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EFFECT OF COLUMN TEMPERATURE ON THE RETENTION OF PERO-PYRENE-TYPE POLYCYCLIC AROMATIC HYDROCARBONS ON VARIOUS CHEMICALLY BONDED STATIONARY PHASES IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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(First received July 27th, 1987; revised manuscript received September 25th, 1987)

SUMMARY

The effect of column temperature on the reversed-phase retention of polycyclic aromatic hydrocarbons has been investigated using various chemically bonded phases. Four solutes, coronene, tetrabenzo[*a,cd,j,lm*]perylene, tetrabenzo[*a,cd,f,lm*]perylene and benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene, were used as the test probes. The temperature dependences of the retention are almost linear (the logarithm of capacity factor is proportional to the reciprocal of the column absolute temperature) with monomeric C_{18}, monomeric C_{18} with endcapping and diphenyl bonded phases, while non-linear behaviour was observed with polymeric C_{18} phases. These differences in behaviour of the stationary phases are interpreted in terms of their structural differences.

INTRODUCTION

In liquid chromatography (LC), especially reversed-phase LC, it is necessary to optimize the separation conditions, hopefully without time-consuming, tedious trial-and-error experiments. In order to pursue this task, the retention mechanism of the separations has to be investigated. Because of their bioactive properties, there is an urgent need for powerful analytical techniques for polycyclic aromatic hydrocarbons (PAHs). Many scientists have tried to study reversed-phase retention mechanism by using PAHs as the sample probes[1–8]. The results indicate that the dominant factors controlling PAH retention in the reversed-phase mode are the sizes and shapes of the solutes[9–12]. Therefore, as indicated in our previous paper[13], various stationary

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phases having different structures give interesting retention behaviours for large PAHs having different degrees of planarity. Unique retention patterns on certain stationary phases, namely polymeric C_{18}, pyrenylethyl and diphenyl bonded phases, were suggested for large PAHs. The composition of the mobile phase[14] and the structures of the stationary phases affect the retention of large non-planar PAHs. This can be explained if it is assumed that the structures of the bonded phases change somewhat with changing composition of the mobile phase.

It is necessary to study in more detail the structural differences between various stationary phases. We have investigated the effect of temperature on the retention of four large PAHs and interpreted the observations in terms of structural differences between the stationary phases.

In recent years, several workers have reported the effect of column temperature on retention[15–19]. For C_{18} bonded silicas, orientational changes under reversed-phase LC conditions were observed[17–22]. It was found that with a totally aqueous mobile phase, the relationship at lower temperature between log k' and the reciprocal of the temperature was linear for selected test solutes and the retention decreased with increasing temperature. At higher temperatures this linear relationship was followed by a sigmoidal decrease[20–22]. The investigation of the temperature effect on the retention of large PAHs seems to of interest in order better to understand the complicated retention mechanism of reversed-phase LC.

EXPERIMENTAL

The chemically bonded stationary phases used were as follows: laboratory-made; polymeric C_{18} (synthesized from dichlorosilanes), monomeric C_{18}, monomeric C_{18} with C_1 endcapping, diphenyl and pyrenylethyl, commercially available; Vydac 201 TPB-5 (Separation Group, Hesperia, CA, U.S.A., described commercially as polymeric C_{18} synthesized from trichlorosilanes). The laboratory-made materials were the same as those used in our previous papers[13,14]. Each stationary phase was packed into a fused-silica capillary tube (400 mm × 0.35 mm I.D.) by the conventional slurry technique.

Four large PAHs, coronene, tetrabenzo[*a,cd,j,lm*]perylene, tetrabenzo[*a,cd,f,lm*]perylene and benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene, were used as the

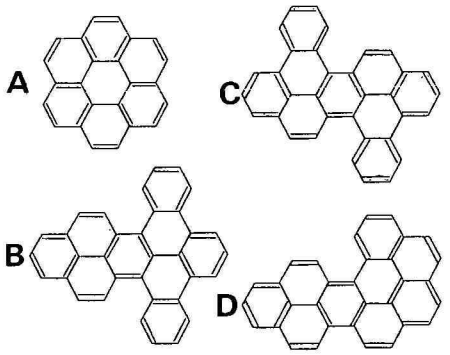

Fig. 1. Structures of the four PAHs: (A) coronene; (B) tetrabenzo[*a,cd,f,lm*]perylene; (C) tetrabenzo[*a,cd,j,lm*]perylene; (D) benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene.

test probes. These compounds (except coronene which is commercially available) were synthesized[23]. The structures of the PAHs are shown in Fig. 1. Three of them have similar shapes and the same ring number (nine) but have different degrees of planarity. Benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene is more planar than the others which are non-polar due to intramolecular steric strain. Coronene has a planar conformation but is smaller than the others.

The microcolumn liquid chromatograph consisted of a microfeeder MF-2 (Azuma Electric, Tokyo, Japan) as a pump, an Uvidec 100-III UV detector (Jasco, Tokyo, Japan) and a 0.08-μl microloop injector Jasco ML-422. The mobile phase used was methanol–dichloromethane (80:20). Chromatographic retention times were measured as the averages from triplicate experiments. Column temperatures were controlled to ± 0.2°C with an air-bath equipped with an hand-made controller.

Thermal analysis was performed with a SSC-5000 and DSC-200 thermal analyzer (Seiko, Tokyo, Japan). The measurement range was between 20 and 200°C, the temperature programming rate being 10°C/min. Although in the usual chromatographic case the stationary phases are in the presence of mobile phase, for convenience the measurements were carried out with no mobile phase environment.

RESULTS AND DISCUSSION

Fig. 2A–F shows plots of log k' *vs.* $1/T$ for the six stationary phases. Very high degrees of curvature are observed with polymeric C_{18} and Vydac columns. This could be due to two linear regions of different slopes. On the other hand, with monomeric C_{18} and other bonded phases, the plots appear linear.

On the diphenyl bonded phase, the elution order of the four PAHs is different from those on the other bonded phases. Benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene which has a more planar conformation is the most weakly retained of the three peropyrene-type PAHs. This solute has a smaller F number and a larger L/B value

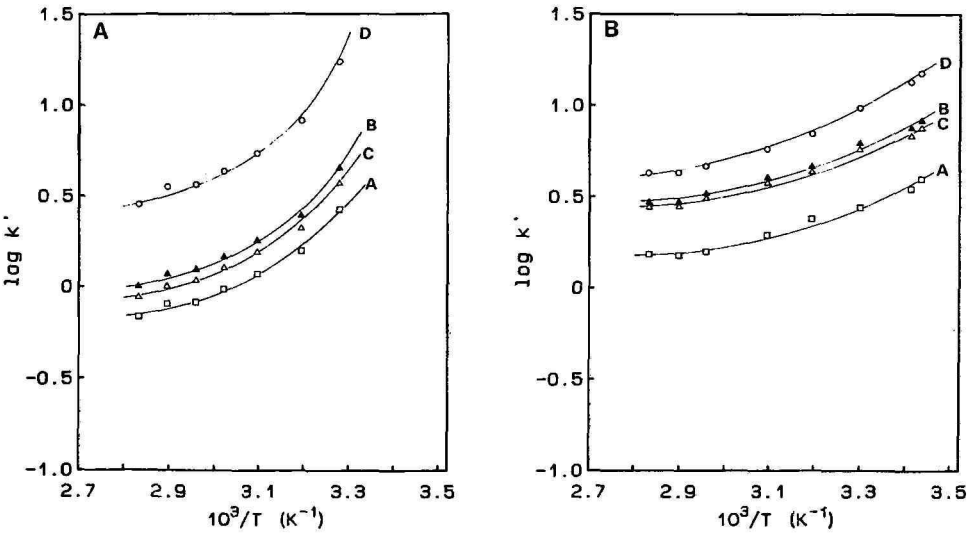

Fig. 2.

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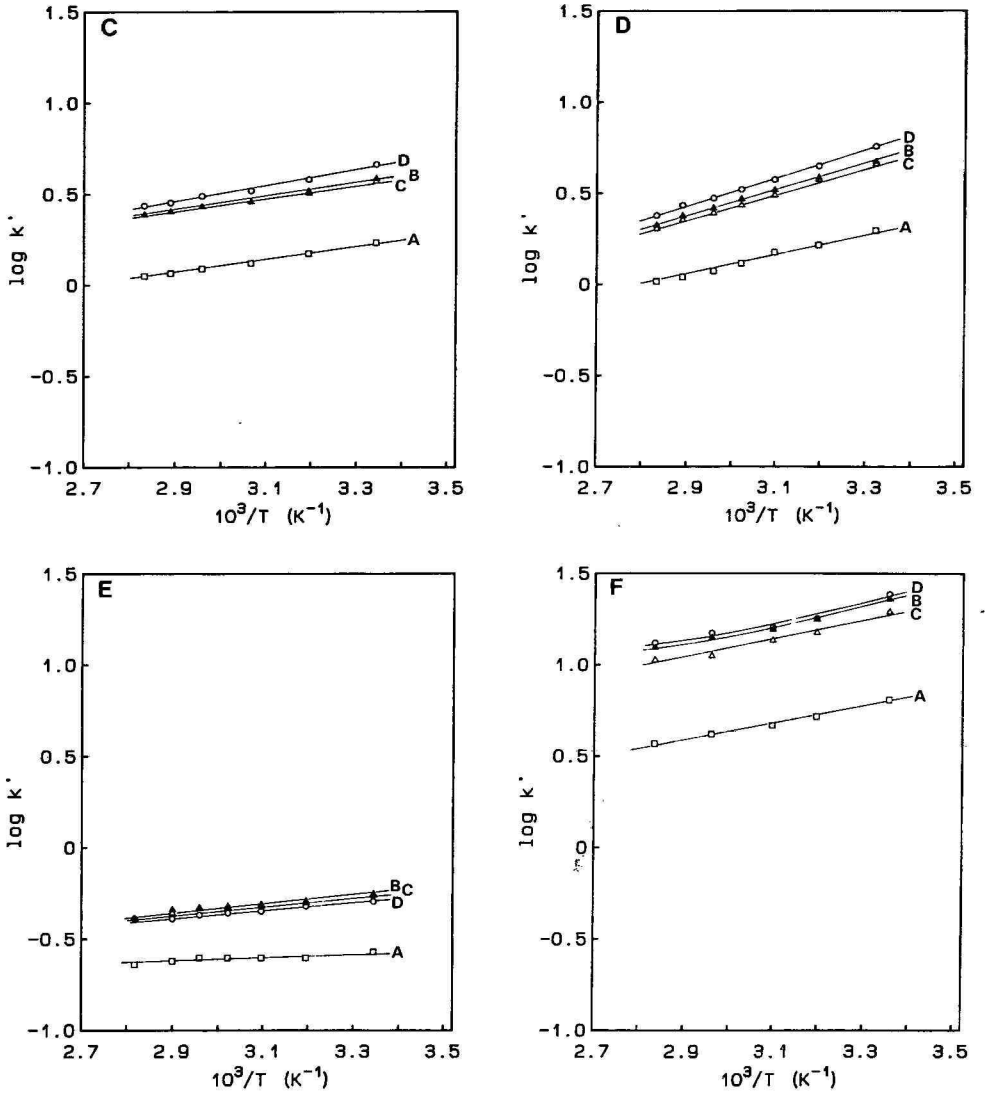

Fig. 2. Plots of log k' *vs.* reciprocal of column temperature: (A) Vydac 201 TPB-5; (B) polymeric C_{18}; (C) monomeric C_{18} with endcapping; (D) monomeric C_{18}; (E) diphenyl; (F) pyrenylethyl. Solutes as in Fig. 1.

TABLE I

F AND *L*/*B* VALUES OF PEROPYRENE-TYPE PAHs

Compound	*F*	*L*/*B*
Coronene	12	1.00
Benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene	16	1.37
Tetrabenzo[*a*,*cd*,*f*,*lm*]perylene	17	1.13
Tetrabenzo[*a*,*cd*,*j*,*lm*]perylene	17	1.21

than those of the tetrabenzoperylene isomers. F is a correlation factor proposed by Schabron *et al.*[24] defined as (number of double bonds) + (number of primary and secondary carbons) − 0.5 × number of non-aromatic rings, and L/B is the maximum length-to-breadth ratio proposed by Wise *et al.*[9] in 1981. Values of these parameters for the PAHs are given in Table I. In our previous works[13,14,25], this type of retention behaviour was found with the diphenyl phase, on which isomers with large F values gave longer retention and isomers with larger L/B and the same F number gave shorter retention than those with smaller L/B values. Bulky stationary phases such as the diphenyl bonded phase cannot discriminate solutes of different planarity, and PAHs are eluted in order of increasing F number and decreasing L/B value.

Table II presents the temperature dependence of the separation coefficients for the three peropyrene-type PAHs relative to coronene. The separation coefficients have a tendency to decrease with increasing column temperature on all the stationary phases. The results suggest that the separation of PAHs may be improved by decreasing the column temperature. We believe that the peculier dependencies of retention on temperature are rather due to corresponding changes with temperature of the conformation of the bonded phase, although it is likely that there are some changes in solute conformation with temperature[26].

From these observations, it seems that there is a definite critical temperature at which some change in the surface structure of polymeric C_{18} phases occurs. According to the concept of Gilpin and co-workers[20,21], the rearrangements of the bonded moiety occur at higher temperature. At lower temperatures, the bonded moieties of polymeric C_{18} phases could be in a folded or associated orientation in this methanol–dichloromethane (80:20) mobile phase, while at higher temperature the structure changes to "bristle" like. The critical temperature is the point in Fig. 2A and B at which curvature occurs, while it seems that the surface structures of monomeric C_{18} and of monomeric C_{18} with endcapping at this mobile phase composition are not changed. With monomeric phases, increasing column temperature causes a similar change but the difference in the original configurations is smaller, because the distance between each C_{18} chain bonded to the base silica should be larger than that in polymeric phases. On polymeric phases, that distance seems to be small[27] and the folded structure is more likely at low column temperature. The experimental data

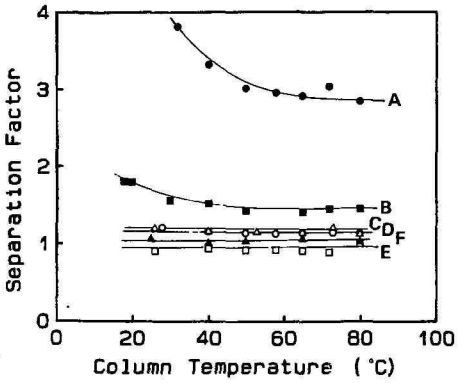

Fig. 3. Plots of the separation coefficients between benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene and tetrabenzo[*a,cd,f,lm*]perylene *vs.* column temperature. Phases as in Fig. 2.

TABLE II

SEPARATION COEFFICIENTS FOR PEROPYRENE-TYPE PAHs WITH VARIOUS STATIONARY PHASES AT DIFFERENT COLUMN TEMPERATURES

Phases: 1 = coronene; 2 = tetrabenzo[*a,cd,j,lm*]perylene; 3 = tetrabenzo[*a,cd,f,lm*]perylene; 4 = benzo[*lm*]phenanthro[4,5,6-*abcd*]perylene.

Phase		*Temperature (°C)*														
		82	*80*	*73*	*72*	*65*	*58*	*53*	*50*	*40*	*32*	*30*	*28*	*26*	*25*	*20*	*18*
Diphenyl	2/1	1.78			1.83	1.88	1.88		1.92	2.00				2.04			
	3/1	1.78			1.92	1.88	1.92		1.96	2.04				2.07			
	4/1	1.78			1.71	1.72	1.76		1.80	1.92				1.89			
Monomeric C_{18}	2/1		1.94	2.05		2.08	2.08		2.05	2.30			2.31				
	3/1		2.03	2.16		2.20	2.25		2.19	2.35			2.38				
	4/1		2.30	2.47		2.50	2.54		2.50	2.72			2.89				
Monomeric C_{18} with endcapping	2/1		2.17	2.18		2.19		2.17		2.14				2.16			
	3/1		2.17	2.18		2.19		2.17		2.19				2.24			
	4/1		2.44	2.44		2.50		2.49		2.54				2.68			
Pyrenylethyl	2/1		2.87			2.68			2.91	2.87					3.02		
	3/1		3.36			3.37			3.35	3.39					3.56		
	4/1		3.56			3.58			3.44	3.44					3.78		
Vydac 201 TPB-5	2/1		1.28		1.25	1.31	1.31		1.31	1.33	1.39						
	3/1		1.46		1.46	1.51	1.50		1.52	1.56	1.69						
	4/1		4.14		4.42	4.40	4.43		4.57	5.18	6.44						
Polymeric C_{18}	2/1		1.80		1.85	1.96			1.93	1.80		1.93				1.94	1.90
	3/1		1.92		1.96	2.08			2.07	1.93		2.09				2.15	2.09
	4/1		2.78		2.84	2.94			2.97	2.93		3.26				3.86	3.79

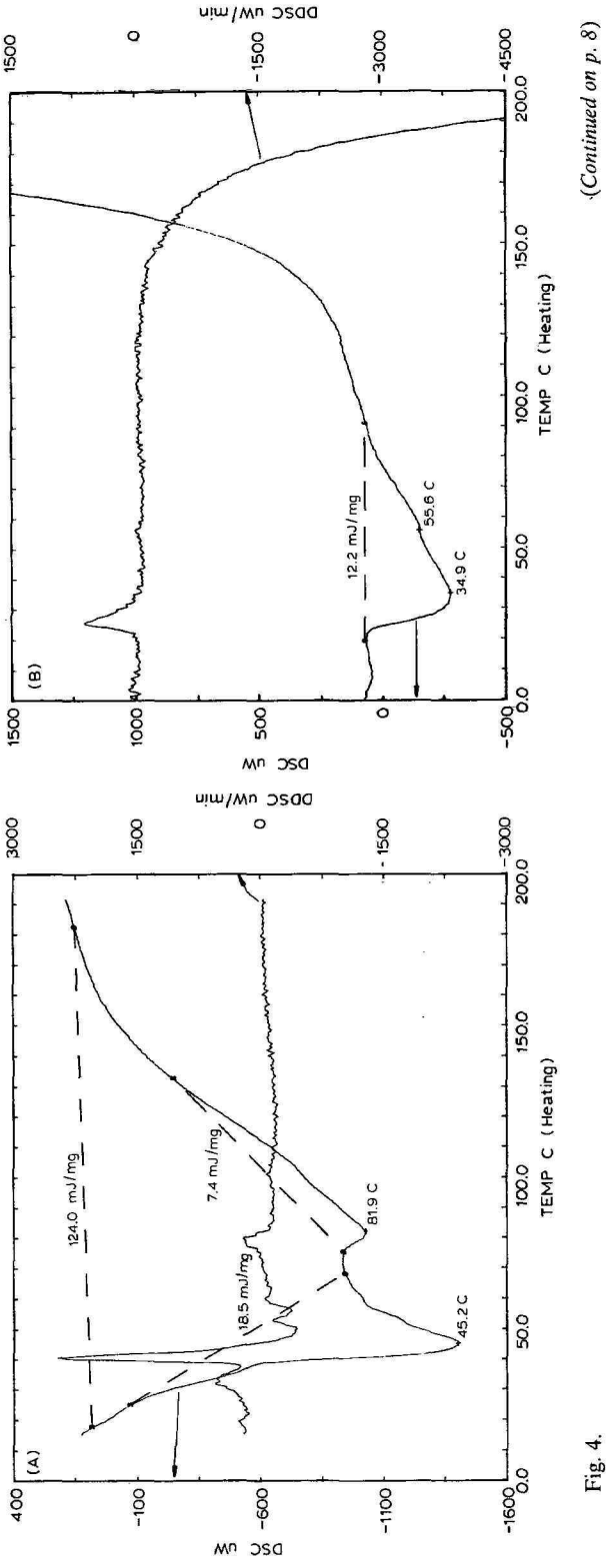

Fig. 4.

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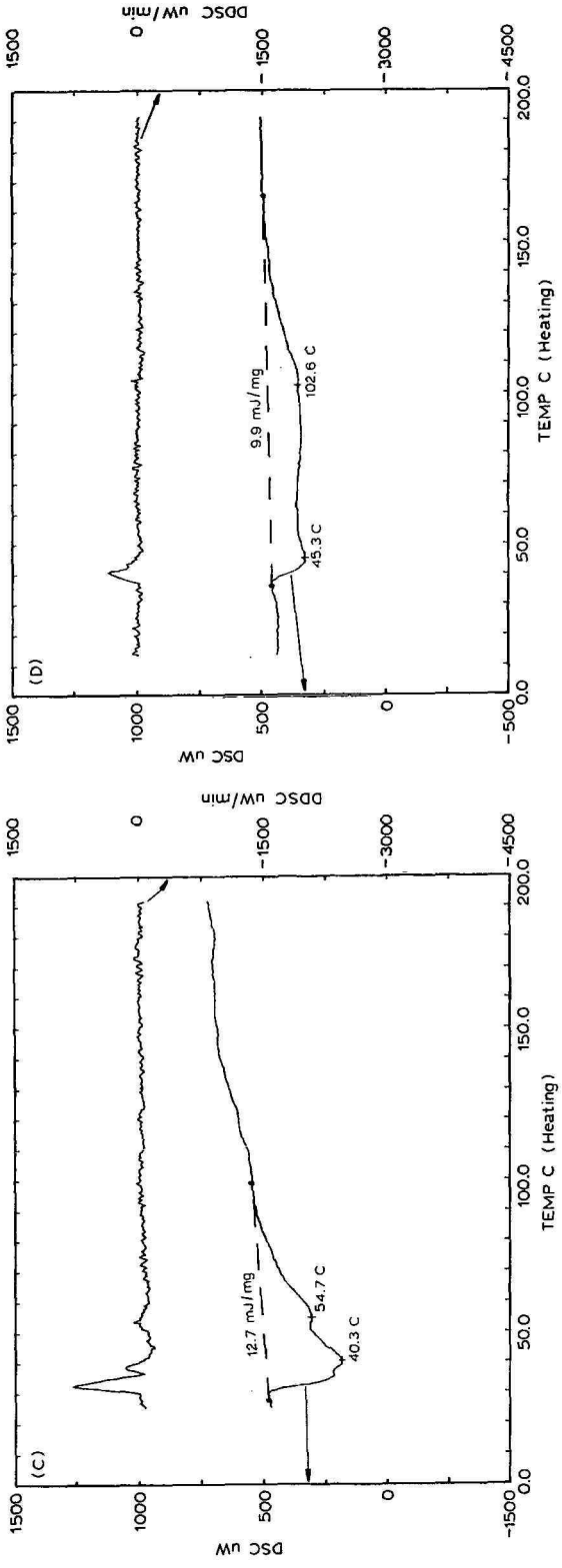

Fig. 4. DSC charts for four different stationary phases: (A) Vydac 201 TPB-5; (B) monomeric C_{18}; (C) diphenyl; (D) pyrenylethyl. Conditions are described in the text.

summarized in Fig. 3 clearly indicates such differences. As the column temperature is increased with polymeric phases, the separation coefficient between planar benzo[*lm*]phananthro[4,5,6-*abcd*]perylene and non-planar tetrabenzo[*a,cd,f,lm*]perylene decreased more than for other stationary phases. This indicates that the planarity discrimination with polymeric phases depends strongly on the changes in surface structure of the bonded moieties with changing column temperature and mobile phase composition.

It is concluded that the ability of polymeric phases to recognize the planarity of PAHs is higher than that of monomeric phases, and this trend is much more marked with polymeric phases synthesized from trichlorosilanes than those synthesized from dichlorosilanes. The difference in planarity recognition among those phases decreases with increasing column temperature, and the critical point should be between 40 and 60°C. This fact might be explained by the existence of phase transitions between 40 and 60°C.

In order to investigate this, thermal analysis has been performed for the stationary phases used. The typical DSC (differential scanning calorimetry) charts are shown in Fig. 4A–D. The thermal changes were observed at 40.3 and 54.7°C with diphenyl, 45.3 and 102.6°C with pyrenylethyl, 34.9 and 55.6°C with monomeric C_{18} and 45.2 and 81.9°C with Vydac, respectively. The magnitudes of these changes were 12.7 mJ/mg with diphenyl, 9.9 mJ/mg with pyrenylethyl, 12.2 mJ/mg with monomeric C_{18} and 124.0 mJ/mg with Vydac. The results of the thermal analysis clearly indicate that Vydac has a strong thermal transition around 40–50°C which accounts for the changes in retention behaviour of large PAHs, which occur to relatively small degrees on other stationary phases.

REFERENCES

1 R. B. Sleight, *J. Chromatogr.*, 83 (1973) 31.
2 A. L. Colmsjo and J. C. MacDonald, *Chromatographia*, 13 (1980) 350.
3 A. Radecki, H. Lamparczyk and R. Kaliszan, *Chromatographia*, 12 (1979) 595.
4 K. Ogan, E. Katz and W. Slavin, *Anal. Chem.*, 51 (1979) 1315.
5 W. R. Melander, J.-X. Hung, Cs. Horváth, R. W. Stout and J. J. Destano, *Chromatographia*, 20 (1985) 641.
6 E. Tomlinson, *J. Chromatogr.*, 113 (1975) 1.
7 R. Kaliszan, *J. Chromatogr.*, 220 (1981) 71.
8 K. Jinno and K. Kawasaki, *J. Chromatogr.*, 316 (1984) 1.
9 S. A. Wise, W. J. Bonnett, F. R. Guenther and W. E. May, *J. Chromatogr. Sci.*, 19 (1981) 457.
10 S. A. Wise and L. C. Sanders, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 248.
11 L. C. Sanders and S. A. Wise, *Adv. Chromatogr. (N.Y.)*, 26 (1986) 139–218.
12 K. Jinno and M. Okamoto, *Chromatographia*, 18 (1984) 495.
13 K. Jinno, T. Nagoshi, N. Tanaka, M. Okamoto, J. C. Fetzer and W. R. Biggs, *J. Chromatogr.*, 386 (1987) 123.
14 K. Jinno, T. Nagoshi, N. Tanaka, M. Okamoto, J. C. Fetzer and W. R. Biggs, *J. Chromatogr.*, 392 (1987) 75.
15 W. Melander, D. E. Campbell and Cs. Horváth, *J. Chromatogr.*, 158 (1978) 215.
16 H. Colin and G. Guiochon, *J. Chromatogr.*, 158 (1978) 183.
17 J. Bowermaster and H. M. McNair, *J. Chromatogr. Sci.*, 22 (1984) 165.
18 K. Jinno and Y. Hitata, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 85.
19 K. Jinno, D. P. Carney and J. B. Phillips, *Anal. Chem.*, 57 (1985) 574.
20 R. K. Gilpin and W. R. Sisco, *J. Chromatogr.*, 194 (1980) 285.
21 R. K. Gilpin and J. A. Squires, *J. Chromatogr. Sci.*, 19 (1981) 195.

22 R. K. Gilpin, *Am. Lab. (Fairfield, Conn.)*, 14(13) (1982) 164.
23 J. C. Fetzer and W. R. Biggs, *J. Chromatogr.*, 386 (1987) 87.
24 J. F. Schabron, R. J. Hurtubise and H. F. Silver, *Anal. Chem.*, 49 (1977) 2253.
25 K. Jinno, N. Anazawa, N. Tanaka, M. Okamoto, J. C. Fetzer and W. R. Biggs, *J. Chromatogr.*, 402 (1987) 173.
26 J. C. Fetzer and W. R. Biggs, *J. Chromatogr.*, 295 (1984) 161.
27 L. C. Sander and S. A. Wise, *Anal. Chem.*, 56 (1984) 504.

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AUTOMATED IDENTIFICATION OF TOXIC SUBSTANCES IN POISONED HUMAN FLUIDS BY A RETENTION PREDICTION SYSTEM IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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(First received July 14th, 1987; revised manuscript received September 19th, 1987)

SUMMARY

An automated identification system based on the retention prediction concept has been constructed for toxic substances. The system performance has been evaluated for the identification of toxic compounds in poisoned human fluids.

INTRODUCTION

When a doctor is confronted with a patient suffering from a drug overdose he must attempt to determine what kind of drugs are involved and then decide what kind of medicines to administer. These very important decisions should be made as early as possible, although this is a difficult task without any instrumental assistance. Recent developments in liquid chromatography (LC) may enable its use for this purpose. However, the disadvantage of this technique is the identification, because only retention information is obtainable. If UV multichannel detectors can be employed instead of conventional single-channel detectors, then spectral information can be obtained but this is still not enough to identify toxic substances because UV spectra are very similar in some cases. Therefore, other identification methods are required[1].

One such method is automated identification in reversed-phase LC, as recently proposed by one of the authors[2], which is based on the concept of retention prediction[3–7]

$$k' = f(P_i) \quad (1)$$

where k' is the capacity factor of a solute and P_i is a physicochemical parameter of the solute. Equations such as eqn. 1 can be derived for various chromatographic systems and then the retention of solutes can be predicted.

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Eqn. 1 can be useful in the confirmation of approximately identified compounds automatically by the use of retention prediction in reverse. Having obtained k' for a peak from an experiment, the system looks for the appropriate descriptors for the peak, using the data files of descriptors for several compounds as the standard basis. In this case, the descriptor is the retention parameter which is defined in the Discussion section (closely related to the hydrophobicity of the compounds) and compounds are medicaments which are typically psychotropic drugs. Then the names of the compounds corresponding to the calculated retention parameter are listed on a cathode ray tube (CRT) or line printer.

It is the purpose of this communication to demonstrate this concept for automated identification of toxic substances in poisoned human fluids by reversed-phase LC.

EXPERIMENTAL

The LC system used comprised a Model 880-PU pump and Multi-320 multichannel UV detector (Jasco, Tokyo, Japan). The column was a Jasco Finepak Sil C_{18} S (250 mm × 4.6 mm I.D.) set at 50°C. The mobile phases were mixtures of 10 m*M* perchloric acid, 10 m*M* sodium perchlorate and acetonitrile and the flow-rate was 1 ml/min. The standard samples were dissolved in acetonitrile to a concentration of 100 μg/ml. Actual human fluids such as gastric contents, sera and urines from poisoned patients were collected at the Critical Care Medical Center (CCMC), Nippon Medical School, Tokyo. The data handling was performed by a NEC 9801 VM2 microcomputer (Nippon Electric, Tokyo, Japan).

RESULTS AND DISCUSSION

Determination of retention parameter

To obtain the retention prediction equation, first one has to find an appropriate descriptor. In this case, it is reasonable to consider that the retention of the toxic substances is controlled by their hydrophobicities[8–10], but accurate estimation of the hydrophobicities is a difficult task even when the methods of Rekker[11] or Hansch and Leo[12] are used. Therefore, we used the most convenient way to perform this task, *i.e.*, the use of a retention parameter determined by the hydrophobic parameters of selected standards. The hydrophobic parameters of barbital ($\log P_d = 0.67$, where P_d is the octanol–water partition coefficient), phenobarbital (1.48), phenacetin (1.58) and triazolam (2.42) obtained from the data base of Hansch and Leo[12] were correlated to their $\log k'$ values measured in buffer–acetonitrile (70:30), −0.058, 0.257, 0.390 and 1.086, respectively. Thus eqn. 2 is obtained:

$$\log k' = 0.6614 \log P_d - 0.571 \quad (2)$$

The capacity factors, k', of 48 representative toxic substances were determined experimentally under the same elution conditions and are summarized in Table I. Eqn. 2 can be converted into

$$\log P_c = (\log k' + 0.571)/0.6614 \quad (3)$$

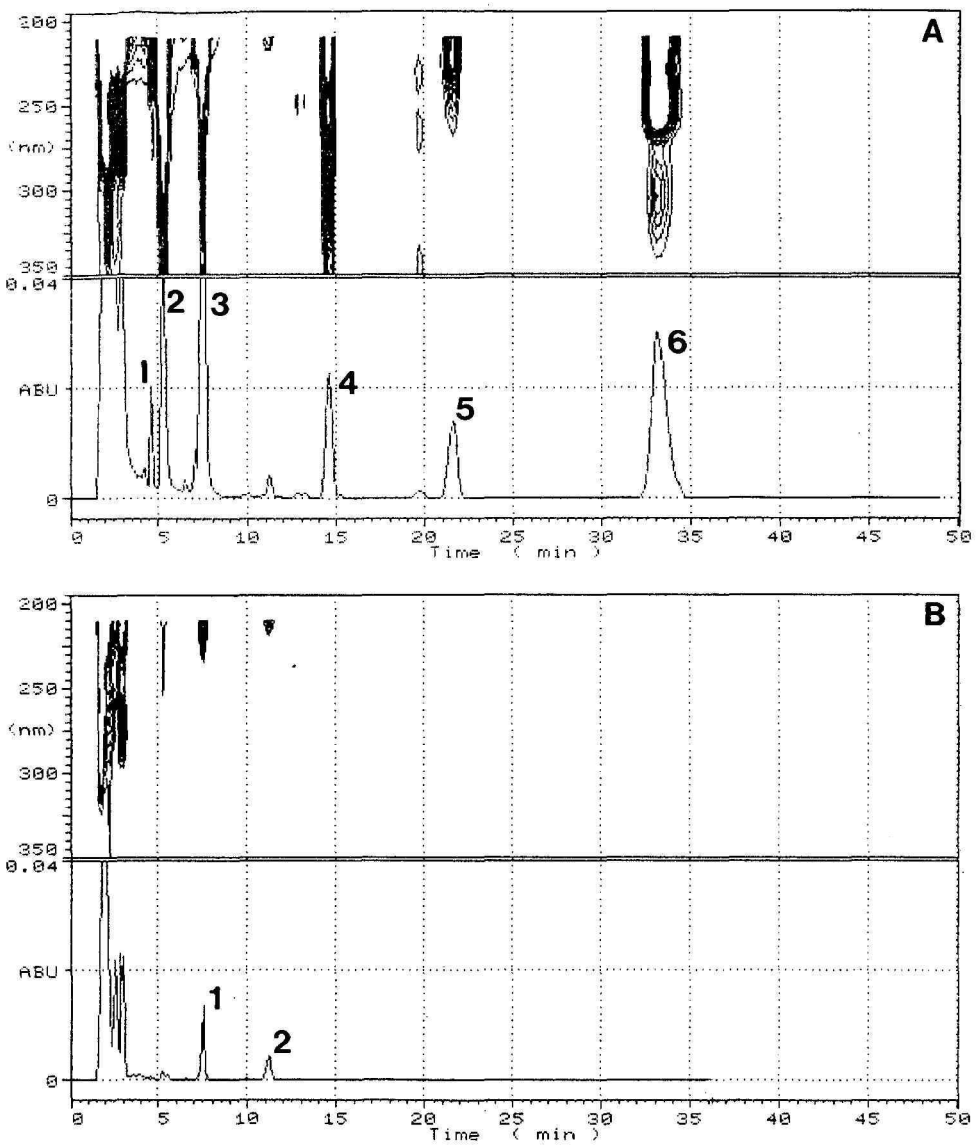

Fig. 1. Chromatograms of human fluids sampled from a patient who had taken some poisons. (A) gastric contents, (B) serum. Mobile phase: buffer–acetonitrile (70:30). Detection: UV, 215 nm.

where log P_e is the retention parameters for each substance obtained by inserting each k' into eqn. 3. These values are also listed in Table I.

Construction of retention prediction system

The construction of the retention prediction system has been described previously[2,7], and therefore only the basics will be mentioned in this work.

If there is an highly correlated relationship between log k' and log P_e for toxic

TABLE I

DATA TABLE FOR 48 TOXIC COMPOUNDS: RETENTION TIME, CAPACITY FACTOR AND ESTIMATED log P_e

No.	*Compound*	*Index*	t_R	k'	*log k'*	*log* P_e
1	Acetaminophen	AA	2.410	0.506	−0.296	0.418
2	Caffeine	CAF	2.870	0.794	−0.100	0.713
3	Barbital	BAL	3.000	0.875	−0.058	0.777
4	Sulpiride	SUL	3.220	1.013	0.005	0.873
5	Acetylsalicylic acid	ASA	4.020	1.513	0.180	1.137
6	Phenobarbital	PHB	4.490	1.806	0.257	1.253
7	Bromvalerylurea	BVU	4.830	2.019	0.305	1.326
8	Etheenzamide	ETM	5.060	2.163	0.335	1.371
9	Bromazepam	BMZ	5.060	2.163	0.335	1.371
10	Phenacetin	PNC	5.530	2.456	0.390	1.455
11	Cloxazolam	CXZ	5.870	2.669	0.426	1.510
12	Oxazolam	OXZ	6.220	2.888	0.461	1.561
13	Chlormezanone	CM	6.450	3.031	0.482	1.593
14	Chlordiazepoxide	CD	7.020	3.388	0.530	1.666
15	Pentobarbital	PTB	7.370	3.606	0.557	1.707
16	Nitrazepam	NTZ	7.370	3.606	0.557	1.707
17	Amobarbital	AMB	7.610	3.756	0.575	1.734
18	Phenytoin	PHT	7.610	3.756	0.575	1.734
19	Secobarbital	SEB	9.100	4.688	0.671	1.879
20	Carbamazepine	CBM	9.100	4.688	0.671	1.879
21	Glutethimide	GLT	9.100	4.688	0.671	1.879
22	Oxazepam	OPZ	9.920	5.200	0.716	1.948
23	Nimetazepam	NMZ	11.880	6.425	0.808	2.086
24	Estazolam	ESZ	12.220	6.638	0.822	2.108
25	Diazepam	DIZ	12.800	7.000	0.845	2.143
26	Flunitrazepam	FNZ	14.650	8.156	0.911	2.243
27	Flurazepam	FLZ	15.650	8.781	0.944	2.292
28	Chlordiazepoxide	CD	15.670	8.794	0.944	2.293
29	Alprazolam	APZ	16.490	9.306	0.969	2.330
30	Medazepam	MDZ	16.710	9.444	0.975	2.339
31	Haloperidol	HP	18.000	10.250	1.011	2.393
32	Prepericyazine	PPC	18.570	10.606	1.026	2.416
33	Triazolam	TRZ	21.100	12.188	1.086	2.507
34	Bromperidol	BP	21.220	12.263	1.089	2.511
35	Promethazine	PM	20.370	11.731	1.069	2.482
36	Desipramine	DEP	21.920	12.700	1.104	2.534
37	Carpipramine	CAP	25.020	14.638	1.165	2.627
38	Maprotyline	MPT	25.370	14.856	1.172	2.637
39	Nortriptyline	NTP	25.490	14.931	1.174	2.640
40	Hydroxyzine	HX	26.180	15.363	1.186	2.659
41	Imipramine	IMP	27.220	16.013	1.204	2.686
42	Trihexyphenidyl	THPH	27.450	16.156	1.208	2.692
43	Amitriptyline	ATP	31.610	18.756	1.273	2.790
44	Trimipramine	TMO	33.100	19.688	1.294	2.822
45	Levomepromazine	LMP	34.370	20.481	1.311	2.848
46	Clocapramine	CCP	42.690	25.681	1.410	2.996
47	Chlorpromazine	CP	43.020	25.888	1.413	3.002
48	Clomipramine	CMP	49.490	29.931	1.476	3.097

TABLE II

EXPERIMENTAL DATA FOR OBTAINING EQN. 4a IN THE TEXT

Standard compound	*log P_e*	*Capacity factor, k'*			
		Mobile phase composition, buffer–acetonitrile			
		60:40	*65:35*	*70:30*	*74:25*
Barbital	0.777	0.581	0.719	0.875	1.106
Phenobarbital	1.253	1.063	1.394	1.881	2.675
Phenacetin	1.455	1.450	1.919	2.554	3.606
Triazolam	2.507	4.183	7.163	12.431	22.866

substances, multiple regression analysis on the data set shown in Table II gives

$$\log k' = f_1(X)\log P_e + f_2(X) \quad (4)$$

(the results of the regression analysis are shown in Table III, where A and B are regression coefficients) where X is the volume fraction of the organic solvent (in this case, acetonitrile) in the mobile phase; the experimentally determined relationship is:

$$\log k' = (-0.0178X + 1.2003) \log P_e - (0.0034X + 0.4562) \quad (4a)$$

Eqns. 4 and 4a mean that, if X and log P_e of a substance are known, the logarithm of the capacity factor can be predicted under given chromatographic conditions. This is the basic concept of the retention prediction for toxic compounds investigated here. Eqn. 4a can be stored in the program of the retention prediction system[3–7] and also in the automated identification system[2].

Automated identification system

Eqn. 4a can be used, in turn, to obtain log P_e for toxic compounds by substituting the measured retention information, k'. This concept can easily be systemized in a microcomputer yielding an interactive identification tool for toxic compounds.

The procedure is as follows: if one measures the k' value of a peak, the computerized system establishes the appropriate log P_e for the peak, using the data file

TABLE III

RESULTS OF REGRESSION ANALYSIS FOR THE DATA SET IN TABLE II

log $k' = A \log P_e + B$. F = statistical F ratio; R = correlation coefficient.

Mobile phase composition	*A*	*B*	*F*	*R*
60:40	0.4899	−0.5904	310	0.997
65:35	0.5742	−0.5753	1306	0.999
70:30	0.6635	−0.5644	7130	1.000
75:25	0.7571	−0.5373	5066	1.000

A SAMPLE : 130GAST **1**

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	4.53 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	0.26
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
phenobarbital	PHB	1.253	0.276	0.692

##########

SAMPLE : 130GSAT **2**

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	5.32 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	0.37
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
ethenzamide	ETM	1.371	0.355	0.732
bromazepam	BMZ	1.371	0.355	0.732
bromvalerylurea	BVU	1.326	0.325	0.081

##########

SAMPLE : 130GAST **3**

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	7.62 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	0.58
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
pentobarbital	PTB	1.707	0.578	0.938
nitrazepam	NTZ	1.707	0.578	0.938
amobarbital	AMB	1.734	0.596	0.516
phenytoin	PHT	1.734	0.596	0.516
chlordiazepoxide	CD	1.666	0.551	0.448

##########

SAMPLE : 130GAST **4**

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	14.50 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	0.91
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
flunitrazepam	FNZ	2.243	0.935	0.323
diazepam	DIZ	2.143	0.868	0.154

##########

Fig. 2.

SAMPLE : 130GAST 5

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	21.60 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	1.10
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
promethazine	PM	2.482	1.094	0.929
triazolam	TRZ	2.507	1.110	0.683
bromperidol	BP	2.511	1.113	0.619
desipramine	DEP	2.534	1.128	0.246

##########

SAMPLE : 130GAST 6

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	33.03 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	1.29
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
levomepromazine	LMP	2.848	1.294	0.981
trimipramine	TMO	2.822	1.277	0.637
amitriptyline	ATP	2.790	1.256	0.188

##########

B SAMPLE : 130SERUM 1

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	5.32 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	0.37
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
ethenzamide	ETM	1.371	0.355	0.732
bromazepam	BMZ	1.371	0.355	0.732
bromvalerylurea	BVU	1.326	0.325	0.081

##########

SAMPLE : 130SERUM 2

########## Search Version 3.0 ##########

Concentration of CH3CN	30.00 vol%
Retention Time of Unknown Solute	7.62 min
Elution Time of Unretained Solute	1.60 min
Logarithm of Capacity Factor	0.58
Maximum Value of Relative Error	10.00 %

---------- Candidates ----------

sample name	index	logP	logk'	coeff.
pentobarbital	PTB	1.707	0.578	0.938
nitrazepam	NTZ	1.707	0.578	0.938
amobarbital	AMB	1.734	0.596	0.516
phenytoin	PHT	1.734	0.596	0.516
chlordiazepoxide	CD	1.666	0.551	0.448

##########

Fig. 2. Output of the automated identification system for peaks in the chromatograms of Fig. 1. (A) For gastric contents, (B) serum. Each number corresponds to the peak number in Fig. 1.

TABLE IV

COMPARISON BETWEEN THE AUTOMATICALLY IDENTIFIED DRUGS AND THE PRESCRIBED DRUGS

Sample fluid	*Drugs identified by the system*	*Drugs prescribed*
Gastric fluid	Phenobarbital	Bromazepam
	Ethenzamide	Flunitrazepam
	Bromazapam	Triazolam
	Pentobarbital	Levomepromazine
	Nitrazepam	Trihexylphenidyl
	Promethazine	Pentobarbital
	Triazolam	Nitrazepam
	Levomepromazine	Thioridazine*
Serum	Ethenzamide	Timiperone*
	Bromazepam	Clofedanol*
	Pentobarbital	
	Nitrazepam	
Urine	Bromvalerylurea	
	Oxazolam	

* Drug not included in the library.

of log P_e for several toxic compounds as the standard basis. Then the names of the compounds having values closest to the calculated log P_e value (within the inputted relative error) are listed on the CRT or the line printer, together with the corresponding correlation coefficients. This coefficient is a measure of the probability of the predicted identification.

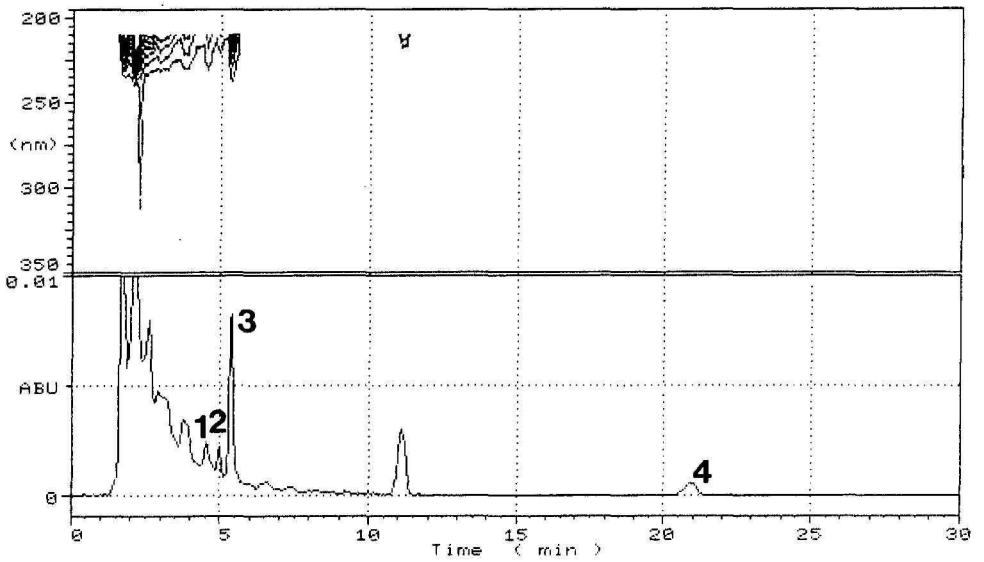

Fig. 3. Chromatogram of gastric contents sampled from a woman who had taken poison. Experimental conditions as in Fig. 1.

```
SAMPLE : 139GAST 1
############################ Search Version 3.0 ############################

Concentration of CH3CN              30.00 vol%
Retention Time of Unknown Solute     4.49 min
Elution Time of Unretained Solute    1.60 min
Logarithm of Capacity Factor         0.26
Maximum Value of Relative Error     10.00  %

---------------------------------- Candidates ----------------------------

 sample name                        index   logP  logk'      coeff.
phenobarbital                       PHB     1.253 0.276      0.549
##########################################################################

SAMPLE : 139GAST 2
############################ Search Version 3.0 ############################

Concentration of CH3CN              30.00 vol%
Retention Time of Unknown Solute     4.98 min
Elution Time of Unretained Solute    1.60 min
Logarithm of Capacity Factor         0.32
Maximum Value of Relative Error     10.00  %

---------------------------------- Candidates ----------------------------

 sample name                        index   logP  logk'      coeff.
bromvalerylurea                     BVU     1.326 0.325      0.994
ethenzamide                         ETM     1.371 0.355      0.289
bromazepam                          BMZ     1.371 0.355      0.289
##########################################################################

SAMPLE : 139GAST 3
############################ Search Version 3.0 ############################

Concentration of CH3CN              30.00 vol%
Retention Time of Unknown Solute     5.40 min
Elution Time of Unretained Solute    1.60 min
Logarithm of Capacity Factor         0.38
Maximum Value of Relative Error     10.00  %

---------------------------------- Candidates ----------------------------

 sample name                        index   logP  logk'      coeff.
bromazepam                          BMZ     1.371 0.355      0.527
ethenzamide                         ETM     1.371 0.355      0.527
phenacetin                          PNC     1.455 0.410      0.170
##########################################################################

SAMPLE : 139GAST 4
############################ Search Version 3.0 ############################

Concentration of CH3CN              30.00 vol%
Retention Time of Unknown Solute    21.00 min
Elution Time of Unretained Solute    1.60 min
Logarithm of Capacity Factor         1.08
Maximum Value of Relative Error     10.00  %

---------------------------------- Candidates ----------------------------

 sample name                        index   logP  logk'      coeff.
promethazine                        PM      2.482 1.094      0.764
triazolam                           TRZ     2.507 1.110      0.363
bromperidol                         BP      2.511 1.113      0.298
propericyazine                      PPC     2.416 1.050      0.248
##########################################################################
```

Fig. 4. Output of the automated identification system for the chromatogram in Fig. 3.

Application of the system to actual samples

The first application is to the analysis of gastric contents, urine and serum of a deceased patient who had been sent to hospital on February 24th, 1987. The chromatograms of these samples are shown in Fig. 1A for gastric contents and in Fig. 1B for serum, respectively. A peak at 11.2 min which appeared in all chromatograms is not due to toxic substances because this peak also appeared in the chromatograms of pooled control samples. In Fig. 2, the output of the automated identification system for peaks in the chromatograms is shown. The gastric sample is the most suitable to judge what kind of toxic substances were present: from the chromatographic data assisted by the automated identification system, it is highly possible that the patient took one or some of the drugs listed in Table IV. In Table IV, drugs which he had been prescribed for his psychiatric disease are also indicated. Since it is considered that he took some of the drugs which had been prescribed, these drugs should be included in the list. Some drugs identified by the automated system are found in the prescribed drugs and therefore the analysis could be assisted by the system output.

The second example is the gastric contents taken from a woman who took a drug on April 19th, 1987. After recovery, she stated she had taken chlordiazepoxide, although it was reported that she had bromvaleryurea. The chromatogram and the output of the automated system are shown in Figs. 3 and 4, respectively. The result of the identification clearly shows that she had taken bromvaleryurea not chlordiazepoxide. The chromatogram of the gastric sample indicates no trace of chlordiazepoxide.

CONCLUSIONS

A microcomputer-assisted automated identification system for toxic compounds in reversed-phase LC has been described. To construct the system, the user or column supplier should conduct some experiments with different mobile phase compositions and selected standards so as to obtain eqn. 4a, and day-to-day fluctuation of retention data can be corrected.

Although the system in its present form will leave many problems to be solved in practice, the concept opens a new dimension of clinical toxicological analysis. If the system is coupled to the spectral-matching function of multichannel detectors, its reliability is expected to increase, and this is under investigation in our laboratory. An expansion of the log P_e data file on toxic substances is also required for much wider application of the system.

REFERENCES

1 A. C. Moffat (Editor), *Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids, and Post-Mortem Material*, The Pharmaceutical Press, London, 2nd ed., 1986.
2 K. Jinno and M. Kuwajima, *Chromatographia*, 21 (1986) 622.
3 K. Jinno, *Proceedings of the 8th International Symposium on Capillary Chromatography*, Vol. 2, Hüthig, Heidelberg, 1987, pp. 1143–1152.
4 K. Jinno and K. Kawasaki, *J. Chromatogr.*, 316 (1984) 1.
5 K. Jinno and K. Kawasaki, *J. Chromatogr.*, 298 (1984) 326.
6 K. Jinno and K. Kawasaki, *ACS Symp. Ser.*, 297 (1986) 167–187.

7 K. Jinno, T. Hoshino, T. Hondo, M. Saito and M. Senda, *Anal. Chem.*, 58 (1986) 2696.
8 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
9 W. Melander, J. Stoveken and Cs. Horváth, *J. Chromatogr.*, 199 (1980) 35.
10 L. Hafkenscheid, *Ph.D. Thesis*, University of Amsterdam, Amsterdam, 1983.
11 F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1977.
12 C. Hansch and A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology*, Wiley, New York, 1979.

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DNA–NITROSOUREA INTERACTIONS

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CROSS-LINKED DINUCLEOSIDES AND SUBSTITUTED DEOXYNUCLEOSIDES

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SUMMARY

A reconstituted mixture of five cross-linked dinucleosides possibly involved in DNA–nitrosourea interactions, and of their degradation products (nucleobases, deoxynucleosides and mono- or disubstituted deoxynucleosides), was analysed by reversed-phase high-performance liquid chromatography using C_{18} columns and an diode-array detector. The chromatographic conditions for separating the twenty-one investigated compounds were optimized, and the compounds were identified by both their retention times and their UV spectra. A structure–retention time relationship was observed under suitable conditions and is discussed. Its validity was confirmed by the prediction of the retention time of a new cross-linked dinucleoside synthesized for this purpose.

INTRODUCTION

Anticancer 2-chloroethylnitrosoureas (CENUs) have an established place in the clinical treatment of human malignancies[1–3]. Their mechanism of action has been extensively studied[4,5], and it is now well established that such alkylating agents form cross-links between DNA strands, especially at the level of dG–dC base pairs. Two cross-linked dinucleosides and some monosubstituted nucleosides have been isolated and identified after incubation of calf thymus DNA with various CENUs and subsequent cleavage of modified DNA[6–10].

However, other cross-linked dimers could be present in such mixtures, even in very low amounts. We recently synthesized[11,12] some new cross-links in order to identify them. In addition, we studied their stability at different pH values by identification of their degradation products[13]. This led us to the extensive use of reversed-phase high-performance liquid chromatography (RP-HPLC). We present here the analysis of a reconstituted mixture of twenty-one compounds potentially present after treatment of DNA with nitrosoureas. After optimization of the chromatographic parameters, detection with an UV–VIS diode-array detector allowed the charac-

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terization of each eluted compound from both its retention time and its UV spectrum, even at the nanogram level.

The relationship observed between the retention times and the structures of these compounds and its application to predict the retention time of other unknown cross-linked dinucleosides are discussed.

EXPERIMENTAL

Instrumentation

The adjustment of chromatographic parameters was performed on a Waters Assoc. instrument, equipped with two Model 510 EF solvent-delivery systems, a Model 720 solvent programmer, a Model U6K sample injector, a Model 481 UV absorbance detector operating at 254 nm, a Model R401 differential refractometer and a Model 730 integrator. Protected by prefilters and a C_{18} Guard Pak precolumn, the analytical reversed-phase column was a C_{18} Radial-Pak (100 mm × 8 mm I.D., particle size 10 μm) in a Waters Assoc. radial compression module RCM-100.

Spectral data for the compounds eluted were obtained with two Model 6000A solvent-delivery systems, a Model 680 solvent programmer, a Model U6K sample injector (Waters Assoc.) and a PU 4021 multichannel detector (Philips). Data were computed by means of a PU 4850 videochromatography centre equipped with a PU 4900/20 printer/plotter (Philips). The ultra-fast reversed-phase column was an Ultrasphere XLODS cartridge (70 mm × 4.6 mm I.D., particle size 3 μm; Beckman) protected by prefilters and an XLODS precolumn (5 mm × 4.6 mm I.D., 3 μm).

The two instruments were placed in a thermostated chamber (Frigor-Kulmobel) which could be controlled between 13 and 24°C with a precision of 0.25°C. Because of this feature, reproducible results ($\Delta t_R/t_R < 1\%$, where t_R is retention time) could be obtained despite significant variations of the ambient temperature.

Chemicals

The water used for the buffers was distilled and purified through a Milli-Q system to give a resistivity of 18 MΩ/cm (Millipore). Acetonitrile was of far-UV HPLC-grade (Fisons). Analytical-grade ammonium acetate and sodium dihydrogenphosphate Rectapur grade were obtained from Merck and Prolabo respectively. The stock solutions of acetate (1 l, 1 *M*) or phosphate (500 ml, 1 *M*) were filtered through a Millipore membrane (Type HA, pore size 0.45 μm). The pH of solvent A (100 ml stock solution diluted to 1 l) and solvent B (100 ml stock solution, 200 ml acetonitrile, diluted to 1 l) was adjusted with acetic or phosphoric acid. The solvents were degassed in an ultrasonic bath.

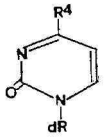
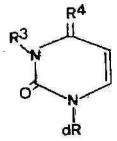
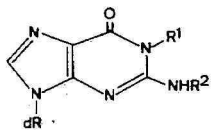
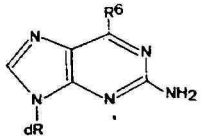
The compounds investigated are presented on Table I. All the derivatives were synthesized in our laboratory[11,12], except nucleobases 1–3 and 2′-deoxynucleosides 4, 7, 11. Each pure sample was dissolved in water and filtered (HV-4, Millipore). The different solutions were mixed in various proportions in order to identify each compound on the chromatogram. The complete mixture was adjusted so that the UV spectra at the apex had the same order of magnitude. Spectral data allowed the verification of the assignment of each peak.

The following linear solvent gradients were employed: I, A to B in 45 min; II, A to B in 75 min; III, A to B in 100 min; IV, step 1, A to 60% A–40% B in 30 min, step 2, 60% A–40% B to B in 5 min.

TABLE I

FORMULAS OF DERIVATIVES INVESTIGATED[11,12]

The retention times expressed in seconds are obtained under the conditions of gradient programme II.

Derivatives investigated							t_R (s)
No.	*Structure*						
	Formula	R^1	R^2	R^3	R^4	R^6	
1	Cytosine						86
2	Uracil						106
3	Guanine						190
4					NH_2		277
5					$NHCH_2CH_2OH$		648
6					$NHCH_2CH_2NH_2$		374
22					OCH_2CH_2OH		936
7				H	O		408
8				CH_2CH_2OH	O		915
9				CH_2CH_2OH	NH		552
10				CH_2–CH_2N			486
11		H	H				822
12		CH_2CH_2OH	H				1103
13		CH_2–CH_2					1192
14						NH_2	1090
15						OCH_2CH_2OH	1610
16						$OCH_2CH_2NH_2$	1480
17	$dC^{N^4CH_2CH_2N^4}dC$						1534
18	$dG^{O^6CH_2CH_2N^3}dC$						1913
19	$dG^{O^6CH_2CH_2N^4}dC$						2244
20	$dG^{O^6CH_2CH_2N^3}dU$						2361
21	$dG^{O^6CH_2CH_2O^6}dG$						2828
23	$dU^{O^4CH_2CH_2O^4}dU$						1950

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Most normal nucleosides are relatively weak acids and bases[14]. Cytidine has the highest pK_{ab} (4.15), uridine and guanosine the lowest pK_{aa} (9.2). Since they are

neutral between their pK_{ab} and pK_{aa}, they can be analysed by RPLC according to their hydrophobicities. This technique has been extensively used for analytical separation and determination of natural and modified nucleosides. Cytidine and uridine derivatives are slightly hydrophobic and are quickly eluted with buffers. Guanosine and adenosine derivatives are retained longer on the stationary phase and the addition of an organic modifier is necessary to elute them in a reasonable time.

Preliminary attempts following literature procedures[15–19] showed that elution on the Radial-Pak 10-μm column with 0.1 *M* sodium phosphate buffer, pH 4.85 does not allow good resolution of the first compounds eluted. The application of 0.1 *M* ammonium acetate buffer improved this separation. Different pH values, 5.9, 6.9, 7.7, and several programs for the addition of organic modifier were investigated.

Acetonitrile was preferred to methanol because of its better UV transmission. The best resolution obtained with a multistep gradient at pH 7.7 left only one overlap (Table II, entry 1).

As expected, under similar conditions except at a reduced flow-rate, the resolution was enhanced by using an Ultrasphere column of smaller particle size (3 μm). We observed several modifications in the elution order in comparison with the Radial-Pak column. Examination of Table II shows that the retention times of compounds 6, 14 and 16 (all containing a primary amino group) and of imino group-containing derivatives 9, 10 and 18 are highly sensitive to the nature of the stationary phase (entries 1, 2).

The remaining overlap of compounds 5 and 9 was suppressed by decreasing the pH of the buffer from 7.7 to 5.9. Comparison of entries 2 and 3 (Table II) shows that the retention times of the imino group-containing compounds 9, 10 and 18 are strongly affected by the eluent pH.

The rate of addition of acetonitrile was further adjusted to reduce the analysis time. When the percentage of acetonitrile linearly increased at 0.44%/min (I), the analysis time did not exceed 35 min but several peaks were overlapped. Increases of 0.27% (II) and 0.20%/min (III) resolved all compounds in analysis times of 45 and 65 min respectively. A compromise for fast routine analysis was found by using a two-step gradient which permitted complete analysis in less than 40 min (IV: 0.27%/min for 30 min, 2.4%/min for 5 min then isocratic conditions). Fig. 1 shows

TABLE II

QUALITATIVE VARIATION OF THE ELUTION ORDER OF ALL THE COMPOUNDS STUDIED

This variation depends on the stationary phase (particle size 10 or 3 μm) (compare entries 1 and 2) and on the eluent pH (compare entries 2 and 3).

Entry	*Particle size (μm)*	*pH*	*Elution order*
1	10	7.7	1 2 3 4 - 7 (6) — — 5 (9) 11 — 8 (10) — 12 (14) 13 — 17 (16) 15 — (18) 20 21 (19)
2	3	7.7	1 2 3 4 (6) 7 - — — 5 (9) - 11 (10) 8 — (14) 12 — 13 (16) 17 — 15 (18) 19 20 21
3	3	5.9	1 2 3 4 (6) 7 - (10) (9) 5 - 11 — 8 — (14) 12 — 13 (16) 17 — 15 (18) 19 20 21

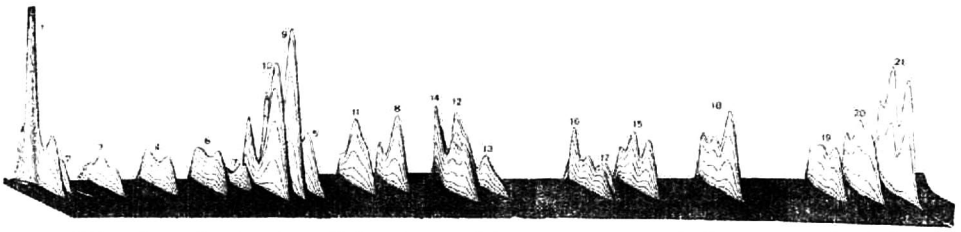


Fig. 1. Three-dimensional pattern of the mixture of the 21 compounds obtained with an XLODS 3-μm column (solvent gradient IV).

the three-dimensional pattern obtained under these optimized conditions with the diode-array detector, from which may be computed the conventional chromatogram at the chosen wavelength (Fig. 2) or the UV spectrum of each compound eluted. These UV spectra were in accordance with our previous data[11,12].

It is known[18,20] that the elution order of nucleosides (C, U, G) or deoxynucleosides dC, dU, dG ($4 < 7 < 11$) follows that of the corresponding nucleobases Cy, Ur, Gu ($1 < 2 < 3$). Under the previously mentioned conditions (Table II), we observed that the related hydroxyethyl-substituted deoxynucleosides ($9, 5 < 8 < 12, 15$) behave similarly. In the same way, dinucleosides 18 21 containing an O^6-hydroxyethyl deoxyguanosine moiety are assumed to be analogues of parent nucleosides (dC, dU, dG) and experimentally follow the same elution order ($18, 19 < 20 < 21$). The same retention behaviour was observed with the two dimers ($17 < 19$) containing the N^4-hydroxyethyl deoxycytidine moiety.

Moreover, under one-step linear gradient conditions I–III, it is noteworthy that when the retention times of nucleobases 1–3, hydroxyethyl-substituted deoxy-

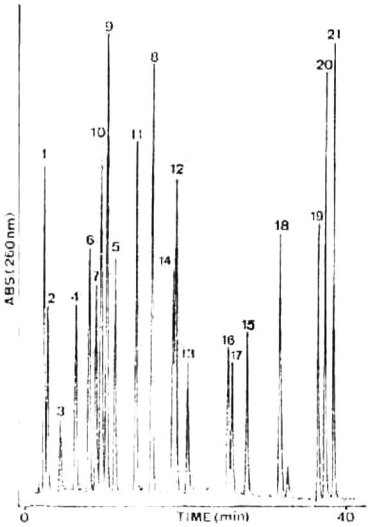


Fig. 2. Chromatogram of the reconstituted mixture of the 21 compounds at 260 nm (solvent gradient IV).

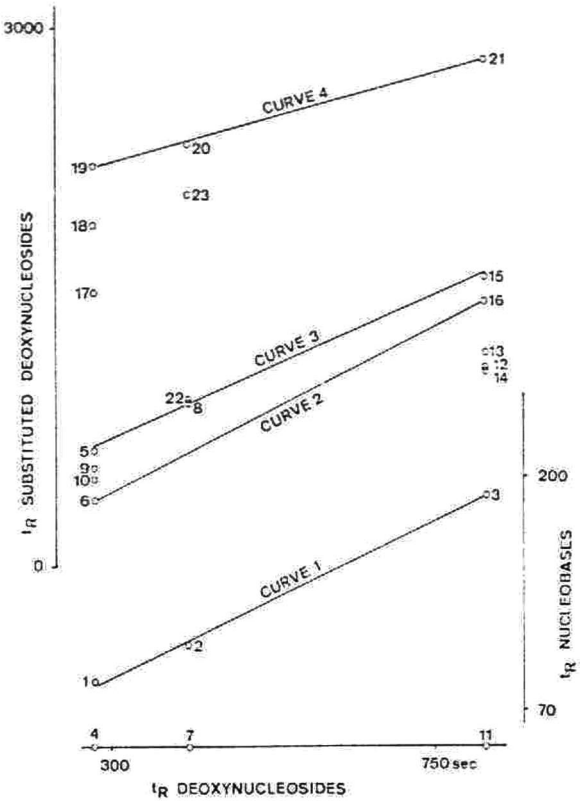


Fig. 3. Linear relationships (curves 1, 3, 4) between the t_R of parent deoxynucleosides (dC, 4; dU, 7; dG, 11) and the t_R of the corresponding nucleobases, hydroxyethyl-substituted deoxynucleosides and dinucleosides. A similar pattern is obtained for aminoethyl-substituted deoxynucleosides (curve 2).

nucleosides 5, 8 and 15, and dinucleosides 19–21 were plotted *versus* the retention times of the parent deoxynucleosides 4, 7 and 11, the plots (curves 1, 3 and 4) were linear; regression coefficients = 0.99 (Fig. 3).

Several plots appeared not to fit these linear relationships. If we exclude for obvious reasons both cyclic compounds 10 and 13 and deoxynucleosides 6, 14 and 16 lacking an hydroxyethyl group, there remain three derivatives 9, 18 and 12 which exhibit a different behaviour.

We then focused attention on the observed difference between N^3-substituted (9 and 18) and N^4-substituted (5 and 19) deoxycytidine derivatives. According to the reported pK_{ab}[14] for deoxycytidine (4.3), N^4-methyldeoxycytidine (4.0) and N^3-methylcytidine (8.7), we concluded that N^3-substituted deoxycytidine derivatives 9 and 18 are mainly in the neutral form at pH 7.7 and protonated at pH 5.9, whereas other derivatives of guanine, uracil and cytosine are neutral in the range of pH 5–8.

This is consistent with the aforementioned observation about the high sensitivity of the retention times of compounds 9 and 18 to the eluent pH (Table II). These differences in neutral and protonated forms could explain the peculiar behaviour of the N^3-hydroxyethyl deoxycytidine derivatives. Hence, N^3-substituted deoxycytidine

derivatives 9 and 18 cannot be grouped with other families of homologous compounds (5, 8 and 15) and dinucleosides (19–21).

With respect to the N^1-hydroxyethyl deoxyguanosine 12 compared to the O^6-hydroxyethyl derivative 15, the required data are not available to account for the non-linearity of their plots.

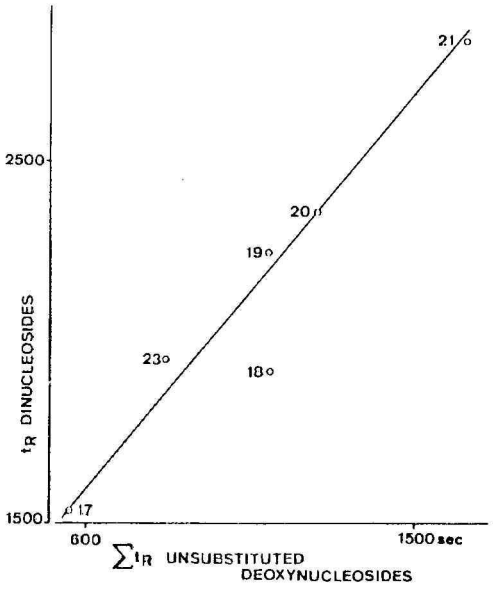
A question arises from these results as to why linear relationships are observed. Under isocratic conditions, a linear relationship may appear between the logarithm of the capacity factors, k', of compounds belonging to a series of related compounds[21]. Otherwise, the eluting capacity of the solvent systems is not usually a direct function of time in linear gradients[22]. Under our experimental conditions, the eluting capacity probably varies in a quasi-exponential manner when the content of the organic modifier increases linearly. Therefore the apparent k' (or approximately t_R) values were linearly incremented according to the substituent for derivatives belonging to the same series. Similar linear relationships between substituted and parent compounds appeared in the three linear gradient programmes investigated.

If this linearity is assumed not to be random, a correlation is expected between the t_R of a given dinucleoside and the sum of the t_R values of its parent compounds:

$$t_R(\text{dinucleoside}) = a\Sigma t_R(\text{parents}) + b$$

This was verified for the dinucleosides 17 and 19–21, from deoxynucleosides (Fig. 4) and from hydroxyethyl or aminoethyl deoxynucleosides (curves 5 and 6; Fig. 5).

An additional control was performed to establish this non-random linearity.



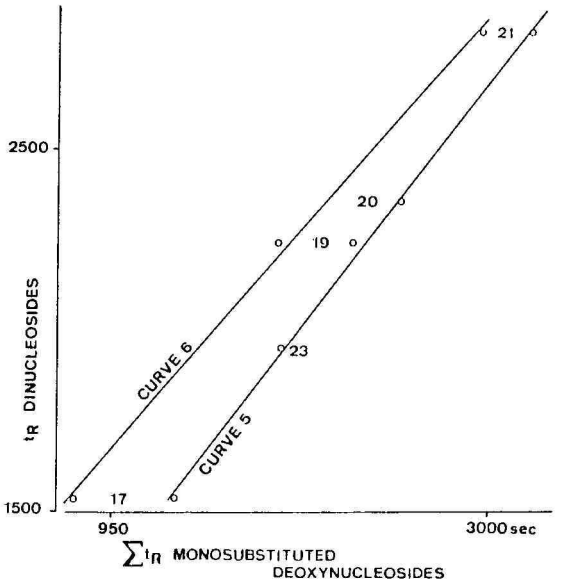


Fig. 4. The t_R of dinucleosides plotted *vs.* the sum of the t_R of unsubstituted deoxynucleosides.

Fig. 5. The linear relationships between the sum of the t_R of hydroxyethyl (curve 5) or aminoethyl (curve 6) deoxynucleosides *versus* the t_R observed for dinucleosides.

We chose a compound expected to be in neutral form at pH 5.9 and attempted to predict its retention time from the experimentally determined curves (Figs. 4 and 5). For this purpose, we synthesized[13] the O^4-hydroxyethyl deoxyuridine 22 and the corresponding symmetrical dimer 23. The monomer 22 gave a t_R (936 s) close to that of N^3-hydroxyethyl deoxyuridine 8 (915 s), whereas the t_R of dimer 23 was 1950 s. The t_R values of compound 23 deduced from Figs. 4 and 5 are 1866 and 1910 s respectively. The experimental and theoretical plots agree fairly well and permitted the prediction of the t_R value of any dinucleoside belonging to the above series from the available commercial deoxynucleosides.

In conclusion, the t_R of dinucleosides exhibit a linear relationship with those of the parent unsubstituted or monosubstituted deoxynucleosides. The t_R of any dinucleoside, in neutral form at pH 5.9, can be accurately estimated.

The gradient conditions developed for separating the twenty-one derivatives investigated may permit the detection of hitherto unidentified cross-linked dinucleosides in mixtures obtained from the reaction of CENUs with DNA.

REFERENCES

1 A. Godin, in B. Serrou, P. S. Schein and J.-L. Imbach (Editor), *Nitrosoureas in Cancer Treatment*, Elsevier, North-Holland, Amsterdam, 1981, p. 3.
2 B. Singer and D. Grunberger, in *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York, London, 1983, p. 45.
3 M. Brendel and A. Ruhland, *Mutat. Res.*, 133 (1984) 51.
4 T. P. Johnston and A. Montgomery, *Cancer Treat. Rep.*, 70 (1986) 13.
5 D. B. Ludlum and W. P. Tong, in F. M. Muggia (Editor), *Experimental and Clinical Progress in Cancer Chemotherapy*, M. Nijhoff Publishers, Boston, MA, 1985, p. 141.
6 W. P. Tong, M. C. Kirk and D. B. Ludlum, *Cancer Res.*, 42 (1982) 3102.
7 W. P. Tong, M. C. Kirk and D. B. Ludlum, *Biochem. Pharmacol.*, 32 (1983) 2011.
8 J. W. Lown, R. R. Koganty, U. G. Bhat, A.-M. Sapse and E. B. Allen, *Drugs Exptl. Clin. Res.*, 12 (1985) 463.
9 S. Parker, M. C. Kirk, D. B. Ludlum, R. R. Koganty and J. W. Lown, *Biochem. Biophys. Res. Commun.*, 139 (1986) 31.
10 J. W. Lown, R. R. Koganty, U. G. Bhat, S. M. S. Chauhan, A.-M. Sapse and E. B. Allen, *IARC Sci. Publ.*, 70 (1986) 129.
11 A.-F. Maggio, M. Lucas, J.-L. Barascut, A. Pompon and J.-L. Imbach, *Nouv. J. Chim.*, 10 (1986) 643.
12 A.-F. Maggio, M. Lucas, J.-L. Barascut and J.-L. Imbach, *J. Heterocycl. Chem.*, 23 (1986) 969.
13 A.-F. Maggio, M. Lucas, J.-L. Barascut and J.-L Imbach, unpublished results.
14 H. A. Sober (Editor), *Handbook of Biochemistry*, CRC Press, Boca Raton, Fl., 1970, Section G.
15 W. Schroeder, III, T. L. Cupps and L. B. Townsend, *J. Chromatogr.*, 254 (1983) 315.
16 S. P. Assenza, P. R. Brown and A. P. Goldberg, *J. Chromatogr.*, 277 (1983) 305.
17 C. Yi, J. L. Fasching and P. R. Brown, *J. Chromatogr.*, 339 (1985) 75.
18 A. Rizzi and H. R. M. Lang, *J. Chromatogr.*, 331 (1985) 33.
19 A. Pompon, G. Gosselin, M.-C. Bergogne and J.-L. Imbach, *J. Chromatogr.*, 388 (1987) 113.
20 C. W. Gehrke, R. A. McCune, M. A. Gama-Sosa, M. Ehrlich and K. C. Kuo, *J. Chromatogr.*, 301 (1984) 199.
21 J. Jacobson, Z. El Rassi and C. Horváth, in I. Molnar (Editor), *Practical Aspects of Modern HPLC*, Walter de Gruyter, Berlin, New York 1982, p. 15.
22 L. R. Snyder, in *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 208.

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α-, β- AND γ-CYCLODEXTRINS AS MOBILE PHASE ADDITIVES IN THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERIC COMPOUNDS

II. OPTIMIZATION OF THE SEPARATION METHOD BY USING α-, β- AND γ-CYCLODEXTRINS IN MIXTURE

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SUMMARY

Mixtures of α-, β- and γ-cyclodextrins (CDs) have been used as mobile phase additives for the separation of isomeric compounds. It was found that by using a mixture of three CDs practically the same retention can be obtained. A CD which does not react with quest molecules or with which a weak reaction is observed has no significant influence on the retention. The selection of the most reactive complexing agent is the base of a simple optimization procedure.

INTRODUCTION

In recent papers[1,2] the application of γ-cyclodextrin (γ-CD) as a mobile phase additive and cyanopropyl-silica as the stationary phase for the high-performance liquid chromatographic (HPLC) separation of different stereo-, positional and optical isomers was reported. The separation of these isomers by means of CDs differing in cavity size (α-, β- and γ-CD) on cyanopropyl-silica has also been studied.

As a continuation of this work, the application of a mixture of the three CDs in the eluent has been tried. The main aim was to develop a simple optimization technique when CD is used as a mobile phase additive for the separation of isomeric compounds. The cavity size of CD has great importance in inclusion complex formation[3,4]; only those guest molecules which can be fitted into the chiral cavity of CD, resulting in intimate contact with the inner surface, can form stable inclusion complexes. If the size of the molecules is too small or too large, only a weak reaction or no reaction is observed, which means that in the presence of these CDs almost the same retention is obtained for the compounds. On the basis of the results it is easy to select the most reactive complexing agent.

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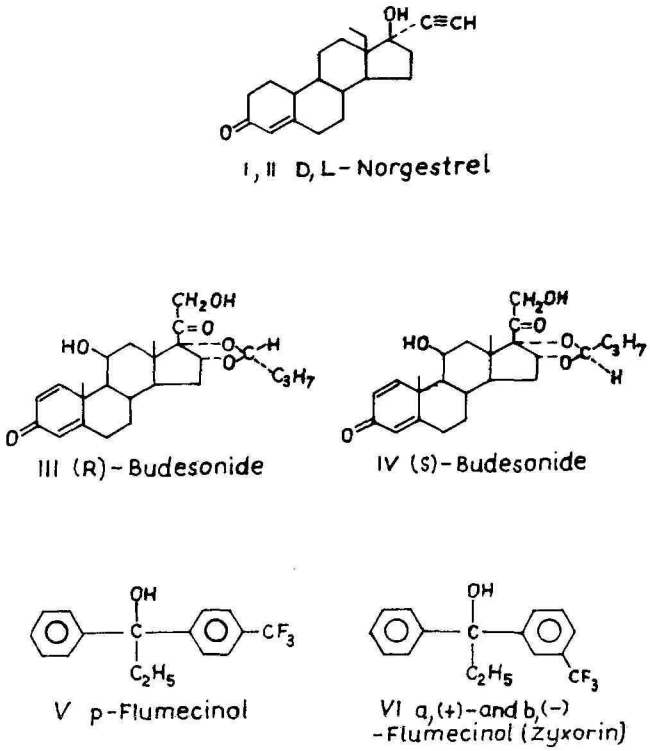

Fig. 1. Structures of the compounds investigated.

EXPERIMENTAL

A Liquochrom 2010 HPLC system (Labor-MIM, Esztergom-Budapest, Hungary) equipped with a variable-wavelength UV detector, a loop injector and a recorder was used. The separations were performed on pre-packed Nucleosil 10 CN (250 × 4.6 mm I.D.) and Hypersil ODS (5 μm) (250 × 4.6 mm I.D.) columns (Chrompack, Middelburg, The Netherlands). The eluents were prepared with HPLC-grade solvents and were degassed prior to use.

α-, β- and γ-CDs were obtained from Chinoin (Budapest, Hungary) and were used without further purification. The compounds to be tested were prepared at the Chemical Works of Gedeon Richter (Budapest, Hungary) and their quality was checked by HPLC before use. Their structures are shown in Fig. 1.

RESULTS AND DISCUSSION

Effect of α-, β- and γ-CD concentrations in the eluent on the capacity ratios of model compounds

The dependence of the capacity ratios of the model compounds indicated in Fig. 1 on the α-, β- and γ-CD concentrations using cyanopropyl-silica as the stationary phase and methanol–water as the eluent has been reported previously[2]. Only

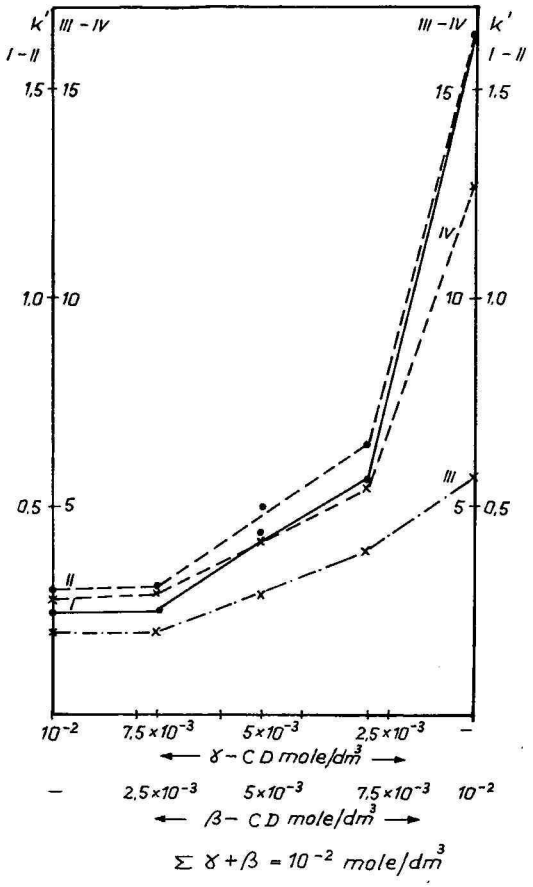

Fig. 2. Dependence of capacity ratios on the ratio of β- and γ-CD in the eluent. Total CD concentration, 10^{-2} mole/dm^3. Column, Nucleosil 10 CN (250 × 4.6 mm I.D.); eluent, methanol–water (1:3); flow-rate, 1 cm^3/min; detection, 254 nm. Compounds as in Fig. 1.

small changes in retention occurred when the α-CD concentration is varied but larger changes were obtained for β-CD and γ-CD. The selectivity of the separation is greatly influenced by both the size of the guest molecules and the cavity size of the CD used. In Figs. 2 and 3 the dependence of the capacity ratios of model compounds on the ratio of β- and γ-CDs is shown for a constant total concentration of CDs and the same mobile and stationary phases.

D- and L-norgestrel isomers and *R*- and *S*-isomers of budesonide form strong inclusion complexes with γ-CD. The enantiomeric forms of norgestrel can only be separated with γ-CD, whereas the (*R*)- and (*S*)-budesonide can be separated with either β- or γ-CD, although in the form of γ-CD complexes these compounds have smaller k' values, indicating stronger inclusion complex formation. The retentions of these four compounds are in good agreement with the data calculated from the k' *versus* CD concentration plot[2].

In Fig. 3 the dependence of the capacity ratios of flumecinol isomers on the

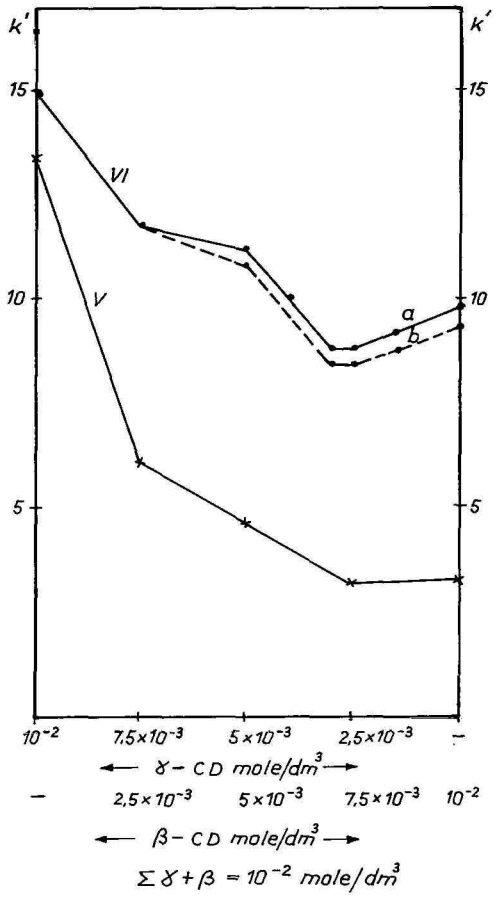

Fig. 3. Dependence of capacity ratios of flumecinol isomers on the ratio of β- and γ-CD in the eluent. Conditions as in Fig. 2.

ratio of β- and γ-CD is shown. The best separation is achieved in the presence of β-CD. Only small changes in retention are observed when γ-CD is present alone in the eluent. In contrast to the curve shown in Fig. 2, a definite minimum of the capacity ratios of the two enantiomers appears, indicating that the most polar complex is formed at about a 1:3 ratio of β- and γ-CD and the retentions of the compounds are significantly lower than when the CDs are used individually at the same concentration (10^{-2} mole/dm^3). This suggests a very complex mechanism for inclusion complex formation; possibly one guest molecule reacts with more CD molecules and both β- and γ-CD may participate in the complex formation between the guest and host molecules, resulting in more stable mixed complexes.

Fig. 4 shows the dependence of the capacity ratios on the CD concentrations when α-, β- and γ-CDs are used together in the eluent. With the enantiomeric forms of norgestrel the same retention is obtained when γ-CD alone, α- + γ-CD, β- + γ-CD and α- + β- + γ-CD are used. α-CD does not form a complex with the molecules and a less polar complex is formed with β-CD. α-CD does not react with budesonide

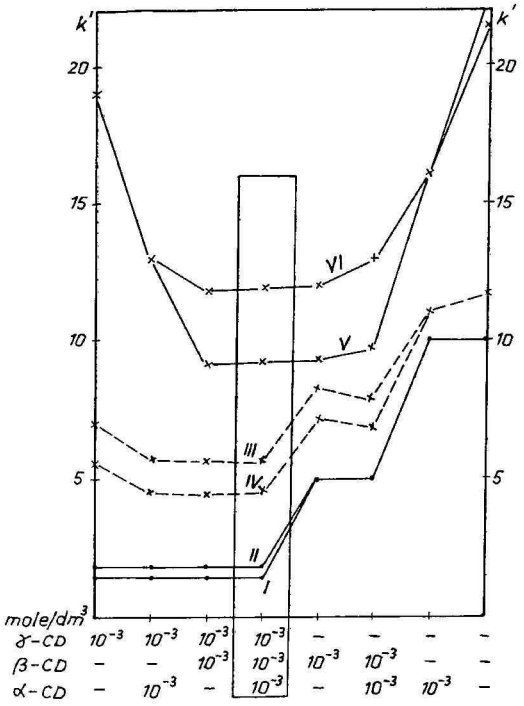

Fig. 4. Dependence of capacity ratios on the presence of different CDs in the eluent. Conditions as in Fig. 2; compounds as in Fig. 1.

isomers, whereas β- and γ-CD can form complexes with these isomers. With budesonide the decreased retention can be explained by the increase in the total CD concentration. Flumecinol isomers can react with γ-CD but only a weak complex is formed. A strong reaction is observed with β-CD, and have the retention is slightly influenced by the presence of other CDs.

In general, the addition of a mixture of the different CDs to the eluent gives information about the estimated changes in retention. A CD that does not react with the guest molecules has no significant influence on the retention, and in the presence of a less active CD the change in retention is small. These results can provide significant help in method optimization.

Effect of pH and ionic strength on inclusion complex formation

To study the influence of the pH and ionic strength of the eluent on inclusion complex formation, the guest molecules indicated in Fig. 1 were used, because these molecules do not contain ionizable functional groups.

In Fig. 5 the results for the dependence of capacity ratios on pH at constant ionic strength (Fig. 5A) and on ionic strength at constant pH (Fig. 5B) are shown.

As shown in Fig. 5A, for budesonide isomers a small decrease in k' with increasing pH is observed, but the selectivity factor measured between the two isomers is almost constant. A similar effect of ionic strength on retention was also found. The separation of norgestrel isomers is hardly affected by pH or ionic strength.

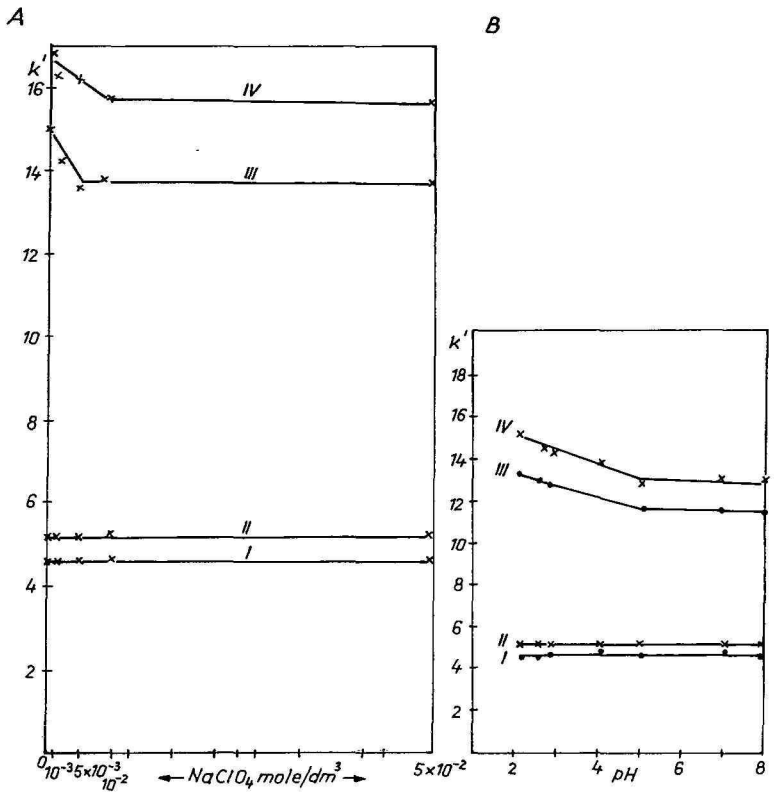

Fig. 5. Dependence of capacity ratios on (A) the ionic strength and (B) the pH of the eluent. Column, Hypersil ODS, 5 μm (250 × 4.6 mm I.D.); eluent, methanol–water (1:1); other conditions as in Fig. 2. (A) γ-CD concentration, 10^{-2} mole/dm³ (pH = 6.0); (B) 10^{-3} mole/dm³ phosphate buffer + 10^{-2} mole/dm³ γ-CD.

In general, it can be stated that the selectivity and efficiency of separation are independent of the salt concentration and pH when guest molecules without ionizable functional groups are examined.

TABLE I

STATIONARY PHASE SELECTION

Type of compound	*Estimated capacity ratio in methanol–water eluent (20–50%)*			*Stationary phase selected*
	C_{18}	*CN*	*Polar*	
Polar	5–15			C_{18}
Medium polar		5–15		CN
Less polar			5–15	Amine, silica
Ionic compounds (using ion-supression media)	5–15	5–15		C_{18} or CN

TABLE II

SELECTION OF STARTING CD CONCENTRATION

Methanol concentration in eluent (%)	*Individual CD concentration (mole/dm³)*		
	α-CD	*β-CD*	*γ-CD*
>40	10^{-2}	$5 \cdot 10^{-3}$	10^{-2}
40–25	$5 \cdot 10^{-3}$	$5 \cdot 10^{-3}$*	$5 \cdot 10^{-3}$
25–5	10^{-3}	10^{-3}	10^{-3}
<5	$5 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	$5 \cdot 10^{-4}$

* To avoid precipitation.

Optimization of the separation using CDs as mobile phase additives for the separation of isomeric organic substances

On the basis of our previous experiments and the above results the different steps in method development and optimization can be summarized as follows.

Stationary phase system selection. The stationary phase can be selected on the basis of the chromatographic properties of the substances examined, based on the chromatographic data with methanol–water as eluent (methanol concentration less than 50%) to obtain the optimal starting retention ($k' = 10$–15). The principles of stationary phase selection are summarized in Table I.

Concentration of CDs. The starting CD concentration (mixture of α-, β- and γ-CD of the same concentration) depends on the methanol concentration in the eluent, as indicated in Table II.

First modification of eluent composition on the basis of the data obtained with the first CD-containing eluent. The mobile phase composition is changed on the basis of the ratio of capacity factors obtained in the presence and absence of CD in the eluent, as indicated in Table III.

Second modification of mobile phase composition. In Table III the possible change in retention using a CD-containing eluent in comparison with the same eluent without CD can be divided into three groups (A, B and C).

TABLE III

CHANGE IN THE MOBILE PHASE COMPOSITION IN THE THIRD EXPERIMENT

Group	*Change in retention*	$\frac{k'_0}{k'_1}$*	*Proposed change in mobile phase composition*
A	Small	1.0–1.2	CD concentration is increased in the eluent or another stationary phase requiring less methanol concentration is tried
B	Medium	1.2–2.0	The next eluent contains only β- and γ-CD at the previous concentration
C	Significant	>2.0	The next eluent contains only α- and γ-CD at the previous concentration

* k'_0 = capacity ratio of the compounds in the absence of CD; k'_1 = capacity ratio in the presence of CD.

In case A, when the change in retention with an increased CD concentration is small, there is virtually no further possibility of increasing the selectivity of the separation by means of CD inclusion complex formation. If an appropriate change in retention was obtained ($k'_0/k'_2 \approx 1.2$–2.0), the experiments are continued according to case B.

Three possibilities can occur in case B. The first is no complex formation, which means that almost the same retention (k'_3) is found as k'_0. In this instance α-CD is responsible for inclusion complex formation but was not present in the eluent. The experiments are then continued with the use of α-CD alone. The second possibility is when the retention is smaller than k'_0 but larger than k'_2. If k'_3 is closer to k'_0 than to k'_2, in the subsequent experiment an α-CD-containing eluent is used (β-CD can also react but a less stable complex is formed. When k'_3 is closer to k'_2 than to k'_0 but less than k'_2, in the next eluent only β-CD is used because it gives the strongest inclusion complex with the guest molecules. If the retention is almost the same as k'_1, only γ-CD is applied in the next eluent.

In case C, if the situation is very similar to that discussed above when there is no complexation ($k_3 = k_0$), the next eluent contains only β-CD. If the retention is between k'_3 and k'_0 the next eluent contains only γ-CD, and finally if k'_3 is almost the same as k'_2 in the next eluent only γ-CD is applied.

Dependence on CD concentration. The CD reagent forming the strongest inclusion complex is selected according to the previous section. The dependence of the capacity factors and selectivity on the CD concentration is studied using the same stationary and mobile phases as in the earlier experiments.

Dependence on pH and ionic strength. If the guest molecules contain an ionizable functional group the pH dependence of the separation can also be studied.

Consideration of the analytical task. The chromatographic system is re-optimized according to the aim of the analytical task involved.

CONCLUSIONS

Mixtures of α-, β- and γ-CDs have been used as mobile phase additives for the separation of isomeric compounds. It was found that by the simultaneous use of the three CDs the most reactive complexing agent easily be selected, which can be the basis of a very simple optimization procedure.

By using this optimization procedure, the separation conditions for a particular analytical task can be selected.

REFERENCES

1 M. Gazdag, G. Szepesi and L. Huszár, *J. Chromatogr.*, 351 (1986) 128.
2 M. Gazdag, G. Szepesi and L. Huszár, *J. Chromatogr.*, 371 (1986) 227.
3 D. W. Armstrong and W. DeMond, *J. Chromatogr. Sci.*, 2 (1984) 411.
4 D. W. Armstrong, A. Alak, K. Bui, W. DeMond, T. Word, T. E. Richl and W. E. Hinze, *J. Inclusion Phenom.*, 2 (1984) 533.

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DERIVATIZATION OF SOME POLYMERS WITH *o*-PHTHALALDEHYDE PRIOR TO HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

The well known OPA (*o*-phthalaldehyde) derivatization commonly used in aqueous media has been modified in order to be able to carry out the reaction with various polymeric materials in tetrahydrofuran, a solvent very often used in size-exclusion chromatography. The modified OPA reaction leads to stable fluorescent derivatives of the investigated analytes. The derivatization procedure has three important advantages: (i) the selectivity of the analytical method is greatly improved since only compounds having a primary amino group are detected; (ii) the sensitivity is increased by at least two orders of magnitude compared to ultraviolet or refractive index detection; (iii) the chromatography is facilitated due to a conversion of the primary amino groups.

INTRODUCTION

High-performance size-exclusion chromatography (HPSEC) is a powerful technique whose use for the analysis of polymer samples has increased considerably during the past years. Nowadays, many types of columns for SEC are commercially available. Several reviews of the theory and applicability of this technique have appeared[1,2].

A disadvantage of SEC is that the peak capacity is relatively small since all compounds are eluted between the exclusion volume and the total permeation volume. This disadvantage becomes more important with increasing complexity of the sample. One solution of this problem is the enhancement of selectivity, *i.e.*, only part of the sample of interest is detected. This can be done by the use of more selective detectors, *e.g.*, a fluorescence detector. Much selectivity can be gained by the use of functional group selective derivatization techniques used either in the pre-column or in the post-column mode[3,4]. However, the use of derivatization techniques for poly-

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mers in combination with HPSEC has not yet been reported. Some derivatization techniques for polymers used in combination with methods other than HPSEC (UV–VIS and fluorescence spectrometry and potentiometric titration) have been described[5–8]. Almost all derivatizations for use with liquid chromatography are carried out in water-containing media. However, water is not compatible with many polymers and with the packing material of most HPSEC columns.

The present paper describes a pre-column derivatization method with *o*-phthalaldehyde (OPA) for polymer samples of molecular weight up to about 10 000. OPA reacts with compounds containing primary amino groups yielding fluorescent derivatives. The conventional reaction conditions were changed so that the reaction could be carried out in tetrahydrofuran (THF) without the necessity of a highly concentrated borate buffer. Optimum derivatization provides information on the presence of primary amino groups in polymers. This information is important since the physical and chemical properties of the polymer are related to the presence of these groups.

EXPERIMENTAL

Chemicals

Tetrahydrofuran (J. T. Baker, Deventer, The Netherlands) filtered over a 1.0-μm membrane filter (TF 1000; Gelman Sciences, Ann Arbor, MI, U.S.A.) was used as the mobile phase. For the derivatization, two solutions were prepared; one was THF (J. T. Baker) containing $1.33 \cdot 10^{-2}$ *M* *o*-phthalaldehyde (Merck, Darmstadt, F.R.G.), the other THF containing $4 \cdot 10^{-2}$ *M* 2-mercaptoethanol (2-ME; Janssen, Beerse, Belgium). Armeen HT ($C_{18}H_{37}NH_2$), Armeen 2HT [$(C_{18}H_{37})_2NH$], Armeen 3HT[$(C_{18}H_{37})_3N$] and Armeen M2HT[$(C_{18}H_{37})_2N$–CH_3] were used as simple, relatively short chain, well defined test analytes. Besides the amino group, only alkyl chains are present.

Versamid 100, 115 and 125 are polyamino acids based on diethylenetriamine (DETA) and dimerized diacids derived from dehydrated fatty acids. Versamid 140 is made from triethylenetetramine instead of DETA. The Versamids are used as a raw material in the coatings industry. The molecular weight of these resins is roughly between 1000 and 10 000 daltons. The versamids were used as model resins. All Armeen and Versamid samples were supplied by AKZO (Arnhem, The Netherlands).

Apparatus

The system for SEC was built from a reciprocating dual-piston pump (M-6000; Waters, Milford, MA, U.S.A.), a laboratory-built six-way injection valve, equipped with a 50-μl loop and a 600 mm × 7.5 mm separation column (PL-Gel, 500 Å, d_p = 5 μm; Polymer Labs., Church Stretton, U.K.). A low-dead-volume filter (Waters) was placed between the injector and the column in order to protect the latter from contamination. Detection was performed with a refractive index detector (R 401; Waters), a variable wavelength UV detector (LC 3 UV; Pye Unicam, Cambridge, U.K.), a fluorescence detector (204A; Perkin-Elmer, Norwalk, CT, U.S.A.) or a PU 4021 multichannel diode-array UV–VIS detector with a PU 4850 video control centre (Philips Analytical/Pye Unicam, Cambridge, U.K.). Sometimes, two detectors were used in series. Chromatograms were recorded with a two-channel strip-chart recorder (BD-9; Kipp and Zonen, Delft, The Netherlands).

Derivatization procedure

All solutions were made in THF and were prepared daily. A 750-μl volume of $1.33 \cdot 10^{-2}$ *M* OPA solution was mixed with 250 μl of $4 \cdot 10^{-2}$ *M* 2-ME solution and 1 ml of the analyte dissolved in THF was added. Then the tube was shaken for 10 s followed by either immediate injection of the contents on the SEC column or storage for a certain reaction time before injection. Reactions at 50°C were carried out using a thermostatted water-bath. In those cases, all solutions were preconditioned at 50°C prior to the reaction.

RESULTS AND DISCUSSION

Column characteristics

Polystyrene standards were used to determine the exclusion volume and the internal pore volume of the column. These were found to be 8.8 and 8.3 ml, respectively. The plate number was determined using toluene as the test solute and was found to be 40 000. During a 6-month period, this value decreased to approximately 30 000.

Kinetics of OPA derivatization

Armeen HT was taken as the test compound for the investigation of the reaction kinetics. The derivatization was carried out as described in the Experimental section. The Armeen HT concentration in the stock solution, *i.e.*, before addition of reagents, was 10^{-2} g/l. Detection took place by measuring the fluorescence intensity

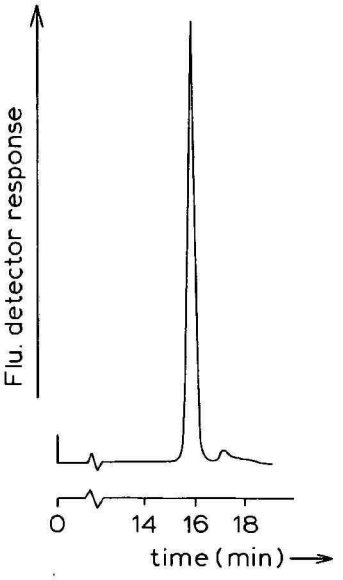

Fig. 1. Chromatogram of Armeen HT after pre-column derivatization with OPA. Conditions: flow-rate of mobile phase (THF), 0.8 ml/min; column, 600 mm × 7.5 mm I.D. packed with PL-Gel, 500 Å, d_p = 5 μm; fluorescence detection, 340 (excitation) and 420 (emission) nm; injection volume, 50 μl; Armeen HT sample (10^{-2} g/l) derivatized with OPA (*cf.*, text); reaction time, 100 min at 50°C.

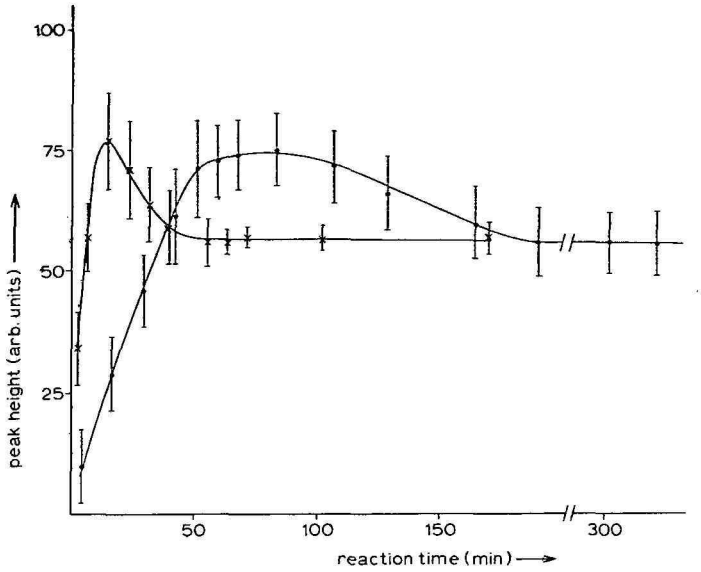

Fig. 2. Height of the Armeen HT peak after pre-column derivatization with OPA as a function of the reaction time at 20°C (●) and at 50°C (×). For derivatization, see text. Conditions as in Fig. 1. The bars indicate the range of the measurements.

using an excitation wavelength of 340 nm. The emission maximum was found to be 420 nm, which is 35 nm lower than that of OPA derivatives in aqueous media.

One peak was observed after injection on the SEC column (Fig. 1) at a retention time of 15.7 min. The height of this peak was measured as a function of the reaction time at 20 and at 50°C. Results are shown in Fig. 2. The bars in Fig. 2 indicate the range of the measurements. At both temperatures, a rapid increase in peak height as a function of the reaction time is observed, followed by a slower decrease towards a final plateau. The level of the plateau at both temperatures is the same. However, simply as a result of faster kinetics at higher temperature, the plateau is achieved after 60 min at 50°C, and only after *ca.* 200 min at 20°C. In addition, the repeatability based on peak-height measurements was better after reaction times of 60 min and longer at 50°C than at any time at 20°C.

A reaction time of 60 min at 50°C was chosen in the standard procedure for OPA derivatization. In all chromatograms of OPA-derivatized Armeen HT, only one analyte peak was observed having a retention time and peak shape that was independent of reaction time and temperature. We thus assumed that only one fluorescent Armeen HT-OPA derivative with the excitation and emission wavelengths mentioned before is formed. An observed in support of this suggestion was that, with a multichannel diode-array UV–VIS detector, identical spectra (190–390 nm) were obtained for the derivative peak independent of the reaction time and temperature. Since the sensitivity of this diode-array detection was worse than that of the fluorescence detector, a sample solution with a ten times higher concentration had to be used.

Application of OPA derivatization to several analytes

Armeen. Armeen samples were derivatized using the method described above. Armeen HT yielded the chromatogram as shown in Fig. 1. Derivatization of an Armeen 2HT sample yielded a small peak at the retention time of the OPA derivative of Armeen HT. This is due to small amounts of Armeen HT that are present in Armeen 2HT. Armeen 2HT itself, Armeen 3HT and Armeen M2HT did not yield fluorescent derivatives, as expected because of the absence of primary amino groups. The present OPA derivatization method offers an attractive possibility for the determination of the Armeen HT content in other Armeen samples.

Versamid. The method was also used for the analysis of Versamid samples. Chromatograms obtained after pre-column derivatization of Versamid 140, 125, 115 and 100 are shown in Fig. 3. The concentration of Versamid in the sample solutions after derivatization was $5 \cdot 10^{-2}$ g/l. For comparison, chromatograms of underivatized Versamid samples with UV detection at 254 nm are shown in Fig. 4, the sample concentration now being 10 g/l. The chromatograms in Fig. 3 show sharper peaks than the ones in Fig. 4 (note the difference in time scale). This may be a result of the fact that, when using OPA derivatization, only those compounds in the sample having a primary amino group will be detected. Moreover, after the derivatization, the interaction between the primary amino groups of the analytes and the column pack-

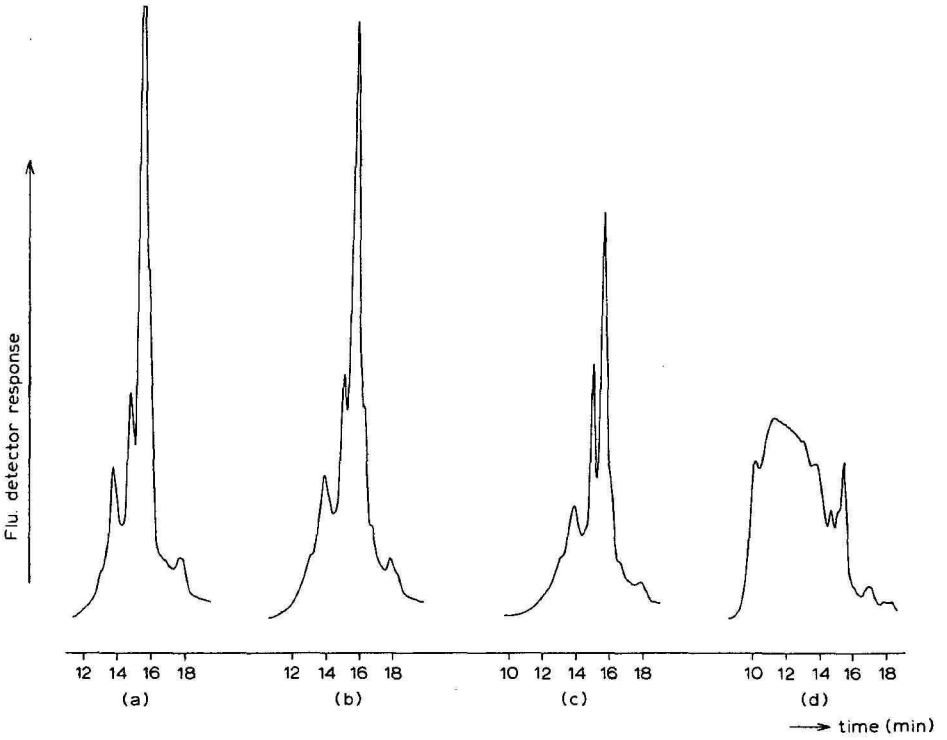

Fig. 3. Chromatograms of Versamid samples after pre-column derivatization with OPA: (a) Versamid 140; (b) 125, (c) 115 and (d) 100. Versamid samples (0.1 g/l) were derivatized with OPA (*cf.*, text); reaction time 60 min at 50°C. All other conditions as in Fig. 1.

ing, *cf.*, the tailing effects in Fig. 4, is suppressed. Versamid 100 which exhibits the highest molecular weight is partly excluded from the pores of the packing. Therefore, its chromatogram obtained after derivatization was found to be of lower quality than that of the other Versamids. Also in the derivatization of Versamid samples with OPA, a plateau is reached after reaction for 60 min at 50°C. The reaction time was varied between 5 and 120 min, and within the range from 50–120 min identical chromatograms were found.

Analytical data

Some relevant analytical data are collected in Table I. For both the Armeen HT and Versamid 125 samples, the repeatability of peak-height measurements after precolumn OPA derivatization was good. With both samples, in addition to the increase in selectivity shown above, a dramatic increase in detectability was observed. The linearity of the response of OPA-derivatized Armeen HT was found to range from the detection limit up to at least a sample concentration (before derivatization) of $3 \cdot 10^{-2}$ g/l ($r = 0.999$, $n = 6$), this being the highest concentration investigated. For OPA-derivatized Versamid 125, the linearity of response ranged from the detection limit up to at least a sample concentration of 1 g/l, the correlation coefficient being 0.997 ($n = 6$). The peak-height measurement was done on the highest peak in the chromatogram.

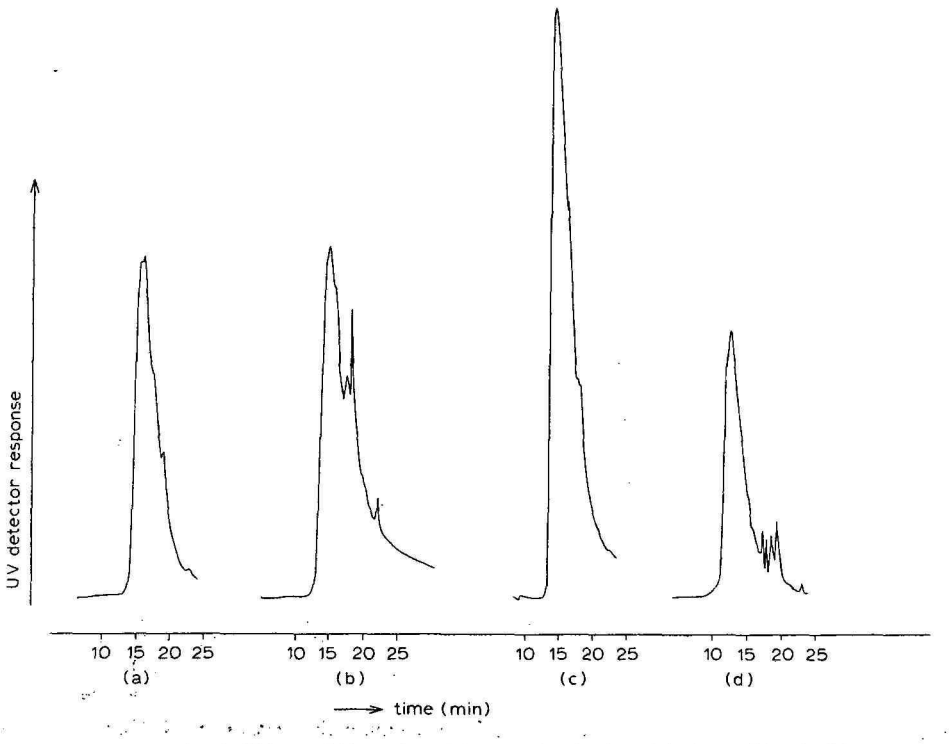

Fig. 4. Chromatograms of Versamid samples without derivatization. (a) Versamid 140, (b) 125, (c) 115 and (d) 100. Sample concentration 10 g/l. UV detection at 254 nm. All other conditions as in Fig. 1.

TABLE I

ANALYTICAL DATA ON ARMEEN AND VERSAMID DERIVATIZATION

Conditions: flow-rate of mobile phase, 0.8 ml/min; column, 600 mm × 7.5 mm I.D., PL-Gel 500 Å, d_p = 5 μm; injection volume, 50 μl; UV detection at 254 nm or RI detection for underivatized sample or fluorescence detection at λ_{ex} = 340 and λ_{em} = 420 nm for derivatized sample (*cf.*, text).

Sample	*Repeatability (% R.S.D., n = 4) of peak height with OPA derivatization*	*Detection limit (S/N = 3)*	
		Without derivatization	*With OPA derivatization*
Armeen HT	1.6	0.1 μmol (RI)	4 pmol
Versamid 125	2.2*	10 μg (UV)	25 ng*

* Based on the highest peak in the chromatogram.

Stability of sample solutions

During the project, it was found that dilute solutions of Versamid in THF were not stable over a long period of time, even when stored in the dark at 4°C. Comparison of chromatograms obtained after OPA derivatization of a fresh Versamid solution (0.1 g/l) and a sample solution of the same concentration which had been stored in the dark at 4°C for 2 days showed that in the latter case the peaks were shifted to longer retention times, *i.e.*, to the lower-molecular-weight section of the chromatogram. This difference was not observed when a concentrated sample solution (10 g/l) was stored in the dark at 4°C for 2 days and was diluted 100-fold prior to derivatization and chromatography. A probable explanation of this phenomenon is that low concentrations of peroxides that are always present in THF react with Versamid resulting in the formation of lower-molecular-weight reaction products. If the sample solution is more concentrated, only a relatively small amount of the sample will be decomposed. The above results suggest that it is best to prepare the sample solution at most several hours before analysis or, alternatively, prepare concentrated solutions that are diluted just prior to analysis.

CONCLUSIONS

This study reveals that pre-column derivatization in organic solution (THF) is a powerful method to increase the applicability of high-performance size-exclusion chromatography for the analysis of polymers. It was shown that with a slight modification of the OPA reagent, *i.e.*, using THF instead of water and adding no borate buffer, amino-group-containing polymers can be derivatized yielding stable fluorescent derivatives. The method is extremely simple and leads to very reproducible results. The increase in selectivity of the method, in combination with a decrease in unwanted interactions between the analytes and the column packing material, results in chromatograms with an increased information content. Though less important than the increase in selectivity, it is noteworthy that the sensitivity of the method is increased by at least two orders of magnitude. The method may well be useful for the quality control of high-molecular-weight compounds, *i.e.*, the determination of the Armeen HT content in crude Armeen 2HT, and for production process control.

Future work will concentrate on the applicability of other reagents for the selective derivatization of other functional groups in polymers and on the use of other (organic) solvents as derivatization media.

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REFERENCES

1 D. D. Bly, in B. Carrol (Editor), *Physical Methods in Macromolecular Chemistry*, Vol. 2, Marcel Dekker, New York, 1972, Ch. 1.
2 W. W. Yau, J. J. Kirkland and D. D. Bly, *Modern Size-Exclusion Chromatography*, Wiley, New York, 1979.
3 I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986.
4 R. W. Frei and J. F. Lawrence (Editors), *Chemical Derivatization in Analytical Chemistry*, Vol. 1, Plenum, New York, 1981, Chs. 3 and 4.
5 K. Schmidt and K. Geckeler, *Anal. Chim. Acta*, 71 (1974) 79–84.
6 L. K. Tan and R. A. Patsiga, *Anal. Lett.*, 10 (1977) 437–449.
7 G. D. B. van Houwelingen, J. G. M. Aalbers and A. J. de Hoog, *Fresenius' Z. Anal. Chem.*, 300 (1980) 112–120.
8 Y. Eckstein and P. Dreyfuss, *Anal Chem.*, 52 (1980) 537–541.

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HIGH-RESOLUTION GAS CHROMATOGRAPHY WITH LIQUID CRYSTAL GLASS CAPILLARIES

X*. SEPARATION OF DIASTEREOMERIC ALKANES

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SUMMARY

The separation of diastereomeric C_8–C_{20} alkanes using mesogenic stationary phases has been studied. A comparison of the retentions of the diastereomers on mesogenic and non-mesogenic phases showed the effect of the mesophase diastereoselectivity. Examples of the separation of some diastereomeric C_9–C_{10} alkanes indicated that liquid crystals may be useful stationary phases in the capillary gas chromatographic separation of diastereomeric compounds.

INTRODUCTION

Liquid crystals as stationary phases in gas chromatography possess shape-selective separation properties for the structural and geometrical isomers of hydrocarbons[1]. The contribution of this effect to the resolution of isomers with small differences in molecular geometry is relative low. For example, this stereospecifity contribution is not more than 3% of the full retention for structural isomers of *n*-dodecenes with a central double bond separated on liquid crystals. Hence the utilization of this effect for separations is possible only in combination with the high separation efficiency of capillary columns.

Separation systems with mesogenic stationary phases permit faster and better separations, mainly of structural isomers, than systems based on non-mesogenic stationary phases. The reason is not only that the mesogenic selectivity for the structural isomers increases with a shift of the functional group towards the end of the carbon chain, but also that the elution order follows the same trend. In a group of geo-

* For Part IX, see ref. 7.

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metrical isomers, *e.g.*, *cis*- and *trans*-*n*-alkenes, mesogenic stationary phases are more selective for *trans*-isomers. Because the elution order of *cis*- and *trans*-isomers of *n*-alkenes is altered with the position of the double bond and the number of carbon atoms, the separation of geometrical isomers on mesogenic stationary phases may not only be easier but may also be more difficult than on non-mesogenic stationary phases. If the retention order on a non-mesogenic stationary phase for geometrical isomers is the *trans*- after *cis*-isomer (or they coelute), their separation on a mesogenic stationary phase will be better.

In this work, the separation of diastereomeric compounds using mesogenic stationary phases was investigated with diastereoisomeric alkanes as model compounds. The results of previous studies carried out on non-mesogenic polar and non-polar stationary phases showed that some diastereomeric alkanes are not separated[2–6]. A comparison of the retention data of sixteen diastereomeric alkanes up to C_{10} measured on squalane and ucon LB-550-X showed a difference in the retention indices of less than 1 index unit for ten alkanes[5]. The object of this study was the separation of diastereomeric alkanes with very similar physico-chemical properties by capillary gas chromatography using liquid crystals as the stationary phase.

EXPERIMENTAL

Model mixtures of diastereomeric C_8–C_{10} alkanes were prepared from the individual C_7–C_9 alkanes using the methylene insertion reaction (MIR)[2], which leads to multi-component mixtures with a statistical distribution of isomers, depending on the individual C–H bonds in the starting alkanes. The prepared mixtures included fourteen out of sixteen possible diastereomeric alkanes up to C_{10}, with the absence of 2,2,3,4-tetramethylhexane and 3-ethyl-2,4-dimethylhexane diastereomers because of the lack of starting alkanes. Isoprenoid diastereomeric C_{18}–C_{20} alkanes were obtained as a distillation cut from petroleum.

The gas chromatographic measurements on model mixtures were carried out with a Perkin Elmer F-11 gas chromatograph with a flame ionization detector. For the separation of diastereomeric C_8–C_{10} alkanes a glass capillary column (100 m × 0.25 mm I.D.) coated with 4-*n*-pentylacetophenone-O-(4-*n*-pentyloxybenzoyloxime) (PBO) liquid crystal was used. The efficiency of this column was 290 000 theoretical and 170 000 effective plates for *n*-nonane with a capacity ratio of $k = 3.3$ at 40°C and a linear velocity of hydrogen of 0.26 m s^{-1}. Isoprenoid diastereomeric C_{18}–C_{20} alkanes were separated in a glass capillary column (100 m × 0.25 mm I.D.) coated with methoxythoxyazoxybenzene (MEAB) liquid crystal with an efficiency of 200 000 plates and in a column (200 m × 0.25 mm I.D.) coated with Apolan 87 with an efficiency of 500 000 plates.

RESULTS AND DISCUSSION

For the separation of the diastereomeric C_8–C_{10} alkanes PBO liquid crystal was chosen as it gave good separations of isomeric C_9–C_{11} *n*-alkenes[7]. This system has a suitable temperature range of the nematic mesophase (63–94°C with undercooling up to 27°C). The lower column temperature and the higher film thickness of the stationary phase are necessary to increase the sorption of the relatively low-boil-

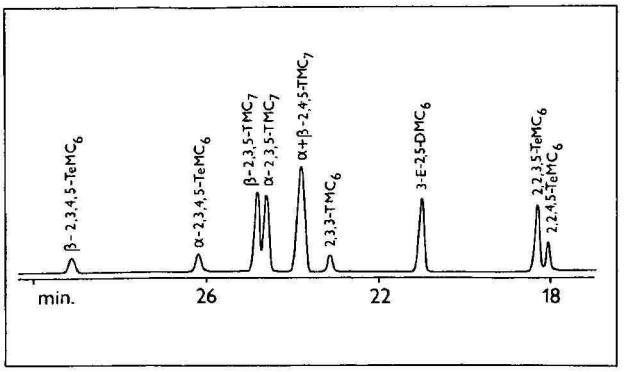


Fig. 1. Chromatogram of methylene-inserted 2,3,5-trimethylhexane on PBO at 40°C. $EDMC_6$ = Ethyldimethylhexane; TMC_6 = trimethylhexane; TMC_7 = trimethylheptane; $TeMC_6$ = tetramethylhexane; α- and β-diastereomers.

TABLE I

RETENTION INDICES, THEIR TEMPERATURE COEFFICIENTS AND SELECTIVITY FACTORS FOR C_8–C_{10} DIASTEREOMERIC ALKANES ON PBO AT 40°C

Alkane	I_{40}^{PBO}	dI^{PBO}/dT	α_{40}^{PBO}
3,4-Dimethylhexane	758.1	0.25	1.000
	758.1	0.25	
3,5-Dimethylheptane	811.9	0.20	1.014
	813.4	0.18	
2,3,4-Trimethylhexane	828.5	0.33	1.043
	832.9	0.33	
3,4-Dimethylheptane	840.7	0.25	1.009
	841.9	0.24	
2,4,5-Trimethylheptane	880.7	0.24	1.002
	881.0	0.24	
2,3,5-Trimethylheptane	885.6	0.22	1.010
	886.9	0.23	
3,5-Dimethyloctane	894.3	0.21	1.046
	898.8	0.21	
2,3,4,5-Tetramethylhexane	894.3	0.38	1.146
	907.0	0.40	
2,3,4-Trimethylheptane	909.3	0.30	1.027
	912.1	0.32	
3-Methyl-4-ethylheptane	911.0	0.30	1.006
	911.6	0.29	
3,6-Dimethyloctane	913.1	0.19	1.007
	914.2	0.17	
3,4-Dimethyloctane	920.8	0.25	1.038
	924.7	0.21	
3,4,5-Trimethylheptane*	923.0	0.27	1.047
	927.9	0.24	
4,5-Dimethyloctane	931.3	0.25	1.016
	932.9	0.22	

* The retention index of the third peak is 924.6, $dI/dT = 0.27$.

ing diastereomeric C_8–C_{10} alkanes. The more selective mesophase MEAB[1] could not be used because its mesomorphic temperature range was too high.

A typical chromatogram of the separation of MIR products on PBO is shown in Fig. 1. The peaks were identified using the calculated composition of reaction products and their statistical distribution, published retention indices on squalane[5] and correlations between the retention indices of model complex alkanes up to C_{10} measured on squalane and liquid crystal stationary phases[8]. In accordance with previous studies[2–6], for all the diastereoisomers we considered only two peaks in each instance investigated. Some of these diastereostructures of alkanes were discussed in detail by Schomburg[3].

The measured retention indices, I, their temperature coefficients, dI/dT, and the selectivity factors, α, of the diastereomeric C_8–C_{10} alkanes on PBO are given in Table I.

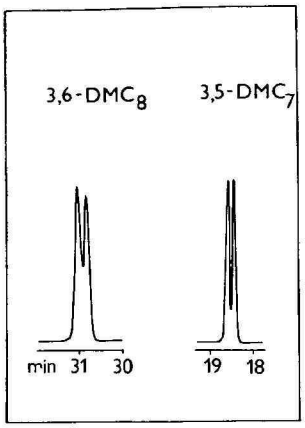
The stereoselectivity effect of the stationary phase on the separation of diastereomeric alkanes was examined by comparing the retention index differences, δI, measured on squalane[5], Ucon LB-550-X[5], silicones (similar to SE-30 and SE-54)[6] and PBO (Table II). We had to use the δI values for this purpose as the selectivity factors, α, of the diastereomeric C_8–C_{10} alkanes on non-mesogenic stationary phases are unknown and the δI values on non-mesogenic stationary phases of various polarity are very similar. This demonstrates that the polarity of the stationary phase has only a small influence on the separation of diastereoisomers. The selectivity of silicone stationary phases was found to be slightly higher than that of the other non-mesogenic stationary phases.

The δI values of diastereomeric C_8–C_{10} alkanes on PBO are mostly higher than those of non-mesogenic stationary phases; ten of the fourteen investigated pairs of isomers were found to have higher δI values than those measured on non-mesogenic stationary phases. The only poorer separation was that of 2,4,5-trimethylhep-

TABLE II

DIFFERENCES IN RETENTION INDICES OF C_8–C_{10} DIASTEREOMERIC ALKANES ON PBO, SQUALANE, UCON LB-550-X AND SE AT 40°C

Alkane	δI			
	PBO	*Squalane*	*Ucon*	*SE*
3,4-Dimethylhexane	0.0	0.0	0.0	0.3
2,4,5-Trimethylheptane	0.3	1.4	1.0	–
3-Methyl-4-ethylheptane	0.6	0.5	0.0	–
3,6-Dimethyloctane	1.1	0.0	0.0	0.8
3,4-Dimethylheptane	1.2	1.6	1.2	0.8
2,3,5-Trimethylheptane	1.2	0.6	0.1	–
3,5-Dimethylheptane	1.5	0.0	0.0	1.1
4,5-Dimethyloctane	1.6	2.0	2.1	–
2,3,4-Trimethylheptane	2.8	0.3	0.4	1.7
3,4-Dimethyloctane	3.9	0.8	2.2	–
2,3,4-Trimethylhexane	4.4	3.1	2.6	–
3,5-Dimethyloctane	4.5	1.5	1.6	2.2
3,4,5-Trimethylheptane	4.9	0.3	0.5	1.1
2,3,4,5-Tetramethylhexane	12.7	10.2	14.0	–



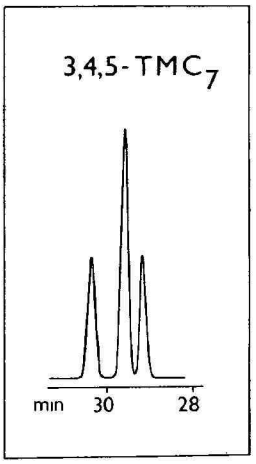


Fig. 2. Separation of diastereomers of 3,5-dimethylheptane and 3,6-dimethyloctane on PBO at 40°C.

Fig. 3. Separation of 3,4,5-trimethylheptane diastereomers on PBO at 40°C.

tane with a δI value of 0.3 i.u. on PBO compared with 1.4 i.u. on squalane and 1.0 i.u. on Ucon. The higher selectivity of the mesophase used and the higher resolution of the separation system is demonstrated on the separation of 3,5-dimethylheptane and 3,6-dimethyloctane diastereomers (Fig. 2). Their separation is one of the most complicated problems on non-mesogenic stationary phases.

We found three peaks in the range where the elution of three possible 3,4,5-trimethylheptane diastereomers[9] is assumed to occur (Fig. 3). In all instances the diastereomeric C_8–C_{10} alkanes had a quantitative ratio of 1:1, but for 3,4,5-trimethylheptane this applied only to the first and third peaks, which are given as the diastereomers in Tables I and II. We did not have any other suitable means of confirming the identification of all three 3,4,5-trimethylheptane diastereoisomers.

The separation of 3,4-dimethylhexane diastereomers failed both on non-mesogenic phases and on PBO. This may be due to the low sorption of these isomers on the PBO column, their capacity ratio at 40°C being only $k = 0.86$ and the effective efficiency 45000 plates. Lubeck and Sutton[6] obtained the same retention index at 60°C, but different dI/dT values for 3,4-dimethylhexane diastereomers measured on silicone stationary phases. The δI values calculated for these diastereomers from their retention indices and dI/dT values show a difference $\delta I = 0.3$ i.u. at 40°C. Desty[10] resolved 3,4-dimethylhexane into its diastereoisomers on silicone oil at 18°C.

There is an interesting group of diastereomeric isoprenoids (norpristane, pristane and phytane) in the range of C_{18}–C_{20} alkanes[11–14]. Their separation on non-mesogenic stationary phases requires 390000–600000 plates[12]. We studied their separation with the mesogenic stationary phase MEAB and the non-mesogenic Apolan 87. The measured α values of diastereomeric isoprenoids on the MEAB are lower than those on non-mesogenic phases (Table III). The highest selectivity for diastereomeric norpristane, pristane and phytane was shown by the non-polar stationary phase Apolan 87; the selectivity factor increases with decrease in column temperature.

The differences in the separation of diastereomeric C_8–C_{10} and C_{18}–C_{20} alkanes on mesogenic and non-mesogenic stationary phases are connected with the different elution orders of individual stereoisomers (similarly to geometrical isomers).

TABLE III

SELECTIVITY FACTORS, α, OF THE DIASTEREOISOMERS OF NORPRISTANE, PRISTANE AND PHYTANE ON VARIOUS STATIONARY PHASES

Stationary phase	*Norpristane*	*Pristane*	*Phytane**
Apolan 87, 140°C	1.0062	1.0076	1.0097 (1.0116)
OV-101[13]	–	–	– (1.0112)
Carbowax 20M[13]	–	–	– (1.0095)
1,4-Butanediol succinate[14]	–	–	– (1.0082)
Methoxyethoxyazoxybenzene (MEAB), 90°C	1.0043	1.0080	1.0107 (1.0080)

* Values in parentheses at 120°C.

There are some differences in the length-to-breadth ratio of molecules of the individual members of the diastereoisomeric pairs. One of the isomers is (usually) "elongated" in comparison to the other. This "elongated" isomer has the higher retention on mesogenic stationary phases because of their shape selectivity. And so the separation of that stereoisomeric pair on a mesogenic stationary phase depends on the retention order of these isomers on a non-mesogenic stationary phase. If the "elongated" isomer is eluted first on a non-mesogenic phase, the separation of that isomeric pair on a mesogenic phase is worse; the reverse of the retention order is less probable. And if this "elongated" isomer is eluted second, (or if both isomers are coeluted) the separation of that isomeric pair on a mesogenic stationary phase is better. Published data[3,11] indicate that for alkanes up to C_{10} with "meso" structures (3,5-dimethylheptane, 3,5-dimethyloctane) have lower retentions than those with a "racemic" form. For the isoprenoid C_{18}–C_{20} alkanes, the "meso" diastereomers (pristane) have higher retentions than the "racemic" isomers. The higher α values of the diastereomeric alkanes up to C_{10} and the lower α values of the diastereomeric C_{18}–C_{20} isoprenoids on mesogenic stationary phases indicate that, in these cases, the liquid crystals selectively retain the "racemic" form of the diastereomers.

REFERENCES

1 L. Soják, G. Kraus, P. Farkaš and I. Ostrovský, *J. Chromatogr.*, 294 (1984) 155.
2 M. C. Simmons, D. B. Richardson and I. Dvoretzky, in R. P. W. Scott (Editor), *Gas Chromatography 1960*, Butterworths, London, 1960, p. 211.
3 G. Schomburg, *Anal. Chim. Acta*, 38 (1967) 45.
4 D. A. Tourres, *J. Chromatogr.*, 30 (1967) 357.
5 A. Matukuma, in C. L. A. Harbourn (Editor), *Gas Chromatography 1968*, Institute of Petroleum, London, 1969, p. 55.
6 A. J. Lubeck and D. L. Sutton, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 328.
7 L. Soják, I. Ostrovský, P. Farkaš and J. Janák, *J. Chromatogr.*, 356 (1986) 105.
8 J. Hrouzek, *Thesis*, Comenius University, Bratislava, 1984.
9 P. Lachance, S. Brownstein and A. M. Eastham, *Can. J. Chem.*, 57 (1979) 367.
10 D. H. Desty, in C. L. A. Harbourn (Editor), *Gas Chromatography 1968*, Institute of Petroleum, London, 1969, p. 73.
11 J. R. Maxwell, R. E. Cox, R. G. Ackman and S. N. Hooper, *Advances in Organic Geochemistry 1971*, Pergamon Press, Oxford, 1972, p. 277.
12 F. Berthou and M. P. Friocourt, *J. Chromatogr.*, 219 (1981) 393.
13 H. Borwitzky and G. Schomburg, *J. Chromatogr.*, 240 (1982) 307.
14 A. F. Shlyakhov, *Gas Chromatography in Organic Geochemistry*, Nedra, Moscow, 1984, p. 74 (in Russian).

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CHROM. 20 141

REQUIREMENTS OF MUTUALLY MISCIBLE LIQUIDS FOR TERNARY CHROMATOGRAPHIC SOLVENTS WITH REFERENCE TO MONOSACCHARIDE PAPER CHROMATOGRAPHY

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SUMMARY

Ascending paper chromatography of three monosaccharides is re-examined using ternary solvent mixtures. These solvents contain water together with a "water-immiscible" organic solvent (*n*-butanol, ethyl acetate or cyclohexanol) made into a monophase with one of four mutually miscible liquids (MMLs). The requirements of such MMLs are defined as a consequence.

INTRODUCTION

Sugars were one of the first types of solutes to be examined by paper chromatography (PC)[1]. A recent review[2] comments that, for sugar PC "monophasic solvent systems came into use (replacing biphasics). They consist of water, a water-miscible and a water-immiscible organic solvent. In such systems the mobility of the sugars, which increases with the water content, is generally the same; namely pentoses (followed by) hexoses ...". The term water-immiscible is imprecise for solvents like butanol, and an important point is that the water-miscible organic should *also* be miscible with the water-immiscible solvent. A better term for it is mutually miscible liquid (MML). Jermyn and Isherwood[3] realised this as long ago as 1949 when they used "three-component systems so that the R_F value(s) can be adjusted ... usually two of the components are immiscible whereas the third is miscible with either of the other two ... a third component which is soluble in both phases" of the biphasic mixture.

Glacial acetic acid was the first MML, used by Partridge[1] for the PC of sugars in 1948 to increase the amount of water which would dissolve in the *n*-butanolic layer of the biphasic mix examined. Robards and Whitelaw[2] comment that "the temperature dependence of the composition of such (biphasic) systems may result in phase separation during use". Betts[4] has pointed out that the problem is more complex; and also biphasic mixtures are unscientific as the mobile phase used is of unknown composition. With the Partridge[1] solvent mixture, phase separation of the separated upper layer definitely occurs in time due to the formation of a fourth component, butyl acetate[4].

A reason for preferring PC over thin-layer chromatography for the laminar

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TABLE I

R_F VALUES OF THREE SUGARS OBTAINED BY ASCENDING PAPER CHROMATOGRAPHY USING SOLVENT MIXTURES OF ONE PART WATER WITH INCOMPLETELY MISCIBLE ORGANIC LIQUIDS, MADE INTO MONOPHASIC TERNARY MIXTURES BY ADDITION OF VARIOUS MML

R, *A* and *G* represent R_F values of rhamnose, arabinose and galactose, respectively. The paper used was Whatman 1 Chr.

Parts (v/v) of organic liquid mixed with one part water	*Volume of MML added to give ternary monophase*		R_F			*Separation 100(R−G)/R*	*Separation 100(A−G)/A*	*Comment*
			Rhamnose	*Arabinose*	*Galactose*			
n-Butanol 3	Ethanol	0.67	0.41	0.26	0.20	51	23	Good
n-Butanol 2	Ethanol	0.67	0.52	0.37	0.34	35	8	*A*/*G* too close
n-Butanol 3	Pyridine	0.67	0.43	0.22	0.15	65	32	Good
n-Butanol 2	Pyridine	0.75	0.52	0.33	0.27	48	18	Good
n-Butanol 2	Acetone	0.50	0.42	0.30 *(ca.)*	0.26	38	13?	Unsatisfactory
n-Butanol 3	Formamide	0.50	0.54	0.43	0.34	37	21	Frontal analysis of solvent
Ethyl acetate 3	Ethanol	1.25	0.50	0.34	0.26	48	23	Good
Ethyl acetate 2	Ethanol	1.00	0.61	0.48	0.40	34	17	Good
Ethyl acetate 3	Pyridine	1.25	0.48	0.29	0.21	56	28	Good
Ethyl acetate 2	Pyridine	1.00	0.62 *(ca.)*	0.46 *(ca.)*	0.38 *(ca.)*	40?	17?	Erratic results
Ethyl acetate 2	Acetone	2.50	Not used, excessive MML required					
Ethyl acetate	Formamide		Does not form monophase in desired proportions					
Cyclohexanol 3	Ethanol	1.00	0.41	0.24	0.18	56	25	Streaky chromatogram
Cyclohexanol 2	Ethanol	1.00	0.48	0.33	0.27	44	18	Streaky chromatogram
Cyclohexanol 3	Pyridine	2.50	Not used, excessive MML required					
Cyclohexanol 2	Pyridine	1.67	0.58	0.40	0.37	36	7	*A*/*G* too close
Cyclohexanol 2	Acetone	1.00	0.45 *(ca.)*	0.18 *(ca.)*	0.17 *(ca.)*	62?	5?	*A*/*G* too close, erratic
Cyclohexanol	Formamide		Does not form monophase in desired proportions					

study of sugars is that "when sugars are chromatographed on silica gel G with ammoniacal solvents ... aminated sugars are formed ... resulting in hexoses (and) pentoses ... being split into at least two different compounds. Silica gel apparently exerts a catalytic effect ..."[2]. These reviewers note that "the PC of carbohydrates is (still being) actively pursued", even as recently as 1985.[5] The Centennial Edition of the *Official Methods of Analysis* AOAC (1984) uses a paper chromatographic method for Glucose (commercial) in honey (Method 31.152). Yet the properties of MML remain to be defined.

Many ternary systems have been previously applied for sugar PC and used by successive workers. Aqueous ethyl acetate has been used with acetic acid as MML and also with pyridine[3], but the latter is toxic and foul-smelling.

Aqueous *n*-butanol has been used with pyridine as MML[6], with ethanol[7] and of course with acetic acid[1] which is reactive with it.

There is no scientific justification for using quaternary monophasic mixtures such as aqueous isopronanol with the *two* MMLs pyridine and acetic acid together[8], an obviously reactive mixture. This bad practice was continued in 1974 when Jarvis and Duncan[9] noted "paper chromatography (of sugars) is still useful for applications in which flexibility and low cost are important". They used quaternary mixes of aqueous ethyl acetate they claimed were "buffered with pyridine–acetic acid". Any necessary buffering (to counteract the pyridine or the acetic acid as sole MML?) could have been achieved with inorganic buffers. In fact, they did use some ternary mixes with only one of these MMLs which were obviously unbuffered. They used "a less polar solvent (how, with acetic acid there?) than usual and running for longer periods" by descending technique. Any changes obtained in sugar R_F values were more likely to be due to changes in ethyl acetate content (from 66 to 49%) than to the proportions of the two MMLs ("the acidity or otherwise of the solvent"). One of their figures, in fact, suggested this.

In this work, attempts are made to evaluate some MMLs from PC results for three sugars, using solvent mixtures of water with three "water-immiscible" organics made into ternary monophases by four different MMLs.

EXPERIMENTAL

The paper used was Whatman 1 Chr, 20 × 20 cm sheets. Ascending chromatography was performed in cylindrical glass tanks. The solvents were normal laboratory grade, from various suppliers. The spray reagent consisted of phthalic acid (Hopkin and Williams) 1.66 g, aniline (Ajax) 0.9 ml, water 10 ml and *n*-butanol (Mallinckrodt) to 100 ml. After spraying, the chromatogram was heated at 100°C for a few minutes to give brown spots, which could be seen (if faint) as blue fluorescent areas under UV light at 366 nm. The sugars used were L(+)-arabinose, D(+)-galactose and L(+)-rhamnose (BDH), spotted from water solutions.

RESULTS AND DISCUSSION

Using water as a pure solvent for PC of monosaccharides results in them all moving to the solvent front as the cellulose is unable to retain any of them selectively. What is needed theoretically is a distribution of the sugars on either side of R_F 0.5,

as this should give the maximum opportunity for resolution of their mixtures. This can be achieved by diluting water with a low polarity solvent, for which an MML is needed. Jermyn and Isherwood[3] realised that sugar "R_F values were directly dependent upon the molar fraction of water".

Table I indicates that as the solvent mixtures are altered to raise the R_F of rhamnose above 0.5 by increasing the proportion of water, the slower moving sugars are pushed closer to it. Thus it appears undesirable to produce a solvent mix which would yield an R_F for rhamnose much above 0.6, *i.e.* rich in water. In fact a mixture of equal parts water and *n*-butanol, with ethanol as MML, gives virtually the same results as the 1:2 mix, so there is likely to be a limiting upper set of R_F values for each ternary mixture.

Ethanol is one of the best MMLs, as usually no more of it is required for a solvent monophase than of any other MML examined. Acetone tends to give unstable mixtures which yield streaky chromatograms, and the toxicity and smell of pyridine are great disadvantages. The latter has probably had a history of use for sugar PC because it is one of the few organic liquids which dissolves sugars. Pyridine as MML gives lower R_F values for arabinose and galactose than does ethanol. Formamide is not a good MML, as it is not fully miscible with ethyl acetate or cyclohexanol, although it can be used with *n*-butanol, but then solvent frontal analysis occurs, and residual formamide interferes with the spot detection.

For the monosaccharides studied L-rhamnose (a desoxy hexose) was the fastest running and the hexose D-galactose the slowest. The resolution obtained between these two indicates the efficiency of any ternary solvent mix; and the difference in their R_F values, expressed as a proportion of the R_F for rhamnose, showed this, with largest values being desirable. The R_F values for glucose (not shown in the results) were always slightly higher than those for galactose, indicating the all-equatorial hydroxy group configuration of the former, compared with the axial 4-OH of galactose. The pentoses L-arabinose and D-xylose (results not shown) always appeared in this sequence between rhamnose and glucose.

CONCLUSIONS

For the three "water-immiscible" organic solvents studied, ethanol and pyridine are the best MMLs, and are most likely to give satisfactory chromatograms. These two MMLs give somewhat different R_F values for the sugars examined, which increase as the proportion of water in the ternary solvent mixture is increased.

The best "separations" of the three sugars studied were obtained with mixtures of water and ethyl acetate or *n*-butanol, made into ternary monophases with the MML ethanol or pyridine. Ethanol is to be preferred, and ethyl acetate yields results quickly.

Consideration of the above leads to definition of the general requirements for MMLs.

An MML in a ternary chromatographic solvent must (i) allow monophasic mixing of two other liquids of differing polarity in proportions which would not be monophasic without it; (ii) be fully miscible with both the other two liquids which show limited miscibility with one another; (iii) be present so that it is not a major component of the solvent mixture, by not being required a higher proportion than

one of the other two liquids of the ternary mixture; (iv) not interact with either of the other two liquids in the solvent mix; (v) not produce a solvent mixture which undergoes obvious frontal analysis during the chromatographic process to yield interfering false fronts on the chromatogram; and (vi) not make the solvent mixture more unpleasant or difficult to handle (*e.g.* in viscosity, toxicity, odour, etc.) than either of the other two liquids of the ternary mixture.

The above requirements are put forward from experience of laminar chromatography, but should apply equally to columnar solvent systems.

REFERENCES

1 S. M. Partridge, *Biochem. J.*, 42 (1948) 238.
2 K. Robards and M. Whitelaw, *J. Chromatogr.*, 373 (1986) 81.
3 M. A. Jermyn and F. A. Isherwood, *Biochem. J.*, 44 (1949) 402.
4 T. J. Betts, *J. Pharm. Sci.*, 53 (1964) 794.
5 P. W. Simon and R. E. Freeman, *Hortscience*, 20 (1985) 133.
6 E. Chargaff, C. Levine and C. J. Green, *J. Biol Chem.*, 175 (1948) 67.
7 K. T. Williams and A. Benevue, *Science (Washington, D.C.)*, 113 (1951) 582.
8 H. T. Gordon, W. Thornburg and L. N. Werum, *Anal. Chem.*, 28 (1956) 849.
9 M. C. Jarvis and H. J. Duncan, *J. Chromatogr.*, 92 (1974) 454.

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ELECTROPHORETIC STUDY OF α-D-GALACTOSIDASES FROM SEEDS OF GLYCINE SOJA AND VIGNA RADIATA POSSESSING ERYTHROAGGLUTINATING ACTIVITY

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SUMMARY

Polyacrylamide gel electrophoresis in an acidic buffer system was used to study the electrophoretic behaviour of two forms of α-D-galactosidase from seeds of soy bean (Glycine soja) and mung bean (Vigna radiata). The interaction of the enzymes with saccharides was monitored by affinity electrophoresis; for the preparation of affinity gels, water-soluble O-glycosyl polyacrylamide copolymers and polysaccharides were used. α-D-Galactosidases from both sources interact with immobilized α-D-galactosyl residues. On the basis of the results of affinity electrophoresis performed in the presence of various free sugars, dissociation constants for the complexes between α-D-galactosidase and free sugars were calculated.

INTRODUCTION

Seeds of Glycine soja and Vigna radiata have been shown to contain two forms of α-D-galactosidase differing in their molecular weights: a tetramer with M_r of about 160 000 and a monomer with M_r of about 40 000[1–4]. The tetrameric forms of the enzyme from both sources display, in addition to enzymatic activity, the ability to agglutinate trypsinized rabbit erythrocytes under defined conditions. The agglutination is inhibited by competitive inhibitors of the enzymatic activity[2–5]. Seeds of soy bean contain, besides two forms of α-D-galactosidase, also other protein capable of interacting with D-galactose: classical lectin, which can easily be separated from α-D-galactosidase by affinity chromatography[6].

In our previous communication[7] we reported the electrophoretic properties of another α-D-galactosidase from seeds of Vicia faba which also displayed erythroagglutinating activity. Contrary to soy bean and mung bean enzymes, both monomeric and tetrameric forms possessed the erythroagglutinating activity and this activity was inhibited by derivatives of D-mannose and D-glucose[8–11].

In the present communication we describe the electrophoretic properties of

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both the tetrameric and the monomeric forms of α-D-galactosidases from mung bean and soy bean, their possible interconversion and their sugar-binding properties according to affinity electrophoresis.

MATERIALS AND METHODS

Seeds of Glycine soja were supplied by Soja (Kolín, Czechoslovakia) and Vigna radiata by Sutten Seeds via a local garden centre in Egham (Surrey, U.K.). Water-soluble poly(glycosyloxyalkenylacrylamide) copolymers used for affinity electrophoresis were prepared as described earlier[12]. Mussel glycogen was obtained from Sigma (St. Louis, MO, U.S.A.).

Two forms of α-D-galactosidase, I and II, from soy bean and mung bean were separated by gel filtration using a Sephacryl S-200 column (92 cm × 2.6 cm) equilibrated with McIlvaine buffer pH 5.5 containing 0.1 *M* sodium chloride. Powdered seeds were initially extracted with McIlvaine buffer pH 5.5. The pH of the extract was lowered to 4.5 with 1 *M* citric acid, precipitated proteins were removed by centrifugation at 10 000 *g* and the 30–70% ammonium sulphate fraction was used for gel filtration. Soy bean lectin was removed by affinity chromatography on an O-α-D-galactosyl derivative of polyacrylamide[6]. For the study of the simultaneous interaction of the two forms of the enzyme with saccharides, the 0.15 *M* sodium chloride extract was first acidified to pH 4.5 and then the protein was precipitated with ammonium sulphate (0–80% saturation).

Effect of pH on the interconversion of α-D-galactosidase I and II

The solutions of the separated forms of α-D-galactosidase I and II from both sources (10 mg/ml) either in 0.05 *M* citrate–phosphate buffer pH 4.0 or in 0.1 *M* phosphate buffer pH 8.0 were dialyzed against the same buffer for 24–72 h. The solutions obtained were used after the addition of glycerol for polyacrylamide gel electrophoresis (PAGE).

Affinity electrophoresis

PAGE of α-D-galactosidase was performed in an apparatus according to Davis[13] in a discontinuous acidic buffer system[14] (omitting the stacking gel layers).

Protein samples (15–30 μl) in 20% glycerol solution (30 μl) were applied to each tube (75 mm × 5 mm) and electrophoresis was performed at 7 mA per tube for 100 min. Gels were stained specifically using 6-bromonaphthyl α-D-galactopyranoside as a substrate[15]. The migration distances of the zones of α-D-galactosidases were measured with an accuracy of ± 0.5 mm.

The dissociation constants, K_i, of the complexes of α-D-galactosidase I and II with immobilized α-D-galactosyl residues were determined, by a modification of our original method[16], from the dependence of $1/d_0 - d$ *vs.* $1/c_i$[17]. The dissociation constants, K, of the complexes of α-D-galactosidase and free sugars were obtained as described in our previous communication[16] from the dependence of $d/d_0 - d$ *vs.* c, where c_i is the concentration of immobilized sugar, c is the concentration of free sugar, d_0 is the mobility on the control gel containing water-soluble polyacrylamide without sugar residues and d is the affinity gel at given c_i and c.

Affinity gels were prepared by the addition of an appropriate amount of the

solution of O-glycosyl polyacrylamide copolymer or polysaccharide to the polymerization mixture to give a desired concentration, c_i, of immobilized sugar residues in the range $3.1 \cdot 10^{-3}$–$12.2 \cdot 10^{-3}$ *M*. For the determination of *K*, solutions of free sugars were added to the polymerization mixture; the concentration, *c*, of free sugars was different ($8 \cdot 10^{-3}$–$240 \cdot 10^{-3}$ *M*) for various sugars according to the strength of the interaction.

RESULTS

Dry seeds of soy bean and mung bean contain two forms of α-D-galactosidase, a high-molecular-weight α-D-galactosidase I and a low-molecular-weight α-D-galactosidase II. These forms differ in their electrophoretic mobilities on polyacrylamide gels in acidic buffer systems; two forms of the enzyme from both sources could be separated by molecular sieve chromatography. The electrophoretic band with the α-D-galactosidase activity having lower mobility corresponds to α-D-galactosidase I and that with higher mobility to α-D-galactosidase II.

In the case of soy bean, the separation of the low-molecular-weight form of α-D-galactosidase (α-D-galactosidase II) could be achieved in the course of the preparation of the crude ammonium sulphate fraction from the soy bean extract after acidification to pH 4.5: after dialysis of the ammonium sulphate fraction against water, only α-D-galactosidase II was detected in the water-soluble fraction, while in the water-insoluble material both forms were present. In the ammonium sulphate fraction obtained from the acidified extract of mung bean, α-D-galactosidase I was predominant.

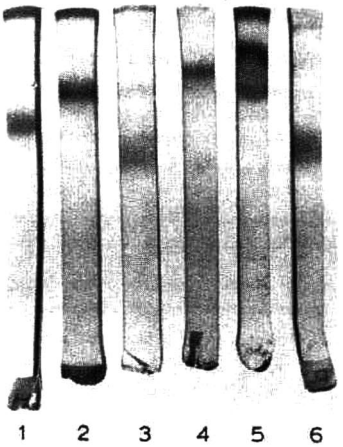

Fig. 1. Affinity electrophoresis of purified forms of soy bean α-D-galactosidases. 1 and 3 = Control gels; 2 and 4 = affinity gels containing O-α-D-galactosylpolyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*); 5 and 6 = affinity gels containing 1% glycogen. α-D-Galatosidase I is present in gels 1, 2 and 5 and α-D-galactosidase II in gels 3, 4 and 6.

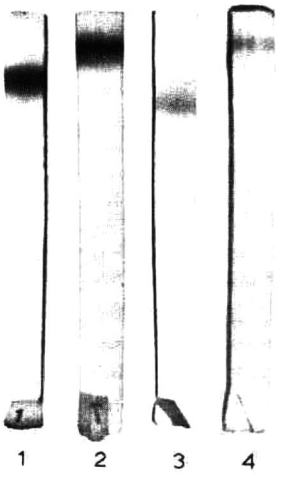

Fig. 2. Affinity electrophoresis of purified forms of mung bean α-D-galactosidase. 1,3 = Control gels; 2 and 4 = affinity gels containing O-α-D-galactosylpolyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*). α-D-Galactosidase I is present in gels 1 and 2 and α-D-galactosidase II in gels 3 and 4.

Fig. 3. Affinity electrophoresis of soy bean α-D-galactosidase. Gels as in Fig. 2. Ammonium sulphate fraction soluble after dialysis is present in gels 1 and 2 and that fraction is soluble after dialysis against water is present in gels 3 and 4.

The electrophoretic behaviour of the purified forms of α-D-galactosidases from both sources was the same as that of enzymes in crude protein fractions.

Interconversion of α-D-galactosidase forms

The high- and the low-molecular-weight forms of soy bean and mung bean α-D-galactosidase can be reversibly interconverted by changes of pH; at pH 4.0 the enzyme consists of a tetrameric form which dissociates to a monomer at pH 8.0. The dissociation and association process has previously been investigated by gel chromatography on Sephacryl or Sephadex[1,5]. In our experiments we used PAGE to follow the effects of pH on the electrophoretic behaviour of both forms of α-D-ga-

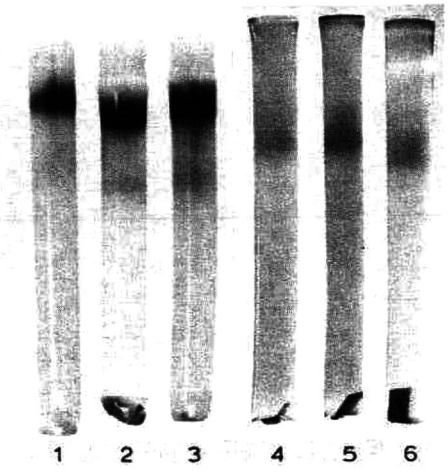

Fig. 4. PAGE of soy bean α-D-galactosidase. Samples without dialysis: 1 and 4. Samples dialyzed for 72 h against buffer: 2 and 5, 0.05 *M* citrate–phosphate buffer pH 4.0; 3 and 6, 0.1 *M* phosphate buffer pH 8.0, α-D-Galactosidase I is present in samples 1–3, α-D-galactosidase II in samples 4–6.

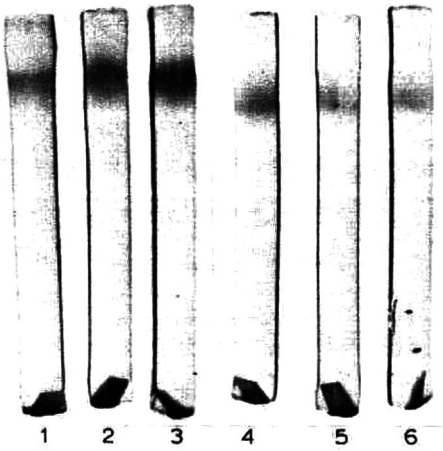

Fig. 5. PAGE of mung bean α-D-galactosidase. Details as in Fig. 4.

lactosidase from mung bean and soy bean. The separated forms of the enzyme (α-D-galactosidase I and II) from both sources were dialyzed for 24–72 h against buffer of pH 8.0 and 4.0. The results are shown in Figs. 4 and 5. No changes in the electrophoretic behaviour of α-D-galactosidase I and II from mung bean and soy bean was observed after dialysis against buffers of different pH. Small amounts of α-D-galactosidase II which could be found in samples of α-D-galactosidase I are formed in solutions of the enzyme independently at the pH used.

Affinity electrophoresis

In our experiments, we used affinity electrophoresis on polyacrylamide containing poly(glycosyloxyalkenyl-acrylamide) copolymers (O-glycosylpolyacrylamide copolymers)[12] to study the sugar-binding properties of the high- and low-molecular-weight forms of α-D-galactosidases from soy bean and mung bean.

The addition of O-α-D-galactosylpolyacrylamide copolymer to polyacrylamide gels caused a decrease in the electrophoretic mobility of both forms I and II of the enzyme from both sources, Figs. 1–3. The decrease in electrophoretic mobility is dependent on the concentration of immobilized D-galactosyl residues in the polyacrylamide gels. The control gels were prepared by the addition of water-soluble polyacrylamide without saccharide residues in the same concentration as the O-α-D-galactosylpolyacrylamide copolymer.

From the dependence of the electrophoretic mobility on the concentration of immobilized sugar, c_i, the dissociation constant of the complex between the enzyme and the immobilized D-galactosyl residue was calculated (Table I). The electrophoretic mobility of both forms of α-D-galactosidase from both plant species was not affected by the presence of N-acetyl-O-α-D-galactosaminyl-, O-α-D-mannosyl- and β-lactosylpolyacrylamide copolymers at $c_i = 2.0 \cdot 10^{-2}$, $1.8 \cdot 10^{-2}$ and $0.9 \cdot 10^{-2}$ *M*, respectively.

With the natural polysaccharides tested (dextran T-500, yeast mannan, glycogen) used in concentrations of 1 and 2%, a marked decrease in the mobility of α-D-galactosidase I of soy bean (Fig. 1, gels 5 and 6) and mung bean (not shown) was observed only in the case of glycogen.

TABLE I

DISSOCIATION CONSTANTS, K_i, OF COMPLEXES OF PURIFIED α-D-GALACTOSIDASE I AND II WITH IMMOBILIZED SACCHARIDE

Average values from ten measurements are presented. No interaction with immobilized D-mannose (at $c_i = 1.9 \cdot 10^{-2}$ *M*), D-glucose (at $c_i = 2.1 \cdot 10^{-2}$ *M*), N-acetyl-D-galactosamine (at $c_i = 1.7 \cdot 10^{-2}$ *M*) and lactose (at $c_i = 0.9 \cdot 10^{-2}$ *M*).

	α-D-*Galactosidase*	$10^3 \cdot K_i$ (*M*)
Vicia faba	I	11.5 (ref. 7)
	II	6.9 (ref. 7)
Glycine soja	I	9.9
	II	3.5
Vigna radiata	I	14.2
	II	5.0

The addition of free sugars, which interact with the enzyme forms, to affinity gels containing immobilized α-D-galactosyl residues reduced the extent of the decrease in enzyme mobility. Only those sugars which inhibit the α-D-galactosidase activity were able to do this (D-galactose, D-xylose, L-arabinose, D-fucose, myo-inositol); sugars which did not inhibit the enzyme activity did not interact with α-D-galactosidases under the conditions of affinity electrophoresis (2-deoxy-D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, D- and L-mannose, L-galactose, L-rhamnose, L-fucose and D-fructose).

The dependence of the electrophoretic mobility of the α-D-galactosidase zones on the concentration of free sugars, *c*, was used to determine the dissociation constants, *K*, of the enzyme–free sugar complexes; values of the dissociation constants are given in Table II. For comparison, values obtained for Vicia faba enzymes[7] obtained previously are included.

TABLE II

DISSOCIATION CONSTANTS, *K*, OF COMPLEXES OF PURIFIED α-D-GALACTOSIDASE I AND II WITH FREE SUGARS

	α-D-*Galactosidase*	$10^3 \cdot K$ (*M*)		
		D-*Galactose*	D-*Xylose*	*myo-Inositol*
Vicia faba	I	11.1	31.2	66.1 (ref. 7)
	II	8.8	30.4	12.9 (ref. 7)
Glycine soja	I	3.4	14.6	131.5
	II	2.5	13.9	34.5
Vigna radiata	I	3.6	–*	–*
	II	2.0	10.2	62.0

* No interaction was observed under the experimental conditions used.

DISCUSSION

α-D-Galactosidases have been reported to occur widely in nature[18]; seeds of leguminous plants are characterized by the presence of multimolecular forms of this enzyme. Shannon *et al.*[19,20] found that α-D-galactosidase from seeds of some leguminous plants exhibit, besides their enzymatic activity, also erythroagglutinating activity; they tested 26 species of leguminous plants and α-D-galactosidase from only 6 species displayed erythroagglutinating activity. In these cases the agglutinating activity was inhibited by the same sugars that inhibited the enzymatic activity; α-D-galactosidases from soy bean and mung bean belong to this group. Another type of α-D-galactosidase with erythroagglutinating activity has been described to be present in seeds of Vicia faba[8–10]: its erythroagglutinating activity was not inhibited by inhibitors of its enzymatic activity, but by derivatives of D-glucose and D-mannose.

In our previous communication[7] we reported the use of affinity electrophoresis to study the sugar-binding properties of α-D-galactosidases from Vicia faba seeds. Under the conditions PAGE (acidic buffer system[14]) we demonstrated an interaction of the Vicia faba enzymes with immobilized D-galactosyl residues, while no interaction was observed with immobilized D-mannosyl residues. The electrophoretic behaviour of two forms of α-D-galactosidases from mung bean and soy bean was very similar to that of the Vicia faba enzymes[7]. The band with higher mobility corresponded in both cases to the low-molecular-weight form of α-D-galactosidase (according to gel chromatography) and the band with lower mobility to the high-molecular-weight form. It is interesting that the electrophoresis was carried out in an acidic buffer[14], in which an association of the low-molecular-weight form of mung bean and soy bean enzyme should be preferred. After electrophoresis in polyacrylamide in alkaline buffers, no band with α-D-galactosidase activity was detected.

It is difficult to explain the results of our experiments on the interconversion of the two forms of α-D-galactosidases upon changes of pH. Even after prolonged dialysis of both forms from both sources we did not observe any change in the electrophoretic pattern of the separated forms of the enzyme.

The results of affinity electrophoresis of mung bean and soy bean α-D-galactosidases were very similar to those obtained with the enzyme forms from Vicia faba seeds[7]. An interesting phenomenon was observed in the cases of affinity gels containing glycogen; again, as in the case of the Vicia faba enzyme, α-D-galactosidase I from mung bean and soy bean was retarded on the affinity gel; the mobility of α-D-galactosidase II was not affected by the presence of glycogen. Dey *et al.*[8–11] have described the precipitation of the Vicia faba α-D-galactosidase with mannan and glycogen, but both high- and low-molecular-weight forms were precipitated, and moreover, in the acidic pH range (which was used in affinity electrophoresis) no precipitation occurred. So our observed decrease in mobility of α-D-galactosidase I on affinity gels containing glycogen from all three species might not be related to the sugar specificity of the agglutinating activity of α-D-galactosidases.

Affinity electrophoresis in the absence and in the presence of free saccharides was used for the determination of dissociation constants of complexes of α-D-galactosidases from soy bean and mung bean with immobilized and free sugars. The values are in quite good agreement with inhibition constants determined kinetically[18]. The values of the dissociation constants determined for the purified forms of

α-D-galactosidase I and II of soy bean and mung bean did not differ from values obtained with crude protein fractions containing both forms. The presence or absence of D-galactose/N-acetyl-D-galactosamine specific lectin from soy bean in the sample has no effect on the strength of the interaction of soy bean α-D-galactosidase with immobilized sugar.

REFERENCES

1 N. Harpaz, H. M. Flowers and N. Sharon, *Eur. J. Biochem.*, 77 (1977) 419–426.
2 E. DelCampillo and L. M. Shannon, *Plant Physiol.*, 69 (1982) 628–631.
3 C. N. Hankins and L. M. Shannon, *J. Biol. Chem.*, 253 (1978) 7791–7797.
4 P. M. Dey, *Eur. J. Biochem.*, 140 (1984) 385–390.
5 E. DelCampillo, L. M. Shannon and C. N. Hankins, *J. Biol. Chem.*, 256 (1981) 7177–7180.
6 J. Nováková, M. Tichá and J. Kocourek, *Biochim. Biophys. Acta*, 670 (1981) 401–407.
7 P. Malý, M. Tichá and J. Kocourek, *J. Chromatogr.*, 347 (1985) 343–350.
8 P. M. Dey, S. Naik and J. B. Pridham, *FEBS Lett.*, 150 (1982) 233–237.
9 P. M. Dey, S. Naik and J. B. Pridham, *Planta*, 167 (1986) 114–118.
10 P. M. Dey, S. Naik and J. B. Pridham, *Phytochemistry*, 25 (1986) 359–365.
11 P. M. Dey, S. Naik and J. B. Pridham, *Phytochemistry*, 25 (1986) 1057–1061.
12 V. Hořejší, P. Smolek and J. Kocourek, *Biochim. Biophys. Acta*, 538 (1978) 293–298.
13 B. J. Davis, *Ann. NY Acad. Sci.*, 121 (1964) 404–427.
14 R. A. Resifeld, V. J. Levis and D. E. Williams, *Nature (London)*, 195 (1962) 281–283.
15 V. Hadačová and K. Beneš, *Biol. Plant.*, 19 (1977) 436–441.
16 V. Hořejší, M. Tichá and J. Kocourek, *Biochim. Biophys. Acta*, 499 (1977) 290–300.
17 M. Tichá, J. Barthová, J. Labský and M. Semanský, *J. Chromatogr.*, 194 (1980) 183–189.
18 P. M. Dey and J. B. Pridham, *Adv. Enzymol.*, 36 (1972) 91–130.
19 C. N. Hankins, J. I. Kindinger and L. M. Shannon, *Plant Physiol.*, 66 (1980) 375–378.
20 C. N. Hankins, J. I. Kindinger and L. M. Shannon, *Plant Physiol.*, 65 (1980) 618–622.

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CHROM. 20 109

GAS CHROMATOGRAPHIC ANALYSIS OF AMINOALKYLPHOSPHONIC ACIDS AND AMINOALKYL PHOSPHATES

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(Received September 21st, 1987)

SUMMARY

A method for the determination of aminoalkylphosphonic acids and aminoalkyl phosphates by gas chromatography has been developed. It involves the conversion of these compounds into their N-isobutoxycarbonyl methyl ester derivatives and chromatography on a 1.5% OV-17 column. The derivatives are volatile enough and stable, giving single and symmetrical peaks. The calibration plots in the range 5–50 μg were linear and sufficiently reproducible for quantitation.

INTRODUCTION

Aminoalkylphosphonic acids and aminoalkyl phosphates are the third class of amino acids next to aminocarboxylic acids and aminosulphonic acids. Some of them have been identified in the free state or as constituents of lipids, proteins and nucleic acids in various species[1–10].

These compounds are non-volatile and thus require conversion into volatile derivatives prior to gas chromatography (GC). Per-trimethylsilyl (TMS)[11–13], N-acetyl TMS ester[12], acetone Schiff base TMS ester[12], isothiocyanate TMS ester[12], N-acetyl alkyl ester[6] and N-trifluoroacetyl alkyl ester[14–16] derivatives have been used for this purpose. However, TMS derivatives are very sensitive to moisture, and therefore inadequate for reliable quantitative work. On the other hand, the preparation of N-acyl alkyl ester derivatives requires a lengthy procedure and anhydrous conditions.

It has been demonstrated in our laboratory[17,18] that a variety of amino acids can be successfully analyzed as their N-isobutoxycarbonyl (N-isoBOC) methyl esters, which are conveniently prepared by a simple procedure involving isobutoxycarbonylation with isobutyl chloroformate in an aqueous medium followed by esterification with diazomethane. In the present work, the analysis of aminoalkylphosphonic acids and aminoalkyl phosphates by the use of this GC technique was investigated.

EXPERIMENTAL

All of the aminoalkylphosphonic acids and aminoalkylphosphates, except

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4-aminophenylphosphonic acid and O-phospho-D,L-threonine, were obtained from Sigma (St. Louis, MO, U.S.A.). 4-Aminophenylphosphonic acid and O-phospho-D,L-threonine were obtained from Aldrich (Milwaukee, WI, U.S.A.) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. Two standard solutions, one containing thirteen aminoalkylphosphonic acids and another containing four aminoalkyl phosphates, were prepared so as to contain 0.1 mg/ml of each compound in distilled water. 9-Bromophenanthrene (Tokyo Kasei Kogyo) was dissolved in ethyl acetate at 0.2 mg/ml and used as an internal standard (I.S.). Isobutyl chloroformate (isoBCF) stabilized with calcium carbonate was obtained from Tokyo Kasei Kogyo and used without further purification. N-Methyl-N-nitroso-*p*-toluenesulphonamide for use in the generation of diazomethane was obtained from Nakarai Chemicals (Kyoto, Japan). All other chemicals were of analytical grade.

Derivatization

An aliquot of the sample solution containing 5–50 μg of aminoalkylphosphonic acids or aminoalkyl phosphates was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. After addition of 0.2 ml of 0.5 *M* sodium hydroxide, the total reaction volume was made up to 1 ml with distilled water if necessary. Immediately after the addition of 0.1 ml of isoBCF, the mixture was shaken with a shaker set at 300 rpm (up and down) for 10 min at room temperature. The reaction mixture was extracted with 3 ml of diethyl ether in order to remove the excess of reagent, and the ethereal extract was discarded. The aqueous layer was saturated with sodium chloride, acidified to pH 1–2 with 2 *M* hydrochloric acid and then extracted twice with 3 ml of diethyl ether containing 10% isopropanol. The pooled ethereal extracts were methylated by bubbling diazomethane, generated according to the micro-scale procedure of Schlenk and Gellerman[19], through this solution until a yellow tinge became visible. After standing at room temperature for 5 min, the solvents were evaporated to dryness at 80°C under a stream of dry air. The residue was dissolved in 0.1 ml of 0.2 mg/ml I.S. solution and 2 μl of this solution were injected into the gas chromatograph.

Gas chromatography

GC analysis was carried out with a Shimadzu 4CM gas chromatograph equipped with an hydrogen flame ionization detector. The column packing, 1.5% OV-17 on Uniport HP (100–120 mesh), was prepared using toluene as a coating solvent according to the solution coating technique[20], and was poured into a silanized glass column (1.5 m × 3 mm I.D.). The packed column was conditioned at 285°C for 24 h with nitrogen at a flow-rate of 30 ml/min. The operating conditions are indicated in the figures.

Gas chromatography–mass spectrometry (GC–MS)

A Shimadzu-LKB 9000 gas chromatograph-mass spectrometer with the same type of column as used for GC analysis was employed under the following conditions: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion-source temperature, 270°C; separator temperature, 245°C; helium flow-rate, 38 ml/min.

RESULTS AND DISCUSSION

Aminoalkylphosphonic acids and aminoalkyl phosphates could be successfully converted into their N-isoBOC methyl ester derivatives by essentially the same procedure as that used in the derivatization of amino acids[17,18]. The isobutoxycarbonylation of the amino function of these compounds proceeded rapidly and quantitatively in aqueous alkaline media at room temperature. Diethyl ether has been used as an extraction solvent for N-isoBOC aminocarboxylic acids[17,18], but the extraction of N-isoBOC aminoalkylphosphonic acids and of N-isoBOC aminoalkylphosphates with this solvent was found to be incomplete. In order to solve this problem, the addition of another solvent to diethyl ether was tested. Isopropanol proved to be the most satisfactory solvent for this purpose, and its optimum concentration was found to be in the range 5–15% (Fig. 1). Thus, diethyl ether containing 10% isopropanol was used in the present method. The subsequent methylation with diazomethane could be successfully carried out in this solvent. The derivative preparation could be performed within 30 min.

The structures of the derivatives were confirmed by GC–MS analysis. As shown in Fig. 2, a molecular ion peak (M^+) was observed for each of the derivatives and other common ion peaks which were useful for structure elucidation were $M^+ - 59$ ($COOCH_3$), $M^+ - 73$ [$(CH_3)_2CHCH_2O$] and *m/e* 109 [$PO(OCH_3)_2$].

The derivatives were very stable to moisture, therefore no precaution to exclude moisture was necessary in their handling and storage. No decomposition was observed even after standing in ethyl acetate for 3 weeks at room temperature.

Typical chromatograms of aminoalkylphosphonic acids and aminoalkyl phosphates were shown in Figs. 3 and 4, respectively. Each compound gave a single and

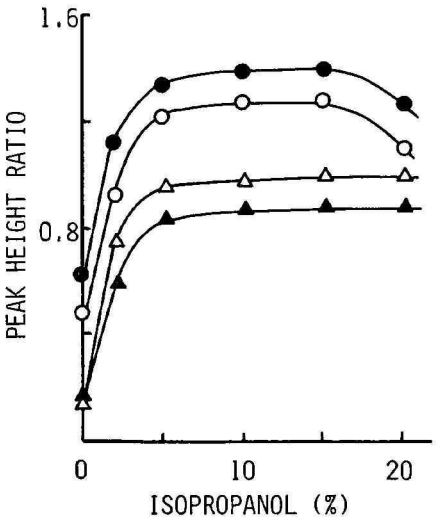

Fig. 1. Effects of isopropanol concentration on the ether extraction of N-isoBOC 2-aminoethylphosphonic acid (○), N-isoBOC phosphoethanolamine (●), N-isoBOC 2-amino-3-phosphonopropionic acid (△) and N-isoBOC O-phospho-L-serine (▲).

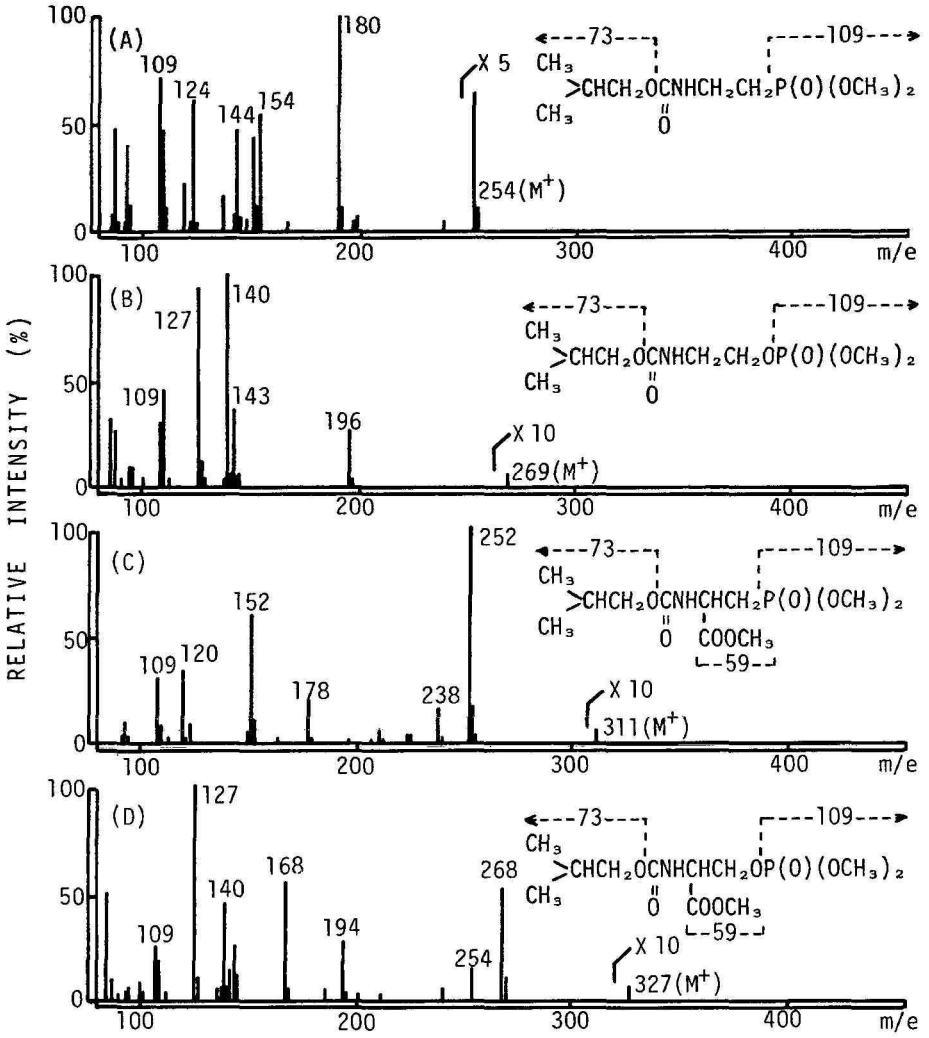

Fig. 2. Mass spectra of the N-isoBOC methyl ester derivatives of (A) 2-aminoethylphosphonic acid, (B) phosphoethanolamine, (C) 2-amino-3-phosphonopropionic acid and (D) O-phospho-L-serine.

symmetrical peak upon GC with a OV-17 column; no extraneous peaks due to thermal decomposition were observed.

In order to test the linearity of the calibration plot, various amounts of aminoalkylphosphonic acids and aminoalkylphosphates ranging from 5 to 50 μg were derivatized in a mixture and aliquots representing 0.1–1.0 μg of each compound were injected for GC. In each case, a linear relationship was obtained and its reproducibility was found to be satisfactory.

These experiments have conclusively demonstrated that the method proposed is suitable for quantitation of aminoalkylphosphonic acids and aminoalkyl phos-

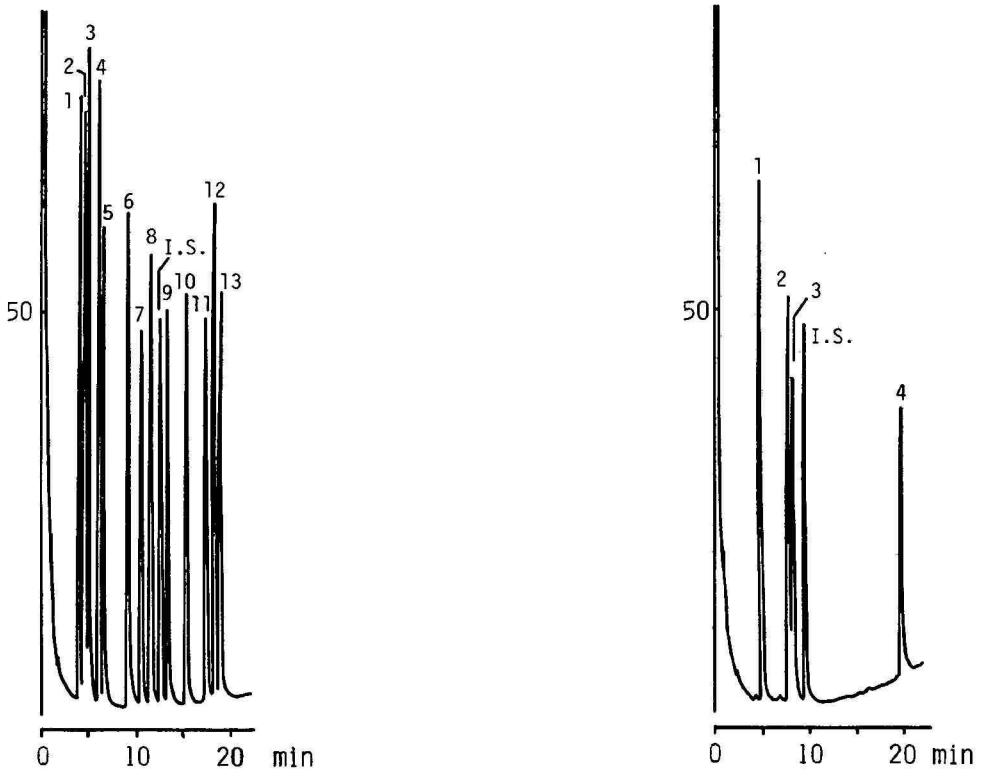

Fig. 3. Gas chromatogram of the N-isoBOC methyl ester derivatives of some aminoalkylphosphonic acids. GC conditions: column, 1.5% OV-17 on Uniport HP (100–120 mesh), 1.5 m × 3 mm I.D., glass; column temperature, programmed at 5°C/min from 150 to 260°C; injection and detector temperatures, 270°C; nitrogen flow-rate, 40 ml/min. Peaks: 1 = aminomethylphosphonic acid; 2 = 1-aminoethylphosphonic acid; 3 = 1-aminopropylphosphonic acid; 4 = 1-aminobutylphosphonic acid; 5 = 2-aminoethylphosphonic acid; 6 = 3-aminopropylphosphonic acid; 7 = 2-amino-3-phosphonopropionic acid; 8 = 4-aminobutylphosphonic acid; 9 = 2-amino-4-phosphonobutyric acid; 10 = 2-amino-5-phosphonovaleric acid; 11 = 2-amino-6-phosphonohexanoic acid; 12 = 4-aminophenylphosphonic acid; 13 = 2-amino-7-phosphonoheptanoic acid; I.S. = 9-bromophenanthrene. Each peak represents 400 ng of aminoalkylphosphonic acid.

Fig. 4. Gas chromatogram of the N-isoBOC methyl ester derivatives of some aminoalkyl phosphates. GC conditions: as Fig. 3, except column temperature, programmed at 5°C/min from 170 to 260°C. Peaks: 1 = phosphoethanolamine; 2 = O-phospho-D,L-threonine; 3 = O-phospho-L-serine; 4 = O-phospho-L-tyrosine; I.S. = 9-bromophenanthrene. Each peak represents 400 ng of aminoalkyl phosphate.

phates. Further investigations on the application of this method to biological samples are in progress.

REFERENCES

1 M. Horiguchi and M. Kandatsu, *Nature (London)*, 184 (1959) 901.
2 J. S. Kittredge and R. R. Hughes, *Biochemistry*, 3 (1964) 991.
3 T. Hori, I. Arakawa and M. Sugita, *J. Biochem. (Tokyo)*, 62 (1967) 67.
4 K. Saito and K. Mukoyama, *Biochim. Biophys. Acta*, 164 (1968) 596.

5 J. S. Kittredge and E. Roberts, *Science (Washington, D.C.)*, 164 (1969) 37.
6 J. A. Alhadef and G. D. Daves, *Biochemistry*, 9 (1970) 4866.
7 M. Kates, *Adv. Lipid. Res.*, 8 (1970) 225.
8 K. J. Stevenson, D. Gibson and G. H. Dixon, *Can. J. Biochem.*, 52 (1974) 93.
9 Y. Takai, A. Kishimoto, Y. Iwasa, Y. Kawahara, T. Mori and Y. Nishizuka, *J. Biol. Chem.*, 254 (1979) 3629.
10 P. Cohen, *Eur. J. Biochem.*, 151 (1985) 439.
11 K. A. Karlson, *Biochem. Biophys. Res. Commun.*, 39 (1970) 847.
12 D. J. Harvey and M. G. Horning, *J. Chromatogr.*, 79 (1973) 65.
13 J. J. Shieh and D. Desiderio, *Anal. Lett.*, 10 (1977) 831.
14 E. D. Korn, D. G. Dearborn, H. M. Fales and E. A. Sokoloski, *J. Biol. Chem.*, 248 (1973) 2257.
15 M. L. Ruppel, L. A. Suba and J. T. Marvel, *Biomed. Mass Spectrom.*, 3 (1976) 28.
16 J. W. Huber, *J. Chromatogr.*, 152 (1978) 220.
17 M. Makita, S. Yamamoto and M. Kōno, *J. Chromatogr.*, 120 (1976) 129.
18 M. Makita, S. Yamamoto, K. Sakai and M. Shiraishi, *J. Chromatogr.*, 124 (1976) 92.
19 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
20 E. C. Horning, W. J. A. VandenHeuvel and B. G. Creech, *Methods Biochem. Anal.*, 11 (1963) 69.

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THIN-LAYER CHROMATOGRAPHIC SCREENING METHOD FOR THE TRANQUILLIZERS AZAPERONE, PROPIOPROMAZINE AND CARAZOLOL IN PIG TISSUES

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SUMMARY

A procedure is described for the detection of azaperone, propiopromazine and carazolol in pig muscle, liver and kidney tissue. The method comprises extraction from an alkaline tissue homogenate with diethyl ether, followed by cleaning up and concentration of the extract on a silica gel solid-phase extraction column. Two-dimensional thin-layer chromatography on a silica plate was used for the detection of the tranquillizers. Detection levels were 25 $\mu g\ kg^{-1}$ for propiopromazine, 50 $\mu g\ kg^{-1}$ for azaperone (or its metabolite azaperol) and 125 $\mu g\ kg^{-1}$ for carazolol. In pigs treated with the usual doses the presence of propiopromazine and azaperol could be established in kidney tissue 8 h after administration, whilst in injection sites all three tranquillizers could be detected.

INTRODUCTION

In pigs, sensitivity to stress may present a serious problem, in particular during transport to the slaughterhouse. Stress in pigs often results in decreased meat quality, and occasionally in premature death.

Apart from improvement of the transport conditions and breeding of more stress-resistant pigs, these problems can be diminished by administration of tranquillizers shortly before transport of pigs to the slaughterhouse. For this purpose, neuroleptic drugs such as azaperone {4'-fluoro-4-[4-(2-pyridyl)-1-piperazinyl]butyrophenone; Stresnil®} and propiopromazine [10-(3-dimethylaminopropyl)-2-propionylphenothiazine; Combelene®] are frequently used. For sedating stressed pigs the beta-blocker carazolol [4-(2-hydroxy-3-isopropylaminopropoxy)carbazole; Suacron®] is sometimes administered as well. The application of tranquillizers to pigs prior to transport, however, may give rise to a residue problem.

For the determination of tranquillizer residues in food of animal origin, methods employed include gas chromatography[1–4], high-performance liquid chromatography[5,6], thin-layer chromatography[7] and direct fluorimetry[8]. A radioimmunoassay for the determination of carazolol in blood and urine was recently de-

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scribed[9]. Most of these procedures are directed towards individual transquillizers, *i.e.*, azaperone[1,3,6], propiopromazine[2,7] and carazolol[8]. However, Scheutwinkel-Reich *et al.*[4] and Etter *et al.*[5] described simultaneous determinations of tranquillizers.

Apart from these methods, it was felt necessary to develop a relatively simple qualitative thin-layer chromatographic (TLC) screening procedure for azaperone (together with its metabolite azaperol[3]), propiopromazine (together with its metabolite propiopromazine sulphoxide) and carazolol in pig meat and kidney tissue. The present paper describes such a method using two-dimensional TLC. For efficient sample clean-up, solid-phase extraction (SPE) was used. The procedure was used for the detection of these tranquillizers in pig tissues 2, 5 and 8 h after intramuscular injection.

MATERIALS AND METHODS

Reagents and chemicals

All solvents used were A.R. grade. Diethyl ether, light petroleum (b.p. 40–60°C), methanol, dichloromethane, acetone, *n*-butanol and sodium hydroxide were from Merck (Darmstadt, F.R.G.), acetic acid (99%) and ammonia solution (25%) from Baker (Phillipsburgh, NJ, U.S.A.). Methanol saturated with hydrochloric acid was prepared by passing hydrogen chloride vapour through the solvent. The saturated solution was diluted in methanol (*ca.* 1:100, v/v); a pH test strip indicated a value of 1.6 (Lyphan L652, pH 1.6–3.7).

Silica gel disposable SPE columns (3 ml) were from Baker. Just before use the column was pretreated with about 10 ml of diethyl ether–light petroleum (1:1, v/v). After this pretreatment the column should not be allowed to run dry.

Azaperone was from Janssen Pharmaceutica (Beerse, Belgium), carazolol from Praemix Wirkstoff (Mannheim, F.R.G.) and propiopromazine from Bayer (Leverkusen, F.R.G.). Azaperol was synthesized from azaperone by reduction with sodium borohydride. The standard solution for TLC was prepared in methanol (100 μg of each compound per ml).

Precoated 20 cm × 20 cm HPTLC aluminium sheets coated with silica gel 60 F_{254} were obtained from Merck. The plates were cut into twelve 6.5 cm × 5 cm sheets. Each sheet was pre-cleaned by development in the longitudinal direction with methanol–25% ammonia (98:2, v/v). To remove impurities, eluted with the front, the upper parts of the plates were cut off, thus reducing their lengths to 5 cm.

The solvent systems employed for TLC were: I, dichloromethane–acetone–25% ammonia (100:100:5, v/v/v, freshly prepared); II, *n*-butanol–acetic acid–water (80:20:100, v/v/v, organic layer[10]).

Apparatus

A table centrifuge (Type TJ6; Beckmann Instruments, Bohemia, NY, U.S.A.), a Moulinette homogenizer (Moulinex, Gouda, The Netherlands), a Vortex mixer (Scientific Industries, Bohemia, NY, U.S.A.), an Ultra Turrax (Janke u Kunkel K.G., Staufen i. Breisgau, F.R.G.) and a shaker bath (Köttermann, Hänigsen, F.R.G.) filled with water were used. A vacuum manifold (Baker) was used for suction of the SPE columns.

The UV chromatographic viewer (254 and 366 nm) and the TLC tank were

from Camag (Muttenz, Switzerland). The 5-μl syringe (SGE, Scientific Glass Engineering, Ringwood, Australia) was fitted with a PTFE nozzle.

Sample preparation

About 20 g of ground tissue were weighed into a 250-ml stoppered erlenmeyer flask. Sodium hydroxide solution (1 mol l^{-1}) was added: 15 ml for kidney and liver tissue and 30 ml for meat tissue. The kidney and liver mass was thoroughly mixed with an Ultra Turrax, whilst the meat suspension could be extracted directly. After shaking at 95°C for 1 h and subsequent cooling, 70 ml of diethyl ether were added. The suspension was thoroughly shaken for 2 min and the ether layer was transferred to a 100-ml erlenmeyer flask. To the ether extract, light petroleum was added (40 ml for kidney tissue, 75 ml for meat and liver tissue).

The entire solution was passed through the pretreated silica gel column [connected with a 75-ml reservoir (Baker)] at a flow-rate of *ca.* 3 ml/min through the manifold. The column was dried in a stream of air for 10 min. A 5-ml syringe was filled with 3 ml of the diluted methanol–hydrochloric acid solution and the solution was pressed through the SPE column.

The entire eluate was collected in a 3.5-ml polypropylene collection vessel and evaporated to 20–50 μl (as soon as the volume had decreased to about 100 μl the solution was homogenized, using a vortex mixer). The solution was centrifuged for 10 min at 2000 *g*; the supernatant was used for TLC.

Thin-layer chromatography

Identification of tranquillizers in the sample solution was performed by two-dimensional HPTLC. About 5 μl of the sample solution were applied on the left angle of the silica gel plate (*viz.*, Fig. 1) by repetitive spotting using a 5-μl syringe and drying in a stream of air. (As propiopromazine may be strongly absorbed to glass surfaces it is recommended to avoid contact of the needle with concentrated solutions; even repeated rinsing with methanol is insufficient to prevent contamination after handling a concentrated solution.) The spot should be as small as possible. A 1-μl volume of the standard solution was applied at the margin of the plate on two sites (*viz.*, Fig. 1). The plate was first developed in solvent system I (saturated tank) over 3 cm. After thorough drying, the plate was turned 90° and developed in solvent system II (saturated tank), again over 3 cm. The solvent was allowed to evaporate from the plate. The plate was examined under UV light at 254 nm for the dark blue spots of azaperone and azaperol and the brown spot of carazolol on a greenish fluorescent background, and at 366 nm for the yellowish orange propiopromazine spot which appears on a purple background. At this wavelength the propiopromazine sulphoxide (light blue to white) can also be observed, but not carazolol and azaperone/azaperol.

Animal study

Eighteen slaughter pigs (weight roughly 110 kg each) were divided into three groups. The pigs from each group (six animals) were intramuscularly injected just behind the right ear with Combelene®, Stresnil® or Suacron®, in amounts as recommended by the manufacturer, *i.e.*, 0.5 mg of propiopromazine, 0.4 mg of azaperone or 0.01 mg of carazolol per kg body weight. Tissue samples (kidney, injection

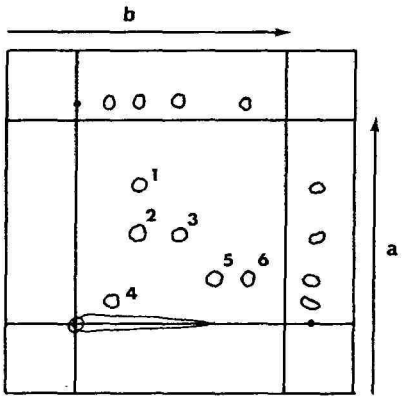

Fig. 1. Thin-layer chromatogram of tissue extract. For chromatographic conditions, see text. a, Direction of first development; b, direction of second development. Spots: 1 = azaperone; 2 = azaperol; 3 = propiopromazine; 4 = propiopromazine sulphoxide; 5 = matrix compound (kidney); 6 = carazolol.

site and pillar of the diaphragm) were obtained after slaughtering 2, 5 and 8 h after application. Each sample was analyzed according to the procedure described.

In order to avoid overloading of the TLC plate it was necessary to dilute some extracts from injection sites. These dilutions were performed directly after the SPE clean-up step. For propiopromazine, dilutions of 1:40, 2 h after application, and 1:10, 5 h after application, were made. For azaperone, only the extract obtained 2 h after application was diluted 1:10. For carazolol extracts, dilutions were unnecessary.

RESULTS AND DISCUSSION

Analytical procedure

A characteristic chromatogram is shown in Fig. 1. Metabolites such as azaperol and propiopromazine sulphoxide are also separated and detected in this system. An indication of the R_F values relative to both eluents is given in Table I. Eluent I, used for the first development, is based on that described by Davis and Harrington[11], toluene–acetone–ammonia (50:50:2.4) for TLC of chlorpromazine in plasma. In our study it proved necessary to replace toluene by dichloromethane in order to obtain

TABLE I

R_F VALUES ON HPTLC SILICA PLATES

Eluents: I, dichloromethane–acetone–25% ammonia (100:100:5, v/v/v); II, *n*-butanol–acetic acid–water (80:20:100 v/v/v, organic layer).

Compound	*Eluent I*	*Eluent II*
Propiopromazine sulphoxide	0.1	0.15
Carazolol	0.2	0.8
Propiopromazine	0.4	0.5
Azaperol	0.4	0.3
Azaperone	0.7	0.3

a good separation of the three parent compounds. Moreover, separation from nearly all matrix components is achieved, as these remain at the starting point. This eluent, however, does not allow a separation between azaperol and propiopromazine. For this reason, two-dimensional chromatography should be performed to complete the separation. Furthermore, it was observed that, in the second development, carazolol was separated from a minor matrix compound present only in kidney samples.

Because of the volatility of the ammonia component in eluent I, some changes in R_F values may occur. To prevent these changes as far as possible, the eluent should be freshly prepared before analysis. Nevertheless, the R_F values given in Table I can only be indicative because of their strong dependence on the ammonia content of the eluent. To prevent the presence of undefined spots on the chromatogram caused by impurities in the plate, pre-development with methanol/ammonia is advisable.

Detection at 254 nm is suitable for any of the individual tranquillizers. Propiopromazine and its sulphoxide, however, are more sensitively detected at 366 nm, whilst the other compounds are not detected at this wavelength.

After administration of any of the tranquillizers under consideration, relatively high concentrations, apart from the injection site, are found in kidney tissue. Therefore this matrix was chosen to develop a method for the detection of tranquillizers in pigs. As, in the kidney, azaperone is mainly present in its reduced form[1,12], it is necessary to include azaperol in the chromatographic system.

Until recently, *i.e.*, when starting these experiments, the occurrence of propiopromazine sulphoxide in tissues from pigs treated with propiopromazine was not observed. In a recent publication[2], however, the presence of this metabolite was demonstrated, but in amounts that were very small compared to those of propiopromazine, and mainly in spare rib tissue. It is also known that propiopromazine is easily oxidized to this compound when exposed to light and air. In the method described here no special precautions, such as the application of nitrogen gas in the evaporation step and for column drying, were taken to prevent this oxidation. In spite of this, oxidation was generally not observed during the analysis of spiked tissues according to the method described. Incidentally, a trace of propiopromazine sulphoxide was found in the analysis of spiked kidney tissue.

In general, tranquillizers are extracted from an alkaline tissue homogenate by means of organic solvents[2,3,5,7,8]. Diethyl ether extraction from a heated alkaline kidney homogenate, as applied for carazolol[8], was found also to be suitable for the extraction of propiopromazine and of azaperone from kidney tissue. Ether extracts, after dilution with a non-polar solvent such as light petroleum, can be directly submitted to solid-phase extraction of tranquillizers by means of a silica column.

For elution of carazolol into an acidified solvent a small volume is essential. For this purpose, methanol–0.1 *M* hydrochloric acid was used earlier[8]. This eluent, however, proved to be unsuitable for subsequent chromatography. It was found that methanol saturated with hydrogen chloride vapour and diluted in pure methanol, as described in Materials and Methods, could be successfully applied to the elution of all tranquillizers in a small volume, whilst interferences caused by hydrochloric acid in the second development could be restricted to an acceptable minimum.

The complete procedure could be also used, with some minor adaptations, for the screening of muscle and liver tissue.

In spiked tissues, the presence of concentrations down to 125 $\mu g\ kg^{-1}$ for

TABLE II

R_F VALUES ON HPTLC SILICA PLATES USING ELUENT I (DISTANCE 18 cm) AND DETECTION LIMITS OBTAINED IN THE ADAPTED PROCEDURE

Compound	*R_F value*	*Detection limit ($\mu g\ kg^{-1}$)*
Carazolol	0.14	80
Acepromazine	0.26	20
Propiopromazine	0.36	60
Chlorpromazine	0.43	80
Haloperidol	0.51	200
Xylazine	0.60	600
Azaperone	0.68	60

carazolol, 50 $\mu g\ kg^{-1}$ for azaperone/azaperol and 25 $\mu g\ kg^{-1}$ for propiopromazine can be demonstrated. For kidney tissue these detection levels are sufficient for azaperol and propiopromazine, but not for carazolol. However, the sensitivity for carazolol is sufficient when injection sites are examined.

This study prompted an extension to a screening method which, apart from the three tranquillizers studied, includes the tranquillizers acepromazine, chlorpromazine, haloperidol and xylazine. Olling and Besamusca[13] thus adapted the method described as follows. The diethyl ether extraction was performed three times, starting with 10 g of tissue. The tranquillizers were eluted from the silica SPE column with 4 ml of the solution of methanol saturated with hydrochloric acid, diluted 5:95 (v/v) in methanol. One-dimensional chromatography was carried out on a 20 cm × 20 cm HPTLC silica plate over a distance of 18 cm, using eluent I. An indication of the R_F values observed is given in Table II. Elution over 18 cm allows reasonable separation of all tranquillizers. The minor matrix compound from the kidney, which interfered after elution over 3 cm, was now somewhat below the carazolol spot, whilst the azaperol spot was situated between those of chlorpromazine and haloperidol. As only one-dimensional chromatography using eluent I was performed, hydrochloric acid did not interfere.

The detection limits obtained are also given in Table II. Despite the greater diffusion caused by the long elution period, *i.e.*, 2 h, and the smaller aliquot used, approximately the same detection limits for azeperone, carazolol and propiopromazine were found as in the original procedure. This can be explained by the more exhaustive extraction in the adapted procedure and the use of a less diluted methanol–hydrochloric acid solution for the SPE column elution. As this extraction is quite laborious, the extraction applied by us in combination with one-dimensional chromatography after elution with less diluted methanol–hydrochloric acid may be a useful alternative.

Apart from this, we studied the possible interferences of xylazine, acepromazine, chlorpromazine and haloperidol in our method. Only chlorpromazine and xylazine show approximately the same R_F value as propiopromazine after two-dimensional chromatography. The acepromazine spot is close to that of azaperol. However, at 366 nm both propiopromazine and acepromazine can be distinguished because of their yellow colour. It should be noted that two-dimensional chromatography as described here is not suitable to demonstrate the presence of all seven tranquillizers.

TABLE III

DETECTION OF PROPIOPROMAZINE, AZAPERONE/AZAPEROL AND CARAZOLOL IN PIG TISSUE

For experimental conditions, see text. +, Detected; −, not detected.

Matrix	*Hours after application*	*Propiopromazine*	*Azaperone/ azaperol*	*Carazolol*
Injection site	2	+	+	+
	5	+	+	+
	8	+	+	+
Kidney	2	+	+	−
	5	+	+	−
	8	+	+	−
Pillar of the diaphragm	2	+	+	−
	5	+	+	−
	8	−	−	−

Animal study

The results of the animal study are given in Table III. In kidney tissue the presence of propiopromazine and azaperol could be established 8 h after administration of the usual dose. As expected[8], carazolol was not detected.

In the injection site no problems arose with respect to the limit of detection. For propiopromazine and azaperone it was even necessary to dilute the extracts. In accordance with Rauws and Olling[12], traces of azaperol were found at the injection site of azaperone-treated pigs.

Although it was known which areas might contain injection sites it was not easy to find these. For propiopromazine, however, the yellow colour of the substance was a help.

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REFERENCES

1 W. Arneth, *Fleischwirtschaft*, 65 (1985) 945.
2 W. Arneth, *Fleischwirtschaft*, 66 (1986) 922.
3 A. G. Rauws, M. Olling, J. Freudenthal and M. ten Ham, *Toxicol. Appl. Pharmacol.*, 35 (1976) 333.
4 M. Scheutwinkel-Reich, H. G. Grohmann, S. Jordan, A. M. Preisz and H. J. Stan, *Fresenius' Z. Anal. Chem.*, 311 (1982) 398.
5 R. Etter, R. Battaglia, J. Noser and F. Schuppisser, *Mitt. Geb. Lebensm. Hyg.*, 75 (1984) 447.
6 B. Hoffmann, H. A. Meemken and W. Groebel, *Lebensmittelchem. Gerichtl. Chem.*, 36 (1982) 104.
7 M. Olling, R. W. Stephany and A. G. Rauws, *J. Vet. Pharmacol. Ther.*, 4 (1981) 291.
8 J. W. Engelsma and J. Simons, *Vet. Quart.*, 7 (1985) 73.
9 E. Rattenberger, P. Matzke and J. Neudegger, *Arch. Lebensmittel-hyg.*, 36 (1985) 85.
10 *Prüfungsvorschrift für Injektionslösung (Suacron®) für Tiere*, Boehringer, Mannheim, 1978.
11 C. M. Davis and C. A. Harrington, *J. Chromatogr. Sci.*, 22 (1984) 71.
12 A. G. Rauws and M. Olling, *J. Vet. Pharmacol. Ther.*, 1 (1978) 57.
13 M. Ollings and P. Besamusca, personal communication.

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Note

Amino acid analysis: buffers and artifacts

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To maintain a standard of excellence in performance with an amino acid analyzer, Beckman Instruments attempt to provide a total package covering the analyzer's operation, *i.e.* installation, service back-up and provision of all buffers and reagents used therein. The supply of buffers and reagents are an integral part of the package, in fact, the instrument manufacturer proposes that if they are not used then they cannot be held responsible for the performance of the analyzer.

Recently it was demonstrated that one of the buffers, supplied for use in the Beckman Model 6300 analyzer, was responsible for the presence of an artifact on the chromatogram[1]. This artifact, under certain circumstances can interfere with the automatic integration of the histidine peak, thereby introducing a calibration error. Further, another of the supplied buffers has been found to cause a similar problem associated with the arginine peak. Thus, in the context that all is not well with some of the commercially available buffers, and as the trend at present is to obtain more and more results without incurring additional expense, it was decided to prepare a substitute for one of the Beckman buffers. The sample dilution buffer was selected for replacement as it could not have any long term effect on analyzer performance because such a small amount was used in dilution of analytical samples. This paper describes the preparation of a substitute sample dilution buffer and compares its effectiveness to that of other commercial preparations. A further examination of buffer-introduced chromatogram artifacts is also carried out.

MATERIALS AND METHODS

A Model 6300 amino acid analyzer supplied by Beckman Instruments (Fullerton, CA, U.S.A.) was used for the tests and a Model SP 4270 plotting/integrator, supplied by Spectra-Physics (San Jose, CA, U.S.A.) integrated the analyzer data. The parameters selected for calibrating the integrator as well as the buffers and reagents used in the analyzer have been given before[1]. The commercial dilution buffers used in this work were obtained from Beckman Instruments (Cat. No. 338083) and Pierce (Rockford, IL, U.S.A., Cat. No. 2716). The substitute dilution buffers were prepared with distilled water that had been pumped through a Milli-Q™ water purification station, supplied by the Millipore (Cat. No. ZD20 230 84).

A volume of 500 ml of substitute dilutions buffer was prepared by the addition

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of 9.8 g trisodium citrate Sepramer™ grade (BDH Chemicals, Poole, U.K.), 7.8 ml of concentrated hydrochloric acid (Merck, Darmstadt, F.R.G.) and 1.4 ml of thiodiglycol (Pierce). After the preparation had been taken to volume it was passed through a Millipore membrane, Type HA, 0.45 μm. The pH was adjusted to a value of 2.22 by dropwise addition of hydrochloric acid. For purification of the thiodiglycol 20 ml of water was added to 10 ml of the concentrate and the solution was passed down an activated charcoal (Norit) column (bed of 2 cm). Aliquots of 4.5 ml were collected and used to prepare substitute buffer in place of using the concentrate. In each test of dilution buffer, 0.2 ml of "Beckman" amino acid calibration mixture (2.5 μml/ml) (Cat. No. 338088) was diluted to 5 ml in a standard flask and 50 μl of the resulting solution was analysed.

RESULTS AND DISCUSSION

When embarking upon a project that involves providing a substitute reagent it is of prime concern that a high grade of purity be established for the proposed substitute. The use of a water purification system, such as that supplied by Millipore is essential for the preparation of buffers. Further, the buffer must be subjected to membrane filtration before being used in the analyzer in order to protect the ion-exchange column from contamination. The analyzer column is of metal construction, identical in appearance to those columns used in high-performance liquid chromatography (HPLC), but whereas a guard column can be used with HPLC, there is no such protective device available for an amino acid analyzer.

Fig. 1 shows four chromatograms of an amino acid calibration mixture. For each chromatogram a different dilution buffer had been used. The sample in chromatogram 1 had been diluted with Beckman Na-S buffer; sample 2 had been diluted with the Pierce buffer; sample 3 with the initial substitute buffer, and sample 4 with substitute buffer that contained thiodiglycol which had been passed through the activated charcoal column. Thiodiglycol concentrate (product of Pierce) is recognized by the company as being a product that is difficult to supply in a pure state. However, there is no great difficulty presented to removing the contamination, in the procedure described above. All the dilution buffers were ideal as far as ionic strength and pH measurement were concerned; any moderate variation in pH would have effected the retention time (t_R) for aspartic acid (peak at 6.07 min on chromatogram 4) and placed the peak outside the integrator window for making the measurement for that peak[2]. The arrow from the encircled letter A indicates a point on the chromatogram baseline at which an artifact peak occurs. Although this peak is absent in the chromatogram obtained with Beckman dilution buffer (chromatogram 1) it is present in chromatogram 2 and to a lesser degree in chromatogram 3, the intended substitute. Chromatogram 4 is entirely free of the contaminant. Further, the contaminant responsible for artifact A on chromatogram 2 also occurs when this dilution buffer is analyzed in the absence of amino acid calibration mixture. In order to establish beyond doubt that the impurity had been completely removed, the calibration mixture used to obtain chromatogram 4 had been further diluted (1:1) with substitute buffer containing the purified thiodiglycol concentrate. In deciding to use an activated charcoal (Norit) column for purifying the thiodiglycol, it had been assumed that the contaminant was an amine (reacting with ninhydrin). Thiodiglycol is included in buffer preparations to prevent oxidation of methionine during the analysis procedure.

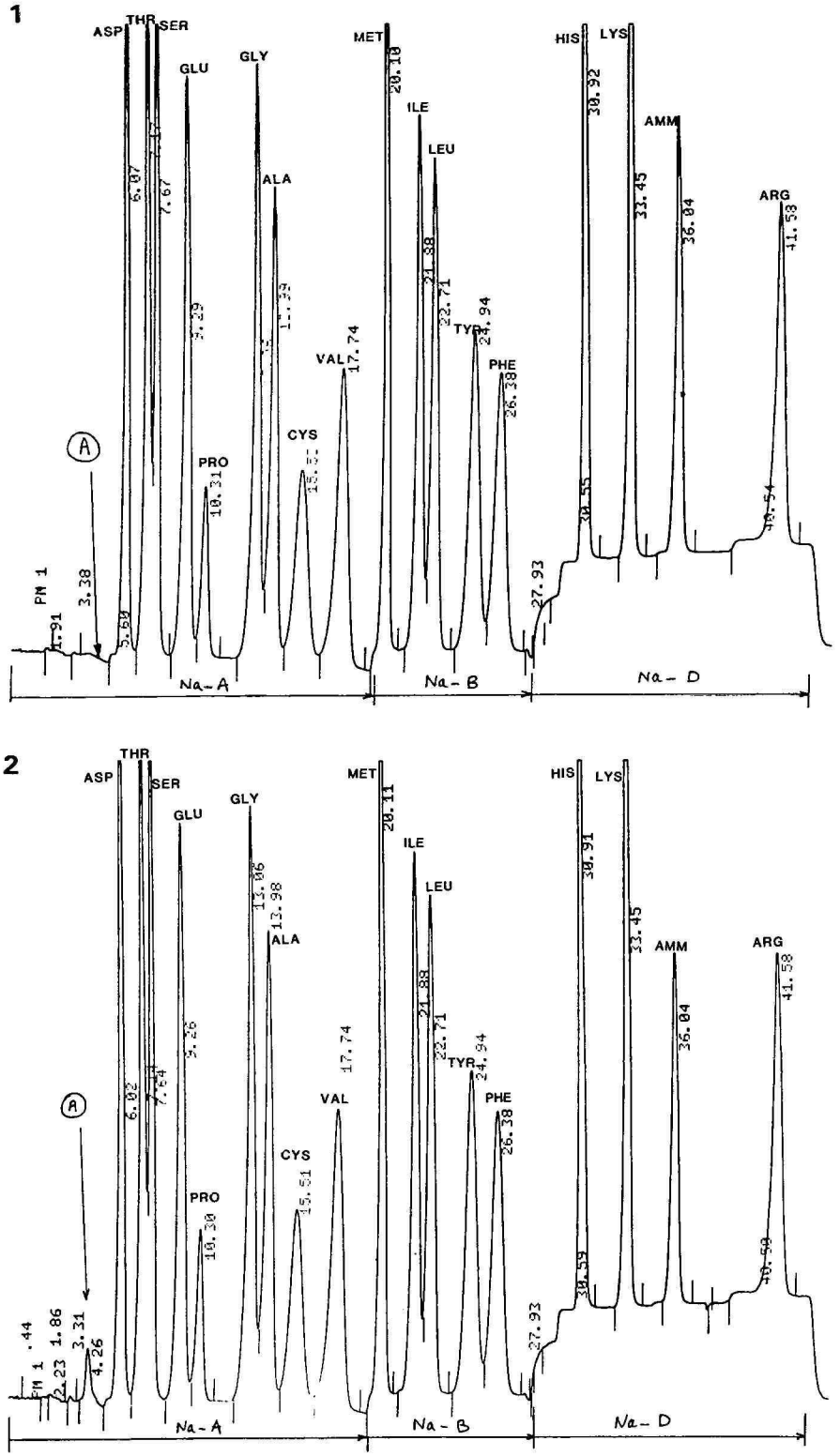
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Fig. 1.

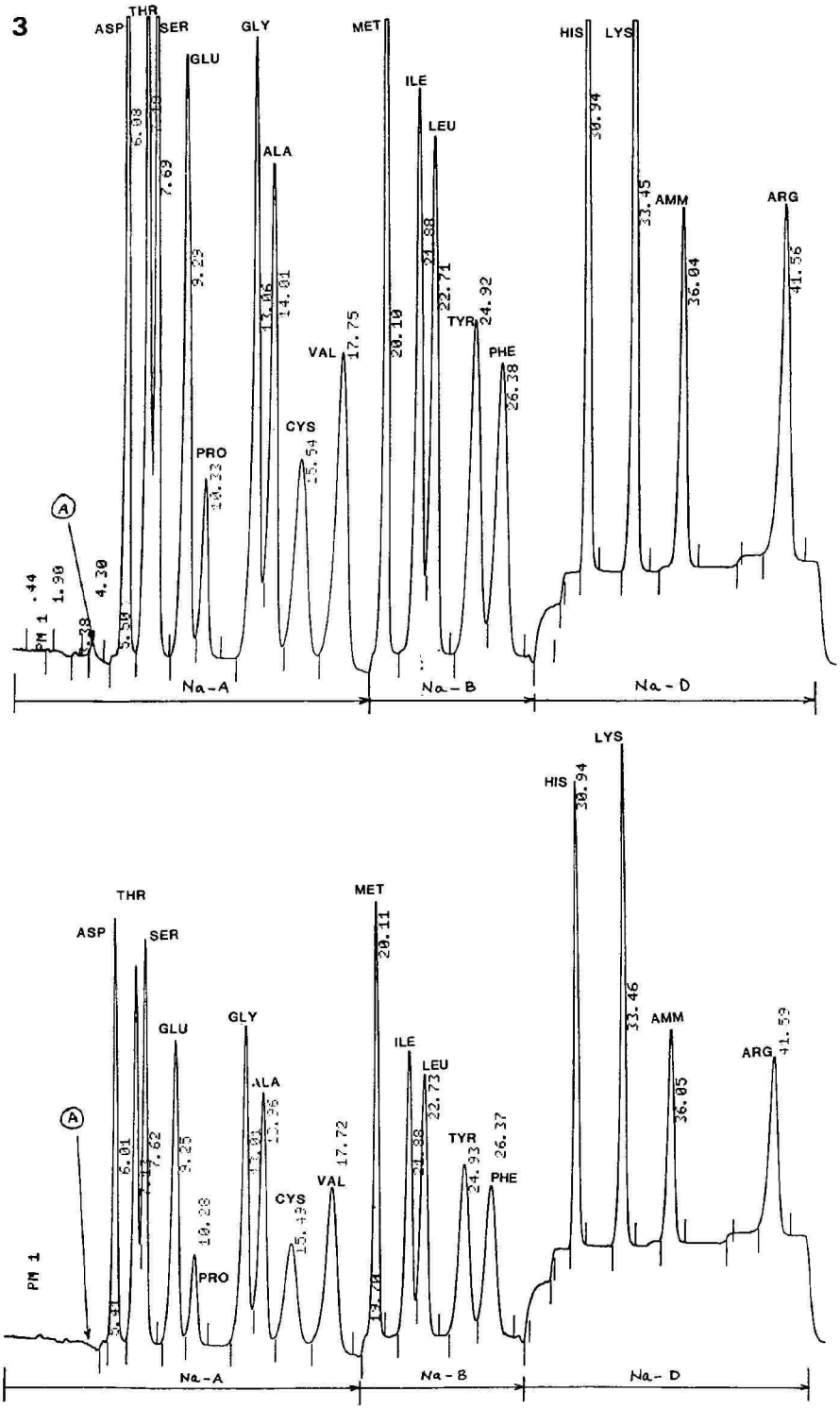

Fig. 1. Chromatograms of amino acid calibration mixture which had been diluted with buffers from divers sources. (1) Sample diluted with Beckman Na-S buffer; (2) sample diluted with the Pierce buffer; (3) sample diluted with the initial substitute buffer; (4) sample diluted with substitute buffer that contained thiodiglycol which had been passed through an activated charcoal column. Time scale in minutes.

Beneath chromatogram baseline printout, the chromatograms have been partitioned into three sections. Amino acid elution in each section being under control of one of the three elution buffers[1]; Na-A, Na-B and Na-D. Where the arrow heads are located the baseline effect of a buffer breakthrough is recorded, shown by an upward shift in the baseline as each succeeding and more concentrated elution buffer enters the analyzer colorimeter. The elution of an amino acid peak close to a buffer breakthrough point can cause a problem when using an automatic integrator; a portion of the baseline shift can be included in the counts attributed to the bonafide peak. Selection of the most satisfactory peak width parameter is paramount to obtaining accurate peak integration. Also when there is a baseline blip close to the leading edge of a peak the blip counts can be likewise attributed to that peak. In all chromatograms shown in Fig. 1, peak markers are used to locate where the integrator recognizes the beginning and end of a peak. For a separated peak a mark is placed below the baseline at the point pf peak emergence and above the baseline at the tailend of the peak. In chromatogram 1, examples of each problem appears to have taken place. The first peak marker for the aspartic acid peak (t_R = 6.07 min) includes a baseline blip and the histidine peak (t_R = 30.92 min) includes a portion of the buffer breakthrough effect. However, all is in order because there has been a time printout by the integrator at the base of each peak; the integrator has "sensed" that the blip and the breakthrough effect are not part of the aspartate and histidine peaks respectively.

In chromatogram 2 the situation is not so clearcut; the blip in front of the aspartate peak (t_R = 6,02 min) would have been added to counts attributed to the aspartate peak if an inadequate peak width parameter had been selected; this is evidenced by the missing time entry at the base of the aspartate peak. As the contaminant causing the blip is present in all chromatograms but is absent when dilution buffer from any source is subjected to analysis, the source of the blip must be the amino acid calibration mixture. Another buffer caused artifact is to be seen immediately preceding the recording of the arginine peak (t_R = 41.58 min on chromatogram 1). The sharp rise in the baseline at the point of entry of buffer Na-D can be accounted for in terms of increased concentration of sodium ion in buffer Na-D, but this buffer also carries a colour producing substance which shifts the baseline (upwards) before the arginine peak is eluted (see Fig. 1). Prolonging the use of batches of buffer Na-D seems to exacerbate this problem.

Threonine and serine have always been difficult to resolve by amino acid chromatography, and the immoderate trend to shorten overall analysis time has compounded this difficulty. In an attempt to obtain better resolution of these two closely related amino acids the practice of adding an alcohol to the relevant buffer, Na-A in this case, was adopted. Initially methanol was used as an additive when operating a Technicon (NJ, U.S.A.) AutoAnalyzer™. Later the addition of isopropanol was introduced for the operation of buffer systems in Beckman amino acid analyzers. It is now possible that the presence of the isopropanol, or rather a contaminant in the isopropanol is the most likely source of the artifact that is co-eluted with the histidine peak (t_R = 30.92 min) after the analyzer has been idle for periods of 24 h or longer[1].

Finally, it is taken as understood that when operating a sophisticated analyzer, such as the Beckman 6300 instrument, alteration of any component or, as in this case, replacement of a buffer can have a profound effect on the analysis result, as

well as future performance of the analyzer. In replacing a buffer the main risk would arise from inducing an increase in back pressure from the ion-exchange resin column due to deposition of particulate matter atop the resin bed. Filtration membranes with pore size of 0.45 μm or even 0.22 μm must be used to prevent column contamination. It is further proposed that the weakness of any enterprise associated with provision of buffers, is in maintaining contaminant free preparations over long periods of time. With commercially prepared buffers expiry dates are given by the manufacturer to draw attention to the detriment that occurs through storage. In the case of dilution buffer, which is adjusted to a low pH value and is composed predominantly of water, storage under refrigeration and discarding unused volumes after a period of three months should ensure a trouble-free operation. However, if bacterial contamination is considered to be a likely event, depending on the circumstances, the addition of a drop of caprylic acid to inhibit bacterial growth would be advisable.

REFERENCES

1 L. B. James, *J. Chromatogr.*, 403 (1987) 271.
2 L. B. James, *J. Chromatogr.*, 369 (1986) 178.

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Note

Use of first derivative conductometric detection in ion chromatography

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Ion chromatography is a widely used, versatile separation system which was originally developed in 1975[1]. The typical applications include environmental analyses[2], industrial analyses[3], biochemical analyses[4], as well as a wide variety of other applications[5]. The availability of good detection systems has assisted in the growth and success of ion chromatography by providing a mechanism whereby trace levels of ions can be determined with a high degree of precision and accuracy. Conductometric, electrochemical, and photometric detectors have all made significant contributions to ion chromatography[6]. However, the detectors most frequently used in ion chromatographic analyses give the best results only when the species of interest are well resolved from any potential interfering ions. The presence of overlapping peaks in an ion chromatogram, as in many other areas of chromatography, increases the difficulty of analysis and makes accurate quantitative analysis more time consuming, the potential for the apparent co-elution of peaks always is of concern to the analytical chemist and must be considered in many analytical situations.

Boeke[7] employed a derivative mode detector in gas chromatography and Essigmann and Catsimpoolas[8] introduced this mode in a UV dual channel detector for use in liquid chromatography. In this paper, we report a simple modification of a commercially available instrument which provides for first derivative conductometric detection. The first derivative detector can measure retention times directly, can easily observe differences in peaks with similar retention times, can provide for accurate quantitative detection especially for overlapping peaks, and can increase the operating efficiency of an ion chromatograph.

EXPERIMENTAL

Chromatographic system

A wide variety of chromatographic components were used in this study. One of the less obvious manifestations of this use of multiple systems is that the detection mode described here is thus applicable to a wide variety of situations and equipment. A general flow schematic could be described as the following using one specific system as an example: a high-performance liquid chromatography pump (Tracor Model 959) was used to pump the eluent through the entire system which was comprised of (in order of appearance) an injection valve (Tracor Model 925), a separator column (250

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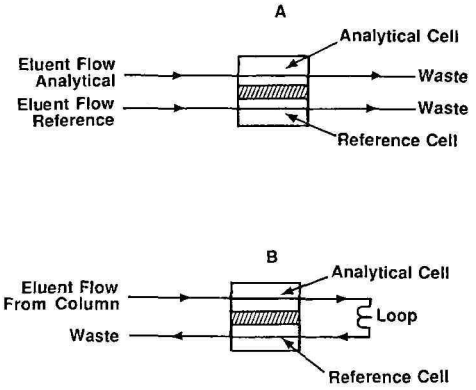

Fig. 1. Flow schematic through dual-channel conductometric detector. (A) Eluent flow as originally designed for the detector. The reference eluent flow is essentially subtracted as a blank value. (B) New flow arrangements whereby the detector will operate in the first derivative mode.

mm × 4.6 mm I.D.; Dionex HPIC-AS4), a suppressor column (optional) (Dionex AMMS), a single-channel conductivity detector (for comparison purposes) (Dionex Model 2010 ion chromatograph), and the first derivative conductivity detector (Tracor Model 965). All of the work was recorded on a dual pen chart recorder to facilitate comparison (Houston Instrument).

Fig. 1 shows the geometry of the dual-channel conductivity detector. Fig. 1A shows the flow schematic of the detector as originally received from the factory and Fig. 1B shows the new flow schematic as used for this work.

Reagents

All reagents were reagent grade or equal quality chemicals. The anion solutions were prepared either from the sodium salts (for most of the inorganic ions) or from the pure material (for the organic acids). The water used to prepare all of the solutions and the chromatographic eluent was building deionized water which was distilled in our laboratory prior to use.

Procedure

The detectors were connected in series for comparison since the development of a new, or modified, detection system must be superior in at least some applications to the current detection modes in order to be of significance. During the course of this work, we operated the chromatographs using normal operating guidelines with the exception of the modification to the dual-channel detector and the use of the detectors in series.

During portions of this research, the chromatographic operating conditions were manipulated in such a manner as to decrease the resolution of the system. This would not be the typical manner for a chromatographic system but it was done here in order to compare the detection modes under less than ideal conditions.

For quantitative determinations, we measured the peak height from the normal conductivity detector and used a triangle technique to calculate the area for the first derivative detector.

Examples of the chromatographic results and the illustration of the methods of calculation will be presented later in the paper. The calibration curves obtained from the normal conductivity detector were then compared with the calibration curves obtained from the first derivative detector.

The operating conditions were optimized for best chromatographic results for the determination of the linear operating range and the evaluation of the detection limits. Also, in the evaluation of the effect on the first derivative detection on the change in the loop volume, the optimum chromatographic conditions for that particular situation were employed.

MATHEMATICAL BASIS FOR DETECTOR RESPONSE

A complete theoretical treatment of first derivative detection has not been included as a very concise, lucid explanation is available in the literature[9].

RESULTS AND DISCUSSION

An example chromatogram is given in Fig. 2. Fig. 2 shows the results of the chromatographic separation of a multiple anion solution followed by both normal conductivity detection as well as first derivative conductivity detection. It is quite easy to see the relationship between the two chromatograms and also to see that the first derivative chromatogram is quite efficient and easy to use.

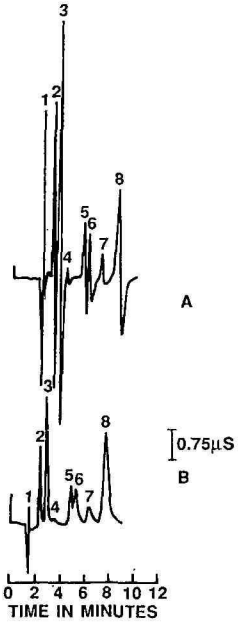

Fig. 2. (A) First derivative conductometric detection of a solution composed of eight inorganic ions. (B) Normal conductometric detection for this same solution. Peak identification is as follows: 1 = 0.4 ppm F^-; 2 = 0.4 ppm Cl^-; 3 = 1 ppm NO_2^-; 4 = 2 ppm PO_4^{3-}; 5 = 2 ppm SO_3^{2-}; 6 = 1 ppm Br^-; 7 = 2 ppm NO_3^-; 8 = 5 ppm SO_4^{2-}. Chromatographic conditions: Dionex AS4 separator column (250 mm × 4.6 mm I.D.), 0.003 *M* $NaHCO_3$ + 0.0024 *M* Na_2CO_3 eluent at a flow-rate of 2.3 ml/min. Chart speed is 0.5 cm/min.

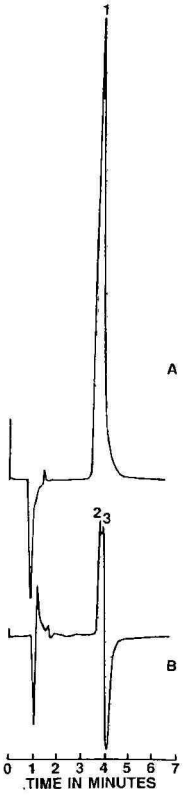

Fig. 3. (A) Normal conductometric chromatogram of a nitrate–sulfate solution purposely co-eluted. (B) Same solution using the first derivative detection mode. Notice that the first derivative detection does indicate that there are co-eluted peaks not just a single peak as seen by the normal conductance detection. The two detectors were connected in series for this experiment so the chromatographic tracings actually represent two different detection modes for the same injection. Chromatographic conditions: Dionex AS4 separator column (250 mm × 4.6 mm I.D.), eluent was 0.0045 *M* $NaHCO_3$ + 0.0036 *M* Na_2CO_3 at a flow-rate of 1.8 ml/min and a chart speed of 1.0 cm/min.

The key advantage to the use of the first derivative conductometric detection is in the evaluation of overlapping peaks. Fig. 3 illustrates a rather extreme case of overlapping peaks. Fig. 3A is the normal conductivity chromatogram showing what appears to be a single large peak. The same solution is shown in Fig. 3B using first derivative conductometric detection (the two detectors were actually linked in series). It is obvious from Fig. 3B that there really are two peaks present and not just the one. Granted, quantitative analysis of the two peaks would be quite difficult; but at least the presence of two peaks would be confirmed, a fact which would most likely have been missed using normal conductivity detection.

The area measurements for this work reported here were performed by approximating the area as a triangle. Table I illustrates the error in quantitative analysis which was obtained from experimental data for overlapping nitrate–sulfate peaks. Data reduction for the normal mode was performed based on peak height while that for its first derivative mode was based on area as discussed later. The first derivative

TABLE I

OBSERVED CONCENTRATION ERRORS FOR OVERLAPPING PEAKS

Actual conc. (ppm)		*Nitrate (%)*		*Sulfate (%)*	
NO_3^-	SO_4^{2-}	*Normal mode*	*First derivative mode*	*Normal mode*	*First derivative mode*
2.5	2.5	6	0.4	18	6
5.0	5.0	6	3.0	12	0
7.5	7.5	3	0.7	11	1
10.0	10.0	4	0.5	13	0.5

mode shows smaller error indicating that the first derivative mode is capable of performing quantitative analysis at least as well as, if not better than, normal conductivity detection for overlapping peaks. Fig. 4 illustrates this kind of overlap where it is obvious, even from normal conductivity detection, that two peaks are present. Fig. 5 is an example illustrating exactly how the quantitative analysis was performed using the first derivative chromatogram.

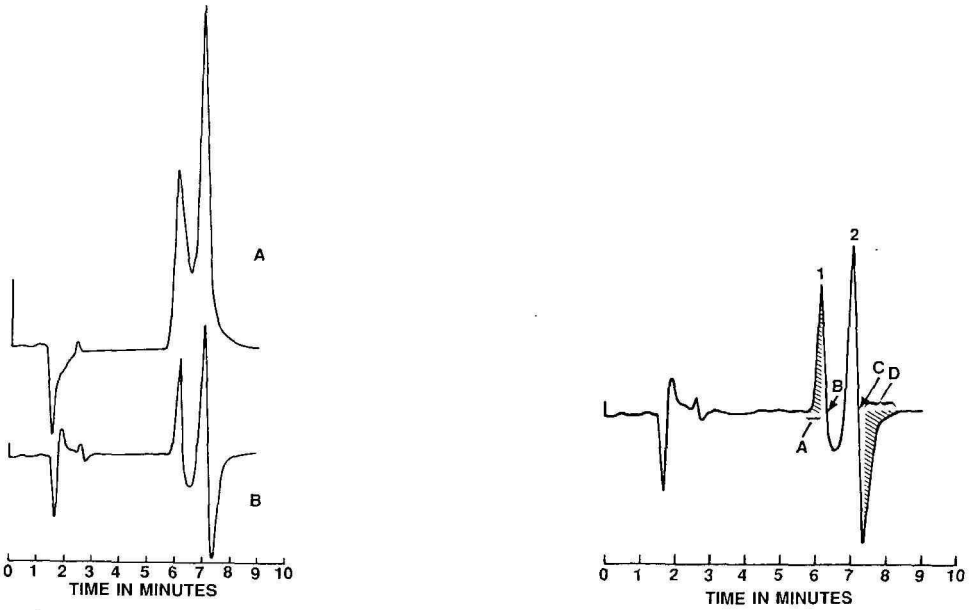

Fig. 4. (A) Normal conductometric detection of a nitrate–sulfate solution where the conditions were such that complete resolution was not obtained. (B) First derivative conductometric detection of the same solution. Chromatographic conditions: Dionex AS4 separator column (250 mm × 4.6 mm I.D.), eluent was 0.00375 *M* $NaHCO_3$ + 0.0030 *M* Na_2CO_3 at a flow-rate of 1.2 ml/min. The chart speed was 1.0 cm/min.

Fig. 5. Example first derivative chromatogram of two overlapping peaks. The first peak is the nitrate ion and the second peak is the sulfate ion. A corresponds to the baseline width under the first peak which is used to determine the area, and hence quantitate, the nitrate ion concentration. B represents the retention time for the nitrate ion. C corresponds to the retention time for the sulfate ion. D corresponds to the baseline width under the second peak used to determine the area and quantitate the sulfate ion concentration.

TABLE II

CALIBRATION CURVE: COMPARISON OF NORMAL AND FIRST DERIVATIVE CONDUCTIVITY DETECTION

Concentration range	*Normal detection*		*First derivative detection*	
	Slope	r^{**}	*Slope*	r^{**}
0.1–2 ppm (5)*	6.86	1.000	6.03	0.999
1.0–20 ppm (5)	5.33	0.999	4.94	0.998
10–200 ppm (6)	7.71	0.999	2.89	0.985
50–600 ppm (6)	8.24	0.999	1.53	0.982
0.1–20 ppm (8)	5.41	0.999	5.06	0.998
10–600 ppm (8)	8.11	0.998	1.14	0.707

* Number of data points in calibration curve.
** Correlation coefficient.

The linear dynamic range for this detector compares favorably with normal conductivity detection. The range is approximately 0.1–400 ppm. The calibration data is shown in Table II where the calibration data is broken down into several linear portions more closely representing the way experimental data would be obtained and manipulated. The change in slope as the concentration increases for the first derivative curve indicates that the calibration curve is actually an arc of large radius. The correlation coefficients confirm that any relatively small segment (a variation in concentration of slightly over one order of magnitude) can be quite adequately considered to be a straight line. The linearity of this method would lead to very acceptable quantitative results when good analytical practices are followed for data collection and manipulation.

One important feature of the first derivative detector is the volume of the loop used to connect the two cells in the detector. Loop volumes of 0.02–0.08 ml were investigated. The sensitivity of the method increases with increasing loop volume since the two conductance readings from which the derivative is made will be farther apart in time. A large loop volume provides for higher sensitivity for sharp peaks with relatively small peak widths. Peaks with a large peak width will show a decreased sensitivity due to the slow rate of change of the detector signal and hence the smaller difference observed by the first derivative detector. In all cases, the loop volume should be much smaller than the peak elution volume.

We have had the opportunity to observe the performance of the first derivative detector under a variety of different analytical operating conditions. The detector response will, of course, vary to some degree from one set of conditions to the next. The detector has worked quite well in a single column (non-suppressed) mode providing that the conductance of the eluant is sufficiently low to allow for adequate detector measurements. This has not been a problem with any of the eluents typically used in single-column ion chromatography with which we have experimented. We have also used the detector in a two-column mode, with a suppressor column, and have found the results to be quite satisfactory. As with the previous material presented here, the first derivative detector seems to work as well as, if not better than, the normal conductivity detector and its application can follow the rules of applications for normal conductivity detectors.

As with any scientific procedure, a gain in one aspect of a method is usually made at the expense of a second aspect. In the case of the first derivative detector, the advantage is that the detector is better at quantifying overlapping peaks than is normal conductivity detection. The disadvantages are that the first derivative conductivity detection has an apparent, although not extremely limiting, loss of linearity in the calibration curves. Another disadvantage, or loss, is that the sensitivity of the first derivative detector decreases as the peak width increases thereby decreasing sensitivity for the broad, later eluting species.

CONCLUSION

The first derivative conductivity detection mode described in this manuscript is an efficient detection mode for the detection and quantification of overlapping peaks in ion chromatography as well as being applicable for non-overlapping peaks. The detector has the ability to ascertain the presence of overlapping peaks which might be missed using other detection modes. In addition, the first derivative detection can be performed by making a small adjustment to a currently existing, commercially available, detector. The wide dynamic operating range and the relatively low detection limits imply that the first derivative conductometric detector should have a wide range of applicability in ion chromatographic analysis.

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REFERENCES

1 H. Small, T. S. Stevens and W. C. Bauman, *Anal. Chem.*, 47 (1975) 1801.
2 P. Pecevich, *Pollut. Eng.*, 17 (1985) 53.
3 L. K. Tan and J. E. Detrizac, *Anal. Chem.*, 57 (1985) 1027.
4 I. Yoshizawa and M. Kaneyama, *J. Chromatogr.*, 338 (1985) 404.
5 J. Weiss, *Handbook of Ion Chromatography*, Dionex Co., Sunnyvale, CA, 1986, pp. 185–221.
6 P. R. Haddad and P. Jandik, in J. G. Tarter (Editor), *Ion Chromatography*, Marcel Dekker, New York, 1986, pp. 87–156.
7 J. Boeke, in R. P. W. Scott (Editor), *Gas Chromatography*, Butterworth, London, 1960, p. 88.
8 J. M. Essigmann and N. Catsimpoolas, *J. Chromatogr.*, 103 (1975) 7.
9 R. P. W. Scott, *Liquid Chromatography Detectors* (*Journal of Chromatography Library*, Vol 11), Elsevier, Amsterdam, 1977, pp. 209–215.

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Note

E-Isozeatin and *cis*-norzeatin riboside as internal standards for cytokinin analyses

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Natural cytokinins are a group of plant hormones which occur in many forms, but all have a common 6-aminopurine structural element[1]. They are known to regulate many important plant functions at very low concentrations. The use of fused-silica capillary columns[2], selective gas chromatographic (GC) detectors[2], high-performance liquid chromatography (HPLC)[3], immunoassay[4], and the increased use of gas chromatography–mass spectrometry (GC–MS)[5] have greatly increased researchers' ability to detect and positively identify trace quantities of cytokinins in small samples of plant materials.

While these techniques provide the means to detect and identify even minor cytokinin components which may be physiologically important in a plant system, analytical procedures still cannot accurately and reproducibly quantitate cytokinins without the routine use of good internal standards[6]. This is due to the chemical nature of cytokinins and their trace quantities present which makes them especially susceptible to large variable losses that can occur in the processes of clean-up, isolation, concentration and derivatization. Glassware surfaces, especially non-silanized surfaces are a particularly significant source of loss during analysis. Losses can also occur in the GC–MS system.

Two of the most useful types of internal standards for cytokinin analysis are stable-isotope analogues and homologues or isomers. The former are chemically the most similar and thus have essentially the same retention time (t_R) on GC and HPLC. They have different *m/e* values. While these are the best internal standards for GC–MS analysis they are expensive, difficult to synthesize, and must be made for every compound of interest. The latter internal standards are chemically similar and can be chosen to have different retention times on HPLC and GC. This allows the extraction efficiency during the HPLC separation and purification steps to be evaluated, and quantitation of the permethylated derivatives with nitrogen–phosphorus detection or MS. They can have similar or different *m/e* values. It is also necessary that the internal standard be readily prepared in high purity. With these requirements in mind, the nor- and isozeatins appear worthy of consideration as internal standards.

An excellent stereospecific synthesis of *cis*-norzeatin exists[7], and the *E*- and *Z*-isomers of isozeatin have been described[8] although the syntheses are tedious. In this work we describe a new synthesis of *E*-isozeatin and study the usefulness of this isomer and the homologue *cis*-norzeatin riboside as internal standards for cytokinin analysis by GC–MS.

EXPERIMENTAL

Reference compounds

All cytokinins used in this study were synthesized at this laboratory by reported procedures[9]. Standard solutions were made up in methanol.

Materials

Potassium *tert.*-butoxide was from Aldrich. Methyl iodide (J. T. Baker, reagent grade) was glass distilled and stored in 3.5-ml vials over anhydrous calcium sulfate in a freezer until needed. Dimethylsulfoxide (DMSO, silylation grade) and dimethyldichlorosilane (DMDCS) were from Pierce. All other solvents were silylation or HPLC-grade.

The methylsulfinyl carbanion was prepared by mixing with a magnetic stirring bar in the ratio of 20 mg potassium *tert.*-butoxide per ml DMSO in a 3.5-ml screw-cap septum vial as needed.

Derivatization

The cytokinin mixtures were dried under a stream of dry nitrogen. The methylsulfinyl carbanion solution (50 μl) was added and mixed. Methyl iodide (10 μl) was added and mixed and the mixture was heated for 1 h at 40°C. The reaction vial was removed from the heat and water (50 μl) and chloroform (100 μl) were added and mixed. The chloroform layer was removed after centrifuging and washed again with 50 μl water. The chloroform layer was taken to dryness and 100 μl of ethyl acetate was added.

Synthesis of internal standards

E-4-Acetoxy-2-methylbut-2-enylphthalimide (1). A mixture of 1-chloro-2-methyl-4-acetoxybut-2-ene (47.0 g, 0.29 mol)[10], potassium phthalimide (53.7 g, 0.29 mol) and dimethylformamide (500 ml) was stirred at 90°C (3 h). The solvent was removed *in vacuo* and the residue was treated with water (500 ml) and ethyl acetate (500 ml). The organic layer was separated, washed with water and dried (sodium sulfate). After filtering, ethyl acetate was removed *in vacuo* and the residue was extracted with boiling hexane (300 ml). Crude 1 (56.0 g; 70.7% yield), m.p. 86–89°C. Anal. calc. for $C_{15}H_{15}NO_4$: C, 65.92; H, 5.53; N, 5.13. Found: C, 66.0; H, 5.52; N, 5.10.

E-4-Hydroxy-2-methylbut-2-enylphthalimide (2). A solution of 5 g of 1 in 300 ml methanol was stirred overnight at room temperature with 3 g of macroreticular Amberlyst A-26 (OH)[11]. The resin was removed by filtration and the filtrate evaporated to dryness *in vacuo*; yield, quantitative. Anal. calc. for $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.2; H, 5.66; N, 5.87.

E-Isozeatin (3). A solution of 2.30 g (10.5 mmol) of 2 and 0.32 g N_2H_4 (10

mmol) in 30 ml ethanol was allowed to stand at room temperature overnight. 0.5 *M* Sulfuric acid (10 ml) was added together with 30 ml water and the resulting precipitate was removed by filtration. The dried filtrate heated overnight on the steam bath with 0.8 g (5 mmol) of 6-chloropurine, 25 ml 1-butanol and 0.5 ml diisopropylethylamine. The solvents were removed *in vacuo* and an excess of saturated potassium bicarbonate solution was added. Again the volatiles were removed *in vacuo*, 30 ml water was added and the process repeated. The dry solid was extracted several times with ethanol. The concentrated ethanol extracts were applied to a silica gel column (50 cm × 25 mm, 200–325 mesh) made up in ethyl acetate–ethanol (9:1). Elution was carried out with a linear gradient of 1 l ethyl acetate–ethanol (3:1) to 1 l ethyl acetate–ethanol (9:1). The main fraction yielded 160 mg after recrystallization from ethanol–diethyl ether; m.p. 184–185°C; ^{1}H NMR (90 MHz dimethylsulfoxide-d_6) δ 8.17 (1H, s, 7 or 8-H), 8.07 (1H, s, 7 or 8H), 7.66 (1H, t, J = 7.5 Hz, N*H*), 5.42 (1H, t, J = 6 Hz; C = C*H*), 4.44 (1H, t, J = 6 Hz, O*H*), 3.8–4.2 (4H, m, 1-H, 4-H), 1.60 (3H, s, CH_3); the peaks at δ = 3.8–4.2 changed on the addition of ^2H_2O to 3.96 (2H, d, J = 6, –CH_2O) and 4.10 (2H, s, CH_2N) and those at 7.66 and 4.44 disappeared. Anal. calc. for $C_{10}H_{13}N_5O$: C, 54.78, H, 5.98; N, 31.95. Found: C, 54.2; H, 5.91; N, 31.9.

cis-Norzeatin riboside (4). 6-Chloropurine riboside (1.79 g, 6.2 mmol) was reacted with 0.6 g (6.9 mmol) of 4-hydroxy-*cis*-but-2-enylamine[7,12], in 15 ml 1-butanol and 1 ml of triethylamine and worked up as described above for 3; yield 0.33 g (16%); decomposed on heating. Anal. calc. for $C_{14}H_{19}N_5O_5$: C, 49.84; H, 5.68; N, 20.76: Found: C, 49.8; H, 5.68; N, 20.7.

Chromatography

Eight naturally occurring cytokinins and the internal standards isozeatin and *cis*-norzeatin riboside were separated by HPLC using a μBondapak C_{18} column (30 cm × 3.9 mm I.D., Waters Assoc.). The Waters HPLC system was programmed with a 1-h concave gradient (No. 7) from 6 to 30% acetonitrile in water at a flow-rate of 1.4 ml/min. The water contained 1% acetic acid. The cytokinins were detected at 264 nm with a Tracor 970 variable-wavelength detector. These same cytokinins were permethylated and analyzed with a Hewlett Packard 5970B quadrupole-based mass selective detector. The detector was operated in both the scan and selected ion monitoring (SIM) mode. The scan mode was utilized to obtain full spectra of the internal standards and naturally occurring cytokinins. The SIM mode was utilized to obtain response ratios. Permethylated cytokinins were introduced into the detector using an HP 5790A gas chromatograph with a capillary direct interface operated at 280°C. A 12 m × 0.2 mm I.D. dimethylsilicone cross-linked column (0.33 μm film thickness) was used in these experiments. The gas chromatograph was operated in the splitless mode with the column temperature programmed from 60 to 180°C at 25°C/min, then 6°C/min to the final temperature of 280°C. The injection temperature was 260°C. The silanized splitless silica liner contained a small silanized fused-silica wool plug placed 1 cm from the bottom of the liner. The ionization voltage was 70 eV, ion source operated at a fixed nominal temperature of 250°C and the head pressure was 5.0 p.s.i. Data was analyzed using a HP9816S computer, 9133 XV Winchester drive and HP59974 GC/MS software.

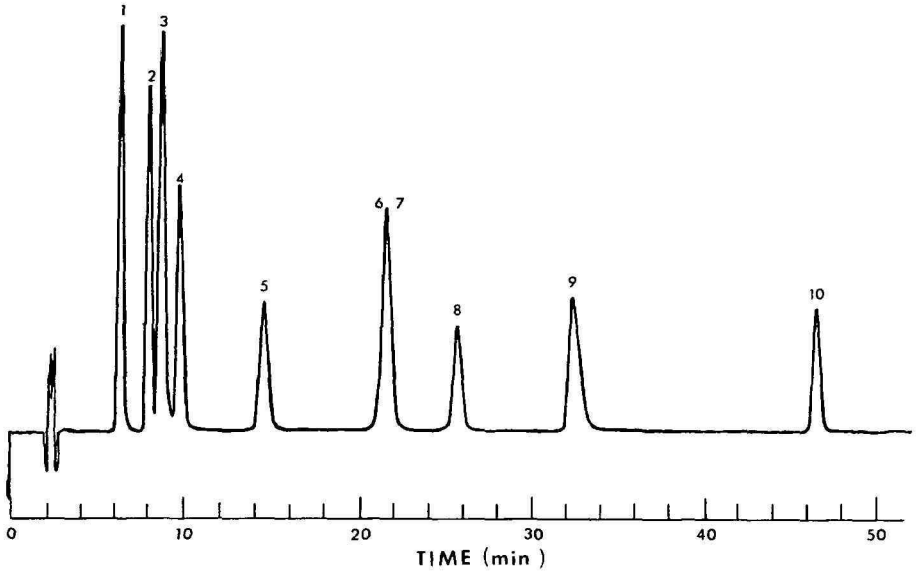

Fig. 1. Chromatogram of cytokinins on a μBondapak C_{18} (30 cm × 3.9 mm I.D.) HPLC column programmed from 6 to 30% acetonitrile in water at a flow-rate of 1.4 ml/min. Peaks (200 ng): 1 = *E*-isozeatin, 2 = *trans*-zeatin, 3 = dihydrozeatin, 4 = *cis*-zeatin, 5 = *cis*-norzeatin riboside, 6,7 = *trans*-zeatin riboside, dihydrozeatin riboside, 8 = *cis*-zeatin riboside, 9 = isopentenyladenine, 10 = isopentyladenosine.

RESULTS AND DISCUSSION

1-Chloro-2-methyl-4-acetoxybut-2-ene, prepared by reaction of *tert.*-butyl hypochlorite with isoprene[10], reacts with potassium phthalimide to give, as a readily

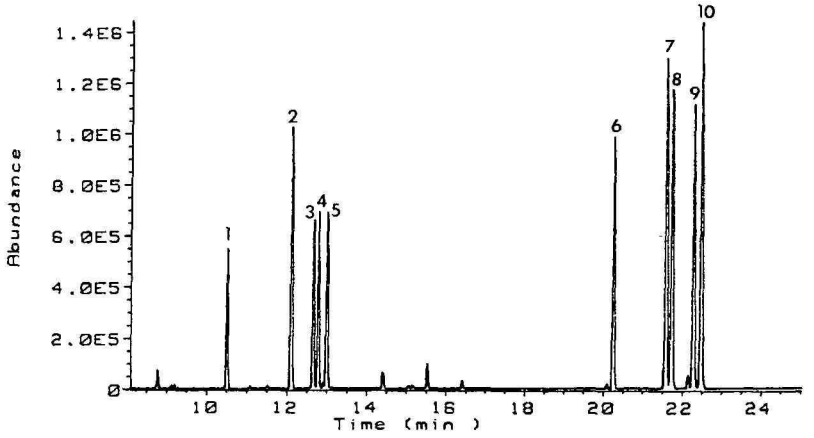

Fig. 2. Chromatogram of the permethylated cytokinins on a 12 m × 0.2 mm I.D. dimethylsilicone cross-linked column programmed from 60 to 180°C at 25°C/min, then 6°C/min to the final temperature of 280°C. Peaks (20 ng): 1 = isopentenyladenine, 2 = dihydrozeatin, 3 = *E*-isozeatin, 4 = *cis*-zeatin, 5 = *trans*-zeatin, 6 = isopentenyladenosine, 7 = dihydrozeatin riboside, 8 = *cis*-norzeatin riboside, 9 = *cis*-zeatin riboside, 10 = *trans*-zeatin riboside.

isolable product, *E*-4-acetoxy-2-methylbut-2-enylphthalimide (1), in 70% yield. Removal of the acetate and phthalimido groups gives *E*-4-hydroxy-2-methylbut-2-enylamine which reacts with 6-chloropurine to yield *E*-isozeatin. The melting point of 184–185°C (lit.[8] 177–179°C) clearly distinguishes it from the *Z*-isomer (m.p. 210–211°C).

The HPLC chromatogram in Fig. 1 shows complete separation of the internal standards from endogenous cytokinins. Thus a semi-quantitative estimation of the recovery of endogenous cytokinins from extraction and clean-up procedures can be made by comparison of the internal standard peak areas in the plant extract to expected peak areas for that quantity added, provided the extract is reasonably clean. The total ion chromatogram (TIC) in Fig. 2 illustrates baseline resolution of ten permethylated cytokinins.

Fig. 3A and C shows plots of the ion current ratios for the free bases, *trans*-zeatin (*m/e* 230), isopentenyladenine (*m/e* 188) and their internal standard *E*-isozeastin at *m/e* 230 *versus* the quantity of *trans*-zeatin and isopentenyladenine with a constant amount (5 μg) of internal standard. Fig. 3B and D shows plots for the ribosides, *trans*-zeatin riboside (*m/e* 216), isopentenyladenosine (*m/e* 174) and their internal standard (5 μg), *cis*-norzeatin riboside (*m/e* 202). Each sample was injected three times with the mean values and standard deviations shown in Table I. The

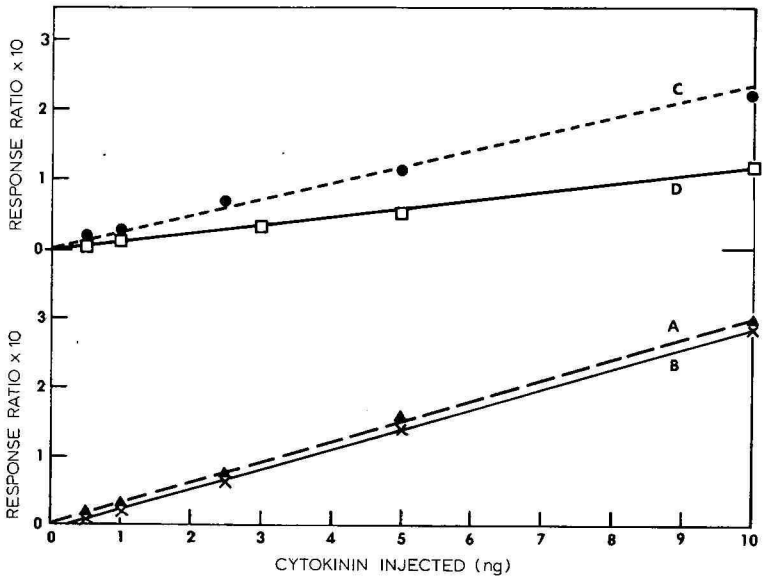

Fig. 3. (A) Response ratios at *m/e* 230 for *trans*-zeatin to *E*-isozeatin internal standard *m/e* 230 *vs.* amount of *trans*-zeatin injected with a constant amount (50 ng) of internal standard. (B) Response ratios at *m/e* 216 for *trans*-zeatin riboside to *cis*-norzeatin riboside internal standard *m/e* 202 *vs.* amount of *trans*-zeatin riboside injected with a constant amount (50 ng) of internal standard. (C) Response ratios at *m/e* 188 for isopentenyladenine to *E*-isozeatin internal standard *m/e* 230 *vs.* amount of isopentenyladenine injected with a constant amount (50 ng) of internal standard. (D) Response ratios at *m/e* 174 for isopentenyladenosine to *cis*-norzeatin riboside internal standard *m/e* 202 *vs.* amount of isopentenyladenosine with a constant amount (50 ng) of internal standard.

TABLE I

RESPONSE RATIOS FOR *TRANS*-ZEATIN AND *TRANS*-ZEATIN RIBOSIDE *VS.* INTERNAL STANDARDS AFTER DERIVATIZATION

Conc. of cytokinin in the sample (ng per 5 μg internal standard)	*Amount of cytokinins injected (ng)*	*Response ratios × 10*	
		Mean	*S.D.*
trans-Zeatin/iso-zeatin			
50	0.5	0.14	0.011
100	1.0	0.31	0.019
250	2.5	0.75	0.018
500	5.0	1.53	0.013
1000	10.0	2.91	0.026
trans-Zeatin riboside/cis-norzeatin riboside			
50	0.5	0.08	0.003
100	1.0	0.21	0.005
250	2.5	0.65	0.011
500	5.0	1.41	0.034
1000	10.0	2.82	0.005
Isopentenyladenine/iso-zeatin			
50	0.5	0.14	0.008
100	1.0	0.24	0.016
250	2.5	0.63	0.023
500	5.0	1.13	0.013
1000	10.0	2.21	0.102
Isopentenyladenosine/cis-norzeatin riboside			
50	0.5	0.04	0.003
100	1.0	0.10	0.003
250	2.5	0.30	0.002
500	5.0	0.56	0.004
1000	10.0	1.15	0.011

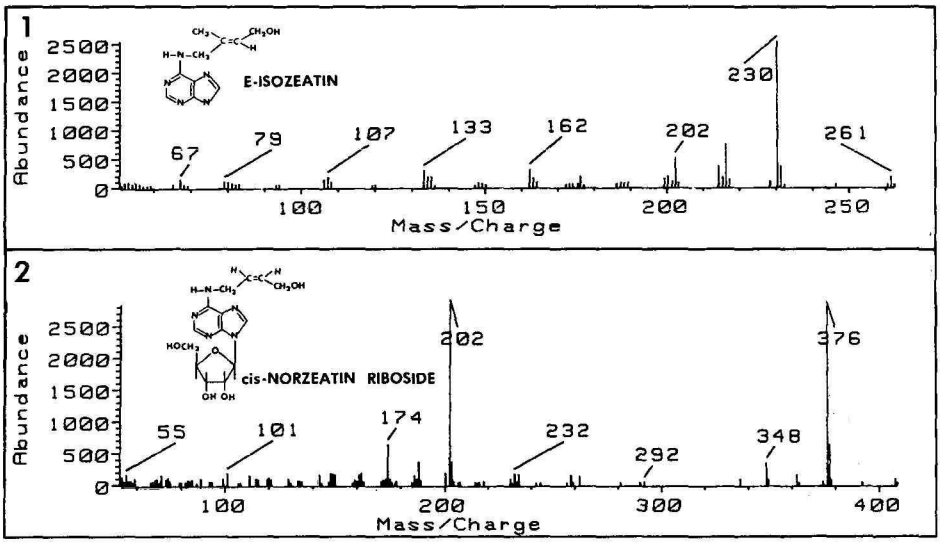

Fig. 4. Mass spectra of permethylated internal standards. 1 = *E*-isozeatin, 2 = *cis*-norzeatin riboside.

TABLE II

RECOVERY OF *TRANS*-ZEATIN AND *TRANS*-ZEATIN RIBOSIDE

Conc. of trans-zeatin and trans-zeatin riboside in the sample (ng)	*trans-Zeatin*		*trans-Zeatin riboside*	
	Mean	*S.D.*	*Mean*	*S.D.*
100	98	1.5	116	3.7
500	488	11.5	470	6.5

curves are linear over the range measured. The mass spectra of the above permethylated internal standards along with their structures are shown in Fig. 4.

To establish that both endogenous cytokinins and internal standards were recovered to the same extent, mixtures of these were taken through isolation and purification steps[13]. Two amounts (100 and 500 ng) *trans*-zeatin and *trans*-zeatin riboside each containing internal standards were used for this study. The results are shown in Table II. Each sample was injected three times.

In this study the base peak ions were monitored to give the greatest sensitivity, although, when the higher mass ions *m/e* 376 and 390 in the zeatin ribosides were monitored the response ratios were also linear.

Both internal standards were used in these experiments for determining response ratios and recoveries. The free base internal standard isozeatin was used *trans*-zeatin and isopentenyladenine and the riboside internal standard, *cis*-norzeatin riboside was used for *trans*-zeatin riboside and isopentenyladenosine. While it may be advantageous in some cases to use both internal standards in this manner, the response ratios were also linear for *cis*-norzeatin riboside *vs. trans*-zeatin and isopentenyladenine and isozeatin *vs. trans*-zeatin riboside and isopentenyladenosine so that only one of the internal standards would be necessary for the quantitation of a sample containing both free bases and ribosides.

While stable isotope internal standards are generally superior for GC–MS analysis, there are some significant problems associated with their use[14,15]. Results from this study indicate that the homologue, *cis*-norzeatin riboside and the isomer *E*-isozeatin provide an acceptable alternative to the use of stable isotope internal standards for quantitation of cytokinins.

REFERENCES

1 D. S. Letham and L. M. S. Palni, *Ann. Rev. Plant Physiol.*, 34 (1983) 163–197.
2 A. E. Stafford and J. Corse, *J. Chromatogr.*, 247 (1982) 176–179.
3 R. Horgan and M. R. Kramers, *J. Chromatogr.*, 173 (1979) 263–270.
4 E. W. Weiler, *Planta*, 149 (1980) 155–162.
5 R. O. Morris, *Plant Physiol.*, 59 (1977) 1029–1033.
6 B. J. Millard, in A. P. Delenkeer, R. R. Roneucci and C. Van Peteghem (Editors), *Quantitative Mass Spectrometry in Life Sciences, II*, Elsevier, Amsterdam, 1978, pp. 83–102.
7 R. Y. Schmitz, F. Skoog, A. J. Playtis and N. J. Leonard, *Plant Physiol.*, 50 (1972) 701–705.
8 N. J. Leonard and A. J. Playtis, *An. Quim.*, (1972) 821–825.
9 J. A. Kuhnle, G. Fuller, J. Corse and B. E. Mackey, *Physiol. Plant.*, 41 (1977) 14–21.
10 W. Oroshnik and R. A. Mallory, *J. Am. Chem. Soc.*, 72 (1950) 4608–4613.
11 L. A. Reed, III, P. A. Risbood and L. Goodman, *J. Chem. Soc. Chem. Commun.*, (1981) 760–761.
12 O. Wichterle and J. Novak, *Collect. Czech. Chem. Commun.*, 15 (1950) 309–321.
13 A. E. Stafford, J. A. Kunhle, J. Corse and E. Hautala, *J. Chromatogr.*, 294 (1984) 485–488.
14 J. L. Caruso and C. S. Zeisler, *Phytochemistry*, 22 (1983) 589–590.
15 B. H. Brown, S. J. Neill and R. Horgan, *Planta*, 167 (1986) 421–423.

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Note

Use of chloroform as a solvent for the formation of N-trifluoroacetyl-*n*-butylamino acid derivatives for gas chromatographic analysis

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The use of gas chromatography (GC) for the quantification of amino acids is a well established procedure and one of the most commonly used derivatives is the trifluoroacetyl-*n*-butyl (TAB) ester introduced by Gehrke and co-workers[1–4]. In their procedure, hydrochloric acid–*n*-butanol is used for esterification of the amino acids, followed by trifluoroacylation with trifluoroacetic anhydride (TFAA) in dichloromethane (20:80, v/v) and injection into the chromatograph for quantification without evaporating the excess of TFAA–dichloromethane. Evaporation of excess solvents is not recommended owing to losses of low-boiling TAB-amino acids (alanine, valine, etc.), possible destruction of sensitive amino acids at trace levels and the extra time and costs involved. Hence, the excess of TFAA and dichloromethane poses a problem in tropical climates owing to their low boiling points. The solvent evaporation problem during the hot and humid summer was so severe that it was not even possible to micropipette the TAB-amino acids dissolved in TFAA–dichloromethane for GC analysis. Hence, we needed a solvent with a much higher boiling point, and in this paper we report our studies on the use of chloroform as a solvent to replace dichloromethane.

EXPERIMENTAL

Apparatus

An Instrumentos Científicos CG Model 37 gas chromatograph equipped with a temperature programmer, a flame ionization detector and an intregator was used.

Reagents and methods

Amino acid standards and TFAA were purchased from Pierce (Rockford, IL, U.S.A.). Hydrochloric acid–*n*-butanol (3 *M*) was obtained from Regis Chemicals. chloroform and dichloromethane were obtained from Merck (Darmstadt, F.R.G.) and were distilled before use. The column packing materials were purchased and prepared as described in the literature[1]. The TAB derivatives were prepared as described[1] using 400 μg of each amino acid, 5 ml of hydrochloric acid–*n*-butanol for esterification and followed by trifluoroacetylation with TFAA (200 μl) + chloroform (1.5 ml).

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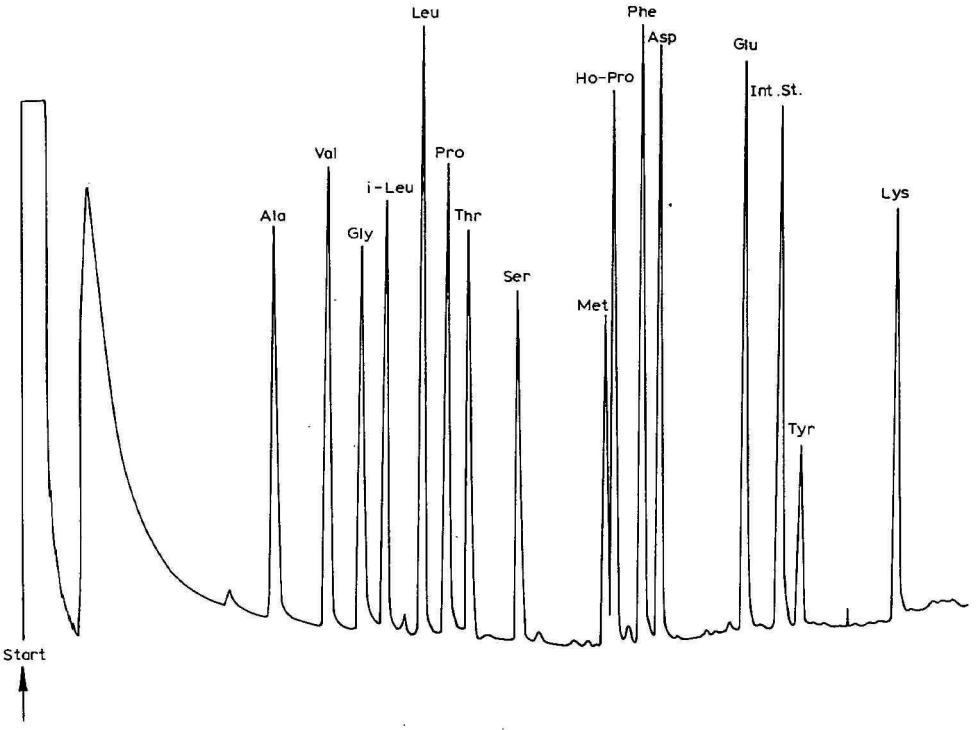

Fig. 1. GC of amino acid standards (1 μg each) on a Tabsorb column.

Chromatographic conditions

The following conditions were used: (a) a Tabsorb column with an initial temperature of 80°C, a final temperature of 210°C, an initial and final hold of 5 min with programming at 4°C/min; (b) a Tabsorb-HAC column with an initial temperature of 140°C, a final temperature of 250°, an initial and final hold of 5 min with programming at 6°C/min. In both instances the injector and detector temperatures were 185 and 240°C, respectively, using a nitrogen flow-rate of 30 ml/min.

RESULTS AND DISCUSSION

As can be seen from Figs. 1 and 2, chloroform worked reasonably well as an acylation solvent and no major differences were noted between chloroform and dichloromethane with regard to the responses and separation of the amino acids. In addition, no major contamination occurred. However, the use of chloroform offered an advantage over dichloromethane since there was no micropipetting problem for injection of TAB-amino acids and hence in our opinion it is a superior solvent for use in tropical climates.

ACKNOWLEDGEMENTS

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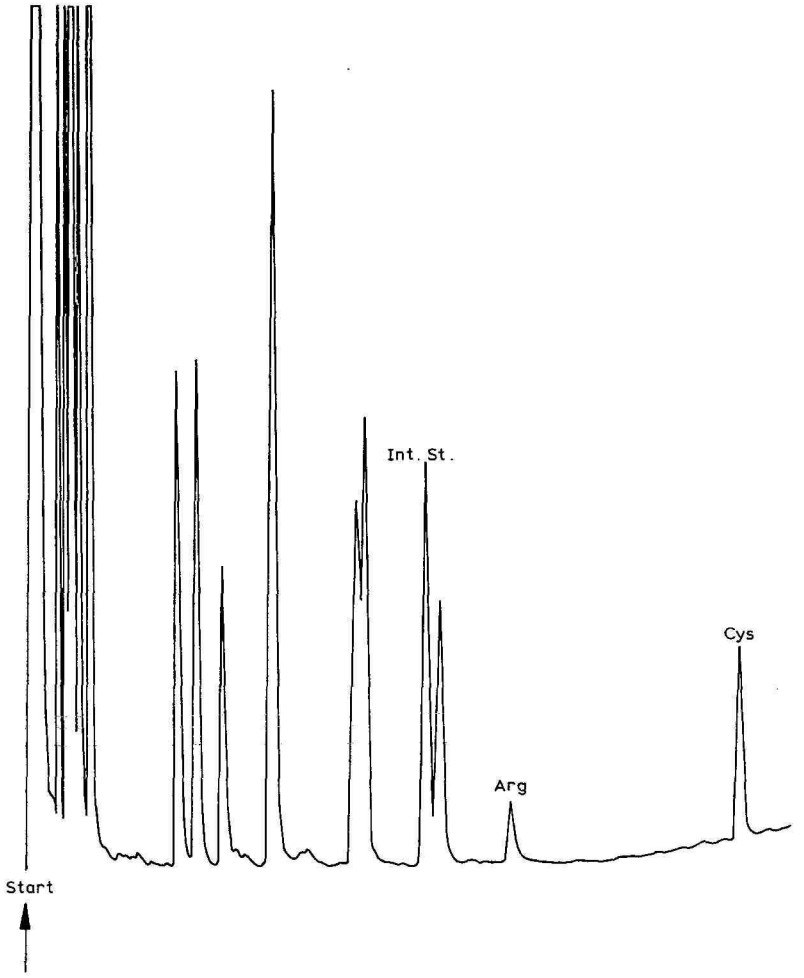

Fig. 2. GC of amino acid standards (1 μg each) on a Tabsorb-HAC column.

REFERENCES

1 F. E. Kaiser, C. W. Gehrke, R. W. Zumwalt and K. C. Kuo, *J. Chromatogr.*, 94 (1974) 113.
2 C. W. Gehrke, D. R. Yonker, K. O. Gerhardt and K. C. Kuo, *J. Chromatogr. Sci.*, 17 (1979) 301.
3 C. W. Gehrke, L. L. Wall, J. S. Absheer, F. E. Kaiser and R. W. Zumwalt, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 811.
4 C. W. Gehrke, F. E. Kaiser and R. W. Zumwalt (Editors), *Amino Acid Analysis by Gas Chromatography*, CRC Press, Boca Raton, FL, 1985.

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Note

Constituents of essential oil of *Thymus carnosus* Boiss.

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The genus *Thymus* (Labiatae) includes more than 200 species distributed throughout Europe, with different sections and subsections. The analysis of its essential oil has been the subject of numerous investigators[1–4].

Thymus carnosus Boiss. belongs to the subsection Thymastra Velen. (Sec. Thymastra Velen.) according to Jalas[5]. It is an endemic species from Huelva and Southern Portugal, and because of its very limited localization, it has been investigated only sparingly, and only preliminary work concerning the analysis of the essential oil is available[6], in which only one constituent was identified (carvacrol).

This paper reports the results of a detailed chemical analysis and the identification of 41 components of the essential oil.

EXPERIMENTAL

Plant material

Thymus carnosus Boiss. was collected from the shore sands between El Terrón and La Antilla, Huelva, Spain, in July 1983, when it was in the flowering stage. Voucher specimens were authenticated and then deposited in the herbarium of the Department of Botany, University of Seville. The essential oil was obtained by steam distillation of fresh plant material in a modified Clevenger apparatus. The yield was 2.28 ± 0.56% (v/w). The values of the determined physical constants were $n_D^{20} = 1.476$, $[\alpha]_D^{20} = -0.92$ and $d_{20}^{20} = 0.949$.

Thin-layer chromatography (TLC)

Silica gel pre-coated plates (Merck) were used with (A) *n*-hexane–diethyl ether (85:15) and (B) benzene–ethyl acetate (85:15) as mobile phases. Vanillin–sulphuric acid was used as the detection agent. The R_F values were as follows: borneol, 0.20 (A), 0.48 (B); bornyl acetate, 0.78 (A), 0.57 (B); linalool, 0.54 (A), 0.30 (B); and camphor (UV detection at 360 nm), 0.60 (B).

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IR spectroscopy

A Perkin-Elmer 1310 infrared spectrophotometer, range 4000–600 cm^{-1}, was used.

Gas chromatography (GC)

Hewlett-Packard 5834A gas chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen at a flow-rate of 30 ml/min. A 2 m × 1.4 in. I.D. Pyrex glass column packed with 3% DEGS + 10% Carbowax 20M on Chromosorb W (AW DMCS) (80–100 mesh) was used. The column temperature programmes were 20 min at 70°C, then increased to 180°C at 3°C/min (programme A), and 6 min at 70°C, 10 min at 100°C, 14 min at 150°C, remainder at 180°C (programme B). Other temperatures were injection chamber 200°C and detector 200°C.

Quantitation was carried out with a Hewlett-Packard 18850A reporting integrator.

Quantitative measurements of camphene, γ-terpinene, *p*-cymene, linalool, camphor, bornyl acetate and terpinen-4-ol were also carried out with *n*-hexanol as an internal standard.

Perkin-Elmer F-30 gas chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen at a flow-rate of 17 cm/s. An 80 m × 0.25 mm I.D. fused-silica capillary column (SE-54) was used. The temperature programme was 10 min at 100°C, then increased to 210°C at 3°C/min. Other temperatures were injection chamber 200°C and detector 200°C.

Peak areas were measured with a Perkin-Elmer M-1 integrator. The relative amounts of the components were calculated from the peak areas without using correction factors.

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett-Packard 5995B quadrupole gas chromatograph–mass spectrometer was coupled to a Hewlett-Packard 9885M data system. The carrier gas was helium at a flow-rate of 1 ml/min. A 12 m × 0.20 mm fused-silica capillary column (cross-linked OV-1) was used. The temperature programme was 2 min at 40°C, then increased to 140°C at 2°C/min. Splitless injection was used. Mass spectra were taken over the range *m/z* 40–600, utilizing an ionization voltage of 70 eV.

The mass spectra were compared with those of authentic samples and with literature spectra[7–9].

RESULTS AND DISCUSSION

The essential oil of *Thymus carnosus* Boiss. was analysed by TLC, GC, GC–MS and IR spectroscopy.

In the gas chromatogram the presence of more than 45 constituents was observed, some only in trace amounts (< 1%).

The IR spectrum from the main component, which crystalized spontaneously during the distillation of the oil (3350 sw, 2990 m, 2950 s, 2850 m, 1060 s, 1460 m, 1390 m, 1370 m, 1390 m, 1370 m, 1450–1500 mw, cm^{-1}) was completely superimposable on that obtained with a sample of borneol (97.74%). Also, its mass spectrum gave a molecular ion at *m/z* = 154, a base peak at *m/z* 95 and other characteristic

TABLE I

COMPOUND IDENTIFIED IN ESSENTIAL OIL OF *THYMUS CARNOSUS*

No.	*Compound**	*Peak area (%)***	*w/w (%)*	*Technique*
1	Unidentified			GC–MS
2	Cyclofenchene	t		GC; GC–MS
3	α-Thujene	1.49		GC; GC–MS
4	α-Pinene	4.53		GC; GC–MS
5	Camphene	10.74	9.56	GC; GC–MS
6	Thuj-3-en-2-ol	t		GC–MS
7	β-Pinene	1.36		GC; GC–MS
8	Myrcene	t		GC; GC–MS
9	Sabinene	t		GC; GC–MS
10	α-Terpinene	t		GC; GC–MS
11	Limonene	1.05		GC; GC–MS
12	1,8-Cineol	t		GC; GC–MS; TLC
13	β-Phellandrene	t		GC; GC–MS
14	Bornylene	t		GC–MS
15	β-Ocimene	t		GC–MS
16	γ-Terpinene	1.23	1.50	GC; GC–MS
17	*p*-Cimene	1.18	1.28	GC; GC–MS
18	β-Terpinene	1.22		GC; GC–MS
19	Terpinolene	t		GC; GC–MS
20	Unidentified alcohol, MW 156			GC–MS
21	β-Terpineol	t		GC–MS
22	Linalool	2.34	2.49	GC; GC–MS; TLC
23	Unidentified			
24	Camphor	6.05	3.75	GC; GC–MS; TLC
25	Verbenol + ?	t		GC–MS
26	Unidentified			
27	Unidentified			
28	Unidentified			
29	*d,l*-Borneol	43.65		GC; GC–MS; TLC; IR
30	Isoborneol	2.16		GC; GC–MS
31	Terpinen-4-ol	8.04	7.83	GC; GC–MS; TLC
32	Dihydrocarvone	t		GC–MS
33	*l*-α-Terpineol	t		GC; GC–MS
34	γ-Terpineol	t		GC; GC–MS
35	Verbenone	t		GC–MS
36	Nerol	t		GC; GC–MS
37	Neral	t		GC; GC–MS
38	Citral	t		GC; TLC
39	Linalyl acetate	t		GC; GC–MS
40	Bornyl acetate	8.57	9.22	GC; GC–MS; TLC
41	α-Terpinyl acetate	t		GC; GC–MS
42	Thymol	t		GC; GC–MS
43	Carvacrol	t		GC; GC–MS
44	β-Caryophyllene	t		GC; GC–MS
45	Aromadendrene	t		GC–MS
46	β-Maaliene + ?	t		GC–MS
47	Elemol + ?	1.36		GC–MS

* + ? = Tentatively identified.

** t = Trace (< 1%).

fragments at *m/z* 41 (relative intensity 27), 55 (16), 67 (13), 93 (10), 96 (11), 110 (23), 121 (7), 136 (6) and 139 (10). By comparison with a data bank and a bibliography, the compound was identified as *d,l*-borneol.

In decreasing order, camphene (5), bornyl acetate (40), terpinen-4-ol (31), camphor (24) and α-pinene (4) were found (Table I). The alcoholic fraction represents 57% of the constituents of the oil, mono- and sesquiterpenic hydrocarbons 24% and esters and ketones 15%. The high content of borneol in the sample studied is not unusual in the genus *Thymus*, as other species such as *T. villosus* sp. *Lusitanicus*[10], *T. satureoides*[11], *T. hiemalis*[12] and undefined *Thymus* oil of Spanish origin[13] have been found to contain 15.6, 26.2, 66–67 and 60%, respectively, of borneol.

It is interesting to note the presence of cyclofenchene (2), bornylene (14), β-terpinene (18), γ-terpinol (34), dihydrocarvone (32), thuj-3-en-2-ol (6), verbenol (25) and β-maaliene (46) as constituents, as they are rarely present in *Thymus* oils and, in the case of β-terpinene, in any essential oil.

Thymol and carvacrol, the main components reported in other species of *Thymus*[14,15], could be detected only in trace amounts.

REFERENCES

1 B. M. Lawrence, *Ann. Techn. VIII Congrèss International des Huiles Essentielles, Parf. et Aröm. Symph. Nat.*, Feradom, Grasse, 1980.
2 T. Adzet, R. Granger and J. Passet, *Biochem. Syst. Ecol.*, 5 (1977) 269.
3 J. Passet, *Parfum. Cosmét. Arôm.*, 28 (1979) 39.
4 J. Passet, *Dragoco Rep. (Ger. Ed.)*, 27 (1980) 234.
5 J. Jalas, *Bot. J. Linn. Soc.*, 64 (1971) 203.
6 A. Fernandes Costa, *Elementos de Flora Aromática*, Junta de Investigaçoes Cientificas do Ultramar, Lisbon, 1975, pp. 342–347.
7 E. Stenhagen, S. Abrahamsson and W. F. McLafferty, *Registry of Mass Spectral Data*, Wiley, New York, 1974.
8 Y. Masada, *Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry*, Wiley, New York, 1967.
9 W. Jennings and T. Shibamoto, *Qualitative Analysis of Flavour and Fragance Volatiles by Glass Capillary Gas Chromatography*, Academic Press, New York, 1980.
10 J. Miqueel, H. M. J. Richard and F. G. Sandret, *J. Agric. Food Chem.*, 24 (1976) 833.
11 M. J. Perez Alonso and A. Velasco Nereguela, *Phytochemistry*, 23 (1984) 581.
12 C. Ranti and G. Bruni, *Boll. Chim. Farm.*, (1960) 435.
13 T. Adzet, R. Granger, J. Passet and R. San Martin, *Plant Méd. Phytothér.*, 10 (1976) 6.
14 C. Mateo, M. P. Morera, J. Sanz, J. Calderon and A. Hernandez, *Riv. Ital. Essenze, Profumi, Piante Off.*, 60 (1978) 621.
15 R. Granger and J. Passet, *C.R. Acad. Sci., Ser. D*, 273 (1972) 2350.

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Note

Determination of trace amounts of iodine in sedimentary rocks by ion chromatography with amperometric detection

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A knowledge of the iodine content of sedimentary rocks is required for a number of purposes, including the determination of ancient salinities, distinguishing between marine and continental deposits, oil prospecting and evaluating environments. Analytical methods for the determination of iodine have in the past involved procedures such as extraction spectrophotometry, X-ray fluorescence spectrometry and the use of ion-selective electrodes[1,2]. These methods, however, suffer from the disadvantages of relatively poor sensitivity and/or interferences, particularly from other halogens. Another procedure is dynamic catalytic analysis[3,4], which suffers from the disadvantages of overelaborate techniques, significant interferences and the use of carcinogenic or poisonous reagents. Ion chromatography with conductimetric detection would seem to be an obvious technique for the determination of iodine, but it suffers from insufficient sensitivity[5].

We have developed an ion chromatographic technique for the determination of soluble trace iodine in sedimentary rocks in which amperometric detection is used.

EXPERIMENTAL

Determinations were carried out on a Dionex 16 ion chromatograph using an amperometric detector with a cell containing a silver working electrode and silver–silver chloride reference electrode. The chromatographic conditions were as follows: columns, Dionex HPIC-AG_3 (50 mm × 6 mm I.D.)–AS_3 (250 mm × 6 mm I.D.); sample loop size, 100 μl; and pump pressure, 280 p.s.i.

RESULTS AND DISCUSSION

Dionex recommend the use of 8 m*M* sodium carbonate and 20 m*M* sodium nitrate as eluents for the determination of iodine[6,7]. However, these eluents did not produce the optimum results (Fig. 1) and 20 m*M* sodium nitrate–0.025 m*M* sodium hydroxide was chosen in order to shorten the retention times and to improve both the sensitivity and peak shape.

The influence of the applied potential on the determination of iodine is shown in Fig. 2. The optimal potential was about 0.17 V, which was used in all subsequent experiments.

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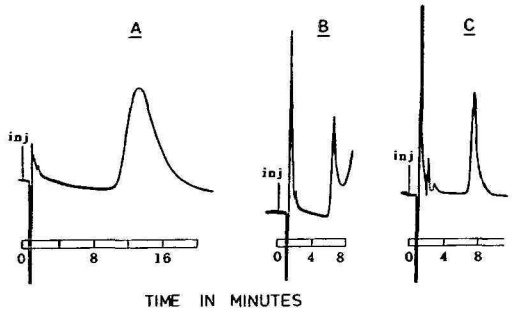

Fig. 1. Elution curves for iodide using different eluents: (A) 8 m*M* sodium carbonate, I^- 20 μg/ml, 300 nA/V; (B) 20 m*M* sodium nitrate, I^- 1 μg/ml, 100 nA/V; (C) 20 m*M* sodium nitrate–0.025 m*M* sodium hydroxide, I^- 50 ng/ml, 100 nA/V.

A typical calibration graph is shown in Fig. 3 and indicates a detection limit of about 10 ng/g iodine.

Interferences from Br^-, Cl^-, S^{2-} and SCN^- were studied and the findings are summarized in Table I. It was clearly possible to determine trace iodide in the presence of vastly greater concentrations of other halides. Separation from S^{2-} and SCN^- was also possible because these two ions had longer retention times than had iodide. When the concentrations of these two interfering ions were very high, it was possible to reduce or eliminate their interference by acidification and boiling of the analyte solutions.

The normal working procedure consists in shaking finely powdered rock samples (−200 mesh) for 3–5 days, filtering off the aqueous phase and injecting the liquid into the ion chromatograph.

The precision of the technique was assessed at 3.5% from ten replicate determinations of 50 ng/g of iodine. The results of the recovery tests are shown in Table II. The recovery averaged 100.8%.

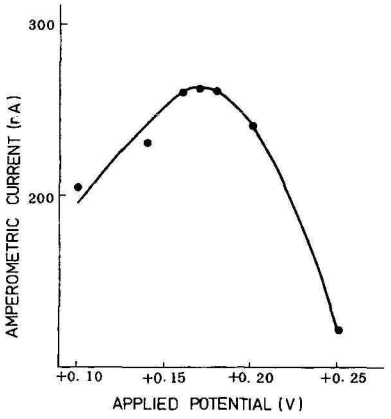

Fig. 2. Amperometric detection of iodide as a function of the applied potential.

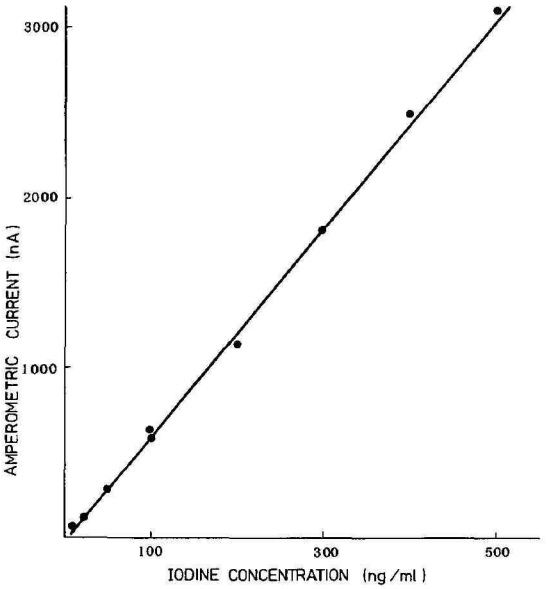

Fig. 3. Calibration graph for the amperometric determination of iodide.

TABLE I

EFFECT OF INTERFERING ELEMENTS ON THE DETERMINATION OF IODIDE

Ion (X)	*X/I^- concentration ratio*	*Recovery of I^- (%)*
Br^-	2	100
	20	96
	40	97
Cl^-	6000	97
	12 000	102
	24 000	98
S^{2-}	10	91
	20	94
	5000*	103
SCN^-	4	97
	20	100
	8000*	100

* Solutions acidified and boiled.

TABLE II

RECOVERY TESTS FOR IODIDE DETERMINATION

I^- added (ng/ml)	*I^- found (ng/ml)*	*Recovery (%)*
10.0	10.5	105
20.0	21.0	105
50.0	45.0	90
50.0	46.5	93
100	110	111

It is concluded that the method described will have wide application for the determination of iodine in sedimentary and other rocks and will prove to be a useful tool in oil exploration.

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REFERENCES

1 E. A. Crecelius, *Anal. Chem.*, 47 (1975) 2034.
2 M. Novkirishka, *Anal. Chim. Acta*, 78 (1975) 63.
3 E. I. Yasinskine, *Zh. Anal. Khim.*, 30 (1975) 1590.
4 Qing Hai Institute of Salt Lakes, Academia Sincia, *Analytical Methods for Brines and Salts*, Science Press, Beijing, 1973, p. 146.
5 J. C. McDonald (Editor), *Inorganic Chromatographic Analysis*, Wiley, New York, 1985, p. 366.
6 *Ion Chromatograph 16 — Operation and Maintenance Manual*, Dionex, Sunnyvale, CA, 1979, p. 103.
7 R. D. Rocklin and E. L. Johnson, *Anal. Chem.*, 55 (1983) 4.

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Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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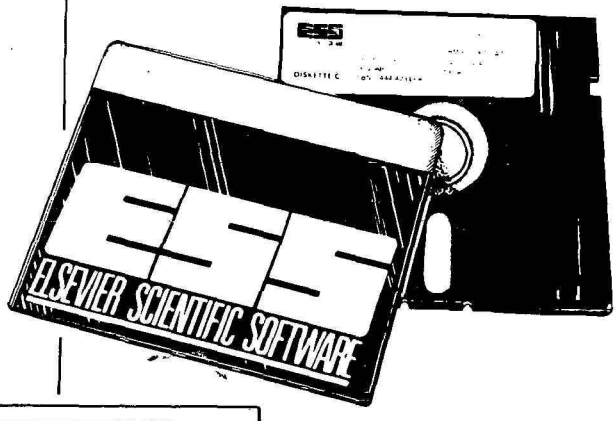

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