ISSN 0021-9673

VOL. 509 NO. 2 JUNE 22, 1990

THIS ISSUE COMPLETES VOL. 509

JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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JOURNAL OF CHROMATOGRAPHY

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0021-9673/90/$03.50

Printed in The Netherlands

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Elsevier Science Publishers B.V., Amsterdam

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2,6-Di-O-pentyl-3-O-trifluoroacetyl cyclodextrin liquid stationary phases for capillary gas chromatographic separation of enantiomers

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(First received January 24th, 1990; revised manuscript received February 23rd, 1990)

ABSTRACT

A series of liquid cyclodextrin derivatives, 2,6-di-O-pentyl-3-O-trifluoroacetyl α-, β- and γ-cyclodextrins (DP-TFA α-, β- and γ-CD), have been used as highly selective chiral stationary phases for capillary gas chromatography. More than 150 pairs of enantiomers were resolved; 120 on DP-TFA-γ-CD, which is the first reported γ-cyclodextrin stationary phase that is more widely useful than the β-cyclodextrin analogue. The enantiomers resolved include chiral alcohols, diols, polyols, amines, amino alcohols, halohydrocarbons, lactones, α-halocarboxylic acid esters, carbohydrates, epoxides, nicotine compounds, pyrans, furans and so on. Identical α values were observed for diol, amine and γ-halocarboxylic acid ester homologues, respectively. The relationship between the unusual selectivity behavior and separation mechanism is discussed.

INTRODUCTION

There is recently a rapidly increasing interest in enantiomeric separation. The significance of chirality is based on the fact that most natural organic products are asymmetric and most life processes proceed stereospecifically. It is not surprising that the stereoisomeric composition of chemical products closely related to human health, particularly pharmaceuticals, has brought increased attention of both the scientific and regulatory communities. In the pharmaceutical market today, a majority of drugs contain one or more chiral centers, and many of these are administered as the racemic mixtures[1]. Scientific studies show that the biological activity of chiral drugs is often related to stereochemistry. For many chiral drugs, further investigations are necessary to understand the activity, toxicity and metabolic pathways of the two enantiomers. Therefore, sophisticated enantiomeric separation techniques are needed not only for separating mixtures of the chiral drugs and their optically active metabolites, but also for the quality control of enantiomerically pure drugs and the development of asymmetric syntheses. The same techniques also are needed in the pesticide

0021-9673/90/$03.50

and herbicide industries because the same principles of stereochemistry hold true and the same problems exist for these chemicals. Chiral separation techniques have been used for the determination of unnatural amino acids in bacterial cell walls and in peptide antibiotics[2,3], the monitoring of amino acid purity during peptide synthesis[4,5], and the determination of enantiomer enrichment (EE). The applications of enantiomeric separations to configurational analysis[6] and archeometric investigations[7], etc. also have been reported.

Two strategies can be considered for the resolution of enantiomeric compounds by gas chromatography (GC). One method involves the conversion of the enantiomers into diastereomeric derivatives with a proper auxiliary, enantiomerically pure derivatization reagent prior to GC analysis. Disadvantages associated with the indirect approach include the requirement of an active functional group for the formation of diastereomeric derivatives, the difficulties in obtaining optically pure reagents, chiral discrimination in the reaction rates of enantiomeric compounds with the chiral derivatizing agent and the requirement of chemical and stereochemical stabilities of the derivatives under GC conditions[8,9].

Another method for the resolution of enantiomers by GC takes advantage of chiral stationary phases (CSPs) which can rapidly and reversibly form diastereomeric association complexes with chiral analytes. Successful chiral stationary phases for wall-coated capillary GC should have some characteristic properties. They need to be highly viscous even at elevated temperatures and have the proper surface tension to wet the capillary wall completely. They should be able to form rapid and reversible diastereomeric association complexes with the chiral analytes via various interactions (*e.g.*, hydrogen bonding, dispersion, dipole–dipole and steric interactions, etc.) to give reasonable chiral selectivity. Other desirable properties include high temperature stability, no racemization at elevated temperatures and low levels of bleeding.

Since Gil-Av *et al.*[10] first reported the chiral separation of N-trifluoroacetyl (TFA)-α-amino acid esters on glass capillary columns coated with N-TFA-L-isoleucine dodecyl ester or N-TFA-L-phenylalanine cyclohexyl ester, a large number of CSPs, based on peptides, amides, diamides and carbonyl bis(amino acid esters)[10] have been developed. However, most of these CSPs had lower operating temperature ranges and exhibited limited chiral selectivities so that they have not found wide applications.

Cyclodextrins (CDs) have been used successfully as chiral stationary phases in high-performance liquid chromatography (HPLC)[11–13]. They also have been used as GC stationary phases by a number of research groups but without much success because native CDs are highly crystalline solids which do not coat well and result in columns with low efficiency[14–18]. Recently, there have been reports on hydrophobic liquid CD derivatives suitable for capillary GC[19–24]. König and co-workers[20–22] have developed two types of derivatized cyclodextrin stationary phases, *i.e.* perpentylated and 2,6-di-O-pentyl-3-O-acetyl CDs. These stationary phases are relatively hydrophobic. Compounds separated on these columns include alcohols, diols, polyols, carbohydrates, halohydrocarbons, amino alcohols, hydroxy acid esters, spiroacetals, etc. Usually 40-m glass capillary columns were utilized. We have recently reported two additional types of CD stationary phases, 2,6-di-O-pentyl CDs, which are hydrophobic[23] and permethyl-O-(S)-2-hydroxypropyl CDs[24] which are hydrophilic. Racemic amines, amino alcohols, carbohydrates, lactones, furans and pyrans, epoxides,

glycidyl analogues and other compounds were separated on these columns. Fused-silica capillary columns of 10 m length were usually used. All these CD-based CSPs previously reported have rigid ring structure and a large number of chiral centers. Many racemic compounds resolved were not aromatic and cannot be separated on any known liquid chromatographic CSP.

This paper describes a new class of chiral GC stationary phases based on 2,6-di-O-pentyl-3-O-trifluoroacetyl liquid derivatives of α-, β- and γ-CDs. These stationary phases, particularly the γ-CD analogue, have extraordinary versatility and chiral selectivity. More than 150 pairs of enantiomers were resolved on wall-coated 10-m fused-silica capillary columns. The enantiomers resolved include chiral alcohols, diols, polyols, halohydrocarbons, lactones, etc. Unique retention behavior was observed for the diol, amine and α-halocarboxylic acid ester homologous series. Possible separation mechanisms and column stability are evaluated as well.

EXPERIMENTAL

Stationary phases

2,6-Di-O-pentyl cyclodextrins (DP-CDs). The synthesis of 2,6-di-O-alkyl cyclodextrins has been reported previously[25]. A 3.0-g amount of the dried CD and excess 1-bromopentane were added to 30 ml dimethyl sulfoxide (DMSO). The reaction was carried out at 60°C for 6 h. Water was then added to the reaction mixture and a waxy precipitate was obtained. The raw product was dissolved in chloroform and the solution was washed with water. Chloroform was removed under vacuum and the product was used for the next reaction without further purification. Previous results have shown that the dialkyl product formed for this reaction is actually a mixture of homologues and isomers and that it is very difficult to obtain the pure 2,6-disubstituted compound[23].

2,6-O-Dipentyl-3-O-trifluoroacetyl cyclodextrins (DP-TFA-CDs). The above material and an excess of trifluoroacetic anhydride were added to 30 ml tetrahydrofuran (THF). The mixture was boiled for 2 h then poured over ice to precipitate the product. The precipitate was washed with cold water and dissolved in chloroform. The chloroform solution was extracted three times with 5% aqueous $NaHCO_3$ and three times with water. The chloroform layer was collected and dried with anhydrous Na_2SO_4. Chloroform was allowed to evaporate in a vacuum desiccator and the final viscous liquid was dried in a vacuum over night.

Columns

Fused-silica capillary tubing (0.25 mm I.D.) was obtained from Supelco (Bellefonte, PA, U.S.A.). Untreated 10-m capillary columns were coated with DP-TFA-α-, β- and γ-CD, via the static method as previously reported[26]. The capillary was placed in a water bath at 36°C. A 0.2% (w/v) diethyl ether solution of the CSP filled the capillary. One end of the capillary was sealed and the other connected to a vacuum line. It took about 4 h to coat a 10-m column. The column efficiency was tested at 100°C by using *n*-hydrocarbons (C_{11} and C_{12}) as test solutes. Only columns that produced $\geqslant$ 3600 plates per column meter were used for this research.

Test solutions

All chemicals were obtained from Aldrich (Milwaukee, WI, U.S.A.), Sigma (St. Louis, MO, U.S.A.) or Fluka (Ronkonkoma, NY, U.S.A.). All alcohols, polyols, amines and other compounds containing hydroxyl and/or amine functionalities were derivatized using trifluoroacetic anhydride. About 1 mg of the racemic analyte was dissolved in 0.5 ml of diethyl ether and 200 μl of trifluoroacetic anhydride were added. After *ca.* 5–30 min, dry nitrogen was bubbled through the solution to remove any excess anhydride. The residue was dissolved in 0.5 ml of ether or methanol for chromatographic analysis.

Apparatus

All chromatographic measurements were performed on a Varian Model 3700 gas chromatograph equipped with a flame ionization detector. Nitrogen was used as the carrier gas and the gas velocity was *ca.* 10–15 cm/s. The injector and detector temperature were held at 200°C. A split ratio of 100:1 was used for all the columns and at all of the column temperatures. The injection volume was 0.5 μl.

RESULTS AND DISCUSSION

Enantioselective properties

Tables I and II show the enantiomeric separation data for compounds resolved

TABLE I

RETENTION AND SELECTIVITY OF RACEMIC ANALYTES ON DP-TFA-CD STATIONARY PHASES

k'_1 = Capacity factor of first-eluted enantiomer. Stationary phases: A = DP-TFA-α-CD; B = DP-TFA-β-CD; G = DP-TFA-γ-CD.

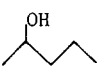
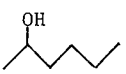
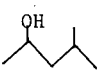
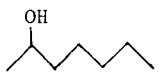
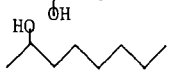
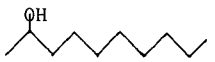
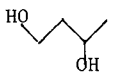
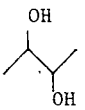
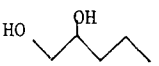
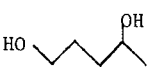
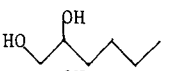
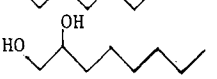
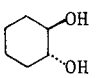
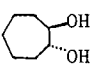
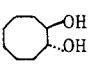
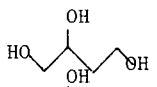
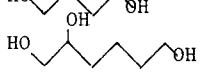
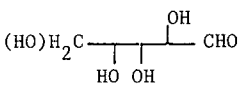
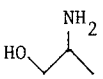
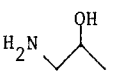
Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationdry phase*
Alcohols, diols and polyols					
	2-Butanol	40	0.69	1.22	B
		40	0.82	1.16	G
	3-Butyn-2-ol	70	0.57	1.08	B
		40	0.77	1.09	G
	2-Pentanol	40	1.31	1.26	B
		40	1.61	1.27	G
	2-Hexanol	40	3.75	1.31	G
	4-Methyl-2-pentanol	40	1.58	1.19	B
		40	2.18	1.17	G

TABLE I *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	2-Heptanol	40	7.85	1.25	B
		40	8.39	1.26	G
	3-Heptanol	40	4.14	1.08	G
	2-Octanol	70	2.80	1.06	B
		40	20.7	1.15	G
	2-Decanol	70	13.4	1.18	B
		40	67.1	1.22	G
	1,2-Propanediol	70	2.06	1.08	A
		70	1.67	1.18	B
	1,3-Butanediol	70	4.92	1.06	A
		70	4.86	1.14	B
		70	4.79	1.21	G
	(2*R*,3*R*)- and (2*S*, 3*S*)-butanediol	70	1.43	1.58	G
	1,2-Pentanediol	70	7.77	1.09	A
		70	3.54	1.03	B
	1,4-Pentanediol	90	5.23	1.06	B
		70	13.6	1.05	G
	1,2-Hexanediol	70	22.5	1.08	A
	1,2-Octanediol	90	10.6	1.05	B
	*trans*-1,2-Cyclohexanediol	70	12.0	1.58	G
	*trans*-1,2-Cycloheptanediol	70	19.6	1.15	G
	*trans*-1,2-Cyclooctanediol	70	36.0	1.12	G

(Continued on p. 308)

TABLE I *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	1,2,4-Butanetriol	110	8.10	1.02	B
	1,2,6-Hexanetriol	110	23.3	1.07	B
	Arabitol	100	20.6	1.06	B
Amino Alcohols					
	2-Amino-1-propanol	100	5.88	1.07	A
		110	3.81	1.16	B
		100	6.53	1.99	G
	1-Amino-2-propanol	100	6.25	1.14	A
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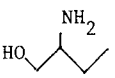	2-Amino-1-butanol	110	7.31	1.08	A
		110	3.42	1.06	B
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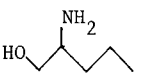	2-Amino-1-pentanol	110	4.33	1.14	B
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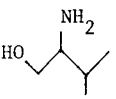
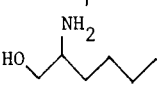
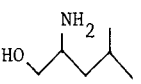
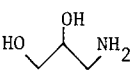
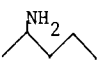
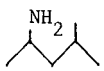
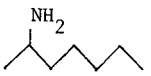
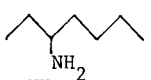
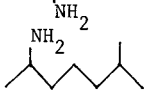
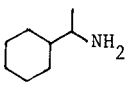
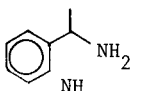
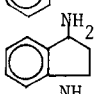
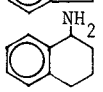
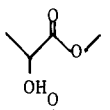
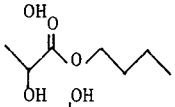
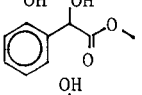
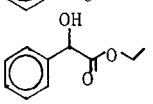
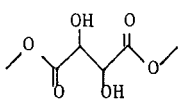
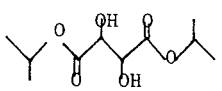
	2-Amino-3-methyl-1-butanol	110	2.95	1.07	B
		100	6.43	1.19	G
	2-Amino-1-hexanol	110	15.0	1.04	A
		110	7.57	1.11	B
		100	10.4	1.1	G
	Leucinol	120	3.81	1.06	A
		110	4.33	1.14	B
	3-Amino-1,2-propanediol	140	6.14	1.12	B
Amines					
	2-Aminobutane	80	4.29	1.04	G
	2-Aminopentane	80	3.75	1.03	A

TABLE I *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	1,3-Dimethylbutyl-amine	80	4.88	1.06	A
	2-Aminoheptane	90 80	10.3 19.6	1.07 1.02	A G
	3-Aminoheptane	90 80	7.19 13.4	1.06 1.03	A G
	1,5-Dimethylhexyl-amine	90 80	10.1 28.4	1.01 1.01	A G
	1-Cyclohexylethyl-amine	100	11.2	1.08	B
	1-Phenylethyl-amine	100	12.9	1.05	B
	1-Aminoindan	140	4.06	1.06	A
	1,2,3,4-Tetrahydro-1-naphthylamine	140 140	6.12 8.21	1.04 1.03	A G
Carboxylic acid esters					
	Lactic acid methyl ester	50	6.79	1.47	G
	Lactic acid butyl ester	60	15.0	1.05	G
	Mandelic acid methyl ester	110	4.00	1.04	B
	Mandelic acid ethyl ester	110 120	3.57 4.11	1.03 1.09	B G
	D,L-Tartaric acid dimethyl ester	90	2.79	1.04	B
	D,L-Tartaric acid diisopropyl ester	90	8.57	1.07	B

(Continued on p. 310)

TABLE I *(continued)*

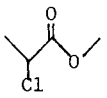
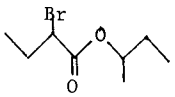
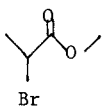
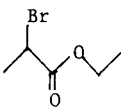
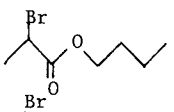
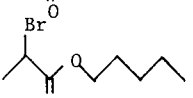
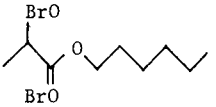
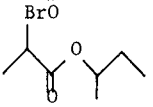
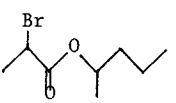
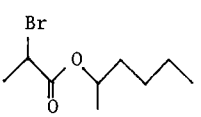
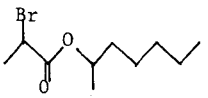
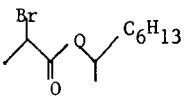
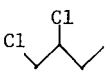
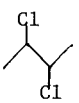
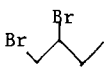
Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	2-Chloropropionic acid methyl ester	60	6.25	2.69	B
		60	5.00	2.14	G
	2-Bromobutyric acid *sec.*-butyl ester[a]	80	10.0	1.22	G
			10.6	1.07	
	2-Bromopropionic acid methyl ester	80	9.38	1.12	B
		80	2.44	1.47	G
	2-Bromopropionic acid ethyl ester	80	3.85	1.14	B
		80	3.50	1.16	G
	2-Bromopropionic acid butyl ester	80	8.00	1.05	G
	2-Bromopropionic acid pentyl ester	80	15.6	1.04	G
	2-Bromopropionic acid hexyl ester	80	30.9	1.04	G
	2-Bromopropionic acid *sec.*-butyl ester[a]	60	12.0	1.25	B
			12.5	1.05	
		80	4.89	1.29	G
			5.39	1.02	
	2-Bromopropionic acid *sec.*-pentyl ester[a]	60	21.5	1.28	B
			22.9	1.12	
		80	8.00	1.24	G
			8.37	1.05	
	2-Bromopropionic acid *sec.*-hexyl ester[a]	60	45.6	1.26	B
			49.5	1.10	
		80	14.7	1.25	G
			15.1	1.07	

TABLE I *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	2-Bromopropionic acid *sec.*-heptyl ester[a]	90	15.1 15.4	1.16 1.04	G
	2-Bromopropionic acid *sec.*-octyl ester[a]	90	29.3 29.6	1.16 1.03	G
Halohydrocarbons					
	2-Bromo-1-chloropropane	40 40 30	4.31 4.00 7.64	1.06 1.12 1.05	A B G
	2-Chlorobutane	30 30	0.73 0.91	1.10 1.12	A G
	3-Chloro-1-butene	30 30	0.72 0.82	1.06 1.13	A G
	2-Bromobutane	30 35 30	1.65 1.74 2.44	1.38 1.04 1.09	A B G
	2-Iodobutane	40 60 30	2.92 1.31 6.36	1.04 1.24 1.06	A B G
	1,2-Dichlorobutane	60 60	2.45 0.89	1.09 1.04	B G
	(2*R*,3*R*)- and (2*S*,3*S*)-2,3-dichlorobutane	60 60	2.15 2.05	1.59 1.60	B G
	1,2-Dibromobutane	90 70 60	4.13 4.54 8.57	1.03 1.03 1.13	A B G

(Continued on p. 312)

TABLE I *(continued)*

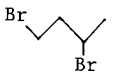
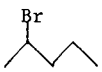
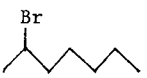
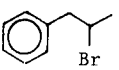
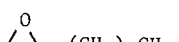
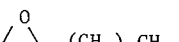
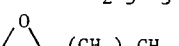
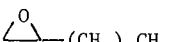
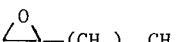
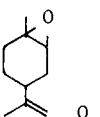
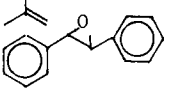
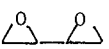
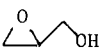
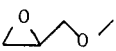
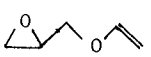
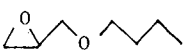
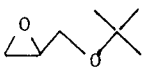
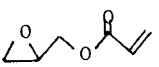
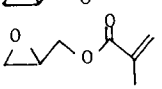
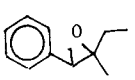
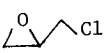
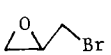
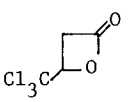
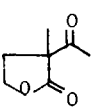
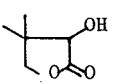
Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	1,3-Dibromobutane	90	6.38	1.02	A
		70	7.38	1.05	B
		60	13.6	1.79	G
	2-Bromopentane	35	3.44	1.03	B
		30	8.27	1.32	G
	2-Bromoheptane	70	4.15	1.07	B
		50	8.57	1.18	G
	2-Bromo-1-phenyl-propane	100	6.39	1.06	G
Expoxides, glycidyl analogues and haloepihydrins					
	1,2-Epoxyhexane	50	3.31	1.02	A
		40	5.36	1.10	G
	1,2-Epoxyoctane	50	22.3	1.04	A
	1,2-Epoxydecane	90	9.88	1.02	A
	1,2-Epoxydodecane	90	108	1.02	A
	1,2-Epoxytetradecane	100	125	1.02	A
	Styrene oxide	80	5.57	1.01	B
		80	10.7	1.57	G
	Limonene oxide[a]	80	8.57	1.06	G
			8.57	1.10	
	*trans*-Stilbene oxide	140	20.0	1.02	G
	(±)-1,3-Butadiene diepoxide	80	3.93	1.11	G
	Glycidol	60	4.29	1.06	G
	Glycidyl methyl ether	40	3.33	1.04	B
		45	5.89	1.16	G
	Glycidyl isopropyl ether	40	6.00	1.04	B
		45	10.5	1.04	G

TABLE I *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	Allyl glycidyl ether	50 45	3.23 21.6	1.02 1.42	A G
	*n*-Butyl glycidyl ether	50 45	11.8 23.2	1.02 1.04	B G
	*tert.*-Butyl glycidyl ether	45	14.5	1.06	G
	Glycidyl acrylate	80	10.1	1.14	G
	Glycidyl methacrylate	80	10.4	1.04	G
	(2*S*,3*S*)- and (2*R*,3*R*)-2-methyl-3-phenyl-glycidol	100	13.6	1.06	G
	Epifluorohydrin	30	2.50	1.02	G
	Epichlorohydrin	60	4.82	1.20	G
	Epibromohydrin	60	4.82	1.20	G
Lactones					
	β-Butyrolactone	110 70 80	2.73 13.7 7.14	1.14 1.62 1.20	A B G
	3-Hydroxy-4,4,4-trichlorobutyric-β-lactone	120 100	3.50 15.6	1.11 1.19	B G
	α-Methyl-γ-butyrolactone	110 110	3.03 11.5	1.29 1.07	A B
	α-Acetyl-α-methyl-γ-butyrolactone	120	6.28	1.59	B
	Pantoyl lactone	120 120	4.25 1.57	1.04 1.05	A B

(Continued on p. 314)

TABLE I *(continued)*

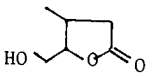
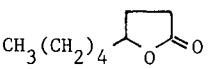
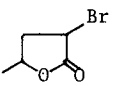
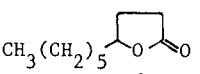
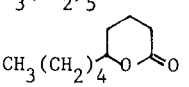
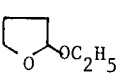
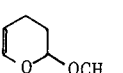
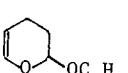
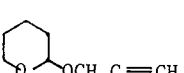
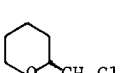
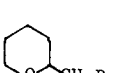
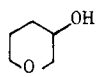
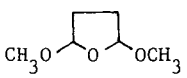
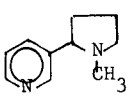
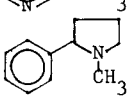
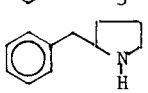
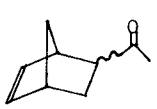
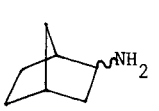
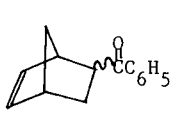
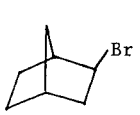
Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	β,β'-Dimethyl-γ-(hydroxymethyl)-γ-butyrolactone	120	12.3	1.10	B
	γ-Nonanoiclactone	160	4.07	1.06	B
	α-Bromo-γ-valero-lactone[a]	150	4.64 5.36	1.09 1.09	G
	γ-Decanolactone	140	9.24	1.05	G
	δ-Decanolactone	140	11.8	1.02	G
Furan and pyran derivatives					
	2-Ethoxytetra-hydrofuran	40 40 45	3.63 3.08 3.04	1.03 1.05 1.20	A B G
	3,4-Dihydro-2-methoxy-2H-pyran	40 40 45	3.38 4.33 4.29	1.07 1.22 1.13	A B G
	3,4-Dihydro-2-ethoxy-2H-pyran	40 40 45	5.85 5.92 6.07	1.04 1.06 1.09	A B G
	Tetrahydro-2-(2-propynyloxy)-2H-pyran	80 70	3.71 8.14	1.01 1.08	B G
	2-(Chloromethyl)-tetrahydropyran	80 70	3.24 8.04	1.28 1.03	B G
	2-(Bromomethyl)-tetrahydropyran	80	6.07	1.25	B

TABLE I *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	3-Hydroxytetrahydropyran	50	7.14	1.18	G
	*trans*-2,5-Dimethyltetrahydrofuran	45	8.57	1.06	G
	*trans*-2,5-Dimethoxytetrahydrofuran	60	1.46	1.92	G
Nicotine compounds					
	Nicotine	160	2.14	1.13	B
	1-Methyl-2-phenylpyrrolidine	160	1.43	1.20	B
	2-Benzylpyrrolidine	140	10.2	1.06	B
Bicyclic compounds					
	*endo*- and *exo*-2-acetyl-5-norbornene[a]	110	1.73 2.45	1.03 1.00	B
		100	4.39 6.32	1.11 1.09	G
	*endo*- and *exo*-2-aminonorbornane[a]	110	4.71 6.21	1.18 1.10	B
	*endo*- and *exo*-2-benzoyl-5-norbornene[a]	120	34.6 41.8	1.00 1.04	G
	*exo*-2-Bromonorbornane	70	11.3	1.02	G

(Continued on p. 316)

TABLE I *(continued)*

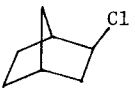
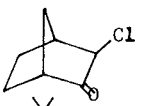
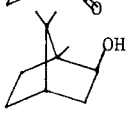
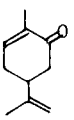
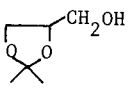
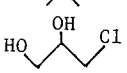
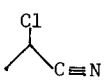
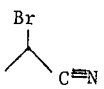
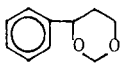
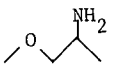
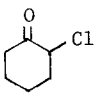
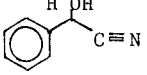
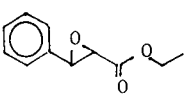
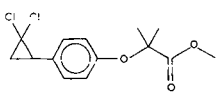
Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	*exo*-2-Chloro-norbornane	70	5.79	1.01	G
	*endo*- and *exo*-3-chloro-2-norbornanone[a]	120	6.57 10.2	1.06 1.04	G
	DL-Isoborneol	70	8.68	1.05	G
Ketones					
	2-Methylcyclo-hexanone	80	5.29	1.08	G
	Carvone	90 110 100	13.6 7.05 11.4	1.04 1.09 1.01	A B G
Miscellaneous					
	3-Chloro-2-butanone	50 60	3.46 2.43	1.62 1.59	B G
	Solketal	90 60	1.57 7.86	1.04 1.07	B G
	3-chloro-1,2-propanediol	90	3.52	1.09	B
	2-Chloropropionitrile	80 70	1.93 1.65	1.07 1.06	B G
	2-Bromopropionitrile	80 70	4.07 4.13	1.14 1.06	B G
	1-Chloro-2-propanol	70	5.21	1.02	G

TABLE I *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	4-Phenyl-1,3-dioxane	120	7.36	1.04	G
	2-Chlorocyclo-pentanone	90 110	12.6 2.47	2.51 1.33	B G
	2-Amino-1-methoxy-propane	90 100	1.90 1.71	1.25 1.08	B G
	2-Chlorocyclo-hexanone	90 110	21.2 4.26	1.06 1.10	B G
	2-Methylpiperidin	100	5.54	1.13	G
	3-Methylpiperidin	100	5.00	1.14	G
	Mandelonitrile	100	5.21	1.10	G
	Ethyl-3-phenyl-glycidate[a]	120	16.6 23.8	1.05 1.04	G
	Ciprofibrate methyl ester	140	67.7	1.03	B

[a] Compound containing more than one chiral center.

on the α-, β- and γ-versions of DP-TFA-CD capillary GC columns. The γ-CD column displays excellent chiral selectivity to alcohols, diols, amino alcohols, α-halocarboxylic acid esters, halohydrocarbons, glycidyl analogues, lactones, bicyclic compounds, pyran and furan derivatives. Racemic amines, as well as alcohols, diols, amino alcohols, halohydrocarbons, lactones, pyran and furan derivatives were resolved on the β-CD column. The α-CD column is especially useful for the enantiomeric separations of long carbon chain epoxides (Table I). In addition, piperidines, α-halocycloketones, as well as some analytes containing nitrile functionality, were also resolved on the γ-CD columns and/or β-CD columns. Large separation factors (α) were obtained for most of the racemates, particularly alcohols, diols, lactones, α-halocarboxylic acid esters and some halohydrocarbons. (*R*)- and (*S*)-2-chloropropionic acid methyl esters (which are precursors in the synthesis of many herbicides) show an α value of 2.69 on

TABLE II

COMPARISON OF RETENTION AND SELECTIVITY OF HOMOLOGOUS RACEMIC AMINES, DIOLS AND α-HALOCARBOXYLIC ACID ESTER ANALOGUES ON DP-TFA-CD STATIONARY PHASES

k'_1 and stationary phases as in Table I.

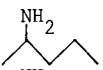
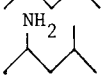
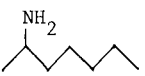
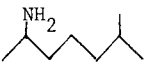
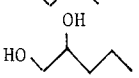
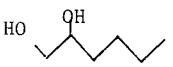
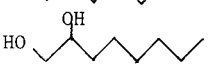
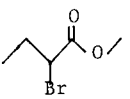
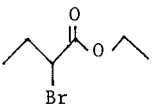
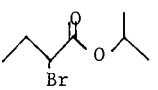
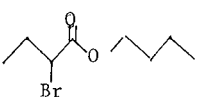
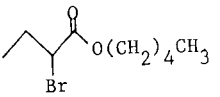
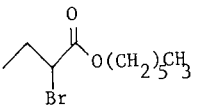
Structure	*Compound*	*Temperature* (°C)	k'_1	α	Stationary phase
	2-Aminobutane	90	1.55	1.14	B
	2-Amino-3,3-dimethylbutane	90	2.10	1.22	B
	2-Aminopentane	90	2.40	1.22	B
	1,3-Dimethylbutylamine	90	2.80	1.22	B
	2-Aminoheptane	90	8.15	1.22	B
	1,5-Dimethylhexylamine	90	12.5	1.22	B
	1,2-Propanediol	70	1.61	1.49	G
	1,2-Pentanediol	70	3.50	1.23	G
	1,2-Hexanediol	70	6.64	1.23	G
	1,2-Octanediol	70	29.3	1.23	G
	2-Bromobutyric acid methyl ester	80 80	507 6.71	1.56 1.57	B G
	2-Bromobutyric acid ethyl ester	80 80	5.25 9.93	1.29 1.16	B G
	2-Bromobutyric isopropyl ester	80	6.57	1.08	G
	2-Bromobutyric acid butyl ester	80 80	15.5 20.5	1.16 1.09	B G

TABLE II *(continued)*

Structure	*Compound*	*Temperature (°C)*	k'_1	α	*Stationary phase*
	2-Bromobutyric acid pentyl ester	80	32.3	1.16	B
		80	39.5	1.08	G
	2-Bromobutyric acid hexyl ester	80	66.4	1.16	B
		80	80.0	1.09	G

DP-TFA-β-CD (Table I), which is unusually large for a GC separation of enantiomers. Therefore, shorter columns and higher column temperatures can be used.

Typical separations are illustrated in Figs. 1–4. Optically active alcohols (Fig. 1) are found naturally in the chemical communication system of insects. For example, 2-heptanol is active as an alarm pheromone in ants. Aliphatic 1,2- and 1,3-diols (Fig. 2) are important chiral building blocks in asymmetric synthesis. All of the compounds in Figs. 1–4 lack the aromatic functionality required for most LC separations. In fact, lower enantioselectivity was observed toward some aromatic compounds. For example, although 1-indanol, 1,2,3,4-tetrahydro-1-naphthol and 1-(1-naphthyl)-ethylamine were resolved on 2,6-di-O-pentyl CD and permethyl-O-(*S*)-2-hydroxypropyl CD stationary phases as previously reported[24,25], none of these compounds was

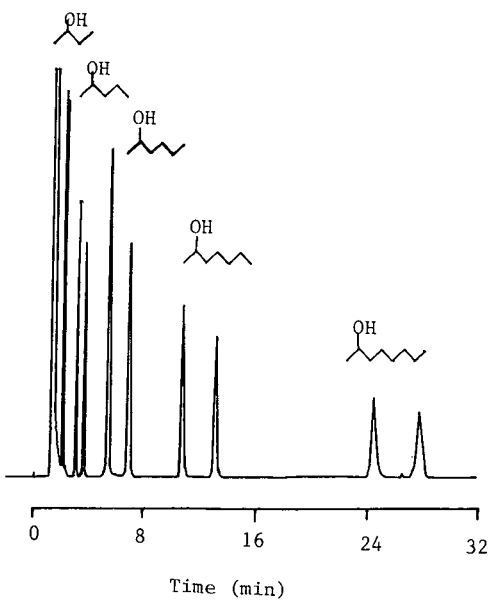


Fig. 1. Enantiomeric separation of alcohols after trifluoroacetylation. Column, 10 m fused silica with DP-TFA-γ-CD; column temperature, 40°C; carrier gas, nitrogen, 3 p.s.i.

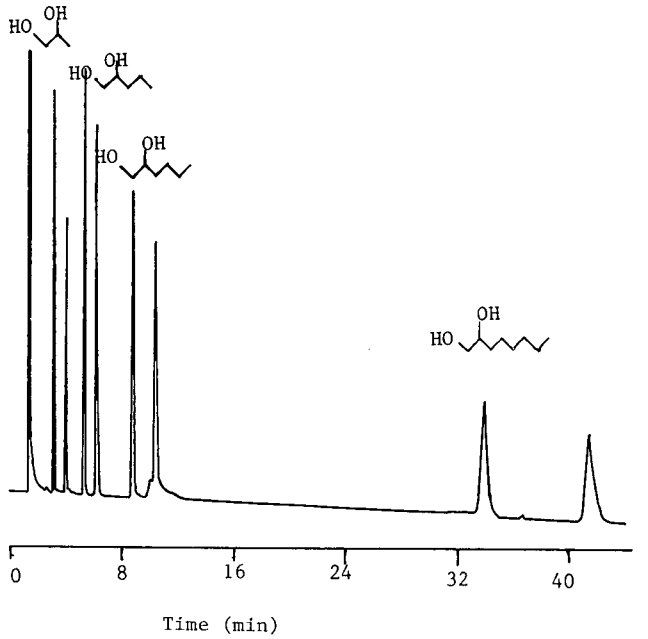


Fig. 2. Enantiomeric separation of diols after trifluoroacetylation. Column, 10 m fused silica with DP-TFA-γ-CD; column temperature, 70°C; carrier gas, nitrogen, 3 p.s.i.

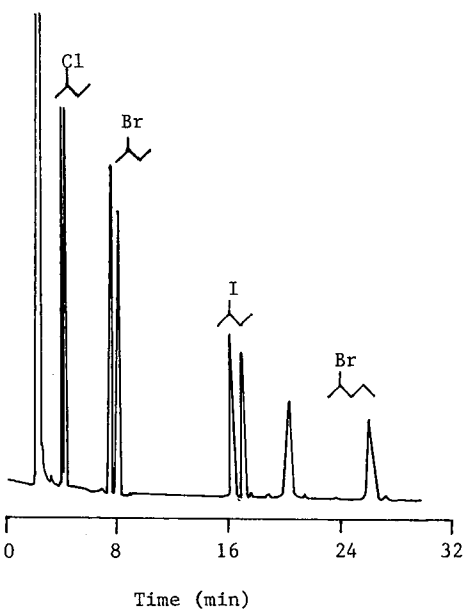


Fig. 3. Enantiomeric separation of monohalohydrocarbons. Column, 10 m fused silica with DP-TFA-γ-CD; column temperature, 30°C; carrier gas, nitrogen, 3 p.s.i.

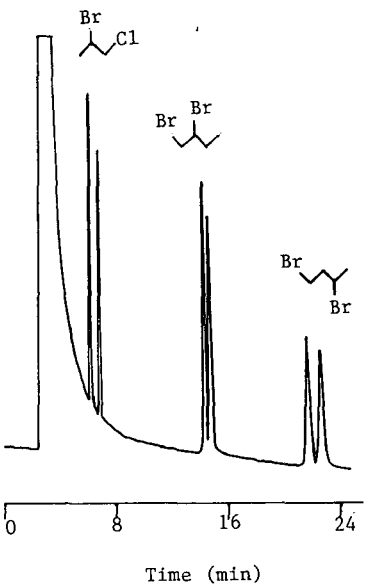


Fig. 4. Enantiomeric separation of dihalohydrocarbons. Column, 10 m fused silica with DP-TFA-β-CD; column temperature, 70°C; carrier gas, nitrogen, 3 p.s.i.

resolved on any of the DP-TFA-CD columns. Of the 150 different racemic compounds injected on the columns, 80% were resolved on the γ-CD column, 60% on the β-CD column and only 30% on the α-CD column. It appears that the DP-TFA-γ-CD column is the most widely useful of the three, although there are certain compounds that resolve only on the β-CD and α-CD analogues. To our knowledge, this is the only CD stationary phase series (in either LC or GC) thus far in which the γ-CD derivative exhibits a wider chiral selectivity and usefulness than the β-CD analogue.

Separation mechanism

There have been few mechanistic retention studies involving derivatized CD GC stationary phases. In contrast to analogous LC separations, the interactions between chiral solutes and GC CSPs are not significantly affected by mobile phase interactions. Feibush and Gil-Av[27] have suggested that association complexes via hydrogen bonding between carbonyl and amide functions were responsible for the GC chiral separation of amino acids on the dipeptide phases. Others claimed that only one significant point of attachment was involved in the formation of the diastereomeric association complex[28]. Cyclodextrins are composed of linked α-1,4-glucose units. Each glucose has three hydroxyl groups designated 2-OH, 3-OH and 6-OH. The 6-OH are primary hydroxyls and are located at the more narrow, "bottom" end of the CD torous. The 2-OH and 3-OH groups are secondary hydroxyl groups and are located at the wide end or "mouth" of the CD molecule. After 2,6-di-O-alkylation and 3-O-trifluoroacetylation, CD molecules are no longer good hydrogen bond donors as are native cyclodextrins. Also, many of the racemates resolved had no hydrogen bond donor groups and relatively poor hydrogen bond accepting

groups (*e.g.*, haloalkanes). Consequently, some of the separations shown in Tables I and II, cannot be explained by hydrogen bonding interactions. Molecules such as halohydrocarbons and lactones (that contain only hydrogen bonding acceptor groups) have permanent dipole moments. Stronger dipole–dipole interactions between analytes and DP-TFA-CD molecules are very likely. Inclusion complexation as well as dispersion, or steric interactions also may play a role in chiral recognition and enantioselective retention.

There are several different ways in which the dipole-dipole interaction may occur. The analytes may enter into the cyclodextrin's cavity via the "mouth" or the more narrow bottom of the CD, or may be adsorbed on the exterior surface of the stationary phase molecules. It is difficult to know exactly where the enantioselective interactions are occurring on the CD stationary phase. However, studies can be devised which provide circumstantial evidence as to some of the relevant interactions. For example, retention data for three homologous series on DP-TFA-β- and γ-CD are gathered in Table II. All of the compounds in this series have a common structural characteristic, *i.e.*, they all have a polar "head" and an non-polar "tail". Chromatographic measurements were carried out under identical conditions for each compound in the series. For the larger racemic homologues, identical α values are obtained regardless of the chain length or branching of the "tail" (Table II). In the amine homologous series, the smallest member has a smaller α value than the higher-molecular-weight members. In the other two series, the smallest members have larger α values than the rest (Table II). It is reasonable to assume that for these homologous series: (a) only part of the carbon chain (one to two carbons) contribute significantly to the chiral recognition because the α values of the larger homologous members are independent of carbon chain length, (b) longer carbon chains affect the retention of the molecules to the CSP (*i.e.*, the longer the chain the greater the retention) but not the enantioselectivity and (c) the orientation of the molecule can be affected by the substituent group on the CD and the size of the CD cavity. The functionality of the analytes may also play a role because other homologous series, such as epoxides, alcohols and amino alcohols did not exhibit the same behavior (Table I).

Column stability and special operational considerations

When considering column stability for GC CSPs three very different problems must be addressed: decomposition of backbone structure, racemization of chiral functionality and disruption of the coated film. All of these processes are irreversible and any one of them can render the column useless for the separation of enantiomers. It has been found[22,23] that the derivatized CD stationary phases do not racemize at temperatures up to 300°C. Also, we have found that DP-TFA-CDs have good wettability to untreated fused-silica capillary wall and that the film was stable up to 180°C. To test the stability of the film on fused-silica capillary wall, the column temperatures were held at 160, 180, 200 and 220°C for 4 h. The changes in column efficiency were monitored at 100°C using *n*-hydrocarbons as test solutes. It was found that the column efficiency dropped dramatically after the column had been used above 200°C. Droplets were observed on the capillary wall by visual inspection under a microscope. The thermal stability of 3-O-trifluoroacetyl functionality on the stationary phases is another concern because the ester linkage is susceptible to hydrolysis. We used every column continuously for 1 to 2 months. Moisture was removed from the carrier gas

by using an in-line gas purifier (Alltech, Deerfield, IL, U.S.A.). Column efficiency and selectivity were continuously monitored during all of the previously mentioned studies. Insignificant changes in the selectivity were observed. However, great care must be taken to prolong the life time of the column. For the wall-coated capillary, only split injection is advised with a split ratio of $\geqslant$ 100:1. A single splitless or on-column injection may permanently damage the column. The selection of sample solvent also is critical. The suggested sample solvent is diethyl ether, which is very volatile and inactive to derivatizing reagents such as trifluoroacetic anhydride. Solvents such as benzene and toluene are not recommended, since they may form inclusion complexes with the stationary phases and interfere with enantiomeric separation. This has occasionally been observed in our laboratory. In addition, like other common GC stationary phases, derivatized CD stationary phases are sensitive to thermal shock. As for all other wall coated GC capillaries, these columns should never be heated or cooled at a rate of more than 20°C/min.

CONCLUSION

The DP-TFA-CD, particularly the γ-CD, stationary phases exhibit wider chiral selectivity and usefulness than other previously reported CD-based GC stationary phases[19–24]. High separation factors have been observed for some racemates, which indicates that it may be possible to utilize the stationary phases in packed GC or preparative GC separations of enantiomers. The enantioselective retention data in this paper suggests a specific space-oriented dipole-dipole interaction between the analytes and stationary phase molecules. However, mechanistic studies are needed to fully understand the separation mechanism.

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Journal of Chromatography, 509 (1990) 325–332
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 438

Long-chain fatty alcohol quantitation in subfemtomole amounts by gas chromatography–negative ion chemical ionization mass spectrometry

Application to long-chain acyl coenzyme A measurement

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ABSTRACT

We describe a simple and sensitive method to identify and quantitate long-chain fatty alcohols. Long-chain fatty alcohols were converted to their pentafluorobenzoyl derivative and analyzed by gas chromatography (GC)–mass spectrometry in the negative ion chemical ionization (NICI) mode with selected ion monitoring. GC resolution was obtained for myristyl, palmityl, heptadecyl, stearyl, oleyl, linoleyl and arachidonyl alcohols. As little as 0.4 fmol of fatty alcohol can be detected, which represents a six order-of-magnitude increase in sensitivity over previously described methods. This assay can be used to measure femtomolar amounts of long-chain acyl coenzyme A thioesters after reduction to the corresponding fatty alcohols with sodium borohydride. Other potential applications of this assay include identification and quantitation of long-chain fatty alcohol production by microorganisms.

INTRODUCTION

Measurement of endogenous cellular levels of long-chain acyl CoA thioesters and the identification of the long-chain acyl composition of these compounds is attracting interest in a variety of settings. Long chain acyl coenzyme A (CoA) thioesters have been implicated as substrates in a number of metabolic reactions at the cellular level[1,2]. Increased amounts of these compounds have been described in certain pathological conditions, including myocardial ischemia, and may contribute to cellular injury in such settings[3,4]. More recently, *de novo* synthesis of diacylglycerol from glucose has been implicated in the pathogenesis of the vascular complications of diabetes mellitus[5]. There is evidence in various tissues, including pancreatic islets and retinal capillary endothelial cells, that this pathway involves metabolism of glucose to triose phosphates, which are then acylated using long-chain acyl CoA as the fatty acyl

0021-9673/90/$03.50

donor. The resultant acyl-dihydroxyacetone-phosphate is then reduced to lysophosphatidic acid, which is hydrolyzed[5–10].

A number of assays have been decribed for the measurement of long-chain acyl CoA thioesters[11–19]. High-performance liquid chromatographic (HPLC) methods achieve sensitivity in the nanomolar range[16]. Recently, Prasad *et al.*[17] have described a technique which takes advantage of the ability of sodium borohydride to reduce the thioester linkage of CoA esters[18] to obtain fatty alcohols, which were then identified and quantitated in the subnanomolar range by gas chromatography (GC) with flame ionization detection. These authors suggested that the sensitivity of this technique might be increased with the use of mass spectrometric (MS) detection. We describe here a GC–negative ion chemical ionization (NICI)-MS method for the measurement of fatty alcohols derived from long-chain acyl CoA esters in the subfemtomolar range.

MATERIALS AND METHODS

Materials

Fatty alcohols were purchased from Avanti (Pelham, AL, U.S.A.). Long-chain acyl CoA esters were obtained from Sigma (St. Louis, MO, U.S.A.). [1-^{14}C]Myristoyl CoA was from Amersham (Arlington Heights, IL, U.S.A.). All other chemicals including pentafluorobenzoyl chloride were obtained from Sigma. All solvents (HPLC grade) were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). Screw-cap septum vials (1.5 ml, No. 13208) were obtained from Pierce (Rockford, IL, U.S.A.).

Derivatization of fatty alcohols

Long-chain fatty alcohols were concentrated to dryness under nitrogen in silanized, acid-washed 1.5-ml glass screw-cap vials. Derivatization was performed by adding 0.1 ml of pentafluorobenzoyl chloride and incubating for 45 min at 120°C. The contents were then concentrated to dryness (nitrogen) and water (0.1 ml) was added. Extraction was performed with methylene chloride (0.2 ml), followed by one water (0.1 ml) wash. The organic extract was evaporated under nitrogen and the sample was reconstituted in heptane (0.1 ml) prior to analysis.

Gas chromatographic–mass spectrometric analysis

GC was performed on a Hewlett-Packard 5890 gas chromatograph interfaced with a Hewlett-Packard 5988 mass spectrometer. A capillary column (Hewlett-Packard Ultraperformance, 8 m × 0.31 mm I.D., cross-linked methylsilicone, film thickness 0.17 μm) was operated with a Grob-type injector in the splitless mode with helium as carrier gas (inlet pressure 4 p.s.i., injector temperature 280°C). The distal end of the column was inserted directly into the ion source (interface temperature 280°C). The GC oven temperature was programmed from 85 to 200°C at a rate of 30°C/min starting 0.8 min after the injection, and maintained at 200°C for 6 min. The mass spectrometer was operated in the NICI mode with methane as reagent gas (source pressure 0.6 Torr). The ionizing potential was 240 eV. Selected ions were monitored which corresponded to the molecular ion of the fatty alcohol pentafluorobenzoyl ester derivative.

Long-chain acyl CoA analysis

Two extraction techniques of long-chain acyl CoA were investigated. The first scheme was described by Prasad *et al.*[17] and involves chloroform–methanol extraction, acetonitrile precipitation, adsorbtion on Al_2O_3, and reduction of the adsorbed fatty acyl CoA to alcohols with sodium borohydride. Fatty alcohols were extracted with pentane, converted to the pentafluorobenzoyl ester, and then analyzed by GC–NICI-MS as described above.

In the second scheme, a chloroform–methanol (1:2, v/v) extract containing long-chain fatty acyl CoA thioesters with heptadecanoyl CoA as an internal standard (3 nmol) was applied to a channeled thin-layer chromatography (TLC) plate (Whatman LK6) which was developed in butanol–acetic acid–water (100:40:60, v/v/v) for *ca.* 4–5 h[20]. Long-chain acyl CoA spots were localized by light iodine staining of the standards run on the same plate and the corresponding silica was scraped into silanized conical 5-ml borosilicate tubes (with PTFE screw caps). Long-chain acyl CoA thioesters were extracted from the silica by adding 0.5 ml of the TLC developing solvent, vortexing 30 min and centrifuging 5 min at 800 *g*. The supernatant was then transferred to a clean 5-ml tube. (The extraction was repeated two more times). Following concentration under nitrogen, the following solution was added to each tube: 1 ml of diethyl ether, 0.05 ml of 0.02 *M* borate buffer pH 8.0, 10 m*M* $CaCl_2$ and 20 units of phospholipase C from *B. cereus*. Tubes were incubated 2 h at 37°C. The contents of each tube were concentrated to dryness (nitrogen); reconstituted in 0.05 ml of ethanol–acetic acid (50:50, v/v); and applied to a channeled Whatman LK6 plate, which was developed in the same solvent system as described above. Long-chain acyl CoA thioester spots were scraped and reduced to their corresponding long-chain fatty alcohols by the addition of 1.25 ml of methanol–water (50:50, v/v) and 16 mg of $NaBH_4$ and were then incubated 60 min at 37°C in a shaking water bath. The reaction was terminated with 0.6 ml of 1 *M* hydrochloric acid. Fatty alcohols were extracted with 2 ml of pentane twice. The pentane fraction containing the fatty alcohols was washed with water (1 ml) and concentrated to dryness (nitrogen). Derivatization and analysis of the pentafluorobenzoyl derivatives of fatty alcohols were performed as described above.*

RESULTS AND DISCUSSION

Fatty alcohol analysis

Long-chain fatty alcohols were converted to their pentafluorobenzoyl derivatives and analyzed by GC–NICI-MS. Fig. 1 shows the total ion current tracing and corresponding NICI-mass spectra for the pentafluorobenzoyl derivative of stearyl alcohol. A strong molecular ion (*m/z* 464) was observed. The mass spectra of the polyunsaturated fatty alcohol arachidonyl alcohol is shown in Fig. 2. A strong molecular ion (*m/z* 484) was noted. Pentafluorobenzoyl derivatives of other fatty alcohols can also be monitored by their molecular ion in the NICI mode: *m/z* 408 (myristyl alcohol), 436 (palmityl alcohol), 450 (heptadecyl alcohol), 460 (linoleyl alcohol), 462 (oleyl alcohol).

As illustrated in Fig. 3, when increasing amounts of the pentafluorobenzoyl derivative of stearyl alcohol were analyzed by GC–NICI-MS, the peak area was a linear function of the amount of stearyl alcohol added (between 0.4 and 400 fmol). As

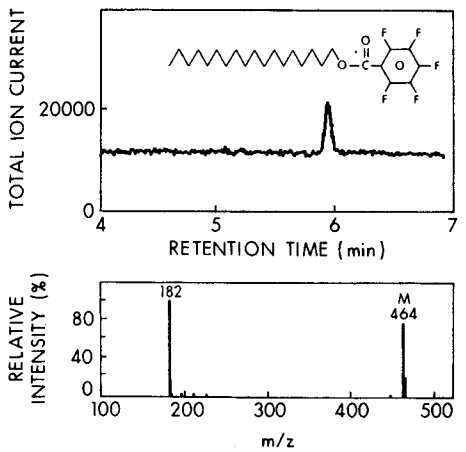

Fig. 1. NICI-MS of the pentafluorobenzoyl derivative of stearyl alcohol. A 3-pmol amount of a pentafluorobenzoyl derivative of stearyl alcohol was injected into the gas chromatograph as described in Materials and Methods. The mass spectrometer was operated in the NICI mode with methane as the reagent gas (source pressure: 0.6 Torr). Top: total ion current chromatogram of the pentafluorobenzoyl derivative of stearyl alcohol. Bottom: mass spectra of the peak eluting at 5.97 min (electron multiplier voltage setting of 1400).

little as 0.37 fmol of stearyl alohol can be detected with a satisfactory signal-to-noise ratio (Fig. 3, lower right panel).

Fig. 4 illustrates a standard curve of a mix of pentafluorobenzoyl derivatives of fatty alcohols (14:0, 16:0, 18:0, 18:1, 18:2, and 20:4) using 4 pmol of the pentafluorobenzoyl derivative of heptadecyl alcohol as the internal standard with analysis by GC–NICI-MS. Fatty alcohols were identified based on their retention time and

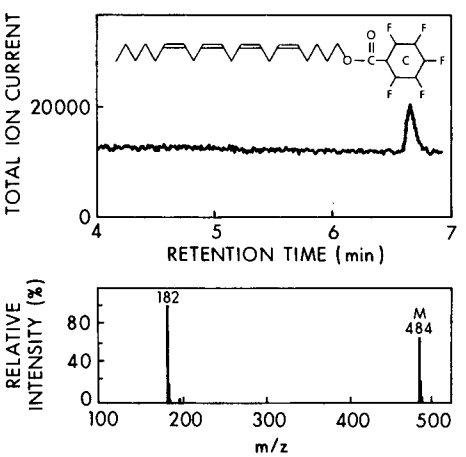

Fig. 2. NICI-MS of the pentafluorobenzoyl derivative of arachidonyl alcohol. A 3-pmol amount of a pentafluorobenzoyl derivative of arachidonyl alcohol was injected into the gas chromatograph as described in Fig. 1. Top: total ion current chromatogram. Bottom: mass spectra of the peak eluting at 6.73 min (electron multiplier voltage setting of 1400).

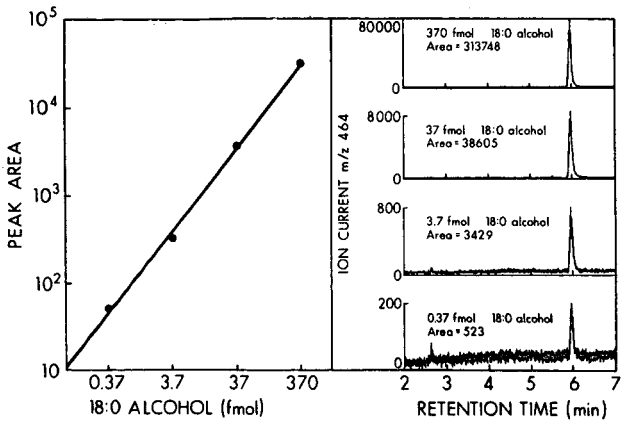

Fig. 3. Response curve of the pentafluorobenzoyl derivative of stearyl alcohol by GC–NICI-MS. Varying amounts of the pentafluorobenzoyl derivatives of stearyl alcohol were injected into the gas chromatograph and eluted as described in Fig. 1. The eluent was monitored by NICI-MS at a *m/z* ion of 464 (mass defect = 0.3). The chromatograms obtained are shown in the right panel. The area under the peak was integrated by the Hewlett-Packard computer and the dilution curve obtained is shown in the left panel as a function of the amount of fatty acid stearyl alcohol (0.4–400 fmol).

molecular ion (Fig. 4, right panel) and quantitated by selected ion monitoring: the area under the peak was expressed as a ratio to that of the internal standard (heptadecyl alcohol) and a standard curve constructed (Fig. 4, left panel). The amount of each fatty alcohol recovered is a linear function of the amount added with a slope (determined by linear regression) approaching 1 (myristyl alcohol: slope = 1.00, intercept = 0, r = 1.000; palmityl alcohol: slope = 1.00, intercept = 0.02, r = 0.997; linoleyl alcohol: slope = 0.99, intercept = 8.48, r = 0.998; oleyl alcohol: slope = 0.96, intercept = 37.40, r = 0.998; stearyl alcohol: slope = 1.00, intercept = 1.29, r = 0.995; arachidonyl alcohol: slope = 0.99, intercept = 4.48, r = 0.996).

These observations demonstrate the feasibility of measuring fatty alcohols by GC–MS with quantitation achievable in the subfemtomolar range. Prasad *et al.*[17] have recently described a GC method for the analysis of fatty alcohols which achieved subnanomolar sensitivity using *tert.*-butyldimethylsilyl derivatives of the fatty alcohols. Problems associated with that technique are the presence of contaminants after silylation which can interfere with the analysis. The use of MS to analyze pentafluorobenzoyl derivatives of fatty alcohols bypasses the contaminant problem associated with GC analysis by flame ionization detection. The GC–MS method also identifies the fatty alcohol derivative based on its molecular ion and achieves a six order-of-magnitude increase in sensitivity. A potential application of this sensitive technique is the identification and quantitation of long-chain fatty alcohol production by yeasts and bacteria[21,22].

Long-chain acyl CoA analysis

Several approaches have been used to assay long-chain acyl CoA thioesters. Enzymatic assays measure coenzyme A released from long-chain acyl CoA with a sensitivity in the subnanomolar range[19]. This method, however, provides no information on the fatty acyl composition of the CoA esters and is subject to a number

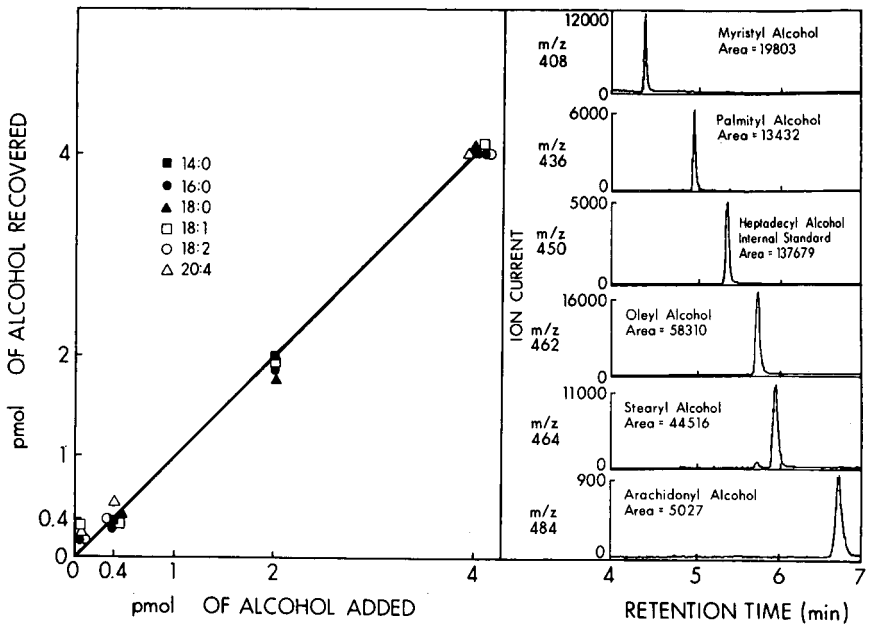

Fig. 4. Standard curve of a mix of pentafluorobenzoyl derivatives of long-chain fatty alcohols. Increasing amounts of pentafluorobenzoyl derivative of long-chain fatty alcohols were injected into the gas chromatograph and eluted as described in Fig. 1. The eluent was monitored by NICI-MS at the following *m/z* (dwell-time 50 ms): 408 (■, myristyl alohol), 436 (●, palmityl alcohol), 450 (heptadecyl alcohol as internal standard), 460 (○, linoleyl alcohol), 462 (□, oleyl alcohol), 464 (▲, stearyl alcohol), 484 (△, arachidonyl alcohol). Left: Standard curve of mixes of fatty alcohols. Linear regression analysis was performed for each long-chain fatty alcohol (see Results and Discussion section for the slope and intercept of the calculated regression line for each long-chain fatty alcohol) and used to calculate the amount of fatty alcohol recovered. The solid line indicates the line of identity. Right: Chromatograms of a mix of pentafluorobenzoyl derivative of fatty alcohols. This tube contained 400 fmol of myristyl alcohol, 400 fmol of palmityl alcohol, 4000 fmol of heptadecyl alcohol, 4000 fmol of oleyl alcohol, 2000 fmol of steaoyl alcohol and 40 fmol of arachidonyl alcohol. Each section of the panel represents the individual chromatogram obtained at the *m/z* ion specific for each long-chain fatty alcohol monitored by NICI-MS as indicated above.

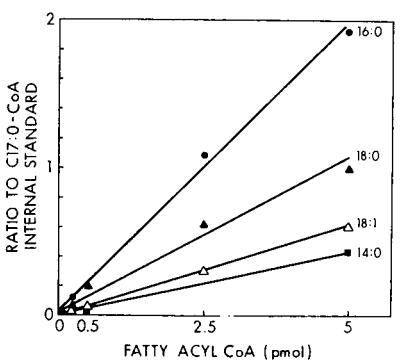

Fig. 5. Standard curve of long-chain acyl CoA thioesters measured as pentafluorobenzoyl derivative of corresponding fatty alcohols. Increasing amounts of long-chain acyl CoA thioesters were purified according to Materials and Methods, second scheme, reduced to their corresponding fatty alcohols, derivatized to pentafluorobenzoyl esters and analyzed as described in Fig. 4. Results are expressed as a ratio of the area under the peak of the corresponding fatty alcohol divided by that of heptadecyl alcohol, the internal standard. ■ = Myristoyl CoA; ● = palmitoyl CoA; ▲ = stearoyl CoA; △ = oleoyl CoA.

of interferences[14]. Recently, a reversed-phase HPLC technique has been described which separates the individual long-chain acyl CoA thioesters. UV detection used with this technique achieves a sensitivity in the nanomolar range[16]. Finally, Prasad *et al.*[17] have used sodium borohydride to reduce long-chain acyl CoA thioesters to their corresponding long-chain alcohols and have quantitated these compounds by GC–flame ionization detection with a reported sensitivity in the subnanomolar range. Using their technique for extracting and reducing long-chain acyl CoA (see Materials and Methods) and measuring the resultant fatty alcohols by GC–NICI-MS as described above, we found that the addition of 21, 19 and 19 pmol of palmitoyl, stearoyl and oleoyl CoA resulted in the recovery of 24, 22 and 11 pmol, respectively, of the corresponding fatty alcohol using heptadecanoyl CoA (3 nmol) as an internal standard. Recovery (as assessed with [^{14}C]myristoyl CoA) was 22%.

We have also developed an alternate long-chain acyl CoA purification scheme aimed at separating long-chain acyl CoA from other lipids, most notably phosphatidylcholine, which is difficult to separate from long-chain acyl CoA species on TLC. Fatty acyl oxoesters in phospholipids are also reduced by sodium borohydride to the corresponding fatty alcohols. This alternate procedure involves an initial TLC separation, which achieves good resolution of fatty acyl CoA (R_F = 0.47–0.51 for C14:0 to C20:4 CoA) from unesterified fatty acids (R_F = 0.86), diacylglycerols (R_F = 0.91), triacylglycerols (R_F = 0.94), phosphatidic acid (R_F = 0.70), cardiolipin (R_F = 0.69), phosphatidylethanolamine (R_F = 0.67) and phosphatidylinositol/phosphatidylserine (R_F = 0.62). Phosphatidylcholine, however, co-migrates (R_F = 0.53) with the long-chain acyl CoA esters. The spot corresponding to long-chain acyl CoA thioesters is scraped, extracted, and digested with phospholipase C (from *B. cereus*) to hydrolyze (>99.8 complete) phosphatidylcholine to diacylglycerol and phosphocholine. Digestion is then followed by another TLC purification. The purified long-chain acyl CoA tioesters are then directly reduced on silica to their corresponding fatty alcohols with sodium borohydride (70% recovery as compared to 55% on Al_2O_3). Fig. 5 shows a standard curve of a mix of long-chain fatty CoA processed in the presence of nmole amounts of lipids. A linear response (ratio of the peak area of the pentafluorobenzoyl derivative of the corresponding fatty alcohol to that of the heptadecyl alcohol internal standard) was obtained in the femtomolar and picomolar range for saturated and unsaturated long-chain acyl CoA esters.

CONCLUSIONS

We have described a GC–MS method to identify and quantitate long-chain fatty alcohols in the subfemtomolar range. Advantages of this simple technique are that it provides identification of each species of long-chain fatty alcohol, is not subject to interferences present with GC–flame ionization detection techniques and is extremely sensitive. Potential applications of this sensitive technique include measurement of the long-chain acyl CoA content of cells and tissues.

ACKNOWLEDGEMENTS

These studies were supported by grants from the U.S. Public Health Service (Grants R01-DK-34388, K04-DK-01553), from the Council for Tobacco Research

(Grant 2364), and from the Juvenile Diabetes Foundation International (Grant 188471). Jane Huth is due special thanks for her expert preparation of the manuscript.

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Journal of Chromatography, 509 (1990) 333–346
Elsevier Science Publishers B.V., Amsterdam

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Preparative separation of sphingolipids and of individual molecular species by high-performance liquid chromatography and their identification by gas chromatography–mass spectrometry

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(First received December 1st, 1989; revised manuscript received February 2nd, 1990)

ABSTRACT

Six fractions containing tri- to pentaglycosylceramides were isolated from the green, fresh water alga *Chlorella kessleri*, grown heterotrophically, by using preparative high-performance liquid chromatography (HPLC). Up to twelve fractions were obtained by further reversed-phase HPLC of each glycosylceramide. The use of a polar capillary column with Supelcowax 10 as the stationary phase allowed an excellent separation of the individual molecular species of ceramides, even though the separation did not occur when the ceramides differed only in the position of the amide bond. The individual molecular species (even if present in mixtures) were identified by gas chromatography–chemical ionization mass spectrometry. The evidence for a complete structure was obtained by enzyme splitting with α- and β-galactosidases (the sequence of monosaccharides) and by negative ionization fast atom bombardment mass spectrometry. More than 400 molecular species of glycosylceramides were identified.

INTRODUCTION

A very interesting group of lipids, so far studied only in mammals, are sphingolipids. Several review papers and monographs have covered their isolation and occurrence[1–3]. The identification of these compounds always includes their separation according to the number of saccharides followed by separation according to the number of carbon atoms in the ceramide part of the molecule. Recently, thin-layer chromatography (TLC) has been superseded by high-performance liquid chromatography (HPLC), which is used either in the normal-phase mode (separation according to the number of monosaccharides) or a modification of reversed-phase (RP) HPLC

0021-9673/90/$03.50

(separation according to the number of carbon atoms in ceramide)[4–10]. The problem of a complete separation into the individual molecular species of glycosylceramides has not yet been satisfactorily tackled, even though the peaks after HPLC were collected and identified by using mass spectrometry (MS)[9] or HPLC couples with MS (LC–MS)[11]. The variability of long-chain bases (LCB) was very small in mammals; in most instances sphingosine prevailed.

In contrast to mammals, the knowledge of sphingolipids in plants and fungi is modest. In a chapter concerning the occurrence of sphingolipids, Hakomori[2] devoted only two pages to plant sphingolipids, in comparison with several tens of pages to animal sphingolipids. In addition, only phosphosphingolipids were included. There are only two reports on glycosylceramides; one of them was discovered in wheat flour and the other in the fungus *Neurospora crassa*[12]. Mono-, di- and trihexosylceramide were isolated from the flour[13], but only trigalactosylglucosylceramide from the fungus. 2-Hydroxy acids and the bases sphingosine and sphinganine represented major components in both the flour and the fungus; phytosphinganine and its C_{20} homologue were another two major compounds identified in the latter material.

In view of the lack of studies on the occurrence of sphingolipids in plants, we attempted to carry out a detailed analysis of these compounds in algae, in which, to our knowledge, sphingolipids had not previously been detected. A knowledge of the occurrence and structure of the sphingolipids molecules can help in understanding phylogenesis of the lower plants to which algae belong.

Working with the green, freshwater alga *Chlorella kessleri*, we previously isolated and identified triacylglycerols[14], wax esters[15] and polar lipids[16]; in this paper, we made a detailed study of sphingolipids present under the conditions of heterotrophic growth.

EXPERIMENTAL

Chemicals

The enzymes α- and β-galactosidase, both from *E. coli*, benzoic anhydride, cellobiose and gluco- and lactocerebrosides were obtained from Sigma ((St. Louis, MO, U.S.A.). Other chemicals were purchased from Lachema (Brno, Czechoslovakia).

Isolation

An amount of 1 kg of the disintegrated, lyophilized biomass of *Chlorella kessleri* obtained from the Department of Autotrophic Microorganisms, Institute of Microbiology, Třeboň, Czechoslovakia, was used for isolation. The extraction procedure according to Bergelson[17] yielded a total of 630 mg of crude sphingolipids.

Derivatization, cleavage and hydrolysis

The total sphingolipids were hydrolysed under alkaline or acidic conditions and, after preparing the methyl esters, were determined by gas chromatography (GC)–MS[2,18,19]. The preparation of the dinitrophenyl (DNP) derivatives of amino alcohols and of FAME (fatty acid methyl esters) from DNP-alcohols was described previously[2]. The preparation of per-O-benzoylglycosphingolipids and their debenzoylation was described by Gross and McLuer[7].

Alditol acetates and partially methylated alditol acetates[2,18], free ceramides (from glycosylceramides) and their trimethylsilyl (TMS) derivatives were prepared by methods described in the literature[2,18,19]. Enzymatic hydrolysis has been described previously[10].

GC–MS

All GC–MS separations were performed by using a Finnigan MAT (San Jose, CA, U.S.A.) 1020 B apparatus with electron impact (EI) or chemical ionization (CI).

Per-TMS-bases. An HP-1, cross-linked methylsilicone (Hewlett-Packard, Palo Alto, CA, U.S.A.) fused-silica capillary column (25 m × 0.2 mm I.D., 0.11 μm film thickness) was used. The injection temperature was 260°C (splitless) and the temperature was programmed from 150 to 300°C at 5°C/min. The linear velocity of the carrier gas (helium) was 45 cm/s and the ionization energy was 70 eV (EI mode).

FAME. A fused-silica capillary column (Supelcowax 10; Supelco, Bellefonte, PA, U.S.A.) (30 m × 0.25 mm I.D., 0.25 μm film thickness) was used. The temperatures were as follows: splitless injection, 240°C; column, 100°C for 1 min, then increased at 20°C/min to 160°C and at 2°C/min to 280°C. The carrier gas was helium at 36 cm/s and the ionization energy was 70 eV (EI mode).

Alditols. Alditol acetates and partially methylated alditol acetates were separated on the HP-1 fused-silica capillary column under the above conditions. For alditol acetates EI was used, and for partially methylated alditol acetates both EI and CI (isobutane; 1.0 Torr) were used.

TMS-ceramides. An injection temperature of 100°C and a Supelcowax 10 column (15 m × 0.25 mm I.D., 0.25 μm film thickness) were used. The temperature programme was as follows: 100°C for 1 min, then increased at 20°C/min to 230°C and at 2°C/min to 280°C, which was maintained for 10 min. The carrier gas was hydrogen at a flow-rate of 120 cm/s. Ammonia (0.6 Torr) was used as the CI reagent gas. The spectra were scanned within the range m/z 250–900.

HPLC

Preparative HPLC was performed with a G-I gradient LC system (Shimadzu, Kyoto, Japan) with two LC-6A pumps (5 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (230 nm), an SIL-1A sample injector and a C-R3A data processor. Preparative columns (25 cm × 21.1 mm I.D.) packed with Zorbax-SIL or ODS (Shimadzu) with 5-μm particles were used.

Per-O-benzoylglycoceramides. These were separated on a preparative column in the normal-phase mode (injection of 27 mg). A linear gradient from 89:10:1 to 50:10:40 hexane–tetrahydrofuran–dioxane in 39 min was used, followed by a 15-min isocratic step. When the analysis was finished, the column was washed with pure dioxane for 30 min and conditioned to the original state by washing with the mobile phase for 1 h. The chromatographic step was repeated 40 times. The individual peaks were collected manually and the solvent evaporated before further use.

RP-HPLC. The individual peaks (A–F) were separated on a preparative column with ODS (see above). The amount injected was 20 mg. A linear gradient from 49:1:50 to 1:49:50 methanol–isopropanol–acetonitrile in 48 min was used, followed by a 15-min isocratic step. When the preparation was finished, the column was washed with pure isopropanol for 30 min and then conditioned with the initial elution

mixture. The individual peaks were collected manually and the solvent evaporated to dryness before further use.

Negative ionization fast atom bombardment MS (NI-FAB-MS)

A 1–5-μg amount of glycosylceramide after debenzoylation [peaks A-4 and (B–F)-6 after RP-HPLC] was dissolved in 10 μl of chloroform–methanol mixture (1:1) and 5 μl of triethanolamine were added. The sample was analysed by using a Hewlett-Packard HP 5988 A apparatus with a FAB source and a high-mass range. Xenon was used as the reaction gas with an energy of 5 keV.

RESULTS AND DISCUSSION

Isolation

Our previous results indicated that the fraction X_{v1} would represent about 0.1% of the total[16], which was consistent with other reports (sum of glycosylceramides in flour = 0.043 ± 0.015% of the total)[13], and consequently a modified procedure of Bergelson[17] was employed to obtain a greater amount of these compounds. To simplify the extraction and reduce the handling of large volumes, a several-fold extraction of the lyophilized biomass was performed (see Experimental). The resulting mass of 630 mg, representing 0.063% of the initial biomass, is in keeping with the above-mentioned data. During the extraction, a partial loss could take place, counter balanced by the subsequent rapid reduction of the volumes handled. If the classical method had been used, volumes of the solvent of up to several tens of litres would have had to be handled, requiring the use of pilot-plant equipment. The mild alkaline hydrolysis is a very suitable step that enabled us to remove most contaminating lipids.

HPLC

Neutral glycosylceramides were mostly isolated by TLC, sometimes after acetylation. As a result of advanced technology, the HPLC of both free sphingolipids and their derivatives, mostly strongly absorbing UV light, has recently been introduced[4–8,10]. As the ceramides had to be in a free form for further analyses, benzoic anhydride, which reacts only with the hydroxy and not the amido groups, was employed for derivatization. The absorption of the benzoyl group at 230 nm is comparable to that of the *p*-nitrobenzoyl groups at 254 nm (ε = 10 000), which exceeds by several orders of magnitude the value for free sphingolipids. Therefore, a wavelength of 210 nm (or preferably even lower) should be used for detection when these compounds are chromatographed.

The analysis of benzoyl derivatives has been reported several times[7,10], but unfortunately not in the preparative or, at least, semi-preparative mode. Therefore, we adapted the classical method of Gross and McCluer[7] by using tetrahydrofuran to prepare a ternary mixture. The addition of tetrahydrofuran resulted in an enhancement of the solubility of benzoyl derivatives, better miscibility of dioxane with hexane and a decrease in the viscosity of the mobile phase. Even though benzoylation was repeated, the expected mass increase was not obtained. Therefore, we assume that other additional lipids are present in crude sphingolipids (alkyl- and alkenylglycerols, sterol glycosides, etc.). The total mass of the peaks A–F (Table I) was 473 mg, which represented a yield of 43.8% (relative to the crude benzoyl derivatives). To obtain this

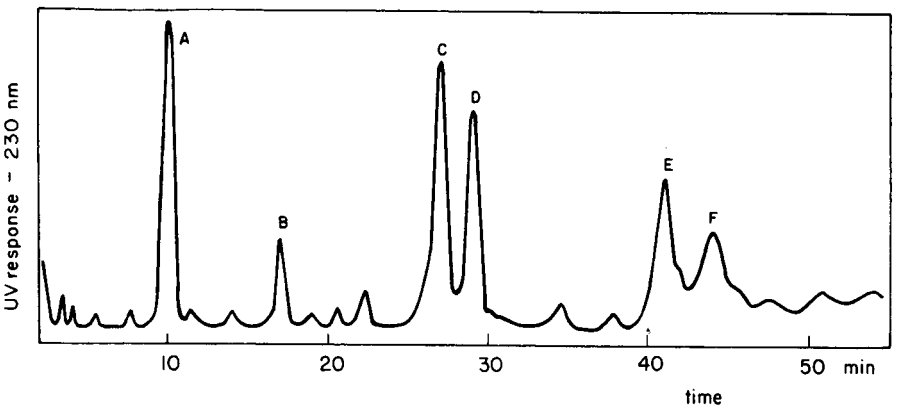

Fig. 1. Preparative HPLC of crude per-O-benzoylglycosylceramides. Peaks A–F are per-O-benzoylglycoceramides. For structures see Table I and for experimental conditions see text.

TABLE I

STRUCTURES AND AMOUNTS OF GLYCOSYLCERAMIDES FROM *C. KESSLERI*

Peak	*Structure of glycosylceramide*	*Proportion (%)*	*Monosaccharides (molar ratio)*	*Structure of partially methylated alditol acetates*
A	Gal–α-1–4-Gal–β-1–4-Glc–1–1-Cer	26.4	Gal,Glc (2:1)	2,3,4,6-Tetra-O-Me-galactitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a]
B	Gal–1–4-Gal–1–4-Glc–1–1-Cer 3 1 Rha	7.2	Gal,Glc,Rha (2:1:1)	2,3,4,6-Tetra-O-Me-galactitol[a] 2,3,4-Tri-O-Me-rhamnitol[a] 2,6-Di-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a]
C	Rha–1–3-Gal–1–4-Gal–1–4-Glc–1–1-Cer	23.2	Gal,Glc,Rha (2:1:1)	2,3,4-Tri-O-Me-rhamnitol[a] 2,3,4,6-Tetra-O-Me-galactitol[a] 2,3,6-Tri-O-Me-glucitol[b] 2,6-Di-O-Me-galactitol[b]
D	Fuc–1–4-Gal–1–4-Gal–1–4-Glc–1–1-Cer	17.8	Gal,Glc,Fuc (2:1:1)	2,3,4-Tri-O-Me-fucitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a]
E	Fuc–1–4-Gal–1–4-Gal–1–4-Glc–1–1-Cer 3 1 Rha	14.2	Gal,Glc,Fuc,Rha (2:1:1:1)	2,3,4-Tri-O-Me-rhamnitol[a] 2,3,4-Tri-O-Me-fucitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a] 2,6-Di-O-Me-galactitol[b]
F	Fuc–1–4-Gal–1–4-Gal–1–4-Glc–1–1-Cer 3 1 Rha	11.2	Gal,Glc,Fuc,Rha (2:1:1:1)	2,3,4-Tri-O-Me-rhamnitol[a] 2,3,4-Tri-O-Me-fucitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a] 2,6-Di-O-Me-galactitol[b]

[a] Identified by standards and GC–MS; Me = methyl.
[b] Identified by GC–MS only.

TABLE II

COMPOSITION OF ALGAL GLYCOSYLCERAMIDES AFTER RP-HPLC

Peak No.	*ECN*	*Glycosylceramides (%)*						*Content of LCB*	*Content of FA*
		A	*B*	*C*	*D*	*E*	*F*		
1	28	5.9	1.0	0.8	0.7	0.9	1.0	15:1 + 16:1 + 18:2	16:1
2	30	10.2	2.4	2.3	1.9	2.5	2.7	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1	18:1 + 16:1
3	32	23.1	3.9	3.5	4.1	4.3	3.9	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1	20:1 + 18:1 + 16:1
4	34	31.5	11.4	14.6	14.1	9.9	10.3	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1	22:1 + 20:0 + 20:1 + 18:1 + 16:1
5	36	22.3	20.3	19.8	22.3	20.1	18.7	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1	24:1 + 22:0 + 22:1 + 20:0 + 20:1
6	38	3.9	22.2	19.4	20.7	25.1	19.9	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1	26:1 + 24:0 + 24:1 + 22:0 + 22:1 + 20:0 + 20:1
7	40	2.0	18.1	18.6	17.4	18.2	19.1	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1 + 24:1	28:1 + 26:0 + 26:1 + 24:0 + 24:1 + 22:0 + 22:1 + 20:0 + 20:1
8	42	0.9	12.4	10.8	12.1	11.7	12.9	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1 + 24:1	30:1 + 28:0 + 28:1 + 26:0 + 26:1 + 24:0 + 24:1 + 22:0 + 22:1 + 20:0
9	44	0	6.2	6.8	5.4	6.1	7.3	16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1 + 24:1	30:1 + 28:0 + 28:1 + 26:0 + 26:1 + 24:0 + 24:1 + 22:0
10	46	0	1.5	2.1	0.9	1.1	1.9	20:1 + 18:0 + 22:1 + 24:1	30:1 + 28:0 + 28:1 + 26:0 + 26:1 + 24:0
11	48	0	0.5	1.0	0.3	0.1	1.4	22:1 + 24:1	30:1 + 28:0 + 28:1 + 16:0
12	50	0	0.1	0.3	0.1	0	0.9	24:1	30:1 + 28:0

amount, the HPLC had to be repeated as many as 40 times (for a chromatogram, see Fig. 1). We tried to increase the mass of the injected mixture, but if the injection exceeded 30 mg of the crude derivatized sphingolipids peaks C–D and E–F were not separated. In contrast, baseline separation was observed on the analytical scale, but the amount of sample injected was about 5 mg. Using this amount for injection would have caused the number of repeated injections to be too high.

Structure of oligosaccharides bound to ceramides

The suggested structures obtained by four independent methods are shown in Table I. First, the proportion of oligosaccharides (*e.g.*, alditol acetate in peaks A–F after normal-phase HPLC; Fig. 1) was determined. Second, partially methylated alditol acetates that were further characterized after capillary GC–MS were prepared by methylation of glycosylceramides followed by hydrolysis, reduction and acetylation of the free hydroxy groups (Table I). The structures of hexitols and 6-deoxyhexitols were determined by using the mass spectra, and those of tetra-O-methyl-Gal, tri-O-methyl-Glc, fucitol and rhamnitol also by comparing their retention times with those of standards. The identification of the inner saccharides (*i.e.*, partially methylated Gal and Glc) was difficult. The tabulated retention data of many tens of derivatives were used for this purpose[20–24].

It was necessary to distinguish only 2,6-di-O-methyl-Gal and 2,3,6-tri-O-methyl-Gal from 2,3,6-tri-O-methyl-Glc, the last of them being available as a standard prepared from cellobiose. Owing to the use of capillary GC–MS, the identification of 2,6-di-O-methyl and 2,3,6-tri-O-methyl alditols was easy. The only problem was to distinguish the 4-linked Gal from Glc. By using the results of McNeil and Albersheim[22], we were able to distinguish these two alditols, employing GC–MS with CI (isobutane). With the Gal derivatives, the proportions of $M+1$, $M+1-32$ and $M+1-60$ ions (M = molecular ion) were 3:1:1, whereas with the Glc derivatives they were 10:1:30. These intensity ratios clearly indicate the suggested structure and are fully consistent with literature data[22].

To obtain independent evidence for the structure of oligoglycosylceramides, we employed NI-FAB-MS[25–27]. During this type of soft ionization, not only fragments were detected (*i.e.*, splitting off the individual saccharides from the molecule) but also a pseudo-molecular ion. Unfortunately, owing to the wide variation of the chain lengths of LCB and FA (fatty acids), peaks A–F could not be used directly for the study and they had to be separated by RP-HPLC [peaks A-4 and (B–F)-6; Table II]. The M peaks overlapped the M − saccharide peaks that had longer LCB and FA. In this way, saccharides having different molecular weights can be distinguished (*e.g.*, Fuc and Glc), but not, for example, Gal and Glc. In spite of this the method yielded excellent results that enabled us to discern peaks E and F. Fig. 2 shows the difference between the spectra of E-6 and F-6, which confirmed the structures given in Table I. In the region below m/z 400, the structure of the spectrum is very complex, resulting from the presence of many ions formed by splitting the saccharide and ceramide part of the molecule. These ions are not essential for the structure determination, so this region was not examined. In this way, the ions representing ceramides became the base peak, in contrast with some other studies[25–28]. Our values determined for the individual ions differ by as many as 2 a.m.u. owing to the isotopic increments. It should be realized that, for a molecule having a molecular weight of about 1400

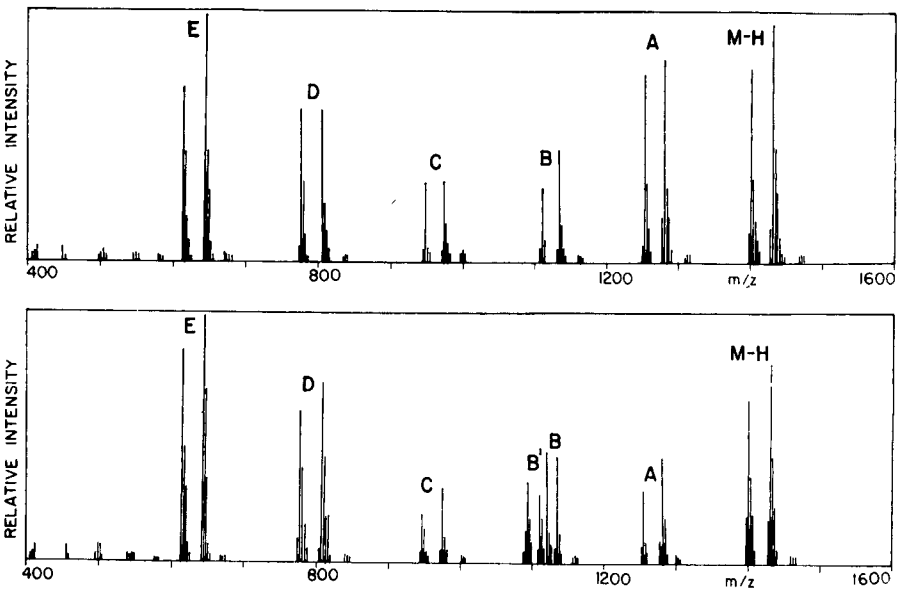

Fig. 2. NI-FAB-MS of peaks F-6 (top) and E-6 (bottom). A = M−H−Fuc or Rha; B = M−H−Fuc−Rha; B′ = M−H−Fuc−Gal; C = M−H−Fuc−Rha−Gal; D = M−H−Fuc−Rha−Gal−Gal; E = M−H − pentasaccharide.

a.m.u., the isotopic increment can be as high as 2 a.m.u. The molecular formula for peaks E-6 and F-6 having LCB 18:1 and FA 24:1 is $C_{72}H_{131}NO_{26}$, *i.e.*, M.W. 1425. $M^+ - H$ has M.W. 1424 whereas, if calculated with the isotopic increment, it is 1426.83.

Another source of information concerning the structure of the oligosaccharides was the enzyme splitting by α- and β-galactosidase. Peak A-4 was found to be suitable for this treatment as the material after the preparation using the normal- and reversed-phase modes amounted to about 39 mg. By using the two enzymes, Gal–Glc–Cer and Glc–Cer were successively obtained; these two compounds had similar chromatographic (HPLC after benzoylation) and mass spectrometric (NI-FAB-MS) properties to the commercial standards. They differed only in the lengths of the LCB and FA chains in ceramide. In our sample, a greater variation in the chain length was observed.

The structures shown in Table I were proposed on the basis of all four methods employed, suggesting that, in all six glycosylceramides, a repeating Gal–Gal–Glc–Cer unit was present, substituted by one or two 6-deoxyhexoses. Our further work will be focused on the complete determination of this structure, including the configuration of the glycosidic bonds and the type (L or D) of the saccharide present.

RP-HPLC

Fig. 3 shows a chromatogram of pentaglycosylceramide (peak F). It clearly indicates that compounds differing in their ECN (equivalent chain number; *e.g.*, chain length minus twice the number of double bonds) were easily separated. Un-

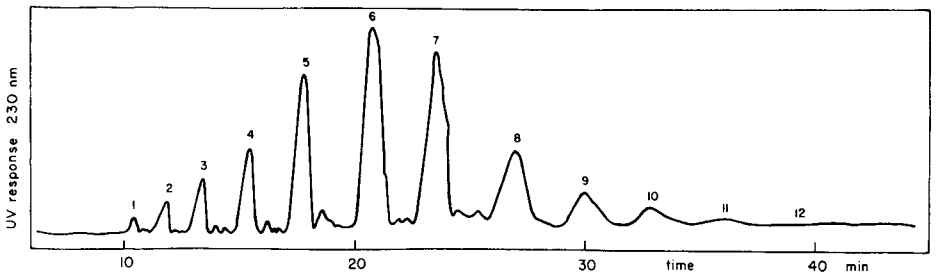

Fig. 3. Semi-preparative RP-HPLC of peak F from Fig. 1. Peaks 1–12 are the molecular species of per-O-benzoylglycoceramides. For structures see Table II and for experimental conditions see text.

fortunately, the great variability in the length of the FA and LCB chains present resulted in the formation of so-called critical pairs when several molecular species were present in one peak, which was indicative of their partial separation. According to the literature, this behaviour can be overcome by changing the temperature[29], by addition of acetonitrile to the mixture[10,30] or by argentation HPLC[31].

In spite of the use of the second method, we were not able to separate the compounds on the semi-preparative scale, which can be explained by the presence of even more than ten molecular species in one peak. The previously described increase in the retention time (tens of minutes)[10], related to the increased number of benzoyl groups in the saccharide, was also observed, but by employing a gradient of the methanol–acetonitrile–isopropanol ternary mixture we were able to eliminate this unfavourable effect almost completely. As a result, *e.g.*, peaks (B–F)-6 had a retention time of 21.3 ± 2.5 min. After collecting all the peaks, acid hydrolysis of the compounds was carried out and the LCB and FA present were identified in each peak. To give a clear arrangement of the results, Table II shows only those LCB and FA whose amounts exceeded 3% of the sum of components of the chromatogram.

Concerning the amount injected, the value of 400 μg indicated in the literature could be exceeded as much as 50-fold, which enabled us to perform a separation on a semi-preparative scale. On using an even greater amount of the compounds separated, overlapping of the peaks was observed[9]. The use of the relative retention time (RRT) in relation to the ECN of the FA and LCB separated, however, did not improve the identification of the individual glycosylceramides because of the great variability in the chain length. Therefore, in addition to the above-mentioned hydrolysis, the collected peaks were also debenzoylated and the free glycosylceramides oxidatively split to obtain ceramides. Periodic acid was chosen as the most suitable reagent for this splitting[18,19]. As other degradations took place during this step, only the peaks isolated by RP-HPLC representing more than 10% of the total amount were identified by GC–MS (Tables III and IV).

GC–MS

The GC of ceramides in the form of their derivatives [most often TMS or *tert.*-butyldimethylsilyl (*t*-BDMS)] has been performed on packed columns[32,33]. Unfortunately, by using these columns, the compounds were separated only in relation to the number of carbon atoms and not the number of double bonds in the molecule. A similar result was obtained when capillary columns with a non-polar stationary

TABLE III

MAJOR CERAMIDES AFTER DEGRADATION IDENTIFIED BY POLAR CAPILLARY GC–MS

Glycosylceramide after HPLC and RP-HPLC		*Proportion (%)*	*LCB-FA Composition*[b]	
A-2	32:1[a]	1.9	16:0–16:1	
	32:2	3:2	15:1–18:1	
	i-34:2	6.7	i-18:1–16:1	
	34:2	85.2	16:1–18:1/18:1–16:1	M/L
	36:3	3.0	18:2–18:1	
A-3	34:1	3.7	16:0–18:1/18:0–16:1	S/M
	35:2	7.4	15:1–20:1	
	i-36:2	3.6	i-18:1–18:1	
	36:2	78.3	16:1–20:1/18:1–18:1/20:1–16:1	L/L/S
	38:3	7.0	18:2–20:1	
A-4	35:1	2.0	15:1–20:0	
	36:1	10.6	16:1–20:0	
	36:1	1.8	16:0–20:1/18:0/18:1	M/S
	37:2	5.3	15:1–22:1	
	i-38:2	4.0	i-18:1–20:1	
	38:2	1.9	18:2–20:0	
	38:2	69.5	16:1–22:1/18:1–20:1/20:1–18:1/22:1–16:1	L/L/S/S
A-5	36:0	0.5	16:0–20:0	
	37:1	1.1	15:1–22:0	
	i-38:1	1.6	i-18:1–20:0	
	38:1	22.1	16:1–22:0/18:1–20:0	L/L
	38:1	2.3	18:0–20:1/16:0–22:1	L/L
	39:2	3.1	15:1–14:1	
	i-40:2	4.3	i-18:1–22:1	
	40:2	1.0	18:2–22:0	
	40:2	61.1	16:1–24:1/18:1–22:1/20:1–20:1	L/L/S
	42:3	2.9	18:2–24:1	

[a] First number, number of carbon atoms in the chains; second number, number of double bond(s); i = isoacid.

[b] S = Small, M = medium, L = large (intensity according to ions $M - a_x$, see text).

phase were employed[34–36]. Much better separation was achieved on polarizable or polar capillary columns, using both the high-temperature phase RSL-300[34] (methylphenylsilicone) or RTX 2330 (cyanopropylsilicone)[37,38]. Unfortunately, no chromatogram of the separation of a mixture of di-TMS-ceramides was published in either paper by Myher *et al.*[37,38]. Thus, only the chromatogram from an RSL-300 column remains[33].

Ceramides were baseline separated, even 16:1–24:1 and 18:2–22:0, *e.g.*, the compounds having identical ECN (36). As can be seen in Fig. 4, we were able to separate on the baseline even the molecular species differing only in the presence of a double bond in LCB or FA, *e.g.*, the peaks 40:1 (16:1–24:0 and 16:0–24:1). The peaks containing branched LCB were also easily separated from those of compounds having a straight chain in LCB. Very similar molecular species may thus be discerned by chromatography on a polar capillary column.

The mass spectra of ceramides, mainly their splitting by electron-impact ionization (EI-MS), were studied in detail[32,33]. Splitting in the region of the molecular

TABLE IV

MAJOR CERAMIDES (FROM PENTAGLYCOSYLCERAMIDE = F) AFTER DEGRADATION, IDENTIFIED BY POLAR CAPILLARY GC–MS

Glycosylceramide after HPLC and RP-HPLC		*Proportion (%)*	*LCB-FA composition*[b]	
F-4	35:1[a]	2.0	15:1–20:0	
	36:1	10.6	16:1–20:0	
	36:1	1.8	16:0–20:1/18:0–18:1	L/M
	37:2	5.3	15:1–22:1	
	i-38:2	4.0	i-18:1–20:1	
	38:2	69.5	16:1–22:1–20:1/20:1–18:1/22:1–16:1	L/L/S/S
	40:3	4.9	18:2–22:1	
F-5	36:0	0.5	16:0–20:0	
	37:1	1.0	15:1–22:0	
	38:1	5.3	16:1–22:0	
	38:1	2.2	16:0–22:1	
	i-38:2	3.5	i-18:1–20:1	
	38:2	15.9	18:1–20:1	
	39:2	3.8	15:1–24:1	
	40:1	2.6	18:0–20:1	
	i-40:2	4.9	i-18:1–22:1	
	40:2	0.9	18:2–22:0	
	40:2	55.7	16:1–24:1/18:1–22:1/20:1–20:1	M/L/S
	42:3	3.7	18:2–24:1	
F-6	38:0	0.7	16:0–22:0/18:0–22:0	L/L
	39:1	2.1	15:1–24:0	
	i-40:1	1.5	i-18:1–22:0	
	40:1	27.9	16:1–24:0/18:1–22:0/20:1–20:0	L/L/S
	40:1	3.2	16:0–24:1/18:0–22:1	L/M
	41:2	1.7	15:1–26:1	
	i-42:2	4.4	i-18:1–24:1	
	42:2	2.0	18:2–24:0	
	42:2	54.9	16:1–26:1/18:1–24:1/20:1–22:1/22:1–20:1	M/L/S/S
	44:3	1.6	18:2–26:1	
F-7	40:0	1.2	16:0–24:0/18:0–22:0	L/L
	41:1	1.6	15:1–26:0	
	i-42:1	4.4	i-18:1–24:0	
	42:1	54.6	16:1–26:0/18:1–24:0/20:1–22:0/22:1–20:0	M/L/S/S
	42:1	3.6	16:0–26:1/18:0–24:1	M/L
	43:2	0.9	15:1–28:1	
	i-44:2	2.1	i-18:1–26:1	
	44:2	1.5	18:2–26:0	
	44:2	29.3	16:1–28:1/18:1–26:1/20:1–24:1/22:1–22:1/24:1–20:1	M/L/M/M/S
	46:3	0.8	18:2–28:1	
F-8	42:0	1.3	16:0–26:0	
	43:1	1.7	15:1–28:0	
	i-44:1	4.5	i-18:1–26:0	
	44:1	49.6	16:1–28:0/18:1–26:0/20:1–24:0/22:1–22:0/24:1–20:0	S/L/M/S/S
	44:1	3.8	16:0–28:1/18:0–26:1	M/L
	45:2	0.8	15:1–30:1	
	i-46:2	2.5	i-18:1–28:1	
	46:2	1.6	18:2–28:0	
	46:2	33.5	16:1–30:1/18:1–28:1/20:1–26:1/22:1–24:1/24:1–22:1	M/L/S/M/S
	48:3	0.7	18:2–30:1	

[a,b] See Table III.

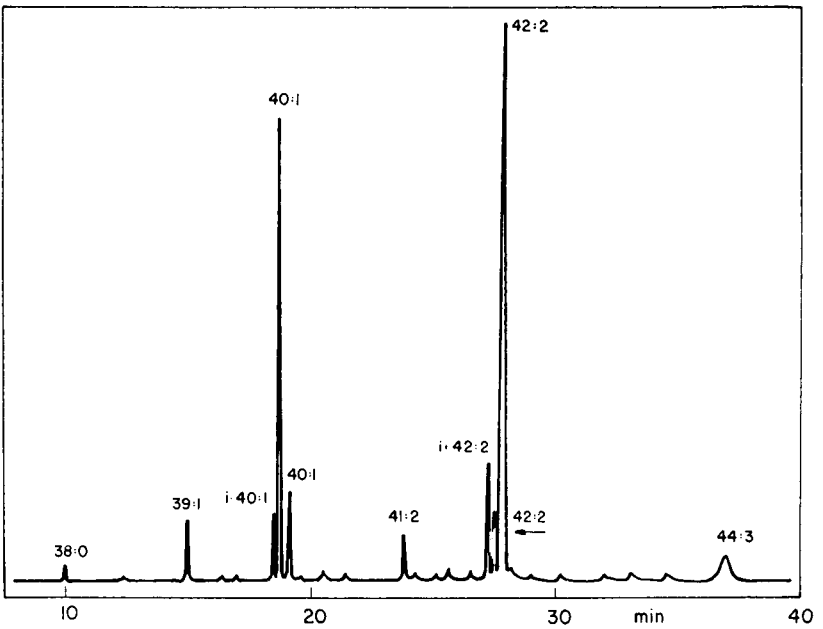

Fig. 4. GC–MS of di-O-TMS-ceramides from glycosylceramides (peak F-6, Fig. 3) after degradation, on a polar column. For conditions see text.

ion (M) resulting in the formation of the ions M − 15, M − 90 and M − 103 was observed, M being mostly undetectable. Another very important group of the ions are those formed through cleavage of the molecule, *e.g.*, the splitting of a C–C bond next to the carbon atoms bearing heteroatoms[32,33].

Being fully aware of the limitations of EI-MS, Oshima *et al.*[39] used GC–CI-MS for the detection of various molecular species as early as 1977. Using this type of ionization, M or, more exactly, the quasi-molecular ion (QM) is always detectable and its intensity depends on the kind of ionizing gas employed. In our case, ammonia was used, QM becoming the base peak. The structure of the other ions is in keeping with the literature data[32,33,39]. The $M - a_x$ ions (see Fig. 5), their structures in the literature[31,32,38] enabled us to determine the structure of the amide-bound FA, whereas the ions $M - d_x$ (see Fig. 5) indicated the numbers of carbon atoms and double bonds present in the molecule of LCB. The interpretation of the data was much hindered by the presence of the isotopic increase. Fig. 5 shows the mass spectrum of the peak 42:2 from Fig. 4. The corresponding molecular weight was 791. Taking into account the isotopic increase, the value was, however, 792.5, which affected the QM value, giving 810.5 a.m.u. On the basis of splitting in MS, we sought not only to identify the individual peaks but also, as far as the peaks containing several molecular species were concerned, to assess their quality and quantity. As far as the qualitative determination is concerned, the length and the degree of unsaturation of the chains of LCB and FA could have been determined on the basis of the ions M − a and M − d. However, to our knowledge, no one has been able to separate ceramides of the same molecular weight, whose LCB and FA would have different

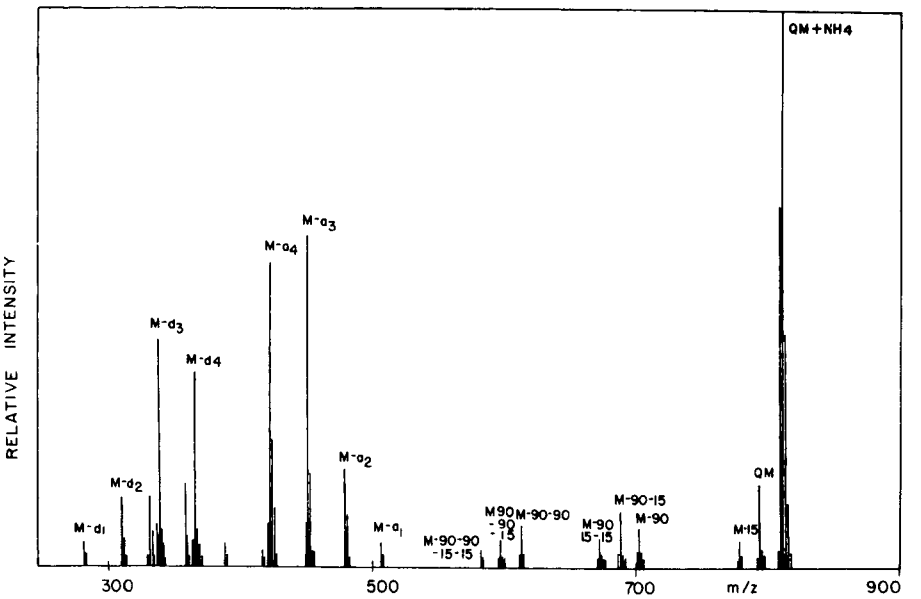

Fig. 5. Mass spectrum of peak 42:2 (major) from Fig. 4. For explanation see text.

chain lengths. We presume that the problem in the separation of these critical pairs is similar to that of wax esters, where some progress has recently been made. Under special conditions, esters differing from each other in the length of the alcohol and acid moieties by more than two carbon atoms can now be separated[15,40].

Irrespective of the fact that we sought to apply previous experience, we were not able to separate the critical pairs in the natural sample. We assume that we could be successful with a synthetic mixture, such as LCB-FA 16:0–20:0 or 20:0–16:0. As far as the proportion of the molecular species is concerned, we attempted to quantify approximately the species present (small, medium, large); however, for a precise determination of the proportion of the individual molecular species, tens of standards would have been necessary for the calibration. In this respect, we do not fully agree with the attempt of Myher *et al.*[35] to assess the amounts of the molecular species on the basis of GC–MS without an adequate calibration.

The combined use of HPLC, RP-HPLC and the polar capillary column GC–MS together with enzyme splitting, TLC and NI-FAB-MS enabled us to identify more than 400 molecular species of sphingolipids, or more precisely glycosylceramides. It is clear that they represent only major molecular species. We conclude that such a combination of modern analytical methods will help to give a better understanding of the structure of lipids and that this complex approach can be also applied to other types of biological material.

ACKNOWLEDGEMENT

We are grateful to Miss Zuzana Dobiášová for excellent technical assistance with the preparative HPLC.

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Journal of Chromatography, 509 (1990) 347–356
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Analysis of δ-L-α-aminoadipyl-L-cysteinyl-D-valine by ion chromatography and pulsed amperometric detection

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(First received September 22nd, 1989; revised manuscript received February 13th, 1990)

ABSTRACT

A novel high-performance liquid chromatography (HPLC) method is presented for the detection and trace level determination of the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV). The tripeptide, an intermediate in penicillin production, is derived from fungal fermentation. The technique relies on ion-exchange separation of the tripeptide on an anion-exchange column followed by detection by reduction on a gold electrode using pulsed amperometry. The sensitivity of direct determination of ACV is increased by employing pulsed amperometric detection (PAD) over direct ultraviolet detection. Choice of the working electrode and optimisation of electrode potentials was based on cyclic voltammograms recorded for the tripeptide in the mobile phase.

A linear regression equation was obtained over the range 0–100 μg ml^{-1}. The detection limit in fermentation broths was found to be 0.1 μg ml^{-1} whereas in buffer the detection limit was found to be 10 ng ml^{-1}. A good correlation coefficient was observed when ACV concentrations determined by ion chromatography–PAD were compared with measurements obtained by pre-column derivatisation with fluoromethylorthochloroformate followed by HPLC separation on a reversed-phase C_{18} silica column with UV detection.

The procedure has been applied to the measurement of natural levels of ACV in fermentation broths of selected strains of *Aspergillus nidulans* and *Penicillin chrysogenum*.

INTRODUCTION

Many of the classical β-lactam sulphur-containing antibiotics are derived from the same amino acid precursors, L-cysteine, D-valine and α-L-aminoadipic acid. In certain micro-organisms these amino acids form the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV), the condensation reaction being catalysed by ACV synthetase[1]. The biosynthetic pathway for the production of penicillin antibiotics in penicillium and cephalosporium species is now well established[2], and the production of ACV in fermentations represents the committed step in the production of these

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antibiotics. As such, its determination is important in the monitoring of antibiotic fermentations, the study of novel β-lactam-producing organisms and the assessment of blocked mutants. The available methods for the determination of ACV by high-performance liquid chromatography (HPLC) rely upon direct ultraviolet (UV) detection[3] or pre-column and post-column derivatisation[4,5] followed by UV or fluorescence detection.

Direct UV detection of ACV is limited in sensitivity because ACV is a poor chromophore with little absorbance above 210 nm. Derivatisation techniques are much more sensitive, although they are difficult to automate and can yield anomalous results when applied to ACV derived from fermentation broths containing large amounts of other derivatisable compounds. Pre-column derivatisation methods also have a major disadvantage in that they require several time-consuming pre-treatment steps which introduce inaccuracies and imprecision into the analysis.

ACV exists in two forms: within the fungal hyphae it is present as a monomer and, on release from the hyphae into the external medium, it is rapidly oxidised to form a dimer in which two tripeptide molecules are linked by a disulphide bond. Recently, sensitive HPLC methods have become available in which detection is based upon the electrochemical activity of the analytes[6,7]. In this report, the basis for the detection of ACV dimer is the reduction of the disulphide bond. Many organic materials have been studied using solid electrodes, however, sulphur-containing compounds rapidly poison noble-element electrodes due to strong adsorption[6]. With pulsed amperometric detection (PAD) the working electrode undergoes a cycle of reverse polarisation steps that serves to detect, clean and regenerate the surface of the electrode.

This report has set out to optimise the electrode materials, potentials and pulse durations for PAD of ACV and to use the optimised conditions for the determination of ACV in fermentation broths. The technique has been applied to the assay of ACV produced in fermentation broths by several fungal species which exhibited a wide variation in the levels of ACV production.

EXPERIMENTAL

Fungal strains

Penicillium chrysogenum strain P2 (kindly donated by Pan Labs.) is a fungal strain which has been used in the industrial production of penicillin antibiotics. The strains were grown in 25 ml of a defined medium described by Jarvis and Johnson[8] for three days in an orbital shaker at 150 rpm and 27°C. *Aspergillus nidulans* wild type is a fungus that produces low levels of penicillin[1]. *A. nidulans* was grown in Aspergillus Complete Medium (ACM)[9] for three days in an orbital shaker (150 rpm at 27°C) at which point the fermentation broths were assayed. *A. niger*, a fungal strain that does not produce penicillin, was used as a blank for recovery and spiking experiments. This species was grown in an orbital shaker (150 rpm at 27°C) in 25 ml of ACM for three days. All cultures were filtered through 0.22-μm Durapore (Millipore Waters Chromatography) filters prior to analysis.

Reagents

Mobile phase solutions consisting of 100 m*M* sodium hydroxide (BDH, Poole,

U.K.) and 25 m*M* sodium acetate (Sigma) were prepared by dilution from a stock solution of 12.5 *M* sodium hydroxide and a stock solution of 1 *M* sodium acetate. HPLC-grade water was prepared from a Nanopure II water system (Barnstead). A standard sample of ACV dimer was a gift obtained from Glaxo U.K. Fluoromethylorthochloroformate (FMOC) was purchased from Sigma (Poole, U.K.). Acetonitrile and acetone, used in HPLC mobile phases and the preparation of the FMOC reagents, were HPLC grade and purchased from Rathburn (Walkerburn, U.K.). Boric acid, sodium dihydrogenphosphate and sodium hydroxide, used for the preparation of buffer solutions, were obtained from BDH and were AnalaR grade. Pentane was purchased from BDH and was chromatographic grade.

Chromatographic apparatus

Equipment supplied by Dionex U.K. used for ACV analysis was based on a Dionex BioLC quaternary gradient pump. The anion-exchange column employed for the separation of ACV was an AS6 column with an AG6 guard column containing the same stationary phase. The detector used was a Model PAD-2 pulsed amperometric detector operated with a gold working electrode. The working electrode potentials used were $E_1 = -0.8$ V, $E_2 = 0.05$ V, $E_3 = -0.95$ V, where E_1 was the sampling voltage, and E_2 and E_3 cleaning and regenerating voltages, respectively, and the durations of these potentials were 300, 60 and 60 ms, respectively. An Ag/AgCl reference electrode was employed.

The eluents were sparged and pressurised with helium using a Dionex Eluent degas unit. Sample injection was achieved using a Dionex autosampler module with a 50-μl injection volume. Chromatographic data were collected and analysed with a Trio 2 data station.

Bioassay of recovered ACV

Bioassays were carried out by a well-plate assay method with partially purified isopenicillin synthetase derived from *P. chrysogenum*[10,11]. The reaction mixture consisted of lyopholized HPLC eluent fractions resuspended in 50 μl of 3-(N-morpholino)propanesulphonic acid (MOPS) buffer (pH 8, 0.1 *M*), 10 μl of dithiothreotol (2 m*M*) and 10 μl of iron sulphate (0.1 m*M*), all purchased from Sigma. To this mixture 160 μl of the enzyme preparation were added and the reaction was allowed to proceed for 10 min at 30°C in 10 cm × 1.4 cm test tubes gently shaken in a reciprocating water bath. Finally, the reaction was quenched by the addition of 10 μl of disodium ethylenediaminotetraacetic acid (40 m*M*).

The production of isopenicillin N was then bioassayed with *Staphylococcus aureus* (strain 750), grown overnight at 36°C for 12 h, by means of the well-plate assay, using 6-mm diameter plug holes.

Cyclic voltammetry

Cyclic voltammetry was carried out with gold, glassy carbon and platinum electrodes. The equipment supplied by Ursar Scientific Equipment (Cannington, Oxford, U.K.) could be ramped between two set voltage values over a variable time period. In this way the rate of voltage change could be varied. The voltage change was measured against a standard calomel electrode.

RESULTS AND DISCUSSION

Calibration and limits of detection

Calibration over the range 0.35–100 μg ml^{-1} ACV in buffer at a sensitivity setting of 1000 nA on the pulsed amperometric detector gave the following linear regression equation: concentration (μg ml^{-1}) $= -1.2 + 2.2 \cdot 10^{-4}$ area counts (arbitrary units).

The limits of detection of ACV in fermentation broth, with a detector sensitivity of 1 nA, was 0.1 μg ml^{-1} (signal-to-noise ratio of 2:1). The calibration range for ACV spiked into fermentation media derived from *A. niger* (ACV-free) was linear between 0.1 and 100 μg ml^{-1}. The linear regression equation obtained for this concentration range took the form: concentration (μg ml^{-1}) $= -1.5 + 3.3 \cdot 1.0^{-4}$ area counts.

When solutions of ACV dissolved in phosphate buffer (pH 9, 10 m*M*) were analysed on a day-to-day basis the correlation coefficients for respective linear calibration equations were in the range 0.954–0.993.

The technique of anion-exchange chromatography has been applied to amino acid analysis[12]. However, amino acid analysers of this type require post-column derivatisation facilities. The detection of amino acids is usually based upon there reaction with orthophthaldialdehyde[13] or ninhydrin[12]. There are now alternative

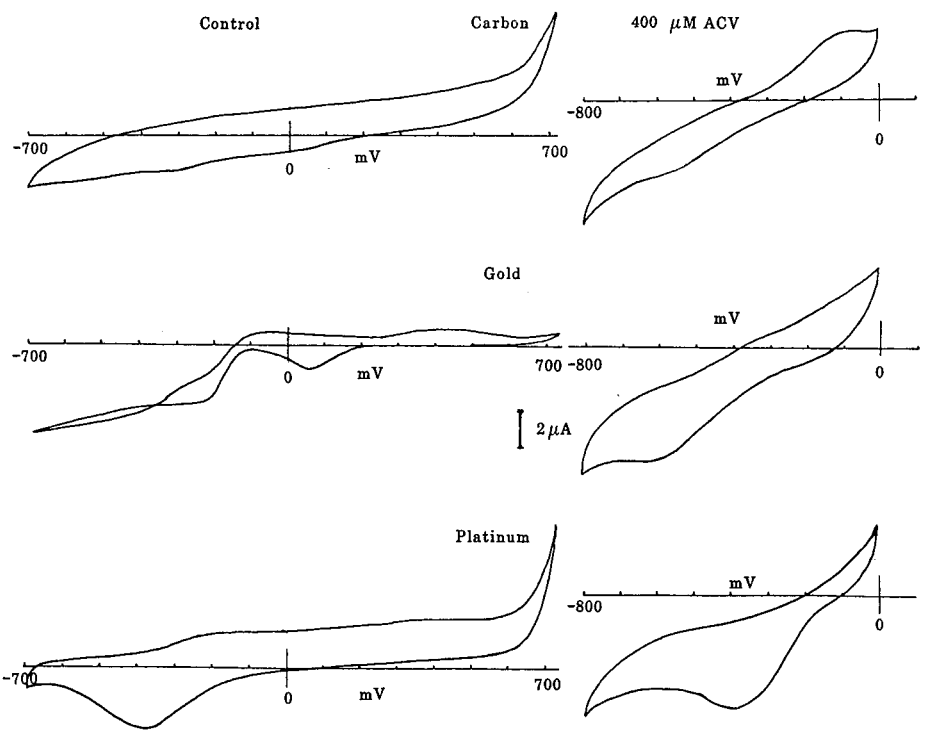

Fig. 1. Cyclic voltammograms for IC buffer and ACV (1.46 mg ml^{-1}) dissolved in IC buffer for different electrode materials. Conditions: scan rate, 20 mV s^{-1}; 25 m*M* sodium acetate, 100 m*M* sodium hydroxide.

pre-column derivatisation methods available for the analysis of amino acids based upon their reaction with FMOC[3], dansylaziridine[5] and phenylisothiocyanate[14]. These methods unfortunately have specific drawbacks in that they either require post-column pumps and reaction coils or require time-consuming sample clean-up, derivatisation and extraction steps.

PAD of biological materials has been demonstrated to be a versatile, sensitive technique[6,7]. This type of detection is based upon either oxidation or reduction at an electrode. The technique can be made selective for particular analytes by operating at electrode potentials at which these analytes are electroactive and interfering components are inactive.

Fig. 1 illustrates the cyclic voltammograms for different electrode materials in the ion chromatography (IC) mobile phase and the mobile phase containing dissolved ACV. Only the gold electrode shows a significantly different cyclic voltammogram for the IC buffer after the addition of ACV. A prominent trough is observed at −600 mV using the gold electrode, whereas glassy carbon only showed a small change in current at −550 mV with ACV present. No detectable change in the cyclic voltammogram for the reference solution and for the ACV solution was observed with the platinum electrode.

Fig. 2 shows the effect of the rate of change of applied potential on the observed current–voltage plot for ACV using a gold electrode. The diagram illustrates that the electrolytic reduction of ACV is a comparatively slow reaction. This necessitates a long E_1 value of 300 ms for the operation of the pulsed amperometric detector to improve the sensitivity of the detection system.

Fig. 3 shows the detector response at different electrode potentials for the reduction of ACV. The final electrode potential of −0.8 V was chosen to give a relatively simple chromatogram, with good separation between analytes and contaminating peaks, for the analysis of ACV in complex fermentation broths. When electrode potentials below −0.9 V were used, a high background noise was observed and other components in the broths, which eluted close to ACV, were detected.

IC patterns of a standard solution of ACV and samples of fungal broths containing ACV are shown in Figs. 4–6. The identity of the ACV peak was confirmed by collecting the column eluate and subjecting the fractions to enzymic reaction; the

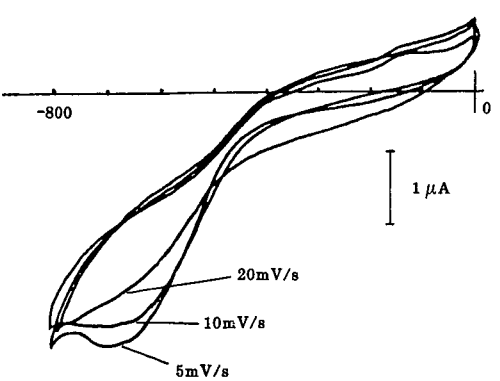

Fig. 2. Effect of voltage scan rate on the response of a gold electrode in the presence of 1.46 mg ml^{-1} ACV dissolved in IC buffer.

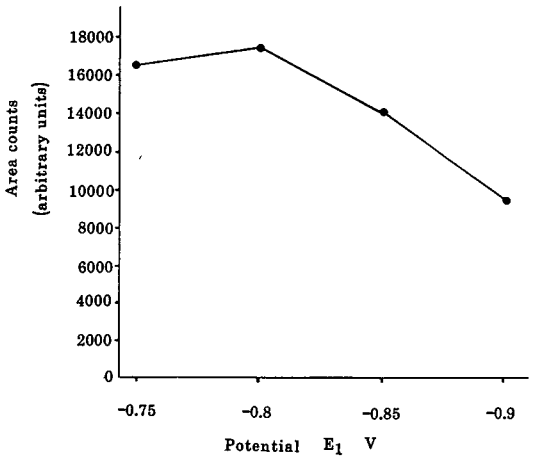

Fig. 3. Detector response for 83 μg ml^{-1} ACV at different E_1 potentials. Conditions: 100 mM sodium hydroxide, 25 mM sodium acetate with a flow-rate of 1 ml min^{-1}, E_1 potentials: -0.75, -0.8, -0.85 and -0.9 V. The potentials E_2 and E_3 were kept constant at 0.05 and -0.95 V, respectively.

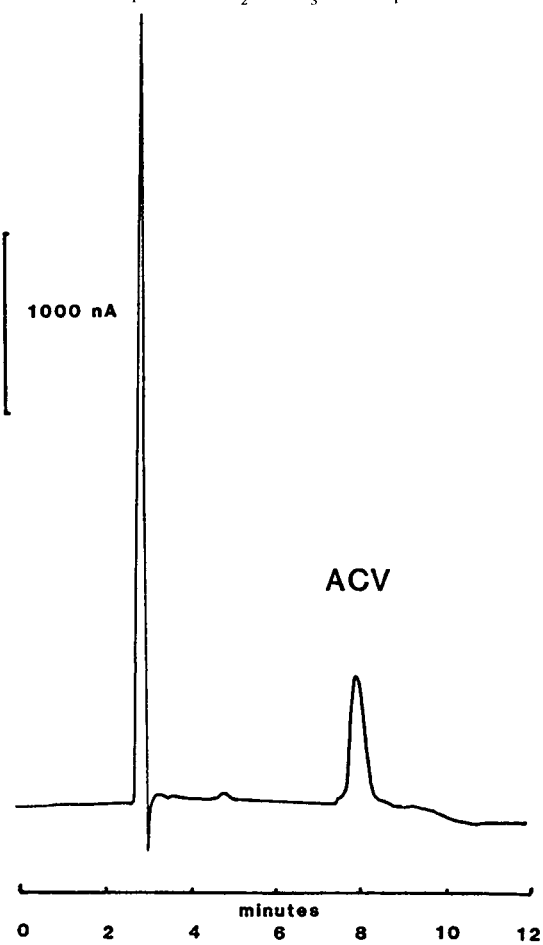

Fig. 4. IC analysis of a standard solution of ACV (300 μg ml^{-1}) determined by IC–PAD. Chromatographic conditions: 100 mM sodium hydroxide, 25 mM sodium acetate with a flow-rate of 1 ml min^{-1}. The chromatography was performed on a AS6 ion-exchange column with an AG6 guard column. The pulsed amperometric detector settings were $E_1 = -0.8$ V, $E_2 = 0.05$ V and $E_3 = -0.95$ V.

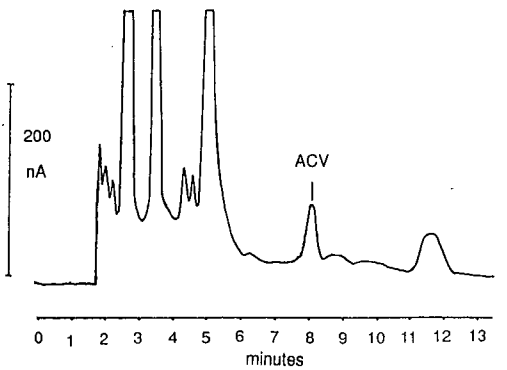

Fig. 5. IC analysis of *P. chrysogenum* grown in shake flasks and assayed for extracellular ACV concentration on day 3 of fermentation. The ACV peak illustrated represents a concentration of 12 μg ml^{-1}. Chromatographic conditions were as described in Fig. 4.

production of isopenicillin was then assayed by a standard well-plate assay technique. The relative simplicity of the chromatograms is an indication of the high selectivity with PAD. *A. nidulans* represents a species of fungus that produces β-lactam antibiotics and β-lactam biosynthetic pathway intermediates at very low extracellular concentrations. To detect and quantify ACV at these low concentrations in broths, derivatisation was previously necessary[4,5]. The IC method presented here allows direct detection of ACV in fermentation broths at concentrations down to 0.1 μg ml^{-1}. *P. chrysogenum* is an industrially important organism used for the production of β-lactam antibiotics. The analysis of ACV derived extracellularly from this species

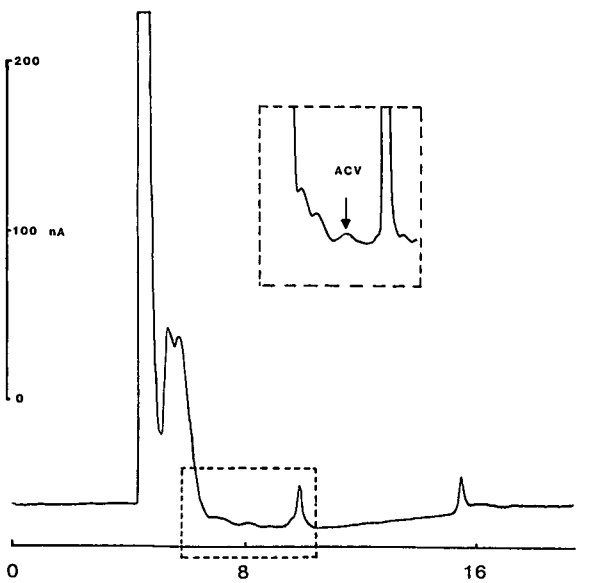

Fig. 6. IC analysis of a fermentation broth derived from *A. nidulans* for the production of extracellular ACV after 72 h of growth. Chromatographic and column conditions were as described in Fig. 4.

TABLE I

RELATIVE REPRODUCIBILITIES FOR THE IC–PAD ANALYSIS OF ACV PRODUCED NATURALLY IN FERMENTATION BROTHS DERIVED FROM PENICILLIN-PRODUCING ORGANISMS.

Chromatographic conditions are as described in Fig. 4.

Sample	*Concentration (mean ± S.D.) (μg ml^{-1})*	*Coefficient of variation (%)*
A. nidulans		
GH 108	2.6 ± 0.14	5.4
GH 1	3.8 ± 0.08	2.1
P. chrysogenum		
P2	56.8 ± 0.70	1.2

of fungus is an important parameter in the monitoring of β-lactam antibiotic production during fermentation processes. Table I shows results obtained for typical fermentation analysis of β-lactam-producing organisms.

IC separation and PAD of ACV has several advantages over previously reported techniques of ACV determination. The method is simple to operate, with a relatively short analysis time (20 min) and lends itself to automation for the analysis of large numbers of samples. Fig. 7 shows the linear regression line for two sets of data for the determination of ACV by the IC–PAD and by a previously reported

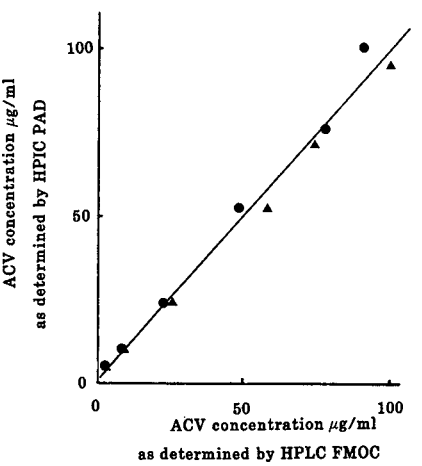

Fig. 7. Correlation between two different techniques for the detection of ACV in buffer. (●) ACV determined by FMOC derivatisation followed by HPLC with UV detection; (▲) ACV determined by IC–PAD. Chromatographic conditions are as described in Fig. 4 for IC–PAD and by Shah and Adlard[4] for FMOC HPLC determination of ACV. Standard solutions containing ACV at different concentrations were determined experimentally by both procedures. The experimentally determined concentrations were then used in regression analysis.

TABLE II

DETERMINATION OF ACV IN FERMENTATION BROTH

ACV was added to blank broth derived from *A. niger* in a range of concentrations after fermentation. Peak areas were used to determine experimentally the ACV concentration. Chromatographic conditions are as described in Fig. 4.

Sample No.	*ACV concentration (μg ml^{-1})*		*Recovery (%)*
	Theoretical	*Experimental*	
1	10	9.8	98.0
2	25	25.9	103.6
3	50	48.1	96.2
4	70	69.3	99.0
5	110	108.0	98.2

technique[4]. The correlation between this IC–PAD method of determining ACV concentrations and the alternative FMOC-HPLC method described by Shah and Adlard[4] gave a correlation factor of 0.947 for standard ACV solutions in buffer (see Fig. 7). The procedure of Shah and Adlard[4] was carried out as described with the exception that detection was achieved by UV absorbance at 275 nm in place of fluorimetric detection. The data presented in Table II indicate that this analytical procedure provides an acceptable level of precision and reproducibility.

CONCLUSION

The IC analysis of ACV in fungal fermentation broths has been demonstrated. The detection of ACV using PAD has been optimised on a gold electrode, and the method relies on the reduction of ACV at the electrode. IC determination of ACV gives good limits of detection for ACV in broths derived from fungal fermentations and compares well with conventional pre-column derivatisation methods for the analysis of ACV.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the assistance of Drs. Cass and Dachyar at Imperial College, (London, U.K.). This work was supported by an SERC Grant GR/D73850.

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Journal of Chromatography, 509 (1990) 357–368
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 386

Separation and indirect detection of small-chain peptides using chromophoric mobile phase additives

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(First received June 2nd, 1989; revised manuscript received February 27th, 1990)

ABSTRACT

Ruthenium(II) 1,10-phenanthroline, $Ru(phen)_3^{2+}$, and ruthenium(II) 2,2′-bipyridyl, $Ru(bipy)_3^{2+}$, salts were evaluated as mobile phase additives for the liquid chromatographic separation of small-chain peptides on a polystyrene–divinylbenzene copolymeric (Hamilton PRP-1) stationary phase. In a basic mobile phase peptides are anions, and retention, resolution and detection occur because of the interactions between the stationary phase, the Ru^{II} complex and the peptide anion. Since the Ru^{II} complex concentration changes in the analyte band relative to the background eluent Ru^{II} complex concentration, the peptide can be detected by indirect photometric detection using the wavelength where the Ru^{II} complex absorbs. Peptide analyte peaks may be positive or negative depending on the counter-anion and its concentration. Small-chain peptides that do not contain chromophoric side-chains are detected without derivatization at about 0.1 nmol injected at a 3:1 signal-to-noise ratio. Factors that affect retention, resolution and indirect photometric detection are the Ru^{II} complex, its mobile phase concentration, mobile phase pH and solvent composition, and the type and concentration of the mobile phase counter-anion and/or buffer anion.

INTRODUCTION

Three liquid chromatographic (LC) strategies are routinely used for the separation of small-chain peptides. Anion and cation exchangers can be used to separate peptides as anions or cations, respectively[1–3]. A second approach relies on a reversed stationary phase for the separation[4–9]. Mobile phase pH and solvent composition are the major parameters adjusted to affect peptide retention, resolution and elution. The third general strategy is to use a charged hydrophobic ion as a mobile phase additive in combination with a reversed stationary phase[10–13]. Charged peptides are retained because of peptide–hydrophobic ion–stationary phase interactions where quaternary ammonium salts are used for anionic peptide separations and alkyl sulfonate or sulfate salts are used for cationic peptides. Mobile phase parameters, such as pH, buffer ion and counter-ion concentration, hydrophobic ion

0021-9673/90/$03.50

structure and concentration, and solvent composition, are manipulated to enhance peptide retention and improve resolution. In all three strategies the amino acid side-chain structure in the peptide chain strongly influences the peptide elution order. These three approaches have been used in a variety of applications and are reviewed elsewhere[1–13].

If the small-chain peptide contains a subunit with a UV chromophoric side-chain, detection is possible at 250–290 nm. In the absence of the chromophore, detection is possible at a low UV wavelength due to the amide and carboxyl groups. However, the low wavelength also limits the kind and concentration of mobile phase components. Recently, it was shown that analyte anions can be separated on reversed stationary phases and indirectly detected by using 1,10-phenanthroline or 2,2′-bipyridyl metal complexes as mobile phase additives[14–16]. The complex is an ion interaction (pairing) reagent that enhances the analyte anion retention, and by manipulation of the mobile phase parameters complex mixtures of analyte anions can be resolved. Since the complexes are chromophoric and the amount of complex in the analyte band changes in proportion to the amount of analyte relative to the background complex concentration in the mobile phase, indirect photometric detection (IPD) is possible[15]. If the fluorescent Ru^{II} complexes are used indirect fluorometric detection (IFD) is possible[17].

This report focuses on using ruthenium(II) 1,10-phenanthroline ($Ru(phen)_3^{2+}$) and ruthenium(II) 2,2′-bipyridyl ($Ru(bipy)_3^{2+}$) salts as mobile phase additives for the separation and indirect detection of short-chain peptides. A polystyrene–divinylbenzene copolymeric stationary phase is used because a basic mobile phase is required to convert the peptides into their anionic forms.

EXPERIMENTAL

Reagents

$Ru(bipy)_3Cl_2(H_2O)_6$ was purchased from G. F. Smith Chemicals and $Ru(phen)_3^{2+}$ salts were prepared as described[14,16,18]; other counter-anion forms were obtained by anion exchange[14]. Peptides (Sigma and Chemalog Chemical Dynamics) were used as received. Disodium 1,5-naphthalenedisulfonate (1,5-NDS) was purchased from Eastman Kodak. Buffer and/or ionic-strength salts were analytical-reagent grade when possible. Organic solvents were of LC quality and water was treated with a Sybron–Barnstead purification unit. Polystyrene–divinylbenzene columns (PRP-1, 10 μm, 150 mm × 4.1 mm I.D.) were obtained from Hamilton. The LC instrumentation consisted of a Waters 6000A or Spectra Physics 8800 pump, a Rheodyne 7125 injector, a Kratos 773 or Spectra Physics 8450 detector, a Hewlett Packard 3390A integrator and a Bioanalytical Instrument temperature controller.

Procedures

Aqueous peptide standards (1.5 mg/ml) were injected as 1–5 μl aliquots by syringe. Mobile phase solvent mixtures are percent by volume. Buffer, ionic strength salts, counter-anion salts and $Ru(bipy)_3Cl_2$ were added by weight while Na_2CO_3, NH_3 or 1,5-NDS solutions were added by volume. Mobile phase pH was adjusted with a dilute NaOH solution and diluted to volume.

Columns were conditioned with at least 50 ml (1 ml/min) of the mobile phase

beyond its breakthrough point[14]. An acetonitrile–water mixture (1:1, *ca.* 200 ml) can be used to remove the retained $Ru(bipy)^{2+}$ salt from the PRP-1 column. The flow-rate as 1.0 ml/min, the column temperature 30°C, the inlet pressure 600–1000 p.s.i. and detection was performed at 448 nm for $Ru(phen)_3^{2+}$ and at 445 nm for $Ru(bipy)_3^{2+}$. Typical column void volumes were 1.1–1.3 ml.

RESULTS AND DISCUSSION

When a mobile phase containing a $Ru(bipy)_3^{2+}$ or a $Ru(phen)_3^{2+}$ salt passes through a PRP-1 column an equilibrium amount of the complex is maintained as a double layer on the PRP-1 surface according to the mobile phase conditions. The $Ru(bipy)_3^{2+}$ occupies the primary layer and a counter-anion, C^-, is present as a diffuse secondary layer. An analyte anion competes with C^- and it is the selectivity difference between the analyte anion and C^- which accounts for analyte anion retention. Peptides ionize under basic conditions, and as an anion, the peptide will also compete with C^- in the diffuse layer. The parameters, which affect these equilibria[14–17], are: (1) Fe^{II} or Ru^{II} complex concentration; (2) type and concentration of counter-anion; (3) mobile phase solvent composition; (4) pH. Optimization of each of these parameters as described elsewhere 14–17 can be used to bring about the retention, selectivity and resolution of small-chain peptide analytes.

The Ru^{II} complexes were used rather then $Fe(phen)_3^{2+}$ salts for the following reasons. (1) The Ru^{II} complexes are stable at basic pH. (2) Absorptivity is high and remains constant at the basic pH; this is required for indirect detection and a favorable detection limit. (3) Retention on PRP-1 follows the order $Ru(phen)_3C_2 > Fe(phen)_3C_2 > Ru(bipy)_3C_2$. Since peptides can be highly retained their retention can be reduced by using a $Ru(bipy)_3^{2+}$ salt. (4) $Ru(phen)_3^{2+}$ and $Ru(bipy)_3^{2+}$ salts are divalent, reversibly retained on PRP-1 and undergo rapid, reversible interaction with analyte anions. (5) Ru^{II} complexes are chromophoric and will fluoresce, thus, both IPD[14,15] and IFD[17] are possible; the origin and parameters affecting IPD are discussed elsewhere[15].

The Ru^{II} complex mobile phase concentration should provide an absorbance of < 0.7. When the absorbance is larger, the detector offset capabilities can be exceeded depending on the detector. Also, IPD sensitivity decreases rapidly as the background absorbance increases.

PRP-1 columns were used because the mobile phases were basic. A given column was used continuously providing efficiency and k' values were reproducible for a standard benzene–phenol sample and acetonitrile–water (9:1) as the mobile phase. In general, column life often exceeded several months.

The IPD strategy produces system peaks (SP), as well as analyte peaks, due to the competing equilibria between the analyte anion and the counter-anion(s) and the effects of these processes on the equilibrium amount of the Ru^{II} complex maintained on the PRP-1 column[15,16]. Each kind of counter-anion present may produce an SP. In general, when other factors are equal, the higher the counter-anion exchange-like selectivity the higher the retention time for its SP. A basic mobile phase contributes to a special SP problem. As the pH is increased to ensure peptides are anions, the OH^- SP moves to a higher retention time and increases in peak area because of the increased OH^- concentration. A compromise is reached by using a pH 9.5 mobile phase. At this

condition the OH^- SP area is small, the SP occurs at a low retention time or prior to the peptide peak, and the fraction of peptide in an anionic form is high. The OH^- SP is minimized further by adding a second counter-anion of high anion-exchange selectivity of suitable concentration to the mobile phase. This also shifts the OH^- SP to a lower retention time because the exchange selectivity for OH^- is small. Adding the second counter-anion also aids the chromatography because peptide anions are highly retained and strong eluent counter-anions are required for their elution. In these studies, except where noted, the OH^- SP appears at a low retention time in the chromatogram.

A second problem is CO_2 absorption by the basic mobile phase which introduces a CO_3^{2-}/HCO_3^- SP. The location (higher retention time than OH^- because of its greater anion selectivity) and peak area are concentration-dependent and depend also on mobile phase preparation and storage. Alternatively, CO_3^{2-}/HCO_3^- can be added to the mobile phase as a counter-anion to fix the CO_3^{2-}/HCO_3^- SP retention time and peak area since the CO_2 absorbed is insignificant compared to the added CO_3^{2-}/HCO_3^-.

Table I lists retention data for a series of dipeptides for three mobile phase conditions. All the mobile phases were basic (pH 9.5), contained the strong eluent 1.5-NDS counter-anion and differed in other parameters. If the pH was increased, the retention increased and appeared to correspond to the fraction of the peptide present in the anionic form. However, the OH^- SP and its affect also became more significant. When the pH was lowered the fraction of peptide as an anion decreased and retention and peak area (detection limit is less favored) decreased rapidly. When organic modifier was increased peptide retention decreased. In Table I the retention difference between mobile phases 1 and 2 is partly due to the solvent composition and partly due to the differences in counter-anion. When the concentration of the 1,5-NDS was increased the retention decreased. Switching to a different counter-anion changed the dipeptide retention according to counter-anion selectivity. Two other trends in Table I are: (1) dipeptide retention is significantly lower in a $Ru(bipy)_3^{2+}$ salt mobile phase compared to one containing a $Ru(phen)_3^{2+}$ salt, (2) dipeptide retention is structure-dependent and correlates to hydrophobicity effects.

In Table I, mobile phase 3, the equilibrium amount of a $Ru(bipy)_3^{2+}$ salt on the PRP-1 surface is 6 μmol per column compared to 34 μmol per column when using a $Ru(phen)_3^{2+}$ salt, mobile phase 2. This difference is responsible for the sharp drop in dipeptide retention when using the $Ru(bipy)_3^{2+}$ salt. A well defined peak shape as well as the favorable feature of IPD and detection limit are still retained. Thus, peptide retention can be reduced by using the $Ru(bipy)_3^{2+}$ complex and by increasing organic modifier and/or counter-anion concentration.

In general, retention increases as hydrophobic amino acid (AA) units are introduced into the dipeptide. When the dipeptides in Table I are grouped into families of similar structure (Gly-AA and AA-Gly) two trends are apparent. First, retention increases as the AA side-chain hydrophobicity increases. This trend, even though it involves anion-exchange-like interactions, is observed in dipeptide retention on reversed stationary phases[6]. Second, the dipeptide with the side-chain AA in position 1, or near the terminal amine group, has a higher retention when compared to the corresponding dipeptide with the AA in position 2. This trend is also observed for reversed-phase dipeptide retention[6]. Acidic side-chains enhance retention of the

TABLE I

RETENTION OF DIPEPTIDES

Analyte	*Capacity factor (k')*		
	Mobile phase[a]		
	1	*2*	*3*
Gly-Gly	2.26[b]		
Gly-L-Thr	2.24		
Gly-L-Ser	2.11[b]		
L-Ser-Gly	2.07[b]		
Gly-L-Ala	2.50		
L-Ala-Gly	2.57		
Gly-L-Pro	3.97		
Gly-L-Val	6.14		
L-Val-Gly	8.46(−)[c]		
Gly-L-Asp	12.4(−)	6.43	1.8[b]
L-Asp-Gly	13.4(−)	6.47	1.7[b]
Gly-L-Glu	13.4(−)	6.43	1.8[b]
L-Glu-Gly	14.0(−)	6.30	1.7[b]
Gly-L-Met	13.1(−)	5.16	2.26
L-Met-Gly	15.1(−)	6.36	2.77
Gly-L-Leu	17.0(−)	7.36	3.37
L-Leu-Gly	27.4(−)	10.1	4.77
Gly-L-Phe		20.7	9.04
L-Phe-Gly		33	14.6(−)[c]
L-Ala-L-Phe		35	15.1(−)
OH^-, Cl^- SP	2.0	2	0.7(−)
CO_3^{-2}/HCO_3^- SP	21	3	1.4(−)

[a] Mobile phase 1: 0.050 m*M* $Ru(phen)_3(HCO_3)_2$, 0.10 m*M* $Na_2CO_3/NaHCO_3$, 0.050 m*M* Na_2-1,5-naphthalenedisulfonate, acetonitrile–water (2:98), pH 9.5; mobile phase 2: 0.050 m*M* $Ru(phen)_3Cl_2$, 0.20 m*M* NH_3, 0.050 m*M* Na_2-1,5-naphthalenedisulfonate, acetonitrile–water (5:95), pH 9.5; mobile phase 3: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.20 m*M* Na_2-1,5-naphthalenedisulfonate, acetonitrile–water, (5:95) pH 9.5.

[b] Overlapped with system peak (SP).

[c] A negative peak direction.

dipeptides through additional ionization at these sites. However, at pH 9.5, ionization is not high and large side-chain hydrophobicity, such as provided by Phe and Leu, is greater than anionic charge contribution due to acidic side-chains. If the pH is increased the acidic side-chain dissociation increases and the difference between the two effects is less pronounced.

Increasing the number of hydrophobic side-chain units increases the retention (see Table II where the retention of $(L\text{-Ala})_n$ peptides for three mobile phases is listed). The effects of counter-anion, concentration of counter-anion and solvent composition on retention are consistent with dipeptide retention. The retention times for the longer-chain peptides can be excessive for the $Ru(phen)_3^{2+}$ salt. These are reduced by using a $Ru(bipy)_3^{2+}$ salt (see mobile phase 3 in Table II).

Table III lists tripeptide retention for two mobile phases containing $Ru(bipy)_3Cl_2$

TABLE II

RETENTION OF (L-ALA)$_n$ PEPTIDES

Analyte	*Capacity factor (k')*		
	Mobile phase[a]		
	1	*2*	*3*
L-Ala	2.60	3.09(−)[b]	2.13
(L-Ala)$_2$	7.09	17.3	8.40
(L-Ala)$_3$	9.00	25.2	14.3
(L-Ala)$_4$	12.2	38.8	24
(L-Ala)$_5$	29.9(−)[c]	67.5	50
OH^- SP	4.5	5.5(−)	3.5
CO_3^{-2}/HCO_3^- SP	34(−)	44(−)	16(−)

[a] Mobile phase 1: 0.050 m*M* $Ru(phen)_3(HCO_3)_2$, 0.10 m*M* L-Trp, acetonitrile–water (1:99), pH 9.5; mobile phase 2: 0.050 m*M* $Ru(phen)_3(ClO_4)_2$, 0.40 m*M* $NaClO_4$, 0.20 m*M* NH_3, aqueous, pH 9.5; mobile phase 3: 0.050 m*M* $Ru(bipy)_3(ClO_4)_2$, 0.40 m*M* $NaClO_4$, 0.20 m*M* NH_3, aqueous, pH 9.5.

[b] A negative peak direction.

[c] Overlapped with CO_3^{-2}/HCO_3^- system peak (SP).

which was used to reduce retention. If acetonitrile or counter-anion concentration is increased, the retention time decreases. The effect of side-chain hydrophobicity is consistent with the trends for dipeptide retention. For the tripeptide series, Gly-Gly-AA and AA-Gly-Gly, retention increases as the AA side-chain hydrophobicity increases. Second, retention is greater when the AA subunit is in position 1 near the terminal amine group rather than position 3 close to the $-CO_2^-$ site. Furthermore, the retention difference between tripeptide pairs, such as AA-Gly-Gly and Gly-Gly-AA, is greater than the difference for AA-Gly and Gly-AA dipeptides. For tripeptides where the subunit is in position 2, or Gly-AA-Gly, retention is intermediate to examples where the AA unit is in position 1 or 3. When two or more side-chains are present (see Table III) retention increases depending on hydrophobicity of the side-chains.

Retention of hydrophobic peptides will still be high even when using a $Ru(bipy)_3^{2+}$ salt. Increasing the organic modifier and/or using a counter-anion of higher anion selectivity reduces peptide retention. However, the equilibrium amount of the Ru^{II} complex retained on the PRP-1 also increases as counter-anion selectivity increases which increases peptide retention. The effect of counter-anion selectivity on exchange is greater resulting in a net decrease in analyte anion retention. Counter-anion selectivity at pH 9.5 was shown to follow the order:

p-OH-benzoate < L-Tyr < carbonate < succinate < phosphate < benzoate < L-Phe < phthalate < ClO_4^- < *p*-CH_3-benzoate < *p*-CH_3-benzenesulfonate < *p*-NO_2-benzoate < L-Trp < *p*-Cl-benzoate < *p*-Cl-benzenesulfonate < 1,5-naphthalenedisulfonate

In Tables I–III L-Trp and 1,5-NDS mobile phases are the stronger eluents when considering only counter-anion effects.

TABLE III

RETENTION OF TRIPEPTIDES

Analyte	*Capacity factor (k')*	
	Mobile phase[a]	
	1	*2*
Gly-Gly-Gly	1.61	2.51
L-Ala-Gly-Gly	1.92	3.31
L-Pro-Gly-Gly	7.31	14.2(−)[b]
L-Val-Gly-Gly	8.62	18[c]
L-Tyr-Gly-Gly	18.2	35
L-Ile-Gly-Gly	29	
L-Leu-Gly-Gly	33	
Gly-Gly-L-Ala		2.97
Gly-Gly-L-His		3.85
Gly-Gly-L-Val	4.1[c]	7.77(−)
Gly-Gly-L-Glu		12.4(−)
Gly-Gly-L-Tyr	8.31	20
Gly-Gly-L-Ile		24
Gly-Gly-L-Leu	12.9	29
Gly-L-Tyr-Gly	11.9	
Gly-L-Ser-L-Ala		2.62[d]
Gly-L-Pro-L-Ala	4.23[c]	7.69(−)[d]
Gly-L-Tyr-L-Ala	16	33[d]
OH^-, Cl^- SP	1.3 to 1.8(−)	1.7 to 2.2(−)
CO_3^{-2}/HCO_3^- SP	2.7 to 3.2(−)	16 to 18

[a] Mobile phase 1: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.20 m*M* Na_2-1,5-naphthalenedisulfonate, 0.20 m*M* NH_3, acetonitrile–water (1:99), pH 9.5; mobile phase 2: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.05 m*M* L-Trp, 0.10 m*M* Na_2CO_3, acetonitrile–water (1:99), pH 9.5.

[b] A negative peak direction.

[c] Overlapped with CO_3^{-2}/HCO_3^- system peak (SP).

[d] Mobile phase was the same as mobile phase 2 except that 0.075 m*M* L-Trp was used.

Two other types of eluent counter-anions were examined. Table IV lists tripeptide retention data using L-Asp-L-Asp, L-Phe-L-Leu and L-Leu-L-Phe (or D,L-Leu-D,L-Phe) as counter-anions. L-Asp-L-Asp has the potential to be highly charged because of the three carboxyl groups. However, this counter-anion also reduces the equilibrium amount of the Ru^{II} complex on the PRP-1; for mobile phase 1 in Table IV the equilibrium amount was 10 μmol of $Ru(bipy)_3^{2+}$ salt per column. The other two dipeptides are strong counter-anions because of side-chain hydrophobicity. Table IV indicates that the more significant retention decrease is caused by the hydrophobic counter-anion rather than the multiple-charged counter-anion. Other hydrophobic dipeptides or longer-chain peptides can be used providing they are soluble at the chromatographic conditions. In Table IV acetonitrile was required to dissolve L-Phe-L-Leu and this contributed to reduced retention.

Short-chain peptide diastereomers can be separated and detected as shown in Table V. Several trends are indicated. (1) The L-L and D-D dipeptide coelute first followed by L-D and D-L coelution. Only the L-Leu-L-Tyr was found to have a higher

TABLE IV

EFFECT OF DIPEPTIDE COUNTER-ANION ON THE RETENTION OF TRIPEPTIDES

Analyte	*Capacity factor (k')*	
	Mobile phase[a]	
	1	*2*
Gly-Gly-Gly	1.8[b]	
Gly-Gly-L-Ala	2.1[b]	
Gly-Gly-L-His	2.67	
Gly-Gly-L-Val	6.60	
Gly-Gly-L-Glu	7.60	
Gly-Gly-L-Tyr	13.5	
Gly-Gly-L-Ile	20	
Gly-Gly-L-Leu	24	
L-Ile-Gly-Gly		4.13
L-Leu-Gly-Gly		4.44
Gly-Gly-L-Phe		6.56
Gly-L-Phe-Gly		14.0
L-Phe-Gly-Gly		13.3
Gly-L-Phe-L-Ala		15.8
OH^-, Cl^- SP	1.5(−)	
CO_3^{-2}/HCO_3^- SP	4.0(−)	1.8

[a] Mobile phase 1: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.10 m*M* L-Asp-L-Asp, acetonitrile–water (1:99), pH 9.5; mobile phase 2: 0.05 m*M* $Ru(bipy)_3Cl_2$, 0.10 m*M* L-Phe-L-Leu, acetonitrile–water (2:98), pH 9.5.

[b] Overlapped with OH^-, Cl^- system peak (SP).

TABLE V

RETENTION OF DIASTEREOMERIC DI- AND TRIPEPTIDES

Analyte	*Capacity factor k'*	*Mobile phase*[a]	*Amount per column*[b] *(μequiv.)*
D,L-Val-D,L-Val[c]	11.6/14.4	1	25
D,L-Ala-D,L-Leu[c]	4.8/5.9	2	36
D,L-Ala-D,L-Phe[c]	19/21	1	25
D,L-Leu-D,L-Tyr[c]	8.9/7.6	3	11
D,L-Leu-D,L-Leu[c]	32/38	3	11
Gly-D,L-Leu-D,L-Ala[c]	6.4/10.2	1	25
L-Ala-L-Ala-L-Ala	4.3	4	11
L-Ala-L-Ala-D-Ala	8.3	4	11
D-Ala-D-Ala-D-Ala	4.2	4	11
CO_3^{-2}/HCO_3^- SP	2 to 3(−)[d]		

[a] Mobile phase 1: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.075 m*M* D,L-Leu-D,L-Phe, acetonitrile–water (1:99), pH 9.5; mobile phase 2: same as 1 except that 0.10 m*M* L-Leu-L-Phe was used; mobile phase 3: same as 1 except that 0.10 m*M* D,L-Leu-D,L-Phe and acetonitrile–water (6:94) were used; mobile phase 4: same as 1 except that 0.10 m*M* D,L-Trp was used.

[b] Equilibrium amount of $Ru(bipy)_3^{2+}$ salt maintained on the PRP-1 surface.

[c] First *k'* value is for the L,L- and D,D-diastereomer.

[d] Location of system peak depends on the mobile phase; a negative peak direction.

retention over the D-L diastereomer. (2) Resolution appears to be dependent on side-chain hydrophobicity. (3) Resolution of tripeptide diastereomers containing two chiral centers is better than for dipeptides with two chiral centers. (4) Resolution of tripeptides containing three chiral centers is possible. Peak confirmation in Table V was established by using diastereomeric standards.

Figs. 1–4 illustrate that resolution is favorable, peak shapes are well defined, IPD is applicable to peptide analytes, and that mobile phase parameters can be altered in a predictable way to affect resolution. In general, peptide quantities injected were in the range 1–3 μg.

In Fig. 1 dipeptides were separated using a basic mobile phase and a $Ru(phen)_3^{2+}$ salt (Fig. 1A and B) or a $Ru(bipy)_3^{2+}$ salt (Fig. 1C). The counter-anion and acetonitrile concentration was low in Fig. 1A because these peptides have low retention. When the concentrations are increased higher retained peptides are more easily separated (Fig. 1B). If the mobile phase in Fig. 1B contains the counter-anion CO_3^{2-}/HCO_3^- instead of NH_3, eluent strength is increased and retention of hydrophobic dipeptides is

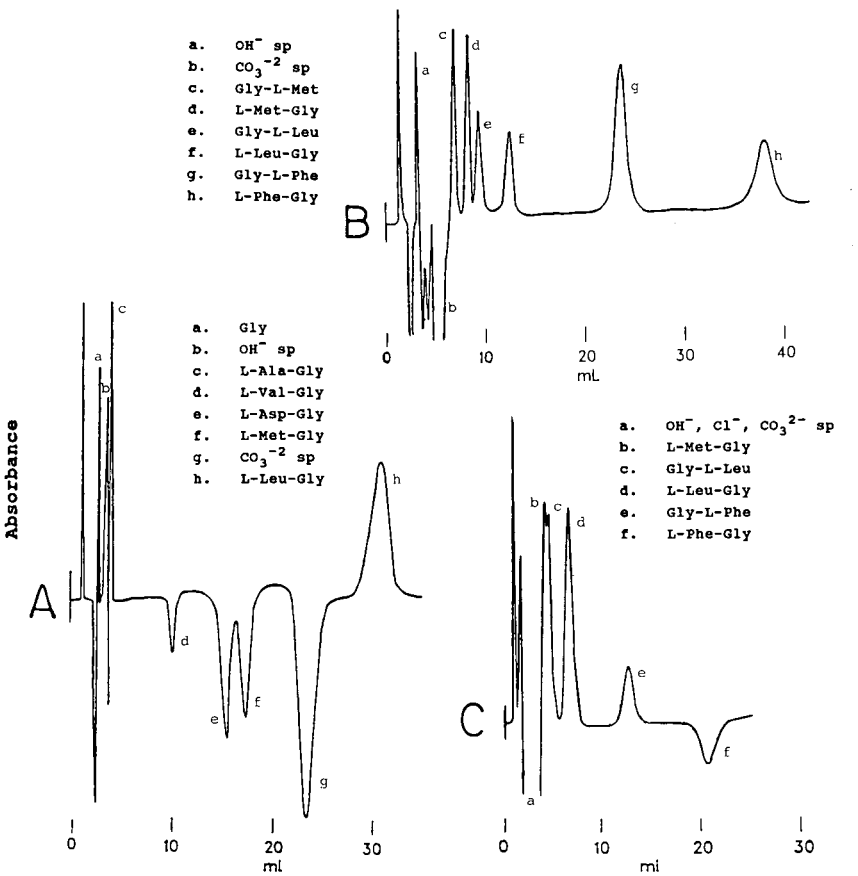

Fig. 1. Separation of dipeptides. (A) Mobile phase 1, Table I; (B) mobile phase 2, Table I except that 0.20 m*M* Na_2-1,5-naphthalenedisulfonate was used; (C) mobile phase 2, Table I, except that 0.050 m*M* $Ru(bipy)_3Cl_2$ was used.

decreased. Fig. 1C illustrates a lowering of retention by about 50% by using a $Ru(bipy)^{2+}$ salt which reduces the equilibrium amount of interaction sites on the PRP-1.

Separation of tripeptides and longer-chain peptides using a $Ru(bipy)_3^{2+}$ salt to reduce retention is shown in Fig. 2. Changes in mobile phase solvent composition and counter-anion concentration can also be used to alter retention. The side-chain effect on separation is illustrated in Fig. 2A where the side-chain structure changes at subunit position 1. The effect of the side-chain at subunit 3 is illustrated in Table III; these tripeptides can also be separated (see Fig. 3A). If 1,5-NDS is the counter-anion all peaks are positive. Fig. 2C shows the separation of $(L\text{-Ala})_n$, where $n = 1$–5, using a $Ru(phen)_3^{2+}$ salt. For a $Ru(bipy)_3^{2+}$ salt retention times are reduced by about 25%. L-Trp, a strong eluent counter-anion with a system peak of » 60 min, is used and this lowers retention.

When L-Asp-L-Asp, which is multiple charged, is the counter-anion (Fig. 3A), resolution is favorable but this counter-anion offers little advantage over a hydro-

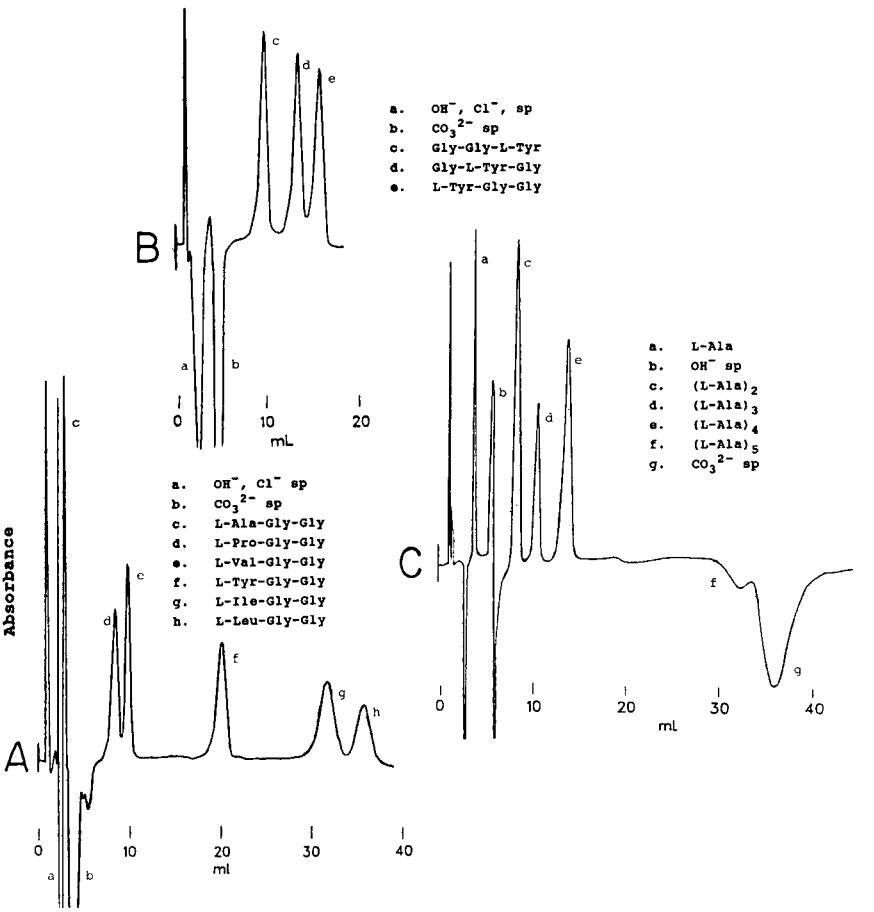

Fig. 2. Separation of small-chain peptides. (A) Mobile phase 1, Table III; (B) mobile phase 1, Table III; (C) mobile phase 1, Table II.

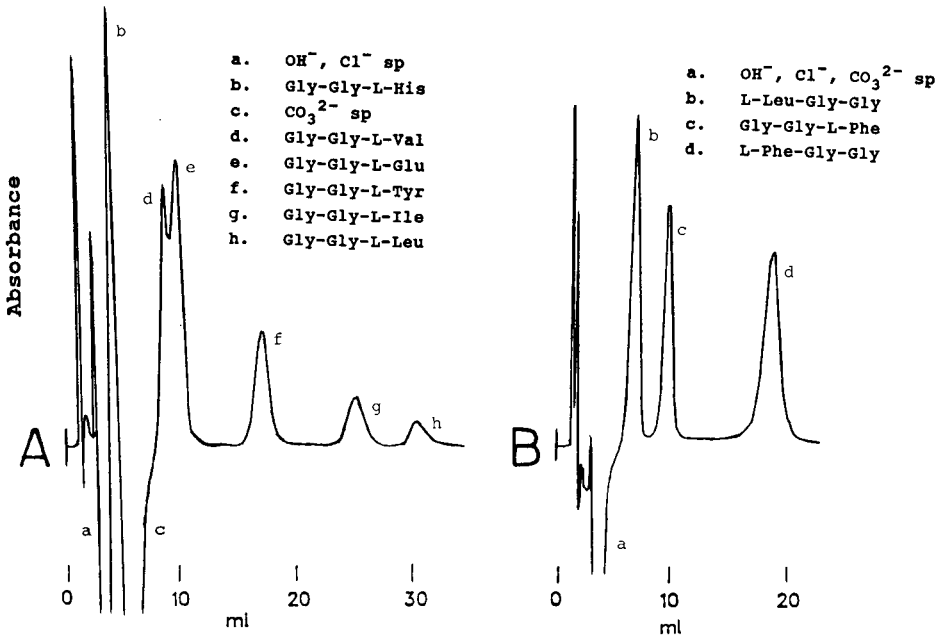

Fig. 3. Effect of counter-anion on the separation of tripeptides. (A) Mobile phase 1, Table IV; (B) mobile phase 2, Table IV.

phobic counter-anion such as L-Trp (see Table III). However, when hydrophobic L-Phe-L-Leu or L-Leu-L-Phe is the counter-anion, the retention is sharply reduced and highly retained tripeptides are resolved in a favorable analysis time (see Fig. 3B). D,L-Leu-D,L-Phe is preferred because it is inexpensive and readily available, and using a dipeptide diastereomer as a counter-anion offers no advantage. In both cases the dipeptide counter-anion system peak is at a high retention time and does not interfere. Fig. 4 illustrates the separation of peptide diastereomers; other examples are listed in Table V.

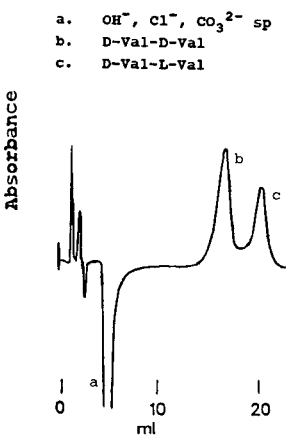

Fig. 4. Separation of peptide diastereomers. Mobile phase 1, Table V.

Calibration curves were prepared using Gly-L-Phe, Gly-Gly-L-Glu, L-Val-Gly-Gly and Gly-Gly-L-Phe standards over the range 0.2–100 nmol per 10 μl. Mobile phases in Fig. 1A (except 0.10 m*M* 1,5-NDS), 1 in Table III, in Fig. 2A and Fig. 3B were used, respectively. Calibration curves were linear and the upper limit of linearity was not determined. Change in peak retention time over this concentration range was modest and corresponded to less than a 10% difference between the low and high sample quantity. The major factor determining the linear calibration curve slope was mobile phase ionic strength; as ionic strength increased, the slope decreased which is consistent with previous studies[15]. In general, the detection limit for the four standards was *ca.* 0.1 nmol at a 3:1 peak height signal-to-noise ratio.

ACKNOWLEDGEMENT

Part of this work was supported by Grant DE 8613 awarded by the National Institute of Dental Research.

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Journal of Chromatography, 509 (1990) 369–376
Elsevier Science Publishers B.V., Amsterdam

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Separation of cations using methacrylate-based low-capacity cation exchangers

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(First received November 16th, 1990; revised manuscript received February 12th, 1990)

ABSTRACT

The possibility of using macroporous methacrylate-based low-capacity ion exchangers in the separation of cations of alkali, alkaline earth, transition and heavy metals was tested. The separation conditions were optimized with respect to the composition of the eluent (content of ethylenediamine, tartaric acid, organic solvent and pH). The retentions of cations on methacrylate ion exchangers were compared with those on derivatives of styrene–divinylbenzene and silica gel. The detection limits obtained using the usual apparatus and method were $2.5 \cdot 10^{-8}$ g.

INTRODUCTION

The determination of cations is not the most significant field of application of ion chromatography (IC), but it has received considerable attention recently. Benson[1] filled short columns with cross-linked polystyrene selectively sulphonated to a low exchange capacity (on the usual ion exchangers the ions are retained too strongly) and separated cations of alkali metals. These cations were separated on a methacrylate cation exchanger also without suppressed conductivity detection[2]. The ammonium cation, which has properties differing from those of alkali metals, can be separated by using eluents with an admixture of organic solvents[3]. The possibility of using organic solvents appears to be a special feature of the discussed columns compared with the Dionex type.

More than one solution has been proposed for the separation of alkaline earth and transition metals. In addition to the classical separation with a strongly acidic cation exchanger[4], complex-forming sorbents or eluents are also employed in the process. A sorbent containing phosphonic groups[5] or an anion exchanger converted into the EDTA form[6] can also be used. In these instances the concentration of exchange groups of the ion exchanger can be higher[3] (up to 0.4 mmol/g).

0021-9673/90/$03.50

A different approach leading to a more universal method utilizes a complex-forming eluent. The complex-forming agents improve the selectivity of separation and the shape of the peaks[7]. Also, for the separation of transition and heavy metals complex-forming eluents such as ethylenediamine[8,9], tartaric acid[8], citric acid[10], oxalid acid[10] or lactic acid[11,12] are recommended. The sensitivity is reported to be as high as 0.1 ppb if peak detection by the suppressed conductivity detection technique is used[13].

In addition to conductivity detection, in particular for transsition metals, other methods are also applied, *e.g*, UV detection after reaction with 4-(2-pyridylazo)-resorcinol (PAR)[14].

This study is concerned with the chromatographic properties of macroporous methacrylate copolymers possessing sulphopropyl groups for the separation of alkaline earth and transition metals.

EXPERIMENTAL

Materials

The sorbent for ion chromatography was prepared by a consecutive chemical transformation of the copolymer (glycidylmethacrylate–ethylenedimethacrylate) (40 wt.-% of the cross-linking agent) obtained by radical suspension copolymerization[15]. Glycidyl groups of the copolymer, particle size 17–25 μm, were hydrolysed and subsequently modified by reaction with propane sultone[2,16]. The properties of the sorbents used are summarized in Table I.

Chromatography

Chromatographic measurements were carried out using a Cvet 306 liquid chromatograph (Khimavtomatika, Moscow, U.S.S.R.) with conductivity (alkali metals) and UV (transition and heavy metals) detection; a solution of the post-column reagent (0.001% PAR + 0.5% ammonia, pH 12.5) was added to the eluate by means of an extra pump. If not stated otherwise, a mixture of ethylenediamine and tartaric acid with the pH adjusted by the addition of 0.1 mol/l of acid or base was used as the eluent. The standard mixture contained $5 \cdot 10^{-4}$ mol/l of cations.

TABLE I

PROPERTIES OF MACROPOROUS METHACRYLATE LOW-CAPACITY IONEXCHANGERS

Exchanger	*Agent/sorbent ratio*	*Conversion (mol.-%)*	*Particle size (μm)*	*Content of groups (mmol/g)*	
				Elemental analysis	*Chromatography*
A	0.31	17.6	19–25	0.42	0.1
B	0.083	71.5	17–20	0.13	0.02
C	0.36	53.2	19–25	0.11	0.02

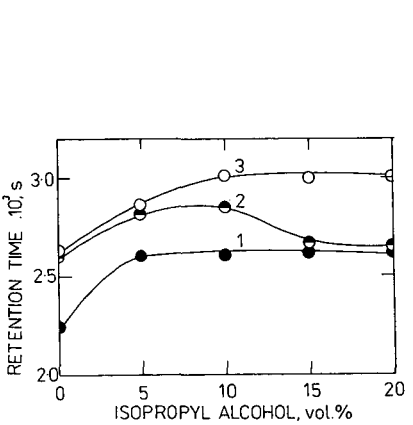
RESULTS

In previous studies[2,3], we demonstrated some possible separations of alkali metals ions and of the ammonium cation on macroporous methacrylate copolymers containing sulphopropyl groups, and the effects of acetone, ethanol and methanol in the eluent. It has been found that methanol can be replaced with isopropanol, which influences the retention of ammonium in a manner different to that of sodium or potassium (Fig. 1). The optimum concentration of isopropanol is 10 vol.-% in 2 mmol/l nitric acid when mixtures of monovalent cations are to be separated within 15 min.

Our methacrylate cation exchanger also makes possible the separation of alkaline earth metals and of transition and heavy metals (Fig. 2). For this purpose, it was necessary to optimize the composition of ethylenediamine and tartaric acid mixtures. The optimization consisted of experimental evaluations of the effects of the ethylenediamine and acetone concentrations in the eluent. The effect of eluent pH was also studied.

At an ethylenediamine concentration below 1 mmol/l the retention times are disproportionately long (Table II); the duration of analysis at a concentration of 2 mmol/l ethylenediamine in the eluate seems to be acceptable. The elution time also increases with increasing pH (Table III), pH 4 being the optimum value.

Similarly to alkali metals, with transition metals an increase in the concentration of the organic solvent (acetone) also increases the retention time; 20 vol.-% of acetone makes the retention time twice as long compared with the use of a reference eluent without the organic solvent. Such long retention times are not advantageous from the practical point of view.



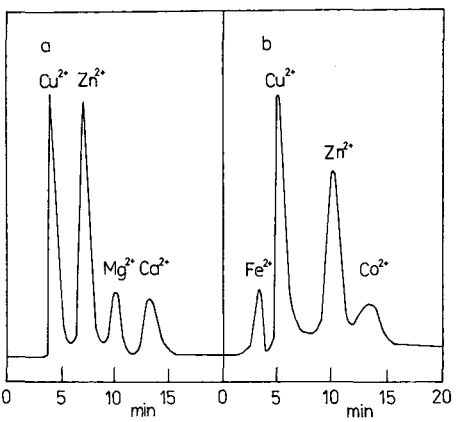


Fig. 1. Dependence of the retention of cations on sample C (0.11 mmol sulpho groups/g) on the content of isopropyl alcohol in 1 mmol/l HNO_3. Conditions: Column, 10 × 0.4 cm I.D.; eluent flow-rate, 1 ml/min. 1 = Na^+; 2 = NH_4^+; 3 = K^+.

Fig. 2. (a) Chromatogram of the separation of alkaline earth and transition metal cations. Conditions: column, 10 × 0.6 cm I.D.; methacrylate cation exchanger (0.11 mmol sulpho groups/g); eluent, 2 mmol/l tartaric acid–2 mmol/g ethylenediamine (pH 4); flow-rate, 1.5 ml/min. (b) Chromatogram of the separation of heavy metal and transition metal cations. Conditions as in (a) except methacrylate cation exchanger (0.42 mmol sulpho groups/g).

TABLE II

EFFECT OF THE ETHYLENEDIAMINE CONCENTRATION ON THE RETENTION TIMES (s) OF TRANSITION METALS

Conditions: Column, 20 × 0.6 cm I.D.; sorbent B (Table I); eluent, 2 mmol/l tartaric acid (pH 4); flow-rate, 2 ml/min.

Cation	*Ethylenediamine concentration (mmol/l)*		
	2	*1*	*0.5*
Cu	310	332	1200
Zn	492	655	1860
Co	587	862	2160

Table IV compares the retention values of alkaline earth metals and transition metals on five cation exchangers for ion chromatography, prepared on the basis of silica gel, styrene–divinylbenzene and glycidylmethacrylate copolymer with different contents of sulphonic groups. The methacrylate copolymers give longer retention times than the ion exchangers based on silica gel and styrene–divinylbenzene copolymer, but they can reliably separate all the cations studies, in particular Mg^{2+} and Ca^{2+}. The time needed for separation varies between 12 and 16 min. The longer retention time can be explained by additional interactions between the cation and further polar groups of the sorbent, *e.g.*, hydroxyl groups. The retention time of cations on methacrylate ion exchangers is proportional to the acidic group content, as follows from Tables I and IV. The efficiency of methacrylate columns, shown as the number of theoretical plates in Table V, is lower than that of the columns in Table IV because of the larger particle size of methacrylate sorbents.

The detection limits (three times the noise) vary between 10^{-4} and 10^{-5} mol/l for concentrations between 10^{-6} and $0.25 \cdot 10^{-7}$ g for the amounts injected. The best and poorest detection limits are observed for Zn^{2+} ($1.5 \cdot 10^{-6}$ mol/l) and Fe^{3+} (10^{-4} mol/l), respectively.

TABLE III

EFFECT OF pH OF THE ELUENT ON THE SEPARATION OF TRANSITION METAL AND ALKALINE EARTH METAL CATIONS

Conditions: Column, 20 × 0.6 cm I.D.; sorbent A (Table I); eluent, 2 mmol/l tartaric acid–2 mmol/l ethylenediamine; flow-rate, 1.5 ml/min.

Cation	*Retention time (s)*		
	pH 4.0	*pH 4.6*	*pH 5.3*
Cu	247	270	255
Zn	420	480	405
Ni	465	495	465
Co	510	600	525
Mg	525	660	645
Ca	705	870	840

TABLE IV

RETENTION TIMES (s) OF TRANSITION METALS ON DIFFERENT SORBENTS

Conditions: Column, 10 × 0.6 cm I.D.; eluent 2 mmol/l tartaric acid–2 mmol/l ethylenediamine (pH 4); flow-rate, 1.5 ml/min.

Cation	*Silica gel*		*Methacrylates*[b]		*Styrene–divinylbenzene IREA*[a]
	Diasorb	*INEOC*[a]	*A*	*B*	
Cu	255	225	225	240	90
Zn	540	225	405	510	142.5
Mg	735	300	570	735	165
Ca	885	300	735	1050	225

[a] Made in U.S.S.R.
[b] See Table I.

TABLE V

SELECTIVITY AND EFFICIENCY OF METHACRYLATE COLUMNS

Conditions[a]	*Cation*	t_R,s	k'[b]	α[c]	R_s[d]	N[e]	
						per column	*per m*
I	Fe	255	4.67			94.4	944
	Cu	300	5.67	1.21	1.45	177	1777
	Zn	480	9.67	1.71	1.67	181	1815
	Co	630	13.00	1.34	1.08	348	3484
	Mn	780	16.33	1.26	1.08	536	5363
II	Cu	255	4.67			94	944
	Zn	465	9.33	2.00	1.88	440	2402
	Mg	630	13.00	1.39	1.37	441	4410
	Ca	840	17.67	1.36	1.40	348	3480

[a] Conditions I: column, 10 × 0.6 cm I.D.; sorbent A (Table I), particle size 19–25 μm; eluent, 2 mmol/l tartaric acid–2 mmol/l ethylenediamine (pH 4); flow-rate, 1.5 ml/min; t_0 = 45 s. Conditions II: column, eluent, flow-rate and t_0 as for I; sorbent C (Table I), particle size 19–25 μm.
[b] Capacity factor $k' = (t_R - t_0)/t_0$.
[c] Selectivity $\alpha = k'_2/k'_1$.
[d] Resolution $R_s = 2(t_{R2} - t_{R1})/(\Delta t_{R2} + \Delta t_{R1})$.
[e] Theoretical plates $N = 16\,(t_R/\Delta t)^2$.

DISCUSSION

Bivalent alkaline earth metal cations and bivalent or trivalent transition and heavy metals are bound by the ion exchanger much more strongly than cations of alkali metals. Their elution from the column requires mineral acids at high concentrations. In practice, two types of eluent components are used in the chromatographic analysis of cations: first diacidic ammonium compounds such as ethylenediamine or phenylenediamine and second di- and tribasic carboxylic acids, such as oxalic, tartaric, lactic and hydroxylactic acid. The di- and tribasic acids form complex compounds with the metals.

The in exchange equation with ethylenediamine as the eluent has the form

$$2\ M^{z+}R_z + z\ E^{2+} \rightleftharpoons z\ E^{2+}R_2 + 2\ M^{z+} \tag{1}$$

where E^{2+} is ethylenediamine, M^{z+} is the metal cation, z is the charge of the metal cation and R is the number of exchange groups of the ion exchanger with the respect to the cation.

The selectivity coefficient is given by[17]

$$K_m^E = \frac{[E^{2+}R_2]^z\ [M^{z+}]^2}{[E^{2+}]^z\ [M^{z+}R_z]^2} \tag{2}$$

At a low metal content in the sample, a condition which is usually met in chromatographic analysis, $[c/2] = [E^{2+}R_2]$ (where c is group content in the ion exchanger) and the capacity of the ion exchanger is then given by $K = [M^{z+}R_z]/[M^{z+}]$. Hence,for the selectivity coefficient we obtain

$$K_m^E = [c/2]^2/[E^{2+}][K] \tag{3}$$

By substituting the retention time of the cation being eluted, $t_R = K\ t_{R_0}$, we obtain

$$K_m^E = \frac{[c/2]^z\ t_{R_0}^2}{[E^{2+}]^z\ t_R} \tag{4}$$

After rearrangement,

$$\log t_R = (z/2) \log (c/2) + \log t_{R_0} - z \log [E^{2+}] - (1/2) \log K_m^E \tag{5}$$

For a cation charge $z = 2$, we have

$$\log t_R = A - \log [E^{2+}] \tag{6}$$

The agreement between the theoretical value ($z = 1$) of the slopes and the experimental values given in Table II suggests an ionic mechanism for separations of cobalt, zinc and copper.

The mechanism of interactions of hydroxy acids with metals is determined by their ability to form complexes of neutral or anionic character. This type of interaction decreases the effective charge of the metal cation and hence also its retention time.

The term $[M^{z+}]$ in eqn. 2 can be replaced with $[M']\alpha_M$, where $[M']$ is the total concentration of both[18] the free and bound metal complex in solution and α_M is that part of metal in solution which is present in the form of free cations. The equation for $\log t_R$ then becomes

$$\log t_R = (z/2) \log \alpha_M + (z/2) \log (c/2) + \log t_{R_0} - (z/2) \log [E^{2+}] - \\ - (1/2) \log K_M^E \tag{7}$$

where α_M is given by the concentration of the complex-forming agent and by the stability constant of the corresponding complex.

Addition of the complex-forming agent to the eluent not only reduces the retention time but also increases the selectivity of separation. For closely eluting metals bearing a charge of the same magnitude (Cu^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}), the distribution coefficient without the use of complexing acids (D_B = constand/[A], where [A] is concentration of ions of the eluent) differs only slightly. In such a case the separation is given by the ratio of the distribution coefficients of the two metals, D_B/D_C, and does not depend on the eluent concentration.

If complexation becomes part of the separation mechanism, it is possible to observe larger variations in retention behaviour. These variations are mainly caused by the large differences in the values of the equilibrium constants for complex formation. The separations including complex formation can be described by

$$\frac{D_{MB}}{D_{MC}} = \frac{D_B}{D_C} \cdot \frac{K_{compl.B}}{K_{compl.C}} \tag{8}$$

where K_{compl} is the constant of complex formation. At $D_B/D_C \approx 1$, $D_{MB}/D_{MC} \neq 1$.

Experimentally, it was found that the separation depends on the pH of eluent (Table III). In a more acidic medium the retention times are longer and the separation becomes poorer. Clearly, with increasing pH the complex-forming ability of hydroxy acids increases, which in the sense mentioned above leads to shorter retention times and to better separations.

The complex formation may be described by the equation

$$M^{z+} + F'^{u-} \rightleftharpoons MF'^{z-u} \tag{9}$$

where z and u are the charges of the metal cation M and hydroxy acid F', respectively, [F] is the total concentration of hydroxy acid in all forms in which metal is not bound.

The effective constant of complex formation is given by

$$K_{MF}^{eff} = \frac{[MF]}{[M]\ [F']} = \frac{K_{MF}}{\alpha_H^F} \tag{10}$$

where K_{MF} is the constant of complex formation.

K_{MF} is independent of pH, being a constant of the reaction

$$M^{z+} + F^{s-} \rightleftharpoons MF^{z-s} \tag{11}$$

Here, [F] is the total concentration and α_H^F is the distribution coeffient of hydroxy acid which occurs in variously protonated forms:

$$\alpha_H^F = 1 + [H]\ K_{HF} + [H^2]\ K_{HF}\ K_{H^2F} + \ldots \tag{12}$$

The logarithms of the constants K_{HF} and K_{H^2F} are identical with the pK values of the corresponding acids ($pK_1^F = \log K_{HF}$; $pK_2^F = \log K_{H^2F}$):

$$\log K_{MF}^{eff} = \log K_{MF} - \log \alpha_H^F \tag{13}$$

With increasing pH, the effective constant of complex formation, K_M^{eff}, approaches K_{MF}, *i.e.*, the stability of the complex increases. These conclusions are of general validity for both cations and anions[19,20].

The results show that cation exchangers based on methacrylate macroporous resins can be applied for separations of complicated mixtures of alkaline earth cations and transition metals.

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Journal of Chromatography, 509 (1990) 377–382
Elsevier Science Publishers B.V., Amsterdam

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Note

Gas chromatographic and mass spectral determination of aloenin in skin-care cosmetics

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Cosmetics generally contain a wide variety of ingredients (*e.g.*, oils, waxes, alcohols, surfactants, fragrances, dyes, preservatives and plant extracts, and their identification and determination are of importance for quality control.

Various kinds of extracts derived from plants, especially aloe extracts, are used in skin-care cosmetics. Among many kinds of aloe species, the following three species are commonly used: *Aloe barbadensis* Miller (*A. vera* Linne), *A. ferox* Miller and *A. arborescens* Miller var. *natalensis* Rerger (Japanese name: Kidachi-Aloe) (Liliaceae)[1]. These three species contain anthrone glycosides, barbaloin, aloinosides A, B, etc., and their aglycone[2]. In general, barbaloin as a glucoside is easily hydrolysed, and therefore cannot be detected in commercial cosmetics, although it is present at high concentrations in aloe extracts[3].

Aloe arborescens Miller var. *natalensis* Rerger (Kidachi-Aloe) contains aloenin (O-glucoside) and barbaloin (C-glucoside); the former compound is very stable in comparison with the latter, even in water or methanol solution. Consequently, we tried to monitor the Kidachi-Aloe extracts in cosmetics by the determination of their aloenin contents.

There have been some reports of the detection of aloenin in aloe leaves[4], foods[4] and skin lotions[5] using thin-layer (TLC) and high-performance liquid chromatography (HPLC). However, the verification of aloenin in cosmetics by TLC and HPLC is not easy as interferences from the matrix substances occur. We have previously reported[3] that the aloenin and barbaloin in cosmetics such as cream and lotion could be measured by gas chromatography–mass spectrometery (GC–MS) as the trimethylsilyl derivatives. However, barbaloin in cosmetics cannot be detected because it is hydrolysed. The detection limit of aloenin in cosmetics was about 2 $\mu g/g$[3].

We report here a highly sensitive method using GC with mass fragmentography (GC–MF) to detect low concentrations of aloenin in cosmetics. This method was

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successfully applied to the determination of aloenin in several commercial skin-care cosmetics.

EXPERIMENTAL

Reagents

Fresh leaves (*ca.* 500 g) of *Aloe arborescens* Miller var. *natalensis* Rerger (Kidachi-Aloe), cut into small pieces, were extracted for 60 min with 500 ml of water–methanol (1:1) in an ultrasonic bath. The extract was concentrated with a vacuum evaporator (Büchi-Sibata RE-111A; Sibata, Tokyo, Japan) at 40°C to about 200 ml and centrifuged at 1600 *g* for 20 min. Aloenin in the supernatant was purified three times by the use of a fully automatic preparative high-performance liquid chromatograph (HLC-837; Tosoh, Tokyo, Japan) connected to a preparative column (300 × 55 mm I.D.) packed with octadecylsilica gel (ODS), and subsequently recrystallized from methanol to yeild aloenin, which was identified by its m.p. and NMR, mass and UV spectra[6].

A 100 μg/ml stock standard solution of aloenin in acetonitrile was prepared. Working standard solutions were prepared by suitable dilution of the stock solution with acetonitrile.

N,O-Bis(trimethylsilyl)acetamide (BSA) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and an ODS cartridge (Sep-Pak C_{18}) from Waters Assoc. (Milford, MA, U.S.A.). Other reagents (methanol, ethanol and acetonitrile) were of special grade reagent from Wako (Osaka, Japan).

Instrumentation

GC–MS system was a JOEL (Tokyo, Japan) JMS D-300 mass spectrometer equipped with a Hewlett-Packard (Palo Alto, CA, U.S.A.) 7710A gas chromatograph and a JOEL MS-MIDO3 multi-ion detector. GC was performed using a glass column (1.0 m × 2 mm I.D.) packed with 2% OV-17 on Chromosorb W AW DMCS (60–80 mesh) with helium as the carrier gas (flow-rate 30 ml/min) with temperature programming (250°C for 2 min, increased at 16°C/min to 280°C, held for 4 min). The injection port temperature was 300°C and the injection volume was 2 μl. The operating conditions for the mass spectrometer equipped with the multi-ion detector were follows: electron impact (EI) mode; ionization voltage, 70 eV; ionization current, 300 μA; ion source temperature, 250°C; ion multiplier 100–220 (−1.0 to −2.2 kV); gain of multi-ion detector 1 or 2; monitoring ion, *m/z* 392.

Analytical procedure

For aqueous and alcoholic cosmetics (lotion and hair tonic), a mixture of each sample (0.5 g) in ethanol (*ca.* 10 ml) was evaporated to dryness under reduced pressure at 40°C. The residue was dissolved in 20 ml of water, followed by passage through an ODS cartridge (previously rinsed with 10 ml of methanol and 10 ml of water) at a rate of about 8 ml/min. After washing with 10 ml of water, 5 ml of methanol were passed through the cartridge to elute aloenin. The eluate was evaporated to dryness *in vacuo* at 40°C, a 200-μl volume of acetonitrile was added to the residue and the mixture was transferred into a 2-ml mini-vial. A 200-μl volume of BSA was added and the vial was stoppered tightly. The mixture was held at 90°C in a water-bath for 60 min. After cooling, a 2-μl volume of the mixture was injected into the GC–MF apparatus.

For oil-rich cosmetics (cream, milky lotions, etc.), a 0.5-g sample was dissolved in ethanol (*ca.* 20 ml) and the mixture was filtered with a filter-paper (No. 2; Toyo Roshi, Tokyo, Japan), followed by evaporation to dryness. After addition of water (*ca.* 20 ml), the solution was passed through an ODS cartridge (previously rinsed as above) at a rate of 8 ml/min. After washing with 10 ml of water, 5 ml of methanol were passed through the cartridge to elute aloenin. The eluate was evaporated to dryness and the residue was dissolved in 200 μl of acetonitrile. The mixture was transferred into a 2-ml mini-vial, 200 μl of BSA were added and the vial was stoppered tightly. The mixture in the mini-vial was held at 90°C in a water-bath for 60 min. After cooling, a 2-μl volume of the mixture was injected into the GC–MF apparatus.

The analytical procedure is summarized schematically in Fig. 1.

RESULTS AND DISCUSSION

Pretreatment by ODS cartridge

For the detection of trace aloenin in skin-care cosmetics containing large

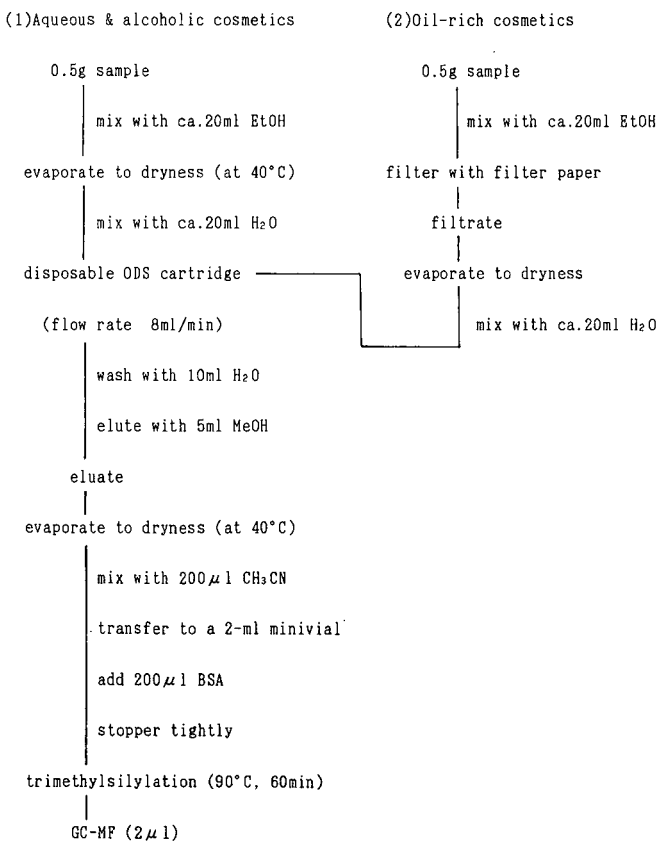

Fig. 1. Analytical procedure for the determination of aloenin in cosmetics. EtOH = Ethanol; MeOH = methanol.

amounts of interfering substances such as surfactants, preservatives or oily constituents, an effective clean-up was necessary.

After removing the volatile substances in a cosmetic sample *in vacuo*, a mixture of the residue in water was passed through a disposable ODS cartridge and the aloenin held on the ODS cartridge was eluted with methanol. Although the very slight content of matrix substances (*e.g.*, surfactants, preservatives) in the resulting solution was confirmed by HPLC with an ODS column of TSKgel ODS-80TM (15 cm × 4.6 mm I.D.) (Tosoh), using water–methanol (1:1) as the mobile phase, these substances did not interfere in the subsequent trimethylsilylation and the detection by GC–MF. These procedures with 0.5-g cosmetic sample resulted in a recovery of nearly 100% in all instances.

Trimethylsilylation

As aloenin is non-volatile, a derivatization procedure was essential. Of the derivatization reactions generally used in the GC determination of substances with hydroxy groups, we chose trimethylsilylation with BSA because of its expediency and sensitivity.

Trimethylsilylation was effected at 90°C for 60 min using BSA with acetonitrile. Trimethylsilyl (TMS) groups were introduced on all five hydroxy groups and as a result, a pentatrimethylsilyl derivative of aloenin was formed. A typical mass spectrum of the derivative is shown in Fig. 2. Ions such as those of m/z 755 (M − 15), 665 (M − 15 − 90) and 575 (M − 15 − 90 − 90) are recognized as the characteristic fragment ions of a TMS derivative, and the base peak (m/z 392) is presumed to be a di-TMS derivative fragment of the aglycone, which arose from cleavage of the TMS-sugar residue from the parent molecule followed by further trimethylsilylation of the resulting aglycone fragment. This phenomenon was observed in the mass spectrum of the penta-TMS derivative of arbutin (4-hydroxyphenyl-β-D-glucopyranoside), one of O-glucosides, which had a base peak (m/z 254) corresponding to a di-TMS derivative fragment of the aglycone[3].

Calibration graphs and recoveries

Calibration graphs obtained by plotting the peak height (ion intensity at m/z 392) against the concentration of aloenin showed good linearity over the ranges 0.2–2.0 and 2–20 μg/g, the regression equations being $y = 1.30 + 11.70x$ ($r = 0.999$)

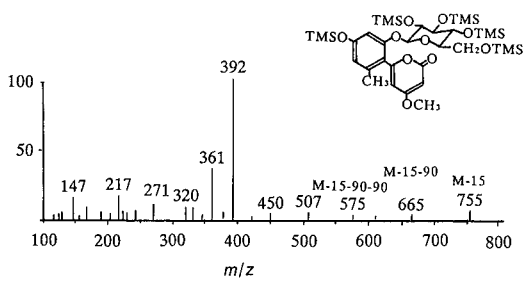

Fig. 2. Typical mass spectrum of the TMS derivative of aloenin obtained by GC–MS using a VG Analytical (Manchester, U.K.) Model 70s mass spectrometer equipped with a Hewlett-Packard 5890A gas chromatograph with a Megabore CB-1 fused-silica capillary column, the same as reported in our previous paper[3]. Vertical axis: intensity.

TABLE I

RECOVERIES OF ALOENIN FROM COSMETICS

Sample	*Added (μg/g)*	*Recovery (%)*	*R.S.D. (%) (n=5)*
Lotion	0.25	95	7.0
	2.5	97	3.9
Cream	0.25	94	7.3
	2.5	98	5.2

and $y = 1.78 + 1.64x$ ($r = 0.999$), respectively (y = peak height, x = aloenin concentration). The determination of aloenin concentration over wide ranges was possible by transforming the ion multiplier voltage of the mass spectrometer and the gain of the multi-ion detector.

Average recoveries of five determinations with addition of aloenin standards to a commercial lotion and a commercial cream are given in Table I. The average recoveries were more than 94% with relative standard deviations of 4–7%. The detection limit was 0.02 μg/g for the standard solutions, 0.05 ng per injection, on the basis of a signal-to-noise ratio of 1:5. This method has a 100-fold higher sensitivity than the previous GC method[3], which was carried out with a Megabore CB-1 fused-silica capillary column (15 m × 0.53 mm I.D.) with flame ionization detection, after treatment with an ODS cartridge and trimethylsilylation with BSA in the present method.

TABLE II

ALOENIN CONTENTS IN COMMERCIAL COSMETIC PRODUCTS

Sample	*Kidachi-Aloe extracts content combined into the products (%)*[a]	*Aloenin content (μg/g)*[b]
Lotions:		
1	1.30	42
2	0.15	1.2
3	0.01	0.80
Milky lotions:		
1	0.25	1.7
2	0.01	0.96
Creams:		
1	0.15	2.3
2	0.30	3.5
3	0.20	4.0
4	0.39	0.25
5	0.25	1.8
Face packs:		
1	0.20	2.3
2	0.50	4.8
Hair rinse	0.25	1.6
Hair tonic	1.50	6.2

[a] Reported by the manufactures.
[b] Detection limit 0.02 μg/g.

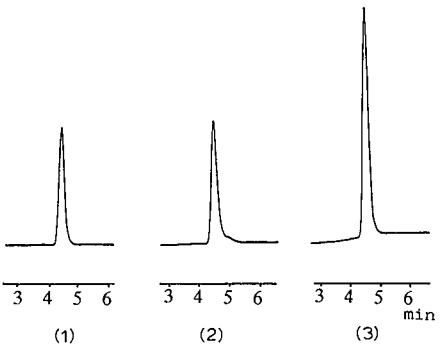

Fig. 3. Typical GC-MF at m/z 392 of commercial skin-care cosmetics. The retention time of each peak is 4.35 min, and the peak height of each sample was obtained with the following ion multiplier voltages (IMV) of the mass spectrometer and gain (G) of the multi-ion detector: (1) cream, IMV = −1.2 kV, G = 1; (2) face pack, IMV = −1.4 kV, G = 1; (3) milky lotion, IMV = −1.6 kV, G = 1. Other GC–MF conditions as under Experimental.

Aloenin content in commercial aloe cosmetics

The aloenin contents of several commercial cosmetics containing 0.01–1.5% of Kidachi-Aloe extracts was determined by the present method. The resulting contents were in the range 0.25–42 μg/g, as shown in Table II. Trace amounts of aloenin in commercial cosmetics containing Kidachi-Aloe extracts were successfully determined with less interferences by the present method. Typical GC–MF results for aloenin in commercial skin-care cosmetics are shown in Fig. 3.

CONCLUSION

Trace amounts of aloenin in commercial cosmetics containing Kidachi-Aloe extracts were determined by GC–MF as the trimethylsilyl derivative. The average recoveries were more than 94% with a relative standard deviation of 4–7% and a detection limit of 0.02 μg/g. This method is suitable for the verification of Kidachi-Aloe extracts in commercial skin-care cosmetics.

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Journal of Chromatography, 509 (1990) 383–389
Elsevier Science Publishers B.V., Amsterdam

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Note

Gas chromatographic determination of 2-ethylhexanol and 2-ethylhexanoic acid as derivatives suitable for electron-capture and nitrogen–phosphorus detection after single reaction with heptafluorobutyrylimidazole

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2-Ethylhexanol (2-EH) enters our environment largely in the form of esters with various poly-functional acids, especially phthalic. Di(2-ethylhexyl)phthalate (DEHP), the most widely used plasticizer, can leach slowly from plastic products. Both DEHP and its hydrolysis product, 2-EH, contaminate the environment[1,2]. The National Toxicology Program is investigating the toxicological properties of 2-EH because of the wide exposure of the general population to its precursor the hepatocarcinogen DEHP[3].

During the analytical methods development to support the toxicology studies, the major metabolite of 2-EH, 2-ethylhexanoic acid (2-EHA)[4], was found to react in quantitative yield with the derivatization reagents N-trifluoroacetyl-, N-pentafluoropropionyl- and N-heptafluorobutyrylimidazole (HFBI), which were being used for acylation of 2-EH. The resultant derivative N-2-ethylhexanoylimidazole (EHI) was shown to have good chromatographic properties. Although N-substituted fluorinated acyl imidazole reagents are known to form esters with phenols, alcohols and amines[5], their reaction with carboxylic acids to form imidazole derivatives has not been reported.

Derivatization of the hydroxyl and carboxy groups by means of fluoroacylimidazoles was accomplished by a one-step reaction.

This finding raised the possibility of simultaneous quantitation of 2-EH and 2-EHA in biological samples by means of gas chromatography (GC) with highly sensitive and selective electron-capture and nitrogen–phosphorus detectors, respectively. This paper describes the simultaneous derivatization of 2-EH and 2-EHA and focuses on the evaluation of HFBI as a reagent for the determination of 2-EHA. The mass spectral properties of the 2-EHA derivative are also discussed.

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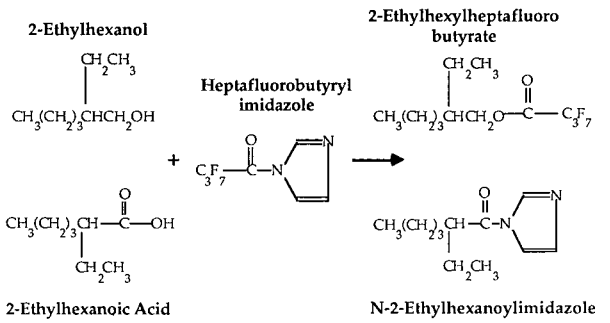

EXPERIMENTAL

Reagents

2-Ethylhexanol, 2-ethylhexanoic acid, octanol and 2-propylpentanoic acid (2-PPA) were obtained from Aldrich (Milwaukee, WI, U.S.A.), and were the highest purity available. HFBI as an imidazole kit (10 × 0.2 g) was obtained from Alltech/Applied Science, Deerfield, IL, U.S.A. All other chemicals were analytical-reagent grade. Solutions of NH_4OH were prepared in deionized water. Standard solutions of 2-EH and 2-EHA, as well as the internal standards, octanol and 2-PPA, were prepared by dissolving appropriate amount of each in *n*-hexane.

Instrumentation

For 2-EH analyses, capillary column GC was performed with a Perkin-Elmer Sigma 2000 gas chromatograph equipped with an ^{63}Ni electron capture detector, a split/splitless capillary inlet system, and a 1.0-μm DB-5 film, fused-silica capillary column (30 m × 0.32 mm; J and W Scientific, Rancho Cordova, CA, U.S.A.). The system was used in the split mode (*ca.* 1:45). The column was operated isothermally at 100°C with the injector and detector at 150 and 250°C, respectively. Helium, at a flow-rate of 1 ml/min, was used as the carrier gas, and argon–methane (90:10) was delivered at 30 ml/min as the detector makeup gas.

For 2-EHA, chromatographic analyses were performed on a Perkin-Elmer Sigma 2000 gas chromatograph equipped with both flame ionization and nitrogen–phosphorus detectors, and a 1.8 m × 2 mm glass column packed with Ultra-Bond FFAP 100–120 mesh (Alltech, Deerfield, IL, U.S.A.). Helium was used as the carrier gas at a flow-rate of 30 ml/min. The nitrogen–phosphorus detector was optimized for maximum selectivity and the bead current was kept below maximum sensitivity in order to increase the lifetime of the bead. The temperatures employed were as follows: oven, 130; injector, 200; detector, 250°C.

A laboratory data system, Hewlett-Packard Model 3359A, was used for data handling. The 0.8-ml autosampler vials capped with viton septa (Sunbrokers, Wilmington, NC, U.S.A.) were used for samples and standard preparation.

GC–mass spectrometry experiments were conducted on a Finnigan-MAT 4500 gas chromatograph–mass spectrometer–data system. The DB-5 capillary column was introduced directly into the ion source. The source temperature was maintained at

200°C and the electron energy was 70 eV. The gas chromatograph was programmed from 35 to 250°C at a rate of 4°C/min, with the injector at 200°C. Helium was used as the carrier gas at 1 ml/min in the splitless/split mode. The scanning speed was 1 sec over a mass range of 35–650 mass units. Data were acquired on an INCOS data system using a Nova 3C computer.

Derivatization

To 200 μl of a solution of 2-EH, 2-EHA and internal standard 2-PPA in a 0.8-ml autosampler vial were added 10 μl of the HFBI and the vial was capped. The solution was shaken briefly and after 5 min, 400 μl water was injected into the vial and vortexed until the organic phase was clear (*ca.* 20 s). The water was removed by syringe and 400 μl of 5% NH_4OH was injected into the vial. After vortexing, the NH_4OH phase was removed and the organic phase was washed twice with 400 μl water. A 150-μl aliquot of the organic phase was removed by syringe and transferred into a new vial. Aliquots of 2.5 μl of the hexane phase were injected into the gas chromatograph.

RESULTS AND DISCUSSION

A major problem in the development of GC methods for biological studies

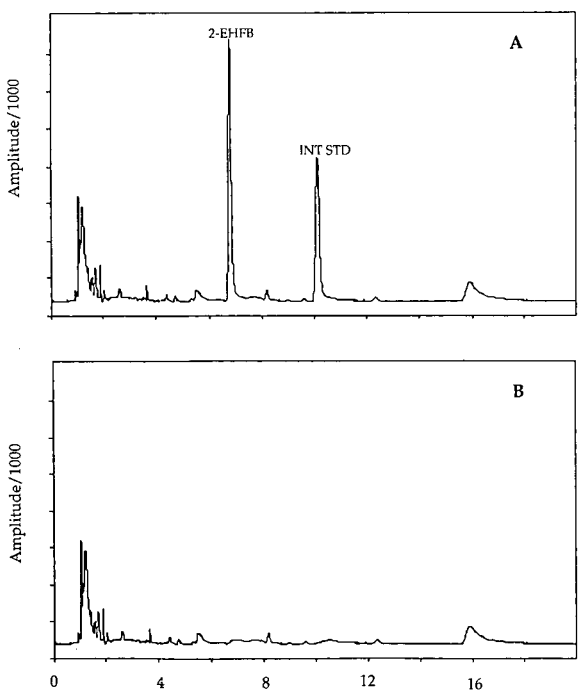

Fig. 1. GC–electron-capture detection of (A) derivatized 2-EH and octanol (internal standard, INT STD) (4 μg/ml) and (B) control. Chromatographic conditions: column, DB-5 FSCC 30 m × 0.32 mm. Temperature program: 100°C isothermal. Injector: split 1:45. Carrier gas: helium at 1.0 ml/min.

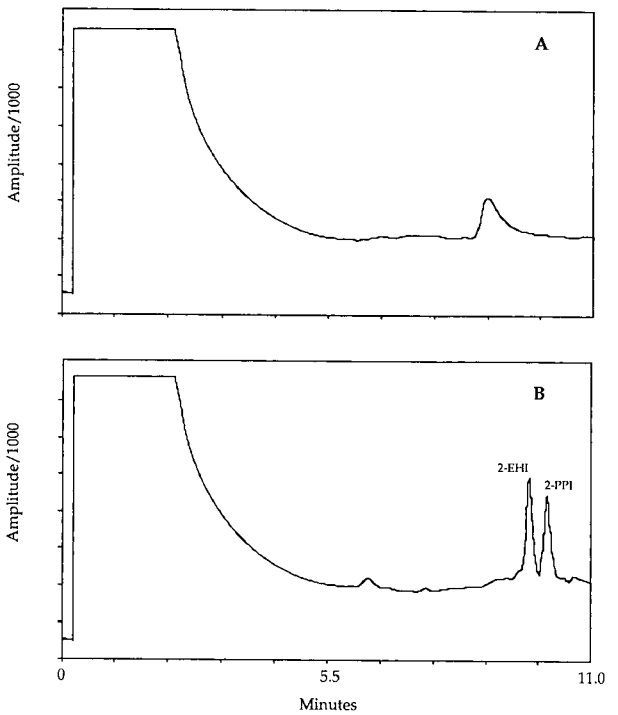

Fig. 2. GC–flame ionization detection of 2-EHA and 2-PPA (0.4 μg/ml) before (A) and after (B) reaction with HFBI. Chromatographic conditions: column, glass, 1.8 m × 2 mm packed with Ultra-Bond FFAP 100–120 mesh. Temperature program: 100°C for 5 min, then to 150°C at 10°C/min hold for 1 min. Carrier gas: nitrogen at 30 ml/min.

involves achieving highly sensitive and selective detection. From literature data[1], as well as preliminary studies conducted in our laboratory, it was apparent that expected levels of 2-EH in blood would be very low. For this reason the procedure described here, involving the formation of the heptafluorobutyryl ester of 2-EH, was chosen for investigation. It is known that HFB esters of alcohols may be formed readily, that these compounds have good GC properties and that the HFB group is detectable with high sensitivity by electron-capture detection[6].

A capillary column was chosen for use in the studies with 2-EH because low levels were expected and because of its potential for high resolution (Fig. 1). A packed column was used for the 2-EHA studies because much higher levels of the metabolite were anticipated (Fig. 2).

During the development work with 2-EH it was observed that the chromatographic properties of 2-EHA also changed. On further evaluation by mass spectrometry the derivative of 2-EHA was shown to be N-2-ethylhexanoylimidazole (EHI). This derivative exhibited good GC properties with sharp peaks, an absence of tailing, no evidence of decomposition and a wide linear response.

In order to optimize the analytical procedure, the kinetic parameters of the exchange reaction were studied. In these experiments 100 μg of 2-EHA and 2-PPA

were reacted with 10 μl HFBI at room temperature, and the reaction yield was measured as a function of time. The yield was calculated by measuring the disappearance of the 2-EHA and 2-PPA with a flame ionization detector. The results obtained indicate that the reaction reached equilibrium (91.0 ± 3.0% yield) in less than 6 min and that the derivatives of 2-EHA and 2-PPA are stable in *n*-hexane for at least 24 h. The effect of temperature on the reaction yield was also studied. No noticeable effect was observed when the reaction temperature was raised to 60°C, a further indication that the reaction is quantitative. The effect of concentration on the reaction kinetics was studied for 2-EHA and 2-PPA by using solutions from 8 to 500 ng/μl. In all cases the yields reached a plateau rapidly and remained constant for hours, indicating no effect of concentration on the total yield over the stated concentration range.

The results obtained from the reaction kinetic studies indicate that HFBI is a good analytical reagent for 2-EHA as well as for the well characterized reaction with alcohols such as 2-EH, since the reaction is rapid, quantitative, and can be easily performed under very mild conditions. Comparison between nitrogen–phosphorus

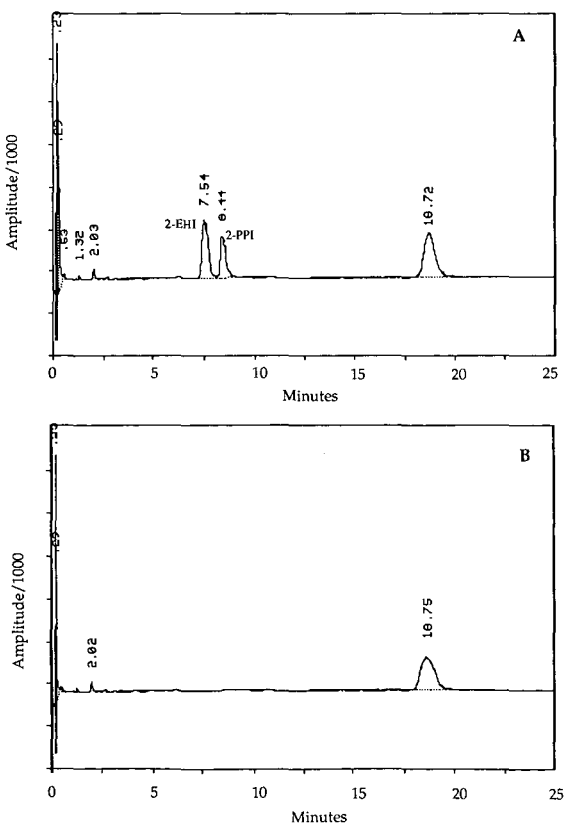

Fig. 3. GC-nitrogen–phosphorus detection of (A) derivatized 2-EHA (0.8 μg/ml) and 2-PPA (0.8 μg/ml) extracted and (B) control. Chromatographic conditions: column, glass, 1.8 m × 2 mm packed with Ultra-Bond FFAP 100–120 mesh. Temperature program: 130°C isothermal. Carrier gas: helium at 30 ml/min.

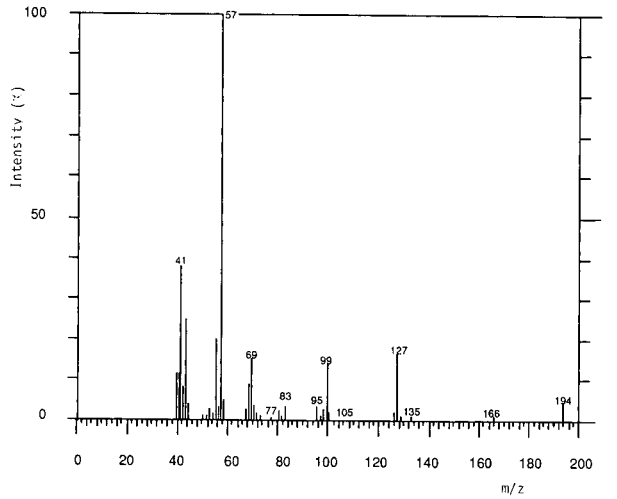

Fig. 4. Electron-impact (70 eV) mass spectrum of EHI.

and flame ionization detectors showed that sensitivity for EHI is *ca.* 10 times greater with nitrogen–phosphorus detection than with flame ionization detection. An example of GC–nitrogen–phosphorus detection of a 2-EHA and 2-PPA after derivatization and of a reagent blank are shown in Fig. 3. Under the routine detection conditions used, the lowest quantity detected was found to be 50 pg.

The mass spectral behavior of the imidazolo derivative of 2-EHA was also studied. The mass spectrum of EHI is shown in Fig. 4. Examination of this spectrum revealed that the spectrum contains sufficient information for structure confirmation and that relatively intense diagnostic ions were observed. The molecular ion of the

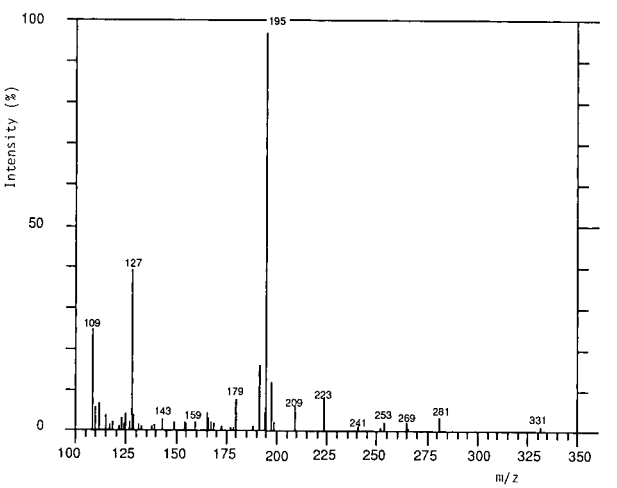

Fig. 5. Methane chemical ionization mass spectrum of EHI.

imidazolo derivative of 2-ethylhexanoic acid was observed at mass 194. A minor loss of 28 mass units observed at mass 166. The ion of mass 127 arose from an α-cleavage of the imidazolo group, with a subsequent cleavage of the carbonyl group to generate the ion of mass 99. Charge retention with transfer of two hydrogen atoms to the imidazolo group generated the ion of mass 69. McLafferty rearrangement, with charge retention on the hydrocarbon fragment of mass 57, was responsible for the generation of the base peak of the mass spectrum. Reinforcement for the assignment of mass 194 as the molecular weight of the imidazolo ester derivative was obtained from the methane chemical ionization mass spectrum (Fig. 5), which was dominated by the $(M + 1)^+$ species at mass 195.

The potential for simultaneous derivatization and analysis of 2-EH and 2-EHA has permitted the development of an analytical method for determining 2-EH and 2-EHA in biological samples which has facilitated our pharmacokinetic studies of 2-EH. Work with this derivatization reagent for other branched chain carboxylic acids is continuing in our laboratory.

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Journal of Chromatography, 509 (1990) 390–395
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 445

Note

Internal surface reversed-phase high-performance liquid chromatographic separation of the cyanobacterial peptide toxins microcystin-LA, -LR, -YR, -RR and nodularin

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(First received January 31st, 1990; revised manuscript received March 14th, 1990)

Several species of freshwater cyanobacteria (blue-green algae), *e.g.*, *Microcystis aeruginosa*, *Oscillatoria agardhii* and *Anabaena flos-aquae*, produce a family of low-molecular-weight peptide liver toxins known as microcystins (MCYST). The general structure of these heptapeptide toxins is cyclo(-D-Ala–L-X–D-*erythro*-β-methyl-Asp–L-Y–ADDA–D-Glu–N-methyldehydro-Ala), where X and Y denote the variable amino acids and ADDA represents β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid[1–3]. Further, the residues D-*erythro*-β-methyl-Asp and N-methyldehydro-Ala can be desmethylated[4]. One brackish water cyanobacterial species, *Nodularia spumigena*, produces a pentapeptide toxin, nodularin (NODLN), with the structure cyclo(-D-*erythro*-β-methyl-Asp–L-Arg–ADDA–D-Glu–N-methyldehydroaminobutyric acid)[5]. Only one type of nodularin has been reported so far[5–8].

Cyanobacterial toxins have been detected and analysed by the use of mouse bioassay, high-performance liquid chromatography (HPLC) and immunoassay. These methods have their advantages and disadvantages. The mouse bioassay is rapid and does detect degree of toxicity, but it does not detect multiple hepatotoxins in a

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sample and it is difficult to detect hepatotoxins in a sample when the faster acting neurotoxins are also present. In addition, there is more variability in reporting toxicity between different laboratories than is desired for accurate toxicity level reporting. Finally, there is the need to minimize the use of laboratory animals in toxicity testing, if other equally accurate or more accurate clinical detection methods are available.

Immunoassay is a rapid, accurate method of detection for determining the presence and amount of the peptide toxins in water, algal cells and animal tissue[9]; however, only total toxin content is determined, not the amounts or types of the individual peptide toxins. It is important to be able to distinguish between the different microcystins because their toxicities vary.

HPLC has been the method of choice for the separation and analysis of the various cyanobacterial peptide toxins[10–12]. Although optimization of the stationary phase and the mobile phase composition has been used successfully to separate the various toxins, there is always a need for more rapid separations of multiple toxin samples from crude field extracts and laboratory-cultured strains. This paper reports the successful use of internal surface reversed-phase (ISRP) HPLC columns to overcome some of the problems with multiple toxins and crude cell extracts.

ISRP-HPLC was originally developed to simplify drug analysis in protein-rich serum samples[13–15]. It combines size-exclusion sample purification and reversed-phase separation. Although the stationary phase, Gly-L-Phe–L-Phe, is basically a reversed phase, the free carboxyl group in the terminal phenylalanine gives the phase additional functionality as a weak cation exchanger. The stationary phase is located on the inner walls of porous silica material. Only small analytes (molecular weight <5000) penetrate the pores and are retained and separated. Macromolecules are unretained and eluted rapidly.

In a previous study[16] we used ISRP-HPLC for the analysis of three (structurally uncharacterized) cyanobacterial peptide toxins. In this work we used the ISRP approach for the separation of four microcystin analogues and nodularin in an isocratic run.

EXPERIMENTAL

Purification of toxins

The peptide toxins were purified from the following sources as described previously: MCYST-LR (Leu and Arg as the variable residues) from *Microcystis aeruginosa* collected from a natural bloom in lake Akersvatn, Norway[17], MCYST-RR (Arg, Arg) from the *M. viridis* culture TAC44[11], MCYST-YR (Tyr, Arg) from the *M. aeruginosa* culture M-228[18], MCYST-LA (Leu, Ala) from a toxic water bloom in Eau Claire, WI, U.S.A.[9] and NODLN from the *Nodularia spumigena* culture L575[6].

Preparation of a field sample

Lyophilized *M. aeruginosa* material from Akersvatn was used to test the extraction efficiency. A 10-mg amount of cyanobacteria in a 1.5-ml polypropylene Eppendorf tube (Treff, Degersheim, Switzerland) was extracted for 5 min in a bath sonicator using 100 μl/mg of acetonitrile–0.1 *M* potassium dihydrogenphosphate (15:85; pH 6.8, adjusted with 10 *M* potassium hydroxide after addition of acetonitrile; chemicals from Merck, Darmstadt, F.R.G.). The sample was centrifuged at about

10 000 *g* for 10 min, after which the supernatant was collected and the pellet was re-extracted once or twice (four samples per treatment). The combined supernatants were spun once more and the sample was ready to be injected in the HPLC system.

Extracts of *M. aeruginosa* material were also used to check how well the toxins were separated from macromolecules and pigments in a natural cyanobacterial sample. The material was extracted twice in the manner described above.

Internal surface reversed-phase HPLC

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-7A pump coupled to an SPD-6A detector set at 238 nm and a CR-5A integrator. The 250 mm × 4.6 mm I.D. GFF-S5-80 ISRP column (serial number 21102) was manufactured by Regis Chemical (Morton Grove, IL, U.S.A.). The column was protected by a Rheodyne (Cotati, CA, U.S.A.) 0.45-μm on-line filter and ISRP precolumn. The mobile phase was acetonitrile–0.1 *M* potassium dihydrogenphosphate (pH 6.8, adjusted with 10 *M* potassium hydroxide after the addition of acetonitrile) (15:85) at a flow-rate of 1 ml/min. Samples of 20 μl of purified toxins or extract were injected with a Rheodyne 7125 injector.

The toxins were first run separately and then as a mixture at concentrations of MCYST-LA 8.5, NODLN 9.5, MCYST-LR 15.5, MCYST-YR 18.0 and MCYST-RR 22.0 μg/ml. The *M. aeruginosa* extracts were run either separately or spiked (1:1, v/v) with the toxin mixture (MCYST-LA, -LR, -YR, -RR and NODLN). The MCYST-LR concentration in the extracts was 16.8 μg/ml, corresponding to 3360 μg/g cyanobacteria.

RESULTS

The third extraction did not improve the toxin yield significantly. More than 97% of MCYST-LR in the *M. aeruginosa* material was extracted after two cycles and the extraction was thus considered to be complete after two cycles.

The four microcystins and nodularin were well separated and had good peak shapes. The retention times were MCYST-LA 3.45, NODLN 4.30, MCYST-LR 5.08, MCYST-YR 5.84 and MCYST-RR 7.45 min (Fig. 1, left panel). The retention times in spiked field sample were MCYST-LA 3.44, NODLN 4.29, MCYST-LR 5.06, MCYST-YR 5.81 and MCYST-RR 7.43 min. The fraction of macromolecules and polar molecules in the beginning of the chromatogram did not interfere with the identification of the toxins (Fig. 1, right panel).

DISCUSSION

Our results together with earlier work[19] show that ISRP-HPLC can be used in applications beyond its original scope, the analysis of drugs in serum samples. Separation of certain peptides seems to be a new area for the ISRP columns. Especially those applications requiring extensive sample pretreatment could benefit from the concept of ISRP to separate macromolecules from analytes.

Analyte selectivity of the ISRP column

A problem in the chemical analysis of cyanobacterial toxins is the number of

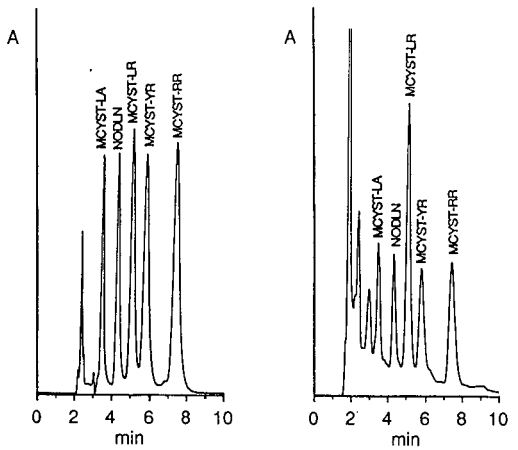

Fig. 1. ISRP-HPLC separation of cyanobacterial peptide toxins. Column, Regis GFF-S5-80 ISRP (250 mm × 4.6 mm I.D.) with an ISRP precolumn; mobile phase, acetonitrile–0.1 *M* potassium dihydrogenphosphate, pH 6.8 (15:85); flow-rate, 1 ml/min; sample size, 20 µl; detection at 238 nm. (Left panel) Separation of the purified toxins MCYST-LA (170 ng), NODLN (190 ng), MCYST-LR (310 ng), MCYST-YR (360 ng) and MCYST-RR (440 ng). The MCYST-LR signal was 41.8 milli-absorbance units. (Right panel), Separation of *M. aeruginosa* extracts spiked (1:1, v/v) with the purified toxins MCYST-LA, -LR, -YR, -RR and NODLN. The amounts of the toxins were MCYST-LA 85, NODLN 95, MCYST-LR 330, MCYST-YR 180 and MCYST-RR 220 ng. The MCYST-LR signal together with the underlying tailing part of the chromatogram was 45.6 milli-absorbance units.

analogues. The ISRP column resolves at least five toxins in a 10-min run. The resolving power of the ISRP column is not surprising as ISRP analyses combine reversed-phased chromatography with cation exchange and size exclusion.

The primary aim of the size-exclusion mode is on-line purification and separation of macromolecules from analytes. Ordinary reversed-phase columns are prone to protein denaturation and column clogging. This is a real problem in the cyanobacterial context because cyanobacteria can contain more than 15% protein (dry weight). Protein binding at neutral pH and small amounts of acetonitrile is not a problem with the ISRP column and the lifetime of the ISRP columns is longer was expected; we used a 15-cm ISRP column with more than 2000 extract and toxin samples without any significant loss of resolution[20].

Reversed-phase and cation-exchange mechanisms in the ISRP phase resolve analytes. Reversed-phase separation is thought to be the primary mode in ISRP-HPLC[13,15,21]. The ISRP material is selective for aromatic and other ring structures. The retention power of ISRP was found to resemble that of a phenyl phase when phenytoin (5,5-diphenyl-2,4-imidazolidinedione) was run on ISRP, phenyl, C_8 and C_{18} columns[13]. In a later study the capacity factors of 36 drugs and related compounds were measured using 0.1 M phosphate–isopropanol–tetrahydrofuran (84:10:6)[15]. In general, single-ring aromatic and aliphatic compounds yielded capacity factors (k') ranging from 0 to 2. Substances with two aromatic rings had $k' = 2$–12 and compounds with three fused rings or diaryl compounds with aliphatic side-chains gave $k' = 14$–26. The obvious selectivity for ring structures is due to the phenylalanine residues in the stationary phase.

However, the apolar residue ADDA, which has a lipophilic side-chain ending in a phenyl ring, seems to play a relatively minor role in the total retention under the conditions used. ADDA is present in all the toxins studied in this work. For example, for MCYST-LA $k' \approx 0.9$ (for bovine serum albumin $k' = 0$). This could indicate that the phenyl ring of ADDA is not available to the ISRP phase. This hypothesis is supported by conformational simulations with the molecular modelling program Chem-X (Chemical Design, Oxford, U.K.). The studies showed that the lipophilic side-chain of the ADDA residue is folded and turned inwards towards the centre of the toxin molecules[22].

Comparison of the separated toxins and work by Pinkerton and Koeplinger[19] showed that cation exchange plays a crucial role in analyte selectivity. The arginine residues are positively charged at pH 6.8 and MCYST-RR is the most cationic of the toxins separated here. It is also the most polar molecule (as indicated by a low capacity factor on a C_{18} phase and a low R_F value on silica gel thin-layer chromatographic plates[11]). However, it is the last to elute ($k' \approx 3.1$). Further evidence of the importance of the cation-exchange mechanism is given by the fact that the tyrosine-containing toxin MCYST-YR is eluted before MCYST-RR.

There are several more microcystins in addition to those studied here. Although speculation, the good separation results obtained in this work suggest that ISRP-HPLC can separate more microcystins. Changes in pH, organic modifier and buffer concentration should provide new separation possibilities. Preliminary studies on toxin separation at lower pH showed that, in general, the retention times become longer and the column has even more resolving power at lower pH. Low-pH separations were suggested by Pinkerton *et al.*[15].

Practical aspects

The retention times in the spiked field sample were slightly shorter than those in the sample with the purified toxins. This difference is probably caused by interfering compounds that block the stationary phase pores so that analytes cannot penetrate all the pores. In most instances the difference is negligible and should not jeopardize identification. However, in critical work the use of a diode-array detector and spectral analysis is justified for identification of peaks.

A diode-array detector can also be used to identify novel toxins and toxins not mentioned in this paper. All the microcystins and nodularin that we isolated had a local maximum at 238–240 nm in their UV spectrum (due to the conjugated double bonds in ADDA).

Although cyanobacterial material can be injected directly without off-line purification, there are samples that require concentration. The detection limit (signal-to-noise ratio = 3) for pure toxin samples is less than 1 ng of toxin per injection in the HPLC system used here. In field samples with interfering contaminants the limit of detection may be about 10 ng per injection. Samples with low toxin levels can be concentrated using C_{18} solid-phase extraction cartridges[11].

Further studies on the ISRP-HPLC of cyanobacterial toxins will be focused on the separation of more analogues and the simultaneous analysis of peptide and alkaloid toxins in cyanobacteria. We are also investigating the ISRP retention mechanisms in detail.

ACKNOWLEDGEMENTS

We thank the Maj and Tor Nessling Foundation, the Academy of Finland (Research Council for Environmental Sciences) and the Oskar Öflund Foundation for their valued financial support. Toxin purification was supported in part by USAMRDC contract DAMD-17-87-C-7019 to W.W.C. We also thank Olav Skulberg, Norwegian Institute for Water Research, for the Akersvatn bloom material.

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Journal of Chromatography, 509 (1990) 396–399
Elsevier Science Publishers B.V., Amsterdam

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Note

Analytical study of phosphorothioate analogues of oligodeoxynucleotides using high-performance liquid chromatography

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(First received October 30th, 1989; revised manuscript received February 2nd, 1990)

There is an increasingly widespread use of synthetic oligodeoxynucleotides, more recently in the field of selective gene regulation by "antisense oligonucleotides"[1], with consequent therapeutic implications. The selection of phosphate backbone modified analogues of oligonucleotides, such as methylphosphonate[2–4], phosphorothioate[5–8] and phosphoramidate[6,7] provides nuclease resistance and has greatly enhanced the possibility of their use *in vivo*. Since the purity and chemical identity of

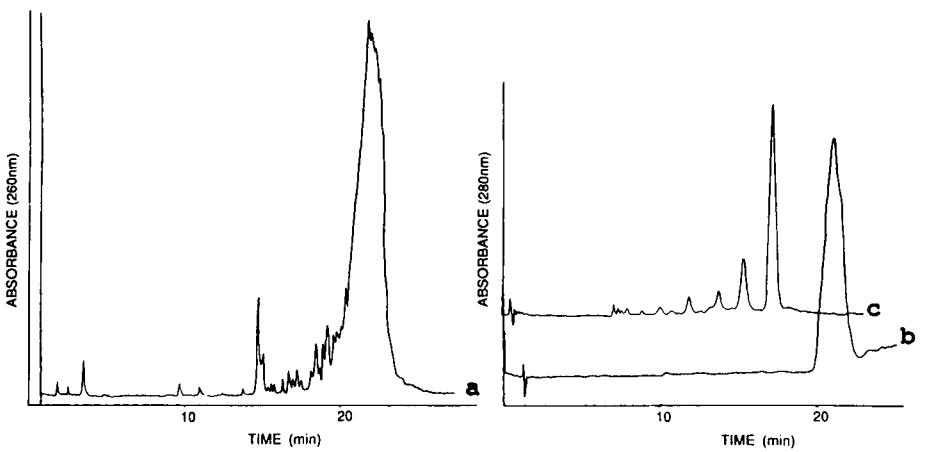

Fig. 1. HPLC traces of (a) crude phosphorothioate octamer, ACTGATGC on reversed phase, (b) purified phosphorothioate octamer on reversed phase and (c) purified phosphorothioate octamer on Partisphere SAX. Conditions: HPLC system consists of Waters 600E system controller, Waters Lambda Max Model 481 detector, Waters 745 data module, reversed-phase column Novapak C_{18} with RCM 100, ion-exchange column Partisphere SAX. For reversed-phase HPLC the buffers were 0.1 *M* ammonium acetate (pH unadjusted) containing (A) 0% acetonitrile and (B) 80% acetonitrile. Gradient was 0% B 2 min, 0–60% B 35 min, flow-rate, 1.5 ml min^{-1}. For ion-exchange HPLC the buffers were prepared from a stock solution of 1 *M* KH_2PO_4 adjusted to pH 6.3 with KOH to give (A) 1 m*M* and (B) 0.4 *M* both containing 60% formamide, flow-rate, 2 ml min^{-1}, gradient was 0% B 2 min, 0–70% B 35 min.

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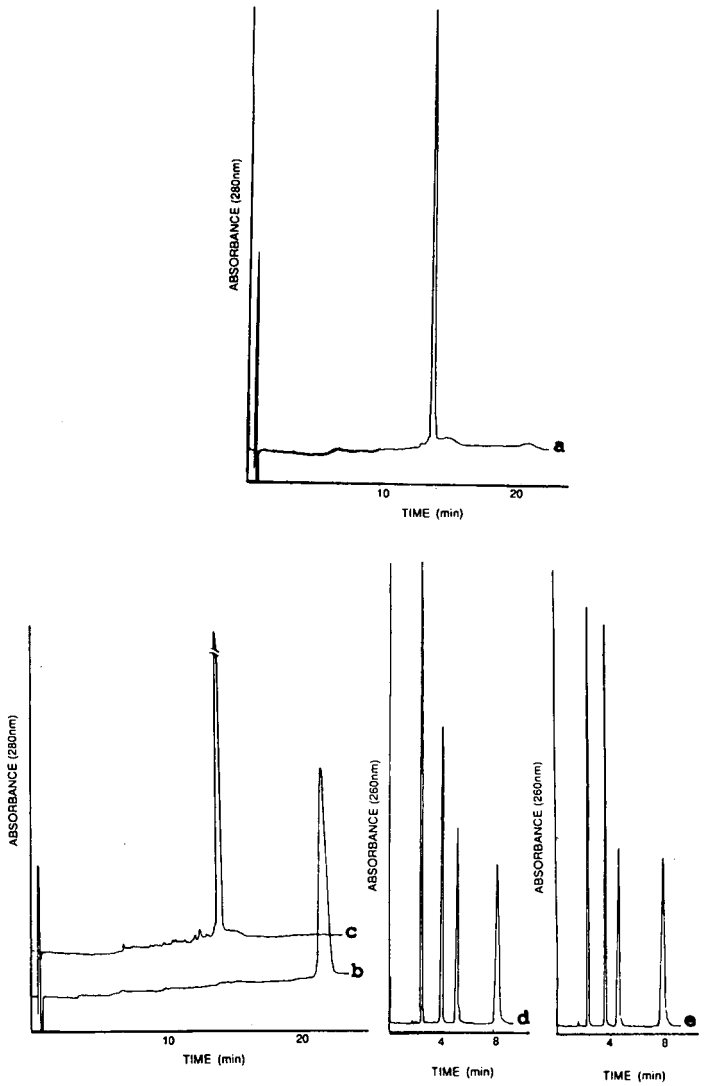

Fig. 2. HPLC traces of (a) purified phosphodiester octamer, ACTGATGC on Partisphere SAX, (b) purified phosphorothioate octamer, ACTGATGC on Partisphere SAX, (c) phosphorothioate octamer oxidised with sodium metaperiodate, (d) and (e) base composition analysis of phosphodiester octamer (authentic) and phosphodiester octamer obtained after oxidation of phosphorothioate octamer, respectively, after digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase. Conditions: HPLC system, buffers and columns were same as in the legend to Fig. 1. Gradient for HPLC (a), (b) and (c) was 0% B 2 min, 0–60% B 40 min, and gradient for HPLC (d) and (e), 5% A in 95% B isocratic.

a particular oligonucleotide is crucial to many applications, the ability to characterize synthetic oligonucleotide analogues on a routine basis is important. However, the analytical study of phosphorothioates has not been reported heretofore.

Phosphorothioate analogue of oligonucleotides can be assembled using either methoxyphosphoramidite[9] or H-phosphate chemistry[6,10]. These are diastereomeric

mixtures due to chirality at phosphorus. As a result, although they migrate like corresponding phosphodiester oligonucleotides on gel electrophoresis, on high-performance liquid chromatography (HPLC) the phosphorothioate oligonucleotides give broder peaks than phosphodiester oligonucleotides and also run slower because of their increased hydrophobicity. Fig. 1a shows the elution of a crude octamer, ACTGATGC, from a reversed-phase C_{18} column and, in Fig. 1b the elution of the purified material. When this is then chromatographed on an ion-exchange column shorter oligomers present are separated, as shown in Fig. 1c, allowing the isolation of the pure octamer. Thus, reversed-phase chromatography in itself is insufficient to purify phosphorothioate oligonucleotides. Phosphorothioate analogues are somewhat resistant to nucleases[11], and for the analysis of the base composition (and if necessary the sequence) conversion to the phosphodiester oligonucleotide is required. We have recently found that phosphorothioate oligonucleotides are rapidly and cleanly oxidised to the corresponding phosphodiester oligonucleotides by sodium metaperiodate in water. Iodine-bicarbonate oxidation is fast and equally effective and has been used for desulphurising oligomers containing one or two phosphorothioate internucleotide linkages[12,13]. When the purified phosphorothioate octamer (Fig. 2b) is oxidised it is converted to the phosphodiester octamer (Fig. 2c) identical in elution time to an authentic sample of the latter (Fig. 2a). The oxidised phosphorothioate octamer when digested with snake venom phosphodiesterase and bacterial alkaline phosphatase[14], gave a nucleoside mixture separable by HPLC (Fig. 2d and e) identical with that derived from the authentic phosphodiester oligomer[a], giving the correct base composition (A–G–T–C, 1:0.98:1:0.97). This procedure has been applied successfully to a number of other phosphorothioate oligomers including several 20-mers.

ACKNOWLEDGEMENTS

This work was supported by grants from: NIAID Cooperative grant U01 A 124846, and The G. Harold and Leila Y. Mathers Foundation to Dr. P. C. Zamecnik, whom we thank for provision of facilities and encouragement.

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[a] Assembly of the octamer was carried out using H-phosphonate chemistry. The intermediate CPG-bound octamer H-phosphonate before oxidation was divided into two portions. One portion oxidised with 2% iodine in pyridine–water (98:2) and used as an authentic sample. The other portion oxidised with 5% sulphur in triethylamine–pyridine–carbon disulfide (1:10:10) and used for oxidation with sodium metaperiodate.

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Journal of Chromatography, 509 (1990) 400–405
Elsevier Science Publishers B.V., Amsterdam

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Note

Chromatographie en couche mince sur gel de silice avec double imprégnation ascendante à sens inversés à l'huile de silicone

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La chromatographie en couche mince de gel de silice est l'une des méthodes les plus utilisées pour l'étude de la lipophilie des substances biologiquement actives[1]. On améliore dans certains cas les séparations chromatographiques en modifiant la nature de l'adsorbant utilisé, par example par une imprégnation des chromatoplaques à l'huile de silicone. Il existe en pratique deux méthodes d'imprégnation utilisées pendant plus de vingt ans dans notre département. (a) Par la première méthode, la plus ancienne, la plaque garnie de gel de silice est immergée dans la solution d'huile de silicone (solution de méthylsilicone, le plus souvent à 5% dans de l'éther). Une fois retirée, on laisse sécher à température ordinaire pour faire évaporer l'éther. (b) La deuxième méthode consiste à imprégner les chromatoplaques par une ascention de la solution de silicone dans une cuve fermée. Chaque de ces deux méthodes présente certaines insuffisances: la première méthode conduit à une répartition non-homogène de la couche imprégnante (couche d'huile de silicone) sur la chromatoplaque. La deuxième méthode permet une répartition plus homogène de la couche imprégnante. Cependant la section de cette couche diminue progressivement de la base plongée dans la solution d'imprégnation vers le sommet. Nous avons très tôt abandonné la première méthode pour son insuffisance qui ne permet pas d'avoir des résultats reproductibles. Plusieurs résultats ont été publiés avec la deuxième méthode. Il s'agit lá des résultats reproductibles avec l'utilisation d'un étalon. Des substances étudiées par cette méthode, nous pouvons citer les phénols[2,3], les dérivés de colchicine[4], les dérivés des acides iodobenzoïque, iodophénylacetique, iodophénylpropionique, iodophenoxy-acetique et iodohippurique[5,6], les dithiooxalamides[7] et les thiobenzamides[8].

L'insuffisance de la deuxième méthode, qui consiste en la diminution progressive de la section de la couche imprégnante, peut être éliminée selon nos expériences actuelles par une double imprégnation ascendante à sens inversés. Nous avons utilisé cette nouvelle méthode pendant l'étude de la lipophilie des thiohydrazides[9,10] et thiobenzanilides[11]. Le but de ce travail est de prouver l'efficacité et les avantages que nous apporte cette troisième méthode en comparaison avec les deux premières. Une autre possibilité est la chromatographie sur couche mince de gel de silice silanisé, fourni par certaines firmes telles que Whatman et Merck.

0021-9673/90/$03.50

PARTIE EXPÉRIMENTALE

Réactifs

Les substances étudiées, thiobenzanilide (I), 4-méthylthiobenzanilide (II), 4-méthoxythiobenzanilide (III), 4,4'-diméthylthiobenzanilide (IV), 4'-chloro-4 méthylthiobenzanilide (V), 4'-chloro-4-méthylthiobenzanilide (VI) et 4-chloro-4'-méthoxythiobenzanilide (VII) ont été décrites et caractérisées dans l'une de nos précédentes publications[11]. Les solvants, méthanol et éther, sont de pureté p.A. (Lachema, Brno, Tchécoslovaquia). La solution d'imprégnation utilisée est une solution à 5% dans de l'éther de méthylsilicone (Lucoil M 100, Lutchebnik, Kolin, Tchécoslovaquie). La phase mobile chromatographique est composée d'une solution tampon phosphate (5,447 g de KH_2PO_4 et 9,541 g de $Na_2HPO_4 \cdot 12\,H_2O$ dans 1000 ml de l'eau, pH 6.900, ajoutée à du méthanol en proportions variables).

Matériels

Les chromatoplaques, 20 × 20 cm, munies d'une couche de gel de silice (Silufol UV 254, Kavalier, Votice, Tchécoslovaquie) ont été imprégnées dans une cuve en verre avec couvercle rodé. Pour une comparaison appropriée nous avons aussi utilisé les chromatoplaques silanisées KC 18F reversed-phase (Whatman, Clifton, U.S.A.). Celles-ci n'ont pas été imprégnées.

Chromatographie

Les plaques Silufol ont été imprégnées de trois manières différentes A, B et C.

(A) La plaque garnie de gel de silice est immergée dans la solution de méthylsilicone; une fois retirée, on laisse sécher 24 h durant à la température du laboratoire, 23°C.

(B) La deuxième méthode consiste en la migration ascendante de la solution d'imprégnation pendant une durée de 15 h dans la cuve en verre soigneusement fermée. La plaque retirée est séchée à la température du milieu pendant 24 h.

(C) Double imprégnation à sens inversés: Après une migration ascendante de la solution d'imprégnation pendant une durée de 15 h et après avoir séché la plaque pendant 7 h, nous avons répété l'imprégnation suivant le principe B, mais avec une migration opposée à la première. La plaque a été ensuite séchée pendant 24 h à la température du laboratoire. Nous avons imprégné 60 chromatoplaques, 20 pour chaque type d'imprégnation.

Le développement et la détection chromatographique ont été plus simples. Nous avons chromatographié par la technique ascendante sur une distance de 15 cm à partir de la ligne de départ. Celle-ci est située à 20 cm du bord inférieur de la plaque. Toutes les substances étudiées (solution à 5% dans du chloroforme) ont été déposées sur la ligne de départ à environ 2 cm d'intervalle les unes des autres. Sur chaque chromatoplaque nous avons toutefois déposé toutes les sept substances. Les plaques ont été déposées simultanément dans la phase mobile (solution tampon phosphate plus méthanol en proportions variables selon la méthode de Biagi *et al.*[12]). Les concentrations de méthanol dans la phase mobile ont été respectivement de 30, 40, 50, 60 et 70%. Les chromatoplaques Whatman ont été utilisables pour les concentrations de 50, 60, 70 et 80% de méthanol dans la phase mobile. L'opération a été répétée quatre fois pour chaque concentration avec variation de la place des substances sur les plaques.

Des valeurs de R_F obtenues après détection sous lampe UV nous avons calculé les valeurs de R_M correspondants par l'équation 1 à l'aide de la calculatrice programmable Texas Instruments TI-59. Pour les équations de regression permettant l'extrapolation à la concentration zéro du méthanol, ainsi que pour toute autre équation pour la comparaison des trois types d'imprégnation nous avons utilisé le mini-ordinateur Sharp PC 1211. L'extrapolation a été faite selon l'équation 2.

$$R_M = \log(1/R_F - 1) \tag{1}$$

$$R_M = a(\%\ \text{méthanol}) + b \tag{2}$$

RÉSULTATS ET DISCUSSION

L'expérience a montré certains avantages de la double imprégnation à sens inversés, c'est-à-dire la méthode C, par rapport aux méthodes A et B. Le front de la phase mobile a migré sur toutes les plaques imprégnées par la méthode C parallèlement à la ligne horizontale de départ. Pour une comparaison objective nous nous sommes servis des variations aléatoires (S)[13] des valeurs de R_M (Tableaux II, III et IV). Pour la plupart les valeurs de S des valeurs de R_M par la méthode C sont inférieures à celles des valeurs de R_M obtenues par les méthodes A et B. L'imprégnation C permet aussi une grande reproductibilité des valeurs de R_M. Il existe cependant une corrélation notable entre les valeurs de R_M des trois types d'imprégnation:

$$R_M^B = 0{,}582 \quad R_M^A + 0{,}895 \qquad r = 0{,}916 \quad s = 0{,}126 \quad F = 26{,}125 \quad n = 7 \tag{3}$$

$$R_M^C = 1{,}044 \quad R_M^A + 0{,}212 \qquad r = 0{,}917 \quad s = 0{,}225 \quad F = 26{,}427 \quad n = 7 \tag{4}$$

TABLEAU I

ÉTUDE DE LA LIPOPHILIE AVEC LES CHROMATOPLAQUES WHATMAN KC 18F

Valeurs de R_M selon les différentes proportions de méthanol dans la phase mobile.

Substances	R_M				
	Proportions de méthanol dans la phase mobile (%)				
	50	*60*	*70*	*80*	*0*[a]
I	0,180	−0,180	−0,504	−0,890	1,949
II	1,090	0,542	0,301	−0,034	2,825
III	0,265	−0,211	−0,530	−0,794	1,955
IV	1,123	0,689	0,358	0,000	2,948
V	0,962	0,065	−0,311	−0,678	3,452
VI	1,038	0,578	0,204	−0,158	2,992
VII	1,123	0,720	0,347	−0,017	3,009

[a] Valeurs extrapolées selon l'équation 2.

TABLEAU II

ÉTUDE DE LA LIPOPHILIE AVEC LES CHROMATOPLAQUES IMPRÉGNÉES PAR LA MÉTHODE A

Valeurs de R_M extrapolées à la concentration zéro du méthanol dans la phase mobile; $n = 4$.

Substances	$R_M \pm S$					
	Proportions de méthanol dans la phase mobile (%)					
	30	*40*	*50*	*60*	*70*	*0*[a]
I	0,892 ± 0,053	0,538 ± 0,011	0,215 ± 0,048	0,065 ± 0,195	−0,408 ± 0,058	1,797
II	1,106 ± 0,008	0,712 ± 0,042	0,383 ± 0,014	0,119 ± 0,149	−0,275 ± 0,288	2,087
III	0,926 ± 0,008	0,544 ± 0,029	0,300 ± 0,175	0,138 ± 0,269	−0,274 ± 0,178	1,730
IV	1,263 ± 0,019	0,959 ± 0,045	0,506 ± 0,019	0,365 ± 0,296	−0,163 ± 0,022	2,309
V	1,372 ± 0,102	1,140 ± 0,565	0,378 ± 0,146	0,378 ± 0,146	−0,173 ± 0,054	2,582
VI	1,700 ± 0,055	0,683 ± 0,252	0,547 ± 0,085	0,351 ± 0,195	−0,190 ± 0,144	2,674
VII	1,534 ± 0,125	1,120 ± 0,048	0,710 ± 0,027	0,457 ± 0,450	−0,112 ± 0,053	2,719

[a] Valeurs extrapolées selon l'équation 2.

$$R_M^C = 1{,}704 \quad R_M^B - 1{,}193 \qquad (5)$$
$$r = 0{,}951 \quad s = 0{,}174 \quad F = 47{,}376 \quad n = 7$$

Les coefficients de corrélation r et les coefficients s (variances des résidus) sont obtenus par un test de Fisher[13].

Les indices A, B, C utilisés dans ces équations désignent les différentes sortes d'imprégnation. Les R_M^A sont les valeurs de R_M obtenues des chromatoplaques Whatman KC 18F.

En comparaison avec les valeurs de R_M obtenues avec les chromatoplaques Whatman KC 18F, la méthode A présente une corrélation $r = 0.885$, la méthode B $r = 0.947$ et la méthode C $r = 0.990$:

TABLEAU III

ÉTUDE DE LA LIPOPHILIE AVEC LES CHROMATOPLAQUES IMPRÉGNÉES PAR LA MTHODE B

Valeurs de R_M extrapolées à la concentration zéro du méthanol dans la phase mobile; $n = 4$.

Substances	$R_M \pm S$					
	Proportions de méthanol dans la phase mobile					
	30	*40*	*50*	*60*	*70*	*0*[a]
I	0,945 ± 0,018	0,682 ± 0,108	0,231 ± 0,023	0,077 ± 0,027	−0,309 ± 0,068	1,882
II	1,107 ± 0,125	0,793 ± 0,128	0,396 ± 0,143	0,111 ± 0,014	−0,279 ± 0,077	2,152
III	0,940 ± 0,065	0,634 ± 0,110	0,253 ± 0,043	0,078 ± 0,040	−0,293 ± 0,078	1,833
IV	1,144 ± 0,137	0,859 ± 0,063	0,498 ± 0,028	0,239 ± 0,010	−0,226 ± 0,017	2,183
V	1,463 ± 0,062	1,074 ± 0,360	0,779 ± 0,045	0,449 ± 0,045	−0,161 ± 0,094	2,657
VI	1,314 ± 0,333	0,990 ± 0,075	0,734 ± 0,012	0,455 ± 0,047	−0,280 ± 0,094	2,507
VII	1,315 ± 0,190	0,999 ± 0,021	0,717 ± 0,017	0,402 ± 0,044	−0,323 ± 0,017	2,559

[a] Valeurs extrapolées selon l'équation 2.

TABLEAU IV

ÉTUDE DE LA LIPOPHILIE AVEC LES CHROMATOPLAQUES IMPRÉGNÉES PAR LA MÉTHODE C

Valeurs de R_M extrapolées à la concentration zéro du méthanol dans la phase mobile; $n = 4$.

Substances	$R_M \pm S$					
	Proportions de méthanol dans la phase mobile (%)					
	30	*40*	*50*	*60*	*70*	*0*[a]
I	0,945 ± 0,012	0,732 ± 0,047	0,253 ± 0,022	0,087 ± 0,055	−0,286 ± 0,060	1,900
II	1,619 ± 0,020	1,298 ± 0,071	1,022 ± 0,057	0,597 ± 0,014	0,285 ± 0,054	2,649
III	0,985 ± 0,014	0,740 ± 0,053	0,348 ± 0,038	0,122 ± 0,015	−0,218 ± 0,050	1,904
IV	1,809 ± 0,029	1,464 ± 0,038	1,165 ± 0,023	0,784 ± 0,007	0,394 ± 0,064	2,878
V	1,728 ± 0,034	1,483 ± 0,094	0,968 ± 0,034	0,631 ± 0,034	−0,057 ± 0,019	3,162
VI	1,550 ± 0,025	1,304 ± 0,071	0,789 ± 0,002	0,378 ± 0,027	−0,082 ± 0,053	2,883
VII	1,640 ± 0,022	1,393 ± 0,089	0,868 ± 0,007	0,593 ± 0,007	−0,064 ± 0,025	2,990

[a] Valeurs extrapolées selon l'équation 2.

$$R_M^A = 0{,}700 \quad R_M^W + 0{,}397 \qquad (6)$$
$$r = 0{,}885 \quad s = 0{,}235 \quad F = 17{,}254 \quad n = 7$$

$$R_M^B = 0{,}479 \quad R_M^W + 0{,}931 \qquad (7)$$
$$r = 0{,}947 \quad s = 0{,}101 \quad F = 43{,}885 \quad n = 7$$

$$R_M^C = 0{,}896 \quad R_M^W + 0{,}176 \qquad (8)$$
$$r = 0{,}990 \quad s = 0{,}081 \quad F = 235{,}566 \quad n = 7$$

Somme toute, les avantages de la double imprégnation ascendante à sens inversés (méthode C) nous ont conduit à préférer cette méthode malgré la proportionalité des équations 4, 5 et 6. Dans notre département de chimie minérale et organique nous avons adopté cette méthode d'imprégnation applicable pendant l'étude chromatographique de la lipophilie des substances. L'étude réalisée sur les plaques Whatman a prouvé une excellente corrélation avec l'étude des mêmes substances sur le plaques de la méthode C. Nous pouvons alors conclure que le gel de silice silanisé peut être valablement utilisé pour l'étude de la lipophile des substances. Une explication profonde des effects physico-chimiques de la double imprégnation C, permettant une répartition homogène de la couche imprégnante, est l'objet de notre prochaine publication.

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Journal of Chromatography, 509 (1990) 406–407
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 418

Book Review

Practical HPLC method development, by L. R. Snyder, J. L. Glajch and J. J. Kirkland, Wiley, Chichester, New York, 1988, XVI + 260 pp., price £ 28.75, ISBN 0-471-62782-8.

This book contains information for chromatographers involved in developing (or improving) separation methods. It discusses the initial stages of method development ("Getting Started", Chapter 1). The basics of the separation (*i.e.*, resolution) are reviewed in Chapter 2. Although it deals with more than just the mobile-phase effects suggested by its title, this chapter is very brief. Chapter 3 is a more satisfactory review of the role of the column. The remaining six chapters deal in some way with systematic method development. Chapters 4 and 5 are arguably the most useful chapters for chromatographers in practice, as the text contains much information on which parameters to consider and on which effects can be expected. Chapter 4 concerns "easy" separations, in which the first approach yields acceptable results rapidly, and Chapter 5 concerns more difficult separations, which require more sophisticated method development or optimization methods. Chapter 6 concerns gradient elution. In Chapter 7 some "special" techniques are discussed, namely ion (exchange) chromatography, size-exclusion chromatography, the separation of enantiomers and trace analysis. Chapter 8 describes some commercial computer programs that may be of aid in the method development process, and Chapter 9 outlines the different procedures suggested in chromatographic rather than computer terms.

Most of the suggested procedures could be useful in forcing the reader into a systematic pattern of method development. Most of the recommendations are definitely sound. I personally believe that less emphasis should be put on the possibilities of adapting the selectivity by varying the solvent strength. While this is sometimes useful (as in the "standard" example of nitro compounds in reversed-phase liquid chromatography) the results often may not warrant the effort. I would also recommend some practical changes in the procedure for characterizing gradient elution equipment (pp. 174–175). Placing a restrictor after the detector can have dramatic consequences with many commercial systems. Also, I would not like to recommend measuring the UV absorption on a steep slope in the spectrum.

All in all, there is a lot of information in this book. In accordance with the title, most of it is very practical and there are many hints which I found useful. I was intrigued by the authors' attempts to use analogous (optimization) procedures in similar manners in what are thought to be very different techniques (reversed-phase, normal-phase, ion-pair). This is not always logical, for example because the sum of the parameters in ion-pair chromatography (Fig. 4.13) is not restricted as in the case of optimizing the overall mobile phase composition (sum of all volume fractions equal to unity). Thus, the triangular design loses its foundation. Nevertheless, this book is about *practical* method development, which should include all methods that work.

The book has obviously been written for chromatographers who prefer to work in the laboratory, rather than to read a book. There may be many such chromatographers, but in some places the brevity of the book becomes restrictive. On a number of complex issues, the book refers to the "classic" text of Snyder and Kirkland (*An Introduction to Modern Liquid Chromatography*, 2nd ed., Wiley, New York, 1979) and it is understandable that the authors did not try to include all the material from their monumental past into the present "practical" book. However, some aspects of contemporary high-performance liquid chromatography (HPLC) are now missing from the book. In the inventory of stationary phases (p. 67), for example, no mention is made of either carbon-based stationary phases or polymer-coated silicas. Both materials have received much interest in the chromatographic world since the publication of Snyder and Kirkland's text and both are being advocated by some as the materials of the 1990s.

To my disappointment, a number of advances in HPLC that are highly relevant to the method development process are not addressed. For example, I am strongly in favour of the use of diode-array detectors in HPLC method development. They allow rapid checks on peak purity and identity and can be used very easily and conveniently in *practical* method development. Diode-array detectors are mentioned only once in the book, in a very specific context (p. 215). Generally, very little attention is paid to detectors other than the "standard" UV spectrophotometer, except in the section on trace analysis (p. 192).

Another important subject that does not receive the attention it deserves is the use of expert systems. In my opinion these have already been proved useful for method development in HPLC, but in the book they are not mentioned at all. Method validation, an area that is of increasing importance in many (industrial) laboratories, is mentioned nowhere. The use of control charts is suggested indirectly (on p. 68, for monitoring the plate count), but the term is not used and the subject is not discussed as such.

This book gives a reasonable introduction to the process of method development in the 1980s, but it does not provide a perspective of the future. There is a need for good procedures, computer programs and literature on method development in HPLC, simply because, as stated on p. 124, "Some samples resist easy separation". I am inclined to direct my co-workers involved in developing HPLC methods to the "classical" Snyder and Kirkland text mentioned above. Using this book, good analysts will remain able to set out their own strategies. However, they will find increasing assistance in the form of hardware (diode-array detectors, data stations) and software (optimization procedures, expert systems), on which the new book does not provide much additional information.

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Journal of Chromatography, 509 (1990) 408–410
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 415

Book Review

Chiral liquid chromatography, edited by W. J. Lough, Blackie, Glasgow, 1989, IX + 276 pp., price £ 45.00, ISBN 0-216-92499-5.

The important role of stereochemistry in the various fields of life sciences has been increasingly recognized over the past few decades. The significance of stereoisomerism in relation to biological activity has become a serious issue in drug development, and regulatory agencies, *e.g.*, the FDA, require good scientific data on the potential differences between the enantiomers of a chiral molecule in terms of their toxicological, pharmacodynamic and pharmacokinetic profiles. This may also include information about drug interactions of the stereoisomers when co-administered in form of the common racemic mixture.

Within the biological environment, which is predominantly chiral, stereoselective recognition mechanisms (*e.g.*, receptor affinity) of chiral xenobiotics will have to be investigated in detail. This includes studies of the stereospecificity of enzymatic reactions, from a scientific point of view, but it may also be of relevance for, *e.g.*, the synthesis of chiral compounds using stereoselective biocatalysts. Questions about *in vivo* and *in vitro* racemization of chiral compounds will have to be tackled and discussed, since this might also have some far-reaching implications. At this point several more relevant subjects under discussion could be mentioned. However, within all these reflections a crucial part is the analytical recording of each individual enantiomer in a mixture thereof and probably down to a mass ratio range of 999:1. The methods for analysing enantiomeric mixtures are many-fold, but all share the general principle that the chiral analytes, selectand (SA), have to come into contact with a chiral source, the selector (SO), via intermolecular but spatially oriented contact points and complementary binding forces forming diastereomeric SO–SA molecule complexes.

This book tries to focus on such aspects by describing and discussing the main liquid chromatographic resolution techniques for enantiomers within separate chapters in order to provide the chromatographer with a broad spectrum of background information and specific references.

The multi-author book is divided into five parts and sixteen chapters, of which the first three are considered to be introductory ones. Chapter 1, "Molecular asymmetry" by S. Matlin, describes very elementarily the elements of chirality; however, the list of references provided to consolidate this knowledge is short and more recent books on stereochemistry have not been considered.

Chapter 2, "The importance of enantiomer separations" by W. De Camp, discusses this subject only in the context of a concerned regulatory agent. The thalidomide tragedy of the early 1960s is described broadly and extensive studies of other drugs are mentioned only briefly or have been neglected, but in my opinion they would have been of considerably more importance in order to broaden the background spectrum and to reinforce the necessity for enantioselective analysis in mod-

ern life sciences. The spectrum of relevant implications for analytical enantioseparations is also much broader, *e.g.*, monitoring of the ratio of enantiomers of, for instance, certain amino acids in archeological samples and the use of this information for dating are not mentioned.

Chapter 3, "Chiral liquid chromatography, past and present" by A. Pryde, gives a good and brief overview of the historical perspectives and the diverse main enantioseparation techniques together with relevant references.

Chapter 4, "Chiral derivatizations" by M. Ahnhoff and S. Einarsson, gives an excellent and useful overview of "indirect" methods of enantioseparation. A few relevant examples are presented that will enable the reader to understand possible advantages and pitfalls of this technique. However, some newer and promising work has not been considered in the tables, and more examples from the challenging bioanalytical field and information about the shelf-life of the chiral reagents in terms of optical and chemical stability are missing. A list of suppliers for the main reagents would have been of value for the reader.

Chapter 5, dealing with "Chiral ligand exchange chromatography (CLEC)", by S. Lam, considers primarily the use of chiral metal coordinating ligands as mobile phase additives, which is, of course, only a special application of the general CLEC technique, including immobilized ligands, as expected from the title of the chapter. Examples of D,L-amino acid resolutions together with some explanations about the possible separation mechanisms are given, but they are more superficial and for a deeper understanding the articles and books by Davankov are highly recommended. The enantioselective thin-layer chromatography approach using CLEC is not mentioned.

In Chapter 6, by T. Doyle, the difficult task of classifying the masses of various chiral stationary phases (CSPs) that have appeared in the literature and of surveying and interpreting the so-called multiple-interaction CSPs was tried. The author, D.Doyle, made a very individual selection and the list of promising CSPs is incomplete, examples of real-life applications are missing and the reference list is not up-to-date (it ends at 1987).

The last aspect also applies in Chapter 7, dealing with "Immobilized proteins as HPLC chiral stationary phases" by I. Wainer. The newer and much better and stabler CSPs have not been considered owing to long time gap between the author's deadline and the appearance of this book. However, the potential of these CSPs could clearly be demonstrated.

Chapter 8, "Cyclodextrin inclusion complexation" by L. Coventry, covers inadequately this promising technique and is poorly referenced.

Chapters 9 and 10, written by D. Johns and dealing with cellulose-based CSPs and other polymer-type CSPs, do not describe adequately the potential and the widespread use of these "first-choice" materials for particular applications. No guidelines are given on how to use and what to expect from such CSPs and relevant references are missing.

Chapter 11, dealing with "Ion pairing", by G. Szepesi, describes this technique in an easily readable and understandable form, although the number of good examples presented is small.

In Chapter 12, by W. Lough, some "Other direct chiral resolution methods" are briefly mentioned, which is a good idea for broadening the background information

of the potential reader of this book. However, it is unlikely that these techniques will be adapted easily for routine work.

In Chapter 13, "Consideration of Other Techniques", by W. Lough and S. Matlin, alternative techniques for determination of enantiomers, such as nuclear magnetic resonance spectroscopy, gas chromatography, supercritical fluid chromatography, and radioimmunoassay have been mentioned very briefly to direct the reader's attention to these quite potent techniques. Unfortunately the enantioselective capillary zone electrophoresis as promising method of the future has been ignored.

Chapter 14, "Choice of chiral LC systems", and Chapter 15, "Optimization", by W. Laugh and I. Wainer and by T. Noctor, A. Fell, and B. Kayn, respectively, should be key articles for chromatographers at the bench attempting to solve a given resolution problem. Unfortunately, there is at the moment no rational approach for how to tackle the problem of selecting the "right or best" CSP or enantioselective system based on the chemical structure of the analytes; however, starting points of considerable value for the practitioner are discussed in this chapter.

Finally, in Chapter 16 the book stresses also the subject of "Future trends and requirements". The opinion of the author (D. Taylor) is subjective, but it is agreed that a deeper understanding of the separation mechanism will eventually lead to better predictions rather than following the "trial and error" approach, as is mostly done. To optimize the instrumentation in all respects is *per se* fundamental.

To summarize, this book was obviously intended for practitioners in the pharmaceutical field and maybe for students. However, as several of the chapters are not written by first-rate authorities in this dramatically evolving field, the broad range of subjects has not received full coverage, in theory and practice. Despite these shortcomings, the book will certainly be of value in laboratories occasionally involved with enantioseparations.

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Journal of Chromatography, 509 (1990) 411–412
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 417

Book Review

Process chromatography —A practical guide, by G. K. Sofer and L. E. Nystrom, Harcourt Brace Jovanovich, Sidkup, U.K., 1989, IX + 145 pp., price £ 19.95, ISBN 0-12-654268-6.

There is a serious ambiguity in this short monograph. It aims to cover all the major practical aspects of preparative chromatography, it looks very much as if it does, yet it does not. It should really have been subtitled: "The view from Pharmacia", and that would have been more honest. This view is certainly a respectable one. It is also a very biased one, and one which is fast becoming obsolete.

The book deals entirely with LPLC (low-pressure liquid chromatography, *i.e.*, low-performance liquid chromatography). At the only index entry for "Pumps, pressure/flow rate", we read, "It is most important that the pump covers the entire flow rate range of the process and that the discharge pressure is adequate over the whole range (Figure 32)". Fig. 32 is a plot of flow-rate (in %, not very informative) *versus* pressure (bar), covering the range from 0 to 3 (three!) bar. Fig. 21 gives plots of mobile phase velocity on a Sepharose 6 Fast Flow column *versus* pressure, up to 0.1 MPa (*i.e.*, 10 atm). Many process equipments can operate at more than ten times this pressure.

Almost all the data regarding packing materials refer to Pharmacia patented and trade-marked products. All Pharmacia trade-names are in the index. The words silica, chemically bonded silica, C_{18}-silica, the common trade-names of silica and the most common trade-names of competitive polymeric products are not quoted in the index. This reviewer looked in vain through the entire volume for any reference to a non-Pharmacia stationary phase.

On the other hand, the book contains some very useful information. The chapters on (1) "Choice of host", (2) "Isolation steps", (5) "Initial purification steps", (9) "Process hygiene" and some of the appendices (Appendix C, "Regulatory considerations", gives a good list of references) contain much information of great practical interest. However, this comment may merely reflect the ignorance of the reviewer. The chapters on (6) "Optimization", (7) "Scale-up" and (8) "Equipment" have a dearth of good practical advice and at the same time are a collection of prejudices and obsolete ideas. The literature published since 1940 on the topic of high-concentration chromatography is completely ignored, although there are a few theoretical insights available which could help the separation engineer to understand what is happening to the process and what should be done to improve its performance.

It is obvious at this stage of preparative chromatography that production rate increases with increasing pressure. Considerable savings could be made by working in the 50–200 atm range rather than below 3 atm. Thus, the supports used should resist that pressure differential. The supports discussed in this book have great qualities which are highly desirable for the separation of proteins, but they do not withstand pressure. The future will belong to those companies which, instead of trying to force

inefficient products down the throats of customers, will supply them with new products which combine the advantages of the old ones with the mechanical resistance newly required (*e.g.*, POROS?).

Knoxville, TN (U.S.A.) GEORGES A. GUIOCHON

Journal of Chromatography, 509 (1990) 413–415
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 416

Book Review

High-performance liquid chromatography, edited by P. R. Brown and R. A. Hartwick, Wiley, Chichester, New York, 1989, XII + 688 pp., price £ 47.95, ISBN 0-471-84506-X.

A wealth of books dealing with modern high-performance liquid chromatography (HPLC) have been published in the last two decades and numerous monographs have been devoted to various aspects of this powerful technique. The present book is intended "to consolidate the basic theories of liquid chromatography along with the more exciting developments in the field" and to give an idea about the current state-of-the-art, the frontiers and possible directions of further developments in liquid chromatography. It should be a "broad-based general book, giving the basic theoretical concepts" and valuable for chromatographers for years to come. To meet these objectives, the individual chapters were contributed by wellknown experts in their fields of activities. All the chapters are written in an erudite manner, but with different degrees of detail.

The book contains 16 chapters covering various fields of HPLC. The first two chapters deal with general aspects of HPLC theory. Weber and Carr present a comprehensive survey of the dynamics of liquid chromatography, including both the widely accepted theoretical models, such as the Giddings theory, and the results of more recent research. Attention is concentrated on band broadening in open and packed columns under isocratic conditions, with both linear and non-linear isotherms. The chapter includes appendices illustrating the derivation of the theoretical equations discussed and gives numerous references to the original literature. In the second chapter, R. P. W. Scott reviews his own "molecular interaction model" of solute retention in liquid chromatography. Unfortunately, the retention models proposed by others workers (*e.g.*, Snyder, Horváth, Martire) are not considered, except for a general criticism of the thermodynamic theory of retention for its inability to predict retention from thermodynamic data. However, the constants of the equations describing the "molecular interaction model" are not more suitable for this purpose.

The next three chapters deal with the stationary phases in HPLC: Unger and Trüdinger describe silica, alumina and other metal hydrous oxides as adsorbents for normal-phase chromatography, Knox and Kaur carbon as the sorbent for reversed-phase, ion-pair and steric-exclusion chromatography and Pietrzyk organic polymeric stationary phases for reversed-phase and liquid–liquid chromatography and for the isolation, stripping and concentration of organic and inorganic analytes in various samples. All are very well written, practically oriented reviews of the preparation and properties and selected representative applications of various column packing materials for HPLC, containing a number of references to the original work.

The following two chapters describe principles, specific instrumental and methodological aspects and a few selected more recent applications of high-performance size-exclusion chromatography (Yau, Kirkland and Bly) and of high-performance

affinity chromatography (Chaiken *et al.*). The chapter by Hermansson and Schill gives an overview of the separation of chiral compounds by HPLC, with special attention to the applications of α_1-acid glycoprotein bonded to silica as the chiral selector. Possibilities of the regulation of stereoselectivity by the control of temperature, pH and the nature and concentration of mobile phase modifiers are discussed.

Five chapters deal with selected important specific aspects of analytical HPLC. Simpson gives a concise review of the theoretical and practical considerations of the instrumentation suitable for performing high-speed liquid chromatography and compares the advantages and disadvantages of this technique with respect to conventional analytical HPLC. Krull *et al.* present a theoretical approach to pre- and post-column on-line derivatization with attention to general limitations, advantages and disadvantages of various techniques, including homogeneous, solid-phase and photochemical derivatization. Examples specific of applications are not given. Grushka and Zamir discuss a very important topic, namely the effects of flow-rate, temperature, mobile phase composition and the function and setting of the integrator on the precision of retention times and peak areas. The sources of decreases in day-to-day and laboratory-to-laboratory reproducibility of the measured chromatographic data are also considered. The chapter by Kaliszan provides a brief description of the types of interactions that take place in HPLC and their effects on the chromatographic behaviour of solutes in reversed-phase systems. Various numerical descriptors of solute structure used for correlations with retention are characterized and possibilities of the determination of hydrophobicity by HPLC and the applications of HPLC in quantitative structure–biological activity relationships are reviewed. On the basis of the statistical theory of component overlap, Sagliano, Raglione and Hartwick justify the need for multi-dimensional systems for complex separations and present a survey of the design principles, instrumental limitations and applications of multi-dimensional separation techniques using on-line coupling of HPLC–HPLC and HPLC–gas chromatography.

The steadily increasing practical importance of preparative and process-scale liquid column chromatography is clearly demonstrated in two excellent reviews. Colin presents the theory of the effects of sample amount on the throughput and recovery of products in linear and non-linear preparative liquid chromatography and surveys possibilities for optimization of the length and diameter of a preparative column, particle size and flow-rate with respect to the injected sample volume and amount. He also considers practical aspects of column design, properties of packing materials and sample injection techniques on the preparative scale and briefly explains the basic principles of displacement chromatography and recycling as ancillary techniques in preparative chromatography. Production rate and column efficiency as a function of sample load in process HPLC are discussed by Skea, who also gives basic characteristics of commercial equipment suitable for process-scale applications and offers a guide for an empirical step-by-step development of a process-scale HPLC separation.

The review of field flow fractionation (FFF) by Kesner and Giddings is very well written, but it seems outside the scope of this book. The theory, instrumentation and application possibilities of sedimentation, thermal, flow and steric FFF are presented. Possibilities for the determination of molecular weight distributions using FFF are compared with the application of size-exclusion chromatography for this purpose.

On the whole, the book is well produced and has been edited with a great care (except for a few uncorrected errors in equations and two or three inprecise formulations in the text), and offers much more than an ordinary textbook on HPLC. The matter is dealt with in an in-depth manner such as is usually found only in specialized monographs or reviews. Unfortunately, the full scope of HPLC is not covered and some important topics are omitted, such as chemically bonded stationary phases and polymeric phases deposited on silica or alumina supports, ion chromatography, computer-assisted optimization of working conditions in HPLC, time-programmed chromatographic analysis, coupling HPLC with spectroscopic methods, data processing and automation of analysis, to mention just a few. Nevertheless, practising chromatographers may find a surprisingly large amount of valuable information concentrated in this book. Advanced students are offered a good idea of the current state and further prospects for of a major part of various aspects of contemporary HPLC.

Pardubice (Czechoslovakia) PAVEL JANDERA

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Errata

J. Chromatogr., 479 (1989) 233–242

Page 234, lines 2–4: Dr. Houghton, first author of ref. 14, points out that GC–MS in the negative ion chemical ionization mode was used in his work and not the electron impact ionization mode. Also steroids were analysed only in urine and not in faeces.

Page 242, ref. 14 should read E. Houghton, M. C. Dumasia, P. Teale and J. K. Wellby, *Biomed. Mass. Spectrom.*, 9 (1982) 459.

J. Chromatogr., 504 (1990) 319–333

Page 319, Summary, 6th line, "15" should read "5".

Page 319, 4th text line from bottom, "Tiselius *et al.*[1,2]" should read "Tiselius *et al.*[1] and others[2]".

Page 326, 13th line, "spectra[23–25]" should read "spectra[23–26]".

Page 333, 7th line, "Fig. 9" should read "Fig. 10".

Page 333, 9th and 10th lines, "Fig. 10" should read "Fig. 11".

PUBLICATION SCHEDULE FOR 1990

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 478, 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.)

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Determination of Beta-Blockers in Biological Material

edited by **V. Marko,** *Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, Bratislava, Czechoslovakia*

(Techniques and Instrumentation in Analytical Chemistry, 4C)

This is the third volume of a sub-series entitled *Evaluation of Analytical Methods in Biological Systems*. (The first two were *Analysis of Biogenic Amines* edited by G.B. Baker and R.T. Coutts and *Hazardous Metals in Human Toxicology* edited by A. Vercruysse). This new volume addresses beta-blockers - an area of research for which a Nobel Prize in Medicine was awarded in 1988. It provides an up-to-date and comprehensive coverage of the theory and practice of the determination of beta-blockers in biological material. Two main fields of research are dealt with in this book: analytical chemistry and pharmacology, and, as it deals with drugs used in clinical practice, it is also related to a third area: therapy. Thus, it offers relevant information to workers in all three fields.

Some 50 beta-blockers and nine methods of analysis are discussed. The methods are divided into three groups: optical, chromatographic, and saturation methods. In addition to the analytical methods themselves, sample handling problems are also covered in detail, as is the information content of the analytical results obtained. Special chapters are directed to those working in pharmacology and pharmacokinetics. Finally, as recent evidence points to the increased importance of distinguishing optical isomers of drugs, a chapter on the determination of optical isomers of beta-blockers in biological material is also included. An extensive subject index and two supplements giving retention indices and structures of beta-blockers complete the book.

This is the first book to treat beta-blockers from the point of view of their determination and to discuss in detail the use of analytical methods for beta-blockers. It will thus appeal to a wide-ranging readership.

1989 xiv + 334 pages
US$ 152.75 / Dfl. 290.00
ISBN 0-444-87305-8

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P.O. Box 882, Madison Square Station, New York, NY 10159, USA