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See page 3 of cover for Publication Schedule, Information for Authors, and information on the News Section and Advertisements.

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CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index)

Protein–protein interactions studied by counter-current distribution. III. Simulation of self-associating systems
by L. Backman (Umeå, Sweden) (Received October 22nd, 1981) 185

Adsorption behaviour of several supports in reversed-phase thin-layer chromatography as demonstrated by the determination of relative partition coefficients of some 4-hydroxycoumarin derivatives
by W. F. van der Giesen and L. H. M. Janssen (Utrecht, The Netherlands) (Received October 22nd, 1981) 199

Comparison of adsorption and reversed-phase partition high-performance liquid chromatography for the separation of androgens
by J.-T. Lin and E. Heftmann (Berkeley, CA, U.S.A.) (Received October 29th, 1981) . . 215

Binding capacities of human serum albumin monomer and dimer by continuous frontal affinity chromatography
by N. I. Nakano, Y. Shimamori and S. Yamaguchi (Otaru, Japan) (Received October 13th, 1981) 225

High-performance liquid chromatography–negative chemical ionization mass spectrometry of organophosphorus pesticides
by C. E. Parker (Research Triangle Park, NC, U.S.A.), C. A. Haney (Research Triangle Park, NC, U.S.A. and Chapel Hill, NC, U.S.A.) and J. R. Hass (Research Triangle Park, NC, U.S.A.) (Received October 6th, 1981) 233

Identification by gas chromatography–mass spectrometry of vinyl chloride oligomers and other low-molecular-weight components in poly(vinyl chloride) resins for food packaging applications
by J. Gilbert, M. J. Shepherd, J. R. Startin and M. A. Wallwork (Norwich, Great Britain) (Received October 12th, 1981) 249

Determination of polycyclic aromatic hydrocarbons in vegetable oils by caffeine complexation and glass capillary gas chromatography
by L. Kolarovič and H. Traitler (La Tour-de-Peilz, Switzerland) (Received October 28th, 1981) 263

Clean-up of polynuclear aromatic hydrocarbons from air particulate matter on XAD-2
by T. Spitzer (Hamburg, G.F.R.) (Received October 14th, 1981) 273

Improved gas–liquid chromatographic method for the determination of protein amino acids
by M. Makita, S. Yamamoto and S. Kiyama (Okayama, Japan) (Received September 28th, 1981) 279

Rapid and highly sensitive method for determination of methamphetamine and amphetamine in urine by electron-capture gas chromatography
by M. Terada, T. Yamamoto, T. Yoshida, Y. Kuroiwa and S. Yoshimura (Tokyo, Japan) (Received September 30th, 1981) 285

Notes

Isoelectric focusing in chloral hydrate
by O. Brenna, E. Gianazza and P. G. Righetti (Milan, Italy) (Received October 21st, 1981) 293

(Continued overleaf)

Some N-acyl derivatives of 1-(α-naphthyl)ethylamine as stationary phases for the separation of optical isomers in gas chromatography
by N. Ôi, H. Kitahara, Y. Inda and T. Doi (Takarazuka-shi, Japan) (Received October 19th, 1981) 297

β-Cyclodextrin as a chiral component of the mobile phase for separation of mandelic acid into enantiomers in reversed-phase systems of high-performance liquid chromatography
by J. Dębowski, D. Sybilska and J. Jurczak (Warsaw, Poland) (Received October 13th, 1981) 303

Water-free solvent system for droplet counter-current chromatography and its suitability for the separation of non-polar substances
by H. Becker, J. Reichling and W.-Ch. Hsieh (Heidelberg, G.F.R.) (Received October 26th, 1981) 307

High-performance liquid chromatography of cytokinin ribonucleoside 5′-monophosphates
by I. M. Scott and R. Horgan (Aberystwyth, Great Britain) (Received November 2nd, 1981) 311

Use of ion-pair, reversed-phase, high-performance liquid chromatography for the analysis of cytokinins
by M. A. Walker and E. B. Dumbroff (Waterloo, Canada) (Received November 27th, 1981) 316

High-performance liquid chromatographic separation of the chlorination products of terephthalamide
by K.-S. Lee, K.-L. Hsio and T.-T. Su (Hsinchu, Taiwan) and K.-T. Kuo (Chung-Li, Taiwan) (Received October 26th, 1981) 324

Chromatographic fractionation of multiple forms of red blood cell hexokinase
by V. Stocchi, A. Stulzini and M. Magnani (Urbino, Italy) (Received October 24th, 1981) 330

Simple detection of steroid sulphatase activity after chromatographic fractionation
by Y. Yamaguchi (Osaka, Japan) (Received September 30th, 1981) 336

Sensitive and rapid amino acid analysis of peptide hydrolysates by high-performance liquid chromatography of *o*-phthaldialdehyde derivatives
by J. P. H. Burbach, A. Prins, J. L. M. Lebouille, J. Verhoef and A. Witter (Utrecht, The Netherlands) (Received October 16th, 1981) 339

Determination of butanilicaine in horse plasma and urine by extractive benzoylation and gas chromatography with a nitrogen–phosphorus detector
by F. T. Delbeke and M. Debackere (Ghent, Belgium) (Received October 15th, 1981) . . 344

Preparative-scale separation of alkene geometric isomers by liquid chromatography
by R. P. Evershed, E. D. Morgan and L. D. Thompson (Keele, Great Britain) (Received November 23rd, 1981) 350

Book Reviews

Actinide separations (ACS Symposium Series, No. 117) (edited by J. D. Navratil and W. W. Schulz), reviewed by M. Lederer 355

Fluorimetrie (by M. Zander), reviewed by J. Wagner 356

Bibliography Section

Gas Chromatography B1
Liquid Column Chromatography B15
Paper Chromatography B47
Thin-Layer Chromatography B52
Electrophoretic Techniques B74

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PROTEIN–PROTEIN INTERACTIONS STUDIED BY COUNTER-CURRENT DISTRIBUTION

III. SIMULATION OF SELF-ASSOCIATING SYSTEMS

LARS BACKMAN
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(Received October 22nd, 1981)

SUMMARY

Biological macromolecules that undergo self-association in solution are widely encountered. As differences in surface properties, such as charge and hydrophobicity, between monomeric and polymeric forms, are very probable it should be possible to use the counter-current distribution technique in two-phase liquid systems for the study of self-association.

The distribution behaviour of a self-associating solute has been simulated in order to establish the boundary conditions and limitations as well as potentials of the counter-current distribution technique as a tool for studying self-associations. The distributions have been calculated for a range of association constants, partition coefficients and initial solute concentration as well as for small zone and moving boundary counter-current distribution.

INTRODUCTION

Self-association of biological macromolecules in solution is a widely encountered phenomenon. The degree of self-association is usually dependent on solution conditions such as pH, ionic strength and temperature.

A self-association can be represented by equilibria of the type

$$j\,P_1 \rightleftharpoons P_j \qquad j = 2, 3, \ldots \tag{1}$$

or

$$j\,P_1 \rightleftharpoons r\,P_m + s\,P_n \tag{2}$$

and related equilibria, where P represents the self-associating solute.

In principle, self-associations can be studied by almost any experimental method that can differentiate between monomer and polymer(s) in some respect, *e.g.*, molecular weights or surface properties. However, sedimentation velocity and

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equilibrium, elastic light scattering, membrane osmometry and molecular sieve chromatography are the most extensively used techniques[1]. Many other methods, such as various spectroscopic techniques, calorimetry and isoelectric focusing, have been described and used for the analysis of associating systems[1]. Single-step partitions[2–4] and counter-current distribution (CCD)[5–7] in aqueous two-phase systems have been used successfully to study heterogeneous protein–protein interactions. Therefore, both partitions and CCD should also be suitable for the analysis of self-associations. In fact, the tetramer–dimer dissociation of human oxy- and methaemoglobin has been studied by Middaugh and Lawson using single-step partitions[8]. The CCD behaviour of self-associating solutes has also been predicted from the concentration dependence of the partition coefficients of the solutes[9]. However, to my knowledge the CCD method has not been used to analyse macromolecular self-associations.

In order to establish boundary conditions and limitations as well as possibilities of the CCD method as a tool for the study self-associations it was necessary to make a thorough theoretical investigation of the method. Therefore, the dependence of the CCD behaviour of monomer–j-mer and monomer–m-mer–n-mer equilibria on association constants, partition coefficients and initial solute concentration was studied. Monomer–single polymer equilibria in small zone CCD have previously been treated theoretically although incompletely by Bethune and Kegeles[10]. However, their approach is not useful for the description and future adaptation to experiments in aqueous two-phase systems owing to the unrealistic values assigned to the partition coefficients and the initial mass of the solute. On the other hand, the theoretical treatments of moving boundary CCD[11,12] are much more extensive. However, these treatments are not primarily aimed at the description of CCD behaviour but rather to incorporate diffusional effects in continuous transport systems, *i.e.*, sedimentation velocity.

In this paper, the behaviour of equilibria of types 1 and 2 in small zone CCD and moving boundary CCD are discussed.

CALCULATIONS

In the following description of the equilibration of a self-associating system between two phases it is assumed that the two phases are immiscible, that no volume change occurs upon mixing and equilibration, that all solutions are thermodynamically ideal and that there is equilibrium both within and between the phases before a transfer. Further, the total concentration of solute in any chamber is expressed as total monomer concentration.

If a monomer–j-mer association occurs in such a biphasic system, then the various equilibria would be

$$\begin{array}{ccc} & \xrightarrow{K^{u}_{1j}} & \\ j\,P_{1,u} & \rightleftharpoons & P_{j,u} \\ \uparrow\downarrow K_{P_1} & & \uparrow\downarrow K_{P_j} \\ & \xrightarrow{K^{l}_{1j}} & \\ j\,P_{1,l} & \rightleftharpoons & P_{j,l} \end{array} \qquad (3)$$

where the subscripts u and l refer to the upper and lower phase, respectively. The equilibria within each phase are described by the association constants, K^{u}_{1j} and K^{l}_{1j}, defined as

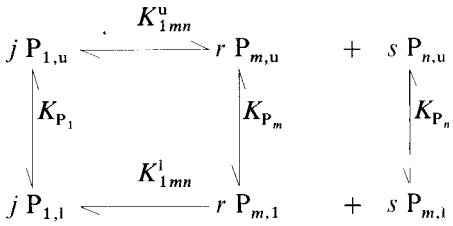
$$K^{x}_{1j} = \frac{[P_j]_x}{([P_1]_x)^j} \tag{4}$$

where x = u or l and the square brackets denote concentrations in moles per litre. The partition coefficient of each solute, i.e., K_{P_1} and K_{P_j}, defined as

$$K_{P_z} = \frac{[P_z]_u}{[P_z]_l} \tag{5}$$

where $z = 1$ or j, represent the equilibrium between the two phases.

Similarly, self-associations of type 2 can be depicted by



(6)

where

$$K^{x}_{1mn} = \frac{([P_m]_x)^r\ ([P_n]_x)^s}{([P_1]_x)^j} \tag{7}$$

where x = u or l. However, the formation of higher polymeric species can be considered to occur in a single-step association or through step-wise associations. In the former case the concentration of any polymeric form can be expressed by equations such as eqn. 4. In the latter case the concentration of any polymeric form is given by

$$[P_m] = [P_1]^m \prod_{\substack{g=0 \\ h=g+1}}^{m-1} K_{gh} \tag{8}$$

where, by definition, $K_{01} = 1$. The subsequent equations are developed in the same manner as previously[13] for repeated partitions, CCD, in which the lower phase is held stationary and the upper phase is transferred after equilibrium to the next chamber in sequence.

The total concentration of solute, expressed as monomeric concentration, in the ith chamber after n transfers is obtained by summation of all species in solution at equilibrium

$$[P_1]^{in}_{tot} = \sum_j (p\, j\, [P_j]^{in}_u + q\, j\, [P_j]^{in}_l) \tag{9}$$

where p and q are the fractions of volume of the upper and the lower phase, respectively. Eqn. 9 can be rewritten by inserting eqns. 4, 5 and 8 as

$$[P_1]_{tot}^{in} = \sum_j j\,([P_1]_l^{in})^j\,\alpha_j \tag{10}$$

where

$$\alpha_j = (p\,K_{P_j} + q)\,K_{1j}^1 \tag{11}$$

or

$$\alpha_j = (p\,K_{P_j} + q) \prod_{\substack{g=0 \\ h=g+1}}^{j-1} K_{gh}^1 \tag{12}$$

depending on the type of self-association.

Small zone CCD

In a small zone CCD experiment the solute is initially introduced into the first m chambers. Thus, the total concentration in these chambers before the first transfer are

$$[P_1]_{tot}^{x0} = \sum_j (p\,j\,[P_j]_u^{x0} + q\,j\,[P_j]_l^{x0}) \tag{13}$$

where $x = 0, 1, \ldots, m$. Given the initial values of total concentration and assigning values to the association constant(s) in the lower phase (or in the upper phase) and to the partition coefficients, the equilibrium concentration of the monomer in each lower phase can be determined. From these values the other equilibrium concentrations in each chamber can be calculated. Each upper phase is then transferred to the next chamber in sequence, except that the upper phase of the last chamber, *i.e.*, chamber n, is transferred to chamber zero. The first transfer is accomplished by calculating the total concentrations in chambers zero to $m + 1$ after the transfer using

$$[P_1]_{tot}^{01} = \sum_j (p\,j\,[P_j]_u^{n0}) + \sum_j (q\,j\,[P_j]_l^{00}) \tag{14}$$

$$[P_1]_{tot}^{x1} = \sum_j (p\,j\,[P_j]_u^{x-10}) + \sum_j (q\,j\,[P_j]_l^{x0}) \tag{15}$$

where $x = 1, 2, \ldots, m + 1$. Eqn. 10 is then solved for $m + 1$ chambers using the new values of total concentrations, yielding $m + 1$ sets of equilibrium concentrations for these chambers. Hence, by repeating this process, the final CCD pattern is obtained for n transfers.

Moving boundary CCD

In a moving boundary CCD experiment a plateau of original concentration

must be maintained throughout the experiment. A plateau is obtained if the number of chambers initially containing the solute is greater than the number of transfers, *i.e.*, $m > n$, and chamber zero is fed with pure upper phase at each transfer.

The equilibrium concentrations before a transfer and the total concentrations after a transfer in each chamber are obtained in the same manner as in small zone CCD. However, the total concentration in chamber zero after n transfers is given by

$$[P_1]_{tot}^{0n} = \sum_j (q\, j\, [P_j]_l^{0n-1}) \tag{16}$$

where $n = 1, 2, \ldots, n$. Correspondingly, the complete moving boundary CCD pattern is obtained by repetition of this process.

The programs, written in Basic for a Hewlett-Packard 9835 desk-top computer and in Fortran for a Cyber 172 computer, were mainly checked by summation of the equilibrium concentrations in all chambers after each transfer. The total amount of solute in all chambers never differed from that initially introduced by more than 1 part in 10^8.

The programs were also checked by letting all partition coefficients be equal or by setting the association constants equal to zero. In both instances the simulated CCD patterns should coincide with appropriate binomial curves, which they also did.

RESULTS AND DISCUSSION

After the completion of a CCD experiment all the extractable information, *e.g.*, pattern position, partition coefficients, homogeneity and distribution behaviour, are concealed in the resulting CCD pattern. Part of this information can be enhanced by taking the first derivative of the pattern. The first derivative or concentration gradient is particularly useful for detecting abnormalities in the distribution behaviour. Thus, the gradient should also be useful for comparing the CCD behaviour of different self-associating systems.

As CCD is a discontinuous process, the concentration gradient must be approximated by taking the differences between solute concentrations in adjacent chambers. Further, for the sake of clarity only the part of the concentration gradient patterns corresponding to the dispersed edges of the CCD patterns have been plotted in the figures.

The CCD pattern of a reversible self-associating solute is dependent on the initial concentration and the association constants as well as on the partition coefficients and the number of transfers. If the polymers migrate more rapidly than the monomer, *i.e.*, $KP_1 < KP_2, KP_3, \ldots$, then the polymeric species in the forward edge of the zone will migrate into chambers of low solute concentration. The equilibrium is thus shifted towards dissociation of polymers to form more slowly migrating monomers. Consequently, a hyper-sharpened boundary is formed at the forward edge, whereas at the backward edge the boundary is extended as dilution by polymer migration continually shifts the solute composition to a higher monomer content. This is analogous to the behaviour of a self-associating solute in molecular sieve chromatography[14,15]. The effects on the boundaries would obviously be reversed if the partition coefficient of the monomer is greater than those of the polymers, as demonstrated in Fig. 1.

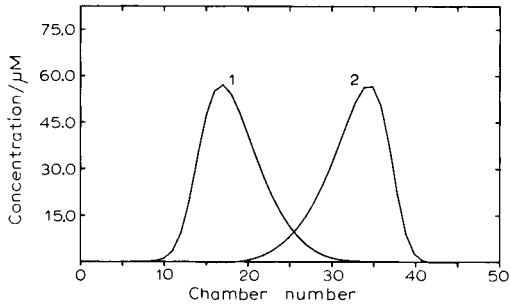


Fig. 1. Influence of the partition coefficients on the CCD behaviour of a monomer–dimer–trimer association. The initial solute concentration was 0.5 mM, the number of transfers was 50 and the association constants in the lower phase, K^l_{12} and K^l_{23}, were both $1 \cdot 10^4$ M^{-1}. The partition coefficients of monomer, dimer and trimer were 0.8, 0.5 and 0.2 (curve 1) and 1.25, 2 and 5 (curve 2), respectively.

As the migration continues the solute spreads into an increasing number of chambers and the relative monomer content of the solute is thereby gradually increased. Accordingly, as the number of transfers increases, the migration rate of the zone slowly approaches that of the monomer but nevertheless the hyper-sharpening and the dispersion of the boundaries persist, as Fig. 2 illustrates.

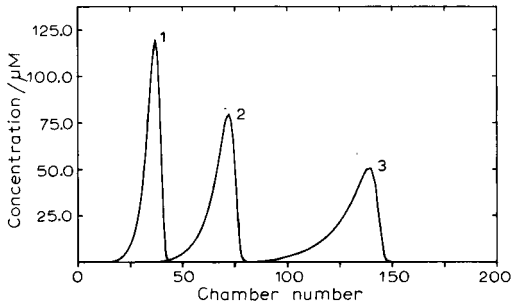


Fig. 2. Influence of the number of transfers on the CCD behaviour of a monomer–dimer association. The initial solute concentration was 1 mM, the association constant in the lower phase was 5000 M^{-1} and the partition coefficients of monomer and dimer were 1 and 5, respectively. The number of transfers was 50 (curve 1), 100 (curve 2) and 200 (curve 3).

The solute composition in any chamber is determined by the total solute concentration in that chamber and by the association constants, whereas the position of the zone is also determined by the partition coefficients. Therefore, the value of the association constants and the initial solute concentration determine the ratio of monomer to polymer and thus the degree of hyper-sharpening and dispersion of the CCD pattern.

Small zone CCD

The CCD behaviour of a series of self-associating systems was simulated and some typical results are shown in Figs. 3–7. The different CCD patterns represent different assignments to the values of the relevant association constants, and the

values for each class were chosen to cover a range from relatively loose to relatively tight association.

Monomer–dimer systems

Fig. 3a shows the CCD patterns produced by simulating the counter-current distribution of a monomer–dimer associating system. For a sufficiently loose association the solute zone migrates at the same rate as the monomer and, hence, the resulting CCD pattern is indistinguishable from a binomial one calculated with the partition coefficient of the monomer. The migration rate of the solute zone will approach that of the dimer as the association constants are increased and the dimer content thus becomes significant. Finally, at infinite association the migration rate will be the same as that of the dimer. Similar behaviour can be observed if the initial solute concentration is varied instead of the association constants. The corresponding concentration gradient patterns are shown in Fig. 3b. The patterns are unimodal and smooth; none of them shows any shoulder or inflection. Further, in all of the calculated gradient patterns the parts corresponding to the dispersed edge of the CCD pattern are appreciably skewed in the same direction, and the degree of skewing increases as the association becomes tighter.

In a previous paper[13] we postulated that the model for heterogeneous 1:1 interactions could be used to simulate dimerization patterns. However, this conclusion has now been found to be incorrect.

Monomer–trimer systems

CCD patterns generated by the model using different values of the monomer–

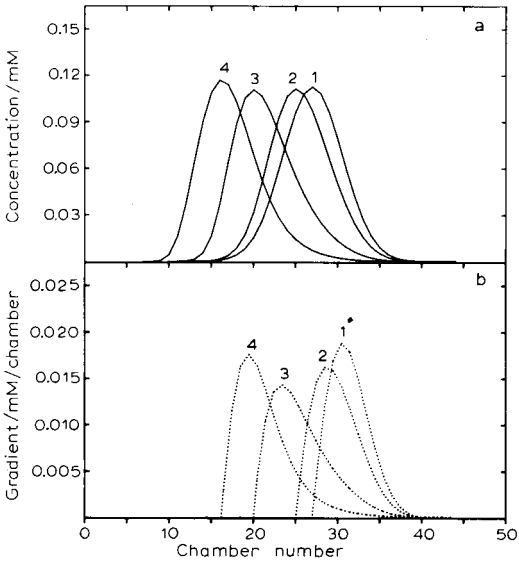


Fig. 3. (a) Simulated CCD patterns and (b) corresponding concentration gradient patterns of a monomer–dimer association. The initial solute concentration was 1 mM, the number of transfers was 50 and the partition coefficients of monomer and dimer were 1.2 and 0.4, respectively. The association constant in the lower phase was 100 M^{-1} (curve 1), 1000 M^{-1} (curve 2), $1 \cdot 10^4$ M^{-1} (curve 3) and $1 \cdot 10^5$ M^{-1} (curve 4).

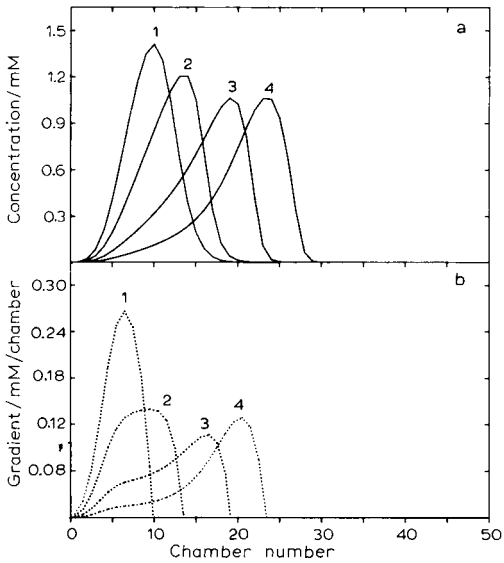


Fig. 4. (a) Simulated CCD patterns and (b) corresponding concentration gradient patterns of a monomer–trimer association. The initial solute concentration was 10 mM, the number of transfers was 50 and the partition coefficients of monomer and trimer were 0.2 and 1.5, respectively. The association constant in the lower phase was 1000 M^{-2} (curve 1), $1 \cdot 10^4$ M^{-2} (curve 2), $1 \cdot 10^5$ M^{-2} (curve 3) and $1 \cdot 10^6$ M^{-2} (curve 4).

trimer association are shown in Fig. 4a. The patterns are very similar to those simulated for a monomer–dimer system; the hyper-sharpening and dispersion progressively increase as the association becomes tighter or the initial concentration increases.

A useful diagnostic according to Bethune and Kegeles[10] for distinguishing between monomer–dimer and monomer–trimer associations in CCD should be the presence of an additional inflection point on the dispersed edge in the latter case. Hence, there should be a third optimum in the concentration gradient pattern.

The gradient patterns in Fig. 4b do not fulfil the predictions of the model of Bethune and Kegeles[10]. Only in gradient patterns 3 and 4 is a tendency to develop an extra optimum clearly apparent. In the part of pattern 2 corresponding to the dispersed edge no shoulder or inflection is visible; the main feature is its strange broadened shape. However, if the values assigned to the partition coefficients are extreme, *e.g.*, $KP_1 = 15$ and $KP_3 = 0.15$, the additional inflection in the CCD pattern will be visible. A similar discrepancy is also found between the asymptotic and diffusion models of sedimentation velocity[16] and molecular sieve chromatography[17].

Monomer–tetramer and monomer–hexamer systems

The simulated CCD patterns of monomer–tetramer associations (Fig. 5a) differ substantially from those patterns obtained for monomer–trimer and monomer–dimer associations. The patterns are not as smooth as before and the additional inflection point is readily apparent in patterns 3–5.

The concentration gradient patterns in Fig. 5b show a corresponding appearance. Patterns 3–5 exhibit distinct minima while pattern 2 shows a very marked shoulder. In pattern 1, for the weakest association, only an indication of a shoulder is

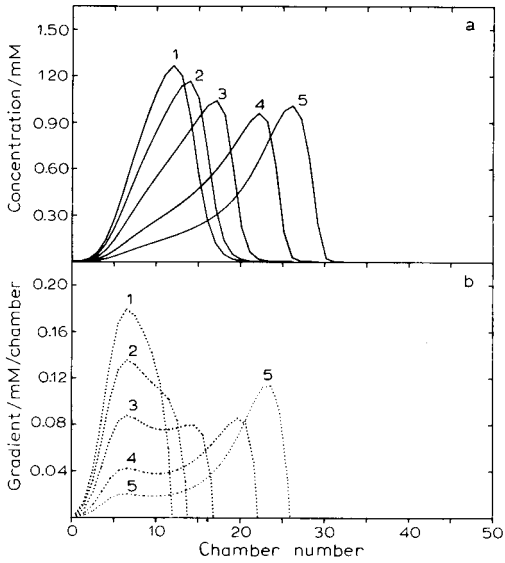


Fig. 5. (a) Simulated CCD patterns and (b) corresponding concentration gradient patterns of a monomer–tetramer association. The initial solute concentration was 10 mM, the number of transfers was 50 and the partition coefficients of monomer and tetramer were 0.2 and 1.5, respectively. The association constant in the lower phase was $1 \cdot 10^6\ M^{-3}$ (curve 1), $2.5 \cdot 10^6\ M^{-3}$ (curve 2), $1 \cdot 10^7\ M^{-3}$ (curve 3), $1 \cdot 10^8\ M^{-3}$ (curve 4) and $1 \cdot 10^9\ M^{-3}$ (curve 5).

evident. The most striking feature of the simulated gradient patterns for monomer–tetramer associations is, however, that all of the first maxima of the various gradient patterns lie nearly at the same position and, further, are very close to the position of the maximum for the monomer.

The effect of varying the association strength for a hexamerizing system resembles that of a tetramerizing system, as can be seen in Fig. 6. However, the presence of an inflection point on the dispersed edge of the CCD patterns is much more obvious. In this instance not only the first maxima but also the minima of the gradient patterns all lie at nearly the same position.

Monomer–dimer–trimer and related systems

These types of systems are much more complex than the other systems, because there are nearly no restrictions on the experimentally possible values of the partition coefficients. Hence there is no relationship between partition coefficient and size of the polymer as there is in molecular sieve chromatography[15].

The earlier approaches[11,12] of letting the velocities and the diffusion coefficients impose on the attainable values of the partition coefficients are not useful for a description of the distribution behaviour. Therefore, only a few simulations were made for these types of associations. Fig. 7 shows the resulting patterns for a monomer–dimer–trimer–tetramer system and for a monomer–dimer–tetramer system.

However, it is not possible to draw any general conclusion for the behaviour of these and related systems. The pattern can probably show any shape, depending on the values of the association constants and the partition coefficients.

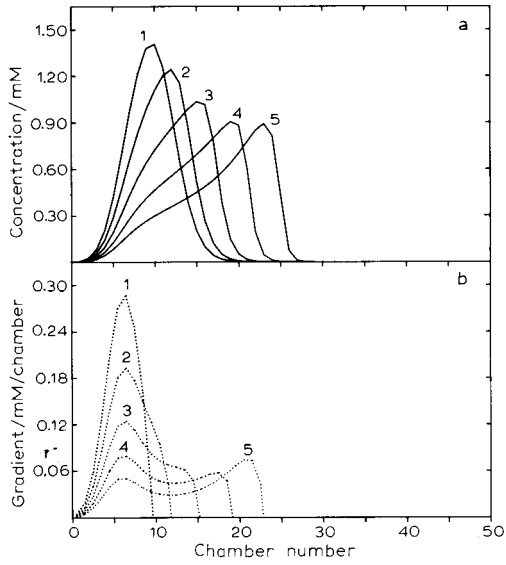


Fig. 6. (a) Simulated CCD patterns and (b) corresponding concentration gradient patterns of a monomer–hexamer association. The initial solute concentration was 10 mM, the number of transfers was 50 and the partition coefficients of monomer and hexamer were 0.2 and 1.5, respectively. The association constant in the lower phase was $1 \cdot 10^{10}$ M^{-5} (curve 1), $1 \cdot 10^{11}$ M^{-5} (curve 2), $1 \cdot 10^{12}$ M^{-5} (curve 3), $1 \cdot 10^{13}$ M^{-5} (curve 4) and $1 \cdot 10^{14}$ M^{-5} (curve 5).

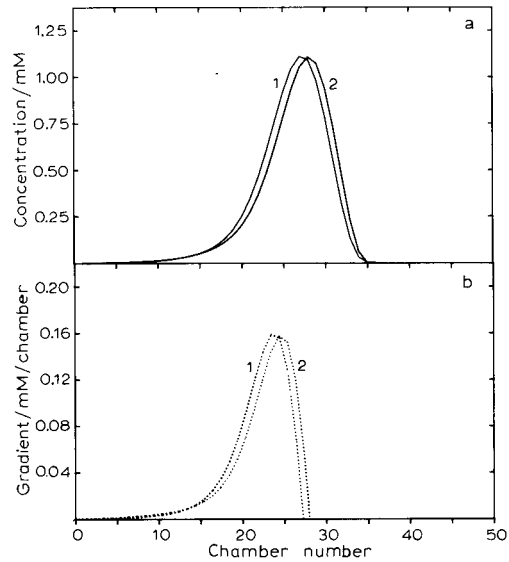


Fig. 7. (a) Simulated CCD patterns and (b) corresponding concentration gradient patterns of monomer–dimer–trimer–tetramer and monomer–dimer–tetramer associations. The initial solute concentration was 10 mM, the number of transfers was 50 and the partition coefficients of monomer, dimer, trimer and tetramer were 0.2, 0.7, 1.1 and 1.5, respectively. The association constants in the lower phase, K^{l}_{12}, K^{l}_{23} and K^{l}_{34}, describing the monomer–dimer–trimer–tetramer equilibrium were all $1 \cdot 10^{4}$ M^{-1} (curve 1). The association constants of the monomer–dimer–tetramer association, K^{l}_{12} and K^{l}_{24}, were both $1 \cdot 10^{4}$ M^{-1} (curve 2).

Moving boundary CCD

In a moving boundary CCD experiment a plateau of constant solute concentration is created and maintained throughout the complete experiment. Therefore, as the solute zone migrates the forward and backward boundaries will be separated by a decreasing number of chambers of constant solute concentration equal to the initial solute concentration. In contrast, in a small zone experiment the initial plateau of solute concentration will not be sustained but will decrease progressively as the zone migrates and thus spreads in an increasing number of chambers. However, as the only substantial difference between small zone and moving boundary CCD is the maintenance of a plateau in the latter instance, the edges of the simulated moving boundary CCD patterns and the corresponding concentration gradient patterns must be very similar in shape to those generated by the small zone model. This is illustrated in Fig. 8, which shows the simulated moving boundary patterns for a monomer–tetramer system. Accordingly, the conclusions concerning small zone CCD are also valid for moving boundary CCD. Likewise, the typical features of the different types of self-associations are similar for both models. Further, the theory of moving boundary CCD of self-associating solutes has been treated fairly extensively previously, although not aimed towards the description of CCD[11,12]. Therefore, only one typical case of self-association is presented here, namely a monomer–tetramer system.

In Fig. 8a, it can be seen that the transformation of the dispersed edge of the CCD pattern as the binding becomes tighter closely resembles the transformation generated by the small zone model (Fig. 5a). Similarly, the concentration gradient

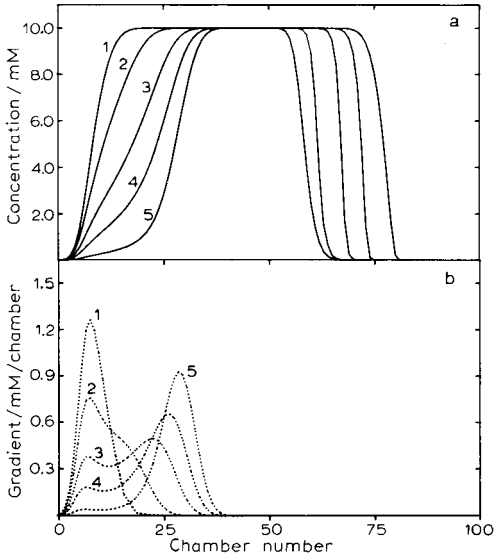


Fig. 8. (a) Simulated moving boundary CCD patterns and (b) corresponding concentration gradient patterns of a monomer–tetramer associations. The initial solute concentration in chambers 0–49 was 10 mM, the number of transfers was 50 and the partition coefficients of monomer and tetramer were 0.2 and 1.5, respectively. The association constant in the lower phase was 1000 M^{-3} (curve 1), $1 \cdot 10^4$ M^{-3} (curve 2), $1 \cdot 10^5$ M^{-3} (curve 3), $1 \cdot 10^6$ M^{-3} (curve 4) and $1 \cdot 10^8$ M^{-3} (curve 5).

patterns in Fig. 8b show the same appearance as before (Fig. 5b), from an indication of a shoulder to a distinct minimum depending on the binding strength.

For a certain degree or type of self-association, the shape of the patterns will depend on the partition coefficients, the association constants, the initial solute concentration and the number of transfers. Therefore, the simulations presented above should not be regarded as valid for all cases. It must be stressed that most of the simulations described here were obtained for one set of partition coefficients. If the difference in the values of the partition coefficients chosen were smaller, the typical features would be less preserved. On the other hand, if the difference between the partition coefficients were greater the characteristics of the patterns would be more pronounced.

Nevertheless, it seems possible to make some general comments on the basis of the calculations presented. Although both small zone and moving boundary CCD are discontinuous transport processes, they show the same general features as continuous transport processes such as sedimentation velocity[16,18] and molecular sieve chromatography[14,15,17,19]. It is reasonable to conclude that a solute self-associates whenever the distribution behaviour of the solute exhibits a concentration dependence. The converse conclusion, however, is not always correct. If the polymeric forms of the solute distribute similarly to the monomer in a particular two-phase system, obviously no hyper-sharpening or dispersion of the CCD pattern would be expected on formation of polymers. In such a case it should be possible to conclude whether the solute self-associates or not by changing the composition of the two-phase system, and thereby the partition of the different forms of the solute.

In a monomer–dimer equilibrium the dispersed edge of both the CCD and the gradient patterns must fall smoothly. Thence, a monomer–dimer association can be eliminated when a shoulder or a definite minimum is present in the experimental concentration gradient pattern. On the other hand, a monomer–trimer association will not necessarily generate a shoulder in the gradient pattern if the binding is relatively loose, as is evident in Fig. 4. The monomer–tetramer and monomer–hexamer cases studied display patterns that are easily distinguishable from monomer–trimer patterns, at least when the polymer content is significant. The additional inflection point is readily apparent and the gradient patterns show distinct minima. The position of the first maximum of the gradient pattern, if it appears, provides a useful diagnostic indicator. If the position is independent of the polymer content, *i.e.*, independent of the initial solute concentration or the binding strength, then monomer–dimer and monomer–trimer equilibria can be excluded. Further, the presence of tetrameric or hexameric species might be distinguished by the position of the minimum. However, these distinct features might not persist if other values of the partition coefficients are chosen.

From the appearance of the experimental CCD patterns and the corresponding concentration gradient patterns it is thus possible not only to detect a self-association but also, under certain circumstances, to determine the type of equilibrium.

Numerical analysis can, of course, be applied to experimental CCD results of self-associating solutes for quantifying the binding strength and partition coefficients of participating species. The method of least squares in connection with an iterative minimization procedure such as Simplex[20] has proved to be very useful for such purposes[8,21]. By generating patterns according to the different models by using the

known initial solute concentration and assigning values to the association constants and the partition coefficients, it is then possible to find a set of parameters that gives the best fitting simulated patterns for each model. The analysis procedure is thus capable of showing whether a particular model and set of parameters can account for the results of a CCD experiment.

Although the typical features of each model are similar for both small zone and moving boundary CCD, there are important distinctions between the two experimental procedures. The typical characteristics of each model are better preserved in moving boundary CCD, and the analysis of experimental CCD results becomes easier. In addition, the lower limit of the detectable binding strength becomes lower than for small zone CCD. However, the major disadvantage of moving boundary CCD is that large amounts of material are needed. Therefore, the choice between small zone CCD and moving boundary CCD is mainly dependent on the amount of material available and on the presumed binding strength.

The choice of two-phase system is not crucial for the theoretical considerations presented here. However, aqueous two-phase systems containing dextran and poly(ethylene glycol) have been shown to be very suitable for work with substances of biological origin[22]. The phases of these two-phase systems are rich in water and have a stabilizing effect on structure and biological activities. In particular, the partition coefficient can easily be adjusted by changing the composition of the two-phase system. Further, both dextran[23] and poly(ethylene glycol)[24,25] stimulate the association of proteins, probably owing to the excluded volume effect. It has been proposed[24,26] that polymers introduced into *in vitro* systems mimic the cell and would thereby provide a more normal cellular milieu than aqueous solutions of standard ionic strength and pH.

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ADSORPTION BEHAVIOUR OF SEVERAL SUPPORTS IN REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY AS DEMONSTRATED BY THE DETERMINATION OF RELATIVE PARTITION COEFFICIENTS OF SOME 4-HYDROXYCOUMARIN DERIVATIVES

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SUMMARY

The adsorptive behaviour of four supports in reversed-phase thin-layer chromatography was investigated. Oleyl alcohol was used as the stationary phase and methanol–water mixtures as the mobile phase. A series of 4-hydroxycoumarins were used as test compounds. Kieselguhr and cellulose show adsorption. Kieselgel also is not a good support, probably because of its large specific surface area. Kieselguhr modified by silanization behaves as a support without adsorptive sites. The R_M values obtained with silanized Kieselguhr as the support correlate very well with the partition coefficients for the octanol–water system.

INTRODUCTION

It is sometimes very difficult or even impossible to determine partition coefficients by means of shake-flask experiments, but instead one can use reversed-phase chromatography. This is a good alternative technique, especially when the compounds are available in small amounts, when they contain impurities or when they are liable to decompose. Sometimes there is a discrepancy between the values of the partition coefficients obtained chromatographically and those obtained with shake-flask experiments. In such instances it has generally been concluded that in addition to a partitioning process there has been adsorption of the compounds on to the solid support[1–5]. However, it is not always possible to compare the results of the two methods and it is clear that the values obtained chromatographically may not always be reliable. Mirrlees *et al.*[6] assumed that if in the case of some test compounds (with known partition coefficients) it is only partitioning that causes retention in a reversed-phase high-performance liquid chromatographic (RP-HPLC) system, then this must also hold for every other compound in that system. However, we think this is rather speculative. Hulshoff and Perrin[7,8] developed a method for examining whether or not adsorption occurs in a reversed-phase thin-layer chromatographic (RP-TLC) system. Using oleyl alcohol as the stationary phase they found that Kieselguhr is a good

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support for phenothiazines and benzodiazepines. Experiments in our laboratory, however, have indicated that Kieselguhr is not an inert support for a series of 4-hydroxycoumarin derivatives. For this reason special attention has been given in this investigation to the choice of support in RP-TLC for some acidic compounds. A series of 4-hydroxycoumarin derivatives were used to demonstrate the influence of several supports.

THEORETICAL

Hulshoff and Perrin[7], using methanol–water mixtures as the mobile phase, derived the relationship between the partition coefficient and a number of chromatographic parameters in an RP-TLC system. They express this relationship as follows:

$$R_M = \log P + \log \phi + bC \tag{1}$$

where P is the partition coefficient of a neutral species in the stationary phase–water system, ϕ is the stationary phase/mobile phase phase-volume ratio, C is the methanol concentration (%, v/v) in the mobile phase and b is a constant depending on the compound and on the chromatographic system used. Of the two variables ϕ and C, one can be kept constant. At a given loading of the support the phase-volume ratio, ϕ, is a constant.

If at a constant loading the methanol concentration, C, in the mobile phase is varied and if the measured R_M values are plotted against C, then one expects to find a straight line with an intercept given by $\log P + \log \phi = R_{M_w}$, *i.e.*, R_M values with 100% water as solvent in the mobile phase. As ϕ is a constant for a given RP-TLC system, R_{M_w}, is a measure of the log P value of a compound.

On the other hand, the composition of the mobile phase can be kept constant and the phase-volume ratio, ϕ, can be varied by using different amounts of stationary phase. If it is assumed that ϕ is a linear function of the amount of stationary phase, then

$$R_M = \log {}_sP + \log k + \log S \tag{2}$$

(see Appendix A for derivation), where ${}_sP$ is the partition coefficient if the mobile phase is not 100% water but is a mixture of water and methanol, k is a constant and S is the amount of stationary phase calculated as grams per gram of unloaded support. A graph of R_M against log S should be a straight line with a slope of unity. If the experimental slopes deviate from unity, this means that processes other than liquid–liquid partitioning are involved in the retention mechanism.

When retention is caused by both liquid–liquid partitioning and adsorption on to the solid support a relationship more complex than eqn. 2 must be used (see Appendix B for derivation):

$$R_M = \log {}_sP + \log k + \log S + \log (1 + K_A S^{-1} s_A d_s) \tag{3}$$

where K_A is the adsorption constant (cm), s_A is the specific surface area of the support (cm^2/g) and d_s is the density of the stationary phase (g/cm^3). If $K_A = 0$, then the

adsorption term in eqn. 3 is zero and eqn. 3 reduces to eqn. 2. In the case of adsorption, $K_A > 0$ and the relationship between R_M and log S is no longer linear.

In order to demonstrate the effect of adsorption as given in eqn. 3 some calculations were performed. In Fig. 1 log S + log $(1 + K_A S^{-1} s_A d_s)$ is plotted against log S. Some calculated curves are shown for some selected values of $K_A s_A d_s$. Curve 1 represents the situation where no adsorption occurs, whereas curves 2 and 3 were calculated using increasing values of K_A. It is clear that if very strong adsorption occurs a straight line with zero slope will be found, because then it will be possible to write eqn. 3 as

$$R_M = \log {}_sP + \log k + \log (K_A s_A d_s) \tag{4}$$

In Fig. 1 the region between the two dashed lines represents the usual loading range of the support. The important conclusion to be drawn from this figure is that the curves in this range can be considered as straight lines independent of the degree of adsorption. In other words, one should not look for deviations from linearity in order to detect adsorption phenomena; instead, one can use the slopes of the lines obtained by plotting R_M against log S to detect adsorption, as suggested by Fig. 1. The degree to which the slope of the line deviates from unity when R_M is plotted against log S is a measure of the adsorption.

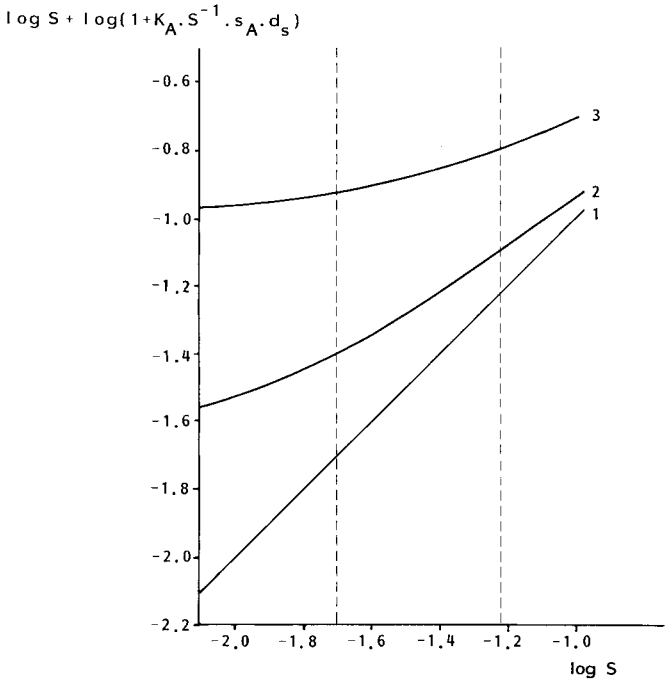

Fig. 1. Theoretical curves of log S + log $(1 + K_A S^{-1} s_A d_s)$ *versus* log S. The broken lines indicate the region of the usual loading range (S = 0.02–0.06). For curves 1, 2 and 3 $K_A s_A d_s$ = 0, 0.02 and 0.10, respectively. These values were chosen so as to obtain values for the slopes of the curves (considered as straight lines) between the broken lines of the same order of magnitude as found in this investigation. See also Table I.

More extended calculations were performed to demonstrate the points just discussed. In Table I it can be seen that in the usual loading range straight lines are indeed obtained, as shown by the values of the correlation coefficient, r. In Fig. 2 values of the slopes of the lines discussed above are given. This figure may be used to obtain the value of $K_A s_A d_s$ for a slope value found experimentally. It should be noted that this relationship is valid irrespective of the kind of support and the kind of stationary phase.

TABLE I

SLOPES OF THE THEORETICAL CURVES OF LOG S + LOG $(1 + K_A S^{-1} s_A d_s)$ *VERSUS* LOG S FOR SEVERAL VALUES OF $K_A s_A d_s$

The values of S used were 0.02, 0.03, 0.04, 0.05 and 0.06. The slopes of the curves in the second column were calculated from the curves given in Fig. 1; a linear behaviour is assumed over the range S = 0.02–0.06. This region is indicated by the broken lines in Fig. 1. The correlation coefficients, r, were obtained from a linear least-squares method.

$K_A s_A d_s$	*Slope*	*Correlation coefficient, r*
0.000	1.000	
0.001	0.971	1.000
0.003	0.917	1.000
0.005	0.870	1.000
0.010	0.771	0.999
0.020	0.630	0.998
0.030	0.534	0.997
0.040	0.463	0.996
0.050	0.410	0.996
0.060	0.368	0.995
0.070	0.333	0.994
0.080	0.305	0.994
0.090	0.281	0.993
0.100	0.260	0.993

In principle there may be another way of detecting adsorption[7]. Eqn. 3 can be rearranged to

$$\frac{1}{R_F} = {}_sPkK_A s_A d_s + 1 + {}_sPkS \tag{5}$$

A graph of $R_F{}^{-1}$ against S will be a straight line. If the intercept with the $R_F{}^{-1}$ axis is unity, then obviously K_A approaches zero. A correlation between the magnitude of the deviation from unity and the magnitude of adsorption is difficult to establish, because ${}_sP$ is one of the factors of the adsorption term of eqn. 5. For this reason we prefer to use eqn. 3 in order to check whether there has been adsorption on to the solid support in RP-TLC.

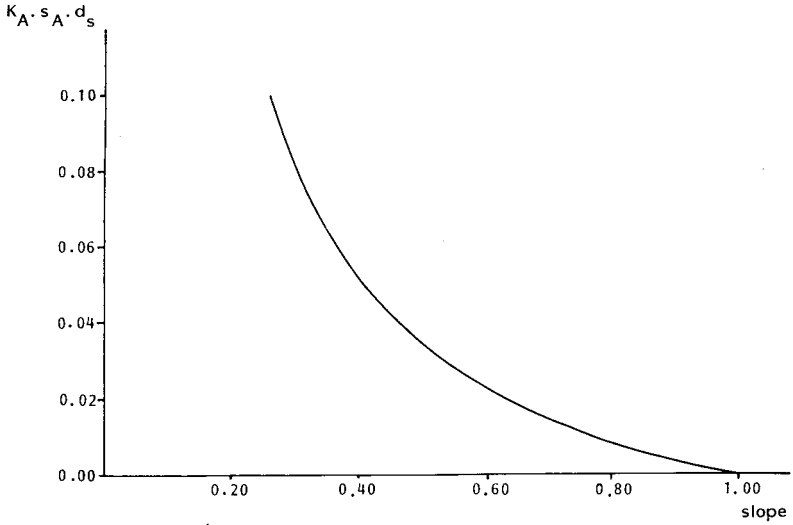

Fig. 2. $K_A s_A d_s$ as a function of the slope (s) using the numbers in Table I. This relationship can be best represented by a sixth degree polynomial function: $K_A s_A d_s = 2.3772\ s^6 - 10.1914\ s^5 + 18.1304\ s^4 - 17.3027\ s^3 + 9.5433\ s^2 - 3.0330\ s + 0.4762$. This figure permits the calculation of $K_A s_A d_s$ if the experimental value of s is known. An example of such a calculation is given in the text.

EXPERIMENTAL

Chemicals were of analytical-reagent grade unless specified otherwise. Demineralized water was used throughout.

Acenocoumarol (Sintrom; Ciba-Geigy, Arnhem, The Netherlands), coumetarol (Dicumoxane; ACF, Maarssen, The Netherlands), ethyl biscoumacetate (Tromexan; Ciba-Geigy) and phenprocoumon (Marcoumar; Hoffman-La Roche, Mijdrecht, The Netherlands) were gifts from the manufacturers.

4-Hydroxycoumarin (Merck, Darmstadt, G.F.R.), warfarin sodium (British Pharmacopoeia quality; ACF), methanol (Merck), 38% hydrochloric acid (Merck), cellulose MN 300 (Machery, Nagel & Co., Düren, G.F.R.), Kieselgel H (Merck), Kieselguhr MN (Machery, Nagel & Co.), dichloromethane (Baker, Deventer, The Netherlands) and chlorotrimethylsilane (Aldrich-Europe, Beerse, Belgium) were used as supplied.

Oleyl alcohol (Merck), containing 95% *cis*-9-octadecen-1-ol, was passed through a glass column (60 × 2.5 cm I.D.), the lower half of which was filled with basic aluminium oxide (Merck) and the upper half with charcoal pellets (Norit, Amsterdam, The Netherlands). The resulting product was colourless and odourless, the density at 25°C being 0.848 g/cm^3.

Silanization of Kieselguhr

Silanized Kieselguhr was prepared by adding 30 ml of chlorotrimethylsilane to a stirred suspension of 300 g of Kieselguhr MN in 2.5 l of dichloromethane at room temperature. Stirring was stopped after 1 h and the silanized Kieselguhr was allowed to settle. The yellow supernatant was decanted and the silanized Kieselguhr was

washed by multiple decantations with methanol until the supernatant was colourless. Finally, the silanized Kieselguhr was dried at 60°C. Three preparations yielded identical results.

Preparation of TLC plates

Using standard TLC equipment (Shandon Southern, Camberley, Great Britain) glass plates (20 × 20 cm) were covered with a 0.25-mm layer of a slurry consisting of a mixture of the support, stationary phase (oleyl alcohol) and ethanol (see Table II). A Warring blender was run at maximum speed for 1 min to homogenize the slurries. The values of the loading, S, ranged from 0.01 to 0.07. The plates were allowed to dry for 16 h at room temperature. The mobile phases consisted of solutions of hydrochloric acid in methanol–water mixtures. The methanol concentration in the mobile phase ranged from 20 to 70% (v/v); 0.1% solutions were made of 4-hydroxycoumarin, acenocoumarin, warfarin and phenprocoumon in methanol and 0.05% solutions were made of coumetarol and ethyl biscoumacetate in dichloromethane.

TABLE II

COMPOSITION OF THE SLURRIES USED TO PREPARE THE TLC PLATES

The amounts given are sufficient for five plates (20 × 20 cm). To each slurry oleyl alcohol was added in an amount dependent on the desired final loading S.

Support		*Solvent*	
Name	*Amount (g)*	*Amount (ml)*	*Ethanol concentration (%, v/v)*
Kieselguhr MN	20	85	70
Cellulose MN 300	15	80	96
Kieselgel H	20	75	90
Kieselguhr, silanized	19	55	90

Volumes of 1 μl of the solutions was spotted on the plates, in varying allocations, at 1.5-cm intervals along a line 2 cm from the lower edge of the plate. Each compound was spotted twice on the same plate. A migration of 10 cm on all plates was obtained by cutting the layer at 12 cm from the lower edge. After development, the spots were revealed by spraying with a freshly prepared mixture of equal volumes of 1% iron(III) chloride in 50% ethanol and 1% potassium hexacyanoferrate(III) in 50% ethanol[9]. This mixture gives blue spots on a yellow background.

The diagonal method described by Hulshoff and Perrin[7] was used to check whether or not the conditions had changed during development. If the plates are equilibrated for 2 h in a chromatographic chamber saturated with the vapour of the mobile phase before development, then one can be certain that the conditions do not alter during development. The temperature was maintained at 25°C.

RESULTS AND DISCUSSION

To examine the possible adsorption of some 4-hydroxycoumarin derivatives on to several supports, the compounds were chromatographed at constant mobile phase composition, but with varying amounts of stationary phase. The R_M values obtained were plotted against log S.

The results for Kieselguhr MN as support are shown in Fig. 3. A linear relationship is found. The values of the slopes of these lines are given in Table III. The slopes are smaller than unity; it is clear that adsorption on to the Kieselguhr plays a significant role in the retention process.

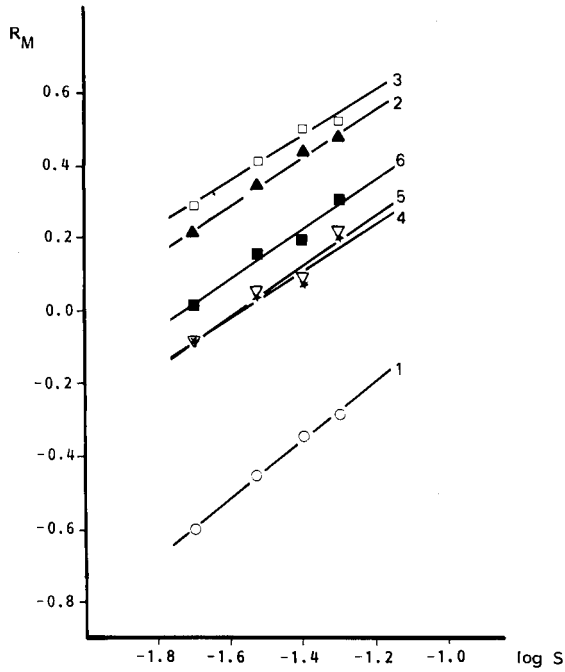

Fig. 3. R_M values of 4-hydroxycoumarins plotted *versus* log S using Kieselguhr MN as support. Every point is the mean of 6–8 determinations. The drawn lines were calculated using the least-squares method for linear regression. 1, 4-Hydroxycoumarin; 2, warfarin; 3, acenocoumarin; 4, phenprocoumon; 5, ethyl biscoumacetate; 6, coumetarol. Mobile phase: 1 M HCl in 30% (v/v) methanol for compounds 1–3 and 1 M HCl in 60% (v/v) methanol for compounds 4–6.

On the other hand, phenothiazines[7] and benzodiazepines[8] as representatives of lipophilic basic and neutral compounds, respectively, did not show adsorption on to Kieselguhr, provided that the loading was above a certain minimum value. Acids are good proton donors for hydrogen-bridge formation with silanol sites of the solid support; this might cause adsorption of the (acidic) 4-hydroxycoumarin derivatives on to Kieselguhr.

With the help of the calculated function given in the legend to Fig. 2, the K_A values of the several 4-hydroxycoumarin derivatives were calculated in the cases where Kieselguhr served as the support. For this calculation the value of the specific

TABLE III

SLOPES OF THE STRAIGHT LINES OF R_M *VERSUS* LOG S, AND THE ADSORPTION CONSTANT K_A FOR KIESELGUHR AS SUPPORT

The experimental slopes were obtained from Figs. 3–6. In the last column the value for the adsorption constant K_A is given, calculated as described in the text. Standard deviations are given in parentheses.

Compound	*Slope*				$K_A \cdot 10^7$ *(cm)*
	Kieselguhr	*Cellulose*	*Kieselgel*	*Silanized Kieselguhr*	
4-Hydroxycoumarin	0.80 (±0.03)	0.46 (±0.02)	0.89 (±0.13)	1.06 (±0.07)	2.4 (±0.4)
Warfarin	0.67 (±0.04)	0.51 (±0.02)	1.19 (±0.03)	1.00 (±0.10)	4.7 (±0.8)
Acenocoumarin	0.62 (±0.06)	0.43 (±0.02)	1.15 (±0.08)	1.02 (±0.11)	5.8 (±1.4)
Phenprocoumon	0.66 (±0.11)	0.69 (±0.03)	1.23 (±0.07)	1.05 (±0.10)	4.9 (±2.0)
Ethyl biscoumacetate	0.70 (±0.11)	0.41 (±0.01)	0.82 (±0.11)	1.09 (±0.10)	4.0 (±1.8)
Coumetarol	0.69 (±0.09)	0.49 (±0.02)	1.19 (±0.06)	1.12 (±0.07)	4.2 (±1.6)

surface area, s_A, of Kieselguhr was assumed to be $4.2 \cdot 10^4$ cm^2/g (ref. 10) and the density, d_s, of oleyl alcohol was taken to be 0.848 g/cm^3. The resulting K_A values are given in the last column in Table III. Asshauer and Halász[11] found K_A values of the same order of magnitude for the adsorption of some organic solvents on to Porasil A as support in a gas–liquid chromatographic system.

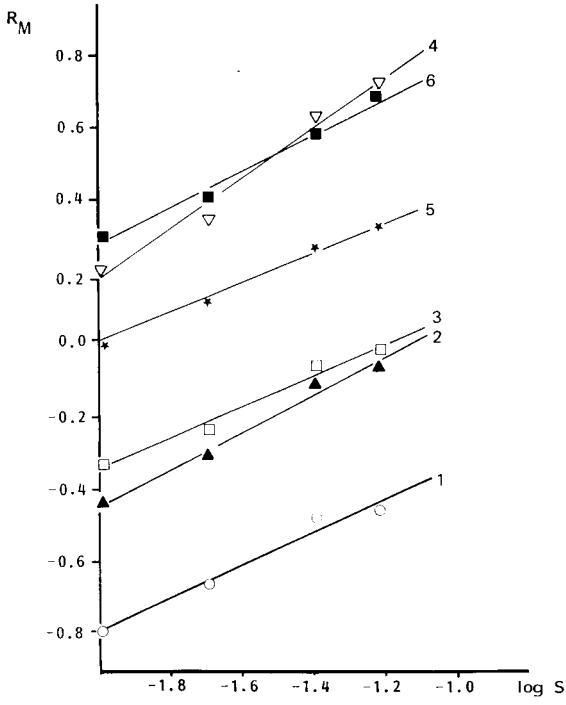

Fig. 4. R_M values of 4-hydroxycoumarins plotted *versus* log S using Cellulose MN 300 as support. Further details as in Fig. 3, except for the mobile phase, which was 0.1 M HCl in 45% (v/v) methanol.

The results for cellulose MN 300 are shown in Fig. 4. As with Kieselguhr, straight lines are obtained when R_M is plotted against log S. The slopes of the lines in Fig. 4, given in Table III, are smaller than those for Kieselguhr. It can be concluded that adsorption plays an important role here also. However, Bird and Marshall[12] found that cellulose had only some very weak adsorptive sites for penicillins.

The results for Kieselgel are shown in Fig. 5. The behaviour of Kieselgel is remarkable: straight lines with slopes smaller and larger than unity are obtained when R_M is plotted against log S (see also Table III). Obviously Kieselgel does not meet the requirements for an inert support. Other reports in the literature point to adsorption phenomena when Kieselgel is used. Kuchař *et al.*[13] found considerable adsorption on to Kieselgel G in an RP-TLC system for two series of aryl-aliphatic acids; nevertheless, they used the R_M values they obtained as substitutes for log P values. Biagi *et al.*[14], using octanol-impregnated Kieselgel G, found the relationship $\log P_{oct} = 0.569\ R_{M_w} + 1.354$ ($n = 39$, $r = 0.838$) between the partition coefficient in the system octanol-water and the R_{M_w} values for a series of benzodiazepines. The correlation coefficient is low and the coefficient 0.569 deviates considerably from unity (see eqn. 1); this suggests that partitioning was not the only process involved in the chromatographic system used.

The specific surface area of Kieselgel is so large that it is of considerable

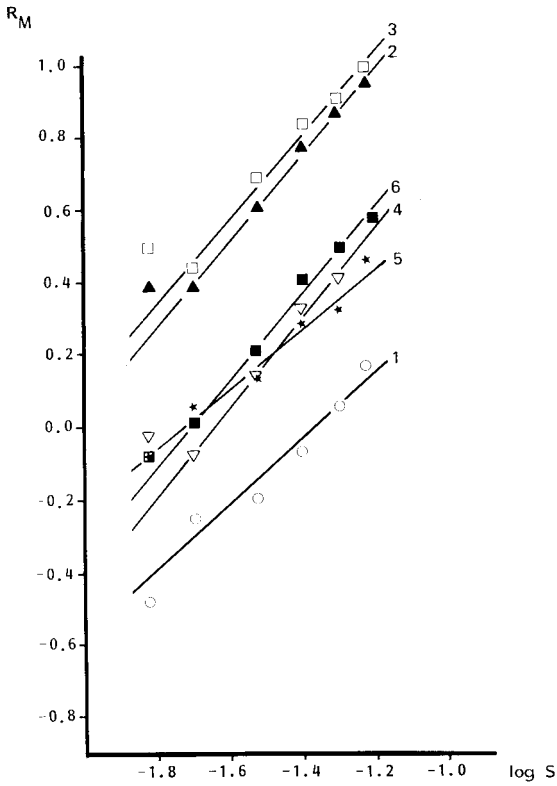

Fig. 5. R_M values of 4-hydroxycoumarins plotted *versus* log S using Kieselgel as support. Further details as in Fig. 3. The points measured at the lowest S value were excluded in the calculation of the drawn lines.

importance. Scott and Kucera[15] found that for some solvents, which had a mean molecular diameter of about 6.6 Å and covered a surface area of about 34.2 $Å^2$, on average $6.6 \cdot 10^{20}$ solvent molecules per gram of Kieselgel are needed to form a monomolecular layer. Assuming for oleyl alcohol a mean molecular diameter of 8 Å, which corresponds to a surface area of 50 $Å^2$, one finds that a mean number of $4.5 \cdot 10^{20}$ molecules or 0.20 g of oleyl alcohol is needed to form a monomolecular layer per gram of Kieselgel. It is reasonable to say that the amount of stationary phase required to form a monomolecular layer depends mainly on the surface area of the support. If values of 4.2 and 620 m^2/g are assumed[10,16] for the specific surface areas of Kieselguhr and Kieselgel, respectively, then it can be estimated that 0.0014 g of oleyl alcohol per gram of Kieselguhr is needed to form a monomolecular layer. As these numbers only give the order of magnitude, it is clear that with Kieselgel the usual amounts of oleyl alcohol in RP-TLC are certainly not enough to form a monomolecular layer, whereas with Kieselguhr a multimolecular layer is formed. It is very unlikely that a monomolecular layer will behave as a bulk phase, whereas it is very likely that a multimolecular layer will do so. This effect, combined with adsorption phenomena, is probably the reason for the strange behaviour of Kieselgel.

The results obtained so far urged us to seek a better support that showed no adsorptive properties. We found this support by blocking the active silanol sites of Kieselguhr MN with chlorotrimethylsilane. The method used to modify the Kieselguhr is in fact a slight modification of the method proposed by Little and co-workers[17,18]. They found that carbon tetrachloride is the best solvent in which to perform

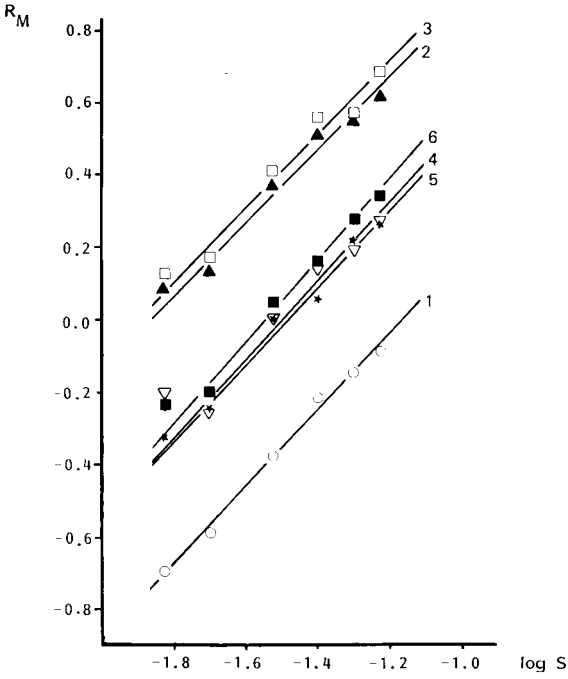

Fig. 6. R_M values of 4-hydroxycoumarins plotted *versus* log S using silanized Kieselguhr as support. Further details as in Fig. 3. The points measured at the lowest S value were excluded in the calculation of the drawn lines.

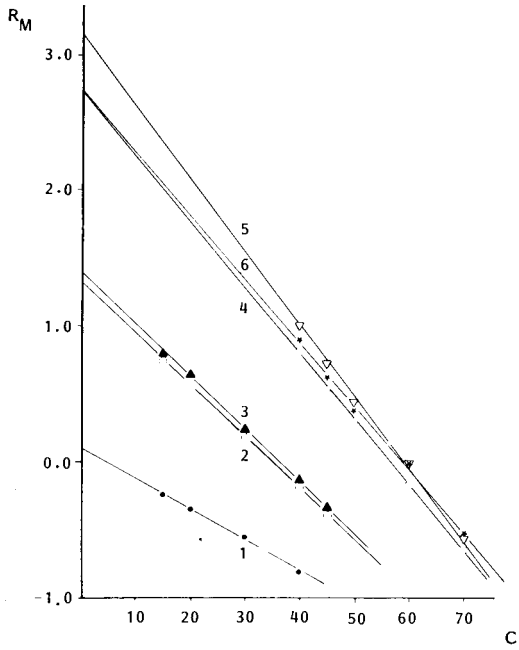

Fig. 7. Effect of the methanol concentration, C (%, v/v), in the mobile phase on the R_M values of 4-hydroxycoumarins. Stationary phase: oleyl alcohol (loading $S = 0.02$) on silanized Kieselguhr as support. Every point is the mean of 6–8 determinations. The drawn lines were calculated using the least-squares method for the linear regression. 1 = Hydroxycoumarin; 2 = warfarin; 3 = acenocoumarin; 4 = phenprocoumon; 5 = ethyl biscoumacetate; 6 = coumetarol.

the chemical reaction. However, we preferred to use dichloromethane as the solvent in order to achieve a better sedimentation rate in the decantation step. The method of silanizing the Kieselguhr is simple. The adherence of the silanized Kieselguhr to the glass plates is adequate and no decomposition of the support occurs even with the very acidic mobile phases we used in our investigations.

TABLE IV

SLOPES (b) AND INTERCEPTS (R_{M_w}) OF THE LINES WHEN R_M IS PLOTTED *VERSUS* THE METHANOL CONCENTRATION OF THE MOBILE PHASE, USING OLEYL ALCOHOL AS THE STATIONARY PHASE (LOADING $S = 0.02$) ADSORBED ON TO SILANIZED KIESELGUHR AS SUPPORT

Also given are the partition coefficients (octanol–water) obtained from shake-flask experiments (log P_{oct}). Standard deviations are given in parentheses.

Compound	b	R_{M_w}	*Correlation coefficient, r*	*Log* P_{oct}
4-Hydroxycoumarin	−2.24 (±0.06)	0.10 (±0.02)	0.9993	2.37
Warfarin	−3.77 (±0.03)	1.32 (±0.01)	0.9999	3.28
Acenocoumarin	−3.84 (±0.08)	1.38 (±0.03)	0.9993	3.24
Phenprocoumon	−4.83 (±0.09)	2.73 (±0.05)	0.9995	4.56
Ethyl biscoumacetate	−5.35 (±0.17)	3.15 (±0.09)	0.9984	4.93
Coumetarol	−4.67 (±0.13)	2.73 (±0.07)	0.9987	4.55

The results obtained with silanized Kieselguhr as support are shown in Fig. 6. Straight lines are found when R_M is plotted against log S. The slopes of the lines and the corresponding standard deviations are given in Table III. These slopes are unity within one standard deviation, so the results for this support are superior to those for any of the other supports discussed above. This proves clearly that silanized Kieselguhr behaves as a support without adsorptive sites.

Using silanized Kieselguhr as support and oleyl alcohol as stationary phase we determined R_M values with several methanol–water mixtures as mobile phases. For all 4-hydroxycoumarin derivatives straight lines are obtained when R_M is plotted against C (see Fig. 7). Extrapolating to $C = 0$ gives R_{M_w} values (see Table IV). Also given in Table IV are the slopes, b, of the lines in Fig. 7, together with the partition coefficients in the octanol–water system obtained from shake-flask experiments[19].

To calculate the partition coefficient, P_1, of a solute in one solvent system when the partition coefficient, P_2, of the same solute in another solvent system is known, a Collander-type equation can be used[20,21]:

$$\log P_1 = a \log P_2 + c \tag{6}$$

where a and c are constants characterizing the solvent systems involved. This type of equation is useful for many solvent systems in which the non-polar phase is varied and in which the polar phase is always water, particularly when the solutes are divided into proton donors, proton acceptors and neutral species[21].

As the R_{M_w} values in this investigation represent the partition coefficient in the oleyl alcohol–water system it should be possible to use a Collander-type equation to correlate log P values in a certain solvent system and R_{M_w} values in another solvent system. We found the following correlation*:

$$\log P_{oct} = 0.859\ (\pm 0.034) R_{M_w} + 2.187\ (\pm 0.074) \tag{7}$$
$$n = 6;\ r^2 = 0.994;\ s = 0.088;\ F = 644$$
$$0.787 < \text{slope}^{**} < 0.931;\ 2.030 < \text{intercept}^{**} < 2.344$$

The constant 2.187 in eqn. 7 is the sum of the constant c in eqn. 6 and the logarithm of the phase-volume ratio, ϕ. When the mobile phase is not pure water but a methanol–water mixture, log P_{oct} and R_M correlate well with each other. As an example, in eqn. 8 the correlation is given when the methanol concentration C in the mobile phase is 30% (v/v):

$$\log P_{oct} = 1.192\ (\pm 0.029) R_{M_{30}} + 3.025\ (\pm 0.030) \tag{8}$$
$$n = 6;\ r^2 = 0.998;\ s = 0.055;\ F = 1684$$
$$1.130 < \text{slope} < 1.254;\ 2.962 < \text{intercept} < 3.088$$

We conclude that a Collander-type equation can also be used when the organic phase is kept constant and the aqueous phase is changed. The Δlog P values in the solvent system stationary phase–water are equal to the ΔR_{M_w} values.

* The standard deviation is given in parentheses; n = number of compounds; r = correlation coefficient; s = standard error of estimate or $s_{y,x}$; F = value of the F-test of significance.

** 90% confidence interval.

However, these $\Delta\log P$ values are not equal to the ΔR_M values obtained using a certain methanol–water mixture as the mobile phase. This would only have been true if the coefficient b in eqn. 1 had been independent of the choice of the solute. It is clear from Fig. 7 and Table IV that b has a characteristic value for every compound. These findings for 4-hydroxycoumarins are similar to the findings for phenothiazines and benzodiazepines[7,8].

RP-TLC has some drawbacks compared with RP-HPLC: a large number of experiments are needed to obtain accurate R_{M_w} values, and detection of the spots can be a serious problem. One great advantage of RP-TLC, however, is the fact that it enables one to examine the adsorption on to the solid support for every solute, following the method described above in which various amounts of stationary phase are used. In RP-HPLC it is very difficult to vary the loading of the support in the column, and the loading is only possible within a very limited range[22].

APPENDIX A

Phase-volume ratio, ϕ, as a linear function of the loading, S

It is assumed here that the total pore volume of the support is filled with stationary and mobile phase. In other words, the pore volume of the support available for the mobile phase decreases with increasing amounts of stationary phase, according to the equation

$$V_m = V_p - V_s = V_p - d_s^{-1}S \tag{9}$$

where V_m and V_s (cm^3) are the volumes of the mobile and the stationary phase, respectively, V_p (cm^3) is the total pore volume of the support, d_s (g/cm^3) is the density of the stationary phase and S is the amount of stationary phase in grams per gram of unloaded support. The phase-volume ratio, ϕ, is given by V_s/V_m, or

$$\phi = \frac{d_s^{-1}S}{V_p - d_s^{-1}S} = \frac{S}{d_sV_p - S} \tag{10}$$

In our investigations the S values range from 0.01 to 0.06. V_p for Kieselguhr is 1.16 cm^3/g (ref. 10) and d_s for oleyl alcohol is 0.848 g/cm^3. Using these values, Table V can be compiled. It is clear from this table that the relative change of $(d_sV_p - S)^{-1}$ is small compared with the relative increase of S. The error is negligible when $(d_sV_p - S)^{-1}$ is considered to be constant. Therefore, the following relationship may be used:

$$\phi = kS \tag{11}$$

This relationship was assumed earlier by Hulshoff and Perrin[7].

TABLE V

DEPENDENCE OF $(d_sV_p - S)^{-1}$ ON S

S	$(d_sV_p - S)^{-1}$	S	$(d_sV_p - S)^{-1}$
0.01	1.0270	0.04	1.0597
0.02	1.0377	0.05	1.0710
0.03	1.0486	0.06	1.0826

APPENDIX B

Derivation of eqn. 3

The predominant mechanism of solute retention in liquid–liquid chromatography is partitioning between the mobile phase and the liquid stationary phase. The solute may also be retained by adsorption on to the solid support. Mathematically this can be represented by the equation

$$P_{obs} = \frac{\Delta V_s c_s + \Delta A c_A}{\Delta V_m c_m} \tag{12}$$

where P_{obs} is the equilibrium constant of a solute in a chromatographic system in which only liquid–liquid partitioning and adsorption on to the solid support take place, c_s and c_m (g/cm^3) are the concentrations of the solute in adjacent volumes of stationary phase (ΔV_s) and mobile phase (ΔV_m) and c_A (g/cm^2) is the concentration of the solute at the part of the surface area of the support (ΔA) adjacent to ΔV_s. Eqn. 12 can be rearranged to

$$P_{obs} = \frac{\Delta V_s}{\Delta V_m} \cdot \frac{c_s}{c_m} + \frac{\Delta A}{\Delta V_s} \cdot \frac{\Delta V_s}{\Delta V_m} \cdot \frac{c_A}{c_s} \cdot \frac{c_s}{c_m} \tag{13}$$

where V_s/V_m is the phase-volume ratio ϕ, c_s/c_m is the partition coefficient ${}_sP$ (where s indicates that a mixed solvent such as a methanol–water mixture is used as the mobile phase) and c_A/c_s is the adsorption coefficient K_A (cm). Substitution of these terms into eqn. 13 gives

$$P_{obs} = \phi \cdot {}_sP \left(1 + K_A \cdot \frac{\Delta A}{\Delta V_s}\right) \tag{14}$$

In TLC R_F can be defined as the ratio of the amount of the solute in the mobile phase and the total amount of solute:

$$R_F = \frac{\Delta V_m c_m}{\Delta V_m c_m + \Delta V_s c_s + \Delta A c_A} \tag{15}$$

Combining eqns. 12 and 15 one obtains the well known equation

$$P_{obs} = \frac{1}{R_F} - 1 = \text{antilog } R_M \tag{16}$$

Combining eqns. 11, 14 and 16 we obtain

$$R_M = \log {}_sP + \log k + \log S + \log \left(1 + K_A \cdot \frac{\Delta A}{\Delta V_s}\right) \tag{17}$$

$\Delta A/\Delta V_s$ can be replaced by $S^{-1} s_A d_s$, s_A (cm^2/g) is the specific surface area of the support and d_s (g/cm^3) is the density of the stationary phase. This results in

$$R_M = \log {}_sP + \log k + \log S + \log (1 + K_A S^{-1} s_A d_s) \tag{18}$$

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COMPARISON OF ADSORPTION AND REVERSED-PHASE PARTITION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF ANDROGENS

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SUMMARY

Sixty-nine androstane derivatives were chromatographed by adsorption and reversed-phase high-performance liquid chromatography (HPLC). The total number of hydroxyl and keto groups on the androstane molecule is the most important factor in determining their chromatographic behavior. An α,β-unsaturated keto group contributes as much to the polarity of the molecule as a hydroxyl group and more than an isolated keto group. Generally, 3-hydroxyandrostane derivatives are more polar in adsorption HPLC with *n*-hexane–ethanol as eluent when the hydroxyl group is equatorial than when it is axial. 17β-Hydroxyandrostane derivatives are generally more polar than their 17α-epimers in reversed-phase partition HPLC with methanol–water as eluent. Compounds inseparable by adsorption HPLC can often be separated by reversed-phase partition HPLC and *vice versa*.

INTRODUCTION

In earlier work on the metabolism of labeled 4-androstene-3,17-dione in pea plants[1], we have felt the need for a fractionation method more efficient than thin-layer chromatography. Since then, we have studied the progesterone metabolites in pea plants[2] by high-performance liquid chromatography (HPLC)[3], which allowed us to isolate eleven labeled products. At present, we are studying the metabolism of androstenedione in male and female cucumber plants. Although we have previously devised a HPLC method for eleven androstane derivatives[4] and the literature contains additional methodology[5], we decided to make a systematic study of the relative merits of adsorption and reversed-phase HPLC of 69 androgen metabolites we may possibly encounter. It includes oxidation ($C_{19}O_3$) as well as reduction products of androstenedione. Most of them can be detected at 205 nm, but since some of the saturated alcohols cannot easily be detected that way, we have made extensive use of a refractive index (RI) detector.

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EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. The adsorption column was a 250 × 4.6 mm I.D. stainless-steel chromatography tube (Altex, Berkeley, CA, U.S.A.), packed with Zorbax BP-SIL (7–8 μm; DuPont, Wilmington, DE, U.S.A.). The reversed-phase column had the same dimensions, but it was packed with Zorbax BP-ODS (7–8 μm, DuPont). The columns were packed in our laboratory. The packing method, detectors, solvents, pump, and sample injection-valve were as previously described[3,6]. The chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

Our results are summarized in Table I. Sixty-nine androstane derivatives are arranged in the order of increasing oxygen content and increasing polarity in adsorption HPLC. The total number of hydroxyl and keto groups plays the most important role in the polarity of the androstane derivatives in both adsorption and reversed-phase partition HPLC. However, testosterone (30), the most polar $C_{19}O_2$ compound in adsorption HPLC, was somewhat more polar than 3α,11β-dihydroxy-5α-androstan-17-one (31), the least polar $C_{19}O_3$ compound. 4-Androstene-3β,17β-diol (23), the most polar $C_{19}O_2$ compound in reversed-phase partition HPLC, was well separated from the least polar $C_{19}O_3$ compound, 5β-androstane-3α,16α,17β-triol (60). Apparently, the effect of the total number of hydroxyl and keto groups on the polarity of androstane derivatives is more important in reversed-phase partition than in adsorption HPLC. Our previous results on triterpenoids[6], estrogens[7], and gibberellins[8] have likewise shown that the number of hydroxyl groups is the most important factor in the separation, but none of these compounds contained more than one keto group.

In adsorption HPLC (Fig. 1), 5β-androstane-3,17-dione (5) was eluted before 5α-androstane-3,17-dione (7) and 17α-hydroxy-4-androsten-3-one (27) was eluted before 17β-hydroxy-4-androsten-3-one (30). This is in agreement with most observations on the chromatographic behavior of epimeric steroids[9], but contrary to our previous results with dichloromethane–acetonitrile–2-propanol (179:20:1) as the eluent and Partisil 5 as the adsorbent[4]. When this eluent was used with the Zorbax BP-SIL column, the following retention times were observed: 5α-androstane-3,17-dione (7) (2.75 min), 5β-androstane-3,17-dione (5) (3 min), 17β-hydroxy-4-androsten-3-one (30) (8 min), 17α-hydroxy-4-androsten-3-one (27) (9 min). Thus, the sequences were the same as in our previous report[4], but the separations were not as good. The different order may be ascribed to the difference in eluents. In the adsorption systems 1 and 2 (Table I, Fig. 1) the 3-keto-5α-androstane derivatives (7, 10, 40) are more polar than their 5β-epimers (5, 8, 36), except 17β-hydroxy-5α-androstan-3-one (13) which is inseparable from its 5β-epimer (14). Thus, generally, the planar (A/B-*trans*) 3-ketosteroids are more strongly adsorbed than the folded (A/B-*cis*) 3-ketosteroids.

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

TABLE I

RETENTION TIMES OF ANDROSTANE DERIVATIVES

Hydroxyl groups are indicated by α and β, depending on orientation, at the position listed. However, at C-5, α and β are used to designate the orientation of hydrogen. Keto groups are indicated by O and double bonds by Δ. Hydroxyl groups at C-18 and C-19 are indicated by OH. Systems: 1, see Fig. 1; 2, see Fig. 2; 3, see Fig. 3; 4, see Fig. 4; 5, see Fig. 3, except that methanol–water (17:3) was used as eluent.

No.	*Substituents*									*Retention time (min)*				
	3	*4*	*5*	*6*	*11*	*16*	*17*	*18*	*19*	*Systems*				
										1	*2*	*3*	*4*	*5*
$C_{19}O$														
1	O	–	α	–	–	Δ	–	–	–	2	–	–	–	20
2	α	–	α	–	–	Δ	–	–	–	2.75	–	>60	–	18.25
3	β	–	Δ	–	–	–	–	–	–	4	–	–	–	23.5
4	β	–	α	–	–	Δ	–	–	–	4	–	–	–	20
$C_{19}O_2$														
5	O	–	β	–	–	–	O	–	–	7.5	–	15.5	–	–
6	O	–	Δ	–	–	–	O	–	–	8	–	17.5	–	–
7	O	–	α	–	–	–	O	–	–	8	–	17.75	–	–
8	O	–	β	–	–	–	α	–	–	8.5	–	23	–	–
9	β	–	β	–	–	–	O	–	–	10	–	14.25	–	–
10	O	–	α	–	–	–	α	–	–	10.25	–	18	–	–
11	α	–	α	–	–	–	O	–	–	12.5	–	16.25	–	–
12	β	–	Δ	–	–	–	O	–	–	12.5	–	11.25	–	–
13	O	–	α	–	–	–	β	–	–	12.5	–	16	–	–
14	O	–	β	–	–	–	β	–	–	12.5	–	15.75	–	–
15	β	–	α	–	–	–	O	–	–	13	–	14.75	–	–
16	α	–	β	–	–	–	O	–	–	13	–	16.25	–	–
17	β	–	β	–	–	–	β	–	–	16.5	–	11.75	–	–
18	α	–	α	–	–	–	β	–	–	18	–	16.75	–	–
19	β	–	β	–	–	–	α	–	–	19.25	–	16	–	–
20	O	Δ	–	–	–	–	O	–	–	20	–	11.5	–	–
21	β	–	Δ	–	–	–	β	–	–	20	–	10.25	–	–
22	β	–	α	–	–	–	β	–	–	20	–	13.5	–	–
23	β	Δ	–	–	–	–	β	–	–	20.25	–	9.75	41.5	–
24	α		α	–	–	–	α	–	–	20.25	–	33	–	–
25	β	–	Δ	–	–	–	α	–	–	20.5	–	11.25	–	–
26	β	–	α	–	–	–	α	–	–	20.5	–	14.5	–	–
27	O	Δ	–	–	–	–	α	–	–	21.5	7.5	12	–	–
28	α	–	β	–	–	–	α	–	–	24	7.0	32	–	–
29	α	–	β	–	–	–	β	–	–	24	7.5	15.5	–	–
30	O	Δ	–	–	–	–	β	–	–	25.75	8.75	10.75	–	–
$C_{19}O_3$														
31	α	–	α	–	β	–	O	–	–	–	8.5	–	19.75	–
32	α	–	β	–	β	–	O	–	–	–	9.25	–	17.5	–
33	β	–	Δ	–	β	–	O	–	–	–	9.25	–	13.25	–
34	O	Δ	–	–	β	–	O	–	–	–	9.5	–	13.75	–
35	β	–	Δ	–	–	β	O	–	–	–	9.5	–	13	–

(Continued on p. 218)

TABLE I *(continued)*

No.	Substituents									Retention time (min)				
	3	4	5	6	11	16	17	18	19	Systems				
										1	2	3	4	5
36	O	–	β	–	O	–	O	–	–	–	10	–	17.75	–
37	β	–	α	–	β	–	O	–	–	–	10	–	16.25	–
38	β	–	Δ	–	–	α	α	–	–	–	11.5	–	20.25	–
39	α	–	β	–	O	–	O	–	–	–	11.5	–	19.75	–
40	O	–	α	–	O	–	O	–	–	–	11.5	–	16.75	–
41	β	–	α	–	–	α	α	–	–	–	11.5	–	27.5	–
42	β	–	Δ	–	–	α	O	–	–	–	11	–	11.75	–
43	β	–	Δ	–	–	O	β	–	–	–	11.5	–	10	–
44	β	–	Δ	–	–	β	β	–	–	–	12	–	16.25	–
45	β	–	Δ	–	O	–	O	–	–	–	12.75	–	11.25	–
46	O	Δ	–	β	–	–	O	–	–	–	14.25	–	10.25	–
47	α	–	α	–	α	–	O	–	–	–	14.75	–	20.75	–
48	β	–	α	–	O	–	O	–	–	–	15.75	–	12.5	–
49	β	–	β	–	–	α	β	–	–	–	17.25	–	11.75	–
50	β	–	Δ	–	–	–	O	OH	–	–	17.5	–	9	–
51	O	Δ	–	–	β	–	β	–	–	–	17.5	–	15.5	–
52	α	–	α	–	β	–	β	–	–	–	18.25	–	18.5	–
53	β	–	Δ	–	–	α	β	–	–	–	18.75	–	8	–
54	β	–	Δ	–	β	–	β	–	–	–	19	–	13.75	–
55	α	–	β	–	O	–	β	–	–	–	19.25	–	22.75	–
56	β	–	α	–	–	α	β	–	–	–	19.5	–	11	–
57	β	–	α	–	β	–	β	–	–	–	20.25	–	17.5	–
58	α	–	β	–	β	–	β	–	–	–	20.5	–	17.25	–
59	β	–	Δ	–	–	–	O	–	OH	–	20.75	–	10.25	–
60	α	–	β	–	–	α	β	–	–	–	21	–	32	–
61	O	Δ	–	–	O	–	O	–	–	–	21.25	–	12.25	–
62	O	Δ	–	–	O	–	β	–	–	–	21.25	–	11.75	–
63	O	Δ	–	–	–	–	β	OH	–	–	21.5	–	19.75	–
64	O	Δ	–	–	–	α	O	–	–	–	23.5	–	11	–
65	O	Δ	–	–	–	–	O	–	OH	–	24.5	–	8	–
66	O	Δ	–	–	–	α	β	–	–	–	25.25	–	9	–
67	α	–	β	–	α	–	β	–	–	–	26.25	–	27.5	–
68	O	Δ	–	–	α	–	α	–	–	–	30	–	10.5	–
69	O	Δ	–	–	α	–	β	–	–	–	30	–	10.75	–

As shown in Table I, the 3-keto-4-androstene derivatives (20, 37, 30) were much more polar than their 5α-,5β-, and Δ^5-analogues (5, 6, 7; 8, 10; 13, 14) in both adsorption and reversed-phase partition HPLC. Steroids containing conjugated carbonyl groups are, as a rule, more polar than steroids with isolated keto groups. Their polarity was found to be comparable to the corresponding hydroxy steroids in both HPLC systems. In reversed-phase partition HPLC, the 3-keto-4-androstene derivatives (20, 27, 30, 34, 51, 61, 62, 66) were more polar than the corresponding 3-hydroxyandrostane derivatives (9, 11, 15, 16; 19, 24, 26, 28; 17, 18, 22, 29; 31, 32, 37; 52, 57, 58; 39, 48; 55; 49, 56, 60) but less polar than the 3-hydroxy-4 (or 5)-androstene derivatives (12; 25; 21, 23; 33; 54; 45; -; 53) (Table I and Fig. 3). We were unable to

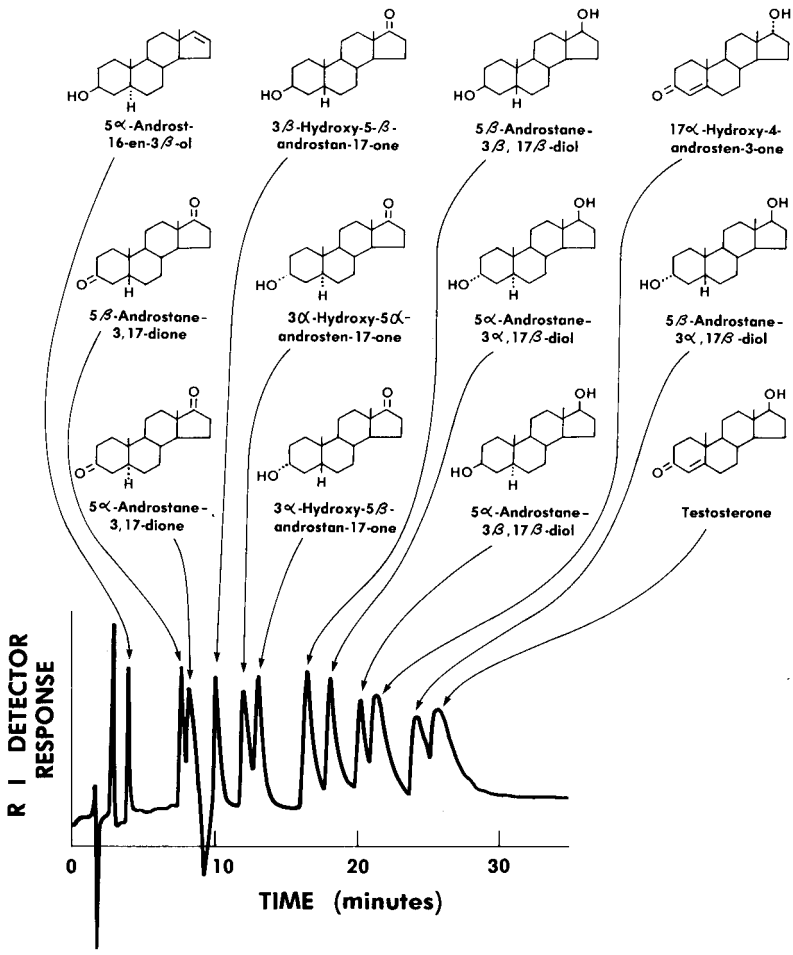

Fig. 1. Adsorption chromatogram of $C_{19}O$ and $C_{19}O_2$ androstane derivatives. Between 50 μg (5α-androst-16-en-3β-ol) and 300 μg (testosterone) of androstane derivatives, dissolved in 250 μl of the eluent, were chromatographed on a column of Zorbax BP-SIL, 250 × 4.6 mm I.D. Eluent *n*-hexane–ethanol (97:3); flow-rate, 2 ml/min; pressure, 600 p.s.i.; RI detector, 16 × ; recorder, speed 12 cm/h, span 10 mV.

separate the 3-hydroxy-4-androstene derivative, 4-androstene-3β,17β-diol (23) from its Δ^5- and 5α-analogues (21, 22), but separated it from its 5β-analogue (17) by adsorption HPLC. However, in reversed-phase partition HPLC, the Δ^4-, Δ^5-, 5α-, and 5β-analogues were readily separated (Table I and Fig. 4).

Table II summarizes the behavior of 3- and 5-epimers of 3-hydroxyandrostane derivatives in our adsorption (Figs. 1 and 2) and reversed-phase (Figs. 3 and 4) systems. Comparison of the effects of axial (a) and equatorial (e) hydroxyl groups (30 examples) showed that in adsorption HPLC steroids with equatorial 3-hydroxyl groups were consistently more polar than their axial epimers, except for 3β-hydroxy-5-androsten-17-one (12), which was inseparable from 3α-hydroxy-5α-androstan-17-one (11). This rule was also followed in reversed-phase partition HPLC, but several

TABLE II

RELATIVE POLARITIES OF 3- AND 5-ANALOGUES OF 3-HYDROXYANDROSTANE DERIVATIVES

> indicates that the first group of steroids is, as a rule, more polar than the second. A = adsorption, R = reversed-phase partition, + indicates that the rule shown in each heading is obeyed, − that it is violated, = indicates that analogues are inseparable. The superior HPLC system for each group of analogue separations is underlined.

Comparison between 3-OH(e) and 3-OH(a)

3α(e),5β > 3α(a),5α		*A*	*R*	*3β(e),5α > 3α(a),5α*		*A*	*R*
16	11	+	=	4	2	+	−
28	24	+	+	15	11	+	+
29	18	+	+	22	18	+	+
31	32	+	+	26	24	+	+
58	52	+	+	37	31	+	+
				57	52	+	+

3β(e),5α > 3β(a),5β		*A*	*R*	*3α(e),5β > 3β(a),5β*		*A*	*R*
9	15	+	−	16	9	+	−
22	17	+	−	28	19	+	−
26	19	+	+	29	17	+	−
56	49	+	+	60	49	+	−

3β(e),Δ⁵ > 3α(a),5α		*A*	*R*	*3β(e),Δ⁵ > 3β(a)5β*		*A*	*R*
12	11	=	+	12	9	+	+
21	18	+	+	21	17	+	+
25	24	+	+	25	19	+	+
33	31	+	+	53	49	+	+
54	52	+	+				

3β(e),Δ⁴ > 3α(a),5α		*A*	*R*	*3β (e),Δ⁴ > 3β(a),5β*		*A*	*R*
23	18	+	+	23	17	+	+

Comparison between 3-OH(e) and 3-OH(e)

3β(e),5α > 3α(e),5β		*A*	*R*	*3β(e),Δ⁵ > 3α(e),5β*		*A*	*R*
15	16	=	+	12	16	−	+
22	29	−	+	21	29	−	+
26	28	−	+	25	28	−	+
37	32	+	+	33	32	=	+
48	39	+	+	53	60	−	+
56	60	−	+	54	58	−	+
57	58	−	−				

3β(e),Δ⁴ > 3β(e),5α		*A*	*R*	*3β(e),Δ⁴ > 3β(e),Δ⁵*		*A*	*R*
23	22	+	+	23	21	+	+

3α(e),5β > 3β(e),Δ⁴		*A*	*R*
29	23	+	−

TABLE II *(continued)*

Comparison between 3-OH(a) and 3-OH(a)							
3α(a),5α > 3β(a),5β		*A*	*R*	*3β(a),Δ⁵ > 3β(a),5α*		*A*	*R*
11	9	+	–	12	15	–	+
18	17	+	–	21	22	=	+
24	19	+	–	25	26	=	+
				33	37	–	+
				38	41	=	+
				45	48	–	+
				53	56	–	+
				54	57	–	+

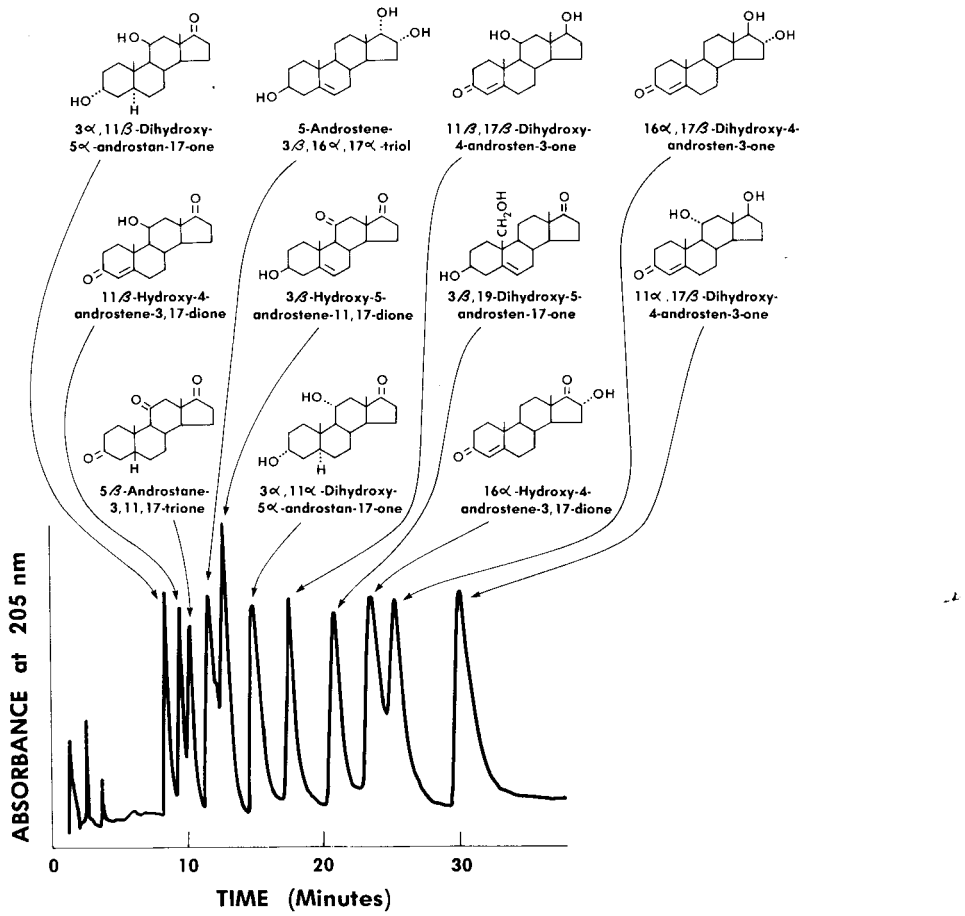

Fig. 2. Adsorption chromatogram of $C_{19}O_3$ androstane derivatives. Conditions as in Fig. 1, except between 5 μg (11β-hydroxy-4-androstene-3,17-dione) and 100 μg (3α,11α-dihydroxy-5α-androstan-17-one) of androstane derivatives, an eluent of *n*-hexane–ethanol (93:7), and a UV detector at 205 nm, range 0.2, time constant 1.0, were used.

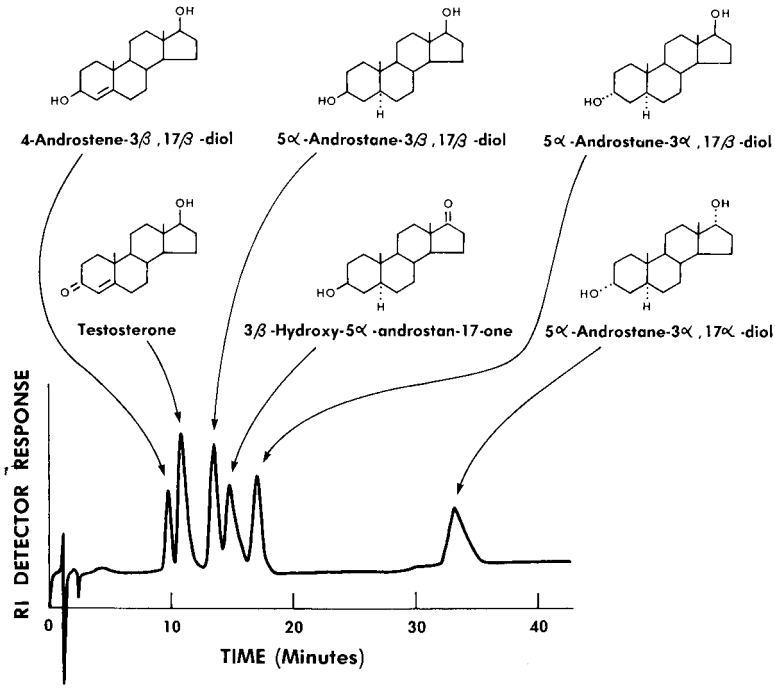

Fig. 3. Reversed-phase partition chromatogram of $C_{19}O_2$ androstane derivatives. About 100 μg of each androstane derivative, dissolved in 250 μl of the eluent, were chromatographed on a column of Zorbax BP-ODS, 250 × 4.6 mm I.D. Eluent, methanol–water (7:3); pressure, 100 p.s.i.; RI detector, 8×. Other conditions as in Fig. 1.

exceptions were noted. We have observed that adsorption HPLC separates 5-epimers better than reversed-phase partition HPLC, while reversed-phase partition HPLC separates 3-epimers better than adsorption HPLC.

Comparison of 5-analogues with equatorial 3-hydroxyl groups (16 examples) showed that, generally, the 3β-hydroxy-5α-androstane derivatives and the 3β-hydroxy-5-androstene derivatives are more polar than the 3α-hydroxy-5β-androstane derivatives in reversed-phase partition HPLC, while in adsorption HPLC the 3α-hydroxy-5β-androstane derivatives are more polar than the 3β-hydroxy-5-androstene derivatives. For this group of steroids, reversed-phase partition HPLC was found to give separations superior to those by adsorption HPLC.

Comparisons of 5-analogues with axial 3-hydroxyl groups (11 examples) showed that, generally, the 3α-hydroxy-5α-androstane derivatives were more polar than the 3β-hydroxy-5β-androstane derivatives in adsorption HPLC, while the 3β-hydroxy-5β-androstane derivatives were more polar than the 3α-hydroxy-5α-androstane derivatives in reversed-phase partition HPLC. The 3β-hydroxy-5-androstene derivatives were more polar than the 3β-hydroxy-5α-androstane derivatives in reversed-phase partition HPLC, but in adsorption HPLC the 3β-hydroxy-5α-androstane derivatives were either more polar than the 3β-hydroxy-5-androstene derivatives or inseparable from them. For this group of steroids reversed-phase partition HPLC was also found to provide greater resolving power.

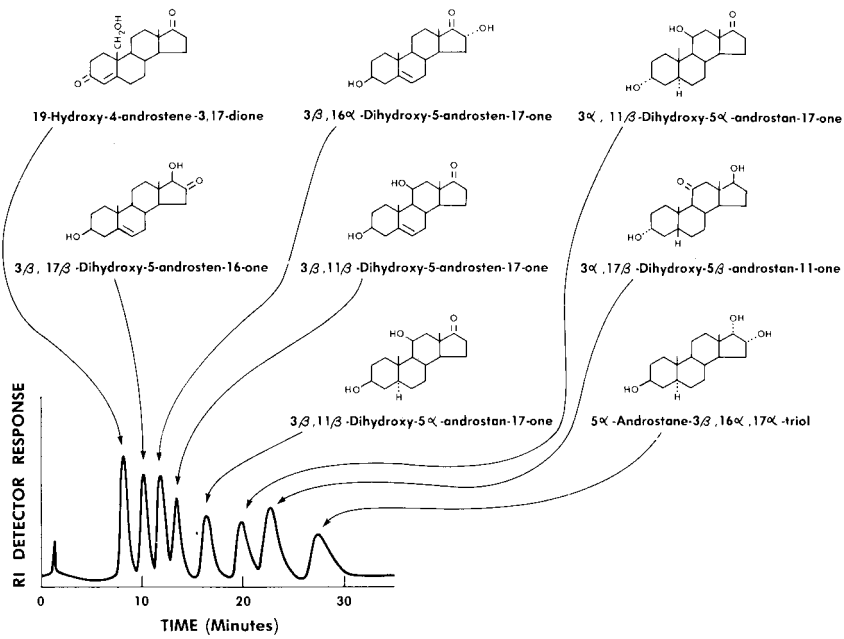

Fig. 4. Reversed-phase partition chromatogram of $C_{19}O_3$ androstane derivatives. Conditions as in Fig. 3 except an eluent of methanol–water (11:9) was used and the pressure was 1100 p.s.i.

All of the 17β-hydroxyandrostane derivatives in Table I (13, 14, 17, 18, 21, 22, 29, 30, 53, 56, 59) were more polar than their 17α-epimers (10, 8, 19, 24, 25, 26, 28, 27, 38, 41, 68) in reversed-phase partition HPLC. 11β-Hydroxyandrostane derivatives (31, 51, 58) were less polar than their 11α-epimers (47, 69, 67) in adsorption HPLC. Adsorption HPLC separated the 11-epimers better than reversed-phase partition HPLC. The 16β-hydroxyandrostane derivatives (35, 44) were less polar than their 16α-epimers (42, 53) in both HPLC systems.

In general, hydroxyl groups contribute more to the polarity of the steroids than keto groups, but an α,β-unsaturated keto group makes them about as polar as a hydroxyl group. The number of hydroxyl groups and keto groups, the locations of both groups and of double bonds, the configuration at all chiral centers, the sorbent, and the eluent determine the elution order of androstane derivatives in HPLC. Adsorption and reversed-phase partition HPLC complement each other, and most androstane derivatives can be separated by a combination of both methods.

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BINDING CAPACITIES OF HUMAN SERUM ALBUMIN MONOMER AND DIMER BY CONTINUOUS FRONTAL AFFINITY CHROMATOGRAPHY

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SUMMARY

Human serum albumin monomer and dimer obtained by fractionation of a commercial preparation were immobilized on CH-Sepharose 4B by covalent coupling. For salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole and sulphonylureas, the binding capacities of the monomer and dimer were compared by continuous frontal affinity chromatography. The salicylate-binding capacities of both monomer and dimer were essentially retained upon immobilization. For these drugs, the dimer showed only about 10–30% less capacity per monomeric unit than that of the monomer, the reduction being associated for most drugs with the intrinsic binding constant rather than with the number of binding sites.

INTRODUCTION

In our laboratories we have been studying the use of albumin covalently coupled to insoluble carriers for the simple and precise *in vitro* evaluation of the relative affinity of drugs to serum albumin in conjunction with frontal affinity chromatography. The latter technique was first demonstrated for the quantitative evaluation of various trypsin-inhibitor complexes using glycylglycyl-L-arginine immobilized on agarose beads[1]. Utilizing bovine serum albumin (BSA) we have previously shown[2,3] that, when the albumin is covalently attached to agarose gel matrix through a six-carbon-atom spacer, the binding properties of albumin are essentially retained. This technique was further extended to the simultaneous binding of two drugs to human serum albumin (HSA) whereby mutual displacement phenomena can be directly observed[4].

This paper demonstrates another application of this technique to proteins that are usually not readily available, using the dimer of HSA as a model, whereby comparison of the binding of many ligands by HSA monomer and dimer is expected to be readily possible. We have already reported that the fractionation of an HSA preparation on Sephadex G-200 columns using 0.1 *M* sodium chloride solution as the eluting medium and shown by equilibrium dialysis that the salicylate-binding capacity of the dimer per monomeric unit was about 80% of the monomer[5]. In order to investigate the generality of this relationship for a variety of drugs that show strong affinity, we

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have immobilized the monomer and dimer under the same conditions and the binding of several drugs to these affinity columns was quantitatively investigated by continuous frontal analysis, which is a modification of the previously employed technique[2].

EXPERIMENTAL

Materials

Human serum albumin (HSA), fraction V (essentially fatty acid free, lot no. 76C-7480), was purchased from Sigma (St. Louis, MO, U.S.A.) and fractionated on Sephadex G-200 columns as described previously[5] using 0.1 *M* sodium chloride solution as the eluting medium. The monomeric fraction contained over 95% monomer, whereas the dimeric fraction contained about 90% dimer. Activated CH-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Chlorpropamide was obtained from Sankyo (Tokyo, Japan). Mefenamic acid and tolbutamide were products of Upjohn (Kalamazoo, MI, U.S.A.) and Ono Pharmaceutical (Osaka, Japan), respectively. Other drugs were as described previously[3]. All other chemicals were of analytical-reagent grade and water was deionized and doubly distilled, the second distillation being performed in all-glass apparatus.

Immobilization of HSA monomer and dimer on CH-Sepharose 4B

To about 75 ml of either the monomer or dimer fraction containing about 1% HSA in 0.1 *M* sodium chloride solution, appropriate amounts of sodium hydrogen carbonate and sodium chloride were added so that the solution became 0.1 *M* and 0.5 *M* with respect to sodium hydrogen carbonate and sodium chloride, respectively. These HSA solutions were mixed with swollen activated CH-Sepharose 4B (15 g) and reacted at room temperature for 1 h in screw-capped test-tubes which were rotated end-over-end.

Determination of the amount of monomer or dimer immobilized on the gel matrix (CH-Sepharose 4B)

Direct spectrophotometric method. HSA-coupled gel (0.5 ml) in water was measured and water was added so that the total volume became 5 ml. This gel suspension (1 ml) was transferred into a 1-cm cell and 2 ml of 75% glycerin were added. The turbidity due to the gel matrix of the sample cell was balanced with that of the reference cell with either Sepharose 4B or CH-Sepharose 4B in 50% glycerine. The difference spectrum was then recorded in the second cell compartment of a Shimadzu Model UV-300 double-beam spectrophotometer (in which the cells were positioned directly in front of the photomultiplier for turbid samples). A molecular weight of 66,250[6] was used for the monometric unit of HSA. Absorbance values ($A^{1\%}_{1\,cm}$ at 278 nm) of 5.30 and 5.47 were used for the monomer and dimer, respectively[5].

Lowry's method[7]. HSA-coupled gel (1 ml) was measured and water was added to make the total volume 100 ml. To 1 ml of this suspension 0.5 ml of 1 *M* sodium hydroxide solution was added and the mixture was boiled for about 10 min. Alkaline copper reagent (5 ml) was added and the mixture was stirred occasionally for 11 min. A 1-ml volume of Folin–Ciocalteu reagent was then added and the mixture was left for about 30 min with occasional stirring. The absorbance of the supernatant solution

at 750 nm was measured. The amounts of the monomer and dimer coupled to the gel were determined from calibration graphs obtained with the monomer and dimer of HSA used for coupling.

The amounts of monomer immobilized as measured by the direct spectrophotometric method and Lowry's method were in fairly good agreement. On average, about 11 mg of HSA were coupled per millilitre of gel. For some batches, particularly those of the dimer, we experienced difficulty in balancing the turbidity due to the gel matrix in the direct method and no satisfactory difference spectrum was obtained. In such instances, the values obtained by Lowry's method were employed in the calculation of the binding parameters.

Determination of drugs bound by continuous frontal affinity chromatography

The HSA-coupled gel was packed into either a Pharmacia column (K16/20) or a Whatman column (MS-PC 1020; Whatman Biochemicals, Maidstone, Great Britain) with flow adaptors. Upward elution was carried out by means of a Pharmacia Model P-3 peristaltic pump at a rate of 10 ml/h. The temperature of the column was maintained at 4°C with water circulated through the jacket of the column by a Haake Model FK 10 constant-temperature circulator. All binding experiments were carried out in 0.05 *M* Tris buffer containing 0.1 *M* sodium chloride (pH 7.87) at 4°C. When the columns were not in use they were constantly eluted with buffer containing 0.01 % sodium azide. Prior to use they were pre-equilibrated with the buffer for about 16 h. The void volume of the column (V_0), the sum of the volume of the gel bed available for eluting media and that of the tubing, was determined by elution of 0.1 % EDTA (disodium salt), which has less affinity to the HSA-coupled gel than the sodium azide employed previously for this purpose[2–4].

Basically the previously described frontal analysis technique was followed except that after application of a known concentration of drug solution, which is the concentration of the free (or unbound) drug in this method, to the equilibrium state, a second solution with a different concentration of drug was directly applied to the column without regeneration of the column with the buffer as had been done previously[2,3]. The column eluate was collected by a fraction collector in pre-weighed test-tubes and exact volume determined by weighing. All of the drugs studied showed negligible affinity towards the gel matrix itself, with the exception of mefenamic acid, for which corrections were made by determining the adsorption isotherm of the drug to the gel by frontal analysis.

RESULTS AND DISCUSSION

Continuous frontal analysis

Fig. 1 shows that two different concentrations of drug solutions can be applied continuously without regeneration of the column with the buffer after application of each drug solution. This is demonstrated for the determination of the amounts of salicylic acid bound to an 8.2-ml gel column of immobilized HSA monomer ($1.34 \cdot 10^{-6}$ mole) corresponding to two different concentrations of free (or unbound) salicylic acid, i.e., $2.25 \cdot 10^{-5}$ and $3.64 \cdot 10^{-5}$ *M*. The amount of salicylic acid bound corresponding to the lower concentration can be calculated from area B to be $1.34 \cdot 10^{-6}$ mole and that corresponding to the higher concentration from the sum of

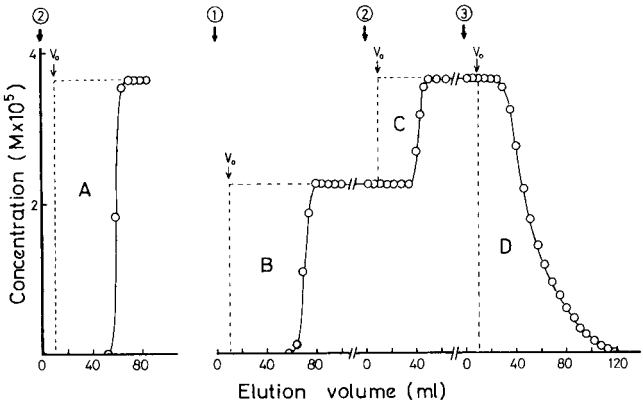

Fig. 1. Determination of the amount of salicylic acid bound by continuous frontal affinity chromatography. Column: 8.2 ml of HSA monomer ($1.34 \cdot 10^{-6}$ mole) immobilized CH-Sepharose 4B gel equilibrated at 4°C with 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87), V_0 = 10.5 ml. The arrows indicate the points of application of the following salicylic acid solutions and the buffer: (1) 2.25×10^{-5} *M*; (2) 3.64×10^{-5} *M*; and (3) the buffer. See text for areas A–D.

areas B and C to be $1.80 \cdot 10^{-6}$ mole. Agreement between the sum of areas B and C and either area A for the direct application of the solution of higher concentration ($1.77 \cdot 10^{-6}$ mole) or area D ($1.77 \cdot 10^{-6}$ mole) corresponding to the amount released following elution with the buffer justifies the present continuous application of drug solutions.

Fig. 2 shows a typical continuous frontal analysis elution pattern for four concentrations of free salicylic acid applied to a 15.2-ml HSA dimer-immobilized column. This procedure about halves the time required for the determination of binding parameters compared with the previous procedure[2].

Drug-binding characteristics of the immobilized HSA monomer and dimer

Salicylate-binding characteristics of the immobilized HSA monomer and dimer

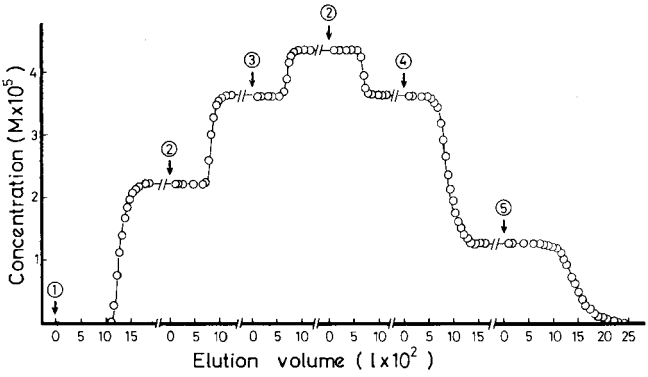

Fig. 2. Elution profile of a consecutive application at 4°C of four solutions of different concentrations of salicylic acid in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87) and the buffer to 15.2 ml of HSA dimer ($2.66 \cdot 10^{-6}$ mole) immobilized column, V_0 = 19.1 ml. The arrows indicate the points of application of salicylic acid solutions and the buffer: (1) $2.22 \cdot 10^{-5}$ *M*; (2) $3.64 \cdot 10^{-5}$ *M*; (3) $4.38 \cdot 10^{-5}$ *M*; (4) $1.29 \cdot 10^{-5}$ *M*; and (5) the buffer.

are compared in Fig. 3, in the form of Scatchard plots, with those of soluble species determined previously[5] by equilibrium dialysis under the same experimental conditions, where r is the number of moles of salicylic acid bound per mole of a monomeric unit of HSA and D_f is the concentration of free salicylic acid. The Scatchard plots show reasonable linearity; the data were treated as previously on the basis of the presence of only one class of n binding sites per monomeric unit, which possess an equal intrinsic binding constant K.

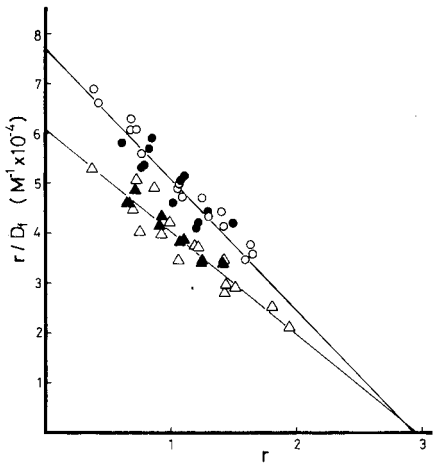

Fig. 3. Scatchard plots for the interaction of HSA monomer and dimer with salicylic acid at 4°C in 0.05 M Tris buffer containing 0.1 M NaCl (pH 7.87). By continuous frontal analysis: HSA monomer (●) and HSA dimer (▲). By equilibrium dialysis: HSA monomer (○) and HSA dimer (△). The straight lines represent the regression lines for the continuous frontal affinity chromatographic data.

These column data were obtained from four monomer and three dimer columns, each consisting of 8–20 ml of albumin-immobilized gel of different batches. Salicylate-binding parameters determined from each column by continuous frontal analysis of three solutions with different concentrations of salicylic acid are presented in Table I.

The monomer column data in Fig. 3 show considerable scatter. The scatter can be seen in Table I to exist between columns, *i.e.*, for each column the data give almost parallel lines, so that K values show much better agreement than n values. It should be pointed out that n depends upon the amount of total albumin present in the column, whereas K does not. The amount of total albumin present in the column is subject to some experimental errors originating, for instance, in the determination of albumin immobilized per millilitre of gel, in the measurement of gel volume before packing and in the leakage of covalently coupled albumin. Less scatter of the dimer data probably means that we could control at least some of these factors as we became used to handling these gels.

The salicylate-binding parameters of soluble HSA calculated previously[5] from the equilibrium dialysis data presented in Fig. 3 are $K = 2.58 \cdot 10^4\ M^{-1}$, $n = 3.03$ for the monomer, and $K = 2.05 \cdot 10^4\ M^{-1}$, $n = 2.96$ per monomeric unit of the dimer. Good agreement between these values and corresponding values for the immobilized

TABLE I

DRUG-BINDING PARAMETERS OF HSA MONOMER AND DIMER

Studied at 4°C in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87) by continuous frontal affinity chromatography. nK, K and n were calculated by the method of least squares from the Scatchard plot of three sets of data points. D_f ranged from $0.8 \cdot 10^{-5}$ to $4.5 \cdot 10^{-5}$ *M*.

Drug	*HSA*	$nK \cdot 10^{-4}$ (M^{-1})	$K \cdot 10^{-4}$ (M^{-1})	n	$-\varrho$*
Salicylic acid	Monomer	8.13	2.64	3.08	0.997
		7.31	2.63	2.78	0.999
		7.87	2.65	2.97	0.999
		7.45	2.65	2.81	0.999
		7.69 ± 0.38**	2.64 ± 0.01**	2.91 ± 0.14**	
	Dimer	5.94	2.00	2.97	0.998
		6.30	2.10	3.00	0.991
		5.98	2.04	2.93	0.999
		6.07 ± 0.20**	2.05 ± 0.05**	2.97 ± 0.04**	
Warfarin$_r$-	Monomer	18.1	8.69	2.08	0.993
	Dimer	16.7	8.17	2.05	0.993
Phenylbutazone	Monomer	32.9	20.8	1.58	0.920
	Dimer	26.5	17.2	1.54	0.999
Sulphamethizole	Monomer	3.73	1.37	2.72	0.998
	Dimer	2.63	0.97	2.71	0.999
Mefenamic acid	Monomer	49.0	5.86	8.36	0.997
	Dimer	42.9	5.07	8.46	0.998
Chlorpropamide	Monomer	3.78	1.57	2.41	0.999
	Dimer	3.00	1.41	2.13	0.999
Tolbutamide	Monomer	8.39	3.90	2.15	0.999
	Dimer	6.79	3.43	1.98	0.998

* ϱ = correlation coefficient.
** Average value (± S.D.).

species (Table I) indicates that the binding of salicylic acid to both HSA monomer and dimer is essentially unaffected by immobilization on the insoluble carrier.

The binding characteristics of several drugs to the immobilized HSA monomer and dimer were evaluated similarly employing two columns each for the monomer and the dimer, and the results are presented in Table I. Variations in r for a fixed value of D_f between columns were corrected by employing salicylic acid as a standard drug. Thus, continuous analysis with three concentrations of salicylic acid was carried out and, employing the average values of K and n given in Table I, the total amount of albumin present in the column was calculated. A standard solution of salicylic acid was always applied before proceeding to a different drug and a check on albumin leakage was made. When leakage of albumin was suspected from such a check, salicylic acid was re-analysed by continuous frontal analysis and when the K value was unchanged a new value of total albumin present was re-calculated. The binding characteristics of these drugs were similar to those of salicylic acid, and the dimer showed slightly lower capacities (about 10–30% less) per monometric unit than the monomer, which for most drugs appears to reflect the greater reduction in K than in n values. This trend is not so clear with the sulphonylureas.

Sollenne *et al.*[8], however, recently observed that HSA dimer did not bind L-tryptophan, nor did it have the esterase activity of HSA monomer, suggesting that the tryptophan-binding site of HSA monomer, in which the esterase activity is considered

to reside[9], is lost upon dimerization. The linkage of two monomer units occurs at or near the tryptophan-binding site. In view of their observations, our present data may suggest that the primary binding site(s) of the drugs studied here is (are) different from the tryptophan-binding site.

Characterization of the specific binding sites present in a HSA molecule has been the subject of recent investigations[10–14]. Although many contradictory results have been reported, it is likely that an HSA molecule has at least two or more major independent drug-binding sites, one of which is the indole–benzodiazepine-binding site[10] (tryptophan-binding site, called site II by Sudlow *et al.*[14]) located around the tyrosine 411 residue of HSA, and the other being warfarin-binding site(s) (called site I by Sudlow *et al.*[14]), located around the lone tryptophan residue of HSA. According to this classification none of the drugs studied here is definitely classified as binding HSA only at the tryptophan-binding site. Therefore, these drugs probably bind to HSA primarily at the warfarin-binding site(s), which is (are) relatively unaffected upon dimerization, and the slight reduction in binding may be due to the allosteric effects. However, HSA dimer was reported to bind indole analogues[15] and progesterone[16] to the same extent as the two monomers.

Although a comparison of binding capacities of the monomer and dimer of HSA is an indirect approach, the present technique may be of value in the elucidation of site specificity of drugs. We are now directing our efforts in this direction by examining the drugs that are reported to bind to HSA at site II of Sudlow's classification.

CONCLUSION

Both HSA monomer and dimer essentially retained the salicylic acid-binding capacity upon immobilization on agarose beads by means of activated CH-Sepharose 4B. The activated beads are particularly suitable for covalent coupling of purified HSA monomer and dimer because no dimer or polymers are expected to be formed during the coupling reaction. Further, the reaction is very rapid under very mild conditions.

As with salicylic acid, for all of the drugs studied here the binding capacity of the dimer was only slightly less than that of the two monomers and for most drugs this reduction was associated with a reduction in K values rather than n values. The binding of these drugs to HSA probably takes place at site(s) other than the site of the linkage of two monomer units in dimer formation.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–NEGATIVE CHEMICAL IONIZATION MASS SPECTROMETRY OF ORGANOPHOSPHORUS PESTICIDES

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SUMMARY

Eighteen organophosphorus pesticides were studied by combined high-performance liquid chromatography–negative chemical ionization mass spectrometry (HPLC–NCI-MS). The LC separation was done on a reversed-phase C_8 column, using acetonitrile–water (60:40) as mobile phase. The MS analysis was done on-line, using a direct liquid-insertion probe LC–MS interface. The negative-ion mass spectra obtained under these LC–MS conditions are very simple and are very similar to those reported for methane-enhanced negative ionization. Molecular ions are generally not present in either mode, but intense fragment ions containing useful structural information are usually observed.

INTRODUCTION

Organophosphorus pesticides are widely used in agriculture, especially in recent years since the use of the more persistent chlorinated hydrocarbon pesticides has been curtailed. As a result, there is much interest in methods for the detection of these compounds in environmental matrices, and analytical methods have been developed for the determination of organophosphorus compounds in animal tissues[1–3], in plant tissues[4–8], water[2,5,9–11], soil[5], sewage sludge[10] and human urine[7].

At present, analysis of organophosphorus compounds is done mainly by gas chromatography (GC)[7,10,12]. Several researchers have studied these compounds by high-performance liquid chromatography (HPLC), using both normal-phase and re-

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versed-phase columns. Normal-phase techniques used include adsorption chromatography on silica gel[13] and diatomaceous earth[8], as well as on polar stationary phases such as β,β′-oxydipropionitrile coated on Zipax[2] or Corasil[14]. Reversed-phase separations have been done on μBondapak/Corasil[9] and Zorbax-ODS[11], both of which are octadecylsilane (C_{18}) stationary phases.

Several mass spectrometric (MS) studies of these compounds have been performed on individual pesticides by direct-probe analysis in the electron-impact ionization mode[6,15], and by direct-probe analysis in the methane positive chemical ionization (CI) mode[16]. Chloride-attachment negative CI (NCI) has also been used for organophosphorus compounds[17]. Combined GC–MS has been done on several organophosphorus pesticides, and the relative sensitivities in various positive and negative ionization modes, with different combinations of methane and oxygen, have been compared[18]. Various on-line LC–MS systems have been developed[19–23] and have been used for a variety of analytical applications[24,25].

Based on the increased sensitivity observed for organophosphorus compounds in NCI modes[18], it was decided to study the applicability of combined LC–NCI-MS to this class of compounds in order to investigate a method for its possible use in trace analysis of organophosphorus pesticides, and/or their possibly more polar or thermally labile metabolites.

EXPERIMENTAL

Equipment

The HPLC system consisted of two Waters 600A pumps (Waters Assoc., Milford, MA, U.S.A.), a Waters 660 Solvent Programmer, and a Perkin-Elmer LC-55 variable-wavelength UV detector (Perkin-Elmer, Norwalk, CT, U.S.A.). The HPLC column used was an RP-8 reversed-phase column, 10 μm particle size, 10 cm × 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase used was acetonitrile–water (60:40), and the flow-rate was 1 ml/min. The UV detector wavelength was 254 nm.

The LC–MS interface was an unmodified Hewlett-Packard Direct Liquid Insertion Probe[26] (Hewlett-Packard, Palo Alto, CA, U.S.A.), which is a variable split-type interface. The usual split ratio is *ca.* 1:99 (mass spectrometer to fraction collector) (Fig. 1). This means that, with an LC flow-rate of 1 ml/min, *ca.* 10 μl/min of mobile phase enters the mass spectrometer source.

The mass spectrometer used in this research was a Finnigan 3300 chemical ionization mass spectrometer (Finnigan-MAT, Sunnyvale, CA), previously modified for NCI operation[27]. The mass spectrometer was interfaced to a Finnigan/Incos 2300 data system. The standard Finnigan 3300 1/4 in. I.D. direct-probe inlet system was replaced by a 1/2 in. I.D. inlet system, and a "desolvation chamber" was threaded into a modified direct-probe inlet-adapter plate on the Finnigan ion source (Fig. 2). The LC–MS interface probe slides into the desolvation chamber, maintaining source tightness for CI. The droplets of the jet are vaporized in the desolvation chamber, and the resulting sample–solvent gas mixture then enters the source, where it is ionized. Since the solvent is present in excess, it acts as a CI reagent gas[19] and as an electron-energy moderator, and the mode of ionization is probably electron capture and dissociative electron capture. No modification of the mass spectrometer pumping system

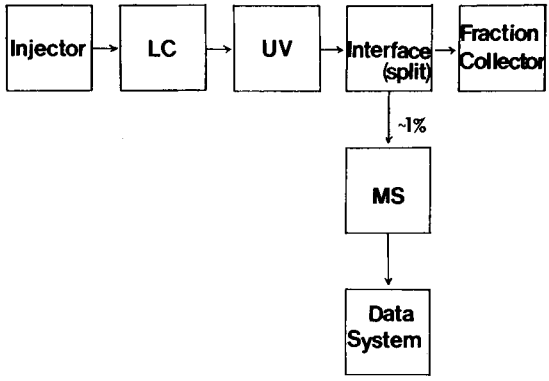


Fig. 1. Diagram of LC–MS system.

was necessary. Details of operation of the LC–MS system have been given earlier[28]. Since source temperature has been shown to have a profound effect on NCI mass spectra[29,30], source temperature was held at approximately 185°C.

Samples and solvents

Samples of the organophosphorus pesticides were obtained from the U.S. Environmental Protection Agency. Standard solutions were prepared using HPLC-grade acetonitrile. All sample solutions were stored in a freezer when not in use. The stated purities of these compounds ranged from 92 to 100%, with the exception of mevinphos, which was stated to be 64.5% pure. Since HPLC was used for sample introduction, no other purification was attempted prior to analysis.

Solvents used in this study were HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and HPLC-grade water (J.T. Baker, Phillipsburgh, NJ, U.S.A.). The acetonitrile was filtered through a 0.5-μm filter; the water was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.).

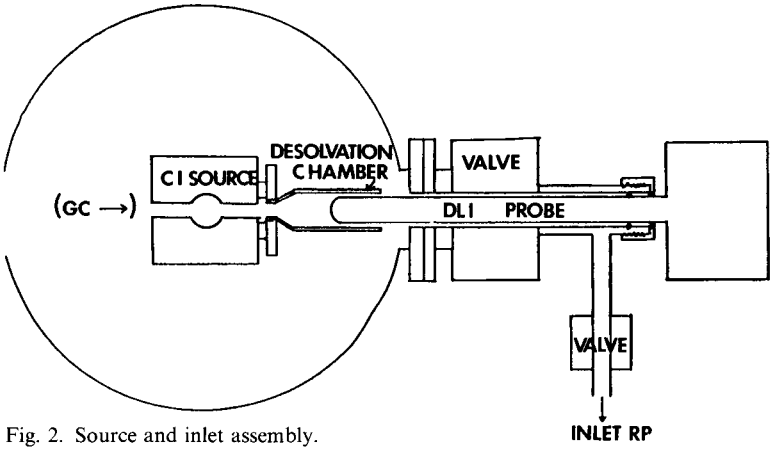


Fig. 2. Source and inlet assembly.

RESULTS AND DISCUSSION

Four classes of compounds were studied: phosphates (monocrotophos, dicrotophos and mevinphos), phosphorothioates (ronnel, bromophos, dichlofenthion, ethyl parathion, chlorpyrifos and diazinon), phosphorodithioates (dimethoate, malathion, azinphos-methyl, azinphos-ethyl, phorate, carbophenthion and menazon), and phenylphosphorothioates (leptophos and EPN).

The mass spectra and LC retention times are shown in Tables I–V. The methane-enhanced negative-ion (MENI) and methane oxygen-enhanced negative-ion (MOENI) mass spectra of most of the compounds studied have been reported previously[18]. The LC–NCI-MS mass spectra were simple and were similar to the reported MENI spectra, with more of the ion current being carried by the lower-mass fragments in the LC–NCI-MS spectra. All major peaks in the LC–NCI-MS spectra are also found in the MENI spectra. Molecular ions are not usually found, but diagnostic fragment ions giving structural information are usually present (Table V). The similarity between the GC–MS and the LC–MS spectra indicates that there is not much thermal decomposition occurring during GC analysis for the compounds studied.

Phosphates

For the three members of this compound class studied, almost all of the ion

TABLE I

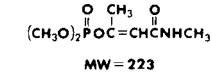
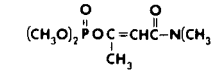
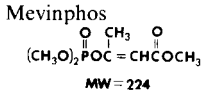
COMPOUNDS STUDIED

Compound class	*Compound*	*MW*	*Retention time (min:sec)*	*Structure*
Phosphates	Monocrotophos	223	1:55	$(CH_3O)_2(PO)OC(CH_3)CHCONHCH_3$
	Dicrotophos	237	7:27	$(CH_3O)_2(PO)OC(CH_3)CHCON(CH_3)_2$
	Mevinphos	224	2:11	$(CH_3O)_2(PO)OC(CH_3)CHCO(OCH_3)$
Phosphorothioates	Ronnel	320	6:46	$(CH_3O)_2(PS)OC_6H_2Cl_3$
	Bromophos	364	6:58	$(CH_3O)_2(PS)OC_6H_2Cl_2Br$
	Dichlofenthion	314	8:04	$(C_2H_5O)_2(PS)OC_6H_3Cl_2$
	Ethyl parathion	291	4:43	$(C_2H_5O)_2(PS)OC_6H_4NO_2$
	Chlorpyrifos	349	8:12	$(C_2H_5O)_2(PS)OC_5NHCl_3$
	Diazinon	304	5:03	$(C_2H_5O)_2(PS)OC_4N_2H_2CH_3CH(CH_3)_2$
Phosphorodithioates	Dimethoate	229	2:11	$(CH_3O)_2(PS)SCH_2CONHCH_3$
	Malathion	330	3:45	$(CH_3O)_2(PS)SCH(COOC_2H_5)CH_2(COOC_2H_5)$
	Azinphos-methyl	317	3:09	$(CH_3O)_2(PS)SCH_2N_3COC_6H_4$
	Azinphos-ethyl	345	4:02	$(C_2H_5O)_2(PS)SCH_2N_3COC_6H_4$
	Phorate	260	5:20	$(C_2H_5O)_2(PS)SCH_2SC_2H_5$
	Carbopenthion	342	9:09	$(C_2H_5O)_2(PS)SCH_2SC_6H_4Cl$
	Menazon	281	2:07	$(CH_3O)_2(PS)SCH_2C_3N_3(NH_2)_2$
Phenylphosphonothioates	Leptophos	410	11:45	$(CH_3O)(C_6H_5)(PS)OC_6H_2Cl_2Br$
	EPN	323	6:05	$(C_2H_5O)(C_6H_5)(PS)OC_6H_4NO_2$

TABLE II

LC–NCI MASS SPECTRA OF PHOSPHATE PESTICIDES

MW = Molecular weight.

Compound	*Mass*	*Relative abundance (%)*	*Tentative identification*
Monocrotophos	114	<0.1	$[M-(CH_3O)_2PO]^-$
	124	0.3	
	125	100.0	$(CH_3O)_2PO_2^-$
MW = 223	126	1.4	
	127	0.3	
Dicrotophos	125	100.0	$(CH_3O)_2PO_2^-$
	127	0.3	
	128	0.8	
	129	0.6	
MW = 237	222	1.9	$[M-(CH_3)]^-$
Mevinphos	125	100.0	$(CH_3O)_2PO_2^-$
	127	0.1	
MW = 224			

current was carried by the peak at *m/z* 125, which corresponds to $(CH_3O)_2PO_2^-$ (Table II). This ion is apparently characteristic of this class of compounds. In addition, the spectrum of monocrotophos contains a small peak corresponding to $[M-(CH_3O)_2PO]^-$, and the spectrum of dicrotophos a peak for the loss of CH_3 from the molecular ion.

Phosphorothioates

Of these compounds, two (ronnel and bromophos) are dimethoxy compounds and four (dichlofenthion, ethyl parathion, chlorpyrifos and diazinon) are diethoxy compounds (Table III). This class of compounds shows common fragments corresponding to $(RO)_2PSO^-$ at *m/z* 141 when R = CH_3, and at *m/z* 169 when R = C_2H_5. Also present are peaks corresponding to the thiophenate ions produced by S migration. The phenoxide ion was observed in the LC–NCI-MS spectra of dichlofenthion, ethyl parathion and chlorpyrifos at *m/z* 161, 177 and 196, respectively. The substitution reaction involving addition of O_2 (from trace amounts of air in the source) and loss of $ClO^{\bullet}$ giving an overall decrease of 19 a.m.u. is seen for the thiophenate ions in ronnel, dichlofenthion and chlorpyrifos.

The *m/z* 79 ion found in the spectra of ronnel, chlorpyrifos and dichlofenthion corresponds to a combination of PO_3^- and PSO^-, PO_3^- probably being the predominant species since the PO_3^-:PSO^- ratio reported for chlorpyrifos was 4:1[31]. The *m/z* 79 ion in bromophos is the base peak in the spectrum, with the ^{81}Br isotope giving a relative intensity of 87%. The intensity difference between the *m/z* 79 and *m/z* 81 peaks is probably due to the PO_3^- and PSO^- contributions to the $^{79}Br^-$ peak. Bromophos also shows a trace of $(M-CH_3)^-$ and $(M-CH_3Br)^-$ at *m/z* 351 and 270, respectively.

TABLE III

LC–NCI MASS SPECTRA OF PHOSPHOROTHIOATE PESTICIDES

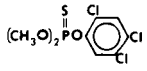
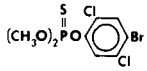
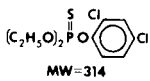
Compound	*Mass*	*Relative abundance (%)*	*Tentative identification*
Ronnel	79	2.3	PSO^-
	95	10.9	PSO_2^-
	110	0.3	$(CH_3O)PSO^-$
	126	0.2	$(CH_3O)PSO_2^-$
MW = 320	139	< 0.1	
	141	100.0	$(CH_3O)_2PSO^-$
	192	1.8	$[SC_6H_2Cl_3^- + O_2 - ClO^{\cdot}]$
	194	1.2	
	196	0.3	
	210	0.4	
	211	82.5	$SC_6H_2Cl_3^-$
	213	86.6	
	215	30.6	
	217	1.9	
	234	0.2	$[M - CH_3 - HCl_2]^-$
	270	0.1	$[M - CH_3 - Cl]^-$
	305	0.1	$[M - CH_3]^-$
Bromophos	77	< 0.1	
	79	100.0	$^{79}Br^-$ and PSO^-
	81	86.1	$^{81}Br^-$
	95	1.2	PSO_2^-
MW = 364	140	0.1	
	141	23.6	$(CH_3O)_2PSO^-$
	143	0.3	
	220	0.2	$SC_6H_3ClBr^-$
	221	< 0.1	
	254	< 0.1	
	255	14.2	$SC_6H_2Cl_2Br^-$
	257	25.5	
	259	13.3	
	261	1.2	
	270	2.1	$[M - CH_3 - Br]^-$
	271	1.5	
	272	0.9	
	273	0.4	
	351	< 0.1	$[M - CH_3]^-$
Dichlofenthion	63	2.4	$COCl^-$
	79	1.1	PSO^-
	93	1.3	
	95	100.0	PSO_2^-
MW = 314	97	4.2	
	124	0.7	$(C_2H_5O)PSO^-$
	157	0.8	
	158	17.3	$[SC_6H_2Cl_2^- + O_2 - CO^{\cdot}]$
	160	4.5	
	161	1.4	$OC_6H_2Cl_2^-$
	163	0.9	
	169	62.1	$(C_2H_5O)_2PSO^-$
	170	1.7	

TABLE III *(continued)*

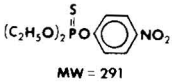
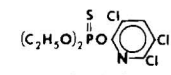
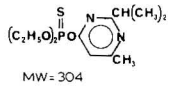
Compound	*Mass*	*Relative abundance (%)*	*Tentative identification*
	177	10.4	$SC_6H_3Cl_2^-$
	179	6.5	
	250	1.7	$[M - C_2H_5 - Cl]^-$
	251	13.6	$[M - COCl]^-$
	253	6.6	
	278	1.7	$[M - HCl]^-$
	280	0.5	
	285	4.2	$[M - C_2H_5]^-$
	287	3.4	
Ethyl parathion	138	5.0	$OC_6H_4NO_2^-$
	154	100.0	$SC_6H_4NO_2^-$
	155	7.7	
	156	4.0	
MW = 291	157	<0.1	
	169	16.1	$(C_2H_5O)_2PSO^-$
	170	0.5	
	171	0.4	
	262	<0.1	$[M - (C_2H_5)]^-$
	290	0.5	$[M - 1]^-$
Chlorpyrifos	79	1.9	PSO^-
	95	62.1	PSO_2^-
	96	2.2	
MW = 349	97	1.4	
	124	0.6	$(C_2H_5O)PSO^-$
	141	2.2	
	161	0.8	
	169	100.0	$(C_2H_5O)_2PSO^-$
	170	3.2	
	171	3.8	
	189	1.3	
	191	0.9	
	193	3.4	$[SC_5NHCl_3 + O_2 - ClO]^-$
	195	2.2	
	196	0.8	
	198	0.8	
	211	1.1	
	212	57.7	$SC_5NHCl_3^-$
	214	56.8	
	216	19.3	
	217	1.5	
	218	1.5	
	288	0.6	
	313	1.0	$[M - HCl]^-$
	315	1.4	
	316	1.4	
Diazinon	151	0.4	$[M - (C_2H_5O)_2PO]^-$
	168	0.5	
	169	100.0	$(C_2H_5O)_2PSO^-$
	170	3.5	
MW = 304	171	2.8	

TABLE IV

LC–NCI MASS SPECTRA OF PHOSPHORODITHIOATE PESTICIDES

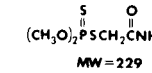
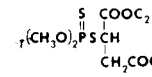
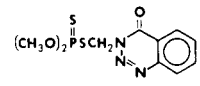
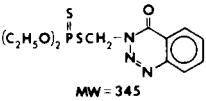
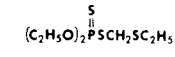
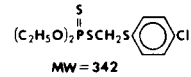
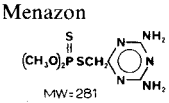
Compound	*Mass*	*Relative abundance (%)*	*Tentative identification*
Dimethoate	104	<0.1	$[M - ((CH_3O)_2PS)]^-$
	141	6.3	$(CH_3O)_2PSO^-$
	142	0.8	
MW = 229	143	0.1	
	155	0.1	
	157	100.0	$(CH_3O)_2PS_2^-$
	159	8.8	
	161	<0.1	
Malathion	110	<0.1	$(CH_3O)PSO^-$
	125	0.3	$(CH_3O)_2PS^-$
	129	0.1	
	142	0.5	
MW = 330	157	100.0	$(CH_3O)_2PS_2^-$
	159	9.1	
	161	<0.1	
	203	<0.1	
Azinphos-methyl	120	0.1	
	125	0.2	$(CH_3O)_2PS^-$
	132	0.6	$CH_2N_3C_6H_4^-$ or $CHNCOC_6H_4^-$
	134	<0.1	
MW = 317	142	0.8	
	146	0.1	$N_3COC_6H_4^-$
	157	100.0	$(CH_3O)_2PS_2^-$
	159	10.1	
	164	0.8	$[M - (CH_3O)_2PS - N_2]^-$ or $[M - (CH_3O)_2PS - CO]^-$
Azinphos-ethyl	95	0.2	PSO^- and PS_2^-
	111	0.1	
	120	0.1	
	132	0.6	$CH_2N_3C_6H_4^-$ or $CHNCOC_6H_4^-$
	134	0.5	
MW = 345	146	0.1	$N_3COC_6H_4^-$
	152	0.1	$(C_2H_5O)_2PS^-$
	154	0.1	
	164	1.1	$[M - (C_2H_5O)_2PS - N_2]^-$ or $[M - (C_2H_5O)_2PS - CO]^-$
	169	0.1	$(C_2H_5O)_2PO^-$
	185	100.0	$(C_2H_5O)_2PS_2^-$
	187	10.4	
	189	<0.1	
Phorate	61	0.8	$SC_2H_5^-$
	79	0.5	PSO^-
	95	3.0	PSO_2^- and PS_2^-
MW = 260	112	<0.1	
	124	0.1	
	153	3.0	$(C_2H_5O)_2PS^-$
	157	<0.1	
	184	0.1	
	185	100.0	$(C_2H_5O)_2PS_2^-$
	187	9.7	

TABLE IV *(continued)*

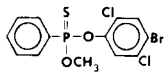
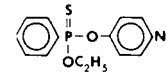
Compound	*Mass*	*Relative abundance (%)*	*Tentative identification*
Carbophenthion	95	0.1	PS_2^-
	112	0.1	
	143	100.0	$SC_6H_4Cl^-$
	145	37.4	
MW = 342	147	0.5	
	153	0.4	$(C_2H_5O)_2PS^-$
	183	0.2	
	185	82.3	$(C_2H_5O)_2PS_2^-$
	187	7.0	
Menazon	157	100.0	$(CH_3O)_2PS_2^-$
	158	4.6	
	159	3.9	
MW=281	280	0.4	$[M - 1]^-$

Phosphorodithioates

Characteristic ions for this class of compounds are $(RO)_2PS_2^-$, giving intense peaks at *m/z* 157 (when R = CH_3) and *m/z* 185 (when R = C_2H_5) (Table IV). In the

TABLE V

LC–NCI MASS SPECTRA OF PHENYLPHOSPHONOTHIOATE PESTICIDES

Compound	*Mass*	*Relative abundance, %*	*Tentative identification*
Leptophos	78	0.2	
	79	100.0	$^{79}Br^-$ and PSO^-
	81	81.8	$^{81}Br^-$
MW = 410	187	6.1	$C_6H_5PS_2OOCH_3^-$
	238	0.3	
	239	21.3	
	241	40.2	$OC_6H_2Cl_2Br^-$
	243	14.6	
	245	1.2	
	255	4.1	
	257	7.5	$SC_6H_2Cl_2Br^-$
	258	0.1	
	259	2.8	
EPN	122	1.6	$C_6H_4NO_2^-$
	136	< 0.1	
	138	100.0	$OC_6H_4NO_2^-$
	140	3.3	
MW - 323	141	0.2	
	153	0.1	
	154	19.1	
	155	1.1	$SC_6H_4NO_2^-$
	156	0.6	
	201	4.3	$C_6H_5PSOOC_2H_5^-$
	203	0.2	
	204	< 0.1	
	322	0.1	$[M - 1]^-$

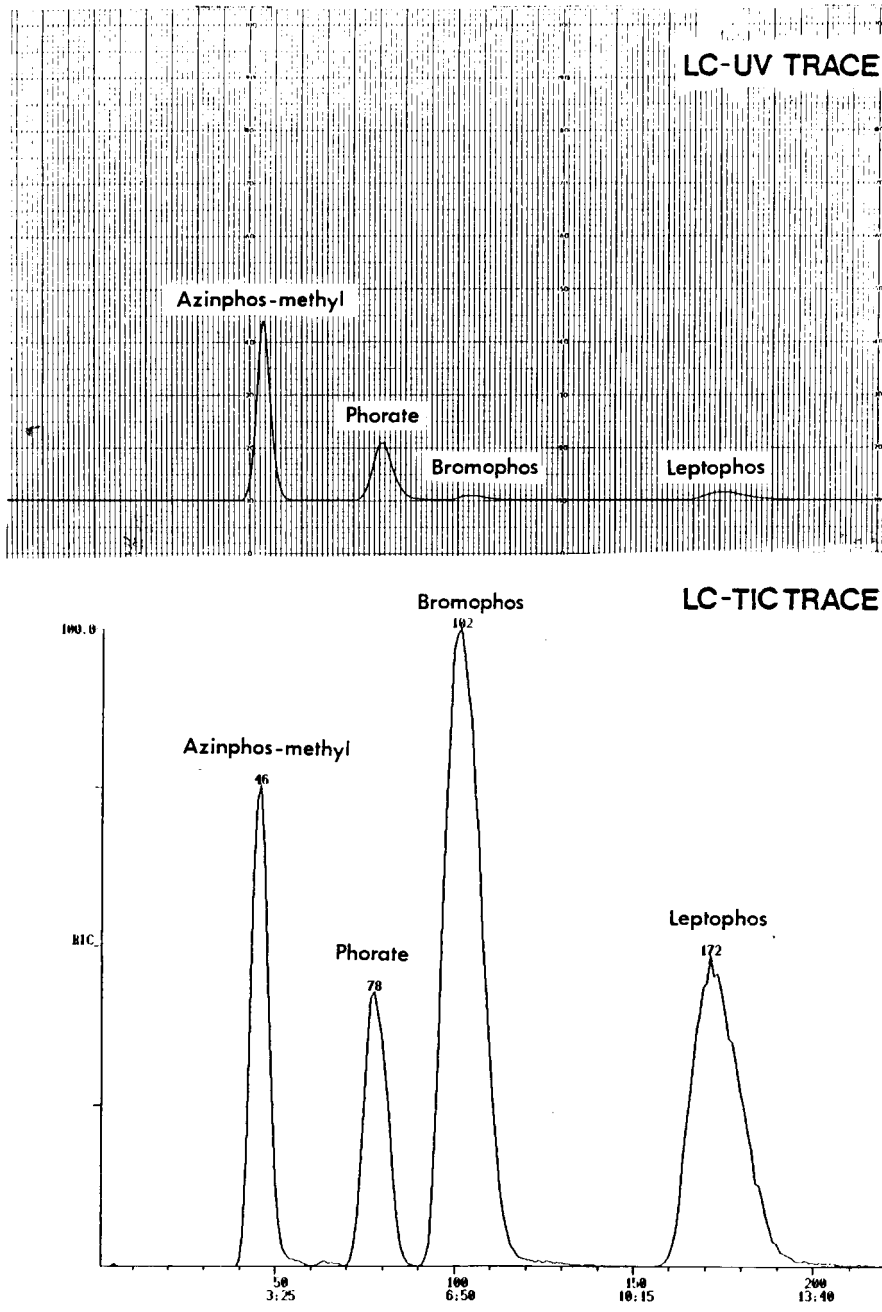


Fig. 3. LC–UV and LC–TIC traces for a mixture of bromophos, azinphos-methyl, phorate and leptophos.

aromatic phosphorothioates, some of the ion current is also carried by the aromatic portion of the molecule, for example, $SC_6H_4Cl^-$ is the base peak for carbophenthion, and there is a trace of $N_3COC_6H_4$ at m/z 146 in azinphos-ethyl and traces of

$CH_2N_3C_6H_4^-$ at *m/z* 132 in both azinphos-methyl and azinphos-ethyl. A peak at *m/z* 132 has been reported in the electron-impact mass spectrum of these compounds and has been ascribed to the loss of CO from the $CH_2N_3COC_6H_4^+$ ions[15]. Another rearrangement ion in azinphos-ethyl, at *m/z* 164, probably corresponds to $[M - (C_2H_5O)_2PS - N_2]^-$ or $[M - (C_2H_5O)_2PSCO]^-$ to give $SCH_2N_3C_6H_4^-$.

Other small but commonly occurring fragments include $[(RO)_2PS^-]$ at *m/z* 125 and *m/z* 152 for R = CH_3 and R = C_2H_5, respectively. This fragment is found in all of the dithioates studied with the exception of dimethoate and menazon. The $(CH_3O)_2PSO^-$ ion, apparently the result of oxygen/sulfur migration, gives a small peak at *m/z* 141 in dimethoate, and the corresponding $(CH_3CH_2O)_2PSO^-$ ion appears as a small peak at *m/z* 169 in azinphos-ethyl.

Other fragments include *m/z* 79 and *m/z* 95, corresponding to PSO^- (ref. 31) and a combination of PSO_2^- and PS_2^- (ref. 32), respectively.

Phenylphosphonothioates

As can be seen in Table V, diagnostic ions for this class of compounds are *m/z* 187 and *m/z* 201, corresponding to $C_6H_5P(OR)SO^-$, where R = CH_3 and R = C_2H_5, respectively. Peaks are also observed for the phenoxide ions from both compounds, $OC_6H_4NO_2^-$ at *m/z* 138 from EPN and $OC_6H_2Cl_2Br$ at *m/z* 239 from leptophos. The corresponding thiophenate-S-migration ions are also observed, at *m/z* 154 and *m/z* 255, respectively. Peaks at *m/z* 79 and 81 in leptophos are probably due to $^{79}Br^-$ and PSO^-, and $^{81}Br^-$, respectively. A small $(M - 1)^-$ ion is observed for EPN.

HPLC chromatograms resulting from UV and MS detection

LC–UV chromatograms and the corresponding LC–total ion current (TIC) traces for two mixtures of organophosphorus pesticides are shown in Figs. 3 and 5; Figs. 4 and 6 show the mass spectra obtained from these runs. Approximately 10–20 μg of each component were injected on to the LC column, which means that approximately 100–200 ng of each component entered the mass spectrometer ion source. Subsequent runs on these mixtures were made to determine approximate mass spectral detection limits. At levels of 100–200 ng injected onto the LC column (1–2 ng into the source), the major fragment ions could be detected in the reconstructed ion chromatograms, whereas no HPLC peak could be detected in the TIC trace. Thus, since at least a 10-fold increase in sensitivity would be expected by the use of single-ion monitoring, LC–MS sensitivities should be the same as those reported for MENI, which is 25–100 pg into the mass spectrometer source[18]. Since, obviously, the mass spectrometric detection limits are determined by the amount of material entering the source, the 99:1 split ratio would mean that about 100 times as much material must be injected on to the LC column. The use of a micro LC probe[25,33–35], where all of the HPLC effluent enters the mass spectrometer source and the need for a 99:1 split is eliminated, would be desirable for trace analysis.

Applicability to analysis of water samples

Oisuki and Takaku[9] reported a procedure for the analysis of Abate in water samples in which the water sample was pumped through the LC column, thus preconcentrating the pesticide at the front end of the column. The solvent was then programmed from 100% water to 100% acetonitrile, and a peak corresponding to the pesticide was observed in the UV trace.

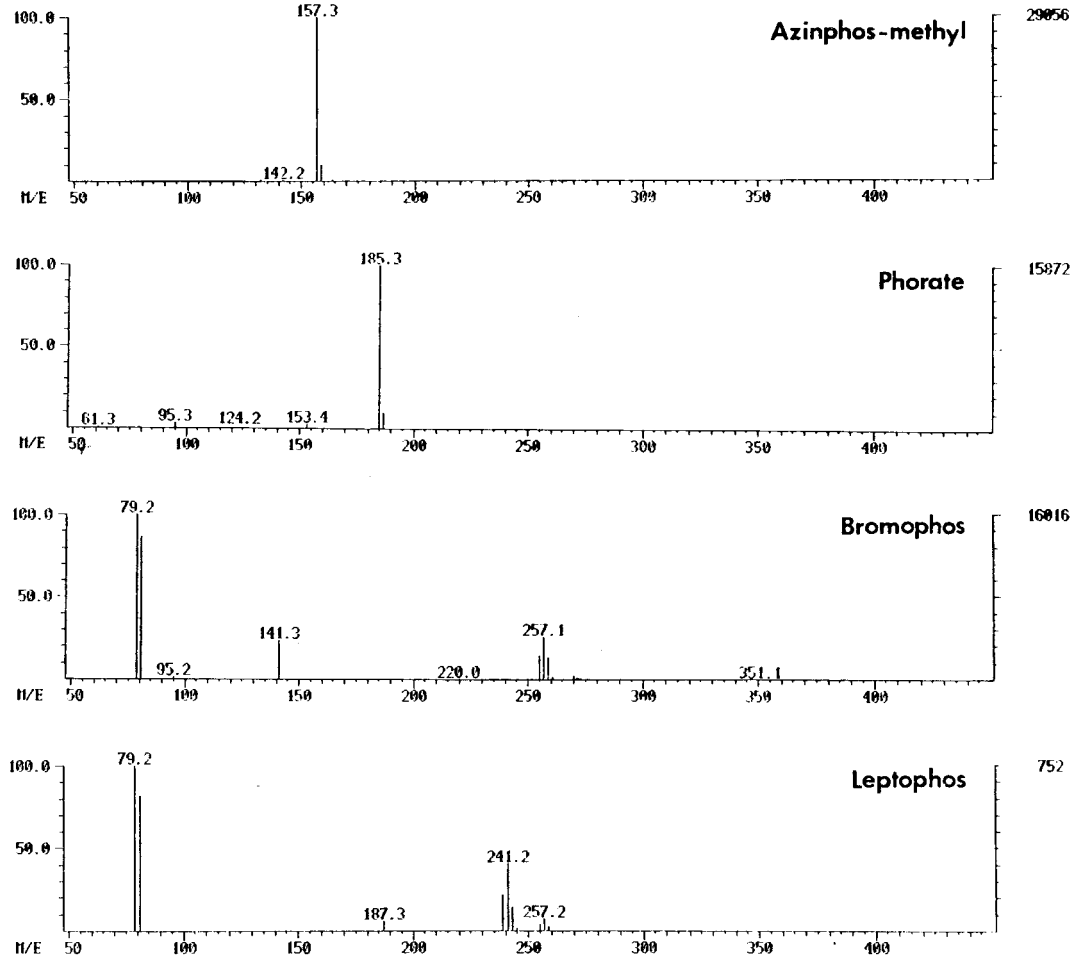


Fig. 4. LC–NCI mass spectra of bromophos, azinphos-methyl, phorate and leptophos.

In the analysis shown in Fig. 7, this idea was extended for use with mixtures of pesticides. The procedure used was to pump through the column 50 ml of water containing *ca.* 100 ng/ml each of monocrotophos, bromophos, leptophos, azinphos-methyl and phorate, followed by another 50 ml of water. The solvent programmer was then stepped to acetonitrile–water (60:40). A slight shift in relative retention times was observed, probably due to the column not having completely equilibrated to the change in solvent, but good LC peak shape and resolution were observed, indicating that little band spreading occurred during the pre-concentration step. There was no change in elution order, and no change in the mass spectra was observed since the solvent mixture, which becomes the mass spectrometer "reagent gas" remains in the same 60:40 ratio.

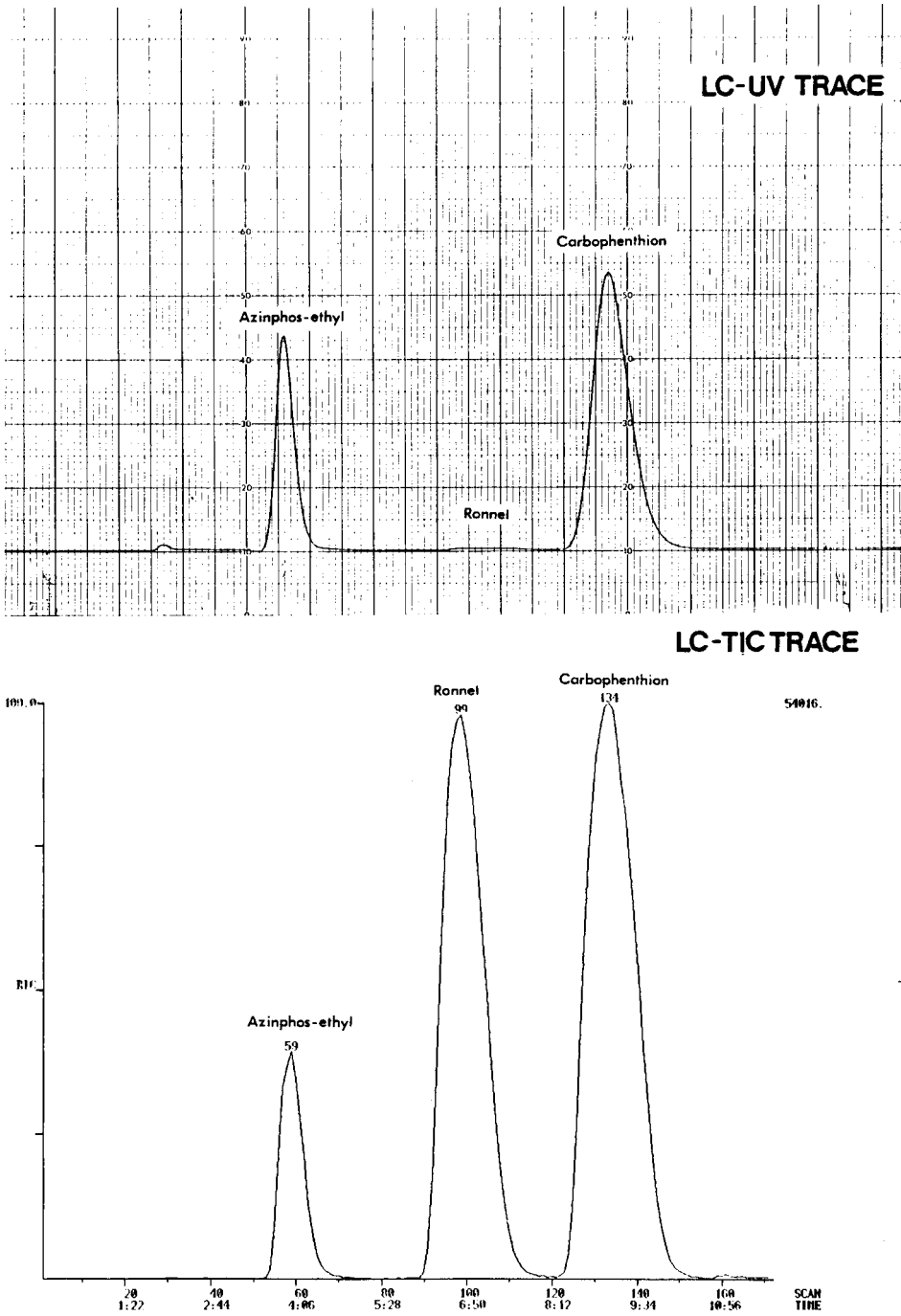


Fig. 5. LC–UV and LC–TIC traces for a mixture of ronnel, carbophenthion and azinphos-ethyl.

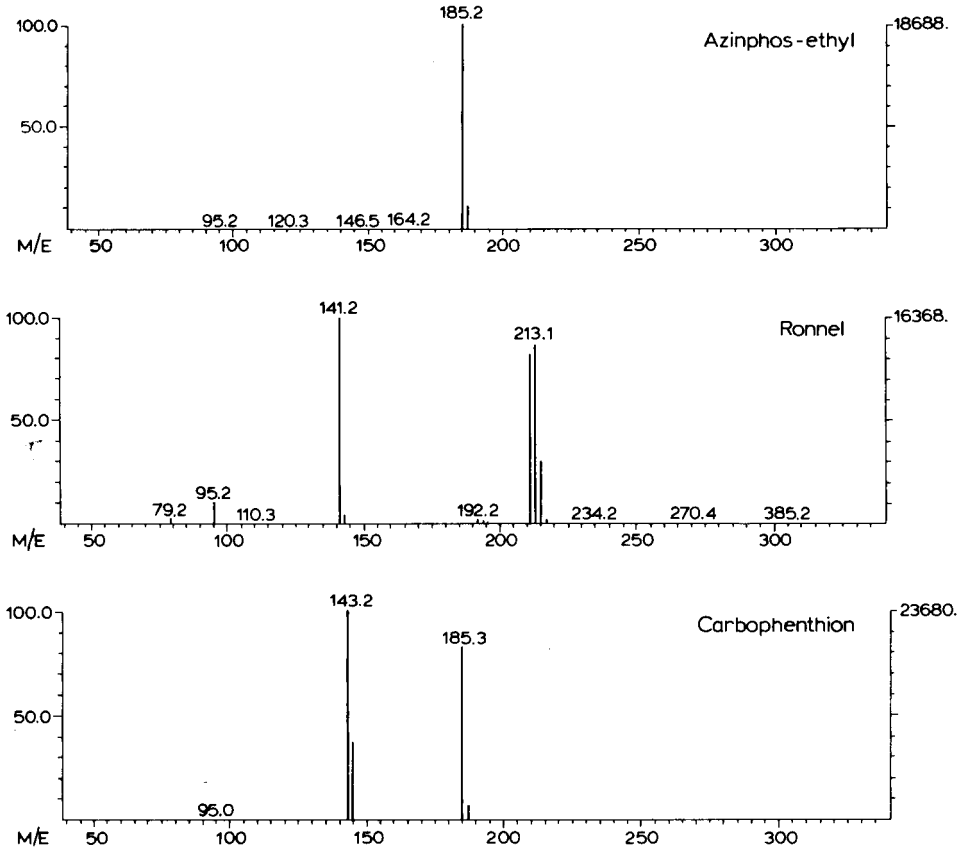


Fig. 6. LC–NCI mass spectra for ronnel, carbophenthion and azinphos-ethyl.

CONCLUSIONS

The technique of combined LC–NCI-MS shows promise for the analysis of organophosphorus pesticides. Combined with suitable extraction procedures, the MS sensitivities should make this technique applicable for residue analysis and the analysis of environmental samples. Since comparison of LC–MS results with those obtained by GC–MS shows that thermal degradation occurred during GC–MS separation, the main advantage of LC–MS over GC–MS would be for analysis of the possibly more polar or thermally labile metabolites of these pesticides. It should also be especially useful in the analysis of water samples, where the HPLC column can be used to pre-concentrate the pesticides in addition to providing the separation for the analysis. By combining LC retention-time data with MS fragmentation reaction data LC–NCI-MS provides a convenient and specific method for organophosphorus pesticides. In addition, it appears that negative ion spectra obtained with methane as reagent gas are sufficiently similar to those obtained with acetonitrile–water that existing information on MENI sensitivities and library spectra may be used.

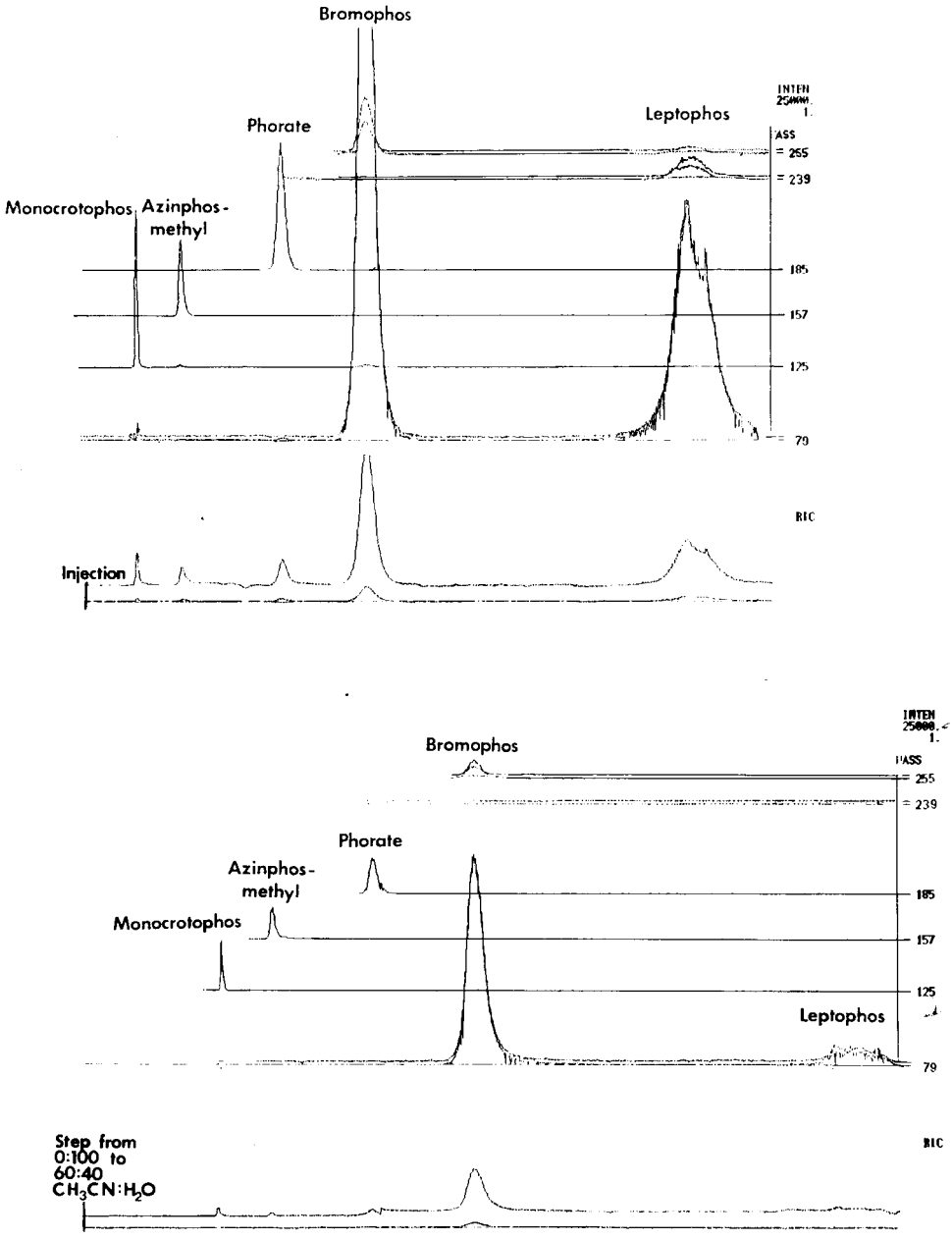


Fig. 7. Reconstructed Ion Chromatogram traces for a mixture of monocrotophos, bromophos, azinphos-methyl, phorate and leptophos under isocratic and "stepped" conditions.

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IDENTIFICATION BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF VINYL CHLORIDE OLIGOMERS AND OTHER LOW-MOLECULAR-WEIGHT COMPONENTS IN POLY(VINYL CHLORIDE) RESINS FOR FOOD PACKAGING APPLICATIONS

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SUMMARY

Material of molecular weight less than approximately 400–500 was isolated from food grade poly(vinyl chloride) resins by initial soxhlet diethyl ether extraction and subsequent size-exclusion fractionation. Analysis by packed-column gas chromatography using Hall electrolytic conductivity detection showed the presence of a series of chlorinated components which by subsequent gas chromatography–mass spectrometry were tentatively identified as vinyl chloride oligomers. The chlorinated constituents in the extracts were selectively isolated from other low-molecular-weight components by silica gel chromatography. From low-resolution capillary gas chromatography–mass spectrometry data (together with evidence from hydrogenated extracts) the structures of these oligomers were postulated as being a complete series ranging from trimer to hexamer (and probably to the octamer), each oligomer being represented by species containing a ring or a double bond and each occurring as a number of structural isomers. Other non-chlorinated compounds identified in the resins included mixed phthalates, alkanes, nonylphenol, and undecyl dodecanoate, the latter being derived from the polymerisation initiator lauryl peroxide.

INTRODUCTION

There have been many studies on the oligomers of styrene both because of their widespread use as calibration standards in size-exclusion chromatography (SEC)[1,2] and because they enable an insight into the mechanisms of polymer initiation and propagation reactions[3–5]. Poly(ethylene terephthalate) (PET) oligomers have also been characterised and have been shown to have undesirable effects in certain technical applications[6,7]. Despite the extent of analytical data available both on the separation of these oligomers from one another[8] and from the polymer[9], and on their structural elucidation[10,11], it is only recently that there has been any interest in them as species capable of migration from plastics into foods[12].

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In marked contrast there has been no published work whatever on the isolation and identification of oligomers from vinyl chloride (VC) polymers, although VC monomer itself has received considerable attention both in terms of residual levels in the plastic[13,14] and as a consequent contaminant in foods[15,16]. In the course of our research programme on plastic packaging materials for food, we have taken a particular interest in the low-molecular-weight components present in poly(vinyl chloride) (PVC) resins, as these represent a considerable potential source for migration. We have previously reported the occurrence of 1,1,1-trichloroethane in PVC resins and bottles used for food packaging[17,18], and more recently have determined on the basis of molecular weight fractionation and micro chlorine estimations, the overall minimum concentrations of VC oligomers in food grade PVC resins[19]. These results did not distinguish between individual oligomer species and it was originally anticipated that the analytical difficulties (particularly thermal instability) which apply to chlorinated paraffins[20,21] would by analogy cause problems in the separation and identification of VC oligomers. However using glass capillary columns and an all-glass coupling line to the mass spectrometer it has now proved possible both to separate and tentatively assign structural identification to the individual oligomers below molecular weight *ca.* 500 which were found to range from trimers to hexamers. Other evidence indicated the presence of heptamers and octamers. In this paper we report this gas chromatography–mass spectrometry (GC–MS) evidence for the occurrence of these previously unidentified species in PVC resins, together with the silica gel column chromatographic "clean-up" of the extracts, and the GC–MS results for the hydrogenated material. Further evidence from chemical ionisation MS and accurate mass measurements (from GC–MS) substantiated the initially postulated oligomer structures.

EXPERIMENTAL

Materials

Six PVC resins (designated A to F) were obtained from a number of European polymer manufacturers as being representative of a wide range of the types of materials used for PVC food packaging. Tris(nonylphenyl) phosphite (TNPP, "Phosclere P315") was obtained from Akzo Chemie (Liverpool, Great Britain), *p*-nonylphenol from Pfaltz and Bauer (Stamford, CT, U.S.A.) and undecyl dodecanoate was synthesized in the laboratory by sulphuric acid catalysed esterification of *n*-dodecanoic acid with *n*-undecanol.

Methods

Solvent extraction of base polymer. Base polymer (20 g) contained in preextracted thimbles was soxhlet extracted with re-distilled diethyl ether for 16 h. After solvent removal on a rotary evaporator and vacuum drying at 40°C the weighed residues were stored at −15° until required.

Size-exclusion chromatography. "Sephadex LH-60" columns were prepared and used as previously described[22]. Column lengths were 33 ± 1 cm and two fractions were routinely collected, standardised at 0–90 ml and 90–190 ml, containing respectively high-molecular-weight and low-molecular-weight material. Total ether extracts were fractionated in 160–180 mg aliquots dissolved in redistilled tetrahydro-

furan (THF) (2 ml), the collected fractions being weighed after solvent removal by blowing down under nitrogen.

"Bio-Beads S-X3" gels were prepared by a slurry technique in THF using Pharmacia SR25/45 columns to give a gel bed of 32 ± 2.5 cm. Aliquots (up to 250 mg) of the low-molecular-weight fraction from the Sephadex column in THF (2.0 ml) were subjected to chromatography in the normal manner[22]. Elution profiles were generated by collecting 2.5-ml fractions (Gilson Microcol TDC 80 fraction collector) in pre-conditioned, pre-weighed 10-ml tubes. After blowing to dryness under nitrogen, the tubes were heated for 30 min at 40°C in a vacuum oven, allowed to re-condition for 2 h and re-weighed.

Fractions intended for subsequent GC analysis were simply blown to dryness under nitrogen without further heating. From calibration of the column the fraction (53–90 ml) nominally containing material of molecular weight 0 to 500 was routinely collected (designated B3 fraction).

Elemental analysis. Elemental microanalysis for chlorine was performed by Butterworth Labs. (Teddington, Great Britain). All samples collected from SEC columns were heated at 40°C in a vacuum oven to constant weight and amounts of not less than 10 mg per determination were sent for analysis.

Purification of oligomer fractions

Silica gel column chromatographic purification of B3 oligomer fractions was carried out using a 10 × 1 cm I.D. bed of silica gel (Merck 7734, 70–230 mesh) packed in *n*-hexane. After the sample (up to 0.08 g B3 in 2 ml hexane) was loaded onto the column, it was washed with *n*-hexane (100 ml) to remove alkanes and the oligomers eluted with toluene (50 ml). The solvent was removed on a rotary evaporator under vacuum at 60°C prior to GC analysis.

Hydrogenation of oligomer fractions

The purified oligomer fraction B3 (up to 0.1 g) dissolved in dry methanol (20 ml) was shaken for 5 min with palladium chloride (0.01 g) under hydrogen (1.5 atm) at ambient temperature. After filtration of the supernatant through a 0.45-μm PTFE filter (Millipore), the methanol was removed on a rotary evaporator under vacuum and the sample blown to dryness under nitrogen prior to GC analysis.

The hydrogenation procedure was tested to show complete saturation of double bonds by converting 2,4-hexadiene to hexane, but was shown to be sufficiently mild to avoid displacing chlorine by leaving dichlorononane unchanged after an identical treatment.

Gas chromatography

Packed-column chromatography was performed with a Pye Series 104 chromatograph using the following columns and conditions:

(a) 2.7 m × 2 mm I.D. glass column packed with 3% OV-1 on Diatomite CLQ (100–120 mesh). Nitrogen carrier gas was used at 25 ml/min. On-column injections were made with the column oven held at 130°C for 2 min and then programmed at 8°/min to 300°C.

(b) 1.5 m × 2 mm I.D. glass column packed with 3% Dexsil 300 on Supelcoport (100–120 mesh). Nitrogen carrier gas was used at 25 ml/min. On-column

injections were made with the column oven at 150°C, immediately programmed at 8°/min to 400°C.

The effluent was split between a flame ionisation detector (FID) (30%) operated at 350° (OV-1) or 450°C (Dexsil 300), and a Tracor model 700 Hall electrolytic conductivity detector (70%) with an interface temperature of 350° (OV-1) or 450°C (Dexsil 300); furnace temperature 850°C; hydrogen make-up gas flow 50 ml/min; and isopropanol–water flow-rate 0.8 ml/min. The conductivity setting was × 100.

Capillary column chromatography used a Carlo Erba model 4160 chromatograph fitted with a 20 m × 0.3 mm borosilicate glass column coated with 0.2-μm OV-101. The carrier gas velocity was 25 cm/sec measured at 200°C. Splitless injections using THF as solvent were made with the injector at 250°C. The split valve was opened to provide an injector purge 20 sec after injection. The column oven was held at 75° for 2 min and then programmed to 240°C at 5°/min. The FID was held at 270°C.

Gas chromatography–mass spectrometry

Capillary column GC–MS was performed with a Carlo Erba 4160 chromatograph operated as above, and connected to a VG7070H mass spectrometer via an all-glass direct coupling interface. The transfer line was held at 250°C. The MS was operated at a source temperature of 200°C with 70 eV electron energy and a 200 μA trap current. Nominal mass spectra were obtained at 1000 resolution (10% valley) scanning from m/z 500–25 at 0.7 sec/decade. Accurate masses were obtained using C_2I_4 as internal reference at 2000 resolution and 1.5 sec/decade. All spectra were processed with a VG2000 data system.

RESULTS AND DISCUSSION

Six different samples of PVC resins intended for food contact applications were obtained from a number of European manufacturers. Resins A, B and F were bottle blowing–rigid foil grades whilst resins C, D and E were designated as film grades. Average molecular weights of resins for films (M_n *ca.* $65 \cdot 10^3$) are generally substantially higher than those of resins for bottle blowing (M_n *ca.* $37–45 \cdot 10^3$). When each of the polymers was soxhlet extracted with diethyl ether and fractionated by SEC the total amount of material obtained with a molecular weight of less than 400–500 (*i.e.* B3 fractions) varied from 500 to 1150 mg/kg, as shown in Table I.

Using elemental chlorine data obtained on these fractions and assuming an empirical formula for all oligomers identical to that of vinyl chloride, it is possible to estimate oligomer concentrations as discussed in an earlier paper[19]. Data generated in this manner requires a number of other assumptions, not least that all the available chlorine is present as oligomers rather than as any alternative chlorinated organic compounds. Nevertheless, these results do indicate that PVC resins probably contain substantial amounts of low-molecular-weight oligomers.

When GC–FID analysis of the B3 fractions of the PVC resins was carried out using packed columns, complex chromatograms were obtained with significantly different profiles from one polymer to another. However using dual detection with the effluent split to a Hall electrolytic conductivity detector operated in a halogen specific mode, the Hall detector showed similar chromatograms for all the resins (Fig. 1).

TABLE I

ESTIMATED TOTAL CONCENTRATIONS OF MATERIAL IN LOW-MOLECULAR-WEIGHT FRACTIONS AND CONCENTRATIONS OF INDIVIDUAL IDENTIFIED COMPONENTS

Polymer	*Concentration (ppm) in base resin*					
	Total non-volatiles mol. wt. 400–500*	*Chlorine** containing mol. wt. 400–500*	*C_{22} Hydrocarbon*** n-docosane*	*Undecyl-dodecanoate****	*Nonyl-phenol****	*Mixed phthalates****
A	500	110	30–40	15–20	200	10
B	840	240	30–40	15–20	<1	<1
C	680	160	<1	<1	<1	40
D	720	250	70	20	<1	<1
E	510	180	<1	<1	<1	<1
F	1150	350	110	30	<1	<1

* Estimated gravimetrically from SEC fraction (B3).
** Estimated by micro-chlorine assuming oligomers of PVC empirical formula.
*** Estimated by gas chromatography using response factor of standard compounds.

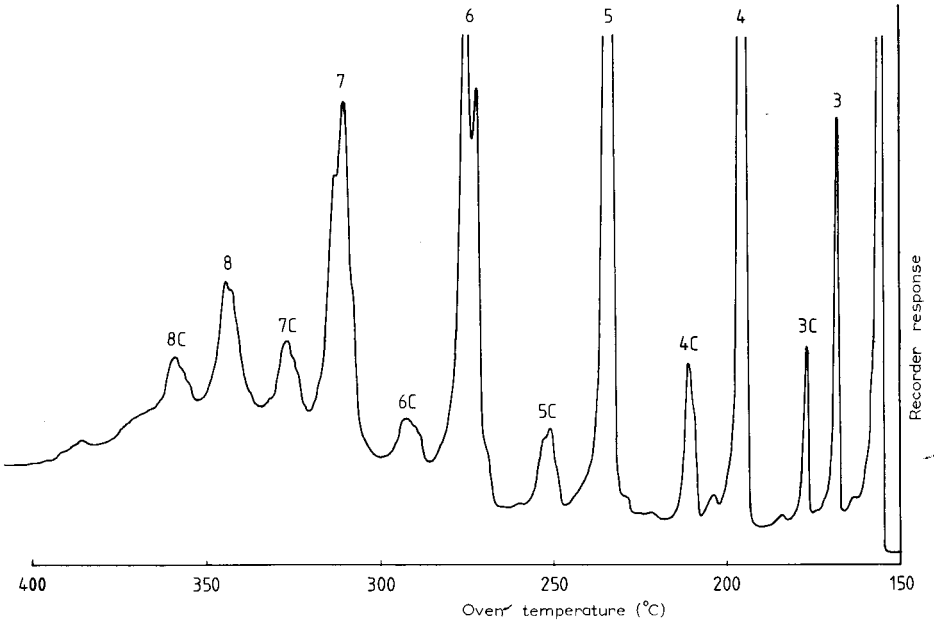


Fig. 1. Hall electrolytic conductivity chromatogram of a B3 fraction from resin A. Dexsil column. For other conditions, see *Methods* section.

Hence the B3 extracts contain essentially the same chlorinated compounds in very similar amounts, but differ from one another only in the presence of other low-molecular-weight non-halogenated components. Glass capillary column GC–MS analysis of the B3 fractions led to the identification of a number of these non-halogenated components, although several still remain unidentified. Alkanes in the range C_{13}–C_{22} at low levels occur widely and in four of the six resins C_{22} is present in

significant amounts (see Table I). Undecyl dodecanoate was unequivocably identified both by comparison of its mass spectrum with the authentic synthesized compound and by agreement of retention indices, and was found in significant amounts in the same resins containing the C_{22} alkane. Both these compounds can be formed by decarboxylation of lauryl peroxide[23–25] and are probably derived from the initiator either during polymerisation or subsequently. A characteristic group of isomeric compounds identified as nonylphenols were found in resin A. These are probably derived from the use of tris(nonylphenyl) phosphite as a pre-stabiliser during polymer drying. Similar but unidentified phenolic mixtures found in resins B and F are thought to be derived from 2,6-di-*tert.*-butyl-*p*-cresol (BHT) or other related phenolic antioxidants. In two of the resins (A and C) complex mixtures of C_8 phthalates were identified and it is possible that these derived from their use as solvents, for example for incorporation of minor additives or in some other aspect of the manufacturing process.

Although good mass spectral evidence is presented here for the establishment of the empirical formulae of the VC oligomers, it has not yet proved possible to make a complete structural elucidation. For clarity, the oligomers referred to in this paper have been numbered using the following system. Oligomers 3, 4, *n* are respectively trimer, tetramer and *n*-mer. This corresponds to $(CH_2CHCl)_n$ *i.e.* vinyl chloride oligomers containing one double bond. A suffix letter C refers to a second oligomer series but in this case cyclic. Thus 3C designates a trimer of the same empirical formula as 3 but where the hydrogen deficiency is thought to be due to a cyclic structure.

The molecular weights, retention indices, empirical formulae (and number of isomers observed by capillary GC–MS) for the oligomers in PVC resin A are given in Table II. A reconstructed total ion chromatogram of a B3 extract from resin C is shown in Fig. 2 in which peaks due to oligomers are marked by arrows. Because of a

TABLE II

PHYSICAL DATA FOR VINYL CHLORIDE OLIGOMERS FROM PVC RESIN A

Oligomer	*Number of isomers observed by capillary GC–MS*	*Empirical formula*	*Mol. wt. (based on ^{35}Cl)*	*Retention index range**
3	1	$C_6H_9Cl_3$	186**	1250
3C	1	$C_6H_9Cl_3$	186**	1360
4	3	$C_8H_{12}Cl_4$	248**	1600–1620
4C	2	$C_8H_{12}Cl_4$	248**	1770
5	4	$C_{10}H_{15}Cl_5$	310	1990–2010
5C	4	$C_{10}H_{15}Cl_5$	310	2140–2170
6	2	$C_{12}H_{18}Cl_6$	372	2350–2410
6C	–	$C_{12}H_{18}Cl_6$	372	2500–2520
7	–	$C_{14}H_{21}Cl_7$	434	*ca.* 2740
7C	–	$C_{14}H_{21}Cl_7$	434	*ca.* 2920
8	–	$C_{16}H_{24}Cl_8$	496	*ca.* 3090
8C	–	$C_{16}H_{24}Cl_8$	496	–

* Retention indices were generated by co-chromatography of B3 fractions with a series of *n*-alkanes.

** Molecular ion observed on mass spectra.

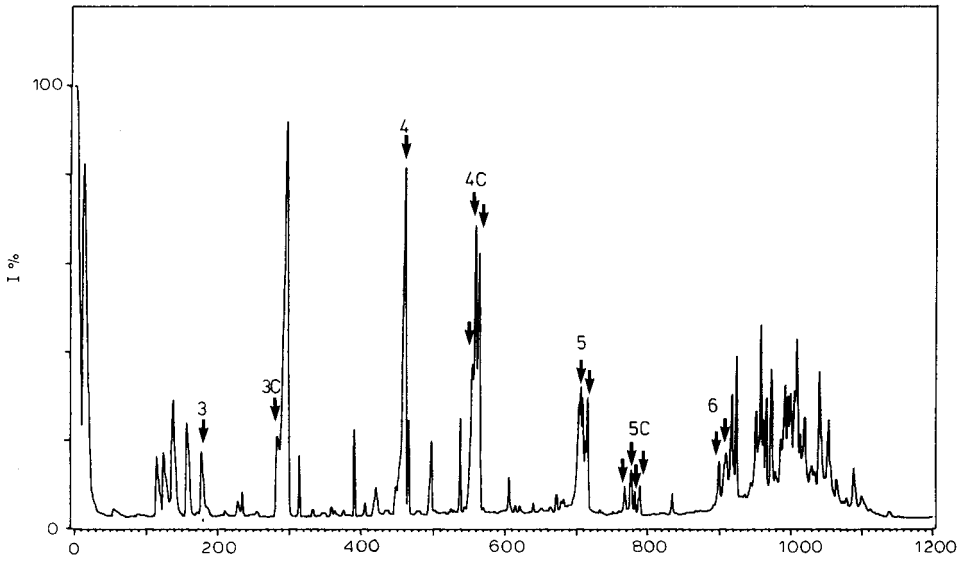


Fig. 2. Reconstructed total-ion capillary GC–MS chromatogram of a B3 fraction from resin C. Isomeric clusters of oligomers are indicated.

temperature limit of 250°C on the GC–MS interface we were unable to obtain spectra for oligomers eluting after the hexamer. In future, however, with improved heating to the GC–MS transfer line, this restriction will not apply.

Some typical oligomer mass spectra are shown in Fig. 3 and the MS evidence of identification is summarised in Table III which shows the assignment of the characteristic fragment ions for the oligomers, except for 6, where the spectra were both mixed and of low absolute intensity. Measurements of "accurate" mass gave good agreement ($\pm$ 5 m.m.u.) with calculated values for most of the more intense high mass fragments. At lower masses the technique was found to be unreliable by analysis of reference compounds giving known fragments.

Molecular ions were either not observed or were of low relative intensity (1–6%). Chemical ionisation (CI) using either isobutane or ammonia failed to give any enhancement, either of oligomers or standard chloroalkanes. Negative-ion CI gave only chlorine ions of great intensity at m/z 35 and 37. This behaviour is consistent with the interpretation that the oligomers are chlorinated aliphatic alkanes as typified by dichlorononane. Standard chloroalkenes could not be obtained.

The most striking features of the oligomer spectra are the clusters of ions due to ^{35}Cl–^{37}Cl combinations, in some instances further complicated by overlapping patterns caused by losses of either Cl or HCl from the same precursor ion. For example, for the tetramer (mol. wt. 248) two fragments at 212 and 213 are produced and these give rise to isotope peaks at 214, 216 and 218 and also 215, 217 and 219 with the overall effect of producing a characteristic cluster of ions at every mass from 212 to 219. It was observed that further fragmentation of such cluster by loss of Cl or HCl frequently occurs in such a fashion that the primary products were due to M $-$ HCl_2 with only a small contribution from M $-$ H_2Cl_2.

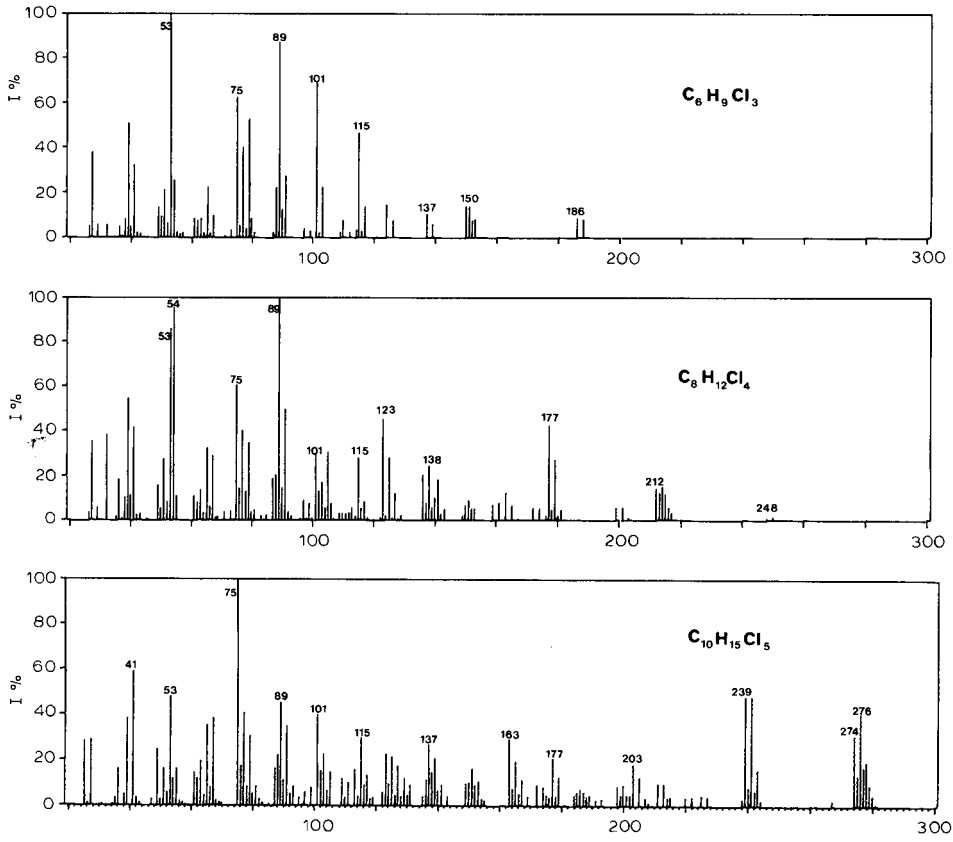
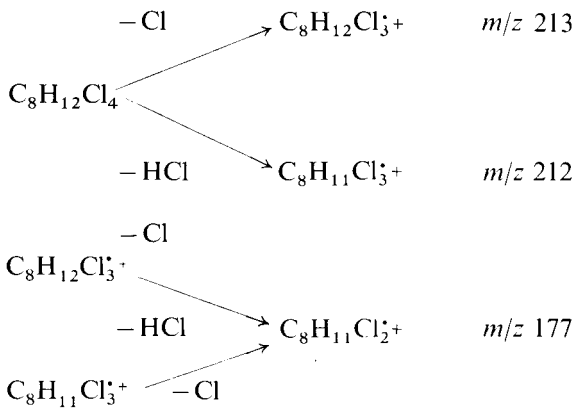


Fig. 3. Typical mass spectra for oligomers 3 ($C_6H_9Cl_3$), 4 ($C_8H_{12}Cl_4$) and 5C ($C_{10}H_{15}Cl_5$).



Several regions of the chromatogram showed partially resolved groups of peaks where the spectra of the individual member peaks were virtually indistinguishable (Fig. 2). It is likely that these groups are due to isomeric families of oligomers arising

TABLE III

CHARACTERISTIC IONS FROM 70 eV FRAGMENTATION OF VINYL CHLORIDE OLIGOMERS

m/z	*Assignment**	*VC oligomers % intensity*					
		3	*3C*	*4*	*4C*	*5*	*5C*
53	C_4H_5	100	34	75	40	35	55
54	C_4H_6	32	5	100	8	10	8
75	$C_3H_4\ ^{35}Cl$	95	100	61	100	61	100
89	$C_3H_6\ ^{35}Cl$	73	48	84	41	29	49
91	–	24	15	31	18	100	37
101	$C_5H_6\ ^{35}Cl$	61	53	33	36	32	46
115	$C_6H_8\ ^{35}Cl$	32	75	28	35	17	29
123	–	0	16	49	18	29	29
127	–	0	3	10	8	62	22
137	$C_5H_7\ ^{35}Cl_2$	11	74	10	27	21	29
150	$C_6H_8\ ^{35}Cl_2$	10	55	6	7	0	15
163	–	0	0	12	28	55	31
177	–	0	0	37	51	16	17
186	$C_6H_9\ ^{35}Cl_3$	(m^+) 6	(m^+) 6	1	2	0	12
199	–			6	0	22	0
212	$C_8H_{11}\ ^{35}Cl_3$			13	22	0	0
239	$C_{10}H_{14}\ ^{35}Cl_3$			0	0	19	42
248	$C_8H_{12}\ ^{35}Cl_4$			(m^+) 1	(m^+) 5	0	0
274	$C_{10}H_{14}\ ^{35}Cl_4$					13	44
275	$C_{10}H_{15}\ ^{35}Cl_4$					0	14

* In all cases ions from ^{35}Cl isotopes only are shown.

from different chlorine substituent position patterns since oligomers which differ in branching would give rise to member peaks more widely separated.

The components designated 3C and 4C give spectra similar to those of 3 and 4 but with some differences in the relative abundancies of certain fragment ions. For example, lower intensities were observed for *m/z* 53 and 54 for 3C and 4C compared with 3 and 4 and conversely, higher intensities for *m/z* 75. In addition the relative intensities of the M – Cl and M – HCl fragments were significantly higher for the C series oligomers. Molecular ions are not observed for either 5 or 5C. In the spectrum ascribed to 5C this was immediately apparent from the presence of overlapping 4 – Cl groups at *m/z* 274 and 275 and although the similar loss from 5 could not be so readily deduced from the pattern of the (less intense) group at *m/z* 274, strong similarities exist between this spectrum and that due to 4. The identification ascribed to 6 was generally consistent with this spectrum but the relative intensities of ions were rather unreliable because of the low absolute intensity of the spectrum and because of the subtractions required due to its elution with a phthalate ester as a partially mixed peak.

On the basis of mass spectral evidence empirical formulae could be obtained for oligomers 3, 4, 5 and 6 and also 3C, 4C and 5C. When the electrolytic conductivity chromatogram of a B3 fraction (Fig. 1) is inspected the regularity of the pattern of peaks immediately suggests the presence of two homologous series of oligomers.

Consequently the tentative assignations of oligomer structures 7 and 8 and also 6C, 7C and 8C have been associated with the later-eluting peaks of this chromatogram. The molecular weights of both 8 and 8C are 496 (all ^{35}Cl) and thus the lack of higher oligomers as evidenced in Fig. 1 is entirely consistent with the estimated molecular weight cut-off point for the B3 fraction of 400–500. It might be expected that considerably greater amounts of 8 and 8C would be found in the B2 fraction.

In further support of this identification of the higher oligomers, it was found that the non-cyclic oligomeric species were absent from a chromatogram of a hydrogenated purified B3 fraction (Fig. 4), being replaced by another, later-eluting, series of peaks of similar relative intensities. This makes it unlikely that the original peak spacings of the non-cyclic oligomer series were fortuitous. Further GC–MS studies with a high temperature interface should settle this point.

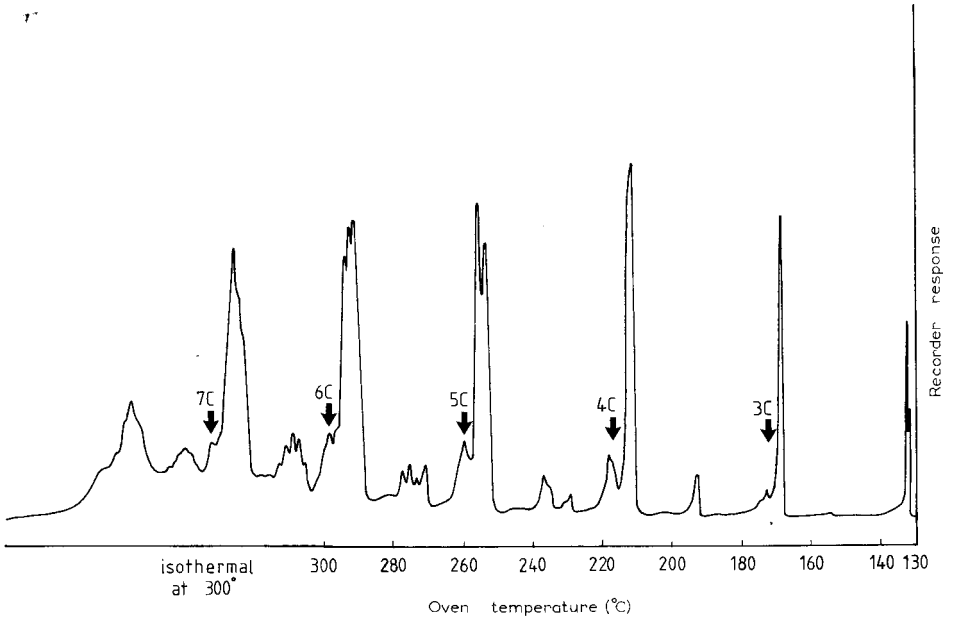


Fig. 4. Hall electrolytic conductivity chromatogram of a hydrogenated purified B3 fraction from resin A. OV-1 column. For other conditions, see *Methods* section.

The major evidence for cyclic structures for the C series also came from hydrogenation studies as 3C, 4C and 5C remained the only oligomers unchanged in both retention time and mass spectra. In the electrolytic conductivity chromatogram of a similar hydrogenated fraction (Fig. 4) peaks were found at the predicted retention times of all the series C oligomers.

Although the conditions of hydrogenation were carefully chosen and tested on a model chloroalkane to avoid any hydrogenolysis, hydrogenation of oligomer fractions led to significant changes in the non-cyclic oligomer series, as described above, which did not represent simple saturation of double bonds. While the products were still chlorine-containing, as shown by both the mass spectra and the electrolytic conductivity–GC traces, the spectra were not easily interpretable and it is possible

that rearrangement reactions did take place during hydrogenation. The apparent survival of intact 3C, 4C and 5C suggests otherwise but "scrambling" of the chlorine substituent patterns in these would not have been detected.

In this context it is interesting that Schwenk *et al.*[26] deduced that certain allylic chlorines were replaced by methoxy groups during hydrogenation of low-molecular-weight PVC at 0°C in methanol. Our experiments were carried out at an ambient temperature of around 20°C and thus a similar substitution can be expected to have occurred but even considering this possibility the mass spectra remained uninterpretable. Micro-hydrogenation of trapped GC peaks may be of help in elucidating the changes occurring on hydrogenation.

The origin of the compounds giving electrolytic conductivity peaks intermediate between the series of cyclic oligomer peaks and the next-eluting major peak (Fig. 4) is unknown but may be artifactual or alternatively derived from the hydrogenation of a non-volatile oligomer in the B3 fraction. Although the silica gel clean-up of this fraction was very effective, more polar oligomers might have been co-eluted, as was the case with undecyl dodecanoate.

One factor suggesting that other, more polar, oligomers were present in the B3 fraction is that the quantitative estimate of total oligomer concentrations (Table I) derived from micro-chlorine and gravimetric data[19] is significantly greater than the sum of the individual oligomer concentrations (Table IV). The latter concentrations were estimated by calibration of the electrolytic conductivity detector with a solution of dichlorononane in order to obtain a response factor per unit mass of chlorine. The numbers of chlorines per oligomer were then deduced; for 3–6 and 3C–5C from the mass spectra and for 7 and 8 and 6C–8C by inference. GC analysis of a known mass of B3 fraction completed the determination. The relative distribution of the different oligomers was very similar for all six resins examined. Because of the number of assumptions involved, the extent of experimental error is difficult to assess and is probably quite high but the difference between the two measures of total oligomers is so great that the presence of non-volatile oligomers in the B3 fraction seems likely. Literature reports[26–28] of end-groups detected in low-molecular-weight PVC by nuclear magnetic resonance (NMR) techniques include:

$$-CH_2-CHCl-CH = CH-CH_2\,Cl$$
$$-CHCl-CH_2-CH = CH-CH_2\,Cl$$
$$-CHCl-CH_2\,Cl$$
$$-CH_2-CH_2\,Cl$$

Polar end-groups have not been found to any appreciable extent. Thus some other source of such non-volatile species is required to explain these observations. This might involve artifacts or alternatively, for example, oligomers retaining initiator end groups.

To summarise, although good evidence is presented for the occurrence of a series of vinyl chloride oligomers at mg/kg levels in a number of different commercial PVC resins, further work still needs to be carried out to establish unequivocably the structures of these compounds. This work is necessary in order to assess whether vinyl chloride oligomers represent potential migratory species in food packaging materials and additionally could possibly provide useful information on the sites of unsatura-

TABLE IV

QUANTIFICATION OF VINYL CHLORIDE OLIGOMERS IN PVC RESINS

Oligomer	*Amount of oligomer found (ppm in base resin)*				
	Resin A		*Resin C*		*Resin F**
	Duplicates	*Mean (% of total)*	*Duplicates*	*Mean (% of total)*	*(% of total)*
3	1.0	1.55	2.4	2.7	3.1
	2.1	(3.2)	3.0	(10.5)	(2.8)
3C	0.7	0.85	2.2	1.9	1.2
	1.0	(1.8)	1.6	(7.4)	(1.1)
4	9.7	10.25	5.4	5.4	13.8
	10.8	(21.4)	5.4	(21.1)	(12.5)
4C	1.7	1.7	4.3	3.75	2.5
	1.7	(3.6)	3.2	(14.6)	(2.3)
5	12.1	11.75	3.8	3.8	23.2
	11.4	(24.6)	3.8	(14.8)	(21.0)
5C	3.3	3.15	1.4	1.4	3.4
	3.0	(6.6)	1.4	(5.5)	(3.1)
6	9.8	10.2	3.8	3.5	24.7
	10.4	(21.3)	3.2	(13.7)	(22.4)
6C	1.7	1.7	1.4	1.4	5.4
	1.7	(3.6)	1.4	(5.5)	(4.9)
7	4.7	4.85	0.8	0.8	28.8
	5.0	(10.1)	0.8	(3.1)	(26.1)
7C	1.7	1.7	1.0	1.0	4.2
	1.7	(3.6)	1.0	(3.9)	(3.8)
Total	46.4 48.8	47.8	26.5 24.8	25.6	110.3
Total found by micro-chlorine analysis	110 (43.5)		160 (16.0)		340 (32.4)

* Oligomers 8 (5.1 ppm) and 8C (1.3 ppm) were quantified in Resin F.

tion in PVC. Possible approaches to the structural elucidation of the oligomers under consideration are, isolation of individual species by preparative GC for subsequent NMR analysis and use of microchemical techniques such as epoxidation or ozonolysis in conjunction with GC for location of the double bonds in these unsaturated structures.

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DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN VEGETABLE OILS BY CAFFEINE COMPLEXATION AND GLASS CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

Glass capillary gas chromatography was used for the determination of polycylic aromatic hydrocarbons (PAHs). Reproducible PAH profiles were obtained from vegetable fats and oils by complexing them with caffeine in formic acid solution. The caffeine-complexable materials were then separated by column liquid and thin-layer chromatography, respectively. Glass capillary gas chromatographic results for PAHs specified by the U.S. Environmental Protection Agency were obtained after split injection within about 25 min. Quantitative and qualitative results are given for several commercial vegetable oils. Recoveries of PAHs were in the range 68–95%. The detection limit was 16 ng of coronene in 100 g of oil. Grapeseed oil was shown to contain the lowest "polycyclic aromatic hydrocarbons burden".

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have been implicated in oncogenesis. The level of their environmental occurrence is one of the variables used in determining the extent of voluntary or involuntary human exposure to the combined action of carcinogens, procarcinogens and syncarcinogens. Consequently, numerous analytical methods have been developed to monitor the total carcinogenic burden by accurate measurements of the PAH level in various environmental sources. A large part of the work has focused on food and food products. It has been established that the average D.D.R. citizen ingests up to 85 mg of benzo[*a*]pyrene during his lifetime[1]. Recently, this carcinogenic compound has also been demonstrated to produce atherosclerotic lesions to chickens when fed on diets at 0.1–10 mg kg^{-1} levels for up to 20 weeks[2]. Fifteen other PAHs are included in the list of "priority pollutants" issued by the U.S. Environmental Protection Agency (EPA)[3].

The traditional techniques for the isolation of PAHs from food usually involve an alkaline digestion followed by liquid–liquid partition with organic solvents and liquid–solid chromatography. These combined procedures have been described with specific methods applicable to different materials[4–7]. Although reproducible results are obtained for relatively small samples analysed for the benzo[*a*]pyrene level

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alone[8–12], the approach remains unsuitable for profile PAH analyses owing to the lack of any reliable definition of the oncogenetic extracts. The liquid–liquid extraction step is often the major cause of poor profile reproducibility, especially with emulsion-forming samples.

Recently, Sagredos and Sinha-Roy[6,7] described two methods involving complexation of PAHs in caffeine–formic acid solution[13]. The principle of these methods represents a particular advantage because it defines the PAH profile as that fraction of the oil which is complexable by caffeine. This approach, similar to that used by Van Heddeghem *et al.*[14], improves the reproducibility of PAH profiles and their evaluation by internal standard methods. We have adapted this rapid procedure for the determination of PAHs in fats and oils by glass capillary gas chromatography (GC).

Several workers[15–19] have presented glass capillary GC profiles PAHs obtained by various extraction and enrichment procedures. Until now, glass capillary GC analyses involving cold on-column injection techniques have been preferred to those linked with the inlet splitter injection system, which was reported to cause sample discrimination[20,21]. In a recent paper[22] we described the glass capillary GC conditions under which the split injection of PAH standards provided satisfactory quantitative results. This paper illustrates the application of the same experimental conditions to the screening of refined vegetable fats and oils.

EXPERIMENTAL

Benzo[*b*]chrysene, benzo[*a*]pyrene, benzo[*e*]pyrene and benzo[*j*]fluoranthene were supplied by the Community Bureau of Reference (Brussels, Belgium) and coronene, dibenz[*a*,*c*]anthracene, perylene, pyrene and triphenylene by Fluka (Buchs, Switzerland). Sixteen PAHs specified by the EPA protocol were supplied by Supelco (Crans, Switzerland). Otherwise analytical materials were as stated elsewhere[6,7].

Isolation of PAH

A 400-ml volume of cyclohexane, 100 g of vegetable oil (or melted fat) and 50 µl of internal standard solution (5 mg of benzo[*b*]chrysene dissolved in 100 ml of toluene) were placed in a 1000-ml separating funnel. The mixture was extracted twice for 120 sec with 100 ml of caffeine–formic acid solution (90% formic acid containing 15% of caffeine). After standing for 15 min, the caffeine–formic acid phase was drawn off into a 3000-ml separating funnel containing 1500 ml of 2% sodium chloride solution. After agitation for 1 min, the sodium chloride phase was extracted twice for 60 sec with 250 ml of cyclohexane. The combined cyclohexane extracts were allowed to stand for 20 min, then the excess of water was drawn off. The extract was further dried over 30 g of anhydrous sodium sulphate (Merck, Darmstadt, G.F.R.), which was added through the top of the 3000-ml separating funnel, then passed slowly through fluted filter-paper (diameter 18 cm) containing *ca.* 5 g of anhydrous sodium sulphate, into a 1000-ml round-bottomed flask. The volume of the sample was reduced to *ca.* 10 ml at 40°C using a vacuum rotary evaporator.

Liquid chromatographic clean-up procedure

A 5-g amount of silica gel for adsorption chromatography (Woelm, Eschwege, G.F.R.) with a 15% water content were packed into a 20 × 1 cm I.D. glass column.

The prepared extract was chromatographed on the column by elution with 110 ml of cyclohexane. After evaporation to *ca.* 3–4 ml, 1 ml of toluene was added to the eluate. A 10-ml conical test-tube was used for further concentration of the sample to *ca.* 200–300 μl under a stream of nitrogen at 30°C. Additional details of the method have been described elsewhere[4,6,7].

Thin-layer chromatographic clean-up procedure

High-performance thin-layer chromatography (HPTLC) plates (Merck) were used for the TLC clean-up of the concentrated column eluate. The eluate was loaded on to the HPTLC plate as a 70-mm band, using a Linomat III apparatus fitted with a 500-μl syringe (Camag, Mutenz, Switzerland). Then the remaining starting area of the plate was loaded with PAH standard solution, after which the plate was eluted twice with isooctane in a darkened TLC tank. Before the second elution, the plate was dried with a stream of nitrogen for about 1 min. The chromatograms were examined under UV light (254 and 366 mm) for approximately 5 sec. The PAH zone was detached using a plate scraper (Camag) and then rapidly homogenized in an 8-ml screw-capped test-tube (Sovirel, Paris, France) with a glass rod. To extract the PAHs, 4 ml of toluene were added to the test-tube, the contents of which were then heated to 45°C. After agitation for 1 min with a Vortex Genie Mixer (Scientific Industry, Bohemia, NY, U.S.A.) the sample was centrifuged at 2300 ± 100 *g* and then the supernatant evaporated in a conical test-tube to *ca.* 200 μl under a stream of nitrogen. The volume of the sample was further reduced to *ca.* 10 μl in a 500 μl Reacti-Vial, prior to glass capillary GC split injection.

Glass capillary GC analysis

Analyses were carried out using an HP 5830 A gas chromatograph fitted with a flame ionization detector (FID) and a home-made inlet splitter injection system, including septum purge. The GC conditions were as follows: 30 m × 0.3 mm I.D. glass-capillary column coated with OV-17–SE-30 (1:1) stationary phase, which was prepared using the ammonia etching pre-treatment[23]; injection temperature, 260°C; detector temperature, 280°C; oven temperature, 150°C, then programmed at 6°C min^{-1} and held at 280°C; pre-set splitting ratio, 1:10; carrier gas hydrogen at 0.55 bar.

The injector port thread was first lined with *ca.* 6 cm PTFE tape and then closed with a modified septum holder[22]. The latter was designed to guide the syringe through a 3 × 0.5 mm diameter hole into the injection chamber. A 5-μl syringe (Hamilton, Reno, NE, U.S.A.) fitted with a cemented needle (0.46 mm O.D.) was used for injection. The split injection of PAHs was carried out as follows. The syringe was pre-washed several times with toluene, so that clean solvent remained in the injection needle. The syringe was loaded successively with 1 μl of air, 1 μl of sample and 1 μl air (air–sample–air injection). The sample was injected immediately after smooth septum penetration. The whole operation was timed with a stop-watch. The syringe was removed from the vaporizing chamber 30 sec after the injection. Chromatograms were recorded and computed using an HP 18850 A GC terminal.

The effect of cold on-column injection on the linearity of PAH profiles was also examined using a Carlo Erba Model 4160 gas chromatograph, fitted with two injectors (cold on-column and split/splitless). The GC conditions were as follows: 30 m ×

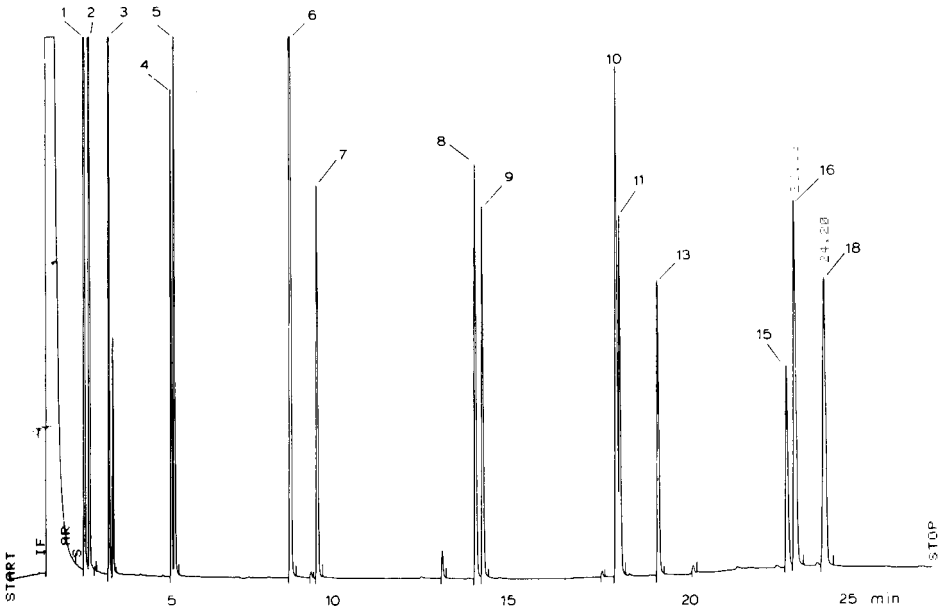

Fig. 1. Glass capillary GC separation of PAH standards from the list of EPA priority pollutants. All identified peaks are listed in Table I. GC conditions: 30 m × 0.3 mm I.D. glass capillary column coated with OV-17–SE-30 (1:1); oven temperature, 150°C, then programmed at 6°C min^{-1} and held at 280°C; FID temperature, 280°C; injector temperature, 260°C; carrier gas, hydrogen at 0.55 bar; pre-set splitting ratio, 1:10.

0.3 mm I.D. glass-capillary column coated with OV-17–SE-30 (1:1) stationary phase; cold on-column injection; 1 min isothermal at 80°C, then programmed at 35°C min^{-1} to 150°C, again 1 min isothermal, then programmed at 6°C min^{-1} and held at 290°C; detector FID; detector temperature, 350°C; carrier gas, hydrogen at 0.65 bar.

RESULTS AND DISCUSSION

Fig. 1 illustrates the glass capillary GC resolution of fifteen PAH standards that conform to the EPA priority pollutants programme[3]. Ammonia etching pre-treatment of uncoated, persilylated glass capillaries, combined with the effect of increased polarity of OV-17–SE-30 mixed stationary phase (similar to OV-7, but containing a gum phase) contributed substantially to the rapidity and efficiency of the glass capillary GC analysis[23]. This, together with data from our previous investigation on the linearity of PAH split injection[22], allowed us to select the principal GC parameters: a higher initial oven temperature (150°C) and a faster programming rate (6°C min^{-1}), which reduced the time required for the analysis of the above PAH standards to *ca.* 25 min. The only rejected target compound was naphthalene (mol. wt. 128). The separation of this compound from the solvent peak could be achieved only with a lower initial oven temperature.

All of the compounds listed in Table I were identified according to retention times recorded for pure PAH standards. High-chart-speed chromatograms were also

TABLE I

LEVELS OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOME COMMERCIAL VEGETABLE OILS

Average values after statistical evaluation of four glass capillary GC measurements.

Peak No.	*PAH*	*PAH content ($\mu g\ kg^{-1}$)****						
		Grapeseed	*Rapeseed*	*Sunflower*	*Cocoa butter*	*Soybean*	*Peanut A*	*Peanut B*
1	Acenaphthylene	3.48	1.09	4.36	1.22	0.86	–	0.90
2	Acenaphthene	2.47	0.59	2.49	0.47	0.81	–	0.93
3	Fluorene	2.10	0.68	1.96	0.43	0.79	–	0.93
4	Phenanthrene	1.57	1.10	2.31	2.71	2.17	–	6.19
5	Anthracene	0.35	t	t	1.35	2.10	–	t
6	Fluoranthene	2.80	1.98	6.70	16.35	8.95	17.14	19.88
7	Pyrene	3.08	0.57	4.97	8.47	2.57	7.17	5.79
8	Chrysene	0.49	1.04	1.70	3.65	17.35	63.31	10.26
8*	Triphenylene							
9	Benzo[*a*]anthracene	0.86	1.69	3.11	6.07	21.93	78.52	15.54
10	Benzo[*b*]fluoranthene	0.56	2.30	2.21	3.19	24.83	85.29	11.63
11	Benzo[*k*]fluoranthene	0.53	1.96	2.01	2.54	27.61	98.77	13.19
11*	Benzo[*j*]fluoranthene							
12*	Benzo[*e*]pyrene	1.89	3.15	4.11?	4.91	25.25	87.63	10.01
13	Benzo[*a*]pyrene	0.60	2.14	1.51	2.58	28.45	105.74	10.69
14*	Perylene	0.46	1.77	0.60	2.76	10.00	36.17	2.92
15	Indeno[1,2,3-*cd*]pyrene	0.27	4.05	1.32	0.32	22.82	80.63	9.01
16	Dibenzo[*a,h*]anthracene	0.24	1.31	t	t	4.74	12.92	2.24
16*	Dibenzo[*a,c*]anthracene							
17**	Benzo[*b*]chrysene							
18	Benzo[*g,h,i*]perylene	0.49	5.90	1.68	0.76	16.86	65.75	8.43
19*	Coronene	0.16	0.39	0.31	t	2.14	7.43	0.92
Σ PAH ($\mu g\ kg^{-1}$)		22.93	34.61	41.35	57.78	220.23	750.25	129.10
S.D. ($\mu g\ kg^{-1}$)		2.45	3.18	3.14	3.89	15.08	29.68	10.59

* Unspecified by EPA.
** Internal standard (22.74 $\mu g\ kg^{-1}$) added in each oil.
*** ? = merged peak; t = trace.

compared. Coronene eluted *ca.* 33 min after injection. This six-ring PAH could be detected and quantitated at levels as low as 16 ng per 100 g of vegetable oil (Table I). A systematic repetition of a whole series of peaks was observed in different PAH profiles. Such a tendency offers the advantage of a fundamental analytical description of the glass capillary GC pattern typical for fats and oils in relation to the method of isolation via caffeine complexes. Erroneous results are often linked to the unexpected components that arise during clean-up procedures and from analytical equipment. In our work, the hot injection chamber generated a limited series of homologues that were missing from the chromatograms obtained by cold on-column injection. Gieger and Schaffner[15] described similar effects as a possible result of processes in the traditional injection system. The effect appears to be more pronounced in relation to the

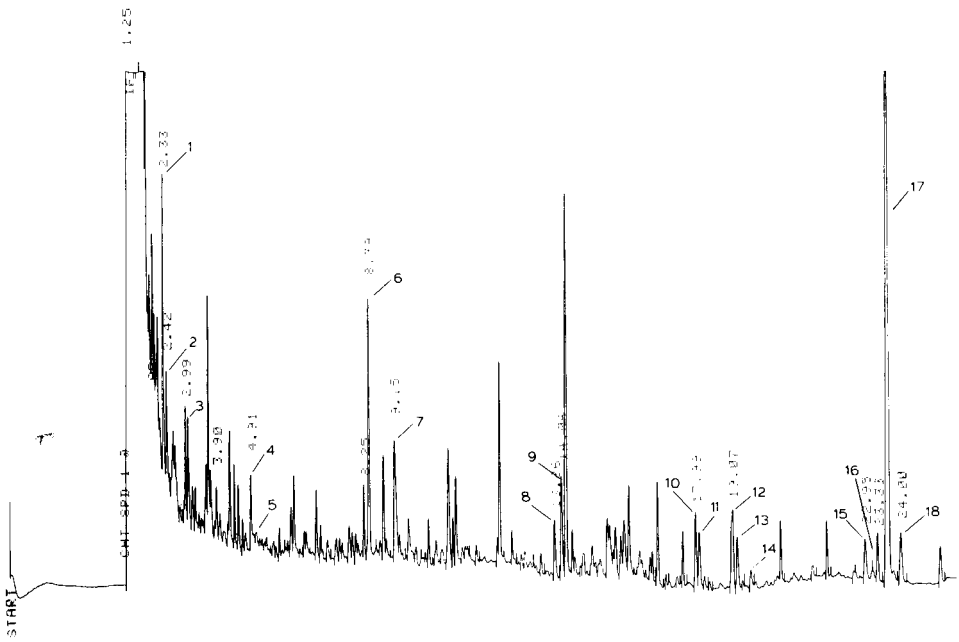

Fig. 2. Glass capillary GC profile of PAHs isolated from sunflower oil. All identified peaks are listed in Table I. GC conditions as in Fig. 1.

hot-needle injection of PAH standards[22]. Fig. 2 shows a non-target compound, benzo[*e*]pyrene, merging with one such artefact.

The original method of PAH isolation via caffeine complexes[6] was modified after unsuccessful attempts to extract PAHs from coffee oils. A small amount of the natural caffeine, even as low as 20–600 ppm, when present in these oils enhances the complexation of PAH and their consecutive solubilization in the 90% formic acid pre-washing phase. As other compounds in fats and oils, so far still unspecified, may induce PAH complexation, some procedures described in the previous method[6] were avoided. The modification resulted in the successful isolation of PAH profiles from the coffee oils (to be published later).

Liquid-adsorption chromatography on silica gel columns has been widely accepted as a rapid and reliable clean-up procedure in PAH analysis[4–6]. A less favourable attitude has been adopted towards *in situ* PAH evaluation by TLC under UV light[24,25]. The rapid TLC clean-up procedure described in this paper was shown to produce no deleterious effects on the stability of PAH standards eluted (see Table II, procedure E). However, rapid, probably photochemical, changes were observed with caffeine–complexable materials, which migrated below rather than along with the PAH band. Their fluorescence took on a red to deep red coloration under UV light within a few seconds. A comparative study, using different TLC supports, *e.g.*, acetylated cellulose, will be useful in establishing whether these differently migrating compounds may be derivatives of the PAH formed during the TLC procedure. Nevertheless, HPTLC plates proved to be useful in the rapid clean-up of caffeine-complexable materials from refined vegetable fats and oils (compare Figs. 2–4 and Fig. 5).

TABLE II

RECOVERY OF POLYCYCLIC AROMATIC HYDROCARBONS AFTER THE DIFFERENT PROCEDURES

(A) PAH standard mixture used in steps B, C, D and E; (B) mixture A after addition of known amount of benzo[*b*]chrysene (solution 1); (C) mixture A recovered after evaporation of 500 ml of cyclohexane and addition of solution 1; (D) mixture A recovered after caffeine complexation, extraction, concentration, LC clean-up and addition of solution 1; (E) mixture A recovered after TLC clean-up and addition of solution 1; (F) total recoveries after steps D and E. The amount of benzo[*b*]chrysene used in steps B, C, D and E (solution 1) was 16.85 $\mu g\ kg^{-1}$. All values are averages of five determinations.

Procedure	*Results*	*Phenanthrene*	*Pyrene*	*Chrysene*	*Benzo[a]pyrene*	*Benzo[b]chrysene*
A	Concentration present (%)	20.97	20.89	18.21	20.13	19.85
	S.D. (%)	0.55	0.50	1.04	0.57	0.96
	Recovery (%)	–	–	–	–	–
B	Concentration present (%)	16.41	16.94	15.53	17.29	33.83
	S.D. (%)	0.70	0.64	0.42	0.47	0.79
	Recovery (%)	–	–	–	–	–
C	Concentration present (%)	11.24	15.66	15.00	16.70	40.21
	S.D. (%)	0.35	0.72	0.66	0.73	0.69
	Recovery (%)	68	92	97	97	84
D	Concentration present (%)	10.82	14.27	14.75	16.42	43.73
	S.D. (%)	1.30	1.80	0.63	0.70	2.70
	Recovery (%)	66	84	95	95	77
E	Concentration present (%)	16.92	16.97	14.58	17.28	33.24
	S.D. (%)	1.09	0.95	0.27	0.42	1.33
	Recovery (%)	103	100	94	100	102
F	Recovery (%)	68	84	89	95	79

Other workers[26–31] have also demonstrated successful TLC separations of PAHs. With crude and heat-abused oils, the approach is lacking in efficiency. We were unsuccessful in obtaining satisfactory PAH recoveries from alumina sheets[6], using toluene as the extraction solvent. At present studies are under way using acetylated cellulose in the TLC clean-up of caffeine-complexable materials from crude or heat-abused vegetable oils.

The data in Table II show that major divergences in the recoveries of PAH were caused after application of procedure C. Procedure D also involves evaporation of cyclohexane: *ca.* 500 ml from extraction and *ca.* 110 ml from LC clean-up. Comparison between the recoveries in procedures C and D suggests that caffeine complexation and extraction, cyclohexane extraction and LC clean-up, have less pronounced effects on recoveries of PAHs than concentration of the cyclohexane.

The relative amounts of "target compounds" in the caffeine-complexable materials are apparent on rapid inspection of HPTLC plates under UV light. This was particularly noticeable when the TLC plates of soybean and peanut oil A were compared, and which reflected the higher PAH burden of the latter (Table I).

While the quantitative results may not always be comparable to previously published data, this may simply reflect the influence of industrial processing, storage and environment. The PAH burden has been shown to decrease after deodorization and bleaching[32–34]. On the other hand, the low PAH burden in the grapeseed oil is

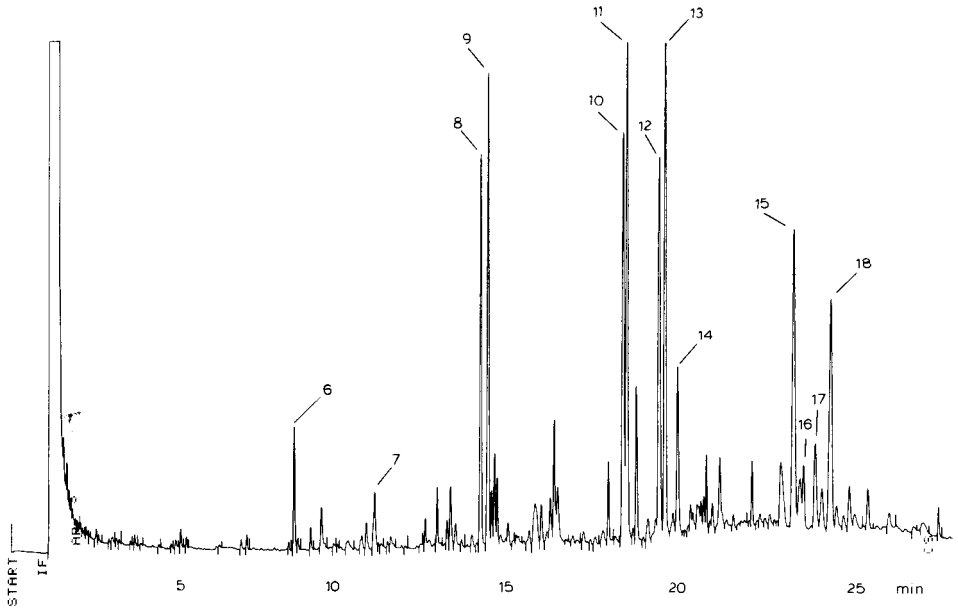

Fig. 3. Glass capillary GC profile of PAHs isolated from peanut oil A. All identified peaks are listed in Table I. GC conditions as in Fig. 1.

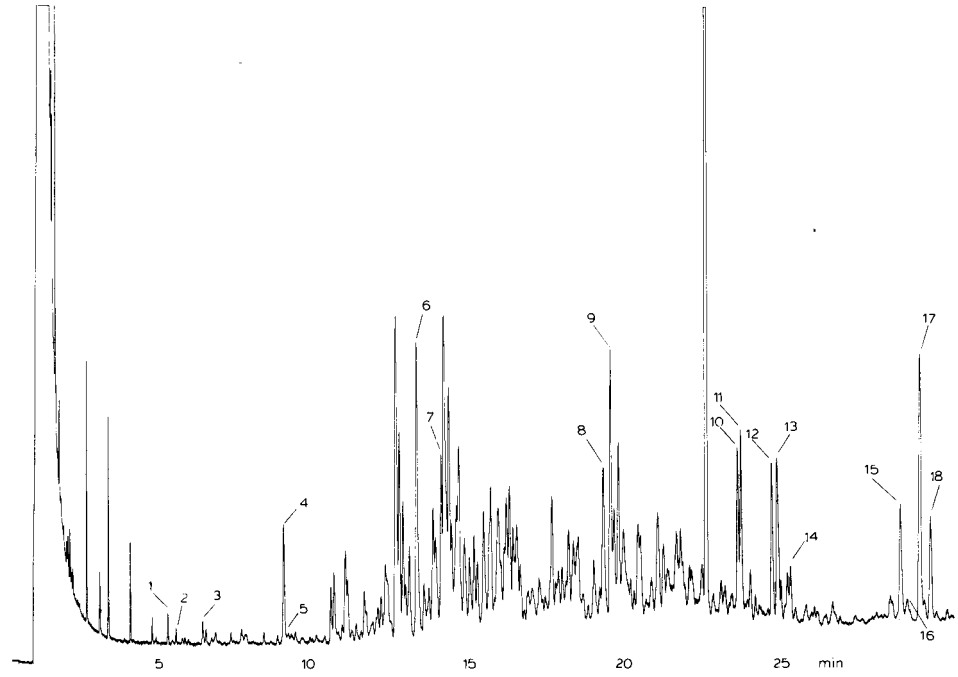

Fig. 4. Glass capillary GC profile of PAHs isolated from peanut oil B. On-column injection. All identified peaks are listed in Table I. GC conditions: column as in Fig. 1; cold on-column injection, 1 min isothermal at 80°C, then programmed at 20°C min^{-1} up to 150°C, 1 min isothermal, followed by 6°C min^{-1} and held at 280°C; FID temperature, 350°C; carrier gas, hydrogen at 0.65 bar.

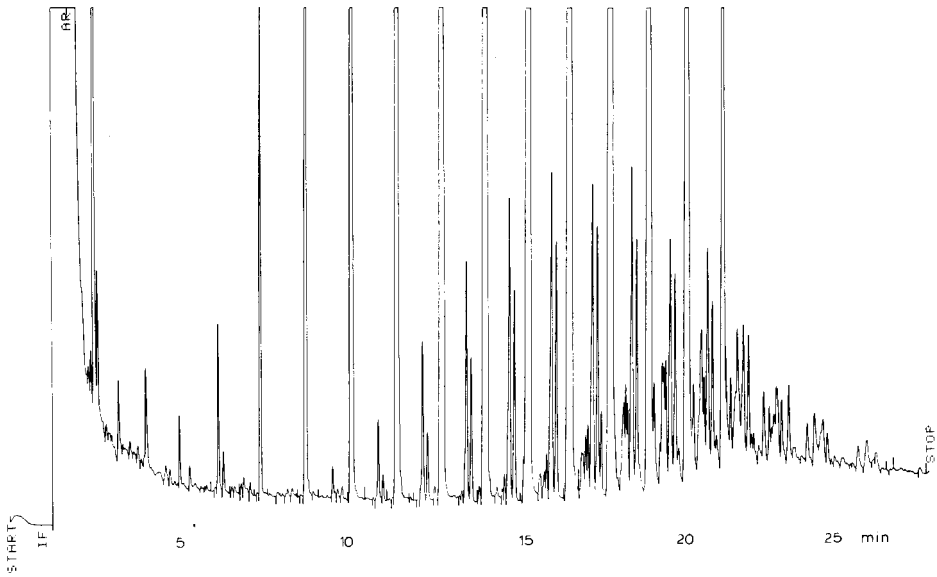

Fig. 5. A typical broad-spectrum glass capillary GC profile of caffeine-complexable materials before TLC clean-up, extracted from vegetable oils. The major homologous series was confirmed by GC–MS to be saturated hydrocarbons. GC conditions as in Fig. 1.

possibly explained by the botanical constitution of the parent fruit.

Fig. 5 represents a typical GC profile of the caffeine-complexable materials before their TLC clean-up. GC–mass spectrometry (MS) analytical data confirmed the major homologous series to be saturated hydrocarbons. The definition of these broad-spectrum profiles requires further and systematic investigation in order to establish qualitative and quantitative changes in caffeine-complexable materials obtained from different oils before and after exposure to thermo-oxidative processes[11]. Sufficient amounts of caffeine-complexable materials for these purposes can be obtained from only a few grams (*ca.* 5 g) of oil. A recent publication summarizes the possible interrelationships between fat and cancer[35]. The potential of the caffeine-complexable materials (see Fig. 5) to affect the oncogenetic response induced by benzo[*a*]pyrene and other carcinogens remains to be studied.

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CLEAN-UP OF POLYNUCLEAR AROMATIC HYDROCARBONS FROM AIR PARTICULATE MATTER ON XAD-2

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SUMMARY

A clean-up procedure is developed for polynuclear aromatic compounds. It is based on adsorption chromatography on XAD-2 and stepwise elution with ethanol, *n*-pentane and toluene. The polynuclear aromatic fraction of air particulate matter is isolated by this method and analyzed by glass capillary gas chromatography. Recoveries of nine polynuclear aromatic compounds are reported. The efficiency of group isolation is demonstrated with mixtures of polyaromatic compounds, phthalates and hydrocarbons.

INTRODUCTION

Polynuclear aromatic hydrocarbons (PAHs) are carcinogenic[1,2] and appear in the environment at concentrations at parts per 10^6 and 10^9 levels. Their determination requires efficient separation from polar and non-polar substrates, which usually appear in the sample at much higher concentrations. This has been achieved by various methods, e.g., column chromatography on silica gel or Sephadex LH-20[3] or thin-layer chromatography[4]. In order to obtain a clean PAH fraction, normally several methods have to be combined. The analysis of air particulate matter is usually carried out by solvent extraction, partition, chromatography on silica gel and Sephadex LH-20 and glass capillary gas chromatography (GC)[5].

Considering the good solubility of PAHs in aromatic solvents, a polymer consisting of aromatic units should adsorb these compounds strongly. One such polymer is XAD-2, a resin consisting of copolymerized styrene and divinylbenzene[6]. This polymer is normally employed in trace enrichment of organic compounds from water[7,8]. In this work, the applicability of XAD-2 to group separation of polyaromatic compounds from polar and non-polar contaminants in air particulate matter was investigated.

EXPERIMENTAL

Reagents and standards

A reference solution was prepared from phenanthrene, anthracene, pyrene

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(Merck, Darmstadt, G.F.R.), benzo(*a*)pyrene, benzo(*e*)pyrene, perylene, coronene, benzo(*ghi*)perylene and chrysene (EGA-Chemie, Steinheim, G.F.R.) at concentrations of 40–300 ppm in toluene. Another stock solution was prepared from *n*-alkanes (C_{16}, C_{18}, C_{20}, C_{22}, C_{23}, C_{24}, C_{25}, C_{26} and C_{28}) (Supelco, Bellefonte, PA, U.S.A.) at concentrations from 180 to 850 ppm and from diethyl and dibutyl phthalate at a concentration of 15,000 ppm in *n*-hexane. Squalane, squalene, *n*-eicosane (Supelco) and nonyl phthalate (Merck) were dissolved in cyclohexane. Dilutions of the desired strength were prepared with cyclohexane, using a Hamilton digital dilutor.

Solvents were glass-distilled through a column 40 cm long. XAD-2 of particle size 150–200 μm was supplied by Serva (Heidelberg, G.F.R.). OV-1 was purchased from Phase Separations (Queensferry, Great Britain).

Sample preparation

Airborne particulates were precipitated on glass-fibre filters (Schleicher & Schüll, Dassel, G.F.R.) with a high-volume sampling system (HVS 100; Sartorius, Göttingen, G.F.R.). The loaded filters (257 mm in diameter) were extracted with toluene or cyclohexane in a Soxhlet extraction apparatus. The extracts were evaporated to dryness at 40°C (rotary evaporator).

A glass column of 1.4 cm I.D. was filled with XAD-2 resin to a height of 9 cm. A solvent reservoir was fitted to the top of the column and a stop-cock to the bottom. The extracts were dissolved in ethanol and transferred quantitatively to the top of the resin bed. The column was then eluted successively with 25 ml of ethanol (polar fraction), 10 ml of *n*-pentane, followed by 10 ml of ethanol (non-polar fraction) and 12 ml of toluene, followed by 10 ml of ethanol (PAH fraction). The solvent flow-rate was adjusted to 2 ml/min. Finally, a further 20 ml of toluene were passed through the column, followed by 25 ml of ethanol, and the column was then ready for the next sample.

The PAH fraction was evaporated to a small volume (0.5 ml). *n*-Dotriacontane was added as an internal standard if quantitative results were to be obtained.

Gas chromatography

Extracts were analysed on a Carlo Erba 4160 high-resolution gas chromatograph, equipped with a flame-ionization detector and a column of OV-1 (30 m × 0.22 mm I.D., WCOT). The capillary was drawn from Pyrex tubing, high-temperature silanized and coated statically. Column bleed was low up to 300°C, and no deterioration occurred during operation.

RESULTS AND DISCUSSION

Desorption of organic compounds from the macroreticular XAD-2 resin is strongly influenced by the type of solvent. Table I lists the volumes of different solvents required for complete elution of *n*-alkanes (C_{16}–C_{28}), phthalates (diethyl and dibutyl) and PAHs at column loads of 40 μg per *n*-alkane, 5 μg per PAH and 1 mg per phthalate. The values were obtained by collecting 20–50-ml portions stepwise from the column eluate and analysis by GC.

n-Pentane is a strong eluent for *n*-alkanes and phthalates, but its drawback is the early elution of phenanthrene and anthracene, which start at 15 ml. On the other

TABLE I

DESORPTION OF COMPOUND CLASSES FROM XAD-2

Column: 9 cm × 1.4 cm I.D.

Solvent	*Elution volume (ml)*		
	n-Alkanes	*Phthalates*	*PAHs*
Ethanol	>150	100	>150
Isopropanol	100	60	>150
Acetone	20	20	20
Toluene	20	20	20
Diethyl ether	20	20	40
n-Pentane	20	20	>150

hand, heavy PAHs such as benzo(*ghi*)perylene require elution volumes in excess of 150 ml. Ethanol is a weak eluent for non-polar compounds and PAHs. Owing to these difficulties, a clean-up with three different solvents was developed, as outlined in Fig. 1. The procedure can remove 200 mg of diethyl phthalate from a sample. Filters can be impregnated with diethyl phthalate[9] in order to improve the recovery of volatile PAHs (phenanthrene, fluoranthene) from air.

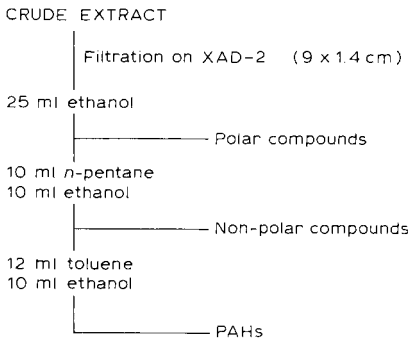

Fig. 1. Scheme for clean-up on XAD-2.

The solvents acetone, toluene and diethyl ether are strong eluents and do not achieve any group separation of alkanes and PAHs on XAD-2 if they are used alone.

Recoveries were determined by applying standard mixtures of PAHs to the column and elution according to the standard procedure outlined in Fig. 1. Values were obtained by capillary GC with an internal standard (*n*-dotriacontane) and are listed in Table II. Repeating the clean-up procedure with a sample did not cause losses of PAHs, apart from phenanthrene and anthracene. When fresh resin was used for the first time, recoveries of some PAHs [*e.g.*, benzo(*a*)pyrene] were low. Nevertheless, irreversible absorption does not occur after repeated use of the column.

The efficiency of clean-up was tested with artificial mixtures containing nine PAHs (amounts as given in Table II) and increasing amounts of six contaminants as listed in Table III.

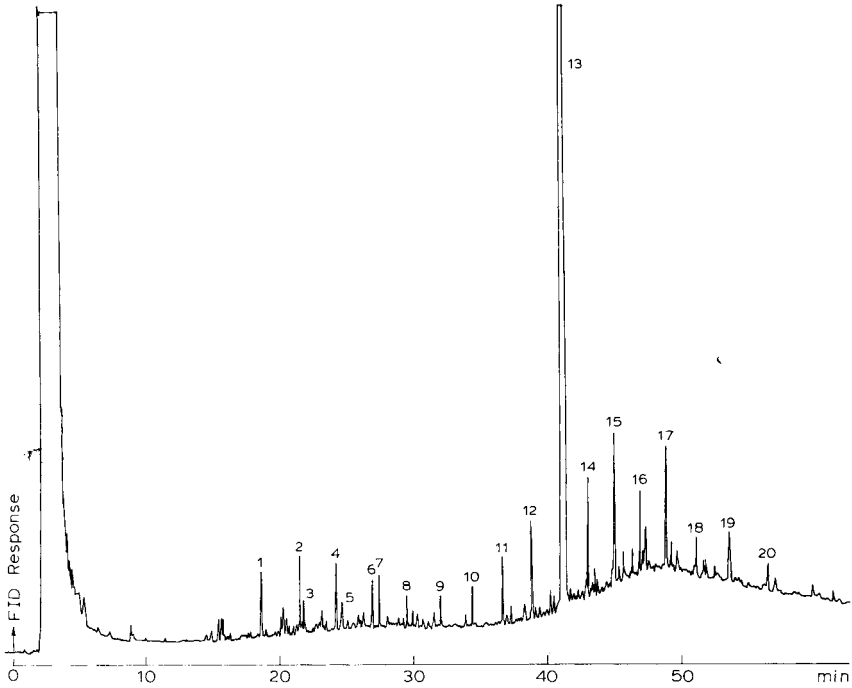

Fig. 2. Gas chromatogram obtained from the crude extract of air particulates. GC conditions: injection, 2 μl splitless; injection port temperature, 290°C; column, 30 m × 0.22 mm I.D.; stationary phase, OV-1; temperature programme, 110 to 300°C at 4°C/min. Peaks: 3 = pristane; 5 = phytane; 7 = unknown; 13 = *n*-pentacosane + phthalate; remaining peaks 1–20, C_{16}–C_{32} *n*-alkanes.

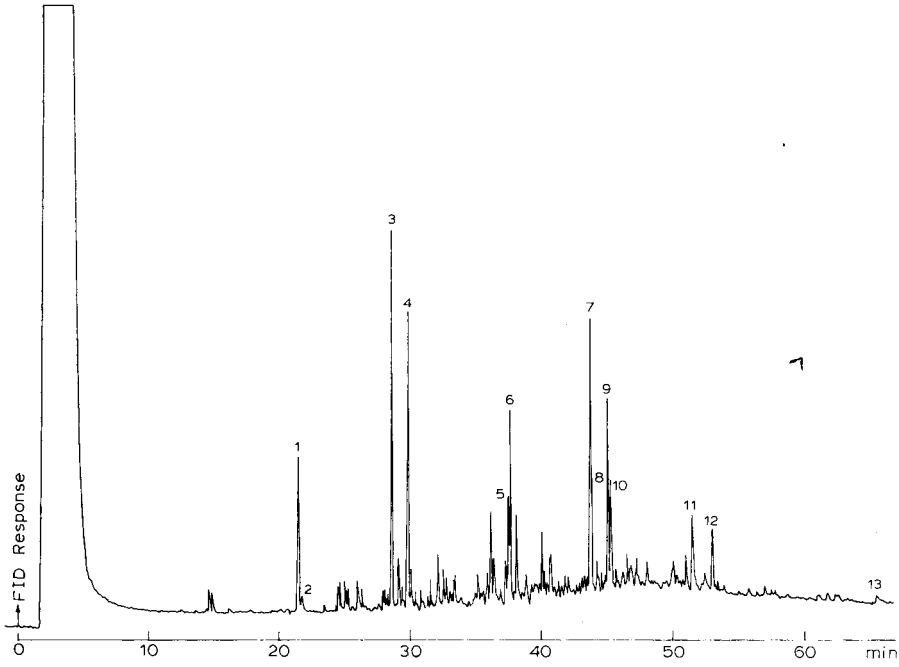

Fig. 3. PAH profile of air particulates after clean-up on XAD-2. Conditions as in Fig. 1. Peaks: 1 = phenanthrene; 2 = anthracene; 3 = fluoranthene; 4 = pyrene; 5 = benz(*a*)anthracene; 6 = chrysene + triphenylene; 7 = benzo(*b*)fluoranthene + benzo(*j*)fluoranthene; 8 = benzo(*k*)fluoranthene; 9 = benzo(*e*)pyrene; 10 = benzo(*a*)pyrene; 11 = indeno(1,2,3-*cd*)pyrene; 12 = benzo(*ghi*)perylene; 13 = coronene.

TABLE II

RECOVERIES OF POLYNUCLEAR AROMATIC HYDROCARBONS AFTER CLEAN-UP ON XAD-2

Compound	*Amount (μg)*	*Recovery (%)*	
		Cleaned-up once	*Cleaned-up twice*
Phenanthrene	14.1	81	69
Anthracene	9.8	81	65
Pyrene	2.8	85	90
Chrysene	5.6	88	87
Benzo(*e*)pyrene	3.5	93	91
Benzo(*a*)pyrene	2.0	85	86
Perylene	5.8	91	83
Benzo(*ghi*)perylene	9.8	95	70
Coronene	2.3	91	82

Contaminants are eluted with the polar or non-polar fraction. At higher column loads, however, they exhibit tailing behaviour on the XAD-2 resin, and less than 1 % of their total amount is co-eluted with PAHs. Repeating the clean-up would purify the PAH fraction completely in such a case.

Fig. 2 shows the gas chromatogram of a cyclohexane extract from a glass-fibre filter after sampling 316 mg of urban particulates in the city of Hamburg. The extract was evaporated to 10 ml. All PAHs overlap with large amounts of co-extractants which are present in much higher concentrations.

Clean-up on XAD-2 removes polar and non-polar contaminants; Fig. 3 shows the PAH fraction from 228 mg of air particulates which had been extracted with toluene. The PAH fraction was evaporated to a volume of 0.5 ml.

TABLE III

AMOUNTS OF SIX COMPOUNDS ELUTED WITH THE PAH FRACTION FROM XAD-2 AFTER A SINGLE CLEAN-UP

Compound	*Column load*			
	10 μg	*100 μg*	*1 mg*	*>1 mg**
1-Eicosene	0	0.2 μg	0.8 μg	4.2 μg (9 mg)
n-Eicosane	0	0.2 μg	0.7 μg	3.9 μg (11 mg)
Squalane	0	1.1 μg	4.6 μg	35 μg (12.5 mg)
Dinonyl phthalate	0	0	4.9 μg	50 μg (15 mg)
n-Octacosane	0	0.7 μg	11.5 μg	46 μg (10 mg)
Squalene	0	0	0.7 μg	128 μg (28 mg)

* The actual column load is given in parentheses.

CONCLUSION

XAD-2 is a suitable adsorbent for group separation of PAHs from polar and non-polar compounds. It readily provides the PAH profile of airborne particulates.

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IMPROVED GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PROTEIN AMINO ACIDS

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SUMMARY

The gas–liquid chromatographic method previously reported by us was improved with regard to the gas chromatographic columns and their operating conditions. It became possible to perform more accurate quantification of the 22 protein amino acids, including asparagine and glutamine, in less than 25 min on a dual set of columns. At the same time, the method allowed determination of the amino acids in the range 0.5–10 μg of each by eliminating the contaminant peaks derived from solvents and reaction vials. A single sample could be analysed in an overall time of 1 h.

INTRODUCTION

We have previously reported a simple and convenient derivatization method for gas–liquid chromatographic (GLC) determination of the protein amino acids[1], in which the volatile derivatives, N(O,S)-isobutyloxycarbonyl (isoBOC) methyl esters, of amino acids except arginine were prepared by two-step procedures involving isobutyloxycarbonylation with isobutyl chloroformate (isoBCF) in aqueous alkaline medium, followed by esterification with diazomethane. Prior to derivatization, arginine, by treatment with arginase, was converted into ornithine, which could be determined as its isoBOC methyl ester. Both asparagine and glutamine could also be derivatized by this method and separated by GLC in the presence of the other protein amino acids[2]. The derivatives could be easily and rapidly prepared and were stable, unlike most derivatives previously proposed for GLC determination of amino acids. However, our previous method was relatively time-consuming since it necessitated the operation of two columns under different thermal conditions for the separation of the 22 protein amino acids including asparagine and glutamine, and it has been subsequently shown that separation of some of the amino acid pairs was not entirely satisfactory especially when one of the pair was present in large excess. For these reasons, an investigation aimed at improving the previous method with regard to GLC columns and their operating conditions has been made. Furhtermore, by eliminating the contaminant peaks caused by solvents and reaction vials, we have been able to carry out the determination of all the protein amino acids in sub-microgram amounts.

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EXPERIMENTAL

Materials and reagents

All amino acids were obtained from Nakarai Chemicals (Kyoto, Japan); *p*-hydroxyphenylacetic acid, used as an internal standard, was supplied by Sigma (St. Louis, MO, U.S.A.). Two standard stock solutions (each 100 μg/ml), one containing the 22 protein amino acids and the other containing the 22 protein amino acids plus ornithine, were prepared in 0.1 *M* hydrochloric acid, and aliquots were taken and diluted with water to make the test mixtures as required. Arginase solution was prepared with some modifications as follows: prior to use, 10 mg of arginase (40 units/mg) (Sigma) were activated in 0.4 ml of 1.25 *M* ammonium acetate and 0.1 ml of 0.05 *M* manganese(II) sulphate at 37°C for 4 h. After the solution had been centrifuged for 1 min at 3000 rpm, the supernatant was separated, to which 0.5 ml of water was added. This solution was stored frozen when not in use.

IsoBCF stabilized with calcium carbonate (Tokyo Kasei Kogyo, Tokyo, Japan), was used without further purification and stored at 4°C. N-Methyl-N-nitroso-*p*-toluenesulphonamide and diethylene glycol monoethyl ether for the generation of diazomethane[3] were obtained from Wako (Osaka, Japan). Purified diethyl ether was prepared as previously reported[1]. The water which was used was treated as follows. Deionized water was distilled in an all-glass system after addition of several pellets of sodium hydroxide to remove the acidic contaminants. Sodium sulphate and sodium chloride were washed with methanol, then with purified diethyl ether and dried at 100°C. All other chemicals and solvents were the purest grades available from standard commercial sources. Materials for GLC were as follows: Poly-I-110, Poly-A-101A and FFAP (Applied Science Labs., State College, PA, U.S.A.) and 100–120 mesh Uniport HP (silanized) (Gasukuro Kogyo, Tokyo, Japan). Prior to coating, the support was treated as follows to attain maximum column efficiency. The support was floated on concentrated hydrochloric acid and complete contact with the liquid was ensured by gentle swirling. The grey and black particles which were precipitated and the acid were removed by pipette. This procedure was repeated three times. The support was again floated on deionized water and a similar procedure to that described above was carried out to neutrality to remove further grey and black particles as well as the remaining acid. The support precipitated by the addition of methanol was then washed four times with the same solvent, and dried at 100°C. The dry support was silanized with 5% dimethyldichlorosilane (DMCS) in toluene[4,5].

Gas–liquid chromatography

Analyses were performed on a dual set of columns using a Shimadzu 4CM gas chromatograph with two electrometers, two hydrogen flame detectors, on-column injection ports and a temperature programmer. Each electrometer was individually connected to a one-pen recorder. The glass columns (1 m × 3 mm I.D.) and quartz wool (used as plugs at each end of the columns) were treated with DMCS–toluene (5:95). The mixed-phase column packings, 1.605% Poly-I-110–Poly-A-101A–FFAP (1200:300:105, w/w/w) and 1.0% Poly-A-101A–FFAP (1:1, w/w), were prepared by the filtration technique[4,5] using chloroform–*n*-butanol (1:1, v/v) as coating solvents. In the case of the former packing, the solution obtained after dissolving the liquid phases in the coating solvents by vigorous shaking for 1 h was filtered to remove

insoluble materials. The column packings were packed into the columns with gentle tapping under suction by an aspirator. The packed columns were preconditioned at 280°C for 20 h with a nitrogen flow-rate of 30 ml/min after being programmed to 280°C at 2°C/min at the same nitrogen flow-rate. Nitrogen (> 99.99%) was passed through a tube (20 × 3 cm I.D.) containing molecular sieve 5A. Other GLC conditions are given in Fig. 1. A 1.0% Poly-A-101A–FFAP column was used for the determination of leucine, isoleucine and arginine, and a 1.605% Poly-I-110–Poly-A-101A–FFAP column for the determination of the other amino acids, as well as ornithine which was present in the original samples.

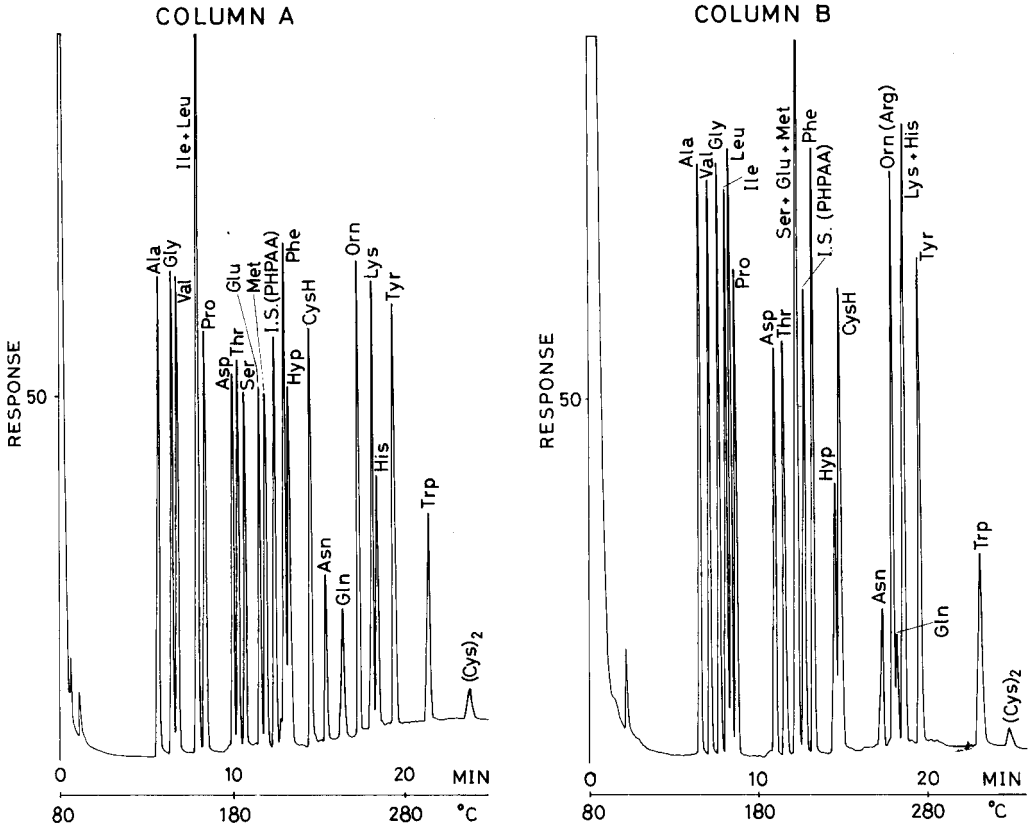

Fig. 1. Gas chromatographic separation of 22 amino acids as N-isobutyloxycarbonyl methyl esters on a dual set of columns. Column A: 1.605% Poly-I-110–Poly-A-101A–FFAP (1200:300:105, w/w/w) on 100–120 mesh Uniport HP, 1 m × 3 mm I.D. glass. Column B: 1.0% Poly-A-101A–FFAP (1:1, w/w) on 100–120 mesh Uniport HP, 1 m × 3 mm I.D. glass. Conditions: injection port and detector temperatures, 280°C; nitrogen flow-rate, 35 ml/min; hydrogen flow-rate, 50 ml/min; air flow-rate, 0.8 l/min; temperature program, linear rise at 10° per min from 80 to 280°C, then held for 5 min. Arginine was separated as the ornithine derivative. Internal standard (I.S.), *p*-hydroxyphenylacetic acid.

In order to obtain a stable baseline in every analysis, the columns were kept overnight at 150°C with a nitrogen flow-rate of 20 ml/min before use. The columns were maintained at 280°C until a suitably low rise in baseline was obtained when they were reconnected to the instrument for the temperature-programmed analysis.

Preparation of derivatives

The details of the principle of the derivatization reactions have been presented previously[1]. The derivatization procedure was modified to make it possible to be performed at sub-microgram levels. Isobutyloxycarbonylation was conducted in a glass screw-top culture tube (Corning No. 9826) whose cap was fitted with a thin PTFE gasket to avoid any contamination which might show as interference peaks on the chromatogram. Test mixtures were used to prepare calibration graphs and to evaluate the reproducibility throughout the overall procedure. Two series of test mixtures (each 1 ml) in the range 0.5–10 μg and 0.5 ml of the internal standard solution (10 μg/ml) were transferred into the vials by pipette. One, not containing ornithine, was treated with arginase according to the procedure of ref. 1 except that 20 μl of arginase solution were added and the resulting solution was centrifuged after incubation for 10 min. The supernatant, after having been subjected to the derivatization procedure, was employed for the assay of arginine, leucine and isoleucine. The other, containing ornithine, was immediately subjected to the derivatization procedure. A 0.5-ml portion of 10% sodium carbonate and 20 μl of isoBCF were added to each vial containing the standard solution or that which had been treated with arginase, and the mixtures were treated as previously reported[1]. It was found that this amount of reagent was enough to perform quantitative isobutyloxycarbonylation of up to 2 mg of a mixture of amino acids. The resulting isoBOC derivatives were extracted five times with 2 ml of diethyl ether from the acidic solution saturated with sodium chloride. Saturation with sodium chloride and extraction five times improved extraction efficiency, especially for the derivatives of threonine, serine, hydroxyproline, asparagine and glutamine. Methyl esterification of the combined ethereal extract was carried out by bubblying through diazomethane without addition of methanol. Evaporation of solvent was accomplished at 40–45°C in a water-bath without use of a current of nitrogen. The residue was dissolved in 20 μl of ethyl acetate and the solution was dried over anhydrous sodium sulphate. A 1–5-μl aliquot was injected on to the gas chromatograph. Peak heights for the amino acids and the internal standard were measured and the peak height ratios were calculated for the construction of calibration graphs.

RESULTS AND DISCUSSION

The 1.605% Poly-I-110–Poly-A-101A–FFAP column provided the desired separation of all the protein amino acids except leucine and isoleucine. Compared to the columns previously developed, separation was better and analysis time shorter, although there still remained the inability to separate the leucine–isoleucine pair. On the other hand, the 1.0% Poly-A-101A–FFAP column did give a good enough separation of this pair when operated under the same thermal conditions as employed for the 1.605% Poly-I-110–Poly-A-101A–FFAP column. This method required a separate analysis if quantitation of ornithine was required at the same time as arginine was being assayed, and it was found that the quantitative determination of methionine, cysteine and tryptophan was interfered with when test mixtures were subjected to the arginase treatment. Therefore, in view of the problems mentioned above, we decided to operate both columns simultaneously and to assay leucine, isoleucine and arginine on the 1.0% Poly-A-101A–FFAP column. In practice, in a sample contain-

ing both arginine and ornithine, the amount of arginine could be determined by subtracting the amount of ornithine determined without arginase treatment using 1.605% Poly-I-110–Poly-A-101A–FFAP column from that determined with arginase treatment using 1.0% Poly-A-101A–FFAP column, and then by converting the remaining ornithine into arginine. Separation of the 22 protein amino acids could be achieved using two columns in a single programmed temperature cycle as shown in Fig. 1, and was complete in less than 25 min. *p*-Hydroxyphenylacetic acid was the most suitable internal standard of the compounds tested. Its O-isoBOC methyl ester was efficiently separated from the other amino acids on both columns, as shown in Fig. 1.

The support used was purified and re-silanized, and this allowed us to overcome fluctuations in performance of the support and to obtain quantitative accuracy, most notably with the amides and cystine. Properly prepared columns retained their ability to separate the amino acids for about three months.

Diethyl ether and water purified by distillation were used after confirming that no contaminant peaks were present. Some makes of reaction vials, the caps of which were fitted with thin synthetic resin gaskets, produced interfering peaks when the chromatograph was operated at high sensitivity. A glass tube with a PTFE-lined gasket was the most suitable reaction vial for isobutyloxycarbonylation.

Conversion yields of arginine to ornithine were almost quantitative in the range 0.5–10 μg under the conditions used. Calibration graphs for all the amino acids, obtained by plotting the ratios of their peak heights to that of the internal standard

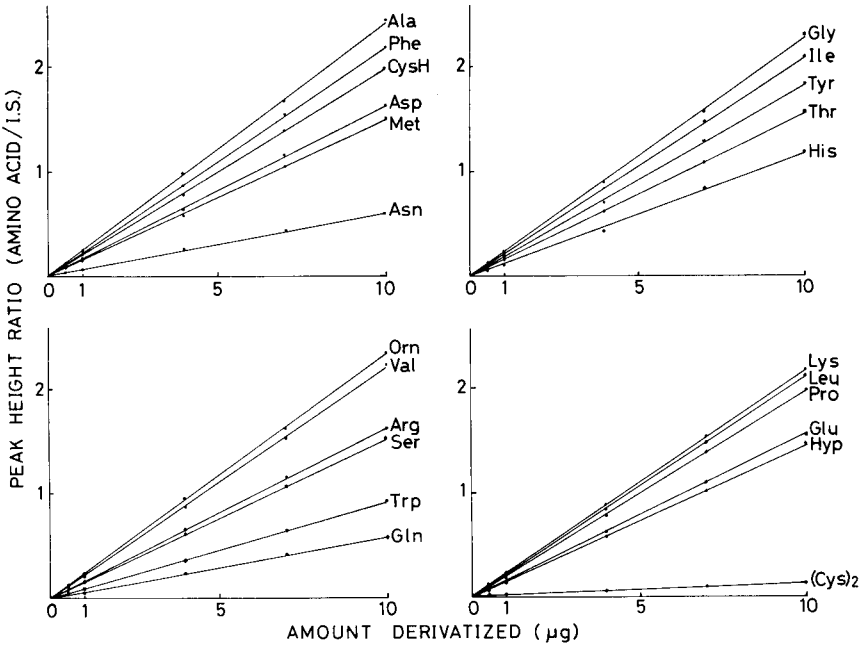

Fig. 2. Calibration graphs for amino acids in the range 0.5–10 μg. Internal standard (I.S.), *p*-hydroxyphenylacetic acid, 5 μg. Arginine was assayed as the ornithine derivative after conversion into ornithine with arginase.

versus the amounts of amino acids (in the range 0.5–10 μg), were linear and passed through the origin, as shown in Fig. 2; reproducibilities were satisfactory. This method permitted detection of even 0.1 μg amounts of each amino acid.

In conclusion, manipulation of these derivatives is easier than the other derivatives which have been proposed, particularly when the operation is carried out on a routine basis. The columns developed in this study gave the desired separation for each amino acid, thus permitting more accurate determinations: the present method allows determination of 0.5 μg amounts of each amino acid. A single analysis, consisting of arginase treatment, derivatization and GLC, could be performed in 1 h. This method has the advantage that both asparagine and glutamine can be determined, unlike N-acetyl or -perfluoroalkyl ester derivatives which require esterification with hydrochloric acid and alcohols under drastic conditions. The method has been applied to biological samples and this will be the subject of a subsequent report.

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RAPID AND HIGHLY SENSITIVE METHOD FOR DETERMINATION OF METHAMPHETAMINE AND AMPHETAMINE IN URINE BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive method for determination of methamphetamine and amphetamine in urine was developed by using electron-capture gas chromatography. The extraction procedure, the experimental conditions for pentafluorobenzoyl derivative formation and the percentage recovery of the drugs from urine are described.

The pentafluorobenzoyl derivative of methamphetamine showed a higher electron-capture sensitivity and was detected in at least 23-fold lower concentration than the heptafluorobutyryl derivative which is commonly used as a derivatizing agent for the amine. The detection limit of pentafluorobenzoyl derivatives of methamphetamine and amphetamine was *ca.* 10 pg. A concentration as low as 10 ng/ml of methamphetamine and amphetamine in urine was easily detected by this method.

INTRODUCTION

Various methods for the identification of methamphetamine and other related amines in biological material have been presented during recent years. These methods include gas chromatography (GC)[1–4], thin-layer chromatography (TLC)[4–7], spectrophotofluorometry[8,9], high-performance liquid chromatography (HPLC)[10,11], gas chromatography–mass spectrometry (GC–MS)[12–14] and immunoassay[11,15–17].

In recent years, the number of abusers of methamphetamine has been markedly increasing again in Japan. Thus, the development of a more simple, rapid and sensitive assay method for methamphetamine has been required.

Using the electron-capture detector (ECD) it has become feasible to analyse

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methamphetamine and related amines at highly sensitive levels. In this paper, we report a more sensitive derivatization of methamphetamine and amphetamine for the ECD, and the experimental conditions for determination of these amines in urine.

EXPERIMENTAL

Apparatus

GC analysis was carried out with a Hitachi 023 gas chromatograph equipped with a ^{63}Ni ECD. A glass column (2 m × 2 mm I.D.), packed with 2% Thermon-3000 on Chromosorb W (AW-DMCS), 80–100 mesh, was used. The temperatures were: oven, 200°C; injection port, 220°C; detector, 200°C. The nitrogen flow-rate was 50 ml/min. The chart speed was 5 mm/min. The mass spectrometric analysis was carried out on a JEOL JMS-D300 mass spectrometer connected to the chromatograph. A glass column (2 m × 2 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q, 80–100 mesh, was used. The temperatures were: oven, 260°C; injection port, 280°C; separator, 250°C; ion source, 240°C. The ion current was 300 μA and the ion voltage 300 eV. Methane was used as reactant gas.

Reagents

Trifluoroacetic, pentafluoropropionic and heptafluorobutyric anhydrides and pentafluorobenzoyl chloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Acetone (specially prepared reagent) and *n*-pentane were obtained from Nakarai and Wako (Osaka, Japan), respectively. These solvents were redistilled and then used in these experiments. Methamphetamine hydrochloride was obtained from Dainippon Seiyaku. All other chemicals were of reagent grade.

Extraction of methamphetamine and amphetamine from urine

To diluted urine (0.2 → 2.0) in a 10-ml stoppered round-bottomed centrifuge tube were added 0.5 ml of 10 *M* NaOH and 6.0 ml of *n*-pentane. The contents were mixed for 10 min and centrifuged for 5 min at 800 *g*. Next, 5 ml of the *n*-pentane layer were transferred to a 10-ml screw-cap round-bottomed centrifuge tube, and 1 ml of 400 nmol/ml pentafluorobenzoyl chloride, dissolved in *n*-pentane, was added to the extract and incubated at 80°C for 20 min. Then the mixture was cooled and washed twice with 2.0 ml of 0.1 *M* $NaHCO_3$ and once with 2.0 ml of distilled water. Following centrifugation for 5 min at 800 *g*, 5.0 ml of the *n*-pentane layer was transferred to a 10-ml stoppered conical-bottomed centrifuge tube, and a small antibubbling granule was added. The sample was evaporated at 50°C until just dry. The residue was dissolved in acetone and an aliquot (1–3 μl) was injected into the gas chromatograph.

Electron-capture response of various methamphetamine derivatives

A mixture of 1.0 ml of methamphetamine (10 μg/ml in *n*-pentane) and 4.0 ml of *n*-pentane was transferred to a 10-ml screw-cap round-bottomed centrifuge tube, to which was added 0.2 ml of heptafluorobutyric anhydride or 0.2 ml of pentafluoropropionic anhydride or 0.5 ml of trifluoroacetic anhydride or 0.2 ml of pentafluorobenzoyl chloride (20 μmol/ml in *n*-pentane). The tubes were incubated at 65°C for 20 min for trifluoroacylation or 60 min for formation of the other derivatives. The tubes were

cooled to room temperature. The *n*-pentane layer was washed twice with 2.0 ml of 0.1 *M* $NaHCO_3$ and once with 1.0 ml of distilled water. The *n*-pentane layer was transferred to a 10-ml stoppered conical-bottomed centrifuge tube and evaporated under nitrogen. The residue was dissolved in acetone and subjected to GC–ECD.

Preparation of pentafluorobenzoyl derivative of methamphetamine

A mixture of 5.0 ml of methamphetamine solution (10 mg/ml in *n*-pentane), 0.8 ml of pentafluorobenzoyl chloride and 50 μl of pyridine was warmed at 65°C for 90 min. The mixture was cooled and washed six times with 1 ml of 10 *M* NaOH and twice with 5.0 ml of distilled water. The *n*-pentane layer was evaporated at room temperature. The crystalline layer was recrysrtallized from *n*-pentane and analysed by a melting point determination and GC–MS. The crystals were dissolved in acetone and used as an authentic standard of the pentafluorobenzoyl derivative of methamphetamine for GC.

RESULTS AND DISCUSSION

Mass spectrum of authentic standard of pentafluorobenzoyl derivative of methamphetamine

The crystals (m.p. 96–97°C) obtained from methamphetamine and pentafluorobenzoyl chloride were white and scaly, and their chemical ionization mass spectrum is shown in Fig. 1. The parent ion of the pentafluorobenzoyl derivative of methamphetamine (MW 343) yielded the following *m/z* fragments, 344 (QM^+, M + 1) and 372 $(M + C_2H_5)^+$. From the resulting spectrum, the crystals were identified as the pentafluorobenzoyl derivative of methamphetamine, and thereafter used as an authentic standard.

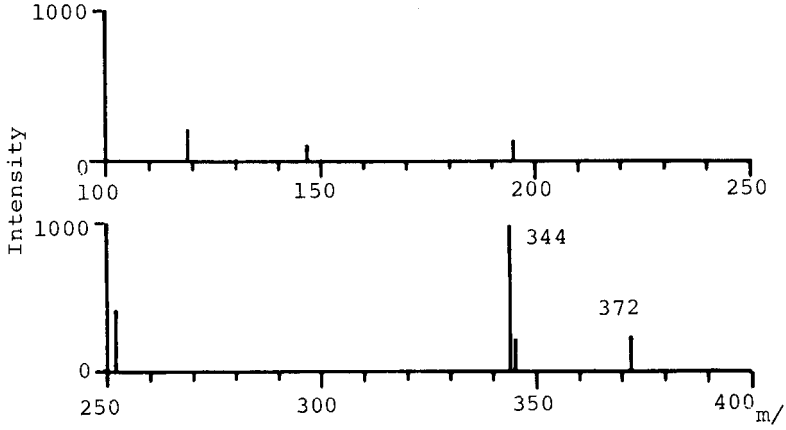

Fig. 1. Chemical ionization mass spectrum of the pentafluorobenzoyl derivative of methamphetamine.

Electron-capture response of various methamphetamine derivatives

Experiments were carried out to compare the electron-capture affinity of the various derivatives of methamphetamine, including the pentafluorobenzoyl derivative of the amine. As shown in Table I, the electron-capture affinity of the penta-

TABLE I

RELATIVE SENSITIVITIES OF VARIOUS METHAMPHETAMINE DERIVATIVES

Derivative	*Sensitivity**
Trifluoroacetyl	0.028
Pentafluoropropionyl	3.19
Heptafluorobutyryl	24.25
Pentafluorobenzoyl	560.00

* Peak height mm/ng methamphetamine.

fluorobenzoyl derivative of methamphetamine was 23 times and 176 times higher than that of the heptafluorobutyryl and pentafluoropropionyl derivatives, respectively. The results indicate that the pentafluorobenzoyl derivative of methamphetamine showed a higher affinity towards the ECD than other derivatives of the amine.

In accordance with the present findings, Wilkinson and Beckett[18] have reported that the pentafluorobenzoyl derivative of phenylalkylamine has a greater electron-capture affinity. Midha *et al.*[19] have also reported that the electron-capture affinity of the pentafluorobenzoyl derivative of ephedrine is higher than those of the heptafluorobutyryl, pentafluoropropionyl and trifluoroacetyl derivatives.

Because the pentafluorobenzoyl derivative of methamphetamine showed a higher electron-capture affinity and thus seemed to be a good derivatizing agent for the determination of the amine by GC–ECD, we examined the detailed experimental conditions for the derivatization of the amine as described below.

Effect of amounts of pentafluorobenzoyl chloride on methamphetamine derivatization

To a mixture of 1.0 ml of methamphetamine (100 ng/ml in *n*-pentane) and 4.0 ml of *n*-pentane was added 1.0 ml of 500, 100, 20, 3, 0.8 or 0.16 nmol/ml pentafluorobenzoyl chloride dissolved in *n*-pentane. The rates of formation of pentafluorobenzamide were calculated from peak heights of the authentic standard of pentafluorobenzoyl derivative of methamphetamine (100 pg).

TABLE II

EFFECT OF PENTAFLUOROBENZOYL CHLORIDE (PFB-Cl) AMOUNTS ON FORMATION OF PFB DERIVATIVE OF METHAMPHETAMINE

Methamphetamine, 100 ng (0.65 nmol); reaction time, 20 min; temperature, 80°C.

PFB-Cl (nmol)	*Formation of PFB-methamphetamine (%)*
500	102.0
100	105.0
20	100.0
4	97.6
0.8	68.6
0.16	30.7

As shown in Table II, when 4 nmol or more of pentafluorobenzoyl chloride was added to the reaction mixture, there was complete formation of pentafluorobenzamide. Thereafter, we used 400 nmol of pentafluorobenzoyl chloride to derivatize methamphetamine.

Effect of reaction time and temperature on methamphetamine derivatization

A mixture of 1.0 ml of methamphetamine standard solution (100 ng in *n*-pentane), 4.0 ml of *n*-pentane and 1.0 ml of pentafluorobenzoyl chloride (4 nmol/ml in *n*-pentane) was warmed at 40°C, 60°C or 80°C for 10, 20, 40, 60 or 90 min.

As shown in Fig. 2, derivatization of methamphetamine was complete within 20 min. Variation of the reaction temperature did not show any appreciable effect on the formation of pentafluorobenzamide under the experimental conditions. Based on these results, the experimental conditions for the derivatization of methamphetamine were chosen as follows: reaction time, 20 min; temperature, 80°C.

The calibration curves for methamphetamine and amphetamine obtained under the experimental conditions are shown in Fig. 3. There was a good linearity between peak height and methamphetamine or amphetamine amount ranging from 10 to 150 pg.

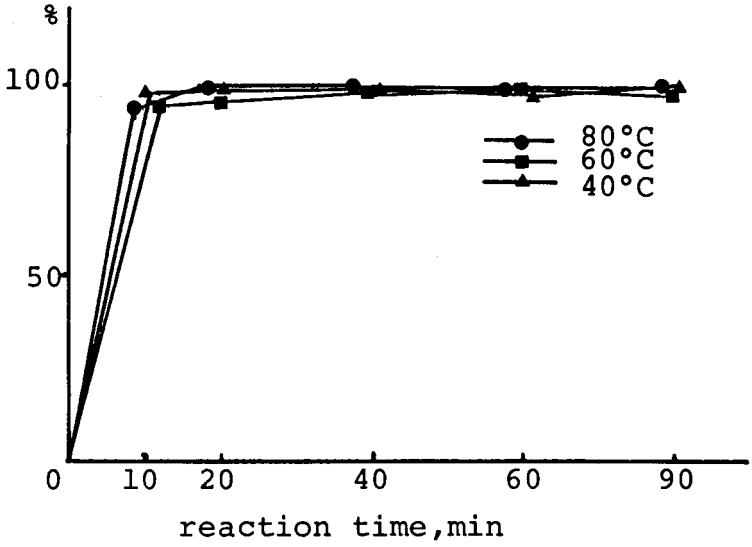

Fig. 2. Effect of reaction time on the formation of the pentafluorobenzoyl derivative of methamphetamine.

Gas chromatograms

Because the pentafluorobenzoyl derivative of methamphetamine is highly sensitive to the ECD, we applied this method to biological material.

Gas chromatograms of pentafluorobenzoyl derivatives of methamphetamine and amphetamine from urine are shown in Fig. 4. Fig. 4A shows a typical gas chromatogram obtained from extracts of blank urine. No extraneous peaks are observed. Fig. 4B shows a gas chromatogram obtained from a standard 100-pg pentafluorobenzoyl derivative of methamphetamine and amphetamine, and Fig. 4C shows a gas chromatogram from an extract of urine containing methamphetamine and amphetamine (50 ng/ml).

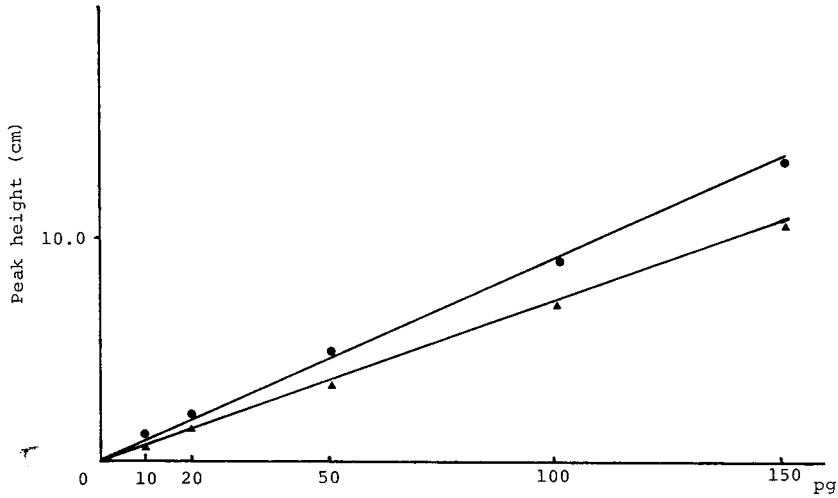

Fig. 3. Calibration curves of the pentafluorobenzoyl derivatives of methamphetamine (●) and amphetamine (▲).

Retention times of the pentafluorobenzoyl derivatives of methamphetamine and amphetamine using various packing materials are listed in Table III. When Thermon-3000 was used as packing material, the pentafluorobenzoyl derivatives of methamphetamine and amphetamine were separated clearly and had retention times of 3.3 min and 6.9 min, respectively. Separation of the pentafluorobenzoyl derivatives

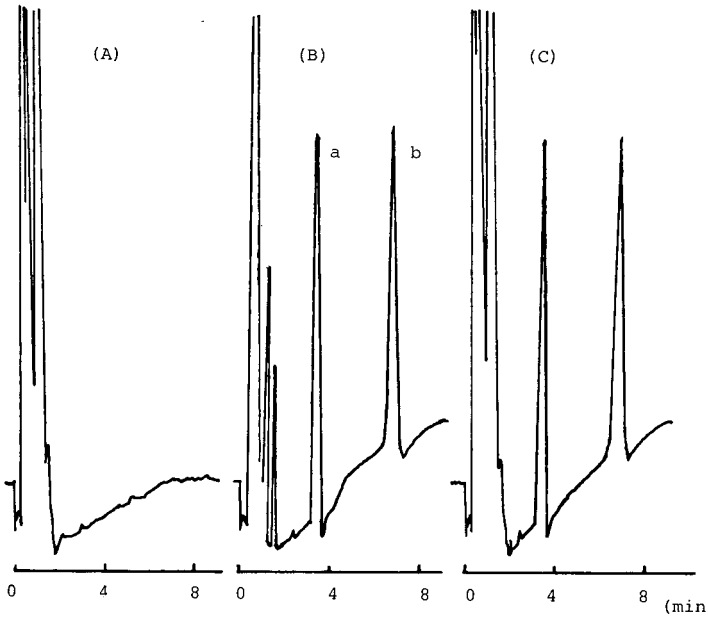

Fig. 4. Gas chromatograms of (A) extract from blank urine, (B) 100 pg of pentafluorobenzoyl chlorides of methamphetamine (a) and amphetamine (b), and (C) extract from urine containing 50 ng/ml methamphetamine and amphetamine. Retention times for (a) and (b) were 3.3 and 6.9 min, respectively.

TABLE III

RETENTION TIMES OF PENTAFLUOROBENZOYL DERIVATIVES OF METHAMPHETAMINE AND AMPHETAMINE

Compound	*Retention time (min)*			
	3% QF-1, 2 m, 200°C	*1.5% SE-30, 2 m, 200°C*	*2% OV-17, 3 m, 220°C*	*2% Thermon-3000, 2 m, 200°C*
Methamphetamine	6.92	5.01	6.98	3.32
Amphetamine	6.65	4.72	6.95	6.86

TABLE IV

RECOVERIES OF METHAMPHETAMINE AND AMPHETAMINE FROM URINE

Methamphetamine and amphetamine added to 0.2 ml of urine	*Recovery (%)**	
	Methamphetamine	*Amphetamine*
5 ng	107.1 ± 0.05	100.2 ± 0.06
50 ng	93.9 ± 0.85	98.7 ± 0.60

* Mean ± standard error.

of methamphetamine and amphetamine was incomplete when OV-17 or OV-225 was used as the packing material.

Recovery from urine

The results of the recovery experiments of methamphetamine and amphetamine, added to urine, are shown in Table IV. The average percentage recoveries for methamphetamine and amphetamine were 93.9–107.1 and 98.7–100.2, respectively. Because the recoveries of both methamphetamine and amphetamine when added to urine were satisfactory, the assay method presented would be applicable for the determination of these amines in biological materials.

Bruce and Maynard[20] have reported the determination of heptafluorobutyryl derivatives of amphetamine and related amines in blood by GC–ECD. The detection limit of methamphetamine and amphetamine by their method was 400 pg. Driscoll *et al.*[21] used the trichloroacetamide derivative for the determination of methamphetamine, with a detection limit of 25 pg. They reported that the derivative of methamphetamine showed a greater sensitivity than those of heptafluorobutyramide and pentafluorobenzamide.

In the present study, the detection limit of the pentafluorobenzoyl derivatives of methamphetamine and amphetamine was shown to be *ca.* 10 pg. This indicates that a concentration as low as 10 ng/ml of methamphetamine or amphetamine in urine can easily be detected by this procedure.

Matin and Rowland[22] have shown that the order of electron-capture sensitivity for primary amines is as follows; pentafluorobenzamide > pentafluorobenzylidine > heptafluorobutyramide, and that primary amines exhibit a greater sensitivity

than secondary amines. In the present study, however, it was found that the sensitivities of pentafluorobenzoyl derivatives of methamphetamine and amphetamine were similar.

In conclusion, GC–ECD of both methamphetamine and amphetamine, after derivatization of the amines with pentafluorobenzoyl chloride, was rapid and highly sensitive and would be suitable for the determination of these amines in biological materials.

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Note

Isoelectric focusing in chloral hydrate

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Non-ionic detergents, especially Triton X-100 and Nonidet P-40 (NP-40), have found widespread application in the disaggregation and subsequent electrophoretic analysis of membrane proteins because, in contrast with sodium dodecyl sulphate, they allow recovery of enzyme activities and of relatively intact protein structures[1]. Recently, using immunoelectrophoretic techniques[2], it has been found that Triton X-100 binds to proteins and this phenomenon has been exploited for the electrophoretic separation of normal adult haemoglobin from foetal haemoglobin[3]. The same type of binding occurs also with NP-40, and this has led to the separation, by isoelectric focusing (IEF), of β and γ human globin chains, since the binding of this ligand alters the pK values of neighbouring groups and thus also the pI values of these two chains, allowing for an increased resolution[4]. Unfortunately, these two detergents also bind to carrier ampholytes, the amphoteric substances used to generate and stabilize the pH gradient in IEF[5]. These complexes are generally insoluble in 10% trichloroacetic acid and can only be leached out from the polyacrylamide gel by long and tedious washings in alcoholic solvents at high temperatures (60°C)[4,5]. Whilst searching for a membrane solvent which would not interfere with subsequent IEF analysis of the solubilized proteins, we noticed that chloral hydrate had been successfully used for the disaggregation and electrophoretic characterization of membrane proteins[6,7], as well as for the isopycnic banding of chromatin in density gradients ranging[8] from 1.4 to 1.6 g/cm^3. As chloral hydrate is a non-ionic compound, freely soluble in water at greater than 100% concentrations, is a strong membrane-disaggregating agent, does not interfere with polyacrylamide gel polymerization and does not seem to alter or modify proteins[6–8], it seemed ideally suited also for isoelectric focusing.

We now report how, contrary to previous reports, chloral hydrate in IEF severely modifies carrier ampholytes and proteins, leading to altered or abolished pH gradients and producing a highly heterogeneous spectrum of bands from homogeneous proteins.

Fig. 1 shows the pH gradients obtained in a control gel (pH 3.5–10) and in gels containing increasing amounts of chloral hydrate, from 30 to 100%. While the control shows a regular and approximately linear pH gradient spanning the pH range 3.5–10, the same gradient is modified to a pH 2.5–6 range in the presence of 30%

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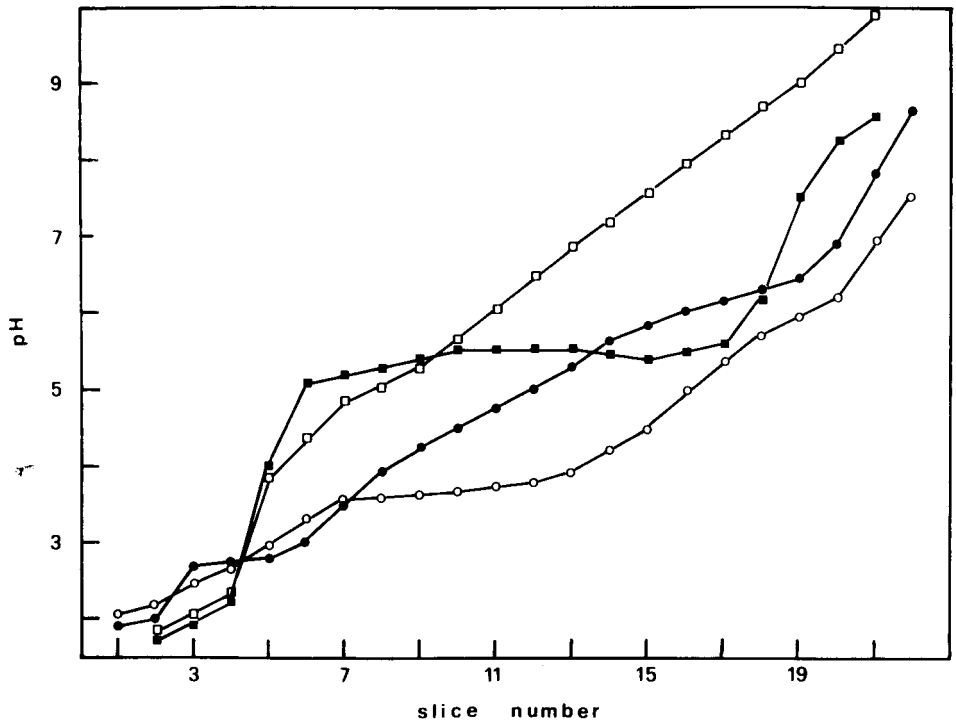

Fig. 1. IEF in the absence (□) and presence of 30 (●), 60 (○) and 100% (■) chloral hydrate. IEF was performed in a thin (0.7 mm) gel slab in an LKB Multiphor 2117 chamber with an LKB constant wattage power supply at 10°C, by applying 10 W (800 V at equilibrium) for 2 h. Gel: 7% acrylamide, 2% Ampholine pH 3.5–10. At the end, the pH gradient was measured at room temperature (22°C) by cutting 0.5-cm gel slices and eluting them with 300 μl of 10 m*M* potassium chloride.

chloral hydrate and to a pH 2.5–7 range in gels containing 60% chloral hydrate. Moreover, in the presence of 100% aldehyde, the pH gradient is completely abolished and, except for the large pH jumps at the extremes (presumably due to 1 *M* orthophosphoric acid and 1 *M* sodium hydroxide electrolyte solution diffusion into the gel), most of the gel length is covered by a wide plateau at pH *ca.* 5.5. We have interpreted this as extensive reaction of the amino groups of carrier ampholytes with the aldehyde, giving rise to Schiff bases. The carrier ampholytes thus lose positive groups and become progressively acidic. This reaction is more severe in alkaline pH ranges, which contain a greater number of deprotonated, and thus more reactive, amino groups, as compared with acidic pH ranges, which contain an average of four to five positively charged groups in an ordered sequence[9–11].

In order to see whether proteins could also be modified by reaction with chloral hydrate, we have performed IEF, in gels containing 8 *M* urea, of purified α, β and γ globin chains either untreated or incubated in 100% aldehyde. The treated samples were incubated either at pH *ca.* 2 (the pH of an aqueous solution of 100% chloral hydrate) or at pH 7 (it is impossible to titrate the aldehyde to pH 8 or above, as it is decomposed into chloroform and formic acid). As shown in Fig. 2, the pH 7 samples react to a great extent (80–90%) producing a highly heterogeneous spectrum of lower

p*I* components. This behaviour is consistent with a loss of positive charges, *i.e.* with reaction of amino groups with the aldehyde. Interestingly, even the samples incubated at pH *ca.* 2 are not completely unreactive, as each globin chain exhibits traces of lower p*I* species, clearly not present to the same extent and at the same p*I* values in the controls. By a densitometric evaluation of the IEF patterns it appears that about 5% protein has reacted, which should put a note of caution also on the use of electrophoretic techniques in lactate buffers at pH 2.5–3.0[6,7].

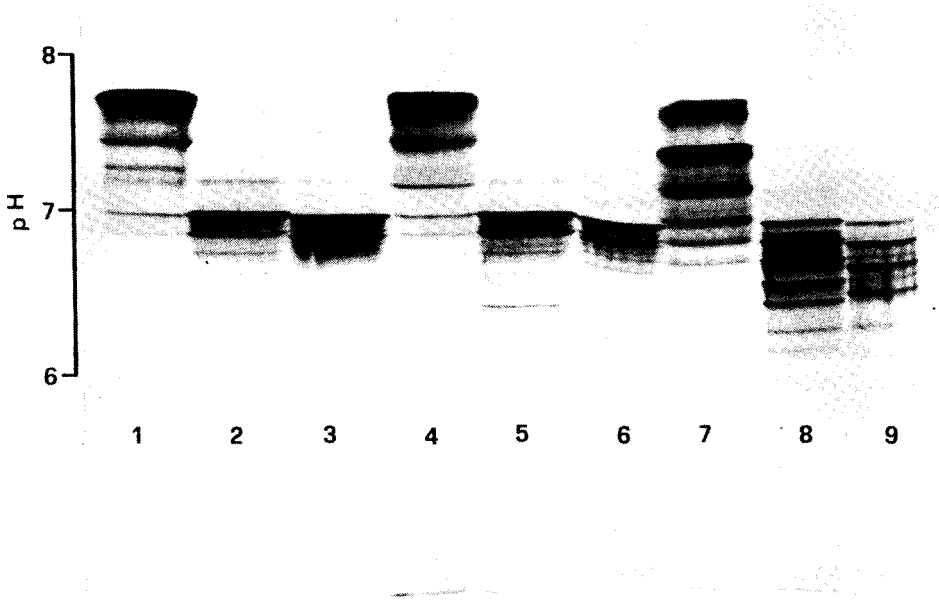

Fig. 2. IEF of α, β and γ human globin chains. The globins were chromatographically pure, haem-free chains. Slots 1–3: untreated α, β and γ chains, respectively. Slots 4–6: as for samples 1–3, except that the chains were incubated for 30 min at 25°C in 100% chloral hydrate, pH *ca.* 2. Slots: 7–9 as for samples 1–3, except that the globins were incubated for 30 min at 25°C in 100% chloral hydrate pH 7. The sample (30 μg) was applied soaked onto strips of filter paper to a pre-focused gel slab, containing 8 *M* urea and 2% Ampholine pH 3.5–10, from the anodic side. Running time: 4 h at 10 W. All other conditions as in Fig. 1. Staining: after the method of Blakesley and Boezi[12].

In conclusion, IEF in 100% chloral hydrate does not appear to be a feasible technique. However, it is of interest to notice that, by treatment with 30 or 60% aldehyde, it is possible to transform a wide Ampholine pH range (pH 3.5–10) into a narrow pH range (*e.g.* pH 2.5–5) which appears to buffer particularly well in the pH region 2–4, where commercial carrier ampholytes behave rather poorly. Thus, this reaction might be exploited to produced narrow-range carrier ampholytes tailored to special separation problems.

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Note

Some N-acyl derivatives of 1-(α-naphthyl)ethylamine as stationary phases for the separation of optical isomers in gas chromatography

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It was reported by Weinstein *et al.*[1] that it suffices for a chiral stationary phase to contain an amide group and an asymmetric carbon atom, attached to the nitrogen atom [RCONHCH(CH_3)R′], in order to show selectivity in its interaction with the enantiomers of amides, and that the best efficiency is obtained when R′ is aromatic, particularly α-naphthyl, as in N-lauroyl-(*S*)-1-(α-naphthyl)ethylamine. During the course of our examinations of the influence on the enantioselectivity when the structure of the amide phase is converted to contain two asymmetric carbon atoms attached to both nitrogen and carbon atoms of the amide group, we[2] have found that N-(1*R*,3*R*)-*trans*-chrysanthemoyl-(*R*)-1-(α-naphthyl)ethylamine shows excellent enantioselectivity compared to that of N-lauroyl-(*R*)-1-(α-naphthyl)ethylamine. This result suggested that other N-acyl derivatives of (*R*) or (*S*)-1-(α-naphthyl)ethylamine should also show high stereoselectivity for enantiomeric amides. In this paper we report the chromatographic properties of four new amides derived from (*R*)- and (*S*)-1-(α-naphthyl)ethylamine with (*S*)-mandelic acid and (*S*)-proline as optically active stationary phases.

EXPERIMENTAL

Synthesis of stationary phases

O-Lauroyl-(S)-mandelic acid (S)-1-(α-naphthyl)ethylamide (phase I). To a stirred solution of (*S*)-mandelic acid (0.015 mol), (*S*)-1-(α-naphthyl)ethylamine (0.015 mol) and 1-hydroxybenzotriazole (0.017 mol) in tetrahydrofuran (30 ml) kept at 0°C, N,N′-dicyclohexylcarbodiimide (0.016 mol) in tetrahydrofuran (10 ml) was added dropwise. The mixture was first stirred in an ice-bath for 2 h and then at room temperature for 2 h, filtered and the solution evaporated under reduced pressure. The residue was dissolved in ethyl acetate and the solution was washed successively with 2 *N* citric acid, saturated sodium bicarbonate solution and water. After drying over sodium sulphate, the crude product was purified by column chromatography on silica gel. The fraction eluted with chloroform was (*S*)-mandelic acid (*S*)-1-(α-naphthyl)ethylamide, which was identified by nuclear magnetic resonance (NMR) and mass spectrometry. Phase I was obtained from the above amide (0.004 mol) by reaction with lauroyl chloride (0.008 mol) in dry dioxan (20 ml) in the presence of

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pyridine (0.01 mol) at 100°C for 4 h. After removal of the solvent under reduced pressure, the residue was dissolved in ethyl acetate and the solution washed successively with 1 *N* hydrochloric acid, saturated sodium bicarbonate solution and water. After drying over sodium sulphate, the crude product was purified by column chromatography on silica gel. The fraction eluted with hexane–ethyl acetate (1:1) was the desired compound (phase I), as demonstrated by NMR and mass spectrometry (found: C, 78.5; H, 8.6; N, 2.9; calculated for $C_{32}H_{41}NO_3$: C, 78.8; H, 8.5; N, 2.9%); $[\alpha]_D^{20}$ + 37° (*c* = 0.16% in chloroform); m.p. 86–88°C.

O-Lauroyl-(S)-mandelic acid (R)-1-(α-naphthyl)ethylamide (phase II). Phase II was synthesized using (*R*)-1-(α-naphthyl)ethylamine instead of (*S*)-1-(α-

TABLE I

GAS CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS

Chromatographed on 40 m × 0.25 mm I.D. glass capillary columns. Carrier gas: helium at 0.7–1.0 ml/min.

Compound	*Column temp. (°C)*	*Optically active stationary phase*		
		Phase I		
		*Retention time (min)**		*α***
		1st peak	*2nd peak*	
N-TFA Amines				
2-Octylamine	100	36.58	37.89	1.036
1-Phenylethylamine	100	115.5(+)	127.5(−)	1.104
1-(α-naphthyl)ethylamine***	150	100.4(+)	110.9(−)	1.105
N-TFA Amino acid isopropyl esters				
Alanine	100	12.03(D)	12.61(L)	1.048
Valine	100	14.89(D)	15.53(L)	1.043
Leucine	100	35.60(D)	36.87(L)	1.036
O-TFA α-hydroxycarboxylic acid isopropyl ester				
Mandelic acid	100	37.62(−)	38.57(+)	1.025
Carboxylic acid tert.-butyl amides				
2-Phenylpropionic acid	130	63.78(+)	68.44(−)	1.073
3,3-Dimethyl-2-ethylbutyric acid	100	34.09		1.000
2-Bromo-3,3-dimethylbutyric acid	100	57.79(−)	63.23(+)	1.094
2-(4-Chlorophenyl)isovaleric acid	150	128.3(+)	136.7(−)	1.065
Carboxylic acid ethyl esters				
trans-Chrysanthemic acid	100	17.36		1.000
cis-Chrysanthemic acid	100	16.65		1.000
trans-3-(2,2-Dichlorovinyl)-cyclopropanecarboxylic acid	100	65.13		1.000
cis-3-(2,2-Dichlorovinyl)-cyclopropanecarboxylic acid	100	50.56		1.000
Nitriles				
2-(2-Fluorophenyl)isovaleronitrile	100	41.89	42.16	1.006
2-(4-Chlorophenyl)isovaleronitrile	120	113.1	115.2	1.019
Alcohol				
Pantoyl lactone	100	46.40	46.92	1.011

* Measured from solvent peak.

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

*** Resolved as N-pentafluoropropionyl derivative.

naphthyl)ethylamine according to the procedure described in the synthesis of phase I. The final product had NMR and mass spectra which agreed with the expected structure (found: C, 78.2; H, 8.8; N, 3.1; calculated for $C_{32}H_{41}NO_3$: C, 78.8; H, 8.5; N, 2.9%); $[\alpha]_D^{20}$ + 51° (*c* = 0.18% in chloroform); m.p. 56–57°C.

N-Lauroyl-(S)-proline (S)-1-(α-naphthyl)ethylamide (phase III). To a stirred solution of N-*tert.*-butoxycarbonyl-(*S*)-proline (0.01 mol), (*S*)-1-(α-naphthyl)-ethylamine (0.01 mol) and 1-hydroxybenzotriazole (0.011 mol) in tetrahydrofuran (20 ml) kept at 0°C, N,N′-dicyclohexylcarbodiimide (0.011 mol) in tetrahydrofuran (10 ml) was added dropwise. The mixture was first stirred in an ice-bath for 2 h and then at room temperature for 2 h, filtered and the solution was evaporated

Phase II			*Phase III*			*Phase IV*		
*Retention time (min)**		α**	*Retention time (min)**		α**	*Retention time (min)**		α**
1st peak	*2nd peak*		*1st peak*	*2nd peak*		*1st peak*	*2nd peak*	
37.57	38.44	1.023	37.36	39.70	1.048	81.30		1.000
94.93(−)	103.7(+)	1.092	168.0(+)	187.6(−)	1.117	234.7(−)	247.0(+)	1.052
111.8(−)	118.5(+)	1.060	88.75(+)	100.2(−)	1.129	214.0(−)	222.4(+)	1.039
10.43(L)	10.78(D)	1.034	8.40(D)	8.82(L)	1.050	17.52(L)	18.04(D)	1.030
13.16(L)	13.60(D)	1.033	9.10(D)	9.62(L)	1.057	19.30(L)	20.14(D)	1.044
31.59(L)	32.36(D)	1.024	26.25		1.000	54.60(L)	57.69(D)	1.057
37.15(+)	38.08(−)	1.025	33.29(−)	34.57(+)	1.038	36.40		1.000
63.82(−)	67.37(+)	1.056	57.95(+)	63.49(−)	1.096	61.43(−)	63.62(+)	1.036
30.38	31.14	1.025	20.96		1.000	49.78	50.90	1.022
55.90(+)	59.33(−)	1.061	45.47(−)	51.49(+)	1.132	109.3(+)	116.0(−)	1.061
136.5(−)	142.6(+)	1.045	105.0(+)	111.4(−)	1.061	218.1(−)	224.5(+)	1.029
16.85	16.78	1.012	8.16		1.000	18.63		1.000
16.01	16.21	1.012	8.16		1.000	18.63		1.000
64.43	65.52	1.017	33.73	34.09	1.011	78.49	79.17	1.009
49.97	50.91	1.019	26.54		1.000	61.65		1.000
40.35		1.000	21.93		1.000	50.98		1.000
135.4	136.8	1.010	65.81	67.45	1.025	158.0		1.000
44.57		1.000	51.60	54.50	1.054	116.4	118.5	1.018

under reduced pressure. The residue was dissolved in ethyl acetate and the solution was washed successively with 2 *N* citric acid and water. The solvent was evaporated under reduced pressure, the residue was dissolved in 4 *N* hydrochloric acid in dioxan (20 ml) and the solution was stirred at room temperature for 6 h. The solution was evaporated under reduced pressure, and the residue was dissolved in chloroform and extracted with water. The aqueous layer was made alkaline with ammonia solution and then re-extracted with chloroform. The extract was dried over sodium sulphate and evaporation of the chloroform gave (*S*)-proline-(*S*)-1-(α-naphthyl)ethylamide, which was identified by NMR and mass spectrometry. Phase III was obtained from the above amide (0.005 mol) by reaction with lauroyl chloride (0.009 mol) in dry dioxan (20 ml) in the presence of pyridine (0.01 mol) at room temperature for 2 h. After evaporation of the solution under reduced pressure, the residue was dissolved in ethyl acetate and the solution was washed succesively with 1 *N* hydrochloric acid, saturated sodium bicarbonate solution and water. After drying over sodium sulphate, the crude product was purified by column chromatography with silica gel. The fraction eluted with chloroform was the desired compound (phase III), as demonstrated by NMR and mass spectrometry (found: C, 77.3; H, 9.6; N, 6.1; calculated for $C_{29}H_{42}N_2O_2$: C, 77.3; H, 9.4; N, 6.2%); $[\alpha]_D^{20}$ − 66° (*c* = 0.23% in chloroform); m.p. 59–62°C.

N-Lauroyl-(S)-proline (R)-1-(α-naphthyl)ethylamide (phase IV). Phase IV was synthesized using (*R*)-1-(α-naphthyl)ethylamine instead of (*S*)-1-(α-naphthyl)-ethylamine according to the procedure described for the synthesis of phase III. The final product had NMR and mass spectra which agreed with the expected structure (found: C, 77.3; H, 9.7; N, 6.2; calculated for $C_{29}H_{42}N_2O_2$: C, 77.3; H, 9.4; N, 6.2%); $[\alpha]_D^{20}$ − 114° (*c* = 0.28% in chloroform) m.p. 67–68°C.

Thermogravimetric analysis

The experiments were performed with a Rigaku Thermoflex instrument provided with a thermobalance. The amount of sample was 8–12 mg. The temperature was raised at a rate of 10°C/min using α-alumina as the reference between 50 and 450°C.

Gas chromatography

The experiments were carried out with a Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector. The glass capillary columns (40 m × 0.25 mm I.D.) were coated with a 5% solution of each stationary phase in chloroform.

RESULTS AND DISCUSSION

The gas chromatographic results are given in Table I. The four stationary phases (I–IV) show excellent enantioselectivity. They separate amino acid, amine and carboxylic acid enantiomers, although they do not show very much higher separation factors than N-lauroyl-(*S*)-1-(α-naphthyl)ethylamine[1] which contains only one asymmetric centre. It should be emphasized that some carboxylic acid ester, nitrile and alcohol enantiomers can be separated on these new amides as well as on N-(1*R*,3*R*)-*trans*-chrysanthemoyl-(*R*)-1-(α-naphthyl)ethylamine which was reported by us previously[2]. It is interesting that phase II partially separates all four isomers of ethyl

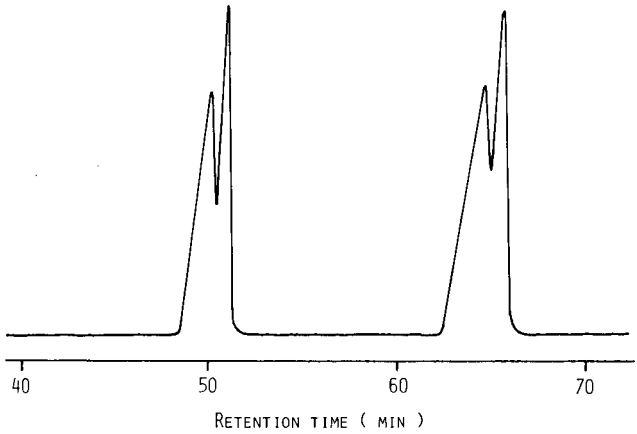

Fig. 1. Gas chromatogram of racemic ethyl *cis,trans*-3-(2,2-dichlorovinyl)cyclopropanecarboxylate. Column, glass capillary column (40 m × 0.25 mm I.D.) coated with O-lauroyl-(*S*)-mandelic acid (*R*)-1-(α-naphthyl)ethylamide; temperature, 100°C; carrier gas (helium) flow-rate, 0.9 ml/min.

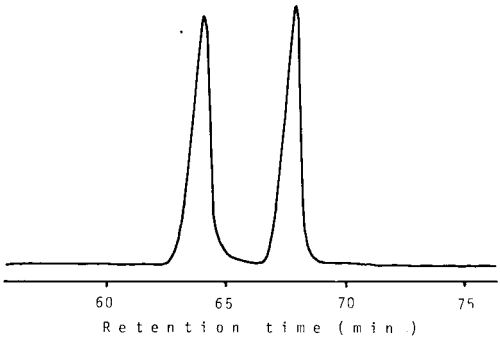

Fig. 2. Gas chromatogram of racemic 2-(4-chlorophenyl)isovaleric acid isopropylamide. Column, glass capillary column (40 m × 0.25 mm I.D.) coated with O-lauroyl-(*S*)-mandelic acid (*S*)-1-(α-naphthyl)ethylamide; temperature, 180°C; carrier gas (helium) flow-rate, 0.7 ml/min.

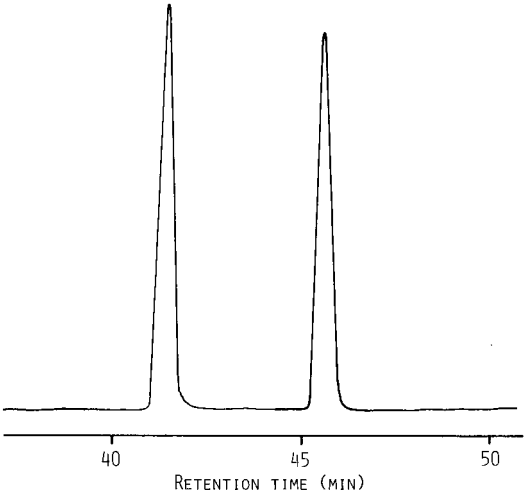

Fig. 3. Gas chromatogram of racemic N-pentafluoropropionyl-1-(α-naphthyl)ethylamine. Column, glass capillary column (40 m × 0.25 mm I.D.) coated with N-lauroyl-(*S*)-proline (*S*)-1-(α-naphthyl)ethylamide; temperature, 180°C; carrier gas (helium) flow-rate, 1.0 ml/min.

chrysanthemate and ethyl 3-(2,2-dichlorovinyl)cyclopropanecarboxylate, to our knowledge for the first time. A typical chromatogram is shown in Fig. 1.

It is also notable that these new amides have excellent thermal stability. Thermogravimetric analysis showed that bleeding should start only at about 200°C. As can be seen in Figs. 2 and 3, stable baselines were obtained when operating at 180°C with the instrument set at 8×10^{11} A full-scale deflection. We suggest these new phases could be useful for the separation of optical isomers of some less volatile N-acyl amines or carboxylic acid amides.

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Note

β-Cyclodextrin as a chiral component of the mobile phase for separation of mandelic acid into enantiomers in reversed-phase systems of high-performance liquid chromatography

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Separation of racemates into enantiomers is an essential procedure in organic chemistry, particularly in the total synthesis of natural products. Although this problem has been studied for a long time in many laboratories it has not so far been solved. High-performance liquid chromatography (HPLC) with the use of chiral substances as additives to the stationary phase[1,2] or to the eluent[3,4] is one of the most important, although not well elucidated, methods for racemate separation. β-Cyclodextrin forms diastereomeric inclusion complexes with many chiral organic compounds and has also been successfully used as a component of the stationary phase for racemate separation by HPLC[5,6]. Aqueous solutions of cyclodextrins have proven to be an effective mobile phase in thin-layer chromatographic (TLC) separations of mixtures of isomers[7,8] and in HPLC separations of prostaglandins[9]. No attempts have so far been made to use such a system for the resolution of enantiomers.

The aim of this work was to determine whether β-cyclodextrin may be appropriate as an optically active component of the mobile phase solutions for the resolution of racemates in reversed-phase HPLC systems. The resolution of mandelic acid, both enantiomers of which are readily accessible, has now been attempted.

EXPERIMENTAL

All reagents were p.a. grade. β-Cyclodextrin (CyD) was supplied by Chinoin, Budapest, Hungary.

Chromatographic measurements were performed using a HPLC unit constructed at the Institute of Physical Chemistry, P.A.N., Warsaw, Poland, equipped with a 5-μl high-pressure injection valve and a Z-shaped detection passage (volume, 8 μl)[10]. Chromatograms were recorded using a Hewlett-Packard 7101 strip chart recorder. For HPLC, use was made of stainless-steel columns (250 × 4 mm I.D.), slurry packed at 435 kg/cm^2 by a "balanced weight" technique with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) as sorbent.

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The mobile phases consisted of aqueous solutions containing various concentrations of CyD and 0.1 *M* phosphate buffer (pH 2.1). All investigated samples were artificial mixtures prepared from pure enantiomers of mandelic acid (MA) dissolved in the mobile phase. The experiments were performed at 22 ± 1°C.

RESULTS AND DISCUSSION

Fig. 1 illustrates the behaviour of the values of the term $(CyD)_m/(V'_0 - V_{obs})$ for (*R*)-(−)- and (*S*)-(+)-mandelic acid as a function of the β-cyclodextrin concentration. The plots enable evaluation of the stability constants of both diastereomeric complexes [(*R*)-(−)-MA–CyD and (*S*)-(+)-MA–CyD], using equation 1, derived by Uekama *et al.*[11]:

$$\frac{(CyD)_m}{V'_0 - V_{obs}} = \frac{1}{V'_0 - V_c}(CyD)_m + \frac{1}{K_c(V'_0 - V_c)} \quad (1)$$

where $(CyD)_m$ is the β-cyclodextrin concentration (moles/l), V'_0, V_c, V_{obs} are the retention volumes of mandelic acid itself, of the inclusion complex of one of its enantiomers [(*R*)-(−)-MA–CyD) or (*S*)-(+)-MA–CyD] and of the corresponding enantiomer observed at a given CyD concentration, respectively, and K_c is the stability constant of a given inclusion complex in the mobile phase:

$$K_c = \frac{(MA\text{–}CyD)_m}{(MA)_m(CyD)_m}$$

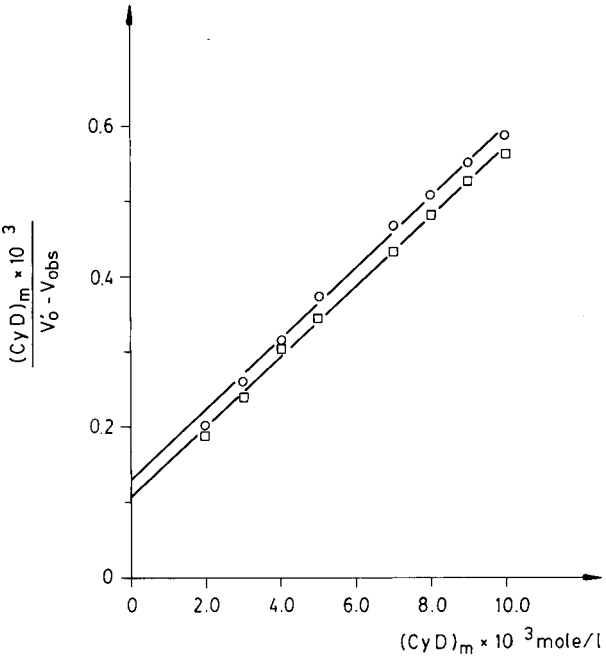

Fig. 1. Values of the term $(CyD)_m/(V'_0 - V_{obs})$ for (*R*)-(−)- and (*S*)-(+)-mandelic acid *versus* CyD concentration in the mobile phase solution.

TABLE I

EQUILIBRIUM DISTRIBUTION COEFFICIENT OF MANDELIC ACID (k), EQUILIBRIUM DISTRIBUTION COEFFICIENTS OF INCLUSION COMPLEXES FORMED WITH CyD BY ITS ENANTIOMERS (k_c) AND THE STABILITY CONSTANTS OF THESE COMPLEXES (K_c)

Compound	k	k_c	K_c
(*R*)-(−)-MA	19.2	5.9	460
(*S*)-(+)-MA	19.2	6.3	410

Table I shows the equilibrium distribution coefficient (k) of mandelic acid, determined in 0.1 *M* aqueous phosphate buffer of pH 2.1, the equilibrium distribution coefficients (k_c) of inclusion complexes formed with CyD by its enantiomers determined in $1.2 \cdot 10^{-2}$ *M* CyD in the same buffer and the stability constants (K_c) of these complexes evaluated from the plots shown in Fig. 1 and from equation 1[11].

Equation 1, originally derived for ion-exchange sorbents[11] was adapted by us for the reversed-phase system on the assumption that only neutral organic species are adsorbed onto the stationary phase. This assumption is based on our findings that the capacity factors of MA and of its inclusion complexes are approximately zero in the mobile phase solution at pH $> pK_a + 2$, since the pK_a of MA amounts to 3.4 at pH > 5.4.

Two chromatograms for two consecutive injections of the mixture of MA enantiomers are shown in Fig. 2. It is evident that the enantiomers were eluted at different rates (selectivity factor $\alpha = k_1/k_2 = 1.05$).

Similar experiments were successfully performed using phenylalanine as an example of an amino acid.

The ability of β-cyclodextrin to form inclusion complexes with various organic compounds of acidic, basic and neutral character, together with knowledge of the

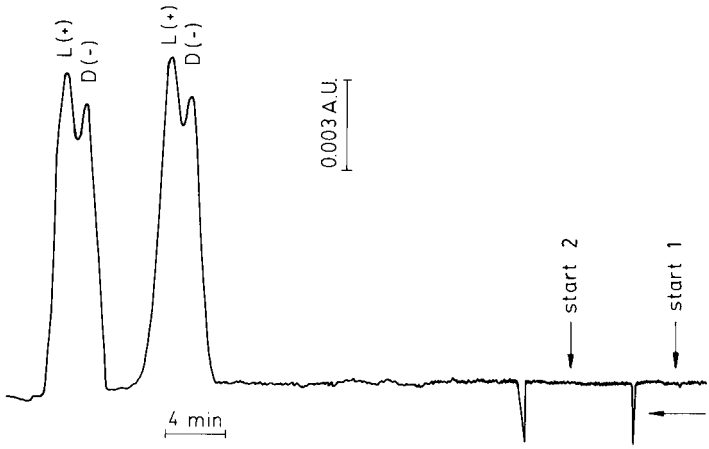

Fig. 2. Elution curves for two consecutive injections of the mixture of $2.10 \cdot 10^{-4}$ *M* mandelic acid enantiomers. Mobile phase composition, $5 \cdot 10^{-3}$ *M* CyD in aqueous phosphate buffer of pH 2.1 (0.1 *M*); flow-rate, 0.6 ml min^{-1}.

kinetics of the inclusion processes, may be of great importance in solving the problem of chromatographic racemate resolution. Attempts at improving the separative power in our system are under way.

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Note

Water-free solvent system for droplet counter-current chromatography and its suitability for the separation of non-polar substances

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With the development of new designs of apparatus, liquid–liquid chromatographic methods[1] have made a come-back. Of these methods, droplet counter-current chromatography[2] (DCCC) has been widely used and has proved successful in the separation of many natural products. Up till now, the limitations of the method have been that only relatively polar substances can be separated and also that there is a danger of hydrolysis occurring in the aqueous solvent systems, especially when relatively long separation times of between 1 day and 1 week are employed. Water-free solvent systems have been difficult to establish because they need to meet the main condition for DCCC: the formation of suitable droplets having more or less the diameter of the tubes. Using as a guide the work by Hecker[3] on partition chromatography, we have recently developed a water-free system that satisfies this condition.

Preliminary experiments[4] have shown the system to be suitable for the separation of non-polar valepotriates and of the essential oil of *Echinacea angustifolia*. However, these results need to be confirmed by the separation of a complex, but nevertheless well defined, mixture of natural compounds. We therefore selected the essential oil of chamomile (*Matricaria chamomilla*), the components of which (Fig. 1) can be qualitatively and quantitatively examined by gas chromatography (GC)[5].

MATERIALS AND METHODS

Apparatus

A DCC-A instrument (Tokyo Rikakikai Co., Tokyo, Japan) with 300 columns (0.2 mm I.D.) was employed. The pressure was 4 kg/cm^2 and each fraction consisted of 120 drops collected at a rate of 4 drops per min.

Essential oil of Matricaria chamomilla

Flowers of *M. chamomilla* (from Argentina) were distilled in 10-g portions, according to the European Pharmacopoeia. The essential oil from each distillation was collected in 2 ml of pentane, the solution was dried, and the pentane was evaporated under nitrogen.

Gas chromatography

The total essential oil and the combined fractions were chromatographed using

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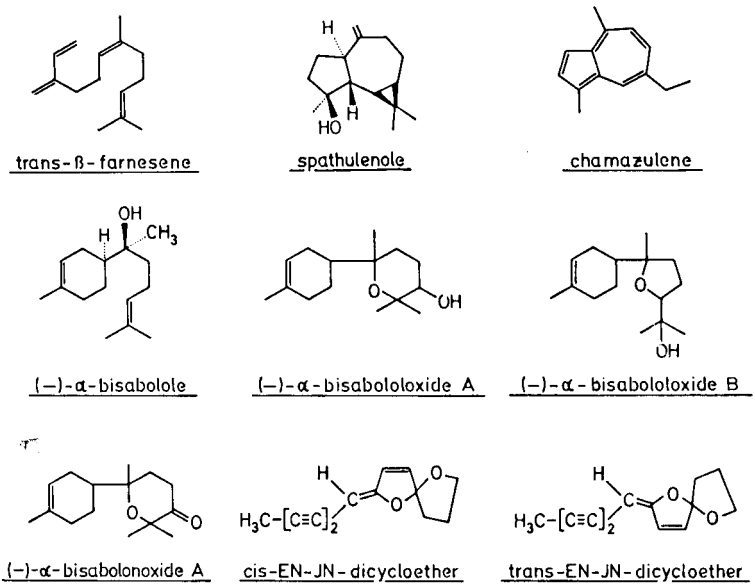

Fig. 1. Known components of the essential oil of *M. chamomilla*.

a Varian chromatograph fitted with a Methyl-Silicon (fused-silica capillary) 19091-60050 column (Hewlett-Packard, Avondale, PA, U.S.A.) of length 50 m. The following conditions were employed: injection temperature, 230°C; detection temperature, 250°C; temperature programmed to 140°C at 4°C/min; pressure, 1.5 bar; sensitivity, $36 \cdot 10^{-11}$; injection volume, 0.2 µl. For the total essential oil, guajazulene was used as internal standard. The purity of the combined fractions was calculated from the peak areas.

Thin-layer chromatography

Each fraction was examined by thin-layer chromatography (TLC) on silica gel 60 G (E. Merck, Darmstadt, G.F.R.) using 10 × 6.6 cm plates. For conditions, see ref. 5. Identical fractions were combined.

RESULTS AND DISCUSSION

The essential oil of *M. chamomilla* was found to have the constitution shown in Table I.

After analysis by TLC, the essential oil was split into ten fractions which were examined by GC. The main components are listed in Table II. As can be seen, four fractions have compounds with a purity greater than 90%. This means after just one run, four of the seven known constituents of the mixture can be isolated almost pure. The amounts of these compounds, starting from 0.75 g of essential oil (*i.e.* farnese, 24.3 mg; chamazulene, 40.5 mg; spathulenole, 15 mg; bisaboloxide B, 36.2 mg) would be enough to enable the necessary spectra for structural elucidation to be run.

If the purity is insufficient for this purpose, a second run can be performed with

TABLE I

COMPOSITION OF ESSENTIAL OIL OF *MATRICARIA CHAMOMILLA*

Guajazulene as internal standard.

Constituent	*Content (mg per 100 ml)*
Farnesene	12
Bisabololoxide B	35
Bisabolole	6
Spathulenole	1.2
Bisabololoxide A	7.8
Chamazulene	6.1
cis-EN-IN-Dicycloether	6.4

either the same or a different solvent system. After pre-screening on a silica plate, according to the method of Hostettmann[2], we chose the system *n*-hexane–ethylacetate–nitromethane–methanol (9:1.5:4.5:1.5, v/v), with the upper phase as mobile phase. Fractions g + h (90–104) from the first run (0.1568 g) gave the fractions shown in Table III.

The essential oil obtained from steam distillation of chamomile always contains fatty acids. After a DCCC separation using 750 mg of volatile oil, 613 mg were recovered from the upper phase; over 90% of the fatty acids (*ca.* 130 mg) remained in the lower phase together with only a small amount of the volatile oil.

TABLE II

COMBINED FRACTIONS OF THE FIRST DCCC RUN

n-Hexane–ethyl acetate–nitromethane–methanol (9:2:2:3) as eluent; upper phase as mobile phase.

Fraction		*Weight (mg)*	*Main component(s)*	*Purity according to GC peak area (%)*
a	(1–21)	153	Farnesene	26
b	(22–38)	24.3	Farnesene	96
c	(39–55)	9.8	Farnesene	58
d	(56–72)	40.5	Chamazulene	96
e	(73–84)	15.0	Spathulenole	93
f	(85–89)	8.7	Bisabolole, bisabololoxide B, spathulenole	–
g	(90–98)	80.0	Bisabololoxide B	69
			Bisabolole	30
h	(99–104)	76.8	Bisabololoxide B	80
			Bisabolole	10
i	(105–106)	17.4	Bisabololoxide B	98
j	(107–109)	18.8	Bisabololoxide B	86
k	(110–113)	12.4	Mixture + bisabololoxide A	5
l	(114–123)	13.9	Mixture EN–IN–dicycloether	–
m	(124–282)	142	Mixture	–

TABLE III

FRACTIONS OF THE SECOND DCCC RUN OF FRACTIONS g AND h

n-Hexane–ethyl acetate–nitromethane–methanol (9:1.5:4.5:1.5) as eluent; upper phase as mobile phase.

Fraction		*Weight (mg)*	*Main component(s)*	*Purity according to GC peak area (%)*
0	(0–71)		–	–
A	(72–75)	0.6	Bisabolole	100
B	(76–78)	2.0	Bisabolole	53
			Bisaboloxide B	10
C	(79–84)	6.2	Bisabolole	78
			Bisabololoxide B	22
D	(85–89)	16.6	Bisabololoxide B	98
			Bisabolole	2
E	(90–93)	15.9	Bisabololoxide B	100
F	(94–102)	8.7	Bisabololoxide B	94
G	(103–113)	1.8	Bisabololoxide A	80

The constituents of the volatile oil showed a somewhat different elution pattern with DCCC from that obtained with GC. DCCC gives a separation according to the polarity of the components, *i.e.* farnesene is eluted first followed by chamazulene and spathulenole, with the oxygenated bisaboloids being eluted last. In GC, farnesene has the shortest retention time, followed by bisabololoxide B, bisabolole, bisabololoxide A, chamazulene and finally *cis–trans*-EN-IN-dicycloether.

On the basis of these results, DCCC with a water-free solvent system appears to be an appropriate method for the separation of apolar substances.

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Note

High-performance liquid chromatography of cytokinin ribonucleoside 5′-monophosphates

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(Received November 2nd, 1981)

The cytokinins, a group of naturally occurring compounds which regulate a variety of aspects of plant development, are N^6-substituted derivatives of adenine, which may occur as the free-base, ribonucleoside or ribonucleotide forms[1]. Although cytokinin nucleotides are common metabolites of exogenous cytokinins in plant tissues[2], and may be of significance in cytokinin biosynthesis[3], there have been few conclusive identifications of endogenous cytokinin nucleotides in contrast to numerous reports of the occurrence of base or nucleoside forms[1].

This situation is probably due to the lack of analytical methods for cytokinin nucleotides. Cytokinin purification procedures commonly involve the use of cation-exchange chromatography to separate the basic and acidic/neutral fractions of plant extracts. However, the latter fraction, in which nucleotides occur, is invariably of a viscous and pigmented nature, with a high content of compounds which are inhibitory in the bioassays widely used to screen for cytokinin activity. A common procedure is to degrade the nucleotides in the acidic/neutral fraction using enzymatic or chemical methods and then to analyse them as nucleosides or bases. However, we have found that in plant extracts these methods are unreliable. Furthermore, if employed at this stage, without any further characterization of the putative cytokinin nucleotides, this approach provides only weak evidence for an identification. For example, the poor performance of the ion-exchange step could result in a misidentification. Similarly, the cytokinin activity remaining in the aqueous fraction after butanol partitioning of an extract is often regarded as being due to nucleotides, which may then be degraded and re-analysed as nucleosides without any controls being performed. However, the partition coefficients of cytokinin glucosides are so low that there is often a residue of these compounds in the aqueous phase[4], and identifications of glucosylated cytokinin nucleotides obtained by such procedures[5] are questionable.

The identification of cytokinin nucleotides would be greatly enhanced by the use of high-resolution chromatographic procedures. High-performance liquid chromatography (HPLC) has been used increasingly in recent years for the analysis of cytokinin bases and nucleosides[6]. We have investigated the HPLC properties of some cytokinin ribonucleoside 5′-monophosphates which exhibit the full range of polarity of N^6-substituents found in nature. The complex nature of acidic/neutral fractions of plant extracts makes the isolation of cytokinin nucleotides a formidable task, and so

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we have attempted to develop a range of HPLC systems based on widely different separation mechanisms in order to maximize the purification achieved by a sequence of HPLC steps. Since we hope to use HPLC for preparative as well as analytical purposes, a further objective was to use solvents containing only volatile components.

MATERIALS AND METHODS

Synthesis of cytokinin ribonucleoside 5′-monophosphates

6-Chloropurine riboside 5′-monophosphate (Calbiochem) was refluxed for 3 h in butan-1-ol in the presence of a molar excess of 4-hydroxy-3-methylbut-*trans*-2-enylamine[7], 4-O-β-D-glucosyl-3-methylbut-*trans*-2-enylamine[8], or 3-methylbut-2-enylamine[9] for synthesis of the ribonucleoside 5′-monophosphates of zeatin (ZMP), zeatin-O-β-D-glucoside (ZGMP) and Δ^2-isopentenyladenine (iPMP) respectively. The reaction mixture was reduced to dryness *in vacuo* and the cytokinin nucleotide product purified by column chromatography on Sephadex LH-20 (Table I), followed by paper chromatography (Table II). Product identity was confirmed by ultraviolet and mass spectrometry, HPLC and thin-layer chromatography following degradation to the riboside form using alkaline phosphatase. Yields were usually greater than 70%.

TABLE I

SEPHADEX LH-20 CHROMATOGRAPHY OF CYTOKININ NUCLEOTIDES

A column of Sephadex LH-20 (71 × 2.5 cm) was eluted with 10 m*M* formic acid at a flow-rate of 30 cm^3 h^{-1} in the descending direction.

Compound	*Elution volume (cm^3)*
ZGMP	184
ZMP	222
iPMP	243

TABLE II

PAPER CHROMATOGRAPHY OF CYTOKININ NUCLEOTIDES

Descending chromatography on Whatman 3MM paper was carried out using butan-1-ol–acetic acid–water (12:3:5).

Compound	*R_F value*
ZGMP	0.16
ZMP	0.34
iPMP	0.50

Chromatographic equipment and materials

HPLC columns (150 × 4.5 mm I.D.) were slurry-packed at 6000 p.s.i. using a pneumatic amplifier pump. The slurry medium was acetone (for Hypersil ODS) or methanol (for Hypersil APS). Column materials were obtained from Shandon Southern, Runcorn, Great Britain.

Chromatography was carried out on a Pye LC3X system with the absorbance detector operating at 265 nm. Samples (70 μl) were introduced via an Altex 905-42 syringe-loading sample injector fitted with a 100-μl loop.

All solvents were glass-distilled prior to use. Triethylammonium bicarbonate was prepared by saturating a 2.5 *M* solution of triethylamine with carbon dioxide. Tetrabutylammonium hydroxide was obtained from Sigma.

Columns were washed and stored in methanol. Ion-pair reagent was flushed out of ODS columns with 0.1 *M* acetic acid prior to washing with methanol[10].

RESULTS AND DISCUSSION

The ribonucleoside 5′-monophosphates of the naturally occurring cytokinins zeatin (ZMP), zeatin-O-glucoside (ZMGP), and Δ^2-isopentenyladenine (iPMP) were synthesized and their HPLC properties were investigated. It was found that these compounds behaved in a much less polar fashion than the common nucleotides on reversed-phase HPLC on a C_{18} bonded stationary phase, and could be resolved without the use of phosphate-buffered solvents. The resolution was marginally improved by the use of 0.1 *M* acetic acid rather than water at pH 7, presumably due to ion suppression effects. Fig. 1 illustrates the separation of the cytokinin nucleotides on a column of Hypersil ODS eluted with a gradient of increasing concentration of methanol in 0.1 *M* acetic acid. iPMP elutes much later than the zeatin-related compounds, and requires a second gradient segment for elution in a reasonable time. It is surprising that ZMP elutes before ZGMP in this system in view of the polar nature of the glucosyl moiety. Possibly there is some shielding of the charged phosphate group by the large N^6-substituent in the case of ZGMP.

Ion-pair reversed-phase HPLC has been used for the separation of the common nucleotides[10]. The inclusion of the volatile ion-pair reagent tetrabutylammonium hydroxide in the reversed-phase system greatly altered the chromatographic

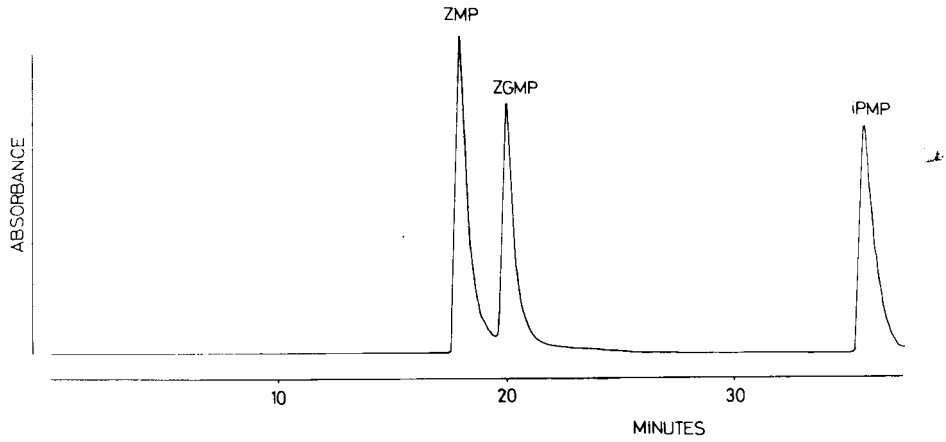

Fig. 1. Separation of cytokinin nucleotides by reversed-phase HPLC. A column (150 × 4.5 mm I.D.) of Hypersil ODS was eluted at a flow-rate of 2 $cm^3\ min^{-1}$ with a 2-segment linear gradient of methanol in 0.1 *M* acetic acid. Segment 1 = 0–15% methanol over 30 min, segment 2 = 15–80% methanol over 10 min. ZMP, ZGMP and iPMP are the ribonucleoside 5′-monophosphates of zeatin, zeatin-O-β-D-glucoside and Δ^2-isopentenyladenine respectively.

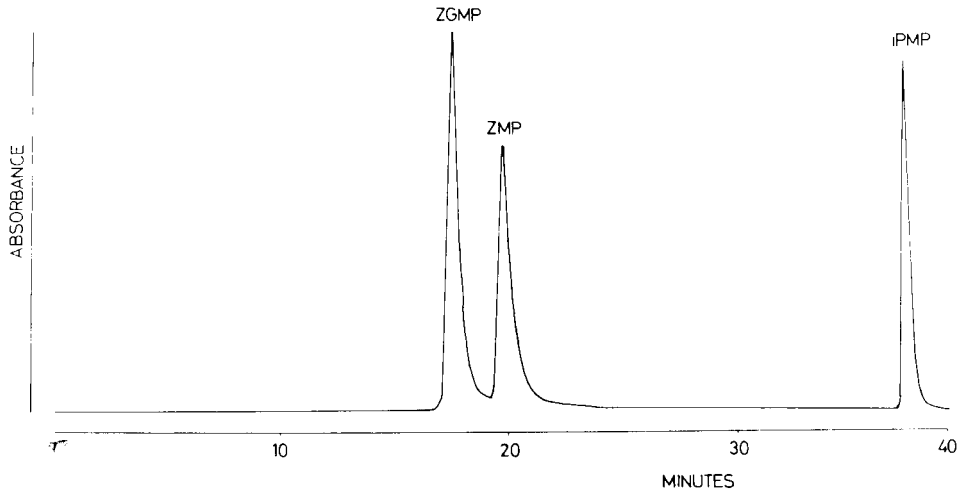

Fig. 2. Separation of cytokinin nucleotides by ion-pair reversed-phase HPLC. A column (150 × 4.5 mm I.D.) of Hypersil ODS was eluted at a flow-rate of 2 cm^3 min^{-1} with a 2-segment linear gradient of methanol in 0.1 *M* acetic acid containing 2.5 m*M* tetrabutylammonium hydroxide. Segment 1 = 15–30% methanol over 30 min, segment 2 = 30–90% methanol over 10 min. Abbreviations as in Fig. 1.

properties of the cytokinin nucleotides, making available a contrasting separation system. As shown in Fig. 2, a much higher concentration of methanol is required to elute the cytokinin nucleotides in the ion-pair system, and the elution sequence of ZMP and ZGMP is reversed. Presumably the counterion nullifies the charge on the phosphate group so that the polar nature of the glucosyl moiety of ZGMP becomes of greater significance in this system.

HPLC of nucleotides in a strong anion-exchange system would involve the use of involatile buffer salts[11], but it was found that the cytokinin nucleotides could be

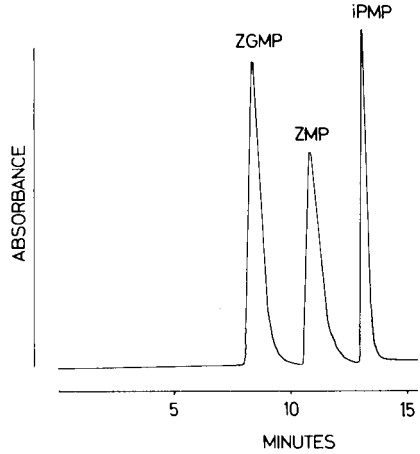

Fig. 3. Separation of cytokinin nucleotides by ion-exchange HPLC. A column (150 × 4.5 mm I.D.) of Hypersil APS was eluted at a flow-rate of 2 cm^3 min^{-1} with a linear gradient of 100 m*M* triethylammonium bicarbonate (0–40% over 20 min) in 10 m*M* ammonium bicarbonate. Abbreviations as in Fig. 1.

separated on a column of Hypersil APS, an aminopropyl-bonded silica with weak anion-exchange properties, using volatile buffers. Fig. 3 shows the separation achieved using a gradient of increasing proportion of 100 m*M* triethylammonium bicarbonate in 10 m*M* ammonium bicarbonate. Complete though slightly reduced resolution was also achieved using 1 m*M* acetic acid in place of ammonium bicarbonate. This system probably operates by gradual suppression of the ionization of the amino groups of the stationary phase as the pH of the solvent rises. The salts in the effluent may be removed by a few evaporations with methanol.

The cytokinin nucleoside di- and triphosphates seem to occur in some tissues[2] but not others[3]. The analysis of these compounds by HPLC would probably require the use of involatile buffer salts.

These HPLC systems have been used to identify chromatographically ZMP as an endogenous cytokinin (detected by bioassay) and as a metabolite of [^{14}C]zeatin in *Vinca rosea* crown gall tissue. We therefore anticipate that HPLC will provide an efficient and reliable method for the analysis of cytokinin nucleotides and should help to remedy the lack of knowledge of these potentially important compounds.

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Note

Use of ion-pair, reversed-phase, high-performance liquid chromatography for the analysis of cytokinins

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High-performance liquid chromatography (HPLC) is a valuable tool for the separation and measurement of plant growth substances. The combination of rapid analysis, high resolution and, frequently, the elimination of a derivitization step gives HPLC a marked advantage over other methods of separation including thin-layer, low-pressure column, and gas–liquid chromatography. In some of the early attempts to separate cytokinins by HPLC, ion-exchange columns were used[1], but the resins did not provide good resolution of the reference compounds, and serious difficulties were encountered in recovering the growth regulators from crude extracts. Improved resolution and increased sample capacity have been achieved using chemically bonded, organic, stationary phases over silica supports in what is commonly referred to as the reversed-phase mode[2–5]. During separation of plant growth regulators on these columns, small amounts of acetic acid are often added to the mobile aqueous phases to improve resolution by suppressing ion formation and reducing tailing of acidic compounds[3–6]. However, the resultant positive charges on the weakly basic cytokinins may mask slight differences in polarity usually associated with small dissimilarities in structure. Under these conditions, resolution of closely related compounds such as the *cis* and *trans* isomers would be expected to decrease. In an attempt to improve the separation of cytokinins with similar polarities, Holland *et al.*[7] investigated the use of ion-pair chromatography. Shortened retention times were achieved but no improvement in peak shape or resolution resulted from the addition of sodium lauryl sulphate to a mobile phase held at pH 5. In the present work, we describe the optimization of an ion-pair system and demonstrate its potential for resolving the geometric isomers of cytokinins isolated from plant material.

MATERIALS AND METHODS

Chromatographic equipment

Gradient-elution chromatography was performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) SP 8000 three-solvent system using two detectors, *viz.*, an SP 8200 dual-beam UV–visible unit and an SP 8400 variable-wavelength UV–visible unit operating at 254 and 269 nm, respectively. Samples were introduced via a nitrogen driven auto-injector port fitted with a 100-μl loop.

Prepacked columns (250 × 4.6 mm I.D. and 150 × 4.6 mm I.D.) of 5-μm

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Ultrasphere-I.P. and a semi-preparative column (250 × 10.0 mm I.D.) of 10-μm Ultrasil-ODS were purchased from Beckman (Berkeley, CA, U.S.A.). Two additional columns (250 × 4.6 mm I.D.) packed with LiChrosorb 10 RP-8 and RP-18 were obtained from Technical Marketing Associates (Mississauga, Canada).

Chemicals

All chromatographic solvents were HPLC grade (Caledon Labs., Georgetown, Canada), and all water was glass distilled and filtered through 0.2-μm MF Millipore (Bedford, MA, U.S.A.) filters.

The ion-pair reagents tested were N-1-heptanesulphonic acid, sodium salt monohydrate (Helix Assoc., Newark, DE, U.S.A.) and 1-octanesulphonic acid, sodium salt (Eastman Kodak, Rochester, NY, U.S.A.).

The cytokinin standards were purchased from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

Seeds of *Acer saccharum* Marsh. were incubated under moist conditions at 5°C for 20 days. Several 26-g samples were homogenized in 80% aqueous methanol, reduced *in vacuo* to the aqueous phase and then partitioned at pH 2.5 against ethyl acetate. After partitioning the ethyl acetate fraction against water-saturated *n*-butanol at pH 8, the butanol fraction was dried and the residue containing the cytokinins[8] was resuspended in a solution of 0.05 *M* KH_2PO_4 at pH 4 and slurried for 20 min with insoluble polyvinylpyrrolidone. The partially purified fraction was loaded onto a 3-ml C_{18} Bond Elut cartridge (Analytichem International, Lawndale, CA, U.S.A.) and washed with distilled water before eluting with methanol. The sample was then passed through a 0.2-μm filter and chromatographed on a reversed-phase semi-preparative column. Fractions with retention times corresponding to those of authentic standards were collected for further analysis by ion-pair chromatography.

RESULTS AND DISCUSSION

Several chromatographic parameters were investigated during development of the method. The data in Table I compare R_S values [resolution between two peaks, P_1 and P_2, where $R_S = 2\Delta t/(W_2 + W_1)$ and Δt = retention time of $P_2 - P_1$, W_2 = width of P_2, W_1 = width of P_1] between cytokinin pairs and demonstrate a marked improvement in resolution of a C_{18}-over a C_8-bonded stationary phase. Higher operating temperatures can also improve resolution by their effects on column efficiency, sample capacity and a decrease in k' values [capacity factor where k' = (peak retention time − void time)/void time] brought about by changes in diffusion coefficients and increased solubility of the sample components in the mobile phase[9]. Although the effects of temperature have not been considered in recent studies of methods development for cytokinins[2,3,10], the results in Fig. 1 show that a clear improvement in peak separation and a reduction in retention times was achieved by increasing column temperatures from 25 to 40°C.

Horgan and Kramers[2] concluded that resolution of cytokinins could be maximized on reversed-phase columns if the growth regulators were maintained in an unionized state at pH levels well above their pK_a values of about 4. Based on that premise, they developed an HPLC method using a triethylammonium bicarbonate

TABLE I

COMPARISON OF C_8 AND C_{18} REVERSED-PHASE HPLC COLUMNS FOR RESOLUTION (R_s VALUES, SEE TEXT) OF CYTOKININ PAIRS

Columns, Brownlee RP-18, 10 μm (250 × 4.6 mm I.D.) and Spectra-Physics RP-8, 10 μm (250 × 4.6 mm I.D.); flow-rate, 2 ml/min; temperature, 25°C. Mobile phase, a linear gradient of water (with 0.2 *M* acetic acid) to 30% acetonitrile (with 0.2 *M* acetic acid) over 30 min. Abbreviations: Z = zeatin; ZR = zeatin riboside; IPA = isopentenyl adenine; IPAR = isopentenyl adenosine.

Cytokinin pairs	*Resolution (R_s)*	
	C_8	C_{18}
trans-ZR + *trans*-Z	1.20	2.24
trans-ZR + IPA	11.23	25.36
trans-ZR + IPAR	12.06	26.25
trans-Z + IPA	11.01	24.70
trans-Z + IPAR	11.14	24.69
IPA + IPAR	0.72	0.76

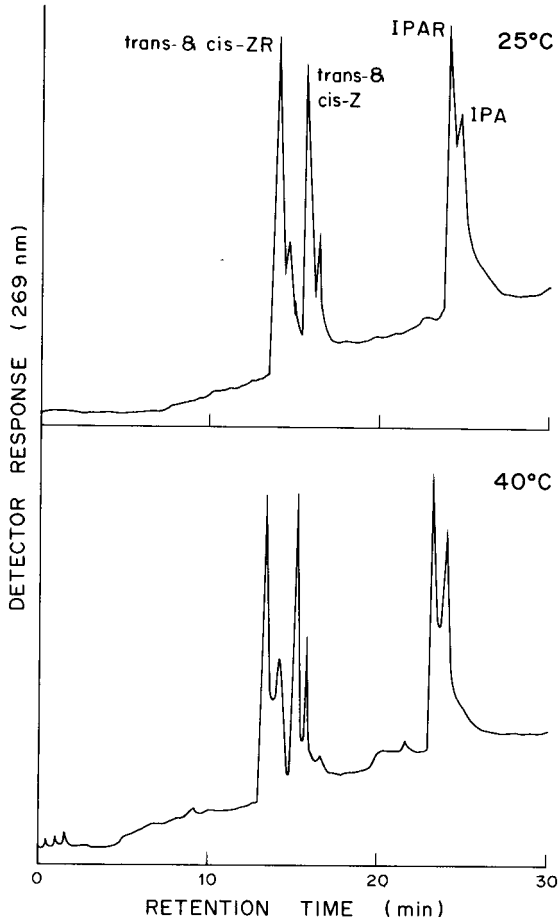

Fig. 1. Separation of cytokinin standards at ambient and elevated temperatures. Column, Brownlee RP-18, 10 μm (250 × 4.6 mm I.D.). Chromatographic conditions and abbreviations as in Table I.

buffer at pH 7 as the mobile phase. The separations achieved during our attempts to duplicate their procedure were not satisfactory due, apparently, to adsorption of cytokinins to the support matrix as evidenced by a drop in peak areas during repeated injections. Good separations were obtained by using ion-pair chromatography with a highly acidic solvent system at a pH of 2.65 and heptanesulphonate as the counter-ion. At higher pH levels, resolution was reduced and the peaks merged (Fig. 2). The use of buffer salts was avoided and the requisite pH maintained by making the solvent system 0.2 *M* with respect to acetic acid. Concentrations of 3–6 m*M* heptanesulphonate provided optimal separation of peaks while a 10 m*M* concentration prolonged analysis time without additional benefits. A larger counter-ion, *i.e.*, a longer alkyl chain, also increased retention time and k' values (see also ref. 11), but separation of the cytokinins was nevertheless reduced (Table II). These observations may explain the report by Holland *et al.*[7] that a C_{12} counter-ion dissolved in a mobile phase at pH 5 failed to provide any significant improvement in resolution or peak shape.

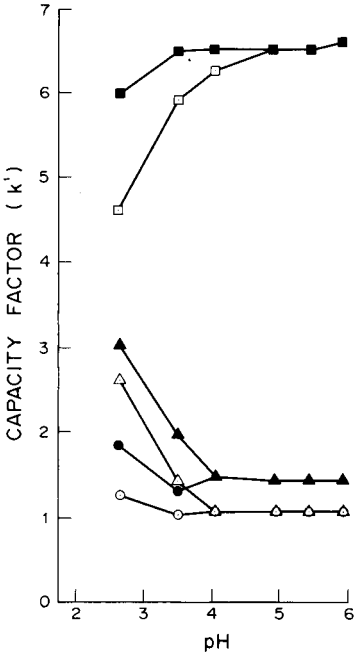

Fig. 2. Effect of pH on the capacity factor. Column, Ultrasphere-I.P., 5 μm (150 × 4.6 mm I.D.); flow-rate, 1 ml/min; temperature, 40°C; pH adjusted with 0.2 *M* sodium acetate buffer. A three-solvent mobile phase with a linear gradient: (A) 75% water with 0.2 *M* acetic acid, pH 2.65, and 6 m*M* heptanesulfonic acid; (B) 6% acetonitrile with 0.2 *M* acetic acid; (C) 19% methanol with 0.2 *M* acetic acid, held for 2 min, then programmed to 55% A, 6% B, 39% C over 6 min and held for 10 min. Symbols: ⊙ = *trans*-zeatin riboside; ● = *cis*-zeatin riboside; △ = *trans*-zeatin; ▲ = *cis*-zeatin; □ = isopentenyl adenosine; ■ = isopentenyl adenine.

Acetonitrile was chosen as the major organic modifier since it proved superior to both methanol and tetrahydrofuran for separation of UV-absorbing contaminants from cytokinin peaks. A microprocessor controlled ternary-gradient system gave

TABLE II

EFFECTS OF TWO COUNTER-IONS WITH DIFFERENT CHAIN LENGTHS ON THE CAPACITY FACTOR (*k'*, SEE TEXT)

Column, Ultrasphere-I.P., 5 μm (250 × 4.6 mm I.D.); flow-rate, 0.8 ml/min; temperature, 40°C. A three-solvent mobile phase with a two segment linear gradient: (A) 93% water with 0.2 *M* acetic acid, pH 2.65, and 6 m*M* heptanesulphonic acid; (B) 5% acetonitrile with 0.2 *M* acetic acid; (C) 2% methanol with 0.2 *M* acetic acid programmed to 70% A, 25% B, 5% C over 30 min, to 50% A, 45% B, 5% C over 8 min and held for 7 min. Abbreviations as in Table I.

Cytokinin	*Capacity factor (k')*	
	Heptanesulphonic	*Octanesulphonic*
trans-ZR	1.26	3.17
cis-ZR	1.85	3.58
trans-Z	2.65	4.12
cis-Z	3.09	4.39
IPAR	4.60	5.12
IPA	6.01	7.39

excellent flexibility in programming the composition of the mobile phase and also minimized solvent waste during methods development.

Bond Elut tubes provided a fast and effective means of removing pigments and UV-absorbing contaminants in the butanol fraction of the seed extracts prior to semi-preparative HPLC. For some applications, sample clean-up with these reversed-phase, disposable cartridges is sufficiently effective to proceed directly to an analytical column. In the case of ion-pair chromatography, however, semi-preparative, reversed-phase HPLC was useful for removing compounds that could interfere with or act as counterions. Fractions from the semi-preparative column that had retention times corresponding to authentic standards (Fig. 3) were bioassayed with the oat-leaf senescence test[12] to confirm cytokinin activity, and the unused portions were then pooled and loaded onto the 5-μm ion-pair column. The estimates of cytokinin content in the seeds indicated concentrations of 78 and 17 ng of *trans*- and *cis*-zeatin and 70 and 24 ng of *trans*- and *cis*-zeatin riboside per gram fresh weight of tissue (Fig. 4). Peaks that co-chromatographed with authentic isopentenyl adenine or isopentenyl adenosine were not observed.

The UV-absorbing contaminants evident in Fig. 4 decrease the reliability of the previous measurements, and more stringent purification procedures for use prior to analytical HPLC are currently under development. Nevertheless, the cytokinin fractions are well-separated and clean enough for further confirmation of both identity and amounts by gas chromatography–flame-ionization detection[13], bioassay or gas chromatography–mass spectrometry. The potential of the ion-pair method is clearly demonstrated by resolution of authentic geometric isomers of cytokinin bases and their ribosyl derivatives at 254 and 269 nm (Fig. 5). The absorbance ratios obtained by monitoring cytokinin levels at these two wavelengths supplement co-injection with authentics and improve the likelihood of correct identification during HPLC of tissue extracts.

Before proceeding to bioassays and other methods of confirmation, the counter-ions from the ion-pair reagent must be removed from the cytokinin fractions.

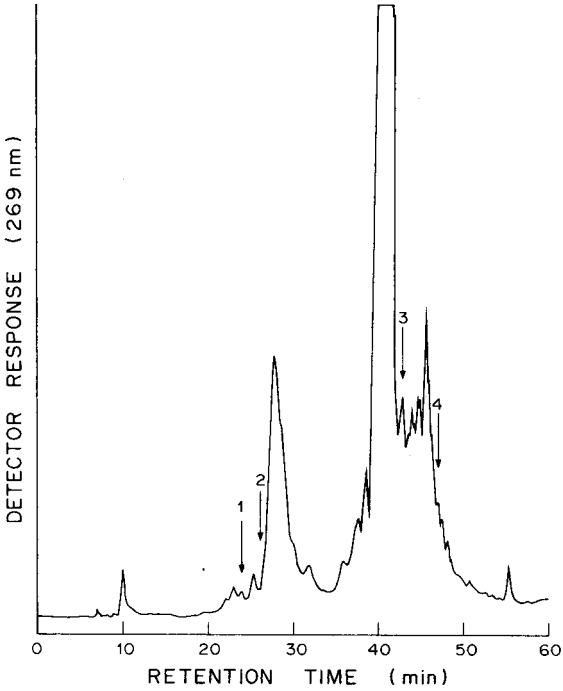

Fig. 3. Fractions collected from a partially purified extract of *Acer saccharum* seeds. Column, Ultrasil-ODS, 10 μm (250 × 10.0 mm I.D.); flow-rate 2 ml/min; temperature, 40°C. A three-solvent mobile phase with a linear gradient: (A) 85% water with 0.2 *M* formic acid: (B) 5% acetonitrile with 0.2 *M* formic acid; (C) 10% methanol with 0.2 *M* formic acid programmed to 10% A, 5% B, 85% C over 50 min. Peaks: 1 = fraction containing *trans* isomers of zeatin and zeatin riboside; 2 = fraction containing *cis* isomers of zeatin and zeatin riboside; 3 = fraction containing isopentenyl adenosine; 4 = fraction containing isopentenyl adenine.

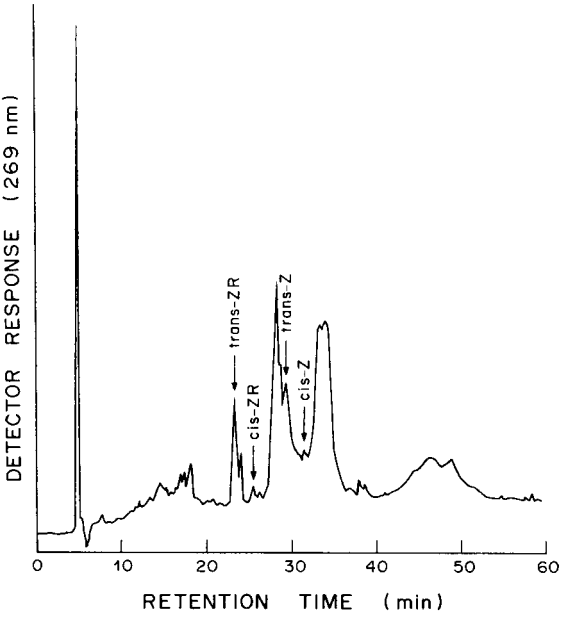

Fig. 4. Ion-pair chromatography of combined fractions 1 and 2 from Fig. 3. Chromatographic conditions as in Table II. Abbreviations as in Table I.

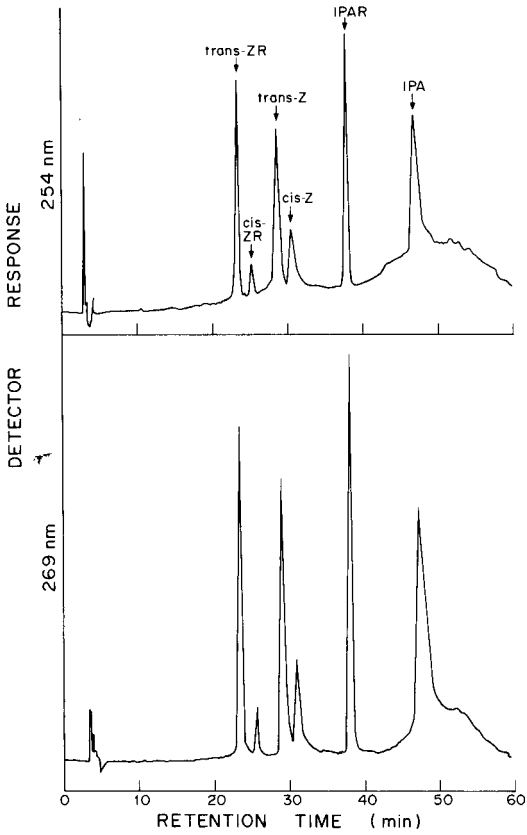

Fig. 5. Separation of cytokinin standards by ion-pair chromatography. Chromatographic conditions as in Table II. Abbreviations as in Table I. Amounts injected: *trans*-zeatin riboside = 4.6 μg, *cis*-zeatin riboside = 0.4 μg; *trans*-zeatin = 4.5 μg, *cis*-zeatin = 0.5 μg, isopentenyl adenosine = 5.0 μg, isopentenyl adenine = 5.0 μg.

This was achieved by adjusting the fractions to pH 8 with 0.1 *M* NaOH and loading onto Bond Elut cartridges. Raising the pH neutralized the charged cytokinins and allowed the counter-ions to be washed from the cartridges with distilled water. Subsequent elution with methanol gave cytokinin recoveries in the range of 90% and higher. To ensure that molecular integrity of the cytokinins was maintained during elution of the ion-pair reagent, chromatographic behaviour, UV-absorption patterns and biological activities were used to compare standards from which counter-ions were removed (treated) to standards not previously exposed to the ion-pair reagent (untreated). Thin-layer chromatography on both cellulose and silica gel plates gave identical R_F valves for treated and untreated standards and the UV absorption patterns measured from 230 to 280 nm were also the same. The oat-leaf senescence test[12] confirmed these results, and for all dilutions tested, over the range of 10^{-3} to 10^{-5} *M*, the biological activity of the cytokinins eluted from the Bond Elut columns remained unchanged.

The present work demonstrates that ion-pair chromatography is readily adaptable for the separation and measurement of closely related cytokinins and that

subsequent removal of the ion-pair reagent to facilitate more definitive identification poses no special problems.

ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatographic separation of the chlorination products of terephthalamide

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p-Phenylenediamine is an important chemical in many areas of industry and research. It is valuable not only in dye chemistry but also in the rubber industry. Recently, it has gained increasing importance as a monomer for new and already commercially utilized groups of high-modulus fibres. In view of the fact that the well-known manufacturing processes for *p*-phenylenediamine require extensive purification to result in a polymer-grade product, Akzo[1–3] have developed a new process for its production from polyester waste, terephthalic esters, or terephthalic acid (TPA).

In order to investigate the chlorination of terephthalamide in Akzo's process, especially the kinetics, it was necessary to develop a rapid and sensitive method for the detection and quantification of the chlorination products.

Because the melting points of terephthalamide and its chlorination products are relatively high and their vapour pressures low, gas chromatography is not suitable for their analyses. Iodometric titration is useful in the determination of the N-chloroamide group content, but it cannot distinguish the monochlorination product from the dichlorination product. As far as high-performance liquid chromatography (HPLC) is concerned, the solubilities of terephthalamide and its chlorination products in water and the most commonly used organic solvents (*e.g.* methanol, ethanol, acetone, acetonitrile, THF, dioxane, ether, chloroform, carbon tetrachloride, benzene) are very low. A more suitable solvent for sample preparation was aqueous NaOH (or NH_4OH) solution, in which the chlorination products are readily soluble, even at low temperatures. However, they are unstable under these conditions, as shown in Fig. 1. Hofmann rearrangement proceeded even at 5°C. Thus, direct analysis of chlorination products was very difficult.

In order to study the chlorination of terephthalamide, the chlorination products were determined by analysing the corresponding products (*p*-phenylenediamine and *p*-aminobenzamide) from Hofmann rearrangement of mono- and dichlorination products. This analysis was accomplished by reversed-phase chromatography using methanol–1 % aqueous acetic acid as the mobile phase.

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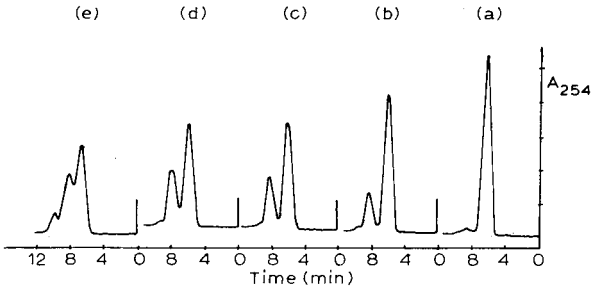

Fig. 1. Variation of chromatogram of chlorination products of terephthalamide with time on a Finepak C_{18} column with methanol–water (1:1) as the mobile phase. The flow-rate was 5 μl/min. (a) Crude chlorination products; (b) crude chlorination products standing for 24 min at 5°C in 8% aqueous NaOH; (c) the same as (b) except the time was 48 min; (d) the same as (b) except the time was 72 min; (e) the same as (b) except the time was 24 h.

EXPERIMENTAL

Reagents and samples

All chromatographic solvents, except water, were of liquid chromatography grade (E. Merck, Darmstadt, G.F.R.). Degassed distilled water was used.

Authentic samples of *p*-phenylenediamine and TPA were used as supplied. *p*-Aminobenzoic acid was produced by the reduction of commercially available *p*-nitrobenzoic acid. *p*-Aminobenzamide was prepared by the reduction of *p*-nitrobenzamide, which was obtained from the reaction of *p*-nitrobenzoyl chloride and ammonia water. Terephthalamide was prepared by the ammonolysis of polyester waste.

The sample of the chlorination products of terephthalamide was prepared by bubbling excess chlorine into a suspension of terephthalamide in water.

Apparatus and chromatographic conditions

A Model ALC/GPC 244 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 6000 solvent delivery system and a Model Familic 100N (JASCO, Japan) equipped with UVEDEC-100-II detector were used. The chromatographic columns used were a μBondapak C_{18}, 10 μm, obtained from Waters, a VerCopak C_{18}, 10 μm (packing material, Nucleosil; particle form, porous beads; surface area, 350–400 m^2/g, pore size, 100 Å, pore volume, 1.5 ml/g), obtained from Vertex (Taiwan), and a Finepak C_{18}, obtained from JASCO. A fixed-wavelength (254 nm) ultraviolet (UV) detector was employed and the chromatograph was operated under ambient temperature conditions. The flow-rate was 1–1.5 ml/min under different cases as specified in the figures. Most chromatograms were recorded on a Omniscribe recorder at a 0.5 cm/min chart speed.

RESULTS AND DISCUSSION

On the μBondapak C_{18} column the effect of the polarity of the mobile phase on the separation of *p*-phenylenediamine and *p*-aminobenzamide was investigated by changing the ratio of methanol and 1% aqueous acetic acid. If the ratio was 50:50 or 25:75 no separation resulted. The retention times of *p*-phenylenediamine and *p*-amino-

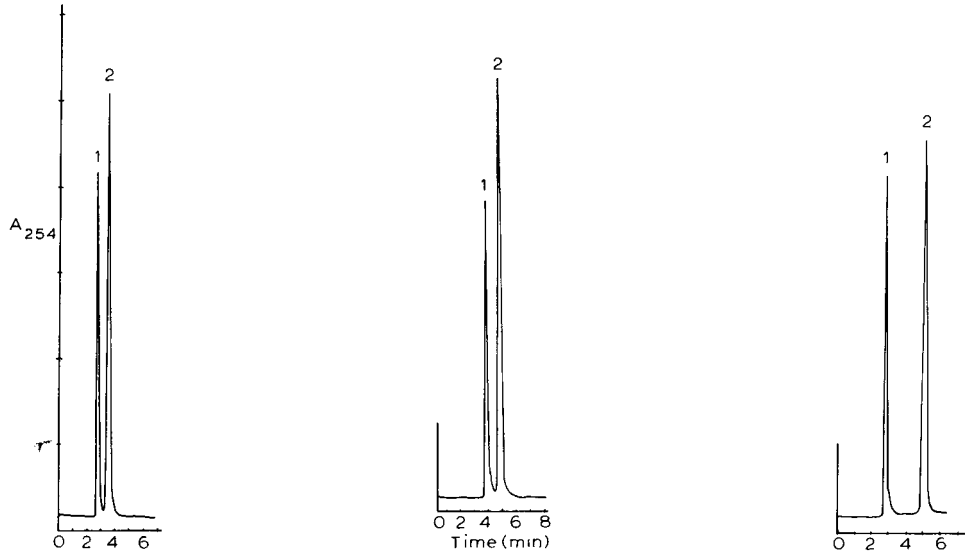

Fig. 2. Separation of *p*-phenylenediamine from *p*-aminobenzamide on a μBondapak C_{18}, 10 μm, column with methanol–1% aqueous acetic acid (10:90) as the mobile phase. The flow-rate was 1.5 ml/min. Peaks: 1 = *p*-phenylenediamine; 2 = *p*-aminobenzamide.

Fig. 3. Experimental conditions were the same as in Fig. 2, except the flow-rate was reduced to 1.2 ml/min.

Fig. 4. Experimental conditions were the same as in Fig. 2, except the mobile phase was 5:95 methanol–1% aqueous acetic acid and the flow-rate was 1 ml/min.

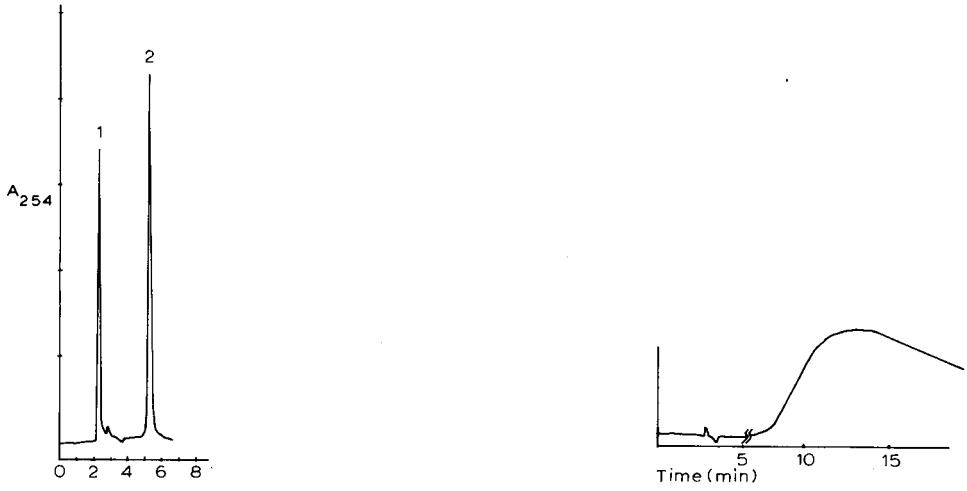

Fig. 5. Experimental conditions were the same as in Fig. 4, except the column used was VerCopak C_{18}, 10 μm.

Fig. 6. The chromatogram of *p*-phenylenediamine on a LiChrosorb RP-18, 10 μm, column. Other experimental conditions were the same as in Fig. 4.

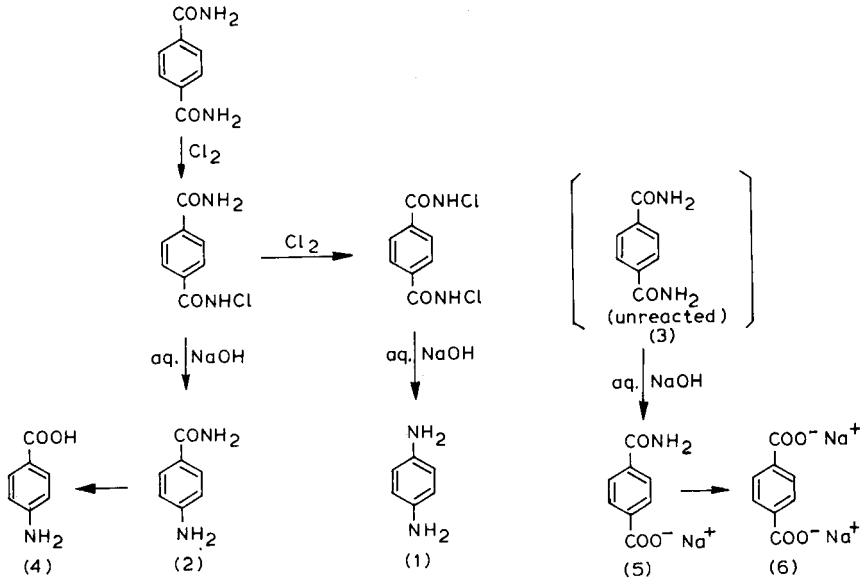

Fig. 7. Possible compounds formed in the chlorination of terephthalamide followed by Hofmann rearrangement.

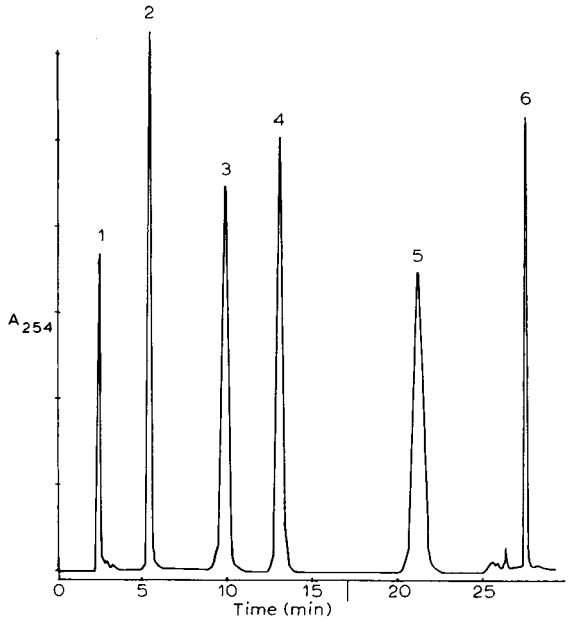

Fig. 8. Separation of *p*-phenylenediamine, *p*-aminobenzamide, terephthalamide, *p*-aminobenzoic acid, *p*-carboxybenzamide, and terephthalic acid on a VerCopak C_{18}, 10 μm, column with 5:95 methanol–aqueous acetic acid as the initial mobile phase. After 17 min the mobile phase was changed to 50:50 methanol–1% aqueous acetic acid. Peaks: 1 = *p*-phenylenediamine; 2 = *p*-aminobenzamide; 3 = terephthalamide; 4 = *p*-aminobenzoic acid; 5 = *p*-carboxybenzamide; 6 = terephthalic acid.

benzamide were about the same (*ca.* 3 min). If the ratio was 10:90, the separation was improved as shown in Fig. 2. At this ratio, a change in the flow-rate did not result in complete separation (Fig. 3). Complete separation was achieved using a mobile phase ratio of 5:95 and a flow-rate of 1 ml/min (Fig. 4).

Complete separation of *p*-phenylenediamine and *p*-aminobenzamide was also achieved on the VerCopak C_{18} column at the same mobile-phase ratio (Fig. 5). When LiChrosorb RP-18, 10 μm, was used *p*-phenylenediamine appeared on the chromatogram as a very broad peak and at a relatively longer time (Fig. 6). Thus it is not suitable for a quantitative analysis of *p*-phenylenediamine.

During kinetic studies of this chlorination reaction, sampling of the reaction mixture followed by Hofmann rearrangement resulted in the formation of six possible compounds, as shown in Fig. 7. Therefore the analytical conditions for satisfactory separation of these compounds had to be developed.

When the VerCopak C_{18} column was used with a mobile phase ratio of 5:95, these six compounds were well separated. However, TPA appeared after a relatively long time and as a broad peak. In order to achieve a good separation in a reasonable time, it is necessary to change the mobile phase ratio, after *p*-aminobenzoic acid has emerged (*ca.* 17 min), to 30:70 or 50:50. Under these conditions all the six possible reaction products can be well separated, as shown in Fig. 8. Instead of 1 % aqueous acetic acid, phosphate buffer can be used, as shown in Fig. 9. In order to shorten the analysis time, the mobile phase ratio can be changed to 10:90 (methanol–1 % aqueous acetic acid) (Fig. 10). The separation was still good.

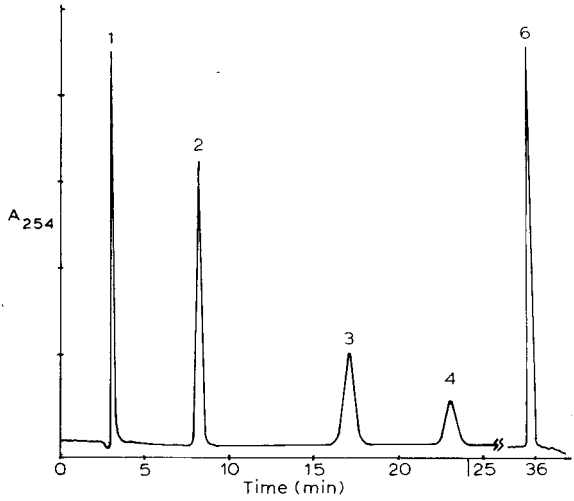

Fig. 9. Separation of *p*-phenylenediamine, *p*-aminobenzamide, terephthalamide, *p*-aminobenzoic acid, and terephthalic acid on a VerCopak C_{18}, 10 μm, column with 5:95 methanol–water containing 0.03 *M* NaH_2PO_4/H_3PO_4 buffer at pH 3 as the initial mobile phase. After 24 min the mobile phase was changed to 50:50 methanol–1 % aqueous acetic acid. Peak identification is the same as in Fig. 7.

On the μBondapak C_{18} column, the six possible Hofmann rearrangement products were well separated. However, on the LiChrosorb RP-18 column, *p*-aminobenzamide and terephthalamide were inseparable. This column was not suitable for kinetic studies of the chlorination reaction of terephthalamide under these conditions.

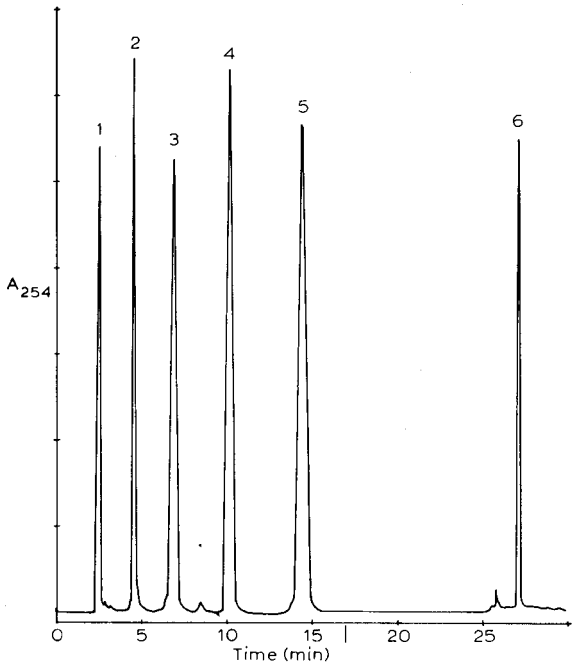

Fig. 10. Experimental conditions were the same as in Fig. 7 except the initial mobile phase was 10:90 methanol–1% aqueous acetic acid. The mobile phase was changed to 50:50 methanol–1% aqueous acetic acid after 17 min as indicated by the arrow signal on the chromatogram.

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Note

Chromatographic fractionation of multiple forms of red blood cell hexokinase

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It is well established that four hexokinase (E.C. 2.7.1.1) isozymes (named I, II, III and IV in order of increasing electrophoretic mobility towards the anode) are present in mammalian tissues[1,2]. Conflicting results have been reported for the hexokinase pattern in the erythrocytes[3–14]. The discrepancies are mainly due to the presence of sub-types or multiple forms of hexokinase I and to the difficulties related to their separation. Electrophoresis on starch gel, agarose gel, cellulose acetate membrane, polyacrylamide gel, and isoelectric focusing or ion-exchange chromatography have been employed as separation techniques of the red blood cells isozymic pattern. Unfortunately starch gel, agarose gel and cellulose acetate have been found unsuitable as media for hexokinase isozymes separation owing to the lack of resolution. Polyacrylamide disc gel electrophoresis or electrofocusing, on the other hand, inactivate some isozymes.

In this paper we propose the use of small DE-52 ion-exchange columns for the resolution and the complete recovery of the hexokinase isozymic pattern in red blood cells. This approach has been used successfully in studies of the hexokinase isozymic patterns of red cells of different ages[15] and of different mammalian species[16,17]

MATERIALS AND METHODS

Materials

Coenzymes, enzymes, substrates and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.). DE-52 was purchased from Whatman (Maidstone, Kent, Great Britain). All other reagents were of analytical grade.

Methods

Rabbit and human blood samples were collected using EDTA as anticoagulant. Red blood cells were washed and haemolysed as previously described[15]. Rabbit reticulocytes were obtained as in ref. 15. DE-52 column chromatography (24 × 0.35 cm I.D. unless otherwise indicated) was performed at 4°C in 5 m*M* sodium potassium phosphate buffer (pH 7.5) containing 1 m*M* glucose, 3 m*M* KF, 3 m*M* 2-mercaptoethanol and 5 m*M* dithiothreitol. Flow-rates were maintained at 5.0 ml/h using a peristaltic pump (Gilson minipuls 2). Fractions (0.7 ml) were collected in a fraction collector (LKB Ultrorac II) and assayed for hexokinase activity; the absorbance was

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monitored at 280 nm. Columns were developed with 280-ml linear gradients by an automatic gradient former (Gilson Mixograd) from 0 to 0.4 *M* KCl in the same sodium potassium phosphate buffer.

Enzyme assay

Hexokinase activity was measured spectrophotometrically at 37°C in a system coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) as previously described[16]. One unit of enzymatic activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of glucose-6-phosphate per minute at 37°C.

RESULTS AND DISCUSSION

As part of the study of the regulatory and biochemical properties of the multiple forms of hexokinase in the erythrocytes, we have developed a method that permits the separation and recovery of hexokinase isozymes starting from haemolysates.

After some preliminary experiments with polyacrylamide disc gel electrophoresis, isoelectric focusing and agarose gel electrophoresis, we decided to approach this study by utilising the DE-52 ion-exchange chromatography method. However, the successful application of this method requires consideration of several parameters and depends on the optimization of all the experimental conditions.

Influence of column dimensions

Fig. 1 shows the experiments carried out to determine the influence of column dimensions on hexokinase resolution. When the ratio (*R*) of the height to the diameter of the column was varied from 20 to 70 the column resolution increased. At higher *R* values the two hexokinase isoenzymes are separated with loss of resolution (Fig. 1D). These results were obtained by loading 1-ml samples; larger volumes of haemolysates significantly affect the separation. Furthermore, correct column packing plays a significant role in separation of the hexokinases. In order to obtain reproducible results the ion-exchanger was pre-equilibrated in the elution buffer degassed by a water pump, loaded into the column at a flow-rate of 5 ml/h and packed for at least 20–30 h.

Influence of gradients

Two different gradients have been tested for separation of the hexokinase isozymes. The first was a pH gradient from pH 8.0 to 6.5. Under these conditions two main problems affect the separation: (a) the bulk of the haemoglobin is strongly bound to the exchanger at the basic pH values with consequent blockage of the column; (b) the enzyme recovery is greatly affected by the pH of the elution buffer (Fig. 2). For these reasons further experiments have been done with salt gradients at a constant pH of 7.5.

Fig. 3 shows the results of experiments at different gradient slope. Peak width decreases with increasing ionic concentration from 0 to 0.5 *M* KCl. The best results were obtained with 280-ml linear gradient 0 to 0.4 *M* KCl in the equilibrating buffer at pH 7.5.

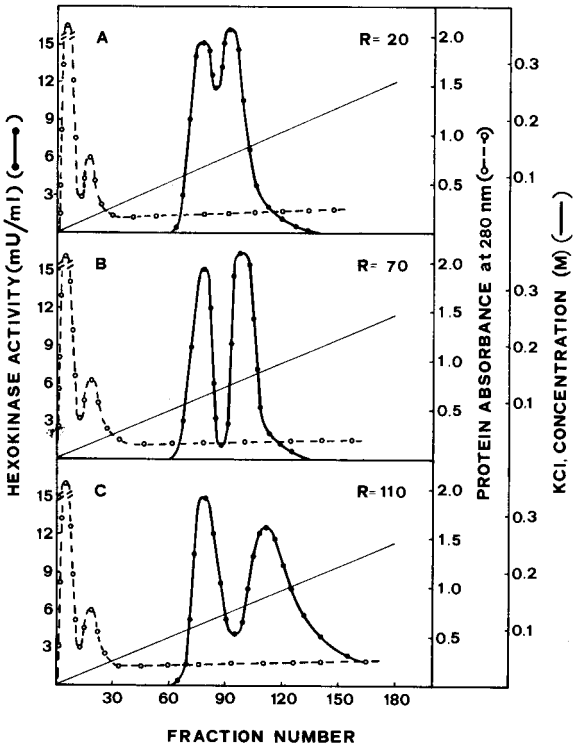

Fig. 1. Influence of column dimensions on resolution of rabbit reticulocytes hexokinase pattern. Haemolysate (1 ml) was applied to three different columns with height/diameter ratios of 20, 70 and 110, respectively. Elution was obtained by a linear gradient of 280 ml from 0 to 0.4 *M* KCl in 5 m*M* sodium potassium phosphate buffer (pH 7.5) contraining 1 m*M* glucose, 3 m*M* KF, 3 m*M* 2-mercaptoethanol and 5 m*M* dithiothreitol. Column packing and equilibration were performed in the same buffer system at a flow-rate of 5 ml/h. Fractions of 0.7 ml were collected and assayed for enzyme activity (●—●) and protein absorbance at 280 nm (O---O).

Enzyme stability

As discussed above, the column chromatography buffer greatly affects hexokinase recovery. Optimal enzyme stability was obtained between pH 7.5 and 8.0. Four different buffer systems have been tested. Complete hexokinase recovery was obtained in sodium potassium phosphate buffer, while with glycylglycine, Tris-HCl and triethanolamine the recovery range was 20–70 % of the activity introduced on the columns. Furthermore, 1 m*M* glucose and 5 m*M* dithiothreitol were essential for good enzyme stability in any buffer system.

CONCLUSION

DE-52 column chromatography, under the conditions described, provides the most efficient method so far reported for the study of the hexokinase isozymic pattern in mammalian red blood cells. Fig. 4 shows that it is possible to resolve as complex a

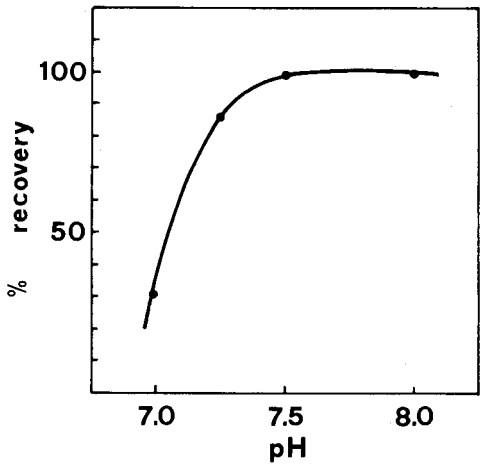

Fig. 2. Hexokinase activity recovery after ion-exchange chromatography. Experiments were carried out as in Fig. 1B except that the pH was varied as reported.

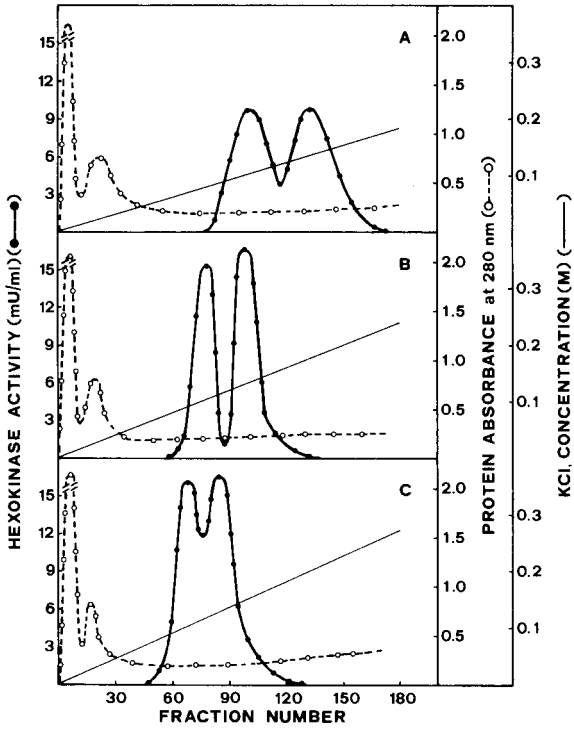

Fig. 3. Effect of salt gradient on the chromatographic fractionation of hexokinase isozymes. Three different gradients, 0 to 0.3 *M* KCl (A), 0–0.4 *M* KCl (B) and 0–0.5 *M* KCl (C), were tested. The column used had an *R* value of 70 (24.5 × 0.35 cm I.D.). Other experimental conditions were as given in Fig. 1.

pattern as that of the hexokinase in human erythrocytes and to weight each of the molecular forms present. This is very important because of the different decay rate of each isozyme during cell ageing. Furthermore, other systems that employ the removal of the bulk of haemoglobin before the study of the red cell hexokinase pattern, inevitably cause a modification of the isozymic pattern, as clearly shown in Fig. 4C. The method described permits the use of the haemolysate as starting material because of the non-retention of haemoglobin.

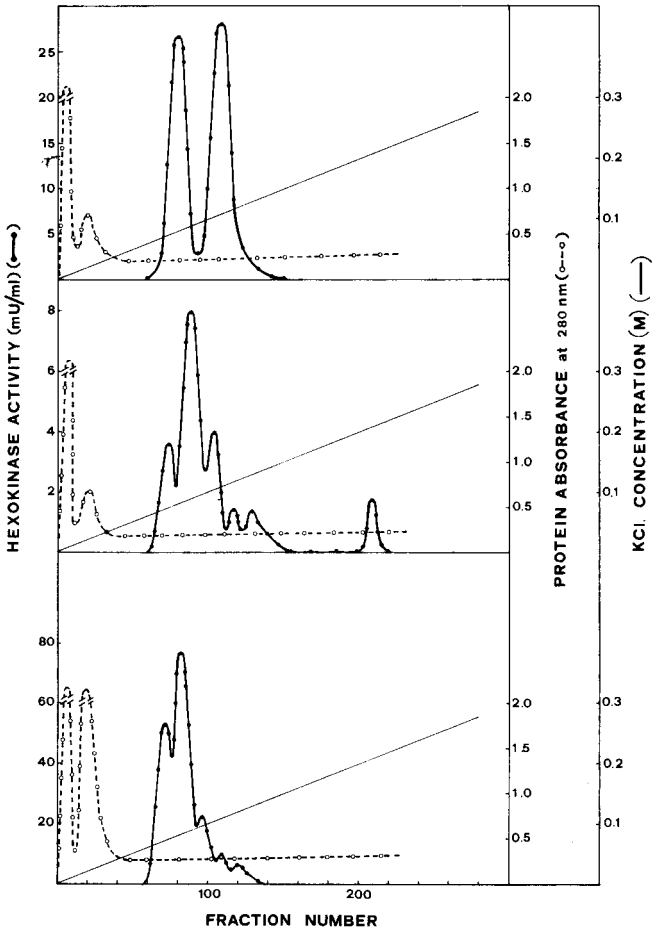

Fig. 4. DE-52 ion-exchange chromatography of hexokinase activity from rabbit reticulocytes (A), human red blood cells (B) and ammonium sulphate fraction, 35–75%, from human red cells (C), prepared as in ref. 18. All the experimental conditions were as in Fig. 1B.

Furthermore this approach could be useful not only in the study of the red cell hexokinase system but also in the investigation of the pattern of many other erythrocyte enzymes and their genetic variants.

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Note

Simple detection of steroid sulphatase activity after chromatographic fractionation

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Determination of steroid sulphatase activity has been performed by isotopic, colorimetric and enzymatic methods[1–4]. Determination of sulphatase activity by colorimetric methods, such as the methylene blue method and the Zimmermann reaction, is time-consuming because removal of the organic solvent is required, making it difficult to treat the large number of samples which are collected in chromatographic fractionation. In this paper, a one-tube detection method for steroid sulphatase activity after chromatography is described.

MATERIALS AND METHODS

All chemicals were of analytical-reagent grade and were commercially available. The different steroid substrates were from Sigma (St. Louis, MO, U.S.A.) and Ikapharm (Ramat Gan, Israel). Sephadex and the column were purchased from Pharmacia (Uppsala, Sweden). A 0.05 *M* acetate buffer (pH 5.0) was employed. The substrate solution contained 0.5 mg/ml of dehydroepiandrosterone sulphate and estrone sulphate in water. The steroid sulphatase used in these studies was sulphatase from *Helix pomatia* (Type H-1, E.C. 3.1.6.1) (Sigma) and 200 U/ml of enzyme solution was prepared. Preparation of an enzyme solution for the colour development of dehydroepiandrosterone has been described previously[4–7]: 20 mg of 4-aminoantipyrine, 100 mg of phenol, 100 U of 3β-hydroxysteroid oxidase (E.C. 1.1.3.6 from *Brevibacterium sterolicum*), 1000 U of peroxidase and 0.1 ml of Triton-X 100 were dissolved in 50 ml of phosphate buffer (0.2 *M*, pH 7.5).

Sephadex-Gel filtration

Sephadex G-200 was swollen by heating a suspension of the particles in acetate buffer (0.05 *M*, pH 5.0) for 4 h at 90°C under constant stirring. The "fines" were removed by several decantations and the slurry was poured directly into the column (60 × 1 cm) which was then washed for 3 h with acetate buffer.

Application of sample to the column

A solution of the enzyme in acetate buffer, *ca.* 200 units/ml, was centrifuged for 5 min at 2500 *g* and the supernatant of 1 ml was applied to the column. Twenty-five fractions (each 1.5 ml) were collected.

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Enzyme activity assay

Steroid sulphatase activity was detected by the use of dehydroepiandrosterone (3β-hydroxy-5-antrosten-17-one) sulphate and estrone sulphate as substrate, and β-glucuronidase activity was detected using phenolphthalein glucuronic acid as substrate.

Steroid sulphatase activity assay by the use of dehydroepiandrosterone sulphate as substrate[4]. A 0.2-ml portion of each fraction was transferred by pipette into a test tube and 0.5 ml of substrate solution and 0.8 ml of acetate buffer (0.05 *M*, pH 5.0) were added. After incubation (*ca.* 5–10 h at 37°C), 1 ml of colour development reagent for dehydroepiandrosterone was added and the mixture was incubated for 15 min at 37°C. The absorbance was measured at 500 nm.

Steroid sulphatase activity assay by use of estrone sulphate as substrate. A 0.2-ml portion of each fraction was pipetted into a test tube, and 0.5 ml of substrate solution and 1 ml of 0.2 *M* acetate buffer (pH 5.0) were added. After incubation (for 30–60 min) at 37°C, the turbidity caused by insoluble released estrone was measured at 400 nm.

β-Glucuronidase activity assay by use of phenolphthalein glucuronic acid. To 0.05 ml of sample, 0.3 ml of phenolphthalein glucuronic acid (0.003 *M*, pH 4.5) was added. After incubation for 30 min at 37°C, 1.5 ml of glycine buffer (0.2 *M*, pH 10.4) was added. The absorbance was measured at 540 nm.

RESULTS AND DISCUSSION

Gel chromatography of sulphatase from *Helix pomatia* (Type H-1) on Sephadex G-200 is shown in Fig. 1. Protein was detected at 280 nm and the steroid

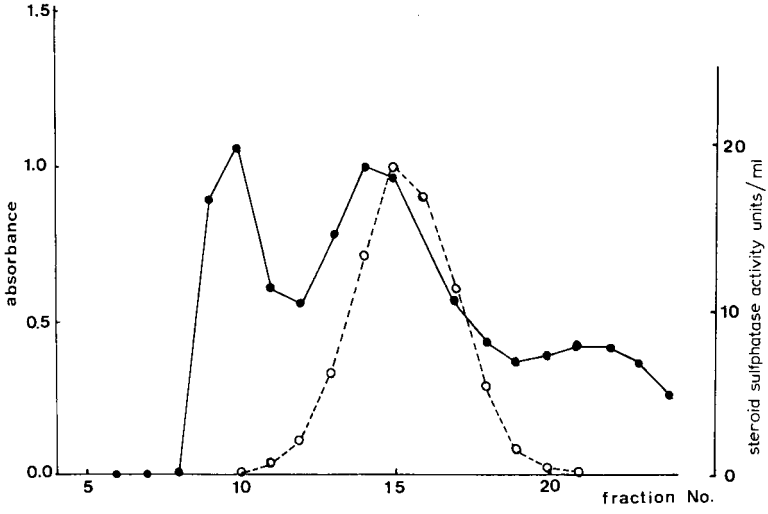

Fig. 1. Gel filtration of steroid sulphatase on Sephadex G-200. A 1-ml portion of steroid sulphatase solution (200 units/ml) from *Helix pomatia* (Type H-1) was applied onto Sephadex G-200 with 0.05 *M* acetate buffer (pH 5.0) as eluent. Fractions (each 40 ml) were collected as described in the text. Protein content was detected at 280 nm (●—●), and steroid sulphatase activity was detected by the present method (○----○; absorbance at 500 nm and calculated activity).

sulphatase activity was measured by use of dehydroepiandrosterone sulphate (absorbance at 500 nm, steroid sulphatase activity calculated) under the optimum conditions previously reported. The activity of β-glucuronidase, a contaminant of sulphatase, is shown in Fig. 2 in which the absorbance at 540 nm is plotted. Steroid sulphatase activity, measured using estrone sulphate as substrate, is also shown in Fig. 2 and was determined by turbidimetric assay (absorbance at 400 nm).

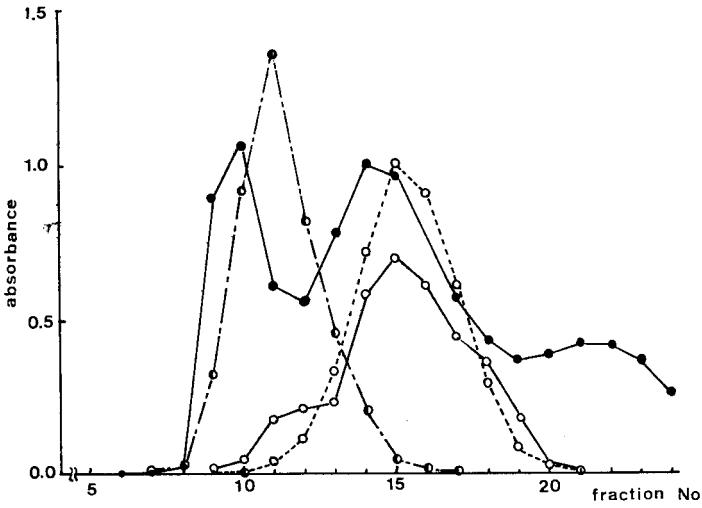

Fig. 2. Gel filtration of β-glucuronidase and steroid sulfatase on Sephadex G-200. Protein content: ●—● (absorbance at 280 nm). β-Glucuronidase: ◑- — - ◑ (absorbance at 540 nm), detected by the method described in the test. Steroid sulphatase activity: O----O (absorbance at 500 nm). Steroid sulphatase activity with estrone sulphate as substrate (O — O) was measured by the method described in the text (absorbance at 400 nm).

The enzymatic determination of sulphatase activity has been reported as a preliminary communication[4]. In this paper, a simple method for determining steroid sulphatase activity after chromatography is described.

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Note

Sensitive and rapid amino acid analysis of peptide hydrolysates by high-performance liquid chromatography of *o*-phthaldialdehyde derivatives

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(First received September 8th, 1981; revised manuscript received October 16th, 1981)

The amino acid composition of peptides isolated from biological sources provides essential information for their chemical characterization. Since such peptides are often obtained in only very small amounts a sensitive and accurate determination of the amino acid composition is essential. Recent reports indicate that amino acids derivatized with *o*-phthaldialdehyde (OPT) can be separated by reversed-phase high-performance liquid chromatography (HPLC) and detected with high sensitivity[1–6]. We found that some of the previously described solvent systems resulted in insufficient resolution when applied to a Nucleosil 5 C_{18} column. In this paper a solvent system optimized for the Nucleosil 5 C_{18} column is described. This system separates OPT derivatives of all amino acids common to peptide hydrolysates with good resolution. An evaluation of chromatographic conditions and quantitative properties is presented, resulting in a reproducible and sensitive method for the amino acid analysis of small amounts of biologically active peptides.

EXPERIMENTAL

Apparatus

HPLC was performed with a Waters Assoc. assembly consisting of two Model 6000A pumps, a Model 660 solvent programmer and a U6K injector; a Nucleosil 5 C_{18} column (250 × 4.6 mm I.D., particle size 5 μm, Chrompack, Middelburg, The Netherlands) was used. The column effluent was monitored with an Aminco Fluoro-Monitor equipped with a Corning 7-51 primary filter (330 nm cut-off) and a Wratten 2A secondary filter (420 nm cut-off). The flow cell volume was 18 μl.

Chromatography

In the standard elution program the mobile phase was formed by a gradient of sodium citrate buffer (A_I or A_{II}) and methanol (B) according to the following schemes:

$$\text{I: } 0\% \xrightarrow{\text{linear (5 min)}} 30\% \xrightarrow{\text{isocratic (5 min)}} 30\% \xrightarrow{\text{linear (20 min)}} 65\% \text{ B}$$

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[solvent A_I was 0.1 *M* sodium citrate (pH 6.5) premixed with methanol (4:1, v/v); solvent B was methanol] or

$$\text{II: } 20\% \xrightarrow{\text{linear (5 min)}} 45\% \xrightarrow{\text{isocratic (5 min)}} 45\% \xrightarrow{\text{linear (20 min)}} 75\% \text{ B}$$

[solvent A_{II} was 0.1 *M* sodium citrate (pH 6.5); solvent B was methanol]

Chromatography was performed at a flow-rate of 1.0 ml/min at ambient temperature.

o-Phthaldialdehyde–2-mercaptoethanol (OPT–ME) reagent

o-Phthaldialdehyde (Sigma, St. Louis, MO, U.S.A.) was dissolved in methanol to a concentration of 56 mg/ml. One volume of this solution was mixed with nine volumes of 0.4 *M* sodium borate buffer, pH 9.5, and 4 μl of 2-mercaptoethanol per ml of reagent mixture were added. Addition of 1 μl of 2-mercaptoethanol per ml reagent each week was sufficient to maintain a constant reagent strength during two months.

Derivatization

The derivatization procedure was standardized in the following way. Samples containing 50–200 pmole of individual amino acid residues were made up in 25 μl of quartz-distilled water, and 50 μl of the OPT–ME reagent was added (t = 0 sec). After thorough mixing at ambient temperature, 50 μl of the reaction mixture were taken up in the HPLC syringe at t = 60 sec. The syringe was placed in the sample loading port of the injector unit and at t = 90 sec the total volume was injected into the sample loading loop. At t = 120 sec the sample was loaded onto the column.

Sample preparation

For HPLC analysis, standard mixtures containing 1–50 pmole of each amino acid per μl were prepared. Peptides were hydrolysed in 50–100 μl 6 *M* hydrochloric acid containing 0.1 % thioglycolic acid in evacuated sealed glass tubes at 110°C for 16 h[7]. Performic acid oxidation of cyst(e)ine-containing peptides to the corresponding cysteic acid peptides was carried out prior to hydrolysis essentially according to a modified procedure of Hirs[8,9].

RESULTS AND DISCUSSION

Chromatography

After attempts with several solvent systems to separate the OPT–amino acid derivatives on a Nucleosil 5 C_{18} column, optimal conditions were found in the use of a gradient of 0.1 *M* sodium citrate buffer, pH 6.5, and methanol. Fig. 1 shows the separation of all amino acid derivatives common to peptide and protein hydrolysates and the change of effective methanol concentration in a typical run.

During evaluation of the present system the influence of buffer composition, buffer concentration and pH has been examined. Unsatisfactory resolution was obtained when the sodium citrate concentration was lowered from 0.1 to 0.05 *M*. Replacement of sodium citrate by sodium acetate or the use of sodium citrate mixed with sodium acetate buffers in various ratios resulted in insufficient separation between

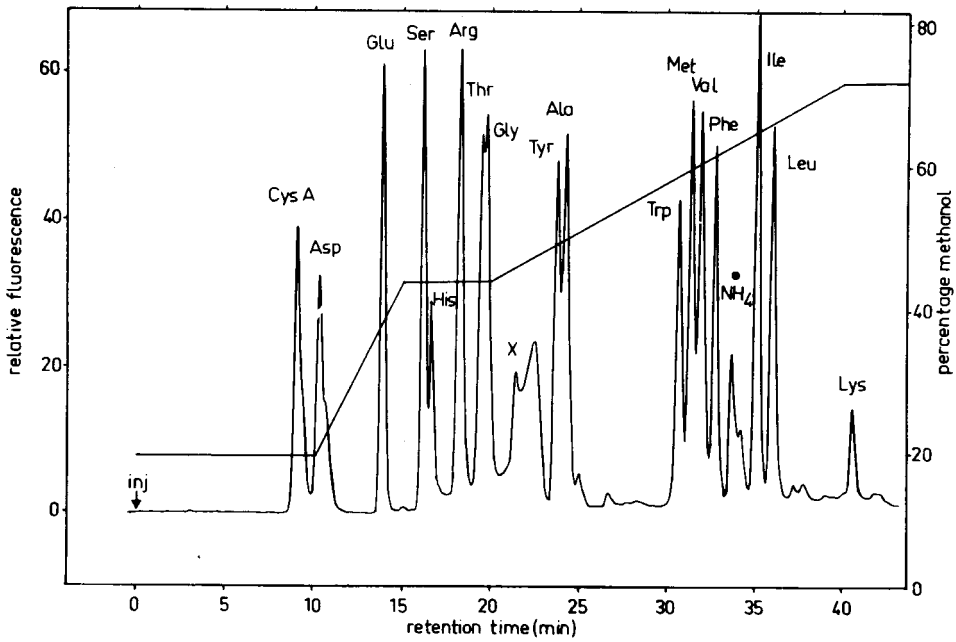

Fig. 1. Elution profile of OPT derivatives of amino acid standards (80 pmole each) on a Nucleosil 5 C_{18} column. The gradient was formed between 0.1 *M* sodium citrate, pH 6.5, and methanol (system II, see Experimental). The calculated actual methanol concentration in the effluent expressed as volume percentage is shown. Chromatography was performed at ambient temperature (22°C). The flow-rate was 1.0 ml/min. Component X originates from the OPT–ME reagent. Amino acids are indicated by conventional abbreviations except cysteic acid (CysA).

OPT–Thr and OPT–Gly. The effect of pH of the 0.1 *M* sodium citrate buffer was examined over the range pH 5.5–6.5. The overall elution profile and resolution of the OPT–amino acids was found optimal at pH 6.5.

In some HPLC systems OPT–Thr and OPT–Gly are not separated or are separated only with low resolution[1,6]. Although in the present system they were sufficiently separated for quantitative determination we tried to improve the resolution by including 1 % tetrahydrofuran in the mobile phase, following the suggestions of earlier reports[3,5]. The addition of 1 % tetrahydrofuran to the sodium citrate solution led to loss of separation between the OPT pairs Ser/His, Thr/Gly, and Tyr/Ala. In our set-up, use of tetrahydrofuran in combination with 0.05 *M* sodium acetate, pH 5.9, resulted in co-elution of OPT–Thr and OPT–Gly, of OPT–Tyr and OPT–Ala, and of OPT–Trp and OPT–Met, while the elution order of OPT–Thr/Gly and OPT–Arg was reversed.

In the present system, OPT–amino acid derivatives were separated with good resolution as single components except for OPT–His. Secondary peaks of OPT–His were present: a relatively small, sharp peak eluted with a retention time of approximately 17 min and a broad asymmetric component eluted between 20 and 23 min (Fig. 1). The first peak was used for quantitation. This phenomenon appeared to be dependent on the pH of the aqueous elution buffer.

Quantitative properties

The reaction time of the OPT-derivatization reaction and the stability of OPT–amino acid derivatives are known to influence the fluorescence intensity of the products after HPLC[2,3,5]. Therefore, a stringently standardized procedure of derivatization and chromatography was employed. For evaluation of the reproducibility of the procedure the retention times and fluorescence responses of consecutive analyses of amino acid standard mixtures were measured. These experiments allowed us to evaluate the deviation in retention times, the linearity of and deviation in the fluorescence responses, and to calculate the fluorescence factor of each OPT–amino acid (Table I). The average deviation in the retention times was *ca.* 0.1 min. A linear relationship between fluorescence response, measured as peak height, and amount of amino acid was found in the tested range of 20–200 pmole. The calculated linear regression of the standard curves had excellent correlation coefficients (Table I). From the slopes of the standard curves the fluorescence factors were calculated and normalized to OPT–Glu (Table I). The average deviation in fluorescence response measured as peak height was 3.3%.

TABLE I

CHARACTERISTICS OF AMINO ACID ANALYSIS BY REVERSED-PHASE HPLC OF OPT DERIVATIVES: RETENTION TIMES, LINEARITY OF FLUORESCENCE, FLUORESCENCE FACTORS AND PRECISION

OPT–amino acid derivative	*Retention time* ± S.D. (min)*	*Correlation** coefficient of linearity*	*Fluorescence*** factor*	*Deviation in§ fluorescence response (%)*
CysA	8.7 ± 0.1	0.9998	0.96	2.0
Asp	10.0 ± 0.2	0.9993	0.59	4.4
Glu	13.7 ± 0.1	0.9987	1.00	1.8
Ser	16.1 ± 0.1	0.9994	1.09	1.1
His	16.7 ± 0.1	0.9974	0.37	6.2
Arg	17.8 ± 0.1	0.9999	1.49	1.5
Thr	19.3 ± 0.2	0.9997	1.23	1.8
Gly	19.7 ± 0.1	0.9997	1.00	3.6
Tyr	23.2 ± 0.2	0.9990	1.06	2.7
Ala	23.9 ± 0.2	0.9994	0.87	2.5
Trp	30.2 ± 0.1	0.9985	0.81	4.6
Met	30.9 ± 0.1	0.9993	1.35	3.5
Val	31.6 ± 0.1	0.9990	1.23	3.7
Phe	32.4 ± 0.1	0.9990	1.03	3.3
Ile	34.9 ± 0.1	0.9998	1.56	3.4
Leu	35.8 ± 0.1	0.9997	1.08	3.9
Lys	40.1 ± 0.1	0.9804	0.26	6.2

* Measured from the chromatograms of seven consecutive runs of 20, 20, 40, 80, 120, 160 and 200 pmole of each amino acid residue.

** The correlation coefficient of linearity of the fluorescence response (measured as peak height) of the individual OPT–amino acid derivatives of the same consecutive runs mentioned under footnote*.

*** The fluorescence factor is defined as the normalized slope of the fluorescence response (measured as peak height) versus the amount of OPT–amino acid (Glu = 1.00). Data were obtained as under footnote*.

§ Calculated from the peak heights of 120 pmole of each amino acid determined from three consecutive runs.

The signal-to-noise ratio obtained from 10 pmole of amino acid was 10, resulting in a detection limit in the order of 3.pmole.

Three synthetic naturally occurring peptides, human β-endorphin, vasopressin, and oxytocin, were subjected to acid hydrolysis and their amino acid compositions were determined using the system described in this paper. The data obtained from a single determination are presented in Table II.

TABLE II

AMINO ACID COMPOSITION OF PEPTIDES

Data were obtained in a single analysis of 187 (a), 164 (b), and 191 pmole (c), respectively. Oxytocin and arginine–vasopressin were oxidized by performic acid treatment prior to acid hydrolysis, resulting in some loss of tyrosine and the simultaneous generation of a component with a retention time similar to that of OPT-Ser. n.d. = Not detectable.

Amino acid residue	*Human β-endorphin*[a]	*Oxytocin*[b]	*Arginine–vasopressin*[c]
CysA	n.d. (0)	1.99 (2)	1.91 (2)
Asp	2.04 (2)	1.00 (1)	1.00 (1)
Glu	3.04 (3)	1.09 (1)	1.07 (1)
Ser	1.62 (2)	0.25 (0)	0.35 (0)
His	n.d. (0)	n.d. (0)	n.d. (0)
Arg	0.03 (0)	0.04 (0)	0.98 (1)
Thr	2.96 (3)	n.d. (0)	n.d. (0)
Gly	2.81 (3)	1.00 (1)	1.11 (1)
Tyr	2.00 (2)	0.54 (1)	0.74 (1)
Ala	1.82 (2)	0.03 (0)	0.06 (0)
Met	1.01 (1)	n.d. (0)	n.d. (0)
Val	1.29 (1)	0.05 (0)	0.06 (0)
Phe	1.98 (2)	0.02 (0)	0.93 (1)
Ile	1.74 (2)	0.91 (1)	0.04 (0)
Leu	1.99 (2)	0.96 (1)	0.04 (0)
Lys	4.87 (5)	n.d. (0)	n.d. (0)

CONCLUSION

The HPLC method described here provides a technique which allows quantitative analysis of amino acids in the low pmol range with good precision and reproducibility. The method is rapid and easy to perform and does not require specialized equipment. Applied to peptide hydrolysates it provides an excellent tool for the chemical characterization of peptides obtained in very small amounts.

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Note

Determination of butanilicaine in horse plasma and urine by extractive benzoylation and gas chromatography with a nitrogen–phosphorus detector*

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(Received October 15th, 1981)

Butanilicaine [(2-(butylamino)-N-(2-chloro-6-methylphenyl)-acetamide)] is an amide-type local anaesthetic for veterinary use, with a faster and longer activity than procaine[1].

A previously described method[2] for the analysis of butanilicaine in urine was based on colorimetry. Earlier studies concerning the metabolism of local anaesthetics[3–5], especially butanilicaine breakdown in the rat[2,6], reveal that this drug is expected to be extensively metabolized in the horse. Therefore the complicated colorimetric method should lack sensivity for the determination of butanilicaine in horse plasma and urine.

Although a gas chromatographic (GC) screening procedure with nitrogen specific detection enabling the detection of eleven local anaesthetics in horse urine and plasma was recently published[7], this method could not been used for the quantitative determination of butanilicaine owing to peak tailing.

In the present study an extractive benzoylation reaction based on the Schotten–Baumann procedure has been utilized for the quantitative determination of butanilicaine in biological fluids.

EXPERIMENTAL

Materials

Butanilicaine triphosphate and the internal standard 2-(butylamino)-N-(2-methyl-4-chlorophenyl)acetamide. HCl were supplied by Hoechst (Frankfurt, G.F.R.). Stock solutions were prepared in double-distilled water.

Pentafluorobenzoyl chloride (PFBCl) was obtained from Aldrich Europe; trifluoroacetic acid anhydride (TFAA) and pentafluoropropionic acid anhydride (PFPA) were purchased from Pierce. The triethanolamine–cyclohexane extraction solvent (CH-TEA) was prepared by briefly refluxing cyclohexane with small amounts of triethanolamine, cooling and separating the two phases. The ammonium buffer was a saturated NH_4Cl solution adjusted to pH 9.4 with NH_4OH.

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All glassware was silanized as described earlier[8], and the organic solvents (analytical grade) were freshly distilled before use. Dilutions were made with a Hamilton digital diluter/dispenser.

Gas chromatography

A Varian 3700 equipped with ^{63}Ni and nitrogen-specific detectors and connected to a Varian CDS 111 integrator was used. The glass column (150 × 0.25 cm I.D.) was packed with 3% OV-7 on Chromosorb W HP, 80–100 mesh. The oven temperature was set at 235°C. The injector and detector temperatures were kept at 250°C and 300°C, respectively. Nitrogen (25 ml/min) was used as carrier gas.

Mass spectrometry

A mass spectrum was obtained on a HP 5995 apparatus, the column being a 25-m SP-2100 fused-silica column.

Methods

For the determination of butanilicaine in horse plasma or urine, 2 ml of the biological fluid, 50 μl of the aqueous internal standard solution (10 μg/ml) and 0.2 ml of ammonium buffer were mixed with 6 ml CH-TEA and 10 μl of PFBCl (5% in cyclohexane) for 5 min. After a brief centrifugation, 5 ml of the organic phase were transferred to a tapered test-tube and evaporated to dryness under nitrogen at 40°C. The residue was redissolved in 50 μl of ethyl acetate, and 1 μl was injected into the gas chromatograph (nitrogen–phosphorus detection mode).

A standard graph was prepared by treating known amounts of butanilicaine triphosphate in plasma according to this procedure.

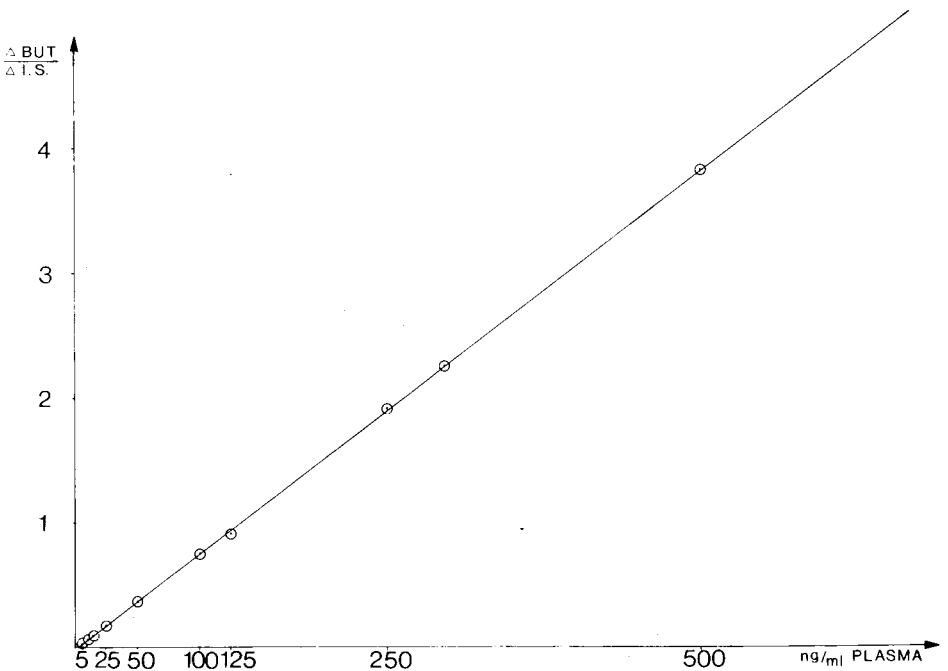
The recoveries were obtained by adding different amounts of butanilicaine triphosphate to 2 ml of plasma or urine and extractive benzoylation (rotating or vortexing) followed by the addition of the internal standard as a methanolic solution, evaporation and subsequent reaction with PFBCl.

RESULTS AND DISCUSSION

Because the gas–liquid chromatographic separation of compounds containing amino groups generally results in peak tailing due to spurious adsorption onto the column, several approaches have been used for the derivatization of the amine function in butanilicaine and the isomeric internal standard.

The acylation with TFAA and PFPA was not complete at room temperature and the compounds formed were easily hydrolysed. Therefore benzoylation of the secondary amino group in butanilicaine was tried.

Benzoyl derivatives can be made from alcohols, thiols and amines by treating the residue obtained by benzene extraction with pyridine and benzoyl chloride and shaking over several hours[9]. The Schotten–Baumann procedure, whereby an alkaline glycerol solution was shaken with benzoyl chloride, followed by extracting the derivatives, was applied by Decroix *et al.*[10]. The Schotten–Baumann type acylation reaction was also used for the determination of phenolic amines in water or plasma[11]. PFBCl derivatives are generally made with trimethylamine as catalyst[12] or at elevated temperatures[13,14]. In this work, however, a Schotten–Baumann reaction was performed



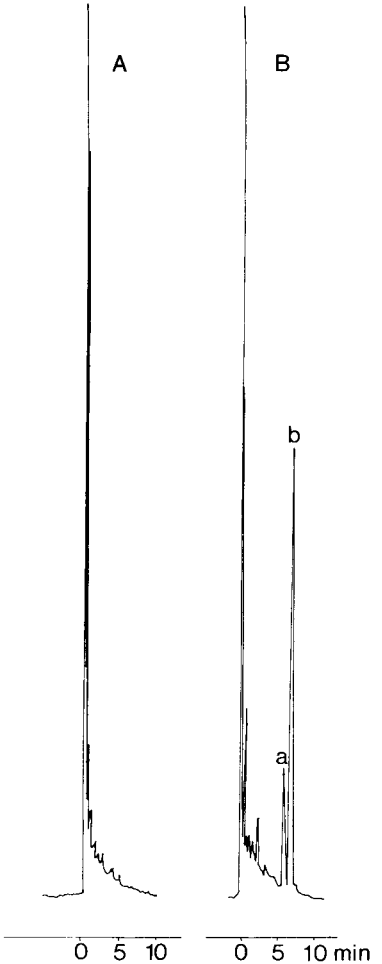


Fig. 1. Standard graph obtained by spiking horse plasma.

Fig. 2. Typical chromatograms of horse plasma. A, Blank control plasma; B, spiked plasma. Peaks a and b are the N-pentafluorobenzamides of butanilicaine and the internal standard, respectively.

using PFBCl with simultaneous extraction at room temperature of the pentafluorobenzamide formed. The basic catalyst was the water-soluble triethanolamine. The derivative has good GC properties, and the organic phase can be washed with 0.01 *N* NaOH without noticeable hydrolysis.

Although the pentafluorobenzoyl group appears to be the one that confers the greatest sensitivity for electron-capture detection of amines[15], this detection method could not be used here. Indeed, the formation of the strongly electron-capturing pentafluorobenzoic acid (PFBH) causes serious GC interference. Although PFBH can be removed by extracting the organic phase with 1 *N* NaOH[16], this procedure could not be used here owing to the hydrolysis of the benzamide and butanilicaine itself at this pH. Nevertheless, the use of the nitrogen-specific detector enables an acceptable detection limit to be achieved.

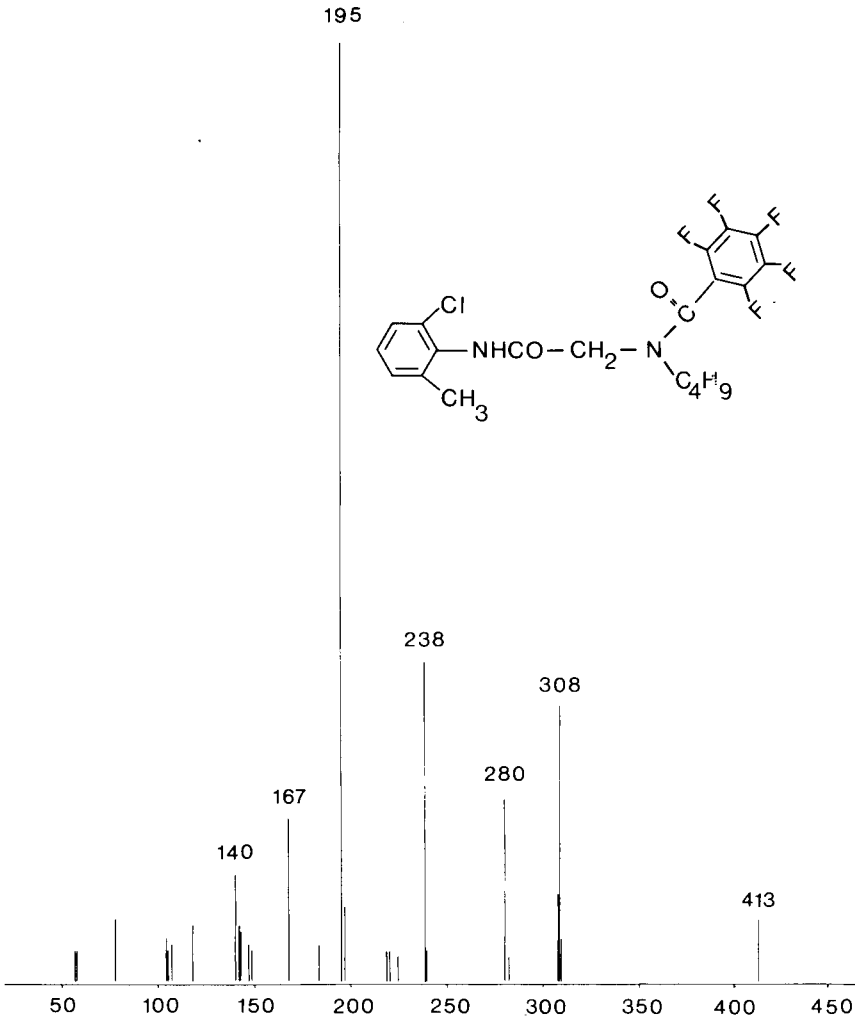


Fig. 3. Electron impact mass spectrum of the pentafluorobenzamide of butanilicaine.

TABLE I

RECOVERIES OF BUTANILICAINE ADDED TO HORSE PLASMA OR URINE

Butanilicaine added to 2 ml	*Recovery*		
	Plasma		*Urine*
	Shaking 5 min	*Vortexing, 1 min*	*Vortexing, 1 min*
250 ng	183 ± 12 ng	193 ± 23 mg	210 ± 10 ng
500 ng	452 ± 36 ng	396 ± 18 ng	389 ± 10 ng
1250 ng	1108 ± 48 ng	944 ± 27 ng	1017 ± 60 ng
Recovered, pipetting 5 ml out of 6 ml	85 ± 9% $n = 17$	77 ± 5% $n = 16$	81 ± 4% $n = 15$
Absolute recovery	±102%	±92%	±97%

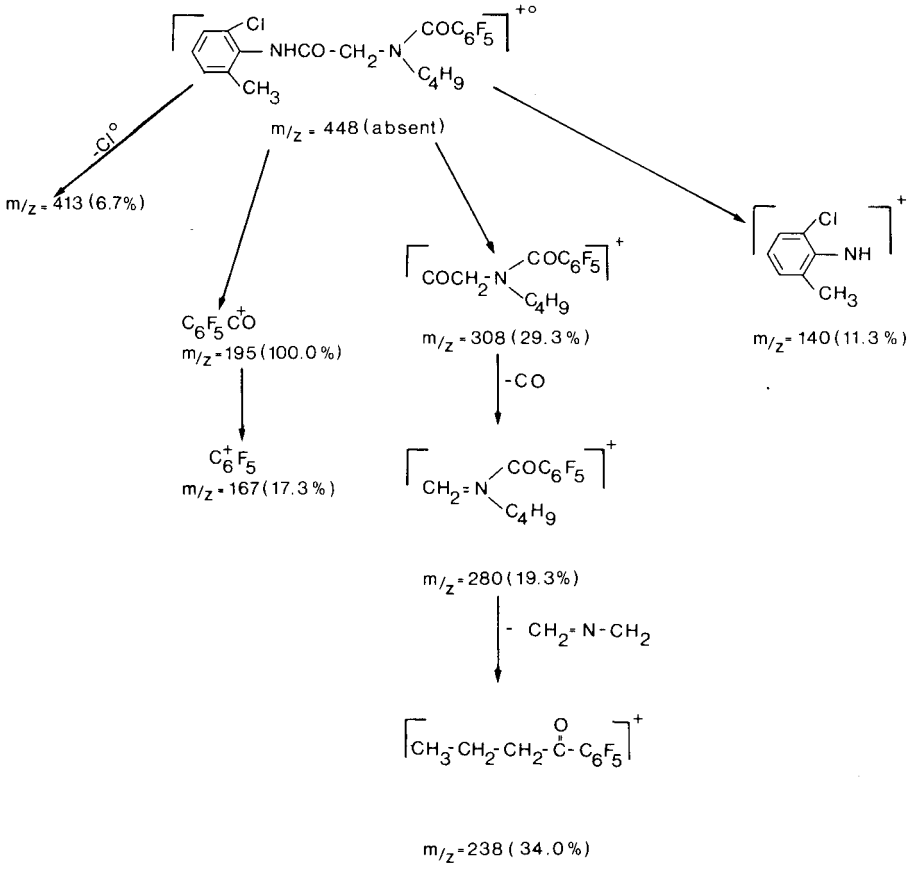


Fig. 4. Postulated mass fragmentation pattern of the N-pentafluorobenzamide of butanilicaine.

The extractive pentafluorobenzoylation of butanilicaine and the internal standard was complete after *ca.* 5 min at room temperature. The recoveries (Table I) were nearly quantitative.

A standard graph with a linear concentration range in the interval 5–500 ng/ml is presented in Fig. 1 ($r = 0.999$, $n = 38$). With 2 ml of plasma, the detection limit was evaluated at 5 ng of butanilicaine triphosphate. Fig. 2 shows a typical chromatogram obtained by processing blank and spiked horse plasma as described in the Experimental section.

The mass spectrum of the pentafluorobenzamide of butanilicaine did not give a molecular ion (Fig. 3). However, diagnostic ions at $m/z = 413$, 308, 280, 238 and 140 were observed. The postulated structures for these ions are shown in Fig. 4.

CONCLUSIONS

With triethanolamine as catalyst, the extractive benzoylation under Schotten–Baumann conditions of butanilicaine with PFBCl and subsequent GC with nitrogen-specific detection is a simple, rapid and sensitive technique for the determination of butanilicaine in plasma and urine.

ACKNOWLEDGEMENT

The authors thank Mrs. G. Demey and Mr. N. Desmet for technical assistance. Thanks are also due to Hoechst AG for a gift of butanilicaine and some isomers. This work was supported by the Wellcome Foundation Centenary Grant.

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Note

Preparative-scale separation of alkene geometric isomers by liquid chromatography

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(Received November 23rd, 1981)

The separation of mixtures of geometrical isomers is often difficult to achieve on a preparative scale, though the acquisition of a single pure isomer may be important. In the field of insect pheromones in particular, isomer purity can be of highest concern and small amounts of contamination by an unnatural isomer may strongly modify the insect response.

A number of techniques have been applied to the separation of geometric isomers on a preparative scale or for the quantitative analysis of mixtures. Silver-loaded thin-layer chromatography (TLC) plates have been widely used and a discussion of this and other types of argentation chromatography is presented in a review by Guha and Janák[1]. More recently the use of silver-loaded supports for high-performance liquid chromatography (HPLC) have become popular. Heath and co-workers[2,3] separated isomers of olefinic insect attractants by HPLC on silica impregnated with silver nitrate. Silver-loaded HPLC supports have also been prepared by loading of silver ions on a strong cation exchanger and insect sex attractants[4–6] and prostaglandins[7] have been separated using these supports. Lam and Grushka[8] separated isomers of C_{16} and C_{18} fatty acids and permethrin (a synthetic pyrethrin) using silver loaded aluminosilicate as a support for HPLC. Silica gel was treated with sodium aluminate to form a polyanionic surface and the counter ions were then exchanged for silver ions. Conversely, silver ions in the mobile phase were used by Vonach and Schomburg[9] to separate olefinic isomers by HPLC on a reversed-phase support.

Gas chromatography (GC) has also been employed for the separation of geometrical isomers. Warthen and Green[10] analysed *cis* and *trans* isomers of fatty alcohol acetates using diethylene glycol succinate (DEGS) in a 300-ft. capillary column. Other liquid phases have been used by Litchfield *et al.*[11,12], Kaufman and Lee[13] and Lipsky *et al.*[14,15] for similar separations.

It is also possible to separate geometrical isomers of some olefins by a non-chromatographic method. Leadbetter and Plimmer[16] have recently used the preferential formation of urea inclusion complexes by *E* isomers to separate alkenes prepared by Wittig condensations. The *Z* isomers did not form inclusion compounds, and the method can be used on a large scale.

In view of the present high cost of silver, the simplest efficient method of separation of geometric isomers: silver nitrate-loaded TLC plates, is unattractive. The silver can be recovered but recovery is neither simple nor efficient. Ideally a

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system is required where the silver salt remains on the support, the column or thin layer can be used repeatedly, and the separation is as efficient as can be obtained by TLC.

We describe here a silver nitrate-loaded silica column which we have found both cheap and efficient for the preparative separation of geometric isomers. By using particles of 50 μm diameter and medium pressure to force the solvent through, efficiencies comparable to TLC are obtained. The method may be described as argentation medium-pressure liquid chromatography (Ag-MPLC).

EXPERIMENTAL

Apparatus

An MPL series II micropump (Metering Pumps, London, Great Britain) with a PTFE diaphragm pumphead (Maximum pressure ≈100 p.s.i.) was used for solvent delivery. Samples were injected (Altex, Berkeley, CA, U.S.A.) onto the column through a 4-way Tefzel slider valve using a 10-ml Hamilton 1010 gas-tight syringe. Fractions were collected with an LKB 2112 Redirac fraction collector and individually analysed on a Pye series 104 gas chromatograph with flame ionisation detector using either a 3 m 5% DEGS on supersorb (100–120 mesh) column or a 1.5 m 3% OV-101 on Chromosorb W (100–120 mesh) column.

Reagents

Diethyl ether and ethyl acetate were dried over Type 4A molecular sieves. Light petroleum (b.p. 40–60° and b.p. 60–80°) was used without further purification.

Preparation of silver-loaded support

Kieselgel 60 (100 g Merck, Darmstadt, G.F.R.) of particle size 40–63 μm (230–300 mesh, ASTM) was suspended in a solution of silver nitrate (20 g) dissolved in acetonitrile. The solvent was then evaporated under vacuum, slowly and carefully in a rotary evaporator, to avoid the formation of finer particles. The silver-loaded silica was finally dried on a fluid bed drier with a stream of nitrogen. Silica gel loaded with 40% of its weight of silver nitrate was prepared similarly.

Column packing

The silver-loaded silica was dry-packed by the tap-and-fill method into a 100 cm × 15 mm glass column (Whatman, Maidstone, Great Britain), and the ends capped with Whatman barrel sleeve assemblies and pistons. The column was wrapped in a covering of thick card, to exclude light, and connected to the pump and valve with 1/16 in O.D. PTFE capillary tubing (Alltech, Arlington Heights, IL, U.S.A.). Adaptor connections were necessary to connect to the 1/16 in. I.D. PTFE tubing at the ends of the Whatman column.

Use of the column

A 1-g amount of a mixture of (*Z*) and (*E*)-8-heptadecene, approximately 80:20 was injected into the capillary line leading into the 20% loaded column and eluted with light petroleum (b.p. 40–60°) at a flow-rate of 1 ml min^{-1}. Fractions of 1 ml were collected and analysed by GC on a 5% DEGS column at 100°C with nitrogen carrier gas at 60 ml min^{-1}. Results are shown in Fig. 1.

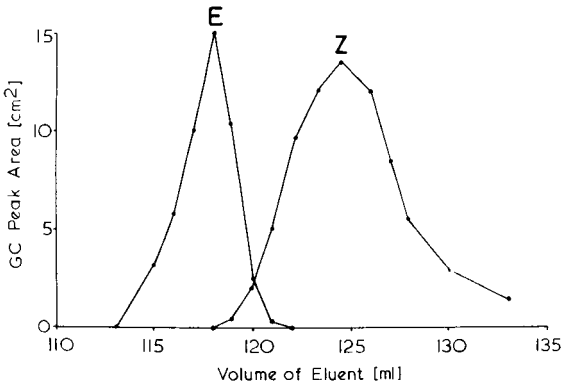

Fig. 1. Separation of (*E*) and (*Z*)-8-heptadecene on a 20% silver nitrate loaded silica gel column. Fraction composition was determined by gas chromatography.

From a mixture of (*Z*,*E*)- and (*Z*,*Z*)-α-farnesene, 1 g was similarly chromatographed on the 20% loaded column, eluting with 10% diethyl ether in light petroleum (b.p. 40–60°C) at a flow-rate of 6.5 ml min^{-1}. Fractions of 15 ml were collected and analysed by GC on a 3% OV-101 column at 120°C and nitrogen carrier gas at 60 ml min^{-1}. Results are shown in Fig. 2.

From a mixture of (*Z*)- and (*E*)-7-methyl-6-nonen-3-one, 1.1 g was similarly chromatographed on the 20% loaded column, eluting with light petroleum (b.p. 60–80°C) containing 2% ethyl acetate, flow-rate 1.6 ml min^{-1}, and also on the 40% loaded column, eluting with light petroleum containing 5% diethyl ether.

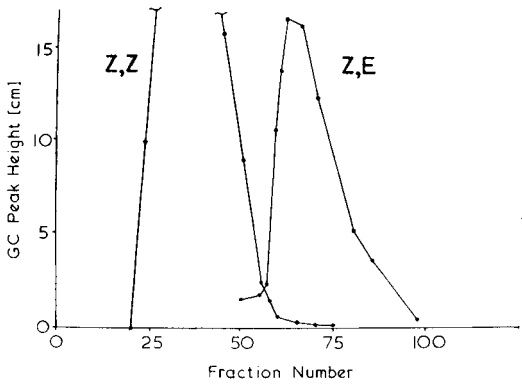

Fig. 2. Separation of (*Z*,*Z*) and (*Z*,*E*)-α-farnesene on a 20% silver nitrate loaded silica gel column.

The columns were prepared for re-use by backflushing with diethyl ether. When not needed for an extended period, the solvent was blown out and the column stored dry.

Pure *Z* and *E* isomers of 8-heptadecene and 9-nonadecene were required for comparison with substances obtained from the Dufour glands of various ant

species[17,18]. A mixture of the two isomers in each case was prepared by Wittig reaction from octanal or decanal, respectively, and triphenylnonylphosphonium bromide. The separation achieved on the silver-loaded silica column is shown in Fig. 1, for 8-heptadecane; 9-nonadecene behaved similarly.

The farnesenes contain four double bonds; in the case of the α-farnesenes which contain all the four bonds in the same positions, 4 isomers exist, namely (*E*,*E*), (*E*,*Z*), (*Z*,*E*) and (*Z*,*Z*)[19,20]. These can be separated by GC only by the most efficient columns. A mixture of (*Z*,*E*) and (*Z*,*Z*)-α-farnesene (Fig. 3) was obtained by a partially stereo-selective synthesis, the final stage of which gave two isomers. These were efficiently separated as shown in Fig. 2. The presence of other double bonds did not greatly complicate the separation.

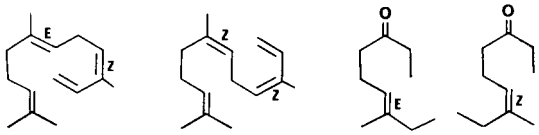

Fig. 3. Structures of farnesene and methylnonenone isomers.

The mixture of (*Z*) and (*E*)-7-methyl-6-nonen-3-one (Fig. 3) was not completely separated on any of our packed GC columns, it was shown by ^{13}C nuclear magnetic resonance (NMR) spectroscopy to consist of a 66:34 mixture of *E*:*Z* isomers. The C-7 carbon of the *E* isomer resonated at 122.5 ppm from tetramethylsilane (TMS) and in the *Z* form at 121.3 ppm. Chromatography on the 20% silver nitrate column gave only partial separation of the isomers. The *E* isomer eluted first, but some contamination with the *Z* isomer was soon detected. The first 16 fractions of 5 ml each, when combined, were found to consist of a 78:22 *E*:*Z* mixture (by ^{13}C NMR spectroscopy). The total material was eluted in 36 fractions. Chromatography on the 40% column did not achieve a significantly improved separation.

DISCUSSION

A silver nitrate-loaded silica column has been used for separations of alkene isomers, on a gram scale. For larger quantities the column size can be scaled up proportionally. By choice of the right particle size, separations can be as efficient as those obtained at much greater cost in time and chemicals by TLC, and without using high pressure pumping. The polyunsaturated farnesenes are rapidly oxidized in air and the loss in handling them on TLC plates and recovering the separated isomers is considerable. By the liquid chromatography method they remain out of contact with air and losses are considerably reduced.

The column can be used repeatedly without any apparent deterioration in performance. The loading of 20% of silver nitrate was chosen by comparison with what can be conveniently used in argentation-TLC. We have not seen any reports of columns or thin layers with loadings as high as 40%. When we failed to separate the ketone isomers on the 20% column we tried a column with 40% loading, but did not achieve any significant improvement. Not too much should be concluded from this failure, for the separation of this particular isomer pair may be inherently difficult,

since it could not be achieved completely on a GC column either. The two isomers differ only slightly in structure.

It is noteworthy that for heptadecene, nonadecene and methylnonenone, the *E* isomer eluted before the *Z* on both the column and TLC. For the farnesenes, the *Z,Z* isomer eluted in both cases before the *Z,E* isomer. This is probably a function of the co-ordination of the somewhat crowded farnesene bonds around the silver ion.

Our own interest has concentrated on the separation of alkenes encountered in our study of ant pheromones, but the method should be equally applicable to other types of compounds, provided the isomers differ sufficiently in their spatial arrangements.

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Book Review

Actinide separations (ACS Symposium Series, No. 117) (based on a symposium sponsored by ACS Division of Industrial and Engineering Chemistry at the *ACS/CSJ Chemical Congress (117th ACS National Meeting), Honolulu, Hawaii, April 3–5, 1979)*, edited by J. D. Navratil and W. W. Schulz, American Chemical Society, Washington, DC, 1980, XIV + 609 pp., price US$ 43.50, £ 23.00, ISBN 0-8412-0527-2.

This volume contains 39 papers presented at the above-mentioned symposium and constitutes a survey of separation science as applied to industrial problems in nuclear energy production.

The book begins with five papers on "Ion exchange and precipitation methods", one of which is a review of plutonium peroxide precipitation. Then follow seven papers on solvent extraction methods, and again there is a review on "Actinide extractants".

The next seven papers deal with "Pyrochemical and photochemical methods". Workers outside the field may find the techniques employing molten salts and liquid metals interesting.

The remaining chapters deal with "Power reactor fuel reprocessing" (7 papers), "Recovery from waste solution" (7 papers) and "General methods—Review papers" (6 papers).

The reviewer found two concepts particularly interesting. A paper by Bilal *et al.* describes an industrial-scale counter-current ion migration technique, which uses the principle of isotachophoresis on a large scale, seemingly with good results. The papers dealing with two-phase extraction between molten salts and alloys in the processing of spent nuclear fuel are also valuable.

The book was prepared by photographic reproduction of typescripts, but is clear and readable. Most figures and graphs are well reproduced.

Lausanne (Switzerland) M. LEDERER

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Book Review

Fluorimetrie, by M. Zander, Springer, Berlin, Heidelberg, New York, 1981, VIII + 127 pp., price DM 68.00, *ca.* US$ 32.40, ISBN 3-540-10512-3.

The great progress in fluorimetry in recent decades was the result of progress in instrumentation and also progress in new measurement principles. It is therefore the aim of the book to transmit knowledge of these new developments but not to transmit methods, and it is from this point of view that the concepts of the book are presented. As an introduction the position of fluorimetry in instrumental analysis and the theoretical basis of the luminescence of organic molecules are discussed. Measurement principles, sources of errors and their prevention are discussed in the chapter "Methodical fundamentals of fluorimetry". Off-line and on-line coupling of chromatographic methods and fluorimetry are treated briefly. Important new techniques in fluorimetry such as low-temperature fluorimetry, Shpol'skii fluorimetry and matrix-isolation fluorimetry are treated. Recommendations for fluorimetric determinations of proteins, enzymes, nucleic acids, polycyclic and heterocyclic compounds, elements and inorganic ions via their chelates conclude the book. Each chapter is completed by adequate reference lists. This recommendable book is addressed to all analysts working in biochemistry, clinical chemistry and environmental chemistry.

Leipzig (G.D.R.) J. WAGNER

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Gas Chromatography

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See also 7, 8.

2. FUNDAMENTALS, THEORY AND GENERAL

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

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23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

23a. Porphyrins and other pyrroles

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See also 1177, 1186, 1294, 1297, 1299, 1319, 1320.

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH		J	F	M	A	M	J	J	A	S	O	N	D
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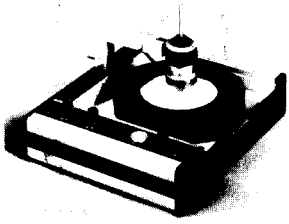

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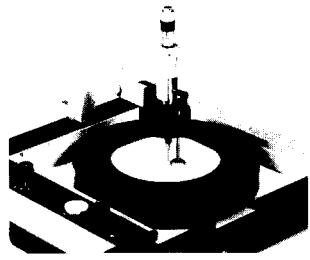

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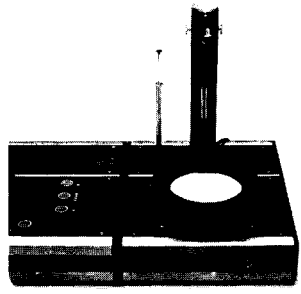

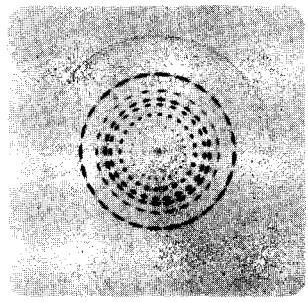

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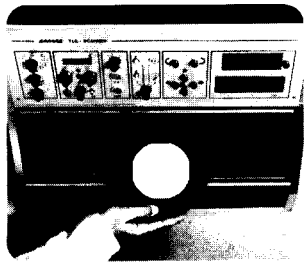

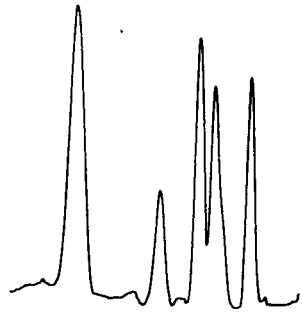

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