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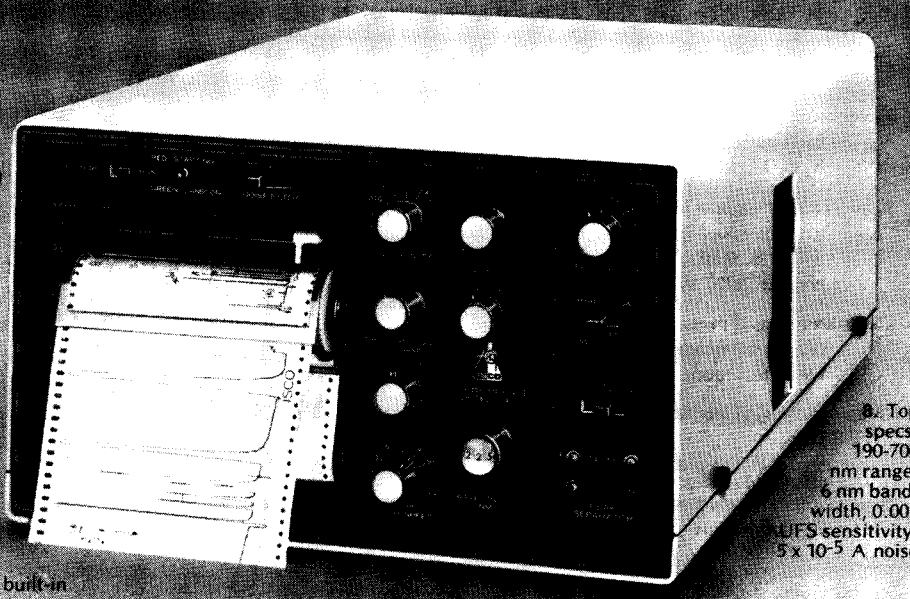

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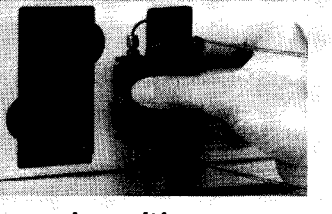

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PRACTICAL OPTIMIZATION OF SOLVENT SELECTIVITY IN LIQUID–SOLID CHROMATOGRAPHY USING A MIXTURE–DESIGN STATISTICAL TECHNIQUE

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(First received November 17th, 1981; revised manuscript received December 16th, 1981)

SUMMARY

A systematic approach is described for the optimization of solvent selectivity in liquid–solid chromatography (LSC), with emphasis on changes in selectivity as a result of variation of mobile phase composition. Major contributions to selectivity are provided by solvent–solute localization and solvent-specific localization. Exploitation of these effects is achieved by the use of a mixture-design statistical technique to minimize the number of experiments to find an optimum solvent mixture for LSC separation. Quaternary-solvent mobile phases are required for difficult separations to invoke the full range of selectivity effects possible for LSC separation. The four preferred solvents for LSC optimization based on localization effects are hexane, methylene chloride, methyl *tert.*-butyl ether and acetonitrile. In the optimization process retention data are required for only seven mobile-phase systems, and an overlapping resolution mapping (ORM) technique of data analysis is used to establish the optimum solvent mixture for the highest resolution of all adjacent bands in the chromatogram.

INTRODUCTION

As the application of liquid chromatography (LC) has become more widespread, increasing interest has developed in practical procedures for optimizing separations. Such separations are increasingly used in areas such as quality control, process control and clinical analysis, where large numbers of samples have to be analysed each day. Such applications place special emphasis on the complete separation of samples of interest in a minimum time per sample. Adequate separation can be measured in terms of the usual resolution function R_s[1], where R_s can be related to other separation variables by

$$R_s = 1/4\,(\alpha - 1)\,(N^{1/2})\left(\frac{k'}{1 + k'}\right) \quad (1)$$

where k' is the average value of the capacity factors k_1 and k_2 of two adjacent bands 1 and 2, α is the separation factor (k_2/k_1), and N is the column plate number. To a first approximation, these three terms of eqn. 1 are independent of each other and can be separately optimized.

Several workers[1–4] have discussed the optimization of plate number N in LC. In previous papers[5,6] we discussed the prediction of solvent strength in liquid–solid chromatographic (LSC) separation, which in turn determines values of k'; optimum values of k' generally lie in the range $1 < k' < 10$. Finally, values of α in LSC can be systematically varied by changing the composition of the mobile phase[7].

It has been demonstrated for reversed-phase LC separations[8] that large changes in values of α are possible as a result of change in the mobile phase organic modifier. For example, a change from methanol–water to acetonitrile–water or tetrahydrofuran–water can greatly influence the α values or the selectivities among solutes. Often, however, various binary solvent mixtures are incapable of separating all compounds in a given mixture. In such cases, it has been found[9–12] that the use of ternary-solvent mobile phases is generally advantageous. However, for these more complex mobile phases, the systematic optimization of values for every pair of adjacent bands in the chromatogram becomes more complicated.

Recently, we have introduced a mixture-design statistical approach for the efficient optimization of selectivity in reversed-phase LC[13]. The potential of this scheme for retention optimization using quaternary-solvent mobile phases has been demonstrated in the successful separation of complex mixtures of both substituted naphthalenes[13] and phenylthiohydantoin (PTH) amino acids[14] using reversed-phase LC. The latter optimization procedure is based on the solvent-triangle classification of solvents according to their separation selectivity[15,16]. For bonded-phase LC, solvents can be categorized according to their proton donor, proton acceptor or dipole interactions. With the three "extreme" solvents from the corners of the solvent triangle (methanol, acetonitrile, tetrahydrofuran) plus water as carrier, the mixture-design procedure then requires a continuous variation of α values for all peak pairs in the chromatogram. The approaches which have been described for reversed-phase LC should also be applicable to liquid–solid chromatography (LSC). We have recently reported[7] on the theoretical background necessary for the optimization of the mobile phase in LSC. We report here experimental results in support of this theory and describe approaches for the systematic optimization of mobile phases in LSC.

EXPERIMENTAL

The apparatus, materials and procedures used in this study have been described previously. In this work, three 15 × 0.46 cm I.D. columns of Zorbax®-SIL chromatographic packings (Du Pont Analytical Instruments Division, Wilmington, DE, U.S.A.) from the same lot were used. Mobile-phase solvents were 50% water-saturated[1]. Solvents were vacuum degassed individually and then mixed before water saturation. Solvents with 50% water-saturation were obtained by adding equal volumes of water-saturated solvent (obtained using 30% water in silica gel as in ref. 1) and water-free solvent.

RESULTS

The basis of selectivity and the variation of α values with change in mobile phase composition is substantially different in LSC than in other LC methods. In bonded-phase LC methods, interactions between solvent and solute molecules in the mobile phase are of primary importance, and the solvent triangle serves as a useful guide for selecting extreme solvents of very different selectivity. However, for most LSC separations, we have shown[7] that localization effects in the stationary phase are of greater importance. These localization effects, which involve competition between mobile phase and sample molecules for a position directly over adsorption sites on the surface of the stationary phase (*e.g.*, silanol groups in the case of silica), can be subdivided into solvent–solute localization and solvent-specific localization. The degree of solvent localization can be measured by a mobile phase parameter *m*, which increases as the concentration of some polar solvent in the mobile phase increases. We have shown previously[7] that methyl *tert.*-butyl ether (MTBE) and acetonitrile (ACN) are solvents that give large values of *m* for mobile phases which contain these solvents.

Solvent-specific localization appears to involve direct hydrogen bonding of a basic, polar solvent with surface sites on the adsorbent. Thus, basic polar solvents such as MTBE yield additional selectivity effects as opposed to less basic polar solvents such as ACN. Therefore, values of α can be further varied by changing the ratio of concentrations of MTBE to ACN while holding *m* constant.

These considerations have led us to the design of an LSC selectivity triangle (Fig. 1) based on solvent localization for use in the present optimization scheme. This selectivity triangle is analogous to that used previously for optimization in reversed-phase LC[13]. In Fig. 1 it can be seen that the corners of the triangle, corresponding to solvents of extreme selectivity, consist of (1) a non-localizing solvent (methylene

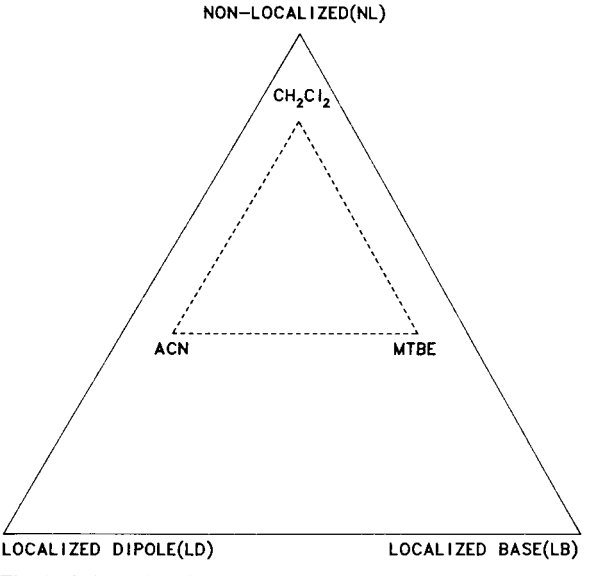

Fig. 1. Solvent localization triangle for major selectivity effects in LSC.

chloride, MC), (2) a basic, localizing solvent (MTBE) and (3) a non-basic localizing solvent (ACN). Adjustment of the ratios of these three solvents in the final mobile phase allows the systematic, continuous variation of α values in LSC over very wide limits. A fourth "inert" solvent such as hexane (HEX) or Freon®-113[17] is the carrier used to adjust the solvent strength of the mobile phase. The four solvents for LSC in Fig. 1 may be compared with the use of methanol, acetonitrile and tetrahydrofuran plus water in reversed-phase LC[13].

Once the extreme selectivity solvents of Fig. 1 have been selected for optimization studies, a strategy for the development of a given separation by LSC can be planned. As with other LC methods, the first step is the selection of a column, flow-rate and temperature for the sample of interest. These variables will be held constant while retention is optimized by a change in mobile phase composition. These initial choices largely determine the plate number N of the column. The optimum solvent strength for the given separation can next be determined by trial-and-error, using the binary-solvent mobile phase MC–hexane and varying the concentration of MC.

After the appropriate concentration of MC in the mobile phase has been determined, the value of ε, the mobile phase strength, can be calculated[5]. This, in turn, defines the various mobile phase compositions in the selectivity triangle of Fig. 1 for that value of ε as calculated by the procedure in ref. 6. Corresponding values of m for this particular selectivity triangle can also be calculated from the various mobile phase compositions represented in the triangle (as in ref. 7). Thus, points within the triangle represent mobile phase compositions that can be described as follows: (1) all compositions have the same value of ε; (2) values of m for these compositions vary linearly from a maximum and equal value for the bottom corners (MTBE and ACN) of the triangle to a minimum value for the top corner (MC); (3) the concentration ratio [MTBE]/([MTBE] + [ACN]) varies linearly along any horizontal line of the

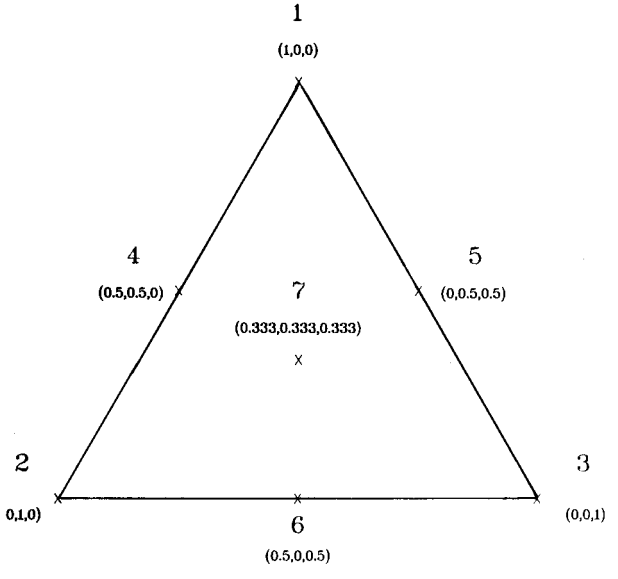

Fig. 2. Simplex design for three solvents (A, B and C) and mixtures. Values for each point are trilinear coordinates of A/B/C.

triangle; (4) all solvent compositions are miscible (note that this particular system requires addition of MC as co-solvent for some mixtures of hexane and ACN; with Freon-113 carrier[17], no MC co-solvent is required). Fig. 2 also classifies the seven standard solvents for the solvent selectivity triangle in terms of values of m and R = [MTBE]/([MTBE] + [ACN]). Here the relative value of m is arbitrarily set equal to 0 for the MC corner and 1 for the MTBE/ACN corners.

The statistical approach used here represents an efficient procedure for identifying the point or points in the selectivity triangle which will optimize the resolution of the sample, *i.e.*, yield the largest value of α and R_s for the most poorly resolved band-pair in the chromatogram. Fig. 2 illustrates the application of the statistical approach in terms of the use of the seven prescribed mobile phases from the selectivity triangle. These seven mobile phases are selected for approximately equal spacing of values of log k' for any solute pair within the sample of interest. For example, solvent 7 in Fig. 2 is a quaternary mixture of the four standard solvents in Fig. 1. The composition of the quaternary mixture is chosen to give approximately equal contributions from the three corner-solvents (Fig. 1) to log k'. Thus, the value of m for this composition is the average of the m for MC, MTBE and ACN or 0.67. The value of [MTBE]/([MTBE]. + [ACN]) is 0.5.

After the compositions of the seven reference mobile phases in Fig. 2 have been calculated according to the above principles, the sample is then separated in each of

TABLE I

k' DATA FOR SEVEN MOBILE PHASES IN FIG. 2 AND OPTIMUM PREDICTED BY PROGRAM

Zorbax-SIL 15 × 0.46 cm I.D. column; 35°C; 2.0 ml/min.

*Molar fractions**	*Mobile phase No.*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8 (Opt.)*
N_A	0.422	0.870	0.958	0.686	0.920	0.768	0.887	0.913
N_B	0.578	0.100	0.000	0.300	0.048	0.220	0.090	0.060
N_C	0.000	0.030	0.000	0.014	0.016	0.000	0.012	0.020
N_D	0.000	0.000	0.042	0.000	0.016	0.012	0.011	0.007
*Solutes***								
2-OCH_3	0.58	0.57	0.59	0.65	0.67	0.54	0.67	0.70
1-NO_2	0.86	1.20	1.62	1.10	1.36	0.90	1.30	1.48
1,2-$(OCH_3)_2$	1.15	0.82	1.00	1.02	0.95	0.91	1.02	1.09
1,5-$(NO_2)_2$	2.37	3.27	3.70	2.98	3.62	2.63	3.63	4.13
1-CHO	2.75	1.69	2.45	2.22	2.11	2.27	2.33	2.20
2-CO_2CH_3	3.29	2.49	2.83	3.00	2.99	2.78	3.25	3.23
1-CO_2CH_3	3.31	2.71	3.07	3.13	3.33	2.85	3.57	3.67
2-CHO	3.97	2.22	3.25	3.19	2.83	3.12	3.17	2.97
1-CH_2CN	4.06	4.73	7.23	4.86	6.09	4.83	6.30	6.55
1-OH	4.44	8.17	6.65	6.77	7.14	6.27	8.00	8.96
1-$COCH_3$	5.17	2.58	3.54	3.71	3.25	3.72	3.72	3.42
2-$COCH_3$	7.33	3.33	4.76	5.14	4.39	5.16	5.10	4.67
2-OH	7.98	11.86	11.35	10.69	11.58	11.57	13.42	14.19

* Molar fractions: N_A = hexane; N_B = methylene chloride; N_C = acetonitrile; N_D = methyl *tert.*-butyl ether.

** Substituted naphthalenes.

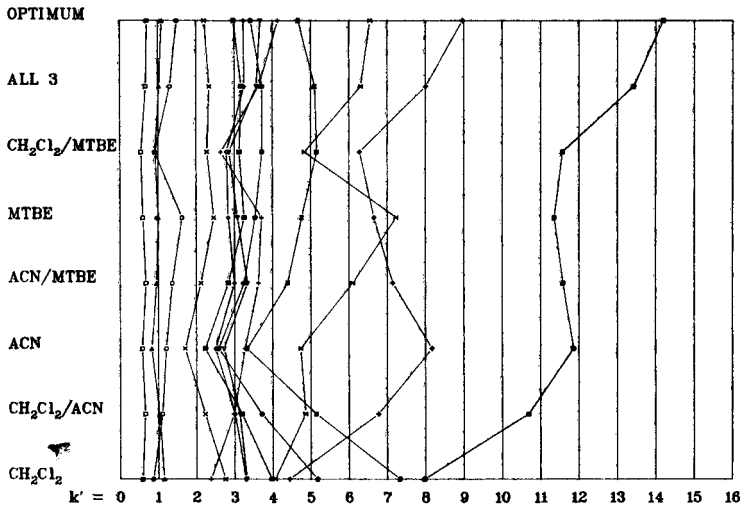

Fig. 3. k' maps for each solute in eight different mobile phases. Each line represents one solute (data in Table I). Mobile phases: optimum, 8; all, 3, 7; CH_2Cl_2–MTBE, 6; MTBE, 3; ACN–MTBE, 5; ACN, 2; CH_2Cl_2–ACN, 3; CH_2Cl_2, 1. Solutes (in order for CH_2Cl_2): □, 2-OCH_3; ○, 1-NO_2; △, 1,2-$(OCH_3)_2$; +, 1,5-$(NO_2)_2$; ×, 1-CHO; ◇, 2-CO_2CH_3; ▽, 1-CO_2CH_3; ■, 2-CHO; ✳, 1-CH_2CN; ◆, 1-OH; ●, 1-$COCH_3$; ⧗, 2-$COCH_3$; ⊠, 2-OH.

these mobile phases and values of k' for each solute in each mobile phase are measured. Where overlapping bands prevent the accurate determination of k' values, individual solutes must be re-run separately.

The above procedure for optimizing the mobile phase solvent was applied to the mixture of thirteen substituted naphthalenes listed in Table I. The optimum value of ε was determined as 0.23 for separating the substituted naphthalene mixture, and the mobile phase compositions for the seven standard solvents of this strength are shown separately in Table I. Two conclusions can immediately be drawn from the data in Table I: (1) none of the seven standard mobile phases alone provides complete separation of the sample components; (2) there are large changes in α values among the various mobile phases. These changes are evident when examining the k' values for the solute bands, which are plotted in Fig. 3.

With the retention data in Table I, sufficient information is available to carry out the data analysis to locate the mobile phase composition within the selectivity triangle of Fig. 2 that provides maximum resolution of sample components. This is accomplished by the resolution mapping procedure illustrated in Fig. 4. The resolution values of peak pairs at the seven points on the triangle (Fig. 1) are calculated from the seven chromatograms for a single peak pair. In this case, peaks 6 and 8 are illustrated. From these seven resolution values, a resolution surface of the solvent triangle can be calculated based upon fitting the data to a second-order polynomial equation[13]. The contour lines in Fig. 4 represent predicted resolutions for this peak pair within the selected solvent triangle. These results are presented in a different manner in Fig. 5. Here, the $R_s = 1.0$ contour is shown, and the shaded area of the solvent triangle indicates mobile phases for which the resolution is less than 1.0. The white area within the solvent triangle represents mobile phases for which a resolution

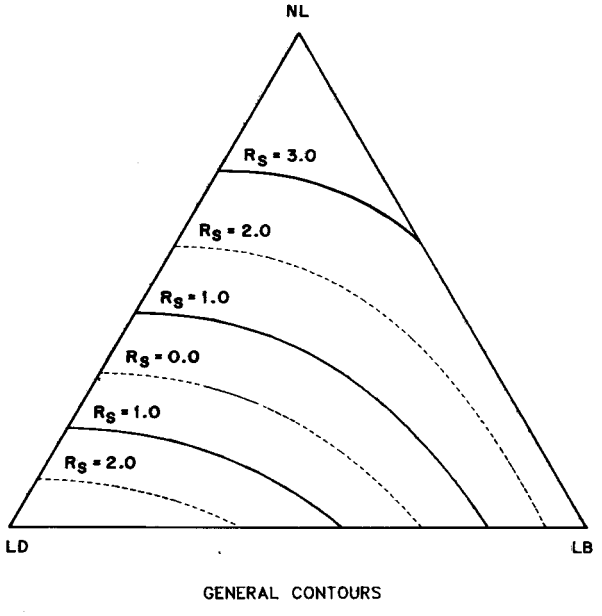

Fig. 4. Resolution map for peaks 6–8. Contours are predicted values of resolution (R_s) between peaks 6–8.

of at least 1.0 is obtained for peaks band 8. Resolution maps are prepared for all peak pairs within the chromatogram, and similar shaded areas in the solvent selectivity triangles are designated as solvents for which the derived resolution is *not* obtained. When these resolution maps for all peak pairs are manually overlayed and shaded

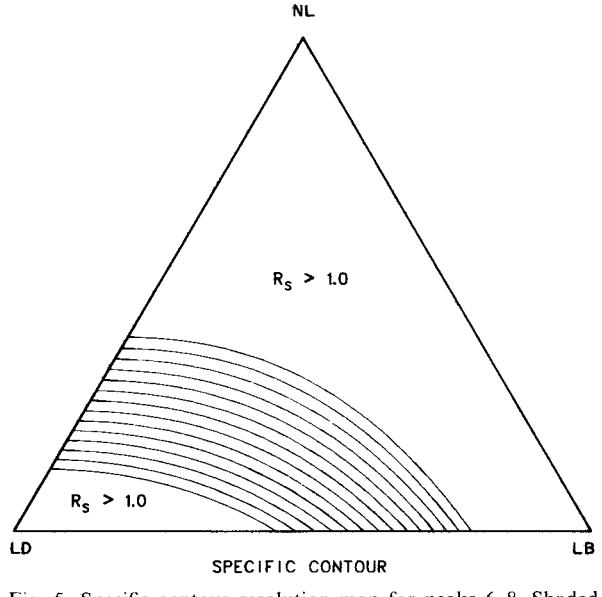

Fig. 5. Specific contour resolution map for peaks 6–8. Shaded area represents mobile phase mixtures where the resolution for peaks 6–8 is < 1.0.

areas intersected, regions which are unshaded (remain white) indicate a mobile phase which resolves *all* peak pairs to at least a resolution of 1.0. For convenience, this overlaying procedure may be carried out with a computer[13] to establish the optimum solvent region.

Two points should be made regarding the resolution of peak pairs by this approach. First, the required resolution for the various peak pairs of interest is arbitrarily established by the operator. Another resolution value would produce similar contour maps, but unshaded resolved areas would be more limited or nonexistent, as in this case, for $R_s = 1.2$. In practice, the computer program used for these calculations determines the maximum R_s value which still predicts some available mobile phase within the solvent triangle that satisfies the resolution requirement for all the peak pairs. This mobile phase is then the optimum for the components in the sample of interest.

The second point of interest is the number of peak pairs which must be considered in the analysis. If there is no change in peak order with changing solvent compositions, it is only necessary to examine adjacent peak pairs for the desired resolution. In most systems of interest, however, some peak crossovers will occur; that is, there will be changes in the solute retention order. This situation appears to complicate the analysis, but in fact can be handled readily by considering *all* pairs (even non-adjacent pairs) for every chromatogram obtained for the seven statistically designed experiments. Although in principle this could be a formidable task, the actual number of possible peak pairs is not overly large and can be predicted as[13]

$$\binom{n}{2} = \frac{n!}{(n-2)!\,2!}$$

where n is the number of peaks. For a thirteen-component system, this corresponds to 78 possible pairs, the data for which can be easily handled by a computer.

In the case illustrated by the data used from Table I, overlapping resolution mapping (ORM) analysis reveals that there is one region of the solvent triangle which predicts a resolution of 1.0 or more for all peak pairs, as shown in Fig. 6. The white area is the acceptable result of the ORM of all of the contour maps (similar to Fig. 5), for all peak pairs in the mixture. Any shaded region of the triangle corresponds to a solvent mixture where at least one peak pair does not have a resolution of at least 1.0. The optimum solvent for this system is indicated by × in Fig. 6, and corresponds to 0.83% ACN, 0.67% MTBE and 3.05% CH_2Cl_2 (by volume) in hexane.

A predicted resolution of 1.0, indicated for the peaks in the chromatograms shown in Fig. 7 for all three columns used in this work, is acceptable for most applications. However, the resolution could be increased, if desired, by increasing the column length by connecting columns in series. The total length of column required to achieve a certain resolution may be calculated from the discussion in ref. 1. In Fig. 7, the observed resolution of 0.9 should be increased to about 1.3 by doubling the column length (constant pressure). However, for this approach to be successful, the columns to be connected must have reproducible values of N[18], and especially values of α and k, to insure reproducible separation. Note that the observed resolution of 0.9 for this experiment is less than the predicted value of 1.0, probably owing to the approximations utilized in the optimization approach plus experimental variation.

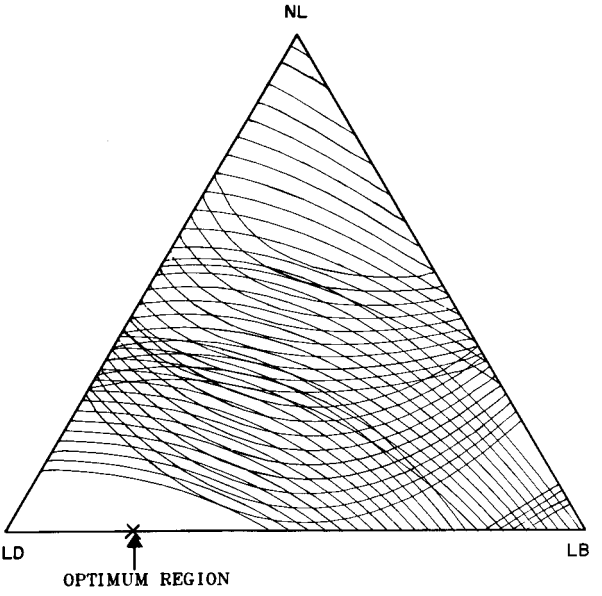

Fig. 6. Overlapping resolution map (ORM) for all peak pairs. The optimum mobile phase region ($R_s \geq 1$) is indicated in white.

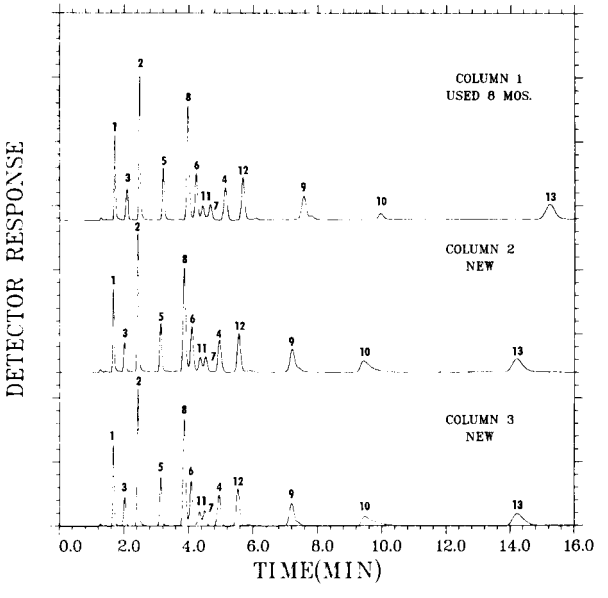

Fig. 7. Column reproducibility for three columns at the optimum mobile phase. Conditions as in Experimental. Peaks: 1 = 2-OCH_3; 2 = 1-NO_2; 3 = 1,2-$(OCH_3)_2$; 4 = 1,5-$(NO_2)_2$; 5 = 1-CHO; 6 = 2-CO_2CH_3; 7 = 1-CO_2CH_3; 8 = 2-CHO; 9 = 1-CH_2CN; 10 = 1-OH; 11 = 1-$COCH_3$; 12 = 2-$COCH_3$; 13 = 2-OH. Minor peaks after peaks 9 and 12 are unknown impurities in the substituted naphthalene standards used.

TABLE II

REPRODUCIBILITY OF k' DATA FOR THREE COLUMNS

Conditions as in Table I.

*Solute**	*Column No.*			*Relative standard deviation (%)*
	1	*2*	*3*	
2-OCH_3	0.70	0.66	0.66	3.4
1-NO_2	1.48	1.42	1.42	2.4
1,2-$(OCH_3)_2$	1.09	1.01	1.02	4.2
1,5-$(NO_2)_2$	4.13	3.95	3.94	2.7
1-CHO	2.20	2.12	2.13	2.0
2-CO_2CH_3	3.23	3.09	3.08	2.7
1-CO_2CH_3	3.67	3.52	3.50	2.6
2-CHO	2.97	2.85	2.86	2.3
1-CH_2CN	6.55	6.21	6.19	3.2
1-OH	8.96	8.42	8.45	3.5
1-$COCH_3$	3.42	3.35	3.33	1.4
2-$COCH_3$	4.67	4.55	4.52	1.7
2-OH	14.19	13.21	13.22	4.2

* Substituted naphthalene derivatives.

In this study, the three different silica columns were tested for reproducibility of k' with the optimum mobile phase predicted by ORM data analysis; Table II shows that k' data obtained for these three columns are sufficiently reproducible to permit the desired resolution increase. Using the optimum mobile phase predicted by the statistically designed experiments, the two newest of the three columns were connected to produce the chromatogram in Fig. 8. The limiting or most closely

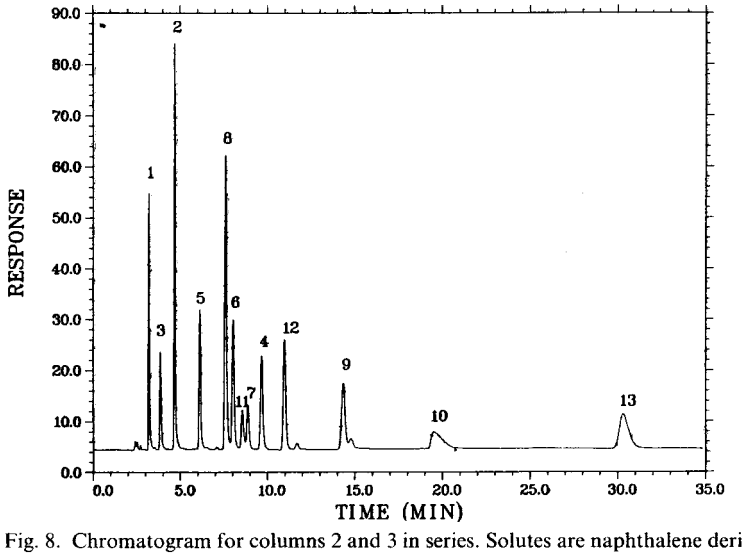

Fig. 8. Chromatogram for columns 2 and 3 in series. Solutes are naphthalene derivatives substituted as in Fig. 7.

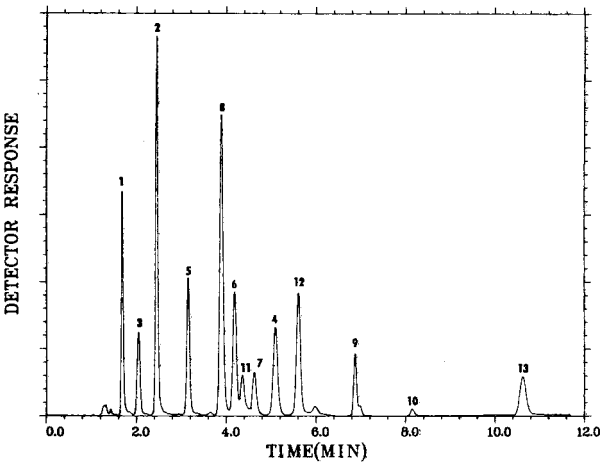

Fig. 9. Chromatogram for column 2 using step-flow programming as described in text. Solutes numbered as in Fig. 8.

spaced peaks (7, 11) now show a resolution of 1.22 compared with the initial resolution of 1.3 as predicted by theory. It should be noted, however, that this increased resolution is obtained only with longer analysis, in this case from 14 min with one column to 30 min with the two-column series. If desired, the analysis time can be decreased by doubling the column pressure to adjust the column flow-rate to 4.0 ml/min. In this case, resolution of the limiting pair 7–11 slightly decreases, but analysis time is also decreased to 15 min.

The analysis time can also be decreased by using step flow programming to increase flow-rates for peaks at the end of the chromatogram that are over-resolved. As illustrated in Fig. 9, the flow-rate is set at 2.0 ml/min to achieve optimum resolution in the early part of the chromatogram, then increased to 4.0 ml/min after 6 min to elute over-resolved later peaks at a much faster rate. In this case a resolution of at least 1.0 is maintained for all peak pairs, but the total analysis time is now 12 min instead of the 15 min in Fig. 7. This refinement for decreasing the separation time can be helpful in developing a final analysis. However, to use this approach effectively it is important that the solvent system first be optimized to achieve the best overall results.

In addition to the determination of optimum solvent composition in the preceding section by overlapping resolution mapping, it is also feasible to generate response surfaces for other separation parameters such as α and k'. The mapping of α values can also be used as a means of determining optimum solvent composition for separating a mixture; our limited experience suggests that results equivalent to resolution mapping are obtained. The mapping of k' values can be of interest in visualizing the absolute effect of various solvent modifiers on the retention of peaks of interest.

Finally, the use of the solvent optimization methods described here is not limited to silica as adsorbent and the four solvents discussed. For example, other adsorbents such as alumina should provide equally useful results with this optimization method. A more basic localizing solvent, such as triethylamine, instead of MTBE may prove to be a desirable alternative for particular mixtures. However,

based on theory and the experimental work carried out to date, we feel that methylene chloride, acetonitrile and MTBE as modifiers should prove to be optimum for most LSC separation systems. 1,1,2-Trifluorotrichloroethane (FC-113) as an attractive alternative to *n*-hexane is currently under study and will be reported shortly[17].

The optimization of mobile phase solvents is especially important in two other widely used forms of adsorption chromatography, namely, thin-layer and preparative chromatography. The present approach should be equally applicable for these areas.

CONCLUSIONS

Quaternary-solvent mobile phases can be used for the systematic optimization of α values in LSC. A mixture-design statistical technique using overlapping resolution mapping requires retention data for only seven mobile phases to predict the mobile phase composition for optimum resolution. This approach greatly reduces the number of separate experiments with mobile phases which are required to establish an acceptable separation. Prediction of α and k' values for any mobile phase is also possible.

Once the mobile phase solvent composition has been optimized, increased resolution and/or decreased analysis time can usually be obtained by increasing the column length and mobile phase flow-rate together.

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GAS CHROMATOGRAPHIC STUDY OF SOLUTION AND ADSORPTION OF HYDROCARBONS ON GLYCOLS

II*. ETHYLENE GLYCOL

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SUMMARY

Adsorption at the gas–liquid interface is demonstrated in hydrocarbon–ethylene glycol systems. Thermodynamic properties of solution and adsorption at the gas–liquid interface of these systems were determined.

The retention behaviour of olefins in ethylene glycol was studied. Values of retention parameters obtained from experimental data are compared with values in the literature; possible causes of discrepancies are discussed.

The contribution of so-called Kelvin retention to the overall retention process was calculated and its significance for different systems is discussed.

INTRODUCTION

Thermodynamic parameters of solution and adsorption at the gas–liquid interface have been determined by several workers employing a chromatographic method[1–4]. Even though the precision thus attained is not as good as that obtained when gas–liquid partition is the only mechanism responsible for the retention process, the results have proved the validity of the approach.

Martin[5] was able to demonstrate that the adsorption at the gas–liquid interface contributed to the retention volume when non-polar solutes were eluted from columns containing a polar liquid phase; taking this effect into account, he formulated the following equation:

$$V_N^0 = K_L V_L^0 + K_A A_L^0 \quad (1)$$

where V_N^0 is the net retention volume per gram of packing, K_L is the gas–liquid partition coefficient, K_A is the adsorption coefficient at the gas–liquid interface, V_L^0 is

* For Part I, see ref. 9.

0021-9673/82/0000–0000/$02.75

the volume of the stationary liquid and A_L^0 is the exposed surface of same liquid, these last two parameters being per gram of packing.

Some other ratios have been proposed[1,6–8] that take into account the effect of additional mechanisms besides those already considered by Martin. The incidence of a given mechanism depends on the nature of the system studied and on the stationary phase to solid support ratio used. Thus, for instance, it has been shown that adsorption on the solid support is meaningless when more than 5 % of a hydroxylated liquid is used as the stationary phase[1,2].

Results obtained for the thermodynamic parameters of solution and of adsorption at the gas–liquid interface of hydrocarbons when using diethylene glycol (DEG) and triethylene glycol (TEG) have been published recently[9]. These two systems, within the range of stationary phase to solid support ratios used, exhibit two main mechanisms which contribute to the retention volume, *viz.*, gas–liquid partition and adsorption at the gas–liquid interface.

The possibility of comparing the solvent properties of ethylene glycol (EG) with those of DEG and TEG, as well as a predictable higher adsorption at the gas–liquid interface, determined the selection of EG as the liquid phase in this work. The thermodynamic properties of solution and of adsorption at the gas–liquid interface of hydrocarbons in EG, calculated on the basis of the experimental data, are given here. The influence of the Kelvin retention effect[10,11] on the adsorption coefficient at the gas–liquid interface is also shown.

EXPERIMENTAL

The equipment employed and the procedure followed have been described in detail in a previous paper[9]; the only difference was the temperature range for the experiments, which in this work was between 18 and 28°C.

Columns and reagents

Stainless-steel columns of 1/4 in. O.D. were used, 50 cm long for aromatic hydrocarbons and 150 cm long for aliphatic hydrocarbons. In order to avoid losses of stationary phase a pre-column of the same material and diameter and 50 cm in length was used, filled with the highest percentage of stationary phase that was employed.

Chromatographic-grade EG (Carlo Erba, Milan, Italy) was used as the stationary phase, and 60–80 mesh Chromosorb P, acid-washed, as the solid support. Packings were prepared as described previously[9]; their characteristics are given in Table I. Hydrocarbons were at least 99 % pure and are listed in Table II.

RESULTS

The retention process for some of the hydrocarbons studied is due to a mixed mechanism, as shown in Fig. 1. When gas–liquid partition is the only effect responsible for the retention process, the graph should consist of straight lines converging to the origin; deviations obey to adsorption at the gas–liquid interface and, as can be seen, this effect is greater for aliphatic than for aromatic hydrocarbons.

Values of the gas–liquid partition coefficients were obtained as described previously[9], using areas calculated according to the proposal of Martire *et al.*[12]; they are

TABLE I

COLUMN CHARACTERISTICS AT 25°C

Parameter	*Value*				
Length	50 cm, for aromatic hydrocarbons				
Stationary phase percentage	23.53	19.85	14.46	9.779	4.719
W (g of packing)	4.6444	4.2552	3.8814	3.6755	3.5480
V_L^0 (cm^3/g)	0.2120	0.1788	0.1303	0.0881	0.0425
A_L^0 (m^2/g)	0.335	0.499	0.756	1.01	1.34
Length	150 cm, for aliphatic hydrocarbons				
Stationary phase percentage	26.19	18.95	14.46	7.373	5.166
W (g of packing)	14.6453	12.7452	12.1827	11.9295	11.4936
V_L^0 (cm^3/g)	0.2359	0.1707	0.1303	0.0664	0.0465
A_L^0 (m^2/g)	0.213	0.546	0.756	1.16	1.30

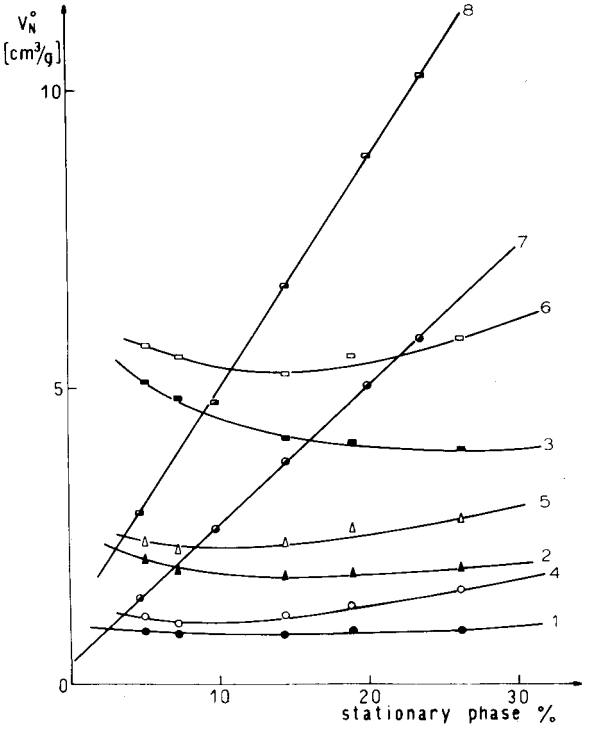

Fig. 1. Representative net retention volumes per gram of column packing as a function of stationary phase percentage: 1 = *n*-hexane; 2 = *n*-heptane; 3 = *n*-octane; 4 = 1-hexene; 5 = 1-heptene; 6 = 1-octene; 7 = benzene; 8 = toluene.

TABLE II

THERMODYNAMIC FUNCTIONS OF SOLUTION AT 25°C

Solute	$K_L \pm \sigma t$	γ^∞	ΔH_s^0 *(kcal/mole)*	ΔH_L^0 *(Ref. 13) (kcal/mole)*
n-Hexane	3.68 ± 0.10	598	5.9	7.5
n-Heptane	7.13 ± 0.22	1020	6.9	8.7
n-Octane	13.7 ± 0.5	1734	7.8	9.9
n-Nonane	27.1 ± 0.9	2853	9.1	11.1
1-Hexene	6.16 ± 0.15	290	6.1	7.4
1-Heptene	11.7 ± 0.3	507	7.2	8.6
1-Octene	21.4 ± 0.6	914	8.0	9.7
Cyclohexane	13.4 ± 0.2	253	6.2	7.9
Methylcyclohexane	16.0 ± 0.3	542	6.4	8.4
Ethylcyclohexane	33.1 ± 0.7	773	7.5	9.7
cis-2-Hexene	8.21 ± 0.19	268	6.1	7.5
Cyclohexene	29.2 ± 0.4	128	6.6	7.9
Isooctane	5.91 ± 0.21	1148	6.7	8.4
Isooctene	12.0 ± 0.4	674	6.8	8.8
Benzene	108 ± 2	32.3	6.8	8.1
Toluene	189 ± 3	61.7	7.6	9.1
Ethylbenzene	309 ± 6	112	8.1	10.1
n-Propylbenzene	482 ± 8	203	8.6	11.0
Isopropylbenzene	393 ± 7	184	8.4	10.8
o-Xylene	470 ± 7	107	8.3	10.4
m-Xylene	322 ± 5	124	8.0	10.2
p-Xylene	311 ± 6	123	8.1	10.1

listed in Table II, together with their respective 95% confidence intervals. Values obtained for the activity coefficients at infinite dilution and the standard partial molar enthalpies of solution (ΔH_s^0) are also given in Table II. Values of ΔH_s^0 were derived from the slope of the graph of ln K_L *versus* $1/T$, after subtracting the correction term $RT(1 - \eta T)$, (where η is the thermal expansion coefficient of the solvent); they correspond to the transfer of 1 mole of solute from an ideal vapour phase, at a pressure of 1 atm, to a hypothetical solution of unit molar fraction but obeying Henry's law. The value of the correction term in EG is 481 cal/mole at 25°C.

The standard molar free energy of adsorption at the gas–liquid interface was obtained from the equation

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

where p^0 is the pressure corresponding to the gaseous state and π^0 is the surface pressure of the adsorbed standard state. The standard states considered here are those defined by Kemball and Rideal[14]. Values of K_A at 25°C with their 95% confidence intervals for all of the hydrocarbons studied are given in Table III, together with the thermodynamic functions of adsorption at the gas–liquid interface.

TABLE III

THERMODYNAMIC FUNCTIONS OF ADSORPTION AT 25°C

Solute	$(K_A \pm \sigma t \cdot 10^5 (cm)$	$-\Delta G_A^0$ *(kcal/mole)*	$-\Delta H_A^0$ *(kcal/mole)*	$-\Delta S_A^0$ *(e.u.)*
n-Hexane	5.36 ± 0.39	4.02	6.7	8.9
n-Heptane	13.4 ± 0.9	4.57	7.6	10.2
n-Octane	34.4 ± 1.1	5.12	8.6	11.8
n-Nonane	84.6 ± 2.3	5.66	9.7	13.6
1-Hexene	5.58 ± 0.48	4.05	6.6	8.5
1-Heptene	14.0 ± 1.2	4.59	7.5	9.9
1-Octene	36.1 ± 1.3	5.15	8.6	11.6
Cyclohexane	4.75 ± 0.83	3.95	5.9	6.5
Methylcyclohexane	10.9 ± 0.7	4.45	6.8	8.0
Ethylcyclohexane	27.1 ± 1.4	4.98	7.8	9.4
cis-2-Hexene	5.96 ± 0.61	4.09	6.6	8.5
Cyclohexene	5.89 ± 0.82	4.08	5.9	6.0
Isooctane	16.1 ± 0.9	4.67	7.5	9.4
Isooctene	19.8 ± 0.8	4.80	7.6	9.3
Benzene	8.94 ± 1.20	4.33	8.8	15.3
Toluene	25.9 ± 3.1	4.96	9.9	16.7
Ethylbenzene	65.4 ± 7.9	5.50	10.6	17.2
n-Propylbenzene	160 ± 20	6.03	11.5	18.2
Isopropylbenzene	130 ± 16	5.91	11.2	17.6
o-Xylene	82.0 ± 12.5	5.64	10.9	17.8
m-Xylene	72.0 ± 9.5	5.56	10.7	17.2
p-Xylene	70.7 ± 9.0	5.55	10.6	16.8

TABLE IV

PARTITION COEFFICIENTS AT 40°C

Solute	$K_{L(1)}$	$K_{L(2)}$	$K_{L(3)}$	$K_{L(4)}$
1-Hexene	3.92	4.18	3.5	2.58
1-Heptene	6.77	7.40	7.3	4.45
1-Octene	11.7	13.2	13.1	7.47
n-Hexane	2.38	2.63	–	1.47
n-Heptane	4.24	4.84	–	2.56
n-Octane	7.58	9.05	–	4.40
cis-2-Hexene	5.22	5.48	–	–
Cyclohexene	17.8	18.2	14.7	–
Benzene	65.1	65.7	47.8	–

DISCUSSION

The evaluation of the adsorption effect at the gas–liquid interface for the hydrocarbon–EG systems confirms and clarifies the suspicions of some authors about the existence of this effect[15].

In order to compare our results with those in the literature, we have extrapolated to 40°C the K_L values obtained from eqn. 1 and those calculated using the simple equation $V_N = K_L V_L$ for the maximum percentage of stationary phase tested.

Table IV lists the following: $K_{L(1)}$ are values of K_L free of surface effects, $K_{L(2)}$ are values of K_L for the maximum percentage of stationary phase, without correction because of adsorption, $K_{L(3)}$ are values taken from Muhs and Weiss[16] and $K_{L(4)}$ are values calculated from net retention volumes per gram of EG, at 40°C, as given by Ligny *et al.*[15].

Comparing values given by Muhs and Weiss[16], who used 22% of stationary phase, it is easy to conclude that it is necessary to consider the effect of adsorption at the gas–liquid interface; nevertheless, the results obtained for cyclohexane and benzene cannot be explained by this effect.

The values of K_L obtained from the data of Ligny *et al.*[15] are completely different from our $K_{L(1)}$ values. They used 40% of stationary phase under experimental conditions similar to those used in this work, except for the support (Kieselguhr) and they prepared the packing using methanol as the solvent, which was discarded in our study owing to significant losses of EG at 40°C.

Considering that the support could not affect their results, the values given by Ligny *et al.*[15] for V_N of hydrocarbons on EG cannot be supported by a rational basis.

Values of the activity coefficients at infinite dilution for the systems *n*-hexane–EG and benzene–EG, given in Table II, were compared with those in the literature at 25°C. Deviations as low as 4.5 and 4.0% were obtained between our values and those of Deal and Derr[17] derived from phase equilibrium data. The same systems showed deviations of 14.5 and 16.4%, respectively, between these reference values and those taken from chromatographic determinations without considering interfacial effects[18].

As in DEG and TEG, activity coefficients at infinite dilution of hydrocarbons in EG show great positive deviations from Raoult's law, the larger ones corresponding to aliphatic hydrocarbons and the smaller ones to aromatic hydrocarbons; the deviations increase with increasing number of carbon atoms within each particular family of hydrocarbons.

Larger deviations in EG than in DEG and TEG could be expected because of the greater association power of the first solvent, through the establishment of hydrogen bonds, thus making more difficult the dissolution of a hydrocarbon in such a medium.

On the other hand, the density of hydroxyl groups, which is higher in EG than in DEG and TEG, makes EG more effective in polarizing delocalized electrons in hydrocarbon molecules, and thus it facilitates the solubility of aromatic hydrocarbons because of their higher polarizability. This is in accordance with the values given in Table II, where it can be seen that the absolute solubility of aromatic hydrocarbons in EG is lower than that in DEG and TEG, as could be predicted from the already mentioned higher association in EG.

The great deviation obtained in the activity coefficients at infinite dilution calcu-

lated using the theory of regular solutions as modified by the equation of Weimer and Prausnitz[19] can be similarly adscribed to the high association of EG molecules.

Values of excess partial molar enthalpies (ΔH^e) calculated according to ref. 20 and excess partial molar entropies are given in Table II. According to these results, it could be concluded that the main energetic effect is due to the rupture of hydrogen bonds among glycol molecules.

Castells[3] obtained analogous results using formamide as stationary phase; he explained the high entropies of solution through a structural rearrangement of the solvent molecules around the solute molecules, similar to the behaviour of hydrocarbons in water reported by Frank and Evans[21].

According to Tyuzyo[22], the association in EG is comparable to that in formamide and, in spite of structural differences between these two solvents, there is some parallelism between the experimental results. It has not been possible to calculate the change in structural entropy with the equation proposed by DeVoe[23] for these two solvents because of the lack of available data to estimate the diameter of rigid spheres.

Selectivity values at 25 and 50°C for *n*-hexane–benzene and *n*-octane–benzene pairs, and capacity values of EG towards benzene, are given in Table V, together with the corresponding parameters in DEG and TEG[9]. The larger selectivities of EG compared with those of DEG and TEG for the two pairs considered, at both 25 and at 50°C, demonstrates its advantage as a solvent when trying to separate aromatic hydrocarbons; nevertheless, its poor capacity for dissolving these hydrocarbons discourages its application on an industrial scale.

Table III gives the values of the adsorption coefficients at the gas–liquid interphase at 25°C for all of the hydrocarbons studied, with their respective 95% confidence intervals. It can be seen that the precision of K_A values is directly related to the magnitude of the interfacial effect in the retention process.

Several workers[10,11,24] have claimed that it is necessary to consider the Kelvin effect in chromatographic beds, as a consequence of the curved surface of the stationary liquid in the pores of the solid support. Devillez *et al.*[10] took into account the Kelvin effect in the retention process for systems that show mixed mechanisms and concluded that in those instances, the so-called Kelvin retention affects the value of the adsorption coefficient at the gas–liquid interface.

TABLE V

SELECTIVITY AND CAPACITY TOWARDS BENZENE

Solvent	*Temperature (°C)*	*Selectivity**		*Capacity:* $x_B = 1/\gamma_B^\infty$
		$\beta_{H/B}$	$\beta_{O/B}$	
Ethylene glycol	25	18.5	53.9	0.031
	50	17.6	48.3	0.037
Diethylene glycol[9]	25	17.0	38.6	0.155
	50	13.8	30.4	0.155
Triethylene glycol[9]	25	17.6	37.7	0.263
	50	13.4	27.8	0.263

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

TABLE VI

CONTRIBUTION OF KELVIN RETENTION (KR)

Solute	*Stationary phase percentage*	$K_L/K_L^0 \cdot 10^2$	$KR/K_A\bar{A}_L \cdot 10^2$
n-Hexane	5.166	100.2	0.57
	26.19	100.5	3.56
n-Nonane	5.166	103.0	0.36
	26.19	100.7	2.24
1-Hexene	5.166	102.1	0.88
	26.19	100.4	5.46
Cyclohexene	5.166	101.7	3.17
	26.19	100.4	19.74
Benzene	4.719	101.9	5.71
	23.53	100.9	33.5
Ethylbenzene	4.719	102.1	3.0
	23.53	100.6	17.7

The Kelvin retention for some hydrocarbons representative of the families studied was determined and compared with the contribution of adsorption at the gas–liquid interface; values are given in Table VI, where ratios between the calculated partition coefficient and that for a plane surface[10,11] are also listed.

The results show, as Devillez *et al.*[10] had already stated, that the curved surface has a minimum effect on the partition coefficient and, consequently, activity coefficients at infinite dilution are practically unaffected because of this phenomenon. In contrast, adsorption coefficients at the gas–liquid interface for solutes of high solubility in the liquid stationary phase are definitely affected by the Kelvin retention; this applies to both aromatic hydrocarbons and cyclohexene, as shown in Table VI, where the Kelvin retention is important when it is compared with the adsorption at the gas–liquid interface. Nevertheless, because of the solubility of these hydrocarbons, adsorption at the gas–liquid interface exerts a very small effect, so that the precision of the resulting values is too poor to consider the correction due to Kelvin retention to be at all reliable.

Values for the Kelvin retention were calculated through the variation of $\bar{A}_L$ with $\bar{V}_L$ ($\bar{A}_L$ being the area of stationary liquid and $\bar{V}_L$ its volume, both per gram of solid support).

There are two reasons which made us to choose this means of calculating the Kelvin retention. On the one hand, there are discrepancies between the values of Conder and Young[11] and those of Devillez *et al.*[10] for the Kelvin retention although in both instances the values were obtained using the data for pore radius for Chromosorb P given by Saha and Giddings[25] and, on the other hand, there is agreement between equivalent series of values which both Conder and Young and Devillez *et al.* obtained through the procedure finally adopted here, taking values of $\bar{A}_L$ and $\bar{V}_L$ from Martin[5] when the behaviour of benzene in thiodipropionitrile was studied.

As already stated, the precision of K_A increases when the solubility of the solute in the stationary liquid phase decreases; the same considerations may be applied to the thermodynamic properties of adsorption given in Table VI.

The precision of the heats of sorption[1] and solution and of the heat of adsorp-

tion at the gas–liquid interface was calculated by applying Castells' reasoning[26] for some hydrocarbons representative of the families studied. Thus, the precision of the heat of sorption is ±220 cal/mole in *n*-nonane, cyclohexane and benzene, and the precisions of the heats of solution are ±300, ±110 and ±90 cal/mole for the same solutes, respectively. The precision of the heats of adsorption, however, is ±1.200 cal/mole for benzene, ±1.000 cal/mole for cyclohexane and ±270 cal/mole for *n*-nonane.

The standard molar enthalpies of adsorption at the gas–liquid interface given in Table VI are smaller than the heat of liquefaction, except for the aromatic hydrocarbons, for which they are slightly higher; this allows us to conclude that hydrocarbons are retained at the glycol surface by very weak forces.

It can be seen that most of the adsorption entropy values at the gas–liquid interface listed in Table VI are more negative than the theoretical values calculated by Kemball and Rideal[14] for the entropic change associated with the transition of a three-dimensional gas to a two-dimensional gas; from this point it is possible to consider the adsorbed hydrocarbon molecules as a two-dimensional gas and include them in the mobile adsorption defined by Kemball[27].

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ADSORPTION PROPERTIES OF SILANIZED AND CARBOWAX-MODIFIED SUPPORTS FOR GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Two of the best supports for gas–liquid chromatography were studied for adsorption activity using adsorbates with different polarities and molecular structures. The thermodynamics of adsorption were determined on the best commercial silanized support (Chromaton N Super) and on Carbowax-modified Chromaton N AW. The experimental data indicate the presence of some specific adsorption sites on the silanized support surface while free hydroxyls are eliminated from this surface. Metal cations seem to be the active sites. When using the modified support the adsorption isotherms are linear, while non-linearity of the adsorption isotherms for oxygen-containing adsorbates is observed on the silanized support.

INTRODUCTION

Creation of the ideal support is one of the most urgent problems in gas–liquid chromatography (GLC). The general properties of many supports have been reviewed[1,2], particular attention being paid to commercial supports. At present, silanized supports such as Chromosorb W HP, Chromosorb 750 and Chromaton N Super seem to be the best for GLC. However, even these supports have some disadvantages for polar solutes on non-polar or weakly polar stationary phases (peaks asymmetry, tailing, etc.). Even after taking into account the non-linearity of the sorption isotherm for these solutes, the "self-modification" effect[3] remains which prevents the determination of reproducible data for mixtures of polar solutes.

Carbowax modified according to the Aue procedure[4,5] has almost ideal properties for GLC with non-polar stationary phases[5]. The "self-modification" effect is not observed on the modified support.

Many papers have reported "bad" and "good" support properties, mainly when qualitative parameters for the supports were compared (for example, peak symmetry, tailing, possibility of elution). No thermodynamic adsorption parameters for the best silanized supports and the Carbowax modified support have been reported, thus preventing discussion of the support surface. This paper describes a comparative thermodynamic study of adsorption on the two best support surfaces: silanized and modified.

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EXPERIMENTAL

Instruments and calculations

The experiment was performed on a Chrom-42 (Laboratorní Prístroje) instrument with a flame-ionization detector. Stainless-steel tubes (2.4 m × 3 mm I.D.) were used as columns and packed with the support and conditioned at 260°C overnight.

Chromaton N AW (Lachema, Czechoslovakia) was chosen as the basic material for the compared supports; Chromaton N Super (Lachema) was the best silanized support. The same basic material (Chromaton N AW) was used for the Carbowax modification; therefore, the compared supports have the same bulk properties. The modification procedure was similar to that described by Aue and co-workers[4,5]: Chromaton N AW was coated with 0.2% polyethylene glycol 15000, and then conditioned at 260°C for 6 h in a helium stream. The extraction step was omitted.

The relative retentions, r, of some solutes were measured using benzene as the standard. The relationship between $\ln r$ and $1/T_c$ (T_c = column temperature in °K) was plotted and the r value at 50°C was calculated from this graph. The same relationship was used to calculate the relative molar heats of adsorption, ΔH_a^0, as described elsewhere.

The entropic selectivity, F^0, was calculated as[6]

$$F^0 = R \ln r + 0.535\, \Delta H_a^0/T \tag{1}$$

where R is the gas constant.

The relative mean standard deviation for r was about 0.1% for the cases with linear isotherms and up to 1% for those with non-linear isotherms; for ΔH_a^0 the corresponding deviations were 3 and 8%, respectively.

When the adsorption isotherm is non-linear, the net retention volume, V_N, depends on the sample amount or peak height, h. The following equation was used to take account of the non-linearity of the adsorption isotherm[7], where A and B are constants:

$$V_N = B + A/\log h \tag{2}$$

The relationship between V_N and $1/\log h$ was plotted using 3–5 experimental points, and the V_N value for a chosen peak height thus calculated.

The recorder full scale was 10^{-11} A (chart width 250 mm); peak height was measured in mm. The chosen peak height for the calculations (isobaric retention) was the value at which $1/\log h = 0.3$. Isobaric thermodynamic functions of adsorption were calculated by using the isobaric retention data as elsewhere[7].

The degree of non-linearity of the adsorption isotherm was calculated by using the following equation[7]:

$$A^* = (V_{N_{0.4}} - V_{N_{0.3}})/V_{N_{0.3}} \tag{3}$$

The subscripts 0.3 and 0.4 relate to the corresponding values of $1/\log h$. The A^* value is expressed in relative units and this parameter is valuable for comparison of adsorbents at chosen vapour pressures of the adsorbate.

TABLE I

ADSORPTION CHARACTERISTICS OF THE ADSORBATES ON THE SILANIZED (S) AND CARBOWAX-MODIFIED (M) SUPPORTS

ΔH_a^0 in kcal/mole, F^0 in cal./°K · mole. The standard temperature was 50°C.

Adsorbate	*S*			*M*		
	r	ΔH_a^0	F^0	*r*	ΔH_a^0	F^0
Carbon tetrachloride	0.735	7.2	11.98	0.902	−9.5	−16.8
Chloroform	0.564	− 4.6	− 9.17	1.78	2.1	4.8
1,2-Dichloroethane	1.331	−14.8	−25.3	2.05	4.5	9.3
Ethyl acetate	9.04	6.5	15.7	0.925	−3.7	− 6.6
Methyl ethyl ketone	10.1	6.6	16.1	1.01	−2.9	− 5.0
n-Propanol	17.1	16.6	17.1	2.97	2.5	6.5

Because of the low adsorption activity of the compared supports the flow-rate of the carrier gas was chosen to be sufficiently low (about 5–7 ml/min) that the retention times could be determined with high precision. These determinations were performed isothermically in the column temperature range 40–70°C.

The adsorbates studied are listed in Table I.

RESULTS AND DISCUSSION

The experimentally determined support properties (S = silanized support, M = the modified one) are listed in Table I. First we discuss the relative molar heats of adsorption which are related to the energy of intermolecular forces at the support surface.

Carbon tetrachloride is a non-polar adsorbate with zero dipole moment. Its ΔH_a^0 on the silanized support is 7.2 kcal/mole which indicates a very low energy of intermolecular forces as compared with benzene. The benzene molecule also has a dipole moment of zero, however it has a π-electron cloud that may interact to form complexes with metal cations which are on the surface of the silanized support. The cations are formed by calcination of white supports, and some metal cations ware absorbed by diatomites from sea water (Fe, Al, etc.)[8–10]. Upon acid treatment of the white support some metal cations are eluted from the surface. However, sodium having a high diffusion coefficient reaches the surface even after acid washing. A small amount of heavy metal cations also remains on the support surface after acid washing. All these cations may form π-complexes with benzene molecules, thus leading to strong interactions between benzene and the support surface. Additional evidence for this is the relative molar heat of adsorption for carbon tetrachloride on the modified support: this value of −9.5 kcal/mole means that benzene is less strongly bonded with the support surface than is carbon tetrachloride. Indeed, the modified surface is coated by a uniform film of the modifier, therefore no specific adsorption centres are present on the surface. A marked difference in the nature of the compared support surfaces is illustrated by the relative retention data for the supports.

The 1,2-dichloroethane molecule has two microdipoles between the atoms of

carbon and chlorine. The orientation forces with the support surface may be revealed for this molecule unlike the non-polar molecules benzene and carbon tetrachloride. When using the silanized support the relative molar heat of adsorption of dichloroethane is 22 kcal/mole less than for carbon tetrachloride although the molar refractions for the two molecules are about the same. This shows the strong adsorbate–absorbent interaction with orientation forces. When using the modified support, the molar heat of adsorption for dichloroethane is 14 kcal/mole more positive than that for carbon tetrachloride, clearly indicating the different natures of the compared supports.

Chloroform may form weak hydrogen bonds, however, the dipole moment of this molecule is low because of shielding C–Cl bonds. On the compared supports the molar heat of adsorption for chloroform is intermediate between those for carbon tetrachloride and dichloroethane, indicating the same change of the intermolecular interaction throughout the series of chlorinated hydrocarbons. In addition, the energy of the hydrogen bond is not large when chloroform is adsorbed on these supports. Indeed, all free hydroxyl groups on the Chromaton N Super surface are eliminated during silanization; the same groups are shielded by the modifier film on the modified support.

The oxygen-containing substances examined are much more polar than the chloro derivatives of hydrocarbons. Ethyl acetate, methyl ethyl ketone and *n*-propanol have different polarities and possibilities for hydrogen bond formation; however, when using the silanized support no marked differences in the molar heats of adsorption are observed for these substances. Moreover, although the dipole moment of the C = O bond is much higher than that of the C–Cl bond the molar heat of adsorption for methyl ethyl ketone is more positive (by about 20 kcal/mole) than that for dichloroethane. This indicates the high adsorption specificity of the Chromaton N Super surface and comparatively little influence of orientation forces on adsorption energy. The large difference in ΔH_a may reflect complex formation of the halogenated adsorbates with metal cations on the silanized support surface.

Comparing the molar heats of adsorption for the three oxygen-containing adsorbates, the greater difference is observed on the modified support: ethyl acetate and methyl ethyl ketone interact more strongly with the modified support surface than does *n*-propanol. Molecules of methyl ethyl ketone and of ethyl acetate have the same microdipole (C = O bond) moment which is higher than that of *n*-propanol (C–OH microdipole); therefore, the data for the modified support show that the orientation forces are the dominant specific (without taking into account dispersive forces) interaction on the surface. Free hydroxyl groups and any groups which can form the hydrogen bond are shielded on the modified support. Free hydroxyl groups at the ends of the Carbowax molecule seem to be bonded with the same groups on the support surface and the whole Carbowax molecule is bonded with the support surface by more stronger forces than physical adsorption. Only these strong bonds can be the reason for the good shielding of any active sites of the Chromaton surface. The data for the silanized support show the presence of hydrogen bonds with some active sites on the support surface when *n*-propanol is used as the adsorbate. Taking into account that all free hydroxyl groups on the silanized surface are converted into ethers, the presence of some non-hydroxyl groups on the surface which form hydrogen bonds is proved by the data.

Table II is of aid when considering intermolecular interactions between the

TABLE II

THERMODYNAMICS OF GAS–LIQUID DISTRIBUTION WHEN USING METHYLPOLYSILOXANE AS THE LIQUID AND BENZENE AS THE STANDARD

Solute	*r*	ΔH_s^0	F^0
Carbon tetrachloride	1.033	0.18	−0.59
Chloroform	0.65	0.26	−0.40
Dichloroethane	0.83	0.08	−0.23
Methyl ethyl ketone	0.54	0.22	−0.84

silanized support and the adsorbates and in the solutions, the interactions in the solutions were determined by GLC. First, the differences in molar heats of solution are less than those in the molar heats of adsorption. The intermolecular interaction in the methylpolysiloxane solution occurs between the $Si–CH_3$ fragments of the stationary phase and the groups of the solute. When using the silanized support only part of the support surface is covered by $–O–Si(CH_3)_3$ groups, the main part being that of the initial calcinated surface of the diatomite. This is the reason for marked differences in interaction of the solution and the support.

Some information on the orientation of the sorbate molecules on the support surface may be obtained from F^0 data (F^0 mainly reflects loss of rotational entropy when the sorbate molecule passes from the gas phase to the support surface). The F^0 values on the modified support for all sorbates, except chloroform and dichloroethane, are smaller than that on the silanized support indicating that the surface of the modified support is more homogeneous[3].

The A^* values for three adsorbates (the remaining ones have $A^* = 0$, *i.e.*, exhibit linear adsorption isotherms) are given in Table III. An appreciable A^* value is

TABLE III

A^* VALUES (RELATIVE UNITS) FOR SOME ADSORBATES ON CHROMATON N SUPER

Adsorbate	*Column temperature (°C)*		
	37.7	*46.0*	*51.5*
Methyl ethyl ketone	1.71	1.39	0.81
n-Propanol	1.69	1.43	1.06
Ethyl acetate	1.64	1.55	1.01

observed only on the silanized support which shows the greater non-homogeneity as compared with the modified support. The A^* values are about the same for all polar compounds studied indicating no preferable interactions for these adsorbates. Thus, such values serve as additional evidence of homogeneity of the compared support surfaces. These data are in good accord with suggestions[4,5] about symmetrical peaks obtained for many polar substances on the Carbowax-modified support.

CONCLUSIONS

The experimental thermodynamic data allow us to make some conclusions on

the nature of the two supports. Even the most optimal silanization procedure does not yield a chemically inert and homogeneous surface, although free hydroxyls are eliminated. Some surface impurities (presumably, metal cations) on the silanized support determine the strong intermolecular interaction with some classes of adsorbates including bonds with π-electrons. The modifier film of thermally bonded Carbowax gives a homogeneous support surface of low polarity with no specific interactions with the adsorbate molecules.

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REVERSE SALT GRADIENT CHROMATOGRAPHY OF tRNA ON UNSUBSTITUTED AGAROSE

I. VARIATIONS IN ELUTION PROFILE AND EVIDENCE FOR TWO FRACTIONATION MECHANISMS

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SUMMARY

E. coli tRNA was fractionated by the application of ammonium sulphate reverse gradients to Sepharose 4B. Variations in elution profile were partly attributable to differences between batches of Sepharose. The profile also varied with column length and gradient parameters. This suggests the existence of two distinct mechanisms which do not separate different tRNAs in the same sequence. The first mechanism, believed to be interfacial precipitation, releases tRNAs progressively as the salt concentration is reduced. A second mechanism introduces adsorptive retardation in which molecules lag behind the solvent. This process, widely believed not to be important in the chromatography of macromolecules with multiple binding sites, is in the present case mainly responsible for the improved resolution of peaks on passage down a long column. Isocratic (constant-salt) fractionation is also feasible. The Sepharose batch variation affects the second mechanism more than the first.

INTRODUCTION

Since the first reports of protein and tRNA fractionation by reverse salt gradients on unsubstituted agarose (Sepharose) there has been controversy about the binding mechanism involved. There have also been unexplained differences in behaviour between published accounts of its use. Since Sepharose carries few charges, ionic interactions are generally thought to be unimportant except in work at very low ionic strengths[1]. Gel filtration is assumed to be inoperative because the solute lags behind the solvent, and because the exclusion limit of the Sepharose used is always much higher than the molecular weights of the molecules being fractionated. Holmes *et al.*[2], working with *E. coli* tRNA, presumed by analogy with earlier work with proteins on valine-substituted Sepharose[3] that only hydrophobic interactions were involved. They were unable to explain on this basis why binding appeared negligible at pH 7.5 compared with that at pH 4.5. Other results from work with acidic proteins

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at pH 6.6 (ref.[4]) were also interpreted as favouring hydrophobic interactions, on the grounds that the method required high concentrations of structure-forming anions such as sulphate.

Work with polynucleotides[5] also led to the conclusion that hydrophobic interactions were dominant above pH 4, though under more acid conditions the irreversible binding of poly(C) suggested that precipitation had occurred. However, von der Haar[6] proposed that the binding of proteins to Sepharose was due not to hydrophobic bonding but simply to interfacial salting out, precipitation being induced by contact with the gel surface; this can occur at a lower salt concentration than that required in free solution. Such effects were observed long ago in the chromatography of proteins on other materials[7].

A similar conclusion was reached by Morris[8], who fractionated yeast tRNA on unsubstituted Sepharose and other media under a wide variety of conditions. He found that the binding strength decreased markedly with rise in temperature, and argued that this made hydrophobic interactions unlikely. He also pointed out that unsubstituted agarose does not contain strongly hydrophobic regions. Morris concluded that "either an adsorptive or a selective solubility retention mechanism appears to be indicated". This does not of course rule out the possibility of multiple weak interactions with the methyl substituents that are present even in unmodified Sepharose.

Our interest in the high-salt Sepharose fractionation of tRNA arose from the fact that the order of elution of species is different from that found with other comparably gentle methods[9]. We started by following the procedure of Holmes *et al.*[2] using Sepharose 4B, which gives a better separation of species than other agarose-based media[8]. We found, however, that under standard conditions of pH and temperature the general form of the profile depended markedly on the length of the column and other parameters. The results presented here suggest that, at least for tRNA, there are two distinct separation processes: one due to interfacial salting-out and the other to differences in migration rate after release from the precipitate. There are also considerable variations between different batches of Sepharose. In Parts II[10] and III[11] the origin of these variations is discussed.

MATERIALS AND METHODS

Sepharose 4B was purchased from Pharmacia. Batches used in the present work were identified as in Table I, the date of manufacture being ascertained where possible from the manufacturer. tRNA from *E. coli* K12 strain CA 265 was obtained from the Microbiological Research Establishment (Porton, Great Britain).

Columns were operated under a hydrostatic head of 2 m in a room kept at 5 $\pm$ 2°C. For work with Sepharose batches C, D, E and F the columns were also insulated to keep the temperature variations within $\pm$0.5°C. Eluent conductivity and ultraviolet absorbance were continuously monitored, and amino-acid acceptor activities determined as described elsewhere[9].

All solutions contained 10 m*M* sodium acetate buffer, pH 4.5, with 10 m*M* magnesium chloride and 1 m*M* EDTA (solution A)[2]. A rise in the apparent pH to *ca.* 4.9 was observed on addition of ammonium sulphate to 1 *M* or higher, but in view of uncertainties about the reliability of the pH electrode under these conditions no

TABLE I

BATCHES OF SEPHAROSE 4B

Designation in text	*Manufacturer's Lot No.*	*Year of manufacture*
A	Not recorded	Before 1977
B	Not recorded	Before 1977
C	Not recorded	Before 1977
D	8237	1975
E	5326	1978
F	11801	1979

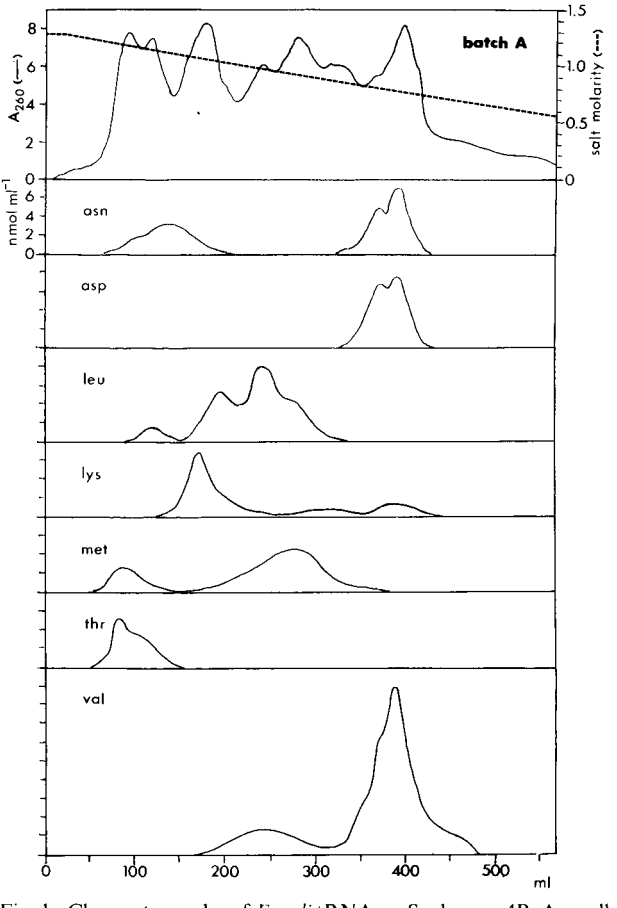

Fig. 1. Chromatography of *E. coli* tRNA on Sepharose 4B. A small volume of solution containing 150 mg tRNA (including *ca.* 25% water) was applied to a 1000 × 16 mm column and eluted with a 2 × 500-ml reverse gradient of ammonium sulphate from 1.3 *M* to zero in solution A. The flow-rate was 9 ml h^{-1}. In this and similar figures, the volume plotted on the horizontal axis is measured from the start of gradient elution from the base of the column. For further details see Materials and methods.

subsequent adjustment was made. The effect was unrelated to the grade of ammonium sulphate used, which in most of the work was "ultrapure" material from Schwarz/Mann. Impurities in this salt normally give an opposite shift in the pH; acetate buffer is also expected to show a slight depression of pH on addition of neutral salt. A comparable shift in pH was not observed when sodium chloride was used.

RESULTS AND DISCUSSION

Batch variations

In our early experiments we followed Holmes *et al.*[2] in using a reverse gradient at pH 4.5 starting at 1.3 *M* ammonium sulphate. We noted that after application of tRNA in solution a zone of light precipitation could be seen over the first few centimetres of the bed; this dispersed on application of the reverse salt gradient. Thus interfacial salting-out seemed certain to be at least one of the factors involved. The results obtained with *E. coli* tRNA (Fig. 1) showed good separation of species; the major peak of valine acceptance showed a particularly high purity of *ca.* 50%. The shape of the absorbance profile was not, however, the same in detail as that obtained (also with *E. coli* K 12) by the earlier workers, nor with that reported by others[12] for

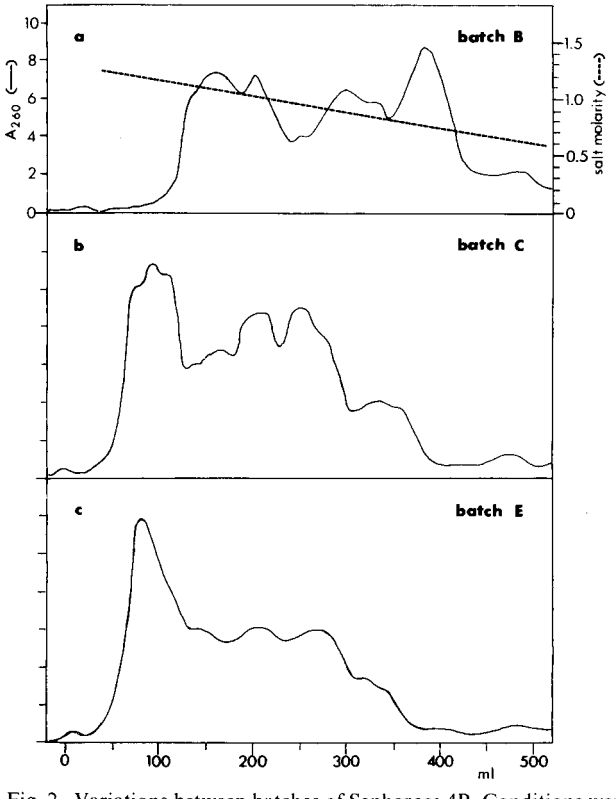

Fig. 2. Variations between batches of Sepharose 4B. Conditions were similar to those in Fig. 1 except that flow-rates were up to 30 ml h^{-1}.

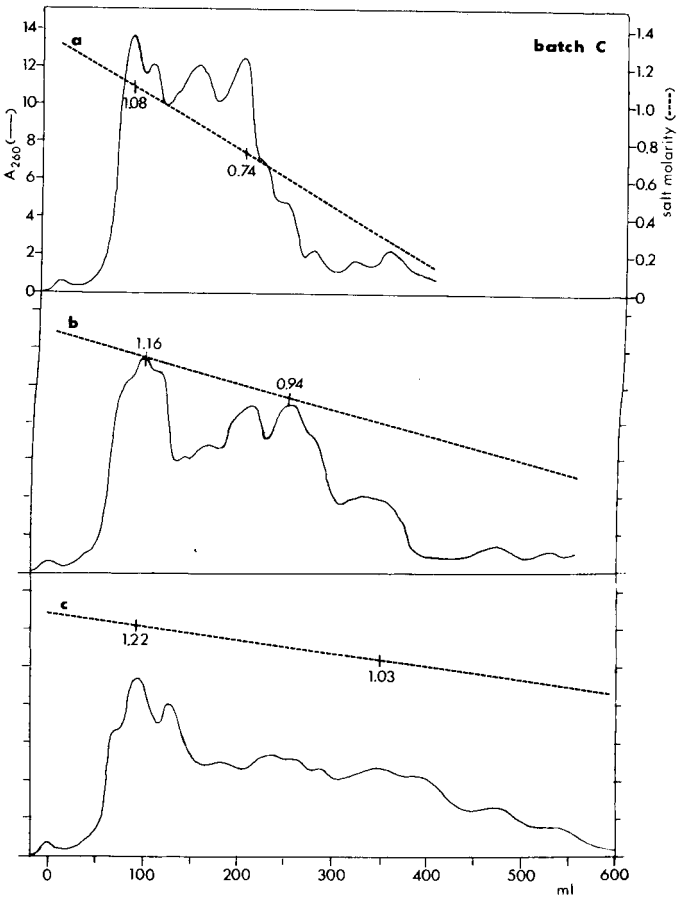

Fig. 3. Variations with gradient slope. Conditions were similar to those in Fig. 1, except that in (a) the gradient was 2 × 250 ml and in (c) it was 2 × 1000 ml. Flow-rates were 15 ml h^{-1}.

E. coli MRE 600. There were also some variations in the assay results; the leucine profile of Fig. 1 was not, for instance, the same as that in Fig. 4 of Holmes *et al.*[2]. Hjertén *et al.*[13] have reported similar discrepancies.

It became apparent after further work that these variations could partly be attributed to differences between the properties of different batches of Sepharose. Fig. 2 shows profiles obtained with three other batches under conditions similar to those of Fig. 1. Tests showed that a given profile was reproducible, even when different columns were prepared from the same batch. The flow-rate was not critical. However, no two batches gave exactly the same profile; both the distribution of peaks and the overall range of elution were different for each batch.

Variations with gradient slope and column length

We also found considerable variations in profile with the volume of the gradient. Fig. 3 illustrates this for batch C. Fig. 3a shows that a steeper gradient (obtained by halving the volume) did not simply compress the profile of Fig. 3b; there

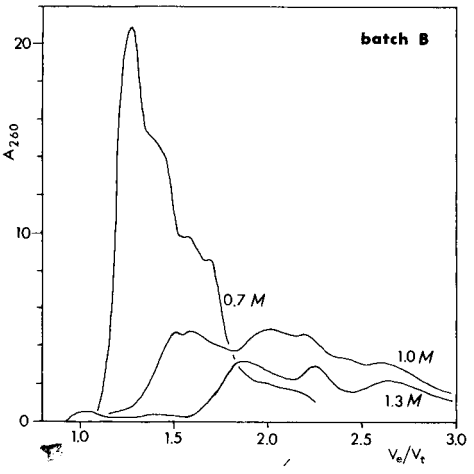

Fig. 4. Isocratic elution of *E. coli* tRNA from Sepharose 4B. A 150-mg sample was applied in a volume of 4 ml to a 1000 × 16 mm column and eluted with a constant concentration of ammonium sulphate as indicated. V_t is the volume of liquid in the column and V_e the volume collected after application of the sample; thus, unretarded material emerges at $V_e/V_t = 1$. Flow-rates were up to 20 ml h^{-1}.

was a redistribution of peaks, and also a shift in the overall range of elution. With the steeper gradient, elution occurred at lower salt concentrations than in Fig. 3b. Conversely, making the gradient more shallow as in Fig. 3c gave elution at higher salt concentrations. Doubling the column length while keeping the gradient volume the same had a similar effect (not illustrated) to halving the gradient volume. Halving or doubling the sample load had minor effects on the shape of the profile but did not change the elution range.

All this suggested that tRNA released from the column matrix lagged progressively behind the solvent as it moved down the column. We shall refer to this as *adsorptive retardation;* there is partition between the immobilized state when bound to the matrix and the free state when travelling with the solvent, and under given conditions an equilibrium distribution between the two states*. Such a mechanism is, of course, the basis of the classical chromatography of small molecules, but it is often stated[14,15] that with macromolecules having multiple strong binding sites there is usually an abrupt transition between high and low distribution ratios. Binding is thus expected to be an "all-or-nothing" process, and molecules once released should suffer little further retardation. Gradient elution is commonly employed to release sequentially the components in a mixture, rather than using isocratic elution at contant salt concentration.

Direct evidence of adsorptive retardation on Sepharose was obtained by isocratic elution at various salt concentrations. Fig. 4 (obtained with batch B, as in Fig. 2a) shows that a substantial fraction of the sample travelled at less than half the rate of the solvent; the higher the salt concentration, the greater the retardation. As expected, more material was released at the lower salt concentrations. What is most interesting is the existence of discrete peaks, suggesting that this might be a useful alternative method of fractionating tRNA.

* The effect is sometimes called "finite-adsorption equilibrium" to distinguish it from "tight adsorption".

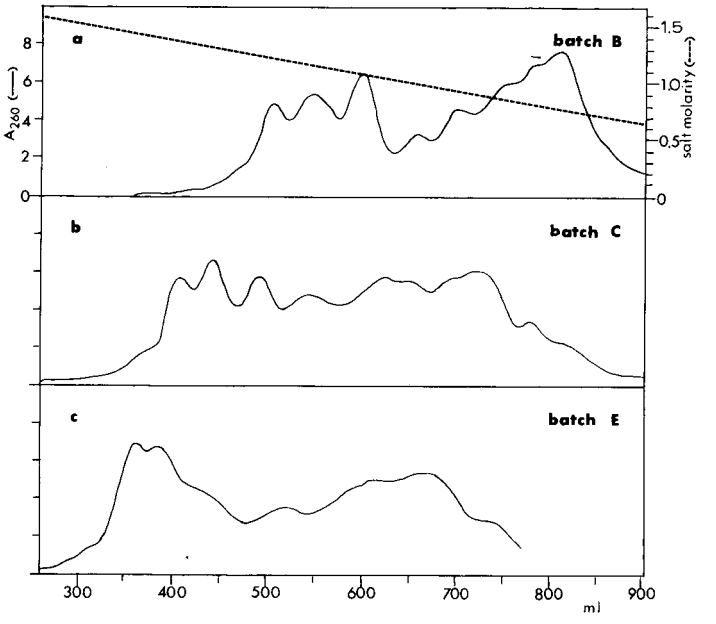

Fig. 5. Batch variations for a 2 × 750 ml reverse gradient of ammonium sulphate from 2.0 *M* to zero. Conditions otherwise similar to those in Fig. 2.

In view of this evidence for retardation it seemed likely that, although very little material eluted with the sample solvent when the gradient started at 1.3 *M* salt, it was not in fact all bound to the matrix at this salt concentration. The experiments illustrated in Fig. 2 were therefore repeated using gradients starting at 2.0 *M*. At this molarity some tRNA precipitated even before addition to the column. Fig. 5 shows that quite different profiles were then obtained; in particular, the large early peak given by batch E in Fig. 2c was resolved into multiple peaks by the more extended gradient in Fig. 5c. Evidently much of the material applied at 1.3 *M* salt was never fully immobilized on the column. Furthermore, even the later regions of the elution profile were not identical in the two cases. This suggested that there might be at least two distinct mechanisms at work, one responsible for "tight binding" and the other for adsorptive retardation after release, and that the various amino-acid accepting species of tRNA were not affected in the same way by the two mechanisms.

To attempt a separation of the two effects we compared elution from a short column with that from a much longer one, and conducted a limited number of assays for amino-acid acceptance. The same batch of Sepharose was used for each column. Elution was, as expected, much earlier in the gradient from the shorter column, and resolution was inferior to that obtained with the longer column. Results for the long column (Fig. 6b) were broadly similar to those shown in Fig. 1 for another long column, even though the batch and the gradient were not the same. However, for the short column (Fig. 6a and unpublished data for other acceptors), although most species eluted in a similar way there were some significant differences. Thus, although threonine acceptors eluted early from both long and short columns, the major valine acceptor eluted earlier in the short-column profile and the leucine profiles were quite different.

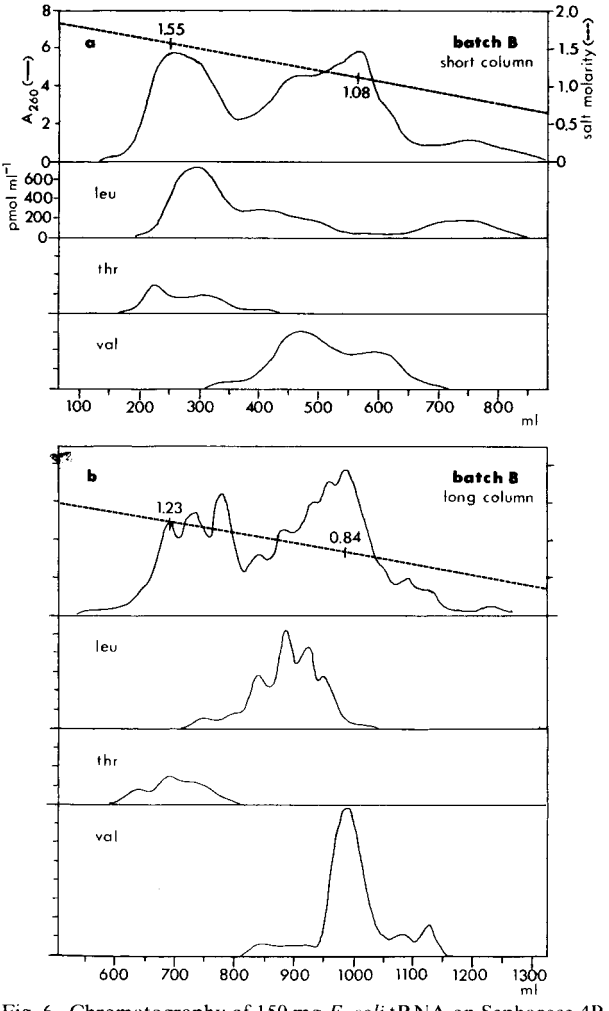

Fig. 6. Chromatography of 150 mg *E. coli* tRNA on Sepharose 4B, using 2 × 750 ml reverse gradients of ammonium sulphate from 2.0 *M* to zero. (a) Column, 100 × 16 mm; flow-rate, 72 ml h^{-1}. (b) Column, 1000 × 16 mm; flow-rate, 7 ml h^{-1}.

There are at least five isoacceptors for leucine[16], and only a full anticodon assay would show exactly what redistribution had occurred, but it is clear that the short and long columns did not retard all acceptors in the same sequence. There must therefore be some differences between the two mechanisms at work; with a single mechanism, changes in column length alone would not be expected to cause a redistribution of peaks.

This conclusion has some interesting corollaries. It must to some extent be possible to change the order of elution of species simply by varying the length of the column; if a species X is released ahead of species Y, it may still be overtaken by Y in its passage down a long column if Y suffers a lesser retardation after release. With a shorter column, however, X will emerge ahead of Y. Similarly, the application of a

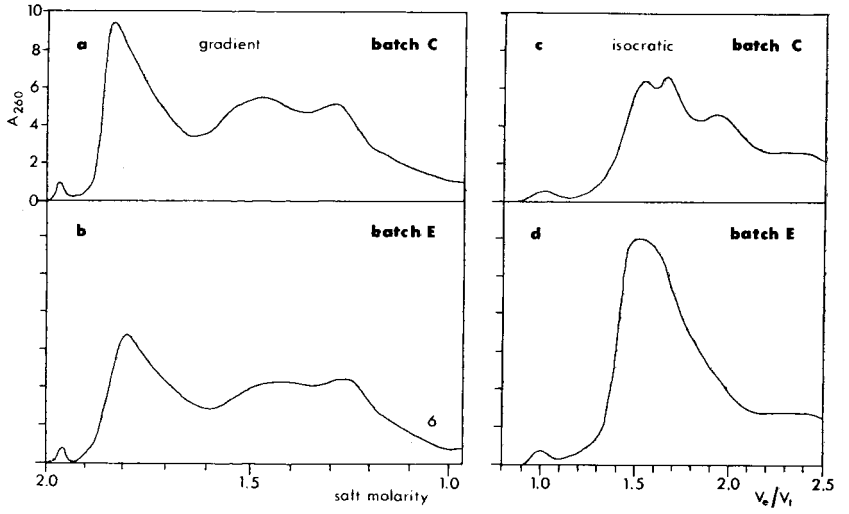

Fig. 7. Comparison of isocratic elution at 1.3 *M* ammonium sulphate, as in Fig. 4, with reverse gradient elution from 100 × 16-mm column as in Fig. 6.

gradient starting at a value lower than the limiting release molarity could change the order of elution, as suggested by comparing Figs. 2 and 5. Simply by varying parameters such as these one might achieve a higher degree of purification by successive refractionation. Yet another variant would be to follow isocratic elution by the application of a reverse gradient to the same column.

Further evidence for two distinct mechanisms is provided by Fig. 7. Between the two batches chosen there was little difference between the profiles obtained when a reverse gradient was applied to a short column: release from tight binding was similar in each case. With isocratic elution, however, batch C retarded more strongly than batch E, and gave a better resolution of peaks. This difference in adsorptive retardation is consistent with the long-column profiles of Fig. 5b and c.

The difference between the isocratic profiles in Fig. 7 is not simply attributable to a shift in the salt concentration associated with a given profile. Experiments at higher salt concentrations (not illustrated) with batches E and F, both of which gave a single peak at 1.3 *M* salt, showed more adsorptive retardation but failed to give the degree of resolution into multiple peaks that was characteristic of the other batches (Figs. 4 and 7, and unpublished data).

For the sake of completeness it would have been useful to perform gradient fractionation under conditions where the precipitation effect was negligible; one would then expect no variation of profile with column length. Unfortunately such experiments are not feasible, because to ensure the absence of precipitation one would have to start the gradient at a salt concentration well below the 1.3 *M* used in our initial experiments. Fig. 4 indicates that under these conditions, even for the exceptionally retarding batch B, the partition retardation would be so reduced in magnitude as to give inadequate resolution of peaks.

CONCLUSION

It is now clear that the differences between profiles obtained under different conditions are traceable to two causes: firstly a considerable batch variation in the range over which elution occurs, and secondly a surprisingly high degree of adsorptive retardation. This has previously obscured the fact that much of the tRNA is released from tight binding at higher salt concentrations than the values often used at the start of a reverse gradient.

The mechanism of fractionation seems to be a combination of two effects. There is evidently interfacial precipitation and sequential release of species by the reverse gradient, but subsequent adsorptive retardation has a profound effect on the profile and contributes much to the resolution of overlapping peaks. The variation between batches appears to lie much more in the adsorptive retardation than in the release from tight binding.

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REVERSE SALT GRADIENT CHROMATOGRAPHY OF tRNA ON UNSUBSTITUTED AGAROSE

II. FURTHER STUDY OF BINDING MECHANISMS

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SUMMARY

The existence of two mechanisms in the fractionation of tRNA on Sepharose 4B, using reverse gradients of ammonium sulphate, was confirmed by comparing yeast and *E. coli* tRNAs, and by extending the range of conditions used. The difference in elution range between the two kinds of tRNA is attributable mainly to variations in tight binding rather than adsorptive retardation. Variations of pH or of temperature produce not only changes in peak distribution, but overall shifts in the elution profile. The shifts correlate closely with changes in the solubility of tRNA in free solution, confirming that tight binding is associated with interfacial precipitation. On the other hand the degree of adsorptive retardation, as indicated by the difference between elution profiles from long and short columns, is quite similar under all conditions. It is if anything slightly greater at 25°C than at 5°C, implying a binding mechanism analogous to hydrophobic bonding. Binding-equilibrium studies suggest that the effect is related to the formation of a monolayer of tRNA on the agarose threads of the matrix. Experiments with hydroxyapatite also demonstrate adsorptive retardation, comparable in degree to that observed with Sepharose. This indicates that the effect may be much more important in ion-exchange chromatography than is normally assumed.

INTRODUCTION

In Part I[1] it was concluded that two mechanisms are involved in the fractionation of tRNA by reverse gradients of ammonium sulphate on Sepharose 4B. The first mechanism, believed to be interfacial precipitation, releases different tRNAs sequentially as the salt concentration is reduced. The second, which contributes largely to the high resolution of peaks obtainable by this technique, is a previously unsuspected adsorptive retardation of the kind more usually associated with the single-site binding of small molecules. The two mechanisms do not separate tRNAs in exactly the same sequence, so that they appear to be different in origin.

Any explanation of these effects must take account of reports[2,3] that the bind-

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ing of tRNA to Sepharose is much reduced at pH values away from the normally used value of 4.5, and that resolution appears to deteriorate as the temperature is raised[3]. In Part I[1] the elution profiles were all obtained at the same values of pH and temperature, so that possible variations in tRNA structure did not complicate the issue. In the present paper two sources of tRNA are compared, the range of conditions is extended, and elution profiles are compared with the solubility curves for tRNA in free solution.

MATERIALS AND METHODS

Sepharose 4B was purchased from Pharmacia. Batches denoted A, B, C etc. in the present paper were as in Table I of Part I[1]. *E. coli* tRNA was as used earlier[1]. Yeast tRNA (depleted of $tRNA^{Cys}$) was the gift of Professor C. J. O. R. Morris. Buffered solutions were adjusted in pH before addition of ammonium sulphate, which was "ultrapure" material from Schwarz/Mann. For work at 5°C, pH 7.5, solutions containing Tris–HCl were adjusted to pH 7.0 at room temperature to allow for the expected variation with temperature.

Solubility measurements were carried out at 5°C, using a water-bath regulated to ±0.5°C. Samples were made up at room temperature to a total volume of 5 ml and left overnight in the water bath; they were then centrifuged for 15 min at about 700 *g*. The supernatant concentration of tRNA was determined by spectrophotometry at 260 nm, diluting with 0.2 *M* ammonium sulphate and assuming a specific absorptivity ($A^{1\%}_{1\,cm}$) of 200.

Sepharose binding was measured in a similar way by adding to the mixture an aliquot from a settled slurry of Sepharose, previously equilibrated in a column with a solution containing 2 *M* ammonium sulphate and the appropriate buffer. The suspension was dispensed with a wide-bore volumetric pipette, specially calibrated to deliver the required volume; this was necessary because Sepharose tended to stick to the walls. Instead of standing in a water bath, tubes were left overnight at 5°C on a tube rotator (Stuart Scientific, Croydon, Great Britain) operating at *ca.* 60 rpm, and centrifuged as described above. From the measured volume of Sepharose after centrifugation, the concentration of agarose in the gravity-settled slurry was estimated to be 2.1 ± 0.2%.

RESULTS AND DISCUSSION

Comparison of yeast and E. coli tRNAs

Fig. 1 compares the fractionation of tRNAs from these two different organisms, using both long and short columns but the same batch of Sepharose throughout. It proved necessary to start the reverse gradient as high as 3.0 *M* ammonium sulphate because yeast tRNAs were less tightly bound, and also because the batch of Sepharose used bound all tRNAs rather less strongly than that used for the profiles in Fig. 6 of Part I[1].

The long-column profile of Fig. 1d differs markedly from that obtained by Morris[3] at pH 4.25, but in view of what is now clear about batch variations and other factors this is not very surprising. A more striking feature is the overall difference between tRNAs from yeast and *E. coli*; yeast tRNAs elute earlier in the gradient, and

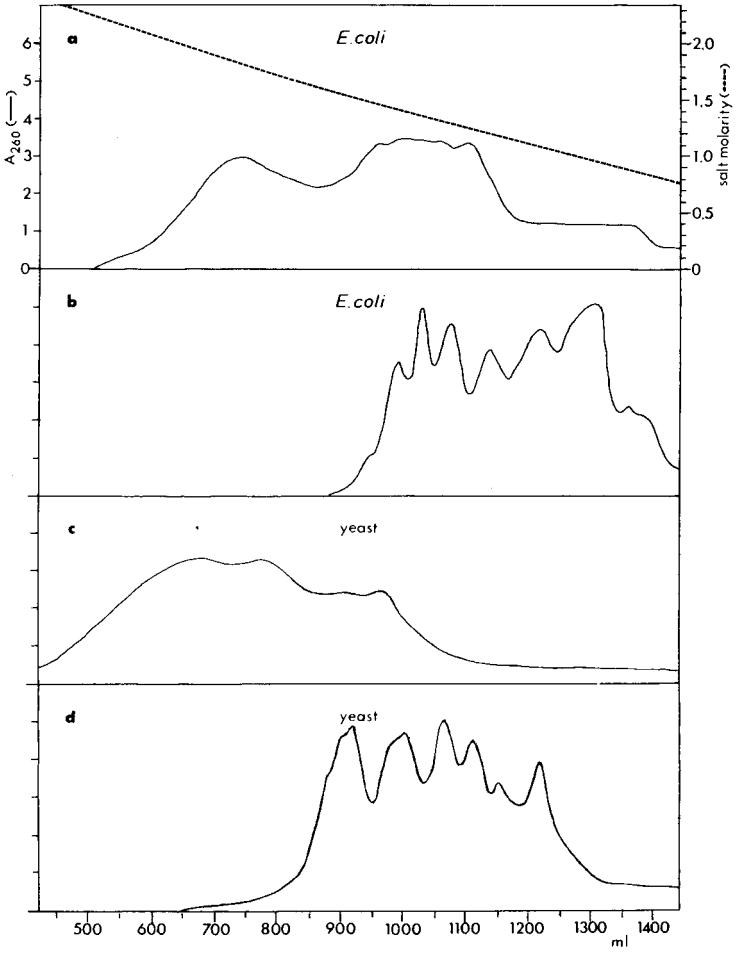

Fig. 1. Comparison of yeast and *E. coli* tRNA chromatography on Sepharose 4B (batch C) at 5 ± 0.5°C. All gradients were 2 × 1000 ml of ammonium sulphate from 3.0 *M* to zero; solutions also contained 10 m*M* magnesium chloride, 1 m*M* ethylenediaminetetraacetate (EDTA) and 10 m*M* sodium acetate buffer, pH 4.5. In (a) and (c) the column was 100 × 16 mm, in (b) and (d) 830 × 16 mm. Loads were 150 mg and flow-rates 15 ml h^{-1}. The plotted salt gradient applies to all experiments, and the volume shown is measured from the start of gradient elution from the bottom of each column.

Fig. 1 suggests that this is due mainly to a difference in the degree of tight binding rather than in the adsorptive retardation after release. The latter effect is equally strong in both cases.

Variations in profile with pH and temperature

Fig. 2 illustrates short- and long-column elution profiles of *E. coli* tRNA at 5°C, pH 4.5; 5°C, pH 7.5; and 25°C, pH 4.5. The apparent pH of the eluate in the second case was *ca.* 6.5 at 5°C, but this may have been an electrode artifact associated with the high concentration of salt[1]. The release of tRNA from the short column occurred earlier in the reverse gradient at pH 7.5 than at pH 4.5, and much earlier at

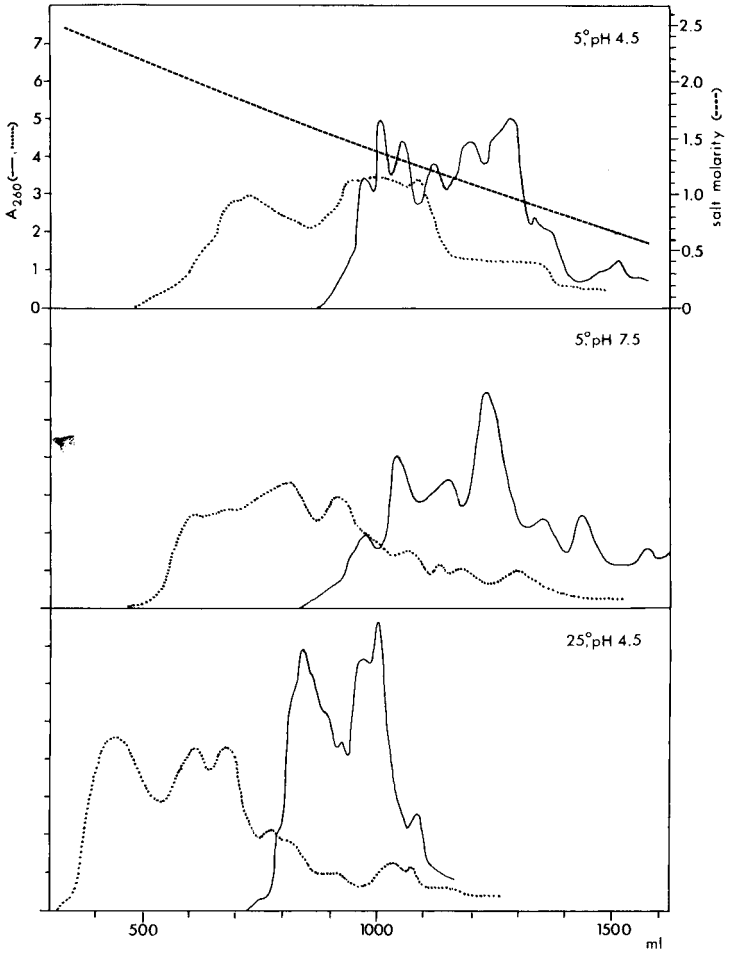

Fig. 2. Chromatography of *E. coli* tRNA on Sepharose 4B (batch C) under different conditions. Temperatures were controlled within ± 0.5°C. In each case the dotted line represents elution from a short column (*ca.* 100 × 16 mm) and the solid line, elution from a longer one (*ca.* 800 × 16 mm). Conditions were otherwise as in Fig. 1, except that for pH 7.5 the buffer was 10 m*M* Tris–HCl.

25°C than at 5°C. However, the degree of adsorptive retardation after release, as shown by the difference between long- and short-column profiles, was similar in all three cases; indeed, the separation of the two kinds of profile was somewhat greater at 25°C than at 5°C. This reinforces the conclusion[1] that there are two distinct binding mechanisms at work.

Fig. 2 shows that resolution is not greatly impaired by elevated pH or temperature, provided that the gradient starts at a sufficiently high salt concentration. The failure of Holmes *et al.*[2] to obtain any fractionation at pH 7.5 is now seen to arise from a combination of two factors; firstly their gradient started at 1.3 *M* salt, so that interfacial precipitation did not occur; and secondly, they used (for these experiments only) a relatively short column of 150 × 15 mm, so that adsorptive retardation was not able to develop. The observations of Morris[3] with yeast tRNA may be similarly

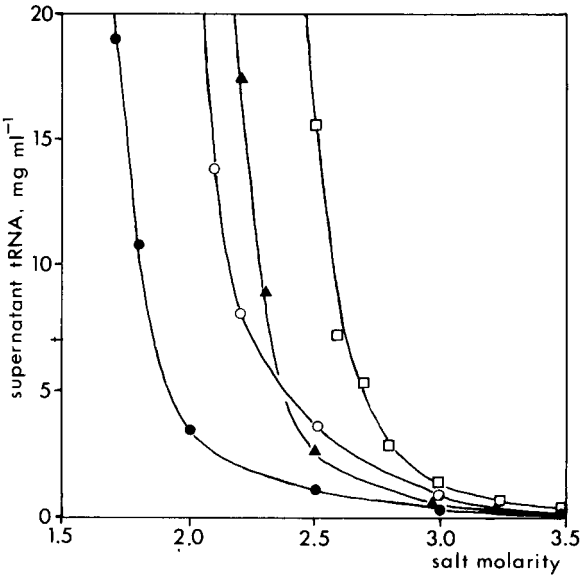

Fig. 3. Solubility of tRNA in ammonium sulphate. Solutions were as in Fig. 1; ●, *E. coli* tRNA, pH 4.5, 5°C; ○, yeast, pH 4.5, 5°C; ▲, *E. coli*, pH 7.5, 5°C; □, *E. coli*, pH 4.5, 25°C.

explained; his gradients all started at 2.0 *M* salt, which was probably too low for optimum resolution under some of the conditions used.

Solubility and binding studies

Fig. 3 illustrates measurements at two pH and temperature values of the solubilities in free solution of *E. coli* and yeast tRNAs, as a function of ammonium sulphate concentration. Because the tRNA from each organism contained a large variety of amino-acid accepting species it did not show a unique solubility; the amount left in solution at a given salt concentration depended on the total concentration of tRNA. The points plotted correspond to the onset of visible precipitation. It is clear that at pH 4.5 and a given salt concentration, yeast tRNA is considerably more soluble than material from *E. coli*; also for *E. coli* tRNA, the solubility is greater at pH 7.5 than at pH 4.5, and much greater at 25°C than at 5°C.

All these results are consistent with the hypothesis that a solubility-related process is important in the binding of tRNA to Sepharose, for the elution from short columns (where interfacial precipitation is presumed to be dominant) follows the same pattern of behaviour. Results reported here and in Part I[1] show that elution of *E. coli* tRNA is earliest at 25°C, pH 4.5 and latest at 5°C, pH 4.5, while similar intermediate behaviour is shown by *E. coli* tRNA at 5°C, pH 7.5 and by yeast tRNA at 5°C, pH 4.5.

The variations with pH are probably due to the combined effects of changes in ionization[4] and consequent changes in conformation[5]. However, comparison of Fig. 3 with the corresponding elution profiles (Figs. 1 and 2) shows that Sepharose induces binding at much lower salt concentrations than those at which tRNA precipitates from free solution. This is confirmed by Fig. 4, which illustrates the equilibria at-

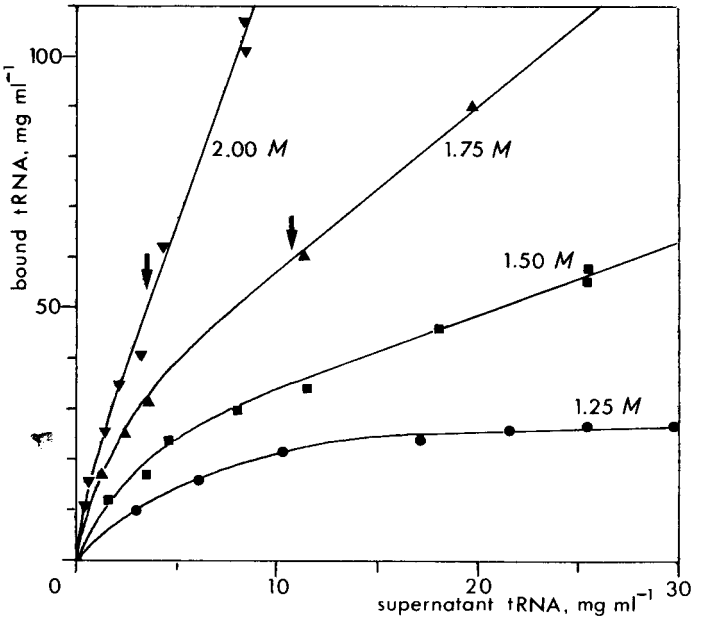

Fig. 4. Binding of *E. coli* tRNA to Sepharose 4B (batch E) at 5°C. All solutions contained pH 4.5 buffer and other salts as in Fig. 1. The ordinate represents the excess tRNA bound by 1 ml settled volume of Sepharose suspension. Arrows indicate the points at which precipitation begins in free solution (Fig. 3).

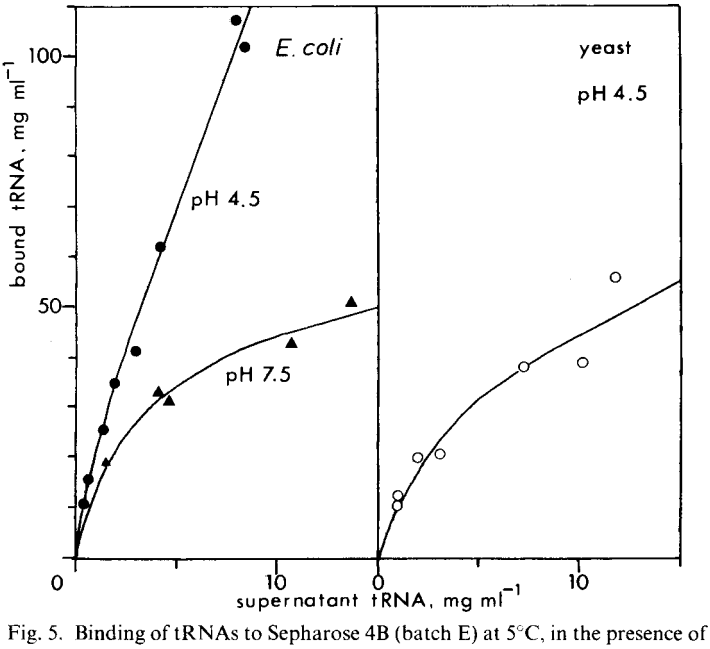

Fig. 5. Binding of tRNAs to Sepharose 4B (batch E) at 5°C, in the presence of 2.0 *M* ammonium sulphate. For further details see Fig. 4.

tained when Sepharose beads are added to solutions of tRNA and ammonium sulphate at pH 4.5. Strong binding occurs even at 1.25 *M* salt, where tRNA is highly soluble in free solution.

Fig. 5 compares the binding in 2.0 *M* salt of *E. coli* tRNA at pH 4.5 and pH 7.5 with that of yeast tRNA at pH 4.5. The last two curves resemble that obtained with *E. coli* tRNA at pH 4.5 and the lower salt concentration of 1.5 *M* (Fig. 4); it appears that a similar family of binding curves applies to each set of conditions.

At pH 4.5 and 1.25 *M* salt, the binding behaviour of *E. coli* tRNA (Fig. 4) approximates to the Langmuir isotherm expected when a monolayer of homogeneous solute attaches to the surface of a stationary phase. At all higher salt concentrations there is no plateau; such behaviour could be taken to indicate multi-layer adsorption, or more than one type of adsorption site, or a range of solute affinities for the substrate[6]. It seems almost certain that at the higher salt concentrations the curves are influenced by interfacial precipitation, but the different effects are not separable.

The origin of adsorptive retardation

Figs. 4 and 5 suggest that a Langmuir adsorption equilibrium, involving no more than a monolayer of tRNA on Sepharose, may be associated with the observed adsorptive retardation. Interfacial precipitation could differ from this because it involved intermolecular interactions between successive layers of tRNA, as well as direct interactions between tRNA and Sepharose. Molecules released from the multilayer precipitate could still then be susceptible to monolayer binding or retardation further down the column. It would thus be likely that tRNAs released together from the precipitate did not suffer the same subsequent retardation.

This hypothesis is strengthened by a numerical calculation from the binding data of Fig. 4. At 1.25 *M* salt, the binding levels off at a value of about 27 mg ml^{-1}. Since the slurry of Sepharose used in these experiments was about 2.1% (w/v) in agarose (see Materials and methods), the binding within a bead of 4% gel would be 51 mg ml^{-1}. If we accept the model for agarose structure proposed by Laurent[7], a 4% gel is composed of fibres with an average radius of 2.56 nm, and 1 ml of gel contains a fibre length of $2.36 \cdot 10^9$ m. Assuming a tRNA molecular weight of $2.6 \cdot 10^4$, this implies that the plateau region corresponds to a binding of one molecule for every 2.0 nm of agarose thread. This is roughly consistent with the known dimensions of tRNA[8], if one assumes that molecules are close-packed in linear arrays along the threads. An alternative calculation gives a thread surface area of 32 nm^2 per molecule.

Regarding the nature of the binding forces involved, we must reconsider the notion that something analogous to "hydrophobic bonding" is responsible. The idea was largely discounted by Morris[3] on the grounds that the observed binding became weaker as the temperature was raised, whereas hydrophobic forces are normally expected to become stronger. However, the data discussed here indicate that this is largely a consequence of changes in tRNA solubility, which influences the release of molecules from interfacial precipitate. The degree of adsorptive retardation *after* release is, as we have seen, at least as great at 25°C as at 5°C.

The binding of tRNA to agarose may be analogous to that occurring between the double helices of the agarose gel; in both cases, alterations in water structure could induce free energy changes favouring cohesion[9]. In agarose, the rigidity of the helices implies the absence of a configurational entropy term that favours solution[10].

Such effects (which could affect the balance either way) have been invoked to explain why flexible polynucleotides are less strongly bound to a substrate than more ordered molecules[11], but for tRNA it is reasonable to assume that under high-salt conditions (which are known to favour crystallization) the structure is stable.

It is significant that hydrophobic column substituents are known to enhance the binding of tRNA[12]; although agarose is hydrophilic, it does contain alternating residues of D-galactose and a more hydrophobic anhydrogalactose[13].

Comparison with hydroxyapatite

Since the use of reverse salt gradients is much less common than ion-exchange chromatography in positive-slope gradients, it was of interest to test whether adsorptive retardation was important in such methods. Comparable experiments were therefore performed with high-resolution hydroxyapatite[14], in which tRNA was bound at low ionic strength and displaced by a positive gradient of phosphate buffer[15]. The results led to the conclusion that adsorptive retardation was significant here as well:

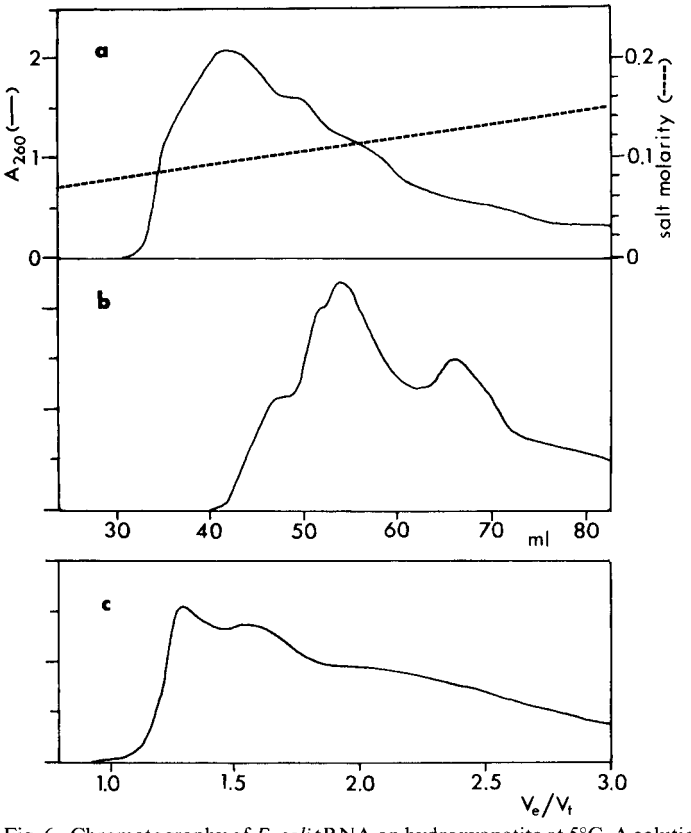

Fig. 6. Chromatography of *E. coli* tRNA on hydroxyapatite at 5°C. A solution containing 4 mg tRNA was applied, and eluted with sodium phosphate buffer, pH 7.0. (a) Gradient 2 × 60 ml of 0 to 0.2 *M* phosphate, column 30 × 9 mm; (b) as above but column 280 × 9 mm; (c) isocratic elution at 0.11 *M* from column 280 × 9 mm. Flow-rate, 3 ml h^{-1}. In (a) and (b) the volume plotted is measured from the start of gradient elution from the bottom of the column; in (c), V_t is the volume of liquid in the column and V_e the volume collected after application of the sample.

the profile for a long column was delayed compared with that of a short one and showed better resolution (Fig. 6a and b), while an isocratic run (Fig. 6c) showed retardation and apparent fractionation comparable to that found with Sepharose. This suggests that the theoretical arguments for neglecting adsorptive retardation in ion-exchange methods are of doubtful general validity. The binding of tRNA to hydroxyapatite should be a classic example of multi-site attraction; there is also evidence that binding is strongest when the linear dimensions of hydroxyapatite microcrystals approach those of a tRNA molecule[15]. The present results are, however, consistent with the empirical tradition among biochemists that even with gradient elution it is generally better to use a long, narrow column to obtain maximum resolution.

There are in fact precedents from the early history of ion-exchange chromatography, mentioned briefly in reviews[16], for the observation of partition retardation. In the development of cellulose derivatives for column chromatography Sober and coworkers[17,18] and Peterson[19] reported that when protein mixtures were applied to a column, some components were separable by elution at a constant salt concentration; most, however, required the application of a gradient of salt concentration and pH. The use of gradients subsequently became general in this field.

CONCLUSION

The unique character of the profile obtained under a given set of conditions shows the remarkable versatility of reverse salt gradient chromatography, and a better understanding of the underlying mechanisms should enable the method to be used to greater advantage. There remains an uncertainty about the origin of the batch variation, which affects mainly the adsorptive retardation effect. This effect seems on present evidence to be associated with Langmuir adsorption to the surfaces of agarose threads, by some mechanism analogous to hydrophobic bonding that presumably involves changes in the water layers surrounding substrate and solute.

The observed batch variations in adsorptive retardation (Part I[1], Fig. 7) could arise from either chemical or physical variations in the structure of the agarose gel. Although they might also arise from variable retention of the emulsifier used in manufacture, this seems to be ruled out by the persistence of the effects during repeated re-use of the material. The studies reported in Part III[20] were designed to test a number of possible causes.

The importance of adsorptive retardation in ion-exchange fractionation on hydroxyapatite raises the question of how general such an effect may be in the chromatography of large molecules, and there is clearly scope here for further study.

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REVERSE SALT GRADIENT CHROMATOGRAPHY OF tRNA ON UNSUBSTITUTED AGAROSE

III. PHYSICAL AND CHEMICAL PROPERTIES OF DIFFERENT BATCHES OF SEPHAROSE 4B

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SUMMARY

Four batches of Sepharose 4B, which gave differing elution profiles in reverse salt gradient chromatography of tRNA, were analysed by a number of techniques. Charged groups were estimated from infrared spectra, and gave 0.04–0.05 sulphates and 0.18–0.27 carboxyls per disaccharide. Nuclear magnetic resonance showed the presence of 0.21–0.23 6-O-methyl substituents per disaccharide. The mass concentrations of the beads in aqueous suspension (including agarose-bound water) ranged from 4.7 to 5.0%. High-resolution scanning electron microscopy demonstrated a three-dimensional network of threads with diameters between 2 and 20 nm. None of these properties varied appreciably between batches. There was, however, a variation in the size distribution of beads. In two batches which induced a high degree of adsorptive retardation during chromatography, very few beads exceeded 100 μm in diameter; two other batches which gave inferior retardation had a preponderance of somewhat larger beads. The significance of this observation remains unclear.

INTRODUCTION

In previous papers[1,2] it was established that two mechanisms operate in the reverse salt gradient chromatography of tRNA on Sepharose 4B. One mechanism involves interfacial precipitation, and varies with conditions in the same way as the solubility of the given tRNA in free solution. The other appears to be largely independent of the source of tRNA and to be very similar in magnitude at temperatures of 5°C and 25°C. It was suggested[2] that this mechanism, which induces adsorptive retardation of molecules after release from interfacial precipitate, involves the formation of a monolayer on the threads of the agarose network. The binding involved is probably analogous to "hydrophobic bonding".

Since adsorptive retardation was found to be subject to considerable variation between batches of Sepharose[1], a number of physical and chemical properties were measured in the hope of establishing a correlation with the variation in binding

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behaviour. The present paper reports these measurements, which are of intrinsic interest because some of the techniques have not previously been applied to Sepharose. The remaining uncertainty about the source of the batch variation is also discussed.

MATERIALS AND METHODS

tRNA was as used earlier[1,2], and batches of Sepharose 4B as specified in Table I of Part I[1]. Blue Dextran 2000 (lot No. 2678) was supplied by Pharmacia.

Infrared (IR) spectra were recorded on a Grubb–Parsons Spectromaster and replotted as absorbance against wavenumber. Samples were prepared from desalted Sepharose dissolved in boiling water and dried under vacuum on calcium fluoride plates. A similar plate was inserted into the reference beam.

Nuclear magnetic resonance (NMR) measurements were carried out by Messrs. D. Welti and S. M. Bociek of the Colworth Laboratory of Unilever Research, using a Bruker WP200 spectrometer. Material in aqueous suspension was first desalted by flushing with water in a small column. Samples in ^2H_2O were studied at 95°C, using 200.1 MHz for ^1H and 50.32 MHz for ^{13}C. Spectral peaks were identified by comparison with those reported for ^1H at 300 MHz (ref. 3) and for ^{13}C at 67.9 MHz (ref. 4). Peak areas were measured from expanded plots by cutting out and weighing the peaks.

Interference microscopy was carried out on a Zeiss WL microscope equipped with Smith–Lebedeff interferometer optics and a calcite Ehringhaus compensator. Material in aqueous suspension was first desalted as above.

Scanning electron microscopy was carried out on a JEOL Model JEM-200CX in the SEI mode, operated at 200 kV with the "spot size" control activated to reduce beam damage. Specimens were resuspended in acetone by sequential centrifugation through graded mixtures of acetone and water. They were then subjected to critical-point drying with carbon dioxide in apparatus E3000 supplied by Polaron (Watford, Great Britain). It was found advisable to allow 3 h for liquid exchange between the two periods of flushing with carbon dioxide. Beads were then sputter-coated with gold for 1 min at 10 mA at a distance of 40 mm from the cathode of apparatus SCD020 (Balzers, Balzers, Liechtenstein), filled with argon at a pressure of *ca.* 0.05 mbar. To ensure a reproducible thickness of coating it was necessary to monitor the absorbance of the deposit on a piece of filter paper: the first deposit obtained after the cathode had been exposed to air for some time was often less than those obtained by repeating the process.

Bead size distributions were obtained by measurement of photographs taken of aqueous suspensions of Sepharose, using bright-field light microscopy.

RESULTS AND DISCUSSION

Estimation of charged groups

Although agarose contains a lower proportion of charged residues than the agar from which it is purified, residual charges remain which are attributed to sulphate and carboxyl groups (including pyruvate)[5,6]. It was initially hoped that these could be estimated by direct titration. Material was first desalted by exhaustive flush-

ing with water distilled after passage through a deionizer. Subsequent addition of salt led to a fall in pH due to release of protons from the agarose. However, the charge concentrations calculated from these experiments were all much lower than those deduced by the second method described below, and it was concluded that the groups were probably not fully protonated under the conditions used.

The second method involved the recording of IR spectra. Sulphate esters of carbohydrates show a strong S = O stretching vibration at 1240 cm^{-1}, though there are many overlapping bands in this region[7]. It is also possible, with samples dried from different pH values, to estimate carboxylic and pyruvic acid residues; when protonated these give a C = O stretch vibration at 1725 cm^{-1}.

Fig. 1 illustrates a typical pair of spectra from one batch of Sepharose. There is only a small peak at *ca.* 1250 cm^{-1}, but a much larger one at 1725 cm^{-1} which is present only at acid pH.

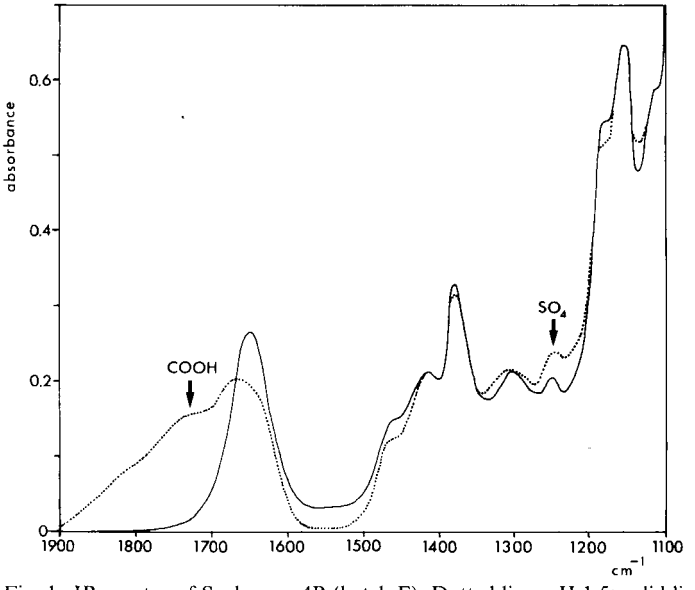

Fig. 1. IR spectra of Sepharose 4B (batch E). Dotted line, pH 1.5; solid line, pH 7.

To calculate an upper limit to the sulphate content, the peak height at *ca.* 1250 cm^{-1} was estimated by assuming a background contribution from neighbouring peaks, and comparing with the absolute height of the peak at 1155 cm^{-1} which arises from the antisymmetric bridge oxygen stretching vibration. The ratio thus obtained was compared with that calculated from the spectrum of alginic acid containing three sulphate residues per disaccharide[7,8]. For carboxyl content, the difference between spectra at pH 1.5 and 7.0 gave a peak at 1725 cm^{-1} which was compared with that shown by hyaluronic acid having one carboxyl residue per disaccharide[7,8]. The absorbance ratio with the peak at 1155 cm^{-1} was again used to normalize the spectra.

Results are summarized in Table I. Sulphate values appeared higher than that obtained by Hjertén[6] from direct sulphur analysis of purified agarose (0.14%, w/w, or 0.013 sulphates per disaccharide), but they were in any case very similar for all

TABLE I

PROPERTIES OF SEPHAROSE 4B

Batch	*No. of groups per disaccharide (mol. wt. 306)*			*Mass concentration of beads (%)*
	*Sulphate**	*Carboxyl**	*O-Methyl***	
C	0.04	0.27	0.21	5.0
D	0.04	0.21	0.22	4.8
E	0.05	0.26	0.23	4.8
F	0.04	0.18	0.22	4.7

* By IR spectroscopy; ± 0.03.
** By NMR spectroscopy; ± 0.01.

batches; there was no variation that could explain the difference in fractionation behaviour[1] between Sepharose batches C and E. Carboxyl values were also similar for these two batches. Total calculated charge contents were, for all batches, *ca.* 0.3 per disaccharide or 1 mequiv. g^{-1}. Since a packed column of wet Sepharose 4B contains about 3% agarose, this means a charge content of *ca.* 0.03 equiv. l^{-1}.

Ion-exchange resins commonly show capacities of the order of 1 equiv. l^{-1}. Thus the values for Sepharose, though much lower, are by no means negligible. For the monolayer of tRNA discussed in the previous paper[2], about twenty charges would be in the vicinity of each tRNA binding site. Against this must be set the fact that the high salt concentrations used will tend to shield the charges. The charges are of course negative, so they would not be expected to contribute to a positive attraction of tRNA to Sepharose. They could, however, modify the effect of some other mechanism. To explain the batch variation in fractionation behaviour, the charges would need to vary in their *distribution* along the threads, but there is no evidence on this point.

Methylation of agarose

NMR spectra showed no major differences between batches. ^{13}C Spectra (not illustrated) were largely as expected for pure agarose[4], but ^{1}H spectra (Fig. 2) indicated appreciable 6-O-methyl substitution[3]. Values are tabulated in Table I. Although this again did not explain the variations in chromatographic behaviour, such methylation must be presumed to contribute to "hydrophobic bonding" between solutes and agarose.

Concentration measurements

Interference microscopy was used both to check the concentration of agarose in the beads of different batches, and to monitor specimen changes during preparation for electron microscopy. In order to convert optical path differences into concentrations it was first necessary to determine specific refractive increments in both water and air. These were obtained indirectly from refractive index and density measurements, using the formula for the increment $\alpha = (n - n_m)/100\varrho$; n and n_m are the refractive indices of suspended solid and liquid medium respectively, and ϱ is the density of the solid. Material was prepared by critical-point drying (see Materials and

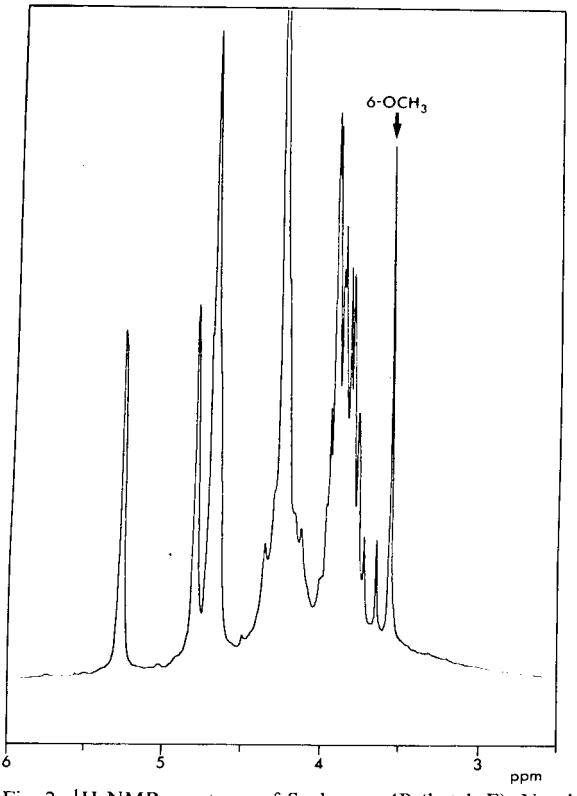

Fig. 2. ^{1}H NMR spectrum of Sepharose 4B (batch E). Number of scans, 200. Shifts are relative to an arbitrary reference point as no internal standard was used.

methods) and n determined to be 1.54 by resuspension of both dry and acetone-suspended beads in a range of oils of known refractive index, ϱ was determined to be 1.36 by flotation of beads in mixtures of acetone and F113 (1,1,2-trichloro-1,2,2-trifluoroethane). The rather low density value suggests that preparation by the method used does not remove bound water, which is believed to lie in the central cavity of each agarose helix[9]. Calculation then gives $\alpha = 1.5 \cdot 10^{-3}$ for a 1% concentration change in water, and $\alpha = 4.0 \cdot 10^{-3}$ in air.

For each batch of Sepharose a range of bead sizes was examined, and for each bead the diameter and optical path difference at the centre were recorded. Assuming the thickness at the centre to be equal to the measured diameter, the optical path difference, Δ, was then plotted against diameter, d, to give a straight line (Fig. 3) whose slope was determined. The percentage concentration c was obtained from the equation $c = \Delta/\alpha d$. Table I shows that for untreated Sepharose beads in water, c was in all cases between 4.7 and 5.0%. These values include the bound water. The minor variations in c did not correlate with variations in fractionation behaviour.

Scanning electron microscopy

Fig. 4 illustrates high-resolution scanning electron microscopy of the surface of Sepharose beads, after critical-point drying. Interference microscopy after drying

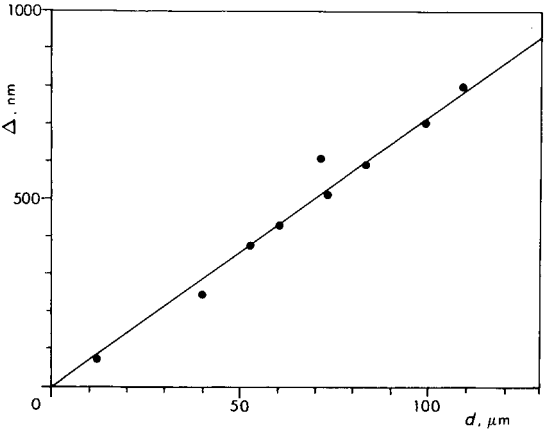

Fig. 3. Plot of optical path difference at 546 nm against bead diameter, for an aqueous suspension of Sepharose 4B (batch E).

indicated concentration increases consistent with a dimensional shrinkage of *ca.* 20 %; on the other hand, conventional freeze-drying produced gross distortions.

The thickness of the gold coating was estimated from separate experiments (not illustrated) in which the time of sputtering was varied and the average thread diameter measured; as a further check, coatings were deposited on glass and their thickness measured by multiple-beam interferometry[10]. For Fig. 4 the coating was calculated to have a thickness of 3 nm, giving an increase of up to 6 nm in the apparent thickness of each thread. The apparent thicknesses ranged between *ca.* 8 and 26 nm, so that the bundles of agarose helices had diameters between 2 and 20 nm. These values are consistent with observations by others[11] of stained and sectioned

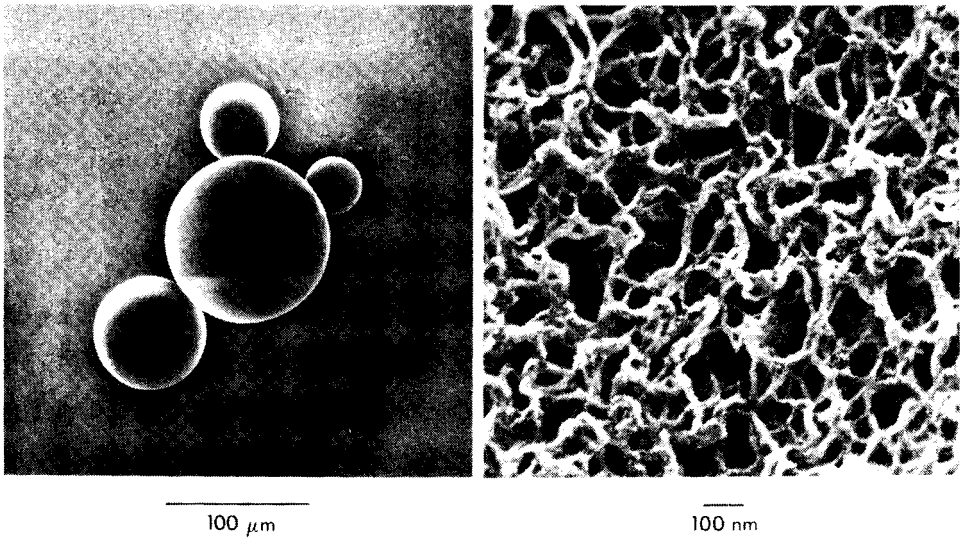

Fig. 4. Scanning electron microscopy of Sepharose 4B (batch E).

beads. There was no obvious variation between batches, nor between large and small beads.

Bead size distributions

Fig. 5 illustrates histograms of bead size distribution, representing the percentage of column *volume* occupied by beads of different diameter. It is apparent that batches E and F differed from C and D, in that they contained a higher proportion of beads with diameter greater than 100 μm. Batch E showed the largest spread in diameters of all the batches. It was previously established that isocratic fractionation profiles of batches C and D were similar, showing the multiple peaks illustrated in Fig. 7c of Part I[1]. In contrast, batch E showed reduced retardation and inferior resolution of the kind illustrated in Fig. 7d of Part I. Batch F (not illustrated) gave intermediate behaviour, with poor resolution but fairly high retardation.

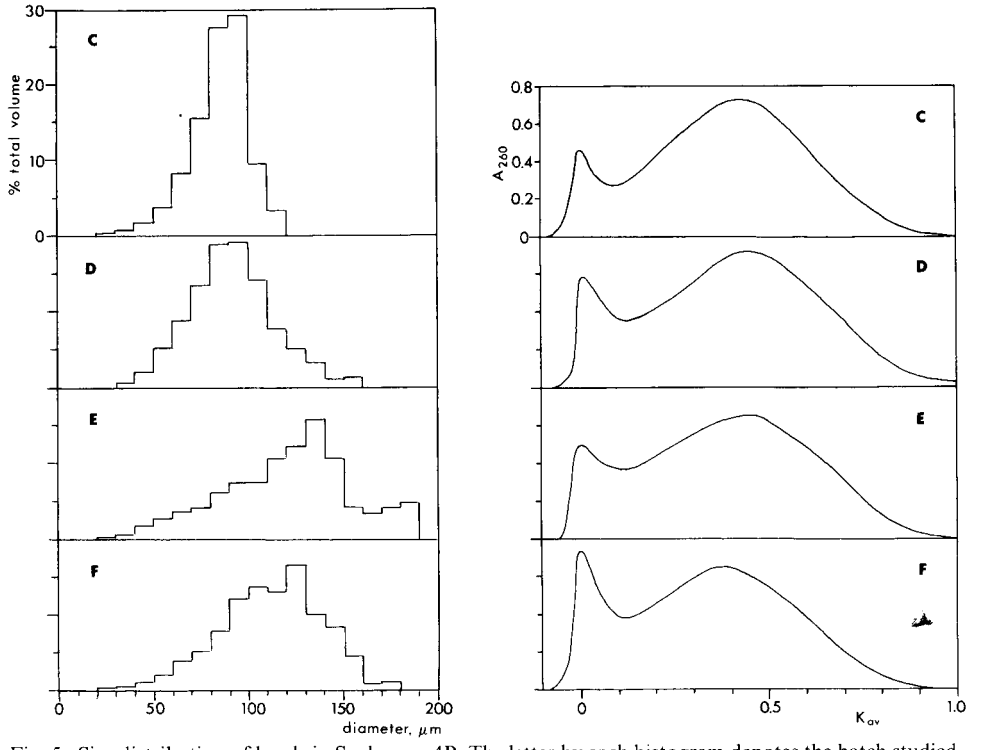

Fig. 5. Size distribution of beads in Sepharose 4B. The letter by each histogram denotes the batch studied. For each range of diameters, the percentage of total volume occupied by the beads is plotted as ordinate. The number of beads measured for each histogram was about 700.

Fig. 6. Blue Dextran 2000 elution profiles. The letters indicate the batch of Sepharose 4B used. In each case a small aliquot of 0.3% blue dextran was applied to a column previously equilibrated at 5°C with 1.3 *M* ammonium sulphate, 10 m*M* magnesium chloride, 1 m*M* EDTA and 10 m*M* sodium phosphate buffer, pH 4.5. Elution was carried out at 15 ml h^{-1} using the same solution. Column diameters were 16 mm and the bed lengths 775, 555, 520 and 830 mm for batches C, D, E and F, respectively. K_{av} is defined as $(V_e - V_0)/(V_t - V_0)$ where V_t = total bed volume, V_0 = excluded (void) volume and V_e = eluted volume. For the four experiments illustrated, V_t/V_0 (a measure of the tightness of packing) had values of 2.9, 2.8, 2.7 and 2.7.

One can only at present speculate on the meaning of these results. In the absence of significant differences in chemical composition or small-scale structure, one might suggest an effect related to the rate of depth of penetration of tRNA into the beads. However, an experiment in which tRNA was stained immediately after binding to a column bed (not illustrated) gave no support to this idea; tRNA seemed uniformly distributed throughout the beads. One would not, therefore, expect a variation of resolution with particle size of the kind experienced when molecules are adsorbed to the surface of solid beads. We have already seen in Part II[2] that the binding data are consistent with the formation of a monolayer of tRNA on the whole of the agarose matrix; the external surface of each bead would offer only a small fraction of the total agarose surface.

Pore size distributions

As a semi-quantitative test both of the exclusion limit and the pore size distribution, Blue Dextran 2000 was applied to the four batches of Sepharose listed in Table I. Results are illustrated in Fig. 6. The manufacturers state that blue dextran has an average molecular weight of $2 \cdot 10^6$, while the exclusion limit for Sepharose 4B is $5 \cdot 10^6$. The peaks in Fig. 6 at $K_{av} = 0$ show that some of the blue dextran exceeded this limit, while most of it ran in a broad peak centred on $K_{av} = 0.4$. Although the four profiles are not identical, no single feature seems to correlate with the batch variation in adsorptive retardation; although the broad peak for batch F has a slightly smaller K_{av} than in the case of batches C and D, batch E does not show the same effect.

CONCLUSION

A further exploration of batch variations will be needed to decide whether the bead size distribution is as relevant as the present results suggest, or whether the correlation is fortuitous. It might also be useful to separate beads in different size ranges from a given batch, and to test each range separately by the techniques described in this series of papers. For the samples studied in the present work there was unfortunately an insufficient quantity to make this feasible. The variation in adsorptive retardation is evidently not related to any gross change in physical or chemical properties. Something in the manufacturing process may have varied; on the other hand, the batches studied were manufactured over a period during which there was a change in the supplier of raw material (personal communication from Pharmacia).

Until there is a clear-cut explanation of the variations, it will be necessary for users of the reverse-gradient technique to test each batch separately. Although there are large batch variations in adsorptive retardation, Fig. 5 of Part I[1] shows that reasonably satisfactory fractionation of tRNA is possible if a sufficiently extended gradient is employed. However, the best resolution is undoubtedly associated with a high degree of adsorptive retardation, and it is unfortunate that recently manufactured batches (such as E and F) were found to be inferior in this respect. For isocratic work, only the earlier high-retardation material would be suitable.

The results in the present paper indicate that bound charges and methyl substituents may both have a minor influence on the binding properties of Sepharose, since they are present in far from negligible amounts. The scanning electron micros-

copy demonstrates the unusual stability of the three-dimensional agarose structure, provided that drying artifacts are avoided; Sepharose beads might well serve as useful model specimens for the preparative techniques used in studying biological structures such as cytoskeletal networks.

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IDENTIFICATION D'ALCANES LOURDS (MODÈLES DE POLYÉTHYLÈNE BASSE DENSITE) PAR LEUR TENSION DE VAPEUR

CORRÉLATION AVEC LA CHROMATOGRAPHIE EN PHASE GAZEUSE

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SUMMARY

Identification of heavy hydrocarbons (models of low-density polyethylene) by their vapour pressure. Correlation with gas chromatography

The retention indices and the vapour pressure (10^{-3} to 10 mm Hg) of mono and branched heavy hydrocarbons, models of low-density polyethylene, are given (C_{11}–C(R)–C_{11}, R = methyl to octyl; C_{10}–C(R)–C_n–C(R)–C_{10} R = methyl, ethyl, butyl, n = 4, 5, 6, 8).

It is shown that for branched heavy hydrocarbons the decrease of the retention indice is mainly due to the length of the substituent group but also to its number in the chain.

A new method for the analysis of higher branched hydrocarbons is proposed.

INTRODUCTION

L'étude d'alcanes légers par chromatographie en phase gazeuse a donné lieu à plusieurs travaux ces dernières années[1–3]. Les résultats obtenus par ces auteurs ont montré qu'il était possible d'établir des relations entre les indices de rétention et les structures et de prévoir par règles incrémentielles les indices de composés de la même famille.

Ces données ont trouvé des applications importantes dans la détermination des programmes d'analyse de mélange complexe d'hydrocarbures. Cependant, si les alcanes légers ont été très étudiés, par contre les alcanes lourds ramifiés n'ont donné à notre connaissance que peu de travaux.

Nous nous sommes intéressés dans ce travail à l'étude d'alcanes lourds (C_{23} à C_{38}) présentant une ou deux ramifications dans la chaine. Ces composés représentant des modèles de polyéthylène basse densité pour des études par spectroscopie infrarouge et par résonance magnétique nucléaire du ^{13}C (Bibl. 4 et 5). Pour cette série de

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composés nous avons déterminé les indices de rétention, puis leur tension de vapeur à partir d'un appareillage récemment mis au point au laboratoire[6]. Une analyse des résultats suivant les branchements a été faite et une comparaison des méthodes est proposée.

EXPÉRIMENTAL

Appareillage

Chromatographie. Les divers composés étudiés ont été chromatographiés (chromatographe Intersmat IGC 16) sur un solvant chromatographique le SE-30 (silicone méthylé) de la Général Electric. Les colonnes ont été réalisées par nos soins. Le support est du Chromosorb NAW de granulométrie 0.16–0.20 mm. Le tube est en acier inox de diamètre 1/8 de pouce et 4 m de longueur. Le taux d'imprégnation du support en solvant est de 2%.

Tension de vapeur. L'appareil de mesure de pression de vapeur utilisant la méthode de saturation d'un gaz inerte (pressiostat) a été fabriqué au laboratoire[6].

Nous rappellerons qu'il se comporte principalement de trois parties: un injecteur, un tube de saturation de la vapeur en soluté enfermé dans un thermostat et un détecteur à ionisation de flamme. L'ensemble est parcouru par de l'azote dont le débit régulé est compris entre 5 et 20 ml/min.

Les deux principes d'utilisation de l'appareil sont: (1) une propreté absolue de l'injecteur et du saturateur afin d'éviter tout phénomène d'adsorption; (2) étant donné que la concentration du soluté dans le gaz inerte est celle obéissant aux lois établies à l'endroit le plus froid de l'appareillage il est nécessaire que l'injecteur, le détecteur ainsi que leurs raccords avec le saturateur soient portés à une température supérieure à celle de la mesure.

La limite inférieure de détection est fonction de la nature chimique du composé (facteur K) et de son nombre d'atomes de carbone. On peut descendre beaucoup plus bas avec des produits possédant de nombreux atomes de carbone qu'avec des produits légers. La limite supérieure est fixée par la non conformité des équations établies pour $P^\circ \ll P$ et le seuil de saturation du détecteur. En moyenne le domaine de mesure s'étend d'environ $5 \cdot 10^{-4}$ à 10 mm de mercure.

Les quantités injectées sont fonction de P', de la température et du débit. Elles peuvent être de quelques dizaines à quelques fractions de microlitre, afin de pouvoir obtenir un palier de saturation de 2 à 10 min.

Origine des produits

Nous avons synthétisé les produits au laboratoire, leur mode de préparation variable selon les séries ainsi que leurs données spectroscopiques ont été décrits dans la littérature[4,5].

Méthodes

Chromatographie. La position des pics chromatographiques est caractérisée par l'indice de rétention de Kováts calculé par la relation:

$$I_x = 100 \frac{\log d'_{R(x)}/d'_{R(z)}}{\log d'_{R(z+1)}/d'_{R(z)}} + 100\, z$$

où $d'_{R(z)}$ et $d'_{R(z+1)}$ sont les distances de rétention réduites (corrigées du volume mort) de deux *n*-alcanes successifs possédant z et $z + 1$ atomes de carbone et dont les pics chromatographiques encadrent celui du composé x de distance de rétention $d'_{R(x)}$. La précision de ces valeurs généralement admise est de ± 2.5 unités d'indice.

Tension de vapeur. Nous rappelerons brièvement la théorie qui nous a permis d'étudier les hydrocarbures lourds faisant l'objet de ce travail.

La fraction molaire x_i d'un composé dans une vapeur est proportionnelle à sa pression de vapeur P_i dont la valeur à saturation est P_i° (pression de vapeur saturante):

$$x_i = \frac{n_i}{n_i + N} = \frac{P_i^\circ}{P} \tag{1}$$

où P est la pression totale, n le nombre de molécules de soluté i et N le nombre de molécules de gaz inerte.

La pression totale étant maintenue constante, la mesure de P_i° se confond avec celle de x_i. Si, de plus, on se limite à la mesure de petites pressions de vapeur, x_i est assimilable au rapport molaire n_i/N:

$$n_i = \frac{P_i^\circ N}{P} \tag{2}$$

On dispose de nombreux dispositifs sensibles à la variation de composition d'un milieu gazeux. Le système choisi est un détecteur à ionisation de flamme. Rappelons, très brièvement, que cet appareil délivre un signal électrique proportionnel au nombre d'atomes de carbone ionisés par unité de temps. La tension délivrée par l'amplificateur opérationnel est:

$$E = k\, n_i\, Z_i \tag{3}$$

où Z_i est le nombre d'atomes de carbone de la molécule et k une constante d'appareillage. Selon l'équation 2

$$E = k \frac{P_i^\circ Z_i N}{P} \tag{4}$$

La réponse du détecteur est donc liée à la pression de vapeur du composé, au débit du gaz vecteur, et au coefficient k lui-même lié à la nature chimique du composé.

On trouvera, dans la littérature, l'étude de la réponse de différentes familles chimiques au détecteur à ionisation de flamme.

Si la mesure est effectuée par rapport à un étalon E, on peut écrire:

$$\alpha = \frac{E_i}{E_E} = K \frac{P_i^\circ Z_i}{P_E^\circ Z_E} \tag{5}$$

En prenant en compte une règle assez générale en série linéaire qui veut que la pression de vapeur soit liée au nombre d'atomes de carbone par:

$$\log P_i^\circ = a\, Z_i + b$$

et en considérant que a est lié à la température par une fonction puissance du type:

$$a = A\ T^B$$

autorisant un plus grand intervalle de température que la classique équation d'Antoine, on arrive à une relation générale permettant l'estimation des réponses obtenues expérimentalement:

$$\log \alpha = A(Z_i - ZE)\ T^B + 100 \log K \frac{Z_i}{ZE} \qquad (6)$$

L'erreur expérimentale est directement liée à la stabilité de la température du thermostat. En effet on peut estimer une variation de 5% par degré.

RÉSULTATS ET DISCUSSION

Chromatographie (Tableaux I et II)

Nous avons rassemblé sur le Tableau I les indices de rétention des alkyl-12, tricosanes (composés monobranchés de formule C_{11}–C(R)–C_{11}, R = méthyl à octyl) et de composés diramifiés de formule C_{10}–C(R)–C_n–C(R)–C_{10} (R = méthyl, éthyl, butyl, n = 4, 5, 6, 8) et sur le Tableau II les incréments d'indice déterminés pour chaque ramification. Avec les colonnes chromatographiques utilisées nous ne pouvons espérer séparer les diastéréoisomères pour les composés dibranchés. Les études effectuées par résonance magnétique nucléaire ^{13}C semblent faire paraître qu'il n'est possible de séparer qu'en dessous de $n = 2$.

On remarque:

(a) sur le Tableau I que les valeurs des indices déterminés à deux températures 220 et 270°C sont très peu dépendantes de la température. Ces résultats sont en accord avec les données de la littérature[1–3];

TABLEAU I

INDICES DE RÉTENTION SUR SILICONE SE-30 DES DÉRIVÉS C_{11}–C(R)–C_{11} ET C_{10}–C(R)–C_n–C(R)–C_{10}

R	*I monobranchés*		$I_{270°C}$ *dibranchés*			
	$I_{220°C}$	$I_{270°C}$	$n = 4$	$n = 5$	$n = 6$	$n = 8$
Méthyle	2340	2340	2670	2774	2867	3068
Éthyle	2405	2414	2809	2903	3008	3205
Propyle	2478	2480				
Butyle	2560	2565	3090	3188	3298	3485
Pentyle	2648	2650				
Hexyle	2735	2745				
Heptyle		2839				
Octyle		2930				

TABLEAU II

INCRÉMENTS D'INDICE ΔI POUR UNE RAMIFICATION (T = 270°C)

R branchement	$\Delta I^{\star;\star\star}_{100}$	$\Delta I^{\star\star}_{270}$ *monobranchés*	$\Delta I^{\star\star}_{270}$ *dibranchés*			
			n = 4	*n* = 5	*n* = 6	*n* = 8
Méthyl	− 68	− 60	− 64	− 63	− 66	− 66
Éthyl	− 95	− 86	− 95	− 98	− 96	− 97
Propyl	−118	−120				
Butyl	−133	−135	−155	−156	−151	−157
Pentyl	−137	−150				
Hexyl		−155				
Heptyl		−161				
Octyl		−170				

* Valeurs de Rappoport et Gaumann[3] sur les alcanes monoramifiés légers (C_7 à C_{10}).
** ΔI = I alcane ramifié − I alcane normal ayant le même nombre d'atomes de carbone.

(b) sur le Tableau II que les incréments d'indice ΔI_R d'un branchement sont différents de ceux des dibranchés; ceci semble indiquer qu'il n'y a pas additivité incrémentielle des effets de substituants lorsqu'on passe d'un dérivé monobranché à un dérivé dibranché (quel que soit le nombre de carbones qui sépare les branchements). La décroissance observée avec la longueur de la chaine est en accord avec les travaux de Rappoport et Gaumann[3] sur les alcanes légers.

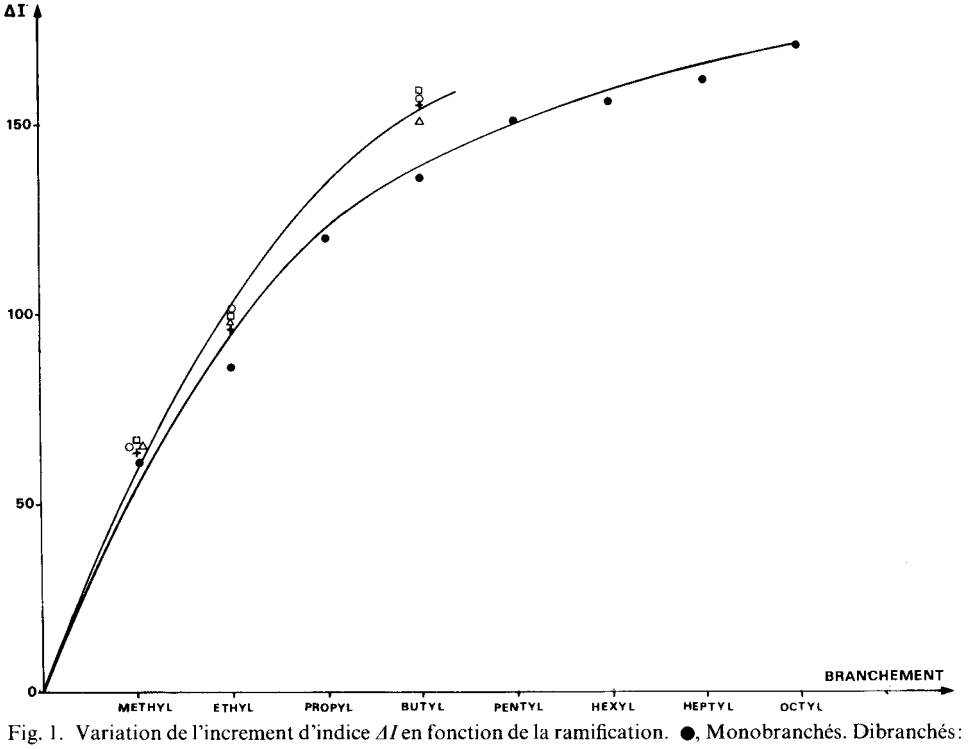

Fig. 1. Variation de l'increment d'indice ΔI en fonction de la ramification. ●, Monobranchés. Dibranchés: +, n = 4; ○, n = 5; △, n = 6; □, n = 8.

TABLEAU III

log α ET PRESSION DE VAPEUR DES COMPOSÉS DE LA SÉRIE C_{11}–C(R)–C_{11} A T = 161.9 ET 180.8°C

Ref.: *n*-octacosane, K = 1.

Composé	Z	*Température (°C)*					
		161.9 ± 0.1			*180.8 ± 0.1*		
		log α	P°	I_{P°	*log α*	P°	I_{P°
Méthyl-12,tricosane	24	0.987	0.28	2336	0.898	0.74	2338
Ethyl-12,tricosane	25	0.854	0.20	2404	0.766	0.53	2408
Propyl-12,tricosane	26	0.700	0.13	2480	0.629	0.37	2481
Butyl-12,tricosane	27	0.528	0.09	2562			
Pentyl-12,tricosane	28	0.334	0.05	2656			
Hexyl-12,tricosane	29	0.121	0.033	2756			
Heptyl-12,tricosane	30	−0.066	0.021	2839			
Octyl-12,tricosane	31	−0.260	0.014	2930			

La Fig. 1 montre l'écart des indices entre l'alcane ramifié par rapport à l'alcane linéaire correspondant. On constate qu'à partir d'une ramification à cinq chainons (pentyl) le ΔI_R demeure constant. Les groupements méthylènes supérieurs possédent alors une valeur indiciaire de 100 comme ceux des chaines linéaires.

Pression de vapeur (Tableaux III et IV). Nous avons pour analyser les composés étudiés dans ce travail pris comme étalon le *n*-octacosane. A partir de valeurs[8] précédemment publiées sur les *n*-alcanes (C_6 au C_{20}), l'équation d'Antoine pour les pressions de vapeur inférieure à 1 mmHg est

$$\log P^\circ = 10.766 - \frac{5383.0}{T\,(^\circ\mathrm{K})}$$

Si l'on estime que les coefficients d'activité sont proches, l'indice de rétention des

TABLEAU IV

log α ET PRESSION DE VAPEUR DES COMPOSÉS DE LA SÉRIE C_{10}–C(R)–C_n–C(R)–C_{10} À 161.9°C

Ref.: *n*-octacosane, K = 1.

n	*R*								
	Méthyle			*Éthyle*			*Butyle*		
	log α	*log α calculé*	P°	*log α*	*log α calculé*	P°	*log α*	*log α calculé*	P°
4	–	0.29	–	–	0.009	–	–	−0.574	
5	0.09	0.07	0.030	−0.19	−0.19	0.015	−0.81	−0.784	0.0031
6	−0.11	−0.12	0.018	−0.39	−0.41	0.009	−1.00	−1.021	0.002
8	−0.49	−0.55	0.007	−0.85	−0.83	0.003	−1.40	−1.422	0.0007

composés étudiés peut être calculé à partir de l'équation suivante:

$$I_{p^\circ} = 100 \frac{\log P_z^\circ / P_x^\circ}{\log P_z^\circ / P_{z+1}^\circ} + 100\, Z$$

x, z et $z + 1$ ayant leur signification précédente.

Un exemple de ces déterminations est donné sur le Tableau III. On constate par comparaison avec les valeurs groupées sur le Tableau I, une bonne concordance des données entre les deux techniques.

Il en résulte que les indices de composés présentant la même structure apparaissent très peu touchés par la température conformément aux résultats de Tourres[1] et Rappoport et Gaumann[3] et que l'hypothèse de l'invariabilité des coefficients d'activité est bien vérifiée.

Inversement la valeur de α peut être calculée à partir des indices de rétention:

$$\log \alpha_i = (I_i - 100\, Z) \frac{k}{100} + \log \frac{Z_i}{Z_E}$$

avec

$$k = \log \frac{P_{z+1}^\circ}{P_z^\circ}$$

Un exemple d'un tel calcul est donné dans le Tableau IV (les valeurs des pressions de vapeur des n-alcanes sont déduites d'un précédent travail[8]).

D'autre part, à partir des données déduites de ce travail, il résulte que la formule générale établie pour les alcanes normaux peuvent être utilisée pour les alcanes branchés sous la forme:

$$\log \alpha_i = \frac{A}{100} |I_i - I_E|\, T^B + \log \frac{Z_i}{Z_E}$$

avec $A = 114{,}400$ et $B = 2.15$. Cette relation permet de connaître les pressions de vapeur saturante à toute température.

CONCLUSION

Si l'on compare les résultats obtenus par les deux méthodes on remarque que la mesure des tensions de vapeur est une méthode plus rapide (environ 5 à 10 min) pour l'étude d'alcanes lourds, cependant elle nécessite des composés très purs. En effet, la présence d'une impureté de tension de vapeur très proche du composé étudié est un écueil pour la méthode (ceci explique l'absence de données sur le Tableau IV pour les composés $n = 4$).

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RESUME

Nous avons déterminé les indices de rétention et les pressions de vapeur (10^{-3} à 10 mmHg) d'une série d'alcanes lourds, mono- et dibranchés, modèles de polyéthylène basse densité. (C_{11}–C(R)–C_{11} R = méthyl à octyl, C_{10}–C(R)–C_n–C(R)–C_{10} R = méthyl, éthyl, butyl, n = 4, 5, 6, 8) Nous avons montré pour les alcanes lourds branchés, que l'indice de rétention était sensible à la longueur du substituant mais aussi au nombre dans la chaine.

Nous proposons une nouvelle méthode d'analyse pour cette série de composés.

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REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF POLYNUCLEAR AROMATIC HYDROCARBONS AND CHLORINATED BIPHENYLS

RELATIONSHIP WITH HYDROPHOBICITY AS MEASURED BY AQUEOUS SOLUBILITY AND OCTANOL–WATER PARTITION COEFFICIENT

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SUMMARY

Reversed-phase thin-layer chromatography with methanol–water as eluent was used to study relationships between retention and hydrophobicity of non-polar aromatic compounds. For polynuclear aromatic hydrocarbons, *n*-alkyl- and chloro-substituted benzenes, good correlation was observed between R_m values and log $P_{octanol}$. Significantly different correlations were obtained for polychlorinated biphenyls (PCBs) containing chlorine atoms in the positions *ortho* to the phenyl–phenyl bond. This effect could not be explained by the measured lower $\pi_{octanol}$ values for *ortho* chlorine in PCBs. Estimation of hydrophobicity from aqueous solubility, S, combined with melting point data is restricted to S values higher than 1 μg/l. A group-contribution approach, including a retention index scale, will be very useful for correlation and prediction of reversed-phase retention, hydrophobicity and environmental and toxicological properties of chemicals.

INTRODUCTION

Hydrophobicity is now recognized as the driving force for the typical distribution processes of non-polar organic chemicals in aqueous environments, such as solubility in water, octanol–water partitioning, bioconcentration in aquatic animals and soil sorption phenomena[1]. It is often directly related to toxicological and pharmacokinetic properties of drugs and other chemicals, as expressed by correlations between the parameter studied and the octanol–water partition coefficient (as log P_{ow}) of structurally related compounds[2]. The abundance of octanol–water partitioning data in the literature and the existence of extensive data compilations have led to methods of calculating partition coefficients, based on the additivity of structural group contributions to the hydrophobicity of a molecule[3,4]. Hydrophobicity may be expressed as an increase in the free energy (ΔG), which is related to log γ_w, the activity coefficient in aqueous solution.

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Correlations between octanol–water partition coefficient and aqueous solubility have been described by several authors[5–9]. Recently, Yalkowsky and Valvani[10] and Mackay and co-workers[11,12] discussed an important improvement in such correlations, made by introduction of a crystal energy term based on the melting point of the substance.

The retention mechanism in reversed-phase liquid chromatography (RP-LC) is also attributed to solvophobic effects[13,14]. In "classical" liquid–liquid chromatography, the partition coefficients between the apolar stationary phase and the polar eluent are related to the capacity factors

$$P = \frac{V_0}{V_s} \times k' = \frac{V_r - V_0}{V_s} \tag{1}$$

where P = partition coefficient, V_r = retention volume, V_0 = volume of mobile phase in column, V_s = volume of stationary phase and k' = capacity factor. Tomlinson[15] has discussed the use of these chromatographic hydrophobic parameters for correlation analysis of structure–activity relationships.

In modern RP-LC, however, the stationary phase is not a viscous liquid coating, but is chemically bonded to an inorganic support. This has made the method accessible to high-performance liquid chromatographic (HPLC) techniques, but, at the same time, it reduces eqn. 1 to a rather theoretical expression without much practical use for the determination of partition coefficients from retention data (and *vice versa*), since partitioning of solutes is now restricted to a very thin "brush" layer of apolar molecular tails (*e.g.*, octadecyl groups)[16]. Direct comparison of RP-LC capacity factors with other, "pure", hydrophobic parameters is also precluded by the use of eluents which often contain water only as a minor constituent.

The first choice for any attempt to correlate RP-LC retention with hydrophobicity now seems to be a methanol–water eluent system, since methanol has nearly the same type of proton donor and acceptor properties as water, which makes it useful as a kind of "organic diluent". A linear increase in log k', often observed when the percentage of methanol in solvent mixtures used for isocratic elution is decreased, is in accordance with this approach. Several authors have found a linear relationship between log k' and alkyl chain length for a homologous series of solutes, and substituents such as carboxylic acid, hydroxyl and phenyl groups also give constant contributions to log k', analogous to the Hansch constants (π) in the octanol–water partitioning system[17–23]. Direct relations between log k' (R_M) and log P_{ow} have been described for chlorinated benzene derivatives in C_{18}-HPLC[24] and for a number of aromatic and organochlorine compounds in C_{18} thin-layer chromatography (TLC)[25,26], whereas HPLC gradient elution (22–75% methanol) resulted in a non-linear relationship[27].

The main purpose of these correlations was to find an alternative to the direct measurement of octanol–water partition coefficients. This is especially useful for highly lipophilic and impure compounds, for which detection and phase separation may become problematic when the classical method is applied, since the concentrations to be measured in the two phases can differ by a factor of 10^5 or more. Several compounds of environmental interest, such as certain polyaromatic hydrocarbons, polychlorinated benzenes, biphenyls and terphenyls, and non-polar polymeric struc-

tures, will fall into this category. A reliable estimation of their hydrophobicity will greatly facilitate prediction and determination of their environmental mobility and bioaccumulation potential[28]. The advantage of hydrophobicity determinations from RP-LC retention parameters alone is clear: compounds with different hydrophobicities would be separated and quantification would not be required. At the same time, a systematic and quantitative approach using linear free energy relationships might provide tools for prediction of the RP-LC retention behaviour of chemicals with known structures[23].

In this study, C_{18} reversed-phase thin-layer chromatography (RP-TLC), using methanol–water mixtures as eluent, was chosen as a relatively simple and inexpensive, neutral reversed-phase system to compare the retention of polynuclear aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) with the octanol–water partition coefficients, and with aqueous solubility as an important physico-chemical property related to hydrophobicity.

EXPERIMENTAL

Measurement of octanol–water partition coefficients

Approximately 5 mg of the test chemical were dissolved in 2.5 ml of water-saturated 1-octanol (Baker, analytical grade). Of this solution, 1 ml was equilibrated with 500 ml octanol-saturated distilled water in a volumetric flask by magnetic stirring during 30 min. Layers were separated by centrifugation at *ca.* 1000 *g* for 10 min, and the octanol layer was then pipetted from the water layer. A 100-ml sample of the water was extracted with *ca.* 100 ml distilled toluene in three portions. Care was taken to prevent contamination of the water sample by octanol droplets. The combined toluene extracts were evaporated to 1 ml to enable quantitation of the chlorinated compounds by gas chromatography with electron-capture detection, whereas a 0.25-ml sample of the octanol phase was sufficiently diluted (*e.g.*, $\times 400$) with distilled toluene to obtain nearly the same concentration.

Partition coefficients were then determined from concentration ratios, measured by successive injections of the concentrated water extract and the diluted sample of the octanol layer on a Tracor 550 GC system equipped with a linearized ^{63}Ni electron-capture detector (ECD) and Spectra-Physics 4100 computing integrator. GC conditions: glass column (1 m $\times$ 2 mm I.D.) packed with 2% Dexsil 300 GC on Chromosorb 750 (100–120 mesh); carrier gas, argon–methane (95:5), flow-rate 25 ml/min; detector make-up, 75 ml/min; isothermal operation at temperatures between 90 and 150°C (injector and outlet, 250°C; detector, 325°C).

For a direct comparison, partition coefficients of isomers were determined simultaneously in one partition experiment, if the GC conditions allowed separate detection and quantification of the compounds.

Reversed-phase thin-layer chromatography

Chemicals were spotted with the aid of glass capillaries on Whatman KC-18 RP-TLC plates (20 $\times$ 20 cm, thickness 0.2 mm), with fluorescent indicator. Chromatograms were obtained by ascending elution with mixtures of methanol (Baker, analytical grade) and de-ionized water in a closed 2-l glass tank system, after equilibration with the aid of wet filter-paper, or by horizontal "sandwich" elution (Camag;

Varo-KS-Kammer). Aromatic, UV-quenching substances were detected as dark spots on fluorescent plates in 254 nm UV-light. The most important retention parameter, R_M, was calculated for each spot

$$R_M = \log k' = \log(1/R_F - 1) \quad (2)$$

where R_F = distance travelled by spot divided by distance travelled by the eluent front.

Chemicals

Polyaromatic hydrocarbons (PAHs), 1,2,3,4-tetra- and pentachlorobenzene were obtained from Aldrich, and *n*-alkylbenzenes from Poly Science Corporation. Polychlorinated biphenyls (PCBs) were purchased from Analabs, except 2,2′,5-tri- and 2,2′,5,5′-tetrachlorobiphenyl, which were synthesized according to Sundström[29], and decachlorobiphenyl which was prepared by perchlorinated[30]. Each chemical was at least 97% pure.

RESULTS

The octanol–water partition coefficients (as log P) of chlorinated biphenyls and of 1,2,3,4-tetrachlorobenzene, measured in this study (Table I), show that substitution of chlorine in the position *ortho* to the phenyl–phenyl bond has a less pronounced effect on log P than substitution in the *meta* or *para* position. π_{Cl} values derived from these measurements are given at the bottom of Table I. They are somewhat lower than the π value (0.66) derived from log P of tetrachlorobenzene, and the values for substitution of chlorine in an aromatic ring given by Rekker[3] and Hansch and Leo[4] (0.71–0.74). Log P values for higher chlorinated biphenyls were calculated using these new differential substitution constants, which resulted in considerably lower values than those estimated previously.

TABLE I

MEASURED log P_{ow} VALUES FOR TETRACHLOROBENZENE, MONO- AND DICHLOROBIPHENYLS

The reproducibility and the accuracy of GC determinations were better than 0.10 log P units.

Compound	*log P_{ow}*
1,2,3,4-Tetrachlorobenzene	4.75 ± 0.1
2-Chlorobiphenyl	4.59 ± 0.1
3-Chlorobiphenyl	4.71 ± 0.1
4-Chlorobiphenyl	4.61 ± 0.1
2,2′-Dichlorobiphenyl	5.00 ± 0.1
3,5-Dichlorobiphenyl	5.37 ± 0.1
3,3′-Dichlorobiphenyl	5.30 ± 0.1
4,4′-Dichlorobiphenyl	5.36 ± 0.1
Calculated π_{Cl} values for PCBs (log P biphenyl = 4.10)	
π_{Cl}-*ortho*	0.46
π_{Cl}-*meta*	0.62
π_{Cl}-*para*	0.59

Correlation with aqueous solubility

As discussed by Mackay *et al.*[11], octanol–water partition coefficients can be correlated with aqueous solubilities of solids if a melting point correction is included. For aqueous solubilities measured at 25°C, the simplest form of the correlation equation is

$$\log P_{ow} = -\log S - 0.01 \times \text{m.p.} + a \quad (3)$$

where S = aqueous solubility (mol/l) and m.p. = melting point (°C). The term a, related to the logarithm of the activity coefficient in octanol, γ_o, is only slightly dependent on the type of compounds in the correlation. Mackay *et al.*[11] found a mean γ_o value of 4.8 for 45 organic, mainly aromatic, compounds, which is in agreement with the value of 0.55 for the term a in eqn. 3 calculated from the PAH data of Yalkowsky and Valvani[10]. The relationship between the calculated log P values of PCBs and log S + 0.01 × m.p. (Table IIa) was fitted to eqn. 3, as shown in Fig. 1 where it is compared with the same correlation for PAHs, mentioned above. The solubility data of PCBs in Table IIa were taken from Weil *et al.*[31], who gave the most complete set obtained by one, reliable method which is also suitable for other almost insoluble compounds. The melting point data in Table IIa were collected by Hutzinger *et al.*[32]. The resulting correlation equation for PCBs containing up to six chlorine atoms is:

$$\log P_{ow} = \log S - 0.01 \times \text{m.p.} + 0.05 \quad (4)$$

The root mean square of the deviation in log P for the 20 points is 0.28, corresponding to a factor of 1.9 in P_{0w}.

The difference between the a terms (eqn. 3) of the PCB and the PAH correlations is 0.5. Following the interpretation of Mackay *et al.*[11], it must be concluded that the mean γ_o of PCBs (up to hexachlorobiphenyl) is about 14, three times as high as γ_o for PAHs.

As shown by Fig. 1, the correlation between P and S failed for PCBs with calculated log P higher than 7.5 or solubilities below 0.5 μg/l (*ca.* 10^{-9} mol/l). Apparently, the hydrophobicity that can be measured by aqueous solubility has reached its maximum here. Similarly, coronene (log P = 7.7, S = 0.14 μg/l or $10^{-9.3}$ mol/l, m.p. = 438°C[33]) was not included by Yalkowsky and Valvani[10] in their PAH correlation.

RP-TLC

Whatman KC-18 TLC plates, compared with other makes, have the advantage of larger surface areas and faster elution[34], which is especially important in ascending tank elution systems. The reproducibility of the ascending tank elution was moderate, presumably due to insufficient acclimatization of the elution chamber, coupled with formation of a solvent gradient on the plate. A better equilibration and reproducibility was obtained by horizontal elution in the sandwich system. Comparison of the behaviour of PAHs in both systems (Table IIb) shows that the retention of relatively fast eluting compounds, at the top of the table, was stronger in the tank system. This might be the result of a decreasing percentage of methanol at the solvent front during

TABLE II

RP-TLC RETENTION, R_M, COMPARED WITH HYDROPHOBIC PARAMETERS OF PCBs (a), PAHs (b) AND SUBSTITUTED BENZENES (c)

m.p. = Melting point (°C); S = aqueous solubility (mol/l); P_{ow} = octanol–water partition coefficient; R_M = log $(1/R_F - 1)$; N_{ab} = n-alkylbenzene retention index. Whatman KC-18 RP-TLC, methanol–water (95:5).

Compound	*m.p.*	$-\log S$	$-\log S + -0.01 \times m.p.$	$\log P_{ow}$	R_M		N_{ab}
(a) PCBs							
Biphenyl	71	4.28	3.57	4.10	−0.21		2.56
2-Chloro-	34	4.66	4.32	4.56	−0.24		2.28
3-	17	5.16	4.91	4.72	−0.10		3.57
4-	78	5.32	4.54	4.69	−0.13		3.29
2,2′-Dichloro-	61	5.45	4.84	5.02	−0.32		1.55
2,4-	24	5.55	5.31	5.15	−0.12		3.39
2,5-	23	5.58	5.35	5.18	−0.05		4.43
3,3′-				5.34	0.02		4.67
4,4′-	149	6.60	5.11	5.28	−0.10		3.57
2,2′,5-Trichloro-	44	5.60	5.16	5.64	−0.11		3.48
2,4,4′-	57	5.99	5.42	5.74			
2,4,5-	78	6.44	5.66	5.77			
2,4′,5-				5.77	−0.01		4.40
3,4,4′-	88	7.23	6.35	5.90			
2,2′,5,5′-Tetrachloro-				6.26	0.00		4.49
2,2′,6,6′-				5.94	−0.22		2.47
2,3,4,5-	92	7.18	6.26	6.39			
2,3′,4′,5-				6.39	0.15		5.87
3,3′,4,4′-	180	8.59	6.79	6.52			
3,3′,5,5′-				6.58	0.34		7.62
2,2′,3,4,5′-Pentachloro-	112	7.86	6.74	6.85			
2,2′,4,5,5′-	77	7.89	7.12	6.85			
2,3,4,5,6-	124	7.68	6.44	6.85			
2,2′,3,3′,4,4′-Hexachloro-	150	8.91	7.41	7.44	0.24		6.70
2,2′,4,4′,5,5′-	103	8.47	7.44	7.44			
2,2′,4,4′,6,6′-	114	8.60	7.46	7.12			
2,2′,3,4,5,5′,6-Heptachloro-	149	8.92	7.43	7.93	0.38		7.99
2,2′,3,3′,4,4′,5,5′-Octachloro-	159	9.19	7.60	8.68	0.58		9.83
2,2′,3,3′,5,5′,6,6′-	162	9.37	7.75	8.42			
2,2′,3,3′,4,4′,5,5′,6-Nonachloro-	206	9.61	7.55	9.14	0.67		10.65
Decachlorobiphenyl	305	10.49	7.44	9.60	0.90		12.77
					Tank	*Sandwich*	
(b) PAHs							
Naphthalene	80	3.61	2.81	3.35	−0.08	−0.25	2.19
Fluorene	116	4.93	3.77	4.18	0.00	−0.10	3.57
Anthracene	216	6.38	4.22	4.63	0.07	−0.03	4.21
Phenanthrene	101	5.15	4.14	4.63	0.03	−0.06	3.94
Fluoranthene	111	5.90	4.79	5.22	0.10	0.05	4.95
Pyrene	156	6.18	4.62	5.22	0.16	0.12	5.59
Chrysene	255	8.06	5.51	5.91	0.22	0.16	5.96
Benzo[*a*]pyrene	175	7.82	6.07	6.50	0.40	0.38	7.99
Benzo[*g*,*h*,*i*]perylene	277	9.02	6.25	7.10	0.54	0.54	9.46
(c) Substituted benzenes							
n-Butyl-				4.18	−0.05		4.03
n-Hexyl-				5.24	0.16		5.96
n-Octyl-				6.30	0.38		7.99
n-Decyl-				7.35	0.60		10.01
1,2,3,4-Tetrachloro-				4.94	0.05		4.95
Pentachloro-				5.69	0.23		6.61

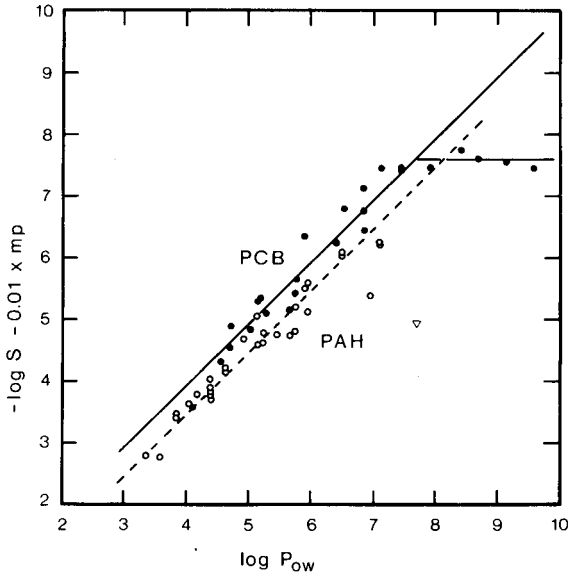


Fig. 1. Relationship between octanol–water partition coefficient, P_{ow}, and aqueous solubility, S (mol/l), when corrected for melting point (m.p.). ●— ●, PCBs; ○----○, PAHs; ▽, coronene.

tank elution. Consequently, the correlation of R_M with log P_{ow} obtained by linear regression analysis was somewhat better for the retention data from the sandwich systems (eqns. 5 and 6):

sandwich

$$\log P_{ow} = 4.52\ R_M + 4.81 \quad (5)$$
$$n = 9,\ r = 0.98,\ s = 0.20$$

tank

$$\log P_{ow} = 5.31\ R_M + 4.39 \quad (6)$$
$$n = 9,\ r = 0.96,\ s = 0.26$$

a non-linear relationship would give a better fit to the tank data. Therefore, the sandwich system was preferred for further experiments.

The relationship between calculated log P_{ow} and R_M for 20 PCBs (Table IIa) is given by:

$$\log P_{ow} = 4.52\ R_M + 5.79 \quad (7)$$
$$n = 20,\ r = 0.95,\ s = 0.51$$

This correlation is far inferior to that of the PAHs (eqn. 5), as demonstrated by the root mean square deviation in log P. Two compounds with equal R_M value have completely different log P values, *viz.*, 4.1 and 5.9 for biphenyl and 2,2′,6,6′-tetrachlorobiphenyl respectively. In addition, eqns. 5 and 7 show a difference of one log P unit.

Important improvements are obtained when the PCBs are distinguished ac-

cording to their number of chlorine atoms in *ortho* positions (numbers 2 and 6 of both phenyl rings). As indicated by the four compounds at the top of Table IIa and the four tetrachlorobiphenyls, substitution of hydrogen by chlorine in one of the *ortho* positions gives no contribution, or even a small negative one, to R_M, in contrast to *meta*- or *para*-chlorine substitution (positions 3, 4 and 5: $\Delta R_{MCl} = 0.13 \pm 0.06$). The new lines in Fig. 2 correspond to the following equations:

no *o*-Cl	: $\log P_{ow} = 4.5\ R_M + 5.2\ (n = 6)$	(8)
one *o*-Cl	: $\log P_{ow} = 4.5\ R_M + 5.6\ (n = 5)$	(9)
two, three or four *o*-Cl	: $\log P_{ow} = 4.2\ R_M + 6.3\ (n = 9)$	(10)

Deviations between calculated and observed values were now generally below 0.25 log P units (factor 1.75 in P); exceptions were 4,4′-di-, 2,2′,6,6′-tetra- and decachlorobiphenyl, with log P deviations of *ca.* 0.5 (factor of 3 in P). This accuracy is comparable to that obtained by the PAH correlation (eqn. 5).

The four *n*-alkylbenzenes (Table II) fitted the equation:

$$\log P_{ow} = 4.85\ R_M + 4.44 \quad (11)$$

Tetra- and pentachlorobenzene were shown to be in intermediate positions between alkylbenzenes and PAHs (Fig. 2).

Direct comparison of aqueous solubility and RP-TLC retention was only

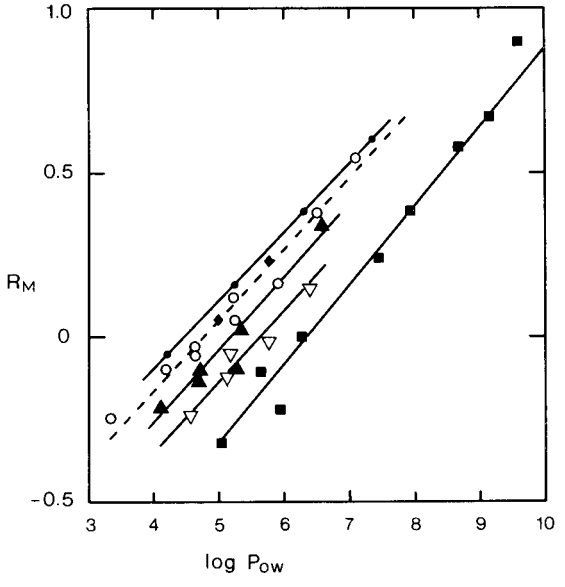


Fig. 2. Relationship between octanol–water partition coefficient, P_{ow}, and reversed-phase retention, R_M. ●, *n*-Alkylbenzenes; ◆, polychlorinated benzenes; ○, polycyclic aromatic hydrocarbons; ▲, polychlorinated biphenyl (PCB) without *ortho*-Cl; ▽, PCB with one *o*-Cl; ■, PCB with two or more *o*-Cl. Whatman KC-18 RP-TLC; methanol–water (95:5).

possible for a restricted number of compounds from Table II (Fig. 3). The correlation between R_M and log S + 0.01 m.p. is evident for the PAHs:

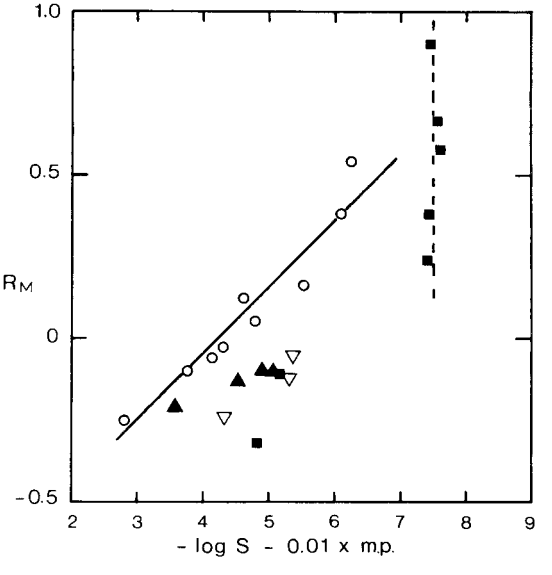
$$-\log S - 0.01 \times \text{m.p.} = 4.3 + 4.7\, R_M \; (n = 9) \tag{12}$$

mean deviation: 0.3 log S units
max. deviation: 0.5 log S units
For the higher chlorinated biphenyls, (log S + 0.01 m.p.) had reached a maximum value, whereas too few data were available for the lower chlorinated biphenyls to allow reasonable correlations according to the different degrees of *ortho*-chlorine substitution.

Changes in the eluent composition resulted in a linear increase of the R_M values of PCBs with the water content of the eluent, in the range of 0–20% (v/v) (Fig. 4)

2,2′,3,4,5,5′,6-heptachlorobiphenyl:	$R_M = 0.06 + 7.6 \times \Phi$	(13)
2,2′,5,5′-tetrachlorobiphenyl:	$R_M = -0.24 + 6.4 \times \Phi$	(14)
2-chlorobiphenyl:	$R_M = -0.34 + 4.5 \times \Phi$	(15)
biphenyl:	$R_M = -0.41 + 4.5 \times \Phi$	(16)

where Φ = the volume fraction of water in the eluent.



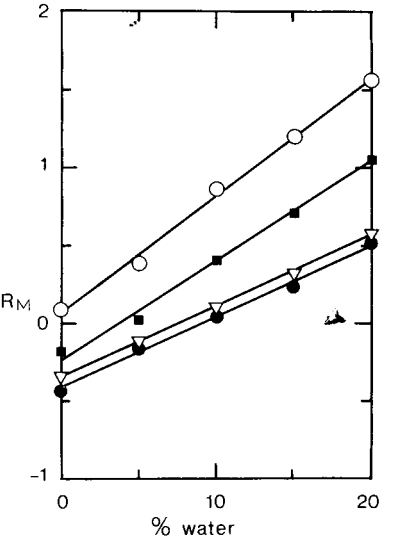


Fig. 3. Relationship between aqueous solubility, S (mol/l), corrected for melting point (m.p.), and reversed-phase retention, R_M. ○, Polycyclic aromatic hydrocarbons; polychlorinated biphenyls: ▲, no *ortho*-Cl; ▽, one *o*-Cl; ■, two or more *o*-Cl. Whatman KC-18 RP-TLC; methanol–water (95:5).

Fig. 4. Retention of polychlorinated biphenyls at different percentages of water in the eluent (Whatman KC-18 RP-TLC, methanol-water). ●, Biphenyl; ▽, 2-chloro-; ■, 2,2′,5,5′-tetrachloro-; ○, 2,2′,3,4,5,5′,6-heptachlorobiphenyl.

CONCLUSIONS AND DISCUSSION

Aqueous solubility and octanol–water partition coefficient can be used to give satisfying and consistent estimations of the hydrophobicity of polyaromatic hydrocarbons and polychlorinated biphenyls, provided that the appropriate correction is made for the crystal energy of the compounds. However, the use of aqueous solubilities is limited to values higher than 0.5 μg/l, corresponding to log $P_{ow} < 7.5$. Estimation of higher log P values by calculation from fragmental or substitution constants is still possible for compounds with known structures, if sufficient log P data are available from smaller, structurally related chemicals. The difference between the π values obtained for chlorine attached to different sites of an aromatic nucleus, previously suggested by Tulp and Hutzinger[9], demonstrates the pitfalls of a too simple application of "literature values".

Especially for mixtures of highly lipophilic unknown chemicals, RP-LC might be a reasonable alternative. However, the results of the present C_{18}-RP-TLC study, by use of methanol–water mixtures, show that pure hydrophobicity data are not obtained. One single correlation of log P_{ow} and R_M for all types of aromatic compounds together will produce systematic errors in log P determinations. The differences may be due to different solvophobic effects, as a result of the high methanol concentrations in the eluent, or to specific interactions with the stationary phase. It is possible that the fit in a semi-crystalline matrix of C_{18}-alkyl chains is affected by any distortion of the planarity of the biphenyl nucleus, resulting from steric hindrance by *ortho*-chlorine atoms. On the other hand, the influence of the other substituting groups (chlorine in other positions, *n*-alkyl, aromatic rings in PAHs) on the RP-TLC retention of aromatic compounds correlates well with π values in the octanol–water system. Therefore, it is expected that R_M values from RP-LC can still be useful for structure–activity correlations of closely related compounds. However, the use of "log P_{ow} derived from reverse phase chromatography" should be avoided, since these values are highly dependent on the type of reference compounds chosen for the basic log P *vs.* R_M correlation.

As a reproducible and reliable intermediate, it is suggested that retention indices be based on the linear relationship between the R_M or log P value and the number of carbon atoms of the components of a homologous series of *n*-alkyl substituted compounds, such as the alkylbenzenes in this study[35,36]. Eventually, "apparent log P_{ow}" values can be calculated for comparison with other hydrophobic parameters, as exemplified by eqn. 17.

$$\log P_{ow} = 2.07 + 0.527 \times N \tag{17}$$

where N represents the *n*-alkylbenzene retention index. It can be considered as the number of carbon atoms in the alkyl chain of an imaginary *n*-alkylbenzene having the same retention; N is determined from R_M by interpolation in a series of *n*-alkylbenzenes.

It should be emphasized, however, that the retention index thus defined is an independent parameter, which can be used directly for structure–activity correlations without conversion into log P. In addition, it will be a practical basis for the determination of relationships between chemical structure and retention in reversed-phase

liquid chromatography. Besides the homologous series on which the retention index is based, only the type of reversed-phase material and the eluent composition should be specified. It is expected that the retention index will show only a minor change with variation in the percentage of methanol in the eluent. The exact determination of this dependence will be simplified by linearity of the relationship between R_M and the methanol percentage over a certain range (see Fig. 3).

The reversed-phase retention index may replace the unreliable log P_{ow} determinations of highly lipophilic chemicals (log $P > 5$), for prediction of biological activity and environmental distribution coefficients, such as bioaccumulation factors and sorption coefficients. For this purpose, more knowledge of different group contributions to RP-LC retention and their influence on environmental and biological parameters is desirable.

Thin-layer chromatography is a rapid, simple and inexpensive method. However, high-performance liquid column chromatography may be preferred for reasons of accuracy, sensitivity and reproducibility. In addition, HPLC offers the possibility of gradient elution, which may be very useful for mixtures of unknown chemicals, as found in chemical wastes, to estimate environmental mobility and accumulation potential[28].

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NOVEL CHEMILUMINESCENCE DETECTOR FOR DETERMINATION OF VOLATILE POLYHALOGENATED HYDROCARBONS BY GAS CHROMATOGRAPHY

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SUMMARY

Based on the chemiluminescence reaction with atomic sodium vapour under reduced pressure, a novel gas-chromatographic chemiluminescence detector (CLD) for the determination of volatile polyhalogenated hydrocarbons (VPHHCs) has been developed; the characteristics of the CLD are compared with those of the electron-capture detector (ECD). The CLD constructed is simple and unique in that it exhibits high sensitivity and good selectivity towards VPHHCs; CCl_4 $ClCH_2CH_2Cl$, $ClCH_2CHCl_2$, $ClCH = CCl_2$, and $Cl_2C = CCl_2$ have low detection limits of the order of 10^{-13} g/sec (in the picogram range) and their molar response ratios to compounds other than VPHHCs exceed 10^4. The CLD can compete in terms of sensitivity with the ECD and also surpasses the ECD with regard to linear dynamic range and selectivity. In addition, it is free from the drawbacks associated with the ECD. These detector characteristics allow us to expect that the CLD will be used in place of the ECD, depending on the kinds of VPHHCs which are to be determined.

INTRODUCTION

To date many classes of compounds associated with human activities have been discharged into the environment in varying amounts. Strong emphasis has been placed on the environmental and health aspects of anthropogenic organic compounds, one class of which, volatile polyhalogenated hydrocarbons (VPHHCs), have a wide variety of industrial applications, notably as refrigerants, aerosol propellants, solvents, chemical intermediates and fire extinguishers. Since the original work of Molina and Rowland[1], a large number of investigators have studied the interaction of VPHHCs (the so-called halocarbons, fluorocarbons or chlorofluorocarbons) with stratospheric ozone. In another field of research, the presence of VPHHCs such as trihalomethanes was recently identified in water samples obtained from several sources[2]: for instance, the average levels of $CHCl_3$, $CHCl_2Br$ and $CHClBr_2$ in samples of drinking water from 80 cities in the United States were found to be 21, 6 and 1.2 μg/l,

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respectively[3]. The main interest in these types of compounds is focused on their possible toxic effects, particularly with regard to carcinogenicity.

Most of the VPHHCs in environmental samples in general involve a mixture of chlorinated hydrocarbons with both other halogenated and non-halogenated compounds. Suitable methods of detection and quantification are therefore essential, and the electron-capture detector (ECD) in gas chromatography (GC) has been commonly used for VPHHC analysis because of its exceedingly high sensitivity, despite a number of drawbacks *viz.* (1) the linear dynamic range is short, in the d.c. and pulsed mode being typically less than two orders of magnitude, although being extended to about four or five orders in the pulse-moldulated constant-current mode; (2) the sensitivity towards individual halogenated hydrocarbons differs and varies over several orders of magnitude, *e.g.*, the response for CH_2Cl_2 is 10^4–10^5 times less than that for CCl_4 (see Table IV); (3) the ECD has poor specificity, *i.e.* it is highly sensitive to electronegative compounds other than halogenated hydrocarbons (such as those containing $-NO_2$, –N, –COCO–, and –COCH=CHCO– groups) and consequently, particular attention must be payed during analysis to avoid contamination of samples; and (4) the radiative sources used are controlled by government regulations.

This work reports attempts to develop a novel chemiluminescence detector (CLD) for GC which is free of the above drawbacks and is capable of selectively detecting VPHHCs in nanogram to picogram amounts, together with a comparison with the ECD. The high CLD response arises from the chemiluminescence reaction between atomic sodium vapour and volatile aliphatic hydrocarbons containing more than two halogen atoms in the molecule[4,5]. The chemiluminescence reaction involves two primary steps, corresponding to successive removals of the halogen atoms X,

$$RX_2 + Na \rightarrow \cdot RX + NaX$$
$$\cdot RX + Na \rightarrow (R) + NaX^{\dagger}$$

followed by a vibrational-to-electronic energy transfer reaction,

$$NaX^{\dagger} + Na(3^2S) \rightarrow NaX + Na^*(3^2P)$$

where † denotes a minimum vibrational excitation of 48.5 kcal/mole, the energy required to excite the *D*-lines. Here R is an aliphatic hydrocarbon group and (R) corresponds to a stable alkene or cyclic hydrocarbon formed by exothermic rearrangement of the biradical during the collision, the stabilization energy being deposited as vibrational excitation in the nascent NaX molecules. The resultant chemiluminescence intensity in the emission of the sodium *D*-lines is monitored.

On the basis of the chemiluminescence reaction, whose reaction mechanism is somewhat different with that above, we had previously reported a new CLD permitting determination of atmospheric N_2O at sub-ppm levels without the need for preconcentration processes[6]. Accordingly, the present work was initially undertaken for preliminary experiments, employing the same type of detector as that used for the N_2O determination; improvements in the construction of the detector were then made.

PRELIMINARY INVESTIGATIONS

The same type of detector as used in the previous work[6] (a CLD made of Pyrex glass tubes) was newly constructed for the preliminary experiments (Fig. 1). As the chemiluminescence reaction described above suggests, the amount of atomic sodium vapour and the detector pressure play important roles in the chemiluminescence intensity. The former, which depends mainly on both the cell temperature and the flow-rate of the atomic sodium vapour carrier gas, must be much greater than the amount of VPHHC admitted into the reaction cell, in order to ensure that the chemiluminescence intensity is proportional to the VPHHC concentration. The latter is correlated with collisional deactivations of vibrationally excited NaX molecules which are thus lost for the subsequent excitation of sodium atoms. The effects of these parameters on the chemiluminescence intensity were examined, and preliminary results, *viz.* lower detection limits (LDLs), linear dynamic ranges (LDRs) and relative molar responses (RMRs), were obtained (Table I). All calibration graphs (log–log plots) gave straight lines; slopes were not always equal to unity, that for the LDRs being more than three orders of magnitude. It is found from the RMRs that the sensitivity exhibits a pronounced dependence on the type of compounds and the number of chlorine atoms in the molecule. In the chloromethane homologous series, for instance, the response increases with an increase in the number of chlorine atoms, while for chloroethanes there is no such tendency. In analogous series, the response cannot be explained in terms of the number of chlorine atoms. This response behaviour will be discussed later. At any rate, the preliminary investigations indicate that the CLD will be capable of completing with the ECD if improved.

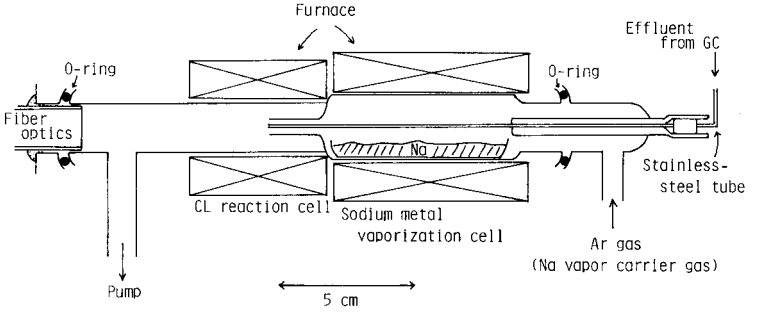

Fig. 1. Pyrex chemiluminescence detector.

However the preliminary experiments reveal that the Pyrex detector suffers the following intrinsic defects derived from both the material and the geometry: many parameters affecting response behaviour, gradual corrosion of the Pyrex glass, contamination of the fibre optics surface owing to deposition of sodium atoms and reaction products, a troublesome procedure for loading the sodium metal, and the inevitable seepage of stray light into the apparatus. Thus, a metallic detector was designed to remove the above defects and to improve the detector characteristics, resulting in a simplified detector construction and easier operation.

TABLE I

PRELIMINARY RESULTS

Operating conditions: sodium vaporization cell temperature, 280°C; chemiluminescence reaction cell temperature, 300°C; sodium vapour carrier gas flow-rate, 17 ml/m; detector pressure, 5 Torr. Background current: $3 \cdot 10^{-10}$ A. Noise current: $1 \cdot 10^{-11}$ A.

VPHHC	*LDL (g/sec)*	*LDR*	*RMR***
CH_2Cl_2	$7.1 \cdot 10^{-10}$	10^3	1
$CHCl_3$	$1.5 \cdot 10^{-10}$	10^4	6
CCl_4	$5.3 \cdot 10^{-12}$	10^5	63
$CFCl_3$	$1.2 \cdot 10^{-11}$	10^4	95
CF_2Cl_2	$2.4 \cdot 10^{-11}$	10^4	69
CF_3Cl	$1.4 \cdot 10^{-9}$	10^3	1
$ClCH_2CH_2Cl$	$4.3 \cdot 10^{-12}$	10^5	112
CH_3CHCl_2	$1.3 \cdot 10^{-9}$	10^3	0.1
$ClCH_2CHCl_2$	$2.0 \cdot 10^{-11}$	10^4	126
CH_3CCl_3	$3.6 \cdot 10^{-10}$	10^3	0.6
$ClCH=CCl_2$	$6.7 \cdot 10^{-12}$	10^4	19
$Cl_2C=CCl_2$	$1.1 \cdot 10^{-11}$	10^4	56

* For a signal-to-noise ratio of 3.

** Normalized with respect to the value for CH_2Cl_2.

EXPERIMENTAL

Apparatus

A Shimadzu Model GC-3A gas chromatograph, equipped with a 10% DC550 column (3 m × 3 mm I.D.) and a thermal conductivity detector (TCD) was used. Pure argon gas from a cylinder was supplied to the chromatograph after having been dried with activated alumina and then with magnesium perchlorate. A three-way micro needle valve (MNVTU-100, Scientific Glass Engineering, North Melbourne, Australia) was installed between the chromatograph and the CLD for controlling the stream of argon gas to the CLD. The remaining exit of the micro needle valve opened into the atmosphere, resulting in a constant flow-rate of argon into the CLD. Consequently, part of the sample eluted from the GC column has to be discarded along with the argon carrier gas. The flow-rate of the argon stream was indirectly determined by calculating the difference between the flow-rate of the vented gas stream before and during the experiment. The CLD was evacuated by a rotary vacuum pump (Model C-150, Tokyo Vacuum Machinery, Tokyo, Japan) and the pressure was measured with a Mcleord gauge. The CL produced was detected by a photomultiplier tube (Hamamatsu TV-R453) through an interference filter (max. 590 nm, Toshiba-KL59). The photomultiplier tube was cooled to *ca.* −20°C in an electronic cooler (Hamamatsu TV-C659B) and operated at −700 V from a high-voltage d.c. stabilizer (Hamamatsu TV-C448A). The signal from the photomultiplier tube passed into an electrometer (tr-8651, Takeda Riken) and was then recorded by a dual-pen recorder along with the signal from the TCD, via a laboratory-built low-pass active filter[7] (frequency cut-off *ca.* 0.1 Hz) between the electrometer and the recorder.

Stainless-steel CLD

A metallic CLD (illustrated in Fig. 2) was constructed in order to improve detector performance. It is composed of stainless steel tubes with Swagelok fittings, *viz.* a "Union Tee", a "Reducer" welded to the Union Tee, and a "Plug". The tube fittings are heated by an aluminium block in which are buried two firerod cartridge heaters (120 V/200 W, Watlow, St. Louis, MO, U.S.A.). The temperature can be regulated up to 400°C by an electronic controller. The inside pressure of the detector is maintained at a few torr. Sodium metal is loaded into the Plug fitting which can be easily detached from the Union Tee, resulting in ease of loading of the sodium metal. No atomic sodium vapour carrier gas is admitted to the detector, unlike the Pyrex detector. As a result of the chemiluminescence reaction, a small diffusion flame due to the chemiluminescence is formed on the nozzle (0.8 mm I.D.) and the chemiluminescence intensity is measured through a quartz window, with no use of fibre optics. In contrast to the Pyrex detector, no stray-light shield was needed.

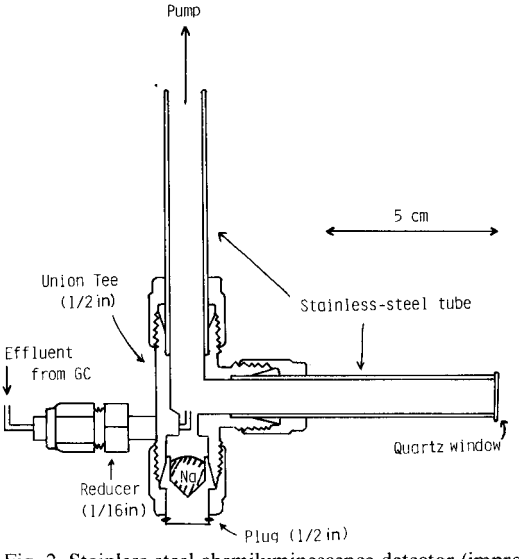

Fig. 2. Stainless-steel chemiluminescence detector (improved detector).

Since the lower detection limit ultimately depends on the noise level, great care was taken over lowering the background signal. This signal involves chemiluminescence based on trace amounts of oxygen in the argon gas stream and the stray light. Some of the oxygen comes from impurities in the argon gas used but the majority comes from air diffusing into the argon gas stream through the analytical lines. The oxygen molecules then react with atomic sodium vapour to give a weak chemiluminescence. This stainless-steel detector gives a lower background noise signal because there is no use of an extra argon carrier gas and because there is complete shielding of stray light because of the material of the detector. Employment of the low-pass active filter further lowers the noise signal.

ECD

A 10-mCi ^{63}Ni ECD in the fixed-frequency pulsed mode (frequency 12 kHz,

pulse width 8 μsec, voltage 50 V), in conjunction with a Shimadzu GC-3AE gas chromatograph, was used for comparison with the CLD. The chromatographic column was the same as that for the CLD study and the carrier gas was pure nitrogen (99.999%).

Reagents

All chemicals were of analytical-reagent grade. Each standard solution of VHHCs, except for the chlorofluoromethanes, was prepared by successive dilution with toluene or *n*-hexane. Low concentrations of chlorofluoromethanes were provided by passing nitrogen gas containing *ca.* 100 ppm of chlorofluoromethanes (Nippon Sanso) and pure nitrogen gas through a standard gas generator (Model SGGU-72, Standard Technology). A 1-ml sample of each prepared gas was introduced into the GC column by means of a gas sampler. Argon gas used had a purity of 99.999%. Sodium metal was boiled enough in toluene before use and *ca.* 1 g was used in each experiment.

RESULTS AND DISCUSSION

As a result of the simplified detector, only two variables, the temperature and pressure of the detector, were examined for detector optimization. Instead of the chemiluminescence intensity, the chemiluminescence intensity-to-noise ratio was plotted for the dependence on the detector temperature (Fig. 3), since the background and noise signals also varied. It can be seen from this figure that the curve for CCl_4 shows a trend opposite to the other two. This trend is also seen in the relation between the detector pressure and the chemiluminescence intensity (Fig. 4). Among the VPHHCs studied (including other halomethanes), only CCl_4 exhibits such behaviour, which was always observed in the preliminary experiments. This behaviour seems unreasonable because a higher detector temperature increases both the amount of atomic sodium vapour and the reaction rate and also because a higher detector

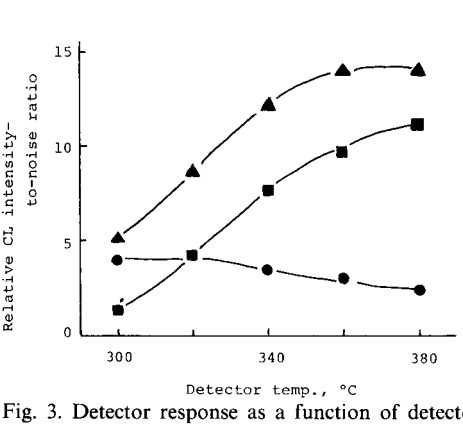

Fig. 3. Detector response as a function of detector temperature. Detector pressure: 2 Torr, ●: CCl_4 ($1.8 \cdot 10^{-10}$ mole), ▲: $ClCH=CCl_2$ ($1.9 \cdot 10^{-10}$ mole), ■: $ClCH_2CHCl_2$ ($1.9 \cdot 10^{-10}$ mole).

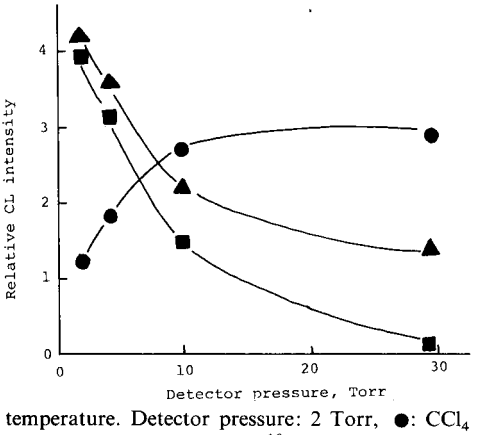

Fig. 4. Detector response as a function of detector pressure. Detector temperature: 350°C. Symbols as in Fig. 3.

pressure causes deactivation of the vibrationally excited NaCl molecules. One of the possible reasons is that CCl_4 is reduced to carbon atoms through the chemiluminescence reaction while other VPHHCs provide hydrocarbon molecules. However, further discussion of this topic is beyond the scope of this article.

Although optimum conditions for most of the VPHHCs studied could not be easily obtained, the detector temperature and pressure were determined, as shown in Table II, for subsequent experiments, taking into account the consumption of sodium metal during the experiment and the ease of operation. The results obtained under these conditions are also summarized in the table. The calibration graphs plotted logarithmically for the VPHHCs exhibited straight lines with slopes of unity. The table shows that the detector characteristics are significantly better than those of the Pyrex detector, *i.e.* the background and noise signals decrease by a factor of 100, and the LDLs and LDRs are 10–100 times lower and longer, respectively; in addition the detector response is arithmetically proportional to the amount of the VPHHCs. Under the optimum conditions, the relative standard deviation was less than 2% for ten successive measurements of a $1 \cdot 10^{-11}$-mole amount of $ClCH_2CH_2Cl$. Although the RMRs show similar tendencies to those for the Pyrex detector, the following favourable trends imply that this type of detector will not suffer geometrical constraint. The sensitivities to chloromethanes, chlorofluorocarbons, and chloroethylenes increase with increasing chlorine numbers. Halomethanes containing equal numbers of chlorine atoms show approximately comparable sensitivies; even replacement of the bromine atoms in bromomethanes by chlorine gives little change. Com-

TABLE II

CHARACTERISTICS OF IMPROVED CLD

Operating conditions: detector temperature, 360°C; detector pressure, 4 Torr. Background current: $5 \cdot 10^{-12}$ A. Noise current: $1 \cdot 10^{-13}$ A.

VPHHC	*LDL (g/sec)**	*LDR*	*RMR***
CH_2Cl_2	$7.3 \cdot 10^{-12}$		1
$CHCl_3$	$2.3 \cdot 10^{-12}$		3
CCl_4	$3.1 \cdot 10^{-13}$		230
CF_3Cl	$1.3 \cdot 10^{-10}$		0.04
CF_2Cl_2	$5.4 \cdot 10^{-12}$		1
$CFCl_3$	$1.4 \cdot 10^{-12}$		5
$CHBr_3$	$4.6 \cdot 10^{-11}$		4
$CHBr_2Cl$	$2.0 \cdot 10^{-11}$		3
$ClCH_2CH_2Cl$	$9.1 \cdot 10^{-14}$		430
$ClCH_2CHCl_2$	$1.0 \cdot 10^{-13}$		540
$Cl_2CHCHCl_2$	$8.0 \cdot 10^{-13}$	10^4–10^6	25
$ClCH_2CCl_3$	$3.7 \cdot 10^{-13}$		26
CH_3CHCl_2	$1.9 \cdot 10^{-10}$		0.4
CH_3CCl_3	$8.6 \cdot 10^{-11}$		0.7
Cl_2CFCF_2Cl	$7.3 \cdot 10^{-12}$		4
cis-$ClCH=CHCl$	$7.2 \cdot 10^{-12}$		2
trans-$ClCH=CHCl$	$2.8 \cdot 10^{-11}$		2
$ClCH=CCl_2$	$1.7 \cdot 10^{-13}$		230
$Cl_2C=CCl_2$	$7.9 \cdot 10^{-14}$		590

* For a signal-to-noise ratio of 3.

** Normalized with respect to the value for CH_2Cl_2.

parison of the sensitivities to chloromethanes and chlorofluorocarbons makes the fluorine atoms appear inactive in the chemiluminescence reaction. This idea is supported by the experimental result that introduction of 1 ml of a gas containing 100 ppm of CF_4, corresponding to *ca.* 10^{-9} mole of CF_4, gives no response. This may be ascribed to the higher energies of the C–F compared to the C–Cl bonds. On the other hand, the sensitivity to chloroethanes varies with the positions of the chlorine atoms in the molecule rather than with the actual numbers of chlorine atoms. This can be interpreted by the following reaction mechanism[5]:

$$\cdot CH_2\text{–}CH_2Cl + Na \rightarrow NaCl + CH_2 = CH_2 \quad \text{(a)}$$

$$CH_3\text{–}CHCl + Na \rightarrow NaCl + CH_2 = CH_2 \quad \text{(b)}$$

(R) in the chemiluminescence reaction described previously is an unsaturated molecule formed either by the closure of a double bond (a) or by the migration of a hydrogen atom (b). Reaction (b), which requires hydrogen atom migration, *e.g.*, for CH_3CHCl_2 and CH_3CCl_3, has a much smaller light yield than that of reaction (a), *e.g.*, for $ClCH_2CH_2Cl$ and $ClCH_2CHCl_2$. Other chloroethanes listed show moderate sensitivity, with both reactions (a) and (b) being responsible for the chemiluminescence reaction. It is found from these detector characteristics that chemiluminescence will occur more smoothly and efficiently in this type of detector rather than in the Pyrex apparatus.

Atomic sodium vapour also reacts with monohalogenated compounds and with some types of nitrogen- or oxygen-containing compounds to produce chemiluminescence, the reaction mechanisms being unknown and light yields low. Thus various kinds of compounds were tested in order to estimate the selectivity of the detector. Table III expresses the results as the RMRs compared with the value for CH_2Cl_2. Zero values mean that compounds concerned show no response at amounts of *ca.* 10^{-7}–10^{-8} moles; *i.e.* the CLD is practically insensitive to them. On the other hand, some compounds show sensitivities of the order of 10^{-2} times that for CH_2Cl_2. The lowest detectable amounts of these compounds are calculated as *ca.* 10^{-9}–10^{-10} moles since that of CH_2Cl_2 is in the order of 10^{-11}–10^{-12} moles. The amount corresponds to a 1-μl intake of solution at a concentration of *ca.* 10–100 μg/ml or a 1-ml intake of gas at a concentration of *ca.* 1–10 ppm. It seems unlikely that these compounds are normally present in the environment in this concentration range or higher. Therefore, it can be said that the CLD is highly selective to the VPHHCs.

Comparison with the ECD

For the purpose of evaluating the CLD characteristics, the CLD was compared with the ECD with regard to LDL, LDR, RMR and selectivity. The LDLs for signal-to-noise ratios noted in Table IV are shown in nanogram units, obtained by measurements of the peak heights. The values listed may vary slightly with the elution times and in the case of the ECD they may also depend on the operating conditions. Although there is therefore no consistency in the experimental conditions in the table, it may be seen that a complementarity is found between the LDLs. For several VPHHCs especially those containing two chlorine atoms in the molecule, the CLD has a comparable or superior LDL to the ECD. For the VPHHCs containing fluorine, bromine, or localized chlorine atoms in the molecule, however, the CLD is markedly

TABLE III

RELATIVE MOLAR RESPONSE OF OTHER COMPOUNDS TO CH_2Cl_2 (= 1)

*Compound**	*CLD*	*ECD***
n-C_3H_7I	0.02	1000
$C_2H_5NO_2$	0.009	1
$CH_3COCOCH_3$	0.007	10000
$(CH_3O)_3P$	0.007	0.06
n-C_3H_7Br	0.007	2
CH_3CN	0.001	0.04
n-C_3H_7Cl	0.0009	0.03
C_2H_5OH	0.0002	0.08
CH_3COCH_3	0	0.03
C_2H_5CHO	0	0.2
$(C_2H_5)_2NH$	0	
$C_2H_5OC_2H_5$	0	0.04
$(C_2H_5)_2S$	0	0
C_6H_5Cl	0	0.06
C_6H_6	0	0.0001

* Elution from GC column was confirmed by TCD signal.
** At 70°C.

TABLE IV

COMPARISON OF CLD AND ECD

S/N = Signal-to-noise ratio.

VPHHC	*LDL (ng)*				*RMR***			
	CLD	*ECD**			*CLD*	*ECD**		
	S/N = 3	*(A)*[8] *S/N = 2*	*(B)*[9] *S/N = 2 or 3*	*(C) S/N = 3*		*(B)*[9]	*(D)*[10]	*(C)*
CH_2Cl_2	0.3		8.6	20	1	1	1	1
$CHCl_3$	0.2	0.003	0.08	0.01	3	150	120	2000
CCl_4	0.003	0.0003	0.002	0.0001	230	7700	2400	280000
CF_3Cl	10				0.04		2	
CF_2Cl_2	0.5	0.002			1		46	
$CFCl_3$	0.1	0.0002			5		1200	
$CHBr_3$	0.2			0.0004	4			140000
$CHBr_2Cl$	0.3			0.0009	3			54000
$ClCH_2CH_2Cl$	0.0009		13	10	430	0.8		2
$ClCH_2CHCl_2$	0.0009		0.07	0.002	540	190	1	18000
$Cl_2CHCHCl_2$	0.03		0.008	0.01	25	2100		2700
$ClCH_2CCl_3$	0.02			0.0005	26			80000
CH_3CHCl_2	0.9			80	0.4			0.3
CH_3CCl_3	0.8	0.002	0.03	0.003	0.7	430		10000
$ClCF_2CFCl_2$	0.2			0.008	4			5800
cis-$ClCH=CHCl$	0.2		13	30	2	0.8	0.3	0.9
trans-$ClCH=CHCl$	0.2		8.4		2	1	0.4	
$ClCH=CCl_2$	0.002		0.02	0.02	230	670	130	1800
$Cl_2C=CCl_2$	0.001		0.003	0.001	590	5600	1100	29000

* ^{63}Ni; (A) = d.c. mode, 300°C; (B) = not specified; (C) = pulsed mode, 70°C; (D) = pulse-modulated constant-current mode, 250°C.
** Normalized with respect to the value for CH_2Cl_2.

less sensitive, as speculated from the chemiluminescence reaction mechanism. Needless to say, the LDR for the CLD is much longer than that for the ECD operated in the d.c. of pulsed mode. The RMRs in the table indicate that the CLD sensitivity to individual VPHHCs in homologous series does not differ as much as that for the ECD. The selectivities, upon which along with sensitivity great emphasis should be laid, are expressed in terms of the RMRs of various other compounds to CH_2Cl_2 (Table III). It is obvious from the table that the CLD is much more selective than the ECD; in contrast to the ECD, no compounds are more sensitive than the VPHHCs. This means that the basic problem of the ECD, the practical impossibility of predicting which compound is giving a particular response, is not present in the CLD. Consequently, the CLD possesses the advantage of permitting a more elegant detection of the VPHHCs in complex mixtures of polar compounds. On the other hand, the CLD suffers difficulties which arise from operation of the detector under reduced pressure and from consumption of sodium metal. However, these difficulties seem trivial when compared with the marked advantages described above: a load of 1 g of sodium metal can allow *ca.* 40 h of operation without any pronounced change in sensitivity, and loading of the sodium metal is easily accomplished by preparing another Plug fitting, in which sodium metal is already loaded, in advance. Thus the CLD with these favourable features succeeds in overcoming the drawbacks of the ECD. This promises to lead to its wide usage as a detector for the determination of VPHHCs by GC.

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DUAL ELECTROCHEMICAL DETECTOR FOR MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO THE SELECTIVE DETECTION OF CATECHOLAMINES

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SUMMARY

A dual electrochemical detector having two working electrodes (anode and cathode) suitable for micro high-performance liquid chromatography has been characterized for the selective detection of catecholamines on the basis of their electrochemical reversibility. The rapid determination of applied potentials for such electrochemical detectors in liquid chromatography by means of cyclic semi-differential voltammetry is described. The dual electrochemical detector has been successfully utilized for the selective determination of catecholamines in human urine injected directly into a micro high-performance liquid chromatograph with an alumina preconcentration micro-column.

INTRODUCTION

Micro high-performance liquid chromatography (MHPLC)[1] using packed columns of I.D. less than 1 mm and packed or open-tubular capillary liquid chromatography (capillary LC)[2,3] are attractive in that the consumption of the mobile phase and sample is much less than that in conventional HPLC. Flow-rates of the mobile phase employed in MHPLC and capillary LC are typically 0.1–30 μl/min. These extremely slow flow-rates require that small volume detectors be designed and this has been achieved for spectrophotometric detection by UV[1] and fluorescence methods[4]. The usefulness of electrochemical detectors in HPLC has already been recognized[5–7].

Goto *et al.*[8] and Hirata *et al.*[9] independently designed sub-microlitre electrochemical detectors having one working electrode for use in MHPLC and capillary LC, and they were successfully utilized for the determination of aminophenol isomers separated on a micro-column and of acidic and neutral metabolites of tyrosine and tryptophan in human urine separated on a packed capillary column, respectively.

In a preceding paper[10], an electrochemical detector having two working electrodes (dual electrochemical detector) was designed for use in MHPLC and was successfully utilized for the selective detection of catecholamines based on their elec-

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trochemical reversibility. The principle of selective detection by the dual electrochemical detector is as follows. The anode and cathode in the twin electrode thin-layer electrolytic cell are set at potentials where the reductant (sample) is oxidized and its oxidant is reduced, respectively. The reductant of reversible or quasi-reversible species is oxidized at the anode and the product of this electrode reaction is re-reduced at the cathode, whereas the reductant of irreversible species is not re-reduced at the cathode. Therefore, the reversible and/or quasi-reversible species present in irreversible species can be selectively detected by measuring the re-reducing or re-oxidizing current.

Care is required in selecting the applied potentials of such electrochemical detectors in LC. To maximize the analytical response and selectivity and minimize background interference, the applied potentials should be held at the minimum potential for oxidation or the maximum potential for reduction, at which currents reach the limiting values for the sample under investigation. The optimum applied potentials can be determined directly by hydrodynamic voltammetry (HDV), in which peak current (i_p) is measured against applied potential (E), point by point, for the sample injected into the stream flowing through the electrochemical detector under the experimental conditions of LC. However, HDV measurements require several hours for completion. Alternatively, the approximate applied potentials for electrochemical detectors can be established, indirectly, much more rapidly by cyclic voltammetry (CV), which may be carried out on a quiet solution (without stirring) in a separate electrochemical cell[11]. Cyclic semi-differential voltammetry (CSV)[12–15] provides higher sensitivity and better resolution than ordinary CV. The technique measures the semi-derivative of current with respect to time (e) *versus* applied potential under the same experimental conditions as in CV. We report here the cyclic semi-differential voltammetric determination of applied potentials for the dual electrochemical detector in MHPLC, using catecholamines in human urine as the test samples.

EXPERIMENTAL

Apparatus

The MHPLC system with a pre-concentration micro-column and a dual electrochemical detector as described previously[10] was used for HDV measurements and the selective determination of catecholamines in human urine with direct injection. The design of the twin-electrode thin-layer electrolytic cell for the dual electrochemical detector is shown in Fig. 2 in a preceding paper[10]. The thin-layer cavity was constructed of two fluorocarbon resin blocks separated by a PTFE sheet 50 μm thick and 2 mm wide. Two working electrodes were made with glassy carbon disks of 3 mm diameter contained in one of the blocks. The silver/silver chloride reference electrode was held in a cylindrical hole in the other block. A platinum tube served as the counter electrode and the exit line. A micro alumina precolumn (2 cm $\times$ 0.5 mm I.D.) and a micro ODS column (15 cm $\times$ 0.5 mm I.D.) were used for enriching catecholamines and separating them.

A cyclic voltammetric instrument (Bioanalytical Systems, Model CV-1B) and a home-made analogue semi-differentiating circuit as shown in Fig. 1 in a previous paper[16] were used for CSV measurements. The output of the cyclic voltammetric instrument was fed to the analogue circuit for semi-differentiation through a voltage

follower and a resistor in order to minimize electrical noise. An *x–y* recorder (Yokogawa Co., Model 3086) was used to record the cyclic *e versus E* curves (cyclic semi-derivative voltammograms). A glassy carbon disk of 3 mm diameter was used as the working electrode. The surface of the disk was polished to a mirror finish with alumina powder (0.05 μm) on an acrylic resin plate before use. A silver/silver chloride electrode and a platinum wire were used for the reference and the counter electrode, respectively.

Reagents

Analytical-reagent grade chemicals were used without further purification. All solutions were prepared from distilled, deionized water. For standard samples, noradrenaline (NA), adrenaline (AD), dopamine (DA) and *l*-dopa (LD) were dissolved in Britton–Robinson buffer (B–R buffer) of pH 1.8. The mobile phase used in MHPLC and the solvent used in CSV were B–R buffer of pH 1.8 containing 0–0.5 m*M* sodium 1-heptanesulphonate (HSA) as the ion-pair reagent and 0–1 m*M* EDTA (disodium salt) as the masking reagent for iron(II) ion. The buffer solution for pre-treatment of the micro alumina pre-concentration column and pH adjustment of the sample in the MHPLC system was 1 *M* Tris buffer of pH 8.7 containing 0.25% EDTA (disodium salt) and 0.05% sodium hydrogen sulphite as the stabilizing reagents for catecholamines.

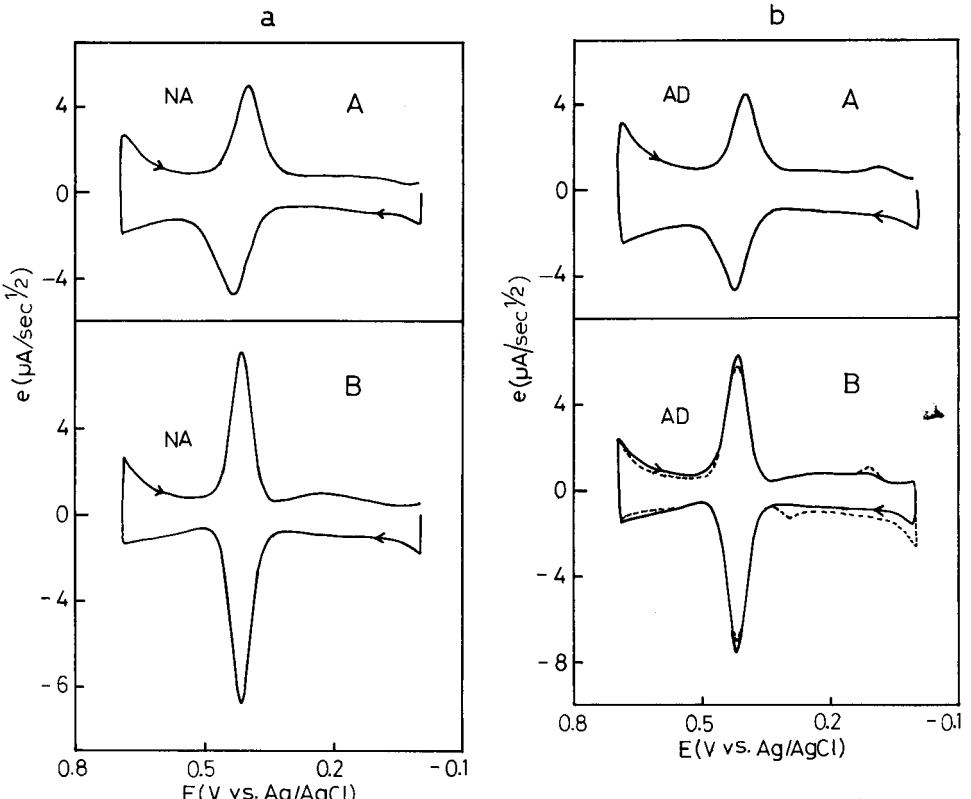

Fig. 1.

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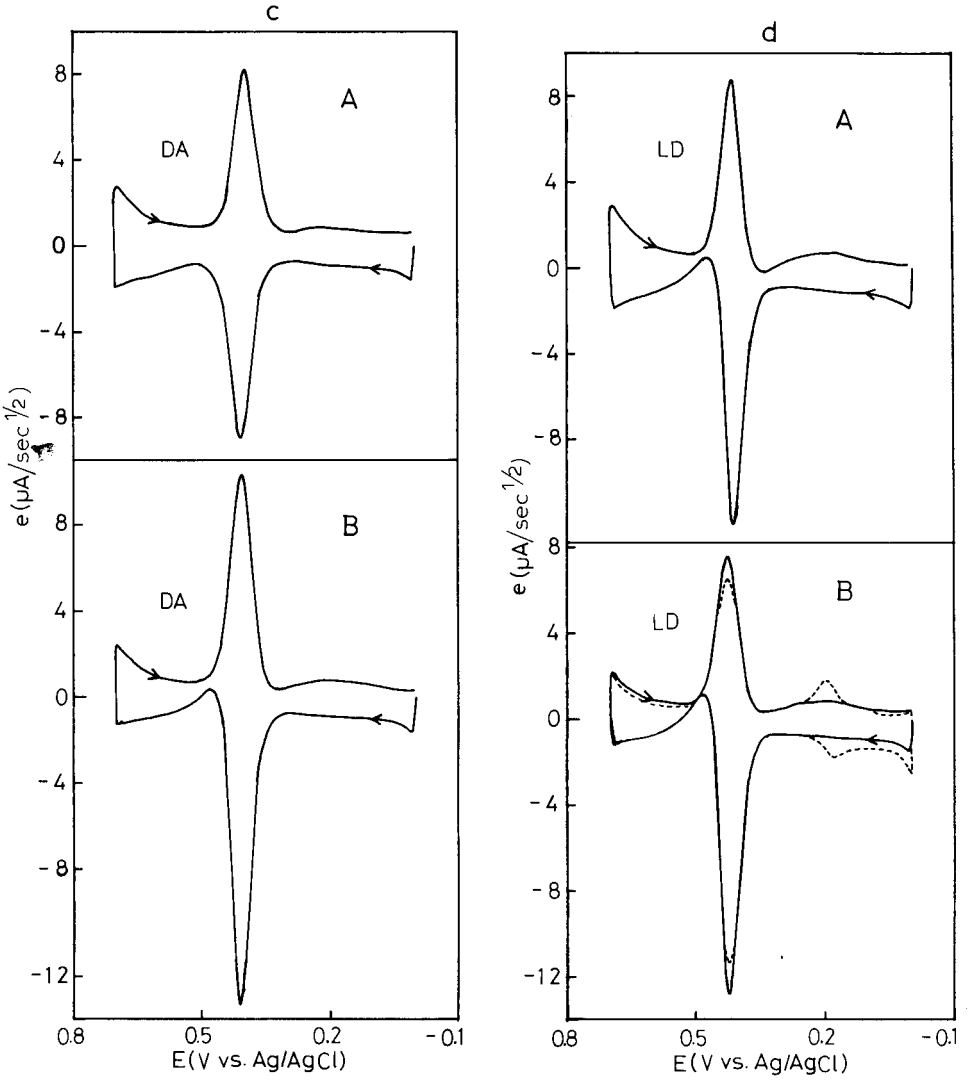


Fig. 1. Cyclic semi-derivative voltammograms of 0.1 m*M* catecholamines in B–R buffer of pH 1.8 alone (A) and containing 0.2 m*M* HSA (B). Scan rate: 100 mV/sec. (a) Noradrenaline; (b) adrenaline; (c) dopamine; (d) *l*-dopa.

RESULTS AND DISCUSSION

Cyclic semi-differential voltammetry of catecholamines

The cyclic semi-differential voltammetric behaviours of NA, AD, DA and LD were studied in B–R buffer alone and containing HSA and/or EDTA. Fig. 1 shows typical cyclic semi-derivative voltammograms of the four catecholamines in B–R buffer of pH 1.8 alone and containing 0.2 m*M* HSA. Theoretically, the anodic peak potential (E_{pa}) in the cyclic *e versus E* curves coincides with the cathodic peak potential (E_{pc}) for the reversible system[13]. The peak potential differences of NA, AD, DA

TABLE I

PARAMETERS FOR CSV OF CATECHOLAMINES IN DIFFERENT MEDIA

Scan rate, 100 mV/sec; sample concentration, 0.1 m*M* each. Medium: (A) B–R buffer of pH 1.8 alone; (B) A plus 0.2 m*M* HSA; (C) A plus 0.5 m*M* HSA; (D) C plus 0.1 m*M* EDTA.

Catecholamine	*Medium*	*Peak potential (V vs. Ag/AgCl)*		*Peak potential differences ($E_{pa} - E_{pc}$) (mV)*
		E_{pa}	E_{pc}	
NA	A	0.43_0	0.41_0	20
	B	0.42_5	0.42_5	0
	C	0.42_0	0.41_5	5
	D	0.41_3	0.41_2	1
AD	A	0.43_0	0.40_2	28
	B	0.42_5	0.42_0	5
	C	0.42_0	0.41_7	3
	D	0.41_7	0.41_3	4
DA	A	0.40_0	0.39_0	10
	B	0.41_0	0.40_5	5
	C	0.40_0	0.39_8	2
	D	0.40_0	0.39_8	2
LD	A	0.41_3	0.41_5	− 2
	B	0.42_3	0.42_7	− 4
	C	0.42_0	0.42_2	− 2
	D	0.42_1	0.42_5	− 3

and LD in B–R buffer of pH 1.8 alone were 20, 28, 10 and 2 mV, respectively (see Table I). This indicates that the electrode reactions of NA, AD and DA are quasi-reversible, while that of LD is reversible in this medium. It is interesting that the electrode reactions of all four catecholamines become reversible on adding ion-pair reagent to the medium, as can be seen in parts B in Fig. 1 and Table I. The asymmetric anodic peak shape for DA and LD in Fig. 1 indicates that they tend to be adsorbed on a glassy carbon electrode. On the first anodic scan for AD, an anodic peak occured at 0.43 V (*vs.* Ag/AgCl), which corresponds to the oxidation of AD to the open-chain quinone. On potential reversal the re-reduction of this quinone was observed at 0.42 V. On subsequent cycles, these peaks were diminished in intensity and another cathodic peak at 0.11 V as well as a new anodic peak at 0.30 V appeared, as shown by the dotted line (the fourth cycle) in Fig. 1b. The former corresponds to the reduction of the cyclized product (adrenochrome) to leucoadrenochrome, while the latter is that of the re-oxidation of leucoadrenochrome to adrenochrome[17]. On subsequent cycles, the similar new cathodic peak at 0.20 V and anodic peak at 0.18 V appeared, as shown by the dotted line (the fifth cycle) in Fig. 1d for LD, while only one anodic and one cathodic peak were observed for NA and DA. The reversibility of catecholamines was virtually independent of the concentration of ion-pair reagent and EDTA present in the medium (see Table I).

Cyclic voltammetric determination of approximate applied potentials for electrochemical detectors in LC

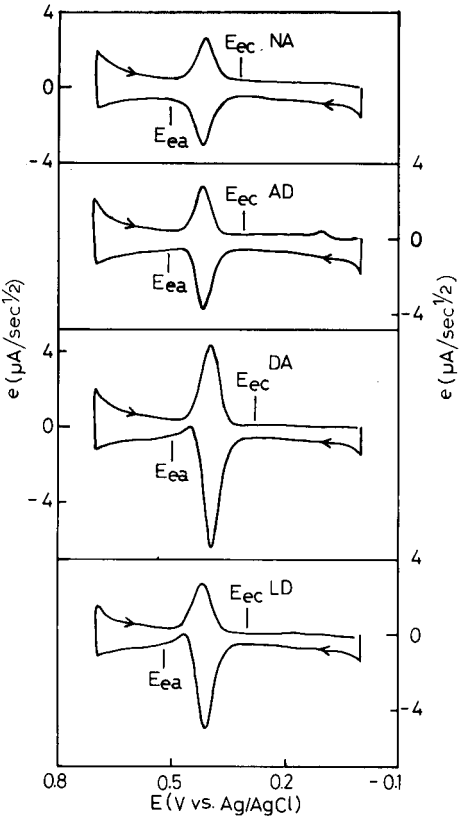
Fig. 2 shows the cyclic semi-derivative voltammograms of catecholamines at a scan rate of 40 mV/sec in the same medium used as the mobile phase in MHPLC. For selective detection of catecholamines by the dual electrochemical detector, the anode and cathode of the twin electrode cell should be set at the end potentials (E_{ea} and E_{ec}), at which the oxidation and re-reduction wave in the cyclic *e versus E* curve are complete, respectively. As shown in Table II, the end potentials of catecholamines tended to shift to larger overpotentials as the scan rate of the potential increased, whereas their peak potentials were virtually constant, independent of the scan rate. It is expected, moreover, that the end potentials in CSV and the minimum or maximum potentials for the limiting currents in HDV for slow electron transfer reactions will shift to larger overpotentials as the scan rate of the potential or the flow-rate of the

TABLE II

PARAMETERS FOR CSV OF CATECHOLAMINES IN B–R BUFFER OF pH 1.8 CONTAINING 0.5 m*M* HSA PLUS 0.1 m*M* EDTA AT DIFFERENT SCAN RATES

Sample concentration: 0.1 m*M* each.

Catechol-amine	*Scan rate (mV/sec)*	*Peak potential (V vs. Ag/AgCl)*		*End potentials (V vs. Ag/AgCl)*	
		E_{pa}	E_{pc}	E_{ea}	E_{ec}
NA	10	0.42	0.42	0.48	0.34
	20	0.42	0.42	0.49	0.33
	40	0.42	0.41	0.50	0.32
	60	0.42	0.42	0.52	0.28
	80	0.42	0.42	0.53	0.26
	100	0.41	0.41	0.55	0.22
AD	10	0.42	0.42	0.49	0.35
	20	0.42	0.42	0.50	0.33
	40	0.42	0.42	0.51	0.31
	60	0.42	0.42	0.52	0.29
	80	0.42	0.41	0.53	0.27
	100	0.42	0.41	0.55	0.22
DA	10	0.40	0.40	0.47	0.32
	20	0.40	0.40	0.48	0.30
	40	0.40	0.40	0.50	0.28
	60	0.40	0.40	0.52	0.26
	80	0.40	0.40	0.54	0.25
	100	0.40	0.40	0.56	0.21
LD	10	0.42	0.42	0.48	0.34
	20	0.42	0.42	0.50	0.32
	40	0.41	0.42	0.52	0.30
	60	0.41	0.42	0.54	0.29
	80	0.41	0.41	0.56	0.28
	100	0.42	0.42	0.59	0.21



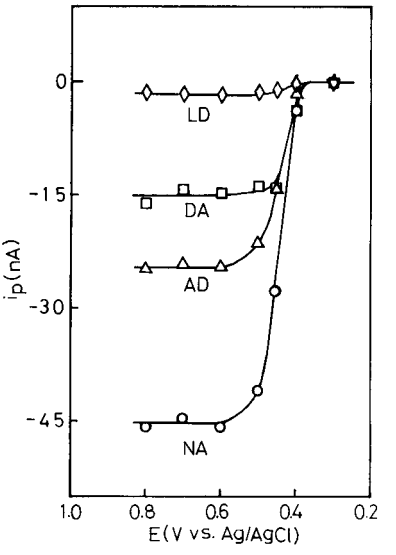


Fig. 2. Cyclic semi-derivative voltammograms of 0.1 m*M* catecholamines in B–R buffer of pH 1.8 containing 0.5 m*M* HSA plus 0.1 m*M* EDTA at a scan rate of 40 mV/sec.

Fig. 3. Hydrodynamic voltammograms of catecholamines using one working electrode. Mobile phase, B–R buffer of pH 1.8 containing 0.5 m*M* HSA plus 0.1 m*M* EDTA; flow-rate, 8.3 μl/min; sample injected, 5 ng each.

mobile phase increases, respectively[11]. Therefore, we recommend that one should determine the approximate applied potentials for electrochemical detectors in LC from the end potentials by CSV at a scan rate of 100 mV/sec and then determine the optimum applied potentials by HDV at a few potentials around them.

Hydrodynamic voltammetric determination of optimum applied potentials for electrochemical detectors in LC

Micro high-performance liquid chromatograms after pre-column enrichment of catecholamines were measured by using a twin electrode thin-layer electrolytic cell at different applied potentials. The mobile phase used was B–R buffer of pH 1.8 containing 0.5 m*M* HSA plus 0.1 m*M* EDTA and its flow-rate was 8.3 μl/min. Fig. 3 shows the i_p *versus* E curves (hydrodynamic voltammograms) of catecholamines using only one working electrode in the twin electrode cell. The minimum potentials, at which their oxidation peak currents reach the limiting values, were about 0.60 V for all four catecholamines.

Fig. 4 shows the hydrodynamic voltammograms using two working electrodes in the twin electrode cell under a constant potential of the upstream electrode (anode) of 0.80 V at different applied potentials of the downstream electrode (cathode or anode). It was found from the responses of the downstream electrode that the maximum potentials, at which their re-reduction peak currents reach the limiting values, were about 0.20 V, independent of the species of catecholamines. Therefore, the optimum applied potentials of the anode and cathode for selective detection of catecholamines in other electroactive species should be 0.60 and 0.20 V, respectively.

The limiting peak current ratios of re-reduction to oxidation of catecholamines were 0.68, 0.68, 0.78 and 0.71 for NA, AD, DA and LD, respectively, under the conditions shown in Fig. 4, in which no electric filter was used to record chromatograms. It should be noted that the limiting peak currents for oxidation of catecholamines in measurements using one working electrode increased by 13–22% when the potential of the downstream electrode was held at 0.20 V or more negative potentials compared with that using two working electrodes. This indicates that the oxidation and re-reduction of catecholamines are repeated between two working electrodes in the thin-layer cell because of the slow flow-rate of the mobile phase, *e.g.*, 8.3 μl/min.

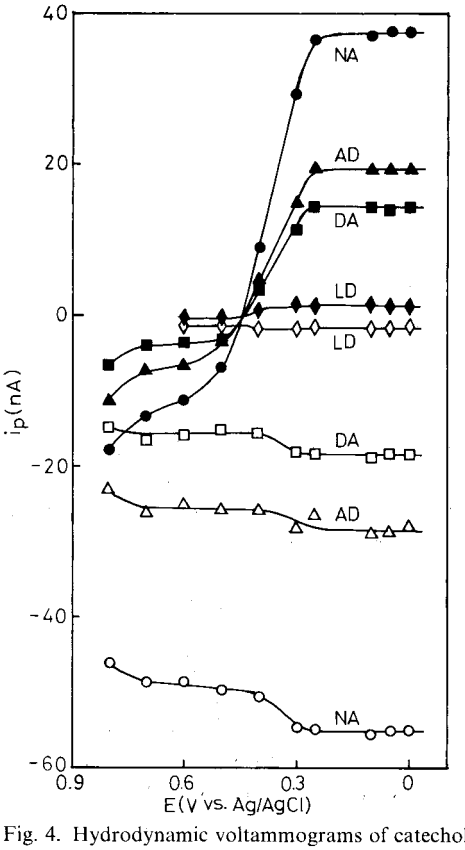


Fig. 4. Hydrodynamic voltammograms of catecholamines using two working electrodes. Upstream electrode potential: 0.80 V *vs.* Ag/AgCl. ○, △, □ and ◇, upstream electrode responses; ●, ▲, ■ and ◆, downstream electrode responses. Other conditions as in Fig. 3.

Kissinger *et al.*[18] predicted amplification of the electrochemical detector response obtained by recycling the electroactive functional group between its reduced and oxidized forms at extremely low flow-rates. One attempt to implement this concept failed because pellicular columns were used at too great a flow-rate[19]. This work is the first experiment in which an amplification effect based on such a concept was achieved.

Selective detection of catecholamines in human urine

Chromatograms of catecholamines in 100 μl of human urine injected directly without any pre-treatment into the MHPLC system with a micro pre-column were measured using the dual electrochemical detector by the procedures described previously[10]. Fig. 5 shows examples of the selective detection of catecholamines in urine from healthy humans under conditions in which the applied potentials of the anode and cathode were held at 0.60 and 0.20 V, respectively. Of particular interest in parts A is the peaks appearing as the background of NA and AD in Fig. 5a and as that of LD in Fig. 5b. By recording the re-reduction current, the interferences from the

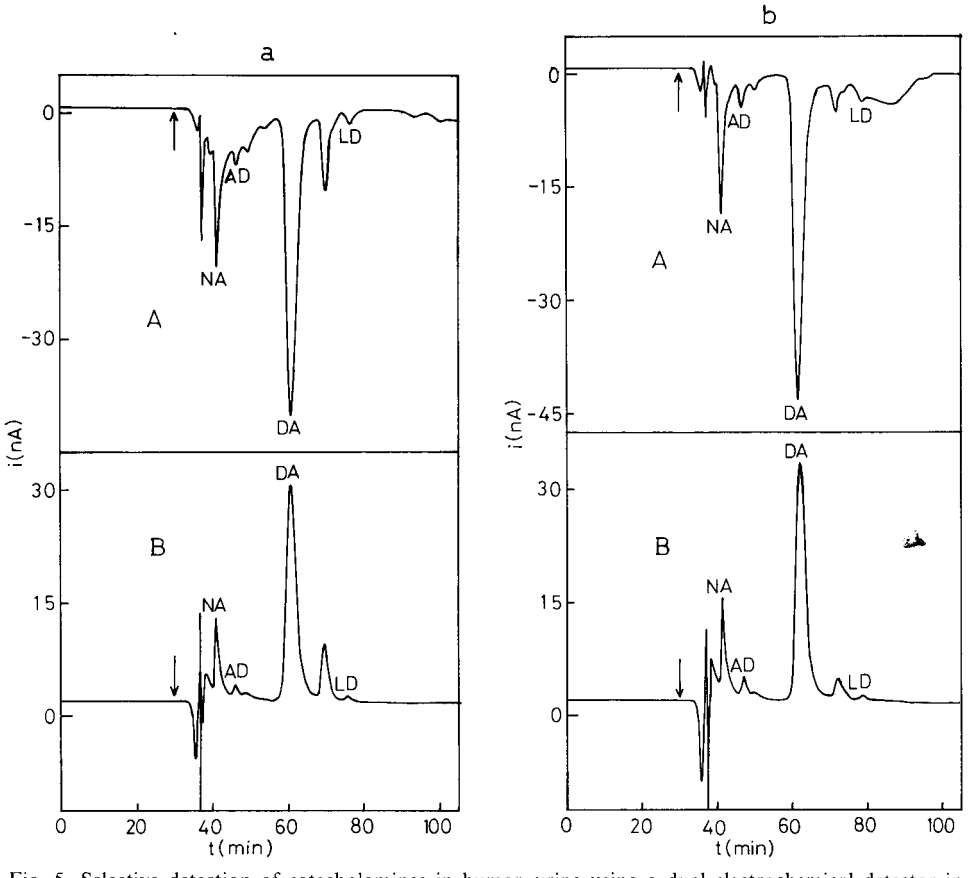


Fig. 5. Selective detection of catecholamines in human urine using a dual electrochemical detector in MHPLC. (A) Anodic response; (B) cathodic response. Sample: 100 μl of healthy human urine. Applied potentials:anode 0.60; cathode 0.20 V *vs.* Ag/AgCl. Other conditions as in Fig. 3.

compounds responsible for these peaks could be removed, as shown in parts B in Fig. 5 on the basis of their electrochemical irreversibility.

CONCLUSIONS

HDV measurements can determine precisely the optimum applied potentials for electrochemical detectors in LC, but require several hours or more for completion, owing to the time required for the background current to stabilize after each change of electrode potential. On the other hand, CV or CSV measurements can be made much more rapidly, requiring less than 1 min for one cycle, and provide both the potentials for oxidation (or reduction) and re-reduction (or re-oxidation) of the samples at the same time. CSV is a more suitable technique than CV for determining the approximate applied potentials for electrochemical detectors, because the peaks observed in CSV are sharp and symmetrical whereas those in CV are broad and asymmetric.

The dual electrochemical detector with an anode and cathode is a powerful instrument for the selective detection of reversible and/or quasi-reversible species present in many irreversible species and may provide an enhancement in sensitivity by recycling oxidation (or reduction) and re-reduction (or re-oxidation) between the anode and cathode for reversible and/or quasi-reversible species at the extremely slow flow-rates.

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GAS CHROMATOGRAPHIC PROPERTIES OF MIXED LIQUID CRYSTAL STATIONARY PHASES

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SUMMARY

The gas chromatographic properties of six stationary phases containing various mixtures of the liquid crystals N,N′-bis(*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine and N,N-bis(*p*-ethoxybenzylidene)-α,α′-bi-*p*-toluidine are reported in both their supercooled and normal nematic temperature ranges. At temperatures at which all mixtures are in their nematic states, separations of 1,3- and 2,3-dimethylnaphthalene, and anthracene and phenanthrene are highest for the stationary phases containing the highest percentage of N,N′-bis(*p*-ethoxybenzylidine)-α,α′-bi-*p*-toluidine. Since the mixed stationary phases can operate at lower temperatures than the pure compounds, it is possible to obtain better separations. Additionally, the liquid crystals interact with the support material, causing the crystal-to-nematic transition temperatures of the mixed-phase column packings to be generally lower than those of the liquid crystal mixtures themselves. This interaction also changes the eutectic composition of the mixed-phase packings. The results demonstrate that the use of mixed stationary phases improves selectivity and extends the usable temperature range of liquid crystal gas chromatographic columns.

INTRODUCTION

The use of nematic liquid crystals as gas chromatographic stationary phases is becoming increasingly popular[1]. Such phases have been successfully employed in a number of difficult separations, including those of polycyclic aromatic hydrocarbons (PAHs)[2], benzene isomers[3], and polychlorinated biphenyls[4]. However, the limited usable temperature range of such phases remains a significant problem, although several methods have been used to overcome this restriction. These include the synthesis of new phases with wider nematic temperature ranges[5], the employment of lower-temperature smectic[5,6] and supercooled-nematic[7,8] thermal regions of some liquid crystalline phases, and the use of mixtures. Generally, blends of two or more liquid crystals have a lower crystal-to-nematic transition temperature than any of the components, and can operate at lower column temperatures when used as gas–liquid chromatographic (GLC) stationary phases. The value of such an approach was demonstrated by Kelker *et al.*[9] and later by Schroeder and co-workers[3,10,11], who em-

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ployed mixed liquid crystalline stationary phases for the improved separation of benzene isomers.

Since the pioneering work of Kelker *et al.* and Schroeder and co-workers, a number of new, more versatile liquid crystalline stationary phases have been developed. The liquid crystal N,N′-bis(*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine (BMBT), for example, has a very wide nematic temperature range, and has been demonstrated to be a useful GLC stationary phase for the separation of 3–5-ring PAHs[12], dimethylnaphthalenes[7], and steroid epimers[13]. The use of BMBT in a mixed stationary phase thus has the potential for even greater versatility. We investigated this potential by measuring the gas chromatographic properties of mixtures of BMBT and its homolog, N,N′-bis(*p*-ethoxybenzylidene)-α,α′-bi-*p*-toluidine (BEBT).

EXPERIMENTAL

Materials

The liquid crystal compounds BMBT and BEBT were synthesized using published methods[12,14] and purified to give constant transition temperatures which matched those reported. The transition temperatures and enthalpies are listed in Table I.

TABLE I

TRANSITION TEMPERATURES AND ENTHALPIES OF THE COMPOUNDS STUDIED

T = transition temperature; ΔH = enthalpy; C → N = crystal to nematic mesophase; N → I = nematic mesophase to isotropic liquid.

Compound	*Transition*	*T (°C)*	*ΔH (kcal/mole)*
BMBT	C → N	181 (181)*	10.4*
	N → I	337 (337)*	1.7*
BEBT	C → N	174 (173)*	9.2*
	N → I	342 (341)*	2.2*

* Data from ref. 14.

Apparatus and procedure

Transition temperatures for all liquid crystal mixtures were determined by differential scanning calorimetry (DSC) using a Perkin-Elmer Model DSC-2 unit.

Gas chromatographic analyses were performed on a Hewlett-Packard Model 7610 gas chromatograph equipped with a flame ionization detector. All of the columns were glass (6 ft. × 2 mm I.D.). The column packings were 2.5% (w/w) of the liquid crystal mixtures on 100–120 mesh Chromosorb W-HP, prepared by dissolving each mixture in chloroform and then coating the support by the solvent slurry technique. The compositions of the liquid crystal mixtures used in each column packing are listed in Table II. Chromatograms were recorded on a 1 mV f.s. strip chart recorder using an electrometer setting of $16 \cdot 10^2$. Carrier gas flow (helium, 20 ml/min) was monitored by a calibrated Brooks 5840 Dual GC Mass Flow Controller, while hydrogen and air flow-rates (40 and 500 ml/min, respectively) were measured by a soap bubble flow meter. Samples (usually 1–2 μl) were injected with a Hamilton 701 N 10-μl syringe.

TABLE II

COMPOSITIONS OF LIQUID CRYSTAL MIXTURES USED IN EACH COLUMN PACKING

Column	*Mole percent BMBT*	*Mole percent BEBT*
1	0	100
2	26.8	73.2
3	43.1	56.9
4	61.6	38.4
5	76.3	23.7
6	100	0

The retention characteristics of 1,3- and 2,3-dimethylnaphthalene were determined while cooling the columns from 180°C to 85°C in 5° increments, while those of anthracene and phenanthrene were determined while heating the columns from 130°C to 205°C also in 5° increments. Retention times were corrected using benzene as the unretained solute, which was experimentally shown to be unretained over the temperature range used. The separation factor, α, was determined by the ratio of corrected retention times ($\alpha = t'_{R2}/t'_{R1}$).

RESULTS AND DISCUSSION

In addition to its potential for increased versatility in GLC analyses, the

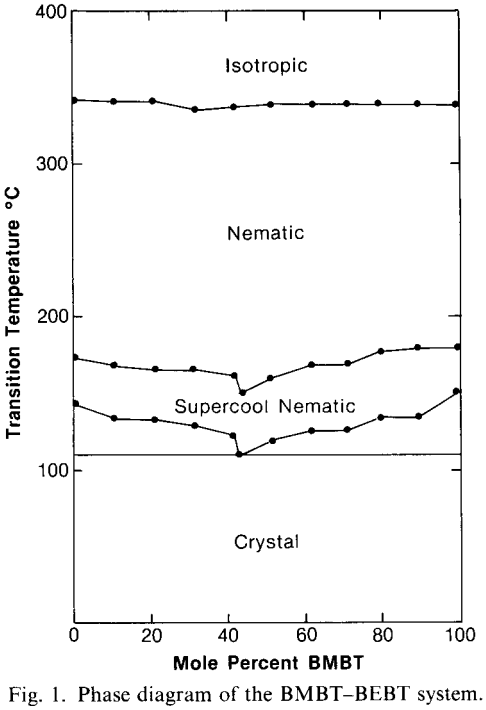

Fig. 1. Phase diagram of the BMBT–BEBT system.

BMBT–BEBT system was chosen for evaluation because it was expected to have predictable physical properties. Being homologs, the two liquid crystals have very similar structures, and therefore should be miscible. Additionally, because they are homologs, mixing of the two compounds should cause little or no disruption of liquid crystalline order. The phase diagram for the BMBT–BEBT system (Fig. 1) confirms these predictions. Both the crystal–nematic and nematic–isotropic transition temperature–composition curves are smooth, with no unexpected discontinuities. In fact, the nematic–isotropic curve is virtually linear, in contrast to the concavity of such curves with binary liquid crystal mixtures of dissimilar structure[11,15].

The experimentally determined eutectic composition and transition temperature for this system is in accord with the values predicted from the Schroder–Van Laar equations[16,17]:

$$-\ln X_1 = \frac{\Delta H_1}{R}\left(\frac{1}{T} - \frac{1}{T_1}\right)$$

$$-\ln (1 - X_1) = \frac{\Delta H_2}{R}\left(\frac{1}{T} - \frac{1}{T_2}\right)$$

where ΔH_1, T_1 and ΔH_2, T_2 are the transition enthalpies and temperatures of pure components 1 and 2 from crystal to nematic mesophase, and T is the eutectic transition temperature for a system containing X_1 mole fraction of component 1. Simultaneous solution of the equations using the data for BMBT and BEBT (Table I) gives a predicted eutectic composition of 43 mole percent BMBT and a transition temperature of 150°C, virtually identical to the experimentally determined values (Fig. 1). The applicability of these equations to the BMBT–BEBT system again indicates the formation of a nearly ideal solution, with little or no interactive forces between the two components.

Mixtures of BMBT and BEBT, as well as the pure liquid crystalline components, exhibit supercooling from the nematic to crystalline state. The supercooling transition temperatures are independent of the cooling rate, in accord with observations by Janini *et al.*[8] and others[7,18] for other supercooled liquid crystals. Additionally, the supercooling nematic-to-crystal transition temperature–composition curve (Fig. 1) parallels that of the normal crystal-to-nematic curve. The eutectic mixture of 43 mole percent BMBT and 57 mole percent BEBT can be supercooled to a temperature of 111°C before it crystallizes.

The existence of supercooled nematic thermal regions in mixtures of BMBT and BEBT gives such mixtures the potential for use of GLC stationary phases at lower column temperatures. The GLC properties of the mixed liquid crystal columns in these supercooled regions were evaluated by determination of the retention characteristics of 1,3- and 2,3-dimethylnaphthalene, solutes which have reasonable retention times at these lower temperatures, and have been successfully separated on other supercooled liquid crystalline phases[7]. At the higher temperatures near the normal (non-supercooled) crystal-to-nematic transitions, the columns were studied by investigation of the retention behavior of anthracene and phenanthrene.

Fig. 2 displays the retention behavior of 1,3- and 2,3-dimethylnaphthalenes (Fig. 2A) and anthracene–phenanthrene (Fig. 2B) in the supercooled and normal

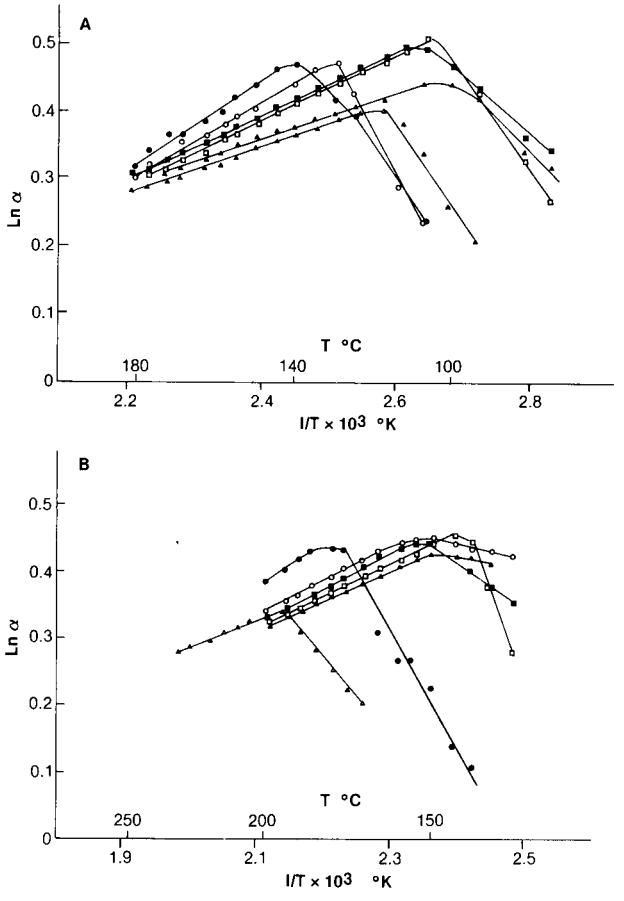

Fig. 2. Behavior of the separation factor with temperature for columns 1–6 for (A) 1,3-dimethylnaphthalene and 2,3-dimethylnaphthalene in the supercooled nematic regions of the columns and (B) anthracene and phenathrene in the normal nematic regions. ●, Column 1; ○, column 2; ■, column 3; □, column 4; ▲, column 5; △, column 6.

(non-supercooled) nematic thermal regions, respectively, for all six columns. The data are presented as plots of the logarithm of the separation factor, α, *vs.* the reciprocal of the absolute column temperature. Since the value ln α has been shown to be an index of relative selectivity for liquid crystal columns[14,19,20], this format provides a direct comparison of column characteristics. The curves are linear for all of the columns in their nematic thermal regions, in accord with Matire's thermodynamic equations[20] relating relative retention and temperature for liquid crystal columns.

At temperatures in which all columns are in their nematic thermotropic regions, selectivity for both 1,3- and 2,3-dimethylnaphthalene and anthracene–phenanthrene is highest for column 1, which consists of a 100% BEBT stationary phase. Pure BEBT has the highest nematic–isotropic transition temperature of all the mixtures evaluated, and thus its superior selectivity is consistent with theories relating increased liquid crystal column selectivity with higher nematic–isotropic transition

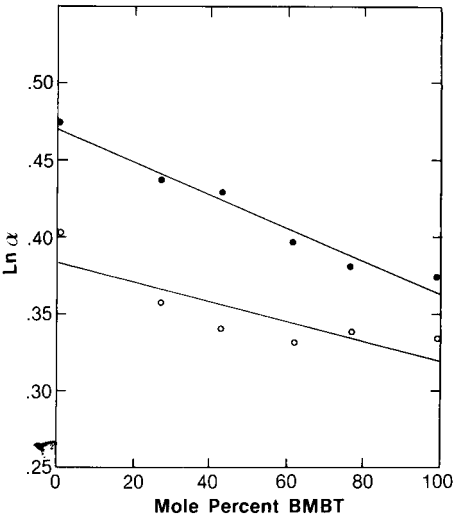

Fig. 3. Behavior of the separation factor with percentage of BMBT in the mixed stationary phase for 1,3-dimethylnaphthalene and 2,3-dimethylnaphthalene at 135°C (●) and anthracene and phenanthrene at 195°C (○).

temperatures[1,14,20]. Additionally, selectivity for the mixed liquid crystal columns is directly related to the composition of the mixture; at a given temperature, columns containing higher proportions of BEBT have greater selectivity. This relationship is displayed graphically in Fig. 3 as plots of the logarithm of the separation factor *vs.* mole percent BMBT at two column temperatures. For both the separations of 1,3- and 2,3-dimethylnaphthalenes and that of anthracene and phenanthrene, these curves are linear within experimental error (correlation coefficients greater than 0.99 for the equation below). At a given column temperature, then, the selectivity of a column composed of a mixed BMBT–BEBT stationary phase is simply a linear combination of the selectivities of the columns composed of the two pure liquid crystals. That is:

$$\ln \alpha_{1,2} = M_1 \ln \alpha_1 + M_2 \ln \alpha_2$$

where α_1, α_2 and $\alpha_{1,2}$ are the separation factors for two solutes on columns consisting of pure component 1, pure component 2, and the mixture, respectively, and M_1 and M_2 are the mole fractions of components 1 and 2 in the mixture, respectively. The above equation allows for the prediction of the separation of solutes for any column using a mixed BMBT–BEBT stationary phase. In addition, the applicability of the equation to the BMBT–BEBT mixed system further indicates the absence of any interactive effects on the separation properties of either of the components of the mixed phases. It should be emphasized that the equation is valid only at temperatures in which all columns are in their nematic states, and only for solutes which separate by the unique geometric factors[1,2,10] associated with liquid crystal columns, such as those used here.

Maximum values of α for both 1,3- and 2,3-dimethylnaphthalenes and anthracene–phenanthrene are shown in Table III. Despite the greater inherent selectivity of

TABLE III

MAXIMUM SEPARATION FACTORS OBTAINED ON EACH COLUMN

α_{max} = maximum separation factor; AN–PH = anthracene–phenanthrene; DMN = 1,3-dimethylnaphthalene–2,3-dimethylnaphthalene; T = column temperature.

Column	*Separation*	α_{max}	T (°C)
1	DMN	1.61	135
	AN–PH	1.54	175
2	DMN	1.61	125
	AN–PH	1.56	155
3	DMN	1.66	105
	AN–PH	1.56	155
4	DMN	1.72	105
	AN–PH	1.57	140
5	DMN	1.53	95
	AN–PH	1.53	150
6	DMN	1.51	115
	AN–PH	1.41	195

the nematic phase of BEBT, lower possible operating temperatures of mixed columns 2–4 allow equal or higher values of α to be achieved at these temperatures. Among the columns evaluated, the highest values of α were obtained on column 4, whose composition apparently strikes a favorable balance between inherent selectivity of its nematic phase and minimum operating temperature.

For virtually all liquid crystal stationary phases, it is well established that the temperature at which the maximum value of α is obtained corresponds to its liquid crystal transition temperature[1,2,14]. Based on this correspondence, crystal-to-nematic transition temperatures for each of the columns were obtained graphically from Fig. 2 as the temperatures of maximum α, in both the supercooled and standard nematic regions. These transition temperatures are plotted as a function of mole percent BMBT in Fig. 4, along with the transition temperatures for the mixtures themselves as determined by DSC. Clearly both the supercooling (Fig. 4A) and standard (Fig. 4B) transition temperatures for the column packings as determined by GLC are different from those obtained by DSC for the liquid crystal mixtures themselves. These differences, generally resulting in lower transition temperatures for the column packings, can only be the result of the interaction of the column support material with the liquid crystalline stationary phase. The support appears to be acting as a third component, generally resulting in further depression of the transition temperatures. The change in the transition temperatures, however, is not the same for each column. As shown in Fig. 4, this results in shifts in the eutectic composition of the mixed phases toward higher percentages of BMBT. Support–liquid crystal interactions have been observed in other liquid crystal columns[21,22]. While the retention characteristics of the mixed BMBT–BEBT columns indicate that these interactions do not significantly alter liquid crystalline order or the separation properties of the mixed stationary phases, they clearly have an important effect on the operating temperature ranges of such phases, which must be considered in the design of any mixed liquid crystal stationary phase.

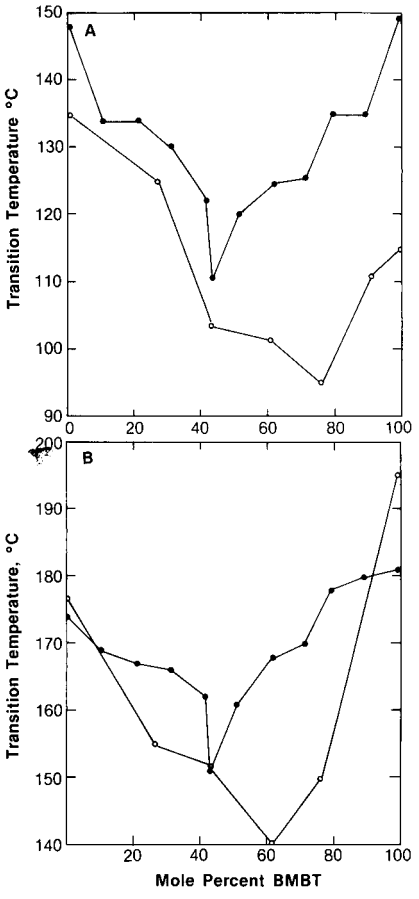

Fig. 4. Behavior of the supercooled (A) and normal (B) crystal–nematic transition temperatures with percentage of BMBT in mixtures of BMBT and BEBT, determined by DSC (●) and BMBT–BEBT mixed stationary phases on Chromosorb W-HP, determined by GC (○). An additional point is included in (A) for a column containing 91.0 mole percent BMBT.

CONCLUSIONS

In this study, gas chromatographic columns consisting of mixed BMBT and BEBT stationary phases have been shown to have greater selectivity than columns made from either of the two liquid crystals alone, due primarily to the lower operating temperatures which are possible. While certain characteristics of such mixed liquid crystalline columns, such as the separation factors of solutes, can often be accurately predicted from data obtained from columns made from the pure components, interactions of the support with the liquid crystalline stationary phase and the existence of supercooled nematic thermal regions make the accurate prediction of minimum operating temperatures virtually impossible. Yet, because these factors generally lower the minimum operating temperatures from the predicted values, the versatility of such columns is increased even further. The use of mixed liquid crystal

stationary phases in gas chromatographic analyses thus remains a powerful tool for the extension of the working temperature range and selectivity of liquid crystal GC columns in general.

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CHROM. 14,623

MICROSCOPIC ANALYSIS OF FOUR COMMERCIAL COLUMN PACKINGS UNCOATED AND COATED WITH A THIN ALGINATE ESTER FILM

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SUMMARY

The structures of four commercial column supports, uncoated and coated with an alginate ester film, have been examined with plain and polarized light microscopy. Some potential advantages of this simple technique for the preparation and use of this water permeable but water insoluble film are assessed.

INTRODUCTION

The efficiency of many solid supports employed in adsorption chromatography is limited by various structural defects in the supports. Some column parameters (*e.g.* flow-rate, exclusion limit and non-specific retention) are influenced by the shape and porosity of polymeric matrices. In addition, many anomalous chromatographic results can be attributed to the structure of the column matrix[1,2]. Nevertheless, microscopic data on many commercial column packings are scant.

The procedure for preparing agarose beads was first reported by Hjertén[3] who provided photomicrographs illustrating the spherical shape of the beads. Neame *et al.*[4] and Gribnau *et al.*[5] reported the presence of vacuoles of various sizes in Sepharose beads. Neame *et al.*[4] described the presence of a large number of highly motile, refractive particles in some of the vacuoles. However, these vacuolar inclusions appeared to be non-motile in the freeze-dried cyanogen bromide-activated Sepharose 4B[4,5]. Some of the vacuoles were shown to be filled with bacteria[4–6]. The difficulty in reconstituting the spherical shape of re-swollen cyanogen bromide-activated Sepharose was attributed to microbial digestion of the internal structure of the beads resulting in a collapsed disc-like structure[4]. Neame *et al.*[4] also published photomicrographs illustrating severe fracture and shrinkage of the CNBr-activated beads. This resulted in major distortion in pore sizes.

In an attempt to prepare improved adsorbents for use in immunoadsorption, we found it necessary to examine the microscopic features of some commonly used

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column packings (*i.e.* Sepharose 6B, Spheron P100,000, Porasil C and silica gel) with the aid of plain light with a green filter and polarized light. Numerous structural aberrations were observed in all the supports examined. This inspired us to coat the solid supports with a propylene glycol alginate ester to obtain a water-insoluble but water-permeable film created by base-catalysed transesterification at 20°C[7]. With the Sepharose and Spheron coated beads, the alginate ester film cross-linked with ethylenediamine proved to be very stable at pH 11.5. However, the durability of the alginate film on the Porasil beads was rather poor. Greater mechanical, chemical and thermal stability was achieved with the cross-linked alginate film. In addition, the non-specific adsorptive properties of Sepharose and Spheron was considerably reduced[7,8].

MATERIALS AND METHODS

Propylene glycol alginate ester (Manucol E/RE, batch 41652) was obtained from Alginate Industries (London, Great Britain). Sepharose® 6B was purchased from Pharmacia (Upsala, Sweden). Spheron® P100,000 was bought from Koch Light (Colnbrook, Great Britain). Silica gel, 66–100 mesh, ethylenediamine, *n*-butyl acetate and cyanogen bromide were obtained from BDH (Poole, Great Britain) and Porasil C was purchased from Waters Assoc. (Milford, MA, U.S.A.). Photomicrographs were taken with a Leitz Wetzlar Orthomat microscope and camera on Ilford PANF ASA 50 professional film using polarized light and plain light with a green filter at 20× magnification.

Encapsulation of solid supports with propylene glycol alginate ester cross-linked with ethylenediamine

Aqueous alginate-solution (5%, w/v) was used for the optimal coating of the following supports:

Silica gel 60–120 mesh. To silica gel (4.0 g) washed with distilled water and suspended in distilled water (20 ml), aqueous ethyleneiamine solution (10 ml, 2% v/v, pH 7.0) and alginate ester solution (20 ml) were added.

Porasil C. To Porasil C beads (2.5 g) washed with distilled water and suspended in distilled water (20 ml), aqueous ethylenediamine solution (10 ml, 2% v/v) and alginate ester solution (20 ml) were added.

Spheron P100,000. To Spheron beads (3.0 ng) were added distilled water (20 ml) aqueous ethylenediamine solution (15.0 ml, 2% v/v) and alginate ester solution (20 ml).

Sepharose 6B. To an aqueous suspension of Sepharose 6B beads (20 ml) were added distilled water (10 ml), aqueous ethylenediamine solution (20 ml, 2% v/v) and alginate ester solution (40 ml).

Coating procedure

To a 500-ml quick fit round bottom flask containing *n*-butyl acetate (150 ml), the alginate ester mixture for a given support was added and the solution stirred vigorously with a vibro-mixer for 10 min before the addition of sodium carbonate solution (100 ml, 10%, v/v) to effect polymerization of the alginate film by transesterification. After settling, the butyl acetate was decanted and the remaining aqueous mixture neutralized with HCl (1 *M*). The encapsulated beads were then

washed free of butyl acetate with phosphate buffered saline (PBS 0.2 *M*, pH 7.2) by successive centrifugation at 2000 *g*. The coated Sepharose, Porasil and silica beads which sedimented freely as discrete particles were stored in PBS. The much lighter coated Spheron beads did not settle easily and had to be filtered on a No. 3 sintered Büchner funnel and dried with acetone before storing in a dry state.

RESULTS

In general, it was observed that the coated beads tended to settle as discrete particles and the smaller uncoated beads such as Spheron tended to clump together. There was also a noticeable increase in the volume of the coated beads compared with the uncoated beads. In view of the observed heterogenicity in the Sepharose bead size, an overall increase in volume is not apparent from the photomicrographs. Nevertheless, an increase in volume was noticeable in several batches of coated beads.

The results of the microscopic observations of the coated and uncoated column packings viewed with plain light and a green filter and with polarized light are illustrated in Fig. 1 and Fig. 2, respectively. With the exception of the uncoated Porasil and silica supports which were examined in the dry state all the other beads were dispersed in PBS during microscopic examination.

DISCUSSION

A comparison of the photomicrographs of the coated and uncoated beads viewed with plain light and a green filter (Fig. 1) reveal a variable bead size in the commercial Sepharose 6B specimen (Fig. 1A and B). The difference between the coated and uncoated Sepharose beads was also less apparent. Nevertheless, some beads were observed with one or more small vacuoles which were optically empty. Other beads possessed larger vacuoles with strongly refractive particles. These observations confirm the findings reported by Gribnau *et al.*[5]. The coated Spheron beads appeared mostly as discrete particles (Fig. 1C) whereas the uncoated particles were clumped (Fig. 1D). The acetone-dried coated Spheron beads were also satisfactorily reswollen.

Structural inhomogeneities similar to those observed on the coated and uncoated Sepharose beads were also noticeable on the coated and uncoated Porasil beads. Some of the Porasil beads were broken and others contained numerous small pits. Other beads had larger cavities containing strongly refracting matter. The coated Porasil beads, unlike the uncoated beads, possessed a well-defined film of alginate ester (Fig. 1E and F). The edges and faces of the coated crystalline silica gel beads were more darkly shaded than those of the uncoated beads. In both the coated and uncoated silica beads less structural inhomogeneities were observed.

In contrast, more convincing evidence of positive coating was observed with the coated beads photomicrographed with polarized light (Fig. 2). The high water content of Sepharose and other polymeric matrices used in affinity chromatography contributes to their low optical diffraction. Consequently, examination of these materials with bright field illumination is unsatisfactory. The use of polarized light afforded a significant improvement in the photomicrographs of Sepharose. A quasi

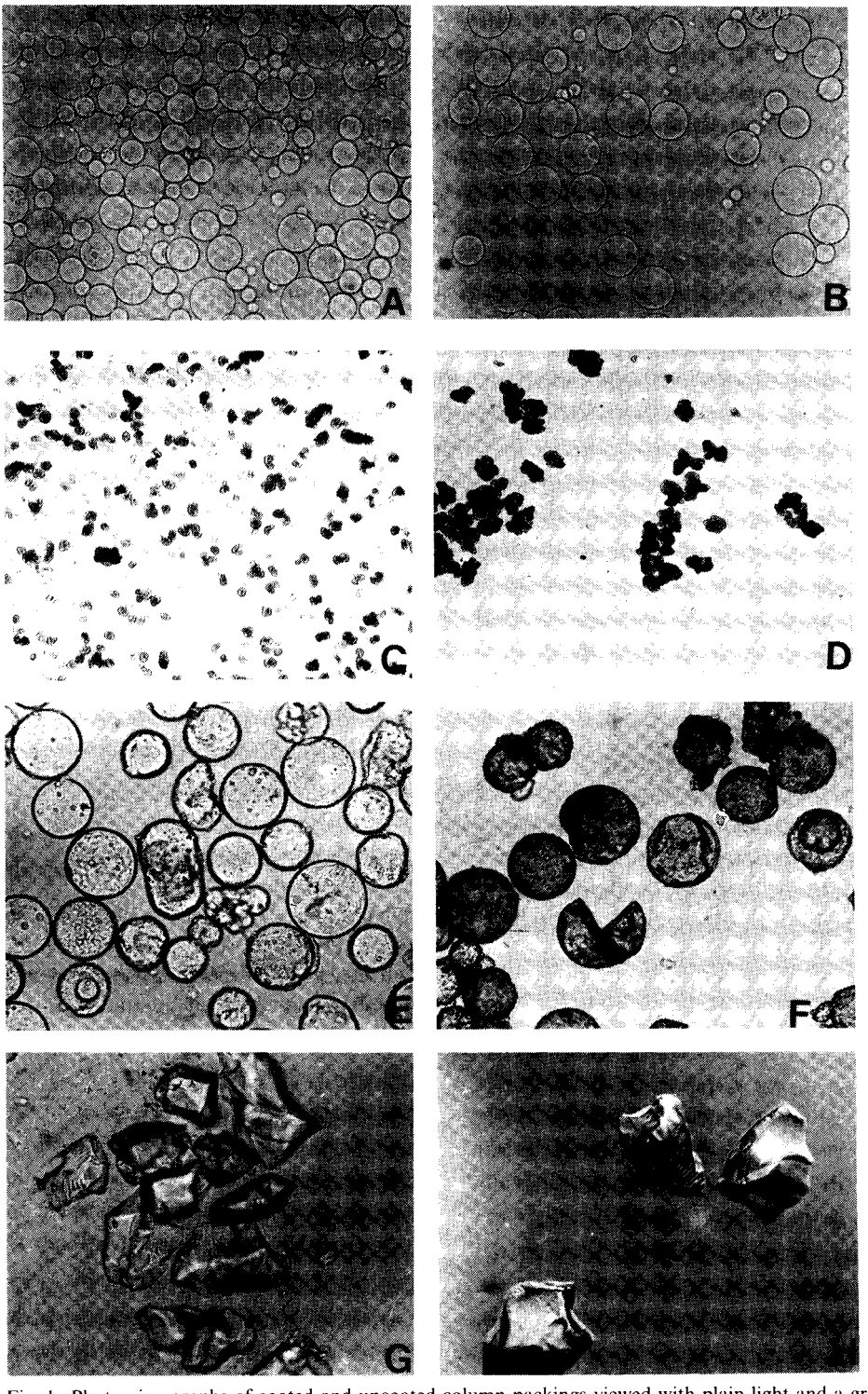

Fig. 1. Photomicrographs of coated and uncoated column packings viewed with plain light and a green filter: Sepharose 6B (A, B); Spheron P100,000 (C, D); Porasil C (E, F); and silica gel (G, H).

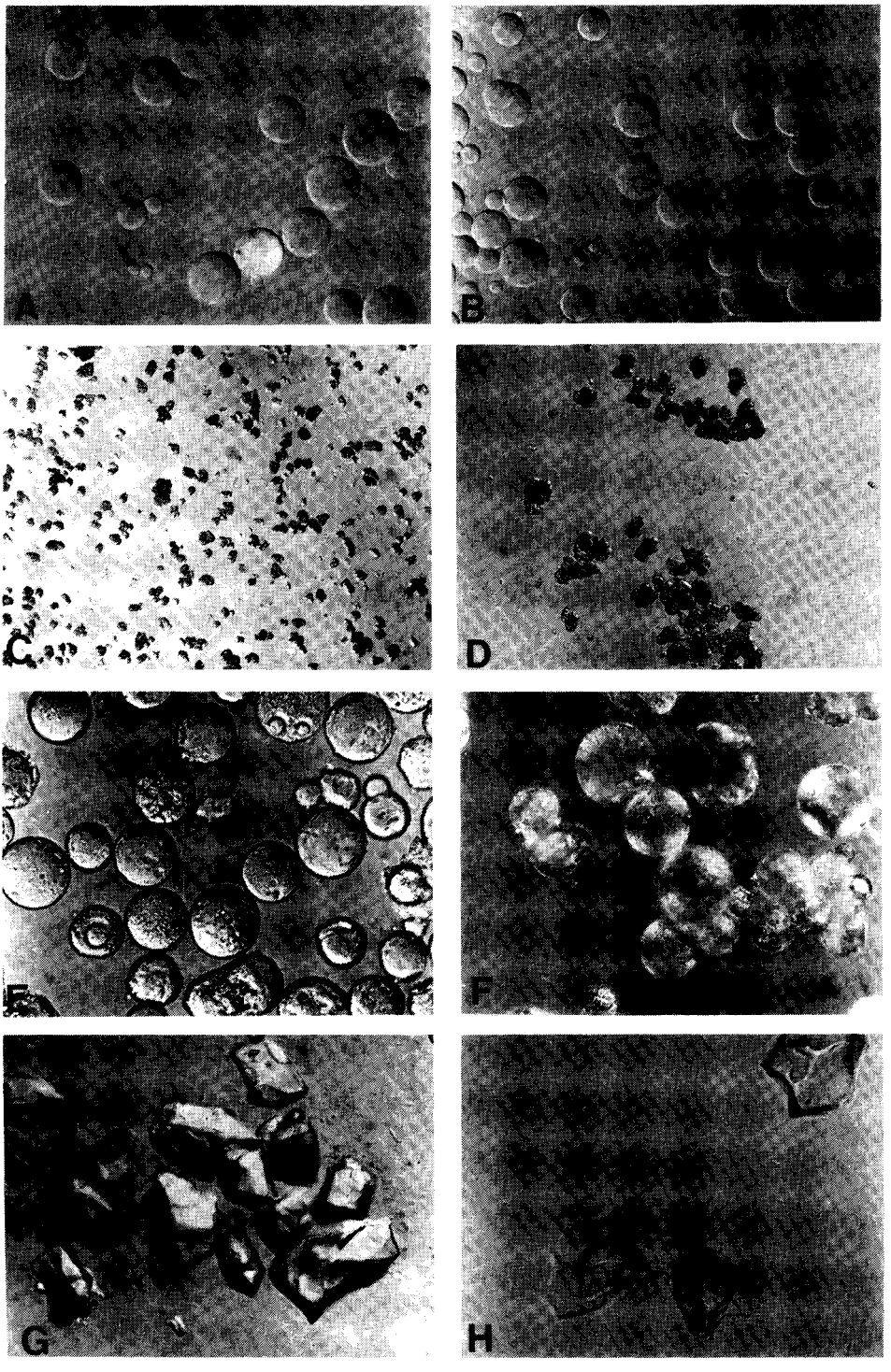

Fig. 2. Photomicrographs of coated and uncoated column packings viewed with polarized light: Sepharose 6B (A, B); Spheron P100,000 (C, D); Porasil C (E, F); and silica gel (G, H).

three-dimensional representation of the beads was obtained and the coated beads were somewhat larger than uncoated beads (Figs. 2A and B). The polarizing effect could be varied reproducibly by rotating the polarizer attached to the microscope condenser. This was dramatically observed with the uncoated Porasil beads which were found to be anisotropic. The distinction between the coated and uncoated isotropic silica gel beads was more pronounced with polarized light (Fig. 2G and H) than with plain light (Fig. 1G and H). The photomicrographs viewed with plain light (Fig. 1) highlight the contrast between the use of plain light and polarized light microscopy (Fig. 2). Moreover, with the exception of Sepharose, there is a significant difference between the coated and uncoated beads in Fig. 1. Positive coating of the beads with an alginate ester film was confirmed by the carbazole test for uronic acid residues.

These observations confirm the widespread occurrence of structural defects in a variety of commonly used column supports. Although the nature of the particulate inclusions in some of the vacuoles of Sepharose beads is disputed[4,5], it is commonly agreed that such structural defects could influence the chromatographic properties of the support materials. The use of column support materials coated with a thin water-permeable but water-insoluble film of propylene glycol alginate ester could afford the following chromatographic advantages:

(1) Improved mechanical, chemical and thermal stability of some supports are achieved by cross-linking the alginate ester with suitable alkyl amines.

(2) The use of Manucol ester E/RE with a high degree of esterification ensures that the esters are not easily precipitated by acids although storage in strong acid conditions could lead to depolymerization of the alginate.

(3) The substituent propylene glycol groups hinder the aggregation of polymer chains and ensure that fewer carboxyl groups are available for interaction with cations[9].

(4) The stability of the propylene glycol alginate film at high pH could make it less susceptible to ligand leakage.

(5) Conservation of a beaded matrix structure which enhances the preparation of supports of high binding capacity.

The structural defects common to many commercial column packings could be circumvented by the use of suitable non-porous beaded solid supports of uniform diameter which could be employed for the synthesis of efficient adsorbents. When such support materials are coated with a propylene glycol alginate ester film, a high binding capacity support with negligible non-specific binding properties can be achieved (unpublished results). An increase in the total bead surface area of a chromatographic column support material increases the capacity for adsorption and ion exchange. Nevertheless, there is also an increase in the sites for non-specific adsorption which, in turn, makes desorption more difficult.

Although agarose is the most popular support material, it exhibits significant non-specific adsorption, partly because of the chemical composition of the polymer and also because of derivatization procedures employed. With the available technology for producing stable, efficient coatings, the use of a suitable hydrophilic polymer which affords a significant reduction in non-specific adsorption should prove very useful.

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ANALYTISCHE CHARAKTERISIERUNG VON BISPHENOL A-EPOXIDHARZEN DURCH HOCHLEISTUNGS-FLÜSSIGCHROMATOGRAPHIE. I.*,**

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(Eingegangen am 25. November 1981)

SUMMARY

Analytical characterization of bisphenol A-type epoxy resins by means of high-performance liquid chromatography. I.

Low-molecular-weight bisphenol A-type epoxy resins can be analyzed by means of reversed-phase gradient elution with water–acetonitrile mixtures. Higher-molecular-weight and to some extent also medium-molecular-weight resins are only incompletely eluted with this system. Silica gel gradient elution with methylene chloride–acetonitrile or methylene chloride–methanol has proved to be generally practicable. In addition to the distribution of the oligomeric bis-glycidyl ethers especially by-products with glycolic, phenolic and chlorohydrinic containing terminal groups can be quantitatively determined by high-performance liquid chromatography. Oligomeric mono-glycidyl ethers with glycolic terminal groups were found in all analyzed epoxy resins and can be separated from the other components as a group of compounds. The chromatograms of low- to medium-molecular-weight resins obtained by high-performance liquid chromatography permit to calculate the epoxide equivalent directly.

EINFÜHRUNG

Die umfassende, für die Technologie und Verarbeitung notwendige Charakterisierung der komplex zusammengesetzten Bisphenol A-Epoxidharze (EPR) stellt hohe Anforderungen an die Analytik. Eine Reihe unterschiedlicher Untersuchungsmethoden muss herangezogen werden. Als leistungsfähig haben sich in letzter Zeit die Ausschlusschromatographie (GPC) und die Hochleistungs-Flüssigchromatographie (HPLC) erwiesen. Besonders letztere Methode liefert eine grössere Zahl von Informationen, die durch andere Methoden nur zum Teil und mit wesentlich höherem Aufwand zu erhalten sind.

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Die GPC von EPR wurde mehrfach beschrieben[1–5]. Bevorzugtes Lösungsmittel ist Tetrahydrofuran (THF); aber auch Dimethylformamid kam zur Anwendung[1].

In jüngster Zeit wird zur Harzcharakterisierung die HPLC eingesetzt[6–11]. Hier erwies sich die Umkehrphasen-Gradientenelution (RP-GE) mit Wasser–Acetonitril (W–AN)[9,10] als vorteilhaft. AN besitzt gegenüber THF u.a. Vorzüge hinsichtlich Selektivität und Reinigungsaufwand[9]. Zur Identifizierung oligomerer Nebenprodukte konnten die IR-, MS- und NMR-Spektroskopie herangezogen werden[10]. Hanuš *et al.*[11] zeigten kürzlich, dass die Trennung niedermolekularer EPR isokratisch mit *n*-Hexan–Chloroform–Isopropanol auch an unmodifiziertem Silicagel möglich ist.

In der vorliegenden Arbeit wurde das chromatographische Verhalten einer grösseren Zahl unterschiedlicher EPR mit den Systemen LiChrosorb RP-8/W–AN (System I) und Silicagel (sphärisch)/Methylenchlorid (MC)–AN (System II, vgl. Tabelle I) eingehend untersucht. Hierbei zeigte sich, dass mit W–AN quantitativ nur nieder- und z.T. mittelmolekulare EPR* untersucht werden können. Bei höhermolekularen Harzen ist die Elution nicht vollständig. Demzufolge sinkt die Trennwirksamkeit der Säulen bei Serienanalysen allmählich ab, wobei der Säulenvordruck ansteigt.

EXPERIMENTELLER TEIL

Es standen ein Flüssigchromatograph des Typs 1084A (Hewlett-Packard, U.S.A.) mit Festwellenlängendetektor, 254 nm, und ein Detektor variabler Wellenlänge des Typs 1030B (Hewlett-Packard) zur Verfügung. Die Trennsäulen (250 × 4.6 mm) wurden im Falle des Systems I mit LiChrosorb RP-8, 10 μm (Merck, B.R.D.)

TABELLE I

BEDINGUNGEN ZUR HOCHLEISTUNGS-FLÜSSIGCHROMATOGRAPHIE VON EPOXIDHARZEN

Die Lösungsmitteltemperaturen für A und B beziehen sich auf den Entgasungsvorgang am Gerät 1084 A, sind jedoch auch für eine störungsfreie Flussregelung erforderlich.

	System I	*System II*
Trennsäule	LiChrosorb RP-8	Sphärisches Silicagel
Korngrösse	10 μm	5.6 μm
Probenlösung	20 mg EPR/5 ml MCS	200 mg EPR/5 ml MC
Lösungsmittel A	W	MC (0.15% W)
Lösungsmittel B	AN	MC–AN (7:3; 0.6% W)
Gradient	50–60% B/20 min; 60-90% B/30 min; 90% B/5 min	5–95% B/75 min
Dosiervolumen	10 μl (40 μg EPR)	10 μl (400 μg EPR)
Fluss	2 ml/min	2 ml/min
Lösungsmitteltemp. A	353°K	303°K
Lösungsmitteltemp. B	333°K	303°K
Säulenraumtemp.	313°K	303°K
Signal	UV, 254 nm	UV, 254 nm
Signalabschwächung	2^4	2^4

* $\bar{E}_n$-Werte für niedermolekulare EPR: *ca.* 170–220, und für mittelmolekulare EPR: *ca.* 300–500. (Einteilung nach Lit. 12).

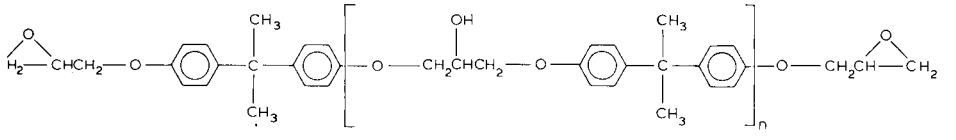
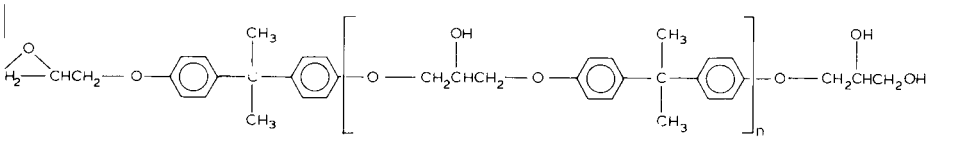
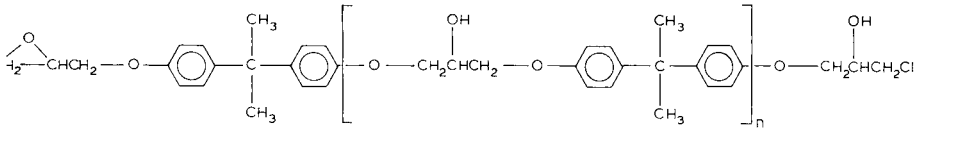
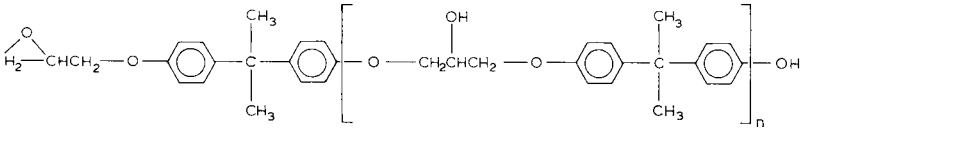
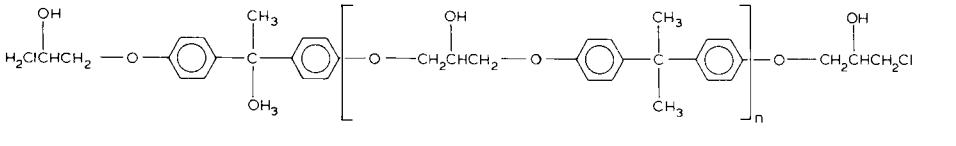
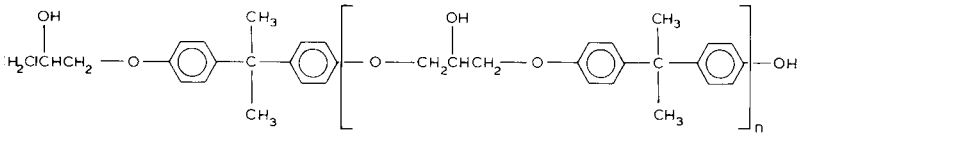
und im Falle des Systems II mit sphärischem Silicagel eigener Herstellung[13] (5.6 μm, $A_a = 440\,m^2\,g^{-1}$, $V_p^* = 1.01\,cm^3\,g^{-1}$, $\bar{D} = 9.2\,nm$*, mittels Schwebesuspensionstechnik[14,15] gefüllt.

Reagenzien

AN, rein (VEB Laborchemie Apolda, D.D.R.), MC, rein (VEB CK Bitterfeld, D.D.R.) und Methanol, rein (VEB Leuna-Werke, D.D.R.) wurden vor der Chromato-

TABELLE II

STRUKTUREN VON BISPHENOL A-EPOXIDHARZEN

Struktur	*Kurzzeichen*	
	n_xEPEP	E_x
	n_xEPG1	G_x
	n_xEPC1	C_x
	n_x EPOH	O_x
	n_xClCl	2
	n_xClOH	1

* Die in Lit. 13 und in Lit. 15 angegebenen Werte für V_p^* und $\bar{D}$ sind mit dem Faktor 0.554 zu multiplizieren.

graphie über eine 40 cm Füllkörperkolonne destilliert*. Frisch destilliertes Wasser wurde nach dem Passieren einer G4-Fritte eingesetzt. Die Vorbehandlung von THF, p.a. (VEB Laborchemie Apolda) erfolgte mit KOH, Perkolation über Silicagel und fraktionierte Destillation. Von Methylcellosolve (MCS), rein (Riedel de Haen, B.R.D.) kam eine mittlere Destillatfraktion zum Einsatz.

Epoxidharze

Zur Untersuchung gelangten EPR-Versuchsprodukte des VEB Leuna-Werke sowie Harzmuster verschiedener Firmen unterschiedlichen Typs (siehe Tabelle V).

Flüssigchromatographie

Tabelle I enthält die wichtigsten Bedingungen für die Chromatographie mit den Systemen I und II.

ERGEBNISSE UND DISKUSSION

Allgemeine Anforderungen für die Trennung

Aussagekräftige Harztrennungen müssen folgenden Forderungen genügen:

(a) hohe Auflösung am Chromatogrammanfang;

(b) Auftrennung der Bisglycidylether (n_xEPEP) bis zu hohen Kondensationsgraden;

(c) individuelle Trennung der Monoglycidylether mit glykolischen Endgruppen (n_xEPG1); sowie

(d) weitgehende Auftrennung der Monoglycidylether mit phenolischen (n_xEPOH) und chlorhydrinischen (n_xEPC1) Endgruppen, und

(e) Abtrennung von epoxygruppenfreien Nebenprodukten (n_xOHCl, n_xClCl) sowie Bisphenol A.

Die in Klammern symbolisierten Strukturen sind in Tabelle II zusammengestellt.

Erfahrungen mit dem System I

Ein typisches Chromatogramm eines mittelmolekularen EPR durch RP-GE mit W–AN zeigt Fig. 1. Die Identifizierung der Hauptkomponenten erfolgte massenspektrometrisch an präparativ isolierten HPLC- und GPC-Fraktionen.

Während sich alle niedermolekularen Epoxidharze mit dem System I ausgezeichnet untersuchen lassen, fiel bei der serienmässigen Untersuchung mittel- und höhermolekularer EPR die bereits erwähnte nachlassende Trennwirksamkeit auf. Eine quantitative Chromatogrammauswertung von *ca.* 70 EPR-Proben ergab den in Fig. 2 dargestellten Zusammenhang zwischen Gesamtpeakflächen und titrimetrisch bestimmter Epoxyäquivalentmasse ($\bar{E}_{mt}$[19,20] mit den Parametern $b = 0.0852$ und $a = 130.8$ für die Ausgleichsgerade[21] $y = a + bx$. Man erkennt eine Abnahme der Gesamtpeakflächen mit steigenden $\bar{E}_{mt}$-Werten, wobei konform damit zu niedrige Epoxyäquivalentmassen aus den Chromatogrammen ($\bar{E}'_{mc}$) errechnet wurden.

* Reinigungen des AN mit Silicagel–Phosphopentoxid–Calciumhydrid[16], mit Aluminiumchlorid–Lithiumcarbonat–Calciumhydrid[17] oder mit Aluminiumoxid–Natriumoctylat[18] waren wenig erfolgreich.

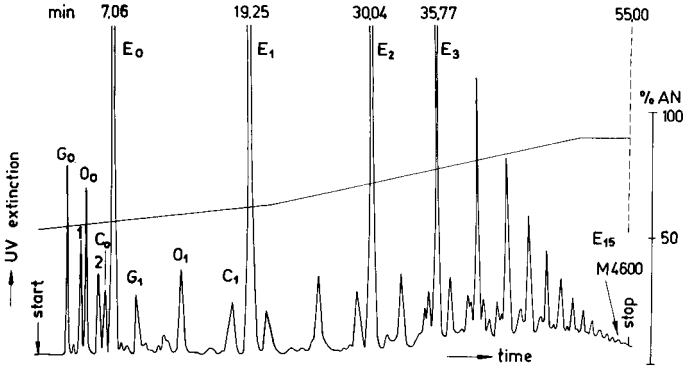


Fig. 1. Chromatogramm eines mittelmolekularen Bisphenol A-Epoxidharzes (M 330–2374, $\bar{E}_{nt}$ = 486, vgl. Tabelle V). Bedingungen (System I) vgl. Tabelle I; LiChrosorb RP-8 (10 μm); Trennsäule 250 × 4.6 mm; W–AN (Gradient); Fluss 2 ml/min; 40 μg EPR/10 μl MCS; UV Detektion, 254 nm; Abkürzungen siehe Tabelle II.

Wir überzeugten uns zunächst, dass dieses Verhalten weder durch systematischen Anstieg der Flussgeschwindigkeit während des Programms[22] noch durch die automatische Peakflächenauswertung verursacht wurde. Auch der Elutionsmittelgradient selbst kam für das Verhalten nicht in Frage. Ferner konnten Fraktionierungseffekte für die Oligomeren n_xEPEP durch Erhöhen der Probenmenge und des Wasseranteils im Elutionsmittel ausgeschlossen werden.

Zur Ermittlung der Flächenprozente ist die individuelle Extinktion E_n der Oligomeren zu berücksichtigen. Wenn man annimmt, dass die aromatischen Ringe voneinander unabhängige Extinktionsbeiträge liefern, lassen sich relative Extinktionswerte errechnen. Auf diese Weise erhält man die Korrekturfaktoren f_n, mit denen die

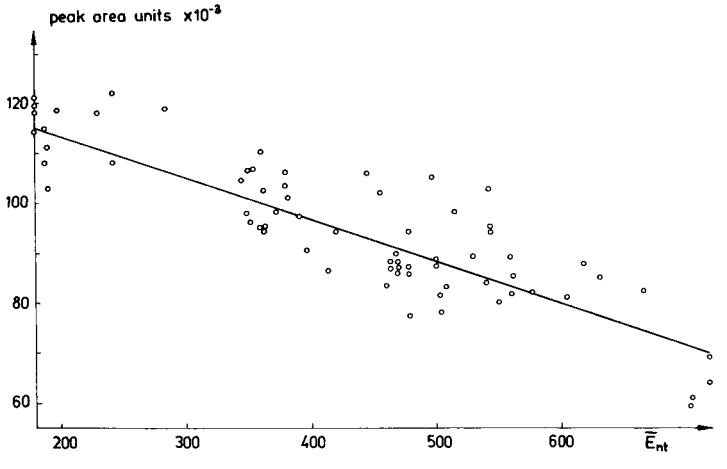


Fig. 2. Funktioneller Zusammenhang zwischen elektronisch integrierten Peakflächensummen (System I, Tabelle I) und titrimetrisch bestimmten Epoxyäquivalentmassen ($\bar{E}_{nt}$, Lit. 19, 20) für Bisphenol A-Epoxidharze

TABELLE III

VERGLEICH EXPERIMENTELL BESTIMMTER (exp.) UND NACH GLEICHUNG 1 BERECHNETER (ber.) EXTINKTIONEN

Verbindung	*M*	*ε (278 nm)*[4] *(1 mol*$^{-1}$ *cm*$^{-1}$*)*	*E(exp.)*	*E(exp.) relativ*	*E(ber.)*
n_0EPEP	340.0	3680	10.82	1.000	1.000
n_1EPEP	624.6	7080	11.33	1.047	1.088
n_2EPEP	908.9	10740	11.81	1.091	1.122
n_3EPEP	1193.2	14190	11.89	1.098	1.139
n_4EPEP	1477.6	17977	12.16	1.123	1.150
n_5EPEP	1761.9	20346	11.54	1.066	1.158
n_6EPEP	2046.2	24210	11.83	1.093	1.163

Peakflächen multipliziert werden müssen, damit gleiche Flächen gleichen Gewichtsmengen entsprechen:

$$E_n = E_0 \cdot \frac{M_0}{M_n}(n + 1) \tag{1a}$$

$$f_n = \frac{E_0}{E_n} = \frac{M_n}{M_0} \frac{1}{(n + 1)} \tag{1b}$$

n ist der Oligomerisationsgrad der Homologen, E_n bzw. E_0 sind die auf ein Gramm bezogenen Extinktionen für den Oligomerisationsgrad n bzw. $n = 0$, M_n und M_0 die relativen Molekülmassen für n_xEPEP und n_0EPEP.

Aus Tabelle III erkennt man, dass ein signifikanter Einfluss der Werte auf die Flächenprozente nur bei den niederen Gliedern zu erwarten ist.

Die Oligomerenverteilung der Bisglycidylether änderte sich bei der Peakflächenregistrierung im Wellenlängenbereich 254–280 nm nicht. Für die laufenden Messungen wurde die Wellenlänge von 254 nm bevorzugt.

Erhöhung der Elutionsmittelstärke bei der RP-GE[23] unter Austausch des AN durch THF erbrachte eine Lösung des "Flächenproblems": Die Gesamtpeakfläche vergrösserte sich durch eine zusätzliche, deutlich als Untergrund ausgebildete Fläche. Sie stand in Relation zu bestimmten Bedingungen während der Herstellung und zur mittleren relativen Molekülmasse der Proben.

Fig. 3 zeigt das Chromatogramm eines höhermolekularen EPR, wobei der Untergrund deutlich zu erkennen ist. In diesem Fall wurde die Trennung bereits an später eingesetztem, unmodifiziertem Silicagel mit MC–M vorgenommen. Die eingezeichnete Grundlinie konnte im Parallelversuch reproduzierbar ermittelt werden. Bei niedermolekularen EPR sind derartige Untergrundflächen nicht vorhanden.

Die untergrundbildenden Verbindungen liessen sich durch fraktionierte Fällung mit AN aus chloroformischer Lösung isolieren. Bei zunehmender Anzahl der Fällungen werden die Bisglycidylether systematisch entfernt, und schwerer lösliche Komponenten reichern sich an (Fig. 4). Nach der 11. Fraktionierung waren die Bisglycidylether vollständig entfernt. Daraufhin wurde der "Untergrund" (a) im System I (Tabelle I), (b) unter Austausch des AN im System I durch THF und (c) an Silicagel

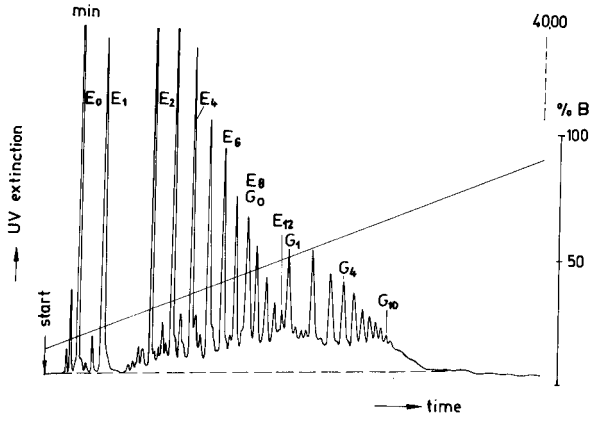


Fig. 3. Chromatogramm eines hochmolekularen Bisphenol A-Epoxidharzes (M 330–516/5A, $\bar{E}_{nt}$ = 720, vgl. Tabelle V). Bedingungen: sphärischen Silicagel (5.6 μm); Trennsäule 250 × 4.6 mm; MC (A)–(MC–M, 95:5) (B); 10–90 % B/40 min; Fluss 1.5 ml/min; 100 μg EPR/10 μl MC; UV-Detektion, 254 nm.

mit MC–M (Bedingungen wie in Fig. 3) chromatographiert. Die Auswertung der Chromatogramme ergab in den Fällen (b) und (c) gleiche Flächen, während im Falle (a) weniger als 50 % dieser Flächen erhalten wurden. Fig. 5 zeigt ein Chromatogramm der Fraktion 11 unter Verwendung von System I. Durch Vergleich mit Fig. 1 erkennt man die starke Verschiebung des Untergrundes nach höheren Retentionszeiten.

Erfahrungen mit dem System II

Die bei der oben dargelegten Beweisführung herangezogene Adsorptionschromatographie an unmodifiziertem Silicagel erwies sich neben der RP-Chromatographie als ausserordentlich brauchbare, allgemein anwendbare Methode zur analyti-

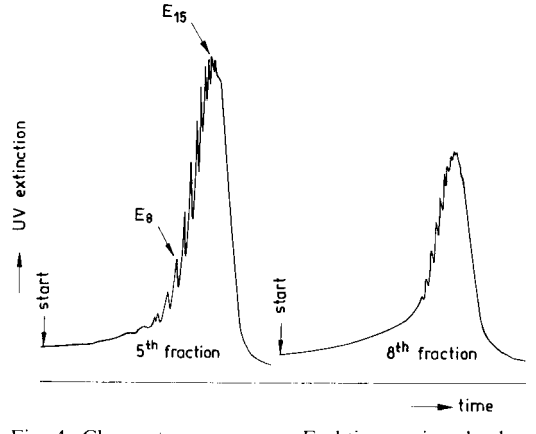


Fig. 4. Chromatogramme von Fraktionen eines hochmolekularen Bisphenol A-Epoxidharzes (M 330–516/5A, $\bar{E}_{nt}$ = 720, vgl. Tabelle V) nach wiederholter Fällung aus chloroformischer Lösung mit AN. Bedingungen: LiChrosorb RP-8 (10 μm); Trennsäule 250 × 4.6 mm; W (A)–AN (B); 70–100 % B/15 min; Fluss 2 ml/min; 5 μl der entsprechenden chloroformischen Lösung + 5 μl MCS; UV-Detektion, 254 nm; Nomenklatur siehe Tabelle II.

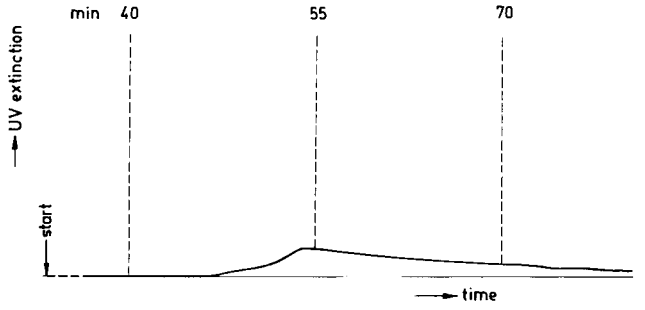


Fig. 5. Chromatogramm der Fraktion 11 im System I. Bedingungen wie in Fig. 1, jedoch unter Verlängerung des isokratischen Teiles mit 90% B auf 90 min.

schen Charakterisierung von EPR. Es erleichtert die Elutionsmittelwahl, wenn auf die Mischbarkeit mit Wasser keine Rücksicht genommen werden braucht. Allerdings ist das von Hanuš *et al.*[11] angegebene ternäre Gemisch *n*-Hexan–Chloroform–Isopropanol (90:5:5) lediglich für niederigmolekulare Harze brauchbar.

Wir verwendeten MC ($\varepsilon^0 = 0.32$, Lit. 24) als Hauptkomponente des Elutionsmittelgemisches. MC besitzt gute Löseeigenschaften für EPR und ist leicht destillativ zu reinigen. Das zunächst benutzte Gemisch (MC–M, 97:3) zeigte bei längerem Gebrauch tendenzielle Retentionszeitveränderungen und zunehmende Peakaufspaltungen am Chromatogrammanfang. Solche Erscheinungen lassen sich, wie bekannt, auf Aktivitätsänderungen des Trägers während des Lösungsmittelgradienten zurückführen, z.B. durch Wasserabgabe von der Trägeroberfläche an das Elutionsmittel. Dieser, dem gewollten Gradienten entgegengerichtete Effekt, äussert sich im Extremfall durch Auftreten von Doppelpeaks[7].

Lange Regenerierungszeiten nach dem Gradienten (bis 120 min) und zu 50%

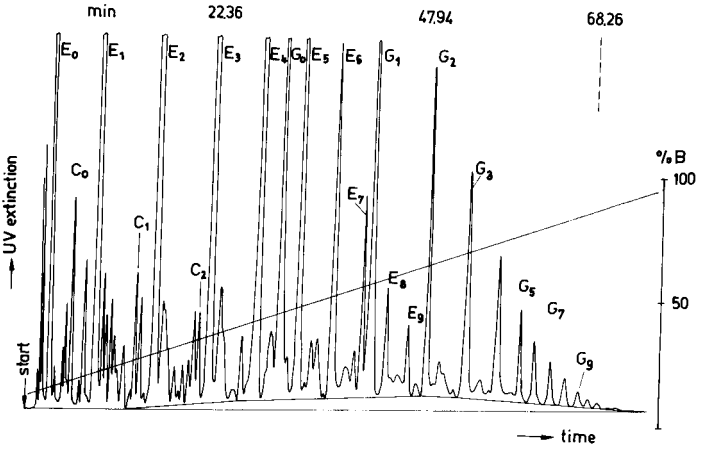


Fig. 6. Chromatogramm eines mittelmolekularen Bisphenol A-Epoxidharzes (EG 1/2497, $\bar{E}_{nt} = 381$, vgl. Tabelle V). Bedingungen (System II) vgl. Tabelle I; sphärisches Silicagel (5.6 μm); Trennsäule 250 × 4.6 mm; MC mit 0.15% W (A)–(MC–AN, 7:3) mit 0.6% W (B); 5–95% B/75 min; Fluss 2 ml/min; 400 μg EPR/10 μl MC; UV Detektion, 254 nm; Nomenklatur siehe Tabelle II.

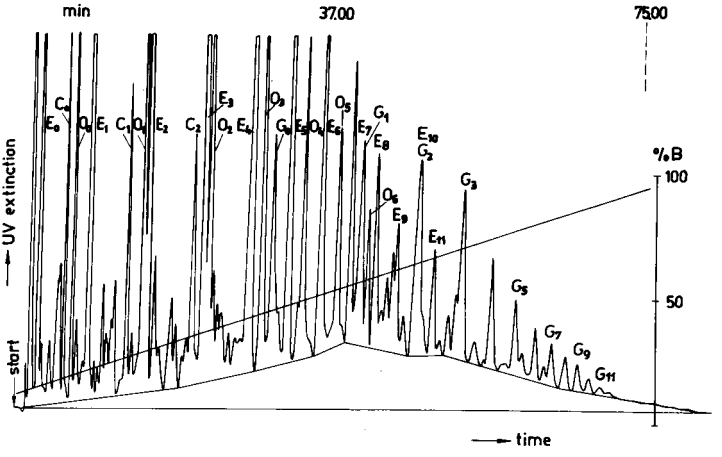


Fig. 7. Chromatogramm eines höhermolekularen Bisphenol A-Epoxidharzes (M 330–516/4UA, $\bar{E}_{nt}$ = 560, vgl. Tabelle V). Bedingungen (System II) siehe Fig. 6 bzw. Tabelle I; Nomenklatur siehe Tabelle II.

wassergesättigtes MC[24] ergaben zunächst kein stabiles Verhalten. Daraufhin wurde das M im Gemisch B durch AN ersetzt, und zwar in einer solchen Menge, dass mit ε^0 = 0.47 wieder ähnliche k_i-Werte erhalten wurden (MC–AN, 6:4). Ein langzeitstabiles Verhalten konnte schliesslich erreicht werden, nachdem der Wassergehalt in A (MC) auf 0.15% und in B (MC–AN) auf 0.6% eingestellt worden war, bei gleichzeitiger Reduzierung des AN-Anteiles in B auf 30%. Nach jeder Trennung musste die Säule mit einem Gemisch der Ausgangszusammensetzung (5% B) regeneriert werden. Hierfür sind 20–30 min ausreichend. Im Falle des Systems I benötigt man ebenfalls *ca.* 15

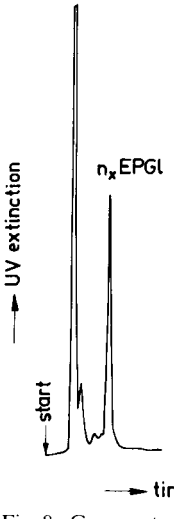


Fig. 8. Gruppentrennung zwischen n_xEPGl-Oligomeren (x = 0 bis 11) und den übrigen EPR-Komponenten eines Bisphenol A-Epoxidharzes (EG 1/2497, $\bar{E}_{nt}$ = 381, vgl. Tabelle V). Bedingungen: sphärisches Silicagel (5.6 μm); MC–M (96:4); Fluss 2 ml/min; 100 μg EPR/10 μl MC; UV-Detektion, 254 nm. Gruppenpeak der n_xEPGl-Oligomeren: 13.0%. Der nach Fig. 6 mit dem System II aus der Summe aller Einzelpeaks ermittelte Wert beträgt 12.9%.

min Spülzeit. Die chromatographischen Bedingungen für System II sind Tabelle I zu entnehmen.

Die Figs. 6 und 7 enthalten Beispiele hochselektiver Trennungen, die mit dem System II erreicht werden konnten. Ähnlich wie in Fig. 3 bestimmen ab Chromatogrammitte die Oligomeren des Types n_xEPGl das chromatographische Bild. Zuletzt eluierte Substanzen sind fast ausschliesslich glykolischer Natur. Die vergleichsweise weniger gute Auflösung in Fig. 3 ist auf den höheren M-Gehalt im Lösungsmittel B zurückzuführen.

Der Gradient mit 5% M-Zusatz bewirkt eine relative Verschiebung der oligomeren n_xEPEP zum Chromatogrammanfang. Während in den Figs. 6 und 7 z.B.

TABELLE IV

ELUTIONSFOLGE NIEDERMOLEKULARER EPR-BESTANDTEILE AN LICHROSORB RP-8 (SYSTEM I) UND SPHÄRISCHEM SILICAGEL (SYSTEM II)

LiChrosorb RP-8	*Sphärisches Silicagel*
Bisphenol A	n_0EPEP
n_0EPGl	n_0EPCl
n_0OHCl	n_0EPOH
n_0EPOH	n_1EPEP
n_0ClCl	n_0ClCl
n_0EPCl	n_0OHCl
n_0EPEP	Bisphenol A
n_1EPGl	n_1EPCl
n_1OHCl	n_1EPOH
n_1EPOH	n_2EPEP
	⋮
n_1ClCl	n_4EPEP
n_1EPCl	n_0EPGl
n_1EPEP	⋮
n_2EPGl	n_7EPEP
	n_1EPGl

n_0EPGl zwischen n_4EPEP und n_5EPEP liegt, fällt es in Fig. 3 mit n_8EPEP zusammen. Eine isokratische Arbeitsweise mit MC–M führt zu einer ausgeprägten Gruppentrennung zwischen n_xEPGl-Oligomeren und den übrigen Komponenten (Fig. 8).

Beim Vergleich von Figs. 6 und 7 erkennt man im übrigen die deutliche Zunahme des Chromatogrammuntergrundes mit steigender Molekül- bzw. Epoxyäquivalentmasse der Proben. Im Falle der Gruppentrennung verteilen sich die Untergrundkomponenten auf beide Peakgruppen.

Tabelle IV zeigt übersichtlich, dass die Systeme I und II ein prinzipiell unterschiedliches Elutionsverhalten für die oligomeren Harzbestandteile besitzen. Durch RP-GE und Adsorptions-GE erhaltene Chromatogramme ergänzen sich in ihrer Aussage.

Berechnung von Epoxyäquivalentmassen ($\bar{E}'_{nc}$)

Zur Berechnung von $\bar{E}'_{nc}$-Werten aus den Chromatogrammen fand folgende Gleichung Verwendung:

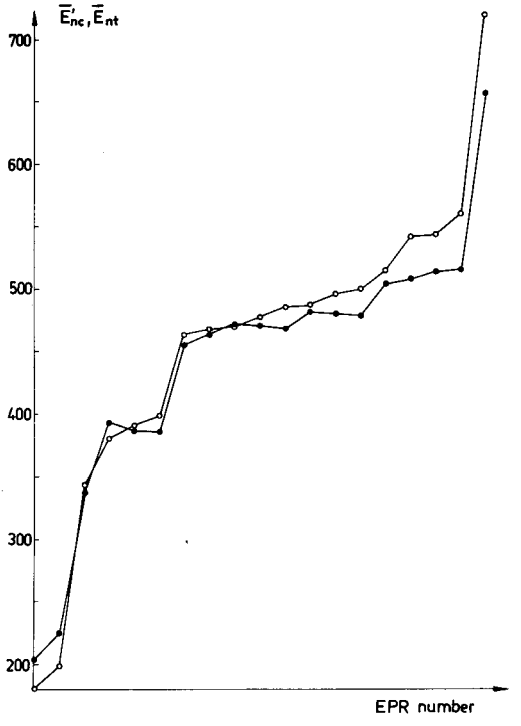


Fig. 9. Vergleich titrimetrisch und chromatographisch (System II) bestimmter Epoxyäquivalentmassen $\bar{E}_{nt}$ (○) und $\bar{E}'_{nc}$ (●).

$$\bar{E}'_{nc} = \frac{100}{2 \sum_{n=0}^{z} \frac{\%\, A_{in}}{M_{in}} + \sum_{n=0}^{z} \frac{\%\, A_{in}}{M_{jn}}} \tag{2}$$

Der $\bar{E}'_{nc}$-Wert ist die relative Molekülmasse pro Epoxyäquivalent, A_n (%) bedeutet korrigierte Flächenprozente für die Homologen i mit 2 Epoxygruppen (Bisglycidylether) bzw. j (n_xEPOH, n_xEPGl, n_xEPCl) mit einer Epoxygruppe im Molekül. M_n sind die zugehörigen relativen Molekülmassen. Bei der Berechnung von $\bar{E}'_{nc}$-Werten wurde allerdings auf die Berücksichtigung individueller Extinktionskoeffizienten verzichtet, da deren Einfluss hier eine untergeordnete Rolle spielt.

Fig. 9 zeigt die Übereinstimmung von $\bar{E}_{nt}$- und $\bar{E}'_{nc}$-Werten bis. *ca.* 470. Bei höheren Werten fallen die Ergebnisse der chromatographischen Auswertung zunehmend zu niedrig aus, wobei die Flächen des Chromatogrammuntergrundes ansteigen. Die prozentualen Anteile der Untergrundflächen an der Gesamtfläche des Chromatogramms wurden für eine Reihe unterschiedlicher Harztypen verschiedener Hersteller in Tabelle V angegeben.

SCHLUSSFOLGERUNGEN

(1) Die Elution höhermolekularer EPR durch RP-GE mit AN–W erwies sich

TABELLE V

ERGEBNISSE DER UNTERSUCHUNG VON EPR DURCH GE AN SPHÄRISCHEM SILICAGEL (SYSTEM II)

Bezeichung	*Hersteller*	$\bar{E}_{mt}$	$\bar{E}'_{nc}$	*Untergrund-fläche (% rel.)*
Epo-Tohto YD 128	Tohto Kasei, Japan	176	204	–
EG 34/210*		198	225	–
ChS-Epoxy 1/8	Spolek, Tschechoslowakei	344	337	4
EG 1/2497*		381	393	5
Lopox 440	CdF Chimie, Frankreich	391	387	12
EG 1/2005*		398	387	9
Araldit 6072	CIBA, Schweiz	463	456	20
Epidian 1	Sarzyna, Polen	468	467	12
Epo-Tohto YD 011(II)	Tohto Kasei, Japan	469	472	11
ChS-Epoxy 1/33	Spolek, Tschechoslowakei	478	462	17
M 330–2374*		486	469	20
M 330–2400*		487	483	13
Rütapox XO 191	Rütgers, B.R.D.	496	481	17
Eposid 1062	Duroplast, B.R.D.	500	478	20
Eporesit K 41-Ö	VSZM, Ungarn	515	504	12
Epo-Tohto YD 011(I)	Tohto Kasei, Japan	542	508	23
Beckopox 301	Reichhold/Albert, B.R.D.	543	514	14
M 330–516/4UA*		560	515	26
M 330–516/5A*		720	656	43

* Versuchsprodukte des VEB Leuna-Werke.

als nicht quantitativ. Bei Serienanalysen der Harze treten deshalb nachlassende Trennsäulenselektivität und allmählich steigender Druckabfall auf.

(2) Die durch RP-GE mit AN–W nicht erfassten Anteile sind keine linearen n_xEPEP-Oligomeren, tragen jedoch Epoxidgruppen. Die Peaks werden selbst unter günstigen chromatographischen Bedingungen nicht aufgelöst und erscheinen als Untergrund. Mit steigender durchschnittlicher relativer Molekülmasse der Proben nimmt der Untergrund zu. Die Struktur der Untergrundkomponenten wird im Teil II der Publikation diskutiert.

(3) Die Chromatographie an unmodifiziertem Silicagel liefert eine brauchbare Alternativvariante zur RP-GE. Gradienten an Silicagel mit dem System MC–AN und optimal eingestelltem Wassergehalt ergeben hohe Chromatogrammauflösung. Die gute Reproduzierbarkeit dieser Methode ist davon abhängig, wie weit es gelingt, Schwankungen der Elutionsmittelzusammensetzung, insbesondere des Wassergehaltes, bzw. Störungen der Gleichgewichtseinstellung zwischen Säule und Eluens zu vermeiden.

(4) An Silicagel als Träger lässt sich mit Elutionsmitteln geeigneter Polarität (Methylenchlorid–Methanol) und isokratischer Arbeitsweise eine Gruppentrennung zwischen n_xEPGl-Oligomeren und den übrigen Chromatogrammkomponenten erreichen. Bei den Oligomeren mit glykolischen Endgruppen handelt es sich um Verbindungen, die in allen untersuchten Epoxidharztypen in wechselnden Mengen vorkommen.

(5) Durch Auswertung der Chromatogramme, zweckmässig mittels Rechner, lassen sich die $\bar{E}'_{nc}$-Werte der Proben ermitteln. Bis zu $\bar{E}'_{nc}$-Werten von 470 wurde eine gute Übereinstimmung zwischen titrimetrisch und chromatographisch bestimmten Werten erhalten.

ABKÜRZUNGEN UND SYMBOLE

AN	Acetonitril
$\bar{E}'_{nc}$	Epoxyäquivalentmasse, errechnet aus dem Chromatogramm
$\bar{E}_{nt}$	Epoxyäquivalentmasse, ermittelt durch Titration
EPR	Bisphenol A-Epoxidharz(e)
GE	Gradientenelution
GPC	Gelpermeationschromatographie
HPLC	Hochleistungs-Flüssigchromatographie
IR	Infrarot
M	Methanol
MC	Methylenchlorid
MCS	Methylcellosolve
MS	Massenspektrometrie
NMR	magnetische Kernresonanz
RP-GE	Umkehrphasen-Gradientenelution
THF	Tetrahydrofuran
W	Wasser
A	Peakfläche
A_a	spezifische Adsorbensoberfläche
$\bar{D}$	mittlerer Porendurchmesser
ε	molarer dekadischer Extinktionskoeffizient
ε^0	Lösungsmittelstärke nach Snyder[24]
$E = \varepsilon/M$	Extinktion pro Gramm
f_n	Korrekturfaktor der Peakflächen
k_i	Kapazitätsfaktor
M	relative Molekülmasse
n	Oligomerisierungsgrad ($n = 0$ bis z)
$n_x \equiv (n = x)$	
V_p^*	spezifisches Porenvolumen

DANK

Für die Überlassung der GPC-Fraktionen danken wir Herrn Dr. K.-D. Müller, für die spektroskopischen Untersuchungen Herrn Dipl.-Phys. E. Müller.

ZUSAMMENFASSUNG

Niedermolekulare Bisphenol A-Epoxidharze lassen sich durch Umkehrphasen-Gradientenelution mit Wasser–Acetonitril-Gemischen untersuchen. Höhermolekulare und z.T. mittelmolekulare Harze werden bei Verwendung dieses Systems nur

unvollständig eluiert. Die Gradientenelution an Silicagel mit Methylenchlorid–Acetonitril bzw. Methylenchlorid–Methanol erwies sich als allgemein anwendbar. Neben der Verteilung der oligomeren Bisglycidylether können durch Hochleistungs-Flüssigchromatographie vor allem Nebenprodukte mit glykolischen, phenolischen und chlorhydrinischen Endgruppen quantitativ bestimmt werden. Oligomere Monoglycidylether mit glykolischen Endgruppen lassen sich in allen untersuchten Epoxidharztypen nachweisen und als Stoffgruppe von den übrigen Komponenten abtrennen. Die mittels Hochleistungs-Flüssigchromatographie erhaltenen Chromatogramme nieder- bis mittelmolekularer Harze erlauben unmittelbar die Berechnung von Epoxyäquivalentmassen.

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ANALYTISCHE CHARAKTERISIERUNG VON BISPHENOL A-EPOXIDHARZEN DURCH HOCHLEISTUNGS-FLÜSSIGCHROMATOGRAPHIE. II.*

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(Eingegangen am 25. November 1981)

SUMMARY

Analytical characterization of bisphenol A-type epoxy resins by means of high-performance liquid chromatography. II.

An improved reversed-phase gradient method with a ternary methylene chloride–methanol–water eluent is described for the characterization of bisphenol A-type epoxy resins. The system shows good dissolving properties, also for higher-molecular-weight epoxy resins. By using an empiric correction, the epoxide equivalent for medium- and higher-molecular-weight epoxy resins up to a relative epoxide equivalent of 1000 can be calculated [s_w (rel.) $\leqslant 1\,\%$]. The chromatogram background with these resins shows branched-chain components.

EINFÜHRUNG

Im ersten Teil unserer Veröffentlichung[1] haben wir die Eignung verschiedener chromatographischer Systeme für die Analytik von Bisphenol A-Epoxidharzen (EPR) diskutiert. Es konnte ferner gezeigt werden, dass insbesondere Silicagel ein für die Chromatographie von Epoxidharzen sehr selektives Trägermaterial darstellt. Die folgenden Ausführungen beinhalten Ergebnisse einer verbesserten Umkehrphasen (RP)-Gradienten-Methode. Ferner wird auf die Natur des bei höhermolekularen Proben mit allen Systemen gefundenen Chromatogrammuntergrundes eingegangen.

EXPERIMENTELLER TEIL

Es stand ein Flüssigchromatograph des Typs 1084 A (Hewlett-Packard, Avondale, PA, U.S.A.) mit Festwellenlängendetektor (254 nm) zur Verfügung. Die Trennsäulen (250 × 4.6 mm) wurden mittels Schwebesuspensionstechnik[2,3] mit LiChrosorb RP-8, 10 μm (Merck, Darmstadt, B.R.D.) gefüllt.

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Reagenzien

Methylenchlorid (MC) (rein; VEB CK Bitterfeld) und Methanol (M) (rein; VEB Leuna-Werke) wurden vor der Chromatographie über eine 40-cm-Füllkörperkolonne fraktioniert. Frisch destilliertes Wasser wurde über eine G 4-Fritte filtriert.

Epoxidharze

Zur Untersuchung gelangten EPR-Versuchsproben des VEB Leuna-Werke sowie Harzmuster verschiedener Firmen unterschiedlichen Typs (s. Tabelle I). Strukturkurzzeichen und zugehörige Strukturen oligomerer Harzkomponenten enthält Tabelle II.

TABELLE I

ERGEBNISSE DER UNTERSUCHUNG AUSGEWÄHLTER BISPHENOL A-EPOXIDHARZE

Bezeichnung	*Hersteller*	$\bar{E}_{nt}$	$\bar{E}_{nc}$**	A_u	$A_c + A_u$***
Epo-Tohto YD128	Tohto Kasei, Japan	176	194	–	120
EG 34/210*		198	219	–	108
Epo-Tohto YD134	Tohto Kasei, Japan	253	258	8	113
Lekutherm X30S	Bayer, B.R.D.	373	360	9	107
EG 1/2497*		381	385	5	113
Lopox 440	CdF Chimie, Frankreich	391	383	15	120
EG 1/2005*		398	393	9	114
ChS Epoxy 1003	Spolek, Tschechoslowakei	434	407	18	109
Epidian 1	Sarzyna, Polen	468	455	17	123
ChS Epoxy 1/33	Spolek, Tschechoslowakei	478	460	14	110
M 330/2400*		487	475	11	117
Rütapox X0191	Rütgers, B.R.D.	496	460	23	119
Eporesit K41-Ö	VSZM, Ungarn	515	474	25	125
Epo-Tohto YDO11(I)	Tohto Kasei, Japan	542	498	25	121
Beckopox 301	Reichold/ Albert, B.R.D.	543	501	23	117
M 330/516/4UA*		560	507	26	118
M 330/2342/G*		667	539	56	127
M 330/516/5A*		720	615	50	122
Epo-Tohto YD014	Tohto Kasei, Japan	983	750	57	123
					$\bar{x} = 117.2$ ($s_w = 6.1$)

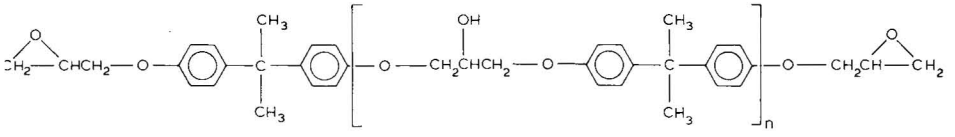
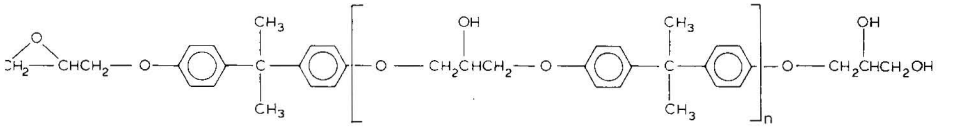
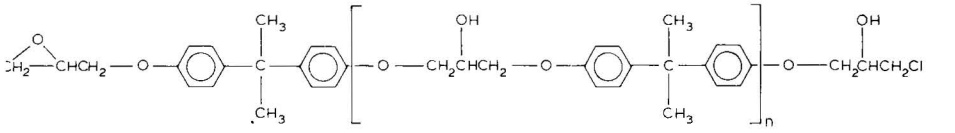
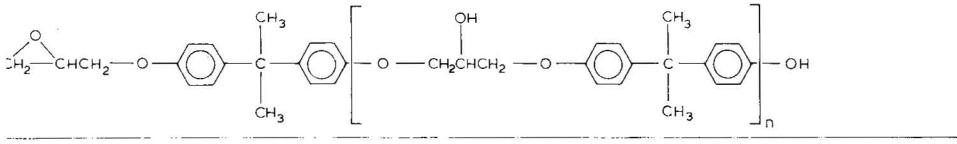
* Versuchsprodukte des VEB Leuna-Werke.
** Chromatographische Bedingungen siehe Tabelle III.
*** In willkürlichen Flächeneinheiten.

Chromatographie

In Tabelle III sind die wichtigsten Bedingungen zur Chromatographie zusammengefasst.

TABELLE II

STRUKTUREN OLIGOMERER BESTANDTEILE VON BISPHENOL A-EPOXIDHARZEN

Struktur	*Kurzzeichen*
	n_xEPEP E_x
	n_xEPG1 G_x
	n_xEPCl C_x
	n_xEPOH O_x

ERGEBNISSE UND DISKUSSION

Auswahl geeigneter chromatographischer Bedingungen

Zwecks Beseitigung der Löslichkeitsprobleme bei Einsatz höhermolekularer EPR entschlossen wir uns aufgrund früherer Erfahrungen[1] zur Erprobung eines ternären Elutionsmittelsystems mit MC. Als Lösungsvermittler zwischen Wasser (W) und MC bot sich M an, da das System MC–M–W eine wesentlich kleinere Mischungslücke als MC–Acetonitril (AN)–W besitzt.

TABELLE III

BEDINGUNGEN ZUR HOCHLEISTUNGS-FLÜSSIGCHROMATOGRAPHIE VON BISPHENOL A-EPR

Trennsäule	LiChrosorb RP-8
Korngrösse	10 μm
Probenlösung	200 mg EPR/5 ml MC
Lösungsmittel A	M–W (65:35, v/v)
Lösungsmittel B	MC–M (65:35, v/v)
Gradient	10–80% (v/v) B, 90 min
Dosiervolumen	10 μl (= 400 μg EPR)
Fluss	2 ml/min
Lösungsmitteltemperatur A	303°K
Lösungsmitteltemperatur B	303°K
Säulenraumtemperatur	303°K
Signal	UV, 254 nm
Signalabschwächung	2^5

Orientierende Versuche zeigten, dass von den für den Gradienten vorgesehenen Lösungsmittelgemischen A und B (Tabelle III) Gemisch A etwa 65 Volumenteile M enthalten muss, um eine ähnliche Anfangselutionsstärke wie mit einem Gemisch aus 1:1 (v/v) AN–W[1] zu erzielen.

Die Optimierung des Gradienten erfolgte unter Berücksichtigung der formulierten Anforderungen an die Trennung[1] sowie der allgemein für die Optimierung von RP-Gradienten gegebenen Hinweise[4,5].

Im Ergebnis dieser Versuche erwies sich der in Tabelle III angeführte Gradient mit 10–80 % (v/v) B/90 min als günstig. Eine Regenerierzeit von *ca.* 20 min zwischen zwei Gradienten war ausreichend.

Die Elutionsfolge der wichtigsten Chromatogrammkomponenten unter diesen Bedingungen zeigt Tabelle IV.

TABELLE IV

ELUTIONSFOLGE UND RETENTIONSZEITEN FÜR NIEDERMOLEKULARE EPR-BESTANDTEILE UNTER DEN IN TABELLE III ANGEGEBENEN BEDINGUNGEN

t_{Ri} wurde unter Wiederholbedingungen (12 Analysen) bestimmt.

Komponente	t_{Ri} *(min)*	*Komponente*	t_{Ri} *(min)*
Bisphenol A	2.3	n_1EPOH	12.4
n_0OHCl*	2.5	n_1ClCl*	14.5
Phenylglycidylether	2.7	n_1EPCl	17.5
n_0EPGl	3.1	n_2OHCl*	19.5
n_0EPOH	3.8	n_1EPEP	21.5
n_0ClCl*	4.7	n_2EPGl	22.8
n_0EPCl	5.6	n_2EPOH	24.8
n_0EPEP	6.5	n_2ClCl*	25.4
n_1OHCl*	9.2	n_2EPCl	29.6
n_1EPGl	10.9	n_2EPEP	35.4

* Siehe Tabelle II, Teil I[1].

Reproduzierbarkeit

Tabelle V lässt erkennen, dass die Reproduzierbarkeit der Retentionszeiten für alle eluierten Substanzen unter Wiederholbedingungen ausgezeichnet ist. Die Werte entsprechen im übrigen den allgemeinen Angaben des Geräteherstellers ($s_w \leqslant 0.5\,\%$ rel.[6]). Auch nach mehr als 100 Analysen war kein Nachlassen der chromatographischen Reproduzierbarkeit und Trennwirksamkeit der Säule feststellbar.

Harzcharakterisierung und Epoxyäquivalentmassen-Bestimmung

Fig. 1 zeigt das Chromatogramm eines relativ nebenprodukthaltigen EPR, das bereits im Teil I[1], Fig. 6, an Silicagel chromatographiert wurde. Die wesentlichen Peaks sind gut zu erkennen. Wiederum ist die geringere Differenziertheit des RP-Chromatogramms im Vergleich zu den Peakfolgen an Silicagel zu erkennen. Die vollständige Substanzelution bereitet jedoch mit dem neuen System im Gegensatz zur RP-Variante mit AN–W[1] keinerlei Schwierigkeiten.

Aus den Fig. 2 und 3 erkennt man eine bemerkenswerte Zunahme des Chroma-

TABELLE V

REPRODUZIERBARKEIT DER RETENTIONSZEITEN t_{Ri}

Komponente	*Analysen-zahl*	$\bar{x}$ *(min)*	s_w *(min)*	s_w *(% rel.)*
n_0EPGl	12	3.11	0.039	1.26
n_0EPOH	11	3.81	0.022	0.59
n_0EPEP	12	6.53	0.032	0.49
n_1EPGl	12	10.86	0.064	0.59
n_1EPEP	12	21.47	0.089	0.41
n_2EPGl	12	22.76	0.084	0.37
n_3EPGl	12	32.38	0.132	0.41
n_2EPEP	12	35.37	0.162	0.45
n_4EPGl	12	39.50	0.157	0.40
n_3EPEP	12	44.14	0.169	0.38
n_4EPEP	12	50.00	0.148	0.30
n_5EPEP	12	54.26	0.147	0.27
n_6EPEP	12	57.52	0.139	0.24
n_7EPEP	12	60.11	0.123	0.20
n_8EPEP	12	62.24	0.120	0.19
n_9EPEP	11	63.97	0.233	0.36
n_{10}EPEP	12	65.49	0.159	0.24

togrammuntergrundes mit steigender Molekül- bzw. Epoxyäquivalentmasse der Harze. Wie Tabelle I ausweist, bleibt die Gesamtchromatogrammfläche, d.h. die Summe aus Einzelpeakflächen (A_c) und Untergrundfläche (A_u) bei gleicher Probenmenge trotz Ansteigens der Untergrundfläche konstant (vgl. Lit.[1]).

Die Übereinstimmung zwischen den titrimetrisch ermittelten Epoxyäquivalentmassen ($\bar{E}_{nt}$) und den ohne Berücksichtigung des Chromatogrammuntergrundes aus den Chromatogrammen nach Gleichung(2) Teil I[1], errechneten Werten $\bar{E}'_{nc}$ darf

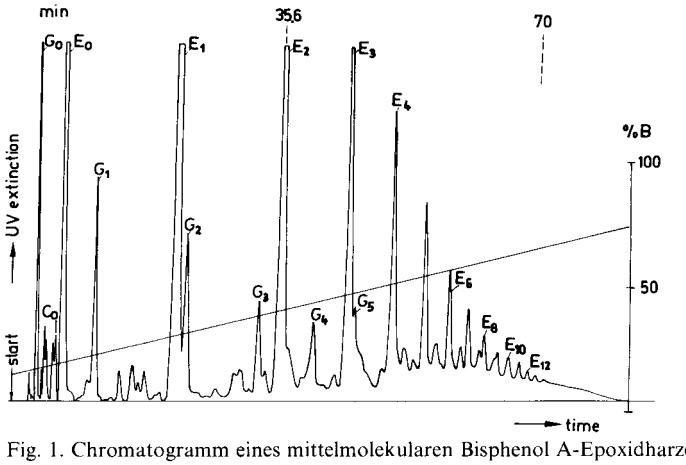


Fig. 1. Chromatogramm eines mittelmolekularen Bisphenol A-Epoxidharzes (EG 1/2497, $\bar{E}_{nt}$ = 381, vgl. Tabelle I). Bedingungen (siehe Tabelle III): LiChrosorb RP-8 (10 μm); Trennsäule 250 × 4.6 mm; W–M (65:35, v/v) (A); MC–M (65:35, v/v) (B); 10–80% B/90 min; Fluss 2 ml/min; 400 μg EPR/10 μl MC; UV-Detektion, 254 nm, Nomenklatur siehe Tabelle II.

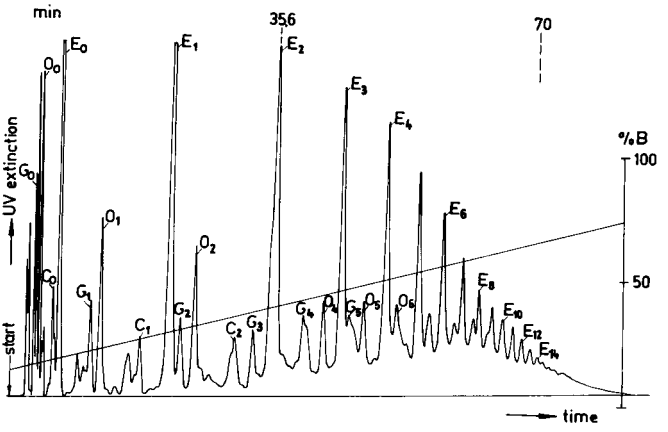


Fig. 2. Chromatogramm eines höhermolekularen Bisphenol A-Epoxidharzes (M 330/516/4UA, $\bar{E}_{nt}$ = 560, vgl. Tabelle I). Bedingungen siehe Fig. 1 bzw. Tabelle III, Nomenklatur siehe Tabelle II. Das Harz besitzt einen hohen Gehalt an Nebenprodukten mit phenolischen, glykolischen und chlorhydrinischen Endgruppen.

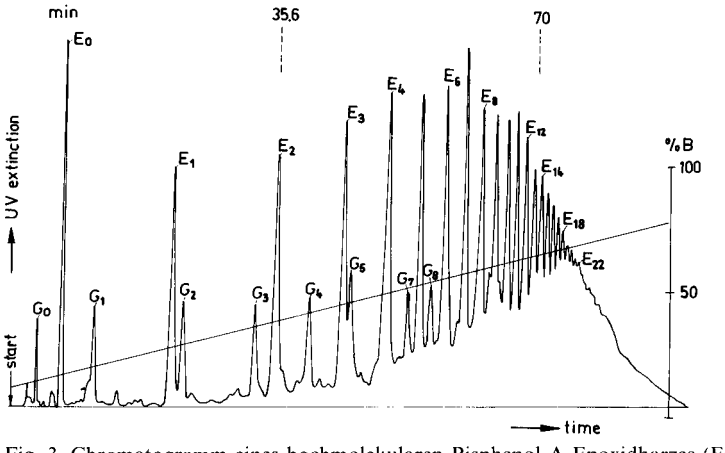


Fig. 3. Chromatogramm eines hochmolekularen Bisphenol A-Epoxidharzes (Epo-Tohto YDO14, $\bar{E}_{nt}$ = 983, vgl. Tabelle I). Bedingungen siehe Fig. 1 bzw. Tabelle III, Nomenklatur siehe Tabelle II.

man in vorliegenden System nur bis zu $\bar{E}_{nt}$ = 400 als gut bezeichnen (Fig. 4). Bei höheren $\bar{E}_{nt}$-Werten muss der Chromatogrammuntergrund berücksichtigt werden.

Die Einbeziehung des Untergrundes in die Auswertung erwies sich als kompliziert. Alle Versuche, eine theoretisch begründbare und gleichzeitig mit vertretbarem Aufwand durchführbare chromatographische Epoxyäquivalentmassenbestimmung zu finden, führten nicht zum Erfolg. Schliesslich entschieden wir uns für eine empirische Berechnungsmethode mit Hilfe eines Korrekturfaktors f_E. Damit liess sich Übereinstimmung zwischen den tritimetrisch und den chromatographisch ermittelten Werten erzielen. Die verwendete Gleichung lautet:

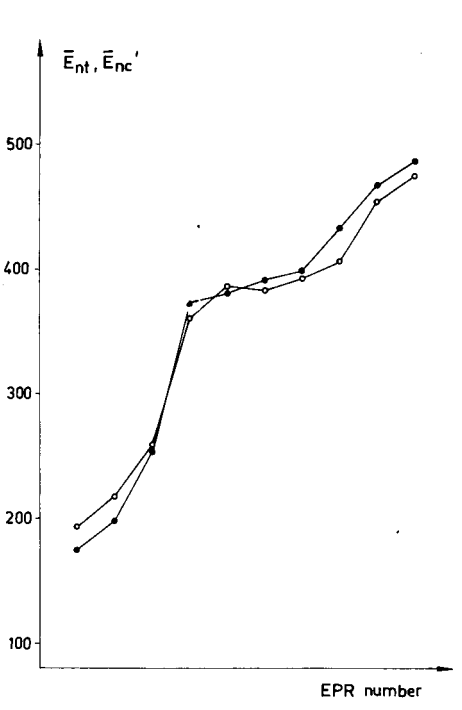
$$\bar{E}_{nc} = \bar{E}_{nt} = \bar{E}'_{nc} \cdot f_E \tag{1}$$

Zunächst hat man die Chromatogrammfläche ohne Untergrund (A_c) auszu-

werten und entsprechend der im Teil I[1] angegebenen Gl. (2) $\bar{E}'_{nc}$ zu berechnen. Nach Integration der Untergrundfläche A_u ergibt sich f_E gemäss:

$$f_E = 1 + \frac{A_u}{2\,(A_c + A_u)} \tag{2}$$

Fig. 5 zeigt die gute Übereinstimmung der titrimetrischen und der aus den Probenchromatogrammen erhaltenen Epoxyäquivalentmassen. Versuche unter Wiederholbedingungen bestätigten die gute Reproduzierbarkeit der Berechnung von $\bar{E}_{nc}$ ($s_w \leqslant 1\,\%$ rel.).



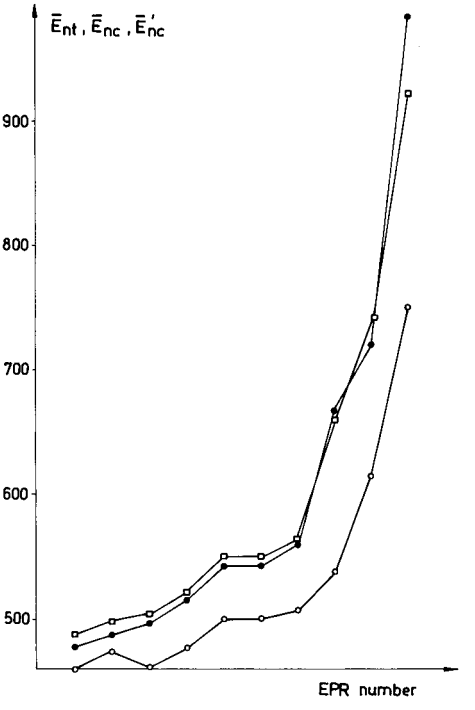


Fig. 4. Vergleich titrimetrisch und chromatographisch bestimmter Epoxyäquivalentmassen ($\bar{E}_{nt}$, $\bar{E}'_{nc}$). ● = $\bar{E}_{nt}$; ○, $\bar{E}'_{nc}$.

Fig. 5. Korrektur von $\bar{E}'_{nc}$ durch Einbeziehung des Untergrundes in die Berechnung. Erläuterungen siehe Text und Tabelle I. ● = $\bar{E}_{nt}$; ○ = $\bar{E}'_{nc}$; □ = $\bar{E}_{nc}$.

Zur Natur der Untergrundkomponenten

Verschiedene Befunde deuten darauf hin, dass die untergrundbildenden Verbindungen eine verzweigte Struktur besitzen. Denkbar ist die Umsetzung von Epoxygruppen mit alkoholischen Gruppen der Kette, wobei mit zunehmender Molekülmasse entsprechend verzweigte Verbindungen entstehen. Für die Trennung der möglichen Isomeren ist die vorhandene chromatographische Auflösung nicht ausreichend. Über die Anwesenheit kettenverzweigter Verbindungen in Epoxidharzen wurde mehrfach berichtet[7,8].

Bei der Verzweigungsreaktion spielen Art und Konzentration des Katalysators sowie die Reaktionstemperatur eine wichtige Rolle[8]. Die Herstellung einer trifunktionellen Epoxyverbindung gelang durch Umsetzung niedermolekularer EPR mit Epichlorhydrin[9]. Auch ein cyclisches Dimeres wurde isoliert[10].

Die Bestimmung der Kettenverzweigungen wird nach Reaktion der aliphatischen Hydroxylgruppen mit Trichloracetylisocyanat durch NMR-Spektroskopie ermöglicht[8,11,12]. Von uns veranlasste NMR-spektroskopische Untersuchungen an isoliertem Untergrund (Fraktion 11, Lit. 1) ohne vorherige chemische Modifizierung bestätigen das Vorliegen entsprechender Kettenverzweigungen.

Der im Chromatogramm mit einfachen Mitteln auswertbare Untergrund besteht infolge ungenügender Chromatogrammauflösung nicht allein aus den Flächen verzweigter Komponenten, sondern auch aus Flächenanteilen der linearen oligomeren Bisglycidylether. Im Verlaufe des Gradienten verschlechtert sich deren Auflösung gerade dann, wenn in zunehmendem Masse verzweigte Komponenten auftreten. Auch die Auswertung der höheren linearen Oligomeren wird dadurch fehlerbehaftet. Zu beachten ist ferner, dass in den spät eluierenden Peaks mehr und mehr Nichthomologe enthalten sind.

Die verzweigten, Epoxygruppen tragenden Untergrundkomponenten besitzen gegenüber den linearen Oligomeren gleicher Retentionszeit eine niedrigere Molekülmasse. Sie werden in allen untersuchten Systemen stärker zurückgehalten als die linearen Oligomeren.

SCHLUSSFOLGERUNGEN

(1) Bei Verwendung eines ternären Lösungsmittelgradienten mit Methylenchlorid–Methanol–Wasser ist die Umkehrphasenchromatographie an RP-8-Trägermaterial für alle Bisphenol A-Epoxidharztypen anwendbar und liefert sehr gut reproduzierbare Ergebnisse.

(2) Die Erweiterung der Berechnung von relativen Epoxyäquivalentmassen aus den Chromatogrammen bis etwa 1000 ist möglich, erfordert jedoch die Berücksichtigung des mit der durchschnittlichen relativen Molekülmasse wachsenden Untergrundes und lässt sich nur mit Hilfe eines empirischen Korrekturverfahrens ausführen.

ABKÜRZUNGEN UND SYMBOLE

AN	Acetonitril
EPR	Bisphenol A-Epoxidharz(e)
HPLC	Hochleistungs-Flüssigchromatographie
MC	Methylenchlorid
M	Methanol
NMR	Magnetische Kernresonanz
RP	Umkehrphase
W	Wasser
A_c	Summe der Einzelpeakflächen ohne Untergrundfläche
A_u	Untergrundfläche
$\bar{E}'_{nc}$	Epoxyäquivalentmasse, ohne Untergrund errechnet
$\bar{E}_{nc}$	Chromatographisch bestimmte korrigierte Epoxyäquivalentmasse

$\bar{E}_{nt}$ titrimetisch ermittelte Epoxyäquivalentmasse
f_E empirischer Korrekturfaktor zur Berechnung von $\bar{E}_{nc}$
s_w Standardabweichung unter Wiederholbedingungen
t_{Ri} Bruttoretentionszeit
$\bar{x}$ arithmetisches Mittel

DANK

Für die NMR-Untersuchungen sind wir Herrn Dipl.-Phys. E. Müller zu Dank verpflichtet.

ZUSAMMENFASSUNG

Für Bisphenol A-Epoxidharze wird eine verbesserte Umkehrphasen-Gradienten-Methode mit einem ternären Elutionsmittel aus Methylenchlorid–Methanol–Wasser beschrieben. Das System besitzt auch für höhermolekulare Epoxidharze gute Löseeigenschaften. Mit Hilfe einer empirischen Korrektur ist die Berechnung von Epoxyäquivalentmassen für mittel- und höhermolekulare Epoxidharze bis zu relativen Epoxyäquivalentmassen von 1000 möglich (s_w (rel.) $\leqslant 1\%$). In den bei diesen Harzen auftretenden Chromatogrammuntergrund gehen kettenverzweigte Komponenten ein.

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MICRO-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH LONG MICRO-PACKED FLEXIBLE FUSED-SILICA COLUMNS

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SUMMARY

Coupling of several 20 cm × 0.25–0.35 mm I.D. micro packed flexible fused silica columns in series is a simple technique for attaining high efficiencies in micro high-performance liquid chromatography. Reversed-phase and gel permeation chromatography using 40–140-cm columns are demonstrated. A 140-cm column packed with G 1000H (5 μm) attained *ca.* 100,000 plates in gel permeation chromatography. These columns were employed to resolve complex polychlorinated biphenyl mixtures or the constituents of water.

INTRODUCTION

For the liquid chromatographic analysis of complex mixtures in the environment or *in vivo*, high efficiency in terms of the theoretical plate number plays an important role as well as optimization of the selectivity of liquid chromatography (LC). A greater number of theoretical plates is generally produced by a longer column[1–3]. Scott and Kucera[1,2] prepared 1 m × 1 mm I.D. microbore packed columns and obtained longer columns (10–14 m) by coupling them in series. Nearly 30,000 plates were produced with a 1-m column in the reversed-phase system, 160,000 plates were produced with a 10-m column in the normal-phase system and 650,000 plates were produced with a 14-m column in gel permeation chromatography[2]. The pressure drop across the column and the time required for the analysis were much greater than for conventional high-performance LC (HPLC).

We have recently examined the employment of flexible fused silica tubing as the column material in micro-HPLC[4] when we found that a 10 cm × 0.25 mm I.D. column attained *ca.* 7000 theoretical plates. In that work[4], the effect of column length on column efficiency was investigated and nearly the same efficiency in terms of height equivalent to a theoretical plate (HETP) was attained with 5–30 cm columns, while a slightly poorer efficiency was observed for an 80-cm column owing to non-uniformity in the packing. However, it may be expected that a desired efficiency can be generated with long columns coupled in series.

A long straight column causes a large separation between the detector and the pump of the chromatograph. Thus, it is desirable for a long column to be coiled.

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However, it has been noted that the coil should not be less than 12 cm in diameter since a reduction in coil diameter causes a significant reduction in efficiency[2]. Flexibility of a fused-silica tubing facilitated adjustment of the coil diameter after packing. Even a long fused-silica tubing column is easy to handle.

In this article, high-resolution flexible fused-silica columns coupled in series were prepared and used for the analysis of complex mixtures by reversed-phase and gel permeation chromatography.

EXPERIMENTAL

The liquid chromatograph was assembled from a Micro Feeder (Azumadenki Kogyo, Tokyo, Japan) with a 250-μl gas-tight syringe as a pump, a JASCO micro valve injector (0.02 μl, Japan Spectroscopic, Tokyo, Japan), a micro pre-column, a micro packed fused silica column, a column oven, a UVIDEC-100 (JASCO) UV spectrometer with a modified flow cell and a back-pressure pump. Fused-silica columns were prepared by the method reported previously[4]. TSK-GEL G 1000H (5 μm), Toyo Soda, Tokyo, Japan) and silica ODS SC-01 (5 μm, JASCO) were selected as packings for gel permeation and reversed-phase chromatography, respectively. Columns were connected by stainless-steel tubing, 0.13 mm I.D., 0.31 mm O.D. and 4–5 mm length, as shown in Fig. 1. The connection volume was only 0.05–0.06 μl and the connecting system shown in Fig. 1 endured 80–100 kg/cm^2. The micro precolumn was composed of PTFE tubing, *ca.* 10 mm × 0.2 mm I.D., packed with Amberlite XAD II and was employed for collecting the organic constituents of water using the technique reported previously[5]. The column oven was constructed in our laboratory and comprised asbestos boards equipped with a heater and a fan. The temperature was adjusted by altering the applied voltage with a sliding rheostat. A micro flow cell was composed of quartz tubing, 1.5 mm × 0.17 mm I.D.. Back-pressure was applied to prevent vaporization of the mobile phase even when the chromatograph was operated at elevated temperatures as described previously[6]. Samples were injected using a modified micro valve injector[7]. All reagents were purchased from Wako (Osaka, Japan) or Tokyo Chemical Industry (Tokyo, Japan). Fused-silica tubings were obtained from Gasukuro Kogyo (Tokyo, Japan) or Scientific Glass (North Melbourne, Australia).

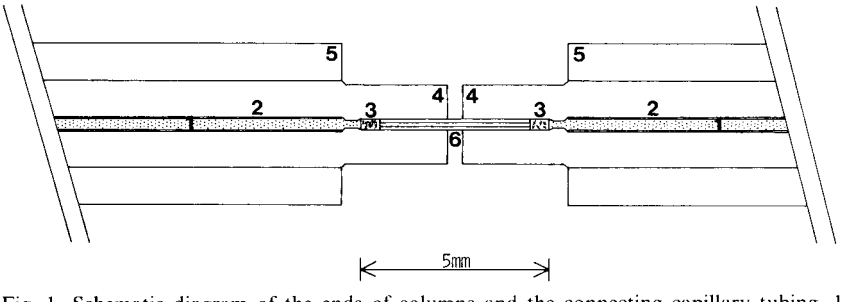


Fig. 1. Schematic diagram of the ends of columns and the connecting capillary tubing. 1 = Packing material; 2 = fused-silica tubing; 3 = quartz wool; 4 = PTFE tubing, 0.2 mm I.D. and 2 mm O.D.; 5 = PTFE tubing, 2 mm I.D. and 4 mm O.D.; 6 = stainless-steel capillary tubing, 0.13 mm I.D. and 0.31 mm O.D.

RESULTS AND DISCUSSION

Reversed-phase chromatography

In previous work[4], 5–30 cm × 0.25 mm I.D. fused-silica columns packed with silica ODS SC-01 (5 μm) gave nearly the same HETP value, 14–20 μm. Thus, 20 cm × 0.25 mm I.D. was adopted as the column dimension in this work, because of the simplicity of preparation.

Table I shows the efficiency of 20-cm columns and coupled columns using aromatic hydrocarbons as test samples and acetonitrile–water (7:3) as the mobile phase. Dependence of HETP on the flow-rates of the mobile phase was low in the region between 0.83 and 3.3 μl/min. Columns of length 20 cm generally produced 10,400–14,100 theoretical plates. The relative standard deviation of HETP with ten 20-cm columns was *ca.* 9%. A 9.9-cm column and coupled columns (39.6 and 59.1 cm) also gave nearly the same HETP value as the 20-cm columns, which indicates that extra-column band broadening in the connection, injection and detection parts is negligibly small.

TABLE I

COLUMN PERFORMANCE OF FLEXIBLE MICRO COLUMNS

Column: 0.25 mm I.D., packed with silica ODS SC-01 (5 μm). Mobile phase: acetonitrile–water (7:3). V_R = Retention volume; N = number of theoretical plates; HETP = height equivalent to a theoretical plate.

Column length (cm)	*Flow-rate (μl/min)*	*Column performance*					
		Biphenyl			*Pyrene*		
		V_R (μl)	N	HETP (μm)	V_R (μl)	N	HETP (μm)
9.9	1.11	11.7	7100	14	24.8	7000	14
20.0	1.04	23.3	10,400	19	42.0	12,300	15
19.7	1.04	23.5	12,400	16	47.2	12,200	16
19.9	1.04	24.0	12,400	16	47.9	10,500	19
19.5	1.04	23.1	12,300	16	47.0	12,500	16
19.5	1.04	23.0	12,200	16	46.3	12,100	16
19.7	1.04	23.0	14,000	14	45.7	14,100	14
19.9	1.04	24.3	14,000	14	49.4	14,100	14
19.9	2.08	24.0	11,700	17	45.8	13,100	15
20.0	2.08	23.0	10,800	19	44.5	12,500	16
19.9	2.08	23.1	11,700	17	43.0	13,100	15
39.6 (19.7 + 19.9)	1.04	47.3	28,400	14	96.2	23,600	17
59.1 (19.7 + 19.9 + 19.5)	1.04	69.6	40,500	15	142.6	40,600	15

Fig. 2 shows the linear relationship between the theoretical plate number and column length. A desired column efficiency can be attained with the columns coupled in series. On the other hand, a single 80-cm column gave a slightly poorer result (HETP = 30–37 μm).

The pressure drop across the 59.1-cm column under the operating conditions

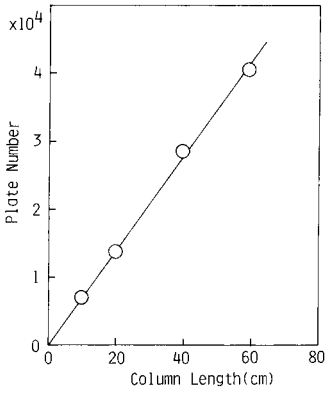


Fig. 2. Relationship between the theoretical plate number and column length. Column, 0.25 mm I.D., packed with SC-01 (5 μm); mobile phase, acetonitrile–water (7:3); flow-rate, 1.04 μl/min; sample, biphenyl; temperature, ambient.

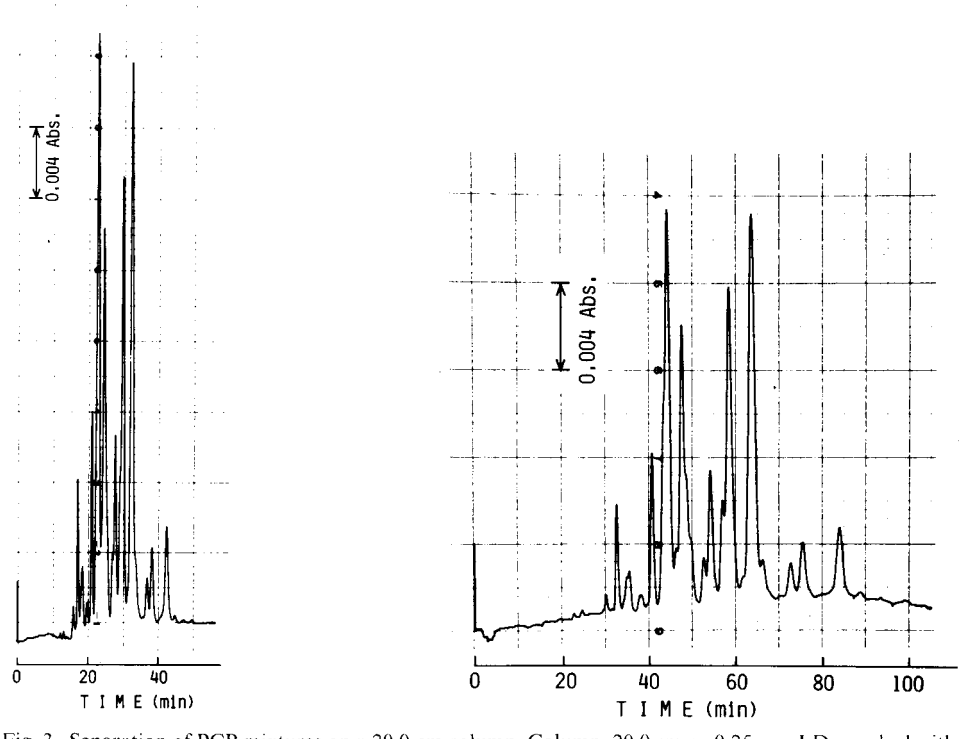


Fig. 3. Separation of PCB mixtures on a 20.0-cm column. Column, 20.0 cm × 0.25 mm I.D., packed with SC-01 (5 μm); mobile phase, acetonitrile–water (8:2); flow-rate: 1.39 μl/min; sample, 1.8% of PCB-48; (Tokyo Chemical Industry, chlorine content *ca.* 48%) sample size, 0.02 μl; temperature, 27 C; wavelength of UV detection, 254 nm.

Fig. 4. Separation of PCB mixtures on a 39.9-cm column. Column, 39.9 cm × 0.25 mm I.D., packed with SC-01 (5 μm). Other operating conditions as in Fig. 3.

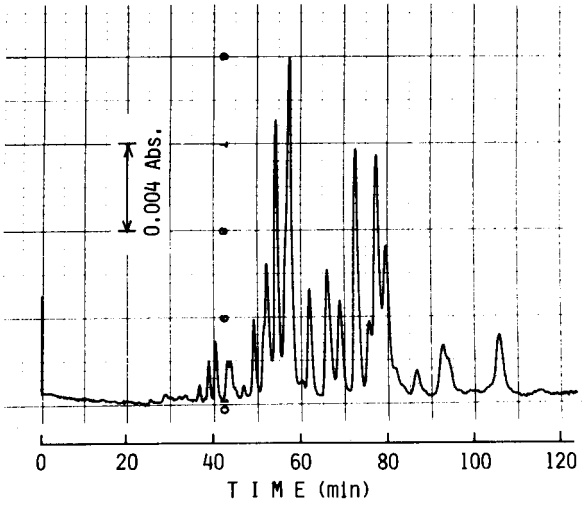


Fig. 5. Separation of PCB mixtures on a 39.5-cm column at elevated temperature. Column: 39.5 cm × 0.25 mm I.D., packed with SC-01 (5 μm); mobile phase, acetonitrile–water (7:3); temperature, 62°C. Other operating conditions as in Fig. 3.

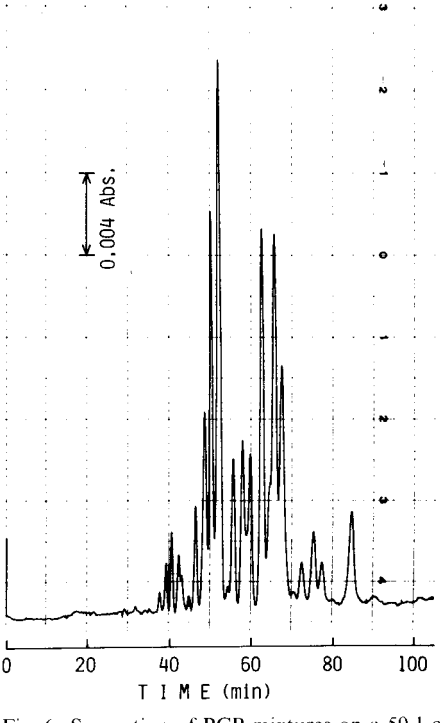


Fig. 6. Separation of PCB mixtures on a 59.1-cm column at elevated temperature. Column, 59.1 cm × 0.25 mm I.D., packed with SC-01 (5 μm); mobile phase, acetonitrile–water (7:3); sample, 2.5% of PCB-48; temperature, 90°C. Other operating conditions as in Fig. 3.

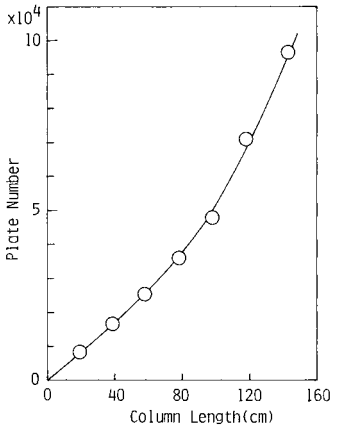


Fig. 7. Relationship between the theoretical plate number and column length. Column, 0.35 mm I.D., packed with G 1000H (5 μm); mobile phase, tetrahydrofuran; flow-rate, 1.04 μl/min; sample, dimethyl phthalate; temperature, ambient.

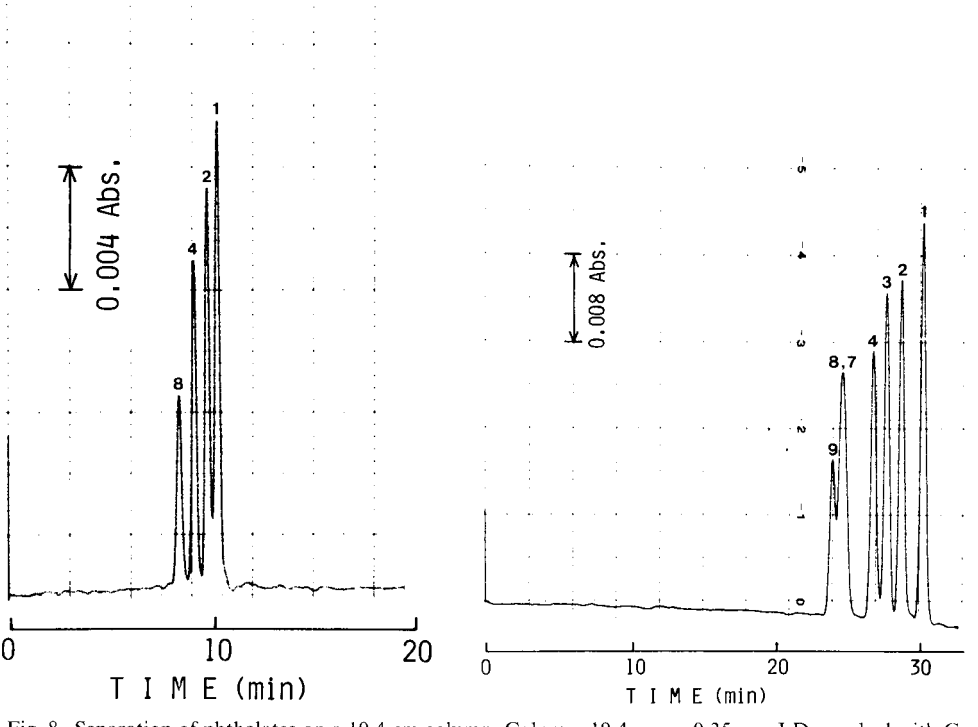


Fig. 8. Separation of phthalates on a 19.4-cm column. Column, 19.4 cm × 0.35 mm I.D., packed with G 1000H (5 μm); flow-rate, 1.04 μl/min. Samples: 8 = di-2-ethylhexyl phthalate; 4 = di-*n*-butylphthalate; 2 = diethyl phthalate; 1 = dimethyl phthalate, each 0.05%. Sample size, 0.02 μl; temperature, ambient; wavelength of UV detection, 235 nm.

Fig. 9. Separation of phthalates on a 57.8-cm column. Column, 57.8 cm × 0.35 mm I.D., packed with G 1000H (5 μm). Samples: 9 = dinonyl phthalate; 8 = di-2-ethylhexyl phthalate; 7 = diheptyl phthalate; 4 = di-*n*-butyl phthalate; 3 = di-*n*-propylphthalate; 2 = diethylphthalate; 1 = dimethylphthalate, each 0.2%. Other operating conditions as in Fig. 8.

indicated in Table I was *ca.* 60 kg/cm^2. The pumping, injecting and connecting systems employed in this work endured 60–80 kg/cm^2. Thus, the chromatograph should be run at lower flow-rates if longer columns are employed, leading to an increase in the analysis time.

Figs. 3–6 demonstrate the analyses of polychlorinated biphenyl (PCB) mixtures on columns of different lengths. Figs. 3 and 4 show separations at room temperature (27°C) on 20.0- and 39.9-cm columns and Figs. 5 and 6 show separations at elevated temperature on 39.5- and 59.1-cm columns, respectively. The longer the column, the more constituents are resolved. The chromatograms obtained on 40-cm columns shown in Figs. 4 and 5 suggest that selectivity at elevated temperatures is higher than at room temperature, which encouraged us to employ the water-rich mobile phase at elevated temperature. In addition, operating at elevated temperature generally decreases the viscosity of the mobile phase, leading to a decrease in the inlet pressure. Thus, operation at elevated temperature facilitates the employment of longer columns and/or separation at higher flow-rates.

Gel permeation chromatography

Several studies on micro-scale gel permeation chromatography have been reported[1,2,8,9], in which 0.5–1-mm bore columns are employed. In this work, TSK-GEL G 1000H (5 μm) and a 0.35-mm I.D. fused-silica tubing were selected as packing and column material, respectively, for gel permeation chromatography.

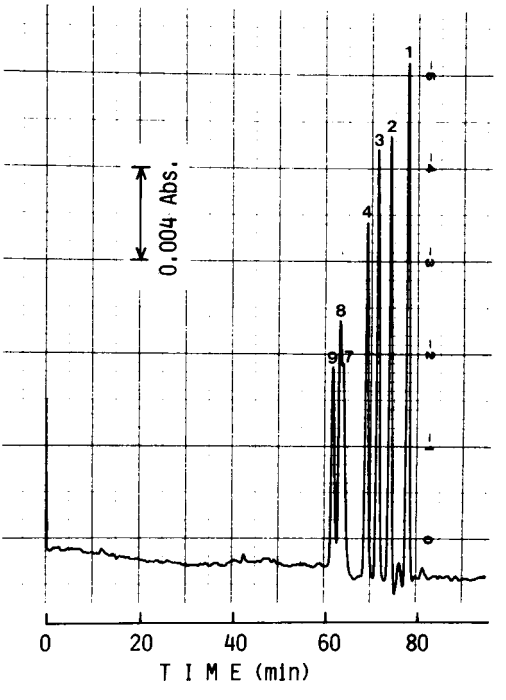


Fig. 10. Separation of phthalates on a 142.6-cm column. Column, 142.6 cm × 0.35 mm I.D., packed with G 1000H (5 μm); samples as in Fig. 9. Other operating conditions as in Fig. 8.

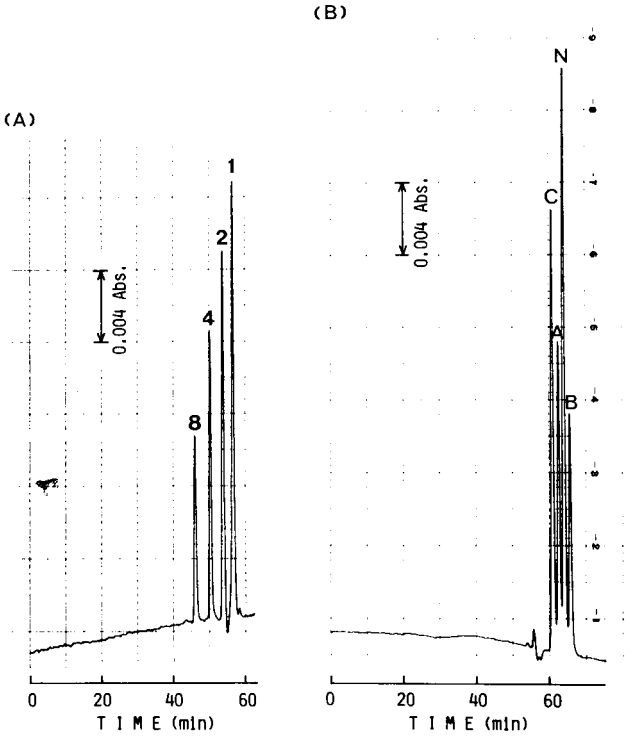


Fig. 11. Separations of phthalates and aromatic hydrocarbons on a 102.8-cm column. Column, 102.8 cm × 0.35 mm I.D., packed with G 1000H (5 μm); mobile phase, tetrahydrofuran; flow-rate, 1.04 μl/min. Samples: (A) phthalates, as in Fig. 8 except for the concentration, each 0.2%; (B) aromatic hydrocarbons: C = 0.034% (w/v) of chrysene; A = 0.0087% of anthracene; N = 0.38% of naphthalene; B = 2.6% of benzene. Sample size, 0.02 μl; temperature, ambient; wavelength of UV detection, (A) 235 nm, (B) 254 nm.

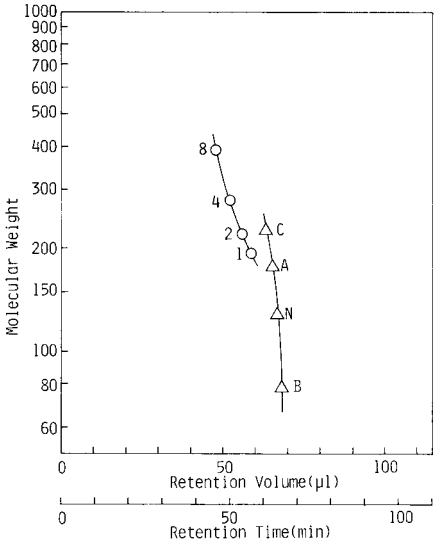
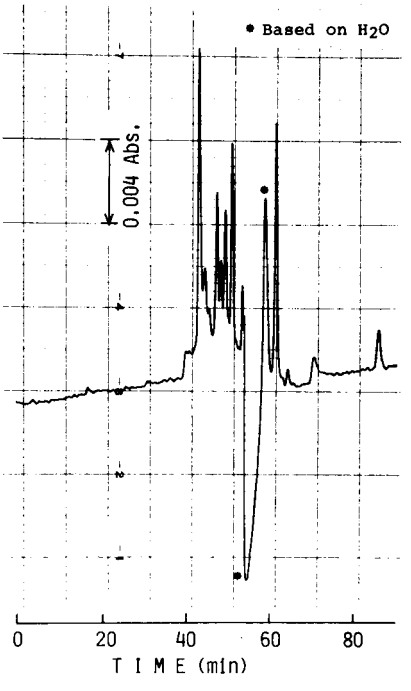


Fig. 12. Relationship between the logarithm of molecular weight and retention volume (or time). Operating conditions as in Fig. 11.

The efficiency of columns coupled in series was examined using phthalates and aromatic hydrocarbons as the test samples and tetrahydrofuran as the mobile phase. Fig. 7 shows the relationship between the theoretical plate number and the column length. The theoretical plate number increases with increasing column length and the relation deviated upwards from linearity. Nearly 100,000 theoretical plates were produced for dimethyl phthalate on a 140-cm column at a retention time of 80 min.

Figs. 8–10 show typical separations of phthalates on columns of different lengths. As the column length increases, the resolution of the solutes becomes greater, *e.g.*, dimethyl and diethyl phthalates are not completely resolved on a 19.4-cm column, whereas baseline separations of these two phthalates are attained on 57.8- and 142.6-cm columns. Fig. 11 shows separations of phthalates and aromatic hydrocarbons on a 102.8-cm column and Fig. 12 shows the relationship between retention volume (or time) and the logarithm of the molecular weight (MW). The compound of higher MW elutes before that of lower MW in the homologues, owing to simple size



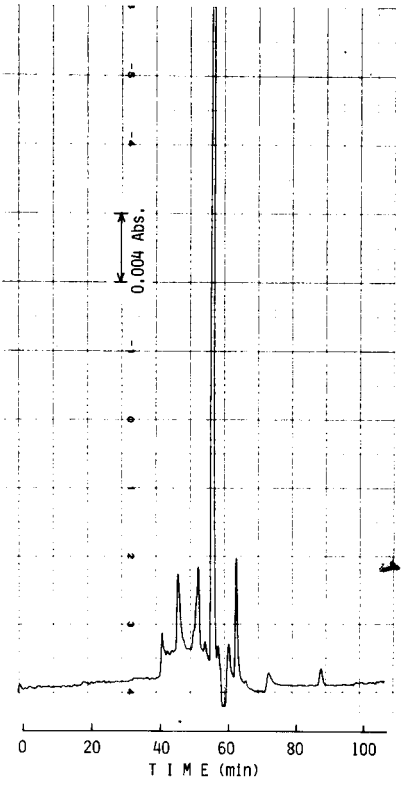


Fig. 13. Analysis of constituents of water. Column, 102.8 cm × 0.35 mm I.D., packed with G 1000H (5 μm); mobile phase, tetrahydrofuran; flow-rate, 1.04 μl/min; pre-column, 1 cm × 0.2 mm I.D., packed with Amberlite XAD II (10 μm); sample, 1 ml of deionized water; temperature, ambient; wavelength of UV detection, 235 nm.

Fig. 14. Analysis of extracts in water from coal. Sample, 1 ml of water in contact with powdered coal. Operating conditions as in Fig. 13.

exclusion. Retention of solutes also depends on the structure of the solutes, *e.g.*, chrysene (MW = 228) elutes after diethyl phthalate (MW = 222) and dimethyl phthalate (MW = 194). Compact compounds generally elute after less compact compounds of the same MW.

High-resolution flexible fused silica columns were applied to some analyses of water samples. Fig. 13 demonstrates the analysis of the organic constituents of de-ionized water. The sample was passed through a pre-column packed with Amberlite XAD II (10 μm) prior to the chromatographic run. After the pre-column containing the organic constituents had been dried *in vacuo*, it was connected to the separation column. A number of peaks were resolved due to the difference in size, although residual water in the pre-column interfered with the detection of some solutes.

The analysis of extracts in water from coal is shown in Fig. 14. MWs of peaks can be estimated by the calibration data indicated in Fig. 12.

CONCLUSION

Micro-packed flexible fused-silica columns coupled in series produced desired efficiencies. A range of 20,000 to 100,000 theoretical plates could be attained on these columns for reversed-phase and gel permeation chromatography, and allowed the resolution of complex mixtures. The technique will be useful for the analysis of various real samples.

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HILFSMITTEL FÜR DIE PRÄPARATIVE HOCHLEISTUNGS-FLÜSSIGKEITSCHROMATOGRAPHIE

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(Eingegangen am 15. Dezember 1981)

SUMMARY

New aids for preparative high-performance liquid chromatography

A new preparative separation system is described. It consists of a column, a splitting system which allows the controlled withdrawal of parts of the eluate for subsequent detection and a peak detector connected to a fraction collector.

EINLEITUNG

Die Hochleistungs-Flüssigkeitschromatographie ist in den letzten Jahren zu einer sehr wirksamen analytischen Trennmethode entwickelt worden. In neuester Zeit ist auch wiederholt und erfolgreich versucht worden, diese Methode für präparative Zwecke einzusetzen. Die wichtigsten diesem Zwecke dienenden Massnahmen sind die Vergrösserung der Kolonnendimension und die Mehrfacheinspritzung.

Die vorliegende Arbeit befasst sich mit der automatischen Chromatographie im Mehrfachlaufverfahren, für die zwei in der Praxis bewährte Systeme im Detail beschrieben werden.

Das hauptsächlichste Problem im Zusammenhang mit der Automation von Mehrfachläufen ist das programmgesteuerte Schneiden der Peaks, das an die Stelle des sehr aufwendigen, ständige Präsenz erfordernden, manuellen Schneidens tritt. Weitere Probleme stellen sich bei der Aufteilung des Eluates in zwei Teile, einen zur Detektion und einen zum Sammeln sowie bei der Uebertragung der Messresultate des Detektors auf den Fraktionensammler.

Zur Lösung dieser Probleme hat sich bei uns das im folgenden beschriebene System bewährt.

Einer mit einer Mehrfacheinspritzautomatik versehenen Säule folgt ein splitting System, das die kontrollierte Abzweigung von Teilen des Eluates für (1) eine direkte Detektion (UV, sichtbar), (2) eine Derivatisierung mit anschliessender Detektion oder (3) eine Verdünnung mit anschliessender Detektion erlaubt, wobei der Detektor mit einem Fraktionensammler gekoppelt ist.

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EXPERIMENTELLER TEIL

Geräte und Materialien

Als Pumpen dienten eine Altex 110 mit präp. Kopf und 28 ml Fluss pro Minute

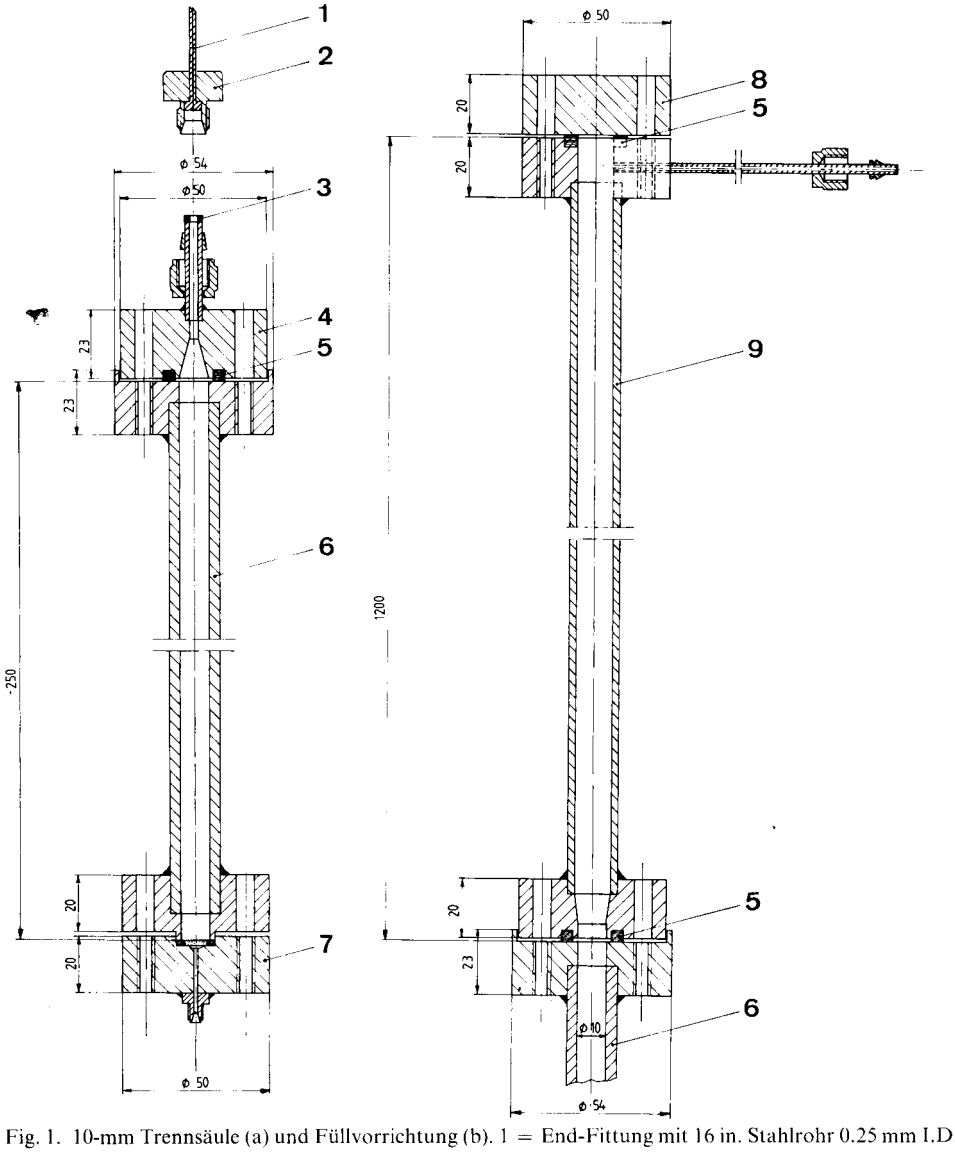

Fig. 1. 10-mm Trennsäule (a) und Füllvorrichtung (b). 1 = End-Fittung mit 16 in. Stahlrohr 0.25 mm I.D. (Argon Schweissung); 2 = Kappe mit einer Bohrung von 1.8 mm I.D. UNF 7/16; 3 = Altex AX 250-21 Fritte; 4 = Verteilerkopf mit 1/4-in. Anschluss und 1/4 Parker Nut und 1/4-in. Parker Ferrule (weitere Einzelheiten zu 1-4[Lit. 2]; 5 = Teflon-Dichtung; 6 = Trennsäule bestehend aus dem Rohr einer 10-mm Altexsäule mit zwei an beiden Enden angeschweissten Flanschen und mit je 6 Bohrungen für Zylinderschrauben mit Innensechskant M 6 × 40 mm; 7 = Säulenabschluss mit einer Altex-Fritte AX 250-22 und einer eingeschweissten 1/16-in. Swagelok-Verschraubung; 8 = Verschlussdeckel; 9 = Füllrohr mit zwei Flanschen mit je 6 Bohrungen und einem Anschluss für die Pumpe.

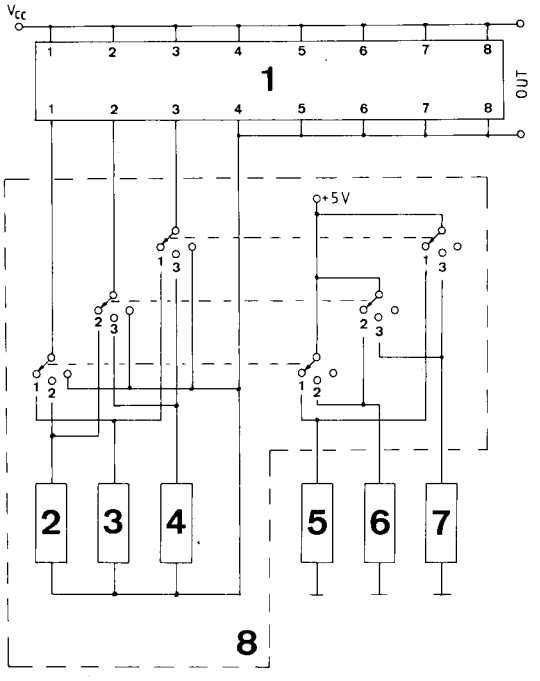

Fig. 2. Kanalwahlschalter. 1 = Mikroprozessor; 2–4 = Relais; 5–7 = Lichtschranken; 8 = Kanalschalter.

für isokratische Chromatographie, für binäre Gradienten zwei Waters M6000 Pumpen mit dem 660 Steuergerät, und für ternäre Gradienten ein Varian HPLC 5060. Für die Säulenfüllung wurde eine Haskel 26980.4 Pumpe verwendet.

Handeinspritzungen erfolgten mit einem Rheodyne 7125 Ventil mit 1-ml Probenschlaufe. Automatisch wurden bis 2 ml mit WISP 710 B Einspritz-Automaten (Waters) eingespritzt. Die Trennsäule und die Vorrichtung zum Packen der Säule wurden im Eigenbau hergestellt (Fig. 1).

Als Detektoren dienten ein Cecil CE 212 variabler UV-Monitor (Cecil, Cambridge, Grossbritannien) mit einer Helma-Küvette von 0.5-mm Schichtdicke, ein variabler UV-Monitor UVIKON 725 (Kontron) und für die Fluoreszenz-Registrierung ein Aminco 4-7439 und ein LC 1000 Detektor (Perkin-Elmer).

Registriert wurde mit WW 312 Zweikanal-Schreibern und einem WW 1100 Einkanal-Schreiber mit Aufbau für die Lichtschranken. Das Zumischen des Derivatisierungs-Reagenz, Fluram oder *o*-Phtaldialdehyd[1], erfolgte mit Metripumpen E 1A/ss3c mit 1–3 Pumpenköpfen (Metering Pumps, London, Grossbritannien).

Für die Aufspaltung des Eluats (splitting) wurde ein zeitgesteuertes Dreiwegventil verwendet (Labomatic, Allschwill, Schweiz).

Die Fraktionen wurden mit folgenden Geräten gesammelt: Büchi 660 für 8 Fraktionen, mit externer Ansteuerung (Büchi Flawil, Schweiz). Labomatic Circular I für 20 und 50 Fraktionen, beide ausgerüstet mit Zeitschaltung, Endabschaltung und externer Ansteuerung und ein Gilson Micro Fractionator für 80 Fraktionen mit Zeitsteuerung. Der Peak-Detektor wird durch einen Mikroprozessor Labtime 1788 (Port-

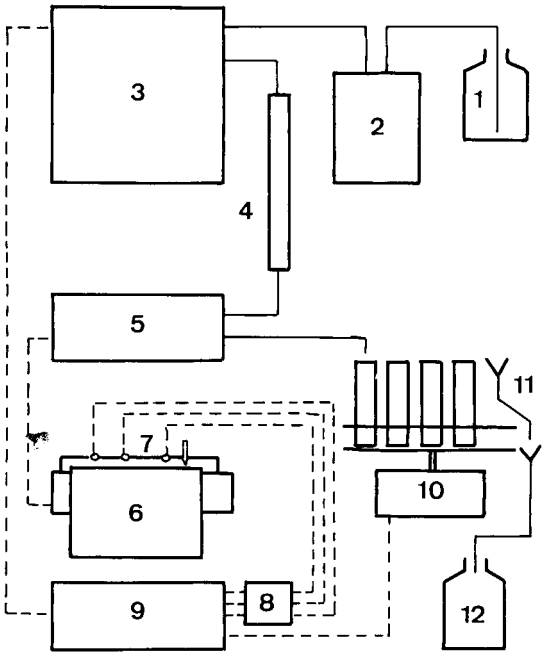

Fig. 3. Vollautomatisches isokratisches oder binäres Trennsystem mit Peak-Detektion. 1 = Lösungsmittel; 2 = präparative Pumpe (Kontron oder Waters) 2 × 600 mit Gradientensteuergerät 660; 3 = WISP Auto-Probengeber; 4 = Trennsäule; 5 = Cecil UV-Monitor; 6 = Schreiber; 7 = Reflexions-Lichtschranken; 8 = Kanalwahlschalter; 9 = Mikroprozessor; 10 = Fraktionensammler mit 7 Probengläser; 11 = Trichter zum Abführen des nicht gebrauchten Eluats; 12 = Sammelflasche.

mann, Therwil, Schweiz) oder einen Labomat 100 (Labomatic) gesteuert. Als Reflexions-Lichtschranken dienten Reflectiv Transducer OBP 125 (Optron, Carrallton, TX, U.S.A.). Die Zuschaltung der Lichtschranken erfolgt über einen Kanalwahlschalter mit Relais (Eigenbau, Fig. 2). Zwei Beispiele für die gewählte Anordnung der Apparate, den Flusslauf sowie die elektrische Schaltung werden in Figs. 3 und 4 schematisch dargestellt.

Trennsäule und Füllvorrichtung

Die 10-mm I.D. Trennsäule (Fig. 1a) wurde am oberen Ende (Säuleneingang) mit einem trichterförmigen Verteiler ausgerüstet (4).

Die Packung der Säule erfolgt in zwei Etappen. In einer ersten Etappe wird mittels der Füllvorrichtung (Fig. 1b) bis zum oberen Ende der 10-mm Säule (6) gefüllt, dann wird in einer zweiten Etappe der Kopf (4) aufgesetzt und mit einem 1/4 in. Anschluss (zum Packen analytischer Säulen) weiteres Material bis zum Ende des 1/4 in. Rohres aufgefüllt. Das Rohr wird oben mit einer Fritte (3) abgeschlossen. Die Füllung erfolgt nach der Viskositätsmethode[3] mit einer grossen Haskel-Pumpe, die über die nötige Kapazität (70 ml Kolben bei 450 bar) verfügt.

Peak-Detektor

Der Peak-Detektor (Fig. 3) besteht aus einem Mikroprozessor (Labtime oder

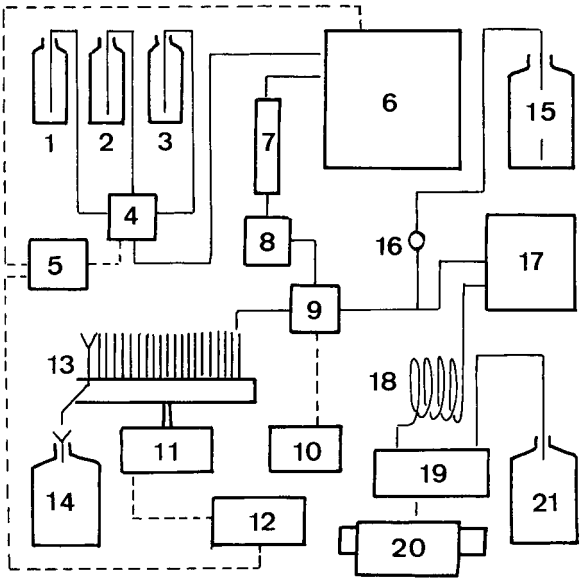

Fig. 4. Vollautomatisches ternäres Trennsystem mit Detektion nach Derivatisierung (ohne Peak-Detektor). 1–3 = Lösungsmittel; 4 = Pumpe mit Vierweg-Ventil (ternäres System, Varian); 5 = Mikroprozessor für die Steuerung des ternären Trennsystem und Start des Fraktionensammlers (Varian); 6 = WISP Auto-Probengeber; 7 = Trennsäule; 8 = UV-Detektor; 9 = Dreiweg-Ventil mit 10 = Zeitsteuerung; 11 = Fraktionensammler (für 20 oder 50 Fraktionen) mit Endabschaltung; 12 = Steuerung; 13 = Trichter (in Pos. 20 resp. 50) zum Abführen des nicht gebrauchten Eluats; 14 = Sammelflasche; 15 = Reagenz; 16 = Schliessventil; 17 = Pumpe; 18 = Reaktionsschlaufe; 19 = Fluoreszenz-Detektor; 20 = Schreiber; 21 = Sammelflasche.

Labomat) einer Schalterkombination (Fig. 2) (Eigenbau) und Reflexions-Lichtschranken, die verschiebbar auf dem Schreiber montiert sind (Eigenbau). Angeschlossen ist ein Fraktonen-Sammler (Büchi) mit 8, respektive 7 Probengläsern. Anstelle des 8. Glases befindet sich ein Trichter, über den das nichtgebrauchte Eluat abgeführt wird. Das Gerät ist mit einer zusätzlichen externen Ansteuerung ausgerüstet.

Der Mikroprozessor wird als Schaltuhr mit einfachen Schliesskontakten verwendet. Eingegeben werden auf die Ausgänge 1–8 je die No. des Ausgangs, sowie die Start- und Stopzeiten des Kontakts. Das Gerät wird für jeden neuen Zyklus durch den Auto-Probengeber gestartet.

Im Beispiel (Fig. 5) werden die für die Ausgänge 4–8 des Mikroprozessors während je 1 sec geschlossen. Die Kontaktkreise der Ausgänge 1–3 werden über die Relais im Kanalwahlschalter (Fig. 2) geschlossen. Sie bleiben während der Passage einer Metallhülse vor den Lichtzellen aktiviert. Die Metallhülse, in der der Schreibstift steckt, ist am Registrierwagen des Schreibers montiert.

Um eventuelle Verschiebungen der Retentionszeit zu berücksichtigen, werden für die Ausgänge 1–3 Zeitfenster eingegeben. Während dieser Zeit bleiben die Reflexionslichtschranken eingeschaltet. Mit dem Kanalwahlschalter können die passenden Lichtschranken zugeschaltet werden.

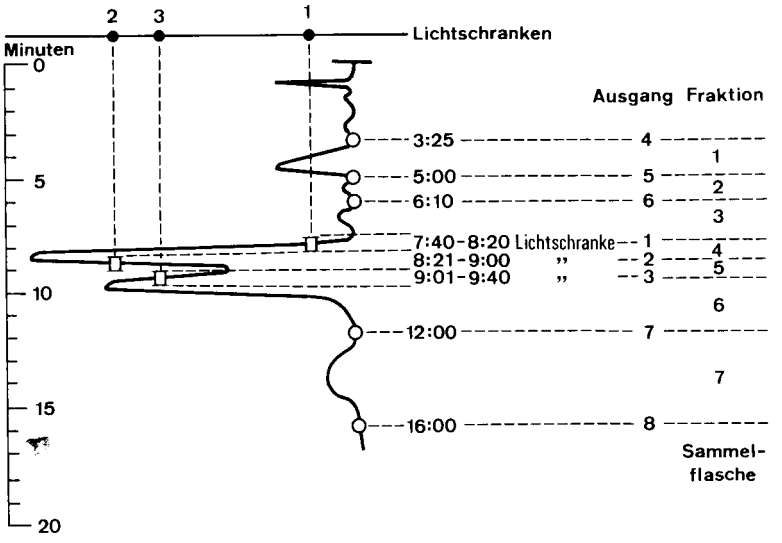

Fig. 5. Programmbeispiel für die Auftrennung eines Gemisches in 7 Fraktionen.

Splitting-System und Derivatisierung

Die Aufteilung (Splitten) des Eluates erfolgt mittels eines elektronisch gesteuerten magnetischen Dreiweg-Ventils. Das Strömungsverhältnis wird durch Zeiteinheiten, z.B. 1:10 bis 1:100 sec. gesteuert.

Bei präparativen Trennungen wird ein kleiner Teil des Eluates dem Detektorsystem zugeführt, während die Hauptmenge in den Fraktionensammler geleitet wird.

Der für die Derivatisierung bestimmte Teil des Eluats wird von einer Pumpe angesaugt (vgl. Fig. 4). In den Leitungsabschnitt zwischen Dreiwegventil und Pumpe wird das Reagenz (z.B. *o*-Phthaldialdehyd oder Fluram) über ein T-Stück zugemischt. Durch diese Anordnung wird das Entstehen eines Vacuums beim Ansaugen durch die Pumpe (bei geschlossenem Ventil) verhindert. Beim Passieren der Pumpe wird das Eluat mit den Reagenz vermischt und über eine Reaktionsschlaufe (0.5 mm I.D. und 8.5 m Länge) dem Fluoreszenz-Detektor zugeführt.

Mit der gleichen Einrichtung kann bei Ueberladung des UV-Detektors ein Teil des Eluates abgetrennt werden. An Stelle des Reagenz wird Lösungsmittel angesaugt, über die Pumpe gemischt und das verdünnte Eluat in den UV-Detektor geleitet.

ERGEBNISSE

Die beschriebenen Trennsysteme sind seit mehr als 2 Jahren im Einsatz. Heute stehen 3 Apparaturen, eine dem Beispiel Fig. 3, eine andere dem Beispiel Fig. 4 und eine dritte, einer Kombination von beiden entsprechend, in Betrieb.

Die Automation, die auch einen Nachtbetrieb ermöglicht, führte zu einem ökonomischen Arbeitseinsatz.

Mit Erfolg wurden Trennungen von Peptiden, Leukotrienen, Interferonen und Isolierungen unbekannter natürlicher Wirkstoffe durchgeführt.

Seit einem Jahr sind auch Säulen mit weiterem innerem Durchmesser mit

Erfolg im Einsatz, nämlich 21 mm I.D. von Latek (Labortechnik-Geräte, Heidelberg) und 25-mm I.D. HIBAR-Fertigsäulen von Merck.

DANK

Für die wertvolle Hilfe beim Bau der beschriebenen Hilfsmittel danken wir den Herren E. Adam und F. Bruder. Herrn Dr. R. Andreatta danken wir für die Ueberarbeitung des Manuskripts.

ZUSAMMENFASSUNG

Es wird ein neues präparatives Trennsystem beschrieben, bestehend aus einer Kolonne und einem splitting System, das die kontrollierte Abzweigung von Teilen des Eluates für die Detektion gestattet. Zum System gehört ferner ein Peak-Detektor, der mit einem Fraktionensammler gekoppelt ist.

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CHROM. 14,584

NEW PROCEDURE FOR GAS CHROMATOGRAPHIC ENANTIOMER SEPARATION

APPLICATION TO CHIRAL AMINES AND HYDROXY ACIDS

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SUMMARY

By reaction with isopropyl isocyanate, chiral amines can be converted into urea derivatives. With the same reagent, urethane derivatives of chiral α-hydroxy acid esters are formed. The enantiomers of both types of derivatives are separated on XE-60–*S*-valine–*S*-α-phenylethylamide with high separation factors.

INTRODUCTION

In our investigations of enantioselective chiral stationary phases we have synthesized several chiral derivatives of the polysiloxanes XE-60 and OV-225[1–3]. The properties of these phases are similar to Chirasil-val, a polymer chiral stationary phase introduced by Frank *et al.*[4,5]. On XE-60–*S*-valine–*S*(*R*)-α-phenylethylamide and on the corresponding OV-225 derivatives we have achieved good enantiomer separations of amino acids[1], amino alcohols[1,2], amines[1,2] and carbohydrates[1,6,7]. Enantiomer separation is not only effected by the enantioselectivity of the stationary phase but also strongly influenced by the type of volatile derivatives employed. This could be demonstrated by our recent separations of chiral alcohols as their urethane derivatives[8]. In this work we describe the formation of corresponding derivatives of α-hydroxy acid esters and amines and their separation on glass capillary columns with XE-60–*S*-valine–*S*-α-phenylethylamide*.

EXPERIMENTAL

Formation of derivatives

Samples consisting of 0.5 mg of racemic mixtures of chiral amines were dissolved in 200 μl of dichloromethane and 100 μl of isopropyl isocyanate (Fluka, Neu Ulm, G.F.R.) in a screw-cap vial. After 10 min at room temperature the excess of

* Fused-silica columns with this stationary phase and the corresponding phase with *R*-α-phenylethylamide are available from Chrompack (Middelburg, The Netherlands).

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reagent was removed with a steady stream of dry nitrogen. The residue was dissolved in 0.5 ml of dichloromethane for gas chromatographic investigation.

Samples, each of 0.1–0.5 mg, of chiral hydroxy acids were esterified in 1 ml of a 1.5 *N* solution of dry HCl gas in isopropanol for 30 min at 100°C. After removing the reagent with nitrogen, 200 μl of dichloromethane and 100 μl of isopropyl isocyanate were added and the sample was heated for 20 min at 100°C. Methyl esters and methyl and *tert.*-butyl urethanes were prepared similarly. Again the reagent was removed with nitrogen and the derivative dissolved in 0.5 ml of dichloromethane for gas chromatography.

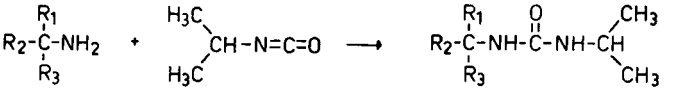
Gas chromatography

The preparation of the stationary phase and of the glass capillary columns has been described in previous communications[1,3,9]. For gas chromatographic investigations Carlo Erba Model 2101 gas chromatographs with flame-ionization detectors and hydrogen as carrier gas were used.

RESULTS AND DISCUSSION

Amines

The enantiomers of N-trifluoroacetylated 2-aminoalkanes have been separated on several monomer and polymer chiral stationary phases with α-values of between 1.01 and 1.02[1–3]. In this work we suggest the application of N-isopropylurea derivatives of chiral amines for enantiomer separation on XE-60–*S*-valine–*S*-α-phenylethylamide as shown in Scheme 1. Urea derivatives can easily be prepared on a



Scheme 1.

microgram scale with isopropyl isocyanate as reagent at room temperature in only 10 min and in excellent yields. (If the reaction is performed at 100°C the urea derivatives again may react with isopropyl isocyanate and derivatives of higher molecular weight are formed which were identified by gas chromatography–mass spectrometry.) These derivatives are less volatile than the trifluoroacetyl derivatives but their separation factors are significantly higher (1.02–1.034, see Table I and Fig. 1). For 2-aminopentane and α-phenylethylamine the *S*-enantiomers have longer retention times than the *R*-enantiomers; the other amines were only available as racemates.

2-Hydroxy acids

In previous publications we have shown that the enantiomers of several 2-(O-trifluoroacetoxy)carboxylic acid isopropyl esters can be separated on chiral stationary phases derived from 2-hydroxy acids[10,11]. These phases lack thermal stability because of their low molecular weight. The α values achieved with 40-m Pyrex glass capillary columns were between 1.01 and 1.02. Unfortunately the enantioselectivity of these phases for hydroxy acids cannot easily be transferred to polymer phases. Frank *et al.*[4] have reported that the cyclohexyl amide derivative of lactic acid is well separated on Chirasil-val. As has been already demonstrated for chiral al-

TABLE I

SEPARATION FACTORS (α) AND OPERATING TEMPERATURES FOR ENANTIOMER SEPARATION OF CHIRAL AMINES AS ISOPROPYL UREA DERIVATIVES

Column: 40-m Pyrex glass capillary coated with XE-60–*S*-valine–*S*-α-phenylethylamide.

Racemate	α *Value*	*Column temperature (°C)*
2-Aminopentane	1.021	170
2-Amino-3-methyl-pentane (diastereoisomers)	1.027 1.024	170
2-Aminohexane	1.025	170
2-Amino-5-methylhexane	1.033	170
2-Aminoheptane	1.030	170
2-Amino-6-methylheptane	1.033	170
2-Aminooctane	1.034	170
2-Phenylethylamine	1.058	200

cohols[8], urethane derivatives can be prepared by reaction of hydroxy groups with isopropyl isocyanate. This reaction can also be applied to hydroxy groups of 2-hydroxy acid esters. Again excellent enantiomer separations are obtained, as demonstrated in Figs. 2 and 3. The α values are given in Table II. The best separations were obtained for 2-hydroxy acid isopropyl esters as isopropyl urethanes. Methyl or isopropyl esters as methyl or *tert.*-butyl urethane derivatives display lower α values.

The order of elution of the enantiomers is consistent: the L-enantiomers have longer retention times than the D-enantiomers.

We have also applied this method to some β-hydroxy acids. The best results were obtained with *tert.*-butyl urethanes of the isopropyl esters, but the separation

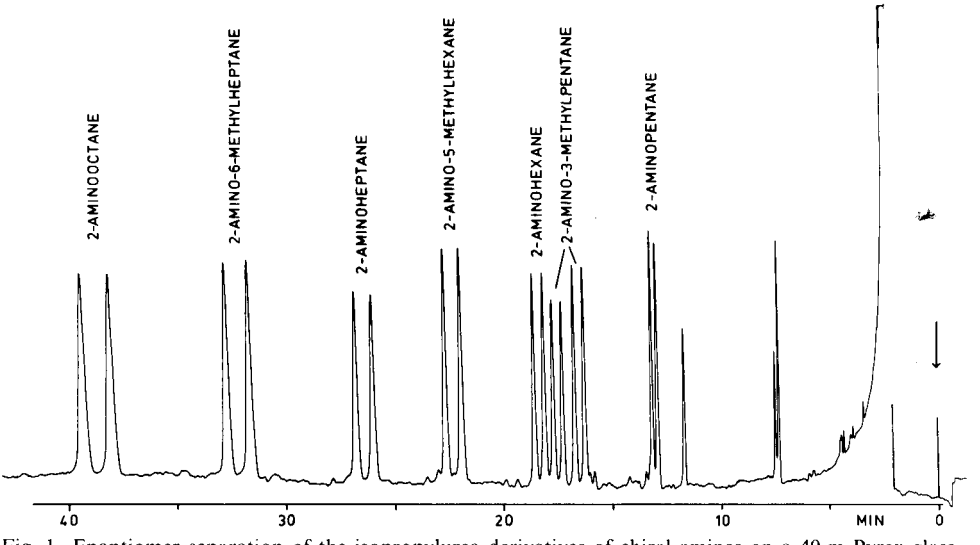


Fig. 1. Enantiomer separation of the isopropylurea derivatives of chiral amines on a 40-m Pyrex glass capillary column coated with XE-60–*S*-valine–*S*-α-phenylethylamide. Column temperature: 170°C (isothermal).

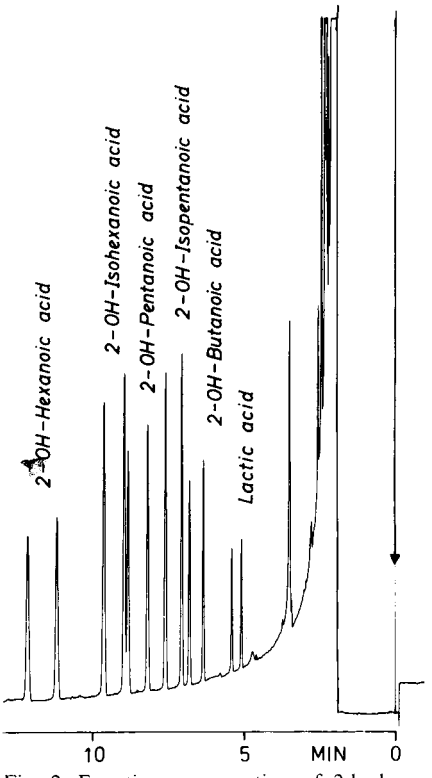


Fig. 2. Enantiomer separation of 2-hydroxy acid isopropyl esters as isopropylurethane derivatives. Column as in Fig. 1. Column temperature: 160°C (isothermal).

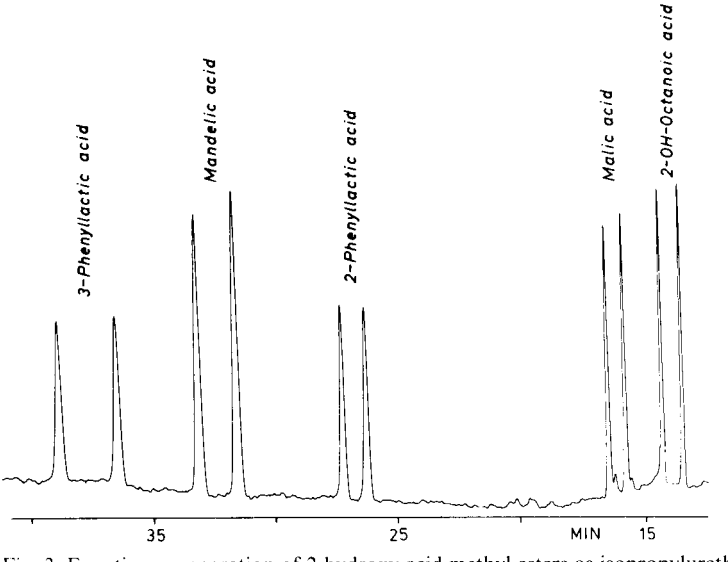


Fig. 3. Enantiomer separation of 2-hydroxy acid methyl esters as isopropylurethane derivatives. Column as in Fig. 1. Column temperature: 170°C (isothermal).

TABLE II

SEPARATION FACTORS (α) AND OPERATING TEMPERATURES FOR ENANTIOMER SEPARATION OF CHIRAL 2-HYDROXY ACID ISOPROPYL ESTERS AS ISOPROPYLURETHANES

Column: 40-m Pyrex glass capillary coated with XE-60–*S*-valine–*S*-α-phenylethylamide

Racemate	α *Value*	*Column temperature (°C)*
Lactic acid	1.095	140
2-Hydroxybutyric acid	1.065	160
2-Hydroxyisopentanoic acid	1.069	160
2-Hydroxypentanoic acid	1.071	160
2-Hydroxyisohexanoic acid	1.069	160
2-Hydroxyhexanoic acid	1.079	160
2-Hydroxyoctanoic acid	1.087	160
2-Hydroxydodecanoid acid	1.084	170
2-Hydroxytetradecanoic acid	1.055	200
2-Hydroxyhexadecanoic acid	1.056	200
Malic acid	1.065	160
Mandelic acid	1.071	160
2-Phenyllactic acid	1.037	170
3-Phenyllactic acid	1.092	160

values are comparably poor and only incomplete separations are achieved (for 3-hydroxybutyric acid, $\alpha = 1.013$ at 160°C).

CONCLUSIONS

Isopropyl isocyanate may be used as an almost universal reagent for the formation of urea or urethane derivatives of chiral compounds with amino or hydroxy groups. The derivatives of amines and 2-hydroxy acid esters can be separated with much larger separation factors than the corresponding trifluoroacetyl derivatives. This increase in enantioselectivity may be ascribed to the additional NH group in the urea and urethane derivatives as a site for hydrogen bonding or dipolar molecular interaction with the chiral sites of XE-60–*S*-valine–*S*-α-phenylethylamide.

ACKNOWLEDGEMENT

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CHROM. 14,622

GAS CHROMATOGRAPHIC DETERMINATION OF METHYL AND ETHYL MERCURY: "PASSIVATION" OF THE CHROMATOGRAPHIC COLUMN

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SUMMARY

A conditioning procedure for chromatographic columns is described in which a benzene solution of mercuric chloride is repeatedly injected onto columns of diethylene glycol succinate; hitherto unparalleled column efficiency is demonstrated for the determination of methylmercuric and ethylmercuric compounds. More than 2700 theoretical plates are attainable, with an absolute detection limit of about 0.2 pg of methylmercuric chloride per injection. After treatment, peak areas are reasonably stable but do tend to decrease about 2–6% over a 4- to 5-h period. The beneficial effects of the treatment are only temporary, however, and it must be repeated daily; the cycle of improvement and subsequent decline in column efficiency and sensitivity seems to be repeatable indefinitely. Fundamental aspects of the chromatography involved and its practical application to the analysis of fish are discussed in detail.

INTRODUCTION

Since the pioneering work of Westöö in Sweden[1–3] and of Sumino in Japan[4,5], considerable effort has been expended in the development of reliable, precise, and sensitive methods for the gas chromatographic determination of methylmercuric chloride (MMC) and ethylmercuric chloride (EMC), particularly in fish and in other biological samples. Recent reports on the chromatographic determination of organic mercury compounds include those of Cappon and Smith[6–8], Watts *et al.*[9], and Goolvard and Smith[10]. The variety and ingenuity of the analytical procedures developed, from sample treatment to the determinative step, attest to the ongoing need for alkyl mercury analyses and the continuing difficulty with analytical methodology. Variations of sample-preparation procedures include use of a radioactive methyl mercury tracer to correct for incomplete recoveries[6,11]; EMC as an internal standard[9,10]; alkaline digestion of the sample[6–8] in contrast to direct acidification and extraction; thiosulfate or cysteine re-extractions to minimize background peaks[1–3,6–8,10,12]; extraction of methyl mercury as the chloride, the bromide, or the iodide; and cupric ion[3,13] and urea[11] to increase recoveries of methyl mercury.

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Despite the diversity of sample-preparation procedures, the determinative step invariably involves gas chromatography with an electron-capture detector. A wide variety of stationary phases have been recommended for use in methyl mercury determinations: diethylene glycol succinate (DEGS)[4,5,9], OV-17 + QF-1[6,8], phenyldiethanolamine succinate[3,12,14–16], ethylene glycol adipate[10], butanediol succinate (BUDS)[4,5,17], Carbowax 20M[1,2,13], and polyethylene glycol succinate[18]. All of these columns have exhibited in some laboratories one or more of the following deficiencies: (a) poor and often variable response to MMC or EMC because of apparent interactions with the column or their decomposition on it; (b) moderate to very severe tailing; (c) poor column efficiency, which can then lead to problems with interferences. On the basis of calculations made with published chromatograms or from statements in reports, the number of theoretical plates for MMC often seems to be only about 100–200[6,8,12–14] or 300–500[1,2,5,15,16]; the highest number of theoretical plates reported is about 900 on 5% BUDS[4] and 1200 on 15% DEGS[9]; (d) very long times to initially condition the column, as much as 3–6 days in some cases[9,10,12,14]; (e) a variable decrease in the peak areas (heights) for MMC and EMC from injections of fish extracts, although MMC and EMC standard solutions furnish good chromatograms before injection of the sample extracts.

In 1979, this laboratory sent samples of fish to a dozen cooperating laboratories to analyze for methyl mercury by the method of Watts *et al.*[9]. Eight of these laboratories experienced little or only moderate difficulty with the method and reported results that agreed very well. Four, however, experienced severe difficulties with the chromatographic column specified (15% DEGS) although excellent results had been obtained previously in this laboratory over a several-year period. For that reason, we decided to investigate in more detail the chromatographic behavior of MMC and EMC on DEGS columns. Our detailed findings are presented in this report.

EXPERIMENTAL

The gas chromatograph used for most of the work was a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 5830A, equipped with Model 18803A ^{63}Ni electron-capture detector; some work was performed on a Hewlett-Packard Model 5710A gas chromatograph with Model 18713A detector. Injector and detector temperatures were maintained at 200 and 300°C, respectively. Carrier gas (argon–methane, 95:5) flow-rates were 30 and 60 ml/min for 2- and 4-mm I.D. columns, respectively. All columns were 0.25-in. O.D. silanized glass. Because the column effluent contains mercury compounds and trace radioactivity, it must be properly vented.

Twelve DEGS columns, differing in length, inner diameter, solid support and loading level were prepared and evaluated during the course of this study with both commercially prepared and "home-loaded" packing. Columns were packed no closer than about 2 cm to the threaded ends of the high temperature injection and detection ports because decomposition of nonstabilized DEGS, in particular, tends to occur at elevated temperatures, resulting in high and noisy baselines. All the columns produced more or less suitable results. Most of the results reported here were obtained with a (6 ft. × 2 mm I.D.) column packed either with 5% (stabilized) DEGS-PS on 100–120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) and operated at 145°C or with 10% (stabilized) DEGS on 90–100 mesh Chromosorb W AW (Analabs,

North Haven, CT, U.S.A.) and operated at 160–170°C because these columns afforded generally the highest performance of those prepared. For convenience, these two particular columns will be referred to as the "5% DEGS" and the "10% DEGS" columns throughout this report.

Stabilized DEGS was conditioned by flushing the column with carrier gas for 0.5 h, heating at 100°C for 1 h, then increasing the temperature at a rate of 4°C/min to 225°C and maintaining it overnight —all with normal carrier-gas flow[19]. Columns of nonstabilized DEGS (HI-EFF-1BP) were conditioned by modifying the recommendations of Watts *et al.*[9]: 0.5 h at room temperature, 2 h at 100°C, overnight at 200°C and 2 h at 225°C— all with normal carrier-gas flow.

Mercuric chloride, bromide, and iodide were reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). The acetone used to wash fish samples was distilled-in-glass (Burdick & Jackson, Muskegon, MI, U.S.A.).

The other chemicals, apparatus, and procedures used in this study have been reported elsewhere[9].

Generally, 5-μl samples were injected into the chromatograph for analysis.

RESULTS AND DISCUSSION

Although a column of nonstabilized DEGS (HI-EFF-1BP) prepared and conditioned in this laboratory in the manner recommended by Watts *et al.*[9] functioned satisfactorily and produced about 950 theoretical plates [$N = 16(t_R/w_b)^2$, where t_R = retention time and w_b = peak width at base] for MMC, a number of other laboratories experienced severe difficulties with this procedure —high and noisy baselines that would not decline even after several days. It was noted that the HI-EFF-1BP column prepared was a light tan after conditioning, and another such column, used successfully by Watts, was quite brown[20]. It seemed, therefore, that the conditioning procedure recommended by Watts, which followed suggestions by Westöö[1,2], probably involved a partial pyrolysis which was somewhat difficult to reproduce. For this reason, we chose to investigate some of the newer, stabilized varieties of DEGS coated on modern high-quality supports.

Initial results with stabilized DEGS were very disappointing. After the initial overnight conditioning, standard solutions of MMC and EMC exhibited very small, broad, tailed peaks and actual overlap of the two peaks with an efficiency of perhaps 30–40 plates.

We then decided to investigate the possible beneficial aspects of treating the column with high levels of mercuric compounds. There are several precedents for this approach: Westöö[3] noted that sample solutions containing sulfur compounds apparently poisoned the chromatographic system, which could be rejuvenated by injection of benzene solutions of methoxyethylmercury iodide or mercuric chloride. Kamps and McMahon[12] reported the necessity of conditioning their column initially by injecting solutions containing high levels of organic and inorganic mercury compounds; degradation of column performance with time could be reversed, for some unknown reason, by injection of extracts of certain blood samples. Uthe *et al.*[13] recommended rejuvenating (Carbowax) columns for MMC determinations by injecting aqueous 3 *M* potassium iodide and waiting for an hour. Schafer *et al.*[15] found it necessary to inject 5 μl of a 1 mg/ml solution of mercuric chloride in benzene twice

before the injection of each sample and several standards in order to obtain reproducible results. Finally, the analytical methods manual of the Environmental Protection Agency[14] specifies an initial 4-day conditioning of the column, including a sequence of 12 injections of a solution containing high levels of organic and inorganic mercury compounds.

Injection of mercuric chloride solutions in benzene at the milligram per milliliter level onto DEGS columns produced a peak with the same retention time as MMC; the peak area was about 0.05 % that of an equal quantity of MMC. (Goolvard and Smith[10] have already noted that mercuric chloride produces a small interferent effect in MMC determinations.) With repeated injections of mercuric chloride this peak shifted to a shorter retention time, sharpened, increased in height considerably, and exhibited much less tailing. Fig. 1 illustrates the effects of mercuric chloride conditioning on a 10 % DEGS column. In this particular case, injections of an MMC and EMC standard solution (5 μl of 0.20 μg/ml of each) onto a new column produced hardly any detector response (Fig. 1A). The very small, broadened peak at 6–9 min corresponds to MMC; EMC elutes at a much longer retention time. After repeated injections of a 1 mg/ml solution of mercuric chloride, the peak corresponding to MMC essentially stabilized; and about an hour later, after equilibration was reached,

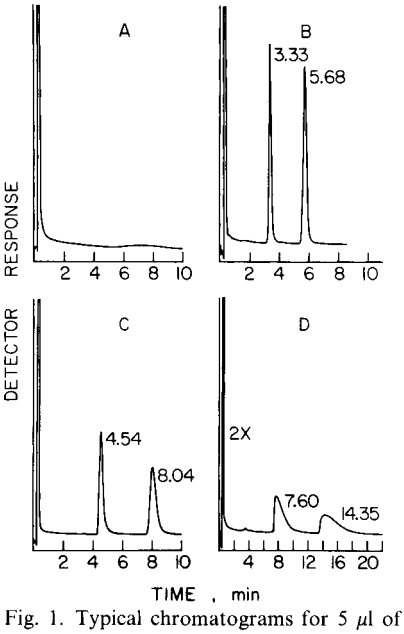

Fig. 1. Typical chromatograms for 5 μl of a standard solution of 0.20 μg/ml methylmercuric chloride (MMC) and ethylmercuric chloride (EMC) in benzene before and after treatment of the column with mercuric chloride (five 20-μl injections of a 1 mg/ml solution of mercuric chloride in benzene). Retention times are listed to the right of the peaks for MMC and EMC. MMC elutes first. Column: 10 % DEGS on 90–100 mesh Chromosorb W AW operated at 170°C and 30 ml/min carrier-gas flow-rate. A, Chromatogram on a freshly (overnight) conditioned column before mercuric chloride treatment; very little response is apparent. B, Chromatogram obtained about 1.5 h after mercuric chloride treatment had begun and column had stabilized; note the dramatic improvement in sensitivity and that only a slight amount of tailing is evident, and only on the EMC peak. C, Chromatogram obtained on the day after the mercuric chloride treatment. D, Chromatogram obtained the second day after mercuric chloride treatment; detector-amplifier sensitivity increased to twice that for the other chromatograms.

injection of standard solutions produced chromatograms such as the one shown in Fig. 1B. Note that there is almost no tailing evident in chromatogram 1B, compared to what is often seen for MMC[1,2,13–15]. A freshly treated 10% DEGS column, such as that used in Fig. 1, often produced upwards of 2700 plates and somewhat more than this for EMC. Resolution between MMC and EMC was typically about 5–6. The net effect of mercuric chloride treatment was a dramatic reduction in the specific and strong interaction of organomercurials with the column, as evidenced by the disappearance of tailing. For this reason, we prefer to call the treatment a *passivation* rather than an "activation", a term sometimes used to refer to a special treatment of a column, or a "conditioning", which usually refers to the initial elevated-temperature purging of a new column.

Generally, about five 20-μl injections of a 1 mg/ml solution of mercuric chloride at 5-min intervals were required to produce the desired degree of passivation for a freshly conditioned DEGS column. Over the next 1.5 h or so, the peak areas for MMC and EMC increased, after which they began to decrease and level off. Peak areas were then stable to within about 2–6% for the next 4–5 h.

In addition to the peak with the same retention time as MMC, injection of mercuric chloride produced several very broad peaks at longer retention times (about 50 min on a 10% DEGS column operated at 170°C, about 120 min for 5% DEGS at 145°C), which sharply decreased in area with successive injections. After elution of these broad peaks, the peaks for MMC and EMC sharpened markedly and increased in height by a factor of 2–3. It is the passage of these broad peaks that finally resulted in a more or less stable column. Although the natures of these peaks are unknown at present, it is suspected that they may represent a flushing of adsorbed compounds (possibly containing sulfur) from the column.

It seems likely, therefore, that at least one important cause for the severe tailing of organomercurials is an interaction with compounds contained within the column in addition to possible interaction with active sites on the solid support. For example, there was little difference in the behavior of columns if the support was silanized (Chromosorb W HP) or simply acid-washed (Chromosorb W AW); after overnight conditioning, columns still required mercuric chloride treatment, and the decreases in peak areas with time after mercuric chloride treatment were similar to those in Fig. 1C and D. Several of the DEGS columns prepared, as well as some others (5%, 1,4-BUDS, 3% ECNSS-M and 10% EGSS-X), exhibited fairly stable chromatograms for standard solutions, but then showed a progressive decrease in the MMC and EMC peak areas with injection of fish-sample extracts. Sometimes this decrease in sensitivity was reversed after overnight standing at operating temperatures or after repeated injection of standards; sometimes it was not. Some columns had a reasonable stability for MMC but exhibited a pronounced drop in sensitivity for EMC on injection of fish extracts.

Interaction with the support cannot be totally excluded, however. The 5% DEGS column tested in this study always showed a small amount of tailing, even with extended mercuric chloride treatment (generally a maximum of 1200–1800 theoretical plates), whereas the 10% DEGS column could be treated to the point where no tailing at all was visible (2200–2700 plates typically; asymmetry, 10% peak height criterion, could usually be reduced to 1.3 or less). In the latter case, the higher loading presumably more effectively insulated the diatomaceous surface.

The range for typical absolute detection limits (S/N = 2 criterion, based on baseline noise levels) for "clean" standard solutions has been about 0.2–0.5 pg of MMC or EMC. Fig. 2 is a chromatogram for the injection of 25 pg each of MMC and EMC. Even at this sensitivity, the baseline noise is hardly visible. The operational limit of quantitation for the overall Watts method (based on a 5-g fish sample, 25 ml final solution volume and injection of 5 μl) is, therefore, about 2–5 ppb in the original sample.

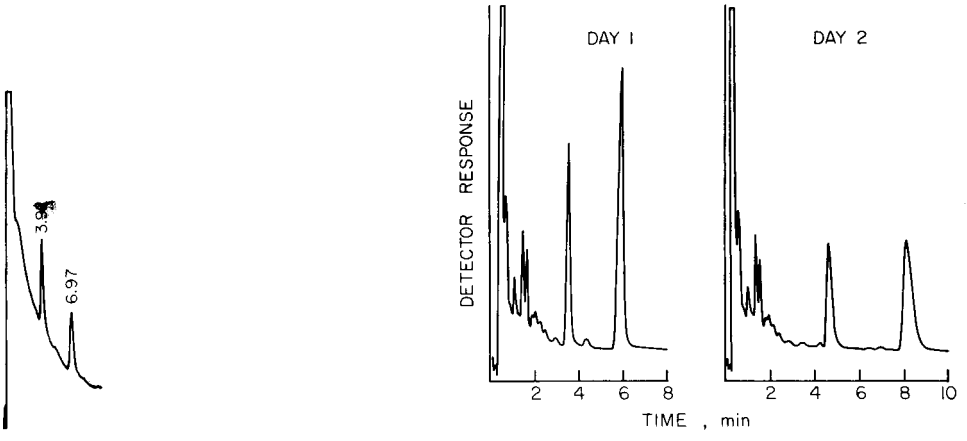

Fig. 2. Chromatogram of 25 pg each of methylmercuric chloride (MMC) and ethylmercuric chloride (EMC) (5 μl of 0.005 μg/ml) in benzene. Column: 5% DEGS-PS on 100–120 mesh Supelcoport operated at 145°C and 30 ml/min carrier-gas flow-rate. Retention times (min) for the peaks are shown. MMC elutes first.

Fig. 3. Chromatograms of a benzene extract of a tuna sample containing a natural level of 0.50 ppm of mercury (as methyl mercury) on the day of (Day 1) and the day after (Day 2) mercuric chloride passivation of the column. A small aliquot of a concentrated solution of ethylmercuric chloride (EMC) was added to the benzene extract to bring its concentration to 0.20 μg/ml. Column: 10% DEGS on 90–100 mesh Chromosorb W AW operated at 160°C and 30 ml/min carrier-gas flow-rate.

Time variation of column sensitivity and resolution

The beneficial effects of mercuric chloride passivation of DEGS columns, unfortunately, are only temporary. Typically, overnight standing of a column at the operating temperature of 170°C (10% DEGS column) caused decreases of about 10–35% in the peak heights, peak areas and the number of theoretical plates. Retention times increased by about 30%, and peak tailing was also much more evident. On the second day after the mercuric chloride treatment, column performance was much worse; peaks were quite small and drawn out. Fig. 1 illustrates these changes for chromatograms of standard solutions.

This deterioration in performance occurred whether or not fish extracts were injected onto the column and was invariably worse for EMC than for MMC. The higher the column temperature, the faster this degradation occurred. For example, a 5% DEGS column maintained at 145°C gave usable results for 3–4 days, whereas a column at 160°C was good for only about 2 days. The entire cycle of treatment with mercuric chloride, the resultant improvement, and the subsequent decline in column performance seemed to be repeatable indefinitely.

Older columns that had been subjected to a number of mercuric chloride injections over a period of time exhibited a better time stability than newer columns.

Although it is rather annoying to have the MMC and EMC peaks shift to longer retention times on the day after mercuric chloride treatment, these shifts do provide one considerable analytical benefit. Particularly for those samples that are apparently very low in MMC, there is always the concern that the "peak for MMC" may actually be due to some other component in the sample —an interferent that happens to have (nearly) the same retention time. Or a particular sample may simply have a very large interferent peak where MMC "normally" occurs. It would be most extraordinary for any interferent peak to shift by exactly the same amount as the MMC (or EMC) peak on the day after mercuric chloride treatment. This procedure provides additional evidence for the presence of MMC or EMC in a sample.

In Fig. 3, the chromatograms of one particular fish extract on the day of and the day after mercuric chloride-column passivation clearly illustrate the MMC and EMC peak shifts. The large, sharp peaks for MMC and EMC at 3.6 and 6.0 min, respectively, shifted to 4.7 and 8.2 min on the second day, whereas the background peaks did not change at all.

Mercuric chloride-treatment procedure to increase sample throughput

The mercuric chloride-passivation procedure recommended and the time for the column to equilibrate afterwards require almost an entire morning. One is therefore limited to about 50–60 injections of sample and standard (if no EMC or late eluters are present) per day. In order to increase the sample throughput, several procedures were tested to improve stability. The deliberate addition of a small, constant level of mercuric chloride to all samples and standards to provide a more or less continuous flow of the passivation agent did not seem to improve long-term column stability. Moreover, mercuric chloride gave a small positive interference for MMC. Loading of a few milligrams of solid potassium chloride into the front end of the column, to serve as a chloride source, also had no discernible effect.

An improvement was obtained, however, by lowering the column temperature to 115°C at the end of a working day, waiting a few minutes, and then injecting 20 μl of a 1 mg/ml solution of mercuric chloride in benzene. The next morning, the column was raised to its operating temperature and was stable and ready for use within about 45 min. The overnight temperature of the column is somewhat critical: Too low a temperature requires a longer time for stabilization the next morning; too high a temperature results in somewhat lower column performance.

Table I illustrates the day-to-day variation in the average peak areas for a standard solution of MMC and EMC on a column injected each night with mercuric chloride. On a day-to-day basis, the peak area for MMC is reasonably constant; the standard deviation of the composite average is only about twice that of the deviation within a day. The peak areas for EMC vary considerably more than those for MMC from day to day; the standard deviation of the composite average is about four times as great as that for MMC. Within a day, however, the precision for EMC peak areas is about the same as that for MMC.

The data in Table II were collected to illustrate that lowering the column temperature to 115°C and injecting mercuric chloride does not adversely affect analyses. In this series of analyses two subsamples (*ca.* 60 g each) of a large sample of

TABLE I

DAY-TO-DAY VARIATION IN AVERAGE PEAK AREAS FOR INJECTION OF 1 ng EACH OF METHYLMERCURIC (MMC) AND ETHYLMERCURIC (EMC) CHLORIDES

Column: 6 ft. × 2 mm I.D. silanized glass, packed with 5% DEGS-PS on 100–120 mesh Supelcoport, operated at 145°C and 30 ml/min carrier-gas flow-rate; 5-μl injections of 0.20 μg/ml standard. At the end of each working day, 20 μl of a solution of 1 mg/ml of mercuric chloride in benzene was injected into the column after the temperature was lowered to 115°C. At the start of the working day, the column temperature was raised to 145°C and allowed to stabilize for about 30 min prior to injections.

Age of column (days)	*Number of injections*	*Average peak area (standard deviation) (integrator counts × 0.01)*	
		MMC	*EMC*
1	3	9902 (71)	9023 (214)
2	6	9350 (402)	7417 (120)
3	8	9469 (261)	8986 (99)
4	5	9675 (93)	10692 (193)
8	6	9553 (115)	11983 (125)
14	7	9124 (141)	–
16	5	9240 (237)	8629 (373)
17	5	9821 (264)	10616 (652)
	5	9271 (199)	9223 (312)
21	4	8754 (51)	8873 (65)
22	6	9148 (154)	9769 (144)
23	3	9209 (68)	10783 (190)
	5	8994 (137)	10165 (162)
24	6	–	11203 (406)
Grand average		9347 (330)	9797 (1252)
Median of standard deviations		(141)	(190)

homogenized oil-packed tuna were taken; one was spiked with MMC. Two splits of each subsample were taken and extracted by the method of Watts *et al.*[9]. The benzene extracts were then analyzed on three separate days. The data illustrate good day-to-day and split-to-split precision for the overall analysis, as well as good recovery of the added MMC. Because there was sometimes a small, gradual decrease (*ca.* 2–6%) in peak areas during the working day, it is recommended that one standard solution of comparable concentration be injected for no more than two injections of sample to maintain accuracy of results.

After a 10% DEGS column is used for 3–4 h at 160–170°C, an additional 20-μl injection of a 1 mg/ml solution of mercuric chloride followed by a 1-h waiting period helps to maintain column sensitivity.

With this overnight-conditioning treatment, the number of sample and standard injections that can be made during one working day is effectively doubled.

Nature of the on-column reaction

A number of reports[1,4,12,17,21] have indicated that small quantities of all methylmercuric compounds elute at nearly the same retention time and with roughly the

TABLE II

DETERMINATION OF METHYL MERCURY IN TWO SPLITS OF NATURAL AND SPIKED OIL-PACKED TUNA

Two jars of a homogenized tuna sample (*ca.* 60 g each) were taken. The spiked sample was prepared by slowly adding a small volume of a concentrated solution of methylmercuric chloride (MMC) in ethanol to one of them with stirring (after weighing) so that the amount added would be equivalent to 0.396 ppm of mercury in the original sample. The level of mercury (in the form of methyl mercury) in the original sample was found to be 0.40 ± 0.05 ppm by eight laboratories in an interlaboratory study using the method of Watts *et al.*[9]. Column: 5% DEGS-PS on 100–120 mesh Supelcoport operated at 145°C and 31 ml/min carrier-gas flow-rate. Each table value is the average from 2–4 injections of sample and each was compared to the average value from 2–4 injections of a standard solution.

Split	*Day*	*ppm Mercury (as methyl mercury)*		*MMC recovery (%)*
		Natural	*Spiked*	
A and A′	1	0.381	0.796	105
	2	0.378	0.763	97
	3	0.399	0.800	101
Mean ± S.D.		0.386 ± 0.011	0.786 ± 0.020	101 ± 4
B and B′	1	0.402	0.775	94
	2	0.383	0.773	98
	3	0.394	0.790	100
Mean ± S.D.		0.393 ± 0.010	0.779 ± 0.009	97 ± 3

same response, regardless of the anion used in making the standard solutions, Johansson *et al.*[21] have reported that the actual molecular species responsible for the "methyl mercury peak" produced by an electron-capture detector was a mixture of MMC and methylmercuric iodide (MMI), regardless of whether the standard solution injected was prepared from the chloride or from the iodide salt. Nishi and Horimoto[22] reported that large injections ($>10^{-5}$ g) of various methyl mercury compounds eluted with different retention times (iodide < bromide < chloride) with stainless-steel columns of 5% DEGS and thermal-conductivity detection, whereas small injections ($<10^{-8}$ g) with electron-capture detection had identical retention times. With larger injections, the small amount of decomposition that occurs is negligible. With glass columns, injections of 10^{-7}- to 10^{-9}-g quantities of various methyl mercury compounds produced peaks of different retention times as well as different heights, depending on the particular compound injected. These results, as well as the severe tailing often seen in methyl mercury chromatograms, clearly indicate that a partial on-column decomposition and anion-exchange process occurs for methyl mercury. Analogous results have been noted for ethyl mercury and other organomercury compounds.

In order to further delineate what may be happening when a DEGS column undergoes mercuric chloride passivation, a column was heated overnight at 225°C and treated with several injections of a 1 mg/ml solution of mercuric bromide or mercuric iodide. Standard solutions of MMC and EMC in benzene were then injected. The data in Table III show that the retention times for the MMC or EMC peak varied, depending on the particular mercuric halide used to treat the column, and increased in the order mercuric iodide < mercuric bromide < mercuric chloride.

TABLE III

RETENTION TIMES FOR METHYLMERCURIC CHLORIDE OR ETHYLMERCURIC CHLORIDE INJECTED ONTO A COLUMN TREATED WITH MERCURIC HALIDE COMPOUNDS AND FOR VARIOUS ORGANOMERCURIC COMPOUNDS INJECTED ONTO A STAINLESS-STEEL COLUMN

MMI = Methylmercuric iodide, MMB = methylmercuric bromide, MMC = methylmercuric chloride, EMI = ethylmercuric iodide, EMB ethylmercuric bromide and EMC = ethylmercuric chloride.

*This work**				*Nishi and Horimoto*[22]**		
Column treatment	*Compound injected*	t_R *(min)*	$t_R/t_{R,Cl}$	*Compound injected*	t_R *(min)*	$t_R/t_{R,Cl}$ *(min)*
Mercuric iodide	MMC	3.30	0.77	MMI	1.60	0.65
Mercuric bromide		3.57	0.83	MMB	2.15	0.88
Mercuric chloride		4.28	1.00	MMC	2.45	1.00
Mercuric iodide	EMC	5.40	0.74	EMI	2.85	0.62
Mercuric bromide		5.92	0.82	EMB	4.00	0.87
Mercuric chloride		7.25	1.00	EMC	4.60	1.00

* Conditions: 6 ft. × 2 mm I.D. silanized glass column packed with 15% (stabilized) DEGS on 80–100 mesh Chromosorb W AW operated at 170°C and 30 ml/min carrier-gas flow-rate. Each value is the average of 4–7 injections. Quantities injected were 10^{-9} g (1 ng).

** Conditions: 0.3 × 200 cm stainless-steel column packed with 5% DEGS on 60–80 mesh Chromosorb W operated at 150°C and 45 ml/min He carrier-gas flow-rate. Quantities injected were $>10^{-5}$ g.

The relative retention times compare quite favorably to those reported by Nishi and Horimoto[22] for injection of large ($>10^{-5}$ g) quantities of authentic MMI, methylmercuric bromide (MMB) and MMC onto a stainless-steel column of 5% DEGS.

Peak heights and areas for MMC and EMC are also affected by the particular mercuric halide used to treat the column (Table IV). On a 15% DEGS column, peak areas increased in the order mercuric iodide < mercuric bromide < mercuric chloride treatment. There is some correspondence of the relative peak heights shown

TABLE IV

COMPARISON OF PEAK HEIGHTS AND AREAS FOR METHYLMERCURIC CHLORIDE (MMC) AND ETHYLMERCURIC CHLORIDE (EMC) INJECTED ONTO COLUMNS TREATED WITH VARIOUS MERCURIC HALIDE COMPOUNDS

Chromatographic conditions are the same as in Table III. Each value is the average of 4–7 injections. Peak areas are integrator counts × 10^{-2}; peak heights are in mm at an attenuator setting of 2^{10}. Quantities injected were 1 ng.

Column treatment	*Compound injected*	*Peak area*	*Relative peak area*	*Peak height*	*Relative peak height*
Mercuric iodide	MMC	7720	0.57	80.4	0.66
Mercuric bromide		8420	0.62	90.7	0.75
Mercuric chloride		13640	1.00	121.1	1.00
Mercuric iodide	EMC	13580	0.69	90.3	0.83
Mercuric bromide		15044	0.77	104.4	0.95
Mercuric chloride		19580	1.00	109.4	1.00

in Table IV to those reported by Nishi and Horimoto[22] for direct injection of MMI, MMB and MMC standard solutions; however, the correspondence of the relative retention times (Table III) is much closer.

Therefore, treatment of DEGS columns with large (10–100 μg) injections of mercuric chloride either: (a) provides a trace level of chloride ion or molecular mercuric chloride in the column that maintains MMC predominantly in its molecular form throughout the column and minimizes decomposition; or (b) temporarily ties up active sites with mercury, chlorine, or some compound containing either or both. The fact that authentic MMI injected onto a column elutes primarily as the corresponding chloride[21] clearly indicates an on-column reaction with some form of chloride bound to or adsorbed on the column packing. The fact that the performance of mercuric chloride-treated columns degrades after standing at an elevated temperature, even if no samples or standards are injected, indicates that a somewhat nonvolatile compound (or compounds) remains in the column to decrease the decomposition of the organomercury halides and the resultant tailing.

The only DEGS column prepared in this laboratory that did not require mercuric chloride treatment to exhibit reasonable efficiency is precisely the one specified by Watts *et al.*[9]: 15% HI-EFF-IBP on Gas-Chrom P. However, some other laboratories have experienced extreme difficulties with this packing, as mentioned earlier. Gas-Chrom P is unique among the solid supports tested in that it is both acid-washed (with hydrochloric acid) and then base-washed (with sodium hydroxide). It seems reasonable to speculate that Gas-Chrom P (or perhaps just some batches) contains a low level of sodium chloride that serves as a source of chloride to maintain organomercury compounds in a molecular form during passage through the column.

A second effect of mercuric chloride injections seems to be the "flushing" of impurities, quite possibly sulfur compounds[12,13], from the column. Certain DEGS (and other) columns which were prepared maintained reasonable efficiency for several days, as long as only "clean" MMC and EMC standard solutions were injected, but performance degraded as soon as fish-extract solutions were injected. Experiments were performed in which six or seven 50-μl injections of fish extracts were made over a 20-min period in an attempt to load the column with impurities. After a 1-h wait to allow the column to clear, injections of MMC and EMC standard solutions were made; decreased efficiency and increased tailing showed that column performance had degraded. Injection of a 1 mg/ml mercuric chloride solution at this point produced large, broad peaks at about 50 min (at 170°C), and column performance was restored. If a mercuric chloride-treated column was simply left to stand at an elevated temperature without injection of fish extracts, column performance degraded and was then restored by further mercuric chloride treatment, but only a small peak was seen at 50 min after injection of the mercuric chloride.

Clearly, therefore, there are things intrinsic to the column —active sites, perhaps— as well as impurities in fish extracts adsorbed or bonded to column materials that cause decomposition and poor chromatograms for organomercury compounds. The multiplicity of these reactons is no doubt one reason why the precise conditions needed for good chromatographic determinations of organomercury compounds are often still difficult to define after nearly two decades of research in methods development.

CONCLUSIONS

The mercuric chloride-passivation treatment of DEGS columns outlined in this report produces extraordinarily efficient columns. The combination of sharp, non-tailing peaks and the sensitivity of the electron-capture detector result in a routine, absolute detection limit of better than 1 pg of MMC per injection. The practical limit of quantitation is about 2–5 ppb in the original sample, and there is a relatively high freedom from interferences because of the high performance of the column.

Fish extracts have been routinely analyzed by using these treated columns, and they should be useful for other types of samples as well. Long-term use has indicated that these columns are serviceable for months, and that the detector is not adversely affected by repeated injections of a 1 mg/ml mercuric chloride solution. New columns require only an initial overnight conditioning, plus about 3 h for the first mercuric chloride treatment and stabilization; thus the several days of conditioning specified by a number of other reports are eliminated. A new column can be packed, conditioned, and ready for use in less than one day.

The only disadvantages of the treatment are a small increase in the complexity of the procedures, and the requirement that standards be frequently re-injected because of a slow decline in sensitivity over 4–5 h.

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GAS CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF TRACE QUANTITIES OF ISOPROPYL METHYLPHOSPHONOFLUORIDATE AND ASSOCIATED COMPOUNDS, *IN SITU* AND IN DECONTAMINATION EFFLUENT

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SUMMARY

Sample preparation procedures and quantitative analytical methodology were investigated for the trace determination (nanograms to picograms) of isopropyl methylphosphonofluoridate (GB) in decontamination media. Systems have been developed wherein residual GB and some related compounds can be extracted from chemically hostile media (compound half-life of seconds) and estimated by gas chromatography using an EGSS-X column and flame photometric detector. Intact GB is determined in the presence of much higher quantities of associated organophosphonates and other compounds.

INTRODUCTION

The primary objective of this work was to develop an accurate, highly sensitive, gas chromatographic (GC) method for determining trace levels of the highly toxic isopropyl methylphosphonofluoridate (GB). The method should be applicable to samples originating from land reclamation, pollution abatement, surveillance, decontaminated sites, and residues and effluent from clean-up processes.

The monitoring requirements established for prudent control of GB associated operations were set as follows: $3 \cdot 10^{-3}$ mg/m^3 in stack effluent; $3 \cdot 10^{-4}$ mg/m^3 maximum-permissible ground-level concentration; < 1 ppm on a w/w basis for solid materials.

Included in the study was the additional requirement for developing suitable sample preparation procedures which would be totally compatible with the analytical method developed here. The requirements for sample preparation included considerations involving possible trace quantity synthesis of GB within a sample workup procedure, or via condensation reaction during GC analysis; also possible decomposition of agent GB which might be contained in original samples prior to workup; and finally the optimization of GB concentration in the analysis medium to insure that minimal levels of residual GB would be determinable.

The very first reported and recommended method for the gas chromatography of GB in the presence of its impurities[1] employed as a substrate DC-LSX-3-0295 on

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60–80 mesh Gas-Chrom P. This system, operating in the thermal conductivity detection mode, was found applicable for the quantitative determination in mixtures of GB, 2-propanol, methylphosphonodifluoride (difluoride), diisopropyl methylphosphonate (DIMP), and the GB pyroester, bis(isopropyl methylphosphonic) anhydride, among others. Furthermore, it was found at that time that isopropyl methylphosphonic acid (IMPA) could disproportionate on a GC column to form DIMP and possibly the methylphosphonic acid (MPA)[1]. Other column systems tested at that time included dioctyl and didecyl phthalate, silicone oil 200, silicone grease, and the Apiezons (J, L, and M), all on Johns-Manville firebrick. Only the Apiezon M approached the versatility of DC-LSX-3-0295 for analysis of GB in the presence of its impurities[1].

Previous related studies on some of the compounds described herein included GC–flame photometric detection[2] and GC–chemical and electron ionization mass spectrometry (MS)[3].

The work reported here, concerning the preparation of samples and the subsequent analytical methods employing gas chromatography, was the result of studies performed in this laboratory on hundreds of the various types of process samples. A large portion of these samples was from stack bubblers, brines, salts, and others related to a demilitarization program and had as their source spray dryer systems after chemical decontamination. The bulk composition of the brines and salts had been studied via infrared and Raman spectrometry[4] as well as by chemical analysis.

All of the GC column systems studied here were found applicable to the trace determination of GB. That found most useful for the determination of GB in media containing relatively large amounts of tributylamine (TBA) is a procedure employing a column packing of EGSS-X on Gas-Chrom Q.

PROCEDURES

Sample type and preparation

Three basic types of samples required analysis in support of a demilitarization program involving agent GB.

Water and stack bubbler samples originated from collection trains installed at various levels of the stack where the residual process gases were vented and dispersed into the atmosphere. The bubblers used for this sampling application contain an aqueous phase adjusted to pH 3.5 to pH 4.5 with sulfuric acid. Pretreatment, utilizing liquid–liquid extraction for concentrating these samples, was required to meet the low-level detection requirements of ambient air quality.

For gas chromatography, a 10-ml sample of the bubbler media was transferred to a clean 15-ml (conical-shaped) centrifuge tube and 2 g of sodium chloride were added and dissolved. Next a 0.1-ml portion of CP grade chloroform was added and shaken for no less than 1 min. The mixture was then centrifuged for 3 min and most of the upper layer (aqueous) was removed and discarded. The thin layer of water remaining inhibits the evaporation of the chloroform sample. A 1–20-μl sample of the chloroform solution was analyzed using the GC procedure described later. Water samples, as from suspected contamination, were handled similarly after adjustment to pH 4.5 with sulfuric acid.

The brine mixture was agitated to assure homogeneity. Brine, as a weighed 10–

15-g sample, was placed into a 100-ml beaker. A 50-ml buret was filled with 0.5 *N* sulfuric acid (precooled in an ice bath to $< 5°C$). A 250-ml beaker containing 20 ml of cooled water was similarly placed in an ice bath positioned on a magnetic stirrer. A pH combination electrode was inserted in the 250-ml beaker which is used as the neutralizer vessel. Brine and cooled acid were slowly but simultaneously added to the water container in the neutralizer vessel. The pH was kept relatively constant at 7 ± 0.5 by varying the addition rates of the respective materials. The remainder of the brine was rinsed (with cold distilled water) into the vessel and adjusted to pH 6.5. The contents of the neutralizer vessel were quantitatively transferred to a graduated cylinder. Aliquots (10 ml) of this solution were transferred to individual centrifuge tubes. A 100-μl volume of chloroform was added to each tube for extraction purposes. The tubes were sealed with corks and shaken vigorously for several minutes. The tubes were then placed in a bench top centrifuge and the chloroform phase settled out after 3 min of operation.

Soil samples that had been decontaminated with sodium hydroxide or sodium carbonate could be similarly treated starting with the formation of a slurry by adding water containing TBA to a 5-g sample of the original soil. These samples were then handled in the same way as the brine mixtures.

After centrifugation was complete, about 9.5 ml of the water layer was removed and small microliter aliquots of the chloroform layer were injected into a gas chromatograph operated under prescribed conditions.

Salt samples were mixed thoroughly to insure homogeneity. Weighed 3.0 ± 0.5 grams of sample were placed into a 100-ml beaker. A 50-ml buret was filled with 0.5 N H_2SO_4 (precooled in an ice bath to $< 5°C$). A 20-ml volume of cooled water and 30 to 50 mg of TBA were added to a 250-ml beaker, mixed and placed in an ice bath positioned on a magnetic stirrer. A pH combination electrode was positioned in the 250-ml beaker which was used as the neutralizer vessel. Salt and cooled acid were slowly but simultaneously added to the water contained in the neutralizer vessel. The pH was kept relatively constant at 7 ± 0.5 by varying the addition rates of the respective materials. The remainder of the salt was rinsed (with cold distilled water) into the vessel and adjusted to pH 6.5. The contents of the neutralizer vessel were quantitatively transferred to a graduated 100-ml cylinder for volume measurement and recorded. Aliquots (10 ml) of this solution were transferred to individual centrifuge tubes. A 100-μl volume of chloroform was added to each tube for extraction purposes. The tubes were sealed with corks and shaken vigorously for several minutes. The tubes were then placed in a bench top centrifuge and the chloroform phase settled out after 3 min of operation.

After centrifugation was complete, about 9.5 ml of the water layer was removed and small microliter aliquots of the chloroform layer were injected into a gas chromatograph.

The TBA added in the above procedure is apparently critical. In all of the salt samples tested, the omission of TBA addition resulted in very low or no recovery of GB "spikes". With the TBA addition, average recoveries of "spikes" of the order of 60% were obtained. This is comparable to the extraction efficiency expected.

An alternate procedure for treating the salts prior to analysis was also developed. This procedure incorporated trituration, with TBA added to the solvent prior to mixing.

The salt was mixed thoroughly to insure homogeneity. A 10.0 ± 0.5-g sample was weighed into a 40-ml screw top vial. A 500 μg TBA/ml chloroform stock solution was prepared for use in trituration (< 25 ml required for each sample). The chloroform stock solution (10 ml) was added to the weighed salt sample. Using a glass "elephant's foot", the mixture was triturated for about 3 min. The mixture was then filtered through a Buchner funnel fitted with a Whatman No. 41 filter pad and the container and filter washed with several small portions (3 to 4 ml each) of the TBA–chloroform solution into a 25-ml graduated cylinder. The contents of the graduated cylinder were evaporated to near dryness and the remaining liquid measured in a 250-μl Hamilton gas-tight syringe (about 100 μl of solvent remained after evaporation in these experiments). Several 10–20-μl aliquots of the chloroform solution were injected into a GC system operated under the prescribed conditions.

The same procedure was applied to concrete and stone samples suspected of being contaminated with GB. The only additional preliminary step taken was to pulverize the sample to expose more surface area to the trituration solvent.

ANALYSIS BY GAS CHROMATOGRAPHY

Procedure for trace analysis of GB samples which contain TBA

The GC procedure developed for the trace analysis of GB samples containing TBA, originating from the demilitarization process where TBA is present, is given below. Any model gas chromatograph adaptable to an all-glass column and on-column injection system and fitted with a flame photometric detector system is suitable for this procedure. The conditions listed below are optimum when applied with a Perkin-Elmer Model 900 chromatograph and minor changes in the conditions listed may be necessary for optimum performance when operating other model instruments.

Column packing: 3% EGSS-X on 100–120 mesh Gas-Chrom Q.
Column dimensions: 6 ft. × 1/4 in. O.D. × 2 mm I.D. Pyrex glass.
Column temperature: Isothermal at 89°C for 2 min, then increasing 8°C/min to 200°C.
Inlet temperature: 180°C.
Detector temperature: 165°C.
Manifold temperature: 200°C.
Detector fuel gas flow-rate: hydrogen, 150 ml/min; oxygen, 15 ml/min; air, 15 ml/min.
Carrier gas (nitrogen) flow-rate: 20 ml/min.

Peak area measurements of the GB peaks are compared with the responses obtained on injecting standard solutions of GB in chloroform for quantitation. Calculations for parts-per-billion* or percent were made on the basis of original sample size and the aliquots used in the determination.

Alternate GC procedures for GB and related compounds

With the exception of the coatings mentioned below on 60–80 mesh Gas-Chrom Q all other parameters including flow-rates were identical to those employed with the EGSS-X column system described above and programmed from 60°C. The

* Throughout this article the American billion (10^9) is meant.

column with the 10% QF-1 coating was programmed at 8°C/min to 220°C. The column of 5% Carbowax 20M terminated with terephthalic acid (TPA) was programmed at 8°C/min to 220°, then isothermal for 4 min. The column of 30% DC-LSX-3-0295 was programmed at 8°C/min to 220°C. The column of 3% FFAP was programmed at 10°C/min to 240°C.

RESULTS AND DISCUSSION

Selection of column systems

The requirement for detection and estimation of extremely low quantities of GB with a high degree of specificity was exacerbated not only by the environmental media but especially by the nature of associated compounds and their decontamination products. Tests with a variety of column packings indicated that the GC methods employed for measuring trace quantities of GB might not be totally satisfactory for all types of samples requiring analysis. Thus, an improved GC method was sought with emphasis placed on moderate to highly polar column packings including Carbowax 20M terminated with TPA, QF-1, DC-LSX-3-0295, Tenax-GC, FFAP, and EGSS-X.

Carbowax 20M with TPA appeared initially to be a promising candidate for this application; however, interference at the elution time of GB was encountered when samples contained large quantitites of tributylamine (TBA, as high as 2 mg/ml), the stabilizer (neutralizer) used in some of the manufactured GB. An additional disadvantage of this carbowax column was its inability to elute the pyroester compound of GB [bis(isopropyl methylphosphonic)anhydride] as a sharp peak because of temperature limitations and retention characteristics.

A QF-1 column system[5–8] that had been adapted to a variety of agents in our laboratory was very successfully used for the analysis of VX and GB[2]. It was found very effective for monitoring low GB concentrations during the initial portion of this work. However, when high TBA concentrations were observed in subsequent samples, significant interference was encountered. The QF-1 column eluted the GB and its pyroester compound as distinct, sharp, resolved peaks in synthetic mixtures with low quantities (1–50 μg/ml) of DIMP. However, although the high DIMP concentrations (1–15 mg/ml) found in some samples did not affect the more rapidly eluting GB, they completely obscured the pyroester measurement area.

Attention was then focused on the slightly more polar fluorosilicone, DC-LSX-3-0295. A glass column, similar to that reported by Sass *et al.*[1] was selected since it had shown the capability for resolving a wide variety of GB-related compounds (including the pyroester) at higher concentrations. This column system, when incorporated into the GC–flame photometric detector (FPD) analyses of standard samples, gave good resolution of all of the volatile compounds, as in the macro system, but did not quite satisfy the need for sensitivity at the picogram level. The Carbowax and QF-1 systems provided detectability of quantities of GB at the 0.5 ng level. The DC-LSX-3-0295 system required more than 5 ng to achieve a comparable signal.

A solid adsorbent packed column containing Tenax-GC appeared very promising during the early phases of its evaluation. GB could be detected reproducibly at the extremely low levels found possible with both Carbowax 20M–TPA and QF-1.

TABLE I

RETENTION CHARACTERISTICS OF THE VARIOUS COLUMN SYSTEMS TESTED

Compound	*EGSS-X*		*QF-1*		*Carbowax 20M–TPA*		*DC-LSX-3-0295*		*FFAP*	
	Time (min)	*Temperature (°C)*	*Time (min)*	*Temperature (°C)*	*Time (min)*	*Temperature (°C)*	*Time (min)*	*Temperature (°C)*	*Time (min)*	*Temperature (°C)*
Chloroform (solvent)	0.4	75*	0.5	64	0.7	65	1.0	68	0.2	62
Diisopropyl-carbodiimide	1.0	75*	2.5	80	3.1	85	7.2	115	0.5	65
Tributylamine	1.4	75*	4.5	96	6.0	108	8.8	130	1.5	75
GB	4.8	104	5.0	100	5.1	101	9.9	140	2.5	85
Diisopropyl-methylphosphonate	7.6	120	9.2	134	9.6	136	13.1	165	5.5	115
Trimethyl-phosphine oxide	9.0	129	8.7	130	9.9	139	12.3	160	6.0	120
Diisopropyl urea	19.0	189	12.5	160	18.4	207	16.8	195	11.0	170
GB pyroester	21.8	206	18.4	207	20+**	220+**	19.0	200***	Did not elute	

* Program preceded by a 2-min isothermal period after sample injection; compounds detected by flame ionization, along with diisopropyl urea.

** Broad peak with gradual leading and tailing edges —not accurately quantifiable or reproducible for sensitive detection.

*** Temperature programmed to 200°C and held there for 4 min.

No column conditioning was required prior to obtaining satisfactory response. Synthetic samples containing TBA (64,000 parts) and GB (1 part) were sufficiently resolved to permit unambiguous GB measurement. Samples of IMPA (isopropyl methylphosphonic acid), the pyro acid bis(hydrogen methylphosphonic acid) anhydride and the pyroester of GB, as well as DIMP, trimethylphosphine oxide (TMPO) and MPA provided no interference to the analysis for compound GB. However, interferences were encountered when sample aliquots were injected from prepared solutions obtained from sulfur dioxide-injected salts and brines. This column packing material was summarily disregarded from further consideration as an analytical column in this application.

The EGSS-X column system performed extremely well in experiments conducted using the gas chromatograph with the FPD system. All of the components of interest were well resolved and detection thresholds were slightly better than those obtained with the Carbowax and QF-1 systems discussed earlier. Unlike the previously employed column systems, the EGSS-X column presented no difficulties in GB measurement or rapid baseline recovery when quantities of sample containing only 1 ng of GB and as much as 1.5 mg of TBA were injected. The polyester phases, of which EGSS-X is a member, are susceptible to hydrolysis at elevated temperatures. When this occurred some change in retention time and ultimate sample sensitivity was observed. The EGSS-X column was replaced with a fresh one when changes in retention time or sensitivity were noted. Rejuvenation of a fouled column using either Silyl-8 or Methelute were found to drastically alter the retention characteristics of this GC phase.

During the course of column screening, it was found that an FFAP system showed promise. Although GC–FPD data obtained using an FFAP column system was limited because of the earlier successes using EGSS-X, the preliminary data indicated this column material could be a satisfactory substitute if new problems arose because of process design changes. In fact, when the EGSS-X column was used in connection with GC–MS^3 applications via electron impact ionization, low-intensity ions were observed at *m/e* values of 99, 100, and 101. Since the *m/e* 99 ion measurement is critical in ascertaining the presence or absence of agent GB using this technique, the presence of these column background ions interfered with the determination. Thus, in GC–MS applications, the FFAP column was used with much success in later MS analysis of GB.

Of the various column materials tested, the five which resulted in the best resolution of components are listed in Table I. Included in this table are the retention times and elution temperatures for each compound on each system. The procedure employed with the EGSS-X column was reported in the experimental portion of this report. The other column systems as indicated were utilized under identical conditions except for the changes noted previously.

Sample preparation

Two of the most difficult sample mediums addressed in this study were brine and dried salt products derived through a caustic destruction process (*i.e.*, chemical disposal of the GB). The brine samples were aqueous slurries composed of approximately 30% solids. Once the brine samples were drum or spray dried, the resultant solids had to be certified that they were free of GB. The composition of these solids

included sodium hydroxide, sodium bicarbonate, sodium carbonate, the sodium salts of IMPA and MPA, sodium fluoride and sodium sulfate. Additionally contained in the solids could be residues of stabilizers such as TBA, diisopropylcarbodiimide, diisopropyl urea, and quantities of DIMP. The pH of the brines and reconstituted salts was greater than 12.8 and for the most part higher than pH 13. Therefore, either type sample provided a very hostile medium from which to manipulate any intact GB molecules into a suitable solvent.

Initial attempts at analysis of the GB salts involved trituration with a variety of solvents. All attempts to add known quantities of GB to the triturated mixtures resulted in little or no recovery of the added intact compound. GB hydrolyzes very readily at pH's of 10 and higher (half life $\approx$ 10 sec) and since small quantities of the compound could readily be reconstituted at pH 4 or lower with HF and IMPA present, the most favorable conditions for sample preparation should have been near neutrality. Even at temperatures controlled to 0–5°C and neutralization to pH 6.5–7.0 with 1 *N* sulfuric acid, "spikes" of GB produced unpredictable results and low recoveries. The cool acid neutralization technique was discarded in favor of a milder neutralization system as described in the procedure.

"Spiking" or adding known quantities of the GB to the material to be analyzed was undertaken to insure that GB was not being degraded by artifact of neutralization or the partitioning method. Its application was very important towards ascertaining the degree of recovery of actual compound that might be present in the substrate being analyzed. Near total recovery of such spikes was considered evidence that the sample preparation procedure would permit GB to be transferred intact to the analysis medium. The partial success observed in recovering GB spikes from brine, as opposed to the poor or no recovery from spiked salts, was suggestive that the spray dryer heat was removing from the salt some component(s) that was present in the brine. Two candidates considered were DIMP and TBA. The latter (TBA) was found in our studies to have the most influence in supporting the GB spike. The spiking of the brine samples was performed early in the preparation procedure. It was found that neutralized brine could support some of the GB spike. GB was added to the 20 ml of cooled water prior to the start of neutralization. The results for some spiked brines are shown in Table II.

The results of two spiking experiments on the salts are given in Table III. The samples (numbered 1–4) were run through the sample preparation procedure without

TABLE II

REPRESENTATIVE RESULTS OF SOME "SPIKING" EXPERIMENTS CONDUCTED WITH BRINES

Brine sample	*GB present (μg)*	*GB added (μg)*	*GB found (μg)*	*Recovered (%)*
A	None detected	9.66	4.50	27.0
B	None detected	9.66	4.56	47.2
C	None detected	9.66	5.76	59.6
D	None detected	9.66	5.76	59.6
E	None detected	10.30	6.28	60.9
F	None detected	10.30	6.19	60.1

TABLE III

RESULTS OF "SPIKING" APPROXIMATELY 3-g SAMPLES OF SPRAY DRY SALTS WITH GB

Sample	*Quantity of TBA added (mg)*	*Quantity of GB added (μg)*	*Quantity of GB found (μg)*	*GB recovered (%)*
Blank	0	0	None detected	–
1	0	19.3	4.1	21.0
2	0	19.3	4.1	21.0
3	0	19.3	4.0	20.4
4	0	19.3	4.3	22.3
Blank	35	0	None detected	–
5	35	482.9	394.9	61.3
6	35	482.9	397.2	61.7
7	35	482.9	384.0	59.6
8	35	120.2	69.8	58.1
9	35	120.2	74.8	62.2
10	35	120.2	72.7	60.5

adding the TBA found to be required in the salt analysis procedure. The other salt samples were "spiked" by adding GB to the cooled water mixture containing TBA prior to the start of neutralization.

In early trituration experiments, attempts to spike the salt directly were unsuccesful. A recovery of 1.6% was obtained in one experiment where a mixture of TBA (50 mg) and GB (500 μg) contained in chloroform was dispersed on a 3.5-g sample of a drum-dried salt.

A vigorous reaction took place during this spiking procedure as the amine odor was quite obvious and the salt (originally just off-white) turned very dark (almost black). The amount of DIMP formed was so great that it overloaded the flame-photometric detector.

Other experiments were then conducted where only 10 ml of TBA-containing chloroform (*ca.* 500 μg/ml) were added to each weighed salt sample. The TBA solu-

TABLE IV

RESULTS OF SOME "SPIKING" EXPERIMENTS CONDUCTED USING THE TRITURATION PROCEDURE WITH TBA ADDED

Approximately 10-g salt sample in each case.

Sample No.	*GB added (μg)*	*GB found*		*Recovery (%)*
		(μg)	*(ng)*	
1	536.6	430.0		79.9
2	0		<20	–
3	536.6	431.2		80.4
4	0		<20	
5	536.6	408.9		76.2
6	536.6	414.8		77.3
7	536.6	430.4		80.3

tion was allowed to wet the salts and then was shaken thoroughly for a few minutes. Exactly 1 ml of a 536.6 μg/ml solution of GB in chloroform was added to four of these samples and the mixture was shaken thoroughly for an additional 2 min. After allowing the solids to settle, an aliquot of the clear chloroform solution was removed for analyses via GC. The results of these experiments are shown in Table IV.

The results shown in Table IV demonstrate that, if intact GB were occluded within the dried salt structure itself (perhaps, within the lattice network of diisopropyl urea crystals), the TBA-containing chloroform solution could provide a means of extraction of the intact GB for subsequent quantitation.

All attempts to add GB to salts in the absence of significant quantities of TBA resulted in complete loss (*i.e.*, destruction) of the GB.

The same trituration procedure employing 500 μg TBA/ml chloroform was used to analyze a representative portion of the spray-dried salts obtained from a variety of sources which incorporated different drying parameters (*e.g.*, drum dryer, operating temperature changes, fuel changes, etc). The most notable conclusion that can be drawn from the found data was that no GB was found in any of the salt samples examined and that the DIMP values obtained were in a relatively constant range of 12 to 26 μg/g of salt irrespective of the operational parameters employed in the drying procedure.

The same trituration procedure has been used with success in analyzing soil, concrete, and exudate samples associated with GB disposal. The ability to maintain and recover a GB "spike" in these additional applications has been used as a gauge in determining the versatility of the developed method.

The results obtained, using the acid-demand brine procedure when applied to several authentic brine samples, are given in Table V.

In addition, water samples originating from a high-pressure concrete removal system were run to establish the presence of absence of agent to insure safe disposal. In this instance, salt (sodium chloride) was purposefully introduced into the water to aid in extraction of GB, and TBA was added in amounts of 5 mg/ml of water in the sample aliquot. This procedure provided for the effective transfer and concentration of known quantities of GB added to blank samples while insuring the agent's stability.

Bubbler solutions were the least difficult to handle and required only a minimum of sample pretreatment prior to analysis. The simulated bubbler solutions

TABLE V

RESULTS OF BRINE ANALYSES

Brine sample	*GB (ng/g)*	*DIMP (ng/g)*	*Apparent pyroester (ng/g)*	*TBA (mg/ml)*	*DICDI* (μg/g)*
1	15	9	5	1.1	–
2	5	10	1	1.5	–
3	<1	20	<1	1.3	<0.2
4	<1	19	<1	<0.1	2.2
5	≈1	31	<1	6.6	<0.2
6	≈2	18	<1	5.4	<0.2

* Diisopropylcarbodiimide.

TABLE VI

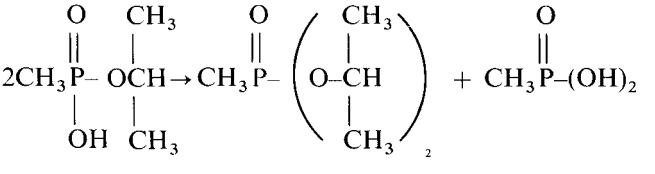
ANALYSES OF KNOWN SOLUTIONS OF GB IN pH 4.5 SULFURIC ACID

Sample	*Quantity of GB (ng)*		*Recovered*
	*Actual**	*Found*	*(%)*
1	96.3	99.3	103.1
2	96.3	98.0	101.8
3	325.0	309.0	95.1
4	192.6	197.0	102.3
5	96.3	96.2	99.9
6	48.2	48.4	100.4
7	96.3	98.9	102.7
8	30.0	27.6	92.0
9	400.0	375.0	93.8
10	96.3	98.1	101.9
11	192.6	190.8	99.1
12	15.0	14.4	96.0
13	48.2	47.0	97.5
14	325.0	304.0	93.0
15	30.0	31.0	103.3
16	400.0	382.0	95.5
17	96.3	100.0	103.8
18	30.0	26.6	88.7

* Corrected to the nanograms expected based on 60% solvent partitioning efficiency.

were dilutions of known amounts of GB in pH 4.5 sulfuric acid solutions. These samples were extracted in the identical manner as the unknown bubbler solutions. The results obtained on a series of the known solutions are listed in Table VI.

Some very interesting aritifacts had been observed in some of our early work[1] using the thermal conductivity detector. It had been found that in-column preparative reactions via disproportionation and condensation did occur and could result in highly misleading information. This becomes especially true and critical when GC is being used for the detection and estimation of nanogram and picogram quantities of substances. IMPA is a hydrolysis product of GB. If not removed during the analytical sample preparation procedure two molecules of IMPA can react to give the disproportionation products DIMP and MPA.



MPA does not elute from the column.

Similarly, it was found that IMPA and HF, if added to the extract, could react in-column to form readily detectable quantities of GB and DIMP. This artifact which could be totally defeating was precluded by following the developed, sample preparation procedure.

Each of the column systems reported here has proved to be highly useful depending on the application areas. The DC-LSX-3-0295 column, for example, was superior to the others in eluting the pyroester of GB. The FFAP column system proved most beneficial in mass spectrometry studies for GB and its associated components with the exception of the pyroester. The Carbowax 20M column was extremely useful when determinations of stabilizer type and quantity were desired. The QF-1 column system showed extremely long-term usefulness and performed satisfactorily in all cases except when TBA was present in gross excess producing a "quenching" effect at the retention time of GB. The Carbowax 20M column was similarly affected by high TBA concentration. The EGSS-X column system reported in the procedure section of this report represented the best all around compromise method for samples from a wide variety of sources. The minimum detectable quantity of GB determined with this system in a variety of different instruments was about 0.3 ng. The accuracy of the method at the 15-ng level was found to be ± 2.2 ng; while the calculated standard deviation at the 100 ng level was ± 1.3.

We have described some misleading artifacts that can occur due to undesired pre-column or in-column preparative reactions that reconstitute molecular species by condensation of their unseparated hydrolysis products. Also observed are new products that are synthesized by in- or on-column disproportionation of residues that have longer retention times in the column. Similar occurrences had been observed by our laboratory in the GC of some arylidinemalononitrile irritants[8] and some esters and amides.

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RAPID AND DIRECT METHOD FOR THE DETERMINATION OF ECDYSTEROID CONJUGATES BY LIQUID CHROMATOGRAPHY

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SUMMARY

The ionic conjugates of ecdysteroids (insect moulting hormones) found in insect ovaries and eggs can be determined quantitatively and quickly by high-pressure liquid chromatography on a reversed-phase system with ultraviolet detection. The mobile phase consists of a gradient of methanol–water containing 0.4 *M* ammonium acetate and tetra-*n*-butylammonium acetate. The conjugates can be determined directly in this way after methanol extraction and two solvent partitions.

INTRODUCTION

The ecdysteroids (insect moulting hormones) are present in large quantities in the ovaries and eggs of insects, both in the free state and as highly polar derivatives[1–5], the latter accounting for 80–90% of the total amount of the hormones in the embryos of several species[2,4–8]. In spite of the relatively large amount of these polar derivatives, their chemical identification has not yet been established. They have been tentatively identified in larvae and eggs as conjugates (sulphates, glycosides, glucuronides or phosphates) on the basis of their electrophoretic behaviour and by enzymic hydrolysis[5–7,9–12]. From our own work[4,5] and that of others[8] it is evident that the conjugates in locust eggs (*Schistocerca gregaria*) consist of anionic derivatives of three ecdysteroids: ecdysone, 2-deoxyecdysone and 20-hydroxyecdysone, which from their enzymic hydrolysis and UV spectra appear to be simple sulphate or phosphate derivatives[5], linked through one of the hydroxyl groups to the ecdysteroids.

A separate group of highly polar ecdysteroid derivatives, not split enzymically, has been reported during late embryogenesis when radioactive cholesterol was injected into adult females *Schistocerca americana*[13]. This group has not been further characterized and is not included in the present study.

The determination of the amounts of the polar ecdysteroid conjugates in ovaries and embryos of different stages of growth is important in the understanding of the physiology of development of insects. At present this is carried out indirectly and laboriously by collecting a polar fraction containing the conjugates by solvent partition or chromatography, then hydrolysing the conjugates with the crude enzyme preparation of the snail *Helix pomatia*, collecting the released ecdysteroids and de-

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termining them directly by high-performance liquid chromatography (HPLC) with UV detection[5,8] or by electron-capture gas chromatography (GC) after derivatisation (refs. 4 and 14).

Because of their ionic character, the conjugates are difficult to analyse directly by liquid chromatography. They are either strongly retained on normal-phase (NP) columns or eluted rapidly in reversed-phase (RP) systems, as broad peaks and poorly separated[15]. Several approaches have been described for separating them as a group from other biological compounds; these include TLC[12,16], reversed-phase chromatography on Amberlite XAD-2[7], column chromatography on DEAE-Sephadex[17] and on silicic acid[8]. Lafont and co-workers have improved their retention and resolution in RP-HPLC systems by buffering the aqueous part of the mobile phase to acid[15] or alkaline[15,16] pH. Another useful technique in handling very polar compounds in reversed phase is to use an aqueous buffered medium containing a large counter-ion (ion-pair chromatography[18,19]). Although all these techniques have been applied in the separation of conjugates, there have been no reports as yet on their use for direct separation, identification and quantification of conjugates, present in insect material.

We have devised a method for the rapid qualitative and quantitative analysis of the conjugates in the eggs of the desert locust (*Schistocerca gregaria*). A methanol extract of the eggs is subjected to two solvent partitions (methanol–water–hexane, and *n*-butanol–water), followed by concentration of the sample and direct ion-paired reversed-phase high-performance liquid chromatography (briefly RP-IPC), on ODS Spherisorb, eluting with a buffered aqueous–methanol gradient. The conjugates, baseline separated, are then quantified by UV monitoring of the effluent.

EXPERIMENTAL

Eggs

Egg pods were obtained from a colony of *Schistocerca gregaria* Forskal reared as previously described[20]. Under the conditions chosen for the incubation of the eggs it required 13 days from oviposition to the hatching of the first instar *Schistocerca* larvae.

Sample preparation

Each batch of eggs (2 g) was ground in methanol in a glazed mortar and the resulting slurry filtered through a sintered glass filter (porosity 3). The filtrate was partitioned between hexane and methanol–water (8:2, v/v), to remove non-polar impurities. The residue from the aqueous methanol phase was then partitioned between countersaturated *n*-butanol and water. Each fraction was backwashed with a small volume of the appropriate counter phase which was added to the main fraction. The free ecdysteroids are partitioned into the butanol phase and the polar conjugates into the aqueous phase which is reduced to dryness *in vacuo* at 40°C. The residue obtained was redissolved in a known volume of water (500 μl) and a portion of this solution (5 μl) was injected into a ODS Spherisorb HPLC column and the eluate monitored by UV detection at 244 nm.

HPLC analyses

The HPLC equipment consists of a LC3XP liquid chromatograph pump (Pye

Unicam, Cambridge, Great Britain) connected to a Pye Unicam gradient elution system, a sample injection valve (Rheodyne, Cotati, U.S.A.) and a Pye Unicam LC-UV variable-wavelength absorbance detector set at 244 nm.

HPLC-grade methanol (Fisons, Loughborough, Great Britain) and glass distilled water were used throughout and degassed by a constant stream of helium.

The analysis was carried out on a 10 cm × 5 mm I.D. column (Shandon Southern, Runcorn, Great Britain) packed with 5-μm particles of ODS Spherisorb (Phase Separations, Clwyd, Great Britain) and eluted with a linear gradient (4% per minute) from 25% to 50% methanol in 0.4 *M* ammonium acetate buffer (pH 7) at a flow-rate of 1 ml/min. An amount of tetrabutylammonium hydroxide (TBA, 0.8 mg/ml) was added to the buffer solution to obtain a concentration of 0.003 *M* of counter ion in the mobile phase. The separation was performed at room temperature (21 ± 1°C).

Quantification was obtained by comparing peak area with a standard curve of peak area against quantity in ng of pure ecdysone (Simes, Milan, Italy), 20-hydroxyecdysone (Simes) and 2-deoxyecdysone (D.H.S. Horn, Melbourne, Australia). For accuracy, comparison must be made between standard and unknown at similar retention volumes, since sensitivity decreases with increasing retention.

For the analysis of the free ecdysteroids two different types of columns were used:

(a) a reversed-phase column 25 cm × 5 mm I.D. packed with 5-μm particles of Hypersil ODS (Shandon Southern) was eluted under isocratic conditions (methanol–water, 60:40, v/v) at a flow-rate of 0.8 ml/min, and

(b) a normal-phase column 25 cm × 5 mm I.D. packed with 5-μm particles of Hypersil (Shandon Southern) was run under isocratic conditions (methylene chloride–isopropanol–water, 125:25:2) at a flow-rate of 1 ml/min. The amount of free ecdysteroids was calculated from standard curves as above.

Comparison of the direct RP-IPC and enzymic hydrolysis methods

The aqueous phase containing ecdysteroid conjugates from eggs after the butanol–water partition, was divided in two equal portions, which were evaporated to dryness. One of them was redissolved in water and analysed for conjugates by RP-IPC, the other portion was taken up in 5 ml of 100 m*M* acetate buffer (pH 5.2) and subjected to overnight enzymatic hydrolysis at 37°C in the presence of sufficient amount of the digestive juice of the snail *Helix pomatia* (10 μl/ml) (Koch-Light Labs., Colnbrook, Great Britain) to give 8000 Roy units per ml of aryl sulphatase, 1000 Fishman units per ml of β-glucuronidase and 200 Sigma units per ml of acid phosphatase. The ecdysteroids, freed by the enzymatic hydrolysis, were purified as follows: the incubation medium was injected into a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) and eluted successively with 5 ml of methanol–water (20:80, v/v) and 7 ml of methanol–water (80:20, v/v). All the different ecdysteroids present in the egg extract were eluted in the methanol–water (80:20) fraction, which was directly analysed for the quantification of the hormones by RP-HPLC with UV monitoring of the eluent from the column.

Identification of the ecdysteroids released from conjugates

The column eluate corresponding to each of the conjugate peaks in RP-IPC

was collected separately under isocratic conditions. The solvent was removed by freeze-drying and the residue redissolved in 100 m*M* acetate buffer (5 ml, pH 5.2), hydrolysed with *Helix pomatia* juice, purified, as described in the previous section, and analysed for ecdysteroids by RP-HPLC and NP-HPLC.

The identification of the individual hormones obtained in this way was confirmed by co-chromatography with the authentic compound on the two different chromatographic systems used, *i.e.* RP-HPLC and NP-HPLC.

RESULTS

The RP-IPC analysis of the hydrolysable conjugates present during embryonic development of *S. gregaria*, was carried out on 1 day old eggs and 11 day old eggs (*i.e.* towards the end of embryogenesis). Each batch of egg pods was extracted and subjected to two successive solvent partitions (as described in the Experimental section). The aqueous layer from the latter partition system was suitable for HPLC analysis without further purification. The conjugates present were separated by RP-IPC and quantified by recording the UV absorbance of the effluent from the column. A typical RP-IPC trace of 1 day old egg extract is shown in Fig. 1.

To verify that this method for the rapid determination of the levels of conjugates in the eggs of *S. gregaria* gave results consistent with the previously used

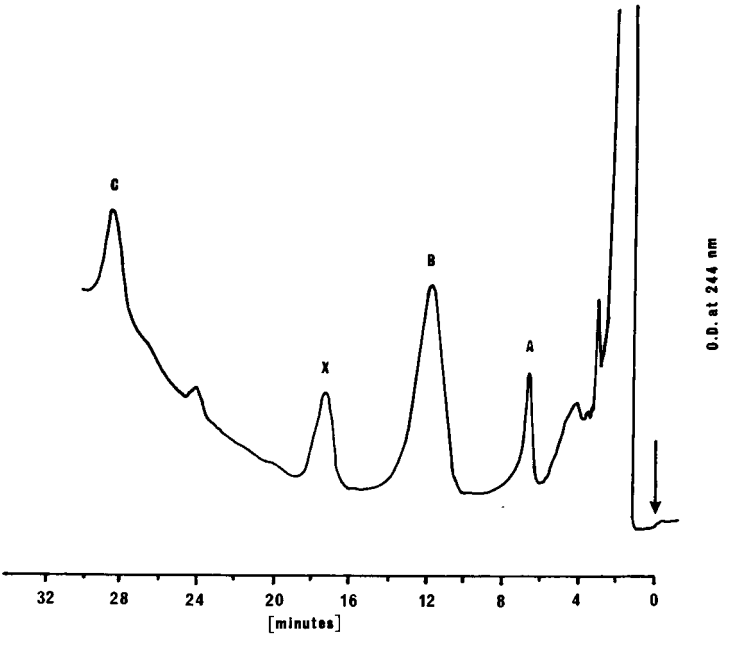

Fig. 1. Typical RP-IPC separation of conjugated ecdysteroids from 1 day old eggs (2 g), using TBA (0.003 *M*). Operating conditions: 10 cm × 5 mm I.D. column of Spherisorb-ODS. Primary solvent: 25% methanol in 40 m*M* ammonium acetate buffer (pH 7). Secondary solvent: 50% methanol in 40 m*M* ammonium acetate buffer (pH 7). Linear gradient: 0 to 100% secondary solvent in 25 min. Flow-rate: 1 ml/min. Peaks: A = conjugated 20-hydroxyecdysone; B = conjugated ecdysone; C = conjugated 2-deoxyecdysone; X = unknown compound, not ecdysteroidal.

TABLE I

COMPARISON OF RESULTS OBTAINED ON 1-DAY-OLD *SCHISTOCERCA* EGGS BY DIRECT RP-IPC METHOD AND BY CHROMATOGRAPHY AFTER HYDROLYSIS

Quantity of ecdysteroids present as conjugates.

Compound	*Direct chromatography (ng/egg)*	*After hydrolysis (ng/egg)*
Ecdysone	382	384
2-Deoxyecdysone	230	218
20-Hydroxyecdysone	33	40

enzymic hydrolysis, coupled with RP-HPLC analysis[5,8], the two methods were compared on the same sample of eggs. The values obtained are given in Table I. The results shown give good agreement between the methods and prove the validity of the RP-IPC titre determination of the hydrolysable conjugates. The limit of detection of the RP-IPC method is the conjugate equivalent to approximately 10 ng of ecdysteroid at a sensitivity of 0.005 absorbance units.

The identity of the compounds separated by RP-IPC analysis was established by collecting the different UV absorbing peaks from the column and then hydrolysing and analysing them on RP-HPLC and NP-HPLC, as described in the Experimental section.

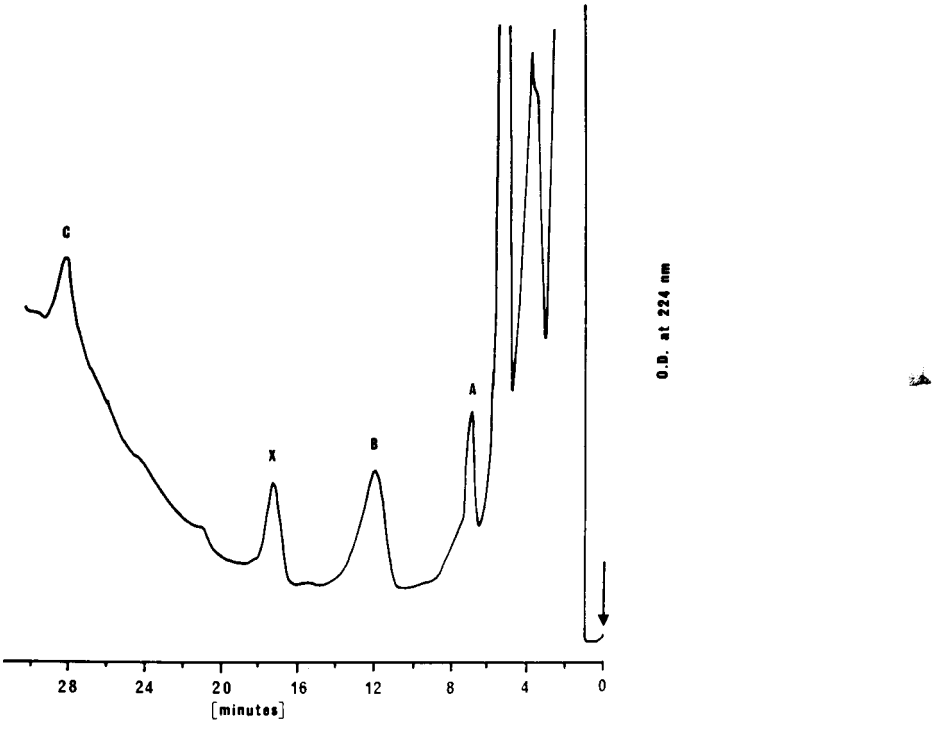

Fig. 2. Typical RP-IPC separation of conjugated ecdysteroids from 11-day-old eggs (2 g). All conditions as in Fig. 1.

After hydrolysis with *Helix pomatia* digestive juice, peak A (Fig. 1) gave 20-hydroxyecdysone, peak B ecdysone, and peak C 2-deoxyecdysone. The amount of ecdysteroid obtained by collecting the effluent, corresponding to each peak in Fig. 1, and then hydrolysing it, corresponded to an efficiency of 82–91 % of the amount expected from the same sample of eggs, when the sample was directly hydrolysed and ecdysteroids determined by HPLC with UV detection.

When a sample of 11 day old eggs was subjected to the RP-IPC analysis, the chromatographic pattern obtained was similar to the 1 day old eggs, with peaks at the same retention volume (Fig. 2), showing that the same hydrolysable conjugates are present at the beginning and at the end of embryogenesis. Moreover, at this time too, good agreement was found between titre determination by our method of RP-IPC and by enzymic hydrolysis coupled with RP-HPLC analysis.

The separation and analysis can also be carried out without added TBA, in which case the peaks are less well resolved (Fig. 3). No deterioration of the column due to TBA was noticed over several weeks of continuous use at pH 7. At more basic pH other authors have found the column performance deteriorated[23,24]. The concentration of buffer and TBA were not found to be critical. The appearance of the chromatogram was not altered if the buffer was changed from 0.4 *M* to 0.04 *M* and the TBA from 0.003 *M* to 0.0015 *M*.

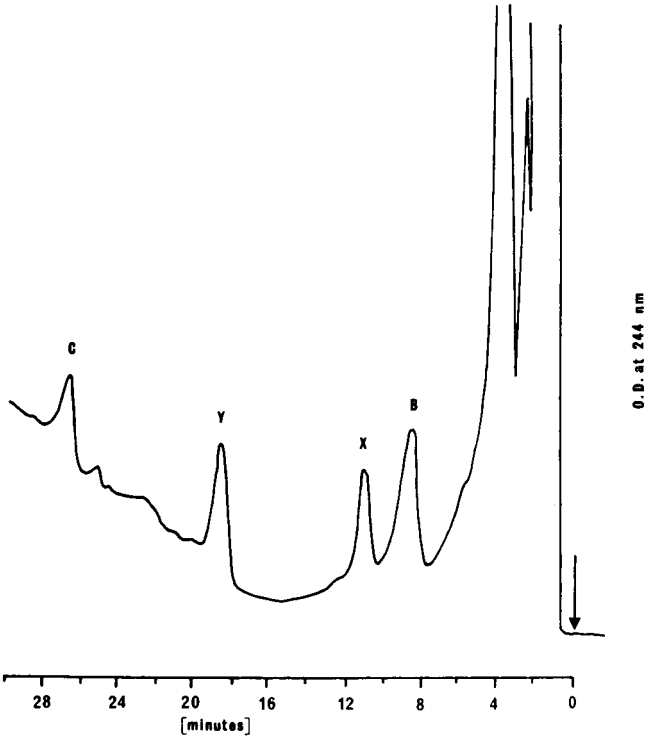

Fig. 3. Separation of conjugated ecdysteroids from 11-day-old eggs (2 g), without TBA in the mobile phase. All other conditions as in Fig. 1. 20-Hydroxyecdysone is not resolved with these conditions. Y = an unidentified substance only appearing in the absence of TBA.

DISCUSSION

The analytical methods used, up to now, for the micro-determination of the ecdysteroids in biological material cannot be applied directly to measure the titre of the polar conjugates of ecdysteroids; they are difficult to analyse by liquid chromatography, inactive in bioassays, and also poorly immunoreactive[21]. Furthermore the radio-immunoassay cannot discriminate between different compounds and it has produced inconsistent results for the determination of the amount of conjugates in 1 day old eggs of *Locusta migratoria*[2,22]. Therefore, quantitative analysis of these substances has been mainly performed on the free hormones released from the conjugates by enzymic hydrolysis. The hydrolysis step not only adds to the time per analysis, but also it causes longer sample manipulation and represents a further source of possible errors as shown in the investigation by Gande and Morgan[4], on the levels of conjugates in eggs of *S. gregaria*. The values reported in that paper were much lower than the quantities present *in vivo*, because of incomplete hydrolysis of the conjugated ecdysteroids[5].

In order to overcome the difficulties connected with the previous analysis of conjugates, a new and rapid method for the separation and quantification of these substances in the eggs of *S. gregaria* has been devised. After the extraction from the eggs and a preliminary purification involving two solvent partition steps, the ionized conjugates were separated as ion-pairs in a single pass through a C_{18} bonded phase column with a solvent gradient. The compounds were detected and quantified by UV monitoring of the eluent. Because UV absorbing impurities are usually present in the biological extracts, UV detection is poorly specific. The 20-hydroxyecdysone peak is associated with, and the ecdysone peak followed by, UV-absorbing substances which are not ecdysteroids. Nevertheless, the method permits accurate and rapid quantification of the substances, suitable for routine analysis.

The level of the conjugates ecdysteroids have been measured (as in the Experimental section) by a standard curve obtained with known amounts of free ecdysteroids. This indicates that the conjugating ionic group itself does not absorb in the ultraviolet region, nor does its attachment involve the unsaturated ketone chromophore.

Furthermore the analysis of eggs of different age suggests that the same hydrolysable conjugates are present at the beginning and at the end of the embryogenesis.

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Note

Separation of homologous series on pre-adsorbed layers

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In a previous communication[1] it was shown that the pre-adsorption of organic vapours on silica gel layers determines the behaviour of organic compounds, all other experimental circumstances being constant. The present paper deals with the separation of homologues on such layers. This form of chromatography has the advantage of both the mobile and stationary phases being volatile liquids. Separated compounds eluted from such layers do not contain any non-volatile components from the chromatographic system and are therefore amenable to identification by infrared or mass spectrometry.

EXPERIMENTAL

The following series were tested: phenylazophenacyl esters of fatty acids, 2,4-dinitrophenylhydrazones of n-alkanals, 2,4 dinitrophenylhydrazones of n-alkenals, 2,4 dinitro-alkylanilides. These derivatives were prepared according to current methods[2].

The following types of silica gel layers were used: silica gel type 60 (E. Merck), silica gel for column chromatography (sieved), silica gel D-O (Camag), silica gel IB-HP_{254} (J. T. Baker). Layers were prepared using the Desaga equipment. Pre-adsorption was effected by placing a sheet of filter paper against the wall of the developing tank. The filter paper was thoroughly wetted with the liquid whose vapours were to be absorbed onto the silica gel layer. The spotted plate was allowed to equilibrate with the organic vapour for *ca.* 5 min. Subsequently, hexane was added carefully to the bottom of the tank, immediately followed by the development. In some cases multiple development was applied in which the plate was taken out of the tank after the first run and the mobile phase was allowed to evaporate for a few minutes. Then the plate was developed again in the same solvent; this procedure can be repeated n times. All ${}^{n}R_F$ values were reduced to ${}^{1}R_F$ values according to the formula

$$ {}^{1}R_F = 1 - \sqrt[n]{1 - {}^{n}R_F} $$

In order to obtain constant R_F values (R_F^c, ref. 3) a mixture of *p*-aminoazobenzene, dimethyl yellow and azobenzene was spotted on at least three different places on the plate. The correction equation of Van Wendel de Joode

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$$\frac{1}{R_{Fi}} = \frac{a}{R_{Fj}} + b,$$

in which R_{Fi} stands for the R_F value of a compound on chromatogram i and R_{Fj} for the same compound on chromatogram j, was applied to all R_F values[4]. The R_F^0 values for the reference compounds were

$$R^0_{F(p\text{-aminoazobenzene})} = 0.041,\ R^0_{F(\text{dimethyl yellow})} = 0.512,\ R^0_{F(\text{azobenzene})} = 0.828.$$

RESULTS AND DISCUSSION

In order to obtain sufficient separation, multiple development was found to be necessary in the systems silica gel–acetonitrile–hexane and silica gel–nitromethane–hexane. The number of developments could be reduced by preparing layers from mixtures of silica gel and Kieselguhr G (E. Merck). The experiments on mixed layers (Fig. 1) showed that the addition of Kieselguhr to the silica gel layer results in a set of

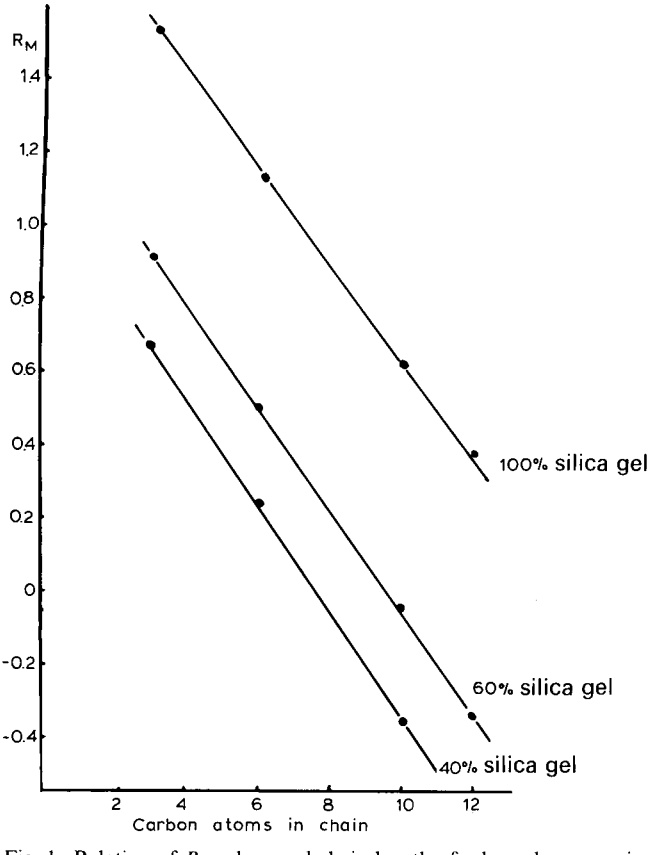

Fig. 1. Relation of R_F values and chain length of a homologous series on silica gel layers and on layers containing mixtures of silica gel with Kieselguhr G, test compounds 2,4-dinitrophenylhydrazones of *n*-alkanals. System: silica gel–acetonitrile–hexane.

parallel lines when R_M is plotted against chain length. In these experiments acetonitrile was used as the impregnating vapour and hexane as the solvent. Constant R_F values were obtained with Van Wendel de Joode's formula, irrespective of the amount of Kieselguhr in the layer. The results of some of these experiments are summarized in Table I.

TABLE I

R_F AND R_F^c VALUES ON SILICA GEL LAYERS OF DIFFERENT ORIGIN AND WITH DIFFERENT AMOUNTS OF SILICA GEL

$n = 5$.

Compound	$R_F \pm$ S.D.	$R_F^c \pm$ S.D.
Dimethyl yellow	0.600 ± 0.104	0.512 ± 0.014
propanal 2,4-dinitrophenylhydrazone	0.133 ± 0.044	0.073 ± 0.005
pentanal 2,4-dinitrophenylhydrazone	0.208 ± 0.071	0.154 ± 0.011
nonanal 2,4-dinitrophenylhydrazone	0.490 ± 0.120	0.405 ± 0.024

The figures show that the Van Wendel de Joode formula can be applied in this case. The R_F values collected during the last few years were obtained on silica gel layers of different origin (*cf.* Experimental section) having different ratios of silica gel: Kieselguhr and being usually run in multiple development. After calculating the ^1R_F values, the Van Wendel de Joode equation was applied, using the R_F^0 values of *p*-aminoazobenzene and azobenzene mentioned above. Some of the results obtained with the homologous series of *n*-alkanal 2,4-dinitrophenylhydrazones are given in Table II. Although these R_F values were obtained under widely different conditions, the standard deviations of the mean R_F^c values did not exceed 0.04 R_F. The regression equations for a number of homologous series in the systems silica gel–acetonitrile–hexane and silica gel–nitromethane–hexane are summarized in Table III.

TABLE II

MEANS AND STANDARD DEVIATIONS OF CORRECTED R_F VALUES (R_F^c) IN THE SILICA GEL–ACETONITRILE–HEXANE SYSTEM

Alkanal 2,4-dinitrophenylhydrazone	$R_F^c \pm$ S.D.	n*
Ethanal	0.035 ± 0.011	10
Propanal	0.073 ± 0.006	13
Butanal	0.111 ± 0.011	13
Pentanal	0.154 ± 0.020	12
Hexanal	0.205 ± 0.026	14
Heptanal	0.261 ± 0.025	7
Octanal	0.331 ± 0.033	13
Nonanal	0.405 ± 0.040	11
Decanal	0.494 ± 0.039	13
Undecanal	0.546 ± 0.020	7
Dodecanal	0.620 ± 0.037	12

* Mean of R_F values.

TABLE III

REGRESSION EQUATIONS FOR SOME HOMOLOGOUS SERIES

Systems: 1 = silica gel–acetonitrile–hexane; 2 = silica gel–nitromethane–hexane

Series	*Type of silica gel (cf. Experimental section)*			
	I	*II*	*III*	*IV*
Phenylazophenacyl esters of *n*-fatty acids	1 $R_M = -0.156C + 1.39$	–	$R_M = -0.160C + 1.33$	–
	2 $R_M = -0.190C + 1.48$	$R_M = -0.191 + 1.49$	$R_M = -0.182 + 1.52$	–
2,4-Dinitrophenylhydrazones of *n*-alkanals	1 $R_M = -0.149C + 1.34$	$R_M = -0.150C + 1.44$	$R_M = -0.152C + 1.43$	$R_M = -0.148C + 1.31$
	2 $R_M = -0.167C + 1.71$	$R_M = -0.172C + 1.84$	–	
2,4-Dinitroalkylanilides	1 $R_M = -0.150C + 1.69$	–	–	$R_M = -0.150C + 1.51$
	2 –	–	–	–
2,4-Dinitrophenylhydrazones of *n*-alkenals	1 $R_M = -0.152C + 1.55$	$R_M = -0.150C + 1.51$	$R_M = -0.148C + 1.50$	–
	2 –	$R_M = -0.162C + 1.94$	$R_M = -0.160C + 1.95$	–

The homologous increment obtained under these conditions remains markedly constant, the mean value for the system containing acetonitrile being −0.151 (S.D. = 0.004) and for the system containing nitromethane −0.175 (S.D. = 0.013). The values of the first two members of each series were not incorporated in the calculations.

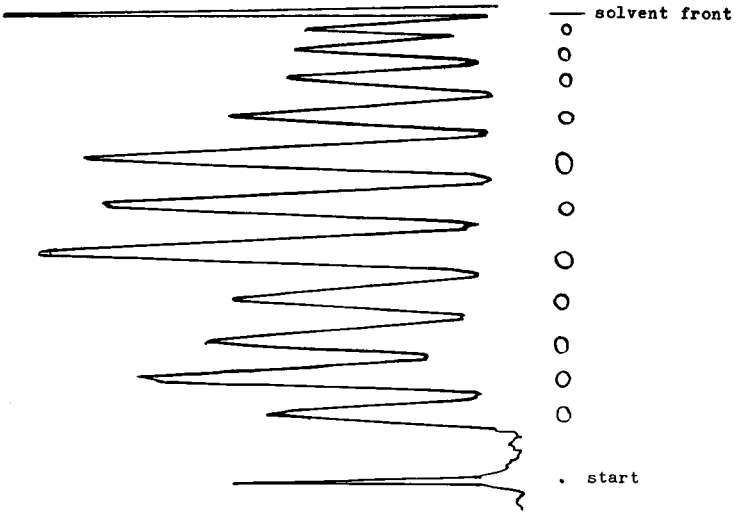

Fig. 2. Separation of a mixture of *n*-alkanal 2,4 dinitrophenylhydrazones. Layer: mixture of 50% silica gel and 50% Kieselguhr G pre-adsorbed with acetonitrile. Two developments in hexane. Distance from start to solvent front: 10 cm. Densitogram: reflection at 450 nm.

CONCLUSION

Homologous series can be separated into their components on silica gel layers pre-adsorbed with either acetonitrile or nitromethane vapour. After elution of the components from the layers with a suitable solvent, the eluted fraction does not contain any non-volatiles from the system.

The corrected R_F values remain fairly constant, even under the widely different conditions applied. It is to be expected that more standardized conditions will result in an increased reproducibility of the corrected R_F values.

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Note

Procedure for the chiral derivatization and chromatographic resolution of *R*-(+)- and *S*-(−)-propranolol

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Propranolol [1-isopropylamino-3-(1-naphthoxy)-2-propanol] (Fig. 1) is an important beta adrenergic blocking agent which has gained widespread usage in the treatment of angina pectoris, cardiac dysrhythmias and hypertension. Currently the commercially available preparation is a racemic mixture of which only the *S*-(−)-enantiomer has beta adrenergic blocking activity[1] while the *R*-(+)-enantiomer has only a membrane stabilizing effect[2]. These isomers were resolved on a preparative scale by repetitive recrystallizations of the dibenzoyl tartaric acid salts[3]. Analysis of each enantiomer in biological fluids has been accomplished by administering a 1:1 mixture of a deuterium-labeled enantiomer and the unlabeled opposite enantiomer and measuring the concentrations of these isomers by gas chromatographic–mass spectrometric (GC–MS) techniques[4]. This method, however, requires the prior availability of the pure enantiomers and the preparation of deuterium-labeled propranolol. A simpler method involves derivatization with an optically pure chiral reagent and the chromatographic separation of the resulting diastereomers. This technique, employing N-trifluoroacetyl-*S*-(−)-prolyl chloride[5], has been used to quantitate each enantiomer of propranolol by GC[6] and high-performance liquid chromatography (HPLC)[7,8] following administration of the racemic drug. Several investigators have noted, however, that this chiral reagent can racemize during storage[5,8].

We have recently examined the applicability of an alternative chiral reagent, *R*-(+)- or *S*-(−)-1-phenylethyl isocyanate[9,*] for converting racemic propranolol to diastereomeric derivatives which can be resolved chromatographically. Each pure

* The Aldrich Chemical Co. catalog (1981–82, p. 629) lists these compounds as *R*-(−)- and *S*-(+)-, with optical rotations measured on the neat liquid. It is clear from previous work[10] that these absolute configurations should be reversed, *i.e.*, the negative rotating enantiomer has the *S* configuration and the positive rotating enantiomer has the *R* configuration.

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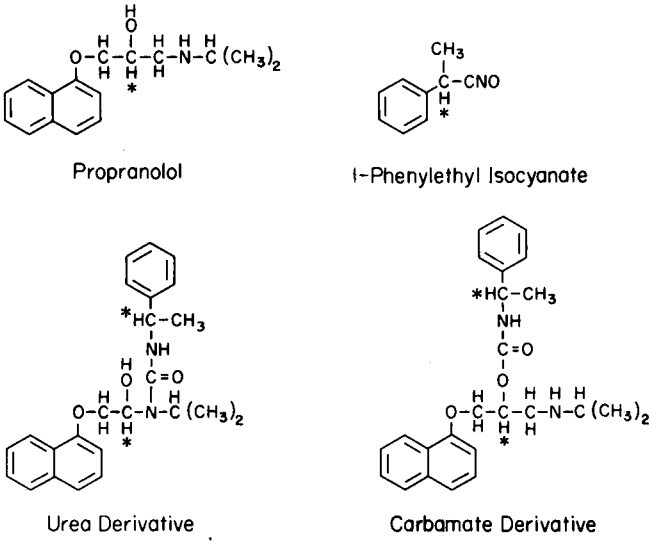

Fig. 1. Structure of propranolol, 1-phenylethyl isocyanate and the possible urea and carbamate derivatives.

enantiomer of 1-phenylethyl isocyanate is commercially available and has been employed for the GC separations of the enantiomers of chiral secondary alcohols after formation of carbamate derivatives[10,11]. Racemization, as described above, does not occur with this reagent and it is chemically quite stable.

In the current study we have found that the reaction with propranolol occurs rapidly at 25°C to form a urea derivative with the secondary amino group of the drug (Fig. 1). The diastereomers have been separated in gram quantities by silica gel chromatography and fully characterized. Subsequent hydrolysis of these isomers produces the optically pure enantiomers of propranolol, thereby providing a method for resolving racemic propranolol without repetitive recrystallizations. This method of resolution is applicable to quantities from the microgram to the gram range. Conditions for GC and HPLC separation have also been developed which demonstrate the potential for using this chiral reagent for micro-scale analyses of the enantiomers of propranolol and of other beta adrenergic blocking drugs in biological fluids.

MATERIALS AND METHODS

R- and *S*-1-phenylethyl isocyanate and *R,S*(+)-propranolol were purchased from Aldrich (Milwaukee, WI, U.S.A.). *R*-(+)- and *S*-(−)-propranolol standards were prepared by the method of Yost and Holtzman[3]. Silica gel (Merck, mesh) was purchased from J. T. Baker (Phillipsburgh, NJ, U.S.A.). Thin-layer chromatography (TLC) plates LK5DF were obtained from Whatman and plastic plates from Kodak (Rochester, NY, U.S.A.). Solvents were ACS reagent grade. Hexamethyldisilazane and trimethylchlorosilane were purchased from Pierce (Rockford, IL, U.S.A.).

Equipment

Infrared (IR) spectra were obtained on a Perkin-Elmer 237B spectrometer. Proton nuclear magnetic resonance (NMR) spectra were obtained at 360 MHz on a

Brucker WH-360 spectrometer at the Middle Atlantic Regional NMR Facility at the University of Pennsylvania*. High-performance liquid chromatography was performed with an Altex Model 110 pump (Beckman, Berkeley, CA, U.S.A.) using a Spherisorb C_{18} 5-μm column (Regis, Chicago, IL, U.S.A.) (25 × 0.46 cm). Peaks were detected with Kratos SF970 fluorometer with a 220-nm excitation and a >340-nm filter.

GC and GC–MS

GC analysis was performed on a Hewlett-Packard (HP) 5710 gas chromatograph equipped with 0.8 m × 2 mm I.D. glass column packed with 5% OV-22 on Supelcoport (80–100 mesh; Supelco, Bellefonte, PA, U.S.A.). Conditions were: helium flow-rate, 30 ml/min; injector temperature, 250°C, and column temperature programmed from 250 to 280°C at 4°C/min and held at the upper temperature. The GC was interfaced via a glass jet separator to an HP 5980 A mass spectrometer and Model 5934A data system. The transfer line was held at 280°C and the ion source at 180°C. The mass spectrometer was scanned repetitively from 50 to 500 a.m.u. using an ionizing energy of 70 eV. Capillary analysis was performed on a HP 5710 gas chromatograph equipped with a Model 18740B capillary inlet system, a nitrogen–phosphorus detector and a 12.5 × 0.2 mm I.D. fused-silica column coated with SP 2100 and deactivated with Carbowax 20M (Hewlett-Packard). The operating conditions were: injector temperature, 250°C; detector temperature, 250°C; and the column temperature was programmed from 230°C to 250°C at 4°C/min and held at the upper temperature.

The diastereomeric propranolol derivatives were trimethylsilylated by treatment with 100 μl of hexamethyldisilazane, 20 μl of trimethylchlorosilane and 100 μl of pyridine at 25°C for 8 h. The resulting derivative was analyzed by GC–MS using the packed column conditions described above. Although no molecular ion was present, M − 15 (*m/z* 463) and M − 90 (*m/z* 388) ions were produced as expected for trimethylsilyl (TMS) derivatives. Other characteristic ions include: *m/z* 373 corresponding to the loss of $\cdot CH(C_6H_5)CH_3$ from M+; *m/z* 359 corresponding to the loss of $NH = C(C_6H_5)CH_3$; and *m/z* 335 corresponding to the loss of the naphthyloxyl group.

RESULTS

Preparation of derivatives of propranolol

Propranolol free base (0.1 g, 0.39 mmol) dissolved in chloroform (30 ml) reacted quickly and quantitatively at room temperature with *R*-(+)-1-phenylethyl isocyanate (40 mg, 0.3 mmol). The chloroform mixture was extracted with 0.1 *M* HCl (50 ml). The pH of the aqueous phase was adjusted to neutrality with 10 *M* NaOH and extracted with chloroform (40 ml). The extract was chromatographed on plastic thin-layer plates developed with acetone. There was a small propranolol spot (R_F 0.24) but no derivative was found (R_F 0.77). Thin-layer chromatography of the reaction mixture on LK5DF plates with development in acetone–acetic acid–water (81:1:1) showed no propranolol (R_F 0.15) or 1-phenylethylamine (R_F 0.26) formed

* The Middle Atlantic NMR Facility is supported by the United States Public Health Service Grant RR 542.

TABLE I

ELUTION OF *R*-(+)- AND *S*-(−)-PROPRANOLOL DERIVATIVE FORMED WITH *R*-(+)- AND *S*-(−)-1-PHENYLETHYL ISOCYANATE FROM A SILICA GEL COLUMN

Volume (ml)	*Propranolol derivative observed*
0– 65	None
65– 87	*R*-(+)-
87– 95	*R*-(+)- + *S*-(−)-
95–127	*S*-(−)-

Column: silica gel (Merck, mesh), 260 × 0.9 cm, eluted with chloroform. The propranolol derivatives were determined by TLC on LK5DF plates developed in chloroform.

from the hydrolysis of the *R*-(+)-1-phenylethyl isocyanate. When LK5DF plates were developed three times in chloroform, the reaction mixture showed a derivative of *R*-(+)-propranolol (R_F 0.067, after three developments R_F = 0.20) and S-(−)-propranolol (R_F 0.053, after three developments R_F = 0.16).

Since the product was not acid extractable, it would indicate that the isocyanate forms a N-substituted urea with the 1-amino group rather than a carbamate with the 2-hydroxyl (Fig. 1).

Three hundred milligrams of the two derivatives were separated on a silica gel column (0.9 × 60 cm) using chloroform as the solvent and following the elution with TLC (Table I). The small region of overlap was discarded.

The IR spectra of the mixture of derivatives and the individual derivatives showed a broad absorption from 1740 cm^{-1} to 1690 cm^{-1}. This region encompasses both expected carbonyl stretching bands for either a carbamate or urea.

The NMR spectrum of the mixture in chloroform shows six doublets of equal intensity in the methyl region, each with J = 7 Hz. The spectrum of each of the separated derivatives had three methyl doublets [*R*-(+)-propranolol (1) (δ 1.49, 1.25,

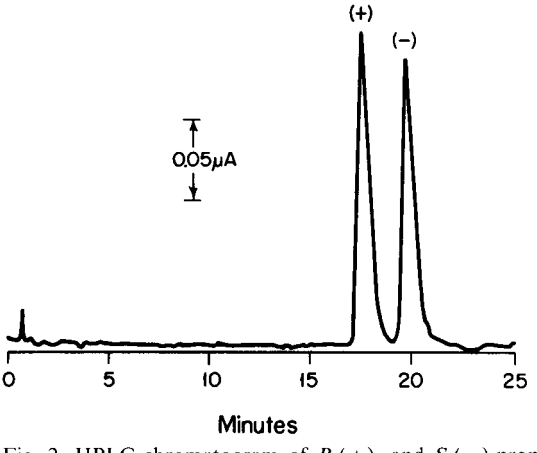

Fig. 2. HPLC chromatogram of *R*-(+)- and *S*-(−)-propranolol derivatized with *R*-(+)-1-phenylethyl isocyanate. The column is a Spherisorb 5-μm C_{18} with an Altex Model 110 pump. The solvent is methanol–water (65:35) runs at 3.2 ml/min (4300 p.s.i.). The detector is a Kratos SF 970 (excitation 220 nm; emission greater than 340 nm).

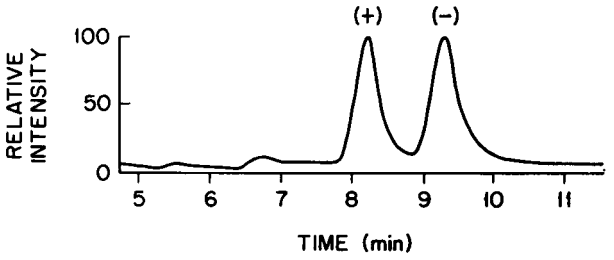

Fig. 3. Total ion current chromatogram from the GC–MS analysis of *R*-(+)- and *S*-(−)-propranolol derivatized with *R*-(+)-1-phenylethyl isocyanate. The column was a 0.8 m × 2 mm I.D. glass column packed with 5% OV-22 on Supelcoport in a HP 5984A GC–MS system. The column was programmed from 250 to 280°C.

1.30), *S*-(−)-propranolol (2) (δ 1.46, 1.27, 1.14)]. The presence of three methyl resonances for each isomer would suggest that the individual methyl groups of the N-isopropyl group were non-equivalent. This is consistent with a urea derivative in which the bulky substituent hinders free rotation.

Chromatographic separation of propranolol derivatives

HPLC of unpurified reaction mixture on C_{18} reversed phase with methanol–water (65:35) at 3.2 ml/min showed two peaks with retention times of 17 min for D-propranolol and 20 min for L-propranolol with a clear baseline separation (Fig. 2). Unreacted propranolol in this system has a retention time of 25 min. The addition of 10–1000 fold excess isocyanate did not affect this pattern, indicating that the carbamate is not formed even when the derivatizing agent is in great excess.

The diastereomeric derivatives of propranolol were separated by GC. The peaks were resolved (resolution, $R_s = 1.49$) in about 10 min using a short packed column as shown in Fig. 3. Further work demonstrated that the peak shape could be greatly improved and the analysis time considerably shortened by using a fused silica capillary column ($R_s = 4.15$). Trimethylsilylation of the free hydroxyl group of the propranolol derivatives yielded diastereomers which eluted from the packed column as one peak with a retention time slightly longer than that of the non-silylated diastereomers.

Hydrolysis of the derivative

The derivatives were hydrolyzed by reflux in 1 *M* H_2SO_4 with 50% recovery of D- and L-propranolol as determined by HPLC.

DISCUSSION

The only previously reported applications of resolution of racemic mixture using the optically pure enantiomers of 1-phenylethyl isocyanate involved analyses of chiral secondary alcohols which form carbamates[10,11]. In the present work, we have shown that a chiral amine can also be derivatized using this reagent. In the case of propranolol, with both secondary hydroxyl and amino groups, the reagents react exclusively with the amino group under the conditions specified even with a 100-fold excess of reagent. The resulting diastereomeric urea derivatives are easily separated

by low-resolution liquid and gas chromatographic methods. The magnitude of the difference in chromatographic properties of the diastereomers is surprising, since there are four atoms separating the chiral centers and these are not included in a ring system. Conversion of the free hydroxy group to a TNS ether drastically reduces the physical differences between these diastereomers as evidenced by the fact that a single GC peak was obtained. On the basis of this information, one can speculate that intramolecular hydrogen bonding between the hydroxyl group and the oxygen of the carbonyl group plays a role in enhancing the physical differences between the diastereomers.

In summary, we have shown that diastereomeric urea derivatives are formed between racemic propranolol and *S*-(−)-1-phenylethyl isocyanate. This chiral reagent can be used to resolve the enantiomers of propranolol by preparative liquid chromatography, followed by acidic hydrolysis of the derivatives. Furthermore, the reagent can be used for the analyses of the propranolol enantiomers on a micro scale by HPLC or GC techniques.

This method is potentially adaptable to the quantitation of the enantiomers in biological fluids and represents a possible convenient alternative to the use of N-trifluoroacetyl-*S*-(−)-prolyl chloride for this purpose. Studies along these lines are currently in progress.

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Note

Adsorption chromatographic separation of testosterone-3-(O-carboxymethyl)oxime tyrosine methyl ester and its ^{125}I-labelled derivative

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As shown previously, iodine-substituted low-molecular-weight substances are retarded on Sephadex LH-20 dextran gel as compared with the parent unsubstituted molecules. The enchanced adsorption of radioiodine-labelled iodothyronines[1,2], 2,3,5-triiodobenzoic acid[3], prostaglandin tyrosine methyl esters[4] and progresterone succinyl tyrosine methyl ester[5] as compared with their parent molecules makes possible the separation of the radioiodine-labelled and unlabelled molecules.

Systematic investigations showed that the distribution coefficient, k, of the radioiodine-labelled substances varies with the organic solvent concentration of the eluent according to

$$\log k = \log k_0 - n \cdot \log S \qquad (1)$$

where S is the concentration of the organic solvent in the binary eluent, k_0 and n are constants for a given binary eluent and iodo compound. Eqn. 1 enables the adjustment of the distribution coefficient of the radioiodine-labelled compounds to any desired value, and removes the need for preliminary investigations aimed at the empirical selection of the optimum composition of binary or ternary eluents.

As anticipated, both radioiodine-labelled and unlabelled testosterone-3-(O-carboxymethyl)oxime tyrosine methyl ester (TCTME) are adsorbed on Sephadex LH-20 gel which enables their separation and validates eqn. 1.

EXPERIMENTAL

The labelling method, apparatus and adsorbent used were described previously[1–5]. TCTME was labelled with ^{125}I by the use of the chloramine-T method. To 25 μg (50 nmole) of TCTME (MW = 530.6) dissolved in 25 μl ethanol–100 μl phosphate buffer (pH 7.6) were added 1–2 mCi (0.5–1.0 nmole) of carrier-free ^{125}I, followed by 50 μl solution containing 200–300 μg of chloramine-T. The labelling reaction was quenched after 30–60 sec with 700 μg of sodium metabisulphite in 100 μl. In the course of the chloramine-T labelling, ^{125}I is introduced via electrophilic substitution in the 3- and/or 5-position of the tyrosine methyl ester residue (Fig. 1).

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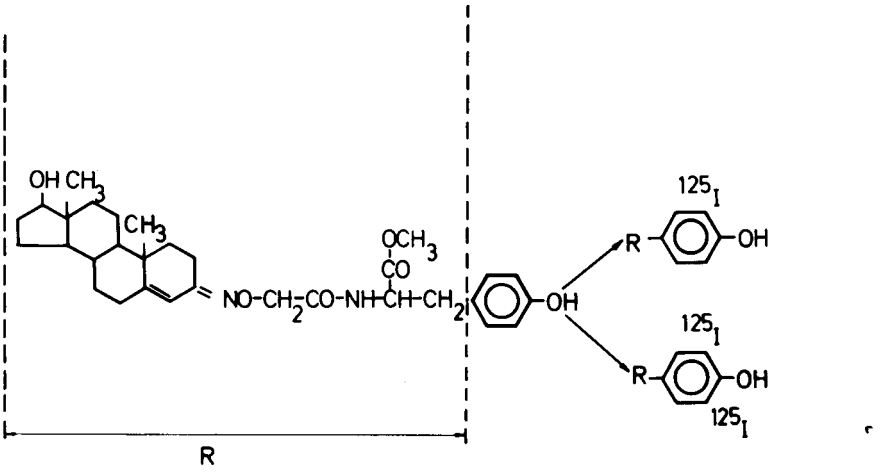

Fig. 1. Mono- and diiodo derivatives of TCTME formed in the course of the chloramine-T labelling.

With such a high excess of TCTME as compared to the amount of radioiodine (*i.e.*, 50 nmole to 0.5–1.0 nmole) the formation of diiodo-TCTME cannot be observed. So, in order to produce TCTME substituted with ^{125}I in the 3 and 5 positions of the phenolic group, 4 μg (32 nmole) inactive iodide were added to the $^{125}I^-$ used for labelling.

Sephadex LH-20 dextran gel was swollen in distilled water prior to being packed in the column (130 × 10 mm I.D.). The height of the packing was 100 mm. In order to check the separation of the starting material (TCTME) from the ^{125}I-labelled TCTME as well as from free radioiodine, tritium-labelled TCTME was also chromatographed separately from the chloramine-T labelling mixture.

In all cases the sample (0.1–0.2 ml) was placed on the top of the column and was allowed to soak in. After 10–20 min, *i.e.*, when adsorption equilibrium had been attained, the free radioiodine was eluted with 10–15 ml aqueous buffer (pH 4) which did not cause displacement of the adsorbed TCTME or [^{125}I]TCTME from the top of the gel. The elution of TCTME and [^{125}I]TCTME was performed with water–ethanol (1.5:1).

When tritium-labelled TCTME was chromatographed the effluent was collected with a fraction collector, and its radioactivity determined by liquid scintillation counting. In the case of the ^{125}I-labelling mixture, the effluent was passed over a NaI (Tl) scintillation crystal and the count rate monitored by a ratemeter and registered by an *x–y* plotter. A peristaltic pump, flow-rate 22–24 ml/h, delivered the eluent.

The distribution coefficient was calculated according to

$$k = (V_e - V_0)/W \tag{2}$$

where V_e, V_0 and W are the elution volume, the dead volume and the weight of the adsorbent, respectively.

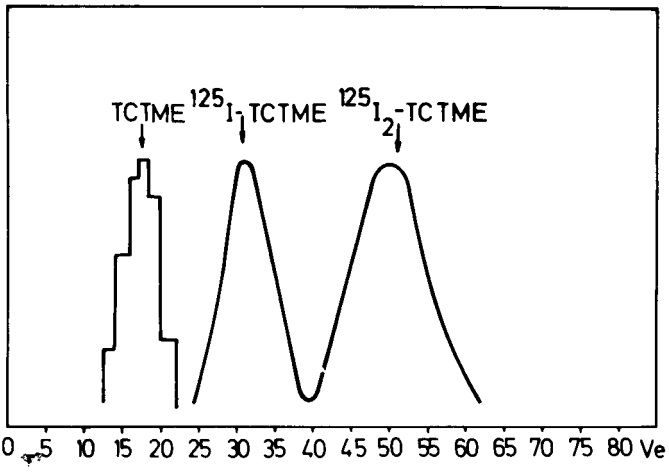

Fig. 2 Superimposed elution pattern of tritium-labelled TCTME, [^{125}I]TCTME and [$^{125}I_2$]TCTME obtained by the use of 40% ethanol as eluent.

RESULTS

Fig. 2 shows the superimposed elution curve of TCTME, [^{125}I]TCTME and [$^{125}I_2$]TCTME when 40% ethanol–water was used as eluent. The elution volume increases in the order TCTME < [^{125}I]TCTME < [$^{125}I_2$]TCTME in full agreement with results for other iodine-substituted compounds[1–5]. The distribution coefficients, k, of TCTME, [^{125}I]TCTME and [$^{125}I_2$]TCTME calculated according to Eqn. 2 as a function of the ethanol concentration of the eluent is presented in Fig. 3, which shows that k increases monotonously with decreasing ethanol concentration. The experimental results in Fig. 3 are shown in Fig. 4 as a log k *vs.* log S plot. The linearity of these plots confirms the validity of eqn. 1.

It should be mentioned that eqn. 1 holds not only for mono- and diiodo-

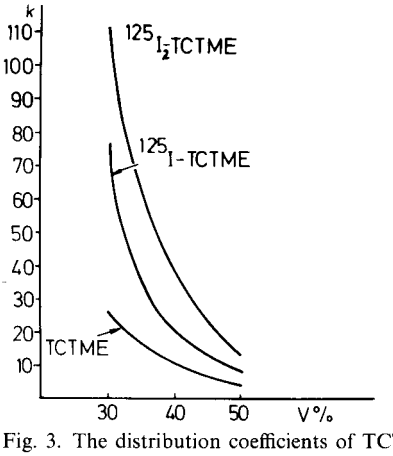

Fig. 3. The distribution coefficients of TCTME, [^{125}I]TCTME and [$^{125}I_2$]TCTME as a function of the ethanol concentration of the eluent.

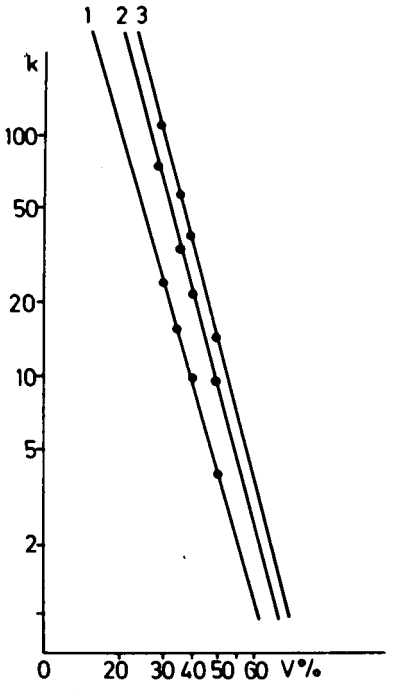

Fig. 4. The log k *versus* log S relationship obtained for TCTME (1), [^{125}I]TCTME (2) and [$^{125}I_2$]TCTME (3).

TCTME, but also for unsubstituted TCTME. Thus, besides the iodo derivatives, the separation of TCTME itself can also be controlled by adjusting the ethanol concentration of the eluent to the value corresponding to the required distribution coefficient.

In radioimmunoassay the monosubstituted TCTME, *i.e.*, [^{125}I]TCTME, is used as tracer. From a practical point of view, to produce [^{125}I]TCTME of high specific activity, radiochemical purity and stability, it is recommended that free radioiodine and unlabelled TCTME be eluted with 30% ethanol followed by 40–50% ethanol which results in considerable increase of the radiochemical concentration.

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Note

Dicyclohexylcarbodiimide as a simple and specific fluorogenic spray reagent for some di- and tricarboxylic acids

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On thin-layer chromatographic (TLC) plates or chromatographic papers, di- and tricarboxylic acids can be detected colorimetrically with pH indicators[1–3], 2,7-dichlorofluorescein–aluminium chloride–iron (III) chloride[4], copper acetate–potassium hexacyanoferrate[5] or dicyclohexylcarbodiimide–hydroxylammonium chloride–iron(III) chloride[6]. These methods detect the acidity or the carboxyl group of the acids, and therefore they will not be specific for the detection of these acids. Lang and Lang[7] recommended a fluorogenic spray reagent composed of pyridine and acetic anhydride, or of acetic anhydride, acetic acid and a small amount of potassium carbonate, for the specific detection of citric, itaconic and aconitic acids. In this paper a sensitive, specific and simpler method for the detection of citric, isocitric, *cis*- and *trans*-aconitic acids and some other biogenic compounds by using dicyclohexylcarbodiimide (DCC) as a spray reagent is described.

EXPERIMENTAL

Reagents and materials

The sources of the test compounds used were Fluka (Buchs, Switzerland; citraconic and itaconic acids), Sigma (St. Louis, MO, U.S.A.; sodium pyruvate, α-ketoglutaric, uric and amino acids), Riedel de Haën (Hannover, G.F.R.; fumaric and glyoxylic acids), Kanto Chemical Co. (Tokyo, Japan; hippuric acid) and E. Merck (Darmstadt, G.F.R.; glycolic and palmitic acids). All of these compounds were used as received.

cis-Aconitic, isocitric and anhydrous citric acids were prepared by common procedures from *cis*-aconitic anhydride, sodium isocitrate (Fluka) and citric acid (Kanto Chemical Co.). *trans*-Aconitic acid (m.p. 206.5–207.0°C) was prepared by the method described by Bruce[8]. Tricarballylic acid (m.p. 163–164°C) and trimethyl citrate (m.p. 79.0–79.6°C) were obtained by hydrogenation of *trans*-aconitic acid with 2.5% sodium amalgam and methylation of citric acid with methanol and sulphuric acid, respectively.

The other test compounds and solvents were of analytical-reagent grade.

The sole reagent for the fluorogenic reaction was DCC (E. Merck). A 0.8 *M* solution of DCC was prepared from *n*-butanol and used as the spray reagent.

Thin-layer and paper chromatography

Pre-coated aluminium TLC sheets (silica gel 60, 5 × 10 cm, 0.2 mm layer) from

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E. Merck and Whatman No. 1 papers were used without any treatment. The sample solutions (0.1–10 m*M* in methanol) were spotted with micro-pipettes (Camag, Muttenz, Switzerland). Ascending chromatography was performed in a glass tank at room temperature (*ca.* 22°C). After development, the air-dried plates or papers were sprayed (twice, if necessary) with the DCC solution in a fume-hood. The spots of aconitic acids could be observed immediately under longwave ultraviolet light, whereas those of the others were observed under the same light after heating in an oven at 70°C for 5 min.

The solvent systems used in TLC and paper chromatographies were freshly prepared and were *n*-butanol–acetic acid–water (3:1:1) and diethylether–formic acid (90%)–water (7:2:1), respectively.

RESULTS AND DISCUSSION

The limits of detection, R_F values and the colours of the fluorescence are listed in Table I. It can be seen that DCC is a sensitive reagent for the fluorometric detection of citric and *trans*- and *cis*-aconitic acids on both TLC plates and papers and can also be used for the semimicroanalysis of leucine, glyoxylic, α-ketoglutaric, isocitric, itaconic, malic and hippuric acids. In addition, DCC behaved as a highly specific reagent for these acids, as demonstrated by a specificity investigation on other carboxylic acids. The following esters and carboxylic acids were negative to DCC: phthalic anhydride, potassium phthalate, trimethyl citrate and benzoic, *p*-hydroxybenzoic, acetic, propionic, palmitic, acetoacetic, succinic, lactic, tartaric, tricarballylic, ascor-

TABLE I

LIMITS OF DETECTION OF THE ACIDS ON TLC PLATES OR PAPERS

Solvent systems: *n*-butanol–acetic acid–water (3:1:1) for TLC and diethyl ether–formic acid (90%)–water (7:2:1) for paper chromatography.

Compound	*Limit of detection (nmole)**	R_F	*Colour of fluorescence*
cis- or *trans*-aconitic acid	0.2 (1.0)	0.52 (0.89)	Yellow-green (blue)
Citric acid	0.5 (1.25)	0.12 (0.54)	Blue-green (blue)
Isocitric acid	25 ((50)	0.34 (0.54)	Blue (blue)
Itaconic acid	50	0.74	Red-orange
Malic acid	100	0.45	Violet
Maleic acid	>150**	0.64	Yellow
Glyoxylic acid	150	0.58	Blue
α-Ketoglutaric acid	150	0.53	Blue-violet
Sodium pyruvate***	150	0.40	Violet
Hippuric acid	40	0.73	Violet
Leucine §	50	0.36	Blue

* The data in parentheses were obtained by paper chromatography.

** Maleic acid was detected as a dark but not fluorescent spot when a small amount of the acid was applied.

*** Before dilution to volume, the sample solution was acidified with 1 *N* hydrochloric acid.

§ The sample solution was prepared with 0.1 *N* hydrochloric acid.

bic, sorbic, orotic, nicotinic, pantothenic, uric, glycolic, malonic and amino acids (glycine, alanine, valine, proline, threonine, serine, methionine, cystine, tyrosine, tryptophan, lysine, arginine, histidine and aspartic and glutamic acids).

The reactions of *trans*- and *cis*-aconitic acids with DCC were rapid and might occur without heating. However, heating was necessary for the other acids. Although the fluorescence intensity of the spots diminished slowly, they remained detectable for several hours when the plates or papers were kept in the dark.

The reaction was investigated in various solvents for citric and aconitic acids. The results, which will be reported elsewhere, reveal that both citric and aconitic acids reacted readily and gave the strongest fluorescence in *n*-butanol. For this reason, *n*-butanol was chosen as a solvent for the spray reagent. In this solvent DCC retained its activity for at least 1 week.

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Note

Substitution of methyl *tert.*-butyl ether for diethyl ether in the standardized thin-layer chromatographic method for lichen products

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A standardized thin-layer chromatographic (TLC) method[1–8] for the identification of lichen products uses three solvent systems, and spots are initially sorted by R_F classes defined on each chromatogram by internal control substances (atranorin and norstictic acid), One of the three solvent systems (solvent B) contains hexane, diethyl ether and formic acid. Lichenologists surveying large numbers of specimens in chemosystematic studies have found this solvent difficult to use because the diethyl ether component of the solution rapidly evaporates. In addition, recent concern over the hazard in the use and storage of diethyl ether has led some workers to discontinue the use of solvent B and to rely upon only solvents A and C. Solvents A and C, however, are much less useful than solvent B for the separation of closely related compounds differing primarily by hydrophobicity. A higher-boiling ether, methyl *tert.*-butyl ether (MTBE), has been shown[9] to be a good substitute for diethyl ether in ether–hexane solvent systems for TLC and normal phase high-performance liquid chromatographic (HPLC) separations of salicylic acid and a variety of monoaromatic phenols that are somewhat similar to the phenolic acid precursors of several categories of di- and triaromatic lichen products. The present note reports a solvent system that contains MTBE instead of diethyl ether and that has chromatographic properties nearly identical to those of solvent B of the standardized method for lichen products.

EXPERIMENTAL

Chromatograms were prepared as described elsewhere[1–3,6] on Merck silica gel 60 F_{254} (0.25-mm layer; No. 5765) plates shortened to 12.5 cm and run to a height of 10 cm from the origin (2 cm from the bottom of the plate). Controls of atranorin and norstictic acid were included at three positions on every plate. Spotted plates were pre-equilibrated 5 min over 60% formic acid[3]. MTBE was HPLC grade (E-127; Fisher Scientific, Pittsburg, PA, U.S.A.) but the product classified as Purified (E-129) gave very similar chromatographic results. A hexane–MTBE–formic acid (140:72:18) solvent gave chromatograms most similar to those obtained with solvent B (hexane–diethyl ether–formic acid; 120:90:20). Chromatograms were air-dried (hood), viewed under short- and longwave UV light and visualized with a spray of 10% sulfuric acid and heat (15–30 min at 110°C). $R_F \times 100$ values were 10–15% higher than normal on the first one or two chromatograms and then decreased only very slowly on sub-

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sequent plates. The level of solution in the tank was maintained near 230 ml with additions of fresh solvent. The $R_F \times 100$ value of norstictic acid was near 30, but values in the range of 26–38 still permitted accurate determinations of R_F classes.

MTBE was compared to diethyl ether as the solvent used for the extraction step in the hydrolysis of depsides[2] using perlatolic acid and barbatic acid to test the procedure. Although the hydrolysis products appeared to dissolve less rapidly in MTBE, this solvent gave satisfactory results in both cases.

RESULTS AND DISCUSSION

Standardized R_F data for solvent B (MTBE) (Table I) determined for examples of all major categories of lichen products (*e.g.*, orcinol and β-orcinol depsides and depsidones, dibenzofurans, xanthones, anthraquinones, pulvinic acids, fatty acids and triterpenes) corresponded closely to the values recently found in our laboratory for solvent B. The useful lifetime of either solvent depends upon the number of chromatograms developed and the time that the solvent is stored in the developing tank. Compared to solvent B, the new solvent system gives satisfactory results for more successive chromatograms and can be left in a closed chromatographic tank (preferably the type with a flanged edge and lid) for a longer time before the R_F values of the atranorin and norstictic acid controls drop below acceptable limits.

The higher boiling and flash points of MTBE (55.2 and −28°C, respectively) compared to diethyl ether (34.5 and −45°C) and the lower tendency to form peroxides greatly reduce fire and explosion hazard. If MTBE is substituted for diethyl ether in solvent B and also used as the extraction solvent in the procedure for the hydrolysis of lichen depsides, there is no need to stock diethyl ether for routine analyses of lichens. This is a special advantage to lichenologists, many of whom work in museums and herbaria that are not equipped to store hazardous materials. We recommend the substitution of MTBE for diethyl ether in solvent B and as the extraction solvent for hydrolysis experiments in chemosystematic studies of lichen-forming fungi.

TABLE I

$R_F \times 100$ VALUES STANDARDIZED AGAINST NORSTICTIC ACID AND ATRANORIN FOR 115 LICHEN PRODUCTS IN SOLVENT B (MTBE)

Compound	R_F *class*	R_F/R_FN, R_FA[a] *(all values × 100)*
Consalazinic	1	1/33, 77
Constictic	1	1.5/32, 74
Erythrin[b]	2	4/33, 74
Salazinic	2	7/32, 73
PQ-4[c]	2	7/32, 77
Stictic	2	9/32, 73
Cryptostictic	2	10/32, 73
Lepraric[d]	2	11/33, 74
Connorstictic	2	11/32, 73
Variolaric[b]	2	11/33, 77

TABLE I *(continued)*

Compound	R_F *class*	R_F/R_FN, R_FA[a] *(all values × 100)*
Porphyrilic	2	12/33, 77
Secalonic A[b,d]	1–2	13/31, 74
Menegazziaic[b]	2–3	15/32, 73
Aspicilin[b]	2–3	17/33, 73
Galbinic[b]	2–3	17/32, 73
Decarboxythamnolic[b]	2–3	18/33, 75
Succinprotocetraric	3	18/31, 73
Protocetraric	3	19/31, 73
Unknown SV-1[d,e]	2–3	20/33, 73
Thamnolic[b]	3	21/33, 75
Strepsilin	3	22/29, 73
Squamatic	3	25/33, 74
Schizopeltic[b]	3	25/32, 74
Hyposalazinic	3	26/32, 77
Fumarprotocetraric	3	26/31, 73
Convirensic[f]	3	28/33, 74
Echinocarpic[b]	4	31.5/33, 75
Endocrocin	4	31.5/32, 74
Alectoronic	4	31/31, 72
Caperatic	4	32/32, 72
Hypostictic	4	32/32, 80
4′-O-Methylpaludosic	4	34/32, 71
Physodalic[b]	4, 5	33/31, 73
4-O-Demethylnotatic	5	33/29, 73
α-Collatolic	5	35/31, 72
Confluentic	5	36/32, 72
Skyrin	5	36/32, 74
Norrangiformic	5	36/32, 73
Diploschistesic	5	37/33, 74
Lividic	5	37/32, 73
Norlobaridone	5	36/30, 72
2′-O-Demethylpsoromic	5	39/33, 74
2,4-Di-O-methylgyrophoric	5	35/28, 72
Physodic	5	35/28, 72
Gangaleoidin[b]	5	38/30, 72
Unknown E-1[g]	5	40/33, 75
Olivetoric	5	41/33, 73
Gyrophoric	5	42/33, 73
Rangiformic	5	41/32, 73
Hypoprotocetraric	5	37/29, 73
2-O-Methylsekikaic	5	40/32, 71
Planaic	5	40/32, 73
Paludosic	5	42/32, 71
Baeomycesic	5	41/33, 74
Microphyllinic	5	42/33, 74
Rhizocarpic	5	42/31, 73
Lecanoric	5	44/33, 73
Methyl gyrophorate	5	39/28, 72
Cryptochlorophaeic	5	45/32, 71
(−)-*allo*-Protolichesterinic	5	43/32, 76

(Continued on p. 486)

TABLE I *(continued)*

Compound	*R_F class*	*R_F/R_FN, R_FA[a] (all values × 100)*
Boninic	5	44/32, 71
Picrolichenic	5	45/33, 73
Notatic	5	46/33, 74
Psoromic	5	46/33, 74
4-O-Methylphysodic	5	45/31, 73
Zeorin	5	47/33, 73
Orsellinic	5	47/33, 72
Lobaric	5	47/32, 73
4,4′-Di-O-methylcryptochlorophaeic	5	47/32, 71
Methyl lecanorate	5	48/33, 72
Unknown Pmc-1[h,i]	5	48/33, 74
Epanorin	5	47/31, 73
4′-O-Methylnorhomosekikaic	5	48/32, 71
Norobtusatic	5	48/31, 74
Protolichesterinic	5	46/32, 77
4-O-Methylgyrophoric	5	50/31, 73
Ursolic	5	48/28, 72
Alectorialic	5	50/32, 73
Merochlorophaeic[h]	5–6	52/32, 71
Unknown CS-1[j]	5–6	52/32, 72
4-O-Demethylbarbatic	5–6	52/31, 74
Thiophanic	5–6	52/31, 73
4-O-Methylhypoprotocetraric	5–6	53/33, 74
Scrobiculin	5–6	55/32, 74
Tenuiorin	5–6	55/33, 72
Anziaic	5–6	55/33, 73
Diffractaic	5–6	55/31, 74
Evernic	5–6	53/28, 73
Lichesterinic	6	58/32, 77
Virensic[h]	6	56/33, 74
4-O-Methylcryptochlorophaeic[h]	6	56/32, 71
Unknown Lgn-1[k]	6	56/30, 72
Emodin	6	58/32, 74
Sekikaic[h]	6	57/32, 71
Grayanic[h]	6	59/33, 74
Vulpinic	6	61/31, 73
Obtusatic	6	63/31, 74
Bourgeanic[h]	6	62/32, 72
Homosekikaic[h]	6	62/32, 71
Pannarin	6	64/33, 74
Diploicin	6	65/30, 72
Lichexanthone	6	66/31, 73
Barbatic	6	67/31, 74
Usnic[d]	6	65/32, 71
Divaricatic	6	65/32, 73
Colensoinic	6	68/32, 73
Parietin	6	69/32, 74
Vicanicin	6	68/30, 72
Didymic[h]	6–7	68/28, 72
Imbricaric	6–7	69/30, 72
Stenosporic	6–7	70/30, 72
Sphaerophorin	7	71/30, 72

TABLE I *(continued)*

Compound	R_F *class*	R_F/R_FN, R_FA[a] *(all values × 100)*
Perlatolic	7	73/30, 72
Methyl barbatate	7	75/32, 74
Calycin	7–8	79/32, 76

[a] The two numbers following the oblique (/) are measurements in millimeters to the norstictic acid line (R_FN) and the atranorin line (R_FA)[1].

[b] R_F relative to standards somewhat lower than with solvent B.

[c] In *Xanthoparmelia quintaria* (Hale) Hale.

[d] A trailing spot difficult to measure.

[e] A common accessory pigment in lichens.

[f] In *Sulcaria virens* (Tayl.) Bystr. *ex* Brodo & Hawksw.; colors yellow with 10% sulfuric acid visualization.

[g] An unknown substance in species with echinocarpic acid.

[h] R_F relative to standards somewhat higher than with solvent B.

[i] An unknown substance in many species with virensic acid (*e.g.*, *Sulcaria virens*) and/or protocetraric acid [*e.g.*, *Parmotrema michauxianum* (Zahlbr.) Hale]; colors gray with 10% sulfuric acid visualization.

[j] An aliphatic unknown in *Cladonia subcariosa* Nyl.

[k] With gangaleoidin in *Lecanora gangaleoides* Nyl.; not the unknown with gangaleoidin in *L. californica* Tuck. and *L. meridionalis* Magn.

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Note

Separation of radiation and photo-induced 5,6-dihydrothymine derivatives by reversed-phase high-performance liquid chromatography

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High-performance liquid chromatography (HPLC), particularly in the reversed-phase mode, has been successfully applied to the separation of DNA and RNA nucleobases and related nucleosides[1–4]. This method was shown to be very efficient for the qualitative and quantitative determination of chemically modified bases and nucleosides resulting from alkylation[5–7] or covalent binding reactions[8–10] with carcinogenic agents.

To the best of our knowledge, the HPLC separation of 5,6 saturated pyrimidines which constitute a major class of radiation or photo-induced DNA lesions has received less attention. One major exception is the recent report on HPLC analysis of the various isomers of cyclobutane dimers of thymine (Thy < > Thy) and thymidine (dThd < > dThd)[11]. This approach may be used as a sensitive method for the quantitation of cyclobutadipyrimidines within the deoxyribonucleic acids (DNA) of living cells exposed to ultraviolet light[12].

We now report the reversed-phase (RP) HPLC separation of various stable 5,6-dihydrothymine derivatives which may be produced by gamma irradiation[13–15] and/or ultraviolet photolysis[16] of aqueous solutions of thymine. Three types of octadecylsilyl (ODS) silica gel, *i.e.*, bulk, monolayer and capped forms, were used throughout this study. Flow-rate gradients appear to be more efficient and more convenient than solvent gradients in a complete separation of the modified pyrimidines which possess a wide range of polarities.

MATERIALS AND METHODS

HPLC instrument

The chromatographic system includes the following Waters Assoc. (Milford, MA, U.S.A.) equipment: two Model M 6000 dual-piston pumps, a Model 660 solvent programmer and a Model U6K universal loop injector. The column was monitored at 220 nm by using a Cecil Model 212 variable-wavelength detector (Cecil Instruments, Cambridge, Great Britain) equipped with a 8-μl flow cell. The chromatographic runs were recorded on a Model Servotrace PE recorder (Sefram, Paris, France) at a chart speed of 0.5 cm/min.

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Columns

Prepacked octadecylsilyl silica gel ODS-2 and ODS-3 columns (25 × 0.46 cm I.D., mean particle size 10 μm) were obtained from Whatman (Maidstone, Great Britain). A third reversed-phase column (25 × 0.47 cm I.D.) was packed with 10-μm ODS Nucleosil C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) by a slurry packing technique[17] using a Haskell pump system (Chromatem, Paris, France). The packing material was homogenized for 3 min by sonication using butanol as the dispersing agent.

Chromatographic procedures

The buffer used either in the flow-rate gradient or in the gradient elution experiments consisted of 0.01 *M* KH_2PO_4 aqueous solutions adjusted to pH 5.5 with phosphoric acid. Aqueous solutions and methanol were filtered using respectively Millipore HA 0.45-μm and cellulose FH 0.5-μm filters (Millipore, Bedford, MA, U.S.A.) prior to use.

The flow-rate gradient experiments were made following the programmed curve 10 from the M 660 programmer. The final flow-rate value, 2.8 ml/min, was reached over a period of 20 min from a starting flow-rate of 0.8 ml/min. The high permeability of the packing materials chosen made possible the use of a relatively high flow-rate. The volume of eluent was estimated using the following equation

$$V\,(\text{ml}) = 0.8\,t + at^6/6$$

where t is expressed in min for $0 < t < 20$ min and $a = 6.25 \cdot 10^{-7}$.

Chemicals

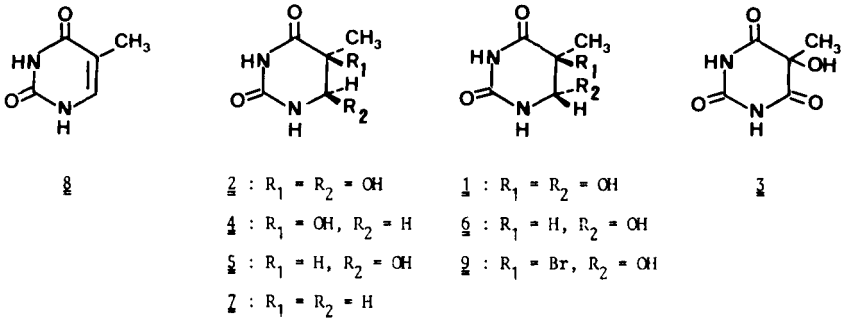
Thymine (8) and 5,6-dihydrothymine (7) were purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. *trans*- and *cis*-5,6-dihydroxy-5,6-dihydrothymine (1,2)[18], 5-hydroxy-5-methylbarbituric acid (3)[19], 5-hydroxy-5,6-dihydrothymine (4)[20], *trans*-6-hydroxy-5,6-dihydrothymine (5)[21], *cis*-6-hydroxy-5,6-dihydrothymine (6)[22] and *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine (9)[23] were prepared according to literature procedures.

RESULTS AND DISCUSSION

Flow-rate gradient separation on an ODS-2 column

The complete separation of a mixture of 5-hydroxy-5-methylbarbituric acid (3), 5,6-dihydrothymine (7) and its five possible 5- and/or 6-hydroxylated derivatives (1, 2, 4–6) (Fig. 1) on a bulky octadecylsilyl silica gel column (ODS-2, Whatman) is illustrated in Fig. 2. Thymine (8) and *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine (9), which is a key intermediate in the chemical synthesis of the *trans* and *cis* thymine glycols (1, 2)[18], as well as in the preparation of thymine "photohydrates" (5, 6)[22], were included in this analytical separation. The convex flow-rate gradient appeared to be very useful for achieving a complete resolution of the mixture of compounds 1–9 in a relatively short period of time. This mixture contained both closely related structural compounds and products exhibiting a wide range of polarities.

The mechanisms of RP-HPLC are still open to debate[24,25]; however, it can



8

2 : $R_1 = R_2 = OH$

4 : $R_1 = OH$, $R_2 = H$

5 : $R_1 = H$, $R_2 = OH$

7 : $R_1 = R_2 = H$

1 : $R_1 = R_2 = OH$

6 : $R_1 = H$, $R_2 = OH$

9 : $R_1 = Br$, $R_2 = OH$

3

Fig. 1. Chemical structure of thymine and its various 5,6-dihydro derivatives. See Table I for names of the compounds.

safely be assumed that solvophobic interactions play a major rôle in the retention of solutes. Accordingly, the observed order of elution, glycols > ketol > 5- or 6-hydroxylated derivative > 5,6-dihydrothymine > bromohydrin (Table I), may be correlated with the increase in the lipophilic character of the molecules. The *trans*

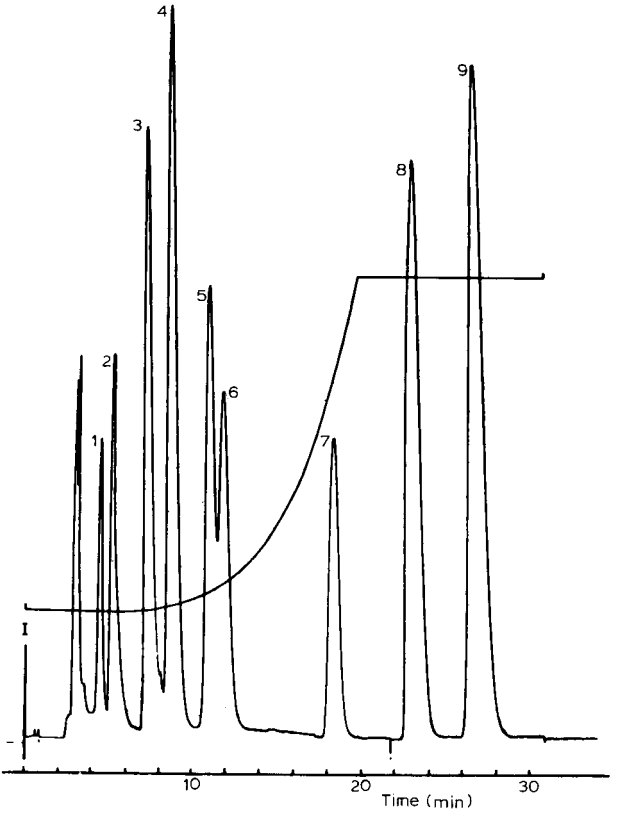


Fig. 2. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Partisil-10 ODS-2 column (25 × 0.46 cm I.D.). Eluent: buffered aqueous solution, pH 5.5; flow-rate gradient, 0.8 to 2.8 ml/min over a period of 20 min.

TABLE I

CAPACITY FACTORS OF 5,6-SATURATED DERIVATIVES OF THYMINE ON THREE ODS COLUMNS USING FLOW-RATE GRADIENT ELUTION

Eluent: phosphate aqueous buffer, pH 5.5; exponential convex flow-rate gradient, 0.8 to 2.8 ml/min over a period of 20 min.

Compound	*ODS reversed-phase packing material*		
	Nucleosil C-18	*ODS-2*	*ODS-3*
1 *trans*-5,6-Dihydroxy-5,6-dihydrothymine	0.73	0.50	0.19
2 *cis*-5,6-Dihydroxy-5,6-dihydrothymine	0.97	0.80	0.40
3 5-Hydroxy-5-methylbarbituric acid	1.44	1.48	0.94
4 5-Hydroxy-5,6-dihydrothymine	1.44	1.95	1.03
5 *trans*-6-Hydroxy-5,6-dihydrothymine	1.99	2.82	1.80
6 *cis*-6-Hydroxy-5,6-dihydrothymine	2.09	3.13	1.94
7 5,6-Dihydrothymine	4.39	6.82	4.58
8 Thymine	5.51	9.44	5.48
9 *trans*-5-Bromo-6-hydroxy-5,6-dihydrothymine	8.51	11.44	8.89

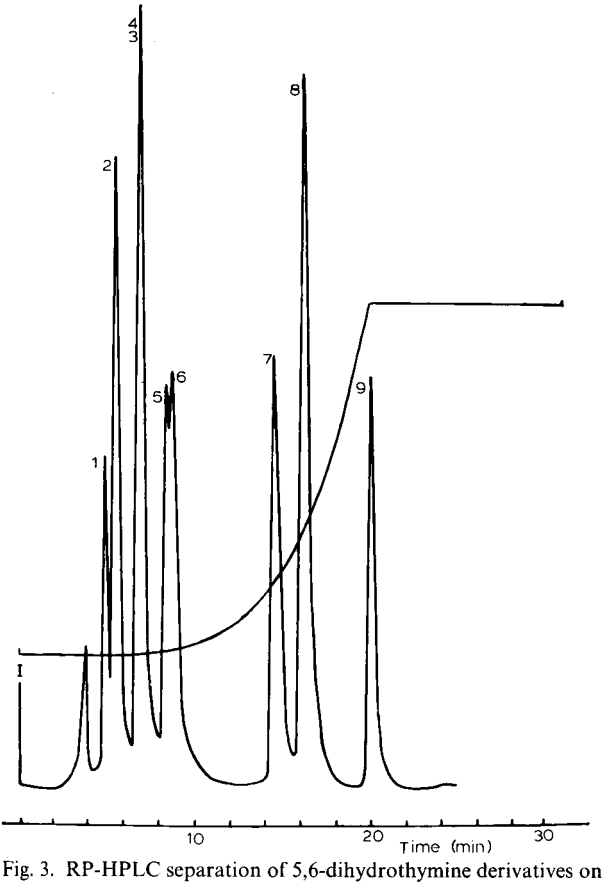


Fig. 3. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Nucleosil-10 C_{18} column (25 × 0.47 cm I.D.); experimental conditions as in Fig. 2.

thymine glycol (1) is eluted faster than the *cis* isomer (2). This may be at least partly explained in terms of lower accessibility for the methyl group of isomer 1 to the hydrocarbon part of the stationary phase. A *gauche* relationship between the methyl substituent and the vicinal hydroxyl group of 1 was deduced from the observation of a pronounced upfield shift (γ effect) of the ^{13}C nuclear magnetic resonance signal of the methyl group[26]. Furthermore, a preferential pseudo equatorial orientation is expected for the methyl substituent of 1, whereas the methyl group of 2 which shows a pseudo axial conformation[27] is less crowded.

The *cis* and *trans* diastereoisomers of 6-hydroxy-5,6-dihydrothymine (5, 6) exhibit higher capacity factors than 5-hydroxy-5,6-dihydrothymine (4) (Table I). The lower retention of 4 may be accounted for by the higher steric hindrance of its methyl group due to the presence of a geminal hydroxyl substituent.

Comparison with other ODS stationary phases

The results of the separation of the various 5,6-dihydrothymine derivatives on a monosubstituted octadecylsilyl silica gel column (Nucleosil C_{18}) and a completely silanized polymeric stationary phase (ODS-3) are shown respectively in Figs. 3 and 4. The elution profiles are similar to those given by the ODS-2 column using identical flow-rate gradients. However, most of the pyrimidines except the hydrophilic *trans* and *cis* thymine glycols (1, 2) are retained longer on the bulky ODS material than on the monomeric substituted ODS column, in agreement with previous findings[28]. This effect is less pronounced for the various compounds 1–9 on the capped ODS-3 column, particularly for thymine (8) (Table I). The complete silanization of the residual silanol groups, which behave as weak acidic cation exchangers[29,30] in neutral aqueous solution, prevents additional interaction between the

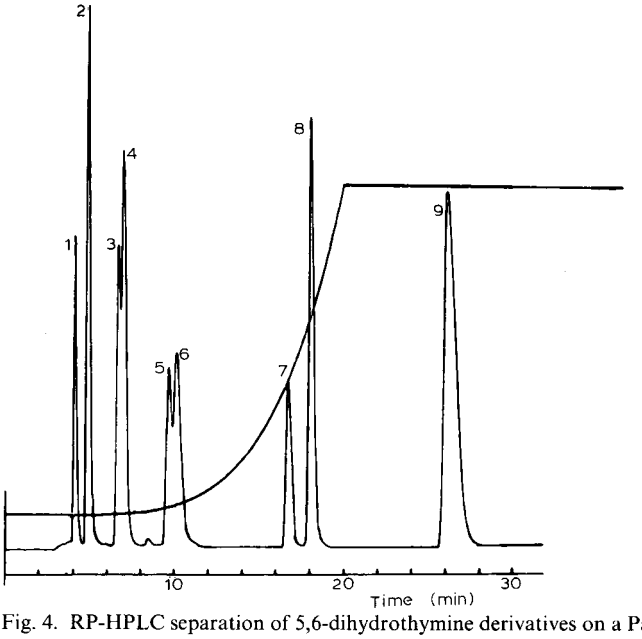


Fig. 4. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Partisil-10 ODS-3 column (25 cm × 0.46 cm I.D.); experimental conditions as in Fig. 2. Peak numbers refer to Fig. 1 and Table I.

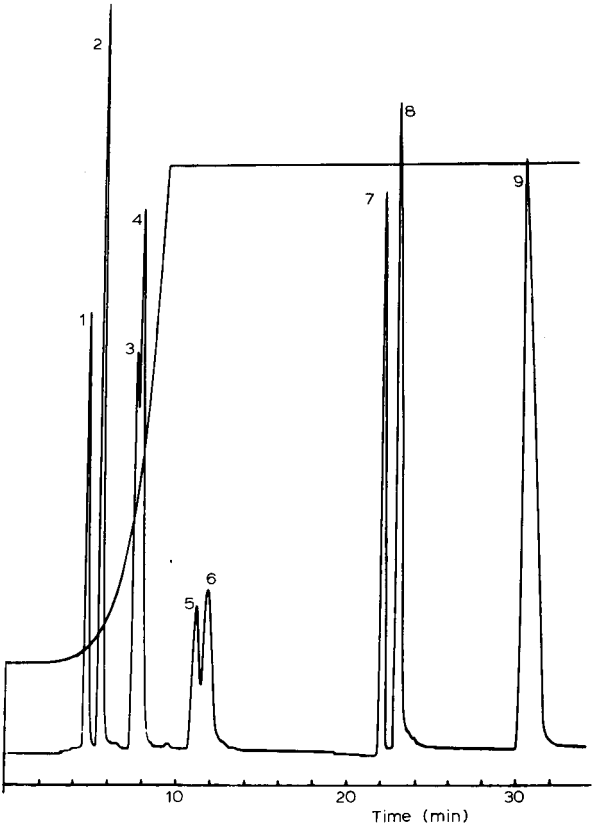


Fig. 5. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Partisil-10 ODS-3 column; solvent gradient of methanol and buffered phosphate buffer (0:100 to 20:80, v/v) over a period of 10 min, followed by isocratic elution. Peak numbers refer to Fig. 1 and Table I.

stationary phase and 5,6-unsaturated 2,4-dioxopyrimidine. Similar adsorption processes are probably involved in the mechanism of retention of heterocyclic compounds which show some aromatic character such as thymine on the slightly negatively charged matrix of dextran gels[31]. This packing appears to be superior to the two other stationary phases used in this study since it presents a higher efficiency (Figs. 2–4) and provides a complete separation of components 1–9 in the shortest period of time.

Solvent gradient elution rate

Fig. 5 depicts a representative separation of compounds 1–9 using a combination of gradient elution and isocratic elution modes. The optimal conditions for the separation of the hydrophilic 5,6-dihydrothymine derivatives 1–4 consist of an exponential convex gradient of methanol and buffered phosphate solution (0:100 to 20:80, v/v) over a period of 10 min. The elution was then pursued in isocratic conditions to resolve the mixture of compounds 6–9. However, this mode of separation requires a longer period of analysis than the flow-rate elution mode to obtain similar

results. Furthermore, the use of isocratic elution is more convenient since it avoids the conditioning of the ODS column after each separation effected in the gradient elution mode.

CONCLUSION

RP-HPLC provides a fast and efficient method of separation of various radiation and photo-induced 5,6-saturated derivatives of thymine (8). This analytical technique can be applied to the quantitation of thymine lesions produced in low yields within nucleic acid chains by exposure to gamma rays[32]. It represents a more efficient and highly sensitive alternative assay for the quantitation of thymine glycol in DNA than liquid chromatographic analysis on a LH-20 column[33].

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Note

Determination of sodium N^6,2′-*O*-dibutyryladenosine cyclic 3′,5′-(hydrogen phosphate) and its hydrolysis products by high-performance liquid chromatography

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DBcAMP*, a biologically active nucleotide (Fig. 1), hydrolyzes readily in aqueous solution into cAMP via 2′-O-MBcAMP and/or N^6-MBcAMP. The kinetics of the hydrolysis reaction has not been examined in detail. Only a column chromatographic method has been presented for the determination of DBcAMP and its hydrolysis products[1]; however, the method is tedious and time-consuming for use in routine work. In order to clarify the kinetics of hydrolysis of DBcAMP a more efficient and reliable method was required.

This paper presents a simple and precise method for the simultaneous determination of DBcAMP, 2′-O-MBcAMP, N^6-MBcAMP and cAMP by high-performance liquid chromatography (HPLC).

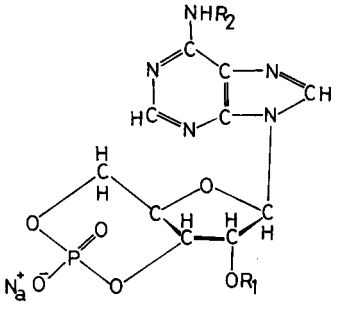

Fig. 1. Structures of DBcAMP and its hydrolysis products.
DBcAMP: $R_1 = R_2 = COC_3H_7$
2′-O-MBcAMP: $R_1 = COC_3H_7$, $R_2 = H$
N^6-MBcAMP: $R_1 = H$, $R_2 = COC_3H_7$
cAMP: $R_1 = R_2 = H$

* Abbreviations: DBcAMP = sodium N^6,2′-O-dibutyryladenosine cyclic 3′,5′-(hydrogen phosphate); 2′-O-MBcAMP = sodium 2′-O-monobutyryladenosine cyclic 3′,5′-(hydrogen phosphate); N^6-MBcAMP = sodium N^6-monobutyryladenosine cyclic 3′,5′-(hydrogen phosphate); cAMP = sodium adenosine cyclic 3′,5′-(hydrogen phosphate).

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EXPERIMENTAL

Materials and reagents

DBcAMP, 2′-O-MBcAMP, N^6-MBcAMP and cAMP were synthesized in our Research Institute. Acetonitrile, specially prepared for HPLC (Nakarai Chemicals, Kyoto, Japan) and acetic acid of analytical reagent grade were used. Water was distilled and all other chemicals were of reagent grade. A column (150 × 6 mm I.D.) packed with Develosil ODS (particle size 5 μm; Nomura Kagaku, Aichi, Japan) was used. It was prepared by a slurry method with slurry solvent B (Machery, Nagel & Co., Düren, G.F.R.). Acetonitrile–1 % acetic acid (30:70) was used as mobile phase.

Apparatus

An Hitachi 638 liquid chromatograph equipped with a variable-wavelength UV-detector UVILOG-5II (Oyobunko, Tokyo, Japan) and Rheodyne 7122 sample injector with 20-μl sampling loop (Rheodyne, CA, U.S.A.) was used. The UV detector was set at 273 nm and 0.08 a.u.f.s.

Ananalytical procedure

Samples were dissolved in the mobile phase to give solutions in the range of about 2–40 μg ml^{-1} for DBcAMP, 2′-O-MBcAMP, N^6-MBcAMP and cAMP. The sample solutions were analyzed by HPLC at a mobile phase flow-rate of 1.0 ml min^{-1}. The concentrations of each compound in the samples were determined from calibration curves prepared by using the peak height method.

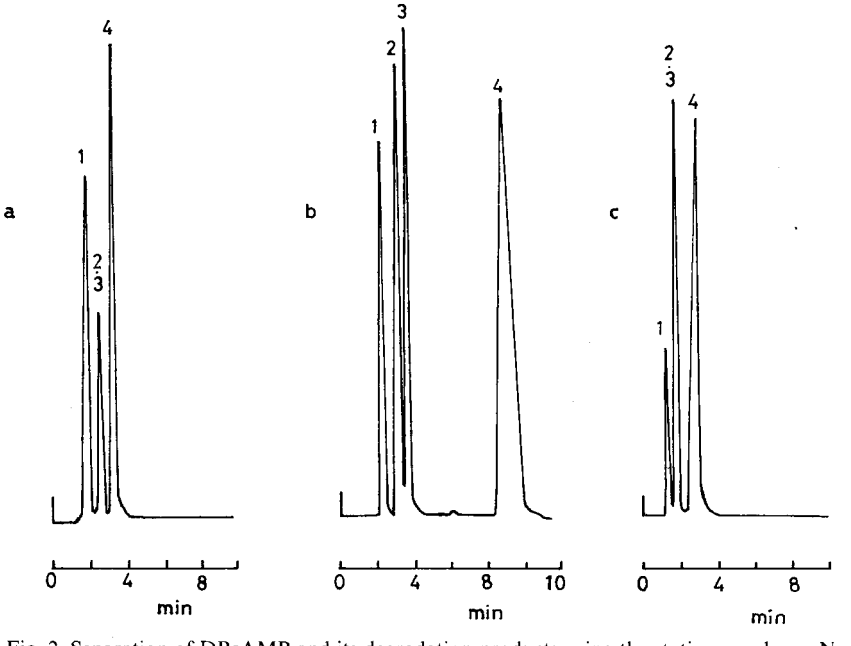

Fig. 2. Separation of DBcAMP and its degradation products using the stationary phases Nucleosil 5 C_{18} (a), Develosil 5 ODS (b) and Cosmosil 5 C_{18} (c). Peaks: 1 = cAMP; 2 = 2′-O-MBcAMP; 3 = N^6-MBcAMP; 4 = DBcAMP.

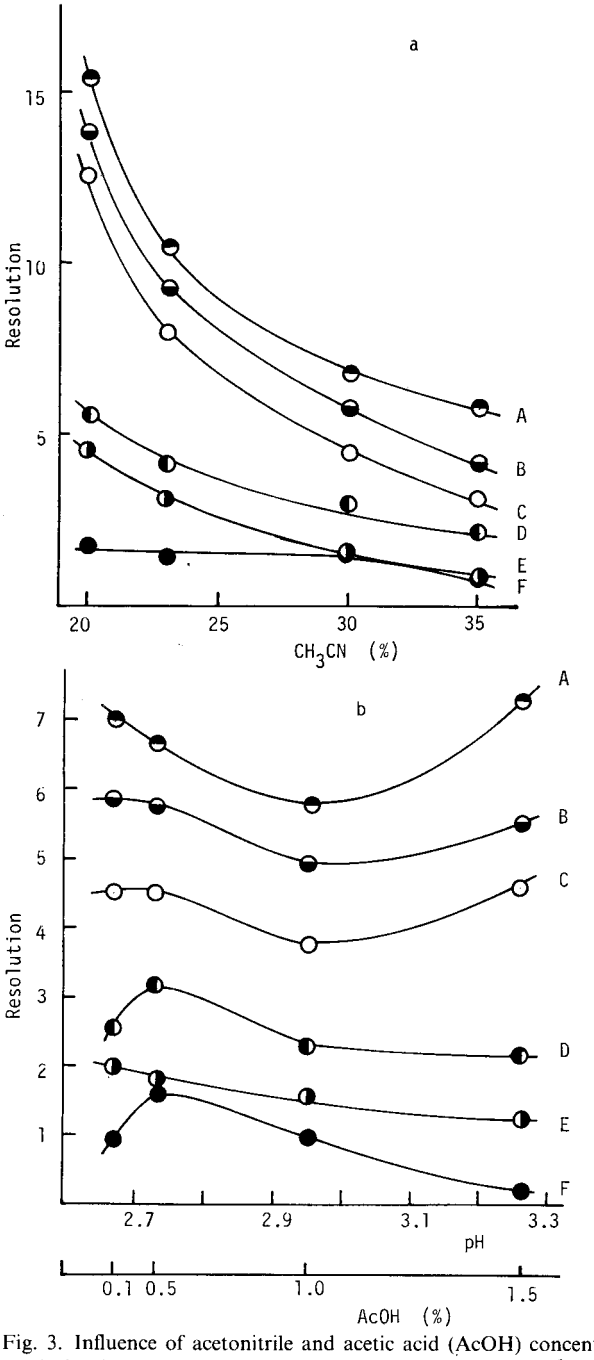

Fig. 3. Influence of acetonitrile and acetic acid (AcOH) concentrations in the mobile phase on mutual resolution between peaks 1 (cAMP), 2(2′-O-MBcAMP), 3(N^6-MBcAMP) and 4(DBcAMP). (a) Acetonitrile concentration in 1 % acetic acid; (b) acetic acid concentration in 30 % acetonitrile A, peaks 1 and 4; B, peaks 2 and 3; C, peaks 3 and 4; D, peaks 1 and 3; E, peaks 1 and 2; F, peaks 2 and 3. Column: Develosil 5 ODS (150 × 6.0 mm I.D.).

RESULTS AND DISCUSSION

Choice of stationary phase and column size

Preliminary attempts to separate DBcAMP and its hydrolysis products were made with three kinds of reversed-phase materials, Develosil ODS, Nucleosil C_{18} (particle size 5 μm, Macherey, Nagel & Co.) and Cosmosil C_{18} (particle size, 5 μm Nakarai Chemicals), in a column of 150 × 4.6 mm I.D. The numbers of theoretical plates, N, of these columns, which were calculated by the usual way[2] from the elution pattern of anthracene using methanol–water (80:20), were similar (N = 6500 for Develosil ODS, N = 6800 for Nucleosil C18, N = 6300 for Cosmosil C18), whereas significant differences were found for separations between DBcAMP and its hydrolysis products as shown in Fig. 2. The most favourable separation was observed on the reversed-phase of Develosil ODS, however it was considered to be not sufficient for the simultaneous determination of DBcAMP and its hydrolysis products. When using a Develosil column of 150 × 6 mm I.D., a more efficient separation was achieved.

Influence of mobile phase

In order to obtain the optimum separation on the Develosoil ODS column (150 × 6 mm I.D.), the influence of the mobile phase was examined with the solvent system of acetonitrile–water–acetic acid. Fig. 3 shows the influence of the concentrations of acetonitrile (a) and acetic acid (b) in on the resolution (R_s) between any two compounds. Each R_s value was calculated in the usual way[3].

In general, efficient and selective chromatographic analyses have been run at separation 6σ corresponding to R_s 1.5. As seen in Fig. 3, a decrease in acetonitrile

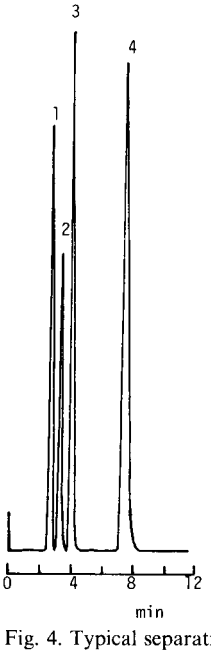

Fig. 4. Typical separation of DBcAMP and its hydrolysis products. Stationary phase: Develosil 5 ODS, 150 × 6.0 mm I.D. Mobile phase: acetonitrile–1% acetic acid (30:70). Peaks as in Fig. 2.

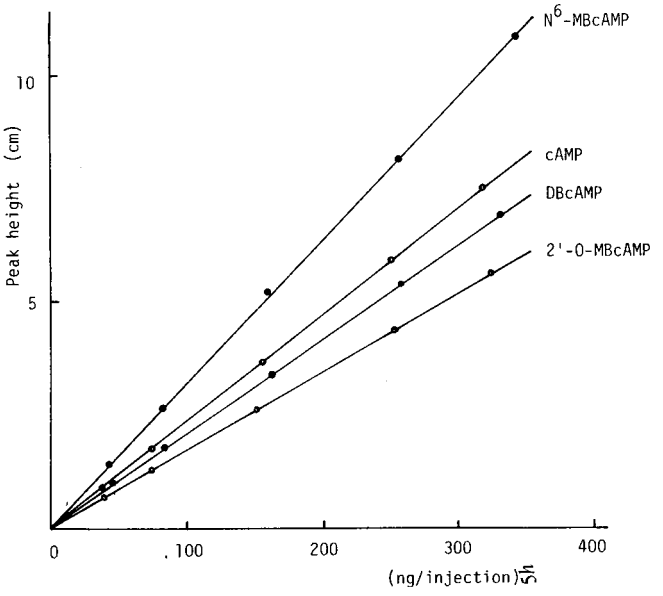

Fig. 5. The calibration curves of DBcAMP and its hydrolysis products. Each peak height is the mean from two determinations.

concentration results in greater R_s for any two nucleotides, however this required a longer analysis time. The optimum concentration of acetonitrile was considered to be 30% from the viewpoint of sufficient separation and length of the analysis time. On the other hand, the influence of acetic acid on R_s is more complicated. A mobile phase of acetonitrile–1% acetic acid (30:70) gave the maximum R_s (1.7) between N^6-MBcAMP and 2′-O-MBcAMP, and this was used for subsequent experiments.

Monitoring wavelength

The UV absorption spectra of the nucleotides in the mobile phase exhibited λ_{max} at 273 nm for DBcAMP and N^6-MBcAMP, and at 257 nm for 2′-O-MBcAMP and cAMP, so the detection was carried out by monitoring the absorbance at 273 nm.

Calibration curves and precision

Fig. 4 shows a typical elution pattern of DBcAMP and its hydrolysis products, and Fig. 5 shows the corresponding calibration curves. The calibration curves were linear. The reproducibility among runs was about 2% or below of the coefficient of variation. The detection limit of each compound was about 1 ng per injection.

The method presented was used successfully for a kinetic study of the hydrolysis of DBcAMP. The results will be presented in a later paper.

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Note

Assay of saccharin and sodium saccharin in animal feed*

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Continued research about possible carcinogenic activity of saccharin necessitated the development of a reliable and accurate assay method for this compound in animal feed diet mixtures used in these studies. It is known that in long-term studies of this nature drug administration to laboratory animals is usually done by incorporation into feed because of the inconvenience of individual manual dosing and the time involved in preparing individual dosages. Analytical methodology is required to determine that appropriate dosages are administered and that the drug is uniformly distributed and stable in the feed mix under the storage conditions used.

Various gas–liquid chromatographic methods were published recently for the assay of saccarin[1–3]. These methods, however, required prior derivatization of the compound. Within the last few years many high-performance liquid chromatography (HPLC) methods have been reported for the quantitation of saccharin. These HPLC methods dealt mostly with biological fluids, beverages and similar samples[4–8] which may not be applicable for animal feed.

Very recently, Holder and Bowman[9] developed an HPLC method for the assay of saccharin in animal feed. This method employed a 2-step extraction of saccharin from the feed sample; first into an aqueous sodium carbonate solution, followed by extraction into ether after acidification. The HPLC step utilized a paired-ion mobile phase and the recovery data were relatively low: 65.0–87.3% for 10.0- and 75,000-ppm samples, respectively. In addition, for samples less than 1000 ppm the feed extract was subjected to a silica gel column cleanup. This paper reports a reversed-phase HPLC method using glacial acetic acid–aqueous methanol mobile phase, after a one-step extraction and a simple cleanup procedure, with sulfathiazole as internal standard. The method resulted in higher percent recovery data.

EXPERIMENTAL

Apparatus

The following were used: a Waters Assoc. Model ALC/GPC 244 liquid chromatograph with a U6K injector, M 6000 pump and M 400 fixed-wavelength UV detector (254 nm); a Houston Instruments (Austin, TX, U.S.A.). Omniscribe Series

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A-5000 strip-chart recorder; a glass chromatography tube, 350 × 15 mm I.D., with a PTFE stopcock: a mixer (Vortex-Genie, Scientific Industries, Springfield, MA, U.S.A.); a centrifuge; a rotary evaporator; and 50-ml centrifuge tubes fitted with polytef-lined screw caps.

Reagent and materials

The following were used: saccharin (Eastman Kodak, Rochester, NY, U.S.A.); sodium saccharin dihydrate (Fisher Scientific, Fair Lawn, NJ, U.S.A.); sulfathiazole (Sigma, St. Louis, MO, U.S.A.); diatomaceous earth (Celite 545 AW; Supelco, Bellefonte, PA, U.S.A.); diatomaceous earth (Celite 545 AW; Supelco, Bellefonte, PA, U.S.A.); animal feed (Purina Laboratory Chow, Ralston Purina, St. Louis, MO, U.S.A.); acetic acid–methanol–water (2.5:100:500); acetic acid–ethyl acetate–chloroform (1:7.5:25); and methanol–water (1:1). All other chemicals used were analytical grade.

HPLC conditions

A 25 cm × 4.5 mm I.D., 10-μm Partisil ODS-II ITP (Jones Chromatography, Columbus, OH, U.S.A.) was used at ambient temperature. An isocratic mobile phase system of acetic acid–methanol–water (2.5:100:500) was delivered at the rate of 1.2 ml/min. This mobile phase was filtered through a 5-μm membrane filter (Miltex Type LS, Millipore, Bedford, MA, U.S.A.) and degassed before used. The detector was attenuated to 0.02 and 0.1 a.u.f.s. for the 200- and 2000-ppm samples, respectively.

Internal standard solution

A solution of 0.02 or 0.1 mg of sulfathiazole/ml of methanol–water (1:1) was prepared for the 200- and 2000-ppm samples, respectively.

Standard solution preparation

About 10 mg of saccharin was weighed accurately into a 10-ml volumetric flask and dissolved in diethyl ether. The solution was diluted to volume with ether. A 1-ml volume of this stock solution (for use with the 2000-ppm sample) or 1.0 ml of a 1:10 diluted stock solution (for use with the 200-ppm sample) was pipetted into a 100-ml boiling flask and carefully evaporated to dryness on the rotary evaporator. Ether traces were removed under a nitrogen stream. The residue was subjected to the same cleanup and chromatographic procedures described for the residue from the sample.

Diatomaceous earth column preparation

A small glass wool plug was placed at the base of the glass chromatographic tube. A 2-g amount of diatomaceous earth AW was transferred to the tube and tamped lightly. A 3-g amount of diatomaceous earth AW and 1.5 ml of 0.1 *M* of sodium hydroxide were mixed gently and transferred to the tube. The tube was tapped on a padded bench to settle the packing and them tamped very gently but evenly.

Sample preparation

About 15 ml of 7.5% (w/v) hydrochloric acid was placed in a 50-ml centrifuge tube. About 5 g of a 200- to 2000-ppm animal feed sample was weighed accurately and transferred into the centrifuge tube. After 20 ml of ether was added, the cen-

trifuge tube was capped and stirred on a vortex mixer for 5 min, then centrifuged at about 2000 *g* for 5 min. With a Pasteur pipet, the clear supernate was transferred carefully into a 100-ml volumetric flask. The extraction was repeated four more times with 20-ml portions of ether. The combined ether extract was diluted to volume with ether, if necessary, and mixed. A 10-ml volume of this solution was pipetted into a 100-ml boiling flask and evaporated to dryness on the rotary evaporator. Ether traces were removed under a nitrogen stream and the residue was dissolved in 10 ml of water-saturated chloroform. The solution was transferred using a Pasteur pipet to the diatomaceous earth column, and the flow-rate was adjusted to 1–1.5 ml/min. The flask was rinsed twice with 20-ml portions of water-saturated chloroform and the rinsing were transferred to the column. The eluate was discarded.

The adsorbed saccharin was eluted with 50 ml of acetic acid–ethyl acetate–chloroform eluent, and the eluate was collected in a 100-ml boiling flask. The eluate was evaporated to dryness on the rotary evaporator at about 45°C. Acetic acid traces were removed under a nitrogen stream. The residue was dissolved in 5.0 ml (2000-ppm sample) or 3.0 ml (200-ppm sample) of the respective internal standard solution. The solution was filtered through a 5-μm membrane filter.

Chromatographic procedure

A 20-μl volume of the standard solution and 20.0 μl of the prepared feed sample solutions were chromatographed under the described HPLC conditions. The chromatogram was quantitated by relating the saccharin–sulfathiazole peak area ratio for the sample to that of the standard solution.

RESULTS AND DISCUSSION

Preliminary studies using a number of solvents and solvent mixtures indicated that the extractant of choice for saccharin was ether. Although saccharin is slightly soluble in ether, its concentration in the animal feed mix was sufficiently low so that multiple extractions could isolate the saccharin quantitatively. Furthermore, with ether the number of feed constituents co-extracting with saccharin was far less than if, for example, acetone was used in a direct liquid–solid extraction as described for the assay of azosemide in animal feed[10]. This resulted in cleaner chromatograms. The extraction with ether was done in the presence of hydrochloric acid so that the method is applicable for either saccharine or sodium saccharin in feed mix. Attempts to extract the compound using a shaker, separatory funnel, Soxhlet extractor, or ultrasonic mixing were unsuccessful; in these cases low recoveries were obtained.

The cleanup procedure was based on the acidic nature of saccharin. In the sodium hydroxide–diatomaceous earth column, saccharin was converted to its sodium salt. To ensure that this salt remained in the column water-saturated chloroform must be used. The sodium hydroxide strength for adsorption on diatomaceous earth and the total amount needed were determined. Under the conditions described the sodium hydroxide–saccharin mole ratio was about 300 and 30 for the 200- and 2000-ppm samples, respectively. Various eluents were investigated for optimum recovery of the trapped saccharin. This was performed by spiking ether extracts of a placebo feed mix with accurately known solutions of saccharin in ether and run through the cleanup steps. The proposed eluent mixture appeared to give optimum

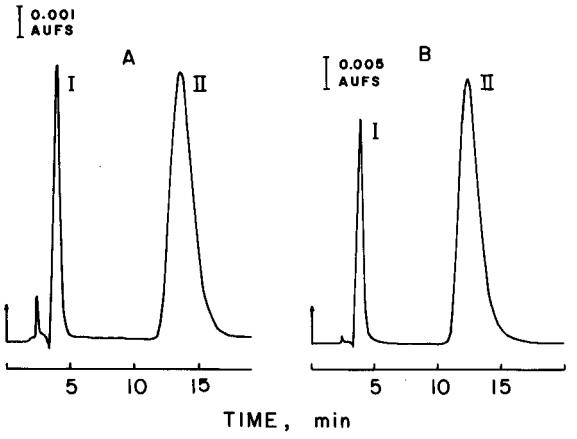

Fig. 1. Chromatograms of the appropriate saccharin (I) and sulfathiazole (II) standard solutions. A, at 0.02 a.u.f.s.; B, at 0.1 a.u.f.s.

recovery. In this mixture sufficient acetic acid was present to supply hydrogen ions which will exchange for the sodium ions of sodium saccharin thus rendering the saccharin in the free and elutable form. The addition of a bottom layer of plain diatomaceous earth in the column helped to break emulsions when they formed in the column.

Under the described HPLC conditions, saccharin and sulfathiazole eluted as symmetrical peaks and were very well resolved from one another. The retention times of saccharin and sulfathiazole were *ca.* 3.8 and 12.3 min, respectively (Fig. 1A and B). The average height equivalent to a theoretical plate (HETP) of the column ($\pm$ S.D.) was 1.116 $\pm$ 0.047 mm for saccharin (n = 10).

Quantitation was based on the drug–internal standard peak area ratio. With these ratios the linearity between detector response at 254 nm and amount of saccharin injected was established. Linearity was obtained between 0.4–5.0 μg of saccharin. A typical regression equation for the saccharin–sulfathiazole peak area ratio

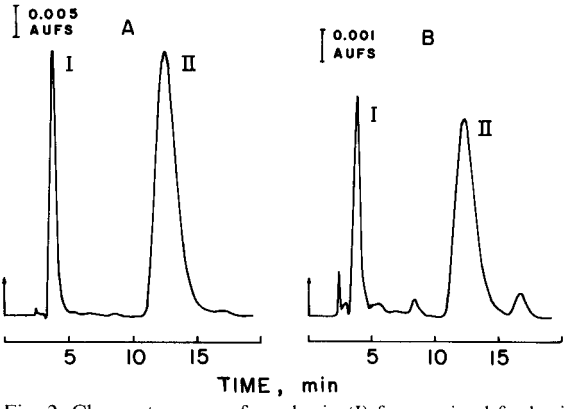

Fig. 2. Chromatograms of saccharin (I) from animal feed mix. II = sulfathiazole; A = 2000-ppm feed sample; B = 200-ppm feed sample.

(A) and amount of saccharin injected (C, expressed in μg) was $A = 0.467\ C + 0.044$ with a correlation coefficient $r = 0.9994$.

The cleanup procedure gave liquid chromatograms for the 2000-ppm sample that resolved saccharin and sulfathiazole from any feed constituents present (Fig. 2A). The liquid chromatograms for the 200-ppm sample showed some interference to the saccharin peak area by the feed constituents (Fig. 2B). This interference, however, amounted to less than 0.6%. For comparison, the liquid chromatograms of blank feed mix run under the conditions for the 200- and 2000-ppm samples, *i.e.* at 0.02 and 0.1 a.u.f.s., respectively, are shown in Fig. 3A and B.

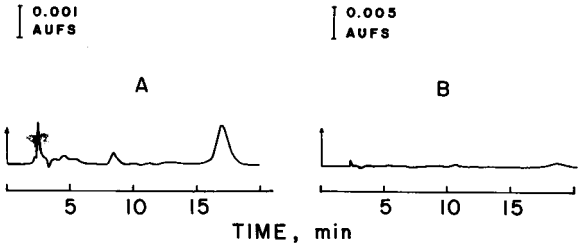

Fig. 3. Chromatograms of placebo feed mix. A, at 0.02 a.u.f.s.; B, at 0.1 a.u.f.s.

To provide better representation of the analytical potency, a concomitant standard was used, *i.e.* the standard was subjected to the same analytical steps in the cleanup procedure. This would eliminate any errors (losses) occuring during the cleanup steps.

Recovery studies were performed on feed samples spiked at 200- and 2000-ppm levels with saccharin or sodium saccharin. The accurately weighed drugs were carefully mixed using a mortar and pestle. Each spiked sample was about 50 g. Table I

TABLE I

RECOVERY OF SACCHARIN FROM SPIKED FEED MIX AT 200- AND 2000-ppm LEVELS

Sample (ppm)	*Amount of saccharin*		*Recovery (%)*
	in analytical sample (mg)	*Found (mg)*	
200 ppm			
214	1.07	1.03	96.3
216	1.08	1.03	95.7
212	1.06	1.06	100.0
Overall recovery (%)			97.6
S.D.			2.1
2000 ppm			
2144	10.72	10.98	102.4
2152	10.76	10.58	98.3
2114	10.57	10.20	96.5
Overall recovery (%)			99.1
S.D.			3.0

shows the recovery data for saccharin from animal feed mix at the 200- and 2000-ppm levels. The overall percent recovery ($\pm$ S.D., $n = 3$) were 97.6 $\pm$ 2.1% and 99.1 $\pm$ 3.0% for the 200- and 2000-ppm samples, respectively. The overall percent recoveries ($\pm$ S.D., $n = 3$) for sodium saccharin from animal feed were 98.9 $\pm$ 2.1% and 100.4 $\pm$ 3.8% for the 200- and 2000-ppm samples, respectively (Table II).

TABLE II

RECOVERY OF SODIUM SACCHARIN FROM SPIKED FEED MIX AT 200- AND 2000-ppm LEVELS

Sample (ppm)	*Amount of sodium saccharin*		*Recovery (%)*
	in analytical sample (mg)	*Found (mg)*	
200 ppm			
288	1.44	1.44	100.0
286	1.43	1.38	96.5
289	1.45	1.46	100.1
Overall recovery (%)			98.9
S.D.			2.1
2000 ppm			
2900	14.50	14.60	100.7
2940	14.70	15.30	104.1
2880	14.40	13.90	96.5
Overall recovery (%)			100.4
S.D.			3.8

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Note

Determination of thiabendazole by ion-pair high-performance liquid chromatography

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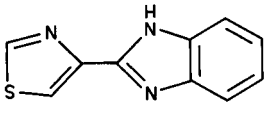
(First received November 2nd, 1981; revised manuscript received November 26th, 1981)

Thiabendazole, 2-(4-thiazolyl)-1*H*-benzimidazole (TBZ), is widely used both as an anthelmintic agent for control of animal and human parasites and as a fungicide for disease control in various crops. Because of the potential for chronic exposure of workers to TBZ at the site of manufacture, it is desirable to have a sensitive and specific analytical method for low levels of this substance.

Numerous methods have been published for the determination of TBZ residues in crops using gas–liquid chromatography (GLC)[1,2], thin-layer chromatography (TLC)[3,4], and high-performance liquid chromatography (HPLC)[5–8]. However, the GLC methods require derivatization and the TLC methods are either qualitative tests or have a limited range of quantitation. The HPLC methods, as well as those using TLC or GLC, require substantial sample clean-up procedures. There have been no reports of methods for collection and analysis of airborne TBZ.

This paper presents a method for collection and analysis of particulate TBZ which requires no extensive sample clean-up when applied to industrial hygiene measurements in a manufacturing environment. At the same time the method is more sensitive and covers a wider analytical range than previously reported methods.



EXPERIMENTAL

Materials

HPLC-grade methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) was used. Deionized water was distilled from an all-glass still. PIC reagent B-7 was purchased from Waters Associates (Milford, MA, U.S.A.). Pure thiabendazole, N-phenyl-4-thiazolecarboxamidine hydrochloride and cyanothiazole were supplied by Merck & Company (Rahway, NJ, U.S.A.).

Equipment

A Waters Model M6000A pump, 710B autosampler, 720 systems controller, and 440 UV detector were interfaced to a Hewlett-Packard 3354 data system. A Vydac 201 TP reversed-phase (C_{18}) column (25 cm × 4.6 mm) was employed. The isocratic mobile phase was methanol–water (59:41) + PIC B-7 reagent prepared according to the manufacturer's directions to give a 0.005 *M* solution of 1-heptanesulfonic acid (pH 3.5). The flow-rate was 1.0 ml/min; the UV absorbance was measured at 313 nm.

Sample collection and extraction

Airborne particulates were collected in the field using personal sampling pumps and 37-mm Gelman type AE glass fiber filters. Samples were collected at a flow-rate of 1.5 l/min for 2–6 h. Surface contamination was examined using Whatman No. 50 smear tabs to collect swipe samples. Both types of collection media were extracted in 20-ml scintillation vials using 5.0 ml methanol in an ultrasonic bath for 15 min and then filtered through 1.0-μm PTFE filters.

RESULTS AND DISCUSSION

Since common problems in the chromatographic analysis of amines are tailing on the columns and non-reproducibility of retention times, the use of an ion-pairing agent was indicated. PIC B-7 reagent (heptanesulfonic acid, pH 3.5) in methanol–water (59:41) was found to give a sharp peak for TBZ. The retention time on the C_{18} reversed-phase column was 4.60 min with a relative standard deviation (R.S.D.) of 0.5%.

A set of working standards of TBZ solutions was prepared by dissolving commercial grade TBZ in methanol, filtering, and making serial dilutions to give a concentration range of 0.2534 ng/μl to 253.4 ng/μl. Multiple aliquots of 5, 10 and 25 μl were injected directly into the chromatograph to generate a calibration plot with a range (amount of TBZ injected) of 1.267 to 6335 ng. The calibration plot (Fig. 1) was

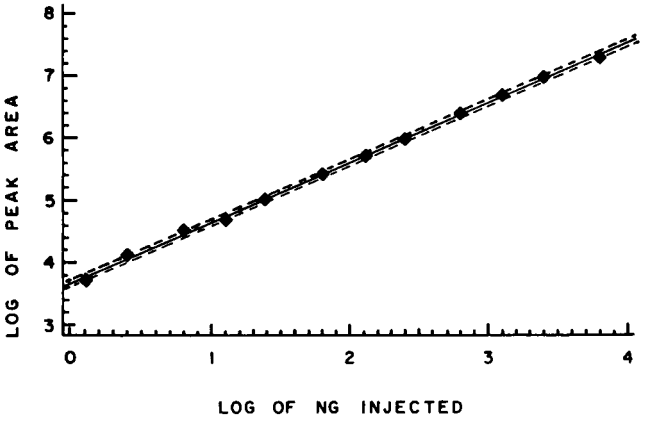


Fig. 1. Thiabendazole (TBZ) calibration curve. Range, 1.27–6335 ng. Correlation coefficient, 0.9987. Dashed lines indicate 95% confidence limits.

found to be linear over the entire range with an R.S.D. (95% confidence limit) of 4.0%. Varying the injection size from 5 to 50 μl had no deleterious effect upon the precision.

The following chemicals, which may be used in the synthesis[9] of TBZ were co-chromatographed and found not to be interferences: 4-cyanothiazole, aniline and N-phenyl-4-thiazolecarboxamidine hydrochloride.

Potential volatilization of TBZ from the filters during collection was checked. Two sets of filters were spiked with TBZ at four different levels each. Laboratory air was drawn through the first set of filters (using a personal sampling pump) for 5 h at a flow-rate of 1.5 l/min. Both sets of filters were then extracted and analyzed. Average recovery for both sets was 91%.

Storage stability of TBZ on glass fiber filters was examined by spiking each of eight filters with 50 μl of a 50 ng/μl TBZ solution. Four were analyzed after 24 h and four after 8 days. Average recoveries were 86 and 88%, respectively.

Application of this method to field samples collected at 1.5 l/min for 6 h gives an effective range of 0.2–9000 $\mu g/m^3$. A typical chromatogram of a sample collected in the field is shown in Fig. 2.

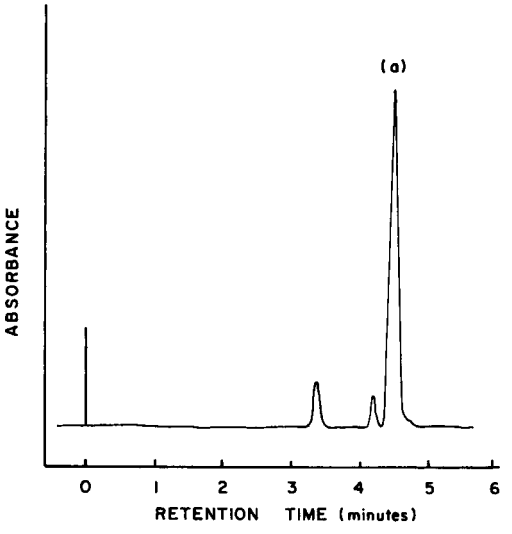


Fig. 2. Representative chromatogram of airborne thiabendazole collected on a glass fiber filter. (a) = TBZ. Solvent, methanol–water (59:41) + PIC B-7 reagent (0.005 *M*); flow-rate, 1.0 ml/min; detection, 313 nm, 0.05 a.u.f.s.; sample size, 50 μl; column, Vydac 201 TP (10 μm).

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Note

Determination of the γ-aminobutyric acid agonist 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol by high-performance liquid chromatography using UV or electrochemical detection

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4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) is a heterocyclic analogue of γ-aminobutyric acid (GABA). It has been observed to be a GABA receptor agonist and may be a drug of clinical value[1,2].

A high-performance liquid chromatographic (HPLC) method for the determination of THIP has been developed in an attempt to establish a procedure for the determination of THIP in biological materials, or in pharmaceutical preparations. The pK_a values of THIP are 4.4 and 8.5[1], and its zwitterionic structure makes it very hydrophilic. Several column packing materials and mobile phase compositions were tried, and the use of an octadecylsilica material and a buffered mobile phase containing perfluorooctanesulphonic acid was found to offer the most stable system as regards constancy of the retention times of THIP and other sample components. As the UV absorption of THIP is maximal at a wavelength of 217 nm and relatively low at wavelengths exceeding 230 nm, electrochemical detection was considered a favourable alternative to measurement of UV absorption for monitoring THIP in the eluate from the column. A slightly modified version of the coulometric detector designed by Lankelma and Poppe[3] was used.

EXPERIMENTAL

Materials

In the final version of the method the following chemicals were used: lithium perchlorate, sodium acetate (both pro analysi grade, Merck, Darmstadt, G.F.R.), perfluorooctanesulphonic acid (Prosynth grade, Riedel-de Haën, Seeltze-Hannover, G.F.R.), tetrahydrofuran (HPLC grade, Rathburn, Walkerburn, Great Britain) and water redistilled in all-glass apparatus; these chemicals were used for the preparation of the mobile phase, consisting of a 90:10 mixture of 0.02 *M* sodium acetate solution (pH 4.5) and tetrahydrofuran, with added perfluorooctanesulphonic acid (5 m*M*) and lithium perchlorate (50 m*M*). The mixture was degassed by occasional purging with helium. The stationary phase was Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.). THIP monohydrate (Lu 2-030) had been synthesized by this company.

During the development of the chromatographic method, the following ion-pairing reagents and column packing materials had also been tried: dioctyl sulpho-

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succinate, sodium salt (Sigma, St. Louis, MO, U.S.A.), perfluorohexanesulphonic acid (Riedel-de Haën), PIC B-7 (Waters Assoc., Milford, MA, U.S.A.), trifluoroacetic acid (Merck-Schuchardt) and LiChrosorb-NH_2 (5 or 10 μm), LiChrosorb RP-2 (5 or 10 μm), Nucleosil 10 CN, Spherisorb S5 CN and Spherisorb S5 ODS.

Equipment

HPLC was carried out with an LC-XPD pump (Pye Unicam, Cambridge, Great Britain), operated at a constant flow-rate, a Rheodyne 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 50-μl loop, a Uvikon 725 variable-wavelength UV absorbance detector (Kontron, Zürich, Switzerland), in most experiments operated at 217 nm, and, placed in series downstream from the UV absorbance detector, an electrochemical detector constructed by our company's engineer, largely according to published instructions[3]. The inlet and outlet tube mountings had been reinforced and made more resistant towards dissolution of the glue by passing the tubes through acrylic 9 mm O.D. bushes cemented to the outside of the auxiliary electrode plate. The holes in this glassy carbon electrode plate were drilled 3 mm wide, permitting a ring-formed liner made from PTFE to be pressed around the end of each metal tube, flush with the polished inner surface of the plate. This liner protected the glue against components of the mobile phases. A spacer of thickness 110 μm separated the glassy carbon electrodes. The working electrode had been conditioned in hot paraffin wax as advised[3]. The wiring of the potentiostat had been modified so as to extend its zeroing capability.

The detector signals were recorded and processed by a Model BD 9 two-channel recorder (Kipp & Zonen, Delft, The Netherlands) and two Model 3390A reporting integrators (Hewlett-Packard, Avondale, PA, U.S.A.). The column materials were packed into Knauer tubes (Knauer, Oberursel, G.F.R.) of I.D. 4.6 mm and length 120 mm by a slurry technique, using the Pye Unicam pump and a reservoir of the Kirkland type[4].

Voltammetry of THIP

To determine a suitable potential for operation of the electrochemical detector, a hydrodynamic voltammogram for THIP was constructed. A 50-μl aliquot of a 100 μM aqueous solution of THIP was injected into the HPLC system at a series of detector electrode potentials. The potential was initially +0.70 V, and was increased in steps of 0.02 V until +1.02 V was applied. The mobile phase flow-rate was 2.0 ml min^{-1}. Two injections were made at each potential, and the resulting mean heights and mean areas of the chromatographic peak from THIP were plotted against applied potential.

Linearity and precision

Aqueous solutions of THIP were prepared at concentrations of 1, 2.5, 5, 10, 50, 100, 200 and 500 μM and 1, 1.5, 2, 3, 4 and 5 mM.

In one series of experiments, 50-μl aliquots of the solutions in the range 1–500 μM were injected into the HPLC system, making six cycles in all. A potential of +0.98 V was applied to the working electrode of the electrochemical detector. In another series of experiments, 10-μl aliquots of all of the solutions were injected, in six cycles, using a detector potential of +0.95 V. At both potential settings the electrode

had been allowed to equilibrate overnight while the voltage was applied and the mobile phase was recirculated. The UV absorbance detector was operated at 217 nm. The mobile phase flow-rate was 2.0 ml min^{-1}.

RESULTS AND DISCUSSION

Chromatographic system

Perfluorooctanesulphonic acid was preferred for use as counter ion for the ion-pairing with THIP because it was able to produce an ion pair that was relatively strongly retained on the column, and the retention of which was reasonably stable towards the influence of changes in the ambient temperature and the qualitative composition of the sample injected. In contrast, systems using a citrate-buffered mobile phase containing 0.15% trifluoroacetic acid and a stationary phase carrying amino or cyano groups were very susceptible to such changes, although an arbitrarily large retention time and a good peak shape could be obtained by adjustment of the concentration of trifluoroacetic acid.

When eluted with a mobile phase containing perfluorooctanesulphonic acid, perfluorohexanesulphonic acid or dioctyl sulphosuccinate, a C_{18} silica retained the ion pair more strongly than did a C_2 silica of the same particle size, as would be expected. Nucleosil 5 C_{18} seemed to offer a better shape of the peak from THIP than did Spherisorb S5 ODS.

Using the final choice of column and mobile phase composition, THIP was eluted from the column in 3.2 min. A chromatogram is shown in Fig. 1. The sample injected was an aqueous solution containing THIP, which had been formed by hydrolysis of a derivative from THIP. The left-hand trace refers to measurement of UV absorption at 217 nm and the right-hand trace is the signal from the electrochemical detector, operated at an electrode potential of +0.98 V.

The application of electrochemical detection involves restrictions in the choice of the composition of the mobile phase. The pH must allow oxidation (or reduction) of the solute of interest. A large proportion of organic solvent in the mobile phase will increase the impedance of the mobile phase (*iR*) and with it the uncompensated resistance between the reference electrode and the working electrode, and this may make an increase in the applied potential necessary in order to avoid an unacceptable limitation of the linear working range[5], although the cell design advised by Lankelma and Poppe[3] reduces the influence of *iR* on the potential of the working electrode. In my experience, lithium perchlorate is useful for simultaneous adjustment of the

Fig. 1. Chromatogram of a sample containing THIP formed by hydrolysis of a derivative from THIP. Mobile phase: 0.02 *M* sodium acetate (pH 4.5)–tetrahydrofuran (90:10) with added perfluorooctanesulphonic acid (5 mmol l^{-1}) and lithium perchlorate (50 mmol l^{-1}); flow-rate, 2.0 ml min^{-1}. Column: 120 × 4.6 mm I.D., packed with Nucleosil 5 C_{18}. Detectors (placed in series): UV absorption, wavelength 217 nm, 0.1 a.u.f.s. (left-hand trace), and electrochemical detector, electrode potential +0.98 V (right-hand trace).

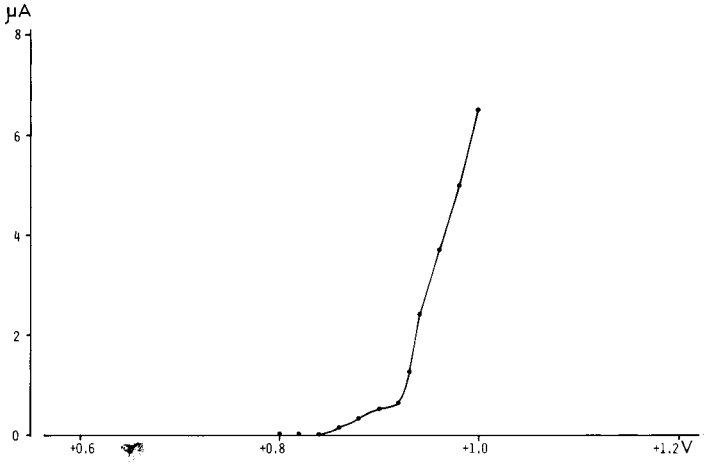

Fig. 2. Peak current (μA) from the electrochemical detector as a function of applied working electrode potential when 5-nmol samples of THIP were injected into the HPLC system.

retention time, suppression of peak tailing and increasing the electrical conductivity of the mobile phase. The use of it as a supporting electrolyte has been reported recently[6].

Choice of electrode potential

The voltammogram obtained from mean peak heights is shown in Fig. 2. No difference was noted when mean peak areas were plotted instead. When a working electrode potential of +0.84 V or less was applied, no measurable oxidation of THIP occurred. Application of a potential exceeding +1.01 V caused the operational amplifiers of the potentiostat to work in the saturated manner, *i.e.*, the amplifier outputs were maximal and thus not able to grade the signal.

An electrode potential of +0.98 V was selected for further experiments in which the detection of low THIP concentrations was desired, whereas a potential of +0.95 V was used for experiments on the linear response range because the lower potential allowed larger amounts of THIP to be detected without amplifier overload.

Calibration graphs: linearity and precision

Fig. 3 shows double logarithmic plots of the response from THIP (mean values) *versus* the amount injected, obtained by the electrochemical detector at electrode potentials of +0.98 and +0.95 V (below), and by the UV absorbance detector (above). The bars indicate standard deviations, but are diminutive at most points when UV absorption was measured. The coefficient of variation (C.V.) ranged from 16.5 to 34.2 in electrochemical detection and from 2.2 to 191.1 in UV detection, but in the latter instance the C.V. did not exceed 12.8 for sample amounts ranging from 0.5 to 50 nmol, whereas high C.V. values were found even with the larger sample sizes when electrochemical detection was used.

The electrochemical response curve obtained from peak areas had a shape similar to that in Fig. 3, but in this instance the C.V. was even larger (20.8–62.2). A reason for the "flatness" of the lower part of the electrochemical detection curves may be addition of a relatively large signal from noise to the detector signal with these

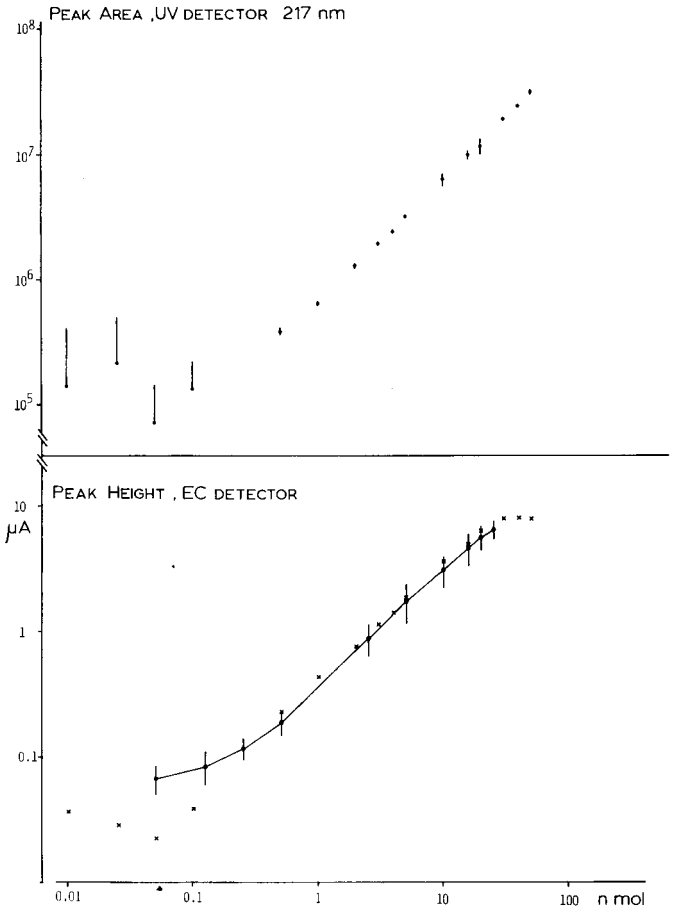

Fig. 3. Detector responses to 0.01–50 nmol of THIP injected into the HPLC system. Above: UV absorbance at 217 nm (peak areas, integrator units). Below: electrochemical (EC) detector operated at +0.98 V (●) or +0.95 V (×) (peak heights, μA). Bars indicate standard deviations (n = 6).

small amounts, and similarly uncertain responses were recorded from the UV detector in this range. The sigmoidal shape of the upper part of the electrochemical detection curves is likely to reflect saturation of processes in the electrochemical detector cell, or amplifier overload.

The electrochemical detector is likely to work in the voltammetric mode at the mobile phase flow-rate and the spacer thickness used[3], and in this instance peak heights should be used for quantitation[7]. In accordance with this, the C.V. was smaller when peak heights were used.

CONCLUSION

The procedure described here is a reliable means for quantitation of the GABA agonist THIP in dilute aqueous solutions. When an electrochemical detector with a large electrode surface area was used, amounts of 50 pmol per injection could be

detected with a C.V. of 25.6 ($n = 6$). Detection by a UV absorbance detector operated at 217 nm is accurate for samples of 500 pmol or more per injection. An advantage of the use of the electrochemical detector is the specificity that can be obtained.

The method has been used successfully in studies on the *in vitro* hydrolytic formation of THIP from THIP derivatives, of which only one was detectable (oxidizable) at the potential used.

ACKNOWLEDGEMENTS

Mr. Hans Mørch, electronic engineer, is thanked for the construction of the electrochemical detector, and thanks are due to Mrs. Elsebeth Johansen for her patience and skillful technical assistance in applying it.

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Note

Rapid extraction and measurement of morphine and opiate antagonists from rat brain using high-performance liquid chromatography and electrochemical detection

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(First received November 26th, 1981; revised manuscript received December 28th, 1981)

A major site of action of opiates is the central nervous system. Following parenteral administration, these drugs penetrate the blood–brain barrier and enter the brain in concentrations high enough to induce the classic opiate effects having a central component (*e.g.*, antinociception, respiratory depression, constipation, etc.). Hence, in mathematical treatments of the relation between drug concentration and observed effect, brain concentration is preferred over administered dose[1,2].

Morphine concentration in brain tissue is often determined by first isolating the drug using a liquid–liquid extraction technique[3] and then quantifying the results using one of several available methods such as thin-layer chromatography, spectrofluorometry, immunoassay, electron capture or mass spectrometry[4,5].

A simplified extraction procedure has been reported for morphine and opiate antagonists[6] in which brain homogenate is adjusted to pH 8.9 by glycine buffer and then suspended on a polar matrix for exposure to the extracting solvent. This method is highly efficient and considerably more rapid than older techniques.

Recently, several authors have shown that solutions or blood extracts containing morphine or certain other opiate agonists and antagonists can be analyzed using high-performance liquid chromatography (HPLC) and electrochemical detection[7–9].

The present report describes a protocol by which the prototype opiate agonist, morphine, and antagonist, naloxone, can be rapidly extracted from rat brain and measured by HPLC with electrochemical detection. By combining and modifying the previous extraction and measurement procedures for compatibility, we found the sensitivity of this process to be sufficient to detect nanogram levels of morphine and naloxone in specific brain regions of rats.

MATERIALS AND METHODS

Extraction

Whole brains or brain sections from male Sprague-Dawley rats were homogenized by Teflon® pestle tissue grinder or sonicated (Bronwill, Rochester, NY, U.S.A.) in 1–4 ml of borate buffer (0.05 *M* boric acid, 0.043 *M* sodium borate) at pH 8.9. Borate buffer was found to be less reactive than glycine buffer at the electrochemical detector. Naltrexone (400 ng) was added to the buffered homogenate to serve as the

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internal standard. This mixture was then placed into extraction columns using Eppendorf pipettes. The extraction columns consisted of an inert hydrophilic material of large surface area, prepacked in disposable polypropylene barrels (ClinElut®, Analytichem International) to which we attached metal valves.

Two minutes after adding the homogenate, spiked with naltrexone, to the extraction columns, 30 ml of solvent was added. Ethanol–chloroform (10:90) is the solvent of choice for morphine, chloroform alone for naloxone[3,6]. The extracting solvent was then drained from the columns into tapered centrifuge tubes. Two additional 2-min exposures to solvent (10-ml volumes each) were followed by a 10-ml wash by solvent. Typically, some solvent remained in the extraction columns.

Special precaution was taken to ensure that solvent only contacted chloroform-resistant plastic, glass or stainless steel. Contact with other materials resulted in unwanted peaks on the chromatogram. Similarly, all glassware was siliconized (Siliclad®; Clay Adams, Parsippany, NJ, U.S.A.) before use to minimize the known problem of morphine adsorption.

The extraction solvent was evaporated from the centrifuge tubes by placing the tubes in a 55°C water-bath and passing filtered air or nitrogen over the open ends. The small amount of residue remaining after evaporation, pale yellowish green in color, was used immediately or refrigerated in the evaporation tubes at 5°C until analyzed. For the analysis, 100 μl of HPLC-grade methanol was added to the tubes using an Eppendorf pipette. The tube was then gently shaken for approximately 1 min, after which samples were injected by Hamilton syringe (No. 705) into the 20-μl sample loop of the HPLC apparatus.

Apparatus

The method used for measuring morphine concentration was adapted from previous reports[7,9]. The HPLC equipment was a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model LC-303 using a Waters M-45 solvent delivery system, Rheodyne sample injector (Model 7125), 20-μl sample loop (Model 7022), LC-3 amperometric detector and glassy carbon electrode. The reverse-phase column (RSil C18HL, 10 μl) had dimensions 25 cm × 1/4 in. O.D. × 4.6 mm I.D. (Alltech, Deerfield, U.S.A.). A potential of +0.6 to +0.8 V across the electrochemical detector provided good peak height, peak shape and signal-to-noise ratio.

Mobile phase

The mobile phase consisted of methanol–water (25:75) containing 50 m*M* tetramethylammonium hydroxide and adjusted to pH 6.1 with phosphoric acid. All chemicals were of HPLC grade and the solution was degassed and filtered (2 μm) before use. The mobile phase was delivered at a rate of 1.5 ml/min at ambient room temperature. Back pressure at this flow-rate was approximately 1500 p.s.i.

Drug standards

Morphine sulfate (Mallinckrodt, St. Louis, MO, U.S.A.), naloxone hydrochloride (Endo Labs., Garden City, NY, U.S.A.) and naltrexone hydrochloride (Endo Labs.) standards were made by dissolving the appropriate salts in HPLC-grade water.

RESULTS AND DISCUSSION

Shown in Fig. 1 are typical chromatograms of rat brain homogenate to which measured amounts of morphine sulfate, naloxone hydrochloride and naltrexone hydrochloride were added. Retention times for morphine, naltrexone and naloxone were approximately 5 min, 6.5 min and 8 min, respectively. Consistent with previous reports, the sensitivity of the electrochemical detector is about four times greater for morphine than for naloxone or naltrexone. As can be seen, at this sensitivity there are relatively few unwanted peaks or interferences with the peaks corresponding to the drugs of interest. At very high sensitivity (10 nA/V, 0.1 V full scale) a peak interfering with morphine (but not naltrexone or naloxone) was sometimes detected. The appearance of this peak was most pronounced when large amounts of tissue in proportion to chloroform were added to the extraction columns. Best results were obtained when tissue homogenate penetrated the column material less than about one fourth of its total length. Pilot studies indicated that per cent recovery was greater than 80% even under poor extraction conditions. Under optimal conditions, recovery would be expected to approach the high values originally reported by Sprague and Takemori[6].

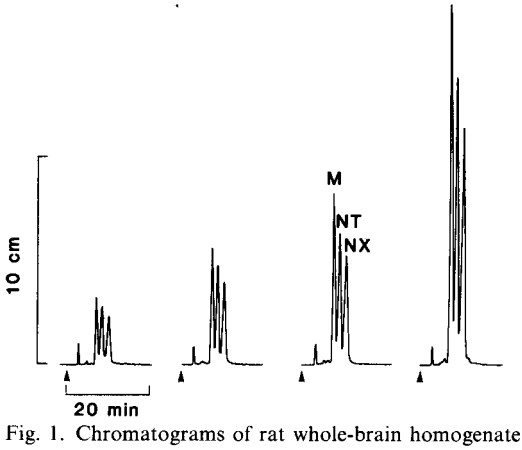
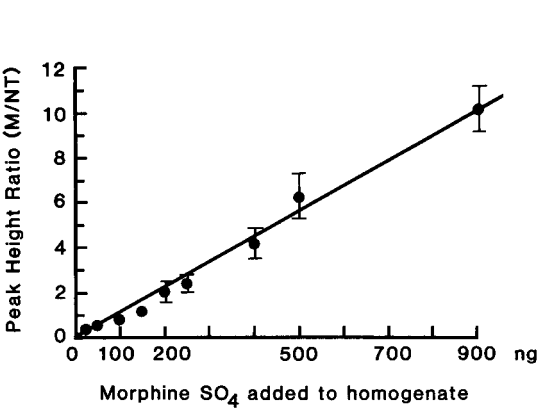


Fig. 1. Chromatograms of rat whole-brain homogenate showing changes in peak height ratios with increasing concentrations of morphine (M), naltrexone (NT) and naloxone (NX) added to the homogenate. Morphine sulfate concentrations are 200, 400, 500 and 900 ng. Naloxone and naltrexone concentrations are four times the concentration of morphine in each case. Chromatograms shown are from a column that had been used in several hundred analyses. With a new column, separation of the three drugs is more complete, with no overlap. Flow-rate = 1.5 ml/min; 10 nA/V, 1 V full scale; +0.6 V; 1400 p.s.i. Arrows indicate time of injection; 10 cm is approximately 5 nA.

For quantifying the amount of morphine present in brain tissue, the ratio of the height of the morphine peak to the height of the naltrexone peak was found to be convenient and reliable. The calibration curve for this measure was obtained by adding known amounts of morphine sulfate and naltrexone hydrochloride to the brain homogenate prior to extraction. The results for morphine are shown in Fig. 2. There is a good linear relationship ($r = 0.96$) between the amount of morphine sulfate added to the homogenate and the ratio of morphine to naltrexone peak heights. The equivalent calibration curve for naloxone is shown in Fig. 3.



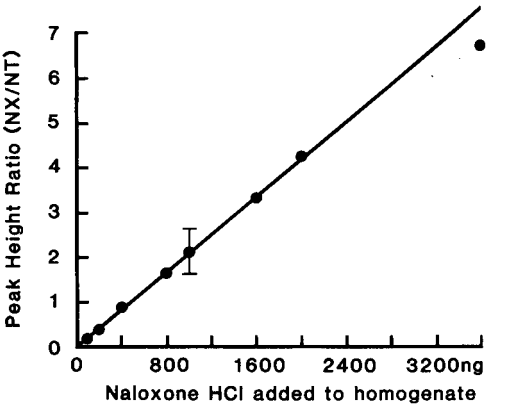


Fig. 2. Relationship between measured peak height ratio (morphine sulfate to naltrexone hydrochloride) and amount of morphine sulfate added to whole-brain homogenates of untreated rats. M = Morphine sulfate; NT = naltrexone hydrochloride. The data points represent the mean (± S.E.M.) of at least four runs. Where not shown, error is within data point symbol.

Fig. 3. Relationship between measured peak height ratio (naloxone hydrochloride, NX, to naltrexone hydrochloride, NT) and amount of naloxone hydrochloride added to whole-brain homogenate of untreated rats.

The procedure described in this paper was applied to measuring morphine levels in specific brain regions following subcutaneous (s.c.) administration of the drug to rats. The regions were dissected by the method of Glowinski and Iversen[10] at various time intervals following drug administration. A representative chromatogram of brainstem tissue is shown in Fig. 4. The other brain regions showed a similar pattern. Preliminary results from rats given morphine sulfate 60 min prior to testing show 223 ± 25 (S.D.) ng/g, 765 ± 206 ng/g and 1085 ± 293 ng/g of morphine sulfate present in brainstem following s.c. administration of 7.5, 35 and 55 mg/kg, respectively.

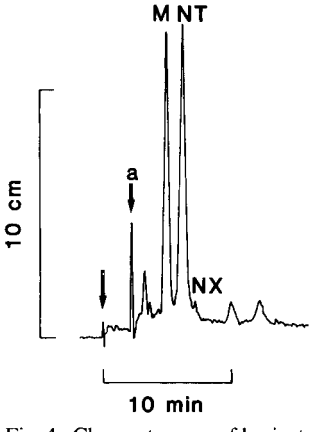


Fig. 4. Chromatogram of brainstem tissue from a rat that received 7.5 mg/kg morphine sulfate s.c. 60 min before and 0.5 mg/kg naloxone hydrochloride s.c. 30 min before testing. Flow-rate = 1.5 ml/min, 10 nA/V, 0.1 V full scale; +0.6 V; 1400 p.s.i. Unlabelled arrow indicates time of injection. Arrow labelled "a" indicates peak produced by solvent front; 10 cm is approximately 5 nA.

Morphine was detected in brain regions following s.c. doses as low as 2.5 mg/kg given 60 min before testing. Naloxone was not as readily detected at the subcutaneous doses normally used (0.05–0.25 mg/kg), hence brain levels were determined by pooling the extractions of several rat brains, by extrapolation from results of higher-than-normal administered doses, or by using the less convenient +0.8 V potential.

Morphine metabolites were not systematically examined in the present study. Normorphine can be separated and detected by HPLC with electrochemical detection, as has previously been reported[9]. Morphine-3-glucuronide analysis has also been described[9]. In a pilot study to measure morphine glucuronide, brain homogenates of rats pretreated with 80 mg/kg morphine sulfate s.c. were incubated with β-glucuronidase (Glucurase™; Sigma, St. Louis, MO, U.S.A.) at 37°C for 20 h and then extracted and measured as usual. Comparison with non-incubated homogenate samples showed a 20% increase in the ratio of morphine to naltrexone peak heights, indicating the presence of morphine glucuronide in amounts consistent with determinations using other methods[11].

In conclusion, the procedure described in the present report appears to offer a rapid and effective means to measure morphine and opiate antagonist levels in brain tissue. Application of this procedure to other types of tissue is presently being investigated.

ACKNOWLEDGEMENTS

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Note

Determination of morphine, diamorphine and their degradation products in pharmaceutical preparations by reversed-phase high-performance liquid chromatography

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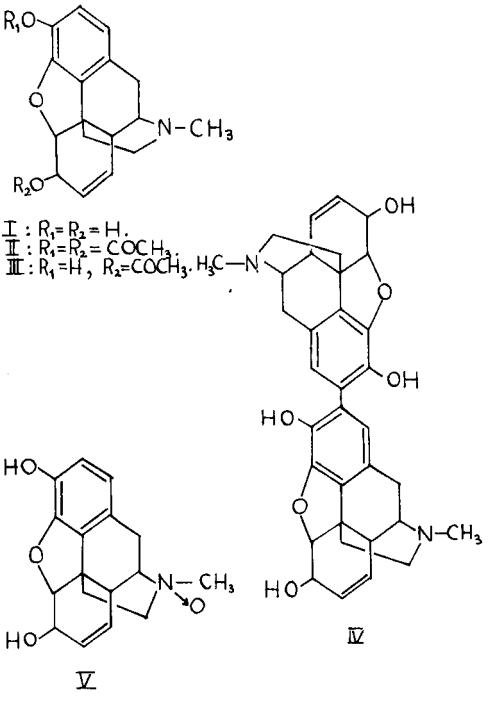
Morphine (I) and its diacetyl derivative diamorphine (II) are frequently used in aqueous oral and parenteral medicines. Diamorphine is known to undergo hydrolysis in aqueous solution to the monoacetyl derivative (III) and morphine itself[1]. Morphine is more stable in aqueous solution than diamorphine, but does decompose on



IV V

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heating in strong base[2] and at milder pH conditions on storage[2,3]. The degradation products of morphine are pseudomorphine (IV) and morphine-N-oxide (V)[2], pseudomorphine being by far the major degradation product.

Methods have been described for the selective determinations of morphine and its degradation products[3] and for the determination of diamorphine and morphine mixtures[4] by high-performance liquid chromatography (HPLC). However, the method described in this report can be used to determine both diamorphine and morphine and their degradation products with only minor variations to the liquid phase. The method, which involves an ion-pair technique, is rapid, easy to perform and highly pH dependant. This method has been used to study the stability of morphine and diamorphine oral mixtures and morphine intrathecal injections.

MATERIALS AND METHODS

Apparatus and materials

The separations of diamorphine and morphine mixtures were carried out on a Pye Unicam LC3 chromatograph coupled to a Hypersil ODS (particle size 5 μm) column, 10 cm × 5 mm I.D., via a Rheodyne 7125 injection valve with a 20-μl loop. Flow-rate was set at 2.0 ml min^{-1} and detection wavelength was 284 nm with a sensitivity of 0.32 a.u.f.s. Peaks were recorded on a Bryans 28000 chart recorder.

Diamorphine hydrochloride and morphine hydrochloride were purchased from Macfarlan Smith Ltd., and 6-monoacetylmorphine was kindly supplied by the Laboratory of the Government Chemist. All standards were used as supplied. The mobile phase, which consisted of 0.01 *M* aq. sodium pentanesulphonate–acetonitrile–orthophosphoric acid (69.5:30:0.5), was prepared freshly each day and degassed before use.

The separations of morphine sulphate and pseudomorphine were performed using an Ultrasphere ODS (particle size 5 μm) column, 25 cm × 4.6 mm I.D., coupled via a Rheodyne 7125 injection valve with 20-μl loop to a single-piston Altex 110A metering pump. The variable wavelength Pye Unicam LC3 UV detector was set at 281 nm and peaks were recorded on a potentiometric Tekman TE200 pen recorder.

Morphine sulphate B.P. (May and Baker) was used as supplied. Pseudomorphine was prepared by the method of Bentley and Dyke[5]; melting point and infrared spectrum agreed with published data[5]. Acetonitrile (Fisons, HPLC grade), sodium pentanesulphonate (Fisons, HPLC grade) and orthophosphoric acid (BDH, AnalaR grade) were used as supplied.

The mobile phase which afforded the best separation of morphine sulphate and pseudomorphine consisted of 0.01 *M* aq. sodium pentanesulphonate–acetonitrile–orthophosphoric acid (69.95:30:0.05) at a flow-rate of 1.0 ml min^{-1} (2000 p.s.i.). The recorder was set at 0.16 a.u.f.s. to record morphine peaks at 80–90% of the full scale deflection.

Procedure

In the study of diamorphine solutions, the following procedure was carried out. Serial dilutions of standard solutions of diamorphine, monoacetylmorphine and morphine were prepared freshly each day in the range of 0.1–2.0 mg ml^{-1}, and calibration curves were produced.

For the calibration of pseudomorphine concentration *versus* peak height and peak height ratio (pseudomorphine/morphine), the following solutions were prepared: A, containing 200 mg of morphine sulphate in 100 ml of water; B, containing 20 mg of pseudomorphine in 100 ml of the mobile phase. From solutions A and B, calibrator solutions C1–C6 were prepared, containing 0, 4, 8, 12, 16 and 20 μg ml^{-1} pseudomorphine respectively and each containing 200 μg ml^{-1} morphine sulphate.

In both studies, each standard solution was injected onto the column twice to give data points which were mean values of two measurements. When assaying samples under test, a full set of calibration points was plotted for each set of samples. The "best fit" straight line being plotted by regression analysis.

The relationship between acid concentration in the mobile phase and retention times of morphine and pseudomorphine was investigated, as was the effect of varying the concentration of the ion-pair reagent.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram produced by a mixture of the three standard materials morphine, diamorphine and 6-monoacetylmorphine under the conditions described for the separation of diamorphine and morphine mixtures. Total analytical time is short, retention times being morphine 37 sec, 6-monoacetylmorphine 58 sec and diamorphine 111 sec. The resolution is good, with an R_s value of 2.79 between 6-monoacetylmorphine and diamorphine, and a capacity factor, k', of 3.11. No internal standard is necessary as the reproducibility of the injection valve system is excellent; a co-efficient of variation of only 0.78 % was found between 20 repeat injections of the same standard. Linearity of the calibration curves is good in the range studied and the limit of detection is 2.0 μg ml^{-1}.

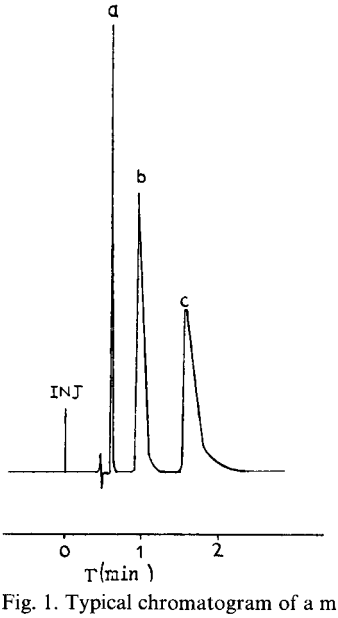

Fig. 1. Typical chromatogram of a mixture of morphine (a), 6-monoacetylmorphine (b) and diamorphine (c) standard solutions.

The plots of peak height ratio (pseudomorphine/morphine) and pseudomorphine peak height *versus* pseudomorphine concentration both gave direct linear relationships, under the adapted conditions described for this assay, with standard deviations of 0.00174 for peak height ratios ranging from 0 to 0.124 and 0.274 mm for pseudomorphine peak height values ranging from 0 to 23.5 mm. The reproducibility of the method, was assessed by running the standard solution C6 (20 μg ml^{-1} of pseudomorphine) five times. The standard deviation of five results was 0.31 μg ml^{-1} and the co-efficient of variation was 1.55%.

The limit of detection of pseudomorphine is 0.4 μg ml^{-1}. At the higher concentrations of pseudomorphine measured a further peak is detectable which is thought to be morphine-N-oxide.

The plot of pseudomorphine peak height (at 0.02 a.u.f.s.) *versus* concentration, between 0 and 1.0 μg ml^{-1} also gives a straight line.

Fig. 2 shows a chromatogram produced by a mixture of morphine and pseudomorphine standard solutions. Morphine-N-oxide is thought to be present as an impurity of pseudomorphine.

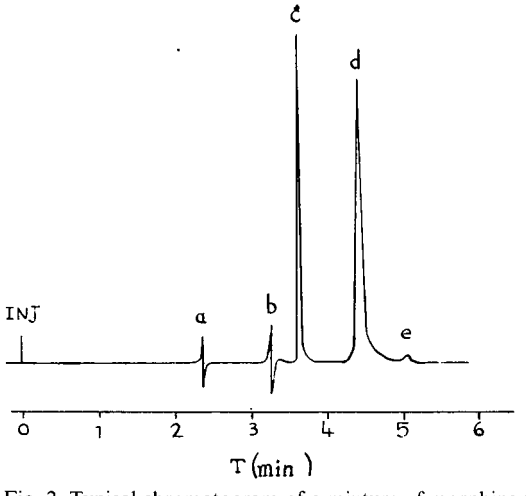

Fig. 2. Typical chromatogram of a mixture of morphine (c) and pseudomorphine (d) standard solutions. Other peaks: a = solvent front; b = unidentified; e = probably morphine-N-oxide.

Table I illustrates the effects of varying the mobile phase. The effect of acid concentration is highly significant giving the optimum R_s value at a pH of about 1.9. However, under these conditions the pseudomorphine is virtually non-retained and the solvent front interferes with the measurement of peak heights. The best chromatographic conditions exist at a pH of about 2.6. At this pH the pseudomorphine is well retained, a good separation of morphine and pseudomorphine occurs and peaks are sharp as shown by the R_s value. A value for N (the number of theoretical plates) of 5100 plates per metre was obtained under these conditions. Above this pH value the peaks tend to broaden as the retention volumes increase, leading to inaccuracies in quantitative measurements. At pH values between 2.10 and 2.20 peaks become inseparable and low R_s values result.

When the ion-pair reagent is removed from the mobile phase, both the mor-

TABLE I

THE EFFECTS OF VARYING THE MOBILE PHASE AND RESOLUTION OF MORPHINE AND PSEUDOMORPHINE

Solvent: acetonitrile–water–orthophosphoric acid.

$$R_s = \frac{t_R\,(B) - t_R\,(A)}{\frac{1}{2}\,[W\,(B) - W\,(A)]}$$

$$k' = \frac{t_R - t_m}{t_m}$$

where t_R (B) = retention time of pseudomorphine in seconds, t_R (A) = retention time of morphine in seconds, W (B) = peak width of pseudomorphine in seconds, W (A) = peak width of morphine in seconds and t_m = retention time of non-retained solute, taken as solvent front.

Solvent	*Concentration of sodium pentane-sulphonate (M)*	*pH*	*Retention volume (ml)*		R_s	*Capacity factor (k') for pseudomorphine*
			Morphine	*Pseudomorphine*		
30:70:0.00	0.01	5.95	4.02	6.18	5.31	1.75
30:70:0.05	0.01	2.58	3.78	4.47	5.13	0.96
30:70:0.10	0.01	2.40	3.53	3.95	4.17	0.77
30:70:0.25	0.01	2.14	3.27	3.28	0.50	0.52
30:70:0.50	0.01	2.10	3.18	3.10	−1.67	0.44
30:70:2.50	0.01	1.92	3.17	2.45	−28.67	0.15
30:70:0.05	Nil	2.20	4.53	8.75	4.96	3.01
30:70:0.05	0.005	2.30	4.02	5.30	3.67	1.39
30:70:0.50	Nil	1.90	3.15	3.05	−1.00	0.408

phine and the pseudomorphine are retained on the column for much longer periods of time and peaks become broader and start to tail. On adding more acid to the mobile phase without ion-pair reagent, peaks again become sharp and less well retained, but their separation is poor. The ion-pair reagent, consequently, is very important in this particular separation.

This method has been used in assessing the stability of an intrathecal morphine injection 2 mg in 10 ml. It has already been shown that this preparation is stable after three autoclave cycles and on storage at ambient and elevated temperatures (37°C) for greater than 3 months. The stability of the product is improved by sealing the ampoules under nitrogen.

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Note

Determination of emetine and cephaeline in Ipecac roots by high- performance liquid chromatography

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Cephaelis ipecachunha Rich (Rubiaceae), commonly known as Ipecac, has been used[1] as an emetic, a diaphoretic and an expectorant since the 18th century. The importance of Ipecac root increased when Rogers[2] reported the use of emetine, one of the major alkaloids of the Ipecac root, as an excellent remedy for amoebic dysentery and amoebic hepatitis[3]. As the roots are marketed directly and there is the possibility of adulteration, most of the pharmacopoeas[4–6] specifically mention the use of authentic roots with a total alkaloid content of at least 2%. As emetine is the most important of the Ipecac alkaloids, the primary task is to determine the emetine content by an accurate and reproducible method. Although several alkaloids have been isolated[7] from Ipecac roots, it appears that the principal constituents of Indian Ipecac roots are emetine, a non-phenolic alkaloid, and cephaeline, a phenolic alkaloid. The total alkaloid content of Indian roots has been reported to be about 2%, including 1.2–1.3% emetine[8]. Several assay procedures are available for the determination of emetine, such as volumetric[9], colorimetric[10–15], thin-layer chromatographic (TLC)[16,17], polarographic[18], densitometric TLC[19,20], oscillopolarographic[21] and combined methods involving column chromatography and spectrometric methods[22–24]. The most common method is the volumetric method as it does not involve sophisticated instrumentation. However, this method gives only the total alkaloid content.

There have been many reports on the quantitative determination of drugs and drug intermediates by high-performance liquid chromatography (HPLC), such as saponins from *Bupleuri radix* (*Bupleurum falcatum* L.)[25], diosgenin from plants[26], alkaloids from opium[27] and tobacco[28] and phenolic lipids from *Anacardium occidentale*[29] etc. to mention a few. Recently, Verpoorte and Baerheim Svendsen[30] separated 24 alkaloids, including emetine and cephaeline, by HPLC using a silica gel column. Gimet and Filloux[31] have described an HPLC method for the determination of three alkaloids, ephedrine, ethylmorphine and codeine, in syrup samples, which has been extended to the separation of sixteen alkaloids beloning to eight different families. This paper describes a simple and rapid direct isocratic HPLC method for the determination of two major alkaloids, emetine and cephaeline, in Indian Ipecac roots.

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EXPERIMENTAL

Apparatus

A Waters Assoc. liquid chromatograph was used, equipped with a Model 6000A solvent pump, a U6K universal injector, a Model 440 UV absorbance detector an Omniscribe recorder. The chromatograph contained a Waters Assoc. stainless-steel column (300 × 3.9 mm I.D.) packed with μPorasil microparticles (10 μm).

Chemicals and reagents

All solvents used were from BDH (Poole, Great Britain) and were glass distilled. Standard samples of emetine and cephaeline used for the preparation of calibration graphs were isolated from Ipecac roots and purified by standard methods. The purity of each alkaloid was confirmed by TLC on silica gel G with the solvent system benzene–ethyl acetate–diethylamine (7:2:1), HPLC, PMR spectroscopy and preparation of their hydrochlorides.

Preparation of standards

A set of ten standard solutions each for emetine and cephaeline was prepared containing 0.95–0.095 mg/ml and 1.0–0.1 mg/ml, respectively, and were stored in air-tight flasks at 20°C in the dark.

Extraction of alkaloids from Ipecac roots for HPLC analysis

Ipecac roots were dried either at room temperature in air or at 40°C in an air oven and then powdered to 40 mesh. Each of the samples (1 g) was extracted with methanol in a glass percolator for 70 h (5 × 15 ml), and filtered quantitatively using Whatman No. 41 filter-paper and washed with methanol (40°C) until the residue was free from alkaloids. The solution was concentrated *in vacuo* and transferred quantita-

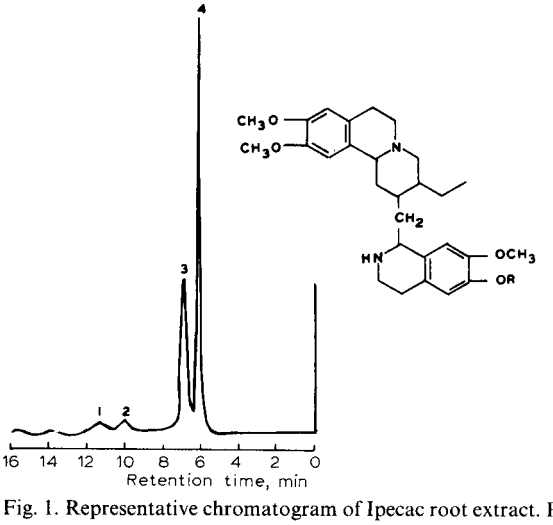

Fig. 1. Representative chromatogram of Ipecac root extract. Peaks: 1, 2 = unknown; 3 = cephaeline, 4 = emetine. Conditions: column, μPorasil (300 × 3.9 mm I.D.); mobile phase, chloroform–methanol–diethylamine (90:10:0.2); flow-rate, 0.5 ml/min; pressure, 150 p.s.i.; detection, UV (280 nm) (1.0 a.u.f.s.); chart speed, 0.5 cm/min; injection volume, 5 μl. Structures: cephaeline, R = H; emetine, R = CH_3.

tively into a volumetric flask using a sample clarification kit consisting of a 10-ml syringe, a Swinney filter holder and millipore filters (0.5 μm). The solution was then made up to a suitable concentration with methanol. A volume of 5 μl of the solution was injected into the chromatograph with a 25-μl Hamilton syringe. A representative chromatogram is shown in Fig. 1.

RESULTS AND DISCUSSION

Separation of emetine and cephaeline was achieved using a μPorasil column (300 × 3.9 mm I.D.) using chloroform–methanol–diethylamine (90:10:0.2) as the mobile phase under isocratic conditions at a flow-rate of 0.5 ml/min and a recorder chart speed 0.5 cm/min. The UV detector was set at 280 nm (1.0 a.u.f.s.). The retention times of emetine and cephaeline were 372 and 420 sec, respectively, and were dependent on the composition of the mobile phase. The determination of emetine and cephaeline was successfully accomplished by comparison with a calibration graph for each alkaloid (Fig. 2) of plotting peak height *versus* amount of compound injected. The relationships were found to be linear over ten measurements at different concen-

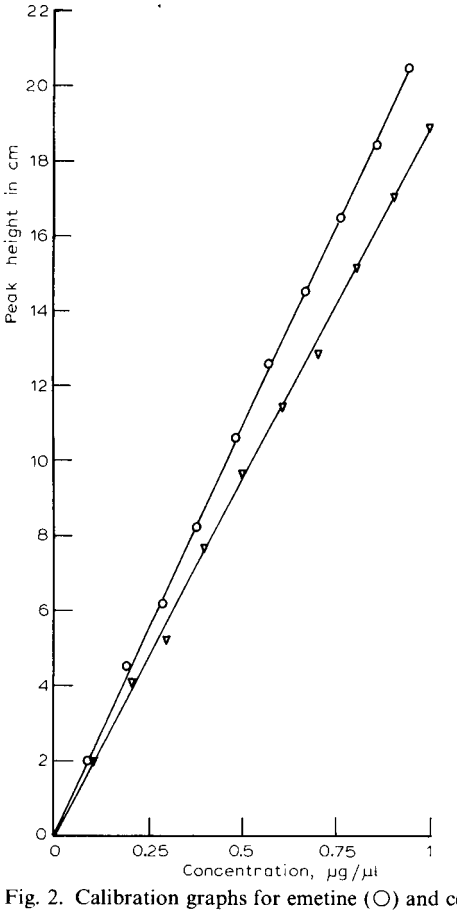

Fig. 2. Calibration graphs for emetine (○) and cephaeline (▽).

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF EMETINE AND CEPHAELINE IN IPECAC ROOTS COLLECTED FROM THE DARJEELING AREA, INDIA

Analysis No.	*Emetine (%)*	*Cephaeline (%)*
1	1.28	0.53
2	1.28	0.53
3	1.30	0.55
4	1.28	0.52
5	1.31	0.55
6	1.25	0.53
7	1.28	0.53
8	1.26	0.50
9	1.31	0.51
10	1.28	0.53
Average	1.28	0.53
Standard deviation	0.02	0.015
Coefficient of variation	1.5	2.8

trations. For analysis 1 g of the sample was used and the method was quantitative and reproducible based on ten measurements. The concentrations of emetine and cephaeline in the samples were found to be 1.28 and 0.53%, respectively. The same results were obtained when more than 1 g of root was used. The coefficient of variation of the method ($n = 10$) was 1.5% for emetine and 2.8% for cephaeline (Table I).

The method is very convenient for the routine simultaneous determination of emetine and cephaeline in Ipecac roots, as the preparation of the sample is easy, the analysis time is short (about 10 min after extraction and preparation of the calibration graphs) and the precision is satisfactory.

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Journal of Chromatography, 238 (1982) 530–534
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Note

Separation of diastereomeric pyrrolizidine alkaloids by chromatography on alkalised silica gel

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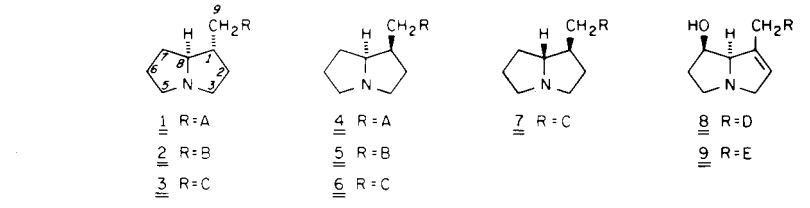
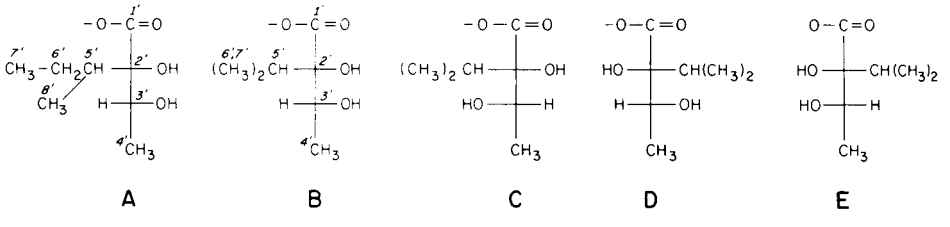
The chemistry of the pyrrolizidine alkaloids is of considerable importance due to their wide distribution in the plant kingdom, their hepatotoxicity and carcinogenicity and their more recent discovery in specialised organs of Lepidoptera[1,2]. Efficient separation techniques are vital because of the frequent co-occurrence among pyrrolizidine alkaloids of several structurally related and diastereomeric bases. Separation of pure diastereomers from mixtures has remained one of the most difficult tasks in this field. For example, the complete separation of the diastereomeric alkaloids intermedine (8) and lycopsamine (9) by column, thin-layer or paper chromatographic methods has not yet been achieved, even on an analytical scale. Resolution satisfactory for analytical purposes is possible by gas chromatography (GC) of the mixed alkaloids as their trimethylsilyl[3] or alkyl boronate[4] derivatives. When first described from *Amsinckia intermedia*, *A. hispida* and *A. lycopsoides*, intermedine and lycopsamine were separated in small amounts by a series of counter-current separations[5].





0021-9673/82/0000–0000/$02.75

During our work on pyrrolizidine alkaloids of *Heliotropium curassavicum*[6] we developed a new chromatographic method to separate the diastereomeric alkaloids curassavine (1) and heliocurassavinine (4), coromandaline (2), heliocoromandaline (5), heliovicine (3), heliocurassavicine (6) and heliocurassavine (7), which is described here. This method involves column chromatography on alkalised silica gel with chloroform–methanol–25% ammonia (17:3.8:0.25) as eluent and is simple and effective.

When our work was completed, a paper by Frahn *et al.*[9] appeared describing the separation of intermedine (8) and lycopsamine (9) as their borate complexes. Their procedures are based on the difference in the degree to which the vicinal glycol groups of different configurations (*erythro* and *threo*) complex with borate and is limited to separation of diastereomers containing such groups. Difficulty was experienced in recovering the free alkaloids in high yields from the borate solutions. Our method is superior in that it involves separation of the alkaloids as such and can be used for separating any diastereomeric pyrrolizidine alkaloids, not necessarily only those alkaloids with vicinal glycol groups of different configurations.

EXPERIMENTAL

Silica gel for column chromatography

Silica gel C or G (TLC grade; ACME, Bombay, India) was shaken well with twice the amount of 0.1 *N* sodium hydroxide solution, poured into an evaporating basin, set aside for 4 h, heated at 120°C for 9 h, powdered well and kept for at least 2 days before use.

Alkaloid mixtures M, P and Q

Extraction and fractionation of the alkaloids of *Heliotropium curassavicum* were as described previously[7,8]. Chromatography of alkaloid fraction A (ether extract before reduction) on a column of neutral alumina gave base M as a mixture of curassavine (1) and coromandaline (2). Alkaloid fraction D (ether extract after reduction) on chromatography over neutral alumina with a chloroform–methanol gradient as eluent gave the alkaloid mixtures P (230 mg) and Q (160 mg). P is a mixture of curassavine (1), coromandaline (2), heliovicine (3), heliocurassavine (4), heliocurassavicine (6) and heliocurassavinine (7). Q is a mixture of curassavine (1), coromandaline (2), heliovicine (3) and heliocoromandaline (5).

Thin-layer chromatography (TLC) was performed on silica gel C or impregnated with *N*/10 sodium hydroxide (20 cm × 5 cm × 0.25 mm) using chloroform–methanol–25% ammonia (17:3.8:0.25) as the solvent system. Alkalised silica gel plates were kept for at least 2 days before use. Spots were visualized with iodine and/or Dragendorff's reagent.

Nuclear magnetic resonance (NMR) monitoring was done on a Varian T-60 instrument and confirmed by using a Bruker HX-270 spectrometer[10].

Column chromatography

Alkaloid mixture P or Q was dissolved in chloroform and applied to a column (2.2 cm I.D.) of alkalised silica gel (50–70 g) set up in chloroform. The column was initially eluted with chloroform and then continuously with chloroform–methanol–

25% ammonia (68:15.2:1) at the rate of 5 drops per minute. 0.5-ml fractions were collected and monitored by TLC followed by NMR.

Structures were established[6–8,10] by high-resolution ^{1}H and ^{13}C NMR, mass spectrometry (MS), GC, rotation and paper electrophoresis of the alkaloids and their hydrolysis products.

RESULTS AND DISCUSSION

Base M (a mixture of curassavine and coromandaline) is homogeneous by TLC on alkalised silica gel with (a) methanol, (b) chloroform–methanol–ammonia (17:2.8:0.2)[11], (c) chloroform–acetone–ethanol–ammonia (10:6:2:2)[12] and (d) benzene–ethyl acetate–diethylamine (14:4:2)[13] as solvent systems. In an attempt to select a solvent system for resolving base M on alkalised silica gel plates, the following systems were tried: various proportions of benzene–ethanol–ammonia, benzene–ethanol–ethyl acetate–ammonia, chloroform–methanol–ammonia, chloroform–methanol–ethyl acetate–ammonia, chloroform–acetone–ethanol–ammonia and benzene–ethyl acetate–diethylamine. Base M showed up as a single spot or at best as an elongated one in all these systems. Finally, however, TLC on silica gel impregnated with 0.1 *N* sodium hydroxide with chloroform–methanol–25% ammonia (17:3.8:0.25) proved to be successful for resolution of base M into two spots, R_F 0.88 (curassavine) and 0.85 (coromandaline). With this system, alkaloid mixtures P and Q could be resolved into more than two spots at low concentrations. This system works best only at temperatures 27–31°C.

The next step was to develop an efficient chromatographic method for the separation of the diastereomeric alkaloids. Preparative TLC was unsuccessful. After standardisation of the conditions (see Experimental), separation of the bases could be effected on a column of alkalised silica gel (TLC grade) with chloroform–methanol–25% ammonia (68:15.2:1) as eluent. To provide an illustration of the method, two typical separations are described herein.

Alkaloid mixture P (220 mg) was chromatographed on alkalised silica gel with the above solvent system as described in the Experimental section with the above solvent system; the results are given in Table I. The first six fractions were pure heliocurassavine (4) followed by mixtures of 4 and curassavine (1). Fractions 9–12 were pure 1 and fraction 13 was a mixture of 1 and heliocurassavine (7). Fractions 14 and 15 were pure 7 followed by a mixture of 7 and heliocurassavicine (6). Pure 6 was eluted next in fractions 17–19, followed by a mixture of 6 and heliovicine (3) and then by pure 3 in fractions 21 and 22. Fraction 23 was a mixture of 3 and coromandaline (2). The last fractions 24 and 25 were pure 2. In this separation, 25 fractions were collected out of which 19 fractions were pure diastereomeric alkaloids. Out of the total recovery (90%), 73% comprised six pure diastereomers and 27% partially separated mixtures.

In a similar way, alkaloid mixture Q (150 mg) was chromatographed; the results are shown in Table II. Fractions 1–5 were pure curassavine (1) whereas fractions 6 and 7 were mixtures of 1 and heliovicine (3). Fractions 8–10 were pure 3 followed by a mixture of 3 and coromandaline (2). Pure 2 was eluted next followed by a mixture of 2 and heliocoromandaline (5). The last fraction was pure 5. In this separation 15 fractions were collected of which 11 represented pure alkaloids. Total

TABLE I

SEPARATION OF THE ALKALOIDS 1, 2, 3, 4, 6 AND 7 FROM ALKALOID MIXTURE P

Fraction No.	*Amount (mg)*	*Alkaloids*	*Approximate percentage abundance (by NMR)*
1–6	25	4	100
7	7	4, 1	60:40
8	9	4, 1	30:70
9–12	35	1	100
13	8	1, 7	65:35
14, 15	15	7	100
16	9	7, 6	30:70
17–19	30	6	100
20	11	6, 3	35:65
21, 22	25	3	100
23	9	3, 2	70:30
24, 25	14	2	100

recovery was 85% of which 69% comprised pure alkaloids and 31% mixtures of partially separated bases.

Fractions were best distinguished by NMR monitoring. Column chromatographic separation with this system works best at temperatures between 27 and 31°C. Humidity may also play a rôle but its importance cannot be specified precisely.

Diastereomeric pyrrolizidine alkaloids have not previously been separated by silica gel chromatography. The method should be applicable to other mixtures of diastereomeric alkaloids with little modification.

TABLE II

SEPARATION OF THE ALKALOIDS 1, 2, 3 AND 5 FROM ALKALOID MIXTURE Q

Fraction No.	*Amount (mg)*	*Alkaloids*	*Approximate percentage abundance (by NMR)*
1–5	40	1	100
6	11	1, 3	60:40
7	12	1, 3	30:70
8–10	29	3	100
11	9	3, 2	65:35
12, 13	11	2	100
14	8	2, 5	40:60
15	7	5	100

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Errata

J. Chromatogr., 234 (1982) 313–320

Page 319, last sentence, and page 320, first sentence, should read "Some observations about the observed inlet adsorption must be made. A more thoroughly deactivated glass column or a fused silica column may be more inert at the injection site".

J. Chromatogr., 236 (1982) 469–480

Page 476, Table I, the missing heading for the last column should be: "*C.V. (%)*".

Page 476, Table I, the value "99.43" in the 4th column under the heading "*Variance*" and in the row "Peak heights" should read "9.43".

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journal of chromatography news section

NEW BOOKS

Gel electrophoresis of proteins: a practical approach, edited by B.D. Hames and D. Rickwood, IRL Press, London, Washington, DC, 1981, XVI + 290 pp., price £ 7.50, US$ 18.00, ISBN 0-904147-22-3.

Reversed-phase high-performance liquid chromatography: Theory, practice and biomedical applications, by A.M. Krstulovic and P.R. Brown, Wiley, Chichester, 1982, *ca.* 187 pp., price *ca.* US$29.95, £16.65, ISBN 0-471-05369-4.

Poycyclic aromatic hydrocarbons in water systems, by D.J. Futoma, T.E. Smith, S.R. Smith and J. Tanaka, CRC Press, Boca Raton, FL, 1981, 200 pp., price US$ 54.95 (U.S.A.), US$ 63.95 (outside U.S.A.).

Organic trace analysis by liquid chromatography, by J.F. Lawrence, Academic Press, New York, 1981, XII + 288 pp., price US$ 34.00, ISBN 0-12-439150-8.

Environmental problem solving using gas and liquid chromatography, by R.L. Grob and M.A. Kaiser, Elsevier, Amsterdam, Oxford, New York, 1982, XIII + 240 pp., price Dfl. 127.00, ISBN 0-444-42065-7.

Environmental speciation and monitoring needs for trace metal-containing substances from energy related processes, edited by F.E. Brinckman and R.H. Fish, U.S. Department of Commerce and National Bureau of Standards, Washington, DC, 1981, XII + 324 pp.

Chemical derivatization in analytical chemistry, Vol. 1, Chromatography, edited by R.W. Frei and J.F. Lawrence, Plenum, New York, 1981, 356 pp., price US$ 39.50.

Ion chromatography, by J.S. Fritz, D.T. Gjerde and C. Pohlandt, Hüthig, Heidelberg, New York, 1982, *ca.* 300 pp., price *ca.* DM 68.00, US$ 38.00, ISBN 3-7785-0760-5.

Benzodiazepines – A handbook, by H. Schütz, Berlin, Heidelberg, New York, 1982, XII + 439 pp., price DM 198.00, *ca.* US$ 92.20, ISBN 3-540-11270-7.

Soft ionization biological mass spectrometry, edited by H.R. Morris, Heyden, London, Philadelphia, Rheine, 1982, XII + 156 pp., price £ 18.00, ISBN 0-85501-706-6.

Data for biochemical research, edited by R.M.C. Dawson, D.C. Elliot, W.H. Elliot and K.M. Jones, Oxford University Press, Oxford, **2nd ed.,** 1982, 600 pp., price £ 25.00, ISBN 0-19-855334-X.

Fourth international symposium Hindelang 1981, edited by R.E. Kaiser, Hüthig, Heidelberg, New York, 1981, 944 pp., price US$ 39.50, ISBN 3-7785-0743-5.

Gmelin, Handbook of inorganic chemistry, U – Uranium, Supplement Volume D2: Solvent extraction of uranium, Springer, Berlin, Heidelberg, New York, **8th ed.,** 1982, XII + 390 pp., price DM 998.00, *ca.* US$464.60, ISBN 3-540-93454-5.

Die Chemische Industrie und ihre Helfer, 1981–1982, Industrieschau-Verlagsgesellschaft, Darmstadt, 1981, 540 pp., price DM 43.00.

Statistics and computer methods in BASIC, by J.D. Lee and T.D. Lee, Van Nostrand Reinhold, New York, 1982, XII + 198 pp., price £ 11.50 (hardback), £ 5.50 (paperback), ISBN 0-442-30474-9 (hardback), 0-442-30475-7 (paperback).

CALENDAR OF FORTHCOMING MEETINGS

June 1–4, 1982
Goslar (near Hannover), G.F.R.

3rd International Symposium on Isotachophoresis
Contact: Dr. C.J. Holloway, ITP 82, Abteilung für klinische Biochemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, G.F.R. (Further details published in Vol. 219, No. 3)

June 6–12, 1982
Frankfurt, G.F.R.

European Meeting on Chemical Engineering and ACHEMA Exhibition Congress 1982
Contact: DECHEMA P.O. Box 970146, D-6000 Frankfurt/M 97, G.F.R.

June 7–11, 1982
Philadelphia, PA, U.S.A.

VI International Symposium on Column Liquid Chromatography
Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Completed program published in Vol. 238, No. 1.)

June 16 + 17, 1982
Szeged, Hungary

Symposium on Advances in Thin-Layer and High-Performance Liquid Chromatography
Contact: Dr. Haleem J. Issaq, Frederick Cancer Research Center, P.O. Box B, Frederick, MD 21701, U.S.A.; or Dr. Tibor Devenyi, Institute of Biochemistry, Hungarian Academy of Sciences, Budapest X1, Hungary. (Further details published in Vol. 216.)

June 18–21, 1982
Lund, Sweden

Flow Analysis II
Contact: Flow Analysis II, c/o The Swedish Chemical Society, Upplandsgatan 6A, 1 tr., S-111 23 Stockholm, Sweden. (Further details published in Vol. 216.)

June 20–23, 1982
Bordighera (near San Remo), Italy

International Symposium on Chromatography and Mass Spectrometry in Biomedical Sciences
Contact: Dr. Alberto Frigerio, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 – 20157 Milan, Italy. Tel.: 35.54.546; Telex: 331268 NEGRI I. (Further details published in Vol. 214, No. 2.)

June 28–30, 1982
East Lansing, MI, U.S.A.

35th American Chemical Society Annual Summer Symposium
Contact: A.I. Popov, Chemistry Department, Michigan State University, East Lansing, MI 48824, U.S.A.

July 11–16, 1982
Washington, DC, U.S.A.

6th International Conference on Computers in Chemical Research and Education (ICCCRE)
Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.

July 11–16, 1982
Louvain-la-Neuve, Belgium

6th IUPAC Conference on Physical Organic Chemistry
Contact: Prof. A, Bruylants, Université Catholique de Louvain, Laboratoire de Chimie Generale et Organique, Batiment Lavoisier 1 Place Louis Pasteur, 1348 Louvain-la-Neuve, Belgium.

July 12–16, 1982
Amherst, MA, U.S.A.

IUPAC Macromolecular Symposium
Contact: James C.W. Chien, Department of Polymer Science & Engineering, University of Massachusetts, Amherst, MA 01003, U.S.A.

July 19–22, 1982
Prague, Czechoslovakia

Prague Microsymposium "Selective Sorbents"
Contact: Dr. F. Svec, c/o Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Heyrovského n.2, 162 06 Prague, Czechoslovakia.

Aug. 11–13, 1982 Hameenlinna, Finland	**6th European Symposium on Polymer Spectroscopy (ESOPS 6)** Contact: Professor Johan Lindberg, Department of Wood and Polymer Chemistry, University of Helsinki, Meritullinkatu 1 A, SF 00170 Helsinki 17, Finland.
Aug. 15–21, 1982 Perth, Australia	**12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.
Aug. 16–20, 1982 Bundoora, Australia	**13th Australian Spectroscopy Conference** Contact: Dr. J.B. Peel, Organizing Secretary, 13th Australian Spectroscopy Conference, Department of Physical Chemistry, La Trobe University, Bundoora, Victoria 3083, Australia.
Aug. 22–28, 1982 Vancouver, Canada	**XIth International Carbohydrate Symposium** Contact: Mr. K. Charbonneau, Executive Secretary, XIth International Carbohydrate Symposium, c/o National Research Council of Canada, Ottawa, Ontaria, Canada K1A 0R6. Tel.: (613) 993-9009; Telex: 053-3145.
Aug. 23–27, 1982 Budapest, Hungary	**22nd International Conference on Coordination Chemistry** Contact: Prof. M.T. Beck, Institute of Physical Chemistry, Kossuth Lajos University, Debrecen 10, H–4010, Hungary.
Aug. 29–Sept. 4, 1982 Kyoto, Japan	**5th International Congress of Pesticide Chemistry** Contact: Rikagaku Kenysho (The Institute of Physical and Chemical Research), 2–1 Hirosawa Wako-shi Saitama Pref. 351, Japan.
Aug. 30–Sept. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A–1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.
Sept. 5–9, 1982 Liège, Belgium	**8th European Workshop on Drug Metabolism** Contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium. Tel.: (32-41)-56.24.80/81. (Further details published in Vol. 225, No. 2.)
Sept. 6–9, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia. (Further details published in Vol. 225, No. 2.)
Sept. 12–17, 1982 Kansas City, MO, U.S.A.	**184th American Chemical Society National Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington DC 20036, U.S.A.
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)

ANNOUNCEMENTS

ANNIVERSARY

The year 1982 marks the 75th anniversary of *Chemical Abstracts*. The *Journal of Chromatography* would like to take this opportunity to congratulate the present and past editors and their assistants on their contribution to the advancement of science and express our gratitude for their work.

60th BIRTHDAY OF PROFESSOR HERIBERT MICHL

On the 15th of May, 1982, Professor Heribert Michl celebrates his 60th birthday. Professor Michl is professor of chemistry at the Agricultural University of Vienna and is one of the pioneers of paper electrophoretic techniques having developed high-voltage techniques as early as 1951. He has also been a contributor to the *Journal of Chromatography* since its inception (a review on "Hochvoltelektrophorese", *J. Chromatogr.*, 1 (1958) 93.

We would like to take this occasion to wish Professor Michl as great a success in his future work as he has achieved up to now.

NEW MEMBER ELECTED TO THE FRENCH ACADEMY OF SCIENCE

Professor Dr. Edgar Lederer was elected member of the French Academy of Science (Académie des Sciences) on the 8th of March, 1982.

The editorial board of the *Journal of Chromatography* would like to take the opportunity to join his many friends in congratulating him on this well-deserved honor.

NOMINATIONS INVITED

THE 1983 STEVEN DAL NOGARE AWARD

The Chromatography Forum of Delaware Valley invites nominations for the *Steven Dal Nogare Award*. This award is given annually for significant contributions to chromatographic theory, instrumentation or applications, and consists of an honorarium and an inscribed plaque. The award will be presented at the 1983 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. The recipient will be expected to give an award address. Nominations should be submitted before June 1, 1982, accompanied by a brief bibliography and a list of accomplishments related to chromatography. Send nominations to Dr. Theodore I. Wishousky, SmithKline Animal Health Products, 1600 Paoli Pike, West Chester, PA 19380, U.S.A.

SHORT COURSE

ANALYTICAL CHEMISTRY SHORT COURSES

The following short course will be held in the Department of Chemistry of the Loughborough University of Technology in Great Britain:

High-performance liquid chromatography (July 5–9, 1982). Fee £195, including residence and all meals.

Further details may be obtained from: Miss C.D. Newton, Department of Chemistry, Loughborough University of Technology, Loughborough, Leics. LE11 3TU, Great Britain. Telephone: (0509) 63171, ext. 351.

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	The publication schedule for further issues will be published later.						
Chromatographic Reviews		251/1		251/2								
Biomedical Applications	227/1	227/2	228	229/1	229/2							

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 review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

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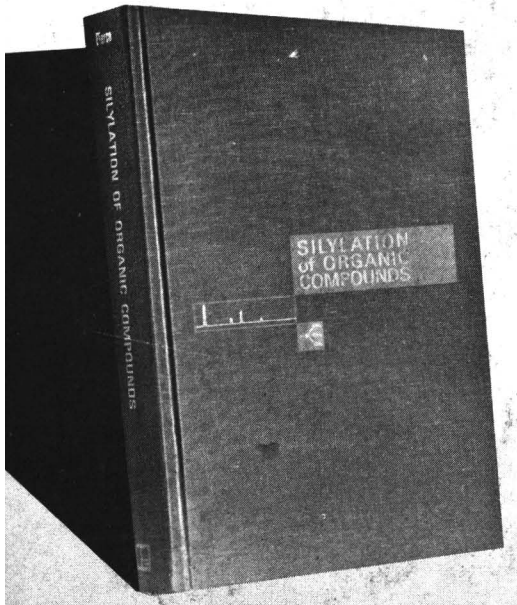

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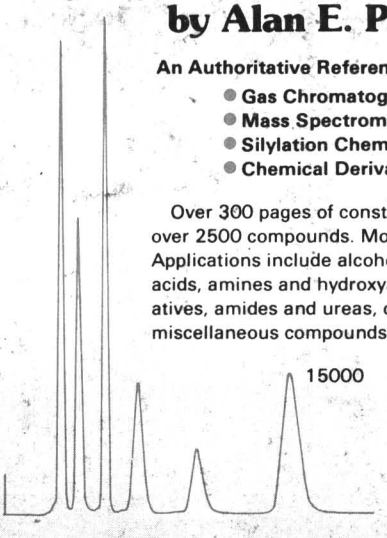

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