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MEASUREMENT OF RETENTION DATA ON OPEN TUBULAR COLUMNS COUPLED IN SERIES

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SUMMARY

An equation is derived which relates the apparent capacity factor of a series of columns to the capacity factors of the individual columns, their lengths and the inlet pressure. This equation, which gives results in excellent agreement with experimental results, permits the correct prediction of the retentions of compounds on combined columns, and hence the calculation of the lengths of the individual columns to be used to obtain optimal separations of complex mixtures. This equation also explains the moderate variation of the column capacity factor with inlet pressure and that resulting from reversal of the carrier gas flow on columns that are not very homogeneous. It is shown that, at least when the difference in the retention indices of compounds on two stationary phases is not very large, the retention index on a series of two columns varies linearly with the length of one of the columns.

INTRODUCTION

The on-line combination of two or more columns is a common approach in gas chromatography (GC) for solving difficult separation problems, either because it is easier to couple in series two columns packed with the same stationary phase than to prepare a very long single column, or because two different stationary phases are needed in order to achieve the separation of a given mixture. In the latter instance two possibilities arise: the use of a series of different columns coupled on-line, each packed with one of the pure stationary phases selected, or the use of one column packed with a mixed stationary phase. Both methods have advantages and drawbacks.

The first method is very practical as it permits the use of columns already available; also these columns can be separated and employed for different analyses later. On the other hand, the individual retention times and the selectivity of the combined column depend on the order of the different units. The problems arising from the combination of packed columns have been thoroughly discussed by various workers[1–5].

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The second method requires that a column be prepared specifically for each new separation, which restricts its use to certain types of routine analysis. In this instance, however, the selectivity of the column is independent of its length and the retention time and resolving power increase with increasing length, as for classical columns[5]. In a series of papers, Purnell and co-workers described the retention behaviour of solutes analysed on packed columns prepared using mixed stationary phases[6–10]. It was shown that the partition coefficient of a solute chromatographed on a mixed stationary phase can be predicted from its partition coefficients on the pure stationary phases and the composition of the mixed phase, assuming a linear variation of the partition coefficient on a binary solvent with the volume fraction of one of these solvents in the mixture. This assumption is controversial[6,11–13] and probably holds only in fairly simple cases. Snyder and Poppe[12] showed that it is probably valid for mixtures of stationary phases on which the partition coefficients are close, such as those used by Laub and Purnell[10], mixtures of polymers that are poorly miscible or mixtures of stationary phases for which the solute–solvent interactions are restricted to 1:1 pairs. Martire[13] showed that the effect observed by Purnell is "... merely an artifact of the insensitive method of data testing used..." Finally, there are many examples of binary solvent GC stationary phases that show poor agreement with this assumption[14,15]. It is therefore doubtful whether in most instances computer calculations can predict accurately enough the optimal composition of the mixed stationary phase, as suggested by Purnell and co-workers[16,17]. Hence the main advantage of this method remains controversial.

As far as open-tubular columns are concerned, it is less difficult to couple them in series than to prepare a single column containing a mixture of stationary phases. Although the static method could be used with a solution of the different stationary phases at the required concentration[18], the stability of the liquid film depends on the wettability of the glass surface, a function of the mode of surface treatment, of the nature of the stationary phase, its surface energy, etc. The best surface treatment is not the same when coating a film of silicone grease or of Carbowax 20M. On the other hand, open-tubular columns have been coupled in series easily, using various types of small-volume connections, the simplest one being shrinkable PTFE tubing[19]. There is no need in the present application for sophisticated column switching systems, which could be applied, however, to the design of variable-polarity columns[3].

The aim of this paper is to point out some of the problems that occur in the use of on-line series of glass capillary columns, with special emphasis on the prediction of retention data.

THEORETICAL

We assume we have in series two open-tubular columns of length L_A and L_B (*cf.*, Fig. 1) and identical inner diameters, and hence the same permeability, k. According to Poiseuille's law[5], the carrier gas velocity at the outlet of this column series is

$$u_0 = \frac{k}{2\eta\,(L_A + L_B)\,P_o} \cdot (P_i^2 - P_o^2) \qquad (1)$$

The intermediate pressure, P_a can be considered either as the inlet pressure of

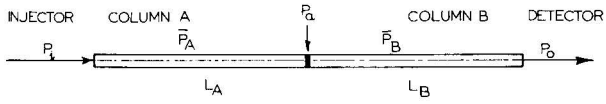

Fig. 1. Schematic diagram of two columns coupled in series. P_i = inlet pressure; P_a = pressure between columns A and B; P_o = outlet pressure; $\bar{P}_A$ = average pressure in the first column; $\bar{P}_B$ = average pressure in the second column; L_A, L_B = column lengths.

the second column or as the outlet pressure of the first column. Mass conservation of the carrier gas demands that the corresponding gas velocity, u_a, be the same, whether it is calculated as the outlet velocity of column 1 or the inlet velocity of column 2:

$$u_a = \frac{k}{2\eta L_A P_a} \cdot (P_i^2 - P_a^2) = \frac{k}{2\eta L_B P_a} \cdot (P_a^2 - P_o^2) \tag{2}$$

Hence

$$\frac{P_i^2 - P_a^2}{L_A} = \frac{P_a^2 - P_o^2}{L_B} = \frac{P_i^2 - P_o^2}{L_A + L_B} \tag{3}$$

The first of these two equations can be solved for P_a^2:

$$P_a^2 = \frac{P_i^2 L_B + P_o^2 L_A}{L_A + L_B} \tag{4}$$

The retention time over a column of length L is given by

$$t_R = \frac{L}{\bar{u}} (1 + k') \tag{5}$$

with[20]

$$\bar{u} = j u_o = \frac{3}{2} \cdot \frac{P_o (P_i^2 - P_o^2)}{P_i^3 - P_o^3} \cdot u_0 \tag{6}$$

The retention time over the series of two columns is similarly given by

$$t_R = \frac{L_A + L_B}{\bar{u}} (1 + k'_{AB}) = \frac{L_A}{\bar{u}_A} (1 + k'_A) + \frac{L_B}{\bar{u}_B} (1 + k'_B) \tag{7}$$

This equation defines a capacity factor for the series of columns, k'_{AB}, which has no thermodynamic meaning as it will depend on the characteristics of the two columns in addition to the two different retention mechanisms used, but at least it is independent of the flow-rate. Combining eqns. 3, 6 and 7, we obtain[4]

$$k'_{AB} = \frac{k'_A L_A \bar{P}_A + k'_B L_B \bar{P}_B}{(L_A + L_B) \bar{P}} \tag{8}$$

where $\bar{P}$ is the average column pressure[4]:

$$\bar{P} = \frac{P_o u_o}{\bar{u}} = \frac{2}{3} \cdot \frac{P_i^3 - P_o^3}{P_i^2 - P_o^2} \tag{9}$$

Using eqns. 4, 8 and 9, k'_{AB} can be calculated. Eqn. 8 can be further simplified by combination with eqns. 3 and 9:

$$k'_{AB} = \frac{k'_A|P_i^3 - P_a^3| + k'_B|P_a^3 - P_o^3|}{P_i^3 - P_o^3} \tag{10}$$

Eqns. 4 and 10 allow the calculation of k'_{AB}, knowing the capacity ratios of the two columns and their lengths. We note that if the same stationary phase is used but the average film thickness is different for the two columns, the apparent column capacity factor becomes a function of the inlet pressure. This is obviously what should happen on a column that is not homogeneous, having a film thickness function of the position along the column.

The apparent column capacity factor for the column series also depends on the column order for a given flow-rate, as does the intermediate pressure, P_a, as was observed by Hildebrand and Reilley[4] for packed columns. As the inner diameter of an open-tubular column sometimes varies along the column[21], as does the film thickness, we may expect that in practice the apparent column capacity factor will deviates from the prediction of eqn. 10 and may even depend on the direction of the gas flow inside each column, their order remaining the same.

In liquid chromatography, the carrier liquid velocity is constant all along the column as the compressibility of liquids can be neglected in calculating this velocity. Then,

$$\bar{u}_A = \bar{u}_B = \bar{u} \tag{11}$$

and eqn. 7 becomes

$$k'_{AB} = \frac{k'_A L_A + k'_B L_B}{L_A + L_B} \tag{12}$$

which is also the limit of eqn. 10 when P_i becomes very close to P_o (and $\bar{u}$ to zero).

Fig. 2 shows plots of k'_{AB}/k'_B as a function of L_A/L_B for different inlet pressures (the outlet pressure is assumed to be atmospheric), for $k'_A/k'_B = 10$ and 0.1. This ratio is expected to be close to 1 at low L_A/L_B and close to either 10 or 0.1 at large L_A/L_B. When $k'_A/k'_B = 1$, k'_{AB}/k'_B is always also equal to 1. The effect of the inlet pressure is important only between 1 and 4 atm. Above about 6 atm, the apparent capacity factor becomes independent of the inlet pressure, and hence of the carrier gas velocity, as shown by the insert in Fig. 2. Assuming that P_0 can be neglected in comparison with P_i, we can rewrite eqn. 4:

$$P_a^2 = P_i^2 \cdot \frac{L_B}{L_A + L_B} \tag{13}$$

and eqn. 10 becomes

$$k'_{AB} = k'_A + (k'_B - k'_A)\left(\frac{L_B}{L_A + L_B}\right)^{3/2} \tag{14}$$

Note that P_o is also negligible compared with P_a only if L_A/L_B is not very large, but then k'_{AB} would be nearly equal to k'_A anyway (*cf.*, eqn. 10).

Fig. 3 shows the variation of k'_{AB}/k'_B as a function of L_A/L_B for different values of k'_A/k'_B. It can be seen that the contribution of the first column (column A) is always more important than that of the second (column B).

For example, if we have $L_A/L_B = 3.16$ with $P_i = 2$ atm ($\Delta P = 1$ atm) and $k'_A = 0.2$, $k'_B = 1$, then $k'_A/k'_B = 0.2$ and $k'_{AB}/k'_B = 0.344$. If we reverse the column order by exchanging columns A and B, L_A/L_B becomes equal to 0.316, k'_A is now equal to 1 and $k'_B = 0.2$. The ratio $k'_A/k'_B = 5$ and with $P_i = 2$ atm again we find $k'_{AB}/k'_B = 2.18$, hence $k'_{AB} = 0.436$, *i.e.*, 27% larger. This is a major change in chromatography and two compounds that have accidentally the same k'_{AB} with different k'_A and k'_B will probably have different k'_{BA}.

Unfortunately, there is no simple relationship between the relative retention of two compounds on the two columns and on their combination, except by writing eqn. 10 for the two compounds and dividing the two corresponding equations. A similar conclusion applies to the retention index.

EXPERIMENTAL

Column preparation and measurements

Glass capillary columns were made from soda-lime glass etched with hydrogen chloride as described previously[18]. Purified Apiezon L[22], hydrocarbon C_{87}[23], polyethylene glycol 20,000 (Carbowax 20M) and polydimethylsiloxane (OV-101) were

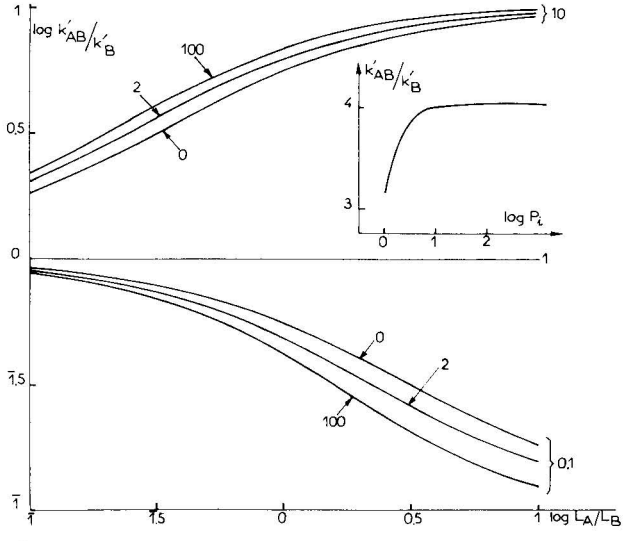

Fig. 2. Logarithmic plots of k'_{AB}/k'_B (ratio of the apparent capacity factor of a combination of two columns A and B to the capacity factor of the second column) against the ratio of the lengths of the two columns. The figure on each curve gives the inlet pressure (atm). The outlet pressure was atmospheric. Upper curves, $k'_A/k'_B = 10$; lower curves, $k'_A/k'_B = 0.10$. If $k'_A/k'_B = 1$, k'_{AB} is always equal to k'_A and k'_B. The insert shows a plot of k'_{AB}/k'_B *versus* log P_i for $k'_A/k'_B = 10$ and $L_A/L_B = 0.316$.

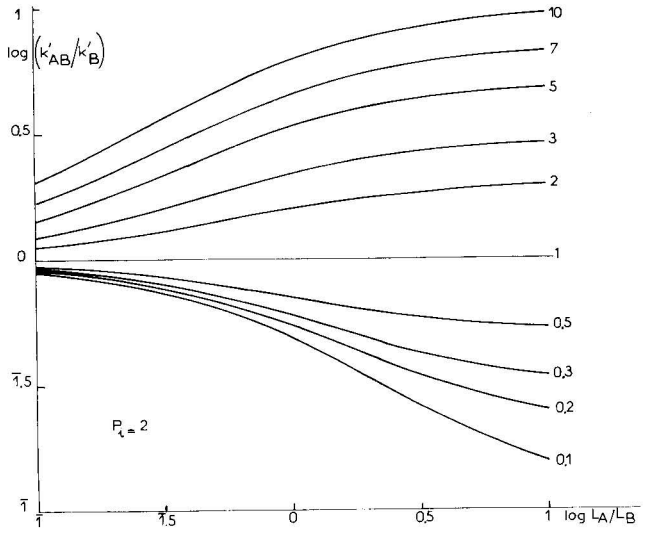

Fig. 3. Plot of log (k'_{AB}/k'_B) *versus* log (L_A/L_B) for various values of k'_A/k'_B, as given on the different curves. P_i = 2 atm; P_o = 1 atm.

used as stationary phases. Columns were coated by the dynamic method using a mercury plug[19]. The solution of stationary phase (0.15–0.30 ml of 5–26% solutions) was sucked into the capillary tube followed by a plug of mercury (*ca.* 30 cm). The vacuum was then disconnected and the mercury plug was pushed through the column by compressed nitrogen at 0.5–5 cm/sec.

The columns were conditioned with nitrogen at a flow-rate of 0.5 ml/min, with temperature programming at the rate of 4°C/min from 50 to 190°C, the latter temperature being maintained for 60 min.

The gas chromatograph was a Fractovap Model 2150 (Carlo Erba, Milan, Italy) equipped with a flame-ionization detector (FID) and an inlet stream splitter. The column tip was carefully inserted into the jet of the FID. Samples were injected using a 10-μl Hamilton microsyringe. Hydrogen or nitrogen was used as the carrier gas.

The capillary columns were characterized by the capacity factors (k') and the efficiency was expressed as the number of theoretical (n) and effective (N) plates and the separation numbers (TZ) for hydrocarbons and polychlorinated biphenyls (PCBs). Most characteristics were derived from distances measured on paper charts; a magnifying glass was used for peak-width measurements. Retention times of the PCBs and of *n*-alkanes used for the calculation of retention indices were measured with a stop-watch.

Column characteristics

Column A (122 m × 0.25 mm I.D.). The column was coated with 0.3 ml of a 15% solution of Apiezon L in *n*-hexane, using a pressure of 30 cmHg and a velocity of 3 cm/sec (other data are given in Table I).

Column B (93 m × 0.25 mm I.D.). The column walls were deactivated with hexamethyldisilazane (HMDS) (2 h at 180°C) and the excess of HMDS was removed

by rinsing the column with 3 ml of dichloromethane. The column was further treated with 0.3 ml of a 5% solution of Carbowax 20M in dichloromethane using a pressure of 30 cmHg and a velocity of 0.3 cm/sec. The excess of Carbowax 20M was removed by rinsing the column with 3 ml of dichloromethane[24]. The column was coated with 0.3 ml of a 5% solution of hydrocarbon C_{87}[23] in *n*-hexane using a pressure of 30 cmHg with a velocity of 3 cm/sec (other data are given in Table I).

Column C (116.4 m × 0.24 mm I.D.). The column was coated with 0.3 ml of a 15% solution of Apiezon L in *n*-hexane using a pressure of 30 cmHg and a velocity of 3 cm/sec. The original column was cut into six parts and the regularity of the film thickness was checked by analysing *n*-octadecane at 180°C. The capacity ratios of the four centre pieces agreed to within ±2% relative[25]. These four pieces when coupled together with PTFE shrinkable tubing gave a 77.6-m column (column C in Table I).

Column D (92 m × 0.25 mm I.D.). The column walls were etched with methyl trifluorochloroethyl ether as described by Tesarik and Novotny[26]. The column walls were deactivated with Carbowax 20M[24] and coated with 0.3 ml of a 10% solution of Carbowax 20M in dichloromethane using a pressure of 30 cmHg and a velocity of 3 cm/sec. The original column was cut into six parts and the four centre pieces were coupled to produce a 56.6-m column (column D in Table I).

TABLE I

CHARACTERISTICS OF THE GLASS CAPILLARY COLUMNS FOR *n*-OCTADECANE (180°C)

Characteristic	*Apiezon L*		*Hydrocarbon C_{87}: column B*	*Carbowax 20M: column D*
	Column A	*Column C*		
$\bar{u}$ (cm/sec)	22.7	13.7*	18.5	11.0*
k'	3.45	3.86	1.07	4.38
n	346,700	289,700	342,000	123,300
n/L (m^{-1})	2840	3730	3670	2670
N	208,400	182,700	91,400	123,000
N/L (m^{-1})	1700	2350	980	2170
TZ	86**	41***	76**	38**

* Measured with nitrogen as carrier gas; hydrogen was used with the other columns.
** Found between *n*-C_{18} and *n*-C_{20}.
*** Found between *n*-C_{20} and *n*-C_{21}.

RESULTS AND DISCUSSION

To test the applicability of eqns. 4 and 10, the capacity factor (k') for *n*-octadecane was determined on two glass capillary columns coated with Apiezon L (column A) and hydrocarbon C_{87} (column B). The capacity ratios measured under the given conditions for C_{10}–C_{20} *n*-alkanes were almost independent of the inlet pressure, which is in contradiction to the results published for packed columns[4] and suggests that the inner diameter of the column and the film thickness of the stationary phase are fairly uniform along the whole column.

Knowing the capacity factors on the individual columns, measurements were

TABLE II

CHARACTERISTICS OF THE COMBINATIONS OF COLUMNS A AND B (180°C)

	Column order	
	AB	*BA*
$\bar{u}$ (cm/sec)	19.7	12.5
k'	2.66	2.31
k'_{calc}	2.71	2.49
n	567,000	736,000
n/L (m^{-1})	2640	3420
N	299,500	342,000
N/L (m^{-1})	1390	1590
TZ*	109	115

* Found between n-C_{18} and n-C_{20}.

performed on the two columns coupled in series, in the two possible orders (AB and BA). The results are given in Table II, together with the capacity factors of n-octadecane (k'_{calc}) calculated from eqns. 4 and 10 and the data in Table I. The agreement between the measured and calculated capacity factors is within $\pm 2\%$ relative for the column combination AB and within $\pm 8\%$ relative for the column combination BA, and thus seems to depend on the column order. It is better when the column having the larger capacity ratio (column A) precedes that with the smaller capacity ratio (B), which agrees with results published for packed columns[4] and corresponds to the fact that the solute spends a much longer time on the first column, on which it is more retained and through which the gas velocity is smaller than on the second column.

Thus, as predicted from eqn. 10, the change in the capacity factor with the

TABLE III

CHARACTERISTICS OF COMBINATIONS OF COLUMN C AND DIFFERENT PARTS OF COLUMN D (180°C)

	Carbowax 20M column (D) lengths (m)			
	15.5	*29.8*	*44.5*	*56.6*
$\bar{u}$* (cm/sec)	12.6	12.8	13.3	11.8
k'**	3.91	3.98	4.09	4.26
k'_{calc}**	3.90	3.94	3.97	4.02
n	323,000	380,000	400,000	420,000
n/L (m^{-1})	3480	3500	3270	3260
N	205,000	242,000	258,000	275,000
N/L (m^{-1})	2200	2200	2100	2050
TZ***	46	48	50	52

* Carrier gas was nitrogen.

** Found for 3,4,4′-trichlorobiphenyl (peak 33 in Fig. 4 and in Table IV), values calculated from eqn. 14.

*** Found between n-C_{20} and n-C_{21}.

column order is due only to the change in the average column pressures $\bar{P}_A$ and $\bar{P}_B$ resulting from reversal of the column order.

It is generally accepted that the separation number (TZ) characterizes the column separation power; however, TZ depends on the n-alkanes chosen for its calculation. For instance, on column A at 100°C we found $TZ = 75$ between n-C_{10}

TABLE IV

RETENTION INDICES OF SOME POLYCHLOROBIPHENYLS AT 180°C ON COMBINATIONS OF AN APIEZON L COLUMN AND CARBOWAX 20M COLUMNS OF VARIOUS LENGTHS (L_B)

Linear relationship between retention indices and Carbowax 20M column length.

Peak No.	*Identification**	*Equation*	*Correlation coefficient*
1	2,2′-	$I = 2.122\ L_B + 1630.2$	0.9996
2	2,3′-	$I = 1.804\ L_B + 1719.9$	0.9999
3	2,4′-	$I = 1.789\ L_B + 1739.9$	0.9997
4	2,2′,6-	$I = 2.305\ L_B + 1731.6$	0.9995
5	2,2′,5-	$I = 1.906\ L_B + 1792.9$	0.9997
6	2,2′,4-	$I = 1.789\ L_B + 1805.8$	0.9997
7	2,3′,6-	$I = 1.910\ L_B + 1809.8$	0.9997
8	2,2′,3-	$I = 2.189\ L_B + 1816.6$	0.9995
9	2,4′,6-	$I = 1.917\ L_B + 1834.8$	0.9997
10	4,4′-	$I = 1.806\ L_B + 1845.7$	0.9997
11	2,2′,5,6′-	$I = 2.042\ L_B + 1881.0$	0.9996
12	2,3′,5-	$I = 1.617\ L_B + 1890.5$	0.9997
13	2,2′,4,6′-	$I = 1.534\ L_B + 1897.4$	0.9996
14	2,5,4′-	$I = 1.925\ L_B + 1903.4$	0.9996
15	2,4,3′-	$I = 2.028\ L_B + 1904.8$	0.9986
16	2,5,4′-	$I = 1.632\ L_B + 1908.8$	0.9997
17	2,3,3′-	$I = 2.234\ L_B + 1911.1$	0.9998
18	2,4,4′-	$I = 1.532\ L_B + 1917.4$	0.9994
19	3,4,2′-	$I = 1.741\ L_B + 1918.5$	0.9997
20		$I = 1.883\ L_B + 1931.2$	0.9997
21		$I = 1.584\ L_B + 1955.0$	0.9996
22	2,2′,5,5′-	$I = 1.732\ L_B + 1954.9$	0.9998
23	2,2′,4,5′-	$I = 1.619\ L_B + 1968.1$	0.9996
24		$I = 1.542\ L_B + 1969.8$	0.9996
25	2,2′,3,5′-	$I = 1.984\ L_B + 1977.9$	0.9997
26	2,2′,4,4′-	$I = 1.517\ L_B + 1983.0$	0.9996
27	2,3′,5′,6-	$I = 1.829\ L_B + 1985.6$	0.9994
28	2,2′,3,4′-	$I = 1.867\ L_B + 1992.4$	0.9997
29	3,4′,5-	$I = 1.953\ L_B + 1998.5$	0.9996
30	3,5,4′-	$I = 1.881\ L_B + 2001.6$	0.9992
31		$I = 2.229\ L_B + 2003.4$	0.9998
32	2,6,3′,4′-	$I = 1.833\ L_B + 2012.8$	0.9997
33	3,4,4′-	$I = 1.789\ L_B + 2035.9$	0.9997
34	2,5,3′,5′-	$I = 1.914\ L_B + 2046.4$	0.9996
35	2,4,3′,4′-	$I = 1.591\ L_B + 2090.0$	0.9997
36		$I = 1.352\ L_B + 2091.5$	0.9995
37	3,4,5,2′-	$I = 1.500\ L_B + 2098.8$	0.9996
38	2,3′,4,4′-	$I = 1.821\ L_B + 2112.2$	0.9997
39	2,3,3′,4′-	$I = 1.716\ L_B + 2119.4$	0.9996
40		$I = 1.512\ L_B + 2131.4$	0.9987

* Positions of chlorine atoms in PCB molecule.

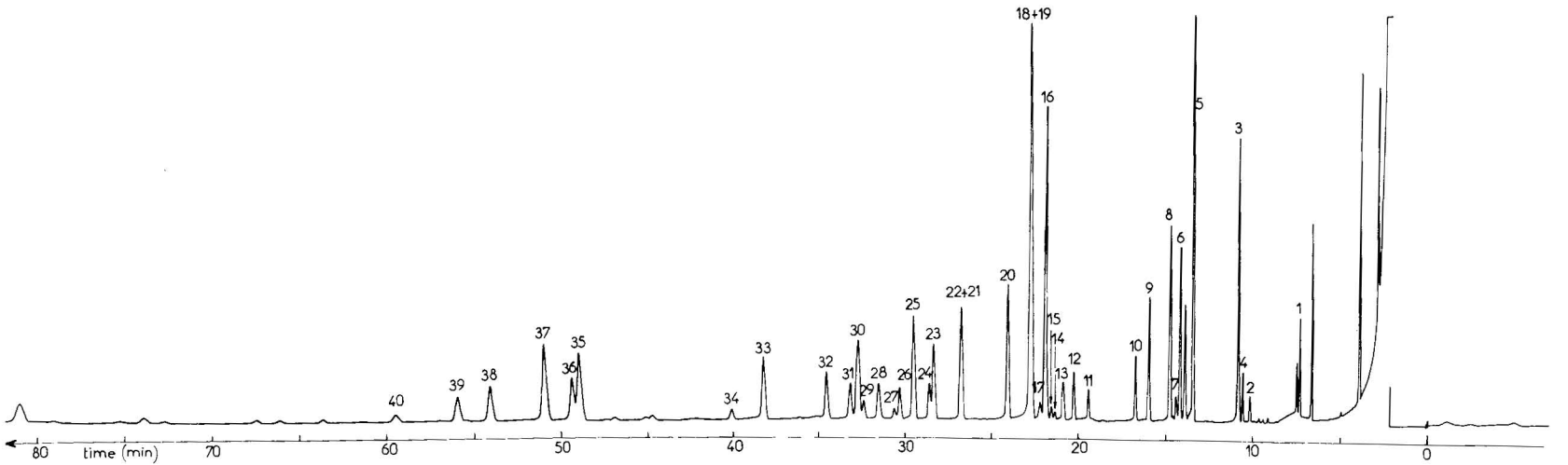

Fig. 4. Separation of a mixture of PCBs and *n*-alkanes on Apiezon L (column C) at 180°C. Peak numbers correspond to those in Table IV.

and n-C_{11} whereas at 180°C we obtained $TZ = 86$ between n-C_{18} and C_{20} (*cf.*, Table I). Therefore, the separation numbers given in Tables I–III do not characterize the maximal separation power of the columns, but permit an estimation of the column performances under the test conditions. As the evaluation of column separation power depends on several factors, our results concerning this problem will be discussed separately[27].

Some characteristics of the combinations of column C and an increasing number of pieces of column D are given in Table III. In this column series the first column (column C) contains Apiezon L but, owing to the smaller film thickness, it has a smaller capacity factor for n-octadecane than the various segments of column D, which are coated with Carbowax 20M. In spite of this the measured and calculated capacity factors are in very good agreement, as shown in Table III (difference 0.2–5%, relative). It can also be seen that the capacity factor does not vary linearly with the length of the Carbowax 20M column added to the Apiezon L column, but markedly faster.

Although there is no theoretical reason for this, it was interesting to examine whether the retention indices which are relatively independent of the average column pressure vary linearly with the length of Carbowax column added to the Apiezon L

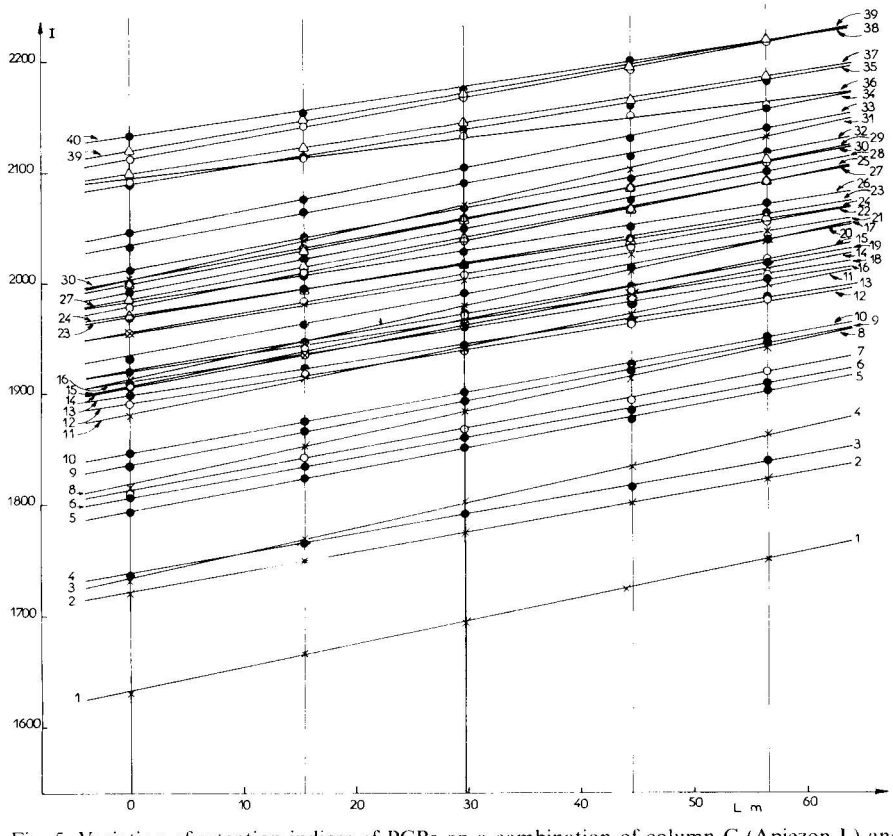

Fig. 5. Variation of retention indices of PCBs on a combination of column C (Apiezon L) and various sections of column D (Carbowax 20M) with the length of the columns (180°C).

column. Fig. 4 shows a chromatogram for the separation of a mixture of PCBs and *n*-alkanes on the Apiezon L column (column C), and the variation in the retention indices with increasing length of the Carbowax 20M column added to the Apiezon L column is given in Table IV. It can be seen that the retention indices increase linearly under the conditions used. The equations derived for these linear relationships are given in Table IV. The values of the correlation coefficients are excellent and there is an extremely small scatter of the experimental results, which originates mainly from experimental errors in the measurements and fluctuations in the experimental parameters during the analysis, as it is of the same order of the reproducibility of retention indices.

The slopes of the linear equations depend on the number of chlorine atoms and their positions on the diphenyl ring system. Use of the data in Table IV or Fig. 5 allows the determination of the optimal length of the Carbowax 20M column that permits complete resolution of the mixture. This optimization also involves the selection of the column temperature and the length of the Apiezon L column.

It must be emphasized that the linear dependence of the retention indices on the length of one of the two columns in the series is an experimental result valid for the particular analysis studied here, and it may be extrapolated to other situations only with caution. This is explained by the fact that the variation of the retention index is relatively slow, the largest change when a 56-m Carbowax 20M column is added to the Apiezon L column being an increase of 131 units, and the smallest change an increase of 85 units. More polar compounds could exhibit larger changes and the plot of I *versus* L_B some curvature.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS

j	Correction factor for gas compressibility (eqn. 6)
k	Column permeability ($= d_c^2/32$ for open-tubular columns) (eqn. 1)
k'	Column capacity factor (eqn. 5)
k'_A, k'_B	Capacity factors for columns A and B, respectively (eqn. 7)
k'_{AB}	Apparent capacity factor for the combination of column A followed by column B (eqn. 7)
L	Column length (eqn. 5)
L_A, L_B	Lengths of columns A and B, respectively (eqn. 1)
$\bar{P}$	Average column pressure (eqn. 8)
$\bar{P}_A$, $\bar{P}_B$	Average pressures of columns A and B, respectively (eqn. 8)
P_i, P_o	Inlet and outlet pressures of the column or column combination, respectively (eqn. 1)
P_a	Pressure between columns A and B (eqn. 2)
t_R	Retention time of a compound (eqn. 5)
$\bar{u}$	Average velocity of the carrier gas (eqn. 5)

$\bar{u}_A$, $\bar{u}_B$	Average carrier gas velocity through columns A and B, respectively (eqn. 7)
u_o	Carrier gas velocity at the outlet of the column or column combination (eqn. 1)
u_a	Carrier gas velocity between columns A and B (eqn. 2)
η	Carrier gas viscosity (eqn. 1)

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GAS–LIQUID–SOLID CHROMATOGRAPHY: EFFECTS OF ADSORPTION AND PARTITIONING

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SUMMARY

The relative effects of gas–solid adsorption and gas–liquid absorption on chromatography are studied. The system is a packed column of porous particles, whose pore surfaces are partially covered with a thin liquid film. Differential equations for the relevant transport processes are used to generate first and second temporal moment expressions for output peaks. These results reduce to more specialized expressions for moments for gas–liquid partition chromatography or gas–solid adsorption chromatography. Effect of partial coverage on retention time and height equivalent to a theoretical plate is discussed.

INTRODUCTION

In gas–liquid chromatography (GLC), a thin layer of liquid is spread on pore surfaces. As the amount of liquid is decreased, partial coverage of pore surfaces may develop so that contact between the gas and solid occurs. For the resulting gas–liquid–solid chromatography (GLSC), gas–solid adsorption effects will be added to the gas–liquid absorption (partitioning) effects. Since GLSC has several advantages over either GLC or gas–solid chromatography (GSC), its continued study is of considerable interest[1]. A significant problem of designing GLSC systems is predicting the optimum liquid–solid ratio of the packing material. A sound theoretical model is obviously desirable for solving this problem. The model presented in this paper describes the essential mass transfer resistances in GLSC, namely, longitudinal dispersion, mass transfer from the mobile phase to the particle surface and intraparticle (pore) diffusion. The rate of adsorption at the gas–solid interface is also included in the model. The equilibrium and rate processes are linear; this facilitates the analysis that allows the properties of the outlet peaks to be derived. While mathematical models that ignore mass transfer resistances may be solved for non-linear equilibrium relations[2], our belief is that the mass transfer resistances are no less important in many cases, and therefore worthy of serious attention.

In what follows we show how the model of gas–liquid partition chromatography[3] may be modified to include gas–solid adsorption effects. The partial differential equations representing mass balances in the interparticle and intraparticle voids are

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solved in the Laplace domain. The temporal moments are calculated from the Laplace transform expression for the gas concentration at the column exit. The normalized first moment is the retention time, which represents the cumulative capacity of the column. Column efficiency in the form of the height equivalent to a theoretical plate (HETP) is related to the second central moment, and the effect of liquid load is discussed.

THEORETICAL

Mathematical model

We consider a column of length L packed with porous spherical particles of radius R. The fraction of voids external to the particles is α, and the porosity of particles is β. The superficial velocity $u_0 = \alpha u$ is the volumetric flow-rate divided by the column cross-sectional area.

The point differential mass balance equation[4] for interparticle concentration $c(t,z)$ in the mobile (gas) phase is:

$$\alpha \frac{\partial c}{\partial t} + u_0 \frac{\partial c}{\partial z} = D_0 \frac{\partial c^2}{\partial z^2} - \frac{3}{R}^{(1-\alpha)} k_f [c - c_i (r = R)] \tag{1}$$

with initial condition:

$$c(t = 0,z) = 0 \tag{2}$$

and boundary conditions:

$$c(t,z = 0) = c_0(t) \tag{3}$$

$$c(t,z \to \infty) = \text{finite} \tag{4}$$

Continuity of flux[4] at the outer particle surface serves as a boundary condition that couples the concentration $c(z,t)$ to the intraparticle concentration $c_i(z,r,t)$:

$$D_i \left(\frac{\partial c_i}{\partial r}\right)_{r=R} = k_f[c - c_i (r = R)] \tag{5}$$

The intraparticle mass balance equation[4] must include absorption into the liquid film as well as adsorption at the exposed solid surface. In terms of the liquid phase concentration $c_l(t)$ and solid phase surface concentration $c_a(t)$, we have:

$$\beta \frac{\partial c_i}{\partial t} = D_i \frac{1}{r^2} \frac{\partial}{\partial r}\left(r^2 \frac{\partial c_i}{\partial r}\right) - V_l \frac{\partial c_l}{\partial t} - A_s \frac{\partial c_a}{\partial t} \tag{6}$$

with initial conditions:

$$c_i(r,t = 0) = 0 \tag{7}$$

$$c_l(t = 0) = 0 \tag{8}$$

$$c_a(t = 0) = 0 \tag{9}$$

In addition to eqn. 5 we have the boundary condition expressing symmetry at the particle center,

$$\left(\frac{\partial c_i}{\partial r}\right)_{r=0} = 0 \tag{10}$$

Instantaneous equilibrium is assumed to be established between the liquid and the gas phase within the pores,

$$K = c_l/c_i \tag{11}$$

From which we have

$$\partial c_l/\partial t = K\,\partial c_i/\partial t \tag{12}$$

Ignoring diffusion in the liquid film will usually be justified for the extremely thin films of GLSC. When the stationary liquid phase is near the monolayer level, its properties may be quite different from the bulk liquid. Here we assume that eqn. 11 applies to the monolayer with the expectation that the equilibrium coefficient K will have different values for monolayer and bulk phases. Such a continuum approach to equilibrium, as well as transport phenomena in thin films has been successful in other contexts (see *e.g.* ref. 5).

A first-order gas–solid adsorption rate expression[4] is assumed:

$$\partial c_a/\partial t = k_a\,(c_i - c_a/K_a) \tag{13}$$

The coefficients k_a and K_a represent the adsorption rate constant (cm/sec) and adsorption equilibrium constant (cm^3/cm^2), respectively.

The set of differential eqns. 1–13 can be solved in the Laplace domain:

$$\bar{c}\,(s) = \bar{c}_0\,(s)\,\exp\,(\lambda Z) \tag{14}$$

where λ is given by:

$$\lambda = \frac{u_0}{2D_0} - \left\{\left(\frac{u_0}{2D_0}\right)^2 + \frac{\alpha}{D_0}\left[s + \frac{3(1-\alpha)\,k_f}{\alpha R}\,(1 - P(s))\right]\right\}^{\frac{1}{2}} \tag{15}$$

with

$$P(s) = \frac{\sin h\,(bR)}{\dfrac{D_i\,b}{k_f}\cos h\,(bR) + \left(1 - \dfrac{D_i}{Rk_f}\right)\sin h\,(bR)} \tag{16}$$

$$bR = \left\{\frac{\beta R^2}{D_i}\,s\left[1 + \frac{V_l\,K}{\beta} + \frac{A_s}{\beta}\,\frac{k_a}{\left(s + \dfrac{k_a}{K_A}\right)}\right]\right\}^{\frac{1}{2}} \tag{17}$$

Moment expressions

Following the usual procedure[4] of using the Laplace transform as a generating function for moments, we obtain the following expressions for the first reduced moment, and second central moment:

$$\mu_1(z) = \mu_1(z = 0) + \frac{z}{u}(1 + \delta_0) \tag{18}$$

where:

$$\delta_0 = \frac{(1 - \alpha)\beta}{\alpha}\left(1 + \frac{V_l K}{\beta} + \frac{A_s K_A}{\beta}\right) \tag{19}$$

and

$$\mu_2'(z) = \mu_2'(z = 0) + \frac{2z}{u}\left[\frac{D_0}{u^2}(1 + \delta_0)^2 + \delta_1 + \delta_2\right] \tag{20}$$

where:

$$\delta_1 = \frac{1}{15}\left(\frac{1}{D_i} + \frac{5}{Rk_f}\right)\left(\frac{1 - \alpha}{\alpha}\right)\beta^2 R^2\left(1 + \frac{V_l K}{\beta} + \frac{A_s K_A}{\beta}\right)^2 \tag{21}$$

$$\delta_2 = \frac{(1 - \alpha)}{\alpha}\frac{K_a^2 A_s}{k_a} \tag{22}$$

In the absence of adsorption, $K_a = 0$, and the moment expressions obtained in this section reduce to those for a model that ignores liquid phase diffusion[3]. In the absence of the liquid phase, we have $V_l = 0$, and the moment expressions reduce to those of the adsorption model of Suzuki and Smith[4].

The change in first moment, $\mu_1(z) - \mu_1(z = 0)$, is the mean retention time t_R for the solute. For GLC, the retention time is:

$$t_R = \frac{z}{u}\left[1 + \frac{(1 - \alpha)\beta}{\alpha}\left(1 + \frac{V_l K}{\beta}\right)\right] \tag{23}$$

while for GLSC combining eqns. 18 and 19 indicates:

$$t_R = \frac{z}{u}\left[1 + \frac{(1 - \alpha)\beta}{\alpha}\left(1 + \frac{V_l K}{\beta} + \frac{A_s K_A}{\beta}\right)\right] \tag{24}$$

That is, the overall retention time is the summation of the individual retention times of the different capacities in the column. The mean region retention time is equal to the holdup in the region divided by the flow-rate through the system as a whole. For example, the retention time for the intraparticle gas phase is gas holdup in the pores divided by the throughput flow-rate:

$$t_g = \frac{z}{u}\frac{(1 - \alpha)\beta}{\alpha} \tag{25}$$

An important result for this system, which has no mass sources or irreversible sinks, is that the overall retention time (first moment) depends only on geometrical and equilibrium properties of the column and the packing and not on mass transfer properties. The first moment, therefore, does not depend on how fast equilibrium is developed in the column, and in fact can easily be established by a mass balance.

Effect of liquid load on retention time

Since retention time in a chromatographic column is the summation of the retention times of the different regions in the column, we may write:

$$t_R = t_m + t_g + t_l + t_a \tag{26}$$

where t_m = retention time of the mobile phase, t_g = intraparticle gas retention time, t_l = liquid phase retention time and t_a = retention time caused by adsorption.

The first effect of submonolayer concentration of a non-volatile liquid on a homogeneous adsorption medium is to decrease the retention time of eluates, because of the reduction of the specific area available to the gaseous adsorbate, *i.e.*, t_a decreases. Under the same conditions if the adsorbing medium is more porous, the rate of decrease of retention time is higher since high surface area is associated with the porous medium[1]. Moreover, the reduction of the solid surface involves the decrease of adsorption configurational entropy owing to a decrease in the number of possible ways of arranging molecules among the surface sites[1]. Hence there is an additional contribution to the decrease of retention of eluate which is significant when the first layer of macromolecules nears completion. Examples of decrease of retention time with increasing liquid/solid ratio are shown in refs. 6 and 7. As soon as a monolayer is formed, no further decrease of the mean retention is observed[1]. On the contrary, a slight but steady increase takes place as the percentage of the liquid is increased, *i.e.*, t_l dominates as δ increases steadily. If the liquid does not cover the solid surface evenly as a monolayer, but instead fills the pores before all the solid is covered, then the minimum obtained in the retention time *vs.* liquid load plot will be reached at a higher value of t_R, because of the gas–liquid partitioning mechanism in the liquid within the pores. Roughened or etched glass surfaces are suggested by Giddings[8,9] to distribute the liquid evenly over the surfaces.

Retention data is widely used in chromatographic studies to extract equilibrium data. Because the retention time for a conservative system (*i.e.*, one without sources or irreversible sinks) is easily written based on a mass balance, the moment method as described here simply conforms with the mass conservation law[3]. However, separation efficiency depends on band spreading, which is quantitatively represented by the second moment, and here the moment technique is of critical importance because of the complex way the transport coefficients appear in μ_2'.

Effect of liquid load on column efficiency

Following the usual procedure[10] we evaluate column efficiency with the expression for the HETP, which is given by:

$$h = L\mu_2'/(\Delta\mu_1)^2 \tag{27}$$

Substituting expressions for first and second moments into the above equation gives us:

$$h = A + B/u + Cu \tag{28}$$

where

$$A = \mu_2' (0)\, u^2/L\, (1 + \delta_0)^2 \tag{29}$$

$$B = 2D_0/\alpha \tag{30}$$

$$C = 2\, (\delta_1 + \delta_2)/(1 + \delta_0)^2 \tag{31}$$

Here we have considered the axial dispersion coefficient to be independent of velocity so that eqn. 28 for h has the standard form. More sophisticated representations for D_0 as a function of u are easily implemented[10].

It is easy to verify that the h *vs.* liquid phase percentage curve has a minimum HETP decreases for low liquid coverage, but a higher liquid percentage leads to an increase in resistance to mass transfer, thus C increases. Optimal liquid phase load will depend on the sample size: at a large sample size A is dominant and one should use a column packing with higher liquid phase concentration. For components retarded longer in the column, and at a higher flow-rate, C is dominant and one should use a column packing with lower liquid phase concentration[11]. The shape of the right-hand branch of the h *vs.* u curve varies with the liquid/solid ratio, *i.e.*, there is a steady increase in the slope of the right-hand branch of the curve as the amount of the liquid is increased[12] (*i.e.*, the C term increases as the liquid load increases).

LIST OF SYMBOLS

A_s	Particle surface area per unit volume, in contact with gas
c	Concentration of solute in interparticle void
c_i	Concentration of solute in intraparticle voids
c_l	Concentration of solute in liquid film
c_0	Concentration of solute at column entrance
D_i	Effective intraparticle diffusion coefficient
D_0	Effective axial dispersion
h	Height equivalent to a theoretical plate (HETP)
k_a	Adsorption rate constant
k_f	Gas mass transfer coefficient external to particles
$K = c_l/c_i$	Equilibrium partition coefficient
K_a	Adsorption equilibrium constant
r	Radial coordinate inside particle
R	Radius of particles
t	Time
t_R	Retention time
$u_0 = \alpha\, u$	Superficial velocity
V_l	Volume of liquid per volume of particle
z	Axial distance
α	Column void fraction

β	Particle porosity
μ_1	Normalized first temporal moment
μ_2'	Normalized second central moment

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CAPILLARY COLUMNS WITH IMMOBILIZED STATIONARY PHASES

II. PRACTICAL ADVANTAGES AND DETAILS OF PROCEDURE

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SUMMARY

Experimentally demonstrated advantages of stationary phase immobilization are considered, namely, prevention of thermal rearrangement, prevention of phase stripping, sharper peaks of solvent and major sample components, regeneration of columns by solvent washing, thick films produced from fluid phases, low bleeding rates, easy recoating and repeated coating to produce sandwich coatings. The cross-linking reaction which produces an immobilized stationary phase is strongly dependent on the structure of the stationary phase. OV-61 is representative of stationary phases having the maximum polarity allowing immobilization. The polarity limit will probably be shifted to higher values. Immobilization as a method of producing stable coatings on difficult to wet support surfaces is limited to support–stationary phase combinations providing nearly perfect wettability. The selection of a suitable peroxide for the cross-linking reaction is discussed. Based on arguments such as activity, cross-linking efficiency, influence of decomposition products, volatility, stability in solution, and safety of handling, a clear preference for a dialkyl peroxide, *e.g.*, dicumyl peroxide, is indicated. Appropriate peroxide concentrations are discussed, and detailed instructions for the immobilization procedure are given. Particular attention is paid to solvent washing of the immobilized stationary phase columns.

INTRODUCTION

Immobilization of stationary phases seems to generate wide interest. Although some of this interest is justified, some is exaggerated and only practical experience will create a realistic picture of the nature and extent of the advantages offered by the technique. To increase the availability of experimental information, the results presented have supplemented those in our previous paper[1].

MERITS OF IMMOBILIZATION

As stated previously[1], immobilization is expected to offer two new possibilities: first, stabilization of the coating in cases where the wettability of a support surface for

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a given liquid is not sufficient to ensure film stability, and second, non-extractability of the immobilized coating.

In our hands progress has proceeded primarily with the second aspect, advances with the first being slow. However, we consider that the modest results of the attempts to overcome the difficulties of insufficiently wettable, inert, thermostable surfaces by immobilization do not indicate an unrealistic expectation but rather the necessity for more fundamental work.

Below we list the advantages of the immobilization we have observed on our laboratory.

Prevention of thermal rearrangement

It is well known that the separation efficiency of freshly coated columns may decrease during the first conditioning, even when the support is sufficiently wettable for the given stationary phase. The decrease is most pronounced in the first few hours, then the efficiency tends to approach a plateau. As a rule, the decrease is smallest on smooth support surfaces and increases with increasing roughness. Further, it is also smallest with gum stationary phases[2] and increases with increasing fluid character of the phase.

The phenomenon is termed thermal rearrangement to account for its probable source. Prolonged heating may cause the stationary liquid to assume its most stable distribution which is, even under ideal wettability conditions, not necessarily identical with a coherent film of constant thickness. Deviation from ideal film symmetry is revealed by a reduction in separation efficiency.

Provided that this model explanation is correct, immobilization is expected to minimize thermal rearrangement as it is the final component in the sequence fluid film/gum film/immobilized film. The empirical verification of this hypothesis will require broad statistical comparisons. We are at present working primarily with gum stationary phases on smooth surfaces. As thermal rearrangement is very small anyway under these conditions, it is not easy to detect significant changes caused by immobilization. The differences will increase when the work expands to more polar stationary phases which are available as fluids and may require roughened surfaces. Our preliminary results with such stationary phases permit us to state that the concept of thermal rearrangement is correct and that immobilization plays the expected role.

Prevention of phase stripping

The danger of damaging a column by allowing an appreciable sample volume to be condensed in the column inlet section is well known. In the course of large-volume on-column or splitless injections, the danger can hardly be totally excluded, even with very careful planning and manipulation. Probably the most direct and clear-cut merit of immobilization is the complete avoidance of this danger. As far as physical film modification is concerned (simultaneous heavy contamination is a separate problem), immobilized coatings are positively resistant to any conceivable stress exerted by sampling.

Behaviour under overlading

Heavy overloading causing large amounts of sample to be dissolved in the

stationary phase is related to but different from the phenomenon discussed in the previous section as long as the influence on the stationary phase is reversible and no dislocation of coating material occurs.

A valuable, though hardly expected, influence of immobilization on overloading was first observed by Blum[3]. The influence has nothing to do with the overloading mechanism causing broadened, in most instances leading, peaks. A large amount of dissolved sample material seems to cause a transient structural modification, possibly swelling, of the stationary phase. It seems that the original structure of the stationary phase may be restored only with some delay. This delay results in tailing, which occurs on all columns, including those from which substances of virtually all classes elute without tailing as long as overloading is avoided. The phenomenon is commonly known from the difficulty of detecting (and, particularly, quantifying) minor sample components eluted soon after a major component (see Fig. 1). The tailing effect is greater for the solvent peak (which may, however, also be caused by unsatisfactory equipment or manipulation).

It seems that an immobilized coating is structurally less influenced by a large amount of solute. Elution is less delayed, resulting in a rapid return to an undisturbed baseline.

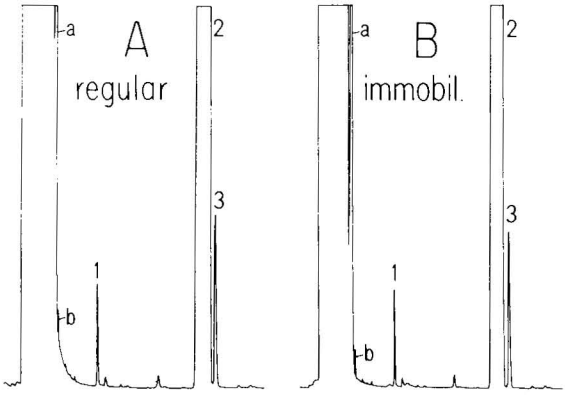

Fig. 1. Difference in resolution following bands with high solute concentration. Columns A and B are identical (16 m × 0.32 mm I.D., persilanized, 1.8-μm OV-1) except that A is not cross-linked and B is immobilized with 0.4% DCUP at 160–180°C. 1 = Toluene; 2 = ethylbenzene; 3 = *p*-xylene; 2 and 3 are present in a ratio of 500:1. Solvent: *n*-hexane (with impurities a, b). Column temperature, 40°C; 2.0 μl injected, splitless, 30 sec; carrier gas, hydrogen; standard flow-rate (0.5 m/sec). Compared with B, the stationary phase in A shows delayed recovery from heavy loading with solute. The effect is best seen at the rear end of the solvent band (peak shape, positions of a and b), and less clearly by the ethylbenzene–*p*-xylene resolution (2; 3). The poorer resolution by A seems to be caused by a generally broadened peak 2.

Solvent washing

Large-volume injections often cause damage to the column, which has nothing to do with phase stripping or overloading. The typical symptom is an unchanged separation efficiency and increased adsorptive activity which may affect only certain sample components. In a very general (probably oversimplified) way, we attribute the damage to low-volatility or non-volatile sample components contaminating the coating. In certain instances a sample clean-up may prevent the phenomenon. In other

instances the only remedy is to break away a length of the column inlet. The lifetime of routinely used columns is often limited by an excessively reduced column length resulting from periodically repeated "therapy".

Fig. 2 shows an example of real "therapy" that can be given to columns with immobilized coatings. The damage, resulting in increased adsorption activity from an unknown cause, could not be repaired by discarding an inlet section of reasonable length (1.5 m). In contrast, a rapid (2 h) wash totally restored the original column quality.

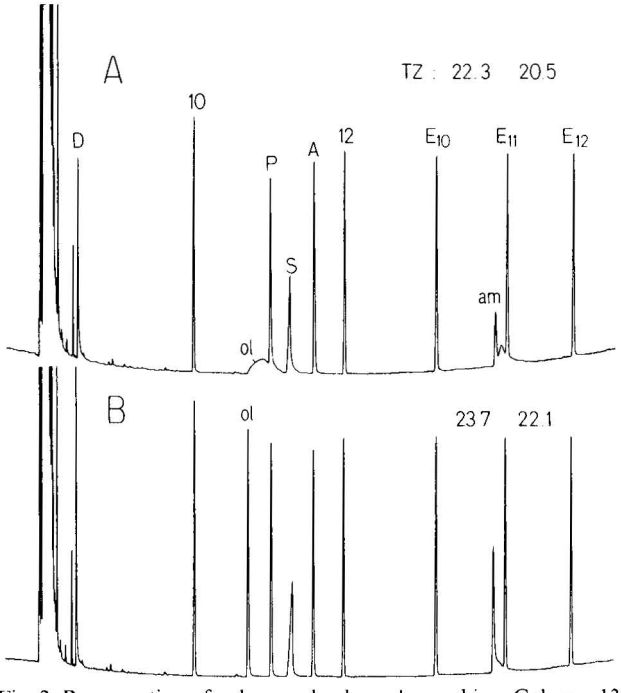

Fig. 2. Regeneration of a damaged column by washing. Column, 13 m × 0.32 mm I.D.; persilylated with HMDS; 0.3-μm OV-1, cross-linked with 0.2% of DCUP at 160°C. Comprehensive, standardized quality test[4]. (A) Test after 3 weeks of continuous analysis of non-ionogenic detergents (free polyethoxylated nonylphenols) extracted from water[5]. Deteriorating results (poorer separation and quantification of detergents) necessitated a quality test, which showed severe adsorption of the slightly basic hydroxyl function (ol = 1-octanol), and weaker adsorption of more acidic hydroxyl groups (D = butanediol; P = dimethylphenol; S = ethylhexanoic acid). The basic substances (A = dimethylaniline, am = dicyclohexylamine) were even less affected. The alkanes (10 = decane; 12 = dodecane) and the esters (E_{10}–E_{12} = decanoic, undecanoic and dodecanoic acid methyl esters) were symmetrically eluted with nearly standard separation efficiency. The nature of damage was unknown; earlier experience had shown that discarding a 1–1.5 m long inlet section did not help, *i.e.*, the damage was not restricted to the inlet section. (B) The same test after washing the entire column with methylene chloride, methanol and *n*-pentane (for details, see text) for 1.5 h. The original quality was restored (note particularly ol), with a slightly increased separation efficiency. The elution temperature (film thickness) was not reduced. The detergents were again analysed with perfect results.

Although this example is not an isolated one, we are not yet able to discuss the range of applicability of the "therapy". We pressume that damage may also be caused by insoluble contaminants. Rules concerning the application of solvent washing (in-

cluding solvent selection; see also the last section) have to be deduced from numerous long-term experiments.

Thick films produced from liquid phases

Gum stationary phases may be considered to be intermediates between fluids and immobilized films. Consequently, gums could be regarded as being dispensable, as immobilized coatings can be obtained directly from fluid phases. The preparation of immobilized coatings from fluid phases even offers a distinct advantage: much thicker films can be produced with fluid phases than with gum phases, owing to the much lower viscosity of solutions of fluid phases.

Our experiments have confirmed these expectations. However, practical realization is not necessarily easy. The period between the static deposition of the fluid film and the cross-linking reaction is a critical time during which the fluid phase may assume an irregular distribution or collapse. Preventing the trouble by adding a peroxide with a low decay temperature is feasible, but this creates the danger of premature cross-linking and consequent column plugging during static coating.

We have had complete success in preparing thick films from fluid phases after modifying three aspects of the procedure developed for gum stationary phases:

(1) Premature film breakage is delayed by adding some gum stationary phase. For example, a 3% coating solution (which can hardly be manipulated when prepared from a pure gum stationary phase) works perfectly when prepared from 2% OV-101 (fluid) and 1% OV-1 (gum).

(2) It is advantageous to use the less stable BP instead of DCUP (see *Selection of the perioxide*) for cross-linking.

(3) To produce an equivalent cross-linking effect, roughly three times more peroxide is required with OV-101 than with OV-1.

Bleeding behaviour

Immobilized coatings have reduced bleed rates. We assume that cross-linking prevents volatilization of fragments of the polymer chains produced by thermal or chemical breakdown.

In addition to this generally different bleeding behaviour, a substantially more important difference is associated with periodic solvent washings during long-term use. Thorough washing may eliminate both causes of bleeding, *viz.*,low-molecular-weight breakdown products of the stationary phase, and active, non-volatile substances (sample by-products, chemically altered stationary phase) which promote polymer breakdown. Accordingly, immobilized coatings offer an entirely new potential in the field of low-bleed capillary gas chromatography (GC), the practical utilization of which is far from being realized at present.

Recoating; repeated coating

The problem of recoating apolar silicone columns has a complex history. For many years very poor columns were obtained when expired silicone columns were washed and recoated. The effect was attributed to so-called autophobicity[6]. It was the success of Schomburg *et al.*[7], who introduced a column preparation procedure based on the recoatability of a silicone-treated surface, that caused us to re-examine the situation. We found that recoating was practically impossible on the more active

support surfaces that we had used for many years. On persilylated surfaces it is feasible, but the quality of the recoated columns is frequently below the original quality.

A clear advantage of immobilization is that it allows the reliable production of high-quality columns on washed support surfaces, even those which have had previous silicone coatings. It seems that only the use of stationary phase immobilization conditions will permit the old "recoatability problem" to be finally overcome.

A second (immobilized) coating may also be produced on a first layer which cannot be extracted because of cross-linking. This process results in an addition rather than in replacement. Consequently, it might be termed repeated coating instead of recoating. Our experience is not sufficient to discuss the potential of repeated coating. We state simply that the technique works easily and reliably. Its primary effect is, of course, an increased film thickness. Additional, more complex effects are observed when the stationary phase used for the second coating differs from the first one. We have produced such mixed phases (sandwich coatings) without difficulty. However, we have not yet investigated their practical applicability.

We repeat that all of the advantages of immobilization listed have been experimentally proved. Of course, this does not mean that the list is complete. An important advantage is expected in HPLC on capillary columns.

ROLE OF THE STATIONARY PHASE

The dependence of immobilization on the structure of the stationary phase is strong. However, this is not surprising in view of the established knowledge in the field of manufacturing silicone rubbers[8]. With adaption for the application to capillary GC, this knowledge may be summarized as follows.

The most frequent cross-linking reaction induced by peroxy radicals occurs between methyl groups of neighbouring silicone molecules. It is probable, though not proved, that it also occurs between methyl groups of the silicone and those of the persilylated support surface. This would then result in surface bonding rather than cross-linking.

The cross-linking between methyl groups is effectively hindered by phenyl side-groups of the polysiloxane chain and, less effectively, also by cyanoalkyl groups. In contrast, vinyl side-groups are even more reactive than methyl groups as points for cross-linking.

The practical importance of these rules may be demonstrated by describing the cross-linking behaviour of several common silicone stationary phases. The model substances for easy cross-linking are pure methyl polysiloxanes such as SE-30, OV-1 and OV-101. The ease of reaction is indicated by the small amount of peroxide [*e.g.*, 0.4% (w/w based on the stationary phase)] required to produce a non-extractable coating.

A 5% degree of phenyl substitution, as present in SE-52 and OV-73, hinders cross-linking to a surprising extent. Roughly three times more peroxide (1.0%) is required to assure a comparable cross-linking effect.

A 2% degree of vinyl substitution, combined with 5% of phenyl substitution, as in SE-54, requires a similar or even lower peroxide concentration compared with that needed for pure methyl silicones. This means that the high reactivity of vinyl

groups fully outweighs the hindering effect of the phenyl groups. It is conceivable that the high durability of SE-54 columns was caused by immobilization which automatically occurred during routine use, probably under the influence of peroxides present in the solvents injected. We know that some of our silicone columns, particularly the best of them, were non-extractable after prolonged use.

With increasing phenyl concentration, cross-linking becomes rapidly difficult or unfeasible. As reported previously[1], the limit may be represented by OV-61 (33% phenyl). Fig. 3 gives a visual indication of the situation. A 0.2-μm film of OV-61, containing 4% of peroxide (*i.e.*, 20 times the amount for OV-1), was deposited and heated to induce cross-linking. Washing with methylene chloride reduced the film thickness to 0.07 μm. Substantial, but only partial, cross-linking had taken place. While it remains an open question whether or not OV-61 should be termed suitable for immobilization, all stationary phases with lower phenyl contents than OV-61 can be reasonably immobilized.

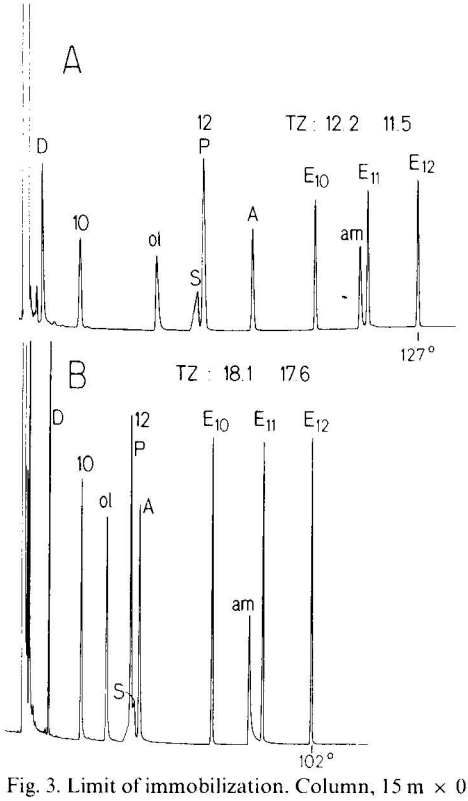

Fig. 3. Limit of immobilization. Column, 15 m × 0.32 mm I.D.; persilylated with pure DPTMDS[9]; 0.2-μm OV-61 containing 4% of DCUP. Comprehensive quality tests; for substances see Fig. 2. (A) Test run immediately after immobilization at 160–180°C. Reasonably symmetrical, but intensely broadened peaks, the early peaks more affected than the later ones; typical result from irregularly distributed stationary phase. Standard separation efficiency should be about TZ = 26–28. (B) Test after washing with methylene chloride. The elution temperature of E_{12} was reduced by 25°C, indicating a 65% loss of stationary phase, *i.e.*, about 35% of the stationary phase is immobilized. The striking increase in separation efficiency demonstrates that the immobilized part of the stationary phase covers the support with a reasonably even distribution, whereas the extractable part formed the bulk of the irregularly distributed material.

Phases with higher phenyl contents, *e.g.*, OV-17, are clearly unsuitable for immobilization. Treatment with 5% peroxide did not result in a significant non-extractable layer. OV-225 differs from OV-17 by having half the phenyl groups replaced with cyanoethyl groups. This change is expected to facilitate cross-linking. Nevertheless, OV-225 can hardly be immobilized by reaction with peroxide.

Introducing vinyl groups into more polar silicones should make their immobilization feasible. It may be challenging work to extend the polarity limit of stationary phases amenable to cross-linking.

WETTABILITY *VERSUS* IMMOBILIZATION

We have looked for experimental evidence to demonstrate the stabilization by cross-linking of a film which is unstable on a given support surface. For this purpose, OV-61 was intentionally deposited on a phenyl dimethyl persilylated surface[9] (Fig. 3) which is not wettable by OV-61. The wettability gap in this surface–stationary phase combination is relatively small, as shown by the fact that the same surface is, at least temporarily, wettable with OV-7. The answer to the question of whether or not this small gap can be bridged by immobilization was not simple.

The coating produced under intense cross-linking conditions showed a separation efficiency of only 40% of the efficiency expected for a column of this geometry. This demonstrates that the lack of intermolecular attraction between the support and the stationary phase was great enough to cause the liquid film to break up before this could be prevented by cross-linking. Some similar experiments supported this conclusion. This leads to the empirical rule that (unfortunately) immobilization produces ideal coatings only with surface–stationary phase combinations that ensure full or almost full wettability without additional stabilization. "Almost full" wettability (without cross-linking) leads to columns that have the standard separations efficiency after mild conditioning but show continuously decreasing efficiency during prolonged use. Only in this particular instance is cross-linking able to replace full wettability.

However, Fig. 3 shows a surprising side effect. We found that the separation efficiency increased by 50% after washing; there is no obvious explanation for this. A decrease in film thickness from 0.2 to 0.07 μm has a negligible effect; the 25°C lower elution temperature causes only an 8–10% higher efficiency. A possible explanation for the increase may be the following: for an unknown reason the immobilization reaction may have been preferentially effective between the support and the stationary phase. The washing then preferentially eliminated the irregular stationary phase accumulations caused by film breakage, whereas a more evenly distributed, and non-extractable, layer remained on the support surface. Of course, this tentative interpretation is a guideline for further experiments rather than a definitive conclusion.

IMMOBILIZATION PROCEDURE: FURTHER INFORMATION

Selection of the peroxide

Of a large number of available peroxides we tested four products with different decay temperatures. Table I lists the temperatures at which half the amount of peroxide was decomposed in 1 h. (Information supplied by ELFA-Oxychemie, Beethovenstrasse 48, 8002 Zürich, Switzerland.)

TABLE I

DECAY TEMPERATURES OF DIFFERENT PEROXIDES

*Product**	*Abbreviation*	*Active oxygen (%)*	*Decay temperature (°C)*
Bis(2,4-dichlorobenzoyl) peroxide	DClBP	4.2	72
Dibenzoyl peroxide	BP	6.4	91
Dicumyl peroxide	DCUP	5.7	136
Di-*tert.*-butyl peroxide	DTBP	10.7	146

* Available from ELFA-Oxychemie, Zürich, Switzerland.

Below we present the practical arguments contributing to the selection of a peroxide.

"*Activity*". There may be the spontaneous idea that peroxides with low decay temperatures are more active and therefore more efficient cross-linking agents. In contrast, we have found the cross-linking efficiency to be surprisingly little dependent on decay temperature, while being roughly proportional to the content of "active oxygen", at least when apolar silicones are treated.

Efficiency of difficult cross-linking. Cross-linking of strongly hindered stationary phases such as OV-61 works slightly better with perioxides that have high decay temperatures. A possible explanation may be that increased molecular mobility counteracts the hindrance.

Role of decomposition products. As reported previously[1], increased amounts of peroxide produce columns of increased activity. It seems reasonable to attribute this activity to the decomposition products of the perioxides. The explanation is supported by the fact that the dialkyl peroxides (DCUP and DTBP) cause less activity than diacyl peroxides (DClBP and BP). The difference may be due to the breakdown products, which are alcohols and ketones from dialkyl peroxides and mainly benzoic acid derivatives from diacyl peroxides.

Volatility. For experimental convenience and in order to remove by-products, some gas flow through the column during the immobilization treatment, even at elevated temperature, should not be precluded (see below). Consequently, relatively volatile peroxides such as DTBP are less desirable.

Stability of solution. It is convenient to add the peroxide to the stationary phase as a 1–2% solution. Alkanes would be the ideal solvents for this purpose because of their minimal contribution to the peroxide decay. However, most peroxides require more polar solvents. Our preferred solvent is toluene. A 2% solution of BP in toluene stored in the dark at 4° C showed signs of alteration (colour, precipitation), whereas a correspondingly treated solution of DCUP was totally unchanged after 3 weeks.

Safety of handling. Peroxides are generally classified as hazardous chemicals. Although the handling of all listed peroxides is safe, provided some elementary precautions are observed, the increasing safety with increasing decay temperature may be of practical interest. For instance, the increased safety may, in certain countries, simplify the shipping conditions.

Summarized comparison. The combination of the listed arguments yields a clear preference for DCUP (high efficiency, inactive decomposition products, low volatility, relatively stable solution, relatively safe handling).

Concentration of the peroxide

We have insufficient experimental evidence to present consistent information about this essential aspect. We suspect that this information will be complex, as different types of application of the columns may require differently conducted immobilization reactions.

One reasonable argument (although probably not the only argument) for optimizing the peroxide concentration is to use the lowest amount of peroxide that just ensures an almost non-extractable coating (see Table II).

TABLE II

MINIMAL CONCENTRATIONS OF DCUP (WEIGHT PER UNIT WEIGHT OF STATIONARY PHASE) PRODUCING A 90–100% NON-EXTRACTABLE COATING

Stationary phase	*Solvent*	*Minimal concentration (%, w/w)*
SE-54	*n*-Pentane	0.2
SE-30, OV-1	*n*-Pentane	0.4
SE-52, OV-73	*n*-Pentane	1.0
OV-3	Diethyl ether	4.0
OV-7	Diethyl ether	10.0

Immobilization reaction

As recommended previously[1], the peroxide is added to the stationary phase solution immediately before static coating. It should be noted that with a critical combination of parameters favouring cross-linking (reactivity of stationary phase, activity and concentration of peroxide, influence of solvent, column temperature during pumping), plugging may occur due to premature cross-linking in solution.

We have tried to eliminate partial evaporation or dislocation of the peroxide by carrying out the immobilization in the closed column. After pumping, we flushed the column with nitrogen and closed it with PTFE caps or sealed it in a flame under vacuum. All of these trials yielded poorer results (reduced cross-linking effect and increased activity). It seems that a by-product (water?) of the reaction should be continuously eliminated.

We have simplified the previously recommended cross-linking procedure. For DCUP, the statically coated column is mounted in a gas chromatograph with the exit end disconnected, and is flushed for 5–10 min using twice the regular carrier gas flow-rate. The flow-rate is reduced to the minimum that can be reliably maintained (0.02–0.1 ml/min). The oven is immediately heated to 160°C and, after 1 h, is heated to 180°C. Depending on the equipment, the carrier gas flow-rate should be checked periodically. After 1 h at 180°C, still with a low carrier gas flow-rate, the flow-rate is increased to the normal level for at least 1–2 h. The temperature may be kept at 180°C

if a fresh test is of interest, or may be increased to any desired conditioning temperature. The column exit is then connected to the detector and a test is run.

We suspected that the column ought to be washed before more intense conditioning to prevent peroxide by-products from undergoing high-temperature reactions. However, we have no experimental evidence that supports this precaution. Thus, it remains up to the user to decide whether he wants to wash the column immediately, *e.g.*, to check the degree of immobilization, or whether he prefers to apply the first washing when extraction of a suspected contaminant becomes desirable.

During the entire treatment we use our regular carrier gas (hydrogen). Our initial thoughts that hydrogen might inactivate some peroxides have not been verified experimentally.

Solvent washing

Contaminants, including non-immobilized polymer molecules, may be captured in the cross-linked network. To facilitate their elimination, we use a solvent that is known to cause swelling of the coating (methylene chloride). For a rapid, general-purpose washing we use the following procedure. Twice the column volume of methylene chloride is forced through the column at a rate of 2 cm/sec and is immediately followed by one volume each of methanol, methylene chloride, and *n*-pentane. *n*-Pentane serves to flush the more effectively retained methylene chloride.

If a contaminant of low solubility is suspected, a large-volume washing overnight is applied.

The selection of solvents to achieve specific cleaning effects may hold considerable potential. Again, our results so far do not permit a consistent discussion of this matter. An example may demonstrate that solvent selection is not just secondary work. An overnight washing of an OV-1 column with acetone produced a column with drastically increased bleeding. The bleeding decreased during conditioning for several days, but did not return to the low value which is typical for this type of column.

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COMPORTEMENT DE PHASES STATIONNAIRES

I. RELATIONS AFFINES ENTRE DONNÉES DE RÉTENTION DE DÉRIVÉS DU BENZÈNE

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SUMMARY

Behaviour of stationary phases. I. Linear relationships between retention data of benzene derivatives

Retention times of about 140 benzenic mono- and polysubstituted compounds have been determined on a collection of stationary phases under isothermic conditions.

The "numerical taxonomic aggregation" methods applied to these data lead to a classification of the stationary phases, in accordance with a measure of association between pairs of variables and an amalgamation rule for clustering. Three classes of phases (polar, non-polar and polyfluorinated) appear on the dendogram.

Using the retention data relative to two phases, φ_1 and φ_2, we show that a linear relationship between the two series of data is obtained whenever the two phases belong to the same cluster.

This relationship

$$\text{Log } t'_{Z0}\ (\phi, Z, \varphi_1, T) = a \log t'_{Z0}\ (\phi, Z, \varphi_2, T) + b$$

(where coefficients a and b are constant in relation to Z, but vary with ϕ, φ_1, φ_2 and T) constitutes a general model, applicable to all benzenic compounds (mono-, di- or trisubstituted) under examination.

The diversity of experimental conditions in the determination of retention data led us to a general updating of the various ways of expressing the retention, by

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simultaneously studying the effects of the temperature T and/or those of the nature of the stationary phase φ. We obtain a linear relationship

$$Gr_1\ (Sq, Z, \varphi_1, T_1) = \alpha\ Gr_2\ (Sq, Z, \varphi_2, T_2) + \beta$$

in which Gr_1 and Gr_2 are either retention indexes, retention volumes or retention times, and Sq represents any carbon skeleton bearing the function Z.

Such a relationship permits the comparison of interlaboratories results. Thus, although the characteristics of the columns and the experimental conditions vary, we obtain very satisfactory linear relationships between our own data expressed as log (reduced time) and those from the literature expressed either as indices or as log (retention volume) for stationary phases belonging to the same taxonomic class.

INTRODUCTION

La comparaison du comportement chromatographique de deux séries ($ROCH_3$ et ROC_2H_5) d'éthers aliphatiques RZ_1 à celui de familles de composés RZ_2 (Z_2 est différent de la fonction alkoxyde), nous a permis antérieurement[1] de mettre en évidence l'existence de relations affines du type

$$\log t'_{R_0}\ (R, Z_1, \varphi_1, T_1) = a_1 \log t'_{R_0}\ (R, Z_2, \varphi_2, T_2) + b_1 \qquad (1)$$

où RZ désigne les produits chromatographiés (Z_1 et Z_2 sont les fonctions chimiques des séries RZ_1 et RZ_2 de composés; R représente la chaîne alkyle de structure quelconque). T_1 et T_2 sont les températures de travail, φ_1 et φ_2 les phases stationnaires. t'_{R_0} représente le temps de rétention réduit relatif du composé RZ, c'est à dire le temps t_{RZ} mesuré expérimentalement rapporté à celui t_{R_0Z}, du composé de réfrence R_0Z:

$$t'_{R_0}\ (R, Z, \varphi, T) = \frac{t_{RZ}\ (\varphi, T) - t_{CH_4}\ (\varphi, T)}{t_{R_0Z}\ (\varphi, T) - t_{CH_4}\ (\varphi, T)} \qquad (2)$$

a_1 et b_1 sont fonction de Z_1, Z_2, φ_1, φ_2, T_1 et T_2, mais sont constants vis à vis de R.

Haken *et al.*[2] ont montré récemment que la relation 1 avait un caractère plus général.

Dans le présent mémoire nous mettons en évidence que, pour une population RZ, où R est fixe et Z une fonction chimique variable, il est possible d'obtenir des relations affines analogues à 1 et/ou de dégager, en fonction de la nature de la phase φ, des similitudes ou des divergences de comportement chromatographique.

Pour réaliser cette recherche nous avons retenu un ensemble de substances benzéniques (R = groupe phényle ϕ). De par leurs propriétés structurales ces composés présentent en effet l'avantage de permettre la détermination simultanée de l'influence des groupes fonctionnels Z sur le phénomène de rétention et de celle des interactions liées à la nature et à la disposition relative de ces substituants.

Les composés aromatiques n'ayant pas été l'objet d'une étude systématique en chromatographic, les information disponibles[3-8], sont très fragmentaires.

Aussi, pour disposer d'un lot de données expérimentales aussi homogène que possible, nous avons procédé à la mesure des grandeurs de rétention de 16 composés monofonctionnels (la majorité des groupes fonctionnels courants de la chimie organique est ainsi représentée), de 110 composés difonctionnels et de 15 trifonctionnels.

A l'aide des données recueillies ainsi à partir d'une population chimique dérivant du benzène, l'examen de l'analogie de comportement des phases stationnaires est alors basé sur la recherche de relations affines à l'aide de la relation 3:

$$\log t'_{Z_0}(\phi, Z, \varphi_1, T) = a \log t'_{Z_0}(\phi, Z, \varphi_2, T) + b \tag{3}$$

où t'_{Z_0} est le temps réduit relatif déterminé par rapport à celui du benzène ϕH, la fonction de référence Z_0 est par conséquent dans ce cas égale à H.

$$t'_H(\phi, Z, \varphi, T) = \frac{t_{\phi Z}(\varphi, T) - t_{CH_4}(\varphi, T)}{t_{\phi H}(\varphi, T) - t_{CH_4}(\varphi, T)} \tag{4}$$

Les coefficients a et b sont fonction de ϕ, φ_1, φ_2 et T, mais constants par rapport aux fonctions chimiques Z.

A partir de l'examen du comportement de 16 dérivés monofonctionnels du benzène vis à vis de 21 phases stationnaires, à l'aide d'une part de la relation du type 3 et d'autre part des méthodes taxonomiques d'agrégation, nous classons les phases stationnaires en trois groupes. Ces résultats nous permettent de sélectionner neuf phases pour l'étude du comportement des substances polyfonctionnelles.

Pour des intervalles de température faibles, les grandeurs de rétention Gr varient linéairement selon

$$\mathrm{Gr}(\varphi, T_1) = f\,\mathrm{Gr}(\varphi, T_2) + \text{constante}$$

D'autre part, pour une même phase stationnaire φ, et à une température T, et par définition, les logarithmes des volumes de rétention ou des temps de rétention sont reliés linéairement entre-eux et aux indices de rétention

$$\mathrm{Gr}_1(\varphi, T) = f\,\mathrm{Gr}_2(\varphi, T) + \text{constante}$$

Une conséquence de ces deux relations et de la relation 3 conduisent à

$$\mathrm{Gr}_1(\mathrm{Sq}, Z, \varphi_1, T_1) = \alpha\,\mathrm{Gr}_2(\mathrm{Sq}, Z, \varphi_2, T_2) + \beta \tag{5}$$

qui résulte de la combinaison de relations affines dont les termes variables sont ramenés à un seul paramètre.

Dans la relation 5 Gr_1 et Gr_2 représentent la donnée de rétention qui peut être exprimée sous diverses formes (indice, volume, temps...), Sq symbolise le squelette carboné fixe, φ_1 et φ_2 sont deux phases stationnaires appartenant à une même classe taxonomique. Comme précédemment, α et β sont constants par rapport à la fonction chimique Z, mais liés à Sq, φ_1, φ_2, T_1 et T_2.

La confrontation de nos propres données avec celles publiées par d'autres chercheurs nous permet d'illustrer par exemple les relations affines

$$\delta I_Z\ (R, \varphi, T_1) = I_{RZ} - I_{RH} = \alpha_3 \log t'_{Z0}(R, Z, \varphi, T_2) \quad (6)$$

dans laquelle deux paramètres varient, ainsi que les relations plus générales où Gr, φ et T varient simultanément

$$\log V_N (\phi, Z, \varphi_1, T_1) = \alpha \log t'_{Z0} (\phi, Z, \varphi_2, T_2) + \beta$$

et qui demeurent valables pour des supports de phases stationnaires divers, des taux d'imprégnation variant entre 6 et 20%, et pour des intervalles de température inférieurs à 70°C.

Toutes ces relations permettent donc de comparer d'une manière pratique des résultats obtenus dans des conditions expérimentales différentes.

PARTIE EXPÉRIMENTALE

Appareillage et conditions standard de travail

Les mesures ont été effectuées dans des conditions isothermes avec un chromatographe en phase gazeuse Varian 3700, équipé d'un détecteur à ionisation de flamme. L'azote est admis dans l'appareillage à travers un manomètre à double détente; le débit est contrôlé par un système de régulation à membrane (Veriflo, Richmond, CA, U.S.A.) et réglé par une vanne à aiguille, placée dans une enceinte thermostatée à 40°C. La pression absolue d'entrée est lue sur un manomètre type Bourdon. Les débits d'hydrogène et d'air sont eux-aussi maintenus constants par une vanne à aiguille.

Le chromatographe est muni d'un enregistreur potentiométrique Servotrace (Sefram) et d'un calculateur-intégrateur Varian CDS 101 qui permet l'acquisition des données. L'injection des échantillons est réalisée automatiquement par un injecteur Varian 8000, dont le volume minimal d'injection est de 1 μl.

Conditions standard de mesure

Températures: four 170 ± 0.5°C; injecteur 230 ± 0.5°C; détecteur 240 ± 0.5°C. Gaz vecteur: azote (pureté > 99.995%, taux d'humidité < 3.5 mg/m^3); débit 30 ml/min (mesuré par un débitmètre à film de savon raccordé à la sortie du détecteur par un tube de PTFE, après coupure de l'hydrogène et de l'air). Débits: d'hydrogène 30 ml/min; d'air 300 ml/min.

Ces conditions permettent d'obtenir un pic chromatographique symétrique pour la plupart des substances étudiées.

Produits et colonnes utilisés

Tous les produits sont commerciaux (Aldrich-Europe, Beerse, Belgique) et ont un taux de pureté annoncé supérieur à 98%.

Les colonnes sont construites avec des tubes en acier inoxydable de diamètre extérieur 1/8 in. et de longueur 10 ft. Le support, de granulométrie 80–100 mesh, est constitué de Varaport 30 (Johns-Manville, Greenwood Plaza, Denver, CO, U.S.A.)

qui est un Chromosorb W ayant subi un tamisage, une neutralisation et une silanisation au DMCS. Le taux d'imprégnation du support a été fixé pour cette étude à 10% (p/p) de phase stationnaire. La masse de phase stationnaire pour chacune des colonnes est comprise entre 0.3 et 0.4 g. Toutes les colonnes ont été conditionnées pendant 12 h et amenées à leur température de conditionnement par une programmation de 4°C/min. En fin de conditionnement on s'assure par pesée sur balance Mettler (précision 10^{-4} g) que la perte de poids durant cette opération est négligeable.

Avant d'effectuer une série de mesures, on attend 24 h, aux conditions standard sélectionnées, afin de stabiliser la colonne.

Les 21 phases stationnaires retenues ont toutes été fournies par Varian (Walnut Creek, CA, U.S.A.). On peut les classer comme suit:

(1) Silicones: SE-30, DC-200, OV-17, OV-210, OV-225, XF-1150, QF-1, DCL5X.

(2) Graisses: Apiezon L (ApL), Apiezon H (ApH).

(3) Esters: néopentylglycolsuccinate (NPGS), éthylène glycolsuccinate (EGS), diéthylèneglycolsuccinate (DEGS), phényldiéthanolaminesuccinate (PDEAS), free fatty acid phase (FFAP), diéthylèneglycoladipate (DEGA) diéthylèneglycolsébacate (DEGSeb).

(4) Polyéthers: Carbowax 20M, Ucon 50 HB 2000 (UP), Ucon LB 550X (ULB 550X), polymétaphényléther 5 Rings (PMPE 5R).

Vérification de la répétabilité des mesures

Pour nous assurer de l'absence de variation dans les conditions opératoires au cours des analyses, nous avons opéré comme suit:

(1) Le temps de rétention de la référence est déterminé à partir d'une série d'injections de 1 μl de benzène pur (six essais par phase stationnaire).

(2) Tous les composés étudiés sont injectés en solution benzénique (25% p/v, 1 μl, six essais dans le cas des mono- et des trisubstitués, trois dans le cas des disubstitués); il est aisé de vérifier pour chaque mesure la constance des conditions opératoires par comparaison de la rétention du benzène avec celle de la série de référence.

(3) Tous les solutés sont injectés directement dans la colonne.

(4) On s'assure en outre de l'invariabilité du volume mort par une injection à intervalles réguliers de méthane.

La limite de répétabilité des temps de rétention du benzène et du méthane est $<2.10^{-2}$ min.

Enfin, nous avons observé que dans un intervalle de concentration variant de 2.5 à 50% de soluté, les temps de rétention de ces derniers restent constants.

Par contre, l'augmentation du volume d'injection entraîne une variation du temps de rétention; ainsi, lorsqu'on passe de 1 à 5 μl, la durée de rétention peut varier jusqu'à $\pm$ 5.10^{-2} min. Selon Nieuwdorp *et al.*[7] cette variation serait la conséquence d'une perturbation de l'équilibre de la colonne liée à la vaporisation d'un volume de liquide trop important.

RÉSULTATS ET DISCUSSION

Les temps de rétention bruts pris en considération sont des moyennes mesurées

TABLEAU I

LOGARITHMES DES TEMPS RÉDUITS RELATIFS AU BENZÈNE DES DÉRIVÉS MONOFONCTIONNELS

Z	*ApL*	*SE-30*	*DC-200*	*PMPE 5R*	*ApH*	*OV-17*	*UP*	*ULB*	*OV-210*	*OV-225*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
CF_3	0.001	0.114	0.137	−0.042	−0.023	0.009	0.047	0.071	0.233	0.042
CH_3	0.332	0.274	0.303	0.302	0.278	0.247	0.239	0.252	0.233	0.234
OCH_3	0.620	0.489	0.552	0.769	0.616	0.681	0.716	0.670	0.627	0.693
Br	0.764	0.569	0.627	0.838	0.750	0.753	0.775	0.735	0.660	0.731
Cl	0.533	0.399	0.433	0.571	0.512	0.532	0.552	0.502	0.495	0.502
F	0.043	0.114	0.107	0.059	0.007	0.009	0.070	0.058	0.127	0.057
I	1.048	0.783	0.842	1.159	1.045	1.025	1.047	1.009	0.844	1.008
CHO	0.737	0.607	0.660	1.031	0.804	0.866	0.970	0.907	0.975	1.010
SH	0.821	0.617	0.677	0.985	0.822	0.855	0.944	0.867	0.729	0.891
$COOCH_3$	0.983	0.835	0.903	1.246	1.039	1.085	1.159	1.122	1.089	1.142
CN	0.745	0.651	0.694	1.106	0.860	0.946	1.088	1.020	1.141	1.153
$COCH_3$	0.949	0.804	0.859	1.270	1.040	1.078	1.175	1.126	1.187	1.229
NH_2	0.746	0.623	0.672	1.086	0.794	0.906	1.196	1.071	0.876	1.133
NO_2	1.015	0.859	0.909	1.369	1.099	1.161	1.295	1.233	1.314	1.358
CH_2OH	0.841	0.732	0.774	1.204	0.922	0.996	1.393	1.270	0.958	1.257
OH	0.637	0.575	0.624	0.995	0.682	0.825	1.520	1.320	0.774	1.263

sur plusieurs essais; les Tableaux I–V (pages suivantes) rassemblent les logarithmes des temps réduits relatifs (log t'_H form. 4) correspondants.

Étude de l'analogie de comportement des phases stationnaires

L'ensemble des résultats des Tableaux I–V constitue la "matrice de données" dont les lignes représentent les données de rétention des substances injectées et les colonnes des phases stationnaires.

Dérivés monofonctionnels. Lorsque le comportement des dérivés monofonctionnels vis à vis de deux phases stationnaires, considérées comme des variables, est analogue, il existe un modèle de relation affine (du type 3) entre ces deux phases, à condition que leur coefficient de corrélation soit voisin de 1 en valeur absolue.

Dans la matrice de corrélation ainsi obtenue (Tableau VI) ces coefficients sont toujours supérieurs à 0.82. Toutefois, lorsqu'ils sont compris entre 0.82 et 0.96, les valeurs des données de rétention recalculées par régression présentent des déviations supérieures à l'écart qui correspond à la précision des mesures. On sera donc amené à regrouper les phases très corrélées entre-elles; pour cela on fait appel à la classification ascendante hiérarchique (CAH), une des méthodes de taxonomie[9] particulièrement adaptée à ce problème. En effet, la CAH a pour but de classer des "objets" à partir d'un indice de ressemblance ou de dissemblance, et d'un critère d'agrégation:

La Fig. 1 représente le dendogramme résultant de l'emploi du logiciel de CAH (BMDP1M) de la Biomedical Computer Programs[10]. Nous avons choisi ici la valeur absolue du coefficient de corrélation comme indice de ressemblance entre phases. Le critère de ressemblance entre deux groupes de phases est la corrélation minimale entre deux "variables-phases" prises dans chacun des groupes.

Le dendogramme révèle l'existence de trois groupes de phases A, B et C que l'on peut identifier très facilement. La corrélation entre deux phases d'un même

NPGS XI	*Carbowax 20M* XII	*XF-1150* XIII	*FFAP* XIV	*PDEAS* XV	*DEGA* XVI	*DEGS* XVII	*EGS* XVIII	*DEGSeb* XIX	*QF 1* XX	*DCL5X* XXI
0.112	−0.011	−0.002	0.048	−0.075	−0.044	0.000	−0.016	−0.029	0.167	0.336
0.271	0.178	0.188	0.240	0.199	0.178	0.301	0.227	0.182	0.199	0.336
0.784	0.688	0.700	0.753	0.712	0.705	0.861	0.788	0.669	0.505	0.813
0.811	0.747	0.713	0.805	0.773	0.729	0.857	0.762	0.725	0.544	0.859
0.589	0.439	0.493	0.571	0.491	0.489	0.623	0.528	0.486	0.520	0.599
0.112	−0.011	0.057	0.108	0.053	0.018	0.186	0.102	0.013	0.094	0.256
1.070	1.023	0.961	1.089	1.069	1.016	1.140	1.035	1.018	0.722	1.076
1.095	1.023	1.105	1.083	1.085	1.040	1.230	1.151	0.955	0.845	1.170
0.990	0.952	0.919	1.031	1.010	0.953	1.086	1.000	0.915	0.613	0.880
1.229	1.132	1.149	1.206	1.154	1.185	1.332	1.266	1.135	0.947	1.265
1.211	1.170	1.270	1.209	1.206	1.168	1.357	1.275	1.074	0.997	1.313
1.301	1.215	1.301	1.267	1.269	1.263	1.429	1.366	1.184	1.043	1.377
1.313	1.280	1.308	1.369	1.311	1.315	1.519	1.446	1.184	0.752	1.076
1.425	1.377	1.439	1.419	1.439	1.402	1.575	1.517	1.312	1.171	1.507
1.457	1.446	1.419	1.534	1.442	1.472	1.661	1.585	1.342	0.814	1.211
1.545	1.598	1.501	1.700	1.600	1.551	1.754	1.657	1.415	0.644	1.035

groupe est toujours supérieure ou égale au niveau de similarité apparaîssant à la formation de ce groupe.

(1) La classe A regroupe les phases "apolaires ou peu polaires" (ApL, SE-30, DC-200, PMPE 5R, ApH et OV-17); la corrélation entre phases de ce groupe est ≥ 0.9661.

(2) La classe B celles qui sont fluorées (OV-210, QF-1 et DCL5X); la corrélation entre phases de ce groupe est ≥ 0.9848.

(3) La classe C l'ensemble des phases dites "polaires" (UP, ULB 550X, NPGS, Carbowax 20M, XF-1150, FFAP, PDEAS, DEGA, DEGS, EGS et DEGSeb). La corrélation entre phases de ce groupe est ≥ 0.9802.

Dans chaque classe le comportement des phases vis à vis des produits étudiés est analogue, bien que leurs structures chimiques puissent être différentes. Des relations affines entre elles sont possibles, par contre il n'existe pas de relations affines de bonne qualité entre phases appartenant à des classes différentes. Le choix d'un solvant de référence représentatif d'une classe peut s'établir suivant un critère objectif à partir du tableau de la matrice des carrés des écarts. La phase stationnaire retenue à ce titre sera celle qui donne par régression la meilleure valeur recalculée des données de rétention des phases de son groupe (minimum de la somme des carrés des écarts au modèle). C'est ainsi que les trois classes A, B et C ont pour représentants respectifs SE-30, OV-210 et NPGS.

Les valeurs des coefficients a et b de la relation 3 sont rassemblées dans le Tableau VII lorsqu'on prend ces trois phases pour références de chacun des groupes. Le coefficient a, caractéristique d'un couple de phases, est indépendant du (ou des) groupement(s) fonctionnel(s). On constate que, pour des phases appartenant au même groupe (A, B ou C) l'écart moyen sur le logarithme des temps réduits relatifs entre valeurs calculées et mesurées σ_e varie entre $2 \cdot 10^{-2}$ et $6 \cdot 10^{-2}$. Par contre, pour des

TABLEAU II

LOGARITHMES DES TEMPS RÉDUITS RELATIFS AU BENZÈNE DES DÉRIVÉS DIFONCTIONNELS/*ortho*

Z_1, Z_2	*ApL*	*SE-30*	*PMPE 5R*	*ULB 550X*	*OV-210*	*OV-225*	*Carbowax 20M*	*PDEAS*	*DEGS*
	I	*II*	*IV*	*VIII*	*IX*	*X*	*XII*	*XV*	*XVII*
CF_3, Br	0.895	0.871	–	–	0.801	0.738	1.283	0.759	0.805
CF_3, NH_2	0.789	0.718	0.987	1.130	0.833	0.925	1.570	1.064	1.242
CF_3, NO_2	–	1.061	1.362	1.335	1.357	1.279	1.768	1.367	1.470
CH_3, CH_3	0.787	0.688	–	0.699	0.478	0.477	1.092	0.479	–
CH_3, Br	1.206	1.021	1.258	–	0.802	0.856	1.371	0.984	0.977
CH_3, CHO	1.179	1.070	1.428	1.249	1.103	1.120	1.630	1.337	1.362
CH_3, $COOCH_3$	–	1.267	–	1.432	1.163	1.194	–	1.324	1.405
CH_3, CN	1.118	1.051	1.415	1.252	1.204	1.201	1.914	1.367	1.402
CH_3, $COCH_3$	1.299	1.183	1.548	1.363	1.192	1.224	1.725	1.374	1.434
CH_3, NH_2	1.167	1.056	1.452	1.366	0.990	–	1.860	1.496	1.597
CH_3, NO_2	1.360	1.235	1.649	1.468	–	–	1.887	1.573	1.603
CH_3, CH_2OH	1.289	1.189	1.622	1.633	1.075	1.372	2.200	1.683	1.815
CH_3, OH	1.013	0.972	1.330	1.568	0.838	1.262	2.079	1.639	1.756
CH_3O, Br	1.502	1.317	1.711	1.521	1.170	1.328	1.892	1.527	1.583
CH_3O, SH	1.537	1.346	1.764	1.581	–	1.393	1.961	1.556	1.677
CH_3O, CHO	–	–	–	1.666	1.504	1.567	–	1.815	1.877
CH_3O, $COCH_3$	1.587	1.478	1.962	–	–	–	2.188	–	–
CH_3O, NH_2	1.372	1.261	1.739	1.588	1.198	1.425	2.103	1.706	1.852
CH_3O, NO_2	1.696	1.599	2.199	1.946	1.815	–	2.485	2.188	2.268
CH_3O, CH_2OH	–	–	–	1.848	1.384	1.636	2.373	1.961	2.109
CH_3O, OH	1.160	1.084	1.468	1.428	1.079	1.227	1.918	1.527	1.667
Br, F	–	–	–	0.960	0.709	0.743	–	0.848	0.979
Br, Br	–	–	–	1.569	1.143	1.322	–	1.544	1.568
Br, Cl	–	–	–	1.353	0.995	1.116	–	1.296	1.355
Br, CHO	–	1.350	1.830	1.595	1.336	1.438	1.966	1.548	1.712
Br, $COOCH_3$	1.786	1.605	–	1.834	–	1.675	–	1.916	1.966
Br, CN	–	–	–	1.780	1.585	1.712	–	1.988	1.997
Br, $COCH_3$	–	–	–	1.740	1.531	1.624	–	1.847	1.895
Br, NH_2	1.549	1.353	1.826	1.742	1.255	1.543	2.238	1.870	1.956
Br, CH_2OH	1.707	1.520	2.036	2.043	1.364	1.751	2.565	2.146	2.223
Br, OH	1.237	1.079	–	–	–	–	–	–	–
Cl, Cl	–	–	–	1.149	0.845	0.913	–	1.044	1.142
Cl, $COCH_3$	–	–	–	1.542	1.370	1.414	1.957	1.600	1.663
SH, NH_2	1.602	1.394	1.911	1.980	–	1.618	2.242	1.873	2.092
CHO, NH_2	1.492	1.352	–	1.792	1.404	1.659	–	2.080	2.137
CHO, NO_2	1.649	1.485	–	1.878	1.789	1.899	2.525	2.238	2.288
CHO, OH	1.118	1.013	1.395	1.272	1.063	1.124	1.716	1.442	1.458
$COOCH_3$, NH_2	0.843	1.011	–	–	1.529	1.763	–	–	2.214
$COOCH_3$, NO_2	1.824	1.735	–	2.044	1.969	2.085	–	–	2.458
$COOCH_3$, OH	1.447	1.322	1.673	–	–	–	3.254	–	–
CN, NO_2	1.897	1.744	–	–	2.237	–	3.120	–	2.813
$COCH_3$, NO_2	1.792	1.682	2.328	2.072	2.022	2.110	2.758	2.385	2.490
$COCH_3$, OH	1.389	1.261	1.671	–	1.301	1.349	1.885	1.655	1.654
NH_2, NO_2	1.879	1.713	2.350	–	1.877	2.185	3.045	–	2.713
NO_2, CH_2OH	1.845	1.704	2.364	2.300	1.827	2.228	3.069	–	2.761
NO_2, OH	1.345	1.197	–	1.380	1.284	1.311	1.872	–	1.677

TABLEAU III

LOGARITHMES DES TEMPS RÉDUITS RELATIFS AU BENZÈNE DES DÉRIVÉS DIFONCTIONNELS/ *meta*

Z_1, Z_2	*ApL*	*SE-30*	*PMPE 5R*	*ULB 550X*	*OV-210*	*OV-225*	*Carbowax 20M*	*PDEAS*	*DEGS*
	I	*II*	*IV*	*VIII*	*IX*	*X*	*XII*	*XV*	*XVII*
CF_3, Br	0.746	0.740	–	0.749	0.669	0.533	1.134	0.479	0.690
CF_3, NH_2	–	0.954	1.215	1.399	–	1.185	1.886	1.376	1.622
CF_3, NO_2	–	0.969	1.218	1.128	–	1.050	1.552	1.119	1.282
CF_3, OH	–	0.923	1.092	1.786	–	1.348	2.218	1.684	1.839
CH_3, CH_3	0.712	0.635	–	0.625	0.388	0.393	1.063	–	0.624
CH_3, Br	1.211	1.031	–	1.125	0.824	0.877	1.387	1.018	1.090
CH_3, CHO	1.172	1.061	1.426	1.252	1.132	1.142	1.644	1.347	1.418
CH_3, $COOCH_3$	1.477	1.325	1.653	1.477	1.222	1.248	1.688	1.417	1.491
CH_3, CN	1.185	1.101	1.489	1.339	1.291	1.258	1.772	1.429	1.527
CH_3, $COCH_3$	1.395	1.267	1.659	1.460	1.311	1.321	1.840	1.517	1.592
CH_3, NH_2	1.169	1.068	1.475	1.414	1.000	1.236	1.915	1.535	1.691
CH_3, OH	1.180	1.026	1.411	1.670	0.891	–	2.144	1.770	1.895
CH_3O, Br	1.500	1.290	1.638	1.463	1.103	1.235	1.760	1.448	1.482
CH_3O, SH	1.581	1.361	1.802	1.606	1.170	1.400	–	–	1.731
CH_3O, CHO	1.446	1.312	1.950	1.570	1.370	1.464	2.026	1.748	1.815
CH_3O, $COCH_3$	1.668	1.513	2.153	1.761	1.545	1.649	–	1.914	1.996
CH_3O, NH_2	1.518	1.390	1.953	1.812	1.345	1.687	2.385	2.037	2.184
CH_3O, CH_2OH	–	–	–	1.988	1.378	1.744	2.554	2.119	2.296
CH_3O, OH	–	1.345	1.854	–	–	–	2.743	2.238	–
Br, F	–	–	–	0.869	0.564	0.615	–	0.718	0.861
Br, Br	–	–	–	1.477	1.059	1.204	–	1.419	1.444
Br, Cl	–	–	–	1.268	0.909	1.005	–	1.175	1.233
Br, CHO	–	1.344	1.861	1.604	1.358	1.487	2.041	1.807	–
Br, $COOCH_3$	–	–	–	1.797	1.480	1.569	–	1.852	1.831
Br, CN	–	–	–	1.639	1.457	1.549	–	1.866	–
Br, $COCH_3$	–	–	–	1.818	1.567	1.665	–	1.919	1.955
Br, NH_2	1.701	1.502	2.064	2.017	1.417	1.818	2.630	2.208	2.329
Br, CH_2OH	1.790	1.578	2.142	2.150	1.437	1.875	2.720	2.308	2.371
Cl, Cl	–	–	–	1.053	0.753	0.787	–	0.921	1.023
Cl, $COCH_3$	–	–	–	1.592	1.419	1.472	2.024	1.719	1.748
CHO, CN	1.518	1.377	–	1.824	1.790	1.867	2.510	2.215	2.280
CHO, NO_2	1.783	–	–	2.004	1.917	2.022	2.682	2.405	2.444
$COOCH_3$, CN	1.763	1.632	–	1.971	1.861	1.879	–	2.217	2.242
$COOCH_3$, NH_2	–	–	–	2.429	1.950	2.357	–	–	–
$COOCH_3$, NO_2	2.033	1.834	–	2.085	2.003	2.054	–	–	2.415
$COOCH_3$, OH	1.818	1.733	2.342	–	–	–	–	–	–
CN, NO_2	1.763	1.564	–	2.015	2.010	2.098	2.745	2.500	2.490
$COCH_3$, NO_2	1.976	1.762	2.477	2.133	2.118		2.795	2.508	2.517
$COCH_3$, OH	1.779	1.677	2.338	2.606	1.738	2.347	–	–	3.004
NH_2, NO_2	2.007	1.840	2.588	2.539	2.041	2.474	3.398	2.970	–
NO_2, CH_2OH	2.085	1.927	2.604	2.589	2.084	2.539	3.437	–	3.063
NO_2, OH	1.984	1.839	–	2.893	1.857	2.649	–	–	3.294

TABLEAU IV

LOGARITHMES DES TEMPS RÉDUITS RELATIFS AU BENZÈNE DES DÉRIVÉS DIFONCTIONNELS/*para*

Z_1, Z_2	*ApL*	*SE-30*	*PMPE 5R*	*ULB 550X*	*OV-210*	*OV-225*	*Carbowax 20M*	*PDEAS*	*DEGS*
	I	*II*	*IV*	*VIII*	*IX*	*X*	*XII*	*XV*	*XVII*
CF_3, Br	0.767	0.735	–	0.791	0.655	0.533	1.157	0.548	0.737
CF_3, $COOCH_3$	–	–	–	–	1.050	0.870	–	0.863	0.990
CF_3, NH_2	0.945	0.968	1.299	–	–	–	–	–	–
CH_3, CH_3	0.711	0.602	–	0.611	0.375	0.413	1.055	–	0.628
CH_3, Br	1.215	1.026	–	1.135	0.824	0.883	1.394	1.044	1.105
CH_3, SH	1.265	1.068	1.371	1.255	0.865	1.015	1.555	1.231	1.293
CH_3, CHO	1.224	1.112	1.464	1.288	1.173	1.174	1.706	1.408	1.470
CH_3, $COOCH_3$	1.459	1.332	1.673	–	1.255	1.273	1.712	1.444	1.513
CH_3, CN	1.199	1.118	1.508	1.379	1.327	1.301	1.786	1.510	1.580
CH_3, $COCH_3$	1.429	1.287	1.689	1.496	1.338	1.358	1.863	1.566	1.643
CH_3, NH_2	1.152	1.049	1.470	1.391	0.985	1.207	1.865	1.561	1.649
CH_3, NO_2	1.515	1.347	–	1.606	1.505	1.511	2.014	1.785	1.791
CH_3, OH	1.042	1.011	1.390	1.659	0.878	1.354	2.157	1.753	1.884
CH_3O, Br	1.533	1.314	1.677	1.497	1.136	1.275	–	1.477	1.532
CH_3O, SH	–	–	–	1.610	1.136	1.404	–	–	1.722
CH_3O, CHO	1.565	1.429	1.962	1.663	1.524	1.637	2.160	1.907	1.998
CH_3O, $COCH_3$	1.779	1.612	2.165	1.840	1.680	1.799	2.327	2.057	2.150
CH_3O, NH_2	1.455	1.330	1.878	1.739	1.303	1.598	2.336	1.965	2.112
CH_3O, NO_2	1.879	1.686	2.299	2.030	1.858	1.967	2.517	2.338	2.330
CH_3O, CH_2OH	1.606	1.484	2.022	1.966	1.390	1.750	–	2.171	2.281
CH_3O, OH	1.356	1.309	1.814	2.044	1.228	–	2.692	2.244	2.391
Br, F	–	–	–	0.879	0.614	0.650	–	0.759	0.920
Br, Br	–	–	–	1.498	1.063	1.226	–	1.444	1.474
Br, Cl	–	–	–	1.290	0.909	1.020	–	1.204	1.265
Br, SH	–	1.412	1.859	–	1.173	1.437	–	–	1.739
Br, CHO	1.585	1.342	1.872	1.622	1.368	1.489	2.050	1.786	1.807
Br, $COOCH_3$	–	1.612	–	1.810	1.456	1.558	–	–	1.829
Br, CN	–	1.366	–	1.694	1.482	1.588	2.150	1.876	1.908
Br, $COCH_3$	–	1.567	2.092	1.834	1.581	1.680	2.235	1.950	1.968
Br, NH_2	1.701	1.494	2.061	1.998	1.442	1.827	2.627	2.268	2.331
Br, CH_2OH	1.790	1.563	2.122	2.132	1.435	1.864	2.700	2.277	2.354
Cl, Cl	–	–	–	1.079	0.770	0.819	–	0.954	1.059
Cl, $COCH_3$	–	–	–	1.625	1.426	1.516	2.028	1.706	1.763
CHO, CN	1.517	1.336	–	–	1.749	1.829	2.454	2.163	2.228
CHO, NO_2	1.782	1.534	–	1.968	1.874	1.985	2.627	2.372	2.399
$COOCH_3$, CN	1.763	1.593	–	1.949	1.812	1.847	–	2.210	2.206
$COOCH_3$, NH_2	–	1.889	2.590	–	–	–	3.264	–	3.017
$COOCH_3$, NO_2	2.034	1.804	–	2.051	1.941	2.059	–	–	2.374
CN, NO_2	1.762	1.527	–	1.995	1.975	2.070	2.691	2.457	2.456
$COCH_3$, NO_2	1.976	1.779	2.509	2.162	2.096	2.169	2.771	2.512	2.526
NH_2, NO_2	–	–	–	–	2.336	–	–	–	3.344
NO_2, CH_2OH	–	1.889	–	2.604	2.105	2.567	3.454	–	3.093
NO_2, OH	–	–	–	2.895	1.993	–	–	–	3.520

TABLEAU V

LOGARITHMES DES TEMPS RÉDUITS RELATIFS AU BENZÈNE DES DÉRIVÉS TRIFONCTIONNELS

	ApL	*SE-30*	*PMPE 5R*	*ULB 550X*	*OV-210*	*OV-225*	*Carbowax 20M*	*PDEAS*	*DEGS*
	I	*II*	*IV*	*VIII*	*IX*	*X*	*XII*	*XV*	*XVII*
Nitro-2, *m*-xylène	1.096	0.931	1.376	1.405	1.306	1.323	1.318	1.463	1.305
Diméthyl-2,4 anisole	0.960	0.767	1.075	1.130	0.824	0.903	0.857	0.976	0.857
Diméthyl-2,5 anisole	0.947	0.767	1.060	1.121	–	0.906	0.857	0.970	0.849
Diméthyl-2,6 anisole	0.874	0.719	0.970	1.022	0.794	0.834	0.748	0.873	0.758
Dichloro-2,6 anisole	1.191	0.969	1.366	1.406	–	1.215	1.225	1.368	1.193
Nitro-3, *o*-xylène	0.319	0.284	0.432	0.655	0.407	0.494	0.380	–	0.505
Diméthyl-3,5 anisole	0.990	0.789	1.126	1.180	0.897	0.976	0.944	1.059	0.944
Nitro-4, *m*-xylène	1.332	1.109	1.662	1.646	1.559	1.576	1.580	1.768	1.577
Dichloro-2,3 anisole	1.414	1.158	1.692	1.703	1.400	1.565	1.639	1.780	1.590
Nitro-4, *o*-xylène	1.490	1.240	1.843	1.809	1.743	1.766	1.769	1.985	1.770
Dichloro-3,5 anisole	1.325	1.044	1.475	1.503	1.195	1.283	1.318	1.469	1.294
Diméthyl-2,3 anisole	1.025	0.815	1.156	1.197	0.915	0.990	0.944	1.084	0.944
Dinitro-3,4 chloro-benzène	1.799	1.520	2.418	2.423	–	2.485	–	–	2.623
Nitro-5, *m*-xylène	1.395	1.165	1.718	1.690	1.628	1.621	1.619	1.811	1.616
Nitro-2 chloro-4 phénol	1.397	1.141	1.724	1.687	1.438	1.553	2.191	1.846	1.725

phases de groupes différents l'écart moyen augmente considérablement et peut atteindre dans le cas de SE 30/DEGS la valeur de 0.25 sur le logarithme des temps réduits relatifs.

L'analyse des corrélations avec les 21 phases stationnaires et le classement taxonomique qui en a résulté nous ont permis de réaliser la suite de l'étude sur 9 phases seulement. Notre choix a porté sur trois phases du groupe A, une du groupe B et cinq du groupe C.

Dérivés polyfonctionnels. De l'examen des Tableaux II–IV il ressort que les dérivés ortho, qui sont susceptibles de former une chélation, ont des temps de rétention plus faibles que les dérivés méta ou para correspondants. Dans l'ensemble, les dérivés méta sont élués plus rapidement que les dérivés para correspondants.

En ce qui concerne la rétention des composés trifonctionnels (Tableau V) on remarque que les temps de rétention décroîssent dans l'ordre 1,2,3 > 1,2,4 > 1,2,5 > 1,2,6 aussi bien avec une phase polaire qu'apolaire.

Nous avons effectué l'étude des régressions sur la matrice de corrélation obtenue à partir de l'ensemble de nos données de rétention (mono- + di- + tri-fonctionnels) sur neuf phases stationnaires. Le tableau VIII regroupe les valeurs des coeffi-

TABLEAU VI

MATRICE DE CORRÉLATION ENTRE "VARIABLES-PHASES"

		ApL	*SE-30*	*DC-200*	*PMPE 5R*	*ApH*	*OV-17*	*UP*	*ULB 550X*	*OV-210*
ApL	I	1.0000								
SE-30	II	0.9826	1.0000							
DC-200	III	0.9858	0.9990	1.0000						
PMPE 5R	IV	0.9661	0.9859	0.9846	1.0000					
ApH	V	0.9933	0.9940	0.9948	0.9862	1.0000				
OV-17	VI	0.9769	0.9901	0.9908	0.9971	0.9927	1.0000			
UP	VII	0.8477	0.8928	0.8902	0.9369	0.8803	0.9233	1.0000		
ULB 550X	VIII	0.8791	0.9238	0.9213	0.9578	0.9105	0.9468	0.9968	1.0000	
OV-210	IX	0.8821	0.9396	0.9341	0.9496	0.9280	0.9467	0.8703	0.8995	1.0000
OV-225	X	0.8893	0.9369	0.9330	0.9730	0.9265	0.9614	0.9846	0.9923	0.9405
NPGS	XI	0.8472	0.8986	0.8952	0.9449	0.8856	0.9302	0.9968	0.9959	0.8979
Carbowax 20M	XII	0.8285	0.8798	0.8765	0.9299	0.8666	0.9136	0.9972	0.9931	0.8758
XF-1150	XIII	0.8286	0.8864	0.8811	0.9394	0.8740	0.9213	0.9878	0.9869	0.9103
FFAP	XIV	0.8183	0.8685	0.8647	0.9214	0.8555	0.9041	0.9977	0.9908	0.8597
PDEAS	XV	0.8439	0.8901	0.8861	0.9412	0.8803	0.9241	0.9958	0.9927	0.8844
DEGA	XVI	0.8399	0.8914	0.8873	0.9403	0.8782	0.9235	0.9968	0.9944	0.8878
DEGS	XVII	0.8260	0.8784	0.8733	0.9335	0.8663	0.9137	0.9934	0.9889	0.8805
EGS	XVIII	0.8210	0.8782	0.8730	0.9320	0.8635	0.9122	0.9920	0.9884	0.8871
DEGSeb	XIX	0.8671	0.9123	0.9090	0.9538	0.9005	0.9403	0.9980	0.9984	0.8954
QF-1	XX	0.8780	0.9318	0.9250	0.9406	0.9230	0.9389	0.8531	0.8828	0.9954
DCL5X	XXI	0.8918	0.9488	0.9436	0.9634	0.9363	0.9583	0.9050	0.9301	0.9950

cients a et b de la relation 3 lorsqu'on prend comme phase de référence SE-30 pour le groupe A et DEGS pour le groupe C. On remarque que l'écart moyen, σ_e, entre valeurs calculées et mesurées est nettement supérieur à ce que nous avons constaté lors de l'étude des dérivés monofonctionnels. Si les deux phases sont prises dans des classes différentes (OV 210/OV 225) le coefficient de corrélation est encore abaissé.

En conclusion, l'approche réalisée à l'aide de la relation 3 [$\log t'_{Z_0}(\phi, Z, \varphi_1, T) = a \log t'_{Z_0}(\phi, Z, \varphi_2, T) + b$] avec φ_1 et φ_2 appartenant à la même classe taxonomique, constitue un modèle général applicable à l'ensemble des dérivés benzéniques mono-, di- et trisubstitués. Pour obtenir une prévision des temps de rétention avec une précision acceptable, il est préférable d'utiliser les coefficients de régression propres à chacune des familles de composés benzéniques mono-, di- ou tri-. Le Tableau IX rassemble à titre d'exemple quelques coefficients de régression concernant l'application de la relation 3. On constate sur ce tableau que les coefficients de corrélation, propres à chacune des familles prises séparément sont meilleurs que les coefficients de corrélation globaux qui figurent sur le Tableau VIII.

Relations affines entre grandeurs de rétention differentes

Il nous a paru intéressant de montrer qu'il est possible d'établir des relations affines, du type 5, de caractère très général

$$\mathrm{Gr}_1(\mathrm{Sq}, Z, \varphi_1, T_1) = \alpha\, \mathrm{Gr}_2(\mathrm{Sq}, Z, \varphi_2, T_2) + \beta \qquad (5)$$

OV-225	*NPGS*	*Carbowax 20M*	*XF-1150*	*FFAP*	*PDEAS*	*DEGA*	*DEGS*	*EGS*	*DEGSeb*	*QF-1*	*DCL5X*
1.0000											
0.9925	1.0000										
0.9857	0.9979	1.0000									
0.9920	0.9964	0.9938	1.0000								
0.9801	0.9963	0.9987	0.9911	1.0000							
0.9892	0.9974	0.9980	0.9950	0.9971	1.0000						
0.9898	0.9993	0.9984	0.9961	0.9977	0.9982	1.0000					
0.9860	0.9967	0.9961	0.9962	0.9965	0.9975	0.9986	1.0000				
0.9868	0.9972	0.9960	0.9974	0.9955	0.9962	0.9986	0.9993	1.0000			
0.9929	0.9986	0.9965	0.9923	0.9952	0.9973	0.9982	0.9948	0.9941	1.0000		
0.9267	0.8815	0.8560	0.8950	0.8417	0.8672	0.8709	0.8642	0.8701	0.8792	1.0000	
0.9626	0.9279	0.9099	0.9367	0.8953	0.9165	0.9191	0.9125	0.9177	0.9261	0.9848	1.0000

entre des séries de mesures caractérisées par des différences pouvant porter simultanément sur la nature de la phase stationnaire φ, la température de travail T, et/ou le mode d'expression des grandeurs de rétention.

Expression des grandeurs de rétention. Dans les relations 5, le choix de Gr_1 et Gr_2 est indifférent entre indice, logarithmes des volumes de rétention ou des temps réduits. Ceci découle des relations affines qui lient ces diverses grandeurs de rétention.

Ainsi, d'après leurs définitions mêmes[11], les volumes de rétention net, V_N, ou spécifique, V_g, sont fonction linéaires du temps réduit de rétention.

$$V(\mathrm{R}, \mathrm{Z}, \varphi, T) = \alpha_1 \left[t(\mathrm{R}, \mathrm{Z}, \varphi, T) - t_{\mathrm{CH_4}}(\varphi, T)\right] \tag{7}$$

avec $\alpha_1 = jD_G$ dans le cas de V_N et $\alpha_1' = jD_G \times 273/w_L T$ dans le cas de V_g (j facteur de compressibilité de James-Martin[16], D_G débit du gaz vecteur, w_L masse de phase stationnaire dans la colonne, T température d'expérience).

Ces coefficients dépendent de la phase stationnaire φ et des conditions expérimentales, mais non du soluté RZ.

De même on peut établir une relation affine entre les indices de Kovats[12] et le temps réduit de rétention, compte tenu de la relation de Evans[13]

$$I(\mathrm{R}, \mathrm{Z}, \varphi, T) = \alpha_1'' \log \left[t(\mathrm{R}, \mathrm{Z}, \varphi, T) - t_{\mathrm{CH_4}}(\varphi, T)\right] + \beta_1'' \tag{8}$$

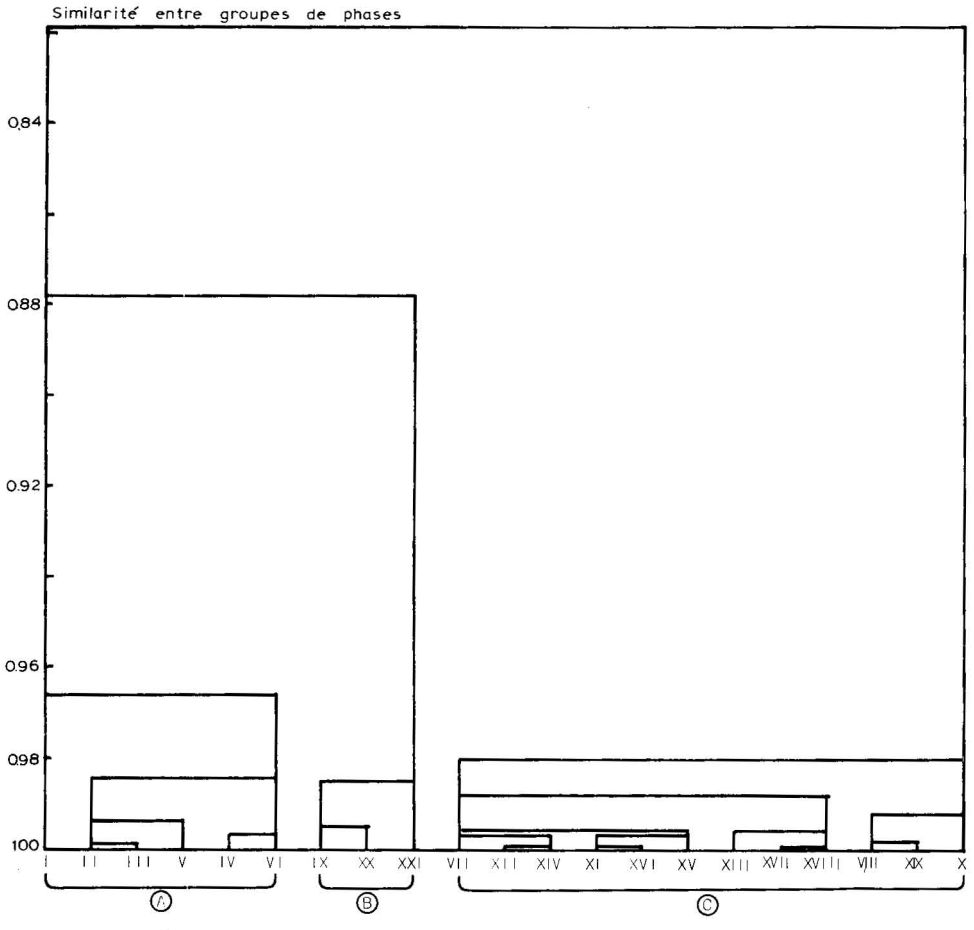

Fig. 1. Classification ascendante hiérarchique des "variables-phases".

avec

$$\alpha_1'' = \frac{100\,(n_1 - n_2)}{\log\,(t_{Cn_1} - t_{CH_4}) - \log\,(t_{Cn_2} - t_{CH_4})}$$

et

$$\beta_1'' = -100\,\frac{n_1 \log\,(t_{Cn_2} - t_{CH_4}) - n_2 \log\,(t_{Cn_1} - t_{CH_4})}{\log\,(t_{Cn_1} - t_{CH_4}) - \log\,(t_{Cn_2} - t_{CH_4})}$$

Cn_1 et Cn_2 représentent les alcanes linéaires ayant respectivement n_1 et n_2 atomes de carbone.

Ces coefficients présentent l'intérêt de n'être fonction que de la phase φ et de la température T; ils sont indépendants du soluté RZ.

TABLEAU VII

APPLICATION DE LA RELATION 3 AUX DÉRIVÉS MONOFONCTIONNELS

r = Coefficient de corrélation; σ_e = écart type moyen de l'erreur d'estimation.

φ_1	φ_2	a	b	r	σ_e
SE-30	ApL	0.734	0.0692	0.9826	0.045
	DC-200	0.939	−0.0081	0.9990	0.012
	PMPE 5R	0.536	0.0981	0.9859	0.041
	ApH	0.668	0.0960	0.9940	0.027
	OV-17	0.635	0.0899	0.9901	0.034
OV-210	QF-1	1.118	0.0275	0.9954	0.035
	DCL5X	0.911	−0.0941	0.9950	0.052
NPGS	Carbowax 20M	0.923	0.1353	0.9979	0.032
	XF-1150	0.935	0.1084	0.9964	0.041
	FFAP	0.931	0.0597	0.9963	0.042
	PDEAS	0.910	0.1189	0.9974	0.035
	DEGA	0.919	0.1279	0.9993	0.018
	DEGS	0.867	0.0413	0.9967	0.039
	EGS	0.876	0.0984	0.9972	0.037
	DEGSeb	1.005	0.1040	0.9986	0.026
	UP	1.031	0.0429	0.9968	0.039
	ULB 550X	1.136	0.0180	0.9959	0.044
	OV-225	1.067	0.0431	0.9925	0.059

TABLEAU VIII

APPLICATION DE LA RELATION 3 À L'ENSEMBLE DES DÉRIVÉS MONO-, DI- ET TRIFONCTIONNELS

φ_1	φ_2	a	b	r	σ_e	*Nombre de points*
SE-30	ApL	0.895	−0.0195	0.9876	0.059	116
	PMPE 5R	0.730	−0.0210	0.9826	0.070	119
DEGS*	Carbowax 20M	0.774	0.2541	0.9682	0.135	112
	ULB 550X	1.095	0.0232	0.9462	0.174	139
	OV-225	1.201	0.0058	0.9592	0.152	141
	PDEAS	0.977	0.0883	0.9793	0.109	128
OV-210	OV-225	0.840	0.0720	0.9379	0.133	138

* La similitude de comportement entre NPGS et DEGS est très grande.

TABLEAU IX

APPLICATION DE LA RELATION 3 —CAS PARTICULIERS

φ_1/φ_2	*Dérivés*		a	b	r	σ_e	*Nombre de points*
SE-30/ApL		*ortho*	0.873	0.051	0.9913	0.034	32
	Disubstitues	*méta*	0.812	0.124	0.9917	0.024	26
		para	0.798	0.160	0.9905	0.038	25
	Trisubstitues		0.867	−0.061	0.9917	0.023	15
DEGS/PDEAS		*ortho*	1.058	−0.013	0.9928	0.043	33
	Disubstitues	*méta*	1.028	0.042	0.9947	0.040	30
		para	0.960	0.152	0.9909	0.051	33
	Trisubstitues		0.931	−0.058	0.9973	0.026	13

Effet de la nature de la phase stationnaire. Il a été mis en évidence dans la partie I (relations de type 3).

Effet de la température. Les grandeurs de rétention varient[15] en fonction de la température selon une loi empirique du type

$$Gr = A + B/(T + C)$$

Cependant, Cook et Raushel[5] et Haken[15] montrent que pour des intervalles de température réduits, la variation peut être considérée comme linéaire.

Partant des données de Cook et Raushel[5] nous avons effectué une série de régressions sur ses indices de rétention pour un intervalle de température de 60°C.

Les coefficients de corrélation rassemblés dans le Tableau X sont très proches de l'unité, justifiant une relation du type

$$I_{RZ}(\varphi, T_1) = \alpha_2 I_{RZ}(\varphi, T_2) + \beta_2 \qquad (9)$$

avec α_2 et β_2 fonction de T_1, T_2, φ, et indépendants de R et Z.

TABLEAU X
APPLICATION DE LA RELATION 9 AUX DONNÉES DE COOK ET RAUSHEL[5]

$I_{\phi,Z}(\varphi, T_1) = \alpha_2 \cdot I_{\phi,Z}(\varphi, T_2) + \beta_2$

φ	T_1/T_2 (°C)	α_2	β_2	r	*Nombre de points*	σ_e
SE-30	160/100	1.047 (±0.014)	−22.106	0.9991	12	2.5
	160/130	1.021 (±0.006)	− 8.493	0.9998	13	2.5
	130/100	1.025 (±0.007)	−13.278	0.9997	13	2.5
ApL	160/100	1.047 (±0.012)	−14.380	0.9995	10	3.8
	160/130	1.025 (±0.006)	− 8.113	0.9998	13	1.9
	130/100	1.022 (±0.007)	− 6.260	0.9998	10	2.1

Les régressions de ce type sont encore très bonnes lorsqu'elles portent sur des produits de nature très différente. C'est ainsi qu'en traitant la totalité (300 composés) des données de McReynolds[14], mesurées à 120°C et à 160°C, on atteint la relation suivante, dans le cas de la phase Carbowax 20M: [$I_{(120°C)} = 0.9972\ I_{(160°C)} - 0.0498$; $r = 0.9983$] (écart type moyen de l'erreur d'estimation sur I: $\sigma_e = 12.3$).

Cette relation 9 reste valable lorsque les deux séries de mesures sont exprimées avec des grandeurs de rétention différentes. En effet, une combinaison des relations 8 et 9 conduit par exemple à une nouvelle relation

$$I(R, Z, \varphi, T_1) = \alpha_3 \log [t(R, Z, \varphi, T_2) - t_{CH_4}(\varphi, T_2)] + \beta_3 \qquad (10)$$

avec $\alpha_3 = \alpha_1''\alpha_2$ et $\beta_3 = \beta_1''\alpha_2 + \beta_2$; α_2 et β_2, définis par la relation 9 sont fonction de φ, T_1 et T_2. α_1'' et β_1'', définis par la relation 8 sont fonction de φ et T_2; α_3 et β_3 sont indépendants de Z.

Cette nouvelle relation permet de situer de façon simple nos propres données, établies en logarithme de temps réduit par rapport à celles de Cook et Raushel[5] par exemple, bien que ses mesures soient exprimées en indices de Kovats. Les résultats sont regroupés dans le Tableau XI.

TABLEAU XI

APPLICATION DE LA RELATION 10 AUX DONNÉES DE COOK ET RAUSHEL[5]

Caractéristiques des colonnes: Cook et Raushel[5], support, W AW DMCS, taux d'imprégnation, 20%; longueur, 6 ft.; ce travail, support, Varaport 30, taux d'imprégnation, 10%; longueur, 10 ft.; $\log(t_{\phi H} - t_{CH_4})$ sur ApL, 0.991; sur SE-30, 0.618. Pour ApL et SE-30 (douze points): R = ϕ et Z = CF_3, F, Cl, Br, I, OCH_3, CN, NO_2, NH_2, CHO, CH_2OH, $COCH_3$. Pour SE-30 (neuf points): R = ϕ et Z = CF_3, F, Cl, Br, I, CN, NO_2, NH_2, H.

φ	T_1/T_2 (°C)	α_3	β_3	r	σ_e	*Nombre de points*
ApL	130/170	425.3	238.3	0.9994	4.0	12
ApL	160/170	436.3	234.9	0.9993	4.6	12
SE-30	160/170	549.5	294.6	0.9964	10	12
SE-30	100/170	499.5	331.0	0.9958	13	9

Nous avons cru utile de compléter ce tableau par le suivant (Tableau XII) où nous reprenons la première régression du Tableau XI et où nous faisons apparaître en particulier les écarts entre les valeurs expérimentales de Cook et Raushel[5] et les indices de rétention calculés par régression à partir de nos propres valeurs. On voit que cet écart n'est jamais supérieur à 6.5 unités d'indice.

TABLEAU XII

CAS PARTICULIER DE L'APPLICATION DE LA RELATION 10

$I_{\phi-Z}$ (ApL, 130°C) = 425.3 $\log(t_{\phi Z} - t_{CH_4})$ (ApL, 170°C) + 238.3; r = 0.9994.

ϕ-Z	$I_{\phi Z}$ *(mesuré par Cook et Raushel[5])*	$I_{\phi Z}$ *(calculé par régression)*	*Écart entre* $I_{\phi-Z}$ *mes. et* $I_{\phi-Z}$ *calc.*
Z = CH_3	800	800.6	−0.6
F	680	677.7	2.3
Cl	886	886.5	−0.5
Br	986	984.3	1.7
I	1109	1105.1	3.9
OCH_3	930	923.5	6.5
CN	971	976.7	−5.7
NO_2	1092	1091.5	0.5
NH_2	972	977.1	−5.1
CHO	969	973.3	−4.3
CH_2OH	1013	1017.1	−4.1
$COCH_3$	1069	1063.5	5.5

La relation 10 et la définition de l'incrément de fonction δI_Z (Bibl. 17) conduisent immédiatement à la relation

$$\delta I_Z\,(R, \varphi, T_1) = I_{RZ} - I_{RH} = \alpha_3 \log t'_{Z_0}\,(R, Z, \varphi, T_2) \qquad (11)$$

avec $\alpha_3 = \alpha'_1 \alpha_2$.

Celle-ci nous permet de confronter nos mesures à celles de West et Hall[6] (Tableau XIII).

TABLEAU XIII

APPLICATION DE LA RELATION 11 AUX DONNÉES DE WEST ET HALL[6]

Caractéristiques des colonnes: West et Hall[6]; support, Gas-Chrom. Q; taux d'imprégnation, 10%; longueur, 6 ft. Ce travail; support. Varaport 30; taux d'imprégnation, 10%; longueur, 10 ft. R = ϕ et Z = CH_3, Cl, Br, OCH_3, CN, NO_2, NH_2, $COCH_3$, I.

φ	T_1/T_2 (°C)	α_3	r	σ_e	*Nombre de points*
ApL	100/170	381.2	0.9995	9.8	9
SE-30	100/170	471.1	0.9994	10	9

On observe une excellente corrélation bien que les auteurs opèrent non seulement avec un support très différent du notre, Gas-Chrom Q, mais également dans un isotherme différent, et avec une longueur de colonne différente.

Intervention simultanée des variables φ et T. La relation générale 5 [Gr_1 (Sq, Z, φ_1, T_1) = α Gr_2 (Sq, Z, φ_2, T_2) + β] est en fait une conséquence des approaches des types précédents, et montre qu'il est possible de comparer des données de rétention obtenues à des températures quelconques, à partir de plusieurs phases stationnaires ressortant de la même classe taxonomique et quelle que soit la forme sous laquelle on exprime les grandeurs de rétention.

Le Tableau XIV résume un certain nombre de régressions que nous avons réalisées entre nos données et celles de Nieuwdorp *et al.*[7].

TABLEAU XIV

APPLICATION DE LA RELATION 5 AUX DONNÉES DE NIEUWDORP *et al.*[7]

Caractéristiques des colonnes: Nieuwdorp *et al.*[7]; support, Gas-Chrom Z; taux d'imprégnation, 10%; longueur, 2 ou 3 m. Ce travail, support, Varaport 30, taux d'imprégnation, 10%; longueur, 10 ft; log ($t_{\phi H} - t_{CH_4}$) sur NPGS, 0.621; sur DEGS, 0.477; sur ApL: 0.991.
Sq = ϕ et Z = H, CH_3, F, Cl, Br, I, OCH_3, CN, NO_2, NH_2, OH.

φ_1/T_1 (°C)	φ_2/T_2 (°C)	α	β	r	σ_e	*Nombre de points*
PEG 20M/165.5	NPGS/170	1.069	0.3932	0.9924	$6 \cdot 10^{-2}$	11
PEG 20M/165.5	DEGS/170	0.931	0.6272	0.9982	$3 \cdot 10^{-2}$	11
PEG 20M/206	DEGS/170	0.772	0.4863	0.9973	$3 \cdot 10^{-2}$	11
Apiezon M/206	ApL/170	0.832	0.1327	0.9958	$2 \cdot 10^{-2}$	11

Nieuwdorp *et al.*[7] a déterminé les log V_N par extrapolation de ses résultats de mesures à dilution infinie. Nos propres données sont obtenues par injection d'une quantité de 1 μl d'une solution benzénique à 25%. Bien qu'il soit probable que l'injection d'une telle quantité puisse affecter les propriétés thermodynamiques d'équilibre à dilution infinie, du fait d'une certaine surcharge de la colonne, cela n'affecte en rien la qualité des régressions.

CONCLUSION

Dans la présent travail, nous avons cherché à examiner sous l'angle des traitements statistiques les valeurs de rétention d'un grand nombre de substances benzéniques. En particulier à partir des données concernant seize dérivés monofonctionnels, nous avons appliqué les méthodes taxonomiques d'agrégation. Il s'agit là de méthodes standards, c'est à dire qui n'ont pas reçu une adaptation particulière à telle ou telle discipline et dont la validation se justifie a postériori par le fait que l'on recoupe un certain nombre de résultats connus antérieurement. Les résultats complémentaires obtenus grâce à elles acquièrent ainsi une crédibilité scientifique réelle. Dans le présent cas, nous avons bien retrouvé la subdivision classique des phases stationnaires en phases "polaires" et "apolaires", mais nous observons en outre l'existence d'une classe particulière de phases, celle des macromolécules polyfluorées. Une telle répartition des phases stationnaires en trois catégories avait déjà été presentie par Rohrschneider[18] lors de ses travaux sur la prévision des temps de rétention.

La relation générale que nous mettons en évidence [Gr_1 (Sq, Z, φ_1, T_1) = α Gr_2 (Sq, Z, φ_2, T_2) + β] applicable dans le cas où les deux phases stationnaires φ_1 et φ_2 appartiennent à la même classe taxonomique devrait permettre de simplifier la tâche du chromatographiste de laboratoire. En effet, grâce à elle, il lui devient possible (a) d'une part de confronter valablement ses propres résultats à ceux d'autres chercheurs, résultats obtenus éventuellement dans des conditions expérimentales très différentes; (b) et d'autre part de réaliser des prévisions de comportements chromatographiques grâce à la proférance sur d'autres phases stationnaires des résultats expérimentaux acquis sur une première ressortant de la même classe taxonomique.

En définitive, même si elle semble inattendue, compte-tenu de la complexité du phénomène de chromatographie et des méchanismes composites (mixed mechanisms) qui le régentent et qui coexistent, l'existence de telles relations affines entre des séries de données, obtenues dans des conditions de travail très différentes, est réconfortante et devrait donner lieu à de nombreux développements.

RÉSUMÉ

Pour un ensemble de phases stationnaires les temps de rétention de près de 140 dérivés mono- et polysubstitués du benzène ont été déterminés dans des conditions isothermes.

Les méthodes taxonomiques d'agrégation appliquées aux données ainsi obtenues permettent de classer les phases stationnaires en fonction d'un indice de ressemblance et d'un critère d'agrégation. Le dendogramme résultant permet de distinguer trois groupes de phases (polaires, apolaires et polyfluorées).

A partir des grandeurs de rétention relative à deux phases φ_1 et φ_2 les auteurs

mettent en évidence une relation affine chaque fois que les deux phases appartiennent au même groupe taxonomique. Cette relation:

$$\log t'_{Z_0}(\phi, Z, \varphi_1, T) = a \log t'_{Z_0}(\phi, Z, \varphi_2, T) + b$$

peut être considérée comme un modèle général, applicable à l'ensemble des dérivés mono- et polysubstitués du benzène ayant fair l'objet de la présente étude. (Les coefficients a et b, constants par rapport à Z, sont fonction de ϕ, φ_1, φ_2 et T).

La diversité des conditions expérimentales dans la détermination des données de rétention nous a conduit à faire une mise au point générale entre les diverses expressions des grandeurs de rétention, en étudiant simultanément les effets de la température T et/ou ceux de la nature de la phase stationnaire φ. Dans ce cas, et quelle que soit la nature de la grandeur de rétention, nous obtenons une relation affine

$$Gr_1(Sq, Z, \varphi_1, T_1) = \alpha\, Gr_2(Sq, Z, \varphi_2, T_2) + \beta$$

dans laquelle Gr_1 et Gr_2 représentent indifféremment des indices de rétention, des logarithmes de volumes de rétention ou de temps réduits, et Sq le squelette carboné de la molécule porteur de la fonction Z.

Une telle relation permet de comparer des résultats interlaboratoires. C'est ainsi que pour des phases appartenant à la même classe taxonomique et bien que les caractéristiques des colonnes soient peu voisines et les conditions expérimentales différentes, nous obtenons des relations affines très satisfaisantes entre nos propres données exprimées en logarithme de temps réduit et celles existant dans la littérature exprimées soit en indices, soit en logarithme de volumes de rétention.

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EFFECT OF MOBILE PHASE PRE-HEATING ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMN PERFORMANCE: A NEW TYPE OF COLUMN THERMOSTAT

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SUMMARY

The use of conventional air-bath thermostats as a means of controlling column temperature in high-performance liquid chromatography is limited to a relatively narrow temperature range if full column efficiency is to be maintained. Particularly at high air-bath temperatures, large axial temperature gradients in the column occur, which are detrimental to the column performance. This was demonstrated by both temperature measurements in the column and determination of column performance.

The design of a new type of column thermostat, utilizing a highly efficient, small-volume mobile phase pre-heater, is described. Suitable adjustment of the heat transfer in the mobile phase heater and air heater result in nearly identical temperatures of the mobile phase when entering the column and of the air flowing around the column. Thus axial and radial temperature gradients in the column are minimized, as temperature measurements in the column bed indicate, leading to a marked improvement of column performance at elevated temperatures.

INTRODUCTION

Column temperature has become increasingly accepted as a separation parameter in high-performance liquid chromatography (HPLC). Consequently, the means of maintaining a constant column temperature is now also considered to be an important aspect of HPLC instrument design.

The influence of temperature on the capacity factor (k') can be significant; *e.g.*, in reversed-phase HPLC changes in k' of 2.5–3% per degree of temperature change can be commonly observed. As the magnitude of this effect is also dependent on the substances to be separated, column temperature can be an important means of optimizing separation selectivity. Chmielowiec and Sawatzky[1] published retention data for polynuclear aromatic hydrocarbons (PAHs) showing reversal of the elution order in some instances when the temperature was changed by 10–55°C.

The influence of temperature on column efficiency (plate height) is mainly determined by the relationship between temperature and the diffusion coefficient of the solutes in the mobile phase, and between temperature and the solute mass transfer

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in the mobile and stationary phases. According to Melander and Horváth[2], the plate height in reversed-phase HPLC may decrease by 30% if the column temperature is increased from 15 to 72°C.

A secondary effect of working at elevated column temperature is a reduction in column pressure due to a decrease in mobile phase viscosity. This may become of interest if large plate numbers are required, and the upper pressure limit of the pumping system limits the column length.

In many commercial HPLC instruments, a constant column temperature is achieved by putting the column into a temperature-controlled "air-bath". It is to be expected that insufficient heat transfer from air to the column wall and through the column wall into the mobile phase will lead to considerable axial and radial temperature gradients at temperatures different from ambient if the mobile phase enters the column at ambient temperature. For example, heating water at 4 ml/min from 20 to 80°C would require a heat transfer of 17 J/sec. As heat transfer from air to the column wall and to the mobile phase will depend on various parameters, such as air flow (velocity, direction) and column walls (surface, thickness, material), comparability of analytical results between laboratories and between different types of instrument would be difficult for data measured at elevated column temperatures. Also, it is expected that axial and radial temperature gradients in the column would impair the column efficiency and peak profiles[3,4].

It was the aim of this work (a) to develop a concept for HPLC column thermostatting that would ensure well defined temperatures and an even temperature distribution in the column bed, with particular attention to safety aspects, and (b) to provide some comparative data on temperature distribution in the column and on separation efficiency for classical air thermostatting *versus* the suggested concept.

DESIGN CONSIDERATIONS FOR AN HPLC COLUMN THERMOSTAT

A useful solution for HPLC column thermostatting should meet the following requirements:

(a) axial and radial temperature gradients in the column bed should not exceed 1–2°C (influence on column efficiency and symmetry);

(b) variation of the column flow-rate in the range 0–5 ml/min must not result in temperature changes in the column bed of more than 1–2°C (influence on k');

(c) injection of large sample volumes must not cause (temporary) temperature instabilities in the column bed of more than 1–2°C; and

(d) no part in a column thermostat must exceed a temperature of 180°C (the spontaneous ignition temperature of diethyl ether is 186°C and that of *n*-heptane is 247°C).

Pre-heating of the mobile phase before it enters the column, in order to avoid too large temperature gradients in the column bed, has been suggested previously[4,5]. However, the volume of such "pre-heater columns" necessitates their use upstream of the sample injector. Hence a decrease in the temperature of the mobile phase may result from the injection device, which, as it is not desirable to heat parts of the injector because this could impair reliability, is usually at room temperature. Injection of large sample volumes would result in a temporary disturbance of the temperature equilibrium in the column.

This work was therefore concentrated on investigating the possibilities of designing a small-volume heat exchanger (volume less than 2 μl) which could be used between the injector and the column. Heat transfer from steel capillary walls into liquid flowing through the capillary at flow-rates typical of analytical HPLC (up to 5 ml/min) was systematically studied for capillary inner diameters from 0.10 to 0.25 mm. In comparing the results of temperature measurements with those of heat transfer calculations, it was found that for I.D. <0.20 mm heat transfer equations for turbulent flow had to be used to match the experimental results. This was surprising because, according to the Reynolds numbers at the flow-rates applied, laminar flow profiles were expected. These results are interpreted such that at least in short capillary tubes (less than 20 cm) of I.D. <0.20 mm radial mass transfer similar to that in turbulent flow profiles occurs at even small Reynolds numbers. Fig. 1 shows temperature variations in water flowing through a capillary tube of I.D. 0.13 mm, calculated with equations valid for turbulent flow. Frictional heat was neglected in these calculations, as its influence is very small. Temperature measurements in the water at the outlet of the capillary matched the end-point of the plots (75 and 47°C) within ± 2°C. The temperature of the (external) wall surface of the capillary tube was maintained by sand casting it into an aluminium block, together with a steel-sheathed heating element with a large contact area.

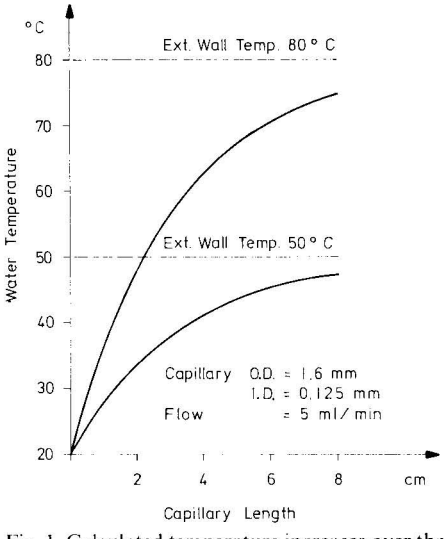


Fig. 1. Calculated temperature increases over the length of a 0.13 mm I.D. capillary for water at a flow-rate of 5 ml/min.

The next task was to ensure near identical temperatures of the column environment (and thus of the column walls) and of the mobile phase liquid at the outlet of the pre-heater capillary. This was achieved by designing the capillary heating block to function simultaneously as the air heater of an air thermostat (column oven). Hence its surface was shaped such that recirculated air flowing across it at a given velocity and surface temperature would be heated (or cooled) enough just to replace its heat loss through the oven walls, and that air temperature and the mobile phase temperature (when entering the column) would be near identical. Fig. 2 illustrates this for

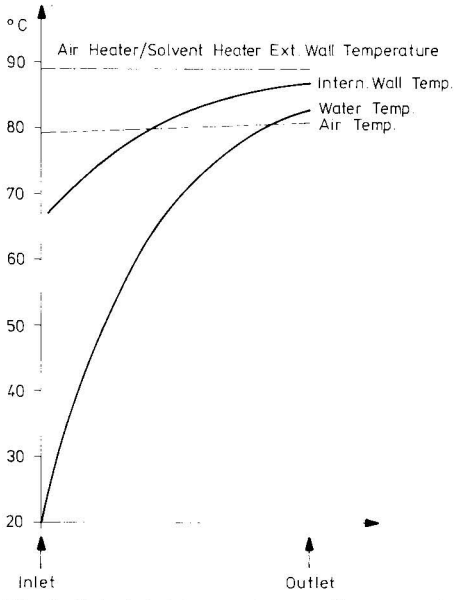


Fig. 2. Calculated temperature profiles over a longitudinal view of the combined solvent and air heater. Water temperature and capillary internal wall surface temperature over capillary length calculated for a flow-rate of 5 ml/min.

certain operating conditions. The surface temperature of this combined solvent–air heater is measured and controlled by a suitable device.

If a stainless-steel tube of suitable dimensions is also die cast into the aluminium block (parallel to the heating element) for circulating cooling fluid, temperature control of the solvent and the column can be extended to ambient and sub-ambient temperatures.

EXPERIMENTAL

Apparatus

Liquid Chromatograph. A Hewlett-Packard 1081B with an automatic injector and UV detector, connected to a 3356 data system, was used.

Columns. A 200 × 4.6 mm RP-8 (10 μm) column was used for temperature measurements in the column bed, and a 100 × 4.6 mm I.D. ODS Hypersil (5 μm) column for chromatographic tests, both supplied by Hewlett-Packard (Waldbronn, G.F.R.).

Temperature sensors. Philips Thermocoax 2ABAC 025 steel-sheathed thermocouples of 0.25 mm O.D. were used for temperature measurements in the column, and a Heraeus WS011 surface resistance thermometer for surface temperature measurements.

Materials

Solvents. Water was used for temperature measurements and acetonitrile–water (70:30) for chromatographic tests.

Samples. Aniline, benzo(*a*)anthracene and 1,2,5,6-dibenzanthracene were dissolved in acetonitrile–water (70:30).

Methods

For temperature measurements in the column bed two steel-sheathed thermocouples, of 0.25 mm O.D., were inserted 1.5 cm deep into the centre of either column end by means of T-junctions with PTFE compression seals. A third sensor was fixed to the outer surface of the column wall to measure the air temperature in the immediate column environment.

Comparative measurements of column performance in an air thermostat (without any solvent pre-heater) *versus* the new solvent + column thermostat (as described in previous section) were performed at room temperature and at temperature settings of 40, 50, 60, 70 and 80°C and at mobile phase velocities of 0.13–0.26 and 0.52 cm/sec. In order to avoid the influence of slow column degradation (a *ca.* 25% decrease in plate number was observed over the measurement duration of 2 weeks) on comparative results, measurements were carried out in the sequence given in Table I. Equilibration times of 1 h and 20 min were allowed after each change of temperature setting and of flow setting, respectively.

Retention times (t_R), capacity factors (k'), theoretical plate numbers (N) and symmetry numbers (S) were determined as follows:

t_R = time at peak maximum − time at injection;

$$k' = \frac{t_R - t_0}{t_0}; \; t_0 = \frac{V_0}{\text{flow-rate}}; \; V_0 = \varepsilon_t \cdot V_{\text{column}}$$

(ε_t obtained from Rozing[6], who measured it according to ref. 7.

$$N = 4\left(\frac{t_R}{W_{0.607}}\right)^2; \; W_{0.607} = \text{peak width at } 60.7\% \text{ of peak height;}$$

$$S = \frac{W_{b0.1}}{W_{f0.1}}; \; W_{b0.1} = \text{width of back part of peak; } W_{f0.1} = \text{width of front part}$$

of peak at 10% of peak height.

Each measurement was repeated five times and the average value and relative standard deviation were calculated. Typical values for the relative standard deviation were 0.5% in retention time measurements, 1% in plate number measurements and 4% in symmetry number measurements.

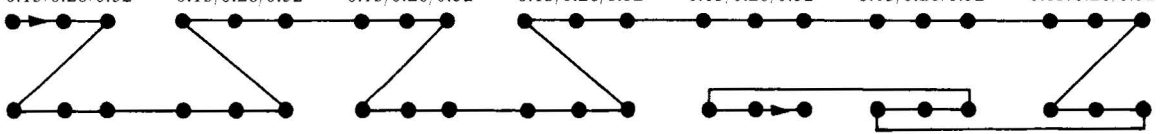
RESULTS AND DISCUSSION

Temperature measurements in the column

As expected, large temperature differences are measured between the inlet, outlet (1.5 cm deep from either end of the column bed) and wall environment of an air-thermostatted column operated at elevated temperatures and flow-rates (Fig. 3). Surprisingly, considerable temperature changes were measured at the column outlet when the flow-rate was changed. Both effects result in axial and radial temperature gradients. When the same column and temperature sensor arrangement is installed in the solvent + column thermostat, the measured temperature differences in the column are reduced to 1.5°C or less (Fig. 4). This confirms that the new combined

TABLE I

MEASUREMENT SEQUENCE

Temperature (°C)	40	50	60	70	80	40	Room (23)
Flow-rate (cm/sec)	0.13/0.26/0.52	0.13/0.26/0.52	0.13/0.26/0.52	0.13/0.26/0.52	0.13/0.26/0.52	0.13/0.26/0.52	0.13/0.26/0.52
Air thermostat Solvent + column thermostat							

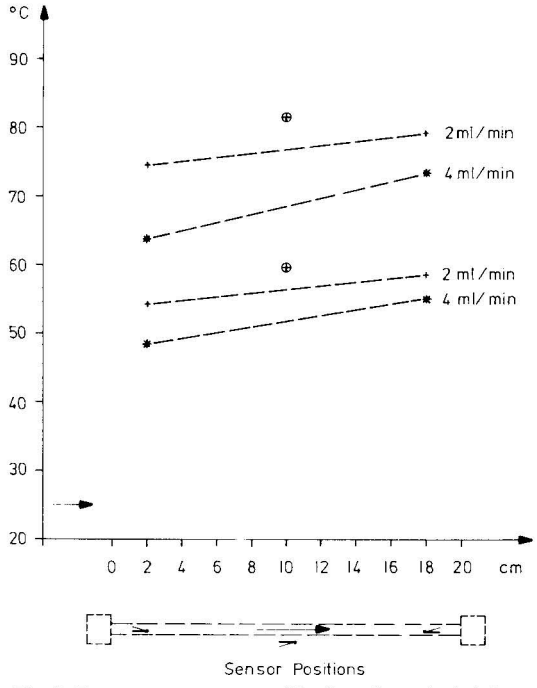


Fig. 3. Temperatures measured in the column bed, 1.5 cm deep from the column inlet and outlet, and in air close to the column wall. Column operated by an air thermostat heating only the column wall. Column: 200 × 4.6 mm I.D. RP-8 (10 μm). Solvent: water.

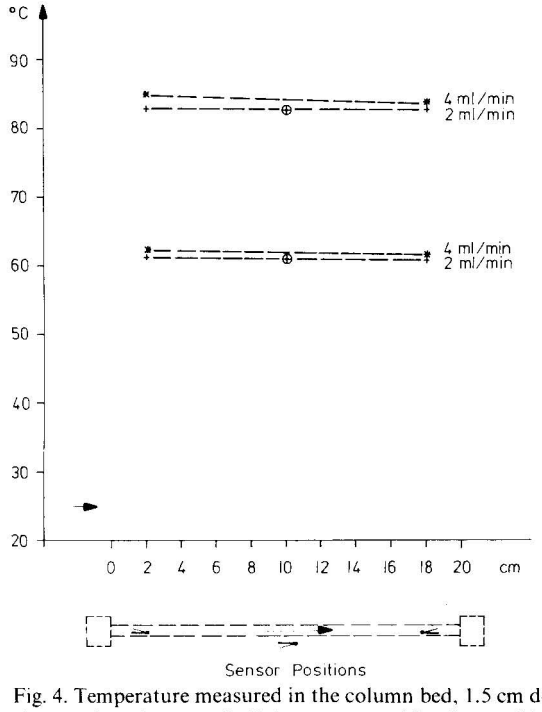


Fig. 4. Temperature measured in the column bed, 1.5 cm deep from the column inlet and outlet, and in air close to the column wall. Column operated by the combined solvent + column thermostat.

solvent + column heater behaves as predicted from the calculated temperature plots presented in Figs. 1 and 2.

Chromatographic tests

After temperature measurements had confirmed the substantial influence of the method of column heating on the temperature distribution in the column bed, the relevance for chromatography was investigated. Column efficiency, peak symmetry and k' were compared for both methods of column thermostatting, as described under Experimental.

Fig. 5 shows percentage ratios of plate numbers under otherwise identical conditions in the air thermostat, relative to the combined solvent + column thermostat. Whereas at room temperature identical plate numbers are measured, at 40°C and 0.5 cm/sec a decrease in column efficiency of 30% in the air thermostat is observed, increasing to a 90% decrease at 80°C and 0.5 cm/sec (examples of chromatograms obtained in the two thermostats under these conditions are given in Fig. 8). This means that "incorrect" column thermostatting can result in up to a 10-fold reduction of column efficiency.

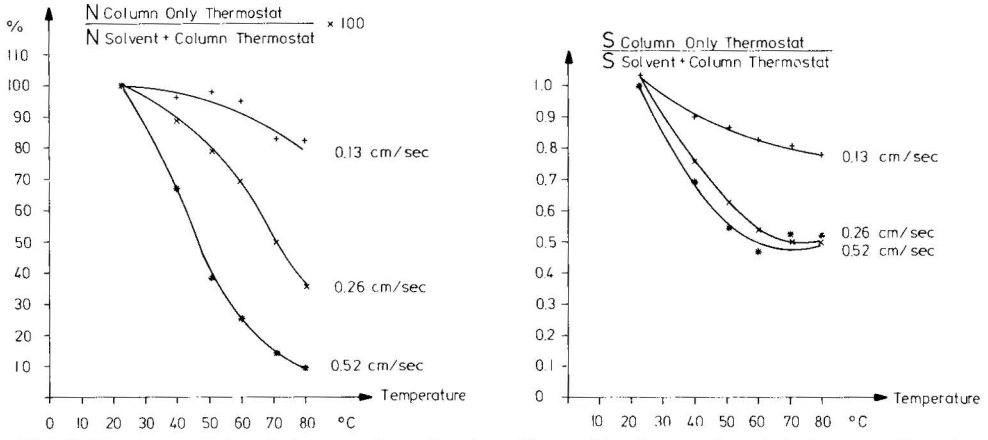


Fig. 5. Percentage ratio of plate numbers showing effects of "column only (air-) thermostat" against "solvent + column thermostat" at various mobile phase flow-rates, plotted against thermostat temperature. Column: 100 × 4.6 mm I.D. ODS Hypersil (5 μm). Solvent: acetonitrile water (70:30). Solute: 1,2,5,6-dibenzanthracene.

Fig. 6. Ratio of symmetry numbers showing effects of "column only thermostat" against "solvent + column thermostat" at various mobile phase flow-rates, plotted against thermostat temperature. Conditions as in Fig. 5.

Fig. 6 shows the ratios of peak symmetries as observed in both types of thermostats; this plot indicates that under the applied chromatographic conditions, temperature gradients occurring in an air thermostatted column will cause a front-leading tendency of the peaks.

In Fig. 7 k' is plotted against flow-rate at various column temperatures for both thermostatting methods. As expected from the results of temperature measurements in the column bed, air thermostatting of the column leads to a considerable increase in k' with increasing flow-rate. Actually an "effective mean bed temperature" could be estimated from the k' values in comparison with those measured in the

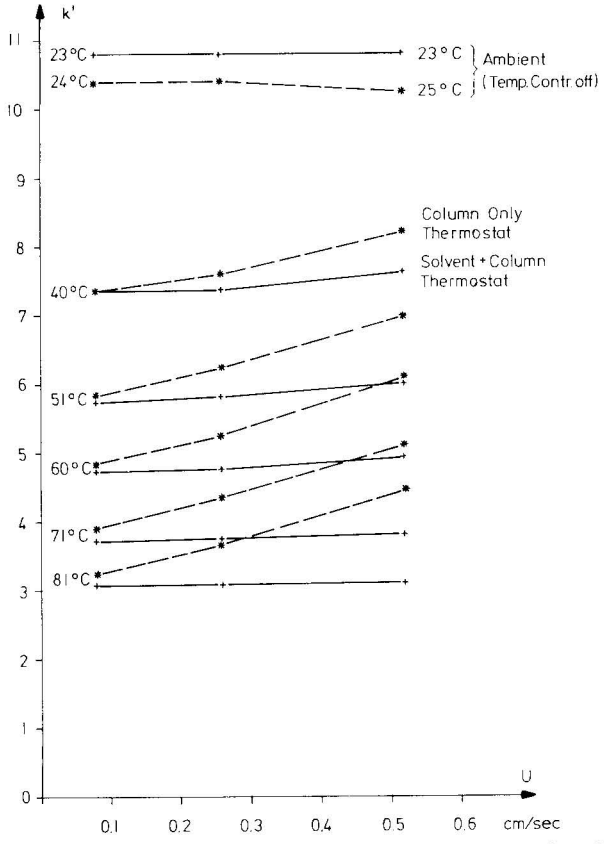


Fig. 7. Comparison of capacity factors showing effects of "column only thermostat" (★---★) against "solvent + column thermostat" (+ +) at various thermostat temperatures, plotted against mobile phase flow-rate. Conditions as in Fig. 5.

combined solvent + column thermostat: *e.g.*, at an air temperature of 81°C and a flow-rate of 0.52 cm/sec, k' in the air thermostat is about 4.45, and this value is reached in the solvent + column thermostat at about 66°C. The conclusion is that under these conditions the "effective mean column bed temperature" in the air thermostat is about 15°C lower than the air temperature around the column. This is consistent with the results of the temperature measurements shown in Fig. 3.

From the results presented here it appears obvious that effective use of the advantages that elevated temperatures would offer in many HPLC analyses can hardly be made in many of the column thermostats that are in use at present. A good column thermostat can save analysis time and improve detection limits, because at higher column temperatures lower plate heights (H) are usually observed and the optimum of the H–u curve shifts to higher mobile phase velocities (band broadening due to axial diffusion increases, but this is more than compensated for by the lower resistance to mass transfer at higher temperatures, while the Eddy diffusion should be little affected by temperature). In the 100-mm ODS Hypersil column, the minimum of the H–u curve was shifted from about 0.1 cm/sec at room temperature to about 0.3 cm/sec at 81°C. Plate heights at 0.5 cm/sec were reduced from about 27 μm at 23°C to about 22 μm at 81°C. These results, however, may be impaired by the influence of extra-column band broadening effects, which are not negligible when this chromato-

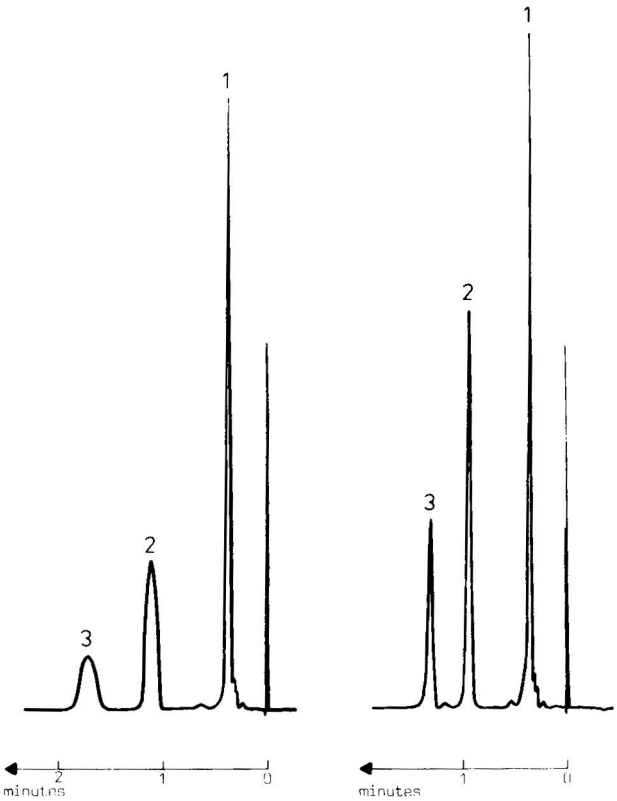


Fig. 8. Examples of chromatograms obtained from "column only thermostat" (left) and from "solvent + column thermostat" (right) under identical chromatographic conditions: Column: 100 × 4.6 mm I.D. ODS Hypersil (5 μm). Solvent: acetonitrile–water (70:30). Flow-rate: 3.8 ml/min. Sample: (1) aniline, (2) benzo(*a*)anthracene and (3) 1,2,5,6-dibenzanthracene dissolved in mobile phase. Injection volume: 5 μl. Thermostat setting: 80°C. UV detector (254 nm), 0.128 absorbance unit full-scale. Chart speed: 1 in./min. Effects of column heating method are less marked on early peaks (k' near 0), partly owing to the influence of extra-column band broadening.

graphic equipment is used with small-volume columns packed with 5-μm material. Similar or better improvements of column efficiency with increasing temperature were reported by Herbut and Kowalczyk[8] for adsorption chromatography. More detailed studies would be necessary to obtain a comprehensive picture of the potential that elevated column temperatures might have in optimizing the analysis time in routine HPLC analysis.

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CONFIRMING THE PRESENCE OF N-NITROSAMINES IN AMBIENT AIR AND CIGARETTE SMOKE BY CONVERTING TO AND PHOTOCHEMICALLY ALTERING THEIR CORRESPONDING N-NITROAMINES

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SUMMARY

An analytical procedure for the detection of N-nitrosamines, which is one of the most sensitive available, has been expanded to make the technique more selective. This procedure involves oxidizing the nitrosamines to their corresponding nitroamines, with subsequent detection of picogram quantities by gas chromatography with an electron-capture detector. The presence of the nitroamines is then confirmed by photochemically altering them using a UV lamp and observing if the resulting decrease in peak height with time of irradiation obeys rate expressions derived by using reference nitroamines. The results of analyses of ambient air and cigarette smoke samples for the presence of nitrosamines are presented.

INTRODUCTION

To detect nitrosamines in ambient air or cigarette smoke, a technique is desirable that is capable of detecting picogram quantities. Of the analytical techniques[1–8] that have been used to detect nitrosamines in a variety of media, few are capable of achieving this sensitivity. In addition to sensitivity, selectivity is an important consideration, especially since questionable results obtained in the past may be attributed to the presence of interfering substances[7,9]. At present, a gas chromatograph with a mass spectrometer[7] and a gas chromatograph with a thermal energy analyzer detector[10] are considered to be the most selective techniques. However, these detectors are not commonly found in analytical laboratories. Therefore, an alternative procedure was developed whereby the more readily available electron-capture detector (ECD) can be used.

One of the most sensitive methods used for the analysis of nitrosamines in foods involves oxidizing the nitrosamines to their corresponding nitroamines; a gas chromatograph with an ECD is then used to detect the resulting nitroamines at the picogram level[11–14]. It is this technique that has been expanded and coupled with a

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newly developed, highly selective, confirmatory test. This confirmatory test is based on the fact that nitroamines, which are strong electron capturers, are converted into relatively weak electron-capturing compounds when irradiated with short-wave UV light[15,16]. By irradiating nitroamine standards under set conditions for different periods, a photochemical rate constant was derived for each nitroamine. These rate constants were then used to characterize the specific nitroamines, since the observed decreases in peak heights were correlated with time of irradiation. Further verification was obtained by two-column confirmation.

EXPERIMENTAL

Apparatus

Gas chromatograph (Tracor MT 220); Kuderna–Danish evaporators (25-ml capacity concentrator tubes with three-stage Snyder columns) and Chromaflex chromatography columns, 30 cm × 1 cm I.D. (Kontes Glass); short-wave (germicidal) UV lamp (Spectroline TF-250); vacuum pump (Metal Bellows MB-41); midget impingers with 75-ml capacity bottles (Ace Glass).

Reagents

Pesticide-grade pentane, heptane and ethyl acetate (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and methylene chloride (Mallinckrodt, St. Louis, MO, U.S.A.); neutral alumina, activity I (ICN Pharmaceuticals, Cleveland, OH, U.S.A.): trifluoroacetic acid (Mallinckrodt, organic reagent); 50% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA, U.S.A.; certified); anhydrous diethyl ether (Fisher ACS).

Standards

Dimethyl- and diethylnitroamines were synthesized by nitrolyzing the corresponding N,N-dialkylformamides with nitric acid in a solvent of trifluoroacetic anhydride[17]. A stock standard solution was prepared by dissolving approximately 10 mg of each nitroamine in 100 ml of ethyl acetate; subsequent dilutions were made in heptane. Standard curves were constructed after injecting standards on to both Reoplex and Carbowax columns. Standards were stored at room temperature, shielded from direct exposure to light.

Irradiation experiment

Standard solutions containing both dimethyl- and diethylnitroamines were irradiated in quartz cuvettes for various periods; the samples were situated 4 cm from a 25-W short-wave UV lamp. After each irradiation, 5 μl was injected on to both Carbowax and Reoplex columns. Plots of the log of the number of picograms per 5-μl injection *vs.* time of irradiation in seconds, were constructed. The resulting slopes were recorded.

Ambient air sampling sites

Ambient air samples were taken at two sites in Rhode Island; four samples at a site in Providence and four at a rural site in West Greenwich. These samples were taken on different days under different meteorological conditions.

Ambient air sample collection

Ambient air was bubbled through a trap containing 50 ml of 1 *N* potassium hydroxide for 90 min at a flow-rate of *ca.* 2 l/min; the trap was shielded from direct sunlight. After collection, the trap was rinsed with 10 ml of KOH, which was added to the 50-ml sample. The sample, stored in an all-glass flask, was returned to the laboratory for analysis.

Sample work-up

(1) The exposed KOH solution was extracted in a separatory funnel three times with 8-ml portions of methylene chloride (CH_2Cl_2). (2) After drying with anhydrous sodium sulfate, the extract was poured into a concentrator tube. The remaining sodium sulfate was rinsed several times with small portions of CH_2Cl_2, which were added to the concentrator tube until a total of 24 ml of CH_2Cl_2 extract had been collected. (3) A 6-ml portion of the CH_2Cl_2 extract was pipetted into a stoppered vial and stored in a refrigerator. (4) The remaining 18 ml of extract was evaporated in a Kuderna–Danish (KD) evaporator to a volume of 1 ml; the temperature of the water bath was maintained between 52 and 55°C. (5) Then 1 ml of distilled water was added to the KD through the Snyder column, and the resulting 2 ml of sample was transferred to a 50-ml stoppered flask using a pasteur pipette. (6) Trifluoroacetic acid (5 ml) and 50% hydrogen peroxide solution (4 ml) were added, and the mixture was swirled for 30 sec, then allowed to react for 24 h. (7) The mixture was poured on to 15 g of crushed ice and neutralized using *ca.* 40 ml of 20% potassium carbonate solution. (8) The nitroamines were extracted in a separatory funnel three times with 8-ml portions of CH_2Cl_2. (9) The extract was dried over sodium sulphate and evaporated to 1 ml in a KD. (10) Pentane (4 ml) was added through the Snyder column, and the mixture saved for column clean-up.

Column clean-up

The following substances were added, in sequence, to a 30 × 1 cm I.D. chromatography column that was half filled with *n*-pentane; each substance was slowly added, with occasional tapping of the column to assist in settling[14].

(1) Neutral alumina (4 g; Brockmann activity III, prepared by adding 6 ml of water to 94 g of alumina activity I).

(2) Magnesium oxide (2 g).

(3) Enough sodium sulfate to fill *ca.* 1 cm of the column. Both the magnesium oxide and the sodium sulfate were purified by baking at 550°C for 6 h. The pentane was drained into the top of the sodium sulfate layer, and the column was washed by eluting with 25 ml of 50% (v/v) diethyl ether in pentane followed by 25 ml of pentane. (The column may not require washing if the components are of sufficient purity.) The sample (step 10 of the sample work-up) was added to the column and drained just into the sodium sulfate layer. The concentrator tube was washed with 2 ml of pentane, which was added to the column and again drained into the sodium sulfate layer. The column was then eluted with 25 ml of a 1% (v/v) solution of diethyl ether in pentane; this eluate was discarded. Next, the column was eluted with 35 ml of 25% (v/v) diethyl ether in pentane, and this 35 ml of eluate was concentrated in a KD evaporator to a final volume of *ca.* 0.5 ml. A water-bath temperature of between 46 and 50°C was used. Heptane was added through the Snyder column of the KD

until the concentrator tube contained 1 ml of sample. The concentrator tube was stoppered and the sample was saved for gas chromatographic analysis.

Gas chromatographic analysis

The concentrated extract was analyzed for nitroamines by using a gas chromatograph equipped with a tritium ECD. The columns and conditions used were: (1) A glass column (6 ft. × 0.250 in. O.D.) packed with 10% of Carbowax 20M on Chromosorb W/HP (80–100 mesh) operated isothermally at 160°C, with a nitrogen flow-rate of 70 ml/min; and (2) a similar column packed with 6% of Reoplex 400 on AW DMCS-treated Chromosorb W (60–80 mesh) and operated isothermally at 140°C, with a nitrogen flow-rate of 70 ml/min.

If the presence of a nitroamine was verified on both columns, then the 6-ml aliquot (from step 3 of the sample work-up) was analyzed for the presence of nitroamines or interfering species that may have been present in the ambient air sample. The 6-ml aliquot was concentrated to 1 ml in a KD evaporator, then 5 ml of pentane were added through the Snyder column, and the sample was again concentrated to 1 ml. This process of adding pentane and concentrating was repeated twice more, and a final 0.5 ml of extract was analyzed on the gas chromatograph.

Cigarette smoke samples

Four cigarettes were smoked, two at a time, with the lit ends inserted into a 250-ml side-arm vacuum flask; each cigarette was puffed once every 30 sec. The side-arm of the flask was connected to the inlet of a midget impinger by a piece of PTFE tubing; the impinger bottle contained 50 ml of 1 *N* KOH. The sample was drawn from the flask at a rate of 2 l/min for a total sampling time of *ca.* 14 min. After collection, the trap was rinsed with 10 ml of KOH, which was added to the 50-ml sample. The sample work-up, column clean-up, gas chromatographic analysis and irradiation procedures were carried out exactly as for the ambient air samples. In addition, two modifications in the overall procedure were used to improve the isolation of the peak corresponding to dimethylnitroamine.

Modification A. A pre-oxidation clean-up of the cigarette smoke extract was performed using the same column as was used for the post-oxidation clean-up of the ambient air extracts[14]. After performing step 4 of the sample work-up, the 1 ml of CH_2Cl_2 extract was diluted with 4 ml of pentane and transferred to the top of the clean-up column. The sample was drained just into the sodium sulfate layer. The concentrator tube was washed with 2 ml of pentane, which was added to the column and again drained into the sodium sulfate layer. The column was then eluted with 50 ml of a 1% (v/v) solution of CH_2Cl_2 in pentane; this eluate was discarded. Next, the column was eluted with 50 ml of a 50% (v/v) solution of CH_2Cl_2 in pentane, this eluate being concentrated in a KD evaporator to a volume of 1 ml. The remaining steps of the procedure were carried out (continuing with step 5 of the sample work-up). In addition, following the oxidation of the sample, a modification to the post-oxidation clean-up was carried out (Modification B).

Modification B. Instead of collecting one 35-ml fraction [25% (v/v) diethyl ether in pentane] from the post-oxidation clean-up column, two separate fractions were collected. The first 20 ml were collected in one flask, while the remaining 15 ml were collected in another. Each fraction was then concentrated and analyzed. In

testing this procedure with nitroamine standards, it was observed that the first (20-ml) fraction quantitatively contained the diethylnitroamine, while the second (15-ml) fraction quantitatively contained the dimethylnitroamine.

Sample analysis and trap efficiencies

Analysis recovery experiments were conducted by adding different amounts of dimethyl- and diethylnitrosamine to 60-ml portions of 1 *N* KOH and carrying out the entire analysis procedure that was used for the ambient air samples. In addition, the following experiment was performed to determine whether the KOH trap was capable of quantitatively trapping both dimethyl- and diethylnitrosamine. To a 250-ml vacuum flask were added 130 ng of dimethylnitrosamine and 91 ng of diethylnitrosamine. The side-arm of the flask was connected to the inlet of the KOH trap, and 180 l of room air were pulled through the vacuum flask and into the trap at a flow-rate of 2 l/min for 90 min. The sample was then analyzed, as were the ambient air samples, and the efficiency of the KOH trap was determined.

Testing for artifact formation

To show that dimethylnitrosamine was not formed during the collection or work-up of the cigarette smoke samples, through the reaction of nitrite ions (or other nitrosating agents present in the sample) with dimethylamine, the following experiment was performed.

Two midget impingers were connected via a "T" to a common side-arm vacuum flask. To one of the impinger bottles was added 0.5 ml of an aqueous solution containing 272 μg of dimethylamine and 0.5 ml of an aqueous solution containing 272 μg of sodium nitrite. Four cigarettes were smoked (two at a time) with tips inserted in the flask for a total sampling time of 14 min. The inlet flow through each impinger was 1.2 l/min. The KOH solutions were then extracted and analyzed, along with a KOH blank to which 272 μg of dimethylamine was added.

RESULTS AND DISCUSSION

Dimethyl- and diethylnitroamine were prepared in good yields. The purity of each was verified by determining its melting or boiling point and IR spectrum. The standard curve obtained using the Reoplex column is shown in Fig. 1. The retention times of dimethyl- and diethylnitroamine on this column are 2.30 and 2.93 min, respectively, while on the Carbowax column the corresponding retention times are 2.62 and 3.16 min.

A standard solution containing 0.0568 ppm of dimethylnitroamine and 0.0714 ppm of diethylnitroamine was irradiated with the UV lamp for different periods. After each irradiation, 5 μl of the resulting solution was injected on to the Reoplex column (Fig. 2). Fig. 3 is a graphic presentation of the decrease in concentration of nitroamines with time of irradiation. This photo-irradiation experiment was conducted six times over a 6-month period. The range of the slope for dimethylnitroamine (Fig. 3) was calculated to be -4.4 to -4.8×10^{-3} (average value -4.7×10^{-3}), while the range for diethylnitroamine was -5.8 to -6.2×10^{-3} (average value -6.0×10^{-3}). By using the average values for the slopes in the following

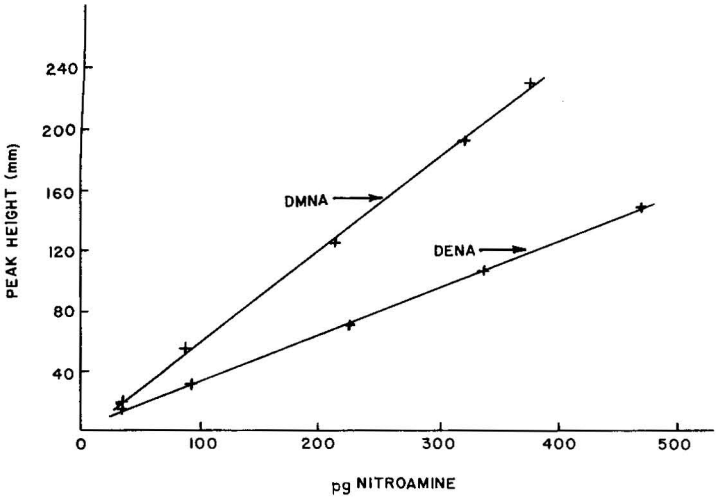

Fig. 1. Standard graphs for dimethylnitroamine (DMNA) and diethylnitroamine (DENA).

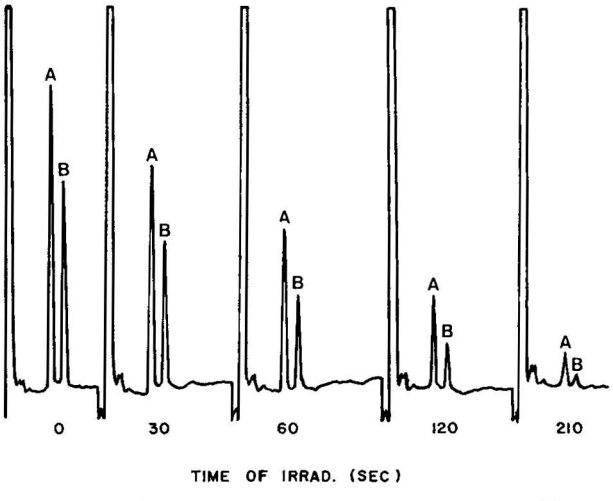

Fig. 2. Irradiation of a standard containing 0.0568 ppm of DMNA (peaks A) and 0.0714 ppm of DENA (peaks B).

equations, it is possible to predict, to within 6%, the decrease in peak height after irradiating a sample for a time (t) of 60 sec.

$$\log \text{dimethylnitroamine content (pg) after irradiation} = -4.7 \times 10^{-3} t + \log \text{dimethylnitroamine content (pg) before irradiation}$$

$$\log \text{diethylnitroamine content (pg) after irradiation} = -6.0 \times 10^{-3} t + \log \text{diethylnitroamine content (pg) before irradiation}$$

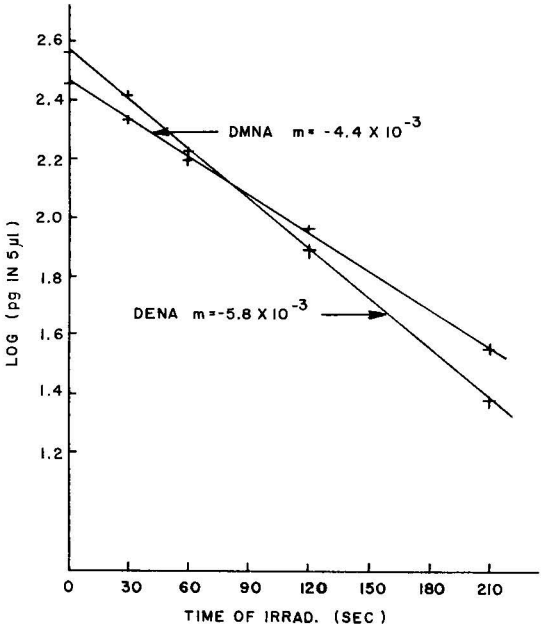

Fig. 3. Photoirradiation of a standard containing 0.0568 ppm of DMNA and 0.0714 ppm of DENA.

To date, only a limited number of air samples has been tested. Of the eight samples that were analyzed, only one was found to contain trace levels of both dimethyl- and diethylnitrosamine. The chromatograms in Fig. 4 show: (a) a blank carried through the total procedure, (b) the air sample with peaks corresponding to

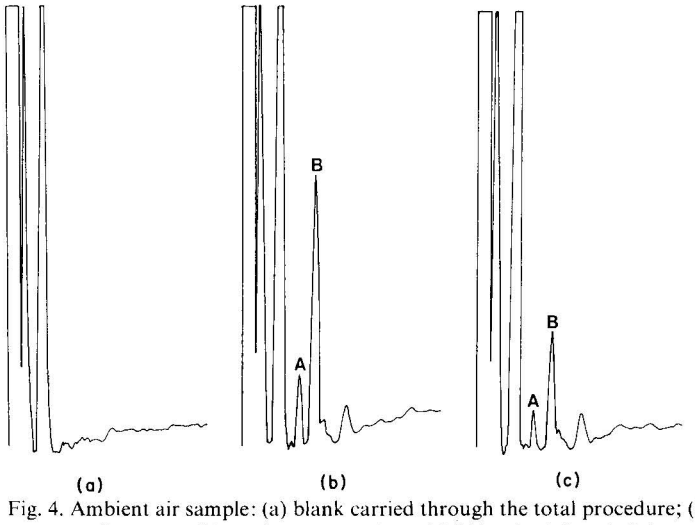

Fig. 4. Ambient air sample: (a) blank carried through the total procedure; (b) ambient air sample: peak A corresponds to an ambient air concentration of 0.044 ppb of dimethylnitrosamine (DMN); peak B to 0.256 ppb of diethylnitrosamine (DEN); (c) ambient air sample after irradiating for 60 sec with a short-wave UV lamp.

ambient air concentrations of 0.044 ppb of dimethylnitrosamine and 0.256 ppb of diethylnitrosamine (confirmed on Carbowax and Reoplex columns), and (c) the air sample after irradiating for 60 sec with short-wave UV light. The resulting peak heights in chromatogram (c) are both within 5% of their theoretically calculated values. Additional sampling is planned at the site where trace amounts were detected. If future samples are found to contain nitrosamines, then the tests for artifact formation, as described by Fine *et al.*[10] and by Krull *et al.*[19], will be performed to ensure that the nitrosamines were not formed during the sample collection or work-up.

The analysis of the extract from the smoke of four American brands of cigarettes showed the presence of dimethylnitrosamine. Fig. 5a is the chromatogram obtained after collecting the last 15 ml of eluate from the post-oxidation clean-up column, concentrating to 1 ml and injecting 5 μl on to the Reoplex column. The labeled peak represents 105.7 pg of dimethylnitroamine. In addition, a 5-μl injection on the Carbowax column gave a peak corresponding to 109.5 pg of dimethylnitroamine. If it is assumed that 10% of the cigarette smoke was trapped and the remaining 90% escaped through the top of the flask, then it can be estimated that each cigarette contained approximately 120 ng of dimethylnitrosamine (side-stream smoke). Upon irradiating this sample for 60 sec, photoreaction rate constants of 2.5×10^{-3} sec^{-1} and 2.7×10^{-3} sec^{-1} were calculated, based on peak-height drops observed on the Carbowax and Reoplex (Fig. 5b) columns, respectively. Since these rates are *ca.* 45% less than the expected average rate of 4.7×10^{-3} sec^{-1}, the peak either does not represent dimethylnitroamine or there is an interfering substance(s) present. To prove or disprove the presence of the nitroamine, the following test was performed: A few μl of a concentrated dimethyl- and diethylnitroamine standard were added to the 1 ml of UV-irradiated cigarette smoke sample mentioned above. Fig. 6a represents a 7-μl injection of the resulting sample on to the Reoplex column. After irradiating this sample for a total of 60, 120 and 240 sec, the reaction rates were calculated to be 2.7×10^{-3} sec^{-1} $\pm$ 0.3×10^{-3} sec^{-1} for dimethylnitroamine and

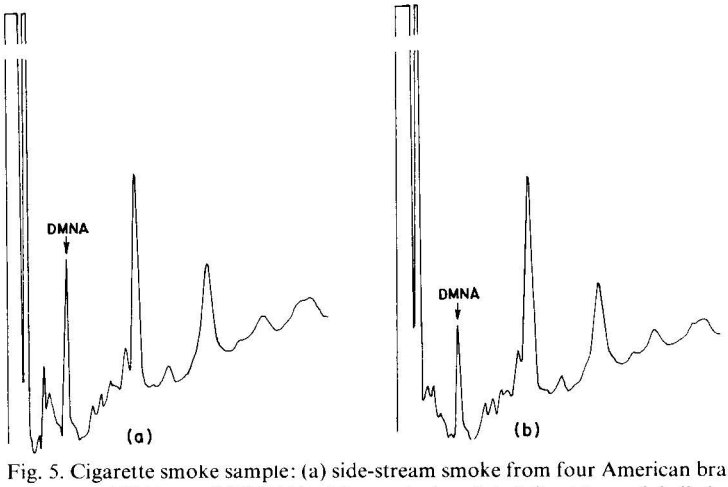

Fig. 5. Cigarette smoke sample: (a) side-stream smoke from four American brand cigarettes (labelled peak represents 105.7 pg of DMNA); (b) sample irradiated for 60 sec (labelled peak represents 72.8 pg of DMNA).

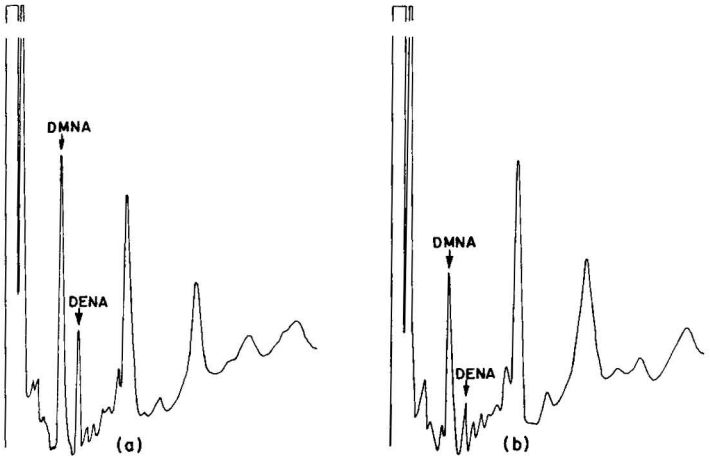

Fig. 6. Cigarette smoke sample: (a) side-stream smoke from four American brand cigarettes irradiated for 60 sec and then "spiked" with a few μl of DMNA–DENA standard (labelled peaks represent 209.7 pg of DMNA and 150.2 pg of DENA); (b) Sample irradiated for 120 sec (labelled peaks represent 106.6 pg of DMNA and 59.3 pg of DENA).

TABLE I

EFFICIENCY OF THE ANALYTICAL PROCEDURE

DMN = dimethylnitrosamine; DMNA = dimethylnitroamine; DEN = diethylnitrosamine; DENA = diethylnitroamine.

Amount DMN added (ng)	*Amount DMNA (ng) (assuming 100% conversion and recovery)*	*Amount DMNA recovered (ng)*	*Recovery (%)*
65.0	79.0	28.2	36
65.0	79.0	35.1	44
65.0	79.0	27.1	34
130.0	158.1	47.7	30
130.0	158.1	53.3	34
130.0	158.1	60.8	38
Amount DEN added (ng)	*Amount DENA (ng) (assuming 100% conversion and recovery)*	*Amount DENA recovered (ng)*	*Recovery (%)*
45.5	52.6	34.3	65
45.5	52.6	36.5	69
45.5	52.6	30.8	59
91.0	105.3	78.4	74
91.0	105.3	59.1	56
91.0	105.3	61.1	58

3.4×10^{-3} sec^{-1} $\pm$ 0.2×10^{-3} sec^{-1} for diethylnitroamine. Fig. 6b shows 7 μl of the sample after being irradiated for 120 sec. Since the rates of photoreaction of both the "spiked" and "unspiked" samples are within experimental agreement, the presence of dimethylnitroamine in cigarette smoke has been confirmed.

Recovery experiments, which were conducted by adding different amounts of dimethyl- and diethylnitrosamine to 60-ml portions of 1 *N* KOH and carrying out the sample work-up and analyses (as was used for ambient air samples), showed that the average recovery for dimethylnitrosamine is 36%, while that for diethylnitrosamine is 64% (Table I). Preliminary tests indicate that the inclusion of modifications *A* and *B* will lower the recovery by several percent. The experiment conducted to determine the efficiency of the KOH trap[10,18] in retaining dimethyl- and diethylnitrosamine showed that, within the limits of experimental variability (Table I), both compounds appear to have been quantitatively trapped.

The test for artifact formation proved that dimethylnitrosamine was not formed during the cigarette smoke sample collection or work-up via the reaction of nitrite ions (or other nitrosating agents present) with dimethylamine. The two samples collected simultaneously were found to contain the same amount of dimethylnitrosamine, after adjustment for trace levels of dimethylnitrosamine detected in the dimethylamine[20].

CONCLUSIONS

The analytical procedure presented has been shown to be suitable for detecting sub-ppb levels of volatile nitrosamines in ambient air and cigarette smoke. The lower detection limit for dimethyl- and diethylnitrosamine is *ca.* 0.05 ppb. It is important to note that this procedure contains four tests that must be considered collectively in order to confirm the presence of the nitrosamine(s). These tests are:

(a) Analysis of the sample extract by gas chromatography with an ECD before and after carrying out the oxidation so that the absence of and then appearance of the nitroamine peaks will be observed.

(b) Injection of the oxidized and cleaned-up sample on to two different gas chromatographic columns and then comparing the retention times and peak-height ratios to nitroamine standards.

(c) Irradiation of the resulting nitroamines with UV light and comparing the calculated rates of photoreaction to the constants derived by irradiating nitroamine standards. If the sample contains impurities that affect the rates of photoreaction, as was observed with the cigarette smoke samples, then nitroamine standards can be added to the sample and new matrix-specific photoreaction rate constants can be calculated.

(d) Conducting tests to check for artifact formation.

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GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF N-ALKYLPYRIDINIUM SALTS

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SUMMARY

Long-chain N-alkylpyridinium salts (I–V) used as detergents and disinfectants were readily determined by the gas–liquid chromatography of the reduction products (VI–X) obtained by treatment with sodium borohydride and nickel(II) chloride. The procedure is useful for the routine analysis of N-alkylpyridinium salts, as the reduction takes place quantitatively and the reagents are relatively safe to handle, instead of the need to use a complicated apparatus for catalytic hydrogenation. The reduction products of I–V were identified as N-alkylpiperidines (VI–X), *i.e.*, the perhydrogenated products, by mass spectrometry and elemental analysis. The reduction system described serves as a novel and convenient method for the synthesis of N-alkylpiperidines.

INTRODUCTION

In continuing studies of the analysis of cationic surfactants, we have now studied the determination of long-chain N-alkylpyridinium salts by gas–liquid chromatography (GLC). The long-chain N-alkylpyridinium compounds (alkyl = C_{10}–C_{18}) are used as disinfectants, and N-cetylpyridinium chloride is well known as an antiseptic detergent.

As these pyridinium salts are non-volatile, their GLC has previously been performed after conversion into their reduction products[1] or thermal decomposition products[2,3]. However, such conversion methods have not been used in quantitative analysis because of the appearance of multiple peaks and the poor response.

In previous papers, we reported that herbicides based on N-alkylbipyridylium derivatives, such as diquat[4], paraquat[4] and morphamquat[5], could be determined by GLC of their perhydrogenated products obtained by reduction with a mixture of sodium borohydride ($NaBH_4$) and a transition metal salt, *e.g.*, nickel(II) chloride

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TABLE I

N-ALKYLPIPERIDINES (VI–X)

No.	*R*	*Boiling point (°C/mmHg)*			*Appearance*	*Formula*	*Analysis (%)*	
		*Experimental**	*Literature*				*Calculated*	*Found*
			Value	*Reference*				
VI	n-$C_{10}H_{21}$	128/8	150–152/13	7	Colourless oil	$C_{15}H_{31}N$	C 79.92	79.85
							H 13.86	14.05
							N 6.21	6.20
VII	n-$C_{12}H_{25}$	161/5	114–116/0.4	8	Colourless oil	$C_{17}H_{35}N$	C 80.56	80.75
							H 13.92	14.20
							N 5.53	5.40
VIII	n-$C_{14}H_{29}$	171/5	186–187**	6	Colourless oil	$C_{19}H_{39}N$	C 81.06	81.04
							H 13.96	14.11
							N 4.98	4.79
IX	n-$C_{16}H_{33}$	176–177/1	148–151/0.1	7	Colourless oil	$C_{21}H_{43}N$	C 81.47	81.41
							H 14.00	14.08
							N 4.52	4.24
X	n-$C_{18}H_{37}$	199–200/5 (32.5-34)***	126–127/8	7	Colourless solid	$C_{23}H_{47}N$	C 81.82	81.37
							H 14.03	13.96
							N 4.15	4.04

* All uncorrected.
** Melting point (HCl salt).
*** Melting point.

($NiCl_2$). This paper deals with the GLC determination of long-chain N-alkylpyridinium salts by use of the reduction system of sodium borohydride and nickel(II) chloride.

EXPERIMENTAL

Apparatus

GLC was carried out with a glass column (1 or 2 m × 0.3 cm I.D.) on a Hitachi Model 073 gas chromatograph equipped with a hydrogen flame-ionization detector (HFID). Mass spectrometry was performed on a Shimadzu Model LKB-9000 mass spectrometer.

Materials

N-Decylpyridinium bromide(I), N-tetradecylpyridinium bromide(III) and N-stearylpyridinium bromide(V) were prepared by reaction of pyridine and the corresponding alkyl bromide in the usual way[6]. N-Laurylpyridinium chloride(II) and N-cetylpyridinium chloride(IV) were commercially available.

Reagents

All chemicals were of analytical-reagent grade. Freshly distilled diethyl ether was used in the extraction of the reduction products.

Synthesis of N-alkylpiperidine (VI–X) from N-alkylpyridinium salts (I–V) by the $NaBH_4$–$NiCl_2$ reduction system

N-Alkylpyridinium salts (I–V: 2.76–4.13 g, 0.01 mol) and $NiCl_2 \cdot 6H_2O$ (2.38 g, 0.013 mol) were dissolved in methanol (300 ml). To the solution was added $NaBH_4$ (11.35 g, 0.03 mol) in small portions at 25°C with stirring, which was continued for 1 h at room temperature. A black precipitate was formed and hydrogen was evolved. The precipitate was filtered off and washed with methanol and the filtrate and washings were combined and concentrated to one third of the initial volume under reduced pressure. To the residue was added water (50 ml) and the mixture was extracted with benzene (three 50-ml volumes). The benzene layer was dried over sodium sulphate and evaporated to dryness. The resulting residue was distilled under reduced pressure to give a colourless oil in 70–75% yield, as shown in Table I.

Reduction of N-alkylpyridinium salts(I–V) with the $NaBH_4$–$NiCl_2$ system on the gas–liquid chromatographic scale

To the aqueous solution (1 ml) of N-alkylpyridinium salt (I–V: 5–140 μg) was added 0.02 *M* $NiCl_2$ (0.5 ml) and 2.6 *M* $NaBH_4$ (0.6 ml) with stirring. The mixture turned black and hydrogen was evolved. The mixture was allowed to stand for 1 h at room temperature, and was then extracted with diethyl ether (four 2-ml volumes). The combined organic layer was dried over sodium sulphate, acidified with a few drops of acetic acid and evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (100 μl), and 1 μl of the solution was injected into the gas chromatograph.

GLC was performed on a glass column (2 m × 0.3 cm I.D.) packed with 5% potassium hydroxide plus 5% PEG 20M on Chromosorb W AW DMCS (60–80

mesh) at 190°C with a nitrogen flow-rate of 30 ml/min (injection port temperature, 250°C; attenuation, 1 × 8). In the determination of IV and V, a shorter column (1 m × 0.3 cm I.D.) was used at 180°C (retention times relative to internal standard: IX = 0.85; X = 1.2). The internal standards were 0.01% *p*-anisidine for I, II and III, 0.02% γ,γ'-dipyridyl for IV and 0.02% diphenylamine for V.

Temperature-programmed GLC was carried out on dual glass columns packed with 1% SE-30 on Gas-Chrom Z, the column temperature being increased at 7.5°C/min from 100 to 250°C.

Rate of reduction of IV–IX with the $NaBH_4$–$NiCl_2$ system

An aqueous solution (1 ml) of IV (1 mg; 3 μmol/ml) was treated with 0.02 *M* $NiCl_2$ (9 μmol; 0.45 ml) and 2.6 *M* $NaBH_4$ (1.5 mmol; 0.58 ml) at room temperature for various times (15 min, 30 min, 1 h and 2 h). The resulting reduction products were examined under isothermal GLC conditions as described above.

Influence of the amount of $NaBH_4$ on the reduction of IV–IX with the $NaBH_4$–$NiCl_2$ system

An aqueous solution (1 ml) of IV (1 mg; 3 μmol) was treated with 0.45 ml (9 μmol) of 0.02 *M* $NiCl_2$ and different amounts of 2.6 *M* $NaBH_4$ (aqueous solutions of 15, 30, 60, 90, 150, 210, 300, 900 and 1500 μmol) at room temperature for 1 h. The resulting reduction products were subjected to GLC as described above.

Influence of the amount of $NiCl_2$ on the reduction of IV–IX with the $NaBH_4$–$NiCl_2$ system

An aqueous solution (1 ml) of IV (1 mg; 3 μmol) was treated with different amounts of 0.02 *M* $NiCl_2$ (aqueous solutions of 0.09, 0.15, 0.3, 0.6, 0.9, 1.5, 2.0, 3.0, 6.0, 9.0 and 12 μmol) and 2.6 *M* $NaBH_4$ (0.58 ml) at room temperature for 1 h. The resulting reduction products were examined by GLC as described above.

RESULTS AND DISCUSSION

Reduction of N-alkylpyridinium salts with $NaBH_4$ has been reported by several investigators[1-9]. On GLC, the reduction products emerged as two peaks composed of dihydro- and tetrahydro-N-alkylpiperidines[1]. On the other hand, the catalytic hydrogenation of these N-alkylpyridinium compounds gave N-alkylpiperidines (perhydrogenated products)[6], but the application of this method in GLC was undesirable because of the tedious procedure involved in handling the apparatus.

Previously we found[4] that the $NaBH_4$–$NiCl_2$ reduction system was the most effective for obtaining the perhydrogenated products in the reduction of N-alkylpyridinium salts with a combination of $NaBH_4$ and the salts of transition metal ions such as Ag^+, Co^{2+}, Ni^{2+}, Mn^{2+}, Fe^{3+}, Pt^{4+} and Cr^{6+}. Using a similar procedure to that reported previously[4], the $NaBH_4$–$NiCl_2$ reduction system was applied to the GLC determination of long-chain N-alkylpyridinium salts (alkyl = C_{10}–C_{18}). The reduction products (VI–X) thus prepared by the treatment of I–V with $NaBH_4$ and $NiCl_2$ were chromatographed in a glass column packed with 5% potassium hydroxide + 5% PEG 20M on Chromosorb W AW DMCS at 190°C. As shown in Fig. 1, all hydrogenated products (VI–X) were readily resolved and each gave a single, symmetrical

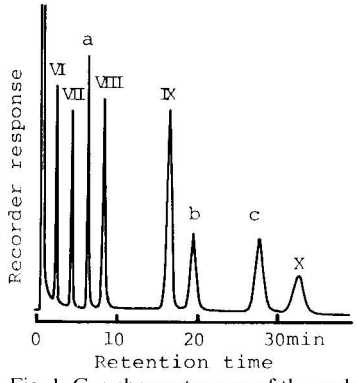


Fig. 1. Gas chromatogram of the perhydrogenated products (VI–X) derived from N-alkylpyridinium salts (I–V). Internal standards: a = *p*-anisidine; b = γ,γ′-dipyridyl; c = diphenylamine.

peak. The peaks emerged in order of the size of the alkyl groups, the retention time doubling for each additional carbon atom in the alkyl groups.

Satisfactory separations were achieved on alkaline columns such as 5% potassium hydroxide plus 5% PEG 20M or PEG 6000, but neutral columns such as PEGS, PEG 20M, PEG 6000, SE-30 and OV-17 exhibited slight tailing under isothermal conditions.

Temperature-programmed GLC of the reduction products (VI–X) was carried out with dual glass columns packed with 1% SE-30 on Gas-Chrom Z, the column temperature being increased at 7.5°C/min from 100 to 250°C. As alkaline columns cannot be used at high temperatures, an SE-30 column was employed. The peaks of the

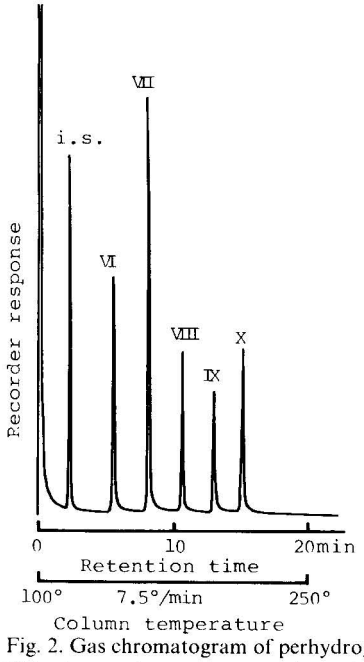
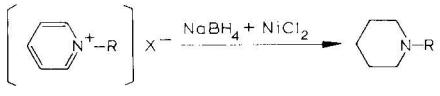


Fig. 2. Gas chromatogram of perhydrogenated products (VI–X) derived from N-alkylpyridinium salts (I–V) by temperature-programmed gas chromatography. Internal standard (i.s.): diphenyl.

products appeared in order of the number of carbon atoms in the alkyl group, the retention time increasing by about 5 min for each additional carbon atom, as shown in Fig. 2. The isothermal and temperature-programmed GLC behaviours of VI–X are similar to those of higher alkanes[10].

The structure of the reduction products (VI–X) was clarified by an independent synthesis in the following manner. When a large excess of $NaBH_4$ was added to a methanolic solution of pyridinium salts (I–V) and $NiCl_2$, a black precipitate of nickel boride[11,12] was immediately formed, with evolution of hydrogen. The reduction of these compounds (I–V) proceeded smoothly with the continuous evolution of hydrogen, and was complete in about 1 h to afford the hydrogenated products (VI–X). The resulting products were purified by distillation under reduced pressure and gave colourless oils, as indicated in Scheme 1.



I: R = n-$C_{10}H_{21}$, X = Br
II: R = n-$C_{12}H_{25}$, X = Cl
III: R = n-$C_{14}H_{29}$, X = Br
IV: R = n-$C_{16}H_{33}$, X = Cl
V: R = n-$C_{18}H_{37}$, X = Br

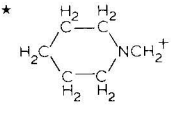
VI: R = n-$C_{10}H_{21}$
VII: R = n-$C_{12}H_{25}$
VIII: R = n-$C_{14}H_{29}$
IX: R = n-$C_{16}H_{33}$
X: R = n-$C_{18}H_{37}$

Scheme 1.

Elemental analyses of the products were consistent with the calculated values for N-alkylpiperidines. The mass spectra of VI–X exhibited a base fragment peak at m/e 98 due to the piperidino-N-methylene ion*, and also showed a parent ion peak corresponding to the N-alkylpiperidine. Based on these results, the reduction products (VI–X) were identified as N-alkylpiperidines, *i.e.* the perhydrogenated compounds of I–V. Thus this reduction serves as a novel and convenient method for the synthesis of N-alkylpiperidines.

The conditions for the perhydrogenation of the N-alkylpyridinium salts (I–V) with $NaBH_4$ and $NiCl_2$ on the analytical (GLC) scale were examined in aqueous solution by use of N-cetylpyridinium chloride (IV). As shown in Fig. 3, the reduction of an appropriate amount of IV (3 μmol in 1 ml of water), which corresponds to a high response in the determination, is dependent on the amount of $NiCl_2$ in the presence of a large excess of $NaBH_4$ (1.5 mmol in 0.58 ml of water). The hydrogenation of IV to VIII proceeded quantitatively when more than 3 μmol of $NiCl_2$ (1.0 mol per mole of IV) were used in the reducion system.

When less than 0.8 μmol of $NiCl_2$ was used, undesirable peaks due to by-products resulting from incomplete reduction of IV appeared. This indicates that the reduction system requires at least 1 mol of $NiCl_2$ per mole of IV for the complete hydrogenation of three double bonds in the pyridinium nucleus. In the presence of a

* 

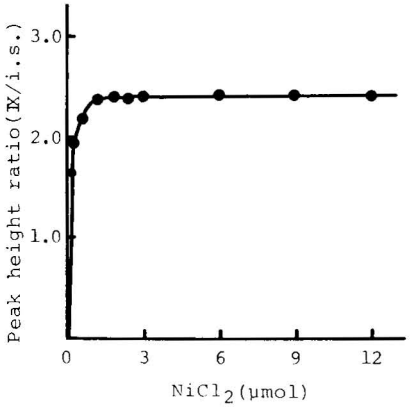
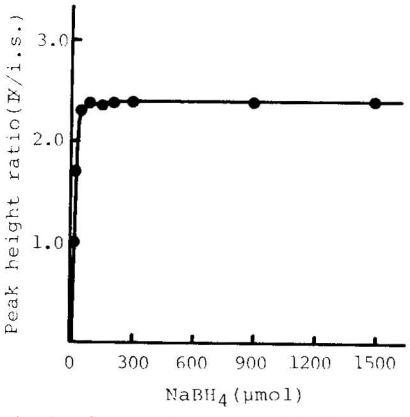


Fig. 3. Influence of amount of $NiCl_2$ on reduction of IV to IX with the $NaBH_4$–$NiCl_2$ system. Internal standard (i.s.): γ,γ'-dipyridyl.

definite amount of $NiCl_2$ (9 μmol), the reduction was complete with amounts of $NaBH_4$ in the range 90–150 μmol, as shown in Fig. 4. To avoid incomplete reduction, a large excess of the reducing agent, such as the combination of 0.5 ml (10 μmol) of 0.02 *M* $NiCl_2$ and 0.6 ml (1.56 mmol) of 2.6 *M* $NaBH_4$, is advisable for the GLC analysis of I–V. Fig. 5 demonstrates that the hydrogenation under these conditions is completed within 1 h at room temperature.



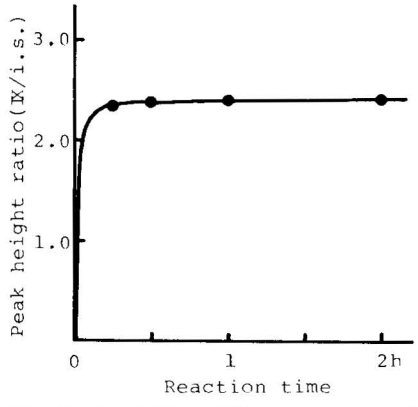
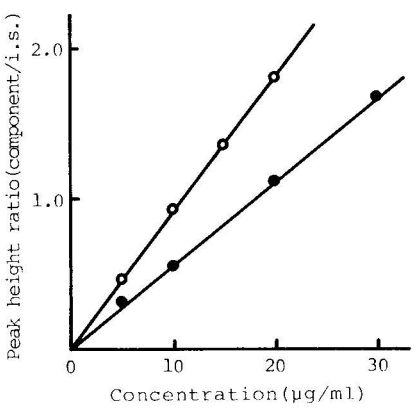


Fig. 4. Influence of amount of $NaBH_4$ on reduction of IV to IX with the $NaBH_4$–$NiCl_2$ system. Internal standard (i.s.): γ,γ'-dipyridyl.

Fig. 5. Reduction of IV with the $NaBH_4$–$NiCl_2$ system. Internal standard (i.s.): γ,γ'-dipyridyl.

The quantitative determination of N-alkylpyridinium salts (I–V) under isothermal conditions was performed by the peak-height ratio method. The calibration graphs obtained with perhydrogenated products for I–V (Figs. 6 and 7) showed good linearity using *p*-anisidine as internal standard for I–III, γ,γ'-dipyridyl for IV and diphenylamine for V.



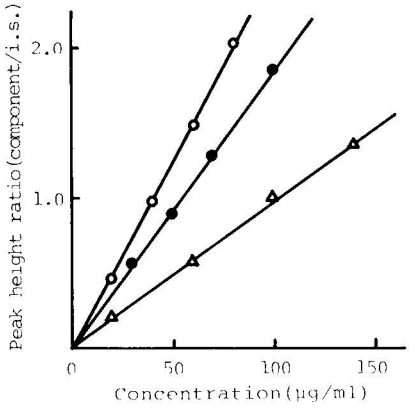


Fig. 6. Calibration graphs for I (○) and II (●). Internal standard (i.s.): *p*-anisidine.

Fig. 7. Calibration graphs for III (○), IV (●) and V (△). Internal standards (i.s.): III, *p*-anisidine; IV, γ,γ′-dipyridyl; V, diphenylamine.

The limits of determination for the N-alkylpyridinium compounds (I–V) using an HFID were 5 μg/ml for I and II, 10 μg/ml for III and 20 μg/ml for IV and V.

The reproducibilities and average recoveries for the analyses of I–V are illustrated in Table II.

TABLE II

PRECISION OF ANALYSIS

Parameter	*Analysis No.*	*Compound*				
		I	*II*	*III*	*IV*	*V*
Amount added (μg)	–	16.9	20.0	12.1	30.0	30.0
Amount found (μg)	1	17.7	19.4	10.6	27.5	27.7
	2	18.2	19.3	11.7	29.2	30.9
	3	16.8	19.9	12.7	29.4	30.6
	4	16.8	20.4	12.2	31.9	31.3
	5	17.5	19.9	12.4	31.2	31.4
	6	16.9	19.6	12.4	30.8	30.2
	7	15.4	19.6	12.1	30.1	30.2
	8	16.0	20.9	11.7	29.2	31.5
	9	17.6	20.0	12.2	30.0	29.3
	10	17.7	21.0	11.8	28.4	30.5
Mean (μg)	–	17.1	20.0	12.0	29.8	30.4
Standard deviation (μg)	–	0.8	0.6	0.6	1.3	1.1

CONCLUSION

Three double bonds in the N-alkylpyridinium nucleus are completely hydrogenated by the $NaBH_4$–$NiCl_2$ reduction system, and I–V are readily determined by

the GLC of their perhydrogenated compounds (VI–X) obtained by this reduction.

The procedure is suitable for the routine determination of N-alkylpyridinium compounds in detergents, disinfectants and drugs, as the reduction takes place cleanly in aqueous medium at room temperature with easily handled reagents, without the need for the complicated apparatus previously employed[6]. Further, our procedure would be applicable to the determination other quaternary ammonium compounds in disinfectants and drugs. Details of these analyses will be reported in the near future.

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SYSTEMATIC SEPARATION OF MEDIUM-SIZED BIOLOGICALLY ACTIVE PEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The systematic separation of medium-sized biologically active peptides by high-performance liquid chromatography (HPLC) is described. Three steps are involved: first, high-performance sodium dodecyl sulphate (SDS) gel chromatography on a newly developed column, TSK-GEL 2000SW; secondly, ion-pair reversed-phase HPLC using stepwise elution with mobile phases containing SDS and tetrabutylammonium phosphate; thirdly, high-performance cation-exchange chromatography on a Partisil SCX column for purification, using stepwise gradient elution with volatile buffers. Removal of SDS was possible in the final step. This systematic method is fast, reproducible and gives excellent separations and recoveries.

INTRODUCTION

In recent years many reports have been published on the application of high-performance liquid chromatography (HPLC) for the separation of proteins and peptides (for a review, see ref. 1). However, the use of HPLC in the field of peptide separation has generally been restricted to the final step of separation[2–8]. The systematic separation of biologically active peptides has not been reported. Until now, tedious and time-consuming traditional methods such as Sephadex gel filtration followed by ion-exchange chromatography have been used for this purpose.

We report here the first HPLC procedure for the systematic separation of medium-sized biologically active peptides having molecular weights above 500.

EXPERIMENTAL

Chemicals

Cytochrome *c* and aprotinin were obtained from Mochida (Tokyo, Japan), ovalbumin from Miles Labs. (Elkhart, IN, U.S.A.), ferritin from Boehringer (Mannheim, G.F.R.), α-chymotrypsinogen A from Sigma (St. Louis, MO, U.S.A.) and porcine MC insulin and glucagon from Novo (Bagsvaerd, Denmark). All other peptides were from the Peptide Institute (Minoh, Osaka, Japan). Sodium dodecyl

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[^{35}S]sulphate ([^{35}S]SDS) (12.4 mCi/mmol) and [^{3}H]leu-enkephalin (26.8 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). [^{32}P]Phosphoric acid (carrier free) was obtained from Japan Atomic Energy Research Institute (Ibaragi, Japan). Aqueous counting scintillant, ACS II, was from Amersham (Arlington Heights, IL, U.S.A.). All other reagents were obtained from Nakarai Chemical Co. (Kyoto, Japan).

All reagents were of analytical or HPLC grade. The solvents used were filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and deaerated before use.

General methods

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used which included a Model SIL injector, a Model SGR-1A step gradient former, a Model GRE-2B gradient former, a Model CRD-5A chemical reaction detector, a Model SPD-2A variable wavelength UV detector equipped with an 8-μl flow cell and a Model RF-500LC spectrofluorometer equipped with a 12-μl flow cell. Fractions were collected in a Gilson Model FC-220K fraction collector equipped with a flow interrupter (Gilson, Middleton, WI, U.S.A.). The column effluent was monitored by UV spectrophotometry at 210 or 280 nm, or by post-column fluorescence derivatization with fluorescamine[9,10]. All chromatograms were run at room temperature (*ca.* 20–23°C). Radioactivity was counted in a Aloka Model LSC-700 liquid scintillation counter (Aloka, Tokyo, Japan).

HPLC (Step I)

A prepacked TSK-GEL 2000SW column[11–14] (particle size 10 $\pm$ 2 μm; 60 $\times$ 0.75 cm) was obtained from Toyo Soda (Tokyo, Japan). According to the manufacturer, this column has fractionation ranges of mol. wt. 500–60,000. Samples were dissolved in deionized, distilled water. Analyses were performed by using a mobile phase of 0.05 *M* sodium phosphate buffer, pH 7.2, containing 0.3% (w/v) SDS, at a flow-rate of 0.3 ml/min. The void volume (V_0), and the total permeation volume, V_t, were determined by using ferritin and alanine, respectively[12]. The distribution coefficient, K_d, is defined as

$$K_d = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume of the sample.

Preliminary experiments revealed that the addition of SDS to the mobile phase alleviated adsorption of peptides on the column. The resolution was dependent chiefly on the concentration of SDS, the most suitable concentration being in the range 0.3–0.5% (10.7–17.3 m*M*). In this method, no pretreatment of samples was required, except for ferritin.

Ion-pair reversed-phase HPLC (Step II)

A prepacked octadecylsilane column, Cosmosil 5C_{18} (particle size 5 μm; 15 $\times$ 0.46 cm), was obtained from Nakarai. Samples were dissolved in 0.05 *M* sodium phosphate buffer, pH 7.2, containing 0.3% SDS, which was used as the mobile phase in Step I. The separation of peptides was performed by a modification of the method

described by Hancock *et al.*[15,16], using stepwise elution at a flow-rate of 1.5 ml/min. The program of mobile phases was as follows: (1) acetonitrile–water (50:50, v/v) containing 10 m*M* phosphoric acid and 15 m*M* SDS, 10 min; (2) acetonitrile–water (60:40) containing 15 m*M* SDS, 10 min; (3) acetonitrile–water (75:25) containing 15 m*M* SDS, 10 min; (4) acetonitrile–water (50:50) containing 15 m*M* SDS and 1 m*M* tetrabutylammonium phosphate (TBA), 10 min.

UV detection was not suitable for flow monitoring over 25 min due to significant baseline drift at high sensitivities.

High-performance cation-exchange chromatography (Step III)

A prepacked Partisil SCX column (25 × 0.46 cm) was obtained from Whatman (Maidstone, Great Britain). Samples were dissolved in 75% acetonitrile–water containing 15 m*M* SDS. The separation was performed by a modification of the method described by Radhakrishnan *et al.*[17], using stepwise gradient elution with pyridine–acetate buffers. The program of mobile phases and flow-rates was as follows: (1) distilled water or 0.005 *M* pyridine–0.04 *M* acetic acid at a flow-rate of 1.5 ml/min, 15 min; (2) 3.0 *M* pyridine–0.5 *M* acetic acid at a flow-rate of 0.5 ml/min, 30 min.

Pyridine–acetate buffers exhibit UV absorbance, therefore the UV detector is unsuitable in this case. The detection system used was the fluorescamine method. Some peptides such as neurotensin having amino terminal prolyl or pyroglutamyl residues are weakly reactive or undetectable with fluorescamine[18]. Fractions obtained were applied in Step II for the detection of these peptides.

Removal of SDS was estimated with [^{35}S]SDS. The sample solutions were prepared by incubating the peptide samples, at concentrations of 5–20 nmol/ml, in acetonitrile–water (50:50) containing 1% SDS and $9.6 \cdot 10^{-4}$% [^{35}S]SDS at *ca.* 20°C overnight.

Recovery

Recoveries of peptides were estimated by using [^{3}H]leu-enkephalin.

RESULTS AND DISCUSSION

Step I

Adsorption of peptides on the column could not be alleviated by adding salts such as sodium hydroxide or sodium sulphate to the mobile phase[12]. Kato *et al.*[14] have reported that the separation range of TSK-GEL columns was not extended below mol. wt. 10,000 even by use of a 2000SW column which has a smaller pore size, and the elution behaviour of samples was greatly affected by sodium phosphate concentration in the eluent. They used a 0.05–0.2 *M* sodium phosphate buffer containing 0.1% SDS as the eluent. Frenkel and Blagrove[19] have reported the gel filtration of proteins and peptides in denaturing solvents (6 *M* urea and 0.5% SDS) over controlled pore glass (CPG) for molecular weight determination in the range of 3500–12,000. Adsorption of samples on the CPG column could be alleviated by increasing the concentration of SDS to 0.5% and including 6 *M* urea in the phosphate buffer. Our preliminary experiments revealed that the addition of 0.3–0.5% SDS was effective in alleviating the adsorption of peptides on the TSK-GEL 2000SW column.

The elution volumes, K_d values and molecular weights of samples are shown in Table I. Pretreatment with SDS had no significant effect on the elution behaviour of samples, except for ferritin. The reproducibility of results was better than $\pm 2\%$ (relative standard deviation). The elution order of samples seems to follow that of the molecular weights. This rule, of course, cannot be generalized. A semilogarithmic plot of molecular weight *versus* K_d for several samples is shown in Fig. 1. The linearity could not be extended below mol. wt. 5000. It was possible to estimate the relative molecular weights of proteins and peptides in the range of 1000–45,000 despite the deviation of several samples.

TABLE I

ELUTION VOLUMES (V_e), K_d VALUES AND MOLECULAR WEIGHTS OF SAMPLES CHROMATOGRAPHED ON A TSK-GEL 2000SW COLUMN

No.	*Sample*	*V_e (ml)*	*K_d*	*Mol.wt.*
1	Ferritin (V_0)	12.6	0	480,000
2	Ovalbumin	12.6	0	45,000
3	α-Chymotrypsinogen A	13.2	0.058	25,700
4	Cytochrome *c*	14.5	0.183	12,400
5	Aprotinin	14.5	0.183	6520
6	Insulin (porcine)	14.6	0.192	5782
7	Glucagon	14.8	0.211	3485
8	Tetracosactide	14.8	0.211	2934
9	α-Endorphin	15.9	0.317	1746
10	Neurotensin	15.0	0.231	1673
11	α-Melanocyte stimulating hormone	15.2	0.250	1665
12	Somatostatin	15.4	0.270	1638
13	Substance P	15.1	0.240	1348
14	Luteinizing hormone releasing hormone	14.9	0.221	1182
15	Bradykinin	15.2	0.250	1060
16	Angiotensin I	15.0	0.231	1297
17	Angiotensin II	15.8	0.308	1046
18	Angiotensin III	15.6	0.288	931
19	Oxytocin	15.0	0.250	1007
20	Vasopressin	15.2	0.250	1056
21	Arg-vasotocin	15.3	0.260	1022
22	β-Casmorphin	17.5	0.471	790
23	β-Casmorphin (1–5)	19.0	0.615	580
24	Thymopoietin active fragment (32 36)	16.8	0.404	680
25	Met-enkephalin	20.2	0.731	574
26	Leu-enkephalin	19.0	0.615	556
27	Tuftsin	16.4	0.365	501
28	Arginine	21.0	0.808	174
29	Alanine (V_t)	23.0	1.000	89
30	Other amino acids	>22.8	–	–

This method is rapid and simple compared with SDS polyacrylamide gel electrophoresis[20,21] in the estimation of peptide molecular weights. The fluorescamine system can detect as little as 10 pmol peptides[9,22], therefore high sensitivity is obtainable.

Regnier *et al.*[23] have reported that the ratio $V_t - V_0/V_0$ in gel permeation

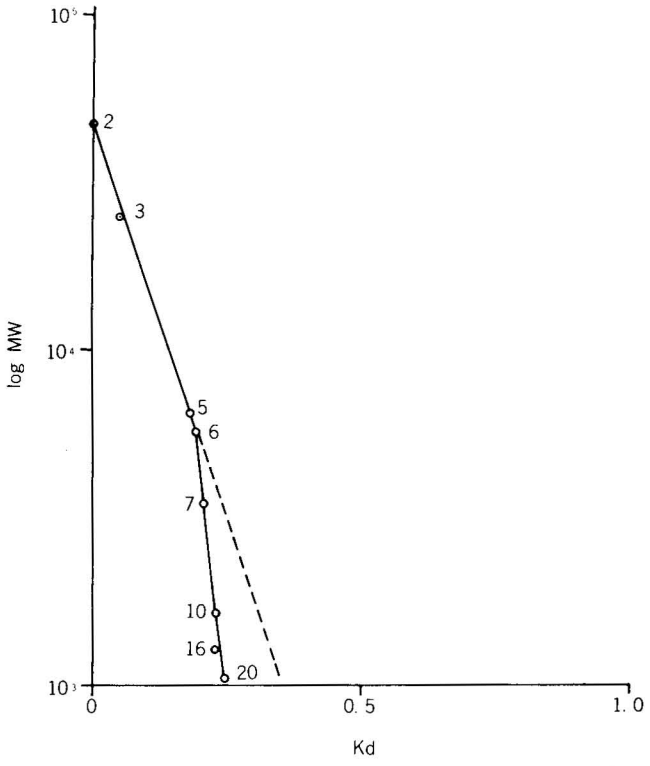

Fig. 1. Semilogarithmic plot of molecular weight (MW) *versus* K_d for several samples chromatographed on a TSK-GEL 2000SW column. Sample numbers as in Table I.

chromatography cannot exceed 1.3. The ratio obtained from our results is about 0.83. However, other phenomena such as partition are more likely to be predominant in the separation of peptides having molecular weights below 5000.

Recently, we have reported[24] the analysis of medium-molecular-weight peptides in normal and uremic body fluids using the method reported here.

Step II

The results are shown in Table II. The reproducibility was better than $\pm 3\%$ (relative standard deviation). Using this program for the stepwise elution, samples having molecular weights above 6000 were not eluted. It was possible, however, to elute these samples using other programs of mobile phases (data not shown). Several linear gradients tested gave poor resolutions and lacked reproducibility. The samples dissolved in solutions containing SDS showed different behaviours from the samples dissolved in distilled water when other ion-pairing reagents such as phosphoric acid[15,25] were used, and some samples were strongly retained on the column. Therefore, we chose SDS as the anionic ion-pairing reagent.

Hancock *et al.*[15] have used lower concentrations of SDS (< 5 mM) in an effort to minimize the problems of removal of SDS from the samples. We used higher concentrations of SDS (15 mM) in order to minimize band broadening or tailing and

TABLE II

RETENTION TIMES OF SAMPLES CHROMATOGRAPHED ON A COSMOSIL $5C_{18}$ COLUMN

Samples as in Table I. ND = Not done; NE = not eluted.

No.	*Retention time (min)*	*No.*	*Retention time (min)*
1	ND	16	25.1
2	ND	17	14.8
3	ND	18	27.1
4	NE	19	3.9
5	NE	20	11.3
6	35.6	21	2.4
7	35.3	22	3.3
8	35.2	23	2.3
9	6.4	24	16.2
10	24.1	25	3.1
11	23.2	26	3.8
12	21.1	27	15.7
13	28.6	28	6.7
14	11.0	29	ND
15	24.2	30	<5.8

for resolution. Hancock *et al.*[16] have also shown that an equimolar mixture of the anionic (SDS) and cationic (dodecylammonium acetate) reagents resulted in rapid elution of peptide and protein samples. Our preliminary experiments, however, revealed that the addition of as little as 1 m*M* of the cationic ion-pairing reagent TBA to acetonitrile–water (50:50) containing 15 m*M* SDS is effective for the elution of larger peptides such as insulin.

Step III

All peptides tested were eluted within 40 min as sharp peaks. SDS was eluted with distilled water, therefore it was readily removed. An example of an elution profile is given in Fig. 2. The baseline elevation and ghost peak were essentially inevitable. The removal of [^{35}S]SDS within 15 min was 98.7 ± 1.4% (mean ± S.D., $n = 3$). Acetonitrile, monitored with a UV detector at 210 nm, was eluted as a sharp peak near the void volume. Moreover, phosphoric acid could be eliminated and the removal of [^{32}P]phosphoric acid within 6 min was 99.4 ± 3.6% (mean ± S.D., $n = 4$) (Fig. 3). The removal of TBA and the removal rate of acetonitrile were not estimated in this study.

However, large peptides such as insulin were eluted as two peaks, that is, a SDS–peptide complex peak and a native peptide peak (Fig. 4A). The dissociation of the SDS–peptide complex may thus be incomplete in distilled water. For these peptides, instead of distilled water, 0.005 *M* pyridine–0.04 *M* acetate buffer was effective in dissociating the SDS–peptide complex (Fig. 4B). The important question to consider is whether or not SDS truly binds to small peptides. Most proteins bind 1.44 g SDS per g of protein[26,27]. However, the degree of binding of SDS to small peptides has not been reported. Assuming that small peptides bind 1.44 g of SDS per g peptide, the net amounts of bound SDS are small on a molar basis compared with large

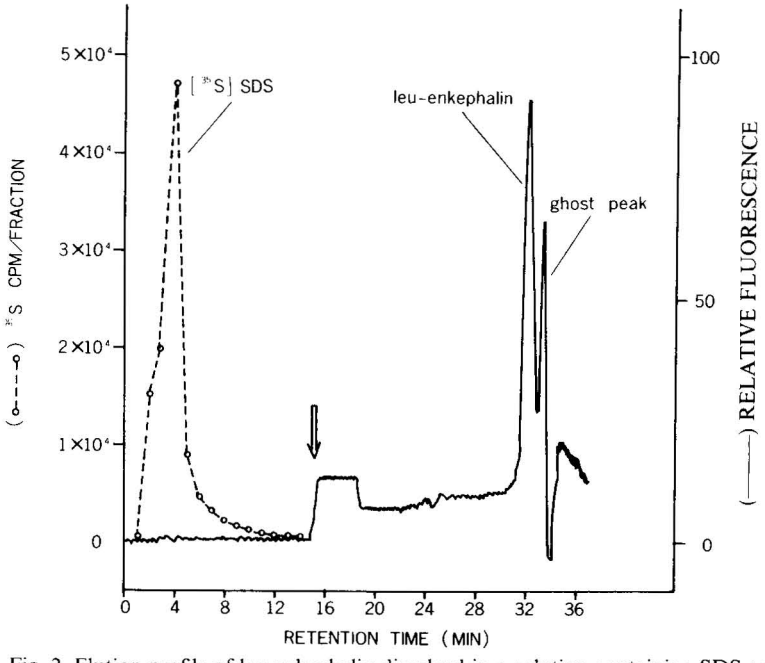

Fig. 2. Elution profile of leu-enkephalin dissolved in a solution containing SDS and [^{35}S]SDS chromatographed on a Partisil SCX column with a stepwise gradient elution. Mobile phase: (1) distilled water at a flow-rate of 1.5 ml/min, 15 min; (2) 3.0 *M* pyridine–0.5 *M* acetic acid at a flow-rate of 0.5 ml/min, 30 min. The arrow indicates the beginning of the elution with phase 2. Radioactivity was determined by collecting eluate fractions at 1-min intervals.

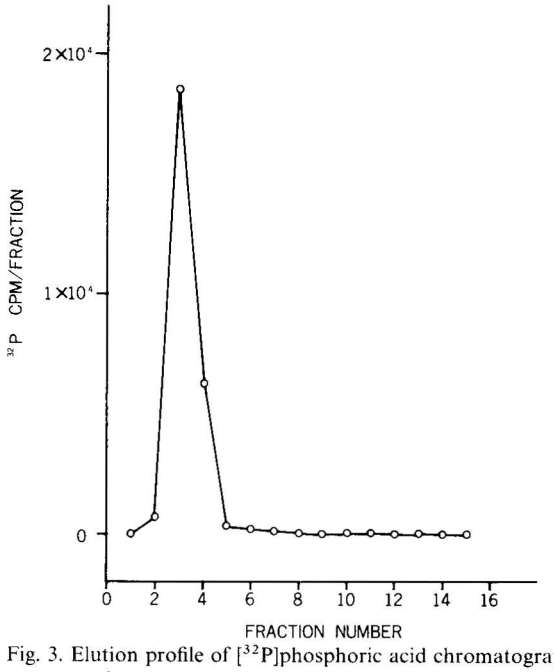

Fig. 3. Elution profile of [^{32}P]phosphoric acid chromatographed on a Partisil SCX column with distilled water at a flow-rate of 1.5 ml/min. Eluate fractions were collected at 1-min intervals.

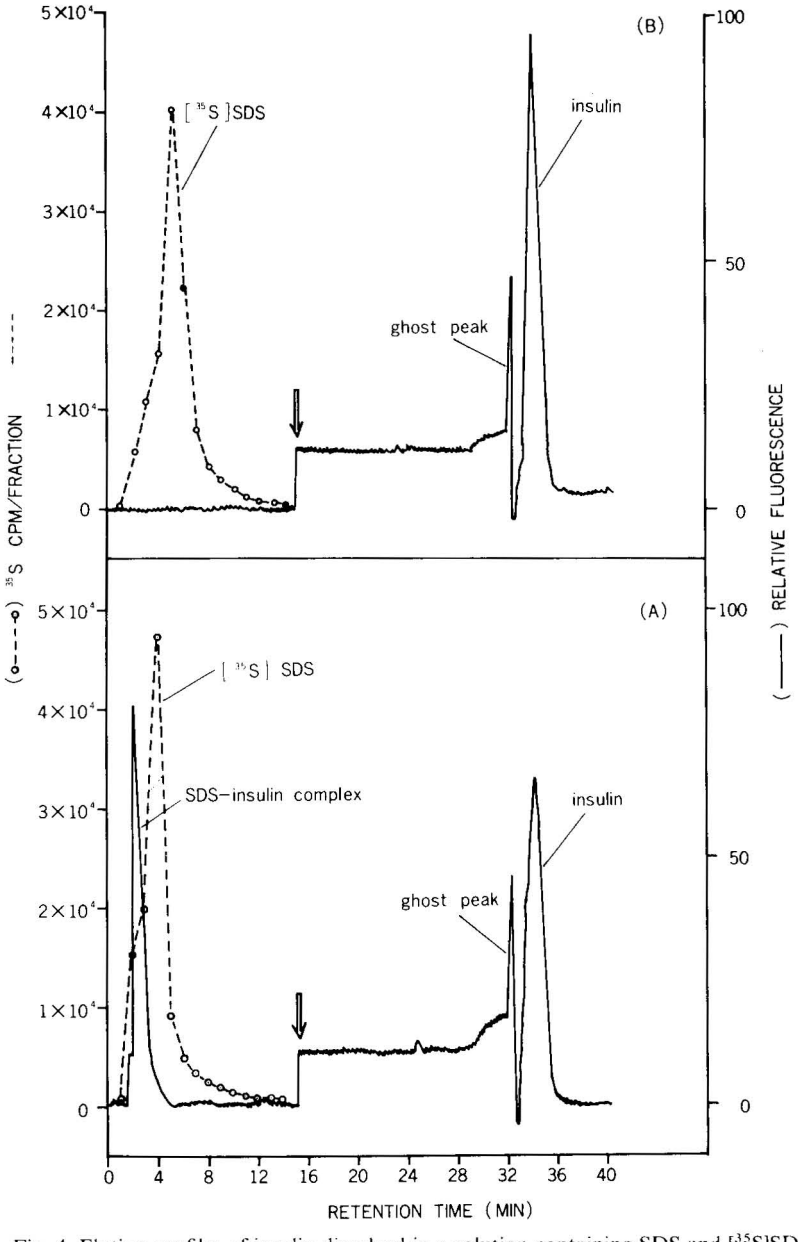

Fig. 4. Elution profiles of insulin dissolved in a solution containing SDS and [^{35}S]SDS chromatographed on a Partisil SCX column with stepwise gradient elution. Mobile phase: (A) (1), (2) as in Fig. 2; (B) (1) 0.005 *M* pyridine–0.04 *M* acetic acid at a flow-rate of 1.5 ml/min; (2) 3.0 *M* pyridine–0.5 *M* acetic acid at a flow-rate of 0.5 ml/min, 30 min. The arrows indicate the beginning of elution with 3.0 *M* pyridine–0.5 *M* acetic acid. Radioactivity was determined by collecting eluate fractions at 1-min intervals.

peptides. Probably, this is the reason why small peptides are not eluted as two peaks, but its proof remains elusive.

SDS is one of the most potent protein denaturants and solubilizing agents and has been widely used. It may be removed by prolonged dialysis. However, it is not applicable to peptide samples. The method reported here is rapid compared with previous methods[28–30]. In addition, the solvents used are readily removed from the eluted samples by lyophilization and facilitate further direct investigations of separated samples. Moreover, isocratic or gradient elutions with pyridine–acetate buffers are useful for further separations of peptides[17]. The application of this method to the removal of SDS from proteins is now under investigation.

The fluorescamine detection system is destructive to samples without the stream-splitting device described by Bohlen *et al.*[9]. This is the only disadvantage of the method.

Systematic separation

Examples of systematic separations of a mixture of samples are shown in Figs. 5 and 6. All peptides tested were well separated. If further separations of peptides are

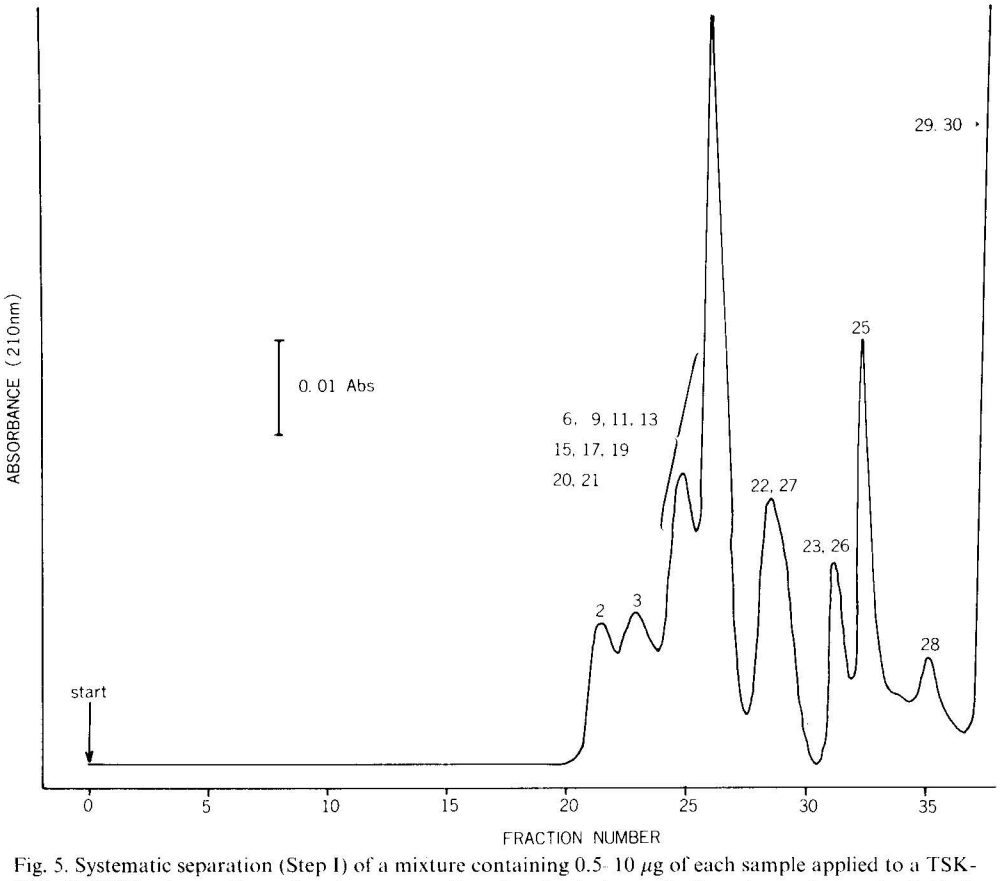

Fig. 5. Systematic separation (Step I) of a mixture containing 0.5–10 μg of each sample applied to a TSK-GEL 2000SW column. Eluate fractions were collected at 2-min intervals. Sample numbers as in Table I. See text for details.

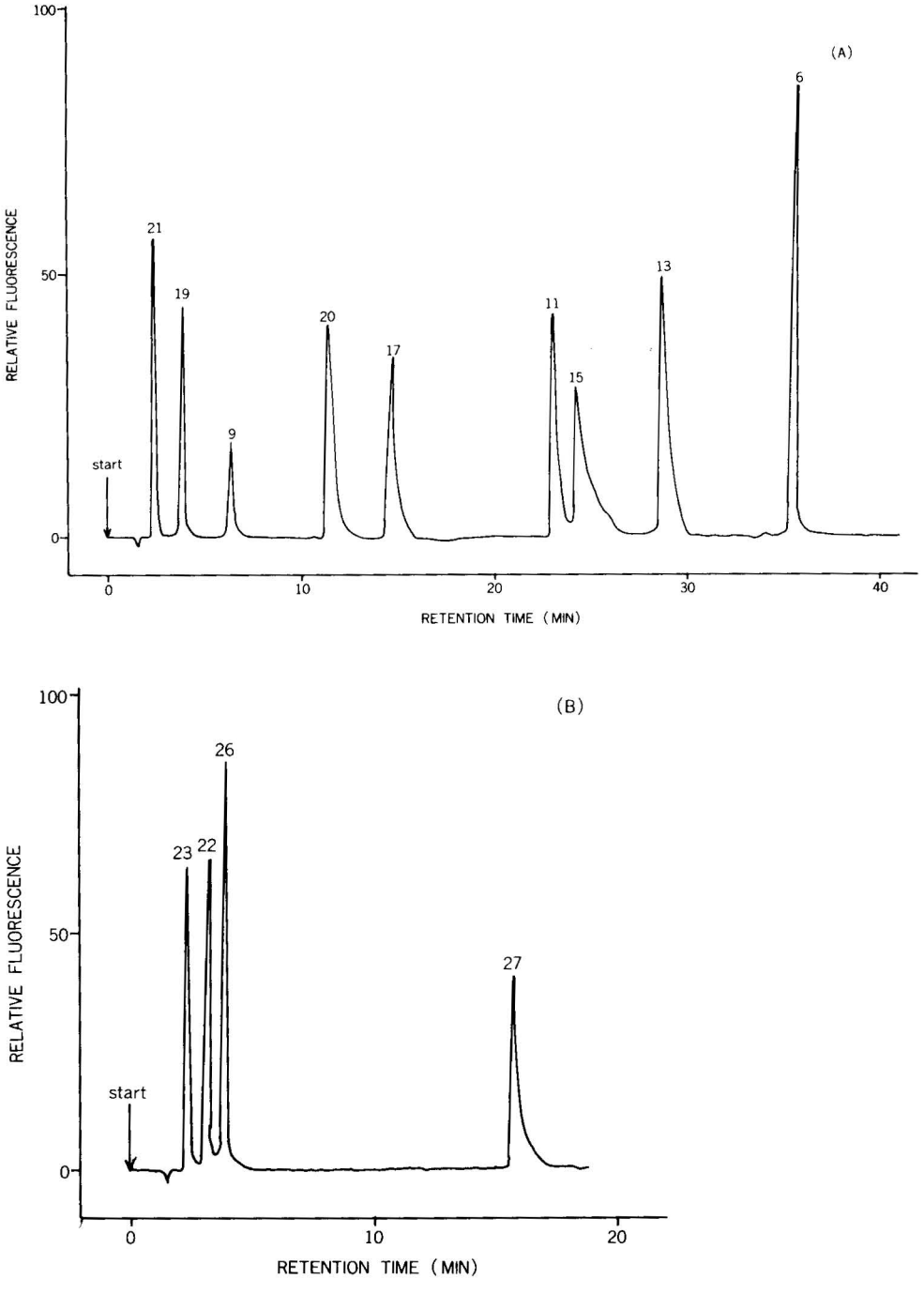

Fig. 6. Systematic separation (Step II) of fractions 25–27 (A) and 28–32 (B) obtained from TSK-GEL 2000SW applied to a Cosmosil $5C_{18}$ column. Sample sizes: 500 μl. Sample numbers as in Table I. See text for details.

necessary, isocratic or gradient elutions with pyridine–acetate buffers on a Partisil SCX column are useful. The three HPLC steps allowed the separation of peptides with an analysis time of about 150 min.

Recovery

The data in Table III indicate that the recovery is in excess of 90% in all the steps. The maximum sample size was not determined, however, as much as 100 μg of oxytocin were eluted without any signs of overloading.

TABLE III

RECOVERIES OF [^{3}H]LEU-ENKEPHALIN

Loading, 20 ng.

Step	*Recovery (%) (mean ± S.D., n = 4)*
I	90.4 ± 1.0
II	99.8 ± 5.5
III	90.0 ± 3.0

In conclusion, this systematic separation and purification of medium-sized biologically active peptides by HPLC may have a wide applicability in biological samples.

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LONG-CHAIN PHENOLS

XXI*. QUANTITATIVE ANALYSIS OF THE PHENOLIC LIPIDS IN TECHNICAL CASHEW NUT-SHELL LIQUID, FROM *ANACARDIUM OCCIDENTALE*, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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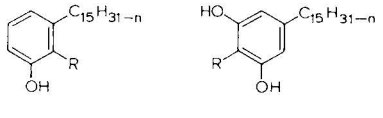
SUMMARY

The novel separation of the constituent phenols in technical cashew nut-shell liquid from the industrial processing of *Anacardium occidentale* has been effected by high-performance liquid chromatography.

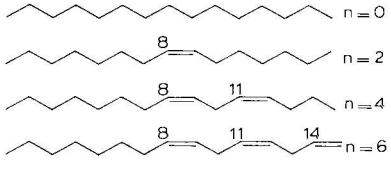
The adsorption mode on columns of 5-μm and 10-μm Partisil and the reversed-phase partition mode with 5-μm and 10-μm Spherisorb bonded with octadecylsilane have been investigated. Determination of relative molar response values for the main constituent phenols and the use of an internal standard have led to a quantitative procedure by isocratic elution under reversed-phase partition, preferably with the solvent acetonitrile–water. Gradient elution with tetrahydrofuran and acetonitrile has also enabled the polymeric material to be estimated in the various types of technical cashew nut-shell liquid examined.

INTRODUCTION

The principal component phenols of technical cashew nut-shell liquid (CNSL) from the industrial processing of *Anacardium occidentale* are cardanol (I; R = H, n = 0, 2, 4 or 6) and cardol (II; R = H, n = 0, 2, 4 or 6), and earlier contributions[1–5] have been concerned with various techniques for quantitative chromatographic determination. Although the trimethylsilylated material can be analysed by gas–liquid chro-



I II



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matography (GLC) on polyethyleneglycol adipate[6], the derivative process and the possibility of polymerisation of the highly susceptible diene and triene constituents led us to examine quantitative analysis by high-performance liquid chromatography (HPLC). A further requirement[7] to monitor rapidly reaction mixtures involving I and II made it desirable to devise a "cold" method. No previous analyses have been described in the field of phenolic lipids, although the basis of the method was established qualitatively some time ago[8].

The separation by HPLC of certain homologous 5-*n*-alkylresorcinols has been described and of C_8–C_{10} alkylphenols[10]. Acetylated urushiol obtained by the use of acetic anhydride under acidic conditions has been examined[11] by HPLC. In any area of natural and other products, quantitative HPLC[12] has received little attention.

With the reversed-phase partition mode under isocratic conditions and the use of an internal standard the relative molar response values of the constituents of the principal phenols in technical CNSL have been determined, and thence the total quantitative composition without the need for any derivative. By gradient elution following this procedure the polymeric material has been separated. The results agree with those of a previous GLC method[13].

The HPLC method revealed a number of hitherto undetected* minor components probably consisting of geometrical and structural isomers of the principal C_{15} unsaturated constituents together with the unsaturated constituents of the C_{17} homologue of cardanol since upon hydrogenation of the technical CNSL the principal phenolic materials observed were only (15:0)-cardanol, (17:0)-cardanol and (15:0)-cardol.

EXPERIMENTAL

Equipment

A self-constructed liquid chromatograph was used, consisting of a Perkin-Elmer ultraviolet variable-wavelength spectrophotometer (Model LC55) equipped with a flow-through cell, an Altex (Anachem) metering pump (Model 110A), a Rheodyne injection system (Model 7120) with a 20-μl loop, a Servoscribe recorder (Model 1S) and an Infrotonics programmable digital integrator (Model CRS 20-1) with a Monroe (1310) printer. Subsequently a Hewlett-Packard printer–plotter (Model 3909) was used as computing integrator giving results in agreement and without the erratic and sometimes unpredictable behaviour of the former system. The units were interconnected in the usual way with standard stainless-steel tubing and unions.

Gradient elution was carried out with a second Altex metering pump of similar type and an Altex programmer (Model 420). Stainless-steel columns (Altex) were 250 × 4.6 mm I.D. For adsorption conditions they had been packed with silica gel (Partisil) and for reversed-phase partition, Spherisorb bonded with octadecylsilane (ODS). In both types columns with particle sizes of 5 μm and 10 μm were used.

* The presence of unsaturated constituents with double bonds at other positions than the 8, 11 and 14 has been shown chemically[14].

Conditions

Generally for detection of phenolic constituents the wavelength used was 275 nm since the absorption maxima were in the range 275–280 nm[15]. The pressure in the system depended on the viscosity of the solvent but was usually in the range 1000–1500 p.s.i. and the flow-rate was generally in the range 1–3 ml/min. Solute was made up in chloroform, and generally 5 μl of a 10% solution was chromatographed, with recorder sensitivity in the middle (5 mV) of the range, and a chart speed of 4 mm/min. In quantitative determinations all HPLC analyses were conducted six times to obtain representative peak areas for integration. Reproducibility was excellent and the standard deviations for each constituent were low. Isocratic elution with the reversed-phase partition mode and solvent acetonitrile–water (66:34) was used for simplicity in the final analysis of the phenolic constituents and subsequently gradient elution for the polymeric material (as discussed later).

Materials

All solvents for HPLC were of liquid chromatography grade. Technical CNSL of three types was used. The first (sometimes termed "raw" CNSL) was kindly made available by 3M Co. (St. Paul, MN, U.S.A.) and was believed to be of Brazilian origin. Distilled CNSL, representing vacuum-distilled "raw" CNSL, was from the same source. Blend 3, initially more polymerised, was material obtained five years ago but stored at 0°C, sealed up and in the dark in the interim.

Cardanol and cardol required for the preparation of a standard calibration solution were obtained by column chromatography as described[16] followed by further purification by preparative thin-layer chromatography (TLC). Analytical and preparative TLC were carried out with silica gel G (type 60) as previously described. The constituents of cardanol were separated by preparative argentation TLC on silica gel G containing 15% silver nitrate with the solvent ethyl acetate–chloroform (20:80) and those of cardol with ethyl acetate–chloroform (50:50). Bands were located by spraying a narrow strip resulting from a spot separate from the rest of the plate with 50% aqueous sulphuric acid and warming in a stream of hot air. The phenolic constituents were eluted with ether–methanol (90:10), isolated by filtration, concentration under reduced pressure, ethereal extraction of the residue, water-washed to remove some silver nitrate, dried and recovered. These materials were then all repurified by ordinary preparative TLC with the solvent ethyl acetate–chloroform (5:95). The purity of all six materials was determined by HPLC examination. All gave single peaks*. Saturated cardanol and saturated cardol were obtained as described[1,16] and purified by recrystallisation from light petroleum (b.p. 40–60°C). *p*-Cresol (BDH, Poole, Great Britain) gave a single peak by HPLC.

For the preparation of the standard containing the unsaturated phenols, cardanol monoene (11.36 mg), cardanol diene (1.55 mg), cardanol triene (11.51 mg), cardol diene (0.75 mg), cardol triene (10.30 mg) and the internal standard, *p*-cresol (2.1 mg), were weighed on a five-place balance. (Unfortunately in the final standard prepared, following a number of trial runs, insufficient cardol monoene was available.) The mixture was prepared in chloroform (2 ml) and stored under nitrogen

* Cardanol monoene contained a trace of peak B1. Preparative HPLC should ideally be used for obtaining pure constituents.

TABLE I

RETENTION TIMES AND RETENTION VOLUMES OF CASHEW-NUT PHENOLS UNDER ADSORPTION CONDITIONS ON PARTISIL (5 μm)

t_R = Retention time (min); V_R = retention volume (ml).

Solvent	*Retention parameter*	*Flow-rate (ml/min)*	*Cardanol*				*Cardol*				*2-Methylcardol*			*Mixed*		
			15:0	*15:1*	*15:2*	*15:3*	*15:0*	*15:1*	*15:2*	*15:3*	*15:1*	*15:2*	*15:3*	*Cardanol*	*Cardol*	*2-Methyl-cardol*
n-Hexane–methanol (100:4)	t_R	1	3.75	4	–	–		22	26.75	32.5	20.3	24.5	30.5	–	–	–
	V_R		3.75	4	–	–		22	26.75	32.5	20.3	24.5	30.5			
n-Hexane–methanol (100:2)	t_R	2	2.5	2.75	3	3.37	–	21.75	25.5	30.25	24	26	31.25	–	–	–
	V_R		5.0	5.5	6	6.74		43.50	51.0	60.5	48	52	62.5			
n-Hexane–ethyl acetate (100:5)	t_R	2	6	6.25	6.75	7.5	–	–	–	–	–	–	–	–	–	–
	V_R															
n-Hexane–iso-propanol (100:4)	t_R	1.5	–	–	–	–		–	–	–	–	–	–	3.5	10.0	5.87
	V_R													5.25	15.0	8.80
Isooctane–methanol (100:4)	t_R	1	3.75	4.25	4.87	5.12	17	22.0	28.1	36.25	26.75	30.1	38.0	–	–	–
	V_R		3.75	4.25	4.87	5.12	17	22.0	28.1	36.25	26.75	30.1	38.0			
Isooctane–methanol–diethyl ether (100:3.6:1.2)	t_R	1	4.75	5.25	5.75	6.5	–	26.25	31.0	37.25	25.75	30	36.25	–	–	–
	V_R		4.75	5.25	5.75	6.5		26.25	31.0	37.25	25.75	30	36.25	–	–	–

at −20°C when not required. A separate standard of saturated cardanol (11.70 mg), saturated cardol (9.84 mg) and *p*-cresol (1.92 mg) was prepared and made up similarly in chloroform solution.

A linearity check with the six constituents and *p*-cresol was made to check the validity of Beer's law for the concentration ranges encountered, and substantially straight-line plots were found for volume (μl) *versus* peak area in all cases.

RESULTS AND DISCUSSIONS

Retention volumes of the constituents of the component phenols

Adsorption mode. A large number of solvents were examined for the separation of the component phenolic constituents of technical CNSL. In these experiments *n*-hexane or isooctane was the major component and a minor proportion of methanol, ethyl acetate, or isopropanol was used. The results in terms of retention time and volume are summarised in Table I. Reduction of polarity in the solvent led generally to the expected increase in retention volume, the minor, more polar component of the binary combination exerting the greater effect. Thus a change from *n*-hexane to isooctane did not greatly affect the complete resolution of the four constituents of cardanol, cardol and 2-methylcardol (II; R = CH_3, n = 0, 2, 4 or 6), but replacement of methanol by a similar proportion of isopropanol resulted in no resolution of the constituents of each phenol, and three collective peaks were observed for cardanol, cardol and 2-methylcardol. Departures from the combinations shown in the table led to exceedingly long retention times or loss of resolution of the constituents of each component phenol. *n*-Hexane, dichloromethane–ethyl acetate (90:10), chlorohexane–ethyl acetate (96:4), *n*-hexane–acetonitrile (100:2) were all ineffective. A typical chromatogram obtained with *n*-hexane–methanol (100:4) is shown in Fig. 1.

Partition mode. The results for a number of reversed-phase partition experiments under isocratic conditions are given in Table II. The use of methanol–water compared with acetonitrile–water resulted generally in lower retention volumes for all the constituents, but frequently lack of resolution of minor components. The resolution of minor constituents was used as a criterion for the effectiveness of the particular binary combination under examination. The solvent acetonitrile–water (66:34) was an improvement generally in giving resolution of the minor components B1 to B7 as in the typical chromatogram illustrated in Fig. 2. Fig. 3 shows the separation in methanol–water (80:20). The lower viscosity of the former solvent enabled the pressure to be reduced considerably by comparison with aqueous methanol combinations. On account of the better resolution effected, the reversed-phase partition mode was the preferred method for use in quantitative analysis. Furthermore, a profusion of large peaks in the adsorption mode at the commencement of the chromatographic run complicated the choice of internal standard. The average height equivalent to a theoretical plate (HETP) for the separations in Fig. 2 was 0.005 cm.

Determination of relative molar response (RMR) values

The reversed-phase partition mode simplified the choice of an internal standard and enabled a lower alkylphenol to be used. *p*-Cresol was available in pure form and was generally more suitable than *m*-cresol, *p*-ethylphenol, *p*-isopropylphenol, or *p-tert.*-butylphenol. The internal standard and the pure monoene, diene and triene

TABLE II

RETENTION TIMES AND RETENTION VOLUMES OF CASHEW NUT-SHELL PHENOLS UNDER PARTITION (REVERSED-PHASE) CONDITIONS ON SPHERISORB BONDED WITH OCTADECYLSILANE (5 μm)

A = Acetonitrile; E = ethanol; M = methanol; W = water; t_R = retention time (min); V_R = retention volume (ml); NR = not resolved.

Solvent	*Retention parameter**	*Flow-rate (ml/min)*	*Cardol*				*2-Methylcardol 15:3*	*Cardanol*				*Other constituents (minor)*						
			15:3	*15:2*	*15:1*	*15:0*		*15:3*	*15:2*	*15:1*	*15:0*	*B1*	*B2*	*B3*	*B4*	*B5*	*B6*	*B7*
A–W (75:25)	t_R	1.5	5.2	9.25	13.75	–	7.62	16.25	22.5	35	68	15.37	17.75	20.75	24.5	27.37	30.25	
	V_R		7.8	13.87	20.62		11.43	24.37	33.75	52.5	102	23.05	26.62	31.12	36.75	41.05	45.37	–
A–W (70:30)	t_R	1.0	11.25	15	22.4	–	12.55	26.5	37.5	58.5	–	25.4	29.75	34.75	41.25	46	51.25	–
	V_R		11.25	15	22.4		12.55	26.5	37.5	58.5	–	25.4	29.75	34.75	41.25	46	51.25	
A–W (68:32)	t_R	1.5	8.0	10.75	16	–	8.87	19.12	27	42.5	–	18.25	21	24.75	29.25	32.75	36.5	–
	V_R		12.0	16.12	24	–	13.30	28.68	40.2	63.75	–	27.37	31.5	37.12	43.87	49.12	54.75	–
A–W (65:35)	t_R	1.5	10.87	14.75	22.75	–	12.37	28.0	40.25	64.5	–	26.5	33.5	–	43.87	50.0	–	61.75
	V_R		16.30	22.12	34.12	–	18.55	42.0	60.37	96.75	–	39.75	50.25	–	65.80	75.0	–	92.6
A–W (66:34)	t_R	1.7	8.75	12.12	18.5	–	10.12	22.5	32.25	51.62	–	21.25	25.12	30.0	35.5	40.0	44.75	49.75
	V_R		14.87	20.60	31.45	–	17.20	38.25	54.82	87.75	–	36.12	42.70	51.0	60.35	68.0	76.07	84.57
A–W (66:34), average (11 runs)	V_R	1.7	14.69	20.11	30.84	55.45	16.86	37.52	53.56	85.85	165.35	35.31	41.77	49.81	58.73	66.59	74.37	81.94
M–W (90:10)	t_R	1.0	5.5	6.37	7.75	–	NR	9.0	10.75	13.87	20.75	NR	–	–	–	–	–	–
	V_R		5.5	6.37	7.75			9.0	10.75	13.87	20.75							
M–W (85:15)	t_R	1.0	8.5	10.5	13.25		NR	16.5	21.12	29.25	49.5	15.5	18.75	NR	NR	25.8	27.87	NR
	V_R		8.5	10.5	13.25			16.5	21.12	29.25	49.5	15.5	18.75			25.8	27.87	
M–W (82.5:17.5)	t_R	1.5	7	7.87	12.5	–	NR	15.0	19.75	28.5	51.25	14.25	16.0	17.25	NR	25.0	26.5	27.25
	V_R		10.5	11.8	18.75	–		22.5	29.62	42.75	76.87	21.37	24.0	25.87		37.5	39.75	40.87
M–W (83:17)	t_R	1.8	6.0	7.5	10.5	–	NR	12.25	16.0	23.12	40.5	11.75*	NR	14.25	NR	20.25	21.5	–
	V_R		10.8	13.5	18.9			22.05	28.8	41.61	72.9	21.15		25.65		36.45	38.7	–
M–W (80:20), average (8 runs)	t_R	1.8	8.1	10.64	15.14	–	NR	18.34	24.72	36.77	75.05	17.32	19.76	20.62	26.64	32.36	33.92	–
	V_R		14.58	19.15	27.25			33.01	44.49	66.19	135.09	31.18	35.57	37.12	47.95	58.25	61.05	–
E–W (70:30)	t_R	0.9	10.75	13.0	17.25	–	NR	23.37	29.5	40.0	68.0	NR	25.25	NR	30.25	36.35	38.0	–
	V_R			11.7	15.52			21.03	26.55	36.0	61.2		22.72		37.22	32.62	34.2	–

* Marginal separation.

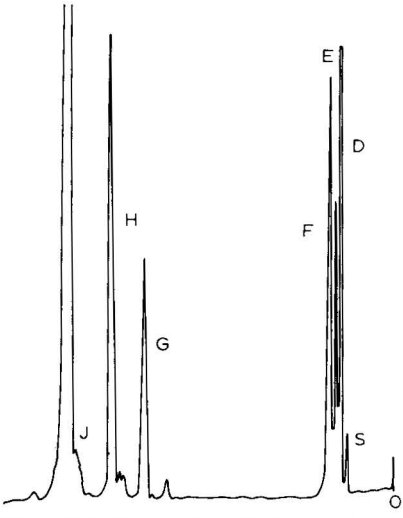

Fig. 1. HPLC separation of mixed cardanol and cardol under adsorption conditions on Partisil (5 μm) with *n*-hexane–methanol (100:4). Flow-rate, 1.0 ml/min. Peaks: D = cardanol monoene; E = cardanol diene; F = cardanol triene; G = cardol monoene; H = cardol diene; I = cardol triene.

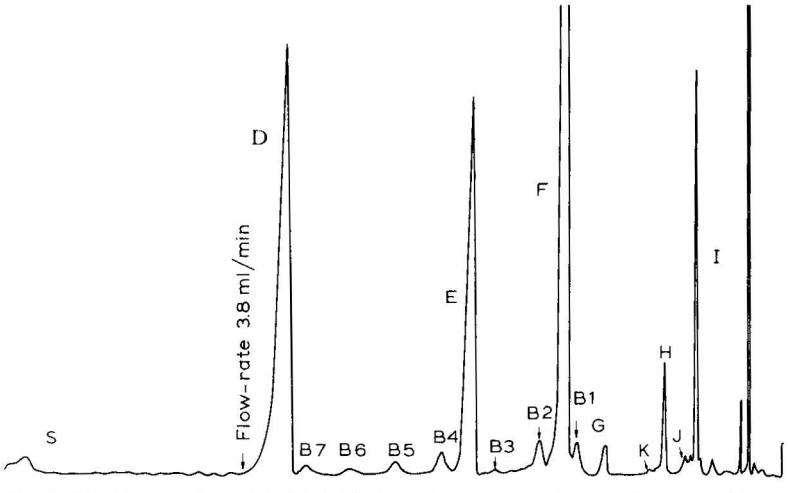

Fig. 2. HPLC separation of distilled CNSL under reversed-phase partition conditions on Spherisorb ODS (5 μm) with acetonitrile–water (66:34). Flow-rate, 1.7 ml/min. Peaks: D–I as in Fig. 1; J = 2-methylcardol triene; K = 2-methylcardol diene; S = saturated cardanol.

constituents of cardanol and cardol were incorporated in one solution which was chromatographically examined twelve times in order to select six representative results. To obtain a measure of agreement between the relative molar response (RMR) values for the monoene, diene and triene it proved necessary for chromatographic reasons or owing to integrator problems to examine four different standards before the required accuracy could be achieved, so that a considerable number of chromatograms were carried out to achieve the final result. A second standard of saturated

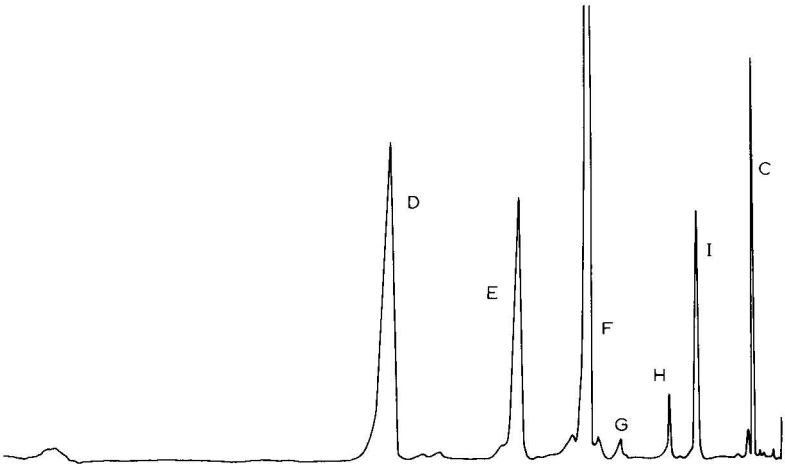

Fig. 3. HPLC separation of distilled CNSL on Spherisorb ODS (5 μm) with methanol–water (80:20). Flow-rate, 1.8 ml/min. Peaks as in Fig. 2. C = *p*-Cresol.

cardanol and saturated cardol with *p*-cresol was prepared, and the results for both are given in Table III. The lower RMR values for the cardol constituents compared with those of cardanol are expected from their respective ε values[14]. RMR values for the constituent phenol $(RMR)_p$ in relation to that for *p*-cresol ($RMR_c = 1$) were calculated from

$$RMR_p/RMR_c = \frac{(\text{peak area})_p}{(\text{g mole})_p} \Big/ \frac{(\text{peak area})_c}{(\text{g mole})_c}$$

TABLE III

RELATIVE MOLAR RESPONSE VALUES OF CASHEW NUT-SHELL CONSTITUENT PHENOLS (WITH REFERENCE TO *p*-CRESOL AS INTERNAL STANDARD)

Phenol	*Weight (mg)*	*Peak areas (normalised %)*	*Relative molar response value*
p-Cresol	2.10	10.35 ± 0.14	1.000
(15:3)-Cardol (cardol triene)	10.30	17.32 ± 0.13	0.992
(15:2)-Cardol (cardol diene)	0.74	1.25 ± 0.014	0.997
(15:1)-Cardol (cardol monoene)	–	–	0.997*
(15:3)-Cardanol (cardanol triene)	11.36	26.29 ± 0.39	1.296
(15:2)-Cardanol (cardanol diene)	7.55	17.13 ± 0.44	1.279
(15:1)-Cardanol (cardanol monoene)	11.51	26.47 ± 0.22	1.305
p-Cresol	1.92	18.07 ± 1.04	1.000
(15:0)-Cardol (cardol saturated)	11.70	31.29 ± 0.08	1.001
(15:0)-Cardanol (cardanol saturated)	9.84	50.61 ± 1.05	1.294

* Calculated value.

TABLE IV

QUANTITATIVE ANALYSIS OF DIFFERENT TYPES OF TECHNICAL CASHEW NUT-SHELL LIQUID

Parameter	*Internal standard (1)*	*(15:3) Cardanol (2)*	*(15:2) Cardol (3)*	*(15:1) Cardol (4)*	*(15:3) Cardanol (5)*	*(15:2) Cardanol (6)*	*(15:1) Cardanol (7)*	*(15:0) Cardanol (8)*	*Total (mg)*
Technical CNSL (new material) (128.86 mg)									
Relative molar response value	1.000	0.992	1.003	0.997	1.296	1.279	1.305	1.294	–
Peak area (normalised %)	7.47 ± 0.186	11.31 ± 0.100	2.92 ± 0.061	0.89 ± 0.077	38.29 ± 0.142	13.81 ± 0.190	22.03 ± 0.240	3.23 ± 0.369	–
Wt. calc. (mg)	(wt. used 3.94)	17.48	4.46	1.38	43.01	15.82	24.90	3.76	110.84
Percentage present (cols. 2–8)	–	13.56	3.48	1.07	33.38	12.28	19.32	2.91	86.02
Distilled CNSL (123.48 mg)									
Peak area (normalised %)	6.45 ± 0.106	5.83 ± 0.118	1.84 ± 0.017	0.75 ± 0.037	33.85 ± 0.287	16.33 ± 0.142	31.05 ± 0.303	3.88 ± 0.532	–
Wt. calc. (mg)	(wt. used 3.60)	9.54	2.96	1.23	40.23	19.80	37.14	4.58	115.61
Percentage present (cols. 2–8)	–	7.72	2.40	0.99	32.58	16.03	30.07	3.81	93.63
Technical CNSL (old material) (128.32 mg) (blend 3)									
Peak area (normalised %)	8.81 ± 0.205	6.36 ± 0.121	2.49 ± 0.085	1.03 ± 0.198	27.75 ± 0.356	16.36 ± 0.334	33.10 ± 0.210	4.05 ± 0.548	–
Wt. calc. (mg)	(wt. used 4.00)	8.46	3.30	1.37	28.83	16.09	32.20	3.99	94.24
Percentage present (cols. 2–8)	–	6.59	2.57	1.06	22.47	12.54	25.09	3.11	73.44

Quantitative analysis of different types of technical CNSL

The reversed-phase partition mode was used to obtain the quantitative composition of a typical good-quality technical CNSL, a vacuum-distilled material and a somewhat polymerised specimen, in terms of the constituents of cardanol and of cardol*. *p*-Cresol was incorporated with each of the types of CNSL and chromatograms obtained under isocratic conditions with the solvent acetonitrile–water (66:34). The objective in each case was to obtain six determinations with reproducible peak areas and low standard deviations. In early experiments sets of asymmetrical peaks were encountered, but subsequently when it was found possible to elute adsorbed polymeric material with 100% tetrahydrofuran, symmetrical peaks were obtained. From the peak areas obtained $(\text{g mole})_p$ for each constituent was calculated from its known RMR_p value, $(\text{peak area})_p$, $(\text{peak area})_c$, $(\text{g mole})_c$ and thence the percentage contribution. The total material accounted for is shown in the final column of Table IV. That unaccounted for includes the minor constituents B1 to B7, 2-methylcardol constituents, the C_{17} bis homologue of cardanol (in the form of the monoene, diene, and triene), polymeric material, and traces of anacardic acid (I; R = COOH, n = 0, 2, 4 or 6)**.

TABLE V

COMPOSITION OF TECHNICAL CNSL SAMPLES IN TERMS OF CARDANOL, CARDOL, 2-METHYLCARDOL, MINOR MONOMERIC CONSTITUENTS AND POLYMERIC MATERIAL

CNSL type	*(Found) cardanol (%)*	*(Found) cardol (%)*	*(Calc.**) 2-methyl-cardol (%)*	*(Calc.*) minor constituents (%)*	*(Calc.) polymeric material (%)*
New	67.82	18.20	3.32	3.28	7.38
Distilled	82.38	11.25	2.05	3.98	0.34
Old (blend 3)	63.13	10.31	1.88	3.05	21.63

* Based on triangulation and proportion of cardanol present. The same RMR value as for cardanol was assumed.

** The same RMR as for cardol was used.

Minor monomeric and polymeric material

Minor monomeric constituents. It was not found possible to use the particular integrator available to determine the peaks B1 to B7, or 2-methylcardol triene and diene. From previous work[2] on the analysis of hydrogenated and methylated technical CNSL the proportion of 2-methylcardol associated with cardol is fairly constant. On the basis of cardol having an associated 18% of 2-methylcardol, the calculated proportion of 2-methylcardol in the three samples of CNSL is given in Table V. By triangulation, the contribution of the minor peaks B1 to B7, due to monomeric substances, in the case of distilled CNSL was found to be 3.97% (in relation to the

* Generally the results show the instability of triene constituents, particularly that of cardol, towards distillation and the increase of the percentage of polymer with age of the material accompanied by diminution of both cardanol and cardol triene constituents.

** Anacardic acid was eluted prior to cardol, the constituents giving tailing peaks. The proportion was negligible in comparison with that reported earlier[17] as 1–1.5%.

peak area of cardanol diene, 16.03%). The final column in the table gives an estimate by difference of the proportion of polymeric material present in the three samples examined.

For a total quantitative analysis ideally it would be preferable to integrate the peak areas of B1 to B7 and to establish their chemical identity. Our work in this direction is as yet incomplete. From their retention data they are mostly related to C_{15} cardanol constituents and to the C_{17} bis homologue, the presence of which in the monoene, diene and triene form has been shown by mass spectroscopy[4].

Upon hydrogenation of the CNSL sample, peaks B1 to B7 substantially disappeared. A new large peak appeared after that for (15:0)-cardanol at the retention expected for saturated C_{17} (17:0)-cardanol, and a small peak preceded that for (15:0)-cardanol. From the retention data found for 3-butylphenol, 3-undecylphenol (log retention time, 1.36) available from synthesis[18] and (15:0)-cardanol (3-pentadecylphenol) (log retention time, 2.05) together with the linear relationship between methylenic carbon chain length and log (retention), the expected relative retention (1.51) compared to that of (15:0)-cardanol, the retention time for (17:0)-cardanol was found. On the reasonable basis that a similar relative retention holds for (17:1)-, (17:2)- and (17:3)-cardanol it seems most probable that peaks B7 and B4, respectively, correspond to the two latter constituents. It is considered that the remaining minor constituents are probably structural and geometrical isomers of (15:1)-, (15:2)- and (15:3)-cardanol. In a study of the effect of using different wavelengths for detection it was observed that at 240 nm certain of the B1 to B7 peaks exhibited maximum absorption consistent with the presence of conjugated side-chains. The small peak preceding (15:0)-cardanol is probably ascribable to C_{13} chain-length material.

Polymeric material. The presence of polymeric material has previously[5] been inferred from quantitative GLC analysis, but by gradient elution it proved possible

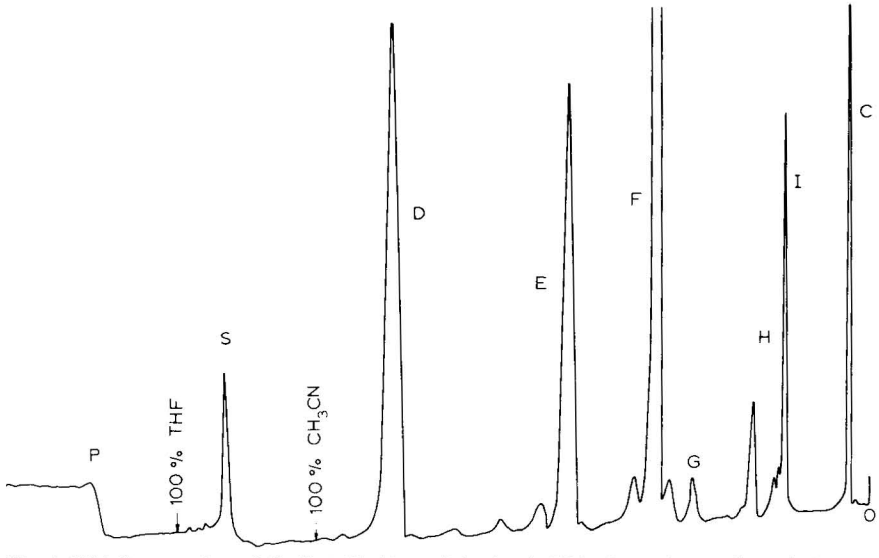

Fig. 4. HPLC separation of distilled CNSL on Spherisorb ODS (5 μm) by gradient elution, starting with acetonitrile–water (66:34). Flow-rate, 1.7 ml/min. Peaks D–I and S as in Fig. 2; P = polymer; C = *p*-cresol.

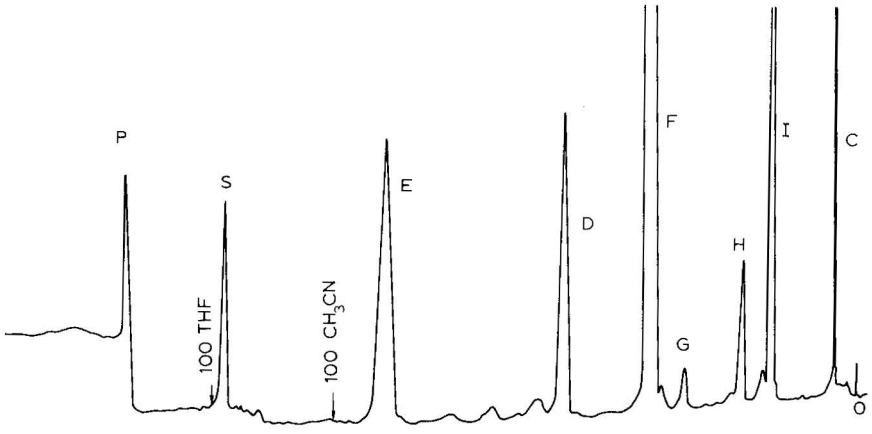

Fig. 5. HPLC separation of new CNSL on Spherisorb ODS (5 μm) by gradient elution, starting with acetonitrile–water (66:34). Flow-rate, 1.7 ml/min. Peaks as in Fig. 4.

by HPLC to demonstrate its presence. Gradient elution was also desirable because of the long retention of (15:0)-cardanol. In the absence of gradient elution, stepwise elution of (15:0)-cardanol was effected with acetonitrile (100%)* and of polymeric material with tetrahydrofuran (100%). With gradient elution a progressive programmed change from acetonitrile–water (66:34) to tetrahydrofuran (THF) (100%) was effected. Figs. 4, 5, and 6 show the complete chromatograms for distilled, new and old (blend 3) technical CNSL, respectively**. The heterogeneous nature of the polymeric material is clear since a number of peaks are produced upon elution with acetonitrile–tetrahydrofuran. The retention information suggests that the material is probably

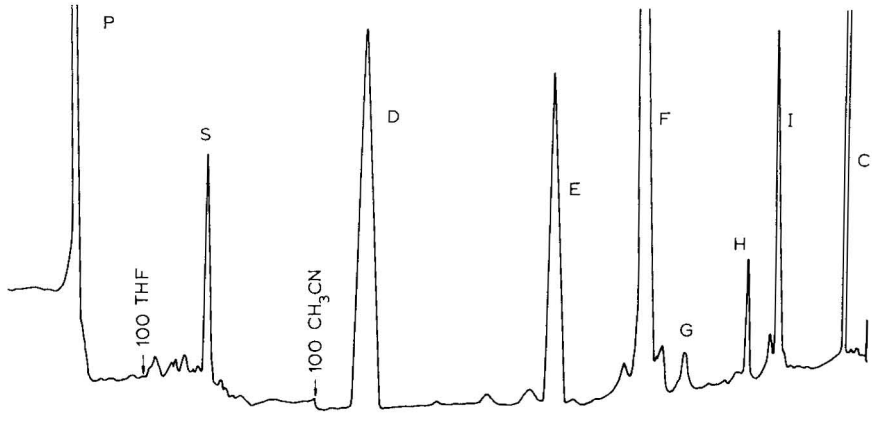

Fig. 6. HPLC separation of old CNSL on Spherisorb ODS (5 μm) by gradient elution, starting with acetonitrile–water (66:34). Flow-rate, 1.7 ml/min. Peaks as in Fig. 4.

* Rapid analysis of hydrogenated technical CNSL was effected with acetonitrile (100%).

** Adsorption conditions were not suitable for demonstrating the presence of polymer.

dimeric and trimeric and relatively saturated. Recovery of the eluted polymeric material and TLC examination (Fig. 7) indicated an increase of complexity and polarity with level of polymerisation.

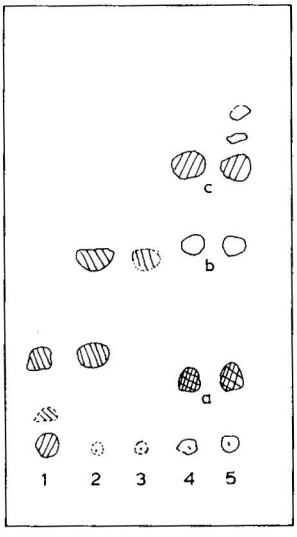

Fig. 7. TLC separation on silica gel G of THF eluted polymer. 1 = From old CNSL; 2 = from new CNSL; 3 = from distilled CNSL; 4 = new CNSL; 5 = old CNSL. Solvent, chloroform–ethyl acetate (90:10). a = Cardol; b = 2-methylcardol; c = cardanol.

Comparison of HPLC with other methods of analysis for phenolic lipids

Previous analyses based essentially on GLC have consisted of two stages, the component phenols being determined after hydrogenation and methylation[2] and the unsaturated constituents of each analysed by preliminary TLC separation of each component phenol followed by GLC on the methyl ethers[1], or mass spectrometry[4] on the component phenols. More recently[6], trimethylsilylated technical CNSL has been examined by GLC on polyethyleneglycol adipate and all the unsaturated constituents were separated. This procedure is somewhat similar to the HPLC adsorption mode in the order of emergence of constituents. The need for derivativisation and the relatively high temperature involved, which may cause some polymerisation, particularly in the preheater of the GLC apparatus, are disadvantages of the "hot" method. The HPLC method, particularly, in the reversed-phase partition mode, avoids the preceding difficulties and gives a higher degree of resolution of all the constituents, although good integration equipment is necessary for all the minor constituents to be determined. A rapid method is now available for the industrial evaluation of technical CNSL in terms of the principal phenols and polymeric material and for this purpose the internal standard is not absolutely necessary.

We have examined natural CNSL and urushiol by HPLC and derivative formation in the latter case appears unnecessary. Bearing in mind the increasing numbers of phenolic lipid types being found[19], most of which contain some highly unsaturated constituents, there is little doubt that for their analysis HPLC is the method of choice.

ACKNOWLEDGEMENTS

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AFFINITY COLUMN FOR SEPARATION OF PYRUVYLATED AND NON-PYRUVYLATED POLYSACCHARIDES

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(Received April 14th, 1981)

SUMMARY

The possibility of separating pyruvylated polysaccharides into pyruvate-rich and pyruvate-poor fractions has been demonstrated using an affinity matrix. Thus in some polymers, a mixture of strand types exists. The affinity matrix was prepared by coupling antibodies to a *Rhizobium* polysaccharide to Sepharose gel. Elution was accomplished by the addition of pyruvate to the eluting buffer. Non-pyruvated polysaccharides were not adsorbed.

INTRODUCTION

There is currently considerable interest in polysaccharides such as that from *Xanthomonas campestris* (xanthan gum) which have commercially useful properties[1]. The physical characteristics of xanthan derive from its cellulosic backbone to which are attached trisaccharide side chains[2,3]. The presence of pyruvate (and acetate) in a molar ratio less than one per trisaccharide repeat unit is an unusual feature of this structure. Many, but not all, microbial exopolysaccharides possess structures in which each repeat unit carries an acetyl and/or a ketal group[4]. As several workers have reported variations in the pyruvate content of xanthan from different strains or prepared by different cultural procedures[5–7], the relationship of pyruvate to carbohydrate structure is of considerable interest. Although a method utilising fractional precipitation with ethanol has been employed to differentiate xanthan preparations into fragments with differing pyruvate content[8], it is most suitable for larger quantities of polysaccharide and is not satisfactory for small aliquots such as might be used in routine analysis or would be available from isotopic studies. It is also unlikely that it would be satisfactory for other partially pyruvylated polysaccharides.

As part of a programme to provide microanalytical procedures for defining microbial polysaccharides, a method was sought to determine whether pyruvate was present on all polymer strands, on alternate repeat units as was earlier found for both acetate and pyruvate[9,10] or whether pyruvate was present on some strands only and absent from others.

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EXPERIMENTAL

Organisms and growth conditions

Xanthomonas campestris strain 646 was grown routinely in a medium containing limiting nitrogen and excess carbohydrate[11] in 100-ml aliquots in 250-ml erlenmeyer flasks shaken at 30°C. The same conditions were used for *Enterobacter aerogenes* and *Escherichia coli* strains. Bacterial cells for preparation of radioactive polysaccharide were grown for 16 h in half-strength nutrient broth at 30°C.

Polysaccharide preparation and purification

Cultures were killed by the addition of 1% (v/v) formalin and bacteria deposited by centrifugation at 10,000 *g* for 30 min. The supernatant fluids were added to two volumes of cold (−40°C) acetone to precipitate polysaccharide. The precipitate was redissolved in a small volume of water, dialysed exhaustively against distilled water, ultracentrifuged at 50,000 *g* for 1 h and lyophilised. Radioactive polymer was prepared by transferring the cells grown in half strength nutrient broth to fresh nitrogen-deficient medium containing 0.1% (w/v) glucose and 0.1 mCi ^{14}C-labelled glucose. After 2–4 h incubation at 30°C, the polysaccharide was recovered in the normal way.

Analytical procedures

Total carbohydrate was assayed by the phenol–sulphuric acid method[12] or, if pyruvate was present, by a micro-modification of the anthrone procedure[13]. The latter method was adopted for column eluates containing pyruvate which interferes in the phenol-sulphuric acid assay. Micromethods for the assay for individual sugars in either intact polysaccharides or in hydrolysates and for acetate and pyruvate were those used in earlier studies[14]. Hydrolysis of the polysaccharides was performed at 100°C in 1 *M* trifluoracetic acid for 12 h. Hydrolysates were dried under vacuum, redissolved in distilled water and again dried. After solution of the hydrolysate in a known volume of distilled water, assays were made for the component sugars identified by paper chromatography. Chromatograms were on Whatman No. 1 paper with butan-1-ol–pyridine–water (6:4:3) as eluent. Paper electrophoresis was performed in pyridinium acetate buffer, pH 5.3 in Locarte (London, Great Britain) equipment. A current of 100 mA was applied for 3 h to 77 × 20 cm strips of Whatman 3MM paper.

Radioactivity measurements

Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer. Aliquots (50 μl) were counted in a dioxane-based scintillant (NE250, Nuclear Enterprises, Edinburgh, Great Britain).

Preparation of the affinity material

The globulin fraction of antiserum prepared against *Rhizobium* strain TA1 polysaccharide was obtained by ammonium sulphate fractionation. After removal of the salt by dialysis, it was coupled to CNBr-activated Sepharose 6MB (Pharmacia, Uppsala, Sweden). The gel material (5 g) was swollen and washed for 15 min on a glass filter with 1 l 10 m*M* HCl. The protein fraction (50 mg) was dissolved in 25 ml 0.1 *M* $NaHCO_3$ buffer containing 0.5 *M* NaCl, mixed with the gel in a glass stoppered

bottle and rotated overnight at 4°C. To remove unbound protein, the gel was washed with coupling buffer and any residual active groups were blocked by treatment with 1 *M* ethanolamine at pH 8.0 for 2 h. The gel was finally washed alternately with acetate–saline buffer and with 0.1 *M* borate buffer (pH 8.0, containing 0.5 *M* NaCl), three times. It was stored until required in acetate buffer containing a few drops of 0.1 % (v/v) merthiolate solution as preservative at 4°C.

RESULTS

Separation of polysaccharides into pyruvate-rich and pyruvate-poor fractions

Although a fractional precipitation procedure has been described for separating *Xanthomonas campestris* polysaccharide into pyruvylated and poorly pyruvylated material[8], it requires relatively large amounts of material, of the order of 500 mg or more for successful results. It was also limited in its applicability, most other bacterial polysaccharides tested requiring different solvent concentrations from xanthan. There was clearly a need to develop a method for use with small amounts of polysaccharide, which could be applied to any polymer and not solely to those from a single bacterial species. The observation of Dudman and Heidelberger[15] that antibodies against the polysaccharide from *Rhizobium* strain TA1 were directed against the pyruvate ketals present in this polymer, indicated the possibility of developing an affinity procedure. Antiserum was fractionated to yield the globulin portion, which was then coupled to CNBr-activated Sepharose 6MB. The gel was employed in 5 or 10 cm × 1 cm columns. Radioactive *X. campestris* polysaccharide was applied to the column in 0.1 *M* acetate buffer (pH 4.0) containing 0.5 *M* NaCl. After thorough elution with this buffer, the eluent was changed to 0.1 *M* acetate buffer at the same pH, containing 0.25 *M* NaCl and 0.25 *M* sodium pyruvate. A typical elution pattern, as measured by the radioactivity in the eluate, is shown in Fig. 1. Clearly, some

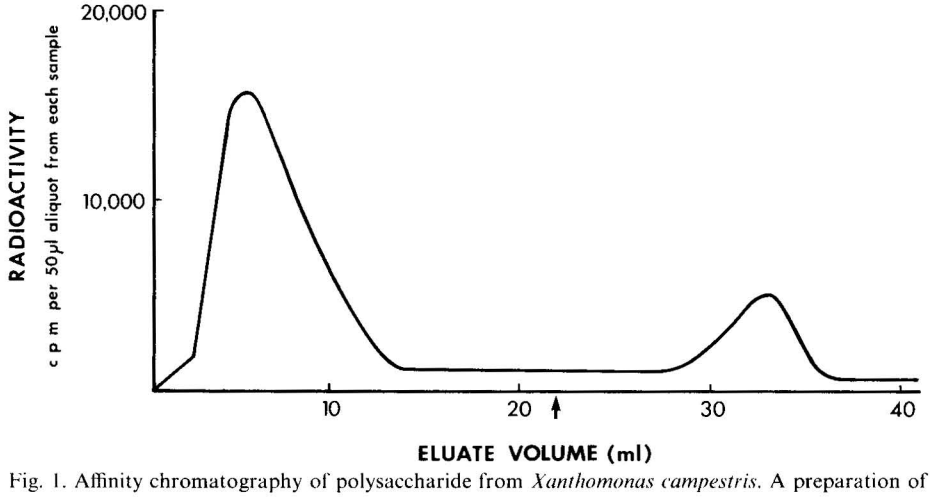

Fig. 1. Affinity chromatography of polysaccharide from *Xanthomonas campestris*. A preparation of ^{14}C-labelled polysaccharide from *X. campestris* strain 646, dissolved in 0.1 *M* acetate buffer (pH 4.0, containing 0.5 *M* sodium chloride) was applied to a column 5 × 1 cm) of Sepharose 6 MB to which *Rhizobium* strain TA1 antiserum had been coupled. The adsorbent had been equilibrated with the same buffer which was then used for elution. After 19 × 1 ml fractions had been eluted, buffer containing 0.25 *M* sodium chloride + 0.25 *M* sodium pyruvate was applied and elution continued.

material failed to adsorb and was eluted in the void volume. The remaining material adsorbed to the column and was eluted by buffer containing pyruvate. When the radioactive solutions were pooled as two separate fractions, the ratio of the radioactivity of the unadsorbed material to that adsorbed was approximately 3.24:1. Recovery of the radioactive material from the column was essentially complete.

After thorough washing with the initial buffer, the adsorbent was used repeatedly to prepare material from unlabelled polysaccharide. The pooled material from the non-radioactive polymer from the same *X. campestris* strain corresponded to 7.5 mg of "fraction 1" (unadsorbed) and 5.54 mg of "fraction 2" (eluted with pyruvate). Analysis of these fractions revealed the expected carbohydrate composition of glucose, mannose and glucuronic acid in the approximate molar ratio of 2:2:1. Both fractions contained about 4.5% acetate, but the pyruvate contents were 2.2% and 9.1% respectively. On treatment with a specific depolymerase enzyme, each fraction was converted to a mixture of two oligosaccharides corresponding to the pyruvylated repeating unit of xanthan and to the non-pyruvylated fragment respectively. These oligosaccharides were separated by paper electrophoresis, eluted and quantified by carbohydrate assay. The ratio of pyruvylated to non-pyruvylated oligosaccharide was 1:4.2 for "fraction 1" and 1:0.6 for "fraction 2". This together with the observed pyruvate content indicated that fraction 1 was "pyruvate-poor" while fraction 2 was "pyruvate-rich".

When materials from the unadsorbed fractions was re-applied to the column, a small amount of material was adsorbed and could be eluted with pyruvate-containing buffer. This was almost certainly due to overloading of the adsorbent in the initial preparative runs. When fraction 2 material was reapplied to the column, care being taken to ensure that it was not overloaded, it was completely adsorbed.

Similar results were obtained with a number of preparations of *Xanthomonas* polysaccharides from different strains or from strains grown under different culture conditions. Each yielded material eluting with the void volume and further material only eluting in the presence of pyruvate. Two exceptions were *X. campestris* polysaccharide from which the pyruvate groups had been removed by treatment with 10 m*M* trifluoracetic acid at 100°C for 90 min, and the polymer from *Xanthomonas phaseoli* strain 1128, a polysaccharide known to contain little, if any, pyruvate under the cultural conditions used for its preparation. Neither of these two preparations was adsorbed to the gel.

Polysaccharides from other bacterial species

Application of other bacterial polysaccharides to the affinity column yielded differing results. None of the polymers lacking pyruvate, which were tested, were adsorbed. Two polymers which, on the basis of their analysis, appeared to contain 1 mole of pyruvate per repeat unit, *Enterobacter aerogenes* type 1 and a pyruvylated form of *E. aerogenes* type 8, were completed adsorbed. So too were a number of preparations of colanic acid, the common product of *Escherichia coli*, *Enterobacter cloacae* and *Salmonella* species. These polysaccharides all eluted as single fractions with pyruvate-containing buffer.

When polysaccharide from *E. aerogenes* type 30 was applied to the column, an elution pattern similar to that obtained with the *X. campestris* polymers was seen (Fig. 2). Part of the material applied was eluted in the void volume and was found to

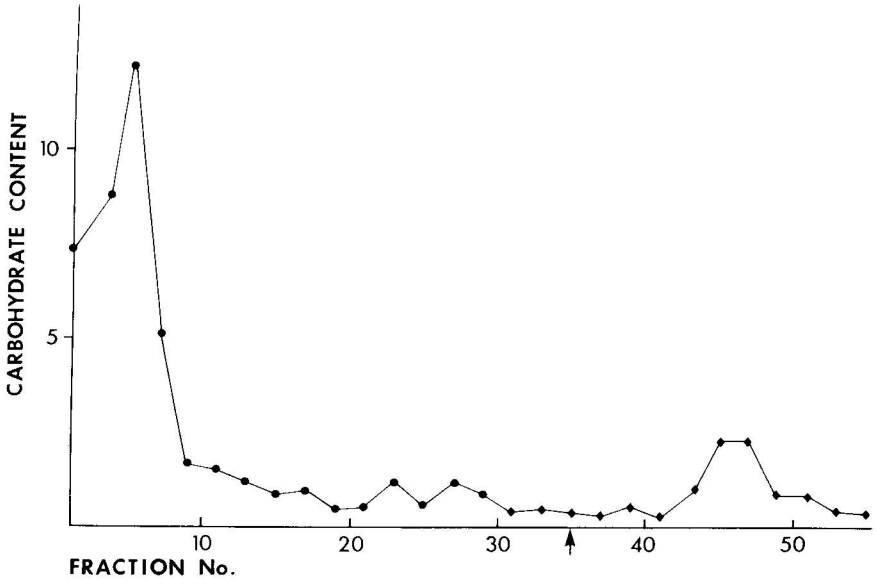

Fig. 2. Chromatography of *Enterobacter aerogenes* type 30 polysaccharide. A polysaccharide preparation (approx. 5 mg) was dissolved in acetate-saline buffer and applied to a column (10 × 1 cm) of adsorbent. The initial eluting fluid was the same buffer and 1 ml fractions were collected and aliquots (100 μl) assayed for carbohydrate by the phenol-sulphuric acid method. At the point indicated by the arrow, elution with pyruvate-containing buffer (0.25 *M*) was started and further assays of eluate made using the anthrone method. The carbohydrate content is given in μg/100 μl.

contain no appreciable pyruvate. The remaining material was adsorbed, but could be eluted with pyruvate-containing buffer. It contained approximately one mole pyruvate per pentasaccharide repeating unit.

DISCUSSION

Although fractionation of polysaccharides with ethanol or other water-miscible solvents is a recognised procedure[8], it has the disadvantage that the conditions must be determined for each polymer. A further disadvantage lies in the considerable variations in molecular weight which may be found in different polysaccharide preparations[16]. These greatly affect the precipitability of the polysaccharides independent of the degree of pyruvylation. In studies of the way in which polysaccharide composition is affected by different culture conditions, a method is needed to determine whether any single batch of material is composed of a mixture of strand types or is uniform in its composition having substituents such as pyruvate occurring irregularly on the molecules. The application of an affinity procedure avoids any molecular weight effects and provides a means of examining small quantities of any pyruvate-containing polysaccharide. A possible drawback might exist if polymers were closely related structurally to the *Rhizobium* TA1 polymer against which the antiserum was prepared. It is also possible that polysaccharides containing different pyruvate configurations[17] might react differently. This remains to be tested. It would

also be an advantage to have an adsorbent with higher capacity. For this, the preparation of an artificial antigen with pyruvate as the immunodominant group might be preferable to the use of *Rhizobium* polysaccharide.

The recognition from the results presented here, that polysaccharides from batch culture, may comprise a mixture of ketalated and non-ketalated molecules indicates that variations in the pyruvate content may depend on the age of the culture, etc. It has already been observed that in continuous culture using different nutrient limitations and other growth conditions, considerable variation in *X. campestris* and related polysaccharides can exist[5,6]. The method described here would enable more accurate examination of such material, particularly when it is possible to use it in conjunction with specific depolymerase enzymes. Recent results suggest that there is much greater inherent variability in polysaccharide structures than has hitherto been recognised[6,18].

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CHROM. 13,871

DISTINCTIVE TEST WITH COPPER(II)–NINHYDRIN REAGENT FOR SMALL α-PEPTIDES SEPARATED BY PAPER CHROMATOGRAPHY

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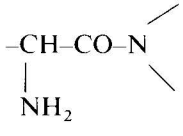
SUMMARY

A Cu^{2+}–ninhydrin reagent was used to distinguish qualitatively small α-peptides and α-amino acid amides from free amino acids on paper after chromatography. With the exception of peptides containing N-terminal L-tryptophan, all the peptides gave a yellow chromophore with a λ_{max} at 395 nm. Protein and non-protein amino acids and polyamines gave different chromophores which varied from one compound to another. Peptides with N-terminal L-proline gave a very faint yellow chromophore or no coloured product at all, while glutathione, a γ-glutamyl peptide, gave a red colour. Polypeptides and proteins did not produce a yellow chromophore. Evidence is provided for a reaction sequence in which peptides first react with Cu^{2+} to form a complex which then reacts with ninhydrin to give the yellow chromophore. These results and also studies with several peptides having N- and C-terminal substitutions suggest that the minimum structural requirement around the α-peptide linkage for the formation of the yellow chromophore with the Cu^{2+}–ninhydrin reagent is as follows:



INTRODUCTION

Until recently, ninhydrin was essentially the only colour reagent used to detect amino acids and peptides on paper. Fluorescamine was introduced a few years ago as a reagent for the qualitative detection and quantitative determination of primary amines, amino acids, peptides and proteins[1,2]. This reagent has been successfully employed to assay the peptide hormones vasopressin and oxytocin[3]. It is also known that *o*-phthalaldehyde reacts with amino acids and peptides in the presence of 2-

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mercaptoethanol to yield highly fluorescent products[4,5]. However, the above reagents are not useful for a general procedure to distinguish small α-peptides from amino acids.

We report here the use of a Cu^{2+}–ninhydrin reagent in an unambiguous qualitative differentiation on paper chromatograms of small α-peptides (with the exception of those containing N-terminal L-tryptophan) from amino acids.

MATERIALS AND METHODS

Most of the peptides, peptide and amino acid derivatives and polyamines were purchased from Sigma (St. Louis, MO, U.S.A.). Hexaglycine, gramicidin-A, -S and thiostrepton were gifts from Professor L. K. Ramachandran (Biochemistry Department, Osmania University, Hyderabad). Poly(L-lysine) (MW 500,000), poly-(L-aspartic acid) (14,000) and poly(L-serine) (11,000) were kindly donated by Professor D. Balasubramaniam (School of Chemistry, University of Hyderabad). Lutropin-releasing hormone (LHRH) and substance-P were gifts from Dr. E. Vijayan (School of Life Sciences, University of Hyderabad). All amino acids were obtained from Calbiochem (Los Angeles, CA, U.S.A.). *cis*-4-Hydroxy-L-proline[6] and *sym*-homospermidine[7] were available in the laboratory. Polyglycine (Cat. No. 901288) was from Schwarz/Mann (Orangeburg, NY, U.S.A.). Ninhydrin of specially purified grade was from Pierce (Rockford, IL, U.S.A.). Cadmium acetate, cupric nitrate, zinc acetate and nickel chloride were of AnalaR grade (BDH, Bombay, India). All the solvents (methanol, isopropanol and acetone) were of analytical grade.

Reagents

Salts of different metals were each dissolved in a mixture of water (3 ml) and glacial acetic acid (1 ml) and the solution was made up to 30 ml with acetone, giving a final metal salt concentration of 25 mmol/l. A 1 % w/v ninhydrin solution in aqueous acetone (95 %) was employed.

Metal–ninhydrin solutions were prepared by dissolving appropriate quantities of the metal salt (final, 25 mmol/l) and ninhydrin (final, 1 % w/v) in a mixture of water (3 ml) and glacial acetic acid (1 ml) and the solution was made up to 30 ml with acetone.

Development of chromatograms

Descending chromatograms (Whatman No. 1 paper, chromatography grade, 20-h run) of the peptides or amino acids were developed with a single phase solvent system, isopropanol–water (4:1). The chromatogram was then dried in air at room temperature for a minimum of 3 h.

Spraying methods

(A) Metal–ninhydrin. The dried chromatogram was uniformly sprayed with the "metal–ninhydrin solution", then air dried and heated at 65°C for 30 min.

(B) Ninhydrin followed by metal. The dried chromatogram was uniformly sprayed with "ninhydrin solution", then air dried and heated at 65°C for 30 min. The paper was sprayed with the "metal salt solution" and finally air dried.

(C) Metal followed by ninhydrin. The chromatogram was uniformly sprayed

with the "metal salt solution" and air dried for 10 min. The paper was then sprayed with ninhydrin solution, air dried and heated at 65°C for 30 min.

The coloured spots and spots of similar areas from an uncoloured portion of the chromatogram (to serve as blanks) were cut out and eluted with 4 ml of methanol–water (80:20). The absorption spectra were recorded on a double beam spectrophotometer (Shimadzu, Model UV 200S).

Unless otherwise stated, 0.01 μmole of each amino compound was used.

RESULTS AND DISCUSSION

Effect of metal ions in the metal–ninhydrin solution

Initial experiments were done with two dipeptides (Gly-L-Leu and L-Leu-Gly) and two tripeptides (Gly-Gly-L-Leu and L-Leu-Gly-Gly). Fig. 1 shows the spectra of the coloured products of the peptides obtained with the combined metal–ninhydrin reagent (spray method A). Of the four metal ions used (Cu^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+}), only Cu^{2+} gave a single yellow chromophore with all the four peptides. The λ_{max} of the chromophore was around 395 nm (Gly-L-Leu, 392 nm; L-Leu-Gly, 396 nm; Gly-Gly-L-Leu, 392 nm; L-Leu-Gly-Gly, 398 nm). Cd^{2+} gave a yellow chromophore with Gly-L-Leu and Gly-Gly-L-Leu (λ_{max}, 390 nm) and a red chromophore with L-Leu-Gly and L-Leu-Gly-Gly. The spectrum of the red chromophore (Fig. 1) showed absorption maxima at 390 nm and 505 nm. Both Zn^{2+} and Ni^{2+} produced a yellow chromophore with Gly-L-Leu and Gly-Gly-L-Leu (λ_{max}, 386 nm) and an orange colour with the other two peptides. This orange chromophore had absorption

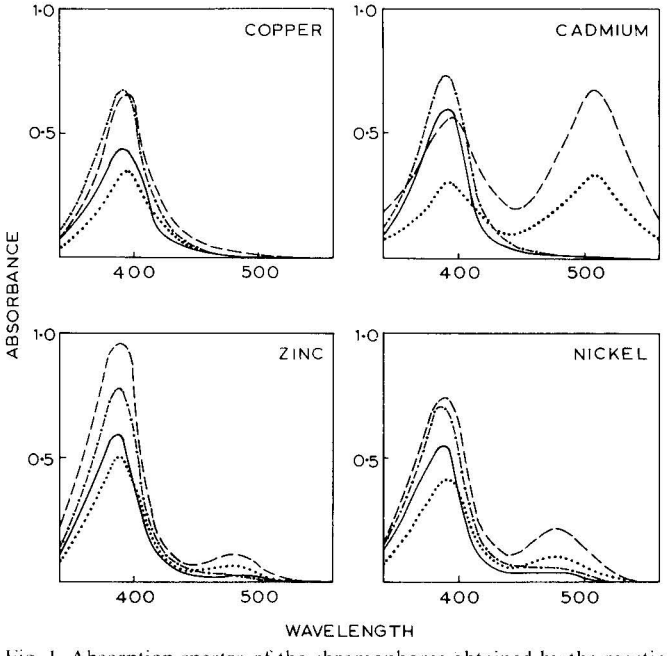


Fig. 1. Absorption spectra of the chromophores obtained by the reaction between peptides and the combined metal–ninhydrin reagents. ——, Gly-L-Leu (0.05 μmole); – – –, L-Leu-Gly (0.1 μmole); —·—·—, Gly-Gly-L-Leu (0.08 μmole); ·····, L-Leu-Gly-Gly (0.05 μmole).

maxima at 390 nm and 480 nm. Since the Cu^{2+}–ninhydrin reagent gave a single yellow chromophore with all the four peptides tested, further studies were carried out with this reagent.

The use of a Cd^{2+}–ninhydrin reagent was suggested for determination of dipeptides as yellow chromophores (λ_{max}, 390 nm) based on a study of peptides containing N-terminal glycine[8]. However, it was found during the present study that this reagent gives a yellow chromophore only with peptides having glycine as the N-terminal amino acid (seventeen dipeptides and eight tripeptides were tested), while in the case of others (thirteen dipeptides and four tripeptides) a red chromophore results (λ_{max}, 390 nm and 505 nm).

Studies with the Cu^{2+}–ninhydrin reagent

(a) Simple peptides, oligopeptides, cyclic peptides, peptide derivatives, polypeptides and proteins. All 157 compounds were tested (Table I). Most of the smaller peptides gave an yellow chromophore (λ_{max} 395 $\pm$ 4 nm) with the exception of peptides containing N-terminal tryptophan which gave a reddish to purple colour.

It is interesting that, while Gly-L-Trp and L-Val-L-Trp gave a yellow colour, L-Trp-L-Leu gave a red colour. Therefore, six other dipeptides with L-tryptophan as the N-terminal residue (Table I) were tested for their reaction with the Cu^{2+}–ninhydrin reagent. All gave a reddish colour. This reagent, thus, may be of use in identifying peptides with L-tryptophan as the N-terminal residue, but obviously more peptides of this type should be tested.

Among the L-proline-containing peptides tested, only Gly-L-Pro produced a visible yellow colour, while peptides with L-proline as the N-terminal residue either gave a faint yellow colour or did not give a coloured product at all. Gly-L-Hyp gave a yellow colour but L-Hyp-Gly did not give a coloured product. The yellow chromophore obtained with Gly-L-Pro had a single λ_{max} at 395 nm. It is to be noted that this chromophore is different from the yellow chromophore which results from the reaction between L-Pro or L-Hyp and ninhydrin which has a λ_{max} at 440 nm.

Glutathione, a γ-glutamyl tripeptide, gave a red colour while peptides with an α-Glu linkage gave an yellow colour. Peptides with a modified carboxyl group such as L-Asp-L-Phe methyl ester, tri-L-Tyr methyl ester and glycyl-L-leucinamide produced an yellow chromophore. Peptides with modified amino groups such as N-CBZ-Gly-L-Leu, N-CBZ-Gly-L-Pro and N-CBZ-Gly-Gly-L-Leu (Table I) gave no colour. Carnosine (β-Ala-L-His) gave a red chromophore.

Oligopeptides such as tetra-L-Ala, penta-L-Ala, hexa-Gly, tuftsin and methionine enkephalin produced a yellow colour. On the other hand, thyrotropin-releasing hormone (TRF) and lutropin-releasing hormone (LHRH) did not give coloured products. Cyclic peptides such as gramicidin-A, -S and thiostrepton also did not produce any colour with this reagent. It is interesting that none of the polypeptides and proteins examined (Table I) produced any colour, except for poly-L-lysine. The latter produced a purple colour which can be attributed to the participation of its ε-amino groups in the reaction.

(b) Protein amino acids and derivatives. All the protein amino acids and the amides Gln and Asn were tested for their reaction with the Cu^{2+}–ninhydrin reagent on paper. Each amino acid produced a different shade of colour but none gave a yellow colour. The spectra of the coloured products of Gly, L-Leu, L-Phe, L-Asp and

TABLE I

COLOURS OBTAINED BY REACTION OF Cu^{2+}–NINHYDRIN REAGENT AND AMINO COMPOUNDS

Compounds are classified according to the colour of the chromophore formed. All compounds were tested at a level of 0.01 μmole.

I. *Compounds yielding yellow chromophores*
Amino acids and derivatives
L-Leucinamide
Dipeptides
Gly-X: X = Gly; L-Leu; L-Ala; L-Ser; L-Met; L-Phe; L-Trp; L-Thr; L-Tyr; L-Glu; L-Ile; L-His; L-Pro; L-Hyp; D-Leu; γ-aminobutyric acid
L-Ala-X: X = Gly; L-Leu; L-Val; L-Ala; L-Met; L-Asp; L-Ser; L-Pro
L-Leu-X: X = Gly; L-Leu; L-Phe; L-Met
L-Met-X: X = Gly; L-Ala; L-Val
L-Phe-X: X = L-Leu; L-Met; L-Pro
L-Tyr-X: X = L-Leu; L-Lys
L-Glu-X: X = L-Ala; L-Val; L-Tyr; L-Glu
L-Val-L-Trp; L-Asp-Gly; L-His-L-Leu; L-Lys-L-Phe
L-Asp-L-Asp; L-Ser-Gly
Tripeptides
Gly-Gly-X: X = Gly; L-Leu; D-Leu; L-Ile; L-Phe; L-Val; L-Ala
Gly-L-Leu-X: X = Gly; L-Tyr
Gly-L-Phe-X: X = L-Phe; L-Ala
Gly-L-Pro-L-Val; Gly-L-Ala-L-Ala; Gly-L-His-Gly;
L-Leu-Gly-Gly; L-Tyr-Gly-Gly;
L-Glu-L-Val-L-Phe; L-Phe-Gly-Gly; D-Ala-Gly-Gly;
Gly-DL-Leu-DL-Ala; tri-L-Leu; tri-L-Ala; tri-L-Tyr;
tri-L-Phe; tri-L-Ser
Tetra and higher peptides
Tetra-L-Ala; penta-L-Ala; hexa-Gly;
L-Thr-L-Lys-L-Pro-L-Arg (tuftsin);
L-Tyr-Gly-Gly-L-Phe-L-Met (Met-enkephalin)
Peptide derivatives
Gly-L-Leu-NH_2; L-Asp-L-Phe methyl ester;
tri-L-Tyr methyl ester

II. *Compounds yielding reddish, purple and similar chromophores*
Amino acids and derivatives
Gly; L-Ala; L-Leu; L-Val; L-Phe;
L-Tyr; L-Ile; L-Lys;
L-Arg; L-Glu; L-Gln;
L-Asp; L-Thr; L-Ser; L-Pro;
L-Orn; L-citrulline; L-2,4-diaminobutyric acid;
L-α,β-diaminopropionic acid; β-Ala;
L-His; L-Cys
Dipeptides
L-Trp-X: X = Gly; L-Phe; L-Ala; L-Tyr; L-Trp; L-Glu; L-Leu
Non-α-peptides
γ-L-Glu-L-Cys-Gly; β-Ala-L-His
Polypeptide
Poly-L-Lys

(Continued on p. 312)

TABLE I *(continued)*

III. *Compounds yielding grey chromophores*
Amino acids and derivatives
cis-L-Hyp; L-Asn; L-Trp

IV. *Compounds yielding no visible colour*
Amino acid derivatives and polyamines
Sarcosine; L-cystathionine; taurine; N-carbamoyl-L-α-Ala; benzoyl-L-Phe; pyroglutamic acid; cadaverine; putrescine; *sym*-homospermidine
Dipeptides
L-Pro-X: X = Gly; L-Leu; L-Ala; L-Trp
L-Hyp-Gly
Tripeptides
L-Pro-Gly-Gly
Peptide derivatives
N-CBZ-Gly-X: X = L-Leu; L-Pro
N-CBZ-Gly-Gly-X: X = Gly; L-Leu; L-Val; L-Ala; L-Ile; L-Met; L-Ser; L-Pro
N-CBZ-Ala-Gly-Gly; N-CBZ-Ile-Gly-Gly
Oligopeptides, polypeptides and proteins
Gramicidin-A; gramicidin-S; thiostrepton; substance-P; LHRH; poly-Gly; poly-L-Asp; poly-L-Ser; bovine serum albumin; carboxypeptidase-A; chymotrypsin; trypsin; pepsin and leucine aminopeptidase

L-Trp are shown in Fig. 2. The chromophore of each amino acid had its own characteristic absorption spectrum and all the chromophores showed two or more absorption maxima. Asparagine gave a greyish colour whereas glutamine gave a red colour. Proline gave a reddish colour while *trans*- and *cis*-4-hydroxy-L-proline gave greyish colours both with a λ_{max} of 460 nm. On the other hand, L-leucinamide, an α-amino acid amide, and glycyl-γ-aminobutyric acid gave a yellow colour. Pyroglutamic acid and other amino acid derivatives such as N-carbamoyl-L-α-alanine and benzoyl-L-phenylalanine gave no colour.

(c) Non-protein amino acids and polyamines. It has been reported that polyamines and certain non-protein amino acids such as β-Ala, γ-aminobutyric acid and taurine behave like peptides on Cu–Sephadex columns[9]. They are not retarded on the column as are protein amino acids. We, therefore, studied the reaction of polyamines and non-protein amino acids with the Cu^{2+}–ninhydrin reagent (Table I). All the non-protein amino acids gave different chromophores but none gave a yellow chromophore. Polyamines such as putrescine and *sym*-homospermidine did not give a coloured product.

Order of addition of reagents

Metal ions have been widely used in the preservation of ninhydrin-stained amino acid chromatograms. Kawerau and Wieland[10] reported a method for the preservation of chromatograms using certain metal ions. When ninhydrin-stained amino acid chromatograms were sprayed with a cupric salt solution, all amino acids

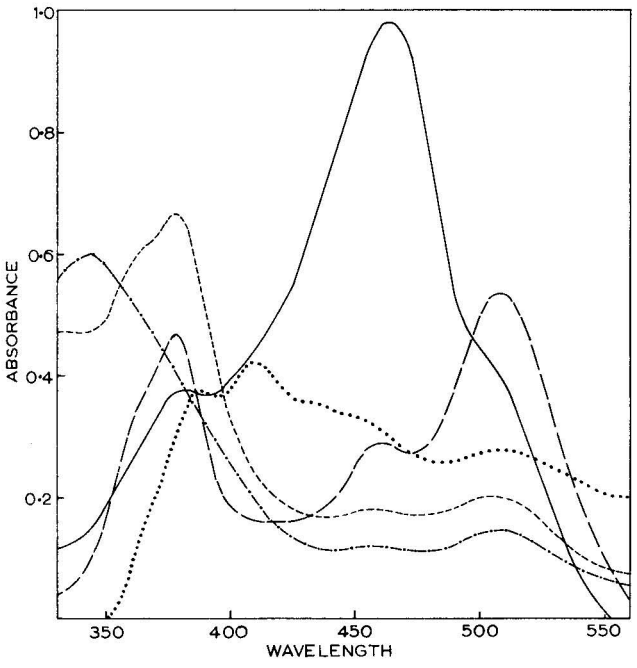


Fig. 2. Absorption spectra of chromophores obtained by the reaction between amino acids and the combined Cu^{2+}–ninhydrin reagent. ——, Gly (0.5 μmole); — — —, L-Leu (1.0 μmole); -------, L-Phe (0.5 μmole); –·–·–·–, L-Trp (0.5 μmole); ·····, L-Asp (1.0 μmole).

produced a reddish colour except for L-Pro and *trans*-4-hydroxy-L-proline. Our earlier study[8] showed that the dipeptides Gly-Gly and Gly-L-Leu also gave a red colour when the chromatogram was first sprayed with ninhydrin, heated at 65°C for 30 min and then sprayed with cupric nitrate solution. However, during the present study it was found that both Gly-Gly and Gly-L-Leu gave a yellow colour with the combined Cu^{2+}–ninhydrin reagent. We, therefore, investigated the individual effect of cupric ions and of ninhydrin on peptides and amino acids by changing the order of spraying (see Materials and Methods). The results of these experiments with three amino acids and three peptides are given in Table II.

TABLE II

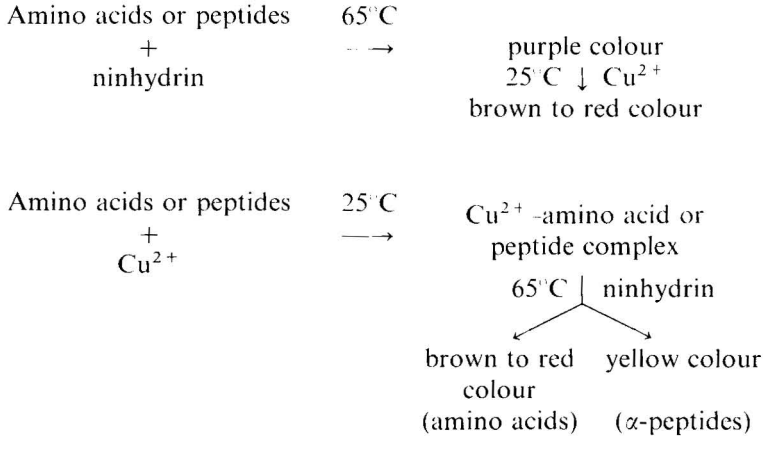
EFFECT OF Cu^{2+} AND NINHYDRIN AND THEIR ORDER OF ADDITION

Amino acid or peptide	*Colour produced*		
	With ninhydrin	*Ninhydrin followed by* Cu^{2+}	Cu^{2+} *followed by ninhydrin*
Gly	Purple	Red	Orange
L-Leu	Purple	Red	Pink
L-Phe	Purple	Red	Pink
Gly-L-Leu	Purple	Red	Yellow
L-Leu-Gly	Purple	Purple	Yellow
Gly-Gly-L-Leu	Purple	Red	Yellow

When a paper containing amino acid and peptide spots was first sprayed with ninhydrin and then with cupric salt solution, both amino acids and peptides gave colours ranging from brown to pink and it was not possible to distinguish peptides from amino acids from the colours of the spots obtained. However, when the paper was first sprayed with cupric salt solution and then with ninhydrin reagent, only amino acids gave colours ranging from brown to pink but *all* the peptides gave a yellow colour. The same result was obtained when the paper containing the spots was sprayed with the combined Cu^{2+}–ninhydrin reagent. This experiment clearly shows that the final products of the reactions between a peptide–ninhydrin complex and Cu^{2+} and between a peptide–Cu^{2+} complex and ninhydrin are not identical.

Small peptides are known to form deep blue complexes with Cu^{2+} (for a review, see ref. 11). However, in the present study, the peptide spots on paper after spraying with cupric salt solution were either colourless or lightly blue since the quantity of the peptides used was very low (0.01 μmole).

The results of this experiment can be summarized as follows:



Thus, it is clear that, when the combined Cu^{2+}–ninhydrin reagent is used, Cu^{2+} reacts rapidly at room temperature with the peptide to yield a complex which then reacts with ninhydrin at 65°C to give a yellow color.

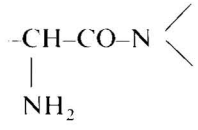
The complex of Gly-Gly with Cu^{2+} was prepared and crystallized according to the procedure of Manyak *et al.*[12]. An aliquot of an aqueous solution of this complex (0.01 μmole) was spotted on chromatographic paper and then sprayed with 1% ninhydrin in aqueous acetone (95%). The paper was heated for 30 min at 65°C. A yellow chromophore with a single λ_{max} at 390 nm was obtained. This experiment clearly shows that the yellow chromophore is the product of the reaction between the copper(II) complex of Gly-Gly and ninhydrin.

Ninhydrin reagent has long been used for qualitative and quantitative analysis of amino acids. However, amino acids cannot be qualitatively distinguished from small peptides using this reagent since both amino acids and peptides give a purple colour with ninhydrin on paper. A Cd^{2+}–ninhydrin reagent was recommended by Atfield and Morris[13] for the detection of amino acids and peptides on paper after

electrophoresis. This reagent was later successfully employed by Ganapathy and Radhakrishnan[8] to quantitate dipeptides having N-terminal glycine. Most of the amino acids gave a red colour with this reagent, while dipeptides containing N-terminal glycine gave a yellow colour. However, the present study shows that peptides having N-terminal residues other than glycine give a red colour with Cd^{2+}–ninhydrin. Thus the use of Cd^{2+}–ninhydrin is limited and it cannot be employed as a general reagent to differentiate small peptides from amino acids.

Minimum structural requirements

From the structure of the compounds employed in this study which are substituted at the N- and C-terminus and α- or other peptide bond, it emerges that the minimum structural requirement around the α-peptide linkage to give a yellow chromophore with the Cu^{2+}–ninhydrin reagent is as follows:



It would appear that the method could be used with confidence to distinguish amino acids and linear peptides containing up to six amino acid residues. Larger linear peptides and cyclic peptides cannot easily be distinguished from amino acids.

The Cu^{2+}–ninhydrin reagent described can conveniently be used to qualitatively differentiate simple α-peptides from amino acids on paper after chromatography or electrophoresis. Work is in progress to determine whether this method can be adapted for quantitation of small α-peptides. Preliminary studies already indicate that reliable linearity between concentration of peptide and absorbance (range, 0.02–0.12 μmole) is obtained for a number of peptides using the 390 nm absorbance, and among several metal ions tested the most sensitive was Zn^{2+} which gives a uniformly higher reading at 390 nm than Cu^{2+}. However, the latter has the advantage of giving a single yellow chromophore.

ACKNOWLEDGEMENTS

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Note

Practical system for polarity rating of packed gas–liquid chromatography columns

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The polarity of the stationary phase is accepted as being a significant factor in obtaining any separation of solutes by gas–liquid chromatography since the initial studies of Rohrschneider[1] in 1959. As absolute values of polarity cannot be obtained. relative values are utilised. Rohrschneider was the first to select squalane as the standard non-polar liquid phase, and he compared another test phase with it utilising two solute probes —an alkane and an alk(di)ene. A logarithmic ratio of retention volumes was involved, but following Kováts' introduction of retention indices[2] these were utilised by Rohrschneider[3] with firstly two solute probes, then five[4] and then seven[5], involving ratios again. In 1970 McReynolds[6] extended the number to ten probes, and worked at 120°C instead of the 100°C used by Rohrschneider. Over 200 stationary phases were compared with squalane, and this study has been much quoted and utilised, at times with only five probes, *e.g.* to obtain an average polarity expression[7]. However, although the probes used differ in polarity, all McReynolds values (ΔI_R, inappropriately termed "constants") are based on the ultimate non-polar series of *n*-alkanes used to determine retention indices (I_R). For any particular probe at 120°C:

$$\Delta I_R = I_{R_{\text{test phase}}} - I_{R_{\text{squalane}}} \text{ (against } n\text{-alkanes)} \quad (1)$$

Manufacturers of stationary phases quote them[8], perhaps for only five probes, most of them being positive numbers, sometimes as large as over 1000. They can be used to forecast whether two different stationary phases will behave in very similar or in quite different fashion, and thus facilitate the choice of substitute columns already possessed for a published procedure, or for the selection of radically different columns in an attempt to improve the separation of a previously poorly resolved mixture. McReynolds values indicate, for example, that various methyl polysiloxanes[8] (*e.g.* SE-30, OV-1, OV-101 and SP-2100) should function virtually identically.

Novák and Růžičková in 1974[9] introduced a "generalization" of the retention index system, pointing out that originally it indicated by a hundredfold the apparent carbon number (usually quoted with an excess of precision to one decimal place) of a solute *if* it were an *n*-alkane, and suggesting that it equally could be based on other homologous series such as 2-ketones, *n*-alcohols, or acetate esters of these. Kováts discussed this as long ago as 1965[10]. Others, too, have recommended polar *n*-al-

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cohols[11,12], and recently *n*-aldehydes[13] have been suggested as the ideal base series, being of intermediate polarity.

Many gas–liquid chromatographic procedures are best performed at temperatures higher than 120°C, and attempts have been made to determine a relationship between I_R and column temperature[14]. It has been claimed that I_R varies less than one unit per degree temperature rise[15], but for ΔI_R determination there is the limitation that the squalane standard phase is too volatile to be used above 140°C. The methyl polysiloxane SE-30 has been used as an alternative standard phase[16] and this withstands high temperatures, whilst hydrogenated Apiezon MH has been used at 160 to 190°C with five aromatic probe solutes[17]. A "g-pack" of six probes, including the monoterpene limonene, the terpene alcohol linalool with its acetate ester, and cinnamyl alcohol which is typical of volatile oil aromatics, has recently been adopted[18] to check —together with *n*-alkanes— that polyethyleneglycol columns are in suitable condition for volatile oil "fingerprinting" at up to 225°C.

The McReynolds system, or variations on it, is designed to characterise the liquid stationary phase, but not a packed column — unless it has been freshly prepared for this experimental purpose. After a period of use, when it may have "mellowed" satisfactorily, there is no easy way to determine the loading of stationary phase remaining in the column. It has been found[19] that nearly 9% of the medium-molecular-weight polyethylene glycol 6000 may evaporate from a support after only 2 h at 100°C.

McReynolds values also imply using identical concentrations of test and standard stationary phases in identical columns. In practice, gas–liquid chromatography is not like this. Polysiloxanes are commonly prepared with a 2 to 5% loading, whilst polyglycols and polyesters are often present initially at 3 to 15% of the weight of column packing. Thus a *practical* system of values is needed to express the relative polarities of the "library" of columns kept by each laboratory. This automatically takes account of the varied history and possible misuse of individual columns.

Eqn. 1 can be adapted for this purpose by selecting a higher temperature with appropriate probes, utilising other standard series besides *n*-alkanes, selecting a higher temperature reference stationary phase (*i.e.* replace squalane), and examining test phases as they have been loaded (and used) in columns. The work reported here involves a study of five of our packed columns (three containing polysiloxanes), with three standard series, and three probes.

EXPERIMENTAL

Apparatus and materials

A Pye 104 gas chromatograph was used, fitted with a flame ionisation detector used at 175°C together with a Linear Instruments recorder.

Columns packed with stationary phase on support were those in the laboratory "library", and are described in Table I.

Solutes were laboratory grade, from various commercial sources.

Procedure

A temperature of 160°C was chosen as being of more general value than 120°C; and three C_{10} volatile oil substances which had short retention times (<9 min) with

nitrogen flow-rate at 40 ml/min, and which represented a range of chemical structures, were selected as probes. They were, in constantly observed retention time (t_R) sequence:

(−)-linalool (alcohol, acyclic, unsaturated (2Δ))	shortest t_R
estragole (aromatic ether, *p*-methoxy-allylbenzene)	medium t_R
(+)-carvone (ketone, cyclic, unsaturated (2Δ))	longest t_R

Each of the five columns, prepared at different times in the past, and used for various purposes, was allowed to stabilise under the standard operational conditions and then received successive and repeated injections of: (i) mixture of the three probes in alcohol, (ii) mixtures of appropriate *n*-alkanes (PolyScience Corp., Niles, IL, U.S.A.), (iii) mixture of *n*-aldehydes in alcohol (C_8 and C_{10} used), (iv) mixture of *n*-alcohols in alcohol (C_8, C_{10} and C_{12} used).

The various retention indices of the three probes were obtained from graphic plots on semi-log paper of t_R (log scale) against carbon number of the three standard series (*n*-alkanes, *n*-alcohols and *n*-aldehydes). Probe I_R against each series is a hundred times the apparent carbon number, by definition. In theory these plots are accepted as straight lines; however a curve was apparent in some cases, especially for the lower alkanes. The graphical determination clearly indicates the limitations of accuracy not apparent in the formula method of Kováts[2] and should be less subject to error[20].

RESULTS AND DISCUSSION

The retention indices observed from repeated observations on the five selected columns are detailed in Table I. Very consistent results were obtained against alkanes on all the polysiloxanes, against alcohols on SP-2250 and PEG 20M, and against aldehydes on OV-1, SP-2250 and DEGS.

In other cases, results showed a surprising variation for what should be a standard procedure. The consistent results obtained with the SP-2250 column led to its selection as the polarity reference base column for *this* laboratory, despite it being not the least (nor most!) polar of the columns studied. Table II gives ΔI_R values against this polarity base column obtained with the other four columns for each probe, referred to each of three standard series (alkanes, alcohols and aldehydes). The average ΔI_R for the set of three probes against each series is also given, together with combined averages against alkanes and alcohols, which seem to provide satisfactory column polarity ratings. Values both negative and positive resulted.

It is notable that the two fully methyl polysiloxane columns, OV-1 and SP-2100, which should behave identically, show different ΔI_R values. This is least apparent when they are rated against the aldehydes series, for which their average values are similar. However, when compared using alkanes and alcohols, they are dissimilar, one being almost twice as non-polar as the other, possibly reflecting their differing history of use, or that the SP-2100 is on a silanized support, which minimises support interaction[21]. The DEGS column is clearly the most polar, but the apparent polarity of the PEG column changes with the reference series used. Against alcohols (which have great affinity for it) it is less polar than the base SP-2250, although it is clearly more polar than the base against alkanes or aldehydes. Using only the classic retention index standard of paraffins, Schomburg observed[22] that a silicone oil stationary

TABLE I

AVERAGE GRAPHICALLY DETERMINED I_R AND t_R OBSERVED AT 160°C WITH NITROGEN FLOW-RATE 40 ml/min

Average results are shown in italics, with the range observed where variation was found.

	Stationary phase				
	OV-1 polysiloxane (fully methyl)	*SP-2100 polysiloxane (fully methyl)*	*SP-2250 polysiloxane (phenyl–methyl, 50:50)*	*PEG 20M polyethylene glycol (20,000 mol. wt.)*	*DEGS polyester (diethyleneglycol succinate)*
	Column details				
	2% on Diatomite CQ 120–150 mesh in glass, 1.5 m × 4 mm I.D. (old)	*2% on Chromosorb W AW DCMS 80–100 mesh in glass, 1.5 m × 4 mm I.D. (recent)*	*3% on Supelcoport, 100–120 mesh in stainless steel, 3.0 m × 5 mm I.D. (recent)*	*10% on Diatomite C AW 100–120 mesh in stainless steel, 1.5 m × 5 mm I.D. (old)*	*15% on Chromosorb W AW 80–100 mesh in stainless steel, 1.5 m × 5 mm I.D. (old)*
I_R vs. n-alkanes					
Linalool	1061—*1068*—1076	1152—*1156*—1160	*1173*	1489—*1500*—1510	1780 approx.
Estragole	*1167*	*1227*	*1342*	1655—*1660*—1665	*
Carvone	1225—*1228*—1231	1258—*1265*—1272	1403—*1405*—1407	1724—*1731*—1738	*
I_R vs. n-alcohols					
Linalool	765— *773*— 781	820— *828*— 837	814— *822*— 830	795— *797*— 800	814— *820*— 832
Estragole	860— *865*— 870	928— *930*— 933	*993*	943— *948*— 955	1035—*1042*—1052
Carvone	943— *945*— 947	987— *991*— 996	1052—*1056*—1060	1017—*1025*—1028	1155—*1161*—1170
I_R vs. n-aldehydes					
Linalool	*875*	836— *861*— 891	879— *882*— 885	988—*1000*—1013	1040—*1045*—1051
Estragole	*962*	950— *975*—1002	*1073*	1182—*1190*—1199	1290—*1293*—1296
Carvone	*1052*	1022—*1045*—1072	1158—*1161*—1165	1278—*1282*—1287	*1427*
Approx. t_R (min)					
Linalool	0.50	1.05	1.50	2.30	3.20
Estragole	0.60	1.20	4.80	3.85	6.05
Carvone	0.70	1.40	6.05	4.95	8.40

* Relevant *n*-alkanes not resolved; determination impossible.

TABLE II

ΔI_R CALCULATED FROM TABLE I*

	Column			
	OV-1	*SP-2100*	*PEG 20M*	*DEGS*
Vs. n-alkanes				
Linalool	−105	−17	+326	+605 approx.
Estragole	−175	−115	+318	**
Carvone	−177	−140	+326	**
Average (P)	−152	−91	+324	+605 approx.
Vs. n-alcohols				
Linalool	−49	+6	−25	−2
Estragole	−128	−63	−45	+49
Carvone	−111	−65	−31	+105
Average (A)	−96	−41	−34	+51
Average of above (P and A)	−124	−66	+145	+330 approx.
Vs. n-aldehydes				
Linalool	−7	−21	+118	+163
Estragole	−111	−98	+117	+220
Carvone	−109	−116	+121	+226
Average	−76	−78	+119	+203
Column polarity rating	← less polar than base* —		— more polar than base* →	

* Base column 3% SP-2250.
** Relevant *n*-alkanes not resolved.

phase was "less polar" than an Apiezon grease with a benzene probe, but that the reverse held for an ester probe solute, confirming that changes in apparent polarity occur.

These results suggest that *n*-alkanes alone, as used by Kováts and McReynolds for I_R and ΔI_R, are not the best choice of standard series as they may exaggerate the relative polarity of the more polar stationary phases, and because paraffin mixtures are not resolved on some highly polar columns (*e.g.* DEGS). According to Martin[23], this lattermost would be due to an adsorption, as well as partition, effect by the polar stationary phase. Others consider this may be a minor influence[24]. Urone and Parcher's work[21] would implicate a column support effect that whilst obviously more relevant at low stationary phase loadings, can still exert an influence at above 10% loads. The DEGS column used here had a nominal 15% loading. Whatever the cause of the results obtained, they indicate that another series of polar compounds such as alcohols should also be used together with alkanes. If one series only is to be chosen as reference, the *n*-aldehydes suggested by Heldt and Köser[13] might be the one of choice, being themselves intermediate in polarity between paraffins and alcohols, although discrepancies may result (see below).

Although the reference base column for our laboratory is an SP-2250 column, any column giving consistent results with good efficiency and reasonably short retention times could have been selected. The method used here is still applicable for

practical comparisons. Other laboratories may make another choice. However, the intermediate polarity of a phenyl–methyl (50:50) polysiloxane should be considered, so that test columns are rated against it on a more or less polar scale. For example, using the procedure here, our 10% polyester SP-1000 column rated identically to PEG using only standard series *n*-alcohols, but at +104 (41 units less polar than PEG) using an average of alkanes and alcohols. Our 5% trifluoropropyl polysiloxane QF-1 column was similar on average to the SP-2250 base using only alcohols, but was +28 more polar using the alkane and alcohol average. Using only *n*-aldehydes, the ratings were +132 for SP-1000 but −104 for QF-1. It seems ridiculous to rate QF-1 as less polar than a fully methyl polysiloxane, so the use of the aldehyde reference series alone is questionable with polysiloxanes, and the double reference of alkanes and alcohols is to be preferred. "A method is needed that will enable an individual chromatographer to characterise his own set of columns"[25]. The method given here may serve this purpose.

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Note

Chromatographic study of optical resolution

VII*. Directional ion-association model for the optical resolution of *cis*-$[Co(O)_2(N)_4]^+$ type of complexes by the antimony *d*-tartrate ion

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For the optical resolution of octahedral metal complexes by ion-exchange chromatography, the antimony *d*-tartrate anion, $[Sb_2(d\text{-tart})_2]^{2-}$, is known to be a very effective eluent. Recently, we have proposed[2,3] the L–J model as a stereoselective ion-association mode between a metal complex cation and the $[Sb_2(d\text{-tart})_2]^{2-}$ anion. The model has been shown to explain nicely the elution behaviour of tripositive complexes of cobalt(III). Because tripositive cobalt(III) complexes contain only electrically neutral ligands, there is no charge localization in such complexes. In contrast, significant charge localization is expected for dipositive or monopositive cobalt(III) complex cations[4], because these complex cations contain anionic ligands. Therefore, when these cations are chromatographed by $[Sb_2(d\text{-tart})_2]^{2-}$, the position where the L-shaped channel[2,3] is situated seems to be very important. In this paper, some monopositive cobalt(III) complexes are subjected to ion-exchange chromatography by $[Sb_2(d\text{-tart})_2]^{2-}$, and the result is interpreted in terms of the L–J model with directional ion-association taken into consideration.

EXPERIMENTAL

The two geometrical isomers, *cis*(O),*cis*(N),*cis*(NH_3) and *cis*(O),*trans*(N), *cis*(NH_3), of $[Co(gly)_2(NH_3)_2]^+$, and the two geometrical isomers, C_1-*cis*(O) and C_2-*cis*(O), of $[Co(gly)_2(en)]^+$ were prepared according to the methods of Kobayashi and Shibata[5] and Dabrowiak and Cooke[6], respectively, where gly stands for the glycinate ion and en for ethylenediamine. SP-Sephadex C-25 resin was packed in a 31.5 cm × 6.5 mm I.D. column. The sample solution containing Blue Dextran 2000, which was used as a marker for the void volume measurement of the column, was charged on the column and was eluted with 0.1 *M* aqueous solution of $K_2[Sb_2(d\text{-tart})_2]$ at a constant rate of 0.33 ml/min. The adjusted retention volumes were measured by a spectrophotometer at a wavelength of the first *d–d* transition region.

* Part VI: ref. 1.

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RESULTS AND DISCUSSION

The L-shaped channel was defined in the previous papers[2,3] as the L-shaped opening between chelate rings, and the J-shaped channel as the mirror image of the L-shaped channel. The optically active $[Sb_2(d\text{-tart})_2]^{2-}$ anion can associate intimately with that enantiomer of a metal complex cation that has the L-shaped channel, but cannot associate closely with the enantiomer that has only the J-shaped channel. If we suppose that there is no charge localization in metal complexes, as in tripositive cobalt(III) complexes, the separation factor when $[Sb_2(d\text{-tart})_2]^{2-}$ is used as an eluent increases with an increase in the difference in the number of L-shaped channels between a pair of enantiomers. On the other hand, in the optical resolution of metal complexes for which charge localization appears to be significant, the position, not the number, of the L-shaped channels in a complex cation may play an important role. In the case of the *cis*-$[Co(O)_2(N)_4]^+$ type of complex, because two oxygen donor atoms carry negative charges, the eluent anion will associate with the complex cation along the direction of the twofold axis and from the side opposite the two oxygen donor atoms. As shown in Fig. 1a, the *cis*-$[Co(O)_2(N)_4]^+$ type of complex has five kinds of edge of an octahedron, which are different stereochemically. Here, rank 1 is the best position to associate with an anion electrostatically, and the ease of access of an anion toward the complex cation decreases from the edge of rank 2 to the edge of rank 5. The L-shaped channel is represented as one edge of an octahedron (Fig. 1b). In other words, there are five stereochemically different kinds of L-shaped channel in the *cis*-$[Co(O)_2(N)_4]^+$ type complexes.

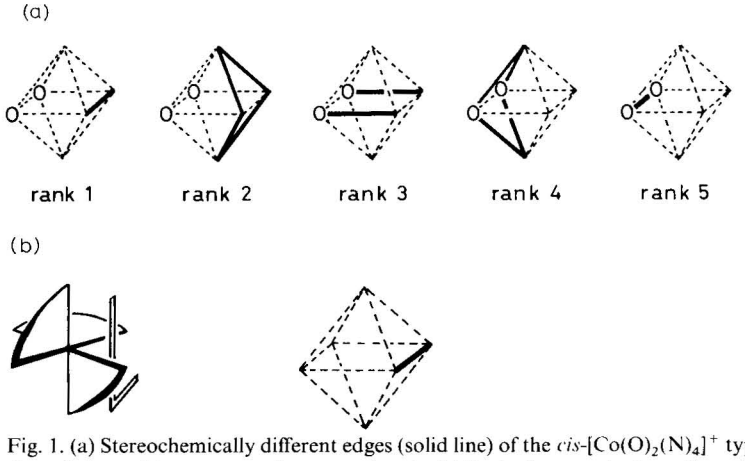

Fig. 1. (a) Stereochemically different edges (solid line) of the *cis*-$[Co(O)_2(N)_4]^+$ type of complex. (b) The L-shaped channel is represented as one edge (solid line) of an octahedron. See ref. 2.

The number and position of the L- and J-shaped channels of the *Λ* enantiomer of the complexes examined in this paper are listed in Table I. The term "LJ" in Table I stands for the opening that can be regarded as both the L- and J-shaped channels. Because the *Λ* enantiomers of complexes 3 and 4 have only the L-shaped channels, the *Λ* enantiomers of these complexes may be expected to be eluted first. Further, because complex 3 has L-shaped channels of higher rank than complex 4 does, the

TABLE I

THE NUMBER AND POSITION (EXPRESSED IN RANK OF FIG.1(a)) OF THE L- AND J-SHAPED CHANNELS OF THE *Λ* ENANTIOMERS

"LJ" stands for the opening that can be regarded as both the L- and J-shaped channels.

Complex	*Rank 1*	*Rank 2*	*Rank 3*	*Rank 4*	*Rank 5*
1 *cis*(O),*cis*(N),*cis*(NH_3)-[$Co(gly)_2(NH_3)_2$]$^+$	1 LJ	2 L 1 J 1 LJ	1 J	1 LJ	1 L
2 *cis*(O),*trans*(N),*cis*(NH_3)-[$Co(gly)_2(NH_3)_2$]$^+$	1 LJ	2 J 2 LJ	2 L	2 L	–
3 C_1-*cis*(O)-[$Co(gly)_2(en)$]$^+$	1 L	2 L	–	–	1 L
4 C_2-*cis*(O)-[$Co(gly)_2(en)$]$^+$	–	–	2 L	2 L	–

separation factor of complex 3 may be better than that of complex 4. For complex 1, because the *Λ* enantiomer has two L-shaped channels and one J-shaped channel, both of rank 2, it is expected that the *Λ* enantiomer is eluted first. In contrast, for complex 2, because the *Δ* enantiomer has two L-shaped channels, the *Δ* enantiomer is expected to be eluted first. Thus, the present model predicts that the absolute configuration of the first eluted enantiomer is *Λ* for *cis*(O),*cis*(N),*cis*(NH_3)-[$Co(gly)_2(NH_3)_2$]$^+$, whereas it is *Δ* for *cis*(O),*trans*(N),*cis*(NH_3)-[$Co(gly)_2(NH_3)_2$]$^+$. Moreover, it may be predicted that the separation factor of complex 2 is better than that of complex 1.

The results are shown in Table II. Although the absolute configurations of complexes 1 and 2 have not been established by single crystal X-ray analysis, the absolute configuration of the first eluted enantiomer of complex 1 is considered to be *Λ* because the sign of the dominant circular dichroism (CD) band is plus, with a maximum at 529 nm, and that of complex 2 may be *Δ* because the sign of the dominant CD band is minus, with a maximum at 520 nm. The absolute configuration of the first eluted enantiomers of complexes 3 and 4 has been assigned as *Λ*[6]. These deductions of the absolute configuration of the first eluted enantiomers are in line with the prediction based on the L–J model described above.

TABLE II

THE RETENTION VOLUMES AND THE SEPARATION FACTORS OF COMPLEXES 1–4 WHEN CHROMATOGRAPHED WITH 0.1 *M* $K_2[Sb_2(d\text{-tart})_2]$

Complex	*Retention volume (ml)*	*Separation factor*
1 *cis*(O),*cis*(N),*cis*(NH_3)-[$Co(gly)_2(NH_3)_2$]$^+$	13.83	–*
2 *cis*(O),*trans*(N),*cis*(NH_3)-[$Co(gly)_2(NH_3)_2$]$^+$	12.20 (*Δ*) 14.03 (*Λ*)	1.150
3 C_1-*cis*(O)-[$Co(gly)_2(en)$]$^+$	13.29 (*Λ*) 15.75 (*Δ*)	1.185
4 C_2-*cis*(O)-[$Co(gly)_2(en)$]$^+$	15.79	–*

* Separation factors could not be obtained because of partial resolution.

Let us compare the retention volumes of complexes 1 and 2. The average number of L-shaped channels of the Λ and Δ enantiomers of complex 1 is one of rank 1 and 2.5 of rank 2, whereas for complex 2 the number of L-shaped channels of the Δ enantiomer is one of rank 1 and four of rank 2 and that of the Λ enantiomer is one of rank 1 and two of rank 2. Complex 2 was optically resolved completely, and the retention volume of complex 1 is closer to that of the Λ enantiomer of complex 2 than that of the Δ enantiomer of complex 2. This result might also support the validity of the reasoning that was based on the L–J model and took directional ion-association into consideration.

The absolute configuration of the first eluted enantiomers is Λ for complexes 3 and 4, and complete resolution was attained for complex 3. The results are in line with the prediction described above. Moreover, the fact that the retention volume of complex 4 is almost equal to that of the Δ enantiomer of complex 3 may support the L–J model with directional ion-association, because the Δ enantiomer of complex 3 and both enantiomers of complex 4 have no L-shaped channel of rank 1 and rank 2.

Therefore, it is concluded that if directional ion-association is considered for complexes having charge localization, the elution order and the degree of separation when eluted with $[Sb_2(d\text{-tart})_2]^{2-}$ are reasonably predicted according to the L–J model.

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Note

Resolution of enantiomers of norepinephrine and epinephrine by reversed-phase high-performance liquid chromatography

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Catecholamines such as norepinephrine and epinephrine are important substances in the fields of biological and clinical chemistry. We have recently reported[1] a simple fluorimetric determination of catecholamines by high-performance liquid chromatography (HPLC). The chromatographic resolution of catecholamine enantiomers, which is important for the elucidation of their biological conversion[2], is dealt with in the present study.

In the previous paper[3], reversed-phase HPLC resolutions of amino acid enantiomers by pre-column chiral derivatization with acetylglycosyl isothiocyanates, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC), were described.

This paper describes the liquid chromatographic resolution of D,L-norepinephrine and D,L-epinephrine on a reversed-phase column using GITC and AITC. Diastereomeric thiourea derivatives which were formed from catecholamines with either GITC or AITC could be resolved on an octadecylsilyl-bonded silica gel column with methanol–10 m*M* phosphate buffer (pH 2.8) as a mobile phase, and detected spectrophotometrically at 250 nm. Excellent resolution was observed without protection of phenolic hydroxyl groups and the procedure was very simple.

MATERIALS AND METHODS

Racemic and L-isomeric norepinephrine and epinephrine were obtained from Sigma (St. Louis, MO, U.S.A.). Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Chemical Industry Company (Tokyo, Japan). All the reagents were of analytical reagent grade. Methanol and water were distilled before use. GITC and AITC were prepared by treatment of α-acetobromoglucose and β-acetobromoarabinose with silver thiocyanate, as described previously[4]; GITC and AITC are commercially available from Polysciences (Warrington, PA, U.S.A.). The 10 m*M* phosphate buffer was prepared from monobasic potassium phosphate and was adjusted to pH 2.8 with perchloric acid.

Equipment

The chromatographic system consisted of a high-pressure pump equipped with a valve universal injector (Sanuki Industry Company, Tokyo, Japan), a Develosil

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ODS column (15 cm × 4.6 mm I.D., particle size 5 μm; Nomura Chemical, Seto-shi, Japan), and an SPD-2A spectrophotometric detector (Shimadzu Seisakusho, Kyoto, Japan).

Derivatization and separation procedure

Each 10 mg of racemic catecholamine and each 5 mg of L-isomer were dissolved in 0.25 *M* aqueous acetic acid to make 100 ml, respectively. Then 50 μl of this catecholamine stock solution was pipetted into a microtube, and evaporated to dryness under reduced pressure in a desiccator at room temperature. To the residue was added 50 μl of 0.2% (w/v) chiral reagent, either GITC or AITC, in dimethylformamide (DMF). This reaction mixture was allowed to stand at room temperature for 10 min, and then 10 μl of 0.5% (v/v) hydrazine hydrate DMF solution were added. The resulting mixture was allowed to stand at room temperature for 10 min, and then a 10-μl aliquot of the mixture was injected directly into the chromatograph. The column was eluted at room temperature and at a flow-rate of 0.9 ml/min, with a mobile phase prepared by mixing methanol and 10 m*M* phosphate buffer, pH 2.8, in an appropriate ratio.

RESULTS AND DISCUSSION

Norepinephrine and epinephrine react readily with chiral reagents, either GITC or AITC, under mild conditions without the formation of by-products. The resulting mixture can directly be injected into the chromatograph. The thiourea derivatives eluted from the column were monitored using the absorption at 250 nm[3,4].

Fig. 1 shows the amount of thiourea derivatives formed from L-norepinephrine with AITC *versus* the reaction time for the two different reaction solvents: DMF and acetonitrile. The amount of thiourea formed is proportional to the peak height. With DMF as the reaction solvent, the reaction proceeded to completion in 10 min. In contrast, more than 50 min were needed when acetonitrile was used, and the yield of

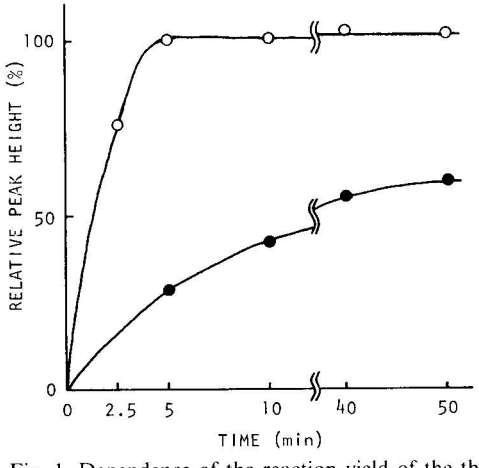

Fig. 1. Dependence of the reaction yield of the thiourea derivative formed from L-norepinephrine with AITC. ○, in DMF; ●, in acetonitrile.

the reaction was low. Recently, Björkqvist[5] reported that the DMF both seemed to catalyse the reaction and also served as a good solvent for the disubstituted urea, in his study of the derivatization of aliphatic and aromatic amines using phenylisocyanate.

Figs. 2 and 3 show the chromatograms of the diastereomeric thiourea derivatives of isomeric norepinephrine and epinephrine when GITC and AITC were used for the derivatization, respectively. Enantiomeric pairs were eluted in the sequence L before D when GITC was used, but in the opposite sequence when AITC was used. These results are in good agreement with those of enantiomeric amino acids[3].

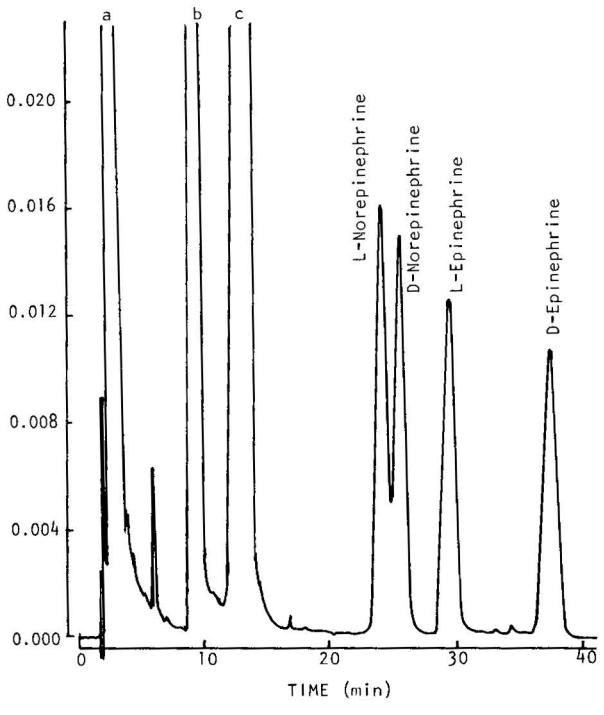

Fig. 2. Separation of diastereomeric thiourea derivatives formed from catecholamines with GITC. Mobile phase, methanol–10 m*M* phosphate buffer (pH 2.8) (35:65). Flow-rate, 0.9 ml/min. About 200 ng of each derivative were injected. Peaks: a = DMF; b, c = reaction products of the excess reagent with hydrazine.

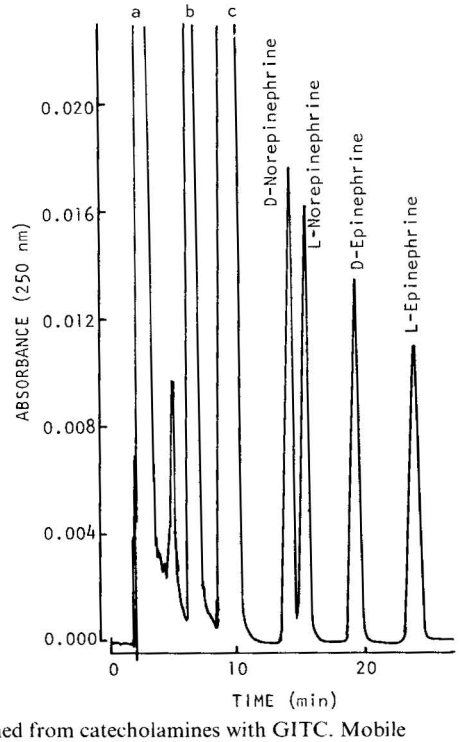

Fig. 3. Separation of diastereomeric thiourea derivatives formed from catecholamines with AITC. Mobile phases, methanol–10 m*M* phosphate buffer (pH 2.8) (30:70). Flow-rate, 0.9 ml/min. About 200 ng of each derivative were injected. Peaks: a = DMF; b, c = reaction products of the excess reagent with hydrazine.

Under the same chromatographic conditions, the reagent peaks are well separated from those of the diastereomers and do not interfere with the detection. However, when the injection of samples is repeated, peaks of the excess reagents interfere with the analysis. Consequently, hydrazine hydrate was added to the derivatization mixture to remove of excess reagents. Hydrazine reacted completely with the excess reagents and the reaction products were eluted faster than any diastereomers (Figs. 2 and 3).

The retentions and resolutions of the diastereomeric GITC and AITC derivatives are listed in Table I; k', α and R_s refer to the capacity ratio, separation factor and resolution respectively for a pair of diastereomers. The resolution of AITC derivatives were favored over that of GITC derivatives.

TABLE I

SEPARATION OF DIASTEREOMERIC THIOUREA DERIVATIVES FORMED FROM CATECHOLAMINES WITH GITC AND AITC

t_0 = 2.0 min. Column, Develosil ODS (15 cm × 4.6 mm I.D.). Mobile phase: methanol–10 m*M* phosphate buffer (pH 2.8) (35:65) (A), (30:70) (B).Flow-rate, 0.9 ml/min. k', α and R_s are defined in the text.

Catecholamine	*GITC*				*AITC*			
	k'	α	R_s	*Mobile phase*	k'	α	R_s	*Mobile phase*
Norepinephrine D	11.40	1.08	1.00	A	6.00	1.10	1.20	B
L	10.60				6.60			
Epinephrine D	17.40	1.30	4.00	A	8.60	1.28	3.69	B
L	13.40				11.00			

Nambara and co-workers[6,7] pointed out that the rigidity of the conformation around the chiral centres, and the proximity between the chiral centres of the diastereomer, are important to secure satisfactory resolution. They have achieved good resolution of amino acid enantiomers using terpene isothiocyanate reagents. However, this reagent required the conversion of the amino acid moiety into the bulky *tert.*-butyldimethylsilyl ester. GITC and AITC facilitated excellent resolution of amino acids without esterification of carboxyl groups[3]. This fact may be attributed to the sterically crowded acetylglycosyl residues of the reagents, which may increase the conformational rigidity. Chiral centres of the catecholamines are located at the β-position from their amino groups, and the distances between the chiral centres of their GITC or AITC derivatives are therefore greater than those of amino acid derivatives. Nevertheless, the catecholamine derivatives were well resolved, presumably owing to the bulkiness of GITC and AITC residues. These bulky groups may fix the conformation around the asymmetric carbon of the catecholamines.

In addition, the bulky hydrophobic group of the present reagent seemed to favour resolution on the ordinary reversed-phase column.

The present method may generally be applicable to the resolution of optical isomers whose chiral centres are too distant from the functional groups to react with the chiral reagents.

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Note

Recovery of HPLC-grade acetonitrile by spinning-band distillation*

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The rising cost of high-purity solvents coupled with the ethical and financial burden of waste solvent disposal have prompted us to investigate the recovery of previously used high-performance liquid chromatographic (HPLC) solvents.

The use of PTFE annular spinning-band distillation columns has been recognized as a valuable analytical technique for the separation of compounds whose boiling points are as close as 0.5°C[1–3]. High-speed rotation of the PTFE band against the column wall produces a thin film of condensate, highly efficient vapor liquid contact, and enrichment (rectification) with low column hold-up. Once equilibrium has been established, solvents may be distilled at rapid boiling rates and low reflux ratios to maximize sample throughput.

This paper describes the recovery of previously used HPLC-grade acetonitrile by distillation without chemical pretreatment on a thirty-plate PTFE annular spinning-band column. Using static, preconcentration, and dynamic (on-column solvent enrichment) evaluation, spectrophotometric and chromatographic data indicate 85–95 % recovery of acetonitrile with purity at or near the specifications of commercially available HPLC-grade solvents.

EXPERIMENTAL

Apparatus

All distillations were carried out on a B/R Instrument Corporation Model 40-T, PTFE-banded still equipped with ground glass joints and PTFE sleeves.

Gas chromatographic data were obtained on a Perkin-Elmer Model 3920B all-glass dual flame ionization detection system, using Carbowax 1540 or Chromosorb 101 (ref. 4) columns.

HPLC baseline absorbance studies were carried out on a Jasco Familic 100 micro HPLC system equipped with a UVIDEC 100-II variable-wavelength detector and a reversed phase (SC-01, C_{18}) column. Dynamic enrichment studies were carried out on a Tracor Model 950/980A gradient elution high-performance liquid chromatograph equipped with a Tracor Model 970A variable-wavelength UV detector and an ISCO Model UA-5 fixed-wavelength (254 nm) detector. The system was interfaced

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with an ISCO Model 1200 fraction collector which allowed for automatic peak separation and collection. Whatman Partisil (5% C_{18}) or Alltech (18% C_{18}) 25.0 cm × 4.6 mm I.D., 10-μm particle size columns were used.

Differential pulse polarograms were obtained using a PAR Model 174A polarographic analyzer.

Materials

Distilled-in-glass grade acetonitrile UV (190 nm UV cutoff) and HPLC-grade water were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Previously used acetonitrile solvent mixtures, originally prepared using Burdick & Jackson solvents, were collected in clean, amber bottles through the cooperation of several local hospital laboratories. The mobile phases, studied for solvent recovery, included acetonitrile–4% aqueous ammonia and acetonitrile–water–acetate buffer (theophyllin or tricyclic antidepressant analyses).

Procedure

All previously used mobile phase mixtures were refluxed for 24 h to ensure equilibrium, followed by distillation at a band speed of 2500 rpm and take-off rate of 125 ml/h. The first 20 ml of distillate at the appropriate boiling range (ambient barometric pressure) were discarded. Acetonitrile was distilled without chemical pretreatment as the pure solvent (81–82°C) or as a binary azeotrope acetonitrile–water (84:16) (77°C).

Solvent purity

Solvent purity was determined from boiling range, refractive index, Karl Fischer titration for water, UV absorbance and fluorescence spectra, as well as from chromatographic information. The specifications of recovered acetonitrile were compared to the specifications of unused HPLC-grade acetonitrile.

Samples of recovered acetonitrile were preconcentrated by reducing 100-ml portions to 10 ml on an unheated rotary flash evaporator. The tenfold preconcentrates were then spectrophotometrically compared to samples of unused HPLC-grade acetonitrile prepared in the same way. The evaporation was done at room temperature to minimize the loss of higher boiling impurities.

Samples of recovered and unused HPLC-grade acetonitrile were also compared after 60 min of on-column HPLC enrichment using acetonitrile–water (30:70) mobile phase at 3.0 ml/min through a C_{18} reversed-phase column. Impurities accumulating on the column were subsequently stripped using a step gradient of acetonitrile–water (70:30) (15 min) followed by a step gradient of 100% acetonitrile (15 min).

RESULTS AND DISCUSSION

Solvent specifications

Table I shows the comparison of specifications for recovered acetonitrile fractions and HPLC-grade acetonitrile. The percentage of water in the pure solvent samples and the binary azeotrope was verified by gas chromatographic analysis on Chromosorb 101 (ref. 4). The total yield of recovered acetonitrile, as pure solvent and/or azeotrope, ranged between 85–95% based upon the compositions of the pot

TABLE I

SOLVENT SPECIFICATIONS

	Acetonitrile		
	HPLC	*Recovered, pure solvent*	*Recovered, azeotrope*
Boiling range (°C)	81–82	82	77–78
Refractive index at 15°C	1.3466	1.3465	–
Water, Karl Fischer	0.07%	0.08%	15.7%
Purity*	99.9%	99.9%	99.7%
UV Cutoff (nm)**	<190	<190	<190

* Gas–liquid chromatography, 8% Carbowax 1540.
** Wavelength at which absorbance equals 1.00.

charge and distillate fractions. The optimum take-off rate for acetonitrile recovery was found to be 120–140 ml/h. Column hold-up was estimated to be 1.2 ml.

Fig. 1 shows that the maximum sensitivity fluorescence spectrum of recovered acetonitrile to be virtually identical to that of unused HPLC grade acetonitrile. The differential pulse polarograms (Fig. 2) show the presence of an impurity, suspected but not confirmed to be acrylonitrile[5], in HPLC-grade but substantially reduced in recovered acetonitrile. The end currents result from supporting electrolyte decomposition.

Baseline stability studies (Fig. 3) show that the recovered solvent has a slightly lower absorbance *versus* time profile but gives essentially the same response as HPLC-grade acetonitrile.

The comparison of static solvent specifications (without preconcentration, or

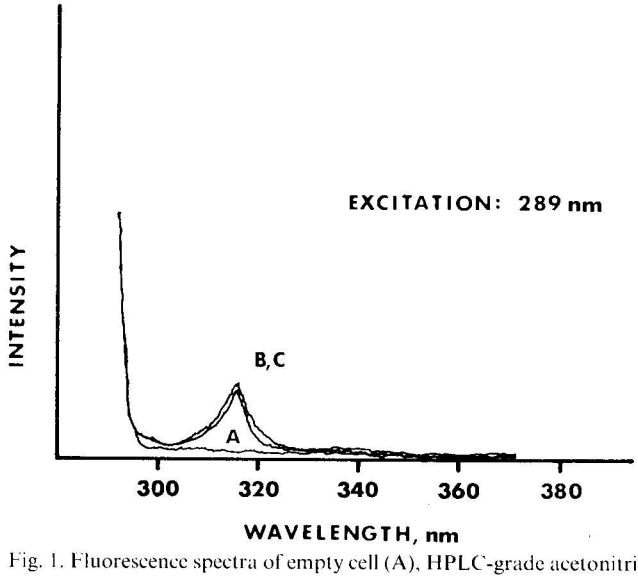

Fig. 1. Fluorescence spectra of empty cell (A), HPLC-grade acetonitrile (B), and recovered acetonitrile (C).

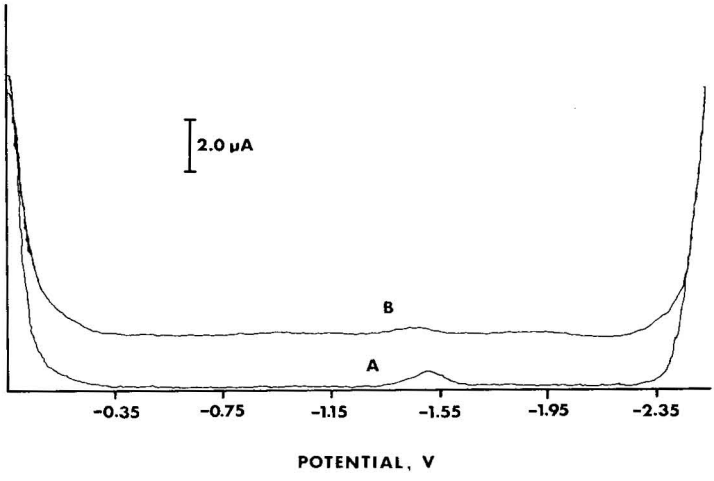

Fig. 2. Differential pulse polarograms of HPLC-grade acetonitrile (A) and recovered acetonitrile (B). Supporting electrolyte: 0.05 *M* tetrabutylammonium iodide; rate: 10 mV/sec, drop-time: 0.5 sec. Curves A and B manually offset 2.0 μA.

enrichment of impurities) suggests that the purity of recovered, previously used acetonitrile is at or near that of commercially available HPLC-grade acetonitrile. However, this comparison does not adequately indicate the presence of impurities which may become potential contaminants in an HPLC determination.

Solvent preconcentration and enrichment

Fig. 4 illustrates the effect of preconcentrating impurities on the UV spectra of acetonitrile samples. Although the static spectra show nearly identical absorbance, the spectra of the tenfold preconcentrates show that the unused HPLC-grade has higher absorbance in the 210–225 nm region which could result in "ghost" peaks during an HPLC analysis.

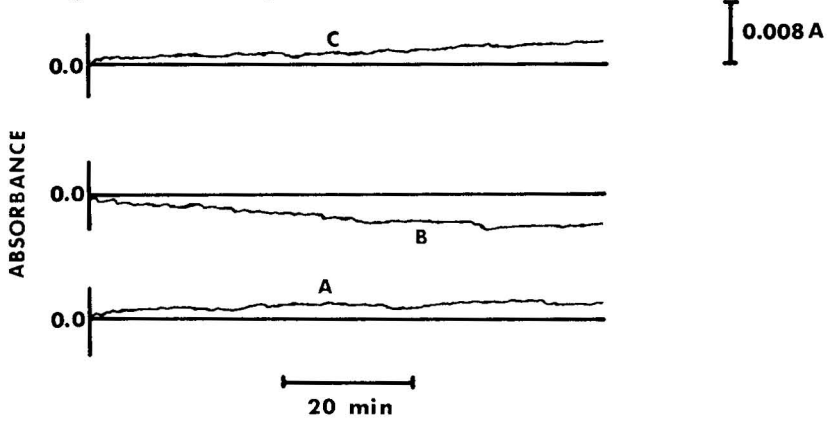

Fig. 3. Chromatograms of 100% HPLC-grade acetonitrile (A), recovered acetonitrile (B), and A rerun (C). Detector: 210 nm; column: C_{18} reversed phase; flow-rate: 4 μl/min.

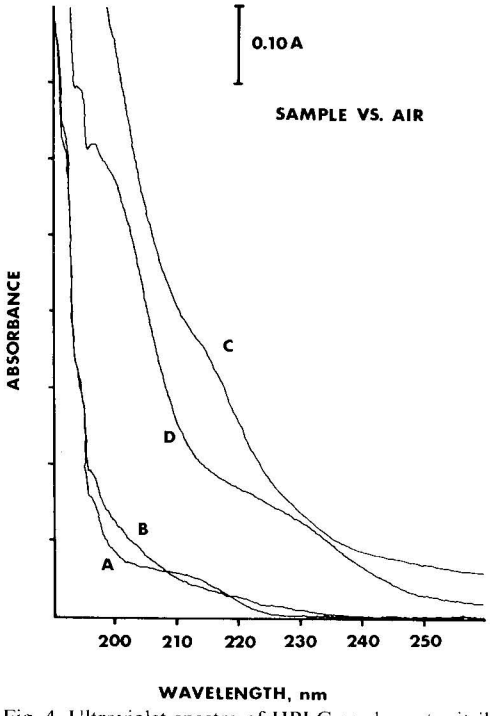

Fig. 4. Ultraviolet spectra of HPLC-grade acetonitrile (A), recovered acetonitrile (B), tenfold preconcentrate of A(C), and tenfold preconcentrate of B(D).

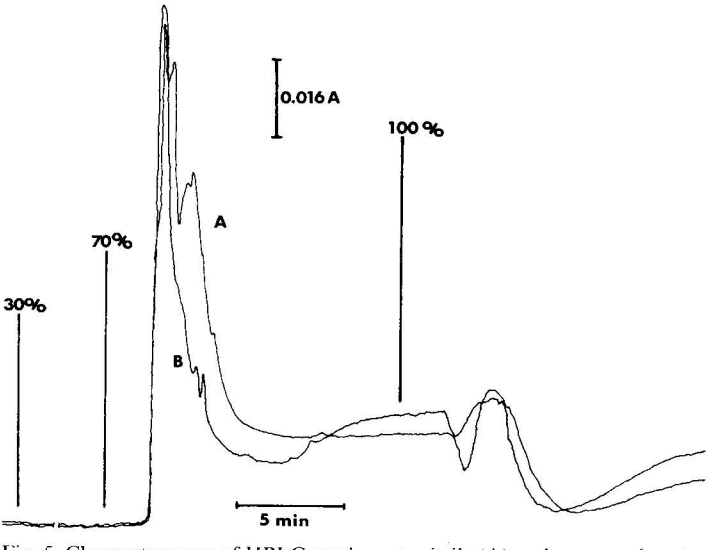

Fig. 5. Chromatograms of HPLC-grade acetonitrile (A) and recovered acetonitrile (B). Detector: 210 nm; column: Whatman C_{18}; solvent program: 30% A or B (60 min) step gradient to 70% A or B (15 min), step gradient to 100% A or B (15 min); flow-rate: 3.0 ml/min.

A more useful assessment of the purity of the recovered acetonitrile can be made by determining the on-column enrichment of impurities during the course of a chromatographic run. Fig. 5 shows that the recovered acetonitrile has a lower level of impurities when compared to unused HPLC-grade acetonitrile after enrichment using acetonitrile–water (30:70) (60 min) followed by step gradients of acetonitrile–water (70:30) (15 min) and 100% acetonitrile (15 min) at 210 nm.

We feel that solvents with sufficient purity for re-use in most HPLC analyses can be recovered using spinning-band distillation. For acetonitrile, both static and dynamic evaluation of the recovered solvent, distilled without prior chemical treatment[6], demonstrates the feasibility of recovering previously used HPLC mobile phases.

In our laboratory, we are now studying the recovery of 2,2,4-trimethylpentane, tetrahydrofuran, methanol, hexane, methylene chloride, dimethyl formamide, and dimethyl sulfoxide by spinning-band distillation. These data should be of interest to high-volume and preparative HPLC users.

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Note

Fluorescence and UV detection of opiates separated by reversed-phase high-performance liquid chromatography

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Considering their importance in pharmacology, toxicology and neurobiology some of the physical properties of the classical (*i.e.*, non-peptide) opiates have lacked adequate investigation. In particular, their fluorescence properties, and the use of these for detection in liquid chromatography have been inadequately reported. On the other hand detection of some natural and synthetic opiates using either their intrinsic or derivatized fluorescence properties has been previously used in paper and thin-layer chromatography[1]. For liquid systems, however, the standard monograph on fluorescence work[2] implies that only reaction products of morphine have appreciable fluorescence. Handbooks have carried through the same information[3] which is repeated in a recent report where electrochemical detection of opiates is discussed[4]. Indeed, a computer search of the high performance liquid chromatographic literature shows that the sole mention of fluorescence detection of opiates was in a recent report where dextrorphan was detected[5]. In that report sensitivities were not mentioned.

Consequently, the purpose of this brief report is to examine some of the relevant properties of some of the more familiar opiate agonists and antagonists. We present a convenient separation and detection scheme and discuss the spectral and chromatographic parameters and give estimates of detection limits.

EXPERIMENTAL

High-performance liquid chromatography (HPLC) was performed on a Micromeritics Model 7000 B chromatograph which was equipped with a Model 785 variable-wavelength ultraviolet detector. The chromatograph was coupled to a Perkin-Elmer Model 204-S dual monochromator fluorescence detector operating in the ratio mode and using the Perkin-Elmer 20-μl detection cell. The source was a high-pressure xenon lamp. Initial fluorescence work on macroscopic samples was done with a Perkin-Elmer Model MPF-3 spectrofluorometer.

Chromatographic separations were worked out initially on a home packed 10-μm RP-18 column (25 cm × 4.6 mm) and the final separations reported here were done with a Supelco 15 cm × 4.6 mm LC-18 column.

All the opiate compounds were used in the form of their water soluble acid salts. Buffer and solvent compounds were of chromatographic grade filtered before

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use and water was from a Millipore "Milli-Q" purifier using house deionized water as its source.

RESULTS

Table I summarizes the UV absorbtion and fluorescence parameters of the opiates studied. We found that ionic strength was very important in developing good HPLC separations for these compounds. The best chromatographic separation parameters are given in Table II along with data on the effect of ionic strength on the separation.

TABLE I

UV AND FLUORESCENCE PROPERTIES OF OPIATES IN ACETONITRILE–PHOSPHATE BUFFER (pH 5.05) (50:50)

Fluorescence spectra taken at 10 μM concentrations.

Parameter	*Compound*			
	Morphine	*Etorphine*	*Naloxone*	*Levallorphan*
$\lambda_{max.}$ (nm)	285	289	280	280
$\varepsilon\lambda_{max.}$ (cm^2/mole)	1652	1807	1638	1062
ε_{255} (cm^2/mole)	14,000	20,400	10,140	3650
λ_{ex} (nm)	290	290	290	290
$\lambda_{ex}^{max.}$ (nm)	340	346	–	309
Rel. emission intensity	1	1.24	0.0	19.5

TABLE II

REVERSED-PHASE HPLC SEPARATION OF OPIATES ON SUPELCO LC-18 (15 cm × 4.6 mm) COLUMN

Flow-rate 2 ml/min. Eluent system: "strong": acetonitrile–phosphate buffer (pH 5.05) (65:35); "weak": acetonitrile–water (80:20).

	t_R *(min)*			
	Naloxone	*Morphine*	*Etorphine*	*Levallorphan*
Retention times, t_R, as a function of eluent composition:				
40% "strong"	1.9	2.3	2.7	7.4
55% "strong"	1.7	2.05	2.5	6.7
60% "strong"	1.7	1.9	2.45	6.5
Effect of ionic strength:				
Final concentration of buffer 3 m*M*		3.2	4.6	
Final concentration of buffer 4 m*M*		2.6	3.9	

DISCUSSION

Our major finding is that for optimal sensitivity detection monitoring of *both* UV absorption at 225 nm and fluorescence emission at 330 nm are desirable. The wavelength of 330 nm was chosen as the best compromise between the emission peaks of the three fluorescent opiates investigated. Studies on a single opiate may benefit from use of the maximum of the emission spectrum (Table I). For example, naloxone does not exhibit detectable fluorescence under our conditions but has a very high extinction coefficient at 225 nm. On the other hand, levallorphan fluoresces very strongly but has low extinction coefficients at 280 and 225 nm. The lowest detectable peak concentrations for levallorphan etorphine and morphine (3:1 signal-to-noise ratio) via fluorescence detection are *ca.* 5 pmoles per peak using our compromise emission wavelength and optimal separation. Naloxone is detectable with the same signal-to-noise ratio at 20 pmoles using UV absorbance at 225 nm. The lowered sensitivity in HPLC detection of levallorphan relative to its fluorescence efficiency is due to peak broadening and the compromise wavelength. However, these lower limits to detectability are essentially the same as those reported for electrochemical detection[4]. Our separation scheme is by no means the only one available for opiate alkaloids. However, we would like to emphasize the ionic strength dependence of separation efficiency which we found and which may be important in individual cases.

The reasons for the presence or absence of fluorescence for these compounds is an intriguing question since the compounds studied all possess a tyramine moiety whose UV absorbance at 280–290 nm gives rise to the fluorescence. Obviously, this is quenched in the case of naloxone and potentiated in levallorphan. The question is worth a theoretical investigation. However in practical terms the varying fluorescence yields may be of value since, for example, one may observe agonists (*e.g.*, morphine) in the presence of a much higher concentration of the antagonist naloxone.

ACKNOWLEDGEMENT

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Note

High-performance liquid chromatographic separation and quantitation of maytansinoids in *Maytenus ilicifolia*

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Maytenus ilicifolia Mart. *ex.* Reiss. (Celastraceae) is a large shrub found in Southern Brazil, Paraguay, Uruguay and Argentina[1–4]. It has been used in Argentinian folk medicine as a sialogogue, antiasthmatic, antiseptic and vulnerary[5], and as an indigenous antitumor remedy in Brazil[6]. The plant, known as Cangorosa, is used by Indian tribes and rural populations in Paraguay as a fertility regulating agent[7–11].

Confirmation of the use of this plant as a menses inducer by the indigenous population in Paraguay was obtained by interviews with a number of individuals in Ascuncion, Paraguay during a field visit in 1979. Among the people interviewed were "Yuyos" (herb women), waitresses, and housewives. The method of use is usually by boiling or steeping the plant material in water and drinking the decoction/infusion at the time of anticipated menses, or shortly after the absence of it is noted. The dose was determined to be approximately nine grams of plant material per day until menstruation begins.

Because of the known presence of maytansine and other antitumor and cytotoxic ansamacrolides in related *Maytenus* species[12–15], the present study was initiated to determine the maytansinoid content in an ethnomedical dose of *M. ilicifolia* tea in order to ascertain its potential adverse effects on the Paraguayan women who continue to use this plant as a fertility regulator.

EXPERIMENTAL

Plant material

Maytenus ilicifolia Mart. *ex.* Reiss was procured in Asuncion, Paraguay in April, 1980 and identified by one of the authors (D.D.S.). Voucher specimens are deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL, U.S.A.

Apparatus

Liquid chromatographic separations were conducted with a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A liquid chromatograph equipped with a

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Rheodyne (Berkeley, CA, U.S.A.) Model 7120 syringe-loading sample injector and 100-μl sample loop, a Waters Assoc. Model 450 variable-wavelength UV spectrophotometer, and a Beckman (Lincolnwood, IL, U.S.A.) 10-in. strip chart recorder. Separations were carried out with a Waters Assoc. 30 × 0.2 cm I.D. μPorasil column.

Chemicals

All chemicals and solvents used in this investigation were reagent grade. Solvents for high-performance liquid chromatography (HPLC) were redistilled in glass.

Reference maytansine, maytanprine, and maytanbutine were generously provided by Dr. D. E. Nettleton, Jr. (Bristol Labs, Syracuse, NY, U.S.A.).

Extraction and fractionation of M. ilicifolia

Decoctions (teas) were prepared from three samples (9.0 g each) of the leaf + twig and three samples (9.0 g each) of the stem + root parts by boiling each sample in 750 ml of distilled water for 5 min in a glass container. The decoctions were cooled, filtered and separately processed for total maytansinoids as illustrated in Fig. 1.

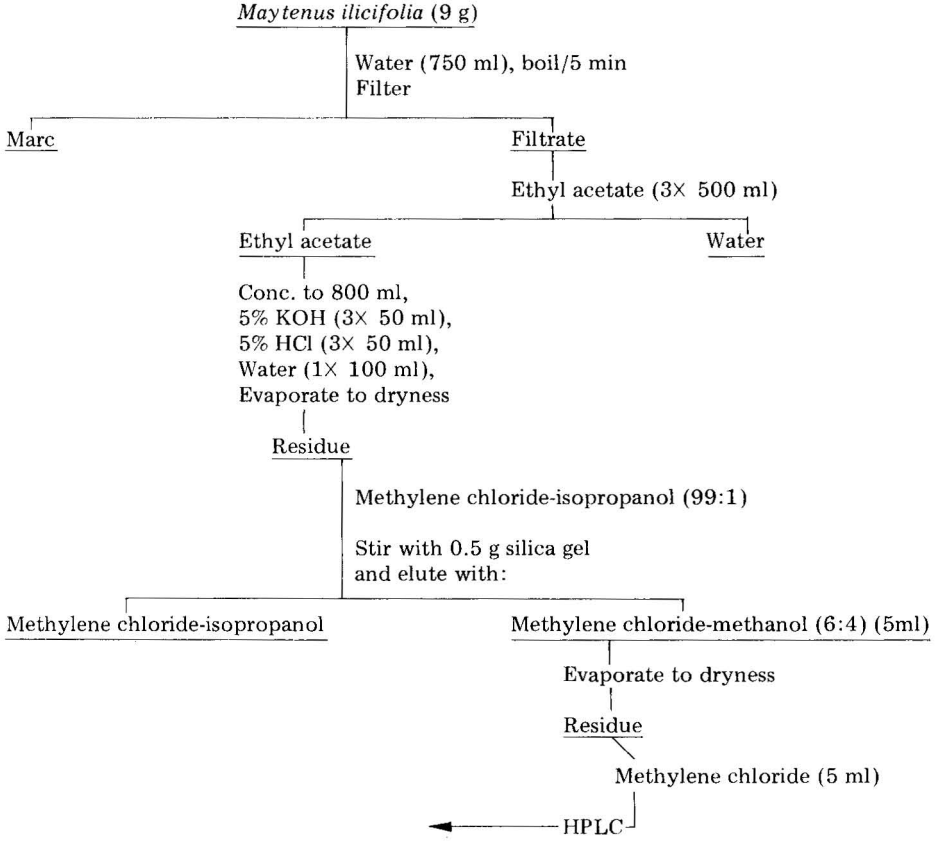
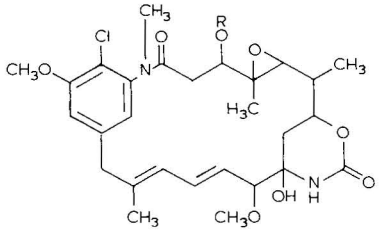


Fig. 1. Extration and purification of *Maytenus ilicifolia*.

RESULTS AND DISCUSSION

The variety and complexity of constituents present in the initial extracts of *M. ilicifolia* leaf + twig and stem + root precluded HPLC detection and quantitation of maytansinoids. Therefore, a purification procedure was employed prior to liquid chromatography. Separation of maytansine (I), maytanprine (II), and maytanbutine (III) was then achieved with methylene chloride–isopropanol–water (96:4:0.5) at a flow-rate of 1.0 ml/min and UV detection at 254 nm. The solvent system is that reported by Nettleton *et al.*[14]. Beer's law curves for I, II, and III showed a linear detection response for concentrations of 12.5 ng to 1 μg. The slopes, *y* axis (peak height) intercepts, and correlation coefficients were calculated by linear regression analyses and are shown in Table I, along with other maytansinoid HPLC characteristics.



Maytansine (I) R=$COCHCH_3NCH_3COCH_3$
Maytanprine (II) R=$COCHCH_3NCH_3CHCH_2CH_3$
Maytanbutine (III) R=$COCHCH_3NCH_3COCH(CH_3)2$

TABLE I

HPLC CHARACTERISTICS OF MAYTANSINOIDS

Compound	*Retention time (min)*	*Slope*	*y axis intercept*	*Correlation coefficient*	*Minimum detectable concentration (ng)**
Maytansine	24.8	0.14	−1.89	0.998	5
Maytanprine	15.6	0.12	+0.91	0.999	5
Maytanbutine	12.0	0.21	−1.09	0.999	5

* UV detector, 254 nm; 0.01 a.u.f.s.

Extracts of leaf + twig, as well as of stem + root, showed satisfactory resolution of I, II and III from other plant constituents. Their presence in both extracts was confirmed by spiking with reference standards. Representative separations of standards, extracts and spiked extracts are shown in Fig. 2. Quantitation of the maytansinoid content of the three leaf + twig samples showed an average total maytansinoid concentration of 5.331 μg (2.162 μg maytansine + 2.377 μg maytanprine + 0.792 μg maytanbutine) per dose of 9.0 g plant material. The average maytansinoid content in the stem + root decoctions was found to be 6.981 μg (2.360 μg maytansine + 2.343 μg maytanprine + 2.278 μg maytanbutine) per dose. In view

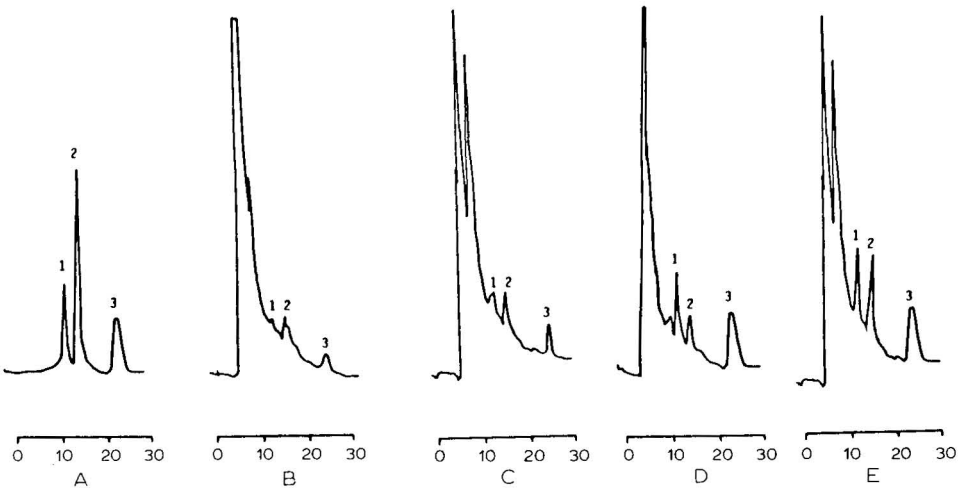


Fig. 2. Chromatograms of (A) mixture of maytansinoids, (B) *Maytenus ilicifolia* leaf + twig extract, (C) *Maytenus ilicifolia* stem + root extract, (D) *Maytenus ilicifolia* leaf + twig extract spiked with maytansinoids, (E) *Maytenus ilicifolia* stem + root extract spiked with maytansinoids. Peaks: 1 = maytanbutine; 2 = maytanprine; 3 = maytansine. Conditions: column, μPorasil (30 × 0.2 cm); solvent, methylene chloride–isopropanol–water (96:4:0.5); flow-rate, 1 ml/min; detector, UV at 254 nm; chart speed, 4 in./60 min.

of the fact that no adverse effects were seen in normal mice given dailly oral doses of 0.5 to 128 μg/kg/9 days in a National Cancer Institute study[16], the low concentration of maytansinoids in the daily dose of Cangorosa tea would probably not cause any adverse effect in the Paraguayan women taking it as a fertility regulator.

Due to the low concentrations of maytansine (0.000001 to 0.00025%)[15] in *Maytenus* species, quantitative analysis of ansamacrolides in less than 1 kg of plant material has not been attempted prior to this study. Thus, the present detection and quantitation of maytansinoids from 9.0-g samples represents the first effort in the quantitative analysis of ansamacrolides from very small samples of plant material.

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Note

Detection and quantification of guayulins A and B in *Parthenium argentatum* (guayule) and F_1 hybrids by high-performance liquid chromatography

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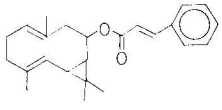
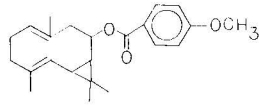
Parthenium argentatum Gray (*Asteraceae*) (common name: guayule) is a small shrub endemic to the Chihuahuan desert of northern Mexico and the south-western parts of Texas, U.S.A., and New Mexico, U.S.A.[1]. Its high rubber content (up to 20% of dry weight) makes it an important agricultural source to partially replace synthetic rubber[2]. Phytochemical studies of *P. argentatum* established the presence of two sesquiterpene phenolic acid esters, germacrene cinnamic acid ester (guayulin A) and germacrene *p*-methoxybenzoic acid ester (guayulin B)[3].



guayulin A



guayulin B

Rodriguez *et al.*[4] recently established that guayulin A is a potent elicitor of contact dermatitis, causing erythremas in sensitized animals at 1.4 nmole. As the cultivation of different strains of *P. argentatum* and crossings with other species of *Parthenium* to increase rubber production is under way, attention should be drawn to the content of the dermatitis elicitors and their inheritance after crossing experiments. High-performance liquid chromatography (HPLC) offers a quick and sensitive method to detect and measure pmole amounts of guayulins A and B in crude plant extracts.

EXPERIMENTAL

Several individual plants of *P. argentatum* and its F_1 hybrids obtained by crossings with *P. tomentosum* var. *stramonium* and *P. fruticosum* var. *trilobatum* were collected during January–March 1981 at the Los Angeles County Arboretum, Arcadia, CA, U.S.A. The plants were separated into leaves and stems, dried, coarsely ground and extracted overnight with distilled acetone. After evaporation, a gummy extract was obtained which was redissolved with acetone for HPLC analysis. Resin

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pouring out of freshly cut stems of *P. argentatum* was collected in vials and dissolved with acetone for HPLC analysis.

A Waters liquid chromatograph was used, equipped with a solvent pump Model 6000A, a universal injector Model U6K and an UV detector Model 440. Detection was achieved at 254 nm. The solvent system was acetonitrile–water (75:25) with a flow-rate of 2.5 ml/min. The HPLC column was Lichrosorb RP-18 (250 × 4.6 mm), pore size 10 μm (Alltech, Los Altos, CA, U.S.A.). Retention times were obtained by measuring the chart (chart speed 0.5 cm/min). Integration of the peak areas was done by height times width at half heights. The quantitative analysis of guayulins A and B in the plant extracts was carried out by using external standards to determine the UV response curves and calculate the linear regression grades. For this purpose guayulins A and B were isolated from *P. argentatum* by means of a SiO_2 column and thin-layer chromatography, solvent system benzene; the guayulins were identified by their ^1H nuclear magnetic resonance spectra[3]. For calculating the capacity factor, k′, and the relative retention, α, the equations $k' = (t_R - t_0)/t_0$ and $\alpha = k'_2/k'_1$ were used[5] (t_R = retention time of compound, t_0 = retention time of solvent, k'_2 = capacity factor of compound 2, k'_1 = capacity factor of compound 1).

RESULTS AND DISCUSSION

Leaf and stem tissues of *P. argentatum* and of F_1 hybrids obtained by crossing with other species of *Parthenium* as well as resin samples were screened for their contents of guayulins A and B by HPLC. The guayulins were resolved with baseline separation in the crude plant extracts within ten minutes by using a reversed-phase C_{18} column and applying an isocratic mixture of acetonitrile–water. The retention times and chromatographic parameters k' and α are given in Table I. The detection limit was in the pmole range, allowing reliable quantification of less than gram samples plant material. Quantification was achieved by using the linear regression grades. The UV responses were tested to the nmole range and shown to be linear, with guayulin B being the better chromophor at 254 nm.

TABLE I

RETENTION TIMES (t_R), CAPACITY FACTORS (k') AND RELATIVE RETENTIONS (α) OF GUAYULINS A AND B AND THEIR CONTENTS IN LEAVES, STEMS AND RESIN OF *PARTHENIUM ARGENTATUM*

The amounts given for guayulins A and B are means of several individual plants.

Guayulin-	t_R *(min:sec)*	k'	α	*Content (μmole/g)*		
				Leaves	*Stems*	*Resin*
B	8:00	4.33	1.20	5	4.4	60
A	9:20	5.22		5.4	13.7	290

The quantitative analysis of the guayulins from leaves, stems and resin samples of *P. argentatum* are given in Table I (for corresponding chromatograms see Fig. 1). The leaf and stem extracts show distinct different patterns. Whereas the amounts of

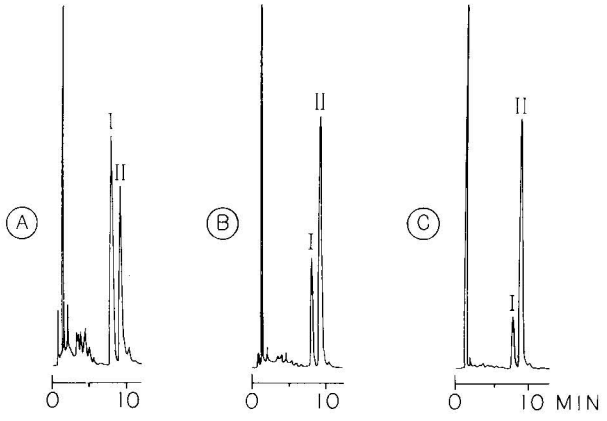

Fig. 1. HPLC resolution of guayulins A (II) and B (I) in (a) leaves; (b) stems; and (c) resin of *Parthenium argentatum* at 0.5 a.u.f.s. For chromatographic conditions see Experimental section.

guayulins A and B are about equal in the leaf tissues (5.4 and 5 μmole/gram dry weight, respectively), guayulin A exhibits more than a two-fold increase (13.7 μmole/gram dry weight) in the stems. Guayulin B is present at slightly lower concentrations (4.4 μmole/gram dry weight) compared to the leaves. The dominance of guayulin A over B is even more significant in the resin that drips out of broken or wounded stems. Ten percent of the resin consists of guayulin A (290 μmole/gram resin) which is five times more than the amount of guayulin B (60 μmole/gram resin).

Analysis of the first filial generations of guayule, obtained by crossing guayule with other species of *Parthenium*, also established the presence of the guayulins. Two hybrid populations of *P. argentatum* × *P. tomentosum* var. *stramonium* and *P. argentatum* × *P. fruticosum* var. *trilobatum* were screened for their contents of guayulins. In all hybrids analyzed the average amounts of guayulins A and B were approximately one fifth to one tenth of the concentrations generally present in *P. argentatum*. The inheritance of the guayulins is in agreement with recent studies on the inheritance of epicuticular alkanes which demonstrated the dominating influence of *P. argentatum* in the F_1 hybrids[6].

Since allergenic reactions of sensitized animals to guayulin A require miniscule amounts of the allergens[4], one can imagine that repeated contact with the resin and its high amounts of guayulins whilst harvesting and processing the plants for rubber extraction could very likely become a serious health hazard. The physiological role of these chemicals within the plant is yet to be determined.

ACKNOWLEDGEMENTS

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Note

Rapid, quantitative separation of chlorophylls and their degradation products by high-performance liquid chromatography

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The quantitative determination of chlorophylls and their degradation products is one of the most frequently performed analyses in aquatic ecology. The most convenient and widely used method is based on the difference in fluorescence yields between chlorophyll *a* and its phaeopigment derivatives (phaeophytin *a* and phaeophorbide *a*)[1]. It is subject to error if significant concentrations of chlorophyll *b* are present[2], but it is not possible to determine whether chlorophyll *b* is present using the fluorometeric technique alone. Chlorophylls can also be determined by absorbance spectroscopy[3], but the method is not sufficiently sensitive and is subject to error if phaeopigments are present. The quantitative separation of all chlorophylls and phaeopigments has been achieved by thin-layer chromatography (TLC)[4]. The technique is time consuming, impractical on shipboard, and may produce artifacts[5]. Finally, a number of high-performance liquid chromatographic (HPLC) procedures have been described for the quantitative separation of chlorophylls and/or their degradation products[6–9]. None of the HPLC methods thus far described combines rapid separation with methodological simplicity, sensitivity, and precision. The major objective of this report is to describe such an HPLC system for the determination of the major chlorophylls and their degradation products, including: chlorophylls *a*, *b*, and *c*, chlorophyllide *a*, phaeophorbide *a*, and phaeophytins *a* and *b*.

EXPERIMENTAL

Reagents

All solvents used for pigment extraction were reagent grade, and all chromatographic solvents were HPLC grade.

Pigments extraction

Pigments were obtained from cultures of the marine diatom, *Skeletonema costatum* (chlorophylls *a* and *c*, and chlorophyllide *a*) and the marine chlorophyte *Dunaliella tertiolecta* (chlorophylls *a* and *b*). Algae were grown in an enriched natural seawater media as previously described[10] and harvested by continuous-flow centrifugation. Pigments were extracted with methanol–water (90:10) at 0 to 4°C by grinding frozen cells (1 to 3 g fresh weight) in a glass tissue grinder with a PTFE pestle. A glass fiber filter (Gelman AE) was added during grinding to promote cell breakage. The

brie was filtered through a glass fiber filter and the pigment extracts were chromatographed on icing sugar columns[11] or by TLC[4], eluted, and stored in 100% methanol in the dark at −20°C. Immediately prior to use the standards were purified by TLC and brought to desired concentrations in methanol–water (80:20). The pigment standards were calibrated with an Aminco DW-2a spectrophotometer, using accepted absorbtivity coefficients[4]. Chlorophyllide *a* was obtained from *S. costatum* by extraction in acetone–water (90:10) at room temperature; conditions which are favorable for the enzymatic hydrolysis of phytol.

For extraction of field samples, 100 to 200 ml of sea water (0.1 to 0.5 μg chlorophyll *a*) were filtered on 25 mm glass-fiber filters. The filters were ground in a tissue grinder with 2 ml of absolute methanol and brie filtered through a glass-fiber filter into a graduated centrifuge tube. The ground glass filters were reextracted on the filter manifold with an additional 1 ml of methanol. A 25-μl volume of pooled pigment extracts was applied to the HPLC column for analysis.

Liquid chromatography

A two-step solvent program was used to resolve chlorophyll *c* from chlorophyllide *a* and phaeophorbide *a* and at the same time allow phaeophytin *a* to elute in a were delivered with an Eldex A-30S pump at 1.5 ml/min. Samples (5–25 μl) were applied to the column with a Valco CV-6-UH Pa-N60 sample injection valve fitted with a 50-μl loop. The detection system consisted of a Farrand Optical Co. A-4 fluorometer equipped with a 10-μl quartz flow cell. Two Corning 5543 filters ($\lambda_{max.}$ 420 nm) were used with an 85 W mercury source to provide excitation energy, and single Corning 2030 was placed on the emission side. The analogue output was recorded directly and also digitized with a Hewlett-Packard (HP) 3437A high-speed digital volt meter. The digital output was accessed and processed with an HP-85 desktop computer.

A two-step solvent program was used to resolve chlorophyll *c* from chlorophyllide *a* and phaeophoribde *a* and at the same time allow phaeophytin *a* to elute in a reasonable time (*ca.* 18 min). Methanol–water (90:10) was applied until phaeophorbide *a* was eluted, followed by methanol–water (98:2). Solvent delivery was automatically programmed with a single-step solenoid valve.

RESULTS AND DISCUSSION

The major chlorophylls and their degradation products were separated in 18 min (Fig. 1). Using the Farrand A-4 fluorometer, less than 0.1 pmole of chlorophyll *a* can be detected (Fig. 2). Fluorescence output is linear with pigment concentration over three orders of magnitude and the coefficient of variation for the estimation of chlorophyll *a* was ±1.3% ($n = 13$, $r^2 = 0.998$).

The HPLC system described provides a rapid and sensitive method for the determination of chlorophylls and their degradation products. It should be considered as a supplement to the simple fluorometric method. For general water analysis, the simple fluorometric method is rapid and precise. In some applications, however, noteable artifacts prevent its sole use. For example, in the analysis of sediment samples, those sediments rich in humic and other organic acids may have a large background fluorescence which is not acid labile. Using the simple fluorometric method alone phaeopigment concentrations would be grossly overestimated. The physical separation of phaeopigements by HPLC prevents such artifacts from occurring.

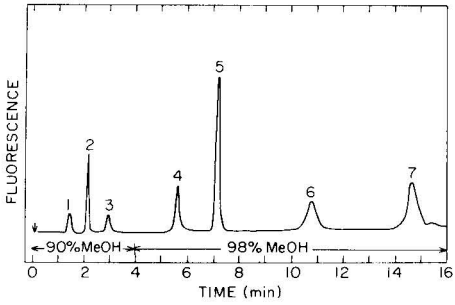

Fig. 1. Elution profile of chlorophylls and degradation products prepared from algal extracts. A 15-cm RP-8 column was used with 1.5 ml/min solvent flow-rate. Peaks: 1 = chlorophyll *c*; 2 = chlorophyllide *a*; 3 = phaeophorbide *a*; 4 = chlorophyll *b*; 5 = chlorophyll *a*; 6 = phaeophytin *b*; 7 = phaeophytin *a*.

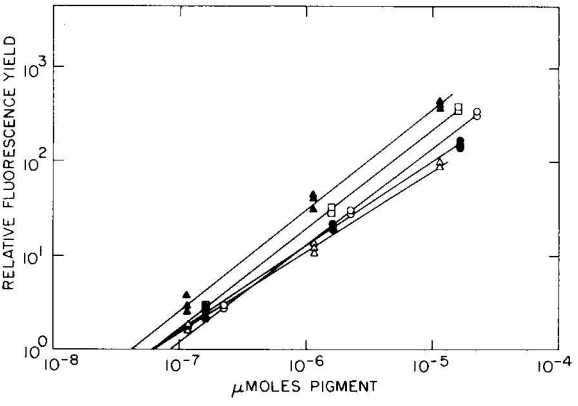

Fig. 2. Calibration curves for five plant pigments using the HPLC system described. ▲ = Chlorophyll *a*; △ = phaeophytin *a*; ● = chlorophyll *b*; ○ = phaeophytin *b*; □ = chlorophyll *c*.

ACKNOWLEDGEMENTS

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CHROM. 13,909

Note

Affinity chromatography of chymotrypsin (E.C. 3.4.21.1) on the potato trypsin inhibitor bound to bead cellulose by the benzoquinone method

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Different affinants were used for the affinity chromatography of chymotrypsin, *e.g.*, 4-phenylbutylamine[1], derivatives of D-tyrosine or D-tryptophan[2–4] and natural protease inhibitors[5,6]. Natural protease inhibitors are little influenced, if at all, by the support matrices and therefore are preferred in some instances.

The affinant is usually bound to the support by the cyanogen bromide method, and the supports most frequently used are agarose gels or modified dextran gels (Sephadex). Cellulose has been used as a support in some instances.

Previously bead cellulose has not been used as a support for affinity chromatography of proteases and the benzoquinone method has not been used for binding of the affinant. The use of both bead cellulose and the benzoquinone method is to be prefered for the industrial separation processes. The benzoquinone method avoids the use of highly toxic reagents such as cyanogen bromide. The good physical properties of bead cellulose[7,8] made high flow-rates in the column possible, thus enhancing the column efficiency.

In this work, the affinity sorbent was prepared by binding the trypsin inhibitor from potatoes to the bead cellulose by the benzoquinone method and its properties were studied.

EXPERIMENTAL

The protein concentration was determined according to the method of Hartree[9]. The protein concentration in the column effluent was measured by a UV monitor (Developmental Workshops of Czechoslovak Academy of Sciences, Prague, Czechoslovakia) at 280 nm. The proteolytic activity was measured by the method of Slavík and Smetana[10] and was expressed in casein units (C.U.); 1 C.U. is the amount of the enzyme that liberates 1 mg of peptides soluble in 0.1 *M* trichloroacetic acid from a 2% solution of casein after 60 min at 30°C and the optimal pH for the protease.

The bead cellulose was activated with the use of benzoquinone by the method described by Brandt *et al.*[11], except that ethanol was used instead of dimethylformamide as a solvent. The bead cellulose (particle diameter range 0.3–0.5 mm, mean 0.42 mm) was obtained from Spolek pro chem. a hutní výrobu, Ústí n.L., Czechoslovakia. The water regain of this support was 7.06 g/g, the coefficient *K* (see ref. 12; from

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the equation $u = K \Delta p/L$, where u = linear flow-rate, Δp = pressure drop and L = bed height) was 10.6 and the sedimentation velocity was 0.28 cm/sec.

Chymotrypsin was obtained from Spofa (Prague, Czechoslovakia), with a specific activity of 4.61 C.U. per milligram of protein and the protein content of 794 mg per gram of product. Trypsin inhibitor from potatoes was prepared as described by Ryan and Kassell[13]. Benzoquinone was prepared as described by Vliet[14].

RESULTS AND DISCUSSION

Trypsin inhibitor prepared from potatoes was bound to the bead cellulose in an amount of 200 mg per gram of the support. The yield of bound proteins was 56.2% (112.4 mg of protein was bound to each gram of support). The static capacity of the sorbent for the chymotrypsin (n_s)[15] was 106 mg per gram of sorbent and the operational capacity (n_0)[15] was 98.5 mg/g. The amount of total bound affinant could not be measured as the affinant was a crude product.

Affinity chromatography was performed in a 36 × 0.9 cm I.D. column a total amount of 1588 mg of protein being used for one run. The starting buffer was 0.1 *M* Tris–glycine (pH 8.01) and the non-proteolytic protein was eluted with this buffer. After the UV absorption had fallen to the starting value, the buffer was changed to

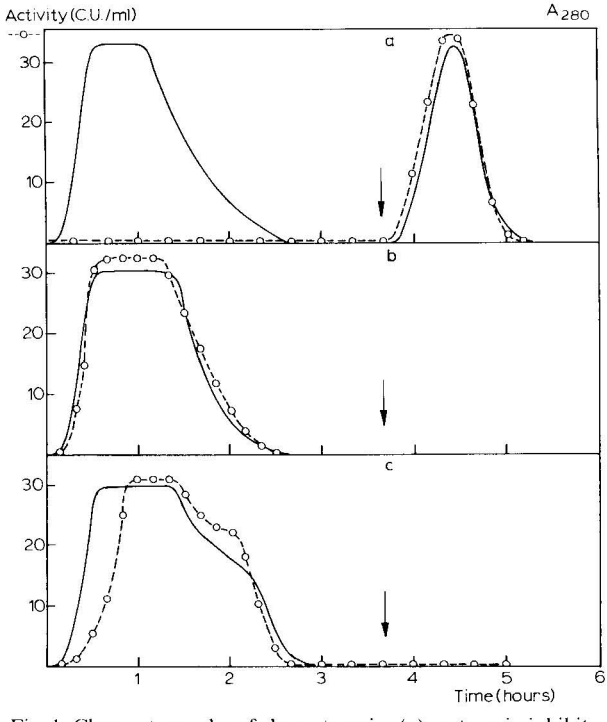

Fig. 1. Chromatography of chymotrypsin: (a) on trypsin inhibitor covalently bound to cellulose beads; (b) on unsubstituted cellulose beads; (c) on cellulose beads activated with benzoquinone in which the reactive groups are blocked with ethanolamine. Arrows indicate the change of starting buffer (0.1 *M* Tris–glycine, pH 8.01) for the elution buffer (0.1 *M* glycine–HCl, pH 2.07). The absorbance (solid lines) at 280 nm is given in arbitrary units, and the proteolytic activity (broken lines) in casein units per millilitre of effluent.

0.1 *M* glycine–hydrochloric acid (pH 2.07) and the chymotrypsin was eluted in a single peak containing 96.2% of the total proteolytic activity used. The specific activity of purified chymotrypsin was 22.4 C.U./mg, which is 4.86 times higher than the original value.

The results are shown in Fig. 1a. The same experimental conditions were used with the unsubstituted bead cellulose (Fig. 1b) and with the activated bead cellulose in which the reactive groups were blocked with ethanolamine (Fig. 1c). From the elution pattern it can be seen that the unsubstituted cellulose is ineffective in the chymotrypsin separation. All of the proteins added were eluted in a single peak. When using activated blocked bead cellulose, the chymotrypsin was partially separated from other proteins during the elution with the starting buffer, but the specific activity of this separated fraction was only 12% higher than the original value.

CONCLUSION

The results demonstrate the usefulness of the benzoquinone method with bead cellulose as a support in affinity chromatography. This method avoids the use of cyanogen bromide and will allow the industrial development of affinity sorption. The use of this method of affinant binding in connection with bead cellulose may be even more advantageous, taking into account the excellent physical properties of the support material.

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Letter to the Editor

Doubly peaked chromatograms

Sir,

Golay and Atwood[1], in their article "Early phases of the dispersion of a sample injected in Poiseuille flow" found doubly peaked chromatograms when the time of flow from the injection point to the observation point was short. Gill and Ananthakrishanan[2] analyzed this same problem using a finite difference method to solve the convective-diffusion equation and they also found that the concentration downstream displayed double peaks under similar conditions.

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1 M. J. E. Golay and J. G. Atwood, *J. Chromatogr.*, 186 (1979) 353.
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Book Review

Analytical methods for pesticides and plant growth regulators, Vol. XI, Updated general techniques and additional pesticides, edited by G. Zweig and J. Sherma, Academic Press, New York, 1980, XIV + 408 pp., price US$ 46.00, ISBN 0-12-784311-6

Volume XI of this seventeen year-old series contains 20 chapters divided into four sections. Part I, General, covers an update on the automated pesticide analytical laboratory, and liquid chromatography and thin-layer chromatography in pesticide analysis. Parts II, III and IV contain analytical methods for formulations and residues on newly developed pesticides in the areas of insecticides, acaricides, fungicides, herbicides, and plant growth regulators. The fungicide section includes an updated chapter on the ethylenebisdithiocarbamates and their degradation products (see Vol. III of this series); important because of the potential toxicity (carcinogen, teratogen) of one of the degradation products ethylenethiourea. There is also a chapter on the analysis of the N-nitroso compounds; important because of N-nitroso impurities that may be present in technical grade herbicide products and must be analyzed by "specific methods to satisfy U.S.A. governmental requirements."

As in some of the previous volumes, the analytical procedures are so detailed and complete that reference to supplemental publications is unnecessary. However, each chapter contains an excellent documented References section for the reader's convenience.

The volume is recommended as a valuable addition to the pesticide chemist's library.

Honolulu, HI (U.S.A.) ARTHUR BEVENUE

Vol. 213, No. 2

journal of chromatography news section

NEW BOOKS

Introduction to high performance liquid chromatography, by R.J. Hamilton and P.A. Sewell, Chapman & Hall, London, **2nd ed.**, 1981, *ca.* 200 pp., price *ca.* £ 12.50, ISBN 0-142-13400-4.

Practice and application of reversed phase thin layer chromatography on Whatman KC_{18} plates, by J. Sherma, Whatman, Clifton, NJ, 1981.

Electrophoresis – Theory, techniques, and biochemical and clinical applications, by A.T. Andrews, Clarendon Press, Oxford, 1981, 400 pp., price £ 28.00, ISBN 0-19-854626-2.

MEETING

WATER'S WESTERN HPLC SYMPOSIUM

Waters Associates, Inc., are sponsoring a symposium on high-performance liquid chromatography to be held at the Marriot Hotel & Tennis Club in Newport Beach, CA, U.S.A., on Oct. 15–16, 1981. More than two-hundred people are expected to attend lectures regarding life science, energy, environmental, polymer, aerospace, organic synthesis, food technology and preparative advancements in high performance liquid chromatography.

Among those presently scheduled to appear are the following: Guest speaker Mr. David Crabtree, Northrup Corporation, presenting a paper titled "LC Analysis of Epoxy Resins for the Aerospace Industry." Dr. Jean Rivier of the Salk Institute will present a paper on advancements in liquid chromatography applications of proteins and peptides. "Collection and Analysis of Benzidine Dye by HPLC" will be presented by Mr. Dave Armitage, OSHA, of Salt Lake City, UT, U.S.A.

For registration details and more information please contact Amy Shangraw, Waters Associates, 34 Maple Street, Milford, MA 01757, U.S.A. Tel. (617) 478-2000.

CALENDAR OF FORTHCOMING MEETINGS

Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC 81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 20 20 20. (Further details published in Vol. 212, No. 2)
Oct. 15–16, 1981 Newport Beach, CA, U.S.A.	**Water's Western HPLC Symposium** Contact: Amy Shangraw, Water's Associates, 34 Maple Street, Milford, MA 01757, U.S.A. Tel. (617) 478-2000.

Oct. 22–23, 1981 Montreux, Switzerland	**Workshop on Liquid Chromatography – Mass Spectrometry** Contact: Prof. Dr. R.W. Frei, Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands (Further details published in Vol. 207, No. 3).
Oct. 27–29, 1981 London, Great Britain	**Petroanalysis 81** Contact: Miss I.A. McCann, Conference Officer, Institute of Petroleum, 61 New Cavendish Street, London W1M 8AR, Great Britain. (Tel: 01–636 1004, Telex: 264380)
Oct. 28–30, 1981 Gatlinburg, TN, U.S.A.	**Resource Recovery and Environmental Issues of Industrial Solid Wastes** Contact: J.S. Watson, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A.
Nov. 9–10, 1981 Berlin, G.F.R.	**Symposium on Practical Aspects of HPLC** Contact: Dr. I. Molnár, Wissenschaftliche Geratebau Dr. H. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37, G.F.R. (Further details published in Vol. 207, No. 2).
Nov. 16–17, 1981 Washington, DC, U.S.A.	**International Symposium on HPLC of Proteins and Peptides** Contact: Ms. S.E. Schlessinger, Symposium Manager, 400 East Randolph, Chicago, IL 60601, U.S.A. (Further details published in Vol. 208, No. 2)
Nov. 23–25, 1981 Barcelona, Spain	**2nd International Congress on Analytical Techniques in Environmental Chemistry** Contact: Dr. Joan Albaigés, General Secretary, Plaza de Espana, Barcelona-4, Spain. Tel. 223 31 01.
Dec. 2–3, 1981 Paris, France	**Journées de Chromatographie en Phase Liquide** Contact: H. Colin, Laboratoire C.A.P., Ecole Polytechnique, Route de Saclay, 91128 Palaiseau Cedex, France.
Jan. 4–9, 1982 Orlando, FL, U.S.A.	**1982 Winter Conference on Plasma Spectrochemistry** Contact: 1982 Winter Conference, c/o ICP Information Newsletter, Chemistry -GRC Towers, University of Massachusetts, Amherst, MA 01003, U.S.A. Tel. (413) 545–2294.
Jan. 19–20, 1982 Amsterdam, The Netherlands	**Symposium on "Detection in High-Performance Liquid Chromatography"** Contact: Mrs. Peschier, Hewlett-Packard Nederland B.V., Analytical Department, van Heuven Goedhartlaan 121, 1181 KK Amstelveen, The Netherlands. (Tel.: 020-47 20 21). (Further details published in Vol. 212, No. 2)
March 8–12, 1982 Atlantic City, NJ, U.S.A.	**1982 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Linda Briggs, Program Secretary, Pittsburgh Conference, Department J-057, 437 Donald Road, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 212, No. 2)
March 28–April 2, 1982 Las Vegas, NV, U.S.A.	**National American Chemical Society Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 5–8, 1982 Las Vegas, NV, U.S.A.	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. (Further details published in Vol. 212, No. 3)
April 14–16, 1982 Amsterdam, The Netherlands	**12th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)

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A NEW JOURNAL

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

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Ionic Hydration in Chemistry and Biophysics

by B.E. CONWAY,
Department of Chemistry, University of Ottawa, Ontario, Canada.

STUDIES IN PHYSICAL AND THEORETICAL CHEMISTRY 12

1981 xxx + 774 pages
Price: US $131.75 / Dfl. 270.00
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This monograph is a comprehensive coverage of all aspects of ionic hydration. Not only is the nature of the phenomenon described, but also its importance in electrochemistry, inorganic and physical chemistry, biophysics and biochemistry. The basic background of techniques and approaches (e.g. electrostatic theory) required for the study and interpretation of ionic hydration in chemistry is included.

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