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Studies in Natural Products Chemistry

edited by
ATTA-UR-RAHMAN
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University of Karachi, Karachi, Pakistan

The field of natural products chemistry is undergoing a rapid change and is becoming both multi- and interdisciplinary. The isolation, structure determination, synthesis, molecular biology, pharmacology, toxicology and therapeutic uses of naturally occurring compounds are becoming the common interest of chemists, biochemists, biophysicists, biologists, pharmacologists, etc. This awareness has encouraged the establishment of a new series of books which will bring together information from diverse fields of physical and biomedical sciences under the title *Studies in Natural Products Chemistry*. The series will comprise a number of volumes under the editorship of Professor Atta-ur-Rahman. Each volume will have a central theme as a link for the various contributed chapters written by renowned scientists.

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An authoritative review... highly recommended...

Optimization of Chromatographic Selectivity

A Guide to Method Development

by **P. Schoenmakers,** *Philips Research Laboratories, Eindhoven, The Netherlands*

(Journal of Chromatography Library, 35)

"The contents of this book have been put together with great expertise and care, and represent an authoritative review of this very timely topic... highly recommended to practising analytical chemists and to advanced students." (Jnl. of Chromatography)

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This is the first detailed description of method development in chromatography - the overall process of which may be summarized as: method selection, phase selection, selectivity optimization, and system optimization. All four aspects receive attention in this eminently readable book.

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Those developing chromatographic methods or wishing to improve existing methods will value the detailed, structured way in which the subject is presented. Because optimization procedures and criteria are described as elements of a complete optimization package, the book will help the reader to understand, evaluate and select current and future commercial systems.

Contents: 1. Introduction. 2. Selection of Methods. 3. Parameters Affecting Selectivity. 4. Optimization Criteria. 5. Optimization Procedures. 6. Programmed Analysis. 7. System Optimization. Indexes.

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IDEAL MIXED CELLS MODEL OF MULTI-COMPONENT PREPARATIVE LIQUID CHROMATOGRAPHY

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(First received July 12th, 1988; revised manuscript received November 2nd, 1988)

SUMMARY

A simple mixed cells model was used for computer simulation of multi-component separation in liquid chromatography. For a description of multi-component adsorption an equation was derived using the concept of adsorption sites blocking by adsorbed molecules in various ratios. With this model the production rate and recovery ratio were followed. At a given load the maximum productivity is attained at the optimum column length. With longer columns with higher loads, higher optimum production rates may be obtained. Optimization of the feed volume increases the productivity only negligibly. If one component in the mixture predominates, the separation is governed by column overloading with this single compound. Higher production rates may be achieved in gradient elution. In displacement chromatography still higher productivities are obtainable, but in this mode not only the overload limitation but also a low limit of injected amount for succesful separation exists. Compounds with crossing isotherms may be separated, contrary to the "golden rule" in displacement chromatography. If the separation of compound pairs with concave and convex hyperbolic isotherms is computed, the two peaks are replaced by a spike at higher loads. This erroneous result is caused by an unlimited increase in the simple hyperbolic (convex) isotherm near the critical solute concentration.

INTRODUCTION

The concept of the theoretical plate model, introduced by Martin and Synge[1], has been complemented by the discontinuous flow model of Mayer and Tompkins[2] and a basis of the theory of column chromatography was developed that has been very fruitful for the understanding of separations of substances. This was preceded by DeVault's[3] attempt to describe the behaviour of solutes on a column by a set of partial differential equations. These two directions in attempts to describe chromatographic separations quantitatively have been followed up to the present.

The first direction in using the description of a continuous model by set of differential equations was further expanded by Glueckauf[4] and by the introduction of the parabolic approximation of the Langmuir isotherm[5], making possible the

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analytical description of peaks in overloaded columns. Numerical methods for the solutions of these equations have been succesfully applied using a digital computer[6]. The Laplace transform of Langmuir's isotherm made it possible to describe multi-component separations in overloaded columns[7]. A more detailed study of the separation of a two-component mixture has been published[8].

The extension of Houghtons' concept of a two-term approximation of Langmuir's isotherm was expanded in recent years[9–11], culminating in a description of the separation of a two-component mixture, where, however, the mutual influence of the components was neglected[12].

The other direction, the discontinuous flow model, was revived by Seshadri and Deming[13], who considered the influence of isotherm curvature on peak shape. Recent application of this concept to a single elution band assuming a Langmuir isotherm demonstrated clearly its applicability to preparative chromatography[14].

Earlier attempts to describe separations by preparative chromatography[15–17] were based on semi-empirical equations and do not enable one to predict the separations of complicated mixtures or to make *ab initio* calculations.

Very interesting from the point of view of preparative chromatography are the gradient and displacements modes of liquid chromatography. The pioneering work of Hagdahl *et al.*[18] has recently been complemented both theoretically and experimentally[19–21]. The elution and displacement modes of chromatography were compared only experimentally; no simple theoretical assessment of these modes was possible until now.

The basic relationship used in all these studies is the Langmuir isotherm. The single-component Langmuir equation was extended to multi-component adsorption by Butler and Ockrent[22]. This isotherm has been criticized because it is inconsistent with the Gibbs adsorption isotherm for ideal adsorbed solution, unless the monolayer capacities are equal. The proposed approximation for binary isotherms is only speculative[23].

Eble *et al.* extended their previous studies[14] to a two-component mixture[24] and were able to extrapolate their results so that gradient chromatography with a moderately overloaded column could be predicted with reasonable accuracy. However, only results from a two-component mixture were presented. In all these previous studies, the purity of the isolated fraction was not defined; only the "separation resolution" was used for the establishment of suitability for preparative separations.

The model based the solution of a two-term Langmuir isotherm expansion was recently extended to a two-component case[25]. The solution of a multi-component case by this method will hardly be possible.

Snyder *et al.*[27] combined the concept of column blockage, formulated by Knox and Pyper[26], with results from the two-component model[24] so that, with empirical equations, even multi-component separations in moderately overloaded column could be formulated.

We start our consideration with the derivation of a multi-component hyperbolic isotherm. Then optimization of production rates and recoveries in multi-component chromatographic separations using isocratic, gradient and displacement modes are examined, using computer simulation.

BASIC RELATIONSHIPS

The equilibrium concentrations of a compound i in the solid and mobile phases can be described by a simple equation:

$$\frac{c_{iF}}{c_i c_F} = K_i \tag{1}$$

where K_i is the equilibrium constant, c_i the concentration of compound i in the mobile phase, c_{iF} the concentration of the adsorbed compound and c_F the concentration of free sorption sites (both in the solid phase). The amount of compound i in one equilibrium cell, G_i, is therefore

$$G_i = c_i V_M + c_{iF} V_s \tag{2}$$

where V_s is the volume of the solid phase and V_M that of the mobile phase. The capacity of the sorbent is the sum of all sorption sites covered by adsorbed compounds plus free sites:

$$G_D = V_s\left(c_F + \sum_{i=1}^{n} c_{iF} R_i\right) \tag{3}$$

where R_i is the blocking factor, defined as the average number of adsorption sites covered by one molecule of compound i bound to one adsorption site. This possibly oversimplified mechanistic approach enables us to describe rationally multi-component equilibria without introducing any artifical empirical coefficients. After simple substitutions we obtain for the concentration of compound j in the mobile phase (in one equilibrium cell)

$$c_j = \frac{G_j}{V_M + G_D K_j \Big/ \left(1 + \sum_{i=1}^{n} R_i K_i C_i\right)} \tag{4}$$

If there is only one compound present, we arrive to the well known Langmuir isotherm:

$$c_{iF} = \frac{c_i K_i G_D / V_s}{1 + K_i R_i c_i} \tag{5}$$

When $R_i = 1$, the classical form of the Langmuir isotherm is obtained. If a linear isotherm describes best the behaviour of an examined compound, then $R_i = 0$. If the isotherm is concave towards the c_{iF} axis, then R_i is negative. In this instance R_i is not related to the number of sites covered by a solute molecule and allows the description of other isotherm systems not identical with the original Langmuir model. Obviously, for positive R_i the maximum adsorbed concentration approaches $G_D/V_s R_i$. If, on the other hand, the blocking factor is negative, then the maximum concentration in the mobile phase is $-1/K_i R_i$. The extension of eqn. 5 to a many-component equilibrium:

$$c_{jF} = \frac{K_j c_j G_D / V_s - \sum_{i \neq j} R_i c_{iF}}{1 + c_j K_j R_j} \quad (6)$$

including a term reflecting the decrease in available adsorption sites caused by other compounds.

This equilibrium model neglects the volume of single components; we assume that molar volumes are zero. The limitation by solubility in mobile or solid phase is also neglected.

The equilibrium cell corresponding to one theoretical plate[1,2] contains equilibrated concentrations of all components. The transport in the chromatographic column is visualized as a discontinuous process, in which the free volume of one theoretical plate (mobile phase volume of the equilibrium cell) is moved step by step, and always equilibrated, with the solid phase contained in one cell. For a low distribution coefficient ($K_i \ll 1$) and a small number of theoretical plates this approximation may lead to severe errors. Another disadvantage of this simulation is that the axial dispersion coefficient is equal and constant for all components and it is impossible to change its value by changing the concentrations of components, as is sometimes the case in real systems.

COMPUTING

For computation the approach of Seshadri and Deming[13] is used, in which the number of mixed cells is equal to the number of theoretical plates[2]. The algorithm for solution of equilibrium eqns. 1–3 by iteration is centred around eqn. 4. The term in parentheses in the denominator is common to all components. Therefore its value is first estimated (from the preceding step), then the concentrations for all components are calculated and finally, the new value of this term and the difference between the new and old values are found. If the relative error is less than $1 \cdot 10^{-5}$, the calculation for this cell is finished. If not, a new value of this term is chosen, according to the rules for iterative calculations, and the whole process is repeated. On average, six steps are needed to solve this set of equations. No approximations are introduced in the equations; the model works with just the arithmetic error in all concentrations and no limit to the concentration or number of components is set. The only limiting factors are technical, *viz.*, the available operating memory and computing time.

With modern personal computers this presents no problem. Eble *et al.*[24], using the same mixed cells model, approximated the two-component Langmuir isotherm by a nine-term polynomial expansion; it was reported that the average error did not exceed 8% for low and moderate surface coverages.

All the computations here were performed with a PDP 11/23 microcomputer (Digital Equipment Co., Maynard, MA, U.S.A.) with a 128K memory under the RT 11 or RSX 11 M system. All programs were written in Fortran F 77. A typical chromatogram [five components, 1000 theoretical plates (T.P.) plus four shorter columns] takes about 15 h of computing time. The values of the concentration of the effluent from all columns in a given computation were stored and subsequently the peak forms, the start and end of all pure fractions, the end of the chromatogram, etc., were evaluated. All fractions were selected so that the purity of separated compound was 99% or higher. In all instances V_M, G_D and V_s were set equal to 1.

RESULTS AND DISCUSSION

To follow the influence of single factors on the separation of a multi-component mixture, a typical example was selected, *viz.*, a sample containing five components with equilibrium constants K_i = 0.5, 1.0, 1.5, 2.0 and 2.5. The volumes of the mobile and stationary phases in one equilibrium cell (theoretical plate) were chosen as 1 and the capacity of the sorbent in this volume was also set equal to 1. The sample amount, the mode of its introduction, values of the blocking factors and column lengths were varied. Three modes of chromatography, isocratic, gradient and displacement, were compared. In some instances, the number of sample components was decreased to three.

From the point of view of preparative chromatography the two most important parameters are the production rate and the amount recovered. The production rate was calculated as the ratio of the amount recovered and the number of steps needed to elute the last component from column, in other words, the amount recovered divided by the time needed to perform the chromatographic separation. The purity of the fractions has to be higher than 99%; in the effluent a step volume containing the greatest amount of component with higher than the required purity was selected and then its volume was increased (forwards and backwards) until the limit of purity was just attained.

Separation of five components with blocking factors of unity

Typical examples of this separation (with increasing sample loads) are displayed in Figs. 1–4. In the first instance, a small amount of sample (1 for all components) was injected in one step. The peak shapes are nearly ideal (Gaussian) with a resolution between adjacent peaks that is so high that the recovery is nearly 100% for all components. If into the same column (1000 T.P.) a 100 times larger sample is injected, only the first and partially the second component can be recovered with the required purity (Fig. 3). All peaks are shifted toward lower volumes, and a mutual influence (*e.g.*, second and third components) on the peak shape is clearly seen. This picture changes drastically if the column length is increased to 2000 T.P. (Fig. 4). Tables I and II give the recoveries and productivities for all five components on two columns that differ only in the number of theoretical plates. With increasing sample load the concentration of the isolated fraction increases and the position of the peak maximum shifts to lower elution volumes (Table I). The productivity initially increases but, after the peaks widen so that the recovery decreases, the productivity starts to decrease.

In Fig. 5 are plotted the production rate and recovery for various lengths of column for the situation when all peaks are Gaussion (and the sample load is constant). Also in this instance the productivity at first increases with increasing resolution, but after the greatest part of the peaks has eluted in high purity, a further increase in the number of theoretical plates only increases the time necessary for a given separation and therefore the production rate declines. When both factors, column length and sample load, are plotted (Fig. 6), the maximum production rates are attained with the longest columns. With decreasing column length the optimum sample load decreases, but the optimum productivity decreases only slightly. The productivity decreases sharply when the column is so short that even at minimum sample loads (Gaussian peak shapes) the resolution between adjacent peaks is less than about 1.5. When a column is this short, its application in the preparative mode is very ineffective.

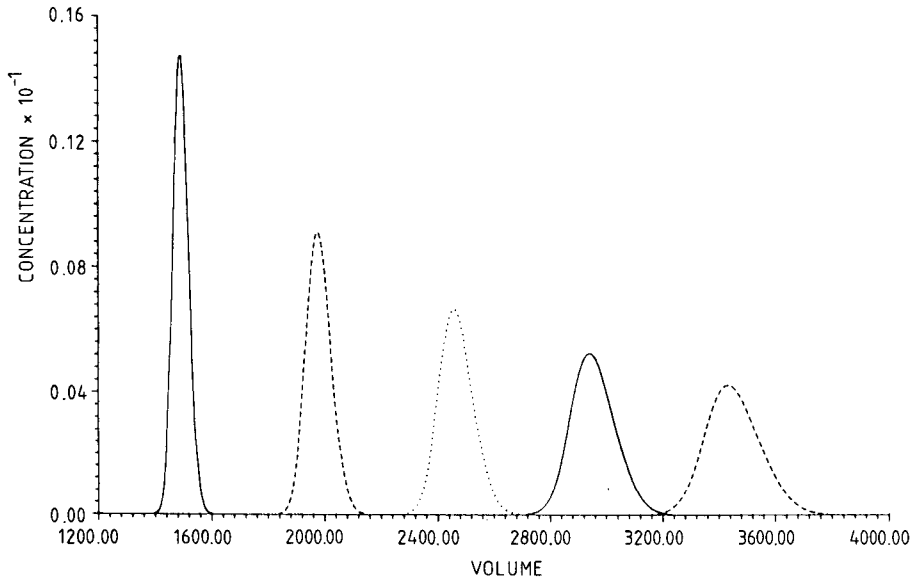

Fig. 1. Separation of a standard five-component mixture. Column, 1000 T.P.; capacity factors, 0.5, 1.0, 1.5, 2.0, 2.5; all blocking factors, 1; volume injected, 1; amount injected (all components), 1.

Knox and Pyper[26] did not directly address the problem of the dependence of production rate (practical throughput in their terminology) on the number of theoretical plates. Their consideration was centred around maximization of the throughput under pressure-limited operation. It should be stressed that in their

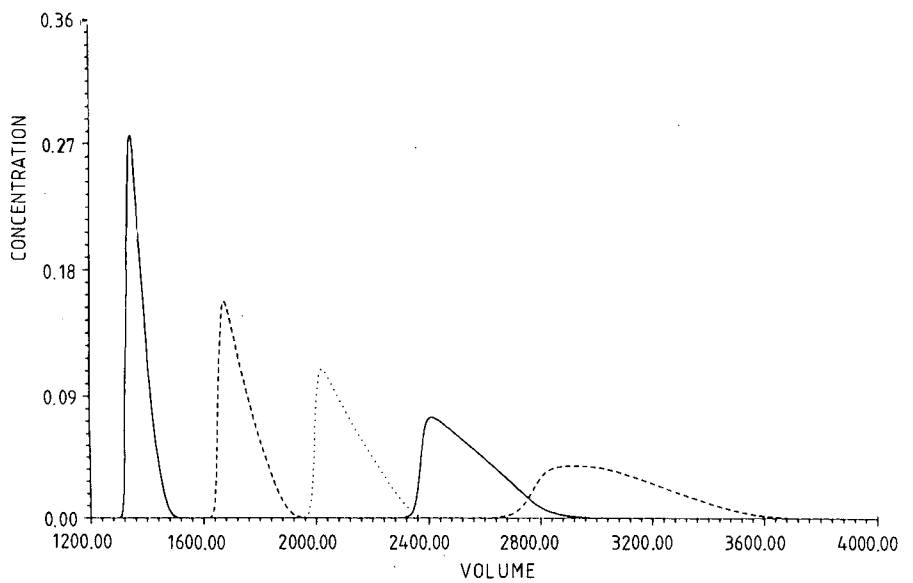

Fig. 2. Separation as in Fig. 1 except amount injected (all components), 20.

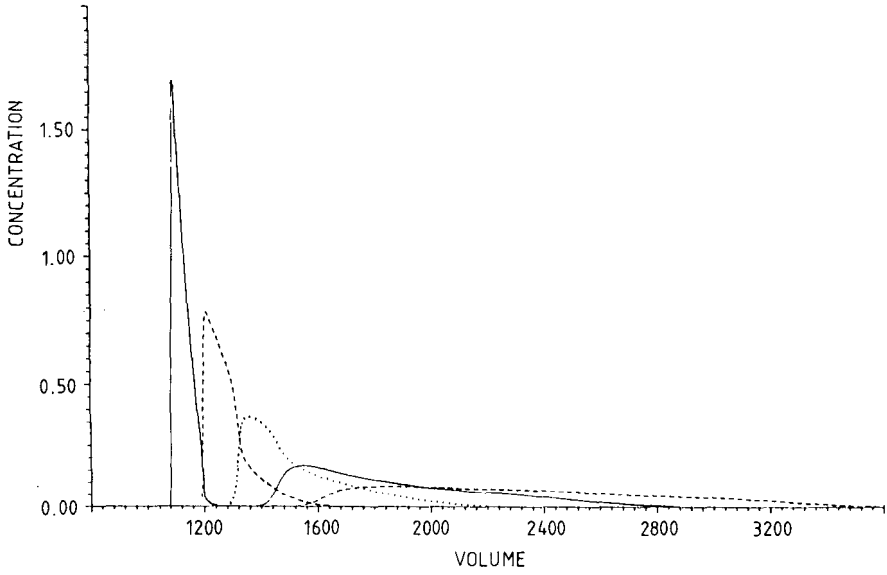

Fig. 3. Separation as in Fig. 1 except amount injected (all components), 100.

discussion it was assumed that there was almost no cross-contamination between adjacent peaks. In our results, optimum throughput is achieved when the peaks strongly interfere and the recovery is around 60%.

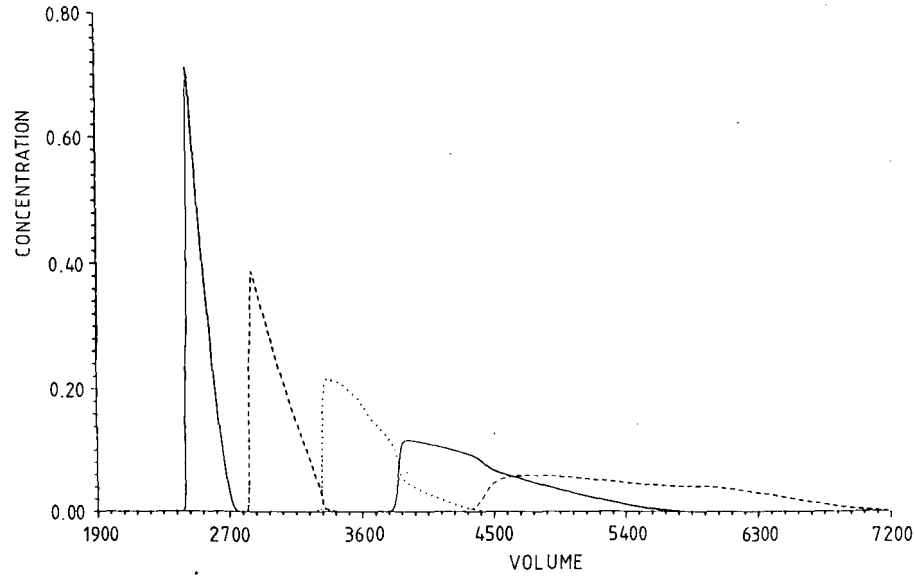

Fig. 4. Separation as in Fig. 3 on a column with 2000 T.P.

TABLE I

INFLUENCE OF SAMPLE LOAD ON RECOVERY AND PRODUCTIVITY

Column, 1000 T.P. Five compounds, capacity factors: 0.5, 1, 1.5, 2.0, 2.5; Blocking factors, 1. REC = Recovery (%); PRD = productivity multiplied by 100; CONC = concentration multiplied by 10^4; P.MAX = peak maximum.

Amount	*Compound 1*				*Compound 2*			
	REC	*PRD*	*CONC*	*P.MAX*	*REC*	*PRD*	*CONC*	*P.MAX*
5 × 1	100.0	0.024	24	1489	100.0	0.024	16	1974
5 × 3	100.0	0.071	73	1469	100.0	0.071	49	1926
5 × 20	100.0	0.472	512	1344	100.0	0.472	432	1674
5 × 25	100.0	0.589	661	1318	100.0	0.589	572	1624
5 × 30	100.0	0.707	852	1294	100.0	0.707	716	1579
5 × 50	100.0	1.179	1866	1215	99.2	1.170	1687	1434
5 × 100	99.0	2.333	7227	1089	51.7	1.218	6152	1202

TABLE II

SEPARATION OF THE SAME COMPOUNDS AS IN TABLE I ON A COLUMN WITH 2000 T.P.

Abbreviations as in Table I.

Amount	*1*		*2*		*3*		*4*		*5*	
	REC	*PRD*	*REC*	*PRD*	*REC*	*PRD*	*REC*	*PRD*	*REC*	*PRD*
5 × 30	100	0.374	100	0.374	100	0.374	99.7	0.373	99.9	0.374
5 × 60	100	0.748	100	0.748	99.2	0.743	65.9	0.493	62.7	0.469
5 × 100	100	1.248	99.7	1.244	78.6	0.981	0	0	38.3	0.478

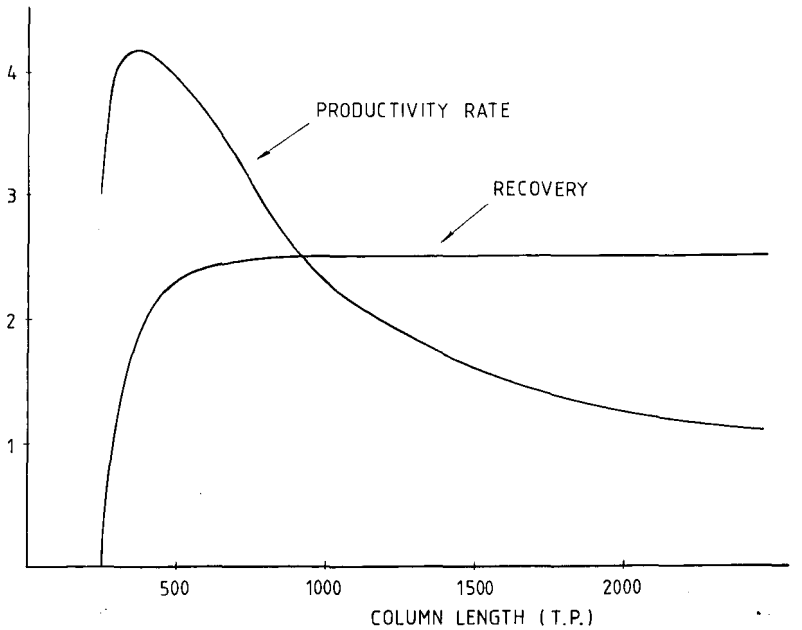

Fig. 5. Productivity rate and recovery from a non-overloaded feed (Gaussian peaks, component **4**) with various column lengths. Capacity and blocking factors as in Fig. 1.

Compound 3				*Compound 4*				*Compound 5*			
REC	*PRD*	*CONC*	*P.MAX*	*REC*	*PRD*	*CONC*	*P.MAX*	*REC*	*PRD*	*CONC*	*P.MAX*
100.0	0.024	15	2454	100.0	0.024	17	2942	100.0	0.024	9	3434
100.0	0.071	47	2383	99.9	0.071	53	2847	100.0	0.071	26	3232
98.8	0.471	460	2022	90.4	0.426	506	2412	81.6	0.385	118	2918
99.3	0.585	635	1952	75.7	0.446	651	2333	66.8	0.394	121	2792
97.7	0.691	842	1891	46.0	0.325	738	2259	56.2	0.398	122	2683
66.9	0.789	1672	1691	0	0	0	1985	34.3	0.405	124	2394
0	0	0	1350	0	0	0	1549	17.4	0.410	125	1846

Influence of feed volume

When the feed is injected in one step in a volume equal to the void volume of one theoretical plate and the amount of components is so high that the productivity of separation is slightly higher than the optimum, then the first parts of the column are strongly overloaded. To offset the overloading and to improve the preparative separation, we tried diluting the same amount of feed in various injected volumes. As can be seen from the results (Table III), the productivity and recovery can be improved

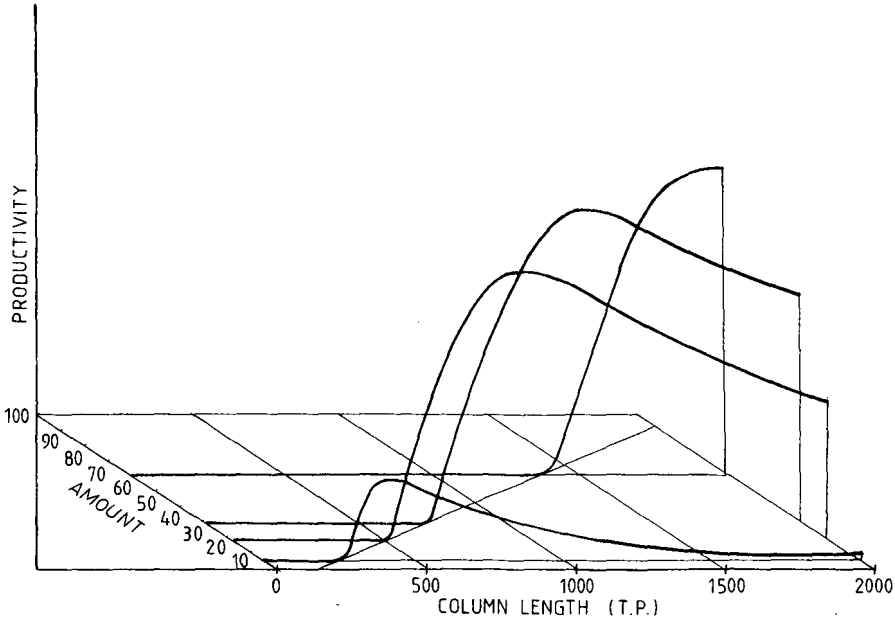

Fig. 6. Production rate of component **4** at various loads (up to 100 for all components) and with various column lengths (up to 2000 T.P.). Capacity and blocking factors as in Fig. 1.

TABLE III

EFFECT OF VOLUME INJECTED

Constant amount: 30 units. Same components as in Table I. Concentrations of all components in feed are identical. Column: 1000 T.P. Abbreviations as in Table I.

Feed volume	*Concentration*	*Component 4*				*Component 3*			
		REC	*PRD*	*CONC*	*P.MAX*	*REC*	*PRD*	*CONC*	*P.MAX*
1	30	46.0	0.325	0.0738	2259	42.0	0.467	0.1701	1023
15	2	54.0	0.381	0.0702	2265	52.4	0.580	0.1621	1035
75	0.4	48.3	0.336	0.0690	2317	38.8	0.421	0.1573	1092
150	0.2	34.2	0.234	0.0670	2377	11.0	0.116	0.1503	1157
30	/0 ... 2/[a]	54.5	0.383	0.0701	2280	53.7	0.592	0.1628	1050

[a] Concentration of feed varies.

only slightly by this approach. If the feed volume is greater than about one tenth of the column void volume, the productivity and recovery decrease. If the concentration of the feed increases linearly during the injection, the separation is about equal to that of uniform injection in half of the volume (see Table III). Chromatograms with two extreme productivities and recoveries of component **4** in Table III are presented in Figs. 7 and 8. It is clear that the separation of other components depends only slightly on the volume injected in the range examined. In both chromatograms the peak shapes are determined predominantly by overloading; the influence of the injection volume is only secondary.

Knox and Pyper[26] predicted that there is a decline in recovery only when the volume injected is increased above about half of the peak volume at the column outlet (without overloading). For component **4**, σ (Gaussian peak) for the peak at the column

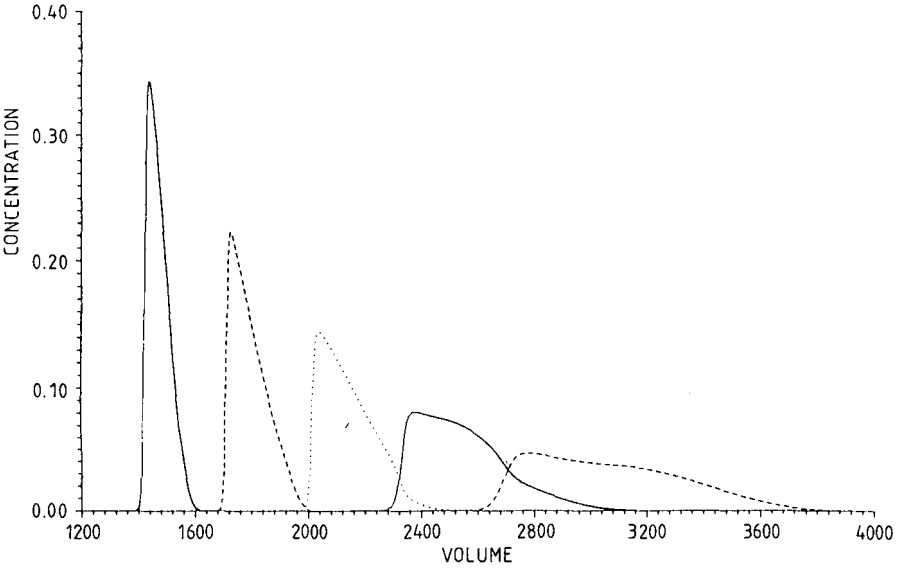

Fig. 7. Separation as in Fig. 1 except amount injected (all components), 30 in volume 150.

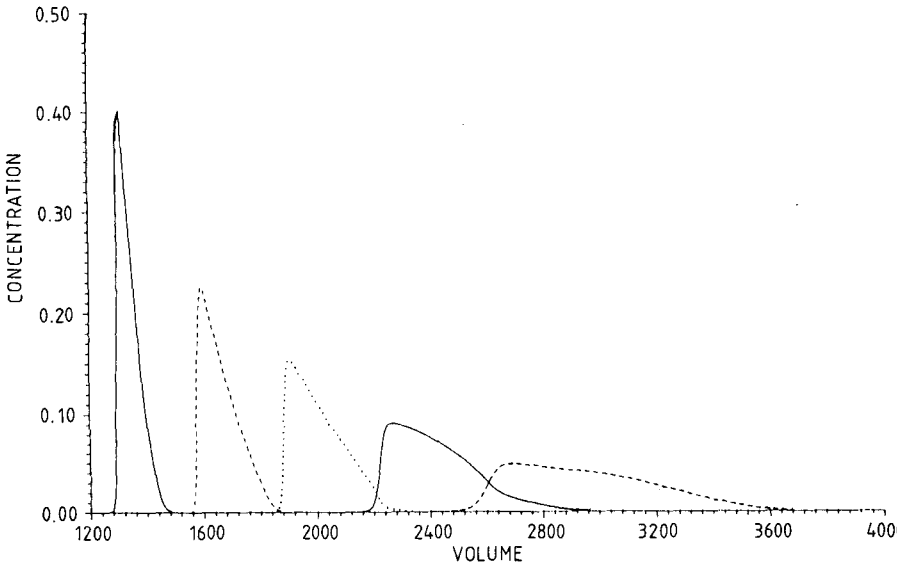

Fig. 8. Separation as in Fig. 1 except amount injected (all components), 30 in volume 15.

outlet (1000 T.P.) is 94.86 and therefore the critical volume should be about 190. Contrary to this prediction, at a feed volume of about 15 an increase of recovery ratio and at a volume of 150 a sharp decline in both recovery and production rate are observed.

Increase in blocking factors

Overloading of the solid phase is determined on the one hand by its capacity and on the other by the sum of the products of the distribution coefficients, concentrations and blocking factors of individual components. Therefore, when the blocking factors of only some of the components are increased, the separation of all components is influenced. In our example, the blocking factors of the first, third and fifth components were increased; the amounts injected and all other parameters remained constant. The recoveries of the second and fourth components decreased on increasing the blocking factors of the surrounding components (see Table IV). In the chromatograms shown in Figs. 9–11 the influence of changing blocking factors is clearly seen. In Fig. 9 the peaks of odd-numbered components would be Gaussian if none of the other components were present. In the example illustrated, however, the third and fifth components are clearly deformed owing to the presence of the fourth component. On the of other hand, the influence of the surrounding components on the peak shape of component **4** is clearly seen. The overloading in the last instance (Fig. 11) is so strong that only the first two components can be isolated; the others are not separated at all. Note also the shift in the peak maxima of all components, even those with constant blocking factors, to lower elution volumes with increase in the blocking factors. This is clear evidence of the mutual influence of sample components in the course of the separation process.

TABLE IV

EFFECT OF INCREASE IN BLOCKING FACTORS

Standard mixture: amount (all compounds), 30. Variable: blocking factors of components **1**, **3** and **5**. All others variables as in Fig. 1.

Blocking factor	*Component 2*		*Component 4*	
	Recovery	*Productivity*	*Recovery*	*Productivity*
0	100.0	0.704	85.2	0.600
1	100.0	0.707	46.0	0.325
2	99.3	0.704	0	0
3	94.5	0.671	0	0
4	86.3	0.613	0	0

Isolation of minor components

If one of the components in the sample predominates, then the recovery is determined not only by its amount but also by its elution volume in relation to other components.

The recoveries listed in Table V vary if the isolated component is eluted before or after the predominant compound. The concentrations of peaks eluted before it are increased, whereas the peaks eluted after the largest peak are smeared and their concentrations are smaller than those which would have been eluted without interference from the predominant component. This is illustrated in Figs. 12 and 13 in comparison with Fig. 1 and confrontation of Tables V and I, too. The effect of

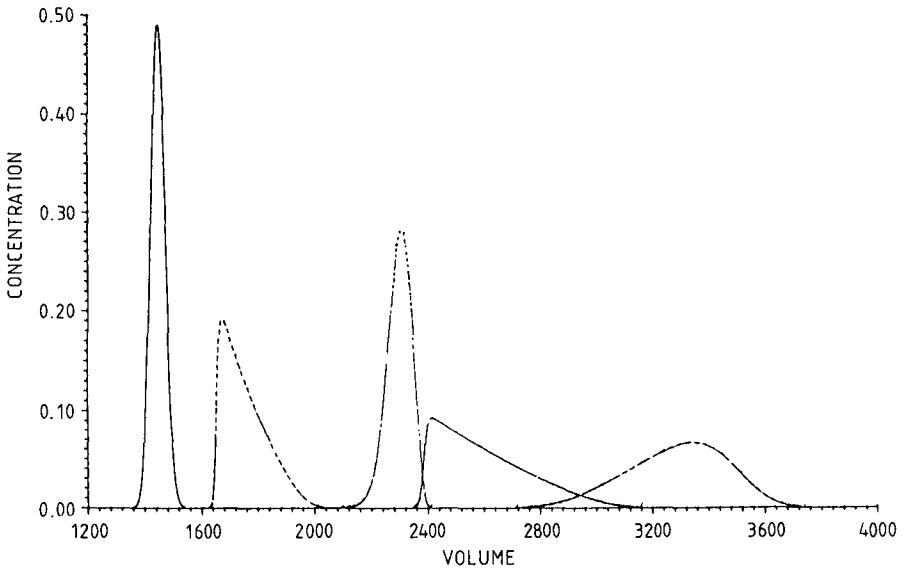

Fig. 9. Separation as in Fig. 1 except amount injected 30; injection volume, 1; blocking factors of components **1**, **3** and **5** = 0.

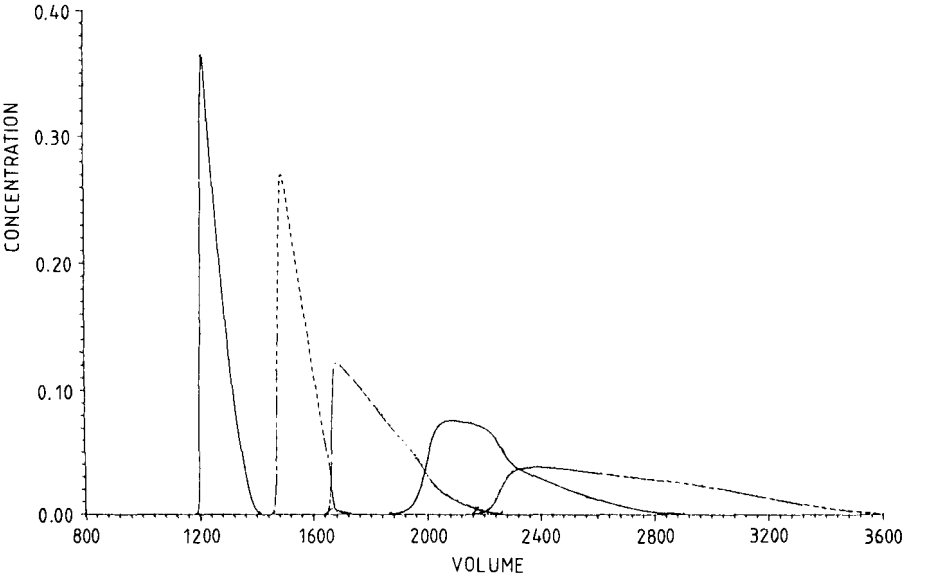

Fig. 10. Separation as in Fig. 9 except blocking factors of components **1**, **3** and **5** = 2.

approximately equal load of first component (475 times 0.5) on recovery is much more adverse than that of the last one (150 times 2.5).

If three components (**1, 3** and **5**) dominate the sample, then the minor components (**2** and **4**), present in the sample in 100 times smaller amounts, can be

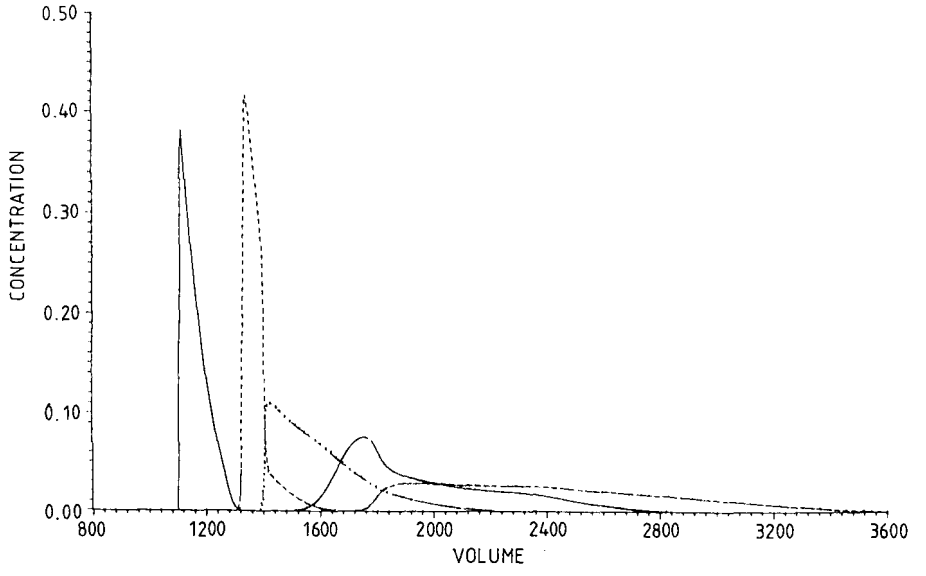

Fig. 11. Separation as in Fig. 9 except blocking factors of components **1**, **3** and **5** = 4.

TABLE V

EFFECT OF A LARGE SURPLUS OF ONE COMPOUND

Column: 1000 T.P.; volume injected, 1; all blocking factors, 1; concentration multiplied by 10^4; k' = capacity factor.

Parameter	***1*** *(k' = 0.5)*	***2*** *(k' = 1)*	***3*** *(k' = 1.5)*	***4*** *(k' = 2.0)*	***5*** *(k' = 2.5)*
Amount	150	1	1	1	1
Peak maximum	1199	1923	2412	2899	3390
Recovery (%)	–	100	99.5	96.0	98.8
Concentration	–	18	18	21	9
Amount	1	1	1	1	150
Peak maximum	1395	1724	1921	1945	1970
Recovery (%)	100	100	44.4	0.0	–
Concentration	28	28	48	–	–
Amount	1	150	1	1	1
Peak maximum	1340	1389	2362	2857	3352
Recovery (%)	98.4	–	14.7	21.9	87.2
Concentration	63	–	16	16	8
Amount	475	1	1	1	1
Peak maximum	1055	1678	2384	2866	3354
Recovery (%)	–	0	0	0	75.2
Concentration	–	–	–	–	7

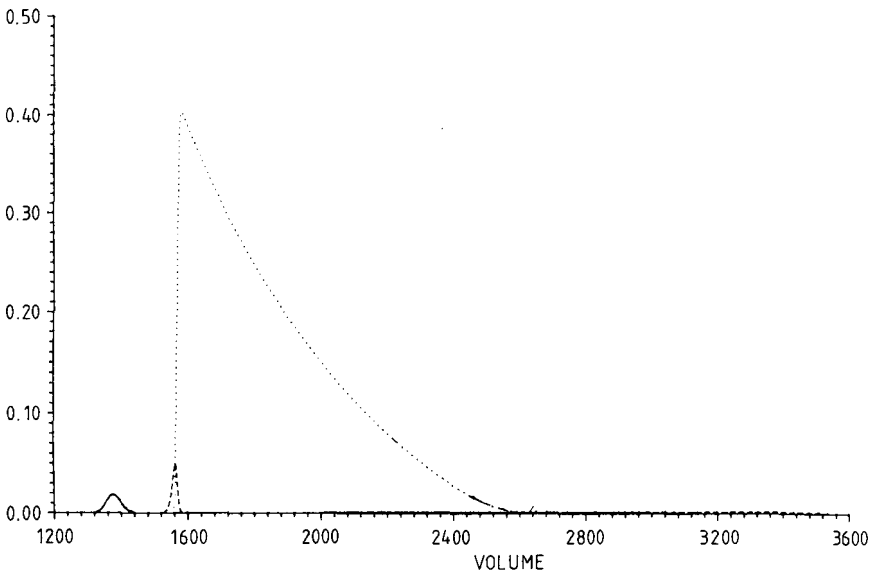

Fig. 12. Separation as in Fig. 1 except amounts injected 1.0, 1.0, 150.0, 1.0, 1.0 (components **1**–**5**).

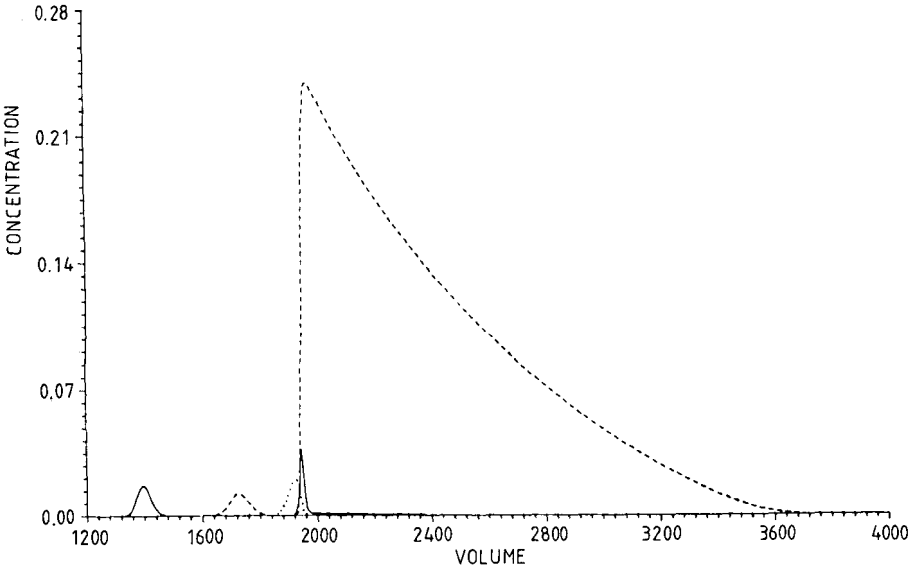

Fig. 13. Separation as in Fig. 1 except amounts injected 1.0, 1.0, 1.0, 1.0, 150.0 (components **1**–**5**).

isolated on the 1000 T.P. column with good recoveries (last entry in Table V). This is true, of course, only when the feed as whole does not overload the column and the separation does not break down.

Gradient elution

To demonstrate the possibilities of gradient elution for preparative separations, three linear gradients were compared. The concentration of the strongest eluent varied from 0 to 1 and the values of its capacity factor were chosen to be 1 (in one instance) and 2 (in another example). The greatest differences are found in the recovery, concentration and productivity of the separation of component **4** when it is isolated from a standard mixture (Table VI). In comparison with isocratic elution, at the same recovery the productivity may be increased by 50% and the concentration of a selected fraction more than doubled (compare the first and fourth separations in Table VI). When the gradient is steeper than the optimum (second line), then the recovery and productivity decrease but the concentration of the selected fraction increases further. It should be stressed that in these computations the eluting agent is not classified as an impurity. It is interesting to follow how its increasing concentration at the column outlet is changed by transport through the column and how the peaks of the separated components are impressed as negative peaks on the trace of eluent concentration (dotted line in Fig. 14).

In an attempt to describe preparative liquid chromatography, Eble *et al.*[24] concluded that gradient elution is equivalent to isocratic elution if "average" capacity factors are equal, but no quantitative treatment of gradient optimization under overload conditions was presented. Therefore, no quantitative comparison with their treatment is possible.

TABLE VI

GRADIENT ELUTION

Column, 1000 T.P.; amount injected, 5 × 20; components, 5. Abbreviations as in Table I.

Capacity factor of eluent	*Length of linear gradient (volume)*	*Component 4*		
		REC	*PRD*	*CONC* ($\times 10^{-5}$)
1	1000	92.2	0.623	1104
2	1000	50.1	0.392	4009
1	2000	92.0	0.567	810
Isocratic	–	90.4	0.426	506

Displacement chromatography

It is generally accepted that displacement chromatography is a more powerful and efficient technique than elution chromatography for the separation of complex mixtures. In an attempt to separate a standard mixture of components, several unsuccessful trials were made to isolate all components from a fully developed train (procession of separated components) in a column with 1000 T.P. or shorter. Only with columns longer than about 1400 T.P. was it possible to isolate all five components (see Table VII). It is characteristic that in all instances when the components could be collected in satisfactory purity, the concentrations of the fractions were higher than in the feed. As in gradient elution, it was assumed that the displacer is not an impurity in

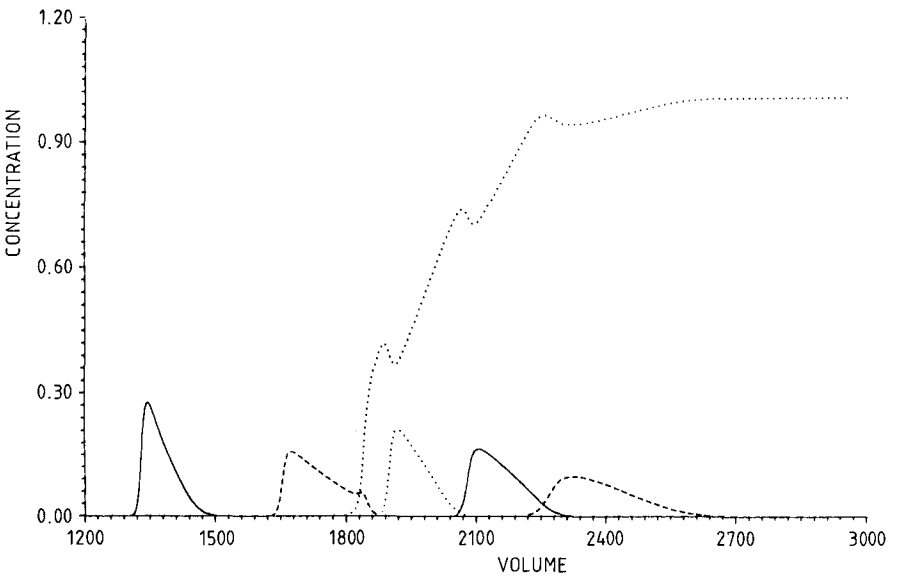

Fig. 14. Gradient elution. Injected amounts: 50 × 20 (volume 1). Capacity and blocking factors of five-component mixture as in Fig. 1. Eluent: capacity factor, 1.0; blocking factor, 1.0. Concentration increasing linearity from 0.0 (volume 0) to 1.0 (volume 1000), later constant (1.0).

TABLE VII

DISPLACEMENT CHROMATOGRAPHY

Feed components: capacity factors, 0.5, 1.0, 1.5, 2.0, 2.5; displacer, 3.0; all blocking factors, 1; injection, 500 × 0.2 (all components); concentration of displacer, 3.0. Productivity is multiplied by 100; recovery is in per cent. Abbreviations as in Table I. CONC = Real values, not multiplied by a quotient.

Column length (T.P.)	***1***			***2***			***3***			***4***			***5***		
	REC	*PRD*	*CONC*	*REC*	*PRD*	*CONC*	*REC*	*PRD*	*CONC*	*REC*	*PRD*	*CONC*	*REC*	*PRD*	*CONC*
100	3.4	0.498	0.0710	0	–	–	0	–	–	0	–	–	0	–	–
300	19.9	2.060	0.1592	0	–	–	0	–	–	0	–	–	0.8	0.078	0.0109
450	31.9	2.726	0.1867	0	–	–	0	–	–	0	–	–	3.0	0.257	0.0362
600	43.2	3.147	0.2048	0	–	–	0	–	–	0	–	–	8.1	0.592	0.0865
1000	74.0	3.886	0.2396	18.5	0.969	2.3067	0	–	–	0	–	–	35.1	1.845	0.3004
1300	95.6	4.156	0.3095	87.5	3.804	2.3036	55.5	2.412	2.6432	0	–	–	61.0	2.651	0.4621
1600	95.6	3.550	0.3452	87.6	3.251	2.3051	71.3	2.646	2.6404	39.3	1.458	2.8058	75.5	2.802	0.5393
1800	96.9	3.278	0.3800	87.9	2.972	2.3121	71.3	2.413	2.6415	47.7	1.613	2.8050	75.7	2.560	0.5329
2000	96.9	3.012	0.4177	87.6	2.723	2.3050	71.3	2.217	2.6416	47.7	1.484	2.8077	78.4	2.438	0.5447

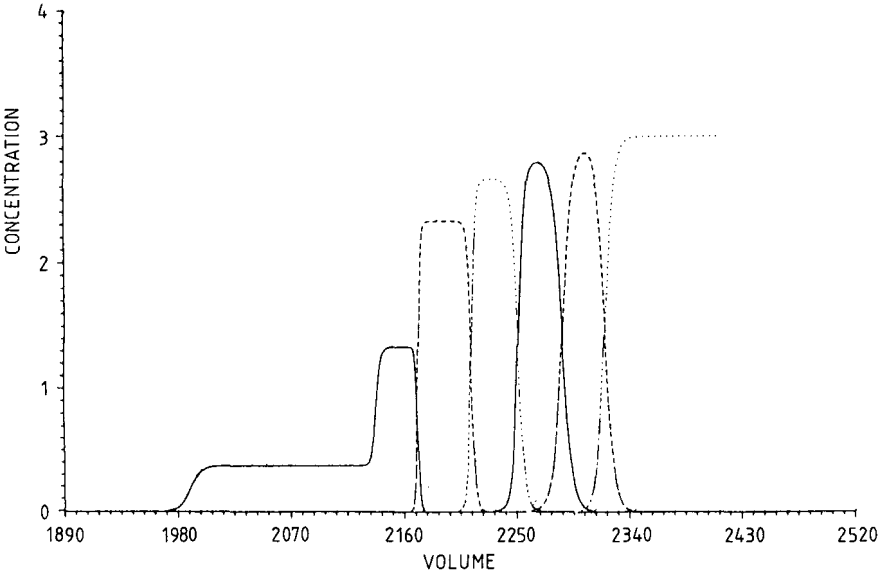

Fig. 15. Displacement chromatography. Column: 1400 T.P. All other parameters as in Table VII.

the collected fractions. This is the reason why the end of last compound fraction reaches far into the volume where predominantly displacer is eluted, and therefore its average concentration is lower than in the preceding fractions. This may be clearly seen from Fig. 15, where a separation on 1400 T.P. column is illustrated. Note that the fourth component cannot be isolated from its separation in satisfactory purity.

The influence of feed volume and amount injected on the recovery of a standard mixture can be seen in Table VIII. The smallest amount injected (30 in volume 1) is not large enough to build up a fully developed train. If a larger amount (100) is injected, then another variable parameter, the volume injected, may influence the recovery ratio of components. From consideration of all the experiments, the optimum volume is 200. In Figs. 16–18 the evolution of separation at three different points in the column (300, 600 and 1000 T.P.) is demonstrated.

TABLE VIII

DISPLACEMENT CHROMATOGRAPHY WITH VARIOUS INJECTED VOLUMES

Standard mixture (see Table VII); column, 1000 T.P. Abbreviations as in Table I.

Feed volume	*Concentration*	*Compound 1*			*Compound 2*		
		REC	*PRD*	*CONC*	*REC*	*PRD*	*CONC*
1	30	86.2	1.663	0.2751	0	0	0
1	100	97.0	6.899	1.2279	39.2	2.786	2.3040
200	0.5	98.0	6.105	0.9337	76.3	4.751	2.3121
400	0.25	81.0	4.488	0.3493	48.4	2.678	2.3034
500	0.2	74.0	3.886	0.2396	18.5	0.969	2.3067

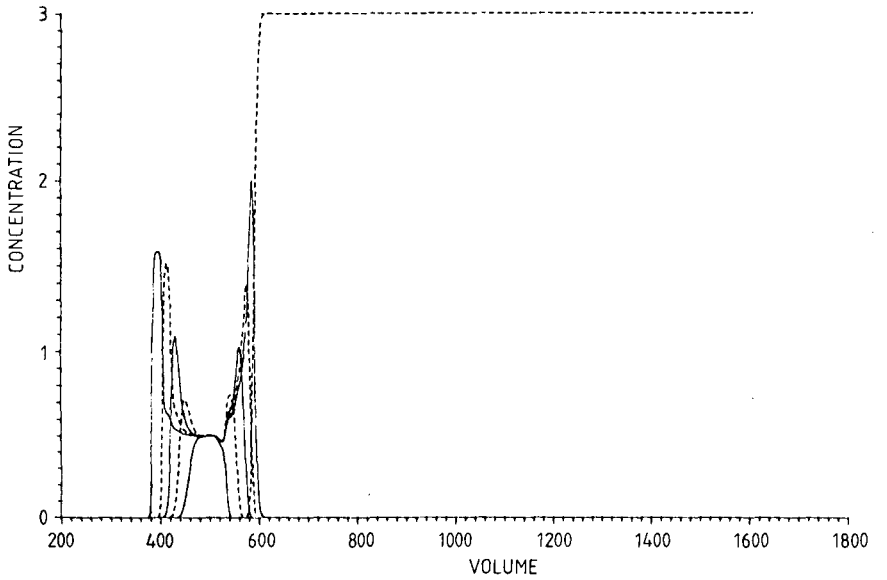

Fig. 16. Displacement chromatography. Components and displacer as in Table VII. Injection: volume 200, concentration 0.5. Displacer concentration, 3.0. Column, 300 T.P.

The recoveries and productivities of separation obtained by elution and displacement chromatography of a three-component mixture are compared in Table IX. From the results we conclude that the productivities using displacement chromatography are about four times higher than those with elution chromatography,

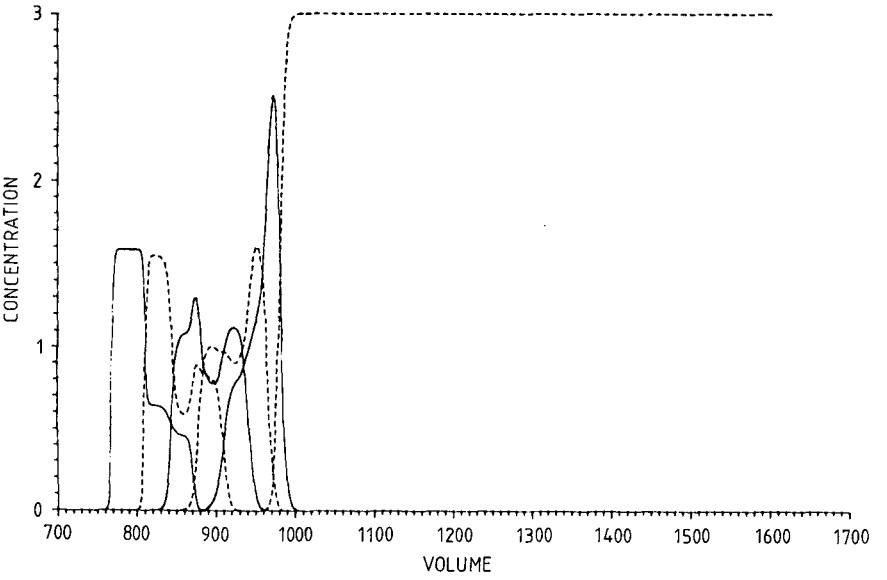

Fig. 17. Separation as in Fig. 16 except column, 600 T.P.

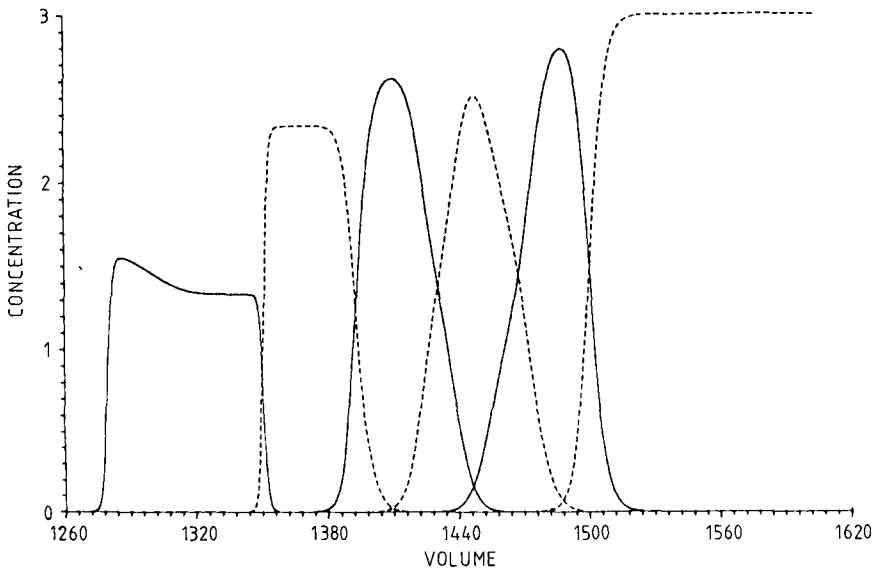

Fig. 18. Separation as in Fig. 16 except column, 1000 T.P.

but the optimization of displacement chromatography is much more difficult. In both elution and displacement chromatography, an upper limit (column overloading) exists. In displacement chromatography, a lower limit exists also; if the amount of sample injected is too small, then the displacement train with a pure component fraction will not be formed and no useful fractions can be isolated. It is interesting that for separation of a standard five-component mixture by displacement chromatography a column longer than 1000 T.P. was needed.

Table X illustrates the separation of a three-component mixture where the isotherms of components **2** and **1** cross each other. This is the case where separation was expected by Frey[28] but was not predicted by the "golden rule"[21].

TABLE IX

COMPARISON OF ELUTION (EL) AND DISPLACEMENT (DI) CHROMATOGRAPHY

Three-component mixture: capacity factors, 0.5, 1.5, 2.5, displacer, 3.0; all blocking factors, 1; column, 1000 T.P.; injection volume, 1; displacer concentration, 3. Abbreviations as in Table I.

Mode	*Amount*	*Compound 1*			*Compound 2*			*Compound 3*		
		REC	*PRD*	*CONC*	*REC*	*PRD*	*CONC*	*REC*	*PRD*	*CONC*
DI	100	99.2	7.047	0.893	89.9	6.387	2.643	93.2	6.623	0.675
DI	200	99.8	14.183	1.559	85.9	12.206	2.6422	93.1	13.235	1.095
DI	300	79.1	16.875	4.964	0	0	0	65.8	14.035	1.135
EL	100	100	2.355	0.2463	99.1	2.334	0.1598	99.4	2.340	0.0462
EL	200	100	4.710	0.9174	66.6	3.139	0.5508	52.1	2.454	0.0480

TABLE X

DISPLACEMENT CHROMATOGRAPHY WITH CROSSED ISOTHERMS

Components: capacity factors, 0.5, 1.5, 2.5; blocking factors, 1, 4, 1; amount injected, 200 (all components); volume, 1. Displacer, capacity factor, 3; blocking factor, 1: concentration, 3. Abbreviations as in Table I.

Compound	*PRD*	*CONC*	*REC*	*P.MAX*
1	0.1420	1.540	99.9	1051.0
2	0.0260	0.665	73.2	1169.5
3	0.138	1.11	97.18	1261.5

The crossing point for both components lies at a concentration of 0.4444. Because injection was made with much higher concentrations and during the whole separation they hardly approached this point, regular behaviour is observed.

Negative blocking factors

The peculiarity of a one-component isotherm with a negative blocking factor lies in the fact that there is not a defined maximum adsorbed concentration, but a maximum attainable mobile phase concentration. If the concentration in the mobile phase were to approach this limit, then the concentration of sample in the solid phase would increase above all limits.

This obviously does not describe any real system; at least the volume of sorbed species would limit the maximum attainable concentration in the solid phase. On the

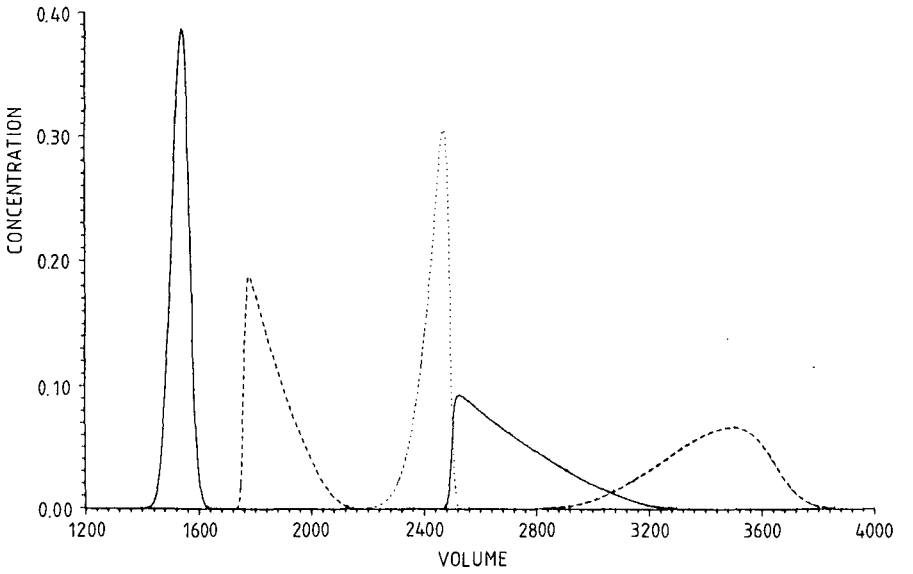

Fig. 19. Isocratic elution chromatography. Column: 1000 T.P. Five components: capacity factors 0.5, 1.0, 1.5, 2.0 and 2.5; blocking factors −0.1, 1.0, −0.1, 1.0, −0.1. Injection (all components): concentration 0.3, volume 1000.

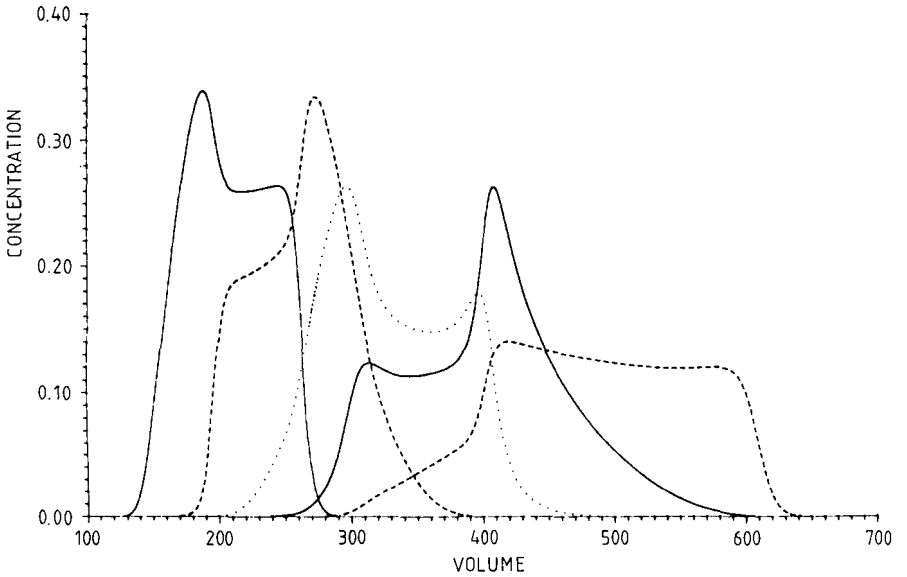

Fig. 20. Separation as in Fig. 19 except blocking factors −1.0, 1.0, −1.0, 1.0, −1.0 and column 100 T.P.

other hand, we may expect that at concentrations much lower than the maximum attainable mobile phase concentration this simple model would describe sorption processes correctly. We have to keep this principal limitation in mind when describing the results of our computations.

In the chromatogram for the five-component mixture with three negative blocking factor components (Fig. 19), the peaks are steeper on the rear side in

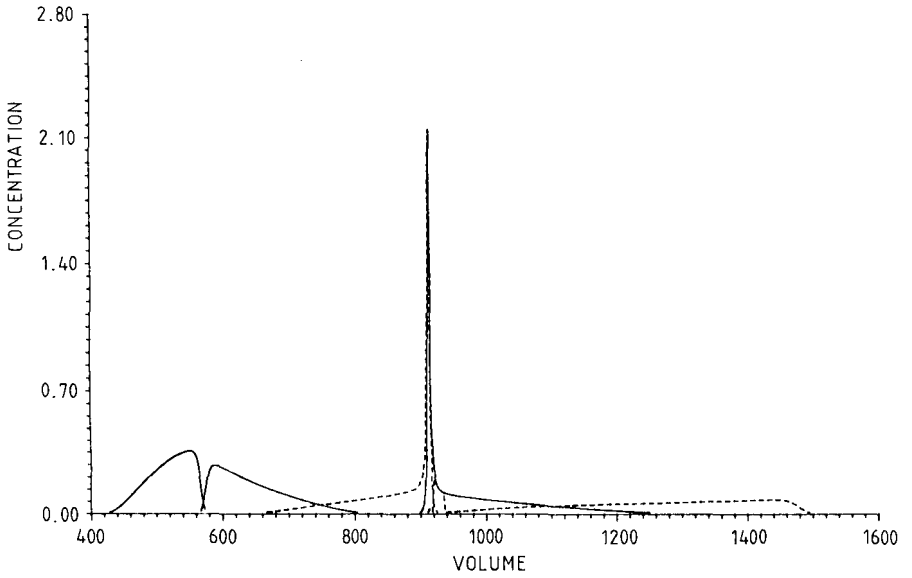

Fig. 21. Separation as in Fig. 20 except column, 300 T.P.

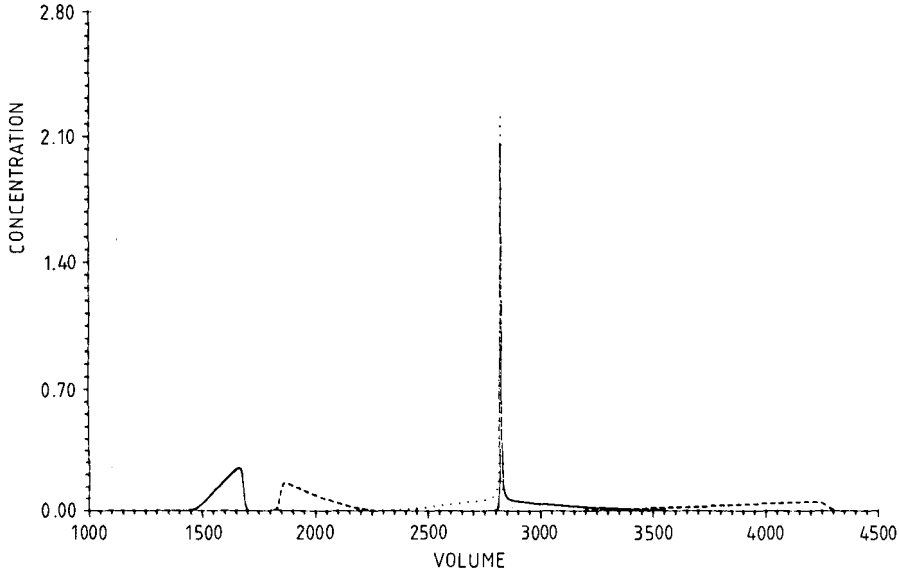

Fig. 22. Separation as in Fig. 20 except column, 1000 T.P.

comparison with a similar chromatogram of components with only positive blocking factors (Fig. 7) and increased peak concentrations of components **1**, **3** and **5** in Fig. 19 are apparent. A shift of the peaks of the second and fourth components toward higher elution volumes is also evident.

When only the chromatogram from a single universal detector is recorded,

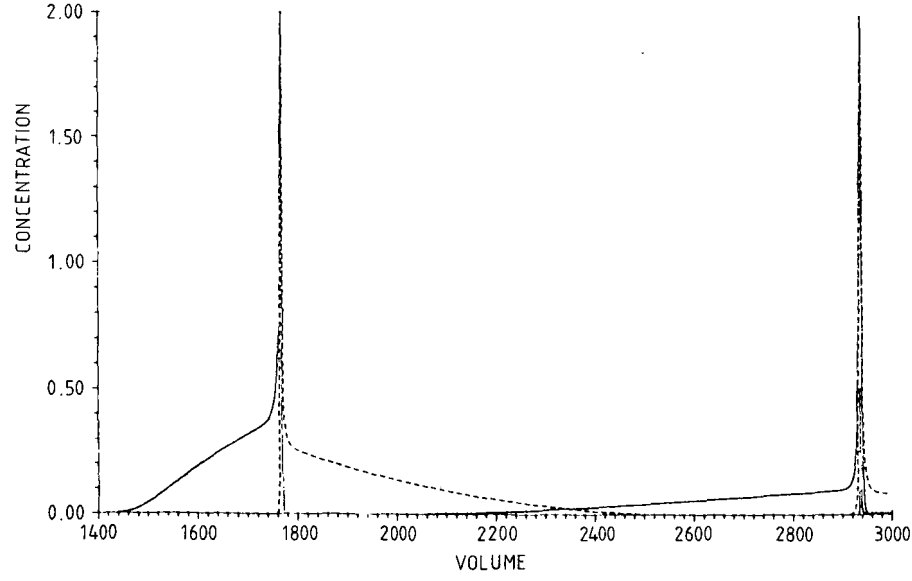

Fig. 23. Separation as in Fig. 22 except amount injected (all components), 100.

components **3** and **4** form a fused peak without any hint of separation. In spite of this, the recovery ratios of these components are fairly high, 87.9 and 81.9%.

When the value of the negative blocking factors is increased, the appearance of the computed chromatogram changes considerably. The third and fourth components form a narrow spike, much thinner than any peak corresponding to a column length of 1000 T.P. The evolution of this peak shape may be traced with three different column lengths. Even with the short 100 T.P. column two areas with higher capacity are formed. These are manifested by secondary peaks at elution volumes of approximately 250 and 400 (Fig. 20). The longer column (Fig. 21) separates components **1** and **2**; the next component pair forms the fused spike; the appearance of the chromatogram does not change substantially with the 1000 T.P. column (Fig. 22). Two virtual peaks are formed when the amount of feed is further increased (Fig. 23).

CONCLUSIONS

It has been demonstrated that a simple ideal mixed cells model is flexible enough to describe all modes of liquid chromatography. Overloading is typical for preparative chromatography. The recoveries and productivities can be easily computed without the introduction of any arbitrary scales as was proposed earlier. Using this model, multi-component systems may be studied if the adsorption of their components can be described by a hyperbolic isotherm. It was shown that under overload conditions the mutual interference of components is always prominent; this is particularly important when trace components are isolated.

The program permits the simultaneous computation of separations with various column lengths. Plotting of effluent concentrations enables the peak forms to be examined closely. From recorded data, production rates and recoveries for various previously defined fraction purities may be computed.

It was demonstrated that isocratic, gradient and displacement modes of preparative chromatography provide the highest throughput when the column is operated in the high overload mode. It is hardly possible to describe the complicated behaviour of these separations with only simple extrapolations from two-component results in a moderately overloaded mode, as was done in recent publications. Our model enables complete chromatograms to be computed with only those data (capacity and blocking factors of every component and capacity and number of theoretical plates of column) which are also necessary for other, oversimplified, models. This work will be supplemented by a comparison of results from the numerical solution of a set of differential equations and will be verified experimentally.

This simple model may lead to erroneous results if components with positive and negative blocking factors are combined in one feed. In a subsequent paper a two-site and two-layer model with a more complicated isotherm shape giving a more accurate picture of components with convex isotherms over the whole concentration range will be described.

REFERENCES

1 A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, 35 (1941) 1358.
2 S. W. Mayer and E. R. Tompkins, *J. Am. Chem. Soc.*, 69 (1947) 2866.
3 D. DeVault, *J. Am. Chem. Soc.*, 65 (1943) 532.

4 E. Glueckauf, *Trans. Faraday Soc.*, 51 (1955) 34.
5 G. Houghton, *J. Phys. Chem.*, 67 (1963) 84.
6 H. Vink, *J. Chromatogr.*, 18 (1965) 25.
7 F. Helfferich and G. Klein, *Multicomponent Chromatography — Theory of Interference*, Marcel Dekker, New York, 1970.
8 J. V. Lankelma and H. C. Smit, *Commun. Math. Chem.*, (1985) 157.
9 H. Poppe and J. C. Kraak, *J. Chromatogr.*, 255 (1983) 395.
10 P. Rouchon, M. Shoenauer, P. Valentin, C. Vidal-Madjar and G. Guichon, *J. Phys. Chem.*, 89 (1985) 2076.
11 A. Jaulmes, C. Vidal-Madjar, H. Colin and G. Guichon, *J. Phys. Chem.*, 90 (1986) 207.
12 M. J. Gonzalez, A. Jaulmes, P. Valentin and C. Vidal-Madjar, *J. Chromatogr.*, 386 (1987) 333.
13 S. Seshadri and S. N. Deming, *Anal. Chem.*, 56 (1984) 1576.
14 J. E. Eble, R. L. Grob, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 384 (1987) 25 and 45.
15 K. P. Hupe and H. H. Lauer, *J. Chromatogr.*, 203 (1981) 41.
16 P. Gareil, C. Durieux and R. Rosset, *Sep. Sci. Technol.*, 18 (1983) 441.
17 G. Gretier and J. L. Rocca, *Chromatographia*, 21 (1986) 143.
18 L. Hagdahl, J. P. Williams and A. Tiselius, *Ark. Kemi*, 4 (1951) 193.
19 P. P. Zolotarev, N. V. Kolosov and N. V. Kurdyumov, *Zh. Fiz. Khim.*, 59 (1985) 1979 and 2837.
20 J. Frenz, C. Horváth, *AIChE. J.*, 31 (1985) 400.
21 Cs. Horváth, A. Nahum and J. H. Frenz, *J. Chromatogr.*, 218 (1981) 365.
22 J. A. V. Butler and C. Ockrent, *J. Phys. Chem.*, 34 (1930) 2841.
23 M. D. LeVan and T. Vermeulen, *J. Phys. Chem.*, 85 (1981) 3247.
24 J. E. Eble, R. L. Grob, P. E. Antle, L. R. Snyder, *J. Chromatogr.*, 405 (1987) 1, 31 and 51.
25 S. Ghodbane, G. Guiochon, *J. Chromatogr.*, 444 (1988) 275.
26 J. H. Knox and H. M. Pyper, *J. Chromatogr.*, 363 (1986) 1.
27 L. R. Snyder, G. B. Cox, P. E. Antle, *Chromatographia*, 24 (1987) 82.
28 D. D. Frey, *J. Chromatogr.*, 409 (1987) 1.

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DEVELOPMENT AND OPTIMISATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR TIOCONAZOLE AND ITS POTENTIAL IMPURITIES

I. SELECTION OF SEPARATION CONDITIONS

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SUMMARY

Statistical mixture design techniques have been utilized to develop an isocratic high-performance liquid chromatographic separation of 1-{2-[(2-chloro-3-thienyl)-methoxy]-2-(2,4-dichlorophenyl)ethyl}-1H-imidazole (tioconazole) and its potential impurities. By using suitable quality criteria such as reduced plate height, peak asymmetry and selectivity for the interrogation of chromatograms the variables producing best column efficiency and greatest selectivity have been identified. The optimum separation for these variables has been located by the sequential simplex approach and confirmed by response surface mapping.

INTRODUCTION

Tioconazole, 1-{2-[(2-chloro-3-thienyl)methoxy]-2-(2,4-dichlorophenyl)ethyl}-1H-imidazole, is an imidazole drug used as a broad-spectrum anti-fungal agent. Three related impurities and one degradation product (Fig. 1) are found in trace amounts[1,2]. Reversed-phase chromatographic separation of related compounds B and C has proved extremely difficult. Indeed, resolution is incomplete for the chromatographic method currently prescribed in the United States Pharmacopeia (U.S.P.)[1]. The resolution enhancement due to peak sharpening, achieved by taking the second derivative of the chromatogram, was previously used to improve quantitation of compounds B and C[3].

The U.S.P. method[1] specifies an eluent containing 0.20% ammonia (sp.gr. 0.88) which results in a high pH. Consequently the column lifetime is short due to dissolution of the silica support. To overcome this problem a pre-column was incorporated to pre-saturate the mobile phase with silica. The 25-cm analytical column employed in this assay gives rise to a long analysis time, while still not providing good resolution of compounds B and C. The U.S.P. method was not developed with the

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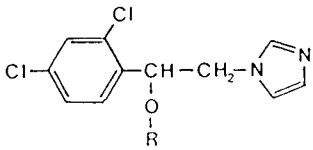
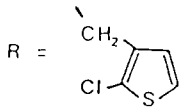
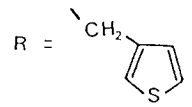
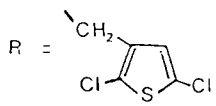




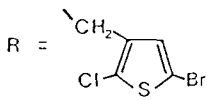
Tioconazole
1-[2-[(2-chloro-3-thienyl)methoxy]-2-
(2,4-dichlorophenyl)ethyl]-1H-imidazole



Related compound A
1-[2-[(3-thienyl)methoxy]-2-
(2,4-dichlorophenyl)ethyl]-1H-imidazole



Related compound B
1-[2-[(2,5-dichloro-3-thienyl)methoxy]-2-
(2,4-dichlorophenyl)ethyl]-1H-imidazole



Related compound C
1-[2-[5-bromo-2-chloro-3-thienyl)methoxy]-
2-(2,4-dichlorophenyl)ethyl]-1H-imidazole

R = H

Compound D - Hydrolysis product
1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)
ethanol

Fig. 1. Structures of tioconazole, related impurities and hydrolytic breakdown product.

quantitation of the hydrolysis product in view. The present work reports the application of systematic optimisation procedures for the development of a high-performance liquid chromatography (HPLC) method to overcome these limitations. A large number of variables are involved in the development of a reversed-phase HPLC separation[4]. The principal chromatographic parameters governing the separation behaviour include: organic modifier, column stationary phase chemistry, pH, buffer and ion-pairing agent. A simultaneous optimisation of all these parameters is beyond any single optimisation procedure. Thus it was decided to explore the rational use of statistical mixture designs and partial factorial designs, to identify the most significant variables in method development for this problem. A sequential search procedure was then employed to optimise the method.

The limitations of the existing assay are that the mobile phase is aggressive towards the packing material, and that the analysis time is long, due to the length of column. Thus the key aims were to develop an assay which would operate at lower pH values and with shorter columns. The analytes in this assay are basic (pK_a for tioconazole is around 6) and exist in two pH-dependent forms. At pH 8 and above, the unionized form is the major species; at pH 4 and below, the protonated form is predominant. The U.S.P. method separates the unionized species, leading to the requirement for an eluent of high pH. Lowering the pH to extend column lifetime involved making a study of the protonated species. For chromatography of these

ionized species an anionic ion-pairing agent would normally be added. This technique therefore formed an essential part of the method development.

EXPERIMENTAL

Measurements were made on two systems. Initial studies used an LKB 2150 pump, a 2151 variable-wavelength detector and a 2210 chart recorder (LKB Instruments, Croydon, U.K.). The sample injection valve was a Rheodyne 7010 fitted with a 20 μl loop (Alltech Asoc., Carnforth, U.K.). Systematic development was based on a Hewlett-Packard 1090A chromatograph incorporating a DR5 ternary pumping system, an autosampler with a Rheodyne 7010 injection valve, an integral 1040A diode array detector (with the 220-nm signal being recorded[1], an integral DPU integrator an HP 7470A plotter and an HP85 microcomputer (Hewlett-Packard, Wokingham, U.K.). The columns used were an Ultrasphere 5-μm ODS (45 × 4.6 mm I.D., packed in-house); a Nucleosil 5-μm cyanopropylsilica (50 × 4.6 mm I.D. Technicol, Stockport, U.K.) a Hypersil 5-μm phenyl (50 × 4.6 mm I.D., Technicol) and a Hypersil 5-μm phenyl (150 × 4.6 mm I.D., Technicol). A summary of these columns is given in Table I.

TABLE I

COLUMNS USED IN THE STUDIES

Particle size, 5 μm; I.D., 4.6 mm.

Column packing	*Chemistry*	*Length (mm)*
Ultrasphere	ODS	45
Nucleosil	Cyanopropyl	50
Hypersil	Phenyl	50
Hypersil	Phenyl	150

Mobile phases were prepared using HPLC grade solvents (Rathburn Chemicals, Peebles, U.K. and Fisons, Loughborough, U.K.). Sodium octylsulphonate was HPLC grade (Fisons). Potassium dihydrogenphosphate was AnalaR grade (BDH, Poole, U.K.) and triethylamine was reagent grade (Hopkins and Williams, Chadwell Heath, U.K.). Buffer pH was adjusted with reagent-grade phosphoric acid (BDH). Tioconazole, the three related impurities and the degradation product were supplied by Pfizer Central Research, Sandwich, U.K.

RESULTS AND DISCUSSION

Selection of columns

The influence of column stationary phase has received only limited attention with regard to separation optimisation. Previous work[5] has suggested that using different column packing materials may provide enhanced selectivity and lead to improved separation. Thus three column types exhibiting differing retention characteristics were investigated during this study. The column packing materials selected

were ODS, phenyl and cyanopropyl, with ODS being the most retentive and cyanopropyl the most polar and thus the least retentive.

The columns utilised for this investigation were all short (5 cm or below) to reduce development time to a minimum. The elution time and re-equilibration time could be significantly reduced, this being an important consideration, as many different mobile phases were to be employed.

Selection of significant mobile phase parameters

After having selected the columns, other variables were considered. The factors most frequently varied to effect a separation are the proportions of organic modifiers. For reversed-phase chromatography, methanol, acetonitrile and tetrahydrofuran (THF) offer the widest range of modifier selectivities and an experimental design aiming to achieve optimum separation should incorporate all three. However, there are many other variables which also affect chromatographic behaviour and they should also be considered.

Preliminary studies with the Ultrasphere 5-μm ODS column (45 $\times$ 4.6 mm I.D.) and simple modifier–water eluents, revealed baseline disturbances on injection of a sample. These perturbations were attributable to changes in local column pH caused by the sample solvent. The inclusion of potassium dihydrogenphosphate buffer in the mobile phase was found to rectify the problems of baseline disturbance. Thus buffered mobile phases were used throughout these studies.

Buffers serve several functions in HPLC; they prevent excessive changes in pH and they may also block the interfering effects of residual silanol groups on the stationary phase[6,7]. Potassium dihydrogenphosphate controls the pH effectively but has only limited action against residual silanols. Another buffer system, triethylamine (TEA) adjusted to the appropriate pH with phosphoric acid, is effective both in controlling pH and in blocking silanol interactions. One of these buffers was included in this study in turn to assess the influence of silanol interaction on the separation.

As discussed above, tioconazole and the potential impurities can exist in two pH-dependent forms. At pH values above 8 the solutes exist as neutral species, while at pH values below 4 the solutes are protonated. These protonated solutes may be eluted as such or, by the addition of an anionic ion-pairing agent, as neutral ion complexes. Thus two other variables are introduced, pH and the presence of an ion-pairing agent.

Once the appropriate variables were selected it was necessary to devise an experimental scheme allowing a rational investigation of the key parameters involved. The experimental scheme proposed was based upon a combination of partial factorial and lattice mixture designs[5,9–12] for each of the three columns (Fig. 2). Many variables were assessed at only two levels (*i.e.*, an upper and lower level, or frequently the presence or absence of the variable). The optimum separation conditions were predicted from the lattice mixture designs for each set of experimental variables. These optimum separation conditions were run in each case and the chromatogram recorded. The importance of each set of variables was determined by measuring a number of parameters for the optimum chromatogram. Column efficiency was described by the reduced plate height for tioconazole and the selectivity was determined by calculating the selectivity factor between compounds B and C. The silanol interaction was assessed by the asymmetry factor.

Throughout these studies the detection wavelength employed was 220 nm, the

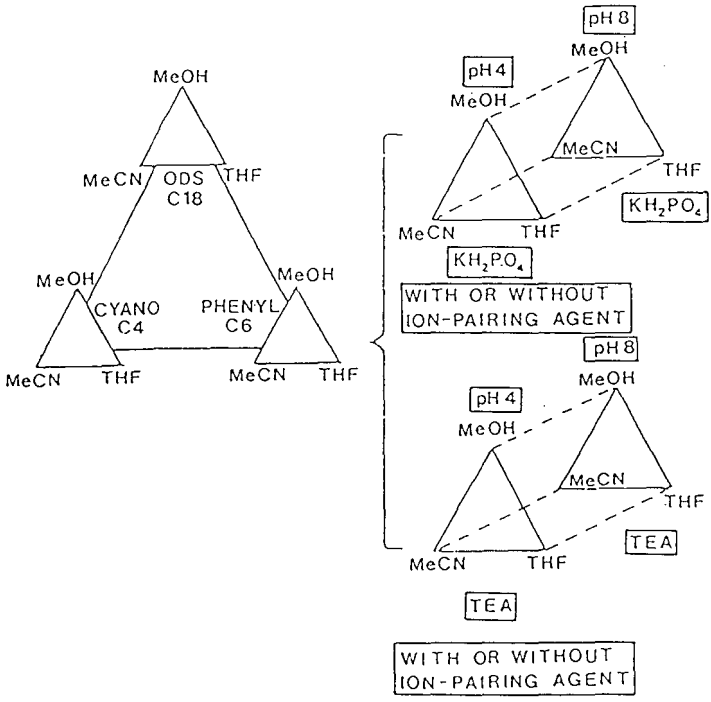


Fig. 2. Experimental scheme employed in the method development. MeOH = Methanol; MeCN = acetonitrile.

flow-rate 1.5 ml min^{-1} and column temperature 40°C. The concentrations of buffer and ion-pairing agent used were 0.05 *M* and 0.025 *M*, respectively. The lattice mixture designs employed for this study all required nominally isoelutropic eluents. The retention time for the last peak yielded by these eluents was approximately 7 min in all cases.

The Hypersil 5-μm phenyl column (50 × 4.6 mm) was investigated using the experimental scheme shown in Fig. 2. The results are recorded in Table II.

The same procedures were followed for the Ultrasphere 5-μm ODS column (45 × 4.6 mm I.D.). Lattice mixture designs were initiated for TEA buffer both with and without ion-pairing agent. However, significant retention time variations occurred on mixing equal proportions of the nominally isoeluotropic binary eluents of methanol–buffer and acetonitrile–buffer. Typically the retention time for tioconazole was 3 min with the methanol binary and 2.7 min with the acetonitrile binary. Mixing these two eluents (1:1, v/v) led to a retention time of 6.9 min. Since the mixed eluents prepared from the isoeluotropic binary eluents yielded retention times for the last peak in excess of those required, the experimental scheme was abandoned for the ODS column. Adjustment of solvent strength for the mixed eluents was not attempted as the lattice mixture designs were run as automated procedures through the method sequence facility of the LC system. It should be noted that this problem was not encountered with the phenyl column.

TABLE II

REDUCED PLATE HEIGHT, BEST PEAK ASYMMETRY AND SELECTIVITY VALUES FROM THE EXPERIMENTAL SCHEME

Column	*pH*	*Buffer*	*Ion-pairing*	*Optimum eluent (%)*			*Minimum reduced plate height*	*Best peak asymmetry*	α_{max} *for B and C*
				Methanol	*Acetonitrile*	*THF*			
Phenyl	4	KH_2PO_4	+	25	16	9	7.2	1.53	1.130
Phenyl	4	KH_2PO_4	–	–	46	–	5.8	1.20	1.202
Phenyl	4	TEA	+	16	39	–	4.9	1.06	1.128
Phenyl	4	TEA	–	55	–	–	8.3	1.20	1.129
Phenyl	8	TEA	–	62	17	–	3.5	1.05	1.153
Phenyl	8	KH_2PO_4	–	47	11	–	6.4	1.35	1.088
Cyanopropyl	4	TEA	+	–	40	–	–	–	1.153

A mixture design was followed for the Nucleosil 5-μm cyanopropyl column (50 × 4.6 mm I.D.) for TEA buffer containing ion-pairing agent. The peak shapes were strongly tailed and this produced high peak asymmetry values and poor resolution. The poor column performance did not warrant continuation of the experimental scheme, thus it was abandoned at this point.

The Hypersil phenyl column results (Table II) showed TEA to be the more appropriate buffer in this separation as compared to potassium dihydrogenphosphate. It yielded better resolution due to the improved peak shape; this was attributable to the effectiveness of TEA in blocking silanol interactions. The three cases where TEA was used as buffer all provided good selectivity, acceptable efficiency and some degree of resolution between peaks B and C. It was felt that consideration of the three sets of variables would benefit, if the best separations possible for each set of conditions were compared. Thus optimum conditions were determined using another strategy. In each lattice mixture design it was observed that THF had no effect on improving selectivity or resolution between compounds B and C. Thus only methanol and acetonitrile were considered. The optimisation strategy selected was the sequential simplex procedure. This optimisation strategy was also applied to confirm that the phenyl column did give the best separations. The optimum separation conditions were determined for both the ODS and cyanopropyl columns for comparison. The variables studied were methanol, acetonitrile and pH 4 TEA buffer containing ion-pairing agent.

Location of the optimum separation

With the variables of interest thus defined, the optimum separation conditions were located using the modified sequential simplex search method developed by Berridge[8]. This is an entirely automated and unattended procedure which does not require retention behaviour to be well defined. For these optimisations a sequential simplex was run with the maximum retention time for the last peak specified as 10 min, to ensure that analysis times were all comparable. The modifiers used were constrained to the following ranges: methanol 0–60%, acetonitrile 0–50%. Minimum retention time = 1 min, maximum retention time = 10 min.

The quality of the chromatographic separations were assessed using the chromatographic response function (CRF) developed by Berridge[8]

$$\text{CRF} = \sum_{i=1}^{n-1} R_i + n^2 - |T_A - T_n| - (T_0 - T_1)$$

where R_i is the resolution between adjacent peak pairs, n is the number of peaks observed in the chromatogram, T_1 is the retention time of first detected peak, T_0 is a specified minimum retention time for the first peak, T_A is the maximum desired retention time for the last peak, and T_n is the retention time for the last peak.

Resolution between adjacent peaks is determined from the data generated by the LC integrator using the following equation:

$$R_s = \frac{2(t_{R,2} - t_{R,1})}{W_1 + W_2}$$

where $t_{R,1}$ and $t_{R,2}$ are retention times of peaks 1 and 2, respectively and W_1 and W_2 are base-widths for peaks 1 and 2, respectively.

TABLE III

SEQUENTIAL SIMPLEX OPTIMA WITH TEA BUFFER

Column	*pH*	*Ion-pairing*	*Optimum eluent*		*Minimum reduced plate height*	*Resolution between B and C*	*CRF*
			Methanol	*Acetonitrile*			
Phenyl	4	+	31	19	5.3	0.45	28.2
Phenyl	4	–	23	33	6.1	0.40	28.5
Phenyl	8	–	29	21	6.6	0.36	28.6
ODS	4	+	20	40	6.2	0.27	28.0
Cyanopropyl	4	+	21	9	21	0.25	28.3

The sequential simplex results (Table III) showed that the phenyl column did yield the best separations, confirming the early decision to abandon studies on the ODS and cyanopropyl columns. The best resolved chromatogram located for the phenyl column corresponded to methanol–acetonitrile–pH 4 TEA buffer containing ion-pairing agent (31:19:50, v/v/v). The movement of this sequential simplex procedure is shown in Fig. 3. Confirmation that this was the global optimum was achieved by using a sequential surface-mapping experiment. Chromatograms were run using conditions representative of the whole response surface, defined by the limits for acetonitrile and methanol levels in the range 0–70%. Both modifier ranges were searched simultaneously in 5% increments, necessitating 120 chromatograms to cover the complete surface. The quality of each separation was assessed using the CRF defined above. The response contours are plotted in Fig. 4. The global optimum separation conditions were located as methanol–acetonitrile–pH 4 TEA containing ion-pairing agent (35:15:50, v/v/v), giving a CRF value of 28.5. A representative chromatogram is shown in Fig. 5.

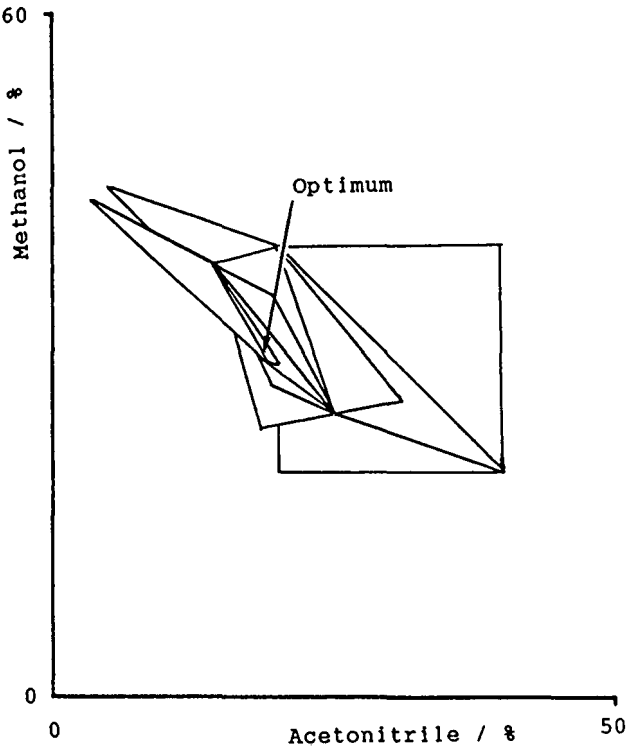


Fig. 3. Movements of the sequential simplex procedure for the phenyl column. Mobile phase consists of methanol, acetonitrile and triethylamine (0.05 *M*) at pH 4 containing ion-pairing agent (0.025 *M*).

Prediction of column dimensions necessary for complete separation

The optimum mobile phase was determined for a short Hypersil 5-μm phenyl column (50 × 4.6 mm I.D.) and, while some resolution of B and C was achieved, the performance of the system was not sufficient for complete separation. Thus, as selectivity had already been optimised, it was necessary to increase column plate count to achieve complete resolution. It was therefore necessary to increase column length. Since capacity factor (k') for the last peak and column length are among the parameters which govern analysis time, minimum analysis time can be attained by using the shortest possible column which provides sufficient theoretical plates for the separation. It was assumed that flow-rate was fixed and need not be considered.

The desired capacity factor for the last peak in this assay was specified as 10. The optimum separation conditions located by sequential simplex and grid mapping procedures both yielded separations where the capacity factor for the last peak was in excess of 10. It was therefore of interest to calculate the reduction in the proportion of buffer in the mobile phase, needed to yield a k' value of 10. It was assumed that selectivity was dependent upon the relative proportions of the modifiers to each other and independent of buffer level. Thus the ratio of methanol to acetonitrile was kept constant (*i.e.*, 70:30, v/v).

As the temperature employed throughout was 40°C, it was also of interest to

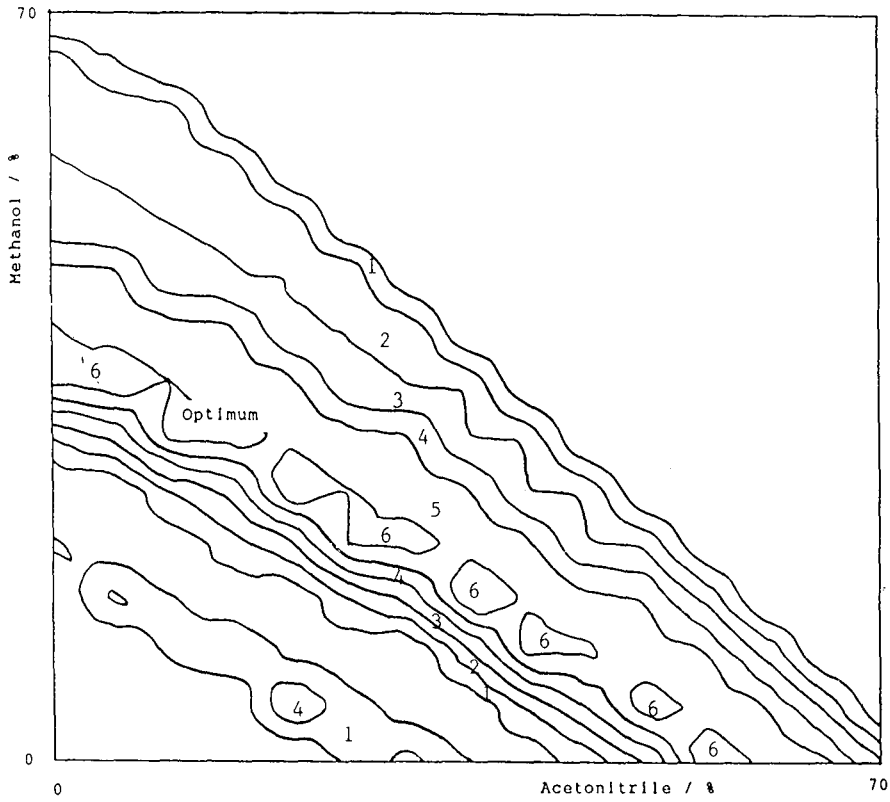


Fig. 4. Contour plot of the response surface. CRF values for contours: (1) 0–5; (2) 5–10; (3) 10–15; (4) 15–20; (5) 20–25; (6) 25–30.

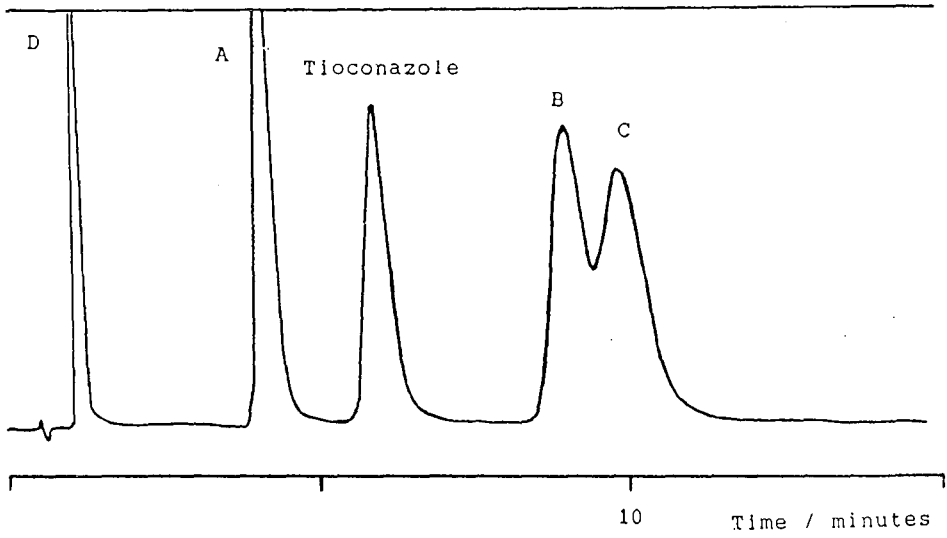


Fig. 5. Separation of tioconazole and related impurities for the 5-cm column. Mobile phase, methanol–acetonitrile–50 m*M* pH 4 TEA buffer (containing 25 m*M* 1-octanesulphonic acid) (31:19:50; v/v/v). Detection wavelength, 220 nm. Flow-rate, 1.5 ml min^{-1}.

TABLE IV

TEMPERATURE AND BUFFER LEVELS FOR INVESTIGATIONS OF INFLUENCE OF TEMPERATURE ON SEPARATION

Temperature (°C)	*Proportion of buffer (%)*	*Capacity factors*	
		B	*C*
50	55	20.0	22.1
40	55	27.4	30.6
30	55	40.5	46.0
50	50	10.9	11.9
40	50	13.9	15.4
30	50	19.7	21.9
50	45	6.4	6.9
40	45	8.0	8.8
30	45	10.4	11.5

calculate the eluent composition required to produce a capacity factor of 10 if the separations were carried out at room temperature (25°C). To calculate these eluent compositions, nine chromatograms with different buffer levels were run at different temperatures to provide the necessary data. Second order equations of the form $y = ax^2 + bx + c$ (where y was equivalent to $\log k'$) were found to fit data both for buffer level at a set temperature and for temperature at a set buffer level. To determine the eluent composition required to produce a capacity factor of 10 for the last peak at 40°C, the equation for compound C linking $\log k'$ and buffer level at 40°C was calculated. Substituting 10 for k' and solving the quadratic for the buffer level showed the proportion of buffer required to be 46% (v/v).

Calculation of the eluent composition required at 25°C was more complicated. The relationship between $\log k'$ for compound C and temperature was determined for each buffer level. A temperature of 25°C was substituted into each function to calculate a $\log k'$ value for that buffer level. Three $\log k'$ values (one for each buffer level) were determined in this way. These data were fitted to a second order equation to describe the dependence of $\log k'$ with buffer level at 25°C. Substitution of 10 for k' was again used to determine the eluent composition required. It was found to be 44% (v/v).

These capacity factor data enabled a prediction of the column length required for complete resolution. An analogous process to that described above was applied to capacity factor data for compound B. The equations linking $\log k'$ and temperature for each buffer level were determined and the $\log k'$ values at 25°C calculated for each one. Fitting these data to a second order equation generated a function relating $\log k'$ to buffer level at 25°C. At this point instead of substituting 10 for k', the proportion of buffer producing $k' = 10$ for compound C was substituted into the equation and k' for compound B calculated. The value of k' determined for compound B was 9.

In this way the k' values for both compounds B and C were known (9 and 10. respectively) and allowed the prediction of the number of theoretical plates necessary for complete separation of all components. The following resolution equation was used for this prediction:

$$R_s = \frac{1}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot N^{0.5} \cdot \frac{k'}{(1 + k')}$$

where R_s is the resolution between B and C, α is the ratio of k' between C and B, N is the number of theoretical plates, and k' is the average of k' for B and C. Complete resolution was assumed to correspond to an R_s value of 1.5.

Substituting the calculated values into the equation produced a value for N of 4400 plates. The length of column required was predicted, based on chromatograms where the 5-cm column generated 360 plates per cm, and found to be *ca.* 12 cm. To allow for a suitable excess the column length of 15 cm was selected.

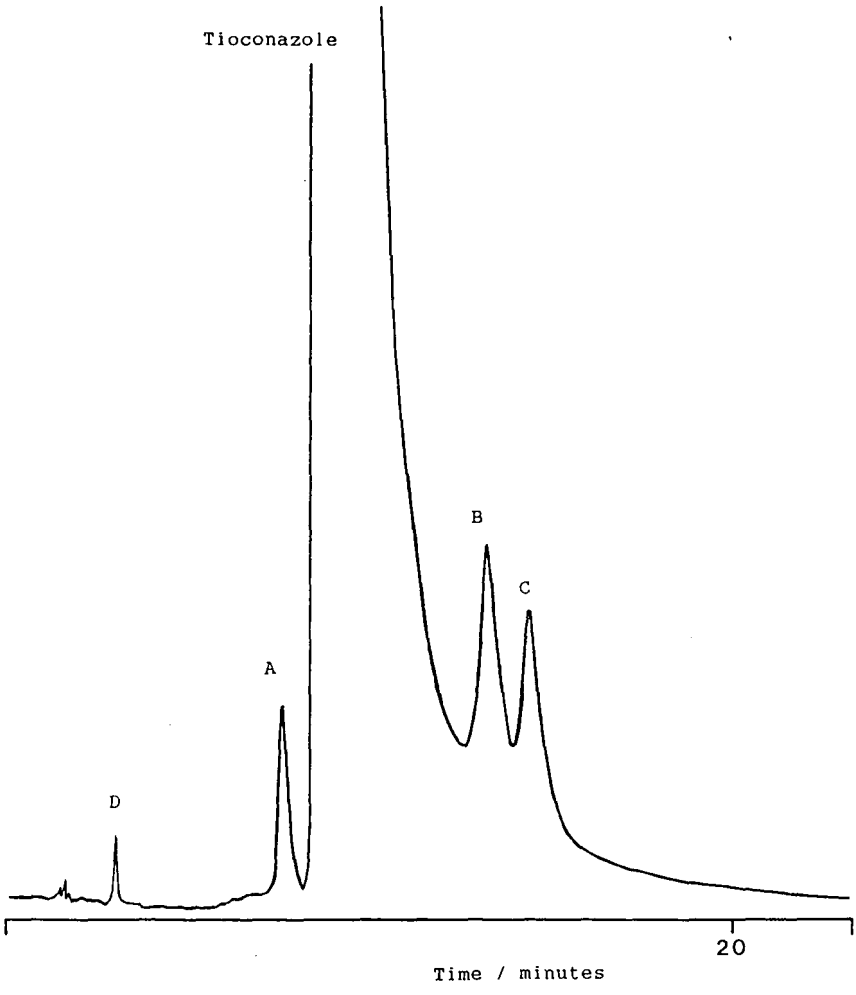


Fig. 6. Separation for the 15-cm column. Mobile phase, [methanol–acetonitrile (70:30, v/v)]–50 m*M* pH 4 TEA buffer (containing 25 m*M* 1-octanesulphonic acid) (54:46, v/v). Detection wavelength, 220 nm. Flow-rate, 1.5 ml min^{-1}.

Validation of method

On reducing the concentration of the related impurities to realistic levels, the question of peak tailing of the major component arises. The extent of this peak tailing prevents accurate determinations of compounds B and C (Fig. 6). This will be considered in a subsequent publication[13].

CONCLUSIONS

In the present work, the initial development criteria were fulfilled as the analysis time was reduced from 25 to less than 15 min, compounds B and C were adequately resolved, the mobile phase did not cause column damage, and the hydrolysis product could also be assayed.

The experimental scheme adopted, incorporating partial factorial designs, lattice mixture designs and sequential simplex has been demonstrated as being suitable for developing a HPLC separation for tioconazole and its related impurities.

ACKNOWLEDGEMENT

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REFERENCES

1 *United States Pharmacopeia XXI*, Supplement 2, 1895, United States Pharmacopeial Convention, Rockville, MD, 1985.
2 J. C. Berridge, P. E. Last and R. V. Platt, *J. Pharm. Biol. Anal.*, 3 (1985) 391.
3 J. C. Berridge and K. S. Andrews, *Analyst (London)*, 109 (1984) 287.
4 J. P. Bounine, G. Guiochon and H. Colin, *J. Chromatogr.*, 298 (1984) 1.
5 J. L. Glajch, J. C. Gluckman, J. G. Charikofsky, J. M. Minor and J. J. Kirkland, *J. Chromatogr.*, 318 (1985) 23–39.
6 J. S. Kiel, S. L. Morgan and R. K. Abramson, *J. Chromatogr.*, 320 (1985) 313–323.
7 W. R. Melander, J. Stoveken and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 111–127.
8 J. C. Berridge, *J. Chromatogr.*, 244 (1982) 1–14.
9 R. D. Snee, *CHEMTECH*, 9 (1979) 702–710.
10 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57–79.
11 J. L. Glajch, J. J. Kirkland and L. R. Snyder, *J. Chromatogr.*, 238 (1982) 269–280.
12 J. L. Glajch and J. J. Kirkland, *Anal. Chem.*, 55 (1983) 319A–336A.
13 A. G. Wright, A. F. Fell and J. C. Berridge, *Analyst (London)*, in press.

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NEW INSIGHTS INTO THE RECOGNITION MECHANISMS OF CHIRAL "BRUSH-TYPE" LIQUID CHROMATOGRAPHIC STATIONARY PHASES

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SUMMARY

It is shown that in some cases the adsorption complex of the enantiomer eluted first is of importance in rationalizing the differences in enantioselectivities between structurally related chiral stationary phases. It is helpful to define the relative capacity factor, $k'^* = k'$(chiral phase)/k'(non-chiral phase) and $k'_{A/B} = k'$(chiral phase A)/k'(chiral phase B) for each enantiomer of interest.

INTRODUCTION

Chiral stationary phases (CSPs) have proved to be a very useful tool for analytical and preparative separation of enantiomers by liquid chromatography. Chiral recognition mechanisms have been proposed for the various types of CSPs including the "brush-type" phases which consist of silica with covalently bonded chiral functional groups. In these discussions generally a complex between the CSP and the more strongly retained enantiomer is suggested[1–5]. The other enantiomer is assumed to be eluted first because it exhibits a poorer fit with the chiral moiety of the CSP, an idea which is not discussed further. In some cases a rather detailed insight into the CSP–sample complex was obtained which allows assessment of the individual interactions[6–10]. Non-enantioselective interactions with silanol groups can be important[11]. Comparisons with gas chromatographic investigations have been made[12].

We propose to describe the retention of chiral compounds on a "brush-type" CSP, expressed as the capacity factor, k', as proportional to the sum of the non-chiral and chiral interactions (the absolute configuration of the CSP must be known, *e.g.*, *R*)

$$k'_R = k'_l \sim \Sigma \text{ interactions with non-chiral sites} + \Sigma \text{ interactions of l complexes}$$
$$k'_S = k'_u \sim \Sigma \text{ interactions with non-chiral sites} + \Sigma \text{ interactions of u complexes}$$

where l and u, meaning like and unlike[13], respectively, describe the relative configuration of the diastereomeric adsorption complex built up from the chiral

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moiety of the CSP and the sample. If the CSP and sample both have the *R* (as above) or *S* configuration, the complex is called like, if they differ in configuration, the complex is unlike. Enantioselectivity, expressed as the separation factor, α^*, then becomes:

$$\alpha^* = \frac{k'_l}{k'_u} = \frac{\Sigma \text{ non-chiral interactions} + \Sigma \text{ l-interactions}}{\Sigma \text{ non-chiral interactions} + \Sigma \text{ u-interactions}}$$

α^* differs from the common definition of the separation factor, α, in that it is the ratio of the capacity factors of the *R* and *S* enantiomers irrespective of their elution order; therefore, α^* is less than 1 if the *S* enantiomer is eluted last. For CSPs I, II and IV discussed in this paper, the enantiomer eluted last is always *R* and therefore $\alpha^* = \alpha$. In the case of CSP III there is an inversion of the elution order, therefore for some enantiomers $\alpha^* = \alpha$, for the others $\alpha^* = 1/\alpha$.

Non-chiral interactions must be taken into consideration when correlating α values with thermodynamic parameters[14]. Retention can be influenced by non-chiral interactions if the loading of the CSP with chiral functional groups is very low. Moreover, if the affinity of the sample to complex with the chiral moiety of the CSP is not so pronounced, *i.e.*, is enhanced only to a low degree relative to the affinity to the non-chiral support, the separation factor, α or α^*, can depend on the loading of the stationary phase. This behaviour was observed, *e.g.*, for N-3,5-dinitrobenzoylphenylethylamide on a naphthylethylamide CSP (phase I)[15]. On the contrary, non-chiral interactions can be neglected and the separation factor is independent of the loading of the stationary phase if the sample has an high affinity to complex with the chiral moiety, as has been reported for the same sample on a different CSP[16].

If non-chiral interactions have been shown experimentally to be negligible, the chiral selectivity, α^*, depends only on the l- and u-interactions. Assuming that for each enantiomer only one single complex with the chiral moiety of the CSP is of importance (because other possible complexes are markedly less stable, this assumption is certainly a simplification in many cases), then α^* can be written as follows:

$$\alpha^* = \frac{\text{l-interaction}}{\text{u-interaction}}$$

Within a series of structurally related compounds, α^* is usually not constant. The most simple approach would be a correlation of the capacity factor of the second eluted enantiomer, k'_2, with the separation factor. This is generally not the case, see the series of homologues[1,17,18] or of π-acceptor amides[19,20]. A low separation factor can be due to rather pronounced non-specific complexation of both the *R* and *S* enantiomer or due to highly specific like and unlike interactions of the two enantiomers to a similar extent.

For the discrimination of these two cases and a discussion of chiral recognition mechanisms in more detail, we propose to use the relative capacity factor, k'^*, which for the two enantiomers is defined as

$$k'^*_u = \frac{k'_u}{k'_N} = \frac{\text{u-interactions}}{\text{non-specific interactions}}$$

$$k_1'^* = \frac{k_1'}{k_N'} = \frac{\text{l-interactions}}{\text{non-specific interactions}}$$

where k_N' is the capactiy factor of the enantiomer on a non-specific, *i.e.*, as low in specificity as possible for the samples investigated, non-chiral reference stationary phase using the same mobile phase, this reflecting mainly the sample polarity.

EXPERIMENTAL

Different non-chiral stationary phases were tested for their suitability as a reference phase: bare silica and silica with chemically bonded nitrile, amine or amide. The silica phase was Spherisorb S5W (Kontron Analytical, Zürich, Switzerland), particle diameter 5 μm, and the nitrile phase was Nucleosil CN (Macherey-Nagel, Oensingen, Switzerland) with 10-μm particles; both phases were used as pre-packed columns. The amine and amide phases were laboratory-made from Matrex Silica Si (Grace, Wallisellen, Switzerland). This 5-μm silica was dried at 100°C and 0.1 mbar for 4 h, then refluxed in a 4% solution of 3-aminopropyltriethoxysilane (Fluka, Buchs, Switzerland) in dry toluene for 16 h; this gave a load of 0.55 mmol/g of aminopropyl groups (calculated with the carbon content obtained by elemental analysis). This amino phase was slurry packed in isopropanol in 25 cm × 4.6 mm I.D. stainless-steel columns. The acetyl amide phase was prepared *in situ* from this amino silica by pumping a solution of 2.5 g of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroxyquinoline (EEDQ, Fluka) and 0.5 g of acetic acid in 50 ml of dichloromethane through an amino column in analogy to a published method[21].

The chromatographic data for the chiral stationary phases used in this study have been published earlier[1,17,18]. The structures of the four different π-donor amide CSPs, I–IV, are given in Fig. 1. The samples used are an homologous series of

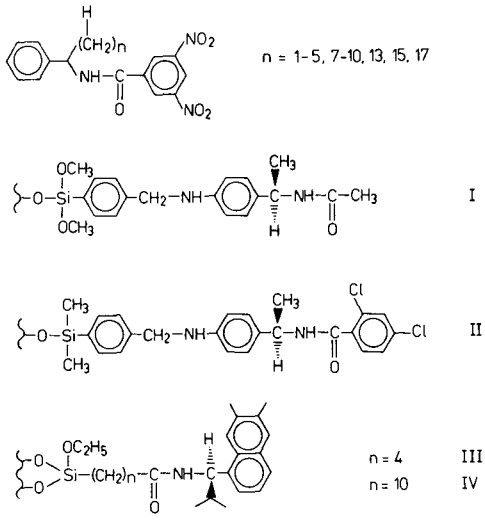

Fig. 1. Structures of the test compounds and of chiral stationary phases I[18], II[18], III[1] and IV[1]. CSP II had been synthesized in the *S* configuration; it is drawn here in the *R* form in accordance with the other phases, and results obtained with it have been adapted.

TABLE I

CAPACITY FACTORS OF THE SERIES OF HOMOLOGUES ON DIFFERENT NON-CHIRAL STATIONARY PHASES

Mobile phase: *n*-hexane–isopropanol (4:1).

n	k'_{Silica}	$k'_{Nitrile}$	k'_{Amino}	k'_{Amide}
1	0.41	2.41	0.57	0.90
2	0.32	2.40	0.48	0.87
3	0.24	2.19	0.41	0.74
4	0.20	2.03	0.38	0.70
5	0.17	1.88	0.35	0.65
7	0.13	1.63	0.31	0.57
8	0.12	1.52	0.29	0.54
9	0.11	1.44	0.28	0.51
10	0.10	1.36	0.26	0.48
13	0.08	1.17	0.23	0.40
15	0.07	1.08	0.21	0.37
17	0.06	0.99	0.19	0.34

3,5-dinitrobenzoylamides of phenyl-*n*-alkylamines, see Fig. 1. The mobile phase was *n*-hexane–isopropanol (4:1) in all cases.

RESULTS AND DISCUSSION

As seen from Table I, all non-chiral stationary phases tested behave similarly in that the capacity factors of the dinitrobenzoylamides decrease with increasing alkyl chain length. Therefore any of these phases can be used as a reference to calculate k'^*; we decided to use the data of the amino phase for reference purposes. The choice is arbitrary and does not influence the quality of the results. A comparison of Tables

TABLE II

CAPACITY FACTORS, SEPARATION FACTORS AND RELATIVE CAPACITY FACTORS, k'^*, ON CHIRAL STATIONARY PHASES I AND II

Mobile phase: *n*-hexane–isopropanol (4:1). $k'_u = k'_1$ and $k'_l = k'_2$ in all cases, therefore $\alpha^* = \alpha$.

n	*Phase I*					*Phase II*				
	k'_u	k'_l	α^*	k'^*_u	k'^*_l	k'_u	k'_l	α^*	k'^*_u	k'^*_l
1	6.72	8.60	1.28	11.8	15.1	13.0	16.3	1.25	22.8	28.6
2	6.87	9.55	1.39	14.3	19.9	13.0	16.3	1.25	27.1	34.0
3	6.25	8.81	1.41	15.2	21.5	13.5	16.5	1.22	32.9	40.2
4	6.18	8.65	1.40	16.3	22.8	12.7	15.6	1.23	33.4	41.0
5	5.71	8.28	1.45	16.3	23.7	12.5	15.3	1.22	35.7	43.7
7	4.94	7.31	1.48	15.9	23.6	10.8	13.3	1.23	34.8	42.9
8	4.71	7.11	1.51	16.2	24.5	10.0	12.6	1.26	34.5	43.4
9	4.32	6.70	1.55	15.4	23.9	9.5	12.0	1.26	34.0	42.9
10	4.09	6.34	1.55	15.7	24.4	8.8	11.1	1.25	34.0	42.7
13	3.48	5.57	1.60	15.1	24.2	7.8	9.9	1.28	33.7	43.1
15	3.32	5.41	1.63	15.8	25.8	7.4	9.4	1.28	35.1	45.0
17	3.01	4.94	1.64	15.8	26.0	6.9	9.0	1.30	36.5	47.5

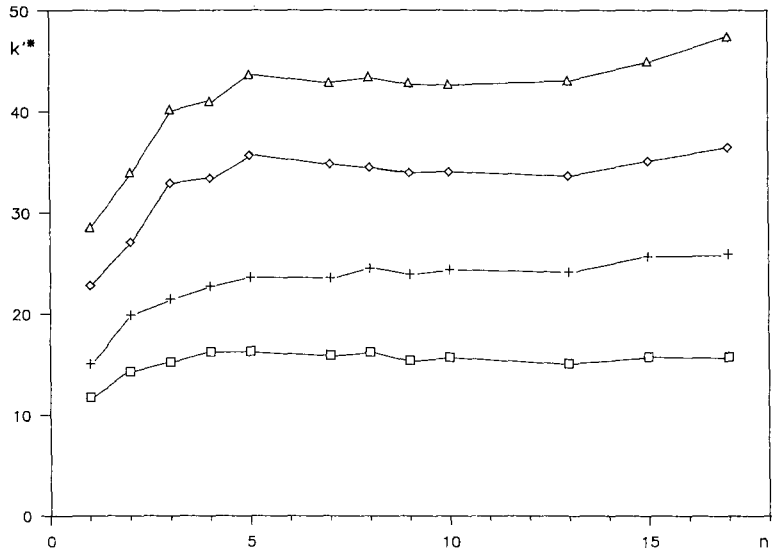

Fig. 2. Relative capacity factors k'^*, on chiral stationary phases I and II. □ = $k'_u{}^*$ (phase I); + = $k'_l{}^*$ (phase I); ◇ = $k'_u{}^*$ (phase II); △ = $k'_l{}^*$ (phase II).

I–III shows that the fraction of retention due to the silanol groups or amine groups in the CSPs is negligible.

Table II gives the capacity factors, k', the separation factors, α^*, and the relative capacity factors, k'^*, for each enantiomer of the samples on CSPs I and II. Relative capacity factors on phase II are almost twice as high as those on phase I. The k'^* values are also presented in Fig. 2. (In Figs. 2–5, points have been connected for clarity, although values between the points have no significance). It is seen that the shapes of the curves are almost the same in all four cases. The decrease of k' with increasing alkyl chain length of the samples is less pronounced on CSPs I and II than on the reference phase. One can speculate that attractive Van der Waals interactions are involved in the retention processes of these samples on phases I and II.

Within a series of compounds, variations of the separation factors are usually rationalized by discussing the complexation of the second enantiomer eluted. This presupposes that the relative capacity factor of the first enantiomer eluted is constant and/or negligible, *i.e.*, that the complexation of this enantiomer does not depend on the chain length (or other parameters) within the series. As is seen from Fig. 2, this is not the case for the homologous series used in this work on CSPs I and II.

Table III and Fig. 3 show the correponding data for CSPs III and IV. The curve for the unlike complexes have similar shapes to those on phases I and II: in contrast, the curves for the like complexes are very different. Beginning at $n = 4$ on phase III or $n = 7$ on phase IV the relative capacity factors decrease drastically instead of reaching a plateau.

The macroscopic relative capacity factor, k'^*, reflects the microscopic diastereomeric adsorption complex; it takes into account the sample polarity. The similar shapes of the k'^* curves indicate a relationship between the microscopic complexes. From Figs. 2 and 3 it can be concluded that the like and unlike complexes on phases

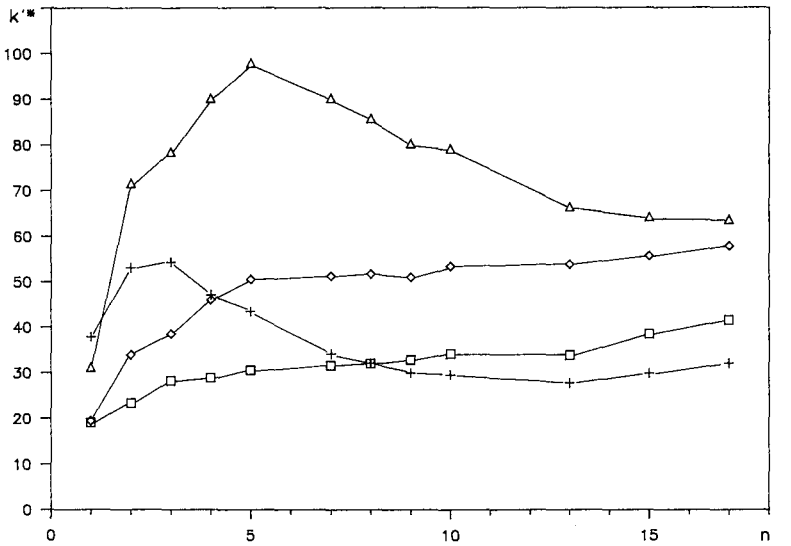

Fig. 3. Relative capacity factors, k'^*, on chiral stationary phases III and IV. □ = $k'_u{}^*$ (phase III); + = $k'_l{}^*$ (phase III); ◇ = $k'_u{}^*$ (phase IV); △ = $k'_l{}^*$ (phase IV).

I and II as well as the unlike complexes on phases III and IV behave similarly. Therefore they must be structurally similar. In contrast, the like complexes on phases III and IV are of another kind. It can be concluded that two structurally different retention mechanisms must be taken into consideration when discussing phases III and IV, as has been proposed earlier[1], whereas this is not necessary for CSPs I and II. With a more simplified discussion based only on the size of the groups arranged near the

TABLE III

CAPACITY FACTORS, SEPARATION FACTORS AND RELATIVE CAPACITY FACTORS, k'^*, ON CHIRAL STATIONARY PHASES III AND IV

Mobile phase: *n*-hexane–isopropanol (4:1). On phase III there is a reversal of elution order at $\alpha^* = 1.00$ ($n = 8$). For $n = 1$ to 7, $k'_1 = k'_u$ and $k'_2 = k'_l$; for $n = 9$ to 17, $k'_1 = k'_l$ and $k'_2 = k'_u$.

n	*Phase III*					*Phase IV*				
	k'_1	k'_2	α^*	$k'_u{}^*$	$k'_l{}^*$	k'_u	k'_l	α^*	$k'_u{}^*$	$k'_l{}^*$
1	10.9	21.6	1.98	19.1	37.9	11.1	17.8	1.60	19.5	31.2
2	11.2	25.5	2.28	23.3	53.1	16.3	34.4	2.11	34.0	71.7
3	11.6	22.3	1.92	28.3	54.4	15.8	32.2	2.04	38.5	78.5
4	11.0	17.9	1.63	29.0	47.1	17.5	34.3	1.96	46.0	90.3
5	10.7	15.2	1.42	30.6	43.4	17.7	34.3	1.94	50.6	98.0
7	9.8	10.6	1.08	31.6	34.2	15.9	28.0	1.76	51.3	90.3
8	9.3	9.3	1.00	32.1	32.1	15.0	24.9	1.66	51.7	85.9
9	8.4	9.2	0.91	32.9	30.0	14.3	22.5	1.57	51.0	80.4
10	7.7	8.9	0.87	34.2	29.6	13.9	20.6	1.48	53.5	79.2
13	6.4	7.8	0.82	33.9	27.8	12.4	15.3	1.23	53.9	66.5
15	6.3	8.1	0.78	38.6	30.0	11.7	13.5	1.15	55.7	64.3
17	6.1	7.9	0.77	41.6	32.1	11.0	12.1	1.10	57.9	63.7

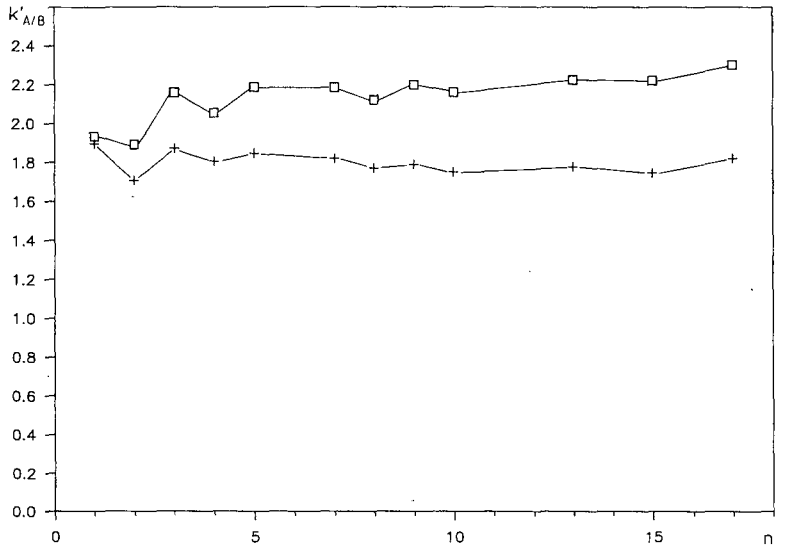

Fig. 4. Relative capacity factors, $k'_{A/B}$ of the unlike (eluted first) and like (eluted last) complexes on chiral stationary phases I and II. + = $k'_l(II)/k'_l(I)$; □ = $k'_u(II)/k'_u(I)$.

chiral centre or on steric interactions[12], it is more difficult to explain the differences between Figs. 2 and 3.

On phase I, α^* increases with n whereas on phase II α^* remains constant. It is useful to define a relative capacity factor of the second kind, $k'_{A/B}$, which is the ratio of the capacity factors on two different CSPs:

$$k'_{A/B} = \frac{k'(A)}{k'(B)}$$

For our purposes we use:

$$k'_{II/I} = \frac{k'(II)}{k'(I)} \quad \text{and} \quad k'_{IV/III} = \frac{k'(IV)}{k'(III)}$$

The corresponding data are given in Table IV and the plots are in Figs. 4 and 5. Note that for CSPs I and II, $k'_{A/B}$ of the like complexes (the enantiomers eluted last) in Fig. 4 is nearly constant whereas the unlike complexes (the enantiomes eluted first) show a slight increase. Any rationalization for the different selectivities of phases I and II for this homologous series therefore needs to be based on the retention of the enantiomer eluted first with the unlike complex. With CSPs III and IV (Fig. 5) the situation is different in that the like complexes show a markedly stronger dependence on n than the unlike ones. Therefore the like complexes must be discussed to explain the different selectivities of phases III and IV.

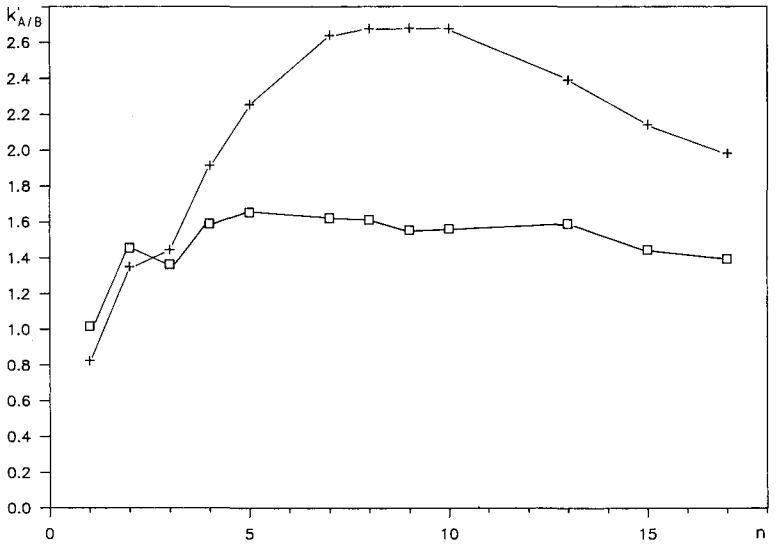

Fig. 5. Relative capacity factors, $k'_{A/B}$, of the unlike and like complexes on chiral stationary phases III and IV. + = k'_l(IV)/k'_l(III); □ = k'_u(IV)/k'_u(III).

CONCLUSIONS

The here defined relative capacity factors, k'*, which is the ratio of capacity factors on a chiral and on a non-chiral stationary phase for the same enantiomer, or $k'_{A/B}$, which is the ratio of capacity factors on different CSPs, allow a more detailed discussion of chiral recognition mechanisms than is possible by merely using the separation factor, α. It may be necessary to consider the complexation of the enantiomer eluted first to explain the differences in enantioselectivity of various CSPs towards a series of samples of related structure; this is the case for CSPs I and II used in

TABLE IV
RELATIVE CAPACITY FACTORS, $k'_{A/B}$

n	$k'_u(II)/k'_u(I)$ *unlike*	$k'_l(II)/k'_l(I)$ *like*	$k'_u(IV)/k'_u(III)$ *unlike*	$k'_l(IV)/k'_l(III)$ *like*
1	1.93	1.90	1.02	0.82
2	1.89	1.71	1.46	1.35
3	2.16	1.87	1.36	1.44
4	2.06	1.80	1.59	1.92
5	2.19	1.85	1.65	2.26
7	2.19	1.82	1.62	2.64
8	2.12	1.77	1.61	2.68
9	2.20	1.79	1.55	2.68
10	2.16	1.75	1.56	2.68
13	2.23	1.78	1.59	2.39
15	2.22	1.75	1.44	2.14
17	2.31	1.83	1.39	1.98

this study. This means that the discussion of the retention mechanism of the enantiomer eluted second alone does not necessarily include the relevant part of a separation.

Possible structures of a complex of the enantiomer eluted first are not given in this paper. It was the aim to propose the concept of relative capacity factors which can help to validate any proposed complexes. These complexes can be found experimentally or theoretically by chromatographic investigations, spectroscopic data[22] or computer methods[3,8,9]. Any structures found by these methods must not be in contradiction with the concept proposed by this paper.

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REFERENCES

1 W. H. Pirkle, M. H. Hyun and B. Bank, *J. Chromatogr.*, 316 (1984) 585.
2 W. H. Pirkle and R. Däppen, *J. Chromatogr.*, 404 (1987) 107.
3 G. Krüger, J. Grötzinger and H. Berndt, *J. Chromatogr.*, 397 (1987) 223.
4 D. A. Nicoll-Griffith, *J. Chromatogr.*, 402 (1987) 179.
5 M. Lienne, M. Caude, R. Rosset and A. Tambuté, *J. Chromatogr.*, 448 (1988) 55.
6 I. W. Wainer, T. D. Doyle, F. S. Fry jr. and Z. Hamidzadeh, *J. Chromatogr.*, 355 (1986) 149.
7 I.W. Wainer and M. C. Alembik, *J. Chromatogr.*, 367 (1986) 59.
8 U. Norinder and E. G. Sundholm, *J. Liq. Chromatogr.*, 10 (1987) 2825.
9 K. B. Lipkowitz, D. A. Demeter, R. Zegarra, R. Larter and T. Darden, *J. Am. Chem Soc.*, 110 (1988) 3446.
10 R. E. Boehm, D. E. Martire and D. W. Armstrong, *Anal. Chem.*, 60 (1988) 522.
11 Y. Dobashi and S. Hara, *J. Org. Chem.*, 52 (1987) 2490.
12 B. Feibush, *J. Chromatogr.*, 436 (1988) 517.
13 D. Seebach and V. Prelog, *Angew. Chem.*, 94 (1982) 696.
14 B. Koppenhoefer and E. Bayer, *Chromatographia.*, 19 (1984) 123.
15 R. Däppen, V. R. Meyer and H. Arm, *J. Chromatogr.*, 361 (1986) 93.
16 W. H. Pirkle and M. H. Hyun, *J. Chromatogr.*, 328 (1985) 1.
17 M. H. Hyun, *Thesis*, University of Illinois, Urbana, IL, 1984.
18 R. Däppen, V. R. Meyer and H. Arm, *J. Chromatogr.*, 295 (1984) 367.
19 R. Däppen, R. M. Wolf and H. R. Karfunkel, Poster presented at the *First International Symposium on Separation of Chiral Molecules, Paris, May–June 1988.*
20 R. Däppen, H. R. Karfunkel and F. J. J. Leusen, *J. Chromatogr.*, submitted for publication.
21 W. H. Pirkle and C. J. Welch, *J. Org. Chem.*, 49 (1984) 138.
22 W. H. Pirkle and T. Pochapsky, *J. Am. Chem. Soc.*, 109 (1987) 5975.

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PARTITION OF HYDROPHOBIC COMPOUNDS BETWEEN TWO LIQUID PHASES OF SIMILAR HYDROPHOBICITY

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SUMMARY

Liquid–liquid two-phase systems have been attained by dissolving pairs of polymers in organic solvents with or without the addition of water. The solvents include dioxane, N,N-dimethylformamide, tetrahydrofuran, dimethyl sulphoxide and acetonitrile. The compositions of the phases have been determined in some cases. Phases (in equilibrium) with nearly the same ratio between the organic solvent and water have been obtained. Hydrophobic compounds have been partitioned within two-phase systems containing dimethylformamide. One of these systems has been used for fractionation of zein (from corn) by counter-current distribution.

INTRODUCTION

Polymeric substances, dissolved together in a solvent, may give rise to more than one liquid phase. This behaviour was observed by Beijerinck[1] and has been a subject of concern to the paint and plastic industry[2,3]. The two liquid phases obtained by using water as a solvent for two polymers were introduced by Albertsson[4] as a tool for fractionation of biomaterial. These aqueous two-phase systems have been useful for the partition of proteins and membrane particles because of the high water content in both phases.

For many biochemical applications it may be valuable to have phases which both, and to the same degree, exhibit a greater hydrophobicity than aqueous systems. If so, it should be possible to partition less water-soluble biochemicals. The present work demonstrates that the replacement, to various degrees, of the water in the aqueous two-phase systems with organic solvents can increase the solubility of hydrophobic compounds in both phases.

MATERIALS AND METHODS

Chemicals

The organic solvents were of analytical grade and were used without further purification. Zein from corn, stearic acid and β-carotene from carrot were obtained from Sigma (St. Louis, MO, U.S.A.). [(1*S*)-*endo*-](−)-Borneol, (1*S*)-(−)-β-pinene and β-estradiol were obtained from Aldrich (Steinheim, F.R.G.). Glycerol tripalmitate

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was from BDH (Poole, U.K.), cholic acid from Schwartz/Mann (Orangeburg, NY, U.S.A.) and benzoic acid from Baker (Phillipsburgh, NJ, U.S.A.). An extract containing chlorophyll was obtained from spinach leaves by treating them with acetone and filtering. The filtrate was evaporated to dryness and the residue was dissolved in acetone and finally filtered. Before use the acetone was evaporated.

Polymers

Polyethylene glycol (PEG) with a molecular weight, M_r = 8000 was obtained from BP Chemicals (Hythe, U.K.). Dextran T500, Ficoll 400 and hydroxypropyl-dextran 500 (with 1.5 mol hydroxypropyl groups per mol glucose) were obtained from Pharmacia (Uppsala, Sweden). Hydroxypropyl-starch (Aquaphase PPT) was obtained from Perstorp Biolytica (Lund, Sweden). Polyvinyl acetate, "medium molecular weight" catalogue No. 18,948-0, was from Aldrich.

Preparation of the two-phase systems

The dry polymers, the solvents and the water were weighed out. In the case of dextran a 20% stock solution was prepared[4]. All concentrations of the polymers and solvents are given in per cent (w/w) of the total system. The mixtures were in most cases heated in a water-bath (50°C) to reduce the time taken to dissolve the polymers. Before equilibration the systems were brought to 22 ± 1°C.

Turbidometric titration

Phase transition points were determined by the addition of solvent mixtures to two-phase systems which were being shaken continuously until the turbidity ceased. Percentage compositions were calculated from the initial and final weights.

Analysis of phase composition

The composition of the phases of a dextran–PEG–dimethylformamide–water two-phase system was analysed by gel chromatography on a Sephacryl S-300 column (25 cm × 1.5 cm) using water as the eluent. The eluate was analysed with a refractive index monitor (Multiref 902B; Optilab, Vällingby, Sweden). Dextran and Ficoll were in some cases analysed by polarimetry[4].

Solubility measurements

The solubility of benzoic acid, in mixtures of organic solvents and water, was measured by the absorbance at 272 nm of the saturated solutions. Cloud points for hydrophobic compounds dissolved in organic solvents (20 g/l) were determined by the dropwise addition of water. For polymers the cloud points were ascertained by the addition of organic solvents to aqueous solutions (10%, w/w).

Partition coefficients

Samples of measured volumes were withdrawn from the phases and diluted in 65% (v/v) dioxane in water. The concentration of the partitioned substance was determined photometrically with an Hitachi 100-60 spectrophotometer. The following wavelengths were used: zein, 277.5 nm; β-carotene, 450 nm; β-estradiol, 280.5 nm; benzoic acid, 272 nm; and chlorophyll a (extracted into acetone from spinach), 665 nm. In some cases the solutes and polymers of the diluted phases (10–20 times) were

separated by gel chromatography (Sephadex LH-20, 50 cm × 1.3 cm) in dimethylformamide and the refractive index of the eluate was monitored.

Counter-current distribution

A nineteen-transfer counter-current distribution was carried out manually as described elsewhere[5]. The volumes in each tube were 2.1 and 2.0 ml for the upper and lower phases, respectively. A sample of 25 mg zein per ml was introduced in system number zero. After the completed transfer, 1.6 ml dimethylformamide were added to each tube to disrupt the systems. The protein content was determined according to Bradford[6]. The yellow constituent in zein was measured by the absorbance at 400 nm.

RESULTS AND DISCUSSION

Solubility of hydrophobic compounds and polymers

The solubility of many organic compounds in water is low while they are very soluble in a number of organic solvents. By adding solvents which mix with water the solubility can be gradually increased. Even a moderate addition of solvent may have a marked effect on the solubility, as demonstrated here for benzoic acid, Fig. 1. A number of hydrophobic substances chosen from groups of compounds of

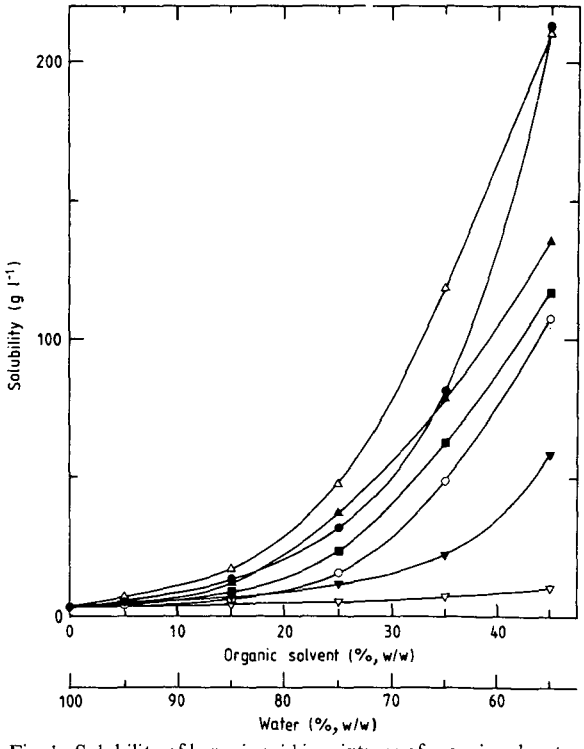

Fig. 1. Solubility of benzoic acid in mixtures of organic solvent and water at 22°C. Solvents: ▽ = ethylene glycol; ▼ = dimethyl sulphoxide; ○ = ethanol; ■ = dioxane; ▲ = acetonitrile; ● = N,N-dimethylformamide; △ = acetone.

TABLE I

SOLUBILITY OF SOME HYDROPHOBIC COMPOUNDS IN A SERIES OF ORGANIC SOLVENTS AT 22°C

+ = The compound is soluble to a concentration of at least 20 g/l; − = the solubility is less then 20 g/l. Values give the cloud points (in per cent organic solvent of total solvent) when solutions of the compounds (20 g/l in the organic solvent) were titrated with water.

Solute	*Solvent*							
	Ethylene glycol	*Dimethyl-formamide*	*Tetrahy-drofuran*	*Aceto-nitrile*	*Dioxane*	*Ethanol*	*Acetone*	*Dimethyl-sulphoxide*
Glycerol tri-palmitate	−	−	+ 75	−	+ 92	−	−	−
Stearic acid	−	+ 92	+ 63	−	+ 82	+ 94	+ 99	−
β-Carotene	−	+ 74	+ 56	+ 58	+ 69	+ 62	+ 63	+ 92
β-Pinene	−	+ 79	+ 61	+ 64	+ 70	+ 61	+ 63	+ 89
β-Estradiol	+ 94	+ 55	+ 33	−	+ 48	+ 55	+ 47	+ 65
Borneol	+ 82	+ 47	+ 34	+ 26	+ 33	+ 30	+ 28	+ 64
Cholic acid	+ 51	+ 23	+ 23	−	+ 23	+ 23	−	+ 39

biochemical interest were tested in eight (water-soluble) solvents, Table I. In several cases 50% or more of the water had to be replaced by the solvent in order to get a reasonable solubility of the hydrophobic solute.

To be able to generate a liquid–liquid two-phase system, with comparable solvent composition in both phases, it is necessary to find two polymers which are soluble in the solvent (or mixture of solvents) but partially exclude each other. Table II shows the solubility of some polymers in a number of solvents. Good solvents for the polymers tested are water, ethylene glycol, glycerol, dimethyl sulphoxide and N,N-dimethylformamide. The last two also have acceptable properties for the hydrophobic substances. Because of the unpleasant properties of dimethyl sulphoxide, dimethylformamide was chosen as the solvent for the systems used to study the partition of hydrophobic substances.

Systems composed of an organic solvent and two polymers

Ficoll 400 and PEG 8000, dissolved together in N,N-dimethylformamide, gave rise to two-phase systems (at 22°C) when the concentration of each polymer exceeded 7.5% (w/w). In these systems the Ficoll was found mainly in the lower phase and the PEG in the upper phase. When PEG was added with hydroxypropyl-dextran 500 (1.5 hydroxypropyl groups per glucose unit), in the same solvent, the concentration of each polymer needed to be higher than 7.1% in order to get two phases. Hydropropyl-dextran is, in contrast to dextran, very soluble in dimethylformamide, Table II.

TABLE II

SOLUBILITY OF SOME POLYMERS IN A SERIES OF SOLVENTS AT 22°C

+ = The polymer is soluble to a concentration of at least 5% (w/w); − = the solubility is less than 5% (w/w). Values give the cloud points (in per cent organic solvent of total solvent) when 10% aqueous solutions of the polymers were titrated with the solvent.

Solvent	*Dextran 500*	*Ficoll 400*	*Aquaphase*	*Hydroxypropyl-dextran 500*	*PEG 8000*	*Polyvinyl acetate*
Water	+	+	+	+	+	−
Glycerol	+	+	+	+	84	−
Ethylene glycol	+	+	+	+	>95	−
Dimethyl sulphoxide	+	+	+	+	+	+
Ethanol	30	61	50	91	+	+
Tetrahydrofuran	30	47	42	59	+	+
Dioxane	46	69	64	80	+	+
Acetonitrile	22	36	31	44	+	+
Dimethylformamide	62	+	+	+	+	+
Acetone	24	46	33	61	+	+

Partition in systems with organic solvents

The partitions of some hydrophobic substances within two-phase systems based on N,N-dimethylformamide are given in Table III. Substances with lower molecular weights showed a somewhat higher affinity for the upper phase with partition coefficients in the range 1–2.5. In the case of an hydrophobic protein, zein from corn, a partition coefficient less than one was observed.

TABLE III

PARTITION OF SOME HYDROPHOBIC COMPOUNDS IN TWO-PHASE SYSTEMS WITH N,N-DIMETHYLFORMAMIDE AS THE SOLVENT

The partition coefficient is defined as the ratio between the concentrations of the compound in the upper and lower phases. Composition of system: A, 8.5% Ficoll 400 and 8.5% PEG 8000; B, 8.1% hydroxypropyl-dextran 500 and 8.1% PEG 8000; C, 10% hydroxypropyl-dextran 500 and 10% PEG 8000. Temperature: 22°C. N.D. = Not determined. Values with an asterisk are partition coefficients obtained via gel chromatography of the phases (on Sephadex LH-20 in dimethylformamide) with values within ±10%. All other partition coefficients (±2%) were determined via photometric measurements.

Partitioned compound	*Molecular weight*	*Concentration (g/l)*	*Partition coefficient in system*		
			A	*B*	*C*
Benzoic acid	122	2	1.28	1.18	1.35
Borneol	154	10	N.D.	1.3*	1.5*
β-Carotene	537	2	2.09	1.69	2.14
Chlorophyll *a*	894	0.05	2.08	1.68	2.43
Cholic acid	387	10	N.D.	1.9*	2.3*
β-Estradiol	272	2	1.45	1.26	1.36
β-Pinene	136	10	N.D.	1.5*	1.9*
Stearic acid	284	30	N.D.	1.4*	2.4*
Zein	≥40 000	10	1.20	0.76	0.54

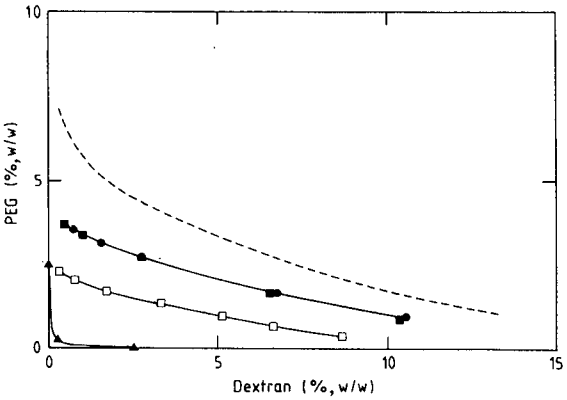

Fig. 2. Transition (one to two phases) curves for systems composed of dextran 500, PEG 8000, water and organic solvent (25%, w/w of total system). Solvents: ■ = dioxane; ● = N,N-dimethylformamide; □ = tetrahydrofuran; ▲ = acetonitrile. Temperature: 22°C. The broken line indicates the corresponding curve with water as the solvent (data from ref. 4).

Systems containing both organic solvents and water

The influence of solvents on the formation of two liquid phases was studied with a dextran–PEG system. Fig. 2 shows the borderline between the one- and two-phase areas (the binodial curve) when 25% (w/w) of the system consisted of organic solvent. The phase transition occurs at lower and lower concentrations of polymers according to the following order of organic solvents: N,N-dimethylformamide = dioxane < tetrahydrofuran < acetonitrile. The concentration range, in which a solvent can be used, was studied by following the transition points (using equal amounts of the two polymers) at increasing concentrations of organic solvent, Fig. 3. In the case of ethylene glycol up to 60% of the water can be replaced by this solvent with only minor changes in the transition point. Dioxane and N,N-dimethylformamide showed identical behaviour up to 25% (of total system) but deviated at higher concentrations.

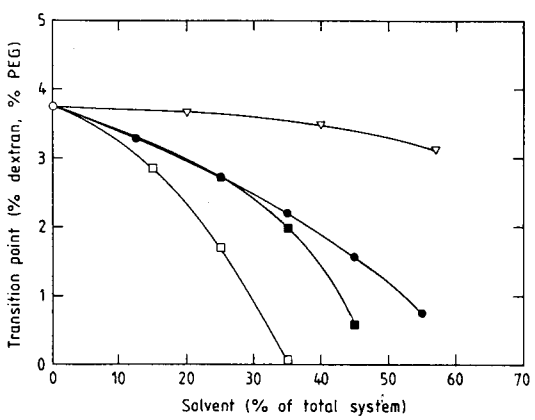

Fig. 3. Transition points (from one to two phases) of phases containing dextran 500 and PEG 8000 (in equal weights) as function of the solvent content (in %, w/w of total system). Solvents: ▽ = ethylene glycol; ● = N,N-dimethylformamide; ■ = dioxane; □ = tetrahydrofuran. Temperature: 22°C.

TABLE IV

RATIOS BETWEEN THE VOLUMES OF THE UPPER AND LOWER PHASES FOR SOME TWO-PHASE SYSTEMS CONTAINING DEXTRAN 500 AND PEG 8000, WITH OR WITHOUT ORGANIC SOLVENT (25% OF TOTAL SYSTEM)

Temperature: 22°C. Data for the solvent-free system have been obtained from ref. 4.

Solvent	*Composition of system (%, w/w)*			*Volume ratio*
	Dextran	*PEG*	*Organic solvent*	
Water	8.0	3.0	–	0.5
	5.0	5.0	–	1.7
	3.0	6.0	–	3.8
N,N-Dimethylformamide	8.0	2.0	25	0.5
	5.0	5.0	25	2.6
	3.0	6.0	25	6.0
Dimethyl sulphoxide	8.0	2.0	25	0.04
	5.0	5.0	25	2.0
	3.0	6.0	25	3.9
Dioxane	8.0	2.0	25	0.4
	5.0	5.0	25	2.5
	1.5	5.0	25	8.4
Tetrahydrofuran	8.0	1.5	25	1.1
	5.0	5.0	25	4.0
	1.5	4.0	25	14
Acetonitrile	1.5	0.25	25	16

The upper limit for the inclusion of N,N-dimethylformamide was around 60%. For dioxane and tetrahydrofuran the limits were 47 and 35%, respectively.

The volume ratios for a number of systems containing 25% organic solvent are shown in Table IV. The volume of the upper phase increased with the concentration of PEG which shows that this polymer was enriched in the top phase. The compositions of the phases may be presented in the form of a phase diagram. Such a diagram is given for the dextran–PEG–water–dimethylformamide system, Fig. 4. The three-dimensional diagram is divided into two parts by a curved surface, the binodial surface. Systems which have compositions above this surface give rise to two phases. The compositions of the two phases are found on the binodial surface. Furthermore, the points representing the total composition of the system and its phases lie on a straight line (the tie-line). The ratio between the weights of the phases is equal to the ratio of the segment lengths of the tie-line (intersected by the point for the total composition). It was found that the upper phases were somewhat richer in dimethylformamide than the lower phases, Table V and Fig. 4. On the other hand, the same is true for water. The relative amount of water and dimethylformamide in the phases did not differ by more than 2 percentage units.

A favourable system would be one in which the ratio between water and organic solvent can be varied within all possible values. It would then be possible to adjust the solvating properties according to the actual mixture of substances to be partitioned.

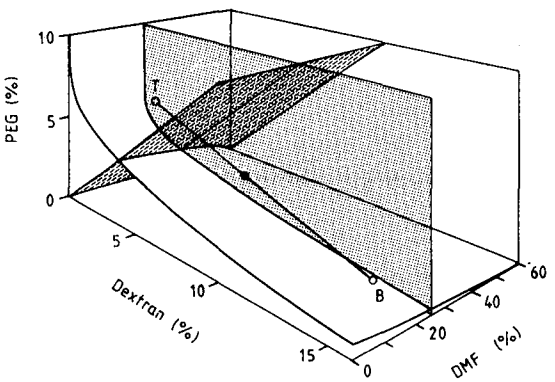

Fig. 4. Phase diagram for the system dextran 500, PEG 8000, water and N,N-dimethylformamide (DMF) at 22°C. The binodial surface is cut by two planes: one where the concentrations of PEG and dextran are equal, the other with constant concentration of DMF (25%, w/w). The total composition of a system (6.9% dextran, 2.7% PEG and 25% DMF) in the latter plane is marked with ●. The composition of the top phase (T) lies behind the plane. The composition of the lower phase (B) is located in front of the same plane. These three points fall on a straight line.

Possible pairs of polymers are Ficoll and PEG as well as hydroxypropyl-dextran and PEG. Both combinations gave two-phase systems in both water and dimethylformamide. The behaviour of the systems when they contained both solvents (in various proportions) can be seen in Table VI. Mixtures of the two solvents enhanced the miscibility of the polymers (in solution), and increasing concentrations were necessary to obtain two phases. At certain proportions of solvents no phase separation was obtained even at high polymer concentrations (20% of each).

Partition in systems containing both solvent and water

The partition of benzoic acid is affected only slightly when the concentration of dimethylformamide is raised from 0 to 45% in a dextran–PEG–water system, Table VII. This is due to the simultaneous and almost equal increase of the hydrophobicity of both phases.

TABLE V

COMPOSITION OF PHASES (IN %, w/w) OF SYSTEMS CONTAINING DEXTRAN 500, PEG 8000, WATER AND N,N-DIMETHYLFORMAMIDE (DMF) AT 22°C

Total system				*Upper phase*				*Lower phase*			
Dextran	*PEG*	*Water*	*DMF*	*Dextran*	*PEG*	*Water*	*DMF*	*Dextran*	*PEG*	*Water*	*DMF*
6.7	4.0	89.3	–	0.5	6.8	92.7	–	12.6	1.1	86.3	–
6.5	3.4	77.6	12.5	0.2	5.9	81.2	12.7	13.1	1.1	74.0	11.8
6.9	2.7	65.4	25.0	0.2	4.8	68.7	26.3	14.0	0.8	61.5	23.7
7.5	2.2	55.3	35.0	0.2	4.1	56.7	39.0	15.6	0.3	51.2	32.9
9.3	1.6	44.1	45.0	0.1	3.2	45.5	51.2	18.4	0.3	39.5	41.8

TABLE VI

TRANSITION POINTS (FROM ONE TO TWO PHASES) AT EQUAL CONCENTRATIONS OF FICOLL 400 AND PEG 8000, OR HYDROXYPROPYL-DEXTRAN 500 (HP-DEXTRAN) AND PEG 8000, RESPECTIVELY, IN MIXTURES OF WATER AND N,N-DIMETHYLFORMAMIDE (DMF)

Temperature: 22°C. Values are in % (w/w)

Solvent mixture		*System composition at phase transition point*				
Water	*DMF*	*Ficoll*	*HP-dextran*	*PEG*	*Water*	*DMF*
100	0	7.7	–	7.7	84.6	0
90	10	8.5	–	8.5	74.7	8.3
80	20	9.3	–	9.3	65.1	16.3
70	30	11.4	–	11.4	54.0	23.2
60	40	16.0	–	16.0	40.8	27.2
50	50	>20	–	>20	<30	<30
30	70	16	–	16	20	28
20	80	10.6	–	10.6	15.8	63.0
10	90	8.0	–	8.0	8.4	75.6
0	100	7.5	–	7.5	0	85.0
100	0	–	14.8	14.8	70.4	0
90	10	–	17.2	17.2	59.0	6.6
80	20	–	>20	>20	<48	<12
40	60	–	>20	>20	<36	<24
30	70	–	15.3	15.3	20.8	48.6
20	80	–	9.9	9.9	16.0	64.2
10	90	–	7.5	7.5	8.5	76.5
0	100	–	7.1	7.1	0	85.8

Counter-current distribution

A water-free system, based on dimethylformamide, was used for multistep partition analyses of the protein zein from corn, Fig. 5. This system was chosen since zein does not dissolve in water or in organic solvents containing more than 50% water. The protein profile shows that zein has at least two components and that the yellow pigment, probably carotenoids, follows one of them. Zein has been shown to consist of two main protein components of equal magnitude[7].

TABLE VII

PARTITION OF BENZOIC ACID IN SYSTEMS CONTAINING DEXTRAN 500, PEG 8000, WATER AND N,N-DIMETHYLFORMAMIDE (DMF)

Concentration of benzoic acid was 2 g l^{-1}. Temperature: 22°C.

System composition (%, w/w)				*Partition coefficient of benzoic acid*
Dextran	*PEG*	*Water*	*DMF*	
6.7	4.0	89.3	0	1.27
6.9	2.7	65.4	25	1.29
7.5	2.15	55.35	35	1.31
9.3	1.6	44.1	45	1.42

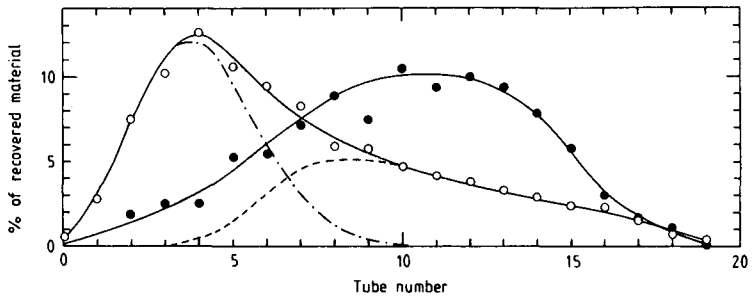

Fig. 5. Counter-current distribution of zein from corn using a system containing 9% hydroxypropyldextran 500, 9% PEG 8000 and 82% N,N-dimethylformamide (DMF). Recovered protein, ○; yellow component, ●. Number of transfers: 19. Temperature: 22°C. The phases were disrupted by the addition of 1.6 ml of DMF. The sample, loaded in tube number zero, contained 100 mg protein. A theoretical curve is included for the left peak, —·—·—, as well as the difference between the experimental protein curve and the theoretical one, — — —.

General remarks

The two-phase systems studied here have comparably low separatory capacity since the substances tested do not partition particularly in favour of one phase or the other. When used in multistep procedure, *e.g.*, counter-current distribution or liquid–liquid chromatography[8] developed for the aqueous two-phase systems, good separation can be foreseen. The new type of two-phase systems may, however, be further improved for specific extractions by introducing chemical groups (ligands) which form strong complexes with the compounds to be extracted. A ligand can be concentrated in mainly one phase by covalently binding to the corresponding polymer. This method of extraction through complex formation in one phase has been applied successfully to proteins in aqueous polymer two-phase systems[4,5]. The increased hydrophobicity is also of interest when the systems are used as enzyme reactors[9,10] (with the enzyme kept in one phase) and the substrate or product has low solubility in water.

CONCLUSIONS

Water-soluble solvents can be included in polymer–polymer (liquid–liquid) two-phase systems and this makes them useful for the partitioning of hydrophobic solutes. The experiments show that liquid–liquid two-phase systems can be attained with great variation in the hydrophobicity but that this property is nearly the same in both phases. Such systems allow the partition of solutes of more hydrophobic character than the usual water–polymer systems do.

ACKNOWLEDGEMENT

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REFERENCES

1 M. W. Beijerinck, *Kolloid Z.*, 7 (1910) 16.
2 A. Dobry and F. Boyer-Kawenoki, *J. Polym. Sci.*, 2 (1947) 90.
3 R. J. Kern and R. J. Slocombe, *J. Polym. Sci.*, 15 (1955) 183.
4 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist & Wiksell, Stockholm, and Wiley, New York, 2nd ed., 1971.
5 G. Johansson and M. Andersson, *J. Chromatogr.*, 291 (1984) 175.
6 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
7 J. Landry and P. Guyon, *Biochimie*, 66 (1984) 451.
8 W. Müller and G. Kütermeier, *Eur. J. Biochem.*, 128 (1982) 231.
9 F. Tjerneld, I. Persson, P.-Å. Albertsson and B. Hahn-Hägerdal, *Biotechnol. Bioeng.*, 27 (1985) 1036.
10 F. Tjerneld, I. Persson, P.-Å. Albertsson and B. Hahn-Hägerdal, *Biotechnol. Bioeng. Symp.*, 15 (1985) 419.

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USE OF THE SURFACTANT 3-(3-CHOLAMIDOPROPYL)-DIMETHYL-AMMONIOPROPANE SULFONATE IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS

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SUMMARY

Isocratic hydrophobic interaction chromatography of five proteins has been carried out using mobile phases containing the surfactant 3-(3-cholamidopropyl)-dimethylammoniopropane sulfonate (CHAPS). Linear relationships were found between log k' and ammonium sulfate concentrations for all the proteins with CHAPS in the submicellar concentration range. The slope of such a plot decreases monotonically as CHAPS concentration is increased. To a first approximation, the effect of CHAPS on protein retention can be explained in terms of a competitive binding model. However, CHAPS does show differential effects on the elution of proteins, substantially altering selectivity. The use of a normalized capacity factor, k'/k'_0, proves useful for comparing retention times of different proteins as a function of CHAPS concentration. The magnitudes of k'/k'_0 were found to be inversely correlated with the slopes of plots of log k' *vs.* ammonium sulfate concentration in the absence of CHAPS. Adsorption isotherms for CHAPS were determined over the working range of ammonium sulfate. The binding of CHAPS to the SynChropak Propyl stationary phase and its effects on retention were found to be readily reversible. For each protein, plots of k'/k'_0 *vs.* surface concentration of CHAPS were superposable for data obtained at different salt concentrations. These findings support a competitive binding model. A simple geometric argument for stationary phase occupancy provides a qualitative explanation for the observed surfactant selectivity.

INTRODUCTION

High-performance liquid chromatography (HPLC) in recent years has become an important technique for the separation of proteins. Most commonly, reversed-phase packings such as alkyl C_{18}, C_8, C_4, and phenyl-type functionalities have been used[1–5]. However, proteins, and particularly membrane proteins, tend to bind very strongly to such packings. Consequently, in the application of HPLC to proteins, the additives commonly used to elute proteins from such columns (*e.g.*, acetonitrile and propanol, often with acids such as trifluoracetic acid or phosphoric acid) are also denaturing toward many proteins. Thus there is a need for combinations of mobile and stationary phases capable of eluting proteins under non-denaturing conditions.

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Recently, increasing emphasis has been placed on hydrophobic interaction chromatography (HIC) as a number of different, commercial high-performance packings have become available for this mode of chromatography[6–10]. Elution in this mode of chromatography is achieved with decreasing salt (usually ammonium sulfate) concentration gradients under relatively mild, non-denaturing conditions.

As might be expected, surfactants were shown to be capable of interacting with HIC and reversed-phase stationary phase surfaces[11–16]. Occasional use of mild surfactants in protein chromatography has been reported[17,18], but systematic studies are lacking. Ionic surfactants are known to be capable of interacting with proteins[19], some even at concentrations below the critical micelle concentration (CMC). In contrast, non-ionic and dipolar ionic (zero net charge) surfactants interact less strongly with proteins, and with less likelihood of denaturation than ionic surfactants[20].

Therefore, zero net charge surfactants are expected to be useful in hydrophobic interaction chromatography either as eluting agents or at least to provide useful selectivity effects[21]. This study is an examination of the effects of the surfactant 3-(3-cholamidopropyl)-dimethylammoniopropane sulfonate (CHAPS) on retention of proteins in high-performance HIC.

EXPERIMENTAL

Materials

Carbonic anhydrase B (human erythrocyte, lot 10F-9320), ribonuclease A (bovine pancreatic, lot 101F-0561), bovine pancreatic trypsin inhibitor (BPTI, lot 104F-8035), and enolase (Yeast Type III, lot 101F-8125) were obtained from Sigma (St. Louis, MO, U.S.A.). Lysozyme (hen egg white, lot 7069) was obtained from Miles Labs. (Elkhart, IN, U.S.A.). The proteins were used without further purification.

Ammonium sulfate (Ultra Pure) was obtained from Schwarz Mann (Cambridge, MA, U.S.A.) and potassium phosphate (ACS reagent) was obtained from Fisher (Fair Lawn, NJ, U.S.A.). HPLC-grade water was produced with a Milli Q purification system.

CHAPS was synthesized according to the procedure of Hjelmeland *et al.*[22] and twice recrystallized from methanol.

Chromatographic procedures and equipment

The chromatograpic system consisted of a Varian Model 5000 pumping system (Palo Alto, CA, US.A.), a Valco (Houston, TX, U.S.A.) six-port injection valve with 25-μl injection loop, a Varian Varichrom variable-wavelength UV detector, and a Hewlett-Packard Model 3390a integrator. The chromatographic column was 15 cm $\times$ 4.6 mm I.D. packed with 6.5-μm SynChropak Propyl HIC packing (SynChrom, Linden, IN, U.S.A.). The column was thermostated with a circulating water bath at 30.0°C.

Ammonium sulfate solutions of varying concentrations were prepared in pairs, one member of the pair containing CHAPS at 0.0015 *M* and the other containing no CHAPS. All mobile phases contained 0.02 *M* potassium phosphate buffer at pH 6.1. CHAPS concentration in the chromatographic mobile phases was controlled by varying the proportion of the CHAPS-containing mobile phase with the HPLC pumping system. Isocratic elution of proteins was accomplished at a flow-rate of

1 ml/min, and monitored at a detector wavelength of 215 nm. Protein solutions were prepared at a concentration of *ca.* 1 mg/ml in mobile phase without CHAPS and filtered through a 0.5-μm filter prior to injection. Equilibration of the column in the presence of surfactant was verified by repeated injections of ribonuclease A until constant retention times were observed. After each series of measurements with surfactant, the column was flushed with water, methanol, and water. The column was then reequilibrated with the starting salt solution and the retention times for the five proteins were remeasured to verify reversibility of the column to surfactant exposure. Adsorption isotherms for CHAPS were measured by frontal chromatography using UV detection at 215 nm. Mobile phase concentrations of CHAPS were chosen to be below the CMC as determined fluorimetrically with a Perkin-Elmer 650-10s spectrofluorimeter (Table I). Mobile phase containing $3 \cdot 10^{-5}$ *M* 7-diethylamino-4-methyl coumarin was titrated with successive additions of a concentrated CHAPS solution, monitoring fluorescence emission at 450 nm (excitation at 370 nm). A rapid increase in fluorescence emission begins at the CMC.

TABLE I

CRITICAL MICELLE CONCENTRATIONS FOR CHAPS AT 30.0 ± 0.5°C

The CMC values were determined fluorimetrically with a estimated uncertainty of ± 0.3 m*M*.

Ammonium sulfate (mol/l)	*CMC (mmol/l)*
0	8.2
0.50	5.3
0.75	3.7
1.00	2.6
1.20	2.1
1.30	1.7
1.40	1.0

RESULTS AND DISCUSSION

Data for the retention of the five proteins in this study were examined by plotting k' *vs.* CHAPS concentration at constant ammonium sulfate concentration (Fig. 1). As can easily be seen from Fig. 1, addition of CHAPS to HIC mobile phases causes definite reductions in k' for all the proteins studied. Similar results were seen for all ammonium sulfate concentrations in the range 1.1–1.4 *M*. Furthermore, it should be noted that, on this stationary phase, the effect of CHAPS on retention is selective in that some proteins are affected to a greater extent than others. For some of the proteins changes in elution order occur as CHAPS concentration is increased.

It is instructive to compare the relative effect of CHAPS at different salt concentrations. In order to do this, k' values were normalized as follows. First k' in the absence of surfactant is defined as k'_0. Then the k' values in the presence of CHAPS are divided by k'_0 to yield the normalized retention parameters, k'/k'_0, which were then plotted as a function of CHAPS concentration in the mobile phase. Typical plots are found in Fig. 2.

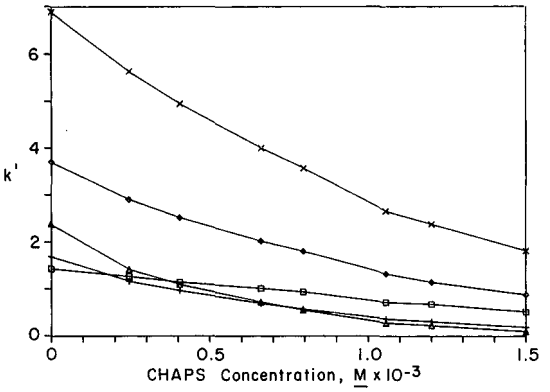

Fig. 1. Isocratic k' dependence on CHAPS concentration for five proteins. (×) Lysozyme; (◇) BPTI; (□) RNase A; (+) carbonic anhydrase; (△) enolase. $(NH_4)_2SO_4$ concentration: 1.30 *M*.

The normalized retention parameter k'/k'_0 can be useful for expressing retention in this mode of HIC, as the relative order of the plots is independent of salt concentration over the range employed in this study. That is, at all salt concentrations examined the relative magnitude of k'/k'_0 for the five proteins increases in the order of enolase < carbonic anhydrase < BPTI < lysozyme < ribonuclease A. Put another way, the effect of addition of CHAPS is always greatest on enolase and least on ribonuclease A. This effect occurs even though changes in order of retention occur as the ammonium sulfate concentration changes. The effect of CHAPS on any given protein's retention increases (*i.e.*, k'/k'_0 decreases) as the salt concentration is increased, as shown in Fig. 2 for RNase and enolase.

In discussing the meaning of these findings, a good starting place is the solvophobic model of retention of proteins. In a number of recent articles retention of proteins in the framework of this model have been discussed[23–25]. In this model, protein retention is related to the change in free energy which occurs on binding of

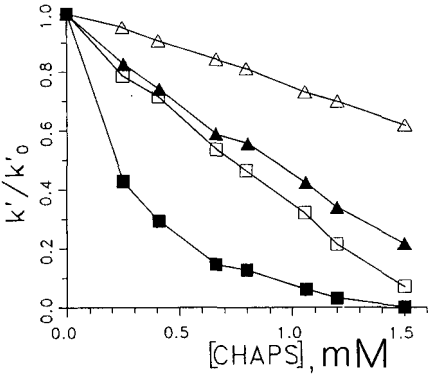

Fig. 2. Normalized retention parameter (k'/k'_0) dependence on CHAPS concentration for RNase A and enolase at two salt concentrations. (□) Enolase at 1.10 *M* $(NH_4)_2SO_4$; (■) enolase at 1.40 *M* $(NH_4)_2SO_4$; (△) RNase A at 1.10 *M* $(NH_4)_2SO_4$; (▲) RNase A at 1.40 *M* $(NH_4)_2SO_4$.

a protein to the stationary phase. From this viewpoint it was shown[23] that retention of proteins in hydrophobic interaction chromatography is predicted to obey an equation of the following form:

$$\ln (k'/k'_0) = Bm^{\frac{1}{2}}/(1 + Cm^{\frac{1}{2}}) + D\mu m + \Delta A_s \sigma m + \nu m + \text{constant} \quad (1)$$

where B, C and D are constants related to the protein, μ is the protein dipole moment, ν is a constant related to the particular salt, protein and stationary phase, m is the salt concentration and ΔA_s is the change in surface area of the stationary phase and protein which occurs on binding of the protein to the stationary phase. Note also that σ is the partial molal surface tension increment of the salt. At sufficiently high salt concentrations, the solvophobic model simplifies to the following equation:

$$\ln k' = \lambda m + Q \quad (2)$$

In this equation m is the salt concentration and Q is the extrapolated value of $\ln k'$ at zero salt concentration. The coefficient λ is a linear function of the hydrophobic area of the protein in contact with the stationary phase; it is also a linear function of the molal surface tension increment, which is characteristic of the particular salt employed. Thus $\ln k'$ is expected to show a linear dependence on salt concentration, with a slope proportional to the protein hydrophobic contact area. It is worth noting that eqn. 2 is essentially the same as the empirical Setschenov equation describing the salting-out of non-electrolytes[26].

To examine our experimental results in the framework of this model, we obtained values of λ and Q by linear regression analysis of plots of $\ln k'$ *vs.* ammonium sulfate concentration. Values of λ and Q were obtained from each of the five proteins at each of the CHAPS concentrations used. Linear plots of $\ln k'$ *vs.* ammonium sulfate concentration were obtained for all five proteins, both with and without CHAPS in the mobile phase, as shown in the example of RNase A, in Fig. 3. This figure shows that increasing CHAPS concentration has the effect of decreasing the slope, λ, and increasing the intercept, Q. In Fig. 4 the values of the slope, λ, are plotted as a function of CHAPS concentration for all five proteins. Here it is shown that for all the proteins

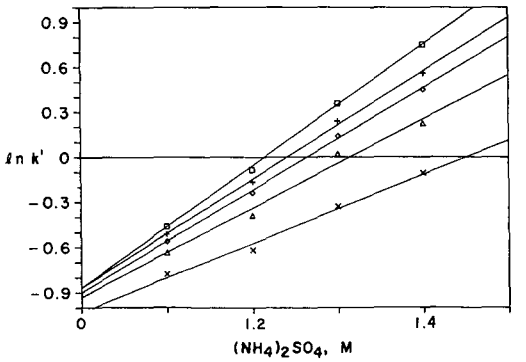

Fig. 3. Retention of RNase A as a function of $(NH_4)_2SO_4$ concentration, at several submicellar concentrations of CHAPS. (□) 0 m*M*; (+) 0.20 m*M*; (◇) 0.60 m*M*; (×) 1.00 m*M*.

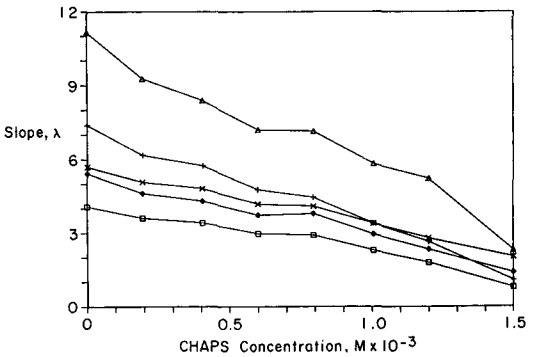

Fig. 4. Effect of CHAPS concentration on λ, the slope of a plot of ln k' *vs.* $(NH_4)_2SO_4$ concentration, for five globular proteins. (×) Lysozyme; (◇) BPTI; (□) RNase A; (+) carbonic anhydrase; (△) enolase. The range of $(NH_4)_2SO_4$ concentration for determining λ is 1.10–1.40 *M*.

an increase in CHAPS concentration leads to a decrease in λ, but to a different degree for each protein. Note that there is an inverse correlation between the magnitude of λ in the absence of CHAPS, and the magnitude of k'/k'_0 in the presence of CHAPS. Proteins whose retention is more strongly dependent on salt concentration are more strongly eluted by the addition of CHAPS to the mobile phase. This generalization is supported both by the data of Table II and Fig. 4. In the framework of the solvophobic model this would mean that the proteins with greater hydrophobic contact area are more strongly eluted by increasing CHAPS concentration.

Can the decrease in k' be accounted for through the surface tension of the mobile phase, as expressed in the solvophobic model? It was shown that surface tension of a surfactant solution can be related to surfactant concentration by a logarithmic relationship[27]. That is, at concentrations below the CMC, a plot of surface tension *vs.* log surfactant concentration will be a straight line. Other workers have shown for HIC in the absence of surfactants[23,24], and eqn. 1 predicts, that log k' is linearly related to the surface tension of the solution. Therefore we would expect to see a linear relation between log k' and log surfactant concentration if mobile phase surface tension is the dominant factor in controlling k'. However, plots of log k' *vs.* log CHAPS concentration are found to be definitely nonlinear. This is observed for the data

TABLE II

INVERSE RELATIONSHIP BETWEEN λ AND k'/k'_0

The slope, λ, from eqn. 2 was determined in the absence of CHAPS, while k'/k'_0 was determined for 1.30 *M* $(NH_4)_2SO_4$ and 0.40 m*M* CHAPS. A similar inverse relationship is found for other salt and CHAPS concentrations.

Protein	λ	k'/k'_0
RNase A	4.08	0.80
BPTI	5.69	0.68
Lysozyme	5.70	0.72
Carbonic anhydrase B	7.40	0.57
Enolase	11.15	0.46

obtained in this study and has also been seen in earlier results from this laboratory[21]. Therefore, with respect to surface tension, the solvophobic treatment of eqn. 1 is clearly inadequate. This is not surprising, given the highly non-ideal nature of surfactant solutions. A physically more realistic argument might be generated in terms of interfacial tensions between stationary and mobile phases. These are difficult to measure, but some encouragement is seen in recent reports[29,30]. Our continued use of eqn. 2 assumes that while the linear surface tension component of λ is here inapplicable, the linear hydrophobic contact area component of λ is valid.

Another possibility for interpreting decrease in k' with increasing CHAPS concentration is in terms of competition of CHAPS for protein-binding sites on the stationary phase, as has been done for reversed-phase chromatography[28]. If CHAPS is bound to the stationary phase and is bound with roughly the same affinity as the protein molecules, an effect similar to that shown in Fig. 5 might be observed. In this model, strength of binding of a protein to the stationary phase is related to the size of the hydrophobic contact area. In general, we expect that large proteins have larger hydrophobic surface patches than small proteins, but exceptions probably exist. The presence of CHAPS decreases the area of stationary phase available for hydrophobic contact with proteins. As the load of CHAPS on the column is increased, the number of large binding sites decreases more rapidly than the total number of binding sites. This is the natural consequence of a statistical distribution of occupancy of surfactant-binding sites. Thus this model predicts that the larger the protein hydrophobic contact area, the greater will be the effect of surfactant in reducing k'. Since the value of λ is proportional to the size of the protein hydrophobic contact area, the greater the value of λ in the absence of CHAPS, the greater will be reduction of k' in the presence of CHAPS (*i.e.* k'/k'_0 will be smaller). Table II shows that this correlation obtains for the five proteins in this study. The same trend is seen over a broader range of conditions in Fig. 4.

The above competitive binding model assumes no association between surfactant and protein in the mobile phase. This appears to be a reasonable assumption in

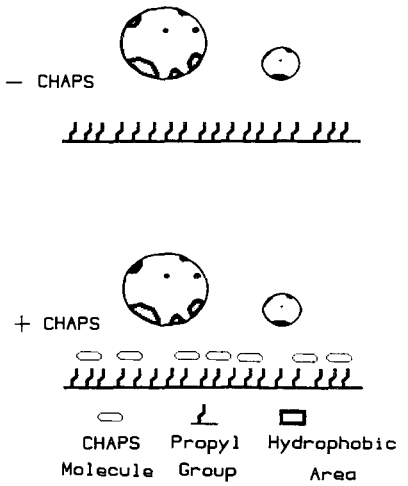

Fig. 5. Scheme to suggest how competitive binding between proteins and CHAPS could lead to selectivity.

view of the results of published binding studies[31]. Attempts to demonstrate mobile phase binding of CHAPS to this group of proteins showed no evidence of such binding[32].

A testable prediction of the above model is that the reduction in retention of a particular protein will depend only on the amount of surfactant bound to the stationary phase, irrespective of the salt concentration. Preliminary to testing this prediction we determined the binding isotherms for CHAPS at different salt concentrations by frontal chromatography (Fig. 6). It can be seen that isotherms of steeper slope are obtained at higher salt concentrations. Clearly the equilibrium between bound and mobile phase surfactant is shifted toward bound by increasing the ammonium sulfate concentration. The first premise of the competitive binding model is that, as CHAPS concentration on the stationary phase increases, decreases in k' for all the proteins will result. Fig. 7 shows this to be the case.

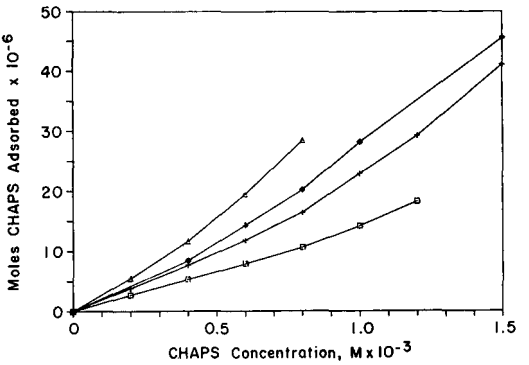

Fig. 6. CHAPS adsorption isotherms at four concentrations of $(NH_4)_2SO_4$. The isotherms were determined by frontal chromatograpy at 30.0°C on a SynChropak Propyl column, 15 × 0.46 cm I.D., pH 6.1. (□) 1.10 *M*; (+) 1.20 *M*; (◇) 1.30 *M*; (△) 1.40 *M*.

Further, if k'/k'_0 values for a protein are plotted against the surface concentration of CHAPS, the data from four different salt concentrations fall on a common curve. Fig. 7 shows that this is the case for each of the five proteins exained. This contrasts with the divergence in curve resulting from similar plots of k'/k'_0 *vs.* CHAPS concentration in the mobile phase, as shown by the examples in Fig. 2. Thus a testable prediction of the competitive binding model has been tested and verified. Considering that values of k' range from two-to-five fold (RNase A, smallest range), and from 14- to >50- fold (enolase, largest range), the fit of the retention data to a single curve for each protein is evidently significant. These results are what would be predicted if a given protein competes for binding sites with bound CHAPS, and if each protein always binds on its same hydrophobic surface or distribution of surfaces. If some other mechanism for decreasing k' were playing a significant role, such as surfactant–protein association in the mobile phase, it is unlikely that the correlations observed in Fig. 7 would obtain.

It should be noted that for simplicity, the competitive binding model discussed here is based on a static picture of the chromatographic interface. A more realistic but

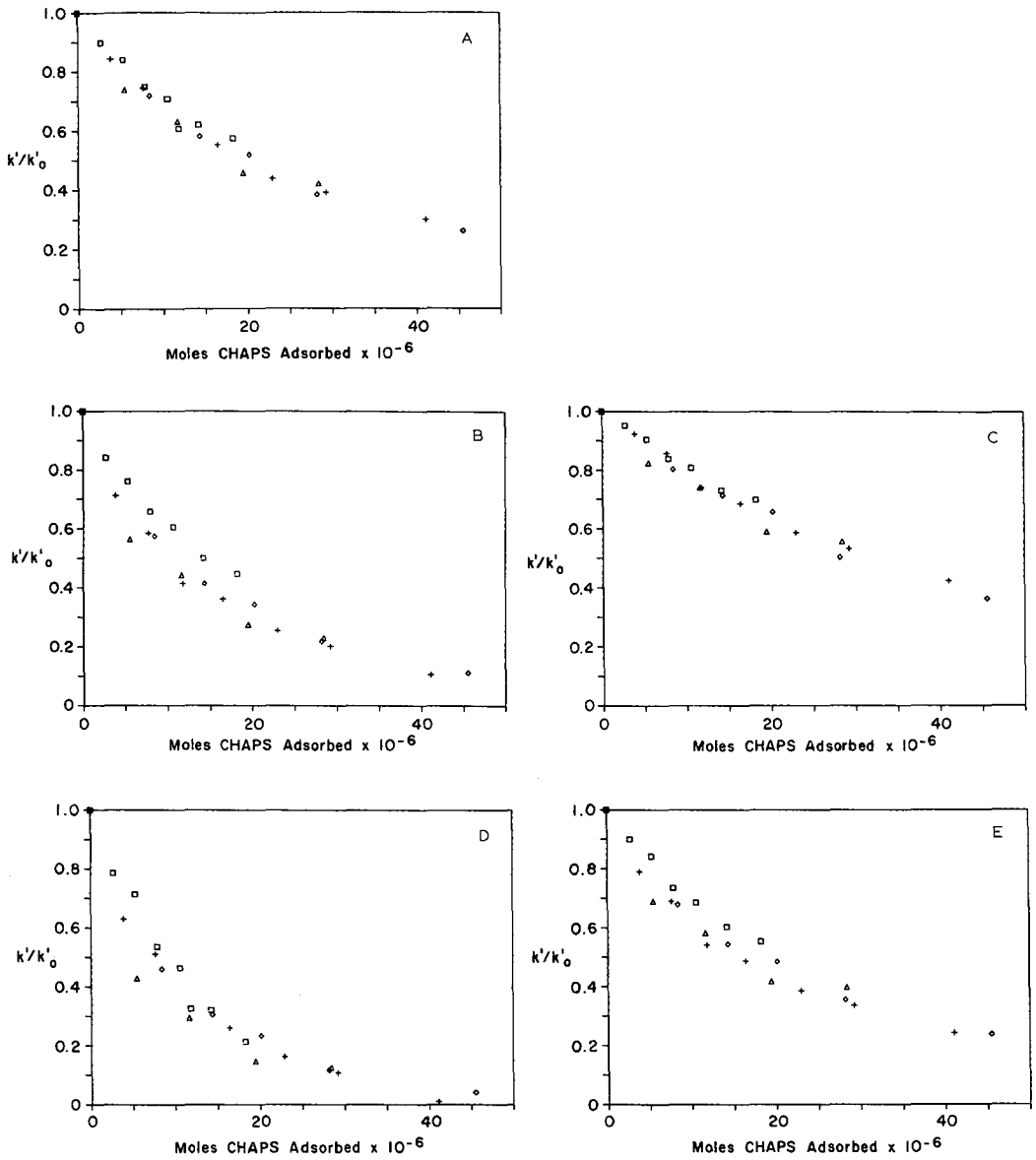

Fig. 7. Normalized retention (k'/k'_0) of five globular proteins as a function of adsorbed CHAPS from Fig. 6. Each panel displays the retention data for a particuar protein at four different $(NH_4)_2SO_4$ concentrations; (□) 1.10 *M*; (+) 1.20 *M*; (◇) 1.30 *M*; (△) 1.40 *M*. (A) Lysozyme; (B) carbonic anhydrase; (C) RNase A; (D) enolase; (E) BPTI.

more complex dynamic model involving the kinetics of association of both CHAPS and protein on and off the stationary phase might be developed; we believe that it would lead to similar conclusions. The possibility of CHAPS binding to some protein

is not excluded by the present model, but it appears unlikely to be significant in the present studies.

The effect of CHAPS on retention was found to be readily reversible, in contrast to the common experience that many surfactants bind irreversibly to reversed-phase columns[11–16,33]. After exposure of the column to CHAPS-containing mobile phases, the column could be restored to original retention characteristics by simply flushing with approximately eight column volumes of water followed by eight column volumes of methanol. Table III gives values of k' obtained on a SynChropak Propyl column before and after exposure to CHAPS-containing mobile phase. After such exposure, the retention characteristics of the column are essentially unchanged. Additional evidence in support of the conclusion that CHAPS is bound reversibly was encountered in the frontal analysis determination of binding isotherms.

TABLE III

REVERSIBILITY OF HIC COLUMNS TO CHAPS-EXPOSURE

Conditions: 1.30 *M* $(NH_4)_2SO_4$; 0.02 *M* phosphate buffer pH 6.1; 1.00 ml/min; UV at 215 nm, 0.5 a.u.f.s.

Protein	*k' before CHAPS exposure*	*k' after CHAPS exposure*
RNase A	2.34	2.35
Carbonic anhydrase	2.82	2.85
BPTI	3.40	3.54
Enolase	6.19	6.10
Lysozyme	7.04	7.12

The use of CHAPS did not lead to any obvious band-broadening in these studies. Mobile phases containing ammonium sulfate and CHAPS appear to be non-denaturing for enolase, carbonic anhydrase, and RNase A[32]. The issue of chromatographic efficiency and the possibility of anomalous chromatographic behavior in the neighborhood of the CMC of CHAPS are presently under investigation. The use of CHAPS in various gradient models is also being explored as is the use of other mild surfactants. These issues will be addressed in forthcoming publications.

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REFERENCES

1 S. A. Cohen, K. P. Benedek, S. Dong, Y. Tapuhi and B. L. Karger, *Anal. Chem.*, 56 (1984) 217.
2 G. Seipke, H. Mullner and U. Grau, *Angew. Chem., Int. Ed. Engl.*, 25 (1986) 535.
3 S. D. Power, M. A. Lochrie and R. O. Poyton, *J. Chromatogr.*, 266 (1983) 585.
4 R. S. Blanquet, K. H. Bui and D. W. Armstrong, *J. Liq. Chromatogr.*, 9 (1986) 1933.

5 W. S. Hancock and J. T. Sparrow, in Cs. Horváth (Editor), *HPLC, Advances and Perspectives*, Vol. 3, Academic Press, New York, NY, 1983, p. 5.
6 N. T. Miller and B. L. Karger, *J. Chromatogr.*, 326 (1985) 45.
7 L. C. Chaplin, *J. Chromatogr.*, 363 (1986) 329.
8 J. L. Fausnaugh and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 131.
9 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1984) 519.
10 S. C. Goheen and S. C. Engelhorn, *J. Chromatogr.*, 317 (1984) 55.
11 W. G. Tramposch and S. G. Weber, *Anal. Chem.*, 58 (1986) 3006.
12 A. Berthod, I. Girard and C. Gonnet, *Anal. Chem.*, 58 (1986) 1362.
13 A. Berthod, I. Girard and C. Gonnet, *Anal. Chem.*, 58 (1986) 1359.
14 A. Berthod, I. Girard and C. Gonnet, *Anal. Chem.*, 58 (1986) 1356.
15 J. G. Dorsey, M. G. Khaledi, J. S. Landy and J. L. Lin, *J. Chromatogr.*, 316 (1984) 183.
16 J. P. Chang, *J. Chromatogr.*, 317 (1984) 157.
17 J. R. Deschamps, *J. Liq. Chromatogr.*, 9 (1986) 635.
18 S. C. Goheen and A. Stevens, *Bio Techniques*, 3 (1985) 48.
19 J. Steinhardt, J. R. Scott and K. S. Birdi, *Biochemistry*, 16 (1977) 718.
20 Alan R. Liss, J. C. Venter and L. C. Harrison (Editors), *Membranes, Detergents, and Receptor Solubilization*, New York, NY, 1984.
21 D. B. Wetlaufer and M. R. Koenigbauer, *J. Chromatogr.*, 359 (1986) 55.
22 L. M. Hjelmeland, D. W. Nebert and J. C. Osborne, Jr., *Anal. Biochem.*, 130 (1983) 72.
23 W. Melander and Cs. Horváth, *Arch. Biochem. Biophys.*, 183 (1977) 200.
24 J. L. Fausnaugh and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 131.
25 W. R. Melander, D. Corradini and Cs. Horváth, *J. Chromatogr.*, 317 (1984) 67.
26 F. A. Long and W. F. McDevit, *Chem. Rec.*, 51 (1982) 119.
27 M. J. Rosen, *Surfactants and Interfacial Phenomena*, Wiley, New York, NY, 1978, pp. 159–171.
28 M. Tang and S. N. Deming, *Anal. Chem.*, 55 (1983) 425.
29 D. R. Absalom and R. A. Barford, *Anal. Chem.*, 60 (1988) 210.
30 E. Vargha-Butler, T. K. Zubovitz, H. A. Hamza and A. W. Newman, *J. Disp. Sci. Technol.*, 6 (1985) 257.
31 C. Tanford, *The Hydrophobic Effect*, Wiley, New York, NY, 1980, pp. 146–164.
32 J. Buckley, J. Gehas and D. B. Wetlaufer, in preparation.
33 I. N. Mefford, *J. Chromatogr.*, 368 (1986) 31.

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EFFECT OF THE STRUCTURE AND DENSITY OF CHEMICALLY BONDED C_{18} PHASE ON THE RECOVERY OF TRYPTOPHAN AND ITS METABOLITES FROM HUMAN URINE[a]

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SUMMARY

A series of materials with chemically bonded C_{18} phase for use as the packings in clean-up columns for solid-phase extraction were prepared. The effects of the monomeric and/or polymeric structure of the chemically bonded phase and of the porous structure of the silica gel support on the recovery of tryptophan and two of its metabolites used as test substances were considered. It appeared that the best recoveries of at least 60% of the three test substances were obtained on material of the "monomer" type containing chemically bonded C_{18} phase characterized by a high coverage density of $\alpha_{RP} \approx 3.8$ μmol/m^2. The use of a silica gel support with a larger pore size and volume permits not only the effective isolation of individual substances, *e.g.*, from urine, but also their 5-fold concentration.

INTRODUCTION

In recent years packings with chemically bonded phase (CBP) have been increasingly used. They are not only good starting materials for the production of high-performance liquid chromatographic (HPLC) columns, but are also used in the preparation of columns for solid-phase extraction This relates especially to the determination of polar substances isolated from biological materials such as urine, serum, blood and tissue[1–6]. These materials possess many advantages and are characterized by high solvolytic, mechanical and thermal resistance. These advantages permit the use of these materials on a large scale in routine clinical and biochemical analysis[1,2–4,6].

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The recovery of substances isolated from biological materials using a clean-up column for solid-phase extraction depends on many factors, the most important being the packing selectivity, the height of the packing bed and the choice of an appropriate eluent[4–6]. These factors significantly influence the reproducibility of analytical data. However, few papers have described the effect of porous structure on the recoveries obtained with off-line clean-up columns for solid-phase extraction. Studies of these effects have been related mainly to typical packings of analytical columns used in HPLC[7–10].

The detailed examination of these effects seemed to be of great interest. In this connection, compounds used in medical diagnostics and characterized by very different chemical natures[11–13] were chosen as test substances.

It is well known that if monochlorosilanes are used in the preparation of CBP packings, then packings with a strictly defined monomeric structure are obtained (a one-point covalent bond between the support surface and the modifier molecule is formed). Di- and trifunctional organosilanes give so-called "polymeric phase" packings with a cross-linked structure of the liquid organic phase[3,6,10,14–17]. The presence of such polymeric phases causes the undesirable screening of unblocked silanols, which represent specific and strong active centres on the support surface. As a result, during elution and/or extraction many effects can appear that are difficult to interpret. These effects can influence significantly efficient isolation by clean-up procedures.

Similar remarks can also be made in relation to the porous structure of CBP supports, which can play a very important role not only in the isolation of compounds but also in the concentration of substances to be determined, and this is connected in turn with the sorption capacity of these packings[6,18,19].

These problems were investigated in this study. Their explanation may permit a more precise description of the mechanism of the interactions between the solute, eluent and stationary phase.

EXPERIMENTAL

Material and reagents

Two types of silica gels were used as the support for the preparation of C_{18} chemically bonded phases used as packings for clean-up columns: Kieselgel Si 60 from Merck (Darmstadt, F.R.G.) and SG-100 prepared in the Polymer Institute, Slovak Academy of Sciences (Bratislava, Czechoslovakia)[20]. The surface characteristics of these bare materials are listed in Table I.

The following organosilanes were used as modifiers: octadecyldimethylchlo-

TABLE I

PHYSICO-CHEMICAL CHARACTERISTICS OF BARE SILICA GELS USED AS CBP SUPPORTS

Silica gel	*Fraction (μm)*	S_{BET} *(m^2/g)*	*D (nm)*	V_p *(cm^3/g)*
Kieselgel Si 60	40–63	348	8.6	0.88
Silica gel SG-100	50–80	196	20.0	2.10

TABLE II
CHARACTERISTICS OF THE ISOLATED AND SEPARATED SUBSTANCES

Compound	*Abbreviation*	pK_a[a]	*Standard concentration (μg/ml)*
Tryptophan	TRP	5.89	10
Serotonin	5-HT	9.8	1
5-Hydroxyindoleacetic acid	5-HIAA	4.7	2

[a] Data from ref. 23.

rosilane (ODMCS) (Petrarch System, Levittown, CA, U.S.A.), octadecylmethyldichlorosilane (ODDCS) (Dynamit Nobel, Transdorf, F.R.G.) and octadecyltrichlorosilane (ODTCS) (Dynamit Nobel). Hexamethyldisilazane (HMDS) (POCh, Gliwice, Poland) was used for secondary silanization (end-capping).

The following solvents were used: dry[21] toluene and morpholine from Reachim (Moscow, U.S.S.R) and benzene, methanol, dimethyl ether, sodium acetate and sodium hydroxide from POCh. Mobile phases used in HPLC analyses were prepared using 0.15 *M* sodium phosphate (pH 4.2) (POCh) and water purified in our laboratory. The substances listed in Table II were used as test substances. All reagents used were of analytical-reagent grade.

Chemically restitant containers of volume of 5 μl made from polyethylene (Chemical Reagents Factory, ZOCh, Lublin, Poland) were used for the construction of the clean-up columns.

Chromatographic analyses were carried out on a 250 × 4 mm I.D. column packed with 7-μm spherical particles of laboratory-made chemically bonded C_{18} phase with a high coverage density of $\alpha_{RP} \geqslant 4$ μmol/m^2[6,24].

Physico-chemical measurements

The parameters characterizing the porosity of the packings, *i.e.*, specific surface area (S_{BET}), pore volume (V_p) and mean pore diameter (D) before chemical modification of these packings were determined by the low-temperature nitrogen adsorption–desorption method using a Model 1800 Sorptomatic apparatus (Carlo Erba, Milan, Italy). The degree of coverage of the surface by alkylsilyl ligands, α_{RP}, was calculated on the basis of the carbon percentage determined with a Model 185 CHN analyser (Hewlett-Packard, Palo Alto, CA, U.S.A.) using the equation

$$\alpha_{RP}\ (\mu mol/m^2) = \frac{10^6 P_c}{1200N_c - P_c(M - n)} \cdot \frac{1}{S_{BET}} \quad (1)$$

where P_c = measured carbon percentage, N_c = number of carbon atoms in the molecule of the bonded silane, M = molecular mass of the silane, S_{BET} = specific surface area (m^2/g) and n = number of functional group substituents in the silane molecule.

Chromatographic measurements were carried out using a liquid chromatograph consisting of an HPP 4001 syringe pump (Laboratorní Přístroje, Prague, Czecho-

TABLE III

CHARACTERISTICS OF THE CLEAN-UP COLUMN PACKINGS AFTER CHEMICAL MODIFICATION

No. of column	*Type of packing*[a]	*Type of CBP structure*	*Coverage density*	
			P_c (%)	α_{RP} ($\mu mol/m^2$)
1	Si–ODMCS	Monomer	10.16	1.39
2	Si–ODMCS + HMDS	Monomer	13.49	4.22
3	Si–ODMCS + A	Monomer	17.26	2.70
4	Si–ODMCS + A + HMDS	Monomer	18.66	3.80
5	Si–ODMCS + A + HMDS	Polymer	21.05	5.12
				2.68[b]
6	Si–ODTCS + A + HMDS	Polymer	21.06	4.98
				2.65[b]
7	SG-100–ODMCS + A + HMDS	Monomer	15.5	4.23
				3.87[b]

[a] A = activator (morpholine).
[b] Data obtained before end-capping with HMDS.

slovakia) and a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.). An ELDEC 102 electrochemical detector (Chromatofield, Chateauneuf-les-Martiques, France) with a glassy carbon electrode working at potentials of +0.9 V and +0.5 V *vs.* an Ag/AgCl reference electrode and a TZ-4200 linear recorder (Laboratorní Přístroje) were also used.

Synthesis of CBP

Bare silica gel samples were placed in glass reactors having an ampoule shape to avoid contact of the reagents with the environment[4–6] and then heated at 200°C under vacuum (10^{-3} Pa) for 6 h.

All packings were synthesised under the standard conditions described previously[6,24,25]. The physico-chemical characteristics of the materials prepared are listed in Table III.

Isolation procedure

Isolation of the test substances was carried out using the procedure described previously[4,6,12] but slightly modified. In the first stage, the packing bed of the clean-up column was washed with 2 ml of methanol and then with 2 ml of 0.1 *M* acetate buffer (pH 5). In the second stage, 2 ml of a mixture containing dissolved standard substances in 0.1 *M* acetate buffer (1:1, v/v) was introduced on to the column, then the bed was washed with 1 ml of water. In the third stage the sorbed substances were eluted with 1 ml of 0.1 *M* ammonia solution–methanol (3:1, v/v). A 10-μl volume of the collected eluate was injected on to the analytical column. It should be pointed out that the simultaneous analysis of serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and tryptophan (TRP) is possible only at potentials of at least to +0.9 V because TRP has a high oxidizing potential of +0.88 V; 5-HT and 5-HIAA can be determined simultaneously at a potential of +0.5 V[5,12].

RESULTS AND DISCUSSION

Table III lists the characteristics of the materials used as the packings in clean-up columns prepared under different conditions. Use of the modifiers containing 1–3-chloro substituents allowed the packings with C_{18} chemically bonded phases with "monomer" and "polymer" type structures to be prepared. According to Unger and Anspach[9], Unger[15] and Bayer *et al.*[14], the surface structures obtained can be represented schematically as shown in Fig. 1a–c.

Considering the data in Table III for packings of the "monomer" type (materials 1–4), we can conclude that depending on the synthesis conditions chemical modification leads to material of increasing coverage density, α_{RP}, and of different layer structure. The presence of an activator influences significantly (see packings 1 and 3) the C_{18} ligand contribution (α_{RP} increases by 60%)[6,24,25]. This effect confirms the opposite increase in the total α_{RP} values after secondary silanization with HMDS of the packings prepared in the first stage (packings 2 and 4). A greater difference in α_{RP} is

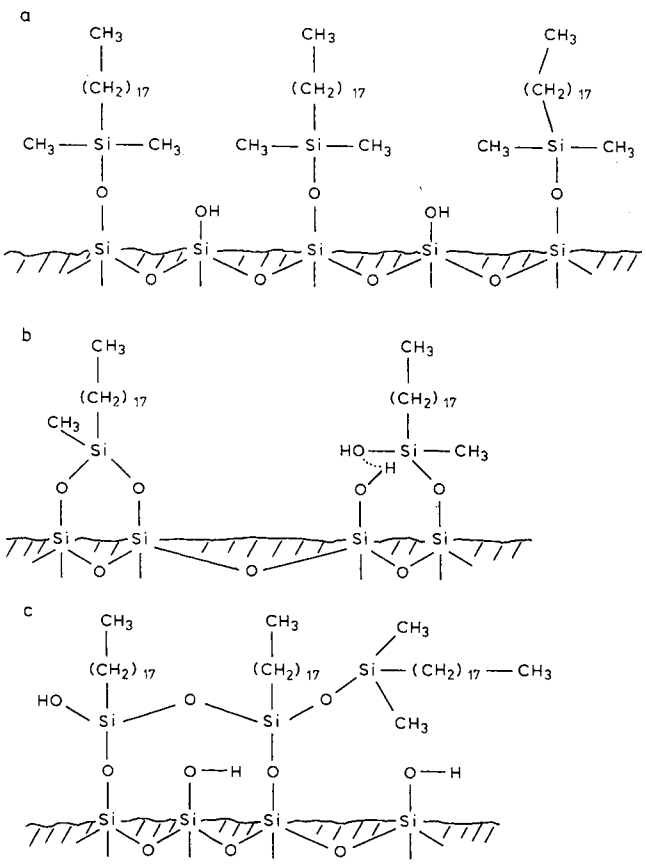

Fig. 1. Possible structures of chemically bonded C_{18} phases which can be formed, depending on the number of chlorine atoms in the modifier molecule. (a) Monochlorosilane; (b) dichlorosilane; (c) trichlorosilane.

observed for materials 1 and 2 than for 3 and 4. This is reasonable considering the greater possibility of spatial penetration of small methylsilyl molecules towards unblocked silanols. For the materials of less dense surface coverage (packing 1, $\alpha_{RP} = 1.39$ μmol/m^2) end-capping is more effective than for the materials of denser surface coverage (packing 3, $\alpha_{RP} = 2.70$ μmol/m^2)[24–26]. It seems that this penetration will be possible mainly as a result of the reduction in steric interactions between individual C_{18} ligands[26] and the change in conformation of the chain in the synthesis medium[27]. Moreover, an additional effect resulting from the porous structure of the silicagel support can be expected.

Materials 1–4 were prepared on an adsorbent characterized by a small pore size and volume (Table I). Material 4 (Table III) should permit the extraction of test substances only in the bonded organic layer without the possibility of penetration into the pores of the substances being determined[6,18]. Comparison with packings prepared under similar conditions with material of larger pore size and volume (Tables I and III, packing 7), significant differences in the α_{RP} values for both primary and secondary silanization are observed. This is in good agreement with our previous results [18].

We have mentioned many times that dense coverages of "monomer" type CBP are obtained by reaction with monochlorosilane in the presence of morpholine as activator and have described the mechanism of this synthesis[6,10,16,18,22,24–26]. Based on the investigations described in the papers cited, it would be expected that the use of di- and trifunctional silanes under conditions analogous to those for the preparation of packings 4 and 7 would permit packings with a more cross-linked structure of the support surface to be obtained (materials 5 and 6, Table III).

Comparison of the α_{RP} values shows that primary silanization by use of di- and trifunctional modifiers is not influenced significantly by the differences in the mechanism of bonding. In both instances the α_{RP} values are comparable to those obtained with a monofunctional modifier (packings 3, 5 and 6). After secondary silanization, however, significant differences appear, which suggests the formation of different CBP structures as illustrated in Fig. 1. Relatively low coverages with C_{18} alkyl ligands in the presence of an activator and their small differences independent of the functionality of the reacting silane modifier (materials 3, 5 and 6) may be explained by steric hindrance to the penetration of large C_{18} molecules into the narrow-pore Kieselgel Si 60 (the surface located outside the pores participates preferentially in the reaction).

The tests on the usefulness of the packings for solid-phase extraction were carried out using tryptophan and its metabolites serotonin and 5-hydroxyindoloacetic acid as test substances. These substances are of interest for two reasons: (i) because of the similar geometric dimensions of the molecules but their completely different chemical properties; and (ii) because of the great importance of these substances in medical diagnosis (optimization of the choice of packings for routine analyses using clean-up columns).

Table IV lists the recoveries of these three compounds after solid-phase extraction. The results indicate that the lowest recoveries of TRP and 5-HT are obtained for the non-end-capped materials synthesized by use of a monofunctional modifier (packings 1 and 3). This is probably due to the greater accessibility of unblocked silanols for the substances isolated. As a consequence, specific interactions between the substances being determined, the eluent and the packing surface occur.

TABLE IV
COMPARISON OF RECOVERIES AND COEFFICIENTS OF VARIATION (C.V.) FOR TRYPTOPHAN AND ITS METABOLITE ISOLATED FROM STANDARD SOLUTION

Off-line column No.	*Recovery (%)*					
	TRP	*C.V. (%)*	*5-HT*	*C.V. (%)*	*5-HIAA*	*C.V. (%)*
1	33.4	12.6	4.0	22.5	80.5	8.6
2	83.8	3.6	43.6	4.1	79.0	3.9
3	48.6	11.6	1.7	23.5	79.5	5.8
4	78.5	3.2	56.1	3.4	62.7	3.5
5	85.0	2.1	35.0	5.4	53.8	5.3
6	70.0	2.2	56.6	3.5	53.0	4.7
7	77.8	3.3	41.3	6.4	94.4	3.3

This supposition may be confirmed by the high values of the coefficient of variation (Table IV).

The secondary silanization (columns 2 and 4) improves the recovery of the above two substances significantly. This is probably due to limited interactions of basic solutes with the silanols, *i.e.*, leakage of free electron pairs or π-electron interactions. The possibility of an additional effect connected with the limited mobility of long C_{18} chains directed towards short trimethylsilyl groups with lower coverages should also be taken into account[25–29].

Owing to these specific interactions a change in the conformation of C_{18} ligands probably takes place, resulting in better screening of the support surface (column 2, Table IV). This may suggest that for the isolation of basic solutes and for dense CBP coverage, partial coverage with the main ligand followed by end-capping may be useful[30].

Because of the leakage of amino groups in the chemical structure of 5-HIAA, the recoveries obtained with sorbents 1–3 by use of a monofunctional silane modifier are relatively high and almost independent of the density of coverage (columns 1–3. Tables III and IV), *i.e.*, an irreversible adsorption effect does not occur. This is in good agreement with our earlier suppositions[31], confirmed recently by Bayer and Paulus[30].

Exhaustive silanization in the presence of an activator and with end-capping leads to a significant reduction in the 5-HIAA recovery (column 4), which is probably the result of blockage of the narrow pores in Si 60 silica gel[6,8,18,31]. A similar explanation may be valid for the recovery results for the three compounds obtained on materials synthesized by use of di- and trifunctional modifiers (columns 5 and 6).

In spite of the higher α_{RP} values, packings of the "polymer" type (materials 5 and 6) show some tendency for irreversible sorption of individual substances. In the examples considered, the adsorption of individual substances is often very high but elution from the surface layer is difficult in many instances (limited mass exchange, Table IV). In this connection, the lack of reproducibility of the recovery on some commercial packings for clean-up columns[4–6] is not surprising. With materials 4 and 7 (Tables III and IV) the effects of shielding of the support surface by hydrophobic ligands of the stationary phase during the isolation of the standard mixture are good. This is confirmed by the relatively small differences in recovery and the small values of

TABLE V
RECOVERY OF TRYPTOPHAN AND ITS METABOLITES ISOLATED FROM HUMAN URINE ON CLEAN-UP COLUMNS WITH DIFFERENT POROSITIES OF THE MATERIAL PACKING

Off-line column No.	*Recovery (%)*					
	TRP	*S.D.*	*5-HT*	*S.D.*	*5-HIAA*	*S.D.*
4	76.0	3.4	45.0	3.6	61.0	3.0
7	74.0	2.3	27.0	4.7	92.7	1.4

the coefficient of variation (except for 5-HT with material 7). On the other hand, material 4 appears to be more selective for the isolation of 5-HT and TRP[18,19]. This is undoubtedly due to the smaller sizes of the pores because, owing to chemical modification, almost all pores are blocked by relatively large C_{18} molecules[6,8–18]. Close packing of C_{18} ligands in narrow pores ($D \approx 6$ nm) significantly reduces the probability of interactions of unblocked silanols with the substances being determined, all the more because interactions between individual ligands (resulting from steric effects) will be preferred here[6–10,14–18,25–27]. This can undoubtedly make mass exchange difficult and also lead to lower sorption capacities[3,4,6]. For the packing 7 the larger pore size permits greater accessibility to unblocked silanols for the molecules of the substance being determined, which probably influences the lower recovery of 5-HT (Table IV). On the other hand, this effect has a very advantagenous influence on the conditions of determination of 5-HIAA, for which the recovery is high and comparable to those obtained previously[4]. The results obtained with both of these packings during the isolation of tryptopan and its metabolites from biological material (human urine, Table V) indicate that our previous suggestions are confirmed. The recovery of 5-HT with packing 4 is higher than that with packing 7, which has comparable properties. Opposite but very similar regularities are observed in the determination of 5-HIAA: in this instance packing 7 is more selective, *i.e.*, possesses a higher sample capacity. The coefficients of variation, which are low in all cases considered (Table V), appear to confirm the above conclusions.

Attempts were made to concentrate the three substances on packings 4 and 7. It appeared that using the material with a large pore size and volume (packing 7) a 5-fold concentration of substances isolated from human urine can be obtained in comparison with packing 4, which has a *ca.* 3-fold smaller pore size and volume (Table I).

The full explanation of the above phenomena is difficult. We may assume that urine contains some other active compounds that undergo preferential adsorption on the CBP surface, reducing the adsorption of the investigated solutes. A denser coverage with C_{18} alkyl ligands followed by end-capping of the wider pore material 7 should reduce such co-adsorption and allow higher recoveries of the very active 5-HT to be obtained during the desorption process.

CONCLUSIONS

For the isolation of substances in biological material (urine, serum, etc.), clean-up column packings with a monomeric structure of chemically bonded C_{18} phase in which the silanol groups are well shielded by C_{18} ligands appear to be most

suitable. End-capping significantly influences the recovery. In this instance the molar ratio of C_{18} to methylsilyl groups should be high if possible, *i.e.*, the maximum coverage with C_{18} ligands should be obtained. More reproducible results are obtained with packings prepared on the basis of a support characterized by a larger pore size and volume, which permits a more compact film of the organic phase, a denser coverage and a lower availability of remaining silanol groups for interaction with solute molecules to be obtained.

A separate question is the hydrolytic stability of end-capped materials, especially in buffer solutions. We did not investigate systematically the lifetime of the end-capped sorbents, but we noted that materials not completely covered with C_{18} monoligands and end-capped gave lower and less reproducible recoveries of 5-HT and 5-HIAA from urine[31], which may be caused by, among other things, a lower sorbent stability. Dense coverage with the use of an activator and end-capped sorbents did not result in such inconvenient phenomena.

REFERENCES

1 B. Tippins, *Int. Lab.*, 17, No. 3 (1987) 28.
2 C. K. Lim, F. Li and T. J. Peters, *Int. Lab.*, 17, No. 6 (1986) 60.
3 C. F. Poole and S. A. Schuette, *Contemporary Practice of Chromatography*, Elsevier, Amsterdam, 1984, Ch. 7, p. 429.
4 B. Buszewski, K. Šebeková, P. Božek, L. Štibranyi, J. Jenrichovský, I. Novák and D. Berek, *Chromatographia.*, 22 (1986) 299.
5 D. Sieńko, B. Buszewski and Z. Suprynowicz, *Anal. Chim Acta*, in press.
6 B. Buszewski, *Ph.D. Thesis*, Slovak Technical University, Bratislava, 1986.
7 L. C. Sander and S. A. Wise, *J. Chromatogr.*, 316 (1984) 163.
8 L. C. Sander and S. A. Wise, *Crit. Rev. Anal. Chem.*, 18 (1987) 299.
9 K. K. Unger and B. Anspach, *Trends Anal. Chem.*, 66 (1987) 121.
10 J. Nawrocki and B. Buszewski, *J. Chromatogr.*, 449 (1988) 1.
11 O. Beck, G. Polmskog and E. Hullman, *Clin. Chim. Acta*, 79 (1977) 149.
12 D. D. Koch and P. T. Kissinger, *J. Chromatogr.*, 164 (1979) 441.
13 P. Božek, O. Uličná, J. Zemek and L. Stibranyi, *Biochem. Clin. Bohemoslov.*, 15 (1986) 175.
14 E. Bayer, K. Albert, J. Reiners, M. Nieder and D. Müller, *J. Chromatogr.*, 264 (1983) 197.
15 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979.
16 B. Buszewski, K. Pilorz and Z. Suprynowicz, *J. Chromatogr.*, submitted for publication.
17 B. Buszewski, *Chem. Stosow.*, 32 (1988) 201.
18 B. Buszewski, D. Berek, J. Garaj, I. Novák and Z. Suprynowicz, *J. Chromatogr.*, 446 (1988) 191.
19 B. Buszewski, D. Berek, I. Novák and J. Garaj, *Chem. Pap.*, in press.
20 I. Novak and D. Berek, *Czech Pat.*, 235250, 1984.
21 B. Buszewski, R. Lodkowski and J. Trocewicz, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 527.
22 B. Buszewski, T. Bleha and D. Berek, *J. High Resolut, Chromatogr. Chromatogr. Commun.*, 8 (1985) 860.
23 D. D. Perrin, *Dissociation Constants of Organic Bases in Aqueous Solution*, Butterworths, London, 1965.
24 B. Buszewski, A. Jurášek, J.Garaj, L. Nondek and D. Berek, *J. Liq. Chromatogr.*, 10 (1987) 2325.
25 B. Buszewski and Z. Suprynowicz, *Anal. Chim. Acta*, 208 (1988) 263.
26 B. Buszewski and Z. Suprynowicz, *Chromatographia.*, 24 (1987) 573.
27 E. Bayer, A. Paulus, B. Peters, G. Laupp, J. Reiners and K. Albert, *J. Chromatogr.*, 364 (1986) 25.
28 S. S. Yang and R. K. Gilpin, *J. Chromatogr.*, 394 (1987) 295.
29 B. Buszewski, K. Šebeková, P. Božek and D. Berek, *J. Chromatogr.*, 367 (1986) 171.
30 E. Bayer and A. Paulus, *J. Chromatogr.*, 400 (1987) 1.
31 Z. Suprynowicz, B. Buszewski, D. Sieńko and J. Gawdzik, *J. Chromatogr.*, 456 (1988) 219.

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MICROBORE LIQUID CHROMATOGRAPHY AND REFRACTIVE INDEX GRADIENT DETECTION OF LOW-NANOGRAM AND LOW-ppm QUANTITIES OF CARBOHYDRATES

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SUMMARY

A refractive index gradient detector is presented as a universal detector in the microbore high-performance liquid chromatography analysis of carbohydrates. Simultaneously, low-ng and low-ppm injected quantities of carbohydrates were detected at the 3 × root-mean-square baseline noise level. A typical microbore high-performance liquid chromatography chromatogram separating fructose from sucrose followed by refractive index gradient (RIG) detection is reported. Use of a position sensitive detector (PSD) in the RIG detector design is reported and experimental considerations discussed. Optimization of the PSD-based RIG detector is addressed. Potential for the device in industrial and clinical applications is considered.

INTRODUCTION

The separation and detection of carbohydrates is an important analytical problem. Analysis of carbohydrates in clinical and industrial settings is commonplace, thus reliable and sensitive analysis techniques are continually being developed and evaluated. The primary approach to carbohydrate analysis for complex samples is high-performance liquid chromatography (HPLC) followed by suitable detection[1]. The separation and detection steps in the analysis are generally independent, and may be examined separately. The state-of-the-art in carbohydrate separations is quite advanced[2–6], especially in the area of ion-exchange chromatography[6–9]. We sought only to demonstrate the refractive index gradient (RIG) detector with a simple microbore normal phase HPLC separation of carbohydrates, although reversed-phase or ion-exchange separations of carbohydrates would have also been appropriate. Thus, we will not emphasize the separation aspect of carbohydrate analysis. The primary problem with the analysis is with detection, especially when coupled with microbore HPLC technology. Detection of carbohydrates following a microbore HPLC separation was the focus of our work.

A variety of carbohydrate detection approaches have been reported, either

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without derivatization[10–23] or with derivatization[24–33]. Since carbohydrate analysis is hampered due to poor absorbance characteristics, refractive index (RI) detectors have been used for carbohydrate determination by conventional HPLC. Commercially available RI detectors provide at best a 1-μg detection limit[3]. Recent advances in detector technology have made improved RI detection for conventional[10,11], microbore[12,13], and capillary[14,15] HPLC possible but detection limits of 40–50 ng were optimum. Electrochemical detection approaches have been reported for microbore HPLC with excellent detectabilities in many cases, often in the low-nanogram range for monosaccharides[16–21]. The problem with EC detection appears to be the application of the detector in a wide variety of liquid environments. A promising approach to obtain 10–20 ng detection limits for sugars is microbore HPLC followed by optical activity detection (OAD), where better than $1 \cdot 10^{-6}$° rotation can be detected[22,23]. The drawback to OAD in general practice is the limitation in routinely manufacturing and tuning the detector. Other approaches such as conductivity[24] and chemiluminescence[25] have been reported, but universal applicability of these techniques is somewhat limited as both of these approaches[24,25] involve carbohydrate derivatization reactions prior to detection. Both pre-column and post-column derivatization[26–30] of carbohydrates followed by either UV–VIS absorbance[27–30] or fluorescence[26] detection have been studied in detail. A comparison between RI and absorbance detection of underivatized sugars yielded comparable detection limits for both approaches of 5–10 μg, which is quite poor[31]. Gas chromatography methods for carbohydrates also generally require pre-derivatization[32,33]. Derivatization reactions are notoriously slow, and are therefore unattractive for many applications where total analysis time must be minimized. Process control in industrial applications and clinical testing are two areas requiring fast turnaround time in carbohydrate analyses. Pre-column derivatization techniques for carbohydrates often have a reaction step of 1–2 h[26–28] and the chromatography must be worked out for the derivatives, with the separation complicated by the common functionality of the derivatization reagent in the carbohydrate reaction product. Post-column derivatization techniques have been developed with the concern for shortening reaction times[30]. Unfortunately, even 20–30-s reaction times[30] may contribute to solute band broadening in microbore HPLC applications. The major advantage of HPLC derivatization techniques for carbohydrates is the excellent detection limits of a few nanograms[26–30].

The most promising HPLC detector for carbohydrate analysis should provide a universal response without derivatization, such as RI, but have the sensitivity obtained in UV–VIS absorbance detection of derivatized samples. Recently, the concept of refractive index gradient (RIG) detection in HPLC has been reported[34–37]. The potential was apparent for a marked improvement in detection sensitivity over conventional RI detection for microbore HPLC. We have designed a RIG detector that provided sub-ng detection limits of polymers in microbore HPLC[36]. The detection was facilitated at a non-absorbing wavelength from a diode laser (780 nm), so the detector is universal. We have recently simplified and improved our RIG detector which now requires only three components: a diode laser, a z-configuration flow cell, and a position-sensitive detector[38–40] (PSD). Previously a PSD was incorporated into a dual-beam absorbance detector[38]. We report here a brief description of the RIG detection principle as pertaining to our RIG detector, and apply the device in the detection of sugars following microbore HPLC. The detection

limit for the sugars was observed to be quite remarkable considering the simplicity of the detector. Detection limits of both low ng and low ppm injected amounts of sugars were obtained and results are presented. These detection limits are three orders of magnitude better than one might anticipate using commercially available RI detectors[3,31]. When injected mass and concentration detection limits are considered collectively, the RIG detector for microbore HPLC is superior to other state-of-the-art RI detectors[10–15].

EXPERIMENTAL

Refractive index gradient detector

The RIG detector shown with respect to the microbore HPLC system in Fig. 1 is a modified version of our original device[36,37] is composed of three primary components. The single-mode DL 25 diode laser output of 350 μW at 780 nm (Physitec, Norfolk, MA, U.S.A.) is focused by a 16.85-mm focal length microscope objective (fitted to the diode laser) through a z-configuration flow cell (made in-house) and on to a Hamamatsu (Hamamatsu City, Japan) S1352 with dimensions of 33 mm × 2.5 mm. The PSD sensitivity to beam position is along the long axis. The distance between the flow cell and PSD, labeled X in Fig. 1, was 25 cm, which was found to be near the optimum signal-to-noise ratio (S/N) for detecting the RIG effect with the PSD. The optimum distance of X in Fig. 1 depends upon the relationship of the analytical signal relative to the baseline noise. The optimization procedure will be described with the experimental results. With a single incident beam, the PSD ratio output readily measures angular beam deflections[38,40]. A detailed inspection of the PSD is shown in Fig. 2. The optical and mathematical configuration of the PSD device with respect to RIG detection will be discussed with the experimental results, also. The PSD ratio output is either collected by a Metra Byte (Taunton, MA, U.S.A.) DASH-8 that facilitated the analog-to-digital (A/D) conversion for subsequent data storage and analysis via a Leading Edge (Canton, MA, U.S.A.) Model D, or recorded directly on a D-5000 chart recorder (Houston Instruments, Austin, TX, U.S.A.).

Microbore HPLC system

The microbore HPLC system applied in our studies consisted of an ISCO (Lincoln, NE, U.S.A.) LC-2600 that delivered acetonitrile–water (90:10 v/v) as eluent

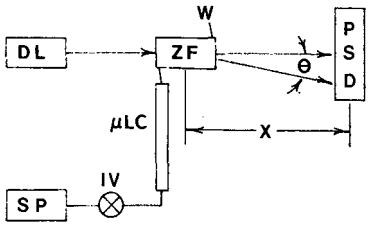

Fig. 1. Microbore HPLC system followed by RIG detection. SP = syringe pump; IV = injection valve; μLC = microbore HPLC column; DL = diode laser; ZF = z-configuration flow cell; PSD = position sensitive detector; W = waste; θ = RIG signal (eqns. 2 and 7); X = distance separating ZF from PSD (eqn. 7).

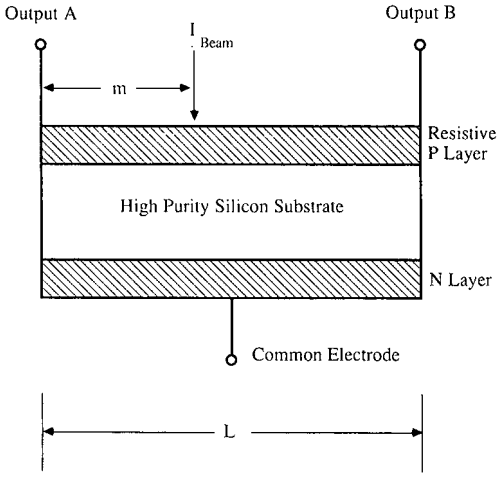

Fig. 2. Single-beam position sensitive detector applied in RIG detection. I_{Beam} (incident probe beam position) separated a distance m from electrode A, where outputs A and B are photocurrents in relation to the common electrode[40].

at 50 μl/min in the normal-phase separation of simple sugars. The eluent was delivered through a Rheodyne (Cotati, CA, U.S.A.), Model 7520 injection valve, fitted with a 0.5-μl loop for subsequent sample introduction to a 250 mm × 1.0 mm I.D., 5 μm particle diameter, Adsorbosphere (Alltech, Deerfield, IL, U.S.A.) silica column. The column effluent was delivered to the z-configuration flow cell, by a short length of 1/16 in. O.D. × 0.007 in. I.D. PTFE tubing, for RIG detection of entering solutes. The z-configuration flow cell is described in detail in a previous report[36], but it is important to note that the flow cell did not contribute significantly to solute band broadening. Due to hydrodynamic considerations, the flow cell has an effective volume of about 1 μl although the total flow cell volume is larger. Band broadening considerations will be discussed in the results.

Carbohydrates studied

RIG detection, analogous to conventional RI detection, universally responds to all solutes with an RI different than the eluent. Thus, we limited the demonstration of the RIG detector with microbore HPLC to relatively simple carbohydrates. Reagent grade sucrose (J. T. Baker, Phillipsburg, NJ, U.S.A.) and D-(−)-fructose (Alfa Products, Danvers, MA, U.S.A.) were chromatographically retained and detected by the system (Fig. 1). Linearity of the detected RIG signal as a function of injected solute mass for the separated sugars ranged from about 10 μg down to the detection limit of about 1 ng. Thus, the linearity of the PSD-based RIG measurement is better than we reported previously[36,37], since beam deflection is measured directly via the PSD rather than by an interferometric mechanism.

RESULTS AND DISCUSSION

Refractive index gradient effect

The RIG effect[34–37] is measured as a beam deflection, θ, depending upon

a cross-sectional distance probed, x, the RI of the eluent, n_0, and magnitude of the RIG, dn/dx, existing perpendicular to the probe beam from the diode laser, which for HPLC conditions is given by

$$\theta = \frac{x}{n_0}\frac{dn}{dx} \tag{1}$$

The RIG is related to the concentration gradient dC/dx by the chain rule[34] yielding

$$\theta = \frac{x}{n_0}\frac{dn}{dC}\frac{dC}{dx} \tag{2}$$

where dn/dC is a sensitivity factor[36] for a given solute in a given solvent. The concentration gradient dC/dx has been quantitatively related to the concentration profile of eluting solute using a Gaussian model, and further related to chromatographic principles[34,35].

Position sensitive detection of RIG effect

Since one is experimentally observing a beam deflection (eqn. 2), a suitable approach to measure the deflection θ is required. Initial studies used an interferometric mechanism translating the beam deflection into an intensity measurement at a single photodiode[36–37]. Use of a PSD provides a direct measurement of the beam deflection θ. Understanding the function and application of the continuous PSD (shown in Fig. 2) is essential to optimization of the measurement of θ of the RIG effect. The RATIO output[38,40] is defined

$$\text{RATIO} = \frac{I_A - I_B}{I_A + I_B} \tag{3}$$

where I_A and I_B are photocurrents measured at electrodes A and B, respectively (Fig. 2). For a single beam

$$\text{RATIO} = 1 - \frac{2m}{L} \tag{4}$$

where m is the position of the incident probe beam on the surface of the PSD relative to electrode A, and L is the length of the photoactive PSD surface (constant). Note that the RATIO output (eqn. 2), is independent of the incident beam intensity. The electronic null condition for the RATIO output occurs when $2m$ equals L, *i.e.*, when the beam is centered on the PSD, so the RATIO output would be zero. The electronic null condition will be defined with m_0, such that m_0 equals $L/2$. A zero RATIO signal is best used as the baseline reading in HPLC, when only eluent is passing through the flow cell. When a deflection, θ, occurs due to the RIG effect, the probe beam is incident upon the PSD at a new position m'. The signal θ (eqn. 2) is derived from the PSD RATIO output, and is observed as a change in probe beam position on the PSD, Δm, defined by

$$\Delta m = m' - m_0 \tag{5}$$

The beam deflection distance Δm relates to X (Fig. 1) and θ (eqns. 1 and 2) by

$$\Delta m = KX\theta \tag{6}$$

where K is a proportionality constant converting RATIO units to distance[38]. The constant K was calibrated for by precisely moving the PSD[38]. Finally, one may solve for θ by rearranging eqn. 6

$$\theta = \frac{\Delta m}{KX} \tag{7}$$

It is important to consider the minimum angular deflection that may be measured with the continuous PSD. The minimum angular deflection defines the detection limit for the RIG detector. A minimum angle of about 2 μrad was routinely measured with the apparatus in Fig. 2 with flowing eluent (operating microbore HPLC system) at a distance X of 25 cm between the flow cell and the PSD. The 2-μrad angle corresponds to a 0.50-μm beam displacement, a factor of 14 better than the manufactured specification of a 7-μm position resolution capability[40]. Readily available low noise electronic components were required to obtain the improved continuous PSD performance. Another report suggests that under ideal conditions a bi-cell PSD[39] may be employed rather than the continuous PSD described by eqns. 3–7. A minimum angular deflection of 50 nrad was reported[39], but the testing system included only a LED light source and the bi-cell PSD. Optics and a chromatography flow cell were not included in the noise study[39]. Yet, the RIG measurement is not presently limited by the choice of PSD since the system (Fig. 1) is not limited by the pointing stability of the light source, but rather subtle fluctuations due to the eluent flow. As the RIG detection technique advances, the bi-cell PSD may be the best choice.

Optimization of the distance X in Fig. 1 and eqns. 6 and 7 was performed empirically. The analytical signal θ is governed by eqns. 1 and 2, but is independent of the PSD position relative to the flow cell, X. Accordingly, the analytical signal is recorded by eqn. 6 as a beam displacement (deflection), Δm, from the initial baseline position. Thus, the signal increases linearly with X for a given θ. Now, the limiting noise must be considered. While changing X, the noise for the system, in terms of Δm, was observed to remain essentially constant until the beam is no longer imaged within the boundaries of the PSD photoactive surface, leading to increased noise as X is increased beyond the imaging limit. The beam necessarily expands after being focused through the flow cell. Unfortunately, refocusing between the flow cell and the PSD cannot be performed because the signal is sacrificed. Thus, when the signal *and* noise are considered collectively, an optimum S/N was observed at 25 cm $\pm$ 3 cm. Thus, the system was operated at the optimum X of 25 cm.

Microbore HPLC RIG detection of carbohydrates

Shown in Fig. 3 is the RIG detection of fructose (6.6 min, 320 ppm and 160 ng injected) and sucrose (9.6 min, 342 ppm and 171 ng injected). The chromatographic data have been plotted in quantitative terms according to eqn. 7. The observed RIG signal is rather unconventional, *i.e.*, derivative of a Gaussian shape[34–37]. Simple integration results in the conventional peak shape[34]. An injection disturbance occurs

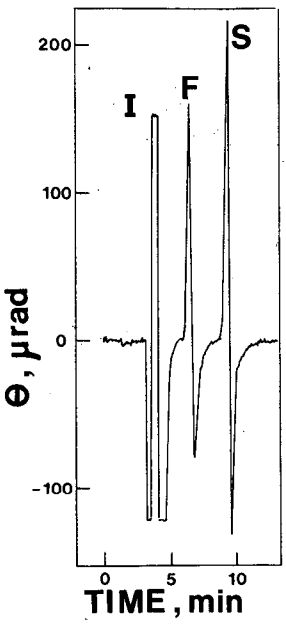

Fig. 3. Microbore HPLC separation and RIG detection of fructose (F) from sucrose (S) with an injection disturbance (I). Quantities injected were 160 ng (320 ppm) fructose and 171 ng (342 ppm) sucrose. Chromatographic conditions given in text.

between 3.0 and 5.2 min due to preparing the sample in pure water. Note that the S/N is quite good for both low injected concentration and low injected mass quantities of the sugars. With the PSD-based RIG detector, solute responses are highly reproducible. For three trials of the separation in Fig. 3 the relative standard deviation of the peak-to-peak RIG signals was 1.8% for fructose and 0.8% for sucrose.

The detection limit was determined from the baseline noise while the microbore HPLC system was functioning with eluent only. Three times the standard deviation of 100-s intervals of baseline noise was used as the detection limit. Several minutes of baseline noise were measured to calculate a reliable detection limit. From eqns. 6 and 7, the deflection distance and angular deflection were measured as 0.50 μm (Δm) and 2.0 μrad (θ), respectively. Extrapolating from the data obtained in Fig. 3, and dilutions from Fig. 3 (not shown for brevity), quite favourable detection limits for the sugars were calculated both for injected concentration and injected mass criteria. For fructose, the detection limits were 3.4 ppm injected concentration and 1.7 ng injected mass. For sucrose, the detection limits were even better at 2.4 ppm injected concentration and 1.2 ng injected mass. The reason sucrose had a better detection limit than fructose is related to the relationship between sensitivity and the detected solute concentration gradient dC/dx (eqn. 2). Band broadening measurements of both solutes revealed that sucrose was less broadened than fructose, necessarily leading to a higher dC/dx at the detector. For fructose the number of theoretical plates, N, was $5.1 \cdot 10^3$, and likewise for sucrose $15.1 \cdot 10^3$. Furthermore, these chromatographic efficiency data are quite reasonable for the normal phase separation of sugars. Thus, it is important to realize that chromatographic efficiency, and the detected solute

information, were not sacrificed by the RIG detector, *i.e.*, the flow cell employed. Other studies have supported this idea[36].

After considering the effect of chromatographic dilution, via band broadening, one may calculate the concentration detection limit of the sugars at the detector. For fructose the concentration detection limit at the detector was 68 ppb or $3.6 \cdot 10^{-7}$ *M*, and for sucrose 65 ppb or $1.9 \cdot 10^{-7}$ *M*. Thus, both sugars have about the same detection limit using the ppb concentration at the detector since both have about the same RI and density. The reported RIG detector is superior to previous reports, for RI[10–15] or RIG[34,35] detection, when one considers both the concentration detection limit and the mass detection limit, collectively.

CONCLUSION

A simple and sensitive approach to carbohydrate detection for microbore HPLC has been presented. Derivatization of the sugars is not required. Simultaneously, detection limits of low-ng and low-ppm injected quantities of sugars were observed. The device is easy to design, tune, and maintain.

ACKNOWLEDGEMENTS

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REFERENCES

1 W. T. Smith, Jr. and J. M. Patterson, *Anal. Chem.*, 60 (1988) 200R.
2 C. H. Lochmüller and W. B. Hill, Jr., *J. Chromatogr.*, 264 (1983) 215.
3 J. G. Baust, R. E. Lee, Jr., R. R. Rojas, D. L. Hendrix, D. Friday and H. James, *J. Chromatogr.*, 261 (1983) 65.
4 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
5 J. K. Palmer, *Anal. Lett.*, 8 (1975) 215.
6 W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 358 (1986) 107.
7 L. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 73.
8 J. K. Duncan, A. J. C. Chen and C. J. Siebert, *J. Chromatogr.*, 397 (1987) 3.
9 J. O. Baker and M. E. Himmel, *J. Chromatogr.*, 357 (1986) 161.
10 S. D. Woodruff and E. S. Yeung, *Anal. Chem.*, 105 (1982) 1175.
11 S. D. Woodruff and E. S. Yeung, *Anal. Chem.*, 54 (1982) 2124.
12 S. A. Wilson and E. S. Yeung, *Anal. Chem.*, 57 (1985) 2611.
13 R. E. Synovec, *Anal. Chem.*, 59 (1987) 2877.
14 D. J. Bornhop and N. J. Dovichi, *Anal. Chem.*, 58 (1986) 504.
15 D. J. Bornhop, T. G. Nolan and N. J. Dovichi, *J. Chromatogr.*, 384 (1987) 181.
16 S. Hughes and D. C. Johnson, *Anal. Chim. Acta*, 132 (1981) 11.
17 R. D. Rocklin and C. A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577.
18 R. E. Reim and R. M. Van Effen, *Anal. Chem.*, 59 (1986) 3203.
19 G. G. Neuberger and D. C. Johnson, *Anal. Chem.*, 59 (1987) 150.
20 G. G. Neuberger and D. C. Johnson, *Anal. Chem.*, 59 (1987) 203.
21 L. M. Santos and R. P. Baldwin, *Anal. Chem.*, 59 (1987) 1766.
22 J. C. Kuo and E. S. Yeung, *J. Chromatogr.*, 223 (1981) 321.
23 D. R. Bobbitt and E. S. Yeung, *Anal. Chem.*, 57 (1985) 271.
24 T. Okada and T. Kuwamoto, *Anal. Chem.*, 58 (1986) 1375.

25 C. A. Koerner and T. A. Nieman, *Anal. Chem.*, 58 (1986) 116.
26 M. Takeda, M. Maeda and A. Tsuji, *J. Chromatogr.*, 244 (1982) 347.
27 A. M. Davies, D. S. Robinson and R. Couchman, *J. Chromatogr.*, 101 (1974) 307.
28 M. Petchey and M. J. C. Crabbe, *J. Chromatogr.*, 307 (1984) 180.
29 J.-K. Lin and S.-S. Wu, *Anal. Chem.*, 59 (1987) 1320.
30 P. Vrátný, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 57 (1985) 224.
31 H. Binder, *J. Chromatogr.*, 189 (1980) 414.
32 G. L. Cowie and J. I. Hedges, *Anal. Chem.*, 56 (1984) 497.
33 G. O. Guerrant and C. W. Moss, *Anal. Chem.*, 56 (1984) 633.
34 J. Pawliszyn, *Anal. Chem.*, 58 (1986) 3207.
35 J. Pawliszyn, *Anal. Chem.*, 58 (1986) 243.
36 D. O. Hancock and R. E. Synovec, *Anal. Chem.*, 60 (1988) 1915.
37 D. O. Hancock and R. E. Synovec, *Anal. Chem.*, 60 (1988) 2812.
38 C. N. Renn and R. E. Synovec, *Anal. Chem.*, 60 (1988) 1188.
39 J. Pawliszyn, *Rev. Sci. Instrum.*, 58 (1987) 245.
40 *Technical Note TN-102*, Hamamatsu, Hamamatsi City, Jan. 1982.

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SEPARATION AND CHARACTERIZATION OF METHYLCYCLOPENTADIENE DIMERS BY GAS CHROMATOGRAPHY AND MOLECULAR SPECTROSCOPY

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SUMMARY

The number and dimeric nature of the components of a commercial mixture of methylcyclopentadiene dimers was established by capillary gas chromatography on different stationary phases and temperatures of 80 and 100°C. The mixture was later fractionated by preparative gas chromatography into fractions containing pure or major components. Some of the dimers are transformed into isomers with an exocyclic double bond. The assignment of structures was carried out on the basis of nuclear magnetic resonance spectroscopic data for the dimers and iso-dimers.

INTRODUCTION

Mixtures of methylcyclopentadiene isomers are frequently obtained by thermal cracking of petroleum and similar hydrocarbon fractions. When isomers were separated by gas chromatography (GC) it was found that the main components of industrial mixtures were the 1-methyl (I) and the 2-methyl (II) forms (Fig. 1). The 5-methyl isomer (III) was always present in very low concentration[1,2].

Diels-Alder reactions of methylcyclopentadienes can theoretically give rise to numerous adducts with *endo*- or *exo*-dicyclopentadiene skeletons, but it is generally accepted that adducts exist mainly in the *endo* form (IV) and arise from isomer I and II reacting as a diene and (or) dienophile[2].

Attempts to elucidate the structure of adducts present in commercial mixtures were made through dimerization of mixtures of I and II of known composition[2] as well as by desdimerization of fractions obtained by preparative GC[3]. However, while Musaev *et al.*[4] supposed hat the dienophile reacted through the double bond close to the methyl group, Franklin[5] and Zharov *et al.*[6] assumed that only the unsubstituted double bond was involved. Consequently, there are few reliable results.

When GC on columns packed with different stationary phases was used for the

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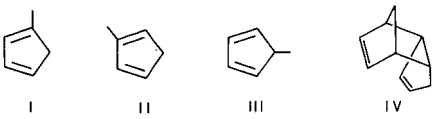
TABLE I

CAPILLARY COLUMNS AND OPERATING CONDITIONS

Parameter	*Column*					
	1[a]	*2*[b]	*3*[b]	*4*[a]	*5*[a]	*6*[b]
Stationary phase	Squalane	SE-54	OV-1701	UCON LB 550X	TXP	OV-215
Length (m)	45	25	25	50	46	25
Internal diameter (mm)	0.50	0.22	0.22	0.25	0.50	0.22
Carrier gas (nitrogen) flow-rate (ml min^{-1})	1.30	1.50	1.00	1.00	2.90	0.85
Splitting ratio	1:91	1:53	1:120	1:92	1:52	1:194
Detector and injector temperatures (°C)	170	170	170	170	170	170
McReynolds polarity	0	337	789	996	1500	1545
Number of theoretical plates	78 000	95 000	118 000	76 500	80 000	94 000

[a] Stainless steel.

[b] Fused silica.



I II III IV

Fig. 1. 1-Methyl- (I), 2-methyl- (II) and 5-methyl-1,3-cyclopentadiene (III); *endo*-dicyclopentadiene (IV).

separation of the resulting adducts, it was clearly established that the dimer arising from I was the least retained while that from II had the highest retention time[2,5]. The same conclusion was made by Langer *et al.*[3] from a study by ^{1}H NMR spectroscopy of three fractions separated by preparative GC. Moreover, these authors assigned structures on the basis of the ^{1}H NMR spectra of the fractions, assuming that these were constituted by only one component. Nevertheless, they did not report either evidence in support of a such a supposition or the spectroscopic data on which the structural assignment was based.

The aim of this work was to further the knowledge of the structures of the dimers present in commercial mixtures. To this end, the number and dimeric nature of the components was first studied by capillary GC on six stationary phases of different polarities (McReynolds scale[7]). The mixture was later fractionated by preparative GC and the composition of the fractions and the structure of the main components was studied by capillary GC and NMR spectroscopy, respectively. The discovery of isomerization reactions involving some of the dimers was of estimable help in the structural assignment.

EXPERIMENTAL

The mixture of methylcyclopentadiene dimers used was supplied by Fluka (technical grade).

Analyses by capillary GC were carried out with Hewlett-Packard 5830A and Perkin-Elmer 8320 chromatographs equipped with flame ionization detection (FID). The capillary columns and operating conditions used are listed in Table I.

Preparative GC was performed on a Perkin-Elmer F21 chromatograph. The methylcyclopentadiene dimer mixture was fractionated using a series of three stainless-steel columns (1 m × 8 mm I.D.), packed with 10% silicone E-301-Chromosorb P N AW (60–80 mesh) with a nitrogen flow-rate of 160 ml min^{-1} and an oven temperature of 100°C.

A mixture with 78% of the isomer of dimer 7 (iso-dimer 7) was obtained by preparative GC from the commercial mixture isomerized by flowing hydrogen chloride into it. A series of three columns (0.90 m × 8 mm I.D.) packed witn 5% Bentone 34 and 5% diisodecyl phthalate on Chromosorb W DMCS (60–80 mesh) was used with a nitrogen flow-rate of 146 ml min^{-1} and an oven temperature of 95°C.

Infrared (IR) spectra were recorded on a Perkin-Elmer Infracord 137 instrument, from samples deposited on NaCl.

^{1}H NMR spectra of samples solved in CCl_4 were obtained on a Varian FT-80 (80 MHz) spectrometer, using tetrmethylsilane (TMS) as the internal standard. ^{13}C NMR spectra were recorded on a Varian FT-80 spectrometer; off-resonance decoupling experiments were also carried out.

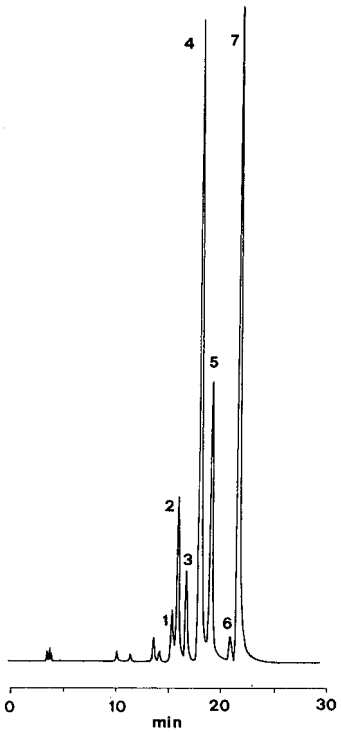


Fig. 2. Chromatogram of a commercial mixture of methylcyclopentadiene dimers on squalane at 100°C. The numbered peaks belong to dimeric compounds.

GC–mass spectrometry (MS) was performed on an Hewlett-Packard 5987 A instrument. Electron impact spectra were recorded at an ionization energy of 70 eV.

RESULTS AND DISCUSSION

Studies on the composition of complex mixtures are usually undertaken by capillary GC using several stationary phases of different chromatographic polarities and by changing the operating conditions. In this way the highest number of components can be detected.

In addition to the possibility of *endo* and *exo* isomers, mixtures of methylcyclopentadiene dimers can be constituted by numerous isomers some of which differ only in the position of one of the methyl groups, including the positions close to the same double bond. For this reason six capillary columns with stationary phases whose polarities range from 0 to 1550 on the McReynolds scale were used for the separation of the dimers at 80 and 100°C (Table I).

The retention times relative to *endo*-dicyclopentadiene of the main components of the mixture (peaks 1–7 in Fig. 2) are shown in Table II. These data reveal that the six stationary phases resolved the mixture into the same seven peaks. Respective peaks have almost identical areas in all chromatograms and the seven components have the

TABLE II

RELATIVE RETENTIONS OF THE METHYLCYCLOPENTADIENE DIMERS IN THE COMMERCIAL MIXTURE ON DIFFERENT STATIONARY PHASES AT 100°C

For chromatographic conditions see Table I.

Peak No.	*Squalane*	*SE-54*	*OV-1701*	*UCON LB 550X*	*OV-215*	*TXP*
	1.00[a]	1.00	1.00	1.00	1.00	1.00
	(9.99)[b]	(7.41)	(5.039)	(5.93)	(4.08)	(8.77)
1	1.49	1.41	1.28	1.27	1.21	1.24
2	1.54	1.44	1.31	1.31	1.24	1.28
3	1.61	1.48	1.35	1.38	1.28	1.37
4	1.74	1.64	1.48	1.47	1.35	1.47
5	1.84	1.66	1.52	1.60	1.40	1.60
6	2.01	1.83	1.63	1.76	1.44	1.73
7	2.09	1.90	1.71	1.82	1.49	1.79

[a] Retention time relative to *endo*-dicyclopentadiene.
[b] Absolute retention time of *endo*-dicyclopentadiene.

same molecular weight [*m/e* 160 (M^+)]. Therefore, it can be stated that the commercial mixture consists of seven components of dimeric nature, of which the principal ones, peaks 2, 4, 5 and 7, were the subject of further study, assuming that they had the structure of *endo*-dicyclopentadiene.

The ^1H NMR spectrum of the commercial mixture shows signals at 6.0–5.7, 5.45 and 5.05 ppm typical of olefinic hydrogens in 1-methylnorbornene[8,9], 2-methylnorbornene[8–10] and methylcyclopentene bound to cycloaliphatic structures[11,12], respectively. Other possible dimeric structures, such as those bearing methyl groups on carbon atoms which join rings of norbornene and cyclopentene (secondary bridgehead), are not present, at less in detectable concentrations. Therefore, it seems apparent that dienophiles mainly react by the unsubstituted double bond. The IR spectrum of the mixture shows only two small bands in the region of the C=C stretching vibration at 1660 and 1630 cm^{-1} compatible with the above mentioned structures.

Dimer 7 was isolated by preparative GC. The most significant feature of its ^1H NMR spectrum (Fig. 3a) is the absence of signals at 6.0–5.7 ppm which rules out structures like 1-methylnorbornene. The 2-methylnorbornene ring of dimer 7 obviously results from a Diels-Alder reaction in which isomer II reacts as a diene. Its highest retention time and concentration in the commercial mixture (Fig. 2) shows, on the basis of previous work[2,3,6], that dimer 7 is a II–II adduct.

Dimer 7 was transformed into a compound with the same molecular weight (MS) and a slightly higher retention time than the parent dimer (Fig. 4). The new compound (peak 10) is an isomer of 7 (iso-dimer 7) different from those present in the commercial mixture. The isomerization progressed until a dimer to iso-dimer ratio of 15:85; the same ratio was found by Weissberger and Page[10] for the isomerization of 2-methylnorbornene to 2-methyleneorbornane. This analogy suggests that the isomerization of dimer 7 results in the formation of an exocyclic double bond at the expense of that in the norbornene ring. The IR spectrum of the dimer to iso-dimer

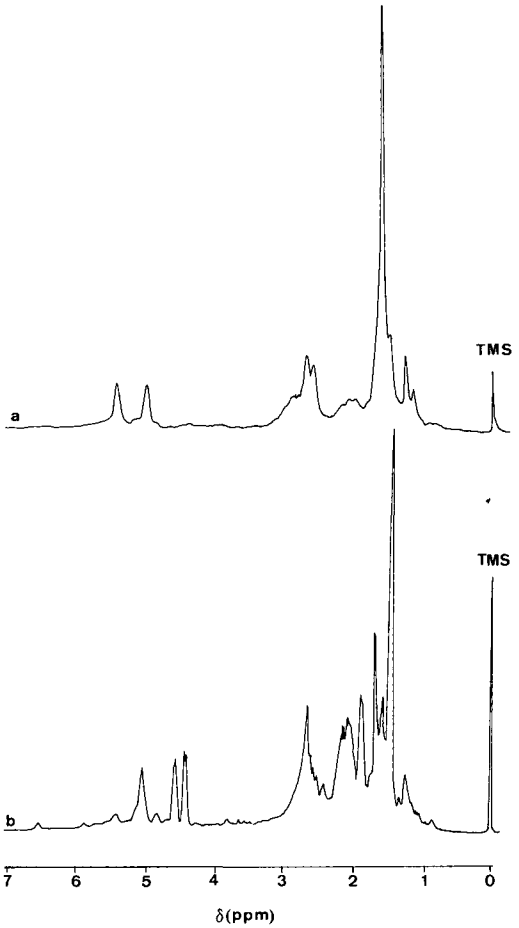


Fig. 3. ^{1}H NMR spectra of dimer 7 (a) and a mixture of dimer 7 and iso-dimer 7 (b).

mixture in Fig. 4b shows a new band at 880 cm^{-1} typical of terminal double bonds. Its ^{1}H NMR spectrum (Fig. 3b) shows that the signal at 5.45 ppm due to the olefinic hydrogen in the 2-methylnorbornene rings is almost extinguished and two new signals at 4.45 and 4.60 ppm have appeared. These new signals may be due to the two olefinic hydrogens of the exocyclic double bond since vinylic hydrogens in the exocyclic methylene group of 2-methylenenorbornane give signals at 4.47 and 4.72 ppm and those of 2-methylene-5-norbornene at 4.67 and 4.95 ppm[10].

Attempts to isolate the pure iso-dimer 7 by preparative GC were not successful since some reactions took place (probably oxidations) resulting in a mixture with 78% of the iso-dimer. Nevertheless, the ^{13}C NMR and off-resonance ^{13}C NMR spectra corroborate the existence of an exocylic double bond by the signals at 104.38, 104.93, 151.76 and 151.86 ppm typical of terminal double bonds, and at 125.89 ppm due to =CH– groups. Cyclic compounds with an exocyclic double bond, such as 2-methylene-5-norbornene[10] and 4,4,7,7-tetramethyl-2-methylenebicyclo[3.3.0]-

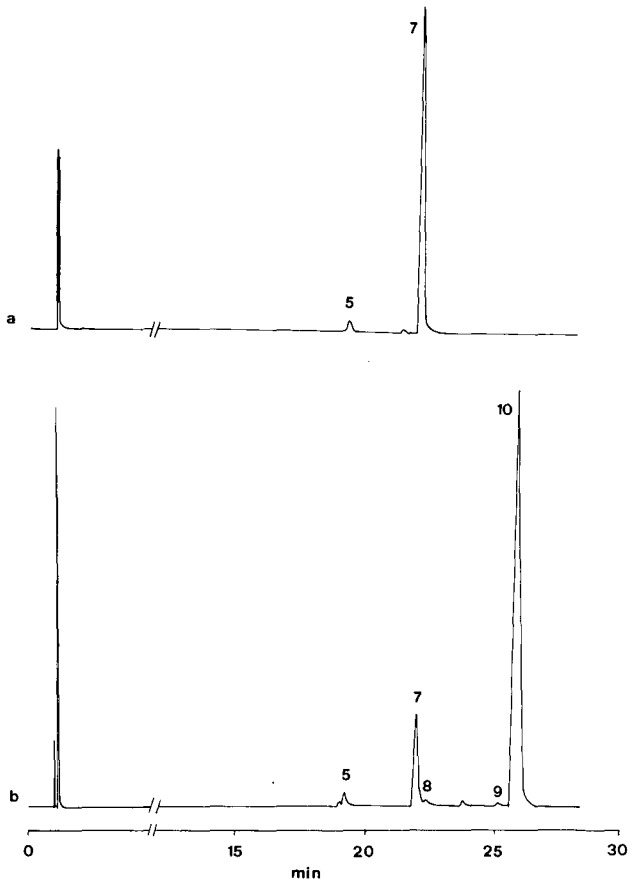


Fig. 4. Chromatograms of dimer 7 (a) and the mixture obtained upon its isomerization (b) on squalane at 100°C.

octane[11] show signals at similar positions. The off-resonance ^{13}C NMR spectrum shows two triplets at 104.38 and 104.93 ppm and two singlets at 151.76 and 151.86 ppm with the same coupling constant, which confirm the existence of carbon atoms in $>C=CH_2$ structures.

A mixture of dimers 4 and 5 (67:33) was isolated by preparative GC. The dimer 4 undergoes the same isomerization as that of dimer 7. The ^1H NMR spectrum of this mixture before isomerization shows identical signals to those of the commercial mixture, which reveals the coexistence of structures with both methylnorbornene rings. After isomerization of dimer 4, the spectrum shows the same signals, at 4.45 and 4.60 ppm, to those of the iso-dimer 7.

The common behaviour of dimers 4 and 7 and the identity of the signals of the olefinic hydrogens of their isomers suggest that 4 and 7 have the same methylnorbornene ring in their structures, differing in the position of the methyl group in the cyclopentene ring. Therefore dimer 4 results from the addition of I (dienophile) to II

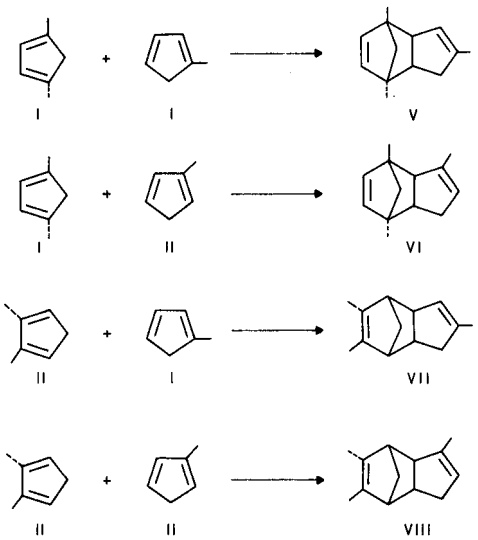


Fig. 5. Probable structures of dimers 2, 4, 5 and 7: I = 1-methyl-1,3-cyclopentadiene; II = 2-methyl-1,3-cyclopentadiene; V = 1,4- (or 4,7-) dimethyl; VI = 1,3- (or 3,7-) dimethyl-; VII = 4,8- (or 4,9-) dimethyl-; VIII = 3,8- (or 3,9-)dimethyl-*endo*-tricyclo[5.2.1.$0^{2,6}$]deca-3,8-diene.

(diene). Consequently, the 1-methylnorbornene ring in the mixture of dimers 4 and 5 belongs to dimer 5 one which should result from the addition of II (dienophile) to I (diene). Finally, judging by its low retention time and concentration, dimer 2 may be the result of a I–I dimerization. The structures in Fig. 5 clearly show why only dimers 4 and 7 (structures VII and VIII, respectively) are able to be transformed into isomers with an exocyclic double bond.

Some insight into the precise position of the methyl group in the norbornene ring can be obtained from the shifts of the signals of olefinic and methyl hydrogens caused by the isomerization. The ^{1}H NMR spectrum of the mixture resulting from the isomerization of dimer 7 (Fig. 3b) shows that the signal of the olefinic hydrogens in the cyclopentene ring shifts from 5.05 to 5.24 ppm, and that due to methyl hydrogens from 1.68 to 1.51 ppm. Since the shifts reflect changes in the displacement of the double bond, the actual position of this in the iso-dimer 7 can be envisaged from molecular models of the two possible structures of dimers 7 and their isomeric forms. It seems that the structure of iso-dimer 7 with the methylene group at C-8 accounts better for the above-mentioned shifts. Nevertheless, other possibility cannot be excluded.

CONCLUSIONS

This study of the composition of a commercial mixture of methylcyclopentadiene dimers carried out by capillary GC on several stationary phases and operating conditions reveals that the mixture consisted of seven components of dimeric nature.

The main dimers were those resulting from Diels-Alder reactions in which the

more reactive of the methylcyclopentadienes, the 2-methyl isomer, reacted as a diene giving rise to structures which include 2-methylnorbornene rings. These structures were the only over able to undergo isomerization, similar to that of 2-methylnorbornene to 2-methylenenorbornane, leading to iso-dimers with an exocyclic double bond.

With regard to the precise position of the methyl group in the 2-methylnorbornene ring, some insight might be gained from the observation of molecular models in relation to the shifts of the ^1H NMR signals of the olefinic and methyl hydrogens caused by the isomerization. It seems that isomerization of 8-methyl structures to 8-methylene ones would account better for the above-mentioned shifts. Nevertheless, other options cannot be excluded.

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REFERENCES

1 K. Alder and H. J. Ache, *Chem. Ber.*, 95 (1962) 503.
2 S. M. Csicsery, *J. Org. Chem.*, 25 (1960) 518.
3 S. H. Langer, H. R. Melton, T. D. Griffith and J. Coca, *J. Chromatogr.*, 122 (1976) 487.
4 M. R. Musaev, S. M. Sharifova and M. A. Aliev, *Azerb. Khim. Zh.*, (1975) 43.
5 W. E. Franklin, *J. Org. Chem.*, 35 (1970) 1794.
6 V. P. Zharov, S. G. Montanari and L. M. Tumanova, *Zh. Anal. Khim.*, 39 (1984) 551.
7 W. O. McReynolds, *J. Chromatogr. Sci.*, 8 (1970) 685.
8 H. C. Brown and K. T. Liu, *J. Am. Chem. Soc.*, 97 (1975) 600.
9 S. Bank, C. A. Rowe, Jr., A. Schriesheim and L. A. Naslund, *J. Am. Chem. Soc.*, 89 (1967) 6897.
10 E. Weissberger and G. Page, *J. Am. Chem. Soc.*, 99 (1977) 147.
11 J. Ipaktaschi and G. Lauterbach, *Angew. Chem., Int. Ed. Engl.*, 25 (1986) 354.
12 L. A. Paquette, R. A. Galemno, Jr., J. C. Caille and R. S. Valpey, *J. Org. Chem.*, 51 (1986) 686.

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PREPARATIVE LIQUID CHROMATOGRAPHY WITH ANALYTICAL SEPARATION QUALITY

INTERVAL INJECTION/DISPLACEMENT REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[a]

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SUMMARY

A method for the enrichment and separation of compounds, called interval injection/displacement reversed-phase high-performance liquid chromatography (HPLC), was applied to several analytical and preparative separations. In principle, the surface of a reversed-phase stationary phase, equilibrated with a weak mobile phase, is "coated" with compounds by stepwise injection of small samples. Distinct time intervals between injections allow the stationary phase to re-equilibrate. In this way, sample enrichment can be achieved in ranges comparable to those in overload elution or displacement HPLC. Sample fractionation proceeds in a similar manner to conventional displacement modes. Also under full mass load conditions, a quality of separation similar to that in analytical elution HPLC is achieved. The results obtained indicate that a very large number of samples can be applied to conventionally sized columns without overload problems, the full mass capacity of a column can be utilized and the method is useful for enriching and separating compounds with a wide range of polarities. Interval injection/displacement reversed-phase HPLC was successfully applied to complex mixtures of natural products and enzyme assay mixtures. Further, this technique is likely to be useful for the analysis of trace compounds and quality control of chemicals. The experiments reported were performed on highly unstable natural products (thiophenic and benzofuran compounds) from *Tagetes* plants.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a highly efficient and versatile method for the analysis of numerous compounds. Analytical separations are typically performed in sample weight ranges below the linear capacity of the stationary phase (less than 1 mg/g of absorbent)[1]. Under these conditions, linear or gradient

[a] A substantial part of this work was presented as a lecture at the *Würzburger Chromatographie-gespräche, Würzburg, F.R.G., September 1988.*

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elution causes the movement of a compound through the column without any interference, and compounds are eluted as quasi-Gaussian peaks[1–5]. By using large columns, scale-up of this technique for preparative purposes is possible with maintenance of the high resolution. However, the preparative throughput (yield of pure compound per unit time) is low. Hence expensive stationary phases are only poorly utilized and compounds leave the column in a highly diluted state[1,2,6]. With conventionally sized columns, higher throughputs can be achieved by volume and/or mass overloading (sample weight ranges higher than 1 mg/g of absorbent) in the elution mode or in the recently reviving displacement mode[7–10]. However, the resolution achieved in this way is poor, and for peak cutting one has to make a compromise between high yield (= low purity) and high resolution (= low yield). Sophisticated techniques, *e.g.*, radial or longitudinal column compression, do not substantially resolve this dilemma[11,12]. As stated by Wehrli[13], it seems that the combination of high sample capacity and high resolution cannot be achieved in the same separation.

Another crucial point in preparative work is the necessity to preconcentrate the samples to a minimum volume prior to injection. A severe concentration procedure (often performed by evaporation), particularly of complex mixtures (*e.g.*, crude extracts) or of bulky aqueous solutions (*e.g.*, subjected to trace compound analysis), entails the risk of the formation of insoluble residues, degradation of unstable compounds or artificial product formation[14,15].

Little and Fallick[16] demonstrated the enrichment of aromatic compounds from bulky aqueous solutions on reversed-phase (RP) stationary phases. In this procedure, the solvent of the sample served as the mobile phase during injection. This enrichment technique was subsequently refined but mainly used for analytical purposes[17–21]. Similarly to the displacement mode described above, compounds are enriched at the top of the column under weak solvent conditions and subsequently displaced by a stronger solvent. Further improvements included column-switching techniques and the use of specially coated precolumns[15,17,18]. This method has been successfully applied to the enrichment and separation of phthalic acid derivatives[15,19], various herbicides[17,18], ergot alkaloids and peptide derivatives[20,21]. Nevertheless, each sample volume or mass overloading of the column or interference between the sample solvent and the mobile phase may cause breakthrough[10,17] of the collected material. This phenomenon results in a similar decrease in resolution to that for scaled-up elution or displacement chromatography.

This paper presents some examples of the application of a new preparative technique for enriching and separating compounds, called interval injection/displacement RP-HPLC. As recently described in a short communication[22], this technique allows the application of a very large number of samples to conventionally sized columns, exploiting their full mass capacity. In contrast to the procedures mentioned above, relatively small sample volumes are injected step-by-step, with distinct time intervals between single injections, which allows the stationary phase to re-equilibrate with the weak solvent. Elution and separation of enriched material take place by application of a special displacement procedure which includes a holding phase[19] to maintain reasonable pressure during the fractionation.

Three examples are presented to demonstrate the suitability of the method for the enrichment, separation and purification of (i) a distinct product from a bulky

ethanolic crude plant extract, (ii) enzymatically formed products from buffered, protein-containing solutions and (iii) compounds eluted in bulk from other chromatographic systems (aqueous and organic solvents). Further examples show some empirically estimated limits of the method for maximum sample volume per single injection, the maximum sample mass and the minimum interval time for a given column size. Finally, a performance test was applied to demonstrate the quality of separation attainable with the new method by comparing the results with those obtained using conventional, isocratic and gradient elution modes. All experiments were performed with highly unstable natural products from *Tagetes* plants (Fig.1)[14,23,24].

EXPERIMENTAL

Apparatus and chemicals

The experiments were performed using a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 400 solvent delivery system, equipped with a Rheodyne (Cotati, CA, U.S.A.) 7125 injector (500-μl sample loop), a Kratos SF 769 spectrophotometer (10

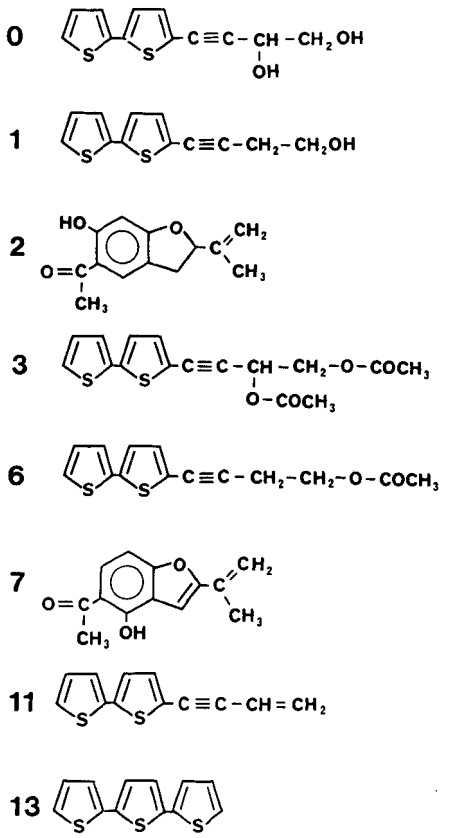

Fig. 1. Natural products extractable from *Tagetes* seedlings[23]. Compound numbers relate to the peaks in Figs. 2–6.

mm pathway cell) and a Spectra-Physics (San Jose, CA, U.S.A.) SP 4100 computing integrator. Columns (250 mm × 4.0, 4.6 or 8.0 mm I.D.) and appropriately sized precolumns were purchased from Knauer (Bad Homburg, F.R.G.). Precolumns were dry-packed with Merck (Darmstadt, F.R.G.) LiChrosorb RP-8 (10 μm). Columns were factory-filled by Knauer with Merck LiChrosorb RP-18 (5 μm) or laboratory-packed (slurry method) with the same material or with Spherisorb (Phase Separations, Queensferry, U.K.) ODS-2 (5 μm). Acetonitrile and water, used as mobile phases, were of HPLC grade (Rathburn, Walkerburn, U.K.). Bithienylbutinene (**11** in Fig. 1) was isolated from *Tagetes* seedlings, as described[23], and α-terthiophene (**13**) was synthesized by Dr. J. Arnason (Ottawa, Canada). Both compounds were used for limit evaluation and performance tests.

Samples

The samples investigated were crude plant extracts, enzyme assay mixtures and affinity chromatography eluates.

Crude plant extracts were prepared from *Tagetes* seedlings by low-temperature extraction[23], concentrated by evaporation and taken up into ethanol.

3,4-Diacetoxybutinylbithiophene:acetate esterase was isolated from *Tagetes* seedlings and was partly purified according to Pensl and Sütfeld[25]. Enzyme assay mixtures (100 μl each) contained 3 nmol of 3,4-diacetoxybutinylbithiophene (dissolved in 10 μl of ethylene glycol monomethyl ether) and the enzyme preparation in 90 μl of potassium phosphate buffer (pH 8.0). The samples were incubated for 10 min and then immediately subjected to HPLC.

A mixture of several compounds from *Tagetes* was immobilized in a Eupergit C (Röhm Pharma, Darmstadt, F.R.G.) column (15 × 8 mm I.D.), equilibrated with 1.0 *M* potassium phosphate buffer (pH 7.5)[26]. Compounds were eluted subsequently with water and with ethylene glycol monomethyl ether (EME) and 100-μl fractions were directly subjected to HPLC.

Mode of interval injection/displacement RP-HPLC and evaluation of limits

Interval injection procedure. Columns of analytical or of semi-preparative size were equilibrated with water–acetonitrile (99:1) using a flow-rate that maintained a constant pressure of about 20 MPa. Under these conditions, any given number of samples could be injected if distinct time intervals were maintained between single injections. The length of the interval time and the maximum applicable sample volume per single injection had to be evaluated empirically for each column size and filling. The maximum applicable sample mass was usually recognizable by a significant increase in pressure after a certain number of injections. After the last injection, enough time was allowed for complete re-equilibration of the column before starting the displacement procedure.

Displacement procedure for separation. After loading the column, the mobile phase was switched to an isocrating holding system[19] containing a strong solvent (acetonitrile) until a constant pressure of about 16 MPa was reached. Previously performed experiments on gradient elution HPLC of thiophenes[23] yielded good results if compounds were eluted in the pressure range 18–10 MPa. Therefore, a similar procedure for displacement was chosen here, using a linear gradient (up to 100% of acetonitrile) and switching to a slightly higher flow-rate to maintain the pressure in this range.

TABLE I

GRADIENT PROFILES EMPLOYED FOR THE SEPARATION AND TRANSFER OF THIOPHENIC COMPOUNDS INTO WATER-FREE MEDIUM

Column: 250 × 4.6 mm I.D. LiChrosorb RP-18 (5 μm) with a precolumn (40 × 4.6 mm I.D.) of LiChrosorb RP-8 (10 μm).

Separation gradient			*Transfer gradient*		
Time (min)	*Flow-rate (ml/min)*	*Water–acetonitrile ratio*	*Time (min)*	*Flow-rate (ml/min)*	*Water–acetonitrile ratio*
0	0.7	99:1	0	0.7	99:1
0.1	0.7	30:70	0.1	0.7	0:100
5.0	0.7	30:70	23.0	1.5	0:100
5.1	1.0	30:70	Indefinite	1.5	0:100
17.0	1.0	0:100			
Indefinite	1.0	0:100			

Displacement procedure for transfer. In order to transfer purified compounds into a water-free medium, material enriched by interval injection was displaced by switching from 1% to 100% of the strong solvent. The expected sudden pressure decrease was compensated for by an increasing flow-rate gradient. Table I gives the gradient profiles employed on a 250 × 4.6 mm I.D. column for the separation and transfer of compounds.

RESULTS

Examples of application

Preparative purification of a product from a crude plant extract. A crude ethanolic extract (5 ml) from *Tagetes* seedlings was applied to an 8.0 mm I.D. RP-18 column by interval injection (10 × 500 μl; interval time, 1 min) and separated as shown in Fig. 2. Fractions of 0.9 ml each were collected and re-chromatographed by analytical HPLC. The re-constructed chromatogram (dilution factor, 1:100) demonstrated the quality of separation of the preparative run. This work was directed to isolate and to purify compound **3**. Therefore, fractions containing **3** were combined (total volume, 4.5 ml) and subjected to a second run under the same conditions (Fig. 3). The peak corresponding to **3** was cut out manually as indicated and this eluate (3 ml) was chromatographed again under transfer gradient conditions (Fig. 4). Cutting out the corresponding peak, as indicated, yielded a product with a purity of nearly 99%, as shown by analytical gradient elution HPLC.

Enrichment and separation of enzyme assay mixtures. Fig. 5 shows the consecutive application of 90 single enzyme assay mixtures (3,4-diacetoxybutinylbithiophene:acetate esterase[25], total volume 9.0 ml, interval time 1 min) to a 4.6 mm I.D. RP-18 column and the separation of products formed (**0** and **3'**, a reaction intermediate[25]) from a given substrate (**3**). The relatively high injection peaks (*cf.*, Figs. 3, 4 and 6–9) suggest that the enzyme protein was eluted during the interval injection period.

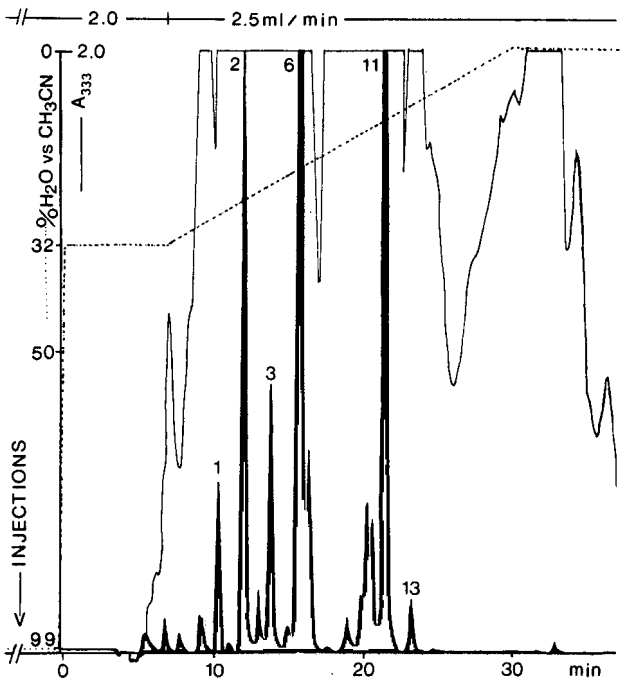

Fig. 2. Fractionation of a crude extract from *Tagetes* seedlings after interval injection. Total extract volume, 5 ml, applied by ten injections of 500 μl each (interval time, 1 min). Solid peaks: re-chromatography of collected fractions by analytical HPLC; dilution factor, 1:100. Column: 250 × 8 mm I.D. LiChrosorb RP-18 (5 μm), with a precolumn (30 × 8 mm I.D.) of LiChrosorb RP-8 (10 μm).

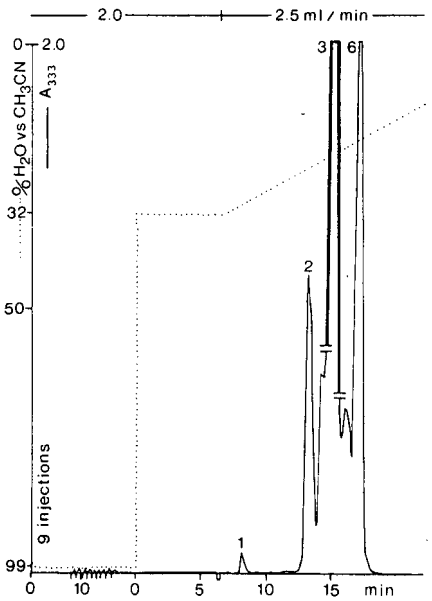

Fig. 3. Interval injection and separation of fractions from the previous run (Fig. 2) containing compound **3**. Nine injections of 500 μl each; total volume injected: 4.5 ml. The fraction indicated by the bold line was collected for subsequent transfer into acetonitrile (Fig. 4). Separation conditions as in Fig. 2.

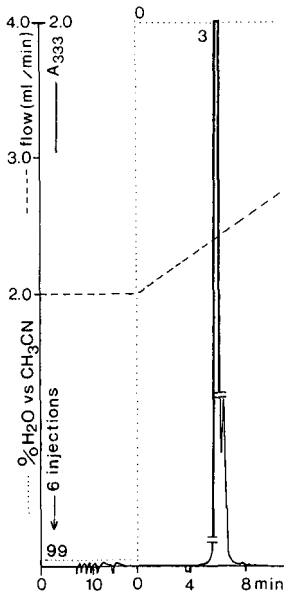

Fig. 4. Interval injection of collected material (Fig. 3) and transfer of compound **3** into acetonitrile. Six injections of 500 μl each; total volume injected: 3.0 ml. The fraction indicated by the bold line was collected for subsequent concentration. Separation conditions as indicated; column and precolumn as described in Fig. 2.

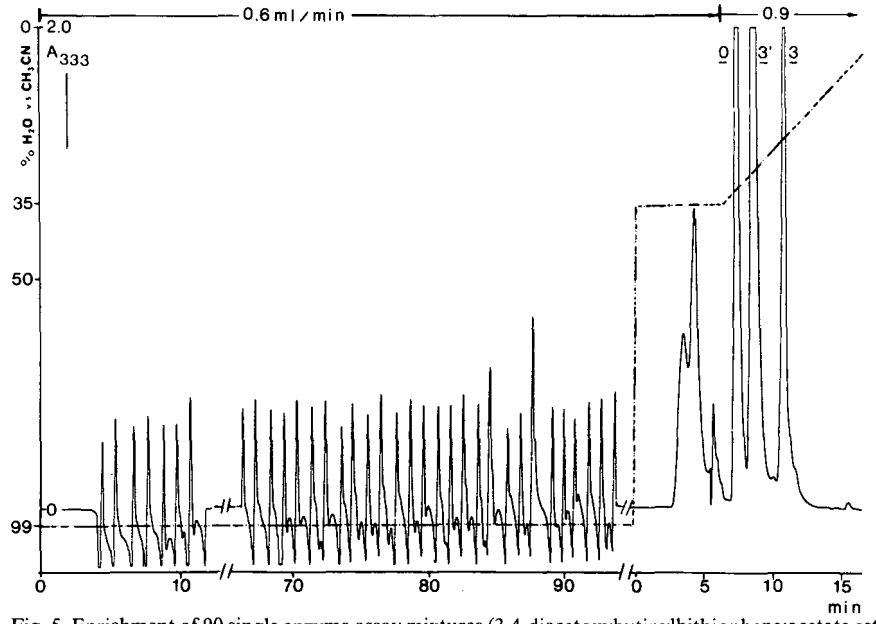

Fig. 5. Enrichment of 90 single enzyme assay mixtures (3,4-diacetoxybutinylbithiophene:acetate esterase[25], total volume 9.0 ml) by interval injection (interval time, 1 min) and separation of products formed (**0** and **3'**) from the substrate (**3**). Compound **3'** supposedly represents 3-hydroxy-4-acetoxybutinylbithiophene[25]. Unnumbered peaks were not further identified. Column: 250 × 4.6 mm I.D. RP-18, 5 μm. Injection: 90 × 100 μl.

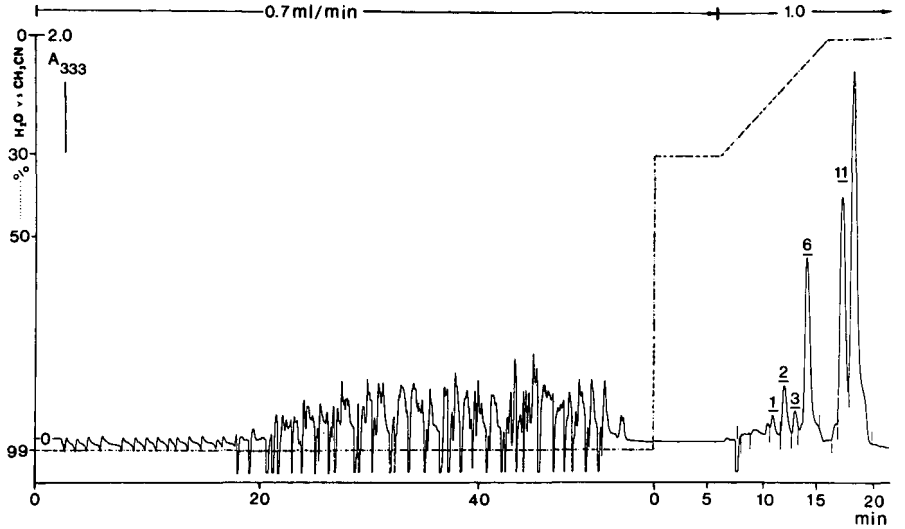

Fig. 6. Enrichment of thiophenic compounds eluted from an affinity chromatography column (Eupergit C). First eluate with 1.5 ml of water, second eluate with 3.2 ml of ethylene glycol monomethyl ether (EME). Interval time, 1 min. Unnumbered peaks were not further identified. Column as in Fig. 5. Injection: 15 × 100 μl eluate (aqueous) + 32 × 100 μl eluate (EME).

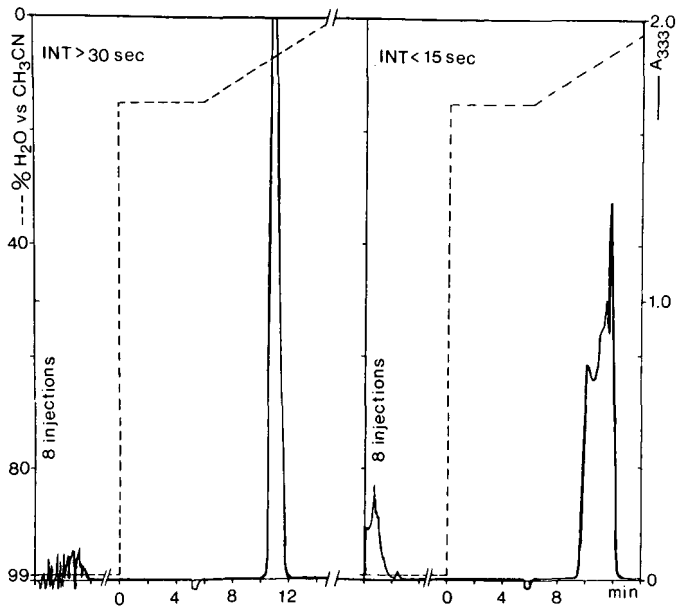

Fig. 7. Influence of interval time on the quality of separation. Sample: α-terthiophene (**13**) (1.5 mg/ml). Column: 250 × 4.0 mm I.D. LiChrosorb RP-18 (5 μm) with a precolumn (11 × 4.0 mm I.D.) of LiChrosorb RP-8 (10 μm). Flow-rate, 0.7 ml/min during injections, 1.0 ml/min during displacement.

Enrichment and separation of bulk eluates from other chromatographic systems. The suitability of interval injection/displacement RP-HPLC for the enrichment and separation of compounds eluted in bulk volumes and changing solvents is demonstrated in Fig. 6. Several natural compounds of *Tagetes* were immobilized in a Eupergit C column[26] and were subsequently eluted with water and ethylene glycol monomethyl ether. No interference between the different solvents was detectable during the injection time and a reasonable quality of separation was achieved.

Limits of interval injection

The experiments shown in Figs. 7–9 were performed with α-terthiophene (**13**), dissolved in ethylene glycol monomethyl ether, as test substance.

Interval time. Fig. 7 shows that a time interval of at least 30 s has to be maintained between single injections if a column with an I.D. of 4.0 mm and a flow-rate of 0.7 ml/min are used. Shorter intervals, *e.g.*, less than 15 s, yielded poor separations or multiple peaks. Similar results were obtained with columns of larger diameters run with correspondingly higher flow-rates.

Volume per single injection. The maximum applicable volume per single injection is strongly dependent on the I.D. of the column in use. Fig. 8 shows that, for a 4 mm I.D. column, this value ranges between 100 and 200 μl per single injection. For an 8 mm I.D. column, a volume of about 500 μl is still applicable. Too high sample volumes per injection lead to the formation of multiple peaks, similar to those shown in Fig. 7.

Total mass. A 1-ml volume of a saturated solution of α-terthiophene (15 mg) was applied to a 4.0 mm I.D. column in ten injections of 100 μl each. After the tenth injection, a sudden pressure increase was observed, indicating a full mass load.

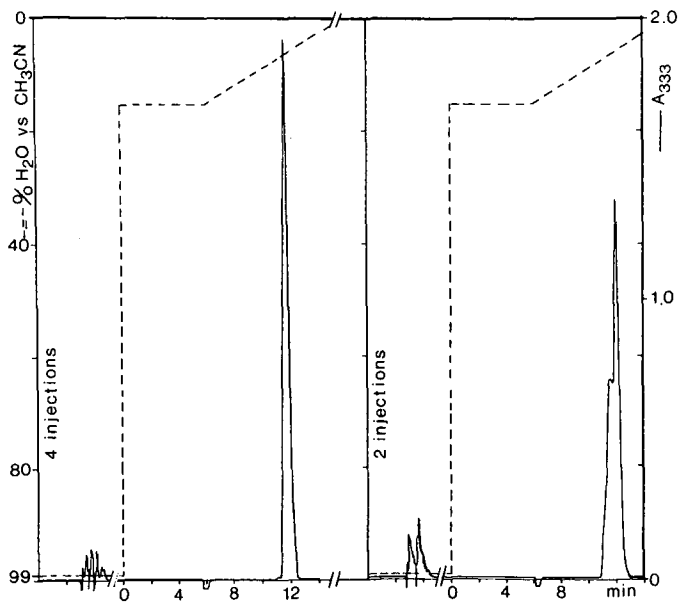

Fig. 8. Influence of the single sample volume on the quality of separation. Sample and separation conditions as in Fig. 7. Left: 4 injections of 100 μl each; right: 2 injections of 200 μl each.

TABLE II

PERFORMANCE TEST USING ISOCRATIC, GRADIENT AND INTERVAL INJECTION/DISPLACEMENT RP-HPLC

Column: 250 × 8 mm I.D. LiChrosorb RP-18 (5 μm) with a precolumn (30 × 8 mm I.D.) of LiChrosorb RP-8 (10 μm). Sample: bithienylbutinene (**11**), 3 mmol/l, 10 μl per injection. Detection, UV (333 nm).

Mode	*Water–acetonitrile ratio*	*Flow-rate (ml/min)*	*Pressure (MPa)*	*Retention time (min)*	*Peak width at half-height (s)*	*N*[a]
Isocratic	10	3.3	14	6.33	11.6	5900
Isocratic	18	2.8	16	11.06	18.0	7500
Isocratic	32	2.5	19	27.60	49.5	6200
Gradient	32–0 (15 min)	2.0	15–8	16.89	13.9	29 000
Isocratic gradient	32 (8 min) 32–0 (15 min)	2.0 2.5	15–9	20.20	14.6	38 300
Interval injection/displacement	99 (indefinite) 32 (8 min) 32–0 (15 min)	 2.0 2.5	22–9	20.39	20.6	19 600

[a] *N*, theoretical plate number, calculated by using retention times and peak widths at half-heights[1,27].

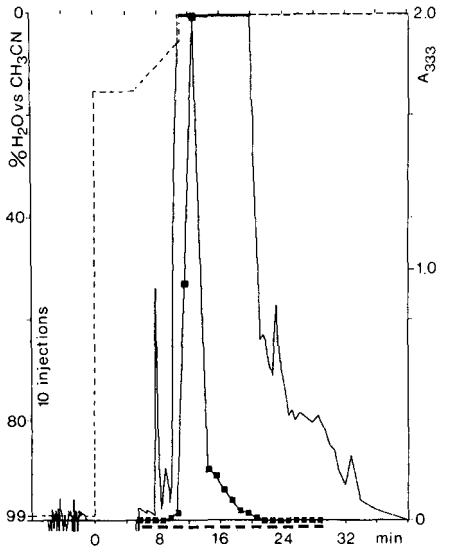

Fig. 9. Interval injection/displacement RP-HPLC under full mass-load conditions. Sample, α-terthiophene (**13**) (15 mg/ml). Separation conditions as in Fig. 7. Fractions were collected as indicated by the black and white bars and re-chromatographed by analytical HPLC at a dilution of 1:100 (■). Ten injections of 100 μl each.

Fractions were collected as indicated in Fig. 9 and re-chromatographed (dilution, 1:100) by analytical HPLC. The reconstructed chromatogram demonstrated the quality of separation in the preparative run. Moreover, the preparative chromatogram gave evidence of the presence of some impurities that could not be observed before.

Performance test

Identical samples of bithienylbutinene (**11**) were chromatographed under various isocratic, gradient and isocratic gradient conditions and also under the conditions of interval injection/displacement HPLC. Table II shows that, according to the calculated theoretical plate numbers, the interval injection method yielded a reasonably high resolution in comparison with the conventional linear and gradient elution methods, which were run under similar mobile phase conditions.

DISCUSSION

Interval injection/displacement RP-HPLC has been shown to be a powerful method for the enrichment and separation of natural plant compounds. It is probably also applicable to a wide range of other natural or synthetic products of similar polarities. Compounds with higher polarities can probably be enriched by modifying the weak solvent on injection, *e.g.*, by acidification or by addition of ion-pair reagents.

With interval injection, concentration of extracts prior to HPLC injection becomes superfluous and hence some new compounds may now be detected that would have been degraded because of severe pretreatment procedures[14]. The results obtained indicate that, with this method, column mass loads can be achieved in ranges

comparable to those in overload elution or displacement modes[1,2,6]. However, employing the interval injection mode, a quality of separation is achieved that resembles analytical runs. It may be assumed that, during interval injections, compounds do not invade the stationary phase but coat the surface of the column as a thin layer. Full mass load is reached if this layer becomes impermeable to the mobile phase, as indicated by a sudden increase in pressure.

Obviously, interval injection/displacement RP-HPLC is able to combine a high preparative throughput with high resolution, in contrast to previous experience[13]. Further, the method is applicable to trace compound analysis and chemical quality control. The new possibility of enriching compounds and transferring them into a water-free medium opens up other prospects for application. For enzymological research, the method has several advantages, *e.g.*, enzyme assay mixtures can be injected without stopping the reaction by treatment with acid, alkali or organic reagents for protein precipitation. The results obtained (Fig. 5) suggest that the native protein is eluted from the column during the interval injections. Because of the possibility of injecting numerous assay mixtures, extremely low enzyme activities can be detected. However, one may argue that a similar enrichment and separation effect could be achieved if the whole of the aqueous solution were to be applied in one step, *e.g.*, by pumping it through the system or by using appropriately sized sample loops as previously described[16–21]. However, in contrast to the disadvantages of these techniques, interval injection does not involve the risk of damage to the solvent delivery system by microparticulate material or by aggressive buffer ions which are often present in enzyme assay mixtures. The size of the sample loop becomes unimportant and hence the number and size of single injections are completely variable.

Interval injection works well if some precautions are taken. Aqueous and organic sample solvents may be injected when they are soluble in the mobile phase (*cf.*, Fig. 6). The length of the interval times should be adapted to the column size (I.D.) and to the sample solvent used in order to prevent compounds from invading the column. The maximum volume per single injection seems to be an important factor, which must be determined and adapted to each individual column size and filling, otherwise, breakthrough[10,17] of collected material will occur, leading to poor separations. Further experiments to apply this method to other compounds and stationary phases are in progress.

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REFERENCES

1 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.
2 M. Verzele and C. Dewaele, *Preparative High Performance Liquid Chromatography —A Practical Guideline*, TEC, Ghent, 1986.
3 R. M. McCormick and B. L. Karger, *J. Chromatogr.*, 199 (1980) 259.

4 F. Riedo and E. sz. Kováts, *J. Chromatogr.*, 239 (1982) 1.
5 Cs. Horváth, J. Frenz and Z. El Rassi, *J. Chromatogr.*, 255 (1983) 273.
6 G. Guiochon and A. Katti, *Chromatographia*, 24 (1987) 165.
7 Cs. Horváth, A. Nahum and J. H. Frenz, *J. Chromatogr.*, 218 (1981) 365.
8 M. Verzele, C. Dewaele, J. van Dijck and D. van Haver, *J. Chromatogr.*, 249 (1982) 231.
9 J. Frenz, Ph. van der Schriek and Cs. Horváth, *J. Chromatogr.*, 330 (1985) 1.
10 V. R. Meyer, *J. Chromatogr.*, 316 (1984) 113.
11 G. Cretier and J. L. Rocca, *Chromatographia*, 16 (1982) 32.
12 E. Bayer and H.-P. Seelmann-Eggebert, *Chromatographia*, 18 (1984) 65.
13 A. Wehrli, *Fresenius Z. Anal. Chem.*, 277 (1975) 289.
14 R. Sütfeld and H. Breteler, *Proc. Int. Conf. NOARC's*, Elsevier, Amsterdam, in press.
15 H. P. M. van Vliet, T. C. Bootsman, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 185 (1979) 483.
16 J. N. Little and G. J. Fallick, *J. Chromatogr.*, 112 (1975) 389.
17 C. E. Goewie, P. Kwakman, R. W. Frei, U. A. Th. Brinkman, W. Maasfeld, T. Seshadri and A. Kettrup, *J. Chromatogr.*, 284 (1984) 73.
18 M. Åkerblom, *J. Chromatogr.*, 319 (1985) 427.
19 A. Otsuki, *J. Chromatogr.*, 133 (1977) 402.
20 P. Schauwecker, R. W. Frei and F. Erni, *J. Chromatogr.*, 136 (1977) 63.
21 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
22 R. Sütfeld, *Fresenius Z. Anal. Chem.*, 330 (1988) 395.
23 R. Sütfeld, *Modern Methods of Plant Analysis*, Vol. 5, Springer, Berlin, 1987, p. 104.
24 R. Sütfeld, *Planta*, 156 (1982) 536.
25 R. Pensl and R. Sütfeld, *Z. Naturforsch., C.*, 40 (1985) 3.
26 R. Sütfeld, in preparation.
27 A. T. James and A. J. P. Martin, *Analyst (London)*, 77 (1952) 115.

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SEPARATION OF BASIC DRUGS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A SILICA COLUMN USING A METHANOL–ETHYLENEDIAMINE BUFFER

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SUMMARY

The application of methanol–aqueous ethylenediamine–ammonium nitrate eluents has been investigated for the high-performance liquid chromatographic separation of basic drugs on silica stationary phases. These eluents were shown to be more reproducible than previously studied systems based on methanol–aqueous ammonia–ammonium nitrate eluents. The effects of different eluent pH and buffer concentrations have been examined.

INTRODUCTION

Basic drugs frequently cause problems when analysed by reversed-phase high-performance liquid chromatography (HPLC) because of their interaction with acidic silanol groups on the surface of the stationary phase. A number of alternative methods have therefore been proposed for their separation and one of the most successful approaches has been the use of silica columns with high pH buffered eluents containing a high proportion of methanol. This method was originally proposed by Jane[1], who used an ammonium nitrate buffer, and has subsequently been studied by Law and co-workers[2,3]. In a recent study Schmid and Wolf[4] have examined a similar high-methanolic system and looked at the effect of sodium acetate buffer and ammonia concentration by using the tricyclic drugs. In all these systems the separation is effectively based on the ion-exchange properties of the silica stationary phase.

There has been some concern that although HPLC is a very widely used technique for analytical separations, intra- and interlaboratory reproducibility of separations can be very poor. As a consequence, we have carried out a series of studies of separations of forensic interest to investigate the sources of these variations and to examine methods to standardise the separations and the recording of the results. As part of this work, the separation of basic drugs on silica using a methanol–aqueous ammonia–ammonium nitrate eluent has been examined in detail.

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In our studies the effects of changes in the composition of the mobile phase and in the operating conditions have been examined[5] and differences in the separation on different batches and brands of silica have been reported[6]. Similar differences between brands have also been noted previously[2,4]. There were significant differences between separations even on a single column when the results were expressed as capacity factors but considerably more reproducible results could be obtained if the results were recorded as relative retentions compared to protriptyline as an internal standard. To test the conclusions of these studies the separation has been examined in national[7] and international collaborative studies[8]. In both these studies the variance of the results was higher than in intralaboratory studies. It was suggested that differences in the operating temperature of the laboratories could have had an effect as few of the laboratories used a thermostated column. However, it appeared that a major factor which could not be controlled was the concentration of the ammonia stock solutions used to prepare the aqueous buffer. This would affect the pH and ionic strength of the mobile phase.

In setting up a standard eluent system to determine the reproducibility of batches of column materials[6], it was demonstrated that consistent results could be obtained over a three-month period using a single source of ammonia, even though its concentration changed due to evaporation. However, deliberate larger changes in the ammonia concentration to 200, 180, 160, 90, 80 or 60% of the original value caused major effects on the relative and absolute retentions. Some of the analyte drugs were more susceptible to the changes than others. These problems with the reproducibility and stability of the concentrated ammonia solution have led to an interest in alternative eluent systems based on less volatile buffer components. If possible the buffer should be prepared by weight or defined volume of a single liquid compound rather than as a volume of a dilute aqueous solution of a volatile base. It is also important to avoid methods which would require pH adjustment as this could cause differences in the ionic strength of the eluent, which were also shown to affect the separations.

The present study reports a system based on the use of a relatively involatile amine, ethylenediamine, as the base, whose proportion in the mobile phase can be precisely defined as the volume of a neat liquid. The effects of differences in the pH and buffer concentrations and of different batches of the stationary phase have been studied.

EXPERIMENTAL

Chemicals and standards

Ammonium nitrate, analytical-reagent grade, and methanol, HPLC grade, were from FSA Laboratory Supplies, Loughborough, U.K. Ethylenediamine was reagent grade from Aldrich, Poole, U.K. Concentrated ammonia solution (sp. gr. 0.880) was laboratory grade from BDH Chemicals, Poole, U.K. Samples of basic drugs were from from the reference collection of the Central Research Establishment, Home Office Forensic Science Service.

HPLC equipment

HPLC separations were carried out using a Pye Unicam LC-XPS pump and an

Altex 153 fixed-wavelength detector at 254 nm. The samples (5 μl) were injected using a 7125 Rheodyne valve fitted with a 20-μl loop onto a Shandon column (250 × 5 mm I.D.) packed with Spherisorb S5W 5 μm (Batch 2752 or 5493, Phase Separations Queensferry, U.K.). The methanol–buffer eluent was pumped at 2 ml min^{-1} and was passed through a pre-column packed with silica, installed between the pump and the injection valve. The pre-column and the analytical column were maintained at 30°C in a circulating-water bath. Peaks were recorded using a chart recorder.

Ammonia buffer solutions

Ammonia–ammonium nitrate buffer was prepared by mixing ammonia (sp. gr. 0.880) (90 ml), ammonium nitrate (27 g) and water (900 ml).

Ethylenediamine–ammonium nitrate buffers

The standard buffer (pH 10.2) was prepared by a ten-fold dilution of a mixture of ammonium nitrate (10.5 g), ethylenediamine (15.0 ml) and water (200 ml).

A buffer of pH 9.47 was prepared by a ten-fold dilution of a mixture of ethylenediamine (5 ml) and ammonium nitrate (10.0 g) in water (200 ml).

A buffer of pH 10.56 was prepared by a ten-fold dilution of a mixture of ethylenediamine (15 ml) and ammonium nitrate (5.02 g) in water (200 ml).

Sample solutions of basic drugs

Solution of mixtures of the basic drugs were made up as described for the collaborative study[7], each including protriptyline hydrochloride as an internal standard, in ethanol–water (90:10, v/v) with concentrations (0.02–8 mg ml^{-1}) chosen to give a similar detector response for each drug.

For much of the work a simplified set of test solutions containing characteristic drugs was used[5]. The detailed composition of these solutions are given below [concentrations in mg ml^{-1} in ethanol–water (90:1, v/v)].

(A) Dipipanone hydrochloride, 0.40; prolintane hydrochloride, 1.24; protriptyline hydrochloride, 0.15; strychnine, 0.07.

(B) Promazine, 0.006; phenylephrine bitartrate, 1.44; protriptyline hydrochloride, 0.15.

(C) Codeine phosphate, 1.07; ephedrine, 2.25; protriptyline hydrochloride, 0.15.

(D) Sodium nitrate, 30 mg ml^{-1} in methanol–water (90:10, v/v) as a column void volume marker.

Calculations

The separations were carried out in triplicate and the mean retention times were used to calculate the capacity factors as $k' = (t_R - t_0)/t_0$.

Relative capacity factors were calculated as k'/k'_p where k'_p is the capacity factor for the protriptyline present as an internal standard in each test solution.

RESULTS AND DISCUSSION

In order to design an eluent that can be prepared reproducibly in different laboratories it must be possible to specify precisely all the constituents, as even small differences may significantly affect the relative retentions of the analytes. Rather than

preparing buffers by adjusting them to a specified pH it is also preferable to use fixed weights or volumes to give a predictable pH. This was demonstrated in earlier studies of the separation of barbiturates in which preparing the buffer by weight from solid salts gave highly reproducible results[9]. In initially investigating the separation of drugs on a silica column, it appeared that the ammonia–ammonium nitrate buffer was robust as the pH was unaffected even by significant changes in the amount of ammonia or ammonium nitrate used in the preparation of the buffer[5]. However, the retentions did appear to be sensitive to the ionic strength of the mobile phase[5]. In collaborative studies[7,8] the relatively poor reproducibility suggested that one area which could not be controlled was the strength of the ammonia solutions used in the preparation of the eluent.

In the present study a limited group of basic drugs was examined. This included dipipanone, pipazethate, phenylephrine and strychnine, compounds which have been found to be particularily sensitive to changes in the experimental conditions[5,6]. The study started by examining the use of other bases as possible alternatives to concentrated ammonia solution for the preparation of buffers with pH of about 10. Piperidine was too insoluble in water to give a pH for the buffer greater than 8. Diethylamine was more satisfactory but it is relatively volatile (b.p. 55°C) and could be lost from the mobile phase during the separation in a similar manner to ammonia. The less volatile amine, ethylenediamine (b.p. 118°C), was also apparently suitable. When it was used in place of ammonia at a similar strength, it gave an aqueous buffer solution with a pH of 10.2. However, when a methanol–buffer (90:10, v/v) eluent was used to separate the limited test set of basic drugs, the drugs were barely retained on the column compared to the corresponding ammonia–ammonium nitrate system. The internal standard, protriptyline, was eluted with a capacity factor of less than 0.5 compared to 2.5 and the retention times of the other analytes were even shorter. These small retentions are insufficient for resolution and identification of the basic drugs.

As the rate of elution is governed by the ionic strength of the mobile phase, this initial buffer mixture was diluted ten-fold with water to give a buffer with a pH of 10.22 and the separation was re-examined. This eluent gave comparable retentions to those achieved earlier (Table I) but the relative retentions for some of the drugs differed from those obtained with the ammonia buffer eluent.

Mobile phases were then prepared using buffer solutions of pH 10.55 and 9.47 by altering the rato of the ethylenediamine and ammonium nitrate (see Experimental) and the separations were repeated. At pH 9.47 the resolution of the test mixtures was very poor. With the pH 10.55 eluent (Table I) a better separation was obtained but the order of elution changed. Strychnine was now eluted much more rapidly than protriptyline. This could cause problems as all the components would be eluted within too short a time span. It was therefore decided to use the pH 10.2 solution as the standard buffer in future studies. Using this buffer, repeated separations on successive days showed that the repeatability of these eluents was satisfactory.

The work up to this point had been carried using a Spherisorb S5W (Batch 2752) column as the stationary phase. However, it has been observed that retention on different batches of Spherisorb S5W gave different selectivities with the ammonium nitrate system[6]. As the initial batch of silica had been exhausted, a second batch of stationary phase (Spherisorb S5W Batch 5493) was examined. The same mixtures of 28 drugs that had been used in the collaborative study[7] were separated using the ethylenediamine eluent and gave good peak samples (Fig. 1).

TABLE I

EFFECT OF DIFFERENT AMINES AND BUFFER CONCENTRATIONS ON THE RETENTION OF BASIC DRUGS ON SPHERISORB S5W

Based on columns prepared from Spherisorb S5W batch 2752. Temperature, 30°C. Eluent, methanol–buffer (90:10, v/v). Values using the ammonia buffer from ref. 6.

Compounds	*Capacity factors*			*Relative capacity factors (× 100)*		
	Buffer			*Buffer*		
	Ammonia	*Ethylenediamine*		*Ammonia*	*Ethylenediamine*	
		pH 10.22	*pH 10.55*		*pH 10.22*	*pH 10.55*
Dipipanone	0.37	0.58	0.62	20.3	27.2	22.1
Promazine	0.71	0.78	0.76	38.8	36.6	27.1
Codeine	0.88	0.92	0.87	48.2	43.2	31.1
Prolintane	0.88	1.11	1.12	48.2	52.1	40.0
Phenylephrine	1.17	1.26	1.43	64.0	59.1	51.1
Ephedrine	1.28	1.50	1.78	70.0	70.4	63.6
Protriptyline	1.83	2.13	2.80	–	–	–
Strychnine	2.65	2.77	2.52	144.4	130.0	90.0

The separation was repeated three times on successive days, using a fresh eluent each day, to determine the repeatability of the separation. The capacity factors and relative capacity factors compared to protriptyline were determined and the standard deviations for the repeated studies were calculated (Table II). The repeatability of the capacity factors and the relative capacity factors were good and in most case better than those obtained for the ammonia-based system[5,6]. The retentions of some analytes could not be distinguished from the solvent front and codeine and dipipanone now

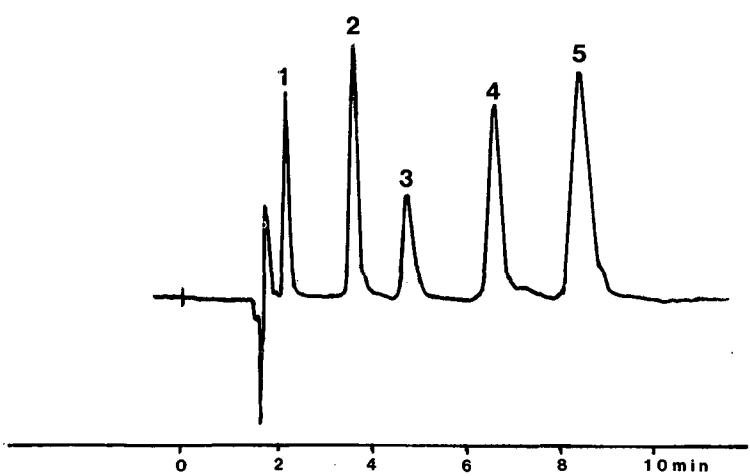

Fig. 1. Separation of drug test mixture on Spherisorb S5W Batch 5493. Eluent: methanol–(ethylenediamine–ammonium nitrate buffer pH 10.2) (90:10, v/v). Basic drugs: (1) procaine; (2) promazine; (3) ethoheptazine; (4) protriptyline (internal standard); (5) strychnine.

TABLE II

REPRODUCIBILITY OF CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS OF BASIC DRUGS DETERMINED USING ETHYLENEDIAMINE BUFFER ELUENT

Based on three separations on successive days on column prepared from Spherisorb S5W Batch 5493. Eluent, methanol–(aqueous ethylenediamine–ammonium nitrate buffer pH 10.2) (90:10, v/v); temperature, 30°C.

Compounds	*Capacity factors*			*Relative capacity factors (× 100)*			*Ammonia*[c]
	Mean	*S.D.*	*C.V. (%)*	*Mean*	*S.D.*	*C.V. (%)*	
Diazepam	[a]	–	–	–	–	–	
Nitrazepam	[a]	–	–	–	–	–	
Papaverine	[a]	–	–	–	–	–	
Caffeine	[a]	–	–	–	–	–	
Dextropropoxyphene	0.09	0.01	11.1	3.5	0.5	14.3	
Cocaine	0.17	0.02	11.8	6.5	0.8	12.3	
Procaine	0.24	0.02	8.3	9.3	0.9	9.7	
Amitriptyline	0.54	0.03	5.5	20.3	0.6	3.0	
Chlorpromazine	0.60	0.04	6.7	22.4	1.0	4.5	
Propranolol	0.68	0.02	2.9	26.1	0.4	1.5	
Imipramine	0.84	0.03	3.6	31.7	0.8	2.5	
Phentermine	0.95	0.03	3.2	35.7	0.5	1.4	
Amphetamine	0.99	0.03	3.0	37.7	0.5	1.3	
Promazine	1.01	0.03	3.0	38.5	0.8	2.1	36.4
Dipipanone[b]	1.06	0.03	2.8	40.1	0.8	2.0	33.5
Codeine[b]							46.0
Morphine	1.22	0.04	3.3	45.9	0.4	0.9	
Pholcodine	1.35	0.05	3.7	51.3	0.8	1.6	
Phenylephrine	1.50	0.01	0.7	56.5	0.1	0.2	60.7
Prolintane	1.62	0.04	2.5	60.9	0.8	1.3	62.8
Ethoheptazine	1.63	0.03	1.8	62.0	0.5	0.8	
Nortriptyline	1.68	0.03	1.8	64.0	0.4	0.6	
Ephedrine	1.85	0.06	3.2	69.6	0.4	0.6	69.9
Methdilazine	1.86	0.05	2.7	70.3	0.7	0.9	
Pipazethate	1.89	0.08	4.2	71.0	1.5	2.1	
Methylamphetamine	2.09	0.08	3.8	79.0	0.7	0.8	
Protriptyline	2.63	0.03	1.1	100.0	–	–	
Strychnine	3.62	0.06	1.7	137.4	0.9	0.7	159.8

[a] Unresolved from solvent front.
[b] Unresolved (in same test mixture).
[c] From ref. 6.

coeluted, whereas previously they had been well separated. The retentions and order of elution of the basic drugs differed markedy from a separation on this batch using the ammonia based eluents[6]. There were also significant differences from the retentions of the smaller group of drugs with the ethylenediamine eluent measured on the older batch of Spherisorb S5W (Table I).

Further studies of this approach to the separation of basic drugs on silica are in progress with the aim of achieving a buffer solution prepared by weight from solid components that will give a highly reproducible mobile phase. This can then be used to examine in detail the differences between batches and columns of the stationary phase.

CONCLUSION

The use of a non-volatile liquid amine to prepare the buffer solution improves the reproducibility of the separation of basic drugs on a silica column. The discrimination of the separation is similar to that obtained with the original ammonia–ammonium nitrate eluent. The selectivity of the separation is susceptible to differences in the silica column material.

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REFERENCES

1 I. Jane, *J. Chromatogr.*, 111 (1975) 227.
2 B. Law, R. Gill and A. C. Moffat, *J. Chromatogr.*, 301 (1984) 165.
3 B Law, *J. Chromatogr.*, 407 (1987) 1.
4 R. W. Schmid and Ch. Wolf, *Chromatographia.*, 24 (1987) 713.
5 R. M. Smith, T. G. Hurdley, R. Gill and M. D. Osselton, *J. Chromatogr.*, 398 (1987) 73.
6 R. M. Smith, T. G. Hurdley, J. P. Westlake, R. Gill and M. D. Osselton, *J. Chromatogr.*, 455 (1988) 77.
7 R. Gill, M. D. Osselton, R. M. Smith and T. G. Hurdley, *J. Chromatogr.*, 386 (1987) 65.
8 R. Gill, M. D. Osselton, R. M. Smith and T. G. Hurdley, *J. Pharm. Biomed. Anal.*, in press.
9 R. M. Smith, T. G. Hurdley, R. Gill and A. C. Moffat, *Chromatographia.*, 19 (1984) 401.

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SUPERCRITICAL FLUID CHROMATOGRAPHY OF SESQUITERPENE HYDROCARBONS ON SILICA PACKED COLUMNS WITH ON-LINE FOURIER TRANSFORM INFRARED DETECTION

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SUMMARY

The on-line coupling of carbon dioxide supercritical fluid chromatography with Fourier transform infrared (FT-IR) spectrometry allows the separation and identification of sesquiterpene hydrocarbon mixtures on bare silica as stationary phase. Separations of these non-polar but extremely thermosensitive organic compounds required low-temperature rather than low-density experimental conditions. These chromatographic conditions preserved structural information about these sesquiterpene hydrocarbons, such as the differentiation of methyl groups (1375–1385 cm^{-1}) from methylene groups and the geminal or single nature of methyl groups (twin bands near 1360–1390 cm^{-1} or a single band near 1380 cm^{-1}). The method was applied to the identification of the sesquiterpene hydrocarbon fraction of several essential oils; important structural features provided by their FT-IR spectra are reported.

INTRODUCTION

Sesquiterpene hydrocarbons occur in detectable amounts in most essential oils. They are generally found as complex mixtures of closely related isomers which are mainly analysed by fused-silica capillary gas chromatography (GC) at high temperatures (150–200°C) and identified by gas chromatography–mass spectrometry (GC–MS)[1]. However, unequivocal identification by MS alone is sometimes difficult; compounds within the same terpenoid structure class have very similar ion fragmentation patterns and differ only in ion intensities[1]; hence GC with Fourier transform infrared (FT-IR) spectrometric detection offers a complementary technique to GC–MS analysis. Kalasinsky and co-workers[2,3] studied the analysis of monoterpenoids by GC–FT-IR on packed stainless-steels columns and concluded that it allows the positive identification of solutes. However, sesquiterpene hydrocarbons are thermosensitive organic compounds[4] and destructive chemical reactions may occur at elevated temperatures.

The increasing interest in supercritical fluid chromatography (SFC) can be at-

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tributed to the more rapid solute mass transfer than in the liquid phase and to the development in SFC of GC- and LC-type detectors[5].

A number of papers have recently appeared on the development of a combined SFC–FT–IR system with a carbon dioxide mobile phase. The two types of interfacing approaches result from the use of solvent elimination techniques and a transmission high pressure-resistant flow cell. Several workers have developed a combined SFC–FT-IR system with a transmission light-pipe flow cell; first, Shafer and Griffiths[6] reported in 1983 the result of FTIR detection in carbon dioxide SFC, Olesik *et al.*[7] in 1984 and Johnson *et al.*[8] in 1985 studied the bands due to Fermi resonance in liquid and supercritical carbon dioxide and particularly their dependence on carbon dioxide pressure[9].

Two important groups of bands are obscured by the carbon dioxide IR spectrum in the 2200–2500 and 3500–3800 cm^{-1} regions, which means that the functional groups of compounds such as alcohols [ν(OH)], amines [ν(NH)], lactams [ν(NH)], amides [ν(NH)], disubstituted alkynes [ν(C≡C)] and nitriles [ν(C≡N)], cannot be identified by their main stretching frequencies[10].

The first band is the asymmetric stretch near 2349 cm^{-1} and the second is due to combination and overtone bands. The fundamental bending vibration mode at 667 cm^{-1} is approximately 300 cm^{-1} wide and consequently rules out spectral information below 820 cm^{-1}. Unfortunately, the spectral range from 800 down to 600 cm^{-1} is important and particularly useful for the identification of *cis*-disubstituted alkenes and substitution patterns of aromatic compounds [the out-of-plane bending γ(CH) modes absorbing near 900–650 cm^{-1} indicate the substitution pattern on the benzene ring]. In addition, organic chlorinated compounds have ν(CCl) stretching frequencies near 750–700 cm^{-1}, brominated compounds show two bands in the 600–500 cm^{-1} region and alkyl iodides absorb in the general region of 500 cm^{-1}; consequently, the carbon–halogen stretching vibration cannot be detected by carbon dioxide SFC–FTIR.

Mercury–cadmium telluride (MCT) detectors differ in spectral bandwidth and broad-band detectors are generally less sensitive than narrow-band detectors. However, the IR transparency of supercritical carbon dioxide avoids the need for a compromise between sensitivity and useful spectral range. In all instances a narrow-band MCT detector with high sensitivity is chosen, but no information can be obtained at wavenumbers below 820–800 cm^{-1}. Moreover, two additional pairs of bands (Fermi resonance bands) appear in the IR spectrum of carbon dioxide in the supercritical state. The intensity and the width of the Fermi resonance bands increase on going from the supercritical to the liquid state[10].

Hence an increase in carbon dioxide density induces a reduction in the IR transparency of the mobile phase in the 2060–2072, 1350–1410 and 1275–1285 cm^{-1} regions. Consequently, some bending frequencies, such as the δ(CH) bending mode of a methyl group or geminal methyl groups, the δ(CH) bending mode of a gem-disubstituted or a monosubstituted olefinic double bond, the δ(CH) bending mode of α-methyl or α-methylene groups in a ketone and finally the δ(OH) bending vibration of an alcohol, which occur in Fermi resonance band region (1350–1410 cm^{-1}), cannot be observed by SFC–FT-IR when the carbon dioxide density is greater than 0.72 g ml^{-1} (10-mm path length cell).

Nevertheless, this direct flow cell approach in SFC–FT-IR is an interesting

technique for the separation and on-line identification of non-volatile compounds such as polycyclic aromatic hydrocarbons[11], free fatty acids[12] and phenolic compounds[13].

Other fluids have been used as the mobile phase in flow cell SFC–FTIR; Freon 23 complements the opaque regions of carbon dioxide by exhibiting transparency from 4000 to 2500 cm^{-1} (ref. 14), and xenon is transparent from the vacuum UV up to the NMR region [15,16].

An interesting and detailed review on the interfacing between SFC and FT-IR spectrometry was published by Jinno[17].

The aim of this work was to determine the retentions of sesquiterpene hydrocarbons by carbon dioxide SFC, especially at low temperatures which minimize the thermal degradation of these solutes, and to establish the capability of carbon dioxide as a supercritical mobile phase to provide solute IR spectra of sufficient quality (spectral data range, signal-to-noise ratio, useful structural information).

EXPERIMENTAL

Instrumentation

SFC–FT-IR experiments were performed on a Nicolet Model 5SX-B FT-IR spectrometer system equipped with a KBr/Ge beam splitter, a liquid nitrogen-cooled MCT-A photodetector ($D = 4.3 \cdot 10^{10}$ cm $Hz^{1/2}$ W^{-1}) with a narrow bandwidth 5000–750 cm^{-1}. This spectrometer was used to collect time-resolved 8 cm^{-1} resolution spectra with a mirror velocity of 2.221 cms^{-1} and a data collection rate of 1.2 spectra per second. All experiments were made employing the standard GC software provided by the manufacturer. A 4X beam condenser (Barnes Analytical, Stramford, U.S.A.) was used to reduce the 4-mm diameter focused beam to an approximate spot diameter of 1 mm.

A Varian Model 5500 liquid chromatograph was modified for SFC[10]. To improve the pump efficiency, the carbon dioxide, kept in a container with an eductor tube, was passed through an ethylene glycol–water cooling mixture which was circulated through a jacket surrounding the pump head. Sample injection was made via a Rheodyne Model 7010 injector with a 10-μl loop. A series of thermocouples (N 225; Thermocoax, Suresnes, France) were used to check the carbon dioxide temperature within the pumping system, the heat exchanger and the outlet of the flow cell. The pressure was controlled by a TESCOM manually adjustable back-pressure regulator (Model 26-3220-24004; GEC Composants, Asnières, France) situated after the FT-IR detector.

The IR flow cell had dimensions of 1 mm I.D. × 10 mm pathlength (8-μl volume) and was designed to maintain a 3500 p.s.i. pressure gradient across the windows by using 2 mm thick ZnSe discs of 13 mm diameter.

A 250 × 4.6 mm I.D. Nucleosil 100 or Spherisorb silica (5-μm particle diameter) packed column was used for the study of sesquiterpene hydrocarbon SFC retention. A constant-temperature water-bath provided temperature control for the column. The capacity factors were calculated by SFC–FT-IR from the retention time of the analyte (t_R) and the retention time of the void volume peak (t_0) using *n*-pentane as an unretained compound.

Carbon dioxide (N 45, 99.995% purity) (Alpha Gaz, Bois d'Arcy, France) was employed.

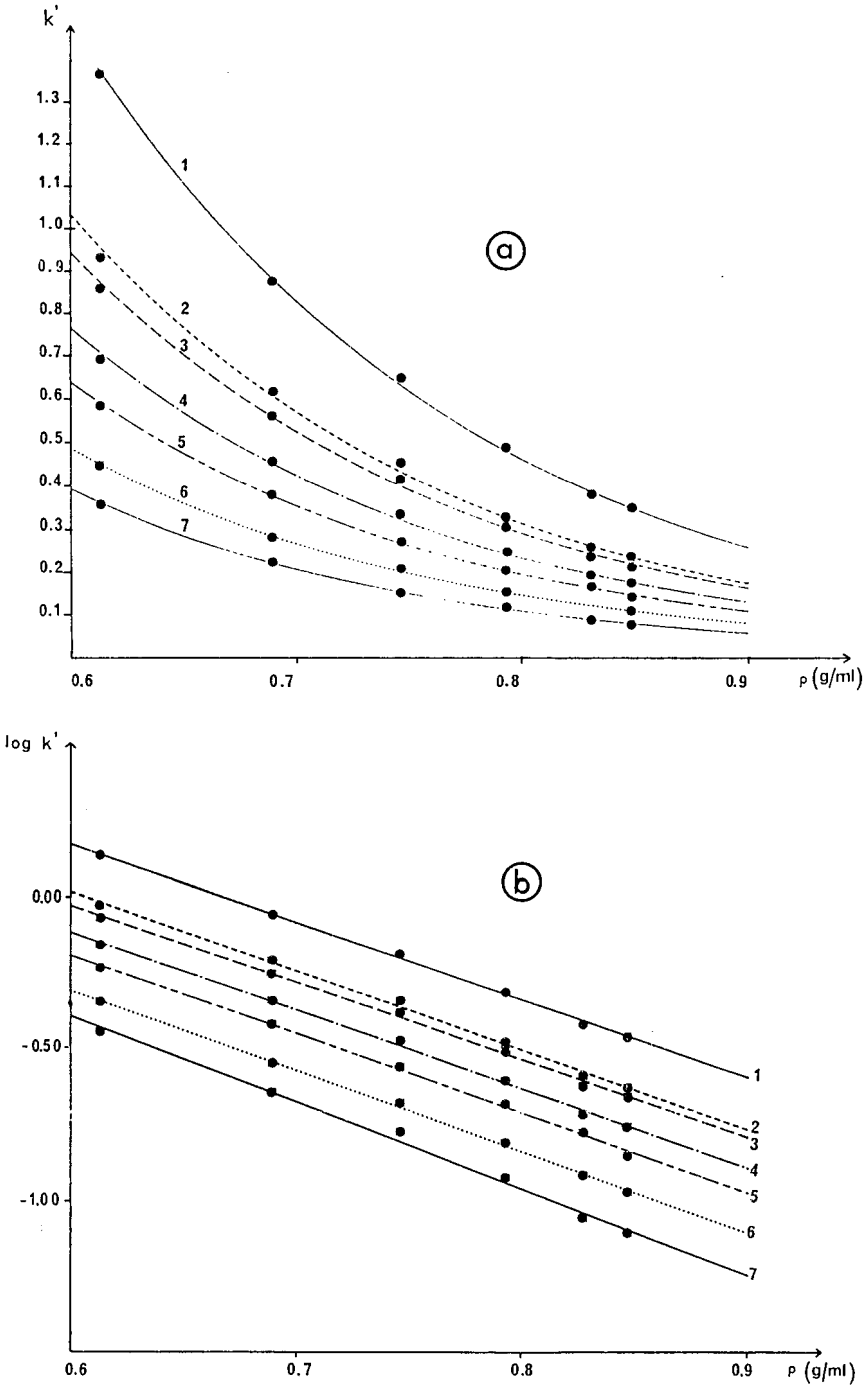

Fig. 1. (a) Influence of supercritical carbon dioxide density on the capacity factors (k') of sesquiterpene hydrocarbons on bare silica. (b) Plots of log k' *versus* carbon dioxide density. Column, 250 × 4.6 mm I.D.; stationary phase, Nucleosil 100 (5 μm); temperature, 40°C; flow-rate, 2 ml min^{-1}; FT-IR detection. Solutes: 1 = humulene; 2 = *trans*-calamenene; 3 = valencene; 4 = ledene; 5 = aromadendrene; 6 = longifolene; 7 = longicyclene.

Sesquiterpene hydrocarbon solutes

γ-Gurjunene, longipinene, thujopsene, longicyclene, aromadendrene and ledene were purchased from Fluka (Buchs, Switzerland), humulene and valencene from Sarsynthex (Merignac, France) and longifolene from Aldrich (Milwaukee, WI, U.S.A.). *trans*-Calamenene, *cis*-calamenene and β-farnesene were from our own collection.

Semi-preparative liquid chromatography

The prefractionation of an essential oil was carried out by semi-preparative liquid chromatography[18]. Reversed-phase chromatography on octadecyl-bonded silica was used and the separation of the essential oil was achieved at ambient temperature, giving four fractions (oxygenated monoterpenes, oxygenated sesquiterpenes, monoterpene hydrocarbons and sesquiterpene hydrocarbons). Finally, the sesquiterpene hydrocarbon fraction was analysed by carbon dioxide SFC–FT-IR.

RESULTS AND DISCUSSION

Chromatographic study

A preliminary study with a synthetic mixture containing sesquiterpene hydrocarbons was carried out to determine their retention behaviour on bare silica with supercritical carbon dioxide. Capacity factors of several sesquiterpene hydrocarbons are reported on Nucleosil 100 silica *versus* carbon dioxide density (Fig. 1a). The experimental variations confirmed the general rule according to which an increase in carbon dioxide density results in enhanced solute solubility and consequently a decrease in retention; on this stationary phase, the capacity factors of these apolar solutes are generally smaller than 2 within the carbon dioxide density range 0.6–0.9 g ml^{-1}. The dependence of the capacity factors on supercritical carbon dioxide density was determined by numerical analysis and confirmed that a logarithmic equation best fits the experimental retention data (Fig. 1b). Hence for each solute at a given temperature:

$$\log k' = a + b\rho \quad (1)$$

where k' = capacity factor and ρ = carbon dioxide density (g ml^{-1}). The numerial constants a and b were determined by linear regression analysis with correlation coefficients greater than 0.998 (Table I).

TABLE I

COEFFICIENTS IN EQN. 1 OBTAINED FROM LINEAR REGRESSION ANALYSIS OF EXPERIMENTAL RETENTION DATA ON SILICA

Solute	*a*	*b*	$\sigma(a)^a$	$\sigma(b)^a$	r^b
Humulene	1.69	−2.54	0.03	0.04	0.999
trans-Calamenene	1.57	−2.59	0.05	0.07	0.998
Valencene	1.51	−2.56	0.04	0.05	0.999
Ledene	1.43	−2.58	0.04	0.06	0.999
Aromadendrene	1.36	−2.58	0.05	0.06	0.998
Longifolene	1.26	−2.62	0.04	0.06	0.999
Longicyclene	1.28	−2.81	0.05	0.06	0.999

[a] Average deviation of experimental data points from the regression curve.
[b] Correlation coefficient.

FT-IR detection

IR window absorbance reconstruction. The power of FT-IR detection results from the ability to reconstruct SFC–FT-IR chromatograms from single-scan interferometric data by various methods. During an SFC–FT-IR separation several hundred interferograms can be collected. However, such a collection of single-scan interferograms gives no indication of which should be transformed in order to obtain the effluent IR spectra. A method is needed to select which interferograms should be transformed and which contain only background information. Two methods are

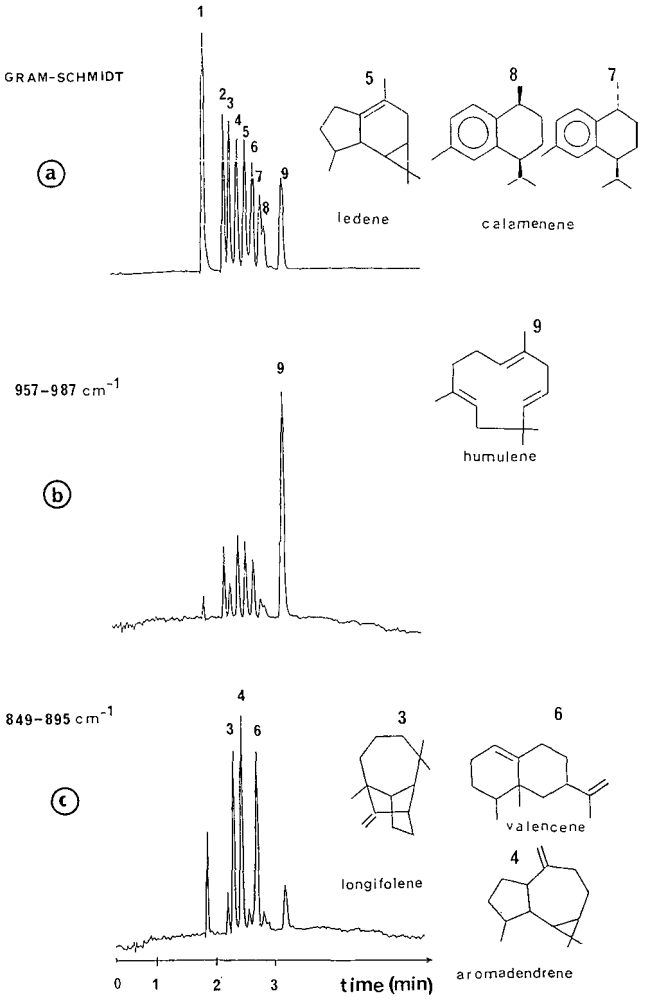

Fig. 2. Gram–Schmidt and spectral window chromatograms of a mixture of eight sesquiterpene hydrocarbons. Two columns in series (250 × 4.6 mm I.D.); stationary phases, Nucleosil 100 and Spherisorb (5 μm); column temperature, 40°C; average pressure, 130 atm; flow-rate, 4 ml min^{-1}. Solutes: 1 = hexane; 2 = longicyclene; 3 = longifolene; 4 = aromadendrene; 5 = ledene; 6 = valencene; 7 = *trans*-calamenene; 8 = *cis*-calamenene; 9 = humulene. (a) Gram–Schmidt total IR chromatogram; (b) IR chromatogram, 957–989 cm^{-1}; (c) IR chromatogram, 849–895 cm^{-1}.

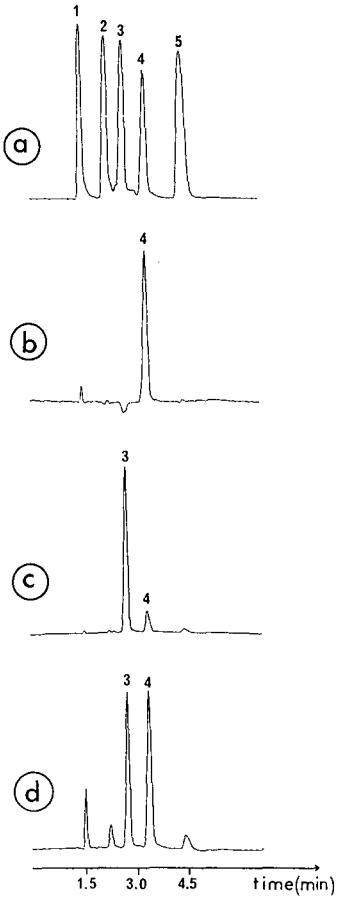

Fig. 3. Gram–Schmidt and spectral window chromatograms of a mixture of four sesquiterpene hydrocarbons. Stationary phase, Nucleosil 100 (5 μm); column temperature, 40°C; average pressure, 107 atm; flow-rate, 2 ml min^{-1}. Solutes: 1 = hexane; 2 = longicyclene; 3 = aromadendrene; 4 = β-farnesene; 5 = humulene. (a) Gram–Schmidt total IR chromatogram; (b) IR chromatogram, 1589–1604 cm^{-1}; (c) IR chromatogram, 1635 cm^{-1}; (d) IR chromatogram, 3086–3101 cm^{-1}.

often used from constructing chromatograms from interferometric data: the first is the Gram–Schmidt orthogonalization procedure[19], which works directly from the interferograms, and the second involves fast Fourier transformation of a 1024-point section of each interferogram to obtain a low-resolution (16 wavenumbers) IR absorbance spectrum which can be integrated to determine a chromatogram detector response[20].

In order to compare these two methods, a standard mixture of eight sesquiterpene hydrocarbons, dissolved in hexane, was separated on a silica column in less than 4 min; 20 μg of each component were injected. Our work involves the use of a 4.6 mm I.D. packed column which has fairly high sample capacities (of the order of 100 μg per component). The Gram–Schmidt reconstruction chromatogram is identical in form with a chromatogram obtained by traditional chromatographic detectors and

shows good resolution, except for compounds 7 and 8 (Fig. 2a); *cis*- and *trans*-calamenene differ only in the position of the methyl and isopropyl substituents on the cyclohexane ring. However, these two stereoisomers can be identified by their supercritical carbon dioxide FT-IR spectra, particularly in the 1400–1000 cm^{-1} region.

A different possibility for calculating IR chromatograms is the construction of IR group-specific or IR window chromatograms. A number of subspectral integration windows can be chosen to monitor specific functional groups simultaneously.

IR absorptions due to bending vibrations of olefinic CH groups are more intense than those caused by C=C stretching vibrations, so they can be used to determine the nature of substituent groups. Fig. 2b and c show chromatograms obtained with an IR window specific to the out-of-plane δ(CH) bending modes of olefinic groups. In the 975–987 cm^{-1} region, the trace is closely related to a *trans* substitution for a disubstituted alkene (humulene) and in the 849–895 cm^{-1} region to a *gem*-disubstituted olefinic group (longifolene, aromadendrene and valencene).

Fig. 3 shows the results of an SFC–FT-IR run on a mixture of longicyclene, aromadendrene, β-farnesene and humulene dissolved in hexane; the trace at the top is the Gram–Schmidt total IR chromatogram. The IR window chromatogram at 1589–1604 cm^{-1} specific to the ν(C=C) stretching vibration for conjugated alkenes, exhibits only one peak for β-farnesene. The 1635 and 3086–3101 cm^{-1} window chromatograms are specific to ν(C=C) and ν(CH) stretching vibrations, respectively, for gem-disubstituted olefinic groups (β-farnesene, aromadendrene).

SFC–FT-IR spectra of sesquiterpene hydrocarbons. Whereas the mass spectra of sesquiterpene hydrocarbons are often similar, their infrared spectra are sufficiently different for positive identification, particularly for *cis–trans* and aromatic positional isomers. Sesquiterpene hydrocarbons can be identified in an SFC–FT-IR separation by their main stretching frequencies (Table II).

The 1375–1385 cm^{-1} band in sesquiterpene hydrocarbons reveals the presence of a methyl group and allows its differentiation from a methylene group. Moreover,

TABLE II

INFRARED ABSORPTIONS OF FUNCTIONAL GROUPS PRESENT IN SESQUITERPENE HYDROCARBONS

Vibrational mode	*Stretching ν(CH)*	*Double bond stretching ν(C=C)*	*In-plane bending δ(CH)*	*Out-of-plane bending γ(CH)*
Alkene				
Monosubstituted (vinyl)	3040–3010 and 3095–3075	1660–1640	1420–1410 and 1300–1290	995–985 and 915–900
Disubstituted, *gem*	3095–3075	1655–1650	1420–1410	895–885
Disubstituted, *trans*	3040–3010	1675–1670	1310–1290	980–965
Disubstituted, *cis*	3040–3010	1660–1655	–	715–665
Trisubstituted	3040–3010	1670	–	840–790
Alkane				
CH_2	2925–2850	–	1485–1445	–
CH_3	2960–2870	–	1470–1430 and 1380–1370	–
gem-Dimethyl	–	–	1385–1380 and 1370–1365	–

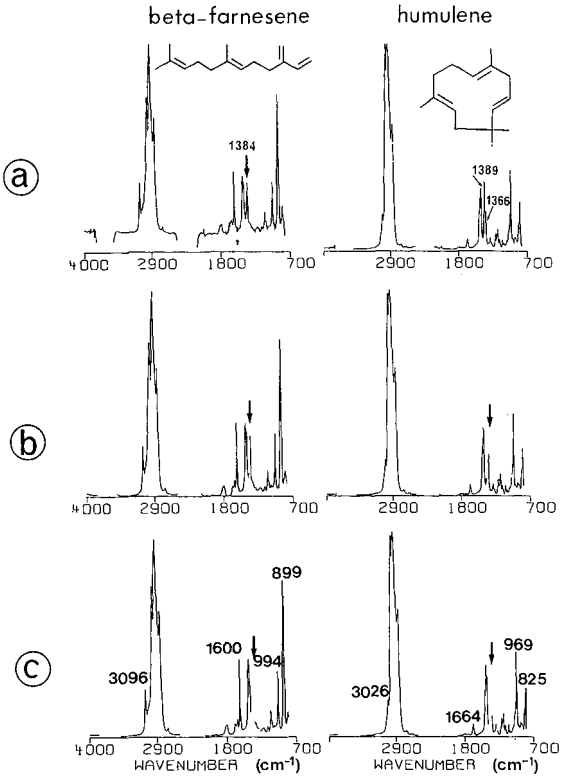

Fig. 4. Influence of carbon dioxide density on carbon dioxide FT-IR spectra of β-farnesene and humulene. Optical resolution, 8 cm^{-1}. Density: (a) 0.45; (b) 0.74; (c) 0.92 g ml^{-1}.

the main information obtained in the Fermi band region is relative to geminal methyl groups (twin bands near 1360–1390 cm^{-1}). Finally, the in-plane δ(CH) bending mode of a *gem*-disubstituted or monosubsituted olefinic group absorbs in the 1410–1420 cm^{-1} region.

With increasing carbon dioxide density, the Fermi doublet in the carbon dioxide spectrum absorbs all the IR radiation so none reaches the MCT detector. As soon as the carbon dioxide density is greater than about 0.74 g ml^{-1} with a 10-mm pathlength flow cell[11], the presence of a single methyl group (single band at 1384 cm^{-1} in the β-farnesene spectrum, Fig. 4) or a *gem*-dimethyl group (twin bands at 1366 and 1389 cm^{-1} in the humulene spectrum, Fig. 4) is not revealed by their IR spectrum. In spite of these restrictions, considerable structural information is given by a carbon dioxide FT-IR spectrum, as follows. For β-farnesene, several IR bands indicate the presence of a vinyl group (3096, 994, 899 cm^{-1}), a methylene geminal group (899 cm^{-1}) and a conjugated olefinic system (1600 cm^{-1}). For humulene, several IR bands prove the presence of a *trans*-disubstituted alkene (3026, 1664, 969 cm^{-1}) and of trisubstituted double bonds (825 cm^{-1}).

In a carbon dioxide FT-IR spectrum of a sesquiterpene hydrocarbon, the substituted nature of a C=C double bond is always detected whatever the carbon dioxide density; indeed, the main stretching [ν(C=C), olefinic ν(CH) and bending (out-

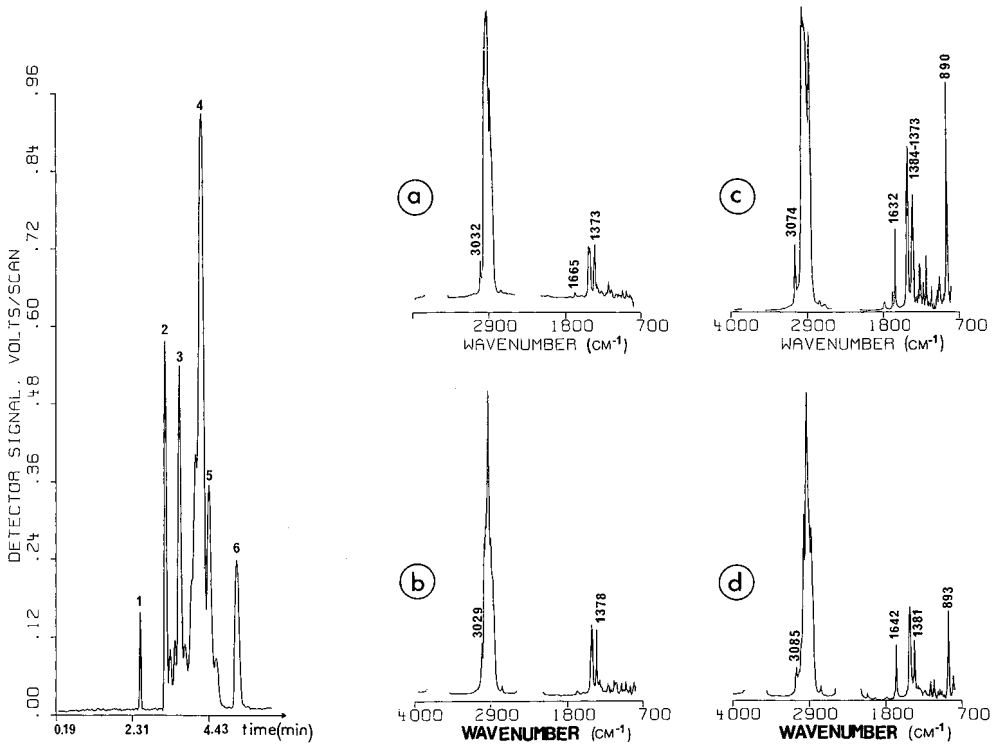

Fig. 5. SFC–FT-IR separation of sesquiterpene hydrocarbons from Copaiba balsam oil. Two columns in series (250 × 4.6 mm I.D.); stationary phases, Nucleosil 100 and Spherisorb (5 μm); average pressure, 140 atm; flow-rate, 4 ml min^{-1}; column temperature, 40°C. Solutes: 1 = hexane (solvent); 2 = α-copaene; 3 = *trans*-α-bergamotene; 4 = β-caryophyllene; 5 = β-bisabolene; 6 = humulene.

Fig. 6. Carbon dioxide FT-IR spectra of sesquiterpene hydrocarbons. Cell pressure, 92 atm; cell temperature, 33°C, carbon dioxide density, 0.71 g ml^{-1}; optical resolution, 8 cm^{-1}. Solutes: (a) α-copaene; (b) *trans*-α-bergamotene; (c) β-caryophyllene; (d) β-bisabolene.

of-plane δ(CH)] vibrational modes absorb in the carbon dioxide transparency domain.

Analysis of sesquiterpene hydrocarbons in essential oils

The sesquiterpene hydrocarbon fraction was isolated at room temperature from essential oils by semi-preparative liquid chromatography on octadecyl-bonded silica as described previously[18]. Prefractionation by liquid chromatography and SFC–FT-IR analysis are well adapted to thermosensitive compounds such as terpenoids and experiments can be made at ambient temperature.

The characterization of the products are carried out by interpretation of their carbon dioxide FT-IR spectra and comparison with those of authentic samples or those published in the literature[21,22].

Copaiba balsam oil. The SFC–FT-IR analysis of the sesquiterpene hydrocarbon fraction of this essential oil[23] was carried out using silica as the stationary phase in less than 5 min with the following chromatographic conditions: inlet pressure, 186 atm; outlet pressure, 92 atm; flow-rate, 4 ml min^{-1}; and column temperature, 40°C.

The Gram–Schmidt reconstruction chromatogram corresponding to the separation is shown in Fig. 5. Several sesquiterpene hydrocarbons (α-copaene, α-*trans*-bergamotene, β-caryophyllene, β-bisabolene, humulene) were identified by comparison of their spectra with those published by Wenninger *et al.*[21,22].

However a change in physical parameters (pressure, temperature) modifies the carbon dioxide mobile phase polarity and consequently alters the IR absorption bands. Stretching vibrations are more disturbed than bending vibrations by variations in carbon dioxide. Frequency shifts are relatively moderate for apolar functional groups such as CH or C=C, but noteworthy with regard to the precision of spectral data measurements; they become more significant for polar functional groups, such as carbonyl or sulphone, where they can reach up to 10 wavenumbers[24].

Fig. 6 shows FT-IR spectra of compounds relative to peaks 2 and 3; the absorption bands centered respectively at 3032, 1665 cm^{-1} for the former and at 3029, 1661 cm^{-1} for the latter indicate the presence of a trisubstituted olefinic group. However, the MCT detector cut-off, near 815 cm^{-1}, prevents us from detecting the out-of-plane γ(CH) bending vibration of this unsaturated functional group in the 780–800 cm^{-1} region. The FT-IR spectrum of the fourth peak matches that of β-caryophyllene; the absorptions centred at 3074, 1632 and especially 890 cm^{-1} relate to a *gem*-disubsti-

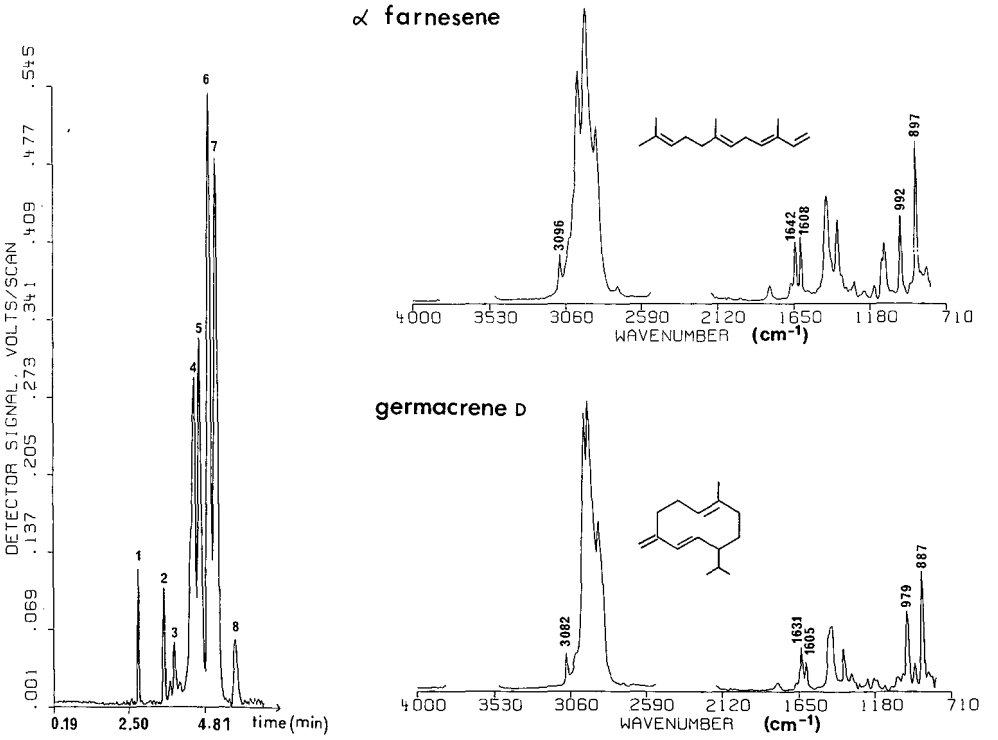

Fig. 7. SFC–FT-IR separation of sesquiterpene hydrocarbons from ylang-ylang oil. Conditions as in Fig. 5. Solutes: 1 = hexane; 2 = α-copaene; 3,4 = unknown; 5 = β-caryophyllene; 6 = α-farnescene; 7 = germacrene D; 8 = unknown.

Fig. 8. Carbon dioxide FT-IR spectra of α-farnesene and germacrene D. Cell pressure, 92 atm; cell temperature, 33°C; carbon dioxide, 0.71 g ml^{-1}; optical resolution, 8 cm^{-1}.

tuted olefinic group, and the twin bands at 1373 and 1384 cm^{-1} to a *gem*-dimethyl group. Finally, the FT-IR spectrum of the fifth peak suggests the presence of a gem-disubstituted olefinic group (3085, 1642, 893 cm^{-1}) and of a trisubstituted olefinic group (3050, 3012, 1674, 830 cm^{-1}), and agrees with that of β-bisabolene.

Ylang-ylang oil. The sesquiterpene hydrocarbon fraction of ylang-ylang 3th[25] was separated by SFC–FT-IR on a silica stationary phase (Fig. 7) and further identified by comparison of the FT-IR spectra with those published in the literature. The presence of β-caryophyllene, α-copaene, germacrene D and α-farnesene was confirmed.

The relevant features of the FT-IR spectrum of the sixth elution peak (Fig. 8a) are absorptions at 3096, 992 and 897 cm^{-1}, indicative of a vinyl group, and two absorption bands at 1642 and 1608 cm^{-1} which indicate the presence of a conjugated diene. Also bands at 1667 and 834 cm^{-1} may be assigned to a trisubstituted double bond. These features agree with those of α-farnesene[26–28].

The FT-IR spectrum of the seventh elution peak (Fig. 8b) suggests the presence of a gem-disubstituted olefinic group (3082, 887 cm^{-1}), a *trans*-double bond (979 cm^{-1}), the presence of a conjugated diene (1631, 1605 cm^{-1}) and a *gem*-dimethyl group (twin bands around 1373 cm^{-1}). The results are in good agreement with those obtained with germacrene D. This sesquiterpene hydrocarbon has been observed to photoisomerize to α- and β-bourbonene or to transform thermally in a GC column or on contact with silica gel into several sesquiterpene hydrocarbons[29], so the different steps of essential oil analysis (prefractionation by LC, separation by carbon dioxide SFC) always achieved at room temperature allow the identification of this extremely thermally labile compound.

CONCLUSION

At nearly ambient temperatures, sesquiterpene hydrocarbons are conveniently resolved by carbon dioxide SFC–FT-IR on bare silica as stationary phase. The IR transparency domain of supercritical carbon dioxide, *i.e.*, 3500–2500 and 2200–800 cm^{-1}, and the chromatographic parameters applied to resolve a mixture of sesquiterpene hydrocarbons allow the successful identification of all IR vibration modes due to functional groups present in sesquiterpene hydrocarbons. Structural information relative to the nature of the C=C double bond will always be provided by the out-of-plane CH bending vibrations. It is important to emphasize the low temperature required for this separation rather than the low-density mobile phase.

REFERENCES

1 M. G. Moshonas and E. D. Lund, *The Flavour Industry.*, 1970, p. 375.
2 V. F. Kalasinsky and J. T. McDonald, *J. Chromatogr. Sci.*, 21 (1983) 193.
3 V. F. Kalasinsky, S. Pechsiri and K. S. Kalasinsky, *J. Chromatogr. Sci.*, 24 (1986) 543.
4 M. I. Garnero, *Parfums Cosmet. Arom.*, 14 (1977) 31.
5 M. Novotny, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 137.
6 K. H. Shafer and P. R. Griffiths, *Anal. Chem.*, 55 (1983) 1939.
7 S. V. Olesik, S. B. French and M. Novotny, *Chromatographia*, 18 (1984) 489.
8 C. C. Johnson, J. W. Jordan, L. T. Taylor and D. W. Vidrine, *Chromatographia*, 20 (1985) 717.
9 M. E. Hughes and J. L. Fasching, *J. Chromatogr. Sci.*, 24 (1985) 535.
10 Ph. Morin, M. Caude, M. Richard and R. Rosset, *Chromatographia*, 21 (1986) 523.

11 Ph. Morin, M. Caude, M. Richard and R. Rosset, *Analusis*, 15 (1987) 117.
12 J. W. Hellgeth, J. W. Jordan, L. T. Taylor and M. Ashraf Khorassani, *J. Chromatogr. Sci.*, 24 (1986) 183.
13 Ph. Morin, M. Caude and R. Rosset, *J. Chromatogr.*, 407 (1987) 87.
14 J. W. Jordan and L. T. Taylor, *J. Chromatogr. Sci.*, 24 (1986) 82.
15 S. B. French and M. Novotny, *Anal. Chem.*, 58 (1986) 164.
16 H. Pichard, M. Caude and R. Rosset, *Analusis*, in press.
17 K. Jinno, *Chromatographia*, 23 (1987) 55.
18 Ph. Morin, M. Caude, H. Richard and R. Rosset, *J. Chromatogr.*, 363 (1986) 57.
19 J. A. de Haseth and T. L. Isenhour, *Anal. Chem.*, 49 (1977) 1977.
20 H. A. Hanna, G. Hangac, B. A. Hohne, G. W. Small, R. C. Wieboldt and T. L. Isenhour, *J. Chromatogr. Sci.*, 17 (1979) 423.
21 J. A. Wenninger, R. L. Yates and M. J. Dolinsky, *J. Assoc. Off. Anal. Chem.*, 50 (1967) 1313.
22 J. A. Wenninger, R. L. Yates and M. J. Dolinsky, *J. Assoc. Off. Anal. Chem.*, 53 (1970) 949.
23 J. A. Wenninger, R. L. Yates and M. J. Dolinsky, *J. Assoc. Off. Anal. Chem.*, 50 (1967) 1304.
24 Ph. Morin, B. Beccard, M. Caude and R. Rosset, *J. High Resolut. Chromatogr. Chromatogr. Commun*, 10 (1988) 697.
25 P. Teisseire and A. Galfre, *Recherches*, 19 (1974) 269.
26 K. E. Murray, *Aust. J. Chem.*, 210 (1969) 1260.
27 F. E. Huelin and K. E. Murray, *Nature (London)*, 210 (1966) 197.
28 G. W. K. Cavill, P. J. Williams and F. B. Whitfield, *Tetrahedron Lett.*, 23 (1967) 2201.
29 K. Yoshihara, Y. Ohta, T. Sakai and Y. Hirose, *Tetrahedron Lett.*, (1969) 2263.

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USE OF CYCLODEXTRINS IN ISOTACHOPHORESIS

VII. RESOLUTION OF STRUCTURALLY RELATED AND CHIRAL PHENOTHIAZINES

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(First received September 12th, 1988; revised manuscript received November 4th, 1988)

SUMMARY

Commercially available phenothiazine derivatives were used for the study of cyclodextrin complex formation by cationic isotachophoresis with α-, β- and γ-cyclodextrin and methylated analogues of β-cyclodextrin as leading electrolyte additives. The relationships between the type of solute substituent in the 10- and/or 2-position and the stability of the created cyclodextrin complex were studied and the results were utilized for the optimization of isotachophoretic conditions suitable for the resolution of the studied phenothiazine derivatives. Successful resolution of three racemic solutes was achieved.

INTRODUCTION

Phenothiazine and its derivatives are an important group of neuroleptic and anti-allergic drugs. More than 100 compounds derived from the fundamental phenothiazine skeleton have been synthesized and pharmacologically tested during the past few decades[1]. The most important from the clinical point of view proved to be derivatives substituted in the 10- and/or 2-position.

From the analytical point of view, commercially produced phenothiazine derivatives (approximately 50 registered drugs) represent a readily available set of structurally related compounds that could be utilized as model solutes for the study of various separation mechanisms and for testing the capabilities of proposed analytical systems to give a precise correlation between structure and retention data.

The favourable mass-to-charge ratio and suitable molecular structure of most phenothiazine derivatives substituted in the 10-position make it possible to utilize

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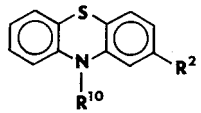
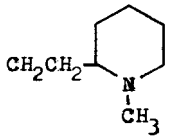
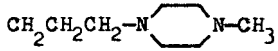
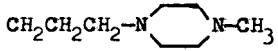
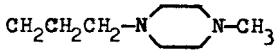
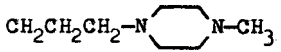
TABLE I

STRUCTURAL FORMULAE OF THE COMPOUNDS INVESTIGATED



Compound	R^2	R^{10}	*Name*
1 [(+)- and (−)-]	SCH_3		Thioridazine = 10-[2-(1-methyl-2-piperidyl)ethyl]-2-methylthiophenothiazine
2 [(+)- and (−)-]	H	$CH_2CH(CH_3)N(CH_3)_2$	Promethazine = 10-[2-(dimethylamino)propyl]phenothiazine
3 [(+)- and (−)-]	CN	$CH_2CH(CH_3)CH_2N(CH_3)_2$	Cyamepromazine = 10-[3-(dimethylamino)-2-methylpropyl]-phenothiazine-2-carbonitrile
4	H	$CH_2CH_2CH_2N(CH_3)_2$	Promazine = 10-[3-(dimethylamino)propyl]phenothiazine
5	Cl	$CH_2CH_2CH_2N(CH_3)_2$	Chlorpromazine = 2-chloro-10-[3-(dimethylamino)propyl]-phenothiazine
6	Cl		Prochlorperazine = 2-chloro-10-[3-(4-methyl-1-piperazinyl)propyl]phenothiazine
7	CF_3		Trifluoperazine = 10-[3-(4-methyl-1-piperazinyl)propyl]-2-trifluoromethylphenothiazine
8	SCH_2CH_3		Thiethylperazine = 2-ethylthio-10-[3-(4-methyl-1-piperazinyl)propyl]phenothiazine
9	$SO_2N(CH_3)_2$		Thioproperazine = N,N-dimethyl-10-[3-(4-methyl-1-piperazinyl)propyl]phenothiazine-2-sulfonamide
10	Cl		Perphenazine = 4-[3-(2-chlorophenothiazin-10-yl)propyl]-1-piperazineethanol
11	CF_3		Fluphenazine = 4-[3-(2-trifluoromethylphenothiazin-10-yl)propyl]-1-piperazineethanol

them as a model compounds for the study of cyclodextrin (CD) inclusion complex formation using cationic isotachophoresis (ITP). Previous experiments showed that cyclodextrins, behaving as specific selectors, may substantially improve the separation of structurally related compounds and various types of isomers[2–7]. The aim of this work was to study the interactions of cyclodextrins with structurally related phenothiazine derivatives and to propose ITP conditions suitable for their effective resolution. Attempts have been made to obtain relationships between the type of solute substituent in the 10- and/or 2-position and the stability of the CD complex formed. Particular attention has been paid to the optimization of ITP conditions suitable for the resolution of three racemic solutes.

EXPERIMENTAL

Chemicals

Redistilled water was used in the preparation of the solutions of the electrolytes and compounds investigated. All chemicals were of the highest quality commercially available: sodium hydroxide, 4-morpholinoethanesulphonic acid (MES), β-alanine (β-ALA), 37% hydrochloric acid, acetic acid, sodium acetate, L(+)-tartaric acid (Merck, Darmstadt, F.R.G.); 6-aminocaproic acid (EACA) (Sigma, St.Louis, MO, U.S.A.); Natrosol 250 HR (hydroxyethylcellulose; HEC) (Hercules, Wilmington, U.S.A.); Zerolit DM-F (indicator) (BHD, Poole, U.K.); α- and γ-cyclodextrins (α- and γ-CD) (Astec, Whippany, U.S.A.); β-cyclodextrin (β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (diMe-β-CD) and heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (triMe-β-CD) (Chinoin, Budapest, Hungary). Natrosol 250 HR solutions were purified by using Zerolit DM-F mixed-bed ion-exchange resin.

The solutes investigated (Table II) were provided by laboratories of the State Institute for the Control of Drugs (Prague, Czechoslovakia). Sample solutions (2 mg/ml) were prepared by dissolving each substance in 50 m*M* hydrochloric acid and were stored in dark bottles in a refrigerator.

Methods

Isotachophoretic experiments were performed using a Tachopor 2127 instrument (LKB, Bromma, Sweden), equipped with a conductivity detector and a poly(tetrafluoroethylene) capillary. The sample solutions were injected with a 10-μl microsyringe (Hamilton, Bonaduz, Switzerland). The operating conditions are given in Table II.

The optical rotation of optically enriched mixtures of enantiomeric phenothiazines (compounds **1**, **2** and **3**) were measured with a Model 241 polarimeter (Perkin-Elmer, Norwalk, CT, U.S.A.).

The ITP separation pattern of the enantiomers was determined indirectly by comparison of ITP and optical rotation measurements for optically enriched samples (obtained by selective precipitation of racemates with γ-CD (compound **1**) or with L(+)-tartaric acid (compounds **2** and **3**).

RESULTS AND DISCUSSION

The preliminary ITP experiments, aimed at selecting suitable ITP conditions,

showed that the optimum pH of the leading electrolyte (LE), ensuring sufficient ionization and optimum ITP resolution of the solutes in the form of uncomplexed ions (unmodified LE) or inclusion complexes (CD-modified LE), lies between 5.2 and 5.7. Two electrolyte systems with MES or acetate counterions were chosen for the following experiments.

To test the influence of the types of CDs used on the effective mobility and the possible resolution of the compounds investigated, the relative step heights, $(h_i)_{rel} = h_S - h_L / h_T - h_L$ [h_S, h_L and h_T = step heights of the sample, LE and terminating electrolyte (TE), respectively] were measured.

The effectiveness of the isotachophoretic resolution was determined on the basis of $(h_i)_{rel}$ differences $[\Delta(h_i)_{rel}]$ of the solutes. According to the given experimental conditions determining the accuracy and reproducibility of the measurements, $(h_i)_{rel}$ differences lower than 0.01 were considered to be negligible and the solute pairs involved to be unresoluted. A value of 0.02 was specified as the lowest $\Delta(h_i)_{rel}$ limit in order to achieve effective and practically significant solute resolution. Intermediate $\Delta(h_i)_{rel}$ values in the range 0.01–0.02 are characteristic of solute pairs that could hardly be separated under given ITP conditions. A low resolution, resulting from closely similar effective mobilities of the solutes, requires the use of long capillary in order to obtain a sufficient maximum load capacity[8] acceptable in practice.

All important data obtained from the experiments with single solutes are summarized in Table III. For a clearer understanding of the experimental data and an easier description of the role of CDs in structural differentation, the compounds studied were divided into several subsets in which only one structural parameter of the molecule was changed. With such an approach, the solutes (**6**, **7**, **8**, **9**), (**4**, **5**) and **10**, **11**) can be included in subset A (with a common alicyclic or heterocyclic-containing 10-substituent but varying 2-substituent). Analogously, the solutes (**5**, **6**, **10**) and (**7**, **11**) form subset B (with a common 2-substituent but varying 10-substituent). The

TABLE II

ELECTROLYTE SYSTEMS AND CONDITIONS FOR ITP

Parameter	*Conditions*
Leading electrolytes:	
LE I	10 m*M* sodium hydroxide including 0.04% HEC adjusted with MES to pH 5.57
LE II	10 m*M* sodium acetate including 0.04% HEC adjusted with acetic acid to pH 5.55
Terminating electrolytes:	
TE I	10 m*M* EACA
TE II	10 m*M* β-Ala
Capillaries:	
I	250 mm × 0.55 mm I.D.
II	520 mm × 0.55 mm I.D.
Current	For capillary I, LE I and II and TE I: 150 μA (5 min), 50 μA for detection For capillary II, LE II and TE II: 100 μA (15 min), 50 μA for detection
Thermostat temperature	18°C
Detection	Conductivity

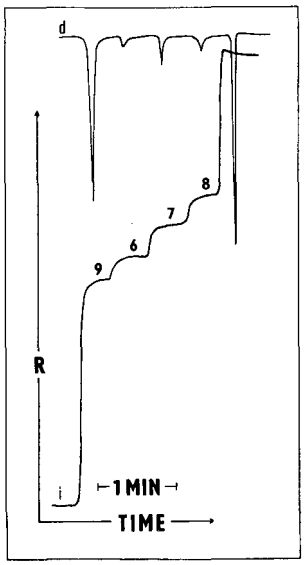


Fig. 1. ITP separation of a mixture of compounds **6–9**. Electrolyte system: LE I with 10 m*M* triMe-β-CD and TE I; capillary I. Injected volume: 2 μl (concentration of each compound = 0.5 mg/ml). *R* = detector response; *i* = conductivity signal; *d* = differentiated conductivity signal.

most interesting from the analytical point of view is probably subset C, containing racemic solutes (**1**, **2**, **3**).

ITP resolution of subset A solutes.

The $(h_i)_{rel}$ difference of solutes **6** and **9** with the unmodified electrolyte system LE I, TE I is not significant and the system does not resolve them. Solute **8** is not able to form a stable zone owing to its low solubility under the given ITP conditions.

The addition of α-CD to LE I significantly improves the solubility of solute **8** and makes it possible to differentiate **8** and **9**, with bulky $-SC_2H_5$ and $-SO_2N(CH_3)_2$ substituents, from 6 and 7, with small Cl and CF_3 groups in the 2-position. Increasing the α-CD concentration results in a significantly stronger retardation of solutes 6 and 7. It can be assumed that the mentioned bulky substituents protect one of the aromatic rings of phenothiazine against complexation with the relatively small α-CD cavity whereas Cl and CF_3 substituents are still dimensionally acceptable for complexation.

The $(h_i)_{rel}$ differentiation of solutes **6**, **7**, **8** and **9** is not achieved with β-CD- and diMe-β-CD-modified LE I, even though the retardation effects are much more significant than with α-CD. This result does not conflict with the mentioned concept of blockage of α-CD binding positions by the phenothiazine 2-substituent. An increase in the diameter of the CD cavity (α-CD < diMe-β-CD < β-CD) makes the differences in the dimensions of the 2-substituents not so critical for inclusion complex formation.

The measurements with γ-CD-modified LE I demonstrated the possibility of the resolution of solute **6**, which is less retarded from the others. Such a reappearance

TABLE III

$(h_i)_{rel}$ VALUES OF COMPOUNDS INVESTIGATED

Values measured in capillary I.

Conditions	*Compound*										
	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***
LE I, TE I	0.300	0.284	0.261	0.279	0.286	0.291	0.325	–	0.292	0.357	0.365
LE I, TE I with 5 m*M* α-CD	0.365	0.309	0.299	0.311	0.344	0.400	0.403	0.345	0.353	0.434	0.436
LE I, TE I with 10 m*M* α-CD	0.439	0.347	0.354	0.356	0.410	0.456	0.453	0.370	0.387	0.498	0.460
LE I, TE I with 20 m*M* α-CD	0.517	0.400	0.417	0.419	0.496	0.549	0.528	0.443	0.450	0.588	0.570
LE I, TE I with 5 m*M* β-CD	0.750	0.600(+) 0.612(−)	0.705(+) 0.711(−)	0.682	0.727	0.745	0.752	0.755	0.737	0.767	0.765
LE I, TE I with 5 m*M* γ-CD	0.558(+) 0.618(−)	0.369	0.396	0.409	0.409	0.602	0.695	0.670	0.672	0.575	0.671
LE I, TE I with 10 m*M* γ-CD	0.637(+) 0.707(−)	0.420	0.441	0.471	0.456	0.683	0.777	0.755	0.756	0.669	0.764
LE I, TE I with 20 m*M* γ-CD	0.700(+) 0.756(−)	0.457	0.480	0.525	0.483	0.728	0.780	0.783	0.786	0.711	0.786
LE I, TE I with 5 m*M* diMe-β-CD	0.776	0.711	0.758	0.758	0.754	0.777	0.789	–	0.777	0.790	0.790
LE I, TE I with 10 m*M* diMe-β-CD	0.794	0.771	0.779	0.780	0.786	0.792	0.806	–	0.824	0.816	0.831
LE I, TE I with 10 m*M* triMe-β-CD	0.514	0.314	0.317	0.344	0.434	0.570	0.633	0.685	0.508	0.574	0.626
LE II, TE I	0.497	0.483	0.442	0.471	0.472	0.553	0.608	–	0.532	0.594	0.668
LE II, TE I with 2.5 m*M* γ-CD	0.849(+) 0.940(−)	0.609	0.648	0.668	0.688	–	1.094	–	1.034	0.899	1.075

of structurally based complexation selectivity, which was unexpected owing to the large size of the γ-CD cavity, indicates a fundamental difference in the structure of the γ-CD–phenotiazine complexes.

Only triMe-β-CD proved to be an efficient LE I additive for complete $(h_i)_{rel}$ differentiation of the solutes studied. It ensures both solubilization and the effect of structural differentiation of the solutes depending on the type of 2-substituent. An example of the successful ITP separation of solutes **6**, **7**, **8** and **9** with a triMe-β-CD-modified LE I is shown in Fig. 1.

Solutes **4** and **5** are able to migrate isotachophoretically in the unmodified LE I, TE I electrolyte system, but they could not be resolved here. The studied CDs added to LE I, except diMe-β-CD, improve the resolution of these compounds. The cyclodextrins could be arranged in the order γ-CD < α-CD < β-CD < triMe-β-CD with regard to their $(h_i)_{rel}$ differentiation effect. The separation pattern of solutes **4** and **5** with α-, β- and triMe-β-CD in LE I is in agreement with our previous experiments; solute **5**, containing a covalently bonded halogen substituent on the aromatic ring, forms stronger inclusion complexes than the unsubstituted solute **4**. A reversed separation pattern was observed at higher γ-CD concentrations (20 m*M*), which supports the idea that the phenotiazine molecule binding sites for γ-CD differ from those for α-, β- and triMe-β-CD.

Similar conclusions could be drawn for solutes **10** and **11**. The $(h_i)_{rel}$ differences in the unmodified and β-CD-containing electrolyte systems is negligible. The addition of α-CD to the LE causes a stronger retardation of solute **10**; γ-CD and triMe-β-CD reverse the separation pattern.

ITP resolution of subset B solutes

From the experiments with subset B solutes it can be generally concluded that complex formation with the CDs studied depends significantly on the structure of the 10-substituent. The weakest retardation was observed for solute **5**, with an alicyclic 10-substituent. Introduction of a piperidine ring into the 10-substituent (solute **6**) had an important effect, supporting inclusion complex formation with the CDs studied. The cyclodextrins could be arranged into the order β-CD < diMe-β-CD < α-CD < triMe-β-CD < γ-CD with respect to their influence on the $(h_i)_{rel}$ differentiation of solutes **5** and **6**.

Extension of the hydrocarbon chain in the 10-substituent and its termination with an OH group [solute **10** (**11**)] is a structural change that alters especially the complex formation with γ-CD. The addition of γ-CD to LE I results in a strong retardation of solute **6** (**7**) and inversion of the separation pattern of solutes **6** and **10** (**7** and **11**) compared with the unmodified elctrolyte system. The other CDs tested did not show any positive resolution effect, their addition resulting in a decrease in the $(h_i)_{rel}$ differences.

Chiral resolution of subset C solutes

Two electrolyte systems, with MES (LE I) and acetate (LE II) as counterions, were used. The experiments showed that the chiral resolution of solute **1** enantiomers could be achieved with γ-CD-modified LE I or LE II only. The other CDs tested proved to be enantiospecifically ineffective. Although it is not possible to compare directly the $(h_i)_{rel}$ differences for the two enantiomers obtained with LE I and LE II, it

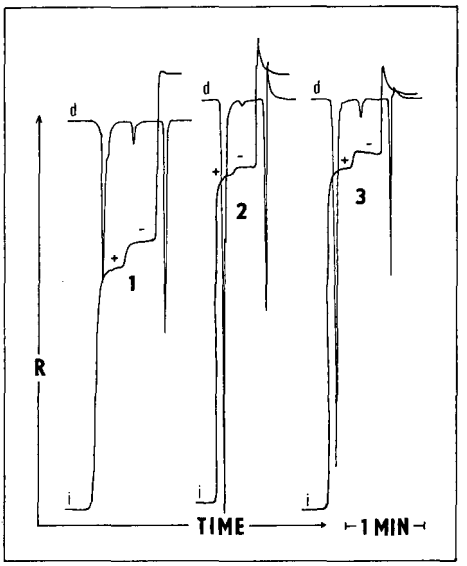


Fig. 2. ITP separation of enantiomers of compounds **1**, **2** and **3**. Electrolyte systems: LE I with 5 m*M* γ-CD for **1**, LE II with 3 m*M* β-CD for **2** and LE II with 1 m*M* β-CD for **3**; TE I for **1** and TE II for **2** and **3**; capillary I for **1** and II for **2** and **3**. Volume injected: 2 μl for **1** and 1 μl for **2** and **3** of racemate solutions. + = Dextrorotatory and − = laevorotatory isomer. *R*, *i* and *d* as in Fig. 1.

can be concluded that the effective chiral resolution of solute 1 with LE II could be achieved with substantially lower γ-CD concentration than with LE I. The possible explanation of this result is based on differences in the stability of the competitive counter-ion inclusion complexes. The small acetate ion, in comparison with the heterocycle-containing MES, is not able to block effectively the CD cavity and thus compete against the CD–solute complex formation.

The choice of ITP conditions suitable for chiral resolution of solutes 2 and 3 is more complicated. β-CD, which seems to be the only enantioselective additive suitable for the resolution of both racemates, provides only a slight differentiating effect and makes the optimization of other ITP conditions critical. It is not possible to achieve chiral resolution with electrolyte system LE I, TE I, probably owing to the above-mentioned counter-ion complex formation which cancels completely the eniantioselectivity of β-CD. Strong retardation effects of β-CD added to LE II requires the use of the slower TE II, which makes it possible to work at optimum β-CD concentrations without the risk of losing the solute in the terminator zone. To improve the maximum racemate load, $(n_r)_{max}$[6], it was necessary to use the longer capillary II.

A practical example of the resolution of racemic solutes **1**, **2** and **3** under optimum ITP conditions is demonstrated in Fig. 2.

CONCLUSIONS

Cyclodextrins, as LE additives, were successfully applied to the improvement of the resolution of various commercially available phenothiazine derivatives. The im-

portant characteristics of the CD-based separation process were studied. The solubilization effect of CDs permits the analysis of poorly soluble phenothiazine derivatives in aqueous electrolyte systems. Structurally related phenothiazine derivatives could be resolved effectively with CD-modified electrolyte systems, depending on the differences in their 2- and 10-substitution.

The chiral resolution of racemic phenothiazine derivatives could be achieved with β- or γ-CD-modified electrolyte systems. Some additional parameters, such as the type of counter ion used, play a crucial role in the quality of the chiral resolution and must be involved in the optimization procedure.

The method developed is very advantageous for practical monitoring of phenothiazine synthesis or drug analysis and broadens the applicability of CD inclusion pseudo-phases in isotachophoresis.

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REFERENCES

1 E. Usdin and D. H. Efron, *Psychotropic Drugs and Related Compounds*, Department of Health, Education and Welfare, Health Services and Mental Health Administration, National Institute of Mental Health, Washington, DC, 1972.
2 I. Jelínek, J. Snopek and E. Smolková-Keulemansová, *J. Chromatogr.*, 405 (1987) 379.
3 J. Snopek. I. Jelínek and E. Smolková-Keulemansová, *J. Chromatogr.*, 411 (1987) 153.
4 I. Jelínek, J. Dohnal, J. Snopek and E. Smolková-Keulemansová, *J. Chromatogr.*, 435 (1988) 496.
5 J. Snopek, I. Jelínek and E. Smolková-Keulemansová, *J. Chromatogr.*, 438 (1988) 211.
6 I. Jelínek, J. Snopek and E. Smolková-Keulemansová, *J. Chromatogr.*, 439 (1988) 386.
7 J. Snopek, E. Smolková-Keulemansová, I. Jelínek, J. Dohnal, J. Klinot and E. Klinotová, *J. Chromatogr.*, 450 (1988) 373.
8 F. E. P. Mikkers, F. M. Everaerts and J. A. F. Peek, *J. Chromatogr.*, 168 (1979) 293.

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DETERMINATION OF RESIDUES OF CARAZOLOL AND A NUMBER OF TRANQUILLIZERS IN SWINE KIDNEY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET AND FLUORESCENCE DETECTION

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SUMMARY

A rapid and sensitive method has been set up for the determination of the beta-receptor blocker carazolol and the tranquillizers acepromazine, azaperone, chlorpromazine, haloperidol, propionylpromazine and xylazine in swine kidneys. The procedure involves extraction with acetonitrile, rapid sample clean-up with a Sep-Pak C_{18} cartridge and high-performance liquid chromatography with ultraviolet and fluorescence detection. The mean recoveries range from 93 to 101%, with the exception of xylazine (52%), and the coefficients of variation from 5.3 to 18.9% at a fortification level of 20 μg/kg. The limits of determination range from 0.3 μg/kg for carazolol to 1–10 μg/kg for the other drugs. The method has been tested in routine monitoring programmes.

INTRODUCTION

In veterinary practice, beta-receptor blocking agents and tranquillizers are used not only therapeutically but also to prevent stress during the transportation of swine and bulls from the farm to the slaughterhouse. Among the compounds claimed to be used, the blocking agent carazolol and the tranquillizers acepromazine, azaperone, chlorpromazine, propionylpromazine and xylazine have priority. Fig. 1 shows the molecular structures of the drugs investigated. It also includes azaperol, which is a major metabolite of azaperone[1], and haloperidol which is mainly used in human medicine.

At the start of this study no relevant data were available about which tranquillizers were used and the frequency of use in The Netherlands. In the F.R.G carazolol is used, but a withdrawal period of 3 days prior to transportation to the slaughterhouse has been established[2]. Still, recent data[3] from the F.R.G. indicated that an high percentage of swine kidneys analysed contained residues of carazolol in concentrations ranging from 0.4 to 10 μg/kg.

The purpose of the present study was to develop a routine multi-method for the determination of all compounds of interest in swine kidneys in the low μg/kg range.

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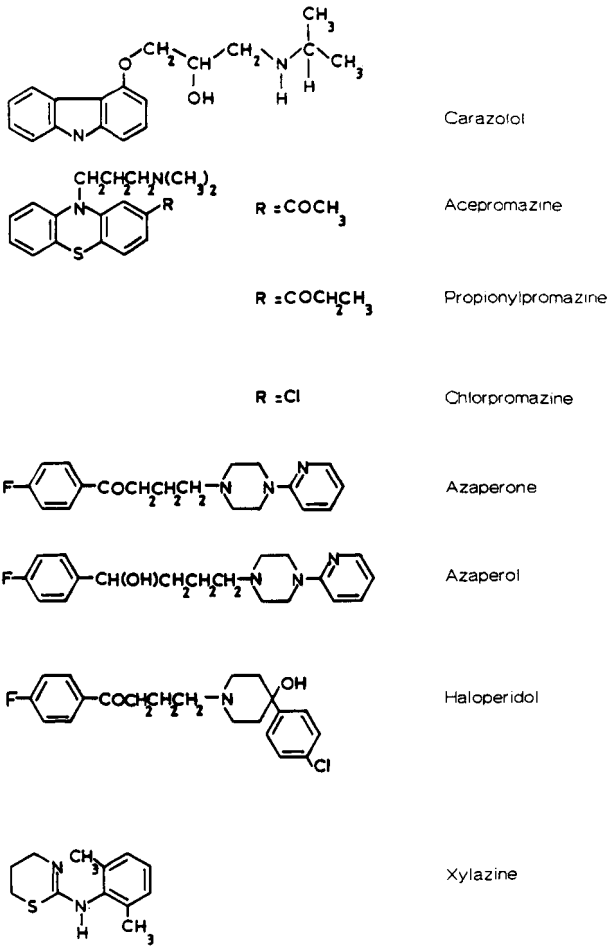

Fig. 1. Molecular structures of the drugs investigated.

Literature data indicated that the kidney was a good target-organ for monitoring the use of tranquillizers[3–6]. To our knowledge, up to now no quantitative multi-method for the determination of all the compounds mentioned has been described in literature. Among the published methods that are mostly applicable only to a single or a few compounds, are a fluorimetric method for carazolol[6], the thin-layer chromatographic (TLC) determination of azaperone and azaperol[1,4], of propionylpromazine[5] and of azaperone, propionylpromazine and carazolol[7], the gaschromatographic (GC) determination of acepromazine, propionylpromazine, chlorpromazine and xylazine[8] and of azaperone[5], an high-performance liquid chromatographic (HPLC) determination of carazolol[2,3] and a radioimmunoassay (RIA) method for carazolol[9]. An HPLC method that was used for the determination of all the relevant tranquillizer drugs except carazolol described by Etter *et al.*[10] showed low recoveries (<60%) and an unsatisfactory precision as indicated by high coefficients of variation.

We developed a simple and rapid method to determine residues of all beta-blocker and tranquillizer compounds mentioned in swine kidneys, using a combination of off-line solid phase extraction clean-up and HPLC separation with UV and fluorescence detection.

MATERIALS AND METHODS

Caution: the compounds investigated are light sensitive; their analysis should be under artificial yellow light using amber glassware.

Standards

Acepromazine, azaperone, chlorpromazine, propionylpromazine and xylazine were a generous gift from the Technical University Berlin (F.R.G.) and azaperol, carazolol and haloperidol from the National Institute of Public Health (Bilthoven, The Netherlands). Stock solutions of 100 μg drug per ml methanol were prepared in amber glassware. These solutions proved to be stable for 6 months when stored in the dark at 6°C.

Working standard solutions contained 100 ng drug per ml 0.01 *M* sulphuric acid except carazolol (50 ng/ml) and xylazine (200 ng/ml). These solutions were stable for at least 1 week when stored in the dark.

Reagents

All chemicals were of analytical grade (Merck, Darmstadt, F.R.G.) except where stated otherwise. A 10% aqueous sodium chloride solution and a 0.01 *M* aqueous sulphuric acid solution were prepared. Acidic acetonitrile was prepared by addition of 1 ml 0.05 *M* sulphuric acid to 100 ml acetonitrile.

The HPLC eluent was prepared by mixing 2.46 g anhydrous sodium acetate, 450 ml water and 550 ml acetonitrile Uvasol. The pH was adjusted to 6.5 with acetic acid. The eluent was filtered before use. The Sep-Pak C_{18} cartridges were obtained from Millipore (Milford, MA, U.S.A.).

Instrumentation

The HPLC system consisted of an automatic sampler Model WISP 710B (Millipore), a solvent-delivery system Model 6000A (Millipore), an UV–VIS detector Model Spectroflow 783 (Applied Biosystems, Foster City, CA, U.S.A.), a fluorescence detector Model 1046A (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a double pen recorder. The analytical column was a 300 mm × 3.9 mm I.D. column packed with Bondapak C_{18} (Millipore) and the guard column (10 mm × 2.1 mm) contained Bondapak C_{18}, 37–50 μm.

The eluent flow-rate was 1.2 ml/min. UV detection was performed at 240 nm with a range setting of 0.002 a.u.f.s. and a risetime of 5 s. Fluorimetric detection was performed at an excitation wavelength of 246 nm and an emission wavelength of 351 nm. The gain setting was 12, the risetime 4 s, the excitation slit width 2 mm and the emission slit width 4 mm. No cut-off filter was used. The recorder speed was 5 mm/min and the mV-setting for both signals was 10.

Analytical procedure

Excessive fat was removed from the kidney sample, which was then cut into small pieces and homogenized with a kitchen grinder. A 5-g amount of homogenized sample was accurately weighed in a polypropylene centrifuge-tube and 20 ml acetonitrile were added with continuous gentle mixing. After closing the tube, the sample was vigorously mixed on a vibromixer for 30 s (1500 rpm). The tube was placed in an ultrasonic bath for 2 min and then centrifuged at 4000 *g* for 5 min.

A Sep-Pak C_{18} cartridge was activated successively with 5 ml methanol and 5 ml water. To 7.5-ml volume of the sample extract were added 40 ml of 10% sodium chloride solution and mixed. This solution was gently pressed through the activated cartridge with a disposable syringe. The cartridge was flushed with 1 ml of 0.01 *M* sulphuric acid and next with 2 ml air. The compounds were eluted with 2 ml acidic acetonitrile and the eluate was collected in a calibrated tube which had been rinsed before use with concentrated ammonia, water and acetone and then dried under a stream of nitrogen.

The eluate was evaporated to 300 μl with a gentle stream of nitrogen at 70°C. After gentle mixing, 1 ml of *n*-hexane was immediately added and mixed for 30 s on a vibromixer. The phases were separated by centrifugation (2000 *g*) and the lower aqueous phase isolated. A 50-μl volume of the latter were injected into the HPLC system. A flow diagram of the analytical procedure is shown in Fig. 2.

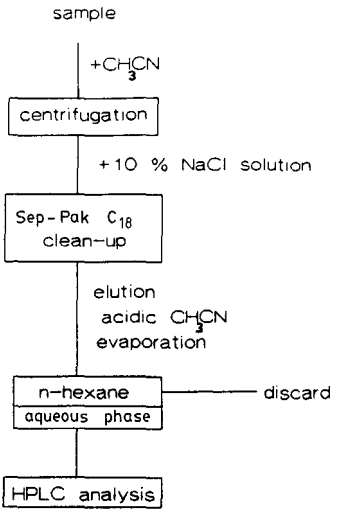

Fig. 2. Flow diagram of the analytical procedure.

RESULTS AND DISCUSSION

Chromatography

The objective was to use isocratic HPLC. An eluent mixture of acetonitrile and an acidic acetate buffer as described by Etter *et al.*[10] in combination with a column packed with a cyanopropyl bonded phase gave good separation for all compounds.

However, the peak heights and retention times of the injected standard solutions were not reproducible. Furthermore the kidney matrix severely influenced the retention times, possibly due to small variations in the pH of the final sample extract.

Therefore the use of C_{18} bonded phases was investigated. Here, the retention characteristics of the tranquillizers were strongly influenced by the endcapping of the column material, especially when acidic eluents were used. Only column materials where the silanol groups are sufficiently endcapped as for instance Bondapak (Millipore) and Supelcosil (Supelco, Bellefonte, PA, U.S.A.) were applicable. Other packing materials such as Chromspher C_{18} (Chrompack, Middelburg, The Netherlands) and LiChrosorb (Merck) showed strong adsorption effects. The promazines were not eluted from these columns when an acidic eluent was used. This was probably caused by strong polar interactions of the protonated basic drugs with available silanol groups.

The most reliable and reproducible results were achieved with a Bondapak C_{18} column with an acidic eluent. It was also essential to use Bondapak material for the guard column. The other materials tested caused strong adsorption of the compounds.

The retention behaviours of the compounds were strongly dependent upon the concentration of the acetate buffer and the pH of the eluent. The retention of all compounds decreased with increasing salt concentrations and at lower pH values, as expected from the basic properties of the drugs. The relationship between the retention time and acetate buffer concentration is shown in Fig. 3 for carazolol, azaperone and propionylpromazine. At a buffer concentration below 0.01 *M* the promazine and xylazine showed strong adsorption resulting in very asymmetric peaks. When the acetate concentration was increased to 0.1 *M* complete resolution was not obtained

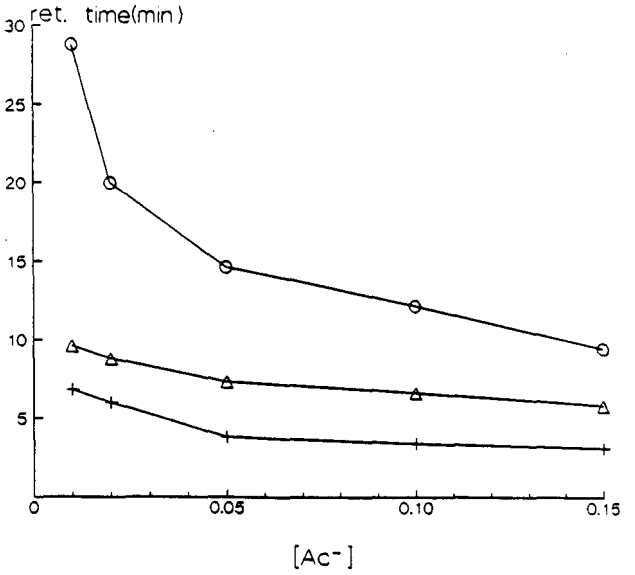

Fig. 3. Relationship between the acetate (Ac) buffer concentration and the retention time for carazolol (+), azaperone (△) and propionylpromazine (○). The eluent was acetonitrile–sodium acetate buffer (45:55, v/v) at pH 7, flow-rate 1.5 ml/min. The column was a Bondapak C_{18} (300 mm × 3.9 mm).

between carazolol and xylazine, even when the percentage of acetonitrile in the eluent was lowered from 55 to 40%. A buffer concentration of 0.03 *M* gave optimum results.

By varying the pH of the mobile phase, the separation of the compounds was further optimized. Depending upon the efficiency of the column used, the pH is set between 6.5 and 7; pH values below 6.5 should not be used. Fig. 4 shows the influence of lowering the pH of the mobile phase from 6.4 to 6.0. Excessive matrix interferences at the retention time of azaperone are observed at these lower pH values.

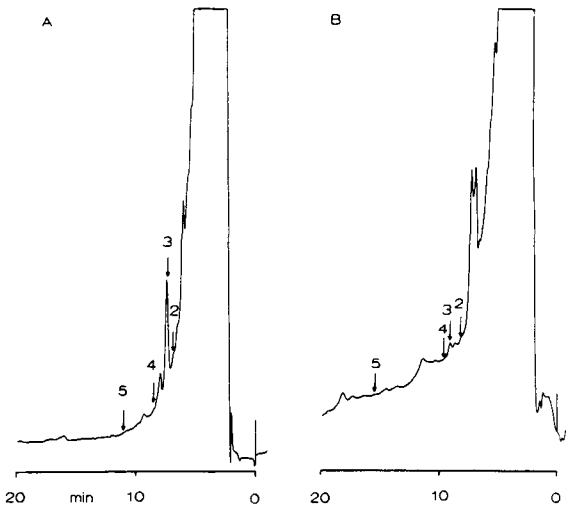

Fig. 4. HPLC chromatograms of a blank kidney sample analysed with an eluent at (A) pH 6.0 and (B) pH 6.4. The experimental conditions were as described in Materials and methods. Peaks: 2 = xylazine; 3 = azaperone; 4 = haloperidol; 5 = acepromazine.

Detection

All the drugs under investigation were detected with UV detection. Absorbance maxima ranged between 240 and 260 nm with the exception of xylazine which showed an optimum below 225 nm. As a compromise, 240 nm was selected as the detection wavelength.

Carazolol can also be determined with fluorescence detection[2,6]. Under the HPLC conditions described above, carazolol showed an excitation spectrum (emission wavelength 351 nm)[6] with a flat optimum between 230 and 246 nm (Fig. 5). This is not in line with the 283 nm observed by Rudolph and Steinhart[2]. An excitation wavelength of 246 nm was chosen because at lower wavelengths the signal-to-noise ratio decreased. In contrast with the findings of Engelsma and Simons[6], a fluorescence signal was also observed for azaperone and azaperol. The latter compound was not fully separated from carazolol. However, the ratio of fluorescence and UV responses for the two drugs can be used to differentiate in case of doubt. Under the experimental conditions used, this ratio was 14.6 for carazolol and 6.4 for azaperol.

Furthermore baseline separation of carazolol and azaperol is possible using an eluent with an acetate buffer concentration of 0.1 *M* and a pH 7.

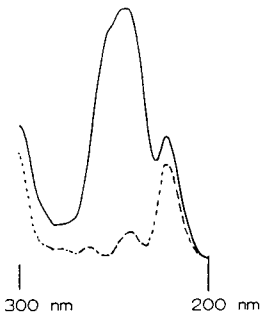

Fig. 5. Fluorescence excitation spectrum for carazolol (———) and the eluent (·····) taken by a stop flow scan at emission wavelength 351 nm; scan speed 1.5 nm/s; paper advance 1 mm/s. The HPLC conditions as described in Materials and methods.

The fluorescence response of azaperone and azaperol allows a very selective determination of these compounds without matrix interferences.

Extraction and sample clean-up

In our hands a number of methods described in the literature for extraction and sample clean-up for some tranquillizers in kidney samples[1,5,7] yielded low and very irreproducible recoveries. The method of Etter *et al.*[10] was not investigated owing to the described irreproducible results.

Therefore a number of methods already in use within our laboratory for the extraction and clean-up of compounds with comparable physicochemical properties as for the substances under investigation were tried on kidney samples spiked with the tranquillizer drugs. First, a combination of an aqueous extraction and an Extrelut® cartridge clean-up was tested because tranquillizers were eluted quantitatively from the cartridge with dichloromethane. This procedure was similar to the one used for the determination of chloramphenicol in meat[11]. The final residue was dissolved in 0.01 *M* sulphuric acid and extracted with light petroleum (b.p. 40–60°C), as described by Etter *et al.*[10] to remove residual fatty material. The recovery percentages were quite acceptable (over 60%) for carazolol, xylazine and azaperone but very low (less than 30%) for haloperidol and the three promazines. In the literature low recoveries for tranquillizers are often attributed to interactions with active sites on glassware surfaces[2,10]. However when using polypropylene materials we observed no significant improvement. Spiking of haloperidol and the promazines at different stages of the sample clean-up showed that these drugs were not completely extracted from kidney samples with water. Therefore, a different approach using a non-aqueous extraction was chosen. Since organic solvents are not compatible with Extrelut, sample enrichment and clean-up was performed on Sep-Pak C_{18} cartridges.

With acetonitrile as the extractant, an aliquot of the organic extract was diluted in water to increase the polarity of the solution in order to retain the compounds of interest. However, it was impossible to retain the polar compounds carazolol, xylazine and azaperol in this way. When water was replaced by a 10% sodium chloride solution, all drugs, except xylazine, were however fully retained on the cartridge.

The use of the sodium chloride solution made it then necessary to flush the

cartridge with water before the actual elution to remove salts. No tranquillizers were lost by this step. At this stage of the study the recoveries obtained were generally acceptably high, but for the promazines not very reproducible. Also, the fluorescence chromatograms showed an interfering peak near carazolol. This interference was caused by the light petroleum used. Replacement by *n*-hexane did not lower the recoveries and no matrix interferences were observed. Typical fluorescence chromatograms for carazolol after extraction with light petroleum and *n*-hexane are given in Fig. 6.

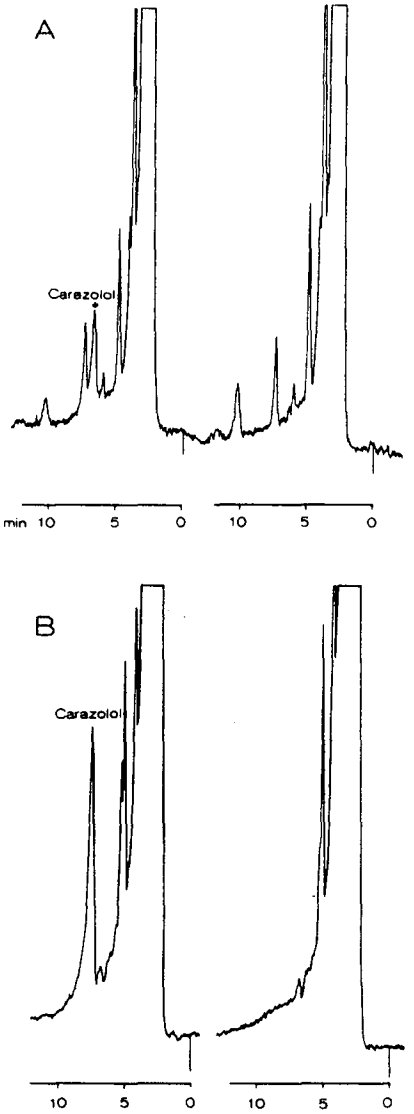

Fig. 6. Fluorescence chromatograms for (A) a blank and carazolol-spiked (1 μg/kg) kidney sample extracted with light petroleum and (B) a blank and a real kidney sample with carazolol (2 μg/kg) extracted with *n*-hexane. The HPLC conditions were as in Materials and methods.

Initially the irreproducible results for the promazines were attributed to losses during evaporation to dryness of the cartridge eluate. To solve this problem, the cartridge eluate was evaporated to a volume of 300 μl, mainly water. However, the solution remaining after evaporation had to be acidic, at least 0.01 *M*, to prevent substantial losses (> 80%) during the partition with *n*-hexane. The most simple way to obtain an acidic solution after evaporation was to flush the cartridge after sample enrichment with 0.01 *M* sulphuric acid instead of water.

This change resulted in more reproducible recoveries but still occasionally low recoveries for chlorpromazine were obtained. When however the hexane partition was performed immediately after evaporation to 300 μl, no losses occurred and the recoveries were generally about 90%, except for xylazine (50%). A possible explanation for the considerable improvement in reproducibility and recovery might be that the three (apolar) promazines are adsorbed to residual fats when the final aqueous extract cools down and are then co-extracted with the fat particles into the hexane phase. Immediate partition of the final (still slightly warm) extract prevents this. The low recovery for xylazine is found because the enrichment of this compound on the Sep-Pak C_{18} is critical. When 5 ml instead of 7.5 ml of the crude acetonitrile extract were applied to the cartridge, the recovery for xylazine was also 90%. Since this would mean an higher limit of detection for the other drugs, this change was not incorporated in the method.

The final procedure, using an acetonitrile extraction, dilution in saline solution, enrichment on a Sep-Pak C_{18} cartridge with mild acidic removal of salts, elution of the drugs with acidic acetonitrile and partition with *n*-hexane showed high recoveries, clean chromatograms and a good precision as shown in the analytical method characteristics below. Typical UV chromatograms of kidney samples are shown in Fig. 7.

Analytical method characteristics and applications

Recoveries and precision. Recovery experiments were carried out on different days by addition of 100 μl of a standard solution in water to the homogenized kidney samples. Carazolol was added at a level of 10 μg/kg, xylazine at a level of 40 μg/kg and all other compounds at a level of 20 μg/kg. The results are shown in Table I.

For azaperol, the azaperone metabolite, mean recoveries of more than 90% were obtained, but it was not routinely included in the standard spike solution. Xylazine showed the highest coefficient of variation caused by the critical sample enrichment on the Sep-Pak cartridge. To improve further the precision, the addition of an internal standard to the final HPLC extract can be recommended, mainly because the calibration of the tubes used in the final evaporation step is not very reliable.

In that case haloperidol can be used as an internal standard. This drug is normally used for human purposes only and is separated from the other tranquillizers in this system.

The stability of spiked kidney samples was tested by storing them at -20°C for 1 month. No loss of recovery was observed. Recoveries are linear over the range of 0.01 to 1 mg/kg.

Limit of determination. Several hundreds of blank kidney samples were analysed with the method proposed. No interferences or significant noise were observed in the UV chromatograms. This made it difficult to carry out a calculation of the limit of

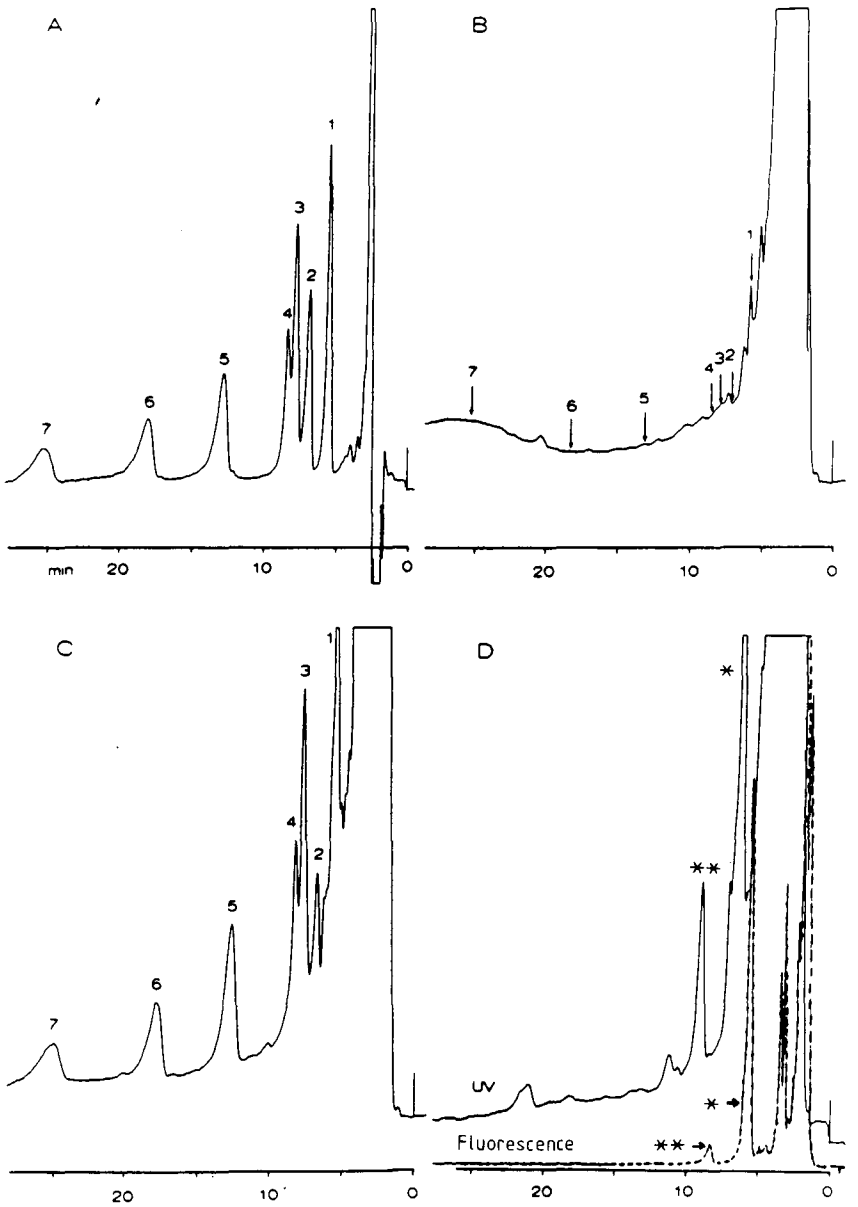

Fig. 7. HPLC chromatograms with UV detection of (A) a mixed standard solution containing 0.2 μg/ml of the drugs except carazolol (0.1 μg/ml) and xylazine (0.4 μg/ml); (B) a blank kidney sample; (C) a blank kidney sample spiked with 50 μg/kg of the tranquillizers except carazolol (25 μg/kg) and xylazine (100 μg/kg) and (D) a practice kidney sample from an azaperone-dosed swine. The HPLC conditions were as described in Materials and methods. Peak: 1 = carazolol; 2 = xylazine; 3 = azaperone; 4 = haloperidol; 5 = acepromazine; 6 = propionylpromazine; 7 = chlorpromazine; (D) only, * = azaperol; ** = azaperone.

TABLE I
RECOVERIES OF TRANQUILLIZERS AND CARAZOLOL IN SWINE KIDNEY SAMPLES ($n = 10$)

No.	*Name*	*Fortification (μg/kg)*	*Mean recovery (%)*	*Coefficient of variation (%)*
1	Carazolol	10	99	5.3
2	Xylazine	40	52	18.9
3	Azaperone	20	99	8.8
4	Haloperidol	20	95	7.6
5	Acepromazine	20	101	8.2
6	Propionylpromazine	20	95	6.7
7	Chlorpromazine	20	93	13.4

detection. This calculation should be based on the mean blank signal plus three times the standard deviation of the blank[12]. Therefore, as an estimation of the practical limit of determination, a peak height of 5 mm for the UV signal at a range of 0.001 a.u.f.s. and 10 mm for the fluorescence signal, in both cases representing more than 10 times the mean noise, was chosen. The results are shown in Table II.

Applicability and interferences. To test further the applicability of the method, a positive kidney sample from a carazolol-dosed swine was analysed as a reference sample in a number of monitoring program sample series. The mean level found was 2.0 μg/kg ($n = 14$) with a coefficient of variation of 14.4%. This gives a good indication of the within-lab reproducibility of the carazolol determination. The somewhat higher C.V. compared with the 10-μg/kg spike results is probably caused by the lower concentration and inhomogeneity of real kidney samples. The C.V. found easily meets the requirements stated by the European Economic Community for the repeatability of methods to be used for detecting residues of groups of veterinary drugs[13]. A value of 20% is considered to be acceptable in the concentration range 2–10 μg/kg.

More than 40 veterinary drugs and other (human) tranquillizers were injected into the HPLC system to check for interferences. Only perphenazine and promazine

TABLE II
LIMIT OF DETERMINATION OF TRANQUILLIZERS IN SWINE KIDNEYS
Results expressed in μg/kg.

No.	*Name*	*Limit of determination*[a]
1	Carazolol	0.3[b]
2	Xylazine	4
3	Azaperone	1
4	Haloperidol	2
5	Acepromazine	2
6	Propionylpromazine	4
7	Chlorpromazine	6
8	Azaperol	2[b]

[a] Defined as described in the text.
[b] Based on fluorescence detection.

showed UV peaks with retention times near propionylpromazine. However, these drugs are not expected in animal tissues and HPLC separation seems possible after optimization.

The method was tested in routine monitoring programmes. Over 1000 samples were analysed. With this method, 30 samples could be analysed per day without technical problems. Although the method was originally developed for kidney samples, it proved also applicable to injection sites, plasma and liver samples. Preliminary drug excretion studies have recently been carried out. The results will be reported elsewhere.

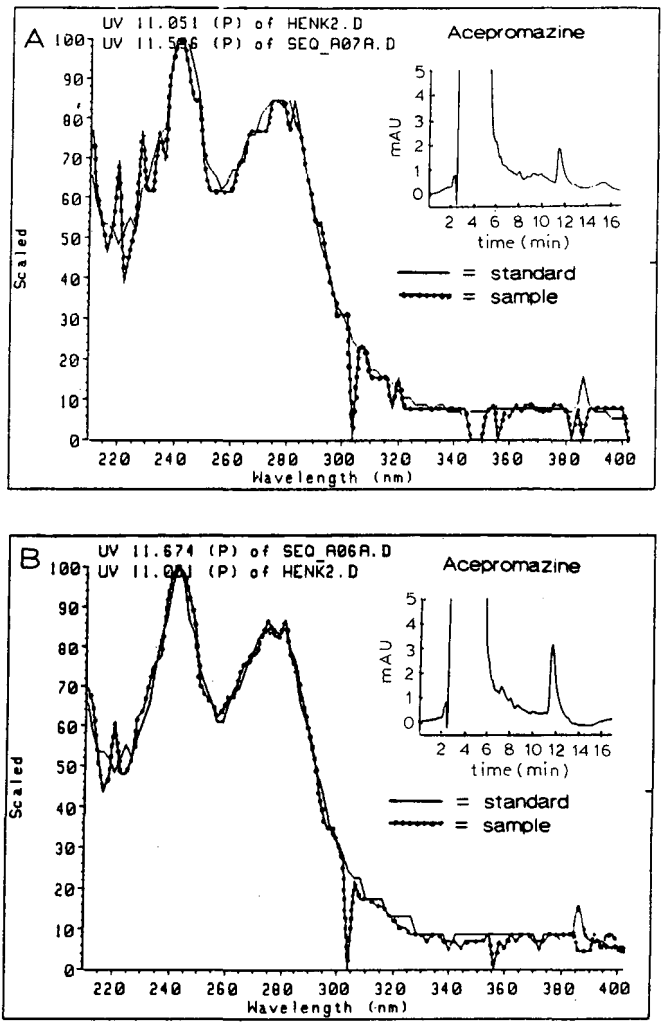

Fig. 8. HPLC chromatograms and co-plotted standard and sample spectra for two practice samples containing 40 and 80 μg/kg acepromazine. The HPLC conditions were as described in Materials and methods; an HP 1040 M UV–VIS detector was used.

Confirmation. When a diode array UV–VIS detector was used (Hewlett-Packard 1040 M), confirmation by co-plotting of UV spectra of the sample peak and standards was possible above a level of about 50 μg/kg. Chromatograms and spectra of two samples containing 40 and 80 μg/kg acepromazine, respectively, are shown in Fig. 8.

CONCLUSIONS

With the method developed, the tranquillizers acepromazine, azaperone (including its metabolite azaperol), chlorpromazine, haloperidol, propionylpromazine, xylazine and the beta-blocking agent carazolol can be determined in swine kidneys with a limit of determination ranging from below 1 μg/kg for carazolol to 1–10 μg/kg for the other drugs.

The analytical recoveries are high (93–101%) except for xylazine (52%) and the precision is excellent (coefficients of variation between 5.3 and 18.9%). The method was tested successfully in routine monitoring programmes. Although developed for kidney samples, the method can also be used for meat, plasma and liver samples. Further research is planned on the confirmation of positive results with diode-array UV–VIS and mass spectrometric detection.

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REFERENCES

1 A. G. Rauws, M. Olling, J. Freudenthal and M. ten Ham, *Toxicol. Appl. Pharmacol.*, 35 (1976) 333.
2 M. Rudolph and H. Steinhart, *J. Chromatogr.*, 392 (1987) 371.
3 M. Rudolph and H. Steinhart, *Dtsch. Lebensm.-Rundsch.*, 83 (1987) 273.
4 A. G. Rauws and M. Olling, *J. Vet. Pharmacol. Therap.*, 1 (1978) 57.
5 M. Olling, R. W. Stephany and A. G. Rauws, *J. Vet. Pharmacol. Therap.*, 4 (1981) 291.
6 J. W. Engelsma and J. Simons, *Vet. Q.*, 7 (1985) 73.
7 N. Haagsma, E. R. Bathelt and J. W. Engelsma, *J. Chromatogr.*, 436 (1988) 73.
8 L. Laitem, I. Bello and P. Gaspar, *J. Chromatogr.*, 156 (1978) 327.
9 E. Rattnberger, P. Matzke and J. Neudegger, *Arch. Lebensmittelhyg.*, 36 (1985) 85.
10 R. Etter, R. Battaglia, J. Noser and F. Scuppisser, *Mitt. Geb. Lebensm. Hyg.*, 75 (1984) 447.
11 H. J. Keukens, W. M. J. Beek and M. M. L. Aerts, *J. Chromatogr.*, 352 (1986) 445.
12 D. Freeman, *Anal. Chem.*, 52 (1980) 2249.
13 Anonymus, *Publication, VI/6290/86-Rev-4 Commission of the European Communities*, Brussels, 1987.

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STABILITY OF TRIMETREXATE, A NEW NON-CLASSICAL ANTIFOLATE, IN INFUSION SOLUTIONS

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SUMMARY

A prerequisite for the prolonged infusion of a drug via a totally implanted drug delivery system is that the drug solution must be sufficiently stable at physiological temperatures to endure the time intervals between drug replacement or pump refills. Consequently, the stability of the chemotherapeutic agents can influence the dosing accuracy and ultimately the achievement of the desired therapeutic goal. The chemical stability of the pharmaceutical preparation Trimetrexate (TMQ) glucuronate, a non-classical, lipophilic antifolate, has been characterized. Incubation of TMQ (prepared in sterile water to a concentration of 5.0 mg/ml) in sterile, amber glass vials at 37°C for 56 days resulted in a degradation rate constant of 0.0134 ± 0.002 day^{-1} and a half-life of 51.6 ± 0.8 days. The major degradation product has been identified as (2,4-diamino-5-methyl-6-carboxaldehyde)quinazoline. Ten percent TMQ degradation would occur by 7.9 days of incubation under these conditions.

INTRODUCTION

Trimetrexate, 2,4-diamino-5-methyl-6[(3,4,5-trimethoxyanilino)-methyl]quinazoline (TMQ), was originally synthesized by Elslager and Davoll[1]. It is a potent inhibitor of dihydrofolate reductase (DHFR) [5,6,7,8-tetrahydrofolate:NADP$^+$-oxido-reductase (EC 1.5.1.3)][2], and has demonstrated significant antitumor activity against murine and human cell lines both *in vitro* and against several murine transplanted tumors[3,4]. TMQ is more lipophilic than the classical antifolate methotrexate (MTX) and has demonstrated an increased uptake into neoplastic cells compared to MTX[5]. Also, TMQ's enhanced lipophilicity may afford its greater penetration into

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the central nervous system (CNS) and, therefore, greater efficacy than MTX against CNS neoplasms.

The importance of antifolate concentration and exposure time in determining toxic and therapeutic effects both *in vitro* and *in vivo* in experimental animal tumors has been established[6,9]. Studies in experimental animal systems have also shown that biological response to antifolates is determined by exposure time and is selective for individual tissues[10–12]. These findings suggest a therapeutic rationale for the prolonged infusion of TMQ at rigidly controlled dose rates which achieve steady-state TMQ plasma levels toxic to neoplastic cells but below the toxic threshold of bone marrow cells from that individual patient. With the advent of totally implanted drug delivery systems, therapy involving prolonged intravenous or, in the case of regional chemotherapy[13], intra-arterial infusions can be accomplished on an out-patient basis with minimal patient risk and significant savings in hospital an patient care expenses.

Portable drug delivery systems, both external and implanted, allow constant drug infusion rates at the desired sites for extended durations. However, a prerequisite for prolonged drug infusion via a totally implanted drug delivery system is that the drug solution must be sufficiently stable at physiological temperatures to endure the time intervals between drug replacement or pump refills. Consequently, the stability of the chemotherapeutic agents can influence the dosing accuracy and ultimately the achievement of the desired therapeutic goal. The present study was designed to characterize the stability of the TMQ drug preparation (Trimetrexate glucuronate) in sterile, amberized glass vials during prolonged incubation (8 weeks) at 37°C.

EXPERIMENTAL

Materials

Trimetrexate glucuronate (50 mg TMQ/vial) and 2,4-diamino-5-methyl-6-carboxaldehyde-quinazoline (AMCQ) were obtained from Warner-Lambert/Parke-Davis, Pharmaceutical Research Devision, Ann Arbor, MI, U.S.A. All other chemicals were high-performance liquid chromatography (HPLC) or analytical grade. Amberized glass vials (10 ml capacity with rubber septa and crimp closures) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Pierce (Rockford, IL, U.S.A.). Sterile plastic (1.0 ml) syringes (Becton, Dickinson & Co., Rutherford, NJ, U.S.A.) were used for sampling.

Kinetic procedures

Trimetrexate glucuronate injection (50-mg vial) was reconstituted in 10.0 ml sterile water to yield a concentration of 5.0 mg TMQ/ml. The reconstituted solution was aseptically transferred to sealed, sterile, amber glass vials. Triplicate sample solutions were prepared. A 0.2-ml aliquot of the samples was removed from each amber vial and signified the samples at zero time. The vials were then incubated in a constant-temperature water bath at 37°C and 0.2-ml aliquots were removed from each vial at the following times: 3, 10, 14, 17, 21, 25, 28, 31, 39, 42, 47, 49 and 56 days. All samples were frozen immediately upon acquisition at −30°C in tightly capped plastic tubes until TMQ analysis.

HPLC assay of TMQ

TMQ standards, quality control and experimental (incubate) samples were diluted 1:5 with methanol–water (50:50, v/v) and 2.0 μl of the diluted samples were injected onto an HPLC reversed-phase column (Hewlett-Packard MOS C_8, 10 cm × 2 mm I.D., 5 μm) using the Hewlett-Packard Model 1090 high-performance liquid chromatograph and autosampler. The chromatographic conditions were: mobile-phase, methanol–acetonitrile–0.05 *M* $(NH_4)H_2PO_4$ buffer (12:18:70, v/v/v); flow-rate, 0.5 ml/min; detector, Hewlett-Packard Model 1040 diode array UV–VIS detector monitoring at 254 nm wavelength and scanning between 200 and 400 nm. The chromatographic peak area integration was acquired using the Hewlett-Packard 3392A integrator/printer.

Calibration curves were constructed by plotting the integrated peak area of TMQ as a function of the drug concentration. The TMQ concentrations of unknown samples were calculated using the results of a least squares linear regression analysis of the calibration data.

Gas chromatography–mass spectrometry

The Hewlett-Packard Model 5987A gas chromatography–mass spectrometry (GC–MS) apparatus equipped with both electron impact (EI) and methane chemical ionization (CI) modes of operation was used in the identification of the major product of TMQ degradation. Separation of the trimethylsilyl (TMS) derivatives of TMQ and its degradation products was accomplished using a fused-silica capillary column (5 m × 0.32 mm I.D.) coated with a cross-linked methyl silicone liquid phase (0.17 mm film thickness). Chromatographic conditions were: injection port and GC–MS interface oven temperatures were 300°C; GC column oven temperature was programmed to hold at the initial temperature of 80°C for 0.1 min, and then increase at a rate of 20°C/min up to the final temperature of 300°C; for EI operation, the ion source temperature was 250°C, electron energy was 70 eV, and the scan range was from 70 to 600 a.m.u.; for methane CI operation, the ion source temperature was 200°C, electron energy was 230 eV, and the scan range was from 130 to 600 a.m.u.

Trimethylsilylation of TMQ and its degradation products was accomplished using MSTFA. Aliquots of 0.1 ml of both the zero-time and 56-day incubation samples were vacuum dried and the residue derivatized in 0.25 ml MSTFA for 2 h at 85°C. A 0.5-μl volume of the reaction mixture was injected for GC–MS analysis.

RESULTS AND DISCUSSION

TMQ assay validation

Under the described chromatographic conditions, the retention time of TMQ was 2.03 min. The integrated peak areas were directly proportional to the drug concentrations over the range of TMQ concentrations tested (0.50–5.00 mg/ml).

Triplicate sets of the calibration standards were assayed on each of three consecutive days. Linear regression of the data from each day was obtained. The reproducibility of the daily standard curves had coefficients of variation which ranged between 1.7 and 4.8%.

The accuracy and precision of the method were assessed by seeding quality control samples at drug concentrations of 1.00 and 4.00 mg TMQ/ml. Triplicate

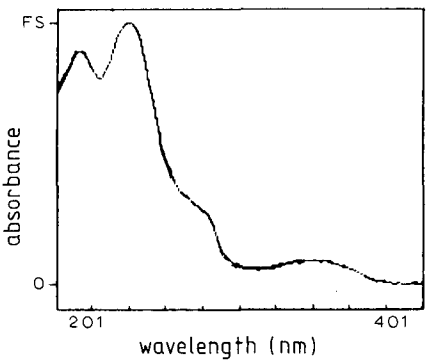

Fig. 1. Results of peak purity evaluation of TMQ peak in HPLC assay procedure. Peak purity is confirmed by three perfectly superimposed UV–VIS absorption spectra.

quality control samples were assayed on each of three consecutive days. The precision of the assay was found to have coefficients of variation ranging between 1.9 and 4.4%. The concentration means for the seeded control samples were found to be within −1.3–+3.2% of the theoretical values.

TMQ assay specificity

The purity of the TMQ peak in the HPLC assay described here was confirmed with a peak purity evaluation program available on the HP 1090 HPLC system equipped with the HP 1040 diode array detector. This program automatically collects UV–VIS absorption spectral data at the upslope, apex and downslope of an eluting peak. In the peak purity analysis, the upslope spectrum of a peak is recorded and becomes a reference spectrum. The apex spectrum of the peak then becomes the reference and the upslope spectrum is discarded. The downslope spectrum of the peak is then plotted against the reference (apex) spectrum. Finally, the end-of-peak spectrum is plotted against the reference (apex) spectrum. Peak purity is established by three perfectly superimposed spectra.

When applied to the TMQ incubation samples, three superimposed spectra

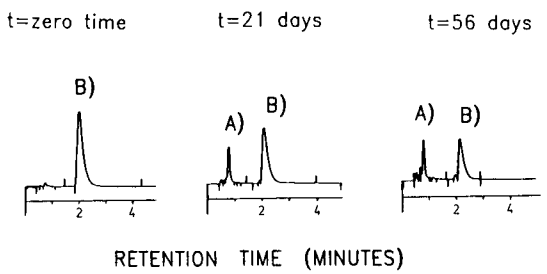

Fig. 2. HPLC chromatograms (2-μl injections) of zero-time, 21-day and 56-day incubation samples showing TMQ (peak B) disappearance and degradation product (peak A) accumulation with increasing incubation time.

(Fig. 1) of the drug peak (peak B in Fig. 2) resulted, thus verifying that the TMQ peak was uncontaminated by any potentially coeluting products.

Degradation kinetics

The integrated peak areas of the incubated samples were compared to those resulting from injections of standard solutions. The concentration *versus* time (in days) data were then entered on semi-log plots as shown in Fig. 3. The stability profile of TMQ can be described by the simple monoexponential decay as given in eqn. 1:

$$C_t = C_0 \exp(-kt) \tag{1}$$

where C_0 is the initial concentration at time zero, k is the rate constant for degradation of TMQ in day^{-1}, t is the time in days, and C_t is the concentration of TMQ at incubation time (t). The parameters of the least squares regression line for eqn. 1 are summarized in Table I. The mean ($\pm$ S.D.) rate constant for drug degradation was 0.0134 ± 0.0002 day^{-1} and, therefore, the mean half-life ($0.693/k$) for TMQ at 37°C in glass was 51.6 ± 0.8 days. If one defines the maximum allowable percent drug loss before either drug replacement or pump refill as 10%, then the maximum allowable incubation (or infusion) time between TMQ refill or replacement based on the rate constant of degradation obtained in this study would be 7.9 days.

Products of TMQ degradation

Since the half-life of TMQ in this preparation at 37°C is only 51.6 days, the degradation product(s) would be expected to accumulate in significant amounts even in the relatively short incubation period of 1–2 weeks. Fig. 2 shows a chromatogram (HPLC) of TMQ samples (2.0 μl injections) from zero-time, 21-day and 56-day incubations. It is evident from Fig. 2 that as the parent drug peak (B) progressively decreases with increasing incubation time, other earlier eluting peaks (primarily peak A) are increasing. It is to be noted that most of the early eluting peaks (including peak A) are absent or only marginally present in the zero-time or TMQ standard solutions.

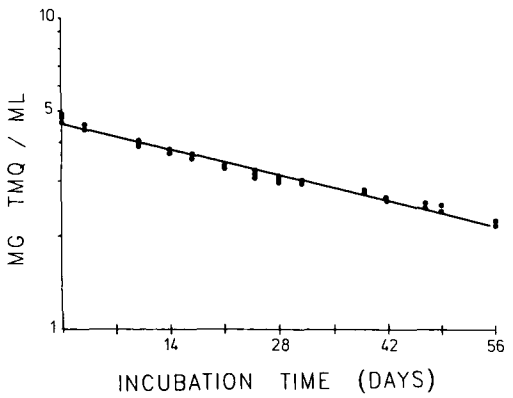

Fig. 3. TMQ concentration *versus* incubation time (in days). Data have been fitted to a monoexponential equation of the form $C_t = C_0 \exp(-kt)$, with the results: $C_t = (4.56 \pm 0.10) \exp -(0.0134 \pm 0.0002)t$ and correlation coefficient $r = 0.993 \pm 0.002$.

TABLE I

SUMMARY OF PARAMETERS OF THE LEAST SQUARES REGRESSION LINE FOR EQN. 1

Concentration of incubate = 5.00 mg/ml.

Drug	C_o	k	$t_{1/2}$ *(days)*
Trimetrexate			
Incubate 1	4.60	0.0136	51.0
Incubate 2	4.63	0.0135	51.3
Incubate 3	4.45	0.0132	52.5
Mean ± S.D.	4.56 ± 0.10	0.0134 ± 0.0002	51.6 ± 0.8

Using the diode array UV–VIS detector, UV spectral data were gathered for both TMQ and its major degradation product, peaks B and A in Fig. 2, respectively. These spectral data are shown in Fig. 4. The major degradation product has a distinctly different UV spectrum, with an absorbance maximum at 253 nm and a secondary peak at 332 nm. TMQ's UV spectrum elicits two primary peaks at 215 and 243 nm and a small secondary peak at 330 nm.

The GC–MS analysis of the vacuum-dried residues from the zero-time and 56-day incubation samples derivatized with MSTFA revealed a large peak (t_R = 5.0 min) in the 56-day sample which was absent in the zero-time sample. Both samples had a large peak eluting at 9.2 min, corresponding to authentic TMQ-bisTMS. When analysed in the EI mode, the mass spectrum of TMQ-bisTMS (Fig. 5) displayed a molecular ion at m/z 513 and the typical TMS derivative fragmentation pattern with loss of a methyl group yielding $[M-15]^+$ at m/z 498, and the prominent $[TMS]^+$ at m/z 73. The base peak at m/z 331 corresponds to cleavage at the anilino–methyl

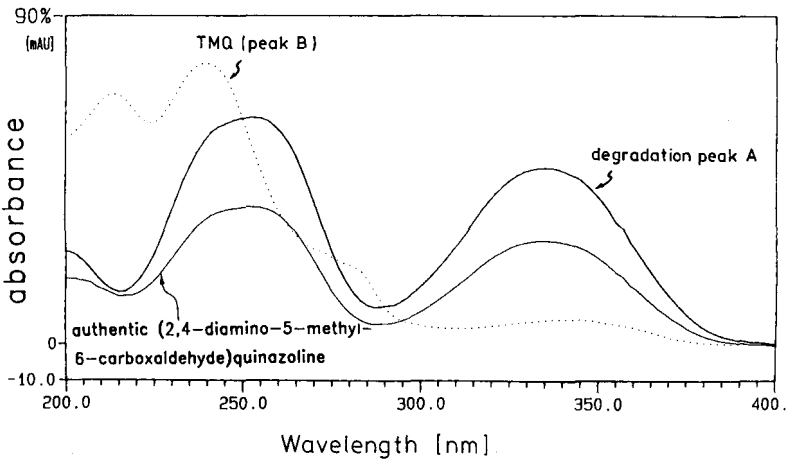

Fig. 4. UV–VIS absorption spectra of TMQ (peak B in Fig. 2), degradation product (peak A in Fig. 2), and authentic AMCQ.

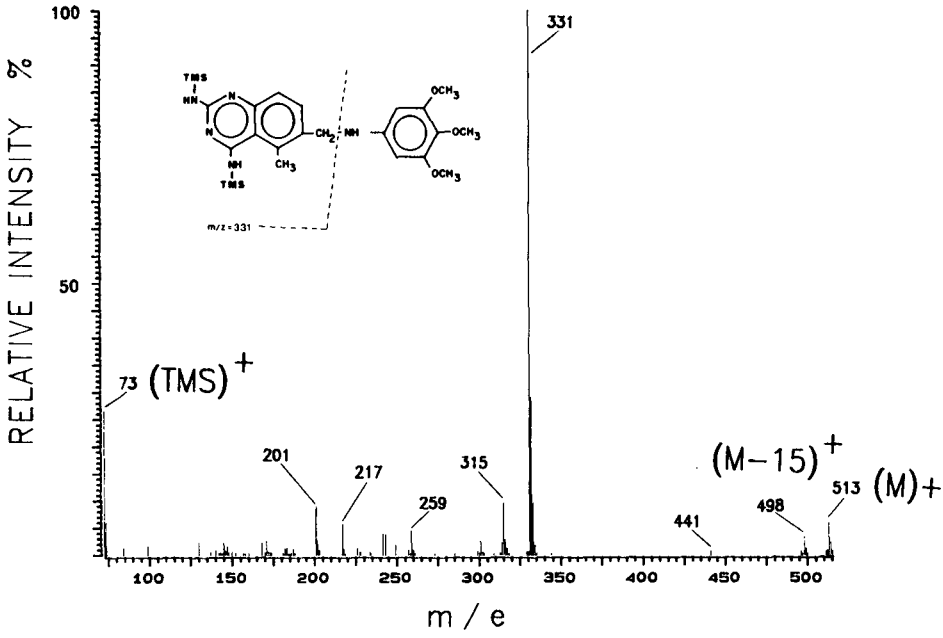

Fig. 5. EI mass spectrum of TMQ-bisTMS (peak B in Fig. 2).

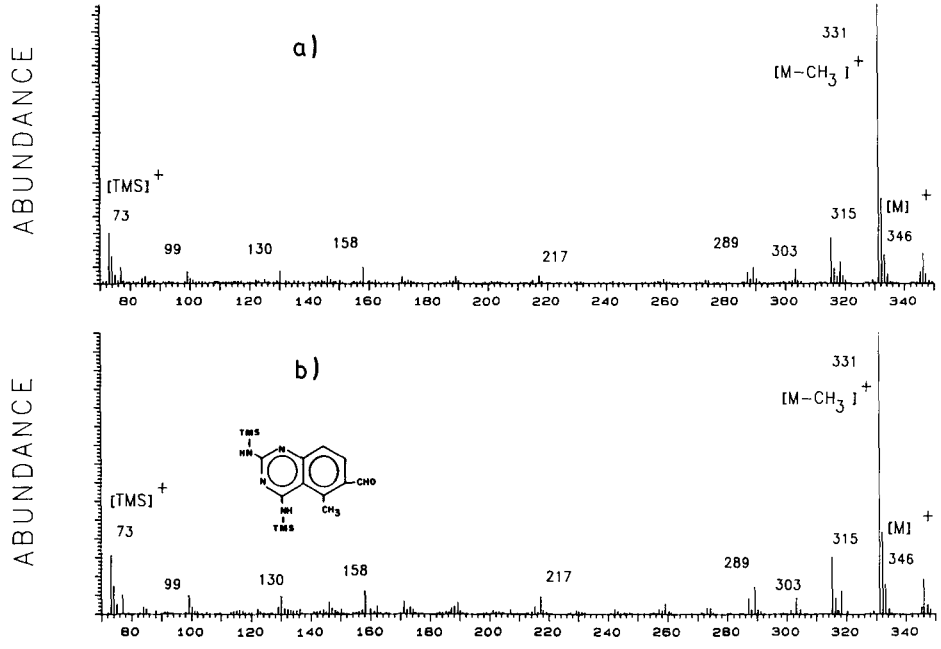

Fig. 6. EI mass spectra of (a) trimethylsilylated degradation product (peak A in Fig. 2), and (b) authentic AMCQ-bisTMS.

linkage. The degradation product's mass spectrum (Fig. 6a) suggests that its molecular ion is at m/z 346, and its base peak is $[M-15]^+$ at m/z 331, in this case due to loss of a methyl group from one of the TMS adducts. Also present were significant peaks at m/z 345 and 318 corresponding to the $[M-1]^+$ and $[M-CO]^+$ peaks which are characteristic of aromatic aldehydes.

GC–MS analysis of these samples in the methane CI mode resulted in base peaks of TMQ and its degradation product at the corresponding $[M+1]^+$ ions (m/z 514 and 347, respectively). Also characteristic of methane CI, each mass spectrum included an $[M+29]^+$ ion, due to the addition of (C_2H_5). Finally, the fragmentation patterns were consistent with TMS derivatives showing a significant $[M-15]^+$ peak. The TMQ-bisTMS CI mass spectrum retained as its second largest peak the ion at m/z 331.

Additional evidence for the presence of an aldehyde in the incubated samples was obtained with the 2,4-dinitrophenylhydrazine test for aldehydes and ketones[14]. The zero-time sample tested negative for aldehydes and ketones (no precipitate formation). However, the 56-day sample elicited a strong positive response with the immediate formation of an orange-red precipitate (the insoluble dinitrophenylhydrazone).

The mass spectral and chemical data suggested the identity of the degradation product as AMCQ. Mass spectral (Fig. 6) and UV–VIS absorption spectral (Fig. 4) analysis of authentic AMCQ confirmed this conclusion.

In conclusion, the chemical stability of the pharmaceutical preparation (Trimetrexate glucuronate) of this non classical antifolate has been evaluated. Incubation in sterile, amber glass vials at 37°C for 56 days resulted in a degradation rate constant of 0.0134 ± 0.0002 day^{-1} and a half-life of 51.6 ± 0.8 days. The major degradation product has been identified as AMCQ. Ten percent TMQ degradation would occur by 7.9 days of incubation under these conditions. Therefore, use of this drug preparation in implanted drug delivery systems would necessitate weekly pump refill or replacement if one sets 10% degradation as the maximum allowed.

ACKNOWLEDGEMENT

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REFERENCES

1 E. F. Elslager and J. Davoll, in R. N. Castle and L. B. Townsend (Editors), *Lectures in Heterocyclic Chemistry*, Vol. II, Hetero Corp., Orem, UT, 1974, pp. 5–97.
2 J. J. McCormack, in W. Pfleiderer (Editor), *Chemistry and Biology of Pteridines*, Walter de Gruyter, New York, NY, 1976, p. 125.
3 J. R. Bertino, W. L. Sawicki, B. A. Moroson, A. R. Cashmore and E.F. Elslager, *Biochem. Pharmacol.*, 28 (1979) 1983.
4 H. Diddens, N. Niethammer and R. C. Jackson, *Cancer Res.*, 43 (1983) 5286.
5 J. R. Bertino, *Cancer Res.*, 39 (1979) 293.
6 G. F. Borsa and F. A. Whitmore, *Cancer Res.*, 29 (1969) 737.
7 G. F. Borsa, F. A. Whitmore, F. A. Valeriote, D. Collins and W. R. Bruce, *J. Natl. Cancer Inst. (U.S.)*, 42 (1969) 235.
8 H. E. Skipper, F. M. Schabel, L. B. Mellett, J. A. Montgomery, L. J. Wilkoff, H. H. Lloyd and R. W. Brockman, *Cancer Chemother. Rep.*, 54 (1970) 431.

9 B. A. Chabner and R. C. Young, *J. Clin. Invest.*, 52 (1973) 1804.
10 H. M. Pinedo, D. S. Zaharko, J. M. Bull and B. A. Chabner, *Cancer Res.*, 37 (1977) 445.
11 D. S. Zaharko, *Pharmacological Basis of Cancer Chemotherapie*, Williams and Wilkins, Baltimore, MD, 1975, pp. 69–84.
12 F. M. Sirotnak, *Pharmacol. Ther. Part A*, 8 (1980) 71.
13 W. D. Ensminger and J. W. Gyves, *Cancer Treat. Rep.*, 68 (1984) 101.
14 D. J. Pasto and C. R. Johnson, *Organic Structure Determination*, Prentice-Hall, Englewood Cliffs, NJ, 1969, p. 382.

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Note

Application of deactivated metal capillaries to the analysis of solvents in varnish

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The analyses of volatile components evolved from varnish while drying is becoming very important from the point of understanding the degree of dryness of the varnish, especially when applied to precision electrical parts, and also from an environmental point of view. For such analysis, the static head space method is a most popular technique, but requires a special attachment on the gas chromatograph and may lack sensitivity towards trace amounts of volatile components and generally towards components having boiling points above 130°C. Some examples[1,2] of solvents having boiling points above 130°C have been analysed by heating the sample, but the necessity of an exclusively designed attachment has prevented general acceptance of the method as a practical technique.

The dynamic head space method is superior in sensitivity to the static head space method because all the volatiles that are trapped are subjected to gas chromatograpic (GC) analyses. Some volatiles from solid materials have been analysed by the dynamic head space method using a combination of a capillary column and a capillary cold trap[3,4]. Special devices were designed in each case for cooling and heating the trap, showing that the method is not yet routine. A special technique for focusing was reported[5] for the head space method, by cooling the column head prior to passage of the sample to the capillary column. There is also a report[6] on focusing with the dynamic head space method in which the volatiles trapped in a Tenax TA cartridge were focused in a fused-silica capillary trap prior to passage to a fused-silica capillary column. Also in this method, special devices must be used for cooling and heating the trap. For the method to become routine, a simple technique for cooling the trap by liquid nitrogen and for rapid heating of the trapped solvents prior to passage to the column should be developed.

The analyses of volatiles in paint has been performed either for the purpose of analysing the solvents in the paint itself or for the purpose of analysing the remaining volatiles in the film[1–5,7,8]. The latter has been performed only in the case of simple components, and, to our knowledge, there is no report on the analyses of the complex mixture of remaining volatiles including alcohols.

Here, a new approach to the dynamic head space method was devised to trace

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the decrease of solvents in varnish. First we will describe how easy cooling and heating is achieved by using a deactivated metal capillary tube[9,10] as a cold trap; secondly, by using a deactivated metal capillary column[11] as a separation column, it was very easy to remove the column from the oven, which enables the ready cooling of the column and facilitates the separation of low boiling solvents; finally, by utilizing the above advantages, it becomes possible to monitor changes in the components of solvents remaining in the varnish during drying which have a complex composition including alcohols.

EXPERIMENTAL

Instrument and sample

For gas chromatography, an Okura Model 701 was used with flame ionization detection (FID), a deactivated metal capillary column, 20 m × 0.25 mm I.D., coated with 0.4 μm OV-1 and a Shimadzu Chromatopac C-RIA as an integrator. The "RAS" metal capillary[9,10] used for the trap capillary, 40 cm × 14 cm × 1 mm I.D. × 2 mm O.D. was supplied by Nippon Chromato (Tokyo, Japan) with the metal capillary column mentioned above. The rapid heating apparatus for the trap capillary included an insulating transformer having an output of 5 V, 15 A, two lead wires between the transformer and both ends of the trap, and an element for measuring the temperature of the trap which was heated electrically as shown in Fig. 1.

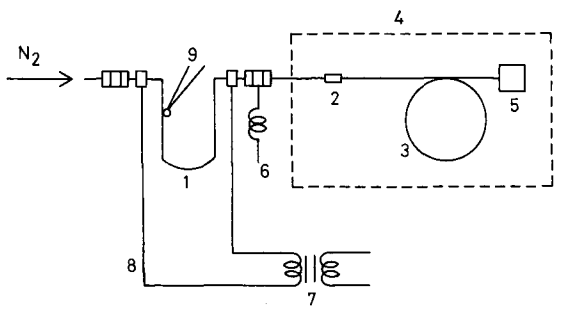

Fig. 1. Method of GC analysis for the samples collected in the trap. 1 = Trap; 2 = PTFE tube; 3 = "RAS" capillary column; 4 = oven; 5 = flame ionization detector; 6 = split; 7 = transformer; 8 = lead wire; 9 = AC thermocouple.

A cylindrical glass vessel, 10 cm × 30 mm I.D., was used as the sample container, to which a glass pipe (10 cm × 6 mm O.D.) is welded to one and an inner joint (29/42) with a glass cap to the other end. A stainless-steel joint (6 mm/2 mm) was used to connect the pipe to the end of the trap.

Collection and GC analyses of volatile components

Onto an aluminium foil (2 cm × 2 cm) was coated a sample of varnish (5–20 mg). After drying for an appropriate time, the foil was put into the sample container. The container was connected to the collection line as shown in Fig. 2, and was evacuated at 1 mmHG for 1 h with the trap dipped in liquid nitrogen so as to collect the volatile components.

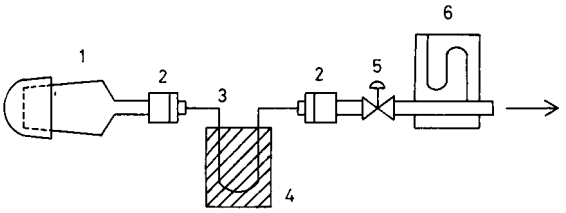

Fig. 2. Collection of volatiles from varnish. 1 = Sample container; 2 = metal joint 6 mm / 2 mm; 3 = trap capillary; 4 = liquid nitrogen; 5 = vacuum cock; 6 = manometer.

The trap with volatile components was connected to the chromatograph as shown in Fig. 1. While cooling the trap, the baseline of the chromatogram was recorded, and then the trap was directly heated by the electric current at 150°C for 5 min in order to transport the volatiles to the chromatograph. Count numbers, which are proportional to peak areas, were measured by the integrator. GC analyses was conducted under the following conditions; nitrogen pressure 1.1 kg/cm^2; column temperature after removal from the oven, held at room temperature for 10 min after the trap heating, then placed in the oven at 60°C followed by programming at 10°C/min; sensitivity $8 \cdot 10^{-11}$; splitting ratio 320:1; velocity 36.6 cm/s.

Calculation of degree of drying and composition of volatiles

When W_0 mg of undried varnish was analyzed by GC to give a total count number, C_0, the count number for 1 mg of the sample is C_0/W_0. For S_0 mg of undried varnish, total count numbers, C_s, can be obtained by $C_s = S_0C_0/W_0$. When S_0 mg of the above undried sample was partly dried to S_1 mg, and the total count number for S_1 mg was obtained, C_d, the degree of drying can be calculated as:

$$\text{Degree of drying} = (1 - C_d/C_s) \cdot 100\%$$

The composition of the volatiles was calculated from the area per cent.

RESULTS AND DISCUSSION

Blank test of the system

A blank test was performed without varnish in the sample container. The trap was heated in order to transport anything trapped for GC analysis. Many large peaks were obtained but there was no reproducibility. By washing the apparatus and joints, and also applying grease to the nearest part of the sample container to the cap, the problem was solved, giving a blank test chromatogram which was almost flat.

Collection time and length of trap

After an hour's collection, the trap was changed to a new one, but another one hour's collection did not give a further appreciable amount, which means that an hour's collection is sufficient. Two traps were connected in series to estimate the necessary trap length; it was confirmed that no appreciable amount of sample was collected in the second trap, suggesting that one trap is enough.

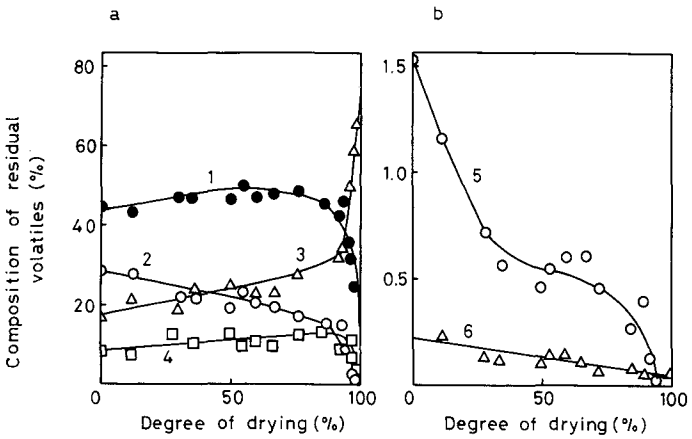

Fig. 3. Residual volatiles in the varnish *vs.* degree of drying: (a) major components; (b) minor components. Curves: 1 toluene; 2 ethyl acetate; 3 isobutanol; 4 isobutyl acetate; 5 methyl acetate; 6 methanol.

Drying time and residual volatiles in the varnish

The drying time was changed to determine the effect on the composition of the residual volatiles. The results are shown in Fig. 3. They show that, at the initial stage, ethyl acetate and methyl acetate vaporize rapidly. Isobutanol becomes predominant in the residual volatiles at the final stage. Fig. 4 shows the chromatogram at 98.4% drying.

Degree of drying

The degree of drying was calculated according to the method described in the Experimental section. It can also be obtained from the weight change of the varnish.

$$\text{Degree of drying} = [1 - (W - W_{100}) / (W_0 - W_{100})] \cdot 100\%$$

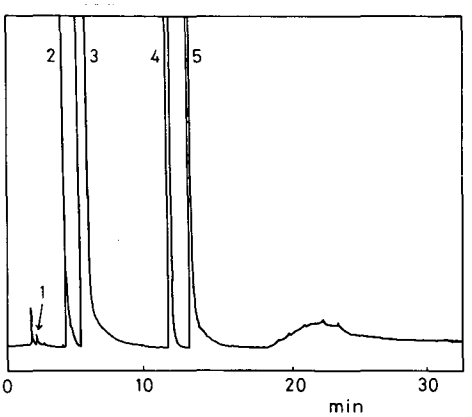

Fig. 4. Chromatogram of the volatiles in the varnish. For GC conditions see Experimental. Degree of drying = 98.4%. Peaks: 1 = methyl acetate; 2 = ethyl acetate; 3 = isobutanol; 4 = toluene; 5 = isobutyl acetate.

where W = weight of varnish after an appropriate drying, W_0 = weight of varnish just after coating and W_{100} = weight of completely dried varnish. The correlation of these two values is as follows:

$$\text{Degree of drying (weight change)} = 0.88 \cdot \text{degree of drying (calculated)}$$

Deactivated metal capillary tube as a cold trap

A naked stainless-steel capillary tube was used as a cold trap[12], and also, desorption was conducted by electrically heating the tube[13]. According to our experiment, however, cold trapped samples, even hydrocarbons, became very hard to recover as the carbon number increased to more than 12, which suggests that quantitative recovery of hydrocarbons that have more than 16 carbons is questionable. We have also found that polar solvents such as alcohols cannot be recovered at all, even those of low molecular weight. The difference between our results and those reported can be attributed to the difference in surface activity of naked stainless-steel capillaries, but considering the fact that the metal surface is active, naked stainless-steel is by no means a good choice for a cold trap.

An alternative is the use of a fused-silica capillary tube as a cold trap[4,5]. However, fused silica has the disadvantage that it is hard to bend to an U shape, especially that of 0.53 mm I.D., and is easy to break.

A solution of this problem was achieved by using a completely inert, deactivated metal capillary tube[9]. The tube can be bent in any way and can be electrically heated . For transport of the trapped volatiles into the capillary column, special devices are necessary with glass or fused-silica capillary. With a metal capillary, the trapped volatiles are easily transferred by only electrically heating of the trap.

Advantage of deactivated metal capillary column

The deactivated metal capillary column withstood continuous use at 450°C in the case of polymethylsiloxane as the stationary phase and retained sufficient mechanical strength for column exchange after use. Thus the column is advantageous as a high-temperature GC column compared with a fused-silica capillary[11].It also has an advantage in the analysis of samples including low boiling compounds. In such analyses, it is desirable to set the initial temperature below 50°C, which is usually difficult due to the remaining heat of the oven and also due to the heat transfer from the flame ionization detector. The metal capillary has a great advantage in that it can be used very easily at initial temperatures of below 50°C, because it can be removed from the oven easily without disconnecting to operate at or below room temperature, and then returned to the oven for further temperature programming. Though fused-silica capillary is flexible to some extent, the elastic limit and the break point are the same, and treatment in this way may lead to breakage of the column.

Potential of dynamic head space method

In the above experiment, volatiles were collected under a vacuum. If gas can be passed through the sample container, the conventional dynamic head space method can be applied. In the case that a small quantity of volatiles is to be analysed, the dynamic head space method[14] is possible, in which the capillary column is removed from the oven, and inlet side of the column is disconnected from the gas chromatograph to

connect it to the sample container and the column is cooled to collect volatiles. Also in this method, a metal capillary has an advantage over a fused-silica one because metals do not break.

CONCLUSIONS

Tracing of the decrease in solvents in varnish was carried out satisfactorily by using a deactivated metal capillary tube as a cold trap and a deactivated metal capillary column as a GC column. The metallic cold trap was rapidly heated directly with an electric current and the capillary column was removed from the oven while it was connected to the gas chromatograph to be chilled to obtain the peaks of the separated low volatile solvents and after an appropriate time it was replaced in the oven for further temperature programming.

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REFERENCES

1 B. Kolb and P. Pospisil, *Chromatographia*, 10 (1977) 705.
2 D. M. Wyatt, *Am. Lab. (Fairfield, Conn.)*, April (1983) 66.
3 S. Jacobsson, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7 (1984) 185.
4 S. Schmidt, L. Blomberg and T. Wännman, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 242.
5 B. Kolb and B. Liebhardt, *Chromatographia*, 21 (1986) 305.
6 M. Termonia and G. Alaerts, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 622.
7 D. A. Buckley, F. G. Belli and P. P. Augostini, *Am. Chem. Soc., Div. ORPL, Paper*, 38 (1978) 737.
8 S. R. Smith, M. A. Krenceski, J. Hubball, E. D. Erickson and J. H. Johnson, *J. Adhesion*, 18 (1984) 157.
9 Y. Takayama and E. Sumiya, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 747.
10 Y. Takayama, E. Sumiya and S. Kawai, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 201.
11 Y. Takayama, T. Takeichi and S. Kawai, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1988) 732.
12 A. Zlatkis, F. S. Wang and H. Shanfield, *Anal. Chem.*, 54 (1982) 2406.
13 J. A. Settlage and W. G. Jennings, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 146.
14 L. A. Fergason, *Macrocontamination*, April (1986) 37.

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Note

Dynamic headspace method for the improved clean-up of gunshot residues prior to the detection of nitroglycerine by capillary column gas chromatography with thermal energy analysis detection

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(Received October 19th, 1988)

The detection of traces of nitroglycerine (NG) and other explosive materials is of forensic importance[1–4], and a gas chromatographic (GC) method with thermal energy analysis detection (TEA)[5] has been in use here for the last 18 months. The system has primarily been employed for the detection of NG in gunshot residues, but other explosives have also been studied. With pure standards it is possible to detect down to about 5 pg of NG but such sensitivity cannot be achieved with extracts from handswabs or vacuumed samples from clothing, for such extracts are substantially contaminated with lipid and other materials. Clean-up is essential to minimise column deterioration and detector noise, and without it detection limits of 5–20 ng of NG/swab are the best attainable. The clean-up procedure previously developed here[6] exploits selective solid phase extraction on to Amberlite XAD-7 polymeric beads and subsequent elution with solvent mixtures of the appropriate polarity and volatility. The clean-up is adequate for many samples, but during experiments on clothing it was frequently found that contamination was too great, and this led to the development of the procedure described in this paper.

The procedure exploits the volatility of NG to provide a primary clean-up. This approach has frequently been used before[7–10], but by trapping out the NG vapour on to the XAD-7 beads, followed by solvent elution a novel and convenient method for a GC–TEA end analysis has been developed. Although mainly applied to the detection of NG in gunshot residue samples, it is apparent that many volatile explosives can be isolated from coextractives by the described method, and hence it could be more generally applicable to the detection of explosive residues. It has been found in the case of NG that after the two-stage clean-up as much as 20% of the final extract can be analysed; this equates with detection limits on filters of about 50–100 pg.

EXPERIMENTAL

Reagents

Nitroglycerine (NG), ethyleneglycol dinitrate (EGDN), butane-1,2,4-triol-trinitrate (BTN), triethyleneglycol dinitrate (TEGDN), 2,4,6-trinitrotoluene (TNT) and RDX (hexogen) were obtained from the Propellants Explosives and Rocket Motors Establishment (PERME) (Waltham Abbey, U.K.). Nitrobenzene (NB), and 4-nitro-

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toluene (4-NT) were obtained from Aldrich (Gillingham, U.K.). 2,4-dinitrotoluene (2,4-DNT) was obtained from Fluka (Glossops, U.K.) and Musk Tibetine was supplied by Givaudin (Whyteleafe, U.K.).

All solvents used were pesticide grade (Fisons, Loughborough, U.K.) except for methyl *tert.*-butyl ether (MTBE) which was HPLC grade (Rathburn Chemicals, Walkerburn, U.K.) and diethyl ether which was Analar grade (BDH, Poole, U.K.).

Sample preparation

Firing was conducted under the conditions described previously[6]. Handswabs and vacuumed clothing samples (sampled at an air flow-rate of 2.6 l/min) were generated using procedures described in the same publication. The filters from vacuuming experiments were spiked by applying known quantities of explosives dissolved in redistilled diethyl ether. The ether was allowed to evaporate by standing at room temperature for about 10 min.

Sample pretreatment

Handswabs were placed in a luer-lock glass syringe fitted with filters identical to that used for vacuuming. The MTBE solvent on the swab was removed under a gentle stream of nitrogen and the swab was then moistened with 400 μl of acetone and allowed to stand for 5 min. The acetone was then removed under a gentle stream of nitrogen before the syringe was inserted in the dynamic head-space apparatus. For other explosives where nitrocellulose was not present the acetone treatment just described was not necessary.

Syringes containing filters on which residues had been trapped by vacuuming were moistened with 200 μl of acetone. Care was necessary to wash the walls of the syringe to ensure complete transfer of particulate matter on to the filter. The filter was allowed to stand for 5 min and the solvent was then removed under a stream of nitrogen. The syringe was then inserted in the dynamic headspace apparatus. The addition of acetone was not necessary if explosives were present in the absence of nitrocellulose.

Dynamic headspace sampling

The equipment used for the headspace clean-up is shown in Fig. 1. The luer-lock glass syringe containing the filter or swab was connected to a glass column containing 18 mg of Amberlite XAD-7 resin; the dimensions of this column were identical to the clean-up columns described in a previous publication[6]. The column and syringe were butt-connected using a short length of PVC tubing (3 mm I.D.,

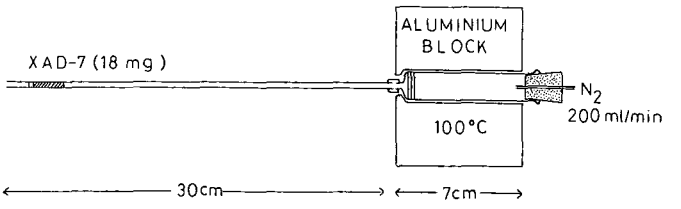

Fig. 1. Dynamic headspace system.

5 mm O.D. transparent tubing from Gallenkamp, Loughborough, U.K.) and the syringe barrel was plugged with a rubber bung (BS No. 13 solid, BDH). Nitrogen (high purity grade, Air Products, Southampton, U.K.) was passed at a rate of 200 ml/min through a stainless-steel hypodermic needle (size 21 gauge × 1.5 in.) inserted through the rubber bung. The apparatus was then inserted into a machined aluminium block held at 100°C which was mounted in a heated unit (Reactitherm, Pierce, Chester, U.K.). After 7 min, the syringe and column were separated, removed from the aluminium block and allowed to cool.

Elution of explosives from the porous polymer

The column containing the XAD-7 beads was mounted vertically and was eluted in the following sequence: (1) 1 ml of pentane to elute NB and 4-NT; (2) 1 ml of pentane–MTBE (1:1, v/v) to elute unwanted coextractives; (3) 0.4 ml of ethyl acetate to elute NG and other explosives. Fraction 1 was reduced in volume to about 50–100 μl by warming on the aluminium block. Fraction 3 was concentrated under a gentle stream of nitrogen to a final volume of 5–20 μl.

Elution of less volatile explosives from the residue in the glass syringe

The syringe containing residual material was clamped vertically and 2 ml of MBTE was passed through it. The solution was then passed through a glass column containing a freshly prepared bed of XAD-7 beads, and a further 1 ml of MBTE was added to remove unwanted coextractives. Explosives were eluted with 400 μl of ethyl acetate and this fraction was evaporated under a stream of nitrogen to a final volume of about 5–20 μl.

Detection of NG and other explosives by GC–TEA

Chromatography was carried out using the chromatographic conditions previously described[5], and 1 μl aliquots of the appropriate fractions were injected. The TEA detector was used in the modified form described in the reference just cited. NG and most other explosives were monitored with a pyrolytic breakdown at 625°C, NB, 4-NT and 2,4-DNT required a pyrolysis temperature of 750°C.

RESULTS AND DISCUSSION

By examining both the XAD-7 beads and the residual material left on filters after dynamic headspacing it was found that effective transfer of NG to the beads from gunshot residues only took place if the filter was moistened with acetone before sampling. This was attributed to the NG being trapped inside nitrocellulose particles, and only becoming available for vaporisation by dissolution of the particles in acetone. The temperature of the aluminium block, the nitrogen flow-rate and the residence time in the block were optimised by experiment. The efficacy of a single XAD-7 trap was confirmed by mounting two traps in series and establishing that no NG was breaking through to the second trap. Recovery of NG from filters spiked at 5 ng was 77% (relative standard deviation, R.S.D., for $n = 5$ was 3.9%).

Although the headspace procedure was effective in separating NG from less volatile compounds it was still found necessary to selectively elute volatile impurities from the XAD-7 beads before injecting samples on to the GC-TEA system. The

two-fold clean-up process permitted the final extract to be reduced to a very low volume and as a result detection levels of about 100 pg of NG per swab or filter could be attained. For quantitation it was found that use of BTN as an internal standard permitted linear calibration plots to be constructed.

The GC-TEA system has been found to be a robust combination and for the detection of NG a pyrolysis temperature of 625°C provides both sensitivity and selectivity. Nitromusks, low-cost nitrated materials extensively used in perfumery, are also detectable under these pyrolysis conditions and being volatile materials are not separated from NG by the headspace procedure. These compounds have frequently been observed in clothing extracts, but selective elution from the XAD-7 beads and substantial GC retention time differences between them and NG suggests that they will not create an interference problem. Chromatograms of a handswab and clothing of a person having fired a hand-gun are shown in Fig. 2. The silica pyrolysis tube mounted between the GC and the TEA detector has proved to be very resistant to contamination and the one used in this work has been in use for over 6 months, whereas three GC columns had to be discarded during the same period because of contamination. With the clean-up procedure which has been developed it is anticipated that the GC columns will also have a greatly extended operational life and the pyrolysis tube may well be usable for a year or more.

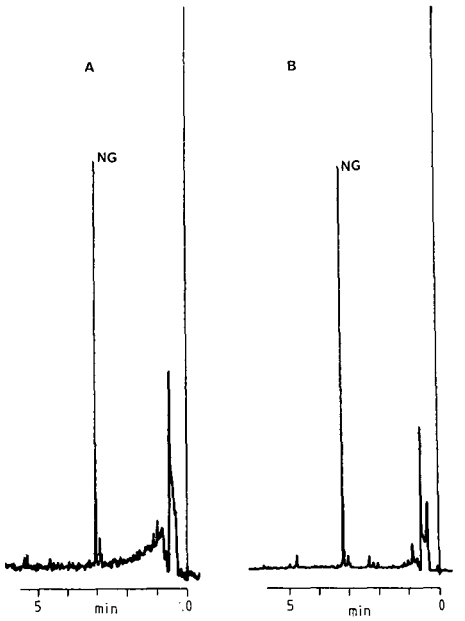

Fig. 2. Chromatograms of handswab and clothing extracts subjected to dynamic headspace clean-up. (A) Handswab extract taken from the firing hand of a subject 1 h after having discharged three rounds of Winchester Super X ammunition from a Smith & Wesson Model 19 handgun. Pyrolysis temperature, 625°C; attenuation, 20 mV; injection volume 1 μl from 114 μl. (B) Clothing extract sampled from shirt of a subject 21.3 h after discharging a handgun as at (A). Pyrolysis temperature, 625°C; attenuation, 50 mV; injection volume, 1 μl from 25 μl.

Although primarily directed at the detection of NG in handswabs many other volatile explosives and nitrated molecules can be separated and trapped by the dynamic headspace procedure described. The retention times for a selection of compounds amenable to this form of clean-up are shown in Table I, together with optimum pyrolysis temperatures. The nitramines, nitrate esters and trinitro-aromatics are all adequately detected at a pyrolysis temperature of 625°C, but the more stable

TABLE I

RELATIVE RETENTION TIMES OF EXPLOSIVES OF FORENSIC INTEREST

GC conditions: chromatograph, Carlo Erba Model 4160; injector, splitless mode at 175°C; column, BP-1 fused-silica capillary 12 m × 0.25 mm I.D. (SGE, Milton Keynes, U.K.), stationary phase film thickness, 0.25 μm; temperature programme, 60°C for 1 min then 39.9°C/min to 250°C, held for 1 min.

Explosive	*Optimum pyrolysis temperature (°C)*	*Fraction*[a]	*Relative retention time*
EGDN	625	b	0.27
NB	750	a	0.34
4-NT	750	a	0.47
NG	625	b	0.57
2,4-DNT	750	b	0.72
TNT	625	b	0.83
RDX	625	c	0.91
Musk Tibetine	625	b	1.00 (4.84 min)

[a] a = Eluted from XAD-7 beads with pentane; b = eluted from XAD-7 beads with ethyl acetate; c = retained on filter and eluted with MBTE.

mono- and dinitroaromatics (NB, 4-NT and 2,4-DNT) require a temperature of 750°C before they give an acceptable signal. Chromatograms of test mixtures analysed under the two alternative pyrolysis conditions are shown in Fig. 3. Fig. 4 shows chromatograms of mixtures of explosives volatilised from spiked filters and subsequently eluted from the XAD-7 trap with pentane (in the case of NB and 4-NT) or ethyl acetate for the more polar explosives. Of the explosives tested only NC and RDX remained behind on the filter after being subjected to the dynamic headspace clean-up procedure and Fig. 5 shows the chromatogram produced when a sample originally spiked with 6 ng of RDX and TNT was analysed by the procedure described under *Elution of less volatile explosives from the residue in the glass syringe* after volatilisation. The chromatogram shows that a trace of TNT also remained on the filter. Fig. 6 compares very dirty vacuum filter extracts spiked at the 5-ng level with NG. One sample (A) was cleaned-up by adsorption on to XAD-7 beads followed by elution with pentane–MTBE (1:1)[6], the other (B) was subjected to the dynamic head spacing procedure described above. It is apparent from the chromatograms that higher signal to noise levels were attainable in sample B and that a higher proportion of the extract could be directly analysed.

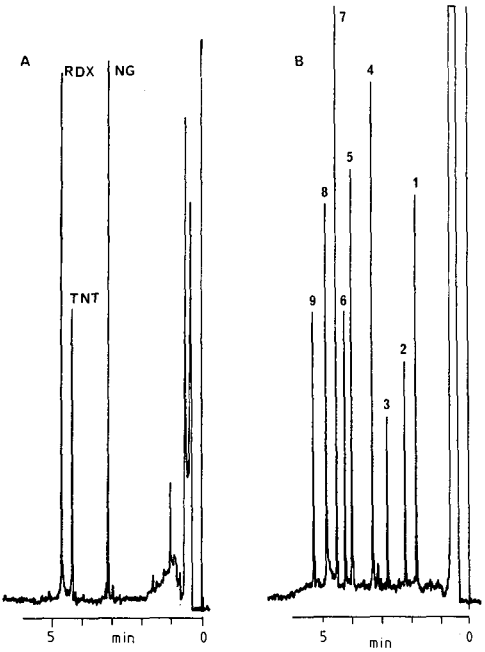

Fig. 3. Chromatograms of test mixtures of explosives. (A) 50 pg of each explosive. Pyrolysis temperature, 625°C; attenuation, 20 mV. (B) 1 = EGDN, 45 pg; all other compounds 90 pg, 2 = NB; 3 = 4-NT; 4 = NG; 5 = 2,4-DNT; 6 = TEGDN; 7 = TNT; 8 = RDX; 9 = Musk Tibetine. Pyrolysis temperature, 750°C; attenuation 20 mV.

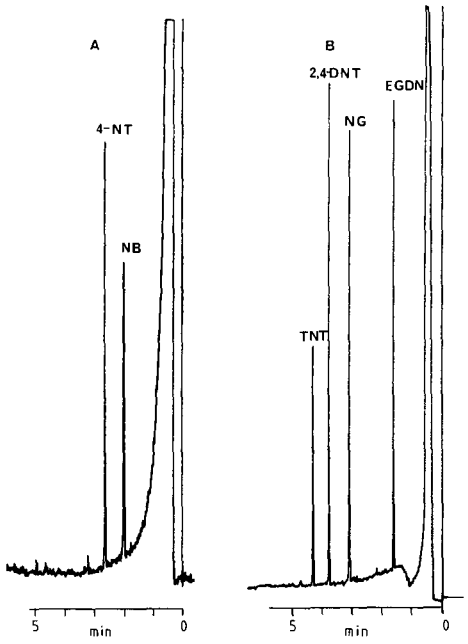

Fig. 4. Chromatograms of explosives volatilised from spiked filters. (A) Filter spiked with 200 ng of each compound. Pyrolysis temperature, 750°C; attenuation, 20 mV; injection volume, 1 μl from 450 μl pentane. (B) Filter spiked at the following levels 15 ng of NG and TNT, 30 ng of EGDN and 2,4-DNT. Pyrolysis temperature, 750°C; attenuation, 20 mV; injection volume, 0.75 μl from 26 μl EA.

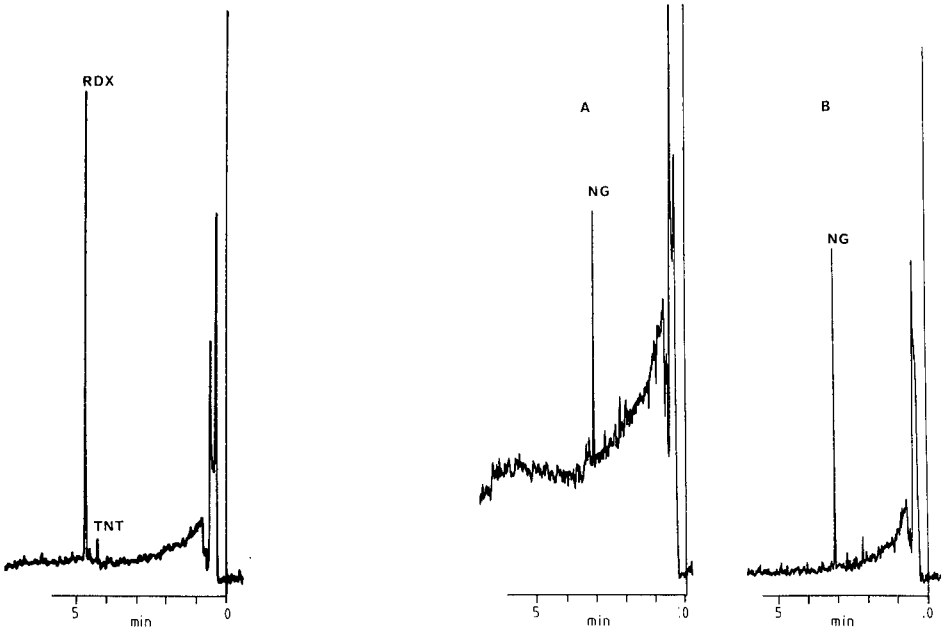

Fig. 5. Chromatogram of explosives retained on a filter after dynamic headspacing. Filter spiked with 6 ng of RDX and TNT. Pyrolysis temperature. 625°C; attenuation, 20 mV; injection volume, 1 μl from 20 μl.

Fig. 6. A comparison of the chromatograms produced from very dirty vacuum filter extracts spiked at 5 ng with NG. (A) XAD-7 clean-up[6]. Injection volume, 0.75 μl from 15 μl (*i.e.*, 5% of extract). (B) Dynamic headspace clean-up. Injection volume, 1 μl from 11 μl (*i.e.*, 9% of extract). Pyrolysis temperature for both samples was 625°C.

CONCLUSIONS

The dynamic headspace procedure described permits volatile explosives such as NG to be effectively separated from involatile impurities that reduce the selectivity and column life of the GC-TEA procedure. By trapping the volatilised explosives on to XAD-7 beads and effecting additional clean-up by selective solvent extraction under the most favourable conditions, detection limits of sub-ng quantities per swab or filter can be achieved. Life of the GC columns is also extended by removal of involatile coextractives before injection.

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REFERENCES

1 A. Beveridge, *J. Energetic Mater.*, 4 (1986) 29.
2 J. B. F. Lloyd, *J. Forensic Sci. Soc.*, 26 (1986) 341.
3 C. M. Selavaka, R. E. Tontarski, Jr. and R. A. Strobel, *J. Forensic Sci.*, 32 (1987) 941.
4 J. M. F. Douse, *J. Chromatogr.*, 445 (1988) 244.
5 J. M. F. Douse, *J. Chromatogr.*, 410 (1987) 181.
6 J. M. F. Douse and R. N. Smith, *J. Energetic Mater.*, 4 (1986) 169.
7 F. J. Conrad, T. A. Burrows and W. D. Williams, *J. Chromatogr.*, 176 (1979) 37.
8 J. E. Chrostwoski, R. N. Holmes and B. W. Rehn, *J. Forensic Sci.*, 21 (1976) 611.
9 R. J. Sullivan and G. W. Watson, in *Proceedings of the International Symposium of the Analysis and Detection of Explosives, Quantico, VA, 1983*, NTIS, Springfield, VA, p. 473.
10 D. P. Lucero and E. M. Boncyk, *J. Energetic Mater.*, 4 (1986) 473.

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Note

Limitations of 2-diphenylacetyl-1,3-indandione-1-hydrazone as a pre-column fluorescence derivatization reagent

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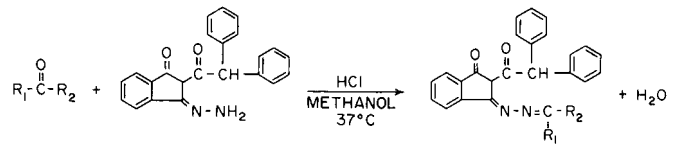
The qualitative and quantitative analysis of clinical and biomedical samples is a challenging problem that demands continual improvement of analytical instrumentation and methodology. The high complexity of such samples necessitates the improvement of separation methods, such as high-efficiency chromatographic and electrophoretic techniques. A promising technique which appears to be particularly well suited for the separation of complex biological samples is microcolumn liquid chromatography (LC)[1,2]. This technique is rapidly gaining recognition because of the very high separation efficiencies that can be achieved[3]. Other benefits of microcolumn LC include the reduced consumption of both sample and solvent[4], as well as the possibility of using novel detection methods[5–7].

Although many analytical techniques have been employed, laser-based detection methods seem especially promising for microcolumn LC[8]. The high intensity and narrow spectral bandwidth of the laser emission can provide sensitive and selective excitation using a wide variety of optical detection methods such as fluorescence, phosphorescence, polarimetry, refractometry, etc. Moreover, the highly collimated laser radiation can be readily focused into small-volume flowcells, as required for chromatographic detection, with minimal loss of radiant power. Although this combination of analytical techniques is still in the preliminary stages of development, microcolumn LC with laser-induced fluorescence detection appears to provide a powerful tool for the characterization of complex clinical and biomedical samples[9–12].

Not all molecules are naturally fluorescent at a given laser wavelength; hence, it may be necessary to chemically modify the analytes of interest to incorporate a fluorescent label[11–14]. In this study, we have examined the application of 2-diphenylacetyl-1,3-indandione-1-hydrazone (DPIH) as a fluorescent probe selective for ketone and aldehyde functional groups.



This reagent has been employed previously for qualitative identification in spot tests[15,16], and as a visualization agent in thin-layer chromatography[17,18]. In addition, Swarin and Lipari[19] have used DPIH for the identification of simple aliphatic ketones

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and aldehydes in automobile exhaust by LC. In this study, we have examined the use of DPIH as a derivatizing agent for ketosteroids, in conjunction with microcolumn LC separation and laser-induced fluorescence detection. This methodology will enhance the separation and selective detection of ketosteroids in the presence of other steroid classes, thus advancing clinical and biomedical analysis.

EXPERIMENTAL

Methodology

Standard solutions (10^{-5} *M*) are prepared by dissolving the ketosteroids (Sigma, St. Louis, MO, U.S.A.) in anhydrous methanol. A saturated solution (*ca.* $3 \cdot 10^{-4}$ *M*) of the derivatization agent is prepared by dissolving DPIH (Aldrich, Milwaukee, WI, U.S.A.) in anhydrous methanol. Equal volumes (3.0 ml) of the standard steroid and DPIH solutions are mixed together in a 10-ml vial, and 0.14 ml concentrated hydrochloric acid (12 *M*, J. T. Baker, Phillipsburg, NJ, U.S.A.) is added as catalyst. In some studies, aluminum(III) chloride, chromium(III) chloride, tin(II) and tin(IV) chlorides as well as magnesium bromide (Aldrich) are added as auxiliary catalysts. The reaction is allowed to proceed without stirring at 40°C for 30 min to 3 h. The solution is then clarified by using a syringe filtration system (Millipore, Bedford, MA, U.S.A.), and the solvent is evaporated under a stream of dry nitrogen. Although the derivatized steroids decompose in several hours at room temperature, they are stable for several days when stored without solvent at 0°C.

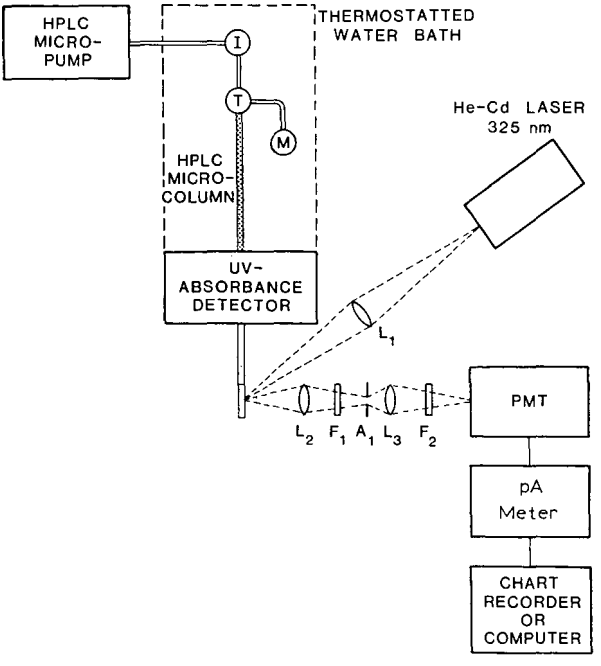


Fig. 1. Schematic diagram of the analytical system using microcolumn liquid chromatography with laser-induced fluorescence detection. I = Injection valve; T = splitting tee; M = metering valve for splitter; L = lens; F = filter; A = aperture; PMT = photomultiplier tube.

The derivatives are analyzed by high-efficiency microcolumn LC with detection by UV absorbance and laser-induced fluorescence methods. The analytical system is shown schematically in Fig. 1, and is described further in the following sections.

Chromatographic system

A single-piston reciprocating pump (Model 114M, Beckman, San Ramon, CA, U.S.A.) is used in the constant-pressure mode for solvent delivery. Samples are introduced by using a 1.0-μl valve injector (Model ECI4W1, Valco Instruments, Houston, TX, U.S.A.), after which the effluent is split (10:1 to 100:1) and applied to the chromatographic column. The microcolumn utilized for this study is prepared from fused-silica capillary tubing (89 cm × 200 μm I.D., Hewlett-Packard, Avondale, PA, U.S.A.) which is packed with an octadecylsilica material (Spheri-5 RP-18, 5 μm, Brownlee Labs., Santa Clara, CA, U.S.A.), as described previously[20]. These microcolumns approach the theoretical limits of efficiency, and are capable of achieving theoretical plate numbers in excess of 100 000 under standard test conditions[20].

UV absorbance detector

A variable-wavelength UV-absorbance detector (Model Uvidec 100-V, Jasco, Tokyo, Japan) is modified for microcolumn LC by using a 25-nl fused-silica flowcell (0.5 cm × 80 μm I.D.). Light throughput from the deuterium lamp source is limited by razor-blade slits mounted parallel to the capillary flowcell.

Laser fluorescence detector

A helium–cadmium laser (Model 3112-10S, Omnichrome, Chino, CA, U.S.A.), with approximately 10 mW of continuous-wave output at 325 nm, is used as the excitation source. The laser radiation is focused directly upon the optically transparent fused-silica microcolumn using a fused-silica lens (2.8 cm diameter, 10.0 cm focal length, A612810, Esco Products, Oak Ridge, NJ, U.S.A.). Sample fluorescence is

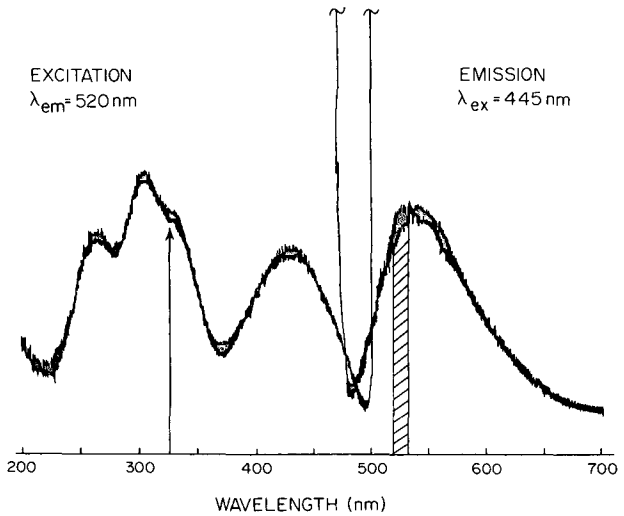


Fig. 2. Fluorescence excitation and emission spectra of ketosteroids derivatized with DPIH in methanol.

collected perpendicular and coplanar to the excitation beam with a fused-silica lens (2.54 cm diameter, 2.54 cm focal length, A110010, Esco Products). The emission is then isolated by appropriate interference filters (FS-204-F, Corion, Holliston, MA, U.S.A.) and is focused onto a photomultiplier tube (Centronic Model Q4249B, Bailey Instruments, Saddle Brook, NJ, U.S.A.). The resulting photocurrent is amplified by a picoammeter (Model 480, Keithley Instruments, Cleveland, OH, U.S.A.) and displayed on a chart recorder (Model 585, Linear Instruments, Reno, NV, U.S.A.).

In preliminary studies, the excitation and emission spectra of the steroid derivatives in methanol were monitored by using a conventional fluorimeter (Model 512, Perkin-Elmer, Norwalk, CT, U.S.A.) with a Xe lamp source. Typical fluorescence spectra are shown in Fig. 2, together with the excitation (325 nm) and emission (520 nm) wavelengths selected for chromatographic analysis. The spectral properties of the steroid derivatives correlate well with those reported previously for the acetone derivative[17], which has maximum absorbance at 297 and 412 nm (ε_{max} 38 800 and 7060 l/mol cm, respectively) and maximum fluorescence emission at 570 nm (φ_F 0.1–0.3).

RESULTS AND DISCUSSION

In initial experiments, fluorescence spectroscopy was used to confirm the formation of fluorescent products upon reaction of the DPIH reagent with simple aliphatic and aromatic ketones. Subsequently, a series of mono-, di-, and tri-ketosteroids was examined under the same reaction conditions. In general, the derivatization agent was reactive with all ketosteroids examined and formed highly fluorescent products. Steroids with conjugated and aromatic ketone sites were found to be far more reactive than the corresponding unconjugated sites. Furthermore,

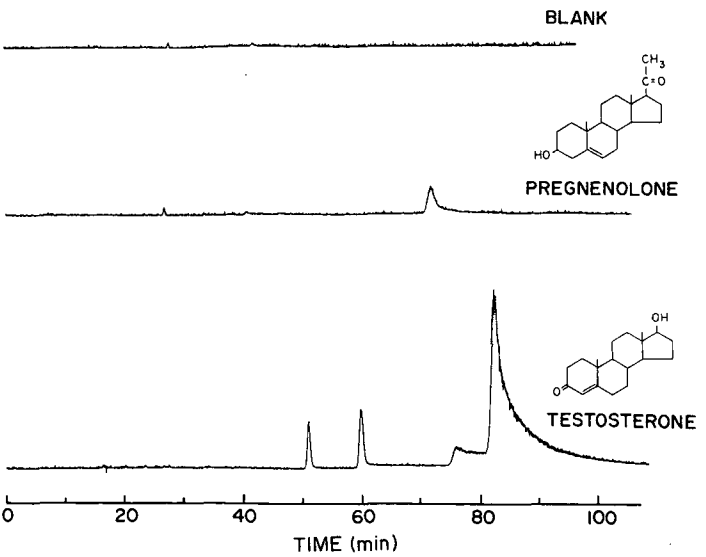


Fig. 3. Chromatograms of pregnenolone and testosterone derivatized with DPIH. Microcolumn: 89 cm × 200 μm I.D. fused-silica capillary, packed with Spheri-5 RP-18. Mobile phase: methanol at 0.9 μl/min. Fluorescence detector: 325 nm excitation, 520 nm emission, sensitivity 1.0 μA/V, 0.1 V full scale.

sterically hindered ketone sites within the steroid rings were less reactive than accessible sites on the side chain. Finally, no fluorescent products were formed for steroids which contained hydroxyl groups but no ketone functionalities.

These preliminary results seemed quite promising, as they appeared to indicate that selective analysis of ketosteroids was feasible using the DPIH reagent. Upon chromatographic separation, however, multiple fluorescent products were observed for monoketosteroids (Fig. 3) as well as polyketosteroids (Fig. 4). Although this phenomenon has been widely reported in the literature for other reagents (refs. 21,22, and references cited therein), it is generally ascribed to the formation of two *syn* and *anti* isomers. With the present reagent, four products were formed with greatly differing chromatographic and spectroscopic properties, as exemplified by testosterone (Fig. 3). Two products, of approximately equal and low concentration, were slightly retained with good peak shape under these chromatographic conditions and showed maximum absorbance at 300 nm wavelength. The other two products, generally of higher and unequal concentration, were more strongly retained with strong tailing under these chromatographic conditions and showed maximum absorbance at 320 nm. Because of the differing chromatographic and spectroscopic

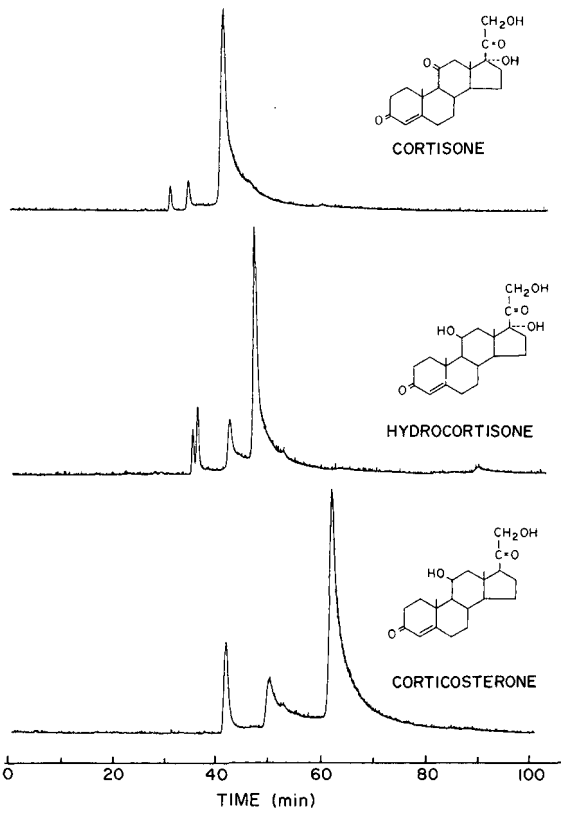


Fig. 4. Chromatograms of cortisone, hydrocortisone, and corticosterone derivatized with DPIH. Chromatographic conditions are described in Fig. 3.

properties, it was initially believed that the multiple products were the results of impurities in the reagents or by-products of the derivatization reaction. Hence, an extensive purification of the materials was performed, followed by a thorough optimization of the reaction conditions using testosterone as a model solute.

Catalyst studies

Several organic and inorganic acids of varying strength were compared as catalysts for the reaction of testosterone with DPIH. No fluorescent products were observed without a catalyst or with a weak organic acid catalyst such as acetic acid. Mild inorganic acids, such as boron trifluoride, appeared to catalyze the reaction but yielded multiple fluorescent products. Similar results were observed for strong mineral acids, such as hydrochloric and sulfuric acids. Although the initial rate of reaction was faster with sulfuric acid than with hydrochloric acid, the decomposition rate of the derivatives was also increased. Hence, hydrochloric acid was chosen for further catalytic studies of the reaction conditions.

Because the rate of addition of the hydrazine nucleophile to the carbonyl group is pH dependent[22,23], the effect of catalyst concentration was examined. The concentration of hydrochloric acid was varied between 0.02 and 0.20 *M*, as shown in Fig. 5, and the optimal concentration appeared to be approximately 0.08 *M*. The amount of catalyst influenced the rate of product formation and decomposition, but did not influence the number of reaction products.

Various Lewis acids, such as $AlCl_3$, $CrCl_3$, $SnCl_2$, $SnCl_4$, and $MgBr_2$ were added as auxiliary catalysts with hydrochloric acid. The Lewis acids were expected to complex selectively with the conjugated diketone group of the DPIH reagent, possibly reducing the number of by-products formed in the derivatization reaction[24]. Although significant differences were observed in the rates and relative amounts of products formed (*vide infra*), Lewis acids were not successful in eliminating the multiple fluorescent products.

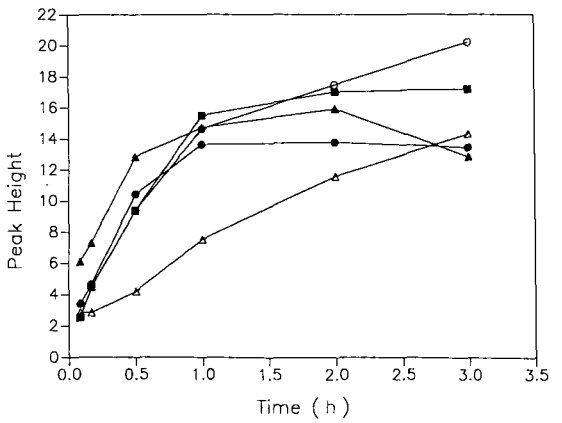


Fig. 5. Effect of hydrochloric acid catalyst concentration on derivatization of testosterone with DPIH. Testosterone $5.2 \cdot 10^{-5}$ *M*; DPIH $3.0 \cdot 10^{-4}$ *M*; catalysts: (△) 0.02 *M* HCl, (○) 0.04 *M* HCl, (□) 0.08 *M* HCl, (●) 0.16 *M* HCl, (▲) 0.20 *M* HCl; temperature 40°C.

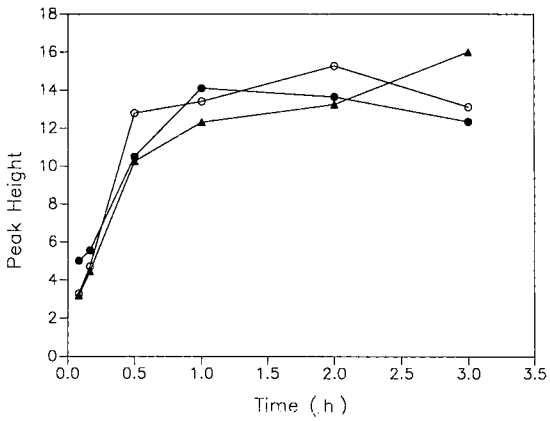


Fig. 6. Effect of temperature on derivatization of testosterone with DPIH. Testosterone $5.2 \cdot 10^{-3}$ *M*; DPIH $3.0 \cdot 10^{-4}$ *M*; catalyst 0.08 *M* HCl; temperatures: (●) 30°C; (▲) 40°C; (○) 50°C.

Finally, to determine whether specific interaction of the catalyst with either the derivatizing agent or the steroid was responsible for the multiplicity of reaction products, the order and timing of reagent addition were systematically varied. However, no change in the amount or number of reaction products was observed.

Temperature studies

The derivatization reaction of testosterone was examined as a function of temperature in the range of 30 to 50°C. As illustrated in Fig. 6, no significant difference was observed in the rate of product formation or decomposition within this temperature range. This indicates that the rate-limiting step in the derivatization reaction is equilibrium controlled, as expected[23], rather than kinetically controlled. Furthermore, the number of reaction products was invariant with temperature, indicating that thermal decomposition of the steroid or steroid derivative was not the source of multiple products.

Additional studies

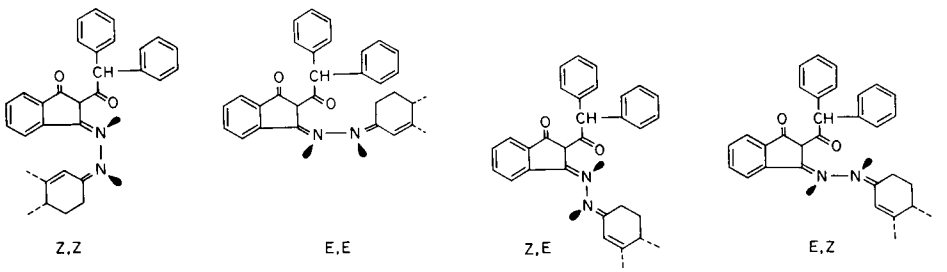
Since water is a product of the derivatization reaction (see Introduction), it may act as a stimulus for product decomposition. This possibility was examined by dehydrating all reagents, solvents, and solutions with a drying agent such as calcium chloride, sodium sulfate, or molecular sieve 4A. No change was observed in the amount or multiplicity of products formed in the presence or absence of water.

Another possible source of interference is the formation and subsequent derivatization of additional ketone sites by tautomerization of hydroxyl groups. This possibility was examined by reacting cholesterol, a steroid with a hydroxyl group in the C-3 position, with the DPIH reagent. No fluorescent products were formed; hence, the possibility of keto–enol tautomerization under the present reaction conditions seems remote.

Finally, an attempt was made to isolate and identify the multiple products formed in the derivatization of testosterone. Fractions corresponding to each of the four products were collected from the liquid chromatograph and reanalyzed to

confirm purity. Mass spectrometric analysis revealed that each fraction had the same molecular ion (*m/z* 624) and similar fragmentation patterns. Upon examination of the fractions by LC, it was discovered that each fraction contained all four products formed in the original reaction mixture. Hence, a dynamic relationship must exist between the reaction products such that interconversion, whether by rearrangement or by isomerization, is possible in the presence of a suitable catalyst.

The most probable source of such isomeric products is the formation of configurational isomers at the azine bond. For simple aliphatic ketones and steroids such as pregnenolone (Fig. 3) that react on the flexible side chain, the configurational isomers may or may not be chromatographically resolved. For steroids such as testosterone (Fig. 3) and the corticosteroids (Fig. 4), however, the rigid ring structure at the C-3 ketone site causes great structural dissimilarity. Four possible *E* and *Z* configurations, shown below, may arise upon derivatization of such steroids with DPIH.



It is possible that the two early eluting derivatization products of testosterone (see Fig. 3) are the *E,E* and *E,Z* configurations. From the examination of molecular models, these products appear to be sterically hindered, and are not expected to be formed in abundance. Furthermore, the lone pairs of electrons on the azine and carbonyl groups are not completely accessible, so tailing of chromatographic peaks due to strong adsorption on silanol groups is not expected and is not observed. Presumably, the later eluting products of testosterone are the *Z,Z* and *Z,E* configurations. From examination of molecular models, the *Z,E* configuration is most stable and is probably the most retained peak, while the other predominant peak is the *Z,Z* conformer. The lone pairs of electrons on the azine and carbonyl groups in these configurational isomers are readily accessible, so concerted adsorption (tailing) would be expected and is observed. To substantiate these configurational assignments, a weak Lewis acid ($MgBr_2$) was added during the derivatization. The *Z,Z* and *Z,E* configurations with accessible electron pairs are expected to bind weakly with the Lewis acid, which will stabilize and enhance formation of these products. Conversely, the *E,E* and *E,Z* configurations which lack the accessible electron pairs cannot be stabilized in this manner. As predicted, a substantial increase in product amount was observed for the later eluting products, whereas no change was discernible for the early eluting products. Hence, the proposed assignment of configurational isomers appears to be justified.

CONCLUSIONS

The reagent DPIH is very reactive and forms highly fluorescent derivatives with simple ketones as well as mono-, di-, and triketosteroids. Unfortunately, its utility as a precolumn derivatizing agent appears to be limited by the formation of multiple configurational isomers. This reagent remains useful as a spray reagent in thin-layer chromatography and is potentially useful as a post-column derivatization agent, where the presence of multiple products is inconsequential.

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REFERENCES

1 M. Novotny, *Anal. Chem.*, 53 (1981) 1294A.
2 M. Novotny, *LC · GC Mag. Liq. Gas Chromatogr.*, 3 (1985) 876.
3 H. G. Menet, P. C. Gareil and R. H. Rosset, *Anal. Chem.*, 56 (1984) 1770.
4 V. L. McGuffin, *LC · GC Mag. Liq. Gas Chromatogr.*, 2 (1984) 282.
5 V. L. McGuffin and M. Novotny, *Anal. Chem.*, 53 (1981) 946.
6 V. L. McGuffin and M. Novotny, *Anal. Chem.*, 55 (1983) 2296.
7 K. Jinno, S. Nakanishi and C. Fujimoto, *Anal. Chem.*, 57 (1985) 2229.
8 E. S. Yeung, *Adv. Chromatogr.*, 23 (1984) 1.
9 E. J. Guthrie, J. W. Jorgenson and P. R. Dluzneski, *J. Chromatogr. Sci.*, 22 (1984) 171.
10 J. Gluckman, D. Shelly and M. Novotný, *J. Chromatogr.*, 317 (1984) 443.
11 V. L. McGuffin and R. N. Zare, *Appl. Spectrosc.*, 39 (1985) 847.
12 S. Folestad, B. Galle and B. Josefsson, *J. Chromatogr. Sci.*, 23 (1985) 273.
13 V. L. McGuffin and R. N. Zare, *ACS Symp. Ser.*, 297 (1986) 120.
14 V. L. McGuffin and R. N. Zare, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 8315.
15 R. Brandt, J. C. Kouines and N. D. Cheronis, *Microchem. J.*, 6 (1962) 519.
16 R. A. Braun and W. A. Mosher, *J. Am. Chem. Soc.*, 80 (1958) 3048.
17 D. J. Pietrzyk and E. P. Chan, *Anal. Chem.*, 42 (1970) 37.
18 E. J. Poziomek, E. V. Crabtree and J. W. Mullin, *Anal. Lett.*, 14(A11) (1981) 825.
19 S. J. Swarin and F. Lipari, *J. Liq. Chromatogr.*, 6 (1983) 425.
20 J. C. Gluckman, A. Hirose, V. L. McGuffin and M. Novotny, *Chromatographia*, 17 (1983) 303.
21 J. Drozd, *Chemical Derivatization in Gas Chromatography* (*Journal of Chromatography Library*, Vol. 19), Elsevier, Amsterdam, 1981, pp. 92–97, 151–165.
22 L. A. Sternson, in R. W. Frei and J. F. Lawrence (Editors), *Chemical Derivatization in Analytical Chemistry*, Vol. 1, Plenum Press, New York, 1981, pp. 183–193.
23 T. H. Lowry and K. S. Richardson, *Mechanism and Theory in Organic Chemistry*, Harper and Row, New York, 1976, pp. 432–439.
24 A. L. Gemal and J. L. Luche, *Tetrahedron Lett.*, 22 (1981) 4077.

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Note

Separation of gibberellins by normal-phase high-performance liquid chromatography

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A major difficulty with the study of endogenous gibberellins (GAs) in plants is to remove substances interfering with the subsequent qualitative or quantitative analyses, for a general review see, *e.g.*, ref. 1. Since vegetative plant tissues usually contain small amounts of GAs together with large amounts of closely related impurities, the efficiences of the chromatographic procedures employed are of great importance. Therefore, high-performance liquid chromatography (HPLC) is now increasingly preferred to other chromatographic techniques[1,2].

Reversed-phase HPLC has proved to be very suitable for the separation of GAs[3–7]. If even higher purity is required, or as an alternative, normal-phase HPLC, can be used. The combination of a reversed-phase HPLC purification step with a normal-phase HPLC purification step increases the probability that the impurities are separated from the GAs and thereby facilitates the subsequent qualitative and quantitative analyses[2].

A frequently used normal-phase HPLC method for GAs was developed by Reeve *et al.*[8] from an open column system described by Powel and Tautvydas[9]. The stationary phase of this system was aqueous formic acid adsorbed to a microparticulate silica gel. The mobile phase used consisted of gradients or certain proportions of *n*-hexane saturated with aqueous formic acid, and ethyl acetate saturated with aqueous formic acid. This system is difficult to handle since the liquid stationary phase is easily stripped off the silica support, resulting in adsorption chromatography rather than partition chromatography. The development of HPLC has, however, resulted in covalently bonded polar stationary phases and such a system would be preferable. Bonded normal-phase HPLC of free GAs has been reported by Yamaguchi *et al.*[10] who used Nucleosil $N(CH_3)_2$ as a stationary phase, isocratically eluted with methanol plus 0.05% acetic acid.

The purpose of the present investigation was to develop a normal-phase HPLC method for semi-preparative and analytical separations of GAs. The method should be used for the purification of GAs in extracts of vegatative conifer tissues.

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MATERIALS AND METHODS

Preparation of radiolabelled GAs

Carbon-14 labelled GAs were prepared from mevalonic acid by enzymatic conversion in cell-free systems from pumpkin (*Cucurbita maxima*) and pea (*Pisum sativum*). Mevalonic acid lactone (1.96 GBq/mmol, Amersham) was hydrolysed with 1 *M* KOH at 30°C for 15 min and was dissolved in 2.35 ml of water. The solution was added to an incubation mixture consisting of 100 μl 0.05 *M* $MgCl_2$, 100 μl 0.1 *M* $MnCl_2$, 100 μl 0.5 *M* ATP, 150 μl 0.5 *M* phosphoenolpyruvate (PEP), 150 μl 0.05 *M* reduced nicotinamide–adenine dinucleotide phosphate (NADPH) and 7 ml of liquid pumpkin endosperm[11]. After 2 h of incubation at 30°C the conversion was stopped and the gibberellins were extracted at pH 3.0 with ethyl acetate. The products were purified by thin-layer chromatography and gradient elution reversed-phase HPLC and their identities were established by gas chromatography–mass spectrometry (GC–MS). In this manner kaurene, kaurenal, kaurenol, kaurenoic acid, GA_{12}-aldehyde, GA_{12} and GA_{15} were obtained.

Cell-free systems from pea seeds (for more details see ref. 12) were used for the preparation of ^{14}C-labelled GA_{19}, GA_{20}, GA_{29}, GA_{44} and GA_{53}. First the [^{14}C]GA_{12} was incubated with the low-speed supernatant (S-2, 2000 *g*) together with NADPH, ATP and PEP to yield [^{14}C]GA_{53}. The GA_{53} was then incubated with the high-speed supernatant (S-200, 200 000 *g*) and Fe^{2+} and ascorbate. This resulted in the production of ^{14}C-labelled GA_{19}, GA_{20}, GA_{29} and GA_{44}. All GAs were further purified by reversed-phase HPLC and their identities were established by GC–MS.

The other GAs were commercially available or obtained as gifts: [^{3}H]GA_1 (New England Nuclear, U.K.), [^{14}C]GA_3 (Amersham, U.K.) [^{3}H]GA_4 (R.P. Pharis, Calgary, Canada), [^{3}H]GA_8 (R.P. Pharis) and [^{3}H]GA_9 (Alan Crozier, Glasgow, U.K.).

Plant material

Seeds of Norway spruce [(*Picea abies* (L) Karst.)] were collected from a stand outside Umeå (lat. 63°50′ N). The seeds were germinated and grown in a greenhouse under artificial long day conditions during the period from July to December. Supplementary light was given 20 h a day with Osram metal halogen lamps, HQI-TS 400 W/DH (*ca.* 100 μmol m^{-2} s^{-1}, 400–700 nm). The seedlings were watered daily and given a complete nutrient solution twice a week. At the age of *ca.* 6 months the actively growing, non-lignified upper part of the seedlings was harvested and frozen in liquid nitrogen. Thereafter the plant material was stored at −80°C until analyzed.

Extraction and purification

Shoot samples of 200 g fresh weight were homogenized in 1000 ml cold (+4°C) methanol. After 2 h of extraction the tissue debris was filtered off and washed with another 500 ml of methanol. The methanol fractions were pooled, and 10 ml of 2% diethyldithiocarbamic acid were added. The organic phase was evaporated *in vacuo* at 35°C and the water phase was adjusted to a volume of 10 ml with 0.5 *M* phosphate buffer and, if necessary, adjusted to pH 8.0 with 6 *M* NaOH. The extract was applied to a 30 cm × 10 mm I.D. column packed with insoluble poly-(N-vinylpolypyrrolidone) (PVPP). The column was eluted with 0.1 *M* phosphate buffer pH 8.0 and the

fraction 0–200 ml was collected. This semipurified extract was then purified by semi-preparative normal-phase HPLC with a covalently bonded stationary phase.

High-performance liquid chromatography

The solvent delivery system consisted of one Waters M 510 pump, one Waters M 45 pump controlled by a Waters M 680 system controller. The samples were introduced to the columns via a Waters U6K injector. The GAs were detected by a Reeve Analytical radioactivity detector equipped with a 500-μl homogeneous flow cell. Instafluor (Packard) liquid scintillation fluid was delivered by a Reeve scintillation pump and mixed with the column effluent in the homogeneous flow cell.

The analytical separations were made with 150 mm × 4.6 mm I.D. steel columns packed with Nucleosil 5-μm particulate NO_2 phase. The phase is bonded to the silica support via a propyl group. The mobile phase consisted of gradients of *n*-heptane half saturated with 1 *M* formic acid to ethyl acetate with 1% water and 0.5% formic acid. Water and formic acid were added as polar modifiers and ion suppression agents in the mobile phase.

The semi-preparative separations were made with a 250 mm × 10 mm I.D. column packed with 5-μm Polygosil NO_2. This system was used for the first separation of GA-like substances in a Norway spruce extract. The GA-like substances were detected by the Tan-ginbozu dwarf rice bioassay according to Murakami[13].

RESULTS AND DISCUSSION

Since our aim was to use the system for preparative purifications of unknown GA-like substances we concentrated on gradient elution, which makes it possible to chromatograph GAs with different chemical characteristics in the same experiment. The mobile phase of choice was gradients of *n*-heptane to ethyl acetate. The use of formic acid and water in the mobile phase was necessary to suppress the ionization of the carboxyl groups and thereby avoid high capacity factors and poor peak symmetry. The water and acid content was very critical for good selectivities and 1% of water and 0.5% of formic acid in the ethyl acetate proved to be optimal. The *n*-heptane was half saturated with 1 *M* formic acid in water. A problem with formic acid in water in *n*-heptane and ethyl acetate is that the two solvents separately hold more water and acid than they do in mixtures. If the water and acid content is too high, water and formic acid will separate during gradient elution thus risking deterioration of the stationary phase.

The separation of the available GAs and GA precursors is shown in Fig. 1. A comparison of the elution order of the γ-lactonic C_{19}-GAs indicates that the number and also the position of the hydroxyl groups on the GAs are very important for the solubility in the stationary phase. GA_9 with no hydroxyl group is eluted first (*ca.* 40 ml) and is followed by GA_4 (*ca.* 56 ml) and GA_{20} (*ca.* 63 ml), each having one hydroxyl group. GAs with two hydroxyl groups, GA_1, GA_3 and GA_{29}, are eluted at *ca.* 85, 88 and 101 ml, respectively. The GA possessing three hydroxyl groups, GA_8, is eluted at *ca.* 117 ml, thus exhibiting the highest solubility in the polar stationary phase. The same effect of the number of hydroxyl groups is observed when comparing the δ-lactonic C_{20}-GAs, GA_{15} and GA_{44}, and the C_{20}-GAs with a C_{20}-methyl group. GA_{12} and GA_{53}. Ga_{15} and GA_{12} have no free hydroxyl group and are eluted earlier

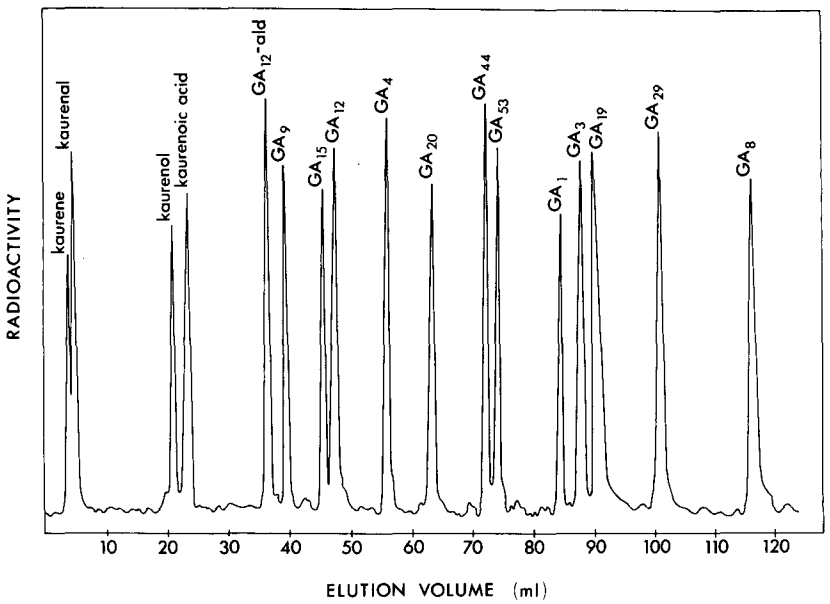

Fig. 1. Analytical normal-phase HPLC of radioactively labelled gibberellins and gibberellin precursors. Kaurene, kaurenol, kaurenal, kaurenoic acid, GA_{12}-aldehyde, GA_{15}, GA_{12}, GA_{20}, GA_{44}, GA_{53}, GA_3 and GA_{29} were ^{14}C-labelled and the other GAs were ^{3}H-labelled. Stationary phase: 5-μm Nucleosil NO_2 packed in a 125 mm × 4.6 mm I.D. Column. The mobile phase was a gradient of *n*-heptane half saturated with 1 *M* formic acid to ethyl acetate with 1% water and 0.5% formic acid. Gradient sweep time: 60 min. Flow-rate: 2 ml/min.

than the corresponding GAs with one hydroxyl group, GA_{44} and GA_{53}. It is also possible to refer the elution order to the position of the hydroxyl groups. By comparing the elution volume of GA_4, having the hydroxyl group in 3-position, with the elution volume of GA_{20}, having the OH group in 13-position, it is observed that GA_4 is eluted earlier, probably because the interaction of the 3-OH group with the stationary phase is reduced by hydrogen bonding to the C-7 carboxyl group. This type of hydrogen bonding is not possible if the OH group is in 13-position. This explains also the difference in elution volumes of GA_1, GA_3 and GA_{29}, all possessing two OH groups. GA_1 and GA_3, hydroxylated in the 3- and 13-positions are eluted earlier than GA_{29} which is hydroxylated in the 2- and 13-positions. The distance between the 2-OH group and the C-7 carboxyl group in GA_{29} is, however, too long for such hydrogen bonding. The slightly higher elution volume of GA_3 compared to GA_1 is probably caused by a higher interaction of the 1,2-double bond of GA_3 in the stationary phase.

By comparing the elution volumes of the γ-lactonic and non-OH C_{19}-GA, GA_9 with the δ-lactonic, non-OH C_{20}-GA, GA_{15}, it can be concluded that the δ-lactone form is more soluble in the stationary phase. The same is observed when comparing GA_4 and GA_{20}, both γ-lactones with one OH-group, with GA_{44} which is a δ-lactone also with one OH-group. The effect of the oxidation state of the C_{20}-carbon on the elution volume is demonstrated by the difference between GA_{53}, with a methyl in the C_{20} position, and GA_{19}, where this position is occupied by an aldehyde. The more

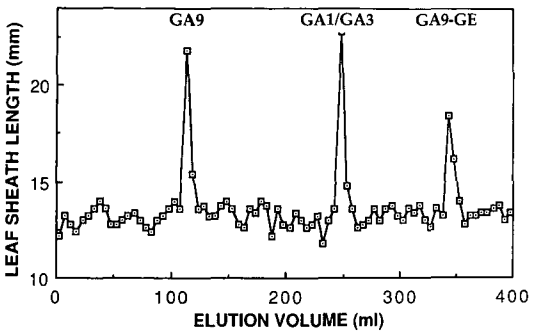

Fig. 2. Separation of gibberellins in a Norway spruce extract by semi-preparative normal-phase HPLC. Gibberellin-like substances detected by Tan-ginbozu dwarf bioassay. Stationary phase: 5 μm Polygosil NO_2 packed in a 250 mm × 10 mm I.D. column. Mobile phase: gradient from *n*-heptane half saturated with 1 *M* formic acid to ethyl acetate with 1% water and 0.5% formic acid. Gradient sweep time: 60 min. Flow-rate: 5 ml/min. Fraction size: 5 ml.

polar nature of the aldehyde group makes GA_{19} more soluble in the stationary phase and thereby increases the elution volume compared with GA_{53}. The difference in retention characteristic between GAs possessing a carboxyl group or an aldehyde group is demonstrated by the difference in elution volume between GA_{12}-aldehyde, with an aldehyde at C-6, and GA_{12}, which has a carboxyl group at C-6. The more polar nature of the carboxyl group increases the solubility of GA_{12} in the stationary phase compared to GA_{12}-aldehyde.

From these observations it may be possible to predict the approximate elution volume also of other GAs by consideration of: (1) the number of OH groups; (2) the position of the OH groups; (3) the type of GA, γ-lactonic, δ-lactonic, and the oxidation state of a retained C_{20}.

The semi-preparative separation of a semi-purified spruce extract corresponding to 400 g fresh weigth resulted in GA-like activity as shown in Fig. 2. The first region of activity (around 110 ml) was, after further purification by reversed phase HPLC, identified as GA_9 by (GC–MS)[14]. The second region (250 ml) was, after further purification by reversed phase HPLC, identified as GA_1 and GA_3 by GC–MS[15]. This means that GA_1 and GA_3 did not separate in the gradient elution preparative system. The third region of activity, 350 ml (not detected when formic acid coated on a silica support was used as the stationary phase), was further purified by reversed-phase HPLC. After cellulase hydrolysis, GA_9 was identified by GC–MS, thus indicating that this compound is GA_9-glucosyl ester[16]. This normal-phase HPLC system is easy to handle, reproducible, efficient, selective and the solvents are easily evaporated which makes it well suited for both semi-preparative and analytical separations of GAs. It has been used for the semi-preparative purification of large extracts (200–400 g fresh weight) of Norway spruce tissue. An additional purification of the GA-like substances by reversed-phase HPLC made it possible to identify them by GC–MS. The analytical system has been routinely used for the separation of GA_9, GA_1 and GA_3 in small extracts (5 g fresh weigth) of Norway spruce prior to quantitation by radioimmunoasay.

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REFERENCES

1 P. Hedden, in L. Rivier and A. Crozier (Editors), *Principles of Plant Hormone Analysis*, Vol. 1, Academic Press, London, 1987, p. 9.
2 R. Horgan, *Prog. Phytochem.*, 7 (1981) 137.
3 G. W. M. Barendse, P. H. Van De Werken and N. Takahashi, *J. Chromatogr.*, 198 (1980) 449.
4 M. G. Jones, J. D. Metzger and J. A. D. Zeevaart, *Plant Physiol.*, 65 (1980) 218.
5 J. T. Lin and E. Heftmann, *J. Chromatogr.*, 213 (1981) 507.
6 M. Koshioka, J. Harada, K. Takeno, M. Noma, T. Sassa, K. Ogiyama, J. S. Taylor, S. B. Rood, R. L. Legge and R. P. Pharis, *J. Chromatogr.*, 256 (1983) 101.
7 E. Jensen, A. Crozier and A. M. Monteiro, *J. Chromatogr.*, 367 (1986) 377.
8 D. R. Reeve, T. Yokota, L. J. Nash and A. Crozier, *J. Exp. Bot.*, 27 (1978) 1243.
9 L. E. Powell and K. J. Tautvydas, *Nature (London)*, 213 (1967) 185.
10 I. Yamaguchi, S. Fujisawa and N. Takahashi, *Phytochemistry*, 21 (1980) 2049.
11 J. E. Graebe, D.-H. Bowen and L. MacMillan, *Planta*, 102 (1972) 261.
12 Y. Kamiya and J. E. Graebe, *Phytochemistry*, 22 (1983) 681.
13 Y. Murakami, *Bot. Mag. (Tokyo)*, 81 (1969) 271.
14 P. C. Odén, B. Andersson and R. Gref, *J. Chromatogr.*, 247 (1982) 133.
15 P. C. Odén, L. Schwenen and J. E. Graebe, *Plant Physiol.*, 84 (1987) 516.
16 P. C. Odén and T. Moritz, in preparation.

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Note

Separation of 1-naphthylamine from the five known impurities and sub-ppm level detection and quantitation of 2-naphthylamine by normal-phase high-performance liquid chromatography[a]

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The free base 1-naphthylamine and certain of its sulphonic acid derivatives serve as diazo and coupling components in the preparation of azo dyes, including some permitted synthetic food colours. Technical 1-naphthylamine may contain some 1,1′-dinaphthylamine, 1,5-diaminonaphthalene, 1- and 2-naphthols and invariably a little of the 2-isomer (about 0.5%, w/w) as impurities (Fig. 1).

2-Naphthylamine has long been recognized as a human carcinogen and as a toxic chemical. Some azo synthetic food colours were actually found to contain

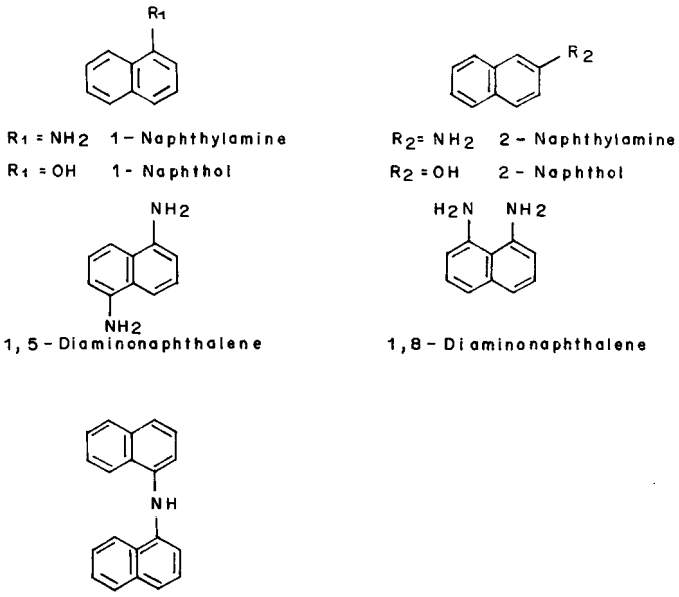

Fig. 1. Structures of 1-naphthylamine and the likely associated impurities.

[a] NCL Communication No. 4508.

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2-naphthylamine as an impurity[1,2], most likely a carry-over from the 1-naphthylamine intermediate used in their manufacture. Therefore, monitoring the 2-naphthylamine impurity in 1-naphthylamine should help to control the quality of such food colours.

High-performance liquid chromatography (HPLC), using a bonded strong cation exchanger[3], a bonded amino phase co-ordinated with Cu(II) as a ligand exchanger[4], a bonded β-cyclodextrin[5], a bonded octadecylsilane[6,7] and silica gel[8] as the stationary phases, has been applied for this isomer separation problem. Further, an open-tubular microcapillary column with β,β-oxydipropionitrile (BOP) as a stationary phase[9] and liquefied alkanes as the mobile phase resolved the isomers completely.

Unlike the previous attempt by Sliwiok and Szulik[8], we report in this paper an high resolution (R_s = 2.5) HPLC method using a silica gel column and 50% water-saturated diethyl ether–*n*-hexane (25:75) as the mobile phase with detection at 280 nm for the determination of underivatized 2-naphthylamine in technical 1-naphthylamine with sub-ppm as the lower detection limit in *n*-hexane solution. Moreover, the method simultaneously separated all the other four potential impurities mentioned earlier (Fig. 2).

EXPERIMENTAL

Liquid chromatography

The work was carried out on a Waters HPLC system (Millipore-Waters Chromatography Division, Milford, MA, U.S.A.) consisting of two Model 6000A dual-head reciprocating solvent-delivery systems controlled by a Model 660 solvent flow programmer, a Model U6K universal injector and a Model 440 dual-channel discrete multi-wavelength absorbance detector operating at 280 nm. The analogue output of the absorbance detector was recorded and processed with a Waters Model 730 data module (a printer, plotter and integrator).

A Waters μPorasil (irregular particles, 10 μm) stainless-steel pre-packed column (30 cm × 3.9 mm I.D.) was used.

Naphthylamine standards

A technical grade 1-naphthylamine sample was purified by repeated crystallizations from light petroleum (b.p. 60–80°C) in which 2-naphthylamine is more soluble. An old reagent grade 2-naphthylamine sample was similarly purified by recrystallizations from ethanol. The 1,5- and 1,8-diaminonaphthalenes ("Purum" grade) were obtained from Fluka (Buchs, Switzerland) and 1,1'-dinaphthylamine was prepared and purified according to a literature method[10].

Mobile phase

Diethyl ether and *n*-hexane were purified to HPLC quality in our laboratory.

A 50% water-saturated diethyl ether–*n*-hexane (25:75) mobile phase was prepared as directed in ref. 11 and was filtered through a 0.45-μm Millipore Fluoropore (PTFE) membrane filter (FHUP 047 00) using a Millipore all-glass filter apparatus (XX 15 047 00) before use.

About 50 ml of 50% water-saturated filtered mobile phase was passed through a Waters μPorasil column to ensure proper equilibration. The mobile phase flow-rate was 1.5 ml/min.

External standard calibration graph

Stock standard solution. About 10 mg of 2-naphthylamine were accurately weighed, dissolved and diluted to volume with *n*-hexane in a 100-ml volumetric flask. A 10.0-ml aliquot of this solution (A) was further diluted up to volume with *n*-hexane in a second 100-ml volumetric flask. This stock solution (B) corresponds to about 10 μg 2-naphthylamine per ml.

Working standard solutions. Aliquots of stock solution (B) ranging from 1 to 9 ml were diluted to volume with *n*-hexane in separate 10-ml volumetric flasks. An 100-μl volume from each working standard solution was injected into the liquid chromatograph to check the linearity of the detector (280 nm) response (peak area and height). This corresponds to *ca.* 100–900 ng 2-naphthylamine per injection.

Sample solution. About 100 mg of a technical sample were accurately weighed, dissolved and diluted to volume in a 100-ml volumetric flask and filtered through a 0.5-μm PTFE membrane filter. An 100-μl volume of this filtered solution was used for each injection.

RESULTS AND DISCUSSION

We were interested in developing a simple, sensitive, reliable and rapid HPLC method to determine 2-naphthylamine as an impurity in a 1-naphthylamine matrix without derivatization. The method was also expected simultaneously to separate other reported impurities such as 1,1′-dinaphthylamine, 1,5-diaminonaphthalene and 1- and 2-naphthols (Fig. 1). All of these compounds have a good solubility in *n*-hexane and other non-aqueous HPLC solvents and mainly differ in the position, number and nature (primary and secondary) of the nitrogen functionality. Therefore, HPLC in liquid–solid adsorption mode, using silica gel as a stationary phase, was expected to provide the selectivity necessary for the separation of these compounds. However, despite its potential, this HPLC mode has been used only sparingly in practice for this separation problem. Although Sliwiok and Szulik[8] achieved a partial resolution of the two naphthylamines on a silica gel column, they neither studied a simultaneous separation of other impurities nor attempted a determination of 2-naphthylamine in 1-naphthylamine.

Initially we performed some scouting experiments for the optimization of the mobile phase selectivity parameters, X_i, using a silica gel column. Thus, we blended *n*-hexane with modifiers like chloroform (proton donor activity, $X_d = 0.41$), dichloromethane (large dipole activity, $X_n = 0.53$) and diethyl ether (proton acceptor activity, $X_e = 0.53$)[12] to produce a series of binary and ternary mobile phases with different selectivities. A diethyl ether–*n*-hexane (20:75) (50% water-saturated) mixture offered one of the best selectivity factors ($\alpha = 1.29$) among various mobile phases tried (Table I). The use of 50% water-saturated mobile phase compositions helped to reduce tailing and offered better reproducibility of retention times. We also noted that, although acetonitrile–chloroform (10:90) offered the highest selectivity ($\alpha = 2.54$) for the separation of 1- and 2-naphthylamines, this mobile phase is not recommended for a routine use because such a mixture of two reactive organic solvents is known to form crystalline products on standing. A typical simultaneous separation of 1,1′-dinaphthylamine, 1- and 2-naphthols, 1- and 2-naphthylamines and 1,5-diaminonaphthalene is shown in Fig. 2 and the HPLC data for these compounds are given in Table II.

TABLE I

MOBILE PHASE SELECTIVITY FOR THE SEPARATION OF 1- AND 2-NAPHTHYLAMINES (NA)

Void volume time, t_0 = 3.15 min at the flow-rate 1.0 ml/min. All the mobile phases were 50% water-saturated.

Mobile phase No.	*Modifiers for n-hexane*	*Capacity factor, k'*		*Selectivity, α*
		1-NA	*2-NA*	
1	40% Diethyl ether	1.31	1.74	1.33
	20% Diethyl ether	2.49	3.21	1.29
2	60% Dichloromethane	1.35	1.54	1.14
3	20% Diethyl ether + 30% dichloromethane	1.09	1.28	1.17
4	45% Chloroform	1.10	1.35	1.22
5	22.5% Chloroform + 30% dichloromethane	1.27	1.53	1.20
6	35% Diethyl ether + 40% chloroform	0.67	0.85	1.27
7	10% Acetonitrile[a]	0.51	1.42	2.78

[a] In chloroform instead of *n*-hexane.

2-Naphthylamine is more basic and more polar than 1-naphthylamine (pK_a 4.14 and 3.92; dipole moment, in benzene at 25°C, 1.77 and 1.49 D, respectively). Further, the 2-isomer is the most linear of the two which means reduced steric hin-

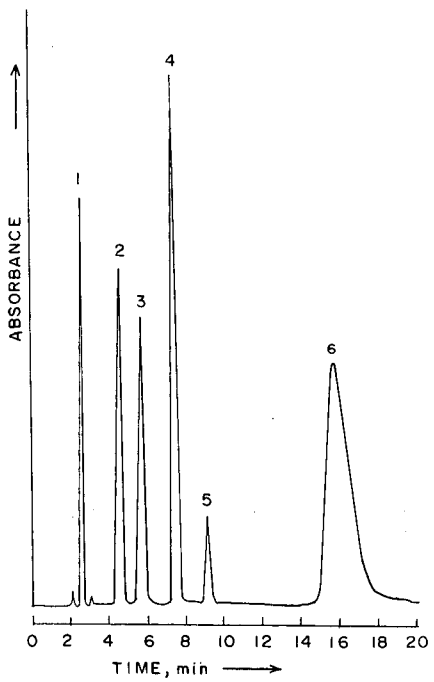

Fig. 2. Normal-phase HPLC separation of an artificial mixture of 1-naphthylamine and the five likely impurities. Column: Waters stainless-steel μPorasil (30 cm × 3.9 mm I.D.). Mobile phase: diethyl ether–*n*-hexane (25:75) (50% water-saturated); flow-rate 1.5 ml/min. UV detection: 280 nm. Peaks: 1 = 1,1'-dinaphthylamine; 2 = 1-naphthol; 3 = 2-naphthol; 4 = 1-naphthylamine; 5 = 2-naphthylamine; 6 = 1,5-diaminonaphthalene.

TABLE II

LIQUID–SOLID CHROMATOGRAPHY DATA FOR A SIMULTANEOUS SEPARATION OF AN ARTIFICIAL MIXTURE OF 1-NAPHTHYLAMINE AND THE POSSIBLE IMPURITIES

Void volume time, t_0 = 2.10 min at 1.46 ml/min.

No.	*Compound*	pK_a	*Retention time (min)*	*Capacity factor, k'*	*Selectivity, α*	*Resolution, R_s*
1	1,1'-Dinaphthylamine	<0.7	2.60	0.24		
					4.83	>5.00
2	1-Naphthol	9.30	4.54	1.16		
					1.50	1.86
3	2-Naphthol	9.57	5.75	1.74		
4	1-Naphthylamine	3.92	7.65	2.64		
					1.31	2.50
5	2-Naphthylamine	4.14	9.36	3.46		
6	1,5-Diaminonaphthalene	1.74	16.26	6.74		
		4.07			1.07	Poor
7	1,8-Diaminonaphthalene	4.29	17.22	7.19		

drance of the fused ring system towards the silica adsorbent. This enables 2-naphthylamine to interact more strongly with polar silanol groups on the surface of silica gel.

A strong and direct interaction of the polar primary amino functional group with a polar silanol adsorption site on the surface of silica (solute localization) demands the use of a modifier with a basic polar group such as diethyl ether (solvent strength parameter, $\varepsilon^0 = 0.38$; $X_e = 0.53$) which competes for a position directly over silanol by hydrogen bonding (solvent localization). The degree of solvent localization can be measured by a mobile phase parameter, m. Mobile phases containing alkyl ethers and acetonitrile are reported[13] to have large values of m. In the case of most liquid–solid chromatographic separations, localization effects in the stationary phase are of greater significance than interactions between solvent and solute molecules in the mobile phase. Thus, the use of a basic polar solvent such as diethyl ether yielded additional selectivity by solvent-specific localization effects leading to excellent resolution of 1- and 2-naphthylamine isomers in real samples too (Fig. 3).

External standard quantitation

A linear relationship was observed on plotting peak areas as well as peak heights against the corresponding injected mass (ng per 100 μl injected) at least up to 1000 ng 2-naphthylamine at the selected detection wavelength of 280 nm. This range covers the reported percentage range (up to 0.5%, w/w) of 2-naphthylamine in commercial 1-naphthylamine samples when the same volume of *ca.* 1 mg/ml sample solution is injected into the liquid chromatograph. We found that as the sample was dissolved in plain *n*-hexane, which is eluotropically much weaker than the mobile phase, there was no appreciable loss of resolution between the two naphthylamines at the sample loading used (100 μg in 100 μl). A regression analysis by the least-squares method ($n = 8$) for a plot of peak area (area units), y, or height (cm), y', against mass

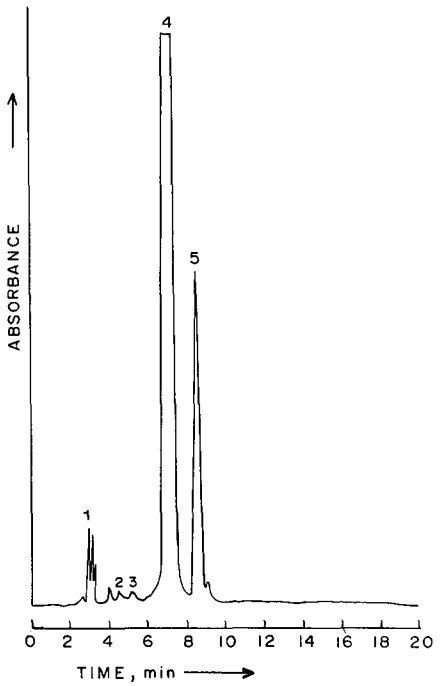

Fig. 3. HPLC chromatogram of a real technical sample of 1-naphthylamine dissolved in *n*-hexane. Details as in Fig. 2. 1,5-Diaminonaphthalene could not be detected in this particular sample.

injected (ng per 100 μl injected), x, for 2-naphthylamine yielded the following calibration equations with correlation coefficients, r, indicating excellent linearity:

Peak area: $y = 7259\,x + 23114$; $r = 0.99931$

Peak height: $y' = 0.015207\,x + 0.086667$; $r = 0.99925$

Using real samples of 1-naphthylamine, we studied the reproducibility of reten-

TABLE III

REPRODUCIBILITY OF RETENTION TIME, PEAK AREA AND PEAK HEIGHT OF 2-NAPHTHYLAMINE IN REAL SAMPLES OF 1-NAPHTHYLAMINE

Number of experiments for each sample, $n = 8$.

Sample	*Retention time*		*Peak area*		*Peak height*	
	Mean ± S.D. (min)	*R.S.D. (%)*	*Mean ± S.D. (area units)*	*R.S.D. (%)*	*Mean ± S.D. (cm)*	*R.S.D. (%)*
A	8.60 ± 0.042	0.49	9 925 519 ± 265 466	2.67	8.60 ± 0.042	0.49
B	8.58 ± 0.054	0.63	7 131 636 ± 148 399	2.08	6.39 ± 0.070	1.09

tion time, peak area and peak height for 2-naphthylamine (Table III). The reproducibility of peak heights was distinctly better than that of peak areas. Despite using a low-boiling mobile phase modifier like diethyl ether (b.p. 35°C), the reproducibility of retention time is good. Substitution of methyl *tert*-butyl ether (MTBE) (b.p. 55°C) for diethyl ether should improve the reproducibility further.

We have estimated that, under the chromatographic conditions employed, it is easy to detect at least 0.06 ppm (w/w) of 2-naphthylamine in *ca.* 1500 ppm (w/w) solution of 1-naphthylamine in *n*-hexane. This lowest detection limit corresponds to *ca.* 0.004% 2-naphthylamine in solid 1-naphthylamine samples and can be improved further by simply increasing the injection volume.

REFERENCES

1 B. Stavric, R. Klassen and W. Miles, *J. Assoc. Off. Anal. Chem.*, 62 (1979) 1020.
2 H. R. Hunzikar, *Mitt. Geb. Lebensmittelunters. Hyg.*, 72 (1981) 216; *C.A.*, 98 (1983) 105 812t.
3 I. Mcfford, W. Keller, R. N. Adams, L. A. Sternson and M. S. Yllow, *Anal. Chem.*, 49 (1977) 683.
4 F. K. Chow and E. Grushkâ, *Anal. Chem.*, 49 (1977) 1756.
5 R. Fujimura, T. Ueda and T. Ando, *Anal. Chem.*, 55 (1983) 446.
6 F. H. Walters and G. Gomez, *Anal. Lett.*, 19 (1986) 1187.
7 H. Matsushita, T. Shiozaki, T. Handa and K. Tanabe, *Bunseki Kagaku*, 36 (1987) 244; *C.A.*, 106 (1987) 226 809q.
8 J. Sliwiok and J. Szulik, *Microchem. J.*, 26 (1981) 294.
9 T. Takeuchi and D. Ishii, *J. Chromatogr.*, 240 (1982) 51.
10 E. Lieber and S. Somasekhara, *J. Org. Chem.*, 24 (1959) 1775.
11 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, Ch. 9, p. 381.
12 L. R. Snyder, *J. Chromatogr. Sci.*, 16 (1978) 223.
13 L. R. Snyder, J. L. Glajch and J. J. Kirkland, *J. Chromatogr.*, 218 (1981) 299.

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Note

Liquid chromatographic determination of hydrazine, carbohydrazide and thiocarbohydrazide in aqueous solutions

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Hydrazine and carbohydrazide [$(NH_2NH)_2CO$] are used extensively as water additives in steam generating plants[1,2]. Thiocarbohydrazide [$(NH_2NH)_2CS$] is used as a metal complexing agent and analytical reagent, especially in the determination of vicinal diols in carbohydrates and proteins[3–7]. Additionally, carbohydrazide and thiocarbohydrazide are used as intermediates in the synthesis of various heterocyclic structures[8,9].

Gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods for the determination of hydrazine, with and without derivatization, have been published[10,11]. The most sensitive method reported to date involves extraction followed by HPLC and electrochemical detection with a pretreated electrode[12]. Another report described derivatization with salicylaldehyde followed by extraction and normal-phase HPLC[13]. In this work, the derivatization method of Matsui *et al.*[14] has been extended to include the concurrent determination of hydrazine and carbohydrazide in aqueous samples.

As hydrazine and carbohydrazide are water-treatment agents, their determination in aqueous solution is advantageous. Similarly, thiocarbohydrazide as a metal complexant and as an analytical reagent requires an assay method for aqueous samples. We know of no reports that have described an HPLC method for either carbohydrazide or thiocarbohydrazide. This paper describes a procedure for the determination of all three compounds in aqueous samples. The procedure avoids extraction steps and the use of specialized detectors while still maintaining the ability to assay samples in the low parts per million range.

EXPERIMENTAL

Apparatus

A Phase Separations (Queensferry, U.K.) 15 cm × 4.6 mm I.D. ODS-1 (5 μm) column was used in a Varian Model 5020 liquid chromatograph equipped with a column oven and a Rheodyne (Cotati, CA, U.S.A.) Model 7125 loop injector with a 10-μl loop. Peak areas were measured electronically with a Spectra-Physics (Darmstadt, F.R.G.) Model 4290 recording integrator. Analytes were detected by ultraviolet spectrophotometry using a Varian Model UV-100 variable-wavelength instrument.

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Reagents

HPLC-grade acetonitrile, synthesis-grade hydrazine hydrate [64% (w/w) hydrazine], synthesis-grade benzaldehyde and extra-pure grade potassium dihydrogenphosphate were supplied by Merck (Darmstadt, F.R.G.) and analytical-reagent grade ethanol by Frutarom (Haifa, Israel). Water was deionized and glass distilled prior to use. Buffer (0.1 *M*) was prepared by adding 13.6 g of potassium dihydrogenphosphate to a 1-l volumetric flask, diluting to mark with water, adjusting the pH to 7.0 with a few drops of dilute potassium hydroxide solution and then filtering through a 4.5-μm filter. The mobile phase was acetonitrile– buffer (45:55, v/v) and was filtered prior to use. Standards of the benzaldehyde derivates of hydrazine, carbohydrazide and thiocarbohydrazide were supplied by the Organic Chemistry Department (Makhteshim, Beer-Sheva, Israel). A stock solution of all three standards was prepared fresh daily by weighing exactly about 50 mg of each into the same 50-ml volumetric flask and diluting to the mark with acetonitrile. Dilutions of the stock solution were made as required and benzaldehyde reagent was prepared as a 15% (w/v) solution in ethanol.

Procedure

The flow-rate was 2.0 ml/min at a column oven setting of 40°C and the column was equilibrated for 20 min. Standard solution was injected and, if necessary, the eluent composition and compound concentration adjusted such that the capacity factor (k') of benzalazine was 14.6 $\pm$ 5% and the absorption was within the desired range. Stock solutions were prepared in duplicate and diluted such that the standard response was $\pm$ 10% of the sample response. Analytes were detected at 310 nm at a detector time constant of 0.5 s and 10 μl of solution were injected.

For samples with an analyte concentration expected to be at or above the 1 ppm range, accurately weigh, in duplicate, about 2 g of sample into a 25-ml volumetric flask, add 1 ml of benazaldehyde solution and place the flask in a 70°C water-bath for 30 min. Cool to the room temperature, add acetonitrile to the mark and make further dilutions of this solution as necessary with acetonitrile. Adjust the concentrations such that the peak areas of the standard and sample are within the linear detector range and agree to within $\pm$ 10%. Inject standard solutions until duplicate injections agree to within $\pm$ 5% at concentrations from 0.1 to about 10 ppm and then start the analytical sequence. Calculate the concentration of analytes in sample as follows:

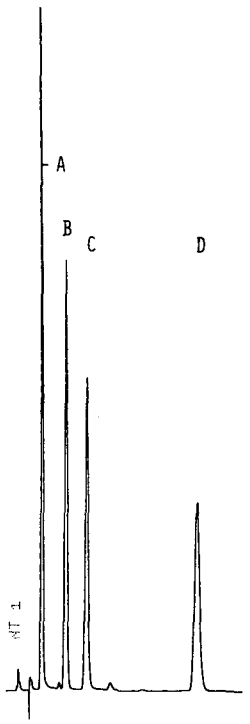
$$\text{Concentration of analyte (\%)} = \frac{(\text{area/g sample})(\text{concentration of hydrazine standard})}{(\text{area/g standard}) \cdot F}$$

where area/g is the integration area divided by weight and the factor F is the molecular weight of the derivative/molecular weight of underivatized material. The values of F are hydrazine 6.56, carbohydrazide 2.95 and thiocarbohydrazide 2.66.

Samples containing low concentrations of hydrazine must be maintained in an oxygen-free atmosphere prior to derivatization. Failure to exclude oxygen leads to low results for hydrazine but not for carbohydrazide.

RESULTS AND DISCUSSION

The chromatographic separations were complete, as shown in Fig. 1. In this system unreacted benzaldehyde eluted first, followed in order by the benzaldehyde derivatives of carbohydrazide, thiocarbohydrazide and hydrazine. The capacity factors were 1.9, 4.1, 5.6 and 14.6, respectively. Absorption maxima in the UV spectra of carbohydrazide derivatives were noted at 300 and 319 nm whereas a maximum at 301 nm was reported for the hydrazine derivative[10]. An effective compromise was found by setting the detector wavelength at 310 nm for all three derivatives. This wavelength was superior to 254 nm because at 310 nm the detection limit was at least one order of magnitude better for all three compounds. The detection limits (signal-to-noise ratio > 3) found under these conditions were 17, 16 and 32 ppb for the hydrazine, carbohydrazide and thiocarbohydrazide derivatives, respectively (Fig. 2). The recoveries of hydrazine, carbohydrazide and thiocarbohydrazide (triplicate determinations) were 101, 106 and 104% respectively, when solutions containing 0.1% of each were reacted with an excess of benzaldehyde. When five samples, each containing 40 ppb of carbo-



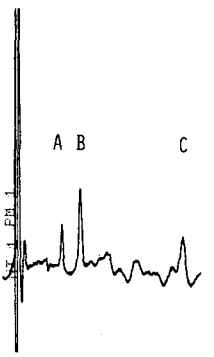


Fig. 1. Chromatogram showing resolution of benzaldehyde (A), carbohydrazide derivative (B), thiocarbohydrazide derivative (C) and benzalazine (D) using a 15-cm OSD-1 column; 10-μl aliquots from samples containing the benzaldehyde derivatives equivalent to 39 ppm of carbohydrazide, 79 ppm of thiocarbohydrazide and 33 ppm of hydrazine were injected with a detector wavelength of 310 nm.

Fig. 2. Detection of (A) carbohydrazide, (B) thiocarbohydrazide and (C) hydrazine in samples containing 16, 32 and 17 ppb, respectively, when 10 μl of the benzaldehyde derivatives were injected, with a detector wavelength of 310 nm (0.004 a.u.f.s.).

TABLE I

STUDY OF DETECTOR RESPONSE FOR BENZALDEHYDE DERIVATIVES OF HYDRAZINE, CARBOHYDRAZIDE AND THIOCARBOHYDRAZIDE

Benzaldehyde derivative of	*Solution[a] concentration (ppm)*	*Response[b] (area/g)*
Hydrazine	0.026	730
	0.052	800
	0.132	804
	13.3	742
Carbohydrazide	0.026	327
	0.052	296
	0.13	321
	13.2	310
Thiocarbohydrazide	0.064	396
	0.128	341
	0.316	328
	32	304

[a] 10 μl of solution injected.
[b] Average of duplicate injections at each concentration.

hydrazide, were analysed the average recovery was 83% with a relative standard deviation of 12%. Six samples containing about 0.4 ppm each of hydrazine were analysed by this method. The average recovery was 87% with a relative standard deviation of 5%.

When preparing or handling samples containing low concentrations of hydrazine, it is essential to prevent oxygen from contacting the sample prior to derivatization. A reaction time of 30 min at a bath temperature of 70°C was used for the derivatization of the analytes, which was in agreement with the derivatization conditions for hydrazine reported previously[14]. The benzaldehyde derivatives were preferred to the corresponding salicylaldehyde derivatives owing to their better solubilities in the solvents used.

A study of the linear response range of the detector for the three derivatives was performed and covered four orders of magnitudes (Table I). All the derivatives exhibited a linear response over three orders of magnitude of concentration from the 0.1 ppm level upward when 10 μl of sample were injected. Linear regression analysis on the points in the linear range, with at least four points for each compound, afforded a correlation coefficient for the resulting lines of at least 0.999 for all the derivatives.

REFERENCES

1 W. J. Ward, P. U. Labine and D. A. Redfield, *Ammonia Plant Saf.*, 21 (1979) 57.
2 M. Slovinsky (Nalco Chemical), *U.S. Pat.*, 4 269 717 1981.
3 M. T. Gonzales Montana, J. L. Gomez Ariza and A. Garcia de Torres, *Am. Quim., Ser. B*, 80 (1984) 123.
4 G. C. Desmukh and A. G. Kulkarni, *Indian J. Chem., Sect. A*, (1981) 314.
5 F. J. Barragan de la Rosa, J. L. Gomez Ariza and F. Pino *Talanta*, 30 (1983) 555.
6 M. Derenzini, F. Farebegoli and V. Marinozzi, *J. Histochem. Cytochem.*, 34 (1986) 1161.

7 F. L. Barragan de la Rosa, J. L. Gomez Ariza and F. Pino, *Mikrochim. Acta*, 1 (1984) 171.
8 V. Bindal and H. K. Pujari, *Indian J. Chem., Sect. B*, 24 (1985) 1227.
9 R. Esmail and F. Kurzer, *Tetrahedon*, 33 (1977) 2007.
10 F. Matsui, D. L. Robertson and E. G. Lovering, *J. Pharm. Sci.*, (1983) 948.
11 A. G. Butterfield, N. M. Curran, E. G. Lovering, F. F. Matsui, D. L. Robertson and R. W. Sears, *Can. J. Pharm. Sci.*, 16 (1981) 15.
12 K. Ravichandran and R. P. Badwin, *Anal. Chem.*, 55 (1983) 1782.
13 P. E. Kester and N. D. Danielson, *Chromatographia*, 18 (1984) 125.
14 F. Matsui, R. W. Sears and E. G. Lovering, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 521.

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Note

Separation of natural product sweetening agents using overpressured layer chromatography[a]

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Overpressured layer chromatography (OPLC) is a modified planar chromatographic technique, in which the vapor phase is eliminated by covering the sorbent layer with an elastic membrane under variable external pressure, and the mobile phase is forced through the sorbent layer with a pump[2,3]. The method is particularly advantageous over thin-layer chromatography (TLC), a capillary-controlled system, in that better resolution and more rapid separation can be obtained[2,3].

OPLC methodology that is applicable to both the analytical and on-line preparative separation of natural products has previously been described[4–7]. In this communication, we wish to report the utilization of OPLC for the separation of a number of potently sweet naturally occurring compounds. Analytical separations have been performed on the eight sweet *ent*-kaurene glycoside constituents of *Stevia rebaudiana* (Bertoni) Bertoni leaves, namely, dulcoside A, rebaudiosides A–E, stevioside and steviolbioside. *S. rebaudiana* extracts and stevioside are used as approved sweetening agents in Japan[8], and great interest is currently being shown in rebaudioside A, a major constituent of the plant that has improved properties as a sweetening agent when compared with stevioside[9]. Separations have also been carried out on some highly sweet compounds recently discovered in this laboratory. Polypodoside A is a steroidal saponin constituent of the rhizomes of *Polypodium glycyrrhiza* D.C. Eaton, that was estimated to possess about 600 times the sweetness potency of sucrose[10]. An analytical OPLC system is described for the separation of polypodoside A from its analogues, polypodosides B and C[1], and a preparative OPLC procedure has been applied to purify polypodoside A when present in a crude *P. glycyrrhiza* column chromatographic fraction. Hernandulcin is an intensely sweet sesquiterpene, about 1000 times sweeter than sucrose, that was initially isolated from the leaves of *Lippia dulcis* Trev., and then synthesized from two commercially available ketones by directed aldol condensation[11,12]. An OPLC method is described for the

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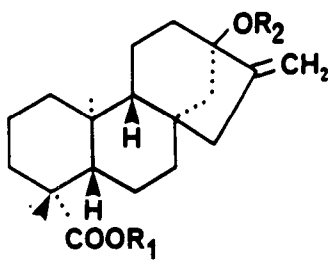
rapid preparative purification of this non-polar compound from an impure synthetic mixture.

EXPERIMENTAL

General procedures

All separations were performed on a Chrompres 25 OPLC instrument (Labor MIM, Budapest, Hungary). The eluting solvent was delivered at a suitable flow-rate with an LDC/Milton Roy (Riviera Beach, FL, U.S.A.) mini-pump VS, and the cushion pressure was kept at 15–17 bar during all separations. Plate-edge impregna-



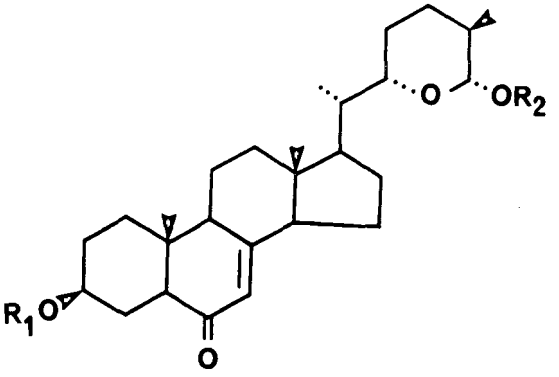
	R_1	R_2
1	β-glc^2-β-glc	β-glc^2-β-glc |^3 β-glc
2	β-glc^2-β-glc	β-glc^2-β-glc
3	β-glc	β-glc^2-β-glc |^3 β-glc
4	β-glc	β-glc^2-α-rha |^3 β-glc
5	β-glc	β-glc^2-glc
6	H	β-glc^2-β-glc |^3 β-glc
7	β-glc	β-glc^2-α-rha
8	H	β-glc^2-β-glc

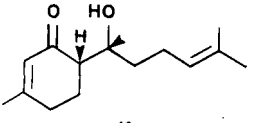
Glc = D-glucopyranosyl; rha = L-rhamnopyranosyl

tion was effected using Impres II polymer suspension (Labor MIM). All solvents used were analytical grade.

Analytical separations were carried out on precoated TLC and high-performance TLC (HPTLC) silica gel 60 F_{254} aluminium- and glass-backed plates, respectively, 10 × 20 cm, 0.2 mm layer thickness (Merck, Darmstadt, F.R.G.). Solute mixtures used in these separations were constituted in a final concentration of about 1 mg/ml with equal quantities of each plant isolate present. Each mixture was applied to the sorbent layers in 5–30-μg quantities. Prior to each separation, the plate was preconditioned with hexane at 2 ml/min for 12 min, to displace air in the adsorbent layers. A flow-rate of 0.2 ml/min was used for all analytical OPLC separations. Chromatograms were evaluated using a Zeiss KM 3 chromatogram spectrophotometer (Zeiss, Oberkochen, F.R.G.).

Preparative separations were made on precoated PSC-silica gel 60 F_{254} plates, 20 × 20 cm, 1 mm layer thickness, with a 4 × 20 cm preconcentration zone (Merck). Such separations were conducted using a previously described on-plate injection technique[7], in which sample injection was accomplished using a Multifit B-D syringe, and, prior to injection, the plates were equilibrated with each solvent system to be used for about 30 min at a flow-rate of 2 ml/min. Eluted fractions were collected in a Buchler (Fort Lee, NJ, U.S.A.) Fractomette Alpha-200 automatic fraction collector. The purity of these fractions was checked by TLC using silica gel 60 PF_{254} aluminium backed plates (Merck).





12

	R_1	R_2
9	β-glc[2]-α-rha	α-rha
10	β-glc	α-rha
11	β-glc	α-rha-3-CH_3

Glc = D-Glucopyranosyl; rha = L-rhamnopyranosyl

Samples

The sweet *ent*-kaurene glycosides, dulcoside A (**7**), rebaudiosides A (**3**), C (**4**), D (**1**), E (**2**) and stevioside (**5**), were isolated in pure form from *S. rebaudiana* leaves as previously described[13,14]. Rebaudioside B (**6**) and steviolbioside (**8**) were prepared from rebaudioside A and stevioside, respectively, by alkaline hydrolysis[13]. Pure polypodosides A (**9**), B (**10**) and C (**11**) were isolated from the rhizomes of *P. glycyrrhiza* collected in Oregon[1,10]. A crude sample of polypodoside A was also obtained from this plant source. (±)-Hernandulcin (**12**) was synthesized from 3-methyl-2-cyclohexen-1-one and 6-methyl-5-hepten-2-one, as published previously[11,12], and was used in this investigation as a highly impure synthetic reaction product.

RESULTS AND DISCUSSION

Separation of the sweet glycosides of Stevia rebaudiana

Thus far, it has not proven possible to resolve all eight sweet diterpene glycosides from *S. rebaudiana* using TLC, and when a published solvent system[14] was employed for this separation, only partial resolution resulted for rebaudiosides D (**1**) and E (**2**), rebaudioside C (**4**) and stevioside (**5**), and rebaudioside B (**6**) and dulcoside A (**7**) (Fig. 1a). When OPLC was applied on normal TLC plates using the same solvent system (Fig. 1b), the development time was reduced by a factor of eight, although no substantial improvement of resolution was apparent between these three pairs of glycosides. However, compounds **4** and **5** were resolved with baseline separation, and compounds **6** and **7** substantially resolved by OPLC in this same solvent system using HPTLC plates (Fig. 1c). Although rebaudiosides D (**1**) and E (**2**) were not separated using this OPLC procedure, it may be pointed out that these two compounds are somewhat more polar than the other *S. rebaudiana* sweet glycosides in being preferentially water soluble, whereas the other six compounds (**3–8**) are extractable into 1-butanol from aqueous solutions[13,14].

*Separations of polypodosides A–C (**9–11**)*

Polypodosides A–C (**9–11**) are polar steroidal saponins that occur in the rhizomes of the North American fern, *P. glycyrrhiza*. Unlike the intensely sweet compound, polypodoside A (**9**), polypodoside B (**10**) is only slightly sweet, while polypodoside C (**11**) is completely devoid of this effect[1,10]. Although a mixture of these compounds was separated easily by TLC on silica gel, this analytical separation took 3 h to complete (Fig. 2a). OPLC on normal TLC plates, using a slightly modified solvent system, enabled the separation of these compounds in only 15 min (Fig. 2b).

An impure sample of polypodoside A (**9**) was obtained after the gravity column chromatographic separation of a 1-butanol extract of *P. glycyrrhiza* rhizomes, by elution with chloroform–methanol (6:1, v/v)[10]. This sample was free of polypodosides B and C, but highly contaminated with other unknown polar plant constituents. The same solvent system as shown in Fig. 2b was chosen for OPLC, although a higher flow-rate of 1.0 ml/min was used. A 55-mg sample of the impure polypodoside was dissolved in 1 ml of mobile phase, filtered, and applied to the preconditioned plate by on-line injection[7]. Altogether 12 10-ml fractions were collected, and 16 mg of pure polypodoside A (**9**) were obtained in fractions 9–11. The total time for this separation was about 2 h.

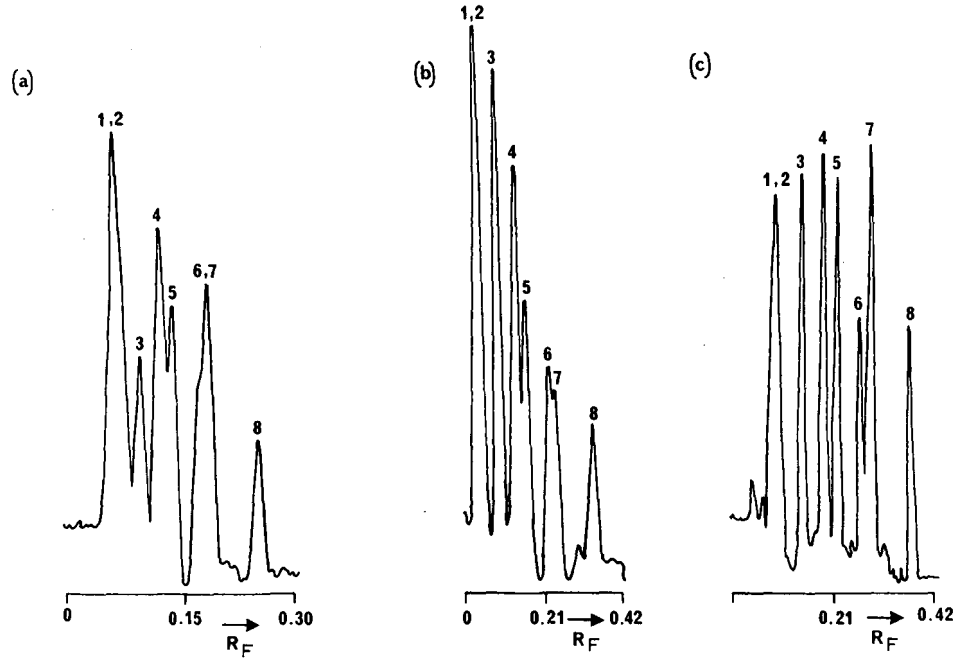


Fig. 1. Analytical separation of sweet *ent*-kaurene glycosides isolated from *S. rebaudiana*. Peaks: 1 = rebaudioside D; 2 = rebaudioside E; 3 = rebaudioside A; 4 = rebaudioside C; 5 = stevioside; 6 = rebaudioside B; 7 = dulcoside A; 8 = steviolbioside. (a) Normal TLC; mobile phase, ethyl acetate–ethanol–water (130:27:20, v/v/v); UV detection (after spraying plate with 60% (w/v) sulfuric acid, and heating for 10 min at 110°C), 325 nm; development time, 135 min; (b) OPLC with TLC plate: solvent and UV detection as for (a); flow-rate, 0.2 ml/min; development time, 16 min; (c) OPLC with HPTLC plate: solvent, UV detection, flow-rate, and development time as for (b).

*Purification of synthetic hernandulcin (***12***)*

When synthetic (±)-hernandulcin (**12**) was prepared by directed aldol condensation from 3-methyl-2-cyclohexen-1-one and 6-methyl-5-hepten-2-one, nearly a 50% yield occurred in this reaction[11,12]. However, in addition to unreacted starting materials, three minor reaction products have also been indentified[12], which must also be removed from the final product. In the present investigation, the work-up of **12** from a mixture of synthetic precursors and reaction products that had been stored for several months was accomplished by OPLC on normal TLC plates, using hexane–ethyl acetate (10:3, v/v) as mobile phase, at a flow-rate of 1 ml/min. The sample (100 mg) was dissolved in 1 ml of mobile phase, and injected onto the preequilibrated sorbent layer. Altogether nine fractions were collected, each of 8 ml, with pure henandulcin (6 mg) collected into fractions 6 and 7. The time of separation was 72 min.

CONCLUSIONS

It has been shown in this investigation that OPLC is effective in both the analytical and preparative separation of three structural classes of high-intensity

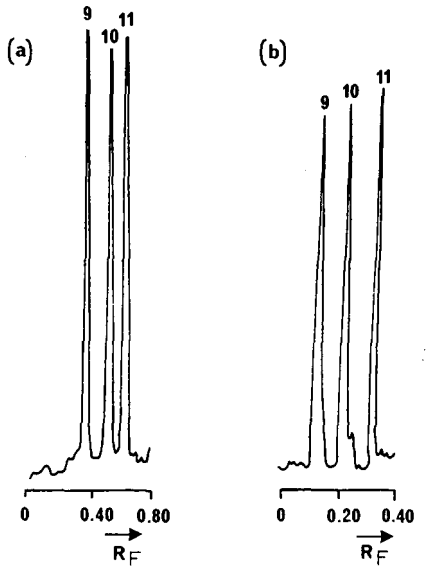


Fig. 2. Analytical separation of sweet steroidal saponins isolated from *P. glycyrrhiza*. Peaks: 9 = polypodoside A; 10 = polypodoside B; 11 = polypodoside C. (a) Normal TLC: mobile phase, chloroform–methanol–water (6:3:1, v/v/v; lower layer); UV detection, 254 nm; development time, 180 min. (b) OPLC with TLC plate: solvent, hexane–chloroform–methanol–water (0.8:6:3:1, v/v/v; lower layer); UV detection, as for (a); flow-rate, 0.2 ml/min; development time, 15 min.

natural sweeteners, which ranged from highly polar *ent*-kaurene glycosides (**1–8**) and steroidal saponins (**9–11**), to a non-polar sesquiterpene (**12**). In analytical separations of mixtures of the first two categories of these substances, the separation speed achieved was 8–12 times faster by OPLC than by conventional TLC. Using an on-line injection technique that avoided the necessity to streak plates with solutes, pure samples of polypodoside A (**9**) and (±)-hernandulcin (**12**) were obtained by preparative OPLC from highly impure starting materials in a period of 2 h or less. In both cases, the use of this preparative procedure avoided the necessity for additional protracted conventional purification techniques. As has been noted previously[4], the greatest utility of OPLC for the separation of bioactive natural products would seem to be for the purification of small (50–100 mg) amounts of partially purified samples.

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REFERENCES

1 J. Kim and A. D. Kinghorn, *Phytochemistry*, 28, in press.
2 Z. Witkiewicz and J. Bladek, *J. Chromatogr.*, 373 (1986) 111.
3 E. Tyihák, *J. Pharm. Biomed. Anal.*, 5 (1987) 191.
4 C. A. J. Erdelmeier, I. Erdelmeier, A. D. Kinghorn and N. R. Farnsworth, *J. Nat. Prod.*, 49 (1986) 1133.
5 K. Dallenbach-Toelke, Sz. Nyiredy, S. Y. Mészáros and O. Sticher, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 362.
6 Sz. Nyiredy, C. A. J. Erdelmeier, K. Dallenbach-Toelke, K. Nyuredy-Mikita and O. Sticher, *J. Nat. Prod.*, 49 (1986) 885.
7 C. A. J. Erdelmeier, A. D. Kinghorn and N. R. Farnsworth, *J. Chromatogr.*, 389 (1987) 345.
8 A. D. Kinghorn and D. D. Soejarto, in H. Wagner, H. Hikino and N. R. Farnsworth (Editors), *Economic and Medicinal Plant Research*, Vol. 1, Academic Press, London, 1985, p. 1.
9 B. Crammer and R. Ikan, in T. H. Grenby (Editor), *Developments in Sweeteners —3*, Elsevier Applied Science, London, 1987, p. 45.
10 J. Kim, J. M. Pezzuto, D. D. Soejarto, R. A. Lang and A. D. Kinghorn, *J. Nat. Prod.*, 51, in press.
11 C. M. Compadre, J. M. Pezzuto, A. D. Kinghorn and S. K. Kamath, *Science (Washington, D.C.)*, 227 (1985) 417.
12 C. M. Compadre, R. A. Hussain, R. L. Lopez de Compadre, J. M. Pezzuto and A. D. Kinghorn, *J. Agric. Food Chem.*, 35 (1987) 273.
13 A. D. Kinghorn, N. P. D. Nanayakkara, D. D. Soejarto, P. J. Medon and S. Kamath, *J. Chromatogr.*, 237 (1982) 478.
14 A. D. Kinghorn, D. D. Soejarto, N. P. D. Nanayakkara, C. M. Compadre, H. C. Makapugay, J. M. Hovanec-Brown, P. J. Medon and S. K. Kamath, *J. Nat. Prod.*, 47 (1984) 439.

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Note

Mobility of some carboxy- and hydroxybenzene derivatives on thin layers of plain and iron(III)-impregnated silica gel

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The mobility of metals, including iron, through soil and sediments is predominantly attributed to complexation with humic substances, mainly via their functional groups such as carboxy and hydroxy[1,2]. Investigations on this subject have been performed with naturally occurring material, and in this way no information could be obtained about the activity of the functional groups with respect to their position in the benzene ring. A kinetic approach involving dissolution of iron (hydr)oxides by biogenic ligands was adopted by Stumm and co-workers[3,4] using different organic acids as ligands.

In previous work[5] we examined the use of thin-layer chromatography with aqueous systems for development on plain and iron(III)-impregnated silica gel and studied the behaviour of some phenolic acids usually found as degradation products of humic material[6,7]. In this work we tested benzene derivatives containing more than one hydroxy and carboxy group in different positions and a carboxy group not directly attached to the benzene ring. All the compounds tested were derived from some of the presumed structures of humic acids or their precursors.

EXPERIMENTAL

The chemicals and procedures were the same as described previously[5]. The parameter R_i [R_F (impregnated) $-R_F$ (plain)] was calculated to make the differences in mobilities more evident. For thin-layer chromatography pre-coated plates of silica gel 60 F_{254} (Merck) were used.

RESULTS AND DISCUSSION

The compounds tested are shown in Fig. 1 and the results of their behaviour on plain and impregnated plates are presented in Table I.

On plain plates the behaviour of the compounds more or less followed their solubility in water, and all substances moved considerably, except phthalic acids. On impregnated plates all acids and pyrogallol had smaller R_F values than on plain plates, regardless of the position of the carboxyl group. On the other hand, the mobility of dihydroxybenzenes was almost the same on impregnated and plain plates. This behaviour can be considered to be a consequence of the characteristics of the iron(III) complexes expected to be formed during the chromatography on iron-im-

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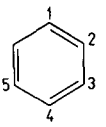

	1	2	3	4	5
I	COOH	COOH	-	-	-
II	COOH	-	COOH	-	-
III	COOH	-	OH	OH	OH
IV	OH	OCH_3	-	CH_2-CH=CHCOOH	-
V	OH	-	-	CH=CHCOOH	-
VI	OH	OH	-	-	-
VII	OH	-	OH	-	-
VIII	OH	-	-	OH	-
IX	OH	OH	OH	-	-
X	OH	-	-	CH_2COOH	-

Fig. 1. Compounds tested.

pregnated plates. The investigations on solutions of such complexes revealed that at pH *ca.* 5 an uncharged catechol–iron complex (3:1) is formed having an equilibrium constant in the range of 10^{40} (ref. 8), what is enormous in comparison with those of iron complexes with other oxygen-containing ligands[9]. The results suggest that catechol ligands may dissolve iron oxides by forming stable soluble complexes; in this category can also be included some phenolic acids[5] having high R_F values on iron-impregnated layers. The behaviour of pyrogallol and gallic acid[10] is different, *i.e.*, they barely moved on impregnated plates. This may be explained by exclusion of the

TABLE I

R_F AND R_i × 100 VALUES OF SOME CARBOXY- AND HYDROXYBENZENE DERIVATIVES ON PLAIN AND IRON(III)-IMPREGNATED SILICA GEL PLATES

Solvents: DW = distilled water; TW = tap water; SW = sea water.

No.	*Compound*	R_F × *100*						R_i × *100*		
		Plain			*Impregnated*			*DW*	*TW*	*SW*
		DW	*TW*	*SW*	*DW*	*TW*	*SW*			
I	*o*-Phthalic acid	24	25	33	11	11	10	−13	−14	−23
II	*m*-Phthalic acid	7	5	7	0	0	0	−7	−5	−7
III	Gallic acid	100	100	100	11	15	12	−89	−85	−88
IV	Ferulic acid	100	100	88	15	16	13	−85	−84	−75
V	*p*-Coumaric acid	100	100	85	17	16	18	−84	−82	−67
VI	Catechol	83	75	63	86	89	70	+3	+14	+7
VII	Resorcinol	88	80	61	94	91	75	+6	+11	+14
VIII	Quinol	100	100	89	93	88	97	−7	−12	+8
IX	Pyrogallol	100	100	91	7	15	11	−93	−85	−81
X	*p*-Hydroxyphenyl-acetic acid	100	100	86	54	59	56	−46	−41	−30

third hydroxy group from the primary complexation and its possible involvement in polymerization of the complex cores[10] resulting in lower solubility.

From the behaviour of the compounds on impregnated plates, some conclusions may be drawn about the activities of the corrresponding functional groups in soil and sediments. The fact that on impregnated plates phenolic acids with a free hydroxy group[5] and dihydroxybenzenes move considerably may indicate that iron and possibly other metals, by complexation with related functional groups in soil and sediments, become movable. On the other hand, benzene dicarboxylic acids and phenolic acids with protected hydroxy groups[5] move slowly or remain almost at the start position on impregnated plates, which suggests the retardation of metals depending on the position and nature of these groups.

REFERENCES

1 M. Schnitzer and S. I. M. Skinner, *Soil Sci.*, 99 (1965) 278.
2 M. Schnitzer and P. A. Poabst, *Nature (London)*, 213 (1967) 598.
3 B. Zinder, G. Furrer and W. Stumm, *Geochim. Cosmochim. Acta*, 50 (1986) 1861.
4 W. Stumm, B. Sulzberger, C. Siffert, D. Suter and S. Banwart, *Mar. Chem.*, in press.
5 O. Hadžija, S. Iskrić and M. Tonković, *J. Chromatogr.*, 402 (1987) 358.
6 J. I. Hedges and P. L. Parker, *Geochim. Cosmochim. Acta*, 40 (1976) 1019.
7 I. Kögel and R. Bochter, *Soil Biol. Biochem.*, 17 (1985) 637.
8 R. C. Hider, A. R. Mohd-Nor, J. Silver, I. E. G. Morrison and L. V. C. Rees, *J. Chem. Soc., Dalton Trans.*, (1981) 609.
9 A. Avdeef, S. R. Sofen, T. L. Bregante and K. N. Raymond, *J. Am. Chem. Soc.*, 100 (1978) 5362.
10 S. A. Kazmi, M. S. Qureshi and Z. Maqsood, *Inorg. Chim. Acta*, 137 (1987) 151.

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Book Review

Ion chromatography in water analysis, by O. A. Shpigun and Yu. A. Zolotov, Ellis Horwood, Chichester, 1988, 188 pp., price £ 32.50, ISBN 0-7458-0020-3.

This volume can definitely be classed among the fine accounts of ion chromatography, of which there are already several available, which can be used as starting points when intending to work with this method.

The Preface states that the book is intended to provide a better coverage of gradient methods than can be found in previous texts, but it would have been helpful if the authors could have stated up to which date the literature was collected, as some references are from 1986 and only a few date from 1987.

Some minor points that struck the reviewer were that in the chapter on the determination of organic acids, a number of competing methods such as high-performance liquid chromatography, gas–liquid chromatography and isotachophoresis are not mentioned and no discussion is given of their relative merits, so lessening the usefulness to the reader. The same applies to the chapter on the determination of aliphatic amines.

In the chapter on the determination of metals, some splendid separations of transition metals are shown, but it is not evident from the book where the usual 40 metals would interfere. This kind of information is, however, unfortunately sadly lacking also in the primary literature.

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(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

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Analytical Artifacts

GC, MS, HPLC, TLC and PC

by **B.S. MIDDLEDITCH,** *Dept. of Biochemical and Biophysical Sciences, University of Houston, Houston, TX, USA*

(Journal of Chromatography Library, 44)

This encyclopaedic catalogue of the pitfalls and problems that all analysts encounter in their work is destined to spend more time on the analyst's workbench than on a library shelf. The author has dedicated the book to "the innumerable scientists who made mistakes, used impure chemicals and solvents, suffered the consequences of unanticipated side-reactions, and were otherwise exposed to mayhem yet were too embarrassed to publish their findings".

Traditionally, the mass spectroscopist or gas chromatographer learnt his trade by participating in a 4-6 year apprenticeship as graduate student and post-doctoral researcher. Generally, no formal training was provided on the things that go wrong, but this information was accumulated by sharing in the experiences of colleagues. Nowadays, many novice scientists simply purchase a computerized instrument, plug it in, and use it. Much time can be wasted in studying and resolving problems due to artifacts and there is also a strong possibility that artifacts will not be recognized as such. For example, most analysts realize that they should use glass rather than plastic containers; but few of them would anticipate the possibility of plasticizer residues on glassware washed using detergent from a plastic bottle.

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