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AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY

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

(Journal of Chromatography Library, 40)

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

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SUPERCRITICAL FLUID CHROMATOGRAPHY WITH ION-PAIRING MODIFIERS

SEPARATION OF ENANTIOMERIC 1,2-AMINOALCOHOLS AS DIASTEREOMERIC ION PAIRS

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(First received December 29th, 1987; revised manuscript received April 12th, 1988)

SUMMARY

Ionizable organic compounds have been separated by supercritical and subcritical chromatography using ion-pairing modifiers. The mobile phase was carbon dioxide mixed with an acetonitrile solution containing a counter ion and a competing ion. The retention and separating efficiency are controlled by pressure and temperature as well as by the concentration and nature of the ionic mobile phase components. These highly versatile systems are applicable to polar solutes of widely different structures. The technique has been applied to the separation of enantiomeric 1,2-aminoalcohols using N-benzoxycarbonylglycyl-L-proline as a chiral counter ion. The maximum resolution and the separating efficiency at higher flow-rates are superior to that obtained in high-performance liquid chromatography.

INTRODUCTION

Supercritical fluid chromatography (SFC) usually gives higher separating efficiency than high-performance liquid chromatography (HPLC) since it operates under more favourable kinetic conditions owing to an higher diffusivity and a lower viscosity of the mobile phase. This gives possibilities of significantly better resolution at higher flow-rates than that obtained with HPLC[1,2].

The improvement of the separating efficiency is of particular importance in the separation of enantiomeric compounds since it has been observed that chiral solutes often give wider peaks than achiral ones. SFC has been used for few chiral separations and exclusively with chiral solid phases[3–6], mainly due to the limited possibilities of applying the eluting properties of the mobile phase to polar solutes. However, this

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study shows that an important improvement of the selectivity and the versatility for ionizable solutes can be achieved by application of the ion-pairing principles that for a long time have been used in normal-phase HPLC[7].

The ion-pairing technique can be used for HPLC separation of enantiomers as demonstrated by Pettersson *et al.*[8–12]. Among the methods that have been developed, the resolutions of enantiomeric 1,2-aminoalcohols are of particular interest since compounds of this kind are of great pharmacological interest[12]. The chiral selector is an antipode of a dipeptide, N-benzoxycarbonylglycyl-L-proline (ZGP), which is added to the mobile phase. It gives diastereomeric ion pairs with the cationic solutes which are retained by an achiral solid phase. These separation principles have been applied to SFC in this study, using carbon dioxide mixed with a solution of the chiral selector in acetonitrile as the mobile phase. The ion-pair distribution principles have not previously been used in SFC and the objective of this work was to study the selectivity and the separating efficiency that can be achieved with SFC as compared to normal-phase HPLC.

EXPERIMENTAL

Apparatus

The SFC equipment used is shown in Fig. 1. The pumps for carbon dioxide

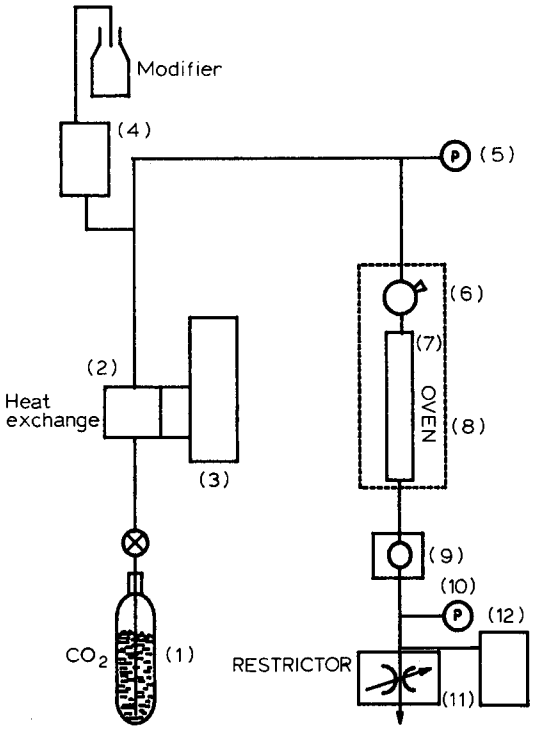


Fig. 1. Equipment for supercritical fluid chromatography: 1 = carbon dioxide cylinder; 2 = heat exchanger; 3 = carbon dioxide pump; 4 = modifier pump; 5, 10 = pressure monitors; 6 = injector; 7 = separation column; 8 = oven; 9 = UV detector; 11 = restrictor; 12 = wash pump.

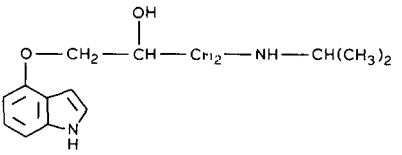
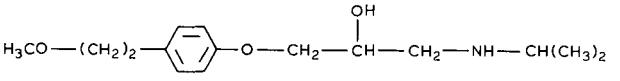
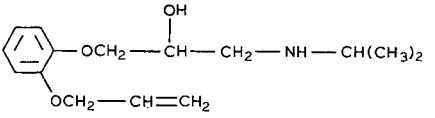
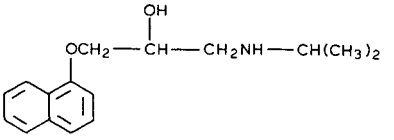
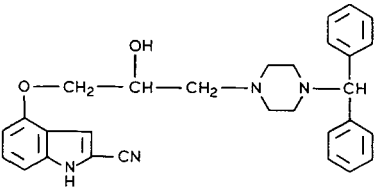
and the modifier were of Type LC240 (Kontron, Zürich, Switzerland). The pumphead for the carbon dioxide delivery was equipped with a heat exchanger which allowed the gas to be cooled below −10°C. The restrictor was continously washed with acetonitrile to prevent precipitation of the ion-pair reagents. The injector was a Rheodyne valve 70-100. An UV detector, UVIKON 720 LC from Kontron, equipped with a laboratory-made high-pressure resistant cell, was used. To maintain the desired system pressure a restrictor 26-1721 (Tescom, Elk River, MA, U.S.A.) was employed. The column inlet and outlet pressure, were measured using pressure monitors LCP 501 (Innovativ Labor, Wallisellen, Switzerland).

Chemicals

Carbon dioxide, 48 grade, was obtained from Carba Gas (Basle, Switzerland), acetonitrile from Rathburn (Walkerburn, U.K.), N-benzoxycarbonylglycyl-L-proline

TABLE I

STRUCTURES OF 1,2-AMINOALCOHOLS

Structure	*Name*
	Pindolol
	Metoprolol
	Oxprenolol
	Propranolol
	DPI 201-106

(ZGP) and triethylamine (TEA) from Fluka (Buchs, Switzerland). The racemic mixtures of the aminoalcohols, pindolol, metoprolol, propranolol, oxprenolol and DPI 201-106 (structures in Table I), were obtained from Sandoz (Basle, Switzerland).

Eluents

ZGP and TEA were dissolved in acetonitrile then mixed with liquid carbon dioxide in 1:4 (v/v) ratio. The concentrations of ZGP and TEA given refer to their concentrations in the modifier solution. This must be considered when comparing the results with those from HPLC measurements.

Columns

The cyano-bonded phase Type CS-GU was obtained from Brownlee Labs. (Santa Clara, CA, U.S.A.). For the thermodynamic measurements (k', α), the column dimensions were 30 mm × 4.6 mm, whereas for the kinetic measurements (H, N), 100 mm × 4.6 mm columns were employed. The void volume was measured with acetonitrile. The total porosity, ε_T, was calculated to be 0.72.

RESULTS AND DISCUSSION

The parameters that control the retention and the selectivity in HPLC separations using ZGP as the selector have been studied in detail by Pettersson and Jeffersson[12]. Some of the results are applicable to SFC, but a series of new problems arises owing to the different solubility and distribution conditions.

Modifier solution

The retention of the 1,2-aminoalcohols can be regulated by the concentration of the counter ion, ZGP, and by the nature and the concentration of a competing amine and a polar solvent in the mobile phase. The ion pairs are formed in the mobile phase and the coupling is disturbed by protic solvents since they compete for the hydrogen-bonding function of the counter ion. The modifier must therefore be aprotic as well as polar and acetonitrile was used in this study.

The solubilities of ZGP and the amine in this solvent are, however, fairly low. The carbon dioxide used as the mobile phase must furthermore have a density above 0.7 g/cm^3 in order to prevent precipitation of the reagent. Mobile phases of this kind gave good chromatographic behaviour of the ion pairs with cyano as well as with phenyl and C_2 phases, whereas DIOL phases gave very long retention times.

Concentrations of ZGP and amine

The addition of a competing amine to the mobile phase is necessary to reduce the retention and prevent peak tailing. Triethylamine was used as in the previous HPLC studies, always in a concentration lower than that of the acidic component, ZGP. The influence of ZGP and TEA on the separation of the enantiomers of the 1,2-aminoalcohol derivative propranolol is demonstrated in Fig. 2. The separation coefficient, α, increases with increasing ZGP concentration and decreases somewhat when the TEA concentration is increased. No chiral separation was obtained at a ZGP concentration below 10 mM.

The retention change of the first enantiomer eluted is demonstrated in Fig. 2:

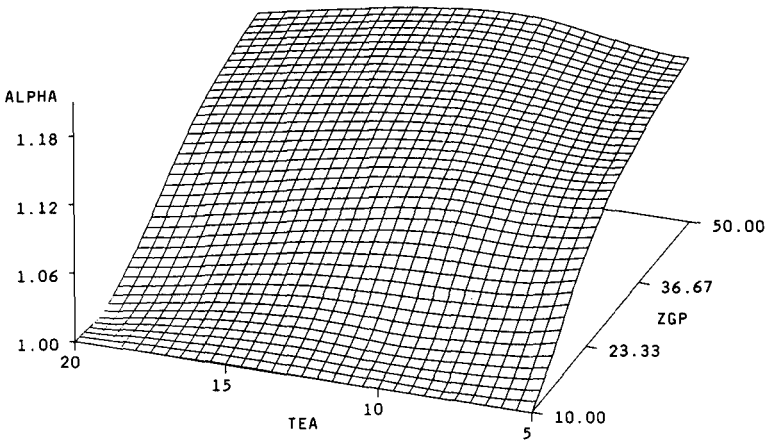


Fig. 2. Influence of ZGP and triethylamine (TEA) on the chiral selectivity, α, for antipodes of propranolol. Conditions: 70°C; 250 bar. Concentration of ZGP and TEA in m*M*.

k' increases with the concentration of the counter ion, ZGP, and decreases with the TEA concentration, in accord with the normal rules for normal-phase ion-pair HPLC.

Influence of pressure

The retention times decrease with increasing pressure as shown in Fig. 4 for four drug substances, all containing an 1,2-aminoalcohol moiety. The increase in the solvating ability of the mobile phase with increasing pressure represents a distinct advantage of SFC over HPLC, giving easy control of the retention. The versatility of the system is demonstrated in Fig. 5 which shows the resolution of two racemic

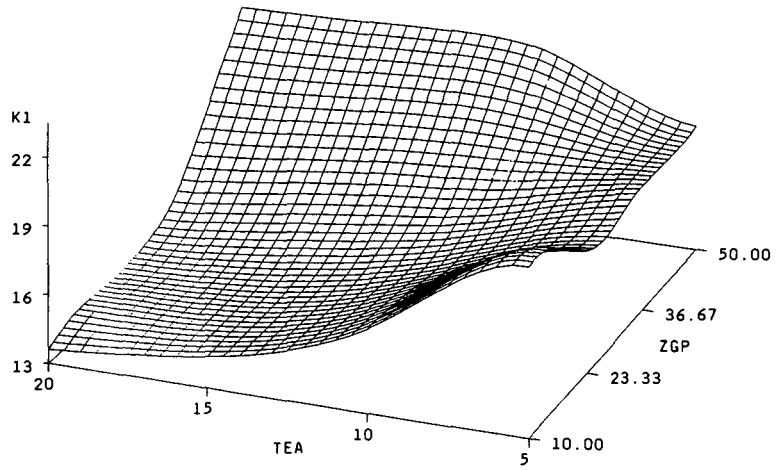


Fig. 3. Influence of ZGP and TEA on the retention of the first antipode of propranolol eluted. Conditions as in Fig. 2.

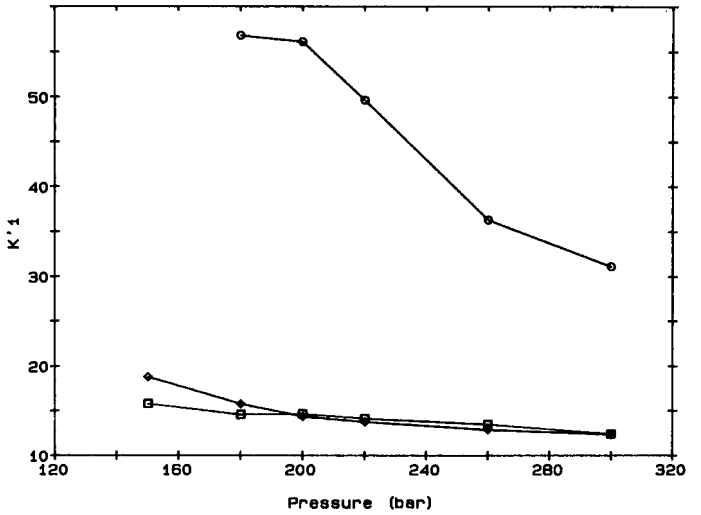


Fig. 4. Influence of pressure on the retention of the first antipode of 1,2-aminoalcohols eluted. Conditions: 30°C; 35 m*M* ZGP; 5 m*M* TEA. ○, Pindolol; □, propranolol; ◇, metoprolol.

aminoalcohols, propanolol and DPI 201-106, under subcritical conditions. The separating efficiency is also positively influenced by high flow-rates as will be discussed later.

The chiral selectivity is somewhat larger at lower than at higher pressure (Fig. 6). This might be due to changes in the stability of the ion pairs with the pressure-induced changes in the hydrophobicity of the mobile, or to changes in the distribution of ZGP in the solid phase.

Influence of temperature

The chiral selectivity decreases with increasing temperature (Fig. 7). The retention mechanism of the 1,2-aminoalcohols in this ion-pairing system has so far not been elucidated. Studies by HPLC[12] have shown that the chiral selectivity might be due to differences in the stabilities of the ion pairs in the mobile phase, as well as to differences in the retetion of the diastereomeric ion pairs by the achiral solid phase. If a similar complex retention mechanism is valid for SFC, both processes are affected by temperature changes. Further studies will be presented in a later paper.

Separating efficiencies in SFC, HPLC and subcritical chromatography

The relationships between the plate height, H, and the linear flow velocity, u, under supercritical, subcritical and HPLC conditions are shown in Fig. 8. The minimum, u_{min}, increases, as expected, drastically since the diffusivities are about 10 times higher in SFC than under HPLC conditions.

The H *vs.* u curves for supercritical and subcritical conditions cannot be well described by the van Deemter equation, probably due to the fact that isocratic conditions do not prevail in SFC. The eluting power of the mobile phase in SFC increases with increasing pressure, giving a shorter retention at the top than at the bottom of

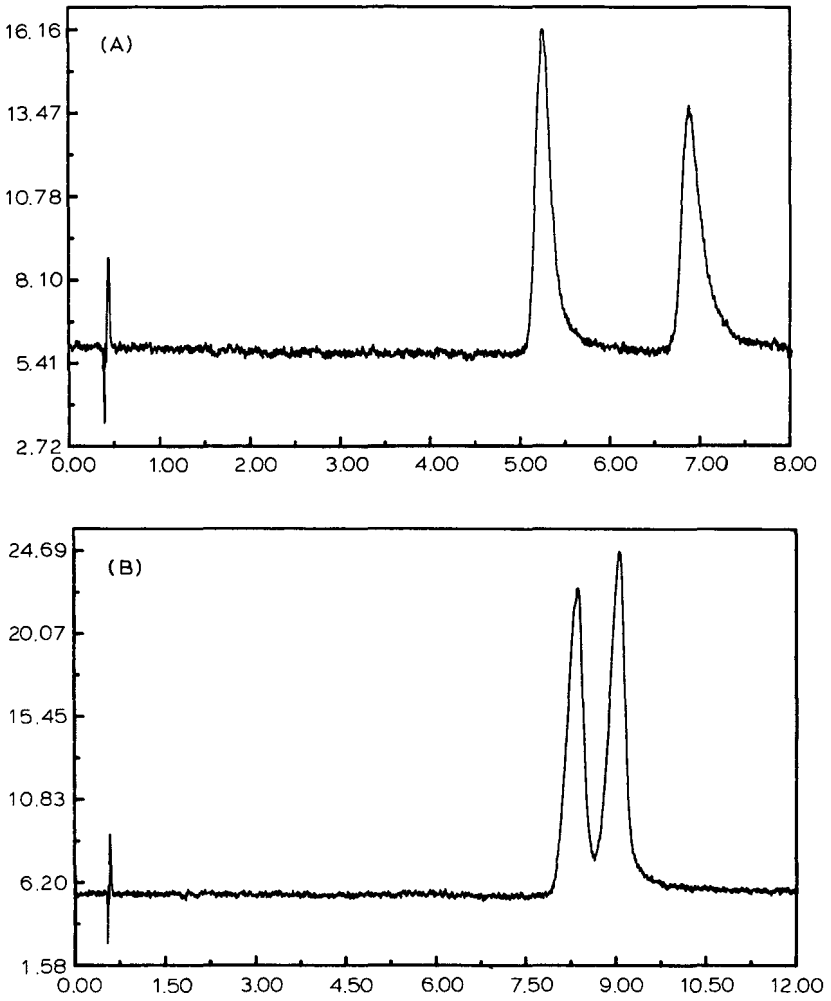


Fig. 5. Separation of antipodes of 1,2-aminoalcohols: (A) propranolol; (B) DPI 201–106. Conditions: subcritical, 21°C, 250 bar; 35 m*M* ZGP; 5 m*M* TEA; column 100 mm × 4.6 mm I.D.

the column. It seems that the peak broadening due to dispersion is superimposed by a peak compression effect similar to that in gradient elution HPLC. Further studies of this effect will be described in a later paper.

Resolution in HPLC, SFC and subcritical chromatography

The relationship between the flow-rate and resolution for the antipodes of propranolol calculated from

$$R_s = \frac{k'_2}{1 + k'_2} \cdot \sqrt{N} \cdot \frac{\alpha - 1}{4\alpha} \quad (1)$$

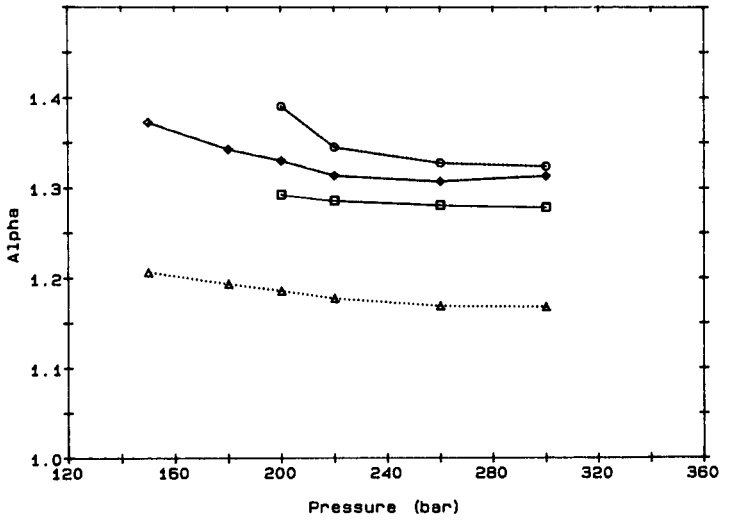


Fig. 6. Influence of pressure on the chiral selectivity for antipodes of 1,2-aminoalcohols. Conditions: 30°C; 35 m*M* ZGP; 5 m*M* TEA. ○, propranolol; ◇, metoprolol; □, pindolol; △, oxprenolol.

is demonstrated in Fig. 9. Subcritical chromatography is obviously superior not only to HPLC but also to supercritical chromatography. However, the difference between the subcritical and the supercritical modes seems to be mainly due to temperature differences since SFC at 32°C and 225 bar gives a curve similar to that obtained

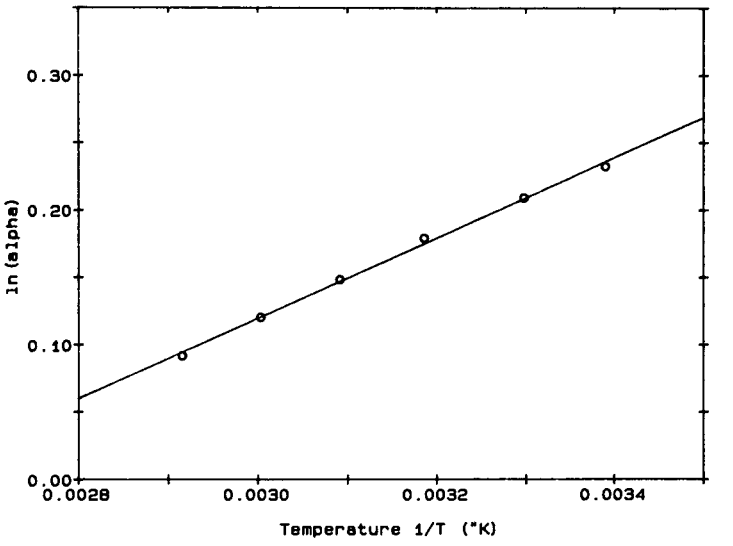


Fig. 7. Influence of temperature on the chiral selectivity for antipodes of propranolol. Conditions: 250 bar; 35 m*M* ZGP; 5 m*M* TEA.

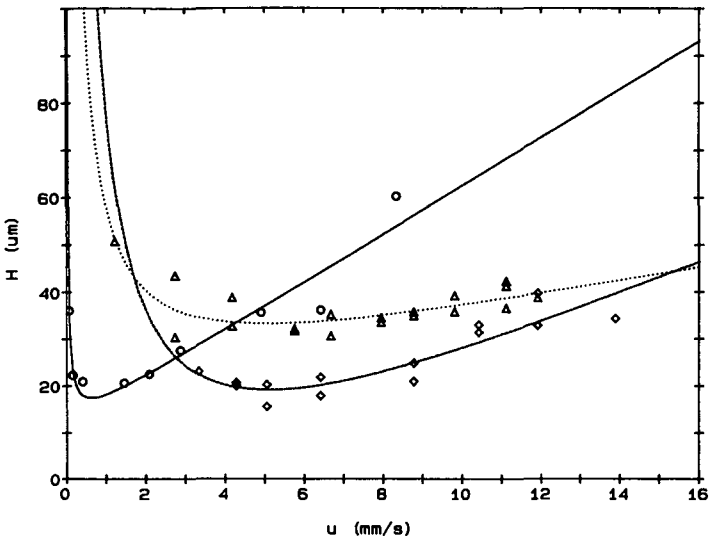


Fig. 8. Relationships between the plate height, H, and mobile phase velocity, u, for propranolol. ○, HPLC (dichloromethane; 2.5 mM ZGP, 1 mM TEA); △, subcritical (21°C, 250 bar; 35 mM ZGP, 5 mM TEA); ◇, supercritical (60°C, 225 bar, 35 mM ZGP, 5 mM TEA).

under subcritical conditions. The results indicate that it is the use of carbon dioxide and acetonitrile as the mobile phase that gives the great advantages of supercritical and subcritical chromatography over HPLC in the resolution of enantiomeric cations by the ion-pair technique.

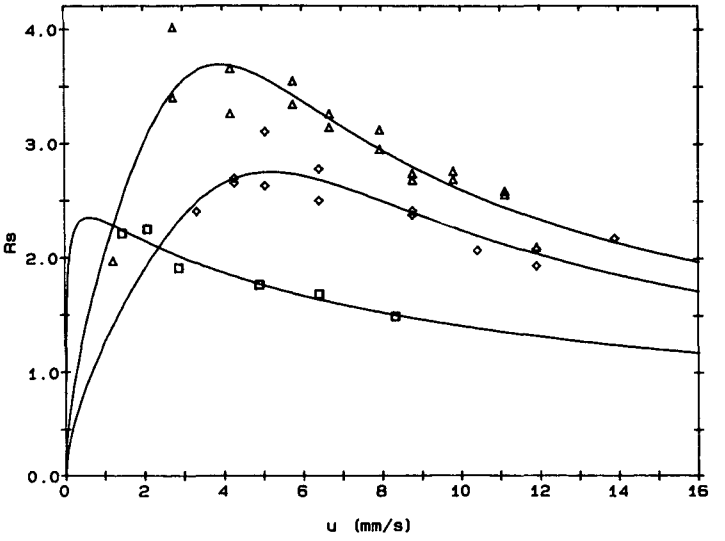


Fig. 9. Influence of the mobile phase velocity on the resolution, R_s, of antipodes of propranolol. □, HPLC (dichloromethane; 2.5 mM ZGP, 1 mM TEA); △, subcritical (21°C, 250 bar; 35 mM ZGP, 5 mM TEA); ◇, supercritical (60°C, 225 bar; 35 mM ZGP, 5 mM TEA).

CONCLUSIONS

Ion-pairing agents are suitable for the control of retention and separating efficiency of ionized organic solutes under supercritical and subcritical conditions in analogy with normal-phase HPLC. Variation of the nature and concentration of the counter ion and a competing ion, present in the mobile phase, makes the systems highly versatile and applicable to solutes of widely different structures.

On application of the technique to the separation of the antipodes of 1,2-aminoalcohols as diastereomeric ion pairs, the maximum resolution is obtained under subcritical and minimum-temperature, supercritical conditions.

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CHROM. 20 589

MICRO-LIQUID CHROMATOGRAPHY AND THE CHIRAL RECOGNITION MECHANISM ON ALBUMIN-COATED SILICA GEL

LARGE SELECTIVITY CHANGES WITH SAMPLE SIZE

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SUMMARY

The suitability of micro-liquid chromatography (micro-LC) (liquid chromatography on packed fused-silica capillary columns) for enantiomer separations was demonstrated for bovine serum albumin (BSA)-coated silica gel. Micro-LC has the great advantage of needing only very small amounts of possibly very expensive chiral stationary phases. The elution order for a series of N-nitroaroylamino acids on BSA-coated silica gel was determined. Column overload effects on BSA-coated silica gel occur at much lower concentrations than on other stationary phases. These overload effects result in strange retention time shifts whereby the later eluting peak is mostly affected. The resulting large selectivity change with sample size is unusual in LC. An explanation is attempted.

INTRODUCTION

One of the disadvantages of direct enantiomer separation by liquid chromatography (LC) on chiral stationary phases (CSPs) is that, with the current "state-of-the-art", it is difficult to predict which one of the several available phases should be used to solve a particular problem. Although with some phases, such as the Pirkle-type[1] or cyclodextrin phases[2], an *a posteriori* rationalization is possible, this is certainly not yet so for protein-based phases as introduced by Hermansson[3] and Allenmark *et al.*[4]. The enantioselectivity observed for a series of N-nitroaroylamino acids suggests that both electronic and steric conditions determine the elution order.

For such studies the miniaturization of LC, resulting in a need for smaller amounts of stationary and mobile phases, is particularly attractive. In this study micro-LC was examined[5–7].

EXPERIMENTAL

Chromatography was performed on a Varian (Walnut Creek, CA, U.S.A.) Model 5000 chromatograph equipped with a split-flow system and a 60-nl Valco

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(Houston, TX, U.S.A.) sample loop injector. A Varian 2050 variable-wavelength detector was modified for micro-LC detection as described previously[8].

Columns were constructed of 320-μm I.D. fused-silica capillaries (RSL/Altech Europe, Eke, Belgium) and packed with wide-pore 5-μm RoSil (a spherical silica gel from RSL/Altech Europe) with a mean pore size of 25 nm and a specific surface area of 50 m^2/g (data from the supplier). After slurry packing with carbon tetrachloride, followed by *n*-hexane at 500 bar, the columns were tested [typically 40 000 plates/m with dimethyl phthalate; *n*-hexane–isopropanol (95:5) at 3 μl/min], rinsed with methanol and water, equilibrated with 0.05 *M* phosphate buffer (pH 5.0) and coated with bovine serum albumin (BSA)[9] using a conventional Valco sample loop injector with a 1-ml loop. Before coating, the columns were painted black to avoid photodecomposition of the adsorbed protein.

All analyses were performed with 0.05 *M* phosphate buffer (pH 7.0) with 2% isopropanol as the eluent at flow-rates of 2.5–3 μl/min.

Analytes (N-benzoyl-, N-*p*-nitrobenzoyl-, N-*m*-nitrobenzoyl- and N-dinitrobenzoylamino acids) were prepared by esterification of commercial amino acids (DL- and L-leucine, DL- and L-alanine, DL- and L-aminobutyric acid) with methanol–thionyl chloride. The methyl esters were derivatized with various nitrobenzoyl chlorides (in acetonitrile at 65–70°C for 2 h in the presence of dimethylaminopyridine). The benzoylated esters were hydrolysed with 0.1 *M* hydrochloric acid and recrystallized from hot water.

RESULTS AND DISCUSSION

In situ stationary phase modification of micro-LC columns

One of the advantages of micro-LC[5–7] is the reduced amount of packing material needed for the column and, consequently, the reduced amount of reagent needed to modify the stationary phase by coating or derivatization. Coating a 15-cm micro-column with BSA required only a few milligrams of protein, as opposed to the several tens of milligrams needed to coat a shorter, conventional column. This approach could be especially interesting for use with expensive proteins or when refined fragments of proteins[10] are used for retention mechanism studies.

Micro-LC as optimized in this laboratory uses inner wall-coated fused-silica capillaries. The inner wall elastic polymer coatings holds the stationary phase in position, which is a very important point for column efficiency and prolonged column stability[11]. In this work with an *in situ* BSA coating, an adverse effect of the presence of an inner wall coating was observed. The results were much better without the inner wall polymer coating. Our interpretation is that, during switching from the apolar solvent used for packing and testing the columns to the polar buffer used for coating and analysis, too large differences in swelling of the inner wall coating results in rupture of the packing and, consequently, in a poor BSA coating and poor chromatography.

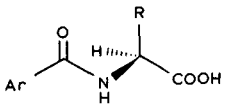
Elution order of some N-nitroaroylamino acids

Elution orders were obtained by comparing the chromatograms of the racemates with those of the corresponding pure L-enantiomers. As can be seen from Table I, the elution order is determined by both the nitroaroyl moiety and the size

TABLE I

ELUTION ORDERS OF N-NITROAROYL-SUBSTITUTED AMINO ACIDS

Eluent: 0.05 *M* phosphate buffer (pH 7.0) + 2% isopropanol.



Ar	*I* (*R* = CH_3)	*II* (*R* = $CH_2CH_2CH_3$)	*III* [*R* = $CH_2CH(CH_3)_2$]
(a) Benzoyl	L < D	D < L	D < L
(b) *m*-Nitrobenzoyl	L < D	D < L	D < L
(c) *p*-Nitrobenzoyl	L < D	L < D	D < L
(d) 3,5-Dinitrobenzoyl	L < D	L < D	L < D

of the alkyl side-chain in the amino acid. A low electron density in the aroyl substituent seems to favour retention of the D-enantiomer whereas a bulkier side-chain favours retention of the L-enantiomer.

It has been argued[1,2] that enantiomer separations on albumin-based phases are governed by hydrophobic interactions, although the role of polar interactions is recognized. Certainly the situation is complex, as is shown by the elution order reversals in Table I. The series that we investigated suggests that hydrophobic interactions may be less important than is generally acknowledged: resolution increases in the alanine series (compound I in Table II) by introducing a 3,5-dinitrobenzoyl group, the L-enantiomers eluting first. This is in agreement with the elution order

TABLE II

CAPACITY FACTORS AND SELECTIVITIES

Eluent and compound numbers as in Table I; 21 ng injected.

Compound	k'_1	k'_2	α
Ia	0.54	0.54	1.00*
Ib	0.68	0.68	1.00*
Ic	0.45	0.85	1.89
Id	0.68	2.86	4.22
IIa	0.66	1.26	0.53**
IIb	0.56	2.06	3.69**
IIc	0.69	2.17	3.13
IId	0.40	1.69	4.24
IIIa	0.87	2.23	2.58**
IIIb	0.95	1.46	1.54**
IIIc	1.55	1.89	1.22**
IIId	1.47	2.13	1.44

* The elution order could be established at lower concentration.

** Elution order reversal (D < L).

found by Allenmark and Andersson[13] for N-(2,4-dinitrophenyl)amino acids. On the other hand, they found that replacing the benzoyl group in Ia by the more electron-dense naphthoyl group caused an elution order reversal, the D-enantiomer eluting before the L-enantiomer[12].

Overload effects

The low capacity for preparative-scale applications of albumin-coated silica gel phases has been mentioned previously[9]. It is common to express sample loading as the amount of analyte injected onto a given amount of stationary phase. Although more fundamental expressions have been proposed[14], this is practical as a rule-of-thumb for daily chromatographic work. When comparing different stationary phases, some conditions are assumed: a fixed specific surface area, as determined by the silica gel, and a fixed adsorption site surface concentration, as determined by the nature of the adsorbing monolayer. With its molecular weight of 65 000, BSA as the active monolayer influences both parameters. The need for larger pores in the silica gel, to make the surface accessible[15], results in a lower specific surface area[16]. In proteins, as opposed to polymers such as cellulose, which are built from repetitive units, it can be assumed that the number of adsorption sites does not increase with increasing molecular weight, as these sites probably occur at a few specific, local geometric configurations. A single BSA molecule will cover a relatively large portion of the surface, but contributes only a single unit, or only very few units, to the number of adsorption sites. With these assumptions, the low capacity of protein phases is understandable.

Figs. 1 and 2 show overload effects, observed here as a decrease in the capacity factors of the enantiomers in a racemic mixture with increasing sample loading. On a conventional 15 × 0.46 cm I.D. column, holding 1–2 g of stationary phase, a 10-μl injection of a 0.1% solution would result in a column loading of 5–10 μg of compound per gram of silica gel. To obtain the same level of loading on a micro-LC column, holding 5–10 mg of stationary phase, only 25–50 ng of injected material are needed, which is arrived at by reducing the injection volume to 60 nl. On most high-capacity stationary phases, such as octadecylated silica gel, even a multiple of that loading level would still be regarded as analytical, *i.e.*, free from mass overload effects (linear chromatography). That this is not so with BSA-coated silica gel simply reflects the low capacity of this kind of phase.

More surprisingly, it is the retention time of the most retained enantiomer that is most drastically changed by higher concentrations (Fig. 2). This resembles the "tag-along" effect, recently described by Ghodbane and Guiochon[17]. However, this would require that the first eluting compound blocks adsorption sites. Being less adsorbed, it could only do so by virtue of a numerical predominance, a situation that is unlikely for a racemate.

Another explanation would be that, superimposed on a more general retention mechanism, similar for both enantiomers, enantioselectivity is obtained through adsorption sites that interact nearly uniquely with one enantiomer. Accordingly, the more retained enantiomer shows an overload behaviour, independent of the first, and without displacement. In turn, this effect could serve as a measure of the chiral specificity of the interactions. This specificity seems to contrast with the broad range of compounds resolvable on a single protein phase, but it is significant that serum

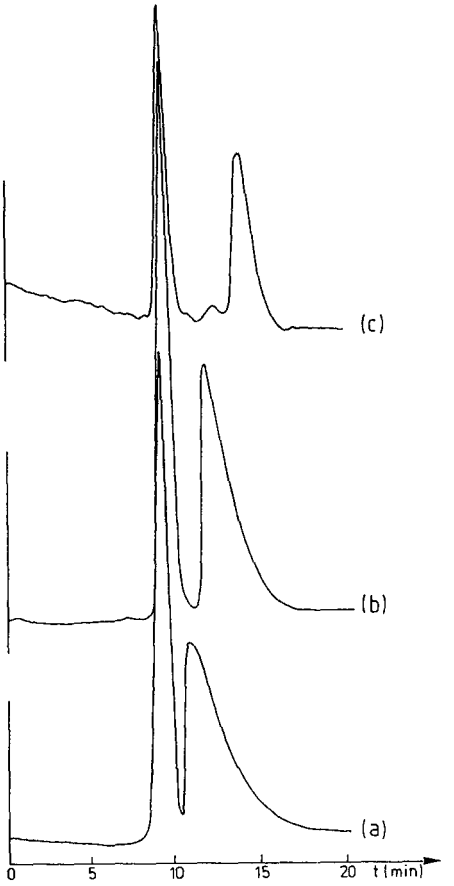


Fig. 1. Resolution of 3,5-dinitrobenzoylleucine on BSA-coated silica gel. Amounts injected: (a) 60; (b) 30; (c) 3.8 ng.

albumin is not an enzyme, with a unique substrate, but acts as a carrier of small molecules and is known to bind many ligands[18].

Apparently, our findings are not restricted to the type of compounds that we investigated, but apply also to other compounds such as DL-benzoin, as can be judged from Fig. 9 in ref. 9. Overloading was observed with relatively small samples (calculated to be below 12.5 μg per gram of silica gel), causing a decrease in resolution, but no comment was made on the unusual nature of the change in selectivity (α). The differential changes in capacity factors, discussed above, lead to changes in α that are dependent on sample size (Fig. 3). A similar case has been described for a preparative separation on a Pirkle phase[19], but in that study, covering a much larger sample load range (1/50 000), a 20% reduction in α also accompanied a 50% reduction in the capacity factor of the first-eluting enantiomer. In at least one instance (DL-3,5-dinitrobenzoylleucine), we found a 30% reduction in α, with only a negligible change in the capacity factor of the first-eluting enantiomer.

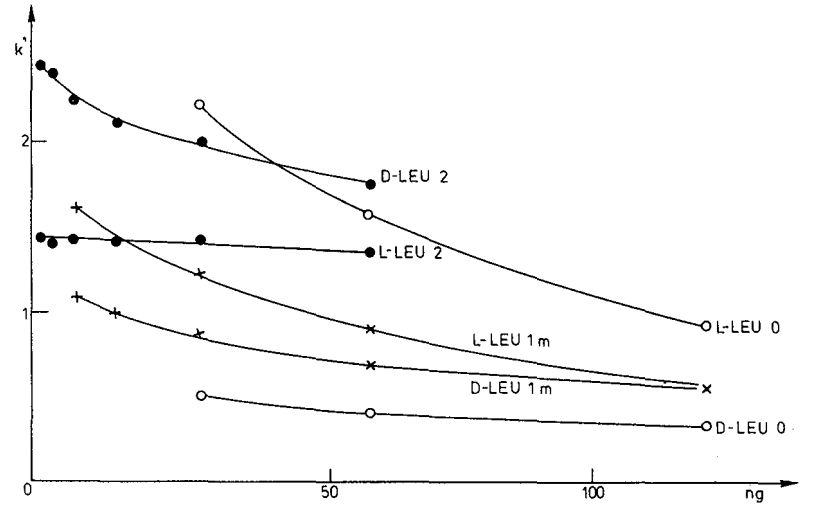


Fig. 2. Influence of amount injected on capacity factors. LEU 2 = 3,5-dinitrobenzoylleucine; LEU 1m = *m*-nitrobenzoylleucine; LEU 0 = benzoylleucine.

Given the large change in the capacity factor with the Pirkle phase, the change in α could be ascribed to subtle differences in the non-linear behaviour that occurs for both enantiomers. On BSA, however, the fact that the two enantiomers behave more independently suggests that at least one of the interactions is more chirally specific in comparison with the Pirkle phase.

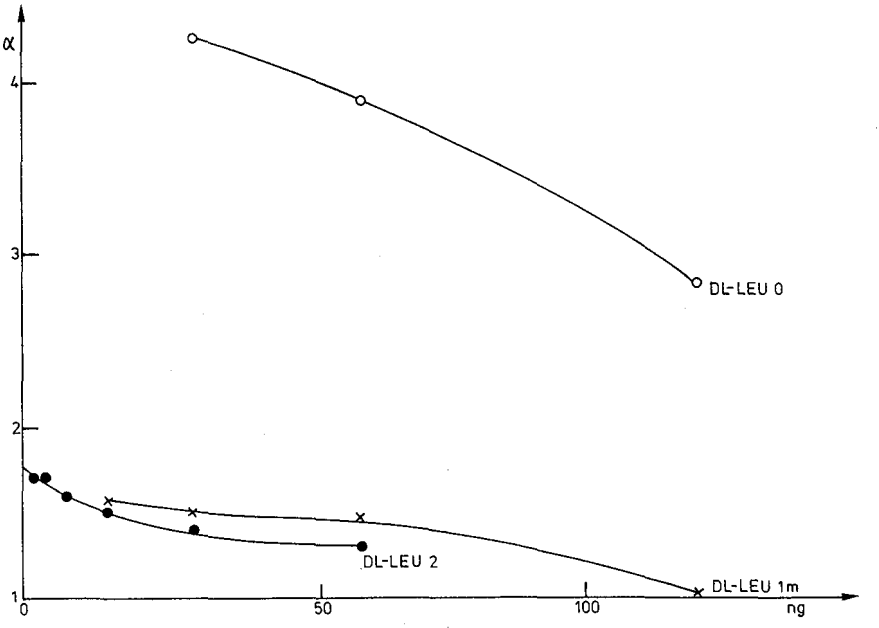


Fig. 3. Influence of amount injected on selectivity. Abbreviations as in Fig. 2.

Unsuspected overload effects at low sample loadings may help to explain why the resolution of some compounds varies with the origin or mode of preparation of the albumin phase[9], without having to assume structural changes in the albumin. Possibly, a quantitative description of overload effects could yield more information on the reversals in elution order described above, and would allow different adsorption sites of BSA, BSA fragments or proteins in general to be recognized. It should be obvious that micro-LC is best suited for such investigations.

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CHROM. 20 586

EQUIVALENT CHAIN-LENGTHS OF METHYL ESTER DERIVATIVES OF FATTY ACIDS ON GAS CHROMATOGRAPHY

A REAPPRAISAL

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(First received February 9th, 1988; revised manuscript received April 22nd, 1988)

SUMMARY

The equivalent chain-lengths of the methyl ester derivatives of synthetic isomeric unsaturated fatty acids in addition to many from natural sources have been determined by gas chromatography (GC) with modern wall-coated open-tubular columns of fused silica coated with Carbowax 20M™, Silar 5CP™, CP-Sil 84™ and a 5% phenyl–methyl silicone. The complete series of *cis*-octadecenoates (3- to 17-, but not the 2-), the methylene-interrupted *cis,cis*-octadecadienoates (3,6- to 14,17- but not the 2,5-isomer) and some *cis,cis*-octadecadienoates with more than one methylene group between the double bonds (5,12-, 6,12-, 7,12-, 8,12-, 6,10- and 6,11-) were used, together with natural fatty acids from pig testis, rich in the $(n-6)$ family, and from cod liver oil, rich in the $(n-3)$ family. There were 79 fatty acids in total. The availability of all these data from a single laboratory at one time allows a reassessment of the value of GC equivalent chain-lengths for the identification of unknown fatty acids, and of stationary phases of particular types for fatty acid analysis in general.

INTRODUCTION

The carbon number[1] or equivalent chain-length (ECL) concepts[2] for the identification of fatty acids separated in the form of the methyl ester derivatives by gas chromatography (GC) have proved of great value to analysts. An enormous amount of data has been produced, and this has been reviewed comprehensively by Jamieson[3] and by Ackman[4,5]. The nature of the stationary phase is the primary factor determining retention values, but nominally identical phases from different manufacturers and even from different batches can vary somewhat. In packed column work especially, it is well established that ECL values are rather susceptible to such factors as the age and conditioning of the column, column dimensions, the loading of the phase on the inert support, and the nature of the support[3]. Operational factors such as column temperature and the nature and flow-rate of the carrier gas are also important. Much smaller but real differences are seen with wall-coated open-tubular (WCOT) columns made from fused silica, as opposed to stainless steel[5]. It is not

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surprising, therefore, that appreciable variation in ECL values are reported between laboratories. Some of the more useful studies of the GC retention properties of fatty acids have been made with synthetic isomeric compounds. For example, data are available for the complete series of *cis*- and *trans*-octadecenoates, all the methylene-interrupted octadecadienoates, and for some octadecadienoates with more than one methylene group between the double bonds[3–5]. Unfortunately, many of the relevant measurements were performed at different times in different laboratories around the world. In addition, a high proportion of the determinations were made twenty years or so ago, and there has been a dramatic change in the nature of the stationary phases in common use and in the physical nature of the columns during the intervening period. For example, fused-silica WCOT columns are now widely available and are likely to become the standard, and the inherent resolution of these is such that there has been a trend towards liquid phases of much lower polarity than is the common practise with packed columns.

The author recently had access to a number of different isomeric C_{18} fatty acids, including most of the isomeric *cis*-octadecenoates and methylene-interrupted *cis,cis*-octadecadienoates, and some *cis,cis*-octadecenoates with more than one methylene group between the double bonds for a study of their mass spectrometric (MS) properties[6,7]. In addition, two natural samples, namely cod liver oil and pig testis lipids, containing a wide range of different unsaturated fatty acids, were characterised by related GC–MS procedures and were available to extend the scope of this study[8,9]. ECL values have now been obtained for all of these fatty acids as the methyl esters over a short period in one gas chromatograph with modern WCOT columns of fused silica and coated with liquid phases in current use. Strict comparisons are, therefore, possible.

EXPERIMENTAL

Isomeric *cis*-octadecenoates (3- to 17-isomers)[10], methylene-interrupted octadecadienoates (3,6- to 14,17-isomers)[11] and 5,12-, 6,12-, 7,12-, 8,12-, 6,10- and 6,11-octadecadienoates[12] had been prepared earlier by total synthesis. Pig testis lipids and cod liver oil samples were from a previous study[8]; these were methylated and then were fractionated according to degree of unsaturation by high-performance liquid chromatography with a silver ion column as described elsewhere[13].

A Carlo Erba Model 4130 capillary gas chromatograph (Erba Science, Swindon, U.K.), fitted with split/splitless injection and equipped with capillary columns of fused silica coated with various stationary phases, was used. Columns coated with Carbowax 20M™, Silar 5CP™ and CP-Sil 84™ (25 m × 0.22 mm I.D.) were obtained from Chrompak (London, U.K.), and one coated with a 5% phenyl–methyl silicone (25 m × 0.2 mm I.D.) was obtained from Hewlett-Packard (Wokingham, U.K.). The Silar 5CP column had been in use for some months, but the remainder were new and were conditioned in the gas chromatograph for 48 h before use. Hydrogen was the carrier gas in each instance at a flow-rate of 1 ml/min. For ECL measurements, isothermal conditions were used, selected so that the longest running component [usually the 22:6 ($n-3$) fatty acid] emerged in about 1 h, *i.e.*, silicone at 175°C, Carbowax 20M at 175°C, Silar 5CP at 170°C, and CP-Sil 84 at 160°C. Samples were injected in hexane, and a small amount of a standard solution of 16:0, 18:0 and

20:0 fatty acid methyl esters was introduced simultaneously. Retention times were measured from the time of elution of the solvent, considered as an unretained solute. Each ECL value was determined in duplicate, but they were rarely more than 0.01 of a unit apart.

For purely analytical runs with the natural lipid materials and the Carbowax 20M column, the temperature was held at 165°C for 3 min, it was temperature programmed at 4°C/min to 195°C, then was held at this point for a further 23 min. With the CP-Sil 84 column, the temperature was maintained at 150°C for 2 min, it was temperature progrtammed at 2°C/min to 180°C, then was held at this point for a further 10 min.

RESULTS AND DISCUSSION

The selection of the most appropriate phases for this study was not easy, because of the range available, even for WCOT columns of fused silica. It was certainly essential to have a column of Carbowax 20M, since Ackman[5] has proposed that phases of the Carbowax 20M™ type should be utilised in the "'standard' reference WCOT column for interlaboratory studies as well as for application in its own right". Phases such as FFAP™, Supelcowax-10™, and SP-1000™, for example, are very similar to this. Silar 5CP was selected as a common phase, slightly more polar than Carbowax 20M, and with somewhat different selectivity. CP-Sil 84 is a much more polar phase, similar in its properties to the widely used polymer of diethyleneglycol-succinate (DEGS). A non-polar silicone phase was selected as some analysts favour its use for fatty acid analysis[14], and its low bleed characteristics render it particularly suitable for GC–MS purposes.

The ECL values of each of the isomeric C_{18} fatty acids were obtained and the results are listed in Table I. While the absolute values differ for each stationary phase, increasing with the polarity of the phase, the elution patterns are broadly similar. Thus the ECL values are lowest when the double bonds are approximately central, *i.e.* in positions 8 or 9. They increase relatively rapidly as the double bonds near the terminal (methyl) end of the molecule, reaching a maximum with 16–18:1, before falling slightly for the 17-isomer. Similarly, the ECL values increase, although rather more slowly, as the double bonds near the carboxyl group and reach a second lower maximum at 3-18:1. They would then be expected to drop for the 2-isomer[15,16]. In addition, the ECL value of the 6-isomer is sometimes slightly out of line, being higher than those of adjacent isomers. The results are similar in nature to those obtained earlier with very different columns[15,16]. A theoretical explanation of the phenomenon in terms of the shapes of molecules and the opportunities for interaction between the double bonds and the walls of a WCOT column has been published[17].

As isomers differing in ECL value by about 0.04 should be separable on most WCOT column, it would be expected that those fatty acids with central double bonds (about 4-18:1 to 9-18:1) will not be easily resolved. Petroselinic (6-18:1) and oleic occur together in some seed oils and are not readily resolved by GC[18]. In the monoenoic fatty acids from animal tissues, there tend to be isomers in which the double bond positions are two carbon atoms apart, because they are formed biosynthetically from homologous fatty acids by chain-elongation or by β-oxidation, in each instance the difference being two carbon atoms. Thus 16:1($n-9$) and 16:1($n-7$), 18:1($n-9$)

TABLE I

EQUIVALENT CHAIN-LENGTHS OF THE METHYL ESTER DERIVATIVES OF ISOMERIC C_{18} MONO- AND DIENOIC FATTY ACIDS

Fatty acid	*Stationary phase*			
	Silicone	*Carbowax*	*Silar 5CP*	*CP-Sil 84*
3-18:1	17.91	18.44	18.47	18.64
4-18:1	17.80	18.19	18.21	18.30
5-18:1	17.72	18.09	18.17	18.29
6-18:1	17.75	18.18	18.25	18.43
7-18:1	17.72	18.14	18.24	18.40
8-18:1	17.72	18.14	18.29	18.43
9-18:1	17.73	18.16	18.30	18.47
10-18:1	17.75	18.19	18.32	18.49
11-18:1	17.78	18.23	18.36	18.54
12-18:1	17.83	18.30	18.46	18.62
13-18:1	17.89	18.37	18.52	18.67
14-18:1	17.95	18.46	18.57	18.76
15-18:1	18.00	18.56	18.62	18.83
16-18:1	18.19	18.84	18.91	19.14
17-18:1	17.94	18.54	18.61	18.82
3,6-18:2	17.73	18.69	18.74	19.14
4,7-18:2	17.58	18.43	18.48	18.82
5,8-18:2	17.53	18.36	18.48	18.85
6,9-18:2	17.59	18.47	18.63	19.04
7,10-18:2	17.57	18.45	18.70	19.02
8,11-18:2	17.61	18.50	18.75	19.12
9,12-18:2	17.65	18.58	18.80	19.20
10,13-18:2	17.73	18.67	18.88	19.30
11,14-18:2	17.81	18.79	18.98	19.41
12,15-18:2	17.89	18.91	19.08	19.51
13,16-18:2	18.12	19.28	19.41	19.90
14,17-18:2	17.90	18.98	19.06	19.52
5,12-18:2	17.56	18.40	18.60	18.93
6,12-18:2	17.60	18.48	18.69	19.06
7,12-18:2	17.56	18.40	18.64	18.99
8,12-18:2	17.63	18.49	18.70	19.07
6,10-18:2	17.58	18.42	18.61	18.94
6,11-18:2	17.52	18.38	18.62	18.93

and 18:1($n-7$), and 20:1($n-11$), 20:1($n-9$) and 20:1($n-7$) are frequently found together, as in the cod liver oil sample, and they are usually separable.

With the C_{18} dienes and each of the phases examined (Table I) also, the ECL values tended to increase with the distance of the double bonds from the carboxyl group, though there are discontinuities for the 3,6- and 13,16-isomers, where the ECL values are higher than those of adjacent compounds (*c.f.* the data for the 3- and 16-monoenoic isomers). The 2,5-isomer was not available, but its ECL values would be expected to be lower than those of the adjacent (3,6-isomer). Again the pattern is qualitatively similar to that obtained in older work with very different columns[19].

As the first natural substrate for analysis, pig testis lipids were selected, as the fatty acids have been well-characterised and have been used as an external standard in the Hormel Institute for some years[20,21]; they contain a wide range of fatty acids of the $(n-6)$ series, encountered typically in animal tissues. The second substrate is cod liver oil, which has also been well-characterised and recommended as an external standard in the analysis of lipids of marine origin[22]; it contains a wide range of fatty acids, and especially those of the $(n-3)$ family. Both of these materials were used by the author in studies of the efficacy of picolinyl ester derivatives of fatty acids for identification by GC–MS[8,9]. Together, they contain most of the fatty acids likely to be encountered in the common animal tissues. The ECL values were determined on fractions obtained by silver ion chromatography in order to simplify identifications and as the retention times of esters are influenced to some extent by components eluting immediately adjacent to them. The data is listed in Table II and is entirely consistent with that for the synthetic standards. Data such as these from WCOT columns of fused silica probably have greater relevance to other laboratories than those from packed columns. Absolute values may vary somewhat among laboratories, but the order of elution should not change.

One major advantage of determining all these ECL values at the same time in a single laboratory is that strict comparisons can be made. Thus, if the fractional chain-length (FCL) values (*i.e.* ECL minus 18) for the complete series of C_{18} monoenes is used to predict the ECL values for the dienes, the calculated value is generally somewhat lower than that actually found. This probably means that there is some interaction (possibly homo-conjugation) between the double bonds or with the diallyl methylene group, that increases the dipole moment of the unsaturated system. Comparable results were obtained in other studies, and the same principle held whether the double bonds were of the *cis*- or the *trans*-configuration[3–5]. The differences between the actual and predicted ECL values for each of the dienes is listed in Table III. With the methylene-interrupted isomers, the discrepancy tends to vary with the position of the double bonds as well as with the stationary phase, and is highest for double bonds in positions 5–11 (0.10–0.15) and diminishes towards either end of the molecule. Similarly, when the FCL values from the monoene data are used to calculate ECL values for the non-methylene-interrupted dienes, the difference between the actual and predicted results was found to be small, *i.e.* of the order of 0.05–0.08, when there are two methylene groups between the double bonds with the Carbowax and silicone liquid phases; it becomes negligible, *i.e.* 0.00–0.02 or even negative, when there are more than two methylene groups with all the phases. Again, these data are similar in kind to those reported earlier.

The FCL values for monoenes together with factors for the interaction with the appropriate methylene groups (the difference between the actual and predicted results for the dienes) have been used by Ackman *et al.*[23] especially for the prediction of ECL values, *e.g.* for the identification of an 18:5$(n-3)$ in a marine alga. If the ECL values obtained here are used in this way, using the data from Tables I and III, it would be predicted that an ECL value for an 18:4$(n-3)$ fatty acid on Carbowax 20M, for example, would be equal to 18 plus the FCL values (0.18 + 0.16 + 0.30 + 0.56 = 1.20) plus the methylene group factors (0.13 + 0.12 + 0.05 = 0.30), *i.e.* ECL = 19.50; the actual value found by direct measurement (Table II) is 19.45. With the silicone phase for this acid, the calculated and actual values were only 0.02 units

TABLE II

EQUIVALENT CHAIN-LENGTHS OF THE METHYL ESTER DERIVATIVES OF SOME NATURAL FATTY ACIDS

No.	*Fatty acid*	*Stationary phase*			
		Silicone	*Carbowax*	*Silar 5CP*	*CP-Sil 84*
1	14:0	14.00	14.00	14.00	14.00
2	14-*isobr*	14.64	14.52	14.52	14.51
3	14-*anteiso*	14.71	14.68	14.68	14.70
4	14:1(*n*−5)	13.88	14.37	14.49	14.72
5	15:0	15.00	15.00	15.00	15.00
6	16:0	16.00	16.00	16.00	16.00
7	16-*isobr*	16.65	16.51	16.51	16.50
8	16-*anteiso*	16.73	16.68	16.68	16.69
9	16:1(*n*−9)	15.76	16.18	16.30	16.48
10	16:1(*n*−7)	15.83	16.25	16.38	16.60
11	16:1(*n*−5)	15.92	16.37	16.48	16.70
12	16:2(*n*−4)	15.83	16.78	16.98	17.47
13	16:3(*n*−3)	15.69	17.09	17.31	18.06
14	16:4(*n*−3)	15.64	17.62	17.77	18.82
15	17:0	17.00	17.00	17.00	17.00
16	17:1(*n*−9)	16.76	17.20	17.33	17.50
17	17:1(*n*−8)	16.75	17.19	17.33	17.51
18	18:0	18.00	18.00	18.00	18.00
19	18:1(*n*−11)	17.72	18.14	18.24	18.40
20	18:1(*n*−9)	17.73	18.16	18.30	18.47
21	18:1(*n*−7)	17.78	18.23	18.36	18.54
22	18:2(*n*−6)	17.65	18.58	18.80	19.20
23	18:2(*n*−4)	17.81	18.79	18.98	19.41
24	18:3(*n*−6)	17.49	18.85	19.30	19.72
25	18:3(*n*−3)	17.72	19.18	19.41	20.07
26	18:4(*n*−3)	17.55	19.45	19.68	20.59
27	19:1(*n*−8)	18.74	19.18	19.32	19.47
28	20:1(*n*−11)	19.67	20.08	20.22	20.35
29	20:1(*n*−9)	19.71	20.14	20.27	20.41
30	20:1(*n*−7)	19.77	20.22	20.36	20.50
31	20:2(*n*−9)	19.51	20.38	20.59	20.92
32	20:2(*n*−6)	19.64	20.56	20.78	21.12
33	20:3(*n*−9)	19.24	20.66	20.92	21.43
34	20:3(*n*−6)	19.43	20.78	21.05	21.61
35	20:3(*n*−3)	19.71	20.95	21.22	21.97
36	20:4(*n*−6)	19.23	20.96	21.19	21.94
37	20:4(*n*−3)	19.-47	21.37	21.64	22.45
38	20:5(*n*−3)	19.27	21.55	21.80	22.80
39	22:1(*n*−11)	21.61	22.04	22.16	22.30
40	22:1(*n*−9)	21.66	22.11	22.23	22.36
41	22:3(*n*−9)	21.20	22.52	22.78	23.25
42	22:3(*n*−6)	21.40	22.71	22.99	23.47
43	22:4(*n*−6)	21.14	22.90	23.21	23.90
44	22:5(*n*−6)	20.99	23.15	23.25	24.19
45	22:5(*n*−3)	21.18	23.50	23.92	24.75
46	22:6(*n*−3)	21.04	23.74	24.07	25.07

TABLE III

DIFFERENCES BETWEEN THE CALCULATED AND ACTUAL EQUIVALENT CHAIN-LENGTH VALUES FOR THE METHYL OCTADECADIENOATES

Fatty acid	*Stationary phase*			
	Silicone	*Carbowax*	*Silar 5CP*	*CP-Sil 84*
3,6-18:2	0.07	0.07	0.02	0.07
4,7-18:2	0.06	0.10	0.03	0.12
5,8-18:2	0.09	0.13	0.02	0.13
6,9-18:2	0.11	0.13	0.08	0.14
7,10-18:2	0.10	0.12	0.14	0.13
8,11-18:2	0.11	0.13	0.10	0.15
9,12-18:2	0.09	0.12	0.04	0.11
10,13-18:2	0.09	0.11	0.04	0.14
11,14-18:2	0.08	0.10	0.05	0.11
12,15-18:2	0.06	0.05	0.00	0.06
13,16-18:2	0.04	0.07	−0.02	0.09
14,17-18:2	0.01	−0.02	−0.12	−0.06
5,12-18:2	0.01	0.01	−0.03	0.02
6,12-18:2	0.02	0.00	−0.02	0.01
7,12-18:2	0.01	−0.04	−0.06	−0.03
8,12-18:2	0.08	0.05	−0.05	0.02
6,10-18:2	0.08	0.05	0.04	0.02
6,11-18:2	0.01	0.03	0.01	0.04

apart, while with the more polar phases the difference was 0.07 in each instance. The results, therefore, confirm the value of this approach to the identification of unknowns provided that the primary data are of sufficient accuracy.

The real value of various phases in WCOT columns for fatty acid analysis can only be judged when they are applied to authentic samples of natural origin. The separation of the methyl esters of the fatty acids of cod liver oil on the Carbowax 20M column in a temperature-programmed run is illustrated in Fig. 1. Each of the main chain length groups is reasonably well resolved. For example, two 16:1 isomers are seen and they are distinct from the C_{17} branched and unsaturated fatty acids. Similarly the important C_{18} components are well separated from each of the C_{20} unsaturated constituents. With the last, the only serious overlap problem is with 20:3($n-3$), which co-chromatographs with 20:4($n-6$); these are, however, just separable on a slightly more polar Silar 5CP column. Others did not find this specific separation to be a problem[24]. Finally, all the biologically-important C_{22} fatty acids are cleanly separated. An equally good results was seen with the pig testis fatty acids.

With the more polar CP-Sil 84 column, there is again excellent resolution of the pig testis fatty acids (Fig. 2). Individual unsaturated esters are particularly well resolved, and for example there is near base-line separation of 18:1($n-9$) and 18:1($n-7$). On the other hand, 18:3($n-6$) emerges after the minor C_{19} fatty acid. The C_{20} group are all well separated from each other but are beginning to run into an area occupied by C_{22} fatty acids. This last effect can be more troublesome with fish oils, which contain appreciable amounts of 22:1 isomers as in the cod liver oil

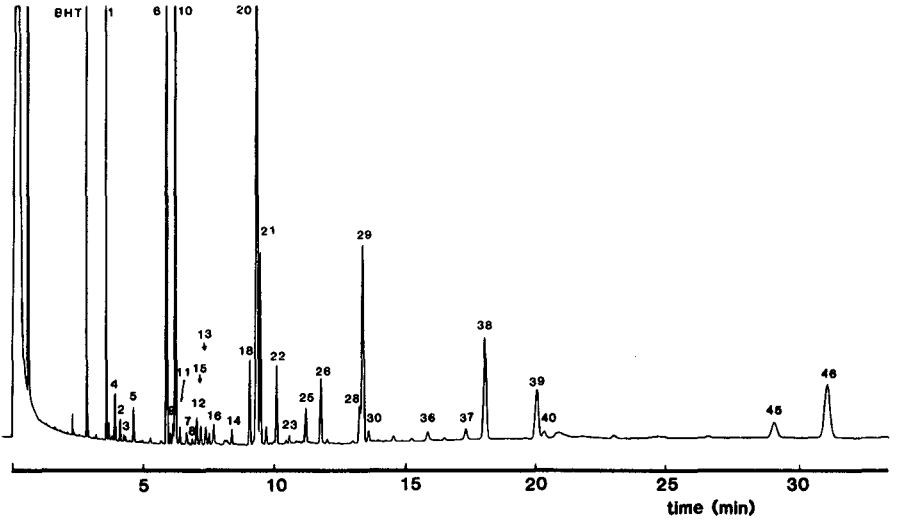

Fig. 1. GC separation of the methyl ester derivatives of the fatty acids of cod liver oil on a WCOT column of fused silica coated with Carbowax 20M. Chromatographic conditions are given in the Experimental section. The number above each peak for identification refer to the listing in Table II.

sample (not shown). The latter compounds emerge just before the C_{20} polyene, 20:4($n-3$). Similarly the C_{16} polyenes elute among the C_{18} fatty acids, and the C_{18} polyenes run into the C_{20} fatty acids. The C_{16} branched and monoenoic constituents tend to co-chromatograph. Nonetheless, with tissue lipids from plants and terrestrial animals especially, the polar column gives excellent results provided that care is taken in identifying the fatty acids.

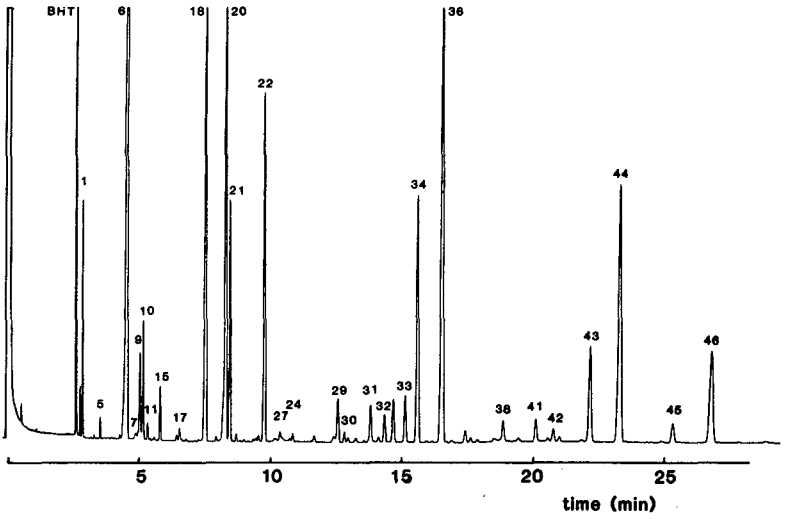

Fig. 2. GC separation of the methyl ester derivatives of the fatty acids of pig testis on a WCOT column of fused silica coated with CP-Sil 84. Chromatographic conditions are given in the Experimental section. The numbers above each peak for identification refer to the listing in Table II.

The nature of the separation attained on non-polar columns is rather different from that with polar columns. Unsaturated components emerge ahead of saturated fatty acids of the same chain-length. Isomeric fatty acids differing in the positions of double bonds are usually clearly resolved, thus 18:1($n-9$) and 18:1($n-7$) are separated as are many of the polyenes. Indeed, the C_{22} fatty acids are possibly almost as well separated as on a polar column of the same length. Unfortunately, there are substantial overlaps among the C_{18} fatty acids, and 18:2($n-6$) is not fully resolved from 18:1($n-9$); 18:2($n-6$) and 18:3($n-3$) merge completely, and this is also true of the corresponding C_{20} and C_{22} compounds. As linoleate and linolenate are essential fatty acids with major nutritional importance, the deficiency in this aspect of the separation is likely to mitigate against a wider use of non-polar column. The order of elution of the C_{22} components is not that which might expected intuitively, *i.e.* 22:5($n-6$), 22:6($n-3$), 22:4($n-6$) and 22:5($n-3$). In this instance, it appears that the position of the double bonds has a greater effect on retention time than does the number of double bonds.

Nearly all analysts then are going to make use of polar stationary phases for the major proportion of their work. The chromatographic traces illustrated here lend support to Ackman's view that Carbowax 20M is the best general purpose stationary phase in WCOT columns for fatty acid analysis. Non-polar phases do have advantages in specific applications, *e.g.* with fatty acids of high molecular weight or containing thermally-labile functional groups and perhaps for certain *cis/trans* separations, where their stability at high temperatures and their considerable degree of inertness are virtues.

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ANION CHROMATOGRAPHY USING A COATED PRP-1 COLUMN AND ELUENTS OF pH > 7

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SUMMARY

A non-polar stationary phase (PRP-1) modified with cetrimide was used for the high-performance liquid chromatographic separation of anions. The retention behaviour of common anions (F^-, Cl^-, NO_2^-, Br^-, NO_3^-, SO_4^{2-} and HPO_4^{2-}) and some weakly dissociated anions ($H_2AsO_3^-$, $HAsO_4^{2-}$, $H_2BO_3^-$, HS^-, HCO_3^- and CO_3^{2-}) was studied using aqueous eluents containing 24% (v/v) methanol, potassium hydrogenphthalate (suitable for indirect UV detection) and tris(hydroxymethyl)-aminomethane (pH > 7).

INTRODUCTION

One of the drawbacks of published methods for the chromatographic determination of weak acid anions is that the eluent is only suitable for fast-running components (*e.g.*, F^-, silicate, borate, arsenite) and is not good enough for bivalent anions (*e.g.*, SO_4^{2-}, arsenate) or, if the eluent is powerful enough for multivalent ions, it gives a poor resolution of anions with short retention times.

In our view, coated anion-exchange columns give more flexibility than anion-exchange columns with a fixed capacity and functional groups. With these columns eluents of pH < 7 have mostly been used[1–3]. Separations with eluents of higher pH were reported by Wheals[4] in 1987. Anions were separated with acetonitrile–citrate at pH 11 on a poly(styrene–divinylbenzene) stationary phase dynamically coated with hexadecyltrimethylammonium hydroxide. Detection was effected by measuring the UV absorbance (220 nm) and with electrochemical cells connnected in series.

The method presented here deals with the separation of anions on a PRP-1 column coated permanently with cetrimide and with indirect UV absorption detection. When a phthalate–Tris eluent of pH > 7 is used, then not only common anions but also anions of weak acids can be chromatographed and monitored with indirect detection.

In a previous paper[5] the reasons for the appearance of system peaks and their

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chromatographic behaviour were investigated. If the mobile phase contains tris-(hydroxymethyl)aminomethane (Tris) buffer in addition to potassium hydrogenphthalate, then only one system peak ($S_{phthalate}$) is obtained. In an eluent of pH 8.5 the retention time of $S_{phthalate}$ is small and, being near the dead volume, it does not affect the chromatographic detection of anions.

EXPERIMENTAL

The laboratory-made instrument used was described previously[5]. The analytical column (150 mm × 4.1 mm I.D.) contained PRP-1 (5 μm) poly(styrene–divinylbenzene) copolymer (prepacked) (Hamilton). The column was dynamically coated with cetyltrimethylammonium bromide (cetrimide).

The PRP-1 column was first washed with 25–30 cm^3 of eluent of pH 7, consisting of 24% (v/v) methanol containing 1 m*M* potassium hydrogenphthalate (KHP) and 1 m*M* Tris. Then 0.2 m*M* cetrimide was added to the eluent. The breakthrough curve of cetrimide was recorded with a refractive index detector connected in series with a UV detector and the amount of cetrimide adsorbed by the column was found to be 210 μmol. The column was then washed again with the cetrimide-free eluent until the retention of the sample ions (F^-, Cl^-, NO_2^-, Br^-, NO_3^- and SO_4^{2-}) did not change in a subsequent injection. (As long as the Br^- ions bound to the quaternary ammonium ions are exchanged with phthalate, the retention of the sample ions decreases.)

The column temperature was maintained as 25 ± 0.5°C by use of a water-bath (Type U10, MLW) and a water-jacket. The eluents were prepared as described earlier[6] with a constant 24% methanol concentration. The pH of the eluents was measured with an OP-208 precision digital pH meter (Radelkis) and a combined glass electrode calibrated with aqueous buffers of pH 4.0 and 7.0.

Standard sample solutions (0.01 *M*) were prepared from AnalaR-grade salts dissolved in deionized water.

RESULTS AND DISCUSSION

The pH of the eluent was changed by increasing of the concentration of the Tris buffer component (2, 10 and 20 m*M* Tris, pH = 7.0, 8.7 and 9.1, respectively) Table I gives the retention volumes (V_R) at these three pH values. As can be seen, the retention volumes of anions which are present in the same form at these pH values do not change. However, the retention volume of the phosphate changes dramatically (from 2.25 to 4.1). The log k' *vs.* pH graph obtained in the pH range 3–9 shows a minimum (Fig. 1). At lower pH values phosphate ions are detected as $H_2PO_4^-$ ions [the increase in retention below pH 5 is due to the decrease in the phthalate ion (HP^-) concentration in the eluent]. At higher pH of the eluent the $H_2PO_4^-$ ions are converted into HPO_4^{2-} ions, which have a higher retention value.

The retention of the arsenate ions increases similarly when the pH of the eluent is increased from 7.0 to 8.7. At pH 7.0, 61.3% of the arsenic is present as $HAsO_4^-$ and 38.7% as $H_2AsO_4^-$, whereas at pH 8.7, 98.5% is present as $HAsO_4^{2-}$ and 1.3% as $H_2AsO_4^-$ ($pK_1 = 11.23$, $pK_2 = 6.77$)[7].

At pH 7 the arsenic elutes as arsenous acid near the dead volume. On increasing

TABLE I

RETENTION, V_R (ml), OF INORGANIC ANIONS AS A FUNCTION OF THE ELUENT pH

Stationary phase: PRP-1 coated with cetrimide (0.21 m*M* cetrimide per column). Mobile phase: 24% (v/v) methanol + 2 m*M* KHP + 1–20 m*M* Tris.

Anion	*Tris (mM)*		
	2 (pH 7.0)	*10 (pH 8.7)*	*20 (pH 9.1)*
Fluoride	1.75	1.75	1.75
Chloride	2.35	2.40	2.40
Nitrite	2.75	2.80	2.80
Bromide	3.45	3.50	3.50
Nitrate	3.90	4.15	4.05
Phosphate	2.25	4.10	4.10
Sulphate	5.35	5.50	5.50
Thiosulphate	8.85	9.45	9.30
Iodate	1.82	1.9	1.70
Bromate	2.75	2.85	2.70
Chlorate	5.74	5.80	5.80
Arsenite	–	1.40	1.50
Arsenate	2.45	4.00	4.25

the pH of the eluent the percentage of the $H_2AsO_3^-$ form increases and so its retention also increases.

Fig. 2 shows the chromatogram of some simple anions obtained at pH 9.3. The peaks occurring between the F^- and Cl^- peaks are positive system peaks due to the HCO_3^-/CO_3^{2-} content of the eluent. Similar peaks were obtained by Ishibashi *et al.*[8]

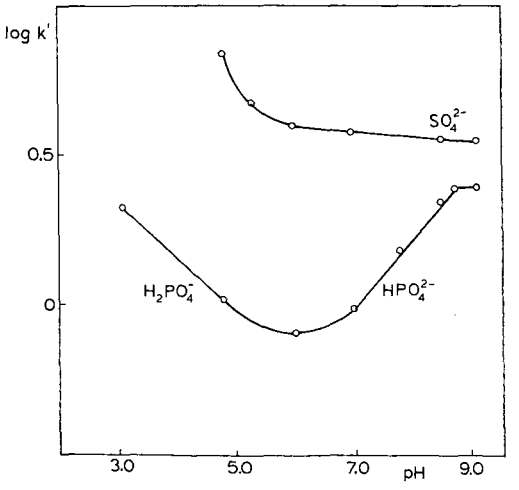

Fig. 1. Retention of phosphate ion as a function of the elutent pH. Mobile phase: 24% methanol in 2 m*M* aqueous phthalate, pH varied with Tris. Stationary phase: PRP-1 coated with cetrimide (210 μmol cetrimide per column).

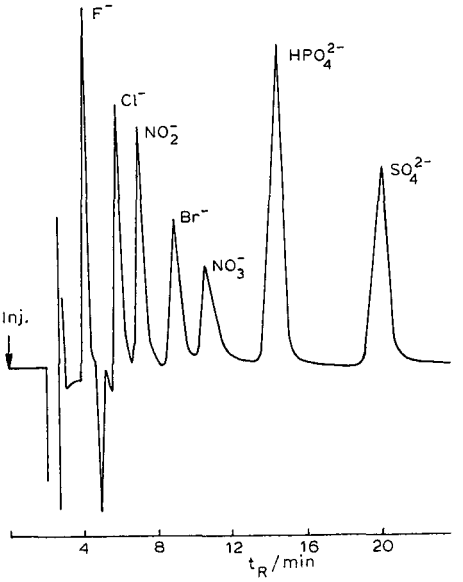

Fig. 2. Chromatogram of inorganic anions. Mobile phase: 24% methanol–1 m*M* KHP–20 m*M* Tris (pH 9.3). Stationary phase: PRP-1 coated with cetrimide. Eluent flow-rate: 0.5 ml/min. Sample size: 10 μl of 10^{-4}–$5 \cdot 10^{-4}$ *M* solute. λ = 282 nm.

with a two-column ion chromatographic system with $NaHCO_3$–Na_2CO_3 as the eluent. They found that the heights, direction and retention of the peaks between the F^- and Cl^- signals depend on the CO_3^{2-}/HCO_3^- concentration ratio in the eluent.

For the identification of our system peaks, HCO_3^-/CO_3^{2-} sample solutions were injected. These ions can be detected as negative peaks as sample ions (Fig. 3a and b).

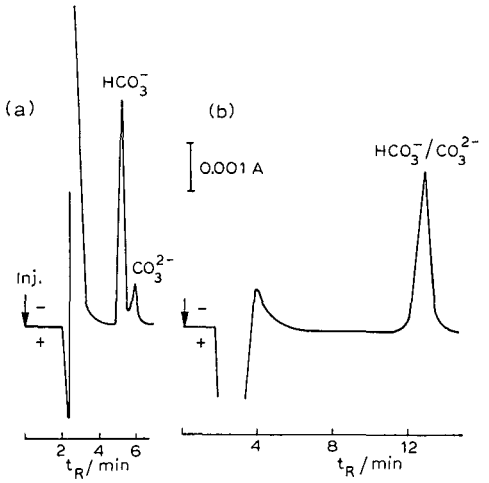

Fig. 3. Chromatogram of HCO_3^- and CO_3^{2-} ions. Mobile phase: 24% methanol–20 m*M* Tris containing (a) 0.8 m*M* KHP (pH = 9.2) and (b) 0.25 m*M* KHP (pH = 9.7). Stationary phase: PRP-1 coated with cetrimide. Eluent flow-rate: 0.5 ml/min. Sample size: 10 μl of 10^{-3} *M* $KHCO_3$. λ: (a) 282 nm; (b) 254 nm.

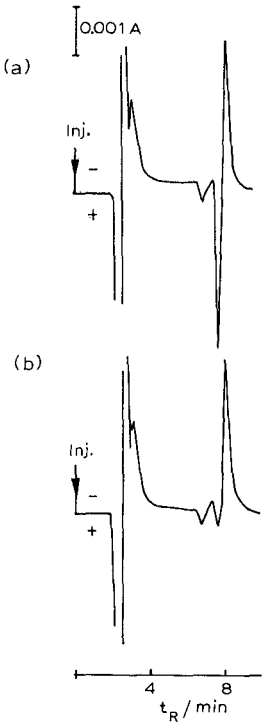

Fig. 4. Chromatogram of HS^- ion. Mobile phase: 24% methanol–0.4 m*M* KHP–20 m*M* Tris. (a) Eluent absorbed carbon dioxide. (b) Eluent prepared with carbonate-free water and measurements were made in a nitrogen atmosphere. Sample size: 10 μl of *ca.* $5 \cdot 10^{-4}$ *M* Na_2S. λ = 262 nm.

Using an eluent of lower pH (9.2) with a higher phthalate concentration (0.8 m*M*), two negative peaks are obtained. At higher pH (9.7) with a lower phthalate concentration (0.25 m*M*), only one peak is obtained. The retention is highly influenced by both the pH of the eluent and its phthalate concentration.

The positive system peak due to the absorption of CO_2 by the eluent is the result of the competition between the sample ions and the HCO_3^-/CO_3^{2-} ions (this situation is very similar to that when bromide and acetate ions were components of the eluent[1]).

The HCO_3^-/CO_3^{2-} system peak may be especially confusing for ions which are eluted with approximately the same retention as the system peak. This occurs when HS^- ions are eluted (Fig. 4a). The HCO_3^-/CO_3^- system peaks can be avoided if provision is made to exclude the absorption of carbon dioxide (Fig. 4b).

Of the anions studied, the retention of some of them (arsenite, arsenate, HS^-, borate, phosphate) can be well controlled by adjusting the pH of the eluent, but the retention of all anions can be controlled by adjusting the phthalate concentration of the eluent. The log *k'* *versus* log c_{KHP} relationships obtained are shown in Figs. 5 and 6. Using the curves for the arsenite and arsenate ions in Fig. 6, a phthalate concentration and pH of the eluent can be selected that are suitable for the simultaneous detection of As^{III} and As^{V} ions (Fig. 7).

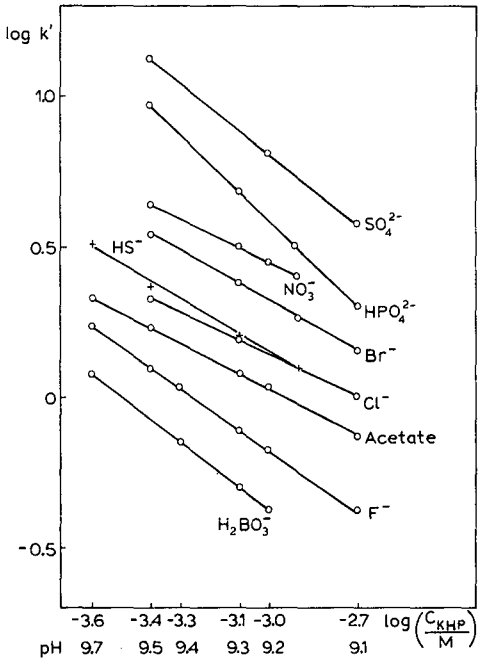

Fig. 5. Retention of inorganic anions as a function of potassium hydrogenphthalate concentration. Mobile phase: 24% methanol–0.25–2 m*M* KHP–20 m*M* Tris. Stationary phase: PRP-1 coated with cetrimide.

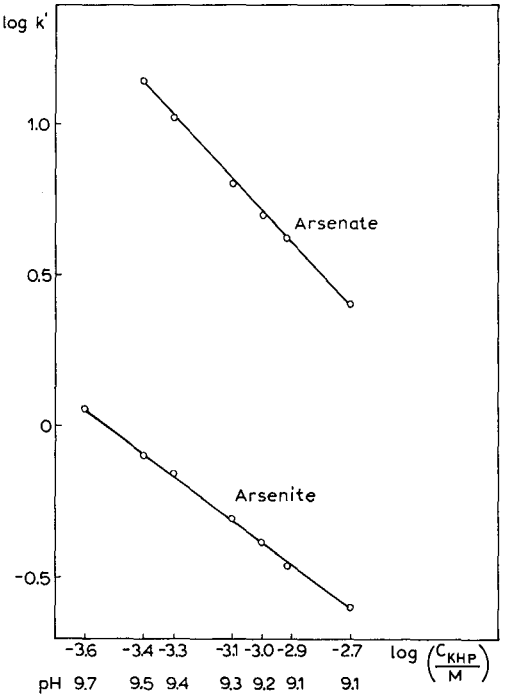

Fig. 6. Retention of arsenite and arsenate as a function of potassium hydrogenphthalate concentration. Conditions as in Fig. 5.

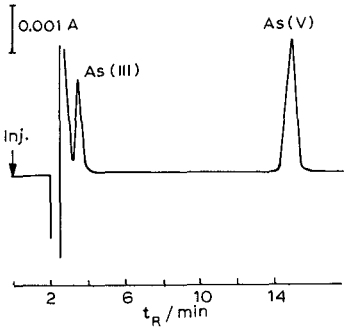

Fig. 7. Chromatogram of arsenite and arsenate ions. Mobile phase: 24% methanol-0.8 m*M* KHP-20 m*M* Tris. Stationary phase: PRP-1 coated with cetrimide. Eluent flow-rate: 0.5 ml/min. Sample size: 10 μl of $5 \cdot 10^{-4}$ *M* Na_2HAsO_4 and $NaAsO_2$. λ = 280 nm.

The study of the slopes of the log k' *versus* log c_{KHP} graphs makes possible a better understanding of the nature of the ion-exchange processes taking place in the system. Making use of the relationship derived by Haddad and Cowie[9]:

$$\log k' = \text{constant} - \frac{y}{x}[B^{x-}]_m$$

where k' is the capacity factor of the sample ion, $[B]_m$ is the concentration of the eluent ion (phthalate), $x = 2$, *i.e.*, the charge on the phthalate ion at pH > 7, and y = charge on the sample ion.

The experimentally obtained slopes of the log k' *versus* log c_{KHP} graphs are given in Table II. As can be seen, the agreement between the experimental and theoretical values (0.5 for monovalent and 1.0 for bivalent ions) is satisfactory for most of the ions studied (*e.g.*, Br^-, HPO_4^{2-}, Cl^-, NO_3^-, $HAsO_4^{2-}$, CH_3COO^-).The difference for the SO_4^{2-}, F^- and HS^- ions, however, is significant. No comparison can be made for the $H_2BO_3^-$ and $H_2AsO_3^-$ ions because the ratio of the neutral and monovalent forms changed as the pH of the eluent was altered.

For the SO_4^{2-} and Br^- ions the slopes were compared with the values published

TABLE II

OBSERVED SLOPES OF LOG k' *VS.* LOG c_{KHP} PLOTS

Anion	*Slope*	*Anion*	*Slope*
Chloride	0.47	HS^-	0.61
Bromide	0.56	$H_2BO_3^-$	0.75
Fluoride	0.69	$H_2AsO_3^-$	0.74
Acetate	0.48	$HAsO_4^{2-}$	1.05
Nitrate	0.48	SO_4^{2-}	0.81
		HPO_4^{2-}	0.96

by Small and Miller[10]. Using an anion-exchange column containing fixed functional groups and a phthalate–borate buffer system (pH 9), the slopes obtained were 0.47 and 0.98 for the Br^- and SO_4^{2-} ions, respectively.

The relatively good agreement between the experimental and theoretical results corroborates the view that the retention of anions on the cetrimide-coated column is governed by the ion-exchange equilibria between the bivalent phthalate and the sample ion when a phthalate–Tris buffer system is used as the eluent.

CONCLUSIONS

Common simple anions and the anions of weak acids can be separated efficiently on a PRP-1 column permanently coated with cetrimide if phthalate ions are used as eluent ions and the pH of eluent is controlled with Tris buffer. Owing to the absorption of carbon dioxide, eluent system peaks(s) occur on the chromatogram. These can be eliminated, however, by the use of carbonate-free eluent and by the use of a nitrogen purge and a nitrogen atmosphere during the measurements.

The column performance was checked after intensive use for 3 months. About a 5% decrease in the retention of Br^- and NO_3^- ions was observed using an eluent of pH 7.

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NON-SUPPRESSED ION CHROMATOGRAPHY OF ARSENIC ANIONS WITH POTASSIUM HYDROXIDE–AROMATIC SALT MIXED ELUENTS

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SUMMARY

The efficiency of mixed potassium hydroxide–aromatic salt solutions as eluents in non-suppressed ion chromatography of arsenic anions was investigated. It was demonstrated that potassium hydroxide–sodium salicylate is the optimum eluent to separate and to detect these arsenic anions because of the formation of internal hydrogen bonding involving the salicylate anion.

INTRODUCTION

Analytical techniques for the separation and the detection of inorganic and organic arsenic compounds have been developed. In one of these methods arsenic species are transferred into volatile arsine compounds[1–4] which can then be separated by gas chromatography[1,3] or thermal volatilization[2,4], and determined mainly by spectroscopic detection.

On the other hand, most other direct methods involve liquid chromatography, which is used for the separation of arsenic compounds[5,6], and voltammetry[5] or absorption spectrometry[6]. Suppressed ion chromatography[7] (IC) was recently used to separate some arsenic compounds[8–16] and conductivity[8,13,14,16], pulse polarography[9], atomic absorption[10–12], inductively coupled argon plasma-atomic emission spectrometry[15] and electrochemical detection[16] are used for their determination.

Suppressed IC with conductivity detection is not able to detect ions having $pK_a > 7$ because the conductance is measured under neutral or acidic conditions[17]. Conversely, it was suggested that non-suppressed IC[18,19] using a basic solution as an eluent[20,21] is able to determine weak acids[20] such as arsenite[21]. However, the analyses of oxysalts such as arsenic salts have not been examined thoroughly.

For these reasons, the efficiency of alkaline eluents such as potassium hydroxide and potassium hydroxide mixed with aromatic salts has been examined for the separation and determination of arsenic anions using non-suppressed IC. It was found that a suitable eluent for the elution of the arsenic compounds is a mixture of potassium hydroxide and sodium salicylate.

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EXPERIMENTAL

Standard solutions

The stock solutions of 1000 μg/ml (as As) sodium arsenite [As(III)], potassium arsenate [As(V)], dimethylarsinic acid (DMA), *o*-aminophenylarsonic acid (o-APA), *p*-aminophenylarsonic acid (p-APA) and phenylarsonic acid (PA) were prepared by dissolving analytical grade salts or acids, respectively. Working standard solutions were obtained by diluting the stock solutions in distilled water. The dissociation constants of the acids are shown in Table I[22,23].

Eluents

The eluents were prepared by dissolving, in distilled water, analytical grade potassium hydroxide and sodium benzoate (pK_{a1} = 4.204[22]), potassium biphthalate (pK_{a1} = 2.950, pK_{a2} = 5.408[22]), sodium salicylate (pK_{a1} = 2.98, pK_{a2} = 12.38[22]), sodium *m*-hydroxybenzoate (pK_{a1} = 4.076, pK_{a2} = 9.85[22]) or sodium *p*-hydroxybenzoate (pK_{a1} = 4.582, pK_{a2} = 9.23[22]). After deaeration, the pH was kept above 11.8.

Apparatus

A Toyo Soda Model HLC-601 non-suppressed ion chromatograph equipped with an anion-exchange column (50 mm × 4.6 mm I.D.) packed with TSKgel IC-Anion-PW (0.03 ± 0.003 mequiv./g) was used, comprising a computer-controlled pump, a conductivity detector, a sample injector (100 μl) and an oven. The flow-rate was maintained at 1.0 ml/min under pressures of 15–30 kg/cm². The separator column and the conductivity detector were placed in an oven regulated at 30°C.

RESULTS AND DISCUSSION

Potassium hydroxide eluent

For anion chromatography, the following equation was derived by Gjerde *et al.*[18]

$$\log t_s = (-y/x)\log [E] - \text{constant} \quad (1)$$

TABLE I

DISSOCIATION CONSTANTS OF ARSENIC COMPOUNDS (25°C)[22]

Anion	pK_{a1}	pK_{a2}	pK_{a3}
(1) Arsenite [As(III)]	9.18		
(2) Arsenate [As(V)]	2.22	6.98	11.50
(3) Dimethylarsinate (DMA)	6.273		
(4) *o*-Aminophenylarsonate (o-APA)	~2*	3.77**	8.66***
(5) *p*-Aminophenylarsonate (p-APA)	~2*	4.02**	8.92***
(6) Phenylarsonate (PA)	3.47§	8.48	

*,**,*** pK_a for $NH_3^+C_6H_4AsO_3H_2 \rightleftharpoons NH_3^+C_6H_4AsO_3H^- + H^+$, $NH_3^+C_6H_4AsO_3H^- \rightleftharpoons NH_2C_6H_4AsO_3H^- + H^+$ and $NH_2C_6H_4AsO_3H^- \rightleftharpoons NH_2C_6H_4AsO_3^{2-} + H^+$, respectively[23].

§ Ref. 23.

where t_s is the retention time of the sample anion, x and y are the "charge" numbers of the eluent anion and the sample anion, respectively, and [E] is the concentration of the eluent anion. The ratio (y/x) is given by the slope of log t_s *vs.* log [E]. Therefore, by making x small or y large, the retention time is shortened upon increasing the concentration of the eluent anion.

Fig. 1 shows an ion chromatogram of five arsenic anions obtained with 6 m*M* potassium hydroxide as the eluent, which was the optimum concentration. The anion of PA was first removed from the sample because it exhibits about the same retention time as that of the anion of p-APA. In this case, the retention times of the sample anions were remarkably different, and the peaks of strongly retained anions such as those of p-APA, o-APA and As(V) were extremely broad.

Moreover, it was found that the retention time of the As(V) anion changed with its concentration; nevertheless the other arsenic anions had constant retention times.

Therefore, it was concluded that the use of potassium hydroxide solution is unsuitable for the elution of arsenic compounds.

Potassium hydroxide and aromatic salts as eluents

From above results, mixtures of 6 m*M* (pH 11.8) potassium hydroxide and aromatic salts were investigated as eluents. At this pH, the "charge" numbers of benzoate and salicylate anions are -1, and those of the other anions are -2.

The relationships between the retention times of the sample anions and the concentrations of aromatic compounds in the mixed eluents are shown in Fig. 2a–d, and can be expressed as

$$a'(\text{DMA}):a'[\text{As(III)}]:a'(\text{p-APA}):a'(\text{PA}):a'(\text{o-APA}):a'[\text{As(V)}] = 1:1:2:2:2:3 \qquad (2)$$

where $a'(\text{A}) = -\text{d log } t_R(\text{A})/\text{d log } C$, $t_R(\text{A})$ is the retention time of the anion of A and C is the concentration of the organic eluent anion. This ratio corresponds to the ratio of the numbers of the "charge" of the sample anions. Therefore, the concentration of the eluent organic anion is very important in the separation of the arsenic anions.

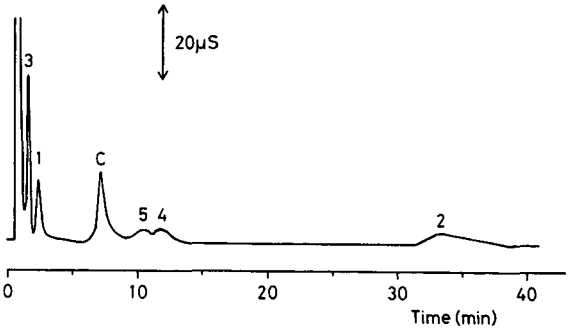


Fig. 1. Ion chromatogram of arsenic anions. Sample: 1 = As(III) (30 μg/ml as As); 2 = As(V) (30 μg/ml as As); 3 = DMA (20 μg/ml as As); 4 = o-APA (10 μg/ml as As); 5 = p-APA (10 μg/ml as As); C = carbonate. Column: TSKgel IC-Anion-PW, 0.03 mequiv./g. Eluent: 6 m*M* potassium hydroxide, pH 11.8.

Subsequently, the properties of individual aromatic anions as the eluents were examined.

Benzoate anion. The retention time of the sample anions in hydroxide–benzoate as the eluent are shown in Fig. 2a. The second dip peak[24] is close to the carbonate peak, and the peaks of the sample arsenic anions were sufficiently isolated and detected in all hydroxide–phthalate mixed eluents used. However, the retention times were little shortened and the resolution hardly improved, compared with the use of the simple hydroxide eluent.

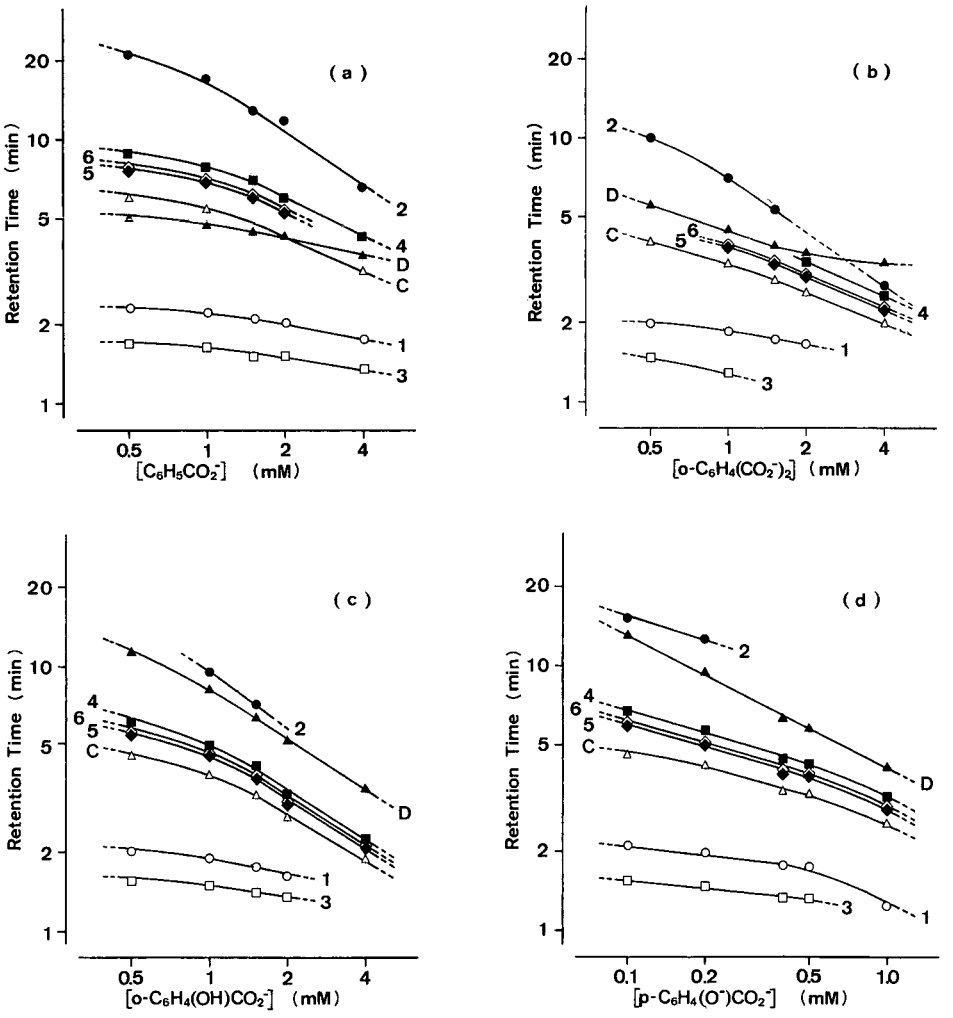


Fig. 2. Retention times of arsenic anions in mixed eluents. Sample: 1 = As(III); 2 = As(V); 3 = DMA; 4 = o-APA; 5 = p-APA; 6 = PA; C = carbonate; D = second dip peak. Column: TSKgel IC-Anion-PW, 0.03 mequiv./g. Eluents: (a) potassium hydroxide–sodium benzoate; (b) potassium hydroxide–potassium biphthalate; (c) potassium hydroxide–sodium salicylate; (d) potassium hydroxide–sodium *p*-hydroxybenzoate, pH 11.8.

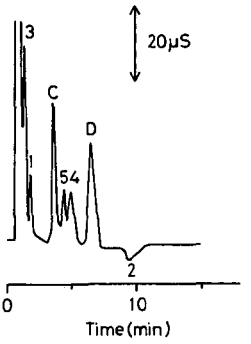


Fig. 3. Ion chromatogram of arsenic anions. Sample as in Fig. 1 except D = second dip peak. Column as in Fig. 1. Eluent: 6 m*M* potassium hydroxide–1.5 m*M* sodium salicylate, pH 11.8.

Phthalate anion. Phthalate anion has a charge of −2 and is more effective as an eluent anion than benzoate anion having a charge of −1. Therefore, as shown in Fig. 2b, the retention time of the arsenic anions is much improved by using phthalate anion as the eluent. However, since the elution behaviour of the second dip peak was so different from that of the second dip peak in the other eluents, it was impossible to detect all of the arsenic anions at once. Therefore, it was conducted that the hydroxide–phthalate mixed eluent was not effective for the separation and detection of the arsenic anions.

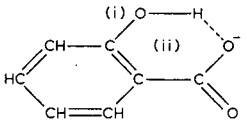
Salicylate anion. Fig. 2c shows the retention times of arsenic anions in hydroxide–salicylate mixed eluents. In this case, the useful concentration of salicylate anion was limited to the detection of the As(V) anion, because of the interference from the second dip peak. However, the retention times of the other arsenic anions were more shortened and their peaks were more sharper than in hydroxide–benzoate eluents. Therefore, this eluent is very effective for the separation and detection of the arsenic anions.

m- And p-hydroxybenzoate anions. Since the behaviours of these organic eluents are very similar, only one of them (*p*-hydroxybenzoate) is discussed. Fig. 2d shows the retention times of arsenic anions in hydroxide–*p*-hydroxybenzoate as an eluent. Assuming that the concentration of *p*-hydroxybenzoate in Fig. 2d was increased by a factor of four, the curves in Fig. 2d are similar to those in Fig. 2c.

TABLE II

QUANTITATIVE RANGES AND DETECTION LIMITS OF ARSENIC ANIONS USING 6 m*M* POTASSIUM HYDROXIDE–1.5 m*M* SODIUM SALICYLATE AS THE ELUENT

Anion	*Quantitative range (μg/ml as As)*	*Detection limit (μg/ml as As)*
(1) As(III)	5–50	3
(2) As(V)	10–50	5
(3) DMA	10–50	5
(4) o-APA	1–50	0.5
(5) p-APA	5–50	0.5
(6) PA	5–50	0.5



Accordingly, the concentration of the eluent aromatic anion should be strictly controlled in order to detect the As(V) anion rather than the salicylate anion.

It was thus found that the hydroxide–salicylate eluent is the most effective for the separation of the arsenic anions. Fig. 3 shows an ion chromatogram of an arsenic sample obtained using 6 m*M* potassium hydroxide–1.5 m*M* sodium salicylate as the eluent, *i.e.*, with the optimum salicylate concentration for 6 m*M* hydroxide (pH 11.8). The quantitative ranges and the detection limits of the arsenic anions using the same conditions are shown in Table II.

Considering the ionic structure of salicylate anion, it is estimated that the phenolic hydroxyl group bonded to benzene ring (i) accelerates the elution of sample anions. Also, because the six-membered ring (ii) is formed by hydrogen bonding between a carboxylic group and a phenolic hydroxyl group, the dissociation of the hydroxyl group is suppressed and it was supposed that the elution is delayed by a decrease in the charge number of salicylate anion.

Moreover, the sample anion peaks were not overlapped by the second dip peak produced by salicylate anion itself.

Therefore, it is concluded that good separations and detections of the arsenic anions were obtained by using hydroxide–salicylate as the eluent.

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EXTRACTIVE PENTAFLUOROBENZYLATION OF FORMIC, ACETIC, LEVULINIC, BENZOIC AND PHTHALIC ACIDS, STUDIED BY LIQUID CHROMATOGRAPHY AND DUAL-OVEN CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

Extractive pentafluorobenzylation of different short-chain carboxylic acids, including formic acid and a dicarboxylic acid, phthalic acid, was examined. Extractive alkylations were carried out in two different two-phase systems, *viz.*, dichloromethane–water and methyl isobutyl ketone–water, at a reaction temperature of 25°C. Quantitative yields were obtained within 50 min for both solvent systems, except for phthalic acid, which required an alkylation time exceeding 65 min in the dichloromethane–water system. Extractive pentafluorobenzylation of the monocarboxylic acids followed second-order rate kinetics.

The derivatives formed have good liquid and gas chromatographic properties. In order to utilize fully the electron-capture properties of the esters formed, a dual-oven capillary gas chromatographic separation procedure with electron-capture detection was developed.

INTRODUCTION

An important class of organic compounds with considerable biological interest and environmental and technical significance is short-chain carboxylic acids. These hydrophilic carboxylic acids are found in various aqueous solutions such as fermenter broth[1,2], plasma and serum[3–5], intravenous solutions[6], resulting from cleavage reactions of carbohydrates[7], sea water[8] and rain and fog samples[9].

The development of methods for the determination of low concentrations of short-chain carboxylic acids has gained considerable interest. The methods have generally followed two lines: either stable derivatives are prepared followed by

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chromatographic analysis[4–6,8–19], or direct analysis of the anion by ion chromatography is performed[7,20,21]. Moreover, the analysis of underivatized carboxylic acids by gas chromatography (GC) have been demonstrated[1–3]. Sensitive methods have been developed involving alkylation of the carboxylic acid with incorporation of a moiety with a good electron-capture response, *e.g.*, pentafluorobenzyl[4,8,14,15] and bromophenacyl[22]. The high sensitivity of the electron-capture detector to formic and acetic acid derivatives, especially the former, is generally obscured by excess of reagent or other matrix-associated compounds, however[4,14,15,22]. Further, halogenated solvents cannot be used with electron-capture detectors. Evaporation of, *e.g.*, dichloromethane followed by dissolution of the residue in *n*-hexane increases the possibility of losses of the derivatives and also prolongs the work-up procedure. However, the good electron-capture response and stability of pentafluorobenzyl derivatives make pentafluorobenzyl bromide a good candidate as an alkylation reagent for short-chain carboxylic acids.

Extractive alkylation, since its introduction as an analytical technique by Ehrsson[23], has been applied to a wide range of compounds. Gas chromatographic determinations of, *e.g.*, carboxylic acids[5,12,15,16,22,24], phenols[23] and theophylline[25] have been reported.

In this paper a study of the extractive alkylation of formic, acetic, levulinic, benzoic and phthalic acids with tetrahexylammonium as the counter ion and pentafluorobenzyl bromide as alkylation agent in two different solvent systems is described. The kinetics of the extractive alkylation were studied in dichloromethane–water and methyl isobutyl ketone–water two-phase systems. The effect of temperature on the reaction rate of formic acid was also investigated. Moreover, in order to circumvent the problems connected with excess of reagent and matrix-associated compounds and to be able to analyse the dichloromethane extract directly, although an electron-capture detector was used, dual-oven capillary GC separation procedures were also demonstrated. To exemplify the feasibility of the extractive alkylation method in combination with dual-oven capillary GC, a heat-sterilized dialysis solution was examined.

EXPERIMENTAL

Chemicals and reagents

Formic, acetic, acrylic and propanoic acid of analytical-reagent grade (Merck) and benzoic and phthalic acid of synthesis quality (Merck) were used. Levulinic acid and the internal standards fluoroacetic acid and *p*-fluorobenzoic acid were obtained at a purity of 98% (Sigma). Tetrahexylammonium hydrogen sulphate was obtained from Niel Clauson-Haas or Fluka (purum quality). The derivatization reagent was 2,3,4,5,6-pentafluorobenzyl bromide (Fluka).

Dichloromethane (Merck) and methyl isobutyl ketone (Merck) of analytical-reagent grade were used without further purification. Milli-Q purified water was distilled after refluxing with potassium persulphate (1 g dm^{-3}) and phosphoric acid (1 cm^3 dm^{-3}) in an all-glass system. Phosphate buffers were prepared from sodium dihydrogenphosphate monohydrate (Merck) and disodium hydrogen phosphate dihydrate (Merck). Orthophosphoric acid (Merck) of Suprapur quality was used.

Preparation of standards

The acids (9 mmol) were derivatized with a mixture of 50 ml of acetone, 2.8 mmol of pentafluorobenzyl bromide and 6 mmol of potassium carbonate, the solution being refluxed for 6 h and then filtered[10]. The solvent was removed in a Rotavaporator and the residue was dissolved in 10 ml of *n*-hexane and washed with 50 ml of water. The hexane extract was then flushed through a silica gel column with *n*-hexane, which removed the remainder of the acid and the reagent. After evaporation, the esters were diluted with dichloromethane, checked by purity and identified by gas chromatography–mass spectrometry.

Pentafluorobenzyl esters of formic, acetic, acrylic, propanoic, levulinic, benzoic, phthalic, monofluoroacetic and *p*-fluorobenzoic acids were prepared.

Apparatus

The liquid chromatographic (LC) equipment used consisted of an LDC Minipump and an LDC UV-III detector. The column (25 cm × 4.6 mm I.D.) was packed with μBondapack C_{18} (mean particle diameter 10 μm) and HPLC-grade acetonitrile (Rathburn Chemicals) and Milli-Q filtered water were used as the mobile phase. Generally, the mobile phase was acetonitrile–0.001 *M* sulphuric acid (30:70) at a flow-rate of 1.0 ml min^{-1}. Peak-area integration was effected with a Spectra-Physics Minigrator.

The gas chromatographs used were connected in tandem by two different laboratory-constructed cold trap/reinjection interfaces. Either an interface based on Deans switching developed for multi-separation GC[26] or a valve-based interface was used. The valve-based interface was a slightly modified dynamic headspace equipment[27].

The first gas chromatograph in the tandem system was a Varian 1400 gas chromatograph modified with a laboratory-constructed glass-lined injector for capillary column GC. The first column, *i.e.*, the precolumn, was 10 m × 0.53 mm I.D. and contained CP-wax 57 (Chrompack) with a film thickness of 3.0 μm. The column temperature was programmed from 50 to 220°C at 10°C min^{-1}. The flame ionization detector temperature was 250°C and the injector temperature 200°C. The interface was maintained at 225°C. The second gas chromatograph was either a Perkin-Elmer Sigma 1 or a Carlo Erba Fractovap 4160. The Perkin-Elmer instrument was equipped with a CP-wax 52 fused-silica capillary column (25 m × 0.22 mm I.D.). The electron-capture detector temperature was 300°C. The column temperature was either maintained isothermally at 90°C or programmed from 90 to 240°C at 10°C min^{-1}. A schematic diagram of this set-up is shown in Fig. 1A.

The Carlo Erba gas chromatograph was equipped with a DB-5 fused-silica capillary column (30 m × 0.25 mm I.D.) with a film thickness of 0.25 μm (J & W Scientific). The column was temperature programmed from 70 to 235°C at 10°C min^{-1}. The electron-capture detector temperature was 260°C. A schematic diagram of this set-up is shown in Fig. 1B.

The integration of peaks generated on the second gas chromatograph was performed on a Nelson 6000 laboratory data system.

Fractions from the first column were transferred to the second column by intermediate cold trapping in order to effect solute band concentration (focusing).

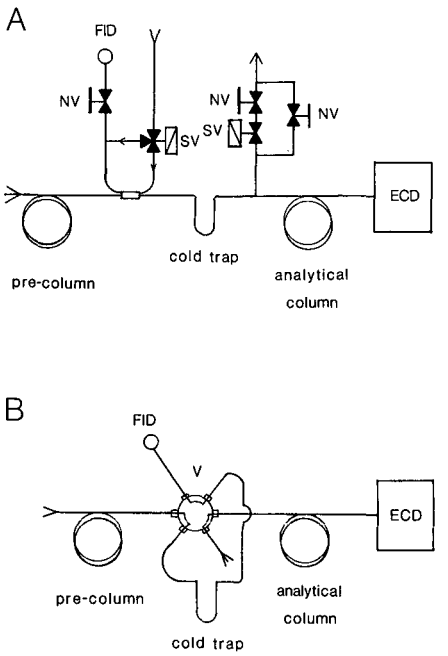

Fig. 1. Schematic diagram of capillary multi-separation configurations. NV = needle valve; SV = solenoid valve; FID = flame ionization detector; ECD = electron-capture detector; V = Valco six-port valve. (A) Capillary multi-separation technique based on flow switching according to Deans. (B) Capillary multi-separation technique based on flow switching with a rotary valve.

Kinetic methods

The acid (1.82 μmol) in 1.00 ml of phosphate buffer (pH 7.4, ionic strength = 0.1) and 1.00 ml of dichloromethane or methyl isobutyl ketone with pentafluorobenzyl bromide (138 μmol), tetrahexylammonium hydrogensulphate (10 μmol) and 0.100 ml of internal standard solution in dichloromethane or isobutyl methyl ketone were shaken in 10-ml tubes on an Evapo-mix (Buchler Instruments). One tube for each measuring interval was prepared. After an appropriate reaction time, the reaction was inhibited by addition of 5 ml 1.0 *M* phosphoric acid to the tube. After centrifugation and phase separation, the organic phase was diluted with 2.0 ml of acetonitrile. The organic phase was reduced to 1.0 ml by evaporation by blowing with nitrogen at room temperature. This was carried out in order to reduce most of the dichloromethane present in the sample, *i.e.*, to make the sample solution more compatible with the reversed-phase LC conditions used. The solution obtained was further diluted with 1.5 ml of acetonitrile and analysed by reversed-phase LC. A typical isocratic LC profile is shown in Fig. 2.

Peak areas obtained with the different measuring intervals were calibrated using the internal standard technique.

Extractive alkylation of dialysis solutions for GC analysis

Samples of the aqueous solution (0.5 ml) were shaken thoroughly with

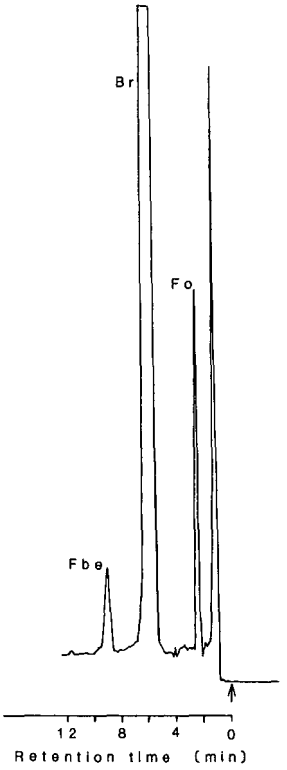

Fig. 2. Isocratic LC separation of a mixture of pentafluorobenzyl bromide (Br), pentafluorobenzyl ester of formic acid (Fo) and pentafluorobenzyl ester of monofluorobenzoic acid (Fbe). Flow-rate, 1.0 ml/min; mobile phase, acetonitrile–0.001 *M* sulphuric acid (30:70). UV detection at 254 nm. Full-scale recorder deflection corresponds to 0.1 absorbance units.

dichloromethane (1 ml) at 30°C for 3–4 h. The organic phase contained the derivatizing reagent, pentafluorobenzyl bromide (25 μl ml^{-1}) and tetrahexylammonium hydrogensulphate (11.7 mg). The dialysis solution was phoshate-buffered (pH = 7.4) prior to the extractive alkylation. After an appropriate reaction time, the organic phase was washed with excess of 1 *M* phosphoric acid, the aqueous layer removed and the dichloromethane solution diluted with 5 ml of *n*-hexane. After centrifugation the organic phase was directly subjected to multi-separation capillary GC. Alternatively, the dichloromethane extract was evaporated to dryness and the residue dissolved in 200 μl of *n*-hexane.

RESULTS AND DISCUSSION

Determination of rate constants

The extractive alkylation can be described as a two-step reaction, which is illustrated by the following equations:

$$Q^+ + A^- \rightleftharpoons QA_{org} \quad (1)$$
(ion-pair extraction)

$$QA_{org} + PFBBr_{org} \rightarrow PFBA_{org} + QBr_{org} \quad (2)$$
(alkylation)

where Q^+ represents the quaternary ammonium ion, A^- the anion of the acid, QA_{org} the ion pair in the organic phase, PFBBr pentafluorobenzyl bromide and PFBA the benzyl ester of the acid. The evaluation of the rate constants follows the principles given by Tivert and Gustavii[28]. The kinetic model used for two-phase alkylation is given by the equation

$$\ln ([PFBA]_\infty - [PFBA]_t) = \ln C_{A,0} - k'_{A,obs}t \quad (3)$$

where $k'_{A,obs}$ is the observed pseudo-first-order rate constant when the alkylation reagent and the quaternary ammonium ion are present in excess. A straight line with a slope equal to $-k'_{A,obs}$ is obtained on plotting $\ln([PFBA]_\infty - [PFBA]_t)$ *versus* t. The observed pseudo-first-order rate constants obtained for the two different alkylation solvent systems are given in Table I.

The extractive alkylations were followed for more than five half-lives. For the two different two-phase systems complete alkylation (*i.e.*, >99% yield) is obtained within 50 min, except for phthalic acid in the dichloromethane–water system (>65 min). The alkylation proceeds throughout more rapidly in the methyl isobutyl ketone–water system than in the dichloromethane–water system. It is not clear why this is so, but it may be attributed to the solution properties of methyl isobutyl ketone. Dipolar aprotic solvents, such as ketones, are known to solvate cations, whereas anions are poorly solvated[29]. As a result, many reactions can be considerably more rapid in dipolar aprotic solvents when the reaction involves anions.

TABLE I

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS FOR TWO-PHASE ALKYLATION

Alkylation reagent, 0.138 *M* pentafluorobenzyl bromide; $C_A = 1.82 \cdot 10^{-3} M$ at pH 7.4; $C_Q = 0.01$ *M*; the alkylations were carried out at 25°C.

Acid	$k'_{A,obs}$ *(min*$^{-1}$*)*	
	Dichloromethane–water	*Methyl isobutyl ketone–water*
Formic	0.097	0.123
Acetic	0.106	0.137
Levulinic	0.123	0.148
Benzoic	0.127	0.143
Phthalic*	0.070	0.101

* Apparent value; see discussion on dicarboxylic acids.

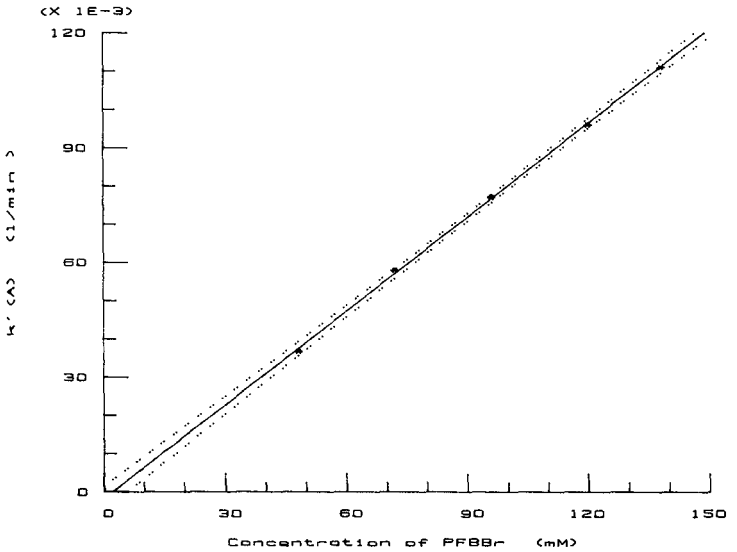

Fig. 3. Extractive alkylation of formic acid with pentafluorobenzyl bromide. Pseudo-first-order rate constant at different concentrations. The dotted lines indicate the confidence limits at the 95% level.

When the concentration of the alkylation reagent PFBBr was increased, $k'_{A,obs}$ also increased. According to Tivert and Gustavii[28], $k'_{A,obs}$ is related to the second-order rate constant k_A and the concentration of PFBBr by

$$k'_{A,obs} = k_A C_{PFBBr} \tag{4}$$

A plot of $k'_{A,obs}$ *vs.* C_{PFBBr} should give a straight line passing through the origin. The slope of the line gives the second-order rate constants. The second-order rate constant for formic acid was 0.81 l mol^{-1} min^{-1} at a reaction temperature of 35°C (see Fig. 3). This result indicates that the reactions follow second-order reaction kinetics and, together with the results in Table I, that the extractive benzylation is complete and that no major side-reactions occur.

The corresponding second-order rate constants for the other acids could be calculated using eqn. 4 and the information given in Table I. For example, the second-order rate constant for acetic acid in the dichloromethane–water system is 0.77 l mol^{-1} min^{-1} at 25°C. This value is considerably higher than the published second-rate order constant (0.018 l mol^{-1} min^{-1}) of acetic acid in a corresponding two-phase alkylation reaction but with tetrabutylammonium as counter ion and phenacyl bromide as alkylation agent[30]. This confirms that for hydrophilic acids a higher degree of extraction is obtained with larger counter ions such as tetrahexylammonium[25]. Further, the comparison gives additional support to the assumption that a highly lipophilic counter ion is needed for extractive alkylation of formic and acetic acid in order to achieve quantitative alkylation and sufficiently short analysis times. It has been reported[31] that an increase in the lipophilic character of the counter ion increases the degradation of pentafluorobenzyl bromide. This was considered to be

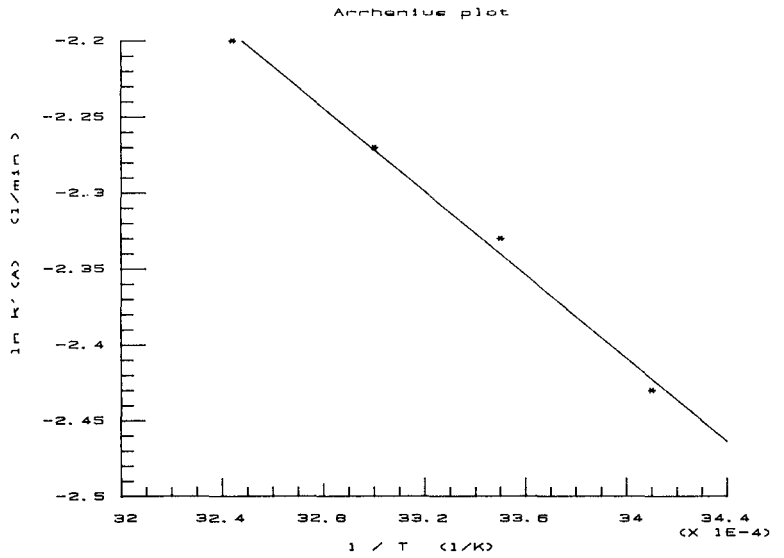

Fig. 4. Extractive alkylation of formic acid with pentafluorobenzyl bromide at different reaction temperatures.

due to an enhanced extraction of hydroxy ions into the organic phase, with a subsequent increase in the hydrolysis rate of the reagent. Those studies were carried out at pH 10 or above, whereas in the present study alkylation was performed at pH 7.4 with a phosphate buffer. This restricts the extraction of hydroxy ions and explains why no apparent degradation of the reagent is observed.

Phthalic acid has two reactive sites, generating a dibenzylated ester. Dialkylation of nonanedioic acid[30] has been proposed to proceed through the extraction into dichloromethane as Q_2A and a monoalkylated intermediate, followed by a reaction forming the dialkylated end product. The steps are governed by the pseudo-first-order rate constants k'_1 and k'_2, respectively. By establishing suitable equations and by plotting the concentration of dialkylated acid *versus* time the constants can be calculated by non-linear curve fitting[30]. However, as the reaction of phthalic acid proceeded approximately according to an observed pseudo-first-order rate, no attempt was made in this work to deconvolute the underlying rate constants.

In order to study the effect of temperature on alkylation, the reaction rate of formic acid in the range 20–35°C was studied. An increase in the reaction temperature from 20 to 35°C increased the pseudo-first-order rate constant from 0.088 to 0.111 min^{-1} (see Fig. 4). The Arrhenius activation energy for formic acid was 11.4 kJ mol^{-1}.

The pentafluorobenzyl esters formed on extractive alkylation have good UV absorption properties and are therefore suitable for LC. However, in order to utilize fully the excellent electron-capture properties of the pentafluorobenzyl derivatives, electron-capture detection (ECD) should be used. The problems associated with attaining good sensitivity when using ECD include chemical nosie due, *e.g.*, to halogenated solvents and the presence of interfering compounds. Owing to decrease in the ECD response when subjected to an excess of, *e.g.*, dichloromethane combined

with overlapping of the formic and acetic acid ester peaks by the pentafluorobenzyl bromide peak, the excess of the alkylation reagent and halogenated solvents must be removed prior the final analysis step. In order to overcome the problems due to the alkylation reagent, Chauhan and Darbre[4] used a column coated with PPSeb stationary phase and a low excess ratio (*e.g.*, 15) of pentafluorobenzyl bromide to the acid. For *p*-bromophenacyl esters, Kawamura and Kaplan[9] separated the alkylation reagent from the esters on a silica gel LC column. This procedure involved two evaporation steps. However, purification on silica gel columns is of limited value because, as pointed out by Davis[14], some decomposition of the pentafluorobenzyl esters occurs.

Brötell *et al.*[32] used two-dimensional GC and heart cutting in order to remove the excess of reagent and to shorten the analysis time in the determination of an amino alcohol in serum after trifluoroacetylation. In this work a similar approach was also utilized.

For removal of the alkylation reagent we developed a capillary precolumn–capillary column system based on multi-dimensional GC technology[26]. By use of the capillary precolumn technique, crude dichloromethane extracts can be analysed without distortion of the ECD performance. A typical example of the GC technique is given in Fig. 5.

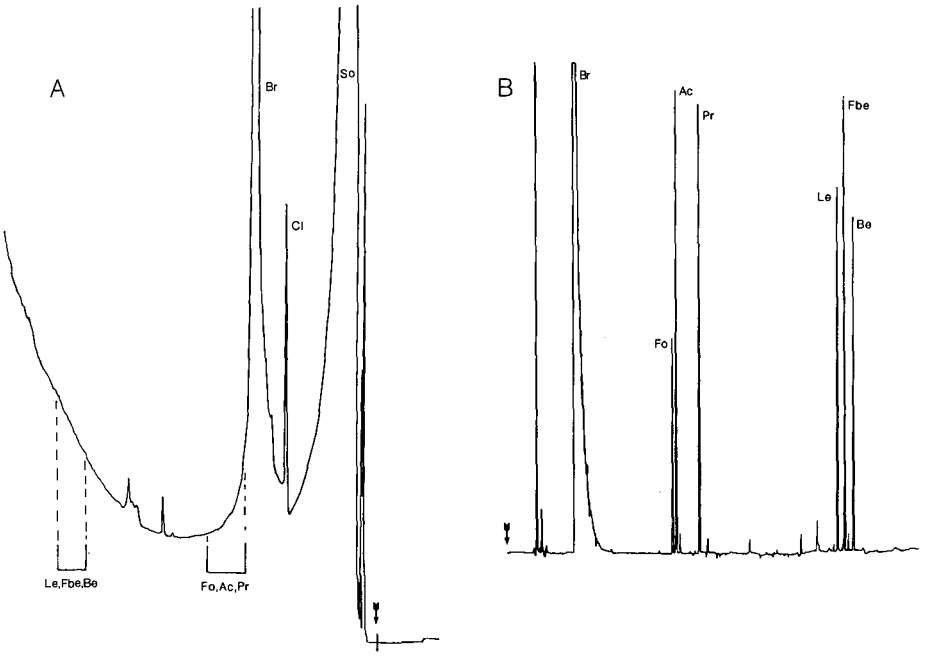

Fig. 5. Chromatograms generated by extractive pentafluorobenzylation of a standard saline solution. So = dichloromethane; Cl = pentafluorobenzyl chloride; Br = pentafluorobenzyl bromide, in chromatogram A also decomposition products of the counter ion; Fo = formic acid; Ac = acetic acid; Pr = propanoic acid; Le = levulinic acid; Be = benzoic acid; Fbe = monofluorobenzoic acid. (A) Chromatogram generated by injection of crude dichloromethane extract with flame ionization detection. Stationary phase, CP-wax 57. (B) Chromatogram obtained by intermediate cryogenic trapping of the indicated fractions from chromatogram A. Detection with electron-capture detector. Stationary phase, CP-wax 52.

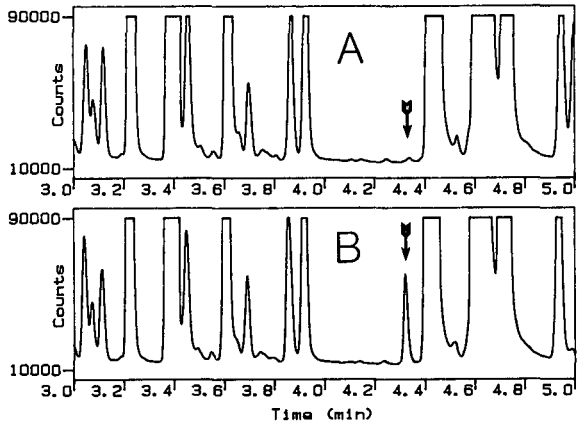

Fig. 6. Part of the chromatograms generated by reinjection on the non-polar DB-5 column of a 60 s wide fraction, with intermediate cryogenic trapping, from the polar CP-wax 57 pre-column. (A) Reinjected fraction from extractive alkylation of dialysis solutions. (B) Reinjected fraction from extractive alkylation of dialysis solutions fortified with acrylic acid (260 ng ml^{-1}).

Apart from nearly complete removal of dichloromethane and the derivatization reagent, the first GC set up, with a polar precolumn and a polar analytical column (see Experimental) is characterized by the resolution of pentafluorobenzyl bromide and the pentafluorobenzyl ester of formic acid. Similar resolutions were not obtained on the less polar column used in the second separation stage. Owing to the cross-linking of the Carbowax stationary phase, low column bleeding is obtained in the working temperature range, with a subsequent low distortion of the ECD performance.

The use of multi-separation procedures utilizing columns of different selectivity has been found to be of a great advantage in the analysis of complex mixtures[27]. In the analysis of dialysis solutions, the increased separation power achieved by using two columns with different selectivities was needed in order to separate the pentafluorobenzyl esters from other matrix-associated compounds with an ECD response. The dialysis solutions contained glucose at up to 44 mg ml^{-1}, sodium lactate at *ca.* 4.5 mg ml^{-1} and sodium chloride at 5.8 mg ml^{-1}. Prior to use, these solutions were heat sterilized. Apart from thermal degradation of the dialysis solution, impurities can migrate from the PVC bag containing the solutions. Lactate and chloride ion are extracted as ion pairs into the organic phase and react with the reagent. Therefore, in order to avoid depletion of the pentafluorobenzyl bromide, the reagent concentration must be in excess of the sum of the molar concentrations of the lactate and chloride. Further, to allow the extractive alkylation to proceed at a sufficient rate, the concentration of the counter ion has to be high. This implies that the molar concentration of the reagent and the counter ion are far above the concentration of the acids under study. For example, the excess ratio of the reagent to some minor acids is $> 10^5$ (w/w) and the the counter ion also $> 10^5$ (w/w). Hence, even very low concentrations of impurities, *e.g.*, formic acid, in the extractive alkylation reaction chemicals could severely affect the analysis results for the dialysis solutions. It was found that it was not possible to determine formic and acetic acid in the low ng ml^{-1} range without further purification of the reagent and counter ion. Especially the latter has to be purified if the

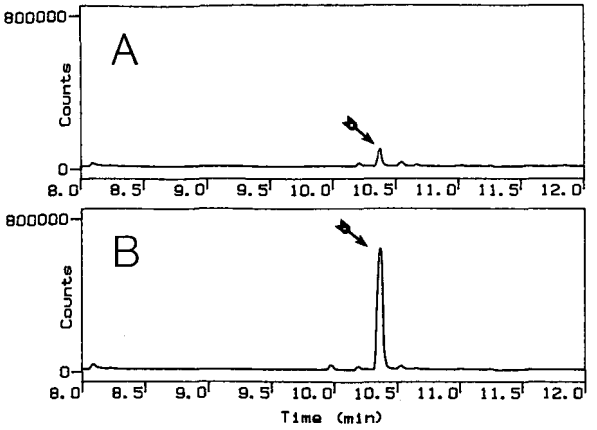

Fig. 7. Part of the chromatograms generated by reinjection on the non-polar DB-5 column of a *ca.* 60 s wide fraction, with intermediate cryogenic trapping, from the polar CP-wax 57 pre-column. (A) Reinjected fraction from extractive alkylation of a dialysis solution. (B) Reinjected fraction from extractive alkylation of a dialysis solution fortified with benzoic acid (284 ng ml^{-1}).

proposed method is to be used for the determination of formic and acetic acid in dialysis solutions. However, acrylic acid could be determined in the low ng ml^{-1} range by the proposed method (see Fig. 6), although the results are strongly dependent on the high resolution properties of multi-dimensional GC. The fraction that contains the ester of acrylic acid is close to the fractions containing the bulk of the reagents and degradation compounds of the counter ion. The situation improves considerably when fractions that are further away from the bulk of the reaction chemicals on the precolumn are analysed on the second column. As an example, the analysis of the pentafluorobenzyl ester of benzoic acid is shown in Fig. 7. These chromatograms show that the detection of short-chain carboxylic acids ($>C_2$) in the low ng ml^{-1} range is possible even in very complex solutions by direct analysis of extractive pentafluorobenzylation extracts by dual-oven capillary GC with ECD.

This work has demonstrated that extractive pentafluorobenzylation of the studied acids in combination with dual-oven capillary GC may be a feasible method for the trace analysis of complex samples. However, in order to validate the method for quantitative analysis, a more complete study of analytical parameters such as impurities in reagents, reproducibility and detection limits has to be carried out.

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TYROSYLATION AND PURIFICATION OF PEPTIDES FOR RADIOIODINATION

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SUMMARY

A simple method for tyrosylation, purification and subsequent radioiodination of peptides which lack suitable acceptor groups for iodine substitution is presented. The reagent, *tert.*-butyloxycarbonyl-L-tyrosine N-hydroxysuccinimide ester, was used for conjugation to the amino groups of peptides. The derivatization was performed with relatively large amounts of reagents to ensure quantitative reactions. The derivatized peptides were purified by reversed-phase high-performance liquid chromatography or by gel filtration. Subsequent radioiodination was performed with sodium [^{125}I]iodide and the sparingly soluble tetrachlorodiphenylglycouril as the oxidative agent to minimize possible oxidative damage to the peptides. The radiolabelled peptides were subsequently purified by isocratic high-performance liquid chromatography.

INTRODUCTION

Radioiodination of peptides is the most commonly used labelling method for the generation of tracers for immunoassays and receptors studies. The iodination is generally performed by chemical substitution of hydrogen in tyrosyl or histidyl residues using oxidative agents such as chloramine-T[1,2], lactoperoxidase[3] or iodogen[4,5]. Techniques have also been developed to protect the peptide from oxidative damage, through separate labelling of a reactive precursor, which is extracted from the iodination mixture and then conjugated to the peptide under mild conditions. The conjugation methods are also useful for the labelling of peptides lacking tyrosyl or histidyl residues. The conjugation reagents include N-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent)[6], methyl-*p*-hydroxybenzimidate[7], diazotized aniline[8] and *tert.*-butyloxycarbonyl(t-BOC)-L-tyrosine N-succinimidine ester (Associan reagent)[9].

Radioiodinated forms of at least the Bolton–Hunter reagent are available commercially. In the reaction with labelled derivatizing reagent, only small, but nevertheless expensive amounts of radiolabelled conjugation reagent and peptide are used. Thus, success is dependent on the absence of chemical scavengers interfering with

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the conjugation reagent or peptide. In organic chemistry laboratories highly purified solvents, etc., are generally available; however, most radioiodinations are performed in physiological and clinical laboratories. We present a simple method for tyrosylation and purification of relatively large amounts of peptides for subsequent routine radioiodination. The method offers an economical alternative to iodination with radioiodinated conjugation reagents. The problems of oxidative damage to the peptide during the iodine substitution are circumvented by using chloroglycouril as the non-soluble oxidative agent, and by subsequent purification of the radioiodinated peptides by isocratic high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Peptides

Bovine pancreatic polypeptide (BPP) was a generous gift from Dr. Ronald E. Chance (Eli Lilly, Indianapolis, IN, U.S.A.). Somatostatin-28 (1-12-fragment) was obtained from Peninsula Laboratories (St. Helens, U.K.) by Drs. F. Baldissera and J. J. Holst. The Cys_0-preproneuropeptide-Y_{83-88} (Cys-Thr-Glu-Asn-Val-Pro-Arg) was synthesized by Cambridge Research Biochemicals (Cambridge, U.K.). Dog pancreatic eicosapeptide was isolated from the duodenal part of the pancreas by acid–ethanol extraction, ether precipitation, gel filtration chromatography and HPLC as described[10,11].

Tyrosylation

Around 50 nmol of BPP, 75 nmol of somatostatin-28 (1–12-fragment) and 1.5 μmol of Cys_0-preproneuropeptide-Y_{83-88} were conjugated, whereas only 5 nmol of the eicosapeptide were used. The peptides were dried under vacuum and reconstituted in 200 μl of 0.1 *M* N-ethylmorpholinę, analytical grade (Serva Feinbiochemica, Heidelberg, F.R.G.), adjusted to pH 8.0 with glacial acetic acid (Merck, Darmstadt, F.R.G.). The tyrosylation reagent was added as 50 μl of a 10 mmol/l solution of t-BOC-L-tyrosine-N-hydroxysuccinimide ester, research grade (Serva), in dimethylformamide, Sequanal Grade (Pierce Chemical, Rockford, IL, U.S.A.). The reaction time was usually 15 min, however in some cases the reaction mixture was analysed by HPLC after various periods. The derivatized peptides were purified either by HPLC or by gel filtration on a 25 cm × 1 cm Bio-Gel P-2 column, eluted at room temperature with 0.05 mol/l phosphate buffer, pH 7.5. The HPLC system used for analytical and preparative purposes consisted of a 25 cm × 0.4 cm reversed-phase Nucleosil 300-5 C_{18} column, 5-μm particles and pore diameter 300 Å. The packing material was obtained from Macherey Nagel (Düren, F.R.G.) and packed into stainless-steel columns (Knauer, Berlin, F.R.G.). The chromatography was performed at 50°C on an Hewlett-Packard 1090 liquid chromatograph with 0.1% (v/v) trifluoroacetic acid (Art. No. 8262, Merck) in water as the aqueous phase and acetonitrile (chromatography grade, Merck) as the organic solvent.

The protecting BOC group was removed from the derivatized, HPLC-purified peptides by treating the dried peptide either with 50 μl of 99% formic acid (Art. No. 264, Merck) for 1 h at room temperature or with undiluted trifluoroacetic acid (Pierce). The solvents were removed under vacuum and the deprotected, derivatized peptide was reconstituted in 50 μl of 0.1% (v/v) trifluoroacetic acid before purification by HPLC.

Sequence determination

The solvent was removed under vacuum from the derivatized and deprotected which was reconstituted in 60 μl of 0.1% (v/v) acetic acid, and subjected to automated sequence analysis by sequential Edman degradation on an Applied Biosystem 470A gas-phase sequenator. The MHTFA1 program of M. Hunkapillar which is a modification of that of Hunkapillar *et al.*[12] of 1983, and available from Applied Biosystems (Foster City, CA, U.S.A.), was used. All chemicals were obtained from Applied Biosystems. The characterization of the phenylthiohydantoin derivatives of amino acids was performed on an Hewlett-Packard 1090 liquid chromatograph with a 25 cm × 0.45 cm CN column with 5-μm particles (IBM Instruments, CT, U.S.A.) and a sodium acetate–acetonitrile gradient elution system as described[13]. Before analysis, the samples from the sequenator were methylated by treatment with acidified methanol (1 *M* hydrochloric acid in methanol, Applied Biosystems) for 10 min at 50°C. Aminobutyric acid was used as an internal standard during HPLC, for correction and for quantitation of the amino acid derivatives.

Amino acid analysis

For hydrolysis, HPLC-purified derivatized peptides were dried under vacuum in 50 mm × 6 mm Culture Tubes (Corning, Stone, U.K.). The tubes were placed in PICO-TAG reaction vials (Waters, Milford, MA, U.S.A.) containing 200 μl of 6 *M* hydrochloric acid with 10 μl phenol. The hydrolysis was performed at 110°C under vacuum for 20–24 h. The excess of hydrochloric acid was removed under vacuum. The free amino acids were analysed as phenylthiocarbamyl derivatives by reversed-phase HPLC on a Merck Supersphere column C_8, 25 cm × 0.4 cm as previously described[14].

Iodination

The derivatized pancreatic eicosapeptide was iodinated with carrier-free $Na^{125}I$ (Amersham, Little Chalfont, U.K.) using the oxidation reagent, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Serva) as described[4]. The chloroglycouril (3.7 mg) was dissolved in 2.0 ml dichloromethane (Sigma, St. Louis, MO, U.S.A.) and the inside of an Eppendorff test-tube (Sarstedt, Nümbrecht, F.R.G.) was coated with 20 μl of this solution by gently turning the tube while the solvent evaporated. The derivatized eicosapeptide (1.5 nmol) was dried under vacuum, reconstituted in 40 μl phosphate buffer, pH 7.38, precision buffer solution (Radiometer, Copenhagen, Denmark) and added to the coated tube. The salt $Na^{125}I$ (1 mCi) was added and the tube was kept on ice. After 5 min, 50 μl of the HPLC solvent (28% acetonitrile in 0.1% trifluoroacetic acid in water) were added before purification on a Nucleosil 300-5 C_{18} column, 25 cm × 0.4 cm, eluted isocratically at 50°C with a flow-rate of 1.0 ml/min. Fractions of 0.5 ml were collected and aliquots of 10 μl counted in a gamma counter.

Test of radioiodinated, derivatized peptide

The derivatized and radioiodinated eicosapeptide was tested for reaction with antisera in radioimmunoassay. Antibodies, standards and labelled peptide were incubated for 48 h at 4°C in tubes containing a total of 1.5 ml of 0.2 mol/l phosphate buffer, pH 7.5, with 2.5 mg/l bovine serum albumin (Sigma). The separation of free and bound peptide was performed by addition of 0.5 ml of a slurry of activated charcoal

(Sigma) 2 g/100 ml in assay buffer supplemented with 10% outdated plasma, and centrifugation (10 min at 4000 *g*) after incubation for 20 min at room temperature.

RESULTS AND DISCUSSION

Reaction time

Pancreatic polypeptide was used to investigate the reaction time. Around one third of the peptide had reacted within 30 s (the time required to inject the sample in HPLC) using 50 nmol of peptide in 0.25 ml and a ten-fold excess of derivatization reagent, Fig. 1a. After a few minutes the reaction was almost complete while the conjugation reagent had started to hydrolyse. The reagent was completely hydrolysed at 40 min, Fig. 1b. When the reagent was dissolved and kept in acetonitrile, no change in elution position, corresponding to the solid arrow in Fig. 1a, was detected even after several hours. However, in 50% acetonitrile in water, the reagent disappeared gradually and a major hydrolysis product appeared with an elution position corresponding to the open arrow in Fig. 1b (data not shown). A reaction time of 15 min was chosen.

Purification

With relatively apolar peptides, *e.g.*, pancreatic polypeptide (PP), the purifica-

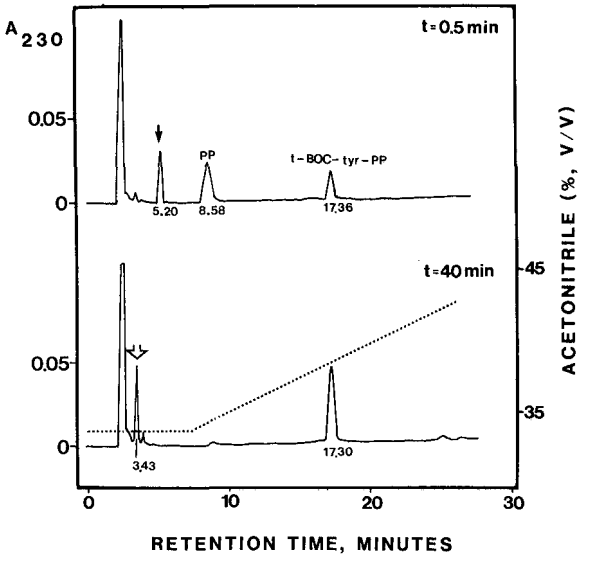

Fig. 1. Derivatization of pancreatic polypeptide (a 36-amino acid, relatively apolar peptide) and hydrolysis of the derivatization reagent. HPLC profile of the reaction mixture after 0.5 min of incubation, upper panel, and after 40 min, lower panel. The elution positions of underivatized pancreatic polypeptide, PP, and of the derivatized peptide, t-BOC-Tyr-PP, are indicated. The solid arrow indicates the elution position of freshly dissolved t-BOC-L-Tyr-N-hydroxysuccinimide ester and the open arrow that of the major hydrolysis product of this reagent. The column (25 cm × 0.4 cm) was packed with Nucleosil C_{18} with a particle size of 5 μm, a pore size of 300 Å and eluted with 0.1% TFA–water and a gradient of acetonitrile as indicated by the dotted line.

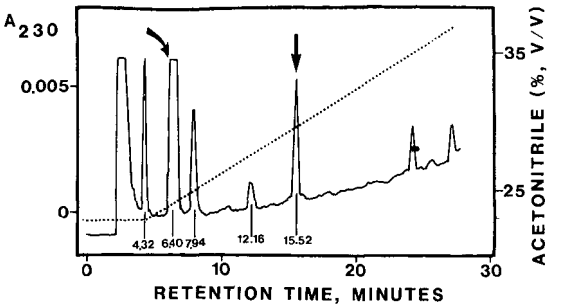

Fig. 2. HPLC purification of derivatized pancreatic eicosapeptide (a relatively polar peptide). Profile of the reaction mixture after 35 min of incubation. In this system the derivatization reagent is eluted after 12.16 min and the major hydrolysis product after 6.40 min; the peaks at 4.32 and 7.94 min are also present when no peptide is added to the reaction mixture. The elution position of the underivatized eicosapeptide is indicated by the curved arrow. The peak at 15.52 min is due to the derivatized eicosapeptide, as demonstrated by amino acid analysis.

tion by HPLC is straightforward since both the normal peptide and the derivatized peptide are eluted later than the conjugation product and its hydrolysis products. However, smaller and more polar peptides may coelute with the reagents which was the case with the canine pancreatic eicosapeptide as shown in Fig. 2. This figure demonstrates that the interpretation of the HPLC elution profile is no longer simple

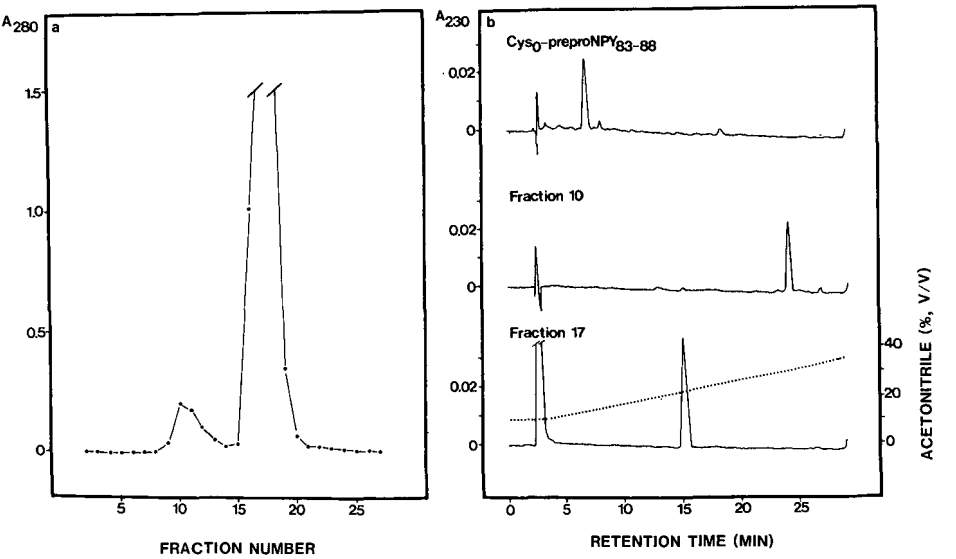

Fig. 3. Purification by gel filtration of a small derivatized peptide, Cys_0-preproneuropeptide-Y_{83-88}. (a) Gel filtration of the reaction mixture separated on a Bio-Gel P-2 column (25 cm × 1 cm) eluted with 0.05 mol/l phosphate buffer, pH 7.5 at room temperature. Fractions 9–13 contain the derivatized peptide. (b) HPLC of the underivatized peptide (upper), purification of gel filtration fraction 10 containing the derivatized, more apolar peptide eluted after 24 min (middle) and profile of gel filtration fraction 17 containing the hydrolysed reagent eluted after 15 min and solvents eluted close to the injection artefact (lower). Column and solvents as in Fig. 1.

when a small amount of peptide is derivatized. We used relatively large amounts of conjugation reagent, 2 mmol/l, to avoid quenching of the reaction by interfering contaminants.

The most generally applicable purification method is gel filtration on, *e.g.*, a Bio-Gel P-2 column. In Fig. 3a is shown the purification of derivatized Cys_0-preproneuropeptide-Y_{83-88} by gel filtration. Through this "desalting" procedure, the reagent and solvent of the reaction mixture are eluted in the total volume of the column and the oligopeptide in the void volume. When desalting is used, it is recommended to test the gel-filtered reaction product by analytical HPLC; a shift towards a more apolar elution position of the derivatized product should be found as compared to the normal peptide, Fig. 3b.

Deprotection of derivatized peptides

The t-BOC group was removed by treatment with concentrated acid, undiluted formic or trifluoroacetic acid. After this treatment the peptide was purified by HPLC as shown in Fig. 4. The deprotected, *i.e.*, tyrosylated peptides are eluted slightly later than the original peptide due to the extra apolar amino acid, tyrosine. After deprotection, the new tyrosyl residue at the amino terminus of the peptides can be verified by amino acid sequence determination, as demonstrated in Table I for PP and the 1-12-fragment of somatostatin-28. The deprotection, particularly with formic acid, can produce heterogeneity of the peptide; in the case of PP a minor fraction of the deprotected peptide is eluted as a separate peak (Fig. 4).

Iodination of derivatized peptides

We have chosen in most instances to iodinate the derivatized peptide directly

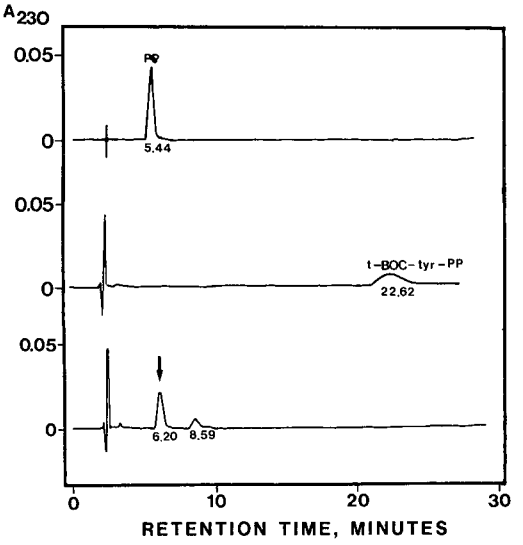

Fig. 4. Deprotection of derivatized, pancreatic polypeptide. Isocratic HPLC analysis of underivatized pancreatic polypeptide (PP), derivatized peptide, t-BOC-Tyr-PP, and the deprotected peptide (lower). The solid arrow indicates the peptide identified as Tyr_0-PP by amino acid sequence analysis, see Table I. The column used in Fig. 1 was eluted with 35% acetonitrile in 0.1% TFA–water.

TABLE I

DETERMINATION OF THE AMINO-TERMINAL AMINO ACID SEQUENCE IN DERIVATIZED, DEBLOCKED PEPTIDES PURIFIED BY HPLC

The amino-terminal sequence of natural, bovine PP (BPP) is Ala-Pro-Leu-Glu-Pro-Glu-Tyr- and the amino-terminal sequence of fragment-1-12 of somatostatin-28 is Ser-Ala-Asn-Ser-Asn-Pro-Ala-.

Sequence cycle	*BPP**		*Somatostatin-28*	
	Amino acid	*Yield (pmol)***	*Amino acid*	*Yield (pmol)***
1	Tyr	79	Tyr	248
2	Ala	65	Ser	44
3	Pro	67	Ala	286
4	Leu	58	Asn	158
5	Glu	31	Ser	26
6	Pro	47	Asn	155
7	Glu	25	Pro	170
8	Tyr	43	Ala	223

* Tyr_0-BPP collected as the peak eluted after 6.20 min in Fig. 4, lower panel.

** Yield of the phenylthiohydantoin derivative of the amino acid in each cycle. Around 200 pmol BPP and 750 pmol somatostatin-28 were applied to the glass filter.

without removal of the protecting t-BOC group. The iodination was performed with the insoluble chloroglycouril as the oxidative agent (Serva), as this causes minimum oxidative damage to larger peptides. The purification of the iodinated, derivatized pancreatic eicosapeptide by isocratic elution in HPLC is shown in Fig. 5a. The peptides are radioiodinated in the "new" amino-terminal tyrosyl residue and therefore react well with antisera which by specific coupling are usually directed towards the carboxy-terminal end of the molecule. In the present case the antisera against the pancreatic eicosapeptide were developed against the peptide coupled to bovine serum albumin (BSA) in its carboxy-terminal α-amino group using difluorodinitrobenzene[10], Fig. 5b and c.

One of the advantages of the original conjugation methods was that the peptide was not in contact with strong oxidants, since the derivatizing reagent was iodinated and purified before it was added to the peptide[6,7,9]. Two different methodological developments within recent years have to some extent made this protection of the peptide superfluous. First, by using the sparingly soluble chloroglycouryl as the oxidative agent, only minimum oxidative damage to peptides occurs[5]. Secondly, because oxidation of methionine residues produces a considerable increase in polarity of the peptide, the purification of the radiolabelled peptides on isocratically eluted HPLC systems will separate oxidized peptides, if at all present, from the non-oxidized forms.

We have chosen to use the Assoian reagent for the introduction of a chemical moiety which can accept radioiodine by substitution. The Bolton–Hunter reagent has previously been used in this way[15–17]. Both reagents use the same active group, N-hydroxysuccinimide ester, for coupling to amino groups on the peptide. Conjugation and subsequent radiolabelling at the amino terminus of the peptides is

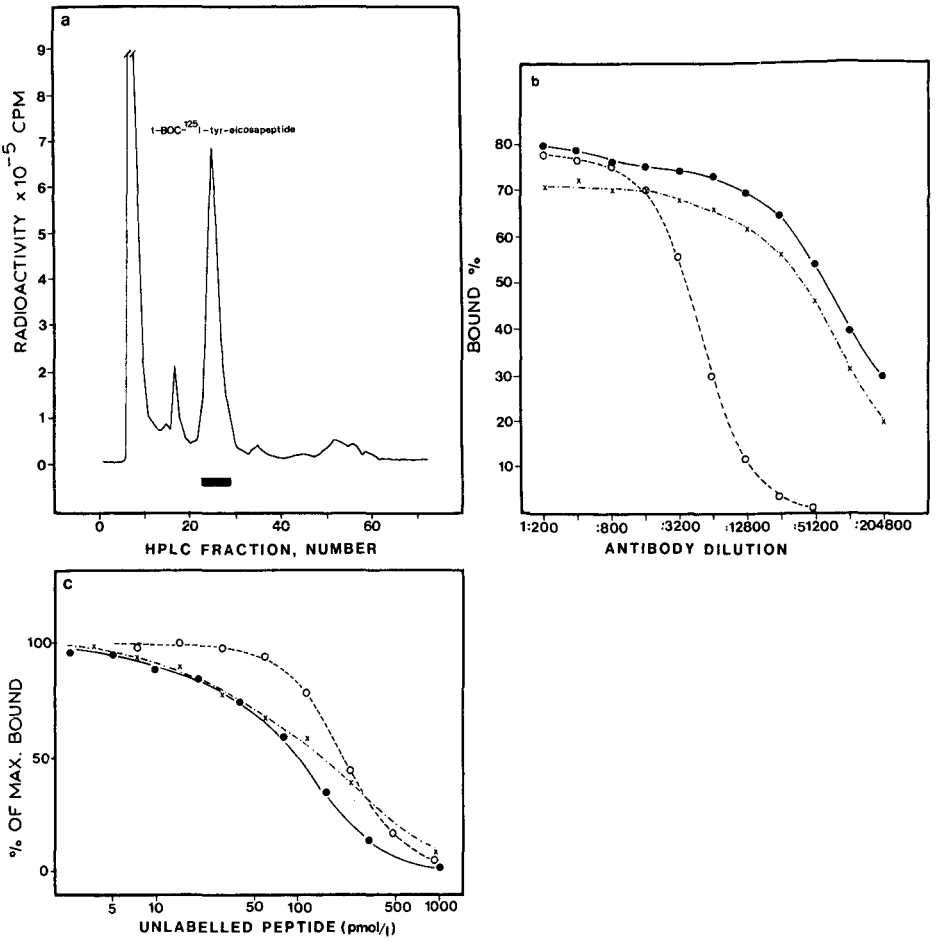

Fig. 5. Radioiodination and radioimmunoassay of derivatized pancreatic eicosapeptide. (a) HPLC purification of t-BOC-Tyr-derivatized pancreatic eicosapeptide radioiodinated by oxidation with 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril. A column similar to the one used in Fig. 1 was eluted isocratically with 28% (v/v) acetonitrile in 0.1% (v/v) TFA in water. The non-iodinated peptide derivative was eluted in fraction 18. The solid bar indicates the fractions used in antibody-binding studies. (b) Binding of radioiodinated, derivatized eicosapeptide to three different antisera, 3201-1 (×), 3202-2 (●) and 3204-L (○). (c) Displacement of natural pancreatic eicosapeptide of radiolabelled, derivatized eicosapeptide binding to different antisera: antiserum 3201-1 diluted 1:6 × 10^4 (×); antiserum 3202-2 diluted 1:1 × 10^5 (●) and antiserum 3204-L diluted 1:2.5 × 10^3 (○).

advantageous since most antisera are raised against peptides coupled to carrier proteins by methods which also use the amino functions of the peptides, *e.g.*, carbodiimide or difluorodinitrobenzene. Hence, it may be advantageous that both the coupling method and the conjugative radiolabelling method leave the carboxy-terminal end of the peptide intact. The Assoian reagent has an extra advantage, as it offers the possibility to regenerate the correct amino group at the beginning of the peptide by removal of the protecting t-BOC group as demonstrated in Fig. 2 and Table

I. This may be important, if the radioiodinated peptide is to be used in cross-linking experiments in receptor studies[18].

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NARROW-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHENYLTHIOCARBAMYL AMINO ACIDS AND CARBOXYPEPTIDASE P DIGESTION FOR PROTEIN C-TERMINAL SEQUENCE ANALYSIS

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SUMMARY

Carboxypeptidase P digestion followed by narrow-bore high-performance liquid chromatography of phenylthiocarbamyl amino acids is employed for polypeptide C-terminal end group and sequence determination. Carboxypeptidase P digestion of polypeptides provides specific cleavage of protein C-terminal amino acids. The digestion offers the advantage that it can be carried out in either 10 m*M* sodium acetate or water at pH 4.0 in the presence of an enzyme activator, Brij-35. The narrow-bore high-performance liquid chromatography of all 20 phenylthiocarbamyl-amino acids has provided quantitative analysis at low picomole levels. This efficient and sensitive procedure is particularly useful for examining *in vivo* excision of protein C-termini and for verifying the integrity of various protein products produced by recombinant DNA techniques.

INTRODUCTION

Analyses of polypeptide C-terminal groups and sequences provide important information on identity and purity of a polypeptide, and on posttranslational processing at the C-terminal end. Methods involving digestion of polypeptides by carboxypeptidases (Cpases) and subsequent identification of the released amino acids by ion-exchange chromatography have often been employed for such analyses[1,2]. This method offers advantages over other approaches which require the use of a specific instrument or multi-step analyses, such as fast atom bombardment (FAB) mapping by mass spectrometry and isolation and characterization of the C-terminal peptide[3,4], in that the Cpase digestion method is performed directly with the intact proteins and the digestion kinetics are determined by time course studies. Chemical degradations, such as hydrazinolysis[5] or the tritium labeling method[6], can also be employed for the C-terminal amino acid determination of proteins; however, these procedures are less sensitive, laborious, and can only release one single C-terminal end group. Although sequential chemical degradations of proteins from the C-terminus using different reactions were reported previously[7,8], these degradations require considerably larger amounts of polypeptide and have not been made routine.

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Cpases A (EC 3.4.12.2) and B (EC 3.4.12.3) from bovine or porcine pancreas and Cpase Y (EC 3.4.12.–) from Baker's yeast have been widely used in the C-terminal amino acid determination[9–11]. However, these enzymes have limited uses due to their narrow specificity for end groups or poor ability to digest a broader range of protein substrates. Recently, an acid carboxypeptidase, Cpase C (EC 3.4.12.1), which was isolated from citrus fruits[13,14], was shown to exhibit broader specificity to sequentially remove various C-terminal residues at various cleavage rates. One drawback of using this Cpase, however, is endopeptidase contamination in the commercial preparations; their further purification may become necessary. On the other hand, Cpase P (EC 3.4.12.–). has been purified to homogeneity from *Penicillium janthinellum*[15,16] and has been observed to exhibit broader specificity for various C-terminal amino acids, including those resistant to Cpase A, B, or Y cleavage. However, Cpase P is not widely used for C-terminal sequence analysis since the specificity of the enzyme is not well understood. The lower detection sensitivity of conventional analytical methods for amino acid analysis also hampers the application of the enzymatic methods for C-terminal analysis.

In this communication, we describe the use of narrow-bore high-performance liquid chromatography (HPLC) of phenylthiocarbamyl (PTC)-amino acids to determine the composition of Cpase P digestion products for protein C-terminal end group and sequence determinations. The narrow-bore HPLC of PTC-amino acids allows resolution of all twenty PTC-amino acids including asparagine and glutamine such that the released end groups can be identified and quantified. The present procedure is able to obtain useful C-terminal sequence information by high sensitivity analysis of amino acids released from limited Cpase P digestion of various proteins and peptides.

MATERIALS AND METHODS

Materials

Cpase P (Cat. No. C-5396), hen egg lysozyme and Brij-35 were obtained from Sigma. Cpase P can also be purchased from Boehringer Mannheim. Recombinant interleukin-2 (IL-2) (Ala-125), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulation factor (GM-CSF), erythropoietin and consensus α-interferon were research products of Amgen and produced by genetically modified *Escherichia coli* except that recombinant erythropoietin was produced by genetically engineered Chinese hamster ovary cells. Bradykinin was a synthetic peptide purchased from Serva. PD-10 columns, containing prepacked Sephadex G-25, were obtained from Pharmacia. The amino acid sequences of these proteins and peptides at the carboxyl terminus are listed in Table I.

Methods

HPLC apparatus. A Hewlett-Packard microbore LC system (HP1090) was used for the identification of PTC-amino acids. The system is equipped with an HP autosampler and a diode array detector for automatic analysis. A Nelson Analytical 4400 system using XTRA-CHROM software is connected to the chromatographic system for data acquisition and processing.

Phenylisothiocyanate (PITC) derivatization of amino acids and separation of

TABLE I

C-TERMINAL AMINO ACID SEQUENCES OF POLYPEPTIDES STUDIED IN THIS EXPERIMENT

The one-letter code for amino acids is as follows: A = alanine; C = cysteine; D = aspartate; E = glutamate; F = phenylalanine; G = glycine; H = histidine; I = isoleucine; K = lysine; L = leusine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine.

Polypeptides	*Sequences**
	160 174
Human rG-CSF	···F-L-E-V-S-Y-R-V-L-R-H-L-A-Q-P
	120 127
Human rGM-CSF	···D-Ç-W-E-P-V-Q-E
	125 133
Human rIL-2(Ala-125)	···A-Q-S-I-I-S-T-L-T
	158 165
Human recombinant erythropoietin	···G-E-A-Ç-R-T-G-D-[R]**
	157 166
Consensus α-interferon	···N-L-Q-E-R-L-R-R-K-E
Henn egg lysozyme	···Ç-R-L
	1 9
Bradykinin	R-P-P-G-F-S-P-F-R

* Ç stands for cysteine involved in disulfide bond formation.

** Arg in the bracket is predicted from gene sequence of erythropoietin but not detected by C-terminal amino acid analysis of the hormone.

PTC-amino acids. PITC coupling of standard amino acids or samples obtained from Cpase P digestion was performed according to our previously reported procedures[17]. Separation of the derivatized PTC-amino acids was then carried out by reversed-phase HPLC using an Altex narrow-bore C_{18} column (25 × 0.2 cm) and a gradient of solvent B in solvent A. Solvent A was 25 m*M* sodium acetate (pH adjusted to 5.0 with phosphoric acid). Solvent B was 25 m*M* sodium acetate (pH adjusted to 6.5 with phosphoric acid)–acetonitrile–methanol (40:50:10). The column was equilibrated in A–B (90:10) and operated at 40°C using a flow-rate of 0.25 ml/min. Separation was achieved using a linear gradient of 10 to 11% B in 1 min, 11 to 47% B in 14 min, 47 to 55% B in 5 min, 55 to 85% B in 1 min, 85 to 100% B in 1 min and followed by an isocratic elution at 100% B for 3 min. Triethylamine (100–200 μl) can be added to solvent A if PTC-Arg is difficult to resolve from PTC-Ala. The PTC-amino acid derivatives were detected at 265 nm.

Cpase A, B and Y digestion. A suspension of diisopropyl phosphorofluoridate (DFP)-treated Cpase A (Sigma) was repeatedly washed with distilled water to remove contaminating free amino acids[9]. The enzyme was then collected by centrifugation and redissolved in 0.2 *M* sodium bicarbonate before use. DFP-treated Cpase B (Sigma) and Cpase Y (Pierce) were used without further purification. Cpase A and/or B digestion was performed in 0.1 *M* N-methylmorpholine (pH 7.6) using an enzyme-to-substrate ratio of 1:100 to 1:50 at 37°C. Cpase Y digestion was carried out in 0.1

M ammonium acetate (pH 5.5) at 37°C using an enzyme-to-substrate ratio of 1:100. A combined digestion by Cpases Y and B was performed at two different pH values (pH 5.5 and 7.6) according to Hayashi[12].

Cpase P digestion. Protein substrates (1–4 nmol) were concentrated by centrifugation using a Centricon-10 (Amicon) microconcentrator with a 10-kilodalton (kD) molecular weight cut-off membrane, and buffer salts were removed by gel filtration over a Pharmacia PD-10 column equilibrated in 10 m*M* sodium acetate, pH 4.0 or distilled water. In the latter condition, the pH of the desalted sample solution was adjusted to 4.0 by diluted hydrochloric acid. Before adding enzyme, a concentrated solution of Brij-35 was added to the protein sample to a final concentration of 0.05% (w/v). The Cpase P digestion conditions varied according to the rate of amino acid release obtained from digestion of different substrates. The normal digestion was performed using an enzyme-to-substrate ratio of 1:400 (w/w) at 25°C in 1 h. When such digestion conditions generated a rapid release rate, the reaction could be slowed down by incubating the sample at 4°C. For samples which were difficult to digest, an enzyme-to-substrate ratio of 1:100 for digestion at 37°C in several hours was used. After digestion, samples at various time points including zero time were taken and immediately acidified with trifluoroacetic acid at a final concentration of 10% to stop the digestion. Acidified samples were dried *in vacuo* by a Speedvac centrifuge and subjected to PITC derivatization and subsequent HPLC analysis as described above. Norleucine (1–2 nmol) was usually added to sample solutions prior to digestion as an internal standard to calibrate recovery of amino acids.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of Cpase P digestion products. Aliquots of Cpase P digestion products at specific time intervals were added to SDS-PAGE sample buffer. The mixtures were immediately incubated at 65°C for 30 min or at 100°C for 5 min to stop Cpase P digestion. The samples were then subjected to SDS-PAGE according to Laemmli[18].

RESULTS AND DISCUSSION

Narrow-bore HPLC of PTC-amino acids

Analysis of amino acids released from Cpase digestion of polypeptides requires a chromatographic procedure that can offer a complete separation of all 20 common amino acids including asparagine, glutamine and tryptophan. The conventional ion-exchange chromatography[19] or HPLC of the precolumn derivatized amino acids[20,21] is usually employed for analysis of samples derived from acid hydrolysis of polypeptides and thus excludes the separation of asparagine and glutamine. Although the ion-exchange chromatography used for the separation of physiological amino acids[22] is well established for C-terminal end group and sequence analysis using Cpase digestion, this method is limited by its lower sensitivity of detection. An improved HPLC procedure using precolumn PITC derivatization and subsequent narrow-bore HPLC was developed to enhance the sensitivity of analysis. Fig. 1 illustrates results of the chromatographic analysis of a standard amino acid mixture at levels of 4 pmol (chromatogram A) and 40 pmol (chromatogram B). The chromatographic conditions allow the separation of all 20 common amino acids. The use of mobile phase A at lower pH effectively resolves Asn from Ser, and Gln from Gly, which otherwise coelute using conditions reported previously[17,20,21]. The addition

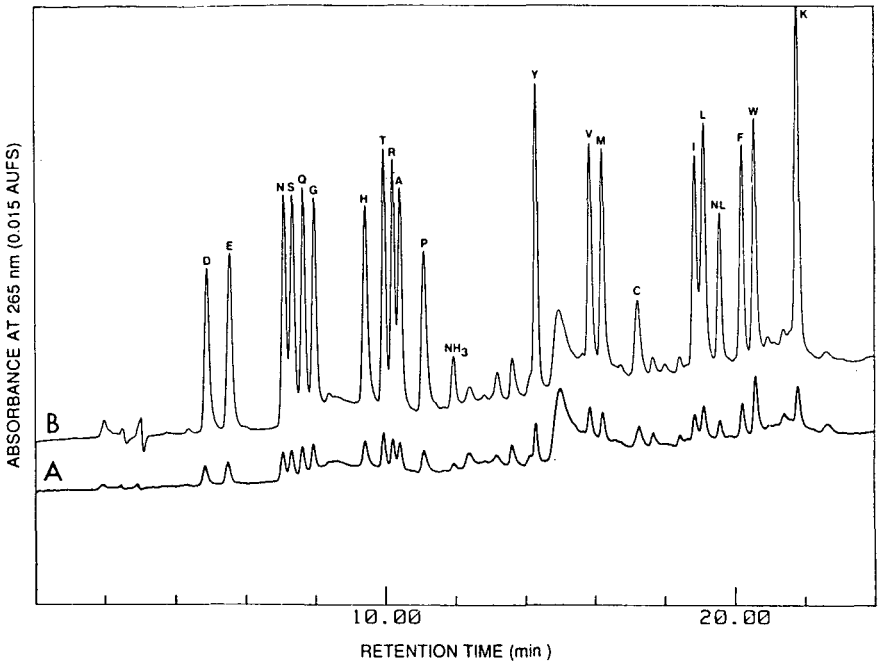

Fig. 1. HPLC separation of 21 PTC-amino acids using an Altex C_{18} column (25 × 0.2 cm, 5 μm). (A) 4 pmol each of standard amino acids including Asn, Gln, Trp and norleucine. (B) 40 pmol of standard.

of triethylamine in mobile phase A facilitates the separation of Arg from Ala. Norleucine completely separates from Leu and Phe, and can be used as an internal standard to evaluate accuracy and reproducibility of PITC coupling and HPLC.

Cpase digestion and amino acid analysis of the digestion products

Cpase A and B as well as Cpase Y digestion have been mostly cited for polypeptide C-terminal analysis[9–11]. Their application in analysis of small peptide sequences was frequently successful. However, it may become more difficult to cleave some of the higher molecular weight protein substrates by these enzymes.

Many lymphokines and hemopoietic factors have been reproduced for human therapeutic uses by the newly developed recombinant DNA technologies. The structural analysis of these highly purified proteins is usually undertaken as a requirement for product application in human clinical use. The purpose of such studies is to confirm the intactness of the molecule, especially at both amino and carboxyl terminal ends. In spite of the successful analysis of protein amino terminal sequences by automated Edman degradation chemistry, we had experienced unsuccessful uses of Cpases A, B and Y to perform C-terminal analyses of many recombinant proteins such as human rIL-2(Ala-125), consensus α-interferon, and erythropoietin (for details on their C-terminal sequences, see Table I) due to specificity and lower activity of these enzymes. Very little information can be obtained even with a digestion using two Cpases together (*i.e.*, Cpases A and B, Cpases B and Y). Moreover, the digestion conditions recommended for these enzymes include buffers containing high concen-

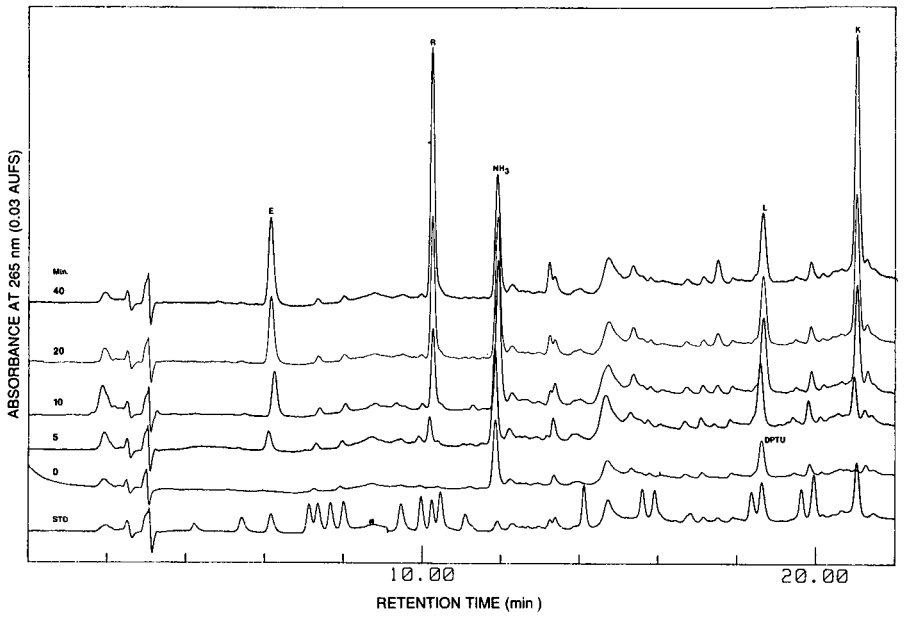

Fig. 2. HPLC analysis of amino acids released from Cpase P digestion of r-consensus α-interferon at various time intervals. Sample (100 pmol) at each time point was subject to analysis after PITC derivatization.

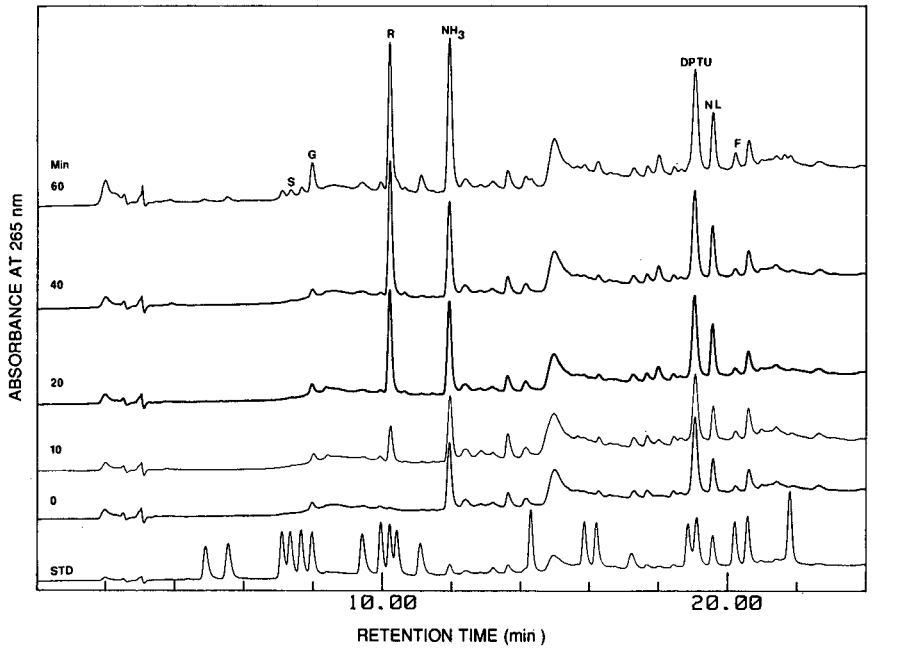

Fig. 3. HPLC analysis of amino acids released from Cpase P digestion of bradykinin at various time intervals. For details, see legend of Fig. 2.

tration of salts, and are not compatible for subsequent PITC derivatization, as buffer salts seriously interfere with the derivatization efficiency and removal of side products during the drying step. These difficulties led us to carry out protein C-terminal analysis using Cpase P digestion in conjunction with narrow bore HPLC.

Fig. 2 shows chromatograms obtained from HPLC of PITC-derivatized digests of r-consensus α-interferon taken at 0, 5, 10, 20 and 40 min. The reaction was carried out using an enzyme-to-substrate ratio of 1:400 at 4°C. At zero time, no amino acid was released by the enzyme, indicating that the enzyme preparation is free from any contaminating amino acids, and that the trifluoroacetic acid (TFA) acidification effectively terminated the digestion. It is apparent that four amino acids, *i.e.*, Glu, Arg, Leu and Lys could be detected and quantified from a cleavage of five consecutive C-terminal amino acids within 40 min. Fig. 3 illustrates the analysis obtained from the digestion of bradykinin. Only one C-terminal Arg was released from digestion of this 9-residue synthetic peptide.

Table II summarizes the quantitative recovery of released amino acids at different time points for proteins and peptides which were treated with Cpase P. All of the samples used in this study are clearly digested by the enzyme at various rates. It is apparent that Cpase P is a very efficient enzyme capable of cleaving most peptide bonds. We found that the appropiate conditions for a successful digestion are to use normal digestion conditions for several time intervals within 1 h, as illustrated in the digestion of human recombinant G-CSF, GM-CSF and erythropoietin (Table II). In the example for the digestion of r-consensus α-interferon, such conditions generated very fast kinetics (results not shown). The experiment was thus conducted at 4°C within 60 min. Bradykinin, human rIL-2(Ala-125), hen egg lysozyme and human r-erythropoietin are more resistant to Cpase P digestion; thus, the enzyme-to-substrate ratio was adjusted to 1:100 and the digestion performed at 37°C for several hours to obtain a good cleavage rate.

For digestion of samples which only release one or two amino acid residue(s), the C-terminal amino acids can be directly identified, as is the case in the digestion of lysozyme and bradykinin for single amino acid release (Leu and Arg, respectively), and rIL-2(Ala-125) as well as rGM-CSF for release of two amino acids (–Leu–Thr and –Gln–Glu, respectively). However, the assignment of the C-terminal sequence for samples with rapid digestion requires quantitative kinetic analysis. Fig. 4 (top) illustrates the plot of amino acid recovery at various time points for r-consensus α-interferon. It is clear that the C-terminal sequence of the recombinant lymphokine can be assigned as ···Leu–Arg–Arg–Lys–Glu. Arginine is released in two residues and its initial release rate is slower than glutamate but faster than Leu. In the case of recombinant G-CSF (Fig. 4, bottom), the first four amino acids can be assigned at 5–10 min digestion (···Leu–Ala–Gln–Pro). At the 30-min time point, more amino acids are released with no further sequence assignment, since histidine at the 5th position is released much more slowly than arginine at the 6th position. However, at least 10 amino acids from the C-terminus up to Tyr-165 are clearly cleaved. The released C-terminal amino acids up to the 10th residue is consistent with the sequence predicted from the cDNA sequence. The mis-assignment at the 5th and 6th positions for histidine and arginine is due to the slower release rate of the His residue. The above analyses indicate that fast kinetic release of amino acids for an unknown protein digestion should be carefully interpreted to prevent mis-assignment.

TABLE II

RECOVERY OF RELEASED AMINO ACIDS FROM CARBOXYPEPTIDASE P DIGESTION OF VARIOUS PROTEIN AND PEPTIDE SUBSTRATES*

(1) r-Consensus α-interferon (enzyme-to-substrate = 1:400 at 4°C)
157 166
C-terminal sequence: ···N-L-Q-E-R-L-R-R-K-E

	Time (min)				
	0	*5*	*10*	*20*	*40*
Glu	0	0.18	0.48	0.75	0.86
Arg	0	0.17	0.57	1.15	1.80
Leu	0	0.11	0.12	0.19	0.23
Lys	0	0.14	0.38	0.53	0.78

(2) Human r-G-CSF (enzyme-to-substrate = 1:400 at 25°C)
160 174
C-terminal sequence: ···F-L-E-V-S-Y-R-V-L-R-H-L-A-Q-P

	Time (min)				
	0	*5*	*10*	*15*	*30*
Gln	0	0.06	0.32	0.74	0.93
His	0	0	0.13	0.41	0.57
Arg	0	0	0.24	0.88	1.45
Ala	0	0.04	0.31	0.91	1.02
Pro	0	0.12	0.55	0.96	1.17
Tyr	0	0	0.02	0.06	0.20
Val	0	0	0.02	0.27	0.59
Leu	0	0.02	0.32	1.30	1.60

(3) Bradkinin (enzyme-to-substrate = 1:100 at 37°C)
Amino acid sequence: R-P-P-G-F-S-P-F-R

	Time (min)				
	0	*10*	*20*	*40*	*60*
Arg	0	0.23	0.72	0.85	0.88
Gly	Trace	0.04	0.04	0.05	0.14
Phe	Trace	0.02	0.02	0.04	0.05

(4) Hen egg lysozyme (enzyme-to-substrate = 1:100 at 37°C)
*C-terminal sequence: ···C-R-L***

	Time (h)			
	0	*1*	*2*	*4*
Leu	0	0.38	0.39	0.44

TABLE II (*continued*)

(5) Human r-interleukin-2(Ala-125) (A: enzyme-to-substrate = 1:400 at 25°C) (B: enzyme-to-substrate = 1:100 at 37°C)

C-terminal sequence: ···A-Q-S-I-I-S-T-L-T (125 … 133)

(A)	*Time (min)*				
	0	*5*	*20*	*40*	*60*
Thr	0	0.09	0.11	0.13	0.18
Leu	0	0	Trace	Trace	Trace

(B)	*Time (h)*			
	0	*1*	*2*	*4*
Thr	0	0.64	0.85	0.91
Leu	0	0.61	0.81	0.90

(6) Human rGM-CSF (enzyme-to-substrate = 1:400 at 25°C)

C-terminal sequence: ···D-Ç-W-E-P-V-Q-E (120 … 127)

	Time (min)				
	0	*10*	*20*	*40*	*60*
Glu	0	0.18	0.75	0.86	0.92
Gln	0	0.15	0.13	0.29	0.40

(7) Human r-erythropoietin (enzyme-to-substrate = 1:00 at 37°C)

*C-terminal sequence: ···G-E-A-Ç-R-T-G-D-[R]*** (158 … 165)

	Time (min)				
	0	*10*	*20*	*40*	*60*
Asp	0	0.09	0.20	0.43	0.57
Gly	0	0.07	0.18	0.34	0.51
Thr	0	0.05	0.08	0.20	0.30
Arg	0	0.01	0.02	0.08	0.12
Ala	0	0.01	0.02	0.08	0.10

* The numbers are expressed in molar recovery of released amino acids. Digestion of all samples was performed in 10 m*M* sodium acetate, pH 4.0, except recombinant consensus α-interferon and r-erythropoietin, which were done in water, pH 4.0.

** Ç stands for cysteine involved in disulfide bond formation.

*** See footnotes in Table I.

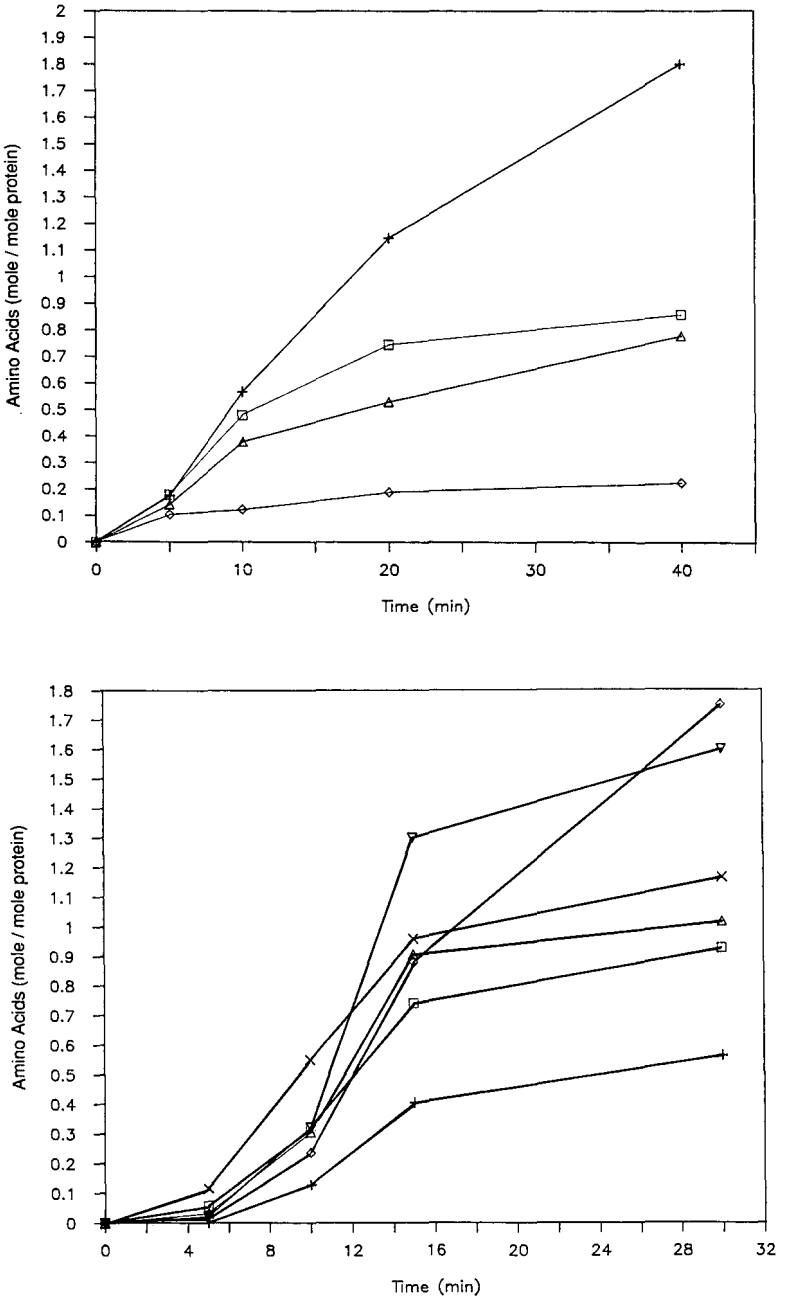

Fig. 4. Kinetic analysis of released amino acids from Cpase P digestion of human recombinant consensus α-interferon (top) and G-CSF (bottom). Top: (□) Glu; (+) Arg; (◇) Leu; (△) Lys. Bottom: (□) Gln; (+) His; (◇) Arg; (△) Ala; (×) Pro; (▽) Leu.

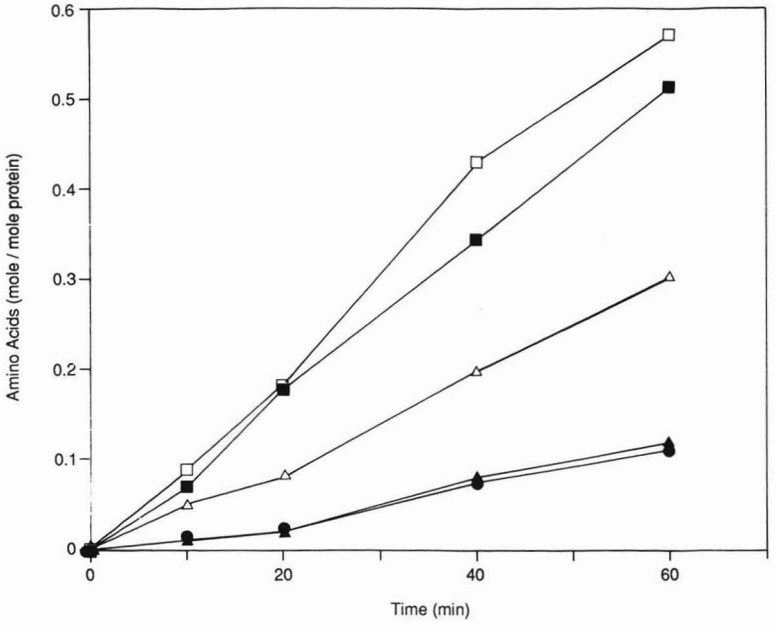

Fig. 5. Kinetic analysis of released amino acids from Cpase P digestion of human r-erythropoietin. (□) Asp; (■) Gly; (△) Thr; (▲) Arg; (●) Ala.

Very slow release of amino acids was observed when the digestion of human r-erythropoietin was done at 25°C with a lower enzyme-to-substrate ratio (1:400). Better results were thus obtained from digestions using an enzyme-to-substrate ratio of 1:100 at 37°C. Fig. 5 illustrates the kinetic plot for the release of amino acids after digestion of erythropoietin, yielding a C-terminal sequence of ···Arg–Thr–Gly–Asp. Much slower release of Arg and Ala was observed, indicating that the Ala–Cys–Arg··· sequence is more resistant to Cpase P attack as found in the digestion of lysozyme. The determined C-terminal sequence indicates that the arginyl residue predicted to be at the C-terminus according to the gene sequence[23] is missing from the recombinant protein. This result is confirmed by the isolation of des-Arg C-terminal peptide and is consistent with the data obtained from Cpase P digestion of human urinary erythropoietin (our unpublished data). It is intriguing to reveal that recombinant erythropoietin expressed in Chinese hamster ovary cells is processed by an endogenous carboxypeptidase in a manner similar to the carboxyl terminal processing of the natural hormone.

The broad specificity of the purified acid carboxypeptidase P has been described previously[16]. The enzymatic properties of this protease are similar to those of Cpase C from citrus fruit; however, their apparent differences were reported previously[15,16]. Cpase C might be contaminated with other amino- or endo-peptidase activity. On the contrary, the commercially available Cpase P does not require further purification prior to use.

As pointed out by Allen[24], endoproteolysis is inherent with all the methods employing Cpases such as Cpases A, B, C or Y. Such non-specific cleavage can be

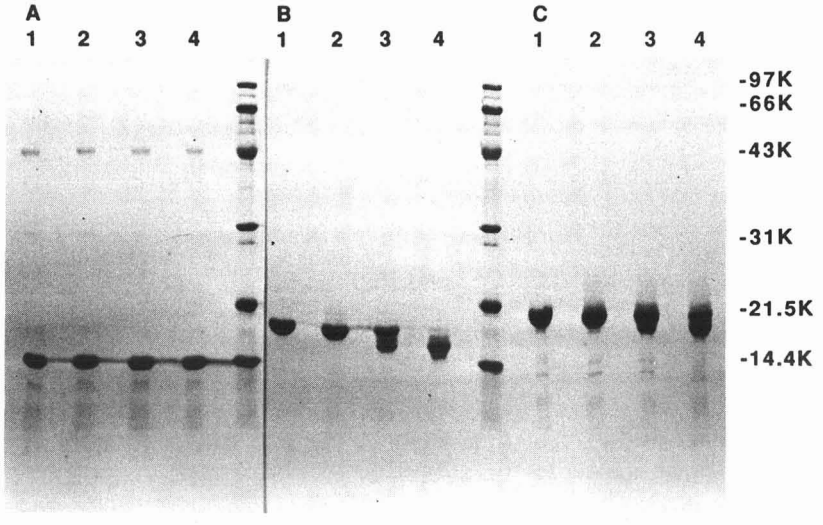

Fig. 6. SDS-PAGE of digested products derived from Cpase P digestion of hen egg lysozyme, human recombinant consensus α-interferon and human rG-CSF. For digestion conditions, see Table I. (A) Lysozyme: lane 1, control: lane 2, 0 time; lane 3, 1 h; lane 4, 4 h. (B) rG-CSF: lane 1, control; lane 2, 0 time; lane 3, 20 min; lane 4, 60 min. (C) Recombinant consensus α-interferon: lane 1, control; lane 2, 0 time; lane 3, 20 min; lane 4, 60 min.

demonstrated by SDS-PAGE of the final digestion product in that significant diminution in the intensity of the original protein band and the appearance of additional bands of lower molecular weight would occur. The non-specific proteolysis may result in interference of sequence assignment as is frequently seen in the literature[25]. In the subsequent studies, Cpase P digestion products obtained from several proteins at various time intervals were examined by SDS-PAGE to examine if the enzyme preparation contained endopeptidase activity. As shown in Fig. 6A, the mobility of protein bands for lysozyme remain unchanged from authentic sample, 0 h, 1 h and 4 h digestion products, indicating that the removal of a single C-terminal amino acid from lysozyme does not alter the apparent molecular weight of the protein. As indicated in Fig. 6B, recombinant G-CSF can be digested by Cpase P in a specific and sequential manner. After 1 h digestion, a new 17.6-kD band is generated by sequential degradation of the original 18.8-kD protein band from the C-terminal end. When the digestion products of recombinant consensus α-interferon were analyzed (Fig. 6C), a protein band at 19 kD appeared together with the 20-kD band within 1 h incubation. The intensity of the 19-kD band increases with the concomitant decrease of the original 20-kD band. It is important to note that no other lower molecular weight degradation bands were observed in the digests of these three proteins. These results also agree with the number of released amino acid residues determined by amino acid analysis (Table I). More importantly, these data indicate that the Cpase P preparation is devoid of endopeptidase activity. Since the PTC-amino acid analysis of Cpase P-treated proteins did not reveal the presence of N-terminal amino acids, especially methionine, it is thus concluded that limited C-terminal exoproteolysis by Cpase P had occurred during a controlled time course digestion.

It has been observed that Cpase P is stable and more reactive in the presence of various non-ionic detergents including Brij-35. The reaction can thus be performed in water containing 0.05% Brij-35 at lower pH. Brij-35 is advantageous to the digestion as it acts as a stabilizer for both Cpase P and protein substrate. The digestion solution containing no buffer or low buffer salt (*i.e.*, 10 m*M* sodium acetate) has great advantage when the analysis is performed using PITC derivatization and HPLC. Salt was found to interfere with subsequent amino acid analysis using narrow-bore HPLC as described previously.

Drawbacks observed during the use of Cpase A and/or B or Y digestion, such as varying cleavage efficiency and resistance of many bonds, have hindered their successful application in C-terminal sequence analysis. For example, recombinant consensus α-interferon, IL-2, and erythropoietin are resistant to cleavage by these enzymes even in combined digestion. On the contrary, Cpase P itself effectively releases C-terminal amino acids from these proteins. Although Cpase P cleaves almost every terminal amino acid, several specific peptide bonds are still resistant to the enzyme. Resistance of Gly–X and Pro–X bonds to Cpase P cleavage has been reported[16]. Similar results are also observed during the digestion of recombinant GM-CSF and bradykinin (Table II). Other peptide bonds such as the Cys–X bond, in which Cys is involved in disulfide linkage, are also resistant to Cpase P digestion. This is evident from the fact that the enzyme can only effectively cleave a single terminal amino acid for lysozyme, since a Cys–Arg bond was found right next to the cleaved Leu. This suggests that S-carboxymethylated or performic acid oxidized samples should be prepared for extended C-terminal analysis.

It is interesting to note that the whole rIL-2(Ala-125) molecule is quite resistant to Cpase P digestion under normal digestion conditions (Table II). The digestion of rIL-2(Ala-125) only releases two amino acids from the C-terminal end of the protein using a 1:100 enzyme-to-substrate ratio at 37°C. The remaining sequence (···Ala–Gln–Ser–Ile–Ile–Ser–Thr) preceding the C-terminal Leu–Thr stays uncleaved for hours. This result seems to suggest that Cpase P is unable to further cleave the Ser–Thr bond.

In summary, the described procedure involving the use of narrow-bore HPLC of PTC-amino acids derived from Cpase P digestion offers an effective and sensitive method for protein and peptide C-terminal sequence analysis. Since no other efficient sequential degradation methods are available for C-terminal analysis, this procedure should be valuable for the determination of the C-terminal sequence of proteins and peptides. The utilization of narrow-bore HPLC for microanalysis of all 20 amino acids greatly enhances the sensitivity required for evaluation of samples available only in minute quantities. Using the described C-terminal analysis methods in conjunction with automated N-terminal sequencing, both amino- and carboxy-terminal sequences can be established for assigning the protein coding region during molecular cloning of genomes. From this study, Cpase P is also best used to compare a native and recombinant protein, given the complexities of its C-terminal exopeptidase activity from one protein to another. Moreover, compared to the more established Cpases A, B, C and Y, Cpase P is a better exopeptidase and has wider applications for limited modification of proteins. It should gain broader application to the study of the relationship between polypeptide structure and function[26].

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CHROM. 20 550

LIQUID CHROMATOGRAPHIC DETERMINATION OF PENICILLINS BY POSTCOLUMN DEGRADATION WITH SODIUM HYPOCHLORITE USING AN HOLLOW-FIBRE MEMBRANE REACTOR

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SUMMARY

A sensitive, high-performance liquid chromatographic method involving postcolumn degradation with sodium hypochlorite and using a hollow-fibre membrane as a reactor is described for the determination of penicillins. Penicillins were separated on a C_{18} column followed by postcolumn reaction with sodium hypochlorite and sodium hydroxide using aminated and sulphonated hollow-fibre membrane reactors immersed in each solution, and detected at 270–280 nm based on the UV absorbances of the degradation products. At penicillin concentrations of 2 μg/ml, the precisions (relative standard deviation) were 2.28–4.78%. The detection limits of the proposed method were 2.5–25 ng for each penicillin at a signal-to-noise ratio of 3. Ampicillin and its metabolites [(5*R*,6*R*)-ampicilloic acid, the (5*S*,6*R*)-epimer and (2*R*)-pierazine-2′,5′-dione] in human serum and urine were simultaneously determined by this method.

INTRODUCTION

Some strategies for the sensitive and selective determination of a drug and its metabolite(s) in body fluids by high-performance liquid chromatography (HPLC) are to perform the sample preparation (which includes enrichment of compounds of interest and/or removal of interfering compounds) and/or to develop a precolumn or postcolumn derivatization method. In previous papers, we developed two HPLC methods for the determination of penicillins and their metabolites in plasma and urine which employed postcolumn reactions with (a) sodium hydroxide, mercury(II) chloride and ethylenediaminetetraacetic acid[1,2], and (b) sodium hypochlorite and sodium hydroxide[3,4]. These methods were sensitive to the metabolites as well as the unchanged penicillins, in contrast to previous postcolumn derivatization methods[5–12] which are sensitive only to the unchanged penicillins. Method (b) has the advantage that it does not require the use of mercury(II) chloride, which is toxic to humans and is an environmental pollutant.

The conventional postcolumn reaction method needs an additional pump(s)

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for delivering the reagent solutions, a mixing unit(s) and a reactor(s). The postcolumn reaction method using an hollow-fibre membrane reactor (HFMR) need not employ the additional reaction devices described above. Previously[13–16], we reported that the HFMR is useful for the postcolumn reaction.

This paper deals with an HPLC method for the determination of penicillins using aminated and sulphonated HFMRs to introduce hypochlorite and hydroxide ions into the main flow stream for the postcolumn degradation reaction. The method was successfully applied to the determination of ampicillin and its metabolites in human serum and urine.

EXPERIMENTAL

Reagents and materials

Ampicillin (ABPC), phenethicillin (PEPC), phenoxymethylpenicillin (PCV) and ciclacillin (ACPC) were kindly donated by Meiji Seika Kaisha (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan). PEPC is a mixture of the (10*R*)- and (10*S*)-epimers. Benzylpenicillin (PCG) was obtained from Sigma (St. Louis, MO, U.S.A.). ABPC metabolites [(5*R*,6*R*)-ampicilloic acid (1), the (5*S*,6*R*)-epimer (2) and (2*R*)-piperazine-2′,5′-dione (3)] were prepared according to the methods reported previously[17,18]. The structures and abbreviations of the penicillins and ABPC metabolites used are shown in Figs. 1 and 2, respectively. Sodium heptanesulphonate and other chemicals of analytical reagent grade were obtained from Nakarai Chemicals

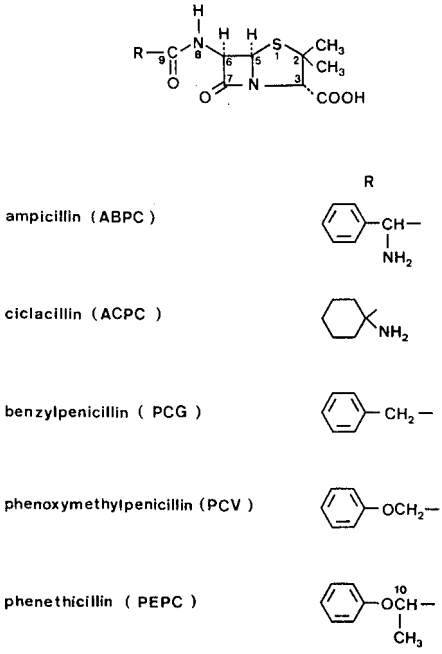

Fig. 1. Structures and abbreviations of penicillins.

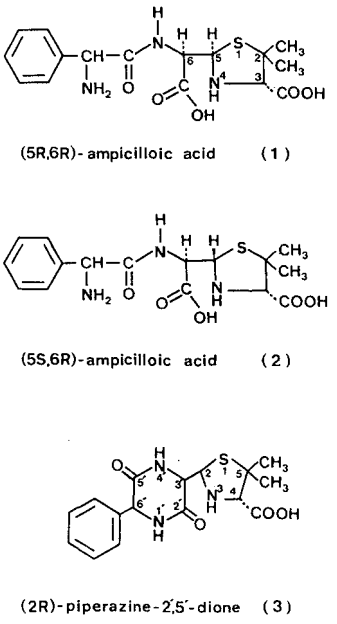

Fig. 2. Structures of ampicillin metabolites.

(Kyoto, Japan). Control serum (Control Serum I Wako) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Sulphonated and aminated hollow-fibre membranes (AFS-2 and CFS1-2 fibres) were obtained from Dionex (Sunnyvale, CA, U.S.A.).

Deionized, glass-distilled water and distilled methanol were used for the preparations of sample solutions and HPLC eluents.

Chromatography

The instrumentation used was as follows: a Model 655 pump (Hitachi, Tokyo, Japan) for eluent delivery; a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A. equipped with a 100-μl loop for sample loading; a main column (125 mm × 4 mm I.D.) and a guard column (4 mm × 4 mm I.D.) packed with LiChrosphere RP-18(e) (particle size 5 μm; E. Merck, Darmstadt, F.R.G.) for the separation of penicillins; a main column (150 mm × 4.6 mm I.D.) and a precolumn (30 mm × 4.6 mm I.D.) packed with Nucleosil C_{18} (particle size 5 μm; Macherey-Nagel, Düren, F.R.G.) for the separation of ABPC and its metabolites; a Model 638-41 variable-wavelength UV monitor (Hitachi) equipped with a 17-μl flow-through cell for detection; a C-R3A recorder-integrator (Shimadzu, Kyoto, Japan) for recording and integrating chromatographic peaks. The eluents used were: A, 10 m*M* sodium dihydrogenphosphate–10 m*M* disodium hydrogenphosphate–methanol (0.9:0.9:1, v/v/v); B, 20 m*M* sodium dihydrogenphosphate–20 m*M* disodium hydrogenphosphate –acetonitrile (3:3:1, v/v/v); C, 15 m*M* sodium heptanesulphonate –21 m*M* phosphoric acid–9 m*M* sodium dihydrogenphosphate–acetonitrile (1.5:1.5:1.5:1). The flow-rate was 0.8 ml/min. Eluent A was used for the separation of penicillins, B for ABPC in serum samples and C for ABPC and its metabolites in urine samples. The aminated and sulphonated HFMRs were connected in series and inserted between the column and the detector. They were immersed in 50-ml beakers containing sodium hypochlorite plus sodium hydroxide solution and sodium hydroxide solution, respectively. The postcolumn reaction conditions (lengths of the HFMRs and concentrations of the reaction solutions) employed for the assay of penicillins and ABPC metabolites are listed in Table I. Separations and postcolumn reactions were carried out at ambient temperature and 50°C, respectively. Detection was performed at 280 nm for unchanged penicillins and 260 nm for ABPC and its metabolites (1, 2 and 3).

Comparison of detection methods

The detection methods were compared as follows: A, detection at 230 nm

TABLE I

OPTIMUM POSTCOLUMN REACTION CONDITIONS

	Penicillins	*ABPC metabolites*
Aminated HFMR length (cm)	50	20
Sodium hypochlorite concentration (%)*	0.2	0.2
Sulphonated HFMR length (cm)	80	80
Sodium hydroxide concentration (*M*)	0.5	3

* Including 1 *M* sodium hydroxide.

without a reactor; B, detection at 280 nm with an open-tubular reactor; C, detection at 280 nm with aminated and sulphonated HFMRs. For method B, the additional reaction devices used were: a double plunger pump (NP-DX-2; Nihon Seimitu Kagaku, Tokyo, Japan) for delivering the postcolumn reagent (0.02% sodium hypochlorite and 0.5 *M* sodium hydroxide) at a flow-rate of 0.2 ml/min; a mixing tee made of Diflon (each angle, 120°) and a reaction coil of 1 m × 0.5 mm I.D. PTFE tube for the postcolumn reaction[3,4]. For method C, the postcolumn reaction conditions were the same as described above.

Pretreatment of serum and urine samples

Serum samples (200 μl) were ultrafiltered using a Molcut II (Nihon Millipore, Tokyo, Japan). A 20-μl portion of the ultrafiltrate was loaded onto a column.

Urine samples, diluted 10-fold in water, were filtered with a 0.45-μm acrylate copolymer membrane (Gelman Science Japan, Tokyo, Japan). A 20-μl portion of the filtrate was loaded onto a column.

RESULTS AND DISCUSSION

Reaction conditions for HFMR

In this study, we attempted to perform the previously reported[3,4] postcolumn reaction by using the HFMRs immersed in sodium hypochlorite and sodium hydroxide solutions. The postcolumn reaction conditions for penicillins, and ABPC and its metabolites, were examined with regard to the lengths of the HFMRs, concentrations of sodium hypochlorite and sodium hydroxide and reaction temperature. Eluents A (for penicillins) and C (for ABPC and its metabolites) were delivered at a flow-rate of 0.8 ml/min. A 20-μl portion of the solution of penicillins or ABPC and its metabolites was loaded onto the column and the peak heights were measured. First the aminated HFMR immersed in sodium hypochlorite plus sodium hydroxide solution was used for introducing hypochlorite and hydroxide ions into the main stream. The UV response obtained was about one fourth to one eighth of that obtained with the conventional postcolumn reaction method reported previously[3]. This is due to an insufficient hydroxide ion concentration for the degradation reaction. Next, the aminated and sulphonated HFMRs were used in series for introducing hypochlorite and hydroxide ions: when the sulphonated HFMR was followed by the aminated one, the UV response was scarcely obtained; on the contrary, when the aminated HFMR was followed by the sulphonated one, the response obtained was about one third of that obtained with the conventional method[3]. At the reaction temperature of 50°C, the peak height obtained was higher than that obtained with the conventional method[3]. When sodium hydroxide was not added to hypochlorite solution, the UV response was hardly obtained. Thus, sodium hydroxide was added to hypochlorite solution at a concentration of 1 *M*.

When an eluent containing acetonitrile as an organic modifier was used, ABPC and its metabolites gave two- to three-times higher UV responses, compared with the use of an eluent containing methanol. Thus, the postcolumn reaction conditions were selected as described under Experimental. The optimum detection wavelength, examined by using the HPLC detector, was 270–280 nm for unchanged penicillins, 255 nm for compounds 1 and 2. Compound 3 had no UV absorption maximum above 230

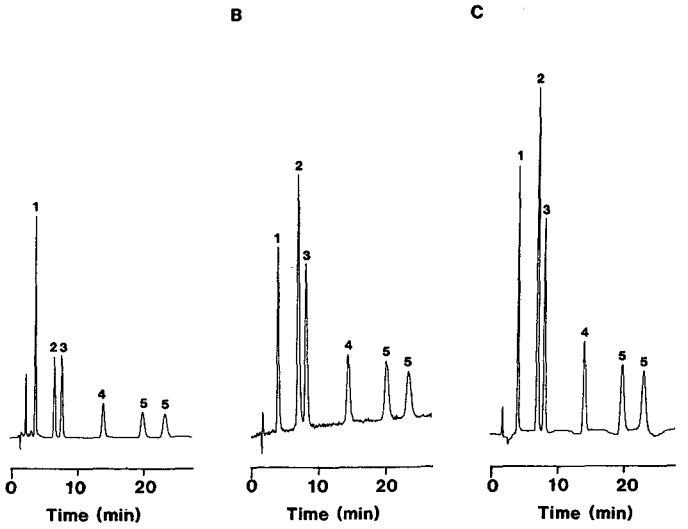

Fig. 3. Comparison of the three detection methods for penicillins: (A) detection at 230 nm without a reactor (method A); (B) detection at 280 nm with an open-tubular reactor (method B); (C) detection at 280 nm with aminated and sulphonated HFMRs (method C). A 20-μl portion of a mixture of ABPC (25 μg/ml), ACPC (25 μg/ml), PCG (25 μg/ml), PCV (25 μg/ml) and PEPC (50 μg/ml) was loaded onto the column. Sensitivity: 0.064 a.u.f.s. Peak assignments: 1 = ABPC; 2 = ACPC; 3 = PCG; 4 = PCV; 5 = (10*R*)- and (10*S*)-epimers of PEPC. Other conditions were given in the text.

nm. The sodium hypochlorite and sodium hydroxide solutions were used for about 10 and 20 h, respectively, without loss of their activity.

Comparison of detection methods

Fig. 3A–C shows the chromatograms of penicillins detected by three different methods (methods A, B and C). Penicillins were more sensitively detected at 280 nm following the postcolumn reaction (methods B and C), compared with direct UV detection at 230 nm (method A); method C gave 1.2- to 4.2-times higher responses than method A. The peak heights of penicillins obtained in method C were 1.2- to 1.5-times higher than in method B, and the resolution between ACPC and PCG (peaks 2 and 3, in Fig. 3) was 1.79 and 2.10 in methods B and C, respectively.

TABLE II

PRECISION OF THE ASSAY OF PENICILLINS

Relative standard deviations (%) of fifteen analyses, are given.

Penicillin	*Concentration (μg/ml)*	
	2.0	*10.0*
ACPC	3.96	2.14
PCG	4.08	2.89
PCV	2.28	1.75
PEPC	4.78	3.14

Reproducibility, linearity and detection limits

Table II lists the precisions [relative standard deviation (R.S.D.)] for measured peak heights of penicillins. The results reveal good reproducibility for all penicillins. Peak heights were found to be scattered at random around a mean value; that is, no trends (constant decrease in peak height with time) were observed. This reveals that the optimum concentrations of hypochlorite and hydroxide ions are maintained in spite of continuous depletion of hypochlorite and hydroxide ions. The calibration graphs of peak height *versus* concentration for each penicillin were linear in the concentration range 0.5–50 μg/ml with a correlation coefficient of $\geqslant 0.999$, and passed through the origin. The detection limits of the proposed method were 2.5–25 ng for each penicillin at a signal-to-noise ratio of 3.

Application to the determination of ABPC and its metabolites in serum and urine

On the basis of the above findings, we attempted to apply the present method to the determination of ABPC and its metabolites in serum and urine. Figs. 4 and 5 show the separation of ABPC, and ABPC and its metabolites, from the background components of serum and urine, respectively; part A, detection at 230 nm without a reactor; B, detection at 260 nm with the HFMRs. ABPC and its metabolites were 1.5- to 3.5-times more sensitively detected at 260 nm following the postcolumn reaction, compared with direct detection at 230 nm. At an ABPC concentration of 2 μg/ml in serum samples, the R.S.D. was 4.45% ($n=15$); and at concentrations of ABPC, 1, 2 and 3 of 5 μg/ml in urine samples, the R.S.D.s were 3.89, 5.14, 5.02 and 5.55% ($n=15$), respectively. The calibration graphs of peak height *versus* concentra-

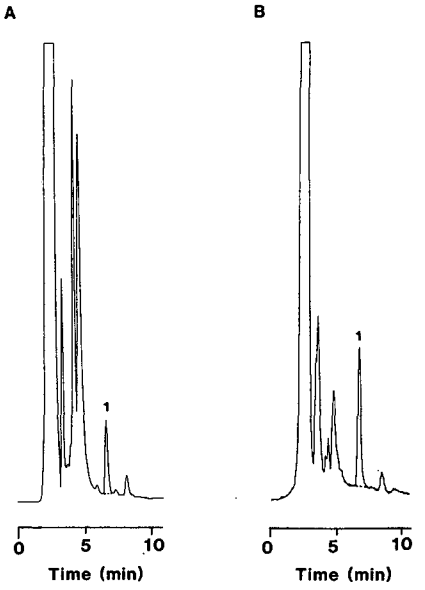

Fig. 4. Separation of ABPC from the background components of serum. A 20-μl portion of the ultrafiltrate of the serum sample was loaded onto the column. (A) Detection at 230 nm without a reactor; (B) detection at 260 nm with aminated and sulphonated HFMRs. Peak 1 is ABPC. Concentration: 5.0 μg/ml. Sensitivity: 0.032 a.u.f.s. The dotted line indicates the serum blank. Other conditions were given in the text.

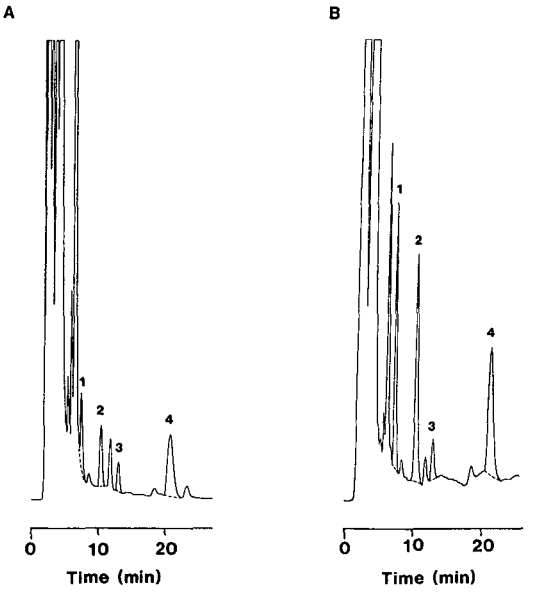

Fig. 5. Separation of ABPC and its metabolites from the background components of urine. A 20-μl portion of the filtrate of the urine sample was loaded onto the column. (A) Detection at 230 nm without a reactor; (B) detection at 260 nm with aminated and sulphonated HFMRs. Peak assignments: 1 = compound 1; 2 = 2; 3 = 3; 4 = ABPC. Concentrations: ABPC, 1 and 2, 20 μg/ml; 3, 10 μg/ml. Sensitivity: 0.032 a.u.f.s. The dotted line indicates the urine blank. Other conditions were given in the text.

tion for ABPC and its metabolites in serum and urine were linear in the concentration range 0.5–50 μg/ml with a correlation coefficient of $\geqslant 0.999$, and passed through the origin. The detection limits were 5 ng for ABPC, 1 and 2, and 20 ng for 3 at a signal-to-noise ratio of 3.

The method proposed is applicable to pharmacokinetic studies of ABPC and its metabolites after therapeutic dosage, and to their determination in bile, cerebrospinal fluid and tissues, upon slight modification.

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ION-EXCLUSION CHROMATOGRAPHY OF CARBOXYLIC ACIDS WITH CONDUCTIVITY DETECTION

PEAK ENHANCEMENT USING A CATION-EXCHANGE HOLLOW-FIBRE MEMBRANE AND AN ALKALINE SOLUTION

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SUMMARY

A sensitive method for the determination of carboxylic acids is described. They were separated using a dilute aqueous solution of sulphuric acid as an eluent on a strong cation-exchange column, passed through a cation-exchange hollow-fibre membrane surrounded by an alkaline solution and detected by electrical conductivity. They gave positive and/or negative chromatographic peaks, depending on the concentration of the alkaline solution used. For most monocarboxylic acids, the response was enhanced 16 to 93 times as both the positive and negative peaks, while the latter gave 1.8 to 3 times higher responses compared with the former. The quantification of carboxylic acids in a wine sample as their negative peaks is also described.

INTRODUCTION

Carboxylic acids are separated usually by three different liquid chromatographic methods: anion exchange[1,2], ion-exclusion[2–5] and reversed-phase[6] chromatography, while the detection methods most commonly used are UV absorbance and conductivity. Due to the lack of selectivity and sensitivity, UV detection is inferior to conductivity detection. A chromatographic method using a dilute acid solution as an eluent on a strong cation-exchange column (that is, ion-exclusion chromatography) and conductivity detection was used for the determination of carboxylic acids. This technique has the advantage that inorganic anions which are completely dissociated, such as chloride, nitrate and sulphate, are excluded from the column. A disadvantage is the low sensitivity towards weak carboxylic acids because of their elution as unionized forms. In order to improve the sensitivity, the background con-

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ductivity of the dilute acid eluant must be reduced prior to detection. A peak enhancement system has been used: the eluent was passed through a cation-exchange membrane (inserted between the column and the detector) surrounded by alkaline solutions[2,7] or neutral salt solutions[6]. Thus, the complete dissociation of carboxylic acids occurred and they were sensitively detected by conductivity.

In this paper, we describe a peak enhancement system using a cation-exchange hollow-fibre membrane and a strong alkaline solution for the detection of carboxylic acids separated by ion-exclusion chromatography. The present system gave negative (decreasing conducitivity) peaks proportional to the amounts of carboxylic acids. The method was successfully applied to the determination of the carboxylic acids in a wine sample.

EXPERIMENTAL

Reagents and materials

All carboxylic acids (free form) and other chemicals of analytical reagent grade were obtained from Nakarai Chemicals (Kyoto, Japan), and used without further purification.

A cation-exchange hollow-fibre membrane (AFS-2) was obtained from Dionex (Sunnyvale, CA, U.S.A.) and used at the desired length.

Water prepared with Nanopure unit (Barnstead, Boston, MA, U.S.A.) was used for the preparation of the eluent and sample solutions.

Chromatography

The QIC analyzer (Dionex) was used for the separation and detection of carboxylic acids. It consisted of a pump, a 50-μl sample loop, a separation column, a cation-exchange hollow-fibre membrane and a conductivity detector. A separation column packed with an high capacity, fully sulphonated styrene–divinylbenzene cation-exchange resin, HPICE-AS1 (250 mm × 9 mm I.D., Dionex), was used. The eluents used were 1 and 2 m*M* sulphuric acid for the separation of carboxylic acids in standard and wine samples, respectively, at a flow-rate of 0.8 ml/min. A cation-exchange hollow-fibre membrane (length 50 cm) was inserted between the column and the detector. The enhancers, which were delivered on the outside of the membrane at a flow-rate of 1.5 ml/min, were 600 and 700 m*M* sodium hydroxide solution for the detection of carboxylic acids in standard and wine samples, respectively. A 50-μl aliquot of the sample solution was loaded onto a column. All the separations and detection were performed at ambient temperature.

Comparison of detection methods

The detection methods were compared as follows: method A, direct conductivity detection without the peak enhancement system; B, conductivity detection with the peak enhancement system using a cation-exchange hollow-fibre membrane (length 250 cm) and a 10 m*M* sodium hydroxide solution; C, conductivity detection with the peak enhancement system using a cation-exchange hollow-fibre membrane (length 50 cm) and a 600 m*M* sodium hydroxide solution. The other chromatographic conditions were the same as those for the separation of the standard carboxylic acids.

Sample preparation

Tamba white wine (Kyoto, Japan) was diluted by twenty-fold in water, filtered with a 0.45-μm microfilter (Gelman Science Japan, Tokyo, Japan) and loaded onto the column.

RESULTS AND DISCUSSION

Separation

It has been reported that carboxylic acids are well separated from strong inorganic anions, *e.g.*, chloride, sulphate, etc., on a strong cation-exchange resin (H^+) using a dilute solution of acids, *e.g.*, hydrochloric acid, sulphuric acid, perchloric acid, octanesulphonic acid, phosphoric acid, benzoic acid[2–5]. The retention times of carboxylic acids were compared by using hydrochloric acid, sulphuric acid or octanesulphonic acid as the eluent. There were almost no differences in their retention times and the column efficiency among the eluents studied. Thus, 1 and 2 m*M* sulphuric acid were used as the eluents for the separation of carboxylic acids in standard and wine samples, respectively.

Detection

Since weak carboxylic acids are only partially ionized in the dilute strong acid eluent, they are not sensitively detected by conductivity. Therefore, it is necessary to enhance the detector response by accelerating the dissociation of the acids and re-

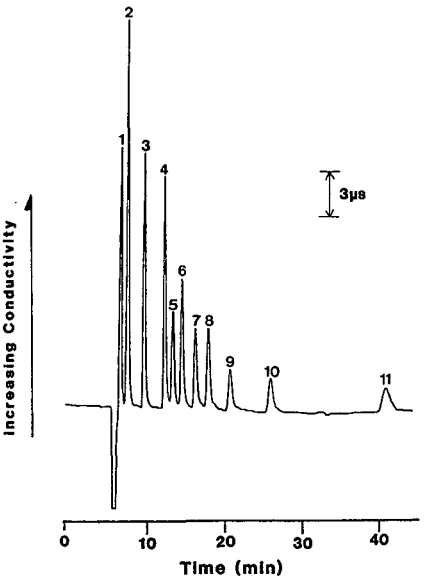

Fig. 1. Chromatogram of carboxylic acids detected at increasing conductivity. Conditions: column, HPICE-AS1 (250 mm × 9 mm I.D.); eluent, 1 m*M* sulphuric acid; flow-rate, 0.8 ml/min; cation-exchange hollow-fibre membrane, 250 cm; enhancer, 10 m*M* sodium hydroxide; flow-rate, 1.5 ml/min; injection volume, 50 μl. Peaks: 1 = citric acid; 2 = malonic acid; 3 = succinic acid; 4 = acetic acid; 5 = levulinic acid; 6 = propionic acid; 7 = isobutyric acid; 8 = *n*-butyric acid; 9 = isovaleric acid; 10 = *n*-valeric acid; 11 = *n*-caproic acid.

ducing the background conductivity. A peak enhancement system using a cation-exchange membrane and an alkaline solution was proposed by Rocklin *et al.*[2] and Slingsby[7]. The concentration of the alkaline solution (tetrabutylammonium hydroxide or potassium hydroxide) used as the enhancer was 2.5–10 m*M*. We tried to detect carboxylic acids more sensitively by using more concentrated alkaline solutions as the enhancers.

Fig. 1 shows a chromatogram of carboxylic acids obtained by conductivity detection after peak enhancement using a cation-exchange hollow-fibre membrane (length 250 cm) and a 10 m*M* sodium hydroxide solution. Carboxylic acids were sensitively detected as their ionic forms. The results obtained were almost the same as those described by Rocklin *et al.*[2] who used octanesulphonic acid as the eluent and tetrabutylammonium hydroxide as the enhancer. Figs. 2A, B and 3 show chromatograms of carboxylic acids obtained by conductivity detection after peak enhancement using the sodium hydroxide solutions of 300, 350 and 600 m*M*, respectively, and a cation-exchange hollow-fibre membrane (length 50 cm). As seen in Fig. 2A and B, the response is observed at increasing and decreasing conductivity, respectively, to give a complicated peak shape. These phenomena may be due to a water molecule produced by the reaction of an hydrogen ion with an hydroxide ion[5]. When a 500 m*M* sodium hydroxide solution was used, the response was observed at decreasing conductivity with a sharp single peak (Fig. 3). The negative peaks in the chromatographic separation of carboxylic acids are due to the lack of hydroxide ion, caused by the greater equivalent conductance of an hydroxide ion compared with that of a carboxylic acid ion.

In line with the main object of this study, that is, the sensitive determination of carboxylic acids in the negative detection mode, the factors affecting the response

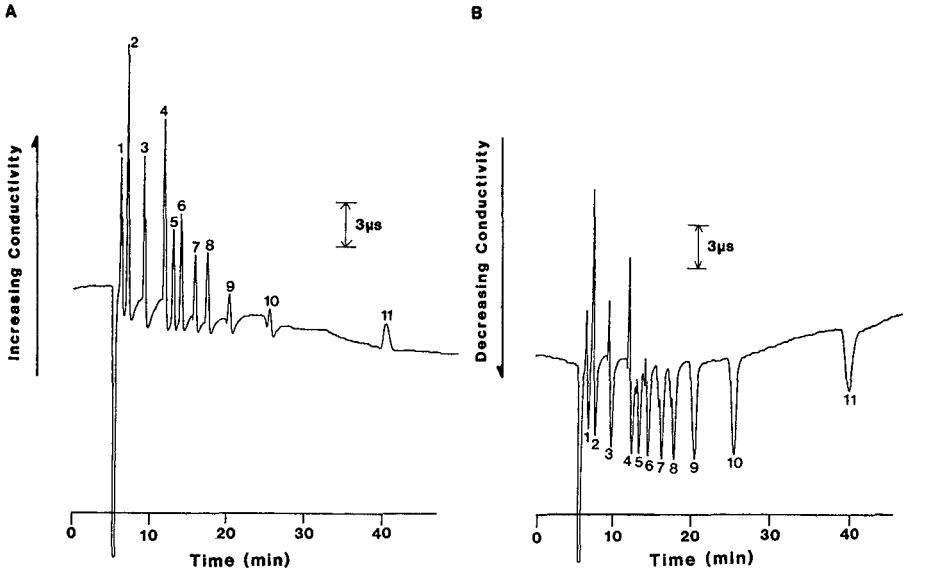

Fig. 2. Chromatograms of carboxylic acids detected at increasing and decreasing conductivity. Conditions as in Fig. 1 except that the length of the cation-exchange hollow-fibre membrane was 50 cm and the sodium hydroxide concentration was 300 (A) and 350 m*M* (B). Peaks as in Fig. 1.

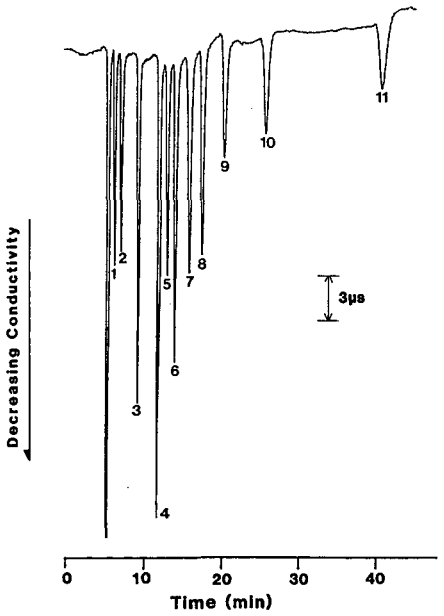

Fig. 3. Chromatogram of carboxylic acids detected at decreasing conductivity. Conditions as in Fig. 1 except that the length of the cation-exchange hollow-fibre membrane was 50 cm and the sodium hydroxide concentration was 600 m*M*. Peaks as in Fig. 1.

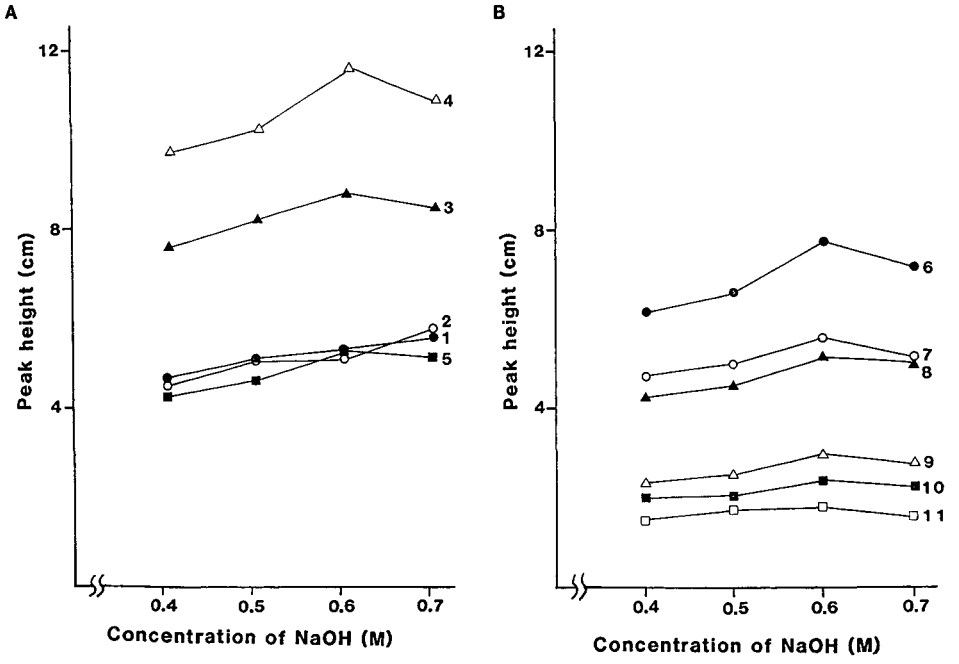

Fig. 4. Effect of the sodium hydroxide concentration on the detector response (decreased conductivity). Chromatographic conditions as in Fig. 1 except that the length of the cation-exchange hollow-fibre membrane was 50 cm. Peak numbers as in Fig. 1.

(decreased conductivity), the length of a cation-exchange hollow-fibre membrane and enhancer concentration, were examined. Lithium, sodium and potassium hydroxides were tested as the enhancers. With a cation-exchange hollow-fibre membrane of 50 cm, all carboxylic acids were observed only as negative chromatographic peaks using lithium, sodium and potassium hydroxides at concentrations of 300–500, 400–700 and 600–900 m*M*, respectively. The enhancer concentration needed for negative peaks increased in the following order of counter cations: lithium < sodium < potassium. These observations are in accord with the fact that the permeability of the hydroxide ion in the cation-exchange hollow-fibre membrane increases with an increase in its concentration in the enhancer and also with a decrease in the radius of the counter cation[5]. When lithium and potassium hydroxides were used as the enhancers, the response obtained changed depending on their concentrations. When sodium hydroxide was used as the enhancer, there was almost no concentration dependence. Thus, sodium hydroxide was used as the enhancer in subsequent experiments.

The length of the cation-exchange hollow-fibre membrane was changed from 25 to 250 cm. Figs. 4 and 5 show the peak heights of carboxylic acids obtained with the various concentrations of enhancer and fibre lengths of 50 and 100 cm, respectively. As the length was increased, the enhancer concentration needed for the negative peaks was decreased. At 250 cm, the peak heights obtained were lower than that with a length of 100 cm. Much the same peak heights were obtained with lengths of 25 and 50 cm. However, the baseline noise at 25 cm was higher than that at 50 cm. Thus, the peak enhancement system selected for the routine assay of carboxylic acids was a cation-exchange hollow-fibre membrane of length 50 cm and a 600 m*M* sodium hydroxide solution.

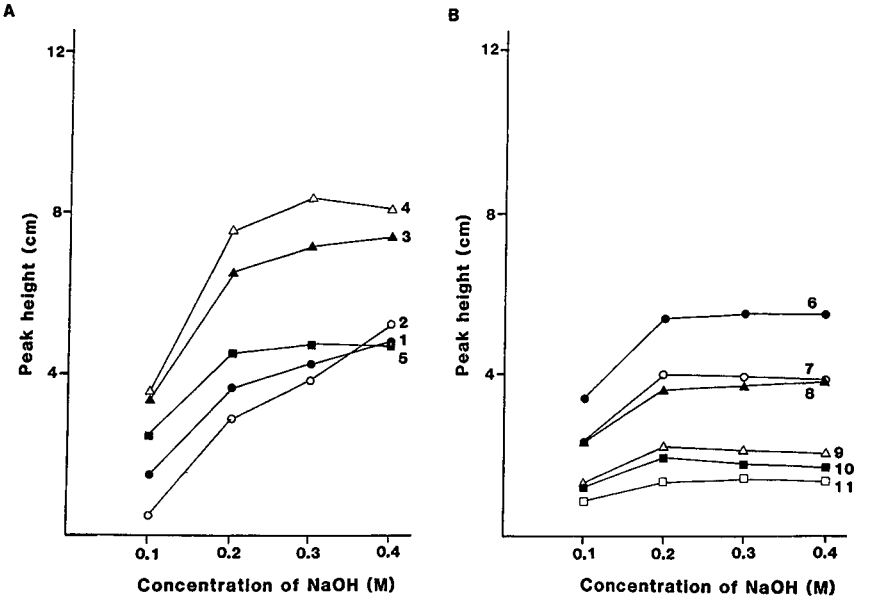

Fig. 5. Effect of the sodium hydroxide concentration on the detector response (decreased conductivity), conditions as in Fig. 4 except that the length of the cation-exchange hollow-fibre membrane was 100 cm.

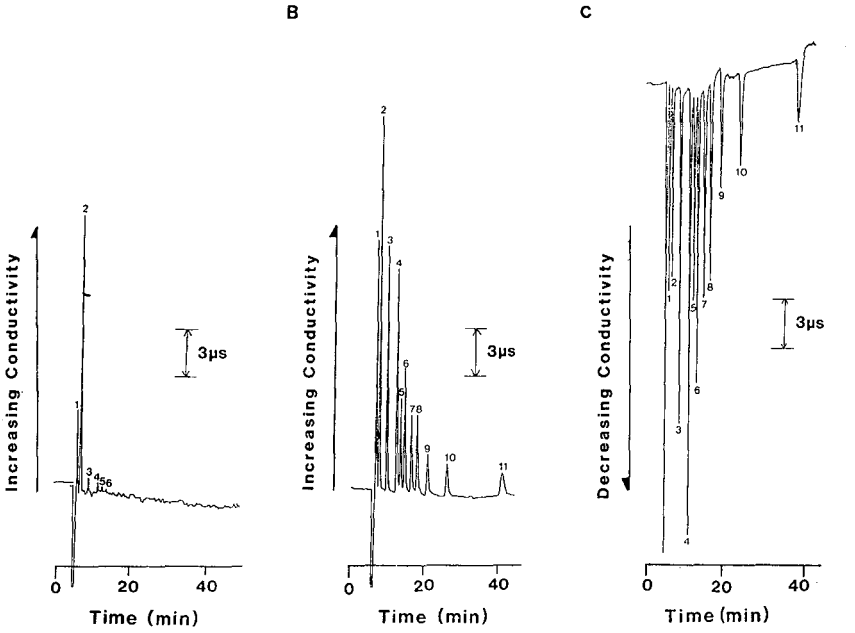

Fig. 6. Comparison of detection methods for carboxylic acids. Methods: (A) direct detection without the peak enhancement system; (B) detection with the peak enhancement system using a cation-exchange hollow-fibre membrane of length 250 cm and a 10 m*M* sodium hydroxide solution; (C) detection with the peak enhancement system using a cation-exchange hollow-fibre membrane of length 50 cm and a 600 m*M* sodium hydroxide solution. Peaks as in Fig. 1.

TABLE I

COMPARISON OF ENHANCEMENT FACTORS FOR CARBOXYLIC ACIDS

Carboxylic acid	*Enhancement factor**	
	Method B	*Method C*
Citric acid	3.1	2.3
Malonic acid	1.4	0.7
Succinic acid	21	27
Acetic acid	28	50
Levulinic acid	16	34
Propionic acid	41	93
Isobutyric acid	25	64
n-Butyric acid	29	69
Isovaleric acid	23	61
n-Valeric acid	32	89
n-Caproic acid	29	86

* The peak enhancement factor is the ratio of the peak areas obtained with and without the peak enhancement system.

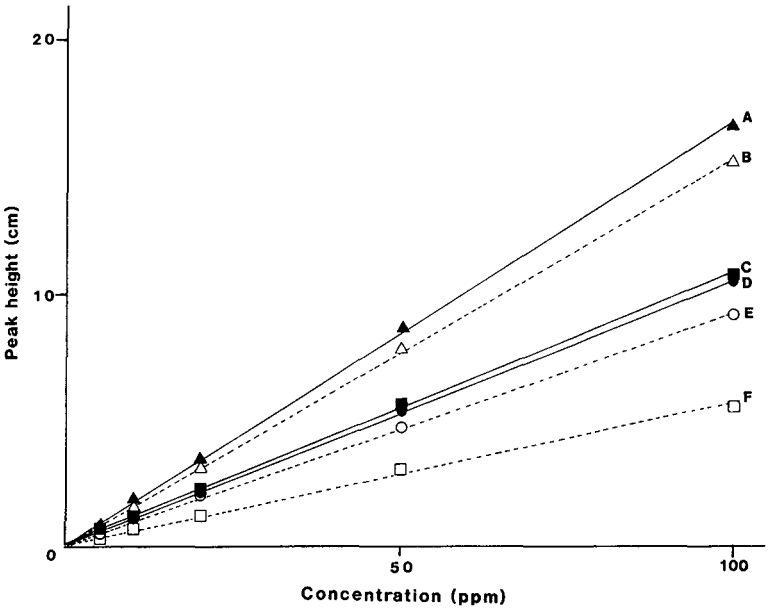

Fig. 7. Typical calibration graphs for carboxylic acids: A, succinic acid; B, propionic acid; C, isobutyric acid; D, citric acid; E, malonic acid; F, isovaleric acid. Chromatographic conditions as in Fig. 3.

Fig. 6 shows a comparison of the detection methods: (A), (B) and (C) correspond to methods A, B and C, respectively. Table I illustrates the peak enhancement factors of methods B (Fig. 6B) and C (Fig. 6C) compared with that of method A (Fig. 6A). This result shows that the present method is 1.8–3-fold more sensitive for

TABLE II

REPRODUCIBILITY AND DETECTION LIMITS FOR CARBOXYLIC ACIDS

The concentration of each carboxylic acid was 20 μg/ml.

Carboxylic acid	*C.V.** (%)	*Detection limit*** (ng)
Citric acid	1.1	90
Malonic acid	1.4	100
Succinic acid	1.8	60
Acetic acid	1.1	40
Levulinic acid	4.1	90
Propionic acid	3.1	70
Isobutyric acid	1.9	90
n-Butyric acid	4.0	100
Isovaleric acid	4.0	160
n-Valeric acid	6.5	200
n-Caproic acid	5.3	280

* Coefficient of variation for five analyses.

** The detection limit was based on a signal-to-noise ratio of 3.

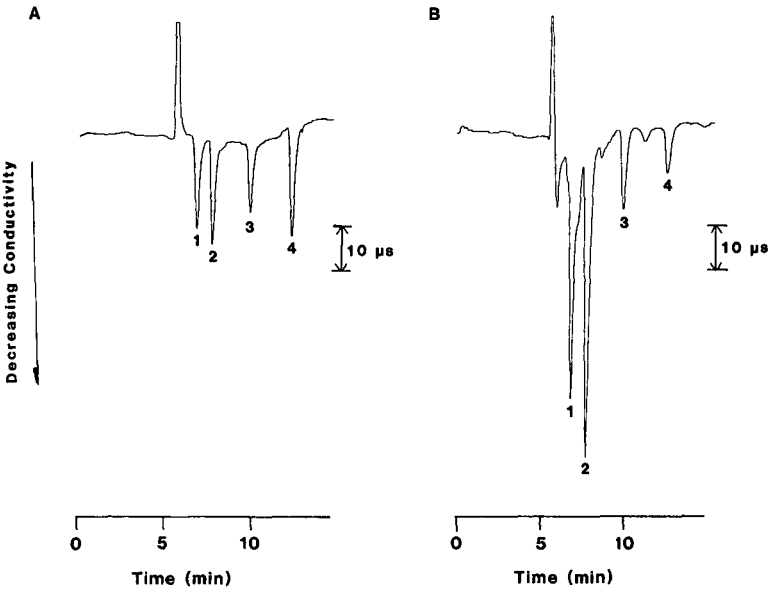

Fig. 8. Chromatograms of carboxylic acids in standard (A) and wine (B) samples. Conditions: column, HPICE-AS1 (250 mm × 9 mm I.D.); eluent, 2 m*M* sulphuric acid; flow-rate, 0.8 ml/min; cation-exchange hollow-fibre membrane, 50 cm; enhancer, 700 m*M* sodium hydroxide; flow-rate, 1.5 ml/min; injection volume, 50 µl. A wine sample was diluted twenty-fold. Peaks: 1 = lactic acid; 2 = tartaric acid; 3 = malic acid; 4 = acetic acid. Concentrations: (A), 50 µg/ml each; (B), peaks 1, 2, 3 and 4 were estimated to contain 97, 139, 63 and 23 µg/ml, respectively.

the detection of monovalent carboxylic acids (acetic acid through *n*-caproic acid) compared with the previously reported one[2].

Calibration graph

Fig. 7 shows typical calibration graphs of peak height *versus* concentration for carboxylic acids. The graphs were linear over the concentration ranges of 5–100 µg/ml with a correlation coefficient >0.99, and passed through the origin.

Reproducibility and detection limits

Table II shows the reproducibility (coefficient of variation for five analyses) and detection limits at a signal-to-noise ratio of 3.

Application

On the basis of the aforementioned results, we attempted to apply the present method to the determination of carboxylic acids in a wine sample. Fig. 8A shows the chromatogram of a standard mixture of carboxylic acids; a chromatogram of a wine sample after twenty-fold dilution is shown in Fig. 8B.

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Note

Capillary tube isotachophoretic separation of heavy metal ions using complex-forming equilibria between cyanide as terminating ion and the metal ions

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Various attempts have been made to improve the separating power of capillary tube isotachophoresis (CITP)[1,2]. The use of complex-forming equilibria is one of the most effective methods for the separation of metal ions, and we have also shown that this method is useful for the separation of alkaline earth metals[3], lanthanides[4], and heavy metals[5]. In most such cases complexing agents were added to the leading electrolyte as a counter-ion or an additive such as a neutral complexing ligand[6,7], and the mobilities of sample ions could be adjusted by the interaction between ligand in the leading electrolyte and the sample ion. On the other hand, the use of complex-forming equilibria between a ligand in the terminating electrolyte and the sample ion has been reported[8,9]. This method was based on using chloride as a terminating ion and the migration of metal ions as metal-chloro complexes with negative charges. In this method, however, a non-aqueous solvent such as dimethylformamide had to be used to suppress the dissociation of hydrogen chloride and make the effective mobility of chloride low enough for it to be used as the terminating ion.

In this study, the isotachophoretic separation of heavy metal ions using the complex-forming equilibria with cyanide as the terminating ion in aqueous solvent was investigated. Cyanide was selected as the terminating ion because it forms complexes strongly with many heavy metal ions in aqueous solvents and its mobility can be adjusted by the pH of the leading electrolyte on basis of the acid–base equilibria. In this operating system, the migrating behaviour of heavy metal ions was studied, and the separation of six metal ions was achieved.

EXPERIMENTAL

Apparatus

A Model IP-1B capillary tube isotachophoretic analyser equipped with a potential gradient detector (Shimadzu, Kyoto, Japan) was used. The PTFE tube for the separation consisted of a main capillary column (150 mm × 0.5 mm I.D.) and a pre-column (40 mm × 1.0 mm I.D.). The current was stabilized at 50–150 μA for 5–15 m*M* leading electrolyte. The capillary tube was filled with electrolyte by carrier gas (nitrogen).

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TABLE I

OPERATING SYSTEM

Parameter	*Leading electrolyte*	*Terminating electrolyte*
Anion	Cl^- or NO_3^-	CN^-
Counter-ion	$TrisH^+$	K^+
Concentration	5–15 m*M*	10 m*M*
Additive	0.005% Poly(vinyl alcohol)	5 m*M* Barium hydroxide

Reagents

Hydrochloric acid and poly(vinyl alcohol) were of analytical-reagent grade and were used without further purification.

Stock standard solutions of metal ion such as mercury(II), cadmium(II), silver(I), zinc(II), cobalt(II) and copper(II) were prepared by dissolving their chlorides or nitrates in water.

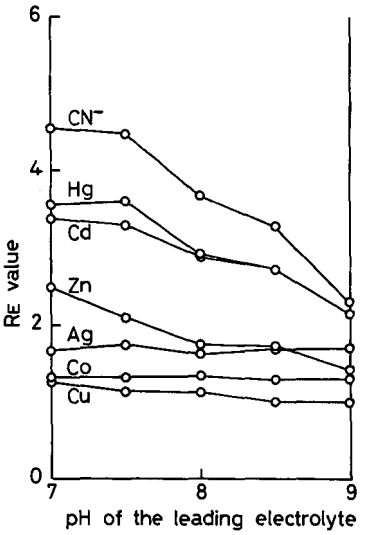
The leading electrolyte was prepared by diluting a stock solution of 2 *M* hydrochloric acid and 1% poly(vinyl alcohol), and the pH was adjusted by adding tris(hydroxymethyl)aminomethane. The terminating electrolyte was prepared by diluting a stock solution of 1 *M* potassium cyanide. Barium hydroxide (5 m*M*) was added to the terminating electrolyte to exclude carbonate ion. The operating system is shown in Table I.

RESULT AND DISCUSSION

In isotachophoretic migration, it is necessary that the difference between the effective mobilities of the leading and terminating ions is sufficiently great. When chloride is used as the terminating ion, a non-aqueous solvent had to be used as the migrating system to suppress the dissociation of hydrogen chloride and lower its mobility[8,9]. Since the cyanide ion is strongly alkaline (its dissociation constant pK_a is 9.4), its effective mobility can be controlled by the pH of leading electrolyte in aqueous solution. At pH *ca.* 7–9 of the leading electrolyte, this mobility is low enough for cyanide to be used as the terminating ion, and there is a sufficient difference in potential gradient between the leading ion and the terminating ion.

When heavy metal ions are injected into this system, they migrate to the terminating side (cathode) because of their positive charge, where they react with cyanide ion to form cyano-complexes with a negative charge, and thence migrate to the leading side (anode). The zones of metal ions could be detected between leading and terminating ions in isotachopherograms.

The effect of the pH of the leading electrolyte on the effective mobilities of cyano-complexes is shown in Fig. 1. The R_E value in the diagram represents the ratio of the potential gradient of sample ions or terminating ion to that of leading ion. The R_E values of most of the cyano-complexes and the cyanide ion decrease with increasing pH. An increase in the pH of the leading electrolyte promotes the dissociation of hydrogen cyanide and increases the concentration of cyanide available for complex formation. Therefore, the conditional stability constants of the cyano-complexes increases with increasing pH, and complexes with a greater negative charge and higher mobility could be formed.



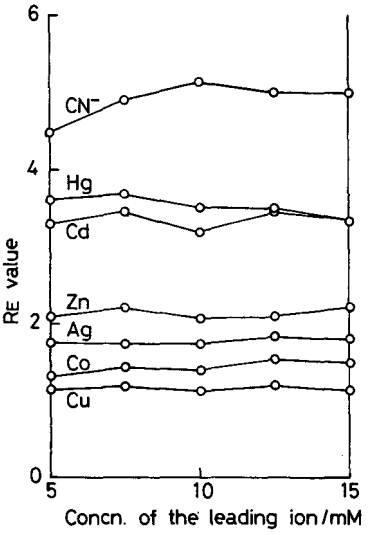


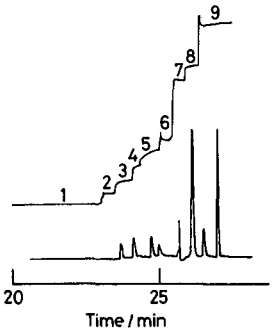
Fig. 1. Effect of the pH of the leading electrolyte on the R_E values of cyano-complexes. The concentration of the leading ion (Cl^-) was constant at 5 mM. Driving current, 50 μA.

Fig. 2. Effect of the concentration of the leading ion on the R_E values of cyano-complexes. The pH was constant at 7.5. Driving current, 50–150 μA.

The effective mobilities of cobalt(II) and copper(II) cyano-complexes below pH 7.0 and of mercury(II) and cadmium(II) above pH 8.0 are similar, and those ions cannot be separated. Also, above pH 8.5, the mobility of copper(II) is similar to that of the leading ion, and the copper(II) cyano-complex cannot be detected. The cyano-complexes of six metal ions can be separated at pH 7.5.

The cyano-complex of copper(II) is reduced to copper(I), and it seems that this reaction occurred in this system.

In isotachophoresis, the concentration of each zone is adjusted with the concentration of leading electrolyte[10]. The concentration of cyanide ion that is supplied from terminating zone and acts as a complex agent may be also affected by the concentration of the leading electrolyte. Therefore, the effect of the concentration of the leading electrolyte on the effective mobilities of cyano-complexes was investigated (Fig. 2). Most of the effective mobilities were constant at concentrations of the leading electrolyte in the range 5–15 mM, although those of the mercury(II) and cadmium(II) cyano-complexes decreased slightly with increasing concentration of the leading electrolyte. This can be explained as follows. The effective mobility of cyano-complexes depends on the fractions of the various polyligand complexes, and the larger the fraction of polyanionic species, the higher the effective mobility. Therefore the differenc of effective mobilities of cyano-complexes become large with increasing the difference between the fractions of anionic complexes of each metal ion. The results calculated using stability constants[11] showed that the difference of fraction for cyano-complexes of mercury and cadmium, which are bivalent anionic species, becomes smaller as the concentration of cyanide increases. This is identical with experimental results, *i.e.* the mobility of each metal complex depends on its stability constant. In



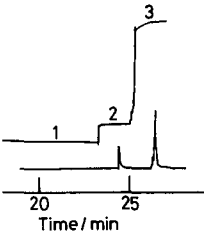


Fig. 3. Isotachopherogram of cyano-complexes: 1 = Cl^-; 2 = copper; 3 = cobalt; 4 = silver; 5 = carbonate; 6 = zinc; 7 = cadmium; 8 = mercury; 9 = CN^-. Leading electrolyte, 5 m*M* hydrochloric acid–Tris, 0.005% poly(vinyl alcohol) (pH 7.5). Sample size, 10 μl of a mixed solution of 0.5 m*M*. Driving current, 50 μA.

Fig. 4. Isotachopherogram of ferricyanate: 1 = NO_3^-; 2 = ferricyanate; 3 = CN^-. Leading electrolyte; 5 m*M* nitric acid–Tris, 0.5 m*M* lanthanum nitrate, 0.005% poly(vinyl alcohol) (pH 7.5). Sample size, 5 μl of 1 m*M* potassium ferricyanate. Driving current, 50 μA.

particular, the consecutive stability constants concerned with the formation of anionic species play an important rule in the separation. The optimum pH for the leading electrolyte, 5 m*M* hydrochloric acid–Tris, is 7.5.

In this system, six metal ions could be separated as shown in Fig. 3. The order of their effective mobilities is mercury < cadmium < zinc < silver < cobalt < copper, which follows the order of the stability constants of their cyano-complexes (except mercury). The zone of carbonate ion was detected between the zones of silver(I) and zinc(II). Carbonate ion could not be excluded completely by addition of barium hydroxide to the terminating electrolyte. Calibration curves for each metal ion were linear in the range 4–20 nmol injected, in 20 μl of sample.

The zones of iron(II), iron(III), and nickel(II) cyano-complexes could not be detected in this system. The effective mobilities of iron(II) and iron(III) cyano-complexes would be larger than that of the leading ion, because their stability constants are sufficiently large to form cyano-complexes with more negative charge than that of other metals ions. The use of ion-pairing equilibria with lanthanum(III) added to the leading electrolyte enabled the zone of the ferricyano-complex to be detected (Fig. 4).

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Note

Determination of chemical composition distribution of styrene–methyl methacrylate copolymers by reversed-phase high-performance liquid chromatography

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Analysis of polymeric compounds by high-performance liquid chromatography (HPLC) is predominantly carried out using the gel permeation mode (GPC), where the interaction between the sample and the stationary phase is negligible. However, with this mode only information concerning molecular volume can be obtained. Recently, normal-phase HPLC, in which the stationary phase is more polar than the eluent, using a polar gel such as silica gel or silica gel modified with cyano groups as the stationary phase, was applied to the determination of the chemical composition distribution (CCD) of copolymers[1–7]. We have also demonstrated that CCDs of styrene–butadiene[8] and styrene–methyl methacrylate (St–MMA) copolymers[9] can be determined by normal-phase HPLC using cross-linked acrylonitrile gel and a non-polar eluent. In normal-phase HPLC, the separation mechanism was found to be adsorption or partition, rather than fractional dissolution (phase separation).

Glöckner and Van den Berg[6,7] used a reversed-phase gel, *e.g.*, a non-polar gel such as octadecylsilica gel, and a non-polar eluent for the separation of copolymers according to composition. They found that in this system the separation was governed by fractional dissolution and that St–MMA copolymer was not well resolved, although St–acrylonitrile copolymer showed good resolution. Recently, Glöckner[10] reported that copolymers can be separated by reversed-phase HPLC on octadecylsilica with methanol–tetrahydrofuran as the eluent. Poor resolution of St–MMA copolymer was observed using cross-linked polystyrene gel and a non-polar eluent[9].

In this paper we report the possibility of determining the CCD of St–MMA copolymer by reversed-phase HPLC using cross-linked polystyrene gel as the stationary phase and a polar eluent consisting of dichloromethane–acetonitrile. The effect of molecular weight on the separation was also examined.

EXPERIMENTAL

St–MMA copolymers (1–5) having different compositions were prepared in bulk using benzoyl peroxide as an initiator. Copolymer samples with different molecular weights (6–8) were prepared by copolymerization with the same monomer in the presence of different amounts of *n*-butyl mercaptan. The copolymers were separated by GPC to obtain samples with narrow molecular weight distributions. The

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TABLE I

POLYMERIZATION CONDITIONS AND MOLECULAR CHARACTERISTICS OF STYRENE–METHYL METHACRYLATE COPOLYMERS

Sample	*Polymerization conditions**		*Properties of copolymer*		
	Styrene in feed, mol-%	*Yield, wt.-%*	*Styrene in polymer, mol-%*	M_n** $\times 10^4$	M_w** $\times 10^4$
1	84.1	5.0	78.3	9.5	13.9
2	70.0	6.9	67.9	8.7	12.4
3	54.9	6.1	53.7	8.8	11.9
4	27.6	7.6	35.4	10.4	13.5
5	14.6	7.6	24.0	13.7	24.3
6	50.0	2.8	49.1	11.0	16.2
7***	50.0	2.2	48.8	2.2	4.3
8***	50.0	1.2	49.1	0.5	1.0
Fraction of 6–8:					
6–1			48.8	18.1	24.7
6-2			48.5	7.1	8.1
7-2			48.3	4.8	5.4
8-2			48.7	2.1	2.6
7-3			48.8	1.1	1.3

* Bulk copolymerization at 60°C using benzoyl peroxide as initiator with a concentration of 0.25 mol-% of monomer.

** M_n = number-average and M_w = weight-average molecular weight. Measured by GPC using calibration graphs for standard polystyrene and poly(methyl methacrylate).

*** Polymerized in the presence of *n*-butyl mercaptan; 0.1 mol-% (sample 7) and 1.0 mol-% (sample 8) of the monomer.

conversions were adjusted to less than 10% in order to obtain samples with narrow CCDs (Table I). The composition was determined from the ^{1}H NMR spectra using the intensity ratio of the phenyl proton in the styrene unit and the α-methyl proton in the methyl methacrylate unit. The molecular weight was determined by GPC using standard polystyrene and poly(methyl methacrylate) as calibration standards.

Styrene (20 ml) and divinylbenzene (49 ml) (containing 45% of ethylvinylbenzene) were copolymerized by conventional suspension copolymerization in the presence of toluene (71 ml) and polystyrene (1.9 g) as diluents and using poly(vinyl alcohol) as a suspension reagent. The resulting copolymer was washed successively with hot water, acetone and chloroform three or four times. The copolymer beads, having diameters of 5–10 μm, were collected by decantation in acetone and packed in a stainless-steel column (25 cm × 4.5 mm I.D.) by the usual slurry method. This column had an exclusion limit of *ca.* $6 \cdot 10^5$ for polystyrene when chloroform was used as the eluent.

HPLC was conducted at ambient temperature (*ca.* 20°C) using two Jasco 880-PU high-pressure pumps, one for providing dichloromethane and the other acetonitrile. The two solvents were mixed after the pump and were delivered to the injector through a filter, which reduced the noise produced by mixing. The flow-rate was set at 0.5 ml/min and the proportion of dichloromethane was increased linearly

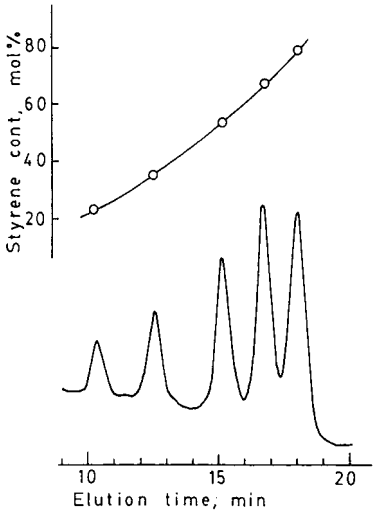
from 20 to 80 vol.-% in 25 min. A 10-μl portion of a dichloromethane solution of the sample (10 mg/ml) was injected through a Reodyne 7125 injector. The column effluent was monitored with a Jasco Uvidec 254 (254 nm) UV detector.

The cloud point of the sample was determined at 20°C. Acetonitrile was gradually added to a dichloromethane solution of the sample (10 mg/ml) and the cloud point was observed visually.

RESULTS AND DISCUSSION

An mixture of equal weights of five St–MMA copolymers containing 24–87 mol-% of styrene units (samples 1–5 in Table I) was separated using acetonitrile–dichloromethane as the eluent in which the proportion of dichloromethane, which is a good solvent for the copolymer, was gradually increased from 20 to 80 vol.-%. These samples were eluted in order of decreasing styrene content and exhibited five peaks, as shown in Fig. 1. This elution order is opposite to that in normal-phase HPLC[9]. It is noteworthy that cross-linked polystyrene gel showed a good resolution of St-MMA copolymer with the use of a polar eluent, although it did not provide a good resolution when a non-polar eluent was used[9].

In order to examine the separation mechanism, the proportion of the good solvent (dichloromethane) in the eluent was plotted against the styrene content of the sample, together with the cloud point (Fig. 2). It was found that the proportion of the good solvent is almost equal to the cloud point for a fractional dissolution mechanism, whereas the former is higher than the latter in an adsorption or partition mechanism[9]. The proportion of the good solvent in the eluent is 20–30% higher than



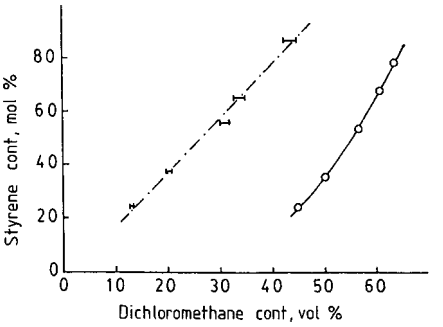


Fig. 1. HPLC trace of a mixture of five styrene–methyl methacrylate copolymers. The dichloromethane content was increased linearity from 20 to 80 vol.-% in 25 min.

Fig. 2. Dichloromethane concentration in the eluent and the cloud point of the styrene–methyl methacrylate copolymer.

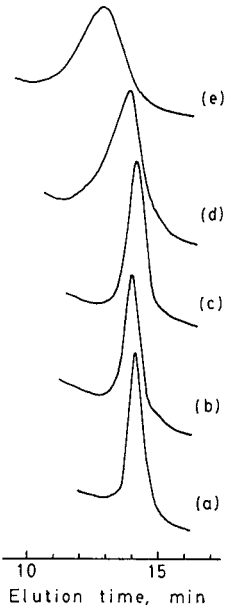


Fig. 3. HPLC traces of styrene–methyl methacrylate copolymers having number-average molecular weights of (a) $18 \cdot 10^4$, (b) $7.1 \cdot 10^4$, (c) $4.8 \cdot 10^4$, (d) $2.1 \cdot 10^4$ and (e) $1.1 \cdot 10^4$.

the cloud point. For comparison, when cross-linked acryronitrile gel was used as the stationary phase, the proportion of the good solvent was almost equal to the cloud point, although the sample concentration was much higher in the cloud point experiment. The relationship between the cloud point and elution behaviour indicates that with a combination of a non-polar gel and a polar eluent the separation was governed by an adsorption or partition mechanism, whereas the sample was eluted by a fractional dissolution mechanism when a polar gel and a polar eluent were used.

Usually the separation of copolymers by HPLC is influenced by the molecular weight of the sample in addition to the composition. Samples with almost the same styrene content and different molecular weights (samples 6–8) were prepared with the same monomer feed and different amounts of chain-transfer reagent (*n*-butyl mercaptan). The samples obtained were fractionated by GPC, giving five fractions having styrene contents of 49–50 mol-% and number-average molecular weights of $1.1 \cdot 10^4$–$18 \cdot 10^4$. Fig. 3 shows the HPLC traces of the five fractions. Samples with number average molecular weights higher than $4.8 \cdot 10^4$ showed a peak at almost the same elution time of 14.1 ± 0.1 min, whereas the samples with molecular weights lower than this critical value displayed peaks at earlier elution times as the molecular weight decreased. The effect of molecular weight on the elution volume is slightly greater in normal-phase than reversed-phase HPLC[9]. The peak width at half-height increased as the molecular weight decreased, as expected from Stockmayer's equation[11]. The abrupt increase in the peak width for samples with number-average molecular weights lower than $2.1 \cdot 10^4$ may be parently attributed to the effect of molecular weight on the elution volume.

The retention volume and peak area for each sample were perfectly reproducible, as in normal-phase HPLC using cross-linked acrylonitrile gel[9]. In addition to the good reproducibility, a long lifetime of the column (more than 1 year) was confirmed. This may be ascribed to the high degree of cross-linking of the gel, which reduced the swelling and shrinkage during the experiments with a solvent gradient. It is concluded that the CCD can also be analysed by reversed-phase HPLC. A detailed comparison of reversed-phase and normal-phase HPLC will be presented in a subsequent paper.

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Note

Resolution of lombricine enantiomers by high-performance liquid chromatography utilising pre-column derivatisation with *o*-phthaldialdehyde–chiral thiols

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Lombricine (Fig.1) is present in a number of invertebrates[1–4] and is thought to act as a regulator of adenosine triphosphate levels[1,5]. Serine, the amino acid in lombricine, occurs in either the L or D configuration depending upon the phylum of the particular invertebrate[3]. Since D-amino acids are not commonly found in nature[6], a convenient and specific method of determining the configuration of the seryl moiety in crude biological extracts was sought as part of our on-going research into novel helminthic high energy phosphates[7,8].

To date, the configuration of the seryl moiety has been laboriously determined by optical rotation measurements on purified isolated lombricine and comparison to authentic D- and L-lombricine whose configuration was unambiguously determined by classical hydrolysis on isolated lombricine[4,9]. The liberated serine was purified by ion-exchange and paper chromatography and the configuration of the purified serine was then established by optical rotation measurements and the action of D-amino acid oxidase.

Recently, there has been interest in enantioselective high-performance liquid chromatographic (HPLC) determination of amino acids[10–14] and amino enantiomers[13,15] by pre-column derivatisation with *o*-phthaldialdehyde (OPA) and chiral thiols to yield diastereoisomeric isoindole derivatives which are separable on reversed-phase HPLC, and can be detected using fluorometry. It was therefore decided to evaluate the use of this new methodology in the development of a new and enantioselective assay for D- and L-lombricine.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical or HPLC grade. Ultra-pure water was obtained by means of a Milli-Q system (Millipore). OPA and standard D- and

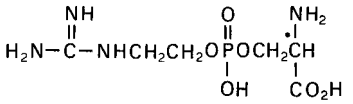

Fig. 1. Structure of lombricine (asterisk denotes chiral centre).

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L-amino acids were purchased from Sigma; N-acetyl-L-cysteine, N-acetyl-D-penicillamine and N-*tert.*-butyloxycarbonyl-S-benzyl-L-cysteine from Fluka. N-*tert.*-butyloxycarbonyl-L-cysteine was prepared as described by Buck and Krummen[13]. Authentic natural D- and L-lombricines were kindly supplied by Y. Robin and H. Rosenberg. Synthetic D/L, D- and L-lombricines were prepared according to the method of Euerby *et al.*[16].

Chromatographic systems

HPLC was performed on an apparatus as described by Euerby *et al.*[8]. A Spherisorb ODS II "EXCEL", 5 μm (25 cm × 4.6 mm I.D.) column was purchased from Hichrom (Reading, U.K.) and fitted with a guard column (5 cm × 2 mm I.D.) packed with Co:Pell ODS sorbent (particle size 40 μm; Hichrom). A fluorescence sensitivity of either 0.2 or 0.1 r.f.u. was employed.

Preparation of standard amino acids and derivatives

Stock solutions of the individual enantiomers were prepared in water at a concentration of 60 μmol/ml and were stable for at least one month of continual use if stored at −14°C. Standard mixtures were prepared by mixing the appropriate stock solutions, followed by dilution with water to yield a final concentration of 30–60 nmol/ml for each individual component.

Mobile phases

Solvents A and B were prepared freshly every other day, filtered through a 0.22-μm membrane filter and degassed by continuous purging with helium. Solvents A and B consisted of 50 m*M* sodium acetate and methanol respectively. The flow-rate was 1 ml/min and the column pressure was approximately 1600 p.s.i. at the beginning of the gradient. The gradient elution programme employed is shown in Table I.

Pre-column derivatisation procedure

The derivatisation reagents were freshly prepared every other day by dissolving 10 mg of OPA and the chiral thiol in 1 ml of methanol (in order to preserve the optical purity of the chiral thiols, the alkaline borate buffer should only be added immediately prior to derivatisation). These reagents were stored at 4°C in the dark until use. The standard amino acid solutions (20 μl) or centrifuged biological extracts (20 μl) were

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF D- AND L-LOMBRICINE

Duration (min)	*From (%solvent A:B)*	*To (%solvent A:B)*
0–30	90:10	75:25
30–35	75:25	70:30
35–45	70:30	70:30
45–50	70:30	90:10
50–60	90:10	90:10

mixed with the derivatisation reagent (40 μl) and borate buffer (60 μl, pH 9.5, adjusted with 2 *M* sodium hydroxide), and incubated for 5 min at ambient temperature in the dark before immediate injection onto the column.

Preparation of biological extracts

The "earthworms" used were collected in the Leicestershire area and were of mixed species but were predominantly *Allolobophora caliginosa*, *Octolasium cyaneum* and *Lumbricus terrestris*[17]. Live pre-weighed earthworms (approximately 10.0 g) were homogenised with ice-cold water (10 ml) in a Polytron® homogeniser (Kinematica) for 5 min. The homogenate was adjusted to pH 3.0 with concentrated sulphuric acid, boiled for 5 min and centrifuged for 25 min at 31 000 *g* using a Beckmann J2-21 centrifuge (JA-20 head). The pellet was re-extracted with two volumes of water and centrifuged as before. The combined supernatants were brought to pH 8.0 with cold-saturated barium hydroxide solution and centrifuged, as before, to remove barium sulphate. The supernatent was then neutralised to pH 7.0 with dilute hydrochloric acid and then lyophilised (Edward EF 4 Module freeze drier operating at −60°C). The prepared extracts were dissolved in water (10 ml) and centrifuged to give the stock extract solutions which were diluted 1 in 1000 when required with water and then derivatised.

RESULTS AND DISCUSSION

We have recently shown that lombricine can be detected in biological extracts using OPA–ethanethiol derivatisation[8]. The seryl moiety of lombricine should, in principle, form diastereoisomers with OPA and the optically pure chiral thiols, N-acetyl-L-cysteine (NAC), N-acetyl-D-penicillamine (NAP) and N-*tert*.-butyloxycarbonyl-L-cysteine (BocC), using the same methodology as described for the amino acids and amino alcohols[10–15]. The resultant diastereoisomers should then be separable on standard reversed-phase HPLC stationary phases, alleviating the need for expensive chiral stationary phases. D- and L-lombricine were, in fact, shown to form highly fluorescent derivatives with all three thiols at pH 9.5 (the NAP adduct had a lower fluorescent intensity than NAC and BocC). The reactions occurred rapidly and quantitatively at ambient temperatures in the dark reaching their maximum fluorescence within 1–2 min and were stable for at least 10 min. Despite the use of the many mobile phase combinations described in the literature[10–15], the separation of the D- and L-lombricine diastereoisomers of NAC and NAP could not be achieved. However, the D- and L-isomers of lombricine could be easily separated, giving near baseline separation (Fig.2A, Table II), by the use of a 50 m*M* sodium acetate–methanol gradient and OPA–BocC derivatisation. The BocC chiral thiol has previously been observed to give better resolution of enantiomers than NAC[12]. A possible explanation for this is that the N-*tert*.-butyloxycarbonyl group of BocC is considerably more bulky than the acetyl groups of NAC and NAP and so forms diastereoisomers with more restricted conformations.

The limit of detection of L-lombricine in a mixture of D- and L-lombricine is better than 0.5% as can be seen in Fig. 2B and a blank run, using water in place of sample for the derivatisation, showed no interfering peaks in this region of interest. It was established that racemisation did not occur since only one enantiomer of

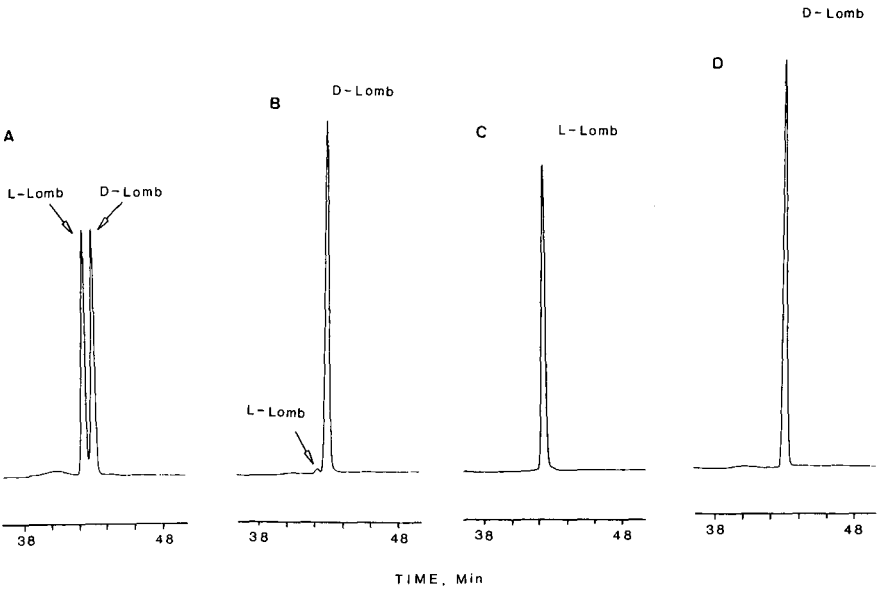

Fig. 2. HPLC of OPA–BocC derivatives of D- and L-lombricine on a Spherisorb ODS II EXCEL reversed-phase column. Chromatographic conditions as in the Experimental section. Non-standard abbreviations used: D-Lomb and L-Lomb = D- and L-lombricine respectively. (a) Synthetic racemic D/L-lombricine; (b) synthetic L- and D-lombricine (0.5:99.5 ratio); (c) L-lombricine from *Urechis caupo*; (d) D-lombricine from *Lumbricus terrestris*.

lombricine was detected on subjecting the single enantiomers to extraction and pre-column derivatisation. This confirms the findings of Pauck and Krummen[12,13]. The initial gradient phase (10 to 25% methanol in 50 m*M* sodium acetate) was vital to avoid co-elution of the diastereoisomers. As observed with other OPA–BocC amino acids[13], the L enantiomer of lombricine eluted before its corresponding D enantiomer. This is probably due to stronger hydrogen-bonds in the D diastereoisomers, resulting in a more hydrophobic molecule which would be expected to interact more strongly with

TABLE II

SEPARATION OF DIASTEREOISOMERIC DERIVATIVES FORMED FROM LOMBRICINE, ASPARTIC ACID AND GLUTAMIC ACID

t_0 = 3.20 min; k', α and R_s are the capacity ratio, separation factor and resolution, respectively, for a pair of enantiomers; chromatographic conditions are as in the Experimental section.

Sample	k'		α	R_s
	L	*D*		
Lombricine	12.16	12.38	1.02	1.17
Aspartic acid	7.97	8.47	1.06	1.74
Glutamic acid	10.21	10.88	1.07	1.77

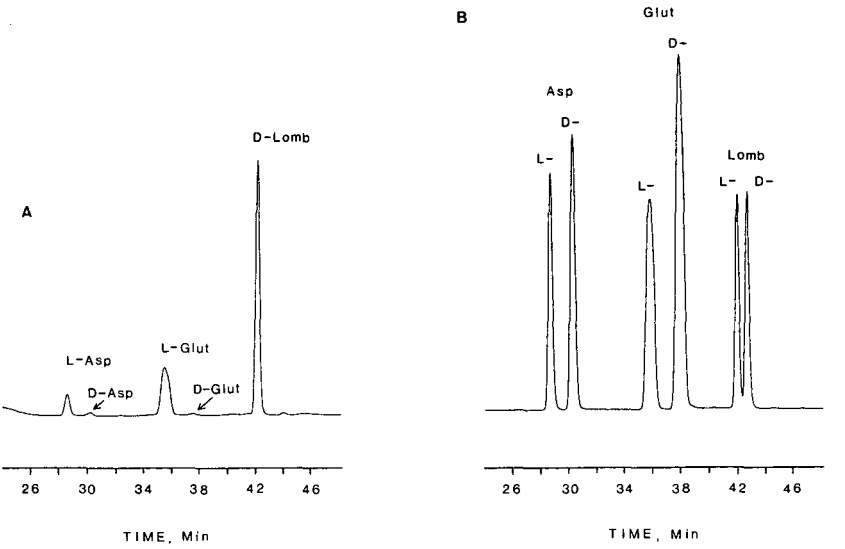

Fig. 3. HPLC of OPA–BocC derivatives in (A) sulphuric acid extract of earthworms and (B) L- and D-lombricine and aspartic acid (120 pmol of each enantiomer injected) and L- and D-glutamic acid (100 and 150 pmol of each enantiomer respectively injected). Conditions and abbreviations as in Fig. 2.

the reversed-phase column and have a longer retention time than its corresponding L diastereoisomer.

The presence of D-lombricine has been previously established in the phylum *Annelida*, whereas L-lombricine is only found in the phylum *Echiuoidea*. Samples of isolated L-lombricine from *Urechis caupo* and D-lombricine from *Lumbricus terrestris* were shown to be enantiomerically pure and had retention times of 42.10 and 42.80 min respectively (Fig. 2C and D) which, on comparison with the retention time of unambiguously synthesised D- and L-lombricine[16], verifies the previously assigned isolated lombricine configurations.

A sulphuric acid extraction of a mixed batch of earthworms [*Allolobophora caliginosa*, *Octolasum cyaneum* and *Lumbricus terrestris* of the *Annelida* phylum, as described by Hoffmann[2] (with the exception that the crude extract was lyophilised instead of being subjected to isolation of lombricine)], was subjected to the assay in order to determine the configuration of lombricine in the crude extract. A typical chromatogram is shown in Fig. 3A. The homogeneity of the assigned peaks (L-Asp, t_R = 28.70 min; D-Asp, 30.30 min; L-Glut, 35.88 min; D-Glut, 38.00 min and D-Lomb, 42.80 min) was confirmed by comparison with the retention times of authentic D and L standards and chromatography of spiked samples. It is of interest to note that in addition to possessing the D enantiomer of lombricine the earthworms studied also possessed a significantly higher level of the D enantiomer of aspartic acid than would be found in mammalian tissues. A standard chromatogram of the enantiomers of aspartic and glutamic acids and lombricine (Fig. 3B) illustrated that elution of their OPA–BocC diastereoisomers occurred in the same order as previously noted for their OPA–ethanethiol adducts[8].

The HPLC assay described represents a specific method for detecting enantiomers of lombricine in crude biological extracts and assessing the enantiomeric purity of synthetic and isolated L- and D-lombricine. The assay also permits the separation and detection of the enantiomers of aspartic acid and glutamic acid in earthworm extract.

ACKNOWLEDGEMENTS

We thank Professor Y. Robin and Professor H. Rosenberg for providing samples of authentic "isolated" D- and L-lombricines, and the C. W. Maplethorpe Trust for a research fellowship (to Dr. M. R. Euerby).

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Note

Chromatographic determination of cyclodextrins on benzoylated polyacrylamide gels

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Cyclodextrins (CDs; specifically α-, β- and γ-CDs) are cyclic dextrins produced enzymatically from starch on an industrial scale. Concomitantly with the increase in the applications of CDs in various fields of science and technology, their analysis is becoming more and more important[1].

CDs have been analysed by complexation with specific colours[2,3], by enzymatic methods[2,3] and by high-performance liquid chromatography (HPLC) with aqueous acetonitrile as the mobile phase[4,5]. The first two types of method may suffer from certain interferences and are most suitable for routine applications where the sample composition maintains relatively constant[3]. Whenever HPLC is applied to the study of enzymatic conversion processes of CDs from starch, the sample preparation is tedious because the other dextrinous side-products must be removed. The hydrolysis of these side-products by glucoamylase[6] to glucose is laborious and contains a risk of partial disappearance of especially γ-CD[3].

We describe here a specific method for the quantitation of individual CDs based on an affinity chromatographic separation mechanism. Bio-Gel P-6 was aminated and the resulting gel benzoylated. This gel effected the separation of CDs from partially hydrolysed starch and linear maltooligosaccharides and can be utilized for the analysis of CD preparations without any special sample preparation.

EXPERIMENTAL

Preparation of benzoylated Bio-Gel

A 25-g amount of Bio-Gel P-6 (Bio-Rad Labs.) was added to 500 ml of ethylenediamine (technical grade, E. Merck) in a round-bottomed flask equipped with a reflux condenser and a magnetic stirrer. The suspension was kept at 110–115°C (oil-batch) with continuous stirring for 4 h. The mixture was cooled, filtered and washed thoroughly with water, 0.2 *M* sodium chloride–0.001 *M* hydrochloric acid, water, 10% ethanol and water. The gel contained 0.57 mmol of amino groups per gram of suction-dried moist gel (potentiometric titration). The method is a modification of that presented earlier[7,8].

Benzoic acid (1 g) was dissolved in 30 ml of warm water and the pH was adjusted to 4.7 with 1 *M* sodium hydroxide solution. If all the benzoic acid does not dissolve, a minimum amount of methanol may be added. Suction-dried aminated

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Bio-Gel P-6 (30 g) was added and the pH was readjusted to 4.7 (with hydrochloric acid or sodium hydroxide). N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma) (1.5 g) was dissolved in 5 ml of water at 0°C and the solution added dropwise to the gel suspension at room temperature during 30 min with continuous stirring. The pH was maintained at 4.7 with hydrochloric acid. Gentle stirring was continued for 4 h and the gel filtered off and washed with water, 50% ethanol, methanol, 50% ethanol, water, 0.2 *M* sodium chloride solution and water (1 l each). According to potentiometric titration, 20–25% of the amino groups were benzoylated.

CD samples

Cyclodextrins were purchased from Sigma and were dissolved in distilled water (5 mg/ml each). The conversion reaction of starch to CDs was carried out by incubating purified cyclomaltodextrin glucanotransferase[9] with pregelatinized potato starch (E. Merck) in 20 m*M* imidazole–hydrochloric acid buffer (pH 6.8) containing 1 m*M* calcium chloride. The starch concentration was 10% (w/v) and the amount of enzyme was 100 units[9] per gram of starch. The reaction took place at 60°C and it was stopped after 2–12 h with 10-min boiling. These mixtures were subjected to analyses for CDs, unless stated otherwise.

Analysis

The equipment involved a peristaltic pump (Pharmacia P-3), a benzoylated gel column (160 × 14 mm I.D.) and a refractive index (RI) detector (LKB Model 2152). Samples of 0.1–0.5 ml were applied and the elution was carried out with distilled water (flow-rate 30 ml/h).

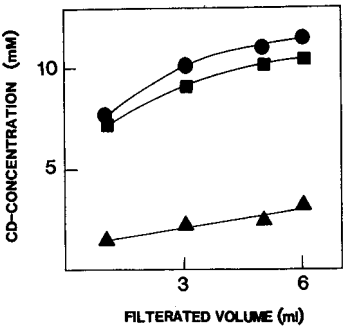
HPLC analyses were performed with an LKB HPLC system including a refractive index (RI) detector as above and a carbohydrate analysis column (Waters-Millipore; No. 84038). Acetonitrile–water (65:35, v/v) was used as the mobile phase at a flow-rate of 1.0 ml/min. The samples (0.5 ml) of the conversion mixtures were prepared by chromatography on Trisacryl GF-05 (LKB) column (40 × 2.1 mm I.D.) combining all fractions containing CDs (100% recovery as measured by specific colour reactions[3]). The elution was carried out with water (flow-rate 30 ml/h).

RESULTS

Studies of the preparation of samples for HPLC

Normally few problems are encountered when pure CDs or mixtures of CDs with maltooligosugars of G1–G8 (glucose = G1, maltose = G2, etc.) are analyzed by HPLC[4,5]. When conversion mixtures of starch are dealt with, at least the bulk of the dextrins should be removed as they are not eluted and hence rapidly degrade the HPLC column. In spite of this fact, HPLC methods with unpurified samples have been reported, however[10].

We studied the sample preparation of conversion mixtures using various techniques. Higher maltosugars were removed by ultrafiltration with an Amicon cell (PM 5 membrane) while the filtrate was analysed by HPLC. Fig. 1 shows that the concentration of CDs in the filtrate is largely dependent on the volume filtered and almost all of the solution must percolate through before reliable results are obtained.



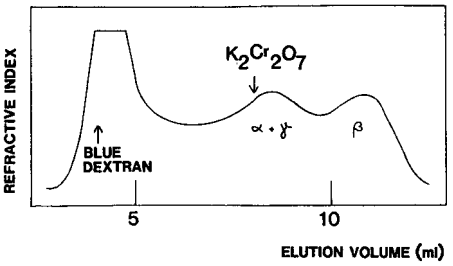


Fig. 1. HPLC analysis of CDs in the permeate through an Amicon PM 5 ultrafiltration membrane as a function of percolating volume. The enzymatic conversion mixture of starch (see Experimental) was diluted 10-fold with water prior to the filtration at room temperature at a rate of about 1 ml/min. ● = β-CD; ■ = α-CD; ▲ = γ-CD.

Fig. 2. Separation of enzymatic conversion mixture of starch, containing all three CD forms, on Trisacryl GF-05 gel (for details, see Experimental). The CDs were identified on the chromatogram by separate runs with pure CDs and by measuring the CDs with specific colour reactions[3]. The elution of Blue Dextran 2000 and potassium dichromate are indicated to show the positions of high- and low-molecular-weight dextrins, respectively. As β-CD is retained from non-excluded sugars it is specifically adsorbed on the gel.

Various gel chromatographic separations were also tested. On Sephadex gels G-10–G-50 (Pharmacia) adsorption of dextrins occurred resulting a poor resolution. Whereas Trisacryl GF-05 as a rule showed a low degree of adsorption of dextrins, β-CD was specifically retarded on this material (Fig. 2). This behaviour is probably due to the formation of an inclusion complex between β-CD- and trishydroxymethylmethane functions which could be of appropriate molecular size to be imbibed. As a result, a long elution time is required. Attempts at the specific precipitation of acyclic dextrins with organic solvents were not satisfactory. With CDs, one should be careful when using commercial disposable HPLC sample preparation kits as the CDs may become specifically attached to their packing materials and hence one or more of the CD forms may be lost.

Affinity separation of CDs

Our study was primarily conducted in order to achieve a convenient method for the determination of CDs in enzymic conversion mixtures of starch. In the early stages commercially available phenyl-glass (μBondapack phenyl; Waters Assoc., product number 27198) was observed to separate CDs with a specific retention from other sugars. Broad diffuse peaks appeared for CDs, and although elution with water was used, sample purification was needed in order to keep the column lifetime reasonable. Therefore, we decided to seek an affinity matrix combining controlled size-exclusion and affinity separation mechanisms. This system should be beneficial as then the capacity for CDs is at its maximum while little binding of large dextrins occurs owing to a limited adsorptive surface.

The problem in designing a gel-type affinity matrix having a high capacity concomitantly with mechanical strength is evident. Polyacrylamide gels were studied in more detail to find the proper degree of substitution and porosity. The degree of

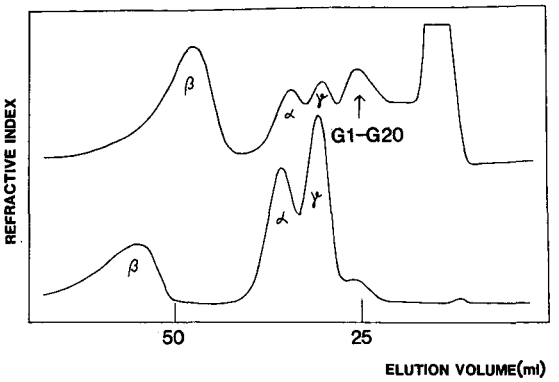


Fig. 3. Chromatography of a standard mixture of pure CDs (bottom curve) and of an enzymatic production mixture of CDs (top curve) on benzoylated Bio-Gel P-6. Sample sizes, 0.25 ml for the pure CD mixture (0.5 mg/ml of each CD) and 0.5 ml for the conversion sample. The region for the elution of oligosugars containing 1–20 glucose units is indicated by G1–G20.

benzoylation was critical; the best results were obtained with gels with 10–25% benzoylation of the amino groups. When the substitution was higher, β-CD could not be eluted from the column with water. The porosity of the gel was another adjustable parameter. A substitution-dependent shrinkage of the gel with concomitant changes in the porosity took place during the syntheses. We found experimentally that the synthesis and starting materials described here produced a satisfactory compromise to be used with water elution at room temperature. However, the support and the method provide several possibilities for further improvement.

Fig. 3 shows the elution patterns of CD standards and of a conversion product from starch on benzoylated polyacrylamide gel. Characteristic of the chromatographic system is the diffuse rear sides of the β-CD peaks and the dependence of the elution volumes on the CD concentration. As suggested above, greater benzoylation increased the elution volumes of CDs. Buffer ions, not competing with CDs for the complexation, increased the elution volumes, as did lowering of the temperature of the chromatography. The tailing of the peak of β-CD appeared in all tested conditions. The flow-rate did not affect the peak shapes and thus the adsorption isotherms of CDs should be convex. In general, such systems are unfavourable for resolution but in the context of the separation of only three molecular species from each other, the situation is not so harmful.

The elution pattern of the standard CDs (Fig. 3) shows a complete separation of β-CD and a partial separation of α- and γ-CDs. It also shows two minute impurity peaks. Direct analysis of the same standards by the HPLC method[4,5] did not reveal their presence. Hence the affinity method provides a positive means of analysing small amounts of impurities among analytical-grade CDs including inorganic salts. This finding should be of considerable importance in analyses of CDs produced for, *e.g.*, medical purposes.

High- and low-molecular-weight dextrins are separated on the gel in Fig. 3 by a size-exclusion mechanism. This is additional information obtained from the starch conversion mixtures and can be utilized in determining the degree of hydrolysis of starch in industrial processes.

TABLE I

COMPARISON OF RESULTS OBTAINED BY AFFINITY AND HPLC METHODS

The conversion mixtures for the production of CDs were prepared as described under Experimental; the reaction times were 2 h (sample 1) and 12 h (sample 2). Prior to HPLC on a Waters Assoc. carbohydrate analysis column, the samples were purified on Trisacryl GF-05. Using HPLC, the recovery of pure CDs was 97–100% (glucose as the internal standard). Results are in mg/ml measured at peak height.

Method	*Sample 1*			*Sample 2*		
	α-CD	*β-CD*	*γ-CD*	*α-CD*	*β-CD*	*γ-CD*
Benzoylated Bio-Gel P-6	0.17	2.90	0.64	0.58	2.15	0.44
HPLC	0.15	2.99	0.67	0.65	2.23	0.51

Table I compares the analyses of two conversion mixtures by the affinity and HPLC methods (for details, see Experimental). The differences between the results are small enough to fall inside presumable experimental errors, even though there is some tendency for higher values to be obtained with the HPLC method.

DISCUSSION

The determination of CDs is important in the quality control of various products containing them and in the enzymatic production of CDs from starch. For these diverse areas the most appropriate analytical methods should be found. A complicating factor is the sample preparation, as many materials adsorb CDs considerably differently depending on their individual forms.

The advantages of the affinity method described here over the existing HPLC method are as follows: (1) sample preparation is unnecessary; (2) positive quantitation of minor impurities (large dextrins, salts) in the CDs is achieved; (3) the whole procedure can be automated more easily; (4) the method does not employ harmful solvents; (5) elution with water increases the sensitivity of refractive index detection; (6) acyclic dextrins of any size do not overlap with CDs; (7) an estimate of the amount of dextrins is obtained in the hydrolysis stage of starch. In contrast, the HPLC method has the following advantages: (1) maltosugars of G1–G10 can be analysed with CDs; (2) the analysis step is faster.

ACKNOWLEDGEMENT

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Note

Lipophilicity measurement of benzodiazepine-receptor ligands by reversed-phase liquid chromatography

Comparison between high-performance liquid and thin-layer chromatography

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Benzodiazepines (BDZs) are a class of drugs endowed with a wide spectrum of anxiolytic, sedative–hypnotic, anticonvulsant and muscle relaxant properties. A few years ago, specific receptors for these drugs were discovered in the mammalian CNS[1,2]. More recently, several new drugs, chemically unrelated to BDZs, have been found that are able to interact with BDZ receptors. They show a spectrum of biological activities ranging from compounds having full BDZ-like properties (agonists), to those with completely opposite actions (inverse agonists), and finally to the antagonists able to bind to the receptor without producing any definite pharmacological effect. These compounds belong to several chemical classes, namely β-carbolines, cyclopyrrolones, pyrazoloquinolines, imidazobenzodiazepines and triazolopyridazines[3].

Many studies have been devoted to the structure–affinity relationships of the compounds interacting with BDZ receptors[4–12]; in particular, BDZs and β-carbolines have been extensively studied.

It has been suggested[12] that BDZ receptor ligands exert their action by interacting with an unique, diffuse and substantially planar recognition site; the main drug–receptor interactions should be mediated by carbonyl or imine groups via hydrogen bonds. The observed differences in pharmacological profiles should be accounted for by the various localizations of the different ligands inside this unique binding site. The lipophilic character seems to play a significant role in determining receptor binding affinities of BDZs and β-carbolines[5,13]. On the other hand, many physico-chemical factors, *e.g.*, capability of forming hydrogen bonds, planarity of the molecules, steric hindrance of substituents and electronic factors[6–13], are involved in the receptor binding affinity of all BDZ receptor ligands. Therefore, it appears very intriguing to single out in a quantitative way the role played by the hydrophobicity of compounds. In this work, by means of reversed-phase thin-layer (TLC) and

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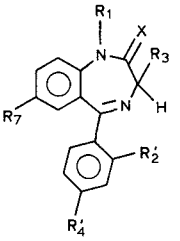
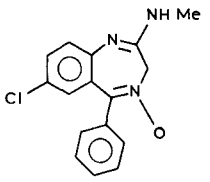
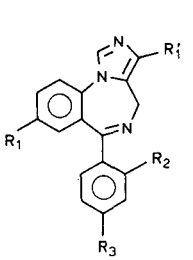
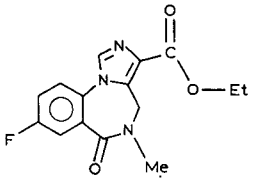
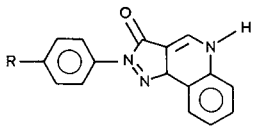
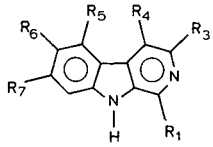
TABLE I

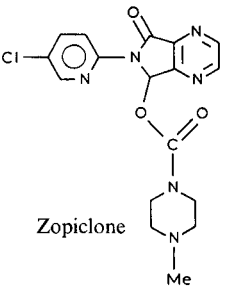
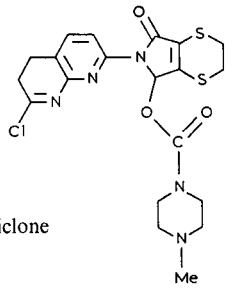
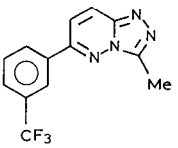
STRUCTURES OF BENZODIAZEPINE-RECEPTOR LIGANDS

Me = methyl; Et = ethyl; Pr = propyl; Ph = phenyl.

Chemical class	*Structure*
Benzo-diazepines	  Chlordiazepoxide Diazepam : R_1 = Me; R_7 = Cl; X = O Oxazepam : R_3 = OH; R_7 = Cl; X = O Prazepam : R_1 = CH_2-cC_3H_5; R_7 = Cl; X = O Medazepam : R_1 = Me, R_7 = Cl; X = H_2 Flunitrazepam : R_1 = Me; R_7 = NO_2; R'_2 = F; X = O
Imidazobenzo-diazepines	  RO 15-1624: R'_1 = $COOC_2H_5$; R_1 = R_2 = R_3 = H RO 15-8670: R_1 = Cl; R'_1 = $COOC_2H_5$; R_2 = R_3 = H RO 22-9735: R_3 = Cl; R'_1 = $COOC_2H_5$; R_1 = R_2 = H RO 21-8384: R_1 = R_2 = Cl; R'_1 = $CONH_2$; R_3 = H
Pyrazolo-quinolines	 CGS 9896: R = Cl CGS 8216: R = H
β-Carbolines	 βCCM : R_3 = COOMe; R_1 = R_4 = R_5 = R_6 = R_7 = H βCCE : R_3 = COOEt; R_1 = R_4 = R_5 = R_6 = R_7 = H PrCC : R_3 = COOnPr; R_1 = R_4 = R_5 = R_6 = R_7 = H Harman : R_1 = CH_3; R_3 = R_4 = R_5 = R_6 = R_7 = H ZK93423 : R_3 = COOEt; R_4 = CH_2OCH_3; R_6 = OCH_2Ph; R_1 = R_5 = R_7 = H

(*Continued on p. 406*)

TABLE I (*continued*)

Chemical class	*Structure*
Cyclopyrrolones	 Zopiclone
	 Suriclone
Triazolo-pyridazines	 CI 218872

high-performance liquid chromatographic (HPLC) systems, we have measured the lipophilic character of some representative compounds belonging to the benzodiazepines, β-carbolines, cyclopyrrolones, triazolopyridazines, pyrazoloquinolines, phenylquinolines and imidazobenzodiazepines (Table I). The purpose was to determine the range of lipophilicity of compounds interacting with BDZ receptors and to compare the chromatographic systems.

EXPERIMENTAL

Materials

Imidazobenzodiazepines were a gift from H. Möhler (Hoffman-La Roche, Basle, Switzerland); β-CCM, β-CCE, PrCC and ZK 93423 were a gift from R. Schmiechen (Schering, Berlin, F.R.G.); Zopiclone and Suriclone were donated by Rhone-Poulenc (Vitry-sur-Seine, France); CGS 8216 and CGS 9896 were donated by Ciba-Geigy (Basle, Switzerland); and CL 218-872 was provided by American Cyanamide (Pearl River, U.S.A.). All other drugs and chemicals (analytical-reagent grade) were obtained from commercial sources.

Chromatography

The HPLC measurements were performed on a Spectra-Physics chromatograph consisting of an SP 87000 solvent delivery system and an SP organizer module. A Varian Aerograph UV detector operated at 254 nm was used. A 30 mm × 3.9 mm I.D. μBondapak C_{18} column from Waters Assoc. was used. The mobile phase was acetonitrile in various mixtures with phosphate buffer (pH = 7.0; ionic strength = 0.05 *M*) in the concentration range 30–70%. The TLC determinations were carried out on Whatman KC18F plates. A Camag Nanomat (Camag, Berlin, F.R.G.) was

used to spot 100 nl of the solute in methanol on the plates. The solutes were detected under UV light (254 nm). Mixtures of acetonitrile–phosphate buffer in the concentration range 30–65% and methanol–phosphate buffer in the 55–90% range were used as mobile phases. The R_M values for compounds 17–21 in the methanol systems had been determined previously[13]. Each HPLC and TLC measurement was replicated at least three times.

RESULTS AND DISCUSSION

In the HPLC system the linear relationship between log k' and acetonitrile concentration in the mobile phase allowed the calculation of theoretical log k' values at 0% acetonitrile (Table II). Similarly, theoretical R_M values were calculated in both TLC systems (Table II).

The log k' and R_M values in Table II were used in order to evaluate eqns. 1–3.

$$R_{M(CH_3CN)} = 0.177\ (\pm 0.177) + 0.885\ (\pm 0.080) \log k'_{(CH_3CN)} \quad (1)$$

$$(n = 21;\ r = 0.931;\ s = 0.142;\ F = 123.87;\ P < 0.005)$$

$$R_{M(CH_3OH)} = 0.033\ (\pm 0.297) + 1.431\ (\pm 0.138)\ R_{M(CH_3CN)} \quad (2)$$

$$(n = 21;\ r = 0.922;\ s = 0.234;\ F = 107.41;\ P < 0.005)$$

$$R_{M(CH_3OH)} = -0.022\ (\pm 0.227) + 1.406\ (\pm 0.102) \log k'_{(CH_3CN)} \quad (3)$$

$$(n = 21;\ r = 0.954;\ s = 0.182;\ F = 190.74;\ P < 0.005)$$

The R_M and log k' values were fairly similar when the mobile phase in both TLC and HPLC systems was based on acetonitrile. In fact, eqn. 1 has an intercept and slope close to 0 and 1, respectively, with a good correlation coefficient. On the other hand, eqn. 2 shows a similar correlation coefficient between the R_M values determined with methanol or acetonitrile in the mobile phase of the TLC system. However, the slope of eqn. 2 is greater than 1, which means that the extrapolated R_M values in the two systems are different. In fact, in the system with methanol in the mobile phase the R_M values range from 1.78 to 4.53, whereas with acetonitrile the R_M values range from 1.34 to 3.17. In other words, in the meethanol system there is a much wider range of R_M values. As a consequence, while describing a good correlation between the TLC and HPLC systems, eqn. 3 indicates again the wider range of R_M values in the TLC system with methanol in the mobile phase.

In our previous studies it was possible to show that the extrapolated R_M values were very similar when the mobile phase contained either methanol or acetone. Therefore, those data were considered as supporting the hypothesis that the extrapolated R_M values were really an expression of the partitioning between water and silicone oil. This does not seem to be so with acetonitrile in the mobile phase. This

TABLE II

LIPOPHILIC CHARACTER OF BENZODIAZEPINE-RECEPTOR LIGANDS

No.	*Compound*	$R_{M(CH_3OH)}$	$R_{M(CH_3CN)}$	*Log* $k'_{(CH_3CN)}$
1	CGS 9896	3.47	2.19	2.30
2	CGS 8216	2.41	1.88	1.96
3	Zopiclone	2.29	1.88	1.80
4	Suriclone	3.23	2.25	2.27
5	CL 218872	3.07	2.38	2.14
6	RO 15 1788	1.78	1.34	1.19
7	RO 15 1624	3.28	1.98	2.22
8	RO 15 8670	3.38	2.35	2.34
9	RO 22 9735	3.58	2.48	2.42
10	RO 21 8384	2.87	1.83	2.16
11	Diazepam	2.91	2.12	2.17
12	Flunitrazepam	2.71	1.95	1.97
13	Chlordiazepoxide	2.82	1.94	2.21
14	Oxazepam	2.30	1.45	1.65
15	Prazepam	3.45	2.42	2.47
16	Medazepam	4.53	3.17	3.26
17	β-CCM	2.92	2.02	1.93
18	β-CCE	3.50	2.12	2.36
19	PrCC	3.54	2.45	2.64
20	ZK 93423	2.93	2.02	2.28
21	Harman	3.35	2.15	2.33

could be due to some kind of interaction between acetonitrile and the stationary phase. However, as eqn. 1 shows that the R_M and log k' values are very similar, such an interaction should act in the same way in both TLC and HPLC. In fact, Braumann *et al.*[14], in describing the relationship between the log k' values of phenylurea herbicides with methanol in the mobile phase and those with acetonitrile, found an equation very similar to our eqn. 2 with a correlation of 0.939.

Obviously this study should be expanded to other series of chemicals in order to find out if there is a more general meaning.

The compounds in Table I belong to six classes. In Table II it can be seen that the examined compounds show quite different R_M values in the methanol system. However, when compounds 16 and 6, with the highest and lowest R_M values, respectively, are excluded the R_M values in the methanol system range from 2.29 to 3.58. This means that the compounds able to interact with the BDZ receptors are characterized by a narrow range of lipophilic character. This could suggest that lipophilic character plays only a secondary role in providing higher binding affinities to BDZ receptors.

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Note

Determination of aflatoxins by capillary column gas chromatography

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Aflatoxins (AFs) are a group of toxins produced by some *Aspergillus flavus* Link moulds[1]. These toxins are potent carcinogens in experimental animals and often contaminate various agricultural commodities such as maize and peanuts[2]. A variety of techniques have been used for the separation and identification of the four major naturally occurring AFs, namely aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) (Fig. 1). The most widely used method has been thin-layer chromatography (TLC). High-performance liquid chromatography (HPLC) has been used since the late 1970s, but gas chromatography (GC), one of the most popular methods of analysis of various mycotoxins, has never been successfully applied to the analysis of mixtures of four aflatoxins. In 1981, Friedli[3] reported that AFB_1 could be analyzed without chemical derivatization by GC using a mass spectrometer as the detector (GC–MS). Subsequently, Trucksess *et al.*[4] and Rosen *et al.*[5] reported that AFB_1 or mixture of AFB_1 and AFB_2 in contaminated peanuts could be determined by GC–MS. We have now succeeded in determining four major AFs (B_1, B_2, G_1 and G_2) using GC with flame ionization detection (FID) with a capillary on-column injector and a fused-silica capillary column.

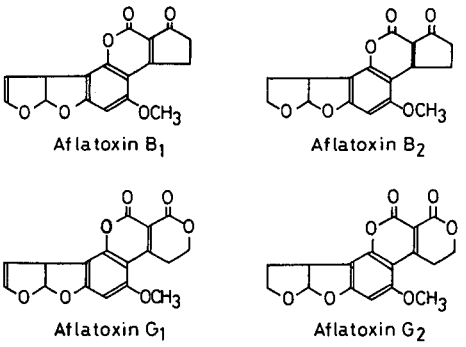

Fig. 1. Structures of aflatoxins.

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EXPERIMENTAL

Gas chromatography

A Shimadzu GC-15A gas chromatograph (Shimadzu, Kyoto, Japan) was used. This system consists of a GC-15A gas chromatograph with an OCI-9A capillary on-column injector, a flame ionization detector and a Chromatopac C-R4A reporting integrator. Fused-silica capillary columns (0.25 mm I.D.) containing a chemically bonded liquid stationary phase (0.25 μm) were purchased from J&W (CA, U.S.A.). The stationary phases were methylsilicone (DB-1) and 5% phenylmethylsilicone (DB-5) and the lengths of the columns were 3, 5, 10, 15 and 25 m. Helium was used as both the carrier gas and make-up gas.

Mass spectrometry

A Shimadzu GCMS QP1000 mass spectrometer was used for the confirmation of each aflatoxin. The ionization voltage was 70 eV, the ionization current was 60 μA and the ion source temperature was 250°C.

Chemicals and samples

Aflatoxin B_1, B_2, G_1 and G_2 were purchased from Makor Chemicals (Jerusalem, Israel) and were dissolved in benzene–acetonitrile (98:2), which was also used to prepare dilutions. Other chemicals and reagents were of analytical-reagent grade and were used without further purification. The AF-producing mould (unidentified) was cultivated in modified Czapek–Dox liquid medium at 27°C for 7 days. A volume of 1-l of this liquid medium contains 30 g of glucose, 3 g of sodium nitrate, 1 g of dipotassium phosphate, 0.5 g of magnesium sulphate, 0.5 g of potassium chloride, 0.01 g of iron(II) sulphate, 0.01 g of zinc sulphate and 0.005 g of copper(II) sulphate. After cultivation, 5 ml of medium were removed and to it were added 250 mg of sodium chloride and 5 ml of methanol. Subsequently, AFs were extracted twice with 3-ml volumes of chloroform. The chloroform extracts were combined and evaporated to dryness under a stream of nitrogen. The residue was dissolved in benzene–acetonitrile (98:2) and used for GC analysis.

RESULTS AND DISCUSSION

Analytical conditions

When the initial temperature of the column and injector was higher than 60°C, all four AF peaks became broad and the sensitivity decreased. Also, either the final temperature was low or the rate of heating was slow, causing an increase in the retention time and a decrease in sensitivity. Therefore, the initial and final temperatures were set at 50 and 300°C and the rate of heating was set at 15 or 20°C/min.

Two types of stationary phases were tested. The methylsilicone column (DB-1, 10 m) did not separate AFG_1 and AFG_2 and barely separated AFB_1 and AFB_2. As a result, the shape of the peaks was distorted. In contrast, a 5% phenylmethylsilicone column (DB-5, 10 m) distinctly separated AFB_1 and AFB_2 and also achieved a 50% separation between AFG_1 and AFG_2 (Fig. 2A). A longer column was used to improve the overall separation. Although the four AFs were completely separated on a 25-m DB-5 column, the sensitivity much lower for AFG_1 and AFG_2 than for AFB_1 and AFB_2.

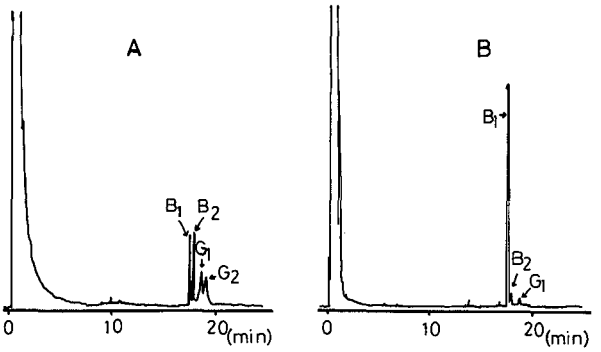

Fig. 2. Gas chromatograms of aflatoxins. (A) Aflatoxin standards: AFB_1, 5 ng; AFB_2, 5 ng; AFG_1, 10 ng; AFG_2, 10 ng. (B) Extract from culture medium.

Determination of the relationship between column length and the sensitivity for AFG_1 and AFG_2

Four columns of different length were used. The relationship between column length and the ratio of the peak areas between the AFB group and the AFG group was calculated by the following method. Each injection contained 25 ng of AFB_1 and AFB_2 and 50 ng of AFG_1 and AFG_2:

$$\text{Ratio of peak area} = \frac{\text{peak area of } AFG_1 + \text{peak area of } AFG_2}{\text{peak area of } AFB_1 + \text{peak area of } AFB_2}$$

As shown in Fig. 3, the ratio of the area attributed to the AFG group decreased depending on the length of column. After considering both sensitivity and separation, a column length of 15 m was selected for practical applications of the method.

Determination of aflatoxins

As shown in Fig. 4, the limit of quantification (signal-to-noise ratio = 5) of each AF was 1 ng; the linear range of this quantitative analysis was from 1 to 50 ng.

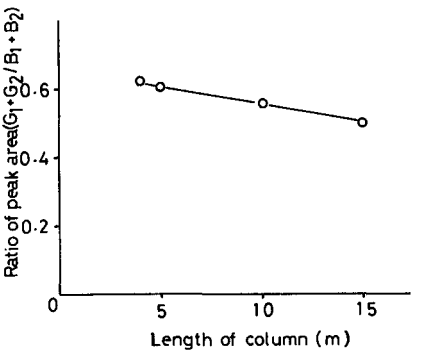

Fig. 3. Ratio of peak areas of the AFB group to the AFG group.

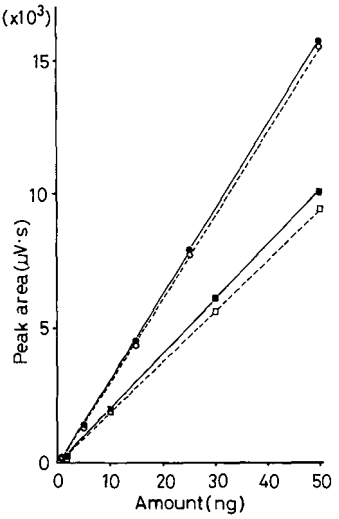

Fig. 4. Calibration graphs for aflatoxins using gas chromatography. ● = AFB_1; ○ = AFB_2; ■ = AFG_1; □ = AGF_2.

The analysis was highly reproducible (Table I) at 2 ng for AFB_1 and AFB_2 and at 4 ng for AFG_1 and AFG_2.

Determination of aflatoxins in culture medium

Extracts of aflatoxigenic mould cultivated in liquid medium were analysed by the method described above (Fig. 2B). AFB_1, AFB_2 and AFG_1 were quantitatively detected, but AFG_2 was not, which agreed with the results obtained using either TLC or HPLC for quantitation. The molecular ions of AFB_1 (*m/z* 312), AFB_2 (*m/z* 314) and AFG_1 (*m/z* 328) were detected at the corresponding retention times in the samples analysed by GC–MS.

Clearly all four major AFs (B_1, B_2, G_1 and G_2), and not only AFB_1 and AFB_2[5], can be determined by GC with FID. The sensitivity of the method is not as high as that of other methods[6,7]. A possible reason for the low sensitivity is the presence of oxygen in the molecule[8]. However, GC does have the advantage of immediate compound identification by using MS detection.

TABLE I

REPRODUCIBILITY OF AFLATOXIN DETERMINATION

Results (n = 7) obtained using a 15-m column with temperature programming from 50 to 300°C at 15°C/min.

	AFB_1 (2 ng)	*AFB_2 (2 ng)*	*AFG_1 (4 ng)*	*AFG_2 (4 ng)*
Average result (ng)	1.98	1.94	3.96	3.93
S.D. (ng)	0.0637	0.0832	0.237	0.149

ACKNOWLEDGEMENT

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Note

Trace determination of sulfide by reversed-phase ion-interaction chromatography using pre-column derivatization

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Sulfide is present in many environmental samples, such as waste waters, where it is often produced from the bacterial reduction of sulfate. This sulfide is readily converted into hydrogen sulfide which causes odour and toxicity problems. For these reasons, the determination of sulfide assumes considerable importance.

Iodine reacts with sulfide in acidic solution and this reaction forms the basis of a titrimetric procedure for sulfide at levels above 1 ppm and in the absence of interfering substances[1]. A more sensitive determination involves reaction of N,N-dimethyl-*p*-phenylenediamine and hydrogen sulfide in the presence of a mild oxidizing agent to produce methylene blue, with spectrophotometric measurement of the amount of methylene blue formed[2]. This reaction, using ferric ion as the oxidant, is shown in Fig. 1; other substituted analogues of *p*-phenylenediamine show a similar reaction. The chief drawback of the spectrophotometric approach is interference by strongly reducing substances which prevent formation of the blue colour, or interference by other ions which also form a blue colour under the reaction conditions used.

Sulfide is difficult to determine by ion chromatography for a number of reasons. First, sulfide exists in solution as neutral hydrogen sulfide (pK_{a1} = 6.99) unless the solution is made alkaline, so anion-exchange separation can be applied successfully only with alkaline mobile phases such as sodium hydroxide. Even under

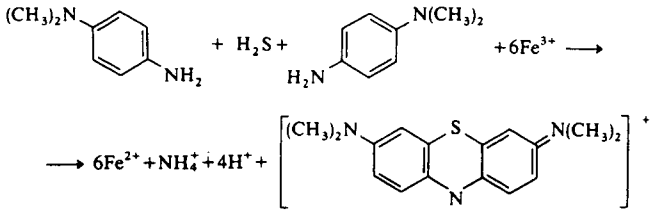

Fig. 1. Reaction of sulfide with N,N-dimethyl-*p*-phenylenediamine and ferric ion to form methylene blue.

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these conditions, sulfide exists predominantly as HS^- and is only weakly retained on anion-exchange columns. Second, sulfide is poorly detected by conductivity measurement when a post-column suppressor device is used because it is converted to a weakly conducting protonated species after passage through the suppressor. Conductometric detection is therefore feasible only for non-suppressed ion chromatography, but the sensitivity attainable by this approach is relatively poor. Finally, sulfide is unstable in aqueous solution and undergoes reaction with oxygen. This reaction can occur both prior to and during the chromatographic analysis.

In view of the limited applicability of conductometric detection, amperometric or potentiometric detection methods are generally recommended for sulfide. Potentiometry using a silver sulfide ion-selective electrode has been reported as a viable detection method for sulfide[3], and amperometric methods using gold[4], mercury-coated platinum[5] or silver[6] working electrodes have also been described. The latter methods exhibit excellent sensitivity for sulfide, with detection limits falling within the 0.1–30 ppb* range, but problems are encountered with non-linearity of calibration at low concentrations and errors due to oxidation of sulfide, impurities in the eluent and adsorption of sulfide onto the ion-exchange columns used.

In this paper we describe a reversed-phase ion-interaction chromatographic method for the determination of trace levels of sulfide after its reaction with N,N-dimethyl-*p*-phenylenediamine to form methylene blue. This process converts sulfide to a stable product and thereby obviates the major problems of the ion-exchange methods. Moreover, the developed method is rapid and simple, and has sensitivity equivalent to that of amperometric detection.

EXPERIMENTAL

Instrumentation and reagents

The liquid chromatograph consisted of a Millipore Waters M590 pump, U6K injector, Model 481 UV–VIS detector operated at 664 nm and M740 data module. The column used was a Waters μBondapak C_{18} (150 × 3.9 mm I.D.), operated at a flow-rate of 1.0 ml/min.

N,N-Dimethyl-*p*-phenylenediamine was obtained from Aldrich (Milwaukee, WI, U.S.A.) and a stock solution was prepared by dissolving 4 g of the free base in 50 ml of 1:1 sulfuric acid. A working solution of the amine was then prepared by diluting 5 ml of stock solution to 100 ml with 1:1 sulfuric acid. Both the stock and working solutions were stored in dark bottles in the refrigerator. The Fe(III) oxidizing solution was prepared by dissolving 25 g of ferric nitrate nonahydrate in 10 ml of water.

A stock solution of sulfide (1000 ppm) was prepared by dissolving an appropriate amount of analytical-grade sodium sulfide nonahydrate in 0.05 *M* sodium hydroxide prepared from boiled-out de-ionized water. This stock solution was stored in a polyethylene bottle in the refrigerator and was used for the daily preparation of standard solutions. These standard solutions were prepared by serial dilution of the

* Throughout the article the American billion (10^9) is meant.

stock solution with de-ionized water (from a Millipore Milli Q apparatus) which had been boiled for 30 min to remove oxygen.

The eluent was prepared by adding 400 ml of HPLC-grade acetonitrile to a 1-litre volumetric flask containing 300 ml of water, after which 5 ml of glacial acetic acid and a vial of Waters low-UV PIC B5 (pentanesulfonic acid) were then added and the flask made up to the mark with water. The eluent was filtered through a 0.45-μm membrane filter and degassed under vacuum in an ultrasonic bath before use.

Procedure

An aliquot (usually 7.0 ml) of sample was added to a 10-ml volumetric flask and 0.5 ml of the working amine solution added with a dispensing pipette, then 3 drops of Fe(III) solution were added with a dropper. The flask was then stoppered and carefully inverted once only. Excessive mixing at this stage can result in loss of hydrogen sulfide, giving low results. The flask was then diluted to the mark with water and allowed to stand for 1 min to permit colour development. After this time, a 100-μl aliquot was injected onto the liquid chromatograph.

RESULTS AND DISCUSSION

Selection of chromatographic conditions

Fig. 2 shows absorption spectra of a 1-ppm sulfide sample and a blank solution, treated according to the procedure described in the Experimental section. These spectra show that the reaction product exhibited maximum absorbance at 664 nm, whilst the unreacted amine showed weak absorbance at 500 nm. It was therefore possible to monitor both the reaction product and the unreacted amine by appropriate wavelength selection.

Methylene blue is a basic compound and under the strongly acidic conditions used in the reaction scheme shown in Fig. 1, it exists as a protonated species. Therefore, an ion-interaction approach using pentanesulfonic acid as the ion-interaction

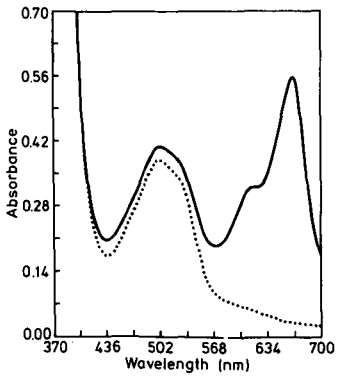

Fig. 2. Absorption spectra of a 1-ppm sulfide standard (solid line) and a blank solution (broken line) treated according to the proposed derivatization procedure. Cells with an optical path length of 1 cm were used.

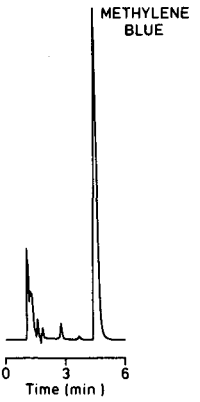

Fig. 3. Chromatogram of a 1-ppm sulfide standard solution derivatized to form methylene blue. Conditions: column, Waters μBondapak C_{18} (150 × 3.9 mm I.D.); mobile phase, acetonitrile–water (40:60, v/v) containing 0.5% glacial acetic acid and 5 m*M* low UV PIC B5; flow-rate, 1.0 ml/min; injection volume, 100 μl; detection at 664 nm; integrator attenuation, 512.

reagent was investigated for separation of methylene blue from the reaction mixture. Acetic acid was added to the mobile phase in order to ensure the protonation of methylene blue and so increase its retention. Fig. 3 shows a chromatogram obtained for a 1-ppm sulfide solution, using a mobile phase comprising acetonitrile–water (40:60), 0.005 *M* pentanesulfonic acid and 0.5% acetic acid, with detection at 664 nm. Methylene blue was eluted as a well-resolved but slightly tailed peak at a retention time of 4.6 min. Injection of an authentic methylene blue standard solution gave a peak at the same retention time as the major peak in Fig. 3. When the chromatogram illustrated in Fig. 3 was run using a detection wavelength of 500 nm, the height of the methylene blue peak decreased in accordance with the absorption spectra shown in Fig. 2, but the height of the peak at 2.7 min increased dramatically. This result suggested that the peak at 2.7 min was due to unreacted amine.

Studies were undertaken to determine the minimum reaction time required to produce the maximum peak height for a given sulfide concentration and it was found that, for a 1-ppm sulfide solution, the maximum peak height was attained after the solution was permitted to stand for 1 min after dilution to the mark. This time was therefore adopted for all future determinations. The effect of temperature was not examined.

It should be noted that the separation shown in Fig. 3 was column-dependent and could not be reproduced on other C_{18} columns tested. In all cases, other columns gave a very tailed peak for methylene blue and care should therefore be exercised if the developed method is to be applied using alternative columns.

Analytical parameters and interferences

Calibration was found to be linear over the range 1–2000 ppb sulfide and the precision for eight replicate determinations at the 10-ppb level was 3.7% relative standard deviation (R.S.D.). The detection limit, determined as the concentration of sulfide in a 100-μl injection giving a signal equal to three times the baseline noise,

TABLE I

INTERFERENCE EFFECTS FOR SOME INORGANIC ANIONS

Interfering ions were added at the indicated concentration to a 1-ppm sulfide standard and peak areas are expressed as a percentage of the area obtained for a 1-ppm sulfide standard injected alone.

Ion	*Area of methylene blue peak (%)*		
	1 ppm	*10 ppm*	*100 ppm*
$Fe(CN)_6^{4-}$	99.6	95.5	32.1
$Fe(CN)_6^{3-}$	100.2	100.1	118.9
I^-	99.4	33.3	16.1
SCN^-	99.7	99.9	99.2
SO_3^{2-}	99.9	100.3	94.0
$S_2O_3^{2-}$	100.1	100.0	53.1

was 0.8 ppb. The accuracy of the method was 98.7%, as indicated by the average recovery obtained for a 500-ppb standard solution of sulfide. Once formed, the methylene blue was quite stable, with the peak area for a 1-ppm sulfide standard solution remaining constant for at least two weeks after performing the derivatization reaction.

Interference studies were conducted for a wide range of species. Common inorganic anions including chloride, bromide, nitrite, nitrate, phosphate, sulfate, carbonate and cyanide showed no interference at 10 000 fold excess over sulfide. Thiosulfate, thiocyanate, sulfite, iodide, ferrocyanide and ferricyanide are known to interfere with the spectrophotometric method, and quantitative data describing their interference effects on the proposed chromatographic method are given in Table I. At a 10 fold excess over sulfide, only iodide showed appreciable interference by inhibiting colour formation, however at the 100 fold excess level, thiosulfate, iodide and ferrocyanide showed negative interference, whereas ferricyanide gave positive interference.

It is interesting to compare the magnitudes of these interferences with those observed in the spectrophotometric method, for which all of the above ions are considered to be strong interferences, either by inhibition of the formation of methylene blue or by formation of interfering coloured products. Whilst the chromatographic method does suffer interference when formation of methylene blue is inhibited, it is less prone to interferences from other coloured products because these are resolved from methylene blue. For example, ferrocyanide and ferricyanide at the 10-ppm level give intensely blue-coloured solutions and represent major interferences to the spectrophotometric method, but at this level did not interfere with the chromatographic method. A further advantage of the chromatographic method is that the purity of the N,N-dimethyl-*p*-phenylenediamine used was not critical, whereas the opposite is true for the spectrophotometric method in which high blank levels can result from impure amine reagent. In addition, residual iron(III) must be decolourized with phosphate in the spectrophotometric method and this step is unnecessary in the chromatographic approach.

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Note

Determination of diphenylamine residues in apples, and 4-aminobiphenyl residues in diphenylamine, by high-performance liquid chromatography and electrochemical detection

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Diphenylamine is a post-harvest fungicide currently used on apples to prevent scald. However, its use is subject to restriction in a number of countries. These restrictions refer to (1) the amount of diphenylamine residues in treated fruit (limited to 3 mg/kg in France for example) and (2) the amount of 4-aminobiphenyl in used diphenylamine (limited to 2 mg/kg in France for example) since 4-aminobiphenyl is a very dangerous impurity as a result of its very high carcinogenic properties.

Such restrictions led us to try to develop simple and rapid methods to determine diphenylamine in apples, and residual 4-aminobiphenyl in diphenylamine.

A number of techniques have been proposed for the determination of diphenylamine involving spectrophotometry, after derivatization[1], thin-layer chromatography (TLC)[2], gas chromatography (GC)[3–7] with electron-capture detection (ECD) (after derivatization), nitrogen-phosphorus detection, or Hall's detector, and high-performance liquid chromatography (HPLC)[8,9] with UV detection.

All these methods have drawbacks. GC requires fairly lengthy sample preparation and, for ECD, a derivatization. TLC is not satisfactory for quantitative determination, and therefore needs an extraction of the spot for a later determination. Liquid chromatography with UV detection lacks sensitivity and needs a pre-concentration of the sample for satisfactory results. For all these reasons, we have turned our attention to liquid chromatography with electrochemical detection (LC–ED).

Because aromatic amines are readily oxidizable, LC–ED offers an attractive approach to their analysis. Its use for similar problems (aromatic amine residues in water, soil or skin) has given excellent results. For that reason we have applied it to the determination of diphenylamine residues in apples.

Determination of 4-aminobiphenyl residues in diphenylamine has not yet been studied. Among different methods proposed for the determination of 4-aminobiphenyl residues in other media, there are electrochemical techniques, such as polarography and coulometry[10], TLC[11], GC[12–14], and HPLC with electrochemical detection[15].

As this technique is particularly well adapted to our problems, we have also applied LC–ED to the determination of 4-aminobiphenyl residues in diphenylamine.

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EXPERIMENTAL

Apparatus

The following equipment was used: a Millipore (Waters) M510 pump; a Rheodyne injection valve, equipped with a 20-μl loop; a μBondapak C_{18} column (10 μm, 30 cm × 4 mm I.D.); an amperometric detector Tacussel PRG-DEL with DEL-1 cell. The working electrode was glassy carbon, the reference electrode was silver/silver chloride/saturated potassium chloride and the auxiliary electrode was platinum. The mobile phase was acetonitrile–water (60:40) containing 2 g/l of lithium perchlorate, and run at a flow-rate of 1 ml/min. Under these conditions, the capacity factors (k') were as follows: diphenylamine, 3.75; 4-aminobiphenyl, 2.08.

Intensity potential curves for 4-aminobiphenyl and diphenylamine

To determine the optimal value of the potential to be applied, we injected a mixture (100 μg/ml) of both compounds to be studied, at different potentials, and measured the intensity of the oxidation peak corresponding to each case. The intensity potential curves observed are indicated in Fig. 1. A study of these curves shows that the limiting diffusion current is reached at +1.2 V for 4-aminobiphenyl as well as for diphenylamine. However, in order to prevent too much residual current and background noise, the potential was fixed at +0.9 V throughout the following procedure.

Separation of 4-aminobiphenyl from diphenylamine

For the determination of residual 4-aminobiphenyl in diphenylamine, we tested a separation method based on the difference in basicity of the two compounds: 4-aminobiphenyl is clearly more basic than diphenylamine.

Procedure. A 1-ml volume of a solution of 4-aminobiphenyl or diphenylamine

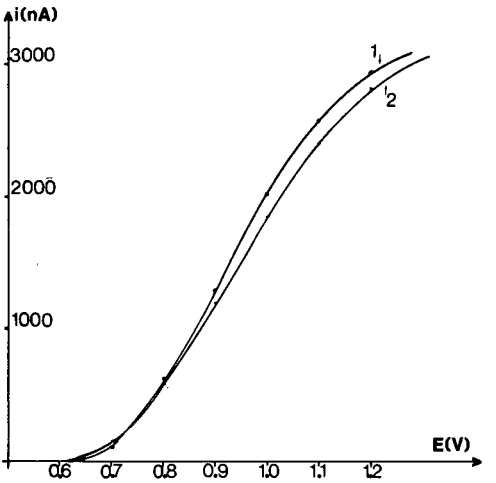

Fig. 1. Intensity–potential curves: 1 = 4-aminobiphenyl; 2 = diphenylamine.

(1 mg/ml) was placed in a 125-ml separatory funnel, and 20 ml of selected pH hydrochloric acid were added. The compounds were extracted with two 20-ml portions of hexane. The hexane phases were combined and evaporated to dryness, and the residue was dissolved into 10 ml of methanol. Then 4-aminobiphenyl or diphenylamine was injected into the chromatograph under the conditions previously described.

A study of the results shows that, although the extraction yield of diphenylamine is close to 100% at any pH, the extraction yield of 4-aminobiphenyl depends strongly on the pH. The lower the pH, the less 4-aminobiphenyl is extracted by hexane. For example, the recovery rates observed varied from 0.9% at pH 0 to 91.7% at pH 3.

Determination of 4-aminobiphenyl residues in diphenylamine

The above results led us to adopt the following procedure for the determination of 4-aminobiphenyl residues in diphenylamine. Exactly 5 g (of diphenylamine) were dissolved in 100 ml of hexane, and the solution was placed in a 250-ml separatory funnel. 4-Aminobiphenyl was extracted by four 20-ml portions of 0.1 *M* hydrochloric acid. The aqueous phases were combined and poured into another 250-ml separatory funnel; the hexane phases were discarded. The aqueous phase was washed with two 40-ml portions of hexane, and neutralized by 10 ml of 1 *M* sodium hydroxide. 4-Aminobiphenyl was extracted with two 40-ml portions of dichloromethane. The organic phases were combined, filtered over anhydrous sodium sulphate, then evaporated to dryness. The evaporation residue was dissolved into 10 ml of methanol, then injected into the liquid chromatograph under the conditions described above.

Determination of diphenylamine residues in apples

A 100-g amount of apples was weighed and peeled carefully. The peel was placed in a 250-ml flask, and 100 ml of methanol and 10 ml of 10% methanolic potassium hydroxide solution were added. The mixture was shaken for 1 h then filtered, and the filtrate was placed in a 1000-ml separatory funnel. Next, 300 ml of distilled water and 30 ml of saturated sodium chloride solution were added, and diphenylamine was extracted with two 60-ml portions of dichloromethane. The organic phases were combined, filtered over anhydrous sodium sulphate, and evaporated to dryness. The residue was dissolved into 10 ml of methanol, filtered on a 0.45-μm PTFE Millipore membrane, then injected into the chromatograph.

RESULTS AND DISCUSSION

Study of the linearity and repeatability of the detector response

In order to study the linearity and repeatability of the method, we repeatedly injected ($n = 10$) several mixtures of 4-aminobiphenyl and diphenylamine at 0.05, 0.5, 5 and 50 μg/ml. We measured the peak heights, and calculated the mean and the standard deviation. The results, given in Table I, show the perfect linearity and the excellent repeatability of electrochemical detection. The calculated correlation coefficients are +0.999 645 for 4-aminobiphenyl, and +0.999 968 for diphenylamine.

Detection limits of diphenylamine and 4-aminobiphenyl

In order to test the sensitivity of the method, we injected smaller and smaller

TABLE I

DETECTOR RESPONSE AND REPEATABILITY

Means and relative standard deviations obtained for ten injections (20 μl) of diphenylamine and 4-aminobiphenyl at different concentrations.

C (μg/ml)	*4-Aminobiphenyl*		*Diphenylamine*	
	I (nA)	*σ (%)*	*I (nA)*	*σ (%)*
0.05	1.2	4.5	0.67	4.1
0.5	12.6	6.6	7.25	6.5
5.0	105	3.5	73.5	2.7
50.0	816	10.6	679	4.1

amounts of each of the two compounds to determine the detection limits. For an injection of 200 pg of both compounds the signal-to-noise ratio is *ca.* 5, so we can estimate the detection limit as *ca.* 100 pg for these compounds.

Determination of 4-aminobiphenyl residues in diphenylamine

The method described has been applied to five samples of diphenylamine, spiked respectively with 0, 1, 5, 10 and 50 mg/kg of 4-aminobiphenyl. The recovery rate was determined for each sample, and the results show that it varies from 88.6% to 100%.

Determination of diphenylamine residues in apples

The recovery rate of diphenylamine has been studied in five samples of apples (Granny Smith type), spiked respectively with 0, 0.1, 0.5, 1 and 5 mg/kg of diphenylamine, according to these method described. Table II indicates that the observed recovery rates were always satisfactory, even for very low concentrations. Also, the chromatogram obtained (Fig. 2) shows that there is no interference for the determination of diphenylamine.

TABLE II

DETERMINATION OF DIPHENYLAMINE RESIDUES IN APPLES

Diphenylamine		*Yield (%)*
Added (mg/kg)	*Recovered (mg/kg)*	
0	0	–
0.1	0.115	115
0.5	0.59	118
1	0.98	98
5	4.6	92

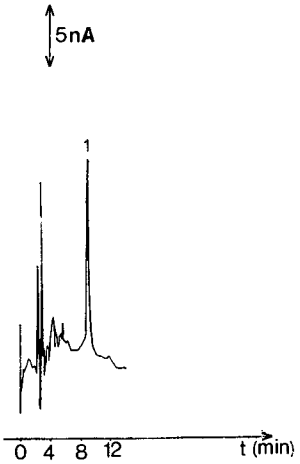

Fig. 2. Determination of diphenylamine residues in apples, obtained with a 0.1 mg/kg spiked sample. Peak: 1 = diphenylamine.

CONCLUSION

The results of this study both for 4-aminobiphenyl and diphenylamine, show that HPLC with electrochemical detection is undoubtedly one of the best methods yet proposed. Its main advantages are (1) extreme sensitivity (detection limit of 100 pg), (2) excellent selectivity, particularly for the determination of diphenylamine in apples, which requires no purifying operation (3) great simplicity and low cost.

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CHROM. 20 621

Note

Liquid chromatographic method for the determination of cyanazine in the presence of some normal soil constituents

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Cyanazine is the ISO common name for 2-chloro-4-(1-cyano-1-methylethylamino)-6-ethylamino-1,3,5-triazine. It is an especially active herbicide, from the triazine group, with pre- and post-emergence activity by photosynthesis inhibition. It is soluble in water at 171 ppm and Sirons *et al.*[1] found significant quantities of it and its metabolic amide, in soils, 12 months after application.

Because of the possible toxicity of cyanazine for man, through contaminated plants and waters, it was decided to study the adsorption–desorption process of cyanazine on soil and its constituents. For this purpose it was necessary to develop an analytical method for the determination of cyanazine in the presence of soil constituents. This study was mainly done on peat since this is the soil constituent that releases the greatest quantity of interfering substances in the presence of aqueous solutions.

To avoid the partial degradation of cyanazine in gas–liquid chromatography (GLC)[2] an high-performance liquid chromatographic (HPLC) method was developed. A reversed phase was chosen, instead of a normal phase, as recommended[3], to eliminate organic solvents of low polarity which would make difficult the interpretation of the adsorption–desorption process of cyanazine on soil.

EXPERIMENTAL

Apparatus

An high-performance liquid chromatograph Hewlett-Packard 1090, equipped with a 4.5-μl spectrometer cell, HP-85 personal computing system, HP-7470A graphics plotter, HP-ThinkJet printer, HP-9121 discs unit, automatic variable-volume injector, diode-array detector and DPU multichannel integrator, was used. The column (Hewlett-Packard 799160D-552) was 100 mm × 2.1 mm I.D., stainless steel, packed with ODS-Hypersil (5 μm).

The Millex filters (Millipore, Bedford, MA, U.S.A.) used were type HV_4, 4 mm, pore size 0.45 μm.

Soil constituents

Kaolinite from Lage (Spain) and Peat from Padul (Spain) were used.

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Reagents

Methanol, HPLC quality, was obtained from Panreac (Madrid, Spain). Water was purified with a Milli-Q water purification system. The eluent was methanol–water (60:40). Cyanazine, as an analytical standard of known purity, was obtained from Shell Research (Sittingbourne, U.K.).

Calibration solutions. First a solution of a cyanazine standard in water was prepared at $10.1988 \cdot 10^{-2}$ g/l and two more solutions were prepared by dilution in water at 1/2.5 and 1/25.

Sample solutions. Approximately 1.0 g of the soil constituent was weighed (to the nearest 0.1 mg). A 20-ml volume of a cyanazine solution at a concentration within the range $0.4 \cdot 10^{-2}$–$10 \cdot 10^{-2}$ g/l was added and shaken mechanically for X min (the time necessary for the study on adsorption–desorption). The solution was then centrifuged at 12 062 *g* for 20 min and an aliquot of the supernatant was filtered through a Millex HV_4 filter into a small vial fitted with a cap.

Chromatography

The chromatographic conditions were as follows: flow-rate, 0.2 ml/min; column temperature, 40°C; wavelength readings at 219 ± 2 nm *vs.* 350 ± 25 nm, and simultaneously at 225 ± 2 nm *vs.* 350 ± 25 nm; range, automatic; injection volume, 2 µl; spectra from the peak, upslope, apex and downslope; stop time, 2.75 min.

Calibration graph

The calibration graph, see Fig. 1, was constructed with a computer software, by the quadratic method, from triplicate injections of the three calibration solutions. Taking into account the low solubility of cyanazine in water (171 ppm), a wider range of concentrations is not feasible.

Quantitation

Triplicate injections of each sample solution were made and the results directly obtained in $g/l \cdot 10^{-2}$.

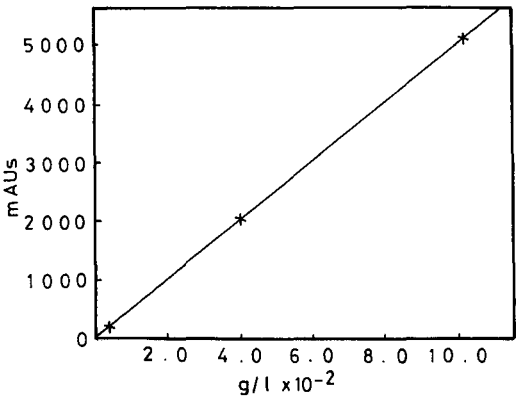

Fig. 1. Calibration graph for cyanazine.

RESULTS AND DISCUSSION

The linear calibration graph (Fig. 1) shows that Beer's law is followed at the tested concentrations.

Fig. 2 shows the chromatography of (a) a kaolinite–cyanazine sample and of (b) a peat–cyanazine sample. In both cases the separation of the cyanazine from impurities seems to be adequate. Fig. 3 shows the chromatograms in Fig. 2a and b plotted in different ways.

Fig. 4 shows the signal plus spectra plot of the chromatogram in Fig. 2b, and indicates the purity of the chromatographic peaks eluted. For this last purpose, the detector performs three scannings at three points (times) of every chromatographic peak: prior to at and after every maximum by reference to the base; (a), (b), and (c) show these spectrochromatograms separately and (d) all three overlaid. The purity of the cyanazine peak is indicated by the similarity of the three spectra which show a maximum at 219 nm and strong absorbance at 225 nm. These are the two wavelengths chosen for simultaneous integration. The ratio of the two signals is shown in Fig. 5 for the chromatogram in Fig. 2b between 0.5 and 2.4 min. The linear plot of the signal ratio, A/B, for the cyanazine peak, is a second demonstration of its purity.

The advantages of this kind of detectors have been pointed out by Lázaro *et al.*[4].

The standard addition method was used to test the accuracy of the method. Four aliquots of a peat–cyanazine supernatant, prepared according to *Sample solutions*, were spiked with cyanazine. Details are given in Table I. Recoveries ranged from 95.14 to 105.17%, with a relative standard deviation of 0.19–2.96.

The relative standard deviation for seven repeated injections of a cyanazine sample at $2.53 \cdot 10^{-2}$ g/l was $S_r = 0.55$.

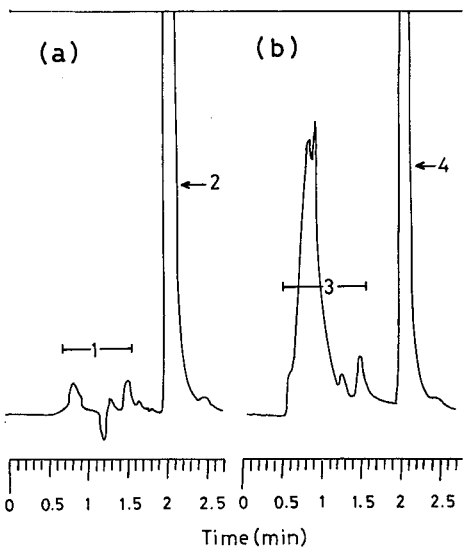

Fig. 2. Chromatography of (a) a kaolinite–cyanazine sample and (b) a peat–cyanazine sample. Peaks: 1 = kaolinite components; 2, 4 = cyanazine; 3 = peat components.

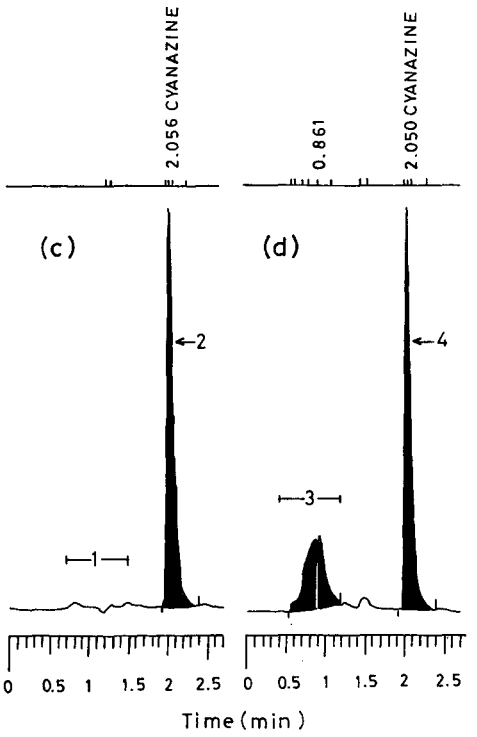

Fig. 3. Replots (c) and (d) of the chromatograms in Fig. 2a and b, respectively.

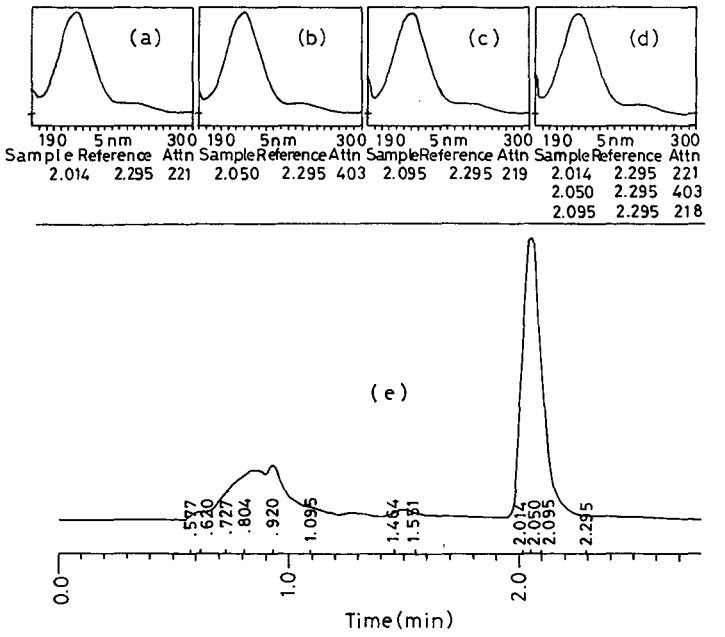

Fig. 4. Signal plus spectra plots of the chromatogram in Fig. 2b. (e) Chromatographic signal; (a), (b) and (c) spectrochromatograms of the cyanazine peak, prior to, at and after its absorption maximum; (d) the three previous spectrochromatograms overlaid.

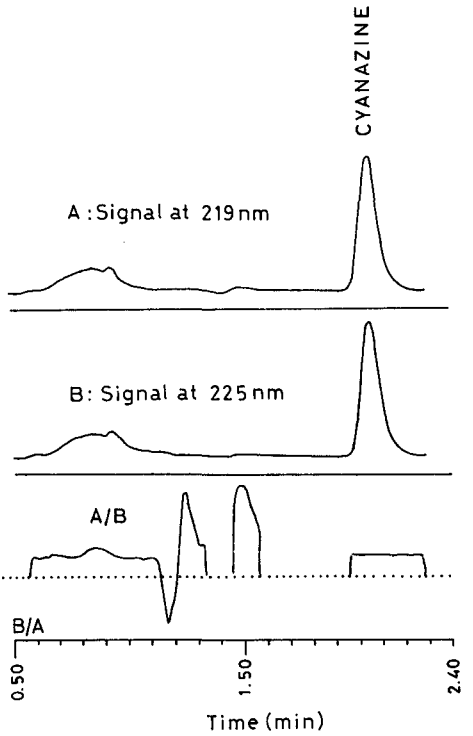

Fig. 5. Ratio of signals in the chromatogram in Fig. 2b between 0.5 and 2.4 min.

The detection limit was 0.253 ng of cyanazine, equivalent to 2 μl of a solution of this chemical at a concentration of $1.265 \cdot 10^{-4}$ g/l.

The method described is specific, accurate and precise and it presents a detection limit comparable to that of the Zweig method[5] using GLC with electron-capture and alkali flame ionization detection.

Other advantages of this method due to the use of a microbore column, a

TABLE I

RECOVERY TEST FOR CYANAZINE

C.L. = confidence limit (P = 0.05).

Cynazine added (ng/2 µl)	*Cyanazine found (ng/2 µl ± C.L.)*	*Recovery (%)*	*S_r**
37.48	35.66 ± 1.49	95.14	1.29
74.96	74.32 ± 6.70	99.15	2.96
112.44	118.25 ± 0.69	105.17	0.19
149.92	151.80 ± 2.32	101.25	0.50

* Relative standard deviation for three determinations.

diode-array detector and a multichannel integrator are: an enormous saving in operating costs, valuable information on the integration process and different tests of the purity or otherwise of every chromatographic peak.

In view of the limited solubility of cyanazine in water, 171 ppm, the concentration range studied, $(0.4–10.20) \cdot 10^{-2}$ g/l, is the most suitable for adsorption–desorption studies of cyanazine on soil and soil constituents.

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CHROM. 20 660

Note

High-performance liquid chromatographic identification of simple β-carboline alkaloids in specimens of Heliconiini butterflies

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The simple β-carboline alkaloids have been identified in twenty-six plant families[1]. They are frequently used in biological research because of their activity as monoamine oxidase inhibitors[2]. Additionally, their activity extends to include being both comutagenic[3] and growth inhibitors of protozoans[4] and lepidopterans[5]. Because of their frequently small concentration in plant material, they have not been quantified in many cases where they are reported[1].

The principal method used for identification of simple β-carboline alkaloids in plant material has been thin-layer chromatography (TLC) utilizing their fluorescence characteristics for identification under long-wave ultraviolet (UV) light[6–9]. The fluorescence excitation maxima are at 375 nm and 368 nm for norharman (β-carboline, 9H-pyrido-[3,4-*b*]indole) and harman (1-methyl-9H-pyrido-[3,4-*b*]indole), respectively[9]. High-performance liquid chromatography (HPLC) coupled with UV absorbance has been used to identify some β-carbolines[10,11]. Two β-carbolines, harman and norharman, have been separated by HPLC and fluorescence detection using 270 nm excitation wavelength[3].

In this investigation, nanogram quantities of seven β-carboline alkaloids were separated on a reversed-phase C_{18} column and identified by means of coupling the HPLC with a fluorescence detector utilizing 370 nm excitation and 425 nm emission wavelengths. Samples of adult butterflies, which fed as larvae on plant material containing β-carboline alkaloids, were used in this study. Dried whole insect samples weighing as little as 100 mg (two or three specimens) were adequate for the identification of these alkaloids, even when the alkaloid concentration was low.

EXPERIMENTAL

Material

Dried samples of seven species of adult Heliconiini butterflies (Family: Nymphalidae) collected in the wild were used.

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Extraction procedure

Amounts of 100 mg to 1 g of dried and ground material were first extracted with methanol. The extract was evaporated to dryness under low pressure. A 10% hydrochloric acid solution was added, the solution filtered, basefied to pH 9, then extracted by partition with dichloromethane. The water fraction was basified to pH 12 with 10% sodium hydroxide and reextracted. The acid–base shake out and partition procedure was repeated. The dichloromethane fractions were combined and evaporated to dryness, and the residue redissolved in a known amount of spectral grade methanol for injection into the high-performance liquid chromatograph.

Standards and chemicals

Standard chemicals of the seven β-carbolines were obtained from Sigma. They were run through the experimental system to check for purity. All solvents in the extraction process were analytical grade. HPLC grade methanol was used with HPLC. Water was twice-distilled.

Apparatus

The HPLC solvent delivery system consisted of a Beckman Model 332 with a 420 Controller driving two 110A pumps. A Perkin-Elmer LC-10 Fluorescence detector used 370 nm excitation, 425 nm emission wavelengths. A Hewlett-Packard 3390A integrator reported retention times and peak areas.

HPLC

The stationary phase was a Varian prepacked analytical 10 μm, 30 cm × 4 mm, reversed-phase C_{18} column. The mobile phase began with methanol–water (65:35), and 0.01% triethylamine and continued in a varying gradient to methanol–water (90:10) at 2 ml/min (Fig. 1). Area determination of quantities of standard compounds were used to determine sample concentrations. Standard sample areas were determined over a 1–100 μg/ml range. Retention times were used to identify compounds.

Mass spectrometry (MS) and TLC

An elution peak identified as norharman by the HPLC method described above was subjected to verification by direct probe high resolution MS (VG Analytic 7070E with an ionizing potential of 70 eV. Accurate mass measurements were carried out by the peak matching method. Silica gel TLC coincident with standard samples of alkaloids was also used to confirm the presence of β-carboline alkaloids. The TLC solvent system used was dihloromethane–methanol–triethylamine (75:25:2).

RESULTS AND DISCUSSION

Fig. 1 represents the HPLC analysts of seven β-caroline standards and a representative chromatogram of *Heliconius erato petiverana.* Baseline separations were obtained except between 6-methoxy harman and harman. Detection limits were as low as 10 ng (harmine) to 0.1 ng (6-methoxyharman) in a 20 μl sample. Total separation time was less than 38 min, with a column recycling time of 10 min. The three major alkaloids identified and quantified by this method in adult specimens of Hel-

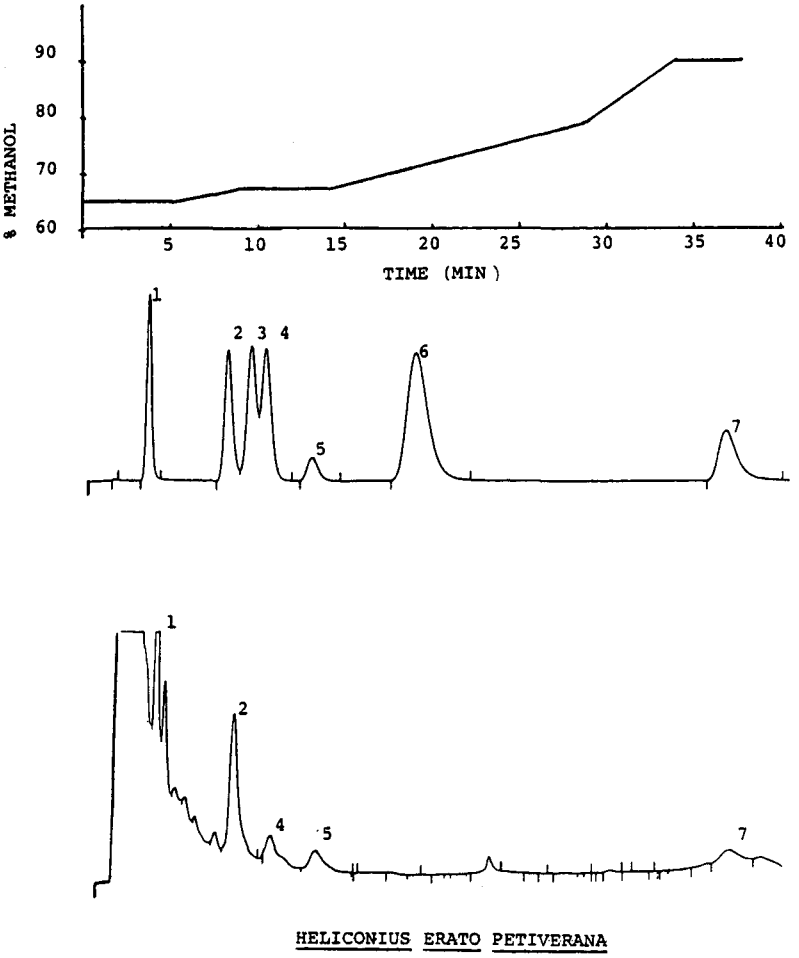

Fig. 1. HPLC analysis of β-carbolines and *Heliconius erato petiverana* extract showing liquid gradient. Peaks: 1 = harmol; 2 = norharman; 3 = 6-methoxy harman; 4 = harman; 5 = harmine; 6 = harmalol; 7 = harmaline.

TABLE I

ALKALOIDS IDENTIFIED IN FIELD COLLECTED SPECIMENS OF HELICONIINI

Species	*n*	*Picomol/individual (μg% dry weight)**			*Total*	*Collection site*
		1	*2*	*3*		
Acraea andromacha	10	13 (5)	106 (52.3)	+	119	Australia
Heliconius sara thamar	6	12 (0.5)	6 (3.3)	9 (6.0)	27	Belem
H. erato petiverana	20	70 (39.5)	14 (8.3)	96 (68.4)	180	UCI (C.R.)**
H. wallacei flavescens	3	+	9 (4.2)	+	9	Belem
H. ethilla eucoma	5	119 (52.3)	+ +	110 (61.2)	229	Belem
H. melpomene rosina	13	55 (22.5)	+	+	55	Pavo
H. Cydno galanthus	7	117 (32.5)	7 (2.2)	11 (4.0)	135	UCI (C.R.)

* 1 = Norharman; 2 = harman; 3 = harmine.

** UCI (C.R.): Colony specimens maintained at UCI, collected from Costa Rica.

iconiini are listed in Table I. Confirmation of peak identification was obtained by direct probe MS of a sample identified by this method as norharman. Peaks of mass 168.0692, 141.0574 and 114.0483 identify the molecular formulae $C_{11}H_8N_2$, $C_{10}H_7N$, and C_9H_8, and correspond to the molecular ion and previously reported major fragments of norharman[12]. TLC confirmed the presence of harman, 6-methoxy harman, harmine and harmaline in butterfly samples.

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Dr. J. T. Smiley graciously provided specimens of adult Heliconiini that he collected. C. Moore of the Chemistry Department (UCI) assisted with the mass spectral analysis.

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CHROM. 20 627

Note

Simple and rapid method for high-performance liquid chromatographic separation and quantification of soybean phospholipids

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The past ten years, numerous methods have been described for the separation of phospholipids. With few exceptions[1,2], silicic acid was used as the stationary phase, the mobile phase being either *n*-hexane–2-propanol–water[3–10], or acetonitrile–methanol–water[11–16]. However, detection of the phospholipids has been a major problem. Although lipids lack specific absorption peaks, UV detection has nevertheless mostly been used. The strong absorption in the 200–214 nm region is caused by the presence of unsaturated centres and functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium. As the extinction coefficient depends on the degree of unsaturation of the phospholipids, UV detection does not allow a quantitative estimation. Furthermore, the mobile phase must be UV-transparent whereas gradients cause a baseline drift. Refractive index detectors are relatively insensitive and incompatible with gradient elution. IR detection on the other hand permits quantification, but necessitates the use of deuterated solvents. Another quantitative detection method is moving-wire flame ionization, but this detector is no longer manufactured commercially.

Recently, the evaporative light-scattering mass detector has been introduced[17,18]. The non-volatile lipids, remaining after evaporation of the nebulised mobile phase by a heated gas stream, form small droplets. Light scattered by this particle cloud is detected by a photomultiplier at an angle of 120° to the incident light beam. Using calibration curves, the absolute amount of lipids can be estimated from the peak areas of the chromatogram. Besides its capability of direct quantification, this detector is also characterized by its insensitivity to solvent changes and gradients. Moreover, the mass detector furnishes a very stable baseline. As all described methods involving this detector for the quantification of phospholipids necessitate gradient elution, it was our aim to develop a simple method for quantification of soybean phospholipids. Moreover, the method was elaborated so that it could easily be automated and scaled up for preparative work.

EXPERIMENTAL

Materials

HPLC-grade *n*-hexane and 2-propanol were supplied by Alltech. The water

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was deionized, distilled and used freshly. Commercial soybean lecithin and phosphatidylcholine (PC) were obtained from Lucas Meyer. Triolein, phosphatidylinositol (PI) and phosphatidic acid (PA) were suplied by Serdary Research Laboratories, and phosphatidylethanolamine (PE), phosphatidylserine (PS) and cerebroside (CER) were delivered by Sigma.

All lipids were dissolved in either chloroform or *n*-hexane–2-propanol (57.8:39) and filtered through a 0.2-μm Dynagard filter.

Instrumental set-up

A Waters Model 590 isocratic HPLC pump equipped with a solvent switcher (Waters) was used. The Waters Intelligent Sample Processor (WISP) could be programmed to inject up to 48 samples. The column consisted of 3 μm Spherisorb, packed in a 125 × 4.9 mm I.D. stainless-steel column (Hichrom Ltd.). To avoid particulate contamination of the column, a 0.2-μm Uptight precolumn filter (Upchurch Scientific) was inserted before the column. The phospholipids were detected by a mass detector (ACS). The peak areas were calculated by a Chromatopac C-R1A integrator (Intersmat). As an alternative, a Quartel data logger (Gulton) could be connected to the mass detector, storing each 5 s the value of the generated voltage. Using the Pronto software these data can be handled by a personal computer and plotted. Finally, a Frac-100 fraction collector (Pharmacia) was used for preparative fractionation. The Model 590 solvent-delivery module is connected to an event in/-event out box, and can be programmed in 40 steps, enabling the pump to be controlled by an external device or vice versa. The configuration is shown schematically in Fig. 1; both the tubings and the electrical connections are drawn.

Chromatographic conditions

The initial part of the separation was carried out with a mobile phase of *n*-hexane–2-propanol–water (57.8:39:3.2, v/v/v). After 9 min another mobile phase, *n*-hexane–2-propanol–water (52.6:42:5.4, v/v/v), was selected by the solvent switcher. After 18 min the first mobile phase was run again, so that a new sample could be

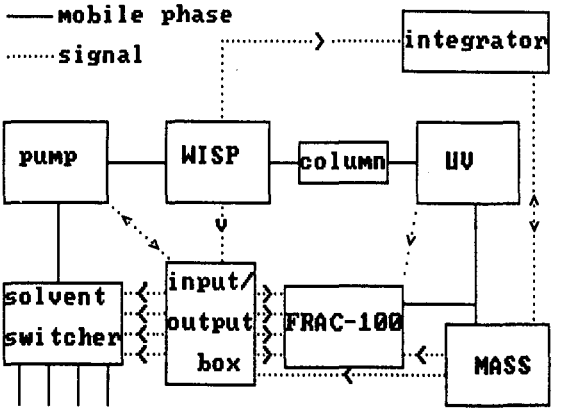

Fig. 1. Schematic representation of the HPLC equipment used. The tubings are shown as solid lines and the electrical connections as dotted lines.

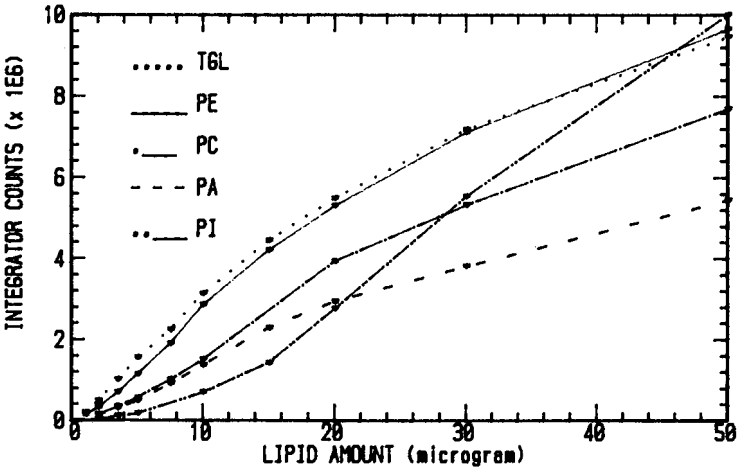

Fig. 2. Calibration curves of triolein (TGL), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA) and phosphatidylinositol (PI).

injected after *ca.* 25 min. From the pressure change it was concluded that newly selected solvent entered the column after *ca.* 3.5 min.

At the end of each day, the column was flushed with *ca* 50 ml of *n*-hexane or 2,2,4-trimethylpentane. Every month the column was rinsed with 100 ml of *n*-hexane–2-propanol–water (42:50:8, v/v/v) to remove polar contaminants.

Degassing of the solvents was achieved by flushing with helium.

RESULTS

To enable direct quantification of phospholipids, calibration curves were elaborated showing the relationship between the peak area as calculated by the integrator and the amount of phospholipids. Oppenheimer and Mourey[19] mentioned that the concentration response curves are sigmoidal when plotted on linear axes, whereas a double logarithmic plot reveals an exponential response. Depending on

TABLE I

COEFFICIENTS FOR THE CALIBRATION EQUATIONS

The equations $Y = aX^b$ and $Y = c(X-d)^e$ were fitted to the calibration curves of neutral lipids (NL), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA) and phosphatidylinositol (PI). Y represents the peak area, whereas X is the amount of phospholipids (in micrograms) and X_1 gives the limit for both equations. The (phospho)lipids are listed according to decreasing height-to-width proportion.

Lipid	*ln a*	*b*	*ln c*	*d*	*e*	X_1
NL	12.2	1.31	14.1	4.4	0.52	5
PE	11.7	1.35	14.1	5.6	0.53	10
PC	10.9	1.43	13.8	7.8	0.55	10
PA	10.7	1.47	13.5	6.7	0.53	10
PI	9.2	1.86	–	–	–	30

TABLE II

ANALYSIS OF CRUDE SOYBEAN LECITHIN AND COMMERCIAL POWDER LECITHIN

The last two columns represent the phospholipid composition of soybeans at 67 and 97 days after flowering, respectively, according to Privett and coworkers[20,21]. Values are in % wt.

	Crude lecithin	*Powder lecithin*	*Soybeans day 67*	*Soybeans day 97*
Neutral lipids	43.1	6.3	–	–
Cerebrosides	2.0	2.7	–	–
Glycolipids	1.0	3.0	–	–
Phosphatidylethanolamine	10.2	14.5	3.5	26.3
Phosphatidylglycerol	1.3	2.6	6.7	3.3
Phosphatidylinositol	10.6	17.1	12.5	14.1
Phosphatidic acid + phosphatidylserine	10.1	14.6	39.2	5.0
Phosphatidylcholine	18.6	33.9	10.2	45.0
Lysophosphatidylcholine	0.7	1.5	–	–
Others	2.4	3.8	27.9	6.3

the concentration range, the flow-rate of the nebulizer gas, and the composition and flow-rate of the mobile phase, the exponent ranged from 0.6 to 2. The calibration curves for neutral lipids (NL), PE, PI, PA and PC are given in Fig. 2. Plotting the response versus the amount of lipids on a double logarithmic scale, the linear region at low lipid concentrations revealed an exponential relation. For larger amounts, the equation $Y = c(X-d)^e$ yielded the best fit. The coefficients of these equations are summarized in Table I.

These results were used to analyse soybean phospholipids: for NL, PE, PI, PA and PC the corresponding equations were applied, whereas for the other components

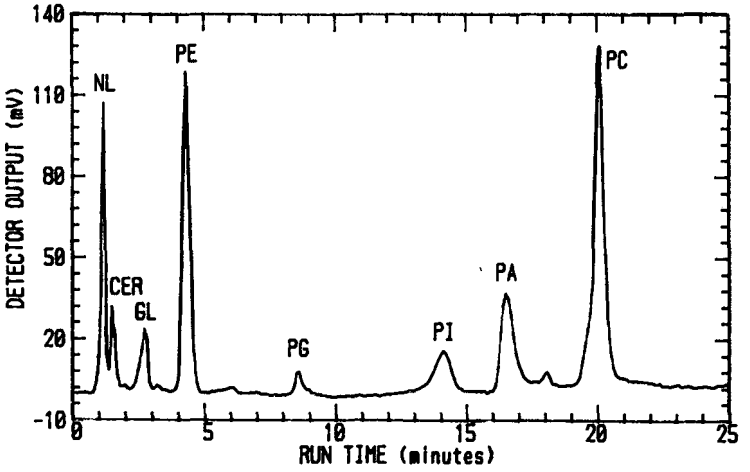

Fig. 3. HPLC separation of commercial powder lecithin; 115 μg were injected, dissolved in 5 μl of chloroform. The experimental conditions are described in the text.

the equation was used of the lipid to which the unknown substance showed most similarity in retention time and peak shape. The compositions of both a crude soybean lecithin and a commercial powder lecithin are shown in Table II, and the separation of the phospholipids of the commercial powder lecithin is represented in Fig. 3. The last two columns of Table II represent the phospholipid composition of soybeans at different stages of maturation, according to Privett and coworkers[20,21]. It can be concluded that our results agree very well with these previously reported values, supposing that the analysed lecithin originated from soybeans harvested about 90 days after flowering.

By repeated injections of the same sample, the standard deviation (S.D.) of this quantification method was shown to be *ca.* 3%. On the other hand, the S.D. of the injection volume was *ca.* 2% for all volumes exceeding 3 μl. Thus, the precision is mainly determined by the automatic injector, whereas the separation process has only minor influence. The detection limit was estimated as the lipid amount that resulted in a peak area of three times the largest background peak. Except for PI, the detection limit of all (phospho)lipids was less than 0.9 μg.

DISCUSSION

Optimization of mobile phase

The method proposed is based upon the procedure described by Nasner and Kraus[6], who used *n*-hexane–2-propanol–water (8:8:1) to separate soybean phospholipids. This solvent mixture results in a poor resolution between PE and neutral lipids, and PA and PI co-elute. The resolution of the latter compounds could be improved by decreasing the water content. However, this resulted in the permanent adsorption of PC on the stationary phase. Using a solvent switcher, two different mobile phases could be used: the first, which had a lower water content, was able to separate neutral lipids, glycolipids (GL) and PE, whereas the second contained more water in order to elute the acid phospholipids. The composition of the latter solvent was mainly of interest for the retention of PC; as already mentioned by Yandrasitz *et al.*[5], increasing both the water and the hexane content resulted in a shorter retention time for PC. The resolution between PA and PI couldn't be improved by altering the composition of the second solvent, but depended to a large extent on the first solvent; if less than 3% water was present, both PI and PA co-eluted with the front of the second solvent. A water content between 3 and 3.5%, however, resulted in a slow migration of PI, with PA remaining fixed to the column. At still higher water contents, PE was no longer separated from the neutral and glycolipids, whereas PI appeared as a broad peak. Besides their composition, the moment of changing the solvents was also very important: the resolution was improved by delaying the switch. Phosphatidylserine always eluted together with phosphatidic acid.

As stated by Tsimidou and Macrae[22], both the sample solvent and the injection volume influence the resolution to a large degree, especially when using chloroform or hexane. Injection volumes smaller than 5 μl did not affect the separation, but larger volumes mainly influenced the PE peak. The retention time was reduced from 3.76 min at 5 μl to 3.47 min at 25 μl and to 3.26 min at 200 μl, and the peak also became increasingly broad. On the other hand, the retention times of the neutral lipids and the glycolipids increased, resulting in a poor resolution of NL, GL and

PE when the injection volume was more than 10 μl. The remaining phospholipids were not influenced. This volume effect was eliminated by dissolving the samples in the first mobile phase used, omitting the water (*i.e.* *n*-hexane–2-propanol 57.8:39). A similar effect resulted if the column was not equilibrated with at least 10 column volumes after storage in *n*-hexane.

Optimization of detector output

The most decisive factors to optimise the detector output were the flow-rate of compressed air and the evaporator temperature. The latter was adjusted to maximize the signal-to-noise ratio. If the temperature was too low, the mobile phase was not fully evaporated, resulting in a baseline drift when the solvents were changed. On the other hand, at higher temperatures some low boiling components, such as some free fatty acids, began to evaporate. An evaporator setting of 70 was selected.

An inverse relationship between the air flow-rate and the peak area was observed. Therefore, the internal air pressure was fixed at 1 bar, whereas the external air pressure was set to 1.6 bar. It was noted that when the internal pressure was increased to 1.5 bar, the peak area decreased by *ca.* 33%, and at double the internal pressure (2 bar) the peak area was reduced by 61%.

From Table I, it may be concluded that an exponential response was obtained for all phospholipids if less than 10 μg was injected, whereas this limit for the neutral lipids amounts to *ca.* 5 μg. The coefficients in the equation $Y = aX^b$ reveal a distinct relationship between the peak area and the peak shape; it follows from the values of ln *a* that the peak area increased as the peaks became sharper, whereas the values of *b* deviated to a larger extent from the theoretical value of 2 (according to the Rayleigh theory) as the peak sharpness increased. For lipid amounts exceeding these limits, a very similar value of the exponent was found for the different (phospho)lipids when the equation $Y = c(X-d)^e$ was fitted to the data using the non-linear Marquardt regression procedure, indicating that the mass detector response is independent of the structure of the compound. An important feature of fitting equations to the experimental data is the greater reliability of inter- or extrapolations. Moreover, the analyses become faster and the results can be printed out in a standard report if these equations are inserted in a computer program.

Automation

One of the major advantages of our procedure is the high degree of automation. Using the automatic injector, up to 48 samples can be injected consecutively. Moreover, upon each injection a pulse is generated to restart the solvent program, and at the same time the integrator is initialized. The solvent program controls not only the flow-rate, but also the solvent switcher and the fraction collector. Safety settings are provided to avoid solvent accumulation in the mass detector when the compressed air flow is interrupted occasionally.

CONCLUSION

A method is described for the direct quantification of the most important soybean phospholipids. A main advantage is that the analyses can be performed with an isocratic HPLC pump using a solvent switcher. The method can be easily scaled

up for preparative fractionation. The mobile phase is also compatible with UV detection.

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Note

Rapid high-performance liquid chromatography method for determination of ethanol and fusel oil in the alcoholic beverage industry

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Ethanol, and its homologues to pentanol have previously been analysed by high-performance liquid chromatography (HPLC)[1–4]. For ethanol, the methods[1,2] have utilised ion-exchange columns at elevated temperatures (*e.g.* 60–80°C). Such columns are expensive, fragile and lead to long elution times (*e.g.* 10–20 min). For the major components of fusel oil [ethanol, 1-propanol, isobutyl alcohol (2-methyl-1-propanol) and amyl alcohols (2-methyl- and 3-methyl-1-butanol)], similar columns have been used[3]. Similar ion-exchange columns have also been used for the separation of various alcohols, carbonyl compounds, acids and "carbohydrates"[4]. This comprehensive paper includes some of the compounds of fusel oil, but not the isomeric methyl-1-butanols which would elute in excess of 30 min by the given HPLC conditions. An on-line chemical abstracts computer search (key-words: fusel, ethanol, HPLC, etc.) was performed over the years 1967–1987. Some 80 references were returned, none related in any way to the composition of fusel oil, the subject of this paper.

In this study, a reversed-phase C_8 column with a methanol–water eluent has been used for both determinations, only the recorder chart speed has been changed. In the case of ethanol determination in distillation column "bottoms", ethanol is eluted after all other components of the fermentation process, such as citric acid, glycerol, methanol, lactic acid, glucose, etc.

In the case of fusel oil analysis, the isomeric amyl alcohols noted above co-elute [as is common with many gas chromatographic (GC) methods]. For most purposes this is not a disadvantage, since GC examination of fusel oils[5] in this laboratory show the five constituents listed above to constitute about 85% of the fusel oil; additionally it contains about 10% water, leaving a small percentage consisting of a mixture of some 40–50 other compounds.

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EXPERIMENTAL

Apparatus

The liquid chromatograph used was a Bio-Rad Labs. system consisting of a Model 1770 differential refractometer, a Model 1330 pump and a column oven (set at 25°C). The detector volume was 12 μl. The column was a C_8, 5 μm particle size, 220 mm × 4.6 mm I.D. cartridge (Brownlee) fitted with a Rheodyne 20-μl syringe-loading sample loop injector. Data acquisition was by a Shimadzu CR3-A recording integrator (Tekscience, Oakville, Canada).

Reagents

The mobile phase was 500 ml HPLC-grade methanol (Caladon Labs., Georgetown, Canada) diluted to 1 l with HPLC-grade water (Caledon Labs.). HPLC-grade methanol was used as solvent. The standards were anhydrous ethanol, 1-propanol, isobutanol and isoamyl alcohol (mixed isomers: 2-methyl and 3-methyl-1-butanol) of known apparent density (BDH Chemicals, Toronto, Canada).

Sample preparation

Samples containing yeast and/or unfermented grains, for ethanol determination were filtered through paper (Whatman grade 4). The analyte solutions were prepared by mixing equal volumes of prepared sample and HPLC-grade methanol and filtering through a 0.45-μm disposable filter and injected.

Analyte solutions for fusel oil determinations were prepared by mixing a 200 μl sample with 4.8 ml of methanol–water (60:40) (Socorex dispensing pipettes 821-200 and 831-5, respectively), filtering through a 0.45-μm disposable filter and injecting.

Chromatographic conditions

Ethanol is satisfactorily resolved from other components of the fermented grain (glucose, organic acid, glycerol, most of which co-elute) using the described solvent at a flow-rate of 1 ml/min (Fig. 1).

The four alcohols in fusel oil are satisfactorily resolved using the above conditions (Fig. 2).

RESULTS AND DISCUSSION

Ethanol in distillation column "bottoms"

The distillation column "bottoms" from the steam distillation of fermented grain or molasses contains residual ethanol, the lower the level, the higher the distillation efficiency.

Fig. 1 shows the chromatogram of a solution of ethanol in laboratory-prepared still "bottoms". The separation is shown to be adequate for quantitation purposes.

Quantifiable levels of ethanol were determined by diluting ethanol to 0.05–0.40% (v/v) (10 nl/20 μl–80 nl/20 μl) and chromatographing under the given conditions. The relationship between area-counts and nanolitres ethanol injected was found to be linear (r = 0.999). The limiting "detectable" concentration of ethanol detectable was 0.01% (based on a pre-set minimum area rejection of 1000 units).

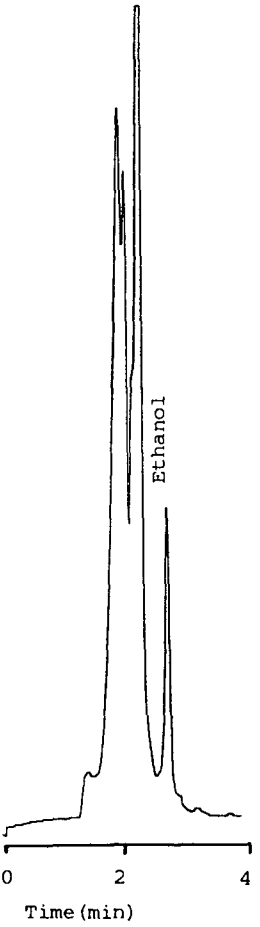

Fig. 1. Chromatogram showing separation of ethanol from other components in still-bottom samples.

TABLE I

RECOVERY OF ADDED ETHANOL (STILL-BOTTOM ANALYSIS)

Amount added (%, v/v)	*Amount found (%, v/v)*	*Recovery (%)*
0.077	0.075	97.4
0.179	0.178	99.4
0.278	0.271	97.5
0.376	0.360	95.7
0.463	0.468	101.1
	Mean	98.2

Recovery of added ethanol was found to be in the range 95.7–101.7% (Table I). Since levels of ethanol below 0.05% are difficult and expensive to obtain the conditions presented therefore provide for more than adequate sensitivity, together with rapid sample preparation and analysis time.

In the case of fusel oil analysis, Fig. 2 shows a chromatogram of the calibration mixture, and Fig. 3, that of a typical fusel oil.

The linearity of detection of the four components was determined for the ranges shown in Table II. For each case, the other three components were kept as constant as possible. All samples were weighed and converted to volume percentages via their appropriate densities. In all cases, the relationship between area counts and percentage (v/v) component was found to be linear (r = 0.999). The limiting detectable concentration of ethanol detectable was 0.2% (based on a preset minimum area rejection of 1000 units). Levels much below 0.5% are difficult and expensive to obtain. For all components, a comparison between the variances found for repeated injection of one sample mixture was compared with that for several sample mixtures. At the 0.05 probability level, there was no significant difference (F = 4.04, 1.13, 1.63 and

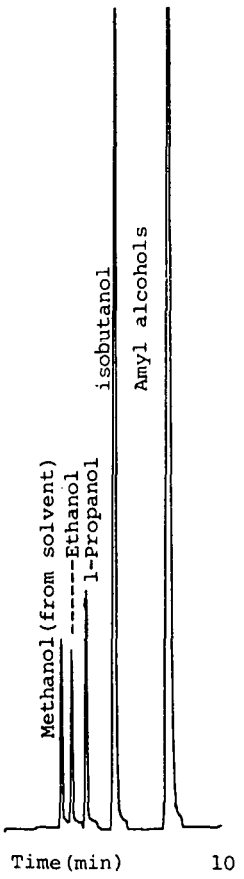

Fig. 2. Chromatogram showing fusel oil calibration mixture (used for fusel oil analysis).

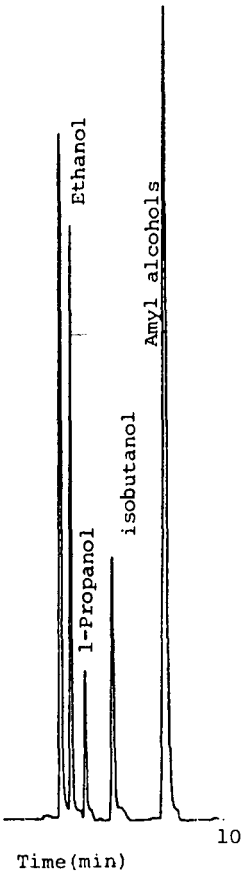

Fig. 3. Chromatogram showing typical fusel oil composition.

TABLE II

COMPONENT RANGES FOR LINEARITY TESTING (IN FUSEL OIL ANALYSIS)

Component	*Percentage range*
Ethanol	0, 2.5, 5, 7.5, 10
1-Propanol	0, 2.5, 5, 7.5, 10
Isobutanol	0, 10, 25, 50
Amyl alcohols	0, 40, 60, 80

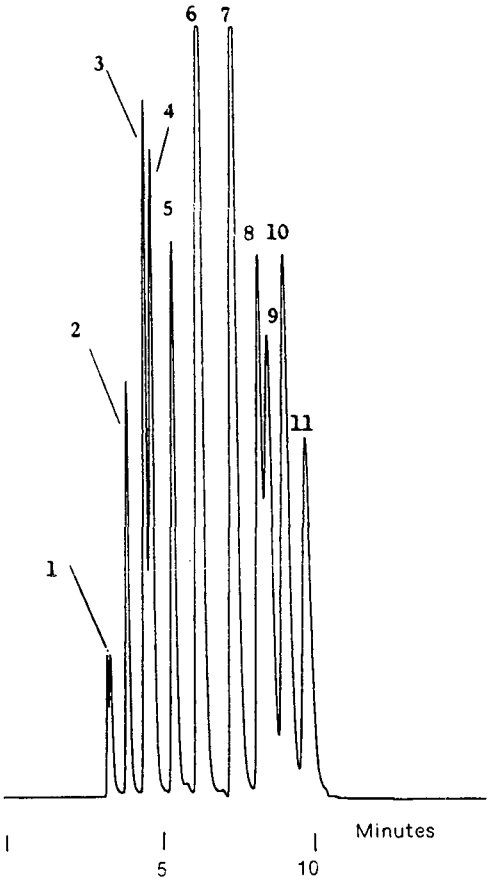

Fig. 4. Chromatogram showing elution of the major and some minor constituents of fused oil. Peaks: 1 = methanol; 2 = ethanol; 3 = 2-propanol; 4 = 1-propanol; 5 = 2-methyl-2-propanol; 6 = 1-butanol + isobutanol; 7 = 2-methyl-2-butanol + 2,2-dimethyl-2-propanol; 8 = 3-pentanol; 9 = 2-pentanol; 10 = 2-methyl + 3-methyl-1-butanol; 11 = 1-pentanol.

4.51 respectively; $n_1 = n_2 = 5$), indicating sufficient confidence in the repeatability of sample preparation.

Some of the more common constituents of fusel oil were chromatographed (Fig. 4). Their elution times, together with that of the solvent and the four major constituents are shown in Fig. 5. Ethanol is not resolved from acetyl methyl carbinol, and isobutanol is not resolved from 1-butanol and ethyl acetate. However, these constituents are found at much lower levels than the four major components and do not constitute significant interference.

The method has also been compared with a GC method developed by Canadian customs and excise designed to be used for the regulation of ethanol in fusel oils. These results are shown in Table III. Exact correspondence was not found between the methods. The difference may be due to the manner in which the GC calibration standards for the external method are prepared: further work to inves-

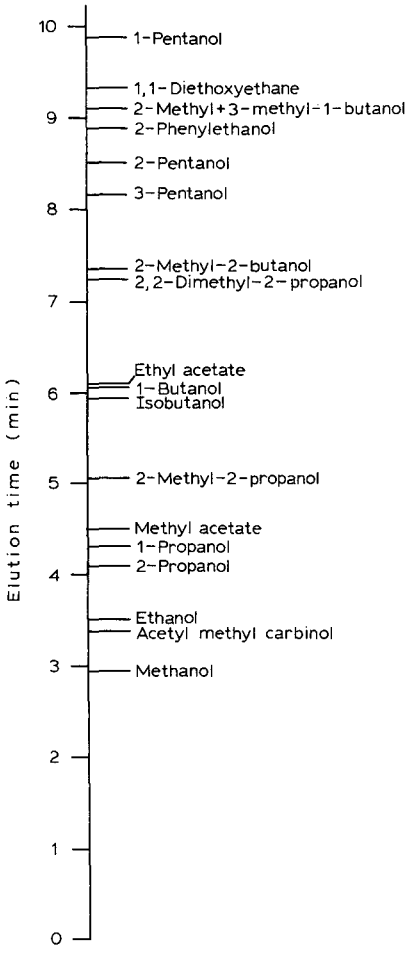

Fig. 5. Chart showing elution times of some fusel oil components.

TABLE III

COMPARISON OF RESULTS BETWEEN PROPOSED METHOD AND EXTERNAL STANDARD GC METHOD (REVENUE CANADA)

All results average of triplicate analyses.

Sample	*Ethanol (%)*		*1-Propanol (%)*		*Isobutanol (%)*		*Amyl alcohols (%)*	
	LC	*GC*	*LC*	*GC*	*LC*	*GC*	*LC*	*GC*
A	8.87	9.76	4.14	4.24	15.20	16.95	51.71	54.50
B	6.89	7.68	3.80	3.96	15.40	17.30	53.26	57.02
C	7.24	7.98	4.17	4.24	14.19	15.66	55.58	58.90
D	5.89	6.72	0.50	0.63	19.00	20.85	54.27	59.38

tigate the discrepancies is beyond the scope of this paper. However, the results by the proposed method will be sufficiently accurate for monitoring fusel oil by-product when many samples have to be analysed, and the simplicity of the method and equipment makes it amenable to use by unskilled operators.

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Erratum

J. Chromatogr., 444 (1988) 165–175
Page 167, second line, "200 mm × 200 mm" should read "200 mm × 20 mm".

corrected, vph

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journal of chromatography news section

NEW BOOKS

Chromatography theory and basic principles (*Chromatographic Science Series*, Vol. 38), edited by J.A. Jönsson, Marcel Dekker, Basel, New York, 1988, 408 pp., price US$ 79.75 (U.S.A. and Canada), US$ 95.50 (rest of world), ISBN 0-8247-7673-9.

On-line process analyzers, by G.D. Nichols, Wiley, Chichester, New York, 1988, *ca.* 416 pp., price *ca.* US$ 60.00, ISBN 0471-86608-3.

Quantitative bioassay, by D. Hawcroft, T. Hector and F. Rowell, Wiley, Chichester, New York, 1988, *ca.* 328 pp., price *ca.* US$ 67.50 (cloth), US$ 67.50 (paperback), ISBN 0471-91400-2 (cloth), 0471-91401-0 (paperback).

Analytical chemistry for technicians, by J. Kenkel, Wiley, Chichester, New York, 1988, *ca.* 482 pp., price *ca.* US$ 44.85, ISBN 087371-128-9.

Particle size analysis 1988, Proceedings of the 6th Particle Size Analysis Conference, University of Surrey, April 1988, edited by P.J. Lloyd, Wiley, Chichester, New York, 1988, *ca.* 360 pp., price *ca.* US$ 95.00, ISBN 0471-91997-7.

Vibrations at surfaces 1987, Proceedings of the 5th International Conference, edited by A.M. Bradshaw and H. Conrad, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, 856 pp., price Dfl. 550.00, US$ 289.50, ISBN 0-444-42944-1.

ANNOUNCEMENTS OF MEETINGS

8th INTERNATIONAL SYMPOSIUM ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS, PEPTIDES AND POLYNUCLEOTIDES, COPENHAGEN, DENMARK, OCTOBER 31–NOVEMBER 2, 1988

The 8th International Symposium on HPLC of Proteins, Peptides, and Polynucleotides will be held at Hotel Scandinavian in the heart of Copenhagen, Denmark, October 31–November 2, 1988.

The scientific programme will include both oral and poster presentations organized into different sessions. Recognized authorities will review current trends and future perspectives in various topics, including: column technology and support materials; protein conformation and chromatographic behaviour; polypeptide structural studies; protein purity and QC of recombinant proteins; polynucleotides; polysaccharides; membrane proteins; affinity chromatography; analytical applications; sample preparation; preparative chromatography of biopolymers; high resolution electrophoresis; integrated purification systems; biospecific detectors; and process monitoring.

Registration fee will be DKK 2600 which covers all scientific and social events. Students will be eligible for a reduced rate of DKK 1300 (a copy of the proceedings is not included). To qualify for student rate, verification of student status by department chairman is a must.

Hotel Scandinavia, with its excellent congress facilities, has a large number of rooms available for the symposium delegates. Budget accommodation can also be found in the down-town area less than 20 min walk from Hotel Scandinavia.

Registration information and full details of the symposium can be obtained from the Secretariat: 8th ISPPP, c/o DIS Congress Service, Linde Allé 48, DK-2720 Vanløse/Copenhagen, Denmark. Tel.: (45) 1-71-22 44, Telex: 15 476 dis dk, Fax: (45) 1-71-60 88.

INTERNATIONAL SYMPOSIUM ON INSTRUMENTAL THIN-LAYER CHROMATOGRAPHY/PLANAR CHROMATOGRAPHY, BRIGHTON, U.K., FEBRUARY 21–24, 1989

The Chromatographic Society, in conjunction with the organizers of the Bad Dürkheim/Interlaken/Würzburg/Selvino series of symposia, are holding a major international meeting on instrumental thin-layer chromatography/planar chromatography at the Grand Hotel Brighton, U.K. on February 21–24, 1989. The chairmen are Professor U.A.Th. Brinkman and Dr. I.D. Wilson and the organizing/scientific committee consists of Professor S. Ebel, Dr. D.E. Jänchen, Dr. D.E.R. Jones, Dr. R.E. Kaiser, Mr. N. McTaggart, Professor C.F. Poole, Dr. H. Read and Dr. H. Traitler.

The scientific programme will comprise of invited plenary lectures by leading workers in the field, supported by both oral papers and posters. The latest developments in apparatus and techniques will also be featured in the accompanying exhibition. The symposium package will include a reception, three nights accommodation/breakfast and lunches, light refreshments, symposium banquet and entry to the exhibition.

Further details may be obtained from the Executive Secretary, The Chromatographic Society, Trent Polytechnic, Burton Street, Nottingham NG1 4BU, U.K.

6th SYMPOSIUM ON ION CHROMATOGRAPHY, SILS-MARIA, SWITZERLAND, APRIL 9–12, 1989

The 6th Symposium on Ion Chromatography will be held in the Hotel Waldhaus in Sils-Maria (in the vicinity of St. Moritz), Switzerland, April 9–12, 1989.

The chromatography of ions has become a topic of wide-spread interest and the whole renaissance of this field has started with the introduction of the suppressor column concept several years ago by Dow Scientists. Since then several European workshops and symposia have been organized in ion-chromatography by the International Association of Environmental Analytical Chemsitry to discuss advances in the field.

The purpose of this three-day symposium (followed by a two-day short course, April 13–14, 1989 probably in Lausanne, Switzerland) is to reassess the current status of this rapidly developing branch of column chromatography. Major topics of interest besides the dual column concept and detection by conductometry will be single column systems, new detection principles, trace enrichment and clean-up procedures for trace ions in complex matrices, new separation mechanisms (such as ion pairing, complexation, ligand exchange) new phases and also new application areas for organic and inorganic ion analysis in biological, environmental and industrial samples. Automation possibilities are also of interest.

The proceedings of the symposium will be published in the *Journal of Chromatography*. The symposium language will be English, no simultaneous translation will be given. The symposium coordinators are (tentatively): R.W. Frei, The Netherlands (Chairman); M. Lederer, Switzerland; P. Haddad, Australia; W. Haerdi, Switzerland; J. Hertz, Switzerland; J.F.K. Huber, Austria; D. Klockow, F.R.G.; R. Rosset, France; J. Slanina, The Netherlands.

For further information, contact: Workshop Office IAEAC, Ms. M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland.

1st INTERNATIONAL SYMPOSIUM ON HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS, BOSTON, MA, U.S.A., APRIL 10–12, 1989

The 1st International Symposium on High Performance Capillary Electrophoresis will be held April 10–12, 1989 at the Boston Park Plaza Hotel. The three-day meeting will include invited lectures, contributed presentations and posters, and ample time will be provided for discussion. There will also be an instrumentation exhibition. Topics will include: zone electrophoresis; isoelectric focusing; isotachophoresis; gel columns; instrumental design; CE–MS; analytical and micropreparative applications for peptides, proteins, oligonucleotides, etc.

Authors are invited to submit an abstract, by November 1, 1988, describing original research in the area of high performance capillary electrophoresis. Papers in related fields such as two-dimensional gel electrophoresis, immuno techniques, capillary liquid chromatography and other bioanalytical methods will also be considered. Papers presented at the symposium will be reviewed for publication in a special issue of the *Journal of Chromatography*. Complete manuscripts will be due at the time of the symposium.

For further information or submission of Abstracts, contact: Shirley E. Schlessinger, Symposium Manager, HPCE '89, 400 East Randolph Drive, Suite 1015, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011.

6th INTERNATIONAL SYMPOSIUM ON PREPARATIVE CHROMATOGRAPHY, WASHINGTON, DC, U.S.A., MAY 8–10, 1989

The 6th International Symposium on Preparative Chromatography (Prep-89) will be held at the Sheraton Hotel in Washington, DC, U.S.A., May 8–10, 1989.

The scientific programme will include invited and contributed oral presentations, poster presentations, and discussion sessions.

You are invited to submit an abstract for consideration for inclusion in the programme. Abstracts should be limited to one, single-spaced typed page, stating title, authors, text and references, and indicating oral or poster reference. Include a complete address and telephone number. Deadline for submission of abstracts is November 30, 1988.

Abstracts and all correspondence should be sent to: Mrs. Janet Cunningham, Prep-89 Symposium Manager, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772

3rd INTERNATIONAL SYMPOSIUM ON QUANTITATIVE LUMINESCENCE SPECTROMETRY IN BIOMEDICAL SCIENCES, GHENT, BELGIUM, MAY 23–26, 1989

The 3rd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences will be held at the Pharmaceutical Institute, Faculty of Pharmaceutical Sciences, of the State University of Ghent, May 23–26, 1989. Both format and atmosphere will be similar to the previous successful meetings.

Plenary, keynote and invited lectures and panel discussions will cover the most important fields of fundamental and applied luminescence spectrometry. The conference envisages a report on the current status techniques applied in drug quality control, clinical analysis, biochemical, toxicological and environmental analysis and in related areas. Plenary, keynote and invited lectures will be presented by outstanding specialists in these fields. Contributed papers (lectures or poster communications) will cover the following topics: drug and bioanalysis via fluorescence, laser fluorescence, delayed fluorescence, phosphorescence (LTP, RTP, micellar) and chemiluminescence; chemiluminescence immunoassays; bioluminescence assays; thermochemiluminescence techniques; luminescence detection techniques in chromatography; chemical derivatization methods; the development of fluorogenic reagents; the development of highly specific and sensitive luminescent systems for the determination of macromolecules in biological samples; the use of expert systems in the analysis of luminescence data; fiber-optic sensors in biomedical sciences; biomedical applications of luminescence in micellar and cyclodextrin media; the development of high-resolution luminescence methods; use of fluorescence and chemiluminescence labels and substrates; the fluorogenic estimation of enzyme activities; the use of three-dimensional fluorescence spectra; the application of computer-aided fluorescence spectros-

copy to the determination of drugs and metabolites; luminescence applications in clinical sciences, biochemistry, biotechnology, pharmacokinetics, toxicology, environmental monitoring, protein tagging, etc.

Those wishing to present a paper (oral or poster, to be specified) are requested to return an abstract of no more than 200 words before February 15, 1989.

As with the earlier Conferences, a special issue of *Analytica Chimica Acta* will be dedicated to the accepted and reviewed Symposium papers, which will be channelled through the usual refereeing system.

For full information, contact: Dr. W. Baeyens, "Luminescence III", Rijksuniversiteit Gent, Laboratoria voor Farmaceutische Chemie en Ontleding van Geneesmiddelen, Farmaceutisch Instituut (F.F.W.), Harelbekestraat 72, B-9000 Gent, Belgium. Tel.: (091) 21 89 51 ext. 254 or 248, telex rugent 12754.

1989 WORKSHOP ON SUPERCRITICAL FLUID CHROMATOGRAPHY, SNOWBIRD, UT, U.S.A., JUNE 13–15, 1989

The 1989 Workshop on Supercritical Fluid Chromatography, sponsored by the State of Utah and Brigham Young University, will be held at Cliff Lodge, Snowbird, UT, U.S.A., June 13–15, 1989.

The purpose of the workshop is to provide a forum for maximum exchange of information on techniques and applications of supercritical fluid chromatography, including analytical supercritical fluid extraction. Scientists, both experienced chromatographers and potential users of SFC, are encouraged to attend.

The workshop will consist of oral presentations, poster sessions, and planned informal discussion sessions. Emphasis will be placed on open discussion of the details of the practice of SFC. The center of focus will be on the various applications to which the technique of SFC can be applied. Attendees are invited to present results of basic research or practical applications of SFC. Both packed column and capillary column topics are appropriate. Informal discussion sessions on important issues are planned. Participation by everyone will be encouraged. Prospective attendees are encouraged to suggest topics for discussion to be included in the programme.

In contrast to abstract books and conference proceedings that are often available at such meetings, no such book will be prepared. Instead, scientists, including but not limited to the attendees, will be asked to send copies of representative chromatograms (including associated operational information) of various applications to be organized and bound for distribution at the meeting. This bound volume will serve as an up-to-date compilation (and companion book to the first workshop volume) of SFC capabilities, and will serve as reference material for planned and informal discussions.

The registration fee (including the workshop application volume and social programme) is: regular attendees, US$ 400.00; graduate students, US$ 150.00.

For further information, contact: Dr. Milton L. Lee, Department of Chemistry, Brigham Young University, Provo, UT 84602, U.S.A. Tel.: (801) 378-2135.

1st INTERNATIONAL SYMPOSIUM AND WORKSHOP ON FIELD-FLOW FRACTIONATION, SALT LAKE CITY, UT, U.S.A., JUNE 14–16, 1989

The 1st International Field-Flow Fractionation (FFF) Symposium will be held June 15–16, 1989; the FFF Workshop will precede the Symposium on June 14, 1989.

The Symposium will have presentations covering all aspects of FFF, ranging from recent theoretical developments to applications in industry, biochemistry, and environmental studies. Invited lectures along with contributed papers and posters will be scheduled. The FFF workshop is intended for those desiring a working knowledge of field-flow fractionation. Topics will include basic theory principles of operation and optimization, and instrumental systems and characteristics. Enrollment will be limited to maintain an informal learning environment.

Authors are invited to submit titles of proposed oral and poster presentations. Posters are particularly encouraged. Final titles will be selected by a scientific committee. The due date for abstracts will be announced later.

For further information, contact: Julie Westwood, Department of Chemistry, University of Utah, Salt Lake City, UT 84112, U.S.A. Tel.: (801) 581-5419

PUBLICATION SCHEDULE FOR 1988

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
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Bibliography Section		460/1		460/2		460/3		460/4		460/5		460/6
Cumulative Indexes, Vols. 401–450												451
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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

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Some Recently Published Papers

Contrasting behaviour of dissolved and particulate nickel and zinc in a polluted estuary, *J.A. Campbell et al. (UK)*

Comparison of salt marsh humic acid with humic-like substances from the indigenous plant species *Spartina alterniflora* (Loisel), *Z. Filip (FRG) et al.*

Cadmium levels in Ontario moose and deer in relation to soil sensitivity to acid precipitation, *V. Glooschenko et al. (Canada)*

Analysis of the real-time measurement of the pH of rainfall at Padova, Italy: seasonal variation and meteorological aspects, *D. Camuffo et al. (Italy)*

Application of linear regressions to the comparison of analytical procedures for the determination of SO_2 in ambient air, *P. Vanderstraeten et al. (Belgium)*

Contamination of urban, industrial and continental waters by chlorinated hydrocarbon pesticides along the Mediterranean coast of Morocco, *M. Kessabi et al. (Morocco)*

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