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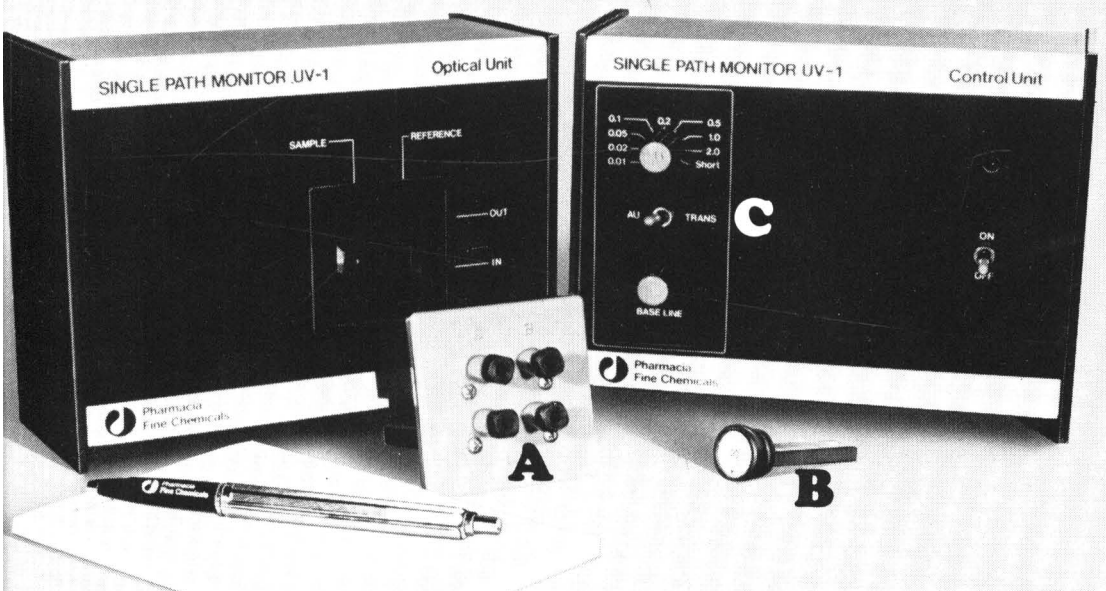

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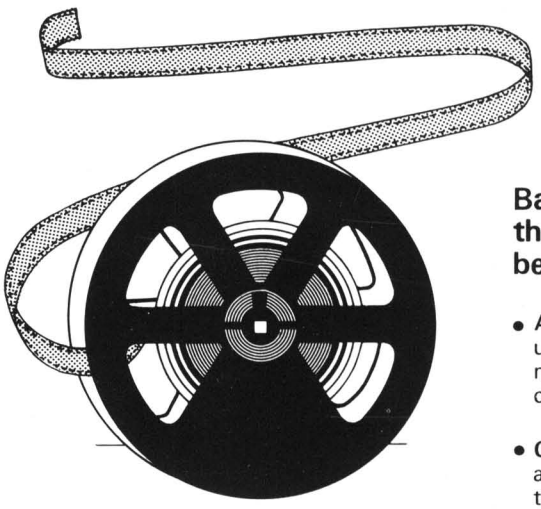

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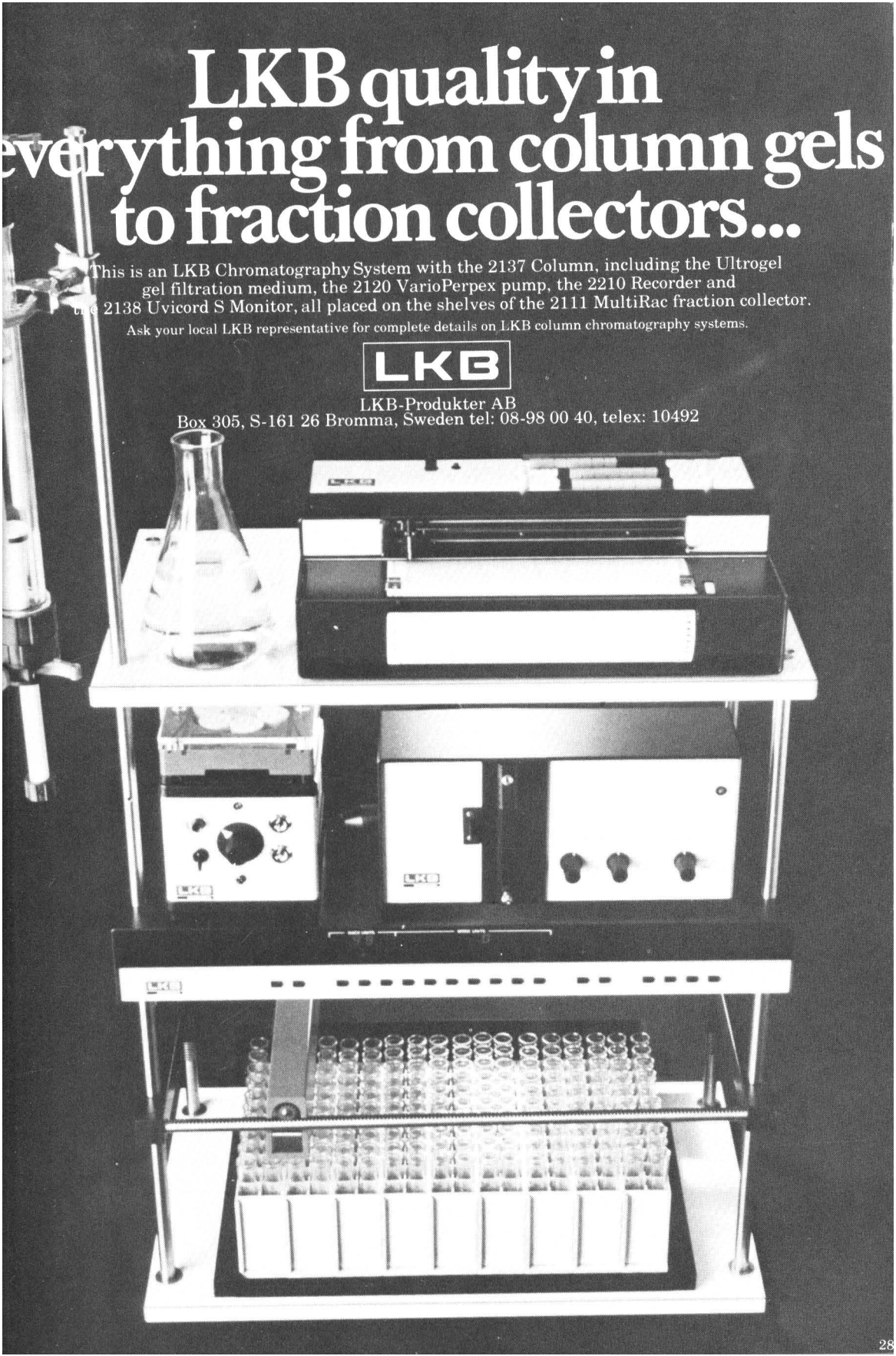
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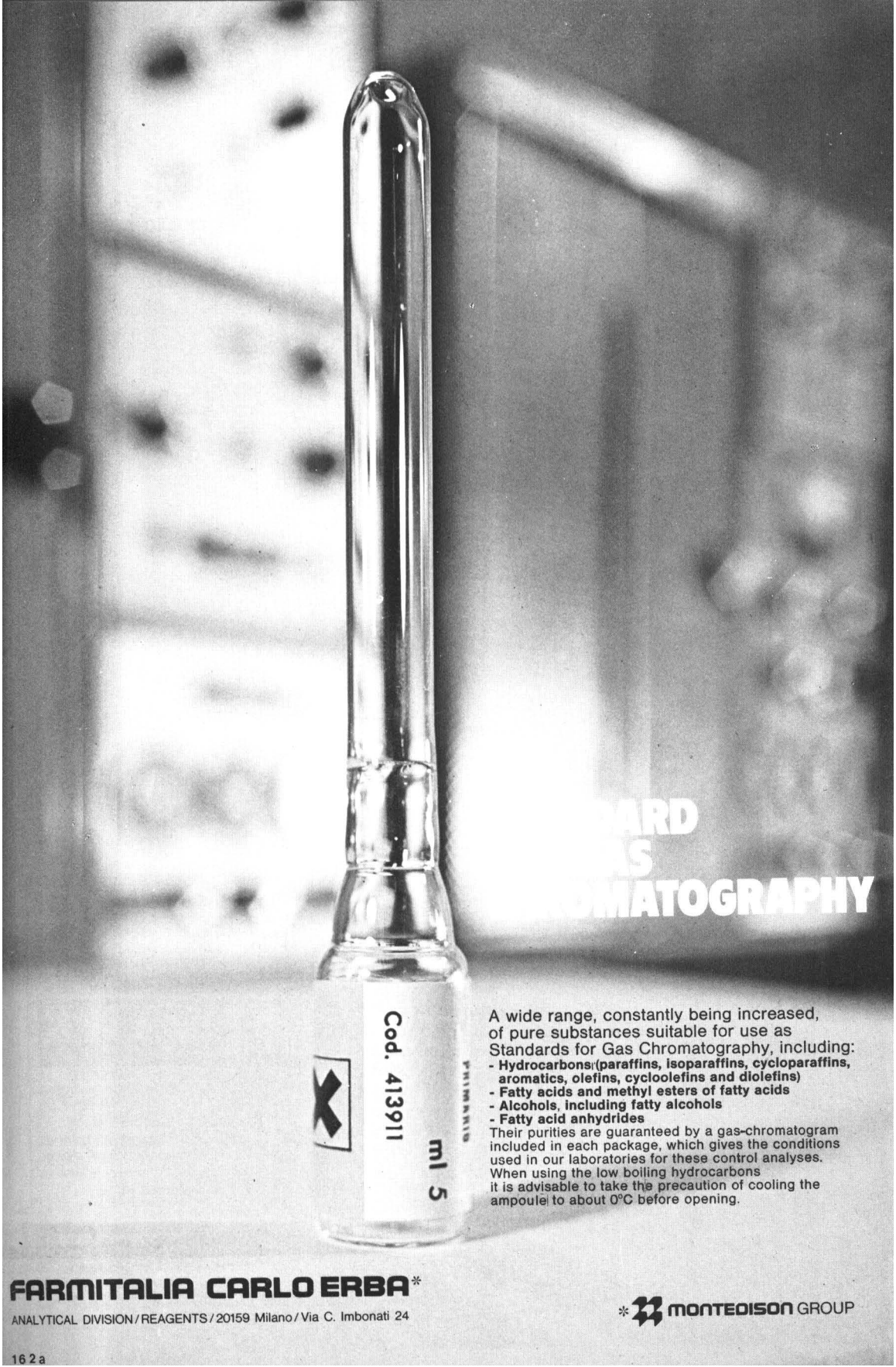

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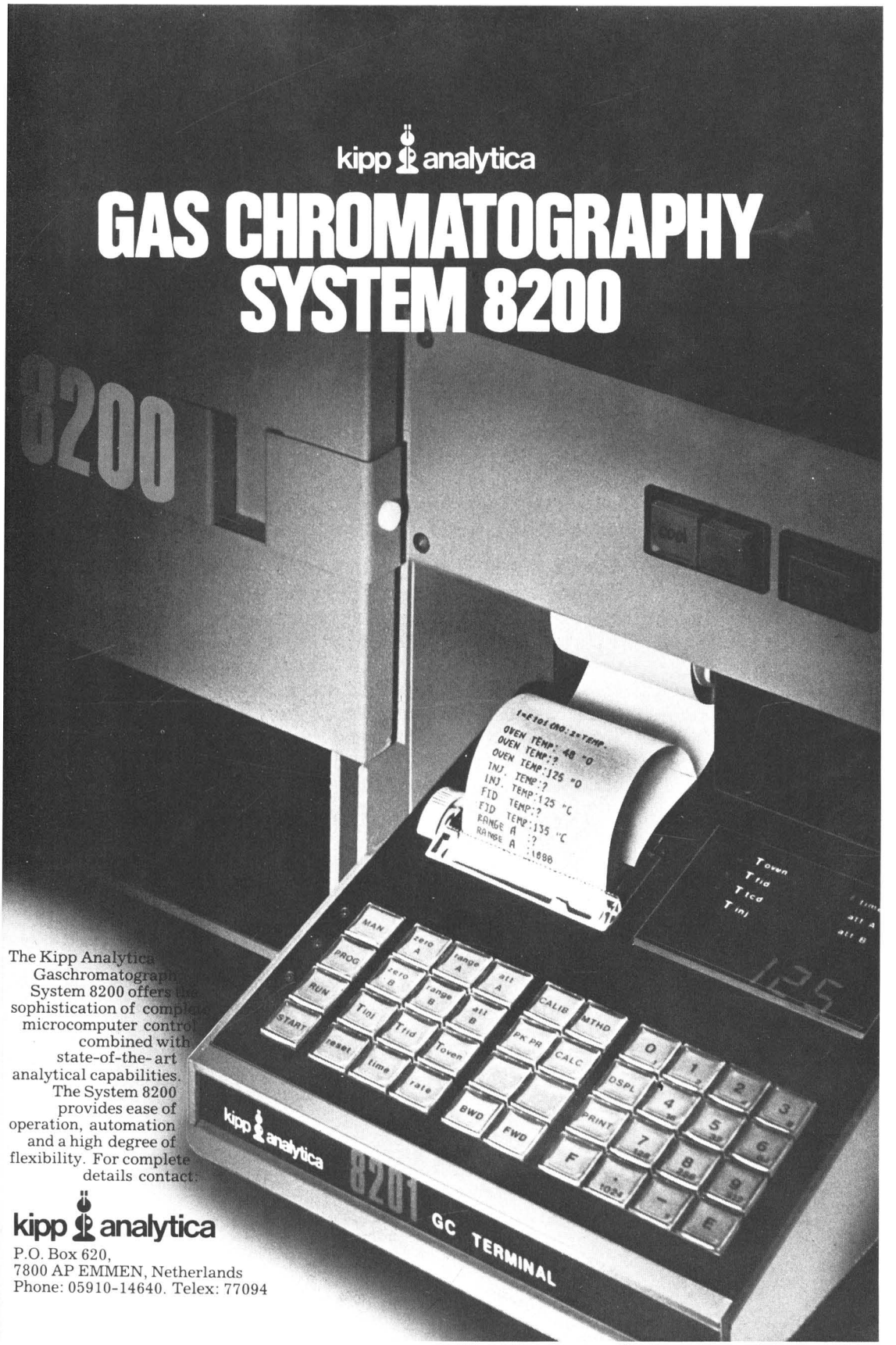

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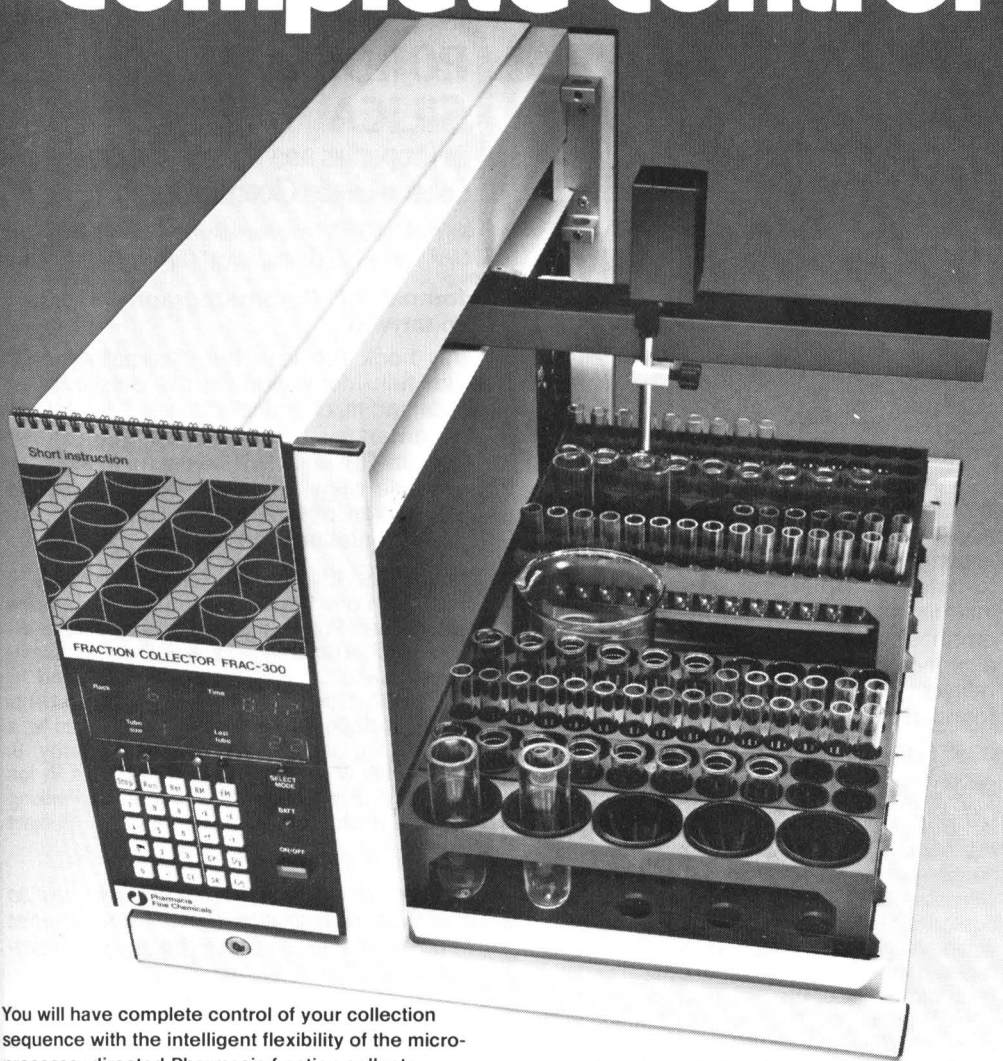

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMNS OF SMALL DIAMETER

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Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, Liaoning (China)
(First received September 26th, 1979; revised manuscript received April 29th, 1980)

SUMMARY

By using a specially designed column system, wall effects can be minimized. An approximately linear relationship between retention volume (V_R) and peak width ($2\Delta V_{1/2}$) (in volume units) for various solutes on columns with different diameters was obtained:

$$2\Delta V_{1/2} = a' + b'V_R$$

Systematic experiments on the influence of the dead volume of the detector system on the values of a' and b' were carried out, which indicated that there is a large influence on a' but little on b'. By using the improved column system and home-made UV detector, a column of I.D. 2 mm and length 10 cm with an efficiency of more than 6000 theoretical plates was obtained.

INTRODUCTION

Currently used commercially available high-performance chromatographic columns with efficiencies of more than 4000 theoretical plates and packed with microparticulate silica have an I.D. of at least 4 mm and a length of 10 cm[1–3]. It has been found that the column efficiency decreases with decrease in column diameter[4–7]. The concept of the "infinite diameter effect" was proposed to explain these results[8,9]. However, small inner diameters with the same column efficiency will require less solvent to achieve the same separation, as the flow-rate of the eluent is proportional to the square of the column diameter. Improvements in the injection system can minimize the wall effect. By using a home-made, specially designed injection system we have carried out a systematic investigation of the influence of column diameter on efficiency. A linear relationship between retention volume and peak width (in volume units) for different solutes for columns with different diameters was obtained. A systematic investigation of the influence of extra-column effects produced by connectors and detectors on the two constants of the above-mentioned linear relationship was carried out and indicated a great influence of the volume of the detector system

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on the column efficiency. By using a home-made UV detector we obtained a column of I.D. 2 mm and length 10 cm with an efficiency of more than 6000 theoretical plates[10].

THEORETICAL

HETP is preferably used to evaluate the column efficiency. Generally the plate equation is very complicated. For example, the equation given by Horváth and Lin[12] for liquid chromatography is

$$H = H_{\text{disp}} + H_{e.\text{diff}} + H_{i.\text{diff}} + H_{\text{kin}} \tag{1}$$

where

$$H_{\text{disp.}} = \frac{2\gamma D_m}{u_e} + \frac{2\lambda d_p u_e^{1/3}}{u_e^{1/3} + \omega(D_m/d_p)^{1/3}} \tag{2}$$

$$H_{e.\text{diff}} = \frac{\kappa(k_0 + k' + k_0k')^2\, d_p^{5/3} u_e^{2/3}}{(1 + k_0)^2\,(1 + k')^2\, D_m^{2/3}} \tag{3}$$

$$H_{i.\text{diff}} = \frac{\theta(k_0 + k' + k_0k')^2\, d_p^2 u_e}{30 D_m k_0\,(1 + k_0)^2\,(1 + k')^2} \tag{4}$$

$$H_{\text{kin}} = \frac{2k' u_e}{(1 + k_0)\,(1 + k')^2\, k_d} \tag{5}$$

and H is the conventional plate height, and u_e, D_m and d_p are the interstitial mobile phase velocity, the diffusivity of the solute in the bulk mobile phase and the particle diameter respectively. The value of k_0 is given by $\varepsilon_i\,(1 - \varepsilon_e)/\varepsilon_e$, where ε_i and ε_e are the appropriate intraparticular and interstitial porosities, respectively. γ, λ, ω and κ are structural parameters of the column packing and θ is the tortuosity factor for the porous particles. k' is the column capacity ratio and k_d is the desorption rate constant.

However, it has been known for almost 20 years that in practice there are some simple and approximately linear relationships between peak widths and retention values, as given in the following equations[13,14]:

$$2\Delta t_{1/2} = a_2 + b_2 t_R' \tag{6}$$

$$2\Delta t_{1/2} = a + b t_R \tag{7}$$

$$2\Delta V_{1/2} = a' + b' V_R \tag{8}$$

where $2\Delta t_{1/2}$ and $2\Delta V_{1/2}$ are the peak width at half-height in time and volume units respectively. V_R, t_R, t_R' t_R^0 and F are the retention volume, retention time, adjusted retention time of the solute, retention time of non-sorbed substance and mobile phase

flow-rate, respectively. The relationships between these parameters and constants are as follows:

$$2\Delta V_{1/2} = 2\Delta t_{1/2} F \tag{9}$$

$$a_2 = a + b t_R^0 \tag{10}$$

$$b' = b_2 = b \tag{11}$$

$$a' = aF \tag{12}$$

From these empirical relationships we can calculate the plate height, H, of the solute with a capacity factor k', and also H^1 and H^∞, which are the plate heights for the solute with a capacity factor, k', equal to unity or approaching infinity, respectively.

$$H = \frac{L}{n} = \frac{L}{5.54\left(\frac{t_R}{2\Delta t_{1/2}}\right)^2} = \frac{L}{5.54\left(\frac{V_R}{2\Delta V_{1/2}}\right)^2} = \frac{Lb^2}{5.54}\left(\frac{k' + 1 + \frac{au}{bL}}{1 + k'}\right)^2$$

$$= \frac{Lb_2^2}{5.54}\left(\frac{\frac{a_2 u}{b_2 L} + k'}{1 + k'}\right) = \frac{Lb'^2}{5.54}\left(\frac{1 + \frac{a'u}{b'LF} + k'}{1 + k'}\right) = H^\infty\left(\frac{\beta + k'}{1 + k'}\right)^2 \tag{13}$$

The three constants in eqn. 13 can be calculated from the following equations:

$$H^\infty = \frac{Lb^2}{5.54} = \frac{Lb'^2}{5.54} = \frac{Lb_2^2}{5.54} \tag{14}$$

$$H^1 = H^\infty\left(\frac{\beta + 1}{2}\right)^2 \tag{15}$$

$$\beta = 1 + \frac{au}{bL} = 1 + \frac{a'u}{Fb'L} = 2\sqrt{\frac{H^1}{H^\infty}} - 1 \tag{16}$$

We can obtain the equations for H^∞ and H^1 when infinity and unity, respectively, are substituted for k' in eqn. 1:

$$H^\infty = \frac{2\gamma D_m}{u_e} + \frac{2\lambda d_p u_e^{1/3}}{u_e^{1/3} + \omega(D_m/d_p)^{1/3}} + \frac{\kappa d_p^{5/3} u_e^{2/3}}{(1 + k_0)^2 D_m^{2/3}} + \frac{\theta d_p^2 u_e}{30 D_m k_0 (1 + k_0)^2} \tag{17}$$

$$H^1 = \frac{2\gamma D_m}{u_e} + \frac{2\lambda d_p u_e^{1/3}}{u_e^{1/3} + \omega(D_m/d_p)^{1/3}} + \frac{\kappa (1 + 2k_0)^2 d_p^{5/3} u_e^{2/3}}{4(1 + k_0)^2 D_m}$$

$$+ \frac{\theta (1 + 2k_0)^2 d_p^2 u_e}{120 D_m k_0 (1 + k_0)^2} + \frac{2u_e}{4(1 + k_0) k_d} + H_{ext.} \tag{18}$$

In developing the high-performance liquid chromatographic column system, the extra-column effects must be taken into consideration. Obviously, the dead volume in the detector and connector must be minimized, and even so the extra-column effects must still be added to the plate height equation. Because for the solute with k' approaching infinity the extra-column effect is comparatively small and can be neglected, we only added this term to the equation for H^1.

Eqn. 14 shows that the constants b, b_2 and b' are determined by H^∞ and, according to eqn. 17, they will not depend seriously on the extra-column effects. Nevertheless, it may have more serious influence on H^1 and also on constants a, a_2 and a'. Further, in these equations no wall effects have been taken into consideration. Only when the wall effect is not the major controlling factor in band broadening will the approximately linear relationship in eqn. 8 between peak width (in volume units) and retention volume be preserved for different solutes on columns with different inner diameters. Especially in such instances it will be very interesting to carry out some systematic experiments on the influence of the dead volume of the detector and connector on a'. It must be remembered that these are only approximate empirical relationships and it is possible that the wall effect would have some influence on b'.

EXPERIMENTAL

Equipment

The K-1 column system (adsorption type) made in our Institute is shown in

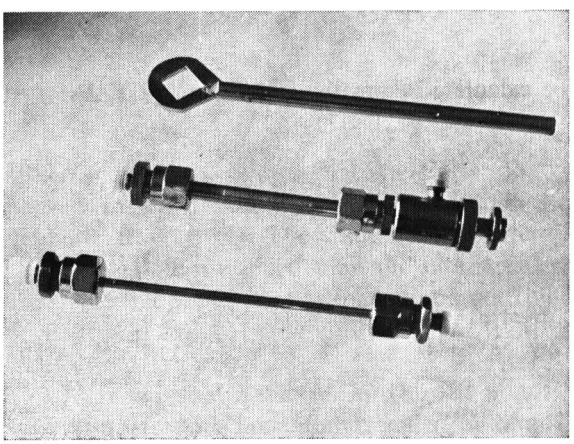

Fig. 1. K-1 (adsorption type) column system made in our Institute.

TABLE I

VOLUMES OF THREE UV DETECTOR SYSTEMS

No. of system	*Cell volume of UV detector (μl)*	*Dead volume of connector (μl)*	*Total volume of the system (μl)*
1	8*	13	21
2	8*	3	11
3	1.2**	0.8	2

* Supplied by Analytical Instruments Factory, Peking, China.
** Home-made in our Institute.

Fig. 1. The microparticulate irregular silica YWG (particle size 3–5 μm) produced by Tsing Tao Ocean Chemical Plant (Tsing Tao, Shandong, China) was used in all experiments. A specially designed central injecting sample injector was used, as shown in Fig. 1. Precision-bore stainless-steel tubing of length 10 cm and with inner diameters of 2, 3, 4, 5 and 6 mm were supplied by the Yan An Steel Tubing Factory, Shanghai, China. The unbalanced high-pressure slurry technique was used to pack these columns; the composition of the slurry was carbon tetrachloride–dioxan–silica (10:5:1, by weight)[11].

A home-made 250-ml syringe pump was used. Three kinds of UV detector–connector systems were used, as shown in Table I.

Mobile phase and samples

n-Hexane without further purification was used as the mobile phase at a linear velocity of 0.9–1 mm/sec.

Sample solutes were ordinary chemical reagents and were dissolved in *n*-hexane. Ethylene tetrachloride was used as the unretained solute. The concentrations of ethylene tetrachloride, benzene, naphthalene, biphenyl and phenanthrene in *n*-hexane were 24, 53, 5, 1.3 and 0.5 μg/μl, respectively. The sample volume should be less than 0.2 μl for the column of 2 mm I.D. in order to prevent overloading.

RESULTS

The peak widths and retention times of different solutes determined on columns with different inner diameters are given in Table II. Fig. 2 shows the linear

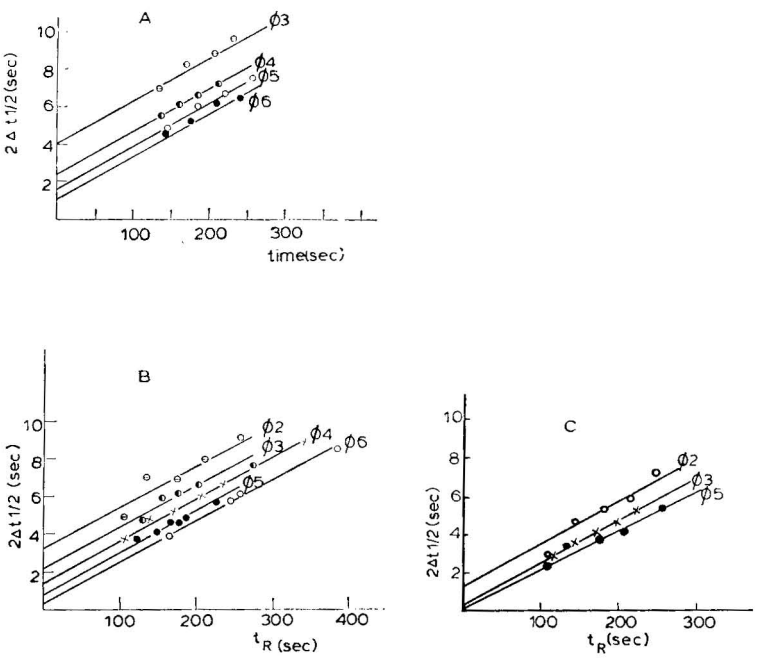

Fig. 2. Peak width *versus* retention time on columns with different inner diameters. Dead volume of detector system: A, 21 μl; B, 11 μl; C, 2 μl.

TABLE II

MEASURED PEAK WIDTHS (IN TIME UNITS) AND RETENTION TIMES OF DIFFERENT SOLUTES ON COLUMNS WITH DIFFERENT INNER DIAMETERS

Average values of more than 3 experiments. Column length, 10 cm.

Dead volume of detector system (μl)	*Column I.D. (mm)*	*Flow-rate of mobile phase (μl/sec)*	*Ethylene tetrachloride*		*Benzene*		*Naphthalene*		*Biphenyl*		*Phenanthrene*	
			t_R *(sec)*	$2\Delta t_{1/2}$ *(sec)*	t_R *(sec)*	$2\Delta t_{1/2}$ *(sec)*	t_R *(sec)*	$2\Delta t_{1/2}$ *(sec)*	t_R *(sec)*	$2\Delta t_{1/2}$ *(sec)*	t_R *(sec)*	$2\Delta t_{1/2}$ *(sec)*
21	3	5.5			134	6.6	169	7.8	205	8.6	231	9.1
	4	9.7			137	5.5	162	6.1	186	6.5	208	7.2
	5	15.0			145	4.8	183	6.0	220	6.7	257	7.4
	6	22.0			141	4.5	175	5.2	210	6.0	242	6.5
11	2	2.5	106	4.8	135	6.0	174	6.9	210	7.9	256	9.5
	3	5.9			125	4.7	151	5.9	175	6.2	198	6.6
	4	10.3	104	3.7	136	4.8	170	5.2	207	6.1	233	6.5
	5	16.3			121	3.7	145	4.0	167	4.4	183	4.7
	6	24.8			135	3.7	175	4.5	222	5.5	251	6.0
2	2	2.5	109	2.9	144	4.6	179	5.2	217	5.8	249	7.3
	3	5.4	112	2.6	139	3.6	172	4.1	193	4.5	224	5.3
	5	16.0	103	2.3	133	3.3	173	3.8	208	4.2	256	5.4

TABLE III

CALCULATED PEAK WIDTHS (IN VOLUME UNITS) AND RETENTION VOLUMES OF DIFFERENT SOLUTES ON COLUMNS WITH DIFFERENT INNER DIAMETERS

Column length, 10 cm.

Dead volume of detector system (μl)	*Column I.D. (mm)*	*Flow-rate of mobile phase (μl/sec)*	*Ethylene tetrachloride*		*Benzene*		*Naphthalene*		*Biphenyl*		*Phenanthrene*	
			V_R (μl)	$2\Delta V_{1\,2}$ (μl)	V_R (μl)	$2\Delta V_{1/2}$ (μl)	V_R (μl)	$2\Delta V_{1/2}$ (μl)	V_R (μl)	$2\Delta V_{1/2}$ (μl)	V_R (μl)	$2\Delta V_{1/2}$ (μl)
21	3	5.5			737	36	919	43	1128	47	1270	50
	4	9.7			1319	53	1571	59	1795	63	2008	70
	5	15.0			2179	72	2747	90	3300	101	3860	111
	6	22.0			3109	100	3859	114	4631	132	5336	143
11	2	2.5	267	12	340	15	438	17	529	20	645	24
	3	5.9			737	28	890	35	1032	37	1174	39
	4	10.3	1068	38	1397	49	1746	53	2132	63	2393	67
	5	16.3			1970	60	2360	66	2720	69	2980	77
	6	24.8			3344	92	4335	111	5500	136	6219	149
2	2	2.5	266	7	353	11	438	13	531	14	610	18
	3	5.4	600	14	745	19	922	22	1035	24	1200	28
	5	16.0	1668	38	2138	54	2800	62	3372	68	4146	88

TABLE IV

CALCULATED VALUES OF a' AND b' IN EQN. 8 USING DIFFERENT DETECTOR SYSTEMS

No. of detector system	*Dead volume of detector system (μl)*	*a' (μl)*	*b'**	*Correlation coefficient*
1	21	20.5 ± 1.5	0.024	0.996
2	11	11.0 ± 1.3	0.023	0.996
3	2	1.7 ± 1.2	0.022	0.995

* Average value: $b' = 0.023$.

relationships between $2\Delta t_{1/2}$ and t_R on columns with different inner diameters and detector systems.

Table III gives the peak widths ($2\Delta V_{1/2}$) (in volume units) and retention volumes (V_R) calculated from $2\Delta t_{1/2}$ and t_R multiplied by the flow-rate of the mobile phase. Fig. 3 shows $2\Delta V_{1/2}$ *versus* V_R for different solutes on columns with different inner diameters but with the same detector system. There are three curves for three detector systems. The values of the constants a' and b' in eqn. 8 calculated by regression analysis on these three detector systems are given in Table IV. It can be clearly seen that the dead volume of the detector system has a great influence on a', but b' remains almost constant for all three detector systems.

The value of a' decreases linearly with decrease in the dead volume of the detector system. Whereas a solute has the same capacity factor, k', using columns with different inner diameters, it will have a smaller retention volume for a smaller

TABLE V

COMPARISON OF CALCULATED (H_{calc}) AND EXPERIMENTAL (H_{exp}) VALUES OF HETP FOR DIFFERENT SUBSTANCES ON THE COLUMNS WITH DIFFERENT INNER DIAMETERS

Column length, 10 cm.

Dead volume of detector system (μl)	*Column I.D. (mm)*	*Flow-rate of mobile phase (μl/sec)*	*Constants in eqn. 8*		*Constants in eqns. 14 and 16*		*Ethylene tetrachloride*		
			a' (μl)	*b'*	H^∞ *(μm)*	β*	k'	H_{calc} *(μm)*	H_{exp} *(μm)*
21	3	5.5	20.5	0.024	9.6	2.52			
	4	9.7				1.85			
	5	15.0				1.54			
	6	22.0				1.37			
11	2	2.5	11.0	0.023		2.37	0	53	37
	3	5.9				1.81			
	4	10.3				1.45	0	20	23
	5	16.3				1.29			
	6	24.8				1.20			
2	2	2.5	1.7	0.022		1.21	0	14	13
	3	5.4				1.13	0	12	10
	5	16.0				1.05	0	10	9

* By using the average value $b' = 0.023$.

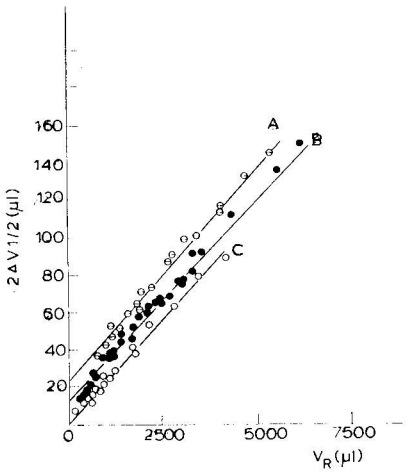

Fig. 3. Peak width (in volume units) *versus* retention volume on columns with different inner diameters by using different dead volumes of the detector system. Dead volume of detector system: A, 21 μl; B, 11 μl; C, 2 μl.

column inner diameter. The efficiency of the column with a smaller inner diameter is greatly improved by decreasing the dead volume of the detector system.

We used average values of b' and different values of a' for three different detector systems to calculate the values of β and H^{∞} from eqns. 14 and 16, respectively. Then, by using these values of β and H^{∞}, we calculated the HETP values for different

Benzene			*Naphthalene*			*Biphenyl*			*Phenanthrene*		
k'	H_{calc} (μm)	H_{exp} (μm)	k'	H_{calc} (μm)	H_{exp} (μm)	k'	H_{calc} (μm)	H_{exp} (μm)	k'	H_{calc} (μm)	H_{exp} (μm)
0.25	47	43	0.57	37	40	0.91	31	31	1.15	28	28
0.25	27	29	0.47	24	26	0.69	22	22	0.88	20	22
0.28	19	20	0.61	17	19	0.94	16	17	1.24	15	15
0.29	16	19	0.61	15	16	0.93	14	15	1.22	13	13
0.27	42	35	0.64	32	29	0.98	27	26	1.42	23	25
0.23	26	26	0.48	23	28	0.72	21	23	0.95	19	20
0.31	17	22	0.63	16	17	0.99	14	16	1.24	14	14
0.21	15	17	0.45	14	14	0.67	13	13	0.83	13	12
0.39	13	14	0.80	12	12	1.29	11	11	1.59	11	10
0.32	14	19	0.64	13	15	0.99	12	13	1.28	12	15
0.24	12	12	0.54	11	10	0.72	11	10	1.00	11	10
0.29	10	12	0.68	10	9	1.02	10	8	1.49	10	8

TABLE VI

INFLUENCE OF THE DEAD VOLUME OF DETECTOR SYSTEMS ON THE PEAK SYMMETRY

Column I.D. (mm)	*Solute*									
	Ethylene tetrachloride		*Benzene*		*Naphthalene*		*Biphenyl*		*Phenanthrene*	
	Dead volume (μl)									
	11	*2*	*11*	*2*	*11*	*2*	*11*	*2*	*11*	*2*
2	5.0	—	3.7	1.7	3.0	1.9	2.4	1.9	2.2	2.2
3	2.6	2.0	2.4	1.8	2.0	1.9	2.0	1.6	1.9	1.5
4	1.6	1.6	2.0	1.5	1.7	1.6	1.7	1.5	1.7	1.6
5	—	—	1.6	—	1.6	—	1.4	—	1.4	—
6	1.8	1.6	1.8	1.4	1.4	1.3	1.3	1.3	1.2	1.1

solutes on columns with different inner diameters for three different detector systems according to eqn. 13 and compared the results with the values determined experimentally, as shown in Table V. The calculated and experimental values are in good agreement.

It must be pointed out that a decrease in the dead volume of the detector

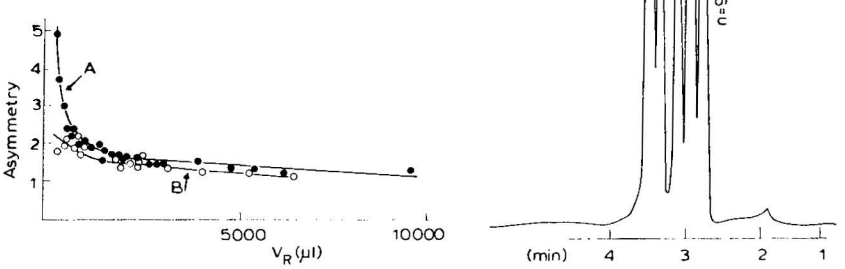

Fig. 4. Influence of the dead volume of the detector system on the peak symmetry. Peak asymmetry is calculated at 1/10 peak height. Dead volume of the detector system: A, 11 μl; B, 2 μl.

Fig. 5. Chromatogram of some benzoates. Column: 15 cm × 2 mm I.D. Packing: YWG-5 (5 μm). Eluent: CH_2Cl_2, 0.17 ml/min. Inlet pressure: 31 atm. Detector: UV, 254 nm (dead volume of detector system = 2 μl). Peaks: (1) isopentyl benzoate; (2) *n*-butyl benzoate; (3) *n*-propyl benzoate; (4) ethyl benzoate; (5) methyl benzoate.

systems also improved the peak asymmetry of columns with a small internal diameter, as shown in Table VI and Fig. 4.

By using the improved home-made injection and detector systems we obtained a column of I.D. 2 mm and length 10 cm with an efficiency of more than 6000 theoretical plates. Some typical chromatograms are shown in Figs. 5–7.

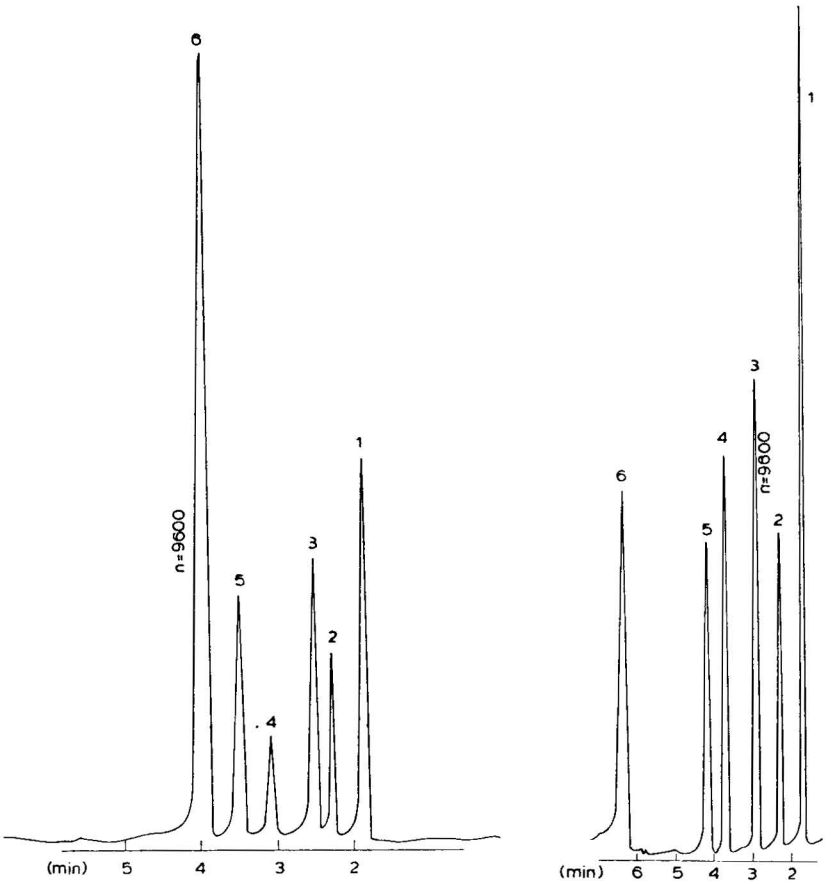

Fig. 6. Chromatogram of some aromatic compounds. Conditions as in Fig. 5. Peaks: (1) naphthalene; (2) benzyl methyl ether; (3) *m*-dinitrobenzene; (4) 2,6-dimethylphenol; (5) methyl benzoate; (6) benzyl ethyl ketone.

Fig. 7. Chromatograms of some aromatic hydrocarbons. Conditions as in Fig. 5, except eluent, *n*-heptane (0.17 ml/min). Peaks: (1) ethylene tetrachloride; (2) benzene; (3) naphthalene; (4) biphenyl; (5) phenanthrene.

CONCLUSIONS

Using the home-made column system, the influence of wall effects on column efficiency can be minimized. An approximately linear relationship between retention volume (V_R) and peak width ($2\Delta V_{1/2}$) (in volume units) for various solutes on columns with different inner diameters was obtained (eqn. 8).

For the three detector systems with dead volumes of 21 μl, 11 μl and 2 μl we obtained values of the constants in eqn. 8 of $b' = 0.024$, 0.023, 0.022 (average 0.023) and $a' = 20.5 \pm 1.5$, 11.0 ± 1.3 and 1.7 ± 1.2 μl, respectively. The volume of the detector system has a great influence on a' but only a slight influence on b'.

Using the average value of b' (0.023) and the above three values of a', we calculated β and H^{∞} from eqns. 14 and 16 and the HETP values according to eqn. 13 for various solutes on columns with different inner diameters for three different detector systems. The calculated values were in good agreement with the experimental values.

By decreasing the volume of the detector system the efficiency of columns with smaller inner diameter and peak asymmetry can be greatly improved.

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QUANTITATIVE ASPECTS OF HEATER DISPLACEMENT CHROMATOGRAPHY

J. P. HORROCKS, J. A. HARRIS, C. S. G. PHILLIPS* and K. F. SCOTT
Oxford University Inorganic Chemistry Laboratory, South Parks Road, Oxford (Great Britain)
(Received April 3rd, 1980)

SUMMARY

This paper describes quantitative developments in heater displacement chromatography. The factors which control the quality of a heater displacement chromatographic separation are discussed, and relationships derived by which the conditions for optimum separation can be determined. An expression is also derived for the maximum sample capacity of a heater displacement column, enabling optimum use to be made of adsorbents, and an equation is generated which describes the concentration profile in the heater zone. This latter function is of importance in the evaluation of the rate of a reaction occurring on the heater displacement column.

The equations developed are tested using a series of model systems.

INTRODUCTION

In a recent paper[1] the technique of heater displacement chromatography was introduced and its ability to achieve preparative-scale separation with efficacy and simplicity was demonstrated. The advantages and disadvantages of displacement chromatography as a preparative method were enumerated, and it was shown how the replacement of the feed of displacer compound in conventional displacement chromatography by a moving heater effectively removed the disadvantages of the technique and transformed it into one of versatility and convenience. It was also shown that by using a catalyst as an adsorbent it was possible to carry out heterogeneously-catalysed reactions with simultaneous separation of products. The use of a displacement chromatographic environment for the conduct of reactions again showed valuable advantages such as the near-quantitative isolation of intermediate products in consecutive processes and the conversion of reactants to points past the thermodynamic equilibrium in cases of reversible reaction.

A heater displacement chromatograph is represented schematically in Fig. 1. It consists of an adsorbent column of length L contained in an oven at temperature T_0 and through which is passed a carrier gas flow F. The sample to be separated is introduced at the beginning of the column where it forms a band of length $\emptyset L$ before it is displaced forward along the column by means of a heater moving at speed U_h.

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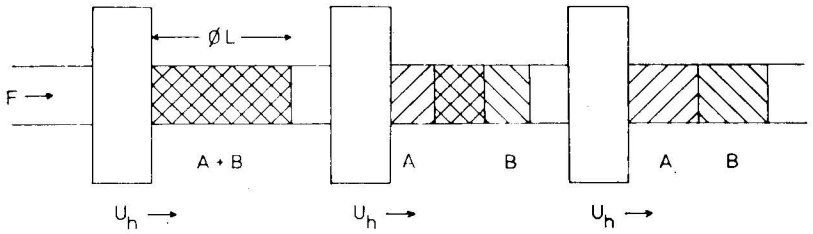

Fig. 1. Schematic representation of a heater displacement chromatograph showing three states of a band of a mixture of two components A and B as it is displaced along a column of length L, with a carrier gas flow-rate F at a heater speed U_h.

By the time it leaves the column the sample has been separated into its individual components ahead of the heater.

In this present paper we seek to place heater displacement chromatography on a quantitative basis. In order that the technique be reduced to one which can be used routinely, the various parameters controlling the separation, *i.e.* carrier gas flow-rate, heater speed, heater temperature and oven temperature, need to be related to the chromatographic (adsorption) properties of the compounds to be separated and the adsorbent used in the column and also the saturated vapour pressures of these compounds at the oven temperature employed. Accordingly in this paper there are three objectives: (a) to determine the limits of the separation parameters insofar as these are controlled by the properties of the adsorbent and adsorbates, so that these parameters can be chosen to allow an optimum heater displacement separation to be achieved; (b) to determine from the properties of the adsorbents and adsorbates, the maximum sample capacity of a heater displacement column to allow optimum use to be made of the adsorbents; and (c) to formulate an expression for the concentration of the adsorbate in the heater zone as a function of temperature, so that in later work the rate of reaction occurring on a catalytic heater displacement column may be predicted. The theoretical aspects of (a), (b) and (c) above are therefore set out and the results demonstrated by a series of experiments using a heater displacement chromatograph.

THEORETICAL

Conditions required for satisfactory displacement

In order to achieve a satisfactory displacement separation of particular adsorbates on a given adsorbent, it is necessary to set the primary heater displacement variables of heater speed and carrier gas flow to the appropriate optimum values. There are four factors which govern the bounds of these variables and these are listed below and illustrated diagrammatically in Fig. 2.

(i) If the ratio of the heater speed to the gas flow-rate (U_h/F) is excessive, the gas phase concentration of the adsorbate ahead of the heater exceeds the saturated vapour pressure of the latter and condensation occurs. This is illustrated by line i.

(ii) If the ratio (U_h/F) is insufficiently large then one or more of the adsorbate bands in the column may elute ahead of the heater. The onset of elution is represented by line ii. This results in possible loss of separation but certainly loss of sample capacity (A).

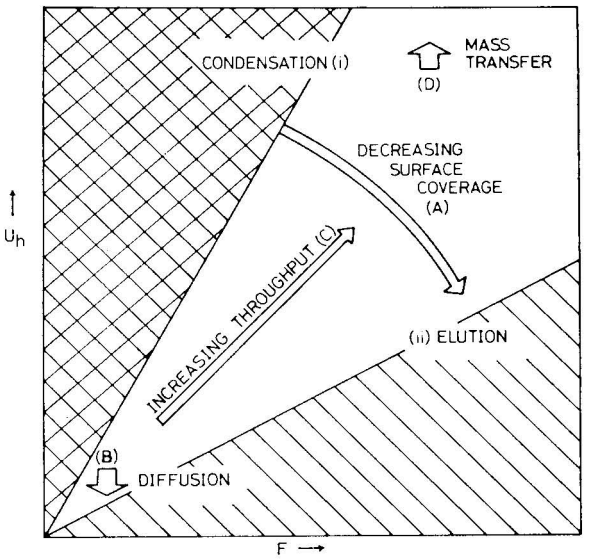

Fig. 2. Operating regime for heater displacement chromatography, with thermodynamic limits (lines i and ii) and kinetic limits (regions B and D) shown. Lines A and C indicate the effect of the heater speed to flow-rate ratio, and the overall speed of separation, on the throughput of material.

(iii) If the heater moves too slowly the regime arrowed B is entered where the separation is impaired by longitudinal diffusion on the column. A slow speed also means the throughput of material will be low (C).

(iv) If the heater moves too rapidly then resistance to mass transfer will impair the separation (D) and thermal gradients across the column may affect the displacement of the last band.

The slope of the condensation limit line (i) can readily be calculated as follows: consider a band of a single component A moving along a heater displacement column. The partial pressure P^A of the adsorbate leaving the column on which it was adsorbed with a concentration C_S^A is given by:

$$P^A = U_h \frac{\varrho C_S^A RT}{Q} \tag{1}$$

where ϱ = adsorbent packing density and Q = total volume flow leaving the column at temperature T. If P_0^A is the saturated vapour pressure of A, then the ratio F/U_h has to be set to a minimum value given by

$$(F/U_h)_{\text{min.}} = \varrho C_S^A RT / \left\{ \frac{P_0 - P_0^A}{P^A} \right\}_0 \tag{2}$$

Eqn. 2 gives the slope of line i, a value which also corresponds to the minimum flow-rate for a multi-component separation where A is the component with the lowest vapour pressure at the separation temperature. The line ii represents a ratio

(U_h/F) below which the least strongly retained adsorbate is eluted ahead of the heater. The slope of this line can be conveniently calculated by setting the flow-rate to the velocity with which a small sample of the adsorbate is eluted as a symmetrical peak. If the measured retention time per unit column length for such a peak is V_R^A then the slope of the line ii is simply $(U_h/F)_{min.} = 1/V_R^A$. In practice, since the isotherm is curved, different concentrations move at different velocities, and it is therefore preferable to operate as close as is praticable to line i, to avoid partial elution of the bands with consequent distortion of band shape and loss of adsorbent capacity.

Sample capacity of a heater displacement column

In order to determine the maximum sample size for a column of given dimensions, use may be made of a modification of the approach introduced by Sillèn[2]. Consider a column in which two substances A and B are being separated by displacement. Suppose A is less strongly held to the surface than B and that they are initially placed on the column as a uniform mixture occupying a fraction ø of the column. To achieve a separation of A and B in a unit column length, substance A must travel unit distance while substance B moves a distance $1-\varnothing$. By considering a small interval of time during the development of the displacement chromatogram, it may readily be shown that the band velocity of substance A, U_A is given by: $U_A = C_G^A F/C_S^A \varrho$. While A moves unit distance along the column substance B moves a distance $1-\varnothing$ at a velocity $U_B = C_G^B F/C_S^B \varrho$ and hence for a separation just to be achieved in unit column length, the initial band must occupy a fraction of the column not greater than

$$\varnothing_{max.} = 1 - C_S^A C_G^B / C_S^B C_G^A \tag{3}$$

which we can write

$$1 - X^{AB}$$

Hence the maximum capacity of a heater displacement column is readily computed from measurements of surface and gas phase concentrations of components from chromatographic measurements obtained at the separation temperature.

Concentration profile in the heater zone

Consider a single substance A being displaced by a moving heater. The isotherm governing the adsorption of the material by the adsorbent has to be of the Langmuir type, *i.e.* concave towards the pressure axis, but it need not necessarily fit the Langmuir equation. However, for the system which we have investigated experimentally the adsorption has followed the Langmuir equation satisfactorily over the relevant concentration ranges, so that we may write:

$$C_S^A = C_S^0 K C_G^A / (1 + K C_G^A) \tag{4}$$

where C_S^0 represents the maximum capacity of the surface and K is the adsorption constant. At a steady state we assume that while the adsorbate is displaced outside the heater zone, it is eluted within it. Moreover, at a steady state, each concentration

within the heater moves at the velocity of the heater. This is similar to the situation prevailing inside the heater of the chromatothermographic technique of Turkeltaub[3]. From the retention volume eqn. 4 which relates the retention volume of a particular finite adsorbate concentration to the slope of the adsorption isotherm at that concentration, we can write the velocity of a concentration C_G^A, $U(C_G^A)$, inside the heater zone as:

$$U(C_G^A) = F/\left\{V_0 + \varrho \frac{\partial C_S^A}{\partial C_G^A}\right\} \tag{5}$$

for a column of unit length, with a dead volume V_0 per unit length. Substituting the differentiated adsorption isotherm (eqn. 4) into eqn. 5, equating $U(C_G^A)$ with the heater speed U_h and rearranging, we obtain,

$$\frac{K}{\{1 + KC_G^A\}^2} = \frac{1}{PC_S^0}\left\{\frac{F}{U_h} - V_0\right\} = a \tag{6}$$

If the adsorption constant K is expressed in terms of a heat of adsorption, ΔH^A,

$$\ln K = \ln K^0 - \Delta H^A/RT$$

substitution into eqn. 6 and rearranging gives:

$$C_G^A = \{aK^0 e^{-\Delta H^A/RT}\}^{-1/2} - \{K^0 e^{-\Delta H^A/RT}\}^{-1} \tag{7}$$

Substituting back into eqn. 4, eqn. 7 becomes:

$$C_S^A = C_S^0\{1 - (a/K^0 e^{-\Delta H^A/RT})^{1/2}\} \tag{8}$$

The shape of this function will be considered in more detail below, but its form is a monotonic decrease of C_S^A with increasing temperature. At a point $T = Tc$, C_S^A passes through zero, and thereafter the function has no physical significance. The fact that, according to this model, the value of C_S^A reaches zero at a finite point in the heater, means that there is no residue on the column after the passage of the heater, an important observation from the point of view of quantitative applications.

The results generated by the above theory may now be applied to experimental heater displacement chromatography.

EXPERIMENTAL

The small-scale heater displacement chromatograph used in these investigations has been described previously, together with operational details[1]. The only modifications carried out for this work were to the heater and the automatic sampling valve.

A temperature-controlled heater was constructed to give the high temperature stability required for these experiments ($\pm$ 2°C). The heater consisted of a brass tube of length 5 cm, wound with approximately 5 m nichrome wire (0.46 mm diameter), which fitted snugly over the heater displacement column. A NiCr–NiA1 thermo-

couple passed through the side of the brass tube halfway along its length and just touched the glass column. The thermocouple was connected to a Pye Ether "Mini" temperature controller (0—1000°C) which controlled the current to the heater, from a variable voltage transformer.

The automatic sampling system was improved by replacing the rotary sampling valve previously described, with a linear valve (type GSV-106-B-V) supplied by Negretti and Zambra (Aviation). This allowed the sampling system to operate reliably at temperatures up to 180°C, an improvement of 100°C on the rotary valve.

The adsorbents used in this work were Alcoa alumina (80–100 mesh) and Waters Assoc. GC Porasil type B (80–100 mesh), both supplied by Phase Separations (Queensferry, Great Britain). Hydrocarbons were supplied by Koch-Light Labs. (Colnbrook, Great Britain).

Isotherms for various adsorbates and stationary phases were extracted from elution chromatographic profiles using the method of Huber and Keulemans[4] as modified by Conder[5]. This was carried out in a conventional gas chromatograph (Pye-Unicam 104) using 1.5 m × 0.4 cm I.D. glass columns.

Surface areas of adsorbents were measured using nitrogen as an adsorbate by the method of Nelsen and Eggertsen[6].

RESULTS

Optimum conditions for displacement

The limits described in Fig. 2 were shown to control the quality of a heater displacement band of *n*-heptane on a Porasil B column (80 × 1.2 cm O.D.) using an oven temperature of 67°C and a heater temperature of 300°C. The heater speed was 3.33 cm min^{-1} and the gas flow-rate was adjusted so that the operating point was just below and just above line i which corresponded to 35 ml min^{-1} at the heater speed used. With a flow-rate of 44 ml min^{-1} a satisfactory band shape was obtained (similar to that shown for *n*-hexane in Fig. 3) while with a flow-rate of 28 ml min^{-1}, distortion was apparent owing to condensation. This was manifested as a sharp rise in. C_S at the centre of the displacement band to a value considerably in excess of C_s^0. This distortion disappeared towards the end of the band as the increasing column temperature in the heater zone raised the saturated vapour pressure of the heptane.

By performing a similar run at a flow-rate of 61 ml min^{-1} the effect of elution on the shape of the displacement band was observed. The band deteriorated into a severely tailing elution peak, the effect being observable even before line ii owing to curvature of the isotherm. This curvature resulted in the onset of elution of high adsorbate concentrations before lower concentrations with only the lowest concentrations being retained sufficiently to be displaced by the heater.

The effects of overall speed of separation (regimes B and D in Fig. 2) were investigated using a constant value for the heater speed–flow-rate ratio. It was found that, for the separation of hexane isomers on an alumina column, the chromatographic efficiency of the heater displacement system followed a Van Deemter type curve. Below a heater speed of about 1.5 cm min^{-1} there was a fairly sharp decrease in efficiency due to longitudinal diffusion, while above this speed there was a slow decrease in efficiency due to mass-transfer effects.

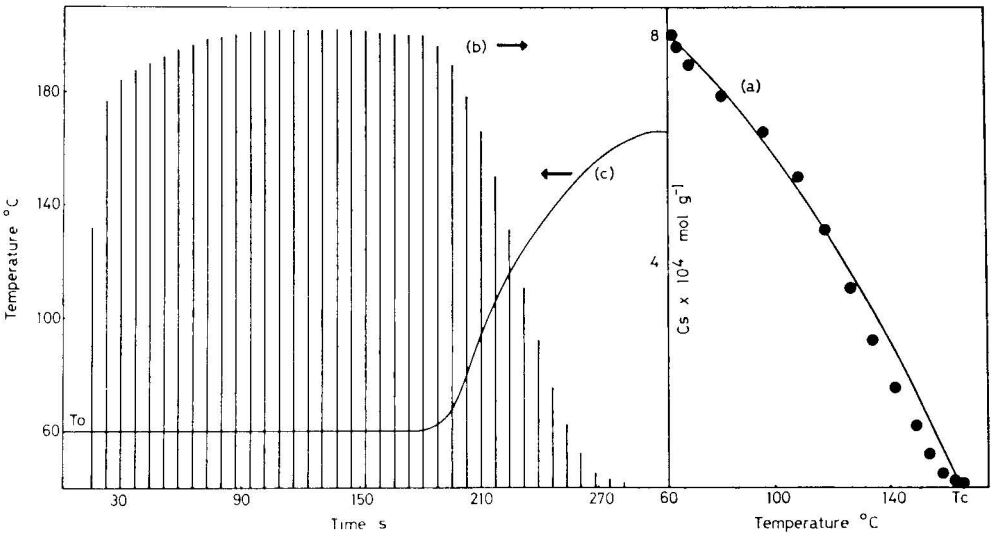

Fig. 3. (a) Theoretical band profile curve (continuous line) for *n*-hexane on alumina with experimental points, obtained from (b) heater displacement chromatogram and (c) heater temperature profile curve, superimposed. Theoretical cut-off temperature (Tc) = 161°C.

Sample capacity

In order to exploit eqn. 3 to determine a theoretical value for the sample capacity of a heater displacement column for a binary separation the value of X^{AB} has to be determined. This was carried out using two methods.

(i) The values of the surface and gas phase concentrations of the components to be separated were determined by a simple static method. Here an amount of the component mixture was injected into a flask sealed with a septum, containing a weighed amount of adsorbent. After shaking and allowing to equilibrate at the displacement temperature, the equilibrium concentration in the gas phase of each component was determined by withdrawing samples and analysing them using elution chromatography.

(ii) Values of the adsorption constant K were also determined by elution chromatography at several different temperatures and extrapolated to the displacement temperature. Although this technique suffered the disadvantage that concentrations were not measured under conditions of displacement, it was found to be a rapid and convenient method of evaluating X^{AB}.

The above methods were applied to determine the maximum sample capacity of a heater displacement column used for the separation of skeletal hexane isomers on Alcoa alumina and on Porasil B. The results are shown in Tables I and II and demonstrate the satisfactory correlation for calculated maximum loadings from methods (i) and (ii) above. They also show that the more difficult separations (*e.g.* that between *n*-hexane and 3-methylpentane) exhibit a correspondingly smaller maximum loading as would be expected. The results also show that an alumina column shows greater selectivity towards the hexane isomer separations than does Porasil B, leading to a greater maximum sample capacity in the former case. This increased selectivity coupled with the larger bulk density of alumina (0.91 g ml^{-1} as

compared with 0.44 g ml^{-1} for Porasil B) means that approximately 3 times as much of the hexane isomers can be separated on an alumina column as on a Porasil B column of the same dimensions.

TABLE I

VALUES OF $\varnothing_{max.}$ FOR HEXANE ISOMERS AND HEXANE ON ALUMINA

Isomers	*Static method*	*Elution chromatography*	*Displacement chromatography*
n-Hexane and 2,2-dimethylbutane	0.47 ± 0.05	0.45 ± 0.05	0.47 ± 0.03
n-Hexane and 2,3-dimethylbutane	0.33 ± 0.02	0.33 ± 0.06	0.29 ± 0.02
n-Hexane and 2-methylpentane	0.24 ± 0.02	0.28 ± 0.06	0.27 ± 0.02
n-Hexane and 3-methylpentane	0.16 ± 0.03	0.19 ± 0.06	0.14 ± 0.02

TABLE II

VALUES OF $\varnothing_{max.}$ FOR HEXANE ISOMERS AND HEXANE ON PORASIL B

Isomers	*Static method*	*Elution chromatography*	*Displacement chromatography*
n-Hexane and 2,2-dimethylbutane	0.43 ± 0.02	0.31 ± 0.02	0.33 ± 0.02
n-Hexane and 2,3-dimcthylbutane	0.34 ± 0.02	0.26 ± 0.02	0.30 ± 0.02
n-Hexane and 2-methylpentane	0.23 ± 0.02	0.19 ± 0.03	0.20 ± 0.02
n-Hexane and 3-methylpentane	0.15 ± 0.02	0.14 ± 0.03	0.17 ± 0.02

Thus while the properties of aluminas and silicas vary a great deal, and the above data only applied to the particular varieties used in this case, it is a relatively simple matter to determine optimum loadings on any adsorbent and use these to determine rationally the most selective adsorbent for any separation.

The loadings predicted in Table I were checked in practice by performing heater displacement separations in which the loading was set to values just above and just below the calculated maximum values. In each case, to provide a uniform initial band at the head of the column, the calculated amount of adsorbent was removed from the column, shaken in a closed flask with the mixture to be separated, and carefully repacked into the displacement column. The heater displacement separation was then carried out. The results are illustrated in Table I. The results of a typical separation obtained in this way on alumina for *n*-hexane and 2,2-dimethylbutane are shown in Fig. 4. It will be observed that exceeding the loading capacity of the column by a very little results in a significant deterioration in the quality of the separation, while consistent separation quality is maintained up to the maximum loading. This is in contrast to elution preparative methods, where the quality of the separation falls progressively as the loading is raised.

The results obtained above were achieved by removal, coating, and replacement of a band of adsorbent at the head of the column. This procedure was found inconvenient for the heater displacement separations, and the samples were normally introduced into the column, either by syringe injection, with the heater at the head of the column or by vapourizing the sample into the carrier gas stream in a small electrically heated saturator placed before the column inlet.

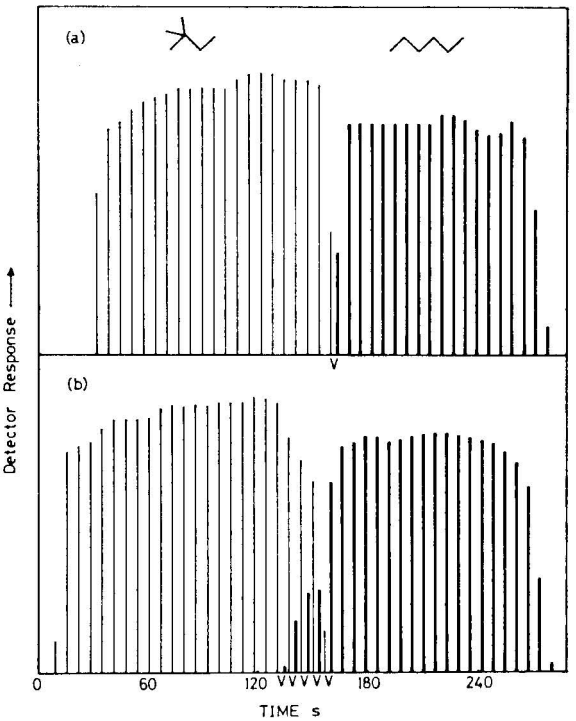

Fig. 4. Separation of 2,2-dimethylbutane and *n*-hexane on alumina at (a) just under the maximum loading ($\varnothing = 0.45$) and (b) just over the maximum loading ($\varnothing = 0.48$). Band overlap can be clearly seen in the latter. The peaks corresponding to a single sample analysis in the overlap regions are marked "V".

Because of the initial separation on the column of the components of the mixture during the development of the band and differences in their volatilities, both these methods result in a slight preliminary separation before the heater displacement run itself. Accordingly the capacity of a column under these conditions is somewhat higher than the predicted value, thus providing a margin of safety over the latter. The effect of initial separation during band formation was illustrated by the direct injection of a mixture of *n*-hexane and 2,2-dimethylbutane onto the alumina column. This gave a value for $\varnothing$ of 0.59 as opposed to 0.47 for introduction of the sample as a band, thereby giving an increase in effective column length of about 25%.

Experimental measurement of the concentration in the heater zone

The experimental validity of eqn. 8 was tested using *n*-hexane as the adsorbate on an alumina column. The parameters of eqn. 8 were measured in a series of preliminary experiments.

The heater temperature profile was measured by means of a thermocouple inserted centrally in the packing material of the column, and the heater allowed to descend over the junction, under conditions of gas flow, heater speed, etc. which prevailed in the intended displacement. The electromotive force (e.m.f.) of the

thermocouple was recorded during the descent of the heater and the temperature profile evaluated for several heater temperatures.

The heat of adsorption was determined from adsorption isotherms obtained from elution chromatography. Isosteric heats of adsorption were obtained at different coverages and the values at the coverages prevailing under heater displacement conditions obtained by extrapolation. Thus for *n*-hexane at a coverage of $4 \cdot 10^{-4}$ mol g^{-1} the heat of adsorption was 33.8 kJ mol^{-1}.

The value of K^0 in eqn. 8 could, in principle, be found by extrapolation of the integrated Van 't Hoff isochore to infinite temperature, but the extrapolation is a long one, and precision is lost in this procedure. As an alternative eqn. 8 was used at $T = T_0$ by setting C_S^A to the measured surface concentration outside the heater, and K^0 evaluated accordingly. For *n*-hexane on alumina a value of $5.6 \cdot 10^{-1}$ ml mol^{-1} was found.

Together with simple determinations of temperatures, gas flow-rate, etc., the above measurements permit the theoretical function of eqn. 8 to be plotted under any given conditions. Such a curve for the displacement of *n*-hexane on alumina at a nominal heater temperature of 200°C is shown in Fig. 3a. Superimposed on these are points determined from the experimental heater displacement profile (Fig. 3b). Relative location of the experimental points and the theoretical curve along the temperature axis was found to be difficult, since the exact positions of the temperature gradient curve (Fig. 3c) and the concentration profile were not precisely known. This problem was overcome by fitting an experimental point to the curve artificially. Approximate calculations on the positions of the experimental and theoretical curves showed that such fitting was entirely reasonable, and the fidelity with which the experimental points fitted the theoretical curve over a wide range of heater profiles indicates the validity of the model for band profile theory.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS

C_G^A, C_G^B	Gas phase concentrations of adsorbates A and B in mol ml^{-1}
C_S^A, C_S^B	Surface concentrations of adsorbates A and B in mol g^{-1}
C_S^0	Maximum capacity of stationary phase, mol g^{-1}
F	Carrier gas flow-rate, ml min^{-1}
K	Distribution coefficient in the Langmuir equation, ml mol^{-1}
K^0	The value of K at infinite temperature
L	Column length, cm
P^A	Partial pressure of adsorbate A, atm
P_0^A	Saturated vapour pressure of adsorbate A at temperature T, atm
Q	Total flux of material leaving column, ml min^{-1}
T	Temperature, °K

Tc	Temperature at which the theoretical band profile curve (eqn. 8) passes through zero
T_0	Oven temperature, °K
U_A, U_B	Band velocities of adsorbates A and B, cm min^{-1}
U_h	Heater speed, cm min^{-1}
V_R^A	Retention volume of adsorbate A per unit column length, cm^2
V_0	Column dead volume per unit length, cm^2
α	Constant defined by eqn. 6, ml mol^{-1}
ΔH	Heat of adsorption, kJ mol^{-1}
ϱ	Column packing density, g cm^{-1}
$\varnothing$	Fraction of column length occupied by adsorbate
$\varnothing_{max.}$	Maximum value of $\varnothing$
X^{AB}	$C_S^A C_G^B / C_S^B C_G^A$

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EFFECT OF THE SENSITIVITY SETTING OF A KATHAROMETER ON RESPONSE FACTORS AT LOW CONCENTRATIONS

C. ROY, G. R. BELLEMARE and E. CHORNET*

Department of Chemical Engineering, Faculty of Applied Sciences, University of Sherbrooke, Sherbrooke, Quebec J1K 2R1 (Canada)

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SUMMARY

A device and procedure are described that permit the easy and precise determination of calibration graphs for the quantitative analysis of gases by gas chromatography The study focuses on the role of the sensitivity of the thermal conductivity detector on the response data, especially when small amounts of gases are to be detected ($< 10^{-6}$ mole). At low sensitivity the response factors for all the gases studied (H_2, N_2, CH_4, CO, CO_2, C_2H_4 and C_4H_{10}) were found to vary with the molar amounts of compound injected. No variation of the relative responses was noted at the normal sensitivity levels except for hydrogen, which exhibited an anomalous chromatographic behavior.

INTRODUCTION

Theoretical[1,2] and experimental[3] methods have been proposed for calculating response factor values in gas chromatography. Tabulated response factor data for a number of pure compounds are also readily available, which can be used with either flame-ionization or thermal conductivity detectors[4–6]. Variables such as carrier gas and carrier gas flow-rate, detector operating temperature, sample concentration, individual sensing unit and recorder attenuation have been studied as a function of the response factors[7–9]. Much less attention however, has been given, to the effect of the detector sensitivity on the relative response data. The object of this work was to determine whether sensitivity could affect the relative responses obtained for the major non-condensable gases (H_2, N_2, CH_4, CO, CO_2, C_2H_4 and C_4H_{10}) originating from the vacuum pyrolysis of solid fuels and which were injected in small amounts (10^{-8}–10^{-5} mole) into the gas chromatograph.

EXPERIMENTAL

Apparatus and materials

A diagram of the apparatus is illustrated in Fig. 1. The main components are a Hewlett-Packard (HP) 5730A gas chromatograph equipped with an HP hot-wire detector coupled to a HP 3380A integrator, a Perkin-Elmer Fluon Rotor gas sampling

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valve (catalogue No. 454-0104), equipped with a 1-ml sampling loop, and MKS Baratron Type 220 electronic manometers with digital readout. Two-way Nupro bellow valves and three-way Whitey ball valve were used in the design. All of the test gases used were of the highest purity available and were provided by Liquid Carbonic Canada, Sherbrooke, Quebec, Canada.

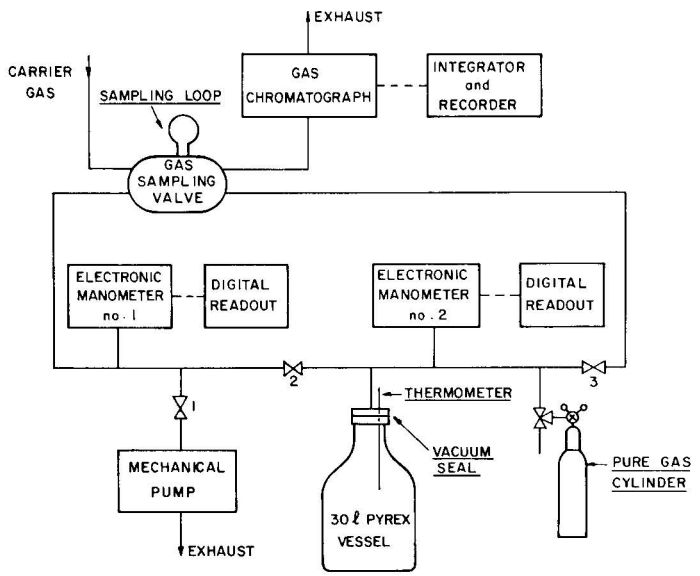

Fig. 1. Calibration apparatus for GC analysis.

The gas chromatograph is equipped with a switching valve to permit the use of two columns for the analysis. The mixture passes through a 1.83 m × 3.2 mm O.D. stainless-steel column packed with 80–100-mesh Porapak Q and a 1.37 m × 3.2 mm O.D. stainless-steel column packed with 60–80-mesh molecular sieve 5A. The carrier gas is a 8 % (v/v) mixture of hydrogen in helium with a flow-rate of 25 ml/min.

Procedure

The gas to be calibrated is first transferred from the high-pressure cylinder to a previously evacuated ($< 10^{-1}$ torr) 30-l Pyrex vessel. This enables precise control of the sample pressure to be injected into the gas chromatograph. Before sampling the gas, the stainless-steel line and the gas sampling loop are evacuated using a mechanical pump through valve V_1 while valves V_2 and V_3 remain closed. Once the vacuum is established, V_1 is closed and the gas is admitted into the sample loop by opening V_3 until the desired pressure is attained. The pressure is then read directly at the manometer head M_1. Thus a known volume of gas at a known pressure and temperature is injected in the gas chromatograph. The pressure was varied in the range 0.3–223 torr, depending on the compound being analysed, which corresponded to molar amounts between $1.6 \cdot 10^{-8}$ and $1.2 \cdot 10^{-5}$ mole. Mixture of gases were prepared by adding successively the individual components, while noting the partial pressures, into the receiving vessel with the manometer head M_2.

When the gas is injected, the Porapak Q and molecular sieve 5A columns are in series. After elution of the hydrogen, the polarity is changed and the Porapak Q is isolated to permit separation of O_2, N_2, CH_4 and CO on the molecular sieve column. After the elution of the last gas present on the molecular sieve, the carrier gas is switched back to the Porapak Q for the analysis of CO_2, light hydrocarbons and a few other components.

The analysis of H_2, O_2, N_2, CH_4 and CO is effected isothermally at 40°C. For the other gases eluting on the Porapak Q column, the temperature is kept isothermal at 40°C for 4 min and is then raised to 200°C at a controlled rate of 8°C/min.

The gas chromatograph was run at two different sensitivity levels which corresponded to the longer life (low sensitivity) and the medium life (normal sensitivity) expectancy of the detector. Low sensitivity of the katharometer was obtained by setting the bridge current to 200 mA with the temperature of the detector controlled at 150°C. Normal sensitivity corresponded to a current of 275 mA while the temperature of the detector was decreased to 110°C. The attenuated signal output from the detector was displayed on a 4–64 mV span recorder. The slope sensitivity of the integrator was fixed at 0.3 mV/min.

RESULTS AND DISCUSSION

The calibration data were obtained for the main pyrolytic gases mentioned above at low and normal sensitivity detector settings. Typical results are presented in Figs. 2 and 3 for CO_2, CO and N_2 where the area response variable from the electronic integrator was plotted as a function of the number of moles of the compound analysed. The calibration graphs in Fig. 2 obtained under normal sensitivity conditions passed through the origin[10,11]. This was not always the case under the low sensitivity conditions, however, where it was noted that the lower portion of the graphs for all of the gases considered was slightly deflected toward the abscissa axis (Fig. 3), At low sensitivity the equations of the straight portion of the lines were calculated using the least-squares method, in which case the correlation coefficient was always better than 0.990 (4 degrees of freedom). At normal sensitivity the slopes of the resulting straight lines were calculated by taking the derivative of S, the sum of squares of the deviations of each value $y = bx$, with respect to b, setting it equal to zero for a minimum, and solving for b. The plots exhibited genuine linear relations with a correlation coefficient which varied between 0.993 and 0.999 (4 degrees of freedom).

The response factors were calculated following the usual procedure with nitrogen as reference. At low sensitivity the factors were found to be strongly dependent on the amount of gaseous compound injected, as shown in Fig. 4 for CH_4, CO, CO_2 and C_2H_4. Such drastic dependences between the response and the concentration were observed with heavier carrier gases at low concentrations only, while the probability of this anomalous phenomenon occurring was thought to be minimal with H_2 or He as carrier gas[12]. At normal sensitivity the response factors were easily derived from the direct ratio of the b values with respect to the reference and obviously were found to be independent of the number of moles injected. The factors are given in Table I for further comparison with other data available in the literature.

Hydrogen has been kept apart as it exhibited anomalous chromatographic behavior even at normal sensitivity, as indicated by the non-linear calibration graphs

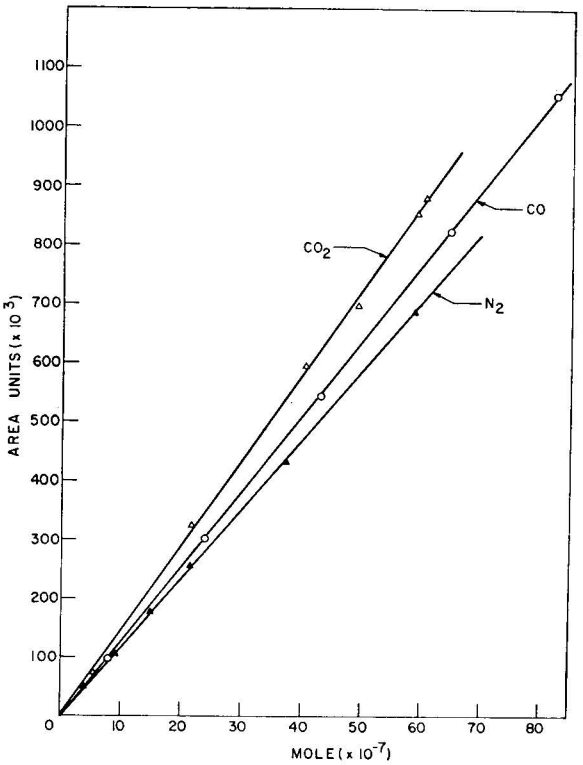

Fig. 2. Calibration graphs for CO_2, CO and N_2. Normal sensitivity.

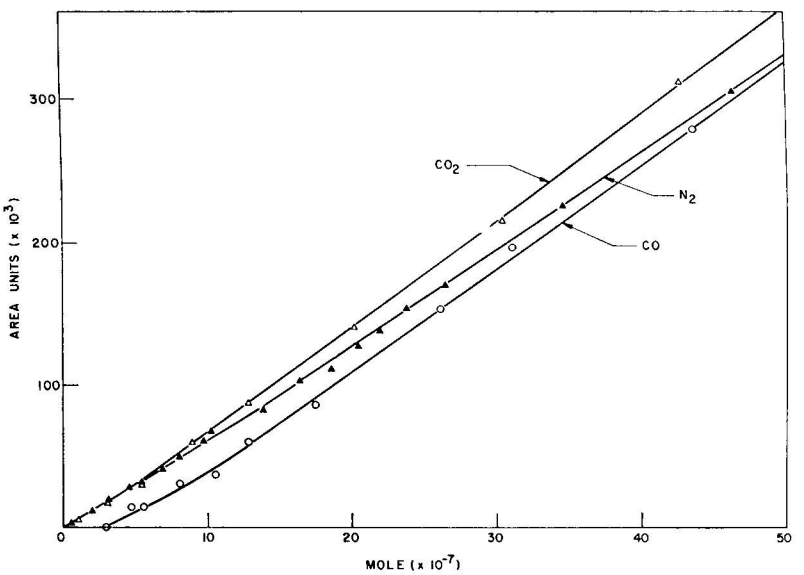

Fig. 3. Calibration graphs for CO_2, CO and N_2. Low sensitivity.

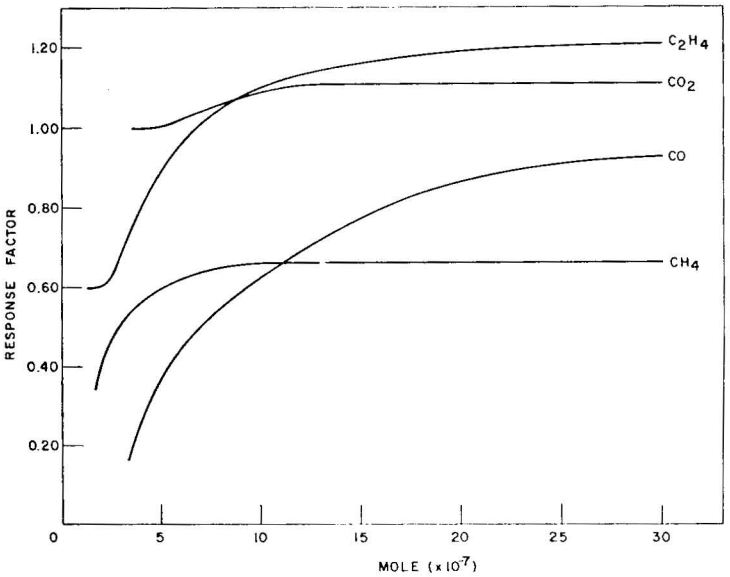

Fig. 4. Response factors for CO_2, CO, CH_4 and C_2H_4. Reference factor: nitrogen = 1.0. Low sensitivity.

in Fig. 5. The concave-type curvature of the H_2 calibration profiles at low concentrations is in sharp contrast with the convex-type curvature expected at higher concentrations[14,15]. The resulting response factors for H_2 are given as a function of the sensitivity levels of the detector in Fig. 6. As expected, the response factor was highly dependent of the amount of gas passing through the detector.

It is necessary to examine the relative responses given by similar detectors, since Messner *et al.*[7] concluded that their data should be applicable to all gas chromatographs using thermal conductivity detectors and helium as carrier gas. A comparison

TABLE I

COMPARISON OF RESPONSE FACTORS TO DIFFERENT THERMAL CONDUCTIVITY DETECTORS

Gas	*Response per mole relative to nitrogen*		
	Present work *	*Messner et al.*[7] **	*Guillemin et al.*[13] ***
H_2	Variable	—	—
N_2	1.00	1.00	1.00
CH_4	0.88	0.86	0.92
CO	1.06	1.00	1.00
CO_2	1.20	1.14	1.20
C_2H_4	1.11	1.14	1.27
C_4H_{10}	2.04	2.02	—

* Carrier gas: 8% H_2 in He. Normal sensitivity (275 mA, 110°C), Hewlett-Packard hot-wire detector.

** Carrier gas: He. Unspecified sensitivity, thermistor-type detector.

*** Carrier gas: He. Normal sensitivity (250 mA, 95°C). Gow-Mac hot-wire detector.

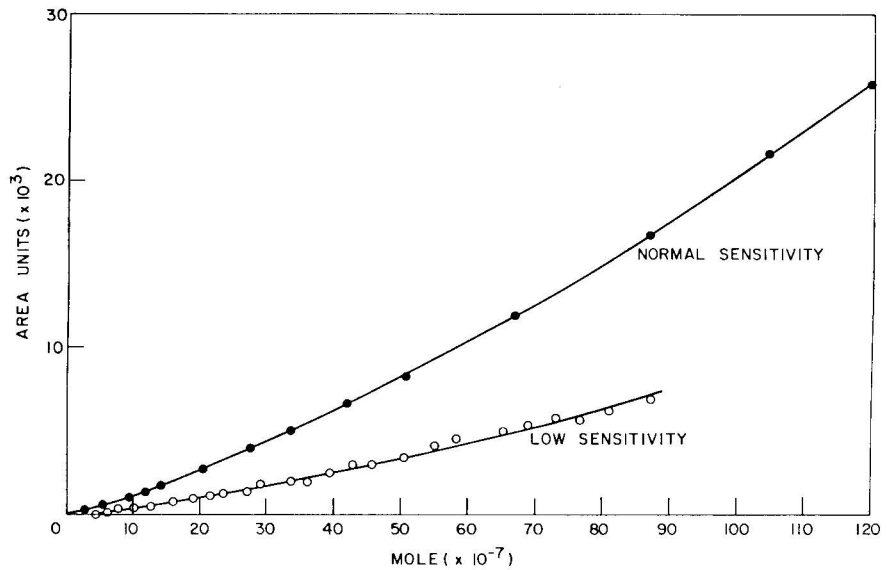

Fig. 5. Calibration graphs for H_2. Low and normal sensitivity.

of their results and those of other investigators using similar instruments is shown in Table I. Although some agreement can be found in the results, there is, as yet, not enough published work on relative responses to show the extent to which the claimed reproducibility exists.

The resulting response factors were tested against a synthetic blend at known composition of H_2, CH_4, CO and CO_2. Five analyses of the same blend were run at a

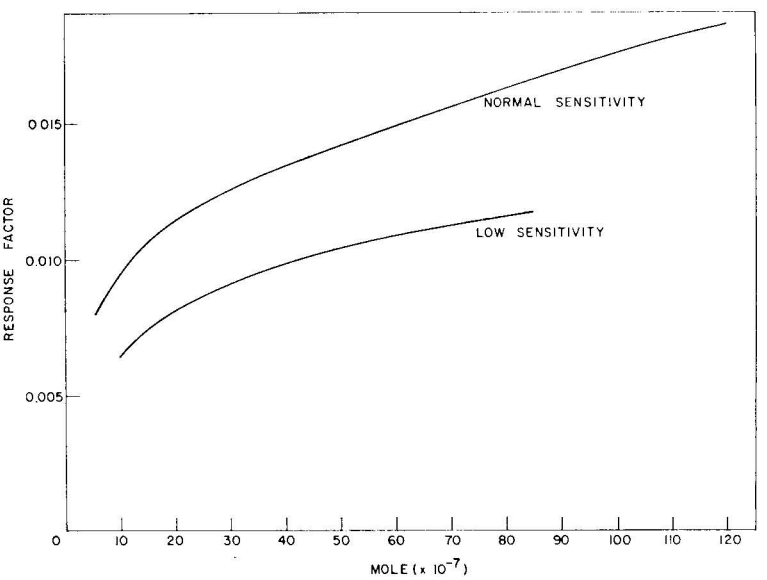

Fig. 6. Response factors for H_2. Reference factor: nitrogen = 1.0. Low and normal sensitivity.

sample pressure of 350 torr with the 1-ml sampling loop. The detection was performed with the normal sensitivity setting of the katharometer. The resulting average values are presented in Table II. Based on the specified true molar percentages, the relative error is not higher than 2.6% in the worst instance.

TABLE II

ANALYSIS OF SYNTHETIC BLEND

Normal sensitivity. Sample volume: 1 ml. Total pressure: 350 torr.

Gas	*True molar %*	*Observed molar %*	*% Error*
H_2	25.70	26.36	2.6
CH_4	18.32	18.53	1.1
CO	22.34	21.79	2.5
CO_2	33.64	33.32	1.0

CONCLUSION

When injecting small amounts of gases into the gas chromatograph the relative responses provided by the katharometer are significantly influenced by the sensitivity of the detector. At low sensitivity, the response factors vary with the amount of compound injected, but at normal sensitivity the relative responses for all the gases considered except H_2 are constant with respect to the reference. In contrast the response factor for H_2 is very sensitive to the carrier gas flow-rate, as its calibration graph is not linear with respect to the reference. Close control of the carrier gas flow-rate is therefore strongly recommended.

ACKNOWLEDGMENTS

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CHROM. 12,940

STUDY OF THE KINETICS FOR A CATALYZED REACTION USING GAS CHROMATOGRAPHY

ROBERT L. GROB*,* and JEAN B. LEASURE
Chemistry Department, Villanova University, Villanova, PA 19085 (U.S.A.)
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SUMMARY

A technique was used which allowed us to study the kinetics of vapor phase catalysis reactions occurring in a gas chromatographic column. The chemical "reactor" is actually the front section of the chromatographic column in the injection port of the chromatograph. The first order decomposition of isopropanol over MnO was the model reaction with which the system is demonstrated. A series of experiments at various flow-rates and temperatures was conducted in order to obtain the kinetic data. Analysis of these data indicated excellent agreement with literature values for the activation energy of the reaction.

INTRODUCTION

In recent years gas chromatography (GC) has been recognized by the catalyst industry as a powerful tool having both analytical and non-analytical applications in the research and development of catalysts. One important non-analytical application is the determination of mechanisms, kinetics and thermodynamics of chemical reactions.

There are two approaches to the utilization of GC in studies of kinetics and catalysis. The first is the microcatalytic technique developed by Kokes *et al.*[1]. In this method the effluent from a micro-reactor chamber is diverted to a gas chromatograph which is fitted with an analytical column used for separating the products and reactants. In the second method, the on-column catalysis or chromatographic reactor approach, both the catalytic effect and the chromatographic separating power of the catalyst are used simultaneously. Bassett and Habgood[2] pointed out that in this technique one could determine the extent of adsorption of the reactant under experimental conditions and thus, the rate constant for the reaction at the catalytic surface.

In the described work a very simple combination of the two techniques was used in order to study the kinetics of a vapor phase catalysis reaction. A small amount of catalyst was placed in the front section of a chromatographic column. The injection port of the gas chromatograph controlled the temperature of the catalyst bed.

* On leave at the University of Hawaii at Manoa as Visiting Professor of Analytical Chemistry.

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The catalyst bed was followed immediately by an analytical column for the separation of products from unreacted starting material. A small glass-wool plug was used to separate the catalyst bed from the analytical column. In order to test the feasibility of this system, the decomposition of isopropanol over MnO was studied. This reaction is frequently used as a model in studies of catalytic activity of non-metallic catalysts[3,4].

EXPERIMENTAL

Chromosorb W AW DMCS (60–80 mesh) obtained from Supelco (Bellefonte, PA, U.S.A.) was used as the inert support for the analytical column. The liquid stationary phases, SP-2401 and Carbowax 1500 also purchased from Supelco, were coated on the support with loadings of 20 and 0.1%, respectively. The coated material was packed in a 12 ft. × 0.25 in. O.D. glass column, leaving only the section that fits into the injection port to be filled with the catalyst. Manganese(II) oxide (99%+) was obtained from Chemetals Corporation (Baltimore, MD, U.S.A.) and sieved to a constant mesh range size (60–80 mesh) suitable for packing in the chromatographic column. The front of the column was packed with 1.110 g of this catalyst. The temperature of the catalyst bed was monitored by a thermocouple pyrometer with parallax correction mirror. For measurements of retention volume of the reactant on the catalyst, a 15 in. × 2 mm I.D. glass column packed with 2.596 g of MnO was used. A Varian Model 1840-1 gas chromatograph equipped with dual flame-ionization detectors was connected to a Vidar Autolab digital integrator. The helium carrier gas was dried and purified over molecular sieve and indicating calcium chloride and regulated by a differential flow controller.

Isopropanol and acetone were "Photrex" reagent-grade (suitable for use in ultraviolet spectrophotometry) and obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Pure reactant vapor was injected by means of a 500-μl Hamilton gas-tight syringe (with a Chaney adapter) after sampling the headspace of a previously evacuated, carefully thermostatted vial fitted with a screw cap Mininert valve. The vial was completely filled with the pure isopropanol liquid and after putting the Minenert value in place, a large bore syringe needle was inserted through the septum. Approximately half the liquid in the vial was withdrawn by vacuum into a trap. This ensures that the entire headspace above the remaining liquid is pure isopropanol vapor in equilibrium with isopropanol liquid. The vial and gas-tight syringe were then thermostatted at 31.0°C, just slightly above ambient temperature. Because of the small difference in temperature, problems such as condensation or adsorption of the reactant vapor on the syringe wall were not encountered. The gas-tight syringe, block heater for thermostatting, vial and Mininert valve were all obtained from Supelco. This headspace sampling was necessary so as to remain within the linear region of the adsorption isotherm and not overload the column and/or the detector.

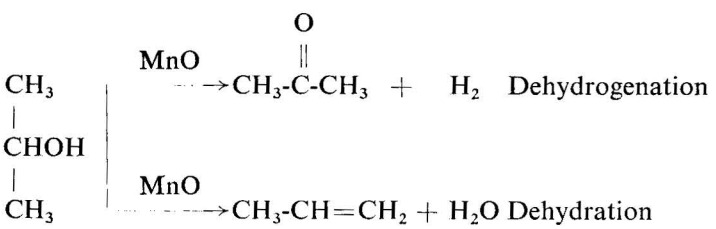
RESULTS AND DISCUSSION

Several workers[5–7] have described experimental set-ups for studying reaction kinetics using GC. These often require special switching valves, pressure regulators,

traps for products and reactants prior to separation and other equipment modifications. Even a set-up such as that described by Choudary[8], in which no modifications of the GC unit itself are made, still requires special valves and design of an appropriate microreactor chamber.

In this work a study was undertaken to determine the feasibility of using a single GC column filled with an analytical packing and a small amount of catalyst in the injection port section of the column, for obtaining kinetic data on vapor phase catalysis reactions. Injections would be made via a gas-tight syringe and no special traps would be necessary. Initial reservations regarding inadequate temperature control of the catalyst bed by the injection port heater were proven to be unfounded.

In using the GC method for kinetic measurements, generally only first-order or pseudo first-order reactions permit quantitative studies. This is because the partial pressure of the reactant varies from point to point through the reactant pulse, and it is only for a first-order reaction that the fractional conversion of reactant to products is independent of pressure. A second criterion for using the GC method for kinetic studies is that the rate of adsorption must be fast relative to the rate of the surface reaction for the latter to be the rate controlling stop. With this in mind the reaction chosen for this investigation was the catalytic decomposition of isopropanol vapor on manganese(II) oxide. The two main paths of this decomposition are:



The dehydrogenation reaction predominates at all temperatures with an average selectivity of 0.80 (ref. 4). However, under the conditions of the reaction in this investigation only the dehydrogenation and not the dehydration reaction was observed.

Initially, a series of injections of varying amounts of pure isopropanol vapor were made in order to determine an amount that would be consistent with remaining on the linear portion of the adsorption isotherm, since this is a requirement for the subsequent data analysis. This study revealed that this could be accomplished only by head-space sampling of pure isopropanol vapor. The amount of reactant (isopropanol) injected is then calculated by using the ideal gas law ($PV = nRT$), where P is the vapor pressure of the pure vapor at the temperature at which the vials are thermostatted and V is the volume injected from the gas-tight syringe. Corrections for deviations from ideality by use of virial coefficients were found to be unnecessary.

The catalysis was carried out at five different flow-rates from 10 to 40 cm^3/min of helium at a constant injector temperature, in order to test the first order rate law and determine the rate constant for the surface reaction. Fig. 1 shows a sample reaction chromatogram. As will be seen later, it was necessary to collect experimental data to obtain values for a Van 't Hoff plot. This experiment consisted of determination of the corrected retention volumes of the reactant on the catalyst at a series of temperatures and constant flow-rate. Values for the Arrhenius plot were determined

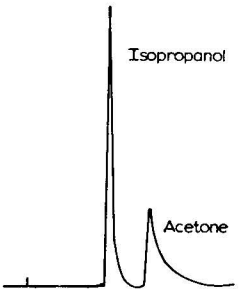


Fig. 1. Chromatogram of unreacted isopropanol plus product (acetone) from the dehydrogenation of isopropanol on MnO. Operating conditions: column, 12 ft. × 1/4 in. O.D. × 2 mm I.D. glass, packed with 20% SP 2401 + 0.1% Carbowax 1500; amount of catalyst, 1.110 g 60–80 mesh MnO. Flow-rate, 28.3 cm^3/min; helium; injector (catalyst) temperature, 198°C; column temperature, 100°C. Amount injected: 400 μl of isopropanol vapor maintained at 31.0°C.

by varying the temperature of the catalyst bed between 150 and 200°C at a constant flow-rate.

Calibration of the resultant chromatographic peaks was accomplished by injection of controlled amounts of acetone at the conditions of each catalysis run. Again, it was necessary to use headspace sampling so as to remain within the linear region of the adsorption isotherm. Acetone may undergo condensation reactions on MnO, but under the conditions used in this investigation (198°C) these were not observed. A material balance of product and reactant was calculated in order to confirm this.

Analysis of the chromatographic data was performed according to the treatment outlined by Bassett and Habgood[2]. Briefly the treatment is as follows. For a rate-controlled surface reaction, the instantaneous rate of reaction of the adsorbed reactant in a section of catalyst is given by:

$$(-)\frac{dn}{dt} = \frac{kKnRT}{(V_g + V_r)} \tag{1}$$

where k is the first-order rate constant of the surface reaction, K is the adsorption equilibrium constant given by $K = V_r/(RT)$, n is the number of moles of reactant, R is the gas constant, and T is the temperature of the catalyst. V_g is the volume of gas space in a section of the reactor containing 1 g of catalyst and V_r is the chromatographic retention volume for 1 g of catalyst, measured at column temperature and corrected for column dead space. Summing over the catalytic reactor and integrating gives an equation of the form:

$$\ln\,[1/(1 - x)] = \frac{kKRT}{(V_g + V_r)}\,t \tag{2}$$

where x is the fractional conversion and t the residence time of the pulse. If the flow-rate of carrier gas in the reactor is F (cm^3/sec) at column temperature, T, and mean pressure, P, and W is the total weight of catalyst, t can be given by:

$$t = \frac{(V_g + V_r)\,W}{F} \tag{3}$$

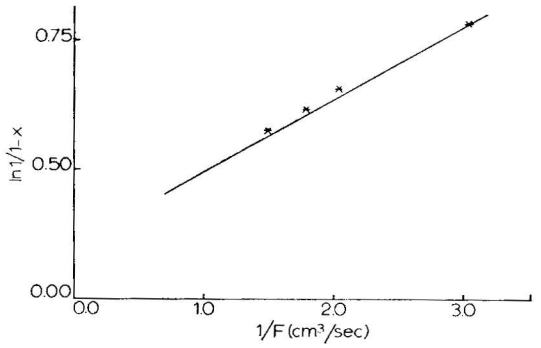


Fig. 2. Plot of ln $[1/(1 - x)]$ *vs.* $1/F$ for decomposition of isopropanol on 1.110 g MnO at 192°C. $kK = 2.72 \cdot 10^{-6}$ mole/atm·g·sec.

Substituting for t gives:

$$\ln [1/(1 - x)] = \frac{RTW}{F} kK \tag{4}$$

This result is analogous to the equation giving the conversion in a conventional flow reactor under steady state conditions, and holds for a catalyst column of any length.

Once x has been calculated from the chromatographic data, it can be seen from eqn. 4 that a plot of ln $[1/(1 - x)]$ *vs.* $1/F$ will yield a straight line whose slope is proportional to the product of the rate constant for the surface reaction and the adsorption equilibrium constant. Fig. 2 depicts this plot, with the product kK equal to $2.72 \cdot 10^{-6}$ mole/atm·g·sec.

In order to determine k one must first find the value of K from a plot of ln V_r^0 *vs.* $1/T$, as shown in Fig. 3. From the slope of this plot one can also determine ΔH_{ads} for the reaction.

The apparent activation energy, E_a, may be obtained from the slope of a plot of ln kK *vs.* $1/T$, and at constant flow-rate, this is equivalent to a plot of ln [ln $1/(1 - x)$] *vs.* $1/T$ (Fig. 4). For the isopropanol decomposition on MnO the apparent activation energy obtained from this graph is 29.1 kcal/mole. One can subseuqently determine

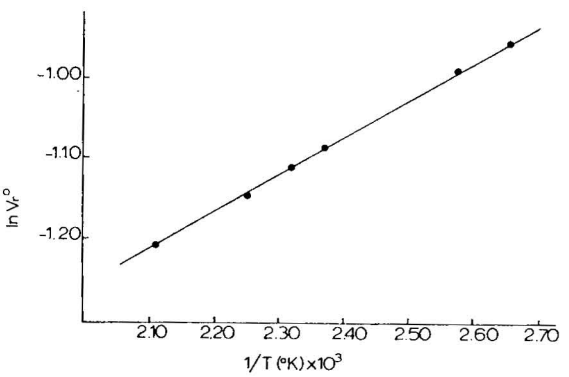


Fig. 3. Plot of ln V_r^0 *vs.* $1/T$. $\Delta H_{ads} = 0.847$ kcal/mole; $K = 3.498 \cdot 10^{-6}\, e^{847/RT}$.

the activation energy of the surface reaction (29.9 kcal/mole) by summing the apparent activation energy and the heat of adsorption from the plot of ln V_r *vs.* $1/T$. This value is in good agreement with the value of 30 $\pm$ 2 kcal/mole found for a steady-state flow reactor[4] and the earlier value of 31.9 kcal/mole from adsorption coefficients[9].

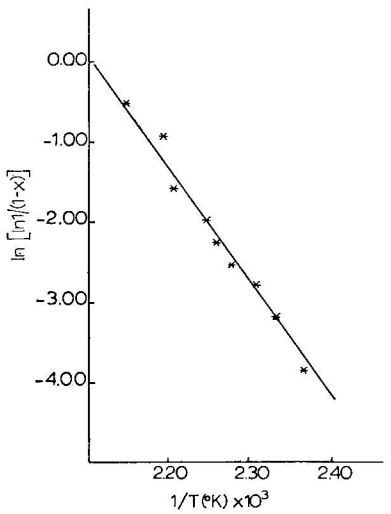


Fig. 4. Arrhenius plot at constant helium flow-rate (27.5 cm³/min). Energy of activation equals 29.1 kcal/mole.

CONCLUSION

The kinetics of a reaction occurring in the vapor phase on a non-metallic catalyst may be determined using a very simple GC technique. Control of the various parameters (*e.g.*, flow-rate, temperature, pressure, weight of catalyst and amount of reactant) enables one to calculate reliable data from a dynamic system. Hopefully, the applications of this technique may be extended to studies of catalyst selectivity and predictions of optimum conditions as well as the best possible catalyst for a given reaction.

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CHROM. 12,921

GAS CHROMATOGRAPHIC STUDY OF SOLUTION AND ADSORPTION OF HYDROCARBONS ON GLYCOLS

I. DIETHYLENE GLYCOL AND TRIETHYLENE GLYCOL

E. L. ARANCIBIA and J. A. CATOGGIO*

División Química Analítica, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 esq. 115, 1900 La Plata (Argentina)

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SUMMARY

Interfacial adsorption is shown to influence the gas chromatographic retention behaviour of hydrocarbons when using glycols as stationary phases. This interfacial effect can be neglected only for lower aromatic hydrocarbons, as demonstrated by the determination of partition and interfacial adsorption coefficients.

Activity coefficients at infinite dilution were calculated; they compare favourably with those determined statically when using the method proposed by Martire *et al.* for the evaluation of exposed areas, whereas the use of experimental values given by Serpinet *et al.* led to erratic results.

Solution and adsorption thermodynamic characteristics of the systems studied are briefly discussed.

INTRODUCTION

Gas chromatography has been used successfully in the determination of thermodynamic parameters of solution, especially when dealing with non-electrolytes, and very precise results are obtained whenever a single retention mechanism occurs. Much work has been published, particularly on infinitely dilute systems[1,2].

The gas chromatographic study of solutions may be complicated by solute adsorption at the gas–liquid interface[3,4]. As the stationary phase is dispersed on a porous solid, the ratio between the interfacial area and liquid volume is large, thus facilitating adsorption processes, especially in systems exhibiting large positive deviations from Raoult's law, where the solute tends to be expelled from the bulk of the solvent towards its surface, basically because solvent–solvent interactions are much more intense than those between solute and solvent molecules.

Mixed processes, involving both dissolution and adsorption, might be expected to govern chromatographic retention in systems constituted by hydrocarbons and highly polar stationary phases; as highly active sites at the surface of the solid

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support are blocked by solvent molecules, it is improbable that adsorption of the hydrocarbon might occur on such a surface.

Under these circumstances, the net retention volume per gram of packing, V_N^o, is expressed by the equation proposed by Martin[3]:

$$V_N^0 = K_L V_L + K_A A_L \tag{1}$$

where K_L is the partition coefficient, equal to the limiting slope in the graph of concentration in the stationary phase *vs.* concentration in the vapour phase when the latter tends to zero, K_A^4 is the adsorption coefficient at the gas–liquid interface, equal to the ratio between the solute concentration in excess at the surface as compared with the bulk concentration in the liquid and the concentration in the gaseous phase, V_L is the volume of the stationary liquid phase per gram of packing and A_L is the exposed surface area of same liquid per gram of packing.

Physico-chemical measurements by means of chromatographic techniques are of special interest in extraction processes on an industrial scale. Thus, the separations of aromatic hydrocarbons from oil fractions in which they are mixed with paraffinic and naphthenic hydrocarbons are conducted with high-polarity solvents, leading to the formation of strongly non-ideal solutions. The study of these systems and the further design of extraction operations are intimately related to activity coefficients at infinite dilution.

When evaluating the selectivity of a given solvent, the limiting separation factor parameter, $\beta_{S/A}^{\infty}$, is used, this being defined as $\beta_{S/A}^{\infty} = \gamma_S^{\infty}/\gamma_A^{\infty}$, where γ_A^{∞} and γ_S^{∞} are the activity coefficients of an aromatic and a saturated hydrocarbon, respectively, both at infinite dilution, in the solvent. The greater the value of $\beta_{A/S}^{\infty}$, the more selective the solvent will be.

For solutions with highly positive deviations from ideal behaviour, the solvent capacity towards aromatic hydrocarbons might be approximated by $X_A = 1/\gamma_A^{\infty}$, where X_A is the molar fraction.

In this work we studied the nature of the phenomena that control the chromatographic behaviour of hydrocarbons within columns containing a glycol (diethylene or triethylene glycol) as the stationary phase, and tried to quantify the individual contributions ascribable to solution and to adsorption at the gas–liquid interface, so as to be able to correct, when necessary, the retention parameters, particularly activity coefficients at infinite dilution, already available in the literature[5].

The use of values for surface areas determined experimentally elsewhere[6] on comparable systems is matched with the reasoning developed and proposed by Martire *et al.*[7] for the calculation of thermodynamic functions of solution and of adsorption on the gas–glycol interface.

EXPERIMENTAL

Apparatus

The equipment used was as described earlier[8]. Columns were thermostated to within 0.05°C by immersion in a modified Lauda U3 bath. Analytical-grade nitrogen, previously purified by passing through a trap charged with 5A molecular sieve, was used as the carrier gas. A Model 8743 Brooks regulator made it possible to control the

flow. In spite of the low vapour pressures of diethylene and triethylene glycol within the experimental temperature range, a 50 cm × $\frac{1}{4}$ in. I.D. pre-column, packed with a filling containing the highest percentage of stationary phase used and submerged in the same bath as the analytical column, was inserted between the flow regulator and the injector[8] so as to keep carrier gas saturated with stationary phase, thus ensuring stability of the packing.

Columns were built from 1/4-in. O.D. stainless-steel tubing, with a length of 25 cm when handling aromatic hydrocarbons and 100 cm for the remaining hydrocarbons. The carrier gas flow-rate was measured by means of a bubble flow meter at the exit of the detector (Hewlett-Packard Model 5750 flame-ionization detector); the exit pressure was always room pressure, and was measured every 2 h.

Columns and reagents

The solvents (stationary phases) used were diethylene glycol (Carlo Erba, Milan, Italy; chromatographic grade) and triethylene glycol (Merck, Darmstadt, G.F.R.; for synthesis purposes), both distilled at reduced pressure under nitrogen and percolated through a column packed with 4A molecular sieve, in an attempt to exclude water as far as possible; Karl Fischer determinations showed that the final products contained, in both instances, less than 0.10% of water. The purity of the solvents was checked by chromatography at 180°C, using a 180 cm × 1/8 in. O.D. stainless-steel column with 4% SE-30 as the stationary phase on 80–100-mesh Chromosorb W.

Acid-washed (with a mixture of concentrated hydrochloric and nitric acids) 60–80-mesh Chromosorb P was used as the solid support. Packings were prepared by carefully weighing both the solid support and the stationary phase in a tightly closed

TABLE I

COLUMN CHARACTERISTICS AT 25°C

Stationary phase	*Parameter**	*Percentage*						
		24.70	*19.70*	*14.73*	*11.97*	*9.696*	*5.778*	*5.095*
Diethylene glycol	W_1	2.3863	2.1328	1.8529	—	1.8272	1.7040	—
	W_2	9.3426	8.4275	8.0258	7.8130	—	—	7.4524
	V_L	0.2220	0.1771	0.1324	0.1076	0.0871	0.0519	0.0458
	A_L (1)	0.281	0.504	0.744	0.883	1.01	1.26	1.31
	A_L (2)	0.95	1.15	1.43	1.62	1.81	2.22	2.32
		Percentage						
		24.44	*23.94*	*19.81*	*18.97*	*14.55*	*9.396*	*9.088*	*4.651*
Triethylene glycol	W_1	2.1286	—	2.0659	—	1.9910	—	1.6620	1.6262
	W_2	—	9.2990	—	8.5296	8.4145	7.8778	—	7.2742
	V_L	0.2183	0.2138	0.1769	0.1694	0.1300	0.0839	0.0812	0.0415
	A_L (1)	0.291	0.315	0.504	0.543	0.747	1.04	1.05	1.34
	A_L (2)	0.97	0.98	1.15	1.18	1.45	1.83	1.85	2.37

* W_1 = grams of packing in a 25-cm column; W_2 = grams of packing in a 100-cm column; V_L = volume of liquid stationary phase at 25°C per gram of packing; A_L (1) = area of liquid stationary phase per gram of packing (m^2/g), calculated according to Martire *et al.*[7]; A_L (2) = area of liquid stationary phase per gram of packing (m^2/g), using experimental data from Serpinet *et al.*[6].

flask, avoiding unnecessary exposure to the atmosphere; the flask was subsequently rotated for 10 h at approximately 50 rpm. The stationary phase to solid support ratios and other column characteristics are given in Table I.

The purity of each hydrocarbon used was greater than 99%; as the solute purity is not a critical factor in this kind of study, they were used without further purification. Twenty-one hydrocarbons of different types were studied (Table II).

Procedure

Hydrocarbons were injected with a 10- or a 50-μl Hamilton syringe, applying the headspace sampling technique. In each instance the sample size was the smallest compatible with the detector noise level. Every solute gave symmetric peaks, thus proving that, wathever the retention mechanism, runs have been conducted within the concentration range for which Henry's law is obeyed.

The hydrocarbons were always injected individually, together with a small amount of methane. Adjusted retention times were measured on the chart between the methane peak and the solute peak maximum. Calculations were made based on three individual values of the retention time for each set of solute, column and temperature. Carrier gas flow-rates were kept between 15 and 80 cm^3/min, so as to obtain reasonable retention times. The columns were operated at four different temperatures within the range 20–31°C.

RESULTS

Graphs of net retention volume per gram of packing *versus* stationary phase percentage are shown in Fig. 1 for some representative hydrocarbons in both solvents. It can be seen that the retention behaviour of none of the systems except benzene could be explained by a single mechanism, as otherwise each pair of straight lines would converge at the origin.

Considering the evidence for mixed processes, the most probable sorption mechanisms are (1) dissolution in the stationary phase, (2) adsorption at the gas–liquid interface, and (3) adsorption on the solid support. The last of these mechanisms would occur either through direct adsorption of solute molecules on bare active sites on the support or through displacement of glycol molecules from some of the active sites. Both processes are highly improbable because, on the one hand, the least percentage of stationary phase used (4.651%) largely exceeds the amount required for a monolayer to be formed and, on the other, in addition to the fact that the amounts of both stationary phases are considerably greater than the small amount of solute, the polarity of the glycol molecules makes it very unlikely that they are replaced from the acidic sites on the surface of the support by hydrocarbon molecules. These arguments are supported by other studies[9–11].

Further study of the behaviour of solutes in solution and on the gas–liquid interface making use of eqn. 1 requires a knowledge of the exposed areas of stationary liquid.

These areas have been calculated using two different approaches: firstly, and on the basis of the already established[6,12,13] fact that the exposed area does not depend on the nature but on the volume of the stationary (liquid) phase, they have been estimated by interpolation and extrapolation between and beyond values measured

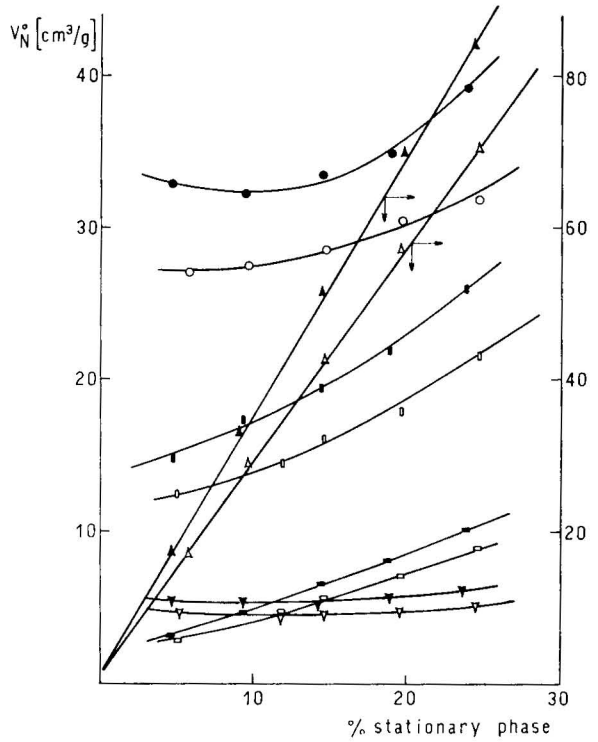

Fig. 1. Representative net retention volumes per gram of column packing as a function of stationary phase percentage: ○, ●, *n*-nonane; △, ▲, benzene; ▯, ▮, 1-octene; ▭, ▬, cyclohexane; ▽, ▼, 2,2,4-trimethylpentane. Open symbols, diethylene glycol; closed symbols, triethylene glycol.

directly[6] for different percentages of glycerol used as the stationary phase on Chromosorb P.

Secondly, they were calculated following Martire *et al.*'s proposal[7], making use of the K_L and K_A values they determined for cyclohexane on thiodipropionitrile in a tightly closed chamber containing a McBain balance and a Du Nouy tensiometer to compare chromatographic data with static values, and retention volumes obtained by Martin[3] for this system, assuming that for the same stationary phase to Chromosorb P ratio both glycols and thiodipropionitrile exhibit the same exposed surface area, an assumption validated by the coincidence of the surface area values obtained when they are measured for two different liquids on the same support[3,14].

The values thus obtained are given in Table I. Stationary phase volumes were calculated by using density data at different temperatures compiled by Riddic and Bunger[15].

Net retention volumes (V_N) were calculated from the adjusted retention times and operating conditions in the usual way[16]. V_N data for each column were adjusted to the following equation:

$$\log V_N = -\Delta H/2.3\,RT + \text{constant}$$

were ΔH is the enthalpy of sorption calculated by the least-squares method.

Experimental values of V_N differ from those calculated by interpolation by at most 1%, the differences being 0.5% or less in most instances.

K_L values were obtained by determining (using the least-squares method) the slopes of the lines resulting from plotting V_N^0/A_L *vs.* V_L/A_L; analogously, K_A values were calculated by the same technique from the slopes of the V_N^0/V_L *vs.* A_L/V_L graphs.

The values obtained are given in Tables II and III, together with the 95% confidence intervals obtained from the standard deviations of the slopes and t factors. K_L values obtained for benzene and toluene in both stationary phases from experimental data, when $K_A = 0$ in eqn. 1, fall within the experimental error for K_L calculated by use of the complete equation. In contrast, and as would be expected because of the rare incidence of adsorption processes, the deviation of the K_A values obtained for both solutes is large.

TABLE II

PARTITION AND ACTIVITY COEFFICIENTS AT 25°C

Solute	*Diethylene glycol*		*Triethylene glycol*	
	K_L	γ_2^∞	K_L	γ_2^∞
n-Hexane	11.7 ± 0.7	110	13.7 ± 0.4	67.0
n-Heptane	25.7 ± 1.4	166	30.8 ± 1.1	98.5
n-Octane	55.7 ± 3.4	250	69.3 ± 3.9	143
n-Nonane	122 ± 2	372	155 ± 9	208
Cyclohexane	39.1 ± 1.5	50.9	45.9 ± 1.2	30.8
Methylcyclohexane	53.5 ± 2.4	79.2	64.4 ± 2.4	46.8
Ethylcyclohexane	128 ± 6	117	159 ± 7	67.1
Cyclohexene	84.3 ± 3.0	26.0	100 ± 3	15.6
cis-2-Hexene	25.5 ± 0.3	50.6	29.8 ± 0.8	30.8
1-Heptene	41.7 ± 2.1	83.5	50.0 ± 1.8	49.5
1-Octene	89.3 ± 5.0	128	110 ± 5	74.1
2,2,4-Trimethylpentane (isooctane)	20.8 ± 1.4	195	24.2 ± 1.0	117
2,4,4-Trimethyl-1-pentene (isooctene)	43.6 ± 2.2	109	52.1 ± 2.1	64.7
Benzene	216 ± 2	6.48	383 ± 6	3.80
Toluene	656 ± 2	10.4	809 ± 10	6.01
Ethylbenzene	1250 ± 2	16.2	1551 ± 15	9.31
n-Propylbenzene	2213 ± 9	25.9	2816 ± 17	14.5
Isopropylbenzene	1740 ± 15	24.3	2193 ± 18	13.7
o-Xylene	1899 ± 10	15.5	2418 ± 22	8.68
m-Xylene	1337 ± 3	17.6	1689 ± 19	9.89
p-Xylene	1280 ± 4	17.5	1617 ± 19	9.85

Activity coefficients at infinite dilution, γ_2^∞, were calculated from the usual expression $\gamma_2^\infty = RT/p_2^0 K_L v_1^0$, where v_1^0 is the molar volume of the solvent and p_2^0 the saturation pressure of pure solute at temperature T (°K). Vapour pressures at each temperature were calculated making use of the constants for the Antoine equation compiled by Riddic and Bunger[15]. No corrections were introduced because of non-ideality in the vapour phase, as it was found to be negligible because positive and negative terms cancelled out.

TABLE III

THERMODYNAMIC FUNCTIONS OF ADSORPTION AT 25°C

Solute	*Diethylene glycol*			*Triethylene glycol*		
	$K_A \cdot 10^5$ *(cm)*	$-\Delta H_A^0$ *(kcal/mole)*	$-\Delta S_A^0$ *(e.u.)*	$K_A \cdot 10^5$ *(cm)*	$-\Delta H_A^0$ *(kcal/mole)*	$-\Delta S_A^0$ *(e.u.)*
n-Hexane	8.85 ± 0.54	7.3	9.9	9.84 ± 0.24	6.6	7.4
n-Heptane	23.4 ± 1.2	8.2	11.1	26.9 ± 0.7	7.7	9.1
n-Octane	62.4 ± 3.0	9.8	14.4	74.0 ± 2.8	9.6	13.6
n-Nonane	164 ± 3	10.3	14.1	199 ± 8	10.7	15.3
Cyclohexane	8.03 ± 1.75	7.3	10.1	8.62 ± 0.67	6.6	7.8
Methylcyclohexane	18.2 ± 2.0	8.1	11.2	20.6 ± 1.9	8.0	10.5
Ethylcyclohexane	48.0 ± 5.6	9.3	13.4	56.0 ± 5.2	9.6	14.0
Cyclohexene	9.19 ± 2.65	8.3	13.3	9.78 ± 2.42	7.6	10.9
cis-2-Hexene	9.76 ± 0.75	6.7	7.7	11.1 ± 0.5	7.0	8.6
1-Heptene	24.8 ± 1.8	8.5	12.1	28.4 ± 1.5	8.2	10.8
1-Octene	65.6 ± 4.6	9.7	14.2	77.5 ± 3.9	9.8	14.0
Isooctane	28.6 ± 1.1	8.2	10.8	33.0 ± 0.7	7.7	8.7
Isooctene	34.7 ± 1.9	8.5	11.2	40.4 ± 1.6	8.6	11.5
Benzene	10.1 ± 2.4	2.8	5.5	8.58 ± 5.53	5.5	4.0
Toluene	27.7 ± 2.0	7.0	6.8	28.4 ± 10.0	8.7	12.5
Ethylbenzene	73.1 ± 4.2	8.7	10.5	84.2 ± 15.3	9.2	12.0
n-Propylbenzene	210 ± 10	10.3	13.8	241 ± 20	10.1	12.9
Isopropylbenzene	172 ± 12	10.5	14.9	201 ± 17	9.7	11.7
o-Xylene	83.7 ± 8.7	10.3	15.6	86.8 ± 18.6	8.0	8.0
m-Xylene	80.3 ± 2.5	9.5	12.9	89.1 ± 15.5	9.0	11.0
p-Xylene	79.0 ± 7.5	9.4	12.7	88.1 ± 15.7	9.0	11.1

Enthalpies of solution, ΔH_S^0, were calculated from the slopes of the ln K_L *vs.* $1/T$ graphs; a correction term, $RT(1 - \eta T)$, was subtracted, where η is the coefficient of thermal expansion of the stationary phase. As Meyer[17] has proved, the enthalpies thus corrected correspond to the transference of 1 mole of solute from an ideal vapour phase at 1 atm to a hypothetical solution at 1 molar fraction but obeying Henry's law. Solute molecules under these conditions are exclusively exposed to interactions with solvent molecules; if one wishes to compare the strength of these interactions with those among solute molecules (solute–solute), the enthalpies of solution obtained should be compared with the enthalpies of condensation, ΔH_L^0, to a pure liquid from an ideal vapour at 1 atm. The difference $\Delta H_S^0 - \Delta H_L^0$ gives the excess partial molar enthalpy of solution and corresponds to the energy change for 1 mole of solute when passing from a pure real liquid to a hypothetical liquid, or from an infinitely dilute ideal solution to an infinitely dilute real solution. The correction terms $RT(1 - \eta T)$ obtained from density data compiled by Riddic and Bunger[15] for diethylene and triethylene glycol at 25°C were 473 and 469 cal/mole, respectively.

Standard molar free energies of adsorption were calculated from the equation

$$-\Delta G_A^0 = RT \ln (p^0/\pi^0) K_A \qquad (2)$$

where p^0 is the pressure in the gaseous reference state and π^0 is the surface pressure in the adsorbed standard state. Following Kemball and Rideal[18], a solute vapour behaving ideally at unit pressure (1 atm) was chosen as the standard gaseous state, and a surface film at a surface pressure of 0.0608 dyne/cm as the adsorbed standard state; this value was deduced by assuming a thickness of 6 Å for the surface layer, this being arrived at by calculating a two-dimensional pressure equivalent to a three-dimensional pressure of 1 atm[26]. Under these circumstances, $p^0/\pi^0 = 1.67 \cdot 10^7$ l/cm.

By applying the Gibbs–Helmholtz ratio to eqn. 2, it can easily be shown that the enthalpy of adsorption, ΔH_A^0, can be obtained from the graph of ln K_A *vs.* $1/T$. Adsorption functions are given in Table III.

The precisions of enthalpies of adsorption and solution were estimated as described by Castells[19]; they depend on the relative importance of the retention mechanism. Thus, for aromatic hydrocarbons, for which a solution mechanism prevails, enthalpies of solution attain the same precision as enthalpies of sorption, *i.e.*, $\pm$ 450 cal/mole for triethylene glycol and $\pm$ 300 cal/mole for diethylene glycol, but the uncertainty in the measurement of enthalpies of adsorption is much greater, because of the minor occurrence of this process and its consequent small contribution to the experimental chromatographic parameters. In contrast, for solutes for which adsorption is the dominant mechanism (alkanes), the precision of the enthalpies of adsorption is of the same order as that of enthalpies of sorption, while that of solution is smaller, even though the uncertainty is less than twice that for enthalpies of sorption.

DISCUSSION

The K_L and K_A values listed in Tables II and III were obtained by using areas calculated according to Martire *et al.*[7]. The few experimental data that we were able to take from Serpinet *et al.*[6] lead to highly erratic results and, when the values thus obtained are compared with those from static phase equilibria given in the literature[20], large deviations occur with systems for which surface effects are meaningful.

Calculated activity coefficients at infinite dilution, as given in Table II, show a mean deviation of 10% relative to data from phase equilibria[20]. Experimental values obtained by chromatography[5], without taking into account surface effects, exhibit mean deviations of about 25% as compared with the same data.

Values of γ^∞ obtained in this work for *n*-alkanes in diethylene glycol were compared with those calculated making use of correlations given by Pierotti *et al.*[21] at 25°C; the mean deviations were less than 2%. The same deviation was found when our value for the activity coefficient at infinite dilution of benzene in diethylene glycol, extrapolated to 50°C, was compared, with that obtained chromatographically[22]; this agreement further validates the extrapolation, especially when the negligible effect of adsorption in this system is taken into account.

Each of the hydrocarbons studied showed a positive deviation from Raoult's law in both solvents; as would be expected, the largest deviations pertain to alkanes and the smallest to aromatic hydrocarbons, olefins and cycloalkanes showing intermediate behaviour. Within each type of hydrocarbon, the deviations increase as the number of carbon atoms increases, whereas they are inversely proportional to the molecular weight of the solvent.

Both glycols are associated liquids, so that it is evident that the dissolution of a given hydrocarbon in such a medium will be the more difficult the higher its molecular volume, owing to the larger number of intermolecular bonds that should be dissociated to provide sufficient room for the guest molecule. On the other hand, as both solvents are highly polar, they must be capable of polarizing delocalized electrons in other molecules, so that energetic interactions in solution will be the more intense the more polarizable is the solute. These criteria agree with the trends found when the hydrocarbons studied are compared. Mean deviations of the $\ln \gamma^{\infty}$ value in both glycols of only 4% for aliphatic hydrocarbons and up to 10% for aromatic hydrocarbons are found between present experimental values and those obtained by applying the Scatchard–Hildebrand theory[23] for regular solutions as modified by Weimer and Prausnitz[24] so as to cover systems in which one of the components is polar, making use of the induction energy terms of Helpinstill and Van Winkle[25].

From Table IV it can be seen that enthalpies of solution in both solvents for alkanes and cycloalkanes are always lower than the enthalpies of condensation given in the literature[15], giving excess partial molar enthalpies of solution of 1–2 kcal/mole. This difference is small, approaching zero for aromatic hydrocarbons. Even though experimental evidence to explain the results is scanty, it can be postulated that the dissolution of a hydrocarbon in these solvents involves the energetic effect

TABLE IV

ENTHALPIES OF SOLUTION AND CONDENSATION

DEG = Diethylene glycol; TEG = triethylene glycol.

Solute	$-\Delta H^0_{S(DEG)}$ *(kcal/mole)*	$-\Delta H^0_{S(TEG)}$ *(kcal/mole)*	$-\Delta H^0_L$ *(kcal/mole)*[15]
n-Hexane	6.3	5.6	7.5
n-Heptane	7.3	6.3	8.7
n-Octane	8.4	7.7	9.9
n-Nonane	9.6	8.9	11.0
Cyclohexane	6.7	6.0	7.9
Methylcyclohexane	7.4	6.7	8.4
Ethylcyclohexane	8.4	7.7	9.7
Cyclohexene	7.5	7.0	7.9
cis-2-Hexene	7.0	6.3	7.5
1-Heptene	7.6	6.9	8.6
1-Octene	8.8	8.0	9.7
Isooctane	7.1	6.3	8.4
Isooctene	7.8	6.5	8.8
Benzene	8.4	8.1	8.1
Toluene	9.2	9.0	9.1
Ethylbenzene	10.1	9.8	10.1
n-Propylbenzene	10.7	10.5	11.0
Isopropylbenzene	10.7	10.5	10.8
o-Xylene	10.4	10.3	10.4
m-Xylene	10.0	9.9	10.2
p-Xylene	10.0	9.9	10.1

necessary to produce a cavity where the solute molecule can lodge, and explain the different behaviour of aromatic hydrocarbons through the strong interactions of hydroxyl groups in the glycol molecule with the electron cloud of the aromatic ring.

Excess entropy values can be evaluated from data in Tables I and IV; all of them are negative, between 1 and 4 e.u. in triethylene glycol and from 4 to 7 e.u. in diethylene glycol, without distinctions for different types of hydrocarbons. The values in triethylene glycol indicate a higher mobility of hydrocarbon molecules as a consequence of the longer paraffinic chain in the glycol, which favours greater solubility of hydrocarbon solutes in this solvent.

Table V gives the limiting separation factor or selectivity for the pairs *n*-hexane–benzene and *n*-octane–benzene, together with the reciprocal of the activity coefficients at infinite dilution for benzene in diethylene and triethylene glycol. It is clear that both solvents are selective, triethylene glycol showing a greater capacity towards benzene. It can be seen, as Weimer and Prausnitz claim[24], that solvent selectivity is affected by the molecular size of the hydrocarbon to be separated. Extrapolation to 50°C of values obtained here is also shown in Table V; they keep almost a constant ratio for each pair considered, although the selectivity decreases.

TABLE V

SELECTIVITIES AND CAPACITIES (TOWARDS BENZENE: $X_B = 1/\gamma_B^\infty$)

H = *n*-hexane; O = *n*-octane; B = benzene.

Solvent	*Temperature (°C)*	*Selectivity*		*Capacity*
		$\beta_{H/B}$	$\beta_{O/B}$	
Diethylene glycol	25	17.0	38.6	0.155
	50	13.8	30.4	0.155
Triethylene glycol	25	17.6	37.7	0.263
	50	13.4	27.8	0.263

Table III includes values of the adsorption thermodynamic functions. In spite of their poor precision, it is possible to detect some common trends and features. Enthalpies of adsorption are, in general, smaller than the corresponding enthalpies of liquefaction, indicating that the gas–glycol interface is a low-energy surface.

Kemball and Rideal[18] calculated the entropy variation associated with the transformation of a three-dimensional into a two-dimensional gas; the variation for the hydrocarbons considered here is about −9 to −10 e.u. The values given in Table III, except for a few, are more negative, suggesting that the adsorbed state corresponds to mobile adsorption as defined by Kemball and Rideal for cases where one degree of freedom (that of translation perpendicular to the surface) is lost.

CONCLUSION

It has been demonstrated that for the hydrocarbons considered, under the experimental conditions employed, it is necessary to take into account surface effects when attempting to ascertain the activity coefficients at infinite dilution by chromatography; among the hydrocarbons studied, these effects can be neglected only with benzene and toluene.

In spite of the association of glycol molecules through hydrogen bonds, the modified theory of the solubility parameter enables the γ^{∞} values to be evaluated with good precision (mean deviation of $\ln \gamma^{\infty} = 4\%$ for alkanes and 10% for aromatic hydrocarbons).

The confidence with which thermodynamic functions at the gas–glycol interface can be determined by chromatography is reasonable for alkanes, intermediate for alkenes, cycloalkanes and propylbenzene, and very poor for cycloalkenes and benzene, either unsubstituted or substituted with low-molecular-weight alkyl chains (C_1 and C_2).

Martire *et al.*'s proposal[7] for the estimation of exposed liquid areas gives much smaller deviations than experimental data from Serpinet *et al.*[6] when used for evaluating γ^{∞} in these systems, after comparing the resulting values with those obtained statically by equilibrating the phases involved.

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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF AROMATIC NITRO COMPOUNDS ON A MACROPOROUS POLYSTYRENE GEL

F. ŠMEJKAL, M. POPL*, A. ČÍHOVÁ and M. ZÁZVORKOVÁ
Department of Analytical Chemistry, Section of Instrumental Analysis, Prague Institute of Chemical Technology, Suchbátarova 5, 166 28 Prague 6 (Czechoslovakia)

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SUMMARY

The retention behaviour of some nitrobenzene derivatives was studied by reversed-phase chromatography on the macroporous polystyrene gel Separon SE with methanol–water, acetonitrile–water, tetrahydrofuran–water and methanol–water with adjusted pH as the eluents. The effect of the mobile phase and the presence of additional polar and non-polar substituents in the molecule of the solute on the retention were investigated.

Using methanol–water (80:20) the contribution of the nitro group to the retention of the molecule is positive for substances containing both polar and non-polar functional groups. The contribution of the nitro group did not change if a mobile phase with adjusted pH was used. With acetonitrile–water and tetrahydrofuran–water the situation was more complicated.

INTRODUCTION

In reversed-phase liquid chromatography (RP-LC) separation of substances can be generally characterized by several fundamental features[1]: (i) hydrocarbons are eluted in order of increasing number of carbon atoms in the molecule; (ii) the retention volume decreases if the molecule is substituted with polar functional groups, except nitro and halogens; (iii) steric shielding of polar functional groups increases the retention volume; (iv) the chromatographic system does not retard ions and compounds in the ionic form.

In RP-LC the contribution of a halogen substituent to the retention of the whole molecule is positive, irrespective of the sorbent used and the composition of the mobile phase[2–4]. Halogen exert a positive mesomeric (+M) and a negative inductive (−I) effect. As a consequence of the competition of these two effects operating in opposite directions, halogens are among the weakest first class substituents[5]. Halobenzenes behave in a similar manner to alkylbenzenes in RP-LC, *i.e.*, the retention increases with the bulk (atomic weight) of the halogens.

The situation is more complicated with the nitro group. This group, exerting

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—M and —I effects on the aromatic system, can be classed as a medium polar substituent[5]. Substances containing a nitro group exhibit selective interactions associated with charge transfer between the substance and the mobile phase. When the substance involves additional polar or non-polar substituents, the electron configuration of the molecule alters appreciably, and so does its chromatographic behaviour.

The relationships between the chromatographic behaviour of nitro compounds and their structure have been studied extensively in thin-layer chromatography (TLC) by adsorption on silica gel[6]. In addition to the effect of the position and type of the substituent[7], the dependences of the R_M values on the mobile phase composition were examined[8] and simple molecular models of adsorption of nitro compounds on silica gel were devised[9,10].

The objective of the present work was to study the retention behaviour of aromatic nitro compounds in RP-LC on Separon SE. The effect of the mobile phase composition and of the presence of additional polar and non-polar substituents in the molecule on the retention were investigated.

EXPERIMENTAL

The chromatographic measurements were carried out on a liquid chromatograph with a stainless-steel column (300 × 6 mm I.D.) packed with Separon SE macroporous gel in the form of spherical particles, 25–32 μm in diamater, with an exclusion limit of molecular weight 300,000 daltons. The sorbent was obtained from Laboratorní přístroje (Prague, Czechoslovakia). The eluent was delivered by means of a VLD 30 high-pressure linear dosing apparatus, manufactured at the Development Workshop, Czechoslovak Academy of Sciences (Prague, Czechoslovakia), and a UV (254 nm) detector with a 10-μl cell and an optical path length of 1 cm was obtained from the same manufacturer.

The mobile phases were methanol–water (MeOH–H_2O) (80:20), acetonitrile–water (AcN–H_2O) (63:37), tetrahydrofuran–water (THF–H_2O) (50:50) and MeOH–H_2O (80:20) of pH = 3.3, 7 and 11.9, and the flow-rate was 60 ml/h.

The standards for chromatography and the solvents for the preparation of the mobile phases were of reagent-grade purity.

RESULTS AND DISCUSSION

Some papers dealing with the effect of the mobile phase composition on the retention behaviour of substances containing polar functional groups have reported in some instances different effects of some functional groups on the retention behaviour of the entire molecule[2,4,11]. The differences found for sorbents with chemically bonded phases are probably due to the presence of unreacted and unshielded silanol groups. Silanol groups exert unfavourable effects on the retention of the substances separated and on the peak symmetry, and their influence must therefore be minimized. Sorbents with chemically bonded phases are usually characterized in terms of the amount of the organic phase on the support surface in μmol/m^2, and the blocking of the silanol groups is expressed through the capacity factor values of some polar substances measured in a non-polar eluent. For instance,

Tanaka *et al.*[4] employed anisole, methyl benzoate and acetophenone, using dry *n*-heptane as the mobile phase.

Separon SE is prepared by copolymerization of styrene with ethylene dimethacrylate. This sorbent does not contain hydroxy groups and behaves as a purely non-polar stationary phase[12]. Comparison of the k' values of anisole, methyl benzoate and acetophenone on Separon SE with the results obtained by Tanaka *et al.*[4] for sorbents with chemically bonded phase indicates that Separon SE acts as a sorbent with a completely covered surface (Table I). The capacity factors were measured by using the procedure reported by Tanaka *et al.*[4].

TABLE I

CHARACTERISTICS OF SORBENTS

Values for Hypersil are taken from ref. 4.

Sorbent	*Size of particles (μm)*	*Column dimensions (mm)*	*Surface coverage (μmol/m²)*	*k' with n-heptane mobile phase*		
				Anisole	*Methyl benzoate*	*Acetophenone*
Hypersil C_8	5	150 × 4.6	3.4	0.2	1.6	5.3
Hypersil C_8 + TMS	5	150 × 4.6	3.4	0.1	0.2	0.55
Separon SE	25–32	300 × 6	—	0.1	0.2	0.19

The results of the chromatographic measurements are given in terms of retention indices (I). By Popl *et al.*'s definition[2], $\log I = 1$, 2 and 3 for benzene, naphthalene and phenanthrene, respectively, and the retention index of any substance is calculated as

$$\log I = \log I_n + \frac{\log R_x - \log R_n}{\log R_{n+1} - \log R_n}$$

where R is the reduced retention volume and the subscripts n, $n + 1$ and x refer to the nearest lower and higher standards and the substance in question, respectively.

The retention volumes and capacity factors of benzene, naphthalene phenanthrene are given in Table II.

TABLE II

CORRECTED RETENTION VOLUMES, V', AND CAPACITY FACTORS, k', OF BENZENE, NAPHTHALENE AND PHENANTHRENE

Compound	*log I*	*MeOH–H_2O (80:20)*		*AcN–H_2O (63:37)*		*THF–H_2O (50:50)*	
		V' (ml)	*k'*	*V' (ml)*	*k'*	*V' (ml)*	*k'*
Benzene	1	13.19	3.07	13.54	4.35	13.23	3.05
Naphthalene	2	49.33	11.49	35.71	11.48	21.81	5.03
Phenanthrene	3	184.49	42.99	97.99	31.51	30.55	7.05

Table III list the retention indices ($\log I_{meas}$) of the aromatic nitro compounds examined, together with the differences, $D = \log I_{meas} - \log I_{theor}$. The $\log I_{theor}$ values were calculated from the contributions of the functional groups involved

TABLE III

RETENTION INDICES FOR SUBSTITUTED NITROBENZENES AND DEVIATIONS (*D*)

Compound	*MeOH–H_2O (80:20)*		*AcN–H_2O (63:37)*		*THF–H_2O (50:50)*	
	log I_{meas}	*D*	*log I_{meas}*	*D*	*log I_{meas}*	*D*
Nitrobenzene	1.31	—	0.84	—	0.97	—
m-Dinitrobenzene	1.78	+0.16	0.86	+0.18	1.87	+0.93
Aniline	0.50	—	0.02	—	−0.83	—
o-Nitroaniline	1.31	+0.50	0.75	+0.89	0.15	+1.01
m-Nitroaniline	1.29	+0.48	0.53	+0.67	0.22	+1.08
p-Nitroaniline	1.18	+0.37	0.37	+0.51	−0.66	+0.20
2,4-Dinitroaniline	1.89	+0.77	0.60	+0.90	0.42	+1.31
Toluene	1.23	—	1.22	—	1.52	—
o-Nitrotoluene	1.52	−0.02	1.06	0.00	1.33	−0.16
p-Nitrotoluene	1.55	+0.01	1.10	+0.04	1.36	−0.13
2,4-Dinitrotoluene	2.03	+0.18	1.10	+0.20	2.52	+1.06
2,4,6-Trinitrotoluene	2.58	+0.42	1.34	+0.60	4.99	+3.56
Phenol	0.42	—	0.05	—	−1.06	—
o-Nitrophenol	1.23	+0.50	0.53	+0.64	0.73	+1.82
m-Nitrophenol	1.15	+0.42	0.18	+0.29	−0.07	+1.02
p-Nitrophenol	1.18	+0.45	−0.42	−0.31	−0.32	−0.77
2-Nitro-4-methylaniline	1.59	+0.55	0.91	+0.83	1.49	+1.83
Chlorobenzene	1.36	—	1.33	—	1.69	—
o-Nitrochlorobenzene	1.56	−0.11	1.10	−0.07	1.12	−0.54
p-Nitrochlorobenzene	1.76	+0.09	1.29	+0.12	2.32	+0.66
2,4-Dinitrochlorobenzene	2.06	+0.08	1.25	+0.24	2.91	+1.28
2,5-Dichloronitrobenzene	2.01	−0.02	1.65	+0.15	2.76	+0.41
Acetophenone	0.79	—	0.40	—	0.23	—
p-Nitroacetophenone	1.32	+0.22	0.52	+0.28	1.06	+0.86

(Table IV), which were obtained for a given mobile phase as the differences between the values for the corresponding monosubstituted benzene derivative and benzene itself. In this manner the effect of the various substituents on the retention behaviour of the entire molecule can be conveniently characterized; this approach, however, is not satisfactory when the various substituents in the molecule interact with each other.

Phenol and nitrophenols exhibited lower log I_{meas} values in the AcN–H_2O and THF–H_2O systems than in MeOH–H_2O because of a strong interaction with the

TABLE IV

Δ log I CONTRIBUTIONS FOR VARIOUS FUNCTIONAL GROUPS IN SUBSTITUTED BENZENES

Functional group	*Mobile phase*		
	MeOH–H_2O (80:20)	*AcN–H_2O (63:37)*	*THF–H_2O (50:50)*
NO_2	+0.31	−0.16	−0.03
OH	−0.58	−0.95	−2.06
NH_2	−0.50	−0.98	−1.83
CH_3	+0.23	+0.22	+0.52
Cl	+0.36	+0.33	+0.69
$COCH_3$	−0.21	−0.60	−0.77

mobile phase, involving dipole–dipole interactions and hydrogen bond formation[11]. As the hydrophobic selectivity of MeOH–H_2O and THF–H_2O is normalized (benzene possesses the same retention volume), it is possible to assess the contribution of the hydroxy group to the retention behaviour of the molecule in the given eluent[4]. The lowest log I_{meas} value for phenol on Separon SE, and therefore also the lowest Δ log I contribution of the hydroxy group, was obtained in the THF–H_2O system. This disagrees with the findings of Bakalyar[3], who observed, using LiChrosorb RP-8, a decrease in the k' values of phenols in AcN–H_2O compared with MeOH–H_2O and, on the other hand, an approximately 50% increase in these values in THF–H_2O.

o-Nitrophenol, in which a strong intramolecular bond forms and the –OH group is sterically shielded, exhibits the highest log I_{meas} value of the three isomeric nitrophenols. In AcN–H_2O and THF–H_2O, the log I values of nitrophenols increase in the order *p*-, *m*-, *o*-; in MeOH–H_2O the order is *m*-, *p*-, *o*-. In AcN and THF [$\delta_b = 15.91 \cdot 10^3$ and $15.49 \cdot 10^3$ $(J/l)^{1/2}$, respectively], their ability to act as proton acceptors obviously plays a part, the probability of formation of the substance–mobile phase hydrogen bond decreasing in the order *p*-, *m*-, *o*-. With MeOH–H_2O, the ability of MeOH to act both as a proton donor and a proton acceptor shows up here [$\delta_a = \delta_b = 34.75 \cdot 10^3$ $(J/l)^{1/2}$][15]. The probability of the two types of interactions is virtually the same for the *p*- and *m*- isomers; for *o*-nitrophenol it is substantially lower because of the intramolecular bonding and steric shielding. This view is supported by the log I_{meas} values of the *p*- and *m*-isomers, which do not differ appreciably. With these substances, dissociation in the system in question plays a role, the elution order in the various eluents being in accordance with the dissociation constants[13].

For nitroanilines, the log I_{meas} values increase in the MeOH–H_2O and AcN–H_2O systems in the order *p*-, *m*-, *o*-, and in THF–H_2O in the order *p*-, *o*-, *m*-. The dissociation has no effect in any of the eluents, owing to the very low dissociation constants[14] (see the measurements in MeOH–H_2O of pH = 3.3 in Table V). With nitroanilines, acceptor–donor and donor–acceptor intermolecular bonds can form with the mobile phase, with nitrophenols. No satisfactory explanation could be found for the behaviour of nitroanilines in THF–H_2O.

For *o*-nitrochlorobenzene, the log I_{meas} value was found to excees log I_{theor}, owing to the strong intramolecular bonding between the two functional groups. On the other hand, *p*-nitrochlorobenzene, in which the substituents have little effect on each other and where, moreover, the –M and –I effects of the nitro group appear to the full extent, exhibits a longer retention time than that which corresponds to the theoretical value. Thus the difference D is negative for *o*-nitrochlorobenzene and positive for *p*-nitrochlorobenzene in all of the mobile phases used.

The greatest differences between the experimental and theoretical log I values were found in THF–H_2O for substances containing two or more nitro groups in a molecule. Their retention behaviour is probably governed by their dipole–dipole interaction. THF, with a rather bulky molecule, for steric reasons is not capable of giving rise to such strong dipole–dipole interactions as, for instance, AcN. This may account for the high log I_{meas} values observed for thos esubstances in THF, the difference D being positive in all instances.

The effect of pH on the retention behaviour of aromatic nitro compounds was investigated in the MeOH–H_2O (80:20) system of pH 3.3, 7.0 and 11.9 (Table V).

TABLE V

RETENTION INDICES FOR SUBSTITUTED NITROBENZENES IN THE MeOH–H_2O (80:20) MOBILE PHASE WITH ADJUSTED pH

Compound	*pH of mobile phase*		
	3.3	*7.0*	*11.9*
Nitrobenzene	1.30	1.31	1.32
m-Dinitrobenzene	1.65	1.78	1.60
Aniline	−0.13	0.50	0.40
o-Nitroaniline	1.26	1.31	1.18
m-Nitroaniline	1.19	1.29	1.26
2,4-Dinitroaniline	1.69	1.89	1.72
Toluene	1.23	1.23	1.25
o-Nitrotoluene	1.41	1.52	1.52
p-Nitrotoluene	1.44	1.55	1.55
2,4-Dinitrotoluene	1.91	2.03	1.86
2,4,6-Trinitrotoluene	2.27	2.58	2.36
Phenol	0.44	0.42	−1.58
o-Nitrophenol	1.19	1.23	−3.88
m-Nitrophenol	1.09	1.15	−1.91
p-Nitrophenol	1.08	1.18	−1.75
2-Nitro-4-methylaniline	1.39	1.59	1.66
Chlorobenzene	1.31	1.36	1.31
o-Nitrochlorobenzene	1.54	1.56	1.54
p-Nitrochlorobenzene	1.62	1.76	1.75
2,4-Dinitrochlorobenzene	1.92	2.06	1.74
2,5-Dichloronitrobenzene	1.90	2.01	1.91
Acetophenone	0.64	0.79	0.67
p-Nitroacetophenone	1.33	1.32	1.34

No changes in the retention indices were observed for substances not capable of dissociation, the differences being within the limits of the measurement precision.

Substances containing an amine or a hydroxy group displayed different log I_{meas} values at pH 3.3 and 11.9 in MeOH–H_2O, in accordance with their dissociation constants.

Based on the data obtained, some general conclusions can be drawn concerning the retention behaviour of aromatic nitro compounds on Separon SE.

In the MeOH–H_2O (80:20) mobile phase the nitro group contribution to the retention of the molecule is positive for substances containing both polar and non-polar functional groups. The contribution of the nitro group is higher (D is positive) for polar substances with basic or acidic functional groups. When assessing the retention behaviour of a substance from the individual contributions of the substituents involved, the highest differences are encountered if the nitro group is in an *ortho* position with respect to a polar functional group; if only non-polar substituents are present in the substance in addition to the nitro group, the retention behaviour can be forecast fairly accurately based on the increments for individual substituents.

The contribution of the nitro group did not change if a mobile phase with adjusted pH was used; the differences found (Table V) lay within the error of measurement.

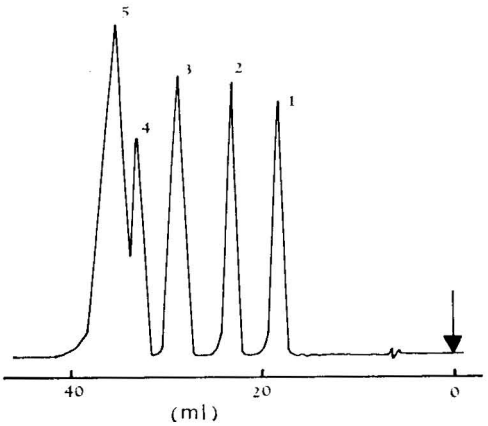

Fig. 1. Separation of chloronitrobenzenes. Column: 300 × 6 mm I.D., Separon SE. Eluent: tetrahydrofuran–water (50:50), 60 ml/h. UV detector at 254 nm. Peaks: 1 = *o*-nitrochlorobenzene; 2 = chlorobenzene; 3 = *p*-nitrochlorobenzene; 4 = 2,5-dichloronitrobenzene; 5 = 2,4-dinitrochlorobenzene.

With AcN–H_2O (63:37) and THF–H_2O (50:50) the situation is more complicated and conclusions cannot be drawn so clearly as in the preceding case. In AcN–H_2O and THF–H_2O the nitro group contributes negatively to the retention of the molecule if the substance contains non-polar groups (halonitrobenzens, alkylnitrobenzenes), whereas if the substance contains medium polar or polar functional groups, basic or acidic, the contribution of the nitro group is positive. The AcN–H_2O and particularly the THF–H_2O systems give rise to specific interactions between the mobile phase and the substance, and are suitable for the separation of aromatic nitro compounds (Fig. 1).

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GENERAL APPROACH TO THE FRACTIONATION AND CLASS DETERMINATION OF COMPLEX MIXTURES OF CHLORINATED AROMATIC COMPOUNDS

PHILLIP W. ALBRO* and CAROL E. PARKER

Laboratory of Environmental Chemistry, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709 (U.S.A.)

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SUMMARY

Among the "inadvertent" environmental pollutants are polychlorinated biphenyls, terphenyls, quadphenyls, naphthalenes, diphenyl ethers, dibenzofurans, dibenzo-*p*-dioxins and benzenes. Mixtures of these classes of compounds also occur in commercial products such as transformer fluids. To analyze such mixtures, gas chromatography–mass spectrometry may be combined with pre-fractionation on basic and acidic alumina columns and semi-quantitative perchlorination techniques. These procedures are illustrated for synthetic mixtures as well as for two samples of stored transformer fluid. Although the described procedure is mainly intended to be applied to the characterization of the major class components of such mixtures, it is also applicable to the determination of trace components such as the dibenzofurans in commercial polychlorinated biphenyls. The mass spectral techniques permit the simultaneous patterning, or "fingerprinting", of the compounds comprising each major class of chlorinated aromatics present.

INTRODUCTION

The environmental chemist is often faced with the necessity of analyzing materials containing hundreds of individual components. For example, the commercial preparations of polychlorinated biphenyls (PCBs), used as heat exchangers, plasticizers, dielectric fluids, etc., usually consist of 40–100 individual compounds[1–3]. Many products are even more complex, in that mixtures of classes of compounds are present. An environmental sample may have been contaminated by PCBs, polychlorinated naphthalenes (PCNs), polychlorinated terphenyls (PCTs), pesticides, chlorinated benzenes (CBs), polychlorinated dibenzofurans (PCDBFs) and/or dibenzo-*p*-dioxins (PCDBDs), as well as other halogenated analogues and alteration products. Although the major interest of environmentalists in these products usually centers around their content of specific compounds having extreme toxicity, carcinogenicity or other undesirable biological activity, direct analysis for specific compounds is usually impracticable. These various classes of halogenated aromatics mutually inter-

fere with routine assay procedures[3–5], and some form of preliminary class fractionation is needed. To be practical, such a fractionation scheme must be applicable in the absence of previous knowledge of the class composition of the sample.

The purpose of the present work was to develop and characterize such a prefractionation scheme, to combine it with methods for the "fingerprinting" of the particular classes present, and to begin to apply it to actual environmental samples. To illustrate the effectiveness of the proposed procedure, we have applied it to the characterization of two samples of transformer fluid which differed in complexity. The complete procedure requires approximately 100 μg of aromatic material after extraction and cleanup. However, this may correspond to as little as 1 μg of any individual compound when complex mixtures such as PCBs are present.

EXPERIMENTAL

Materials

The following chromatographic adsorbents were used throughout this work: Florisil PR, 60–100 mesh (no lot number given) from Supelco (Bellefonte, PA, U.S.A.); basic alumina, 80–200 mesh, Fisher No. A-540 (lot No. 783941; Fisher Scientific, Pittsburgh, PA, U.S.A.); acidic alumina, 80–200 mesh, EM No. 1078 (lot No. 690691; EM Labs., Elmsford, NY, U.S.A.); and silica gel 60, 70–230 mesh, EM No. 7734 (lot No. 7956928; EM Labs.). Although we have not seen any significant lot number variations, the substitution of other types of alumina, Florisil or silica gel would require considerable modification of the procedures to be described. These adsorbents were all activated at 130°C; activation periods between 16 and 72 h gave comparable results, but shorter or longer heating periods, should be avoided.

Granular, anhydrous sodium sulfate was Fisher No. S-421. Sea sand was 50 mesh Sargent-Welch (Skokie, IL, U.S.A.) No. S-73845-8. Chromatographic columns were essentially similar to uncalibrated burettes, equipped with PTFE stopcocks, and plugged with Pyrex glass wool. Sintered glass frits caused adsorption problems and were avoided. Columns having an I.D. of 7 mm were used with 1–2 g of packing; 9 mm I.D. columns were used for 2–4 g of adsorbent; 1 cm I.D. columns for up to 7 g, and 1.5 cm I.D. columns for 7–10 g of adsorbent.

All solvents were distilled-in-glass, either from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) or Matheson, Coleman & Bell (East Rutherford, NJ, U.S.A.). All except methylene chloride and *n*-hexane were used as received; the former was stored over molecular sieve 5A and the latter over activated Davidson type 06 silica gel to ensure dryness. Concentrated sulfuric acid was reagent grade from Mallinckrodt (St. Louis, MO, U.S.A.). Perchlorination reagents sulfuryl chloride, sulfur monochloride and aluminum chloride were from Aldrich (Milwaukee, WI, U.S.A.). Antimony pentachloridewas from Alfa division of Ventron (Beverly, MA, U.S.A.); lot No. 112275 was free of significant bromide contamination[6,7].

Adsorption chromatography was monitored using either an Isco Model UA-5 UV monitor or an LDC No. 1280 monitor set at 280 nm. Fractions were collected on a drop-count basis. Infrared spectra of neat films on sodium chloride plates were run using a Perkin-Elmer Model 621 instrument.

Gas chromatography (GC) was performed using a Varian Model 1200 instrument equipped with a hydrogen flame ionization detector (FID), or a Hewlett-

Packard Model 5750 with a ^{63}Ni electron capture detector (ECD) in the pulse mode with 5% methane in argon as make-up gas. Peak areas were measured using an Autolab system IV computing integrator. Column packings for GC were from Applied Science Labs. (State College, PA, U.S.A.) or from Analabs (North Haven, DE, U.S.A.).

Compounds used as reference standards were obtained from a variety of sources. Chlorobenzenes were from Aldrich or Eastman-Kodak (Rochester, NY, U.S.A.); individual PCBs, polychlorinated diphenyl ethers (PCDPEs), hydroxy PCBs, PCDBDs and the like were from either Analabs or RFR (Hope, RI,, U.S.A.). Various PCBs, PCDPEs and PCDBFs were synthesized here by methods described previously[8,9]. Aroclors 1016, 1221, 1242, 1248, 1254 and 1260 were kindly donated several years ago by Monsanto (St. Louis, MO, U.S.A.). Aroclor 5460 (a mixture of polychlorinated terphenyls), various Halowaxes (mixtures of PCNs), and Kanoclor 400 (PCBs) were from Analabs. A mixture of polychlorinated diphenyl ethers having from 5–8 chlorine atoms per molecule was synthesized by reacting diphenyl ether (Eastman-Kodak) with eight equivalents of antimony pentachloride at 155°C in a sealed tube with chloroform as solvent, for 16 h. Polychlorinated quadphenyls (PCQs) were made by heating Kanoclor 400 (1 g) and activated copper powder (300 mg) under nitrogen in a sealed ampoule at 300°C for 48 h. The PCQs could easily be separated from PCBs on silica gel; PCBs were eluted with hexane, and PCQs with 5% diethyl ether in hexane. Fully perchlorinated compounds were obtained from Analabs or RFR. Samples of "used" transformer fluids were obtained locally.

GC–mass spectrometry (MS) was performed using a Finnigan 3300 mass spectrometer which was interfaced to a Finnigan/Incos 2300 data system. The ionization mode was electron impact at 70 eV and the separator temperature was 270°C. A 4-sec scan cycle was used throughout the GC–MS runs. Exact mass measurements were made using a VG Micromass ZAB-2F double focusing mass spectrometer at a resolution of 5000.

Methods

Preliminary enrichment. Commercial products may either enter the fractionation scheme (Fig. 1) directly if they are exclusively or predominately composed of halogenated aromatic compounds, or, if significant amounts of aliphatics are present they may be subjected to gel permeation chromatography (GPC)[10,11] or partitioned between isooctane and dimethyl sulfoxide[12] to provide a fraction highly enriched in aromatics. Environmental samples (soil, tissues, etc.) are first extracted by a procedure appropriate to the matrix (extraction methods are beyond the scope of the present paper). The extracts are largely freed of lipids and other aliphatics either by GPC[10,11,13] or by partitioning between carbon tetrachloride and sulfuric acid[14].

In order to remove the solvent in which the sample is at this point contained, we add 10–50 μl of 1,3-propanediol and concentrate at 40°C using a rotary evaporator. We observe no loss of PCBs from Aroclor 1254 if this precaution is taken; in the absence of the propanediol, up to 60% of the PCBs may be lost upon rotary evaporation.

The residue from rotary evaporation is taken up in 2 ml of *n*-heptane and washed with 5 ml of water to remove the propanediol. The upper phase, after centrifugation, is ready for fractionation according to the scheme shown in Fig. 1.

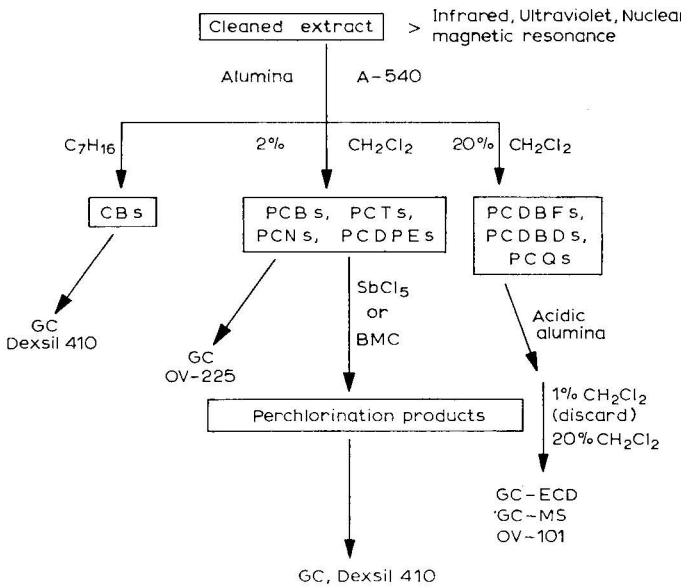

Fig. 1. Routine fractionation scheme.

Fractionation. The initial chromatography on A-540 basic alumina requires 5 g of alumina per mg of halogenated aromatic material present in the sample. The column is dry-packed with alumina (previously activated at 130°C) as soon as the alumina has cooled to room temperature in a desiccator over Drierite. The packing is settled by tapping gently (not tamping). A well-packed column will contain 1 g of alumina per 1.15 ml of packed volume. The void volume will be approximately 0.77 ml per gram of alumina. Before loading the sample, the column is topped with a 2 cm layer of anhydrous, granular sodium sulfate.

The sample is loaded in *n*-heptane, using less than 1 ml of solvent per 3 g of alumina in the column. As soon as the solvent has sunk to the surface of the sodium sulfate, the stopcock is closed for 30 sec to permit binding to become complete. Then the elution of the first fraction may be started, using the first two ml of *n*-heptane to rinse the sample container.

The amount of solvent needed to elute a given fraction depends upon the composition of the sample. In most cases, the first fraction (aliphatic hydrocarbons and chlorobenzenes) will be eluted by the time 3–4 ml of heptane per gram of alumina has passed through the column; however, PCBs and PCNs will begin to elute immediately after the chlorobenzenes and a sharp separation will require that the effluent be continuously monitored for UV absorbance. As soon as the emergence of a second UV-absorbing peak is detected, the eluting solvent is changed to *n*-hexane–methylene chloride (98:2, v/v). It is essential that the second solvent does not contain more than 2% methylene chloride if PCDBFs are to be separated from PCBs.

If the amount of aromatic material available is too small for UV monitoring, the alumina column can be precalibrated by running 50 μg of 2,3,5,6,2′,3′,5′,6′-octachlorobiphenyl in *n*-heptane. This compound will permit detection of the eluate volume corresponding to first appearance of PCBs, and can be completely eluted

with heptane. Since heptane (dry) does not deactivate the column, the calibrated column can then be used to process the low-level unknown sample. The elution solvent will be changed to 2% methylene chloride when an amount of heptane corresponding to the PCB breakthrough volume has been collected.

In general, the amount of 2% methylene chloride in hexane that will be required is at least twice that of the heptane used in eluting chlorobenzenes. If only the more highly chlorinated PCBs are present (analogous to Aroclor 1254 or 1260), fraction 2 will require 10 ml of 2% methylene chloride per gram of alumina. Less chlorinated PCBs (mono-, di- and trichloro) will require 20 ml/g, while PCTs would require 40 ml/g. Unfortunately PCDBFs will begin to elute if more than 10–15 ml of 2% methylene chloride per gram of alumina is used. If both PCTs and PCDBFs are present it will be necessary to run two columns, one from which PCTs are eluted in fraction 2 with 40 ml of 2% methylene chloride per gram of alumina (in which case PCDBFs will be impossible to purify) and one from which only a portion of the PCTs are eluted in fraction 2 with 10 ml solvent per g alumina so that the PCDBF fraction may be eluted and further purified.

Fraction 3, containing PCDBFs, PCDBDs, PCQs and part of the PCTs, is eluted with *n*-hexane–methylene chloride (80:20, v/v), 10 ml/g alumina. This fraction is further purified, to remove small amounts of the less chlorinated PCBs and PCNs, on a small (usually 1–3 g) column of acidic alumina prepared as described for the A-540 alumina. A well-packed column of 70–230 mesh acidic alumina will occupy a volume of 1.06 ml per gram of packing and have a void volume of 0.74 ml per gram. Fraction 3 from the basic column can generally be blown dry under nitrogen with no loss of its contents, since quadphenyls and the cyclic ethers are much less volatile than the PCBs. The residue is loaded onto the acidic alumina in *n*-hexane–methylene chloride (99:1, v/v) and eluted with 10 ml of the same solvent per gram of alumina. The purified PCQ–PCDBF–PCDBD fraction is then eluted with 6 ml of *n*-hexane–methylene chloride (80:20, v/v) per gram of alumina and blown dry under nitrogen at room temperature.

More polar materials may be recovered from the A-540 alumina column if desired. Phthalate diesters are eluted as fraction 4 with *n*-hexane–methylene chloride (1:1, v/v), 10 ml/g alumina. Phenols can be eluted with acetone, but recovery is poor. If the sample is expected to contain phenols, a portion should be run initially on acidic alumina, discarding (or combining) the fractions eluted through 20% methylene chloride, and eluting the phenols with acetone. Better recoveries of phenols will be obtained if the column is run in the dark (*e.g.* wrapped in aluminum foil) and all solvents are deoxygenated before use.

Analysis of the chlorobenzenes (fraction 1). Conditions used for GC of all the fractions are given in Table I. Dexsil 410 gave reasonably acceptable resolution of the various chlorobenzene isomers, although there were two unresolved pairs (Table II). Accurate quantitation with an ECD requires that a standard curve be prepared for each component. The hydrogen FID decreases in sensitivity as the degree of chlorination increases, but the response is nearly independent of the location of the chlorine atoms, so only six standard curves are needed.

Analysis of fraction 2. Fraction 2 commonly contains PCBs, PCNs, PCDPEs, DDE and part or all of the PCTs. Whether or not this mixture of classes can be further resolved into individual classes depends upon the complexity of each class. In general,

TABLE I

GAS CHROMATOGRAPHY CONDITIONS

Parameter	*Chlorobenzenes*	*PCB, PCN, PCDPE*	*PCDBFs, PCDBDs*	*PCT, PCQ*	*Perchlorination products*
Column length (m)	2	2	2	1	2
Column diameter (mm)	2	2	2	2	2
Liquid phase	Dexsil 410	OV-225	OV-101	OV-210	Dexsil 410
Percentage liquid	3	3	5	0.4	3
Solid support	Anakrom AS	Gas-Chrom Q	Gas-Chrom Q	TGB*	Anakrom AS
Mesh range	90–100	100–120	100–120	80–100	90–100
Helium flow-rate (ml/min)	35	25	20	25	35
Column temperature (°C)					
Initial	80	150	220	200	120
Final	150	250**	—	275	250
Linear rate (°C/min)	2	6	—	6	6
Initial hold (min)	3	—	—	—	—

* TGB = textured glass beads.

** To 300°C if PCTs are present.

TABLE II

GC OF CHLOROBENZENES ON DEXSIL 410

GC conditions as described in Table I. Samples of transformer fluid, heptane fraction from basic alumina. ND = none detected.

Component	*Retention*		*FID area (%)*	
	Time (min)	*Temp. (°C)*	*Fluid A*	*Fluid B*
Monochloro	0.86	80.0	ND	ND
m- + *p*-dichloro	2.44	80.0	0.26	0.73
o-dichloro	3.20	80.8	0.62	0.64
1,3,5-Cl_3	6.32	93.3	0.13	trace
1,2,4-Cl_3	7.10	96.4	75.23	67.23
1,2,3-Cl_3	8.60	102.4	23.75	12.30
1,2,3,5 + 1,2,4,5-Cl_4	11.82	115.3	trace	2.32
1,2,3,4-Cl_4	12.90	119.6	ND	13.51
1,2,3,4,5-Cl_5	17.80	139.2	ND	2.32
Hexachloro	23.62	162.5	ND	0.95

we find it more practical to functionally distinguish members of each class by MS techniques than to proliferate fractionation procedures with their attendant handling losses. The GC conditions used for introduction of the fraction 2 components are shown in Table I. Electron impact (70 eV) mass spectra are continuously generated in the scanning mode, after which the computer is used to generate reconstructed, single-ion chromatograms at each of the *m/z* values shown in Table III. These *m/z* values correspond to the molecular ion containing one ^{37}Cl atom and show much less cross-class interference than the all-^{35}Cl molecular ion peaks. Selectivity was further increased by only considering the peaks seen in specific scan number ranges for each single ion chromatogram, as also shown in Table III. In this way interference from the ($M - Cl_2$) fragment from higher analogues could be avoided. The appropriate ranges were determined from chromatograms of various Aroclor, Halowax, and PCDPE mixtures.

TABLE III

m/z VALUES AND SCAN RANGES FOR RECONSTRUCTED SINGLE ION CHROMATOGRAMS

Scan numbers are at 4 sec per scan.

PCN	*m/z*	*Scan numbers*	*PCB*	*m/z*	*Scan numbers*	*PCDPE*	*m/z*	*Scan numbers*
3-Cl	232	1–60	4-Cl	292	40–82	6-Cl	376	80–120
4-Cl	266	30–90	5-Cl	326	60–110	7-Cl	410	110–140
5-Cl	300	60–110	6-Cl	360	80–140	8-Cl	444	140–180
6-Cl	334	90–150	7-Cl	394	95–200	9-Cl	478	180–200

Since the composition of fraction 2 is commonly much too complex to permit quantitation on the basis of standard curves for individual compounds, only approximate methods of quantitation could be used. The relative peak areas for the PCDPEs correlated well with corresponding relative hydrogen FID peak areas, which

by analogy with previous observations on PCBs[15] were tentatively considered to approximate molar percentages. The single ion peak areas were related to the flame detector areas by: single ion peak area = 0.58 (hydrogen FID area) + 0.45, with a correlation coefficient of 0.9888.

Relative peak areas from the reconstructed single ion chromatograms for a variety of PCB standards unfortunately did not correlate well with the corresponding FID peak areas. However, the single ion relative molar responses for PCBs correlated well with the total number of chlorines, such that the relative molar responses (RMR) fit the equation: RMR = 0.132 (No. of Cl) + 0.202 with a correlation coefficient of 0.9956 ($n = 8$). This equation applied when RMR for 2,4,5,2′,5′-pentachlorobiphenyl was set equal to 1.0.

We did not have a collection of individual PCNs or PCTs available, so quantitation of individual components of these classes was not attempted. Considered over a variety of mixtures of Aroclors, Halowaxes and PCDPEs, on the average the total peak area attributed to PCDPEs × 3.4, that due to PCNs × 0.8 and that attributed to PCBs × 1.14 (from the single ion chromatograms) gave relative molar percentages of each class. These correction factors allowed only a first approximation of the class composition, of course, and, except for PCNs, more satisfactory measurements could be made by perchlorination of fraction 2. In general, the reconstructed single ion chromatograms were most useful as "fingerprint" patterns of class composition. PCTs do not begin to elute from Dexsil 410 until after scan 200 (about equivalent to the beginning of elution of nonachlorobiphenyls), and are thus in a region of the chromatograms reasonably free of interferences. Any m/z value in the molecular ion cluster can be used to monitor PCTs.

Perchlorination of fraction 2. Fraction 2 was routinely divided into three portions depending on the amount of material available. One portion was reserved for GC–MS as discussed above. Another portion, which could contain between 50 μg and 1 mg of total organic material after removal of solvent, was dissolved in 0.1 ml of chloroform and perchlorinated with 0.2 ml of antimony pentachloride for 16 h at 170°C as described by Armour[16]. This procedure quantitatively generates decachlorobiphenyl, the three isomeric tetradecachloroterphenyls and hexachlorobenzene from PCBs, PCTs and chlorobenzenes respectively, but destroys PCNs and PCDPEs. Mirex is not affected by perchlorination and may be added as an internal standard.

Perchlorination of the final portion of fraction 2 was performed using "BMC Reagent" (sulfuryl chloride, sulfur monochloride and aluminum chloride)[17]. To the dried residue containing less than 1 mg of aromatic compounds in a 15 ml screw cap test tube was added 0.5 ml of sulfuryl chloride, 10 μl of sulfur monochloride and 10–20 mg of aluminum chloride. It is essential that the aluminum chloride not be exposed to atmospheric moisture for more than a few seconds, and the final concentration of aluminium trichloride should not exceed 5%. The tubes were closed with PTFE-lined screw caps, and placed in a 70°C bath such that only the portions of the tubes containing reagent were immersed. After heating for 3 h, the tubes were cooled, opened, and the samples worked up as described in the original reference[17]. Again, Mirex was used as internal standard.

GC analysis of the perchlorination products under the conditions described in Table I is illustrated using standards in Fig. 2. Relative detector responses to the various pure components are indicated in Table IV.

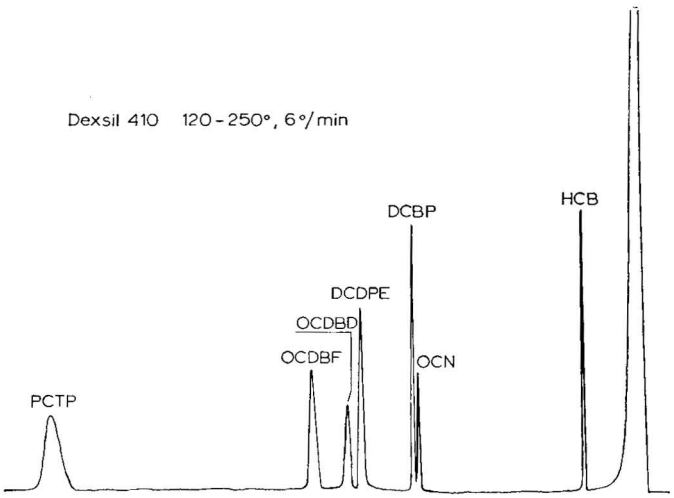

Fig. 2. Gas chromatogram of fully perchlorinated compounds on Dexsil 410. HCB = hexachlorobenzene; OCN = octachloronaphthalene; DCBP = decachlorobiphenyl; DCDPE = decachlorodiphenyl ether; OCDBD = octachlorodibenzo-*p*-dioxin; OCDBF = octachlorodibenzofuran; PCTP = tetradecachloroterphenyl (*para* isomer). OCN may elute before or after DCBP depending upon the column length under these programming conditions.

If the amount of available sample is below 50 μg, it will probably be undesirable to perform both perchlorination procedures. In such a case, the entire residue of fraction 2 not used for MS, augmented with 1 μg of Mirex, is processed according to the antimony pentachloride procedure, using 50 μl of chloroform and 0.1 ml of antimony pentachloride in a 1 ml Reactivial. Since chlorobenzenes have been separated in fraction 1, the occurrence of hexachlorobenzene in the perchlorination products will suggest that diphenyl ethers and/or naphthalenes were present in fraction 2 prior to their decomposition by the reagent. Further information on these classes of compounds would then have to be derived from the MS data.

Analysis of the 20% methylene chloride fraction. Quadphenyls do not elute from GC columns in the range of the PCDBFs or PCDBDs and may be examined

TABLE IV

HYDROGEN FLAME IONIZATION DETECTOR RESPONSE TO PERCHLORINATION PRODUCTS

Response is based on relative peak areas.

Compound	*Relative molar response*	*Relative weight response*
Mirex	1.000	1.000
Hexachlorobenzene	0.775	1.498
Octachloronaphthalene	1.032	1.395
Decachlorobiphenyl	0.979	1.080
Octachlorodibenzo-*p*-dioxin	0.473	0.561
Octachlorodibenzofuran	0.620	0.769
Decachlorodiphenyl ether	1.192	1.274
Tetradecachloroterphenyl	1.446	1.118

without interference by GLC on the low-loaded column described in Table I. If PCTs are absent, the PCDBFs and PCDBDs can be examined using the OV-101 column and an ECD, in which case quantitation will be based upon individual standard curves. The complete families of PCDBF and PCDBD isomers have not been successfully resolved, even using capillary columns[18,19]. Selectivity may be achieved through the use of negative ion chemical ionization MS[20], but because of the inability to distinguish all possible isomers it is necessary to limit characterization to chemical class and number of chlorine atoms in most cases. The occurrence of interferences on the OV-101 column will usually reflect failure to completely eliminate PCBs and/or PCTs, and may be grossly detected by taking note of the peak widths. The PCBs and PCTs give much narrower peaks than do the PCDBFs and PCDBDs at a given elution position.

APPLICATIONS AND ILLUSTRATIONS

Synthetic mixtures

A mixture of Aroclor 1254 (PCBs), Halowax 1013 (PCNs), and PCDPEs (synthetic mixture with 5–8 chlorine atoms per molecule) gave only a single, asymmetrical peak on A-540 alumina (fraction 2, 2% methylene chloride). This mixture was subjected to GC–MS and perchlorination as described above. Reconstructed single ion chromatograms are shown in Fig. 3, while the yields of fully perchlorinated products are given in Table V.

The yields of decachlorobiphenyl were essentially quantitative when the antimony pentachloride procedure was used. PCNs and PCDPEs were broken down to small fragments under these conditions. Yields were incomplete for decachlorobiphenyl and decachlorodiphenyl ether using the BMC reagent, and this reagent has also been found to be unsuitable for derivatizing PCTs. However, in all experiments to date, PCDPEs have been derivatized by the BMC reagent to the same extent as the PCBs; therefore the application of antimony pentachloride and the BMC reagent to separate aliquots of a sample permits the determination of PCDPE content.

Chlorinated naphthalenes break down to a variety of products including hexachlorobenzene when the perchlorination conditions are too vigorous. This occurs, in BMC reagent, if the aluminium trichloride concentration exceeds 5% (w/v). Under the conditions described above, with 2–5% aluminium trichloride, the yields of octachloronaphthalene approach 90%, depending upon the degree of protection of the reagent from atmospheric moisture. This is difficult to reproduce, and we do not presently recommend the use of perchlorination as a strictly quantitative assay for PCNs.

A mixture of Aroclor 1254, Aroclor 5460 (PCTs) and 1,2,3,4-tetrachlorobenzene gave the chromatographic pattern on A-540 alumina shown in Fig. 4. Perchlorination with antimony pentachloride gave essentially quantitative conversion of all three classes of compounds to their fully chlorinated analogs.

The application of these chromatographic procedures in association with chemical ionization negative ion MS to determine the chlorinated dibenzofuran content of commercial PCBs has been described previously[3] and will not be repeated here. In general, the non-oxygenated classes, PCBs, PCTs, etc. do not interfere with determination of the dibenzofurans in this mass spectral technique, so the contamination of fraction 3 by PCTs is not a serious problem. The use of both basic and acidic

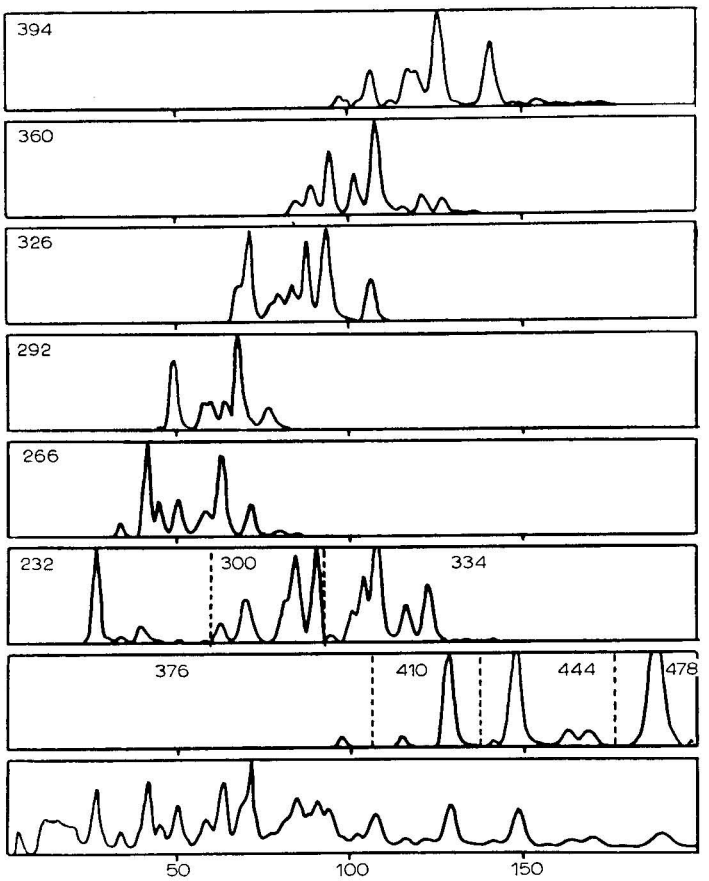

Fig. 3. Reconstructed single ion chromatograms for a mixture of PCBs, PCNs, and PCDPEs. The lowest trace is the total ion current. For GC conditions see Table I.

alumina columns in sequence is, however, essential if PCDPEs are present in the sample, since these compounds yield $M-Cl_2$ fragments that interfere with the determination of the dibenzofurans.

TABLE V

YIELDS OF PERCHLORINATION PRODUCTS FROM A MIXTURE

Theoretical yield is given as mean ± S.D. ($n = 5$).

Component	*Class*	*nmoles per sample*	*Theoretical yield of perchloro derivative (%)*	
			$SbCl_5$	*BMC*
Aroclor 5460	PCT	460	92.4 ± 3.6*	0
Aroclor 1254	PCB	654	95.9 ± 2.1	73.5 ± 6.5
Halowax 1013	PCN	843	0	c**
PCDPE (5–8 Cl)	PCDPE	516	0	74.9 ± 5.1

* Sum of *o*-, *m*- and *p*-isomers.

** Critically dependent upon the amount and activity of the aluminum chloride. Yields of from 0–98% have been seen.

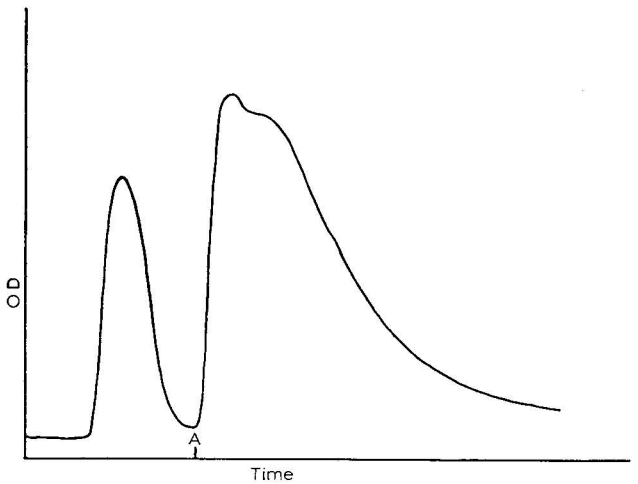

Fig. 4. UV monitor output during chromatography of a mixture of CBs, PCBs, and PCTs on A-540 alumina. Approximately equal amounts of 1,2,3,4-tetrachlorobenzene, Aroclor 1254 and Aroclor 5460 were chromatographed as described in the text. Solvent changed from heptane to 2% methylene chloride in hexane at point A.

Transformer fluids

Infrared examination of the two samples of transformer fluid indicated the absence of significant aliphatic components; accordingly, the samples could enter the analytical scheme at the basic alumina chromatography step. No C=O or -OH was detectable in the infrared spectra, indicating that phenolic compounds and phthalate esters would not comprise as much as 1% of the material. Therefore, only the initial three fractions from the alumina columns were studied in detail.

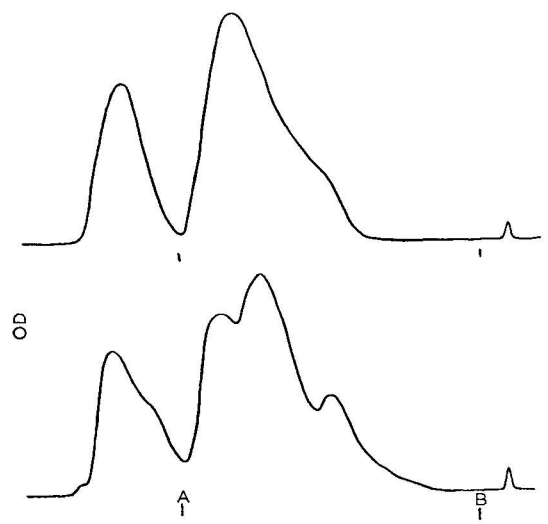

Fig. 5. UV monitor output during chromatography of transformer fluids on A-540 alumina. Upper trace, fluid A; lower trace, fluid B. Solvent changed from heptane to 2% methylene chloride in hexane at A, and to 20% methylene chloride in hexane at B.

The UV monitor outputs for the chromatography on A-540 alumina are shown in Fig. 5. Major elution peaks appeared in the heptane and the 2% methylene chloride fractions, with minor peaks in the 20% methylene chloride fraction.

Fluid A, fraction 1 (heptane), contained exclusively chlorobenzenes as shown in Table II. Fraction 2 contained PCBs similar in chromatographic pattern to Aroclor 1254. Other classes of compounds amounting to more than 0.1% of the sample weight were not detected. Fraction 3 (20% methylene chloride) from the A-540 alumina, after further cleanup on acidic alumina, contained a range of PCDBFs in trace amounts consistent with the reported PCDBF content of Aroclor 1254[21]. These PCDBFs were mainly tetra- and pentachloro isomers (Table VI), as would be expected.

TABLE VI

CHLORINATED DIBENZOFURANS IN TRANSFORMER FLUIDS

Upper limits measured with an ECD. ND = not detected.

No. of Cl	*Fluid (ng/g)*	
	Fluid A	*Fluid B*
4	580	ND
5	115	ND
6	45	16
7	ND	200
8	ND	4500

Fluid B was more complex than fluid A. Fraction 1 contained a wider range of chlorobenzenes than was seen in fluid A (Table II). Perchlorination of fraction 2 from fluid B gave decachlorodiphenyl ether in addition to decachlorobiphenyl. The chlorinated dibenzofuran in fraction 3 were not only in higher total concentration in fluid B than in fluid A, but were also more highly chlorinated than those in fluid A. The compositions of the two samples of transformer fluid are summarized in Table VII.

TABLE VII

COMPOSITIONS OF TWO SAMPLES OF TRANSFORMER FLUID

ND = none detected.

Component	*Fluid A*		*Fluid B*	
	Mol.%	*Pattern*	*Mol.%*	*Pattern*
PCBs	60.3	1254*	50.8	1260*
Chlorobenzenes	39.7	2–3 Cl	41.1	1–6 Cl
PCDPEs	ND	ND	8.0	6–8 Cl
PCNs, PCTs	ND	ND	Trace	—
PCDBFs, PCDBDs	ND	ND	ND	ND

* PCB pattern given as the Aroclor it most nearly resembled.

To confirm the identification of diphenyl ethers in fluid B, exact mass measurements were made on three peaks in the molecular ion cluster of the best resolved components of this class. The results are shown in Table VIII. In addition, this fraction was treated with the silylation reagent described above under conditions

giving quantitative silylation of 2,3,4,5,6,3′,5′-heptachloro-4′-hydroxybiphenyl to check for the occurrence of hydroxybiphenyls. No silyl-reactive components were detected. MS properties of trimethylsilyl derivatives of hydroxybiphenyls have been described[22].

TABLE VIII

EXACT MASS MEASUREMENTS ON COMPONENTS OF TRANSFORMER FLUID B

Transformer fluid B had been in service for at least 20 years prior to sampling.

Scan No.	*Formula*	*No. of ^{37}Cl*	*Exact mass*		*Error (ppm)*
			Theory	*Measured*	
97	$C_{12}H_4OCl_6$	0	373.8393	373.8412	5
		1	375.8364	375.8362	1
		2	377.8334	377.8334	0
127	$C_{12}H_3OCl_7$	0	407.8003	407.8025	5
		1	409.7974	409.7981	2
		2	411.7944	411.7938	2
149	$C_{12}H_2OCl_8$	0	441.7614	441.7600	3
		1	443.7584	443.7612	6
		2	445.7555	445.7561	1

DISCUSSION

The classes of aromatic compounds discussed here, PCBs, PCNs, PCDPEs, PCTs, PCQs, PCDBFs, PCDBDs and CBs, represent "inadvertent" pollutants in that, unlike the pesticides and herbicides, they have not been deliberately disseminated in the environment. The PCDBFs and PCDBDs have entered the environment as unintentional impurities in PCBs and products derived from chlorophenols, as well as being pyrolysis products of PCBs and possibly PCDPEs under appropriate conditions[23,24]. The PCTs are rarely found as environmental pollutants, possibly because they are rarely sought. PCQs and higher "polymers" of PCBs are found in used heat exchange fluid such as lead to the "Yusho Oil" contamination in Japan[25]. Many commercial PCB preparations reportedly contain traces of PCNs[26], and the Halowaxes, mixtures of PCNs, share many of the applications of the PCBs and thus are likely to be found in similar matrices. At the present time, means to adequately determine concentrations of total PCNs in the presence of excess PCBs do not exist (for some of the problems involved see ref. 3).

Throughout the work presented here we have emphasized use of the hydrogen FID rather than the ECD. Not only is the response of the FID much more uniform and predictable than that of the ECD, but the flame sensitivity is closely comparable to that of the mass spectrometer in the scanning mode. Any sample not sufficiently cleaned up to use a FID is also not sufficiently clean for perchlorination. The minimum amount of *e.g.* a perchlorination product that can be accurately quantitated using the FID under the GC conditions described is approximately 5–10 ng at an amplifier setting of $16 \cdot 10^{-12}$ A/mV. This is quite adequate sensitivity relative to the limitations of the mass spectrometer and the obtainable purity of perchlorination reagents.

The procedures described here could give indications of the presence of aromatic compounds other than those specifically discussed. For example, polybromi-

nated biphenyls would show as a discrepancy between the apparent amount of PCBs seen by MS and by GC of the perchlorination products. However, we have not at this time specifically included methods for the separate quantitation of brominated or unsubstituted compounds. The effects of major quantities of pesticides in the samples have also not been examined. The nearly ubiquitous pesticide DDE, which also elutes in fraction 2 under the conditions described, can be determined without interference from PCBs, PCNs, PCTs or PCDPEs by monitoring its m/z 318 ion in the MS procedure. DDE yields no significant interference with any of the ions listed in Table III. PCTs would interfere with mass spectral determination of PCBs, were it not for the fact that they elute from the GC column after the PCB scans have been completed.

The use of chlorination procedures to screen for chlorodibenzo-*p*-dioxins in the presence of PCBs, PCNs and PCDBFs has been suggested previously[27]. In our experience, although the general principal seems reasonable, perchlorination reactions are not sufficiently "clean" to permit the quantitative determination of the trace components of these mixtures. Moreover, toxicological interest at the present time centers on a few specific PCDBF and PCDBD isomers; therefore we prefer to analyze for these classes of compounds following a cleanup procedure rather than a purely qualitative screening procedure that seems to offer considerable potential for false positives.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF CHORINATED DIBENZODIOXINS AND DIBENZOFURANS IN CHICKEN LIVER AND WOOD SHAVING SAMPLES

JOHN JOSEPH RYAN* and JEAN CLAUDE PILON
Food Directorate, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario K1A 0L2 (Canada)
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SUMMARY

High-performance liquid chromatography (HPLC) has been used to cleanup chicken liver and wood shaving samples for their subsequent analysis by mass spectrometry (MS). With a reversed-phase system and discrete fraction collection, hexa-, hepta-, octachlorodibenzodioxin and octachlorodibenzofuran have been detected in wood shavings and chicken livers to a lower level of 25 pg/g. With the use of HPLC as a cleanup tool, the MS determination both on the probe and by gas chromatography–MS resulted in an improved peak shape and a stronger more accurate signal for the dioxins and furans.

INTRODUCTION

For some time, we have been engaged in the analysis of the toxic polychlorinated dibenzodioxins (PCDD) and dibenzofurans (PCDF) in food and environmental samples. Our procedure, taken from several sources and modified to suit particular samples, used extraction with chloroform or chlroform–methanol mixtures in a neutral system similar to the extraction procedure as described by Albro and Corbett[1]. As we were interested in measuring the higher chlorinated congeners from pentachlorophenol contamination, as well as the tetrachlorodibenzodioxins, the use of an alkaline digestion[2–4] for defatting was specifically avoided. For cleanup, most of the lipid material was removed by partitioning with concentrated sulfuric acid. Further cleanup was affected by mini-columns of either Florisil or alumina which were sufficient to remove most of the polychlorinated biphenyls (PCB), DDE and remaining lipid material[3,5].

These techniques were suitable for analysing levels of PCDD and PCDF as low as 1 ng/g using gas chromatography with electron capture detection (GC–ECD). For the detection and determination of lower levels as would be found in most food and environmental samples, recourse had to be made to mass spectrometry (MS). This presented us with problems for the following reasons. The higher chlorinated dioxins, particularly octachlorodibenzodioxin (OCDD) did not pass efficiently through our GC–MS system using a separator based on diffusion through porous glass. When the

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sample extract was placed directly on the probe at low levels, the MS peak shape was distorted due to background interference of co-extracted material. To overcome these problems, we investigated the use of high-performance liquid chromatography (HPLC) to purify the sample further.

This report shows the effect on the probe MS signal of the use of HPLC to purify food and environmental samples so that low levels (< 1 ng/g) can be determined. We find that HPLC with discrete fraction collection purifies the sample greatly as evidenced on the MS probe by the improved peak shape and a more accurate signal. In addition the technique is applicable to several types of dioxins and furans. At the same time, the introduction of a HPLC step increases the specificity of the measurement when used either alone with MS on the probe or in combination with capillary GC.

EXPERIMENTAL

Precautions

Chlorinated dibenzodioxins and -furans are extremely toxic substances. Personnel working with these compounds should be aware of the hazard. All work should be carried out in an isolated laboratory following a definite protocol designed to minimize possible exposure. The laboratory protocol followed at the Health Protection Branch, Ottawa, is available on request. Wastes should be treated separately from ordinary waste and be kept to a minimum. Disposal is best affected by high temperature (>1000°C) incineration.

Equipment

HPLC. A Waters Model 6000 chromatographic pump was used along with a Schoeffel variable-wavelength absorbance detector, Model SF-770, with an 8-μl flow cell, 10 mm pathlength and 0.3 mm I.D. tubing.

Sample injection was carried out using a Valco 6-port universal inlet injector for HPLC with a loop size of 100 μl.

Conditions. A LiChrosorb reversed-phase C_8 analytical column, 250 mm $\times$ 3.2 mm I.D., 10 μm particle size, was used with methanol–water (9:1; degassed) as the eluent at a flow-rate of 0.5 ml/min (pressure was usually less than 1000 p.s.i.). The wavelength maximum for measurement of the dioxins varied among congeners and was 233, 245, 225–245 (broad peak), 250, and 233 and 250 nm for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,6,7,8- and 1,2,3,6,7,9-hexachlorodibenzodioxin (HCDD), 1,2,3,4,6,7,8-heptachlorodibenzodioxin (HpCDD), OCDD and octachlorodibenzofuran (OCDF) (two peaks), respectively, in methanol. Routinely, a value of 235 nm was chosen at an absorbance of 0.01 a.u.f.s. for standards (5–15 ng) and 0.1 a.u.f.s. for samples.

Mass spectrometer. A Varian-Mat 311A high-resolution instrument with electron impact ionization was used. The source was maintained at 250°C with an ionization voltage of 70 eV. The resolution was at least 5,000 (10% valley) for HCDD, HpCDD, OCDD and OCDF and 10,000 for TCDD. The machine was equipped with a gold leaf probe which could be heated from 25 to 225°C in 20 sec. The single ion display response was monitored on a dual pen 10 mV recorder with range between

TABLE I

IONS MONITORED WITH MS

Peaks mentioned are base molecular ion peaks of full scan.

Dioxin	*m/z monitored*
TCDD	321.89
HCDD	389.82
HpCDD	423.78
OCDD	459.73
OCDF	443.74

0.01–1.0 V for full scale deflection. The total ion monitor between 0–100 V was made on the same recorder. The ions monitored are shown in Table I.

Materials

The solvents employed were chloroform, hexane, methanol, methylene chloride, and acetonitrile and were glass distilled grade. Water was doubly distilled. Anhydrous sodium sulfate (Baker analytical; J. T. Baker, Phillipsburgh, NJ, U.S.A.), Florisil (Floridin, Pittsburgh, PA, U.S.A.), alumina (A-540; Fisher Scientific, Pittsburgh, PA, U.S.A.) and glass wool were all extracted in a soxhlet with methylene chloride for 6 h to remove impurities. Sulfuric acid was Baker analytical grade. All glassware was routinely rinsed sequentially with toluene and methylene chloride before use.

Standards

Solids. TCDD was kindly supplied by Dow Chemical (Midland, MI, U.S.A.). OCDD and OCDF were purchased from New England Nuclear (Montreal, Canada); 1,2,3,6,7,8- and 1,2,3,6,7,9-HCDD were a generous gift of J. A. Moore (Research Triangle Park, NC, U.S.A.); HpCDD was obtained from R. Pike, (Canada Agriculture, Ottawa, Canada).

Solutions. GC: 0.1 ng/μl in hexane diluted from 20 ng/μl of either hexane or toluene stock solution. OCDD is slowly soluble in hexane to a maximum concentration of 20 ng/μl.

HPLC: 5 ng/μl in acetonitrile prepared by evaporation under pure nitrogen and redilution of 20 ng/μl hexane solution.

MS: 20 pg/μl in toluene diluted from 20 ng/μl hexane or toluene solution.

Procedure

Extraction. For liver samples, a homogenized ground sample (10 g) was blended with 30 ml chloroform and 60 ml methanol for 2 min. An additional 30 ml of chloroform were added and blended for 30 sec. A volume of 40–45 ml of water was added and a third blend carried out for 30 sec. The mixture was transferred to a 250-ml separatory funnel and the chloroform layer drawn off. The water and the insoluble material at the interface were returned to the blender jar and blended for 1 min with an additional 60 ml chloroform. The separated chloroform was then combined with the first fraction.

For wood shavings and litter, the ground material (10 g) was soaked with water (100 ml) for 1 h and blended with 50 ml chloroform. Shavings were filtered through a funnel containing a glass plug into a separatory funnel and litter was

centrifuged to effect phase separation. The non-chloroform phases were blended a second time with 50 ml additional chloroform and the chloroform fractions combined.

Acid partition. The chloroform phase from the neutral extraction was crudely dried by filtration under suction through a glass filter containing *ca.* 30 g disodium sulfate and the reagent rinsed with a few ml of chloroform. The organic solvent was evaporated to 7–10 ml in a rotary evaporator under vacuum at 30–40°C and 20 ml of hexane were added. After shaking and transferring to a separatory funnel, the non-hexane layers were re-extracted with a second 20 ml portion of hexane. The combined hexane phases were then shaken with 10 ml portions of conc. sulfuric acid until the acid was clear and pale yellow (2–6 times). The first extraction was shaken lightly to avoid strong emulsions. The hexane was washed with a little water, dried and concentrated to 5 ml in a rotary evaporator under vacuum at 30–40°C. The extract was finally reduced to 1 ml (not dryness) under nitrogen in a 15 ml centrifuge tube with washing of the sides of the tube during concentration.

Cleanup. Florisil column chromatography. A Pasteur pipette containing a small plug of glass wool, 5 cm (*ca.* 1 g) of Florisil, and a second plug of wool, was activated at 140°C overnight. The column was prewashed with 5 ml methylene chloride and 2 ml hexane both of which were discarded. The sample in 1 ml hexane was adsorbed onto the column and the container washed twice with 2 ml portions of hexane which were added to the column. All the dioxins and furans were eluted with 8 ml of methylene chloride which was evaporated to a small volume under nitrogen in a 15 ml centrifuge tube with washing of the walls of the container. The sample was then transferred to a 0.5 ml conical tapered vial with two to three washings of 50–100 μl methylene chloride. The organic solvent was then evaporated just to dryness under nitrogen at room temperature and the sides of the vial washed once more with a little methylene chloride and the latter taken again to dryness.

HPLC procedure. To ascertain the retention time of the dioxins for sample fraction collection, a mixed standard containing 5–15 ng each of TCDD, HCDD, HpCDD, OCDD, and OCDF in 50 μl acetonitrile and 50 μl mobile phase was injected onto the column via the 100-μl loop. These amounts of standards at 0.01 absorbance gave deflections of 15–40%. The sample loop was then thoroughly cleaned with three 100 μl injections of methylene chloride followed by three 100 μl injections of methanol.

The partially purified sample in a small tapered vial was completely dissolved in 40 μl acetonitrile. A 100-μl injection syringe was charged with 20 μl of methanol–water (9:1), then 40 μl of sample in acetonitrile. The sample vial was rinsed with a further 10 μl of acetonitrile and the syringe charged with this 10 μl wash and, finally, 10–15 μl methanol–water (9:1). The entire syringe load including the sample and flush volume was injected via the loop onto the column. At the retention time of each dioxin, a fraction of 1.5–2.0 ml (3–4 min elution time) was taken, usually 0.5 min before and 0.5 min after the standard peak. The fraction was collected in a 25-ml volumetric flask containing 1 ml of hexane. The flask was then made to volume with water, shaken several times, and the hexane transferred in steps with a pre-rinsed Pasteur pipette into a 0.5 ml conical vial. The aqueous phase was extracted with an additional 1 ml of hexane. The hexane washings were taken to dryness under nitrogen and the residue put into 25–40 μl of toluene for MS.

The HPLC analytical column was reconditioned after every second or third

sample by flushing the entire system with methylene chloride for 5–10 min followed by methanol–water (9:1) for 30 min.

Mass spectrometry procedure. A standard (20 pg/μl in toluene) of the dioxin of interest was injected onto the probe, a vacuum attained and heat applied. This standard of 100 pg typically gave 50–70% full scale on a 10-mV recorder at the 0.03-V range with a total ion current of less than 10^{-8} A corresponding to 1 V on the machine meter. The same procedure was repeated for a sample and the range adjusted if necessary. Sample concentration was estimated using peak heights taken from a standard curve. Each dioxin or furan from a single food or environmental sample required a separate probe analysis. Recovery values of the above HPLC procedure were obtained using glass capillary GC–MS. The column was a 15 m wall-coated open tubular column (0.25 mm I.D.) SP-2100 operated at 200, 220 and 250°C for TCDD, HCDD, OCDD, respectively with a flow of helium of 50 cm/sec. MS resolution in this case was 1000. The glass capillary GC system was interferfaced directly to the MS source via a platinum wire.

RESULTS AND DISCUSSION

Initial experiments in the analysis of PCDD and PCDF showed that the use of a neutral extraction combined with sulfuric acid and Florisil column chromatography (especially good for separation of dioxins from PCB[3,5] was sufficient to purify food and environmental samples so that they could be measured with GC–ECD down to 1 ng/g. For lower levels, it was necessary to use MS or GC–MS. With GC–MS with packed columns, we had problems of retention–adsorption on the porous-glass separator with the higher chlorinated congeners. With the MS alone on the probe, the samples were still so dirty that the total ion monitor was often above 50 V, the peak was broad and often multiple, and suppression of signal was evident, obviating quantitative measurements. The use of a second mini-column such as alumina for further purification did not appreciably reduce the total ion output of the sample extract on the mass spectrum. To circumvent these problems, we investigated the use of HPLC to purify the samples.

The analysis of dioxins by HPLC with adsorption chromatography is restricted. These compounds elute in such a short time[6,7] that especially dry hexane as the elution solvent must be used. However, dioxins are more commonly separated and quantitated by HPLC using reversed-phase chromatography. Samples analysed have been pesticide formulations[8,9] for high levels ($> \mu$g/g), and, more recently, fish[10] where the single dioxin TCDD was measured using an elevated column temperature. When we used HPLC with reversed-phase systems, initially our standards of dioxins for retention time analyses were dissolved in methylene chloride since this solvent is one of the best for the higher chlorinated dioxins. However the use of as little as 20 μl of methylene chloride as standard solvent on our reversed-phase system caused premature elution and distortion of the dioxin peak. This effect on chromatography is illustrated in Fig. 1 for HCDD and OCDD (lower diagram for methylene chloride). When the standards were in either methanol, ethanol or acetonitrile (upper diagram), the peak shape was good and retention times consistent even when up to 50 μl of these injection solvents were used. Greater amounts of injection solvent resulted in unacceptable

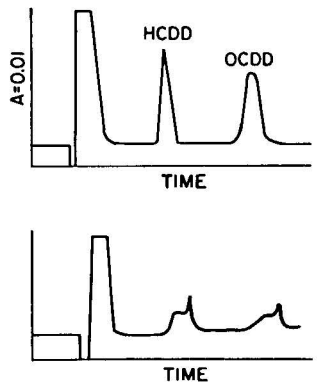

Fig. 1. Solvent effect on elution of dioxin standards (10–25 ng) on reversed-phase HPLC at 235 nm with methanol–water (9:1), 0.5 ml/min, on LiChrosorb C_8 column, 250 mm × 3.2 I.D., 10 μm. Upper diagram, with methanol or acetonitrile as standard solvent (50 μl injection); lower diagram, with methylene chloride as solvent (20 μl).

peak broadening. Acetonitrile was chosen as the solvent since it had better solvent properties than alcohol for partially purified samples.

To ensure high recovery of dioxins and accuracy of the procedure, there are several important points. Firstly, the injection valve must be thoroughly cleaned after the standards are run and before the sample is injected. Otherwise, a small amount of contamination in the valve (*e.g.* 0.1 ng corresponding to 10 pg/g) will give a positive result and preclude meaningful measurements at low levels. We have overcome this cross contamination problem by using two injection valves connected in parallel; one for the standards and one for the samples. Secondly, the retention time of the standards must be reproducible and stable otherwise recoveries will be low; constant flow, temperature, and pressure are necessary to achieve this. Thirdly, when analysing high levels of dioxins (*e.g.* in a formulation or grossly contaminated sample), the insolubility of the higher chlorinated congeners in the HPLC eluting solvent must be taken into account (*e.g.* OCDD has a solubility of 0.1 μg/ml in methanol–water (9:1)). In these cases, the analyst must make a judgement on how much sample to inject into the HPLC. Recoveries of 1 ng quantities of the three dioxins, TCDD, 1,2,3,6,7,8-HCDD and OCDD through the HPLC portion of the method alone as measured by glass capillary GC–MS at 1000 resolution are listed in Table II. The recoveries for spiked liver previously shown to be negative are good for TCDD and HCDD but lower and more variable for OCDD. The reason for the lower recovery for OCDD is not certain but the "lost" OCDD is not found in earlier or later eluting fractions of the HPLC as these were negative when monitored. This data shows there is little or no loss of dioxins through the HPLC step.

Fig. 2 illustrates the HPLC elution pattern of PCDD and PCDF in a standard solution (upper diagram) and of residues detected in a cleaned up extract of contaminated wood shavings used as poultry litter (lower diagram). With the wood shavings sample, due to the high levels present, the actual peaks for OCDD, OCDF, and HpCDD can be detected by their absorption at 235 nm. However, with liver and other samples containing low levels of contamination, this detection by UV is not possible. It is to be noted that the wood shavings sample has a UV peak on HPLC

TABLE II

RECOVERY DATA OF 1.0 ng OF THREE CHLORINATED DIBENZODIOXINS FROM THE HPLC PROCEDURE

Sample		*Recovery (%)*		
		TCDD	*1,2,3,6,7,8-HCDD*	*OCDD*
Solvent	$\bar{x}$	100	95	92
	S.D.	7	20	16
	n	3	5	5
Chicken liver (10 g)	$\bar{x}$	102	101	94
	S.D.	13.8	14.5	26.9
	n	5	5	4

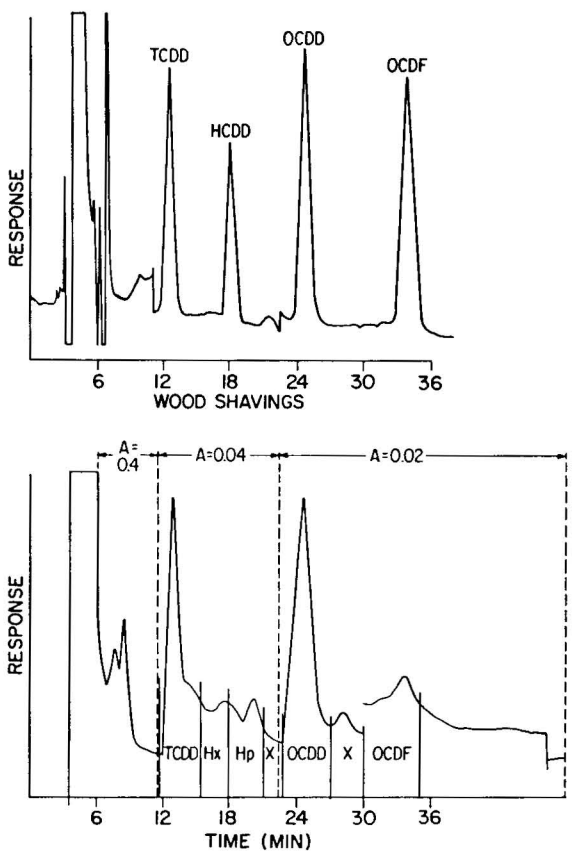

Fig. 2. Method used to collect dioxin fractions from HPLC prior to analysis by MS. Upper diagram, elution pattern of 10–25 ng standards at 235 nm and $A = 0.01$ to obtain retention times; lower diagram, actual tracing of extract of wood shavings at 235 nm and various absorbance values. Vertical lines are collection points of fractions for MS starting at about 11 min and ending at 35 min with five fractions collected. Hx = HCDD; Hp = HpCDD.

near the retention time of TCDD but subsequent analysis by MS gave a negative result.

The effect on the MS by probe analysis of the HPLC cleanup on liver samples is illustrated in Fig. 3. In this case the total ion monitor and peak shape of the MS both before (upper diagram) and after (lower diagram) HPLC is shown from three chicken liver samples taken from a flock reared on wood shavings contaminated with dioxins. The marked decrease in the total ion monitor and the improvement in peak shape is evident after HPLC. The measured levels of several dioxins and OCDF in two of the livers are listed in Table III both before and after HPLC. Before HPLC cleanup, the quenching of the signal at high resolution (attested by the high total ion current) gave either a lower value or no measureable value. This was rectified after HPLC to a more accurate value approaching more closely the standard since both the total ion decreased and the peak shape inproved. The nature of the interfering material is not certain except that it does give a strong total ion peak on MS and is removed by the above HPLC procedure. In this connection, Baughman and Meselson[11] found that the MS signal for TCDD was suppressed 50% by the presence of 5 μg of the

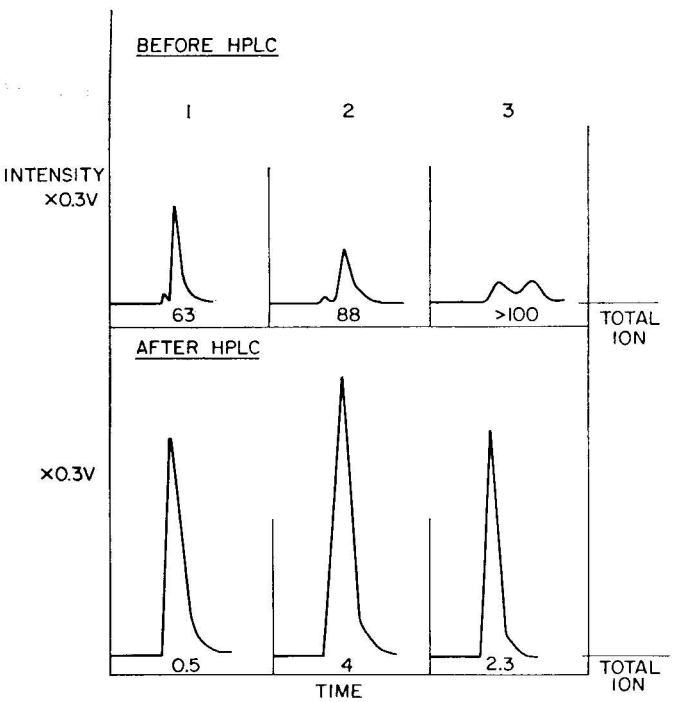

Fig. 3. Mass chromatograms of three liver samples for OCDD at m/z 459.7; Resolution 5000 both before (upper) and after (lower) HPLC. 1 g total sample extract on probe heated from 25 to 225°C in 20 sec; measurements on 10-mV recorder and total ion monitor in volts shown below peak.

TABLE III

PCDD AND PCDF LEVELS OF TWO LIVER SAMPLES ANALYSED BY MS BOTH BEFORE AND AFTER HPLC

Dashes indicate no analysis was carried out.

Compound	*Before HPLC*		*After HPLC*	
	Concentration (pg/g)	*Total ion (V)*	*Concentration (pg/g)*	*Total ion (V)*
Liver 1				
HCDD	0	90	43	5
HpCDD	26	40	243	3
OCDD	169	63	433	3.5
OCDF	—	—	87	3.5
Liver 2				
HCDD	—	—	35	2
HpCDD	—	—	82	3
OCDD	92	88	673	4
OCDF	—	—	130	2.3

lipid squalane. The cleanliness of the final extract was further attested to by its response on GC with flame ionization detection where no positive peaks were observed after the solvent peak. The limit of detection in 10 g liver samples varied depending both on the dioxin and the specific sample. Using a criterium of signal-to-noise ratio of 3:1, it was at least 25 pg/g and in most cases lower.

This HPLC procedure was also used in analysis of TCDD in fish. Application to over thirty samples showed that it was possible to inject large amounts (1–2 g of extracts) onto a glass capillary column directly interfaced with a MS system without affecting either GC or MS. Injection of such large amounts of concentrated extract into the MS without prior HPLC cleanup was not possible either due to poor GC resolution and rapid column deterioration or contamination of the MS source. Although the procedure of manually collecting fractions from an HPLC column and evaporating the solvent before analysis by MS or GC–MS is tedious, the technique not only adds specificity to the measurement, but also improves the purification of the sample so that low levels can be detected, and is applicable to many types of PCDD and PCDF. Until the commercial models for achieving direct interfacing of HPLC with MS are more reliable and versatile[12], we believe this described procedure has definite advantages. A possible improvement would be automatic collection of sample fractions.

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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF AROMATIC SULPHONIC ACIDS AND OTHER STRONGLY POLAR COMPOUNDS WITHOUT ADDITION OF AN ION-PAIRING COUNTER-ION

PAVEL JANDERA* and JAROSLAV CHURÁČEK
Department of Analytical Chemistry, University of Chemical Technology, Pardubice (Czechoslovakia)
(Received April 30th, 1980)

SUMMARY

Efficient separations of aromatic sulphonic acids, which are important as dye intermediates, can be achieved on chemically bonded reversed-phase columns using mobile phases containing strong inorganic electrolytes instead of organic ion-pair-forming substances. The system is highly selective and allows the rapid separation of a number of isomeric aromatic mono-, di- and trisulphonic acids. Other ionic or strongly polar substances, such as linear alkylbenzenesulphonates and water-soluble vitamins, can also be separated using this technique.

INTRODUCTION

Aromatic sulphonic acids, used as intermediates in the production of a large number of important dyes, are most often prepared using sulphonation of simple aromatic hydrocarbons or their derivatives. This operation usually yields mixtures of isomers and compounds with different numbers of sulphonic groups. Appropriate adjustment of reaction conditions, such as temperature and concentration of the sulphonation agent in the reaction mixture, can be used for control and optimization of the ratio of the individual products. For this purpose, as for the control of intermediates and technical products, a precise and rapid analytical method is necessary.

For the analysis of sulphonic acids, modern high-performance liquid chromatography is potentially superior to other methods, which are either time consuming and of insufficient efficiency and accuracy (paper and thin-layer chromatography) or require the preparation of volatile derivatives (gas chromatography).

Chromatography on ion-exchange columns has not been very successful for the analysis of aromatic sulphonic acids owing to the low selectivity and efficiency and especially the very strong retention on styrene–divinylbenzene ion-exchange resins[1].

Recently, ion-pair chromatography has been introduced for the separation of ionic and strongly polar organic substances[2–5]. This technique makes use of the

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formation of ion pairs with appropriate counter-ions, either in the liquid stationary phase anchored on an appropriate support[6] (silica or a short alkyl-chain reversed phase) or in the aqueous–organic mobile phase in reversed-phase systems[4,7–10].

Good separations of some aromatic sulphonic acids have been achieved using reversed-phase ion-pair chromatography on chemically bonded packing materials[9,10], bleeding of the stationary phase being avoided. Retention of sample compounds and the selectivity, to a certain extent, can be controlled by adjusting the type and concentration of the ion-pair-forming counter-ion added to aqueous–organic mobile phase (usually a tetraalkylammonium salt, such as cetyltrimethylammonium bromide or tetrabutyl-, tetraethyl- and tetramethylammonium phosphate or sulphate) and by selection of the type and concentration of the organic solvent in the mobile phase[4,7–10]. Addition of inorganic electrolytes in minor concentrations to the mobile phase or adjustment of its pH can also be used to improve the separation[8,9,11–13]. An appropriate choice of the separation conditions in reversed-phase ion-pair chromatography is critical for the achievement of good separations. As the separation mechanism in ion-pair chromatography is complex and not fully understood yet, it is not always easy to find good separation conditions for each separation problem, and with certain ion-pair-forming counter-ions or certain organic solvents the compounds may be either strongly retained or, on the contrary, remain almost unretained over the whole composition range of the mobile phase used[7,9]. Peak splitting at certain compositions of the mobile phase was occasionally observed with some compounds[9]. Moreover, tetraalkylammonium salts and other ion-pair-forming substances added to the mobile phase in this method are expensive.

Most experiments with reversed-phase chromatography of ionic and strongly polar substances without the addition of ion-pair-forming counter-ions failed because pure water or aqueous–organic solutions were used, with subsequent addition of a buffer or other salt in relatively low concentrations. Then, the ionic and strongly polar compounds were eluted near or even prior to the column void volume, usually as strongly distorted or split peak with irreproducible shapes, owing to ionic-exclusion effects[10]. As we showed elsewhere[10,14], this effect can be overcome by addition of a strong electrolyte (inorganic or certain organic salts) in a relatively high concentration (usually 0.1 *M* or more) to the mobile phase. Then, most strongly polar and ionic compounds become retained, even strongly, and can be eluted as narrow, symmetrical peaks, like non-polar substances[14]. Retention (and the selectivity, to a certain extent) can be controlled conveniently and simply by adjusting the concentrations of the electrolyte and the organic solvent. An increase in the concentration of the electrolyte or a decrease in the concentration of the organic solvent promotes retention on reversed-phase columns[14]. No peak splitting was observed using this method[14]. Lastly, the mobile phases used in this system are much cheaper than those in ion-pair chromatography.

EXPERIMENTAL

The equipment consisted of an M6000 pump (Waters Assoc., Milford, MA, U.S.A.), an injection port which made possible direct syringe injection on the top of the column and an M440 UV detector (Waters Assoc.). A PPM 68005 gradient-forming device (Workshops of the Czechoslovak Academy of Sciences, Prague,

Czechoslovakia) connected to the inlet of the M6000 pump was used in gradient elution[15].

The reversed-phase column packing material was prepared from LiChrosorb SI 100 (10 μm) (Merck, Darmstadt, G.F.R.) by reaction with *n*-octadecyltrichlorosilane[16]. The chemically bonded reversed phase (C_{18}) prepared in this way contained 16.5% (w/w) of carbon and was slurry-packed[17] into a stainless-steel column (300 × × 4.2 mm I.D.). The void volume of the column was determined as the retention volume of ^2H_2O measured with aid of a differential refractometer (R-401, Waters Assoc.), and was 3.05 ml.

The solvents used as the components of the mobile phase were prepared by dissolving the calculated amount of sodium sulphate or lithium sulphate in water or in previously prepared aqueous methanol or by mixing water with methanol. Methanol and water were distilled in glass before use. The salts used were of analytical-reagent grade. The samples of acids were obtained from East-Bohemian Chemical Works Synthesia (Semtin, Czechoslovakia), and were dissolved in the mobile phase used as solvent A.

RESULTS AND DISCUSSION

Firstly, a separation of technically important sulphonic acids potentially present in reaction mixtures after the sulphonation of naphthalene was attempted. As the retention of sulphonic acids decreases with increasing number of sulphonic groups in the molecule of the acid, naphthalenetri- and -tetrasulphonic acids are only slightly retained on an octadecylsilica column; 0.4 *M* sodium sulphate in water without the addition of any organic solvent had to be used as the mobile phase in order to achieve their retention and separation. Under these conditions, naphthalenedi- and -monosulphonic acids are strongly retained and a decrease in concentration of sodium sulphate together with addition of an organic solvent (methanol) to the mobile phase was used for their elution.

Thus, the separation of a mixture containing one naphthalenetetrasulphonic, three naphthalenetrisulphonic, four naphthalenedisulphonic and naphthalene-1- and -2-sulphonic acids could be achieved using the elution of the tetra- and trisulphonic acids with 0.4 *M* sodium sulphate followed by linear gradient elution of di- and monosulphonic acids using 0.4 *M* sodium sulphate as solvent B. Fig. 1 shows the separation under these conditions, which took *ca.* 28 min.

Anthraquinonemono- and -disulphonic acids are more strongly retained than naphthalenesulphonic acids. A rapid separation of a five-component mixture of the acids produced in the sulphonation of anthraquinone could be achieved in 9 min using stepwise elution. Fig. 2 shows that anthraquinonedisulphonic acids were eluted with 0.133 *M* sodium sulphate in methanol–water (40:60) and the elution of anthraquinone-1- and -2-sulphonic acids followed using methanol–water (60:40) without the addition of a salt in the second step.

Fig. 3 shows the separation of five isomeric 1-naphthylaminesulphonic acids in 12 min. 1-Naphthylamine-5- and -4-sulphonic acids were eluted in an isocratic step using 0.32 *M* sodium sulphate in methanol–water (12:88) as the mobile phase, followed by linear gradient elution of the remaining three isomeric acids using methanol–water (60:40) as solvent B.

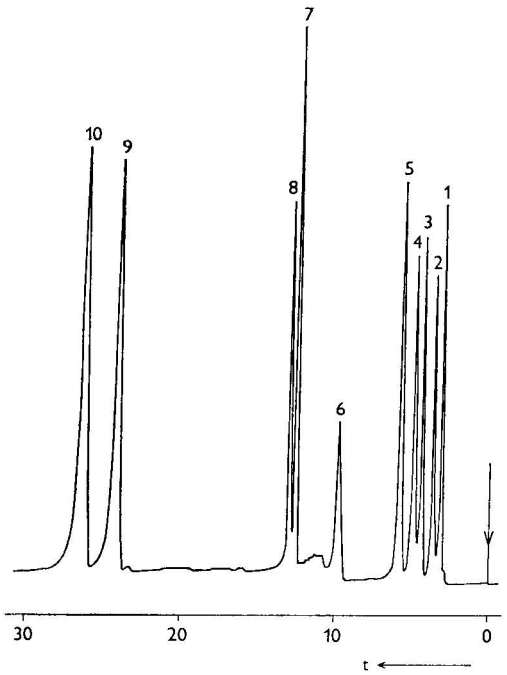

Fig. 1. Reversed-phase separation of naphthalenemono-, -di-, -tri- and -tetrasulphonic acids. Column: C_{18}, 10 μm, 300 × 4.2 mm. Mobile phase: step 1, isocratic elution with 5 ml of 0.4 *M* Na_2SO_4 in water; step 2, linear gradient elution, 0–90% B in 13.5 min. Solvent A, 0.4 *M* Na_2SO_4 in water; solvent B, methanol–water (40:60). Flow-rate: 1.0 ml/min. Detection: UV, 254 nm, 0.5 a.u.f.s. *t* = time elapsed (minutes). Compounds: 1 = naphthalene-1,3,5,7-tetrasulphonic acid; 2 = napthalene-1,3,6-trisulphonic acid; 3 = naphthalene-1,3,5-trisulphonic acid; 4 = naphthalene-1,3,7-trisulphonic acid; 5 = naphthalene-1,5-disulphonic acid; 6 = naphthalene-2,6-disulphonic acid; 7 = naphthalene-1,6-disulphonic acid; 8 = naphthalene-2,7-disulphonic acid; 9 = naphthalene-1-sulphonic acid; 10 = naphthalene-2-sulphonic acid.

The method described can be used for the separation of linear alkylbenzenesulphonates, which are the major surfactants present in household detergents. These compounds can be separated using gas chromatography after desulphonation prior to the separation step[18], on organic gels[19–21] or using reversed-phase ion-pair chromatography with tetramethylammonium[7] or cetrimide[22] as the counter-ion added to the mobile phase[7]. Normal reversed-phase chromatography on chemically bonded phases using methanol–water as the mobile phase without further components was claimed to fail in the analysis of linear alkylbenzenesulphonates[7], but the separation can be achieved with the addition of an inorganic salt to the mobile phase, as demonstrated in Fig. 4, where chromatography of a dilute commercial household detergent is shown under gradient elution conditions.

Not only strong sulphonic (and carboxylic) acids can be separated using this technique, but also basic compounds or mixtures containing both basic and acidic compounds, as it demonstrated in Fig. 5, where the separation of five water-soluble vitamins was achieved in 13 min using as the mobile phase 0.5 *M* lithium sulphate in methanol–water (5:95) for isocratic elution.

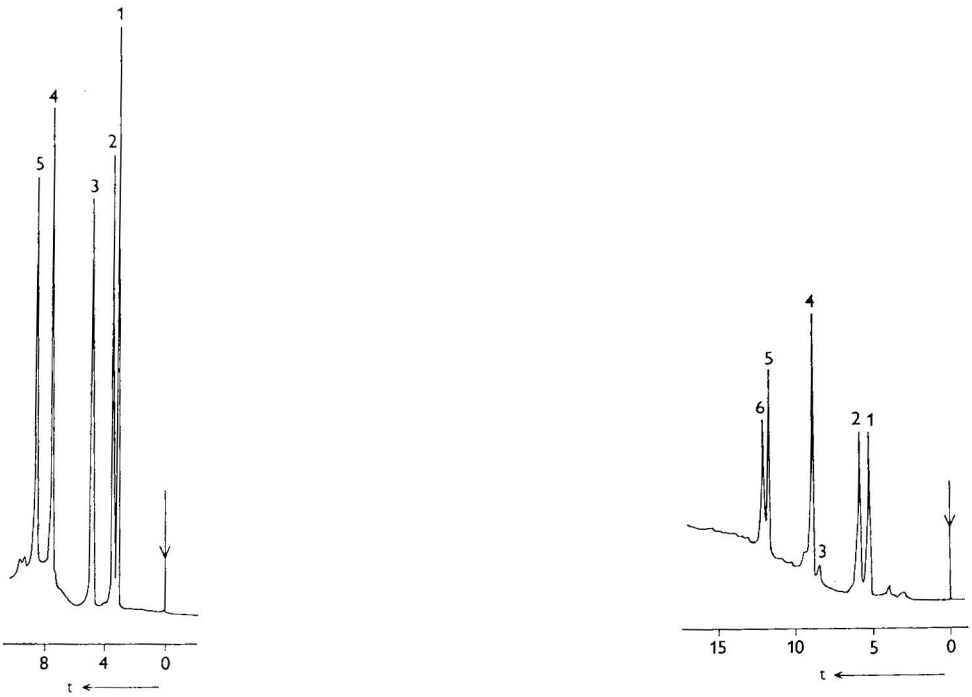

Fig. 2. Reversed-phase separation of anthraquinonemono- and -disulphonic acids. Column as in Fig. 1. Mobile phase: step 1, isocratic elution with 5 ml of 0.133 *M* Na_2SO_4 in methanol–water (40:60); step 2, isocratic elution with methanol–water (60:40). Flow-rate: 1.0 ml/min. Detection: UV, 254 nm, 0.1 a.u.f.s. t = time elapsed (minutes). Compounds: 1 = anthraquinone-1,5-disulphonic acid; 2 = anthraquinone-2,6-disulphonic acid; 3 = anthraquinone-1,8-disulphonic acid; 4 = anthraquinone-1-sulphonic acid; 5 = anthraquinone-2-sulphonic acid.

Fig. 3. Reversed-phase separation of isomeric 1-naphthylaminemonosulphonic acids. Column as in Fig. 1. Mobile phase: step 1, isocratic elution with 5 ml of 0.32 *M* Na_2SO_4 in methanol–water (12:88); step 2, linear gradient elution, 20–100% B in 12 min. Solvent A, 0.4 *M* Na_2SO_4 in water; solvent B, methanol–water (60:40). Flow-rate: 1.0 ml/min. Detection: UV, 254 nm, 0.5 a.u.f.s. t = time elapsed (minutes). Compounds: 1 = 1-aminonaphthalene-5-sulphonic acid; 2 = 1-aminonaphthalene-4-sulphonic acid; 3 = impurity; 4 = 1-aminonaphthalene-6-sulphonic acid; 5 = 1-aminonaphthalene 7-sulphonic acid; 6 = 1-aminonaphthalene-8-sulphonic acid.

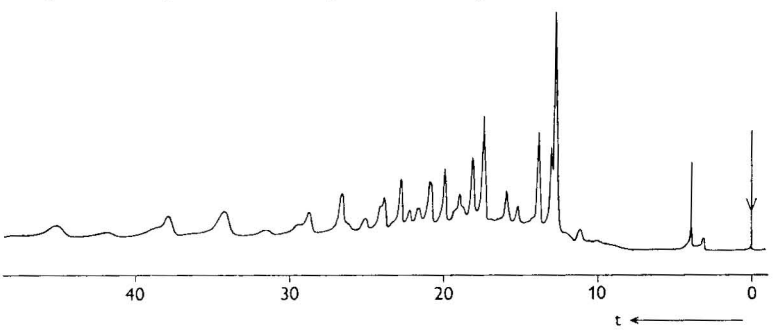

Fig. 4. Reversed-phase separation of alkylbenzenesulphonate surfactants in a commercial household detergent (10 μl of a sample diluted 1:10 with water). Column as in Fig. 1. Mobile phase: linear gradient elution. 0–100% B in 15 min. Solvent A, 0.4 *M* Na_2SO_4 in water; solvent B, methanol–water (60:40). After the end of the gradient, isocratic elution with pure solvent B followed. Flow-rate: 1.0 ml/min. Detection: UV, 254 nm, 1.0 a.u.f.s. t = time elapsed (minutes).

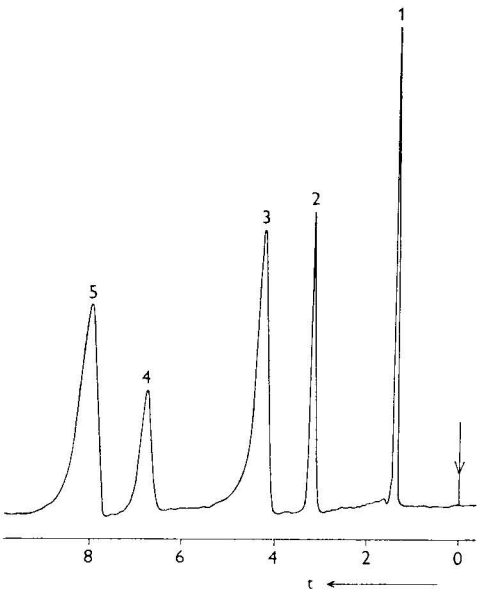

Fig. 5. Reversed-phase separation of a mixture of water-soluble vitamins. Column as in Fig. 1. Mobile phase: 0.5 *M* Li_2SO_4 in methanol–water (5:95) (isocratic elution). Flow-rate: 1.5 ml/min. Detection: UV, 254 nm, 0.05 a.u.f.s. *t* = time elapsed (minutes). Compounds: 1 = ascorbic acid; 2 = nicotinic acid; 3 = pyridoxine; 4 = nicotinamide; 5 = thiamine.

CONCLUSIONS

A few examples of the chromatographic separation of mixtures of aromatic sulphonic acids and other strongly polar compounds demonstrate the high selectivity and efficiency of reversed-phase chromatographic separations using high concentrations of salts in the mobile phase. Here, the ionic strength of the mobile phase is the most important factor controlling the separation. The retention and selectivity can, of course, be modified by selection of different salts used as the electrolytes in the mobile phase, but the type of salt seems to have only a minor effect on the separation[11]. A more detailed study would be necessary, however, for definite conclusions to be drawn.

The proposed method seems to be promising for the rapid separation of ionic and strongly polar compounds, and may compete successfully with reversed-phase ion-pair chromatography.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BEER BITTER ACIDS

C. DEWAELE and M. VERZELE*

Laboratory of Organic Chemistry, State University of Ghent, Krijgslaan, 271 (S. 4), B-9000 Ghent (Belgium)

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SUMMARY

The high-performance liquid chromatography of the six beer bitter iso-α-acids on different modified silica gel stationary phases is described. Octadecyl-, nitrophenyl- and cyanopropyl-substituted and buffered silica gels were studied with a variety of solvent systems. The aim was to find a system suitable for the routine isocratic analysis of the beer bitter compounds. Although the six iso-α-acids can be separated, it is concluded that a separation in three groups for each *cis* and *trans* pair corresponding to the three α-acids is preferable for this purpose. This is possible with an ion-pairing method on octadecyl-silica gel.

INTRODUCTION

The bitter-tasting compounds in beer mostly occur in concentrations around 25 ± 10 ppm. They are formed during the brewing process by isomerization of the three hop α-acids: humulone, adhumulone and cohumulone. Each α-acid yields a *cis* and a *trans* isomer and therefore there are six major iso-α-acids in beer. Their bitter tastes are almost identical and therefore it has been convenient to determine them as a group. Many contributions to this analysis can be found in the literature, but a really satisfactory method for iso-α-acids in beer is still lacking. Numerous ring analyses, inter-laboratory exercises and comparative evaluations organized by the European Brewery Convention (EBC), the American Society of Brewing Chemists (ASBC) or others have given inadequate results, considering the efforts made.

It is advantageous today to turn to high-performance liquid chromatography (HPLC) to obtain the required separation efficiency for the type of analysis involved. This investigation was a study of the HPLC of beer bitter acids. Our main aim was to establish the potential of commercially available derivatized silica gel HPLC stationary phases for the routine analysis of iso-α-acids. A similar study, mainly on the unisomerized hop bitter acids, the α- and β-acids, has recently been published[1].

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EXPERIMENTAL AND RESULTS

All separations were carried out on a Varian 5000 LC chromatograph, equiped with a 10-μl Valco 7000 sample loop injector and a Varichrom variable-wavelength detector. The columns were 25 $\times$ 0.46 cm I.D. Lichroma tubing unless otherwise specified. The stationary phases, all 10 μm, were obtained from RSL (Eke, Belgium) and were used without further treatment. The columns were packed either with a carbon tetrachloride or a glycerol–methanol suspension slurry. All analyses were optimized in the isocratic mode as this greatly facilitates eventual routine analysis.

The chemicals used were pure or were appropriately purified. Special attention was paid to the quality of the solvents (water and methanol).

Beer bitter substances were extracted from acidified beer with 2.5 times their volume of isooctane. Iso-α-acids in the extract form were obtained from PRB (Wetteren, Belgium). Pure individual bitter compounds were prepared from hops according to the techniques developed in this laboratory[2,3].

Reversed-phase chromatography

Reversed-phase C_{18} silica gel or octadecyl-silica gel is generally the most successful of all derivatized silica gels. An example of a separation of hop acids on RSil-C18-HL is shown in Fig. 1. This trace contains only one iso-α-acid peak, namely that of *trans*-isohumulone. The other peaks normally do not occur in beer, or in only very small concentrations, but for some applications it is interesting to know the elution sequence of Fig. 1.

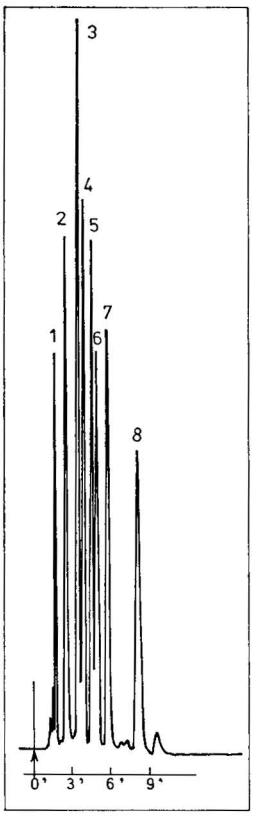
When the percentage of water is increased it becomes possible to separate nearly all of the iso-α-acids. A chromatogram obtained on isomerized hop extract is shown in Fig. 2. The analysis time is about 30 min. Complete resolution, which would permit easy quantitation, is not attained, however. With the high water and buffer concentrations, a high counter pressure is generated (*ca.* 300 kg/cm^2 at 100 ml/h) and a slight loss in column efficiency occurs[4]. With 5-μm RSiL-C18-HL the separation can be complete, but the analysis takes 1 h and is clearly more difficult. A trace of an isomerized hop extract in these conditions is given in ref. 1.

Another possibility is to use four coupled 10-μm columns. The resolution is much improved, as shown in Fig. 3. However, the analysis time is excessive.

As the complete resolution of the six iso-α-acids appears to be impractical, we can aim for partial or group separation, *e.g.* of all the *trans*- and all the *cis*-iso-α-acids together. The complete separation of the *cis–trans* pairs derived from each α-acid would even be better, as this can be related to hop origin and quality. This last aim can be partly achieved on octadecyl-silica gel by using a buffer, as shown in Fig. 4. The isoadhumulones are co-eluted with the isohumulones in Fig. 4. The analysis time is fairly long.

Although our interest was in isocratic analyses, in this instance it is worth mentioning what can be achieved by judicious gradient elution. Such an optimized separation of a large number of six- and five-membered hop and beer bitter acids is shown in Fig. 5.

It must also be emphasized that the octadecyl-silica gel must be of high quality in order to be able to obtain the separations shown here and that it must especially have a very low concentration of trace metal impurities. Trace metals can



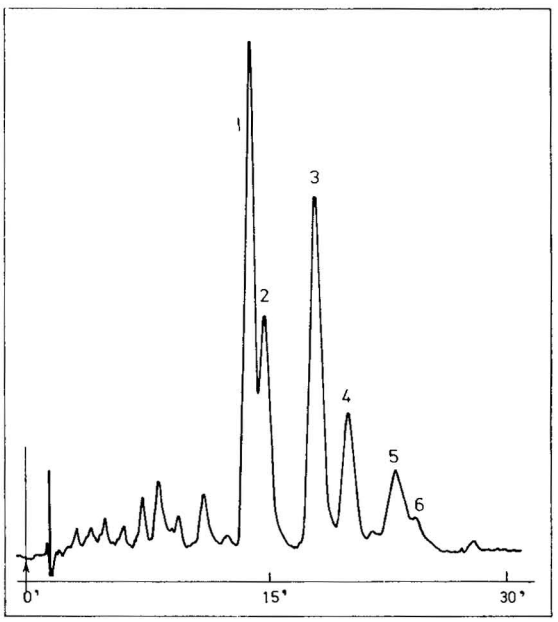


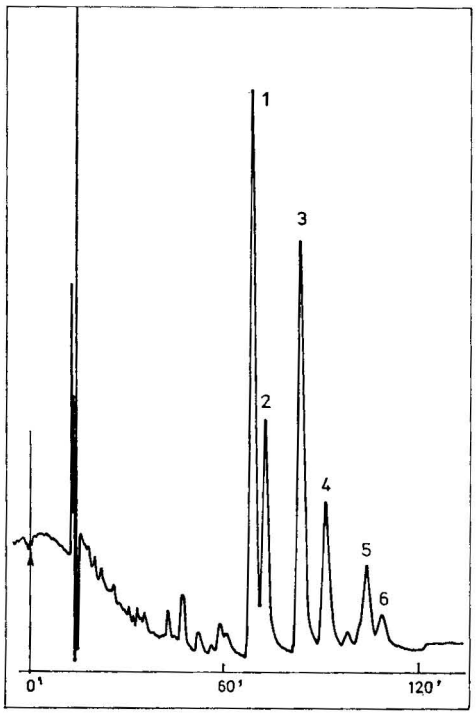
Fig. 1. Reversed-phase chromatogram of a synthetic mixture of hop bitter acids. Eluent: methanol–water–85% orthophosphoric acid (90:10:0.25) at 2 ml/min and 160 kg/cm^2. Detection wavelength: 280 nm. Peaks: 1 = deacylated anti-isohumulone; 2 = *trans*-humulinic acid; 3 = *trans*-isohumulone; 4 = humulone; 5 = *exo*-tricyclooxycolupulone; 6 = colupulone; 7 = lupulone; 8 = hexahydrocolupulone.

Fig. 2. Reversed-phase chromatogram of isomerized hop extract. Eluent: methanol–0.5 *M* citric acid buffer, pH 3.0 (60:40) at 100 ml/h and 250 kg/cm^2. Detection wavelength: 280 nm. Isomerized hop extract showing the six major iso-α-acids mentioned in the text in the sequence 1 = *cis*-isocohumulone, 2 = *trans*-isocohumulone, 3 = *cis*-isohumulone, 4 = *trans*-isohumulone, 5 = *cis*-isoadhumulone and 6 = *trans*-isoadhumulone.

be removed from the reversed-phase material by repeated boiling in 1 *N* or stronger hydrochloric acid–methanol (1:1) mixtures, followed each time by extensive washing with methanol[5]. For identification purposes the elution sequence and relative retention times in Table I are useful.

Ion-pair chromatography

Whitt and Cuzner[6] developed an ion-pair chromatographic group separation of the isohumulones and the isocohumulones. The isoadhumulones are co-eluted with the isohumulones. Whitt and Cuzner[6] used 0.005 *M* tetrabutylammonium phosphate and a methanol gradient from 50 to 60%. This separation, which takes only 8–10 min, looks promising for the routine HPLC analysis of the beer bitter acids.



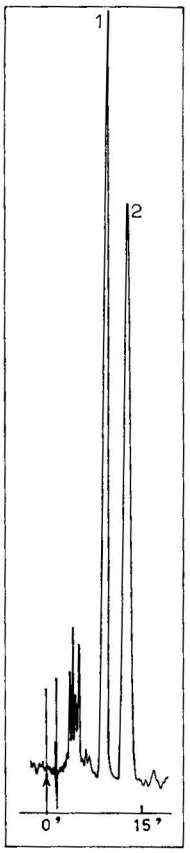


Fig. 3. Reversed-phase chromatogram of isomerized hop extract on a 1-m column in four 25 × 0.2 cm sections. Solvent as in Fig. 2 at 10 ml/h and 360 kg/cm². Peaks as in Fig. 2.

Fig. 4. Reversed-phase chromatogram of isomerized hop extract. Eluent: methanol–0.5 *M* citric acid buffer, pH 4.0 (60:40) at 100 ml/h and 250 kg/cm². Detectionwavelength: 280 nm. Peaks: 1 = *cis*- and *trans*-isocohumulone; 2 = the four other iso-α-acids.

We have studied systematically changes in the nature (cetyltrimethylammonium, tetrabutylammonium, tetraethylammonium, tetramethylammonium and ammonium) and concentration (0.1, 0.01, 0.005 and 0.001 *M*) of the counter ion. The retention decreased with size and concentration, but no substantial changes in resolution were noted. The selectivity, however, was sometimes dependent on very small changes in eluent composition. With the system methanol–water–85% orthophosphoric acid (72.5:27.5:1 –0.02 *M*) tetrabutylammonium salt the chromatogram shown in Fig. 6 is obtained isocratically (β-phenylchalkone was used as a possible internal standard).

The isoadhumulones were separated from the isohumulones. The slightly longer analysis time was compensated for by the separation of the isoadhumulones from the other two pairs. The difference from the Whitt and Cuzner[6] procedure is thus that the separation is run isocratically and that three peaks (for each pair of

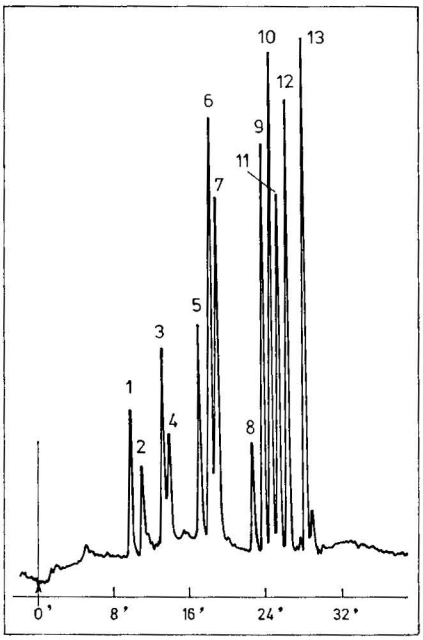


Fig. 5. Reversed-phase gradient chromatography of a synthetic mixture of hop and beer bitter acids. Eluent: from methanol–water–85% orthophosphoric acid (50:50:0.5) to methanol–85% orthophosphoric acid (100:0.5) in 30 min at 2 ml/min and with pressure from 300 to 150 kg/cm^2. Detection wavelength: 280 nm. This trace can be produced only with very pure components of the eluent on a suitable demineralized reversed-phase material. Peaks: see Table I.

cis–trans-iso-α-acids) are obtained instead of two. For routine beer analysis this is considered to be important.

Normal-phase chromatography

Silica gel. Gill[7] has used di-*n*-butylammonium acetate as an ion-pair former

TABLE I

RETENTIONS RELATIVE TO HUMULONE FOR HOP AND BEER BITTER ACIDS IN THE GRADIENT CONDITIONS IN FIG. 5

Peak No.	*Compound*	*Relative retention time*
1	Deacylated *anti*-isohumulone	0.410
2	*trans*-Cohumulinic acid	0.465
3	*trans*-Humulinic acid	0.550
4	*trans*-Adhumulinic acid	0.595
5	*trans-allo*-Isohumulone	0.718
6	*trans*-Isohumulone	0.769
7	*trans*-Isoadhumulone	0.785
8	Cohumulone	0.957
9	Humulone	1.000
10	*exo*-Tricyclooxycolupulone	1.056
11	Colupulone	1.073
12	Lupulone	1.111
13	Hexhydrocolupulone	1.188

in chloroform–light petroleum as the carrier solvent and with silica gel as the stationary phase. The separation of the iso-α-acids was incomplete, however. Buffered silica gel has been studied extensively for the separation of hop bitter acids[8,9], and it is possible to separate the six iso-α-acids in this way. The three *cis*-iso-α-acids elute before the three *trans*-iso-α-acids. Chromatograms of this separation can be found in the references cited. Buffered silica gel is not easy to work with, however.

The reproducibility and lifetime of the columns are unsatisfactory. It is almost impossible to keep the water content of the columns constant. With progressive drying out of the column, the resolution of the iso-α-acids decreases. At some stage (not specifically investigated) it becomes possible to elute all *trans*- and all *cis*-iso-α-acids together in seemingly well resolved peaks (Fig. 7). This result is important in relation to the isomerization procedure leading to synthetic or natural iso-α-acids, and the *cis*/*trans* ratio is dependent on this factor. In our hands, however, normal-phase liquid chromatography with buffered silica gel did not seem suitable for the routine determination of the iso-α-acids.

Nitro-silica gel. Nitro-silica gel (in this case we also used Nucleosil 5 μm NO_2)

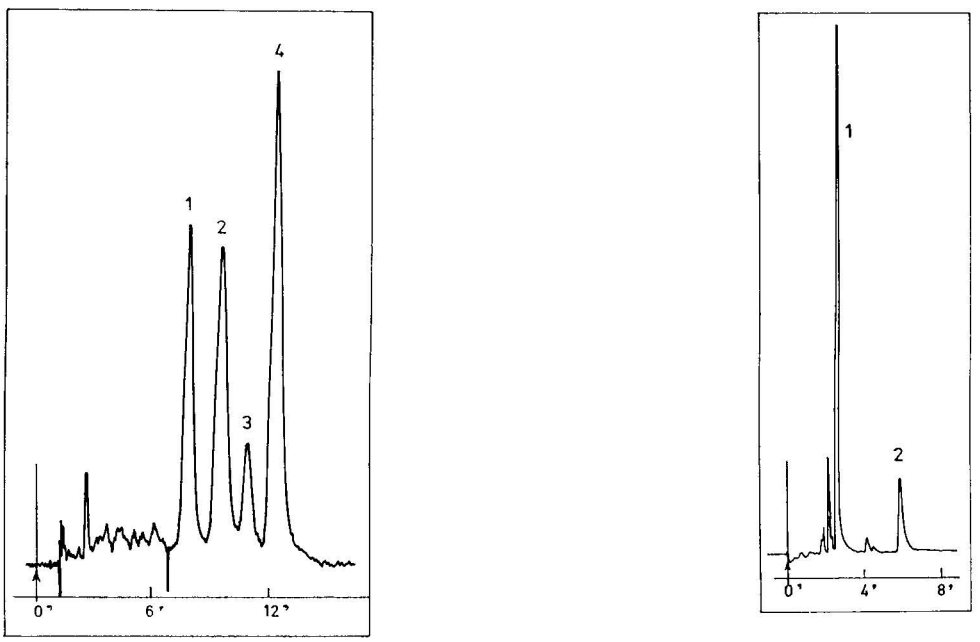


Fig. 6. Ion-pair chromatography of an evaporated isooctane extract of beer containing the six major iso-α-acids. Eluent: prepared by mixing 72.5 ml of methanol, 27.5 ml of water, 1.29 g of 40% tetrabutylammonium hydroxide (making the mixture 0.02 *M*) and 1 ml of 85% orthophosphoric acid; flow-rate, 120 ml/h. Detection wavelength: 280 nm. Peaks: 1 = *cis*- and *trans*-isocohumulone; 2 = *cis*- and *trans*-isohumulone; 3 = *cis*- and *trans*-isoadhumulone; 4 = β-phenylchalkone as possible internal standard.

Fig. 7. Normal-phase partition chromatogram of isomerized hop extract. Fairly dry 5-μm RSiL buffered with citric acid–potassium hydroxide buffer at pH 3.0. Eluent: 10% diethyl ether in isooctane at 100 ml/h. Detection wavelength: 280 nm. Peaks: 1 = the three *cis*-iso-α-acids; 2 = the three *trans*-iso-α-acids. This separation is heavily dependent on the state of hydration and is very difficult to reproduce.

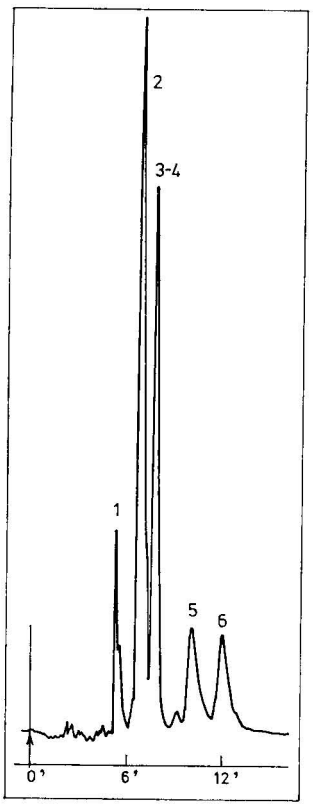


Fig. 8. Adsorption chromatography of isomerized hop extract on Nucleosil 5 μm NO_2. Eluent: isooctane–0.2% isopropanol at 120 ml/h. Detection wavelength: 280 nm. Peaks: 1 = *cis*-isoadhumulone; 2 = *cis*-isohumulone; 3 = *cis*-isocohumulone; 4 = *trans*-isoadhumulone; 5 = *trans*-isohumulone; 6 = *trans*-isocohumulone.

is obtained by derivatization of silica gel with a nitrophenyl group. This phase is very polar and is intended to be used in the normal-phase mode. This leads to the usual difficulties of a substantial influence of trace concentrations of small polar molecules (modifiers, water) and the great difficulty of keeping retention times and selectivity

TABLE II

INFLUENCE OF ISOPROPANOL CONCENTRATION IN ISOOCTANE ON THE RETENTION OF ISO-α-ACIDS

k' values calculated against solvent peak.

Isopropanol concentration, C%	k'*					
	cis-ad	*cis-h*	*cis-co*	*trans-ad*	*trans-h*	*trans-co*
1	1.35	1.40	1.55	1.65	1.75	1.95
0.5	2.25	2.35	2.60	2.95	3.15	3.50
0.25	4.00	4.35	4.80	5.75	6.30	7.00
0	Long					

* ad = isoadhumulone; h = isohumulone; co = iso-cohumulone.

constant. By first washing the column with methanol–water–85% orthophosphoric acid and then with methylene chloride, before applying isooctane–methylene chloride–isopropanol as the eluting solvent, the trace shown in Fig. 8 can be produced for a relatively short number of analyses. The retention of the iso-α-acids increases gradually and becomes excessive.

Cyanopropyl-silica gel. To compensate for the disadvantages of buffered silica gel and very polar nitro-silica gel, we examined a cyanopropyl derivatized silica gel (RSiL-CN). Isooctane, methylene chloride or their mixtures always gave tailing peaks. By washing with methanol–water–85% orthophosphoric acid (90:10:1) followed by methylene chloride, and then applying isooctane–isopropanol, a fairly good separation was obtained. The influence of the isopropanol concentration was studied (Table II).

Separations with 0.2% isopropanol in isooctane of a mixture of iso-α-acids and of an isomerized extract are shown in Fig. 9.

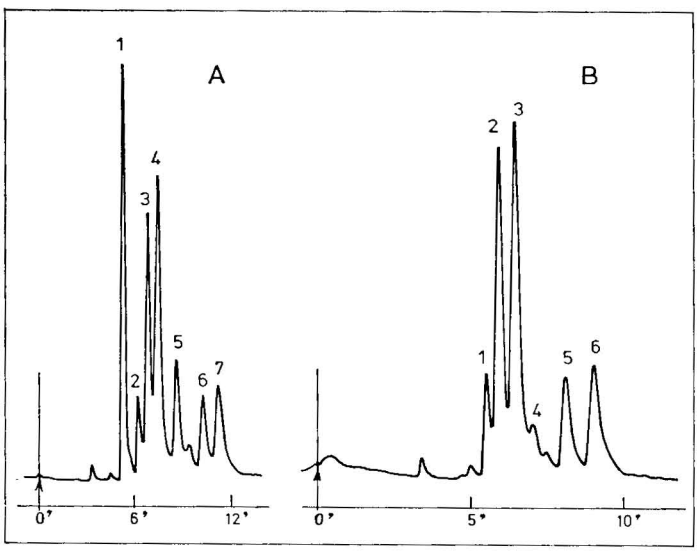


Fig. 9. (A) Adsorption chromatography on RSiL-CN of a synthetic mixture of bitter acids. Eluent: isooctane–0.2% isopropanol at 120 ml/h and 60 kg/cm². Detection wavelength: 280 nm. Sequence of elution: 1 = chalkone; 2 = *cis*-isoadhumulone; 3 = *cis*-isohumulone; 4 = *cis*-isocohumulone; 5 = *trans*-isoadhumulone; 6 = *trans*-isohumulone; 7 = *trans*-isocohumulone. (B) The same as in A but on isomerized hop extract and with isooctane–0.25% isopropanol. Peaks as in A.

The attractive feature of this method is that the counter pressure is very low compared with the usual pressure in reversed-phase liquid chromatography and therefore the analysis times can be controlled. The elution time can then easily be less than 10 min with virtually complete separation of the six iso-α-acids. The elution sequence is completely different from that on reversed-phase silica gel. Considering the low counter pressure, the smaller 5-μm packings could easily be used. We studied this in detail, but it was found that the typical difficulties of normal-phase liquid chromatography (reproducibility and influence of modifiers, as explained above) must be considered too great for cyanopropyl-silica gel be adopted in a routine method for iso-α-acids.

CONCLUSION

Although reversed-phase silica gel does not separate the six iso-α-acids completely, it is concluded that it is suitable for the routine analysis of beer bitter iso-α-acids. A modification of the Whitt and Cuzner[6] ion-pair technique, separating the isohumulones, isocohumulones and isoadhumulones, appears to be the best choice. Complete development of such a procedure is currently in progress[10].

ACKNOWLEDGEMENTS

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SEPARATION OF GLUCOSINOLATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

PER HELBOE

National Board of Health, Drug Standardization Laboratory, 378 Frederikssundsvej, DK-2700 Brønshøj (Denmark)

and

OLE OLSEN and HILMER SØRENSEN*

Chemistry Department, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Copenhagen V (Denmark)

(First received April 24th, 1980; revised manuscript received May 13th, 1980)

SUMMARY

The separation of a series of glucosinolates by reversed-phase ion-pair liquid chromatography is reported. A rapid and adequate separation was achieved on a column of Nucleosil 5 C_{18}, with 0.01 *M* phosphate buffer at pH 7.0–methanol (3:7) containing 0.005 *M* tetraheptylammonium bromide as mobile phase. The modifier concentration, the nature of the counter-ion and the pH greatly influence the separation of the glucosinolates from each other and from non-ionic impurities. The isolation of the total glucosinolate fraction was performed by a newly developed ion-exchange chromatographic method. By combination with the separation method described here, acidic and basic conditions (which would lead to decomposition of the glucosinolates) are completely avoided. The proposed method is briefly discussed in relation to methods previously used for the isolation, identification and quantitative determination of glucosinolates.

INTRODUCTION

Several different methods for the isolation, separation and quantitative determination of components of the complex mixtures of naturally occurring glucosinolates have been reported[1–3]. All are based on the determination of one or more of the enzymatic hydrolysis products or of derivatives of the glucosinolates, but detection and determination of some of the glucosinolates are unsatisfactory[4].

Recently, a method was developed for the quantitative isolation of intact glucosinolates by ion-exchange chromatography[5]. This method, followed by gas chromatography (GC) of the trimethylsilylated derivatives of the glucosinolates, offers a reliable procedure for the qualitative and quantitative analysis of complex glucosinolate mixtures[6,7].

The determination of polar compounds such as glucosinolates by high-

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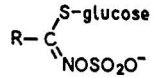
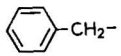
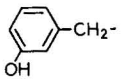
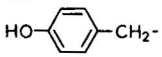
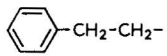
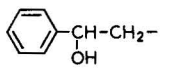
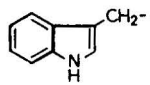
TABLE I

CHEMICAL STRUCTURES OF THE GLUCOSINOLATES



Compound No.	*Nature of group R*	*Value of k'**	*Name of glucosinolate*	*Trivial name of the salt*
1	CH_3–	3.3	Methylglucosinolate	Glucocapparin
2	CH_2=CH–CH_2–	4.2	Allylglucosinolate	Sinigrin
3	CH_2=CH–CH_2–CH_2–	5.0	But-3-enylglucosinolate	Gluconapin
4	CH_2=CH–CH_2–CH_2–CH_2–	5.5	Pent-4-enylglucosinolate	Glucobrassicanapin
5	CH_2=CH–CH(OH)–CH_2–	3.4	2-Hydroxybut-3-enylglucosinolate	Progoitrin
6	CH_2=CH–CH_2–CH(OH)–CH_2–	3.7	2-Hydroxypent-4-enylglucosinolate	Napoleiferin
7	CH_3–SO–CH_2–CH_2–CH_2–	2.1	3-Methylsulfinylpropylglucosinolate	Glucoiberin
8	CH_3–SO–CH_2–CH_2–CH_2–CH_2	—	4-Methylsulfinylbutylglucosinolate	Glucoraphanin
9	CH_3–SO–CH_2–CH_2–CH_2–CH_2–CH_2–	—	5-Methylsulfinylpentylglucosinolate	Glucoalyssin
10	CH_3–SO_2–CH_2–CH_2–CH_2–	2.9	3-Methylsulfonylpropylglucosinolate	Glucocheirolin
11		6.7	Benzylglucosinolate	Glucotropaeolin
12		6.4	*m*-Hydroxybenzylglucosinolate	Glucolepigramin
13		5.0	*p*-Hydroxybenzylglucosinolate	Sinalbin
14		9.8	Phenethylglucosinolate	Gluconasturtiin
15		6.2	2-Hydroxy-2-phenylethylglucosinolate	Glucobarbarin
16		9.8	Indol-3-ylmethylglucosinolate	Glucobrassicin

* Experimental conditions as in Fig. 5.

performance liquid chromatography (HPLC) seems obvious. However, the lack of suitable methods for separation of the glucosinolate fraction from other plant constituents prior to HPLC has prevented the use of this technique. This problem has now been overcome by use of the above-mentioned ion-exchange method. The glucosinolates are obtained as their pyridinium salts contaminated only by small amounts of other plant constituents.

The application of reversed-phase ion-pair liquid chromatography as a convenient alternative to ion-exchange chromatography for the separation of ionic compounds was demonstrated in 1975[8], and its advantages in separating mixtures of ionic and non-ionic compounds have also been shown[9].

This paper presents a reversed-phase ion-pair liquid chromatographic method, which, in combination with the new isolation procedure for the total glucosinolate fraction, allows fast and reliable determination of individual glucosinolates. Until now, HPLC has been used only for the determination of glucosinolate-degradation products[10–14], and this paper is the first report of an HPLC method for determining intact glucosinolates.

EXPERIMENTAL

Chemicals

Tetraalkylammonium bromides were obtained from Fluka (Buchs, Switzerland); all other reagents were of analytical grade from E. Merck (Darmstadt, G.F.R.).

Compounds investigated

Table I lists the glucosinolates studied; they were isolated from natural sources, purified and identified as previously described[5,6]. The total glucosinolate fraction from 2 g of seeds of *Brassica napus* L. cv. Tower was isolated as described elsewhere[7].

Chromatography

The liquid chromatograph used consisted of an Altex Model 110 solvent metering pump, a Pye-Unicam LC-UV spectrophotometer detector, and a Rheodyne Model 7120 injection valve. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder, and retention times and peak areas were measured by means of a Hewlett-Packard Model 3353A laboratory data system.

Preliminary experiments were performed on a column (12 cm × 4.65 mm I.D.) (Knauer, Berlin, G.F.R.) packed with Nucleosil 5 C_{18} (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.). For the analytical separations, two columns in series, each packed with the same material, were used; both columns were packed as described earlier[15]. The efficiency of the columns, expressed as the number of theoretical plates (N) measured for naphthalene when eluted by 90% methanol in water (capacity factor $k' = 1.0$) at a linear solvent velocity of 1.5 mm sec^{-1}, was 5500 for the single column and 12,900 for the two columns in series.

The mobile phase was 0.01 M phosphate buffer (pH 7.0) modified with 60% or 70% of methanol. Different tetraalkylammonium bromides at a concentration of 0.005 M were used as sources of counterions.

Pyridinium salts of glucosinolates were dissolved in water to give 0.01–0.04% solutions and 10-μl samples were injected on to the column.

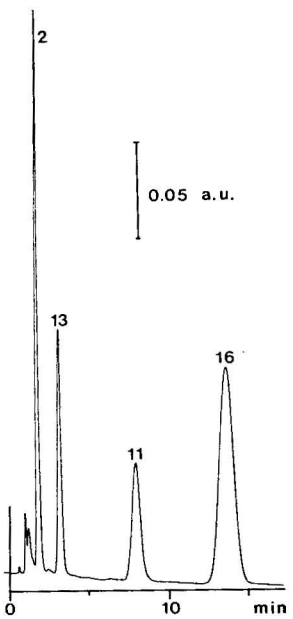
RESULTS AND DISCUSSION

For the elaboration of the HPLC method, solutions of the glucosinolates identified by previous methods[5–7] were used alone and/or in mixtures. Reversed-phase chromatography on Nucleosil 5 C_{18} was investigated. Since glucosinolates are unstable in both acidic and basic solutions, a phosphate buffer (0.01 *M*; pH 7.0) modified with methanol was used as mobile phase.

Detection was at 235 nm, at which wavelength the contribution to the absorbance from the glucosinolate group (Table I) is almost at its maximum, whereas the contribution from interfering aromatic R groups will be of limited magnitude, leading to similar absorptivity values for all glucosinolates.

A reasonable separation of a mixture of the compounds 2, 11, 13 and 16 (Table I) was achieved with a modifier concentration of 5%, but peak shapes were not optimal (Fig. 1). Further, non-ionic impurities originating from the plant material, as well as pyridine originating from the glucosinolate pyridinium salts, were completely retained on the column. This accumulation would in time alter the characteristics of the column.

A change of the modifier concentration to 60% produced rapid elution of pyridine and non-ionic impurities, and, by adding tetraalkylammonium bromides as sources of counter-ions, retention of the glucosinolates was achieved (Fig. 2). As



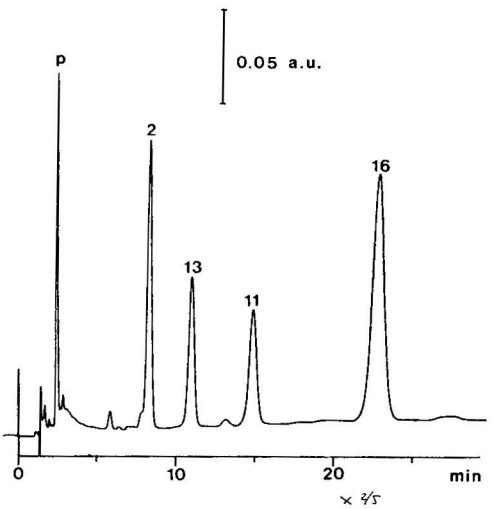


Fig. 1. Chromatogram of glucosinolates 2, 11, 13 and 16 (see Table I) on a column (12 cm × 4.6 mm) of Nucleosil 5 C_{18}. Mobile phase: phosphate buffer (0.01 *M*; pH 7.0)–methanol (95:5). Solvent velocity: 1.5 mm/sec. Detection wavelength: 235 nm.

Fig. 2. Chromatogram of glucosinolates 2, 11, 13 and 16 and pyridine (p). Mobile phase: phosphate buffer (0.01 *M*; pH 7.0)–methanol (4:6) containing tetraheptylammonium bromide (0.005 *M*); other conditions as in Fig. 1.

stated by Sood[16], three factors are important in controlling retention in reversed-phase ion-pair chromatography, *viz.*, pH, counter-ion concentration and nature of the counter-ion.

As mentioned above, a pH of *ca.* 7 is required to ensure stability of the glucosinolates. At this pH, the glucosinolates are totally ionized, owing to the low pK_a value of the sulphate group, and have a negative charge[6].

As earlier reported for the ion-pair separation of amines, with alkanesulphonates as counter-ions[17], a counter-ion concentration of 0.005 *M* was also chosen for the separations described here.

The influence of the nature of the counter-ion is demonstrated in Fig. 3. An increase in the carbon number of the tetraalkylammonium ions, and thereby in their lipophilic properties, caused an increase in the retention of the ion-pairs, but did not affect the retention of pyridine. Fig. 3 shows that the separation of the glucosinolates was optimal within a reasonable time when tetraheptylammonium bromide was used as a source of counter-ions. However, further investigations showed that, when handling more complex mixtures of glucosinolates (see below), the selectivity of the chromatographic system was improved by changing the modifier concentration to 70% and using tetraoctylammonium bromide (Fig. 4).

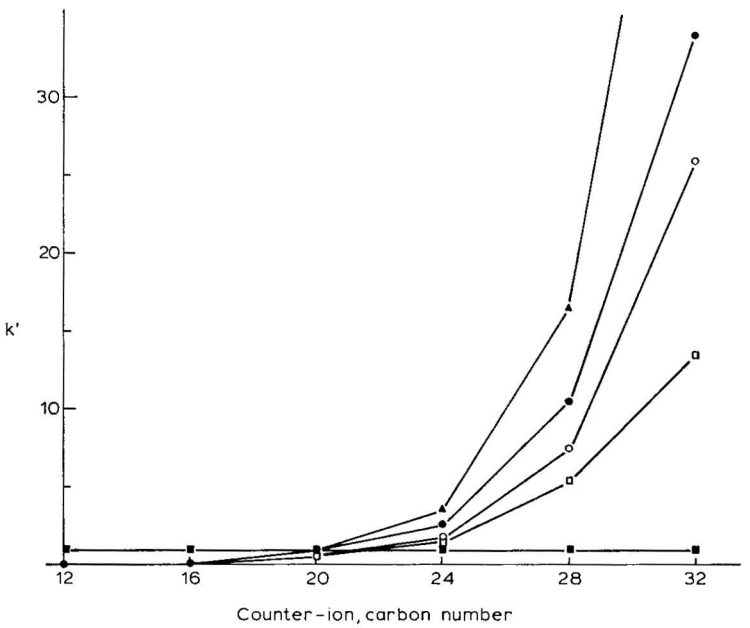


Fig. 3. Influence of nature of counter-ion on retention of the compounds studied (see Table I); ■ = pyridine; □ = 2; ● = 11; ○ = 13; ▲ = 16. Chromatographic conditions: single column, phosphate buffer (0.01 *M*; pH 7.0)–methanol (4:6) containing different tetraalkylammonium bromides (0.005 *M*).

Fig. 5 shows the chromatogram of an artificial mixture of glucosinolates isolated from different plants[6,7] and used as reference compounds; a few of these compounds were not separated. The trend in the separation was similar to that of paper

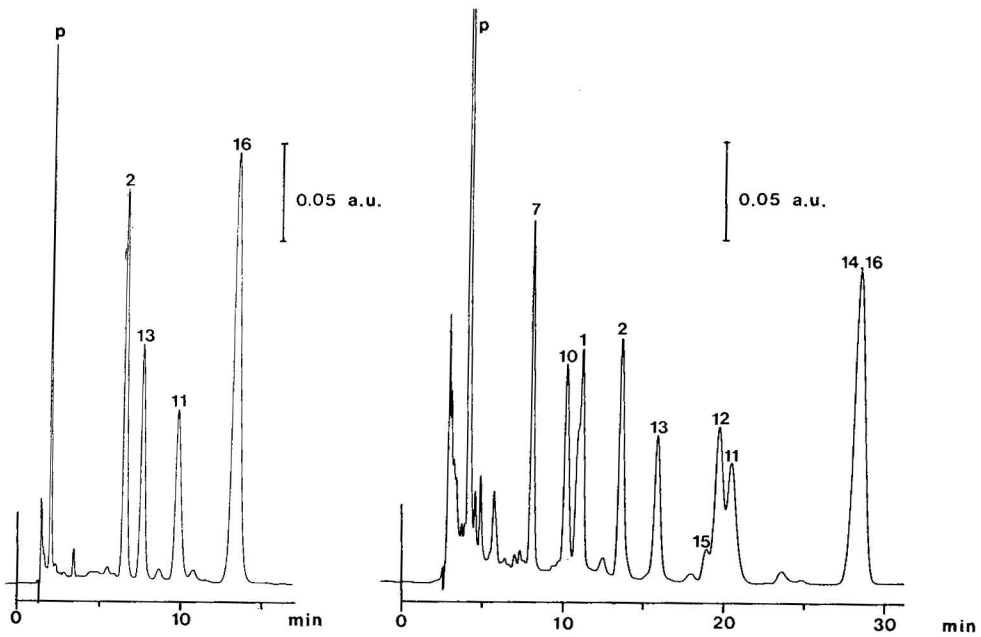


Fig. 4. Chromatogram of glucosinolates 2, 11, 13 and 16 and pyridine (p). Mobile phase: phosphate buffer (0.01 *M*; pH 7.0)–methanol (3:7) containing tetraoctylammonium bromide (0.005 *M*); other conditions as in Fig. 1.

Fig. 5. Chromatogram of artificial mixture of glucosinolates (1–4 μg of each) on a column (24 cm × 4.6 mm) of Nucleosil 5 C_{18}; conditions as in Fig. 4. The value of k' for pyridine (p) is 0.6; k' values for the other compounds are shown in Table I.

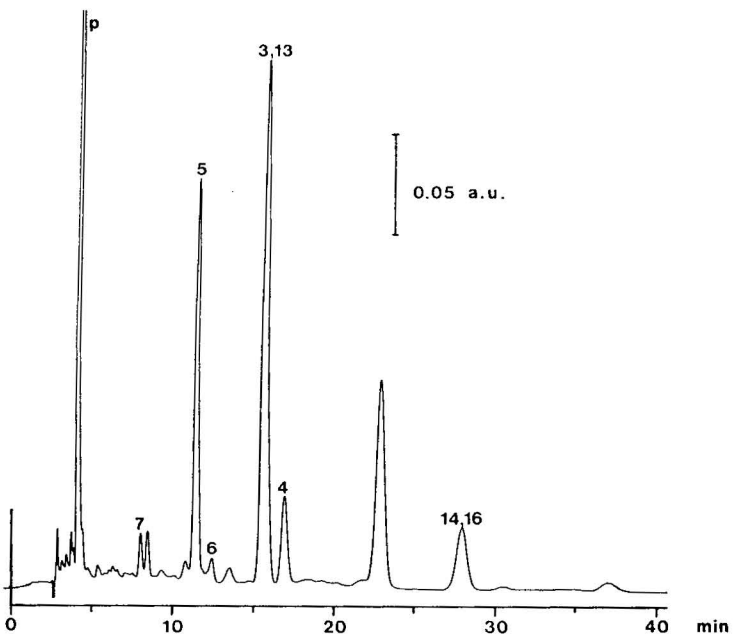


Fig. 6. Chromatogram of glucosinolates isolated from seeds of *B. napus* L. cv. Tower; conditions as in Fig. 5.

chromatography (PC)[5,6], but the efficiency of the HPLC separations was far superior to that of PC.

Fig. 6 shows the separation of the mixture of glucosinolates isolated from seeds of *B. napus* L. cv. Tower. The seeds were from the same lot as used before, and the results were in accordance with those previously reported[7]. The dominant peak, with a retention time (t_R) of 4.1 min, is due to pyridine. The major peaks in the HPLC chromatogram correspond to the compounds 5 (t_R = 11.5 min), 3 + 13 (t_R = 15.7 min), 4 (t_R = 16.9 min) and 14 + 16 (t_R = 27.9 min). As indicated in the chromatogram, some of the remaining peaks might be due to methylsulphinylglucosinolates (*e.g.*, compounds 7, 8, and 9), but the identity of these and some other unidentified glucosinolates in Tower seeds[7] has not yet been unequivocally established.

CONCLUSION

The HPLC method described permits the rapid separation and quantitative determination of intact individual glucosinolates under gentle conditions, and is therefore an important supplement to the GC method, in which trimethylsilylated desulfoglucosinolates are quantitatively determined[7]. Both the GC and the HPLC method avoid problems associated with determination of the many products of enzymatic degradation of glucosinolates.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MYOSIN'S SUBFRAGMENT 1 AND LIGHT CHAINS ON SPHEROGEL TSK-TYPE COLUMNS

ANTHONY P. TOSTE*
Cardiovascular Research Institute, University of California, San Francisco, CA 94143 (U.S.A.)
(First received February 20th, 1980; revised manuscript received May 23rd, 1980)

SUMMARY

Myosin's subfragment 1 and three light chains were chromatographed rapidly at room temperature on Spherogel TSK-type columns, recently developed for steric-exclusion high-performance liquid chromatography, with no adsorption of the proteins to the columns or loss of enzymic activity. Crude preparations of subfragment 1 were extensively purified on Spherogel TSK SW-3000 columns. Unresolved α-chymotryptic-subfragment 1 was separated into its two isoenzymes despite only a small difference in molecular weights, presumably because of conformational differences. Light chain 2 was purified from a mixture of all three light chains by chromatography on a Spherogel TSK SW-2000 column; light chains 1 and 3 eluted as two distinct complexes having ratios of 2:1 and 1:1.

INTRODUCTION

Contractile proteins such as myosin, its enzymatically active subfragments [subfragment 1 (S1)** and heavy meromyosin (HMM)], or its three light chains (lc_1, lc_2, and lc_3) are typically purified from crude protein preparations by steric-exclusion chromatography[1], ion-exchange chromatography[2,3], or, most recently, affinity chromatography[4,5], all on columns filled with carbohydrate gels. Unfortunately, these gels are extremely fragile to changes in pressure, pH, and ionic strength[6]. The mandatory slow flow-rates and gentle pH or ionic gradients result in slow separations, typically 15–20 h for myosin, its subfragments, or its light chains[1–3].

Conventional steric-exclusion chromatography is not only time consuming, but also completely inadequate for resolving the two isoenzymes of α-chymotryptic S1

* Present address: Battelle, Pacific Northwest Laboratories, Earth and Planetary Chemistry Section, Building 329/300 Area, P.O. Box 999, Richland, WA 99352, U.S.A.

** Abbreviations used: S1 = subfragment 1; αct-S1 = α-chymotryptic subfragment 1; lc_1 = light chain 1; lc_2 = light chain 2; lc_3 = light chain 3; αct-S1 (lc_1) = α-chymotryptic subfragment 1 isoenzyme containing light chain 1; αct-S1 (lc_3) = α-chymotryptic subfragment 1 isoenzyme containing light chain 3; HMM = heavy meromyosin; αct = α-chymotrypsin; DTT = DL-dithiothreitol (Cleland's Reagent).

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(αct-S1), αct-S1 ($1c_1$) and αct-S1($1c_3$), or separating myosin's three light chains; these proteins are too similar in size. Affinity chromatography supports capable of such specificity have yet to be developed. Fortunately, conventional ion-exchange chromatography may be used to resolve αct-S1's isoenzymes or the light chains on the basis of charge differences but, again, 15–20 h are required[2,3].

Until recently, most efforts at speeding up protein separations by using fast flow-rates, *i.e.*, high column pressures, and pressure-stable silica supports in high-performance liquid chromatography (HPLC) systems have been only marginally successful. Steric-exclusion and ion-exchange HPLC supports have been tested, but the reactive silica surface of these supports irreversibly adsorbs and/or denatures many proteins[7]. Recently, efficient separations of serum proteins by steric-exclusion HPLC have been reported using a support which consists of a chemically-modified silica, with little adsorption and no apparent denaturation occurring[8,9].

The next step is to test the usefulness of steric-exclusion HPLC for biologically important proteins, most of which remain to be tested. In this study, we tested columns filled with the recently marketed Spherogel TSK-type steric-exclusion HPLC-support, a pressure-stable, hydrophilic, chemically-modified silica, for purifying myosin's subfragments (S1 and HMM), and myosin's three light chains ($1c_1$, $1c_2$, and $1c_3$), in an attempt to speed up existing purification methods. We evaluated the Spherogel TSK-type columns on the basis of their separating powers and the recovery of protein and enzymic activity, specifically S1's ATPase activities and $1c_2$'s ability to be phosphorylated before and after HPLC. In the process of analyzing the columns' separating powers, we also examined the chromatographic properties of S1 and myosin's light chains on the Spherogel TSK-type columns in the hope of elucidating some of the proteins' hydrodynamic properties, *e.g.*, their conformations in solution and possible interactions with each other.

EXPERIMENTAL

Protein preparation

All of the proteins used in the HPLC analyses were extracted from the white skeletal muscle of rabbit. Myosin was purified by the method of Tonomura *et al.*[10] as modified by Crooks and Cooke[11]. Heavy meromyosin was purified by the method of Weeds and Taylor[2]. Myosin subfragment 1 (S1) was purified either by papain digestion of myofibrils by the method of Cooke[1] or by α-chymotryptic digestion of myosin by the method of Weeds and Taylor[2]; the crude, unresolved S1 applied to the HPLC column consisted of the S1-containing supernatant solution after sedimentation of papain-digested myofibrils or α-chymotrypsin-digested myosin. The two isoenzymes of αct-S1, αct-S1($1c_1$) and αct-S1($1c_3$), used as HPLC standards, were separated by ion-exchange chromatography[2]. A fraction containing all three of myosin's light chains was isolated from myosin as described by Holt and Lowey[3]. An aliquot of this light chain mixture was fractionated into pure $1c_2$ and a mixture of $1c_1$ and $1c_3$ by Blue Sepharose affinity chromatography according to the method of Toste and Cooke[4]. All of the proteins were dialyzed against the HPLC column buffer, 50 m*M* sodium phosphate (pH 7.4), 0.2 *M* ammonium sulfate, 1 m*M* ethylene diaminetetraacetic acid (EDTA), 0.2 m*M* dithiothreitol (DTT), and 0.02% sodium azide for 3 h before HPLC.

HPLC analysis

Two Spherogel TSK-type columns (Altex Scientific) were used for the HPLC analyses. S1 and HMM were chromatographed on a Spherogel TSK SW-3000 column (600 × 7.5 mm). Myosin's light chains were chromatographed on a Spherogel TSK SW-2000 column (600 × 7.5 mm). Each protein preparation was recycled through the column three more times in an attempt to improve the separation of individual proteins.

Each Spherogel TSK-type column was calibrated by chromatographing protein standards and plotting the log mol.wt. of each standard against its retention time. Ferritin (mol.wt. 450,000), aldolase (mol.wt. 158,000), hexokinase (mol.wt. 104,000), bovine serum albumin (BSA) (mol.wt. 68,000), ovalbumin (mol.wt. 45,000), chymotrypsinogen A (mol.wt. 25,000), and cytochrome *c* (mol.wt. 12,500) were chromatographed on the SW-3000 column; aldolase, hexokinase, BSA, ovalbumin, chymotrypsinogen A, and cytochrome *c* were chromatographed on the SW-2000 column. The columns were equilibrated with the HPLC column buffer at room temperature before the proteins were chromatographed. The contractile proteins and the protein standards were then chromatographed at room temperature in the HPLC column buffer at flow-rates ranging from 12–60 ml/h.

Each Spherogel TSK-type column was attached to an Altex Model 320 advanced research chromatograph consisting of a Model 100A dual piston analytical pump (flow-rate accuracy of $\pm 1\%$, or 0.005 ml/min, up to 10,000 p.s.i.), a Model 153 UV detector, a Model 210 injection valve and a Model 155 recorder. The injection valve was replaced by a Model 100A recycle chromatography port for the recycle chromatography. The low piston displacement volume (0.1 ml) of the 100A pump, makes it especially well-suited for recycle chromatography.

Assays

The protein recoveries and purity of the HPLC-eluted proteins were determined by assaying their concentrations and composition before and after HPLC. The protein concentrations of the protein preparations and the HPLC-eluted proteins were measured spectrophotometrically at 595 nm with the Bradford assay[12], a protein specific assay which is based on the binding of the dye Coomassie brilliant blue G-250 to proteins. Simple absorption measurement of protein concentration at 230 or 280 nm was judged too non-specific for our purposes; UV absorbance by non-proteins, *e.g.*, nucleotides, masked the extent of the protein separations achieved by HPLC. The protein composition of the protein preparations and the HPLC-eluted proteins was determined by gel electrophoresis according to the method of Ames[13]. We used 1.5-mm slabs of polyacrylamide–BIS–SDS and the discontinuous buffer system of Laemmli[14]. The protein bands on the Coomassie blue-stained gels corresponding to myosin's light chains and S1 light and heavy chains were quantitated by densitometry; the dried gels were scanned at 550 nm.

The recovery of the proteins' enzymic activity after HPLC was measured by assaying S1's ATPase activities and $1c_2$'s phosphorylation activity. S1's ATPase activities were measured colorimetrically by the method of Martin and Doty[15]. The "K^+-ATPase" activity was assayed in 0.6 *M* potassium chloride, 1 m*M* EDTA, and 1 m*M* adenosine triphosphate (ATP) at pH 8, 25°C; "Ca^{2+}-ATPase" activity in 0.6 *M* potassium chloride, 4 m*M* calcium chloride, and 1 m*M* ATP at pH 8, 25°C; "Mg^{2+}-

ATPase" activity in 50 m*M* potassium chloride, 1 m*M* magnesium chloride, and 1 m*M* ATP at pH 8, 25°C; and actin-activated ATPase activity in 30 m*M* potassium chloride, 2 μM actin, 4 m*M* magnesium chloride and 1 m*M* ATP at pH 8, 25°C. lc_2 was phosphorylated as described by Pires and Perry[16] using myosin's light-chain kinase purified in Dr. J. T. Stull's laboratory (Department of Pharmacology, University of Texas, Dallas, TX, U.S.A.) from skeletal muscle. lc_2's phosphorylation activity was determined by gel electrophoresis on 7.5% polyacrylamide–urea slab gels by the method of Pires and Perry[16]. The protein bands on the Coomassie blue-stained gels corresponding to phosphorylated and unphosphorylated lc_2 were quantitated by densitometry.

Materials

The α-chymotrypsin and papain used in the S1 preparations were purchased from Sigma (St. Louis, MO, U.S.A.) and Worthington (Freehold, NJ, U.S.A., respectively. The protein standards used to calibrate the Spherogel TSK-type columns were obtained as a kit from Boehringer (Mannheim, G.F.R.). All other reagents were of analytical reagent-grade or of the best grade available.

RESULTS

Chromatographic properties

Protein standards. All of the protein standards tested were retarded by the Spherogel TSK SW-3000 column (flow-rate, 60 ml/h) to some extent, eluting at the following times: ferritin, 9.5 min; aldolase, 12.6 min; hexokinase, 13.8 min; BSA, 15.2 min; ovalbumin, 16.3 min; chymotrypsinogen, 18.2 min and cytochrome *c*, 20.3 min. Plotting the log mol.wt. of each protein against its retention time (min) yielded an absolutely straight line, giving the TSK SW-3000 column a mol.wt. separation range of at least 12,500–450,000. The protein standards eluted from the Spherogel TSK SW-2000 column (flow-rate, 60 ml/h) at the following times: ferritin, 10.9 min; aldolase, 10.9 min; hexokinase, 10.9 min; BSA, 11.8 min; ovalbumin 13min; chymotrypsinogen, 14.6 min and cytochrome *c*, 16.5 min. Ferritin, aldolase, and hexokinase were occluded from the TSK SW-2000 column; they all eluted in the column's void volume. The rest of the protein standards were retarded to some extent; plotting the log mol.wt. of these standards *vs.* their retention time (min) yielded an absolutely straight line, giving the TSK SW-2000 column a mol.wt. separation range of approx. 12,500–80,000.

S1 and HMM. Papain-S1 and αct-S1 each eluted from the Spherogel TSK SW-3000 column as a single peak at column flow-rates of 30 ml/h or faster. Papain-S1 eluted slightly earlier than unresolved αct-S1 (11.78 min *vs.* 13.03 min, at a flow-rate of 60 ml/h). Comparison of these retention times with the calibration curve for the SW-3000 column yielded effective mol.wts. of 204,000 and 137,000 for papain-S1 and αct-S1, respectively. When the flow-rate was slowed to 12 ml/h, papain-S1 still eluted as a single peak, but αct-S1 separated into its two isoenzymes (Fig. 1). Peak a eluted at 63 min, ocrresponding to an effective mol.wt. of 158,000; peak b eluted at 64.8 min, corresponding to an effective mol.wt. of 140,000. Gel electrophoresis indicated that peak a consisted of S1 (lc_1) and peak b consisted of S1 (lc_3). In contrast, the protein composition across the papain-S1 peak (chromatographed at

12 ml/h) was homogeneous, with respect to both S1 heavy chains and to its light chains Recylcing both S1's through the SW-3000 column three more times did not improve the separations.

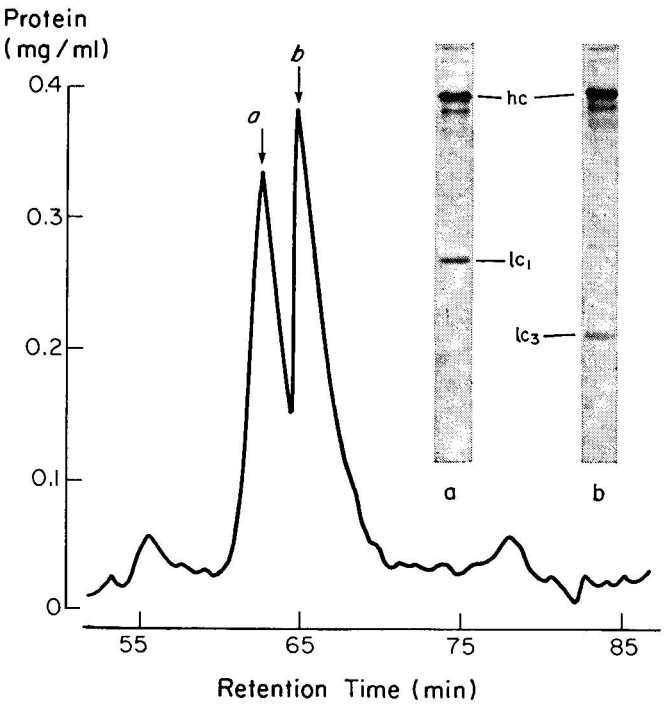

Fig. 1. HPLC separation of αct-S1 isoenzymes, αct-S1 (1 mg, 2.5 mg/ml) was applied at room temperature to a Spherogel TSK SW-3000 column (600 × 7.5 mm) that had been equilibrated with the HPLC column buffer, 50 m*M* Na_3PO_4 (pH 7.4), 0.2 *M* $(NH_4)_2SO_4$, 1 m*M* EDTA, 0.2 m*M* DTT and 0.02% NaN_3. Fractions (300 μl each) were collected at a flow rate of 12 ml/h. The protein concentration of each fraction was monitored at A_{595nm} by the Bradford assay[12]. Figure inset: Polyacrylamide–SDS gel profile of the HPLC-resolved αct-S1 peaks. The positions of αct-S1's heavy chain (hc) and two light chains (lc_1 and lc_3) are noted. Aliquots of the top fraction (a and b) from each protein peak were prepared for gel electrophoresis[14], from which 40 μl (10 μg protein) were applied to a 12.5% polyacrylamide–0.1% SDS gel[13].

HMM eluted in the column's void volume, indicating that it was completely occluded from the Spherogel TSK SW-3000 column.

Light chains. HPLC of the preparation containing all three of myosin's light chains on the Spherogel TSK SW-2000 column at higher flow-rates, *e.g.*, 60 ml/h, yielded a single, poorly-resolved peak containing 2 distinct shoulders. HPLC of the same preparation at 12 ml/h yielded 3 peaks (Fig. 2). Peak a eluted at 61.6 min, peak b at 68.5 min, and peak c at 73.9 min, corresponding to effective mol.wts. of 58,000, 36.000, and 23,500, respectively. Gel electrophoresis of aliquots from the column fractions across the 3 peaks revealed that peak a consisted mainly of lc_1, with some lc_3 present, and peak b consisted mainly of lc_3, with a considerable amount of lc_1 also present, but peak c consisted almost exclusively of lc_2. Qualitative examination of the gel suggested that peaks 1 and 2 consisted of two distinct lc_1–lc_3 complexes.

Densitometry of the gel confirmed this suggestion: the protein composition across peak a consisted of a 2:1 complex of lc_1 and lc_3 and peak 2 consisted of a 1:1 complex. Recylcing the light-chain peaks through the SW-2000 column three more times did not improve the separations.

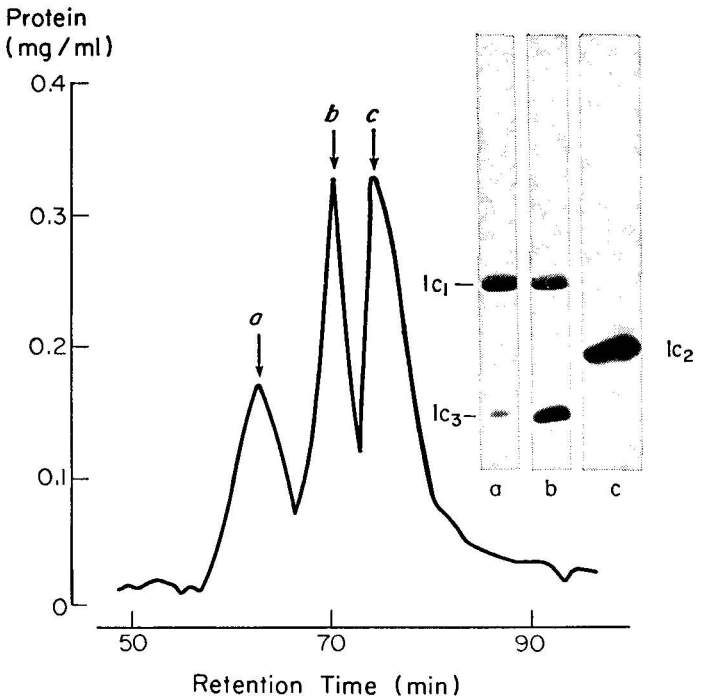

Fig. 2. HPLC separation of a mixture containing myosin's three light chains. The light-chain mixture (2 mg, 4 mg/ml) was chromatographed on a Spherogel TSK SW-2000 column as described in Fig. 1. Figure inset: Polyacrylamide–SDS gel profile of the HPLC-resolved light-chain peaks. Aliquots of the top fraction (a, b and c) from each protein peak were prepared for gel electrophoresis[14], from which 40 μl (6–10 μg protein) were applied to a 15% polyacrylamide–0.1% SDS gel[13].

HPLC of a mixture of lc_1 and lc_3 and HPLC of a sample of lc_2, both separated from the original light-chain preparation by Blue Sepharose chromatography, gave the same results as the HPLC of the mixture of all three light chains. The mixture of lc_1 and lc_3 chromatographed as two peaks, corresponding to peaks a and b of the mixture of all three light chains. lc_2 eluted exactly like peak c of the mixture of all three light chains.

Purification

HPLC of crude papain-S1 and αct-S1 yielded highly purified Sl's. Comparison of gel electrophoresis profiles of papain-S1 before and after HPLC indicated that the HPLC-purified papain-S1 lacked numerous protein contaminants present in the crude papain-S1 preparation, specifically actin and other proteins; these other proteins appeared to be proteolytic fragments of myosin's heavy chains (Fig. 3A). HPLC-

purified αct-S1 (Fig. 3B) lacked several heavy-chain fragments present in crude αct-S1. The protein recoveries from the Spherogel TSK-type columns were 96–98% for all of the proteins studied.

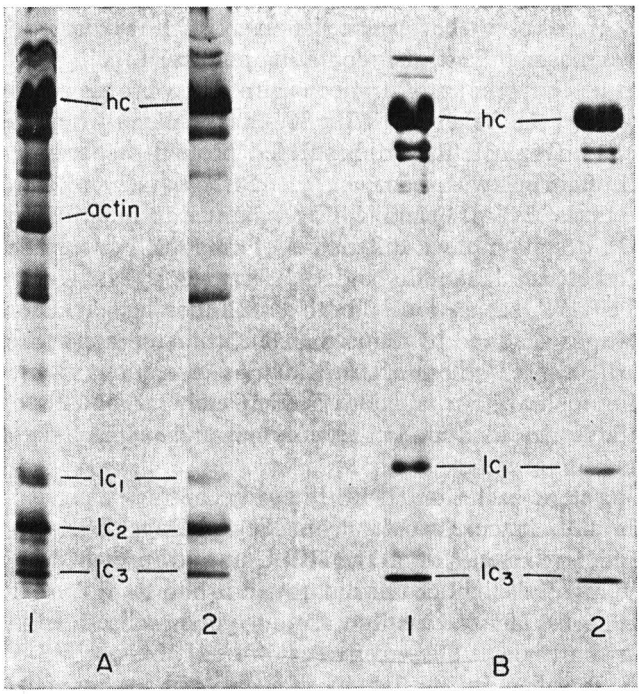

Fig. 3. Polyacrylamide–SDS gel profiles of S1 purified by HPLC. Crude papain-S1 and αct-S1 (2 mg each) were chromatographed separately on the Spherogel TSK SW-3000 column at 60 ml/h in the HPLC column buffer. Protein recoveries from each column run were 96–98%. Each purified S1 was collected as a single fraction. Aliquots of each S1 (10–15 μg) were set aside before and after HPLC and prepared for gel electrophoresis (see Fig. 1). (A) Papain-S1: (1) before HPLC, crude papain-S1 consisted of papain-S1's heavy chains (hc) and light chains (lc) (85.2%), actin (7.2%), and proteolytic fragments (7.6%); (2) after HPLC, purified papain-S1 consisted almost exclusively of papain-S1 heavy chains (hc) and its three light chains. (B) αct-S1: (1) before HPLC, crude αct-S1 consisted of αct-S1's heavy chains and light chains (89%) and proteolytic fragments (11%); (2) after HPLC, purified αct-S1 consisted almost entirely of αct-S1 heavy chains (hc) and its two light chains.

The ATPase activities (in μmol/g·sec) of the HPLC-purified S1's were "K^+-ATPase", 80–97; "Mg^{2+}-ATPase", 0.1; "Ca^{2+}-ATPase", 9; and actin-activated ATPase (1 μM actin), 5.8–6.4.

Only peak c of the HPLC chromatogram of the mixture of all three light chains (Fig. 2) consisted of a purified, single component, lc_2. SDS- and urea-gel electrophoresis indicated that the HPLC-lc_2 was intact and was completely phosphorylated by myosin's light-chain kinase.

The Spherogel TSK-type columns used to purify S1 and the light chains (600 × 7.5 mm) were not designed for HPLC on a preparative scale *per se*. Nevertheless, 40 mg of an equimolar mixture of the protein standards used to calibrate the

Spherogel TSK SW-3000 and SW-2000 columns were chromatographed on the two columns without any apparent overloading or loss of resolution.

DISCUSSION

Steric-exclusion HPLC promises to be an excellent way of purifying contractile proteins rapidly, for two reasons. First, the separating powers of the Spherogel TSK-type columns were quite impressive. Extraneous proteins were separated from both papain-S1 and act-S1 on the Spherogel TSK SW-3000 column within 13 min, yielding highly purified S1's (Fig. 3). Even the purified protein preparations were further separated by HPLC. The two isoenzymes act-S1(lc_1) and act-S1(lc_3) were separated from unresolved act-S1 (Fig. 1) and lc_2 was separated from a mixture of all three light chains (Fig. 2), despite mol.wt. differences of only 8% between the two S1 isoenzymes and of 11% between lc_2 and lc_1 and 14% between lc_2 and lc_3 (see refs. 3 and 17 for mol. wts.). The HPLC separations of both act-S1 and the light chains compare favorably with separations achieved by ion-exchange chromatography[1–3,17], but require only a fraction of the time (70–80 min *vs.* 15–20 h). Conventional steric-exclusion chromatography using sepharose or sephadex is completely inadequate for separating proteins of comparable size like act-S1's isoenzymes and myosin's light chains.

A second reason for using steric-exclusion HPLC to purify contractile proteins is that the recovery of protein and enzymic activity from the Spherogel TSK-type columns was excellent. The protein recoveries of all the HPLC-purified proteins were 96–98%, indicating that S1 and myosin's light chains did not adsorb to the Spherogel TSK-type columns. The recovery of ATPase activities was quite good, indicating that S1 was not denatured. Comparison of the ATPase activities of the HPLC-purified S1 (see Results) with those of S1 purified in our lab by conventional ion-exchange chromatography indicates that the HPLC-purified S1's "K^+-ATPase" activity was 28% higher, the "Mg^{2+}-ATPase" and actin-activated ATPase activities were identical, and the "Ca^{2+}-ATPase" activity was 18% lower. The ATPase activities of the HPLC-purified S1 also compare well with published values[2]. The light chains also appeared to be undamaged by the HPLC. The light chains' electrophoretic mobilities remained unchanged after HPLC, and the HPLC-purified lc_2 was completely phosphorylated by myosin's light-chain kinase.

The reason that no enzymic activity was lost even though S1 and the light chains were chromatographed at room temperature is undoubtedly that the proteins remained in the Spherogel TSK-type columns for short times. In contrast, the long residence times of proteins on conventional ion-exchange and steric-exclusion columns make it necessary to work at cold-room temperatures, *e.g.*, 4°C.

In addition to being useful for purifying contractile proteins, steric-exclusion HPLC is useful for characterizing the proteins' conformations in solution. The HPLC-derived mol.wts. on the unresolved act-S1 (137,000) and particularly the papain-S1 (204,000) are significantly higher than S1's accepted mol.wt. of 115,000–120,000[1,2,17], suggesting that S1 is elongated, which makes it behave like a larger, spherical protein on the Spherogel TSK SW-3000 column. This explanation agrees well with direct evidence that S1 is an elongated ellipsoid[17].

S1's HPLC-derived mol.wts. suggest conformational differences between

papain-S1 (204,000) and unresolved act-S1 (137,000) and between act-S1(lc_1) (158,000) and act-S1(lc_3) (140,000). The differences in mol.wt. between papain-S1 and unresolved act-S1 (49%) and between act-S1(lc_1) and act-S1(lc_3) (13%) are significantly greater than those predicted from the known mol.wts. of myosin's light chains (16 and 5%, respectively). A number of explanations are possible for these unexpected high differences. Perhaps the differences in chromatographic behavior are due to differences in the S1's light-chain complements which result in conformational differences among the various S1's. Or perhaps, the exaggerated differences in mol.wts. between papain-S1 and unresolved act-S1 are due to conformational differences between the two S1's induced by differences in papain's and α-chymotrypsin's cleavage of myosin. Finally, the unexpectedly high difference in mol.wts. between act-S1(lc_1) and act-S1(lc_3) could be due to differences in the spatial arrangement of the light chains on the two heads.

The HPLC-derived mol.wts. of myosin's light chains are closer to the reported mol.wts. than are those of the S1's. lc_2's mol.wt. (23,500) is a bit higher than the reported value of 18,000, presumably because lc_2 is elongated[18–20]. Computation of the mol.wts. for lc_1 and lc_3 from the mol.wt. 58,000 2:1 lc_1–lc_3 complex (Fig. 2, peak a), and the mol.wt. 37,000 1:1 lc_1–lc_3 complex (Fig. 2, peak b) yields mol.wts. of 21,000 and 16,000 for lc_1 and lc_3, respectively. These values agree exactly with published values, suggesting, perhaps, that lc_1 and lc_3 adopt nearly spherical shapes when compexed to each other.

Too much emphasis should not be placed on the absolute values of the HPLC-derived mol.wts. of the various S1's and myosin's light chains because of the acknowledged non-spherical shapes of these proteins. The exaggerated differences in mol.wts. between papain-S1 and act-S1 and particularly between act-S1(lc_1) and act-S1(lc_3), however, are noteworthy and strongly suggest conformational differences between the different S1's.

We have shown that steric-exclusion HPLC can be very useful for purifying and characterizing S1 and myosin's light chains. Other types of HPLC appear promising as well, but remain to be tested. Further refinement of ion-exchange HPLC, specifically the elimination of protein adsorption and denaturation from the ion-exchange supports, should prove invaluable to separations of S1 and of light chains, in that conventional ion-exchange chromatography is superior to conventional steric-exclusion chromatography for separating these proteins[2,3]. Unlike ion-exchange HPLC, reversed-phase HPLC is not likely to be useful for S1 chromatography because the acidic pH values and organic solvents used with reversed-phase HPLC generally denature proteins; but reversed-phase HPLC may prove an excellent way to separate myosin's light chains. They appear inherently more stable than S1 because they can be extracted from myosin by urea or guanidine–HCl denaturation, followed by alcohol precipitation[3]. It seems unlikely, therefore, that elution of the light chains on a reversed-phase column with a 10% methanol–water eluent, for example, would denature them further. Smaller proteins, comparable in size to the light chains, have proven amenable to reversed-phase HPLC analysis[21], whereas larger proteins like S1 tend to adsorb to reversed-phase HPLC supports[22]. It is difficult, of course, to predict exactly how successful these specific approaches will prove. Nevertheless, the potential of HPLC as a rapid, efficient method of separating proteins, particularly contractile proteins, is significant.

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REVERSED-PHASE SEPARATION OF BENZO[*a*]PYRENE METABOLITES BY THIN-LAYER CHROMATOGRAPHY

MILTON V. MARSHALL*,**

Biochemistry Department and Division of Cancer Prevention, The University of Texas System Cancer Center, 6723 Bertner Avenue, Houston, TX 77030, and Biological Sciences Department, North Texas State University, Denton, TX 76203 (U.S.A.)

MELISSA A. GONZALEZ

Biochemistry Department and Division of Cancer Prevention, The University of Texas System Cancer Center, 6723 Bertner Avenue, Houston, TX 77030 (U.S.A.)

THEODORE L. McLEMORE

Department of Medicine, Baylor College of Medicine, Houston, TX 77030, and The Veterans Administration Hospital, 2002 Holcombe Blvd., Houston, TX 77211 (U.S.A.)

DAVID L. BUSBEE

Biological Sciences Department, North Texas State University, Denton, TX 76203 (U.S.A.)

NELDA P. WRAY

Department of Medicine, Baylor College of Medicine, Houston, TX 77030, and The Veterans Administration Hospital, 2002 Holcombe Blvd., Houston, TX 77211 (U.S.A.)

and

A. CLARK GRIFFIN

Biochemistry Department and Division of Cancer Prevention, The University of Texas System Cancer Center, 6723 Bertner Avenue, Houston, TX 77030 (U.S.A.)

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SUMMARY

The use of reversed-phase thin-layer chromatography for the separation of benzo[*a*]pyrene metabolites has been investigated. Two systems are described for the separation of the major metabolites of benzo[*a*]pyrene, including sulfate and glucuronide conjugates.

INTRODUCTION**

Adequate resolution of the major metabolite of B(*a*)P has not been obtained by normal-phase TLC using solvents such as benzene–ethanol[1–4]. The advent of re-

* Address for correspondence: Division of Cancer Prevention, The University of Texas System Cancer Center, 6723 Bertner Avenue, Houston, TX 77030, U.S.A.

** Abbreviations: B(*a*)P = Benzo[*a*]pyrene; NADH = nicotinamide–adenine dinucleotide, reduced; NADPH = nicotinamide–adenine dinucleotide phosphate, reduced; THF = tetrahydrofuran; TBAH = tetrabutylammonium hydrogen sulfate; UDPGA = uridine-3′-phosphoglucuronic acid; S.D. = standard deviation; TLC = thin-layer chromatography; HPLC = high-performance liquid chromatography; S-9 = 9000 *g* supernatant.

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versed-phase HPLC offered rapid separation of the metabolites with increased resolution of the phenols and quinones[5–7]. Use of microparticulate columns has increased resolution and decreased analysis time. In addition, greater resolution of the more polar compounds, including triols and tetrols, has been observed[8,9].

Most studies involving the metabolism of B(*a*)P use HPLC for quantitation of the metabolites. In this report, an inexpensive system utilizing reversed-phase TLC and an ion-pairing reagent is described for the analysis of B(*a*)P metabolites. This procedure offers rapid separation with good resolution of the more polar metabolites, including conjugates.

EXPERIMENTAL

Materials

B(*a*)P metabolite standards were obtained from Dr. David Longfellow, Division of Cancer Cause and Prevention, National Cancer Institute (Bethesda, MD, U.S.A.) Whatman $KC_{18}F$ reversed-phase TLC plates were obtained from Whatman (Clifton, NY, U.S.A.). Solvents were HPLC grade from Fisher Scientific (Fairlawn, NJ, U.S.A.). Aroclor 1254 was a generous gift of Monsanto (St. Louis, MO, U.S.A.). B(*a*)P, α-tocopherol, *E. coli* β-glucuronidase, UDPGA, NADPH, NADH and TBAH were products of Sigma (St. Louis, MO, U.S.A.). [G-^{3}H]Benzo[*a*]pyrene, specific activity 18,000 mCi/mmol, was obtained from Amersham (Arlington Heights, IL, U.S.A.). Silica gel Sep-Paks were purchased from Waters Assoc. (Milford, MA, U.S.A.).

TLC sheets

Whatman $KC_{18}F$ TLC plates were unwrapped just prior to spotting samples to minimize contact with moisture in the air. The tanks were allowed to equilibrate in the solvent for 30–60 min prior to development of the chromatogram. The use of filter paper at the top of the tank was not found to be necessary but does afford better reproducibility. For development of the solvent system, 20 × 5 cm TLC plates were used; for radioactive samples, 20 × 20 cm sheets were used. Generally, 10–20 μl were spotted for each sample.

Solvent system

For separation of the major metabolites of B(*a*)P, a solvent system consisting of acetonitrile–THF–0.071 *M* TBAH (85:1:14) (solvent A) was used. To separate sulfate and glucuronide conjugates from the major metabolites, acetonitrile–methanol–THF–0.042 *M* TBAH (65:10:1:24) (solvent B) was used.

Metabolite formation

Male Sprague-Dawley rats were induced with 500 mg/kg Aroclor 1254 for 3 days according to Fang and Strobel[10]. [^{3}H]B(*a*)P was diluted to a specific activity of 1000 mCi/mmole and purified by silica gel chromatography just prior to incubation[11]. The incubation mixture has been previously described[6]. Briefly, 200 μg rat liver S-9 was added to 100 nmoles [^{3}H]B(*a*)P in 0.05 *M* Tris (pH 7.5), 0.003 *M* $MgCl_2$ with 0.75 mg NADH and 0.75 mg NADPH in 1 ml. Samples were incubated in 5 tubes for 0, 15, 30 or 60 min.

Extraction of metabolites

Following incubation, 0.03 ml of 0.1 *M* α-tocopherol in ethanol was added to each tube prior to extraction with an equal volume of acetone and an equal volume of ethyl acetate[9]. The aqueous layer was reextracted with an equal volume of ethyl acetate. The organic layers were pooled from each of 5 tubes, dried over anhydrous $MgSO_4$, and evaporated under nitrogen. Samples were stored in the dark at 4°C prior to chromatography.

Removal of [^{3}H]Benzo[a]pyrene

[^{3}H]B(*a*)P was removed from the metabolites by dissolving the evaporated organic extract in benzene then applying the sample to a Waters silica gel Sep-Pak. [^{3}H]B(*a*)P was eluted with 5 ml benzene, and the metabolites were eluted with 5 ml methanol. 30 μl of 0.1 *M* α-tocopherol was added to the metabolite fraction prior to evaporation under nitrogen.

TLC

Dried metabolites were dissolved in 100 μl and 15 μl were spotted in two different lanes, along with 5 μl of a mixture of unlabeled metabolite standards. Development times were 55 min for solvent A and 80 min for solvent B.

Autoradiography

After development, the TLC sheets were dried, marked with ^{14}C-labeled ink and a sheet of LKB [^{3}H]Ultrofilm was placed on top. The sheets were developed for 7–14 days.

RESULTS AND DISCUSSION

Polar solvent selection

In attempting to develop a solvent system to separate the B(*a*)P metabolites, efforts were centered around use of a methanol–water system that was analogous to solvent systems used with by HPLC and octadecyl silane column packing. However, this solvent system did not yield adequate separation of the B(*a*)P phenols and quinones (Table I). Thus, the ion-pairing agent TBAH was added (Table I). Increased

TABLE I

R_F VALUES OF BENZO[*a*]PYRENE METABOLITES

Metabolite	*Solvent system*		
	Methanol–water (95:5)	*Methanol–0.04 M TBAH (95:5)*	*Methanol–0.10 M TBAH (95:5)*
B(*a*)P	0.27	0.24	0.27
Quinones*	0.37	0.35	0.37
3-OH	0.43	0.39	0.43
9-OH	0.43	0.40	0.44
4,5-epoxide	0.43	0.40	0.45
7,8-dihydrodiol	0.57	0.55	0.59
4,5-dihydrodiol	0.61	0.60	0.64
9,10-dihydrodiol	0.73	0.72	0.75

* Mixture of 1,6-, 3,6- and 6,12-quinones.

resolution of the quinones and phenols was observed at higher concentrations of TBAH, but adequate resolution of 3-OH- and 9-OH-B(*a*)P was not achieved. Thus, a system using acetonitrile–water was tried (Fig. 1). Increasing the acetonitrile concentration from 80% to 85% or the addition of 1% THF still did not adequately resolve the phenols, but better separation was obtained than with methanol–water (Fig. 1A). Very poor separation was observed for the 4,5-epoxide from the phenols and quinones in the first five solvents. The best separation of all the metabolites was obtained with acetronitrile–THF–0.071 *M* TBAH (85:1:14) as shown in Fig. 1 (solvent 6). Adequate separation of 3-OH- and 9-OH-B(*a*)P was obtained but not between 7-OH- and 3-OH- or 9-OH-B(*a*)P. The 4,5-epoxide was separated from the quinones and 3-OH-B(*a*)P in this system, with an R_F value of 0.45 (Table II). The quinones were not well separated from each other and tended to migrate as a large, diffuse spot.

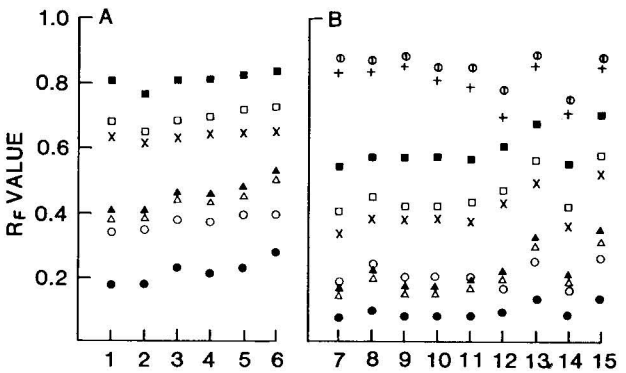

Fig. 1. A, Reversed-phase TLC of major B(*a*)P metabolites in various solvents. Solvent systems: 1 = acetonitrile–water (80:20); 2 = acetonitrile–THF–water (80:1:19); 3 = acetonitrile–water (85:15); 4 = acetonitrile–0.007 *M* TBAH (85:15); 5 = acetonitrile–0.07 *M* TBAH (85:15); 6 = acetonitrile–THF–0.071 *M* TBAH (85:1:14) (solvent system A). B, Reversed-phase TLC of primary B(*a*)P metabolites with sulfate and glucuronide conjugates in various solvents. Solvent systems: 7 = acetonitrile–methanol–water (50:25:25); 8 = acetonitrile–methanol–THF–water (50:25:1:24); 9 = acetonitrile–methanol–THF–water (50:25:2:23); 10 = acetonitrile–methanol–THF–0.004 *M* TBAH (50:25:2:23); 11 = acetonitrile–methanol–0.004 *M* TBAH (50:25:25); 12 = acetonitrile–methanol–0.04 *M* TBAH (50:25:25); 13 = acetonitrile–methanol–THF–0.004 *M* TBAH (50:25:1:24); 14 = acetonitrile–methanol–THF–0.04 *M* TBAH (50:25:1:24); 15 = acetonitrile–methanol–THF–0.04 *M* TBAH (65:10:1:24) (solvent system B). Metabolites: ● B(*a*)P; ○, quinones; △, 3-OH; ▲, 9-OH; ×, 7,8-dihydrodiol; □, 4,5-dihydrodiol; ■, 9,10-dihydrodiol; +, 3-SO_4; ⊙, 3-glucuronide.

Separation of conjugates

A great deal of attention has focused on the detoxification of B(*a*)P through conjugation. Thus, a system was developed to simultaneously identify the presence of sulfate and glucuronide conjugates. The use of acetonitrile–water solvent systems with a concentration of water of greater than 40% led to the TLC sorbent detaching from the glass; therefore, a mixture of acetonitrile–methanol–water was used. The solvent systems of acetonitrile–methanol or acetonitrile–methanol–THF did not yield

TABLE II

R_F VALUES OF MAJOR BENZO[*a*]PYRENE METABOLITES IN SOLVENT SYSTEM A

Metabolite	$\bar{X}^* \pm S.D.$
B(*a*)P	0.27 ± 0.03
1,6-quinone	0.37 ± 0.03
3,6-quinone	0.38 ± 0.03
6,12-quinone	0.38 ± 0.03
4,5-epoxide	0.45 ± 0.03
3-OH	0.50 ± 0.03
7-OH	0.52 ± 0.02
9-OH	0.53 ± 0.02
7,8-dihydrodiol	0.65 ± 0.04
4,5-dihydrodiol	0.73 ± 0.02
9,10-dihydrodiol	0.84 ± 0.03

* Mean value from eight different samples on separate TLC sheets.

adequate separation of the B(*a*)P metabolites in the quinone–phenol region, as shown in Fig. 1B. Addition of TBAH at 0.001 *M* was not much different from acetonitrile–methanol alone. Increasing the concentration of TBAH to 0.01 *M* led to a retardation of the quinones; however, the separation of 4,5-epoxide and 3-OH-B(*a*)P was not accomplished. A combination of acetonitrile–methanol–THF–TBAH was also used, as shown in Fig. 1B, solvents 13, 14, and 15. It appears that 0.001 *M* TBAH gives the best separation at lower organic solvent concentrations (50% acetonitrile and 25% methanol, compared to 85% acetonitrile). The R_F values obtained in solvent system B are shown in Table III.

TABLE III

R_F VALUES OF MAJOR BENZO[*a*]PYRENE METABOLITES IN SOLVENT SYSTEM B

Metabolite	$\bar{X}^* \pm S.D.$
B(*a*)P	0.13 ± 0.02
Quinones	0.22 ± 0.03
4,5-epoxide	0.25 ± 0.03
3-OH	0.27 ± 0.04
7-OH	0.28 ± 0.03
9-OH	0.30 ± 0.04
7,8-dihydrodiol	0.47 ± 0.04
4,5-dihydrodiol	0.53 ± 0.04
9,10-dihydrodiol	0.66 ± 0.04
3-SO_4	0.82 ± 0.04
3-glucuronide	0.86 ± 0.03

* Mean value from thirteen different samples.

Analytical application

Both solvent systems A and B were used to separate the metabolites of [^{3}H]-B(*a*)P. The results are shown in Figs. 2 and 3. Fig. 2 illustrates the separation of the B(*a*)P metabolites in solvent system A. The 3-OH- and 9-OH-B(*a*)P spots are not as well resolved due to the presence of other phenols (*i.e.* 7-OH-B(*a*)P). Fig. 3 illustrates

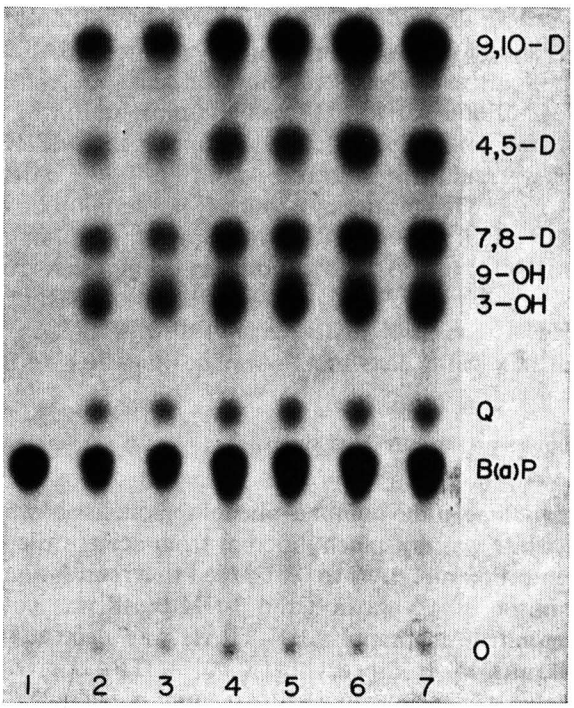

Fig. 2. Chromatography of [^{3}H]B(*a*)P metabolites in solvent system A. Autoradiogram of [^{3}H]B(*a*)P metabolites produced by rat liver S-9 in the presence of 2.5 m*M* UDPGA. Incubation times are 0 min (lane 1), 15 min (lanes 2, 3), 30 min (lanes 4, 5) and 60 min (lanes 6, 7). Autoradiography development was for 14 days. 9,10-D = 9,10-Dihydrodiol; 4,5-D = 4,5-dihydrodiol; 7,8-D = 7,8-dihydrodiol; 9-OH = 9-OH-B(*a*)P; 3-OH = 3-OH-B(*a*)P; Q = mixture of 1,6-, 3,6- and 6,12-quinones; B(*a*)P = benzo[*a*]pyrene; O = origin.

the separation of the B(*a*)P metabolites obtained with solvent B. Lanes 1, 3, 5, and 7 are from the incubation without UDPGA; lanes 2, 4, 6, and 8 were from the incubation with 2.5 m*M* UDPGA. Exposure time was 7 days for lanes 1, 3, 5, and 7, and 14 days for lanes 2, 4, 6, and 8. The glucuronide and sulfate conjugate spots were too faint to be observed, but they are visible following longer autoradiography times. Better resolution is obtained for the 3-OH- and 9-OH-B(*a*)P compared to solvent system A (Fig. 2).

Hydrolysis of the water soluble (glucuronide) conjugates was obtained with *E. coli* β-glucuronidase. Separation of these metabolites in solvent system B is shown in Fig. 4. As can be observed, the major conjugate is 3-OH-B(*a*)P, as previously observed by Nemoto and co-workers.[12,13] The large amount of quinones probably arise from the oxidation of 6-OH-B(*a*)P after hydrolysis. Other hydrolyzed conjugates are observed which migrate between the 7,8-dihydrodiol and 9-OH-B(*a*)P. Another spot is observed which migrates between the quinones and B(*a*)P.

Several chromatography systems have been developed for the quantitation of B(*a*)P and its metabolites. Early techniques include paper partition chromatography utilizing aqueous or organic solvents[14–16]. These techniques were unsatisfactory for resolving the large number of metabolites of B(*a*)P which have subsequently been

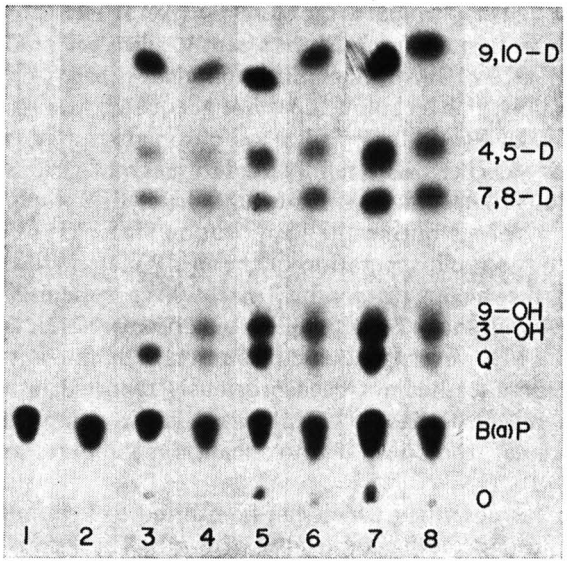

Fig. 3. Chromatography of [^{3}H]B(*a*)P metabolites in solvent system B. Autoradiogram of [^{3}H]B(*a*)P metabolites produced by rat liver S-9 in the absence of UDPGA (lanes 1, 3, 5, 7) or with 2.5 m*M* UDPGA (lanes 2, 4, 6, 8). Incubation times are 0 min (lanes 1, 2), 15 min (lanes 3, 4), 30 min (lanes 5, 6) and 60 min (lanes 7, 8). Autoradiographic exposure was for 7 days for the samples without UDPGA and 14 days for the samples with UDPGA.

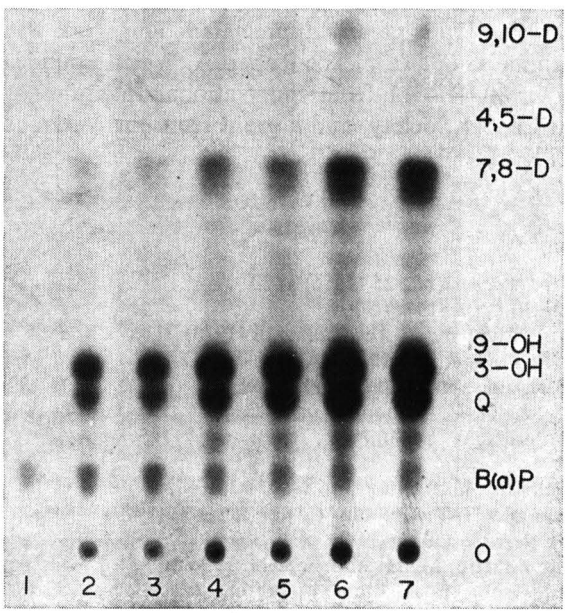

Fig. 4. Hydrolyzed glucuronide conjugates of [^{3}H]B(*a*)P chromatographed in solvent system B. Autoradiogram of hydrolyzed [^{3}H]B(*a*)P conjugates produced by rat liver S-9 in the presence of 2.5 m*M* UDPGA. Following incubation and extraction with ethyl acetate, the aqueous layer was digested with 500 μ/ml *E. coli* β-glucuronidase for 4 h then re-extracted with ethyl acetate as previously described. The evaporated samples were dissolved in 50 μl methanol and 15 μl were spotted in two different lanes, along with 5 μl of a mixture of unlabeled metabolite standards. Incubation times for the various lanes are identical to those in Fig. 2. Autoradiography development was for 14 days.

detected by HPLC[17]. In addition the paper chromatography takes several hours to complete and the R_F values are sensitive to moisture[18]. More recently, silica gel TLC has been used to separate the metabolites of B(*a*)P (including conjugates) based on their relative polarities[19,23]. Unfortunately, this technique does not adequately resolve the phenolic metabolites. The stability of B(*a*)P during silica gel chromatography is also doubtful[24,25]. Gas chromatography has been successfully used to separate various polycyclic aromatic hydrocarbons but this technique is inadequate for hydroxylated metabolites which are destroyed by pyrolysis during the vaporization stage. HPLC was thus the method of choice due to the rapid separation of the B(*a*)P metabolites, particularly phenols and quinones, the major metabolites in rat liver systems[26]. Reversed-phase TLC allows a more rapid analysis of samples, but the resolution is not as great as that obtained by HPLC. However, adequate resolution of sulfate and glucuronide conjugates of B(*a*)P metabolites has not been previously reported in a single chromatographic step. Thus reversed-phase TLC allows a more complete analysis of B(*a*)P metabolism with greater resolution of detoxification metabolites as well as activation metabolites of B(*a*)P.

In summary, a TLC procedure is described which can be utilized to separate both primary and secondary metabolites, including conjugates, of B(*a*)P. The separation of both sulfate and glucuronide conjugates is useful in that these are the primary means of detoxification of B(*a*)P[11–13,19–23,26].

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Note

Effect of derivative structure on flame-ionization detector response of amino acid oxazolidinones

VLADIMÍR FELT and PETR HUŠEK*
Research Institute of Endocrinology, Národní třída 8, CS-116 94 Prague 1 (Czechoslovakia)
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A simple procedure for the conversion of all protein amino acids into cyclic derivatives, suitable for determination by gas chromatography, has been introduced in previous studies[1–4]. The exact derivatization conditions were determined for amino acids possessing the same or similar reactive groups and the particular mixtures, mostly amino acids in homologous series, were analysed with the use of a flame-ionization detector (FID)[1,4].

In order to evaluate the influence of the derivative structure on the response behaviour, the approach used by Islam and Darbre[5] for the trifluoroacetylated (TFA) amino acid methyl esters was used in this work. However, the absolute molar responses in coulombs per mole were determined directly from the known values for the attenuation ($4 \cdot 10^{-10}$ A for 250 mm full-scale deflection of the integrator) and the chart paper speed (10 mm/min), *i.e.*, a hypothetical area of 250 mm^2 corresponds to $4 \cdot 10^{-10}$ A $\cdot$ 6 sec $= 2.4 \cdot 10^{-9}$ C. When compared with the peak area of an exact amount of the derivatized compound injected (*n*-hexadecane was used as the internal standard), the absolute molar responses in coulombs per mole were obtained (Table I). By plotting the found responses against the carbon number of the derivatives in the homologous series, it was possible to determine by extrapolation the responses for compounds with higher carbon numbers that were not recorded by the FID (Fig. 1). The difference between a hypothetical response, being proportional to the actual number of carbon atoms in the molecule, and the lower response found, indicates the presence of specific groups or atoms joined to a carbon atom that prevent it from being recorded as a methyl group.

As the response of hydrocarbons in the FID is generally accepted as being maximal and directly proportional to the number of carbon atoms in the molecule, the dashed line with *n*-hexadecane passes through the origin and the found value of its molar response for C_{16} (3.60 C/mol) agrees with the previously published value[5].

The regression line for the homologous series of the oxazolidinones of amino acids with (A) a carbon-linked chain and also (B_1) a thio-ether bond cuts the abscissa at a "non-effective" value of 2.4. Because of the identical lines for S-alkylated amino acids (Nos. 12–14 in Table I) and amino acids with a carbon-linked side-chain (Nos. 1–11), it is obvious that the thio-ether bond does not reduce the responses of the adjacent carbon atoms, *i.e.*, the compound behaves in the FID like the same compound without the sulphur atom. The same was found by Islam and Darbre[5]

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TABLE I

MOLAR RESPONSES OF DERIVATIZED AMINO ACIDS (N,O-HFB AND N,O-TFA OXAZOLIDIDONES AND *n*-HEXADECANE IN A FLAME-IONIZATION DETECTOR

No.	*Compound*	*Number of carbon atoms**	*Molar response (coulomb/mole)*
—	*n*-Hexadecane	16	3.60
	(A) Amino acids with carbon-linked chain		
1	Glycine (Gly)	5	0.59
2	Alanine (Ala)	6	0.82
3	α-Aminobutyric acid (Aba)	7	1.04
4	α-Aminoisobutyric acid (Aiba)	7	1.08
5	Norvaline (Nval)	8	1.26
6	Valine (Val)	8	1.28
7	Norleucine (Nleu)	9	1.48
8	Leucine (Leu)	9	1.51
9	Isoleucine (Ile)	9	1.53
10	α-Aminocaprylic acid (Aca)	11	1.93
11	Phenylalanine (Phe)	12	2.16
	(B) Amino acids with alkyl substituent on polar group		
12	S-Methylcysteine (Cysm)	7	1.05
13	Methionine (Met)	8	1.26
14	Ethionine (Eth)	9	1.46
15	Sarkosine (Sar)	6	0.69
16	Proline (Pro)	8	1.11
17	Pipecolic acid (Pipa)	9	1.42
18	Tryptophan (Trp)	14	2.34
	(C) Diaminodicarboxylic acids		
19	Diaminosuccinic acid (Dasca)	10	0.98
20	Diaminopimelic acid (Dapa)	13	1.62
21	Diaminosuberic acid (Dasba)	14	1.84
22	Lanthionine (Lan)	12	1.43
23	Cystine (Cys)	12	1.25
24	Homocystine (Hcys)	14	1.51
	(D) Diaminomonocarboxylic acids		
25	Diaminobutyric acid (Daba)	(a) 9	0.91
		(b) 11	1.32
26	Ornithine (Orn)	(a) 10	1.10
		(b) 12	1.55
27	Lysine (Lys)	(a) 11	1.30
		(b) 13	1.75
	(E) Hydroxyamino acids		
28	Serine (Ser)	(a) 8	0.80
		(b) 10	1.25
29	Threonine (Thr)	(a) 9	1.02
		(b) 11	1.47
30	Tyrosine (Tyr)	(a) 14	1.94
		(b) 16	2.33
31	α-Methyltyrosine (α-CH_3-Tyr)	(a) 15	2.13
		(b) 17	2.50
32	Hydroxyproline (Hyp)	(a) 10	1.14
		(b) 12	1.48
	(F) Other amino acids		
33	Histidine (His)	13	1.58
34	Arginine (Arg)	(a) 13	1.53
		(b) 17	1.79
35	Homoarginine (Harg)	(a) 14	1.84
		(b) 18	2.10

* (a) Acylation with TFAA; (b) acylation with HFBA.

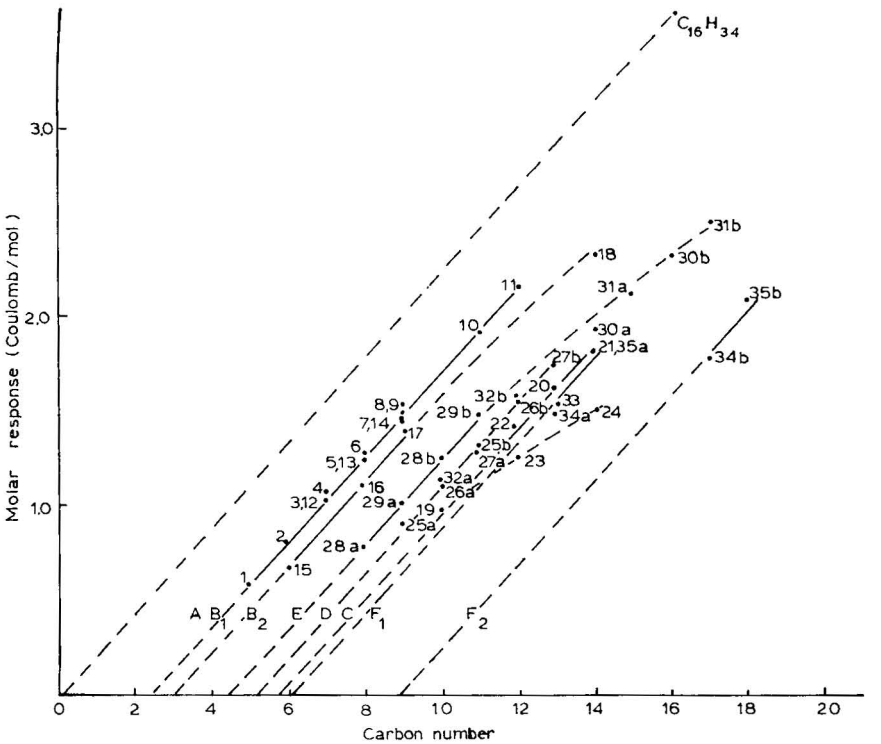
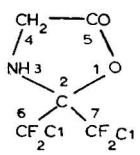


Fig. 1. Relationship between FID molar responses and number of carbon atoms in derivatized compounds. On extrapolation the regression lines cut the abscissa at a point that gives the value of the non-effective carbon number. For identification of compounds, see Table I.

with the N-TFA methyl esters of amino acids, where the responses for Met and Nval were nearly identical, as they were in our work. In contrast, the reason for a lower value for Met as occurred for example, with the N,O-heptafluorobutyryl (HFB) alkyl esters[6–8], probably lies in the derivatization procedure used.

After subtracting the corresponding "non-effective" value of 2.4, a response value of 2.6 will be obtained for the oxazolidinone ring, *i.e.*, glycine after condensation. According to some earlier studies, summarized by Islam and Darbre[5], a carbon atom in the neighbourhood of an oxygen atom (ester bond or carbonyl group), *i.e.*, the second and the fifth carbon atoms of the ring, has a zero response. Therefore, the fourth carbon atom and both chlorodifluoromethyl groups are responsible for the response of the ring.



As the N-containing groups also lower the response of an adjacent carbon atom, *e.g.*, to a value of 0.25–0.75 of its full response, as was found by Sternberg

et al. (ref. 9, p. 231), or to a value of about 0.3–0.4 for imino acids in our work (see further), it follows that the two perhalogenated methyl groups have a full or nearly full response (if we assign them the value of 2.0, there still remains a value of 0.2–0.3 for the carbon atoms in the ring). Identically, as the molar responses of O-TFA and O-HFB esters of Ser and Thr oxazolidinones (regression line in Fig. 1) are close to that of Ala and Aba and to Nval and Nleu in the latter instance, it follows that the terminal groups $-CH_2-O-CO-CF_3$ and $-CH_2-O-CO-C_3F_7$ have the same response as 1 and 3 carbon atoms, respectively. Provided that the two oxygen atoms hinder the adjacent carbon atoms to be recorded, the trifluoromethyl and, as mentioned above, also the chlorodifluoromethyl groups seem to have responses identical with those of their aliphatic equivalents. However, this strange phenomenon (the fluoroalkanes have been repeatedly confirmed to yield a lower detector response than alkanes[10]) is nowadays not exceptional, as discussed later. A possible explanation in our work is that owing to the presence of oxygen in the neighbourhood of a perhalogenated carbon chain, the negative effect of the halogen presence on the response may be reduced.

The hydroxylated aromatic amino acids (Tyr, α-CH_3-Tyr) afford, contrary to expectation, approximately a one carbon atom lower response (curving of the regression line E at the top), which is probably caused by incomplete esterification of the phenolic group (yield about 90%) under the specified reaction conditions[4]. However, it is interesting that a similar decline in the response of the N-TFA methyl ester of Tyr was found[5] even when a breakdown of the derivative in the column was said to be responsible for the partial losses.

The N-TFA and N-HFB acylated oxazolidinones of diaminocarboxylic acids (regression line D) also show a one carbon atom lower response in comparison with the O-TFA and O-HFB acylated forms with an equal number of carbon atoms. Regression line D cuts the abscissa at a value of 5.1. After subtracting the non-effective carbon number for the oxazolidinone ring (2.4), this leaves 2.7 for the $-CH_2-NH-CO-CF_3$ group and 4.7 for the $-CH_2-NH-CO-C_3F_7$ group as the unrecorded carbon portions, *i.e.*, only 0.3 and 2.3 carbon atoms in the latter instance are recorded. As the secondary amino group causes a carbon number reduction of 0.6, as follows for the imino acids (Nos. 15–17) by comparison of the regression lines A (B_1) and B_2, or perhaps of 0.7 in this instance, the recorded value of 0.3 belongs to the methylene group, whereas the $-CO-CF_3$ moeity, connected to a carbon linkage by a nitrogen bond, has a zero response. Considering some previous work on amino acid derivatization[6,11] equally differing values of molar responses of the N,N-diacylated amino acids in comparison with the N,O-diacylated forms can be found. These findings support the preceding tentative interpretation that a response of a halogenated alkyl (carbonyl) chains seems to be influenced by the heterogeneous atom in the neighbourhood.

The diaminodicarboxylic acids (regression line C) have a lower response than twice the response of one oxazolidinone ring, *i.e.*, the response of Dasca (4.3) is not equal to twice the response of Gly (5.2). This means that the response of the methylene group in the oxazolidinone ring is reduced by connection of the antipole. The response of Lan corresponds to a response of a hypothetical diaminoadipic acid (not commercially available), which supports the fact that the thioether bond does not lower the responses of adjacent carbon atoms. The decreased values for Cys

(−10%) and Hcys (−20%) are not caused by the disulphide bond present, but they indicate partial absorption of the derivatives in the column packing used[4]. The same applies to Trp (with a decrease in response of about 5%).

The molar response of the N^{im}-IBOC oxazolidinone of His fits the values of Arg and Harg TFA-oxazolidinones, finding themselves on regression line F_1 (the HFB derivatives form regression line F_2), and this is the only reason for putting His on this line with a non-effective carbon number of 6.0. Considering the structrue of the derivatized His[4], the response should, in fact, be approximately one carbon atom higher, and this also follows from the sharp elution curve (ref. 4, Fig. 8) for this compound. Even under the best analytical conditions about 10% of His is absorbed by the packing. The N,N-diacylated guanidino group in the molecules of Arg and Harg oxazolidinones was expected to have, according to the preceding results, a response identical with that of the acylated terminal amino group in the molecules of Orn and Lys oxazolidinones. However, N,N-diacylated guanidine actually has a higher response with the TFA groups (effective carbon number equal to 2.1) and a lower one with the HFB groups (3.3). We cannot give any explanation for this phenomenon, but a similar response of Arg can also be found with the other derivatized forms[6,11].

In accordance with the results of Islam and Darbre[5], we noted with the oxazolidinones the same response behaviour as that of amino acid isomers. The isomers show a higher response with an increase in branching of the carbon chain in the order Ile > Leu > Nleu, Val > Nval, Aiba > Aba. However, as was shown with hydrocarbon compounds (ref. 9, p. 307), the differences are small and statistically not significant.

In conclusion, the found values of the molar responses can be helpful in reconsidering several previous erratic representations of response–structure relationships[6–8] and they seem to confirm some recent studies[12–14] dealing with the unexpectedly high responses of some halogenated compounds in the FID. Thus, the high carbon number fluorocarbons showed response values similar to those of the corresponding hydrocarbons and their relative response values were almost independent of the operating conditions, provided that higher hydrogen flow-rates than usual for hydrocarbons were employed[12]. In another instance, the low carbon number 2-chloro-2-bromo-1,1,1-trifluoroethane was found to have almost the same response as ethane under the usual FID conditions[13]. Other workers have found that by varying the flame composition in favour of a hydrogen-rich flame and with oxygen as the combustion supporter, a completely different response behaviour of halogenated hydrocarbons is to be expected[10,14]. All of these findings indicate that one must be very careful in generalizing the behaviour of the FID towards halogenated compounds.

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Note

Konzentrationsabhängige Veränderungen der Methylene Units bei Steroidanalysen (MU-Shift)

K. HERKNER* und W. SWOBODA

Ludwig Boltzmann Institut für pädiatrische Endokrinologie, Währinger Gürtel 74–76, A-1090 Wien (Österreich)

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Um in der Gaschromatographie (GC) kostspielige Detektionssysteme, wie sie zum Beispiel IR-Spektrometer und Massenspektrometer darstellen, zu umgehen, und trotzdem qualitativ zuverlässige Aussagen machen zu können, haben sich verschiedene "Identifikationssysteme" wie die Kováts-Indices (KI), die Steroidnummern (SN) oder die Methylene Units (MU) durchgesetzt. Besonders bei Analysen von Steroidhormonen mittels Kapillargaschromatographie dienen derartige Einheiten, speziell die MU- und T-Werte, zur Aufklärung von Molekülstrukturen [1],da sie sich, abgeleitet aus den relativen Retentionszeiten (RRT), additiv aus dem Retentionsverhalten des Molekülskeletts und der einzelnen funktionellen Gruppen zusammensetzen[2,3].

Bei der GC Analyse von Steroiden hat sich die qualitative Zuordnung der Peaks eines Chromatogramms über die MU's durchgesetzt. Bei den dafür hauptsächlich verwendeten polaren flüssigen Phasen dienen langkettige aliphatische Kohlenwasserstoffe (KW) als Bezugssubstanzen für die Berechnung der MU-Werte. Dabei wird die Retentionszeit (t_R) der KW auf einer logarithmischen Basis verglichen. Bei der praktischen Anwendung dieser Zuordnungsmethode muss jedoch darauf geachtet werden, dass diese Kenngrösse nich uneingeschränkt unter diversen Chromatographiebedingungen, bzw. bei verschiedenen Geräteparametern, verwendet werden kann. Mehrere Komponenten führen zu Veränderungen der MU-Werte für verschiedene Substanzen. In der Literatur wird hauptsächlich auf eine Temperaturabhängigkeit hingewiesen[2–5].

Die Identifizierung von Substanzen im Chromatogramm einer unbekannten Probe kann also nur dann vorgenommen werden, wenn die Probe unter identen Temperaturbedingungen wie der Eichstandard analysiert wurde. Eichkurven weisen besonders bei Verwendung eines Flammenionisationsdetektor (FID) oft über einen grossen Konzentrationsbereich ein lineares Verhalten auf, sodass eine Mehrpunkteichung in diesem Falle nicht von absoluter Notwendigkeit ist. Es kann aber zu einer Verschiebung der Werte für die MU's in den verschiedenen Konzentrationsbereichen kommen, zum sogenannten "MU-Shift".

In der vorliegenden Arbeit wird nun diesem konzentrationsabhängigen "MU-Shift" Rechnung getragen. Die Kenntnis der Variabilität der MU's in Abhängigkeit von der Konzentration, bei der sie berechnet werden, ist für die Peakidentifizierung wichtig, um bei Chromatogrammen, in denen Substanzen quantitativ sehr unterschiedlich auftreten, keine falschen Zuordnungen zu treffen.

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MATERIAL UND METHODIK

Die GC Retentionswerte wurden mit einem Carlo Erba, Modell Fractovap 2900 Gaschromatograph ermittelt. Detektor war ein FID. Die Auswertung der Chromatogramme und die Zuordnung der relativen und absoluten Retentionszeiten erfolgte über einen Digitalintegrator SP 4100 (Spectra-Physics).

Das Trennsystem war eine Glaskapillare (OV-101; 25 m × 0.5 mm I.D.). Die Temperatur (Injektor und Detektor) war 300°C; Trägergas, N_2.

Die Analysen erfolgte temperaturprogrammiert gemäss folgendem Schema:

$$65°C\ (2\ min) \xrightarrow{35°C/min} 220°C\ (15\ min) \xrightarrow{1.5°C/min} 250°C.$$

Als Probesubstanzen wurden willkürlich gewählte Steroide der Nebennierenrinde eingesetzt, deren Retentionsverhalten ermittelt wurde. Es waren dies:

3α,17β-Dihydroxyandrost-5β-an	(1)
3α,17β-Dihydroxyandrost-5α-an	(2)
3β,17β-Dihydroxyandrost-5α-an	(3)
3α-Hydroxyandrost-5β-an-17-on	(4)
3α-Hydroxyandrost-5α-an-17-on	(5)
3β-Hydroxyandrost-5α-an-17-on	(6)
17β-Hydroxyandrost-5α-an-3-on	(7a)
17β-Hydroxyandrost-5α-an-3-on	(7b)*
17-Hydroxyandrost-4-en-3-on	(8a)
17-Hydroxyandrost-4-en-3-on	(8b)*
3α,11β-Dihydroxyandrost-5β-an-17-on	(9)
3α,17β-Dihydroxyandrost-5α-an-17-on	(10)
17α-Hydroxypregn-4-en-3,20-dion	(11)

Alle Reinsubstanzen wurden von Steraloids (Wilton, NH, U.S.A.) bezogen. Die Proben wurden als MO–TMS-Derivate chromatographiert[6,7].

Als Bezugssubstanzen für die Berechnung der RRT und MU dienten die Alkane *n*-$C_{24}H_{50}$ (*n*-Tetracosane) und *n*-$C_{32}H_{66}$ (*n*-Dotriacontane) (Applied Science Labs., State College, PA, U.S.A.).

ERGEBNISSE

Die oben angeführten Steroide wurden in drei verschiedenen Konzentrationen chromatographiert: A, 200 ng/μl; B, 50 ng/μl und C, 25 ng/μl. Für jede Konzentration wurden vierfache Einspritzungen vorgenommen.

In Fig. 1 ist das Chromatogramm des Steroidgemisches zu sehen. Tabelle I gibt die Ergebnisse als MU- und RRT-Werte der einzelnen Komponenten wieder.

In Tabelle II sind die Unterschiede (ΔMU- und ΔRRT-Werte) zwischen den zu den verschiedenen Konzentrationen einer Substanz gehörenden Werte angegeben.

---

* Die MO–TMS-Derivate dieser Steroids erscheinen in Chromatogramm als Doppelpeak. Da das analytische System eine Allglasausführung ist, ist eine Zersetzung nicht zu vermuten. Wahrscheinlicher erscheint die auch in der ref. 6 angeführte Erklärung von *syn–anti*-Isomeren der Methoximderivate.

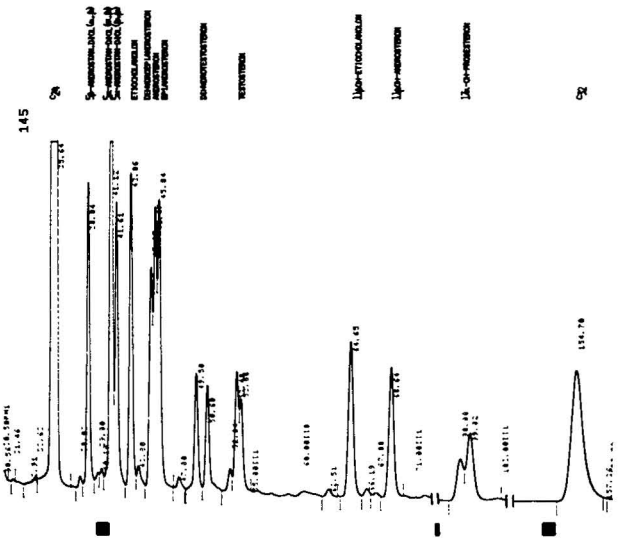

Fig. 1. Kapillargaschromatogram von Steroidhormonen. Säule: 25 m × 0.5 mm I.D. OV-101; Detektor- und Injektortemperatur: 300°C; Oventemperaturprogramm: 65°C (2 min) $\xrightarrow{35°C/min}$ 220°C (15 min) $\xrightarrow{1.5°C/min}$ 250°C.

TABELLE I

ERGEBNISSE DER BESTIMMUNGEN VON MU UND RRT FÜR STEROIDHORMONE IN DREI VERSCHIEDENEN KONZENTRATIONBEREICHEN NACH KAPILLAR-GC AUFTRENNUNG

Substanz Nr.	*MU/RRT ($\bar{x}$*: n = 4)*		
	200 ng/µl	*50 ng/µl*	*25 ng/µl*
1	24.257/1.061	24.200/1.048	24.193/1.047
2	24.563/1.134	24.438/1.115	24.473/1.113
3	24.660/1.157	24.560/1.134	24.547/1.131
4	24.843/1.201	24.783/1.186	24.767/1.184
5	25.203/1.286	25.108/1.264	25.087/1.261
6	25.257/1.299	25.175/1.279	25.150/1.276
7a	25.790/1.426	25.728/1.411	25.715/1.409
7b	25.950/1.464	25.885/1.449	25.870/1.451
8a	26.393/1'569	26.395/1.552	26.305/1.550
8b	26.473/1.589	26.398/1.571	26.390/1.570
9	27.603/1.858	27.538/1.843	27.543/1.851
10	27.880/1.923	27.823/1.909	27.770/1.899
11	29.607/2.339	27.583/2.329	29.000/2.345

* SE $\leqslant$ 0.01 für alle Proben.

DISKUSSION

Bei der Analyse von Steroidhormonen mittels GC bzw. Kapillar-GC werden für die qualitative Zuordnung der Peaks im Chromatogram haptsächlich die MU-Werte verwendet. Als Bezugssubstanzen für diese Berechnungen dienen vorwiegend

TABELLE II

ΔMU- UND ΔRRT-WERTE BEI PEAKZUORDNUNG IN DREI VERSCHIEDENEN KONZENTRATIONSBEREICHEN

"–" vor den Zahlen soll die Abnahme des Wertes anzeigen.

Substanz Nr.	*ΔMU/ΔRRT (200 → 50)*	*ΔMU/ΔRRT (50 → 25)*	*ΔMU/ΔRRT (200 → 25)*
1	−0.057/−0.013	−0.007/−0.001	−0.064/−0.016
2	−0.080/−0.019	−0.010/−0.002	−0.090/−0.021
3	−0.100/−0.023	−0.013/−0.003	−0.113/−0.026
4	−0.060/−0.015	−0.016/−0.002	−0.076/−0.017
5	−0.095/−0.022	−0.021/−0.003	−0.116/−0.025
6	−0.082/−0.020	−0.025/−0.003	−0.107/−0.023
7a	−0.062/−0.015	−0.013/−0.002	−0.075/−0.017
7b	−0.065/−0.015	−0.015/−0.002	−0.080/−0.013
8a	−0.002/−0.017	−0.090/−0.002	−0.088/−0.019
8b	−0.075/−0.018	−0.008/−0.001	−0.083/−0.019
9	−0.065/−0.015	−0.005/−0.008	−0.060/−0.008
10	−0.057/−0.014	−0.053/−0.010	−0.110/−0.024
11	−0.024/−0.010	−0.017/−0.016	−0.007/−0.006

langkettige *n*-Alkane, wenn auch z. T. schon andere Moleküle, wie Äther oder Ketone von *n*-Paraffinen vorgeschlagen wurden[8].

Bei Verwendung der MU-Werte ist auf gleiche Temperaturverhältnisse bei Eichung und Analyse zu achten, da diese Zuordnungsmethode stark temperaturabhängig ist. Neben dieser Variabilität rufen auch Konzentrationunterschiede einen gewissen "MU-Shift" hervor. Wenn die MU-Differenzen bei den angeführten Konzentrationen auch sehr gering erscheinen (siehe Tabelle II/Ergebnisse), muss man doch beachten, dass benachbarte Substanzen ebenfalls nur sehr geringfügige Unterschiede in den MU-Werten aufweisen. So kann man zwischen den Substanzen 2 und 3 einen MU-Wert-Unterschied von 0.097 feststellen; zwischen 5 und 6 bzw. 8a und 8b betragen die Differenzen 0.054 und 0.080 MU-Einheiten. Auf Grund der Werte für den "MU-Shift" durch Konzentrationsveränderungen (siehe Tabelle II) kann man erkennen, dass die Identifizierung von Probenpeaks dann zu falschen qualitativen Aussagen führen kann, wenn die Eichung, und somit die Berechnung der einzelnen Komponenten in einem anderen Konzentrationsbereich durchgeführt wird. Dieser oben aufgezeigten Daten wegen muss die Empfehlung abgegeben werden, bei Peakidentifizierung über MU und RRT nach einem Standardchromatogramm unbedingt den Konzentrationsbereich bei Eichung und Analyse zu beachten, um qualitativ richtige Zuordnungen treffen zu können.

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Note

Fractionation of ^{32}P-labeled animal RNA on Sephadex–Sepharose columns

H. KUBINSKI
Division of Neurosurgery, University of Wisconsin, Madison, WI 53706 (U.S.A.)
(Received April 23rd, 1980)

Sepharose (agarose) is used for separation of various macromolecules including nucleic acids[1–3]. A mixture of macromolecules is fractionated in this gel according to molecule size. Thus, it was rather surprising to find that at least some small molecules, namely the low-molecular-weight phosphates, were not separated from RNA of high molecular weight, apparently due to the formation of complexes between ^{32}Pi and RNA in the presence of agarose. I report here on the observation that a Sepharose column topped with a layer of Sephadex satisfactorily fractionates the RNA and removes the inorganic phosphate. Moreover, such columns have several characteristics superior to those built with Sepharose alone.

MATERIALS AND METHODS

Materials

Sources of RNA: RNA was obtained either from Ehrlich ascites cells incubated with the radioactive precursor *in vitro* or from mouse livers after the animals were injected 90–120 min before sacrificing with 500 to 800 μCi of ^{32}P.

Ehrlich ascites tumor cells were collected from peritoneal cavities of one or several mice inoculated 7–10 days earlier with the Hamburg strain of this tumor[4]. The cells were washed once in a sterile Hanks solution (Microbiological Associates, Bethesda, MD, U.S.A.) and suspended in an Eagle's medium low in phosphate (Grand Island Biological Company, Grand Island, NY, U.S.A.). Radioactive phosphorus was added to the concentration of 20 μCi/ml and the cells were incubated at 37°C and constant pH as previously described[5]. After 30–120 min of incubation the cells were harvested, lysed, and RNA extracted with hot phenol[6,7]. RNA from livers was extracted in a similar manner, atfer the organs had been washed with saline and minced with scissors.

Several lots of Sephadex G-25 and Sepharose 2B were purchased from Pharmacia, Piscataway, NJ, U.S.A. over a period of almost 10 years. No appreciable differences between lots were observed.

Building of Sepharose and Sephadex–Sepharose columns; chromatography of RNA

Sepharose columns were prepared according to the specifications given by the manufacturer. The combined Sephadex–Sepharose columns were prepared as follows: in most cases, a commercial glass column was used. However, equally good

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results were achieved with a section of bottom-stoppered glass tubing. Several centimeters of Sepharose gel were built and the column material allowed to settle. Sephadex was then carefully added. In most cases, the total length of the Sephadex–Sepharose column bed was about 50 cm and the Sephadex layer occupied some 40% of this length. We have repeatedly noted that in order to achieve a satisfactory separation of various RNA fractions it was necessary to avoid mixing of the two gels when building the column: the borderline between the two should be uniformly sharp and even.

Both types of columns were then equilibrated with a sodium acetate–acetic acid buffer, pH 5.1[7]. Subsequently, we found that a better fractionation of RNA is achieved on Sephadex–Sepharose columns if the ionic strength of the buffer is increased to 0.6 *M* NaCl.

The purified RNA was layered on the top of the column, allowed to enter the column material and then eluted with the same buffer as the one used for equilibration. The effluent was monitored for the presence of UV-absorbing (Uvicord, LKB, Rockville, MD, U.S.A.) and radioactive materials (Ratemeter, Nuclear Chicago, presently Tracor Analytic, Elk Grove Village, IL, U.S.A.). The outputs from these two instruments were automatically recorded. Fractions collected during the experiment were subsequently individually assayed for the presence of ^{32}P and UV absorbing materials.

Base ratio analysis

RNA was precipitated and digested as described before[5,8]. The nucleotides were separated by high-voltage electrophoresis according to Click[9].

RESULTS AND DISCUSSION

RNA extracted from Ehrlich ascites cells or, in other experiments, from mouse livers, was applied to and eluted from a Sepharose 2B column (46 × 2.5 cm). The material that eluted in the void volume had levels of specific radioactivity significantly higher than those which could be expected on the basis of other experiments performed with the same material (*e.g.* chromatography on methyl esterified albumin[5,8,10]). Further experimentation revealed that only a very small percentage of this radioactivity, usually less than one percent, was precipitable by trichloroacetic acid (ACT). This apparent low molecular weight of the bulk of the radioactive material eluted in the void volume was confirmed by still another technique. The RNA was layered on the top of a 5–20% sucrose gradient, centrifuged for 2.5 hours at 4°C and at 45,000 rpm in a Spinco SW 56 rotor and the contents of the tubes collected from the bottom. Under these conditions, ribosomal 18 and 28S RNAs are recovered in the middle of the gradient. In contrast, most of the radioactive material present in our "void volume" RNA samples remained close to the top of the gradient. A substantial portion of this radioactivity diffused smoothly into the gradient. The large excess of the low-molecular-weight, highly contaminating radiophosphorus made subsequent analyses of this RNA material often difficult and unreliable (*e.g.* base composition analysis).

It was noted that removal of ^{32}Pi from the samples of crude RNA extract by pre-filtration on Sephadex G-25 prevented the formation of complexes between the

phosphates and the RNA. It became obvious that building a column composed of Sepharose topped with Sephadex may achieve the same purpose.

Fig. 1 shows the elution pattern of RNA chromatographed on such a combined Sephadex–Sepharose column. The first peak contained the high-molecular-weight DNA-like RNA which, during centrifugation in a sucrose density gradient, preceded both the ribosomal RNA bands (Table I). RNA with a base ratio and mobility in sucrose gradients characteristic for ribosomal RNA was found around fraction 28; the material between these two peaks had the base ratio intermediate between DNA-like and ribosomal. Its composition changed from one lot of RNA to another, depending on the conditions of incubation at the time of labeling with ^{32}P (ref. 5). The large peak of radioactivity between fractions 30 and 40 was for more than 99% not precipitable by TCA and presumably contained mostly inorganic phosphate. Its position in the eluate was independent from the other RNA peaks and was related to the length of the top layer of Sephadex: the deeper this layer was, the later the ^{32}Pi peak was recovered in relation to RNA fractions.

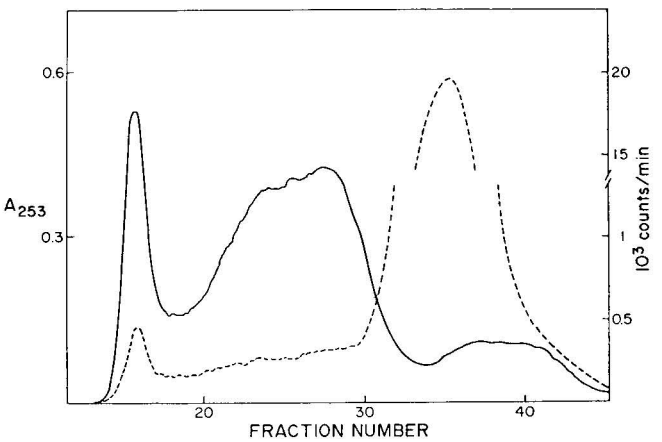

Fig. 1. Elution diagram of ^{32}P-labeled RNA from Ehrlich ascites tumor cells. The Sephadex–Sepharose columns (30 cm of Sepharose 2B, covered with 18 cm of Sephadex G-25, diameter: 2.5 cm) was loaded with approximately 2 mg of RNA and eluted with the 0.6 *M* NaCl solution buffered with pH 5.1 acetate buffer[7]. The effluent was monitored for the presence of UV-absorbing (continuous line) and radioactive materials (broken line) as described in the Materials and methods section. Fractions of 200 drops were collected and base ratio analysis was carried out on the material contained in some of these fractions (Table I).

TABLE I

BASE RATIO ANALYSIS OF RNA ELUTED IN THE VOID VOLUME AND AROUND THE "RIBOSOMAL RNA" PEAK (SEE FIG. 1)

C, A, G and U: cytidylic, adenylic, guanylic and uridylic acids, respectively.

Fractions	*C*	*A*	*G*	*U*
15–17	22.2	27.9	20.9	28.9
27–29	30.0	19.2	29.7	21.2

The Sephadex–Sepharose columns are useful for chromatography of other nucleic acids, including bacterial RNA and DNA from various sources (see, for example, ref. 11). Serum and other body fluids may also be fractionated. Such

columns are easy to build and to operate: they are resistant to the changes in column bed size and to shrinkage due to excessive pressure and/or flow-rate, normally seen with Sepharose. A column may be used repeatedly for several months and even years. The results are reproducible and meaningful comparisons can be made between analyses performed over extended periods of time. For the last 10 years, *i.e.* since we started using the combined Sephadex–Sepharose columns, our laboratory applies them to routine fractionation of cellular macromolecules such as nucleic acids and large proteins.

ACKNOWLEDGMENTS

I thank Dr. David Choie and Dr. Paul Sandok for help. This work was supported in part by U.S. Public Health grants CA-08959 and CA-16989; and by the Consultation Practice Plan of the University of Wisconsin Medical School.

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Note

Improved gas chromatography of amygdalin and its diastereomer

YUZI TAKAYAMA*
Materials Science, Toyohashi University of Technology, Tempaku-cho, Toyohashi 440 (Japan)
and
SATOSHI KAWAI
Gifu College of Pharmacy, Mitahora-Higashi, Gifu 502 (Japan)
(Received May 19th, 1980)

In recent years, amygdalin (D-mandelonitrile-β-D-gentiobioside) has become important in the treatment of cancer[1]. However, it is thought that its diastereomer, neoamygdalin (L-mandelonitrile-β-D-gentiobioisde), has no effect against cancer. Nahrstedt[2] attempted to separate isoamygdalin (a mixture of amygdalin and neoamygdalin) by gas chromatography with a packed column, but failed. The retention time was about 90 min, but the separation was not good enough to be used as a practical analytical method. In this work, we tried the separation on a capillary column using a high temperature and a high flow-rate.

EXPERIMENTAL

Reagent

Amygdalin, supplied by Merck (Darmstadt, G.F.R.), was used without further purification. Isoamygdalin was prepared from amygdalin and aqueous ammonia as described by Fischer[3]. Amygdalin was added to 10 ml of 0.005 *N* ammonia solution and the mixture was kept overnight at room temperature. During this period, D–L isomerization took place and equilibrium between amygdalin and neoamygdalin was attained. In this process, various impurities were formed, but we used the reaction product without further purification. Triphenylbenzene (TPB), supplied by Tokyo Kasei (Tokyo, Japan), was used as an internal standard. Trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were supplied by Kishida Chemical (Osaka, Japan). Pyridine, from commercial sources, was dried with potassium hydroxide pellets.

Trimethylsilylation

Trimethylsilylation of glycosides was carried out as described by Nahrstedt[2]. Glycoside (1–3 mg) and TPB (0.5–2 mg) were weighed accurately and dissolved in 0.7 ml pyridine in a vial. After adding 0.2 ml of HMDS and 0.1 ml of TMCS, the mixture was kept for 10 min at room temperature. The silylated glycosides were contained in the supernatant, of which a 1-μl aliquot was injected on to the gas chromatograph.

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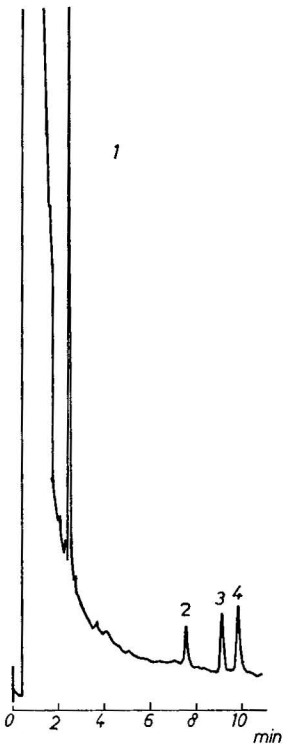
Gas chromatography

An Okura Model 103 gas chromatograph, equipped with a soda-glass capillary column and two hydrogen flame-ionization detectors, was used. The capillary column was prepared by Nippon Chromato (Tokyo, Japan) as described by Alexander and Rutten[4]. The operating conditions are given in Fig. 1.



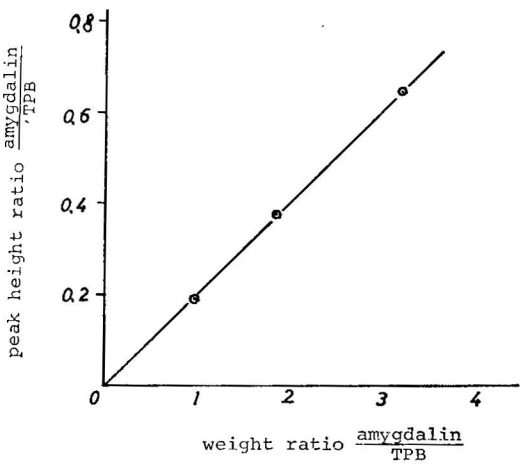


Fig. 1. Gas chromatogram of TMS-amygdalin and TMS-neoamygdalin, obtained from amygdalin. Soda-glass capillary column (10 m × 0.28 mm I.D.), treated with HCl vapour and coated with OV-1. Column temperature, 280°C isothermal. Inlet temperature, 350°C. Carrier gas (nitrogen) linear velocity, 27.8 cm/sec. Splitting ratio, 87.6. Injection volume, 1 μl. Peaks: 1 = TPB; 2 = unknown; 3 = TMS-neoamygdalin; 4 = TMS-amygdalin.

Fig. 2. Relationship between peak-height ratio and composition of mixtures of amygdalin and internal standard (TPB).

RESULTS AND DISCUSSION

Analysis of glycosides

Fig. 1 shows a chromatogram of TMS-isoamygdalin. The complete separation of TMS-amygdalin and TMS-neoamygdalin is achieved, in contrast to the unsatisfactory separation with a packed column. A considerable reduction in the retention time of TMS-amygdalin was achieved by keeping the column at 280°C and using a much higher flow-rate of the carrier gas than usual (about 30 cm/sec). These operating conditions decrease the separation efficiency, but not seriously.

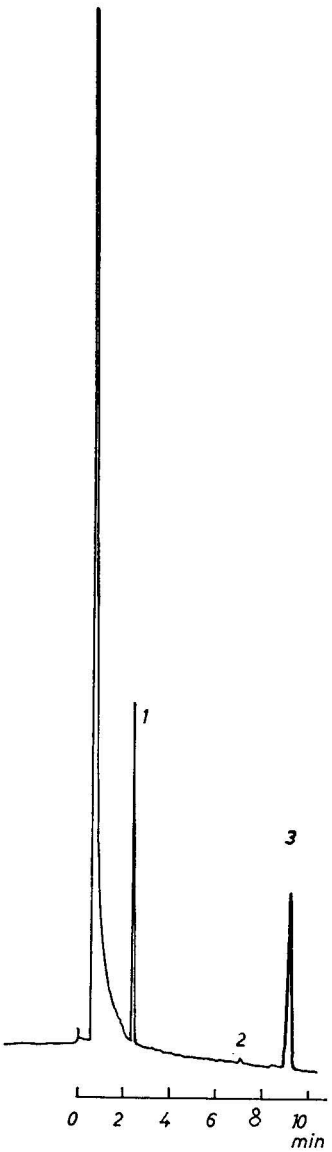


Fig. 3. Gas chromatogram of TMS-amygdalin obtained from commercial tablets containing amygdalin. Conditions and peak numbers as in Fig. 1.

TABLE I

ANALYSIS OF COMMERCIAL TABLETS AND A SOLUTION CONTAINING AMYGDALIN

Parameter	*Tablets*	*Solution*
Nominal content (mg/tablet)	325	—
Average weight (mg/tablet)	380.5	Solid content 3.19 g per 20 ml
Calculated amygdalin content (%)	84.5	—
Observed amygdalin content (%)	85.7	*ca.* 30% in solid
Observed neoamygdalin	Trace	Comparable to amygdalin

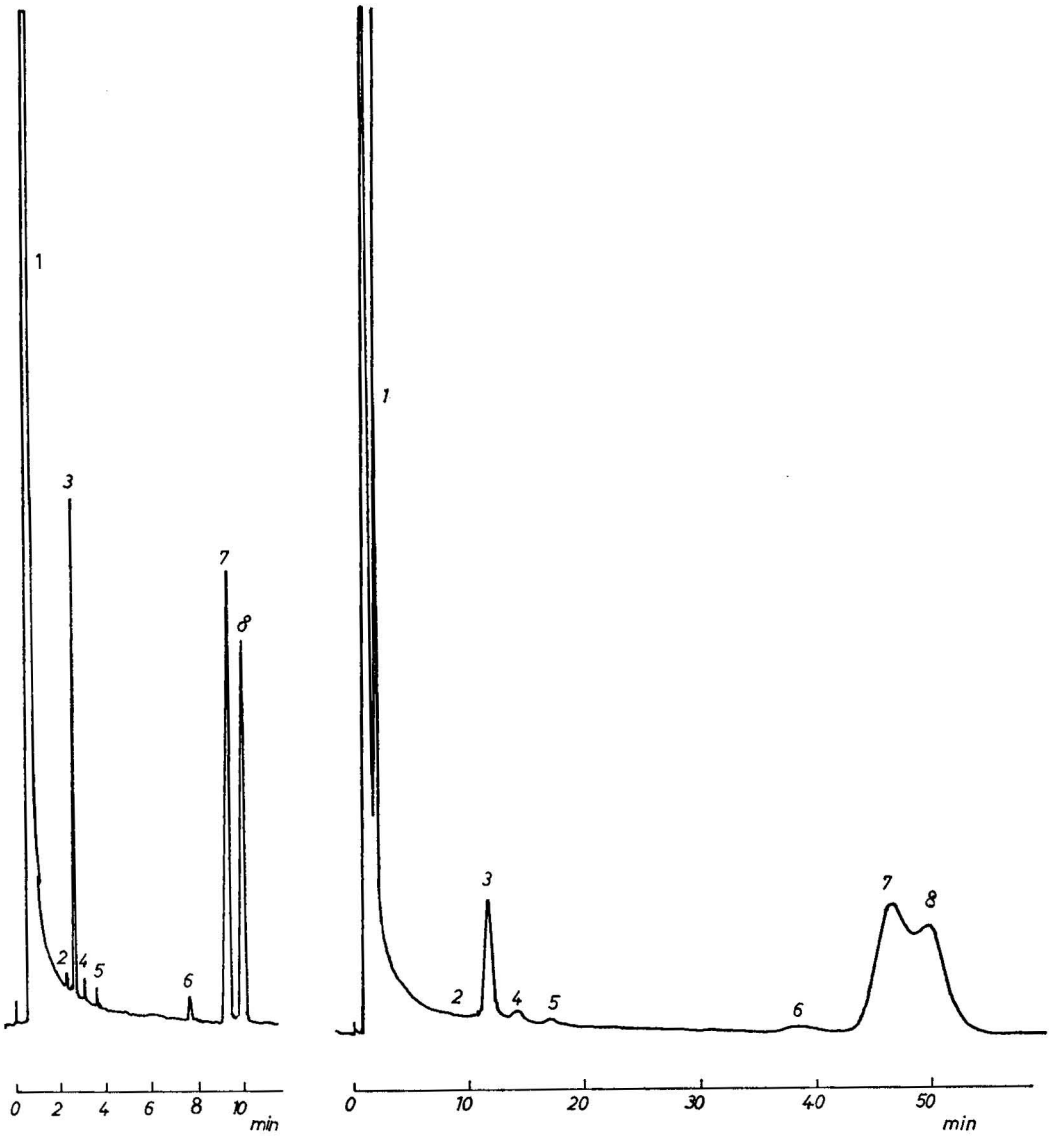


Fig. 4. Gas chromatogram of TMS-amygdalin and other compounds from commercial solution containing amygdalin. Conditions as in Fig. 1. Peaks: 1, 2, 4, 5 and 6 = unknown; 3 = TBP; 7 = TMS-neoamygdalin; 8 = TMS-amygdalin.

Fig. 5. Gas chromatogram of TMS-amygdalin and other compounds obtained from commercial solution containing amygdalin. Stainless-steel packed column (3 m × 3 mm I.D.), 3% OV-1 on silylated Celite (80–100 mesh). Column temperature, 280°C isothermal. Inlet temperature, 350°C. Carrier gas (nitrogen) flow-rate, 25 ml/sec. Injection volume, 1 μl. Peak numbers as in Fig. 4.

A calibration graph for amygdalin in shown in Fig. 2; the linearity is satisfactory.

In order to check the method, commercial tablets with a well defined composition were analysed (Fig. 3). The results are given in Table I.

Subsequently, a commercial aqueous solution of amygdalin without a specified amygdalin content was analysed, and the results are given in Fig. 4 and Table I. In this instance, the aqueous solution was evaporated to dryness. In order to verify that the evaporation process does not change the composition, a tablet was dissolved in water, the solution was evaporated to dryness and the composition of the residue was analysed. The composition was identical with that of the original tablet.

Comparison of capillary and packed columns

A packed column was prepared according to the literature[2], but replacing Chromosorb AW DMCS with Celite silylated with HMDS vapour[5] as the support, and increasing the flow-rate from 30 to 40 ml/min. The chromatogram obtained was similar to that described in the literature[2]. The packed column was then compared with the capillary column, the temperature of the former being kept at 280°C isothermal (Fig. 5).

The results are given in Table II, and indicate that the capillary column is far superior to the packed column.

TABLE II

COMPARISON OF GAS CHROMATOGRAMS

The data were obtained from Figs. 4 and 5.

Parameter	*Capillary column*	*Packed column*
Resolution, R_S	2.3	0.6
Adjusted retention time of TMS-amygdalin, t_R (min)	10	50
Separation factor, α	1.07	1.07
Number of effective theoretical plates of TMS-amygdalin, N_{eff}	1560×10	430×3
Shape for small amounts of components	Sharp, easily detected peaks	Broad, readily missed peaks

Reduction of measurement time

The value of resolution (2.3) given in Table II is unnecessarily large. By reducing it to 1.5, the measurement time, which is nearly equal to the adjusted retention time of TMS-amygdalin, is to be reduced.

The retention time, t_R, is given by the equations[6]

$$R_S = \frac{1}{4}\left(1 - \frac{1}{\alpha}\right) N_{eff}^{1/2}$$

and

$$t_R' = \frac{N_{eff}}{1560} \cdot \frac{t_{R10}'}{10}$$

where R_S is the mean resolution, N_{eff} the number of effective theoretical plates, $N_{eff}/1560$ the column length (L) necessary for obtaining a certain R_S value, α the separation factor and t'_{R10} the adjusted retention time in a capillary column of length 10 m. Then, if we put $R_S = 1.5$, we obtain $N_{eff} = 7785$, $L = 5$ m and $t'_R = 5$ min.

After using the capillary column for 3 months, no decrease in the efficiency of the capillary column or shift in retention time was observed, indicating the practical applicability of the method.

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Note

Liquid chromatography of amitriptyline and related tricyclic compounds

SHELDON H. PRESKORN*, KELLY LEONARD and CHARLES HIGNITE

Departments of Psychiatry and Pharmacology, University of Kansas School of Medicine, Kansas City, KS 66103 (U.S.A.)

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Tricyclic antidepressants are widely prescribed. However, the large variability in the elimination rates for these drugs between individuals hampers their use. The differences in these rates can be as high as 30–40 fold; thus, patients on the same dose can have steady-state levels varying from 25 ng/ml to 1000 ng/ml[1,2]. While the former is therapeutically ineffectual, the latter can be seriously toxic. Thus, measurement of drug concentration in a readily available compartment such as plasma permits rational dosage adjustment.

Mellström and co-workers[3,4] have reported on the use of ion-pair partition chromatography to measure these drugs. Although their system appears to be adequate, modification presented in this report resulted in an improved system with several advantages. First, the solvent contains only two components simplifying solvent preparation and enhancing daily reproducibility. Second, the diisopropyl ether is not used, thus making the solvent less hazardous for routine use. Moreover, trials of their system showed that several different brands of diisopropyl ether contained substances which interfered at 254 nm and therefore appreciably reduced the assay's sensitivity. Finally, this assay achieves good separation in less than 15 min at lower pump pressure thus extending longevity of the system. This assay has been used to quantitate amitriptyline and its metabolites as well as the other commercially available tricyclic antidepressants.

EXPERIMENTAL

Apparatus

The chromatograph from Waters Assoc. (Milford, MA, U.S.A.) consisted of a U6K injector, a Model 6000A solvent delivery system, and a Model 440 absorbance detector operated at 254 nm. The stainless steel separation column (300 × 3.9 mm I.D.) was packed with μBondapak C_{18} (Waters Assoc.).

Chromatographic system

The mobile phase consisted of acetonitrile and a perchlorate solution at pH 2.5 (44:56). The perchlorate solution was prepared by mixing 0.005 *M* perchloric acid with 0.045 *M* sodium perchlorate (9:1). The solvent was degassed by filtering through organic:aqueous filter paper (Millipore FH UP 04700) primed with methanol. The flow-rate was 1.0 ml/min.

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Chemicals and materials

The acetonitrile used was Baker HPLC grade. The water used in making the perchlorate solution was HPLC grade. All other chemicals were analytical-reagent grade. The internal standard, 2-(dibenz[b^5]azepin-5-yl)-N-methylethylamine, obtained from Ciba-Geigy (Basel, Switzerland), was used as a 1 μg/ml solution. Standard solutions of amitriptyline, nortriptyline, and the internal standard were prepared in 0.005 *M* sulfuric acid.

Analytical method

Plasma was separated from the heparinized whole blood by centrifugation at 2000 rpm for 20 min. A 3.0-ml sample was spiked with 100 μl of the internal standard solution. A double extraction was then performed. First, the drugs were extracted into 4 ml of hexane–isoamyl alcohol (99:1) by alkalinization using 200 μl of 1.5 *M* sodium hydroxide and gentle shaking for 60 min. The organic phase was separated from the plasma by centrifugation. The drugs were then back-extracted and concentrated by addition of 200 μl of 0.1 *M* perchloric acid and shaking for 20 min. The organic phase was discarded. A variable quantity of the aqueous phase, usually 50 μl, was injected onto the column. Larger volumes can be delivered for dilute samples without altering peak resolution.

Standard curves using five different concentrations of the drugs to be measured were determined daily immediately preceding experimental sample analysis. These standards were prepared by the addition of known amounts of the tricyclics to drug-free plasma. These samples were analyzed using the method described, and the ratio of peak height of drug to peak height of internal standard was plotted *versus* the drug concentration in the spiked samples. The resulting standard curves were then used for quantitation of the clinical samples. For clinical assays, the concentrations used were: 50, 100, 150, 200 and 250 ng/ml. The therapeutic range for amitriptyline is 150–250 ng/ml.

RESULTS AND DISCUSSION

The theory underlying ion-pair chromatography is available in the literature[4,5]. However, this procedure, which utilizes perchlorate as the counter ion, has several advantages over earlier methods. The solvent contains only acetonitrile and the perchlorate solution, making it less complex and less hazardous than previous solvents requiring diisopropyl ether[4]. Furthermore, several brands of diisopropyl ether contained substances which absorbed light at 254 nm thus reducing assay sensitivity. Unlike the method described by Kraak and Bijster[6], the mobile phase is acidic thus extending column longevity. The same column, used only for this assay, has been in service for over six months without loss of resolving capacity. The peaks of even the most retained tricyclic compounds remain symmetrical and return to baseline.

The resolving capacity of this method permitted quantification of amitriptyline and its metabolites as well as other commercially available tricyclic antidepressants (Figs. 1 and 2). The assay time was less than 15 min for all of these drugs. No interfering substances were detected in blank human or laboraroty rat plasma samples. Chromatograms of amitriptyline and its metabolites from a patient within the therapeutic range and from an overdosed patient are presented in Fig. 3.

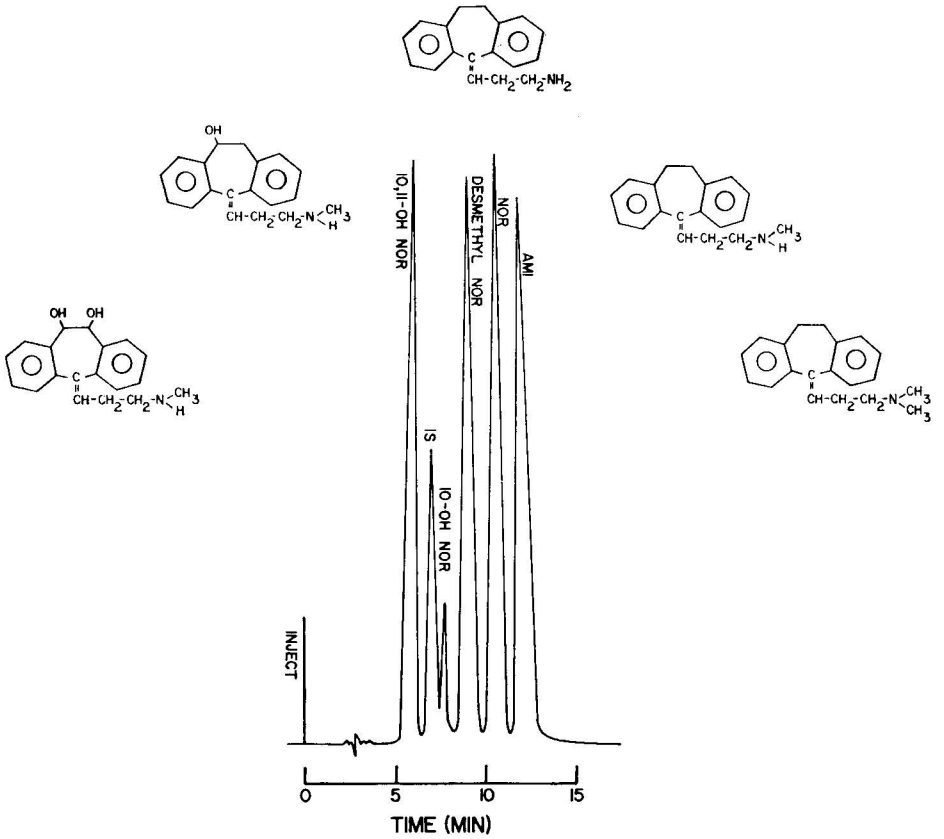

Fig. 1. Separation of amitriptyline and its metabolites. Mobile phase: 0.01 *M* perchlorate solution (pH 2.5)–acetonitrile (56:44); flow-rate 1.0 ml/min. From left to right, the chemical structure and corresponding peak (structure of internal standard, not illustrated). NOR = nortriptyline, AMI = amitriptyline. 10-hydroxyamitriptyline was not available for testing.

The reproducibility of the method for clinical purposes was assessed by correlating results from the measurement of 35 duplicate human plasma samples spiked with known amounts of drug to obtain concentrations from 5 to 400 ng/ml ($r = 0.9887$ for nortriptyline; $r = 0.9984$ for amitriptyline). Drug recovery exceeded 80% over this concentration range. The within-run variation for five determinations of the same plasma sample spiked with 100 ng/ml of drug was 100 $\pm$ 1.3 S.E. for nortriptyline and 100 $\pm$ 2.6 S.E. for amitriptyline. Nine consecutive daily assays of five plasma samples (range: 50–250 ng/ml) had a coefficient of variation of 6.4% for nortriptyline and 7.7% for amitriptyline. The correlation coefficient for these standard curves were 0.9969 for nortriptyline and 0.9967 for amitriptyline.

Similar reproducibility results were obtained for samples from laboratory animals. This method has been used to quantitate amitriptyline in brain, heart, and liver tissue samples from laboratory animals following intraperitoneal drug administration[7]. The sensitivity of the method ($<$ 5 ng/ml) has permitted repeat determination in laboratory mice on plasma samples as small as 100 μl.

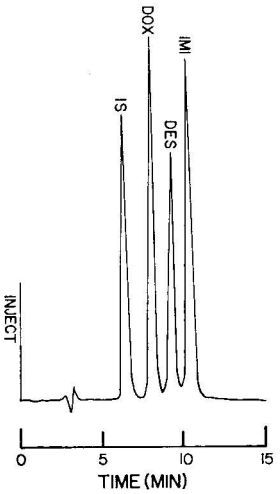

Fig. 2. Separation of other tricyclic antidepressants. Same conditions as in Fig. 1. IMI = imipramine, DOX = doxepin, DES = desipramine, IS = internal standard.

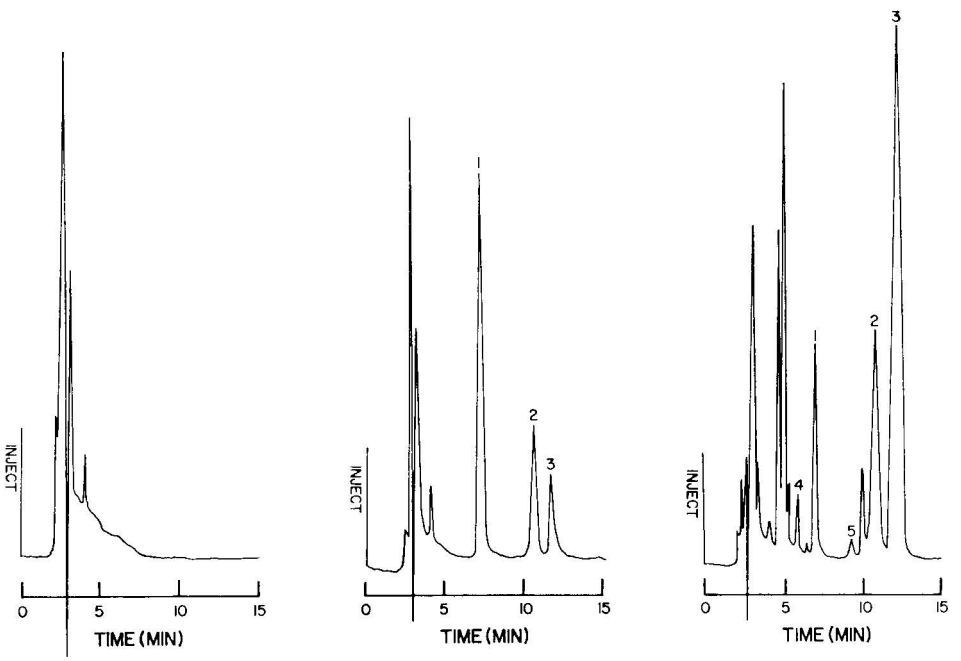

Fig. 3. Chromatograms from human plasma samples. Same conditions as in Fig. 1. From left to right, a blank sample, a sample in the therapeutic range, and a sample in the toxic range. 1 = internal standard, 2 = nortriptyline, 3 = amitriptyline, 4 = desmethylnortriptyline and 5 = 10,11-dihydroxynortriptyline.

Although desipramine and protriptyline elute simultaneously (Fig. 2), such interference should not represent a limitation for either clinical or experimental assays, since these drugs are rarely used concomitantly. Doxepin can be substituted for the internal standard used in this study, if the latter is unavailable. Of a variety of commonly used medications, only diazepam elutes near the tricyclic antidepressants (Table I).

TABLE I

RELATIVE RETENTION (K') FOR TRICYCLIC AND OTHER DRUGS ASSAYED BY THIS METHOD

$K' = (V_1 - V_0)/V_0$, V_1 = retention time of compound, V_0 = void volume retention time.

Drug	K'	*Drug*	K'
Tricyclic antidepressants		*Cardiac drugs*	
Amitriptyline	2.93	Propranolol	1.00
Nortriptyline	2.53	Quinidine	0.33
Doxepin	1.73	Procainamide	0.20
Protriptyline	2.07	*Anticonvulsants*	
Desipramine	2.07	Phenobarbital	0.67
Imipramine	2.47	Carbamizapine	1.07
Neuroleptics		*Analgesics and others*	
Chlorpromazine	3.87	Aspirin	0.60
Mesoridazine	1.20	Acetaminophen	0.07
Fluphenazine	2.33	Codeine	0.20
Benzodiazepines		Theophylline	1.20
Diazepam	2.40		
Flurazepam	1.47		
Oxazepam	1.20		

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Note

Separation behaviour of some isomeric organic compounds on sugars, sugar alcohols and their mixed phases by gas–liquid chromatography

AOI ONO*

Chemical Laboratory, Takada Branch, Faculty of Education, Niigata University, Nishishiro-cho, Joetsu-shi 943 (Japan)

and

YOSHIO MASUDA

General Education Department, Niigata University, Niigata 950-21 (Japan)

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Although Kreicberger *et al.*[1] and Novrocík *et al.*[2] have reported the separation of 3- and 4-nitro-*o*-xylenes (2,3- and 3,4-dimethylnitrobenzenes) by gas–liquid chromatography (GLC), there have been few systematic separations of dimethylnitrobenzene isomers. Further, sugar alcohols were found to be effective liquid stationary phases for separating xylenol (dimethylphenol) isomers[3] and separations of nitroxylene isomers were performed on some sugar alcohols.

Fitzgerald[4], Brooks[5], Kolšek and Matičíc[6], Sassenberg and Wrabetz[7,8], Karr *et al.*[9], Jamieson[10], Mortimer and Gent[11], Janák and Komers[12], Payan[13], Bhattacharjee and Basu[14], Husain *et al.*[15] and Hrivnák and co-workers[16,17] have described separations of alkylphenol, cresol and dimethylphenol isomers by GLC. Brooks[5] separated all the dimethylphenol isomers on 2,4-xylenyl phosphate, Kolšek and Matičič[6] separated them on di-(3,3,5-trimethylcyclohexyl) phthalate, Janák and Komers[12] on salicylideneaminoguanidine and Bhattacharjee and Basu[14] on rubidium benzenesulphonate (clathrate-forming stationary phases), and Mortimer and Gent[11] separated *m*- and *p*-cresols on Bentone-34 and tolyl phosphate.

In spite of these studies, systematic studies of the separation of the dimethylphenol isomers have never been reported. 2,4,7-Trinitro-9-fluorenone[18], sugar alcohols and mixed columns of 2-nitrofluorene and dulcitol or inositol[3] have been shown to be effective liquid stationary phases. For the above reasons, the separation behaviour of nitroxylene and xylenol isomers on sugars, sugar alcohols and mixed columns has been investigated.

EXPERIMENTAL

Apparatus

A Shimadzu Model GC-5A gas chromatograph equipped with a flame-ionization detector was used.

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Chromatographic procedure

The separation column was a 2.25 m × 3 mm I.D. stainless-steel U-tube packed with acid-washed Sil-O-Cel C_{22} firebrick (60–80 mesh) support (Johns-Manville, Denver, CO, U.S.A.; purchased from Gaskuro Kogyo, Tokyo, Japan) coated with 20% (w/w) of liquid. The column and injector temperature were 140 and 270°C, respectively. Nitrogen was used as the carrier gas at 25 ml/min.

The support coated with the liquid phase was heated in an electric oven at the column temperature for 10 h in order to remove the solvent; subsequently it was kept under a stream of nitrogen for 6 h at a temperature *ca.* 20°C higher than the column temperature used.

Liquid stationary phases

Dioctyl phthalate (DOP), dulcitol (DUL), inositol (INO), sorbitol (SOR), mannitol (MAN), xylitol (XYT), glucose (GLU), mannose (MAS), sucrose (SUR), lactose (LAC), fructose (FRU), maltose (MAL), xylose (XYL), starch (soluble) (STA), Span-85 (SPA-85), diglycerol (DIL), galactose (GAL) and sorbose (SORB) of guaranteed grade from Nakarai Chemical Co. (Kyoto, Japan) were used without any purification.

2-Nitrofluorene (2-NF)[19,20], 2-nitro-9-fluorenone (2-NFO)[19–21], 2,7-dinitrofluorene (2,7-DNF)[21–23], 2,7-dinitro-9-fluorenone (2,7-DNFO)[21–23], 2,5-dinitro-9-fluorenone (2,5-DNFO)[21–23], 2,4,7-trinitro-9-fluorenone (2,4,7-TNFO)[24–26] and 2,4,5,7-tetranitro-9-fluorenone (2,4,5,7-TNFO)[25,26] were synthesized in the laboratory and their purities were confirmed by TLC and IR spectrometry.

Samples

3,5-Dimethylnitrobenzene (3,5-DMNB)[27], 2,5-dimethylnitrobenzene (2,5-DMNB)[28], 2,3-dimethylnitrobenzene (2,3-DMNB)[29], 3,4-dimethylnitrobenzene (3,4-DMNB)[30], 2,6-dimethylnitrobenzene (2,6-DMNB)[31], 2,4-dimethylnitrobenzene (2,4-DMNB)[31], 2,3-dimethylphenol (2,3-DMP)[32], 2,6-dimethylphenol (2,6-DMP)[33], 3,4-dimethylphenol (3,4-DMP)[34], 2,4-dimethylphenol (2,4-DMP)[35], 2,5-dimethylphenol (2,5-DMP)[36] and 3,5-dimethylphenol (3,5-DMP)[37] were synthesized and their purities were confirmed by GLC and IR and NMR spectrometry.

RESULTS AND DISCUSSION

Separation of DMNB isomers

In the separation of DMP isomers, it was found that hydrogen bonding plays an important role. It was expected that hydrogen bonding would also be effective in separating DMNB isomers, because the nitro function of DMNB isomers will interact with the hydroxy function of the stationary phase.

The results of the separation of DMNB isomers on nitrofluorenes, nitro-9-fluorenones, sugars, sugar alcohols, polyols and mixed columns are given in Tables I–III. From Table I it can be seen that 2-NF, 2-NFO, 2,5-DNFO, 2,7-DNF, 2,7-DNFO, 2,4,7-TNFO and 2,4,5,7-TNFO resolved effectively all six isomers. In these separations, effective separations were performed at a temperature below the melting points of the liquid stationary phases[38–41].

Norman[42] reported the separation of nitrotoluene isomers by GLC on 2,4,7-

TABLE I

RELATIVE RETENTION TIMES IN THE SEPARATIONS OF DMNB ISOMERS USING NITROFLUORENES AND NITROFLUORENONES

Stationary phase	*2,6-DMNB*	*2,5-DMNB*	*2,3-DMNB*	*2,4-DMNB*	*3,5-DMNB*	*3,4-DMNB*
2-NF	1.00	1.61	1.69	2.10	2.47	3.56
2-NFO	1.00	1.73	1.91	2.21	2.83	3.49
2,5-DNFO	1.00	1.81	1.97	2.29	2.97	3.70
2,7-DNF	1.00	1.70	1.89	2.27	2.63	3.52
2,7-DNFO	1.00	1.75	1.92	2.31	2.71	2.67
2,4,7-TNFO	1.00	1.93	2.13	2.46	2.86	4.06
2,4,5,7-TNFO	1.00	1.81	2.02	2.59	2.89	4.08
2,4,7-TNFO + 2,4,5,7-TNFO	1.00	1.87	2.25	2.25	2.58	3.88

TABLE II

RELATIVE RETENTION TIMES IN THE SEPARATIONS OF DMNB ISOMERS ON MIXED COLUMNS

Stationary phase	*2,6-DMNB*	*2,5-DMNB*	*2,3-DMNB*	*2,4-DMNB*	*3,5-DMNB*	*3,4-DMNB*
2-NF + DOP	1.00	1.75	2.06	2.06	2.33	2.27
2-NF + DUL	1.00	1.91	2.27	2.27	2.59	3.95
2-NF + INO	1.00	1.79	2.16	2.16	2.16	3.53
2-NF + SOR	1.00	1.81	2.17	2.30	2.30	3.61
2-NF + MAN	1.00	1.91	2.26	2.26	2.49	4.09
2-NF + XYT	1.00	1.80	2.00	2.25	2.80	3.95
2-NFO + DUL	1.00	1.86	2.20	2.23	2.59	5.77
2-NFO + MAN	1.00	2.00	2.29	2.29	2.82	4.24
2-NFO + SOR	1.00	1.81	2.13	2.38	2.75	4.19

TNFO at 200°C, but they were resolved effectively at 140°C below its melting point[43].

From Table II, it can also be seen that the mixed column of 2-NF + XYT gave effective separations of all six isomers. Further, the mixed columns of 2-NF and sugar alcohols showed various kinds of separation modes and the mixed columns of 2-NFO and DUL or SOR separated all of the isomers. From Table III, it can be seen that sugars (mono-, di- and polysaccharides) gave effective resolutions of all

TABLE III

RELATIVE RETENTION TIMES IN THE SEPARATIONS OF DMNB ISOMERS USING SUGARS

Stationary phase	*2,6-DMNB*	*2,5-DMNB*	*2,3-DMNB*	*2,4-DMNB*	*3,5-DMNB*	*3,4-DMNB*
GLU	1.00	1.66	1.89	2.06	2.44	3.17
MAS	1.00	1.76	1.95	2.19	2.57	3.71
SUR	1.00	1.59	1.82	1.94	2.24	3.00
FRU	1.00	1.65	1.85	2.05	2.40	3.30
MAL	1.00	1.73	1.93	2.13	2.40	3.40
LAC	1.00	1.75	2.00	2.17	2.50	3.25
STA	1.00	1.59	1.73	2.05	2.35	3.15

TABLE IV

RELATIVE RETENTION TIMES IN THE SEPARATIONS OF DMNB ISOMERS USING SUGAR ALCOHOLS AND POLYOLS

Stationary phase	*2,6-DMNB*	*2,5-DMNB*	*2,3-DMNB*	*2,4-DMNB*	*3,5-DMNB*	*3,4-DMNB*
XYT	1.00	1.91	2.27	2.36	2.64	3.82
MAN	1.00	1.77	1.91	2.33	2.67	3.60
SOR	1.00	1.72	2.11	2.11	2.33	3.56
DUL	1.00	1.70	2.00	2.20	2.50	3.40
INO	1.00	1.37	2.94	2.94	3.15	4.42
INO + SOR	1.00	1.86	2.29	2.29	2.29	3.86
DUL + SOR	1.00	1.90	1.90	2.57	3.15	3.93
INO + DUL	1.00	1.32	1.79	2.00	2.32	3.05
DUL + XYT	1.00	1.94	2.50	2.50	2.50	4.28
SPA-85	1.00	1.65	1.65	2.17	2.36	2.91
DIL	1.00	1.78	2.25	2.25	2.25	3.56

six isomers with almost identical relative peak separations[44]. It seems that hydrogen bonding of the hydroxy group of the stationary phase with the nitro group of the sample plays an important role.

From Table IV, in can be seen that MAN, DUL and XYT separated all of the isomers. Of the mixed columns, only INO + DUL separated all of the isomers. DIL and SPA-85 were ineffective and DUL and XYT are of specific configuration (sugar alcohols). Further, PEG 1540 (polyether) and EGA (polyester) gave effective separations[18].

Separation of DMP isomers

Table V shows that although none of the mixed columns of 2-NF and sugar alcohols or polyols gave good separations of all of the DMP isomers, the mixed columns of 2-NFO and sugar alcohols or polyols separated the isomers effectively. The mixed columns of 2-NF and DUL, INO or XYT separated all of the isomers but DUL, XYT and INO are of specific configuration (sugar alcohols); hence the carbonyl group of 2-NFO seems to be effective in these separations.

TABLE V

RELATIVE RETENTION TIMES OF DMP ISOMERS ON MIXED COLUMNS

Stationary phase	*2,6-DMP*	*2,5-DMP*	*2,4-DMP*	*2,3-DMP*	*3,5-DMP*	*3,4-DMP*
2-NF + SOR	1.00	1.73	1.73	2.26	2.52	4.91
2-NF + MAN	1.00	1.94	1.94	2.34	3.08	3.86
2-NF + DUL	1.00	1.95	2.05	2.64	3.32	4.23
2-NF + INO	1.00	1.91	2.14	2.41	3.65	4.28
2-NF + XYT	1.00	1.80	1.90	2.20	3.00	3.55
2-NFO + DUL	1.00	1.97	2.09	2.68	3.34	4.28
2-NFO + MAN	1.00	1.89	2.04	2.61	3.36	4.18
2-NFO + SOR	1.00	1.92	2.00	2.56	3.24	4.12
2-NFO + INO	1.00	1.71	1.93	2.14	2.54	3.43
2-NFO + XYT	1.00	1.97	2.09	2.67	3.36	4.33

TABLE VI

RELATIVE RETENTION TIMES OF DMP ISOMERS ON SUGAR ALCOHOLS AND POLYOLS

Stationary phase	*2,6-DMP*	*2,5-DMP*	*2,4-DMP*	*2,3-DMP*	*3,5-DMP*	*3,4-DMP*
DUL	1.00	2.13	2.38	2.63	4.13	4.62
SOR	1.00	2.05	2.19	2.74	3.69	4.62
MAN	1.00	2.20	2.53	2.95	4.37	5.00
INO	1.00	2.00	2.23	2.23	4.03	4.29
XYT	1.00	2.24	2.38	2.86	4.09	4.95
DIL	1.00	1.72	1.72	2.23	2.60	3.16
DUL + SOR	1.00	1.90	1.90	2.57	3.15	3.93
INO + DUL	1.00	2.09	2.09	2.82	3.56	4.78
INO + SOR	1.00	2.27	2.27	2.73	3.82	4.91
DUL + XYT	1.00	2.03	2.03	2.68	3.38	4.35
INO + MAN	1.00	2.05	2.15	2.45	3.60	4.15

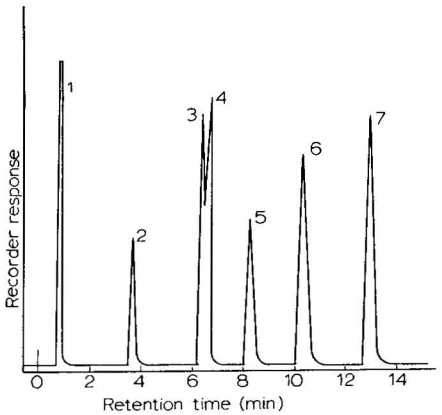
Table VI indicates that all of the sugar alcohols except INO separated all of the isomers. DIL did not give effective separations. INO is a different polyol from DUL and the others used, and DIL is a tetrahydric polyol, but not with vicinal tetrahydroxy groups. Further, the mixed columns of sugar alcohols except for INO and MAN did not give useful separations. Table VII shows that SUC, FRU, MAS, GLU, GAL, MAL, LAC and SORB separated all of the isomers effectively.

Although STA is a polyol, it could not separate 2,3-DMP from 2,4-DMP. The mixed columns of two kinds of sugars separated all of the isomers effectively. Although the retention times of DMP isomers on sugars or sugar alcohols are too short, an improvement was obtained on the mixed columns of 2-NF and INO or DUL and of 2-NFO and DUL or MAN. The improved chromatograms obtained on the mixed liquid stationary phases (2-NFO and DUL or MAN) are shown in Figs. 1 and 2.

TABLE VII

RELATIVE RETENTION TIMES OF DMP ISOMERS ON SUGARS AND POLYOLS

Stationary phase	*2,6-DMP*	*2,5-DMP*	*2,4-DMP*	*2,3-DMP*	*3,5-DMP*	*3,4-DMP*
SUC	1.00	2.13	2.31	2.69	4.00	4.62
FRU	1.00	1.63	1.73	1.97	2.63	2.97
MANS	1.00	1.96	2.24	2.50	2.74	4.33
GAL	1.00	1.94	2.18	2.47	3.65	4.12
GLU	1.00	1.79	2.05	2.32	3.36	3.79
MAL	1.00	1.80	2.00	2.33	3.20	3.66
LAC	1.00	2.13	2.44	2.63	4.00	4.38
SORB	1.00	1.62	1.85	1.92	2.31	3.31
STA	1.00	1.86	2.04	2.04	3.51	3.89
GLU + GAL	1.00	1.68	1.84	2.11	3.11	3.63
MAL + FRU	1.00	2.00	2.25	2.50	3.50	4.00
FRU + GAL	1.00	1.65	2.25	2.50	3.50	4.00
MAL + GLU	1.00	2.13	2.50	2.63	3.00	4.00
GAL + MAS	1.00	1.94	2.12	2.41	3.47	3.88
SPA-85	1.00	1.65	1.65	2.17	2.36	2.91



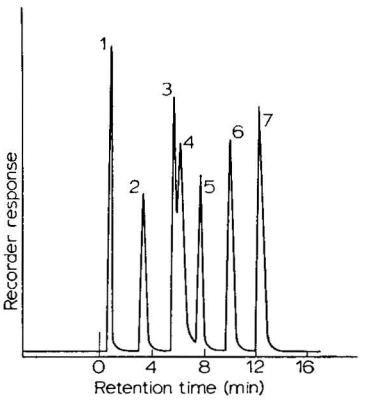


Fig. 1. Separation of DMP isomers on 2-NFO + DUL. Peaks: 1 = solvent; 2 = 2,6-DMP; 3 = 2,5-DMP; 4 = 2,4-DMP; 5 = 2,3-DMP; 6 = 3,5-DMP; 7 = 3,4-DMP.

Fig. 2. Separation of DMP isomers on 2-NFO + MAN. Peaks: 1 = solvent; 2 = 2,6-DMP; 3 = 2,5-DMP; 4 = 2,4-DMP; 5 = 2,3-DMP; 6 = 3,5-DMP; 7 = 3,4-DMP.

In the separation of DMP isomers on the mixed columns of 2-NF and INO and of 2-NF (or 2-NFO) and sugar alcohols, the properties of the component stationary phases are additive. The functional group combined with sugars or sugar alcohols for the improvement of retention is generally preferable, if it can separate the isomers individually.

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Note

Gas chromatographic–mass spectrometric analysis of polybrominated biphenyl constituents of Firemaster FF-1

EDWARD F. DOMINO* and STEVEN E. DOMINO

Department of Pharmacology, M6414 Medical Science Building I, Box 035, The University of Michigan, Ann Arbor, MI 48109 (U.S.A.)

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Sundstrom *et al.*[1], De Kok *et al.*[2], Jacobs *et al.*[3], Dannan *et al.*[4] and Willett *et al.*[5] have all reported on the chromatographic characteristics of polybrominated biphenyls (PBBs); surprisingly little has been reported on the gas chromatographic–mass spectrometric (GC–MS) characteristics of the PBBs. Firemaster FF-1 was the principal contaminant in the food chain in the State of Michigan in 1973–1974[6], and it has commonly been assumed that the PBBs alone were the primary toxic agents. We have had an opportunity to examine the GC–MS characteristics of a hexane extract of Firemaster FF-1 and have observed mixed polybromo- and chlorobiphenyls (PBCBs) as minor contaminants in addition to the well-known PBBs.

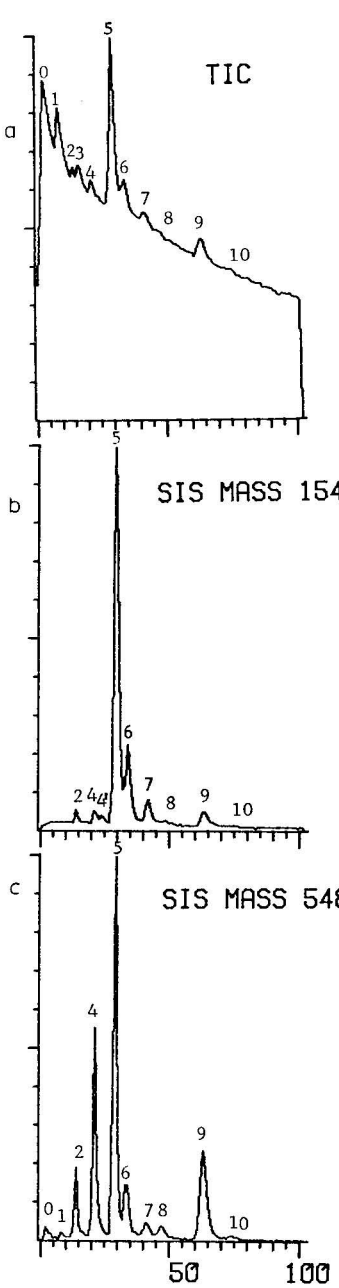
MATERIALS AND METHODS

Firemaster FF-1, lot No. FF1312-FT, was obtained from the National Institute of Environmental Health Sciences. A stock solution (1 mg/ml) in hexane was diluted for subsequent GC–MS analysis. A Finnigan 3200 gas chromatograph–quadrupole mass spectrometer with a 6100 data system was utilized in these studies. The GC–MS conditions were a column of 3% of SE-30 on Gas-Chrom Q (100–120 mesh) operated with a column temperature of 180°C, port and separator temperature 220°C, analyzer temperature 50°C and 100% helium (20 ml/min) as the carrier gas. The electron energy was 70 eV, and the mass spectrometer was calibrated by using perfluorotributylamine (FC43) from 34 to 614 *m/e*. The manufacturer[7] states that only masses up to 800 a.m.u. are accurately calibrated using FC43. Inasmuch as the heptabromobiphenyls (HpBBs) have a molecular weight of 706.3, this was deemed adequate for determining the major components of PBBs. Next, the high end of the mass spectrum was checked using bis(pentafluorophenyl)phenylphosphine, better known as decafluorotriphenylphosphine (DFTPP, Ultramark 433) as recommended by Eichelberger *et al.*[8].

RESULTS AND DISCUSSION

The total ion chromatogram (TIC) of 1.5 μg of Firemaster FF-1 in hexane is illustrated in Fig. 1a. Peaks were labelled 0 through 10 and their retention times were

0021-9673/80/0000–0000/$02.25



Peak Number	Retention Time (min)	Scan Max	Scan Background
0	0.66	2	1
1	1.46	8	7
2	2.26	14	13
3	2.53	16	15
4	3.19	21	20
5	4.25	29	27
6	4.92	34	32
7	5.98	42	40
8	not clear	-	-
9	8.77	63	61
10	not clear	-	-

0	not present		
1	not present		
2	2.26	14	13
3	not present		
4	3.19	21	20
4'	3.59	24	23
5	4.25	29	27
6	4.92	34	32
7	5.98	42	40
8	6.78	48	46
9	8.77	63	61
10	not clear	-	-

0	0.66	2	1
1	1.46	8	7
2	2.26	14	12
3	not clear	-	-
4	3.19	21	19
5	4.25	29	27
6	4.92	34	32
7	5.85	41	39
8	6.65	47	45
9	8.77	63	60
10	not clear	-	-

Fig. 1. Total ion chromatogram (TIC) and selected ion scans (SIS) of PBBs in Firemaster FF-1. The upper panel shows the TIC and the lower panels the SIS at *m/e* 154 and 548. Peak numbers are as shown.

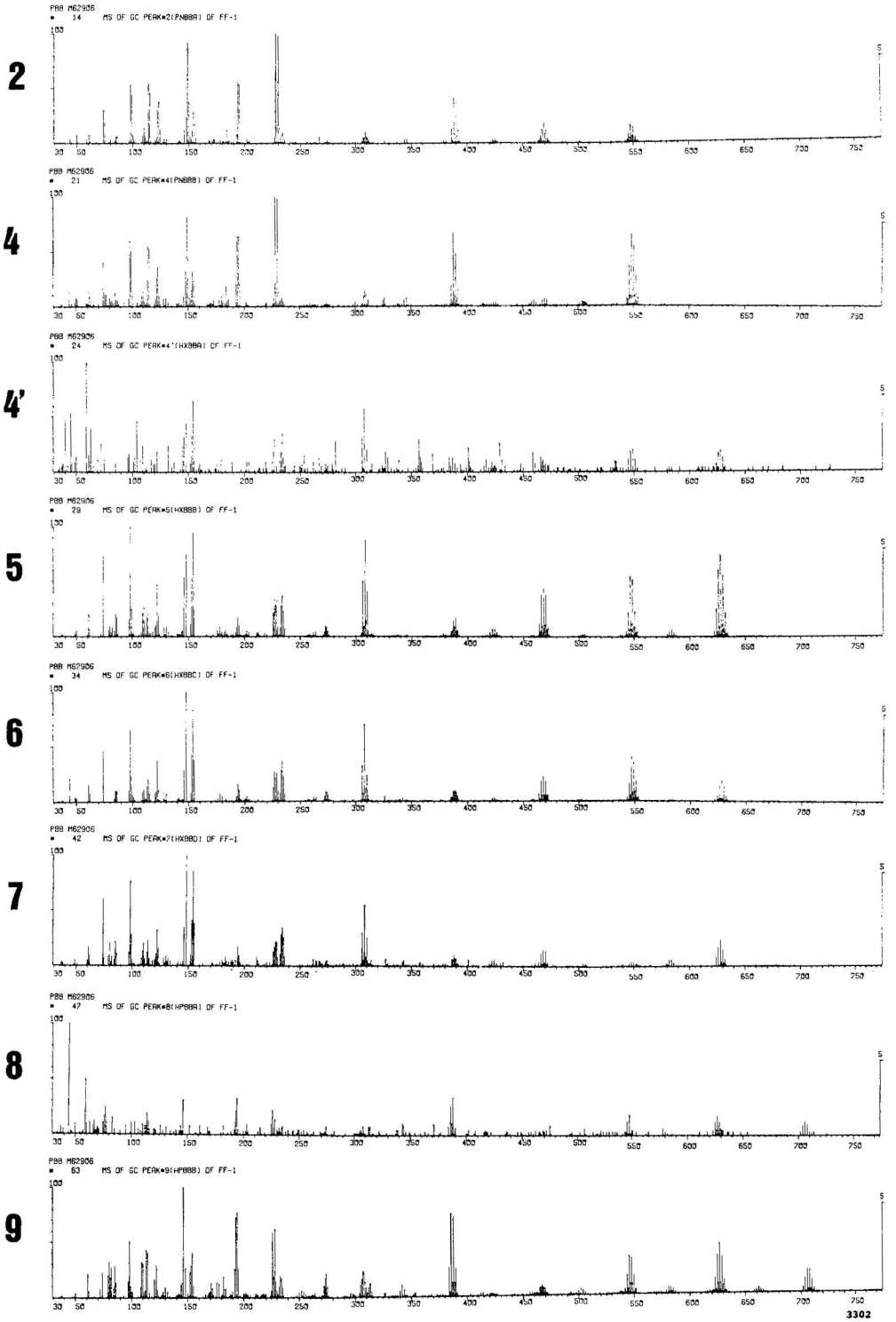
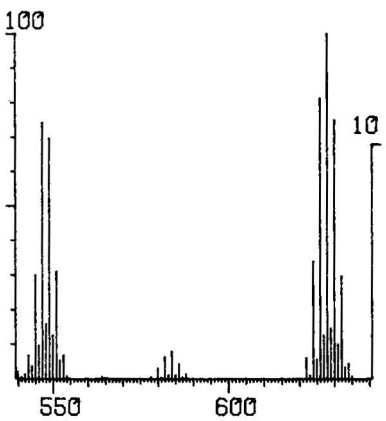


Fig. 2. Mass spectra of the major PBBs in Firemaster FF-1. The mass spectra of the major numbered peaks of the PBBs from Fig. 1 are shown. Note the presence of a chlorine contaminant in the PBBs. See text for details.

determined. Subsequently, selective ion scans for a.m.u. 98, 146, 147, 148, 149, 150, 154, 308, 547, 548 and 549 were carried out in order to better determine the retention times of ion fragments contained in PBBs, and to better define the specific compounds run as a TIC. The structures of the postulated positive-ion fragments were identified. Ions 154 and 548 were especially helpful, as shown in the selected ion scans (SIS) in Fig. 1b and 1c. The mass spectra for 8 of the 11 peaks are illustrated in Fig. 2. Peaks 0, 1, and 3 are possible unidentified contaminants. Peaks 2 and 4 are 2,2′,4,5,5′-pentabromo-1,1′-biphenyl (PnBBa) and 2,3′,4,4′,5-pentabromo-1,1′-biphenyl(PnBBb), respectively. Peak 4′ is a hexabromo-1,1′-biphenyl (HxBBa) whose bromine positions are yet to be assigned. Peak 5 is 2,2′,4,4′,5,5′-hexabromo-1,1′-biphenyl (HxBBb). Peak 6 is 2,2′,3,4,4′,5′-hexabromo-1,1′-biphenyl (HxBBc) and peak 7 is 2,3′,4,4′5,5′-hexabromo-1,1′-biphenyl (HxBBd). The heptabromobiphenyls appear as peaks 8, 9 and 10, known as HpBBa,b, and c, respectively; HpBBb is 2,2′,3,4,4′,5,5′-heptabromo-1,1-biphenyl.

Typical molecular ions of the PBBs can be noted in the spectra in Fig. 2. For example, the lowest molecular ion x should be the sum of ^{12}C, ^{1}H and ^{79}Br, which, for HxBBb, is 622. The number of additional molecular ions is the sum of the bromines[9]. Especially interesting is that chlorine contaminants are observed in many of the PBBs. For example, HxBBb (peak 5) has the expected molecular ions (622, 624, 626, 628, 630, 632, and 634). The [M $-^{79}$Br] groupings 543, 545, 547, 549, 551, and 553 a.m.u. are also observed. However, an unexpected set of ions (578, 580, 582, 584, 586, 588 and 590 a.m.u.) occurs in the spectrum of HxBBb. An enlargement of the [M] and [M $-^{79}$Br] ions of peak 5 is shown in Fig. 3; similar analyses can be done for the other peaks. The analysis of relative abundances of the mass spectrum of peak 5 is consistent with a pentabromomonochlorobiphenyl contaminant that has a GC retention time similar to that of HxBBb. Capillary column separation of the various compounds in FF-1 is an essential task for the future. It can be observed in the mass spectra of Fig. 2 that many of the PBBs have minor chlorine contaminants. To our knowledge, we are



Mass	Relative Abundance	
	Obtained	Theoretical
578	0.08	0.08
580	0.38	0.42
582	0.77	0.89
584	1.00	1.00
586	0.55	0.60
588	0.17	0.19
590	0.02	0.02

Fig. 3. Enlargement of the [M] and [M $-^{79}$Br] molecular ion spectra of the major hexabromobiphenyl of Firemaster FF-1 indicating a pentabromomonochlorobiphenyl contaminant. Note that the obtained and theoretical abundances of the various molecular ions for a pentabromomonochlorobiphenyl contaminant are similar. The theoretical abundance for a $ClBr_5$ compound was obtained from Beynon *et al.*[9] and calculated with mass 584 as 1.00.

the first to point this out, although it is certainly expected that minor amounts of chlorine will contaminate commercial bromine.

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Note

Direct determination of propoxur in plant tissues by gas chromatography with an alkali flame-ionization detector

J. M. VAN DER POLL* and D. VAN DE LAGEMAAT

Division for Nutrition and Food Research TNO, Institute CIVO-Analysis TNO, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

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Propoxur, the common name for 2-isopropoxyphenylmethyl carbamate, is a non-systemic insecticide with rapid knock-down and long residual action. It is particularly destructive to insects such as cockroaches, flies and mosquitoes. Foliage sprays have resulted in effective control of aphids, lygus bugs, grasshoppers and other insects that attack various crops.

Residues of propoxur in various substrates can be determined by gas–liquid chromatography (GLC) with an electron-capture detector after hydrolysation to the corresponding phenol, and derivatization with 1-fluoro-2,4-dinitrobenzene[1–3] or trifluoroacetylation[4].

However, as these methods are time consuming, we have devised an alternative technique based on the direct GLC determination of propoxur. Direct GLC of N-methylcarbamates is often complicated by their tendency to decompose on many columns[5]. Lorah and Hemphill[6], however, demonstrated that a column packing of Chromosorb W, surface modified with Carbowax 20M, has excellent properties for the GLC of intact carbamates (carbaryl, methiocarb, promecarb, mexacarbate).

The utility of support-bonded Carbowax 20M column packings for the direct GLC of carbamates was further confirmed by Moseman[7] and by Hall and Harris[8]. The latter authors determined carbamate residues in soil following this principle.

In our method, Ultrabond, a commercially prepared Carbowax 20M-modified column packing, is used. The extraction and clean-up procedure developed is less laborious than the often quoted method of Holden[2]. This fact, in combination with the more rapid, direct GLC determination, makes the method reported especially suitable for determining propoxur residues in vegetable and fruit samples obtained, for instance, in field trials.

EXPERIMENTAL

Gas–liquid chromatography

A Tracor 550 gas chromatograph with a Tracor 702-N-P nitrogen detector was used, with a glass column (1.8 m × 2.7 mm I.D.) packed with Ultrabond 20M (100–120 mesh), stock number 4904, obtained from Alltech (Arlington Heights, IL, U.S.A.).

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The flow-rates of the carrier and detector gases were 20 ml/min for helium, 3.5 ml/min for hydrogen and 100 ml/min for air. The temperatures of the column oven, injector and detector were 175, 225 and 270°C, respectively. A 1-mV f.s.d. recorder, chart speed 1 cm/min, was used.

Reagents and apparatus

Propoxur standard was obtained from Dr. Siegmund and Irmengard Ehrenstorfer (Fritz Hinternayer Strasse 3, D-9800 Augsburg, G.F.R.). Standard solutions in dichloromethane are stable for at least 1 year when stored in a refrigerator at 4°C. Silica gel 60 [0.05–0.2 mm, 70–270 mesh (ASTM)] for column chromatography was obtained from Machery, Nagel & Co. (Düren, G.F.R.). The silica gel was heated overnight in an oven at 130°C. After cooling, 95.0 g of the gel was deactivated with 5.00 g of water. The mixture was homogenized and, before use, allowed to equilibrate overnight in a tightly stoppered bottle. All other chemicals were of analytical-reagent grade and were checked for interfering impurities by means of blank determinations. The fruit and vegetable samples were macerated in an Ultra-Turrax mixer with solvent.

A centrifuge (830 *g*) with a centrifuge beaker was used. For evaporation a rotary evaporator was used (water-bath at 40°C). A chromatographic column (350 × 6 mm) with a reservoir (50 ml) packed with silica gel, was used to separate propoxur from impurities.

Procedure

Extraction. A 50-g ground and homogenized sample was weighed in a centrifuge beaker. Dichloromethane (100 ml) was added and the mixture was blended for 2 min. The lower layer, dichloromethane, was separated and dried over anhydrous sodium sulphate. To 20 ml (≡ 5 g of sample) were added 10 ml of isooctane and the mixture was concentrated to about 2 ml. Then 5 ml of isooctane were added and the mixture was concentrated to *ca.* 2 ml.

Column chromatographic clean-up. A plug of glass-wool was tamped into the bottom of a chromatographic column, which was filled with approximately 10 ml of *n*-hexane. Subsequently, 1.00 g of silica gel (deactivated with 5% of water) and 0.5 g of anhydrous sodium sulphate were slowly poured in, in succession and allowed to settle. The *n*-hexane was drained until the level had reached the top of the silica gel. The isooctane extract was transferred into the column and allowed to sink in. The flask and column were rinsed three times with about 1 ml of isooctane, then the column was eluted with 55 ml of 10% ethyl acetate in *n*-hexane.

The first 20 ml were discarded and the remaining 35 ml were concentrated to approximately 5 ml and transferred into a graduated test-tube. The liquid was further concentrated to 0.50 ml with the aid of a gentle stream of dry air. Then aliquots of the propoxur standard and of the concentrate were alternately injected into the gas chromatograph: one standard, one concentrate, one standard, one concentrate.

The propoxur concentration in the sample was calculated on the basis of the mean peak heights obtained for the sample and the standard.

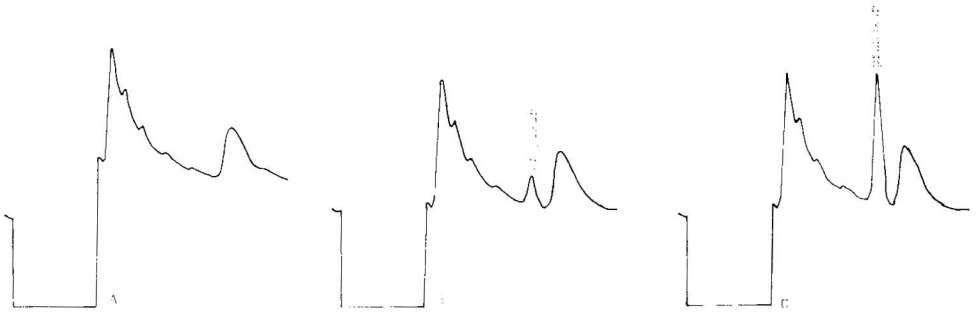

Fig. 1. Chromatogram obtained on a column packed with Ultrabond 20M (100–120 mesh). (A) 12.5 mg of cauliflower; (B) 12.5 mg of cauliflower fortified with 0.02 mg /kgof propoxur; (C) 12.5 mg of cauliflower fortified with 0.1 mg/kg of propoxur.

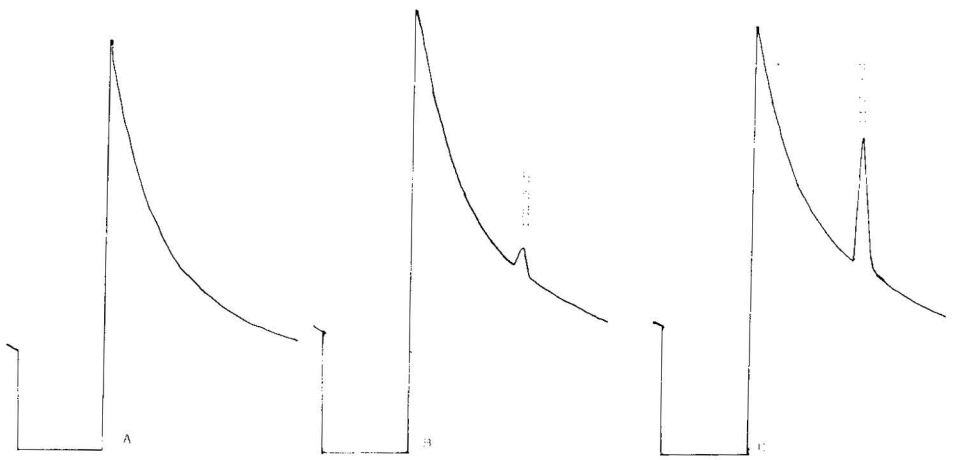

Fig. 2. Chromatogram obtained on a column packed with Ultrabond 20M (100–120 mesh). (A) 12.5 mg of cherries; (B) 12.5 mg cherries of fortified with 0.02 mg/kg of propoxur; (C) 12.5 mg of cherries fortified with 0.1 mg/kg of propoxur.

RESULTS AND DISCUSSION

Recovery experiments were carried out by adding known amounts of propoxur to untreated samples prior to extraction. The results are given in Table I.

It was necessary to condition the GLC columns by injecting a few positive samples until the response of the detector towards propoxur stabilized. It is further advisable to inject alternately standards and samples giving approximately the same peak heights into the gas chromatograph.

In Figs. 1 and 2 typical chromatograms are shown of untreated fortified samples of cauliflower and cherries, analysed with the method described.

In order to check the repeatability of the method, a homogenate of a positive sample of cherries was analysed six times. The following results for propoxur were obtained: 0.105, 0.094, 0.087, 0.099, 0.108 and 0.095 mg/kg. The coefficient of variation was 7.8%.

TABLE I

RESULTS OF RECOVERY EXPERIMENTS

Results given are percentage recoveries.

Sample	*Propoxur added (mg/kg)*					
	0.05	*0.10*	*0.25*	*0.50*	*1.0*	*2.0*
Apples					88	97
Cauliflower		100,110*		90,111*	84	
Cherries		99		90	108	
Currants					86	102
Peas	120			100	106,108*	
Radishes			100			
Savoy cabbage				100		

* In order to check the reproducibility recoveries were determined in duplcate.

In order to find out whether propoxur is detected as such when the GLC system described is applied, the following experiment was carried out. A 500-ng amount of propoxur was injected into the gas chromatograph. The compound eluting at the retention time of the relevant peak was collected in a micro-trap, connected to the outlet of the column. The fraction collected was analysed by thin-layer chromatography according to the technique described by Ernst and Schuring[9]. One spot was found, which had the same R_F value as propoxur. From this experiment, it was concluded that the chromatographic peak used for the determination can be attributed to propoxur.

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Note

Determination of residues of methazole and its metabolites, 1-(3,4-dichlorophenyl)-3-methylurea and 1-(3,4-dichlorophenyl)urea in soil by high-performance liquid chromatography

E. G. COTTERILL

Agricultural Research Council Weed Research Organization, Begbroke Hill, Yarnton, Oxford OX5 1PF (Great Britain)

(Received June 2nd, 1980)

The herbicide methazole, 2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione, is rapidly degraded in soil[1,2]. The major metabolite is 1-(3,4-dichlorophenyl)-3-methylurea (DCPMU) which further degrades to 1-(3,4-dichlorophenyl)-urea (DCPU). It has been suggested that the major phytotoxic agent is DCPMU[3]. Bond and Roberts[4] have shown that there is little loss of activity of residues over winter and that caution should be taken in choice and timing of following crops, hence it is important to be able to measure residues of both the parent compound and its metabolites. Previously reported methods have measured methazole by gas chromatography and its metabolites by derivatization with heptafluorobutyrylimidazole prior to gas chromatography[5]. High-performance liquid chromatography (HPLC) should allow the separation and determination of methazole, DCPMU and DCPU without derivatization or clean-up and the work described here explores this possibility.

Methanol was selected to extract the compounds because of the high recovery of ^{14}C-labelled methazole obtained by Walker and Roberts[1] and because the experience in this laboratory is that methanol is a generally effective solvent but usually extracts less extraneous materials that may interfere with chromatography than other solvents. Walker and Roberts also reported that DCPMU was not completely extracted from soil and that the difficulty of extraction increased with time after application. It is not clear from their paper whether they extracted wet or dry soil. Therefore a further experiment was included to assess the effect of water on the extraction efficiency of methanol although routinely in this laboratory soils are usually extracted without drying.

MATERIALS AND METHODS

Soils

Soils from two locations were used. Table I gives some details of their composition. They were air dried and passed through a 3-mm sieve prior to fortification.

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TABLE I

SOME PROPERTIES OF THE SOILS USED

	Soil	
	1	*2*
Organic Carbon (%)	4.1	1.6
pH	5.1	7.0
Clay (%)	16	16
Silt (%)	16	11
Sand (%)	68	73
Field capacity (% moisture)	27	16.6

Soil fortification

Aqueous dispersions of methazole, DCPMU and DCPU were prepared from methanolic solutions containing 1 mg ml^{-1} herbicide or metabolite. The concentration of the solutions was such that, when sufficient solution was added to dry soil to achieve 75% field capacity, the concentration was 1.0, 0.5 or 0.1 ppm herbicide or metabolite. All samples were prepared in triplicate and allowed to stand for 48 h before extraction.

Further samples of soil 2 were fortified at 1 ppm with a 1 mg ml^{-1} methanolic solution of DCPMU. Subsamples of this dry soil were extracted immediately and at intervals up to 120 h after fortification.

Extraction

Wet soil: 25 g of soil was shaken with 50 ml of methanol for 1 h using a wrist action shaker. After shaking, the slurry was filtered through a Whatman No. 42 filter paper. A 25-ml aliquot of the filtrate was concentrated to about 1 ml by evaporation under reduced pressure in a water bath at 50°C. The remaining solvent was removed by gentle blowing with air. The residue was redissolved in 1 ml of the eluent used for chromatography.

Dry soil: samples taken at each time interval up to 120 h after fortification were extracted as above. An additional set of three subsamples taken at 120 h was extracted using 50 ml of methanol–water (4:1, v/v).

Chromatography

Reversed-phase isocratic high-performance liquid chromatography was used[7]. A constant-flow pump was connected to a 100 × 5 mm I.D. stainless-steel column packed with Hypersil-ODS (Shandon Southern, Cheshire, Great Britain). Injections were made using a Rheodyne valve. Methazole and its metabolites were measured using a Cecil 212 variable-wavelength UV monitor at 250 nm and 0.1. a.u.f.s.d. Methanol–water (1:1, v/v) was used as the eluent at a flow-rate of 0.5 ml min^{-1}. Peak area was evaluated using a Perkin-Elmer Sigma 10 Chromatography Data Station and was found to be proportional to the concentration in the range 2.5 ng/5 μl to 100 ng/5 μl injection. The optimum wavelength for methazole, DCPMU and DCPU was determined by scanning methanolic solutions between 200 and 300 nm prior to chromatography. Using these conditions retention times for methazole,

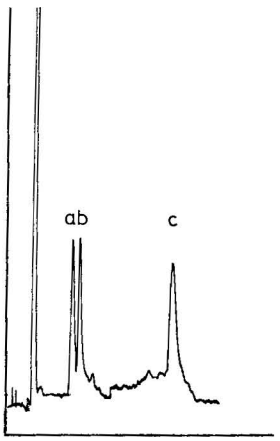

Fig. 1. Typical chromatogram of 10 ng of (a) DCPMU (b) DCPU and (c) methazole.

DCPMU and DCPU were 19.4, 6.7 and 7.5 min, respectively. Fig. 1 shows a chromatogram of 10 ng of DCPMU, DCPU and methazole.

RESULTS AND DISCUSSION

Table II shows that the recovery of methazole, DCPMU and DCPU from wet soil using methanol was satisfactory. Table III shows the comparison between DCPMU extracted from dry soil with methanol and that from wet soil, both fortified at 1 ppm. The results for dry soil show close agreement with those obtained by Walker and Roberts with DCPMU becoming less extractable with time, whereas those for wet soil are essentially constant for the period of the experiment. If the dry soil was extracted after 120 h with methanol–water (4:1, v/v) then the recovery was 93.4%, comparable with that from wet soil. It seems likely therefore either that the presence of water helps to breakdown the soil structure allowing the extractant to work on a greater surface area or that aqueous methanol is simply a better solvent for DCPMU.

TABLE II

RECOVERY (%) OF METHAZOLE, DCPNU AND DCPU FROM SOIL

Figures in parenthesis are standard deviations.

Fortification (ppm)	*Methazole*	*DCPMU*	*DCPU*
Soil 1			
0.1	101.5 (2.4)	92.3 (13.6)	92.2 (5.9)
0.5	95.8 (4.2)	88.8 (3.2)	95.0 (4.5)
1.0	88.3 (4.8)	91.4 (4.0)	100.7 (6.1)
Soil 2			
0.1	98.2 (5.2)	97.4 (8.4)	97.2 (3.1)
0.5	93.2 (0.8)	89.2 (3.9)	96.2 (5.7)
1.0	92.2 (4.8)	89.2 (3.9)	98.6 (6.9)

TABLE III

RECOVERY (%) OF DCPMU FROM WET AND DRY SOIL

Time (h)	*Dry soil*	*Wet soil*
0	91.4	95.9
1	97.1	100.4
2	91.4	101.9
4	71.4	98.9
24	68.6	95.9
48	68.6	96.4
120	70.6	98.9

Walker[8] found that DCPU never accounted for more than 1% of the initial herbicide so it is unlikely that DCPU will be present in the soil in sufficient quantities to determine after normal field application rates. In this case the analysis time can be shortened by using methanol–water (7:3, v/v) as the eluent when retention times for DCPMU + DCPU and methazole become 3.95 and 7.43 min, respectively. The practical limit of detection for this method based on the smallest detectable peak being twice the background signal is about 0.04 ppm for each compound.

This method is not suitable for the less phytotoxic degradation product of DCPMU, 3,4-dichloroaniline as methanol is not an effective extractant.

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Note

High-performance liquid chromatography of vitamin D: enhanced ultraviolet absorbance by prior conversion to isotachysterol derivatives

D. A. SEAMARK and D. J. H. TRAFFORD

Steroid Laboratory, Department of Chemical Pathology, The London Hospital Medical College, Turner Street, London E1 2AD (Great Britain)

P. G. HISCOCKS

Applied Chromatography Systems Ltd., Luton, Bedfordshire LU2 0JE (Great Britain)

and

H. L. J. MAKIN*

Steroid Laboratory, Department of Chemical Pathology, The London Hospital Medical College, Turner Street, London E1 2AD (Great Britain)

(Received April 8th, 1980)

There has been a steadily increasing use of high-performance liquid chromatography (HPLC) in the assay of vitamin D in biological specimens[1]. HPLC is now routinely used for the purification of vitamin D and its metabolites prior to quantitation by ligand binding assay[2,3]. It has also been applied recently to the simultaneous analysis of a number of metabolites of vitamin D in human plasma[4] by the use of ultraviolet (UV) detection for quantitation. Until relatively recently, UV monitoring of HPLC effluent has been carried out with fixed-wavelength detectors at 254 nm (ref. 3). The use of variable-wavelength detectors slightly increases the sensitivity by selection of UV maxima. This communication describes the simple and rapid formation of isotachysterol derivatives[5] prior to HPLC which enhances the sensitivity by a factor of two.

MATERIALS AND METHODS

Vitamin D_3 (D_3) was obtained from Koch-Light Labs. (Colnbrook, Great Britain); 25-hydroxyvitamin D_3 (25-OH-D_3) was a generous gift from Dr. N. Eve (Roussel Laboratories, Wembley Park, Great Britain) and Dr. J. A. Campbell (Upjohn, Kalamazoo, MI, U.S.A.), and 24,25-dihydroxyvitamin D_3 (24,25-OH-D_3) was from Dr. N. T. Pollitt (Roche Products, Welwyn Garden City, Great Britain). Other reagents used were as specified by Seamark *et al.*[5] and were analytical reagent grade whenever possible. Solvents for HPLC were obtained from Rathburn, Peebleshire, Great Britain. HPLC was carried out with a Model 750/03 pump, a Rheodyne 7125 injection valve, and a Model SF770 variable-wavelength (190–700 nm) detector (Schoeffel Instruments) all supplied by Aplied Chromatography Systems, Luton, Great Britain. A Zorbax-SIL (5 μm, 250 $\times$ 0.46 mm, DuPont (U.K.), Hitchin, Great Britain) column was eluted with a solvent system hexane-isopropanol (9:1)[3]. UV spectra were recorded on a SP1700 spectrophotometer (Pye-Unicam, Cambridge, Great Britain).

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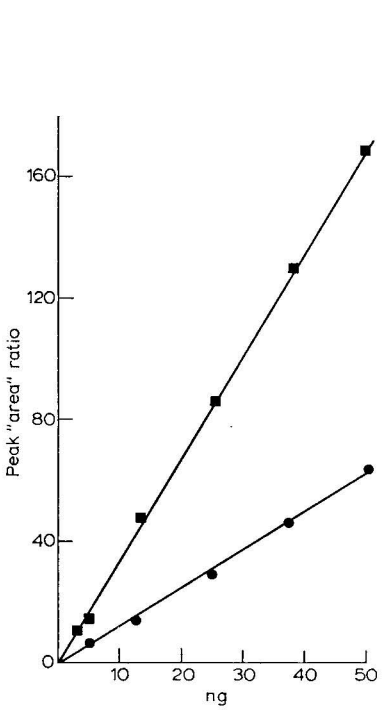
Aliquots of D_3, 25-OH-D_3, and 24,25-OH-D_3, containing amounts of each compound ranging from 5–50 ng, were pipetted into 1-dram vials (4 cm height × 1.3 cm diameter, FBG-Trident, Bristol, Great Britain). Carrier vitamin D_2 (200 ng) was added to tubes containing 25-OH-D_3 and 24,25-OH-D_3. The solvent was removed in a vacuum oven at 37°C and isotachysterol derivatives formed by the method of Seamark *et al.*[5]. Chloroform (75 μl) was added to the *seco*-steroid residue and hydrochloric acid gas was blown into the tube. The tube was stoppered and incubated at 0°C for 5min. The residual solvent was then evaporated under a gentle stream of nitrogen. Equal amounts of the internal standard (500 ng of 1α,25-dihydroxyvitamin D_3) were added to each tube, the contents of which were injected into the HPLC sample valve. The effluent was monitored with the UV detector set at 290 nm.

Aliquots of vitamin D_3, 25-OH-D_3, and 24,25-OH-D_3 were mixed with the same amount of internal standard (500 ng) and subjected, without isomerisation, to HPLC, monitoring the effluent at 264 nm.

Peak "area" ratios (peak "area" of vitamin D/peak "area" of internal standard × amount of internal standard) were calculated. "Area", a quantity proportional to peak area, was calculated by multiplying retention time by peak height[6]. To correct for the differing absorption of the internal standard at the different wavelengths used,



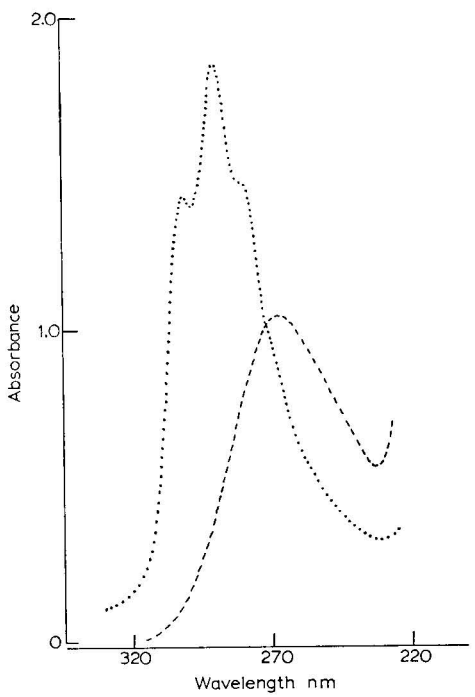


Fig. 1. Relationship between mass and peak "area" ratio before and after isotachysterol formation. D_3, 25-OH-D_3, and 24,25-OH-D_3 measured at 264 nm (●); isotachysterol$_3$, 25-OH-isotachysterol$_3$ and 24,24-OH-isotachysterol$_3$ measured at 290 nm (■). See text for definition of peak "area" ratio.

Fig. 2. The UV absorption spectra of equimolar amounts of D_3 (– – –) and isotachysterol$_3$ (· · ·), measured in hexane–isopropanol (9:1).

a UV spectrum of 1α,25-OH-D_3 in hexane–isopropanol (9:1) was obtained, and a factor relating the UV absorption at 290 nm to that at 264 nm was calculated. Peak "area" ratios were then corrected by using this factor. Standard curves for D_3, 25-OH-D_3, and 24,25-OH-D_3 before isomerisation, monitored at 264 nm, and after isomerisation, monitored at 290 nm, were obtained and are illustrated in Fig. 1.

DISCUSSION

Plasma levels of the majority of the so-far recognised metabolites of D_3 are sufficiently high to enable physico-chemical determination by HPLC with UV detection, using reasonable (3–5 ml) volumes of plasma[3]. The concentrations of 24,25-OH-D_3, and 25,26-OH-D_3 are, however, almost at the minimum detectable limit. Any procedure which can enhance the sensitivity of UV detection is therefore of interest. It is clear from an examination of the UV spectra of equimolar concentrations of D_3 and its isotachysterol$_3$ isomer (see Fig. 2), that the isotachysterol$_3$ has an enhanced absorption at 290 nm in comparison to the absorption of D_3 at its maximum, 264 nm. Most fixed-wavelength detectors monitor at 254 nm, which is not the maximum for any of the vitamin D metabolites examined here. Prior conversion to isotachysterol and use of a variable-wavelength detector enhance the sensitivity of detection by a factor of two.

The isomerisation of vitamin D derivatives, not containing a 1α-hydroxyl, has been carefully evaluated by Seamark *et al.*[5], who showed quantitative conversion down to 100 ng levels. Below this it was necessary to add carrier amounts (200 ng) of vitamin D_2 to all the vitamin D metabolites examined with the exception of D_3 itself which gave quantitative conversion at 10 ng even in the absence of added carrier D_2 (ref. 5). For the vitamin D metabolites examined here, conversion to isotachysterol isomers has not affected the separation in the HPLC system used.

ACKNOWLEDGEMENTS

We are very grateful to Applied Chromatography Systems for the loan of the HPLC apparatus used in this investigation and to all colleagues (acknowledged in the text) who kindly donated samples of vitamin D metabolites.

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Note

Analysis of phytic acid by high-performance liquid chromatography

B. TANGENDJAJA, K. A. BUCKLE* and M. WOOTTON
School of Food Technology, University of New South Wales, P.O. Box 1, Kensington, N.S.W. (Australia)

(Received May 13th, 1980)

Many workers[1–4] have determined phytic acid (inositol hexaphosphoric acid) in cereals by modifications to the original method of Heubner and Stadler[5], which depends upon the extraction of phytic acid from finely ground cereals and precipitation of phytic acid in the extract by addition of ferric chloride. A chromatographic method using a column containing an ion-exchange resin was first introduced by Smith and Clark[6] and has been used subsequently for the determination of phytic acid in soil[7] and blood[8,9]. The precipitation method, however, has been reported to be unreliable, and column chromatography cannot be used for routine analysis, since it is too time-consuming[10].

Recently, high-performance liquid chromatography (HPLC) has been attempted for the determination of phytic acid in pig manure. Separation, however, was unsatisfactory and prior chromatography of the extract on Sephadex was necessary. In the present investigation, a method is described for the HPLC determination of phytic acid in rice bran, and of inositol and inorganic phosphate as the end products of hydrolysis.

EXPERIMENTAL

Materials

Phytic acid solution (*ca.* 50% in water) was purchased from T.C.I. (Tokyo, Japan). Inositol (analytical-reagent grade) was obtained from Sigma (St. Louis, MO, U.S.A.) and sodium acetate (analytical-reagent grade) from Ajax Chemicals (Sydney, Australia). Acetonitrile was purchased from Waters Assoc. (Sydney, Australia) and water was double distilled in glass. Prior to use, all solvents were filtered through a 0.45-μm Millipore filter and degassed for 5 min. During degassing, solvents were shaken in an ultrasonic bath. Rice bran (variety Calrose) was obtained from the Ricegrowers' Cooperative Mills (Leeton, Australia).

Equipment

HPLC was carried out with Waters Assoc. (Milford, MA, U.S.A.) equipment consisting of a Model M6000A pump for solvent delivery, Model 440 ultraviolet (UV, 254 nm) and Model K401 differential refractive index (RI) detectors and a Model U6K injector. The Model 252 recorder was from Linear Instrument Corp. (Costamesa,

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CA, U.S.A.). μBondapak carbohydrate and C_{18} (30 cm × 4 mm) columns were obtained from Waters Assoc., and the syringes used for injection were from Hamilton (Reno, NV, U.S.A.).

Methods

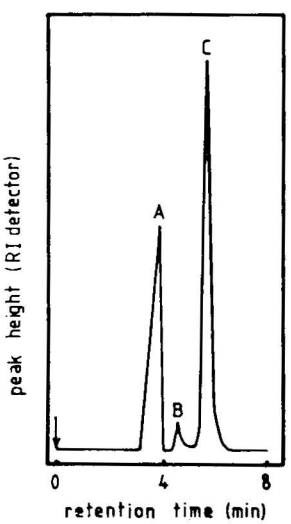
Aqueous solutions of phytic acid (0.5%, 10 μl) and inositol (1%, 5 μl) were mixed and injected onto a μBondapak C_{18} column. Sodium acetate (0.005 *M*) was used as solvent at a flow-rate of 0.5 ml/min; UV and RI detectors were used simultaneously.

Inositol and inorganic phosphate, obtained by heating 0.5% aqueous phytic acid solution for 48 h at 100°C and pH 4.3, were separated by injecting 25 μl on to a μBondapak carbohydrate column and developing with acetonitrile–water (60:40) at a flow-rate of 2 ml/min.

Phytic acid was extracted from rice bran (1 g) with 3% trichloroacetic acid (25 ml) for 30 min in a mechanical shaker, and the slurry was centrifuged for 20 min at 40,000 *g*. The supernatant liquid was filtered through a 0.22-μm Millipore filter, and an aliquot (25 μl) was injected into a μBondapak C_{18} column; the development solvent was sodium acetate (0.005 *M*) at a flow-rate of 2 ml/min.

RESULTS AND DISCUSSION

The ferric chloride precipitation method cannot be used for the determination of phytic acid in all foods and feeds because the presence of interfering substances such as reducing compounds leads to high results.



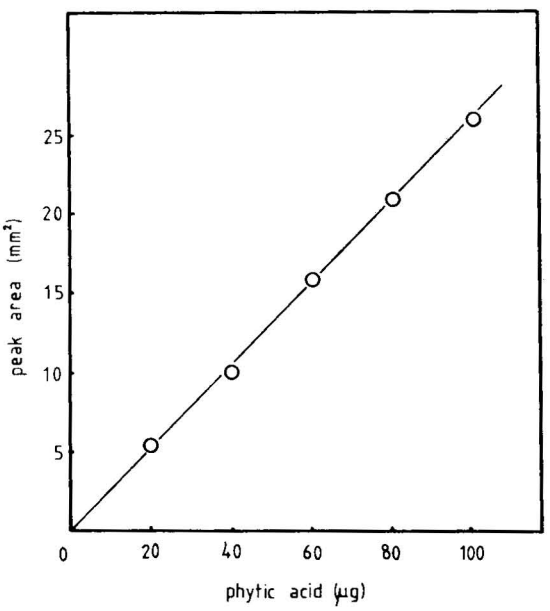
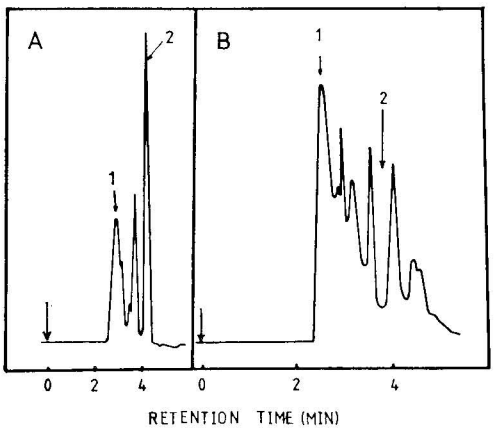


Fig. 1. Separation of phytic acid and inositol on a μBondapak C_{18} column developed with 0.005 *M* sodium acetate. Peaks: A = phytic acid; B = impurities from phytic acid; C = inositol.

Fig. 2. Standard curve for estimation of phytic acid.



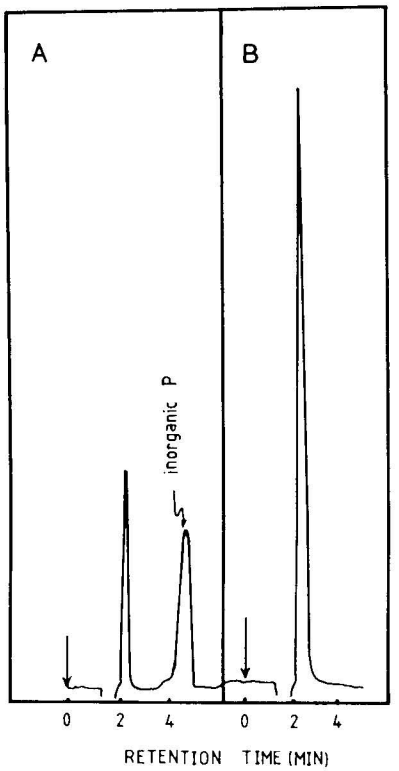


Fig. 3. Chromatogram of phytic acid solution after heating for 12 h at 100°C and pH 6.2. Separation was on a μBondapak C_{18} column developed with 0.005 *M* sodium acetate: A, RI detector; B, UV detector. Peaks: 1 = phytic acid; 2 = position for inositol.

Fig. 4. Chromatogram of phytic acid solution after heating for 48 h at 100°C and pH 4.3. Separation was on a μBondapak carbohydrate column developed with acetonitrile–water (60:40): A, hydrolysed phytic acid; B, inositol.

Ion-exchange chromatographic analysis[7] is not only time-consuming, but fails to detect inositol liberated from phytic acid during hydrolysis. Separation and estimation of both phytic acid and inositol is important for following the rate and extent of hydrolysis.

Fig. 1 shows the chromatogram of separation of phytic acid and inositol by HPLC on a μBondapak C_{18} column; separation was complete *ca.* 7 min after injection. The standard curve for phytic acid estimation is shown in Fig. 2.

The chromatogram of the hydrolysate of phytic acid produced by heating for 12 h at pH 6.2 is shown in Fig. 3. A UV detector gave more peaks than did the RI detector, but the former could not detect inositol. Inositol and inorganic phosphate, however, were separated on a μBondapak carbohydrate column with acetonitrile–water (60:40) as solvent (Fig. 4); increasing the acetonitrile concentration in the solvent gave better resolution, but broader peaks.

Phytic acid in rice bran can be estimated by separation on a μBondapak C_{18} column developed with 0.005 *M* sodium acetate (Fig. 5). Phytic acid was eluted from

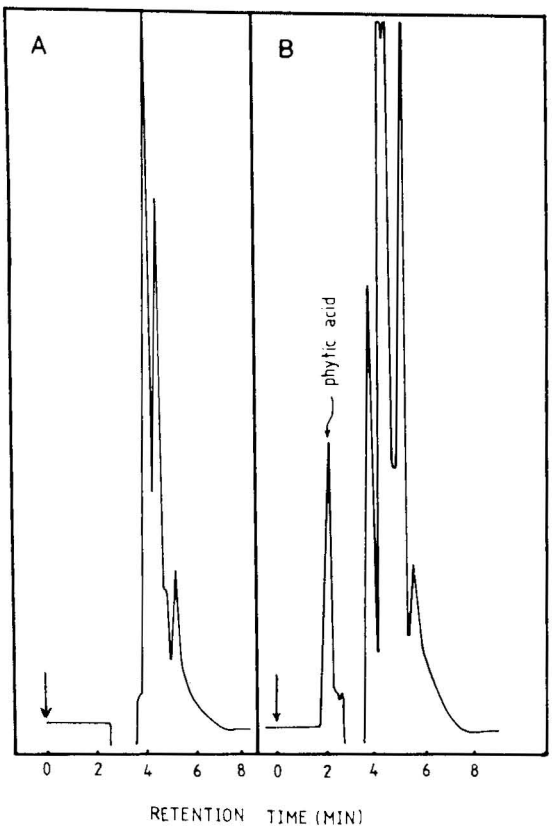


Fig. 5. Chromatogram of separation of phytic acid obtained from rice bran: A, trichloroacetic acid solution; B, rice-bran extract.

the column before trichloroacetic acid. Extraction of phytic acid with ethylenediaminetetraacetic acid solution as reported by Gerritse[11] requires prior separation by Sephadex gel chromatography before injection of the phytic acid extract on to a HPLC column.

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The authors acknowledge the helpful suggestions of Ray Francke, Technical Officer in the School of Food Technology.

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Note

Application of high-speed aqueous gel permeation chromatography to insect venoms

BO RENCK and ROLAND EINARSSON*
Analytical Research, Pharmacia AB, S-751 04 Uppsala (Sweden)
(Received May 1st, 1980)

Gel filtration has been one of the most important techniques in biochemical research since its introduction[1] for separation and purification purposes and in the measurement of the molecular weight and distribution of water-soluble macromolecules. However, the matrices of the gels used are soft and weak, and consequently they do not withstand the high pressures required for high-speed analysis. A considerable amount of protein is needed, the separation procedures are often very time consuming and the resolution may be poor.

Many attempts have been described for the application of high-speed aqueous gel filtration of macromolecules[2]. However, many problems have been found, such as low column efficiencies and significant adsorption of the solute on the gel packing materials[2,3].

For the fractionation and analysis of insect venoms different gels have been used, such as Sepharose and various types of Sephadex[4–6]. In this paper we report on the use of a hydrophilic polyester packing (Shodex OHpak B-804/S) for aqueous gel permeation chromatography (GPC) for the separation and characterization of different insect venoms.

EXPERIMENTAL

Honeybee venom was purchased from Mr. C. Mraz, Middlebury, VT, U.S.A., and was obtained by the electric shock method. The venoms of wasp, white-faced hornet, yellow hornet and yellow jacket were purchased from Dr. A. W. Benton, Pennsylvania State University, Spring Mills, PA, U.S.A., as lyophilized material. The vespid venoms were extracted from the venom sacs.

High-speed aqueous GPC was performed on a chromatograph consisting of a Labotron pump, loop-type sample injector (Waters Assoc., Milford, MA, U.S.A.) and UV detector (Perkin-Elmer flow-through type). Shodex OHpak B-804/S gel was packed in a stainless-steel tube (250 × 8 mm) (Showa Denko, Tokyo, Japan). The eluent (0.02 *M* ammonium acetate buffer, pH 4.75) was generally delivered at 0.3 ml/min (2–3 kg/cm^2). Insect venoms were dissolved in the eluent to a concentration of approximately 0.3–0.5%. The injection volume was varied between 0.05 and 0.2 ml. The effluent was monitored at 254 nm. Blue Dextran 2000 and alanine were

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used as the marker components for totally excluding (V_0) and totally permeating (V_t) molecules.

RESULTS AND DISCUSSION

The high resolving power obtainable with the Shodex OHpak column was demonstrated with five different insect venoms. Figs. 1–5 show typical chromato-

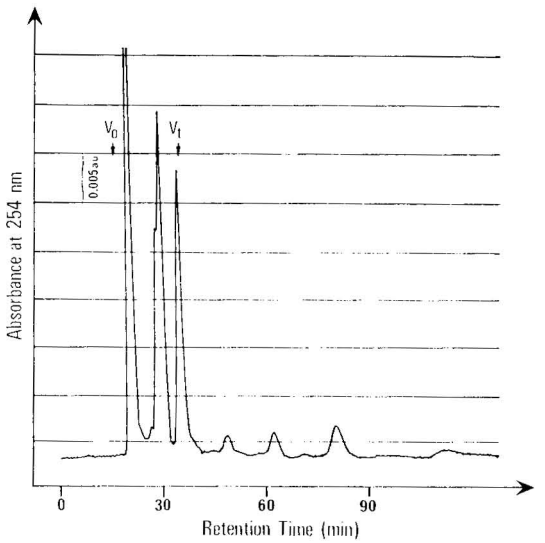

Fig. 1. High-speed aqueous gel permeation chromatogram of honey bee venom (986 μg). Operating conditions: packing, Shodex OHpak, B-804/S; column, 25 cm × 8 mm I.D.; mobile phase, 0.02 *M* ammonium acetate, pH 4.75; flow-rate, 20.0 ml/h; temperature, ambient; detection, absorbance at 254 nm.

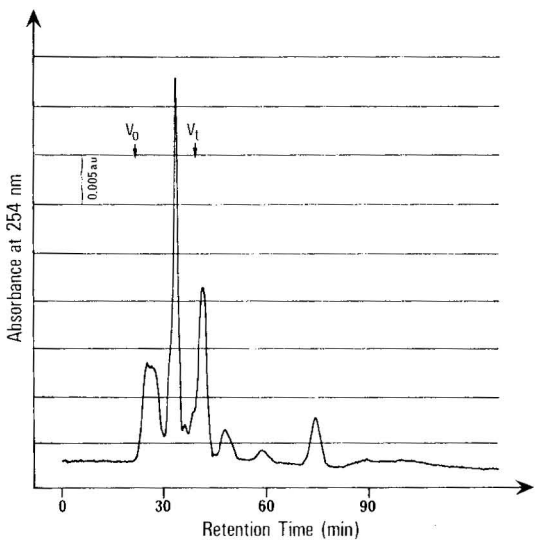

Fig. 2. High-speed aqueous gel permeation chromatogram of yellow jacket venom (648 μg). Operating conditions as in Fig. 1.

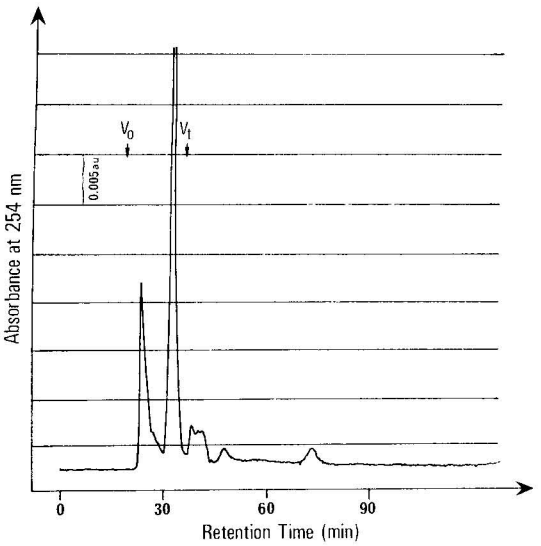

Fig. 3. High-speed aqueous gel permeation chromatogram of white faced hornet venom (500 μg). Operating conditions as in Fig. 1.

grams of the insect venoms eluted with 0.02 *M* ammonium acetate (pH 4.75). Samples of 0.1–1 mg of each insect venom species were applied to the column. On comparing the individual elution patterns it is evident that each species exhibits a characteristic elution profile and that the elution pattern is relatively independent of the amount applied and the flow-rate. Well resolved peaks eluted in the high-molecular-weight range appeared between V_0 and V_t, while peptides appeared in the

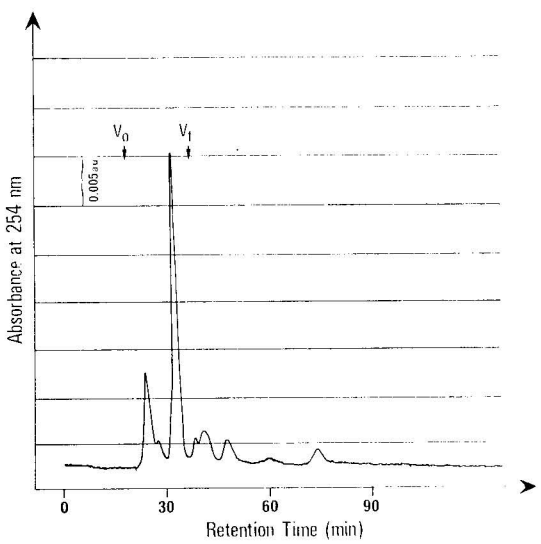

Fig. 4. High-speed aqueous gel permeation chromatogram of yellow hornet venom (390 μg). Operating conditions as in Fig. 1.

chromatogram after V_t, indicating significant adsorption on the matrix. This strong adsorption might be due to the fact that the protein/peptide components in the insect venoms studied are highly basic. Unlike the elution profiles obtained with conventional gel filtration[4–6], the protein/peptide peaks are well resolved in high-speed GPC. Results obtained to date suggest that it is possible to differentiate between the individual insect venoms tested provided that comparable concentrations are used. Further, the reproducibility of high-speed aqueous GPC is such that each species chromatographed on various occasions shows peaks occurring at similar retention times, rendering this technique suitable for identification purposes.

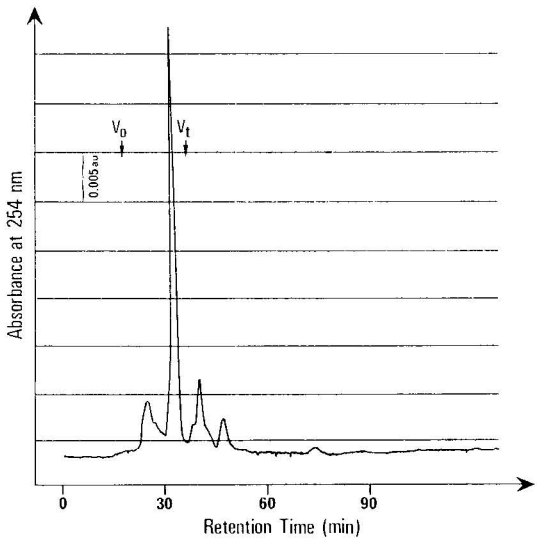

Fig. 5. High-speed aqueous gel permeation chromatogram of wasp venom (356 μg). Operating conditions as in Fig. 1.

These preliminary data show the potential use of Shodex OHpak columns in the hydrophilic aqueous GPC of insect venom proteins at low pressure. High-speed GPC with high resolution and sensitivity might greatly facilitate the increasing demands of the characterization of complex protein mixtures exemplified here by allergens.

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Erratum

J. Chromatogr., 194 (1980) 69–75

Page 75, the following Acknowledgement should be added: "This work was supported by the National Research Council (C.N.R.), program: New Protein Foods, contract No. 79.00102.76.115.0950."

PUBLICATION SCHEDULE FOR 1980

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	197/1 197/2 198/1	198/2 198/3 198/4 199	200/1 200/2	The publication schedule for further issues will be published later.
Chromatographic Reviews			184/1	184/2					184/3			184/4	
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3	183/4		

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