not id.	–
α-HCH	3	16.69	86	86	16.68	Not id.	–	56	16.62
β-HCH	4	18.30	34	34	18.30	Not id.	–	50	18.25
Lindane	5	18.65	45	45	18.64	78	18.56	72	18.56
Quintozene	6	18.97	72	72	18.98	Not id.	–	Not id.	–
Heptachlor	7	23.09	83	83	23.09	Not id.	–	Not id.	–
Heptachlorepoxid-*trans*	8	26.83	58	58	26.83	Not id.	–	47	26.75
o,p-DDE	9	27.70	99	96	27.70	96	27.64	96	27.64
Chlorfenson	10	28.26	94	94	28.26	91	28.21	91	28.21
p,p-DDE	11	28.71	99	99	28.71	Not id.	–	99	28.66
Dieldrin	12	28.83	53	89	28.83	47	28.76	47	28.77
o,p-DDD	13	29.00	96	96	29.00	50	28.94	96	28.94
p,p-DDD	14	30.11	64	64	30.11	Not id.	–	58	30.05
o,p-DDT	15	30.25	91	91	30.25	Not id.	–	90	30.19
p,p'-DDT	16	31.50	90	90	31.50	Not id.	–	Not id.	–
Tetradifon	17	35.08	94	90	35.08	Not id.	–	32	34.98
Mirex	18	36.54	78	83	36.54	Not id.	–	Not id.	–

performing manual evaluation usually starts by checking the largest peaks, which in Fig. 2 are labelled by their retention times. Comparing the abundance of these peaks with those of the standard mixture makes it clear that the pesticides are to be found among the smaller peaks. Using automated integration, a total of 64 peaks were integrated, which would make manual evaluation a time-consuming task. AUTARG Level 1 has shortened this procedure to a few minutes, recognizing five out of eighteen pesticides, even though these are among the smaller peaks, as documented in Table II. When not checking recovery samples, there is the risk of overlooking these small pesticide peaks because the analyst does not expect any particular pesticide in the sample.

AUTARG Level 2 using a control-file with its dedicated search for chlorinated hydrocarbons succeeded in recognizing twelve pesticides. This example illustrates the merits of Level 2: firstly, the recognition of peaks covered by matrix compounds and, secondly, the recognition of peaks due to the much better signal-to-noise ratio found with single-ion chromatograms. It is obvious that advantage can be taken of this fact when searching for defined pesticides. The results of the evaluation of the spiked groundwater sample No. 24 obtained by automated library searching using first AUTARG Level 1 and then the AUTARG Level 2 program is also compiled in Table I.

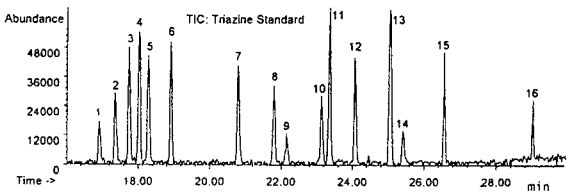

Fig. 3. TIC of the triazine standard mixture (each at 1 ng/μl).

Triazines

The performance of AUTARG was next tested using a standard mixture of sixteen triazines. Fig. 3 shows the TIC of the triazine standard mixture containing 2 ng of each pesticide injected. Fifteen out of the sixteen pesticides were identified with the "Autointegration" option of the integration software. However, the small peak labelled 9 was not recognized in the inte-

TABLE II

RESULTS OF THE ANALYSES OF THE TRIAZINE STANDARD MIXTURE AND THE RECOVERY OF GROUNDWATER SAMPLE 23 BY MEANS OF AUTARG LEVELS 1 AND 2

Pesticide	Peak No.	Expected t_R (min)	Triazine standard mixture			Recovery (sample 23)			
			Level 1	Level 2	t_R (min)	Level 1	t_R (min)	Level 2	t_R (min)
Simeton	1	16.93	78	78	16.93	Not id.	–	Not id.	–
Atraton	2	17.37	91	91	17.38	Not id.	–	58	17.36
Prometon	3	17.76	91	97	17.76	68	17.73	91	17.76
Atrazine	4	18.04	95	95	18.04	38	18.00	53	18.01
Propazine	5	18.30	90	90	18.30	94	18.30	94	18.3
Terbuthylazine	6	18.93	94	94	18.93	94	18.90	97	18.9
Sebuthylazine	7	20.81	90	90	20.81	59	20.79	53	20.8
Desmetryn	8	21.81	78	78	21.82	Not id.	–	Not id.	–
Metribuzine	9	22.17	Not id.	40	22.17	Not id.	–	Not id.	–
Ametryn	10	23.14	72	64	23.15	46	23.13	76	23.12
Prometryn	11	23.37	95	95	23.37	90	23.35	90	23.35
Terbutryn	12	24.09	40	40	24.09	7	24.06	53	24.08
Metolachlor	13	25.06	78	78	25.06	50	25.04	59	25.03
Triadimefon	14	25.43	Not id.	Not id.	–	32	25.38	32	25.38
Metazachlor	15	26.57	90	83	26.57	Not id.	–	Not id.	–
Methoprotryne	16	29.03	72	64	29.03	Not id.	–	Not id.	–

gration procedure, because it was excluded by the automatic threshold setting. While this peak was correctly identified with AUTARG Level 2 as metribuzine, neither AUTARG Level 1 nor Level 2 was able to find triadimefon under peak 14. When investigating the mass spectrum manually, however, several ions characteristic of triadimefon can be revealed, namely *m/z* 208, 181 and 57. In this particular case, the problem is not with the AUTARG program but with the search software, which for some reason comes to the conclusion that the spectrum recorded does not suffice for a positive library search. Even multiple manual attempts did not reverse this unwanted result. The triadimefon case appears even more confused when examining the recovery sample produced with the same standard mixture added to groundwater containing many matrix compounds. In the gas chromatogram of this particular sample many matrix compounds elute at similar retention times to triadimefon but are well separated, so that AUTARG Level 1 and Level 2 both identified the small peak at 25.38 min as triadimefon.

This case is reported as a representative example of our daily routine work to illustrate the well-known problem in environmental trace analysis with GC–MS and cyclic scanning that trace compounds may be overlooked by unfavourable parameter setting. On the other hand, data processing and storage capacity makes it necessary to filter out unnecessary information from the acquisition signals.

The chromatogram of the triazine recovery sample (Fig. 4) is again dominated by peaks of matrix compounds (peaks labelled), even though AUTARG Level 1 is able to recognize ten out of sixteen pesticides. Once again the specific analysis of Level 2 gives a better result, recognizing eleven pesticides with mostly a better quality of library search than that of Level 1 (see Table II).

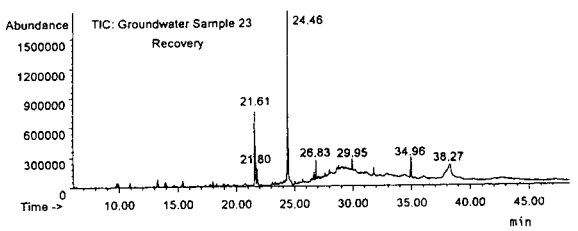

Fig. 4. TIC of groundwater sample No. 23, spiked with 100 ng/l of the triazine standard mixture.

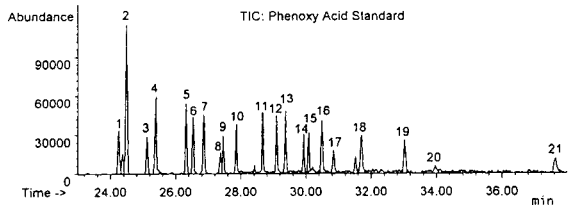

Fig. 5. TIC of the phenoxycarboxylic acid standard mixture (1 ng/μl each, except for the internal standard, which was 2 ng/μl).

Phenoxycarboxylic acids and other acidic herbicides

This group of pesticides has to be derivatized prior to GC analysis. With pentafluorobenzylic esters generated from the standard mixture, the chromatogram shown in Fig. 5 was produced. 2,4-Dichlorobenzoic acid (peak No. 2) was used as internal standard with a concentration twice as high as the other compounds. Fig. 6 shows a chromatogram for the ion trace *m/z* 181, which is typical of all pentafluorobenzylic esters, and also flurenol-butyl. Usually the ion at *m/z* 181 is base peak in the mass spectra of these compounds; flamprop is the only exception, with the ion at *m/z* 105 as base peak.

Analysing this group of pesticides using this type of GC–MS system, the nearness to the detection limit is plain to see from the chromatograms shown in Figs. 5–7. As can be deduced from Table III, 4 out of 21 compounds could not be identified by either AUTARG Level 1 or Level 2. The reason for this has already been

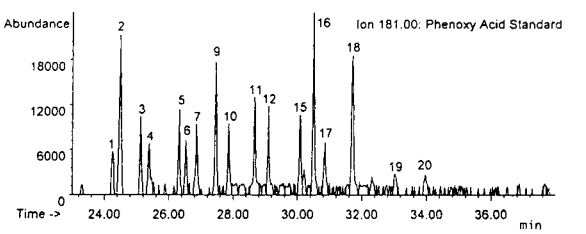

Fig. 6. RIC for *m/z* 181 of the phenoxycarboxylic acid standard, characteristic of all pentafluorobenzylic esters and flurenol-butyl.

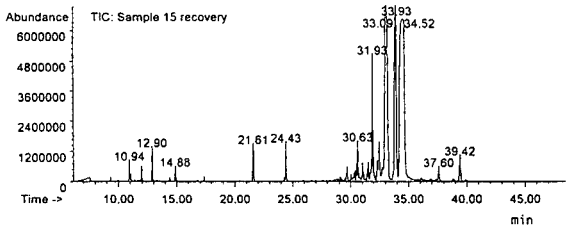

Fig. 7. TIC of the groundwater sample No. 15, spiked with 100 ng/l of the phenoxycarboxylic acid standard mixture and 200 ng/l internal standard.

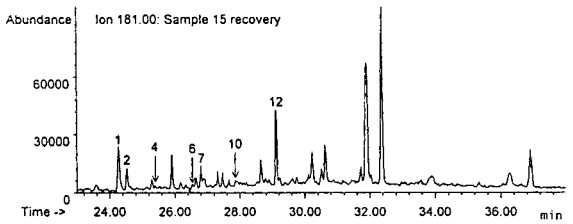

Fig. 8. RIC of m/z 181 of the recovery of groundwater sample No. 15.

referred to in the Triazines section. The compound peaks appear clearly in the chromatogram of the TIC (Fig. 5), but the mass spectra of these four compounds are not sufficient for a successful recognition in the mass spectral library. These spectra consist of 3–5 characteristic masses, but the information is obviously not sufficient for the library search to give positive results.

In Figs. 7 and 8, the TIC and the corresponding reconstructed ion chromatogram (RIC) for m/z 181 of the recovery experiment with groundwater sample No. 15 are shown. With AUTARG Level 1, not one single pesticide was

TABLE III

RESULTS OF THE ANALYSES OF THE PHENOXYCARBOXYLIC ACID STANDARD MIXTURE AND THE RECOVERY OF GROUNDWATER SAMPLE 15 BY MEANS OF AUTARG LEVELS 1 AND 2

2,4-DB = 4-(2,4-dichlorophenoxy) butyric acid; MCPA = 4-chloro-*o*-tolyloxyacetic acid; MCPB = 4-(4-chloro-*o*-tolyloxy)butyric acid.

Pesticide	Peak No.	Expected t_R (min)	Phenoxycarboxylic acid standard			Recovery sample 15			
			Level 1	Level 2	t_R (min)	Level 1	t_R (min)	Level 2	t_R (min)
2-(4)-Chlorophenoxy-2-methylpropionic acid	1	24.26	83	83	24.26	Not id.	–	98	24.28
2,4-Dichlorobenzoic acid	2	24.49	99	98	24.50	Not id.	–	96	24.53
Clopyralid	3	25.13	83	83	25.13	Not id.	–	Not id.	–
Mecoprop	4	25.39	97	97	25.40	Not id.	–	83	25.41
Dicamba	5	26.33	95	95	26.32	Not id.	–	Not id.	–
MCPA	6	26.54	94	94	26.54	Not id.	–	42	26.56
Dichlorprop	7	26.87	91	91	26.86	Not id.	–	58	26.87
Chlorflurenol	8	27.37	90	90	27.39	Not id.	–	Not id.	–
Flurenol-butyl	9	27.46	83	83	27.47	Not id.	–	Not id.	–
2,4-D	10	27.86	94	95	27.87	Not id.	–	50	27.88
Triclopyr	11	28.67	95	95	28.67	Not id.	–	Not id.	–
Fenoprop	12	29.10	91	91	29.11	Not id.	–	40	29.12
Fluazifop-*p*-butyl	13	29.38	94	94	29.38	Not id.	–	Not id.	–
Flammprop-isopropyl	14	29.94	Not id.	Not id.	–	Not id.	–	Not id.	–
2,4,5-T	15	30.09	83	91	30.09	Not id.	–	Not id.	–
MCPB	16	30.50	74	74	30.50	Not id.	–	Not id.	–
Fluroxypyr	17	30.86	72	83	30.87	Not id.	–	Not id.	–
2,4-DB	18	31.71	Not id.	Not id.	–	Not id.	–	Not id.	–
Fluazifop	19	33.04	59	59	33.03	Not id.	–	Not id.	–
Picloram	20	33.98	Not id.	Not id.	–	Not id.	–	Not id.	–
Flamprop	21	37.68	Not id.	Not id.	–	Not id.	–	Not id.	–

recognized, as can be deduced from Table III. Although the abundance of the pesticides is low and some peaks are covered by matrix compounds, with Level 2 seven out of 21 compounds were recognized. The limitation of positive identification by AUTARG Level 2 is again determined by the inadequacy of the mass spectral data passing the noise filter.

Fig. 8 demonstrates how AUTARG Level 2 works. In the RIC of ion trace m/z 181 the pentafluorobenzylates of the acidic pesticides appeared mostly as relatively small peaks but their integration was still possible. The compounds that were found by AUTARG Level 2 are labelled. The 2-(4)-chlorophenoxy-2-methylpropionic acid (peak No. 1) and the internal standard (peak No. 2) were hidden in the TIC by a phthalate peak at 24.43 min. In the reconstructed ion trace, the overlaying of this phthalate peak is not visible. Fenoprop, the large peak No. 12, is covered by a matrix peak in the TIC. The peaks representing three other pesticides (4, 6 and 10) seem to disappear in the noise. Since, as described in Part I, Level 2 works with two ion traces for each compound, the second trace may be even more characteristic, but is generally lower in abundance than mass 181. This example demonstrates the advantages of Level 2 in comparison with Level 1.

Finally, the results of analysing the compounds of three different pesticide classes by means of AUTARG Levels 1 and 2 are summarized in Table IV. The results do not only support our estimation of the value of these tools in daily routine but also demonstrate the differing detection limits observed with the various classes of chemical structures using GC–MS. The experimental approach applied in this study to demonstrate the merits of the macro program AUTARG is also useful for reviewing the detection sensitivity of any target compound group using full-scan mode, identifying those that absolutely require selected-ion monitoring (SIM) for achieving the detection sensitivity needed.

The results obtained using AUTARG for pesticide residue analysis also give a brief overview of the percentage of those pesticides that can be found in water samples above the tolerance level established by the EEC for drinking water when applying cyclic scan mode with our GC–MS system, which represents the first generation of mass selective detectors. All other pesticides or target substances not meeting these criteria have to be analysed separately by means of a SIM programme using defined time windows. This means not only additional analysis time depending on the number of target compounds, but also loss of information with a possible reduction in reliability.

As a consequence, for use in a field study now under way, in addition to the methods described here, a SIM method has been developed for phenoxyalkanoic acids [3] achieving much lower detection limits with the same instrumentation. Furthermore, sample preparation by solid-phase extraction has been improved, with excellent recovery rates for 30 acidic herbicides being reported [4]. This method used for routine analysis offers detection limits for these compounds in drinking water of 1–10 ng/l.

TABLE IV

RESULTS OF THE ANALYSES OF THREE PESTICIDE CLASSES AT A CONCENTRATION LEVEL OF 100 ng/l USING AUTARG LEVEL 1 AND AUTARG LEVEL 2

Classification	Number	Standard/recovery	AUTARG Level 1		AUTARG Level 2	
			No. identified	Identified (%)	No. identified	Identified (%)
Chlorinated hydrocarbons	18	Standard	18	100	18	100
		Recovery (sample 24)	5	28	12	67
Triazines	16	Standard	14	88	15	94
		Recovery (sample 23)	10	63	11	69
Phenoxycarboxylic acids	21	Standard	17	81	17	81
		Recovery (sample 15)	0	0	7	33

CONCLUSIONS

AUTARG Level 1 is a valuable and reliable tool for the automated evaluation of GC–MS data. It replaces the time-consuming manual evaluation by providing similar reliable results. It can be performed immediately after data acquisition using the time elapsed before the next injection.

AUTARG Level 2 is a powerful addition to Level 1, especially in trace level analysis, when looking for specific compounds by using dedicated control files. It has been proved that the use of ion traces by Level 2 makes possible the detection of target compounds hidden in the chromatographic background. This is because the signal-to-noise ratio is much better than that of a TIC and, secondly, because the ion traces suppress overlaying matrix compounds, so that hidden target compound peaks can be found.

In our investigations using an older GC–MS system it has been shown that the limits of AUTARG are determined by the detection limits. Today, new GC–MS systems promise much lower detection limits. Using AUTARG for automated evaluation of scan chromatograms to analyse water samples according to the tolerances for drinking water of the European Community should present the analyst with no problems.

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Chemically bonded chelates as selective complexing sorbents for gas chromatography

II. Ketones, ethers and nitroalkanes

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ABSTRACT

Specific interactions of ketones, ethers and nitroalkanes with Cu(II) and Ni(II) acetyloacetonates chemically bonded to silica surfaces were investigated. A number of relations between the structure of adsorbates and the retention parameters were discovered and described. The differences in specific interactions were sufficient to enable the separation of isomer-containing mixtures.

INTRODUCTION

In the last 20 years a significant number of papers on the application of β-diketonates of the *d*- and *f*-block elements in gas chromatography have been published. These complexes are actively added to liquid stationary phases (in particular, squalane and SE-30). Such systems have been studied by Schurig and co-workers [1–5], Picker and Sievers [6,7] and others [8–13].

A characteristic feature of the packings described in the above-mentioned papers is a proper metal β-diketonate dissolved in a non-polar or weakly polar liquid stationary phase.

Such packings are used in both classical analytical columns and capillary columns as well as in precolumns. In the last case the packings were used to retain strongly nucleophilic compounds (such as alcohols, amines, ketones and aldehydes). The utility of these sorbents was demonstrated by the analysis of cigarette smoke, volatile constituents of urine [7–9] and a cologne essence [6].

Another example of an application testifying to the high selectivity of metal β-diketonates dissolved in squalane is separation of the geometric isomers of butene and pentene [14].

Packings containing nickel(II) bis[3-(heptafluorobutanoyl)-(1*R*)-camphorate] are capable of separating enantiomers, which indicates their high enantioselectivity [5]. Columns containing lanthanide chelate sorbents are also effective in the analysis of volatile compounds of waste water [6]. It should be emphasized that this type of packing is characterized by high thermal stability, which permits their application at a temperature of 225°C, and in some cases even at 300°C [8].

As follows from a brief survey of the properties of packings containing metal β-diketonates, by setting appropriate parameters for the operation of the columns these packings may be used in two different ways. Firstly, as they are highly selective they may be employed in direct chromatographic analysis (including analysis of optically active compounds). Secondly, they may

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be used as selective traps for a wide range of organic compounds. The latter application —because of the high selectivity of these packings— enables the analysis of complex mixtures, and to obtain a number of simpler chromatograms instead of one complex chromatogram. If particular compounds are of interest, it is possible to analyse only the trap-sorbent on which they are retained.

The sorbents studied by us [15] differ from those mentioned above in that appropriate metal β-diketonates [$Cu(acac)_2$ and $Ni(acac)_2$, where acac = acetylacetonate] were chemically bonded with the silica surface (Porasil C) via diphenylphosphine groups. Packings containing such bonds, despite having a considerably lower amount of active component (β-diketonate), proved to be able to interact specifically with unsaturated hydrocarbons. In the present paper their interactions with ethers, nitroalkanes and ketones are examined.

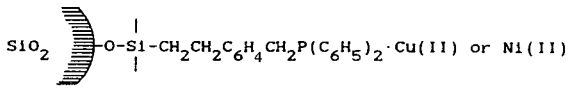
EXPERIMENTAL

Apparatus

Chromatographic measurements were carried out on a GCHF 18.3 gas chromatograph manufactured by Chromatron (Berlin, Germany), equipped a with flame ionization detector and a digital thermometer (Slandi, Warsaw, Poland) for measuring the column temperature. Argon dried over a molecular sieve 4 Å was used as the carrier gas. The flow-rate of the carrier gas was measured using a digital flowmeter (J & W Scientific, Folsom, CA, USA).

Packings of columns

Copper(II) and nickel(II) acetylacetonates were bonded to the surface of silica (Porasil C, 80–100 mesh (177–149 μm), Waters, Milford, MA, USA) via diphenylphosphine groups. Phosphinated silica was obtained as described previously [15]. The specific surface areas of the investigated packings were 85 and 83 m^2/g for packings containing Cu(II) and Ni(II), respectively. The surface concentration of silanes was 4.01 $\mu mol/m^2$. The method of preparing packings containing chemically bonded β-diketonate phases has been published previously (in Part I [15]). The investigated packings can be shown schematically as follows:



RESULTS AND DISCUSSION

From the literature, chemically bonded β-diketonates of transition metals are used in three different types of studies: (i) study of the structure of the chemically bonded β-diketonates and of the adducts formed with additional ligands (CO, pyridine) [16], (ii) application in gas chromatography and high-pressure liquid chromatography as a component of chemically bonded phases [15,17], (iii) application as trap-packings for a wide range of organic compounds [6–9].

The ability to form adducts with additional ligands showing nucleophilic properties was used in the analysis of alkenes [15]. As can be seen in chromatograms shown in Figs. 1–3, these packings may also be used in the analysis of ketones, ethers and nitroalkanes.

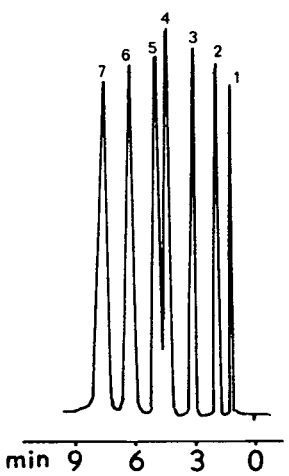


Fig. 1. Separation of a mixture of ketones on a $Ni(acac)_2$ packing. Column temperature, 155°C; carrier gas flow-rate, 20.2 ml/min. Peaks: 1 = propanone; 2 = 2-butanone; 3 = 3-pentanone; 4 = 3-methyl-2-butanone; 5 = 2-pentanone; 6 = 4-methyl-2-hexanone; 7 = 2-hexanone.

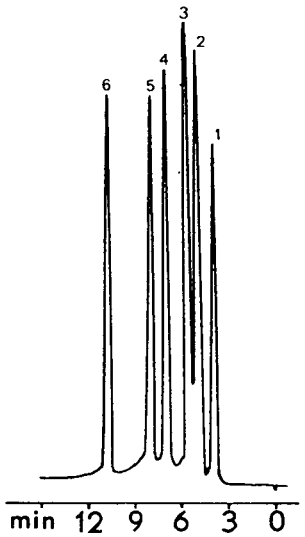


Fig. 2. Analysis of nitroalkanes on a $Cu(acac)_2$ packing. Column temperature, 118.1°C; carrier gas flow-rate, 20 ml/min. Peaks: 1 = nitromethane; 2 = nitroethane; 3 = 2-nitropropane; 4 = 1-nitropropane; 5 = 2-nitrobutane; 6 = 1-nitrobutane.

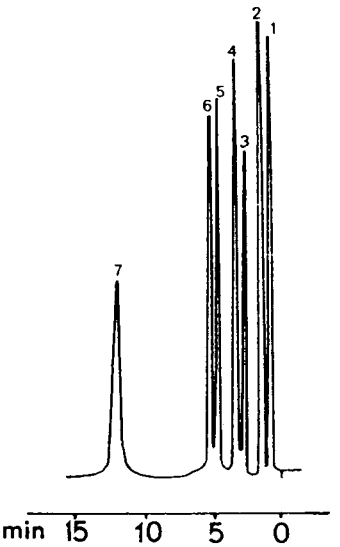


Fig. 3. Separation of mixture of cyclic and aliphatic ethers. Packing as in Fig. 1. Column temperature, 156.7°C; carrier gas flow-rate, 18.8 ml/min. Peaks: 1 = furan; 2 = thiophene; 3 = diethyl ether; 4 = 3,4-dihydro-2H-pirane; 5 = methyl butyl ether; 6 = dipropyl ether; 7 = tetrahydrofuran.

Ketones

Owing to free electron pairs at the oxygen atom, the carbonyl group in ketone molecules is able to interact specifically with the electron-acceptor centre on the surface of packings. These interactions are affected by at least three factors: location of a carbonyl group in the molecule, the presence of additional electron-donor centres (*e.g.* unsaturated bonds) and substituents in the hydrocarbon chain. The more the carbonyl group is shifted towards the centre of the molecule, the weaker are the interactions. This can be deduced from a comparison of k', I, ΔM_e and V_g for such pairs of ketones as 2- and 3-pentanone, 2- and 3-hexanone and 2- and 3-heptanone. This effect is particularly pronounced for the first two pairs. On the other hand, in the case of linear C_7 ketones, the difference in ΔM_e values for 2-heptanone-2 and 3-heptanone is significantly smaller than that for C_6 ketones and is similar to that for C_5 ketones. This leads to the conclusion that chains longer than C_6 causes a considerable steric hindrance (Table I).

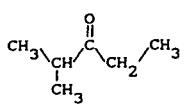
Solutes are listed in Table I by increasing boiling point. However, comparing V_g, k' or I values of both packings studied results in a different sequence that depends on solute structure and electron-donor properties. For this reason compounds with lower boiling points are frequently eluted after compounds with higher boiling points.

This also holds true for branched ketones (*e.g.* 2-methyl-3-pentanone is eluted before 4-methyl-2-pentanone). Introducing methyl substituents into the hydrocarbon chain, because of the increased steric effect, reduces the degree of interaction between the carbonyl group and the metal. This is illustrated by a pair of ketones, 3-methyl-2-butanone and 3,3-dimethyl-2-butanone, for which the ΔM_e values are 30.7 and 20.1 for $Cu(acac)_2$ and 80.9 and 61.1 for $Ni(acac)_2$, respectively. Moreover, it was found out that the greater the distance between the substituent and the >C=O group, the weaker its influence (*e.g.* 4-methyl-2-hexanone interacts more weakly with the packing than 5-methyl-2-hexanone). By comparing the values of ΔM_e for two isomers, 2-methyl-3-pentanone (a) and 4-methyl-2-pentanone (b):

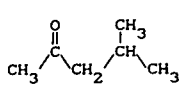
TABLE I

RETENTION PARAMETERS: SPECIFIC RETENTION VOLUMES (V_g), CAPACITY FACTOR (k'), RETENTION INDICES (I), MOLECULAR RETENTION INDICES (ΔM_e) AND MOLAR REFRACTION FOR LINEAR, BRANCHED AND UNSATURATED KETONES

	Packing								Molar refraction (R_M)
	$Cu(acac)_2$				$Ni(acac)_2$				
	V_g (cm^3/g)	k'	I	ΔM_e	V_g (cm^3/g)	k'	I	ΔM_e	
Propanone	3.30	1.92	753	49.5	9.24	4.49	1064	93.2	16.18
2-Butanone	4.39	2.56	815	44.2	11.20	5.44	1109	85.4	21.30
3-Buten-2-one	4.64	2.70	826	47.8	11.49	5.88	1115	88.2	20.69
3-Methyl-2-butanone	4.47	2.61	819	30.7	15.09	7.33	1177	80.9	25.24
3-Pentanone	4.83	2.82	835	33.0	10.47	5.08	1094	69.3	25.21
2-Pentanone	5.54	3.23	864	37.1	15.52	7.54	1183	81.8	25.33
3,3-Dimethyl-2-butanone	5.02	2.92	843	20.1	13.15	6.11	1135	61.1	30.07
2-Methyl-3-pentanone	5.62	3.27	867	23.4	12.58	6.39	1145	62.4	29.74
4-Methyl-2-pentanone	6.51	3.79	900	28.1	19.14	9.30	1269	79.8	30.08
3-Hexanone	6.68	3.89	905	28.8	15.31	7.41	1180	67.4	29.80
2-Hexanone	8.20	4.78	946	34.5	24.72	12.02	1349	91.1	30.01
4-Methyl-3-penten-2-one	6.38	3.72	895	29.4	31.79	11.45	1428	104.1	30.08
Cyclopentanone	11.58	6.75	1017	60.5	35.59	17.29	1482	125.7	23.12
4-Methyl-2-hexanone	10.19	5.94	981	26.8	21.13	10.27	1309	71.4	–
4-Heptanone	9.25	5.39	988	24.1	22.68	11.02	1327	73.9	34.40
5-Methyl-2-hexanone	11.72	6.82	1020	30.1	37.64	18.29	1506	99.1	31.60
3-Heptanone	10.05	5.86	1002	27.8	24.43	11.87	1346	76.6	34.47
2-Heptanone	10.71	6.24	1047	28.3	31.06	15.09	1417	86.3	34.18
Cyclohexanone	14.58	8.50	1066	53.4	51.64	25.09	1624	131.6	27.85
5-Methyl-3-heptanone	12.86	7.44	1039	19.5	32.13	15.66	1434	74.9	38.94
2-Methylcyclohexanone	16.11	9.39	1088	42.4	54.82	26.64	1648	120.9	32.48
3-Methyl-2-heptanone	15.34	8.94	1077	24.8	49.22	23.81	1605	98.9	39.24
2,6-Dimethyl-4-heptanone	15.19	9.85	1075	10.5	31.33	15.22	1421	59.1	41.85
3-Methylcyclohexanone	18.04	10.22	1111	46.6	58.05	28.21	1670	124.0	32.66
4-Methylcyclohexanone	18.89	11:02	1121	47.1	62.51	30.38	1699	128.1	32.69



(a)



(b)

one can seen that they indeed reflect the abilities of particular adsorbates to interact with the packings. In the case of 2-methyl-3-pentanone (a) two factors contributed to the inhibition of the contact between the carbonyl group and metal. The carbonyl group is centrally located, while the methyl substituent is in the close vicinity. In the case of compound (B), the carbonyl group and the substituent take extreme positions, which is reflected in the values of retention parameters.

If an additional function (such as an unsaturated bond) is introduced into the ketone molecule, estimation of the influence of particular parameters on retention becomes more difficult and complicated. As follows from Table I, the presence of an unsaturated bond causes an increase in specific interactions. The strongest interactions were observed for cyclic ketones, as shown by high values of molecular indices of retention. In the case of linear ketones, the interactions become weaker with increase in the length of carbon chain, the strongest being observed (taking into account ΔM_e) for 2-pro-

panone. On the basis of the data obtained for both studied packings, it was found that the packing modified with nickel(II) acetylacetonate interacts more strongly with nucleophilic adsorbates than the packing modified with $Cu(acac)_2$. The dependence of log k' on molar refraction, R_M, shown in Fig. 4 (which is a function of the molecule polarizability), is linear, and the distance between the straight lines for both packings under study points to a relative increase in specific interactions when going from $Cu(acac)_2$ to $Ni(acac)_2$. For the sake of comparison, the series of straight lines for *n*-alkanes (C_5–C_9) is presented.

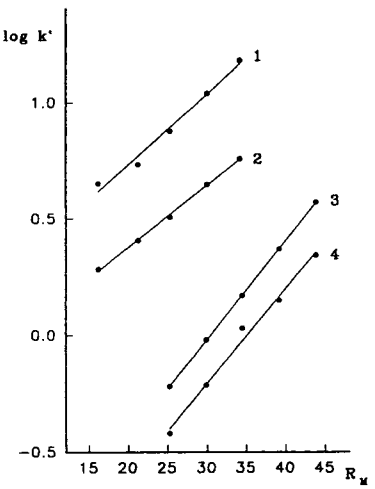


Fig. 4. The dependence of log k' on molar refraction for *n*-ketones C_3–C_7 (lines 1 and 2) and *n*-alkanes C_5–C_9 (lines 3 and 4). Lines 1 and 4 are for $Ni(acac)_2$ packing, and lines 2 and 3 are for $Cu(acac)_2$ packing.

Nitroalkanes

A nitrogen atom in the nitric group has an sp^2 hybridization, yielding three co-planar σ bonds (one C–N and two N–O bonds). The remaining two p electrons at the nitrogen atom and individual electrons at two oxygen atoms form a π bond. Owing to such an electron distribution in the nitric group, the nitrogen atom is positively charged, while the oxygen atoms are negatively charged, according to the scheme given below:

Because of the polar character of the nitric group, nitroalkenes are characterized by high dielectric constant ε (in the range 25–35) and high dipole moments of about 3.6 D, which is reflected by long retention times. The results obtained are presented in Table II.

The high electron density on the protruding part of the molecule provides favourable conditions for electron donor–acceptor interactions. This is shown by high values of retention parameters, and in particular in the values of the

TABLE II

RETENTION PARAMETERS AND DIELECTRIC CONSTANTS (ε) FOR NITROALKANES

Solute	ε	Packing							
		$Cu(acac)_2$				$Ni(acac)_2$			
		V_g (cm^3/g)	k'	I	ΔM_e	V_g (cm^3/g)	k'	I	ΔM_e
Nitromethane	35.87	9.31	1.99	781	50.5	23.72	10.4	1242	115.1
Nitroethane	28.06	10.45	2.24	806	39.9	29.15	12.77	1301	109.4
1-Nitropropane	23.24	12.97	2.78	852	32.4	36.15	15.84	1365	104.3
2-Nitropropane	25.52	10.48	2.25	807	26.1	31.32	13.72	1322	98.3
1-Nitrobutane	–	18.68	4.01	903	29.3	47.55	20.83	1452	102.5
2-Nitrobutane	–	14.73	3.16	880	22.3	39.37	17.25	1392	94.1
1-Nitropentane	–	24.19	5.15	984	22.8	63.43	27.80	1547	101.8
2-Nitropentane	–	19.54	4.16	938	16.4	48.12	21.09	1456	89.1

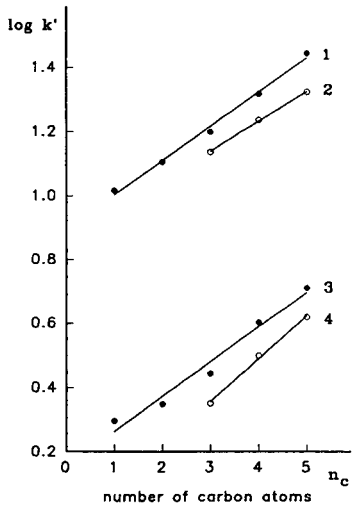


Fig. 5. Log k' of nitroalkanes *vs.* number of carbon atoms. Lines 1 and 2 are for $Ni(acac)_2$, and lines 3 and 4 are for $Cu(acac)_2$. ● = 1-Nitroalkanes (C_1–C_5); ○ = 2-nitroalkanes (C_3–C_5).

molecular retention index. The order of elution is affected by the position of the nitro group in the adsorbate molecule; thus the isomer with the functional group located inside the molecule is eluted first, *i.e.* 2-nitropropane before 1-nitropropane, or 2-nitrobutane before 1-nitrobutane, etc.

As for ketones, the strongest interactions were observed for small adsorbate molecules, and they tended to decrease from nitromethane to nitropropane or, in other words, with increasing length of carbon chain.

The k' values increased in the direction from $Cu(acac)_2$ to $Ni(acac)_2$, on average five-fold, while the values of the retention indices changed by 400–500 retention index units. Examples of chromatograms presented in Fig. 2, obtained for mixtures of nitropropane and nitrobutane isomers, show that the selectivity of acetylacetonate-containing packing is high enough to obtain a complete separation of isomers in a very short period of time. The separation is better than that obtained on packings containing SE-30, polyethylene glycol adipate (PEGA) or Porapak R [18]. The dependence of log k' on the amount of carbon atoms for particular nitroalkanes is shown in Fig. 5.

Ethers

Aliphatic and cyclic ethers belong to a class of monodentate ligands, owing to the basic nature of oxygen, with two electron pairs. There are known stable complexes of ethers (ethyl ether, furan) with boron(III) and aluminium(III) fluorides as well as magnesium(II), tin(II) and titanium(IV) halides [19]. Examples of ethers used in the present paper as adsorbates are listed in Table III.

The results obtained enabled us to establish a

TABLE III

RETENTION PARAMETERS FOR LINEAR AND CYCLIC ETHERS

Solute	Packing							
	$Cu(acac)_2$				$Ni(acac)_2$			
	V_g (cm^3/g)	k'	I	ΔM_e	V_g (cm^3/g)	k'	I	ΔM_e
Diethyl ether	3.75	0.80	588	10.36	10.90	8.73	1011	69.79
Butyl methyl ether	6.35	1.35	702	12.32	18.80	15.06	1119	70.83
Dipropyl ether	7.52	1.60	735	2.92	2.92	22.36	1153	61.56
Dibutyl ether	18.69	3.98	929	2.12	28.77	8.38	1079	51.27
Diisoamyl ether	34.52	7.36	1058	−7.86	31.58	25.30	1212	13.72
Diamyl ether	46.97	10.01	1123	1.24	45.08	36.12	1274	22.41
Furan	3.26	0.69	557	12.06	1.29	1.03	585	15.98
Tetrahydrofuran	47.50	10.13	1125	87.69	45.45	36.41	1280	103.50
3,4-Dihydro-2H-pirane	6.30	1.28	690	14.67	9.30	7.45	980	55.36

few relationships between the retention parameters and the structure of ethers. Because the angle C–O–C in aliphatic ethers is 110°, free access to a lone electron pair will be the more difficult the longer are the carbon chains connected to oxygen. This will undoubtedly affect the value of specific interactions, which will decrease with increasing (or branching) of the hydrocarbon chains. As can be seen in Table III, the ΔM_e values for isoamyl ether are negative for $Cu(acac)_2$ and low (13.7) for $Ni(acac)_2$. This testifies to a significant influence of the steric effect on specific interactions between ethers and a metal complex chemically bonded to SiO_2.

The strength of the influence of electron pairs at the oxygen atom on specific interactions can be seen by comparing the values of retention parameters for furan and tetrahydrofuran. In the case of the former, oxygen, by assuming the sp^2 hybridization, becomes a donor of two electrons to the aromatic sextet. The presence of these electrons in this sextet implies that they will be partly distributed all over the ring, as a result of which the ring will have a negative charge, while the oxygen will assume a partial positive charge. Therefore, specific interactions with a planar ring of aromatic compounds will be much more dependent on the steric effects than it is in the case of tetrahydrofuran, in which electron pairs are located on oxygen. The chromatogram of a mixture of linear and cyclic ethers, shown in Fig. 3, is characterized by sharp and symmetric peaks, which is indicative of a large degree of homogeneity of the packing surface.

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Development of hydrocarbon gas standards

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ABSTRACT

Methodology is described for the gravimetric preparation and analytical evaluation of accurate, stable, multicomponent gas standards in compressed gas cylinders containing C_2–C_{10} alkane, alkene and aromatic hydrocarbons in pure nitrogen or air. Standards have been prepared containing up to fourteen hydrocarbons in a single mixture at concentrations ranging from 5–2000 nmol/mol (ppb). Analysis of hydrocarbons at the low ppb level requires cyrogenic preconcentration. Depending on the combination of hydrocarbons in any one gas mixture, several analytical gas chromatographic columns may be required to achieve baseline separations of all the compounds. The sum of preparative and analytical error components of the uncertainty associated with the concentrations of the hydrocarbons at the 95% confidence level typically ranges from 0.5–5.0%. This total uncertainty depends on the concentration level and the hydrocarbon. Intercomparative analyses of new and previously prepared standards have verified that such mixtures are stable for at least nine months, with the exception of ethyne (acetylene) which has a stability of less than nine months.

INTRODUCTION

Interest in the measurement and determination of ambient non-methane hydrocarbons has increased over the past decade. This increased interest is due to the fact that these non-methane hydrocarbons, along with nitrogen oxides, are primary precursors of ozone (O_3) and other oxidants which are major constituents of photochemical smog [1]. Regulators, such as the Environmental Protection Agency (EPA) and state governments, require data from baseline measurements of ambient hydrocarbon concentrations to help determine the level of reduction in ambient hydrocarbon concentrations required to achieve the national ambient air quality standards for ozone [2,3]. The photochemical reactivity of hydrocarbons differs between the different compounds, thus making it desirable to know the ambient concentrations of individual hydrocarbons. This aids regulators in developing control measures and can also be used as input to urban atmosphere models [4,5]. These models are then used to estimate increases in pollution based on growth of industry and in the numbers of automobiles as well as decreases resulting from anti-pollution measures.

Many studies have been conducted over the years to measure the concentrations of various hydrocarbons in the atmosphere. One example is given by McAllister *et al.* [6] who conducted a study from 1984–1988 where they measured the concentrations of hydrocarbons in 69 cities representing 27 states and the District of Columbia. Their data showed that over that time period average site non-methane organic compounds (NMOC) concentrations decreased in 39 cases while increasing in 19 and remained constant in 11. The total NMOC concentration for any one city was typically less than 1 μmol/mol (ppm). The concentrations of individual hydrocarbons in these studies vary depending on the sampling site. For instance, Lonneman *et al.* [7] report

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concentrations of individual hydrocarbons in the Lincoln Tunnel in New Jersey that ranged from 10 to 400 nmol/mol (ppb) in 1982. The concentrations in an outside air tunnel ventilation sample ranged from 1 to 50 nmol/mol.

In order to determine the concentration of hydrocarbons at these levels, and to follow long term trends, it is essential to have accurate and stable gas standards. Researchers have used several different sources of standards to calibrate instruments. Greenberg and Zimmerman [8] have used their own laboratory standard of 2,2-dimethylbutane (neohexane) in air for their remote continental studies. Nelson and Quigley [9] calculated hydrocarbon concentrations in their Sydney, Australia study using National Institute of Standards and Technology (NIST) methane and propane in air Standard Reference Materials (SRMs). Stump and Dropkin [10] used a vaporization system into which a pure hydrocarbon was injected and mixed with a controlled flow of air. A sample was withdrawn with a gas tight syringe and injected into a Tedlar bag containing a known volume of zero air for dilution to the desired concentration.

A considerable technical effort has been undertaken over the past 10 years at the NIST to develop stable trace level standards of volatile organic compounds in a pure matrix gas. These mixtures have been prepared in compressed aluminum gas cylinders using a microgravimetric technique developed at NIST [11–13]. This paper describes the research and development leading to the preparation of hydrocarbon gas standards in treated aluminum gas cylinders using the microgravimetric technique and other methods. The analytical methods as well as sample preparation for analysis will be discussed.

EXPERIMENTAL

Chemicals

The hydrocarbon compounds were purchased from commercial suppliers. The hydrocarbons were analyzed for impurities by gas chromatography–mass spectrometry (GC–MS) and gas chromatography–flame ionization detection (GC–FID) at NIST. The ultrahigh purity nitrogen (99.9995%) and ultra pure air used as diluent gases, were obtained from commercial sources. These gases were analyzed by NIST for any of the hydrocarbons of interest.

Gas cylinders

New aluminum gas cylinders with CGA-350 stainless steel valves were used to prepare the hydrocarbon standards. Cylinder sizes of 3.4, 5.8 and 30 l were used. The cylinders were precleaned by a commercial supplier in a manner that excluded contamination with trace hydrocarbons and halocarbons, and then treated to deactivate the internal walls.

Weighing apparatus

The gaseous hydrocarbon compounds (C_2–C_4) were weighed into a size 3.4-l cylinder using a two-pan balance with a sensitivity of 0.001 g. The cylinder to which these hydrocarbons were added was weighed against a tare cylinder. The liquid hydrocarbon compounds (C_5–C_{10}) were sealed into glass capillary tubes and weighed on an ultra microbalance, then introduced into a 3.4-l cylinder. The balance used has a mechanical tare capacity of up to 2.99 g, an electrical weighing range of 15 mg, and a readability of 0.1 μg. When using the 5.8-l cylinders, an electrical top-loading balance having a capacity of 15 kg and a 0.1 g sensitivity was used for weight determinations. A floor balance with a 54 kg capacity and a 1 g sensitivity was used to weigh the 30-l cylinders. The sensitivity of this balance was improved to 0.5 g using NIST calibrated weights to minimize "round up" errors.

Gravimetric procedure for preparing gas standards

The gas standards to be developed were to contain hydrocarbons of which some are gases (C_2–C_4) and the others liquids (C_5–C_{10}) at room temperature. We started with those compounds which are gases at room temperature. A new 3.4-l aluminum gas cylinder was evacuated and weighed. Each gaseous hydrocarbon was added through a manifold system in an amount yielding 0.5% in 12.4 MPa of air or nitrogen, starting with the lowest vapor pressure compound. The cylinder was weighed on the two-pan balance after each individual addition. After all

gaseous hydrocarbons of interest were added to the cylinder, diluent gas was added to the appropriate pressure (12.4 MPa) and the cylinder reweighed on the two-pan balance. Gravimetric concentrations of the hydrocarbons in the mixture were then calculated on a mol/mol basis using the data from the weighings. The cylinder was then heated (less than 70°C) to create temperature gradients within the cylinder to thoroughly mix all the constituents.

The next step in the preparation sequence included the use of a microgravimetric technique developed at NIST [11–13] followed by a dilution step. The goal was to prepare a standard at the 2 μmol/mol (ppm) level that contained the gaseous and liquid compounds. Calculations were made to determine the amount of each liquid hydrocarbon needed to prepare a mixture in 13.8 MPa (2000 p.s.i.) of nitrogen or air resulting in 2 μmol/mol of each compound. The high purity hydrocarbons, which are liquid at room temperature, were weighed into thin-walled borosilicate glass capillary tubes. The appropriate nut and nipple, usually CGA-350, was attached to an evacuated preweighed aluminum gas cylinder. A short piece of PTFE tubing was attached to the nut and nipple using the appropriate fittings. The capillary tube containing the liquid hydrocarbon was inserted into the PTFE tubing. The cylinder valve was opened and the end of the capillary tube closest to the cylinder valve was broken. The cylinder vacuum pulls in the liquid as heat is applied to the capillary if necessary to facilitate vaporization. After addition of all the liquids, the cylinder was weighed. A precalculated amount of the 0.5% C_2–C_4 standard was then added to the cylinder. The cylinder was again weighed to determine the amount of the 0.5% standard transferred. The cylinder was then pressurized to 13.8 MPa of nitrogen and the cylinder reweighed. The concentrations were calculated on a μmol/mol basis using the weight data.

The last step to achieve a gravimetric standard at the 5–50 nmol/mol (ppb) level was accomplished by dilution. A new aluminum gas cylinder was evacuated and weighed. A precalculated amount of the 2 μmol/mol hydrocarbon standard needed to result in a 5–50 nmol/mol standard was added to the evacuated cylinder followed by a weight measurement. The cylinder was then pressurized to 13.8 MPa with nitrogen or air and the cylinder reweighed. The concentrations of the hydrocarbons were calculated on a nmol/mol basis.

Several standards were prepared at the 0.5% and 2 μmol/mol levels followed by preparation of the ppb level standards from the 2 μmol/mol standards. This procedure was used so as to eliminate any bias that might occur from using just one initial standard from which all other standards would be blended.

Four different groups of hydrocarbon standards were developed, each group containing a different combination of compounds. Table I lists the compounds in each group of standards and gives the nominal concentrations. The preparation procedure previously described was used to prepare groups 1 and 2. Since the compounds

TABLE I

GROUPS OF HYDROCARBON MIXTURES STUDIED

Compound	Nominal concentration in nmol/mol (ppb)			
	Group 1	Group 2	Group 3	Group 4
Ethane	20	50		
Ethene		50		
Ethyne (acetylene)		50		
Propane	20	50		
Propene		50		
n-Butane	20	50		
Isobutane	20	50		
1-Butene		50		
Isobutene		50		
n-Pentane	20	50		
Isopentane	20	50		5.0
1-Pentene		50		
n-Hexane	20	50	100	
3-Methylpentane	20			5.0
2-Methyl-2-butene		50		
n-Heptane	20			
n-Octane	20		50	
n-Decane			25	
Benzene	20		100	5.0
Toluene	20		100	5.0
meta-Xylene			50	5.0
para-Xylene				5.0
ortho-Xylene	20			5.0

in the third and fourth groups are all liquids at room temperature, the first step in the preparation procedure, blending of a 0.5% standard, was eliminated. Several 2 μmol/mol standards were prepared and then the ppb level standards were prepared from the 2 μmol/mol standards.

Measurement apparatus

Analysis of the hydrocarbon gas standards were conducted using a gas chromatograph equipped with a flame ionization detector. Several different columns and conditions were used to obtain optimum baseline separation for each hydrocarbon in the gravimetric standards.

Method 1. A 25 m × 0.53 mm I.D. open tubular capillary column coated with a 10 μm thick film of aluminum oxide/potassium chloride (Al_2O_3/KCl) was used. The initial temperature was held at 35°C for 12 min then programmed to 45°C at 2°C/min, then to 180°C at 10°C/min and held at this final temperature for 30 min. The column carrier flow-rate was 2.8 ml/min and the detector make-up flow-rate was 28 ml/min (both nitrogen). An example of a chromatogram generated by this method is shown in Fig. 1a.

Method 2. A 1.8 m × 3.2 mm I.D. stainless-steel column packed with phenylisocyanate on 80/100 mesh Porasil C was used. The initial temperature was held at 30°C for 7 min then programmed to 60°C (the maximum allowable) at 10°C/min and held at this final temperature for 40 min. The column carrier flow-rate was 25 ml/min of nitrogen. An example of a chromatogram generated by this method is shown in Fig. 1b.

Method 3. A 30 m × 0.53 mm I.D. open wide bore capillary column containing GS-Q (a porous polymer) was used. The initial temperature was held at 60°C for 7 min then programmed to 240°C at 5°C/min. The column carrier flow-rate was 10 ml/min and the detector make-up flow-rate was 25 ml/min (both nitrogen). An example of a chromatogram generated by this method is shown in Fig. 1c.

Method 4. A 60 m × 0.75 mm I.D. open tubular capillary column coated with a 1 μm thick film of polyethylene glycol was used. The initial temperature was held at 50°C for 10 min then programmed to 185°C at 6°C/min. The column carrier flow-rate was 5 ml/min and the detector make-up flow-rate was 30 ml/min (both nitrogen). An example of a chromatogram generated by this method is shown in Fig. 1d.

Method 5. A 60 m × 0.75 mm I.D. open tubular capillary column coated with a 1 μm thick film of dimethylpolysiloxane phase was used. The initial temperature was held at 30°C isothermal for 8 min then programmed to 200°C at 10°C/min. The column carrier flow-rate was 5 ml/min and the detector make-up flow-rate was 25 ml/min (both nitrogen). An example of a chromatogram generated by this method is shown in Fig. 1e.

The flame ionization detector was operated at 250°C for all the above methods. Due to the low analyte concentrations, it was necessary to concentrate the sample before injection onto the GC column. Several methods of concentrating have been reported, including "on-column enrichment" at cryogenic temperatures [14]. Other techniques include collecting the sample on cartridges packed with charcoal [15], graphitized carbon black [16] and Tenax [17], followed by desorption techniques. The use of an automatic cryogenic trapping unit set at −170°C and equipped with a trap packed with glass wool [10] has been used. Another method has been the use of traps packed with small diameter glass beads using liquid argon or oxygen as the cryogen [9,18]. The authors used techniques similar to those others have reported [8,13,19,20]. The hydrocarbon gas sample was cryogenically trapped either manually using liquid argon or with an automated system using liquid nitrogen programmed at a cryogenic temperature of −180°C. The sample flow-rate was controlled at 50 ml/min using a mass flow controller. The sample was cryogenically trapped in a 0.1-ml sample loop on a six-port gas sampling valve for 5 min. When manually trapping, hot water (90°C) was used to vaporize the sample. Two 0.1-ml stainless-steel sample loops were employed for the study: one was used empty and the other one was packed with 100–120 mesh dimethylchlorosilane treated glass beads. In the automatic trapping system the sample was vaporized by programming to a 150°C purge tempera-

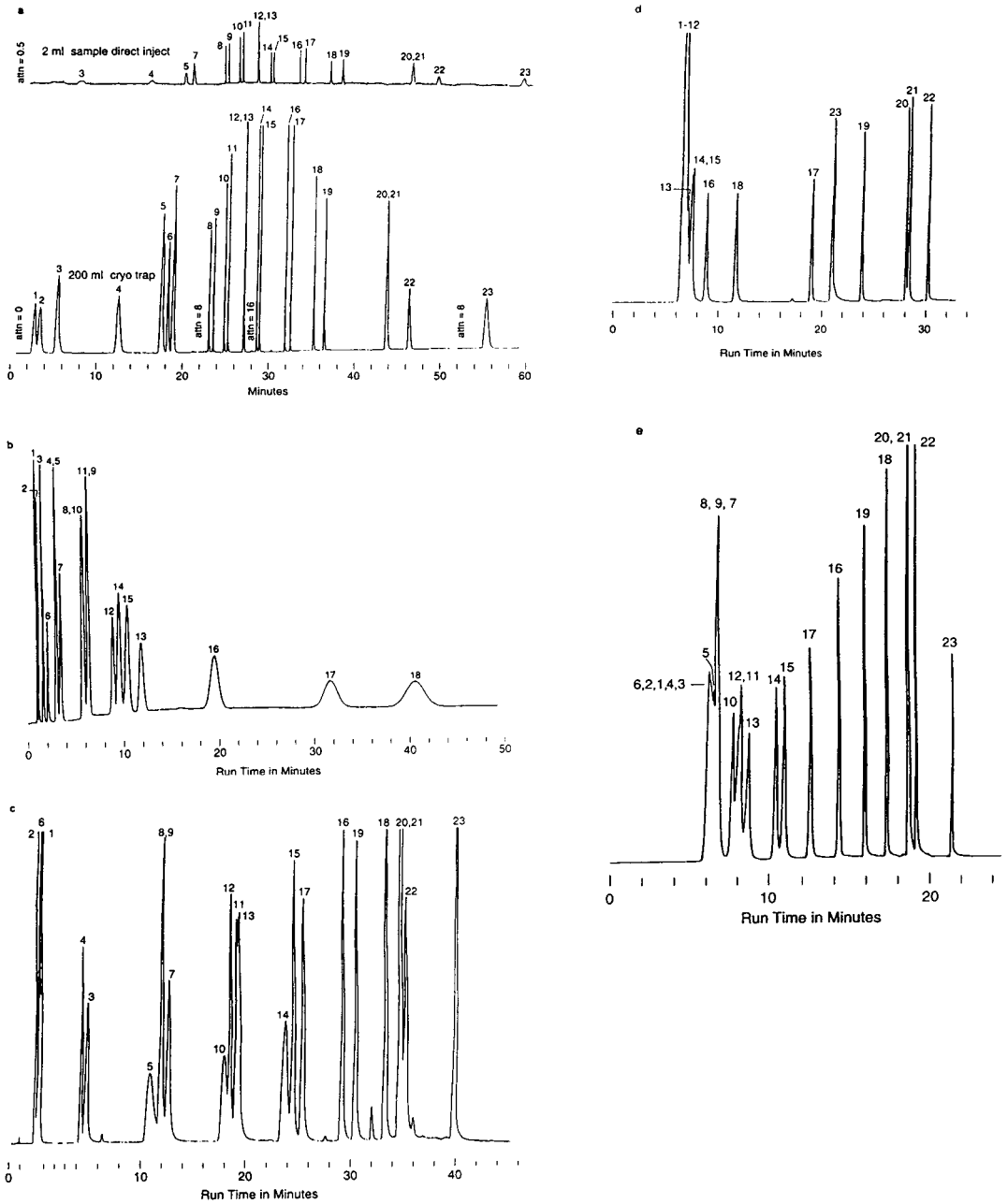

Fig. 1. (a) 25 m × 0.53 mm I.D. capillary column coated with 10 μm thick film Al_2O_3/KCl, 35°C for 12 min to 200°C at 2°C/min and hold. (b) 1.8 m × 3.2 mm I.D. stainless-steel column packed with phenylisocyanate on 80–100 mesh Porasil C, 30°C for 7 min to 60°C at 10°C/min and hold. (c) 30 m × 0.53 mm I.D. porous polymer capillary column, 60°C for 7 min to 240°C at 5°C/min and hold. (d) 60 m × 0.75 mm I.D. capillary column coated with 1 μm thick film polyethylene glycol, 30°C for 10 min to 180°C at 4°C/min. (e) 60 m × 0.75 mm I.D. capillary column coated with 1 μm thick film dimethylpolysiloxane phase, 30°C for 8 min to 200°C at 10°C/min and hold. Peaks: 1 = ethane; 2 = ethene; 3 = propane; 4 = propene; 5 = isobutane; 6 = ethyne (acetylene); 7 = *n*-butane; 8 = 1-butene; 9 = isobutene; 10 = isopentane; 11 = *n*-pentane; 12 = 1-pentene; 13 = 2-methyl-2-butene; 14 = 3-methylpentane; 15 = *n*-hexane; 16 = *n*-heptane; 17 = benzene; 18 = *n*-octane; 19 = toluene; 20 = *para*-xylene; 21 = *meta*-xylene; 22 = *ortho*-xylene; 23 = *n*-decane.

ture. The sampling procedure was computer controlled so as to maximize reproducability.

RESULTS AND DISCUSSION

The objective of this research was to determine if accurate and stable gas standards containing alkane, alkene, alkyne and aromatic hydrocarbons in a nitrogen or air matrix could be developed. The study involved a total of 23 hydrocarbons of interest to the US Environmental Protection Agency (EPA) and the State of California Air Resources Board (CARB), at concentrations ranging from 5–2000 nmol/mol (ppb). The standards were to be used to certify mixtures of hydrocarbons for use in EPA's and CARB's programs for studying ozone precursors in automobile exhaust.

Uncertainty in standards preparation

Taking the weighing imprecisions and the determination of impurities in the pure hydrocarbons and matrix gases into account, the uncertainty in the gravimetric concentrations of each compound ranged from 0.2–0.5% (1 standard deviation) at the 10 nmol/mol level. This uncertainty includes the standard deviations in the mean weight measurements and the uncertainty in the impurities in the pure hydrocarbon compounds and the diluent gas.

Analysis of standards

Analyzing hydrocarbons at the 5–100 ppb range using GC–FID is difficult. When using a capillary column of 0.53 mm I.D. or larger, 2 ml is the maximum amount of gaseous sample that can be directly injected onto the column without having problems with peak distortion. The amount of each compound injected under these conditions is low, resulting in poor precision from replicate analyses ranging from 2–10% depending on the hydrocarbon. However, when using cryogenic preconcentration, trapping of 250 ml of sample can be easily obtained resulting in a 100-fold increase in the amount of each compound injected, which improved the imprecisions to 0.1–1.5%. Fig. 1a illustrates the increase in signal when using cryogenic preconcentration *versus* direct injection of 2 ml of sample.

Differences in the results of using the two different stainless steel sample loops, unpacked *vs.* packed with dimethylchlorosilane treated glass beads, was most noticeable for the C_2 hydrocarbons. Table II lists the GC responses for the C_2 compounds using the different sample loops. In each case, the response is greater using the packed trap. However, the ethyne (acetylene) response does not increase proportionally to the ethane and ethene. Given that ethane, ethene and acetylene all have equal FID response [21], the lower relative response of acetylene must be a result of poor trapping efficiency, probably due to its possible reactivity and volatility.

For the analysis of the first group of compounds, the capillary column coated with Al_2O_3/KCl separated all the compounds within 48 min except for the *meta-* and *para*-xylenes (see Fig. 1a). The packed column containing phenyliso-

TABLE II

GC–FID RESPONSES FOR C_2 COMPOUNDS USING PACKED AND UNPACKED TRAPS

Compound	GC response	
	Stainless-steel empty 15 cm × 1.6 mm I.D.	Stainless-steel packed with glass beads 15 cm × 1.6 mm I.D.
Ethane	4593	11 152
Ethene	3560	11 603
Ethyne (acetylene)	1535	2363

cyanate on Porasil C separated all the compounds up to *n*-octane in less tan 45 min (see Fig. 1b). However, due to its low maximum temperature of 60°C, the toluene and xylenes take over 50 min to elute. The later eluting peaks are also very broad on this column resulting in poor precision in the peak integration. The porous polymer capillary column separated all the compounds, except for the xylenes, in less than 40 min (see Fig. 1c). However, the isobutane and isopentane tend to have broad peaks which tail into the *n*-butane and *n*-pentane, respectively. Overall, the best separations and peak shapes were obtained on the Al_2O_3/KCl capillary column. The xylenes could be made to elute earlier by ramping the temperature at a high rate after the elution of toluene. This resulted in a reasonable GC run time. Of all the columns studied, the xylenes are best separated and analyzed using method 4 which employed the polyethylene glycol capillary column (see Fig. 1d).

The second group of compounds has fewer higher boiling hydrocarbons but has more of the volatile compounds, which complicates the analysis. The packed column has a run time of less than 15 min, but was incapable of separating six compounds contained within three peaks (see Fig. 1b). Lower starting temperatures and ramping rates could not resolve these compounds. The capillary column coated with Al_2O_3/KCl separated most of the compounds and resulted in all around good peak symmetry (see Fig. 1a). One precaution to be noted is that the authors found that with increased column use, the retention times of most of the compounds increased, most notably acetylene. Over a period of hours of use the acetylene peak eventually merged with the *n*-butane peak. Lowering the starting temperature again resulted in the acetylene eluting after the *n*-butane. The reason for this is not known. Though the 1-pentene and 2-methyl-2-butene were not separated using this column, they were separable by the packed column. Therefore, both methods 1 and 2 were utilized for analyzing the second group of compounds.

Measurements of the third and fourth groups of compounds were relatively simple. Four of the five methods described earlier will accomplish the analysis of the third group. Method 5 using the capillary column coated with dimethylpolysiloxane was the technique used to analyze this group of standards (see Fig. 1e). The run time was shortened to less than 18 min by using different conditions stated below since only six compounds were present in this group. The initial temperature was held at 45°C for 8 min then programmed to 145°C at 10°C/min. Method 4 was used to separate the compounds in the fourth group (see Fig. 1d). The *meta*- and *para*-xylenes were not completely separated under these conditions. The separation of these compounds can be improved using a longer hold time at the initial oven temperature and a slower ramping rate, but this results in a longer analysis time. Despite some drawbacks method 4 was employed for the measurement of the xylenes.

Intercomparison of standards

The agreement between the 0.5% hydrocarbon standards was excellent. These standards were then used to prepare the 2 μmol/mol mixtures. These 2 μmol/mol standards were compared with each other along with other primary standards in the NIST inventory. The data were plotted with gravimetric concentration on the *x*-axis and GC response on the *y*-axis and analyzed by linear regression. Table III shows the results of the linear regression analysis of the propane data. The agreement between the new 2 μmol/mol standards and the older standards was excellent. The average residual was 0.20%, and the correlation coefficient (r^2) was 0.9999. This suggests that the preparation method was accurate and that there was no known bias. Table IV shows the linear regression for the 2 μmol/mol *n*-pentane standards. There were no NIST primary standards available for *n*-pentane at this level with which to compare. This set of standards compared very well with $r^2 = 0.9998$. Similar results were obtained for the other hydrocarbons studied.

After determining that there was good agreement between the 2 μmol/mol standards, the nmol/mol level standards were prepared for the respective groups. The standards from all the

TABLE III

LINEAR REGRESSION OF 2 μmol/mol (ppm) PROPANE STANDARDS

Sample number	GC response	Gravimetric concentration	Predicted concentration	Percent difference
x207013	102 660	9.502	9.501	−0.01
x207064	30 807	2.839	2.838	−0.05
000611	27 946	2.571	2.572	+0.05
ALM-009011	25 376	2.326	2.334	+0.35
ALM-008390	24 276	2.236	2.232	−0.18
CAL-8711	23 140	2.123	2.127	+0.17
000690	12 179	1.117	1.110	−0.61
			average absolute difference =	0.20

Correlation coefficient = 0.9999
y-Intercept = 207
Standard error of estimate of y = 58

TABLE IV

LINEAR REGRESSION FOR 2 μmol/mol (ppm) *n*-PENTANE STANDARDS

Sample number	GC response	Gravimetric concentration	Predicted concentration	Percent difference
ALM-009011	46 488	2.385	2.386	+0.03
CAL-8711	40 532	2.083	2.080	−0.16
ALM-008390	38 818	1.989	1.992	+0.13
			average absolute difference =	0.11

Correlation coefficient = 0.9998
y-Intercept = 72
Standard error of estimate for y = 83

groups were compared and the data pooled where there were common hydrocarbons between sets of standards. Linear regression was then applied to the data for each hydrocarbon. Table V shows the results for the standards containing *n*-hexane. The agreement between standards is good with a r^2 of 0.9997 and an average residual of 1.4%. These results show very good agreement when considering the uncertainty in the preparation of the standards is 0.2–0.5% (1 S.D.) and the imprecision of replicate analyses is at least 1.4%. Similar results were obtained for the other 23 compounds studied, which validated the accuracy in the preparation procedure. Table VI gives the gravimetric and analytical concentrations for each hydrocarbon in each standard prepared. In each case, the analytical concentrations, as determined from linear regression of the complete set of standards, agrees very well with the gravimetric concentrations.

Stability of hydrocarbon standards

The stability of a gas mixture is determined by periodic intercomparison to a set of standards. A new standard is prepared to compare with the "aged" set to assure that the standards are not decaying in concentration. Table VII shows stability data for a hydrocarbon standard from

TABLE V

LINEAR REGRESSION FOR nmol/mol (ppb) *n*-HEXANE STANDARDS

Sample number	GC response	Gravimetric concentration	Predicted concentration	Percent difference
ALM-008392	859.99	123.5	124.3	+0.6
ALM-009003	682.78	99.86	98.57	−1.3
ALM-009012	548.80	79.45	79.12	−0.4
ALM-009016	338.51	48.75	48.60	−0.3
ALM-009006	337.20	47.74	48.41	+1.4
ALM-009026	327.06	46.46	46.93	+0.8
ALM-009010	273.86	38.61	39.21	+1.6
X138312	152.57	21.75	21.61	−0.7
FF9755	68.22	9.66	9.36	−3.1
X138362	37.85	5.17	4.96	−4.2
			average absolute difference =	1.4

Correlation coefficient = 0.9997
y-Intercept = 3.71
Standard error of estimate of *y* = 4.58

TABLE VI

GRAVIMETRIC CONCENTRATION *VERSUS* PREDICTED CONCENTRATION FROM LINEAR REGRESSION OF DATA FOR EACH HYDROCARBON IN A GROUP II STANDARD

Compound	Gravimetric concentration[a]	Predicted concentration[a]
Ethane	50.64 ± 0.41	51.2 ± 2.0
Ethene	53.25 ± 0.43	53.6 ± 1.0
Ethyne (acetylene)	53.99 ± 0.43	54.1 ± 1.0
Propane	55.99 ± 0.45	56.1 ± 1.0
Propene	49.08 ± 0.39	49.2 ± 1.0
n-Butane	50.89 ± 0.41	51.0 ± 0.9
Isobutane	60.64 ± 0.49	60.8 ± 1.1
1-Butene	51.89 ± 0.42	52.0 ± 0.9
Isobutene	52.78 ± 0.42	52.9 ± 0.9
n-Pentane	49.81 ± 0.40	49.6 ± 0.9
Isopentane	47.45 ± 0.38	47.4 ± 0.8
1-Pentene	48.77 ± 0.39	48.9 ± 1.1
n-Hexane	47.74 ± 0.38	48.0 ± 1.0
2-Methyl-2-butene	51.32 ± 0.41	51.5 ± 1.1

[a] Concentrations are in nmol/mol (ppb). The uncertainty is at the 95% confidence interval.

the first group of compounds and one from the second. The uncertainties following the concentrations are at the 95% confidence interval. The data show that the hydrocarbons have remained stable for 10 months with the exception of ethyne. The ethyne has decreased by 20% in the standard. Decreases in ethyne concentration have been noticed in other NIST gravimetric standards at the ppb levels. However, the ethyne has remained stable in the higher concentration 2 ppm standards. Further stability checks of these NMOC standards are planned in the future.

CONCLUSIONS

The results of this study show that primary NMOC gravimetric standards can be accurately and precisely prepared at the 5–100 ppb range with uncertainties in the preparation of ±1% (95% confidence interval). These standards have been found to be stable for a period of at least 10 months. These standards will be used to certify gas mixtures containing hydrocarbons which can then be used in various research and environmental programs, such as ozone precursor studies and automobile exhaust. This research has provided background work for the future development of a light hydrocarbon SRM.

TABLE VII

STABILITY DATA FOR HYDROCARBON STANDARDS FROM THE FIRST AND SECOND GROUPS OF COMPOUNDS

Compound	Concentrations in nmol/mol (pbb)[a]			
	Group 1		Group 2	
	June 1990	January 1991	July 1990	May 1991
Ethane	18.9 ± 0.9	20.1 ± 1.0	51.2 ± 2.0	50.7 ± 2.0
Ethene			53.6 ± 1.0	53.2 ± 1.0
Ethyne (acetylene)			54.1 ± 1.0	43.4 ± 1.0
Propane	20.5 ± 1.4	21.5 ± 1.0	56.1 ± 1.0	55.8 ± 1.0
Propene			49.2 ± 1.0	49.3 ± 1.0
n-Butane	20.9 ± 1.0	20.9 ± 1.0	51.0 ± 0.9	50.7 ± 0.9
Isobutane	20.4 ± 1.0	21.2 ± 1.3	60.8 ± 1.1	60.6 ± 1.1
1-Butene			52.0 ± 0.9	51.7 ± 0.9
Isobutene			52.9 ± 0.9	52.6 ± 0.9
n-Pentane	20.4 ± 1.0	20.9 ± 1.0	49.6 ± 0.9	49.7 ± 0.9
Isopentane	20.3 ± 1.0	20.7 ± 1.0	47.4 ± 0.8	47.4 ± 0.9
1-Pentene			48.9 ± 1.1	49.1 ± 1.1
n-Hexane	20.0 ± 1.0	20.3 ± 1.0	48.0 ± 1.0	47.8 ± 1.0
3-Methylpentane	20.0 ± 1.0	20.3 ± 1.0		
2-Methyl-2-butene			51.5 ± 1.1	51.5 ± 1.1
n-Heptane	20.0 ± 1.0	20.2 ± 0.9		
n-Octane	19.8 ± 1.0	19.9 ± 1.0		
Benzene	20.7 ± 1.4	20.2 ± 1.0		
Toluene	19.9 ± 1.0	20.1 ± 1.0		
ortho-Xylene	19.3 ± 1.4	19.1 ± 1.0		

[a] The total uncertainties are the estimated upper limit error of the respective concentrations and are at the 95% confidence interval.

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Supercritical carbon dioxide extraction of polycyclic aromatic hydrocarbons from sediments

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ABSTRACT

A supercritical fluid extraction (SFE) method using carbon dioxide was developed for the determination of the 16 US Environmental Protection Agency polycyclic aromatic hydrocarbon (PAH) priority pollutants in naturally contaminated sediments. While carbon dioxide is less efficient for the heavier PAHs than other fluids such as nitrous oxide and Freon-22, its deficiency was remedied by the use of a mixture of water, methanol, and dichloromethane as modifiers, a higher extraction temperature of 120°C, as well as repetitive extractions. Extraction time can be further reduced to *ca.* 70 min per sample if a high-pressure pump is used for the delivery of the modifiers during dynamic extraction. Except for naphthalene, the SFE results for the 16 PAHs obtained from several certified reference materials and sediments samples were comparable to certified or Soxhlet values in terms of both precision and accuracy. The SFE recoveries of naphthalene as well as methylnaphthalenes which were coextracted alongside other PAHs and methyl-PAHs, ranged from 150 to 125% of their respective Soxhlet values due to higher evaporative losses in the Soxhlet procedure.

INTRODUCTION

Polycyclic or polynuclear aromatic hydrocarbons (PAHs/PNAs) are ubiquitous environmental pollutants that are present in large numbers and varying quantities in air, water, and sediment samples. PAHs are formed naturally by many routes such as forest fires and volcanic activities, however, the recent build-up of the aromatic hydrocarbons in densely populated areas is likely related to the incomplete combustion of coal and other fossil fuels. While the occurrence of alkyl- and nitro-substituted as well as other heteroatom-containing PAHs are often reported, the most abundant and routinely monitored PAHs are the 16 listed in Method 610 [1] by the United States Environmental Protection Agency (US EPA). These compounds are also included in the Priority Substances List under the Canada Environmental Protection Act (CEPA). Many of these hydrocarbons as well as

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their derivatives and metabolites are suspected mutagens and carcinogens. Due to the low solubilities and persistence of the PAHs, they are readily adsorbed and accumulated in sediments at levels from μg/g to ng/g in many sites of the Great Lakes Basin [2].

Numerous analytical methods have been reported for the final analysis of PAHs. Flame ionization, photoionization, electron capture and other detectors have been used with capillary column gas chromatography [3,4]. In the case of high-performance liquid chromatography (HPLC), ultraviolet, fluorescence and photodiode array detectors are generally used in conjunction with reversed-phase columns [5,6]. Mass spectrometry interfaced to either GC or HPLC is also a popular choice for many workers because of its selectivity and sensitivity for PAHs [7]. In contrast, extraction of sediment samples in many cases is accomplished by the Soxhlet technique using various mixtures of organic solvents [8]. This mode of sample preparation, while exhaustive in terms of recovery, requires a lengthy extraction time from 8 to 48 h, consumes large amounts of solvent, and generates a large amount of coextractives which necessitate two to three subsequent column cleanup steps before final analysis. The cleanup procedures further aggregate the time and solvent problem.

More recently, applications of supercritical fluid extraction (SFE) for the determination of PAHs from soil and sediments have been demonstrated [9–11]. The new extraction technique practically eliminates the use of solvents and produces extracts suitable for analysis with selective detectors such as the mass spectrometer without cleanup. The early work with supercritical carbon dioxide at room temperature produced quantitative recovery for five PAHs in an urban dust standard reference material (SRM 1649) distributed by the National Institute of Science and Technology, however, the extraction time was still a lengthy four hours [9]. Subsequent work with a shorter extraction time produced incomplete recovery, particularly for those PAHs with a molecular mass of 252 or higher [12]. While raising the extraction temperature from 50 to 200°C greatly increased the recoveries of PAHs by using pure carbon dioxide [13], improved results for the heavier PAHs were obtained by extraction with supercritical carbon dioxide pre-modified with a polar solvent such as methanol [14]. In contrast, quantitative recovery of PAHs was achieved by the use of supercritical nitrous oxide modified with methanol [11]. Presumably due to its higher dipole moment, Freon-22 (chlorodifluoromethane) has recently been shown to yield higher extraction efficiency than non-modified nitrous oxide and carbon dioxide [15]. Though Freon-22 has a lower ozone-depletion potential and is used as an interim substitute for Freon-11, a common refrigerant, it was estimated that the former had a third to a quarter of the global warming effect as the latter [16]. At a time when government agencies are legislating on the drastic reduction of the use of many chlorofluorocarbons (CFCs) in industrial applications and domestic products, the advocacy of a freon as a SFE solvent is a step backward in environmental protection. Notwithstanding the higher extraction efficiency, Freon-22 is only suitable for research purposes since it is less commonly available, more expensive and poses a greater health risk in the working environment than carbon dioxide. For routine applications, there is still a need to investigate how the efficiency of supercritical carbon dioxide can be improved for the extraction of native PAHs in sediment samples. In this work, we shall discuss the factors affecting the extraction recovery of the 16 PAHs from naturally contaminated sediments using supercritical carbon dioxide. Procedures to further shorten the extraction time by the use of a high-pressure pump to deliver mixtures of modifiers during the extraction will also be introduced.

EXPERIMENTAL

Reagents and chemicals

PAHs and methyl PAHs were obtained from Aldrich (Milwaukee, WI, USA) and Ultra Scientific (North Kingstown, RI, USA). Deuterated PAHs were obtained from Aldrich and MSD Isotopes (Pointe Claire, Canada).

Individual stock solutions of PAH, methyl PAH, and deuterated PAH internal standards at 1000 or 500 μg/ml were prepared in toluene.

Mixtures of the 16 PAHs in US EPA Method 610 [1] at 2 and 0.5 μg/ml, as well as the ten methyl PAHs (see Results and Discussion and Table III) at 500 and 100 ng/ml were prepared in isooctane. Another mixture of six deuterated PAHs was also prepared in isooctane at the following concentrations: [$^2H_8$]naphthalene, 100 μg/ml; [$^2H_{10}$]phenanthrene, 100 μg/ml; [$^2H_{10}$]pyrene, 100 μg/ml; [$^2H_{12}$]chrysene, 50 μg/ml; [$^2H_{12}$]benzo[*a*]pyrene, 50 μg/ml; and [$^2H_{12}$]benzo[*ghi*]perylene, 30 μg/ml. Appropriate amounts of the native and deuterated PAH solutions were mixed and used as calibration standards for GC–MS analysis.

SFE-grade carbon dioxide without a helium head pressure was obtained from Praxair Canada (Mississauga, Canada) and Air Products (Nepean, Canada).

Sediment samples

The samples used in this work are either certified reference materials (CRM) (EC-1 and HS-3) or lake or harbour sediments naturally contaminated with PAHs. EC-1, developed and distributed by Environment Canada, is a lake sediment derived from Hamilton Bay, a heavily industrialized location in western Lake Ontario. The certified PAH concentrations in this material were obtained by over 75 in-house Soxhlet extractions followed by GC–flame ionization detection (FID), GC–MS, or HPLC–ultraviolet/fluorescence detection [17]. These values were further confirmed by an interlaboratory study involving 15 laboratories across Canada. HS-3 is a marine sediment CRM purchased from the National Science and Engineering Research Council of Canada. The other two samples also used in this work were obtained from Vancouver Harbour (VAN-4) and Detroit River (DET-1). All samples were freeze-dried, crushed, ground and sieved before extraction.

SFE of sediment samples

All extractions were done by either the Hewlett-Packard 7680A or 7680T SFE module, both controlled by an IBM compatible personal computer and a dedicated SFE software. The latter extractor is an upgraded model of the 7680A with an eight-position thimble-holder for automated sequential extraction of up to eight samples. Before the extraction, two layers of Whatman GFC filter paper cut to the diameter of the thimble were placed just above the cap at the bottom of the thimble. Then 200 mg of Celite and 1 g of freeze-dried or air-dried sample were weighed into the thimble. In the absence of a modifier pump, the modifier was spiked onto the sediment and the mixture was mixed on a vortex mixer for 1 min. The sample was typically extracted with CO_2 at 36 MPa and a flow-rate of 2 ml/min for 25 min (5 min static and 20 min dynamic) and at various temperatures. For quantitative recovery of nearly all PAHs, three consecutive extractions of the same sample at 120°C in the presence of 500 μl of a 1:1 mixture of methanol and dichloromethane (DCM) were performed. During the dynamic extraction, the PAHs were collected by an octadecylsilane (ODS) trap maintained at 15°C. At the end of the extraction, the trap was heated to 40°C before the PAHs were eluted from the trap with two 1.5-ml rinses of a 1:3 (v/v) mixture of isooctane and DCM. The extracts were combined and the solvent was exchanged into pure isooctane for GC–MS analysis. Using this procedure, each extraction cycle required *ca.* 50 min.

The modifier pump

A Hewlett-Packard 1050 quaternary LC pump was used for the delivery of up to four different modifiers to be mixed with the supercritical carbon dioxide. The outlet tubing from the modifier pump was interfaced to the check valve weldment downstream of the 7680T SFE pulse damper through a 1/16 in. (0.16 cm) stainless-steel tubing with Swagelok and Valco fittings. The pump and the SFE apparatus were both controlled by the same 80386-based personal computer running a pre-release SFE control software (version A.00.00) under the Microsoft Windows 3.1 environment. Before the operation of the modifier pump, the solvent modifiers were first degassed and the channels being used were primed at 5 ml/min. The software switches the pump on and off at the prescribed time as well as controls the percentages (up to a total of 20%)

of the modifiers to be blended with the extraction fluid.

For the SFE of PAHs from sediments using the modifier pump, 500 μl of water was spiked to a 1-g sample and it was thoroughly mixed prior to extraction. A three-step extraction outlined below was used for all sediments. The sample was first extracted with pure carbon dioxide at 34 MPa for 7 min (2 min static and 5 min dynamic) at 120°C and a flow-rate of 4 ml/min. The extract was rinsed off the ODS trap by 1.5 ml of a 1:3 mixture of isooctane and DCM. (Note: if PAHs are analyzed by HPLC methods, the rinse solvent should be replaced by a 1:1 mixture of tetrahydrofuran and acetonitrile.) Without depressurization of the thimble, the extraction was continued at the same temperature and pressure with CO_2 mixed with 1% methanol and 4% DCM at a flow-rate of 2 ml/min for 31 min (1 min static and 30 min dynamic), followed by the final step with pure CO_2 again for 2.5 min at 4 ml/min. At the end of the extraction, the trap was rinsed again with 1.5 and then 1.2 ml of the same solvent mixture, before and after thimble depressurization. The combined extract was also solvent exchanged into isooctane prior to analysis. The entire extraction cycle took *ca.* 70 min.

GC–MS analysis of PAHs

The SFE extracts for each sample were combined and solvent exchanged into a suitable volume (typically between 1 and 10 ml) of isooctane. A 1-ml aliquot of this extract was removed and mixed with 20 μl of the six deuterated internal standard solutions. The extract was then analyzed by capillary column GC–MS using a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a Model 5971 Mass Selective Detector. A 30 m × 0.25 mm I.D. DB-5 column and the following temperature programs were used: initial oven temperature 70°C, initial hold and valve time 1.0 min, oven temperature programming rates 30°C/min (from 70 to 120°C) and 4°C/min (from 120 to 280°C). Carrier gas (helium) head pressure was 35 kPa. Splitless injections (1 μl) were made by a Model 7673 autosampler. Data were acquired in selected ion monitoring mode for the molecular ions of the PAHs and methyl PAHs as listed in Tables I and III. As outlined in US EPA Method 625 [1], response factors for the native PAHs were calculated by using an appropriate deuterated internal standard as described below: (1) [$^2H_8$]naphthalene (*m/z* 136) for naphthalene, all methyl naphthalenes, acenaphthene, and acenaphthylene; (2) [$^2H_{10}$]phenanthrene (*m/z* 188) for fluorene, phenanthrene, and anthracene, and their methyl derivatives; (3) [$^2H_{10}$]pyrene (*m/z* 212) for fluoranthene and pyrene; (4) [$^2H_{12}$]chrysene (*m/z* 240) for benzo[*a*]anthracene and chrysene; (5) [$^2H_{12}$]benzo[*a*]pyrene (*m/z* 264) for benzo[*b*]- and benzo[*k*]fluoranthenes, as well as benzo[*a*]pyrene; and (6) [$^2H_{12}$]benzo[*ghi*]perylene (*m/z* 288) for indeno[1,2,3-*cd*]pyrene, dibenzo[*ah*]anthracene and benzo[*ghi*]perylene.

RESULTS AND DISCUSSION

Effect of modifiers on the SFE of PAHs

There are many examples of incorporating modifiers to increase the recovery of organic compounds under SFE conditions [14,18–20]. In many cases, these modifiers are either water, acids or polar organic solvents. The purpose is either to modify the sample matrix so that the organics are freed for extraction or to increase the solubility of the organics in the supercritical fluid, particularly the non-polar carbon dioxide. The SFE recovery of PAHs can also be benefited by the use of modifiers. Fig. 1 depicts the results for seven PAHs in EC-1 obtained by spiking 500 or 750 μl of the modifier directly onto 1 g of EC-1 prior to extraction at 80°C with 36 MPa of CO_2. These seven compounds, ranging from naphthalene to indeno[1,2,3-*cd*]pyrene, cover the entire mass range for the PAHs in Method 610 and thus their results are truly representative for the whole group of PAHs. As shown in Fig. 1, relatively small amounts of PAHs of mass 252 and above could be recovered by pure carbon dioxide. The presence of 500 μl of either water, methanol, and DCM as modifiers improves the recovery of all PAHs, although the modifiers had the least effect on the recovery of phenanthrene and the largest on indeno[1,2,3-*cd*]pyrene. Among various modifiers, the difference in recovery is small for all PAHs between methanol

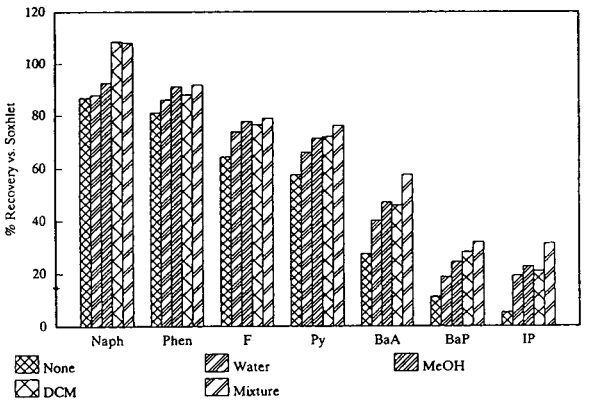

Fig. 1. Effect of modifiers on the SFE recovery of selected PAHs in the certified reference material EC-1. For every PAH, the SFE result (mean of three extractions) is expressed as a percentage of its Soxhlet or certified value. Each extraction was carried out with a 1-g sample at 80°C and CO_2 of 36 MPa. Prior to extraction, each sample was spiked with 500 μl of water, methanol or DCM, or in the case of the mixture, 250 μl each of the above three solvents. Naph = naphthalene, Phen = phenanthrene, F = fluoranthene, Py = pyrene, BaA = benzo[*a*]anthracene, BaP = benzo[*a*]pyrene, IP = indeno[1,2,3-*cd*]pyrene.

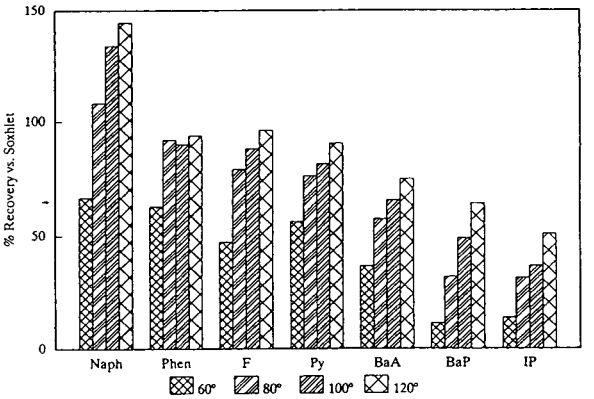

Fig. 2. Effect of extraction temperature (in °C) on the SFE recovery of selected PAHs in EC-1. For every PAH, the SFE result (mean of three extractions) is expressed as a percentage of the Soxhlet or certified value. Each extraction was carried out with a 1-g sample in the presence of 250 μl each of water, methanol and DCM and CO_2 of 36 MPa. For abbreviations see Fig. 1.

and DCM, although they were both more efficient than water. While the mechanism was not clearly understood, 750 μl of a 1:1:1 mixture of the three modifiers definitely gave the highest results for all PAHs, particularly for those PAHs of molecular mass 228 and higher. Apparently, the higher (750 *vs.* 500 μl) modifier volume in the case of the mixture was not responsible for the improved PAH recovery since experiments with either 500 or 1000 μl of the single modifier produced very similar results.

Effect of extraction temperature on the SFE of PAHs

Earlier we have examined the recovery of PAHs after they were spiked to sediment samples and found that quantitative recoveries of PAHs could be obtained at an extraction temperature of 80°C or lower and an extraction time of about 30 min with either methanol or DCM as a modifier. With real world samples such as EC-1, however, it is obvious from Fig. 1 that we were not able to get full recovery of all PAHs even with the most efficient modifier at 80°C. A study of the recovery of the seven PAHs in EC-1 at 60, 80, 100 and 120°C (Fig. 2) clearly indicated that a higher extraction temperature provided a better recovery for *all* PAHs, although going from 80 to 120°C the relative percentage improvement for naphthalene (mass 128), phenanthrene (mass 178), fluoranthene and pyrene (mass 202) was obviously smaller than the higher-molecular-mass PAHs. Thus for the benefit of better extraction efficiency of the heavier PAHs, all subsequent extractions were done at a temperature of 120°C. A closer examination of the results in Fig. 2 also indicated that the SFE recovery for naphthalene at 100 and 120°C were both significantly (30 to 40%) higher than the Soxhlet result. Since the SFE extract required minimal evaporation and no cleanup, the lower naphthalene result is more likely due to higher evaporative loss of the volatile hydrocarbon in the concentration steps for the Soxhlet extract before and after the column cleanup rather than a reflection of the extraction efficiency of the two techniques.

Effect of consecutive SFE on the same sample

As shown in Fig. 2, the recovery of naphthalene, phenanthrene, fluoranthene and pyrene was close to their Soxhlet values (90% or above) after a single extraction at 120°C in the presence of modifiers. However, recovery for the heavier PAHs under the same conditions was still less

than quantitative. In our work, the modifier was spiked directly onto the sample prior to extraction. This method to introduce the modifier is simple and very flexible during method development stages since each extraction can be done with a different modifier, if required. However, it has the drawback that the modifier is quickly consumed in the early part of the dynamic extraction. Since it is not replenished during the extraction, the latter part of the dynamic extraction is virtually done with non-modified carbon dioxide only. This problem can be easily solved by using supercritical fluids premixed with a fixed amount of modifier of choice, yet the latter are more expensive and under some storage conditions, the modifier can separate from the supercritical fluid, resulting in an extractant other than the one labelled and producing unexpected results. A premixed fluid is also not flexible enough for method research purposes since the extractant is confined to a single, predetermined composition. Another way to overcome the problem of incomplete recovery is to perform more than one extraction and spike additional modifier to the sample again before extraction. As shown in Fig. 3, the second and even the third extractions are clearly beneficial to the recovery, percentagewise, of PAHs such as benzo[*a*]pyrene and indeno[1,2,3-*cd*]pyrene.

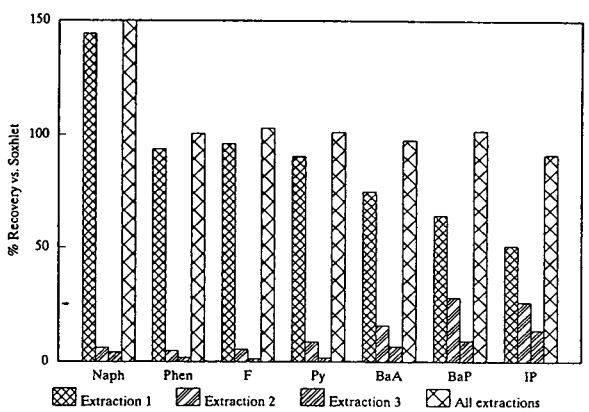

Fig. 3. Effect of consecutive SFE on the recovery of PAHs in EC-1. SFE was carried out on a 1-g sample at 120°C with CO_2 at 36 MPa. Aliquots of 250 μl each of water, methanol and DCM were added to the sample prior to the first extraction and 250 μl each of methanol and DCM were added to the sample prior to the second and third extractions. For abbreviations see Fig. 1.

The combined recovery of each of the seven PAHs in EC-1 after three extractions were all better than or equal to the Soxhlet results with the exception of indeno[1,2,3-*cd*]pyrene which was *ca.* 90% recovered.

Introduction of modifiers by means of a pump

Instead of spiking the modifier directly onto the sample prior to SFE, a better way to introduce the modifier is to mix it with the extraction fluid at a constant rate. This provides a continuous and uniform supply of modified carbon dioxide through the entire dynamic extraction period and thereby improves the recovery of all PAHs. Note that in the modifier pump method, the sediment was wetted to a 50% moisture content before SFE. To improve extraction and trapping efficiency, our extraction method consisted of three extraction and three rinse steps. In extraction step one, the more readily extractable aliphatic hydrocarbons and some of the lower molecular PAHs were first removed from the sediment by a short extraction with pure CO_2 and they were rinsed off the trap before the subsequent steps. The rest of the lighter PAHs and the bulk of the heavier PAHs were extracted in step two when modified CO_2 was introduced. In order to reduce the amount of solvents accumulated in the trap causing degraded adsorption efficiency, a 1:4 instead of a 1:1 mixture of methanol and DCM was used as modifiers. In this case, all of the DCM and some of the methanol were evaporated at the nozzle with a temperature set at 45°C during the depressurization of CO_2. The last step was a short extraction designed to remove any residual modifier in the CO_2 pump and lines before the extraction of the next sample. Using this procedure with the modifier pump, we obtained the same recovery for all PAHs in EC-1 as the Soxhlet or certified values (Table I). By eliminating the second and third extractions of the same sample as in the case without the pump, we were also able to fully automate the entire extraction sequence and reduce the sample extraction time by over 50% from 150 to 70 min. As indicated by the standard deviations for all PAHs in replicate determinations in Table I, the precision of the

TABLE I

PRECISION AND ACCURACY OF THE SFE METHOD USING THE MODIFIER PUMP FOR THE RECOVERY OF PAHs IN EC-1

Standard deviations given for SFE values were based on six replicate extractions.

PAH	*m/z*	Certified value (μg/g)	SFE value (μg/g)	% of certified
Naphthalene	128	(27.9)[a]	41.3 ± 3.6	(148)
Acenaphthylene	152	(0.8)	0.9 ± 0.1	(112)
Acenaphthene	154	(0.2)	0.2 ± 0.01	(100)
Fluorene	166	(15.3)	15.6 ± 1.8	(102)
Phenanthrene	178	15.8 ± 1.2	16.1 ± 1.8	102
Anthracene	178	(1.3)	1.1 ± 0.2	(88)
Fluoranthene	202	23.2 ± 2.0	24.1 ± 2.1	104
Pyrene	202	16.7 ± 2.0	17.2 ± 1.9	103
Benzo[*a*]anthracene	228	8.7 ± 0.8	8.8 ± 1.0	101
Chrysene	228	(9.2)	7.9 ± 0.9	(86)
Benzo[*b*]fluoranthene	252	7.9 ± 0.9	8.5 ± 1.1	108
Benzo[*k*]fluoranthene	252	4.4 ± 0.5	4.1 ± 0.5	91
Benzo[*a*]pyrene	252	5.3 ± 0.7	5.1 ± 0.6	96
Indeno[1,2,3-*cd*]pyrene	276	5.7 ± 0.6	5.2 ± 0.6	91
Benzo[*ghi*]perylene	276	4.9 ± 0.7	4.3 ± 0.5	88
Dibenzo[*ah*]anthracene	278	(1.3)	1.1 ± 0.2	(85)

[a] Values in parentheses were obtained from or compared to Soxhlet extraction results which were not certified.

SFE procedure is virtually the same as the Soxhlet method.

Application of the SFE method to other types of sediments

So far, all the results were based on the work of a single CRM. In order to test the SFE method using the modifier pump for general applicability, it was further examined on several other different types of sediment, including a marine sediment CRM (HS-3) and sediment samples collected in Vancouver Harbour (VAN-4) and Detroit River (DET-1). For HS-3, 13 out of the 16 SFE results (Table II) were within one standard deviation from their certified values. Also in consistency with our findings, all of our PAH results for HS-3 were a few times higher than those obtained on the same CRM by other workers using pure carbon dioxide at 350 atm (1 atm = $1.01 \cdot 10^5$ Pa) and 60°C for 20 min [21]. No certified PAH concentrations were available for DET-1 and VAN-4, however, it is obvious from Table II that the SFE and Soxhlet values are very comparable in nearly all cases. The above results suggested that our proposed SFE procedure is suitable for the extraction of PAHs in environmental samples.

SFE of methyl PAHs in sediments

Lower-molecular-mass PAHs are more readily metabolized in the environment to give methyl or alkyl derivatives. Methylated derivatives of naphthalene and a few other PAHs are commonly found in sediment samples. Our current SFE method can also be applied to the extraction of methyl PAHs which are metabolites of their parent compounds. Because of the availability of authentic standards, our work was only limited to the five methyl and dimethyl naphthalenes, three methyl and dimethyl anthracenes and one each of methylfluorene and methylphenanthrene as listed in Table III. Similar to the findings for naphthalene in EC-1 and other samples in Table II, the SFE recoveries for methyl and dimethyl naphthalenes in DET-1 and VAN-4 were *ca.* 150 and 125%, respectively of their Soxhlet values. Again, the lower results can be attributed to the evaporative losses of these semi-volatile com-

TABLE II

COMPARISON OF PAH RESULTS IN SEVERAL SEDIMENT OR SOIL SAMPLES USING THE SFE AND MODIFIER PUMP TECHNIQUE *VS.* CERTIFIED OR SOXHLET VALUES

All in-house SFE results were the mean of triplicate determinations.

PAH	HS-3 (μg/g)		DET-1 (ng/g)		VAN-4 (μg/g)	
	Certified	SFE	Soxhlet	SFE	Soxhlet	SFE
Naphthalene	9.0 ± 0.7	7.4 ± 0.6	450	695 ± 73	7.1	11.6 ± 1.2
Acenaphthylene	0.3 ± 0.1	0.4 ± 0.1	139	144 ± 13	2.5	2.8 ± 0.3
Acenaphthene	4.5 ± 1.5	3.3 ± 0.3	65	58 ± 3	<0.1	<0.1
Fluorene	13.6 ± 3.1	10.4 ± 1.3	140	135 ± 11	4.8	5.1 ± 0.4
Phenanthrene	85 ± 20	86.2 ± 9.5	1044	1018 ± 88	25.4	25.8 ± 2.6
Anthracene	13.4 ± 0.5	12.1 ± 1.5	379	303 ± 46	4.5	4.3 ± 0.5
Fluoranthene	60 ± 9	54.0 ± 6.1	2345	2067 ± 157	20.7	20.5 ± 2.2
Pyrene	39 ± 9	32.7 ± 3.7	2189	2039 ± 176	30.9	29.4 ± 2.5
Benzo[*a*]anthracene	14.6 ± 2.0	12.1 ± 1.3	2116	2104 ± 235	12.4	12.4 ± 1.4
Chrysene	14.1 ± 2.0	12.0 ± 1.3	1754	1644 ± 149	10.7	9.4 ± 0.9
Benzo[*b*]fluoranthene	7.7 ± 1.2	8.4 ± 0.9	1080	1240 ± 117	4.0	4.2 ± 0.5
Benzo[*k*]fluoranthene	2.8 ± 2.0	3.2 ± 0.5	1142	1198 ± 158	2.1	2.1 ± 0.2
Benzo[*a*]pyrene	7.4 ± 3.6	6.6 ± 0.8	1296	1277 ± 135	9.3	8.5 ± 0.9
Indeno[1,2,3-*cd*]pyrene	5.0 ± 2.0	4.5 ± 0.6	1209	1113 ± 105	4.1	4.0 ± 0.5
Benzo[*ghi*]perylene	5.4 ± 1.3	4.4 ± 0.6	853	626 ± 71	4.2	3.9 ± 0.5
Dibenzo[*ah*]anthracene	1.3 ± 0.5	1.1 ± 0.3	304	288 ± 19	1.8	1.6 ± 0.2

pounds in the Soxhlet procedure. For the methyl derivatives of fluorene, phenanthrene and anthracene, the SFE results were only 5 to 10% higher than the Soxhlet results.

CONCLUSIONS

The 16 PAHs listed in the US EPA Method 610 can be recovered from naturally contami-

TABLE III

CONCENTRATIONS OF SOME METHYL PAHs IN DET-1 AND VAN-4 OBTAINED BY SFE AND SOXHLET EXTRACTION

All SFE results were the mean of triplicate determinations.

Methyl PAH	*m/z*	DET-1		VAN-4	
		Soxhlet (ng/g)	SFE % Soxhlet	Soxhlet (ng/g)	SFE % Soxhlet
2-Methylnaphthalene	142	56	150	1470	149
1-Methylnaphthalene	142	50	152	3760	147
2,6-Dimethylnaphthalene	156	42	131	2520	126
2,3-Dimethylnaphthalene	156	9	125	1540	120
1,2-Dimethylnaphthalene	156	N.D.	N.D.	746	125
1-Methylfluorene	180	36	109	1360	110
2-Methylanthracene	192	115	104	3210	105
1-Methylanthracene + 1-methylphenanthrene	192	221	107	4780	110
9,10-Dimethylanthracene	206	N.D.	N.D.	160	106

nated sediments including certified reference materials by SFE with carbon dioxide at rates similar to Soxhlet extraction. This was achieved by three consecutive extractions on the same sample at 120°C and a carbon dioxide pressure of 36 MPa in the presence of a mixture of water, methanol and DCM as modifiers. Yet, the same results were also obtained for less than half of the time if the modifiers were introduced by a pump during the dynamic extraction stage. Simultaneously, this SFE method using the modifier pump also provided quantitative recovery of several methyl PAHs in sediments. The elimination of cleanup steps and nearly all solvent evaporation, the substitution of carbon dioxide for the less desirable nitrous oxide or Freon-22, fast extraction times (*ca.* 70 min per sample) and Soxhlet-like recovery are the major advantages that make this technique a method of choice among other Soxhlet and SFE procedures developed for PAHs.

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Laser-induced fluorescence detection of 9-fluorenylmethyl chloroformate derivatized amino acids in capillary electrophoresis

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ABSTRACT

Laser-induced fluorescence (LIF) was applied to the detection of 9-fluorenylmethyl chloroformate (FMOC-Cl) derivatized amino acids separated by capillary electrophoresis. Fluorescence excitation was provided by a pulsed, KrF laser operating at 248 nm. A limit of detection of $5 \cdot 10^{-10}$ *M* was obtained for FMOC-alanine ($S/N = 2$). Separation of FMOC-derivatized proline, hydroxyproline, and sarcosine was achieved with a 20 m*M* borate buffer (pH 9.2), and the separation of FMOC-derivatized amino acid standard mixture was obtained using a 20 m*M* borate buffer (pH 9.2) containing 25 m*M* sodium dodecyl sulfate.

INTRODUCTION

Capillary electrophoresis (CE) is a rapid and highly efficient separation technique for a large variety of compounds [1]. UV absorption is the most widely used detection mode in CE. To improve sensitivity, fluorescence detection is also used. Detection of native amino acids is difficult because most of them do not possess strong spectrophotometric or fluorogenic properties. Therefore, chemical derivatization is necessary for sensitive determination of amino acids. Fluorescamine, *o*-phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), and naphthalene-2,3-dicarboxaldehyde (NDA) are some of the common fluorogenic reagents that undergo rapid derivatization reaction with amino acids [2–5], but only FMOC-Cl forms fluorogenic derivatives with both primary and secondary amines. Albin *et al.* [6] have evaluated the applications of fluorescamine, OPA and FMOC-Cl for the fluorescence detection of amino acids in CE using conventional excitation sources. For ultrasensitive detection, laser-induced fluorescence (LIF) detection can be used [7]. LIF detection of fluorescamine-derivatized marine toxins [8], OPA- and NDA-derivatized amino acids [9,10] has been shown with a helium-cadmium laser. To the best of our knowledge, LIF detection of FMOC derivatives has not been done. The goal of this study is to demonstrate that high-sensitivity LIF detection of FMOC-derivatized amino acids in CE can be achieved by utilizing a rugged, affordable, pulsed-laser operating at 248 nm.

EXPERIMENTAL

Apparatus

The home-built CE–LIF system that we used was similar to that described previously [11]. Separations were performed with 70 cm (60 cm to detector) × 50 μm I.D. fused-silica capillaries

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(Polymicro Technologies, Phoenix, AZ, USA). A 1-cm portion of capillary coating was removed by flame for on-column detection. Positive-polarity high voltage for separation was provided by a Glassman power supply (Whitehouse Station, NJ, USA). Gravity injection was carried out by raising the high voltage end of the capillary by 10 cm for 20 s. Fluorescence excitation was provided by a pulsed-UV laser operating at 248 nm (Model GX-500; Potomac Photonics, Lanham, MD, USA). The laser beam was spectroscopically filtered with a line filter (No. 250-S-1D; ARC, Acton, MA, USA) and then focused onto the capillary with a 25 mm f.1., UV-graded bi-convex lens. The laser power after the lens was *ca.* 0.5 mW, which was measured with a power meter (Model 815; Newport Corp., Fountain Valley, CA, USA). Fluorescence emission was collected at a 90° angle to the incident laser beam with a 10×, numerical aperture (NA) = 0.5, UV-graded microscope objective (Model Fluar; Carl Zeiss, Thornwood, NY, USA), or as otherwise specified, with a 20×, NA = 0.4, glass objective (Model 13590; Oriel, Stratford, CT, USA). After passing through a bandpass (FWHM = 60 nm, No. 310-B-1D, ARC) and a cut-off filter (WG-306; Melles Griot, Irvine, CA, USA), the collected emission was detected by a photomultiplier tube (PMT; Model 70680, Oriel). The current output of the PMT was fed into a boxcar averager (Model 4100; EG&G, Princeton, NJ, USA) and its voltage output was displayed in a PS/2 computer via an A/D interfacing module (Model 406; Beckman Instruments, Fullerton, CA, USA).

The amino acid standard mixture containing 17 amino acids (100 pmole/μl each) was obtained from Hewlett-Packard (Palo Alto, CA, USA). Sodium dodecyl sulfate (SDS; Kodak, Rochester, NY, USA) was washed with ether before use. FMOC-Cl (Sigma, St. Louis, MO, USA) was prepared in acetonitrile as a 5 m*M* solution. All other chemicals were obtained from Sigma. Electrophoresis buffers were prepared in high purity water generated with a NANOpure water system (Barnstead, Dubuque, IA, USA) and filtered through a 0.2-μm membrane filter before use. The amino acids were derivatized by mixing 500 μl of dilute sample, 100 μl of 0.4 *M* boric acid-sodium hydroxide (pH 9.3), and 400 μl of FMOC-Cl for 1 min. Excess FMOC-Cl and its hydrolyzed products were extracted twice from the reaction mixture with 2 ml pentane. The aqueous portion was diluted to the appropriate concentration with water before CE analysis. A filtered urine sample was diluted 10-fold with water and the diluted sample was then subjected to the FMOC derivatization described above. The aqueous portion was diluted 100-fold before CE analysis.

RESULTS AND DISCUSSION

FMOC-Cl reacts rapidly (*ca.* 1 min) with primary and secondary amines to form highly fluorescent, stable derivatives [12]. FMOC-Cl and its hydrolyzed products fluoresce, but they can be readily extracted with pentane. Sensitive fluorescence detection of FMOC-derivatized amino acids in CE were achieved using conventional excitation sources such as xenon or deuterium lamps [6]. To further improve sensitivity, LIF detection could be used. The absorption maximum of FMOC derivatives is at approximately 265 nm; thus, the UV laser (248 nm) should provide adequate excitation for these molecules. Since the emission of FMOC is at approximately 315 nm, a UV-graded microscope objective from Carl Zeiss (10×, NA = 0.5) was used for emission collection. We found that this objective provided at least a 5-fold increase in sensitivity as compared to a conventional glass objective (*e.g.* 20×, NA = 0.4 from Oriel). This enhancement was mainly due to the higher transmission of light in the 300 nm region. Fig. 1 shows the high-sensitivity LIF detection of $1.2 \cdot 10^{-8}$ *M* FMOC-alanine ($2 \cdot 10^{-5}$ *M* before derivatization). The limit of detection (LOD) is approximately $5 \cdot 10^{-10}$ *M* (*S*/*N* = 2) which is *ca.* 100-fold more sensitive than that obtained using a xenon lamp (260 nm) as the excitation source [6]. This level of detection is comparable to that of NDA and about 40-fold more sensitive than that of OPA in other CE–LIF studies [9,10]. Derivatization at a low sample concentration is subject to the possibility of an inefficient derivatization reaction and sample loss. A 100-fold less concentrated alanine solution (*i.e.* $2 \cdot 10^{-7}$

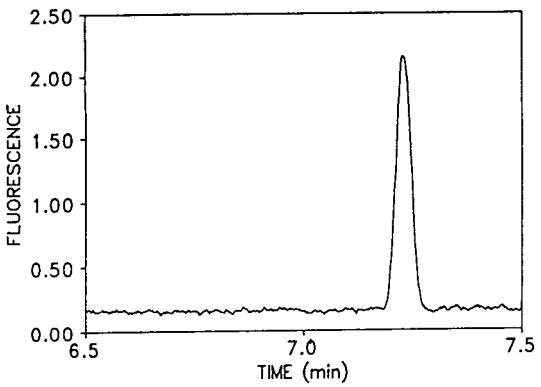

Fig. 1. Electropherogram of $1.2 \cdot 10^{-8}$ *M* FMOC-alanine. Capillary: 70 cm × 50 μm; running buffer: 20 m*M* borate (pH 9.2); voltage: 20 kV; gravity injection: 10 cm, 20 s.

M) was derivatized and then diluted to $1.2 \cdot 10^{-8}$ *M* for injection (Fig. 2). As a comparison, the blank peak is also shown in Fig. 2. The LOD obtained in this case agrees with that obtained from Fig. 1; this suggests that the problems mentioned above did not occur for sample levels of at least 10^{-7} *M*. A 5-point peak-height calibration curve of FMOC-alanine was constructed between $1.3 \cdot 10^{-9}$ and $1.3 \cdot 10^{-7}$ *M*. The response of the LIF detection was linear for at least 2 orders of magnitude ($r^2 = 0.9996$).

Proline (Pro), hydroxyproline (Hyp), and sarcosine (Sar) are secondary amino acids that show important pathological and biological properties [13–16]. Determination of these compounds has been achieved using thin-layer chromatography, gas chromatography or high-performance liquid

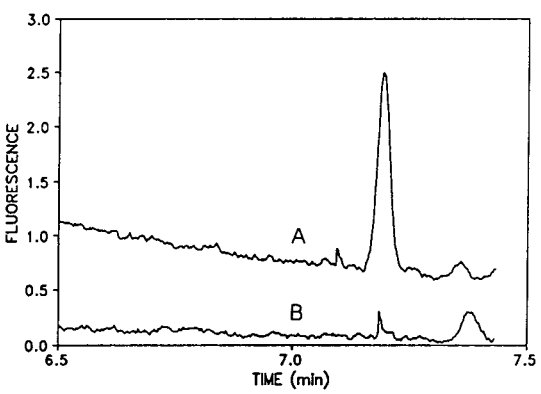

Fig. 2. Electropherograms of (A) $1.2 \cdot 10^{-8}$ *M* FMOC-alanine, and (B) blank. Running conditions as in Fig. 1.

chromatography with various spectroscopic, radiometric, or electrochemical detection methods [17–22]. Most of these methods are time-consuming. Recently, Guzman *et al.* [23] used CE to separate fluorescamine-derivatized Pro and Hyp. Because fluorescamine does not form fluorogenic derivatives with secondary amino acids, absorption detection (214 nm) was used in their study. These authors obtained a detection limit at the 10^{-5} *M* level, which is typical for absorption detection in CE. Fig. 3 shows the electropherogram for the separation of FMOC-derivatized Pro, Hyp and Sar. With LIF detection, an LOD of each amino acid was obtained at the 10^{-9} *M* level. With a 75 μm capillary, Pro and Hyp were partially resolved at the indicated running conditions, but were baseline separated when a 50 μm capillary was used. A borate buffer containing SDS greatly enhanced the separation of Pro and Hyp (below).

Determination of amino acids in biological samples [12] and protein hydrolysates [24] has been performed with precolumn derivatization using FMOC-Cl, and subsequent analysis of the derivatives by reversed-phase HPLC with gradient elution. Alternately, efficient separation of FMOC-derivatized amino acids can be achieved by CE using an SDS micellar buffer [6,25]. Fig. 4 is the electropherogram for the separation and high sensitivity LIF detection of amino acid

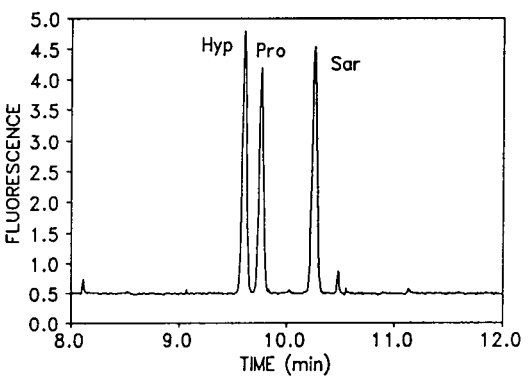

Fig. 3. Electropherogram for the separation of proline (Pro), hydroxyproline (Hyp) and sarcosine (Sar). Capillary: 70 cm × 50 μm; running buffer: 10 m*M* phosphate (pH 7.0); voltage: 25 kV; gravity injection: 10 cm, 20 s. The concentration of each amino acid is $4 \cdot 10^{-8}$ *M*. A glass microscope objective was used.

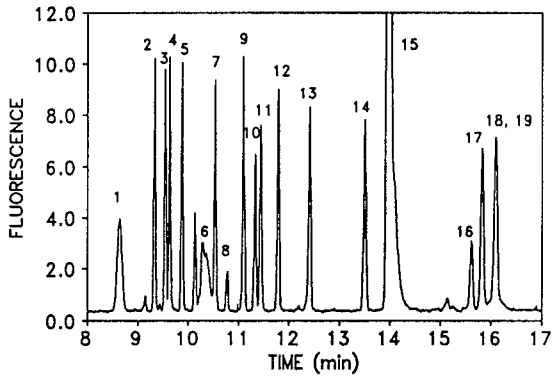

Fig. 4. Electropherogram for the separation of FMOC-derivatized amino acid standard mixture + Hyp. Capillary: 70 cm × 50 μm. Running buffer: 20 m*M* borate (pH 9.2)–25 m*M* SDS; voltage: 20 kV; gravity injection: 10 cm, 20 s. The concentration of each amino acid is $3 \cdot 10^{-8}$ *M*. Peaks: 1 = hydroxyproline; 2 = serine; 3 = threonine; 4 = alanine; 5 = glycine; 6 = proline; 7 = valine; 8 = tyrosine; 9 = glutamic acid; 10 = methionine; 11 = aspartic acid; 12 = isoleucine; 13 = leucine; 14 = phenylalanine; 15 = FMOC background; 16 = cystine; 17 = arginine; 18 = lysine; 19 = histidine.

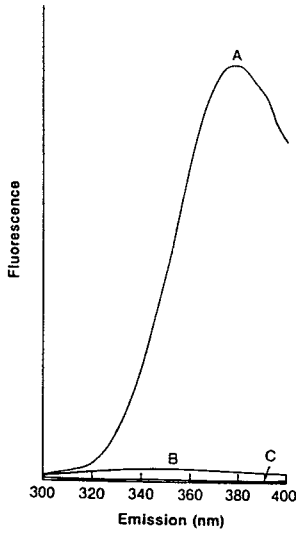

Fig. 5. Fluorescence spectra of (A) SDS, (B) SDS washed with diethyl ether, and (C) water. The SDS concentration was about 10 m*M*.

standard mixture + Hyp that was derivatized with FMOC-Cl. The concentration of each amino acid injected was $3 \cdot 10^{-8}$ *M*. Milofsky and Yeung [26] have pointed out that fluorescence of the impurities associated with SDS degraded detection at 300–400 nm significantly, and the authors purified the SDS by recrystallization before use. We found that most of the SDS impurities were also efficiently removed by washing with diethyl ether (Fig. 5). Background fluorescence was further reduced by the 310-nm bandpass filter. As a result, our detection was degraded by only a factor of about 2 with the addition of 25 m*M* SDS in the running buffer. In Fig. 4, the peaks are identified by spiking of individual derivatized amino acids. With the indicated running conditions, almost all of the amino acids were resolved in under 17 min. McLaughlin *et al.* [25] have show the separation of 21 amino acids with a higher concentration SDS buffer; this highlights the potential application of CE as an alternative separation tool to HPLC in the analysis of FMOC-derivatized amino acids. Since the Hyp peak eluted prior to all of the other tested amino acids, the CE system may facilitate the determination of Hyp in certain biological applications [23]. As an example to show the analysis of a real sample, FMOC-derivatized human urine (1:1000 dilution) was subjected to CE–LIF analysis, as shown in Fig. 6. Identification of the individual peaks was not performed because this was not the goal of this study; rather, it demonstrates the potential application of this CE–LIF system for the high-sensitivity determination of biological amino compounds.

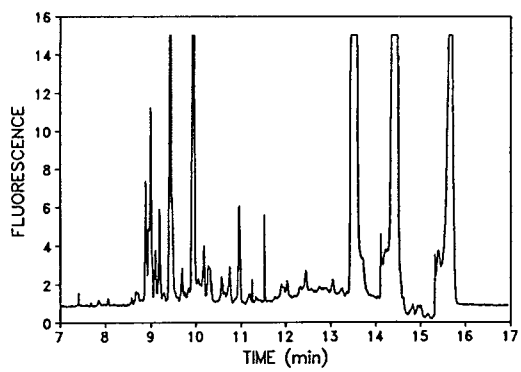

Fig. 6. Electropherogram for the separation of FMOC-derivatized urine (1:1000 dilution). Running conditions as in Fig. 4.

CONCLUSIONS

This study demonstrates that high-sensitivity LIF detection of FMOC-derivatized amino acids separated in CE could be achieved by using a pulsed-laser operating at 248 nm. An LOD of $5 \cdot 10^{-10}$ *M* for FMOC-alanine was obtained, which was comparable to the LOD obtained with NDA and better than those obtained with OPA in other LIF studies. Although not as sensitive as the FMOC derivatives, we could detect OPA derivatized amino acids at the 10^{-8} *M* level with the 248-nm laser. The combination of efficient separation of CE and high-sensitive detection of LIF should be very useful for the analysis of FMOC-labeled amino acids in protein hydrolysates and the amino compounds in biological samples. The use of the combined OPA-FMOC derivatization for the selective detection of secondary amines [17] in CE with LIF detection is currently being studied in this laboratory.

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Determination of aromatic choline esters by micellar electrokinetic capillary chromatography

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ABSTRACT

Micellar electrokinetic capillary chromatography (MECC) using sodium dodecyl sulphate (SDS), alkyltrimethylammonium bromide (C_{10} to C_{18} TAB) or sodium cholate (NaCh) as the micellar phase has been investigated for separation and quantitation of individual aromatic choline esters. MECC based on NaCh was found to be suitable for determination of these choline esters. The influence of changes in separation conditions were evaluated according to migration times, peak areas, and separation efficiency for the compounds considered. Up to 485 000 theoretical plates per meter were obtained and relative standard deviations were 0.4–0.7% for relative migration times. Repeatability, linearity and detection limits for the developed method were determined with the detection limits found to be 25–60 pg. It is shown that efficient separations of structurally closely related compounds are possible within a few minutes in test solution as well as in samples prepared from plant material.

INTRODUCTION

Choline is present in all living cells as phosphate and carboxylic acid esters of which aromatic choline esters form a well defined group of natural products occurring in plants [1]. These compounds are all derivatives of benzoic or cinnamic acids containing phenolic groups or other substituents on the aromatic ring [2–9]. Sinapine is the best known in *Brassica* [9], where it can accumulate to an appreciable level in the seeds and co-occur with several other aromatic choline esters [10]. These compounds call for special attention owing to their effects on rapeseed quality and the physiological effects they may have [10–12].

Methods of analyses for determination of individual aromatic choline esters are needed for efficient studies of the above mentioned problems. Available methods include high-performance liquid chromatography (HPLC), based on ion-pairing chromatography [13,14]. However, HPLC methods suffer from some disadvantages compared to the potential possibilities with use of high-performance capillary electrophoresis (HPCE) for determination of various plant constituents [15,16]. Recent developments indicate thus, that the HPCE technique is an attractive alternative to HPLC determination of aromatic choline esters.

Separation and determination of cations have been achieved in capillary zone electrophoresis of mono- and divalent metal ions, amines [17,18], oligopeptides [17] and ammonium salts [19]. Moreover, the technique of micellar electrokinetic capillary chromatography (MECC) [20] has proved to be successful for catecholamines [21–23] using sodium dodecyl sulphate (SDS) micelles. However, strong ionic interaction of cationic compounds to the polar group of the SDS micelles may constitute a problem for certain cationic species [21–24]. Bile salts, on the other hand, have proved to be applicable as

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detergents in MECC for the separation of basic solutes as well as hydrophobic compounds [24–26].

This paper aims at an efficient HPCE method of analysis for the aromatic choline esters evaluated. Various types of detergents for MECC of these compounds have been tested. A method for qualitative and quantitative analysis, using an anionic bile salt surfactant as the micellar phase supplemented with a zwitterionic compound to prevent adsorption of analytes to the capillary wall, is presented. A test solution containing six structurally closely related compounds was used for evaluation of the influence of different separation conditions on various separation parameters. With the combined technique of group separation, purification, and MECC now developed, a rapid, simple and efficient method of analysis of naturally occurring aromatic choline esters is obtained as illustrated for the complex mixture of these compounds occurring in seeds of *Hesperis matronalis* L. and *Sinapis alba* L.

EXPERIMENTAL

Apparatus

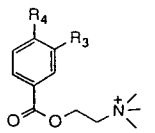
The apparatus used was an ABI Model 270A capillary electrophoresis system (Applied Biosystems, Ramsey, NJ, USA), with a 760 mm × 0.05 mm I.D. fused-silica capillary tube. Detection was performed by on-column measurements of UV absorption at a position 530 mm from the injection end of the capillary. For data processing, a Shimadzu (Kyoto, Japan) Chromatopac C-R3A was used.

Samples and reagents

Aromatic choline esters studied were isolated or synthesized in our laboratory according to methods described elsewhere [13,27].

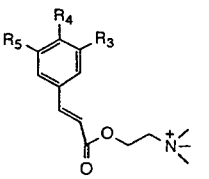
The names and structures of the benzoic- and cinnamic acid derivatives are presented in Fig. 1, together with numbers used in the text as well as in the other figures and tables.

Disodium hydrogenphosphate, disodium tetraborate, taurine, SDS, alkyltrimethylammonium bromide (C_{10} to C_{18} TAB) and sodium cholate (NaCh) were obtained from Sigma (St. Louis, MO, USA). 2-Propanol was from Merck (Darmstadt, Germany). All chemicals were of analytical-reagent grade.



Benzoic acid derivatives

No.	R_3	R_4	Name
2	- H	- OH	4 - Hydroxybenzoylcholine
3	- OCH_3	- OH	Vanillylcholine
9	- OH	- OCH_3	Isovanillylcholine
10	- OCH_3	- OCH_3	Hesperaline



Cinnamic acid derivatives

No.	R_3	R_4	R_5	Name
4	- H	- OH	- H	p - coumaroylcholine
5	- OCH_3	- OH	- H	Feruloylcholine
6	- OCH_3	- OH	- OCH_3	Sinapine
7	- OCH_3	- OCH_3	- H	3,4 - dimethoxycinnamoylcholine
8	- OH	- OCH_3	- H	Isoferuloylcholine

Fig. 1. Structures and names of benzoic and cinnamic acid derivatives used in MECC analyses. Numbers indicated are used in connection with the other figures and tables. Trigonelline amide serves as internal standard and is numbered **1**.

Procedure

The separation buffer in the SDS system was prepared with 50 m*M* SDS, 100 m*M* disodium hydrogenphosphate, 10% 2-propanol and, if added, 1 *M* taurine. Investigations with different chain lengths of alkyltrimethylammonium bromide (50 m*M*) were performed with a buffer containing 30 m*M* disodium hydrogenphosphate, 18 m*M* disodium tetraborate and 5% 2-propanol in addition to the detergent. pH was adjusted to 7.0 in all systems.

The different separation buffers tested in the NaCh system were prepared with variations in concentrations of 2-propanol, NaCh, taurine and

phosphate. pH of the buffers was 7.3, except when the pH dependence was investigated.

Common for all systems was filtration of buffers through a 0.20 μm membrane filter prior to use. Washing of the capillary was performed with 1.0 *M* NaOH for 2 min and with buffer for 5 min before each analysis. When buffer composition was changed, the washing procedure was extended to 4 min with 1.0 *M* NaOH and 10 min with the new buffer. Buffers were changed manually.

In the SDS and NaCh systems, the samples were introduced from the positive end of the capillary and, in the C_{10} to C_{18} TAB systems, from the negative end by vacuum for 1 s. On-column detection at 235 nm was applied. Unless otherwise stated, the separations were performed at 30°C and 20 kV (SDS and NaCh) or 40°C and 18 kV (C_{10} to C_{18} TAB).

Calculations of relative migration times (*RMT*), normalized area (*NA*), theoretical plates (*N*) and resolution (R_s) were performed as described by Michaelsen *et al.* [15]. Repeatabilities were estimated from the means and relative standard deviations (R.S.D.). The linearity of the method was determined from linear regression analysis based on least-squares estimates. Approximate detection limits for the compounds investigated were determined from the linearity analysis and a signal-to-noise ratio of 2:1.

RESULTS AND DISCUSSION

Preliminary trials with SDS as a surfactant in MECC resulted in long migration times (*MT*) and poor separation of the aromatic choline esters studied (Fig. 1). Strong ionic interactions between the positively charged analytes and the anionic part of the surfactant seemed to be the dominating factor, as taurine addition were unable to shorten *MT*. Taurine is a zwitterionic compound, reducing interactions of cations with the negatively charged capillary wall by simple competition mechanism [28].

Ion pairing between analytes and surfactants was prevented by use of long chain cationic detergents as C_{10} to C_{18} alkyltrimethylammonium bromides. These detergents also create a double layer on the capillary wall changing the negatively charge here to a positive double layer [15]. Different chain lengths were tested (C_{10} to C_{18}), with tetradecyltrimethylammonium bromide (TTAB) giving the best separations (Fig. 2). However, the separations obtained were not considered satisfactory and changes of temperature, voltage and concentration of 2-propanol did not lead to sufficient improvements.

The separation using NaCh as pseudo-stationary phase is, as for SDS, based on the hydrophobic and ion pairing interaction of the positively charged aromatic choline esters and the negatively charged micelles. However, micellar structure of NaCh compared to SDS are thought to prevent too strong ionic bonds, and NaCh, at concentrations higher than the critical micelle concentration (CMC), has been suggested to form rod-like or cylindrical micelles

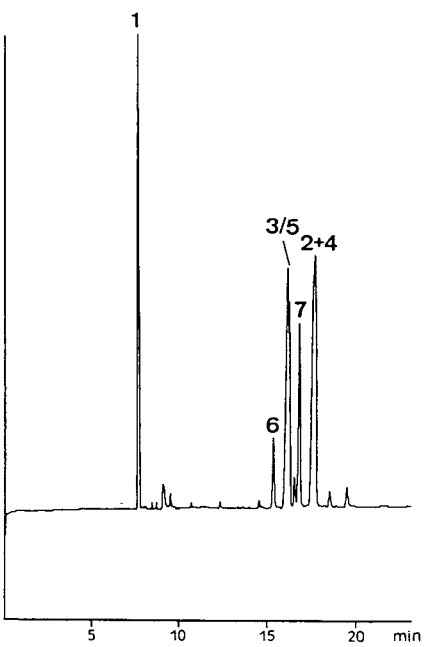


Fig. 2. Electropherogram of the mixture of aromatic choline esters separated by the MECC method (TTAB). Numbers as in Fig. 1. Separation conditions: buffer composition, 30 m*M* disodium hydrogenphosphate–18 m*M* disodium tetraborate–50 m*M* TTAB–5% 2-propanol; temperature, 40°C; voltage, −18 kV; total length of capillary, 760 mm; detection 530 mm from injection end; UV detection at 235 nm. Vacuum injection for 1 s.

with the hydrophobic part situated on the surface and the hydrophilic portions turned inward [26,29]. This should be compared to the likely spherical form of alkyltrimethylammonium bromide or SDS micelles with the charged groups situated on the surface of the micelles and having a hydrophobic core [15,20,30]. Addition of taurine to the buffer is thought to reduce interactions between the negatively charged capillary wall and the positively charged analytes.

Under the separation conditions applied, micelles formed by NaCh move toward the anode, whereas the direction of electroosmotic flow (EOF) will be toward the cathode. Injection of the positively charged aromatic choline esters at the positive end of the capillary results in an increase of analyte speed due to EOF, whereas micelles act retardingly. The selective retention obtained is thus a result of differential partitioning of the aromatic choline esters between the aqueous buffer and the hydrophobic micellar phase.

The electropherogram in Fig. 3 shows the elution order of the aromatic choline esters investigated. Identification of the peaks corresponding to the individual compounds was done from mixing of the authentic compounds [13,27], and according to their UV spectroscopic properties with use of detection at various wavelengths in MECC. In addition, *RMT* under the given separation conditions were determined for the individual compounds when required. *MT* for benzoic acid derivatives is seen to be less than *MT* for cinnamic acid derivatives, indicating less interaction of the benzoic acid derivatives with the micellar phase at the applied conditions. The internal standard appears as peak no. 1, whereas the unnumbered peaks late in the electropherogram are due to the solvent front. The first peak in the solvent front represents EOF as determined by the injection of methanol.

Variation of the separation parameters in the NaCh system implies changes of temperature, voltage and composition of the separation buffer including pH and concentrations of 2-propanol, NaCh, taurine and phosphate. A systematic investigation of the influence of those parameter changes on *MT*, *RMT*, *NA*, R_s and *N*, as an expression for efficiency, have been carried out.

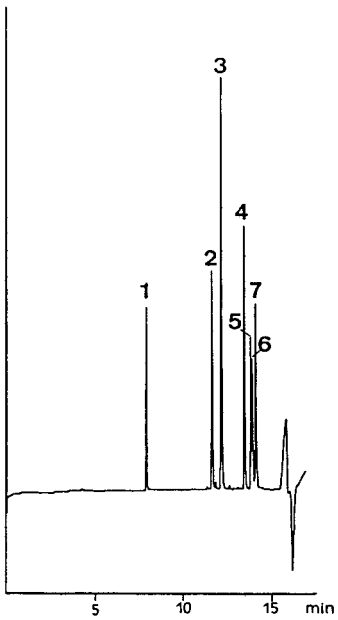


Fig. 3. Electropherogram of the mixture of aromatic choline esters separated by the MECC method (NaCh). Numbers as in Fig. 1. Separation conditions: buffer composition, 100 m*M* disodium hydrogenphosphate–600 m*M* taurine–50 m*M* NaCh–10% 2-propanol; temperature, 30°C; voltage, 20 kV; total length of capillary, 760 mm; detection 530 mm from injection end; UV detection at 235 nm. Vacuum injection for 1 s.

2-Propanol concentration

An increase in the concentration of 2-propanol in the separation buffer from 0 to 12% considerably increased *MT* for all of the solutes. *RMT* values, with trigonelline amide (**1**) as the reference compound, were nearly unaffected or only slightly decreased. A reduction in *NA* was observed for **3** with increasing concentration of 2-propanol in the buffer. For the other compounds, only a little decrease was observed with higher concentration of the organic modifier. R_s values were unaffected by the content of 2-propanol in the buffer, whereas *N* increased for all of the aromatic choline esters tested.

Presence of an organic modifier in the buffer affects EOF, due to reductions in the zeta potential [31,32]. This reduction in EOF correlated well with the observed increase in *MT* for the compounds analysed.

NaCh micelles tolerate high concentrations of organic modifier, which is probably due to the bile salt micelle structure, having the hydropho-

bic portion of the monomers turned outward the micelle (see above). A more stable micelle [26,29] may thus be the result, when an organic modifier such as 2-propanol is added to the buffer. This may again affect partitioning of aromatic choline esters between the aqueous and micellar phase, leading to the observed changes in *NA* as well as separation efficiency. It is thus seen, that the modifier increases the rate of association/dissociation between micelles and analytes.

Addition of 2-propanol was shown to lower the solubility of taurine, resulting in precipitation of taurine crystals in the buffer at high concentrations of 2-propanol. As a relatively high concentration of taurine was necessary in obtaining a satisfactory separation (see below), it was only possible to test up to 12% 2-propanol. For further studies, a concentration of 10% 2-propanol was chosen, as good separation here was obtained within a reasonable period of time and coelution of the last analytes with the peak caused by buffer constituents was avoided.

NaCh concentration

The increase in *MT* and *RMT* with increasing NaCh concentration from 10 to 75 m*M* was most pronounced for the compounds appearing late in the electropherogram. *NA* was nearly unaffected. Appreciable improvement of R_s was seen for **3–4** with increasing NaCh concentration, whereas *N* changed in a non-systematic way.

An increase in *MT* values of the compounds late in the electropherogram with increasing NaCh concentration is anticipated due to increasing the phase ratio, *i.e.* the ratio of the volume of micellar phase to that of the aqueous phase. Moreover, alterations in the EOF may contribute to the effect observed [24]. With a CMC of NaCh at 13 m*M* [33], the coelution of analytes found when 10 m*M* NaCh was used, indicates the necessity of micelles for separation of aromatic choline esters. Increasing concentration to 25 m*M* NaCh gave a good baseline separation but too narrow separation between the two groups of benzoic and cinnamic acid choline esters. Additional increase of NaCh concentration gave improved separation of the benzoic acid derivatives from the cinnamic acid derivatives as also reflected in R_s for **3–4** and for further studies 50 m*M* NaCh was chosen. The influence of NaCh concentration on *N* and R_s was probably due to changes in the micellar concentration and structure, the interaction of compounds depending on the properties of the individual aromatic choline esters.

Taurine concentration

MT and *RMT* were slightly decreased with increasing taurine concentration (200–600 m*M*). *NA* for **3** and **7** was largely reduced, when taurine content in the buffer was increased from 200 to 400 m*M*, whereas only weak decreases were seen for the other compounds. From 400 to 600 m*M*, *NA* was nearly unaffected. Changes in *N* with increasing taurine concentration was remarkable for **7**, which more than doubled from 200 to 400 m*M* taurine. Other changes were only small and non-systematic. R_s only increased for **3–4** with increasing taurine concentration, indicating a more effective separation between cinnamic and benzoic acid derivatives without changing the separation of compounds within each group.

The importance of having a zwitterion included in the system was shown from results without taurine added to the buffer, giving very late elution and poor separation of analytes. The effect on *MT* with increasing concentration of taurine is probably a combination of reduced association of the positively charged aromatic choline esters with the negatively charged capillary wall and decreased EOF due to the zwitterion associated with the capillary wall and to some extent higher ionic strength of the buffer [33]. A possible explanation for the large reduction in *NA* for **3** and **7** could be decreasing response factors at low taurine concentration. The response factors of aromatic choline esters vary also in HPLC according to the individual compounds and separation conditions [34]. The effect of taurine on the viscosity seem not to be appreciable, as no general effect on the *NA* values was found.

Separation efficiency are affected by changes in the interaction between solutes and micellar phase, which are thought to occur as a result of altered micellar properties, and taurine may affect the binding of counterions to the cholate micelles and hereby change CMC. Moreover,

the CMC value as well as the aggregation number of micelles may be affected by the high ionic strength caused by the added zwitterion [30,35]. 600 m*M* taurine was chosen for further studies due to the short *MT* and high *N* and R_s.

Phosphate concentration

A positive relationship was found between *MT* for compounds late in the electropherogram and increasing concentration of phosphate in the buffer (pH = 8.0). *RMT* was nearly unaffected by increased phosphate concentration. The *NA* values remained relatively constant for the different aromatic choline esters except for **3** which got greatly reduced *NA* by increasing phosphate concentration from 100 to 150 m*M*. *N* and R_s were improved with increasing electrolyte concentration except for **5**, where *N* decreased a little.

A change in buffer composition to 50 m*M* phosphate plus 50 m*M* borate giving 100 m*M* of electrolyte resulted in poorer baseline separation, especially for the cinnamic acid derivatives. It was furthermore observed, that less efficient separation was obtained when adjusting the pH to 8.0, instead of using an unadjusted buffer with a pH around 7.3. To avoid heat damage caused by a high electrical current when 150 m*M* phosphate was used, the following separations took place with 100 m*M* phosphate in the buffer.

pH in separation buffer

Changing the pH from 6.0 to 8.0 resulted in faster migration of analytes. The decrease in *MT* was highest for the cinnamic acid derivatives, indicating that altered interaction with the micellar phase was of importance. Differences in *RMT* for the two groups of derivatives were reduced as the pH was increased. *NA* was unaffected except for **3**, which showed a considerably increase when going from pH 7.0 to 8.0. An increase in pH resulted in increased *N* although the number of theoretical plates for **6** decreased strongly when changing pH from 7.0 to 8.0. On the other hand, the best R_s was achieved at the lowest pH, where the separation gave complete baseline separation for all of the compounds considered. However, the separation buffer with low pH was unstable, resulting in precipitations of cholic acid after only few runs.

An investigation was made on this stability problem, using a buffer system containing 50 m*M* NaCh, 600 m*M* taurine, 100 m*M* phosphate and 10% 2-propanol with different pH increasing 0.2 units from 6.0 to 6.8 (both included). The results showed that only buffers with pH above 6.8 were stable. The explanation for this has to be found in the pK_a value of 6.4 for NaCh. As pH in the buffer approaches pK_a for NaCh, protonization of the carboxyl groups will occur. Cholate hereby becomes uncharged and hence more hydrophobic resulting in precipitation. To avoid this problem, pH of the buffer has to be above 6.8.

Voltage

In accordance with theory, increasing the voltage from 15 to 25 kV reduced *MT* considerably, but this had only little effect on *RMT*. *NA* was nearly unaffected. *N* changed in a non-systematic way, though giving the highest values at 15 kV. R_s was only affected for separation of **3–4** and **5–6** with the best results at 20 kV. The influence of voltage on *N* and R_s values has been discussed further in Bjergegaard *et al.* [16]. An acceptable separation was obtained using a voltage of 20 kV.

Temperature

MT was reduced in a non-linear way as the temperature increased, the reduction in *MT* being lowest at high temperatures. The same phenomenon was obtained analysing flavonoids in a similar buffer system containing NaCh [36]. *RMT* remained unchanged. A reduced viscosity of the solvent in the capillary caused by higher temperature explains the overall faster analysis. Although attempts were made to avoid evaporation, *NA* increased as temperature rose (Fig. 4). Changes in *NA* for cinnamic and benzoic acid derivatives were as discussed previously [16]. *N* was increased for **6** at temperatures above 30°C, whereas a slight decrease was seen for the other compounds tested. R_s was unaffected except for **3–4**, where a decrease was seen with increasing temperatures. Changed separation efficiency at higher temperatures may be explained by

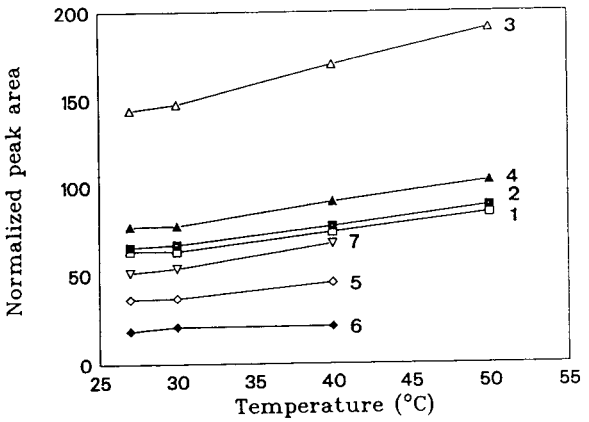


Fig. 4. Relationship between temperature and normalized peak areas of aromatic choline esters. Numbers as in Fig. 1. Other conditions as in Fig. 3.

changed interactions of analytes with the micellar phase [30]. A temperature of 30°C was chosen for the separation of aromatic choline esters, as it resulted in an acceptable separation and total time of analysis.

Adjustment of the buffer system

As a final adjustment of the buffer system, small changes in combinations of NaCh and 2-propanol concentration were performed. Moreover, taurine concentration was lowered from 600 to 500 m*M*. With negligible variations, it was possible to affect separation efficiency markedly. The optimal buffer solution found gave baseline separation of the six structural closely related aromatic choline esters in the test mixture within a detection range of less than 2 min and a total time of analysis about 10 min (Fig. 5). The numbers of theoretical plates per meter of capillary under the chosen separation conditions were 287 000 (**2**), 233 000 (**3**), 403 000 (**4**), 485 000 (**5**), 388 000 (**6**) and 281 000 (**7**), respectively. R_s values for **2–3**, **3–4**, **4–5**, **5–6** and **6–7** were calculated to be 5.0, 8.0, 6.7, 3.3 and 1.5, respectively.

Repeatability

Determination of the repeatabilities of *MT*, *RMT*, *NA* and relative normalized peak area (*RNA*) values (trigonelline amide as reference compound) were performed in the adjusted

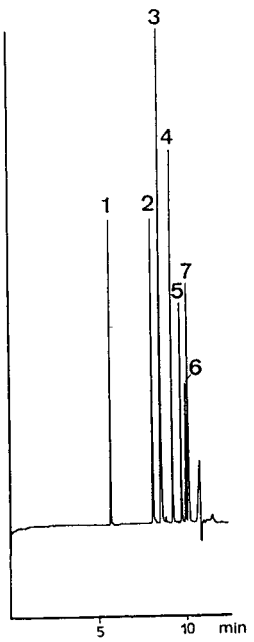


Fig. 5. Electropherogram of the mixture of aromatic choline esters separated by the MECC method (NaCh). Numbers as in Fig. 1. Buffer composition, 100 m*M* disodium hydrogenphosphate–500 m*M* taurine–35 m*M* NaCh–2% 2-propanol. Other separation conditions as in Fig. 3.

buffer system (see above) with the test solution used for variation of separation parameters. Results are shown in Table I.

The experiments were done by changing the buffer at the inlet side between each analysis and at the outlet side after five analyses. Uncertainty caused by evaporation from sample vials during the test [15] was minimized using anti-evaporation septa on the vials. Under the conditions mentioned, the instrument performed very well with respect to repeatabilities. When *RMT*, *NA* and *RNA* values were used compared to *MT* and uncorrected peak areas, the repeatabilities expressed as R.S.D. values were reduced considerably.

Linearity

The linearity was determined from 2 different tests. Test 1 determined the correlation between injection time of a mixture of six different aromatic choline esters and the corresponding *NA* values. Test 2 determined the correlation between increasing concentrations of the same

TABLE I

RELATIVE STANDARD DEVIATION (R.S.D.) OF MIGRATION TIMES (*MT*), RELATIVE MIGRATION TIMES (*RMT*), NORMALIZED PEAK AREAS (*NA*) AND RELATIVE NORMALIZED PEAK AREAS (*RNA*) FOR AROMATIC CHOLINE ESTERS

Separation conditions as in Fig. 5. Numbers in bold are aromatic choline ester numbers (see Fig. 1). For all calculations $n = 8$.

Aromatic choline ester	Relative standard deviation (%)			
	MT	*RMT*	*NA*	*RNA* [a]
2	1.44	0.38	1.68	1.02
3	1.51	0.45	1.73	0.64
4	1.59	0.54	1.67	0.66
5	1.67	0.63	1.31	1.11
6	1.71	0.66	1.60	2.43
7	1.73	0.68	1.55	1.03

[a] Relative to trigonelline amide (**1**)

solution as used in test 1 and the corresponding *NA* values. The results from both tests are shown in Table II.

Both tests showed good linearity with correlation coefficients ranging from 0.9950 to 0.9984. The linear increase in *NA* with increasing injection time as well as increasing concentrations of aromatic choline esters injected shows, that the method now developed may be used to quantitate aromatic choline esters. However, this provides use of an internal standard as trigonelline amide and response factors determined from the results obtained when testing the linearity.

Detection limits

Approximate detection limits have been determined from a signal-to-noise ratio of 2:1 using various dilutions of the aromatic choline ester stock solution. The detection limits found here correspond to the conditions applied, which were a 1-s sample injection into a 760 mm long capillary with an internal diameter of 50 μm. Detection limits were between 16 and 49 μM of each aromatic choline ester in the sample. According to Harbaugh *et al.* [37] and Vinther [38], the injected volume was calculated to be 4.28 nl, assuming a viscosity in the buffer and sample identical to water and with a capillary temperature of 30°C. This results in detection limits between 69 and 282 fmol for each of the aromatic choline esters, which again corresponds to 26–60 pg.

MECC of samples from plant materials

Aromatic choline esters were isolated from seeds of *Hesperis matronalis* L. and *Sinapis alba* L. by the procedure described elsewhere [10], but with the use of a vacuum system (Supelco) for the group separation. Thereby, the group

TABLE II

LINEARITY TESTS

Correlation coefficients (r^2) from linear regression analyses by least squares method of normalized peak areas (*NA*) for various injection times and various concentrations of aromatic choline esters, respectively. Conditions as in Table I and Fig. 5.

Aromatic choline ester	Test 1		Test 2	
	Injection time (s)	r^2	Concentration range (m*M*) [a]	r^2
2	1–9	0.9970	0.049–1.977	0.9968
3	1–9	0.9979	unknown	0.9971
4	1–9	0.9968	0.023–0.921	0.9982
5	1–9	0.9970	0.017–0.663	0.9984
6	1–9	0.9950	0.008–0.325	0.9976
7	1–9	0.9957	0.022–0.863	0.9980

[a] $n = 9$.

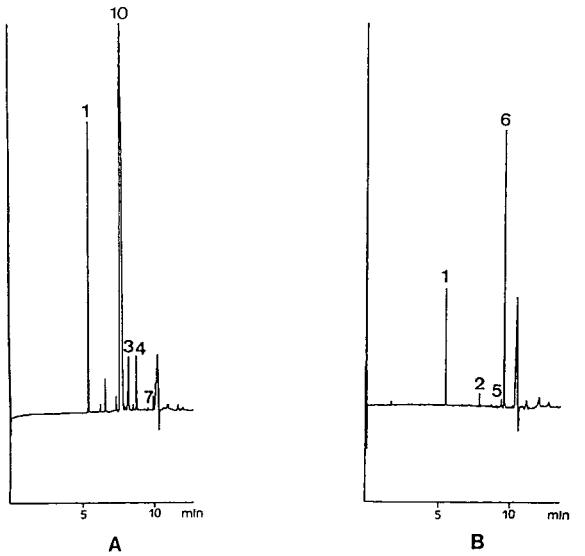


Fig. 6. Electropherogram of the aromatic choline esters accumulated in seeds of *Hesperis matronalis* L. (A) and *Sinapis alba* L. (B). Numbers as in Fig. 1. Separation conditions as in Fig. 5.

separation and purification were reduced in time to only a few minutes. A sample of the eluate (500 μl) was evaporated to dryness and redissolved in water. This solution was used directly for the MECC, resulting in separations of individual aromatic choline esters as shown in Fig. 6. The individual aromatic choline esters in the sample have been identified previously (see above), and as revealed from the electropherograms, it is easy to detect both the quantitatively dominating and minor components in the samples by the applied method.

CONCLUSIONS

Analysis of aromatic choline esters with SDS as the detergent was unsuccessful, probably because of too strong ionic interaction between the positively charged analytes and the negatively charged capillary wall. Use of a positive detergent system, here TTAB, reduced these problems and provided a method which, however, showed much poorer and longer lasting separations compared to the NaCh system tested. Therefore, the MECC technique performed with a bile salt as the surfactant and taurine as the zwitterion is recommended. This system was proven to be highly effective for the separation of the individual aromatic choline esters, having a polar as well as an apolar portion.

The method now developed can be used for qualitative as well as quantitative analysis. Relative migration times are to be known for identification, providing an internal standard to be present in the samples. For quantitation, internal standard and normalized peak areas shall be used in combination with the use of an anti-evaporator on the samples and changing of the buffer at the inlet side preferably after each analysis and at the outlet side after 5 analyses. Taking these precautions, a very reproducible, effective (up to 485 000 theoretical plates per meter) and quick (10 minutes) analysis is provided.

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Separation of aniline derivatives by micellar electrokinetic chromatography

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ABSTRACT

The separation of ten aniline derivatives was investigated by micellar electrokinetic chromatography. The pH dependence of their migration behaviour was determined. The capacity factor for each aniline derivative was calculated and its pH dependence was elucidated from the pK_a value of the aniline derivative. In the case of *p*-anisidine, the pH dependence can be explained by a simple model. The complete separation of ten aniline derivatives was achieved by optimizing the pH and the concentration of sodium dodecyl sulphate, and by adding γ-cyclodextrin. Separation of diphenylamine and N-nitrosodiphenylamine, which cannot be easily distinguished by gas chromatography because of thermal degradation, was achieved.

INTRODUCTION

Micellar electrokinetic chromatography (MEKC) [1] is an attractive, recently developed analytical method. MEKC has a high separation efficiency in comparison with high-performance liquid chromatography, and unlike capillary zone electrophoresis can be used to separate electrically neutral substances. The principle of separation in MEKC is based on differential partition of the solute between ionic micelles and the surrounding aqueous phase [2,3]. MEKC has attracted much attention with regard to the separation of various substances [4–7]. We have previously reported preliminary studies on the analysis of environmental pollutants by MEKC [8,9].

Aniline derivatives are frequently found in environmental waters. In Japan, eight aniline derivatives have been detected in environmental water by capillary gas chromatography–mass spectrometry (GC–MS) [10]. In GC–MS analysis, however, diphenylamine and N-nitrosodiphenylamine cannot be directly distinguished because N-nitrosodiphenylamine decomposes at the temperatures of GC injection ports or columns to produce diphenylamine [11]. Therefore, these compounds are separated from the others by column chromatography prior to GC, and are identified and measured as diphenylamine. For other aniline derivatives, extraction with organic solvents is necessary [10]. These procedures are complex and time-consuming. High-performance liquid chromatography (HPLC) does not require prior separation and has also been used for analysis of aniline derivatives [12,13]; however, the separation efficiency is not as good as in capillary GC.

In this paper, conditions for separation by MEKC analysis of ten aniline derivatives, including eight aniline derivatives which have been found in environmental water, was studied. The separation of a series of aniline derivatives by

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MEKC has not been previously reported. In order to completely separate these ten aniline derivatives by MEKC in a single run, their migration behaviour under various analytical conditions was investigated. The pH of the micellar solution was the most significant factor affecting the migration behaviour of the aniline derivatives. The effects of the micellar concentration and the addition of cyclodextrin (CD) on the migration behaviour were also investigated.

EXPERIMENTAL

Apparatus

MEKC was performed with a Model 270A analytical capillary electrophoresis system (Applied Biosystems, San Jose, CA, USA). A fused-silica capillary tube (720 mm × 50 μm I.D., GL Sciences, Tokyo, Japan) was used as the separation tube. The migrating solute bands were detected at a position 500 mm from the positive end by on-column UV absorption measurements (210 nm). A Chromatopac C-R6A (Shimadzu, Kyoto, Japan) was used for data processing.

Reagents

All aniline derivatives, CDs (α-CD, β-CD and γ-CD) and sodium dodecyl sulphate (SDS) were obtained from Nacalai Tesque (Kyoto, Japan). All reagents and solvents were of analytical grade and were used without further purification. SDS solutions were prepared by dissolving SDS in a mixture of 0.02 *M* sodium dihydrogenphosphate solution and 0.02 *M* sodium tetraborate solution adjusted to the appropriate pH.

Procedure

Stock solutions contained 10 g/l of the aniline derivatives dissolved in methanol. A standard solution of a mixture of the ten aniline derivatives was made by diluting the stock solutions with SDS solution. The concentration of each aniline derivative was 50 mg/l. Samples were injected by vacuum injection [5 in.Hg (1 in.Hg = 3386.379 Pa), 0.2 s] and the injection volume was about 1.5 nl. The set-up voltage and temperature were 20 kV and 30°C, respectively, throughout all experiments.

RESULTS AND DISCUSSION

pH dependence of migration behaviour

The separation of the ten aniline derivatives listed in Table I was investigated by MEKC. The effect of pH on their migration behaviour was investigated using 0.05 *M* SDS solutions whose pH values were 6.2, 7.0, 8.0 and 9.0. Separation could not be obtained with a solution of pH 5.0, due to broadening and delaying of the peaks. This may have been caused by the greatly decreased electroosmotic mobility. In order to investigate the pH dependence of the migration behaviour in detail, we calculated the capacity factors, $\tilde{k}'$, of the aniline derivatives from our experimental data.

In MEKC, the capacity factor of an electrically neutral solute is given by [2,3]:

$$\tilde{k}' = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})} \quad (1)$$

where t_R, t_0 and t_{mc} are the migration time for the solute, methanol (used as a tracer for the electroosmotic flow) and Sudan III (used as a tracer for the micelle), respectively. The dependence of the values of $\tilde{k}'$ calculated from eqn. 1 for each aniline derivative on the pH of the SDS solution is shown in Fig. 1. While $\tilde{k}'$ decreased significantly with increasing pH for *p*-anisidine

TABLE I

LIST OF THE INVESTIGATED ANILINE DERIVATIVES AND THEIR pK_a VALUES

Name of compound	pK_a [a]
Aniline[b]	4.63
o-Anisidine[b]	4.52
m-Anisidine[b]	4.23
p-Anisidine	5.34
o-Chloroaniline[b]	2.65
m-Chloroaniline[b]	3.46
p-Chloroaniline	4.15
N-Methylaniline[b]	4.848
Diphenylamine[b]	0.79
N-Nitrosodiphenylamine[b]	–

[a] Logarithm of the reciprocal of the conjugate acid dissociation constant taken from the literature [14].

[b] Detected in the environmental water of Japan [10].

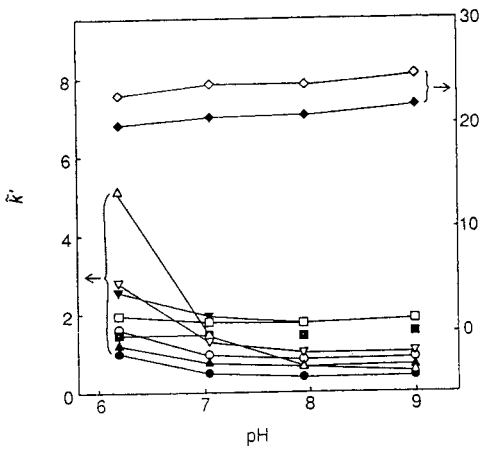

Fig. 1. Dependence of capacity factor $\tilde{k}'$ on pH at 0.05 *M* SDS. ● = Aniline; ○ = *o*-anisidine; ▲ = *m*-anisidine; △ = *p*-anisidine; ■ = *o*-chloroaniline; □ = *m*-chloroaniline; ▼ = *p*-chloroaniline; ▽ = N-methylaniline; ◆ = diphenylamine; ◇ = N-nitrosodiphenylamine.

and N-methylaniline, $\tilde{k}'$ was almost constant for *o*-chloroaniline and *m*-chloroaniline in the same pH range.

This behaviour can be considered to be related to the pK_a values of the aniline derivatives, listed in Table I [14]. *p*-Anisidine and N-methylaniline have relatively high pK_a values, whereas *o*-chloroaniline and *m*-chloroaniline have relatively low pK_a values. The degree of protonation of the former derivatives is larger than that of the latter derivatives at the lower pH values investigated, so the former derivatives interact more strongly with the negatively charged SDS micelle.

To explain these considerations, we have applied a simple model for the migration behaviour of the aniline derivatives. The model shown in Fig. 2 is a simplified version of the complex model for cationic solutes in MEKC [15,16]. In Fig. 2, "B" represents the base form of the aniline derivative and "BH^+" represents its conjugate acid. μ_{eo} is the electroosmotic mobility and μ_{mc} and μ_{BH^+} are the electrophoretic mobility of the micelle and BH^+, respectively. Both B and BH^+ are partitioned between the micelle and the solvent (surrounding aqueous phase). The partition coefficients of B and BH^+ ($K_{mw,B}$ and K_{mw,BH^+}) differ because of the difference in their charges.

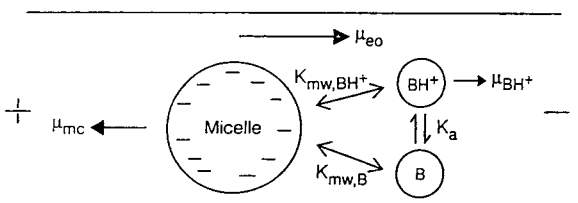

Fig. 2. Interaction of a solute acid/base pair with a micelle in MEKC.

The capacity factor of the aniline derivative was taken as the weighted average of the capacity factors of the base $\tilde{k}'_{BH^+}$ and its conjugate acid $\tilde{k}'_B$ as follows [16]:

$$\tilde{k}' = F^{aq}_{BH^+}\tilde{k}'_{BH^+} + F^{aq}_{B}\tilde{k}'_{B} \quad (2)$$

The F values are the mole fractions of the base and conjugate acid in the aqueous phase. The F values can be expressed in terms of the concentration of H^+ and the acid dissociation constant K_a:

$$F^{aq}_{BH^+} = \frac{[H^+]}{[H^+] + K_a} \quad (3)$$

and

$$F^{aq}_{B} = \frac{K_a}{[H^+] + K_a} \quad (4)$$

Substitution of eqns. 3 and 4 into eqn. 2 yields an equation expressing the pH dependence of $\tilde{k}'$:

$$\tilde{k}' = \frac{\tilde{k}'_{BH^+}[H^+] + \tilde{k}'_{B}K_a}{[H^+] + K_a} \quad (5)$$

Eqn. 5 predicts sigmoidal plots of $\tilde{k}'$ *vs.* pH, as shown schematically in Fig. 3. If the observed $\tilde{k}'$ values show this type of behaviour, $\tilde{k}'_{BH^+}$, $\tilde{k}'_B$ and K_a can be estimated from the experimental data and eqn. 5.

However, calculation of $\tilde{k}'$ by eqn. 1 is valid only for electrically neutral solutes, whereas the degree of ionization of aniline derivatives increases with a decrease in pH. $\tilde{k}'$ can be expressed for ionized solutes by the electrophoretic mobility of the solute μ as follows [5,16]:

$$\tilde{k}' = \frac{\mu - \mu_0}{\mu_{mc} - \mu} \quad (6)$$

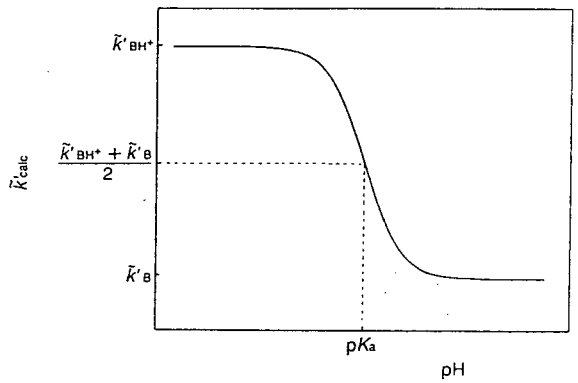

Fig. 3. Illustration of pH dependence of capacity factor $\tilde{k}'$ calculated from eqn. 5.

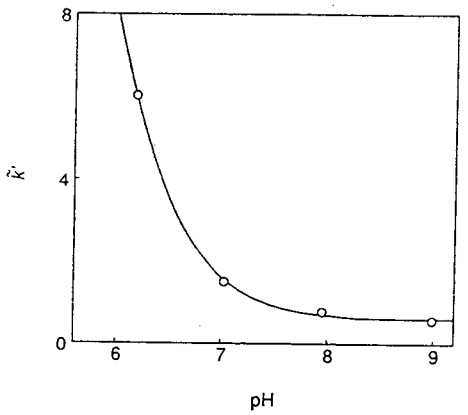

Fig. 4. Least-squares fitting of experimental data of *p*-anisidine for eqn. 7. ○ = $\tilde{k}'_{obs}$; —— = $\tilde{k}'_{calc}$.

where μ_0 is the observed overall mobility of the solute in the aqueous phase, given by:

$$\mu_0 = \frac{[H^+]}{[H^+] + K_a} \mu_{BH^+} \quad (7)$$

For neutral solutes, $\mu_0 = 0$ and eqn. 6 is equivalent to eqn. 1. The contribution of μ_0 to $\tilde{k}'$ is negligible in the pH range of this experiment except for the case of *p*-anisidine at pH 6.2. According to Otsuka *et al.* [5], it is difficult to estimate the value of μ_0 for ionized solutes in micellar solutions, and it is reasonable to assume that the value of μ_0 is the same as that in the buffer solution containing 5 m*M* SDS.

For *p*-anisidine, the least-squares fitting of the experimental data for eqn. 5 using the above assumption is shown in Fig. 4. The resulting estimated values of $\tilde{k}'_{BH^+}$, $\tilde{k}'_B$ and pK_a are shown in Table II, along with the literature value of pK_a. The good fit of the curve and the closeness of the estimated pK_a value to the literature value indicate that the model can be considered to be valid for this case. For the other aniline derivatives, such fitting was unsuccessful, because the experimentally acceptable pH variation is restricted to a narrow range, and the pK_a values of the aniline derivatives are too small for the estimation to be carried out over the entire range of pH values investigated.

Optimization of the analytical conditions for the complete separation of ten aniline derivatives

Optimization of the pH of the SDS solution was carried out by observing the migration behaviour of ten aniline derivatives. Complete separation of eight of the aniline derivatives was achieved at pH 6.2, but the separation of two other compounds, diphenylamine and N-nitrosodiphenylamine, was incomplete. In order to separate these last two compounds completely, the effects of SDS concentration and addition of CD were investigated as follows.

The capacity factor $\tilde{k}'$ is related to the overall partition coefficient, K_{mw}, by the following equation [3]:

$$\tilde{k}' = K_{mw} \frac{V_{mc}}{V_{aq}} \quad (8)$$

$$\approx K_{mw} \bar{v} (c_{sf} - CMC) \quad (9)$$

where V_{mc}/V_{aq} is the volume ratio of the micelle and the aqueous phase, $\bar{v}$ is the partial molar volume of the micelle, c_{sf} is the total concentration and CMC is the critical micellar concentration of the surfactant.

TABLE II

ESTIMATED VALUES OF $\tilde{k}'_{BH^+}$, $\tilde{k}'_B$ AND pK_a OF *p*-ANISIDINE BY LEAST-SQUARES FITTING OF THE EXPERIMENTAL DATA

	Estimated value	Literature value
$\tilde{k}'_{BH^+}$	26	–
$\tilde{k}'_B$	0.58	–
pK_a	5.6	5.34

In the case of neutral solutes, as can be seen from eqn. 3, the order of their capacity factors is independent of the SDS concentration. Some of the aniline derivatives, however, are partially ionized at low pH. Accordingly, the dependence of capacity factor on SDS concentration at pH 6.2 was investigated; the results are shown in Fig. 5. The capacity factor of the aniline derivatives varies with SDS concentration, and some peaks are overlapped at high (0.08–0.15 *M*) SDS concentration. This variation in capacity factor seems to be caused by the partial ionization of some of the aniline derivatives. At 0.03 *M*, the separation behaviour was almost the same as that observed at 0.05 *M*, but slight peak broadening was observed. Consequently, the optimum concentration of SDS was taken to be about 0.05 *M*.

However, the separation of diphenylamine and N-nitrosodiphenylamine was incomplete at all SDS concentrations. Since diphenylamine and N-nitrosodiphenylamine are relatively hydrophobic compounds, their partition into the micelle is too large to separate them completely. Therefore, three kinds of CD (α-, β- and γ-CD) were added to the SDS solutions in order to aid in achieving complete separation of the ten aniline derivatives, especially diphenylamine and N-nitrosodiphenylamine. CD-modified MEKC has been used in order to separate hydrophobic compounds that are almost totally partitioned into the micelle [17]. Addition of CD reduces

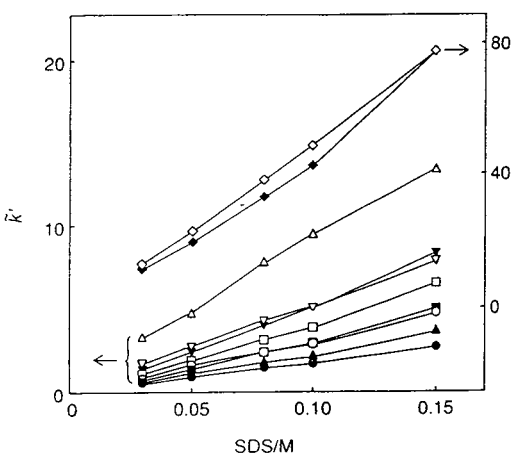

Fig. 5. Dependence of capacity factor $\tilde{k}'$ on SDS concentration at pH 6.2. Symbols as in Fig. 1.

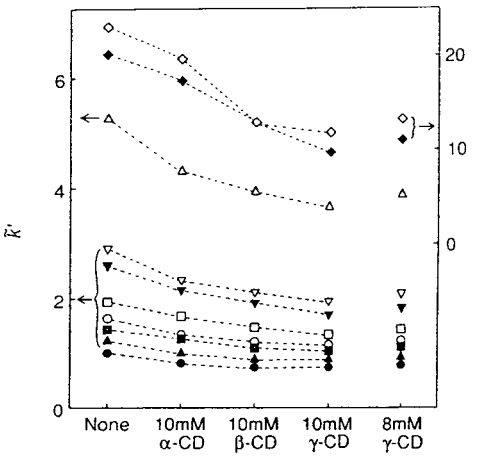

Fig. 6. Effect of CD addition on the capacity factor $\tilde{k}'$ at pH 6.2, 0.05 *M* SDS. Symbols as in Fig. 1.

partition into the micelle because of inclusion in the cavity of CD, which migrates with the electroosmotic velocity.

The effect of CD addition on the migration behaviour of the aniline derivatives is shown in Fig. 6. The capacity factor of all aniline deriva-

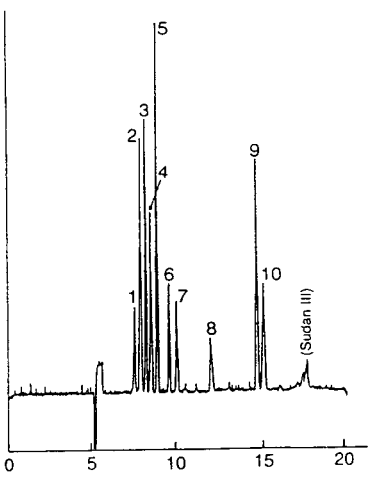

Fig. 7. Complete separation of ten aniline derivatives in MEKC. Peaks: 1 = aniline; 2 = *m*-anisidine; 3 = *o*-chloroaniline; 4 = *o*-anisidine; 5 = *m*-chloroaniline; 6 = *p*-chloroaniline; 7 = N-methylaniline; 8 = *p*-anisidine; 9 = diphenylamine; 10 = N-nitrosodiphenylamine. Micellar solution, 8 m*M* γ-CD and 0.05 *M* SDS in 0.02 *M* borate–phosphate buffer (pH 6.2); separation column, 720 mm × 0.05 μm I.D.; effective length to the detector, 500 mm; applied voltage, 20 kV; current, 20 μA; detection wavelength, 210 nm; temperature, 30°C.

tives decreased upon addition of 10 m*M* of any type of CD, and the separation behaviour of diphenylamine and N-nitrosodiphenylamine depended on the type of CD. This dependence may be due to differences in their cavity sizes. Complete separation was achieved only when γ-CD was used. The migration times of some aniline derivatives were too close with 10 m*M* γ-CD, and better separation was obtained with 8 m*M* γ-CD.

The chromatogram of ten aniline derivatives in MEKC obtained using optimum analytical conditions is shown in Fig. 7. The complete separation of all of the aniline derivatives was achieved in a single run, and the peaks show high separation efficiency (their theoretical plate numbers are about 100 000). This demonstrates that MEKC is a powerful technique for the analysis of these aniline derivatives. Their concentration in environmental waters, however, is at the ppb level or less. Therefore, in order to apply MEKC to the analysis of real samples of environmental waters, more sensitive and qualitative detection methods are required.

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Short Communication

Application of topological indices to chromatographic data

Calculation of the retention indices of anthocyanins

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ABSTRACT

The HPLC retention times (t_R) of anthocyanins were calculated using a simple equation of the form $t_R = a[W/(pn_{OH})] + b$. The Wiener number, W, and polarity number, p, were used as structural descriptors. The other factor that controls the magnitude of the retention times is the number of OH groups (n_{OH}) attached to the flavylium core. The calculated t_R values of several classes of anthocyanins are in excellent agreement with the experimental values.

INTRODUCTION

The anthocyanins are part of the very large and widespread group of plant constituents known collectively as flavonoids. They are glycosylated polyhydroxy derivatives of 2-phenylbenzopyrylium or flavylium salts and are responsible for most of the red, pink, mauve and blue coloration of fruits, flowers and leaves [1]. Hundreds anthocyanins are known to exist in nature. Differences between individual anthocyanins occur in the pattern of hydroxylation or methylation and the number and type of sugar(s) or acylated sugar(s) attached to the flavylium nuclei. The most common anthocyanidins (aglycones) are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. They differ only by the number and position of hydroxyl and/or methoxyl groups in the B-ring (see Fig. 1).

The importance of anthocyanins is reflected in their properties. They appear to be convenient food colouring materials [2–4], though their use is restricted to those foods and beverages whose pH is below 4. Additionally, they are substances with pharmaceutical properties [5–9].

Despite considerable efforts during the last

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decade, qualitative and quantitative analysis of unknown mixtures of anthocyanins still remains difficult [10]. It is the structure and properties of the anthocyanins that make their separation difficult. The basic anthocyanidin skeletons of most of these pigments differ only little in molecular weight and substituents, making clearly defined separations of anthocyanin mixtures difficult. At present, the most satisfactory method for analysing mixtures of anthocyanins is the multistep method of quantification, separation and isolation by HPLC and peak identification by fast atom bombardment (FAB) MS and high-field NMR.

In this paper we investigate the relationship between HPLC retention indices and the structural properties of anthocyanins. Quantitative structure–chromatographic retention relationships (QSCRRs) with topological (graph-theoretical) indices represent a convenient model for studying the correlations between chromatographic and structural properties of molecules [11]. Topological indices in this approach act as molecular descriptors, which can be used to predict the HPLC retention indices via an appropriate statistical equation. Many graph–theoretical (topological) indices have been considered in the literature [12]. The Wiener number has been used extensively in structure–property–activity modelling. The Schultz index is another topological index that is successfully used in quantitative structure–property relationship (QSPR) modelling of properties of molecules.

It was shown by Hosoya [13] that the Wiener number [14], $W = W(G)$, of structure G can be defined as half of the sum of the elements of the distance matrix $\mathbf{D}(G) = \mathbf{D}$:

$$W(G) = \frac{1}{2} \sum_i \sum_j (\mathbf{D})_{ij} \quad (1)$$

where $(\mathbf{D})_{ij}$ are off-diagonal elements of the distance matrix. The Wiener number appears to be a convenient measure of the compactness of the molecule [15].

The molecular topological index (MTI) or the Schultz index [16] is based on the adjacency matrix **A**, the distance matrix **D** and the valency matrix **v** of a molecule [17]:

$$\mathrm{MTI} = \sum_i \mathrm{e}_i \quad (2)$$

where e_i are the elements of the row matrix **v** [**A** + **D**]. Both Wiener number and Schultz index may be extended to heterosystems by replacing the elements of the adjacency matrix and of the distance matrix corresponding to heteroatoms and heterobonds with the values incorporating the corrections due to the changes induced when the carbon atom is replaced by the heteroatom [18].

Wiener also introduced the polarity number, p, which is equal to the number of pairs of atoms separated by three bonds [14,19]. The polarity number $p = p(G)$ of a structure G is given by:

$$p = \frac{1}{2} \sum_i (p_3)_i \quad (3)$$

where p_3 is the number of the paths of length 3 or the number of off-diagonal elements of **D** with the distance 3. Originally described as a polarity number, p is also related to steric aspects of a structure [20].

The topological indices used in this study were calculated using an Atari 1040ST computer and a modified computer program [21].

RESULTS AND DISCUSSION

Structural characteristics of molecules are one of the most important factors responsible for molecular migration in the chromatographic process. The Wiener number is a molecular descriptor that reflects well the structure of molecule. The smaller the Wiener number, the larger the compactness of a structure (in terms of structural features such as branching and cyclicity). Hence, it can be reliably used for correlations with those physical and chemical properties that depend on the ratio of the volume to the surface of the molecule. Chromatographic retention data are typical molecular properties for which the QSPRs with the Wiener number yield correct predictions [22].

Experimental results [23,24] have shown that the polarity of the anthocyanins and anthocyanidins is most important factor affecting the HPLC retention times. Of the anthocyanidins, delphinidin was the first pigment

eluted, followed by cyanidin, petunidin, pelargonidin, peonidin and malvidin, showing the order of retention times to be in order of decreasing polarity of the compounds. Anthocyanins showed the same elution order. A topological index appropriate for modelling of molecular polarity is polarity number.

Of the individual substituents, OH groups have the greatest influence on retention times. As the number of hydroxyl groups on the B-ring increases, the retention time of the flavyliums decreases. Thus, delphinidin, with three hydroxy groups in the B-ring, is less retained than cyanidin with its two hydroxy groups. Methylation of the hydroxy groups increases the retention time: of the methylated flavyliums, petunidin is retained for a shorter time than peonidin. Malvidin shows the longest retention time. A reasonable explanation appears to be the formation of hydrogen bonds, which can greatly influence retention and separability. The number of hydrogen atoms bonded to oxygen atoms is a possible parameter describing hydrogen bonding. Thus, the more OH groups in the flavylium structure, the stronger the bonding with polar eluent and the smaller the retention time.

On the basis of the experimental results [23,24] we developed a very simple relationship between experimental HPLC retention times (t_R) and Wiener number, W, polarity number, p, and number of OH groups, n_{OH}, in the flavylium structure:

$$t_R = a[W/(pn_{OH})] + b \quad (4)$$

where a and b are statistical parameters to be determined by least-squares regression.

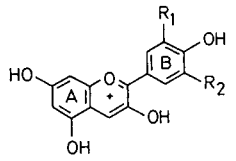
Eqn. 4 indicates that t_R is proportional to the Wiener number. This is confirmed in the QSCRR studies between the t_R and the Wiener number for alkanes (*e.g.* ref. 25). However, the linear relationship between the t_R and W for anthocyanidins is rather poor ($r = 0.4850$, $s = 5.40$, $F = 1.23$). Therefore, the Wiener number alone cannot be used to predict the t_R values of this class of compounds.

Experimental observation indicates that the t_R decreases with the polarity of the molecule. The simple rationale behind this observation is that the polar molecule is better linked to a polar eluent and thus the retention time is shorter. We used the polarity number, p, as a measure of the polarity of the studied molecules. Thus, the t_R is inversely proportional to p. The linear relationship between t_R and W/p is again rather poor ($r = 0.6003$, $s = 4.94$, $F = 2.25$).

It is also observed that the t_R decreases with an increase in the number of the OH groups on the flavylium core. This can be rationalized by considering the formation of the hydrogen bond between the flavylium salts and the polar eluent. The consequence of this is that more OH groups on the flavylium core result in stronger hydrogen bonding with the polar eluent and shorter retention time. This situation is introduced to the model through the number of the OH groups (n_{OH}). Hence, the t_R is inversely proportional to n_{OH}. The linear relationship between t_R and W/n_{OH} is somewhat better than those above ($r = 0.9247$, $s = 2.35$, $F = 23.59$), but still not particularly good. However, when we considered the linear relationship between the t_R and $W/(pn_{OH})$, the model improved considerably, as will be seen below. The point to note here is that W and p are not particularly intercorrelated quantities. For example, in the case of 159 undecanes, the correlation coefficient for the linear correlation between W and p is only 0.869.

We tested the validity of eqn. 4 for different types of flavylium salts: anthocyanidins, anthocyanin 3-glucosides and anthocyanin 3,5-diglucosides. The structures of the studied anthocyanidins, anthocyanin 3-glucosides and anthocyanin 3,5-diglucosides are shown in Figs. 1 and 2. Table I gives the Wiener numbers, W, Schultz indices, MTI, polarity numbers, p, and experimental ($t_{R,exp}$) and calculated ($t_{R,calc}$) retention indices. Experimental HPLC retention times for anthocyanidins were taken from a paper by Wilkinson *et al.* [23], and for anthocyanin 3-glucosides and for anthocyanin 3,5-diglucosides from a paper by Williams *et al.* [24].

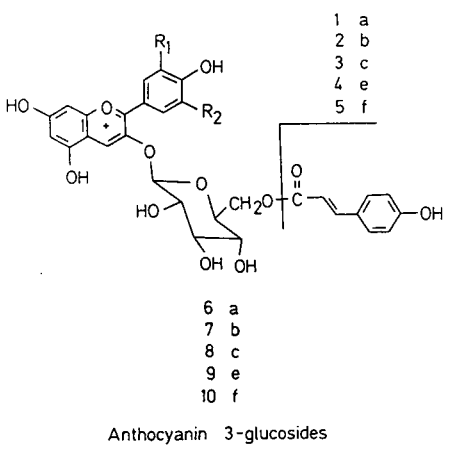
The values of the statistical parameters are given in Table II. The statistical characteristics of all three correlations are of very good quality. Tables I and II show that the quality of fit between observed and calculated retention times is very good. This is confirmed by both high



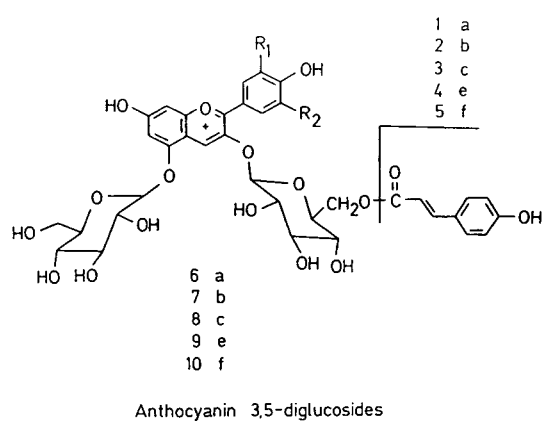
		R_1	R_2	
Delphinidin	(Dp)	OH	OH	a
Cyanidin	(Cy)	OH	H	b
Petunidin	(Pt)	OCH_3	OH	c
Pelargonidin	(Pg)	H	H	d
Peonidin	(Pn)	OCH_3	H	e
Malvidin	(Mv)	OCH_3	OCH_3	f

Fig. 1. Structures of the six most common anthocyanidins. Symbols in brackets represent the two-letter abbreviation of their names. The letters in the last column denote the B-ring substitution patterns.

correlation coefficients and the correctly predicted elution sequences. The high accuracy of the predicted models is also shown in Figs. 3–5,



Anthocyanin 3-glucosides



Anthocyanin 3,5-diglucosides

Fig. 2. Structural formulae of anthocyanins studied.

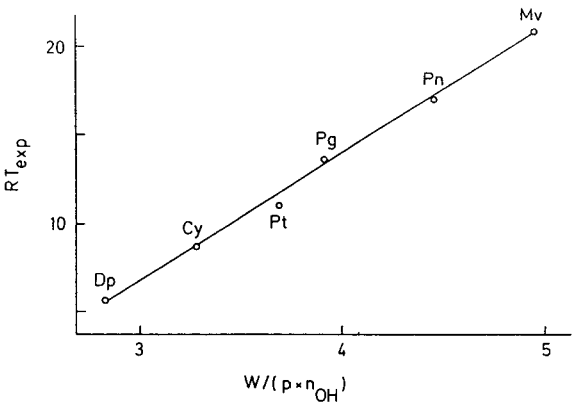


Fig. 3. Plot of $t_{R,exp}$ (RT_{exp}) *vs.* $[W/(pn_{OH})]$ for anthocyanidins.

in which the observed t_R *vs.* $[W/(pn_{OH})]$ relationship are plotted.

The agreement between observed and calculated retention indices indicates that this model describes quite well the retention indices for a given class of flavylium salts: anthocyanidins, anthocyanin 3-glucosides and anthocyanin 3,5-diglucosides. There was a possibility of validating our model by comparison of measured retention times with calculated retention times. The retention times observed by Wulf and Nagel [26] for eleven acylated anthocyanin 3-glucosides correlate to a high degree with the retention times obtained using eqn. 4 ($r = 0.9869$, $s = 1.26$, $F = 314.5$). Further, recently reported [27] retention times for six common anthocyanin 3-glucosides and their six corresponding malonyl esters are also quite well reproduced ($r = 0.9848$, $s = 1.07$, $F = 320.8$).

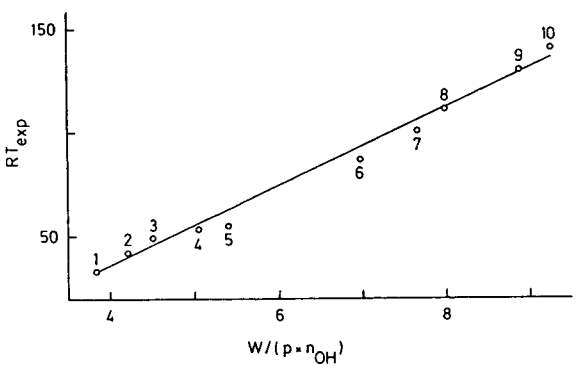


Fig. 4. Plot of $t_{R,exp}$ (RT_{exp}) *vs.* $[W/(pn_{OH})]$ for anthocyanin 3-glucosides.

TABLE I

WIENER NUMBER, SCHULTZ INDEX POLARITY NUMBER, p, EXPERIMENTAL RETENTION INDICES AND CALCULATED RETENTION INDICES USING EQN. 4

Compound	W	MTI	p	$t_{R,exp}$	$t_{R,calc}$
Anthocyanidins					
1 Delphinidin (Dp)	663.02	2829.99	39	5.7	5.5
2 Cyanidin (Cy)	588.18	2531.71	36	8.7	8.7
3 Petunidin (Pt)	754.09	3195.28	41	11.1	11.6
4 Pelargonidin (Pg)	516.33	2243.92	33	13.6	13.3
5 Peonidin (Pn)	675.66	2884.17	38	17.0	17.1
6 Malvidin (Mv)	849.63	3577.04	43	20.8	20.7
Anthocyanins					
Anthocyanin 3-glucosides					
1 Dp 3-O-glucoside	2136.90	9025.11	62	33.2	31.5
2 Cy 3-O-glucoside	1986.87	8429.26	59	42.1	38.8
3 Pt 3-O-glucoside	2311.56	9723.13	64	49.3	44.6
4 Pn 3-O-glucoside	2157.94	9114.44	61	53.2	55.0
5 Mv 3-O-glucoside	2490.56	10436.98	66	55.1	61.4
6 Dp 3-O-*p*-coumarylglucoside	5369.47	22958.74	77	87.0	91.7
7 Cy 3-O-*p*-coumarylglucoside	5096.20	21860.23	74	100.9	104.7
8 Pt 3-O-*p*-coumarylglucoside	5675.61	24193.91	79	111.0	111.0
9 Pn 3-O-*p*-coumarylglucoside	5398.76	23082.57	76	129.9	128.2
10 Mv 3-O-*p*-coumarylglucoside	5986.09	25444.91	81	140.2	135.0
Anthocyanin 3,5-diglucosides					
1 Dp 3,5-diglucoside	4817.79	20300.56	85	22.0	23.0
2 Cy 3,5-diglucoside	4572.51	19323.26	82	26.0	27.8
3 Pt 3,5-diglucoside	5095.95	21414.52	87	30.1	31.3
4 Pn 3,5-diglucoside	4847.08	20424.39	84	37.8	37.4
5 Mv 3,5-diglucoside	5378.44	22544.31	89	42.1	41.1
6 Dp 3-O-*p*-coumarylglucoside-5-glucoside	9649.73	41069.65	100	64.5	60.0
7 Cy 3-O-*p*-coumarylglucoside-5-glucoside	9281.22	39589.70	97	70.0	67.5
8 Pt 3-O-*p*-coumarylglucoside-5-glucoside	10059.38	42720.76	102	72.0	70.9
9 Pn 3-O-*p*-coumarylglucoside-5-glucoside	9687.27	41227.98	99	79.1	80.1
10 Mv 3-O-*p*-coumarylglucoside-5-glucoside	10473.35	44387.71	104	79.1	83.7

In a recent report [28] on molecular descriptors derived from the distance matrix, it was found that the Wiener number, W, and the Schultz index, MTI, are strongly linearly correlated ($r = 0.9999$) distance indices for alkanes. This result suggests the existence of a formal relation between W and MTI for alkanes. Such a relation was indeed found by Klein *et al.* [29]. We have also correlated the Wiener indices and the MTI for anthocyanins. The correlation coefficient obtained ($r = 0.9999$) is indicative that in this case also there may exist a formal relation between these two distance indices. The above result also suggests that the QSCRR with the MTI should be identical to those with the Wiener number. Some numerical experiments confirmed the above.

It was interesting to see how our model works in the case of some other classes of molecules. We tested eqn. 4 for calculating retention times of ten flavonoids [30]. If we consider that studied flavonoids possess different structures from anthocyanins, the correlation coefficient is satisfactory ($r = 0.843$). Many anthocyanins contain

TABLE II

STATISTICAL CHARACTERISTICS OF THE RELATIONSHIP BETWEEN EXPERIMENTAL AND CALCULATED RETENTION TIMES OF FLAVYLIUMS USING EQN. 4

Parameter	Anthocyanidins	Anthocyanin 3-glucosides	Anthocyanin 3,5-diglucosides
n	6	10	10
a	7.1794 ± 0.1870	19.1354 ± 0.7039	13.6883 ± 0.5346
b	−14.7954 ± 0.7309	−41.7504 ± 4.6820	−41.6262 ± 3.7626
r	0.9986	0.9946	0.9940
F	1474.6	739.1	655.6
s	0.32	4.25	2.66

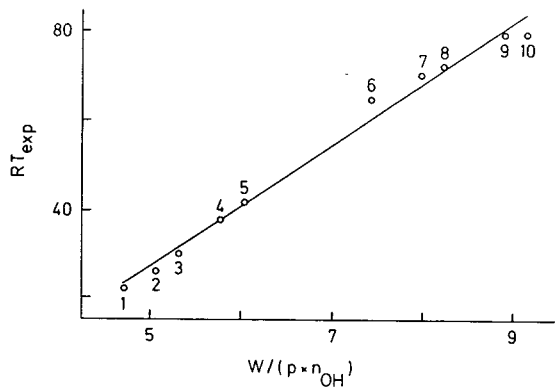


Fig. 5. Plot of $t_{R,exp}$ (RT_{exp}) *vs.* $[W/(pn_{OH})]$ for anthocyanin 3,5-diglucosides.

acylated sugar moieties. The acyl groups are mostly derivatives of cinnamic acid. Our model works quite well in the case of eight cinnamic acid derivatives [31] ($r = 0.972$).

Finally, an attempt was made to test the validity of our model in predicting the correct elution sequence for a mixture of twenty anthocyanin 3-glucosides and anthocyanin 3,5-diglucosides [24]. Unfortunately, it must be pointed out that in this case our model was less successful in reproducing the experimental elution sequence for all twenty anthocyanins ($r = 0.825$).

CONCLUSIONS

We have demonstrated that a very simple model, based on the structural properties of molecules in terms of topological indices (Wiener number, W, and polarity number, p) and number of OH groups in the flavylium structure (a possible parameter describing hydrogen bonding), can be used to predict successfully the HPLC retention indices of flavylium salts. With this model it is possible to reproduce quite accurately the experimental retention indices for different classes of flavylium salts such as anthocyanidins, anthocyanin 3-glucosides and anthocyanin 3,5-diglucosides.

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CHROM. 25 461

Short Communication

Application of an enzyme-based stationary phase to the determination of enzyme kinetic constants and types of inhibition

New high-performance liquid chromatographic approach utilizing an immobilized artificial membrane chromatographic support☆

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ABSTRACT

The application of an immobilized enzyme HPLC column to the qualitative and quantitative determination of enzyme kinetics has been investigated. The enzyme used in this study was α-chymotrypsin (ACHT) which was immobilized by absorption into a commercially available immobilized artificial membrane (IAM) interphase. The resulting IAM-ACHT phases were enzymatically active and catalyzed the hydrolysis of L-tryptophan methyl ester to L-tryptophan. The interaction between the IAM-ACHT phase and known reversible inhibitors of ACHT has been studied with hydrocinnamic acid (HCA) and β-phenylethylamine (BPEA), and the results demonstrate that displacement chromatography can determine the type and degree of enzyme/inhibitor interactions. In addition, an inhibition constant (K_I) of 1.8 m*M* for the competitive inhibition by HCA was calculated which is consistent with the previously reported value of 4.5 m*M* determined using non-immobilized ACHT. For BPEA the calculated K_I was 8.5 m*M* and the inhibition was mostly noncompetitive. This indicates that the IAM-ACHT can be used to quantitatively determine the enzyme kinetic constants associated with enzyme/substrate and enzyme/inhibitor interactions. The same immobilized enzyme was repeatedly used over a 10-day period to study enzyme kinetics.

INTRODUCTION

In the past few years there has been a rapid growth of high-performance liquid chromatography (HPLC) chiral stationary phases (CSPs) based upon immobilized proteins [1–4]. The success of immobilized protein supports as

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HPLC phases has prompted the investigation of the chromatographic utility of another class of biopolymers, enzymes. For example, Wainer and co-workers have covalently immobilized α-chymotrypsin (ACHT-CSP) [5–7] and trypsin (TRYP-CSP) [8] on a silica-based HPLC support. The resulting phases were used in the stereochemical resolution of amino acids, amino acid derivatives and dipeptides. The observed chiral resolutions were based upon two mechanisms: (1) stereoselective enzymatic hydrolysis of only one of the isomers of an enantiomeric substrate, *e.g.* hydrolysis of L-tryptophanamide but not D-tryptophanamide; (2) relative stabilities of the diastereomeric enzyme/pseudo-substrate complexes formed during the chromatographic process [6].

It was also reported [6] that the hydrolytic activity of the immobilized ACHT could be inhibited by injecting 4-nitrophenyl trimethylacetate (4-NTA) onto the ACHT-CSP. 4-NTA is a reversible inhibitor of ACHT and the enzymatic activity of the ACHT-CSP was regenerated by washing the column with the appropriate phosphate buffer. These results indicate that the covalently immobilized ACHT-CSP retained its sensitivity to enzyme inhibitors.

One of the problems associated with the covalent immobilization of enzymes on silica-based HPLC supports is the possible restriction of the conformational mobility of the enzyme by the immobilization process. In order to avoid this potential problem, Chui and Wainer [9] immobilized ACHT and TRYP on an immobilized artificial membrane (IAM) HPLC support to create the IAM-ACHT and IAM-TRYP phases.

The IAM support was developed by Pidgeon *et al.* [10], and produced through the covalent immobilization of a diacylphosphatidylcholine on an aminopropyl silica. The synthetic lipid was 1-myristoyl-2-[(13-carboxyl)tridecanoyl]-*sn*-3-glycerophosphocholine which was covalently attached to the silica support through the ω-carboxyl group on the C-2 fatty acid chain. In the resulting support (IAM·PC), the phosphatidylcholine headgroups form the surface of the support and the hydrocarbon side chains produce a hydrophobic interphase which extends from the charged headgroup to the surface of the aminopropyl silica. The residual amino groups in the IAM stationary phase may be free (IAM·PC), or endcapped with methylglycolate (IAM·PC·MG).

In addition to ACHT and TRYP, the IAM·PC chromatographic support has been used to immobilize lipase [11] and hepatic microsomes [12]. Both phases were enzymatically active. For example, the microsome-based immobilized enzyme reactor was active in catalysing on-line production of phase I and phase II biotransformation metabolites including 7-hydroxycoumarin formed by O-deethylation of 7-ethoxycoumarin in the presence of NADPH (P-450 catalyzed monooxygenation) [12] and glucuronides produced by O-glucuronidation of 7-hydroxy-4-methylcoumarin and 4-nitrophenol in the presence of uridine 5′-diphosphoglucuronic acid (UDPGA) [13].

The IAM-ACHT and IAM-TRYP supports also retained the hydrolytic activity of the native enzymes —IAM-TRYP catalyzed the hydrolysis of Nα-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and IAM-ACHT catalyzed the hydrolysis of a number of substrates including L-tryptophan methyl ester [9]. The hydrolytic activity of the immobilized enzyme could be determined from the resulting substrate/product ratios obtained either directly from IAM-ACHT chromatograms or from chromatograms produced by a coupled column system. In addition, the activities of both supports were decreased by known enzyme inhibitors and the activity of the IAM-ACHT was affected by changes in pH and temperature [9].

The results of the initial study suggested that IAM-ACHT and IAM-TRYP could be used as chromatographic probes of enzyme/substrate and enzyme/inhibitor interactions. The present study continues this investigation and examines the utility of the IAM-enzyme columns in the determination of the qualitative and quantitative aspects of enzyme kinetics. In particular, the hydrolysis of L-tryptophan methyl ester on the IAM-ACHT was examined alone and in the presence of eight known reversible ACHT inhibitors.

The results of this study indicate that the

IAM-ACHT can be used to qualitatively determine the type and degree of enzyme/inhibitor interactions and quantitatively determine the enzyme kinetic constants associated with enzyme/substrate and enzyme/inhibitor interactions.

EXPERIMENTAL

Chemicals

α-Chymotrypsin (ACHT, 57 U/mg protein, 84% protein) and the ACHT inhibitor indole were purchased from ICN Biochemicals (Cleveland, OH, USA). L-Tryptophan methyl ester·HCl (L-Trp-OMe) was purchased from American Chemicals (Montreal, Canada). D-Tryptophan methyl ester·HCl (D-Trp-OMe), L-tryptophan (L-Trp), ACHT inactivated with DFP (diisopropylphosphofluoridate), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and the remaining seven ACHT inhibitors tested: hydrocinnamic acid (HCA), β-phenylethylamine·HCl (BPEA), indole-3-propionic acid, α-acetamidocinnamic acid, hippuric acid, N-benzoyl-DL-methionine and N-benzoyl-DL-phenylalanine were all obtained through Sigma (St. Louis, MO, USA).

Apparatus

The chromatographic experiments were performed with a modular HPLC system which consisted of a Beckman 110B solvent module pump (Beckman Instruments, Houston, TX, USA), a Rheodyne 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, CA, USA), a 783 programable UV absorbance detector (ABI Analytical, Ramsey, NJ, USA) and a DataJet integrator (Spectra-Physics, San Jose, CA, USA).

Chromatographic columns

The frontal elution experiments were performed with 15 cm × 4.6 mm I.D. IAM·PC (12 μm, 300 Å) chromatographic columns. For the displacement chromatography experiments, chromatographic cartridges (1 cm × 3.0 mm I.D.) and columns (3 cm × 4.6 mm I.D.) packed with the same IAM·PC support were used. The samples from the experiments with non-immobilized ACHT were analyzed on a 15 cm × 4.6 mm I.D. Rexchrom Octyl (5 μm, 100 Å) column. All the columns and cartridges were obtained from Regis (Morton Grove, IL, USA).

Chromatographic conditions

On the IAM·PC columns the mobile phase consisted of a sodium phosphate buffer (0.1 *M*, pH 6.8), and the flow-rate was 0.5 ml/min. L-Tryptophan and L-tryptophan methyl ester were quantitated by UV absorption at 280 nm. On the octyl column (used for the determination of substrate conversion with the non-immobilized enzyme), the mobile phase consisted of 0.1 *M* sodium dihydrogenorthophosphate adjusted to pH 3.0 with orthophosphoric acid–acetonitrile (9:1, v/v). The flow-rate was 0.5 ml/min. The experiments were performed at ambient temperature.

Experiments with the immobilized ACHT

Frontal affinity chromatographic approach. The IAM·PC-ACHT (15 cm × 4.6 mm I.D.) column was prepared as described earlier [9]. For each inhibitor, a range of concentrations in a sodium phosphate buffer (0.1 *M*, pH 6.8) were perfused through the column and the elution volume (V) determined for each concentration from the breakthrough elution profile [14]. After each experiment, the inhibitor was washed out from the column using the sodium phosphate buffer (0.1 *M*, pH 6.8).

Identical sets of experiments were performed on an IAM·PC column without the enzyme and on an IAM·PC-ACHT·DFP column. The latter column was prepared in a same way as IAM·PC-ACHT, except that the ACHT used was irreversibly inactivated with diisopropylphosphofluoridate (DFP). An IAM stationary phase containing ACHT irreversibly inactivated with TPCK, was prepared to conduct a single frontal elution experiment with N-benzoyl-DL-phenylalanine.

The enzymatic activity in all the tested columns was checked by injecting 20 μl of 1 m*M* L-tryptophan methyl ester.

Displacement chromatography approach. A range of L-tryptophan methyl ester concentrations (0.5, 1, 2, 3 and 4 m*M*) were injected onto

an IAM · PC-ACHT 1-cm cartridge connected in series to an IAM · PC 3-cm column. The experiment was repeated with several fixed inhibitor concentrations (1, 2, 5 and 10 m*M* HCA; 5 and 10 m*M* BPEA) in the mobile phase. The product formed (L-tryptophan) and the unhydrolyzed substrate were monitored at 280 nm. The injected sample volume was 20 μl, the mobile phase flow-rate was 0.5 ml/min, and all the experiments were performed at room temperature (25°C).

Calculations

The experimentally obtained data (average values from 2–5 HPLC determinations) were processed in terms of Michaelis–Menten kinetics. Kinetic constants were calculated from double reciprocal Lineweaver–Burk plots $1/s$ *versus* $1/v$ (where s is the substrate concentration and v the rate of enzymatic reaction). The initial L-tryptophan methyl ester concentrations were plotted against the specific activity of ACHT. These parameters were determined by the rate of the enzymatic conversion of L-tryptophan methyl ester to L-tryptophan. The rate of enzymatic reaction is presented by the amount of L-tryptophan formed (or the decrease in L-tryptophan methyl ester) during the reaction time. The reaction time is defined by the time the substrate resides in the IAM-ACHT chromatographic column, which is dependent upon the flow-rate in the chromatographic system. The flow-rate was held constant at 0.5 ml/min throughout the study.

RESULTS AND DISCUSSION

The experimental approaches used in this work included both frontal elution and displacement chromatography techniques. The results of the frontal affinity chromatographic experiments with the eight inhibitors indicated that the calculated affinities (K_M) of the inhibitors for the IAM · PC are as great or greater than the K_M calculated for the supports containing active or inactivated enzymes.

Pidgeon *et al.* [10] have demonstrated that retention on the IAM support involves both hydrophobic and electrostatic interactions and both moieties are present in the inhibitors used in this study. From the experimental results with the enzymatically active and inactivated form of ACHT, it appears that the interactions between the IAM support and these compounds are so great that they mask the interactions between the immobilized enzyme and the solutes. In the ideal situation the chromatographic support for frontal affinity chromatography should be negligibly retentive or not retentive at all for the compounds tested. Thus, the frontal elution approach could not be used with the IAM · PC-ACHT to determining the K_M of the inhibitors for ACHT. However, new supports under development by Pidgeon [15] should address this problem and will be tested when they are available.

Bearing in mind that chromatographic supports are always more or less retentive under applied conditions, it should also be mentioned that injecting a mixture of a substrate and an (reversible) inhibitor onto a column with immobilized enzyme will give us a real picture of their interaction only if the contact between all the "reactants" is maintained through the whole length of the chromatographic column. The enzyme being uniformly present in the chromatographic support, this means that both the substrate and the inhibitor should have the same retention time.

These problems were circumvented by the use of displacement chromatography. In this approach, the inhibitor is added to the mobile phase and equilibrated with the chromatographic support, *i.e.* the enzyme. In this technique, the substrate displaces the inhibitor from the enzyme and the extent of the observed enzymatic reaction is a function of both the K_I of the inhibitor and the K_M of the substrate. Different affinities of the substrate and the inhibitor for the chromatographic support itself will not affect the rate of reaction, because the inhibitor is supplied in constant concentration in the mobile phase. The reaction time is equal to the retention time of the substrate in the column with the immobilized enzyme, and is generally not affected by the presence of inhibitors. Thus, the product formed is the real picture of the enzyme/inhibitor relationship in the chromatographic column.

The necessity of placing the inhibitors in the mobile phase eliminated from further study all but two of the inhibitors, HCA and BPEA. Unlike the other inhibitors, these compounds do not display a significant UV absorption at 280 nm, the wavelength used to monitor the substrate and product. Although not an impossible task, the background absorption of the high inhibitor concentration in the mobile phase could make difficult to monitor the subtle increase in the UV absorbance generated by the enzymatically produced product peak in the initial stage of the enzymatic reaction. The substrate chosen for this study was L-Trp-OMe which is converted by ACHT to L-Trp. While L-Trp-OMe is a substrate for ACHT, D-Trp-OMe is not enzymatically cleaved. However, both enantiomers hydrolyze spontaneously in 0.1 *M* sodium phosphate buffer pH 6.8 which can be a source of error in these studies. To overcome this problem, the extent of hydrolysis of D-Trp-OMe was used as a blank for the spontaneous hydrolysis of the L-enantiomer and storing the L- and D-Trp-OMe standard solutions at −20°C reduced the spontaneous hydrolysis to less than 1% in 10 days.

In order to follow the Michaelis–Menten kinetics of the ACHT hydrolysis of L-Trp-OMe, not more than 30% of the initial substrate should be hydrolyzed during each experimental observation; this keeps the experimental conditions within the linear range of the enzyme kinetics [16]. The amount of ACHT immobilized on the 15 cm × 4.6 mm I.D. column was too large for the substrate and inhibitor concentrations used in these studies and a column with a less ACHT was necessary. The required immobilized enzyme concentration was achieved using a 1-cm IAM · PC cartridge. The amount of ACHT on the IAM cartridge was adjusted using a 1 mg/ml solution of ACHT which was either perfused through the cartridge or introduced by repeated bolus injections. After each loading, the enzymatic activity was checked using L-Trp-OMe and the amount of ACHT was adjusted to keep the maximum hydrolysis of the substrate less than 30%.

A baseline separation of L-Trp and L-Trp-OMe could not be achieved on the 1-cm IAM · PC-ACHT which acts as an enzymatic reactor rather than a chromatographic column. In order to obtain a total chromatographic resolution of the substrate and product, a 3-cm IAM · PC column was attached in series. Fig. 1 presents some typical chromatograms from this coupled column system.

The results of the displacement chromatographic studies were analyzed using Lineweaver–Burk plots which are presented in Fig. 2. The K_I values for HCA and BPEA and the K_M values for L-Trp-OMe determined from the Lineweaver–Burk plots are presented in Table I.

The initial qualitative examination of the data revealed a competitive type of ACHT inhibition for HCA which is consistent with the previously reported mode of HCA inhibition [17,18]. The K_I values determined in this study, 1.8 and 6.1 m*M* (Table I), are also consistent with the previously reported value of 4.5 m*M* [17,18]. The literature value was calculated using a single concentration of the substrate, acetyl-L-tyrosinamide, a pH of 7.8, 25°C and the assumption of competitive inhibition. The kinetic measurements reported in this paper were performed using a range of L-Trp-OME concentrations (0.5 to 4.0 m*M*), a pH of 6.8 (the previously determined optimal value for the IAM-ACHT column) and 25°C.

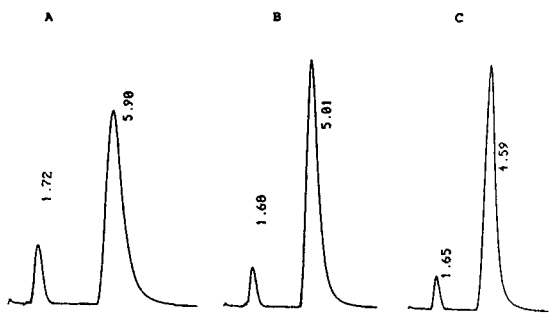

Fig. 1. Chromatograms following injection of L-tryptophan methyl ester 3 m*M* onto an IAM · PC-ACHT (1 cm × 3.0 mm I.D.) cartridge connected in series to an IAM · PC (3 cm × 4.6 mm I.D.) column. The first eluting peak corresponds to the product, L-tryptophan, the second eluting peak corresponds to L-tryptophan methyl ester. (A) No inhibitor in the mobile phase; (B) mobile phase containing 5 m*M* β-phenylethylamine; (C) mobile phase containing 10 m*M* β-phenylethylamine. Values at peaks are retention times in min. See text for chromatographic conditions.

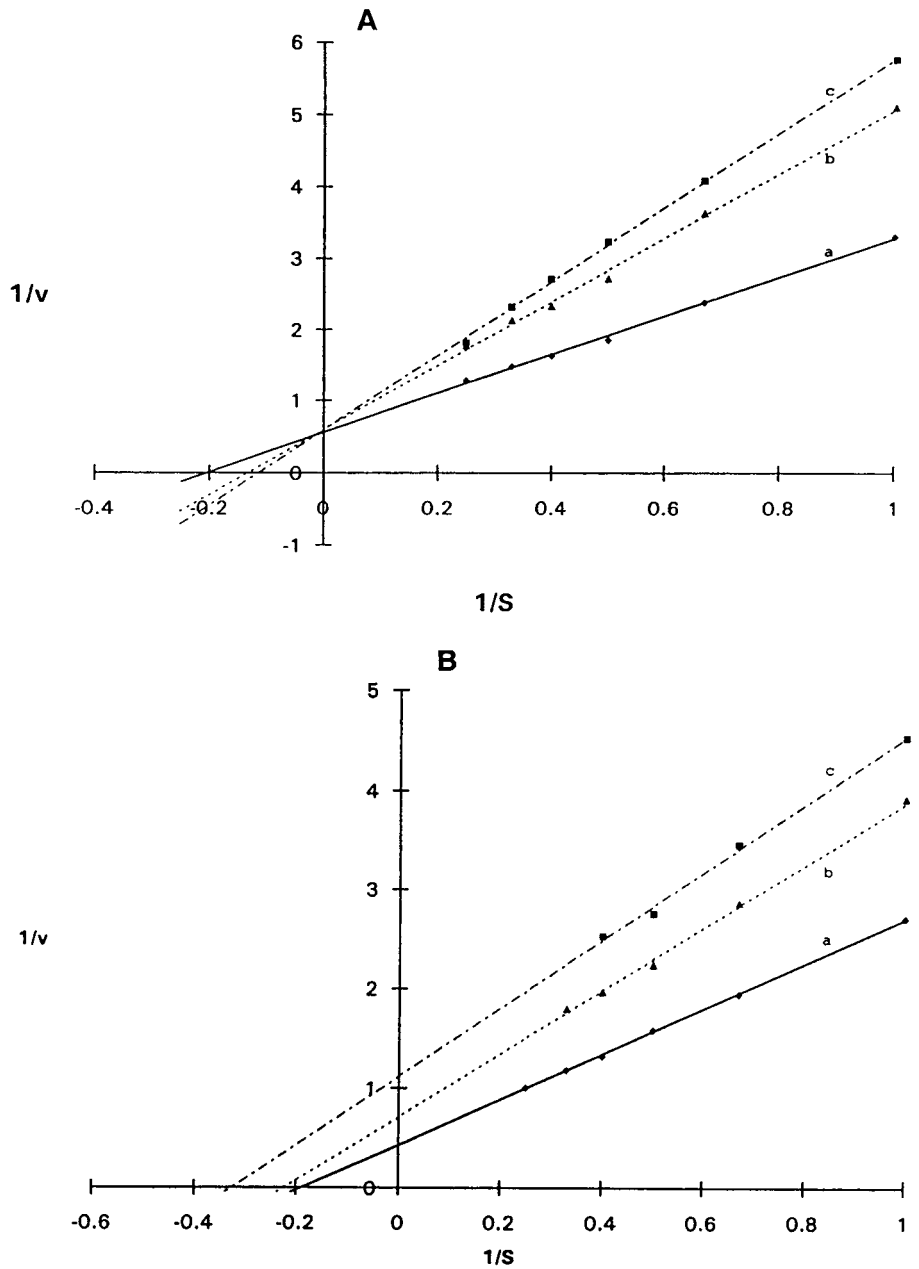

Fig. 2. (A) Lineweaver–Burk plots of the inhibition of enzymatic hydrolysis of L-tryptophan methyl ester by HCA on an IAM · PC-ACHT column. a = Control without addition of inhibitor to the mobile phase; b = mobile phase containing 1 m*M* HCA; c = mobile phase containing 5 m*M* HCA. Abscissa in 1/m*M*, ordinate in min/m*M*. (B) Lineweaver–Burk plots of the inhibition of enzymatic hydrolysis of L-tryptophan methyl ester by BPEA on an IAM · PC-ACHT column. a = Control without addition of inhibitor; b = mobile phase containing 5 m*M* BPEA; c = mobile phase containing 10 m*M* BPEA. Abscissa in 1/m*M*, ordinate in min/m*M*.

TABLE I

CALCULATED AFFINITY CONSTANTS FROM STUDIES WITH THE IAM-ACHT STATIONARY PHASE

Inhibitor and concentration (m*M*)	L-Trp-OMe K_M [a] (m*M*)	V_{max} (mmol/min)	K_I (calc.) (m*M*)	K_I (lit.) (m*M*)	Type of inhibition
HCA 0	4.9	1.8			
1	(7.6)[b]	(1.7)[b]	1.8	4.5 [17,18]	Competitive
5	(8.8)[b]	(1.7)[b]	6.1		
Day 1 (24%)[c]					
BPEA 0	5.3	2.3			
5	(4.7)[b]	(1.5)[b]	8.5	Not	Mixed
10	(3.1)[b]	(0.9)[b]	6.3	available	
Day 11 (11%)[c]					
BPEA 0	19.4	2.7			
5	(22.0)[b]	(1.8)[b]	10.2		Non-competitive
10	(23.0)[b]	(1.6)[b]	15.0		

[a] K_M for L-Trp-OMe calculated using non-immobilized ACHT = 1.5 m*M*.
[b] Apparent values of K_M and V_{max} in the presence of inhibitor.
[c] Activity of immobilized ACHT reported by percent of substrate (L-Trp-OMe, 4 m*M*) converted to L-Trp.

However, it should be mentioned that the consecutive increases in HCA concentration in the mobile phase from 2 to 5 to 10 m*M* did not result in a corresponding increase of ACHT inhibition, as monitored by the product formation in the HPLC column. This effect could be ascribed to either a mass-transfer limitation which has been observed in other immobilized enzyme systems [19], or, less probably, to a mixed type of inhibition.

The inhibition of ACHT by BPEA is depicted in Fig. 2B and the Lineweaver–Burk plots indicated a mixed type of inhibition. With 5 m*M* BPEA in the mobile phase, the inhibition appeared to be non-competitive; when 10 m*M* BPEA was present in the mobile phase, a straight line almost parallel to the one obtained for 5 m*M* BPEA was obtained which is characteristic of uncompetitive inhibition. The K_I calculated from these experiments were 8.5 and 6.3 m*M* (Table I). Unfortunately, there was no previously reported K_I value for BPEA inhibition of ACHT. BPEA has been studied, but the K_I was not determined due to background interference [17,18].

When the BPEA experiment was repeated 10 days later in the same column, the different results were obtained. In the interim, the activity of the IAM · PC-ACHT support had decreased from a 24% conversion of substrate to 11% (Table I). This resulted in changed Lineweaver–Burk plots and consequently different K_I values of 10.2 and 15.0 m*M*. The K_I values were not the only parameters which had changed, the initial K_M values calculated for L-Trp-OMe from the experiments with HCA and BPEA were 4.9 and 5.3 m*M*, respectively, relative to a K_M of 1.5 m*M* calculated using the non-immobilized ACHT. At day 11, this value had risen to 19.4 m*M*.

The addition of inhibitors to the mobile phase affected k' as well as enzymatic activity; the addition of 10 m*M* HCA to the mobile phase increased the k' of L-Trp-OMe by 5% while the presence of 10 m*M* BPEA in the mobile phase decreased the k' of L-Trp-OMe by 30%. These results are consistent with two observations by Pidgeon and co-workers [10,20] regarding chromatographic retention on the IAM · PC phase: (1) It was observed that an important aspect of the retention mechanism on the IAM · PC phase is due to ionic interactions with uncapped primary amines on the aminopropyl silica and that

capping these sites increases the retention; possibly by allowing the solute to penetrate deeper into the hydrophobic cavities. Thus addition of the anionic HCA to the mobile phase should increase k' by blocking some or all of the uncapped primary amines through ionic bonding. (2) It was also observed than an important aspect of the retention mechanism on the IAM · PC was solute adsorption to the polar head groups which contain both anion- and cation-exchange sites. The addition of a cationic modifier, *i.e.* BPEA, to the mobile phase should reduce the interactions between the positively charged solute and negatively charged phosphonate moiety on the stationary phase.

Based upon the results of this study, it is clear that the properties of IAM · PC-ACHT phase will change over time. However, the observed changes between days 1 and 11 in enzymatic activity and the resulting changes in the calculated kinetic constants (Table I) are not that dramatic when one considers the fact that the column was in constant use over the 10-day period. Thus, the IAM · PC-ACHT appears to be relatively stable.

It has been reported that the IAM · PC phase can be regenerated by washing the soluble proteins off the support with detergents [10]. Our experience confirmed this and we have reloaded regenerated IAM · PC supports with ACHT and other enzymes producing enzymatically active phases. In addition, we have found the immobilized enzyme supports to be relatively stable and several 15 cm × 4.6 mm I.D. IAM · PC columns containing ACHT and one column containing lipase have been in constant use in our laboratory as immobilized-enzyme reactors for more than four months.

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Short Communication

Chromatographic stability of glucose-silica

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ABSTRACT

Glucose-silica was prepared and packed into five 100 × 4.6 mm I.D. columns. These columns were tested initially with selected protein mixtures. Each of the columns was stored in one combination of buffer (100 m*M* sodium sulfate, 20 m*M* sodium phosphate, pH 6.8), temperature (4 or 20°C), methanol and sodium azide. After a year, we found that storing glucose-silica columns in the buffer containing sodium azide or methanol at 4°C was necessary to prolong stability in storage. However, glucose-silica also exhibited some dynamic instability.

INTRODUCTION

An important feature of modern high-performance liquid chromatography (HPLC) columns is greater stationary phase stability which increases their useful chromatographic lifetime. Alkaline buffer (*i.e.*, pH > 8) is known to reduce lifetime of columns packed with silica-based materials. Biodegradability is another problem associated with stability of HPLC packing materials [1].

Gel filtration is size-exclusion chromatography that uses mild aqueous solvents and neutral hydrophilic packings suitable for protein separations under non-denaturing conditions. We [2] and others [3,4] have reported the synthesis of glucose-bonded silica for such usage. In most cases, glucose was covalently coupled to aminopropyl-derivatized silica through Schiff's base formation and reduction with $NaCNBH_3$. Although the exact conditions used for synthesis varied among the researchers, we, in particular, concluded that glucose-silica was a well-behaved gel-filtration medium for HPLC of proteins. Later, after prolonged (about 6 months) column usage, we became aware that the performance of glucose-silica columns degrades with time. Our experience and that of others [4,5] suggested that we should further investigate this stability issue. Accordingly, this study addresses the static and dynamic stability of these columns. While the results obtained with glucose-silica stability only strictly apply to this chromatographic support, the results obtained may also suggest strategies for preserving the performance of other biocompatible columns.

EXPERIMENTAL

Chromatography

The chromatograph was a Rainin Rabbit-HP solvent-delivery system outfitted with a Knauer variable-wavelength monitor, a Macintosh SE

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computer, and the Rainin Dynamax version 1.2 software for data collection and analysis. Chromatography was at room temperature (20°C) with a 1 ml/min flow-rate throughout.

Preparation of glucose-silica

Glucose-silica was prepared as previously described [2]. However, in order to obtain glucose-silica consistently Cd–ninhydrin [6] negative at room temperature, we always carried out the reaction twice. Glucose-silica after the first reaction was washed with water only, and after the second reaction was first washed with acetone, then with water. All silica-based supports used were 7 μm Macherey–Nagel Nucleosil silicas obtained from Alltech (Deerfield, IL, USA) and had 50 Å pores.

Column testing

Glucose-silica supports were packed into five 100×4.6 mm I.D. columns by Alltech. The mobile phase was buffer A (100 m*M* sodium sulfate, 20 m*M* sodium phosphate, pH 6.8) throughout. The same sensitivity was used to record chromatograms for a particular test mixture throughout. After testing in each period, columns were flushed with appropriate storage buffers for about ten column volumes and stored (see *Storage conditions* below). The time table for retesting was 1 month, 3 months, 6 months and 1 year after the initial tests.

Proteins with opposite extremes of isoelectric pH (p*I*), were used to test the columns. Neutral but relatively hydrophobic tryptophan (M_r 204) was used as a low-M_r marker for total included column volume (V_T). Proteins of various molecular masses were also included to assess their sensitivity to stationary phase matrix instability. The individual proteins were typically made up as 1 mg/ml stock solutions and various mixtures were then prepared. The mixtures were aliquoted for long-term uses to prevent problems associated with repeated freezing and thawing. Each protein was also injected individually onto the various columns to confirm the identity of each peak in a mixture. The injection volume was 10 μl and detection was by absorption at 220 nm throughout.

Storage conditions

The five storage conditions were as follows: (A) 10 m*M* sodium azide in buffer A at 4°C; (B) 10% methanol in buffer A at 4°C; (C) 100% methanol at 4°C; (D) buffer A alone at 4°C; (E) 10 m*M* sodium azide in buffer A at room temperature.

Chemicals and reagents

The mobile phase buffer contained sodium phosphate (Mallinckrodt, Paris, KY, USA) and sodium sulfate (Sigma, St. Louis, MO, USA). Chicken egg lysozyme (Lyz, M_r 14 000, p*I* 11), horse heart cytochrome *c* (Cyt c, M_r 13 000, p*I* 9.4), bovine serum albumin (BSA, M_r 66 000, p*I* 5), soybean trypsin inhibitor (STI, M_r 20 000, p*I* 4.5), and L-tryptophan (Trp) were from Sigma; L-arginyl-L-phenylalanine (Arg–Phe) was from Cyclo, Los Angeles, CA, USA. Porcine brain calmodulin (CaM, M_r 16 700, p*I* 4) was purified as described [7]. (M_r and p*I* of these proteins are from references 2, 7 and 8.)

RESULTS AND DISCUSSION

Of the five storage conditions tested over a year's time, all of the data fit one of two patterns: those conditions which maintained reasonable chromatographic performance for about 6 months and those which showed rapid degradation of column performance. All of the data need not be shown to illustrate these findings; rather the results of the best and worst storage conditions only are shown in Fig. 1A and B, respectively. Fig. 1A shows that refrigeration in buffer A containing sodium azide maintained reasonable chromatographic performance for about 6 months. Quite similar results were obtained for columns that were refrigerated with either 10 or 100% methanol although the former showed somewhat better stability than the latter (data not shown). The worst storage condition found is illustrated in Fig. 1B, room temperature storage in buffer A containing sodium azide. This condition was only marginally worse than storage in buffer A (without azide) under refrigeration which also gave rapid degradation of column performance. Thus, our results with glucose-silica column stability indicate that both

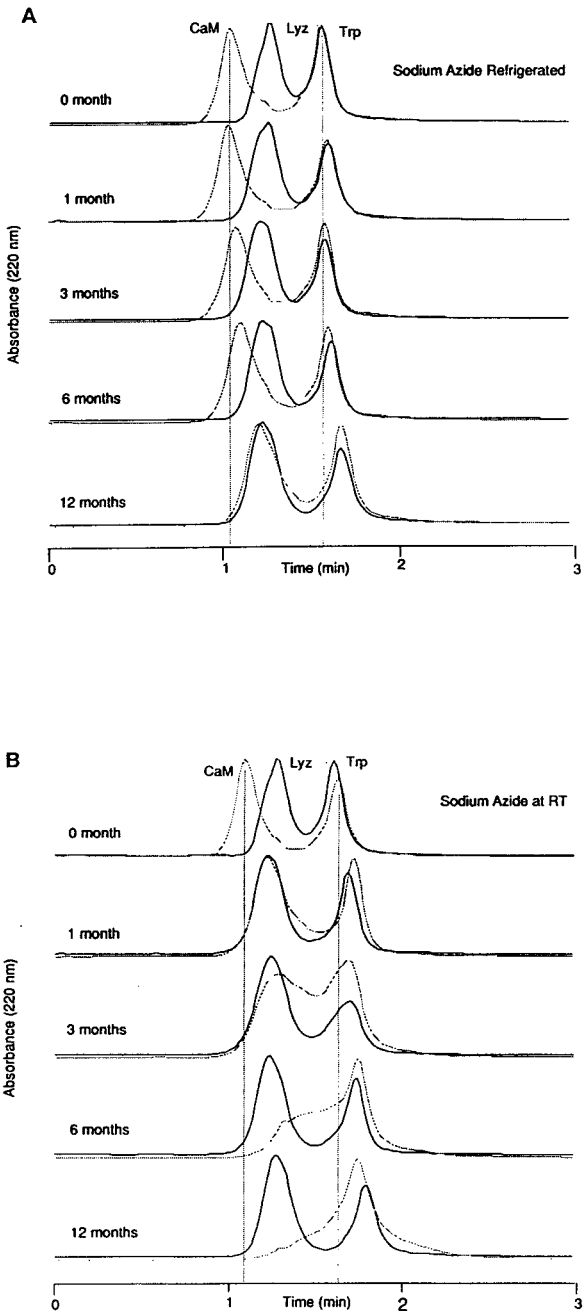

Fig. 1. Chromatographic behavior of acidic and basic proteins for columns stored in 10 m*M* sodium azide at 4 or 20°C. Two test mixtures: (1) Trp and acidic CaM seen as dotted line and (2) Trp and basic Lyz indicated as solid line, were injected onto the column at the indicated period. In Fig. 1B, RT = room temperature. (A) Column stored in 10 m*M* sodium azide at 4°C; (B) column stored in 10 m*M* sodium azide at RT.

refrigeration and inclusion of either methanol or sodium azide in the storage buffer are necessary to maintain relatively stable chromatographic performance; either treatment alone is not sufficient.

The data in Fig. 1A and B also demonstrate that the retention times of all tested substances were not affected the same during column failure. The retention times and peak shapes for Lyz were maintained under all storage conditions throughout a year. Calculations indicated less than 3% changes in retention times of Lyz for all columns. In contrast, CaM, an acidic protein of similar size to Lyz, exhibited peak shapes and retention times with more variation among columns stored differently. Fig. 1A shows that CaM peak shapes were reasonably constant for about 6 months for columns stored refrigerated in 10 m*M* sodium azide (Fig. 1A), 10% methanol or 100% methanol (data not shown). However, columns stored in buffer containing sodium azide at room temperature (Fig. 1B) or in refrigerated buffer alone (data not shown) gave CaM peak shapes characteristic of column degradation within 1 month. The column that gave the largest changes in the CaM peak was the column stored at room temperature (Fig. 1B) even though sodium azide was included as an anti-bacterial agent.

Fig. 2 shows that the chromatographic behavior of BSA, a relatively large acidic protein, was less affected under all storage conditions throughout a year. This result can be contrasted with the greater variation seen with the smaller acidic proteins CaM (Fig. 1) and STI (see below). The likely cause of BSA's stable behavior is that its large size excludes it from the silica pores where more of the changes related to column stability are occurring.

The retention times of Cyt c (Fig. 2), a small basic protein, were not significantly affected, in agreement with the data obtained for Lyz (Fig. 1). Both Cyt c and Lyz are basic and have similar M_r. Small, hydrophobic markers were actually more sensitive to column changes. The geometric volume, V_T, of the 100×4.6 mm columns used is 1.66 ml, which at this flow-rate corresponds to a retention time of 1.66 min. For gel-filtration chromatography, all species should

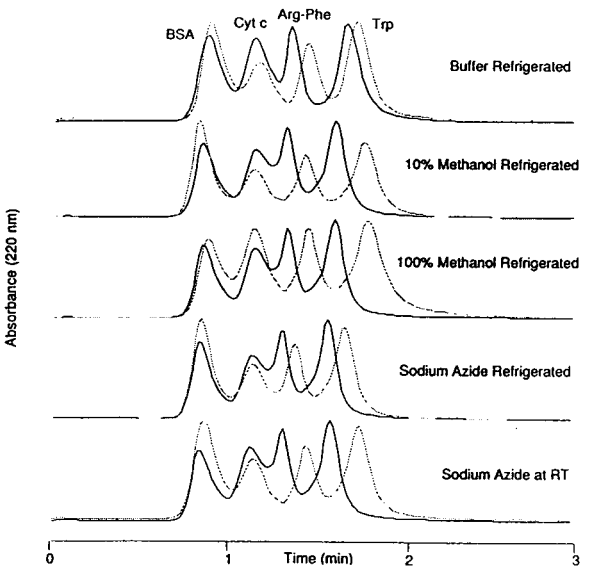

Fig. 2. Chromatographic behavior of a four-component mixture. Chromatograms were overlaid so that one can compare chromatographic changes before (solid line) and after (dotted line) a year of storage under the different conditions shown.

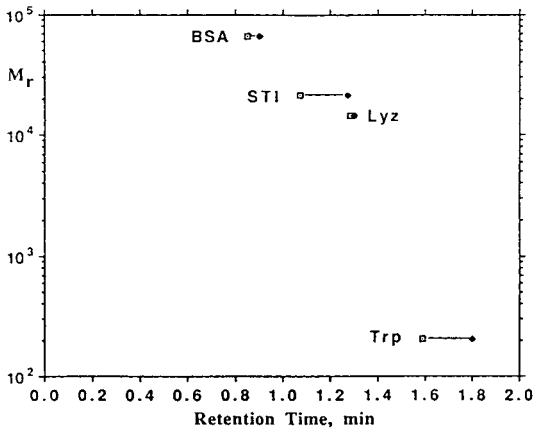

Fig. 3. Dynamic stability of glucose-silica. STI was used as an alternative source of low-M_r acidic protein for this study. The open squares represent day 1 (except for STI which began at day 2) and the closed diamonds represent day 7. 0.05% Sodium azide was added to the mobile phase buffer for the study. The column was stored overnight in this buffer at 4°C.

elute with smaller retention times than V_T. In general, as these columns began to fail, retention volumes of Trp, a small hydrophobic molecule, became greater than V_T. Arg–Phe, a basic, hydrophobic dipeptide, exhibited similar behavior (Fig. 2). These results indicate that as these glucose-silica columns begin to fail, other chromatographic separation modes begin to operate.

The increases in retention times throughout the year shown in Figs. 1 and 2 are not due to changes in the chromatograph over the year. Flow-rates of pumps can be affected by wear, tubing lengths can change, etc. and all of these could affect measured retention times. However, these factors were controlled for and do not account for the differences shown. The changes shown are due to time-dependent changes in the columns themselves.

Fig. 3 shows the results of a dynamic stability study of glucose-silica. For this study, a column was subjected to continuous chromatography each working day for a week with 0.05% sodium azide at room temperature, and the changes that occurred in retention times are illustrated. The greatest changes occurred with the small acidic protein (STI) and Trp, whose retention times were significantly increased throughout. The retention time of Lyz, a basic protein, was constant throughout. BSA, though also an acidic protein, was not much affected probably because of its large molecular size relative to the 50 Å pores. This observation and that of Fig. 2 for BSA suggest that totally excluded proteins are less susceptible to stationary phase changes in HPLC gel-filtration columns. While our long-term study of glucose-silica was necessary to determine the effects of a variety of storage conditions on stability, the data in Fig. 3 show that a dynamic stability study can be used to more rapidly detect unstable columns. This was useful in other studies where we have found that chemically modifying glucose-silica increases its stability [9].

Overall, Figs. 1–3 demonstrate that the observed chromatographic changes of glucose-silica over time were mainly consistent with the increasing presence of cationic and hydrophobic interactions on the support surface as storage is prolonged. This was detected in our study by the changing chromatographic behavior of acidic CaM and STI, the basic and hydrophobic Arg–Phe, and Trp throughout a year. CaM appeared

to bind cationic groups in an ion-exchange interaction with degraded columns.

The mechanism by which these changes occur is not at all certain; however, it seems likely that microbial contamination may be the cause. Removing glucose from glucose-silica would expose the underlying propylamine layer on the support surface, causing the observed increased retention times of CaM and STI. Increased retention of Arg–Phe and Trp suggests that hydrophobic interaction also accompanies column degradation.

Our data suggest that storing columns in sodium azide or methanol at 4°C was effective in maintaining the original chromatographic behavior for glucose-silica columns throughout at least 6 months of storage. This is based mostly on the observations of chromatographic peak shapes and retention times of separated mixtures. As new chromatographic media are synthesized with the goal of biocompatibility, the stability of these materials must be of special concern. Indeed, we have also found glycidiol-silica columns (*e.g.*, Alltech's Macrosphere GPC) to degrade within about 6 months when used daily for chromatography of CaM (unpublished results). The results with glucose-silica suggest that storage under refrigeration and inclusion of either 10 m*M* sodium azide or 10% methanol in mobile phases may be effective methods to prolong the useful lifetime of biocompatible columns. Refrigeration of these columns was also shown to be important, probably because it significantly slowed down microbial growth inside the glucose-silica columns; methanol- and NaN_3-resistant organisms may be responsible for this requirement.

Our data also suggest that mixtures used to test column performance should contain as many diverse kinds of proteins and other biochemicals as is feasible. For example, the results obtained with a test mixture containing only neutral and basic proteins may not be relevant to an investigation involving acidic proteins, testing with large proteins may not reveal changes which occur as readily as proteins which penetrate and interact with porous surfaces, etc.

This study demonstrates effective storage methods, which prevent the degradation of glucose-silica columns for about 6 months. However, the overall instability of glucose-silica observed in this study limits its usefulness. Since glucose-silica was shown to possess many excellent chromatographic properties [2], this instability suggests that while the approach of making polyhydroxylated silica coatings is a good one, these coatings will need to be designed in the future to enhance stability. We have tried the chemistry with maltose instead of glucose but found that such coating with disaccharide was inferior probably because of lower coupling efficiency under the same conditions we used to synthesize glucose-silica (unpublished results). Huisden *et al.* [10] recently also coupled maltose to aminopropyl-silica and cross-linked maltose on the surface. This treated maltose-silica appears to be more stable dynamically than glucose-silica. However, we believe that the proper storage conditions reported in this note may also be applied to prolong its static stability.

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Short Communication

High-performance liquid chromatographic preparation of oxybutynin enantiomers on a chiral stationary phase☆

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ABSTRACT

A method for the preparation of oxybutynin enantiomers by high-performance liquid chromatography (HPLC) on a chiral stationary phase (Chiralpak AD) was developed. Enantiomers were separated directly and rapidly without the need for any derivatization. Good optical resolution was obtained, with separation and resolution factors of 1.43 and 1.28, respectively. This method would be a useful alternative to synthetic preparation of the enantiomers.

INTRODUCTION

Oxybutynin (Fig. 1) is used as a racemate for the treatment of urinary incontinence due to detrusor instability. It has two enantiomers, which show different pharmacological properties [1,2], as well as many pharmaceuticals [3], although pharmacokinetic studies of oxybutynin have been carried out using the racemate [4,5]. Simple preparation and determination of the two enantiomers would be expected to facilitate further investigation of their properties. Chiral stationary phases for high-performance liquid chromatography (HPLC) have been introduced for use in the separation of many racemates [6].

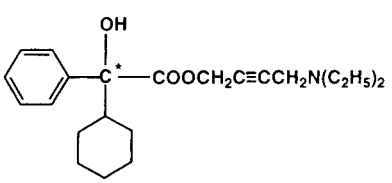

Fig. 1. Structure of oxybutynin.

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However, HPLC resolution of oxybutynin enantiomers has not been reported.

In this study, direct preparation of oxybutynin enantiomers was attempted using carbamate derivatives of amylose coated on silica gel as a chiral stationary phase for HPLC.

EXPERIMENTAL

Materials

Oxybutynin chloride (Sigma, St. Louis, MO, USA) and all other chemicals were of reagent grade obtained commercially and used without further purification.

Preparation of oxybutynin free base

Oxybutynin free base was prepared as follows. Oxybutynin chloride, 1 g, was dissolved in 100 ml of phosphate buffer (pH 8) and extracted twice with 100 ml of *n*-hexane. The organic phase was evaporated under reduced pressure at 30°C. The recovery of oxybutynin as the free base was more than 90%. It was dissolved in the HPLC eluent to give a concentration of 5 mg ml^{-1} and filtered through a 0.45-μm membrane filter (AcroLC, Gelman Sciences Japan, Tokyo, Japan) before injection.

Apparatus and procedure

The liquid chromatograph was equipped with a pump (Model 510, Waters, Milford, MA, USA), an absorbance detector (254 nm, Model 440, Waters), a Model C-R1B integrator (Shimadzu, Kyoto, Japan) and a Rheodyne 7125 injector valve with a 100-μl sample loading loop. The separation was performed on a Chiralpak AD column (25 × 1 cm I.D., 10 μm particle size, Daicel, Tokyo, Japan) connected to a precolumn (Chiralpak AD, 5 × 1 cm I.D.) thermostated by a column oven (L-5030, Hitachi, Tokyo, Japan) at 25.0°C and eluted with *n*-hexane–2-propanol (90:10, v/v) at a flow-rate of 1.0 ml min^{-1}. The sample volume injected was 100 μl for each 20-min period. The eluates of each peak were collected and evaporated under reduced pressure at 30°C. The circular dichroism (CD) spectrum of each sample ($1.0 \cdot 10^{-3}$ mol l^{-1} in eluent) was measured from 210 to 300 nm (Jasco-J-500C spectropolarimeter, Japan Spectroscopic, Tokyo, Japan). The concentration as total oxybutynin was then determined [7]. The dead time of the column was estimated using toluene.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram of the resolution of oxybutynin racemate. The racemate used contained the same amount of each enantiomer, as estimated from the ratio of their peak areas, 0.995 ± 0.003. The HPLC resolution, capacity factor (k'), separation factor (α) and resolution factor (R_s) were calculated as 0.64 (k'_1) and 0.91 (k'_2), 1.43 and 1.28, respectively. The sample for each enantiomer was analysed by HPLC, and each was confirmed to be a single

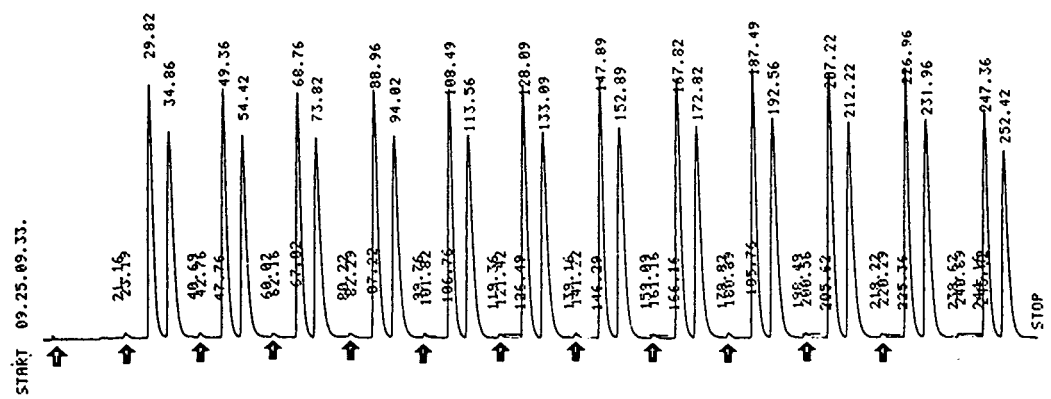

Fig. 2. Chromatographic resolution of oxybutynin racemate injected 100 μl (5 mg ml^{-1}) for each 20-min period. Values at peaks indicate retention times in min.

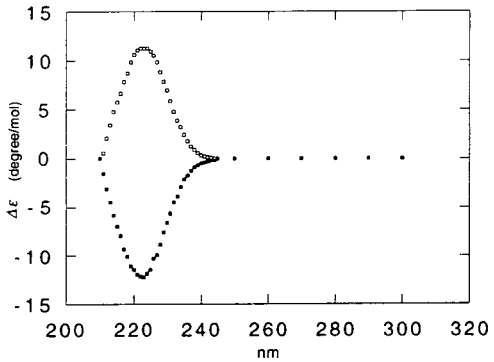

Fig. 3. CD spectra of oxybutynin enantiomers in *n*-hexane–2-propanol (90:10). ■ = Front peak; □ = second peak.

peak. As shown in Fig. 3, the CD spectra of the enantiomers were characterized by a Cotton effect at about 220 nm ($\Delta\varepsilon = -12.0$ mol l^{-1} cm^{-1} for the front peak, +11.4 mol l^{-1} cm^{-1} for the second peak). Racemization of both enantiomers was not observed in the eluent or in methanol during at least 6 months in a refrigerator at 4°C, and both enantiomers were also stable for 2 days in aqueous solution between pH 1.0 and 7.4 at 37.0°C. The contractile response of isolated rat bladder detrusor muscle to concentrations of $1 \cdot 10^{-6}$, 10^{-5} and 10^{-4} mol l^{-1} was compared by measuring the isomeric contractions. The enantiomer prepared from the second peak showed higher potency than the other peak [9].

The HPLC preparation of oxybutynin enantiomers reported here was simple in comparison with synthetic methods [8]. We are now applying this method for simultaneous quantitative determination of enantiomers in biological fluids in order to investigate the pharmacokinetics of oxybutynin enantiomers.

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Short Communication

Direct resolution of racemic drugs using cellulase silica as a chiral stationary phase

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(First received March 9th, 1993; revised manuscript received July 9th, 1993)

ABSTRACT

The enantiomers of propafenone, flecainide and its two analogues 4-hydroxyflecainide and 5-hydroxyflecainide, betaxolol, doxazosin, and terazosin and its analogue A-65297 were separated on a cellulase silica column (TrichSep-100). The effects of pH and the concentration of the organic modifier, 2-propanol, were studied.

INTRODUCTION

Immobilized proteins have been extensively used as chiral stationary phases for the direct resolution of racemates. α_1-Acid glycoprotein (AGP), bovine serum albumin and human serum albumin, immobilized on silica have been successfully used in the separation of racemic drugs and related substances [see refs. 1–3 for reviews]. Furthermore, the use of ovomucoid, a glycoprotein from chicken egg white, in enantioseparations has also been reported [4–6].

More recently, Erlandsson *et al.* [7] and Marle *et al.* [8] have reported on direct resolution of enantiomers using immobilized cellulase, a glycoprotein produced by the fungus *Trichoderma reesei*, as a chiral stationary phase. Also, two recent studies on this chiral phase were performed by Vandenbosch *et al.* [9,10]. The substances investigated on this new chiral column are mainly β-adrenergic blocking agents and local anaesthetics such as prilocaine and analogues.

In this communication, we report on the use of a cellulase silica column for the chiral separation of eight primary and secondary amines which have not previously been baseline resolved on cellulase.

EXPERIMENTAL

Apparatus

The liquid chromatographic system used was a Jasco Model PU-980 pump, a Jasco Model 851-AS autoinjector and a Jasco Model UV-975 variable-wavelength detector. A Barspec Data System was used to collect and process the chromatographic data.

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Chemicals

Terazosin hydrochloride and A-65297 were obtained from Abbott Labs. (North Chicago, IL, USA). Doxazosin mesylate was obtained from Pfizer (Karlsruhe, Germany). Flecainide acetate, 4-hydroxyflecainide acetate and 5-hydroxyflecainide were all obtained from 3M Health Care (Loughborough, UK). Betaxolol hydrochloride was obtained from Alcon-Couvreur (Puurs, Belgium), and propafenone hydrochloride was obtained from Knoll (Ludwigshafen, Germany). The structures of these substances are given in Fig. 1.

All other chemicals and solvents used were of analytical grade, available through normal commercial channels.

Chromatographic conditions

The column used was a TrichSep-100, particle size 10 μm, 10 cm × 4.6 mm I.D. (Skandinaviska GeneTec, Kungsbacka, Sweden).

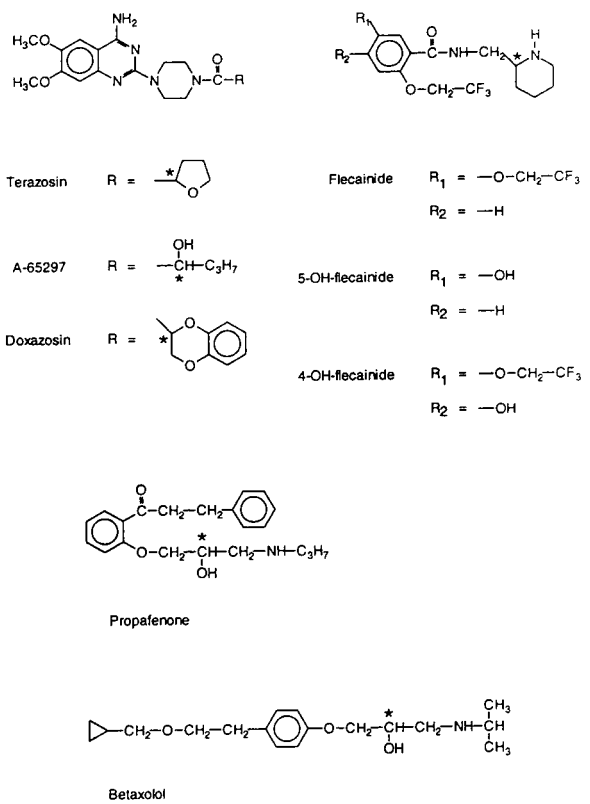

Fig. 1. Structures of the solutes.

The chromatographic experiments were performed at room temperature (20–25°C). Phosphate buffers were prepared from sodium dihydrogenphosphate and disodium hydrogenphosphate, and acetate buffers from sodium acetate and acetic acid. All mobile phases were filtered through a Millipore filter, type HV 0.45 μm, and degassed in an ultrasonic bath prior to use. The solutes were all dissolved in mobile phase at concentrations of 0.1–0.2 mM, and 20–50 μl of these solutions were injected. The flow-rate was 0.7 ml/min, and the detector was set at 240 nm.

The retention time, t_R, was used to calculate the capacity factor, $k' = (t_R - t_0)/t_0$, where t_0 was determined by injection of water, that was assumed to be non-retarded. The enantioselectivity, α, was calculated as k'_2/k'_1, where k'_2 is the capacity factor of the more retained enantiomer. The peak symmetry was measured at baseline: the projection of the intersection point of the two peak tangents divided the baseline in two parts, a, the front side and b, the rear side. The asymmetry factor, *asf*, was then calculated as b/a. The resolution of incompletely resolved peaks was calculated as Kaiser's peak separation function, f/g [11,12]: one line was drawn joining the maxima of the two peaks; a second line was drawn from and perpendicular to the baseline through the valley between the peaks and up to meet the first line. This distance is defined as g. The distance between the valley and the intersection of the two lines is defined as f. The ratio $f/g = 1$ corresponds to complete separation. The resolution of completely resolved peaks was calculated using the equation

$$R_s = 2(t_{R2} - t_{R1})/w_1 + w_2$$

where w_1 and w_2 are the peak widths at baseline for the first and the second eluting band, respectively.

RESULTS AND DISCUSSION

The racemic drugs analyzed are shown in Fig. 1. The results of the analyses of these solutes are shown in Tables I and II. Generally speaking, good separations could be obtained when proper conditions were used and two factors, pH and

TABLE I

INFLUENCE OF VARIATION OF pH ON THE SEPARATION OF ENANTIOMERS

Mobile phase: 0.05 *M* acetate buffer with 0.065 *M* 2-propanol; flow-rate: 0.7 ml/min. asf_2 = Asymmetry factor for the second eluting peak; nc = not calculated.

Compound	Parameter	pH				
		3.5	4.5	5.0	5.5	6.5
Terazosin	k'_2	1.69	10.4	15.7	39.6	102.8
	α	[a]	1.21	1.29	1.36	1.46
	asf_2	nc	1.46	1.69	1.77	nc
	f/g	nc	0.49	0.95	0.94	1.63[b]
A-65297	k'_2	2.06	10.9	19.4	27.1	42.4
	α	1.43	1.59	1.68	1.75	2.15
	asf_2	1.67	1.76	1.87	1.82	1.44
	f/g	0.69	0.91	2.83[b]	2.76[b]	3.59[b]
Doxazosin	k'_2	5.96	34.0	72.6	103.1	152.7
	α	1.0	1.20	1.39	1.41	2.19
	asf_2	–	1.69	2.45	1.73	1.45
	f/g	0	0.41	0.89	0.89	2.93[b]
Flecainide	k'_2	0.19	0.71	1.40	2.89	12.9
	α	1.0	1.90	2.29	2.89	3.70
	asf_2	–	1.66	2.17	1.95	1.36
	f/g	0	0.51	0.93	0.94	3.00[b]
4-Hydroxyflecainide	k'_2	0.43	1.11	2.25	5.59	11.6
	α	1.0	2.46	3.20	4.20	4.89
	asf_2	–	1.96	2.16	2.54	2.98
	f/g	0	0.87	3.49[b]	3.99[b]	5.06[b]
5-Hydroxyflecainide	k'_2	0.29	0.42	0.93	1.69	5.67
	α	1.0	[a]	1.47	1.87	2.06
	asf_2	–	nc	2.00	1.88	1.73
	f/g	0	nc	0.89	1.97[b]	3.11[b]
Propafenone	k'_2	0.35	1.65	2.90	5.53	21.2
	α	1.0	1.2	1.38	1.73	2.42
	asf_2	–	1.27	3.11	2.94	1.47
	f/g	0	0.18	0.62	0.74	0.93
Betaxolol	k'_2	0	0.83	1.71	4.22	10.2
	α	1.0	1.76	2.02	2.59	3.00
	asf_2	–	2.23	2.69	2.73	2.63
	f/g	0	0.60	2.20[b]	2.62[b]	3.66[b]

[a] Separation tendency; *i.e.* $\alpha > 1.0$.
[b] Calculated as R_s.

2-propanol concentration in the mobile phase were found to be important for the regulation of enantioselectivity and retention.

Influence of pH on chiral selectivity and retention

An increase in pH of the mobile phase resulted in increased retention for all the amines studied in this report, as is shown in Table I. Similar findings have been reported for β-blockers [8,10] and local anaesthetics [8]. The isoelectric point of the cellulase protein is 3.9 and an increase in pH of the mobile phase would result in increased electronegativity of the protein. The

TABLE II

INFLUENCE OF CONCENTRATION OF 2-PROPANOL ON THE SEPARATION OF ENANTIOMERS

Mobile phase: 2-propanol in phosphate buffer pH 6.0 (ionic strength 0.01). Flow-rate: 0.7 ml/min. asf_2 and nc as in Table I.

Compound	Parameter	Concentration of 2-propanol (M)			
		0.13	0.39	0.65	1.3
Terazosin	k'_2	∞	68.1	52.5	23.9
	α		1.43	1.39	1.36
	asf_2		1.95	1.88	2.20
	f/g		0.97	0.99	0.96
Flecainide	k'_2	13.8	12.2	12.2	12.5
	α	3.56	3.73	3.79	3.45
	asf_2	2.67	1.92	2.12	2.26
	f/g	3.70[a]	4.21[a]	4.71[a]	4.61[a]
4-Hydroxyflecainide	k'_2	22.9	19.1	17.9	14.4
	α	5.28	4.82	4.17	2.55
	asf_2	3.81	2.16	2.14	2.55
	f/g	5.13[a]	5.56[a]	5.31[a]	3.69[a]
5-Hydroxyflecainide	k'_2	9.06	9.42	10.2	11.9
	α	1.92	1.61	1.34	1.1
	asf_2	3.63	1.86	2.10	nc
	f/g	2.21[a]	2.14[a]	0.97	0.12
Betaxolol	k'_2	13.8	15.4	15.7	15.6
	α	2.96	3.42	3.84	4.53
	asf_2	3.24	2.55	2.54	2.98
	f/g	2.88[a]	3.80[a]	4.56[a]	4.80[a]
Propafenone	k'_2	21.6	17.1	16.1	12.5
	α	2.14	2.38	2.38	2.12
	asf_2	3.23	2.17	2.62	3.15
	f/g	0.86	0.93	0.97	0.96

[a] Calculated as R_s.

amines studied would be predominantly protonated in the pH range 3.5–6.5 and the increased retention observed at higher pH might therefore at least in part be explained by increased electrostatic attraction between the solute and the protein. Table I also demonstrates that the enantioselectivity, α, increased with increasing pH. Most of the enantiomeric pairs eluted as single peaks at the lower pH values studied. In some cases, *e.g.* terazosin at pH 3.5, a peak shoulder was clearly seen, an indication for a minor separation of the enantiomers. Since pure enantiomers were not available it was not possible to calculate these α values with sufficient accuracy, and in Table I they are given as a "separation tendency".

In order to obtain baseline separation of the enantiomeric pairs studied, the pH had to be around 6. For some of the solutes, an increase in 2-propanol content was also required. Examples of the separations obtained are shown in Figs. 2 and 3.

Influence of organic modifier, 2-propanol, on retention and chiral selectivity

The retention and enantioselectivity can also be controlled by the addition of an uncharged organic modifier to the mobile phase. On the AGP column, the modifier most extensively studied is 2-propanol [13]. The influence of 2-propanol has also been studied on the cellulase column, where an increase in the concentration

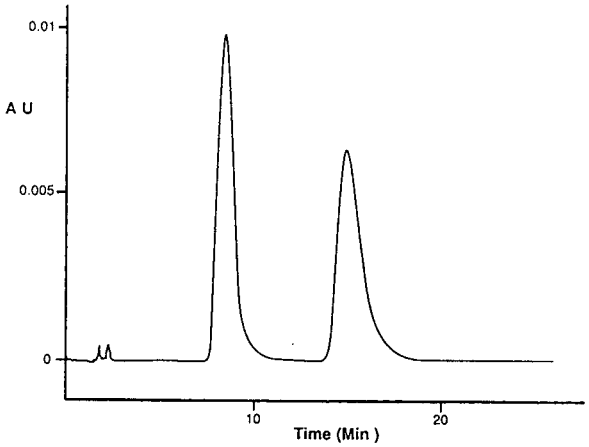

Fig. 2. Resolution of the enantiomers of 5-hydroxyflecainide. Column: TrichSep-100 (100 × 4.6 mm I.D.); mobile phase: 0.05 *M* acetate buffer pH 6.5 containing 0.065 *M* 2-propanol. Amount injected: 2 nmol.

of the alcohol improved the enantioselectivity and peak symmetry for the amines metoprolol, propranolol and prilocaine, while the enantioselectivity was almost unaffected for warfarin and omeprazole (acid and ampholyte, respectively) [8].

For this part of the study we chose phosphate buffer instead of acetate buffer, as phosphate buffer has been reported to give slightly higher stereoselectivity, peak symmetry and resolution for propranolol as compared to acetate buffer [8].

In this study, the effect of 2-propanol concentration was investigated for six of the solutes, as presented in Table II. Note that two of the solutes are being structurally related to meto prolol and propranolol. Compared to metoprolol and propranolol, these two solutes, betaxolol and propafenone, showed a slightly different retention pattern. The highest enantioselectivity for betaxolol was obtained at high 2-propanol content, while the enantioselectivity of propafenone was almost not affected. Interestingly, the increasing enantioselectivity of betaxolol was caused by a decrease in retention of the first eluting enantiomer, while the retention of the second eluting enantiomer initially increased and eventually reached a plateau. The partial resolution ($f/g = 0.85$) of propafenone enantiomers on cellulase at pH 5 was recently reported by Vandenbosch *et al.* [9].

The enantioselectivity of the two analogues of flecainide was also strongly dependent on the concentration of 2-propanol. As can be seen in Fig. 4, the first eluting enantiomer of 5-hydroxyflecainide was strongly retained by an increased

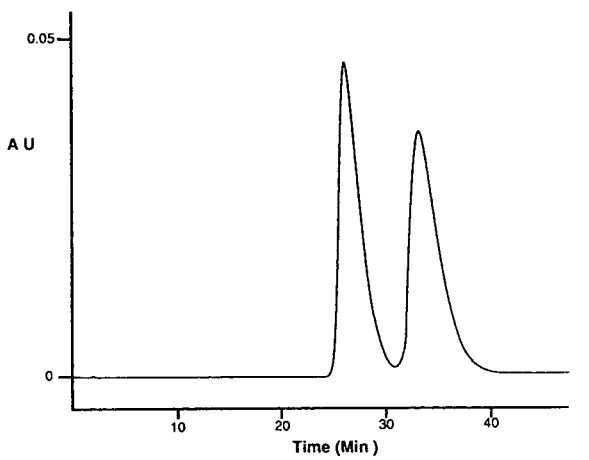

Fig. 3. Resolution of the enantiomers of terazosin. Column: TrichSep-100 (100 × 4.6 mm I.D.); mobile phase: 0.05 *M* acetate buffer pH 5.0 containing 0.065 *M* 2-propanol. Amount injected: 3 nmol.

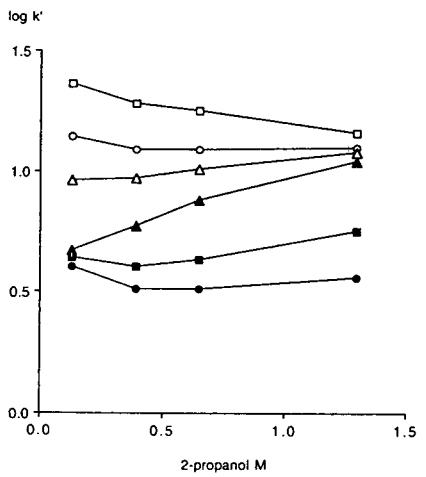

Fig. 4. Influence of mobile phase content of 2-propanol on the capacity factors of the flecainides. Column: TrichSep-100 (100 × 4.6 mm I.D.); mobile phase: phosphate buffer (ionic strength 0.01) pH 6.0 containing different amounts of 2-propanol. ●, ○ = flecainide; ■, □ = 4-hydroxyflecainide; ▲, △ = 5-hydroxyflecainide.

concentration of 2-propanol. The retention of the second enantiomer increased also, but not as dramatically, and thus the largest enantioselectivity was obtained at a low content of 2-propanol. A low concentration of 2-propanol was also favourable for the enantioselectivity of 4-hydroxyflecainide. This was however due to a decrease in retention of the second eluting enantiomer, while the first eluting one showed a complicated dependence on 2-propanol content. Interestingly, the first eluting enantiomers of all three flecainides were eluted close together at low 2-propanol concentration, while an increase in 2-propanol separated these three peaks. The opposite was true for the second eluting enantiomers and thus an intermediate concentration should be chosen to resolve all six peaks. This might be an interesting pharmaceutical application since 4-hydroxyflecainide and 5-hydroxyflecainide are regarded as impurity and degradation product, respectively, of flecainide. The observed differences in the effect of 2-propanol on the flecainides demonstrate that the enantioselectivity is sensitive to the substitution pattern on the aromatic ring, as has also been reported for β-blockers [8].

CONCLUSIONS

The enantiomers of eight chiral compounds were resolved on the cellulase silica phase, TrichSep-100. The enantioselectivity obtained with this column was high, *e.g.* $\alpha = 5.28$ for 4-hydroxyflecainide.

The retention and enantioselectivity can easily be regulated by changing the pH of the mobile phase and the concentration of the uncharged organic modifier 2-propanol. The effects of these changes, however, are strongly dependent on solute structure. Further studies are needed to elucidate the complex nature of the chiral recognition properties of this new chiral stationary phase.

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CHROM. 25 403

Short Communication

Example of pitfalls in the UV detection used in the resolution of racemic compounds by liquid chromatography

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ABSTRACT

The commercially available nitrile (**1**) and the epoxide (**2**) that we used as racemic compounds in previous studies each contained an impurity. Unfortunately, both impurities have a high molar absorptivity at 254 nm, the wavelength at which HPLC detection was carried out. This led us to consider the impurity as one of the enantiomers. Both compounds have been identified. The nitrile (**1**) contains 1,1,1-trifluoroacetophenone (**3**) as impurity and the expoxide (**2**) contains 4-chlorobenzophenone (**4**). The identification of these compounds is described.

INTRODUCTION

In the course of our research into the chromatographic behaviour of several chiral stationary phases of the "brush" type, compounds **1** [1,2] and **2** [1–3] (Fig. 1) were used as test compounds among other racemics. A routine purity control of both commercial products (GC for **1** and ^{1}H NMR for **2**) was carried out. A small quantity of an impurity was detected in

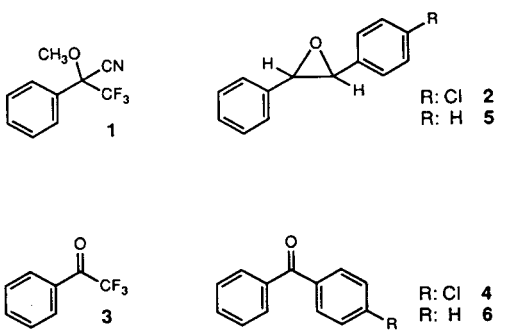

Fig. 1. Structures of racemic compounds and their impurities.

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each product, but it was considered to be negligible. Therefore the nitrile (**1**) and the epoxide (**2**) were used as received from suppliers without further purification. Unfortunately, in both cases the molar absorptivities of compounds **1** and **2** at 254 nm were much lower than those of the accompanying impurities, so racemics and impurities produced similarly important peaks. This fact led us to consider the resolution of two racemics instead of the separation of the impurity from the racemic.

When the attribution errors were discovered, an attempt was made to identify the impurities. Before finishing this study Pirkle *et al.* [4] pointed out the mistake. This identification is now reported.

EXPERIMENTAL

Rotatory power was measured with a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Uberlingen, Germany). GC–MS spectra were performed in an HP 5988A apparatus (Hewlett-Packard, Palo Alto, CA, USA). The UV spectra were recorded on a Uvikon 940 spectrometer (Kontron AG Instruments, Zurich, Switzerland). The analytical liquid chromatographic experiments were carried out on a Hewlett-Packard HP 1090 liquid chromatograph equipped with a PU4020 UV detector (Philips, Cambridge, UK) and the preparative separation was carried out using a chromatograph consisting of a Gilson 302 pump and a UV 115 detector (Gilson, Villiers le Bel, France) and a Valco injection valve (Valco, Houston Instruments, TX, USA). The chiral stationary phases were packed into stainless-steel tubes (analytical, 100×4.6 mm I.D.; preparative, 250×20 mm) by the slurry method. The flow-rate of the pump was 1 ml/min in analytical experiments and 9 ml/min in the preparative separation. The detection wavelength was 254 nm. The mobile phases consisted of a mixture (95:5, v/v) of *n*-heptane and chloroform (to which 0.5% methanol was added). Gas chromatographic experiments were carried out on a Perkin-Elmer 8600 apparatus equipped with a capillary column BP1 ($12 \text{ m} \times 0.22$ mm).

Chemicals and reagents

Compound **1** was purchased from several suppliers: Aldrich (Saint-Quentin Fallavier, France), Janssen (Beerse, Belgium) and JPS (Bevaix, Switzerland). Compounds **2** and **4** were supplied by Aldrich, **3** by Fluka (Saint-Quentin Fallavier, France), **5** by Sigma (Saint-Quentin Fallavier, France) and **6** by Prolabo (Paris, France). Compounds **4** and **6** were purified by crystallization from ethanol 96% and **3** by distillation prior to use.

Chiral stationary phases

Analytical columns packed with CSP-1 (Chirachrom D1) were obtained from Interchim (Montluçon, France). The CSP used in the preparative chromatography (CSP-1p) was prepared as CSP-1 [3] but using 25–45 μm silica. CSP-2 was obtained from N-(3,5-dinitrobenzoyl)-(S)-cyclohexylalanine [5].

RESULTS AND DISCUSSION

Nitrile 1

Because of the high selectivity factors observed for this racemic compound on CSP-1 (CSP-6c in ref. 3), we studied the possibility of effecting a preparative separation using a chiral stationary phase with the same chiral selector. When a sample of **1** was subjected to preparative chromatography on CSP-1p, the two products collected had no action under polarized light. Therefore, no enantiomeric resolution had taken place. The first fraction had a UV spectrum identical to that of **1**. The UV spectrum of the second fraction was different, stable in heptane and changed as a function of time in ethanol. The rate of this evolution increased with the increased amount of water contained in the solvent.

The racemic nitrile **1** was isolated from the first fraction by evaporating the solvent, but the product in the second fraction could not be isolated in the same way. This fact, together with the evolution of the UV spectrum, seemed to point to an unstable product. However, the characterization of the compound was undertaken.

GC–MS of the two fractions collected allowed us to identify the product in the first fraction as the nitrile **1**, but three different products (*m/e* 174, 235 and 229) were detected in the second. Careful examination of the GC–MS spectrum of **1** before chromatography showed only a small quantity of the product with a molecular mass of 174, besides nitrile **1**, at a very short retention time. The other compounds seemed to be aliphatic impurities coming from the solvent used in the preparative separation. The MS spectrum of the product contaminating compound **1** seemed to correspond to 1,1,1-trifluoroacetophenone (**3**, Fig. 1). The UV spectrum of **3** in ethanol evolved in the same way as the product in the second fraction. Such evolution may be due to the hydration of the carbonyl group. This assignation was confirmed by comparing spectral (MS, UV and kinetic evolution in ethanol) and chromatographic data (HPLC and GC) with a real sample of **3**. Thus, the evolution kinetics of **3** in absolute ethanol (Merck)–ethanol 96% (100:1, v/v) is of first order relating to **3** ($k_{observed} = 0.57$/min at 24°C). The presence of this compound in **1** could be expected because it is a synthetic precursor of the nitrile [6].

Molar absorptivities of **1**, purified by distillation, and **3** in heptane were calculated (Fig. 2). Compound **1** had an ε value of 415 l mol^{-1} cm^{-1} at $\lambda_{max} = 261$ nm and **3** had an ε value of 13 190 l mol^{-1} cm^{-1} at $\lambda_{max} = 251$ nm. At 254 nm, the wavelength at which UV detection was carried out, these ε values were 322 for **1** and 12 330 for **3**. Therefore the same absorption (chromatographic peaks with the same area) could be obtained with only 1 mol of **3** in 38 mol of the nitrile, **1**, *i.e.* 2.1 g of **3** in 100 g of contaminated **1**.

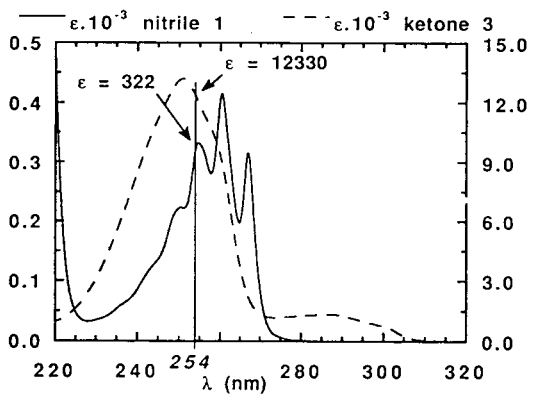

Fig. 2. UV spectra of **1** and **3** in *n*-heptane.

Epoxide **2**

On testing a new chiral stationary phase with N-(3,5-dinitrobenzoyl)-(*S*)-cyclohexylalanine as chiral selector (CSP-2, Fig. 3) [5], an impurity of compound **2** was detected while the partial resolution of the racemic was taking place. Epoxide **2** was easily purified by crystallization from hexane. The impurity was identified from the GC–MS spectrum of the mother liquors from the purification. Thus the major impurity of the sample was a chlorine-containing compound with a molecular mass of 216/218, which corresponds to 4-chlorobenzophenone (**4**, Fig. 1). As in the previous case, the assignation was confirmed by comparing spectral (MS and UV) and chromatographic data (HPLC and GC) with a real sample of **4**.

Molar absorptivities of **2** and **4** were also calculated (Fig. 4). Compound **2** had an ε value of 28 060 l mol^{-1} cm^{-1} at $\lambda_{max} = 234$ nm, and **4** had an ε value of 21 970 l mol^{-1} cm^{-1} at $\lambda_{max} = 255$ nm. At 254 nm, the wavelength at which UV detection was carried out, these ε values are 2180 for **2** and 21 960 for **4**.

The same kind of impurity was detected in a sample of commercially available epoxide **5**.

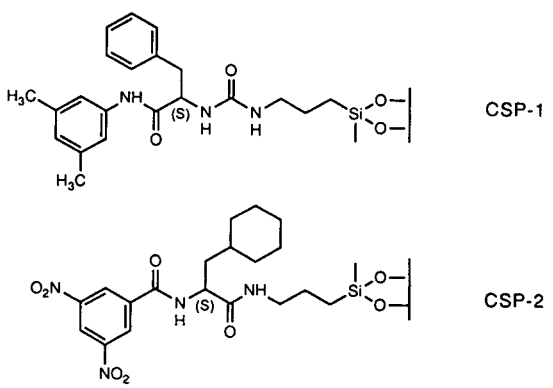

Fig. 3. Structures of stationary phases.

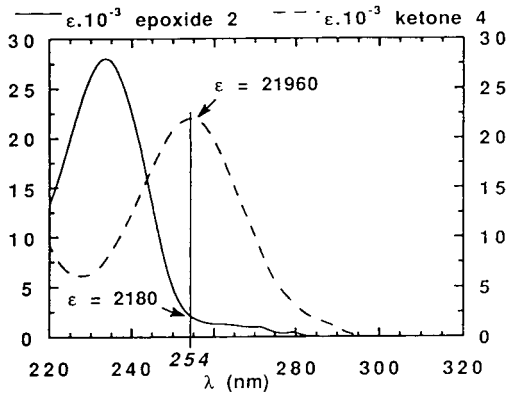

Fig. 4. UV spectra of **2** and **4** in *n*-heptane.

Thus, ketone **6**, with a high molar absorptivity, could produce important peaks in HPLC while simple UV detection is carried out.

CONCLUSIONS

Our errors show two specific examples in which a small quantity of impurity with a strong absorption in the UV range, contained in a product with a weak absorption in the UV range, could produce a peak as important as the principal product in HPLC coupled with UV detection.

Certainly, there are technical resources that provide protection against errors of interpretation in chiral liquid chromatography, such as the use of chiroptic detectors (polarimetric or circular dichroism detector) or multi-wavelength detectors (diode-array detectors) [4], but these are expensive and not available to a number of researchers. However, risks can be limited by carrying out the detection at the absorption maximum of the racemic compound. The spectra in Figs. 2 and 4 show that, in the present case, the surface of peaks belonging to compounds **1** and **2** should have been multiplied by a coefficient equal to the ratio of molar absorptivities of these compounds at the wavelength of their absorption maximum and at the wavelength at which detection was carried out, 254 nm (1.3 and 12.9, respectively), and those of the impurities **3** and **4** divided by 1.4 and 2.8 ($\varepsilon_{254\ nm}/\varepsilon_{\lambda_{max}}$), respectively, carrying out the UV detection at 261 nm for **1** and at 234 nm for **2**.

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Short Communication

Anion-exchange high-performance liquid chromatography with post-column detection for the analysis of phytic acid and other inositol phosphates

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ABSTRACT

The use of gradient anion-exchange HPLC, with a simple post-column detection system, is described for the separation of *myo*-inositol phosphates, including "phytic acid" (*myo*-inositol hexaphosphate). Hexa-, penta-, tetra-, tri- and diphosphate members of this homologous series are clearly resolved within 30 min. This method should facilitate analysis and quantitation of "phytic acid" and other inositol phosphates in plant, food, and soil samples.

INTRODUCTION

Inositol phosphates, currently of considerable interest to biochemists [1], have long been studied by soil scientists and food chemists because of the metal-chelating properties of *myo*-inositol hexaphosphate, also known as "phytic acid" [2,3]. A recent review by Xu *et al.* [4] summarizes the methods which have been used for analysis of these molecules in foods. Nutrition-related studies have indicated that it may be important to know relative amounts of *myo*-inositol hexaphosphate (IP6) *vs.* the penta-, tetra-, tri-, di- and monophosphate esters (IP5, IP4, IP3, IP2 and IP1, respectively), since even limited dephosphorylation of phytic acid can reduce its inhibitory effect on mineral absorption [5,6]. These researchers used ion-pair reversed-phase high-performance liquid chromatography (HPLC) to identify and quantitate IP6, IP5, IP4 and IP3. Ion-pair reversed-phase methods [7,8] give adequate separation of the above inositol phosphoesters (the mono- and diphosphates are not resolved). However, sample extracts must first be passed through anion-exchange resin to remove inorganic phosphate and concentrate the inositol phosphates. Acidic column effluent is then evaporated to dryness (to remove HCl) and reconstituted in water prior to injection onto a reversed-phase HPLC column. Direct analysis of sample extracts by anion-exchange HPLC would eliminate the need for these sample preparation steps.

Ion-exchange chromatography has long been used to fractionate and purify *myo*-inositol phosphoesters produced by the hydrolysis of phytic acid or its phytate salts [7,9,10]. To date, published procedures involving anion-exchange HPLC fall into two categories. Isocratic, ion-

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chromatographic techniques [11–13] do not resolve less-phosphorylated species as well as the ion-pair reversed-phase methods cited earlier. In contrast, the elegant, isomer-specific gradient elution protocols (with post-column detection) developed by Mayr [14] and Phillippy and Bland [15] can provide more information than what was needed for our purpose, which is to screen a variety of plant materials for phytic acid and its partially-hydrolyzed homologues. The method described in this paper affords a compromise, enabling one to clearly resolve and, potentially, quantitate the hexa-, penta-, tetra-, tri- and diphosphate esters of *myo*-inositol with minimal sample preparation and equipment.

EXPERIMENTAL

Materials

Sodium phytate (from corn) and phytase (crude; from wheat) were obtained from Sigma (St. Louis, MO, USA). (Moisture content of the former was 14.5% and this was taken into account when preparing solutions.) Pure *myo*-inositol phosphates used as chromatographic standards were the pentaphosphate from Calbiochem (San Diego, CA, USA); 3,4,5,6-tetraphosphate, 1,4,5-triphosphate, 1,4-biphosphate and 2-monophosphate from Sigma. (Isomeric composition of the phosphoesters was not important for our purposes, but is given here for the sake of completeness.) Also purchased from Sigma were ferric chloride hexahydrate and sulfosalicylic acid. Aldrich (Milwaukee, WI, USA) supplied 1-methylpiperazine (99%), and sodium nitrate (reagent grade) was from J.T. Baker (Phillipsburg, NJ, USA). Distilled, deionized water was used for the preparation of solutions.

Due to the expense of commercially-purified *myo*-inositol phosphoesters IP5, IP4, IP3, and IP2, sodium phytate (10 mg/ml) was hydrolyzed with phytase (1 mg/ml; 10:1 phytate:phytase) following the preparative enzymatic method of Phillippy *et al.* [10]. This produced a mixture of *myo*-inositol phosphoesters which was used for preliminary examination of chromatographic conditions. The exact composition of this mixture varied depending upon initial incubation time and age of the hydrolysate. (For example, IP-5 produced during a 24-h incubation was completely converted to lower phosphoesters during a two-month storage period.) Once optimum separation parameters had been established, the commercially-purified, individual *myo*-inositol phosphates described above were used to verify identity of each hydrolysate component.

Instrumentation

Chromatography was performed with a Varian 5000 liquid chromatograph (Varian, Palo Alto, CA, USA) equipped with a Model 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA). Post-column reagent was pumped with an Eldex Model A-60-S metering pump (Eldex, Menlo Park, CA, USA), and mixed with eluate from the analytical column via a Valco tee and a coil consisting of a 290 cm length of 0.76 mm I.D. polyether ether ketone (PEEK) tubing (1.3 ml volume). Detection was at 500 nm (0.1 AUFS) using an Isco V^4 absorbance detector (Isco, Lincoln, NE, USA). Check valves (SSI soft seat; Alltech, Deerfield, IL, USA) were installed prior to both inlet ports of the tee to prevent backflow of HPLC eluents into the low-pressure system or contamination of the analytical column with detection reagent. An externally adjustable pressure relief valve (Nupro "R3A" series; Indianapolis Valve and Fittings, Indianapolis, IN, USA) was installed in front of the reagent pump as a precaution against exceeding the pressure limit of this pump. An empty 5×0.41 cm column used to plumb this valve into the system also served as a pulse dampener. A diagram of the complete system is shown in Fig. 1.

Chromatography

Separation of the *myo*-inositol phosphates was achieved using a 5×0.46 cm I.D. polystyrene-based strong anion-exchange column (1000 Å PL-SAX; Polymer Labs., Amherst, MA, USA). (A PL guard cartridge system containing a 5×3 mm I.D. cartridge of PL-SAX packing was subsequently added to protect the analytical column during chromatography of plant extracts.) Optimum elution conditions were a

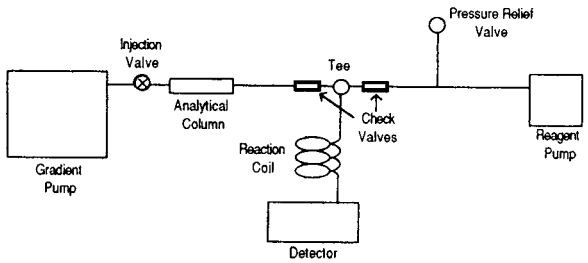

Fig. 1. Schematic diagram of HPLC/post-column detection system.

30-min linear gradient from 0.01 *M* 1-methylpiperazine (pH 4.0) to 0.5 *M* $NaNO_3$ in 0.01 *M* 1-methylpiperazine (pH 4.0) at a flow-rate of 1 ml/min. Effluent from the analytical column combined with a post-column reagent (also pumped at 1 ml/min) which consisted of 0.015% $FeCl_3 \cdot 6H_2O$ (w/v) plus 0.15% (w/v) sulfosalicylic acid [12]. The decrease in absorbance at 500 nm, as eluting phosphates complex with iron in the post-column reagent, was recorded as "positive" peaks by reversing polarity of the detector-chart recorder connections. Both HPLC mobile phase and post-column reagent solutions were filtered through 0.45-μm nylon 66 filters and degassed thoroughly prior to use. The post-column reagent, stored in an amber bottle at room temperature, is stable for several weeks.

Extraction procedure

Extraction of phytic acid and lower inositol phosphates from plant materials was based on the procedures of Cilliers and Van Niekerk [12] and others [4]. To 1.0 g of finely ground, dry sample in a 50-ml, screw-cap centrifuge tube (Nalgene 3119-0050; Nalge, Rochester, NY, USA) was added 10.0 ml of 0.5 *M* HCl. The tubes were shaked mechanically (*ca.* 150 rpm) for 2 h at room temperature. At the end of this time, the tubes were centrifuged for 20 min at 20 000 rpm (48 400 *g*). Supernatant was passed through a 0.2-μm porosity membrane filter (and stored at 4°C, if necessary) prior to injection.

RESULTS AND DISCUSSION

The HPLC column and mobile-phase conditions described in the preceeding section gave

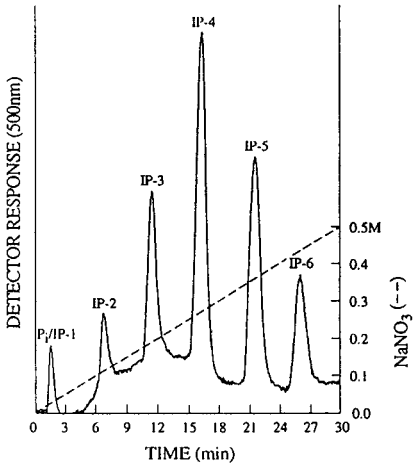

Fig. 2. Resolution of hexa-, penta-, tetra-, tri-, di-, and monophosphate esters of *myo*-inositol (IP6, IP5, IP4, IP3, IP2, and IP1, respectively.) (Inorganic phosphate, P_i, co-elutes with IP1.) A sample of hydrolyzed sodium phytate, spiked with pure IP5 and unhydrolyzed phytic acid, IP6, was chromatographed on a strong anion-exchange column (1000 Å PL-SAX; 5 × 0.46 cm). Elution conditions were a 30-min linear gradient from 0 to 0.50 *M* sodium nitrate in 0.01 *M* 1-methylpiperazine buffer (pH 4.0) at a flow-rate of 1 ml/min. "Detector response" denotes the change in absorbance at 500 nm as explained in Experimental.

good resolution of the hexa-, penta-, tetra-, tri- and diphosphates of *myo*-inositol (Fig. 2). Inorganic phosphate and *myo*-inositol monophosphate co-elute as non-retained species. Resolution of this homologous series of phosphate esters by gradient anion-exchange chromatography exceeds that achieved by isocratic ion chromatography [12] or ion-pair reversed-phase HPLC [7,8], with IP6 through IP2 all separated by at least 3 min. Maintaining the mobile phase at pH 4.0 insures that each phosphate moiety possesses only one negative charge, since six of the 12 replaceable protons in the phytic acid molecule are strongly dissociated ($pK_a \leq 2$) with the remainder being more weakly acidic ($pK_a \geq 5.7$) [2]. The use of a polystyrene-based strong anion-exchange column [16,17] prevents the potential problem of packing material deterioration which could be encountered during prolonged use of a silica-based column at this mobile-phase pH.

Minear *et al.* [18] also employed gradient elution (at pH 10) with a polymeric strong anion-

exchange column (25 × 0.4 cm I.D.) to achieve good resolution of *myo*-inositol phosphates, IP6–IP1. However, longer elution times (58 min for IP6) and off-line detection (fractions were collected and analyzed for phosphorous) are definite disadvantages of that procedure. The gradient ion-chromatographic method of Phillippy and Bland [15], which is potentially applicable to food samples, can resolve isomers of the various *myo*-inositol phosphoesters (*e.g.* all four pentaphosphates). However, the chromatographic information provided may be more appropriate to qualitative or kinetic studies than to quantitation of relative amounts of phytic acid *vs.* partially-hydrolyzed species regarding mineral bioavailability [5,6].

A standard curve for the quantitation of *myo*-inositol hexaphosphate, IP-6, was obtained by injecting various quantities of sodium phytate. The plot of peak area *vs.* amount injected (not shown) was linear from 2.5–100 μg ($r > 0.999$). Precision for replicate injection (10 μl; 100 μg load) of sodium phytate was *ca.* 1% for retention time and 2% for peak area (calculated as $h \times w_{1/2}$). A recovery study was performed with wheat flour that contained 0.1% IP-6 as determined by HPLC. An aliquot of concentrated sodium phytate solution was added to each dry sample so as to provide an addition of 1.0% phytic acid. Samples were then extracted and chromatographed as previously described. Recovery was 97 ± 1% ($n = 3$).

To date, the method described in this communication has been applied to extracts of cowpea seeds (Fig. 3) and leaves, rice grain, and taproots from four species of perennial forage legumes. (Other components of these samples did not interfere with detection, although the high sugar content of taproot extracts necessitated periodic cleaning of the column with 1 *M* sodium chloride.) Results of this work will be the subject of a future publication.

The detection method employed involves a ligand-exchange reaction between the iron(III)-sulfosalicylic acid complex and eluting inositol phosphates. It was first described by Wade and Morgan [19] for the detection of phosphate esters on paper chromatograms, and has subsequently been applied to both colorimetric [20]

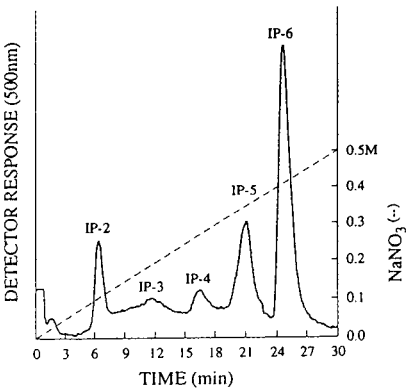

Fig. 3. Chromatogram of *myo*-inositol phosphates extracted from cowpea seed. An aliquot of 20 μl of acidic extract (see Experimental) was chromatographed using the column and elution conditions described in Fig. 2. (Peak identification abbreviations are the same as in Fig. 2.)

and chromatographic [12] methods for the determination of "phytate" or phytic acid, *i.e.* *myo*-inositol hexaphosphate. Although Cilliers and Van Niekerk [12] detected the lower phosphate esters via post-column reaction with this reagent, chromatographic resolution of these homologues was relatively poor, owing to isocratic elution.

In summary, the use of gradient anion-exchange HPLC in conjunction with post-column detection via "Wade reagent" offers several advantages over previously published procedures for the analysis of *myo*-inositol phosphates in non-physiological samples, *i.e.* where discrimination between phosphate isomers is not needed. Minimal preparation of samples is required —acidic sample extracts need only be centrifuged and filtered prior to injection. Resolution of the lower phosphoesters and peak shape of strongly-retained components is considerably better than that afforded by isocratic elution from an ion chromatography column. This HPLC protocol can be implemented in laboratories with a gradient pumping system and a variable-wavelength absorbance detector; all hardware needed to construct the post-column detection system is commercially available. The addition of an electronic integrator would facilitate routine analysis of large numbers of samples, but is not absolutely necessary. Given appropriate sample extraction procedures and

calibration curves, this chromatographic method should be applicable to the analysis and quantitation of homologous inositol phosphates in various plant, food and soil samples.

ACKNOWLEDGEMENTS

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Short Communication

Ion chromatography of polyphosphates and polycarboxylates using a naphthalenetrisulfonate eluent with indirect photometric and conductivity detection

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ABSTRACT

The potential of naphthalenetrisulfonate as a powerful displacing anionic eluent has been extended to the separation and detection of multivalent inorganic polyphosphates and organic polycarboxylates using a low-capacity polystyrene–divinylbenzene ion-exchange column. Four polyphospates (pyro-, trimeta-, tripoly- and tetrapolyphosphate) and three polycarboxylates (nitrilotriacetate, ethylenediaminetetraacetate and citrate) can be separated in about 20 min with indirect photometric and conductivity detection. Indirect photometric detection was found to be superior to conductivity detection because early-eluting anions are well resolved from the injection peak and flow programming can be done with a minimum disturbance of the baseline. Detection limits for the analytes investigated range from 2 to 100 ng which are at least an order of magnitude better than those previously reported using postcolumn derivatization methods. Linear response ranges from 500 or 75 mg/l to the limit of detection for longer and shorter retained analytes, respectively.

INTRODUCTION

Polyphosphates and polycarboxylates have long been of interest to chemists because of their multifunctional characteristics [1]. They act as color preservatives and food stabilizers and therefore are of considerable significance to the food and beverage industry. Polyphosphates, in particular, are also used as sequestering agents in various detergent formulations.

A variety of analytical methods for the determination of these anions have been explored. Some of these for carboxylic acids were gas chromatography after ester derivatization [2,3] and polarography [4]. An ion-selective electrode method has been reported [5] for the determination of polyphosphates. These methods are relatively slow and/or can give erroneous results in quantitation. A better approach is indirect flow injection analysis of polyphosphates based on their complexing ability of methylthymol blue derived from the corresponding magnesium complex [6]. For polyphosphates mixtures, gradient ion chromatography using a sodium hydroxide eluent with suppressed conductivity detection [7] has been effective.

Ion chromatography with postcolumn derivatization has been used for the determination of both polyphosphates and polycarboxylates [8];

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however, this method is more commonly applied to just the former class of compounds [9–15]. After either isocratic [11] or gradient [12,14] separation, ortho (P_1)-, pyro (P_2)-, tripoly (P_3)- and tetrapoly (P_4)-phosphate were hydrolyzed to orthophosphate and reacted with molybdate to form a molybdenum blue complex which is then detected photometrically at 830 nm. A variation of this scheme is to add molybdovanadate to the formed orthophosphate for subsequent product detection at 340 nm [13]. A mixture of P_2- and P_3-phosphate was separated in 12 min using iron(III) nitrate as a derivatizing agent but no detection limits were determined [15]. Similar approaches for the determination of P_1–P_4 polyphosphates using a low-capacity ion-exchange column with a nitric acid gradient and post-column detection with iron(III) perchlorate at 320 nm can be found in the manufacturer's literature. Ion-exchange chromatography in conjunction with phosphorous-selective detection by atomic emission spectroscopy has also been used [16,17] for the determination of polyphosphates. Chester and Smith [18] have also reported an ion-interaction chromatographic method for polyphosphates coupled to a flame photometric phosphorous-selective detector. Thus, in numerous cases, somewhat complicated postcolumn reaction methods have been adopted for these phosphorous compounds.

Naphthalenedisulfonate (NDS) and 1,3,6-naphthalenetrisulfonate (NTS) have recently been applied as pH-independent mobile phases for non-suppressed ion-exchange chromatography in which ions are separated on a polymethacrylate ion-exchange column and detected by using conductivity and indirect photometric detection (IPD). This technique has shown to offer a great deal of promise for the simple, rapid and sensitive determination of inorganic and organic anions [19,20] as well as the highly retained sulfur oxide anions [21]. Aliphatic sulfonate and sulfate surfactants have also been separated on mixed-mode reversed-phase ion-exchange columns using NDS as the eluent with IPD and conductivity detection [22,23].

The purpose of this report is to describe an analogous study for the separation and detection of multivalent polyphosphates such as P_2-, P_3-, P_4- and trimeta-phosphate as well as polycarboxylates such as nitrilotriacetate (NTA), ethylenediaminetetraacetate (EDTA) and citrate in which no chemical modification of the analyte is necessary. Using NTS as the eluent and a polystyrene–divinylbenzene (PS–DVB)-based ion-exchange column, these multivalent anions can be separated in about 20 min and then detected by IPD or non-suppressed conductivity detection. These detection methods have not been previously explored in detail for polyphosphates [24,25]. Using sodium trimesate, only P_2- and P_3-phosphates have been separated by ion-exchange chromatography with IPD [26,27]. The technique reported here provided limits of detection in the nanogram range which are at least an order of magnitude better than the most popularly used postcolumn derivatization methods.

EXPERIMENTAL

Reagents

All reagent-grade chemicals were used without further purification. NTS salt was purchased from American Tokyo Kasei (Portland, OR, USA). A 10 m*M* stock solution of mobile phase was prepared and used after further dilution with 5% acetonitrile (ACN). This percentage of ACN was found to be effective in reducing any possible lipophilic interaction between the PS–DVB stationary phase and the naphthalene moiety of the mobile phase [28]. The disodium salts of EDTA and NTA, as well as the trisodium salt of citrate were of analytical-reagent grade and obtained from Fisher Scientific (Fairlawn, NJ, USA). Tetrasodium P_2-phosphate, hexaammonium P_4-phosphate (both analytical-reagent grade), trisodium trimetaphosphate and pentasodium P_3-phosphate, (both technical grade), were all purchased from Sigma (St. Louis, MO, USA). The stock solutions (1000 mg/l) of the analytes of interest were prepared in the mobile phase and then diluted with doubly distilled deionized water for further use.

Instrumentation

The liquid chromatograph consisted of a Model 510 HPLC pump, a Model MU6K injector with 20-μl sample loop, a Model 441 fixed-

wavelength detector and a Model 430 conductivity detector, all from the Waters Chromatography Division of Millipore (Milford, MA, USA). A Model LP-21 Lo-Pulse dampener from Scientific System (State College, PA, USA) was connected between the injector and pump outlet to eliminate the noisy baseline particularly obvious with conductivity detection [20]. The analyte anions were separated using a Hamilton PRP-X100 low-capacity (0.20 mequiv./g) anion-exchange column (150 × 4.1 mm I.D.) while in some initial experiments a Hamilton PRP-X500 high-capacity (1.60 mequiv./g) anion-exchange polyether ether ketone (PEEK; 50 × 4.6 mm I.D.) column was used. Both conductivity and UV detector outputs were simultaneously displayed on two Model 1500 Fisher Recordall Chart recorders (Austin, TX, USA).

RESULTS AND DISCUSSION

Naphthalene sulfonate derivatives such as NDS and NTS were compared in a preliminary study using the PRP-X100 anion-exchange column. Although NDS can separate a mixture of polycarboxylates containing NTA, EDTA and citrate in 12 min, we choose to pursue work with NTS as this eluent seemed potentially more useful for the separation of mixtures of both polycarboxylates and polyphosphates in a shorter time with lower detection limits . In particular, the greater charge on NTS provided sharper peaks and shorter retention times for the elution of P_3-phosphate, P_4-phosphate and citrate.

Capacity factors (k') of polyphosphates such as P_2-, trimeta-, P_3- and P_4-phosphates and various polycarboxylates such as NTA, EDTA and citrate as a function of NTS concentration using the PRP-X100 column are shown in Fig. 1a and b, respectively. The capacity factors for polyphosphates (P_2-, trimeta- and P_3-phosphate) range from 0.3 to 27, with NTS concentration changing from 0.05 to 0.30 mM, while for polycarboxylates k' ranges from only 0.5 to 2.5 with the same range of NTS concentration. Interestingly, using the high-capacity hydrophilic PRP-X500 column, the retention order of trimeta- and P3-phosphate are reversed compared to that of the PRP-X100 column. However,

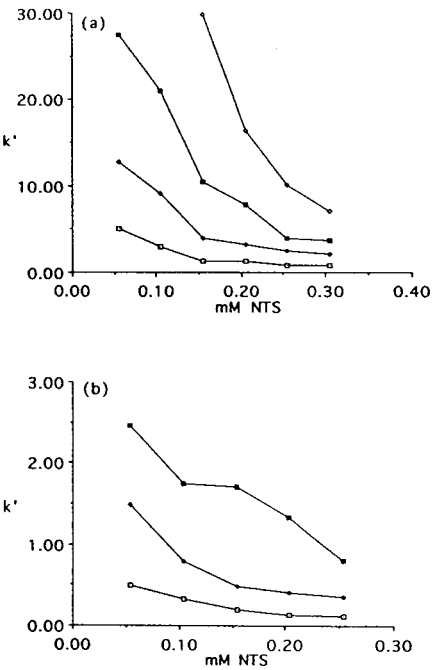

Fig. 1. Retention of polyphosphates (a) and polycarboxylates (b) as a function of eluent concentration using the PRP-X100 column. All mobile phases contained 5% acetonitrile. Flow-rate 1 ml/min for all analytes except for P_4-phosphate (2 ml/min). Analyte concentration 10 mg/l each of (□) P_2-, (◆) trimeta-, (■) P_3- and (◇) P_4-phosphate (a) and 20 mg/l each of (□) NTA, (◆) EDTA and (■) citrate (b).

peaks were broad using the PRP-X500 column and better resolution and detectability were clearly evident in the chromatograms generated using the PRP-X100 column. All future work was carried out on this column. In general, mobile phase concentrations of 0.2 and 0.1 mM NTS were best for the resolution of polyphosphates and polycarboxylates, respectively.

Experimental data relating log k' *versus* log [eluent] were obtained and plots were prepared using the equation $\log k' = -(y/x) \log E + \log B$, where y is the charge of the analyte anion, x is the charge of the eluent, E is the eluent concentration and B is a constant dependent on ion-exchange equilibria and resin capacity [26]. Using linear regression statistical analysis, correlation coefficients of 0.98 or higher were noted for all the polyphosphate anion plots. The observed values for P_2-, P_3- and P_4-phosphate were found to be −1.55, −1.38 and −2.14, respective-

ly which were considerably higher than the values −0.66, −1.00 and −1.33 predicted from theory. The theoretical values were obtained by calculating the predominant charge on the P_2-, P_3- and P_4-phosphate anions which were −2.0, −3.0 and −4.0, respectively, at a mobile phase pH of 5.5. One explanation for the high positive deviation of the observed slope values is that the interaction between the total charge of the analytes or NTS and the ion-exchange groups of the resin is not stoichiometric.

In non-suppressed ion chromatography, using any indirect detection mode, sensitivity is dependent on flow-rate as well as concentration of the eluent. Furthermore, the measured sensitivity using a flow-rate of 1 ml/min at an optimized mobile phase concentration of 0.20 m*M* NTS is about twice as high than that at 2.2 ml/min. As a compromise between resolution and analysis time, we have chosen flow programming for the separation of P_2-, trimeta-, P_3- and P_4-phosphate with 0.2 m*M* NTS and IPD. At a constant 2.2 ml/min, the P_2-phosphate peak overlaps with the injection peak but P_4-phosphate elutes in only 20 min (Fig. 2a). A change in flow-rate from 1 to 2 ml/min at the arrow effectively increased the retention time of P_4-phosphate to 24 min (Fig. 2b) with better retention of P_2-phosphate. However, an analogous separation with conductivity detection was more problematic. First, highly retained anions such as P_3- and P_4-phosphates produce very intense injection peaks which will obscure the P_2-phosphate anion peak. Secondly, as previously noted [20], conductivity detection causes a pronounced conductivity baseline disturbance to occur with any change in flow-rate. Therefore, a standard mixture of polyphosphates containing only P_2-, trimeta- and P_3-phosphate could be determined with conductivity detection as shown in Fig. 2c. The peak direction in conductivity detection depends on the relative ionic equivalent conductance of the eluent and the solute anions. In this case, P_2- and trimetaphosphate have higher equivalent conductance so they appear as positive peaks while the P_3-phosphate anion with a lower equivalent conductance than NTS produces a negative peak. The direction of conductivity signal is also dependent on the solute concentration of some

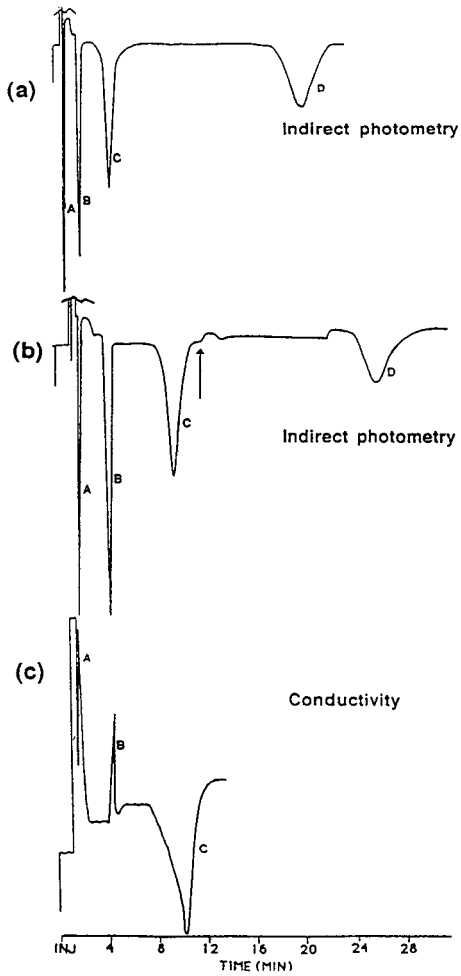

Fig. 2. Separation using the PRP-X100 column of a standard mixture of polyphosphates with (a) IPD at 280 nm, 0.05 AUFS, flow-rate 2.2 ml/min. (b) Similar conditions but flow-rate initially 1 ml/min, then changed to 2 ml/min at the arrow. (c) Conductivity detection (0.2 μS FS), flow-rate 1 ml/min. Mobile phase: 0.20 m*M* NTS–ACN (95:5), Peaks: 15 mg/l each of (A) P_2- and (B) trimetaphosphate, and 25 mg/l each of (C) P_3- and (D) P_4-phosphate.

analytes. When the concentration of P_4-phosphate was 100 ppm, a positive signal was noted while an analyte concentration below 50 ppm caused the peak to become negative.

Fig. 3 shows the separation of polycarboxylates in about 6 min with both IPD and indirect conductivity detection. The chromatogram shows no interference from the injection peak and all analyte peaks are well resolved. However, NTA

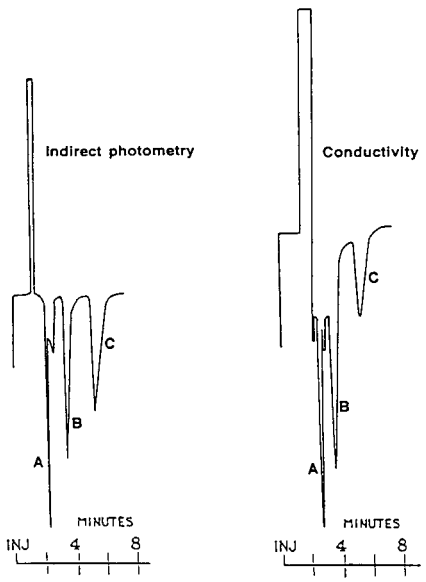

Fig. 3. Separation of a standard mixture of polycarboxylates on the PRP-X100 column with IPD (280 nm, 0.05 AUFS) and conductivity detection (0.2 μS FS). Mobile phase: 0.1 m*M* NTS–ACN (95:5), flow-rate 1 ml/min. Peaks: (A) 10 mg/l of NTA, 25 mg/l each of (B) EDTA and (C) citrate.

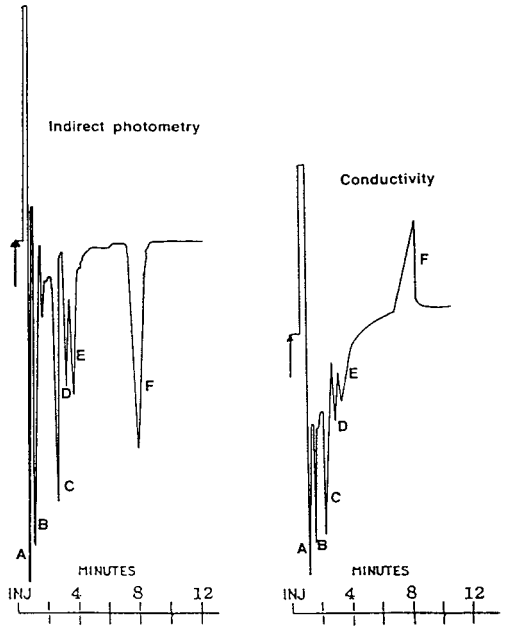

Fig. 4. Separation of a standard mixture of polyphosphates and polycarboxylates on the PRP-X100 column with IPD (280 nm, 0.05 AUFS) and conductivity detection (0.1 μS FS). Other conditions as in Fig. 3 except that the flow-rate was 1.5 ml/min. Peaks: 10 mg/l each of (A) P_1-phosphate and (B) NTA, (C) 25 mg/l of EDTA, (D) 10 mg/l of P_2-phosphate, (E) 20 mg/l of citrate, (F) 15 mg/l of trimetaphosphate.

did show a side impurity peak which was not identified. Using less competitive mobile phases than NTS in an ion-exchange mode, citrate can be retained quite long [24]. Fig. 4 shows a comparison between two chromatograms for the separation of a mixture of polyphosphates and polycarboxylates using IPD and conductivity detection. The order of elution was consistent with the trends in which retention is directly proportional to the effective charge, ionic radius and polarizability and inversely proportional to hydration energy [24,25]. At this mobile phase concentration of 0.1 m*M* NTS, the P_1-phosphate peak overlapped slightly with the injection peak. This anion can be easily separated using NDS as the mobile phase. Raising the mobile phase concentration to 0.15 m*M* NTS and using flow programming, a separation of a mixture of polyphosphates and polycarboxylates which now included P_3-phosphate instead of P_1-phosphate was possible with IPD (Fig. 5). Again, conductivity detection was troublesome because the P_2-phosphate peak becomes positive at this NTS concentration and more baseline disturbance was observed resulting in some distortion of the chromatogram.

A comparison of the detection limits of various anions using NTS as the mobile phase with both IPD and conductivity detection modes is shown in Table I. The detection limits of NTA and EDTA are the same with both detection modes. However, the citrate detection limit using conductivity detection is 40% lower than that for IPD. Conversely, the trimetaphosphate detection limit using IPD is 50% better than that for conductivity detection. These differences in detection limits are due to the fact that using a higher mobile phase concentration of 0.2 m*M* NTS for the determination of trimetaphosphate results in higher background conductance of 64 μS/cm, as opposed to only 32 μS/cm when a lower concentration of 0.1 m*M* NTS was used for citrate. P_2-Phosphate and P_3-phosphate gave essentially the same detection limits using both

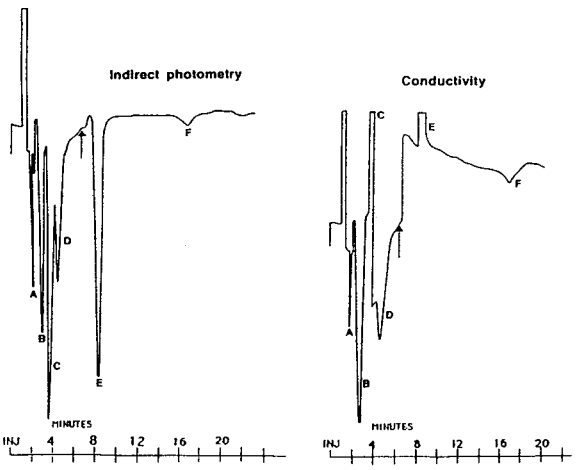

Fig. 5. Flow gradient for the separation of a standard mixture of polyphosphate and polycarboxylates on the PRP-X100 column with IPD (280 nm 0.05 AUFS) and conductivity detection (0.1 μS FS). Flow-rate of 1 ml/min changes to 2 ml/min at the arrow. Mobile phase: 0.15 m*M* NTS–ACN (95:5). Peaks: (A) 10 mg/l of NTA, 20 mg/l each of (B) EDTA, (C) P_2-phosphate, (D) citrate and (E) trimetaphosphate, (F) 10 mg/l of P_3-phosphate.

detection methods. The main reason for the higher detection limit for P_2-phosphate is the interference with the injection peak which increases in size with sample dilution. The detection limit of P_2-phosphate can be improved with a lower mobile phase concentration which causes no interference from the injection peak. P_4-Phosphate could be determined better by IPD rather than conductivity detection. This result is not surprising as P_4-phosphate is a much larger anion and has a lower ionic mobility resulting in lower conductance as compared to other members of the polyphosphate series. The detection limits obtained in our work range from 2 to 100 ng for polyphosphates and from 4 to 10 ng for polycarboxylates. Both ranges are at least an order of magnitude lower than those previously reported using other ion chromatography systems. In ion-interaction chromatography employing benzenetricarboxylic acids with indirect UV detection at 257 nm, detection limits of 60 and 100 ng for EDTA and citrate, respectively, were cited [29]. Detection limits for polyphosphates using the iron(III) postcolumn derivatization method are only in the 1–5 μg range [30].

TABLE I

DETECTION LIMITS COMPARISON FOR POLYPHOSPHATES AND POLYCARBOXYLATES ANIONS USING INDIRECT PHOTOMETRIC AND CONDUCTIVITY DETECTION MODES

Mobile phase 0.1 m*M* NTS–ACN (95:5) for nitrilotriacetate through citrate and 0.2 m*M* NTS–ACN (95:5) for pyrophosphate through tetrapolyphosphate. Detection limits: $S/N > 3$ based on peak height. Injection volume 20 μl.

Analyte	Detection limits, mg/l (ng)	
	IPD	Conductivity
Nitrilotriacetate	0.50 (10)	0.50 (10)
EDTA	0.25 (5)	0.25 (5)
Citrate	0.50 (10)	0.20 (4)
Pyrophosphate	1.0 (20)	1.25 (25)
Trimetaphosphate	0.10 (2)	0.20 (4)
Tripolyphosphate	1.0 (20)	1.0 (20)
Tetrapolyphosphate	2.5 (50)	5.0 (100)

Calibration plots starting from the detection limits for some of these polyvalent anions using NTS as the mobile phase with both detection modes are shown in Fig. 6. The average relative standard deviations of the slope of the calibration graphs were 1.0 and 0.9% for IPD and conductivity detection, respectively. The correlation coefficient varied from 0.9994 to 0.9999 for both detection methods. Reproducibility of three replicate injection of most analytes ranged from 1.1 to 1.8% R.S.D. As can be seen for all the analytes except for P_4-phosphate, the sensitivity (slope of the calibration line) obtained with conductivity detection is higher than that obtained with IPD. Fig. 6a shows the calibration runs for P_2-phosphate and trimetaphosphate. Their slope values are 16 and 2.5 times higher with conductivity detection than those by IPD, respectively. The upper limit of linearity of the P_2-phosphate anion (25 or 10 mg/l) is relatively short because of the interference with the injection peak which increases in size with solute concentration. The trimetaphosphate ion linear range extended to 75 and 50 mg/l with IPD and conductivity detection, respectively. Citrate showed a similar linear response to that of trimetaphosphate from the detection limit up to 75 mg/l. Similarly, the P_3-phosphate slope with conductivity detection is 7.6 times higher than

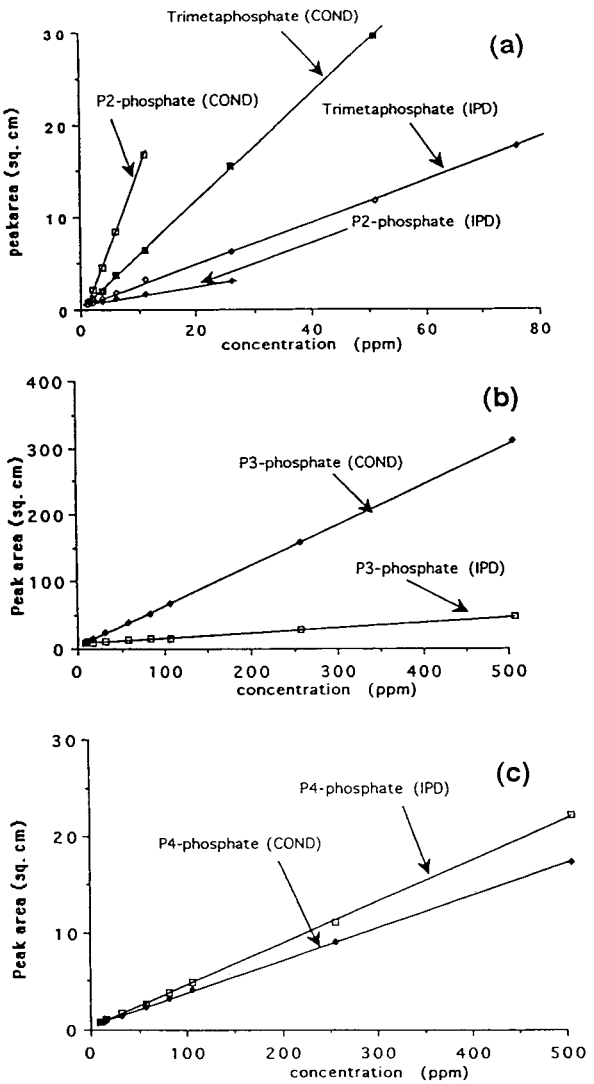

Fig. 6. Linearity comparison for (a) P_2-phosphate and trimetaphosphate, (b) P_3-phosphate and (c) P_4-phosphate using 0.2 m*M* NTS–ACN (95:5) as the mobile phase, with both IPD and conductivity detection.

that for IPD (Fig. 6b). On the other hand, the slope for P_4-phosphate is about 1.8 times higher for IPD rather than conductivity detection (Fig. 6c). Late eluting anions such as P_3- and P_4-phosphates have a much higher upper limit of quantitation with a dynamic concentration range to 500 mg/l.

Real samples such as citrate in diet Coke and P_3-phosphate in detergent can also be analyzed in less than 10 min using a NTS eluent. To expediate the IPD assay of citrate, the caffeine in Coke was removed by passing the sample through a disposable solid-phase C_{18} cartridge in tandem with a disposable 0.45-μm filter in a single operation. Caffeine did not interfere with the conductivity detection of citrate. No sample clean-up was done for the detergent sample.

The results obtained in this work are consistent with a previous comparison study of inorganic anions using naphthalenesulfonate mobile phases with both IPD and conductivity detection [20]. The low detection limits obtained in our work using both IPD and conductivity detection can be attributed to a number of useful properties associated with the use of a NTS eluent. Some of these include: (i) the large effective charge of NTS results in good elution capability of polyphosphates and polycarboxylates, (ii) high molar absorptivity and detection at a longer wavelength permits good IPD and (iii) lower background conductance allows non-suppressed conductivity detection to be more sensitive than a pH dependent eluent.

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Short Communication

High-performance liquid chromatography of the alkaloid perivine from *Catharanthus roseus* after derivatisation with dansyl chloride

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ABSTRACT

The Madagascar periwinkle *Catharanthus roseus* produces an unusually large number of alkaloids which are often difficult to analyse because of their similar physical properties and because of their multiplicity. The majority of these "Vinca alkaloids" contain only indolyl and tertiary nitrogen substituents which are not amenable to derivatisation. However, we found that the 2-acylindole alkaloid perivine does react with dansyl chloride to give a stable, fluorescent derivative with good chromatographic properties. Here we report a reversed-phase high-performance liquid chromatography procedure to selectively detect perivine as its dansyl derivative in the presence of all other co-extractable Vinca alkaloids. The method is applied to detect perivine in different tissues of *C. roseus*, and in *Agrobacterium*-transformed cultures of *C. roseus*.

INTRODUCTION

Catharanthus roseus G. Don. (Madagascar periwinkle) produces more than one hundred

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different indole alkaloids (reviewed in ref. 1) the so-called Vinca alkaloids, two of which, vincristine and vinblastine, are important in the treatment of leukemia [2]. Analysis of these alkaloids from plant extracts can be a formidable problem because of their large number and the very different amounts of each component [3,4]. Thin-layer chromatography utilizing ceric ammonium sulphate spray reagent and UV detection has been used with some success [3], as has reversed-phase HPLC with UV [4–6] or MS [7] detection, and supercritical fluid chromatography with MS detection [8]. However, none of these methods are selective, and the last at least requires rather specialised equipment.

The reaction of dansyl chloride with amino compounds gives highly fluorescent derivatives with good chromatographic properties [9] which are stable enough to allow subsequent spectroscopic analysis. This has been used previously to isolate a novel alkaloid, hydroxynorcytisine, from *Laburnum anagyroides* [10], and to assay cephaeline, emetine, ephedrine and morphine [11,12]. However, many Vinca alkaloids contain only tertiary amino groups which do not react with dansyl chloride. An exception is perivine [13], a Vinca alkaloid which contains an imino group that can be derivatised (Fig. 1). Pre-column dansylation of crude methanolic extracts from *C. roseus* and subsequent separation by reversed-phase high-performance liquid chromatography (HPLC) allowed selective detection of perivine by fluorescence in the presence of all co-extracted alkaloids.

Unlike most other alkaloids from *Catharanthus roseus*, perivine is localised in the leaf tissue [1]. Here, a further study of the tissue-specific location of perivine has been made using the described dansylation method. Furthermore, various *Agrobacterium*-transformed cultures derived from *C. roseus* leaf tissue were established, from a variety of diverse *Agrobacterium* strains, and an application of the method to determine their perivine content is described.

EXPERIMENTAL

Materials

Reagent grade chemicals were from Sigma (Poole, UK). HPLC-grade methanol (Romil, Leicester, UK) was used. Glass-distilled water was used throughout. An authentic sample of perivine was a gift from Professor N.R. Farnsworth.

Sources of plant material

Seeds of *C. roseus* cv. Magic Carpet were obtained from Thompson and Morgan (Ipswich,

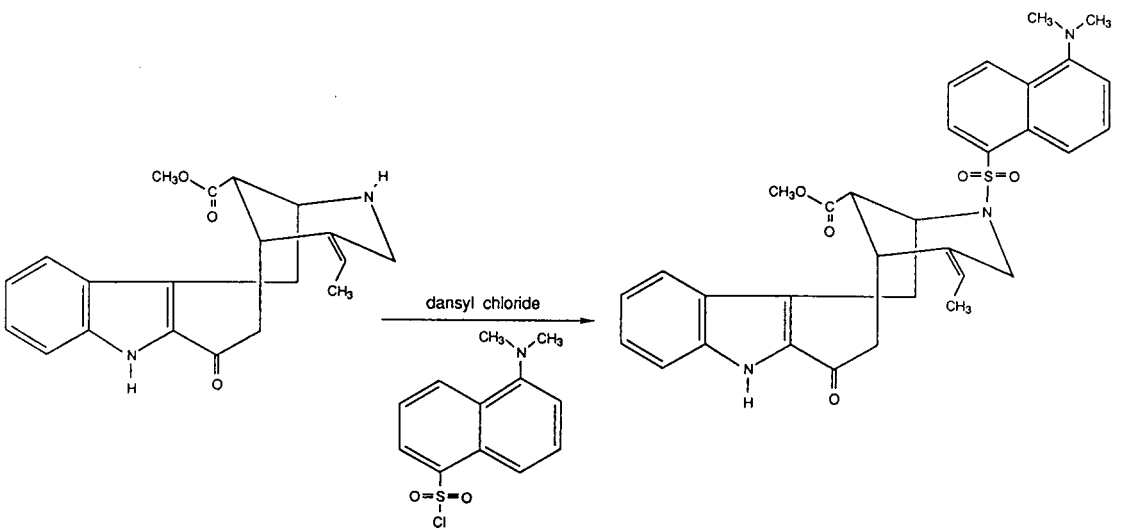

Fig. 1. Reaction scheme for the N-derivatisation of the Vinca alkaloid perivine with dansyl chloride.

UK) and mature plants were grown under conventional greenhouse conditions. Untransformed callus tissue was cultured on Murashige and Skoog [14] medium solidified with 0.7% agar (M–S agar) and supplemented with indole acetic acid (10 μg/ml) and kinetin (0.1 μg/ml). Mature plants were infected with different strains of *Agrobacterium tumefaciens* by wounding with an infected needle. Crown gall tumours developed after four weeks and were excised. Bacteria-free callus cultures were established on phytohormone-free M–S agar containing vancomycin (100 μg/ml) and carbenicillin (100 μg/ml). Phytohormone-free growth and opine production were taken as evidence of transformation [15]. Bacteria-free *Agrobacterium rhizogenes* strain LBA 9402 transformed root cultures of *C. roseus* were maintained in liquid culture in half strength Gamborg B5 medium [16].

Extraction and derivatisation of the basic nitrogenous fraction

The basic nitrogenous fraction was recovered from fresh tissue (<2 g fresh mass) by extraction with methanol–aqueous 0.3 *M* HCl (70:30, v/v; 5 ml) in a Potter homogeniser. After filtration, the supernatant was concentrated by evaporation under vacuum. Aliquots equivalent to 0.1 g fresh mass of tissue were redissolved in water (0.2 ml) and mixed with a solution of dansyl chloride in acetone (0.8 ml, 5 mg/ml). After saturating with solid sodium hydrogencarbonate, the mixture was left to react in the dark at room temperature for 15 h. Excess acetone was removed by warming to 60°C for 10 min, and the mixture was diluted to 1 ml with water. Dansylated derivatives were extracted by vortex mixing with toluene (20 s; 3 × 2 ml). The phases were separated by centrifugation (2700 g, 10 min) and the toluene fractions carefully removed, combined, and evaporated to dryness under a stream of air. At all stages exposure to light was kept to a minimum. Amine standards (20 nmoles) were derivatised by the same procedure. Dansylated residues were redissolved in methanol (500 μl) and centrifuged (9000 g; 4 min) prior to HPLC analysis. The HPLC autoinjector made 30 μl injections of this dilution, equivalent to 60 mg fresh mass of plant tissue.

High-performance liquid chromatography

The HPLC procedure was essentially as described previously [17]. The instrumentation consisted of a Gilson 401 diluter and 231 autoinjector (Gilson, Villiers-le-Bel, France), a Rheodyne Model 7010 injection valve fitted with a 50-μl loop (Rheodyne, Cotati, CA, USA), and two LDC Constametric IIIG pumps (Milton Roy, Riviera Beach, FL, USA). A Brownlee Spheri-5 RP18 reversed-phase column (250 × 4.6 mm, 5 μm particle size) was used (Brownlee Labs., Santa Clara, CA, USA), protected by a Brownlee RP18 guard column (30 × 4.6 mm). Detection was accomplished with an inline Perkin-Elmer (Beaconsfield, UK) LS4 fluorescence spectrophotometer (3-μl cell) set at 340 nm excitation and 540 nm emission. A BBC Goerz Metrawatt SE130 dual pen recorder set at 0.5 cm/min monitored output.

A two step methanol–water solvent gradient was used, consisting of linear sections 60–67% methanol (15 min) and 67–95% methanol (39 min). A 10-min period was allowed to re-equilibrate the system to 60% methanol. Flow-rate was kept constant at 1.0 ml/min.

RESULTS AND DISCUSSION

Acidified methanolic extracts from *C. roseus* leaf tissue were derivatised with dansyl chloride to screen for alkaloids containing primary or secondary amino functions. Non-polar derivatives were separated from the hydrolysis product sodium dansyl sulphonate by toluene extraction and analysed by reversed-phase HPLC (Fig. 2). By this procedure it was possible to detect specifically just one alkaloid, perivine, as its dansylated derivative. A number of biogenic amine derivatives also extracted under these conditions, but in spiking experiments dansyl-perivine did not co-chromatograph with any of forty dansylated amine standards used for comparison [17,18], either by HPLC or by two-dimensional TLC.

An authenticated sample of pure perivine was also dansylated and separated by reversed-phase HPLC and gave a major peak attributable to dansyl-perivine (t_R = 42 min) plus minor peaks representing dansyl-ammonia, dansyl-methyl-

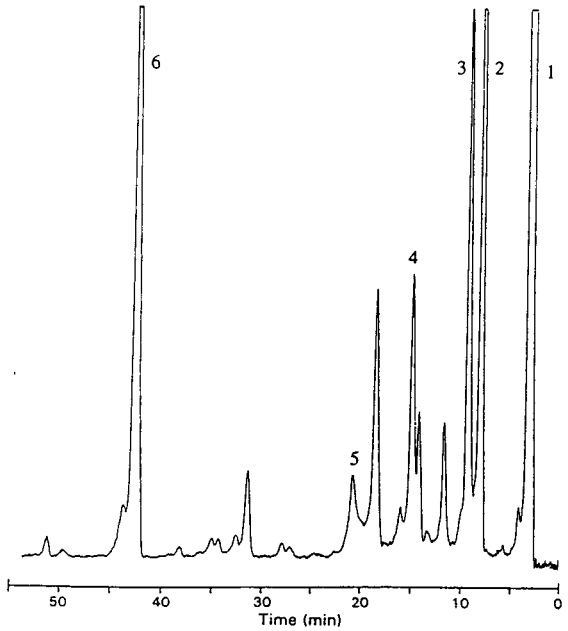

Fig. 2. Reversed-phase HPLC profile of dansylated derivatives recovered from leaf tissue of *Catharanthus roseus*. Injection volume (30 μl) was equivalent to 60 mg fresh mass. Extraction, derivatisation and HPLC conditions are described in the Experimental section. Peaks: 1 = dansyl sulphonate (3.0 min); 2 = dansyl-ammonia (7.9 min); 3 = dansyl-ethanolamine (9.3 min); 4 = dansyl-methylamine (15.0 min); 5 = dansyl-dimethylamine (20.2 min); 6 = dansyl-perivine (42.5 min).

amine, dansyl-dimethylamine, dansyl sulphonate, and excess dansyl chloride. All these minor components arise as by-products of the dansylation reaction [9], and none obscured the dansyl-perivine peak. Only one peak was observed for dansyl-perivine itself indicating that only one major derivative is formed (Fig. 1). At the chosen reaction conditions dansylation does not occur on hydroxy groups or on the indolic nitrogen [17]. This suggested that the perivine was mono-N-dansylated, and this was confirmed by NMR spectroscopy (data not shown).

Phenolics and thiols can also interfere with the dansylation reaction but the derivatives tend to fluoresce at longer wavelengths [9]. On TLC plates dansylated phenolic spots appear orange under 265 nm UV light, whereas dansylated amines and the dansyl-perivine derivative exhibited green fluorescence. Thus, further selectivity was achieved by using a HPLC fluorescence detector optimised to detect dansylated amino derivatives [18].

The major alkaloids in *C. roseus* leaf tissue are catharanthine, vindoline, and 3′,4′-anhydrovinblastine [8], while perivine is present at only about 3–4% of the total alkaloids [16]. Dansylation of perivine renders it less polar, allowing selective isolation from other, underivatised alkaloids by solvent extraction into toluene. HPLC analysis of the spent aqueous phase after solvent extraction suggested that the recovery was better than 90% for this step. Large-scale extraction of perivine from leaf tissue affords recoveries of about 80 μg/g fresh mass [19]. Assuming that the recovery of dansyl-perivine is similar to that of dansylated amines, *i.e.* generally more than 90% [17], this indicates that dansyl perivine can easily be detected in the low μg range. The injection in Fig. 2 (=60 mg fresh mass of leaf tissue), for example, was equivalent to about 5 μg of dansyl-perivine, and suggests that much greater sensitivity is possible. This compares favourably with detection limits of Vinca alkaloids by the ceric ammonium sulphate method [3]. Furthermore, 2-acylindole alkaloids such as perivine and periformyline do not react at all with this reagent [20]. Other workers [4–6] have not reported minimum detection limits.

Location of perivine in C. roseus tissues

Perivine is generally considered to be confined to leaves of *C. roseus* and our method confirmed that the highest concentrations were indeed in leaf tissue. However, we also analysed isolated roots, stems, and flower petals by the same methodology and detected perivine in all three tissue types (Fig. 3), albeit at lower concentrations. Assuming similar recoveries for the different tissues the results indicate that perivine concentration is approximately 10-fold higher in leaves than in other parts of the plant.

Agrobacterium species are plant pathogenic bacteria and convenient tools for plant genetic engineering. Wild type *A. tumefaciens* induces crown galls and *A. rhizogenes* adventitious hairy roots on infected plants [21], and they often have a profound effect on plant secondary metabolites [16]. HPLC–MS analysis of pharmaceutically

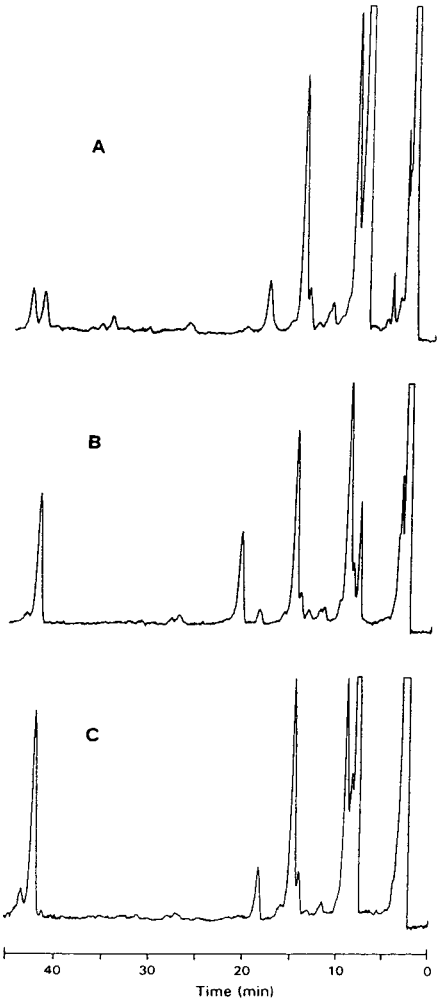

Fig. 3. Location of perivine in *C. roseus* tissues by HPLC after dansylation. (A) Root tissue; (B) flower petals; (C) stem tissue. In each case the injection volume was equivalent to 60 mg fresh mass. The peak attributable to dansyl-perivine is evident at 42 min. Other peaks were assigned as in Fig. 2.

active alkaloids from *Agrobacterium*-transformed *C. roseus* has been investigated previously [7,16] and, as found with other secondary metabolites [22] transformation was shown to generally increase alkaloid biosynthesis.

To investigate further the effect of tissue morphology on endogenous perivine levels the dansylation method was used to detect perivine in hairy root or crown gall cultures originally derived from *C. roseus* leaf tissue. The HPLC trace of the dansylated extract from a *C. roseus–A. rhizogenes* LBA 9402 hairy root culture is shown as an example (Fig. 4).

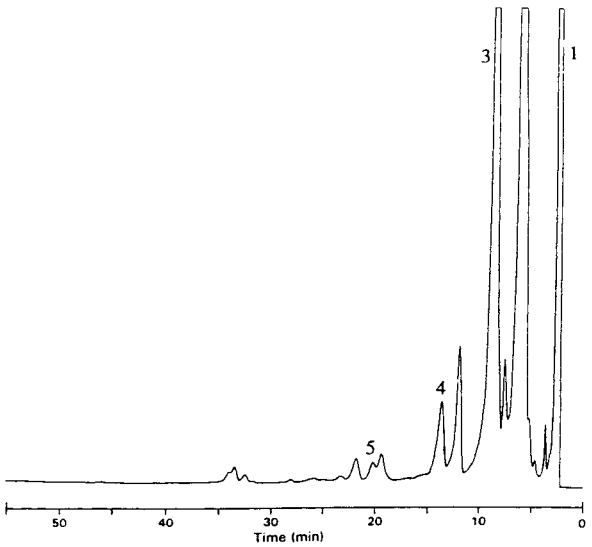

Fig. 4. HPLC chromatograph of the dansylated extract from a hairy root culture (injection = 60 mg fresh mass) incited on *C. roseus* leaf tissue by *Agrobacterium rhizogenes* LBA 9402. Experimental conditions are as described in the Experimental section, and peak assignments as in Fig. 2.

Although co-extractable biogenic amines were present, no dansyl-perivine was detected in either this culture or in *C. roseus–A. tumefaciens* (pTiT37), *C. roseus–A. tumefaciens* (pTiC58), *C. roseus–A. tumefaciens* (pTiAch5), or *C. roseus–A. tumefaciens* (pTiBo542) crown gall cultures, despite the fact that all were derived from transformed leaf tissue. This suggests not only that the majority of perivine is localised in *C. roseus* leaf tissue, but also that the leaf morphology is a prerequisite for perivine synthesis or storage. In contrast, by themospray LC–MS Mellon [7] found more than 20 alkaloids in a *C. roseus* transformed root extract, one of which was assigned as perivine. However, this assignment was made on molecular mass alone (m/z 341) without comparison with an authentic perivine standard. Furthermore, the quantity detected was rather low (>200 ng) and this may represent differences in culturing conditions and/or age of the culture.

More than one hundred different alkaloids have been isolated from *C. roseus* (L.) G. Don, most with similar physical properties, and this

complexity hinders the analysis of individual alkaloids. Pre-column dansylation of *C. roseus* extracts allows selective detection of the imino alkaloid perivine, even in the presence of complicated mixtures of other Vinca alkaloids. In principle, this approach should be equally applicable to the detection of minor amino or imino type alkaloids in other plants which may have been overlooked by more classical methods of alkaloid analysis.

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Short Communication

Potential of three different α-cyclodextrin modifications for the gas chromatographic evaluation of constituents of volatile oils

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ABSTRACT

Twenty-two typical constituents of volatile oils were studied by gas chromatography at 110 and 140°C using capillaries of three different α-cyclodextrin modifications. The monotrifluoracetyl, dipentyl form gave the highest relative retention times against linalol for monoterpene carbonyls and hydrocarbons. It was used for the analysis of dill oil, containing such constituents, which is illustrated, with methofuran as a specific feature. The dipentyl (monohydroxy) derivative previously studied gave highest relative retention times for monoterpenols and ethers (including aromatics). The permethylated hydroxypropyl α-cyclodextrin tested as the most polar phase gave lowest values of the three modifications for most solutes except four alcohols and two others. It was otherwise non-discriminatory over structure, but gave best resolution of certain solute pairs, which emerge close together from some conventional phases.

INTRODUCTION

"Chiraldex" modified cyclodextrins in capillaries have previously been used as stationary phases for the gas chromatography of volatile oil constituents [1,2] and some oils [3]. 2,6-O-Dipentyl monohydroxy (DA) cyclodextrins gave some structurally informative response patterns for monoterpenoids such as bicyclic or acyclic molecules, when relative retention times to *n*-undecane were compared on different sized (α- and β-) modified cyclodextrins [2]. Temperature change on the α-modification also gave results suggesting the position of polar groups in the monoterpenoids. It was now of interest to compare their behaviour and that of aromatics on two other different α-cyclodextrins to see if more information could be obtained. These are available from the same commercial source in the 3-O-monotrifluoroacetyl, dipentyl (TA) and the permethylated-hydroxypropyl (PH) modifications. These cyclodextrin derivatives were originally devised to resolve enantiomeric pairs, but may offer advantages for the gas chromatographic separation of the mixtures which form some volatile oils, even though only one enantiomer usually occurs naturally. They were introduced in 1990 by Armstrong and co-workers, DA first [4], then TA which resolved carvones and various other substances [5], and PH which handled the limonene enantiomers as well [6].

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EXPERIMENTAL

Apparatus

A Hewlett-Packard 5790A gas chromatograph was used, fitted with a capillary control unit, and a splitter injection port and flame ionisation detector both set at 235°C. A Hewlett-Packard 3380A recorder/integrator was attached.

The "Chiraldex" capillaries were purchased from Advanced Separation Technologies (Whippany, NJ, USA) and were 10 m × 0.25 mm I.D. with film thickness given as 0.125 μm ± 10%. They were heated and cooled at less than 10°C min^{-1} to preserve the phases. Helium was the mobile phase, used at 0.9–2.1 ml min^{-1}, and as "makeup" gas to the detector.

The GC–MS apparatus used as an adjunct has been recorded before [3]. The capillary used with this was from J & W Scientific (Folsom, CA, USA) and was 30 m × 0.25 mm I.D. with a film thickness of 0.15 μm DB-23 (cyanopropylmethylpolysiloxane).

Materials and methods

Solutes used were from various commercial sources including Aldrich, BDH, Dragoco (Holzminden, Germany), Eastman, Fritzsche-D.O., Koch-Light, Sigma and T.C.I. (Tokyo, Japan). The dill oil was of unspecified geographic origin, from Faulding (Perth, Australia). Injections of solutes were made with the trace residue in a microsyringe which had been filled, then "emptied"; apart from dill oil, where 0.2 μl was injected. Holdup times were deducted, obtained by extrapolating to methane the retention times for *n*-heptane and *n*-hexane plotted on semi-logarithmic graph paper.

RESULTS AND DISCUSSION

Results are presented in Table I, arranged in descending sequence, so far as possible. Fig. 1, where the A-PH phase is not shown, indicates some changes in relative retention times to linalol, from A-TA to A-DA. (The A prefix indicates α-cyclodextrins, modified chemically as described in the Introduction.) Only citronellol, estragole, 4-terpineol, citronellal and fenchone values fit perfectly at both the temperatures used into this arbitrary arrangement. Four solutes do not "fit" into the sequence at the same position for both temperatures, even after ignoring one result in a set of three. Only α-terpineol and citronellol have two sets of relative retentions to linalol at 110 and 140°C which are each quite similar on all three phases, with low standard deviations.

Inspection of Table I reveals that relative retention values on Chiraldex-A-PH are usually the lowest of the three phases. The exceptions to this are the solutes safrole, pulegone and the alcohols geraniol, citronellol and α-terpineol, together with borneol at 140°C. Alcohols are polar and this phase responds as the most polar of the three modified α-cyclodextrins, even though it is only of intermediate polarity, similar to OV-225. The table shows that PH favours the retention at 110°C of polar α-terpineol (in relation to the arranged sequence of solutes) but does not show affinity for non-polar caryophyllene, as is to be expected. More than half the seventeen solutes showed an unusual response of an increase in relative retention times on going to the higher temperature —not only the quicker emerging substances, but also caryophyllene, piperitone and carvone. The PH phase gave the best resolution here of the diverse solute pairs safrole/anethole, piperitone/carvone, α-terpineol/menthol and *p*-cymene/cineole, which are aromatics and terpenoids. Lemberkovics [7] records that this second terpenone pair is not resolved on polyethylene glycol (PEG) 20M, nor the lattermost pair on OV-17.

Chiraldex-A-TA gave highest values in the sets of three phase results in Table I for carbonyl-containing solutes (ketones and aldehydes) and hydrocarbons. This is displayed in Fig. 1 (in relation to the A-DA phase) where these solutes are marked "CO" and "H", respectively. A-TA particularly favours the retention of cuminal (aromatic aldehyde), caryophyllene and *p*-cymene. It is of intermediate to low polarity, apparently decreasing with temperature increase (as does A-PH). Here only the bicyclic ketones fenchone and camphor showed a distinct increase in relative retention times with temperature, but they gave this on all three Chiraldexes.

TABLE I

RELATIVE RETENTION TIMES (LINALOL = 1.0) ON THREE MODIFIED α-CYCLODEXTRIN "CHIRALDEX" CAPILLARIES "DA", "PH" AND "TA" AT TWO TEMPERATURES (°C)

Average results in best possible decreasing order. Suffix arrows indicate values out of sequence and the direction where they occur. Four solutes, named at the sides, do not fit a common sequence for both temperatures.

Solute	Chiraldex phases at 110°C			Solute	Chiraldex phases at 140°C			Solute
	PH	TA	DA		PH	TA	DA	
Geraniol	4.44	4.12	4.60[a]					
	3.84	3.68↓	4.17[a]	Safrole	3.70	3.32	3.82[a]	
	3.49	3.81↓	4.03[a]	Anethole	3.31↓	3.33	3.72[a]	
					3.62	3.16	3.64[a]	Geraniol
	2.19↓	4.03	3.77[a]	Caryophyllene	2.41↓	3.80↑	3.61[a]	
					2.95	3.72↑	3.22[a]	Cuminal
	3.42	3.31	3.66	Citronellol	2.87	2.72	3.00[b]	
Cuminal	3.00	4.46↑	3.25[a]					
	2.30	2.90	2.34↓	Piperitone	2.40	2.82↑	2.31[b]	
	2.14	2.82	2.23[b]↓	Carvone	2.25	2.69	2.26[b]	
Borneol	1.96	2.00↓	2.49					
	1.98↑	2.20	2.44	Menthol	1.90↓	2.04	2.26[a]	
	2.21↑	2.20	2.29[a]	α-Terpineol	2.14	2.04	2.18[a]	
					2.05	1.99	2.38[b]↑	Borneol
	1.81	2.18	1.74[b]↓	Pulegone	1.89	2.20↑	1.79[b]↓	
	1.71	1.89	2.03[a]	Estragole	1.77	1.84	2.04[a]	
	1.50	1.77	1.81[b]	4-Terpineol	1.58	1.78	1.83[b]	
					1.08	1.35↓	1.26[b]	Camphor
	0.89	1.43	1.16[b]	Citronellal	0.94	1.39	1.14[b]	
Camphor	0.87	1.21	1.12[b]					
	0.51	0.73	0.67[b]	Fenchone	0.64	0.89	0.79[b]	
γ-Terpinene	0.36	0.45	0.44[a]					
p-Cymene	0.34	0.42	0.38[a]					
Limonene	0.28	0.39	0.39[a]					
Cineole (1,8)	0.25	0.39	0.44[a]↑					
α-Terpinene	0.24	0.34	0.32[a]					
Polarity[c]	1.03	0.83	0.65		0.92	0.73	0.67	

[a] Literature value [1].

[b] Graphic interpolation from ref. 3.

[c] Expressed by c ratio corrected retention times 3(cuminal)/4(caryophyllene) [16]. Values below 0.8 indicate low polarity phases; those above this to 1.2 are of intermediate polarity. Thus the TA phase changes from minimal intermediate to the upper region of low polarity on heating from 110 to 140°C.

TA gave best resolution of the terpenoid solute pair piperitone/α-terpineol, which emerge close together from 20M [7].

Chiraldex-A-DA has shown highest values in the sets of three results for ethers ("E" in Fig. 1) and also for alcohols ("OH"), despite rating as the lowest polarity Chiraldex. A-DA is particularly unfavourable for the retention of the monocyclic ketones carvone, piperitone and pulegone —the lattermost is distinctive in giving the lowest relative retention times in both sets of three on this phase, as does piperitone at 140°C. It gave best resolution of the solute pairs borneol/α-terpineol, menthol/pulegone, γ-terpinene/p-cymene and cineole/limonene. Lemberkovics [7] records these four pairs being unresolved by PEG 20M.

The value of using relative retention times

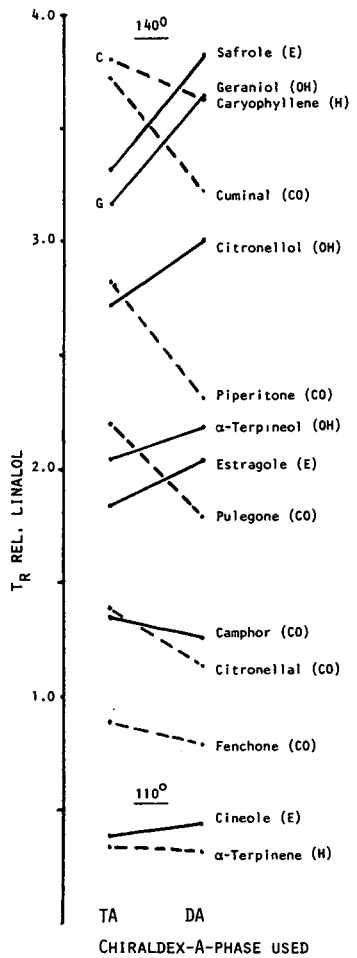

Fig. 1. Relative retention time (T_R REL.) (linalol = 1.00) changes on Chiraldex-A phases going from TA to DA at 140°C, except for plots below 0.5 which are at 110°C. CO = Carbonyl group (terpenoid ketones and aldehydes including an aromatic); E = ether (aromatic or terpenoid); H = hydrocarbon (mono- or sesquiterpene); OH = terpene alcohol.

against the polar solute linalol for this work was confirmed by determining some retention indices by graphical means. Indices for the phases Chiraldex-A-TA and -DA were similar, and only values for the PH phase were distinctly bigger, confirming that it is of higher polarity than the other two (see the bottom line of Table I). Thus no useful structural indications emerged from a consideration of retention indices, which are based on non-polar *n*-alkanes. Illustrative indices for citronellol on the phases Chiraldex-A-PH, -TA and -DA respectively are at 110°C 1500, 1360 and 1357, and at 140°C 1505, 1354 and 1360.

Chiraldexes-A-DA and -TA provide most discrimination in relation to solute structure, and so are displayed in Fig. 1. The implications are that DA should be chosen for ether-containing oils (*e.g.* anise, cajuput, eucalyptus, fennel, mace, nutmeg and sassafras) or those with alcohols (*e.g.* coriander, geranium, lavender, peppermint, rose, rosemary and teatree); whilst TA should be best for aldehyde- or ketone-containing oils (*e.g.* caraway, dill, lemongrass and spearmint) or those with hydrocarbons (*e.g.* ginger, juniper, lemon). The higher affinities of these phases for the types of solutes indicated should ensure the best results. The manufacturers of the Chiraldex capillaries record the resolution of *dextro-* and *laevo*-isomers of carvone on A-TA phase, and of 4-terpineols on A-DA, which agree with the above selectivity conclusions. "Unique selectivity has been noted on the TA series for carbonyl-containing molecules" [8]. I have reported the analysis of sweet fennel and mace oils on Chiraldex-A-DA [1], which was thus appropriately selected. Fig. 2 shows the temperature-programmed analysis of an Indian dill oil using Chiraldex-A-TA, although good isothermal results can be obtained in five minutes at 150°C. The main values obtained correspond well with average results from conventional and liquid crystal capillaries —43.6% impure "limonene", 3.1% anethofuran and 48.9% carvone. A polyethylene glycol 20M or a DB23 capillary was needed to resolve the 41.2% limonene from only 2.1% *p*-cymene, and to separate 0.4% dillapiole from other late peaks. This lattermost was not seen on methyl-polysiloxane, which also failed to resolve a trace of dihydrocarvone from anethofuran —Chiraldex-A-TA achieved both these resolutions (Fig. 2).

Anethofuran was named in 1979 by Goeckeritz *et al.* [9], and is an interesting constituent of dill fruit oil, which serves to distinguish it from caraway oil. This "tetrahydrocoumaran" derivative was detected in dill oils by two independent groups in 1977 [10,11], and found to form about 3% of American source oils a year later, where it was also referred to as a dimethyl-

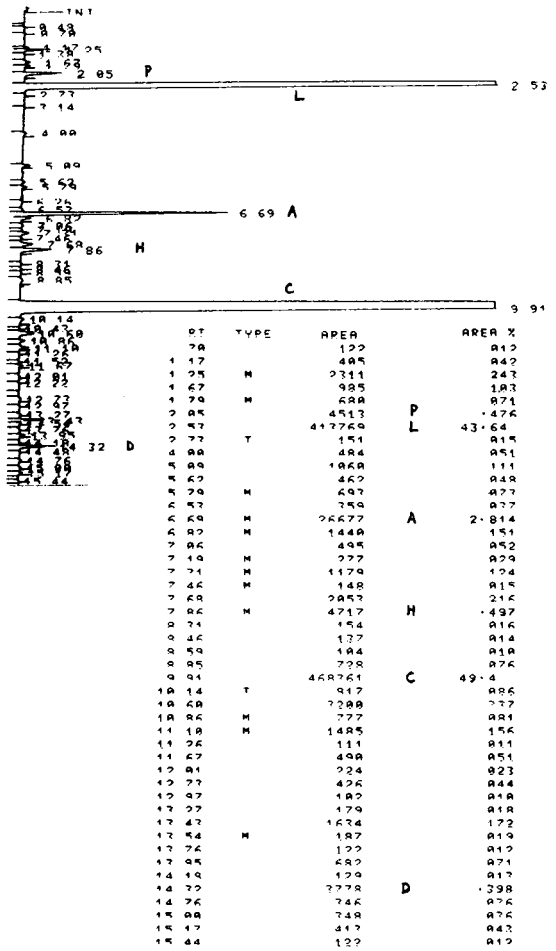

Fig. 2. Chromatogram of 0.2 μl dill oil on Chiraldex-A-TA capillary with 1.6 ml min^{-1} helium flow. Conditions 75°C initially for 1 min followed by programming at 4°C min^{-1} to 110°C (at 9.75 min), then at 10°C min^{-1} to 150°C (at 13.75 min) with concluding isothermal period. RT = retention time in min; peak TYPE = good unless merged (M) with previous one or tangent (T) skimmed baseline. Peaks: A = anethofuran; C = carvone; D = dillapiole; H = *E*-dihydrocarvone; L = limonene (including about 2% unresolved *p*-cymene); P = α-phellandrene. These named constituents form over 97% of the dill oil.

hexahydrobenzofuran [12]. This confusion was compounded in 1981 when it was recorded in dill herb as an "epoxy" (incorrect!)-menthene [13]. Although the benzofuran derivation is the preferred chemical name, anethofuran is clearly a monoterpenoid, best considered as 3-9-(furan)-oxy-*p*-menth-1-ene. It has been found to be a major component of the oil from the flowering umbels, declining as the fruits develop [14], forming 0.4–11.9% of Tasmanian dill oils [15]. I observed an anethofuran peak in each of several other samples of dill oil. Its identity was confirmed by GC–MS [principal ions *m/z* 137, 69, 109 and (trace) 152] from a DB23 capillary —a procedure also used to check other major and minor peaks. The anethofuran peak gave approximately 40% increase in relative retention times to undecane at 150°C on Chiraldex-B-DA compared to A-DA, indicating its cyclic structure [2]. On A-TA it gave almost exactly the same relative retention to linalol as on A-DA, a response not seen here for any other type of solute, and indicating a different structure to those examined in Table I, where there are no furans. The peak identified as dihydrocarvone by GC–MS gave an increase in relative retention times to linalol from 1.53 on A-DA to 1.87 on A-TA at 110°C, very appropriate for a carbonyl compound.

Two of the three α-cyclodextrin modifications used here thus appear to be of value for the analysis of selected volatile oils, and together can suggest the chemical nature of an unknown peak. Under the operating conditions used, there was no possibility of any chiral separations. Chiraldex-A-TA was best able to achieve this for carvones or 4-terpineols when programmed up to about 100°C.

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Short Communication

Preparation of benzo-18-crown-6 ether side-chain polysiloxane used as open tubular column gas chromatographic stationary phase

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ABSTRACT

A new kind of poly(crown ether), 3-propylbenzo-18-crown-6-substituted polysiloxane, was synthesized and used as a stationary phase for gas capillary column chromatography. The efficiency, polarity and selectivity of this phase were characterized. The data showed that fused-silica capillary columns coated with this stationary phase possess a weak polarity and higher selectivity for some positional isomers such as cresol and dimethylphenol isomers.

INTRODUCTION

Side-chain crown ether polymers were first used as open tubular column gas chromatographic stationary phases in 1985 by Fine *et al.* [1]. Since then several types of side-chain crown ether polysiloxanes have been studied as capillary gas chromatographic stationary phases [2–10]. 18-Crown-6-substituted polysiloxane was synthesized and used as a stationary phase coated on fused-silica capillary columns by Rouse *et al.* in 1988 [2]. They then prepared a series of oligoethylene oxide-containing polysiloxanes for use in capillary gas supercritical fluid chromatography in 1989 [3]. Wu *et al.* in 1990 [4] synthesized *n*-undecyloxymethyl-18-crown-6 polysiloxane and used it to coat fused-silica capillary columns, which were similar to Carbowax-20M in terms of polarity and selectivity. Wu synthesized a stationary phase by substituting 2,3-benzo-11-[(propyloxy)methyl]-18-crown-6 onto a polysiloxane backbone [5]. Azo-crown ether, ω-undecylene azo-18-crown polysiloxane and ω-undecylene azo-15-crown-5 polysiloxane were used by Wu *et al.* [6] as stationary phases on fused-silica capillary columns in 1992. These columns have the unique characteristic of separating aniline and other basic compounds without derivatization. We developed 3-propylbenzo-15-crown-5-substituted and 4-propylbenzo-15-crown-5-substituted polysiloxane and

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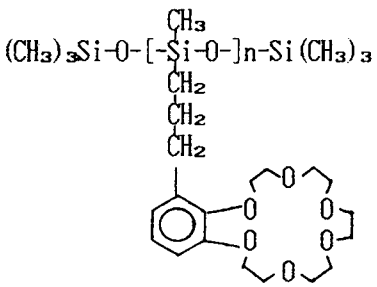

Fig. 1. The structure of PSO-B-18C6.

dibenzo-14-crown-4 polysiloxane used as open tubular column gas chromatographic stationary phases [7–10]. The results showed that these stationary phases have unique selectivity for the separation of some geometric isomers such as cresol, dimethylphenol, dinitrotoluene, etc.

In this work a new kind of poly(crown ether), 3-propylbenzo-18-crown-6-substituted polysiloxane, was synthesized and used as the capillary column stationary phase and compared with the other (crown ether) polysiloxane stationary phases. The structure of poly(crown ether) siloxanes studied is shown in Fig. 1. The aim of this work was to determine the effect of the structure of poly(crown ether) siloxane on the retention behaviour of different solutes.

EXPERIMENTAL

Synthesis of poly(methylsiloxane) containing a 3-propylbenzo-18-crown-6 side-chain (PSO-B-18C6)

PSO-B-18C6 was prepared by a hydrosilylation reaction between poly(methylhydrosiloxane) and 3-propylenebenzo-18-crown-6 (B-18C6). A 0.3524-g (*ca.* 0.001 mol) amount of B-18C6 (obtained from Department of Environmental Science, Wuhan University) and 0.06463 g (*ca.* 0.001 mol) of poly(methylhydrosiloxane) [Merck–Schuchardt (Hohenbrunn, Germany); degree of polymerization 35] were placed in 25 ml of dry, freshly distilled toluene in a dry three-neck round-bottom flask. The mixture was stirred and heated to 110°C under reflux in a nitrogen atmosphere until the solid reactant was dissolved in the toluene. A 30-μl volume of fresh catalyst solution (0.87 mg of chloroplatinic acid dissolved in 1 ml of isopropyl alcohol) was then added to the reactant solution and the solution was continuously heated and stirred for 6–15 h until the Si–H bond was no longer detectable by IR spectroscopy (2140 cm^{-1}). Generally, after 11 h the Si–H bond had almost disappeared and ethylene was then bubbled through the mixture for 1.0 h to react with all of the residual Si–H units. After the mixture was cooled it was left alone for 2 days. The brown precipitate that had formed was then taken out and dissolved in a small amount of methylene dichloride. After adding a small amount of methanol to the polymer solution a brown product was obtained. The product was centrifuged at *ca.* 700 *g* for 30 min dried in air for 24 h, removing the solvent. It was then dried in a vacuum oven at 40°C for 24 h to obtain the final product.

Column preparation

Fused-silica capillaries (0.25 mm I.D., Yongnian Optical Fibre Factory, Hebei, China) were used. Capillaries were purged with nitrogen at 240°C for 6 h before coating. The PSO-B-18C6 was dissolved in methylene dichloride at a concentration of about 4 mg/ml using the static coating procedure. The columns coated with PSO-B-18C6 were conditioned under nitrogen at 140, 160, 180, 200, 220 and 240°C for 2 h at each temperature.

Column evaluation

Column evaluation was carried out with a Model SP-3700 gas chromatograph (Beijing Analytical Instrumental Factory, Beijing, China) and an HP-5890-II gas chromatograph (Hewlett-Packard, China) equipped with a flame ionization detector using nitrogen as carrier gas. Solutes were injected using the split mode (the split ratio was 80:1). Time measurements were obtained using an HP-3390 A integrator. The efficiency of columns was determined as the number of plates per metre for *n*-octanol at 140°C. For evaluation of the selectivity of this stationary phase some standard mixtures of phenols and some positional isomers were used.

TABLE I

CHARACTERISTICS OF PSO-B-18C6 CAPILLARY COLUMNS

Column size: 10 m × 0.25 μm I.D.; O = 1-octanol.

Column No.	Stationary phase	Film thickness (μm)	Capacity factor	Column temperature (°C)	Flow-rate (cm/s)	Column efficiency (plates/m)	Compound tested
1	PSO-B-18C6	0.28	1.03	140	12.8	2890	O
2	PSO-B-18C6	0.19	1.97	140	13.3	6090	O
3	PSO-B-18C6	0.26	1.05	140	14.7	6160	O
4	PSO-B-18C6	0.26	1.04	140	18.0	5700	O
5	PSO-B-15C5[a]	0.28	1.58	140	13.6	4240	O

[a] Poly(crown ether) siloxane, from ref. 7.

RESULTS AND DISCUSSION

Table I shows the characteristics of the four columns coated with PSO-B-18C6. For comparison the efficiency of 3-propylbenzo-15-crown-5-substituted polysiloxane (PSO-B-15C5) is also given [7]. The data indicate that this stationary phase can be easily coated on fused-silica capillary columns and that generally very high efficiency is achieved. The efficiency of column 1 is lower than the efficiency of the other columns because the temperature was not well controlled during the coating. To test the effect of temperature on the column efficiency coated with PSO-B-18C6, the height equivalent to a theoretical plate (HEPT) was determined at different temperatures and at a constant flow-rate of carrier gas. Fig. 2 illustrates a plot of HEPT (h) *vs.* column temperature (T) for *n*-octanol on the columns. It indicates that the column efficiency has a maximum value at about 140°C, which is equal to the transition temperature. In Fig. 3 a plot of the relative retention (α) of nonanol and octanol against the column temperature shows a change in the slope at *ca.* 140°C. This phenomenon occurs in all poly(crown ether) siloxanes, and was called the liquid–liquid transition point by Fine *et al.* [1]. The polarity of PSO-B-18C6 represented by McReynolds constants is listed in Table II, and for comparison the McReynolds constants of SE-30, PEG-20M and PSO-B-3-18C5 (studied by Wu *et al.* [6]) are also given. The average polarity of the liquid phase investigated is lower than that of PEG-20M and the other poly(crown ether) siloxanes. On the other hand, the polarity of this phase is higher than that of SE-30. Thus it is a low-polarity liquid phase. PSO-B-3-18C6 prepared by Wu *et al.* [6]

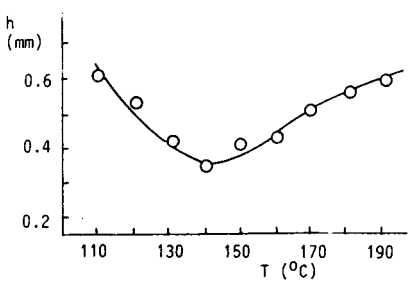

Fig. 2. Plot of HETP against column temperature at a constant flow-rate of carrier gas. Test compound: *n*-octanol. Column 2.

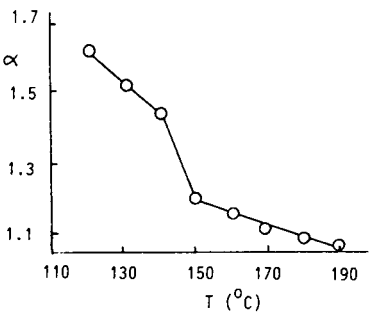

Fig. 3. Plot of α (nonanol/octanol) *vs.* column temperature on column 2.

TABLE II

McREYNOLDS CONSTANTS OF PSO-B-18C6 COLUMNS[a]

X' = Benzene; Y' = 1-butanol; Z' = 2-pentanone; U' = nitropropane; S' = pyridine.

Stationary phase	McReynolds constants					
	X'	Y'	Z'	U'	S'	Average polarity
PSO-B-18C6						
Column 1	167	254	201	399	358	276
Column 2	174	257	194	369	331	265
Column 3	181	260	191	377	341	270
Column 4	177	242	190	372	336	264
PSO average	175	253	194	379	342	269
Carbowax-20 M[b]	322	536	368	572	510	462
PSO-B-15C5	198	390	196	371	434	318
PSO-B-3-18C6[c]	216	381	452	461	465	394
SE-30[b]	15	44	53	64	41	43

[a] From ref. 8.
[b] From ref. 11.
[c] From ref. 5.

has higher polarity than PSO-B-18C6: perhaps the benzene on PSO-B-18C6 is not linked to the spacer, being distant from the main chain.

The selectivity of PSO-B-18C6 is shown by the separation power of disubstituted benzene isomers. Table III lists the relative retention values of isomers of dichlorobenzene, dibromobenzene and cresol. It is well known that generally the separation of isomers of cresol is very difficult, although poly(crown ether) columns possess a unique ability to separate isomeric phenol compounds. The data in Table III show that the $\alpha(p/m)$ value of *p*- and *m*-cresol isomers reached 1.02, which is similar to that of PSO-B-15C5. In Table IV the α value of dimethylphenol isomers is given, and the data for PSO-B-15C5 are also listed for comparison. The data indicate that 2,4/2,5-dimethylphenol isomers are better separated on PSO-B-18C6 than on PSO-B-15C5. These columns have an excellent capacity to separate di- and trinitrotoluene isomers, as shown in Figs. 4 and 5.

TABLE III

RELATIVE RETENTION (α) OF DISUBSTITUTED BENZENES ON THE PSO-B-18C6 COLUMN

Compound	Column temperature (°C)	Isomer	k' value on PSO-B-18C6	α (p/m)
Dichlorobenzene	120	*m*-	0.85	
		p-	1.00	1.18
		o-	1.23	
Dibromobenzene	120	*m*-	3.46	
		p-	3.62	1.05
		o-	4.40	
Cresol	140	*o*-	1.48	
		p-	2.00	1.02
		m-	2.03	

TABLE IV

RELATIVE RETENTION (α) OF DIMETHYLPHENOL ISOMERS ON PSO-B-18C6 AND PSO-B-15C5

Column temperature (°C)	Isomer	α value			
		PSO-B-18C6		PSO-B-15C5	
150	2,6-	1.00		1.00	
	2,5-	1.58	$\alpha(2,5/2,6) = 1.58$	1.65	$\alpha(2,5/2,6) = 1.65$
	2,4-	1.62	$\alpha(2,4/2,5) = 1.02$	1.65	$\alpha(2,4/2,5) = 1.00$
	2,3-	1.96	$\alpha(2,3/2,4) = 1.21$	2.11	$\alpha(2,3/2,4) = 1.28$
	3,5-	2.14	$\alpha(3,5/2,3) = 1.09$	2.31	$\alpha(2,5/2,3) = 1.09$
	3,4-	2.54	$\alpha(3,4/3,5) = 1.19$	2.73	$\alpha(3,4/3,5) = 1.18$
140	2,6-	1.00			
	2,5-	1.81	$\alpha(2,5/2,6) = 1.81$		
	2,4-	1.84	$\alpha(2,4/2,5) = 1.02$		
	2,3-	2.31	$\alpha(2,3/2,4) = 1.26$		
	3,5-	2.54	$\alpha(3,5/2,3) = 1.10$		
	3,4-	2.79	$\alpha(3,4/3,5) = 1.10$		

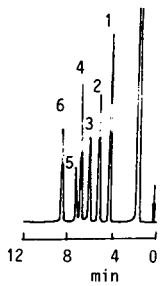

Fig. 4. Chromatogram of the separation of dinitrotoluene (DNT) isomers at 200°C. Peaks: 1 = 2,6-DNT; 2 = 3,5-DNT; 3 = 2,5-DNT; 4 = 2,4-DNT; 5 = 2,3-DNT; 6 = 3,4-DNT.

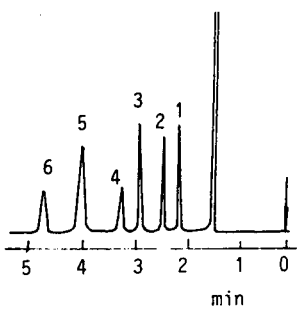

Fig. 5. Chromatogram of the separation of trinitrotoluene (TNT) isomers at 180°C. Peaks: 1 = 2,4,6-TNT; 2 = 2,3,6-TNT; 3 = 2,3,5-TNT; 4 = 2,4,5-TNT; 5 = 2,3,4-TNT; 6 = 3,4,5-TNT.

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Short Communication

Analysis of N-acyl aminonaphthalene sulphonic acid derivatives with potential anti-human immunodeficiency virus activity by thin-layer chromatography and flame ionization detection

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(First received May 24th, 1993; revised manuscript received July 12th, 1993)

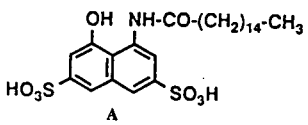
ABSTRACT

A method for checking the purity of N-acyl aminonaphthalene disulphonic acid derivatives was required for a systematic study of the anti-human immunodeficiency virus activity of these agents. We describe the use of thin-layer chromatography and flame ionization detection for the separation of these compounds, which are difficult to analyse by conventional methods. All the samples were prepared in methanol solutions (1 μl) containing 5 μg of aminonaphthalene derivative. These samples were applied to each type SIII Chromarod by a single injection and developed with pure methanol or a methanol–chloroform–ammonium hydroxide (35:55:10, v/v/v) solvent system.

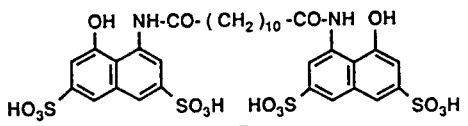
INTRODUCTION

In the quest for molecules with anti-human immunodeficiency virus (HIV) activity [1,2] new antiviral mechanisms have been discovered, and this has spurred the development of novel non-nucleoside agents. These agents inhibit both reverse transcriptase and absorption of virus [3]. Among such agents, the long-chain A, or bolaform B, naphthalene sulphonic acids have been shown to possess activity against both HIV-1 and HIV-2 [4,5].

In an attempt to alter the lipophilicity of these derivatives and optimize their activity, we carried out a study of the effect of the length of the



A



B

alkyl chain linked to the naphthalene disulphonic acid. We developed a new route [6] (Fig. 1) to the required derivatives **3a**, **b** and **i–k** in yields ranging from 25 to 60%. These yields are consistently higher than those obtained by conventional acylation [4]. The derivatives **3** are highly hygroscopic and are thus not readily purified or

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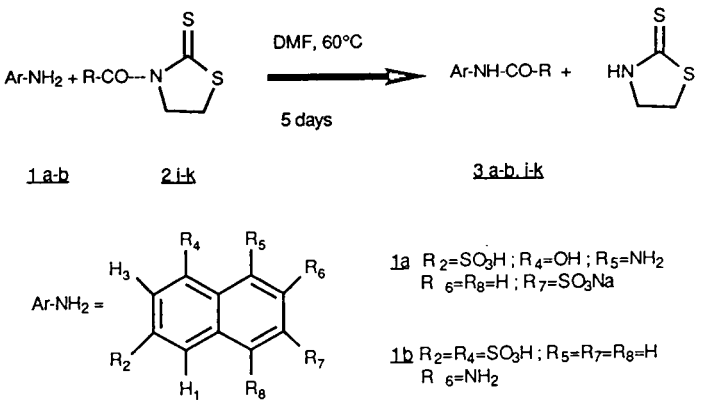

R	2	3
$C_{13}H_{27}$	2i	3a-b,i
$C_{15}H_{31}$	2j	3a-b,j
$C_{17}H_{35}$	2k	3a-b,k

Fig. 1. Acylation of aminonaphthalene disulphonic acids **1** by N-acyl thiazolidine-2-thiones.

crystallized [5]. Elemental analysis of compounds **3** produced inconsistent results, probably due to partial combustion of these high-molecular-mass substances. We therefore decided to employ thin-layer chromatography with flame ionization detection (TLC–FID) [7] to check the purity of the compounds prepared. This method has been used to analyse compounds of similar structure, such as sulphanilic acids [8,9] and various amphiphilic derivatives [10].

EXPERIMENTAL

TLC–FID was carried out using an Iatroscan TH-10 instrument (Iatron Labs., Tokyo, Japan), and the data were analysed by BOREAL software. The flame ionization detector was set up using the following parameters: hydrogen flow 160 ml/min, air flow 1 l/min, scan rate 4 mm/s, paper speed 28 mm/min. Samples (1 μl of a 0.5% solution of **1** or **3** in methanol) were placed on type S III Chromarods, which were activated immediately before use. The rods were developed with pure methanol (system 1) for compounds **3a**, **b** and **i–k**, or methanol–chloroform–ammonium hydroxide (35:55:10, v/v/v) (system 2) for compounds **1a** and **b**, in a chamber previously saturated with the solvent system.

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 2. The values of t_R obtained in the two solvent systems for both the starting compound **1b** and the derivatives synthesized, **3b** and **i–k**, are listed in Table I. Similar results were obtained with compounds **1a** and **3a** and **i–k**. It can be seen that the values of t_R were little affected by the nature of the alkyl chain linked to the aminonaphthalene sulphonic acid. On the other hand, the starting aminonaphthalene sulphonic acid **1** did not migrate in pure methanol ($t_R = 0.45$ min, see Table I). A mixture of methanol, chloroform and ammonium hydroxide was therefore employed to analyse the derivatives **1** ($t_R = 0.16$ min, see Table I). These compounds, like most

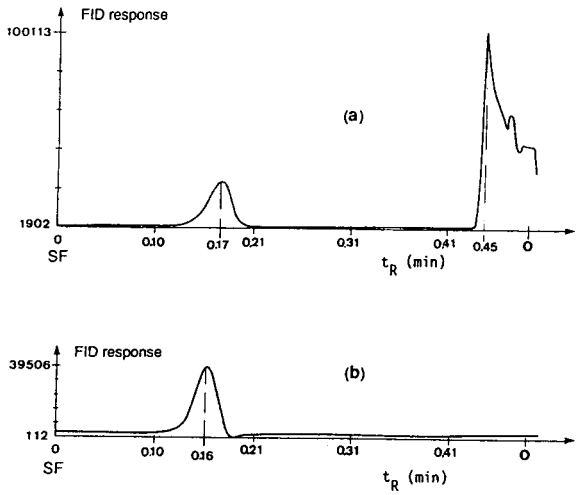

Fig. 2. TLC–FID analysis of mixture of compounds **3b** and **k** and **1b** in solvent system 1 (a) and pure compound **1b** in solvent system 2 (b). SF = Solvent front, $t_R = 0.00$; O = origin, $t_R = 0.50$ min.

TABLE I

TLC–FID ANALYSIS OF SEVERAL NAPHTHALENE SULPHONIC ACID DERIVATIVES

Compounds	t_R (min)	
	System 1	System 2
1b		
technical	0.17[a] + 0.45	0.16 + 0.27[a]
2 recrystallized	0.45	0.16
3b, i	0.18	–
3b, j	0.17	–
3b, k	0.17	0.12

[a] Oxidation product.

aminophenols, oxidize spontaneously in air [11]. They were thus recrystallized before use, and their purity was checked by TLC–FID, which readily detected oxidation products (Table I).

CONCLUSIONS

The Iatroscan system is both convenient to use and of high performance. It was successfully employed to analyse N-acyl aminonaphthalene disulphonic acid derivatives, which are not readily quantified by conventional methods.

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Short Communication

Thin-layer chromatographic detection of carbaryl using phenylhydrazine hydrochloride

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(First received February 17th, 1993; revised manuscript received May 24th, 1993)

ABSTRACT

A new chromogenic spray reagent for the detection of the commonly misused carbamate insecticide carbaryl, is described. Carbaryl, on alkaline hydrolysis, yields 1-naphthol, which in turn reacts with phenylhydrazine hydrochloride to give a red complex. This reagent is selective for carbaryl. There is no interference from other carbamate insecticides or from organophosphorus, organochlorine and pyrethroid insecticides or from constituents of visceral extracts (amino acids, peptides, proteins, etc.). The limit of detection of the reagent is *ca.* 0.1 μg per spot (*i.e. ca.* 350 ng/cm^2) observed after development.

INTRODUCTION

Carbaryl, 1-naphthyl N-methyl carbamate, is a good contact insecticide with occasional systemic activity. It is used for pest control in India and many tropical countries. Its use is continually increasing, and this is reflected in the increasing number of criminal cases referred to forensic science laboratories concerning the misuse of carbamates. Hence, its selective characterization is necessary. A number of reagents have been used for its detection by thin-layer chromatography (TLC), namely diazophenol (after alkaline hydrolysis) [1], alkaline fast blue B [2] and Tollen's reagent [3]. However, these reagents are normally used for phenolic compounds or cannabinoids, and are susceptible to biological impurities such as amino acids, proteins and peptides and are not specific. Although a copper (II) chloride followed by ammonium metavanadate reagent [4] is reported to be specific for carbaryl, it has a low sensitivity of detection.

In this paper we report the use of 1% phenylhydrazine hydrochloride in an alkaline medium for the detection of carbaryl by TLC, yielding an intense red colour.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. Distilled water was used throughout.

Alkaline phenylhydrazine hydrochloride reagent. Equal volumes of 1% (w/v) aqueous phenylhydrazine hydrochloride solution and 10% (w/v) aqueous sodium hydroxide solution are mixed together just before use.

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Extraction of carbaryl from biological materials

Portions of *ca.* 50 g each of various types of visceral tissue (stomach, intestine, liver, spleen and kidney) containing carbaryl were individually minced in 50 ml of aqueous solution. The insecticide was then extracted with 200 ml of diethyl ether and the solvent was evaporated at room temperature. The residue was dissovled in 1–2 ml of ethanol. A known volume (10 μl) of the solution was spotted on an activated TLC plate together with the standard solution of insecticide. The plate was then developed as described in the *Procedure* section and sprayed with alkaline phenylhydrazine hydrochloride reagent.

Procedure

A standard glass TLC plate was coated with a slurry of silica gel G in water (1:2) to a thickness of 0.25 mm. The plate was activated at 110°C for about 1 h. A 10-μl volume of a standard solution of carbaryl in ethanol (1 mg/ml) was spotted on the plate, which was then developed in a previously saturated TLC chamber using *n*-hexane–acetone (4:1) as the solvent up to a height of 10 cm. The plate was removed, dried in air and sprayed with alkaline phenylhydrazine hydrochloride reagent. An intense red spot was observed immediately on the TLC plate at an R_F value of 0.45.

RESULTS AND DISCUSSION

Recovery experiment

A 1-mg amount of carbaryl was added to 50 g of minced visceral tissue, mixed well and kept for a day. The insecticide was then extracted with diethyl ether, the solvent was evaporated at room temperature and the residue was dissolved in 1 ml of ethanol. A 10-μl volume of this solution was spotted on an activated thin-layer plate together with 10 μl each of standard technical carbaryl solutions containing known concentrations of 9, 9.5 and 10 mg per 10 ml in ethanol. The plate was then developed as described in the *Procedure* section and sprayed with alkaline phenylhydrazine hydrochloride reagent. The intensity of the red spots developed from the visceral extracts was compared with those of the known standards and found to agree with the spot resulting from a cabaryl concentration of 10 mg/10 ml (average of three experiments). Hence the recovery was *ca.* 100%.

This reagent is selective for carbaryl. Other carbamate insecticides, such as baygon, carbofuran and Zineb, organophosphorus insecticides, such as malathion, parathion, dimethoate, quinalphos, phorate, fenthion, fenitrothion and monocrotophos, organochlorine insecticides, such as endrin, aldrin, dialdrin, endosulphan, DDT and benzene hexachloride, and pyrethroid insecticides, such as fenvalerate, cypermethrin and deltamethrin, do not give a coloured spot. Moreover, constituents of viscera (amino acids, peptides, proteins, etc.), which are generally coextracted with the insecticides, do not interfere. The sensitivity of the reagent is *ca.* 0.1 μg per spot (*i.e. ca.* 353 ng/cm^2) observed after development.

On alkaline hydrolysis carbaryl yields 1-naphthol [5,6], which then reacts with phenylhydrazine hydrochloride to give red complex III, as shown in Fig. 1. Technical-grade carbaryl and 1-naphthol give one spot at R_F 0.45 and 0.54, respectively, whereas carbaryl in formulation and extracts of biological materials from patients with carbaryl poisoning give two spots with R_F values of 0.45 and 0.54, demonstrating that they contain the hydrolysis product,

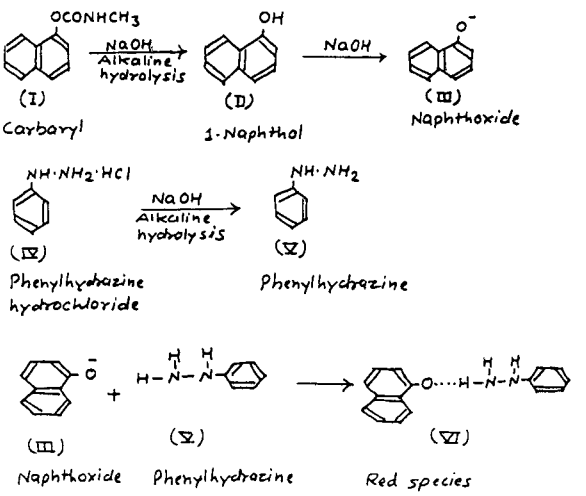

Fig. 1. Proposed reaction for formation of coloured species.

1-naphthol. The colour of the spots is stable for a couple of days.

The reagent described here is very sensitive and specific for carbaryl and hence can be used routinely for the detection and determination of carbaryl and its breakdown product, 1-naphthol, in biological and non-biological materials in forensic toxicology.

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Short Communication

Determination of glycyrrhizin and glycyrrhetinic acid in traditional Chinese medicinal preparations by capillary electrophoresis

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(First received May 25th, 1993; revised manuscript received July 13th, 1993)

ABSTRACT

A simple, rapid, accurate and reproducible capillary electrophoretic method was developed for the assay of glycyrrhizin and glycyrrhetinic acid in traditional Chinese medicinal preparations. The buffer solution used in this method was acetonitrile and 0.02 *M* sodium dihydrogenphosphate solution adjusted to pH 7.5 with 0.05 *M* sodium hydroxide. The linear calibration range was 0.04–2.00 mg/ml ($r = 0.9988$) for glycyrrhizin and 0.007–0.35 mg/ml ($r = 0.9985$) for glycyrrhetinic acid and recoveries were 98.1–101.3% for glycyrrhizin and 98.5–101.4% for glycyrrhetinic acid. The relative standard deviations were 1.02% ($n = 6$) for glycyrrhizin and 0.91% ($n = 6$) for glycyrrhetinic acid. The content of these two acids in Glycyrrhizae Radix and Glycyrrhizae Radix-containing Chinese medicinal preparations was successfully determined within 10 min.

INTRODUCTION

Glycyrrhizae Radix is a Chinese herbal drug commonly used as an expectorant, detoxicant and spleen tonic and to restore vitality, reduce fever, arrest coughing, comfort the stomach, alleviate urgency and potentiate the effect of various other herbs. It is known to contain mainly glycyrrhizin (20β-carboxy-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranuronosyl-α-D-glucopyranosiduronic acid) and its aglycone, glycyrrhetinic acid [1]. It is widely found in Chinese medicinal preparations such as tonic, surdorific, coordinative, vitality-regulating, blood-regulating, chill-dispelling and moistening formulas [2]. At present, the best method of measuring the equivalence of Glycyrrhizae Radix-containing Chinese medicinal preparations is to determine the content of glycyrrhizin by HPLC [3]. However, owing to complicated components in Chinese medicinal formulas, the use of HPLC is restricted by its lengthy analysis time (about 50 min), poor resolution and the fact that the chromatographic column is easily contaminated and hard to clean. Capillary electrophoresis (CE) is a recently developed method that has the following advantages: analysis time is short, only a small sample is required, thorough cleaning of the column is easy and autosampling is possible [4]. Used in the analysis of Chinese herbs, it gives very good results [5–9]. This study has also found that using CE to analyse various Glycyrrhizae Radix-containing preparations can

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offer very satisfactory results. Hence, it is a suitable method for analyses of traditional Chinese medicinal preparations, especially when analysis of large numbers of samples is required and for quality control in pharmaceutical plants.

EXPERIMENTAL

Reagents and materials

Glycyrrhizin and glycyrrhetinic acid were purchased from Tokyo Kasei (Tokyo, Japan) and sodium dihydrogenphosphate from Wako (Osaka, Japan). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions. Acetonitrile and methanol were of HPLC grade. Glycyrrhizae Radix-containing Chinese medicinal preparations were provided by Sun-Ten Pharmaceutical (Taipei, Taiwan).

Preparation of Chinese medicinal preparation extracts

A 1.0-g sample of a Chinese medicinal preparation was extracted with 70% methanol (7.0 ml) by ultrasonic agitation at room temperature for 15 min, then centrifuged at 1500 *g* for 10 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter paper. After the addition of a 1.5 ml of internal standard solution (2.5 mg of cinnamic acid in 1 ml of 70% methanol), the Chinese medicinal preparation extract was diluted to 25 ml with 70% methanol. This solution was passed through a 0.45-μm filter and *ca.* 0.8 nl (5 s hydrostatic sampling) of the filtrate were injected into the capillary electrophoresis system directly.

Apparatus and conditions

All analyses were carried out on a Waters Quanta 4000 capillary electrophoresis system equipped with a UV detector set at 254 nm and a 70 cm × 75 μm I.D. uncoated capillary (Millipore, USA) with the detection window placed at 62.5 cm. The conditions were as follows: sampling time, 5 s hydrostatic; run time, 10 min; applied voltage, 25 kV (constant voltage, positive to negative polarity); temperature, 25.0–25.5°C. The electrolyte was a buffer solution that contained acetonitrile and 0.02 *M* sodium dihydrogenphosphate solution, which was adjusted to pH 7.5 with 0.05 *M* sodium hydroxide.

Solution for linearity response

Eight concentrations of glycyrrhizin, which ranged from 0.04 to 2.00 mg/ml, and eight concentrations of glycyrrhetinic acid, which ranged from 0.007 to 0.35 mg/ml, were prepared. Each concentration was analysed three times.

Solution for recovery studies

Fixed amounts of glycyrrhizin and glycyrrhetinic acid standard were added to three samples of Chinese medicinal preparations of known acids content and the mixture was extracted and analysed using the proposed procedure.

RESULTS AND DISCUSSION

Analytical conditions

Glycyrrhizin and glycyrrhetinic acid contain a carboxyl group and should be analysed as anions in a capillary electrophoresis system. Hence we used sodium dihydrogenphosphate solutions with different pH values as buffer solutions. We found that a 0.02 *M* sodium dihydrogenphosphate solution that was adjusted to pH 7.5 with 0.05 *M* sodium hydroxide gave the best result. At higher pH, the separation was good but the analysis time was longer, whereas at lower pH the peak of glycyrrhizin was found to be overlapped by other peaks. However, further improvements were required to minimize the noise. Addition of acetonitrile to the buffer solution made the peaks sharper, the baseline smoother and also produced a better separation. After a series of trials, a concentration of 20% acetonitrile was selected; a lower concentration of acetonitrile gave only a slight improvement and higher concentrations prolonged the analysis time. An electrolyte containing 80% 0.02 *M* sodium dihydrogenphosphate (pH 7.5) and 20% acetonitrile was found to produce the best resolution. Fig. 1 is an electropherogram showing the separation of the authentic glycyrrhizin and

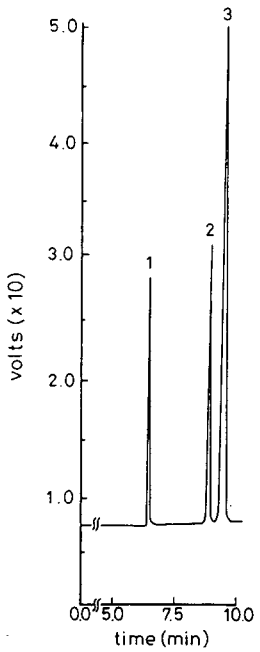

Fig. 1. Capillary electropherogram of a mixture of glycyrrhizin and glycyrrhetinic acid. Peaks: 1 = glycyrrhetinic acid; 2 = internal standard (cinnamic acid); 3 = glycyrrhizin.

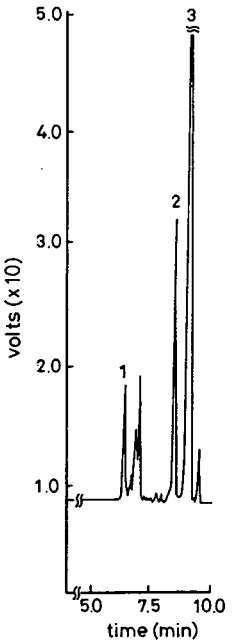

Fig. 2. Capillary electropherogram of the extract of a Glycyrrhizae Radix sample. Symbols as in Fig. 1.

glycyrrhetinic acid with migration times of 6.5 min for glycyrrhetinic acid, 8.8 min for the internal standard and 9.5 min for glycyrrhizin. The analysis of these two constituents can be completed within 10 min. As the methanol–water extracts of the crude drug and Chinese medicinal preparations were injected directly and analysed, the results were as good as those obtained with pure chemical samples without any interference with other peaks, and the analyses could also be completed within 10 min, as shown in Figs. 2 and 3.

Calibration graph for glycyrrhizin and glycyrhetinic acid

Calibration graphs (peak-area ratio, y, *vs.* concentration, x, mg/ml) were constructed in the range 0.04–2.00 mg/ml for glycyrrhizin and 0.007–0.35 mg/ml for glycyrrhetinic acid. The regression equations of these curves and the correlation coefficients were calculated as follows:

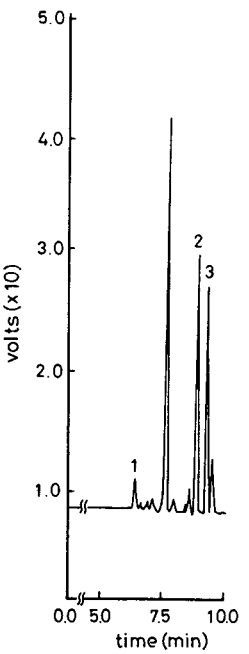

Fig. 3. Capillary electropherogram of a Chinese medicinal preparation, *Chia-wei-hsiao-yao-san*. Symbols as in Fig. 1.

Glycyrrhizin $y = 2.348x - 0.1033$ ($r = 0.9988$)

Glycyrrhetinic acid $y = 4.386x - 0.01125$ ($r = 0.9985$)

System suitability test

The reproducibility (relative standard deviation) of this proposed method, on the basis of peak-area ratios for six replicate injections, was 1.02% (intra-day) and 1.12% (inter-day) for glycyrrhizin and 0.91% (intra-day) and 1.07% (inter-day) for glycyrrhetinic acid.

The results of standard addition recovery studies of glycyrrhizin and glycyrrhetinic acid from sample composites of Chinese medicinal preparations are shown in Table I. The ranges of recovery were 98.1–101.3% for glycyrrhizin and 98.5–101.4% for glycyrrhetinic acid. The tailing factors of the three peaks (internal standard, glycyrrhizin and glycyrrhetinic acid) are very close to 1.

Determination of glycyrrhizin and glycyrrhetinic acid in Chinese medicinal preparations

When the test solutions of Chinese medicinal preparation extracts were analysed by CE under the selected conditions, the calculated contents of glycyrrhizin and glycyrrhetinic acid as shown in Table II were obtained. There was no interference with any peak of the extracts in various Chinese medicinal preparations (total 21 preparations were successfully examined). These data indicate that the proposed CE method is suitable for the determination of glycyrrhizin and glycyrrhetinic acid in Chinese medicinal preparations. Moreover, this analytical method not only needs no pretreatment, but also offers autosampling. In addition to its speed and accuracy, it allows a second injection within 12 min with a thoroughly cleaned column. Therefore, it should

TABLE II

THE CONTENT OF GLYCYRRHIZIN AND GLYCYRRHETINIC ACID IN GLYCYRRHIZAE RADIX AND CHINESE MEDICINAL PREPARATIONS (mg/g) ($n = 6$)

Sample	Glycyrrhizin	Glycyrrhetinic acid
Glycyrrhizae Radix	52.06	1.56
Chia-wei-hsiao-yao-san	5.43	0.38
Kuei-pi-tang	3.51	0.22
Pai-tu-san	2.05	0.13

TABLE I

RECOVERY OF GLYCYRRHIZIN AND GLYCYRRHETINIC ACID FROM VARIOUS CHINESE MEDICINAL PREPARATIONS ($n = 6$)

Sample [9][a]	Amount added (mg)		Recovery (%)	
	Glycy[b]	Gly · acid[c]	Glycy	Gly · acid
Chia-wei-hsiao-yao-san (bupleurum and peony formula)	0.1219	0.05	101.3	101.4
Kuei-pi-tang (ginseng and longan combination)	0.1219	0.05	99.2	100.8
Pai-tu-san (rehmannia and lonicera formula)	0.1219	0.05	98.1	98.5

[a] Name and composition of the Glycyrrhizae Radix-containing Chinese herbal formulas: *Chia-wei-hsiao-yao-san:* Angelicae Radix, Paeoniae Radix, Atractylodis Rhizoma, Poria, Bupleuri Radix, Moutan Radicis Cortex, Gardeniae Fructus, Glycyrrhizae Radix, Zingiberis Rhizoma, Menthae Herba; *Kuei-pi-tang:* Astragali Radix, Ginseng Radix, Atractylodis Rhizoma, Poria, Zizyphi Spinosi Semen, Longanae Arillus, Angelicae Radix, Zingiberis Rhizoma, Zizyphi Fructus, Polygalae Radix, Glycyrrhizae Radix, Saussureae Radix; *Pai-tu-san:* Rehmanniae Radix, Platycodi Radix, Forsythiae Fructus, Moutan Radics Cortex, Trichosanthis Radix, Scrophvlariae Radix, Lonicerae Flos, Bupleuri Radix, Glycyrrhizae Radix, Phellodendri Cortex, Menthae Herba, Paeoniae Radix, Gypsum Fibrosum, Arctii Fructus.

[b] Glycy = Glycyrrhizin.

[c] Gly · acid = Glycyrrhetinic acid.

be especially useful for bulky samples and also for quality control in the pharmaceutical plants.

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