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JOURNAL OF CHROMATOGRAPHY

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STRAIGHT-PHASE ION-PAIR CHROMATOGRAPHY OF ZIMELIDINE AND SIMILAR DIVALENT AMINES

II. THE CHROMATOGRAPHIC SYSTEM

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(Received February 16th, 1981)

SUMMARY

The properties of a straight-phase ion-pair liquid chromatographic system are described. The system is based on perchlorate as the anion component in a strongly acidic stationary phase with methylene chloride–*n*-butanol as the mobile phase, and has been used for the separation of zimelidine, a divalent hydrophobic amine, and related compounds.

Batch distribution data for some of the amines as bases and as 1 + 1 and 1 + 2 ion pairs with perchlorate are presented and used to calculate capacity ratios, which were found to be in good agreement with the experimental chromatographic data. It is concluded that the retention mechanism is based mainly on liquid–liquid distribution and that selectivity factors can be calculated from batch extraction constants. The ion-pair equilibria were found to include an association of a 1 + 2 ion pair in the aqueous phase and also dissociation of the 1 + 1 ion pair in the organic phase.

The relationship between chemical structure and selectivity is discussed, and it is emphasized that it is complicated because of the possible existence of two kinds of ion pairs with the divalent amines. The baseline separation of four compounds that are both geometric and bromo-positional isomers demonstrate the excellent selectivity of the system in practice.

The capacity ratios increase both with increasing flow-rates and at very low flow-rates, but with maintained selectivities, and possible reasons are discussed.

The effects of the injection of large sample volumes (up to 500 μl) on chromatographic efficiencies and resolutions are demonstrated, and linear relationships between the standard deviation (σ) of the dispersion and the injected volume were obtained.

INTRODUCTION

Ion-pair extraction is a well established technique in analytical chemistry, and its fundamental properties regarding theory and applications in batch extractions and

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in modern liquid chromatography have recently been treated in a book[1] and several reviews[2–5].

This paper describes some chromatographic properties of a straight-phase ion-pair system based on a strongly acidic perchlorate solution as the stationary phase on irregular silica gel microparticles (5 μm) with a mixture of methylene chloride and *n*-butanol as the mobile phase. The compounds studied were a divalent amine, zimelidine, and some chemically related compounds.

The application of this system to the analysis of the compounds in biological material has been described earlier[6].

EXPERIMENTAL

Apparatus

The equipment used for chromatography, photometric measurements, pH measurements and ultrasonic homogenizations has been described elsewhere[6]. Two-phase titrimetric experiments and potentiometric titrations were performed with equipment obtained from Radiometer (Copenhagen, Denmark), namely a pH meter (PHM 26) with glass (9202 B) and calomel (K 401) electrodes or, in the determinations of extraction constants at ionic strength 1, an Orion Model 90-02 double-junction electrode, with 1 *M* sodium chloride solution as the salt bridge. The titrant was added with a 0.5-ml Agla micrometer syringe (Wellcome Reagents, London, Great Britain) or a Dosimat E 535 (Metrohm, Herisau, Switzerland) with an E 552-1B micro-exchange unit.

Chemicals

Most of the chemicals, including the chromatographic support (Partisil 5), have been described previously[6]. Zimelidine, norzimelidine, zimelidine N-oxide, zimelidine *E*-isomer, norzimelidine *E*-isomer, compounds I, II, III, V, VI, XI, XII and XIII were obtained from the Department of Organic Chemistry, Astra Läkemedel AB (Södertälje, Sweden), and compounds IV, VII, VIII, IX and X from AB Hässle, Gothenburg, Sweden (see Table IX). They were in the form of bases or salts with chloride, oxalate or maleate and were used as received.

Sodium chloride, sodium sulphate, sodium perchlorate and perchloric acid were of analytical (pro analysi) quality from Merck (Darmstadt, G.F.R.); sulphuric acid (concentrated) was Chemtam® (P-H Tamm, Gothenburg, Sweden).

Column packing and coating

The procedure has been described in detail elsewhere[6]. With methylene chloride–*n*-butanol (89:11) as the mobile phase the volume of the stationary phase (V_s) on the column (150 × 4 mm) was 0.79 ml, as determined by eluting the column with anhydrous methanol and measuring the water content by a Karl Fischer titration. The interstitial volume (V_m) was determined to be 1.02 ml by the injection of an unretained sample (toluene).

The contents of perchlorate in the stationary and mobile phases were determined by a quantitative extraction with dimethylprotriptylin (MPT) into methylene chloride and photometric measurements, according to the principles described by Borg[7].

Determination of distribution data

The batch extractions were carried out in centrifuge tubes either at room temperature or in a thermostated bath at 23.0°C. The approximate shaking time for the determination of extraction constants was 30 min or more, and for the determination of distribution constants for bases 60 min or more. The tubes were then centrifuged at 1600–1900 *g* for 5–10 min. The concentrations were determined by photometry in both the aqueous and the organic phase. Molar absorptivities of the compounds are given in Table I.

TABLE I

MOLAR ABSORPTIVITIES

Aqueous phase: 0.5–1 *M* $HClO_4$. Organic phase: methylene chloride *n*-butanol (89:11), saturated with aqueous phase.

Compound	*Aqueous phase*		*Organic phase*	
	Wavelength (nm)	*log ε*	*Wavelength (nm)*	*log ε*
Zimelidine	250	4.278	250	4.342
Zimelidine *E*-isomer	220	4.281	239	4.230
Norzimelidine	250	4.296	250	4.294
Norzimelidine *E*-isomer	222	4.303	241	4.281
Chlorpheniramine	265	3.922	266	3.896

In the two-phase titrations the pH set was calibrated before and after every titration with two commercially available buffers (pH 4.01 ± 0.02 and 7.01 ± 0.02) (Radiometer). The titrations were performed in a closed vessel to prevent disturbance by carbon dioxide from the air. The vessel was kept in a thermostated water-bath (23.0 ± 0.2°C). The two phases were saturated with each other prior to the titration in order to avoid volume changes.

A 10^{-2} *M* solution of the amine in 30 ml of the organic phase (methylene chloride–*n*-butanol, 89:11) was prepared by extraction from a small volume of an alkalinized aqueous solution of the amine salt. After centrifugation, 25.0 ml of the organic phase were transferred into the titration vessel and 25.0 ml of aqueous phase were added. The two-phase system was then titrated with 1 *M* perchloric acid for ion-pair extraction constants and 0.5 *M* sulphuric acid for base distributions.

The titrant was delivered in equal portions with vigorous stirring. The pH was measured, with stirring, about 60 sec after the addition. Blank titrations were performed in both instances and calculations were made according to Johansson and Gustavii[8,9].

RESULTS AND DISCUSSION

Distribution data

The compounds studied are divalent amines and in the chromatographic system used, which contains a strongly acidic stationary aqueous phase, they can be distributed both as 1 + 1 (HAX_{org}) and 1 + 2 (H_2AX_{2org}) ion pairs. Constants for the distribution of the compounds as bases and as ion pairs to the mobile phase, methylene chloride–*n*-butanol (89:11), are given in Tables II–IV.

TABLE II

CONSTANTS FOR DISTRIBUTION AS BASES

Determined by two-phase titrations. Organic phase: methylene chloride–*n*-butanol (89:11) equilibrated with the aqueous phase. Number of experimental points: 9–12. Aqueous phase: 0.033 *M* Na_2SO_4; titrant, 0.5 *M* H_2SO_4 (I = 0.1).

Amine	$C_A^\circ \cdot 10^3$	$C'_A \cdot 10^3$	*pH*	$-\log k_d \cdot K'_{HA}$	pK'_{H_2A}
Zimelidine	5.01	3.04–3.26	4.52–3.45	3.97	3.76*
Norzimelidine	5.05	3.76–3.94	5.13–3.83	5.36	3.83**
Norzimelidine *E*-isomer	5.03	2.87–3.86	5.38–3.51	5.55	4.29
Zimelidine *E*-isomer	5.02	2.93–3.07	4.55–3.58	4.07	4.25
Chlorpheniramine	5.02	3.13–3.51	5.10–3.33	4.92	3.81

* 3.78 ± 0.03 (n = 11) by potentiometric titration.
** 3.92 ± 0.01 (n = 11) by potentiometric titration.

The distribution of the compounds as bases is negligible at pH < 2 (equal phase volumes), as is the case with increasing ionic strength, as it can be expected that the base distribution will then decrease, in a similar manner to the results for the 1 + 1 ion pairs (Table III) where the difference is about two-fold. Acid dissociation constants of zimelidine and norzimelidine at an ionic strength of 1 were found to be 4.08 ± 0.01 and 4.24 ± 0.01 (n = 11), respectively, as determined by potentiometric titrations, *i.e.*, the acidities are about halved on increasing the ionic strength 10-fold.

The ion-pair extraction constants at an ionic strength of 1.0 were determined according to the principles outlined by Modin and Schill[10]; 1 + 1 ion-pairs could be determined without any influence of 1 + 2 ion pairs at pH 2.88 and 4.15 for zimelidine and norzimelidine, respectively. In the determinations of 1 + 2 ion pairs (Table IV) the co-extraction of the 1 + 1 ion pairs was compensated for by using the determined constants (Table IIIB). At high ionic strength the 1 + 1 ion pairs were found to dissociate in the organic phase (Table IIIB); the determinations of constants at low ionic strength (Table IIIA), however, were performed with much higher concentrations, that is, under conditions where dissociation of an ion pair in the organic phase does not occur.

With variation of the perchlorate concentration under acidic conditions (1 + 2 ion pair, Table IV) the conditional extraction constants for zimelidine and norzime-

TABLE III

EXTRACTION CONSTANTS FOR 1 + 1 PERCHLORATE ION PAIRS

(A) Ionic strength = 0.1

Determination technique: two-phase titrations. Aqueous phase: 0.1 *M* $NaClO_4$; titrant, 1 *M* $HClO_4$.

Compound	$C'_A \cdot 10^3$	$C''_A \cdot 10^5$	*pH*	$\log K_{ex(HAX)}$
Zimelidine	10.06	3.48–6.27	6.19–5.63	3.06
Norzimelidine	10.05	5.77–12.24	7.41–6.79	2.74
Zimelidine *E*-isomer	9.97	5.90–12.52	6.11–5.47	2.73
Norzimelidine *E*-isomer	9.99	19.84–32.44	6.96–6.46	2.33
Chlorpheniramine	10.10	1.73–3.88	7.54–6.80	3.27

(B) Ionic strength = 1

Determination technique: batch extraction and UV photometric measurements. Aqueous phase: 0.2 *M* $HClO_4$ + $NaClO_4$ and NaCl to give $I = 1.0$.

Compound	$C'_A \cdot 10^5$	$C''_A \cdot 10^5$	*pH*	$\log K_{ex(HAX)}$	$-\log k_{diss}$
Zimelidine	2.25–28.36	1.049–15.60	2.88	2.82	5.23★
Norzimelidine	2.21–22.60	0.479–5.67	4.15	2.48	4.75★★

★ $r = 0.9986$.
★★ $r = 0.9888$.

TABLE IV

EXTRACTION CONSTANTS FOR 1 + 2 PERCHLORATE ION PAIRS

Determination technique: batch extractions and UV photometric measurements. Organic phase: methylene chloride–*n*-butanol (89:11). Aqueous phase: 0.2 *M* $HClO_4$ + $NaClO_4$ and NaCl to give $I = 1.0$.

Compound	$C'_{Aorg} \cdot 10^5$	$C''_A \cdot 10^4$	C''_{ClO_4}	$K_{ex(HAX_2)}$	$k_{a(H_2AX_2)}$	*n*
Zimelidine	6.68–8.20	1.20–1.42	0.4–0.8	7.5	14.5	14
Norzimelidine	1.92–5.42	1.72–2.05	0.2–1.0	3.1	13.1	8

lidine decreased with increasing concentration of perchlorate in the aqueous phase. This may be due to dissociation of an ion pair in the organic phase or association reactions in the aqueous phase. The assumption of an association between the amine in divalent form and two molecules of perchlorate to form an ion pair (H_2AX_2) in the aqueous phase gave the best fit to the data as treated by slope analysis. Other possibilities tested were extraction of a 1 + 1 ion pair only and association of amine and perchlorate to form the ion pair HAX in the aqueous phase; extraction of a 1 + 1 ion pair and dissociation of this ion pair in the organic phase; and extraction of a 1 + 2 ion pair and dissociation in the organic phase to form either H_2AX_{org} and X_{org} or

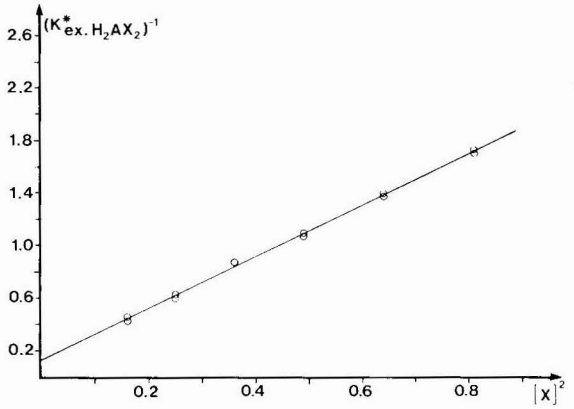


Fig. 1. Ion-pair association in aqueous phase. Organic phase: methylene chloride–*n*-butanol (89:11). Aqueous phase: 0.2 *M* $HClO_4$ + $NaClO_4$ and NaCl to give I = 1.0. Calculation of data from Table IV according to eqn. 1 (r = 0.9991).

H_2A_{org} and 2 X_{org}. Computations according to these principles gave, however, low correlation coefficients and in many instances improbable values of the constants. As an example, the association of zimelidine with two perchlorate molecules in the aqueous phase is illustrated in Fig. 1 according to the equation

$$(K^x_{ex(H_2AX_2)})^{-1} = (K_{ex(H_2AX_2)})^{-1} + k_a [X]^2 (K_{ex(H_2AX_2)})^{-1}$$

The extraction and association constants obtained from the slope and intercept for the two compounds are reported in Table IV.

The magnitude of the association constants means that the ion-pair formation in the aqueous phase is significant at perchlorate concentrations above $7 \cdot 10^{-4}$ *M*. The dissociation of the 1 + 1 ion pairs in the organic phase for zimelidine and norzimelidine is of importance at organic phase concentrations $\leqslant 10^{-5}$ *M*.

The determinations of constants for the 1 + 2 ion pairs were carried out under conditions where the concentrations in the organic phase were low, but as perchloric acid is extracted (see below) the dissociation of the amine–perchlorate ion pairs in the organic phase may be suppressed by perchlorate ions from the acid[11] and remain undetected.

The distribution ratios for actual amines were also determined by batch extractions under conditions identical with those used in the chromatographic system (Table V). As remarked earlier, in this system both 1 + 1 and 1 + 2 ion pairs will distribute simultaneously and complicate discussions on selectivity, as discussed in depth later (see *Chemical structure and selectivity*).

Data on extraction selectivity, expressed as the difference between the logarithmic values for the constants, are given in Table VI. In base distribution high selectivity is obtained between tertiary and secondary amines, which obviously depends on a combination of differences in both distribution constants and acid dissociation constants. High selectivity for geometrical isomers is obtained by 1 + 1 ion-pair extractions, where the selectivity between the tertiary and secondary amines is also

TABLE V

DISTRIBUTION OF ION PAIRS BETWEEN PHASES USED IN THE CHROMATOGRAPHIC SYSTEM

Determination technique: batch extraction and UV photometric measurements. Organic phase: methylene chloride *n*-butanol (89:11). Aqueous phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$.

Compound	$C'_{Aorg} \cdot 10^5$	$C'_A \cdot 10^5$	*Distribution ratio*	*s*	*n*
Chlorphenir-amine	1.768–9.005	1.938–10.123	0.8706	0.0135	8
Zimelidine	0.614–3.062	1.138–5.796	0.5329	0.0067	10
Zimelidine *E*-isomer	0.4710–2.420	1.241–6.430	0.3709	0.0066	4
Norzimelidine	0.4417–2.198	1.183–5.987	0.3745	0.0045	5
Norzimelidine *E*-isomer	0.3766–1.899	1.244–6.00	0.3258	0.0168	5

good. For 1 + 2 ion pairs data only for zimelidine and norzimelidine are available and for this pair of compounds the selectivity is similar to the 1 + 1 ion pairs. In phases used in the chromatographic system D (Table VI), however, where both 1 + 1 and 1 + 2 ion pairs are distributed, the selectivities are lower; this system was, however, preferred for the chromatography mainly because of higher stability and buffer capacity[12].

Capacity ratios

In a chromatographic system based on the distribution of ion pairs, the capacity ratios can be calculated according to

$$k'_c = V_s (V_m D_{A(X)})^{1} \quad (2)$$

The determined capacity ratios are 1.5–2 times higher than the calculated values, as demonstrated in Table VII. The difference may be due to the influence of the support on the properties of stationary phase or on the sample. Larger differences have been found earlier in similar systems for amines[13] and steroidal conjugates[14], and a close correspondence was obtained for some phenylacetic acid derivatives[15].

A quantitative determination of perchlorate in the stationary phase showed that its concentration increased during the course of the equilibration from 1 to 1.23 *M*, which is analogous to the results found for quaternary ammonium compounds in similar systems[14,15]. The chromatographic results indicated in those instances that the additional amount of the counter ion did not take part in the ion-pair distribution. This also seems to be the case in the present system. A significant amount of perchlorate is also extracted into the mobile phase, which was found to have a concentration of $2.01 \cdot 10^{-3}$ *M*, probably mainly consisting of perchloric acid, as this species has a much higher distribution than sodium perchlorate[12].

TABLE VI

EXTRACTION SELECTIVITY

Calculated from values in Tables II–V.

Compounds	*A, base distribution:* $\Delta \log k_d \cdot K'_{HA}$ *(I = 0.1)*	*B, 1 + 1 ion-pair distribution:* $\Delta \log K_{ex(HAX)}$		*C, 1 + 2 ion-pair distribution:* $\Delta \log K_{ex(H_2AH_2)}$ *(I = 1)*	*D, mixed ion-pair distribution:* $\Delta \log D_A$ *(I = 1)*
		I = 0.1	*I = 1*		
Zimelidine–norzimelidine	1.39	0.32	0.34	0.34	0.15
Zimelidine *E*–norzimelidine *E*	1.47	0.39			0.06
Zimelidine–zimelidine *E*	0.11	0.34			0.16
Norzimelidine–norzimelidine *E*	0.19	0.41			0.06
Zimelidine–chlorpheniramine	0.96	0.24			0.21

TABLE VII

DETERMINED AND CALCULATED CAPACITY RATIOS

Support: Partisil 5. Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: methylene chloride–*n*-butanol (89:11). V_s/V_m = 0.77. k'_f and k'_c = determined and calculated (Table V) capacity ratios, respectively.

Compound	k'_f	k'_f/k'_c
Chlorpheniramine	1.78	2.02
Zimelidine	2.36	1.63
Norzimelidine	3.10	1.50
Zimelidine *E*-isomer	3.25	1.56
Norzimelidine *E*-isomer	3.78	1.60

The good agreement between determined and calculated capacity ratios indicates that the retention is due mainly to liquid–liquid distribution. This conclusion is supported by the fact that selectivity factors can be calculated from extraction constants obtained by batch extractions, as demonstrated in Table VIII. It is noteworthy that about the same selectivity is obtained between *Z*- and *E*-isomers as between tertiary and secondary amines.

TABLE VIII

SELECTIVITIES FROM EXTRACTION CONSTANTS AND CHROMATOGRAPHIC DATA

The selectivity factors (α) were calculated from Tables V and VII.

Compounds	α *calculated from*	
	k'_f	$D_{A(X)}$
Zimelidine/zimelidine *E*-isomer	1.38	1.44
Zimelidine/chlorpheniramine	1.33	1.63
Zimelidine/norzimelidine	1.31	1.42
Zimelidine *E*-isomer/ norzimelidine *E*-isomer	1.16	1.14
Zimelidine/norzimelidine *E*-isomer	1.05	1.01
Norzimelidine/norzimelidine *E*-isomer	1.22	1.15

Chemical structure and selectivity

The discussion of factors that determine the selectivity in this chromatographic system is complicated by the possible existence of two kinds of ion pairs in the organic phase, H_2AX_2 and HAX, when the compounds concerned are divalent amines. The ratios of the concentrations of these ion pairs are obtained by the equation

$$R = \frac{\text{total concentration of 1 + 2 ion pair in organic phase}}{\text{total concentration of 1 + 1 ion pair in organic phase}}$$

$$= K^x_{ex(H_2AX_2)}\, a_{H^+}\, [X]\, [K^x_{ex(HAX)}\, K'_{H_2A}]^{-1} \qquad (3)$$

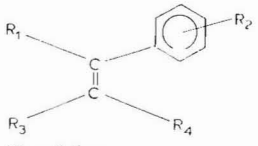
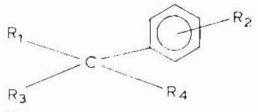
As the pH and the counter ion concentration are kept constant in the chromatographic system, the ratio, R, depends on the quotient of the extraction constants and the acid dissociation constant of the pyridine-nitrogen. R values for zimelidine and norzimelidine, using data from Tables II, IIIB and IVA, are 1.94 and 2.53, corresponding to 66 and 72% of the 1 + 2 ion pair in the chromatographic mobile phase, respectively. The R values are calculated assuming that the dissociation of the 1 + 1 ion pair is negligible as the co-extraction of significant amounts of perchloric acid (see above) will probably suppress this reaction. It can be estimated that for the compounds mentioned in Tables II–V between 60 and 99% of the species present in the organic phase will correspond to the 1 + 2 ion pair.

Capacity ratios for zimelidine and related compounds are summarized in Table IX, and some selectivity factors calculated from these figures are given in Table X.

TABLE IX

CAPACITY RATIOS

Chromatographic conditions: see Table VI.

Compound	R_1	R_2	R_3	R_4	*log* k'_f*
					
Zimelidine	3-Pyridyl	*p*-Br	$-CH_2N(CH_3)_2$	H	0.373
Norzimelidine	3-Pyridyl	*p*-Br	$-CH_2NHCH_3$	H	0.491
I	3-Pyridyl	*p*-Br	$-CH_2NH_2$	H	0.821
II	3-Pyridyl	*p*-Br	$CH_2NHCOCH_3$	H	≤ -0.52
Zimelidine N-oxide	3-Pyridyl	*p*-Br	$CH_2N(CH_3)_2$ ↓ O	H	0.137
III	3-Pyridyl	*p*-Br	COOH	H	−0.523
IV	3-Pyridyl	H	$CH_2N(CH_3)_2$	H	0.807
Zimelidine *E*-isomer	3-Pyridyl	*p*-Br	H	$CH_2N(CH_3)_2$	0.512
Norzimelidine *E*-isomer	3-Pyridyl	*p*-Br	H	CH_2NHCH_3	0.577
V	3-Pyridyl	*o*-Br	$CH_2N(CH_3)_2$	H	0.316
VI	3-Pyridyl	*o*-Br	H	$CH_2N(CH_3)_2$	0.599
VII	2-Pyridyl	H	$CH_2N(CH_3)_2$	H	0.398
VIII	2-Pyridyl	*p*-F	$-CH_2N(CH_3)_2$	H	0.433
IX	2-Pyridyl	*o*-OCH_3	$-CH_2N(CH_3)_2$	H	0.097
X	2-Pyridyl	*p*-CH_3	$-CH_2N(CH_3)_2$	H	0.000
					
XI	3-Pyridyl	*p*-Br	OH	$CH_2CH_2N(CH_3)_2$	0.508
XII	3-Pyridyl	*p*-Br	OH	$CH_2CH_2NHCH_3$	0.665
XIII	3-Pyridyl	*p*-Br	=O	–	−0.984
Chlorpheniramine	2-Pyridyl	*p*-Cl	H	$CH_2CH_2N(CH_3)_2$	0.250
Brompheniramine	2-Pyridyl	*p*-Br	H	$CH_2CH_2N(CH_3)_2$	0.146

* k'_f values are means of two or three experiments.

TABLE X

CHEMICAL STRUCTURES AND SELECTIVITIES

Structural difference	*Compounds*	α
Secondary/tertiary amine	Norzimelidine/zimelidine	1.31
	Norzimelidine/zimelidine *E*-isomers	1.16
Primary/secondary amine	I/norzimelidine	2.14
Geometric isomerism	Zimelidine *E*/zimelidine	1.38
	Norzimelidine *E*/norzimelidine	1.22
Tertiary amine/amine oxide	Zimelidine/zimelidine N-oxide	1.72
Primary amine/acetylated primary amine	I/II	>21.9
Tertiary amine/carboxylic acid	Zimelidine/III	7.87
>C(OH)-CH_2-/>CH=CH< (*Z*-isomers)	XI/zimelidine	1.49
(*E*-isomers)	XI/zimelidine *E*-isomer	1.22
3-Pyridyl/2-pyridyl	IV/VII	2.56
H/*p*-Br	IV/zimelidine	2.72
p-F/H	VIII/VII	1.08
H/*o*-CH_3O	VII/IX	2.00
H/*p*-CH_3	VII/X	2.50
p-Cl/*p*-Br	Chlorpheniramine/brompheniramine	1.27

Owing to the complicated nature of the chromatographic system presented above, the following discussion on selectivities obtained is mainly of a qualitative nature and difficult to compare with other extraction systems.

Amines of different degrees of substitution are easy to separate; the selectivity, however, is strongly dependent on the content of alcohol in the mobile phase, as demonstrated in Fig. 2. The order of elution is reversed with 30 % of *n*-butanol in the mobile phase, the secondary amine being strongly solvated by the alcohol. The selectivity factors decrease continuously (1.34, 1.16, 1.00 and 0.95, respectively) with increasing concentration of *n*-butanol in the mobile phase, as does the number of theoretical plates (from about 7000 to 6000, 4500 and 3000, respectively). The mobile phase volume, V_m (toluene), was constant, indicating that the same amount of stationary phase is adsorbed to the support in all instances.

The excellent selectivity between two pairs of geometrical isomers is illustrated in Fig. 3. A and D are geometric isomers as well as B and C; A and B are furthermore bromo-positional isomers as well as C and D. Selectivity factors for positional isomerism are 1.14 (B/A) and 1.22 (D/C), respectively, while the geometric isomerism gives α = 1.92 (D/A) and α = 1.38 (C/B) in this instance.

Zimelidine N-oxide (k' = 1.37) elutes before zimelidine (k' = 2.36) in this chromatographic system, which is probably mainly a consequence of protolytic properties (the formation of an N-oxide often decreases the basic character of the compound by 4-5 pK_s units[16]), which increases the possibilities for the extraction of a 1 + 1 ion pair.

Acetylation of a primary amine usually decreases the polarity of a compound, but it also eliminates the protolytic properties. Compound II (Table IX) is consequently extracted as a 1 + 1 ion pair only, which accounts for the high selectivity

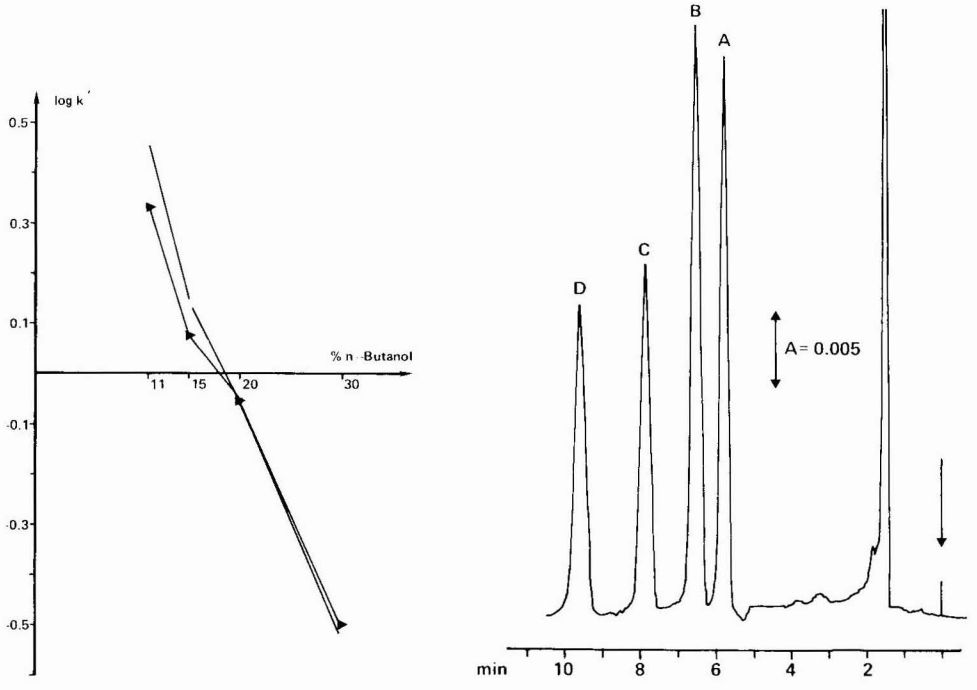


Fig. 2. Retention with different contents of *n*-butanol in the mobile phase. Support: Partisil 5. Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: methylene chloride–*n*-butanol. Sample: 600 pmol of each substance in 40 μl of mobile phase. Each point is the mean of three or four determinations, and the chromatographic system was allowed to equilibrate for not less than 20 h before the experiments. ▼, Zimelidine; ○, norzimelidine.

Fig. 3. Separation of geometric and positional isomerism. Support: Partisil 5. Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: methylene chloride–*n*-butanol (89:11) saturated with stationary phase. A, No. V in Table IX; B, zimelidine; C, zimelidine *E*-isomer; D, No. VI in Table IX.

against the primary amine (compound I) in this instance.

A further illustration of the higher extraction of 1 + 1 ion pairs is given by the carboxylic acid (III), which has a very low retention. The introduction of the polar carboxylic group should normally have increased the capacity ratio.

The exchange of –CH=CH– for $-CH(OH)CH_2-$ in the propyl chain (zimelidine → XI and norzimelidine → XII) increases the affinity to the aqueous phase, as expected. The increase in the capacity ratios (0.13–0.17 log units) is, however, considerably smaller than on introduction of an alcohol group in an alkyl chain of a 1 + 1 ion pair. Extraction constants for choline picrate into 1-pentanol and methylene chloride are, for example, 0.6 and 1.0 log units lower, respectively, than the corresponding constants for the trimethylammonium ion pair[17].

The high selectivity between 2- and 3-pyridyl-substituted compounds (IV and VII) is remarkable and is probably an effect of differences in hydrophobicity and/or affinities to the support, as pK'_{H_2A} values for the two kinds of structures seem to be similar, as indicated, for example, by the pK'_{H_2A} of the 3-pyridyl derivative zimelidine and the 2-pyridyl derivative brompheniramine of 3.84 and 3.93, respectively[9].

Effects of variation in flow-rate

The dependence of H on the flow-rate for norzimelidine and the primary amine (I) is demonstrated in Table XI. H is constant at flow-rates between 0.4 and 1.8 mm/sec but increases at higher velocities. The reason behind this performance is not, however, a simple consequence of a velocity increase because the retention is also affected; the capacity ratios for both compounds are minimal at a flow-rate of about 0.8 mm/sec, as illustrated in Fig. 4. The selectivity is, however, only slightly affected, suggesting that the basic retention mechanism is maintained at all velocities. The retention is temperature dependent, as demonstrated in Table XII, which shows capacity ratios and selectivity factors obtained at 23.0 and 25.7°C. At the higher temperature the capacity ratios increase by 25–30% (0.027 unit per 0.1°C) but the

TABLE XI

INFLUENCE OF FLOW-RATE ON CHROMATOGRAPHIC PERFORMANCE

Amounts injected: 140 ng of norzimelidine and 600 ng of I. Volume injected: 20 µl. Chromatographic conditions: see Fig. 2.

Flow-rate (mm/sec)	*Compound*	*HETP (µm)*	*As**
0.41	Norzimelidine	23	1.38
0.41	I	23	1.26
0.81	Norzimelidine	21	1.22
0.81	I	22	1.21
1.81	Norzimelidine	23	1.14
1.81	I	23	1.14
2.50	Norzimelidine	34	1.13
2.50	I	34	1.25
3.28	Norzimelidine	41	1.23
3.28	I	39	1.20

* Asymmetry factor = back/front of peak at the baseline level.

TABLE XII

TEMPERATURE DEPENDENCE OF CAPACITY AND SELECTIVITY FACTORS

Compound	*23.0°C*		*25.7°C*	
	log k'_f	*α*	*log k'_f*	*α*
Chlorpheniramine	0.250		0.348	
		1.33		1.39
Zimelidine	0.373		0.491	
		1.31		1.31
Norzimelidine	0.491		0.607	
		1.22		1.17
Norzimelidine *E*-isomer	0.577		0.676	

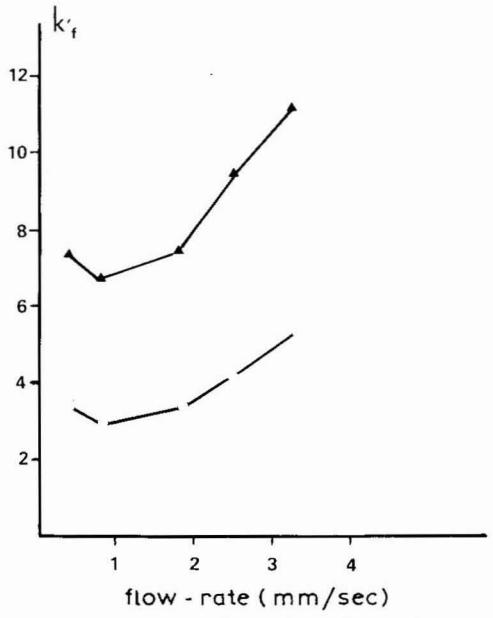


Fig. 4. Dependence of capacity ratios on flow-rate. Chromatographic conditions: see Fig. 3. Amounts injected: 140 ng of norzimelidine and 600 ng of I (primary amine). ○, Norzimelidine; ▲, compound I (Table IX).

selectivity is unchanged. It is known that heat due to friction develops within a column with large pressure drops[18], but such temperature effects can be responsible for only part of the observed difference in retention times in the present instance.

In an ion-pair retention mechanism the capacity ratios are directly proportional to V_s, the volume of the stationary phase (eqn. 2). Data obtained for V_m (the volume available for an unretained compound), however, increased from 1.026 to 1.066 with increasing flow-rate from 0.83 to 3.26 mm/sec, indicating that V_s decreases by about 5% with this flow-rate change. As the capacity ratios increase with increasing flow-rate another retention mechanism must be responsible, possibly an increased availability for adsorption of the compounds by the support with decreasing V_s. The increase in capacity ratios at the lower flow-rates may, however, partly depend on the increasing stationary phase volume.

Injection of large sample volumes

A perfect injection results in a plug with no mixing of the sample and mobile phase occurring but simply a displacement of mobile phase at the top of the column. However, depending on the flow pattern in the injection device, a more or less asymmetric injection profile is obtained in practice.

The dispersion starts as soon as the sample hits the column and results in band broadening at the top of the column as the delivery of the sample takes a certain amount of time. The magnitude of the dispersion also depends on the capacity ratio, as a more retained compound occupies a smaller volume at the top of the column, provided that rapid equilibrium between mobile and stationary phase is established.

The effective volume (V_e) occupied by the sample under these conditions is described by $V_e = V_i (1 + k')^{-1}$, where V_i is the volume injected.

The total dispersion, σ_t^2, is the sum of band-broadening effects at the injection, σ_i^2, and on the column, σ_c^2, provided that other contributions are negligible. It has been demonstrated in some chromatographic systems[19–21] that under certain conditions σ_i^2 is directly proportional to the volume injected by computing the total dispersion against V_i^2 according to

$$\sigma_t^2 = V_i^2/K^2 + \sigma_c^2 \tag{4}$$

where K is a constant representing the effects of the injection valve design and the capacity factor on the dispersion. Plots of eqn. 4, for three compounds with different k' values, X, norzimelidine and I with injection volumes ranging from 10 to 500 μl gave no straight lines, however.

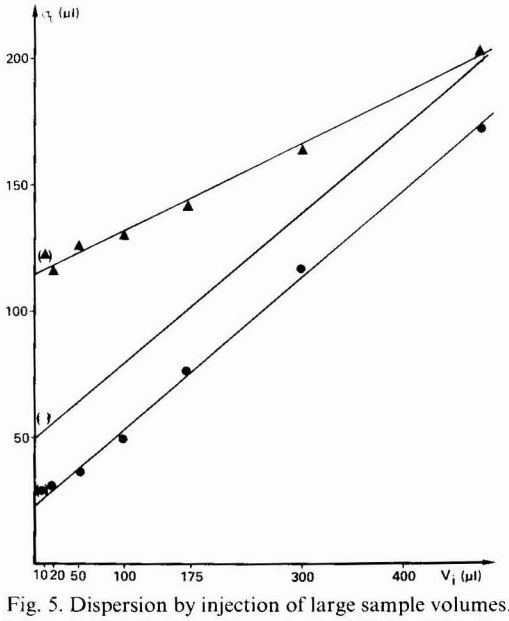


Fig. 5. Dispersion by injection of large sample volumes. Chromatographic conditions: see Fig. 3. Volumes injected: 10–500 μl. ●, Compound X (Table IX); ○, norzimelidine; ▲, compound I (Table IX).

Straight-line relationships were obtained by computing V_i against σ_t (Fig. 5), illustrating that the peak widths increase in proportion to the injected volumes according to the following empirical equations:

Compound X ($k'_f = 1.023 \pm 0.032$):

$$\sigma_t = 0.3040\ V_i + 22.15\ (r = 0.9993) \tag{5}$$

Norzimelidine ($k'_f = 3.57 \pm 0.046$):

$$\sigma_t = 0.3019\ V_i + 49.08\ (r = 0.9986) \tag{6}$$

Compound I ($k'_f = 8.49 \pm 0.053$):

$$\sigma_t = 0.1748\ V_i + 113.58\ (r = 0.9981) \quad (7)$$

Data for $V_i = 10\ \mu l$ are not included in the computation of the equations as they seem to deviate from a straight line, possibly because of excessive dispersion effects by the injection valve on such small volumes.

Replacing σ_t with the more frequently used parameter H by utilizing the relationship $\sigma_t = t_0 (1 + k') H^{1/2} L^{-1/2}$ and plotting against V_e (Fig. 6) gives a common straight line for the two compounds with low capacity ratios (< 3.6) while the third compound ($k' = 8.49$) seems to deviate at large V_e values.

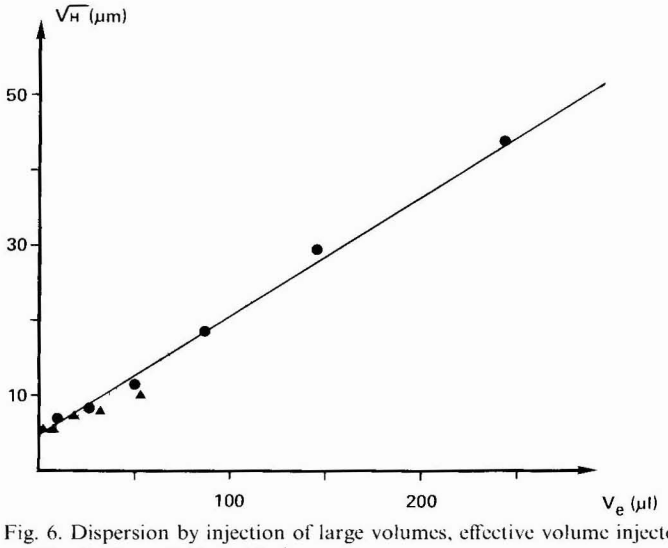


Fig. 6. Dispersion by injection of large volumes, effective volume injected against efficiency. Conditions: see Fig. 5. $V_e = V_i (1 + k')^{-1}$.

Similar results have been obtained for penicillins in a reversed-phase system[22].

The influence of increasing injection volume on the asymmetry factor, illustrated in Fig. 7, initially shows an increasing tailing tendency that stabilizes at a characteristic level for each compound when $50\ \mu l \leqslant V_i \leqslant 200\ \mu l$. With a further increase in V_i the asymmetry decreases for moderately and highly retained compounds, whereas it increases rapidly for the least retained compound. This effect is probably due to the asymmetric profile of the volume injected; for norzimelidine and compound I the asymmetric profile is smoothed out when the compounds are retained at the top of the column.

The influence of the sample volume on the resolution between the geometrical isomers of norzimelidine, which represents a very difficult separation[6], is illustrated in Fig. 8. The resolution decreases rapidly if $V_i > 20\ \mu l$ and is only about 50% of the maximal when 200 μl are injected. The efficiency and selectivity of the chromatographic system are so great, however, that a baseline separation ($R_s = 1.5$) is still achieved at $V_i = 190\ \mu l$.

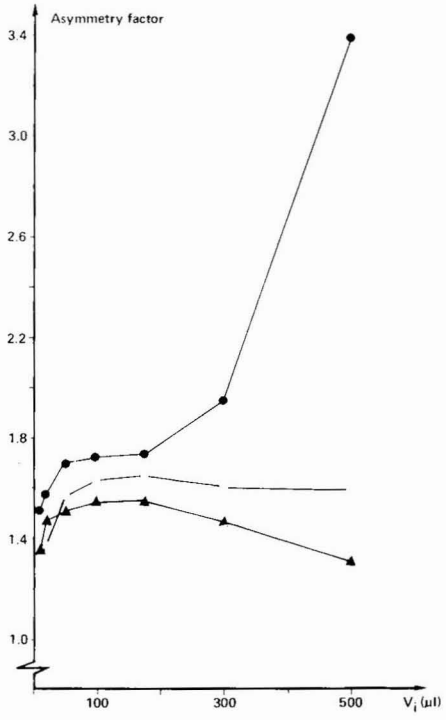


Fig. 7. Dependence of asymmetry factors on volumes injected. Conditions: see Fig. 5.

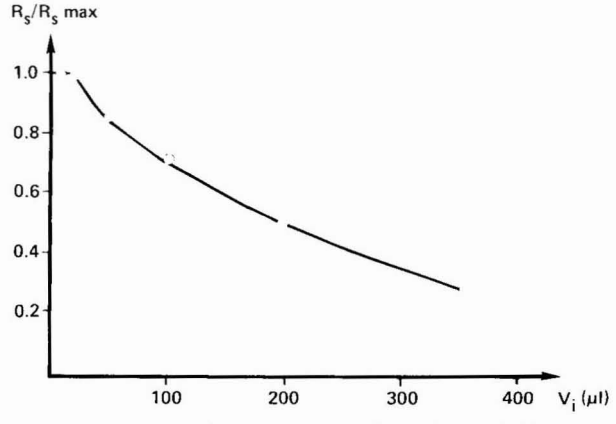


Fig. 8. Dependence of resolution on volumes injected. Chromatographic conditions: see Fig. 2. Injected compounds: norzimelidine and its geometric isomer. $R_s = R_{smax}$ when $V_i \leq 10$ µl.

Application to studies of purity

Although the chromatographic system was developed mainly for use in bioanalytical work, it was utilized in some instances for control of the purity of some batches of zimelidine and norzimelidine hydrochloride. Fig. 9 shows an example of a chromatogram obtained from a batch of norzimelidine hydrochloride, which con-

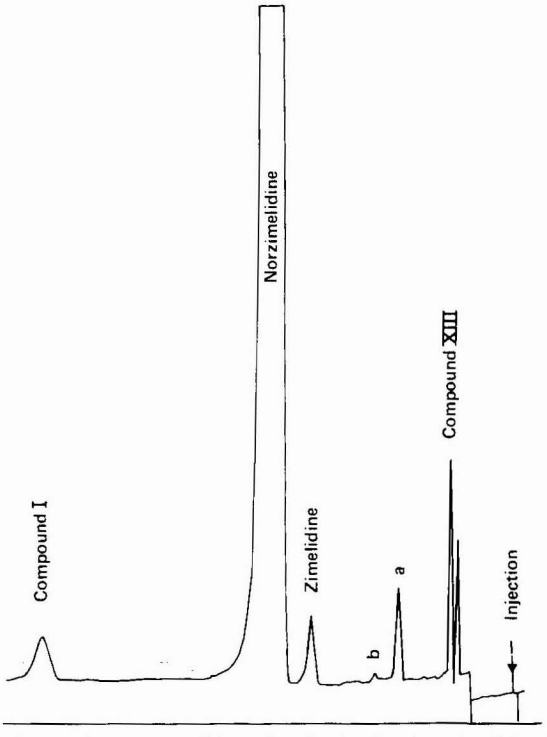


Fig. 9. Chromatographic purity of a batch of norzimelidine hydrochloride. Sample: 3 μg of salt in 100 μl of mobile phase. Chromatographic conditions: see Fig. 3. Estimated degree of impurity: zimelidine 0.2%, compound I 0.5%.

tained zimelidine, the primary amine (compound I), the ketone (compound XIII) and some unknown compounds (a, b) as impurities. The amount of the ketone (XIII) is difficult to interpret reliably as it has a very low capacity ratio (*ca.* 0.1) and is frequently interfered with by front disturbances that occur at $k' \leq 0.5$.

The high selectivity between geometrical isomers can be utilized for studies on isomeric purity, as illustrated in Fig. 10. In an injected amount of 2.4 μg of zimelidine, 0.01% of its *E*-isomer can be detected. However, it is an advantage if the impurity elutes before the main peak, because otherwise it may be masked by the tail of the large peak, so the detection limit of zimelidine as an impurity in the *E*-isomer (see Fig. 10B) is still lower.

SYMBOLS

ε = molar absorptivity;

C_A°, C_X° = initial concentrations of amine and perchlorate, respectively;

C'_{Aorg}, C'_{Xorg} = equilibrium concentrations of amine and perchlorate, respectively, in organic phase;

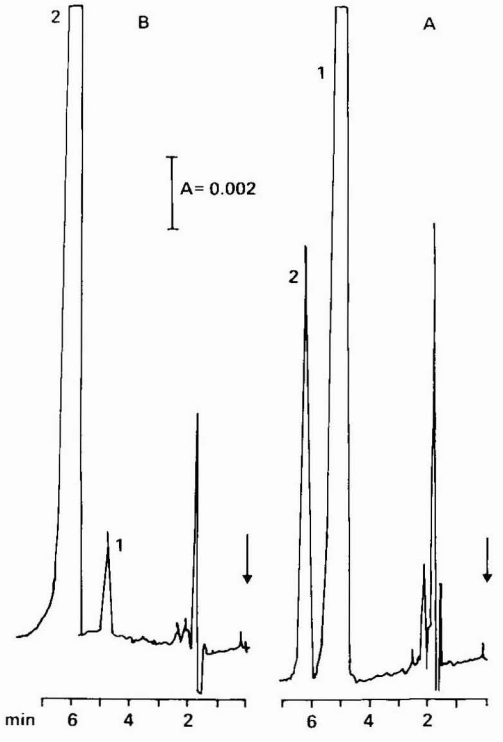


Fig. 10. Studies on purity from geometric isomers. A, Zimelidine (1) containing 2% of *E*-isomer (2); B, zimelidine *E*-isomer (2) containing 0.3% of zimelidine (1). Chromatographic conditions: see Fig. 3. Amounts injected: 2.4 μg in 80 μl of mobile phase.

C'_A, C'_X = equilibrium concentrations of amine and perchlorate, respectively, in aqueous phase;

$[H_2A]$, $[HA]$, $[X]$, $[H_2AX_2]$, $[HAX]_{org}$, $[H_2AX_2]_{org}$ = concentration of respective species in the appropriate phase;

$$k_d = \frac{[A]_{org}}{[A]} = \text{base distribution coefficient;}$$

$D_{A(X)}$ = distribution ratio of A as ion pair with X;

$$K'_{H_2A} = \frac{a_{H^+}[HA]}{[H_2A]} = \text{first apparent acid dissociation constant;}$$

$$K'_{HA} = \frac{a_{H^+}[A]}{[HA]} = \text{second apparent acid dissociation constant;}$$

$$K_{ex(HAX)} = \frac{[HAX]_{org}}{[HA][X]} = \text{extraction constant (1 + 1);}$$

$K^{x}_{ex(HAX)} = \dfrac{C'_{Aorg}}{C'_{A}\,C'_{X}}$ = conditional extraction constant (1 + 1);

$K_{ex(H_2AX_2)} = \dfrac{[H_2AX_2]_{org}}{[H_2A][X]^2}$ = extraction constant (1 + 2);

$K^{x}_{ex(H_2AX_2)} = \dfrac{C'_{Aorg}}{C'_{A}\,(C'_{X})^2}$ = conditional extraction constant (1 + 2);

$k_{a(H_2AX_2)} = \dfrac{[H_2AX_2]}{[H_2A][X]^2}$ = ion-pair formation constant;

k'_f = determined capacity ratio;

k'_c = calculated capacity ratio.

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APPLICATION OF STATISTICAL OPTIMIZATION METHODS TO THE SEPARATION OF MORPHINE, CODEINE, NOSCAPINE AND PAPAVERINE IN REVERSED-PHASE ION-PAIR CHROMATOGRAPHY

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(Received February 26th, 1981)

SUMMARY

The separation of morphine, codeine, noscapine and papaverine in reversed-phase ion-pair chromatography has been investigated by means of statistical optimization methods. The value of the capacity factor, as a function of the methanol–water ratio, pH and the concentrations of buffer and ion-pair reagent, including their interactions, was studied for each compound. It was shown that, by using such methods, conditions could be chosen which essentially improved the separation and that a quantitative characterization of the optimum region was facilitated.

INTRODUCTION

In this paper we will discuss the application of factorial design and response surface calculations to the optimization of a reversed-phase ion-pair chromatographic system. This analytical technique, pioneered by Schill and co-workers[1–3], has been shown to be a powerful tool for ionizable organic substances. During the last few years the reversed-phase mode has become the separation method of choice for many pharmaceutical products. Much interest has been focused on the mechanisms involved[4–6] and on the influences of various additives in the mobile phase, counter ions, buffers, pH and ionic strength on the selectivity[7–10]. One difficulty in evaluating the retention mechanisms arises from the variations in chromatographic behaviour due to the different properties of the reversed-phase materials. A thorough discussion of ion-pair high-performance liquid chromatography (HPLC) has recently been given by Bidlingmeyer[11].

Our analytical problem was to find a method for use in routine work which would accomplish a separation good enough for quantification of the four alkaloids

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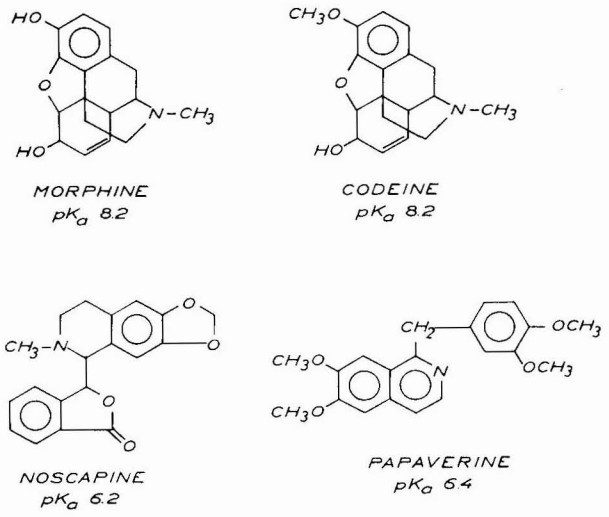

Fig. 1. Structures and pK_a values of the compounds.

morphine, codeine, noscapine and papaverine (Fig. 1) in a commercial solution. Similar problems have been dealt with by Knox and Pryde[12] who separated morphine, codeine and papaverine on a 125 × 5 mm, 6-μm, Hypersil SAS silica column with methanol–water (1:1), containing 25 mM ammonia as the eluent. Wu and Wittick[13] separated all these four alkaloids using two 300 × 4 mm μBondapak C_{18} columns in series and an aqueous mixture containing 25% v/v acetonitrile and 100 mM NaH_2PO_4, pH 4.8, as the eluent. However, with this system it was not possible to quantify morphine since this compound was eluted too close to the solvent front. Besides, the time of analysis was very long, about 40 min for the last compound eluted. For routine analyses the toxic and expensive acetonitrile is also less suitable.

Optimization of chromatographic conditions can be achieved by use of two principally different approaches. The most usual, but perhaps not always the most successful, is to base the choice of chromatographic conditions on knowledge and experience with the sample and the available chromatographic methods. This approach is useful if all relevant factors are known and their importance in the actual case is well understood. The other possible route, which is useful when all circumstances are not fully known, is to seek the optimum via a mathematical-statistical method. The great advantage of the latter approach is that no extensive chemical assumptions have to be made and therefore the results are not subject to chemical model errors. However, chemical knowledge must still be used in the consideration of the choice of variables, in order to reduce the number of experiments. The application of statistical optimization methods, *e.g.*, factorial designs and simplex, has been reviewed[14].

A number of optimization methods has been proposed for use in chromatography. Watson and Carr[15] applied the simplex algorithm to optimize a gradient elution separation, and used the chromatographic response function (CRF), originally proposed by Morgan and Deming[16], as a measure of the quality of the sepa-

ration. The CRF is a function of the experimental peak separation for each pair of peaks (originally defined by Kaiser[17]), the desired peak separation, and the actual and acceptable time of analysis. Smits and co-workers[18,19] optimized an ion-exchange separation using the simplex method and chose "the information content", P_{inf}

$$P_{inf} = \sum_{i=1}^{n} {}^{2}\log S_i$$

as a criterion. Here n is the number of peaks in a fixed period of time and S_i expresses the overlap between neighbouring peaks. These authors also emphasized the difficulties in the choice of the quality criterion. Gant *et al.*[20] developed an optimization method based on semiempirical estimates of the capacity factor and the selectivity. These values are then combined with theoretical values of plate number for the Knox equation to allow calculation of the resolution as a function of all experimental parameters. The consistency of theoretical and experimental values is excellent. However, the general validity of the theoretical assumptions in this approach is doubtful for more complex LC systems. Another question is the applicability of this approach when dealing with incompletely gaussian peaks. As discussed by Christophe[21], this markedly affects the reliability of resolution and plate number calculations. Recently, some authors[22–24] have suggested that, instead of reducing the chromatograms to one single figure, the optimization can be carried out by means of values obtained from each pair of peaks. In this way the risk of losing significant information is diminished.

EXPERIMENTAL

Apparatus

A Laboratory Data Control Constametric II pump, an Altex 152 UV-detector operated at 254 nm, a Rheodyne 7010 injection valve equipped with a 20-μl loop and a Vitatron recorder were used. One 300 × 4 mm μBondapak C_{18} column (Waters Assoc.) was used throughout the experiments.

Reagents and solutions

Methanol, p.a. quality, was from May & Baker (Dagenham, Great Britain); water was obtained from a Millipore Super-Q system. Camphorsulphonic acid (CSA), synthesis quality, from E. Merck (Darmstadt, G.F.R.), was used without purification. Morphine, codeine, noscapine and papaverine were of pharmacopoeial grade. All other chemicals used were of reagent grade and used as received. The water–methanol solutions were prepared by weighing, and the volume ratios given were calculated from the apparent densities. pH values were measured directly in the eluent because reproducible results were given priority over formally correct measurements.

Procedure

The sample was injected and the hold-up volume was determined by the first detector signal deflection from the baseline. The resulting mean for all injections was

2.71 ml (S.D. 0.08 ml). This corresponds to a total column porosity of 0.72 which is in good agreement with the literature value[25].

All measurements were made on equipment contained in a thermostatted room at 21.5°C. Approximately twenty column volumes were pumped through after each change in eluent. Each capacity ratio, k', is the mean from three measurements.

To avoid biasing the results, all experiments were performed randomly. The experiment numbering in Table I is thus only for clarity and does not represent the order in which the experiments were made.

Factorial design

In order to investigate the effect of each component (variable) in the eluent as well as their possible interactions, a full factorial design (FD) was adopted as the optimization strategy. In a FD each variable, v, is given two values, denoted by + or −, defining the experimental domain, see Fig. 2. For M variables, 2^M experiments have to be performed to allow calculation of the magnitudes of the effects and their interactions ($M = 3$ in Fig. 2). The numerical values for the effect of one variable are obtained by subtracting the response at the minus level from another experiment on the plus level. The magnitude of the mean effect of one variable, *e.g.*, v_1, is then equal to the mean of these response differences. The interaction effect between two variables, *e.g.*, v_1 and v_2, is calculated as the difference between those response differences where the variable v_2 has a high value and those where v_2 has a low value. Thus if the effect of v_1 is different at the two levels of v_2 this is seen from the numerical value for the calculated interaction $v_1 \times v_2$. A comprehensive treatment of the method has been given previously[26,27].

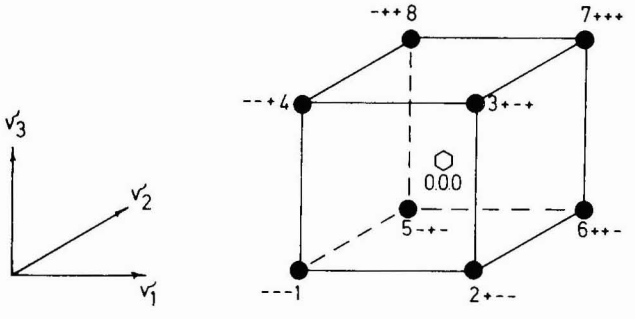

Fig. 2. Schematic representation of a full factorial design for three variables.

Response surface

A response surface was generated in order to get a better picture of the probable optimum[28]. Our approach has three basic steps. (1) The experiments were carried out according to a "central composite design". In this design the experiments are situated at the centre and on the perimeter of a circle. All peripheral experiments are placed with the same distance from each other. This design was later expanded with four experiments because the first evaluation of the data indicated that the optimum

was located at the border of the experimental domain. (2) The data were fitted to a mathematical model for the capacity factor

$$k' = a_0 + a_1 x_1 + a_2 x_2 + a_3 x_1^2 + a_4 x_2^2 + a_5 x_1 x_2$$

where x_1 = concentration (mM) of ion-pair reagent and

$$x_2 = 10^{V_{\text{methanol}}}/(V_{\text{methanol}} + V_{\text{water}}) \text{ (where } V = \text{volume)}$$

(3) This model was used to generate a response surface contour plot.

RESULTS AND DISCUSSION

Screening experiments

As mentioned in the Introduction, our analytical problem was to find a method for use in routine work which would achieve a separation good enough to enable quantification of the four alkaloids morphine, codeine, noscapine and papaverine in a commercial solution. A 300 × 4 mm μBondapak C_{18} column was the natural choice, not least in view of the promising results reported by Wu and Wittick[13]. Besides, most pharmaceutical analyses are performed using C_{18} columns.

The screening experiments showed that it was difficult to separate morphine from codeine without obtaining excessively high k' values for noscapine and papaverine. Camphorsulphonic acid (CSA) was chosen as counter ion and pH of the eluent was kept between 2.0 and 7.5 to avoid destruction of the column. Bad tailing was noted when approaching pH values close to the pK_a, and therefore we chose to avoid this situation when both protonated and unprotonated alkaloids were present in the mobile phase. The values of pK_a in Fig. 1 are for aqueous solutions; the corresponding values in methanol–water mixtures with different autoprotolysis are probably somewhat higher[29].

The best situation achieved after the screening experiments is shown in Fig. 3. Our problem now is to look for conditions for which the time of analysis is reduced without causing any significant loss in resolution. It is also desirable to increase the retention of the first compound eluted to avoid possible interference effects due to the solvent front.

The optimization

The choice of general quality criteria is fraught with problems since the requirements are often ambiguous and difficult to express in quantitative terms. In order to solve the optimization problem we adopted a somewhat different strategy to that used by some previous authors[15,16,19]. It was convenient to study the influence of the various variables on the k' values of the compounds since this quantity is well-defined and simple to evaluate. Moreover, a measure of the time of analysis is obtained from the k' value. These values can then be compared with each other in order to establish the conditions which best correspond to the set requirements. However, by using k' no measure of the separation between adjacent peaks is obtained. This is not a serious drawback because, for a given separation system, there is a correlation between the resolution and the differences in the capacity factors of consecutive peaks.

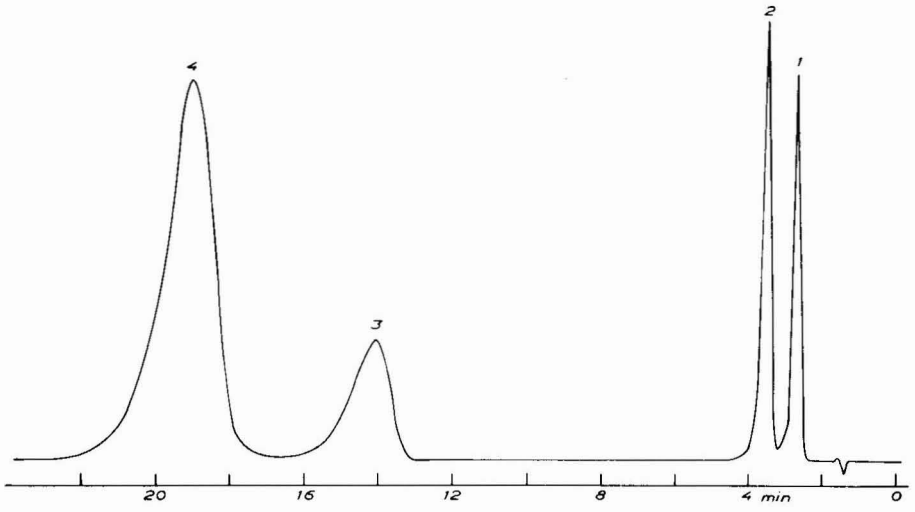

Fig. 3. Separation achieved after screening experiments. Eluent: methanol–water (35:65), 0.050 *M*, phosphate buffer, 0.005 *M* CSA, pH 3.0. Column: μBondapak C_{18}. Flow-rate: 1.50 ml min^{-1}. Elution order with k' values in parentheses: 1 = morphine (0.5); 2 = codeine (1.0); 3 = noscapine (6.8); 4 = papaverine (9.6).

The selection of variables and their values is a critical part of the optimization procedure and here chromatographic experience has to be used. If too many variables are included there will be too many experiments to perform; on the other hand, if too few or the wrong variables are chosen, valuable information will be lost. Our choice of variables included the eluent strength, the pH and the concentrations of phosphate buffer and camphorsulphonic acid. There are also practical limits to the variable range. For example, the buffer concentration must be sufficiently high to maintain a constant pH. The pH range was chosen so that the ion-pair mode should prevail during the experiments. The methanol and camphorsulphonic acid concentrations were selected to give a reasonable change in retention from each variable.

The best eluent from the screening experiments was taken as the origin of a four-dimensional cube spanned by the coordinates (values) of the four variables. In Table I the coordinates for each variable in all the eluents are shown together with the resulting values of the capacity factor for each alkaloid. The effects of the variables on the k' values of the alkaloids were calculated as described in *Factorial design*, and the results are given in Table II. A positive value indicates that the compound has a higher retention at the positive level and a negative value shows higher retention at the negative level. It is seen that the effect of a variable is greater for a substance having a higher capacity factor, and that the methanol–water ratio and the CSA concentration have the greatest numerical values and thereby importance. The effects of the other variables are smaller and can be neglected from an optimization point of view. The interaction effects were also calculated and the two-variable interactions are shown in Table II. Higher order interactions were very small and are therefore omitted. It is noted that the interaction between solvent strength and the CSA concentration is of some importance, which means that the alteration in k' caused by the change in counter ion concentration is dependent on the methanol–water ratio. Fur-

TABLE I

ELUENT COMPOSITIONS AND k' VALUES OBTAINED FROM THE FACTORIAL DESIGN EXPERIMENTS

Expt. no.	*Methanol–water ratio (v/v)*	*pH*	*Buffer concn. (M)*	*CSA concn. (M)*	k'			
					Morphine	*Codeine*	*Noscapine*	*Papaverine*
1	38:62	4.0	0.09	0.010	0.44	0.81	5.19	6.79
2	38:62	4.0	0.09	0.000	0.30	0.58	3.71	5.11
3	38:62	4.0	0.01	0.010	0.65	1.16	7.32	9.34
4	38:62	4.0	0.01	0.000	0.27	0.54	3.53	4.83
5	38:62	2.0	0.09	0.010	0.57	1.02	6.05	7.82
6	38:62	2.0	0.09	0.000	0.24	0.48	2.29	4.13
7	38:62	2.0	0.01	0.010	0.73	1.28	7.42	9.55
8	38:62	2.0	0.01	0.000	0.24	0.48	2.89	4.10
9	32:68	4.0	0.09	0.010	0.64	1.35	12.4	17.8
10	32:68	4.0	0.09	0.000	0.42	0.86	7.62	11.3
11	32:68	4.0	0.01	0.010	0.95	1.88	16.4	22.5
12	32:68	4.0	0.01	0.000	0.35	0.78	7.37	10.8
13	32:68	2.0	0.09	0.010	0.88	1.78	16.0	22.3
14	32:68	2.0	0.09	0.000	0.32	0.69	6.01	9.33
15	32:68	2.0	0.01	0.010	1.08	2.16	19.3	26.5
16	32:68	2.0	0.01	0.000	0.29	0.66	6.18	9.42

TABLE II

CALCULATED EFFECTS FROM THE FACTORIAL DESIGN SHOWING THE VARIATION IN k' CAUSED BY THE CHANGE IN THE ELUENT (CALCULATED FROM TABLE I)

Variable	*Morphine*	*Codeine*	*Noscapine*	*Papaverine*
1 Methanol–water ratio	−0.19	−0.48	−6.6	−9.8
2 pH	−0.42	−0.072	−0.31	−0.58
3 Buffer concn.	−0.096	−0.17	−1.4	−1.6
4 CSA concn.	0.44	0.79	6.3	7.9
Interaction 1/2	0.011	0.033	0.59	0.69
Interaction 1/3	0.011	0.029	0.41	0.57
Interaction 1/4	−0.11	−0.25	−2.9	−4.1
Interaction 2/3	–	–	–	–
Interaction 2/4	−0.11	−0.19	−1.5	−1.8
Interaction 3/4	−0.13	−0.21	−1.3	−1.7

thermore, for all compounds the k' values increase with increasing CSA concentration and decrease with increasing methanol–water ratio, as expected. If, for each compound, the magnitudes of these two effects are compared, it is seen that for noscapine and papaverine they are roughly the same. It should be noted, however, that for morphine and codeine the CSA-concentration effect is approximately twice as large which means that an increase in the CSA concentration will have a greater influence on the first compounds eluted.

These results show that it should be possible to change selectively the capacity factors by appropriate modification of the eluent. Our initial separation problem was to decrease the k' values of noscapine and papaverine without affecting the values for morphine and codeine. This cannot be accomplished simply by increasing the solvent

strength since the capacity factors for morphine and codeine would still decrease. However, the latter effect can be compensated for by a corresponding increase in the CSA concentration. According to Table II, an increase in the CSA concentration will have a smaller influence on the later compounds eluted, the net effect being an improvement in the separation.

When the coordinates from the screening experiment [methanol–water (35:65), 5 m*M* CSA] were reflected through the best point from the factorial design [methanol–water (38:62), 10 m*M* CSA] new coordinates were obtained at [methanol–water (41:59), 15 m*M* CSA]. As is seen in Fig. 4, the experimental results and the calculations are in good agreement. The k' values for noscapine and papaverine are 4.6 and 5.7, respectively, compared with 6.8 and 9.6 in Fig. 3. The resolution, as well as the distance to the solvent front, are still good enough to allow quantification of all the peaks.

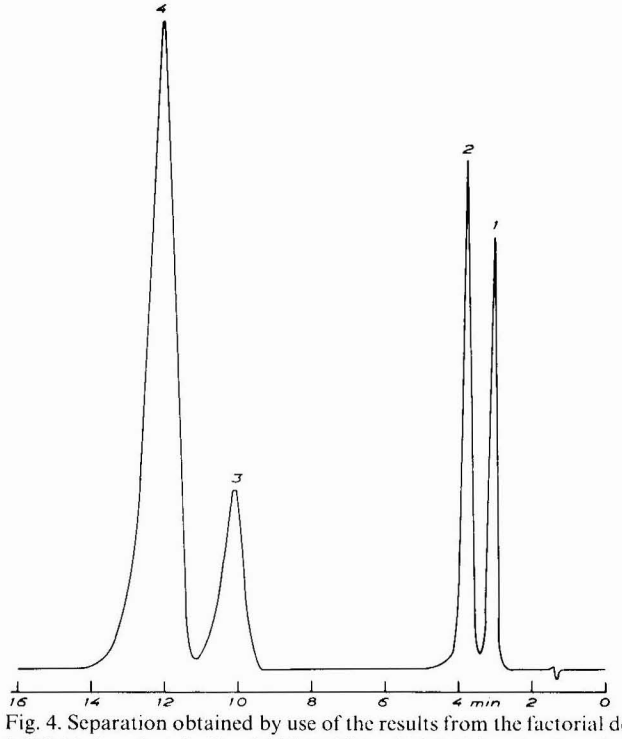

Fig. 4. Separation obtained by use of the results from the factorial design. Eluent: methanol–water (41:59), 0.010 *M* phosphate buffer, 0.015 *M* CSA, pH 2.0. Other details as in Fig. 3. k' values: 1, 0.6; 2, 1.1; 3, 4.6; 4, 5.7.

The response surface

In order to test the validity of the optimum found in the factorial design a response surface was generated with respect to the two most significant variables. The buffer concentration and pH were set at favourable levels (10 m*M* phosphate, pH 2) and the methanol–water ratio and the CSA concentration were varied in the area where the optimum was assumed to be situated. To avoid masking of the optimum, the increments of methanol and CSA were reduced to 1.5 v/v-part and 2.5 m*M*, respectively. Since the optimum was not in the centre of the experimental design, this

was enlarged with four additional experiments. Multiple regression analysis was carried out with k' as the dependent variable. The independent variables were the methanol–water ratio and the CSA concentration, their interaction term and their quadratic terms.

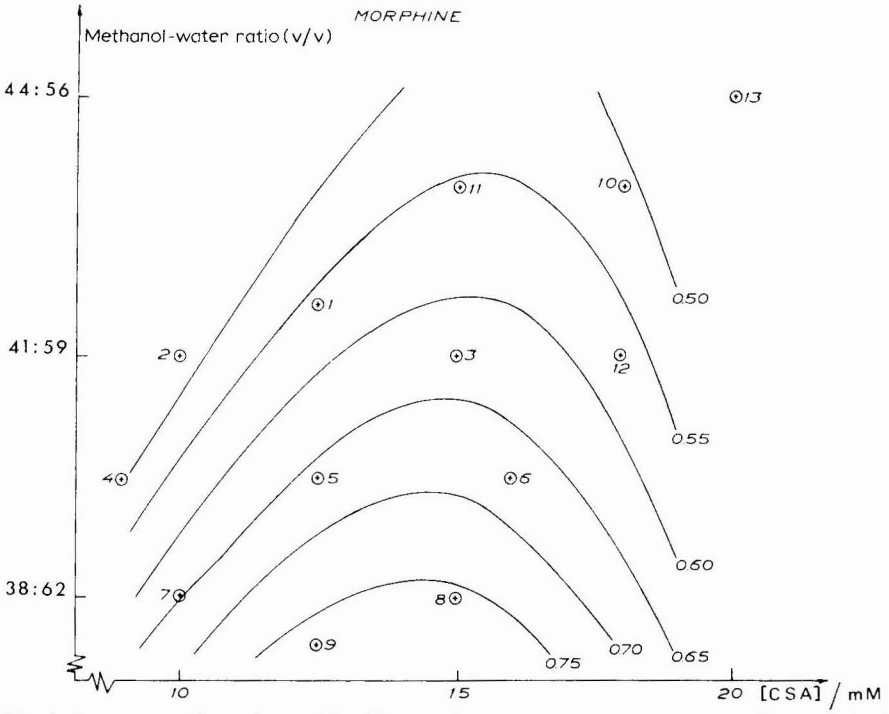

Fig. 5. Response surface of morphine. The numbers at the isoresponse contour lines are the k' values. Experimental points are marked with numbered rings.

From the morphine response surface in Fig. 5 it is seen that, for a fixed methanol–water ratio, k' first increases and then decreases with increasing CSA concentration. This behaviour has been noted before in ion-pair reversed-phase systems and has been studied by Horváth *et al.*[9]. It should be noted that the scale on the ordinate axis is exponential. Also, that the surface has a narrow ridge-like feature which means that morphine is comparatively more sensitive to the CSA concentration than the other alkaloids (see Figs. 6–8). These results are in agreement with those obtained from the factorial design (see Table II). The increasingly shallower curves in Figs. 6–8 demonstrate the diminishing effect of the counter-ion concentration in the order: morphine, codeine, noscapine and papaverine. Maximum k' values are obtained for all four alkaloids at *ca.* 15 m*M* CSA. For this concentration, changes in the methanol–water ratio have less effect on k' than at, *e.g.*, 10 or 18 m*M*.

By superimposing the plots from the first and last peaks eluted as shown in Fig. 9, it is apparent that strong retention of morphine and at the same time a weak retention of papaverine will be achieved somewhere in the shaded area. Outside this, k' for morphine decreases faster than for papaverine. We note that the eluent composition suggested by the factorial design [methanol–water (41:59 v/v), 15 m*M* CSA] lies within this area.

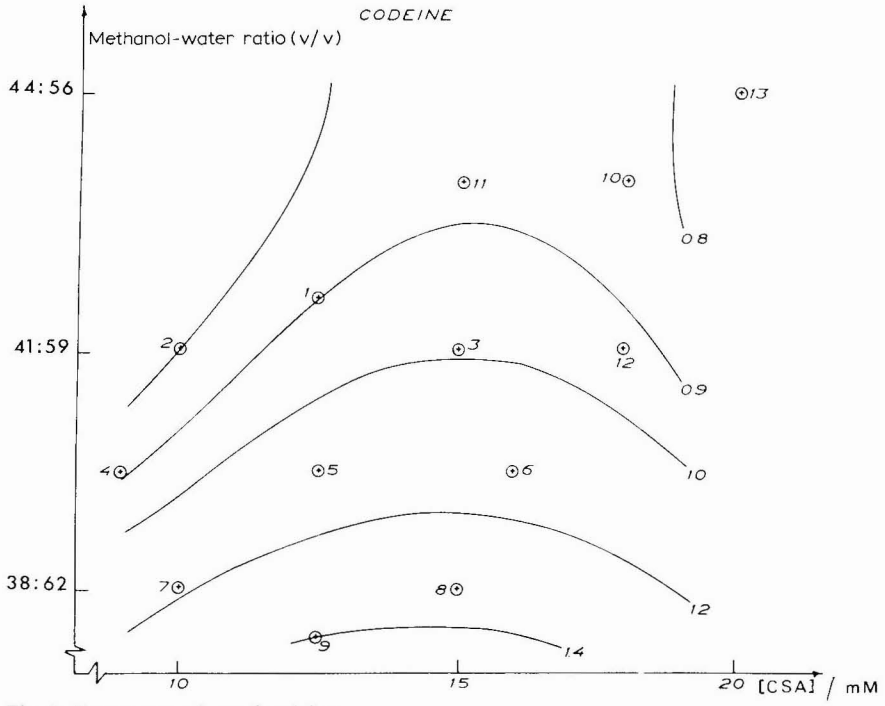

Fig. 6. Response surface of codeine.

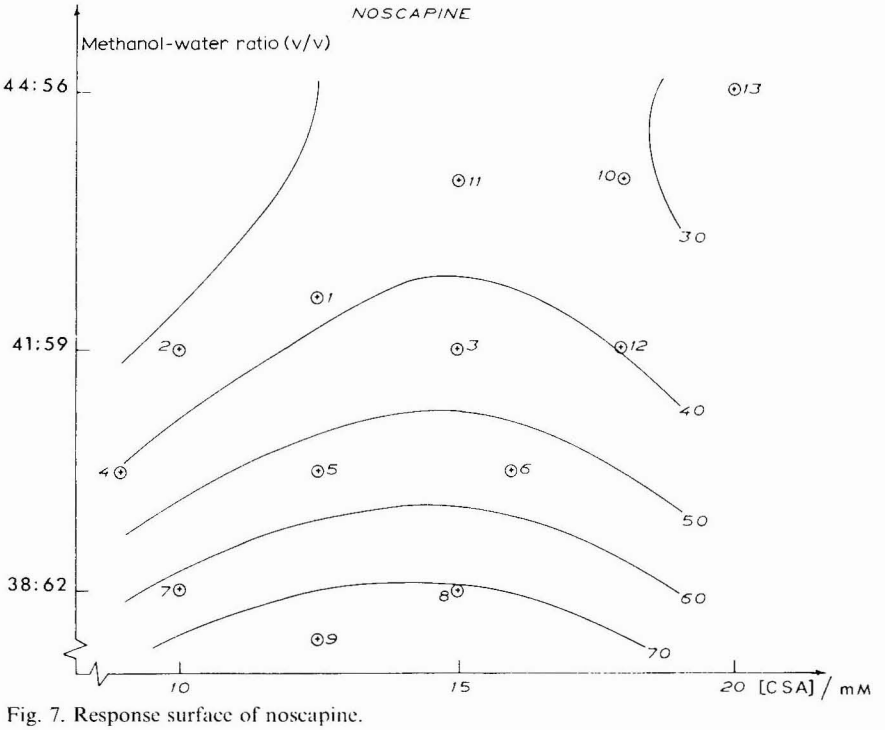

Fig. 7. Response surface of noscapine.

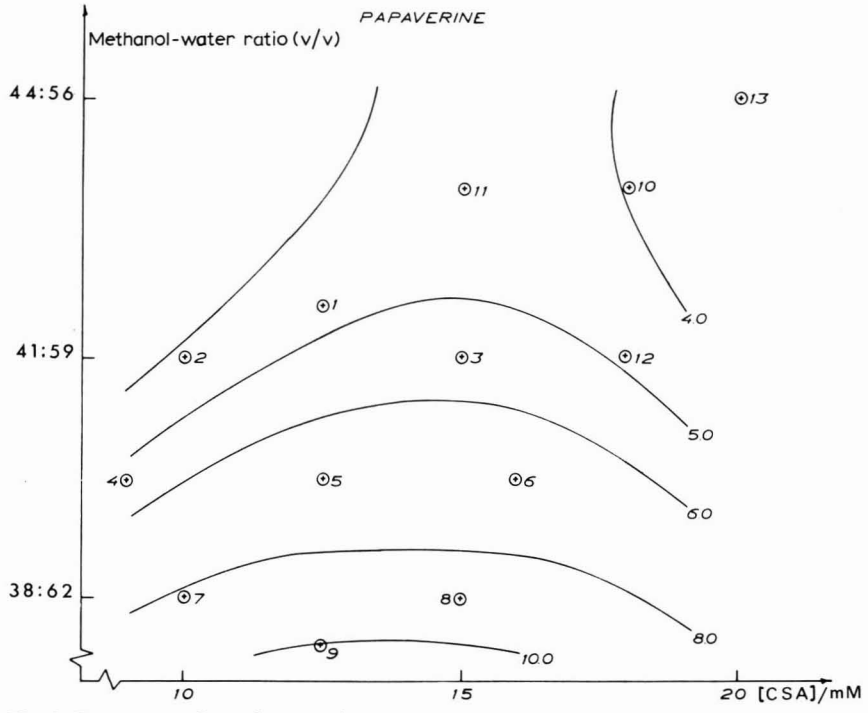

Fig. 8. Response surface of papaverine.

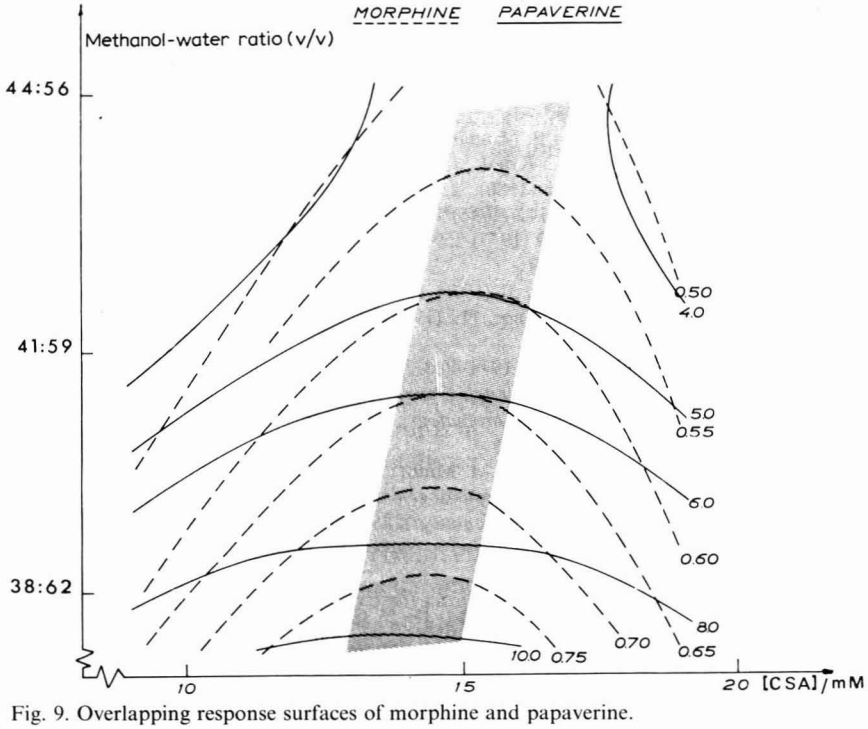

Fig. 9. Overlapping response surfaces of morphine and papaverine.

The plot shown in Fig. 9 also illustrates that the concept of "optimum conditions" depends on the purpose of the separation. Chromatographers requiring high speed of analysis and/or low detection limit should concentrate on the upper part of the shaded area. On the other hand, accurate measurements are favoured by higher resolution and thus higher k' values might be advantageous, as found in the lower shaded parts. Additionally, with increasing retention the probability of separating and detecting possible impurities or decomposition products is higher.

CONCLUSIONS

The utility of statistical methods for optimization purposes in reversed-phase ion-pair chromatography has been illustrated. Such methods offer unique possibilities for judging the qualitative importance of the considered variables, as well as for providing a quantitative picture of the optimum region.

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QUANTITATIVE ION-PAIR EXTRACTION OF 4(5)-METHYLIMIDAZOLE FROM CARAMEL COLOUR AND ITS DETERMINATION BY REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure for quantitative ion-pair extraction of 4(5)-methylimidazole from caramel colour using bis(2-ethylhexyl)phosphoric acid as ion-pairing agent has been developed. Furthermore, a reversed-phase ion-pair liquid chromatographic separation method has been established to analyse the content of 4(5)-methylimidazole in the extracts. A rapid and adequate separation was achieved on a column of Nucleosil 5 C_8 eluted with methanol–0.2 *M* potassium dihydrogen phosphate–water (32.5:25:42.5) containing 0.005 *M* sodium dodecanesulphonate.

This method of determination is superior in speed and repeatability to, and at higher contents of 4(5)-methylimidazole gives a better accuracy than, the World Health Organization method currently official in Denmark. The limit of detection is estimated at 4 μg/g.

INTRODUCTION

Caramel colours are among the most widely used food and drug colouring matters. Commercial caramel colour is mainly manufactured by a sugar–ammonia or by a sugar–ammonia–sulphite reaction procedure, during which imidazole and pyrazine derivatives are formed. The content of 4(5)-methylimidazole (4-MeI) has attracted special attention due to its possible toxicity. At the present time, as a precautionary measure, the World Health Organization has specified the acceptable limit of 4-MeI as 200 ppm based on a caramel colour having a colour intensity of 20,000 European Brewery Convention (EBC) units[1].

Since caramel colour is produced on a large scale, a test for 4-MeI is often done

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and a fast and reliable method is therefore required. So far several methods and many improvements concerning both the extraction and the final determination have been published. The official method in Denmark[1] is based on the work of Wilks *et al.*[2] and involves solvent extraction of a semi-dry mixture of the sample and diatomaceous earth followed by a gas chromatographic (GC) analysis. The extraction step was subsequently changed by Wilks *et al.*[3]. Other isolation techniques are also used, such as ion exchange[4–6] and solvent extraction[7–9]. The analysis of the extract has also been the subject of modifications, for instance through the introduction of 2-methylimidazole as internal standard[3], the conversion into the acetyl derivative before GC[5,7], the use of a nitrogen specific detector[8] or application of reversed-phase ion-pair liquid chromatography[9]. None of the modifications, however, constitutes decisive improvement. For instance, the altered procedure of Wilks *et al.*[3] was found to be subject to interference from an unknown extracted from some batches of caramel colours, biasing the internal standard[10]. Furthermore, none of the published methods was found to have a dynamic range of determination exceeding 200 μg/g of 4-MeI.

All of the above methods are very time-consuming. In particular, when a high accuracy is needed, the workable methods require an analysis time equivalent to not more than four samples per person per day. This problem is mainly caused by insufficient extraction, justifying a closer examination. This paper describes an ion-pair extraction procedure and a high-performance liquid chromatographic (HPLC) separation and their validation for the determination of 4-MeI.

EXPERIMENTAL

Apparatus

Concentrations of 4-MeI in aqueous phases were determined by absorbance measurements at 215 nm using a Beckman Acta III spectrophotometer. pH values of the aqueous phases were read from a Radiometer Model PHM 64 pH-meter.

The liquid chromatograph comprised a Kontron Model 410 LC pump, equipped with a Kontron Model 811 pulse damper, a Rheodyne Model 7125 injection valve and a Pye Unicam LC–UV detector. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder, and retention and area data were measured and processed by means of a Hewlett-Packard Model 3353A laboratory data system.

The gas chromatograph was a Hewlett-Packard Model 5840A equipped with a flame ionization detector and a Hewlett-Packard Model 7672A automatic sampler. Chromatograms were recorded on the plotting integrator of the gas chromatograph, but the peak heights were measured and contents calculated manually.

Chemicals and reagents

4-MeI was obtained from Fluka (Buchs, Switzerland). Bis(2-ethylhexyl)-phosphoric acid (DEHPA) was obtained from BDH (Poole, Great Britain). DEHPA was purified and chloroform was freed from ethanol, both by repeated extraction with 0.1 *M* phosphoric acid. Chloroform as well as all the other reagents were of analytical grade and were obtained from E. Merck (Darmstadt, G.F.R.). Phosphate buffers had an ionic strength of 0.2 *M* and contained potassium as the only cation.

Information about the caramel colours investigated is provided in Table I. The isoelectric points were determined according to White and Munns[11], whereas the

TABLE I

THE CARAMEL COLOURS INVESTIGATED

Sample no.	*Manufacturing procedure*	*Isoelectric point, pH*	*Colour intensity (EBC units)*
1	Ammonia process		43,340
2		4 5	46,660
3		6	32,000
4		4 5	20,000
5		5	36,000
6			29,340
7	Ammonia sulphite process	1.5	20,660
8		1.5	31,340
9		1.5	26,000
10		1.5	46,660
11		1.5	43,340
12		1.5	48,000
13		1.5	45,340
14		1.5	30,000

colour intensities were measured in EBC units in accordance with the standard of the European Brewery Convention[12].

Ion-pair extraction

A 2.50-g amount of caramel colour was diluted with 15 ml of 0.2 *M* phosphate buffer, pH 6.0, in a 20-ml measuring cylinder. After mixing, the pH was adjusted to 6.0 by dropwise addition of a potassium hydroxide solution. Finally the cylinder was filled to the 20-ml mark with the phosphate buffer.

Four millilitres of this sample solution (equivalent to 0.5 g caramel colour) were extracted in a screw-capped centrifuge-tube with 4.00 ml of 0.1 *M* DEHPA in chloroform by shaking for 0.5 min. After separation by centrifuging, 3.00 ml of the chloroform phase were transferred to a new centrifuge-tube containing 3.00 ml of 0.1 *M* phosphoric acid. By shaking for 0.5 min the content of 4-MeI was re-extracted into the aqueous phase, which after separation by centrifuging is ready for the final determination.

The resulting aqueous phase of the back-extraction contains three quarters of the 4-MeI content originally present in the amount of caramel colour sampled. The ion-pair extraction procedure is illustrated in Fig. 1.

Ion-pair chromatography

Stainless-steel columns (120 × 4.6 mm I.D.) from Knauer (Berlin, G.F.R.) were packed with Nucleosil 5 C_8 and 5 C_{18} (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.) according to a previously described procedure[13]. The efficiency of the columns, expressed as the number of theoretical plates, *N*, measured for naphthalene when eluted by 80% and 90% methanol, respectively, in water at a flow-rate of 1 ml/min, was 9000 for the C_8 and the C_{18} column alike.

The eluent was methanol–0.2 *M* potassium dihydrogen phosphate–water (32.5:25:42.5), and sodium dodecanesulphonate was added as counter ion at a con-

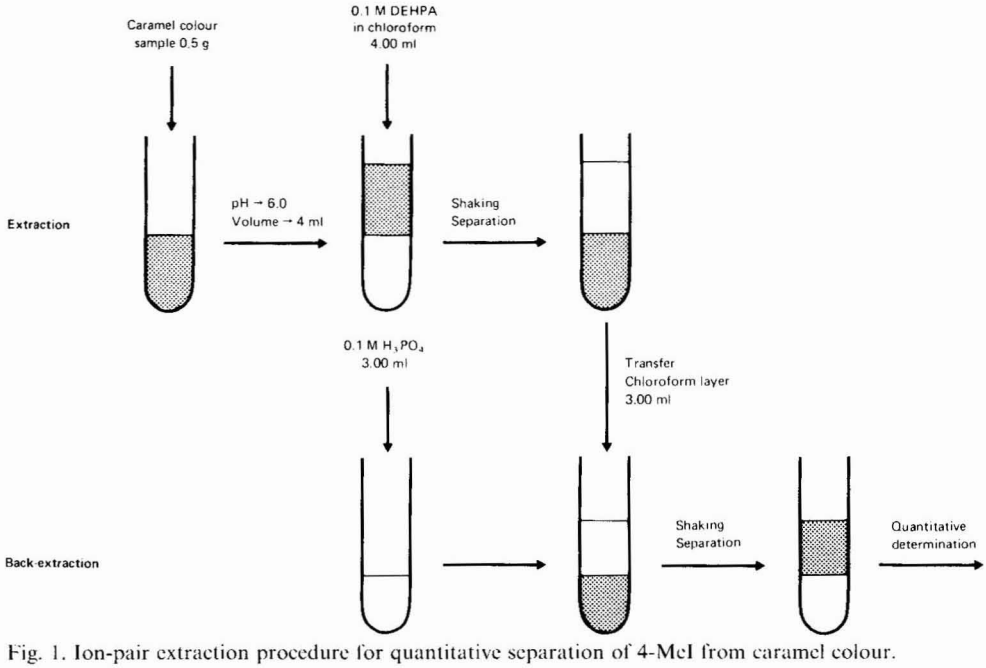

Fig. 1. Ion-pair extraction procedure for quantitative separation of 4-MeI from caramel colour.

centration of 0.005 *M*. A 20-μl volume of the resulting aqueous phase from the back-extraction was injected.

Official method

The extraction was performed according to the official standard[1] modified by using two re-extractions with 10 ml sulphuric acid instead of one. A glass column (1800 × 2.0 mm I.D.) was packed with 5% Carbowax 20M and 2% potassium hydroxide on Chromosorb W AW DMCS (80–100 mesh) (Macherey, Nagel & Co.). The temperatures of the injector, column and detector were 240, 180 and 240 °C respectively. The carrier gas was nitrogen at a flow-rate of 30 ml/min. A 2.5-μl volume of sample solution was injected.

RESULTS AND DISCUSSION

Ion-pair extraction

The determination of 4-MeI in caramel colour requires a prior isolation step due to the nature of the colouring matter. The hydrophilic character of 4-MeI leads to a low efficiency of extraction with most organic solvents, making a quantitative isolation difficult. Using an ion-pair technique, particularly in combination with adduct formation, a more hydrophobic product can be obtained.

Working with a similar problem, Modin and Johansson[14] used DEHPA for the isolation of aminophenols and amino alcohols as ion pairs. But even though the pK_a value of 4-MeI is known to be 7.6[15], it was not possible to apply the technique to 4-MeI on the basis of the general procedures proposed by Modin[16]. It was necessary

to determine experimentally the optimum pH for the extraction in order to achieve full recovery with one extraction only. This was done by measuring the net distribution ratio, D, of 4-MeI between the organic and aqueous phases using a standard solution of 4-MeI extracted at different pH values and determining the contents spectrophotometrically. The resulting relationship between the logarithm of D and pH can be seen in Fig. 2. The extraction with 0.1 M DEHPA in the organic phase is virtually complete at pH 6.0 in the aqueous phase. Under these conditions 4-MeI is assumed to form an (1 + 1) ion pair with DEHPA combined with an adduct consisting of 2 moles of DEHPA[17]. This means that groups containing 48 alkyl carbon atoms in all are coupled to the cation, thus allowing full recovery by only one extraction into chloroform. Furthermore, this procedure has the further advantage of yielding a clean extract, due to the selectivity in the co-extraction and the detention of the counter ion in the organic phase during the back-extraction.

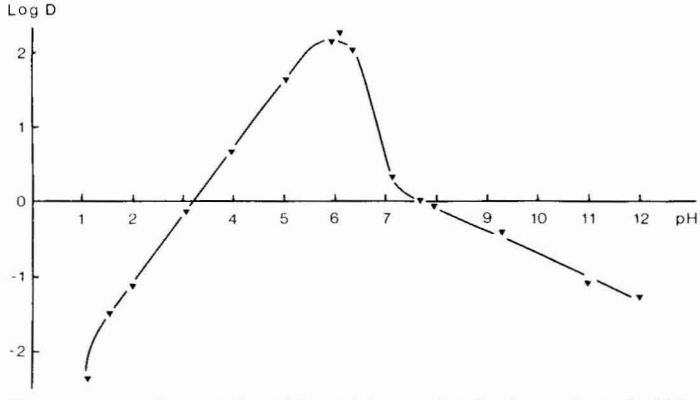

Fig. 2. Relation between logarithm of the net distribution ratio and pH for 4-MeI. The extractions were performed with equal volumes, the organic phases initially comprising 0.1 M DEHPA in chloroform and the aqueous phases comprising 4-MeI in a phosphate buffer.

Ion-pair chromatography

The use of an ion-pair technique for the chromatography of a hydrophilic and ionic substance such as 4-MeI is an obvious approach. Davis and Hartford[9] used heptanesulphonate as the ion-pairing agent and an octadecylsilyl bonded silica as support. In our view the chromatographic system should in this case be based on dodecanesulphonate as counter ion and octylsilyl bonded silica as support, according to a study of Helboe and Thomsen[18]. Using Nucleosil 5 C_8 as the support, a suitable composition of the eluent was found to be methanol–0.2 M potassium dihydrogen phosphate–water (32.5:25:42.5) with 0.005 M sodium dodecanesulphonate.

The separation could also be carried out on octadecylsilyl bonded silica, for instance Nucleosil 5 C_{18}, but the methanol content of the eluent could not be increased, due to interference by a peak from chloroform with which the aqueous phase is saturated during the extraction. The chromatographic behaviour of 4-MeI and chloroform in the systems present can be seen in Fig. 3. The difference leading to intersection of the curves in the range of interest is due to different retention mechanisms of the two substances. 4-MeI seems to be retained partly as an ion pair by a

reversed-phase mechanism and partly by a cation-exchange mechanism. The non-ionic, hydrophobic substance chloroform seems to be chromatographed according to a pure reversed-phase mechanism. The different retention mechanisms are influenced to dissimilar extents by changes in the elution strength of the eluent, leading to intersections of the curves. Additionally, Fig. 3 shows that octylsilyl bonded silica is the more powerful of the two supports with respect to selectivity of the two substances in the range investigated.

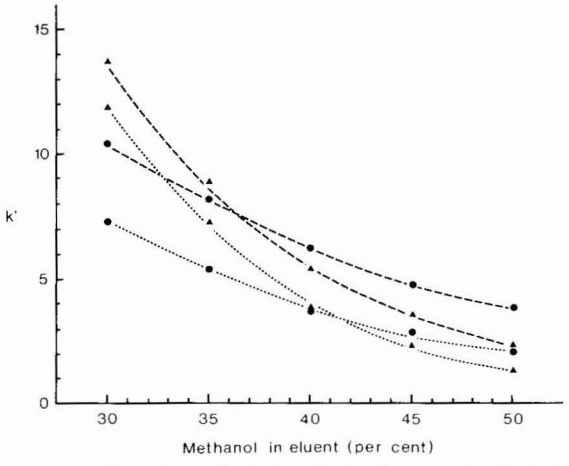

Fig. 3. The behaviour of 4-MeI (▲) and chloroform (●) on Nucleosil C_8 (------) and C_{18} (– – –) columns, expressed by their capacity factors, k', as a function of the percentage of methanol in the eluent containing 0.05 *M* potassium dihydrogen phosphate and 0.005 *M* dodecanesulphonate.

The detection wavelength was chosen on the basis of the absorption spectrum of 4-MeI dissolved in the eluent; the maximum absorption was found at 215 nm. Chromatograms of caramel colours manufactured by the ammonia and by the ammonia–sulphite process respectively are depicted in Fig. 4.

Linearity, recovery and detection limit

The linearity of the detector response and the capability of the extraction procedure were investigated at the same time. Samples of caramel colour from the same batch and equivalent to 1/4, 1/2, 1, 3/2 and 2 times the usual quantity were extracted and analysed according to the described procedures. The results obtained are shown in Table II; a regression analysis showed a small intercept and a satisfactory correlation coefficient.

The recovery of 4-MeI from caramel colour was investigated using a standard amount with different additions of 4-MeI. The results obtained are in Table III. On the basis of these results, which demonstrate an excellent linearity and a quantitative recovery, it was decided to perform the quantitations using standard solutions of one concentration only.

The detection limit of the ion-pair method was investigated by several consecutive determinations on a sample containing a small amount of 4-MeI. From the

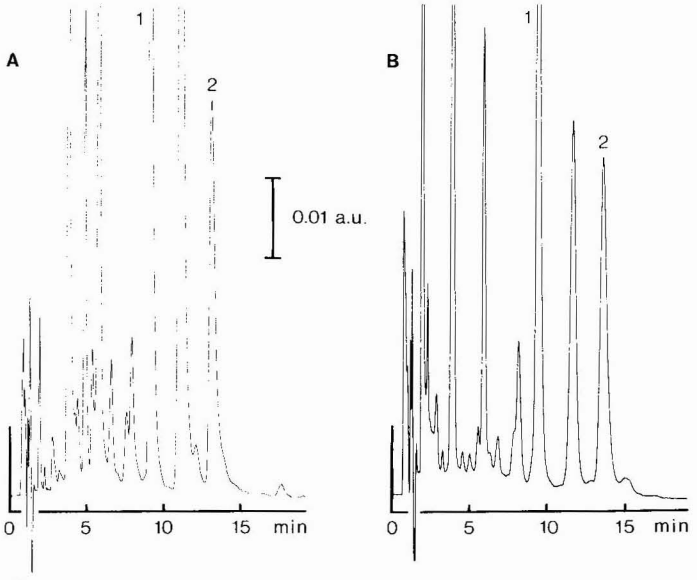

Fig. 4. Chromatograms of caramel colours manufactured by the ammonia process (A) and by the ammonia sulphite process (B). Support: Nucleosil 5 C_8. Eluent: methanol–0.2 *M* potassium dihydrogen phosphate–water (32.5:25:42.5) containing 0.005 *M* dodecanesulphonate; flow-rate 1 ml/min. Detection: 215 nm. Peaks: 1 = chloroform; 2 = 4-MeI (A, 170 μg/g; B, 142 μg/g).

TABLE II

LINEARITY OF EXTRACTION AND CHROMATOGRAPHIC DETERMINATION FOR DIFFERENT QUANTITIES OF THE SAME CARAMEL COLOUR

Linear regression analysis: correlation coefficient, $r = 0.999$; intercept $= -0.82$.

Sample size (g)	*4-MeI found (μg)*
0.120	9.0
0.241	18.5
0.481	41.8
0.722	61.7
0.963	79.5

results obtained the detection limit was estimated as three times the standard deviation of the results, 4 μg/g for a single determination.

Repeatability

The repeatability of the ion-pair method was compared with that of the official method[1] by carrying out ten subsequent extractions and quantitations according to both methods and using the same caramel colour sample. The results obtained are in Table IV, from which it appears that the ion-pair method surpasses the official method in respect of the repeatability. Concerning the speed of the methods, the ion-pair method is about three times faster than the official one.

TABLE III

RECOVERY OF 4-MeI FROM SPIKED CARAMEL COLOUR SAMPLES AFTER EXTRACTION FOLLOWED BY CHROMATOGRAPHIC DETERMINATION

4-MeI (μg)		*Recovery (%)*
Added	*Found*	
0	42	
13	55	100.0
53	96	101.0
132	178	102.3
265	313	102.0
529	593	103.8

TABLE IV

COMPARISON OF THE REPEATABILITY OF THE ION-PAIR METHOD WITH THE OFFICIAL METHOD

Method	*Average,* $\bar{x}_{10}$ *(μg/g)*	*Standard deviation,* S_{rel} *(%)*
Ion-pair	92.6	1.5
Official	92.7	4.1

TABLE V

4-MeI CONTENTS OF VARIOUS CARAMEL COLOURS DETERMINED BY THE ION-PAIR METHOD AND BY THE OFFICIAL METHOD

Caramel colour no.	*4-MeI (ppm per 20,000 EBC units)*	
	Ion-pair method	*Official method*
1	351	310
2	143	119
3	82	83
4	170	170
5	6.6	
6	10.1	
7	139	136
8	66	62
9	123	109
10	341	295
11	272	246
12	145	125
13	149	121
14	62	56

Analyses

The content of 4-MeI in various caramel colours was determined by duplicate determinations by both the ion-pair method and the official method. The results are presented in Table V and are expressed by reference to a standard colour intensity of 20,000 EBC units, as recommended by the European Brewery Convention.

The results of both methods were tested for homogeneity of variance at the 5% level with Bartlett's test according to Youden[19], using the relative standard deviations from duplicate determinations. The test confirms that homogeneity of variance exists for both methods.

Comparison of the results with the confidence intervals determined on the basis of the pooled relative standard deviations from each method shows that, at the 99% level and at contents of 4-MeI higher than 300 μg/g, the ion-pair method gives results significantly greater than those of the official method. Moreover, from the recovery results in Table III, it appears that at high levels of 4-MeI the ion-pair method has a better accuracy than the official method.

ACKNOWLEDGEMENTS

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DETERMINATION OF THE RELATIVE AMOUNTS OF THE B AND C COMPONENTS OF NEOMYCIN BY ION-EXCLUSION CHROMATOGRAPHY USING REFRACTOMETRIC DETECTION

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SUMMARY

Refractometric detection can be used as a convenient alternative to ninhydrin colorimetric or polarimetric detection in ion-exclusion chromatography of neomycin. The determination of the relative amounts of neomycin B and C using different detection methods is examined. The use of a resin of smaller granulometry and a medium-pressure chromatographic apparatus reduces the analysis time to less than 25 min.

INTRODUCTION

Neomycin is a complex mixture of basic water-soluble antibiotics produced during fermentation of *Streptomyces fradiae*[1]. The main active components of this mixture are neomycin B (Fig. 1a) and its stereoisomer neomycin C (b)[2]. Another component, neomycin A, isolated from the mixture[3] and proved to be identical with neamine (g), can be obtained by partial hydrolysis of neomycin B or C[4,5]. Other derivatives isolated from the mixture are neomycin LP-B and LP-C (LP = low potency), which are the mono-N-acetyl derivatives (c, d) of components B and C[6,7]. Related products, isolated from commercial samples of neomycin, are paromamine (h), paromomycin I (e) and paromomycin II (f)[8]. Preparative chromatography of commercial samples in our laboratory confirmed these findings[9]. In addition to the constituents already mentioned, mono-N-acetylneamine (i) was isolated together with minor components designated as G and K. Component G is a O-(diaminodideoxyhexosyl)myoinositol, K is a neomycin B or C molecule lacking the neosamine C part linked to deoxystreptamine.

The antimicrobial potency of component C is lower than that of neomycin B. The potency ratio varies with the microorganism and experimental conditions used in the microbiological assay[10]. An acceptable precision for the latter can only be obtained if the composition of the unknown preparation is fairly similar to that of the standard preparation. Thus a determination of the relative amounts of neomycin B and C has to be included in analysis of commercial neomycin. Chromatographic

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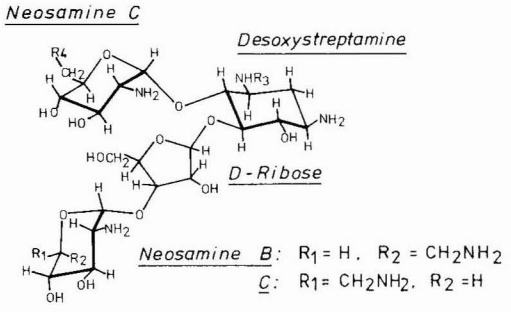
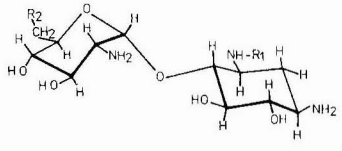


			R_1	R_2	R_3	R_4
a	Neomycin	B	H	CH_2NH_2	H	NH_2
b	Neomycin	C	CH_2NH_2	H	H	NH_2
c	Neomycin	LP-B	H	CH_2NH_2	Ac	NH_2
d	Neomycin	LP-C	CH_2NH_2	H	Ac	NH_2
e	Paromomycin	I	H	CH_2NH_2	H	OH
f	Paromomycin	II	CH_2NH_2	H	H	OH



		R_1	R_2
g	Neamine	H	NH_2
h	Paromamine	H	OH
i	Neomycin LP-A	Ac	NH_2

Fig. 1. Structure of different neomycin components.

separation of the stereoisomers neomycin B and C is quite difficult. Some paper and thin-layer chromatographic systems are successful as mentioned in a recent review[11]. More suitable for routine determinations of neomycin B and C are column chromatography on strongly basic ion-exchange resin (referred to as ion-exclusion chromatography), gas–liquid chromatography (GLC) of trimethylsilyl derivatives[12–14] and high-performance liquid chromatography (HPLC) after dinitrophenylation[15]. In our opinion, chromatography on an ion-exchange column, which requires no pre-column derivatizations, seems to be the simplest procedure. It has been described for the analysis of framycetin[16] (which is neomycin with less than 3% neomycin C) and kanamycin using colorimetric detection after reaction with ninhydrin[17,18], polarimetric detection[19,20] and conductometric detection[21]. It was found that refractometry could be used for the detection of these aminoglycoside antibiotics. So we decided to determine the relative amounts of neomycin B and C in neomycin by the last method and to compare the results with data obtained by previous methods.

EXPERIMENTAL

Column and pump

(A) Pyrex glass columns (40 × 0.6 cm I.D. or 20 × 0.6 cm I.D.), with jackets allowing temperature control by circulation of water, were provided with a glass tube outlet (2 mm I.D.) sealed with PTFE and a stainless-steel capillary. The outlet was plugged with acid-washed glass wool. The top of the column was also plugged with glass wool and closed with a rubber septum. Carbon dioxide-free distilled water was delivered at a constant rate by a Pharmacia P 3 peristaltic pump through a side-inlet at the top of the column (Fig. 2).

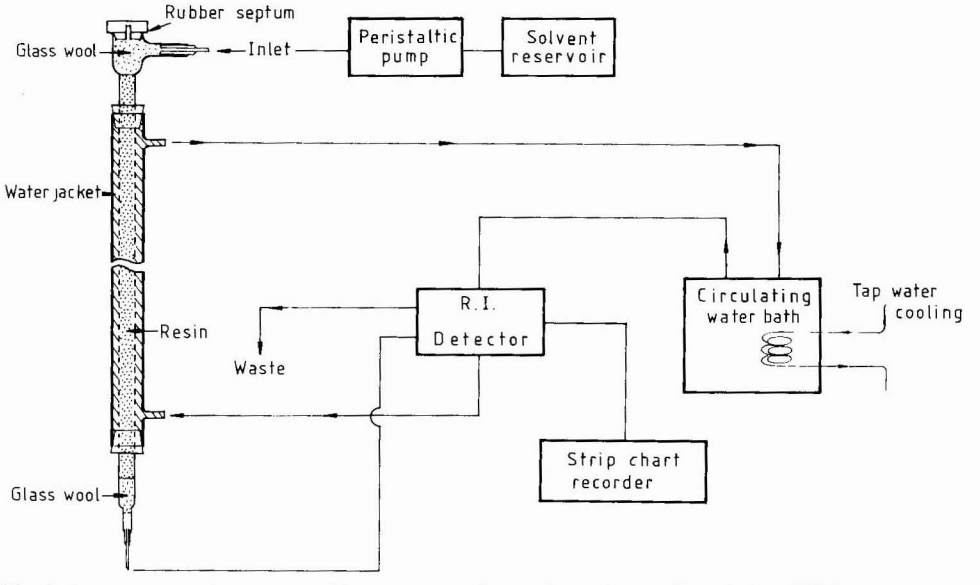


Fig. 2. Low-pressure chromatographic apparatus using a glass column (40 × 0.6 cm I.D.).

(B) A stainless-steel column (Li-Chroma 30 × 1.0 cm I.D.; Alltech Europe, Eke, Belgium) was provided with the necessary end fittings and low dead volume metal tubing. The usual fritted metal discs were replaced by porous polyethylene discs of the same diameter, which had a considerably lower back-pressure. Sheets of this material were purchased from Alltech Europe. The column inlet was connected to a Model CV-6-UHPa-N60 injector (Valco, Houston, TX, U.S.A.). Carbon dioxide-free water was delivered at a constant rate (46–460 ml/h) by a Milton-Roy reciprocating piston Mini pump, provided with a Bourdon-type pressure gauge, which also served as a pulse-damper. A stainless-steel column (10 × 1.0 cm), filled with AG 1-X2 resin (OH^-), was placed between the pressure gauge and injector to remove all traces of CO_2 from the eluent. The column temperature was held constant by immersing the column vertically in a bath of circulated water.

Detector

Glass and metal columns were connected by PTFE tubing (0.3 mm I.D.) to a Waters R-403 Differential Refractometer. The refractometer was thermostatted at 10 or 20°C using a Varian 4100 water-bath, which operated continuously to avoid base-line drifting. For work at 10°C, cooling of the water-bath by a Haake-Cryostat instead of tap-water was necessary. The trapped reference cell of the refractometer was filled with water. With a usual sample load of 10 mg neomycin sulphate, the attenuation setting was $\times 8$. The refractometer is a semi-preparative type which has the advantage of a lower back-pressure than the analytical R-401 model (*ca.* 4 kg/cm^2 at a flow-rate of 270 ml/h). The analytical type can be used with steel columns when the connection between the column outlet and refractometer is made of metal tubing instead of PTFE. Both types gave the same results.

The detector signal was recorded on a Kipp BD40 recorder with a chart speed of 10 mm/min. This high speed was chosen because the precision is higher when measuring larger peak areas. The peak areas were approximated by triangulation or determined with a HAFF 317 Polar Planimeter.

A Thorn PL Type 243 photo-electric polarimeter was provided with a flow-through cell (2 × 0.4 cm I.D.) which was thermostatted at 10°C (to avoid bubble formation in the cell). The output of the polarimeter was shunted to give a 1-cm deviation on the recorder for a $\Delta\alpha$ of 0.001°.

Reagents and materials

Neomycin sulphate was obtained from Roussel-Uclaf (Romainville, France), SIFA (Paris, France) and Upjohn (Kalamazoo, MI, U.S.A.). The free bases of neomycin B and C were isolated as described previously[9]. The weight loss on drying these products was 10.0 and 5.5%, respectively.

The concentration of sample solutions was 100 mg/ml. Bio-Rad AG 1-X2 resin (Cl^-), 200–400 mesh and −400 mesh, was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Carbon dioxide-free water was obtained by boiling and cooling double glass-distilled water.

Procedure

The resin (Cl^-) was suspended in carbon dioxide-free water and poured into a glass column (3 cm I.D.), provided with a fritted glass disc (porosity No. 2) and a 500-ml solvent reservoir. The settled resin bed was washed with 1 *N* sodium hydroxide (*ca.* 500 ml per 15 g resin) until the eluate was free from Cl^-. In order to exclude CO_2 from the atmosphere, a soda-lime trap was placed on top of the column. The flow-rate was adjusted to 20–25 ml/min by moderate vacuum or nitrogen pressure. After removing the chloride ion, the resin was washed with carbon dioxide-free water until the eluate was neutral to universal indicator. A slurry of the resin in carbon dioxide-free water was used immediately for filling the analytical columns.

(a) The top of the glass analytical column was connected to a glass tube (70 × 0.6 cm), in which the slurry was poured. When the resin-bed had settled, the excess of resin above the inlet was discarded and replaced by acid-washed glass wool. The opening was closed with a tightly fitting serum cap. Sample injections (100 µl solution) were made through this septum with a 100-µl Hamilton syringe.

(b) A 50-cm metal tube was connected to the metal analytical column. Five

millilitres of carbon dioxide-free water, followed by the slurry of resin (OH^-), were poured into the column. Carbon dioxide-free water was pumped for about 20 min at a maximum flow-rate of 460 ml/h.

RESULTS

All chromatograms were run on anion-exchange resins (OH^-), Dowex 1-X2 or the analytical grade (AG) produced by Bio-Rad. These resins consist of a polystyrene lattice cross-linked with 2% divinylbenzene, carrying quaternary ammonium groups, $-CH_2-N^+(CH_3)_3$. The chromatogram obtained on a column (40 × 0.6 cm) of Bio-Rad AG 1-X2 (200–400 mesh) at 20°C with a flow-rate of 60 ml/h, requiring 1 h 45 min, is shown in Fig. 3a. Lowering the column temperature to 10°C increases the

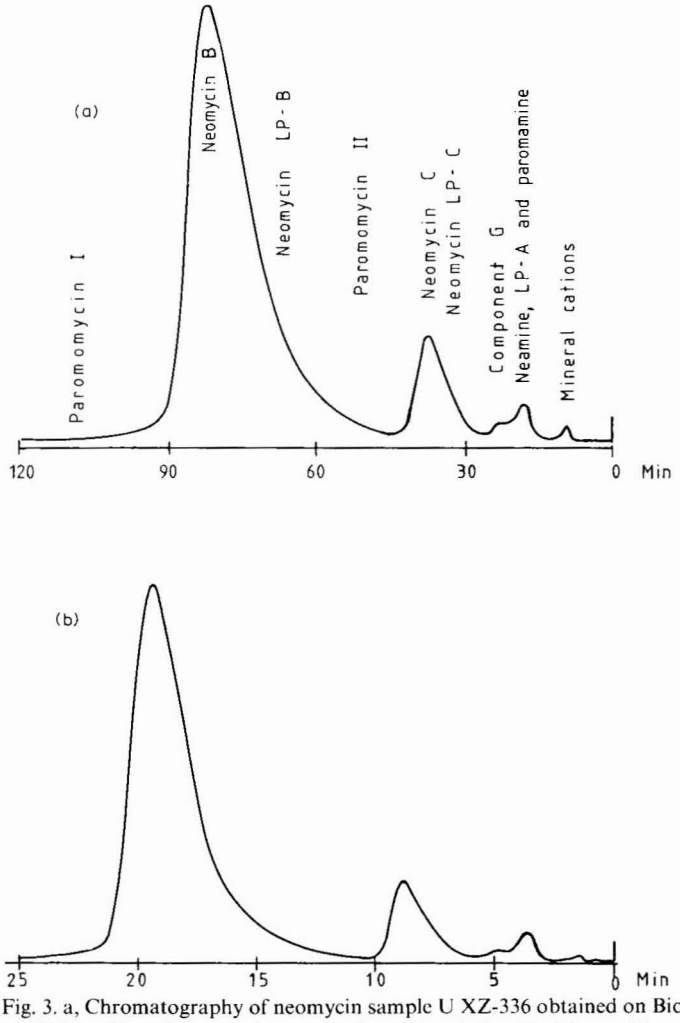


Fig. 3. a, Chromatography of neomycin sample U XZ-336 obtained on Bio-Rad AG 1-X2 (OH^-, 200–400 mesh) in a 40 × 0.6 cm column at a flow-rate of 60 ml/h. The position of the different neomycin components is indicated. b, Chromatography of neomycin obtained on Bio-Rad AG 1-X2 (OH^-, −400 mesh) in a 30 × 1 cm column at a flow-rate of 270 ml/h.

capacity factors, resolution between neomycin B and C, the number of theoretical plates, but also the analysis time. The use of a shorter column (20 cm) at 10°C reduces the elution time (Table I), but in our opinion the increased column efficiency at lower temperature does not justify a more complicated thermostating procedure. The analysis times in Table I include the elution of paromomycin I, which may be present in some samples. Since only the neomycin B and C peaks are of interest in our experiments, two columns can be used alternately, one being connected to the detector while the other is rinsed.

TABLE I

CHROMATOGRAPHIC PARAMETERS ON BIO-RAD AG 1-X2 (OH^-) UNDER DIFFERENT EXPERIMENTAL CONDITIONS

	40 × 0.6 cm column, 200–400 mesh resin, 60 ml/h, 10°C	*40 × 0.6 cm column, 200–400 mesh resin, 60 ml/h, 20°C*	*20 × 0.6 cm column, 200 400 mesh resin, 60 ml/h, 10°C*	*30 × 1 cm column, −400 mesh resin, 270 ml/h, 20°C*
Capacity ratio, neo B	13.6	8.0	13.1	7.9
Capacity ratio, neo C	4.9	3.0	5.0	3.2
Resolution, (neo B–neo C)	2.9	1.9	2.1	2.9
Peak symmetry factor (neo B)	0.74	0.80	1.0	0.77
Number of theoretical plates (neo B)	205 (513/m)	102 (255/m)	105 (525/m)	277 (923/m)
Analysis time	2 h 15 min	1 h 45 min	1 h 45 min	25 min

Separation of the main components can also be improved by using the Bio-Rad resin (−400 mesh). With this resin a metal column and a medium-pressure pump must be used. The improved separation of neomycin B and C allows higher flow-rates and a reduction of the analysis time to 25 min (Table I). A chromatogram obtained at a flow-rate of 270 ml/h on a column (30 × 1 cm) at 20°C is shown in Fig. 3b.

The mobile phase for all chromatographic systems is carbon dioxide-free water at a pH of *ca.* 6.5. We observed that adjustment to pH 7.0, 10.0 and 11.5 by adding sodium hydroxide has no influence on column parameters such as resolution, capacity factors and peak symmetry. It should be noted that capacity factors and resolution decrease with time. This is due partially to retention of sulphuric acid from the neomycin sulphate, but also to degradation of the functional groups of the resin in the OH^- form. A continuous flow of water, even when no samples are applied, has a favourable effect on column lifetime. The resin should be replaced when the calculated resolution is lower than 1.5, since in this case the skewed peaks of neomycin B and C are no longer baseline-separated. This replacement should take place after 1–2 weeks of use or application of 25–30 samples of 10 mg neomycin sulphate. Regeneration of the inexpensive resins is not recommended because of increased peak asymmetry.

Analysis of samples of known composition confirmed the identical response of neomycin B and C to the refractometric detection. These samples were prepared by dissolving known amounts of the pure neomycin B and C free bases in water, taking into account their weight loss on drying.

TABLE II

EXPERIMENTAL CONDITIONS FOR ION-EXCLUSION CHROMATOGRAPHIC ANALYSIS OF NEOMYCIN

Column dimensions and resin	*Flow-rate (ml/h)*	*Temperature (°C)*	*Sample load (mg)*	*Detection*	*Analysis time*	*Ref.*
A 27 × 2.5 cm Dowex 1-X2 (200–400 mesh)	300	Ambient	15	Conductimetric*	*ca.* 2 h	21
B 15 × 1 cm Bio-Rad AG1-X2 (200–400 mesh)	1.5–1.7	20	10	Ninhydrin (manual)	18–21 h	16
C 15 × 1 cm Bio-Rad AG1-X2 (200–400 mesh)	4.5	20	75	Polarimetric	7 h	20
D 40 × 0.6 cm Bio-Rad AG1-X2 (200–400 mesh)	60	20	2	Ninhydrin	1 h 45 min	23
E 20 × 0.6 cm Bio-Rad AG1-X2 (200–400 mesh)	60	10	10	Polarimetric	1 h 45 min	This paper
F 40 × 0.6 cm Bio-Rad AG1-X2 (200–400 mesh)	60	20	10	Refractive index	1 h 45 min	This paper
G 30 × 1 cm (metal) Bio-Rad AG1-X2 (−400 mesh)	270	20	10	Refractive index	25 min	This paper

* Determination of peak height instead of area.

Relative amounts of neomycin B and C in neomycin sulphate were also determined with polarimetric detection. The sample load (10 mg) is lower than in a previous study[20] where 75 mg were used. This is possible because of the use of a larger flow-cell (inner volume of 250 μl instead of 32 μl), with which a more stable baseline is obtained. The difference in $[\alpha]_D$ values of neomycin B and C requires a correction for the peak areas. Instead of the $[\alpha]_D$ values measured in 0.02 *N* H_2SO_4 ($+83^\circ$ for neomycin B and $+121^\circ$ for neomycin C) employed by de Rossi[19], we used the values determined in 0.02 *N* NaOH (neomycin B, $+71^\circ$; neomycin C, $+110^\circ$) as reported by Ford *et al.*[22]. We confirmed these values for neomycin B and C prepared in our laboratory.

A comparison of the methods described in this report with other detection methods is possible, since some samples have been examined in other laboratories also. The experimental conditions for ion-exclusion chromatographic determination of neomycin C are given in Table II. Good correlation was found between the results obtained with ninhydrin detection (Dr. A. Sezerat, Roussel-Uclaf), polarimetric detection (Dr. J. Lightbown, National Laboratory for Biological Standards and Control, London, Great Britain) and our method. This is illustrated in Table III which gives the relative amounts of neomycin C in eight commercial samples, determined with different detection methods. The confidence limits of the mean were calculated with a *t*-test. Conductometric detection[21] used previously in our laboratory gives a systematic overestimation of neomycin C, possibly due to the non-linear response of the detector. Four rather old samples were analysed by preparative chromatography on a carboxylic ion-exchange resin[9]. The percentages of neomycin C found in that

TABLE III

RELATIVE AMOUNTS OF NEOMYCIN C IN COMMERCIAL SAMPLES

Values expressed as:

$$\frac{\text{neo C}}{\text{neo C} + \text{neo B}} \times 100$$

Sample	*Method*						
	A	*C**	*D***	*E*	*F*	*G*	*Preparative chromatography on Amberlite CG-50*[9]
U. XZ-336	11.6	10.8	9.8	9.1–10.3***	9.5–10.6***	9.7–10.5***	9.5
S. 52001	39.0	32.2	37.8	34.5–37.0***	–	–	34.5
U. TRO-32	12.2	9.8	9.6	8.2–9.2***	–	–	8.4
R. 7S-1251	12.5	8.9	10.0	9.2–10.6***	11.0	9.7–10.4***	9.1
R. 9S0560	–	–	12.4	–	11.8	11.0–11.8***	–
R. 9S0572	–	–	–	–	11.8	–	–
R. 9S0581	–	–	15.3	–	15.1	14.0–14.7***	–
R. 9S0594	–	–	–	–	16.5	–	–

* Figures furnished by Dr. J. Lightbown.

** Figures furnished by Dr. A. Sezerat.

*** 95% confidence limits.

study are included in Table III. A comparison between GLC of trimethylsilylated neomycins, HPLC of N-dinitrophenylated neomycin and ion-exclusion chromatography with refractometric detection (Table IV) was made possible by analysis of samples kindly provided by Mr. Tsuji (Upjohn, Kalamazoo, MI, U.S.A.). Our results are intermediate between the values obtained by GLC and HPLC reported by Tsuji *et al.*[15].

TABLE IV

COMPARISON BETWEEN HPLC, GLC AND ION-EXCLUSION CHROMATOGRAPHY

The relative amount of neomycin C is expressed as in Table III.

Sample	*Method G*	*HPLC*[15]	*GLC*[15]
6	8.9–10.4*	11.6	9.7
9	12.4	13.1	8.8
13	11.6–12.4*	13.4	7.8
16	17.9–20.1*	19.9	17.2
17	11.9–12.9*	13.9	8.9

* 95% confidence limits.

It should be mentioned that, in all chromatograms using an anion-exchange resin, the acetyl derivatives of neomycin B and C (LP-B and LP-C) are located in the ascending parts of the neomycin B and C peaks (Fig. 3a). These components, which may be present in some samples at concentrations up to 5%, are determined together with the non-acetylated main products.

CONCLUSION

The results reported show that refractometric detection can be used as an alternative to ninhydrin colorimetry or polarimetric detection in ion-exclusion chromatography of neomycin. It is simple to perform and gives an identical response for the B and C components. Medium-pressure chromatography using a −400 mesh resin permits a reduction of the analysis time to *ca.* 25 min. This makes the ion-exclusion chromatographic determination of neomycin C competitive with the HPLC method described recently by Tsuji *et al.*[15].

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APPLICATION OF POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS TO THE STUDY OF MULTIPLE COMPONENTS OF CONCANAVALIN A AND RELATED LECTINS

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SUMMARY

Polyacrylamide gradient gel electrophoresis was used for analysing different Concanavalin (Con A) preparations. The results indicated one predominant band and various minor extra bands which were not revealed by polyacrylamide disc gel electrophoresis. None of the bands disappeared after repeated affinity chromatography on Sephadex, which suggests that the heterogeneity may depend on the complexity of Con A itself. Con A with different metal content also showed the same electrophoretic components.

The major faster migrating component, which is identical in position with the single band in disc gel electrophoresis, represents intact dimers. The minor components are suggested to be different molecular species, which are separated by means of polyacrylamide gradient gel electrophoresis, probably depending on a combined effect of electrophoretic mobility and molecular sieving.

INTRODUCTION

Electrophoresis has been continuously developed by the introduction of improved supporting media, *e.g.*, polyacrylamide. Polyacrylamide gel electrophoresis[1–3] was first applied to serum proteins using a weakly basic buffer and subsequently to the fractionation of basic proteins and peptides by performing the electrophoresis in acidic buffers[4]. For the characterization of jack bean phytoagglutinin Concanavalin A (Con A), the latter conditions were applied and one band was found at pH 4.5 whereas three bands were observed in the presence of 8 *M* urea at the same pH[5]. Further studies on Con A in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis gave evidence for several components at the subunit level corresponding to the subunit itself and some naturally occurring fragments[6,7]. The occurrence of different electrophoretic components possessing nearly equal hemagglutinating abilities has been demonstrated by isoelectric focusing[8,9].

In an earlier paper it was shown that different batches of highly purified Con A with different metal content were indistinguishable in polyacrylamide gel electrophoresis, all being represented by one band[10].

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In the present study polyacrylamide gradient gel electrophoresis was applied and revealed multiple components of Con A and related lectins. This is a simple, accurate and sensitive method for testing protein homogeneity[11] and the significance of the results is discussed.

EXPERIMENTAL

Materials

Jack bean meal (*Canavalia ensiformis*) was obtained from Sigma (St. Louis, MO, U.S.A.) and Worthington Biochemicals (Freehold, NJ, U.S.A.). Whole beans of *Canavalia rosea* were obtained from Paul Müggenburg (Hamburg, G.F.R.). Commercial Con A was obtained from Calbiochem (Los Angeles, CA, U.S.A.) (grade A, lyophilized in sodium chloride, batch 940022), Miles-Yeda (Rehovot, Israel) (recrystallized twice from saturated sodium chloride solution, batch 79-001), Sigma (grade III, lyophilized in sodium chloride solution, batch 910-5010 No. C-2631) and Pharmacia (Uppsala, Sweden) (lyophilized powder, batches 3059 and 4000). All salt-containing samples were dialysed against distilled water and subsequently against the appropriate electrophoretic buffer. Demetallized Con A was produced by extensive dialysis of the native protein against 0.05 *M* formate buffer (pH 3)[10]. Three different proteins with respect to their metal contents and carbohydrate-binding abilities (A, B and C) were isolated and lectin was isolated from *C. rosea* seeds by specific adsorption on Sephadex according to previous work[10]. Polyacrylamide gradient gel slabs (PAA 4/30) were obtained from Pharmacia.

Polyacrylamide gradient gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in a gradient system with a monomer gradient of 4–30%. A complete system for polyacrylamide gel electrophoresis developed by Pharmacia was used, including pre-formed gel slabs 2.7 mm thick in glass cassettes (82 × 82 × 4.9 mm). Gradients of 8.5–31% were also tried. All slabs were pre-equilibrated with the appropriate electrophoretic buffer. To protein solutions containing 10–30 mg/ml of lectin was added up to 10% of sucrose and 5–15 μl were applied to the sample well using a Hamilton microlitre syringe. Two buffer systems were compared by performing the electrophoresis either in 0.02 *M* acetate buffer (pH 4.0) or according to Reisfeld *et al.*[4]. In the latter instance the gel slabs were equilibrated with 0.06 *M* potassium hydroxide–0.37 *M* acetic acid buffer (pH 4.3) and the electrode compartments were filled with 0.35 *M* β-alanine–0.14 *M* acetic acid buffer (pH 4.5). The running time was 3–5 h at 40 mA.

At the end of the electrophoretic run the gel slabs were stained with 0.5% Amido Black in 7% acetic acid for 10–30 min. Some slabs were stained with Coomassie Brilliant Blue (0.16 mg/ml) in 7.5% acetic acid and heated for 1.5–2 h at 80–85°C.

Slabs stained with Amido Black were electrophoretically destained in 7% acetic acid at 2 A and 36 V for 30 min. Slabs stained with Coomassie Brilliant Blue were destained in water–methanol–acetic acid (6:3:1) for 24 h.

Protein analyses

All protein determinations on purified lectins were estimated from the absorbance at 280 nm using $A^{1\%}_{1\,cm} = 11.4$[12].

Metal analyses

Analyses for manganese and calcium were performed by atomic-absorption spectrometry on a Varian-Techtron Model AA-5 instrument using manganese chloride and calcium chloride as standards.

RESULTS

Homogeneity in polyacrylamide gel electrophoresis is usually taken to be a good indication of the purity of proteins. Using 7.5% polyacrylamide gel electrophoresis (pH 4), all preparations included in this study showed homogeneity and never revealed more than a single band. Samples containing various amounts of metal ions (Mn^{2+} plus Ca^{2+}) have previously been reported to be indistinguishable by polyacrylamide gel electrophoresis, although they could be separated into three subclasses by means of affinity chromatography on Sephadex[10].

In this study polyacrylamide gradient gel electrophoresis was applied, which is a more sophisticated separation method in which proteins are driven through pores of progressively decreasing size until they are brought nearly to a stop according to their size[11]. Fig. 1 shows such an electrophoretic pattern of six different samples of Con A, clearly demonstrating several bands for each species. Irrespective of the metal content of the proteins, all preparations gave one major band and several minor extra bands. The faster migrating predominant component, identical in position with the single

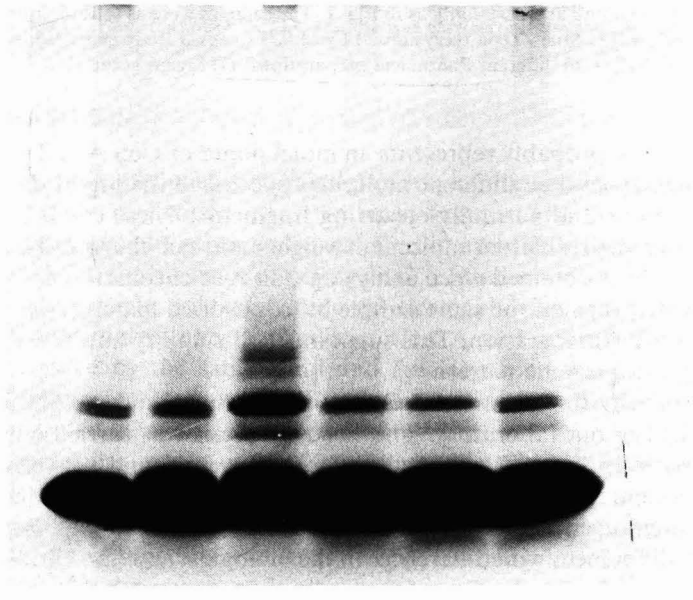

Fig. 1. Polyacrylamide gradient gel electrophoresis of six different samples of Con A containing different amounts of metal. The electrophoresis was performed in a monomer gradient of 4–30% gel in 0.02 *M* sodium acetate buffer (pH 4.0) at 40 mA for 4 h. The plate was stained with 0.5% Amido Black in 7% acetic acid and destained at 2 A and 36 V for 30 min in 7% acetic acid. The samples had the following metal contents in percent from left to right: (1) 0.25 Mn^{2+}, 0.23 Ca^{2+}; (2) 0.16 Mn^{2+}, 0.18 Ca^{2+}; (3) 0.0007 Mn^{2+}, 0.001 Ca^{2+}; (4) 0.003 Mn^{2+}, 0.009 Ca^{2+}; (5) 0.0007 Mn^{2+}, 0.003 Ca^{2+}; (6) 0.17 Mn^{2+}, 0.21 Ca^{2+}.

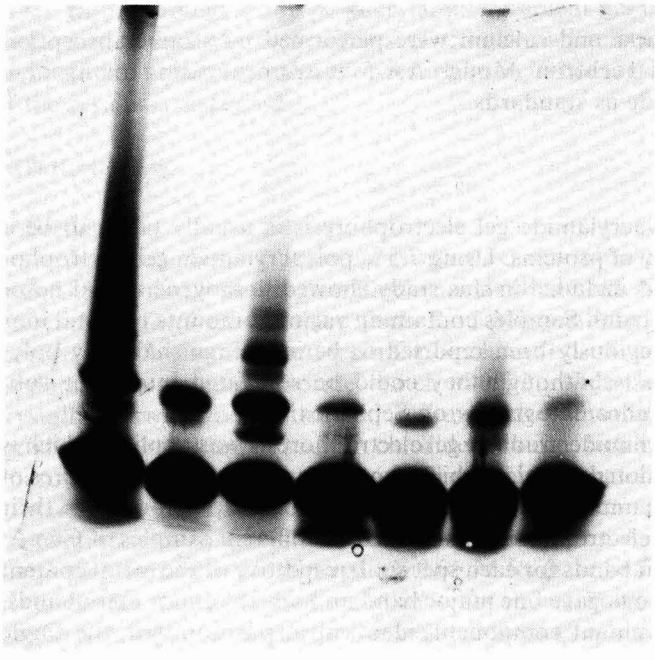

Fig. 2. Polyacrylamide gradient gel electrophoresis of commercial Con A preparations and *C. rosea* lectin. Conditions for electrophoretic run, staining and destaining as in Fig. 1. The samples were as follows from left to right: (1) Calbiochem grade A; (2) Miles-Yeda recrystalized twice; (3) *C. rosea* lectin prepared by affinity chromatography on Sepahdex; (4–6) different Pharmacia preparations; (7) Sigma grade III.

band in disc gel electrophoresis, probably represents an intact dimer of Con A[10]. The minor components are suggested to be different molecular species consisting of different combinations of subunits and naturally occurring fragments. These could be distinguished on the basis of their different molecular weights and net charges. The same electrophoretic pattern was obtained when analysing Con A re-chromatographed on Sephadex, and repeated runs on the same sample of freeze-dried material were reproducible several years after preparation. This suggests good stability and is evidence of a very distinct electrophoretic pattern.

Fig. 2 shows some results for commercial Con A preparations and a related lectin from *C. rosea* isolated in our laboratory. The electrophoresis was carried out under the same conditions as in Fig. 1. Very small differences were found between most samples, except for the batch of Con A from Calbiochem and the *C. rosea* lectin. These two samples contained even more bands than the other species. It was impossible to associate these differences with differences in the biological activity. Ultracentrifugal and gel filtration studies on the *C. rosea* lectin have demonstrated that this lectin has a slightly higher molecular weight than Con A (to be published).

Fig. 3 shows the electrophoretic pattern of the same samples as in Fig. 2. These samples were developed in a modified disc electrophoretic system according to Reisfeld *et al.*[4] using a gradient of 8.5–31 %. The results confirmed the previous observations, *i.e.*, the occurrence of one major and a varying number of minor components.

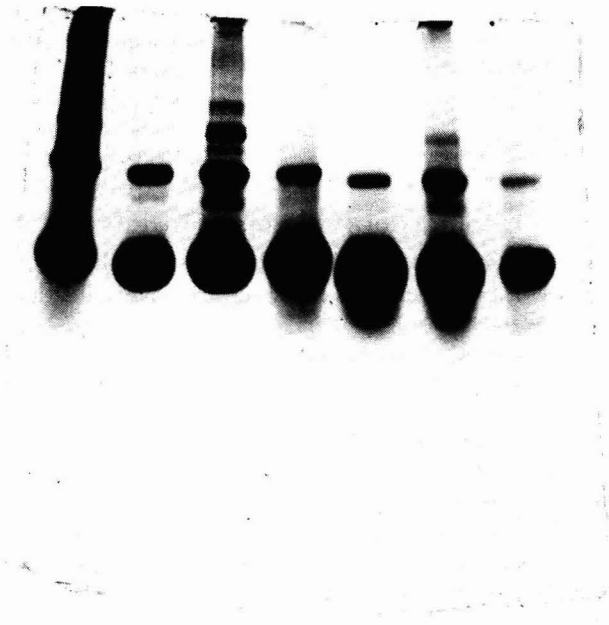

Fig. 3. Polyacrylamide gradient gel electrophoresis of the same samples as in Fig. 2 in a gradient of 8.5–31%. The electrophoresis was performed in 0.14 *M* acetic acid–0.35 *M* β-alanine buffer (pH 4.5) according to Reisfeld *et al.*[4]. The plate was stained with Coomassie Brilliant Blue (0.16 mg/ml) in 7.5% acetic acid and heated for 1.5–2 h at 80–85°C. Destaining was carried out in water–methanol–acetic acid (6:3:1) for 24 h.

DISCUSSION

All previously published polyacrylamide gel electrophoretic data on Con A can generally be divided into two main groups. The first includes runs performed in buffers without other additives[9,10,12,13] and the second includes runs performed in buffers in the presence of detergents[5–7]. Studies with highly purified Con A showed a single band in polyacrylamide gel electrophoresis when the pH was below 7[5,10,12]. Further, native and demetallized Con A and mixtures of these two forms are represented by the same band and are indistinguishable in polyacrylamide gel electrophoresis[10,14]. Using SDS polyacrylamide gel electrophoresis Wang *et al.*[6] demonstrated three electrophoretic components, one representing a uniform subunit with a molecular weight of 27,000 and the other two being naturally occurring fragments of this subunit. The occurrence of different electrophoretic components possessing nearly equal hemagglutinating abilities has been demonstrated by isoelectric focusing[8,9] and suggested the term "isophytohemagglutinins"[8].

In this work multiple components were revealed by using polyacrylamide gradient gel electrophoresis. This method has a very high resolution capacity owing to a combination of the electrophoretic mobility and the resistance of the supporting medium as a molecular sieve. The microheterogeneity was very apparent in all samples tested and no detergents were needed for the separation. To confirm that the extra minor bands were not impurities, several samples were re-chromatographed on

Sephadex and re-used in polyacrylamide gradient gel electrophoresis. None of the bands disappeared under this treatment, which suggests that the heterogeneity may depend on the complexity of lectin itself. It would be interesting to study this phenomenon in detail by preparing larger amounts of the extra bands and testing their chemical composition and biological activity.

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Note

Conversion of linear retention indices into logarithmic retention indices

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In contrast to logarithmic retention indices (I), there is no need of logarithmic calculation or of determination of the elution time of the non-sorbing gas for estimating the values of linear retention indices (I_A). The latter yield fine correlations between structure, temperature dependence of retention and differences in retention on different stationary phases[1,2]. Moreover, Saha and Mitra[1] and Soják and Vigdergauz[2] have demonstrated that, under identical experimental conditions, I_A values can be measured more accurately than I values; the standard deviations of I_A values on polar phases are significantly smaller than those of I values[2].

The major draw-back with the I_A system, is that, unlike the I system, it does not reflect the thermodynamics of the processes involved in elution during gas chromatography. However, this can be eliminated if accurate I values can be determined directly and easily through I_A values. The mathematical relationship between I_A and I given by Vigdergauz and Martynov[3] has the form:

$$J = \frac{\sigma^{\frac{\delta I}{100}} - 1}{\sigma - 1} \tag{1}$$

where

$$\sigma = \frac{t'_{Rn+1}}{t'_{Rn}} = \frac{t'_{Rn}}{t'_{Rn-1}} = \frac{t_{Rn+1} - t_{Rn}}{t_{Rn} - t_{Rn-1}}$$

$$J = \frac{t_{Rx} - t_{Rn}}{t_{Rn+1}\ t_{Rn}} + n = \frac{I_A}{100}$$

and

$$\delta I = I - 100n$$

The adjusted and non-adjusted retention times of normal paraffins with carbon number n and of sample component x are denoted by t'_{Rn}, t_{Rn} and t'_{Rx}, t_{Rx} respectively.

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Eqn. 1 has been found to be incorrect. In this paper a simple graphical method followed by a modified mathematical relation is proposed for the of I values directly from I_A values, predetermined from experimental unadjusted retention data.

EXPERIMENTAL

Retentions were determined at 50 ± 0.01 °C on a stainless-steel capillary column (100 m × 0.25 mm I.D.) coated dynamically with 10% w/v squalane in *n*-hexane. A device for high accuracy measurement of retention time was used[4]. The carrier gas was nitrogen at 2.5 atm. Sample components were injected as a 0.1-μl mixture with a splitting ratio of 1:400. The retention data used in the calculations of I_A values were averages from three injections. The same values of retention data, were used for the calculation of experimental I values.

RESULTS AND DISCUSSION

Mathematical relation between the two indices

The logarithm of σ is defined as the slope of a plot of the adjusted retention values of normal paraffins on the log scale against carbon number[1]. Since σ is related both to the adjusted and non-adjusted retentions of paraffins between which a component elutes, it may be used as a basis of correlation of I_A and I. The modified mathematical relation which results has the form

$$\frac{I_A - 100n}{100} = \frac{\sigma^{\frac{I-100n}{100}} - 1}{\sigma - 1} \qquad (2)$$

where I_A and I are defined as the arithmetic index or linear retention index[5] and as the Kováts' retention index or logarithmic retention index[6] respectively.

The validity of eqn. 2 can easily be tested by assuming the values of I_A and I to be 600 for *n*-hexane and the carbon number of the lower paraffin to be 5 ($n = 5$). Linear and logarithmic retention indices of various types of hydrocarbons have been determined and are shown in Table I. The values determined from non-adjusted retention did not match those determined from adjusted retention of normal paraffins. This deviation is due to the very small retention of methane, used as a non-retentive peak in the FID system. Thus the σ value of 2.66 determined from non-adjusted retention (see Table I) may be used for the interconversion of the two index systems. Furthermore, the actual instrumental dead time was calculated to be 481.5 sec using this σ value. When a dead time of 481.5 sec was used for adjusting the retention times of paraffins, the pre-calculated σ values of 2.73 and 2.70 became 2.66. Thus it was supposed that methane is retained for 9 sec (490.5–481.5) on the liquid phase of the capillary column. Therefore it is quite likely that the I values calculated through the I_A values may be more accurate (see Table I).

Graphical methods of conversion of I_A into I values

In order to determine I values from the predetermined I_A values, graphical methods were developed as follows.

TABLE I

COMPARISON OF THE TWO RETENTION INDICES OF HYDROCARBON COMPONENTS ON SQUALANE

Compound	*Retention time (sec)*	*Linear retention index, I_A*	*Logarithmic retention index, I*		
			*Experimental**	*Graphical***	*Analytical****
Methane	490.5	–	–	–	–
n-Pentane	645.5	500.00	500.00	500.00	500.00
2,3-Pentadiene	700.6	520.48	530.00	530.00	530.00
4-Methylpentene-2 (*cis*)	763.5	543.80	555.95	556.0	555.85
4 Methylpentene-2 (*trans*)	782.8	550.80	562.59	563.0	562.52
Hexene-1	845.5	574.21	581.92	582.0	582.07
n-Hexane	915.0	600.00	600.00	600.00	600.00
2,2-Dimethylpentane	1037.2	616.81	625.60	625.0	625.15
Benzene	1108.8	626.67	638.02	638.0	637.56
Cyclohexane	1279.0	650.10	662.61	662.0	661.80
2-Methylhexane	1310.0	654.45	666.60	666.0	665.83
3-Methylhexene-3 (*cis*)	1470.0	676.40	684.55	684.0	684.68
1,2-Dimethylcyclopentane (*trans*)	1514.4	682.50	689.06	688.0	688.26
Heptene-3 (*cis*)	1535.7	685.42	691.12	690.0	690.26
n-Heptane	1631.6	700.00	700.00	700.00	700.00

* Calculated from Kováts' equation using experimental data from methane injection.

** Calculated from linear retention index data using graphs in Fig. 1.

*** Calculated from linear retention index data using $t'_H/t'_P = 2.73$; $t'_{Hp}/t'_H = 2.70$; $(t_{Hp} - t_H)/(t_H - t_P) = 2.66$, where t'_P, t_P; t'_H, t_H and t'_{Hp}, t_{Hp} are the adjusted and non-adjusted retention times of *n*-pentane, *n*-hexane and *n*-heptane, respectively; and $\sigma = 2.66$.

The value of σ was determined from the non-adjusted retention data of three consecutive normal paraffins. By assigning any arbitrary adjusted retention value to the lower normal paraffin, *e.g.*, unity ($t'_{Rn-1} = 1.0$), the adjusted retention value of the next higher normal paraffin will be increased by a factor of σ ($t'_{Rn} = \sigma$).

The adjusted retention values of any two consecutive paraffins (in this case 1 and 2.66) were now plotted against ($I_A - 100n$) on linear graph paper (Fig. 1A) and against ($I - 100n$) on semi-log graph paper (Fig. 1B). From the ($I_A - 100n$) value of a component (*e.g.*, 50.1 for cyclohexane), the corresponding adjusted retention time (1.84) is determined (see Fig. 1A) and subsequently this value is used in Fig. 1B to determine the respective ($I - 100n$) or I values (*i.e.*, 62 or 662 for cyclohexane). The I values can also be calculated from Kováts' formula[6], once the adjusted values of the components have been determined.

It is worth noting that, from the same two graphs, I values of components eluting between the higher or lower paraffins can also be calculated because the value of σ, once determined, remains constant.

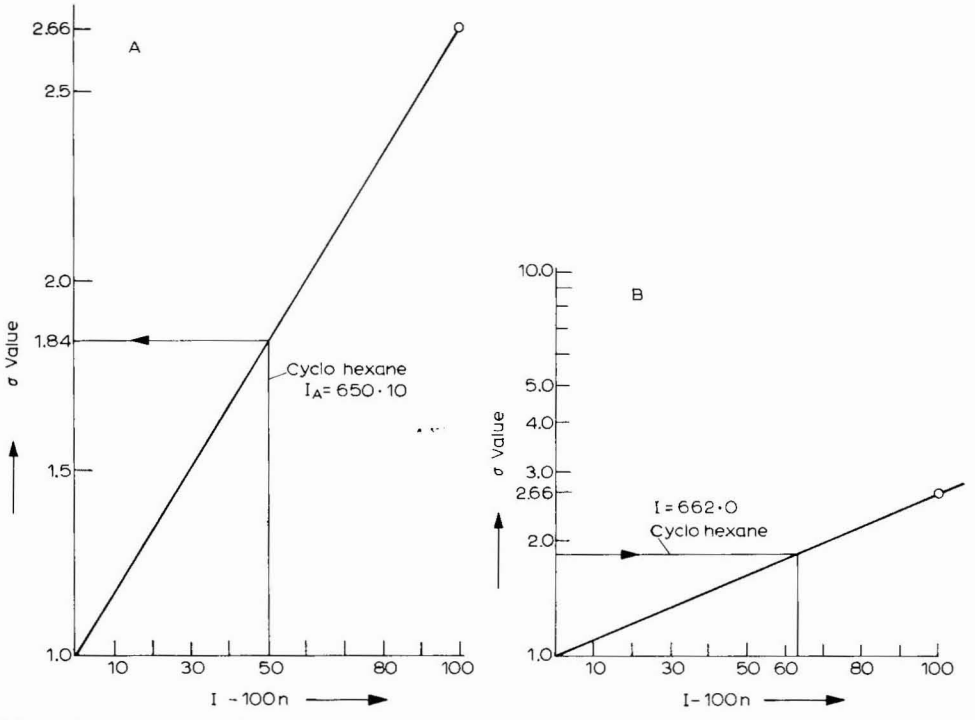

Fig. 1. Graphical conversion of linear retention indices into logarithmic retention indices: A, linear plot; B, logarithmic plot.

From a comparison of all three logarithmic retention index values (Table I), it is seen that the experimental value, using methane injection, deviates from the mathematical and graphical values which are similar. Therefore I values determined through I_A values seem to be more accurate than those determined directly from experiment, using methane injection.

ACKNOWLEDGEMENT

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Note

Capillary columns with immobilized stationary phases

I. A new simple preparation procedure

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Immobilized coatings in capillary columns offer two advantages. The first has to do with insufficient *wettability*. When a liquid does not permanently spread on a given solid support, bonding the molecules, or at least part of them, to the support surface may solve the problem. The same result may be achieved by cross-linking instead of surface bonding. In many cases of insufficient wettability an ideally uniform liquid film can be deposited for a short time or at low temperature. If cross-linking within the liquid is feasible before the film starts breaking up, the film may be perfectly stabilized. The second advantage is to have a *non-extractable* coating which cannot undergo phase stripping due to large splitless or on-column injection and which permits solvent rinsing to free the column from non-volatile sample by-products or from active breakdown products of the liquid phase.

As far as we know, K. Grob[1], in 1966–1967, was the first to attempt experimentally both bonding to the support surface and producing a cross-linked coating. He achieved bonding of terminal hydroxyl groups and of lithium–organic groups on Si–Cl groups obtained by treating the glass surface with thionyl chloride. Non-extractable coating was produced by *in situ* polymerization of polyolefins such as butadiene with boron trifluoride as a catalyst. Although the basic aims could be attained, the work was discontinued in 1968 because the coating was insufficiently thermostable. The main reason for the failure, as we know today, was the untreated glass surface, the leaching of which we introduced only in 1977[2].

In 1976 Madani and co-workers[3] were the first to re-attack the problem by an entirely new approach. They studied *in situ* synthesis of cross-linked methyl- and phenyl methyl polysiloxanes and developed their work to an impressive level[4]. In addition to realising the two above-mentioned advantages, they were able to tailor a given polarity by preselecting the phenyl:methyl ratio. In 1978 Blomberg and co-workers[5] entered the field on a similar line and developed their own synthetic and cross-linking techniques[6].

The column preparation procedures of both groups are relatively sophisticated and laborious. We tried to achieve equivalent results by applying the far simpler methods which have been used industrially for more than twenty years for the pro-

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duction of silicone rubber from silicone oils. These methods are comprehensively described in textbooks, for instance in the one by Noll[7]. Our trials based on this literature were almost immediately successful.

SURFACE BONDING *VERSUS* CROSS-LINKING

We have not been able to find a clear-cut definition for the widely used term "bonded phase"[4,8]. As deduced from its common use, we feel that it emphasizes a covalent bonding between the support surface and the stationary phase, while cross-linking is treated as a secondary aspect or is even suppressed.

We think that instead of theoretically selecting an exact meaning of the term "bonded phase", a proper term should be selected which exactly describes the *experimentally proven* facts involved. From this viewpoint we have first to state that no direct proof has been forwarded so far for the existence of covalent bonds between phase and support, whereas cross-linking within the liquid is an amply proven phenomenon[7]. This statement does not at all lower the merits of the underlying technical progress, since both advantages, film stabilization on an insufficiently wettable support, and non-extractability, are achieved by cross-linking alone.

The results of our simplified procedure are identical to those of the procedures of Madani and co-workers[3,4] and Blomberg and co-workers[5,6], namely cross-linking, and very probably also bonding to the support. We feel that the practical effects combined with their structural interpretation may properly be described by the term "immobilized phase".

SIMPLIFIED PRODUCTION OF IMMOBILIZED PHASES

Silicone rubber articles are manufactured from liquid silicones by cross-linking induced by organic *peroxides*[7]. It is hard to see why this established and simple technique should not be used to immobilize stationary phases, since, if properly applied, it does not influence the organic coating in ways other than cross-linking and probable bonding to the support surface. According to Noll[7], the peroxide transforms a methyl group into a radical which then reacts with a methyl group of a neighbouring molecule to form a covalent bond. Corresponding reactions between other groups occur less frequently. The reaction products of most peroxides are volatile or, in case of a side reaction producing a heavier or more polar reaction product, can be extracted from the immobilized phase. The latter case may explain the frequent observation that solvent-extracting a freshly immobilized coating slightly lowers the adsorption activity and the polarity of the column.

Preparation of an immobilized coating simply involves adding a peroxide to the liquid phase before carrying out the regular static or dynamic coating and changing slightly the regular procedure for conditioning the freshly coated column. Instead of programming the temperature to the upper temperature limit of the column, the temperature has to remain for one or a few hours at a level (100–140°C) favouring the cross-linking reaction. Conditioning should then be continued to evaporate the peroxide breakdown products.

The standard peroxide is dibenzoyl peroxide (DBP). Further study may reveal a different peroxide that is even more suitable. A somewhat disadvantageous feature

of DBP is its volatility; possibly it causes some overlapping of the cross-linking reaction with evaporation of the peroxide. Bis(dichlorobenzoyl)peroxide may be advantageous in this respect.

Reasonable immobilization may be obtained by a fairly broad range of peroxide concentrations. Excessive amounts of peroxide produce increased column activity; amounts which are too low result in increased losses of stationary phase upon rinsing. It may be surprising, however, that strongly varied amounts of peroxide hardly influence column characteristics such as polarity, separation efficiency and loading capacity (apparent film thickness). Thus, the density of cross-links does not seem to be a critical factor provided it exceeds a certain minimum.

Immobilization by reaction with a peroxide is, at the present time, limited to silicones of low or very moderate polarity. We have not yet successfully immobilized phases such as OV-17 and OV-225. However, this is not surprising since, for the same reason, these phases are not available as gums.

PRACTICAL PROCEDURE

Column

Immobilization works, as far as cross-linking is concerned, on any kind of support surface. However, maximum advantage in terms of column quality and thermostability is obtained with persilanized glass or fused silica. Persilanization[9] is carried out the same way as for conventional inert columns.

Phases

All silicones of low polarity (the polarity limit may be represented by the phase OV-61) can be used with the same solvents (*e.g.*, pentane, methylene chloride) and concentration as used without cross-linking. As soon as the peroxide has been added, the solution should be kept dark and cool for a minimum time period.

Peroxide

DBP was used as purchased (*e.g.*, No. 33581 from Fluka, Buchs, Switzerland). We prepared a 2% (w/v) solution in benzene which was kept dark and cool. The concentration of the peroxide in the pure phase was 0.1–1.0% (w/w). An example may eliminate possible misinterpretations. To 10 ml of a 0.2% solution of OV-1 in pentane, 4 μl of the 2% DBP solution are added with a regular syringe to yield a concentration of 0.4% DBP in pure OV-1.

Conditioning

After regular static or dynamic coating the column is mounted into a gas chromatograph without the outlet to the detector. For 15–20 min a high carrier flow-rate of 2–4 ml/min for a 0.3 mm I.D. capillary is applied. The flow-rate is then reduced to 0.1–0.2 ml/min to prevent premature evaporation of DBP. The temperature is quickly raised to 100°C and is programmed at a rate of 0.2°/min to 130°C. In case the programmer does not offer this low program rate, the temperature is raised manually by keeping it for 1 h each at 100, 110 and 120°C. After maintaining 130°C for at least 1 h, the carrier flow-rate is increased to 2 ml/min, and the temperature is simultaneously set at 200°C for 1 h. The column is cooled, the exit is connected to the

detector, and the first quality test is run, which has to include a capacity ratio or an exact elution temperature in the case of our comprehensive test[10]. To check the degree of immobilization, as well as to eliminate possible impurities, the column may be rinsed with two column volumes of methylene chloride and one volume of pentane. The test is repeated. The column may show a slightly reduced adsorption activity. Proper immobilization is demonstrated by a loss of stationary phase of 5% or less, as calculated from the capacity ratio, or by an elution temperature lowered by 1°C at most. Further treatment is as for conventional inert columns.

In case of thickfilm (0.8 μm) columns we have repeatedly observed that prolonged rinsing (twenty column volumes of methylene chloride, overnight) produced a slight apparent increase of film thickness.

To prevent any premature evaporation of DBP we have sealed some columns after short conditioning at room temperature. The reaction at 100–130°C produced surprisingly active columns, however. We suspect that this is due to the water content of the commercially available DBP.

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Note

Couplage d'une technique d'échantillonnage sous basse pression à un chromatographe en phase gazeuse

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(Reçu le 6 février 1981)

Un certain nombre de dispositifs expérimentaux pour l'échantillonnage de gaz sous basse pression en vue d'une analyse chromatographique ont été décrits dans la littérature (par exemple, Bibl. 1–4). En effet l'utilisation des vannes à gaz proposées par les différents constructeurs ne permet pas en général d'effectuer des prélèvements à faible pression à cause des taux de fuites résiduelles qui ne sont absolument pas négligeables et du manque de sensibilité par suite de la faible quantité de matière qui est injectée.

Pourtant l'analyse des systèmes en cours de réaction, dans des conditions de pression et de température souvent critiques (réactions à températures élevées, flammes) nécessite que l'échantillonnage se fasse à plus basse température et sous basse pression afin de figer les réactions parasites et d'obtenir un échantillon représentatif du milieu à étudier. Il est alors indispensable de recueillir un volume important de gaz sous faible pression et de le comprimer avant de l'injecter dans le chromatographe.

Le dispositif que nous décrivons ici a été conçu et utilisé pour étudier la structure de flammes stabilisées sur brûleur en combinaison avec une analyse simultanée des atomes ou radicaux par résonance paramagnétique électronique[5]. Il peut être utilisé pour l'analyse de tout autre système réactionnel stabilisé en phase gazeuse.

DISPOSITIF EXPERIMENTAL

Le schéma de principe du dispositif utilisé est représenté sur la Fig. 1. Il permet d'extraire sous faible pression dans un cylindre muni d'un piston une partie des gaz du milieu à analyser par l'intermédiaire d'une sonde, de les comprimer dans une boucle d'échantillonnage puis de les injecter dans le chromatographe.

La sonde (S) est en quartz, son extrémité en forme de cône de 40° présente un trou dont le diamètre est adapté à l'étude à réaliser. Elle permet un prélèvement ponctuel des échantillons à analyser. Dans le cas de l'analyse de la structure des flammes méthanol–air, stabilisées à 80 Torr sur un brûleur à flamme plate, le diamètre du trou était voisin de 100 μm, conduisant à un débit molaire d'extraction de l'ordre de $1.7 \cdot 10^{-5}$ mole sec^{-1}. Par suite de la différence de pression régnant de part et d'autre de la microfuite, l'écoulement des gaz à travers celle-ci se fait à très grande vitesse et

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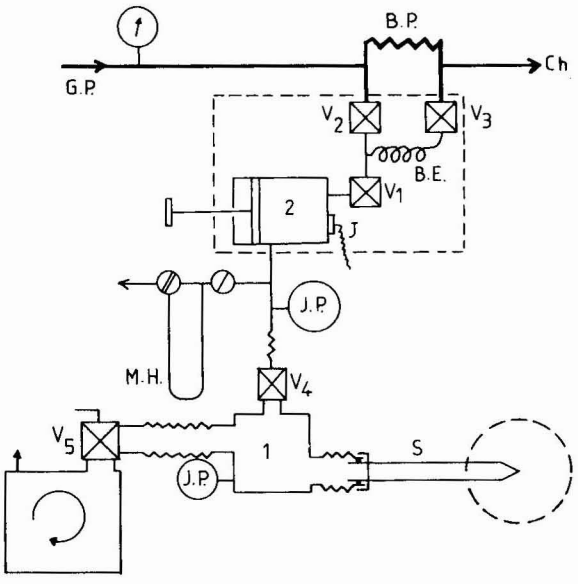

Fig. 1. Schéma de principe pour l'échantillonnage sous basse pression et le couplage à un chromatographe. S = Sonde d'extraction; V_1, V_2, V_3, V_4, V_5 = vannes à soufflets; G.P. = gaz porteur; B.P. = by-pass; Ch = chromatographe; B.E. = boucle d'échantillonnage; J = jauge à semi-conducteur; J.P. = jauge de Pirani; M.H. = manomètre à huile; 1 = chambre d'extraction; 2 = cylindre de compression.

le mélange gazeux atteint très rapidement les parois froides du dispositif de prélèvement ce qui entraîne un blocage efficace des réactions.

La sonde est reliée à la chambre d'extraction (1) par l'intermédiaire d'un soufflet métallique. L'étanchéité est assurée par un système de bagues et de joints toriques permettant le démontage rapide des sondes d'extraction. La chambre d'extraction est connectée à une pompe à palette de 30 m^3/h au moyen d'un soufflet métallique de diamètre suffisant pour réduire au maximum les pertes de charge et qui permet "d'absorber" les vibrations créées par la pompe rotative. La pompe peut être isolée du dispositif par une vanne à soufflet V_5. La pression dans la chambre d'extraction est mesurée par une jauge de Pirani (J.P.).

Le couplage du système de prélèvement au dispositif d'analyse par chromatographie est assuré par la vanne V_4. Le dispositif comprend l'ensemble cylindre-piston, la boucle d'échantillonnage (B.E.), les vannes V_1, V_2 et V_3 et les dispositifs de mesure des pressions (J.P., M.H., J).

Le cylindre en acier inoxydable de 100 mm de diamètre intérieur, de 700 cm^3 environ de volume utile est muni à chaque extrémité de deux flasques amovibles entre lesquelles se déplace le piston. Le guidage et le contrôle du mouvement de translation du piston est réalisé par une vis, solidaire d'un roulement à billes logé dans le corps du piston et tournant dans un filetage situé sur l'une des deux flasques. L'étanchéité lors du déplacement du piston dans le cylindre est assurée par deux joints toriques. En choisissant judicieusement la distance entre ces deux joints en relation avec le diamètre de l'orifice de remplissage latéral, on utilise le piston pour isoler le volume échantillonné avant compression. On évite ainsi de remplir le cylindre par l'autre flasque et d'utiliser une vanne d'arrêt qui augmente notablement le volume mort à la

compression. Cette flasque comporte déjà la vanne d'isolement V_1 et la jauge à membrane affleurante J. Il s'agit d'un capteur de pression absolu à jauges à semi-conducteur 0–15 p.s.i. (Precise Sensors Inc., No. 70143).

Les vannes V_1, V_2, V_3 permettent d'isoler la boucle d'échantillonnage (B.E) et d'injecter son contenu (5 cm^3) dans le chromatographe. Par suite des différences de pression importantes (3 à 7 kg/cm^2) pouvant régner de part et d'autre de ces vannes et afin de diminuer au maximum les volumes morts, nous avons utilisé des vannes à soufflet, testées à l'helium, à taux de fuite négligeable et de petites dimensions (Hocke 4151 G4B). L'ensemble de ces pièces sont raccordées l'une à l'autre par des raccords Swagelok.

Le cylindre, les vannes (V_1, V_2, V_3), la boucle d'échantillonnage et la jauge à membrane affleurante sont placés dans un compartiment isolant, bourré de laine de verre et chauffé à 120°C, ce qui supprime les risques de condensation des composés peu volatils lors de la compression.

MODE OPERATOIRE

Lorsque le vide est établi dans l'ensemble de l'installation et que le débit d'extraction est nul, la pression est de l'ordre de 3 à $4 \cdot 10^{-3}$ Torr avec un taux de fuite résiduel tout à fait négligeable. Lors de l'extraction en continu des gaz du milieu à analyser, la pression limite est fonction du débit molaire de fuite. Dans le cas précédemment cité cette pression est voisine de $4 \cdot 10^{-2}$ Torr.

Afin d'augmenter la sensibilité de la détection, la prise d'échantillon se fait à pression plus élevée par fermeture de V_5. Les vannes V_4 et V_1 étant ouvertes et le piston en butée vers la gauche, la pression dans l'enceinte croît. Quand la pression de 10 Torr est atteinte, le système est isolé par fermeture de V_4 et les gaz sont comprimés dans la boucle d'échantillonnage. Après fermeture de V_1 puis ouverture successive de V_2 puis de V_3, l'échantillon à analyser qui est d'abord comprimé par le gaz porteur est entraîné par celui-ci sous pression élevée dans le chromatographe directement en tête de colonne.

Pour éviter des discontinuités et des interruptions dans l'écoulement du gaz porteur ainsi que d'importantes variations de pression dans les colonnes chromatographiques lors des opérations d'injection, qui se traduisent par des instabilités notables au niveau de la détection, nous utilisons un by-pass (B.P) dont la perte de charge est environ 100 fois plus grande que celle de la boucle d'échantillonnage et qui assure un passage permanent du gaz porteur dans le chromatographe.

Le contrôle de la quantité de matière injectée est réalisée de deux façons: (1) soit en opérant à volume de compression constant et en mesurant la pression avant compression avec précision avec un manomètre à huile (M.H); (2) soit en mesurant directement la pression par la jauge absolue à membrane affleurante. La combinaison des deux méthodes permet de vérifier l'absence de condensation des produits lors de la compression.

APPLICATION

A titre d'exemple nous présentons quelques résultats obtenus dans le cas de l'analyse de la structure d'une flamme stoechiométrique méthanol–air stabilisée sur brûleur à 80 Torr.

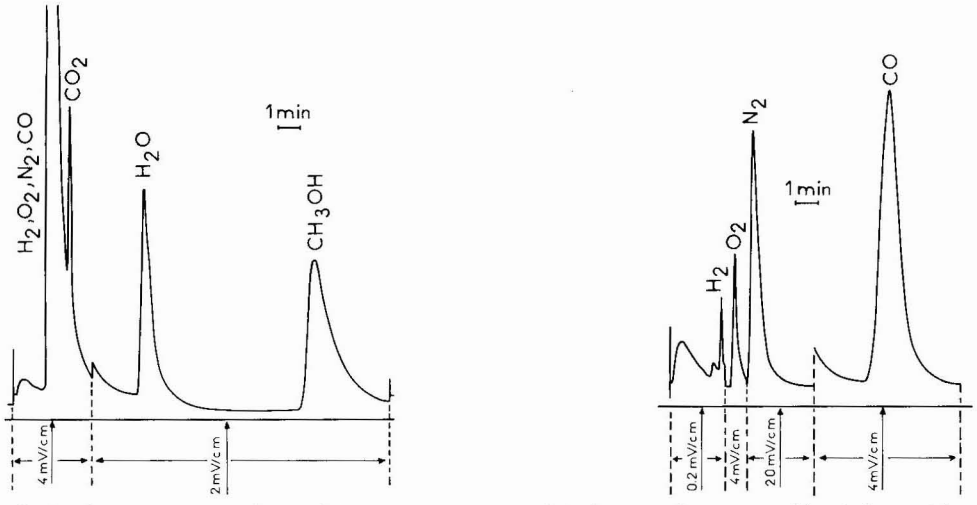

Fig. 2. Chromatogramme des produits sur colonne Porapak Q dans une flamme stoechiométrique méthanol–air stabilisée à 80 Torr, distance entre le front de flamme et la sonde d'extraction d = 1.95 mm.

Fig. 3. Chromatogramme des produits sur tamis moléculaire 5A dans une flamme stoechiométrique méthanol–air stabilisée à 80 Torr, distance entre le front de flamme et la sonde d'extraction d = 1.95 mm.

Les échantillons sont extraits en continu par la sonde jusqu'à une pression de 10 Torr et injectés selon la méthode décrite précédemment dans deux chromatographes à catharomètre placés en série et séparés par un piège refroidi permettant de condenser la vapeur d'eau. Les colonnes utilisées sont une colonne de Porapak Q, 3.6 m × 1/4 in. maintenue à 105°C et une colonne de tamis moléculaire 5A, 4 m × 1/4 in. maintenue à 35°C. Le premier chromatographe permet de séparer CO_2, H_2O et CH_3OH; le second H_2, O_2, N_2 et CO.

Les chromatogrammes correspondants à l'analyse des produits à 1.95 mm au-dessus du front de flamme, dans la zone des gaz brûlés, sont représentés sur les Figs.

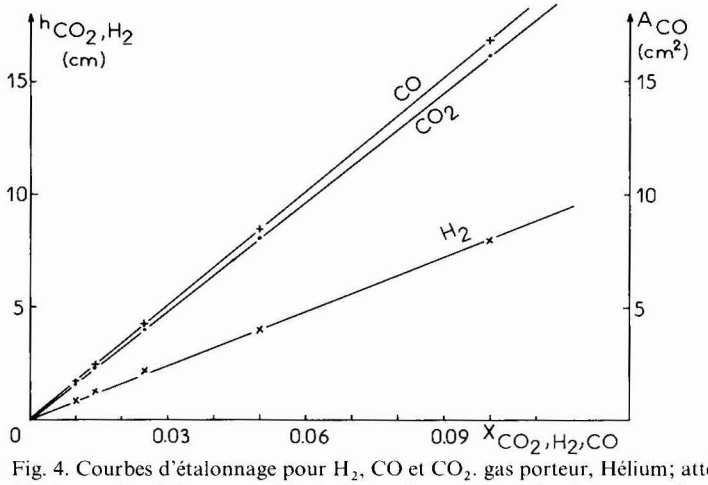

Fig. 4. Courbes d'étalonnage pour H_2, CO et CO_2. gas porteur, Hélium; atténuation, 1; sensibilités: CO_2, 10 mV/cm; H_2, 0.4 mV/cm; CO, 1 mV/cm. h = Hauteur du pic; A = surface du pic; X = fraction molaire dans le piston (10 Torr).

2 et 3. A titre d'exemple nous présentons également (Fig. 4) la courbe d'étalonnage pour CO, CO_2 et H_2. La méthode permet de déterminer sans difficulté des fractions molaires de 10^{-2} dans le mélange de 10 Torr et par conséquent dans la flamme.

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Note

Photometric monitoring of thiols by means of a thiomercurimetric detector

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(Received March 3rd, 1981)

The selective monitoring of thiols after their chromatographic separation is of importance in the analysis of complex mixtures, and procedures based on the change in potential of a platinum electrode in the presence of the iodide–iodine system[1] or the change in potential of a mercury electrode[2] have been suggested. The relationship between change in potential and thiol concentration is not linear and depends on pH. Thiols in natural gas have also been monitored by automatic titration with coulometrically generated silver ion[3].

The method proposed in this paper is based on the displacement of dithiofluorescein from its complex with *o*-hydroxymercuribenzoic acid (HMB)[4]. In alkaline solution dithiofluorescein has a blue colour, which disappears on adding HMB and reappears on adding thiols or hydrogen sulphide.

EXPERIMENTAL AND RESULTS

Materials

Solution of the HMB–dithiofluorescein complex. Dissolve 0.16 g of *o*-hydroxymercuribenzoic acid anhydride (POCh, Poland) in 100 ml of 5% (w/v) diethanolamine solution. Dissolve separately 0.1 g of dithiofluorescein (POCh) in 100 ml of 5% (w/v) diethanolamine solution. Take 20 ml of the first solution and add slowly with mixing the second solution until a distinct blue colour persists, filter rapidly, add 500 ml of 96% ethanol and dilute to 1 l with 5% (w/v) diethanolamine solution. The solution should have a slight blue colour, otherwise add a portion of filtered dithiofluorescein solution. The complex solution is $2 \cdot 10^{-4}$ *M* with respect to HMB and gives a linear absorbance response on adding thiols or hydrogen sulphide until *ca.* 90% of the dithiofluorescein is displaced. The solution should be protected from light.

Thiol solutions. Solutions of thiols were prepared in *n*-hexane and standarized by titration with HMB in the presence of dithiofluorescein as indicator[4].

Thiomercurimetric detector in gas chromatography

Details of the combination are illustrated in Fig. 1. The thiols emerging from the column in a stream of carrier gas are absorbed in vessel 3 in a solution of the complex which is forced from vessel 1. The solution containing absorbed thiol passes

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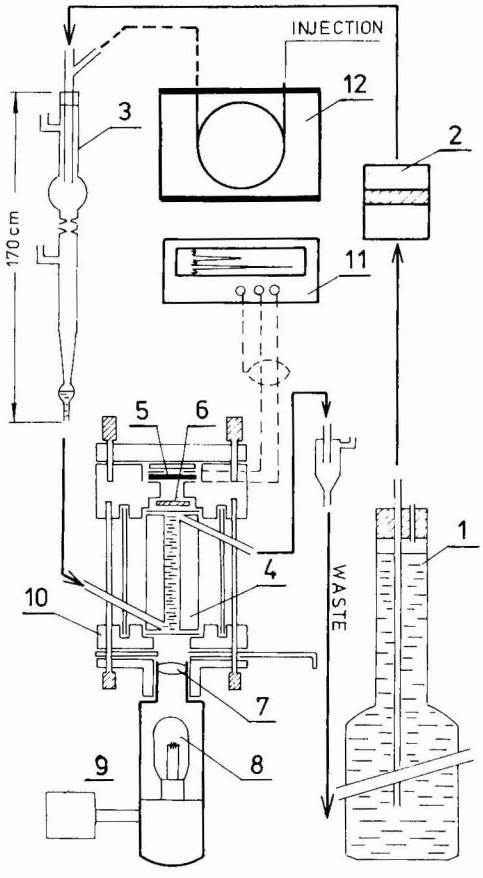

Fig. 1. Thiomercurimetric detector combined with a gas chromatograph. 1, Container for complex solution; 2, pump; 3, absorption column; 4, flow-through cell, 50-mm light path, 3 mm I.D.; 5, photocell; 6, optical filter (580 nm); 7, collimator; 8, light source, 6 V/15 W bulb; 9, transformer; 10, cell holder made of hard resin; 11, TZ-216 recorder (Czechoslovakia); 12, Chrom 41 chromatograph (Czechoslovakia).

to the lower part of vessel 3 and then to the flow cell, while the gas is removed by the two side-tubes. The current of the photocell is compensated by an anti-current and the recorder is then at zero. When the absorption of light in the flowing solution increases the current of the photocell will be decreased and the deflection of the recorder corresponds to the difference between the photocell current and anti-current. Let H be the full deflection of the recorder from the zero line and h the actual deflection. Then the absorbance will be expressed by $\log [H/(H - h)]$, and the concentration of thiol by $\log [H/(H - h)] \cdot$ constant. The total amount of the thiol can be calculated from the equation

$$m = K\Delta V \cdot \log[H/(H - h_m)]$$

where m = total amount of thiol (moles), K = a constant for a given detector valid for all thiols, h_m = peak height and ΔV = basis of the peak (ml).

The value of the K has to be found by standardization which is demonstrated in Table I and Fig. 2. Known amounts of *n*-propanethiol were injected on to the column of the gas chromatograph and the resulting peaks were recorded by the thiomercurimetric detector. The mean value of K thus found was 14 nmole/ml.

TABLE I

STANDARDIZATION OF THE THIOMERCURIMETRIC DETECTOR WITH *n*-PROPANETHIOL ACCORDING TO RESULTS IN FIG. 2

Full-scale deflection of recorder = 200 mm.

Injected (nmole)	ΔV *(ml)*	h_m *(mm)*	K *(nmole/ml)*
5.2	15.7	11	13.7
10.4	12.6	25	14.2
20.8	10.5	55	14.2
31.2	10.5	78	13.9
41.6	10.4	97	14.1
52.0	10.5	112	13.8

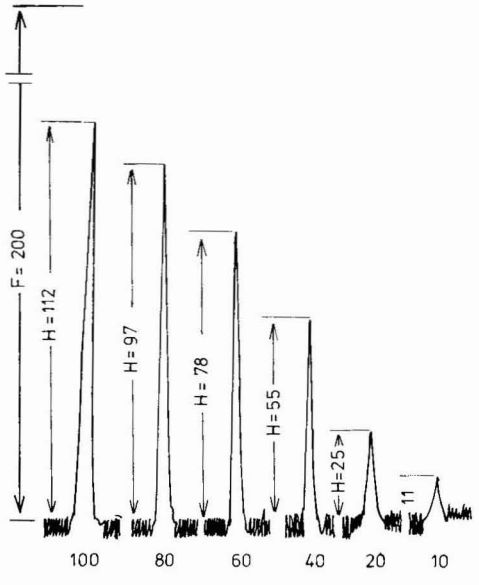

Fig. 2. Standardization of the detector by injection of 10, 20, 40, 60, 80 and 100 μl of $5.2 \cdot 10^{-4}$ M *n*-propanethiol in *n*-hexane. Details as in Fig. 3.

Fig. 3 shows a typical chromatogram for a thiol mixture obtained with the thiomercurimetric detector, and Table II gives the corresponding amounts of thiols calculated for K = 14 nmole/ml. The deviations ranged from -2 to -11%. For more accurate analysis internal standardization is necessary.

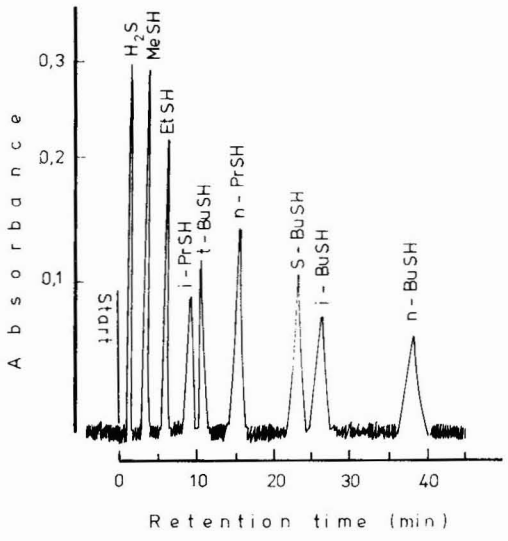

Fig. 3. Gas–liquid chromatogram of standard mixture of thiols in *n*-hexane. Column, 2.5 m × 3 mm I.D., glass, packed with 25% tricresyl phosphate on 80–120-mesh Celite. Temperature, 327°K; injector temperature, 353°K; detector, thiomercurimetric; complex solution flow-rate, 4.2 ml/min; carrier gas, nitrogen at 17 ml/min.

TABLE II

CALCULATION OF THIOL CONTENTS FROM FIG. 3

Thiol	*AV (ml)*	h_m *(mm)*	*Injected (nmole)*	*Found (nmole)*
Methanethiol	4.2	96	16.8	16.5
Ethanethiol	4.6	77	14.7	13.5
Isopropanethiol	8.6	35	10.2	9.8
tert.-Butanethiol	9.2	45	14.7	14.1
n-Propanethiol	9.5	53	18.2	17.6
sec.-Butanethiol	11.5	42	18.2	16.3
Isobutanethiol	12.6	30	13.7	12.2
n-Butanethiol	16.5	26	15.1	13.8

Monitoring of thiols in high-performance liquid chromatography

The liquid eluate leaving the chromatograph is mixed with the complex solution and the mixture is passed to the detector. The chromatograms obtained are shown in Figs. 4 and 5. It can be concluded that the detector is suitable for recording thiols separated by liquid chromatography.

DISCUSSION

The detector can also be used for silylated thiols because in the complex solution the sulphydryl group is quickly regenerated. In the presence of dimethylamine thiol esters are also recorded. Other compounds to which the detector can be applied

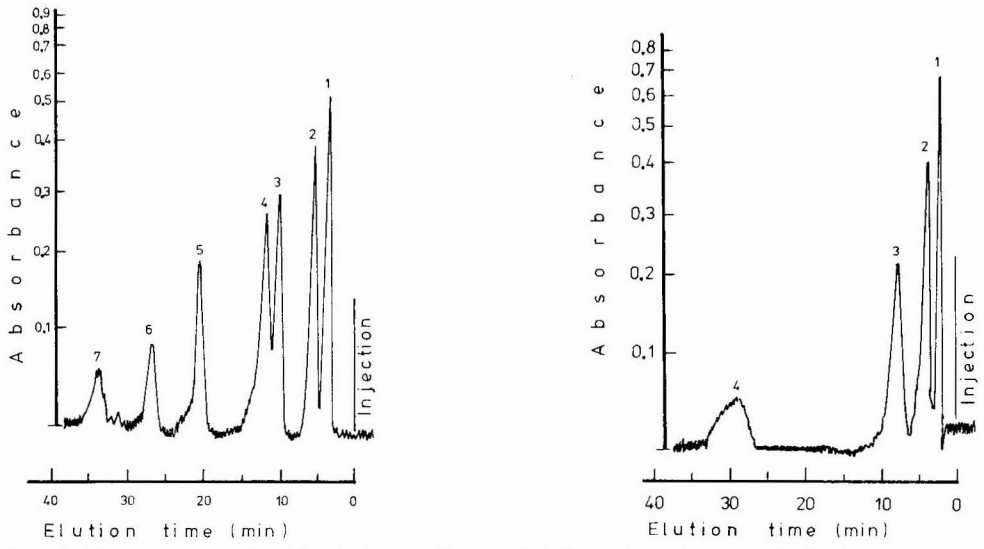

Fig. 4. Chromatogram of thiols obtained with an ALC-204 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.). Column, 30 cm × 3.9 mm I.D., packed with μBondapak C_{18}. Temperature, 295°K; detector, thiomercurimetric; complex solution flow-rate, 1.4 ml/min; eluent, 75% methanol, flow-rate 4 ml/min. Peaks: 1 = CH_3SH; 2 = C_2H_5SH; 3 = *iso*-C_3H_7SH; 4 = *tert.*-C_4H_9SH; 5 = *n*-C_3H_7SH; 6 = *sec.*-C_4H_9SH; 7 = *n*-C_4H_9SH.

Fig. 5. As Fig. 4. Peaks: 1 = *n*-$C_5H_{11}SH$; 2 = *n*-$C_6H_{13}SH$; 3 = *n*-$C_9H_{19}SH$; 4 = *n*-$C_{12}H_{25}SH$.

are dithiocarbamates, carbon disulphide, carbonyl sulphide and compounds that can be readily converted to thiols or hydrogen sulphide.

The sensitivity of the detector is about 2 nanomole of thiol.

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Note

Simple gel apparatus for horizontal polyacrylamide and agarose gel electrophoresis

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(Received February 2nd, 1981)

Vertical gel systems are widely used in agarose and polyacrylamide gel electrophoresis. Air bubbles generated while pouring the gel and leakiness of the buffer chambers are problems that often occur. Restriction analysis of DNA fragments[1] and the Southern blotting technique[2] require good separations and sharp bands. In vertical gel systems the bands usually are not sharp owing to diffusion of the samples in the direction of separation. This can be overcome by using horizontal slab gels. As polyacrylamide does not polymerize when exposed to air, no system was previously available for pouring horizontal polyacrylamide slab gels. We describe here a simple apparatus and demonstrate its use in separating DNA restriction fragments.

EXPERIMENTAL

Chemicals

The agarose used was Type ME from Seakem (Marine Colloids Div., FMC, Marcus Hooke, PA, U.S.A.). Acrylamide (recrystallized twice, p.a. grade) was purchased from Biomol (Ilvesheim, G.F.R.), and N,N′-methylenbisacrylamide from Serva (Heidelberg, G.F.R.). DNA standards II and III are restriction enzyme digests of bacteriophage λ DNA with ECO RI and HIND III and were obtained from Boehringer (Mannheim, G.F.R.). Other chemicals were of analytical-reagent grade.

Apparatus

All parts were assembled from commercial plexiglass in the institute's workshop. A scheme of the gel holder is shown in Fig. 1.

For pouring gels the seals (A) and the edges of the cover (C) are greased with Vaseline and connected as indicated in Fig. 1. The gel is poured through the slit (D) in the cover (C) using a disposable 50-ml syringe. The sample comb (E) is inserted into the slit (D). After polymerization the seals (A) and the sample comb (E) are removed carefully and the apparatus is placed in the buffer chamber, which is then filled to the cover (C) with Tris–borate buffer (89 m*M* Tris, 8.9 m*M* boric acid, 2.5 m*M* EDTA, pH 8.0). Samples are dissolved in 15 μl of 25 m*M* EDTA (pH 8.0) containing 0.05% of bromphenol blue and 0.2% of agarose and heated for 10 min at 56°C before loading.

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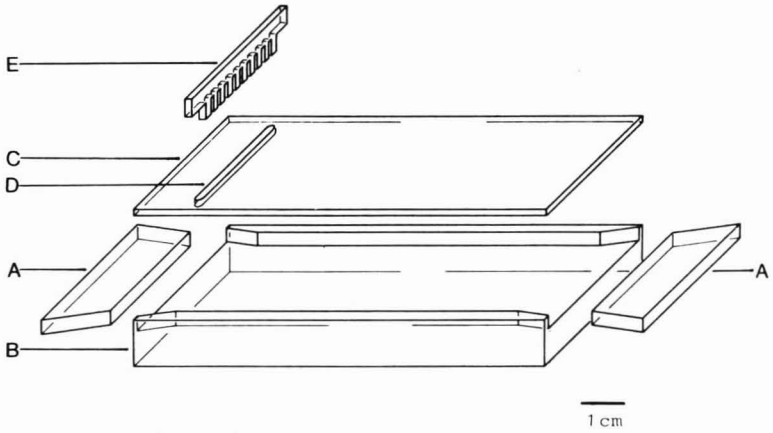

Fig. 1. Design of the gel holder. The parts are assembled as indicated. Prior to assembly the seals (A) and the edges of the cover (C) are greased with Vaseline. The gel is inserted through slit (D) using a disposable syringe. The sample comb (E) is inserted through slit (D). After polymerization of the gel, the comb and the seals (A) are removed carefully. The gels are 9 cm long and 2 or 4 mm thick, depending on the gel holder (B). In 2-mm gels the sample slots are 1 mm deep and in 4-mm gels 2 mm deep. For electrophoresis the gel holder (B) connected with the cover (C) is placed in a buffer chamber (see Fig. 2). Buffer is added up to the cover. After electrophoresis the cover is removed and the gel is stained in the gel holder to prevent damage to the gel.

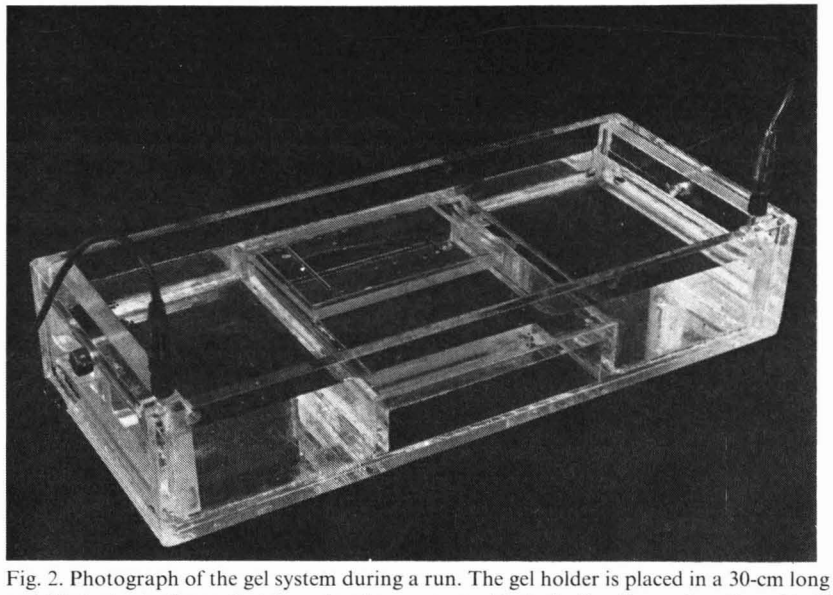

Fig. 2. Photograph of the gel system during a run. The gel holder is placed in a 30-cm long buffer chamber and filled with buffer so that the gel makes contact with the buffer. Even when the gel holder is completely overloaded with buffer good separations are obtained. Direction of separation is from left (−) to right (+). The buffer chamber is not described in detail as other similar chambers can be used. During electrophoresis the buffer chamber is closed with a cover to prevent evaporation of buffer (not shown).

Agarose gels

Agarose was dissolved in Tris–borate buffer (see above) and heated for 3 min using a microwave oven (Philips Model 810 D). After cooling to 70°C the gel was

poured as described above. After cooling to room temperature the samples were loaded and electrophoresis was run at a constant voltage of 1 V/cm for 12 h at room temperature.

Polyacrylamide gels

A 50-ml volume of 5% acrylamide and 0.17% bisacrylamide in Tris–borate buffer was filter-sterilized and degassed for 15 min using water suction, then 50 μl of N,N,N′,N-tetramethylenediamine (TEMED) and 500 μl of 10% ammonium peroxidisulphate in water were added. The gel was poured as described above. After polymerization, the samples were loaded and electrophoresis was run at a constant current of 30 mA for 4 h at room temperature.

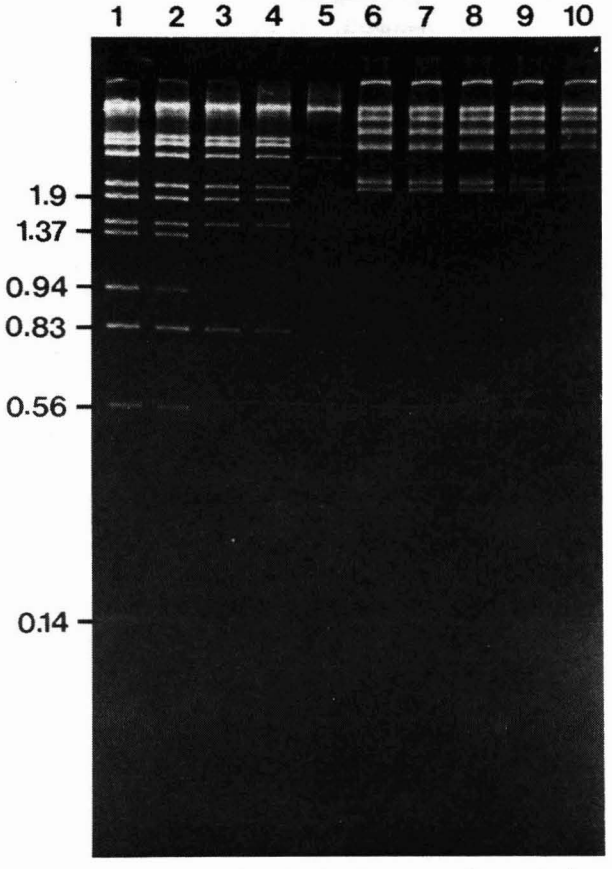

Fig. 3. Separation of DNA restriction fragments of bacteriophage λ DNA on 5% polyacrylamide gel, In lanes 1–5 were loaded 0.5, 1.0, 1.5, 2.0 and 2.5 μg of DNA digested with the restriction enzymes ECO RI and HIND III as described under Experimental. In lanes 6–10 were loaded 0.5, 1.0, 1.5, 2.0 and 2.5 μg of DNA digested with HIND III only. Electrophoresis was performed at 30 mA for 4 h. After the run the gel, in the gel holder, was stained with ethidium bromide solution (4 μg/ml) for 30 min. The bands were revealed under short-wave UV light and photographed with a Polaroid camera. The size of the fragments was given by the supplier of the standards and is indicated adjacent to the bands (kilobases). The gel was 4 mm thick and 9 cm long. Direction of electrophoresis is from top (−) to bottom (+).

Staining

The gels were stained for 30 min in 4 μg/ml ethidium bromide solution and the bands were revealed by illumination with short-wave UV light.

Photography

The gels were photographed with a Polaroid camera using Type 55 film and an orange filter to reduce background.

RESULTS AND DISCUSSION

The apparatus was applied to the separation of DNA restriction fragments. As shown in Figs. 3 and 4, the bands are much sharper than those on conventional gel slabs. Problems with polymerization of gels did not occur. The relatively small size of the gel layer (7 × 10 cm) reduces the costs of chemicals. Under the conditions used 4-

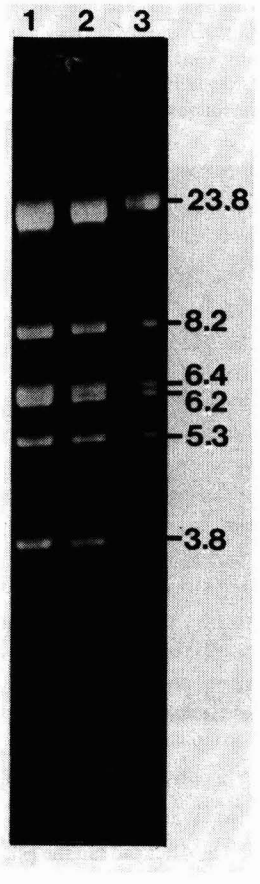

Fig. 4. Separation of DNA restriction fragments of bacteriophage λ DNA on a 0.7% agarose gel. In lanes 1–3 were loaded 1.0, 0.6 and 0.3 μg of DNA digested with the restriction enzyme ECO RI. Electrophoresis was carried out at 1 V/cm for 12 h. Other details as in Fig. 3.

mm thick gels gave the best results for polyacrylamide and 2-mm thick gels gave the best results for agarose.

Southern blots[2] routinely carried out gave excellent results (data not shown). The simplicity and convenient handling of the apparatus makes it applicable to most gel techniques, including discontinuous electrophoresis. Gels can be stained when still in the gel holder, which is advantageous when using low percentage gels or radioactively labelled samples.

As the apparatus is not available commercially, detailed construction plans can be obtained from the authors on request.

ACKNOWLEDGEMENTS

We thank Rudi Borchard for constructing the apparatus, Karen Roberts for technical assistance and Mechthild Ziemer for photography.

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Note

An efficient chemically bonded reversed-phase tin-layer plate permitting the use of water-rich mobile phases*

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(Received February 20th, 1981)

Within the last few years, several new commercially available chemically bonded reversed-phase thin-layer chromatography (RPTLC) plates have led to a renaissance in TLC. Problems associated with the wettability of these RPTLC plates by the solvent, however, have prevented the use of water-rich mobile phases and detection techniques[1].

In an effort to overcome these problems, we first silanized pre-coated TLC plates with dodecyltrichlorosilane according to Gilpin and Sisco[2]. We also tried to coat glass plates with dodecylsilanized silica gel prepared in our laboratories[3], but the layer obtained was not stable enough. Finally, using our silanized silica gel, Antec AG (Bennwil, Switzerland) developed a pre-coated RPTLC plate, which is commercially available under the trade-name OPTI-UP C12**.

During the last 3 years, we have compared their efficiency with those of (eight) other commercially available pre-coated RPTLC plates in systems containing more than 40% of water and using several classes of substances.

EXPERIMENTAL

Materials

The following commercially available TLC plates were tested: 20 × 20 cm plates pre-coated with OPTI-UP C12 containing a fluorescent indicator; 10 × 10 cm plates, pre-coated with LiChrosorb RP-2, RP-8 and RP-18, containing a fluorescent indicator (Merck, Darmstadt, G.F.R.); 10 × 10 cm plates pre-coated with NanoSil C18 (100%, 75% and 50%), containing a fluorescent indicator (Macherey, Nagel & Co., Düren, G.F.R.); 20 × 20 cm pre-coated KC 18 thin-layer plates (Whatman, Maidstone, Great Britain); and 20 × 20 cm RPS Uniplates, which have a support layer impregnated with long-chain hydrocarbons (Analtech, Newark, DE, U.S.A.).

* Dedicated to the late Professor R. B. Woodward, deceased July 8th, 1979, who shared with us his experience of using OPTI-UP C12 plates.

** The plates are also available through Tridom Chemicals (Hauppauge, NY, U.S.A.), Fluka (Neu-Ulm, G.F.R.), Siccap-Emmop (Marseille, France) and Antec (Bennwil, Switzerland).

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Chemicals

All chemicals were of analytical-reagent grade and were purchased from Fluka (Buchs, Switzerland).

RESULTS AND DISCUSSION

In our experience, only OPTI-UP C12 can be used conveniently with mobile phases containing more than 40% of water. They are, in fact, applicable irrespective of the water concentration and without sampling or detection limits.

Neither aqueous sprays nor aqueous test solutions can be applied to the Merck and Macherey, Nagel & Co. plates.

With RPS Uniplates we encountered the same problems as with all impregnated or silanized plates, namely that the solvents were limited to liquids that were relatively immiscible with the stationary phase, and the stability and reproducibility were poor.

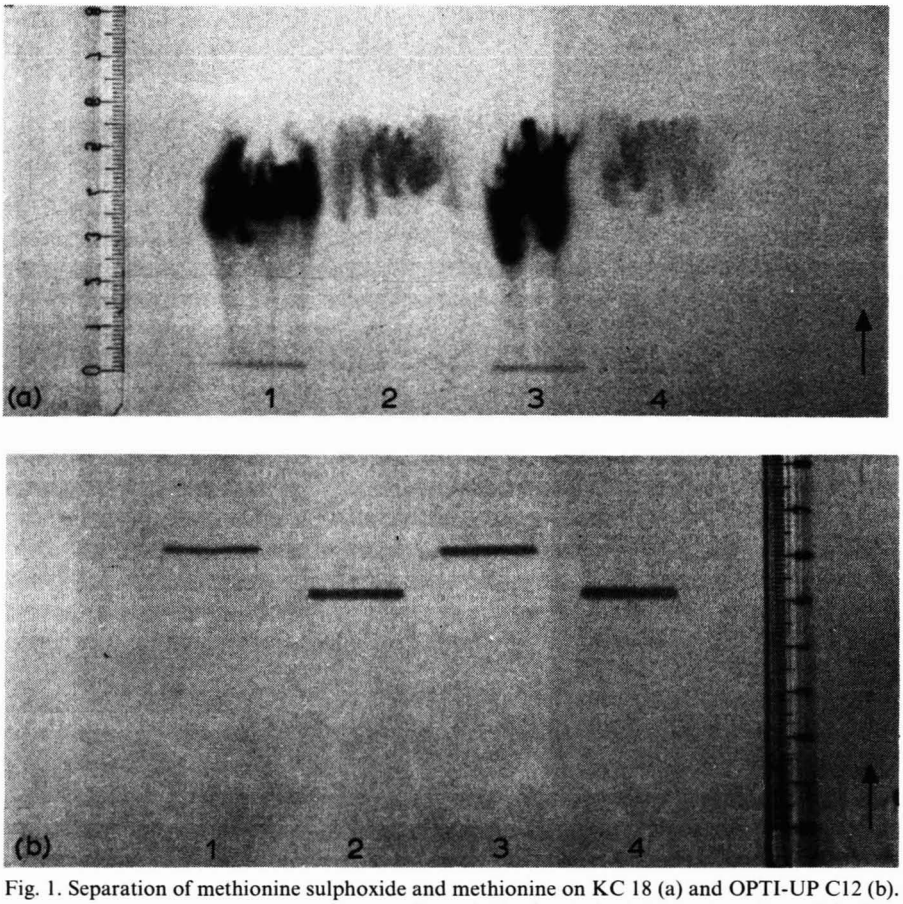

Fig. 1. Separation of methionine sulphoxide and methionine on KC 18 (a) and OPTI-UP C12 (b). Eluent: 3% NaCl in water. Development: (a) 180 min and (b) 45 min for 8.5 cm. Detection: ninhydrin. 1, 3 = Methionine sulphoxide; 2, 4 = methionine.

The Macherey, Nagel & Co. and Merck plates have disadvantages such as the application limits described by Siouffi *et al.*[1] and the Whatman KC 18 plate was useful only at water concentrations up to 38%. With the Whatman plate, the addition of 3% of sodium chloride to the solvent system prevented the dislodgement of the chemically bonded phase from the glass backing and a higher water concentration could be used, but other problems due to the salt arose (Fig. 1). Salt effects in RPTLC on silica gels have been described by Thijssen[4].

Several examples of the application and performance of OPTI-UP C12 plates have recently been published, for the separation of diastereoisomers[5], the quantitative radiometric assay of nucleosides[6], the preparative separation of penems[7,8] and the bioautoradiography of antibiotics[9,10] using solvent systems containing 40–100% water.

In our laboratory, we have been able to separate five dansyl amino acids using 50% water (Fig. 2), strongly polar Cibacron dyes using 70% water (Fig. 3) and two catecholamines using 95% water (Fig. 4).

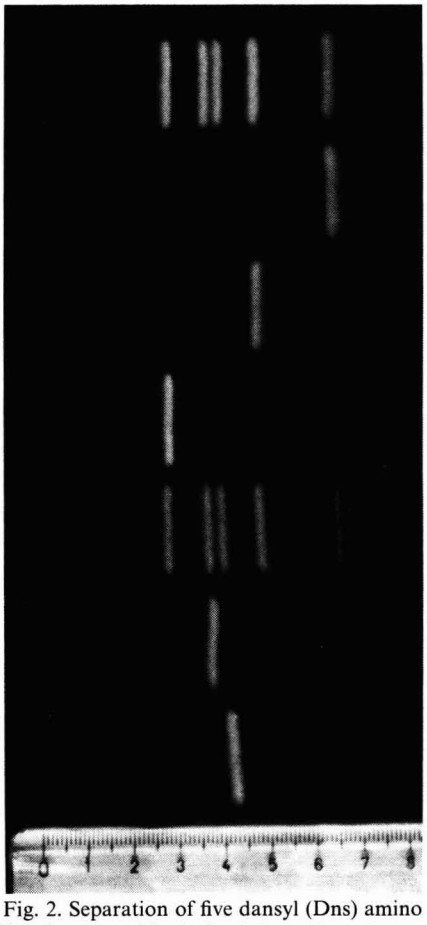

Fig. 2. Separation of five dansyl (Dns) amino acids on OPTI-UP C12. Eluent: 50% acetonitrile in water. Development: 45 min for 8.5 cm. Detection: UV, 366 nm. 1 = Mixture of 1–5; 2 = Dns-$CySO_3H$ (1 μg); 3 = Dns-Asn (1 μg); 4 = Dns-Arg (1 μg); 5 = mixture of 1–5; 6 = Dns-L-α-aminobutyric acid (1 μg); 7 = Dns-Ala (1 μg).

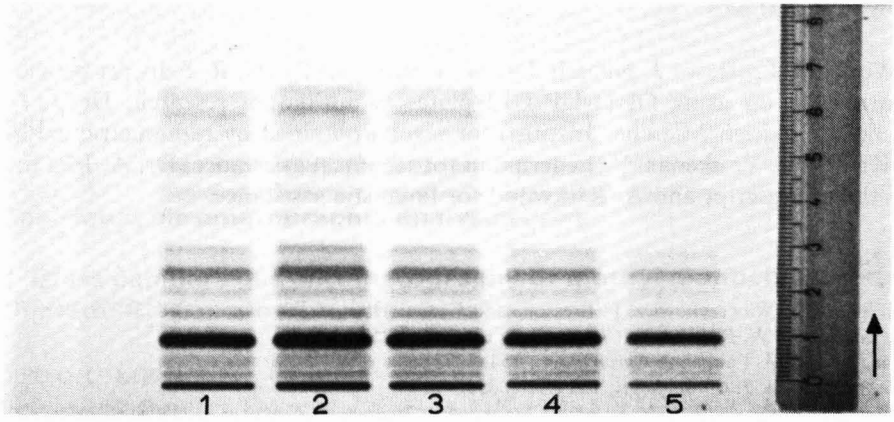

Fig. 3. Separation of a mixture of strongly polar Cibacron dyes on OPTI-UP C12. Eluent: 30% acetonitrile in water. Development: 60 min for 8.5 cm. Detection: daylight. 1 = 25 μg; 2 = 30 μg; 3 = 25 μg; 4 = 20 μg; 5 = 15 μg.

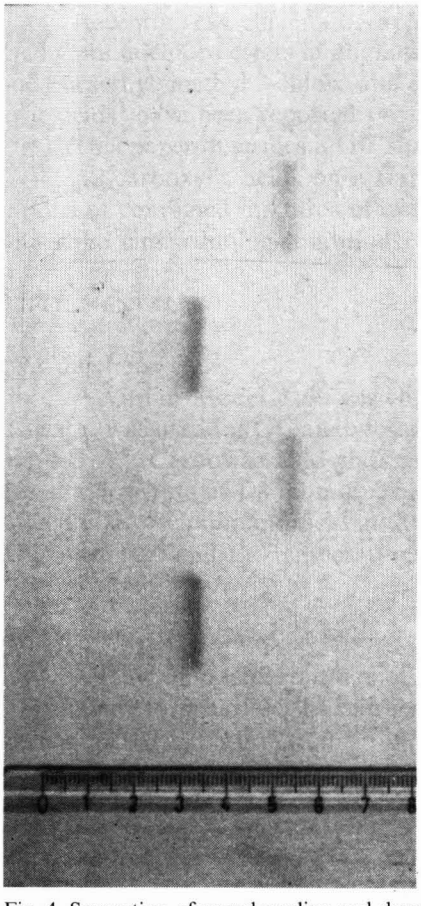

Fig. 4. Separation of noradrenaline and dopamine on OPTI-UP C12. Eluent: 5% formic acid in water. Development: 45 min for 8.5 cm. Detection: Pauly's reagent[11]. 1, 3 = Noradrenaline (30 μg); 2, 4 = dopamine (30 μg).

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Note

Gas–liquid chromatographic analyses

II. Glass capillary gas chromatography of methyl monochloro esters of aliphatic C_2–C_{18} *n*-carboxylic acids

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(First received January 29th, 1981; revised manuscript received February 27th, 1981)

Recently, gas chromatographic (GC) separations of combined mixtures of methyl monochloro esters of aliphatic C_3–C_6 (ref. 1) and C_7–C_{10} *n*-carboxylic acids[2] and of methyl, methyl 2-chloro and chloromethyl esters of aliphatic C_2–C_{20} *n*-carboxylic acids[3] have been reported.

This paper describes a GC study of all methyl monochloro esters of aliphatic C_2–C_{18} *n*-carboxylic acids on a Carbowax 20M glass capillary column. The separations of combined mixtures of even- and odd-carbon-number esters were studied under the same running conditions.

EXPERIMENTAL

GLC analysis

A Varian Model 2400 gas chromatograph, equipped with a flame-ionization detector, was used for GC analyses. The chromatograph was fitted with a 90 m × 0.3 mm I.D. 3% Carbowax 20M glass capillary column. Nitrogen was used as the carrier gas at a flow-rate of 1.8 ml/min. The column temperature was programmed from 50 to 200°C at 4°C/min and held at 200°C until elution of peaks ceased. The splitting ratio was 1:20 and the temperatures of injector and detector were 220 and 240°C, respectively.

Samples

Methyl monochloro esters of aliphatic C_3–C_6 *n*-carboxylic acids were synthesized in pure form as described earlier[4]. Methyl monochloro esters of the C_7–C_{18} acids were prepared by chlorination[2,5] of the corresponding methyl esters. The crude chlorination mixtures were used for GC analyses.

RESULTS AND DISCUSSION

The polarity of the isomeric monochloro esters increases with increasing distance between the chloro and ester groups, which leads to the better separations of isomers on polar than on non-polar columns.

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The gas chromatograms of the combined mixtures of even-carbon-number C_2–C_{18} and odd-carbon-number C_3–C_{17} methyl monochloro esters are illustrated in Figs. 1 and 2 and the relative retention times of the compounds are presented in Tables I and II. All retention times were measured from sample injection and are tabulated relative to unchlorinated methyl esters = 1.00 (Table I) and C_{10} derivatives = 1.00 (Table II). Fig. 3 illustrates the same data as Table I.

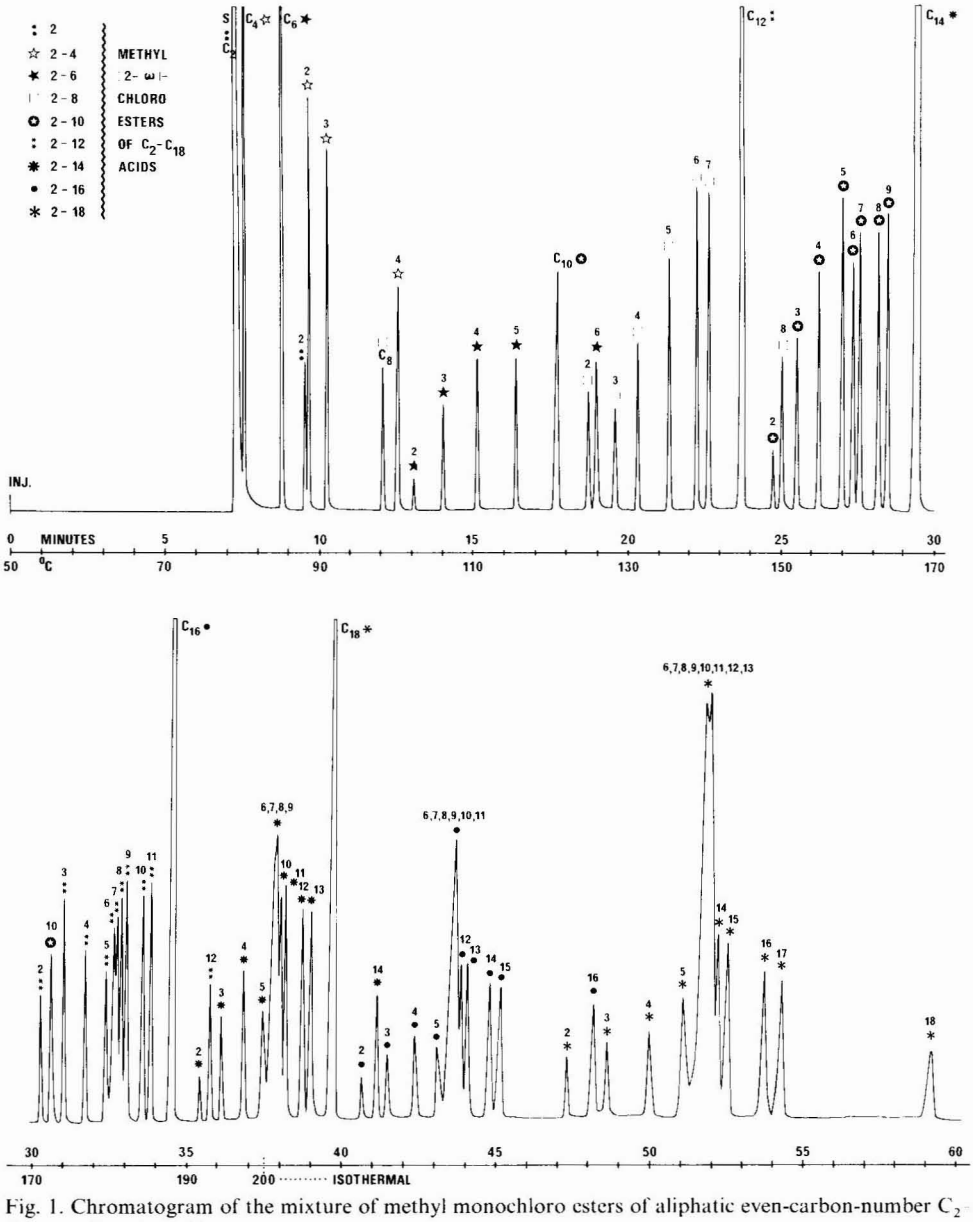

Fig. 1. Chromatogram of the mixture of methyl monochloro esters of aliphatic even-carbon-number C_2–C_{18} *n*-carboxylic acids.

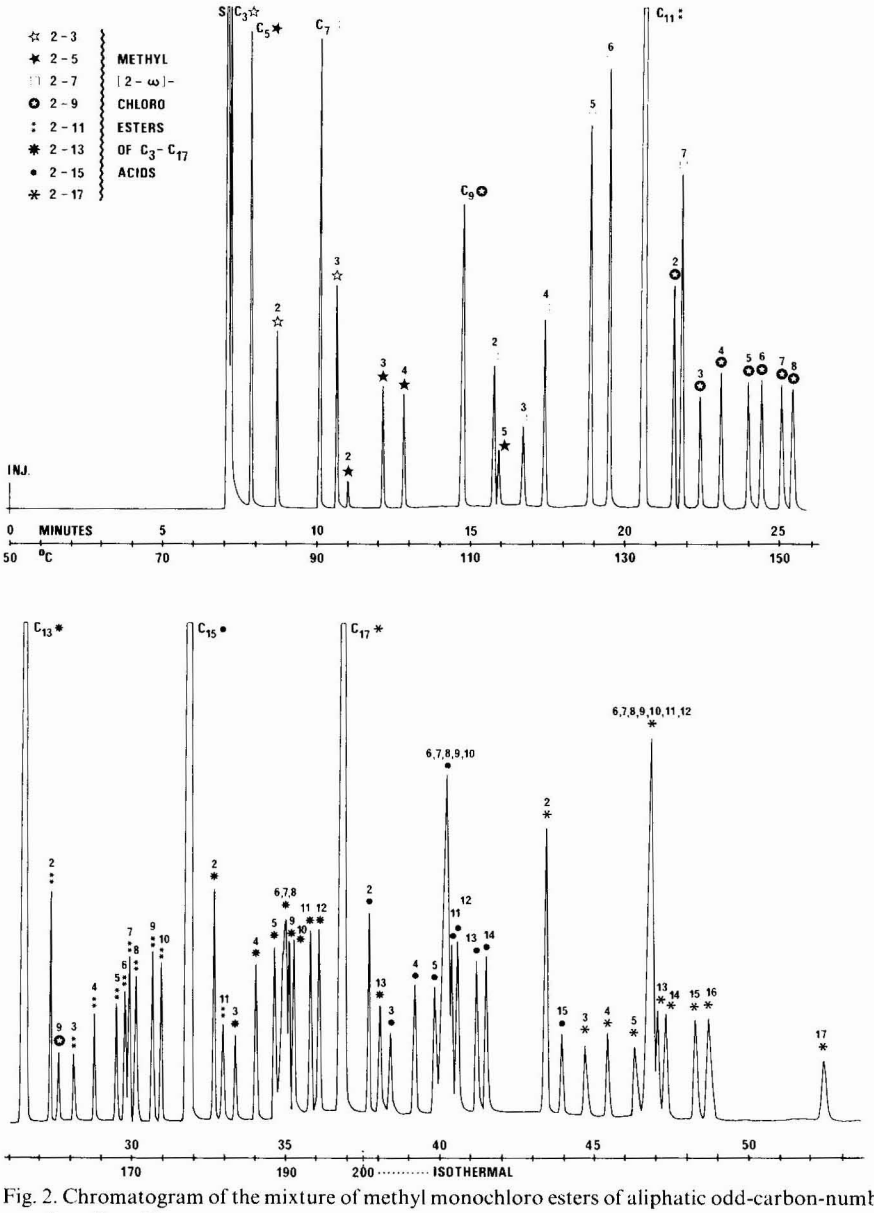

Fig. 2. Chromatogram of the mixture of methyl monochloro esters of aliphatic odd-carbon-number C_3–C_{17} *n*-carboxylic acids.

The chromatograms show that the mixtures of even- and odd-carbon-number esters can be separated. However, the GC analysis of combined mixture of all C_2–C_{18} methyl monochloro esters resulted in several overlapping peaks, particularly with long-chain isomers.

All of the isomeric monochloro esters are resolvable up to a chain length of C_{12}; for longer chain lengths up to C_{18}, the peaks of the mid-chain isomers from 6-

TABLE I

RELATIVE RETENTION TIMES FOR METHYL MONOCHLORO ESTERS OF ALIPHATIC C_2-C_{18} *n*-CARBOXYLIC ACIDS

Isomeric monochloro esters	*Relative retention time**																
	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}
Methyl ester	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2-Cl	1.31	1.21	1.28	1.41	1.49	1.56	1.55	1.46	1.40	1.33	1.28	1.24	1.21	1.19	1.17	1.18	1.19
3-Cl		1.48	1.36	1.55	1.60	1.65	1.62	1.53	1.44	1.36	1.31	1.26	1.23	1.21	1.20	1.21	1.22
4-Cl			1.66	1.63	1.72	1.72	1.68	1.57	1.48	1.40	1.34	1.29	1.26	1.23	1.22	1.23	1.26
5-Cl				2.03	1.87	1.87	1.77	1.63	1.53	1.43	1.37	1.31	1.28	1.25	1.25	1.26	1.28
6-Cl					2.16	1.93	1.84	1.66	1.55	1.44	1.38	1.32	1.29	1.26	1.26	1.27	1.30
7-Cl						2.16	1.88	1.70	1.56	1.45	1.38	1.32	1.29	1.26	1.26	1.27	1.30
8-Cl							2.07	1.73	1.59	1.46	1.39	1.32	1.29	1.26	1.26	1.27	1.30
9-Cl								1.88	1.61	1.49	1.40	1.33	1.29	1.26	1.26	1.27	1.31
10-Cl									1.73	1.50	1.42	1.34	1.30	1.26	1.26	1.27	1.31
11-Cl										1.60	1.43	1.35	1.31	1.27	1.26	1.27	1.31
12-Cl											1.51	1.36	1.32	1.28	1.27	1.27	1.31
13-Cl												1.44	1.33	1.29	1.28	1.28	1.31
14-Cl													1.40	1.30	1.29	1.29	1.31
15-Cl														1.38	1.31	1.31	1.32
16-Cl															1.39	1.32	1.35
17-Cl																1.42	1.37
18-Cl																	1.49
Methyl ester**	7.23***	7.28	7.60	7.87	8.81	10.12	12.12	14.75	17.75	20.66	23.80	26.52	29.42	31.90	34.70	36.90	39.85

* Relative retention times for unchlorinated methyl esters taken as 1.00.

** Absolute retention times (min) for unchlorinated methyl esters.

*** Retention time determined using methyl hexanoate as solvent.

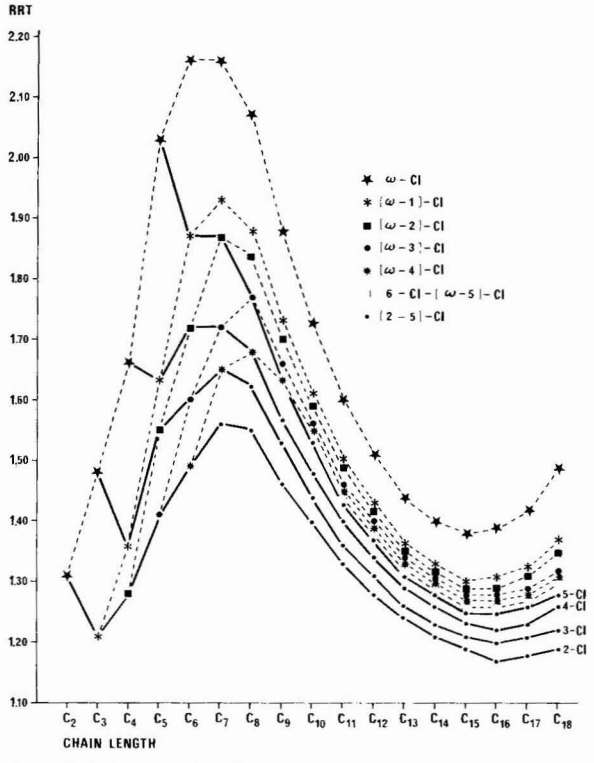

Fig. 3. Relative retention times (RRT) for methyl monochloro esters of aliphatic C_2–C_{18} *n*-carboxylic acids. Retention times were measured from Figs. 1 and 2 and are presented relative to unchlorinated methyl esters = 1.00.

chloro to (ω-5)-chloro compounds always overlap. Several columns and various running conditions were used but the mid-chain isomers of the C_{13}–C_{18} esters could not be separated. Although the methyl monochlorododecanoates are fully resolved[5] and the separations of longer chain isomers are slightly better when the column temperature is programmed slowly, long retention times and broad peaks of long-chain isomers are obtained.

From Fig. 3 it can be seen that isomeric monochloro esters leave the column in direct sequence from the 2-chloro to the ω-chloro compound. Terminally chlorinated products are the most polar, leading to relatively long retention times compared with other isomers.

Under the running conditions used the greatest relative retention times are observed for C_6–C_8 isomers (Fig. 3, Table I) and the values decrease with increasing chain length. With long-chain isomers, however, the relative retention times increase with increasing chain length owing to the isothermal running conditions after 37.5 min.

As shown in Table II, the relative retention times for isomeric monochloro esters from the 2-chloro to the ω-chloro compound are constant, particularly for long-chain esters. With short-chain compounds (C_2–C_6), however, the 2-chloro and

TABLE II

RELATIVE RETENTION TIMES FOR METHYL MONOCHLORO ESTERS OF ALIPHATIC C_2–C_{18} *n*-CARBOXYLIC ACIDS

Chain length	*Relative retention time**										
	Methyl ester	*Isomeric monochloro esters*									
		2-Cl	*3-Cl*	*4-Cl*	*5-Cl*	*6-** (ω-5)-Cl*	*(ω-4)-Cl*	*(ω-3)-Cl*	*(ω-2)-Cl*	*(ω-1)-Cl*	*ω-Cl*
C_2	0.41	0.39									0.31
C_3	0.42	0.35	0.42							0.31	0.35
C_4	0.43	0.39	0.40	0.48					0.34	0.36	0.41
C_5	0.44	0.45	0.48	0.49	0.59			0.40	0.43	0.45	0.52
C_6	0.50	0.53	0.55	0.58	0.61	0.69	0.48	0.51	0.54	0.58	0.62
C_7	0.57	0.64	0.65	0.66	0.70	0.71	0.61	0.63	0.67	0.68	0.71
C_8	0.68	0.76	0.77	0.78	0.79	0.82	0.74	0.77	0.79	0.80	0.82
C_9	0.83	0.87	0.88	0.88	0.89	0.90	0.88	0.88	0.89	0.89	0.90
C_{10}	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C_{11}	1.16	1.11	1.10	1.10	1.09	1.09	1.09	1.09	1.09	1.09	1.08
C_{12}	1.34	1.22	1.22	1.21	1.20	1.20	1.20	1.20	1.19	1.19	1.17
C_{13}	1.49	1.32	1.31	1.30	1.28	1.28	1.28	1.28	1.27	1.27	1.24
C_{14}	1.66	1.43	1.41	1.40	1.39	1.39	1.39	1.38	1.37	1.37	1.34
C_{15}	1.80	1.52	1.50	1.49	1.47	1.48	1.47	1.47	1.46	1.45	1.43
C_{16}	1.95	1.64	1.63	1.61	1.59	1.60	1.60	1.60	1.59	1.58	1.57
C_{17}	2.08	1.75	1.75	1.73	1.71	1.72	1.72	1.71	1.71	1.71	1.71
C_{18}	2.25	1.91	1.90	1.90	1.89	1.90	1.90	1.90	1.90	1.90	1.93
C_{10}***	17.75	24.80	25.59	26.32	27.11		27.48	27.69	28.29	28.59	30.70

* Relative retention times for C_{10} derivatives taken as 1.00.

** Values for C_6–C_9 compounds determined from retention times of 6-chloro isomers and for C_{10}–C_{18} compounds from the average retention times of 6-(ω-5)-chloro isomers.

*** Absolute retention times (min) for C_{10} derivatives.

ω-chloro isomers give rise to the greatest disparities, which can clearly be seen from the first value in each column.

ACKNOWLEDGEMENT

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Note

Gas chromatographic separation of some enantiomers on optically active copper(II) complexes

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Schurig[1] accomplished the first gas chromatographic (GC) resolution of a chiral olefin on an optically active rhodium(I) complex in 1977. In principle, such complexation gas chromatography[2] should be applicable to all conceivable ligand–metal interactions. Indeed some enantiomers of epoxy compound were resolved[3,4] on optically active nickel(II) or europium(III) complexes. Recently we have found that α-hydroxy carboxylic acid ester enantiomers could be resolved on copper(II) complexes of optically active Schiff's bases[5,6]. In the present paper we wish to report the first direct separation of some chiral amino alcohols, amines, amino acid esters and alcohols by GC on optically active copper(II) complexes.

EXPERIMENTAL

Chemicals

1-Dimethyl, 1-diethyl and 1-dibutylamino-2-propanol were purchased from Wako (Osaka, Japan). Their O-trimethylsilyl (TMS) and O-acetyl (Ac) derivatives were prepared by silylation with hexamethyldisilazane and trimethylchlorosilane in pyridine, and by acylation with acetic anhydride in pyridine, respectively. 3-(2,2,2-Trifluoroethoxy)- and 3-methoxy-1-*tert*.-butylamino-2-propanol were prepared from 3-chloro-1-*tert*.-butylamino-2-propanol by treatment with 2,2,2-trifluoroethanol or methanol in alkaline solution. The 3-chloro-1-*tert*.-butylamino-2-propanol was derived by aminolysis with *tert*.-butylamine from epichlorohydrin. Amino acid isopropyl esters were prepared from the corresponding amino acids by esterification in an acidic isopropanol. 2-Ethylpiperidine, 1-phenylethylamine, tetrahydrofurfuryl alcohol and α-phenylpropargyl alcohol were purchased from Tokyo Kasei (Tokyo, Japan) and 4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one (allethrolone) was provided by Dr. Itaya of our laboratory.

The binuclear copper(II) complexes of N-salicyliden-(*R*)-2-amino-1,1-bis(5-*tert*.-butyl-2-octyloxyphenyl)-propan-1-ol (R-1648-Cu) and N-salicyliden-(*S*)-2-amino-1,1-diphenylpropan-1-ol (S-1600-Cu) were prepared by Dr. Nagase as described previously[7].

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Gas chromatography

The experiments were carried out with a Shimadzu Model GC-7A gas chromatograph equipped with a flame ionization detector.

Glass capillary columns (40 m or 20 m × 0.25 mm I.D.) coated with the mixture of R-1648-Cu and silicone OV-101 (1:1) or the mixture of S-1600-Cu and Ucon oil 50-HB-5100 (1:1) were used. Packed columns (2 m × 3 mm I.D.) filled with Chromosorb W AW DMCS (80–100 mesh) coated with 6% of the mixture of R-1648-Cu and silicone OV-101 (5:1) were also used.

RESULTS AND DISCUSSION

The results of the GC separation of amino alcohol, amine, amino acid ester and alcohol enantiomers are summarized in Table I.

Racemic amino alcohols were resolved into their antipodes with good separation factors. A typical chromatogram is shown in Fig. 1. As these optically active copper(II) complexes show high selectivity for amino alcohols, their enantiomers can be also separated on a packed column as shown in Fig. 2. The mass spectra obtained from two separated peaks were identical and consistent with the standard spectrum of the racemic compound.

Hitherto, racemic amino alcohols were resolved generally in the form of N-acyl-O-ester derivatives using amino acid or amine derivatives as the chiral stationary phases[8,9]. It is notable that amino alcohols were separated without any pretreatment such as acylation. It was shown that nitrogen-attached and oxygen-attached hydrogen are not always necessary for the separation of these amino alcohol enantiomers.

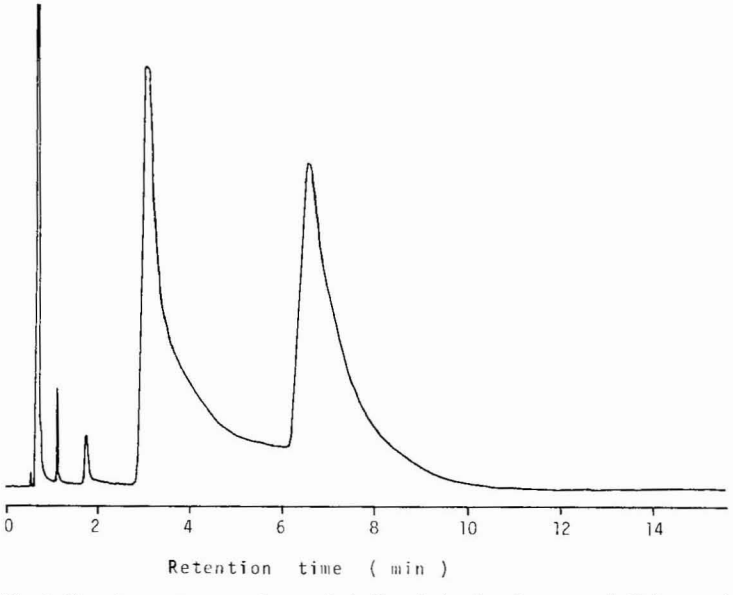

Fig. 1. Gas chromatogram of racemic 1-dimethylamino-2-propanol. Column: glass capillary (20 m × 0.25 mm I.D.) coated with a mixture of R-1648-Cu and silicone OV-101 (1:1). Temperature: 60°C. Carrier gas (helium) flow-rate: 1.2 ml/min.

TABLE I

GAS CHROMATOGRAPHIC SEPARATION OF AMINO ALCOHOL, AMINE, AMINO ACID ESTER AND ALCOHOL ENANTIOMERS

Glass capillary columns, 20 m × 0.25 mm I.D. Carrier gas, helium at a flow-rate of 1.2 ml/min.

Compound				*Stationary phase*							
				R-1648-Cu + Silicone OV-101 (1:1)				*S-1600-Cu + Ucon oil 50-HB-5100 (1:1)*			
				Temp. (°C)	*Retention time* (min)*		*α***	*Temp. (°C)*	*Retention time* (min)*		*α***
					First peak	*Second peak*			*First peak*	*Second peak*	
Amino alcohols $Y-CH(OX)-CH_2-N(R_2)-R_1$											
X	R_1	R_2	*Y*								
H	CH_3	CH_3	CH_3	20	2.38	5.94	2.496	60	2.63	4.88	1.856
TMS	CH_3	CH_3	CH_3	60	1.76	2.09	1.188				
Ac	CH_3	CH_3	CH_3	60	2.79		1.000				
H	C_2H_5	C_2H_5	CH_3	60	2.89	3.91	1.353	60	3.28	3.50	1.067
TMS	C_2H_5	C_2H_5	CH_3	60	5.24	5.64	1.076				
Ac	C_2H_5	C_2H_5	CH_3	60	7.13		1.000				
H	n-C_4H_9	n-C_4H_9	CH_3	60	42.76	46.59	1.097	60	32.20		1.032
TMS	n-C_4H_9	n-C_4H_9	CH_3	60	72.61	74.16	1.021				
Ac	n-C_4H_9	n-C_4H_9	CH_3	60	97.66		1.000				
H	t-C_4H_9	H	OCH_2CF_3	85	70.91(−)	107.56 (+)	1.528	80	50.80(+)	71.80 (−)	1.413
H	t-C_4H_9	H	OCH_3	85	75.65	114.25	1.510	100	41.28	50.24	1.217

Amines								
2-Ethylpiperidine	60	37.39	43.45	1.162	60	60.00	78.40	1.307
1-Phenylethylamine	80	35.84	37.33	1.042	120	10.40	11.00	1.058
Amino acid isopropyl esters								
Alanine***	70	96.0(D)	111.6(L)	1.160	80	25.87	28.52	1.102
α-Aminobutyric acid***	70	74.6	91.6	1.230				
Valine***	70	91.6(D)	105.2(L)	1.150				
Alcohols								
Tetrahydrofurfuryl alcohol	60	3.58	3.84	1.072				
Allethrolone	85	135.12	136.72	1.012	120	68.19	69.26	1.010
α-Phenylpropargyl alcohol	80	23.06	25.59	1.110				

* Measured from solvent peak.

** Separation factor calculated by second peak/first peak.

*** Chromatographed on 40 m × 0.25 mm I.D. glass capillary column using helium at a flow-rate of 0.7 ml/min.

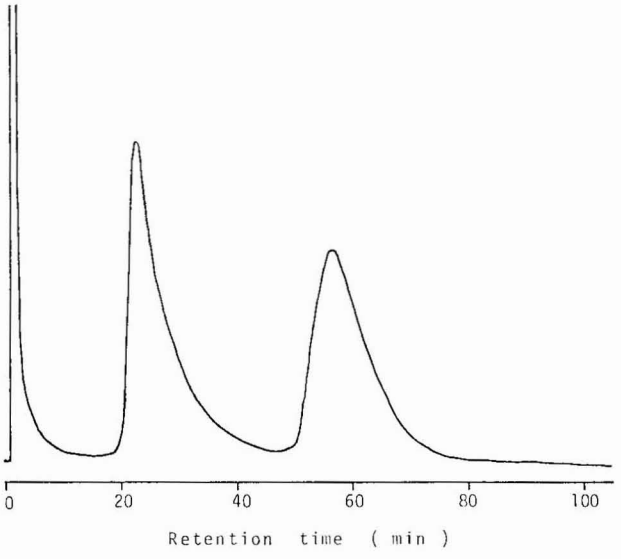

Fig. 2. Gas chromatogram of racemic 1-dimethylamino-2-propanol. Column: glass (2 m × 3 mm I.D.) packed with 80–100 mesh Chromosorb W AW DMCS coated with 6% of a mixture of R-1648-Cu and silicone OV-101 (5:1). Temperature: 60°C. Carrier gas (helium) flow-rate: 40 ml/min.

However, the free hydroxy group should be effective for the enantiomeric separation because separation factors apparently decreased when the hydroxy group was trimethylsilanized, and no separation was observed when the hydroxy group was acylated.

It is also worth noting that some racemic amines and amino acid esters could be directly separated without N-acylation although the peak shape accompanied by tailing was rather broad. An example of this is shown in Fig. 3. Moreover, it is

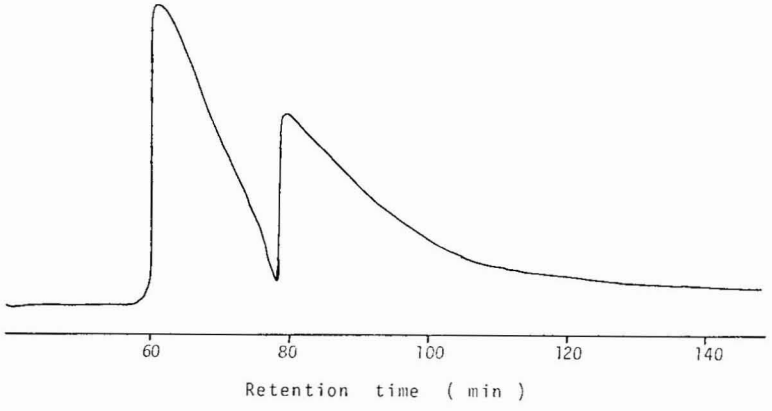

Fig. 3. Gas chromatogram of racemic 2-ethylpiperidine. Column: glass capillary (20 m × 0.25 mm I.D.) coated with a mixture of S-1600-Cu and Ucon oil 50-HB-5100 (1:1). Temperature: 60°C. Carrier gas (helium) flow-rate: 1.2 ml/min.

interesting that some chiral alcohols were resolved into their antipodes although no baseline separation had been obtained.

We consider that these results on optically active copper complexes give new light for complexation GC.

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Note

Essential oil composition of *Aframomum korarima*

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Aframomum korarima (Per.) Engler, family Zingiveraceae, is a perennial plant endemic to Ethiopia[1]. Its seeds, which are brown and shiny, have a diameter of 3–4 mm, and have a strongly aromatic but slightly burning taste that could be matched closely to Indian cardamoms.

Cufodontis[1] reported that the name for the spice crop in Ethiopia is *Aframomum korarima* (Per.) Engler and that the name *A. angustifolium* Schum has been used by the Kew Herbarium and by Mooney[2], but that he himself had seen no specimen corresponding to *A. angustifolium*. *A. angustifolium* has been used interchangeably with *A. korarima* by Mooney[2]. It therefore seems that *A. angustifolium* was used in error and further studies by Cufodontis on the plant in Ethiopia around 1969 seem to have established that the two species are distinct.

Aframomum spp. yield the rarer essential oils applicable in perfumery[3], but little chemical analysis on the essential oils of these species has been carried out. The oil of *A. amoniense* ("natural geraniol") from Tanzania was reported to have constituents similar to those of commercial geranium oil[4]. Analysis of the oils of *A. mala* and *A. amaniense* by Lee and Worsely[5] showed that it was composed of kajene, caryophylene, β-pinene, terpineol, cineol, geraniol, geranyl acetate and other unidentified compounds. The composition of the essential oil of *A. angustifolium* was studied by Coomes *et al.*[6], who reported the results of the analysis of seeds collected from Tanzania. The seeds were found to contain 1.1% of volatile oil and the constituents identified were α-pinene (9.9%), β-pinene (22.8%), limonene and dipentene (8.4%), cineole (18.1%), alcohols (10.7%), sesquiterpenes (12.6%) and esters (1.4%).

This paper reports the results of the gas chromatographic (GC) and gas chromatographic–mass spectrometric (GC–MS) analysis of samples of *Aframonum korarima* Engler.

EXPERIMENTAL

Materials

Essential oil was obtained from *A. korarima* by steam distillation of commercial-grade seeds (moisture content 13.7%) from the Gamo-Gofa Administrative Region of Ethiopia.

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Gas chromatography

A Hewlett-Packard Model 5710A gas chromatograph equipped with a flame-ionization detector was used. The operating conditions that gave the best separation are shown in Table I.

TABLE I

OPERATING CONDITIONS FOR HEWLETT-PACKARD MODEL 5710A GAS CHROMATOGRAPH

Stationary phase	Carbowax 20M (10%)
Solid support	Chromosorb W AW, DMCS treated, 80–100 mesh
Column length	6 ft.
Column diameter	1/8 in. O.D.
Column material	Stainless steel
Column temperature:	
Initial	70°C
Final	200°C
Programming rate	4°C/min
Detector	Flame ionization
Injection port temperature	200°C
Detector oven temperature	200°C
Carrier gas (N_2) flow-rate	66 ml/min
Hydrogen flow-rate	33 ml/min
Air flow-rate	330 ml/min
Sample size	0.5 μl

Combined gas chromatography–mass spectrometry

The GC–MS system was a Finnigan Model 3200 instrument equipped with a linear temperature programmer and a Model 6400 data system. The operating conditions of the GC–MS system are given in Table II. The ion beam current was recorded and used as the gas chromatography trace. Mass spectra (at 70 eV) corresponding to the peak maxima were recorded. Each component was identified by comparison of the retention times and mass spectra with those of standard samples.

TABLE II

OPERATING CONDITIONS FOR FINNIGAN MODEL 3200 GC–MS INSTRUMENT

Stationary phase	Carbowax 20M (10%)
Solid support	Chromosorb W AW, DMCS treated, 80–100 mesh
Column length	6 ft.
Column diameter	1/8 in. O.D.
Column material	Glass
Column temperature:	
Initial	70°C
Final	200°C
Programming rate	4°C/min
Detector	MS ion beam detector
Injection port temperature	200°C
Carrier gas (He) flow-rate	*ca.* 40 ml/min
Sample size	0.5 μl

RESULTS AND DISCUSSION

The essential oil composition of *A. korarima* determined by comparison of retention data and MS fragments with those of authentic samples and the percentage composition calculated from peak areas are given in Table III.

TABLE III

ESSENTIAL OIL COMPOSITION OF *AFRAMOMUM KORARIMA*

Peak No.	*Retention time (min)*	*m/e*	*Composition (%)*	*Compound*
1	2.22	136	2.2	α-Pinene
2	3.31	136	21.4	β-Pinene
3	3.64	136	0.9	Myrcene
4	4.14		1.0	
5	4.49	136	9.1	Limonene
6	4.86	154	33.9	Cineole
7	5.23	136	2.7	γ-Terpinene
8	7.73		0.8	
9	5.94		0.5	
10	9.78		0.3	
11	11.48		0.2	
12	11.91		0.4	
13	12.31		0.2	
14	13.36	154	3.2	Terpinene-4-ol
15	15.84	154	9.3	Terpinyl acetate
16	17.02		4.5	Geranyl acetate
17	19.52	154	3.9	Geraniol
18	28.06	204	5.4	(Sesquiterpene)

It was found that *A. korarima* yields a higher percentage of essential oil (3.2%) than previously reported by Coomes *et. al.*[6] in other *Aframomum* spp.

GC analysis showed eighteen separate peaks and the major constituents (eleven peaks comprising 91.1% of the total) were characterized. Of these peaks, five were monoterpenes, two were monoterpene alcohols, two were esters, one was an ether and one was a sesquiterpene whose structure was not identified. The major components of the oil, cineole, limonene, terpinyl acetate and the pinenes, were present at approximately the same concentrations as in Indian cardomom seeds[8]. In addition, the difference in chemical composition between *A. angustifolium* and *A. korarima* indicate that the two species are indeed distinct.

ACKNOWLEDGEMENTS

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Note

Sephadex LH-20 chromatography of extracts of marine sediment and biological samples for the isolation of polynuclear aromatic hydrocarbons

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Determination of hydrocarbons in environmental samples customarily requires at least three basic steps: (a) extraction of the components from the sample matrix, (b) isolation of compounds of interest from naturally occurring interfering compounds by chromatography of the extract, and (c) analysis of the isolated extract to detect and measure the components[1–4]. To permit trace-level detection of hydrocarbons in sample extracts by current highly sensitive gas chromatographic (GC) techniques, extremely efficient isolation procedures are needed[5,6].

Several procedures for separating hydrocarbons from interfering compounds have been reported. Gel permeation–adsorption chromatography, using Sephadex LH-20 with a low-boiling alcohol or solvent, has been utilized to separate polynuclear aromatic hydrocarbons (PAHs) from lipids and pigments[7,8]. Silica gel chromatography[9] has been used to isolate aromatic hydrocarbons in marine substrates, but this procedure alone provided inadequate sample clean-up for analysis by current GC techniques. Giger and Blumer[10] reported a two-step procedure, using silica gel–alumina chromatography followed by Sephadex LH-20 chromatography (using benzene–methanol, 1:1), to improve the isolation of PAHs from soil and sediment extracts. However, recent data concerning the carcinogenicity of benzene[11,12] make substitution of a safer solvent system desirable.

We report a two-step procedure that combines silica gel chromatography and Sephadex LH-20 chromatography using safer, contaminant-free solvent systems to isolate PAHs from extracts of sediments and tissues from the marine environment.

EXPERIMENTAL**

Preparation of solvents

Commercial, distilled-in-glass solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.; J. T. Baker, Phillipsburg, NJ, U.S.A.) were purified prior to column

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** Reference to a company or a product does not imply endorsement by the U.S. Department of Commerce to the exclusion of others that may be suitable.

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chromatography by redistillation in the azeotropic ratios in which they were to be used: cyclohexane–methanol (3:2) and cyclohexane–isopropanol (2:1). The distillation of azeotropic mixtures resulted in a higher degree of solvent purity than was possible by distillation of the individual solvents. Cyclohexane and methanol are not miscible at room temperature, therefore a small portion of dichloromethane was added to dissolve both solvents into a single phase, resulting in a mixture of cyclohexane–methanol–dichloromethane (6:4:3). In addition, the dichloromethane increases the solubility of sample extract components in the solvent mixture.

Extraction and hydrocarbon fractionation

Sediment samples were extracted with methanol, then with dichloromethane–methanol[7,13]. Biological samples were digested with caustic solution (4 *N* NaOH), then extracted with diethyl ether[4]. The extracts were filtered through a short column packed with granular copper and silica gel to remove elemental sulfur and many interfering polar compounds, including some lipids. The filtrate was concentrated and hexane was added; then the filtrate was concentrated again and transferred to another silica gel column for separation into saturated hydrocarbon fractions[7,13].

Rechromatography of unsaturated fraction

Sephadex LH-20 gel (25–100 μm/size; Sigma, St. Louis, MO, U.S.A.) was prepared for column chromatography by swelling *ca.* 20 g overnight in the elution solvent: I, cyclohexane–methanol–dichloromethane (6:4:3), or II, cyclohexane–isopropanol (2:1). The gel was then poured into 300 × 19 mm I.D. columns. These columns were calibrated by eluting a mixture of azulene and perylene of sufficient concentration to be visible under UV light. If there was a distinct separation between azulene and perylene, the columns were further characterized by eluting a sample extract spiked with PAH standard. PAHs eluted from the gel in the 40–100-ml fraction using solvent I and in the 40–140-ml fraction with solvent II. The unsaturated fraction from the silica gel separation of the sample extracts was then rechromatographed on each of the Sephadex LH-20 columns to isolate the PAHs. Eluates were concentrated to *ca.* 1 ml and the solvents exchanged with hexane.

Gas chromatography and mass spectrometry

Portions (2 μl) of the unsaturated hydrocarbon and PAH fractions were each injected, splitless, into a Hewlett-Parkard 5840A gas chromatograph equipped with a flame-ionization detector and fused-silica capillary column coated with SE-54 (30 m × 0.25 mm; J & W Scientific, Orangevale, CA, U.S.A.). Helium carrier was adjusted to a linear velocity of *ca.* 28 cm/sec at 150°C; split ratio was 20:1. Injections were made at 50°C, and column temperature was programmed from 50–280°C at 4°/min. GC–mass spectrometry (MS) was performed using an identical GC system interfaced with a Finnigan 3200 mass spectrometer used with an Incos 2300 data system.

RESULTS

Following calibration of the Sephadex LH-20 columns using solvent I or II, recoveries of the added standards were determined (Table I). In addition, the extract from a highly contaminated environmental sample was analyzed by GC–MS before

TABLE I

RECOVERY OF PAHs FROM COLUMN CHROMATOGRAPHY ON SEPHADEX LH-20

PAH	*Amount added (μg)*	*Solvent I*		*Solvent II*	
		Amount recovered (μg)	*Recovery (%)*	*Amount recovered (μg)*	*Recovery (%)*
Naphthalene	5.45	5.07	93	5.12	94
2-Methylnaphthalene	5.00	4.60	92	4.59	92
Biphenyl	5.75	5.23	91	5.25	91
Phenanthrene	6.10	5.98	98	5.67	93
1-Methylphenanthrene	5.20	5.01	97	5.04	97
Fluoranthene	5.85	5.56	95	5.55	95
Chrysene	7.50	7.48	100	7.41	99
Benzo[*e*]pyrene	5.40	5.24	97	4.72	87
			$\bar{x}$ = 95		$\bar{x}$ = 93.5

and after Sephadex LH-20 column chromatography (solvent I). PAH percent recoveries for the environmental sample, determined by comparison of concentrations before and after Sephadex, were similar to those for the added standards. Elution volumes of several PAHs on a Sephadex LH-20 column using solvent I are given in Fig. 1.

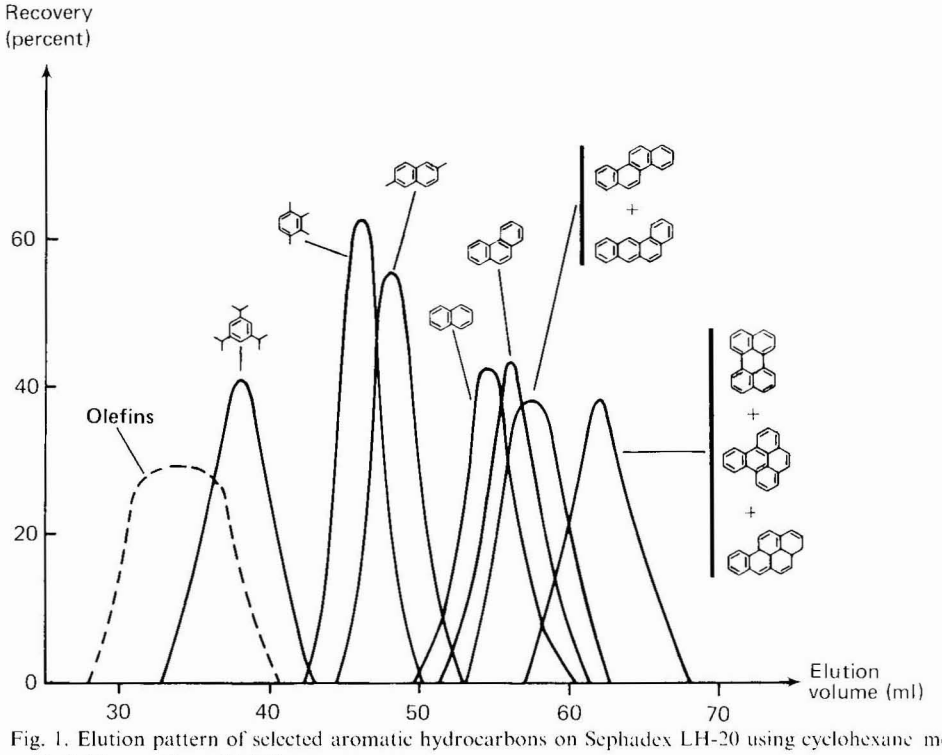

Fig. 1. Elution pattern of selected aromatic hydrocarbons on Sephadex LH-20 using cyclohexane–methanol–dichloromethane (6:4:3).

GC analysis of the unsaturated hydrocarbon fraction of both a sediment extract (Fig. 2a) and a crab hepatopancreas extract (Fig. 3a) following silica gel chromatography revealed a large number of compounds that coelute with PAHs and interfere with their determination by GC. These compounds were shown by GC–MS analysis to be large polyunsaturated aliphatic hydrocarbons. The interfering compounds were removed by chromatography of this fraction on a Sephadex LH-20 column, as shown in Figs. 2b and 3b.

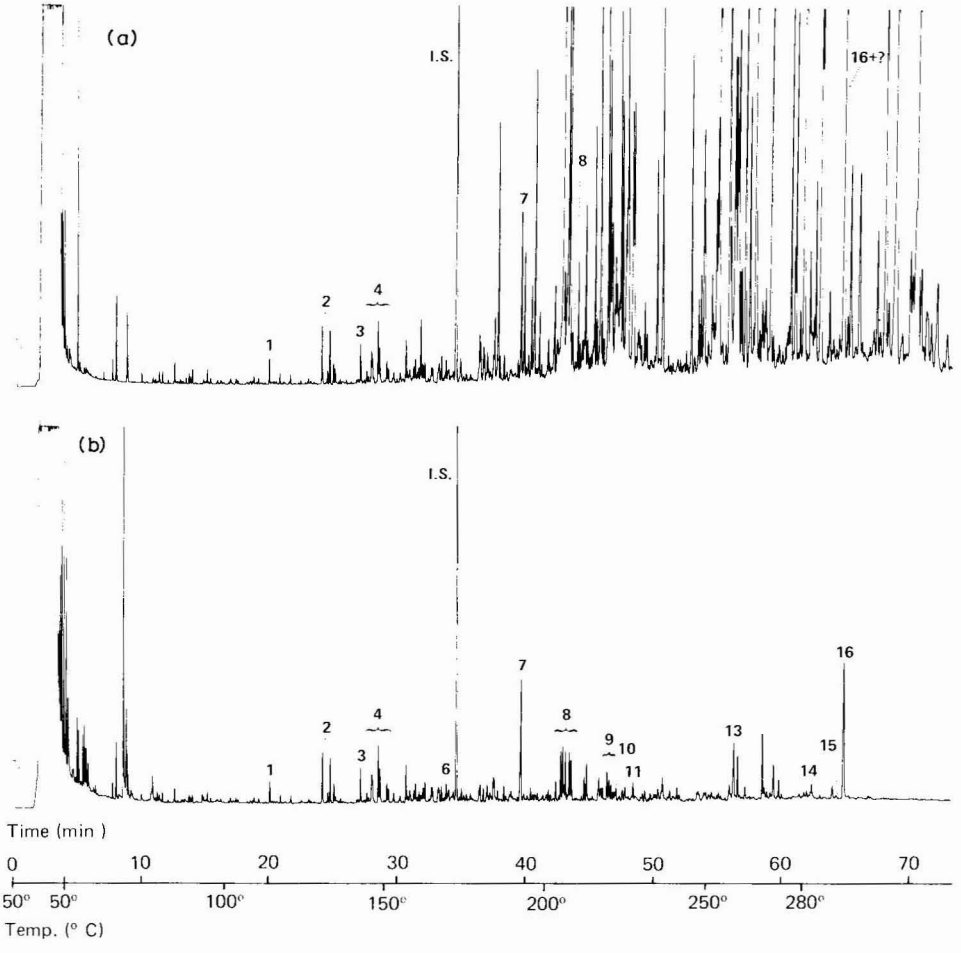

Fig. 2. Gas chromatogram of an intertidal sediment extract following chromatographic clean-up with (a) silica gel, and (b) silica gel, followed by Sephadex LH-20 using cyclohexane–methanol–dichloromethane (6:4:3). See text for GC parameters. Peak identities were verified by GC–MS. Labelled peaks are: I.S. = internal standard; 1 = naphthalene; 2 = methylnaphthalenes; 3 = biphenyl; 4 = dimethylnaphthalenes; 6 = fluorene; 7 = phenanthrene; 8 = methylphenanthrenes; 9 = dimethylphenanthrenes; 10 = fluoranthene; 11 = pyrene; 13 = chrysene; 14 = benzo[*e*]pyrene; 15 = benzo[*a*]pyrene; 16 = perylene.

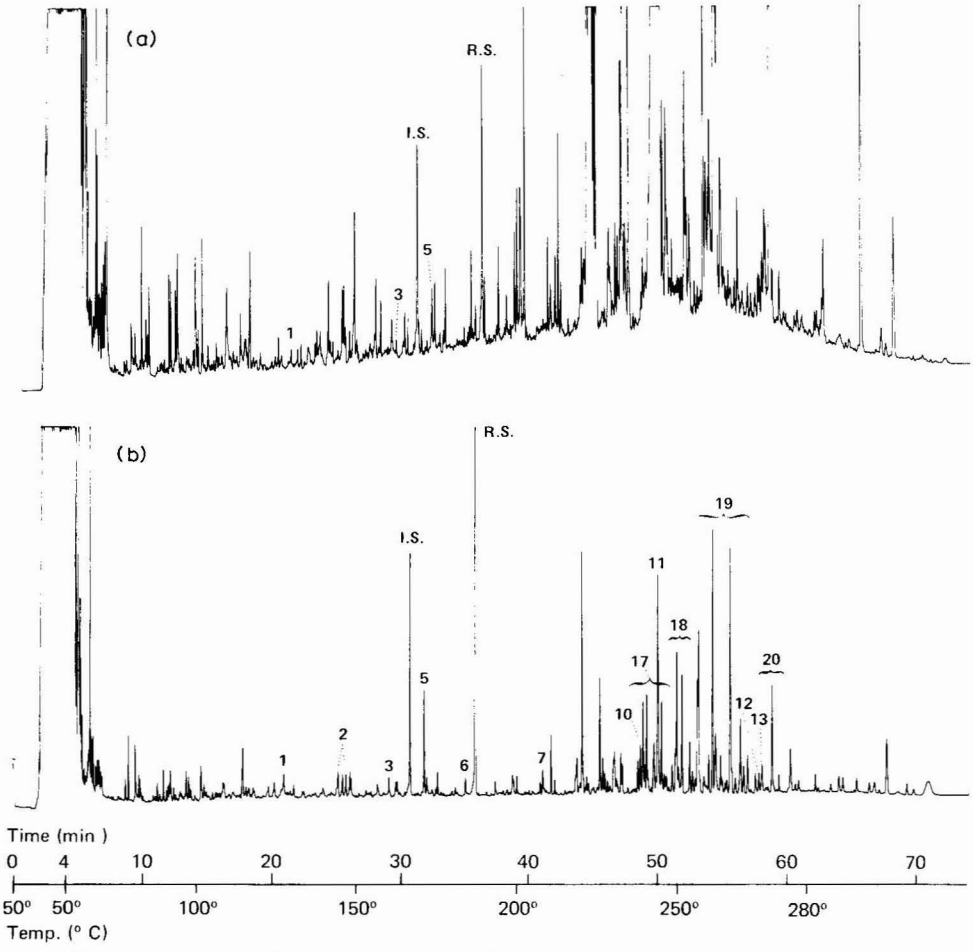

Fig. 3. Gas chromatogram of a crab (*Cancer gracilis*) hepatopancreas extract. See Fig. 2 for analytical conditions. Additional labelled peaks are: R.S. = recovery standard; 5 = acenaphthene; 12 = benz[*a*]anthracene; 17 = Cl_4-polychlorinated biphenyls (PCBs); 18 = Cl_5-PCBs; 19 = Cl_6- and Cl_7-PCBs; 20 = Cl_8-PCB.

DISCUSSION

Previous isolation methods have either failed to separate adequately the PAH fraction from interfering substances or have used benzene which is both carcinogenic and difficult to purify. Our two-step procedure replaced benzene with contaminant-free azeotropic solvent mixtures while achieving the desired isolation of PAHs. A comparison of the chromatograms of a sediment extract (Fig. 2a) and a crab hepatopancreas extract (Fig. 3a) taken before Sephadex LH-20 chromatography with those taken after clean-up (Figs. 2b and 3b) shows the virtually complete removal of interfering compounds from the aromatic hydrocarbon fraction. In addition, this method gave high PAH recoveries (Table I) and eliminated the need for MS techniques to

separate target compounds from coeluting substances before quantitations could be made.

The cut-off point between the olefinic fraction and the PAH fraction was fairly critical with solvent I, as shown in Fig. 1. Although certain alkylated benzenes (*e.g.*, triisopropylbenzene) may not totally separate from the olefins, most PAHs were, however, isolated from interfering compounds. Solvent II separated PAHs from interfering compounds better than solvent I (*e.g.*, no elution overlap between olefins and triisopropylbenzene), so that the fraction end-point was not as critical. However, solvent II eluted PAHs only half as fast as solvent I under the same pressure, and 50% more solvent was required. Also, the boiling point for solvent II is 15°C higher than that for solvent I, increasing the possibility of evaporative losses or oxidative changes of components during concentration steps. Solvent I was preferred for routine analyses because of its speed of elution and low boiling point.

In summary, our two-step isolation procedure separated PAHs in marine samples from interfering compounds with good recoveries of target compounds. The relatively simple gas chromatograms of PAH fractions isolated by this procedure allowed direct quantitation. Finally, to improve the safety of the procedure, the carcinogenic solvent, benzene, was replaced with safer, contaminant-free solvent systems.

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Note

Separation and determination of thiamine-binding proteins in rats by high-performance liquid chromatography

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Ample evidence has accumulated to indicate that thiamine has a specific electrophysiological function in nervous tissues independent of its coenzyme function[1–5]. From several studies[4,6,7], we assume that thiamine-binding proteins play an important rôle in the former function. However, properties and actions of thiamine-binding proteins in animals are quite obscure because of their complexity.

Recently, high-speed gel filtration using high-performance liquid chromatography (HPLC) has been developed[8], and we have explored a new analytical method for the separation of thiamine-binding proteins that can be applicable for studies of thiamine-binding proteins in animals.

EXPERIMENTAL

Apparatus

The system consists of a LC-3A pump for liquid chromatography, a SIL-1A injector, a TSK-Gel G-3000 SW column, a SPD-2A UV detector, a PRR-2A proportioning pump, a RF-500 LCA spectrofluorophotometer and a strip chart recorder. The TSK-Gel column was purchased from Toyo Soda (Tokyo, Japan), and all other equipment was purchased from Shimadzu (Kyoto, Japan).

Preparation of samples

Male Wistar rats were killed and tissues removed. The tissues were homogenized with nine volumes of 0.1 *M* sodium acetate (pH 7.5) containing 1% Triton X-100 and centrifuged at 44,000 *g* for 60 min. The supernatant was used as the sample.

Procedures

The HPLC system is schematically shown in Fig. 1. A 50–100-μl aliquot of sample was injected onto the column, after the zero time being marked, using 0.1 *M* sodium acetate (pH 7.5) as mobile phase at a flow-rate of 0.5 ml/min. The absorbance at 280 nm was monitored continuously with a UV detector. A solution containing 0.01% potassium hexacyanoferrate(III) and 15% sodium hydroxide was applied and mixed with the column eluate at a flow-rate of 0.5 ml/min with a proportioning pump to convert thiamine-binding proteins into fluorophores. The fluorescence was measured using a 12-μl flow cell with a spectrofluorimeter (excitation wavelength, 375 nm; emission maximum, 450 nm) and recorded graphically.

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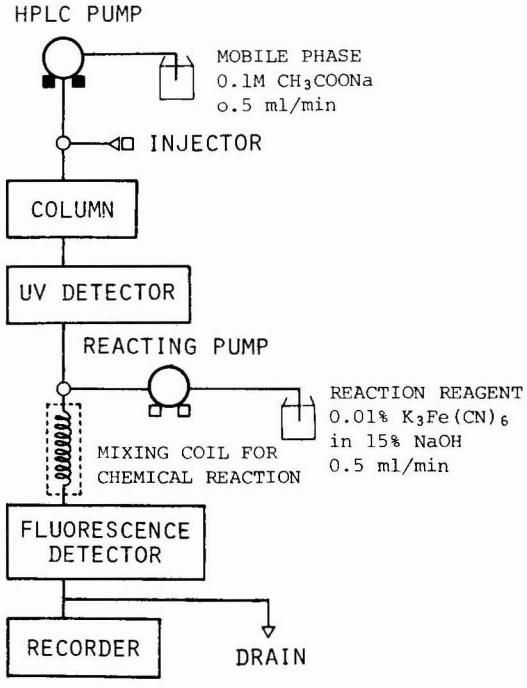

Fig. 1. Schematic diagram of the HPLC system.

Determination of thiamine and transketolase activity by conventional methods

Thiamine was determined by the thiochrome method of Fujiwara and Matsui[9]. Transketolase (E.C. 2.7.1.1) activity was assayed by the method described by Itokawa[10].

RESULTS AND DISCUSSION

Fig. 2 shows separation patterns of thiamine-binding proteins from rat brain. Nine peaks with UV absorption and three peaks with fluorescence were observed. A blank study was performed by adding a solution without hexacyanoferrate(III), and a small peak at the position of the first fluorescent peak (indicated by the broken line) was observed. When the free form of thiamine or each thiamine phosphate ester was applied on this system, the substance yielded was found in a fluorescent peak at the position corresponding to the third fluorescent peak of rat brain proteins.

Thiamine concentration and transketolase activity of pooled fractions of each peak were determined. Thiamine content in 50 μl of brain sample was 5.78, 6.00 and 6.09 ng in the first, second and third peak, respectively. Transketolase activity was detected only in the second fluorescent peak.

Elution patterns of thiamine-binding proteins from sciatic nerve are shown in Fig. 3. Similar to the case of brain proteins, three fluorescent peaks were observed. The height of the first peak increased and those of the second and third peaks decreased as compared with the pattern of brain proteins. As the distribution of

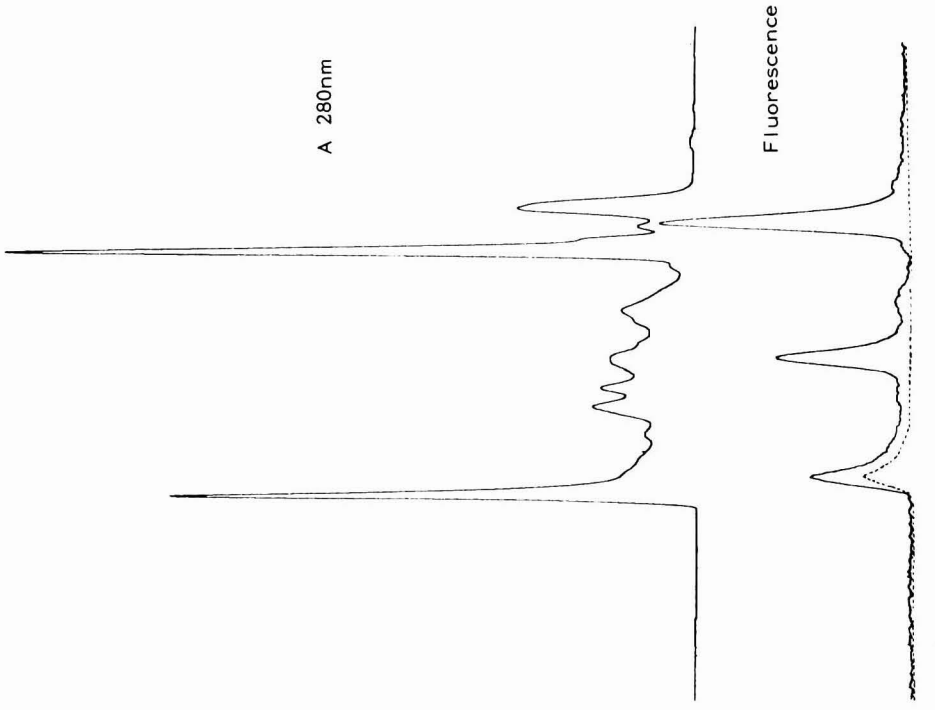

Fig. 2. Elution profiles of thiamine-binding proteins in rat brain.

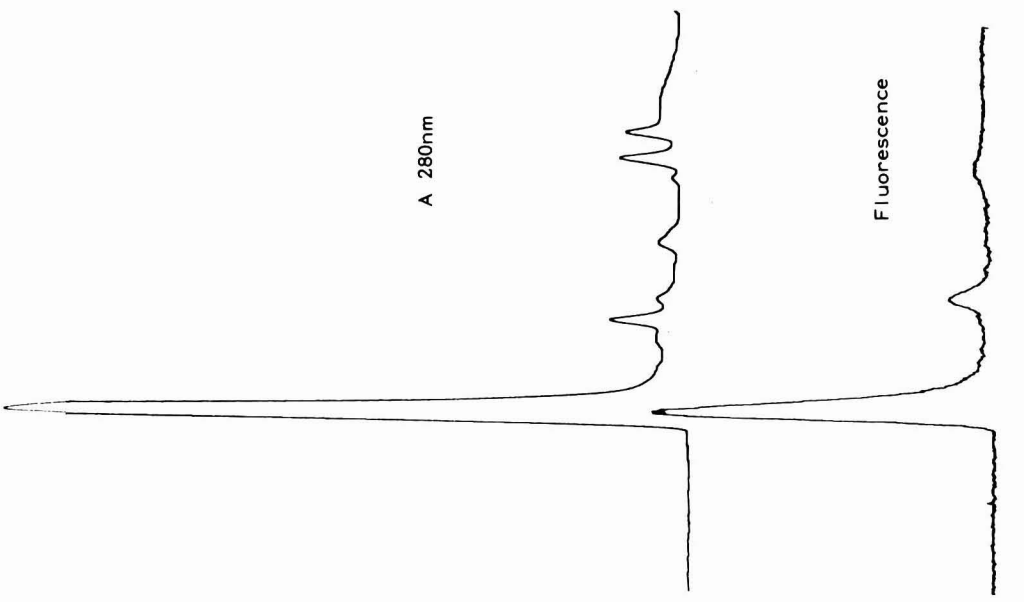

Fig. 3. Elution profiles of thiamine-binding proteins in rat sciatic nerve.

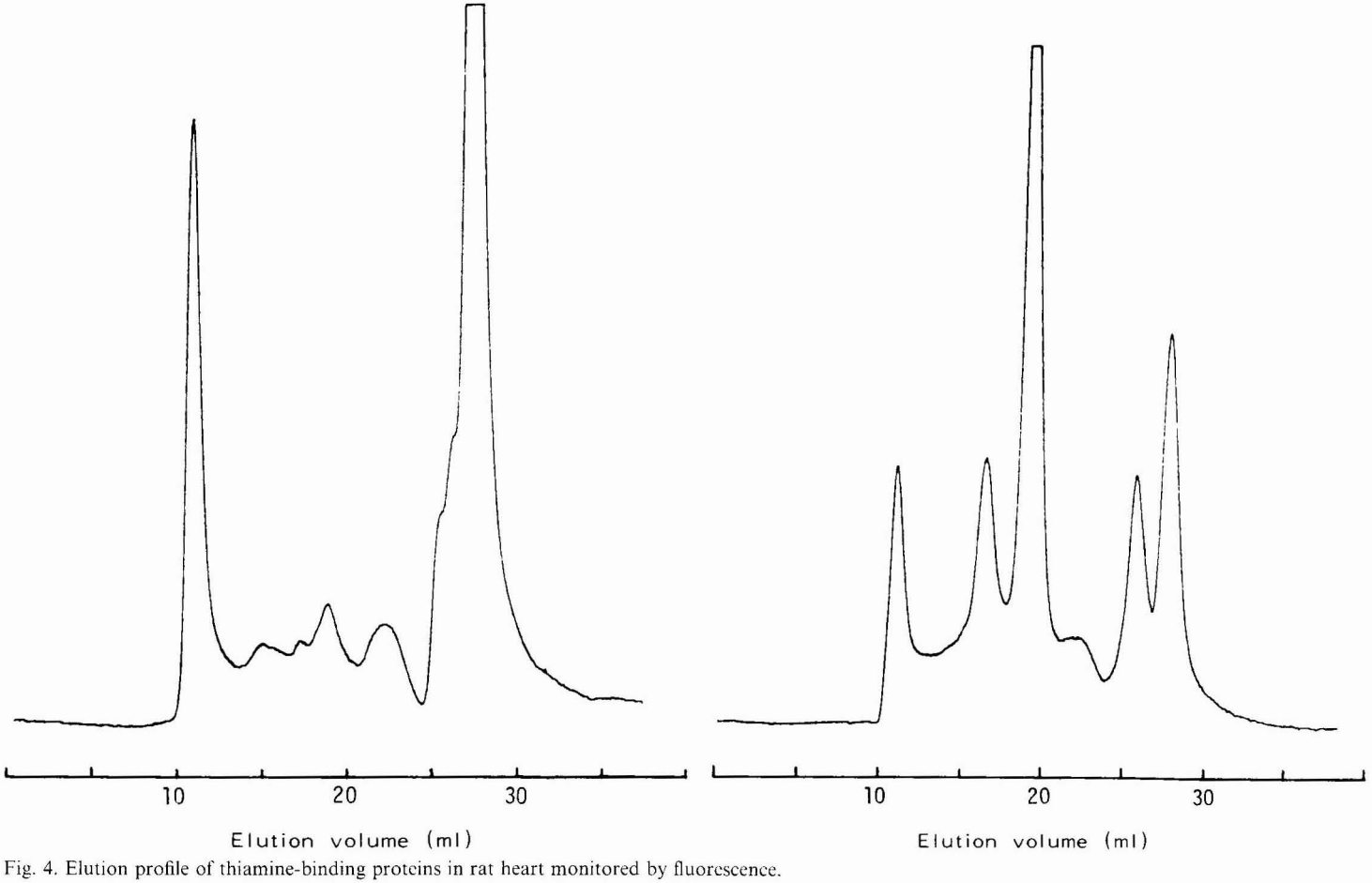

Fig. 4. Elution profile of thiamine-binding proteins in rat heart monitored by fluorescence.

Fig. 5. Elution profile of thiamine-binding proteins in rat liver monitored by fluorescence.

thiamine-binding proteins in membrane structure seemed to be higher in sciatic nerve rather than in brain, we presumed that the first fluorescent peak included thiamine-binding proteins in membranes.

The elution profiles of thiamine-binding proteins of heart and liver monitored by fluorescence are shown in Figs. 4 and 5, respectively. In both cases, six fluorescent peaks were observed. The third and highest peak of liver contains transketolase activity.

From these elution patterns of samples from various tissues, we can assume that the first peak (high-molecular-weight substances) contains thiamine-binding proteins in membrane structure, the middle peak includes transketolase and the last peak (low-molecular-weight material) corresponds to free forms of thiamine phosphate esters.

Although this study is at the preliminary stage at present, HPLC seems to be one of the most useful tools to identify the various thiamine-binding proteins in animal tissues since this method is sensitive and rapid.

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Note

Separation of pinitol and some other cyclitols by high-performance liquid chromatography

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Pinitol (1D-3-O-chiro-inositol) was recognized to be a major cyclitol in foliage and stems of many legume species only a few years ago[1], although it has been known to exist at low levels in certain other plants. Generally, separation of pinitol and various other cyclitols from plants has been a difficult problem. Separation of inositols and polyhydric alcohols has been attempted by partition chromatography with ion-exchange resins[2–4], and by thin-layer chromatography. Some examples of the later method include two-dimensional chromatography of carbohydrates on silica gel impregnated with boric acid[5], sodium borate[6], by 1-butanol–aqueous boric acid mixtures[7], and by use of lead(II) on silica gel[8]. More recently, gas–liquid chromatography (GLC) has been successfully utilized for separation and quantitation of these compounds. Phillips and Smith[9] reviewed this technique comprehensively and evolved a faster method for separation of pinitol from plant extracts. During the past few years high-performance liquid chromatography (HPLC) has been used for the separation of sugars in various foods and food products[10–13]. Data on the use of this technique for separation of plant cyclitols is limited although separation of some polyhydric alcohols in fruits[14] and of phytic acid in rice bran[15] has been reported.

Research on the metabolism of pinitol required a fast and accurate method to isolate and purify radioactively labelled pinitol from legume plant extracts treated with [^{14}C]myo-inositol. This was of importance because radioactively labelled pinitol was not available from commercial sources. The more widely used GLC methods, although very precise, were useless since these methods required cyclitol derivatization prior to chromatography, making the cyclitols unavailable for further metabolic studies. This report describes an HPLC method developed in our laboratory for separation, purification and concentration of [^{14}C]pinitol from soybean plant extracts.

MATERIALS AND METHODS

Apparatus

The analysis was carried out using a Micromeritics (Norcross, GA, U.S.A.) solvent delivery system (Model 750) connected to a universal syringe loading sample injector (Model 730, equipped with a 100-μl loop) and a refractive index (RI) detector

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(Model 771) set at $0.05 \cdot 10^{-3}$ RI unit sensitivity. Chromatograms were recorded on a Heath Schlumberger (Benton Harbor, MI, U.S.A.) recorder (2100 series, 10 mV, full scale range) set at a chart speed of 0.4 cm/min. The chromatographic column was a pre-packed silica-bonded stainless-steel column (Microsil) purchased from Micromeritics. A 5 cm × 0.46 cm stainless-steel precolumn, packed with 10-μm silica gel (Whatman, Clifton, NJ, U.S.A.) using a column packer (Model 705, Micromeritics) was connected in series to protect the main column. The void volume of the system was about 5.8 ml.

Reagents

The following reagents were used: (a) HPLC-grade acetonitrile (Fisher Scientific, Fairlawn, NJ, U.S.A.); (b) water, glass redistilled and degassed for 5 min; (c) myo-inositol was purchased from Sigma (St. Louis, MO, U.S.A.). Pinitol and chiro-inositol were prepared in our laboratory and the purity was checked by gas chromatography–mass spectrometry. Sequoyitol was kindly supplied by Dr. Laurens Anderson of the Department of Biochemistry, University of Wisconsin, Madison, WI, U.S.A. [^{14}C]Myo-inositol (specific activity 278 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

Standard solutions were prepared by dissolving 50 mg of each compound in 5 ml of the solvent except sequoyitol which, due to a limited quantity, was used at a lower concentration (15 mg/5 ml).

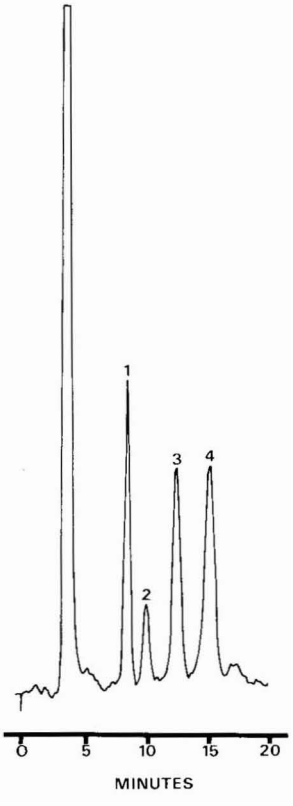
Liquid chromatography

The mobile phase employed acetonitrile–water (78:22). The solvent was degassed under vacuum for 5 min and filtered through a 0.22-μm Millipore filter. The column was equilibrated for about 45 min with the mobile phase. A 25-μl aliquot of each standard solution or mixture was chromatographed at a flow-rate of 2 ml/min. The identity of standard peaks was checked by GLC according to the method of Phillips and Smith[9].

RESULTS AND DISCUSSION

Fig. 1 shows the separation of various cyclitol standards using a mobile phase of acetonitrile–water (78:22) at a flow-rate of 2 ml/min. The elution times of pinitol, sequoyitol, chiro-inositol and myo-inositol were 8 min 38 sec, 10 min 8 sec, 12 min 45 sec, and 15 min 23 sec, respectively. Each peak was collected and identified by GLC[9]. This system provided a rapid and baseline resolution of all cyclitols. Increasing the acetonitrile concentration in the solvent beyond 78% reduced the retention time but the peak widths were increased while decreasing concentration of acetonitrile or the flow-rate delayed the elution time unnecessarily.

Fig. 2 shows the chromatographic profile of the [^{14}C]myo-inositol-treated soybean plant extract which had been cleared of the sugars by running through a set of ion-exchange columns before injecting on the HPLC column. There was a very good separation between [^{14}C]pinitol (peak 2) and myo-inositol (peak 6); consequently, it was quite easy to collect the radioactively labelled pure pinitol from the rest of the intermediates of cyclitol metabolism. These samples contained small quantities of methanol, which eluted immediately after the solvent front, resulting in a



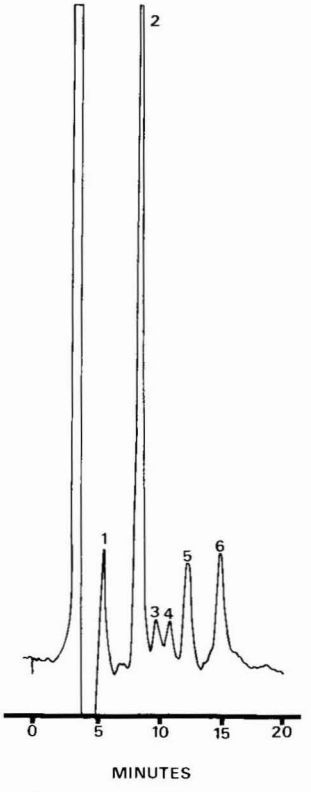


Fig. 1. Chromatogram of cyclitol standards [concentration 10 $\mu g/\mu l$ except for sequoyitol (3 $\mu g/\mu l$)] on a Microsil column at room temperature, with mobile phase acetonitrile water (78:22) at a flow-rate of 2 ml/min, chart speed 0.4 cm/min. Peaks: 1 = pinitol; 2 = sequoyitol; 3 = chiro-inositol; 4 = myo-inositol.

Fig. 2. Separation of pinitol and related cyclitols from soybean plant extract which had been run through the ion-exchange columns. Mobile phase acetonitrile water (78:22) at a flow-rate of 2 ml/min, chart speed 0.4 cm/min. Peaks: 1 = unknown; 2 = pinitol; 3 = sequoyitol; 4 = unknown; 5 = chiro-inositol; 6 = myo-inositol.

negative peak. Peaks 1 and 4 are currently unidentified, although it should be possible to collect and identify these peaks by co-chromatography with known standards or by gas chromatography–mass spectrometry.

A problem encountered during these analyses was that glucose, if present in the plant extract, eluted with sequoyitol since these compounds had the same capacity ratio. Therefore it is absolutely necessary to remove sugars before sequoyitol determinations could be accomplished.

No attempt was made to quantify the amount of each cyclitol present in these samples. However, it is possible to quantify the compounds by comparing the peak height of the sample to the corresponding linear standard curves constructed by running different concentrations of the standard solutions through the system. The proposed HPLC method is relatively rapid and is quite useful for the separation and

quantitation of pinitol and other cyclitols in situations where these compounds have to be saved for further biochemical work.

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Note

Antioxidants and stabilizers

LXXXVI*. Use of chromatography in the investigation of the mechanism of action of amine antidegradants

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Rubbers are protected against atmospheric ageing mainly by additives of the 1,4-phenylenediamine (1,4-PD) type, which possess antioxidant and/or antiozonant properties. In order to understand the mechanism of their action, one ought to have a knowledge of changes in the composition of the additives during ageing and the properties of the reaction products. The identification and determination of the original amines and especially of their transformation products directly in the polymers or in extracts of aged polymers are very difficult. The analysis of rubbers, which contain, in addition to amine stabilizers, other processing and vulcanizing additives, is particularly difficult. Basically, these compounds impede the total analysis of mixtures in rubbers, and thus the obtaining of data needed for the determination of the mechanism of action of amines under specific ageing conditions. Chromatographic methods are to be preferred to other methods for the solution of these problems, which are important from both theoretical and technical points of view[1,2].

One of these problems consisted in developing analytical methods for amine additives and their transformation products under model conditions. We investigated the thermal oxidation, photo-oxidation and ozonization of low-molecular-weight liquid hydrocarbons[3] and examined possibilities for the determination of changes in the composition of the additives during these processes by using thin-layer (TLC) and liquid chromatographic (LC) methods.

EXPERIMENTAL

Chemicals

The following compounds were used for the stabilization of hydrocarbons: N,N′-diphenyl-1,4-phenylenediamine (DPPD) (Altofane DIP; S.A. Française des Matières Colorantes, St. Denis, France), N-isopropyl.N′-phenyl-1,4-phenylenediamine (IPPD) (Santoflex IP; Monsanto, St. Louis, MO, U.S.A.) and N-cyclohexyl-N′-phenyl-1,4-phenylenediamine (CHPPD) (ASM 4010; Bayer, Leverkusen, G.F.R.).

* Part LXXXV: ref. 3.

0021-9673/81/0000–0000/$02.50

Pure compounds were obtained by repeated crystallization of commercial samples. For the 1,4-benzoquinonediimines (BQDI), the symbols denoting substitution in the N,N′-positions are the same as those used for derivatives of 1,4-PD; they were prepared by the oxidation of diamines with potassium hexacyanoferrate(III)[4] or silver oxide[5].

Methods

Solutions of purified tetralin, cyclohexene, squalane and squalene in chlorobenzene and pure oxygen were used in the oxidation experiments. Thermal oxidation was initiated with azobisisobutyronitrile (AIBN) at 60 °C, and photo-oxidation was examined at 35 °C with irradiation with light of wavelength 300–400 nm. A chlorobenzene solution of squalene was used at 25 °C for ozonization with oxygen–ozone mixtures with a rate of ozone generation of 1 g/h. Atmospheric ageing of diamines and BQDI was carried out in chlorobenzene solution and in the solid phase at 25 °C. Samples for analyses were taken during the oxidation processes.

Chromatographic analyses

TLC was carried out using Silufol UV 254 silica gel sheets (Kavalier, Votice, Czechoslovakia) and detection with hexacyanoferrate(III) reagent, acidic potassium permanganate solution and a UV lamp. The elution systems were S_1 = benzene–acetone–26 % aqueous ammonia solution (95:5:0.1) and S_2 = light petroleum–diethyl ether–triethylamine (50:60:0.1).

LC was carried out using an LC Chrom 50 chromatograph (Laboratory Instruments, Prague, Czechoslovakia) with a 300 × 4 mm I.D. glass column packed with SG-10 silica gel (Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) at a flow-rate of 33–64 ml/h and a pressure of 1–1.5 MPa; detection was effected with a differential flow UV analyser at 254 nm. The elution systems were mixtures of isopropanol and *n*-hexane in various proportions containing 0.1 % of triethylamine.

RESULTS AND DISCUSSION

The results showed that the individual derivatives of 1,4-PD and BQDI could be adequately determined by using either TLC or LC (Table I). BQDI derivatives prepared independently as standards by the oxidation of 1,4-PD form in solution a

TABLE I

R_F VALUES OF DERIVATIVES OF 1,4-PD AND BQDI IN SYSTEMS S_1, S_2

Compound	R_F	
	S_1	S_2
N-Isopropyl-N′-phenyl-1,4-phenylenediamine (IPPD)	0.47	0.47
N-Isopropyl-N′-phenyl-1,4-benzoquinonediimine (IPBQDI)	0.15 + 0.30	0.28 + 0.45
N,N′-Diphenyl-1,4-phenylenediamine (DPPD)	0.51	0.49
N,N′-Diphenyl-1,4-benzoquinonediimine (DPBQDI)	0.22 + 0.31	0.38 + 0.46
N-Cyclohexyl-N′-phenyl-1,4-phenylenediamine (CHPPD)	0.54	0.50
N-Cyclohexyl-N′-phenyl-1,4-benzoquinonediimine (CHPBQDI)	0.18 + 0.29	0.30 + 0.37

mixture of *syn*- and *anti*-stereoisomers[6]; in LC, they form two peaks with similar elution times (Fig. 1), and in TLC they yield two spots with similar R_F values. This finding complicates the investigation of transformations of 1,4-PD in rubber extracts.

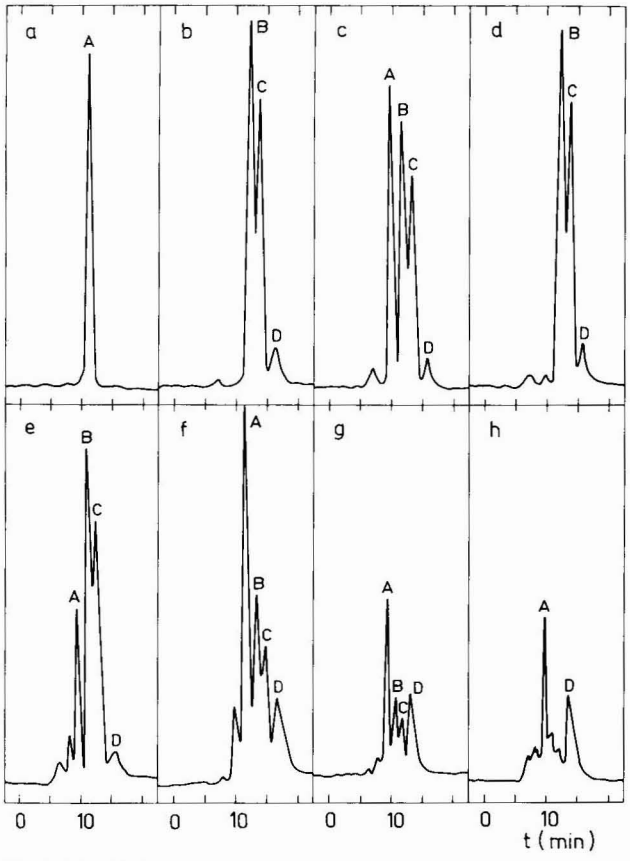

Fig. 1. Liquid chromatograms of transformation products of IPBQDI after long-term ageing. Eluent, 15% isopropanol in *n*-hexane + 0.1% triethylamine; UV detection (254 nm). Peaks: A, IPPD; B, C, isomers of IPBQDI; D, acetone. (a) Recrystallized IPPD; (b) recrystallized IPBQDI; (c) freshly prepared mixture of IPPD + IPBQDI. Ageing of IPBQDI: (d) 3 days; (e) 14 days; (f) 60 days; (g) 151 days; (h) 203 days.

In the long-term atmospheric ageing (1 year) of IPPD, DPPD and CHPPD in solution, chemical changes occurred in which a mixture of several compounds with lower V_e or R_F values than those of the initial amine and a small amount of the corresponding BQDI were formed. In the solid state, 1,4-PD derivatives are more stable. A perceptible change takes place only in crystalline IPPD (change in colour; a small spot at the start appears in TLC). On the other hand, CHPPD and DPPD were stable. Interesting data were provided by an investigation of the long-term ageing of pure BQDI. DPBQDI was very stable; no visible changes occurred during the investigation. CHPBQDI changed very slowly to a corresponding amine (which appeared in the mixture after 80–100 days). IPBQDI was transformed very quickly into IPPD and a mixture of other amine compounds with a lower V_e or R_F values (Fig. 1).

After 342 days, IPBQDI completely disappeared from the reaction mixture. With respect to the mechanism of action of these additives, this result suggests an important role played by the character of the N,N-disubstitution of 1,4-PD. Data on the composition of the mixture of products thus obtained cannot be generalized for the various derivatives of 1,4-PD because of the different reactivities of the derivatives of BQDI, which are an important primary transformation product of 1,4-PD.

Reaction mixtures were analysed in detail during the oxidation of tetralin, cyclohexene, squalane and squalene stabilized with derivatives of 1,4-PD. LC demonstrated in all instances that during the induction period the content of 1,4-PD gradually decreased, while the content of derivatives of BQDI increased. During the induction period (determined kinetically by oxygen absorption measurements) and in the oxidation stages after the induction period the reaction mixture contained only derivatives of BQDI in all instances, the original amine having disappeared. The chromatograms in Fig. 2 provide an example: they represent the composition of products after the oxidation of squalene. On the one hand, this result confirms the transformation of 1,4-PD as a consequence of the role played by its chain-breaking properties during the autoxidation, and on the other, it is one of the pieces of evidence for retardation of the oxidation stages after the induction period.

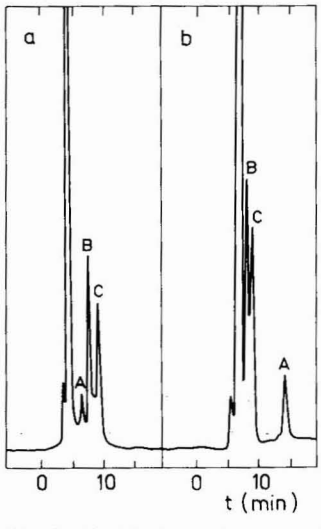

Fig. 2. Liquid chromatograms of the reaction mixture in the induction period range in the inhibited oxidation of squalane. Eluent, 4 % isopropanol in *n*-hexane + 0.1 % triethylamine; UV detection. Content of 1,4-PD derivatives: (a) $5 \cdot 10^{-3}$ mol/l IPPD; (b) $5 \cdot 10^{-3}$ mol/l DPPD. Peaks as in Fig. 1.

The investigation of changes in the derivatives of 1,4-PD during the photooxidation of various hydrocarbons revealed differences in the rate of formation of BQDI arising in systems during the induction period. No other transformation products of amines could be detected. The chromatograms were more complicated, because the transformation products of the substrate were present in much higher numbers and amounts than in the thermal oxidation.

The results of analyses of the samples after ozonization differed considerably from those obtained in earlier investigations, where oxygen or $RO_2^{\cdot}$ radicals and

hydroperoxides derived from the hydrocarbon substrate were the oxidizing agents. Ozonation yielded a varied mixture of products both from the substrate and from the additives. According to chromatograms and colour tests, the original amines disappeared and no corresponding BQDI derivatives were formed. The V_e or R_F values did not correspond to any of the model products of the oxidation transformations of derivatives of 1,4-PD prepared so far and having the structures of substituted amino derivatives of 1,4-PD, BQDI, benzoquinonemonoimine or phenazine[7]. The presence of 8–10 compounds was observed on the chromatograms of ozonized amines in an inert solvent. Ozonation of mixtures of squalene with amines gave rise to even more complicated mixtures. This finding of differences in the reaction products between diamines and ozone or squalene ozonide arising in the ozonization of the mixture indicates the complicated character of the chemical transformations that ought to be considered in rubbers (squalene is a model of polyisoprene). At the same time, it was found that chromatograms of freshly ozonized samples and those of older samples differ from each other, which causes difficulties in the interpretation of data.

The results obtained in this study demonstrate the character of primary transformations that an amine stabilizer undergoes under defined conditions of oxidation ageing of hydrocarbons. Derivatives of BQDI, which are the cause of the retarded oxidation of hydrocarbons, are formed in the thermal or photo-initiated oxidation[3]. The absence of BQDI from the mixture arising after the ozonolysis of hydrocarbons indicates a completely different mechanism of the process. The methods used have also proved to be useful in extensive studies of the mechanism of the stabilizing action of diamines that are in progress and also involve syntheses and identifications of unknown transformation products.

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Note

Detection of benzidines on thin-layer chromatograms with fluorescamine

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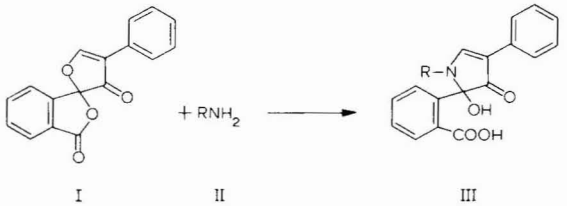
In general, the qualitative and quantitative gas chromatographic (GC) analysis of amines as free bases at low concentrations is limited by adsorption and decomposition in the column and tailed peaks. In order to overcome these limitations, the amino groups and other functional groups if present in the molecule have been masked by different types of derivative formation reactions prior to GC analysis. The derivatives used include acetyl[1–3], trimethylsilyl[4], enamines[3,5], trimethylsilylenamines[6], trimethylsilylheptafluorobutyryl[7], trifluoroacetyl[8], pentafluoropropionyl[9], heptafluorobutyryl[10], *p*-tosylamides[11], and isothiocyanates[12,13]. Amino acids have been determined by GC as alkyl chloroformates with derivatizing agent[14,15]. Alkyl chloroformate reacts readily with amino, imino, phenolic, hydroxyl, sulphydryl, and imidazolic NH groups in aqueous alkaline media at room temperature to provide corresponding compounds with N-, O-, and S-substituted alkyloxycarbonyl groups. A recent report in this journal based upon this chemistry for the determination of specific phenolic amines has been reported[16].

Another approach to the measurement of primary amines is the formation of a fluorescent derivative. The reaction of fluorescamine with a primary amine is shown in Fig. 1. Fluorescamine (I) reacts with primary amines (II) to form intensely fluorescent substances (III), providing the basis for a rapid and highly sensitive assay for compounds containing a primary amine group, such as amino acids, primary amines, peptides, and proteins[17]. Application of fluorescamine as a rapid spot test for solid dosage exhibits in forensic toxicological analysis has been reported[18]. The fluorescamine test only yields a bright aquamarine (blue-green) fluorescent product with primary amines; thus, this test makes a clear cut distinction between amphetamine



I II III

Fig. 1. The reaction between fluorescamine (I) and a primary amine (II) yielding a highly fluorescent derivative (III).

and methamphetamine. Previous common spot tests yielded the same results with these two amines. Fluorescamine is 100 times more sensitive in detecting amphetamine extracted from urine on thin-layer chromatograms than ninhydrin[18,19].

Reported here is a sensitive thin-layer chromatographic (TLC) spray development based on fluorescamine for benzidine (4,4′-diaminobiphenyl) and 3,3′-dichlorobenzidine. These compounds are members of the Environmental Protection Agency (EPA) priority pollutant list. These compounds present variability and difficulty in performing their measurement with direct analysis by GC–mass spectrometry (MS).

EXPERIMENTAL

Reagents

Fluorescamine TLC spray, prepared commercially in 1,2-dichloroethylene, was purchased from Whatman, Clifton, NJ, U.S.A., Cat. No. 4911-109. Precoated TLC plates (LKGF Linear-K), 5 × 20 cm glass plates coated with a 250-μm thickness of silica gel, were also purchased from Whatman.

Known amounts of benzidine and 3,3′-dichlorobenzidine were spotted on TLC chromatograms using a Drummond (Broomall, PA, U.S.A.) 0–10-μl Microdispenser, Cat. No. 210.

Standard solutions were purchased from Supelco, Bellefonte, PA, U.S.A., "Standards for EPA Consent Decree Water Protocol", Cat. No. 4-8808.

All mobile phase solvents were purchased from Burdick & Jackson Labs., Muskegon, MI, U.S.A.

Fluorescence examinations were performed in a Chromato-Vue® box (manufactured by Ultra-Violet Products, San Gabriel, CA, U.S.A.) using the long wavelength (366 nm) for excitation.

Procedure

Appropriate amounts of authentic compounds were spotted on precoated silica gel plates 2 cm from the bottom of the plate and developed in hexane–methyl *tert.*-butyl ether (50:50) until the solvent front migrated 18–19 cm up the plate. Chromatogram development time was approximately 40 min. After being air dried, the plate was placed in a fume hood and sprayed with the fluorescamine solution (note the recommended safety precautions of avoiding skin contact and breathing) and viewed for fluorescence. The compounds appeared as yellowish spots within minutes after spraying.

RESULTS AND DISCUSSION

The mobile phase hexane–methyl *tert.*-butyl ether (50:50) resolved 3,3′-dichlorobenzidine from benzidine. R_F values were 0.57 for 3,3′-dichlorobenzidine and 0.30 for the slower migrating benzidine. Recently, methyl *tert.*-butyl ether has been reported to provide more resolving power than diethyl ether in TLC and high-performance liquid chromatographic systems[20]. Also, this higher boiling ether (boiling point 55–56 °C compared to 34–35 °C for diethyl ether) has been reported to have a relatively small tendency to form dangerous peroxides[21].

By analyzing different amounts of benzidine and 3,3′-dichlorobenzidine, it was

observed that 50 ng can be easily detected using this technique. The colors of the fluorescent products were different for the two condensation products. 3,3′-Dichlorobenzidine was the usual aquamarine fluorescence, but benzidine yielded a butter yellow fluorescent product. Possibly, the reaction product with benzidine contains two molecules of fluorescamine. Previous experience has shown that aquamarine fluorescent products were formed with a wide variety of low-molecular-weight primary amines[18].

Spiking 50 ng/μl of each into complex industrial effluent extracts revealed no interference in their detection by TLC. Base-neutral methylene chloride extracts were prepared according to recommended EPA protocol[23].

Application of this screening technique to industrial effluents requires fluorescent examinations before spraying the developed chromatogram with fluorescamine to avoid misinterpreting results. Many organic compounds contain endogenous fluorescence. Accordingly, it is important to examine the developed plate for both short-wavelength (254 nm) and long-wavelength (366 nm) spots in order to detect any interferences with the fluorescamine spray development for benzidines. It is convenient to circle the short-wavelength spots and draw a dashed line around long-wavelength fluorescent spots with a sharp pencil. When screening an industrial effluent, it is important to analyze a standard of the benzidines on the same TLC plate as a control. Also, it is recommended that the industrial extract be analyzed in duplicate. To one of the duplicate spots, a known amount of benzidines, same as control, should be overlayed as a standard addition before development to aid the analyst in the interpretation of results obtained. The control functions to document the performance of the overall procedure. The standard addition functions to demonstrate detection of the target compounds in a complex matrix and monitor any chromatographic development abnormalities due to the industrial extract.

Recently, fluorescamine has been described as an aid to detect primary polycyclic aromatic amines (PPAAs) in synthetic crudes such as shale oil and coal liquids[24]. The health and environmental effects of these petroleum substitutes are being extensively investigated to determine whether the use of these supplemental energy sources presents significant hazards. At least three classes of organic compounds have been isolated from synthetic crude oils which possess mutagenic activity: polycyclic aromatic hydrocarbons (PAHs), aza-arenes, and PPAAs. PPAAs appear to be present at relatively low concentrations, but the potency of this class of mutagenic compounds is much greater than that of PAHs. These authors pointed out that a successful analytical procedure must be capable of handling: (a) the trace concentrations of PPAA; (b) the complexity of the sample matrix; and (c) the potentially high molecular weights and polar nature of the individual components.

The procedure described in this paper provides a sensitive, quick, and inexpensive method to detect benzidine and 3,3′-dichlorobenzidine. Experience in our laboratory analyzing standard commercial solutions of these two compounds by GC–MS, without derivatization, as recommended by the EPA[23] has demonstrated highly variable detection limits for these two primary amines. It is the opinion of this author that the organic environmental chemist has not used TLC and spot tests as frequently as other scientific areas such as clinical, toxicological, or forensic science. Fluorescamine is a reagent which should be utilized for TLC and spot tests in environmental measurements of primary amines.

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Note

Electrofocusing of stroma-free haemoglobin and its derivatives in agarose isoelectric focusing gels

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In a previous paper[1] we showed that isoelectric focusing of haemoglobin subfractions can be achieved in thin-layer gels of purified agarose C (Pharmacia, Uppsala, Sweden); however, the reproducibility of the results was not as satisfactory as with polyacrylamide gels[2] because of electroendosmosis. Rosén *et al.*[3] used gels of a new variant of agarose EF (LKB, Bromma, Sweden)[4] with extremely low electroendosmosis for the reproducible electrofocusing of various proteins in an Ampholine (LKB) pH gradient. Several advantages of electrofocusing in agarose EF gels were pointed out.

In this paper we describe the electrofocusing of haemoglobin and its derivatives in agarose IEF (Pharmacia)[5] gels and Pharmalyte (Pharmacia) pH gradient.

EXPERIMENTAL

Stroma-free haemoglobin (SFH) was a standard sample stored at −20°C for 1 year. Crude haemolysate of fresh human erythrocytes (either treated or untreated with carbon monoxide) was prepared by the addition of 4 volumes of distilled water to 1 volume of packed erythrocytes washed five times with sodium chloride solution (9 g/l)[2]. The reaction of oxyhaemoglobin (50 g/l) with a solution of pyridoxal-5-phosphate (Roche, Basle, Switzerland) was performed at 10°C for 30 min at a molar ratio of 1:1 and pH 7.4. The reaction of deoxyhaemoglobin (50 g/l) with glutaraldehyde was performed under a flow of nitrogen at 10°C for 60 min at a molar ratio of 1:5 and pH 7.1.

Prior to electrofocusing all haemoglobin samples were desalted on a 9 × 0.9 cm column of Sephadex G-25 Superfine in distilled water.

Isoelectric focusing in agarose IEF was performed with a Pharmacia FBE 3000 flat-bed apparatus and an ECPS 3000/150 constant-power supply following the procedure recommended by the manufacturer[5]. Pharmalyte was used to form a gradient of pH 5–8.

Procedure[5]

To prepare agarose gel, a mixture of 0.3 g of agarose IEF, 3.6 g of sorbitol and

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28 ml of distilled water was heated in a boiling-water bath until the agarose was dissolved (90°C). Then 1.9 ml of Pharmalyte (pH 5–8) were added and the mixture poured on a specially hydrophilized plastic film (Celbond) placed on a levelling table, pre-heated to 60–70°C. The gel was left to cool for 15 min on the levelling table and then transferred to a closed plastic box with moist paper, where it was stored overnight at room temperature.

Porous electrode strips were dipped in electrode solutions (anode solution, 0.05 *M* sulphuric acid; cathode solution, 1 *M* sodium hydroxide solution) and placed on a filter-paper for 1 min to remove excess of liquid. Volumes of 20 μl of desalted samples (haemoglobin concentration in the range 10–30 g/l) were applied on small pieces of filter-paper and laid on the surface of the gel. The constant-power supply was set to deliver a maximum of 15 W and 1500 V. The experiment was run for 90 min; after 45 min the run was interrupted and the sample applicators were removed. After the separation was completed the gel was immediately put into the fixing solution (5% sulphosalicylic acid + 10% trichloroacetic acid in distilled water) and left there for 30 min. Then the gel was washed twice for 15 min with the destaining solution (35% ethanol + 10% acetic acid in distilled water), dried using three pieces of filter-paper and a hair dryer, stained 10 min in the staining solution (0.2% Coomassie Blue G-250), destained for about 5 min and finally dried with a hair dryer.

RESULTS AND DISCUSSION

Fig. 1 shows that thin-layer electrofocusing in agarose IEF gel makes it possible to detect easily even slight differences between the patterns of variously treated human haemoglobin samples. There is a general similarity between the results and their reproducibility achieved in agarose IEF gel and in polyacrylamide gel[2]; however, work with agarose is easier and less hazardous[1,3,4]. Moreover, concentrated

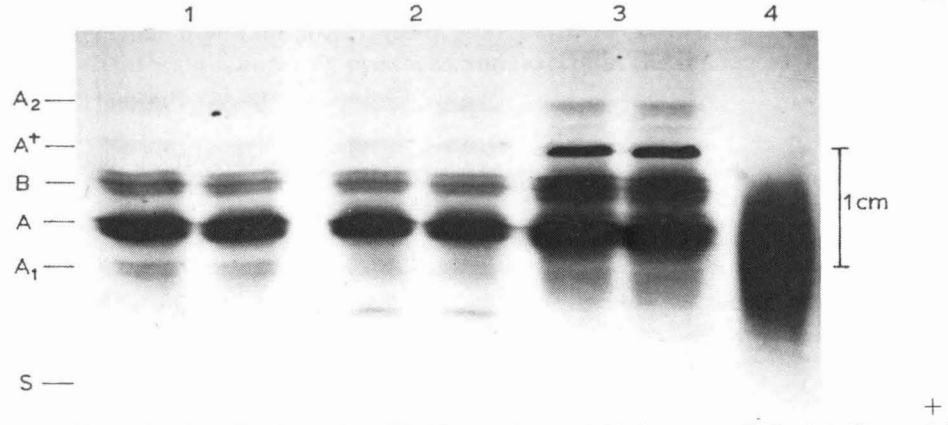

Fig. 1. Electrofocusing of native and modified human haemoglobin in agarose IEF gel. 1, Haemoglobin from fresh lysed erythrocytes; 2, as 1, treated with pyridoxal-5-phosphate; 3 = stroma-free haemoglobin (SFH) stored at −20°C for 1 year; 4 = SFH treated with glutaraldehyde. Ampholyte: Pharmalyte (pH 5–8). s = Position of the start. All samples were treated with carbon monoxide before electrofocusing; staining with Coomassie Blue G-250. Capital letters indicate the positions of main haemoglobin subfractions[1,2].

"overloaded" protein zones (haemoglobin A) seem to be more stable in agarose gels during fixation and staining than in polyacrylamide gels[2]. The pattern of native fresh haemoglobin consisted of 10–12 subfractions as usual[1,2]. After treatment with pyridoxal-5-phosphate a new distinct zone appeared in a more acidic region, due to the reaction of free amino groups of haemoglobin with the aldehydic group and to the induction of the phosphate group. In contrast to sample 1, the zones of the A_1 haemoglobin subfractions became diffuse. Samples 1 and 2 also formed about ten zones of non-haemoglobin proteins, mostly in the region of about pH 5–6.5. Stroma-free haemoglobin (sample 3) stored for 1 year at $-20°C$ formed distinct zones of A_2, of methaemoglobin A and A_2 and of carbonanhydrase, and also some faint unidentified zones. Non-haemoglobin protein fractions in the pH region of about 5–6 were significantly less intense than in samples 1 and 2 owing to the extensive purification of SFH. Reaction of haemoglobin with glutaraldehyde caused a marked change in the pattern characterized by the disappearance of all individual zones[2].

ACKNOWLEDGEMENTS

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Book Review

Ullmanns Encyklopädie der technischen Chemie, Band 5, Analysen- und Messverfahren, edited by H. Kelker, Verlag Chemie, Weinheim, 1980, XVI + 1010 pp., price DM 670.00, ISBN 3-527-20005-3.

This volume contains 35 chapters on analytical techniques, including the chapters reviewed here, on "Basic concepts in chromatography" (26 pp.), "Gas chromatography" (32 pp.), "Liquid chromatography" (34 pp.), "Thin-layer chromatography" (34 pp.) and "Electrophoresis" (28 pp.). Thus there are 154 pages devoted to chromatography, as much as in many short introductions to the subject. It would have been a good opportunity to write concise texts summarizing in a clear form the work of the last 30 years or so. Instead, the text is too often turgid and confusing, as if the authors are trying to steamroller the reader with science: "this is too complicated to understand" is the general trend of many sections.

Under the heading "What is chromatography", the reader is offered 19 lines instead of the very simple definition given years ago by A. J. P. Martin. The paragraph "explaining" isotachophoresis is written in such a way that a reader can not possibly work out its rationale. There is a section on continuous electrophoresis in which something went wrong altogether. First the method is credited to Fawcett (1973) and then previous work in 1949 is mentioned? But why describe continuous electrophoresis at all in such a short chapter? It has never gained popularity and there are various reasons for this (and they are not stated)! On the same page there is a schematic drawing of a Tiselius apparatus, which is fine if you know the technique but unintelligible if you do not.

The book is superbly produced and, except in the references, there are hardly any typographical errors.

Lausanne (Switzerland) M. JANOVSKY

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Book Review

Methods of plasma protein fractionation, edited by J. M. Curling, Academic Press, London, New York, Toronto, Sydney, San Francisco, 1980, XIV + 326 pp., price £ 23.20 (Great Britain), US $ 53.50, ISBN 0-12-199550-X.

A team of 35 outstanding specialists has managed to compile and classify in 326 pages an enormous bulk of information summarizing the present knowledge of the production of plasma protein fractions for therapy and prophylaxis. The thematical content of the book is divided into five methodologically different sections: (1) Fractionation by precipitation; (2) Fractionation by ion-exchange chromatography; (3) Fractionation by gel filtration and affinity chromatography; (4) Removal of solvent and salts from plasma fractions; (5) In-process and product filtration.

The practical and methodological aspects of plasma fractionation are emphasized throughout; however, sufficient space in each chapter is devoted to theory. A reader (whether a biochemist or technologist) will find detailed instructions and flowsheets on each individual technique of protein fractionation and purification. The authors also describe and discuss the limitations and possible drawbacks of a given technology and they indicate new possibilities and trends for future development and amelioration of plasma fractionation. There is a lot of material and ideas in each chapter to inspire further research and innovations in this field. The clear and concise style and the many figures, tables, schemes and photographs reflect the professional experience of the authors and their ability to formulate and present technological problems in an attractive manner.

This excellent book will be greatly appreciated by plasma fractionators and by all having interest in this field. It will surely serve as a manual for technologists and researchers alike. For a chromatographer, this publication offers further proof that chromatography continues to find new fields of application.

Prague (Czechoslovakia) T. I. PŘISTOUPIL

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