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Optimization of Chromatographic Selectivity

A Guide to Method Development

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

(Journal of Chromatography Library, 35)

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

Chapter 1 gives a short introduction, describes chromatographic theory and nomenclature, and outlines the method development process. Chapter 2 describes guidelines for method selection, and quantitative concepts for characterizing and classifying chromatographic phases. Selective separation methods, from both gas and liquid chromatography are given in Chapter 3; the main parameters of each method are identified and simple, quantitative relations are sought to describe their effects. Criteria by which to judge the quality of separation are discussed in Chapter 4 with clear recommendations for different situations. The specific problems involved in the optimization of chromatographic selectivity are explained in Chapter 5. Optimization procedures, illustrated by examples, are extensively described and compared on the basis of a number of criteria. Suggestions are made both for the application of different procedures and for further research. The optimization of programmed analysis receives special attention in Chapter 6, and the last chapter summarizes the optimization of the chromatographic system, including the optimization of the efficiency, sensitivity and instrumentation.

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

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

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INTRODUCTION OF MULTI-CRITERIA DECISION MAKING IN OPTIMIZATION PROCEDURES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS

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Research Group Chemometrics, Pharmaceutical Laboratories, A. Deusinglaan 2, NL-9713 AW Groningen (The Netherlands)
(First received December 23rd, 1985; revised manuscript received July 2nd, 1986)

SUMMARY

A method is proposed which eliminates the necessity of making preliminary assumptions about the relative importance of criteria used in the optimization of high-performance liquid chromatographic separations. This leads to the introduction of a new concept of optimality in analytical chemistry and more specifically in separation methods: the Pareto-Optimality. An example is given for the separation of five sulphonamides.

INTRODUCTION

Much work has been done in developing criteria for judging the quality of a chromatogram. Such criteria are needed in optimization procedures for high-performance liquid chromatographic (HPLC) separations. Recently[1] some of these criteria were critically evaluated, including the chromatographic response function (CRF), the chromatographic optimization function (COF), the informing power (IP), the separation number (SN) and the product resolution (PRES).

Some of these criteria have been refined, Drouen *et al.*[2] designed a sophisticated product resolution criterion. Glajch *et al.*[3] used overlapping resolution maps (ORMs) to establishing the mobile phase composition. Relationships between the resolution and mobile phase composition have been described by Jandera *et al.*[4]. Laub and Purnell[5] used the separation factor as an optimizing criterion, leading to window diagrams. Hsu *et al.*[6] also used a window-diagram technique. However, this technique does not take into account, explicitly, the analysis time. So, some of these criteria measure only the quality of a separation, *i.e.*, ORM, SN, PRES, while others combine two distinct aspects of a chromatogram, *i.e.*, the resolution between peaks and the analysis time (COF, CRF). This sometimes leads to ambiguous results, *e.g.*, Fig. 1 where the CRF is used to assess the chromatograms A, B and C.

When using the expression

$$CRF = a\Sigma R_i + b(T_{max.} - T_p)$$

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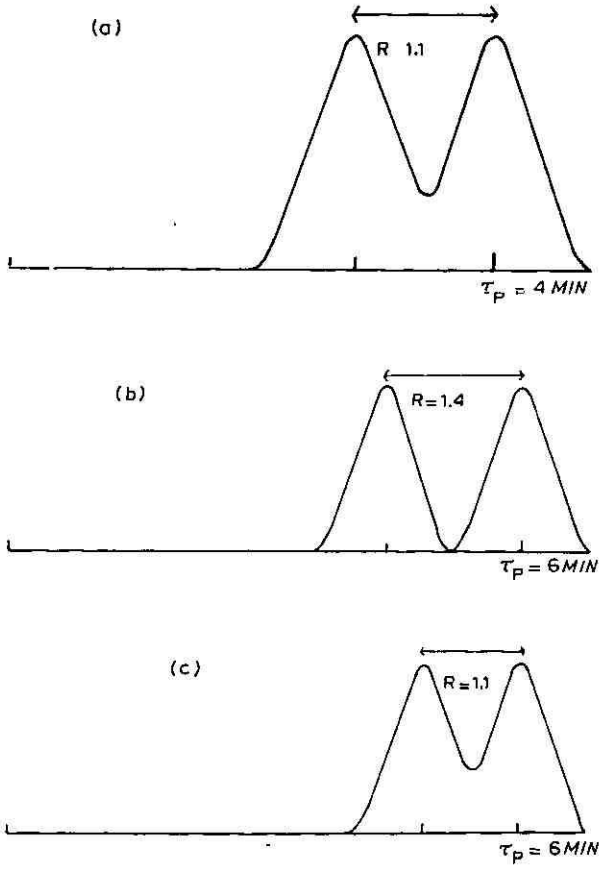

Fig. 1. Chromatograms of two components. R = Resolution between the compounds; T_p = retention time of compound eluted last.

where a and b are weighting factors, R_i is the resolution, $T_{max.}$ the maximum acceptable retention time of the last peak and p is the number of peaks, values for a, b and $T_{max.}$ have to be chosen before the start of the optimization procedure.

Example 1: let $a = 5$, $b = 1$ and $T_{max.} = 10$ min, then the CRF values are 11.5 for A, 11.0 for B and 9.5 for C. So chromatogram A is judged to be of higher quality then B or C (Fig. 1).

Example 2: let $a = 7$, $b = 1$ and $T_{max.} = 10$ min. Only the weighting of the resolution is changed, but a different result is obtained; the CRF values are 13.7 for A, 13.8 for B and 11.7 for C. So B is judged to be of highest quality.

It is obvious that the judgements made depend on the weighting factors. (Note that in both cases chromatogram C is the worst.) So when choosing the weighting factors at the start of the optimization procedure, a decision is made as to the relative importance of the different aspects of a separation. This is done without knowledge of

the behaviour of the different aspects when changing the mobile phase composition. Likewise, *a priori* selection of $T_{max.}$ influences the outcome of the CRF values.

The method we propose does not make preliminary assumptions about the weighting factors and $T_{max.}$. In our implementation of multi-criteria decision making (MCDM) both aspects are considered explicitly.

THEORETICAL

For illustrative purposes we consider only two criteria, the analysis time and the minimum resolution. When using a three-component system (water, organic modifier 1, organic modifier 2) as mobile phase the factor space can be represented by a triangle[7], each vertex of which is occupied by a different solvent. Measurements of the retention time of each solute are made at regular points in the design space, a part of the factor space (The design space can be determined by gradient elution, ensuring that all components are eluted within certain limits.)

The capacity factor of each solute can be related to the mobile phase composition of the design space. The relationship between ln k and the solvent composition is described by a seven-term special cubic equation, the coefficients of which can be calculated by polynomial regression. This requires the measurement of the individual capacity factors at at least seven mobile phase compositions, which are located in the design space according to an extreme vertices design. For a detailed discussion see ref. 8.

Restriction of the chromatographic system to mobile phase mixtures ensuring that the capacity factors of all solutes are, for example, within the interval 1–20, leads to a subset of the design space. This subset is called the feasible or available factor space. For each solute the capacity factor can be predicted at every mobile phase composition within the feasible factor space.

As a measure of the analysis time we have chosen the capacity factor of the last solute eluted. The resolution is calculated with the formula:

$$\mathrm{RES}\ (1,2) = \sqrt{N}(k_2 - k_1)/2(k_2 + k_1 + 2)$$

where N is the column plate number. For every mobile phase composition within the feasible factor space, the predicted capacity factors of all solutes are available. The capacity factor of the last solute to be eluted and the minimum resolution between adjacent peaks are predicted straightforwardly for every mobile phase composition within the feasible factor space. So far our approach is analogous to the "ORM" method. However, we consider not only the resolution but also the analysis time as a criterion for optimization.

Although we could now proceed by overlapping the resolution map with an analysis time map, we have chosen a different approach. In this approach it is not necessary to preselect acceptable values of the minimum resolution and maximum analysis time, which are needed for the construction of the maps. All predicted values at each solvent composition are used and are presented in a two-dimensional picture, Fig. 2.

Each point within or on the boundary of the "egg" in Fig. 2 relates to a pair

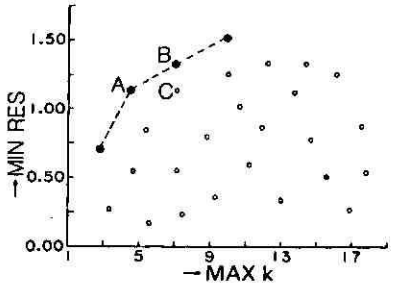

Fig. 2. Plot of the feasible criteria space. It should be emphasized that this plot does not represent a direct functional dependence of MIN RES on MAX k.

of criteria values (MIN RES, MAX k). These values are predicted as the outcome of a chromatographic experiment with a certain mobile phase composition within the feasible factor space. For obvious reasons we call the "egg" the feasible criteria space or feasible objective space. When using one criterion to judge a chromatogram, clear comparisons between two mobile phase compositions can be made (mobile phase composition 1 leads to better results than composition 2 if criterion value 1 is greater than criterion value 2). Such clear comparisons cannot be made when using two (or more) criteria. However, not every point in the feasible criteria space has the same status.

The points (●) are called non-inferior solutions or Pareto-Optimal points. All other points in the feasible criteria space are inferior to these points (or solutions). A point in the feasible criteria space is a Pareto-Optimal point if there exists no other point in that space which yields an improvement in one criterion without causing a degradation in the other criterion. So A and B are Pareto-Optimal points, but C is not (A, B and C correspond to the three situations in Fig. 1).

A consequence of this method is that there is no longer one optimum point but there are several Pareto-Optimal points; there is no longer one optimum mobile phase composition, but a choice can be made between the Pareto-Optimal points. The inferior points within the egg need not be considered and the analyst can base his choice between the Pareto-Optimal points by evaluating quantitatively the pay-off between minimum resolution and analysis time from Fig. 2. The method will be illustrated with an example of the separation of sulphonamides. For an introduction to the theory of MCDM see ref. 9.

EXPERIMENTAL

Methanol was analytical grade (Merck). Tetrahydrofuran was Lichrosolv quality (Merck). Deionized water was used throughout. All mobile phases were acidified using reagent-grade acetic acid (Merck). The five sulphonamides were of pharmaceutical quality, obtained from various manufacturers and used as received: 5-methylsulphadiazine, sulphamerazine, sulphamoxole, sulphadimidine and succinylsulphathiazole (see Fig. 3a–e).

The instrument used was a Model SP740 pump (Spectra-Physics) with pump control and pressure monitor Model 3400, fitted with a dual-wavelength detector

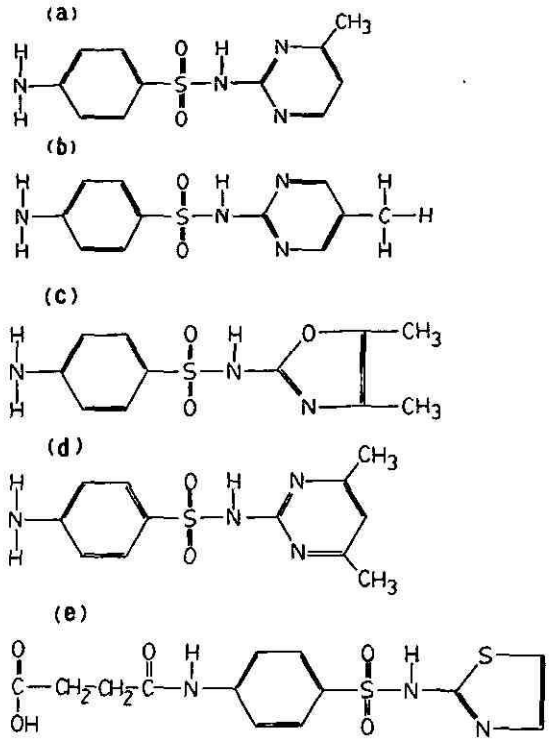

Fig. 3. Structures of the sulphonamides used: (a) sulphamerazine; (b) 5-methylsulphadiazine; (c) sulphamoxole; (d) sulphadimidine; (e) succinylsulphathiazole.

(Chromatronix Model 220), an injection valve (Rheodyne) fitted with a 20-μl injection loop and an Omniscribe recorder (Houston Instrument).

Data acquisition and integration were performed with an Autolab System IVb chromatography data analyser (Spectra-Physics). The column used was 15.0 cm × 4.6 mm stainless steel, packed with Nucleosil RP-8, particle size 5 μm (N = 3500).

The retention times quoted are the averages of three measurements; the dead time was measured as the first baseline distortion caused by the injection of a mobile phase slightly enriched with water. The flow-rate was 0.80 ml/min. Calculations were performed on the CDC 170/160 computer of the Groningen University Computing Centre, using programs written in Fortran IV and Pascal.

RESULTS AND DISCUSSION

The retention times of the five sulphonamides and the dead times werd measured at nine mobile phase compositions, regularly spaced in the design space. The boundaries of the design space are shown in Fig. 4a as line A and line I, chosen such that the capacity factors vary between 1 and 8. The selection of the boundary was based on an extensive study by our group on the reversed-phase (RP)HPLC behaviour of sulphonamides in several mobile phase systems, in which more experimental details and data are given[6].

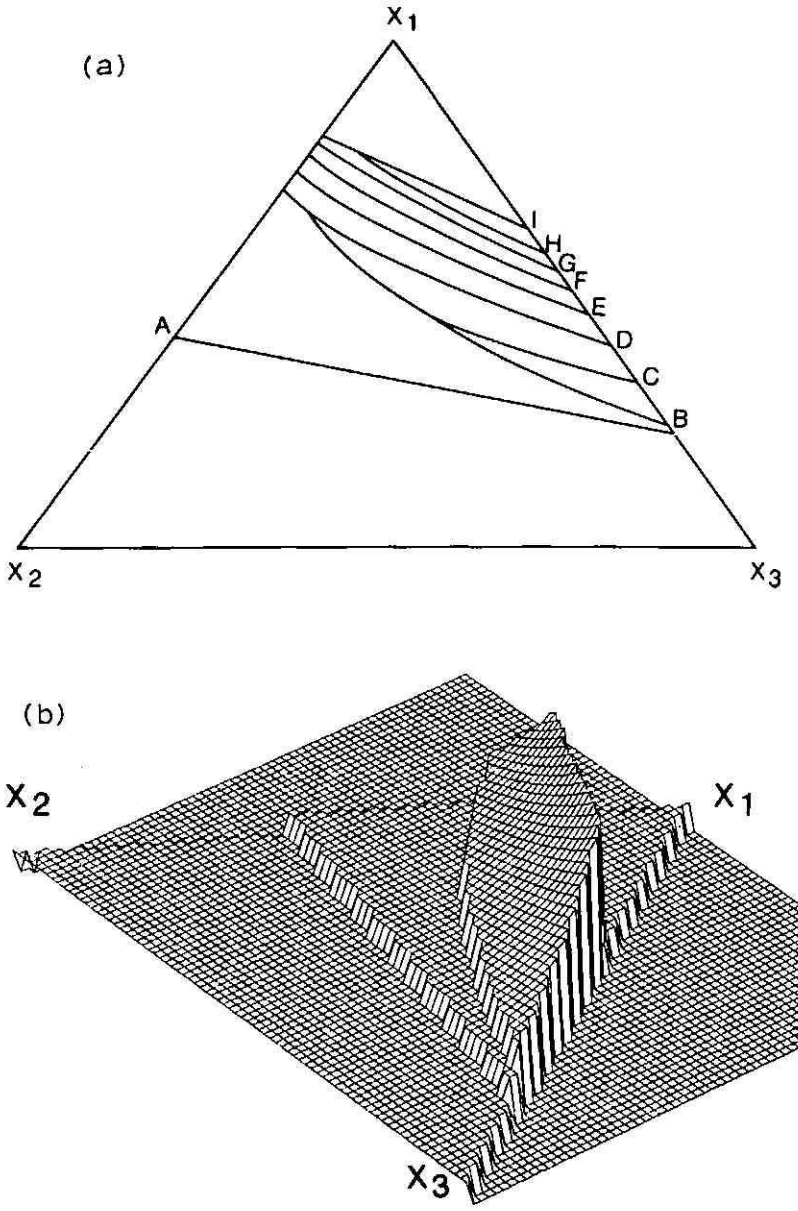

Fig. 4. (a) Contour plot of the maximum capacity factor; X_1 = water; X_2 = tetrahydrofuran–water (50:50); X_3 = methanol–water (50:50). Lines A and I: the boundaries of the design space. A–B: at least one capacity factor smaller than one. Maximum capacity factor: between 1 and 2 (B–C), 2 and 3 (C–D), 3 and 4 (D–E), 4 and 5 (E–F), 5 and 6 (F–G), 6 and 7 (G–H) and 7 and 8 (H–I). (b) Three-dimensional plot of max. k; X_1, X_2 and X_3 as in Fig. 4a.

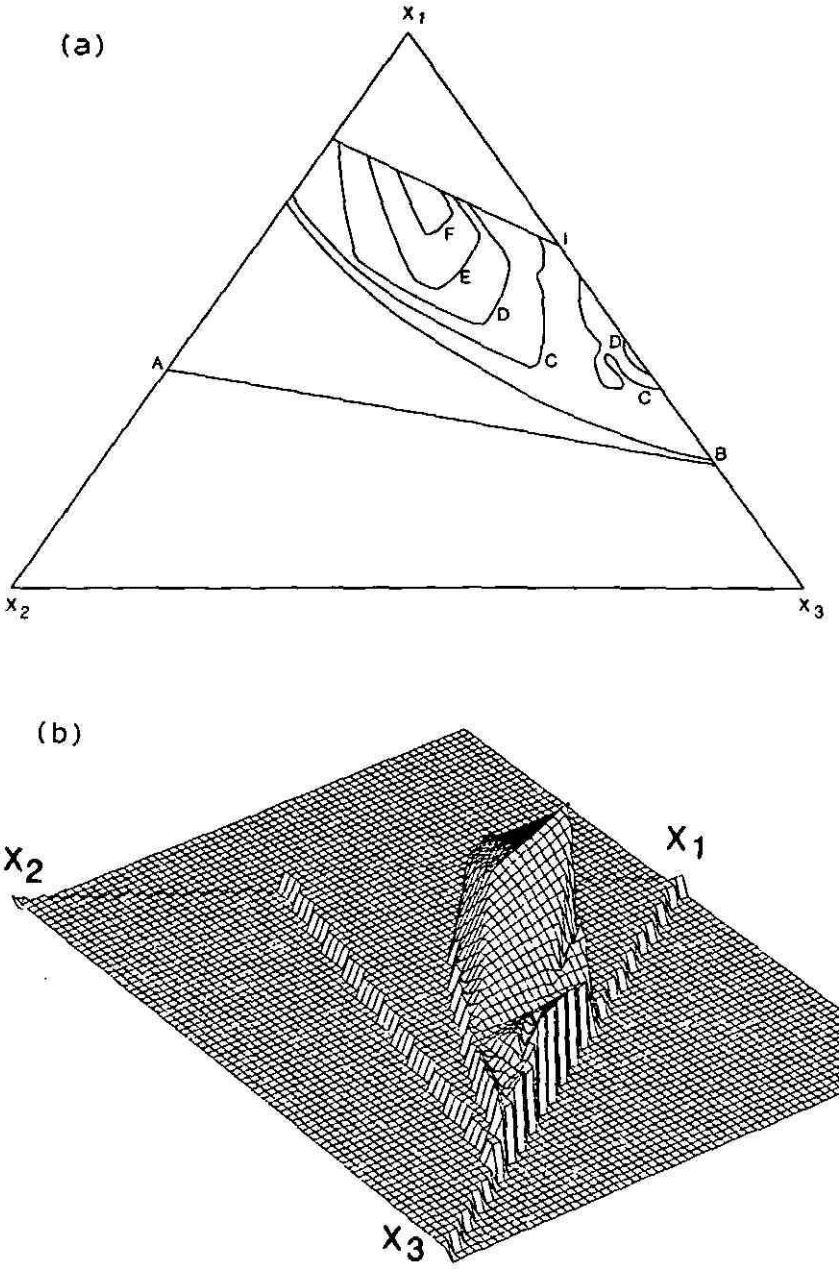

Fig. 5. (a) Contour plot of the minimum resolution; X_1, X_2 and X_3, lines A, I and A–B as in Fig. 4a. Minimum resolution: between 0.00 and 0.20 (B–C), 0.20 and 0.60 (C–D), 0.60 and 1.00 (D–E), 1.00 and 1.40 (E–F) and greater than 1.40 (F–...). (b) Three-dimensional plot of minimum resolution; X_1, X_2 and X_3 as in Fig. 4a.

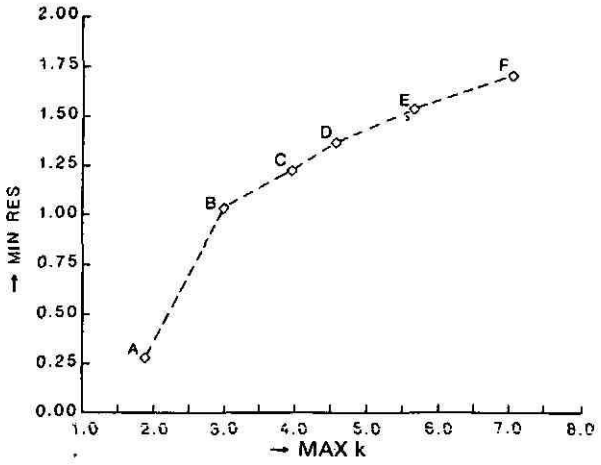

Fig. 6. MCDM plot using seven max. k classes. The points A, B, C, D, E and F are the Pareto-Optimal points. The corresponding mobile phase compositions (in volume percentages of X_1, X_2, X_3) are: A, 0.65:0.00:0.35; B, 0.77:0.10:0.13; C, 0.80:0.09:0.11; D, 0.81:0.08:0.11; E, 0.83:0.07:0.10; F, 0.85:0.06:0.09.

The measured capacity factors for each component were fitted to a special cubic model (eqn. 7 of ref. 8) and the minimum resolution and the maximum capacity factors were predicted for all solvent compositions within the available factor space, using a grid of 2% increments in the water content and 1% increments in each organic modifier. Predicting the maximum capacity factor over the available factors space results in Fig. 4a and b. Similarly, the minimum resolution can be predicted, resulting in Fig. 5a and b.

The next step in the MCDM procedure consists in establishing the Pareto-Optimal points. A plot like Fig. 2 can be made but since only the boundary of such a plot is interesting we calculate only the boundary. The possible maximum capacity

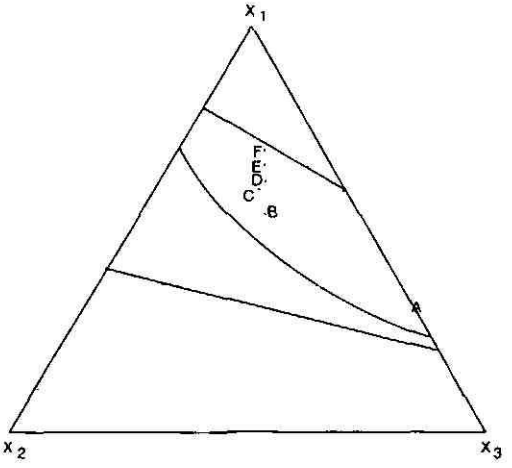

Fig. 7. Pareto-Optimal (PO) plot; X_1, X_2 and X_3 as in Fig. 4a. The points A–F correspond to those in Fig. 6.

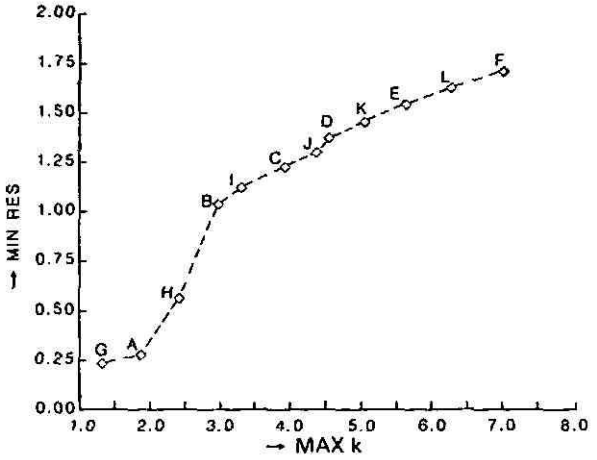

Fig. 8. MCDM plot using fourteen max. k classes (max. k between 1 and 1.5, between 1.5 and 2, ...). Six new PO points are obtained: G, H, I, J, K and L. The points A–F are maintained. The corresponding mobile phase compositions (units as in Fig. 6) are: G, 0.62:0.01:0.37; H, 0.73:0.085:0.185; I, 0.78:0.095:0.125; J, 0.81:0.085:0.105; K, 0.82:0.075:0.105; L, 0.84:0.065:0.095.

factors are divided into seven classes (max. k = 1–2, ..., max. k = 7–8) and in each class we look for the best minimum resolution. The result of this procedure is shown in Fig. 6, the MCDM plot. The points A, B, C, D, E and F are Pareto-Optimal (PO). When these points are plotted in the original solvent triangle we obtain a PO plot (Fig. 7).

The advantage of this procedure is clear from the MCDM plot: the pay-off between the two criteria (analysis time and resolution) is visualized, and a more rigorous decision regarding the mobile phase composition can be made. Because information with respect to both criteria and their pay-off is available, the analyst

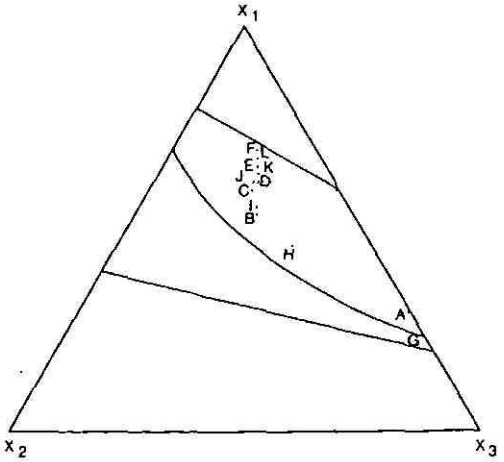

Fig. 9. PO plot; X_1, X_2 and X_3 as in Fig. 4a. The points A–F correspond to those in Fig. 6–8. The new points G–L correspond to those in Fig. 8.

can decide whether or not he is willing to pay for an increase in resolution of 0.2 between points B and C, an increase in the maximum capacity factor of 0.94. No preselection of a minimum resolution or analysis time is necessary. After the choice is made, the corresponding solvent composition is printed out by the program.

An interesting aspect is that allowing the maximum capacity factor to change from 7 to 8 does not guarantee a better separation (there is no Pareto-Optimal point in the max. k class from 7 to 8).

When using smaller max. k classes more information is available. This is illustrated in Fig. 8, another MCDM plot but with smaller max. k classes. The corresponding PO plot is shown in Fig. 9. The ultimate decision as regards which mobile phase composition to be used can be made by the chromatographer, after the optimization is completed. No *a priori* decisions have to be made, but it should be borne in mind that when mixing the mobile phase small errors can be made; examination of Figs. 4 and 5 indicates the impact of such errors on the chromatographic process.

In our opinion such a decision can be made rigorously when using the MCDM approach. Further research on this topic is in progress, including an extension to more than two criteria.

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STEPWISE GRADIENT DEVELOPMENT IN THIN-LAYER CHROMATOGRAPHY

I. OPTIMIZATION OF GRADIENT PROGRAM

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SUMMARY

The application of an equilibrium sandwich chamber with a glass distributer for stepwise gradient development in thin-layer chromatography is described. Six to ten fractions of the eluent having increasing concentrations of a polar modifier are programmed graphically and introduced directly under the distributer with a micropipette. A comparison of the gradient profile with the obtained chromatogram permits adjustment of the gradient program in order to improve the positions of the spots on the chromatogram. The technique is relatively simple and permits the use of multicomponent gradients; it is especially suitable for the separation of complex samples, *e.g.*, plant extracts, including zonal micropreparative separation. It is possible to improve the spot separation in a fragment of the plate without changing the positions of the remaining well separated spots.

INTRODUCTION

Complex mixtures containing components having a wide range of capacity factors cannot be separated by isocratic elution owing to the "general elution problem"[1]: eluents of low strength separate the less strongly retained solutes, while the strongly retained components are eluted much later as low intensity, diffuse peaks. On the other hand, strong eluents do not separate weakly retained components, which are eluted together as a common or partly resolved peak. In high-performance liquid chromatography (HPLC) it is usual to apply gradient elution, analogous to temperature programming in gas chromatography (GC) of solutes which have a wide range of volatilities.

Gradient elution has also been employed in thin-layer chromatography (TLC). The general elution problem in TLC is illustrated in Fig. 1. Gradient elution in TLC can be carried out by several methods[2–6].

(a) Eluent demixing, especially with multicomponent eluents which form a gradient of eluent strength leading to the so-called multizonal chromatography, see ref. 3, p. 177.

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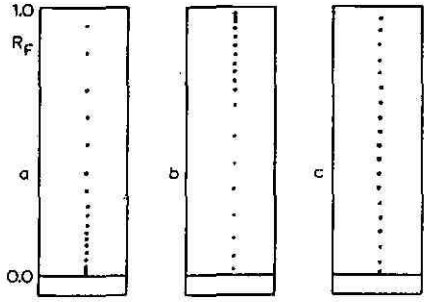

Fig. 1. The general elution problem in TLC: isocratic development (a,b) is not sufficient to separate all components. Eluent: ethyl acetate in chloroform; (a) 10%, (b) 50%, (c) gradient elution, 30 to 70%.

(b) Preadsorption of vapours in chambers of the Vario-KS type where the plate is placed over a tray containing a solvent. Thus, Geiss demonstrated that a plate with preadsorbed benzene vapours and developed with acetone has a very steep gradient of solvent strength (ref. 3, p. 247). These two methods of generation of gradients are relatively simple, however the actual composition of the gradient in the layer is virtually unknown and the control of its shape is difficult.

(c) Controlled changs of the eluent composition in a manner similar to gradient HPLC. Several earlier methods of this type have been discussed in monographs on liquid chromatography[3–6]; also a review by Berezkin *et al.*[7]. In a recent paper[8] a miniaturized device[9] for gradient elution is equilibrium sandwich chambers equipped with a glass distributer was described; the component solvents, A (weaker) and B (stronger), were chosen so as to ensure spontaneous mixing due to molecular interactions and differences in densities, *e.g.*, A = chloroform, B = ethyl acetate.

Another method is to introduce the eluent in small portions[10–12], *e.g.*, six to ten, having increasing concentration of the stronger solvent. The stepwise gradient thus obtained is analogous to a continuous gradient because the steps become diffuse in the development process. Any gradient programs, including multicomponent ones, can be generated in this way and although the gradient shape in the layer may be somewhat distorted relative to the initial program, it is possible to vary the program to fit the actual analytical problem.

EXPERIMENTAL AND METHODS

Apparatus

An equilibrium sandwich chamber (for 20 cm × 5 cm plates) with a glass distributor was used (Polish Reagents POCh, Lublin, Poland)[7–12]. The distributor was 5 or 10 mm wide; in the latter case the volume between the distributor and the carrier plate was about 0.2 cm^3, which for 0.3-mm layers corresponded to development along a distance of 3 cm; six to seven distributor volumes were thus required for full development. The narrower distributor corresponded to a volume of 0.1 cm^3 and about fifteen distributer volumes were necessary to complete the development. To simplify the procedure, a special microfunnel (Fig. 2) was used. It could be filled with up to 0.5 cm^3 of the eluent which was introduced gradually under the distributer by capillary forces.

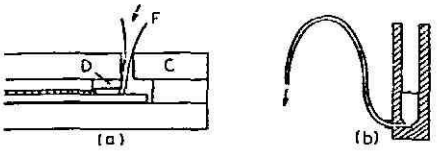

Fig. 2. Cross-section of the equilibrium sandwich chamber: (a) Introduction of eluent between the margin of the carrier plate (cleaned of adsorbent) and the distributor (D) through a microfunnel (F), *e.g.*, the tip of an automatic micropipette, placed in the orifice of the cover plate (C). (b) Another type of microfunnel (0.5–1.0 cm^3) made of PTFE with a capillary elastic PTFE siphon. The siphon connection with the distributer is produced by temporarily lifting the container above the plate level or producing an over-pressure in the container with a syringe.

Gradient programming

A graphical method of gradient programming can be used. A rectangle 20 cm × 10 cm is drawn on millimetre paper (Fig. 3): the vertical side represents solvent composition (and partial volumes of solvents; in Fig. 3 the total volume is 5 cm^3); the horizontal side represents consecutive fractional volumes of the eluent. A line representing the gradient shape is drawn from the starting to the final concentration of the stronger solvent. Vertical lines are divided by this line into parts corresponding to the contents of solvents A and B in the consecutive portions of the eluent; the volumes of A and B necessary to prepare 5-ml portions of the eluent can be read directly from the right-hand ordinate. This is very convenient especially for complex gradient programs as illustrated below.

After development the first eluent fractions are near the end line while the last fractions are at low R_F values; thus, the gradient of the mobile phase composition in the layer is obtained by juxtaposing the chromatogram and the gradient program as shown in Fig. 4.

The calculation of the consecutive spot positions *a priori* is rather difficult and requires knowledge of the quantitative relationships between k' and the composition (see the studies of Golkiewicz and co-workers[13–17] relating to stepwise elution). Moreover, the actual gradient profile in the layer gradually becomes distorted in compar-

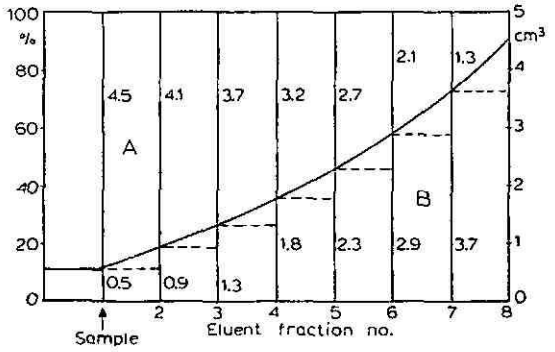

Fig. 3. Graphical programming of an eight-step gradient. After choosing the suitable gradient shape, the volumes of the components A and B are determined from the lengths of the vertical lines dissected by the gradient profile. The sample is spotted after introduction of one fractional volume of the eluent to eliminate eluent demixing.

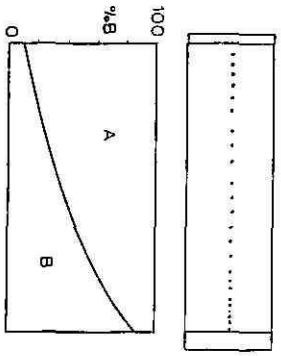

Fig. 4. Comparison of the gradient profile and the resulting chromatogram: the first eluent fractions correspond to the upper part of the plate.

ison to the initial one due to (a) eluent demixing (frontal chromatography of the mobile phase)[2–6,18] and (b) exchange of the stagnant mobile phase in the pores for the mobile phase of increased concentration. The first effect may cause strong deformation of the gradient profile and formation of steep gradients[12,18] especially for low-percentage B[18,19]; it is thus advantageous to start the program not from zero concentration of solvent B but from, *e.g.*, 10% and to spot the sample behind the solvent front. Prewetting with one distributor volume is usually sufficient to displace the deformed part of the gradient profile ahead of the leading zones[12,18].

In spite of the complexity of the gradient development, simplified, general, quantitative rules of gradient optimization can be formulated, based on the analysis of the distribution of the spots along the chromatogram corresponding to the gradient program used. The procedure can be useful in the separation of multicomponent samples, *e.g.*, plant extracts.

Choice of eluent strength range

The sample is chromatographed using solvents of different eluent strengths. On silica, the following series can be recommended: heptane (0.0), trichloroethylene; toluene (0.22); dichloromethane (0.30) or chloroform (0.26); diisopropyl ether (0.42); ethyl acetate (0.94); isopropanol (1.8) (eluent strength, ε^0 values[1], in parentheses).

The gradient program is started from the solvent (A) with which low R_F values are obtained for most components of the sample; with the second solvent (B), most components should have high R_F values and even the strongly retained components should have $R_F > 0$.

The eluent strength range can be chosen more accurately by determination of the R_F values of the sample components in several mixtures of A and B. A plot of R_F *vs.* % B yields the optimum range of the gradient. For instance, Fig. 5 shows that a gradient of 10 to 80% B should be suitable; the mixture in Fig. 5b cannot be separated by a gradient of 10 to 100% B because some of the components have too low R_F values even with pure solvent B and it is necessary to use a wider eluent strength range by the addition of a third solvent C ($\varepsilon_A^0 < \varepsilon_B^0 < \varepsilon_C^0$).

Gradient elution and correction of gradient program

The gradient program chosen from the preliminary experiments may require

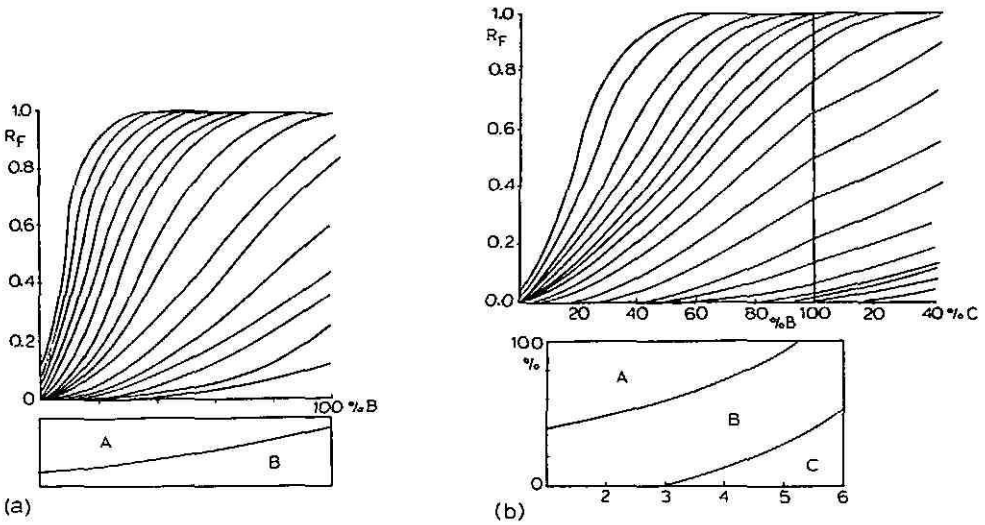

Fig. 5. Examples of the relationships between R_F and the modifier concentration for multicomponent samples and the corresponding gradient profiles required for their separation ($\varepsilon_A^0 < \varepsilon_B^0 < \varepsilon_C^0$).

correction of its eluent strength range and profile. Comparison of the graphical program and the resulting chromatogram according to the principles illustrated in Fig. 6a shows the changes in the gradient shape required to improve the distribution of zones along the plate. Several examples of the correction of gradient profiles are illustrated in Fig. 6a–c.

(a) For the program 10% ethyl acetate (in chloroform) to 100% ethyl acetate most of the spots are accumulated in the upper part of the chromatogram: the range of eluent strength was too high or the profile too steep. Suggested change: replace chloroform and ethyl acetate by weaker solvents, *e.g.*, trichloroethylene and diisopropyl ether, respectively or use a less steep gradient, *e.g.*, 5% ethyl acetate (in heptane) to 30% ethyl acetate.

(b) Most zones are accumulated in the lower part of the chromatogram. Suggested change: use stronger component solvents (B and C instead of A and B) or a ternary gradient A + B + C.

(c) Upper spots are well separated, accumulation of spots having low R_F values with a gap between the two groups of components. Suggested change: steeper gradient of B in the middle fractions and addition of a stronger modifier C to the last fractions of the eluent.

(d) Most spots are accumulated in the central part of the plate. Suggested change: increase slope at the beginning and the end of the gradient and use isocratic elution in the middle. This will decrease the distances between vicinal zones near $R_F = 0$ and $R_F = 1$ and increase the distances near $R_F = 0.5$.

Analogous rules can be formulated for reversed-phase systems. The type of relationship between the k' values and the modifier concentration should be taken into account[20–23]. For plain silica (and generally for polar adsorbents, *e.g.*, diol or aminopropyl silica)

$$\log k' = \text{constant} - n \log (\%\ \text{B})$$

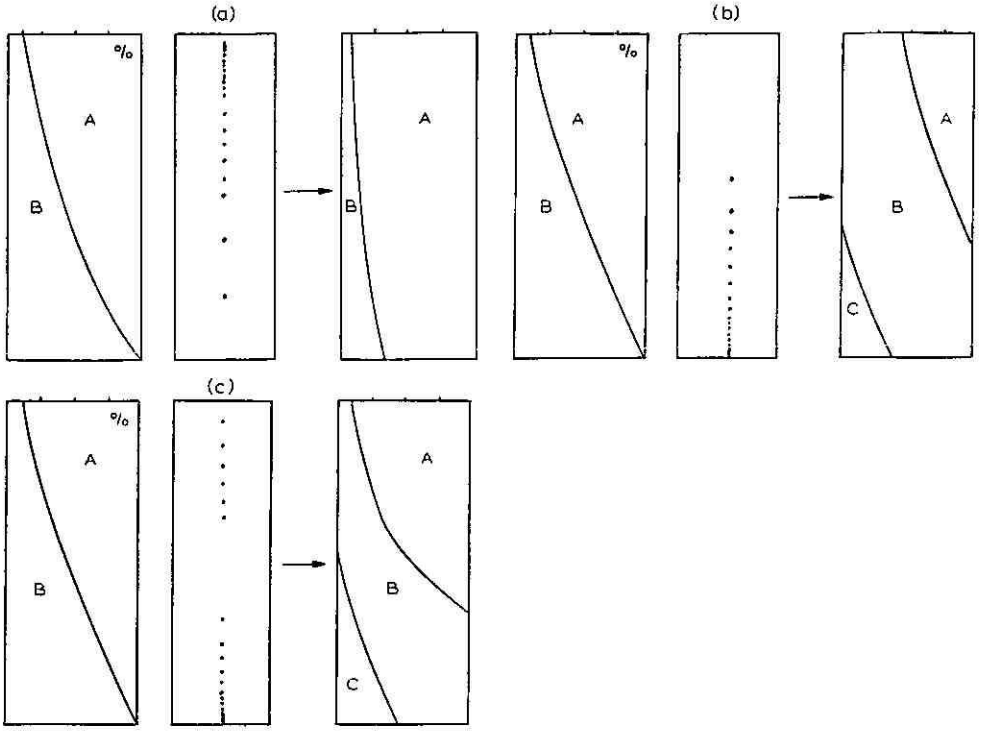

Fig. 6. Improvement of the distribution of spots along the chromatogram by adjustment of the gradient profile. (see text).

and a linear increase in eluent strength is obtained for exponential gradient profiles. For reversed-phase adsorbents and aqueous–organic eluents

$$\log k' = \text{constant} - n\,(\%\ \text{B})$$

and the eluent strength, ε^0, of the mixed solvent increases linearly with the concentration of the modifier B, so that a linear gradient profile is recommended. Owing to the limited wettability of most reversed-phase adsorbents at higher water concentrations, the program should be started from a suitable content of the modifier, *e.g.*, 60% B. It is recommended to prewet the layer with pure modifier; the sample is spotted 1 cm behind the eluent front.

It is advantageous to spot various volumes of the sample solution, *e.g.*, in the proportion 1:2:4, which enables an estimate of the optimum sample size for the detection of the maximum number of spots.

Gradient development of TLC plates is especially favourable in zonal separations carried out to isolate single components from complex mixtures. Even for mixtures of unknown components it is frequently possible to compare the chromatograms obtained from different elution programs owing to the presence of compounds that have a characteristic fluorescence under UV light. The high efficiency of gradient elution is caused by the flattening of the spots by the gradient and good

conditions for mutual displacement. Displacement effects are presumably strongest in the case of numerous adsorption–desorption processes of individual solute molecules, *i.e.*, in the range of moderate k' values. With increasing eluent strength of the mobile phase, consecutive components gradually attain this condition and separation takes place close to the start line[8]. The application of the sample from the edge of the layer (frontal + elution chromatography)[24,26] greatly increases the capacity of the system: even wide starting zones are partly separated and during gradient elution form narrow, well separated bands.

An important advantage of gradient TLC should be emphasized: for isocratic elution, a change in the eluent composition improves the separation in a fragment of the plate at the cost of a deterioration in the resolution in another fragment; all R_F values are changed (Fig. 1a,b). In gradient elution it is possible to vary the R_F values in a poorly separated fragment of the plate without changing those in the remaining part (Fig. 6c).

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STEPWISE GRADIENT DEVELOPMENT IN THIN-LAYER CHROMATOGRAPHY

II. TWO-DIMENSIONAL GRADIENTS FOR COMPLEX MIXTURES

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(First received March 27th, 1986; revised manuscript received June 30th, 1986)

SUMMARY

A modified technique of two-dimensional thin-layer chromatography using an equilibrium sandwich chamber with a glass distributer is described. The sample is introduced from the edge of the layer as a rectangular zone 1.5 cm long and developed using a stepwise gradient of eluent composition. After preconcentration of the rectangular spots in the second direction, the chromatogram is divided into four zones, each being developed in the second direction using eluents of solvent strengths suitable for the individual fractions. In the first stage the sample capacity is significantly increased and the separation of components having large differences in capacity factors is possible, being enhanced by strong displacement effects occurring under gradient conditions; in the second stage the preconcentration of the zones and adaptation of the solvent strength to the individual groups of solutes ensures the distribution of the components over the whole plate area. Owing to the greatly increased sample size, trace components can also be isolated from complex samples such as plant extracts.

INTRODUCTION

Two-dimensional thin-layer chromatography (2D-TLC) is a very effective method of separation of multicomponent samples such as protein hydrolysates or plant extracts; its numerous applications have recently been reviewed by Zakaria *et al.*[1] who also discussed the general principles of optimization of adsorbent–eluent systems for the two perpendicular directions of development. In the ideal case, the number of spots separated is equal to the square of the number of spots separated in a single direction[2,3].

The limitations of 2D-TLC result from three main causes:

(1) The "general elution problem"[4]: solutes having great differences in polarity accumulate in the proximity of either the start line or the end line.

(2) Increased sample size leads to large initial spot diameters and deterioration

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of the separation of spots in the resulting chromatogram. For instance, if the limiting capacity is assumed to be 1 mg of sample per g of adsorbent, then the initial spot containing 1 mg sample should have an area of 10 cm^2 corresponding to 1 g of a 0.2-mm layer.

(3) For analogous adsorbent–eluent systems used in the two directions, the spots tend to accumulate near the straight or curved diagonal line[5–7], leaving the opposite angle areas free of spots. One solution to this problem is to use layers composed of two different adsorbents, *e.g.*, silica and silanized silica RP-2, RP-8 or RP-18[1,8].

The three limitations are illustrated in Fig. 1.

The use of the horizontal sandwich chamber with a glass distributer, constructed by one of the authors (E.S.)[9–13], permits complete or partial elimination of the limitations of conventional TLC.

"The general elution problem" is solved by multistep gradient elution according to a predetermined program, as described in the preceding paper[14]. The space under the distributer of the equilibrium sandwich chamber (*ca.* 0.4 ml for a 190 mm × 190 mm layer, distributer dimensions 185 mm × 5 mm and slit height 0.4 mm) is consecutively filled with 0.4-ml volumes of eluents of increasing strength; thus, the first eluent fraction is 10% ethyl acetate in chloroform, and the last is pure ethyl acetate[13–16]. Twelve eluent fractions are necessary for development along a distance of 180 mm, since a single distributer volume (0.4 ml) develops the 0.25-mm layer along a distance of 1.5 cm. Each eluent fraction is introduced under the distributer after the complete absorption of the preceding fraction (Fig. 2a). The concentration steps are relatively small and are partially smoothed in the chromatographic system so that the gradient profile is approximately linear (Fig. 2b, c; the graphical program corresponds to the compositions of the eluent fractions in the layer).

For gradient elution (single-direction development, continuous or stepwise program), approximately double the number of separated spots were obtained for complex plant extracts[15,16].

To increase the sample size without excessive zone spreading, the sample is introduced as a zone 15 mm in length; in parallel, the eluent is delivered as a zone 175 mm long which prevents radial spreading of the initial zone[17]. In the rectangular initial zone (Fig. 3a) the components are partially separated owing to the mutual displacement characteristic of frontal chromatography[17–19]. Further development, using a single distributer 185 mm long, separates the initial zone into a series of rectangular zones 1.5 cm in length (Fig. 3b), the resolution being significantly improved by gradient development[15–16]

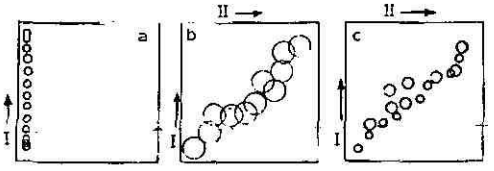

Fig. 1. Limitations of 2D-TLC due to the general elution problem (a), to a large starting zone for point application of a large sample (b) and to the tendency of the spots to accumulate along the diagnonal line (c).

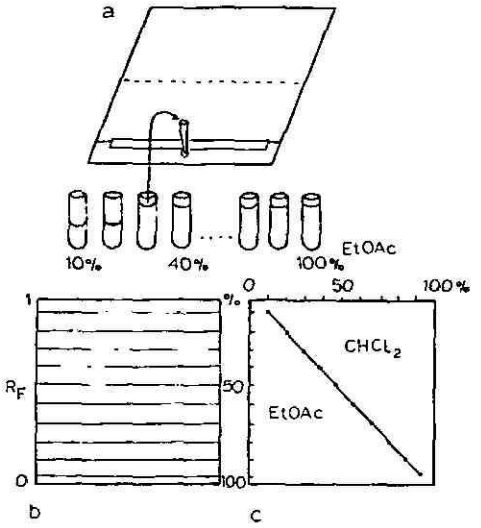

Fig. 2. Stepwise gradient elution in sandwich chambers with a glass distributer of the eluent: (a) 0.4-ml portions of eluents of increased solvent strengths are introduced under the distributer and from the edge of the layer; (b) developed chromatogram with zones of the mobile phase and a stepwise profile of the gradient; (c) corresponding graphical representation of the (approximated) continuous gradient.

Before development in the second (perpendicular) direction the long zones should be focused by elution with a volatile, strong eluent under the narrow cover plate (preconcentration step, Fig. 3c).[20] The preconcentrated zone is developed in the second direction using a suitable eluent or gradient program (Fig. 3d).

To avoid the accumulation of spots along a straight or curved diagonal line, after the preconcentration step (Fig. 3c) the chromatogram is cut (in the direction of the second development) into several, *e.g.*, four, zones. This is easy for precoated

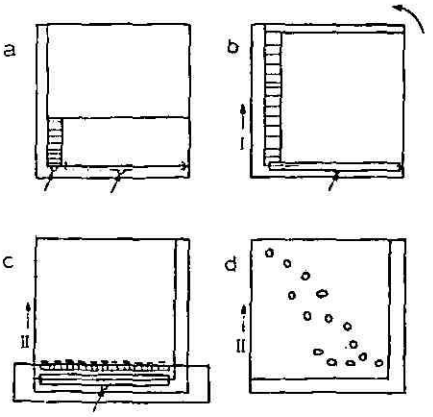

Fig. 3. (a) Increasing the capacity of the system by zonal application of the sample with the short distributer; (b) elution in the direction I; (c) preconcentration of the wide zone in the second direction by development under the narrow cover plate; (d) elution in the direction II. In (a)–(c) the cover plates are omitted; only the positions of the distributer are shown.

aluminium or plastic foils. The zone borders should be chosen between spots visible under an UV lamp so that a single component is not divided between two zones. Each point of the chromatogram is considered as a separate analysis with the initial spots applied on the preconcentration line: the top zone A (relative to the first direction), containing the least polar components, is developed with the weak eluent, *e.g.*, 10% ethyl acetate in chloroform; the middle zones B, C, with a moderately strong eluent, *e.g.*, 20 and 40% ethyl acetate, and the bottom zone, containing the most polar solutes, with a strong eluent, *e.g.*, 10% ethanol in ethyl acetate. Since the eluent strengths are individually chosen for the four fractions obtained by gradient elution in the first direction of development, each fraction will be separated into spots in the optimum range of moderate R_F values. In the integral square chromatogram the spots are thus spread over the whole area of the plate, which is advantageous for a high degree of separation.

EXPERIMENTAL AND RESULTS

Precoated 0.2-mm layers of silica on 20 cm × 20 cm aluminium foil or glass carrier plates with a preconcentration zone were used (E. Merck, Darmstadt, F.R.G.).

The gradient program was optimized for zonal application of the sample which comprised non-volatile compounds of Azulane (extract of Matricaria chamomila flowers; Herbapol, Warsaw, Poland) dissolved in ethyl acetate (3.45%, w/v). one distributer volume (*ca.* 100 μl) of the solution was introduced from the edge of the layer on a 200 mm × 50 mm plate. The first stepwise gradient program in the range 5–30% ethyl acetate in chloroform resulted in the accumulation of zones in the lower part of the chromatogram (Fig. 4a), which indicated that a steeper gradient program

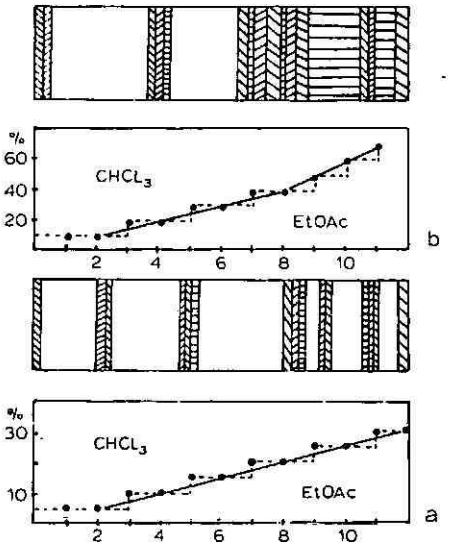

Fig. 4. Zonal chromatograms of an Azulane extract. Stepwise gradients: (a) 5–30%; (b) 5–70% ethyl acetate in chloroform.

was necessary. A steeper gradient, 5–70% ethyl acetate in chloroform, resulted in a better separation of the zones (Fig. 4b).

Although the TLC conditions were rather complex owing to variations in the vapour composition over the layer, the reproducibility was satisfactory as illustrated in Table I. However, the reproducibility of the R_F values depends on the type of eluent used. For non-polar and weakly polar solvents and their mixtures, *e.g.*, heptane, benzene, chloroform, the R_F values depend strongly on the water content of the adsorbent (its activity) and the solvent. In the case of eluents containing polar solvents, *e.g.*, ethyl acetate (such eluents were used in this study), the water content is less critical owing to the reactivation of the adsorbent by the polar solvent and the low activity of water in the mobile phase. The diffusion of vapours over the layer and their adsorption and desorption may be reduced by decreasing the space between the layer and the cover plate to a fraction of a millimetre. For gradient programs at lower eluent strengths, greater care should be taken regarding the standardization of the experimental conditions, *e.g.*, rapid transfer of the plate to the chamber, control of the water content in the solvent, etc.

TABLE I

REPRODUCIBILITY OF $100 \cdot R_F$ VALUES OF COMPONENTS OF A CHAMOMILE EXTRACT IN STEPWISE GRADIENT TLC

Program: 10, 10, 20, 30, 40, 50, 100% ethyl acetate in chloroform. Precoated HPTLC aluminium foil (silica) with a preconcentration zone. Two experiments were carried out in six consecutive days.

Solute No.	*Colour in daylight*	*Colour in* UV_{254}	$100 \cdot R_F$					
			1	*2*	*3*	*4*	*5*	*6*
1	–	Pale green	79 79	83 81	84 82	79 80	80 81	81 79
2	Green	Pink	76 75	80 80	81 79	76 76	76 76	72 73
3	Pale green	Pink-violet	72 72	76 75	77 75	73 73	72 72	70 72
4	Pale green	Pink	54 54	57 56	58 56	53 53	55 54	54 54
5	Pale yellow	Blue-violet	43 42	40 40	45 44	41 41	40 40	43 42
6	Yellow-green	Brown	30 30	29 29	32 30	27 28	29 30	30 30
7	Pale yellow	Pink	18 18	21 20	24 22	19 19	18 19	21 20
8	Pale green	Red	17 15	14 14	16 15	13 14	15 15	14 15

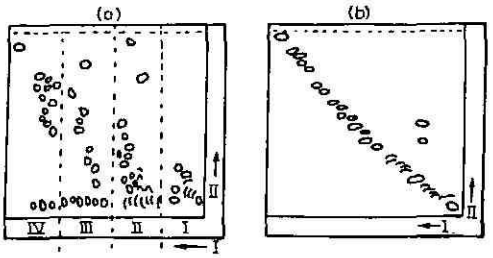

Fig. 5. 2D-TLC chromatograms of an Azulane extract on glass plates (200 mm × 200 mm) with a preconcentration zone (silica): (a) stepwise gradient elution in the first direction (3 × 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% ethyl acetate in chloroform) and isocratic elution after preconcentration of four zones in the second direction (I, 80%; II, 40%; III, 20%; IV, 10% ethyl acetate in chloroform); (b) stepwise gradient development in both directions (2 × 10%, 2 × 20%, 2 × 30%, 2 × 40%, 2 × 50% and 2 × 70% ethyl acetate in chloroform).

The second, steeper gradient was used in 2D-TLC experiments. Margins 1 cm wide were cleaned of adsorbent on two adjacent sides of the foil. To introduce the sample and solvent in the first (19 cm) direction a two-part distributer (15 + 170 mm) was used (Fig. 3a). The sample was applied as a 1.5-cm wide band parallel to the eluent to secure an even linear flow of the mobile phase. After introduction of a *ca.* 2-cm long zone, the small cover plate was replaced with another one with a single 185-mm distributer. The elution was carried out (Fig. 3b) using portions of the eluent having increasing contents[14] of the polar modifier (ethyl acetate), to the farther end of the layer thereby completing the development in the first direction. The volume of the sample corresponding to the 2 cm × 1.5 cm starting zones was *ca.* 20 μl which corresponds to 0.7 mg of dry sample. After preconcentration to narrow bands by development with acetone for a short distance in the second direction (Fig. 3c), the chromatogram was cut into four zones corresponding to hR_F values in the ranges 0–25, 25–50, 50–75 and 75–100.

The four chromatograms were developed in four separate 10 cm × 5 cm sandwich chambers using eluents expected to be suitable for the four fractions, *i.e.*, 80% (I), 40% (II), 20% (III) and 10% ethyl acetate in chloroform (IV). The criterion of success was the number of spots visible under UV light (360 nm) or after exposure to iodine vapours (Fig. 5a).

To compare the results obtained with those from the traditional 2D-TLC technique, in the second experiment the whole plate was developed in the second direction using the same gradient program. The spots were mostly accumulated along a diagonal line (Fig. 5b) and their number was somewhat greater than that obtained using development in a single direction, but much lower in comparison to those in Fig. 5a. The good separation of spots indicates that a greater sample load could be applied.

CONCLUSIONS

The chromatographic technique proposed is somewhat more complex and time-consuming in comparison to the traditional technique. However, the analysis time for low-viscosity solvents is short, about 40 min, and it is possible to carry out several parallel experiments using several sandwich chambers and an automatic mi-

cropipette to introduce the eluent fractions. The greatly increased separation efficiency and purity of the separated components permits direct structure determination of the extracts by mass spectrometry[21]. The zonal application of greatly increased sample sizes (frontal + elution chromatography) together with gradient elution permits the isolation of trace components.

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FACTOR ANALYSIS AND EXPERIMENT DESIGN IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

V. SELECTIVITY OF CHALCONE CONFIGURATION ISOMERS ON 23 REVERSED-PHASE PACKINGS

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SUMMARY

The selectivity of 22 pairs of configuration isomers of X,Y-substituted chalcones ($X–C_6H_5–CH=CH–CO–C_6H_5–Y$) on 23 reversed-phase high-performance liquid chromatographic (HPLC) packings has been studied. The main factors influencing the selectivity of these isomers were analysed with principal component analysis (PCA). The chromatographic behaviour of the *E*-s-*cis* and *Z*-s-*cis* chalcones was found to be well related to the substitution pattern on the chalcone core. PCA helps to formulate a general rule for selection of stationary phases suitable for separation of the configuration isomers and enables a classification of the HPLC packings.

INTRODUCTION

The transmission of electronic effects of the X,Y substituents of chalcones through the chain of conjugated double bonds has been intensively studied. The influence of the substituents on the infrared carbonyl stretching frequencies[1–6] and integral intensities[4–6], on the dipole moment[7–11], the ultraviolet spectra [12,13], the half-wave potential [14–17] and the ^{13}C NMR chemical shifts[18–20] have been reported.

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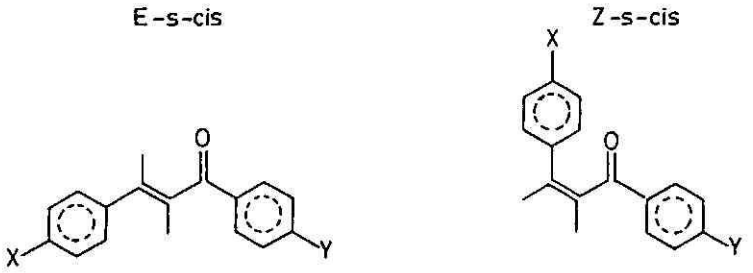

The substituent effects on the chromatographic behaviour of a model series of 53 chalcones have also been analysed by us by reversed-phase high-performance liquid chromatography (RP-HPLC)[21] and by normal-phase HPLC[22–24]. The different contributions of the substituents to the partition coefficient, depending on their position in the 4,4′-substituted chalcones, have been stressed. Additionally, it appears, from our investigations, that these effects depend on the *E*-s-*cis* and *Z*-s-*cis* configuration of the chalcone core, so the separations of these configuration isomers change according to the chemical nature of the substituents.

The separation of the non-substituted *E*-s-*cis* and *Z*-s-*cis* chalcones differs from the separation of the substituted isomers, *e.g.*, methyl, methoxychalcones. Furthermore, and simultaneously, differences are observed in the selectivity of the positional isomers, *e.g.*, CH_3 in position 4 and CH_3O in position 4′ or CH_3O in position 4 and CH_3 in position 4′.

Changes in the configuration of the chalcone core induce changes in molecular volume, contact surface area, polarizability and dipole moment of these isomers. So, it is very difficult to predict the differences in the solubility of the *Z*-s-*cis* and *E*-s-*cis* 4,4′-substituted chalcones. A general rule, which could describe the substituent effects on the separation of the isomers and which could predict their chromatographic behaviour, would be of interest both from the chemistry of chalcones and the chromatographic point of view.

In the present paper, the substituent effects on the selectivity of 22 pairs of the configuration isomers of chalcones are discussed. A study of the ability of 23 RP-HPLC packings to separate the *E*-s-*cis* and *Z*-s-*cis* chalcones was undertaken. The data were analysed with the help of factor analysis methods, which we have used previously to determine trends in the behaviour of various hydrocarbon compounds in gas–solid chromatography[25] or for the model series of 53 chalcones in RP-HPLC[21] or normal-phase HPLC[22–24]. However, instead of using correspondence factor analysis (CFA) to grasp the main trends in selectivity of these configurational chalcone isomers, we will use principal component analysis (PCA) which keeps the information content related to the absolute value of the selectivity parameter analysed.

EXPERIMENTAL

Reagents

The 4- and/or 4′-substituted chalcones considered are listed in Table II. *E*-s-*cis* chalcones were synthesized[26] while the corresponding *Z*-s-*cis* isomers (in our work denoted by asterisks) formed spontaneously in the dichloromethane solution.

The mobile phase consisted of HPLC-grade methanol purchased from Merck (F.R.G.) and of Millipore purified water.

Chromatographic procedure

Prior to the measurements, the columns were washed with the methanol–water (7:3) mobile phase until a constant value was obtained for the retentions of the compounds. Sample solutions (2 mg per 25 ml) were prepared in dichloromethane. All data were collected by averaging three reproducible separations. The same mobile phase and 1 μl of 10^{-3} *M* sodium nitrate, detected at 210 nm, were used to determine the dead time, t_0, for each system. The capacity factor, k', was calculated from the retention time of the solute, t_R, according to the equation $k' = (t_R - t_0)/t_0$. The results are presented in form of selectivity parameters, $\alpha_{X\text{-}Y/X\text{-}Y^*} = k'_{X\text{-}Y}/k'_{X\text{-}Y^*}$, for *E*-s-*cis* (X-Y) and *Z*-s-*cis* (X-Y*) isomer pairs.

Instruments and columns

The HPLC equipment included the following components: a Bruker LC-31 pump, a Rheodyne Model 7125 injection valve, a Schoeffel Model SF 770 spectrophotometer set at 300 nm and a Shimadzu C-RIB recorder.

The commercial columns or columns prepared in our laboratory by slurry-

TABLE I

COLUMN PACKINGS

No.	*Column packing*	*Dimensions (cm × mm)*	*Supplier*	*End-capped*
1	RSIL C_{18} LL*	9 × 4	Alltech	Yes
2	RSIL C_{18} HL*	9 × 4	Alltech	Yes
3	Partisil ODS*	9 × 4	Whatman	No
4	Partisil ODS-2*	9 × 4	Whatman	No
5	Partisil ODS-3*	9 × 4	Whatman	Yes
6	Spherisorb ODS-2*	9 × 4	Phase Separations	Yes
7	μBondapak C_{18}*	9 × 4	Waters	Yes
8	Hypersil C_{18}*	9 × 4	Shandon	Yes
9	Spherosil XOA 600 C_{18}*	9 × 4	Prolabo	No
10	Nucleosil C_{18}*	9 × 4	Macherey-Nagel	Yes
11	Nova-Pak C_{18}	10 × 5	Waters	Yes
12	Resolve C_{18} Radial Pak	10 × 8	Waters	No
13	μBondapak C_{18} Radial Pak	10 × 8	Waters	Yes
14	Zorbax ODS	15 × 4.6	DuPont	No
15	Zorbax C_8*	9 × 4	DuPont	Yes
16	Zorbax TMS	25 × 4.6	DuPont	Yes
17	Zorbax Phenyl	25 × 4.6	DuPont	Yes
18	Zorbax CN	15 × 4.6	DuPont	No
19	Resolve C_8 Radial Pak	10 × 8	Waters	No
20	Resolve CN Radial Pak	10 × 8	Waters	No
21	μBondapak Phenyl	30 × 4.6	Waters	Yes
22	Spherisorb C_6*	9 × 4	Phase Separations	Yes
23	Spherisorb C_8*	9 × 4	Phase Separations	Yes

* Packed in our laboratory.

TABLE II

SELECTIVITY PARAMETERS, $\alpha_{X\text{-}Y/X\text{-}Y^*}$, FOR THE *E*-s-*cis* (X-Y) AND *Z*-s-*cis* (X-Y*) CHALCONES CHROMATOGRAPHED ON 23 RP-HPLC SYSTEMS

No.	*X*	*Y*	*Stationary phase*										
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
1	H	CF_3	1.49	1.55	1.36	1.59	1.47	1.56	1.49	1.48	1.66	1.53	1.57
2	H	*tert.*-C_4H_9	1.56	1.62	1.44	1.66	1.52	1.61	1.54	1.52	1.75	1.63	1.60
3	H	iso-C_3H_7	1.55	1.62	1.44	1.65	1.52	1.62	1.54	1.52	1.76	1.63	1.60
4	H	H	1.52	1.55	1.38	1.64	1.44	1.58	1.46	1.46	1.75	1.60	1.54
5	F	H	1.34	1.33	1.25	1.39	1.29	1.33	1.30	1.27	1.45	1.36	1.34
6	H	F	1.48	1.54	1.37	1.60	1.44	1.56	1.47	1.45	1.69	1.54	1.54
7	H	C_2H_5	1.58	1.63	1.45	1.65	1.52	1.65	1.53	1.53	1.76	1.63	1.64
8	H	CH_3	1.60	1.66	1.45	1.76	1.53	1.68	1.54	1.55	1.88	1.68	1.65
9	F	CH_3	1.40	1.41	1.29	1.46	1.36	1.40	1.37	1.33	1.54	1.42	1.43
10	F	F	1.28	1.34	1.20	1.32	1.32	1.34	1.34	1.28	1.38	1.30	1.37
11	CH_3O	CH_3	1.58	1.51	1.43	1.68	1.42	1.52	1.45	1.41	1.78	1.60	1.46
12	CH_3	CH_3O	1.71	1.74	1.51	1.94	1.58	1.78	1.60	1.62	2.09	1.77	1.73
13	F	CH_3O	1.44	1.46	1.31	1.55	1.38	1.44	1.38	1.36	1.63	1.46	1.46
14	H	NO_2	1.44	1.52	1.30	1.55	1.44	1.56	1.45	1.45	1.66	1.47	1.55
15	F	NO_2	1.20	1.31	1.13	1.22	1.31	1.32	1.33	1.28	1.28	1.20	1.38
16	NO_2	CH_3	1.36	1.49	1.22	1.46	1.43	1.51	1.43	1.43	1.57	1.38	1.57
17	NO_2	H	1.31	1.43	1.20	1.41	1.38	1.44	1.35	1.36	1.48	1.34	1.50
18	CH_3O	CH_3O	1.60	1.57	1.44	1.73	1.44	1.54	1.46	1.42	1.84	1.62	1.51
19	NO_2	F	1.24	1.44	1.12	1.32	1.42	1.46	1.41	1.38	1.38	1.26	1.57
20	NO_2	CH_3O	1.40	1.53	1.26	1.54	1.45	1.54	1.44	1.43	1.65	1.42	1.61
21	NO_2	NO_2	1.12	1.46	1.00	1.20	1.44	1.48	1.45	1.44	1.29	1.15	1.61
22	H	OH	1.52	1.61	1.38	1.71	1.48	1.59	1.48	1.48	1.78	1.60	1.58

packing at 6000 p.s.i. with carbon tetrachloride, followed by methanol, are listed in Table I. The information on the packings comes from the supplier's sourcebooks and gives only a rough idea about the percentage of derivatization.

Data processing

The data were processed by PCA[27,28]. The data matrix, [*D*], with *i* rows and *k* columns is created with the selectivity parameters $\alpha_{X\text{-}Y/X\text{-}Y^*}$. The elements, α_{ik}, of the data matrix are assumed to have the form

$$\alpha_{ik} = \alpha_i + \sum_{j=1}^{n} r_{ij}c_{jk} + e_{ik}$$

where α_i are the averages of the corresponding variables of the *i*th column of the data matrix, r_{ij} and c_{jk} are the PCA parameters (cofactors) and e_{ik} is the residual error.

In PCA the eigenvectors are calculated consecutively so as to minimize the residual error, $\sum_i \sum_k e_{ik}$, in each step. Thus each successive eigenvector accounts for a maximum of the variation in the data. The appropriate number of components, *j*,

12	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*
1.57	1.54	1.59	1.36	1.38	1.33	1.28	1.34	1.20	1.24	1.17	1.28
1.64	1.55	1.62	1.36	1.52	1.31	1.38	1.40	1.25	1.23	1.18	1.31
1.65	1.55	1.65	1.38	1.40	1.31	1.35	1.40	1.24	1.22	1.18	1.32
1.58	1.48	1.60	1.41	1.26	1.31	1.25	1.40	1.18	1.21	1.19	1.33
1.35	1.33	1.36	1.21	1.15	1.19	1.20	1.19	1.13	1.12	1.10	1.17
1.57	1.50	1.58	1.38	1.27	1.28	1.27	1.35	1.18	1.19	1.16	1.28
1.69	1.56	1.68	1.40	1.36	1.31	1.33	1.43	1.23	1.22	1.20	1.34
1.70	1.56	1.70	1.43	1.34	1.30	1.31	1.45	1.21	1.21	1.21	1.36
1.43	1.39	1.43	1.22	1.23	1.19	1.35	1.21	1.16	1.13	1.11	1.18
1.33	1.36	1.37	1.21	1.21	1.18	1.22	1.15	1.14	1.12	1.09	1.16
1.62	1.48	1.55	1.33	1.34	1.21	1.28	1.39	1.20	1.13	1.31	1.28
1.85	1.66	1.81	1.43	1.45	1.27	1.36	1.46	1.24	1.19	1.21	1.36
1.47	1.40	1.50	1.25	1.22	1.18	1.28	1.22	1.18	1.12	1.12	1.21
1.56	1.51	1.58	1.37	1.23	1.29	1.26	1.29	1.11	1.21	1.15	1.25
1.28	1.35	1.35	1.20	1.17	1.20	1.20	1.11	1.11	1.16	1.09	1.14
1.45	1.47	1.54	1.23	1.21	1.17	1.28	1.11	1.18	1.14	1.11	1.17
1.36	1.40	1.48	1.23	1.09	1.17	1.22	1.10	1.14	1.12	1.02	1.16
1.64	1.48	1.60	1.35	1.63	1.20	1.31	1.39	1.21	1.14	1.16	1.29
1.34	1.47	1.50	1.26	1.21	1.19	1.24	1.00	1.13	1.16	1.11	1.17
1.47	1.49	1.61	1.25	1.20	1.17	1.29	1.00	1.17	1.14	1.00	1.18
1.29	1.48	1.51	1.30	1.23	1.23	1.20	1.00	1.09	1.20	1.13	1.18
1.49	1.51	1.62	1.43	1.24	1.28	1.28	1.34	1.15	1.21	1.17	1.33

for a given data set is determined using the imbedded error function (IE)[27]. For the jth factor in the sum, row cofactor r_{ij} is associated with the ith row designee, or identifier of the ith row, of the data matrix and c_{jk} is the corresponding column cofactor associated with the kth column designee (or identifier of the kth column), of the matrix. The row cofactors are called scores and the column cofactors are called loadings.

By factor analysis, we obtain a score matrix which depends solely upon the characteristics of the row designee, and a loading matrix which depends solely upon the column designee.

The teminology used here, designee, score matrix, loading matrix, is in accord with that proposed by Malinowski and Howery[27].

In our study, as all the experimental data are expressed in the same units, the canonic PCA was used, and the matric subjected to analysis was the covariance matrix about the mean. *This means that the eigenvectors and eigenvalues, which* emerge from the factor analysis of the data matrices $[D]$ and $[D]^{-1}$, are not exactly the same.

If we are interested in the differences between RP-HPLC column packings used for the separation of the configuration isomers of chalcones, the chromatographic systems must be treated as the row designees. If the influence of the X,Y substituents

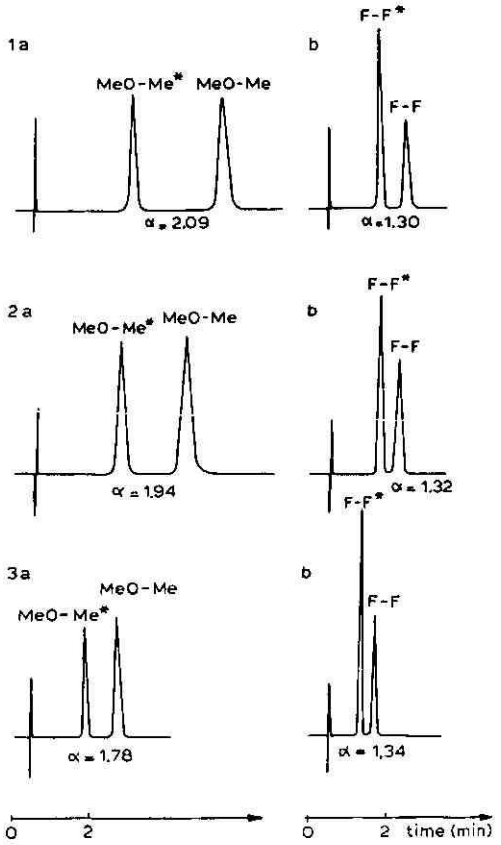

Fig. 1. Separation of (a) CH_3O-CH_3O and CH_3-CH_3O* and (b) F-F and F-F* chalcones on the stationary phases Spherosil XOA C_{18} (1), Partisil ODS-2 (2) and Spherisorb ODS-2 (3). Mobile phase: methanol–water (7:3); flow-rate 1.5 ml/min. Detection: UV at 300 nm. Me = Methyl; MeO = methoxy.

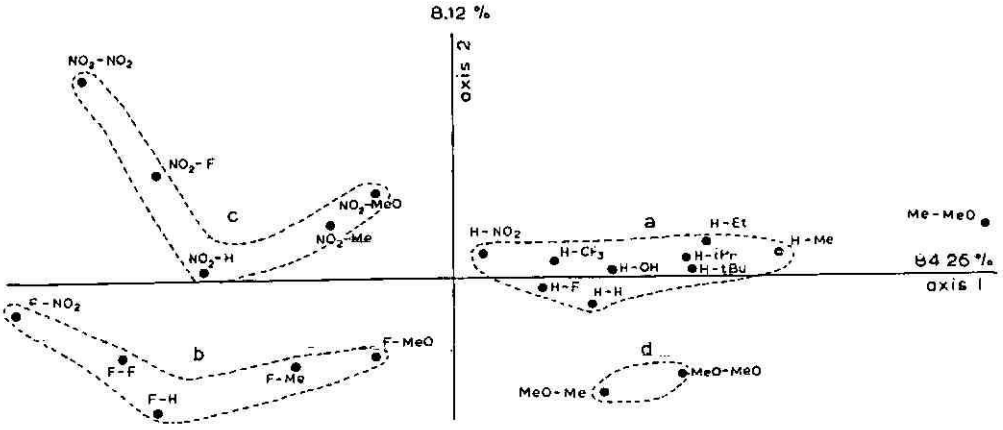

Fig. 2. Projection of 22 pairs of configuration isomers with X,Y substituents onto the plane determined by the two main axes extracted from principal component analysis.

on the separation of the *E*-s-*cis* and *Z*-s-*cis* chalcones is our main object, the pair of X,Y substituents are the row designees. In the first case the score matrix is associated only with the chromatographic systems, in the second, only with the pair of substituents.

RESULTS AND DISCUSSION

The selectivity parameters, $\alpha_{X\text{-}Y/X\text{-}Y^*}$, defined as the ratios of the k' value of the *E*-s-*cis* (X-Y) and *Z*-s-*cis* (X-Y*) chalcones, are listed in Table II. These parameters change from one chromatographic system to another, but in each system they change in a similar manner depending on the substituent pattern of the chalcone core, *e.g.*, in Fig. 1 the separations of the CH_3O-CH_3 and CH_3O-CH_3^* or F-F and F-F* isomers on three different stationary phases are presented.

The average ratio of the capacity factors for *E*-s-*cis* and *Z*-s-*cis* chalcones is 1.39.

To characterize the "true" substituent effect on the isomer separation, independently of the properties of the chromatographic system, and the "true" system ability to separate the configuration isomers, PCA was applied.

Influence of X,Y substituents on the selectivity of E-s-cis and Z-s-cis chalcones

To determine the influence of the X,Y substituents on the selectivity of the *E*-s-*cis* and *Z*-s-*cis* chalcones, the pairs of substituents were treated as the row designees in the data matrix of the $\alpha_{X\text{-}Y/X\text{-}Y^*}$ selectivity parameters. In this case only two main axes create the primary set of factors. The projections of the X,Y substituent pairs onto the plane defined by these axes are presented in Fig. 2.

The X,Y pairs are not grouped in any obvious manner, but some trends in the changes of the $\alpha_{X\text{-}Y/X\text{-}Y^*}$ parameters are seen more easily if one distinguishes the following subclasses of the row designees: a, H,Y; b, F,Y; c, NO_2,Y; d, CH_3O,Y. Within these subclasses only one substituent varies (substituent in position 4'), and the $\alpha_{X\text{-}Y/X\text{-}Y^*}$ parameters increase in the following orders, depending on the nature of this Y substituent:

a : $NO_2 < F < CF_3 < H < OH <$ iso-$C_3H_7 <$ *tert.*-$C_4H_9 < C_2H_5 < CH_3$

b : $NO_2 < F < H < CH_3 < CH_3O$

c : $NO_2 < F < H < CH_3 < CH_3O$

d : $CH_3 < CH_3O$

The influence of the Y substituent on the $\alpha_{X\text{-}Y/X\text{-}Y^*}$ parameters for the H-Y chalcones (subclass a) can be described with only one "abstract" parameter (factor 1). For X-Y chalcones (subclasses b, c) the second "abstract" parameter is necessary (factor 2). The best description of the effect of the Y substituent on isomer selectivity is given by the Hammett parameters, $\sigma_p^{\pm} - \sigma_p$ (ref. 29).

The influence of the X substituent on the $\alpha_{X\text{-}Y/X\text{-}Y^*}$ parameters seems to be more complicated, and two "abstract" factors are required to describe it. Based on isomers with the same Y substituent but with different X substituents, e.g. F-CH_3O, NO_2-

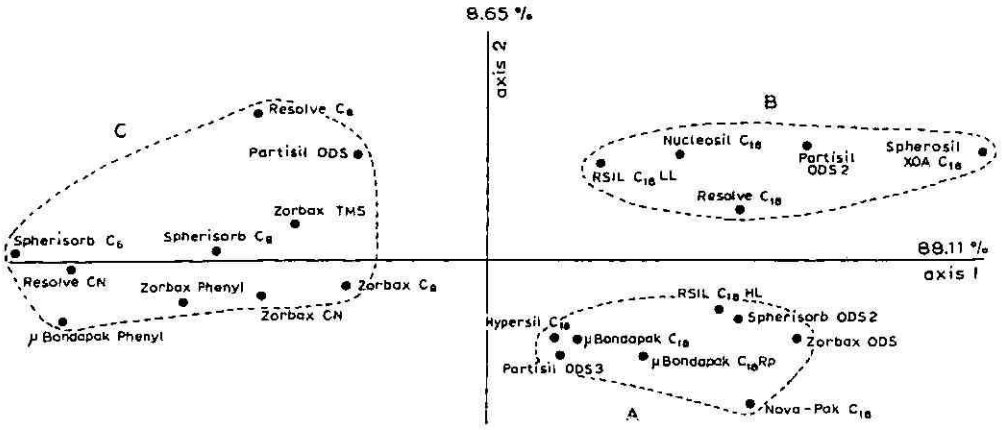

Fig. 3. Projection of 23 chromatographic systems onto the plane determined by the two main axes extracted from principal component analysis.

CH_3O, CH_3O-CH_3O and CH_3-CH_3O, one can order the X substituents as follows:

$$F < NO_2 < CH_3O < CH_3 \text{ (along factor 1)}$$

and

$$CH_3O < F < CH_3 < NO_2 \text{ (along factor 2)}$$

Their effects can be described with the help of Hammett constants σ_I and σ_R^0 (ref. 29).

Hence, the selectivity of *E*-s-*cis* and *Z*-s-*cis* chalcones is well related to their substitution pattern on the chalcone skeleton, and one can generalize the substituents effects in the following way. The selectivity $\alpha_{X\text{-}Y/X\text{-}Y'}$ is highest if both substituents, in positions 4 and 4', are electron-donor groups. The greatest negative influence is observed when electron-acceptor substituents are in position 4.

Influence of the properties of RP-HPLC packings on the selectivity of E-s-cis and Z-s-cis chalcones

To obtain the score matrix, which depends solely upon the characteristics of the stationary phases, these phases were regarded as the row designees in the data matrix. The first four eigenvectors, create a primary set of eigenvectors and the remaining ones account for experimental error only. The factors 3 and 4, associated with smaller eigenvalues, reflect relatively "unique" behaviour associated with only a single designee (Zorbax TMS and Zorbax CN) and will not be taken into consideration below. The results are presented in Fig. 3.

The first eigenvector accounts for the major fraction of the variance in the data and represents a sort of common factor averaged over all the row designees. It defines the best one-factor model for the data. The second principal axis is orthogonal to the first eigenvector. These two factors define a plane passing through the greatest concentration of data points (Fig. 3) and the stationary phases are grouped into the

following subclasses: A, octadecylsilyl endcapped stationary phases; B, octadecylsilyl uncapped or partially capped stationary phases; C, trimethylsilyl, C_6, C_8, phenyl, cyano stationary phases and Partisil ODS which is an uncapped and a low carbon-loaded packing.

The selectivity, $\alpha_{X\text{-}Y/X\text{-}Y'}$, increases along the axis 1 with increasing RP chain length, and with decreasing phase polarity, for the same silica material:

Spherisorb C_6 < Spherisorb C_8 < Spherisorb ODS-2

Resolve CN < Resolve C_8 < Resolve C_{18}

μBondapak Phenyl < μBondapak C_{18}

The additional differences among similarly labelled ODS columns (subclasses A and B) and the differences among the columns within the subclasses A, B and C are also reflected along axis 2.

The selectivity, $\alpha_{X\text{-}Y/X\text{-}Y'}$, seems to increase along the axis 2 with increasing silanol group accessibility: for two phases which have the same coordinates on the axis 1, the selectivity increases in the positive direction of the axis 2, *e.g.*:

Resolve C_{18} > Spherisorb ODS-2

Partisil ODS-2 > Zorbax ODS

Partisil ODS > Zorbax C_8

Resolve C_8 > Zorbax CN

The only common properties of the phases from group A are that they are C_{18} and endcapped. Group B also comprises C_{18} phases and includes uncapped packings: Spherosil XOA C_{18}, Partisil ODS-2 (75% of derivatization) and Resolve C_{18}. The presence of the RSIL C_{18} LL phase in this group can be explained in the following way. This phase is described in the supplier's sourcebook as a capped packing, but as Unger[30,31] and Berendsen and De Galan[32] have shown, a large proportion of the total number of silanol groups, originally present on silica surface, remains underivatized even after an "exhaustive" silanization and can cause a dual retention mechanism. The lower the carbon loading the stronger is the effect of underivatized silanol groups. Probably the same effect causes one of the C_{18} phases to be included in group C. This is the uncapped and low-loaded Partisil ODS. The presence of the Nucleosil C_{18} packing within group B cannot be explained in any obvious way. It is the only polymeric phase in our set of packings.

It is worth stressing that this classification of the stationary phases does not depend on the specific surface area, pore-size distribution, shape of the silica particles or on the character of the organic layer (mono- or multilayer), *e.g.*, subclass A includes Hypersil C_{18} (monolayer) and Partisil ODS-3 (multilayer), RSIL C_{18} HL (irregular shape of silica) and Zorbax ODS (spherical silica) or Nova-Pak C_{18} (surface area 120 m^2/g) and RSIL C_{18} HL (surface area 550 m^2/g).

More generally, the PCA results show that the nature and concentration of the organic "bristle" chemically bonded on the silica surface and the percentage of derivatization of the silica gel play a major rôle in the separation of the *E*-s-*cis* and *Z*-s-*cis* chalcones. (Derivatization is defined as the percentage of the available hydroxyl groups of the silica gel which have reacted with the silane. It is the result both of bonding and sometimes of a final silanization with, *eg.*, methylsilanes in order to minimize unreacted hydroxyl groups.)

CONCLUSIONS

Basing on Rekker's concept[33] of hydrophobic fragmental constants, one cannot predict the difference in solubility of the configuration isomers (*E*-s-*cis* and *Z*-s-*cis*) of chalcones. The present experimental data allow a calculation of the average ratio of the hydrophobicities of the *E*-s-*cis* and *Z*-s-*cis* chalcones, *i.e.*, 1.39.

Due to the transmission of the electronic effects of the substituents, in the 4,4'-substituted chalcones, through the chain of conjugated double bonds, deviations from the additivity of the partition coefficients are observed for the series of *E*-s-*cis* and *Z*-s-*cis* chalcones. These deviations are different for the two series of isomers, so the selectivity of the *E*-s-*cis* and *Z*-s-*cis* isomers changes, and it is difficult to predict their chromatographic behaviour in the RP-HPLC systems. Nevertheless, from the PCA results it is clear that these deviations are well related to the substitution pattern on the chalcone core: the selectivity, $\alpha_{X\text{-}Y/X\text{-}Y^*}$, is highest when both substituents in positions 4 and 4' are electron-donor groups. The greatest opposite influence is observed for the electron-acceptor substituents in position 4.

The ability of 23 commercially available RP-HPLC packing materials to separate configuration isomers has also been compared. The nature and the concentration of the organic "bristle" chemically bonded on the surface of silica and the concentration of the polar silanol groups play a major rôle in the separation of *E*-s-*cis* and *Z*-s-*cis* chalcones.

Based on the PCA results (Fig. 3), one can formulate the following general rule for selection of the stationary phase to be used for separation of configurational isomers: the lower the polarity of the organic "bristle", the greater the carbon loading and the lower the percentage of derivatization, the better is the separation of the configurational isomers.

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RETENTION AND RESOLUTION IN DENSITY-PROGRAMMED SUPERCRITICAL FLUID CHROMATOGRAPHY

II. EXPERIMENTAL RESULTS

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SUMMARY

Experimental retention time data have been obtained from density-programmed supercritical fluid chromatography of dodecyl phenyl ether, using Perisorb RP-8 and Spherisorb ODS-2 as stationary phases and carbon dioxide as a mobile phase. They are compared with values calculated from a theoretical treatment of linear sample velocities during a density programme proposed recently. The numerical integration method presented earlier is confirmed for different density gradients. The effect of a density programme on the peak width, σ_L, was also investigated; under the conditions of the experiments, σ_L does not change much with density.

INTRODUCTION

In supercritical fluid chromatography (SFC), optimized separations of components differing considerably in capacity ratios can be obtained by density programming. Here the density programme influences the retention as well as the resolution.

In a previous paper[1], theoretical relationships were derived for the case where detection takes place before pressure release of the mobile phase. From this theory the retention time, t_R, of the components in a density programme can be calculated from easily obtainable parameters. This has been shown for linear density gradients and packed columns, the sample always being injected before the start of the gradient. Smith *et al.*[2], however, developed a theory for capillary columns with detection after pressure release, the relationships derived by Smith *et al.*[2] and us[1] being in accordance, as well as the methods of calculation of the retention times.

In the present work additional results are presented that were obtained in experiments using linear density gradients and packed columns. Here two stationary phases differing in particle sizes were investigated and the samples were injected at different times. From the measured baseline width and the calculated velocity of the sample during elution, the standard deviation, σ_L, of the peak in length units can be obtained. It is the aim of the present investigations to study the influence of different factors on peak compression for packed columns.

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EXPERIMENTAL

Apparatus

The SFC apparatus for isobaric measurements has been described in detail elsewhere[1]. Only some modifications made for the density-programmed experiments will be described.

High pressure was created with an Altex 100 A double plunger pump that allows isobaric experiments, the upper pressure limit being adjusted by a built-in pressure feedback unit. This unit can also be operated externally by a microcomputer CBM 8032 SK. Using a program written in BASIC and an adequate equation of state for the mobile phase (here carbon dioxide)[3], optional density gradients can be produced. An 8-bit digital-analog converter was used as an interface between the computer and pump, the pressure ranging from 90 to 200 bar and the resolution being 0.43 bar. For packed columns the pressure drop is taken into account and mean values of the pressure and density are produced by the program.

The pressure was measured before and after the column by high precision strain gauges (Sensotec Super TJE). The column was mounted in an air thermostat (Perkin-Elmer LC 100). Samples were injected with a Rheodyne 7120 injector using a 20-μl loop. In contrast to the isobaric measurements, no buffer volume was used in the density-programmed experiments. Detection was effected with a Kontron Uvikon 720 LC UV/VIS spectrometer using an high-pressure flow cell. The mobile phase was expanded after the detector by a reducing valve, giving an adjustable constant mass flow-rate independent of the inlet pressure. For details see refs. 4, 6.

Substances and columns

Carbon dioxide (purity >99.995%; Messer-Griesheim, Düsseldorf, F.R.G.) was used as a mobile phase. It was taken from the gas phase in the container and purified mechanically by a 2-μm in-line metal filter. The sample was dodecyl phenyl ether dissolved in heptane (1%). About 1 μl of the solution was injected in each experiment.

A column individually packed with Perisorb RP-8 (Merck, Darmstadt, F.R.G.; for packing procedure see ref. 5) and a commercial column packed with Spherisorb ODS-2 (Kontron-Analytik, Eching, F.R.G.) were used. The stationary phases differed considerably in specific surface area and particle size; for details see Table I.

RESULTS AND DISCUSSION

In the present work investigations were performed with several density gradients differing in steepness and different flow-rates of the carbon dioxide mobile phase.

As an example, the mean density in the column as a function of time obtained in a characteristic experiment is shown in Fig. 1. Whereas the nominal steepness was 0.0507 g cm^{-3} min^{-1}, the real experimental slope was 0.0509 g cm^{-3} min^{-1} with a delay time of 0.15 min. Thus Fig. 1 demonstrates that good results can be obtained with an 8-bit digital–analog converter.

For each gradient the sample was injected at different times before, during and after the pressure increase. A typical chromatogram is shown in Fig. 2. Here the

TABLE I

COLUMN DATA

No. of column	1	2
Length (mm)	125	256
Inner diameter (mm)	5.0	4.5
Packing	Spherisorb ODS-2	Perisorb RP-8
d_p (μm)	5	30–40
S_{BET} ($m^2\ g^{-1}$)*	110.2	7.3
Mass (g)	1.82	6.12
Void volume, V_0 (cm^3)	1.404	1.68
Porosity, ε	0.413	0.572

* Present measurements.

detector baseline decreases with increassing density of carbon dioxide. In spite of the fact that the same amount was always injected, the peak areas differ considerably. The peaks eluted at constant density have about the same area; the peaks detected during the increase in density, however, are by far too small. This indicates that, in density-programmed SFC, normal UV detection is not applicable for quantitative analysis.

In Table II the measured retention times for dodecyl phenyl ether on Perisorb RP-8 are compared with values calculated from the known column and gradient data. According to a theory published recently[1], the linear velocity, u_i, of a sample zone was taken as

$$u_i = \frac{1}{1 + k_i'} \cdot \frac{\dot{m}_0 + [(L - z)A + V_2] \cdot d\rho/dt}{A\rho} \tag{1}$$

where k_i' = capacity ratio of sample i, $\dot{m}_0$ = mass flow of eluent out of the system, L = column length, z = position of sample zone in the column, A = free internal area of the column and V_2 = volume between the column outlet and reducing valve. The capacity ratio was calculated from

$$k_i' = a(\rho/\rho^0)^{-b} \tag{2}$$

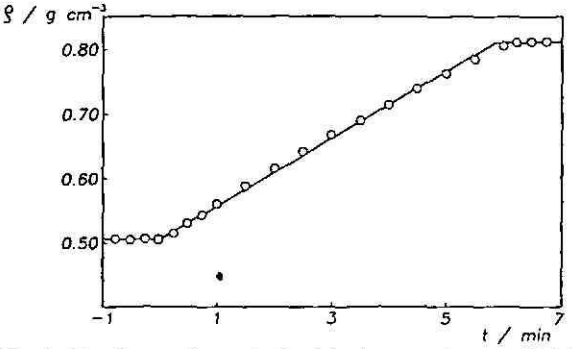

Fig. 1. Density gradient obtained in the experiments. Mobile phase: carbon dioxide. $\bar{p}_A$ = 88.6 bar, $\bar{p}_E$ = 161.8 bar, $\overline{\Delta\rho}$ = 0.302 g cm^{-3}, $d\rho/dt$ = 0.051 g cm^{-3} min^{-1}, $\dot{V}$(NTP) = 312.1 cm^3 min^{-1}, T = 38.9°C. Stationary phase: Spherisorb ODS-2. ———, theoretical; O, calculated.

TABLE II

EXPERIMENTAL AND CALCULATED RETENTION TIMES FOR DODECYL PHENYL ETHER ON PERISORB RP-8 USING CARBON DIOXIDE AS A MOBILE PHASE

$\dot{V}$(NTP) = cm³ of CO_2 per minute at outlet (NTP). $\bar{p}_A$ and $\bar{p}_E$ = mean pressures before and after density programme respectively; ρ_A and ρ_E = mean densities of CO_2 before and after density programme respectively; t_g = duration of density programme; $\Delta\rho/t_g$ = steepness of density gradient during density programme; t_a = delay time; t_R = retention time.

Exp. No.	$\dot{V}$*(NTP)* *(cm³ min⁻¹)*	$\bar{p}_A$ *(bar)*	$\bar{p}_E$ *(bar)*	ρ_A *(g cm⁻³)*	ρ_E *(g cm⁻³)*	$\Delta\rho/t_g$ *(g cm⁻³ min⁻¹)*	t_a *(min)*	t_R *(exptl.)* *(min)*	t_R *(calc.)* *(min)*	*Deviation in %*
1	484.4	85.1	162.6	0.399	0.807	0.198	9.5	4.34	4.27	−1.6
							5.06	4.34	4.27	−1.6
							3.06	3.76	3.50	−6.8
							1.55	2.57	2.33	−9.2
							0.05	1.26	1.06	−15
							−0.94	0.59	0.72	−24
							−1.61	1.00	1.05	+5.0
							−3.00	1.62	1.62	0
2	487.0	83.3	163.3	0.435	0.808	0.093	7.00	3.37	3.33	−1.2
							2.51	3.20	3.02	−5.6
							1.24	2.57	2.32	−9.6
							0.01	1.76	1.56	−11
							−1.57	1.01	1.10	+8.9
							−2.87	0.97	1.00	+3.0
							−7.5	1.67	1.70	+1.7
3	489.2	86.3	163.0	0.438	0.808	0.046	5.51	3.25	3.18	−2.2
							2.50	3.20	3.07	−4.1
							0.01	2.31	1.97	−14
							−2.01	1.58	1.37	−13
							−4.00	1.20	1.22	+1.7
							−5.96	1.22	1.23	+0.8
							−9.00	1.61	1.62	+0.6

4	490.2	86.4	162.7	0.443	0.807	0.023	10.4	4.79	4.72	−1.5
							3.71	4.78	4.70	−1.7
							0.22	3.70	3.38	−8.6
							−2.79	2.85	2.68	−6.0
							−5.79	2.16	2.13	−1.4
							−8.80	1.95	2.03	+4.1
							−11.8	2.00	2.05	+2.5
							−16.69	2.55	2.57	+0.8
5	489.2	86.3	163.0	0.438	0.808	0.046	5.51	3.25	3.18	−2.2
							2.50	3.20	3.07	−4.1
							0.01	2.31	1.97	−14
							−2.01	1.58	1.37	−13
							−4.00	1.20	1.22	+1.7
							−5.96	1.22	1.23	+0.8
							−9.00	1.61	1.62	+0.6
6	317.2	86.2	163.3	0.436	0.808	0.047	9.08	5.26	5.17	−1.7
							3.08	4.80	4.40	−8.3
							0.09	3.04	2.70	−11
							−1.93	2.07	2.02	−2.4
							−3.92	1.53	1.82	+19
							−5.90	1.68	1.78	+6.0
							−9.00	2.57	2.58	+0.4
7	124.4	87.9	167.5	0.479	0.812	0.043	16.28	10.25	10.50	+2.4
							4.26	7.47	6.78	−9.2
							0.29	4.45	4.03	−9.4
							−2.70	2.55	3.23	+26
							−4.73	2.55	3.25	+27
							−9.00	6.40	6.41	+0.2

TABLE III

EXPERIMENTAL AND CALCULATED RETENTION TIMES FOR DODECYL PHENYL ETHER ON SPHERISORB ODS-2

For symbols see Table II.

No.	$\dot{V}(NTP)$ ($cm^3\ min^{-1}$)	$\bar{p}_A$ (*bar*)	$\bar{p}_E$ (*bar*)	ρ_A ($g\ cm^{-3}$)	ρ_E ($g\ cm^{-3}$)	$\Delta\rho/t_g$ ($g\ cm^{-3}\ min^{-1}$)	t_o (*min*)	t_R (*exptl.*) (*min*)	t_R (*calc.*) (*min*)	*Deviation in %*
8	475.0	83.9	161.4	0.376	0.807	0.027	24.0	31.46	31.43	−0.1
							6.96	16.2	16.19	−0
							0.42	10.5	10.43	−0.7
							−4.37	6.60	6.60	0
							−7.84	4.72	4.88	+3.3
							−18.0	3.60	3.58	+0.6
9	477.1	85.9	161.9	0.437	0.808	0.048	36.25	26.92	26.92	−0.1
							16.19	20.24	20.21	−0.
							3.26	9.25	9.41	+1.7
							2.06	4.67	4.78	+2.4
							−5.25	3.30	3.47	+5
							−10.0	3.61	3.60	−0.3
10	475.3	85.7	161.9	0.422	0.807	0.099	35.0	25.18	25.10	−0.3
							20.19	22.04	21.93	−0.5
							8.19	11.29	11.63	+3
							−0.30	3.81	4.16	+9
							−2.31	2.85	3.18	+11
							−10.00	3.61	3.60	−0.3
11	477.8	85.1	161.2	0.407	0.806	0.204	33.78	30.36	30.13	−0.8
							20.08	21.33	21.61	+1.3
							10.05	12.05	11.41	+0.3
							1.11	3.93	4.38	+11
							−1.70	2.45	3.02	+23
							−4.00	3.61	3.60	−0.3
12	476.5	84.4	159.9	0.395	0.806	0.302	37.0	30.72	32.6	+6.1
							24.98	26.00	26.0	0
							15.31	16.36	16.9	+3.3
							3.51	7.16	6.78	−5
							−0.06	1.78	3.03	+7
							−3.00	3.69	3.68	−0.3

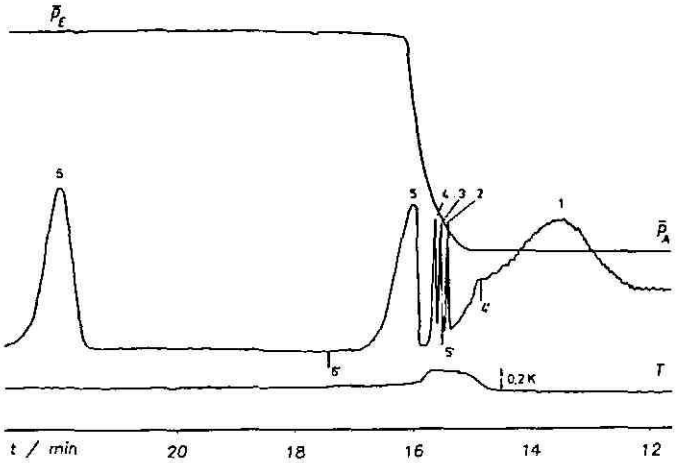

Fig. 2. Chromatogram for density-programmed SFC. Mobile phase: carbon dioxide. $\bar{p}_A$ = 86.3 bar, $\bar{p}_E$ = 164.6 bar, $d\rho$ = 0.434 g cm^{-3}, $\dot{V}$(NTP) = 124.6 cm^3 min^{-1}, T = 38.9°C. Injection times: peak 1, 0.0 min; peak 2, 8.0 min; peak 3, 12.1 min; peak 4, 14.8 min (= 4'); peak 5, 15.7 min (= 5'); peak 6, 17.5 min (= 6'). Stationary phase: Perisorb RP-8. Sample: 1 μl dodecyl phenyl ether in hexane (1%).

where ρ^0 is a standard density of 1 g cm^{-3} and the parameters a = 0.024 and b = 5.96 for the sample on Perisorb RP-8. In order to calculate retention times, t_R, the integral

$$L = \int_0^{t_R} u_i \, dt \tag{3}$$

was solved numerically[1].

In each of the experiments in Table II the peak corresponding to the first injection left the column before the start of the density gradient, and the last injection was made after the density had reached the final value. For these isobaric experiments the calculated retention times agree very well with the times found experimentally. However, discrepancies between calculated and experimental retention times occur when the sample is injected near the start of the gradient. These deviations become larger with increasing steepness of the gradient (experiments 1–4) as well as with decreasing eluent flow-rate at the outlet (experiments 5–7). This means that the eluent velocity at the sample position after the start of the gradient differs more and more from the value before the increase in density. In the range of velocities used in this work and in a previous publication[6], for the isobaric measurements (90–1200 cm^3 min^{-1}) the capacity ratios were scattered by less than 5%. Therefore the discrepancies are probably not produced by disturbance of the partition equilibrium of the sample between the mobile and the stationary phases, respectively, but by a disturbance of the equilibrium of mobile phase velocities along the column.

In Table III our results for the stationary phase Spherisorb ODS-2 are com-

TABLE IV

COMPARISON OF BASELINE WIDTHS, B, DURING DIFFERENT DENSITY GRADIENTS

For symbols see Table II and text.

Stationary phase	$\dot{V}(NTP)$ ($cm^3\ min^{-1}$)	ρ_A ($g\ cm^{-3}$)	ρ_E ($g\ cm^{-3}$)	$\Delta\rho/t_g$ ($g\ cm^{-3}\ min^{-1}$)	t_a (*min*)	B (*min*)	u_t ($cm\ min^{-1}$)	Bu_t (*cm*)
Spherisorb ODS-2	475	0.376	0.807	0.027	24.0	2.10	0.40	0.83
					6.96	0.51	1.68	0.86
					0.42	0.30	2.34	0.70
					−4.37	0.28	2.76	0.77
					−7.84	0.23	3.24	0.75
					−18.00	0.21	3.60	0.76
Spherisorb ODS-2	475.3	0.422	0.807	0.099	35.0	1.81	0.49	0.89
					20.19	0.40	2.28	0.91
					8.19	0.19	4.20	0.80
					0.30	0.18	4.02	0.72
					−2.31	0.20	4.02	0.80
					−10.00	0.20	3.66	0.73
Spherisorb ODS-2	476.5	0.395	0.806	0.302	37.0	2.18	0.41	0.89
					24.98	0.19	4.80	0.91
					15.31	0.14	4.14	0.58
					3.51	0.25	3.48	0.87
					−0.06	0.23	3.48	0.80
					−3.00	0.22	3.48	0.77
Spherisorb ODS-2	168.8	0.511	0.807	0.025	34.64	0.95	0.96	0.91
					25.51	0.71	1.26	0.89
					19.48	0.60	1.50	0.90
					3.50	0.50	1.80	0.90
					−2.50	0.68	1.26	0.86
					−12.75	0.78	1.26	0.98
Spherisorb ODS-2	168.6	0.509	0.810	0.100	35.12	0.40	2.44	0.98
					27.96	0.37	2.58	0.95
					19.54	0.60	1.32	0.79
					15.52	0.62	1.20	0.74
					− 0.91	0.70	1.20	0.84
					−5.15	0.78	1.26	0.98

Spherisorb ODS-2	167.7	0.537	0.810	0.258	35.0	0.30	2.94	0.88
					27.01	0.27	3.00	0.81
					20.1	0.52	1.68	0.87
					9.5	0.61	1.44	0.88
					2.0	0.65	1.28	0.83
					−3.5	0.70	1.20	0.84
Perisorb RP-8	314.8	0.491	0.804	0.240	10.0	0.75	6.5	4.88
					2.51	0.15	28.0	4.20
					0.05	0.15	32.3	4.85
					−1.02	0.40	11.0	4.40
					−10.0	0.42	11.0	4.62
Perisorb RP-8	319.0	0.456	0.790	0.148	12.1	0.91	5.3	4.82
					2.60	0.24	19.6	4.70
					0.01	0.18	23.0	4.14
					−1.53	0.43	11.2	4.82
					−2.41	0.45	11.0	4.95
Perisorb RP-8	371.9	0.370	0.789	0.046	7.45	0.57	6.8	3.88
					0.01	0.39	11.1	4.33
					−2.95	0.32	13.6	4.35
					−6.50	0.31	14.9	4.62
					−8.05	0.43	11.0	4.73
					−14.3	0.43	10.9	4.69
Perisorb RP-8	2.18.1	0.439	0.747	0.016	11.3	1.71	2.8	4.79
					4.0	0.83	5.0	4.15
					−6.0	0.56	7.6	4.26
					−11.0	0.50	8.5	4.25
					−16.0	0.53	8.8	4.66
					−20.0	0.60	7.4	4.44
Perisorb RP-8	381.9	0.424	0.745	0.015	10.0	1.01	4.7	4.75
					3.0	0.70	6.2	4.34
					0.01	0.55	8.6	4.77
					−4.0	0.43	10.7	4.60
					−8.0	0.35	12.8	4.48
					−14.0	0.33	13.3	4.39
					−23.0	0.35	13.3	4.66

piled. Here we used the parameters $a = 0.682$ and $b = 4.841$ in eqn. 2. The conclusions are the same as for Table II.

Our experiments were also designed to study the effect of a density gradient on the peak width of a sample in the column. As is seen from Fig. 2, the peaks recorded during a gradient are much narrower than those recorded at constant densities. The width of a sample zone in the column in length units, σ_L, is related to the width of the detected peak in time units, σ_t

$$\sigma_L = \sigma_t u_i \tag{4}$$

where u_i is the linear velocity of the sample at the end of the column, which was calculated according to eqn. 1.

As we were not interested in the absolute values of σ_L but rather in the relative variations, we did not determine σ_t from the chromatograms but measured the baseline widths, B, of the peaks; B is proportional to σ_t, *e.g.*, for a Gaussian peak the baseline width is $4\sigma_t$. The results for both columns tested are given in Table IV.

If values at low and high densities are compared, σ_L does not change much with density under the conditions of the experiments. During the gradients, however, smaller values of the products are found; since the capacity ratios are rather large and the density increase is rather small, this finding is not very conclusive. Smith *et al.*[2], however, performed their elution experiment at very small values of k' and large density gradients; there, peak compression by compression of the mobile phase evidently played a more important rôle.

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LIQUID CHROMATOGRAPHY WITH CROWN ETHER-CONTAINING MOBILE PHASES

VIII. RETENTION BEHAVIOUR OF AMINO COMPOUNDS IN CATION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The use of crown ethers in the mobile phase with cation-exchange chromatography has been investigated. The capacity factors of primary amino compounds, which form host–guest complexes with 18-membered crown ethers, were significantly increased when 18-crown-6 or dicyclohexyl-18-crown-6 was added to the mobile phase. The degree of retention enhancement varied with the molecular structure of the guest compound, and also with the concentration of the crown ether and protons, the composition of the organic modifier and the nature of added electrolytes in the mobile phase. The retention mechanism in this particular system is discussed and the practical applicability to the separation of amino compounds is demonstrated.

INTRODUCTION

Recently, in high-performance ion-exchange liquid chromatography, ion exchangers in which the ion-exchange group is chemically bonded to totally porous silica microspheres have been widely used instead of cross-linked polystyrene ion-exchange resins because of the large number of theoretical plates, adequate ion-exchange capacity, high pressure resistance and rapid equilibration of the ion-exchange reaction. However, these ion exchangers allow limited selection of pH compared with the latter. It is well known in ion-exchange chromatography that the proton concentration, the nature and ionic strength of added electrolytes and the concentration of the organic solvent in the mobile phase affect the retention of sample solutes. For instance, ionic organic compounds can be separated by controlling the pH, ionic strength and/or concentration of the organic solvent of the mobile phase in high-performance ion-exchange chromatography, though there may also be a slight hydrophobic effect[1,2].

0021-9673/86/$03.50

The use of crown ethers in liquid chromatography was first demonstrated by Cram and co-workers, who achieved optical resolution of amino acids and their ester salts through chiral recognition by a crown ether which was contained in the mobile phase[3] or immobilized on the stationary phase[4,5]. Since then the specific cation-anchoring ability of crown ethers used as stationary ligands or as components of mobile phases has been utilized for the separation of various inorganic and organic cations as well as of anions with a common cation. Shono and co-workers separated alkali and alkaline-earth metal ions with poly(12-crown-4)-modified silica which has a great affinity for Na^+[6], and separated nitrophenol isomers with poly(vinylbenzo-18-crown-6)-modified silica and a potassium chloride-containing mobile phase, where the electrostatic interaction between the K^+ complexed with the benzo-18-crown-6 moiety and the negative charges or dipoles of the nitrophenol isomers is predominant[7]. Furthermore, they separated alkali metal ions by using dodecyl-18-crown-6, which is highly lipophilic, dynamically coated on the stationary phase by hydrophobic interaction[8]. Igawa *et al.*[9] prepared a polyamide-type crown ether resin coated on silica and separated some anions. Detailed investigations of the retention behaviour of various amino compounds in reversed-phase liquid chromatography with mobile phases containing crown ethers were made by Nakagawa *et al.*[10–15].

In high-performance cation-exchange chromatography with crown ether-containing mobile phases, it is expected that the retention behaviour of amino compounds depends on the changes in hydrophobic interaction with the resin matrix as well as on ionic adsorption onto the ion-exchange site, because not only the hydrophobicity but also the ionic properties of protonated primary amino compounds can be affected by complex formation with crown ethers. The present study deals with the retention behaviour of amino compounds (aromatic amines, amides) in high-performance cation-exchange chromatography with mobile phases containing crown ethers and various alkali metal chlorides or ammonium chloride. The analytical applicability of the proposed method is demonstrated.

EXPERIMENTAL

Reagents and materials

The amino compounds and inorganic salts of reagent grade were obtained from commercial sources. They were used as supplied. 18-crown-6 (18-C-6) and dicyclohexyl-18-crown-6 (DC-18-C-6) were from Nippon Soda (Tokyo, Japan). DC-18-C-6 was used without separation of the A,B-isomers. Glass-distilled deionized water and glass-distilled methanol for high-performance liquid chromatography (HPLC) (Katayama Chemicals) were used to prepare the mobile phases. Reagent grade hydrochloric acid was used to adjust the pH of the mobile phase.

Chromatography

A liquid chromatograph (LC-3A; Shimadzu, Kyoto, Japan) equipped with a refractive index detector (SE-51; Showa Denko, Tokyo, Japan) or a variable-wavelength UV detector (SPD-2A; Shimadzu) was used for the measurements of the capacity factors of crown ethers and amino compounds. The stationary phase was a cation exchanger (Nucleosil 10SA, propyl phenyl sulphonate type; Macherey Nagel, Düren, F.R.G.) packed in a stainless-steel tube (50 mm × 4.6 mm I.D.). The operating conditions are given in Table I.

TABLE I

HPLC CONDITIONS

Column temperature: 40°C. *Flow-rate:* 1.0 ml/min.

Experiment (Detection)	*Mobile phase*	*Data given in*
k' of crown ether (RI)	Methanol–water (60:40); [LiCl], [NaCl], [KCl], [NH_4Cl] or [RbCl] = 20 mM or no salts	Table II
k' *vs.* crown ether concentration (UV 254 nm)	Methanol–water (60:40); [18-C-6] or [DC-18-C-6] = 0–15 or 0–20 mM; [LiCl], [NaCl], [KCl], [NH_4Cl] or [RbCl] = 20 mM	Figs. 1–3
k' *vs.* pH (UV 254 nm)	Methanol–water (60:40); [LiCl] = 20 mM; [18-C-6] = 5 mM or [DC-18-C-6] = 3 mM or no crown ethers	Figs. 4, 5
k' *vs.* % methanol (UV 254 nm)	Methanol–water (40:60 to 90:10); [LiCl] = 20 mM; [18-C-6] = 5 mM or [DC-18-C-6] = 3 mM or no crown ethers	Fig. 6

The samples were dissolved in methanol, and the minimum amount required for detection was applied to the chromatograph. The capacity factors, k', were calculated from $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the sample and of a non-adsorbed substance (water or methanol), respectively, averaged over repeated measurements at the top of the elution curve. Both water and methanol gave almost identical t_0 values.

RESULTS AND DISCUSSION

Capacity factors of crown ethers

The capacity factors of 18-C-6 and DC-18-C-6 were measured by the use of mobile phases containing 20 mM LiCl, NaCl, NH_4Cl, RbCl or no salts. The results are shown in Table II. It was found that the two crown ethers were scarcely retained on the cation-exchange stationary phase when the mobile phase contained lithium chloride or no salts. However, the addition of NaCl, RbCl, KCl or NH_4Cl to the mobile phase resulted in increased capacity factors in this order, which reflects the stability of the complex formed between the cations and the 18-membered crown ethers. Thus, it follows that the magnitude of the increase in the capacity factors for the crown ethers depends on the stability of the complex, and that the hydrophobic

TABLE II

CAPACITY FACTORS, k', OF 18-CROWN-6 AND DICYCLOHEXYL-18-CROWN-6

Stationary phase: Nucleosil 10SA (50 mm × 4.6 mm I.D.). Mobile phase: methanol–water (60:40) (pH 3.0 with hydrochloric acid). Flow-rate: 1.0 ml/min. Column temperature: 40°C. Detection: RI.

	No salts added	*20 mM LiCl*	*20 mM NaCl*	*20 mM KCl*	*20 mM NH_4Cl*	*20 mM RbCl*
18-crown-6	0.58	0.36	2.49	2.82	3.02	2.73
Dicyclohexyl-18-crown-6	0.68	0.44	1.98 2.82	2.78	3.13	2.43

interactions between the crown ethers and the resin matrix of the stationary phase are very weak under these HPLC conditions. It is not clear from Table II why DC-18-C-6, which is a mixture of A,B-isomers, gave two peaks only when the mobile phase contained 20 m*M* sodium chloride.

Effect of crown ether concentration

The capacity factors of various amino compounds are shown in Fig. 1 as a function of the concentration of 18-C-6 or DC-18-C-6 in the mobile phase (pH 3.0) containing 20 m*M* lithium chloride. The capacity factors of primary amines increased with increasing concentration of the crown ethers, and gradually approached maxima. There was almost no difference in the effects of 18-C-6 and DC-18-C-6, in contrast to reversed-phase liquid chromatography where the capacity factors of primary amines were increased much more by addition of DC-18-C-6 than 18-C-6[10]. Thus, it follows that the ionic interaction between the protonated amino group of the guest molecule and the cation-exchange site of the resin is enhanced by complex formation with the crown ether, and that the contribution of the ionic effect predominates over that of the hydrophobic interaction with the resin matrix. On the other hand, the

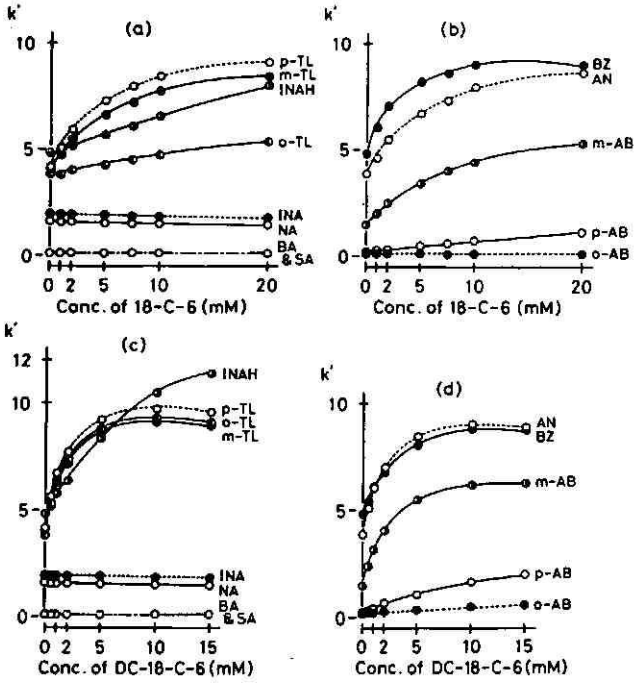

Fig. 1. Effect of the concentrations of 18-crown-6 and dicyclohexyl-18-crown-6 on the capacity factors of amino compounds. Stationary phase: Nucleosil 10SA (50 mm × 4.6 mm I.D.). Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing 20 m*M* lithium chloride and <20 m*M* crown ether. Flow-rate: 1.0 ml/min. Column temperature: 40°C. Detection: UV at 254 nm. Abbreviations: AN = aniline hydrochloride; BZ = benzylamine hydrochloride; SA = salicylamide; BA = benzamide; TL = toluidine; AB = aminobenzoic acid; INAH = isonicotinic acid hydrazide; INA = isonicotinamide; NA = nicotinamide.

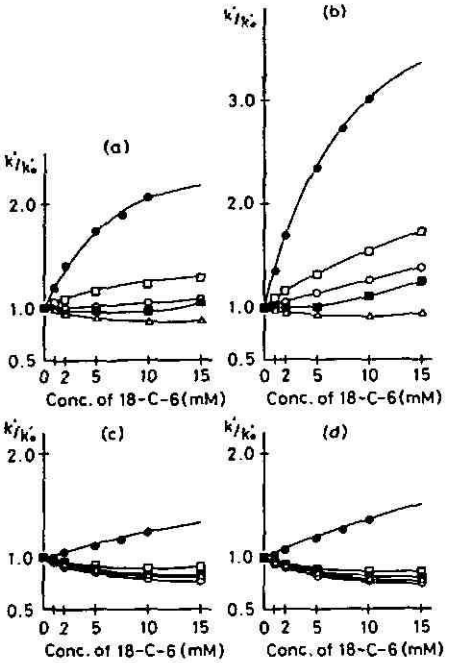

Fig. 2. Effect of added salts on the degree of retention enhancement *vs.* 18-crown-6 concentration for (a) aniline, (b) *m*-aminobenzoic acid, (c) *o*-toluidine and (d) isonicotinic acid hydrazide. Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing 20 m*M* LiCl (●), NaCl (□), NH_4Cl (○), KCl (△) or RbCl (■) and 0–15 m*M* 18-C-6. For other conditions, see Fig. 1.

capacity factors of amides such as isonicotinamide, nicotinamide, salicylamide and benzamide were almost unchanged, because they were not protonated under these mobile phase conditions.

With toluidine and aminobenzoic acid isomers (Fig. 1a and b) it is noticeable that the capacity factors of *ortho*-isomers in the 18-C-6-containing mobile phase are much lower than those of other isomers. This demonstrates the steric effects on the complexation with the crown ether. However, when DC-18-C-6 was used, the capacity factor of *o*-toluidine became as large as those of other isomers (Fig. 1c), whereas that of *o*-aminobenzoic acid remained much lower (Fig. 1d). This suggests that hydrophobic interaction may occur between the methyl group of toluidine and the cyclohexyl groups of DC-18-C-6, and that the carboxyl group of aminobenzoic acid tends to interfere with complex formation.

The effects of added salts on the degree of retention enhancement, k'/k'_0, caused by 18-C-6 are shown in Fig. 2, where k'_0 is the capacity factor observed with the mobile phase not containing 18-C-6. Fig. 2a and b indicates that the capacity factors of aniline and *m*-aminobenzoic acid underwent greater increases than did those of *o*-toluidine and isonicotinic acid hydrazide (INAH) upon the addition of salts, the magnitude of the increase depending on the kind of salt, in the order $Li^+ > Na^+ > NH_4^+ \geqslant Rb^+ > K^+$. For *o*-toluidine and INAH the corresponding sequence was $Li^+ > Na^+ \geqslant Rb^+ \geqslant K^+ \geqslant NH_4^+$. It is also found that the capacity factors for these solutes decreased upon addition of 18-C-6 in the presence of cations, except

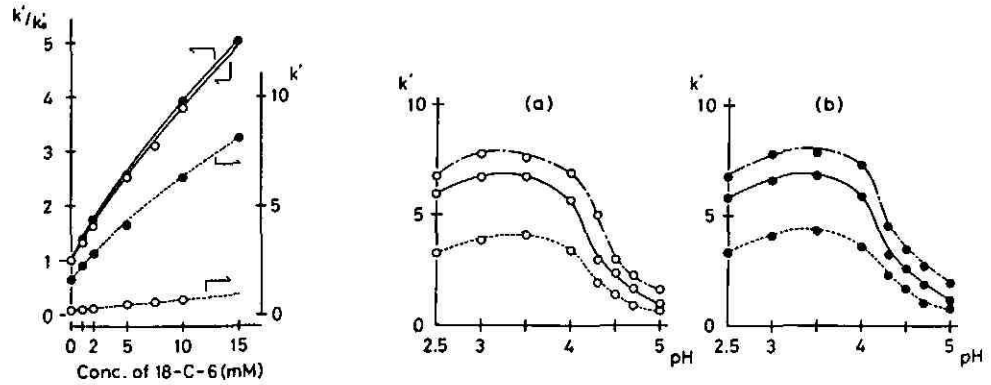

Fig. 3. Effect of added Li^+ on the capacity factor and the degree of retention enhancement *vs.* 18-crown-6 concentration for *p*-aminobenzoic acid. Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing 0 m*M* (●) or 20 m*M* (○) lithium chloride and 0–15 m*M* 18-C-6. For other conditions, see Fig. 1.

Fig. 4. Effect of pH on the capacity factors of (a) aniline and (b) *m*-toluidine. Mobile phase: methanol–water (60:40, v/v) containing 20 m*M* lithium chloride and 5 m*M* 18-C-6 (——), 3 m*M* DC-18-C-6 (– · –) or in the absence of crown ether (------). For other conditions, see Fig. 1.

Li^+. Therefore, it is considered that, except for Li^+, the cations compete with the solutes in complex formation with the crown ether. It is interesting that when the mobile phase contained KCl or RbCl the capacity factors slightly decreased with an initial increase in 18-C-6 concentration, followed by a gradual increase at higher concentrations.

When the mobile phase contained no salts, the capacity factors of the solutes, except salicylamide and benzamide, were much larger than those obtained with the mobile phase containing 20 m*M* lithium chloride. The capacity factor and the degree of retention enhancement for *p*-aminobenzoic acid are shown in Fig. 3 as a function of the concentration of 18-C-6 in the mobile phase containing 20 m*M* lithium chloride

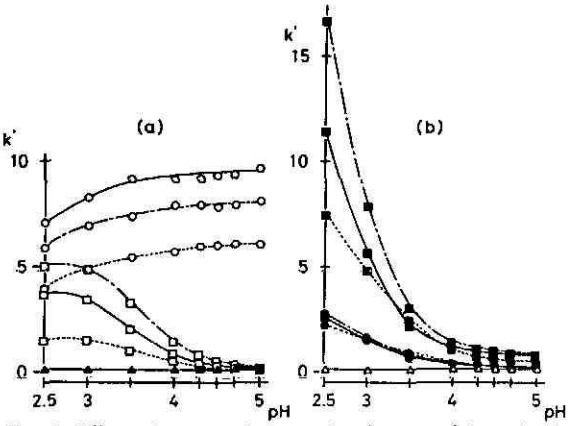

Fig. 5. Effect of pH on the capacity factors of benzylamine (○), isonicotinic acid hydrazide (■), *m*-aminobenzoic acid (□), nicotinamide (●), salicylamide (▲) and benzamide (△). For other conditions, see Fig. 4.

or no salts. There is almost no difference in the degree of retention enhancement between the cases of 20 *mM* lithium chloride and no salts, indicating that Li^+ does not compete with the solutes in complex formation with the 18-membered crown ether.

Effect of pH

The dependence of the capacity factor on pH between 2.5 and 5.0 was investigated by using mobile phases containing 5 *mM* 18-C-6, 3 m*M* DC-18-C-6 or no crown ethers in the presence of 20 m*M* lithium chloride. The results are shown in Figs. 4 and 5. In all cases, the observed profiles of *k'* *vs.* pH were almost unaffected by the presence of a crown ether in the mobile phase.

Under the chromatographic conditions employed in this experiment, protonation of the amino group of the guest molecule affects not only the ionic adsorption onto the cation-exchange site but also the complex formation with the 18-membered crown ether. Therefore, the capacity factors are generally expected to increase with increasing proton concentration. The capacity factors for aniline, *m*-toluidine (Fig. 4) and *m*-aminobenzoic acid (Fig. 5a) increased markedly with an initial decrease in pH of the mobile phase, followed by a gradual approach to maxima between pH 3 and 4. This behaviour is opposite to that observed in reversed-phase liquid chromatography[10], where the capacity factors decreased with increasing proton concentration. The slight decrease in the capacity factors for aniline and *m*-toluidine at low pH may be due to the competition of protonated bases with hydronium ions for the cation-exchange site[16,17]. The *k'* *vs.* pH profile of benzylamine in Fig. 5a exhibited a decrease in the capacity factor at pH < 3.5, because its pK_a value is larger than the pH examined. On the contrary, INAH and nicotinamide (Fig. 5b) showed a

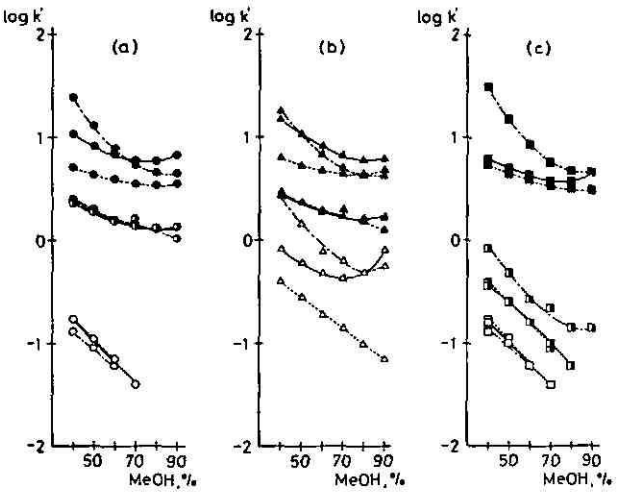

Fig. 6. Effect of methanol concentration on the capacity factors of amino compounds. Mobile phase: methanol–water (40:60 to 90:10, v/v) (pH 3.0 with hydrochloric acid) containing 20 m*M* lithium chloride and 5 m*M* 18-C-6 (——), 3 m*M* DC-18-C-6 (– · –) or in the absence of crown ether (------). For other conditions, see Fig. 1. Key: (●) aniline hydrochloride; (▲) benzylamine hydrochloride; (■) *o-toluidine*; (◑) nicotinamide; (▲) isonicotinamide; (◨) *o*-aminobenzoic acid; (○) salicylamide; (△) *p*-aminobenzoic acid; (□) benzamide.

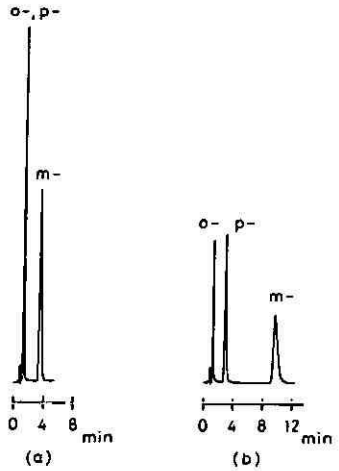

Fig. 7. Separation profile of aminobenzoic acid isomers. Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing (a) 20 m*M* LiCl and (b) 20 m*M* LiCl and 10 m*M* 18-C-6. For other conditions, see Fig. 1.

monotonous increase in k' with decreasing pH, because their pK_a values are lower than the pH. The capacity factors of salicylamide and benzamide (Fig. 5a and b) remained low and constant over the whole pH region examined, suggesting that there are no appreciable interactions with the 18-membered crown ethers. These k' *vs.* pH profiles indicate that when the capacity factor of an amino compound depends on the crown ether concentration it is also equally affected by the proton concentration.

Effect of solvent

The solvent effect on the capacity factor was investigated using mobile phases having various ratios of methanol–water (40:60 to 90:10, v/v) containing 5 m*M* 18-C-6, 3 m*M* DC-18-C-6 or no crown ethers at pH 3.0 in the presence of 20 m*M* lithium chloride. The results are shown in Fig. 6. In general, the log k' values decreased with increasing concentration of methanol, when the mobile phase contained no crown ethers. On the other hand, when a crown ether was added to the mobile phase, the decrease in log k' was followed by a slight increase at $>80\%$ methanol.

In ion-exchange liquid chromatography it is generally expected that the capacity factor of an organic cation is affected not only by the ionic adsorption on the stationary phase but also by the hydrophobic adsorption onto the resin matrix[18,19]. When the dielectric constant of the mobile phase decreases with increasing methanol concentration, it is expected that the ionic adsorption becomes much stronger and the hydrophobic adsorption weaker. Accordingly, the capacity factor varies depending on the balance of these opposing effects. The decrease in log k' values indicates that the elution ability of the mobile phase was enhanced with increasing methanol concentration and exceeded the ionic interaction with the ion-exchange site. At high methanol concentrations, the ionic interaction becomes stronger, so that the log k' values tend to increase. The magnitude of the decrease or increase in log k' is amplified by using mobile phases containing crown ethers, where both the ionic and hydrophobic properties of the guest compounds become stronger upon complex for-

mation. Salicylamide and benzamide, which are not retained on the cation-exchange stationary phase and do not form complexes with crown ethers, exhibited extremely small log k' values because of a slight hydrophobic interaction. Therefore, they showed linear decreases in log k' as in the reversed-phase mode, and the log k' values were almost unchanged despite the presence of crown ethers. Similar results were obtained with nicotinamide and isonicotinamide.

The elution profiles of aminobenzoic acid isomers obtained by using mobile phases with and without 18-C-6 are compared in Fig. 7. It is seen that *ortho*- and *para*-isomers are completely separated within 10 min by addition of 18-C-6.

CONCLUSIONS

In high-performance cation-exchange chromatography with mobile phases containing crown ethers the capacity factor of a guest compound bearing a primary amino group was increased by complex formation with the 18-membered crown ethers, because the hydrophobic adsorption onto the resin matrix as well as the ionic adsorption onto the ion-exchange site become stronger. When various alkali metal cations or ammonium were added to the mobile phase, except for Li^+ they competed with the guest compound in complex formation with the crown ether, resulting in a suppression of the increase in the capacity factor.

Complex formation with the crown ether increased with an initial decrease in the pH of the mobile phase, so that the capacity factor of the guest compound increased. However, a slight decrease in the capacity factor was observed at lower pH owing to the lowering of the ion-exchange ability of the stationary phase.

When methanol is added to the mobile phase, the ionic adsorption becomes stronger and the hydrophobic adsorption becomes weaker. Therefore, the capacity factor varied depending on the balance of these effects.

Thus, the capacity factor of a primary amine depends on the concentrations of the crown ether, proton and methanol in the mobile phase. On the contrary, that of an amide is affected only by the methanol concentration. Generally, the complexation of crown ethers with cations is weaker in water, which is a highly polar solvent, compared with methanol. Lithium does not compete with amino compounds in complex formation with 18-membered crown ethers. Therefore, it is considered that high-performance cation-exchange chromatography with water–methanol mobile phases containing 18-membered crown ethers and lithium chloride is useful for separating amino compounds.

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ISOTACHOPHORETIC DETERMINATION OF MOBILITY AND pK_a BY MEANS OF COMPUTER SIMULATION

IV. EVALUATION OF m_0 AND pK_a OF TWENTY-SIX AMINO ACIDS AND ASSESSMENT OF THE SEPARABILITY

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SUMMARY

Isotachophoretic qualitative indices, R_E, of twenty-six amino acids were measured at several pH_L values in the range of 8.6–9.6. The absolute mobility, m_0, and pK_a values were evaluated by the use of a least-squares method utilizing a simulation of the isotachophoretic steady state. The pK_a values were in good agreement with values cited in the literature. The R_E values simulated using the evaluated constants were in good agreement with R_E values converted from step heights observed previously. By comparing the previously observed separation behaviour of amino acids with their simulated effective mobilities, it is concluded that when the effective mobility of samples differs by *ca.* $1 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} at the steady state, *ca.* 10-nmol samples can be separated by the use of a 80 cm × 0.5 mm I.D. tube. The simulated effective mobilities of twenty-two amino acids were tabulated to assess the separability under some typical electrolyte conditions.

INTRODUCTION

As reported previously[1,2], the isotachophoretically steady state can be treated theoretically and isotachopherograms can be simulated, when the absolute mobilities, m_0, and the thermodynamic acid dissociation constants, pK_a, of the samples and electrolyte constituents are available. This technique can be used for estimation of the optimum separation condition[3,4]. A microcomputer program, SIPS (simulation of isotachophoretic separation), based on a data base including the m_0 and pK_a values of *ca.* 500 ionic species, has been developed[5] and can be used for the practical purpose stated above.

However, in our data base many important samples such as amino acids are not included due to the lack of the physico-chemical constants, especially m_0. This

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is due to the fact that the conventional conductivity method cannot be applied simply to the measurement of m_0 for amphoteric electrolytes such as neutral amino acids. Therefore, among the natural amino acids, the m_0 values of only two acidic amino acids, Asp^- and Glu^-, have been reported. In contrast, the pK_a values of amino acids have been extensively studied, although the thermodynamic values obtained are not always available. In isotachophoresis the ionic strengths of the leading, sample and terminating zones are always different. Therefore, in simulations, the thermodynamic pK_a values must be corrected for the ionic strength by using the Debye–Hückel equation.

The m_0 and pK_a values can be evaluated as reported[6,7] by use of the least-squares method to fit the observed potential gradient ratios of the sample zones separated isotachophoretically. To increase the utility of the SIPS program, in the present study, the m_0 and pK_a values of twenty-six amino acid were evaluated and then added to our data base. Further, the effective mobilities of twenty-two amino acids under several typical electrolyte conditions were simulated using the valuated values to clarify the limitation of separability, taking into account the previous systematic experimental studies by Kopwillem and Lundin[8] and Everaerts *et al.*[1].

EXPERIMENTAL

The amino acids treated were DL-Ala, β-Ala, α-amino-*n*-butyric acid (DL-α-Amin), L-Arg, L-Asn, L-Cys, L-Glu, L-Gln, Gly, L-His, L-Hyp, 3,5-I_2-L-Tyr, DL-Ile, L-Leu, L-Lys, DL-Met, L-Orn, L-Phe, L-Pro, DL-Ser, Tau, DL-Thr, DL-Trp, L-Tyr and DL-Val (guaranteed grade, Tokyo Kasei Co.). Sample solutions (3–10 m*M*) were prepared by dissolving these amino acids in distilled water or diluted sodium hydroxide solution (Cys). CysH was not considered since it is converted in to Cys in the alkaline solution.

Most of the treated amino acids are neutral amino acids and the pK_a values of their cationic forms are in the range of 1.5–3.6 and those of their anionic forms are 9.5–10.5. The cationic amino acids are not very mobile at pH = *ca.* 3.5, the lower limit of isotachophoretically "safe" pH in cationic analysis*, except for some basic amino acids, *e.g.*, Arg, Lys and Orn, and a neutral amino acid β-Ala with relatively large pK_a (3.6). Therefore qualitative indices, R_E, of the anions of neutral and acidic amino acids were measured in the pH_L (pH of the leading electrolyte) range of 8.6–9.6. The R_E is the ratio of the potential gradient, E (V cm^{-1}), of a sample zone, E_S, to that of the leading zone, E_L, which corresponds to the ratio of the effective mobility of the leading ion $\bar{m}_L$, to that of the sample ion, $\bar{m}_S$, *i.e.*, $R_E = E_S/E_L = \bar{m}_L/\bar{m}_S$.

For the R_E measurements of neutral and acidic amino acids the electrolyte systems used were as follows (Nos. 1–6 in Table II): the leading electrolytes were 10 m*M* hydrochloric acid solutions and the pH_L was adjusted to 8.64, 9.00 and 9.40 by adding 2-amino-2-methyl-1,3-propanediol (amediol) and to 9.03, 9.3 and 9.62 by

* According to our simulation, when a leading electrolyte of 10 m*M* potassium hydroxide buffered by formic acid (pH of leading electrolyte, pH_L = 3.5) is used, model cations of $m_0 > ca.\ 45 \cdot 10^{-5}$ ($pK_a > 6$) can migrate isotachophoretically. For the others, H^+ migrates instead. A neutral amino acid β-Ala, for which the pK_1 is the largest of those presently treated, the isotachophoretically steady state is not achieved when formic acid is used as the buffer. When glutamic acid is used as the buffer, the limiting pH for the analysis of β-Ala is *ca.* 4.

adding ethanolamine, respectively. The low pH limit was chosen in order that the effective mobilities would not be too small. If this were not the case, the temperature increment in the zones could not be neglected. The terminator was 10–30 *mM* β-Ala and the pH was adjusted to *ca.* 10 by adding barium hydroxide to suppress the disturbance caused by HCO_3^-. For precise measurement of R_E, the asymmetric potential of the potential gradient detection (PGD) used must be corrected[6]. Gly was used as an internal standard for this purpose, since its precise thermodynamic pK_a has been reported (9.7796 at 25°C)[9]. The m_0 value of Gly was first evaluated by isotachophoresis using similar electrolyte conditions as with the other samples. The internal standards were propionate and caproate ions. The evaluated m_0 value was $37.4 \cdot 10^{-5}$cm^2 V^{-1} s^{-1} (pK_a value fixed at 9.7796 in the least-squares method). The R_E values of Gly simulated using these constants were used for the correction of asymmetric potential under the electrolyte conditions. When Gly was unsuitable for this purpose because of mixed zone formation, Thr, Asn and β-Ala were used indirectly. Since Pro was not very mobile under these conditions, and the R_E values were large, β-Ala was used as the standard and Pro as the terminator.

For the analysis of basic amino acids, Arg, Lys and Orn, the leading electrolytes used were 10 *mM* potassium hydroxide solutions (Nos. 7–10 in Table II). The pH_L was adjusted in the range of 6.4–9.4 by adding 2-(N-morpholino)ethanesulphonic acid (pH_L = 6.43) and Phe (8.84, 9.03, 9.37). The terminator was tris(hydroxymethyl)aminomethane (Tris). The internal standards used were His and Tris and the R_E values are listed in Table II. All of the leading electrolytes contained 0.02% hydroxypropylcellulose to suppress electrode reactions and electroendosmosis.

The isotachopherograms were obtained using a Shimadzu isotachophoretic analyzer, IP-1B, equipped with PGD. The temperature was thermostatted at 25°C. The separating tube used was *ca.* 40 cm × 0.5 mm I.D. The driving current applied was 50 μA and a single experiment took *ca.* 35 min. The pH measurements were carried out using an Horiba expanded pH meter, Model F7ss.

Table I shows the m_0 and pK_a values of the electrolyte constituents used in the calculations. These values were taken mainly from the literature[10–15], but most of the

TABLE I

PHYSICO-CHEMICAL CONSTANTS USED IN THE SIMULATIONS (25°C)

m_0 = Absolute mobility (cm^2 V^{-1} s^{-1}) · 10^5; pK_a = thermodynamic acid dissociation constant; Tris = tris(hydroxymethyl)aminomethane; amediol = 2-amino-2-methyl-1,3-propanediol; MES = 2-(N-morpholino)ethanesulphonic acid; BDB = 5-bromo-2,4-dihydroxybenzoic acid.

Cation	m_0	pK_a	*Anion*	m_0	pK_a
K^+	75.72	–	Cl^-	79.08	–
Histidine	29.5*	6.042	Butyric acid	33.8	4.820
Imidazole	52.0*	7.15	MES	28.0*	6.15
Tris	29.5*	8.076	BDB	27.6*	3.0**
Amediol	32.0*	8.78		50.7*	7.60*
Ethanolamine	44.3*	9.498			

* Obtained isotachophoretically; other constants were taken from refs. 9–13.

** Assumed value.

mobilities used were determined by our isotachophoretic method. The m_0 value of amediol was changed from the previously used value of $29.5 \cdot 10^{-5}$ to $32.0 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}, taking into account the result of the conductivity measurement for the leading electrolyte. The observed conductivity of the leading electrolyte (10.02 m*M* hydrochloric acid solution, pH_L = 8.64, amediol buffer) was 0.998 mS cm^{-1} cm and the simulated value was 0.997 mS cm^{-1}. The conductivity meter used was a TOA Electronics Model CM-30ET.

Table II summarizes the leading electrolyte conditions together with the calculated concentrations, effective mobilities of the leading electrolyte constituents and the R_E values of the internal standard. Fig. 1 shows two typical isotachopherograms obtained by the use of electrolyte systems 1 and 2 in Table II.

For the data processing and the simulation, SIPS programs on SORD M223 MkIII and NEC PC9801E microcomputers and the SIPS-LSQ program on a NEC minicomputer MS120 were used. For plotting the figures, a Watanabe X-Y plotter WX4671 and a Roland DXY-980 were used.

RESULTS AND DISCUSSION

Table III summarizes the observed R_E values for all amino acids treated under the electrolyte conditions 1–10 in Table II. The R_E values, measured from the electropherograms for several completely separable combinations of the amino acids under each electrolyte condition (see Fig. 1), were the averages of at least three experimental determinations. The experimental errors were less than *ca.* ± 0.05 R_E units.

TABLE II

EXPERIMENTAL CONDITIONS FOR THE EVALUATION OF ABSOLUTE MOBILITIES AND pK_a VALUES OF AMINO ACIDS, CALCULATED CONCENTRATIONS AND EFFECTIVE MOBILITIES OF LEADING ZONE CONSTITUENTS

pH_L = pH of leading electrolyte; C_L = total concentration (m*M*) of leading ion; $\bar{m}_L$ = effective mobility (cm^2 V^{-1} s^{-1}) of leading ion · 10^5; $C_{B,L}$ = total concentration (m*M*) of buffer ion; $\bar{m}_{B,L}$ = effective mobility (cm^2 V^{-1} s^{-1}) of buffer ion · 10^5; Std(R_E) = internal standard used for correction of asymmetric potential and the corresponding R_E value. Leading ions: chloride (systems 1–6); potassium (systems 7–10).

System	*Buffer*	pH_L	C_L	$\bar{m}_L$	$C_{B,L}$	$\bar{m}_{B,L}$	Std(R_E)
Anionic analysis							
1	Am	8.64	10.02	74.69	16.57	17.28	Gly (6.12)
2	Am	9.00	10.02	74.69	25.04	11.45	Gly (5.02)
3	Am	9.40	10.02	74.69	47.79	6.004	Gly (3.89)
4	EA	9.03	10.02	74.69	13.11	31.08	Gly (4.01)
5	EA	9.30	10.02	74.69	15.78	25.85	Gly (3.66)
6	EA	9.62	10.02	74.68	22.07	18.51	Gly (3.20)
Cationic analysis							
7	MES	6.43	9.85	71.43	14.51	16.75	His (4.28)
8	Phe	8.84	10.21	71.36	30.82	7.988	Tris (6.23)
9	Phe	9.03	10.21	71.36	23.51	10.47	Tris (7.08)
10	Phe	9.37	10.21	71.36	16.26	15.12	Tris (8.64)

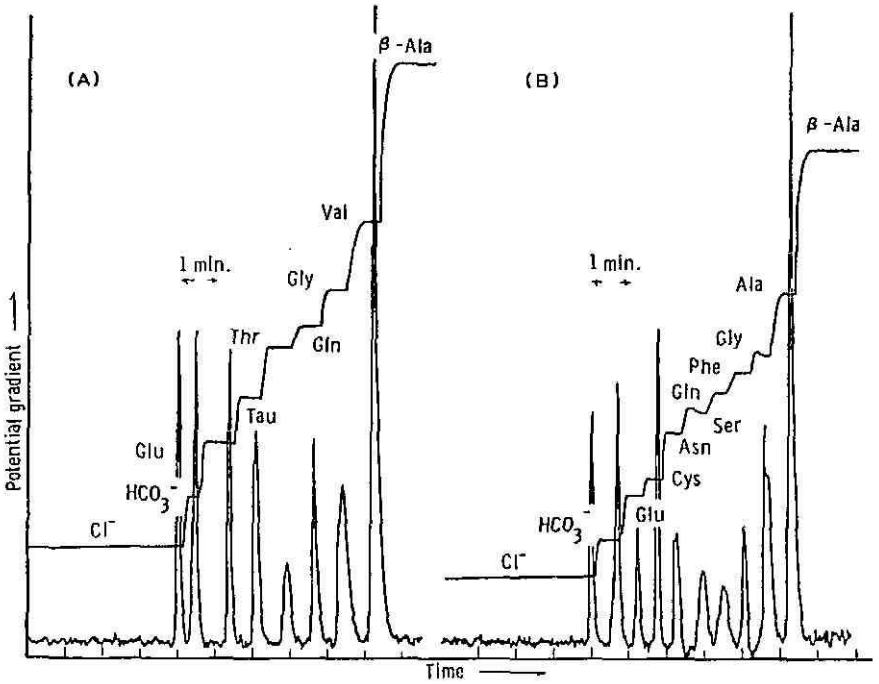

Fig. 1. The observed isotachopherograms for Glu, Tau, Thr, Gln, Gly and Val at pH_L = 9.00 buffered by amediol (A), and for Glu, Cys, Asn, Ser, Gln, Phe, Gly and Ala at pH_L = 6.84 (B). The leading ion was 10 m*M* chloride and the terminator was 30 m*M* β-Ala (pH = *ca.* 10 by adding barium hydroxide). The sample amounts were *ca.* 5–10 nmol and migration current was 50 μA.

The black circles in Figs. 2 and 3 show the pH_L dependence of the observed R_E values of the anionic amino acids in the pH_L range of 8.6–9.6 (buffers: amediol and ethanolamine). Using these R_E values, the m_0 and pK_a values were determined by the least-squares method. The curves in Figs. 2 and 3 were plotted using such values. The discontinuities in the curves are due to the different buffers. Even if the pH_L value is the same, the pH of the separated sample zones, and consequently the effective mobilities and R_E values, will depend on the mobility and pK_a of the buffers used. Table IV shows the observed and the best-fitted R_E values, the effective mobilities and the concentrations of the zone constituents, Ala, Glu, Gln, Leu, Thr, Val and Lys. The observed and the simulated R_E values were in good agreement, the mean error being in the range of 0.46(Glu)–1.43%(Tyr). The evaluated m_0 and pK_a were listed in Table V together with the pK_a values obtained by previous methods. In the least-squares method, several pK_a values were fixed at the literature values as shown in Table V, taking into account the pH range used in the R_E measurement. If the R_E values for in the completely dissociated state could be measured, m_0 and pK_a could be evaluated independently. This is not the case, since the pK_a values of the anionic forms of the samples are large and isotachophoretic equilibria could not be achieved at the completely dissociated state. Concerning 3,5-I_2-Tyr, the reported pK_a values of pK_2 = 6.48 and pK_3 = 7.12 were used as the initial values in the least-squares method; however, our best-fitted pK_3 = 9.69 was significantly different

TABLE III

OBSERVED R_E VALUES OF TWENTY-FIVE AMINO ACIDS

Electrolyte systems numbered as in Table II. R_E = Ratio of potential gradients, E_S/E_L.

Sample	*Electrolyte system and* pH_L					
	1 *8.64*	*2* *9.00*	*3* *9.40*	*4* *9.03*	*5* *9.30*	*6* *9.62*
Asp	2.59	2.41	2.20	2.37	2.24	2.03
Glu	2.84	2.61	2.31	2.52	2.35	2.11
Cys	3.21	2.72	2.28	2.50	2.33	2.11
3,5-I_2-Tyr	3.49	–	2.79	2.99	2.75	2.46
Tau	3.94	3.30	2.76	2.97	2.77	2.52
Asn	4.27	3.63	3.10	3.34	3.14	2.88
Ser	4.77	3.99	3.27	3.46	3.26	2.90
Thr	4.81	4.06	3.38	3.63	3.38	3.04
Gln	5.21	4.42	3.66	3.90	3.65	3.28
Met	5.58	4.65	3.76	4.00	3.77	3.36
Phe	5.70	4.78	3.91	4.28	3.95	3.52
His	5.64	4.78	3.87	4.14	3.88	3.46
Tyr	5.92	4.71	3.88	4.20	3.69	3.17
Gly*	6.12	5.02	3.89	4.02	3.66	3.20
Val	7.47	6.07	4.67	5.01	4.51	3.92
Ala	7.49	5.94	4.69	4.80	4.41	3.82
Trp	7.59	6.11	4.91	5.23	4.73	4.12
Hyp	7.74	6.19	4.78	4.93	4.54	3.94
α-Amin	7.67	6.16	4.80	4.88	4.56	3.93
Ile	8.22	6.64	5.11	5.43	4.94	4.25
Leu	8.00	6.57	5.12	5.36	4.90	4.27
β-Ala	10.64	8.45	6.38	6.18	5.65	4.86
	7 *6.43*	*8* *8.84*	*9* *9.03*	*10* *9.37*		
Arg	2.98	4.01	4.27	5.02		
Lys	3.00	3.77	3.94	4.56		
Orn	2.81	4.20	4.55	5.49		

* Internal standard; simulated value.

from the reported value. For 3,5-I_2-Tyr, Tyr and Cys, the pH_L conditions were unsuitable for the independent evaluation of two pK_a values and two m_0 values; we therefore assumed $m_2 = 2m_1$ in order to decrease unknown constants in the least-squares method. Except for the pK_a values obtained by means of this assumption, the evaluated pK_a agreed well with the values reported previously. Fig. 4 shows the pH dependence of the effective mobility of several amino acids, Asp, Glu, Cys, Tau, Gly, Ala, β-Ala, Leu, His, Orn, Arg and Lys. The curves were plotted using the evaluated absolute mobility and thermodynamic constants and are not for the isotachophoretic steady state. Fig. 5 shows the simulated isotachopherograms for Glu, Cys, Asn, Ser, Gln, Phe, Gly and Ala at $pH_L = 8.64$ and for Glu, Tau, Thr, Gln, Gly and Val at $pH_L = 9.00$ (amediol buffer). The terminator is β-Ala. The simulated and the observed isotachopherogram (Fig. 1) are in good agreement.

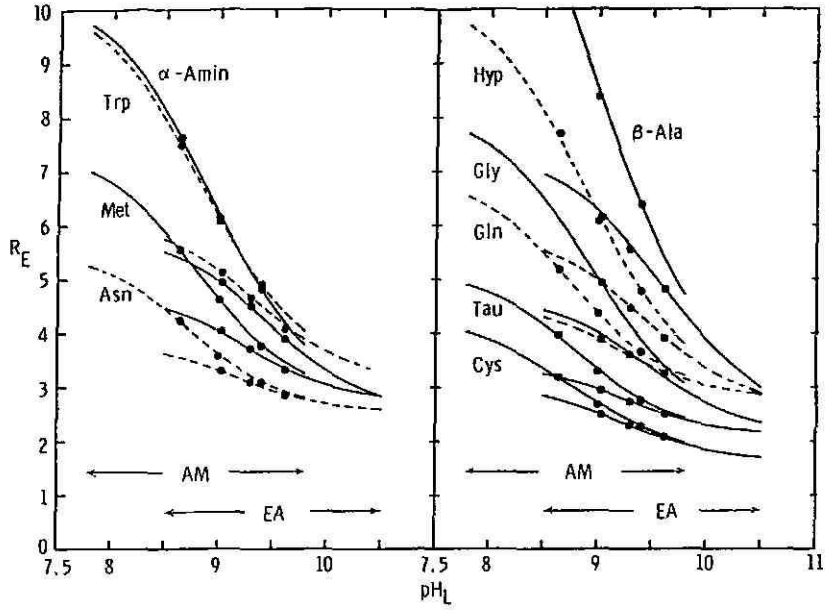

Fig. 2. The observed R_E values of Asn, Met, Trp, Amin, Cys, Tau, Gln, Hyp and β-Ala. The leading ion was 10 mM chloride. The curves were plotted using the best-fitted mobility and pK_a. The pH_L dependence of the R_E values of the internal standard, Gly, is also shown. AM and EA denote the buffers used, amediol and ethanolamine.

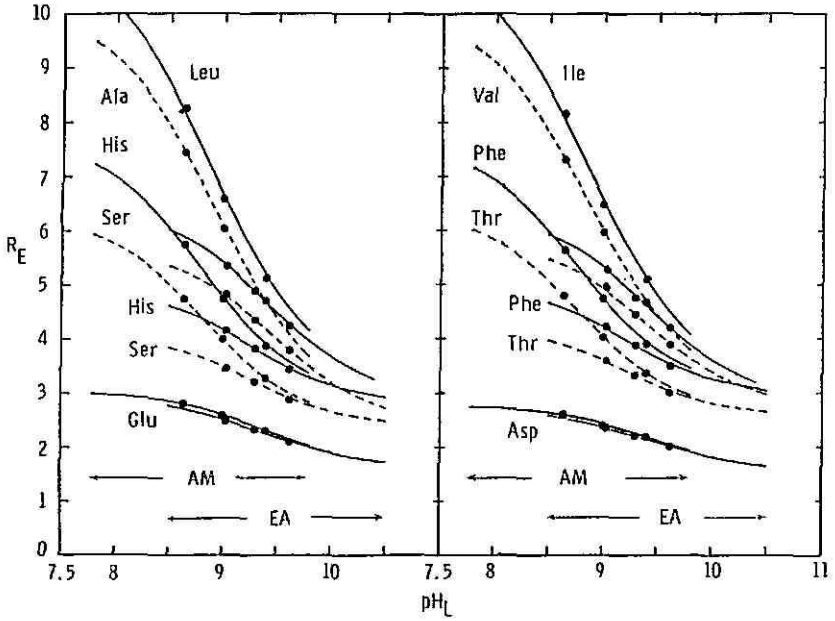

Fig. 3. The observed R_E values of Glu, Ser, His, Ala, Leu, Asp, Thr, Phe, Val and Ile. The leading ion was 10 mM chloride. Other details as in Fig. 2.

To confirm the evaluated m_0 and pK_a values, the step heights of eighteen amino acids observed by Everaerts *et al.*[1] using a conductometric detector were converted into R_E and these values were compared with the simulated R_E values. The amino

TABLE IV

OBSERVED AND SIMULATED R_E VALUES OF SEVEN AMINO ACIDS, EFFECTIVE MOBILITIES AND CONCENTRATIONS OF ZONE CONSTITUENTS (25°C)

System = electrolyte system (see Table II); dev./% = percentage deviation; $\bar{m}_S$ = effective mobility ($cm^2\ V^{-1}\ s^{-1}$) of sample ion · 10^5; pH_S = pH of sample zone; C_S^t = total concentration (mM) of sample; $C_{B,S}^t$ = total concentration (mM) of buffer ion; $\bar{m}_{B,S}$ = effective mobility ($cm^2\ V^{-1}\ s^{-1}$) of buffer ion · 10^5; I = ionic strength · 10^3.

System No.	R_E		*dev./%*	$\bar{m}_S$	pH_S	C_S^t	$C_{B,S}^t$	$\bar{m}_{B,S}$	I
	Obs.	*Calc.*							
Ala									
1	7.49	7.44	0.69	10.04	9.525	6.573	13.78	4.824	2.195
2	5.94	6.07	−2.17	12.31	9.666	6.533	22.28	3.643	2.695
3	4.69	4.67	0.44	15.99	9.883	6.440	45.10	2.316	3.497
4	4.80	4.79	0.12	15.58	9.858	6.006	9.995	13.36	3.170
5	4.41	4.36	1.14	17.13	9.950	5.960	12.70	11.51	3.478
6	3.82	3.79	0.68	19.68	10.11	5.852	19.10	8.704	3.970
	Mean error		0.87						
Glu									
1	2.84	2.83	0.32	26.39	8.862	5.793	13.39	13.82	6.919
2	2.61	2.62	−0.51	28.47	9.205	5.433	22.26	8.453	7.538
3	2.31	2.32	−0.57	32.15	9.577	4.972	45.53	4.310	8.617
4	2.52	2.51	0.30	29.73	9.338	4.932	10.05	25.18	7.359
5	2.35	2.33	0.77	32.03	9.557	4.707	13.06	20.07	8.042
6	2.11	2.12	−0.27	35.30	9.824	4.451	19.73	13.98	8.997
	Mean error		0.46						
Gln									
1	5.21	5.22	−0.12	14.32	9.259	6.408	13.20	7.814	3.452
2	4.42	4.40	0.42	16.97	9.442	6.375	21.69	5.619	4.102
3	3.66	3.67	−0.36	20.33	9.720	6.293	44.51	3.253	4.907
4	3.90	3.91	−0.15	19.12	9.606	5.819	9.301	19.04	4.237
5	3.65	3.62	0.81	20.63	9.743	5.774	12.01	15.84	4.561
6	3.28	3.31	−0.91	22.56	9.966	5.665	18.42	11.17	4.943
	Mean error		0.46						
Leu									
1	8.00	8.03	−0.43	9.296	9.486	5.886	13.09	5.198	2.247
2	6.57	6.56	0.11	11.38	9.640	5.834	21.59	3.836	2.752
3	5.12	5.12	−0.08	14.58	9.876	5.715	44.45	2.355	3.504
4	5.36	5.34	0.36	13.98	9.829	5.258	9.284	13.98	3.079
5	4.90	4.86	0.88	15.38	9.935	5.195	12.02	11.79	3.370
6	4.27	4.26	0.18	17.52	10.12	5.051	18.49	8.586	3.785
	Mean error		0.34						

TABLE IV *(continued)*

System No.	R_E		dev./%	$\bar{m}_S$	pH_S	C_S	$C_{B,S}$	$\bar{m}_{B,S}$	I
	Obs.	Calc.							
Thr									
1	4.81	4.81	0.08	15.54	9.241	6.682	13.45	8.043	3.625
2	4.06	4.07	−0.12	18.37	9.424	6.654	21.93	5.814	4.300
3	3.38	3.40	−0.56	21.97	9.700	6.582	44.74	3.385	5.141
4	3.63	3.61	0.43	20.66	9.586	6.116	9.550	19.51	4.462
5	3.38	3.35	0.74	22.26	9.721	6.077	12.25	16.32	4.801
6	3.04	3.07	−1.00	24.32	9.943	5.981	18.64	11.62	5.211
	Mean error		0.49						
Val									
1	7.47	7.43	0.55	10.06	9.470	6.179	13.31	5.362	2.361
2	6.07	6.08	−0.18	12.28	9.622	6.134	21.81	3.979	2.887
3	4.67	4.75	−1.79	15.71	9.855	6.030	44.65	2.458	3.681
4	5.01	4.96	1.08	15.07	9.808	5.573	9.489	14.41	3.249
5	4.51	4.51	−0.07	16.55	9.913	5.519	12.21	12.23	3.556
6	3.92	3.96	−1.14	18.84	10.09	5.392	18.64	8.995	4.005
	Mean error		0.80						
Lys									
7	3.00	3.01	−0.49	23.69	6.250	6.415	11.09	14.60	6.408
8	3.77	3.72	1.36	19.19	8.548	6.701	30.14	4.363	5.382
9	3.94	3.98	−1.11	17.91	8.684	6.690	21.85	5.606	5.004
10	4.56	4.56	0.08	15.66	8.879	6.660	13.65	7.799	4.346
	Mean error		0.76						

acids were L-Ala, β-Ala, L-Asp, L-Cys, L-Glu, Gly, L-His, L-Hyp, 3,5-I_2-L-Tyr, L-Ile, L-Leu, DL-Met, L-Phe, L-Ser, L-Thr, L-Trp, DL-Tyr and L-Val. 5-Bromo-2,4-dihydroxybenzoate anion (BDB) was used as the leading ion to prevent the disturbance caused by HCO_3^-*. The buffers were ethanolamine and Lys, and the pH_L was in the range of 9–9.5. Table VI summarizes the seven leading electrolyte conditions used together with the calculated concentrations and effective mobilities of the electrolyte constituents. Since the given step heights (mm) were those from the leading zone to sample zones in recorder traces (Tables 13.2 and 13.3 in ref. 1), to convert these step heights into R_E values, the step heights of the leading zones, h_L, were estimated by

$$h_L = h_{std}/(R_{E,std} - 1) \quad (1)$$

* The BDB ions are divalent under the conditions used in ref. 1 and the effective mobility is almost the same as with HCO_3^-. Therefore the zone of HCO_3^- cannot be distinguished from that of BDB by a conductometric detector.

TABLE V

ABSOLUTE MOBILITIES AND THERMODYNAMIC DISSOCIATION CONSTANTS OF TWENTY-SIX AMINO ACIDS (25°C)

Amino acid	*Present method*		*Other methods* pK_a
	m_0	pK_a	
Ala	−32.2	9.857	9.866[9,11], 9.87[10,12,13]
β-Ala	−30.8	10.241	10.240[9,11]
α-Amin	−30.5	9.827	9.830[9,11], 9.833[10]
Arg	26.9	8.919	9.143[10], 8.991[11], 9.05[12], 8.994[13]
Asp	−30.1	3.900*	3.900[9–11], 3.63[12], 3.86[13]
	−55.4	10.002*	10.002[9–11], 9.47[12], 9.82[13]
Asn	−31.6	9.030	8.870[10,11], 8.85[12,13] (20°C)
Cys	−27.0	8.405	7.854[10], 8.00[11]
	−53.9	9.845	9.854[10], 9.850[12]
Glu	−27.0	4.324*	4.288[9], 4.324[10,11], 4.25[12]
	−54.3	9.960	9.387[9], 9.475[10], 9.96[11], 9.67[12]
Gln	−28.8	9.224	9.131[10,11]
Gly	−37.4	9.7796*	9.780[9], 9.7796[10,12,13], 9.778[11]
His	29.6*	6.04*	6.04[10,12,13], 6.00[11]
	−28.3	9.330	9.33[10], 9.17[11,13], 9.12[12]
Hyp	−30.1	9.816	9.662[9–11], 9.58[12] (20°C)
3,5-I_2-Tyr	−21.0	6.5*	6.48[9–11]
	−42.0	9.469	7.12
Ilcu	−26.7	9.765	9.758[9,13], 9.761[10], 9.752[11]
Leu	−26.4	9.728	9.744[9,11,13], 9.748[10], 9.77[12]
Lys	26.4	9.127	8.951[10,12], 9.18[11,12]
	−26.4*	10.79*	10.53[10,13], 10.79[11], 10.72[12] (0.01 *M*, 20°C)
Met	−29.3	9.344	9.210[10,11,12], 9.27[13]
Orn	28.4	8.712	8.65[10,12], 8.690[11,13]
	−28.4*	10.755*	10.76[10], 10.755[11], 10.67[12] (0.02 *M*)
Phe	−26.9	9.262	9.119[10], 9.13[11,12]
Pro	−25.4	10.640*	10.640[9,10,13], 10.643[11], 10.60[12]
Ser	−33.6	9.302	9.208[9–11,13]
Tau	−37.9	9.182	9.061[9–11,13]
Thr	−30.9	9.200	9.100[9,10,13], 9.099[11]
Trp	−25.4	9.594	9.377[10], 9.39[11], 9.55[12] (0.01 *M*, 20°C), 9.43[12] (1 *M*, 20°C), 9.44[13]
Tyr	−20.0	9.099	9.108[10], 9.11[11–13], 9.19[12]
	−40.0	10.189	10.07[10,12], 10.13[11,13], 10.47[12]
Val	−28.4	9.710	9.719[9,13], 9.722[10], 9.716[11], 9.62[12]

* Value fixed in the least-squares method.

where h_{std} is the observed step height of a standard sample and $R_{E,std}$ the R_E value of the standard. Using the estimated h_L, the R_E values of the sample zones, $R_{E,S}$, can be evaluated

$$R_{E,S} = (h_S + h_L)/h_L \qquad (2)$$

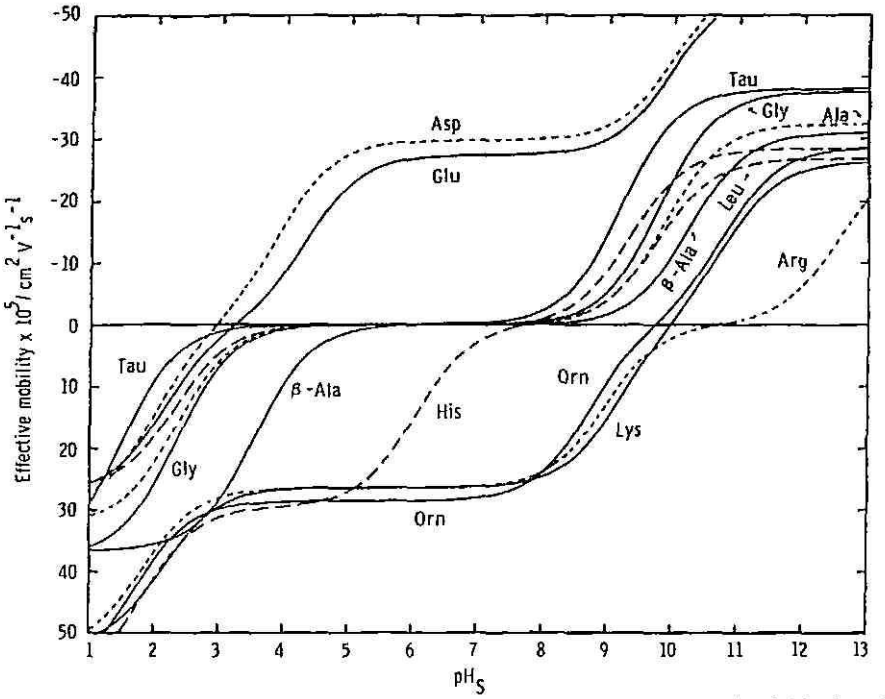

Fig. 4. The pH dependence of the effective mobility of Asp, Glu, Tau, Gly, Ala, β-Ala, Leu, His, Orn, Arg and Lys, *not for the isotachophoretically steady state*. The ionic strength is zero. pH_S = pH of sample zone.

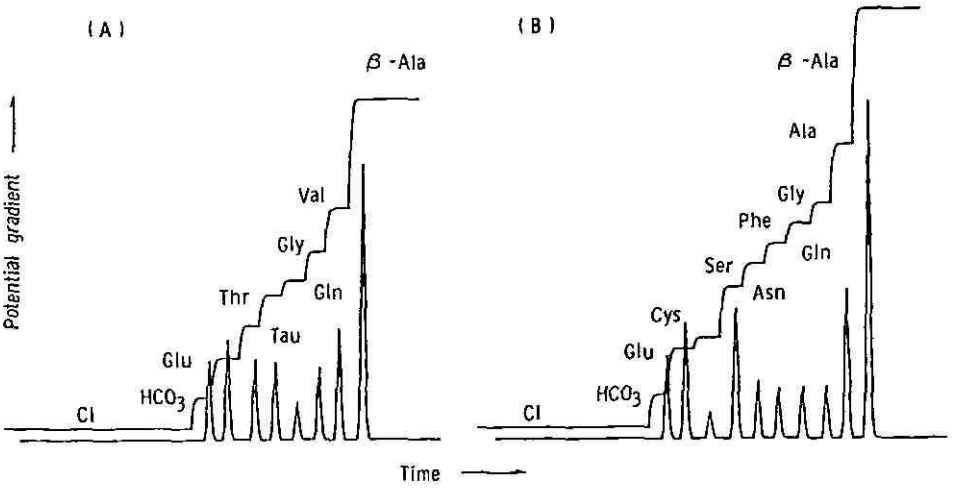

Fig. 5. The simulated isotachopherograms of Glu, Tau, Thr, Gln, Gly and Val at pH_L = 9.00 buffered by amediol (A), and Glu, Cys, Asn, Ser, Gln, Phe, Gly and Ala at pH_L = 8.64 (B). The leading ion was 10 m*M* chloride. The sample amounts were 10 nmol of each acid. Migration current: 50 μA.

TABLE VI

EXPERIMENTAL CONDITIONS USED FOR THE MEASUREMENT OF STEP HEIGHTS FOR EIGHTEEN AMINO ACIDS BY EVERAERTS *et al.*[1], CALCULATED CONCENTRATIONS AND EFFECTIVE MOBILITIES OF LEADING ZONE CONSTITUENTS

For the abbreviations used, see Table II. The leading ion is 5-bromo-2,4-dihydroxybenzoate.

System	*Buffer*	pH_L	C_L	$\bar{m}_L$	$C_{B,L}$	$\bar{m}_{B,L}$	I
1	Lys	9.07	4.00	42.05	14.18	12.74	12.0
2	Lys	9.22	4.00	42.12	17.13	10.58	12.1
3	Lys	9.42	4.00	42.09	23.84	7.613	12.6
4	EA	9.00	4.00	42.21	10.14	31.47	11.8
5	EA	9.20	4.00	42.40	11.52	27.86	11.9
6	EA	9.36	4.00	42.51	13.16	24.47	11.9
7	EA	9.55	4.00	42.61	16.07	20.12	12.0

where h_S is the step height of the sample from the leading to the sample zone. The simulated values of $R_{E,std}$ for the standard Gly ware shown in Table VI. Substituting $R_{E,std}$ and the reported h_{std} (135, 124.5 and 117 mm for systems 1–3 in Table VI) into eqn. 1 gave the estimated h_L values of 80.8, 83.0 and 92.1 mm respectively. Table VII

TABLE VII

CORRECTED AND SIMULATED R_E VALUES OF EIGHTEEN AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS 1–3 IN TABLE VI

The original step heights were measured by Everaerts *et al.*[1]. Corr. = Corrected R_E values; Sim. = simulated R_E values using the evaluated m_0 and pK_a.

Amino acid	pH_L = 9.07			pH_L = 9.22			pH_L = 9.42		
	Corr.	*Calc.*	*dev./%*	*Corr.*	*Calc.*	*dev./%*	*Corr.*	*Calc.*	*dev./%*
Asp	1.40	1.39	0.7	1.35	1.35	0	1.30	1.28	1.5
Cys	1.50	1.53	−2.0	1.43	1.44	−0.7	1.34	1.33	0.7
Glu	1.46	1.49	−2.1	1.40	1.44	−2.9	1.36	1.35	0.7
I_2-Tyr	1.75	1.77	−1.1	1.70	1.70	0	1.62	1.60	1.2
Ser	2.15	2.19	−1.9	1.99	2.06	−3.5	1.85	1.90	−2.7
Thr	2.18	2.24	−2.8	2.05	2.12	−3.4	1.96	1.97	−0.5
Tyr	2.51	2.62	−4.4	2.34	2.43	−3.8	2.13	2.18	−2.3
Met	2.47	2.53	−2.4	2.33	2.38	−2.1	2.15	2.19	−1.9
Gly	2.67	–	std	2.50	–	std	2.27	–	std
His	2.51	2.58	−2.8	2.55	2.43	4.7	2.18	2.23	−2.3
Phe	2.61	2.62	−0.4	2.46	2.47	−0.4	2.26	2.28	−0.9
Ala	3.18	3.17	0.3	2.99	2.96	1.0	2.68	2.67	0.4
Val	3.09	3.19	−3.2	2.93	2.97	−0.4	2.66	2.68	−0.8
Trp	3.12	3.26	−4.5	2.95	3.04	−3.1	2.69	2.74	−1.9
Hyp	3.10	3.25	−4.8	2.95	3.03	−2.7	2.66	2.73	−2.6
Ile	3.33	3.44	−3.3	3.17	3.20	−0.9	2.86	2.87	−0.3
Leu	3.35	3.40	−1.5	3.15	3.16	−0.3	2.85	2.84	0.4
β-Ala	3.97	–	–	3.81	–	–	3.23	–	–
Mean error	(%)		2.4			1.9			1.3

shows the converted R_E values from the observed step heights for the electrolyte systems using Lys as buffer (electrolyte systems 1–3) together with the simulated R_E. The mean deviations, except for β-Ala, between the observed and the simulated R_E values were 2.4, 1.9 and 1.3% respectively. However, the simulation of the steady state of β-Ala as the terminator failed: the pH of the β-Ala zone increased over the isoelectric point of Lys buffer corresponding to conversion of Lys cations into anions. This may suggest that β-Ala was no longer the actual terminator and that hydroxide ions may fulfil this rôle[1].

Table VIII summarizes the converted and the simulated R_E values for the electrolyte systems 4–7 (ethanolamine buffer). The mean deviations were 3.5, 3.2, 2.1 and 3.9% respectively. Except for system 6, the deviations were about twice as large as those found in the the electrolyte systems buffered by Lys. Apparently, from Table VIII, these relatively large mean errors are caused by the large deviations between the observed and the simulated R_E of Trp, Hyp, Ile, Leu and β-Ala. Since ethanolamine was also used in our R_E measurement, such deviations for the electrolyte systems 4, 5 and 7 were not expected.

We confirmed these experimental facts for twenty-two amino acids using a leading electrolyte of 4 m*M* BDB buffered by ethanolamine at pH_L = 9.06. The mean error between the observed and the simulated values was 2.2% for all samples, and 4.1% for Amin, Hyp, Trp, Ile, Leu and β-Ala. Since the migration current was

TABLE VIII

CORRECTED AND SIMULATED R_E VALUES OF EIGHTEEN AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS 4–7 IN TABLE VI

The original step heights were measured by Everaerts *et al.*[1]. For the abbreviations used, see Table VII.

Amino acid	pH_L = 9.00			pH_L = 9.20			pH_L = 9.36			pH_L = 9.55		
	Corr.	*Sim.*	*dev/%*	*Corr.*	*Sim.*	*dev/%*	*Corr.*	*Sim.*	*dev/%*	*Corr.*	*Sim.*	*dev/%*
Asp	1.40	1.37	2.1	1.34	1.32	1.5	1.32	1.28	3.0	1.34	1.22	9.0
Cys	1.50	1.49	0.7	1.41	1.40	0.7	1.38	1.33	3.6	1.30	1.25	3.8
Glu	1.48	1.47	0.7	1.42	1.40	1.4	1.36	1.35	0.7	1.31	1.27	3.1
I_2-Tyr	1.74	1.75	−0.6	1.66	1.66	0.0	1.55	1.59	−2.6	1.45	1.51	−4.1
Ser	2.10	2.04	2.9	1.92	1.95	−1.6	1.89	1.86	1.6	1.75	1.76	−0.6
Thr	2.10	2.12	−1.0	2.03	2.02	0.5	1.95	1.94	0.5	1.83	1.84	−0.5
Tyr	2.32	2.46	−6.0	2.19	2.30	−5.0	2.13	2.16	−1.4	1.92	1.99	−3.6
Met	2.30	2.37	−3.0	2.21	2.25	−1.8	2.19	2.15	1.8	2.03	2.03	0.0
Gly	2.35	–	std	2.24	–	std	2.12	–	std	1.97	–	std
His	2.41	2.43	−0.8	2.29	2.31	−0.9	2.25	2.21	1.8	2.08	2.08	0.0
Phe	2.44	2.48	−1.6	2.33	2.36	−1.3	2.28	2.26	0.9	2.11	2.14	−1.4
Ala	2.76	2.82	−2.2	2.61	2.68	−2.7	2.55	2.53	0.8	2.31	2.35	−1.7
Val	2.79	2.92	−4.7	2.66	2.76	−3.8	2.60	2.61	−0.4	2.36	2.43	−3.0
Trp	2.86	3.04	−6.3	2.68	2.88	−7.5	2.66	2.73	−2.6	2.41	2.55	−5.8
Hyp	2.80	2.93	−4.6	2.69	2.77	−3.0	2.60	2.63	−1.2	2.35	2.43	−3.4
Ile	2.98	3.17	−6.4	2.80	3.00	−7.1	2.72	2.84	−4.4	2.47	2.63	−6.5
Leu	3.00	3.14	−4.7	2.80	2.97	−6.1	2.74	2.81	−2.6	2.49	2.61	−4.8
β-Ala	3.33	3.68	−10.5	3.19	3.48	−9.1	3.12	3.29	−5.4	2.67	3.03	−13.5
Mean error (%)			3.5			3.2			2.1			3.9

TABLE IX

SIMULATED EFFECTIVE MOBILITIES OF TWENTY-TWO AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS OF pH_L = 8.6, 9.0, 9.4 AND 9.7 BUFFERED BY AMEDIOL

The leading ion is 10 m*M* chloride. The values cited are mobility · 10^5 ($cm^2V^{-1}s^{-1}$). The diagonal values are the effective mobilities and the others are the differences.

	Asp	*Glu*	*Cys*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Met*	*His*	*Phe*	*Tyr*	*Gly*	*Val*	*Ala*	*Trp*	*Amin*	*Hyp*	*Leu*	*Ile*	*β-Ala*
pH_L = 8.6																						
1 Asp	28.8	2.6	5.8	7.5	10.1	11.5	13.3	13.5	14.7	15.6	15.9	15.9	16.4	16.8	18.9	19.0	19.1	19.2	19.2	19.7	19.8	21.9
2 Glu		26.2	3.2	4.9	7.5	9.0	10.8	11.0	12.2	13.0	13.3	13.3	13.8	14.3	16.4	16.4	16.5	16.6	16.7	17.1	17.3	19.3
3 Cys			23.0	1.7	4.3	5.7	7.6	7.8	8.9	9.8	10.1	10.1	10.6	11.0	13.1	13.2	13.3	13.4	13.4	13.9	14.1	16.1
4 I_2-Tyr				21.3	2.6	4.1	5.9	6.1	7.3	8.1	8.4	8.4	8.9	9.3	11.5	11.5	11.6	11.7	11.8	12.2	12.4	14.4
5 Tau					18.7	1.5	3.3	3.5	4.7	5.5	5.8	5.8	6.3	6.8	8.9	8.9	9.0	9.1	9.2	9.6	9.8	11.8
6 Asn						17.3	1.8	2.0	3.2	4.0	4.3	4.4	4.8	5.3	7.4	7.4	7.5	7.7	7.7	8.1	8.3	10.3
7 Ser							15.5	0.2	1.4	2.2	2.5	2.6	3.0	3.5	5.6	5.6	5.7	5.9	5.9	6.3	6.5	8.5
8 Thr								15.3	1.2	2.0	2.3	2.4	2.8	3.3	5.4	5.4	5.5	5.7	5.7	6.1	6.3	8.3
9 Gln									14.1	0.9	1.2	1.2	1.6	2.1	4.2	4.2	4.3	4.5	4.5	4.9	5.1	7.1
10 Met										13.2	0.3	0.3	0.8	1.2	3.4	3.4	3.5	3.6	3.7	4.1	4.3	6.3
11 His											12.9	0.0	0.5	0.9	3.1	3.1	3.2	3.3	3.4	3.8	4.0	6.0
12 Phe												12.9	0.5	0.9	3.0	3.1	3.2	3.3	3.3	3.8	3.9	6.0
13 Tyr													12.4	0.5	2.6	2.6	2.7	2.8	2.9	3.3	3.5	5.5
14 Gly														12.0	2.1	2.1	2.2	2.4	2.4	2.9	3.0	5.0
15 Val															9.9	0.0	0.1	0.3	0.3	0.7	0.9	2.9
16 Ala																9.8	0.1	0.2	0.3	0.7	0.9	2.9
17 Trp																	9.7	0.1	0.2	0.6	0.8	2.8
18 Amin																		9.6	0.0	0.5	0.6	2.7
19 Hyp																			9.6	0.4	0.6	2.6
20 Leu																				9.1	0.2	2.2
21 Ile																					9.0	2.0
22 β-Ala																						6.9

	Asp	*Glu*	*Cys*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Met*	*His*	*Phe*	*Tyr*	*Gly*	*Ala*	*Val*	*Trp*	*Amin*	*Hyp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.0																						
1 Asp	30.6	2.2	3.3	6.8	8.2	10.2	11.9	12.3	13.7	14.5	14.9	15.0	15.1	15.8	18.3	18.3	18.6	18.6	18.7	19.2	19.4	21.9
2 Glu		28.5	1.2	4.6	6.0	8.0	9.8	10.1	11.5	12.4	12.8	12.9	12.9	13.6	16.2	16.2	16.4	16.5	16.5	17.1	17.3	19.7
3 Cys			27.3	3.5	4.9	6.8	8.6	8.9	10.3	11.2	11.6	11.7	11.7	12.4	15.0	15.0	15.2	15.3	15.3	15.9	16.1	18.5
4 I_2-Tyr				23.9	1.4	3.4	5.1	5.5	6.9	7.8	8.1	8.2	8.3	9.0	11.6	11.6	11.8	11.8	11.9	12.5	12.6	15.1

5 Tau				22.4	2.0	3.7	4.1	5.5	6.4	6.7	6.8	6.9	7.6	10.1	10.1	10.4	10.4	10.5	11.0	11.2	13.7
6 Asn					20.5	1.7	2.1	3.5	4.4	4.7	4.9	4.9	5.6	8.2	8.2	8.4	8.5	8.5	9.1	9.3	11.7
7 Ser						18.7	0.4	1.7	2.6	3.0	3.1	3.1	3.8	6.4	6.4	6.6	6.7	6.7	7.3	7.5	9.9
8 Thr							18.4	1.4	2.3	2.6	2.8	2.8	3.5	6.1	6.1	6.3	6.4	6.4	7.0	7.2	9.6
9 Gln								17.0	0.9	1.3	1.4	1.4	2.1	4.7	4.7	4.9	5.0	5.0	5.6	5.8	8.2
10 Met									16.1	0.4	0.5	0.5	1.2	3.8	3.8	4.0	4.1	4.1	4.7	4.9	7.3
11 His										15.7	0.1	0.1	0.9	3.4	3.4	3.6	3.7	3.8	4.3	4.5	7.0
12 Phe											15.6	0.0	0.7	3.3	3.3	3.5	3.6	3.6	4.2	4.4	6.8
13 Tyr												15.6	0.7	3.3	3.3	3.5	3.6	3.6	4.2	4.4	6.8
14 Gly													14.9	2.6	2.6	2.8	2.9	2.9	3.5	3.7	6.1
15 Ala														12.3	0.0	0.2	0.3	0.3	0.9	1.1	3.5
16 Val															12.3	0.2	0.3	0.3	0.9	1.1	3.5
17 Trp																12.1	0.1	0.1	0.7	0.9	3.3
18 Amin																	12.0	0.0	0.6	0.8	3.2
19 Hyp																		12.0	0.6	0.8	3.2
20 Leu																			11.4	0.2	2.6
21 Ile																				11.2	2.4
22 β-Ala																					8.8

	Asp	*Cys*	*Glu*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Tyr*	*Met*	*Gly*	*His*	*Phe*	*Ala*	*Val*	*Amin*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.4																						
1 Asp	33.8	1.4	1.7	6.7	6.9	10.0	11.1	11.9	13.5	14.1	14.2	14.6	14.7	15.1	17.8	18.1	18.3	18.3	18.6	19.2	19.4	22.1
2 Cys		32.4	0.3	5.3	5.5	8.6	9.7	10.5	12.1	12.7	12.8	13.3	13.3	13.7	16.5	16.7	16.9	16.9	17.2	17.8	18.0	20.7
3 Glu			32.2	5.0	5.3	8.3	9.4	10.2	11.8	12.5	12.5	13.0	13.0	13.4	16.2	16.4	16.6	16.7	16.9	17.6	17.8	20.4
4 I_2-Tyr				27.1	0.2	3.3	4.4	5.2	6.8	7.4	7.5	7.9	8.0	8.3	11.1	11.4	11.6	11.6	11.9	12.5	12.7	15.4
5 Tau					26.9	3.0	4.1	4.9	6.5	7.2	7.2	7.7	7.8	8.1	10.9	11.2	11.3	11.4	11.7	12.3	12.5	15.1
6 Asn						23.9	1.1	1.9	3.5	4.1	4.2	4.7	4.7	5.1	7.9	8.1	8.3	8.4	8.6	9.3	9.4	12.1
7 Ser							22.8	0.8	2.4	3.1	3.1	3.6	3.6	4.0	6.8	7.0	7.2	7.3	7.5	8.2	8.3	11.0
8 Thr								22.0	1.6	2.2	2.3	2.8	2.8	3.2	6.0	6.2	6.4	6.5	6.7	7.4	7.5	10.2
9 Gln									20.3	0.6	0.7	1.2	1.2	1.6	4.4	4.6	4.8	4.9	5.1	5.7	5.9	8.6
10 Tyr										19.7	0.1	0.5	0.6	0.9	3.7	4.0	4.2	4.2	4.5	5.1	5.3	8.0
11 Met											19.6	0.5	0.5	0.9	3.7	3.9	4.1	4.2	4.4	5.0	5.2	7.9
12 Gly												19.2	0.0	0.4	3.2	3.5	3.6	3.7	4.0	4.6	4.8	7.4
13 His													19.1	0.4	3.2	3.4	3.6	3.6	3.9	4.5	4.7	7.4
14 Phe														18.8	2.8	3.0	3.2	3.3	3.6	4.2	4.4	7.0
15 Ala															16.0	0.3	0.4	0.5	0.8	1.4	1.6	4.2
16 Val																15.7	0.2	0.2	0.5	1.1	1.3	4.0
17 Amin																	15.6	0.1	0.3	1.0	1.1	3.8
18 Hyp																		15.5	0.3	0.9	1.1	3.7

(*Continued on p. 74*)

TABLE IX (*continued*)

	Asp	*Cys*	*Glu*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Tyr*	*Met*	*Gly*	*His*	*Phe*	*Ala*	*Val*	*Amin*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
19 Trp																			15.2	0.6	0.8	3.5
20 Leu																				14.6	0.2	2.9
21 Ile																					14.4	2.7
22 β-Ala																						11.7

	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Tyr*	*Gly*	*Gln*	*Met*	*His*	*Phe*	*Ala*	*Amin*	*Val*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.7																						
1 Asp	36.9	0.6	1.3	7.1	7.4	11.1	11.3	12.6	13.9	13.9	14.4	14.8	15.4	16.1	17.6	18.3	18.3	18.4	19.3	19.7	19.8	22.2
2 Cys		36.3	0.7	6.5	6.7	10.4	10.7	12.0	13.2	13.3	13.8	14.2	14.8	15.4	17.0	17.6	17.7	17.7	18.6	19.0	19.2	21.6
3 Glu			35.6	5.8	6.0	9.7	10.0	11.3	12.5	12.6	13.1	13.5	14.1	14.7	16.3	16.9	17.0	17.0	17.9	18.3	18.5	20.9
4 Tau				29.8	0.3	4.0	4.3	5.5	6.8	6.8	7.3	7.7	8.3	9.0	10.6	11.2	11.3	11.3	12.2	12.6	12.7	15.1
5 I_2-Tyr					29.6	3.7	4.0	5.3	6.5	6.6	7.0	7.5	8.1	8.7	10.3	10.9	11.0	11.0	11.9	12.3	12.4	14.9
6 Asn						25.9	0.3	1.6	2.8	2.9	3.3	3.7	4.4	5.0	6.6	7.2	7.3	7.3	8.2	8.6	8.7	11.1
7 Ser							25.6	1.3	2.5	2.6	3.1	3.5	4.1	4.7	6.3	6.9	7.0	7.0	7.9	8.3	8.5	10.9
8 Thr								24.3	1.2	1.3	1.8	2.2	2.8	3.4	5.0	5.6	5.7	5.7	6.6	7.0	7.2	9.6
9 Tyr									23.1	0.1	0.5	0.9	1.6	2.2	3.8	4.4	4.5	4.5	5.4	5.8	5.9	8.3
10 Gly										23.0	0.5	0.9	1.5	2.1	3.7	4.3	4.4	4.4	5.3	5.7	5.9	8.3
11 Gln											22.5	0.4	1.0	1.7	3.2	3.9	3.9	4.0	4.9	5.3	5.4	7.8
12 Met												22.1	0.6	1.2	2.8	3.4	3.5	3.6	4.4	4.9	5.0	7.4
13 His													21.5	0.6	2.2	2.8	2.9	2.9	3.8	4.2	4.4	6.8
14 Phe														20.9	1.6	2.2	2.3	2.3	3.2	3.6	3.7	6.1
15 Ala															19.3	0.6	0.7	0.7	1.6	2.0	2.1	4.6
16 Amin																18.7	0.1	0.1	1.0	1.4	1.5	4.0
17 Val																	18.6	0.0	0.9	1.3	1.5	3.9
18 Hyp																		18.6	0.9	1.3	1.4	3.8
19 Trp																			17.7	0.4	0.5	2.9
20 Leu																				17.3	0.1	2.5
21 Ile																					17.1	2.4
22 β-Ala																						14.7

TABLE X

SIMULATED EFFECTIVE MOBILITIES OF TWENTY-TWO AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS OF pH_L = 9.0, 9.2, 9.4 AND 9.6 BUFFERED BY ETHANOLAMINE

Other details as in Table IX.

	Asp	*Glu*	*Cys*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gln*	*Gly*	*Met*	*His*	*Tyr*	*Phe*	*Ala*	*Val*	*Amin*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.0																						
1 Asp	31.4	1.9	2.0	6.3	6.5	9.1	10.1	10.9	12.4	12.9	13.1	13.5	13.6	13.9	16.0	16.5	16.5	16.5	17.1	17.5	17.7	19.6
2 Glu		29.5	0.1	4.4	4.6	7.2	8.2	9.0	10.5	11.0	11.2	11.6	11.7	12.0	14.1	14.6	14.6	14.6	15.2	15.6	15.8	17.7
3 Cys			29.4	4.3	4.4	7.1	8.1	8.9	10.4	10.9	11.0	11.5	11.5	11.9	14.0	14.4	14.4	14.5	15.0	15.5	15.6	17.5
4 Tau				25.1	0.2	2.8	3.8	4.6	6.1	6.6	6.8	7.3	7.3	7.6	9.7	10.2	10.2	10.3	10.8	11.2	11.4	13.3
5 I_2-Tyr					*24.9*	2.7	3.7	4.5	6.0	6.5	6.6	7.1	7.1	7.4	9.5	10.0	10.0	10.1	10.6	11.1	11.2	13.1
6 Asn						22.3	1.0	1.8	3.3	3.8	3.9	4.4	4.4	4.8	6.9	7.3	7.3	7.4	8.0	8.4	8.5	10.5
7 Ser							21.3	0.8	2.3	2.8	2.9	3.4	3.4	3.8	5.9	6.3	6.3	6.4	6.9	7.4	7.5	9.4
8 Thr								20.5	1.5	2.0	2.1	2.6	2.6	3.0	5.1	5.5	5.5	5.6	6.1	6.6	6.7	8.7
9 Gln									19.0	0.5	0.6	1.1	1.1	1.5	3.6	4.0	4.0	4.1	4.6	5.1	5.2	7.1
10 Gly										18.5	0.1	0.6	0.6	1.0	3.1	3.5	3.5	3.6	4.1	4.6	4.7	6.6
11 Met											18.4	0.5	0.5	0.8	2.9	3.4	3.4	3.5	4.0	4.5	4.6	6.5
12 His												17.9	0.0	0.3	2.4	2.9	2.9	3.0	3.5	4.0	4.1	6.0
13 Tyr													17.9	0.3	2.4	2.9	2.9	3.0	3.5	4.0	4.1	6.0
14 Phe														17.5	2.1	2.6	2.6	2.7	3.2	3.6	3.8	5.7
15 Ala															15.4	0.5	0.5	0.6	1.1	1.5	1.7	3.6
16 Val																15.0	0.0	0.1	0.6	1.1	1.2	3.1
17 Amin																	15.0	0.1	0.6	1.1	1.2	3.1
18 Hyp																		14.9	0.5	1.0	1.1	3.0
19 Trp																			14.3	0.5	0.6	2.5
20 Leu																				13.9	0.1	2.0
21 Ile																					13.8	1.9
22 β-Ala																						11.8

(*Continued on p. 76*)

TABLE X (*continued*)

	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gln*	*Gly*	*Met*	*Tyr*	*His*	*Phe*	*Ala*	*Amin*	*Val*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.2																						
1 Asp	32.8	1.5	1.7	6.4	6.5	9.4	10.3	11.2	12.8	13.2	13.4	13.5	13.9	14.3	16.4	16.9	16.9	17.0	17.5	18.0	18.1	20.2
2 Cys		31.3	0.2	4.9	5.0	7.9	8.8	9.7	11.3	11.6	11.9	12.0	12.4	12.8	14.8	15.3	15.4	15.4	16.0	16.5	16.6	18.6
3 Glu			31.1	4.7	4.8	7.7	8.6	9.5	11.1	11.5	11.7	11.8	12.2	12.6	14.7	15.2	15.2	15.3	15.8	16.3	16.4	18.5
4 Tau				26.5	0.1	3.1	4.0	4.8	6.4	6.8	7.0	7.2	7.5	7.9	10.0	10.5	10.5	10.6	11.2	11.6	11.8	13.8
5 I_2-Tyr					26.3	2.9	3.8	4.7	6.3	6.7	6.9	7.0	7.4	7.8	9.9	10.4	10.4	10.5	11.0	11.5	11.6	13.7
6 Asn						23.4	0.9	1.8	3.4	3.7	4.0	4.1	4.5	4.9	6.9	7.4	7.4	7.5	8.1	8.6	8.7	10.7
7 Ser							22.5	0.9	2.5	2.8	3.1	3.2	3.6	4.0	6.0	6.5	6.5	6.6	7.2	7.7	7.8	9.8
8 Thr								21.6	1.6	1.9	2.2	2.3	2.7	3.1	5.1	5.6	5.7	5.7	6.3	6.8	6.9	8.9
9 Gln									20.0	0.4	0.6	0.7	1.1	1.5	3.6	4.1	4.1	4.2	4.7	5.2	5.3	7.4
10 Gly										19.7	0.2	0.4	0.7	1.1	3.2	3.7	3.7	3.8	4.4	4.8	5.0	7.0
11 Met											19.4	0.1	0.5	0.9	3.0	3.5	3.5	3.6	4.1	4.6	4.7	6.8
12 Tyr												19.3	0.4	0.8	2.8	3.3	3.3	3.4	4.0	4.5	4.6	6.6
13 His													18.9	0.4	2.5	3.0	3.0	3.0	3.6	4.1	4.2	6.3
14 Phe														18.5	2.1	2.6	2.6	2.7	3.2	3.7	3.8	5.9
15 Ala															16.5	0.5	0.5	0.6	1.2	1.6	1.8	3.8
16 Amin																16.0	0.0	0.1	0.7	1.1	1.3	3.3
17 Val																	16.0	0.1	0.7	1.1	1.3	3.3
18 Hyp																		15.9	0.6	1.1	1.2	3.2
19 Trp																			15.3	0.5	0.6	2.6
20 Leu																				14.8	0.1	2.2
21 Ile																					14.7	2.0
22 β-Ala																						12.7

	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gln*	*Gly*	*Tyr*	*Met*	*His*	*Phe*	*Ala*	*Amin*	*Val*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.4																						
1 Asp	34.6	1.0	1.5	6.6	6.7	10.0	10.6	11.7	13.3	13.3	13.5	13.8	14.4	14.9	16.7	17.3	17.3	17.4	18.1	18.5	18.7	20.7
2 Cys		33.5	0.5	5.5	5.6	8.9	9.6	10.6	12.3	12.3	12.5	12.8	13.4	13.8	15.7	16.2	16.3	16.3	17.1	17.5	17.6	19.7
3 Glu			33.0	5.0	5.2	8.4	9.1	10.1	11.8	11.8	12.0	12.3	12.9	13.4	15.2	15.7	15.8	15.8	16.6	17.0	17.1	19.2
4 Tau				28.0	0.1	3.4	4.1	5.1	6.7	6.8	7.0	7.3	7.8	8.3	10.2	10.7	10.8	10.8	11.5	12.0	12.1	14.2
5 I_2-Tyr					27.9	3.3	3.9	5.0	6.6	6.6	6.8	7.2	7.7	8.2	10.0	10.6	10.6	10.7	11.4	11.8	12.0	14.1
6 Asn						24.6	0.7	1.7	3.3	3.4	3.6	3.9	4.4	4.9	6.8	7.3	7.4	7.4	8.1	8.6	8.7	10.8
7 Ser							23.9	1.0	2.7	2.7	2.9	3.2	3.8	4.3	6.1	6.6	6.7	6.7	7.5	7.9	8.0	10.1
8 Thr								22.9	1.6	1.7	1.9	2.2	2.7	3.2	5.1	5.6	5.7	5.7	6.4	6.9	7.0	9.1
9 Gln									21.3	0.0	0.2	0.5	1.1	1.6	3.4	4.0	4.0	4.1	4.8	5.2	5.4	7.4
10 Gly										21.2	0.2	0.5	1.1	1.6	3.4	3.9	4.0	4.0	4.8	5.2	5.3	7.4

11 Tyr										21.0	0.3	0.9	1.3	3.2	3.7	3.8	3.8	4.6	5.0	5.1	7.2
12 Met											20.7	0.6	1.0	2.9	3.4	3.5	3.5	4.3	4.7	4.8	6.9
13 His												20.2	0.5	2.3	2.9	2.9	3.0	3.7	4.1	4.3	6.3
14 Phe													19.7	1.8	2.4	2.4	2.5	3.2	3.6	3.8	5.9
15 Ala														17.8	0.5	0.6	0.6	1.4	1.8	1.9	4.0
16 Amin															17.3	0.1	0.1	0.8	1.3	1.4	3.5
17 Val																17.2	0.0	0.8	1.2	1.3	3.4
18 Hyp																	17.2	0.7	1.2	1.3	3.4
19 Trp																		16.5	0.4	0.6	2.6
20 Leu																			16.0	0.1	2.2
21 Ile																				15.9	2.1
22 β-Ala																					13.8

	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gly*	*Tyr*	*Gln*	*Met*	*His*	*Phe*	*Ala*	*Amin*	*Hyp*	*Val*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.6																						
1 Asp	36.5	0.7	1.4	6.9	7.1	10.8	11.0	12.3	13.4	13.5	14.0	14.4	15.1	15.7	17.0	17.6	17.7	17.8	18.7	19.1	19.2	21.2
2 Cys		35.8	0.7	6.2	6.4	10.1	10.4	11.6	12.7	12.9	13.4	13.8	14.4	15.0	16.3	17.0	17.1	17.1	18.1	18.4	18.5	20.5
3 Glu			35.1	5.5	5.8	9.4	9.7	11.0	12.0	12.2	12.7	13.1	13.7	14.3	15.6	16.3	16.4	16.4	17.4	17.7	17.8	19.9
4 Tau				29.6	0.2	3.9	4.2	5.4	6.5	6.6	7.1	7.5	8.2	8.8	10.1	10.7	10.8	10.9	11.8	12.2	12.3	14.3
5 I_2-Tyr					29.4	3.7	3.9	5.2	6.3	6.4	6.9	7.3	8.0	8.6	9.9	10.5	10.6	10.7	11.6	12.0	12.1	14.1
6 Asn						25.7	0.3	1.5	2.6	2.8	3.3	3.7	4.3	4.9	6.2	6.9	7.0	7.0	8.0	8.3	8.4	10.4
7 Ser							25.4	1.3	2.3	2.5	3.0	3.4	4.0	4.6	6.0	6.6	6.7	6.7	7.7	8.0	8.1	10.2
8 Thr								24.2	1.1	1.2	1.7	2.1	2.7	3.4	4.7	5.3	5.4	5.5	6.4	6.8	6.9	8.9
9 Gly									23.1	0.2	0.7	1.1	1.7	2.3	3.6	4.3	4.4	4.4	5.4	5.7	5.8	7.8
10 Tyr										22.9	0.5	0.9	1.5	2.1	3.5	4.1	4.2	4.2	5.2	5.5	5.6	7.7
11 Gln											22.5	0.4	1.0	1.6	3.0	3.6	3.7	3.8	4.7	5.1	5.2	7.2
12 Met												22.1	0.6	1.2	2.6	3.2	3.3	3.4	4.3	4.6	4.8	6.8
13 His													21.4	0.6	1.9	2.6	2.7	2.7	3.7	4.0	4.1	6.2
14 Phe														20.8	1.3	2.0	2.1	2.1	3.1	3.4	3.5	5.5
15 Ala															19.5	0.6	0.7	0.8	1.7	2.1	2.2	4.2
16 Amin																18.9	0.1	0.2	1.1	1.5	1.6	3.6
17 Hyp																	18.8	0.1	1.0	1.3	1.5	3.5
18 Val																		18.7	1.0	1.3	1.4	3.4
19 Trp																			17.8	0.3	0.4	2.5
20 Leu																				17.4	0.1	2.1
21 Ile																					17.3	2.0
22 β-Ala																						15.3

25 or 50 μA, the behaviour observed under such conditions is not be due to a temperature effect. This was apparent from the fact that the observed R_E value for the other standard, propionate ion, was 1.25 and the simulated value is the same when Gly was used as the internal standard. Since such a situation was not found in the BDB–Lys system, the nature of the BDB–ethanolamine system is not properly reflected by the simulation.

Separability assessment

Using the evaluated constants, the separability of the amino acids can be assessed by the use of the SIPS program and the results can be compared with those from experiments. The order of appearance and the separability of samples are determined by the magnitude of the mobilities and the differences between them in the transient mixed zone. They are a complicated function of the m_0 and pK_a of the samples, the selected buffer and pH_L, the pH of the injected mixture, etc. Therefore, strictly speaking, a discussion of the order of appearance and the separability should take account of these factors besides steady state information. However, for such complicated sample systems as treated in this paper, the analysis of the mixed zones is a difficult problem. At present, even for a three-component system, no practical elucidation of the separation process has been reported, although two-component systems (monovalent ions) have been relatively well analyzed[14,15]. Although the SIPS program has a routine which is applicable to general multivalent ions, at present its utility is limited to two-component systems (the details will be published in due course). As a first approximation, the difference between the effective mobilities of samples at the steady state can be a good measure of their separation, since the pH of the mixed zone lies in the middle of the pH values of the adjacent separated zones and the effective mobilities in the mixed zone are not very different from those in the steady state.

Table IX summarizes the differences between the simulated effective mobilities of the twenty-two amino acids at the steady state when amediol was used as the pH buffer. The four pH conditions (pH_L = 8.6, 9.0, 9.4 and 9.7) were the same as those used by Kopwillem and Lundlin[8]. In the simulation, the leading ion concentration was 10 m*M*. Table X also summarizes the differences between the simulated effective mobilities in another conveniently used leading electrolyte, 10 m*M* hydrochloric acid–ethanolamine (pH_L = 9.0, 9.2, 9.4 and 9.6). Apparently, from these tables, the differences for adjacent samples are very small and sometimes zero, suggesting that the separation of all of them is not practical as long as a pH effect on the effective mobility is utilized. In comparison with Table IX and X, amediol may be superior in separability.

Kopwillem and Lundin[8] studied the pH dependence of the separation of seventeen amino acids, using thermometric and UV detectors. The samples (each 2.5–10 nmol) were Ala, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr and Val. They found that fourteen amino acids can be separated at pH_L = 8.6 buffered by amediol. The leading ion used was chloride (5–10 m*M*). A capillary tube (81 cm × 0.5 mm I.D.) was used and a single experiment took *ca.* 70 min (driving current = 50 μA).

At pH_L = 8.6 (amediol buffer) the observed order of appearance of the samples in their experiment, the simulated R_E values and the effective mobilities, $\bar{m}$, of the

amino acids were Asp (R_E = 2.60, $\bar{m}$ = 28.8 · 10^{-5}cm^2 V^{-1} s^{-1}), Glu (2.85, 26.2), Cys (3.25, 23.0), Asn (4.32, 17.3), [Ser (4.83, 15.5), Thr (4.89, 15.3)], Gln (5.31, 14.1), Met (5.65, 13.2), Tyr (6.01, 12.4), [His (5.78, 12.9), Phe (5.79, 12.9)], Gly (6.23, 12.0), Trp (7.67, 9.74), Val (7.57, 9.87), Ala (7.59, 9.84), [Leu (8.18, 9.12), Ile (8.33, 8.96)] and β-Ala (10.76, 6.95). The pairs of samples in square brackets could not be separated when the injected sample amounts were 2.5 and 5 nmol of each. It was also reported that the pairs [Ser, Thr], [Tyr, His], [His, Phe], [Ala, Leu] and [Ile, Leu] could not be separated when the sample amounts were 7.5 and 10 nmol of each. For these pairs the differences between the simulated effective mobilities of the individual components are 0.2 · 10^{-5}, 0.5 · 10^{-5}, 0.0 · 10^{-5}, 0.72 · 10^{-5} and 0.16 · 10^{-5}cm^2 V^{-1} s^{-1} respectively. The reported order of appearance is in approximate agreement with both the increasing order of R_E values and the decreasing order of effective mobility, except for Tyr, His, Phe, Trp and Val. The behaviour of the last compounds is contradictory to the estimation not only from the simulation but also to independently observed R_E values with experimental errors of *ca.* 0.05* (5.92, DL-Tyr; 5.70, L-Phe; 5.64, L-His; 7.59, DL-Trp and 7.47, DL-Val). However, UV observation supported the first appearance of Tyr when the separation of Tyr, Phe and His, for example, was attempted at pH_L = 8.64 (amediol buffer). These facts can not be explained as the result of errors in the observed R_E values or the evaluated constants. Most probably, they were caused by the enforced phenomena**.

A tentative simulation of the separation process for the two-component system gave evidence which supported this estimation. When a 10 m*M* hydrochloric acid solution buffered by amediol (pH_L = 8.6) was the leading electrolyte and the pH of the injected 1:1 mixture was 9 (amediol buffer), for example, the simulated pH of the transient mixed zone was 9.255 and the effective mobilities of His and Tyr in the zone were 12.39 · 10^{-5} cm^2 V^{-1} s^{-1}. The mobility of Tyr was larger than that of His, contrary to the steady state, suggesting that enforced migration is occurring. The time needed for the resolution, t_{res}, of 10-nmol samples was 2060 s, when the migration current was 50 μA. The simulated t_{res} for the other inseparable pairs (10 nmol of each) were 2814 (Ser, Thr), 3344 (His, he), 7047 (Ala, Leu) and 2124 s (Ile, Leu). When the other samples coexist, it was confirmed experimentally that the observed t_{res} is larger than the simulated value for a two-component system. The t_{res} values for the amino acids in the electropherogram of Fig. 1 were simulated. The pH_L was 8.64, the pH of the mixture was 9 (amediol buffer respectively) and the migration current was 50 μA. The estimated values were 530 (Glu, Cys), 206 (Cys, Asn), 348 (Asn, Ser), 1055 (Ser, Gln), 745 (Gln, Phe), 228 (Phe, Gly) and 280 s (Gly, Ala).

At pH_L = 9.4 (amediol buffer), the order of appearance reported was Asp (R_E = 2.18, $\bar{m}$ = 34.7), [Glu (2.30, 33.0), Cys (2.28, 33.3)], Asn (3.10, 24.5), Ser (3.25,

* The R_E values could be measured repeatedly within an error of *ca.* ±0.05 R_E units for the completely separable sample combinations when the internal standard was selected properly. However, we found that the R_E value of some samples, *e.g.*, Tyr, varied over a greater range according to the selected combination of the samples, in spite of no mixed zone formation (usually this means that an isotachophoretic steady state is being achieved). The reason for this small but significant fluctuation is not yet known.

** In isotachophoresis the order of appearance of samples usually agrees with the decreasing effective mobilities. When this is not valid in relation to the pH of a sample zone and the preceding zone, it is called an enforced isotachophoretic system (see also ref. 1).

23.3), Thr (3.36, 22.6), Gln (3.62, 20.9), Tyr (3.71, 20.4), Met (3.75, 20.2), His (3.85, 19.7), Phe (3.91, 19.4), Gly (3.84, 19.8), [Trp (4.78, 15.9), Val (4.64, 16.4)], Ala (4.56, 16.6), [Ile (5.03, 15.1), Leu (4.97, 15.3)] and β-Ala (5.97, 12.7). The samples in square brackets could not be separated when the injected amounts were 2.5 nmol of each. When the sample amounts were 10 nmol, the pairs [Glu, Cys], [Tyr, Met], [His, Phe], [Trp, Val], [Val, Ala] and [Leu, Ile] could not be separated. The differences between the simulated effective mobilities are $0.3 \cdot 10^{-5}$, $0.2 \cdot 10^{-5}$, $0.3 \cdot 10^{-5}$, $0.5 \cdot 10^{-5}$, $0.2 \cdot 10^{-5}$ and $0.2 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ respectively. A discrepancy between the reported order of appearance and the order of R_E values was found for several samples. Similarly to the preceding case (pH_L = 8.6), some of these could be attibuted to enforced phenomena.

Comparing the observed separations of the amino acids in the electrolyte systems of pH_L = 8.6, 9.0 and 9.4[8] and the differences in the simulated mobilities, it was found that when the difference in the simulated effective mobilities of the samples were less than *ca.* $1 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ they could not be separated. The sample amounts were 10 nmol or less and the separating tube used was *ca.* 80 cm × 0.5 mm I.D.[8].

At pH_L = 9.7, several exceptions to the above mentioned rule were found for the pairs Thr, Tyr, (mobility difference = $1.2 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$), Gly, His (1.5), Gly, Phe (2.1) and Ala, Trp (1.5). All of these pairs have not been separated. By simulation of the separation process, when 5 m*M* hydrochloric acid solution buffered by amediol (pH_L = 9.7) was the leading electrolyte and the pH of the injected 1:1 mixture was 9, the t_{res} (s) were estimated as 2518 (Thr, Tyr), 3213 (Gly, His), 1916 (Gly, Phe) and 1618 (Ala, Trp).

When the length of the separation tube, l, is less than 80 cm, the threshold value of the difference in effective mobility can be simply estimated as $(80/l) \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$.

As previously concluded experimentally[1], eight to ten amino acids can be separated simultaneously in a single experiment, which is in good agreement with the estimation from the simulated mobility differences. It should be noted that, in some cases, samples having the same effective mobility at the steady state could be separated, and samples with different effective mobilities at the steady state could not be separated[14,15]. In the separation of amino acids, a similar situation can be found in Table IX. However, the separation of amino acids for which the effective mobilities and R_E values are almost the same is not practical. Even when separated, the dynamic range of the separable amount may be small and the separation process may be time-consuming.

So long as only the amino acids are treated, the separability of isotachophoresis using the pH effect on the effective mobility is not competitive with ion-exchange chromatography. To improve this situation, use of Schiff base formation with propanal may be effective for several amino acids[1]. For the separation of mixtures of amino acids and other anionic samples, however, isotachophoresis can be a powerful technique. Especially by utilizing the SITPS program[5], the separability can be assessed and the optimum separation conditions can be estimated conveniently. An example will be found in the succeeding paper on several oligo-peptides[16].

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ANALYSIS OF ORGANOPHOSPHORUS INSECTICIDES IN BIOLOGICAL SAMPLES BY SELECTIVE ION MONITORING GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Gas chromatography with chemical ionization or electron impact mass spectrometry and selected ion monitoring provided a simple and sensitive method for measuring organophosphorus insecticides. Chemical ionization produced higher-mass ions which might increase the selectivity and sensitivity of the assay. The recovery of organophosphates from saline and urine was greater than 75%. The recovery of these compounds from plasma was less than the saline because of the binding of insecticides to plasma protein. Insecticides with lower LD_{50} values showed lower recovery from plasma than organophosphates with higher LD_{50} values.

INTRODUCTION

Organophosphorus insecticides are widely used to control household, agricultural and forestry pests. The application of insecticides for controlling insect pests has recently shown a tendency towards combination of two or more insecticides for diverse reasons, such as for increasing their effectiveness, for controlling more than one pest at a time, and for increasing the storage life of insecticides. The insecticidal property and the mammalian toxicity of organophosphorus insecticides are believed to be because of their inhibition of acetylcholinesterase[1]. Other esterases present in blood and tissue of mammals are also inhibited by the organophosphates[2,3]. The reaction of organophosphates with esterases (other than acetylcholinesterase) may serve to reduce the amount of free organophosphates available for inactivation of acetylcholinesterase[4]. Along with esterases, mammalian tissue also contains certain phosphorylphosphatase activity which hydrolyses and inactivates organophosphorus compounds[5–7]. The amount of free organophosphates in tissues is further reduced by these enzymes. As a result, the amount of organophosphates excreted in the urine will be considerably less than the amount of exposure. These factors may also affect the amount of active residue if organisms are exposed to multiple organophosphorus insecticides. Therefore, a simple and sensitive method for the quantification of or-

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ganophosphorus insecticides is required to monitor animals exposed to several organophosphorus insecticides. Several gas chromatographic (GC) methods have been developed for the analysis of insecticides[8–12]. The GC methods are time consuming, since they always require clean-up of crude extract by either silica gel or charcoal columns. The purpose of this investigation was to develop a highly sensitive, simple and rapid assay for determining the levels of organophosphorus insecticides in aqueous and biological samples by using a chemical ionization (CI) and electron impact (EI) gas chromatography–mass spectrometry (GC–MS). Unlike GC methods, this method does not require the cleanup of crude extract.

EXPERIMENTAL

Materials

The GC–MS system used was a Hewlett-Packard Model 5987 with electron impact (EI) and chemical ionization (CI), and an integral gas chromatograph. The column used in the analysis of organophosphorus insecticides was a DB-5 fused-silica column (25 m) purchased from PolyScience. Other chemicals were purchased from Sigma.

Extraction procedure

Plasma. Plasma samples containing various amounts of organophosphate mixture (10–200 ng/ml) were mixed with ethyl acetate (5.0 ml/ml plasma) and rotoracked (50 rpm) for 15 min. The samples were centrifuged at 1500 *g* for 5 min at 4°C. The organic layer was separated into another tube. The aqueous layer was extracted twice with ethyl acetate and the organic layer was pooled with the previous ethyl acetate extract. The organic layer was dried at 50°C under nitrogen and the dried residue was redissolved in 100 μl of ethyl acetate. A 1.0-μl volume of extract was injected directly into the GC–MS system. The recovery from plasma samples was determined by comparing the amount of organophosphates added and the amount recovered. The extraction efficiency of insecticides was determined by a double extraction method described by Singh *et al.*[4]. Plasma samples (containing 100 ng/ml of organophosphate mixture) were extracted with ethyl acetate as described above. The aqueous and organic layers were separated. The organic layer was dried and subjected to GC–MS analysis. The aqueous layer was extracted again with ethyl acetate. The amount of organophosphates present in the second ethyl acetate extract was determined. It was consistently found that the second extraction contained an additional 20% of the free extractable organophosphates. These observations indicate that *ca.* 75–80% of the free extractable organophosphates were extracted by the first ethyl acetate extraction.

Urine. Urine samples were mixed with various amounts of organophosphate mixture (10–40 ng/ml). The pH of each sample was adjusted to 7.4 with phosphate buffer (1.0 *M*). Urine samples were centrifuged at 1000 *g* for 30 min. Clear samples were transferred into another tube, mixed with ethyl acetate (10 ml/ml urine), and rotoracked for 15 min. Samples were centrifuged for 5 min, the organic layer was transfered into another test tube, and dried at 50°C under nitrogen. The dried residue was redissolved in 100 μl of ethyl acetate and 1.0 μl of extract was injected directly into the GC–MS system. Recovery was determined by comparing the amount of organophosphates added and the amount recovered after extraction. Extraction ef-

ficiency was determined by the double extraction method described for plasma.

Saline. The procedure for the extraction of organophosphorus insecticides from saline or other aqueous solutions was similar to that for urine.

GC–MS conditions

The following GC conditions were used: inlet temperature, 200°C; initial oven temperature, 70°C; oven temperature program, isothermal at 70°C for 1.0 min, then increasing at 10°C/min to 280°C; run time, 30 min. For electron impact ionization the mass spectrometer's source pressure was $4.5 \cdot 10^{-6}$ torr, the source temperature was 200°C, and the electron energy was 70 eV. For chemical ionization using methane as the reagent gas, the source pressure was $2.0 \cdot 10^{-4}$ torr, the source temperature was 150°C, and the electron energy was 200 eV. Injections into the GC inlet were performed using Hamilton microliter syringes. The concentrations employed in generating the mass spectrum of each organophosphate were from 10 to 40 ng/μl.

Quantitative analysis

To establish a standard curve, known amounts of organophosphorus insecticides, ranging from 10 to 50 nmol/ml, were added to 1 ml of ethyl acetate, and 1.0 μl of the various concentrations were injected into the GC–MS system. The integrated areas of the peak were directly proportional to the amount of organophosphates injected upto a concentration of 250 nmol/ml. A standard curve was drawn by plotting to concentration of standards at the x axis, and the integrated peak area at the y axis. The concentration of organophosphates in various samples was determined by using a linear regression program which compared the integrated area of the sample with that of the standard curve.

RESULTS AND DISCUSSION

Thirteen insecticides, ranging in LD_{50} 2–1300 mg/kg, were selected for this study. The three groups represented in these insecticides are: (i) phosphorodithioates (Phorate, Cygon, Guthion, Di-syston, Malathion, Ethion, and Zolone); (ii) phosphorothionates (Diazinon, Ronnel and Co-ral); and (iii) phosphates (DVVP, Phosdrin, and Naled) (Fig. 1).

EI ionization of organophosphorus insecticides

The ions formed from these insecticides by EI ionization are shown in Fig.

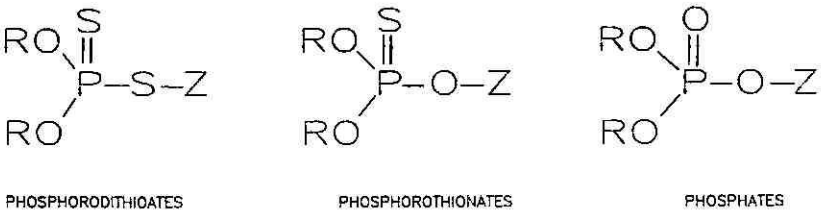
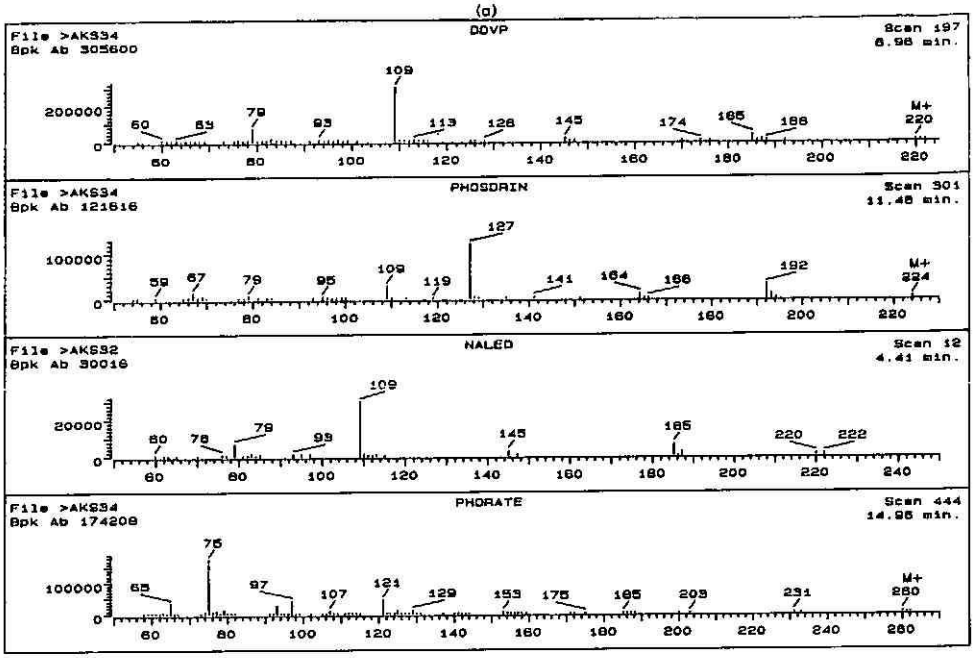


Fig. 1. Basic structures of the three groups of organophosphorus insecticides used in this study.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6.98 min.
PHOSDRIN
File >AKS34
Bpk Ab 121816
Scan 301
11.48 min.
NALED
File >AKS32
Bpk Ab 30016
Scan 12
4.41 min.
PHORATE
File >AKS34
Bpk Ab 174208
Scan 444
14.96 min.

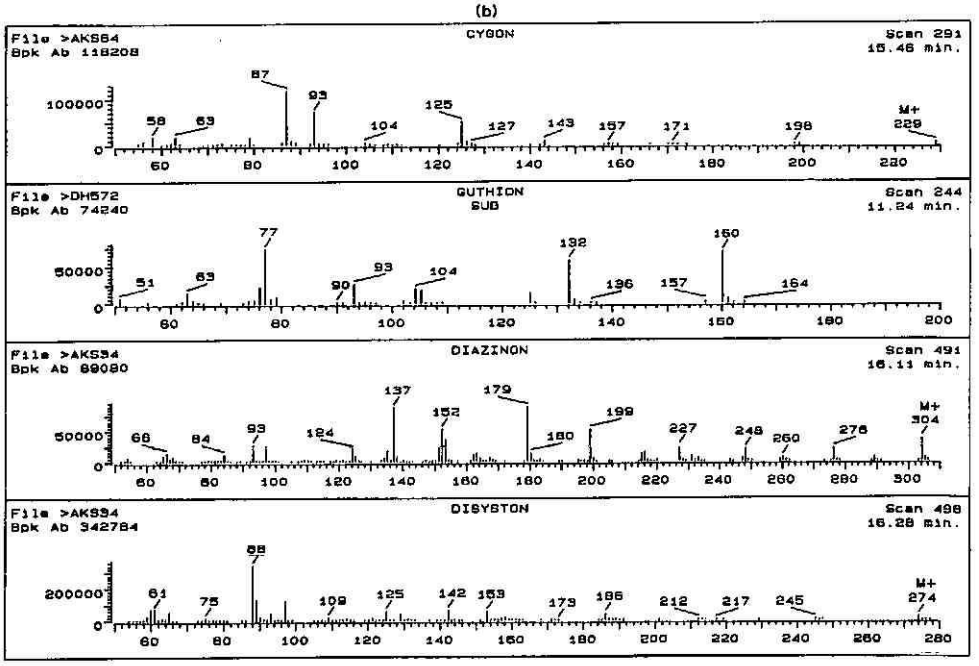

(b)
CYGON
File >AKS64
Bpk Ab 118208
Scan 291
15.46 min.
QUTHION
SUB
File >DH572
Bpk Ab 74240
Scan 244
11.24 min.
DIAZINON
File >AKS34
Bpk Ab 89080
Scan 491
16.11 min.
DISYSTON
File >AKS34
Bpk Ab 342784
Scan 498
16.28 min.

Fig. 2.

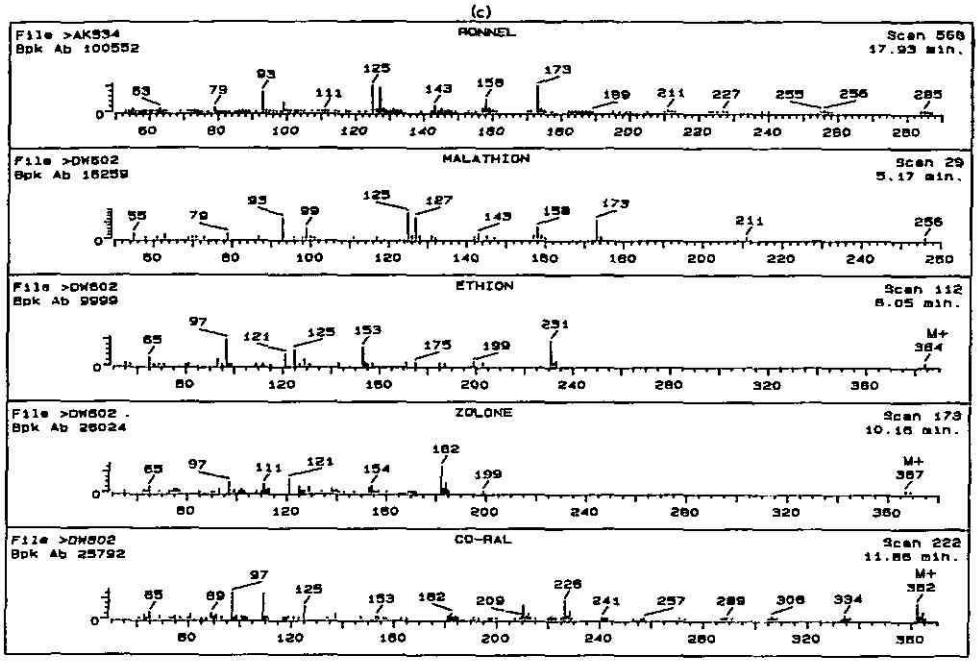


Fig. 2. *EI mass fragmentation patterns of* (a) DVVP, Phosdrin, Naled and Phorate; (b) Cygon, Guthion, Diazinon and Di-syston; (c) Ronell, Malathion, Ethion, Zolone and Co-ral.

2a–c. These ions are produced by many processes, such as rearrangement, alkyl and hydrogen migration, and alpha- and beta-cleavage[13].

The ion *m/z* 77 for Guthion represents a phenyl ion formed through the fragmentation of the alkyl tail and subsequent hydrogen migration. The ion of *m/z* 87 for Cygon is thought to be N-methylthioacetonitrile $[(SCH_2CNCH_3)^+]$ formed through both alpha-cleavage and subsequent dehydration. In Di-syston, rearrangement of the alkyl tail yields the tetrahydrofuran ion of *m/z* 88 $[(C_4H_8S)^+]$. The ion of *m/z* 97 is characteristic of both phosphorodithioates and phosphorothionates formed through the Quayle rearrangement $\{[(HO)_2PS]^+\}$[14]. An ion common to the phosphates is *m/z* 109 $\{[(CH_3O)_2PO]^+\}$. Ion *m/z* 125 is the thio analogue of *m/z* 109$\{[(CH_3O)_2PS]^+\}$ and is found in the spectra of Cygon, Ronnel, Malathion, and Co-ral. Double hydrogen migration is postulated for the *m/z* 127 ion found in Phosdrin $\{[(CH_3O)_2(OH)_2]^+\}$ while the *m/z* 137 ion of Diazinon and Co-ral is formed through simple cleavage $\{[(C_2H_5O)_2PO]^+\}$[13]. Characteristic of both phosphorodithioates and phosphorothionates with ethoxy groups is ion *m/z* 153 $\{[(C_2H_5O)_2PS]^+\}$ formed through simple cleavage and found in the spectra of Phorate, Diazinon, Disyston, Ethion, Zolone, and Co-ral[15,16]. The base peak in the spectra of Guthion is *m/z* 160 which represents the alkyl group $[C_8H_6N_3O]^+$ as does the base peak of Malathion $\{[C_8H_{13}O_4]^+$, *m/z* 173$\}$, both the result of beta-cleavage. Ion *m/z* 179 for Diazinon presents a more complex situation where an ethyl migration to the alkyl group following beta-cleavage is proposed $\{[C_{10}H_{15}ON_2]^+\}$. Naled and DDVP both exhibit ion *m/z* 185 from the same partial fragmentation of the alkyl tail

$\{[(CH_3O)_2POOCHCCl]^+\}$. Phorate and Ethion are similar in sharing a *m/z* 231 ion of identical structure through the same process $\{[(C_2H_5O)_2PSSCH_2S]^+\}$. Phorate's molecular ion *m/z* 260, Ronnel's M-Cl ion *m/z* 285, and Co-ral's molecular ion *m/z* 362 were all present in sufficient abundance.

Chemical ionization of organophosphorus insecticides

Fig. 3a–c show the CI mass spectra of 13 organophosphorus insecticides. The chemical ionization process is a softer ionization which produces higher-mass ions than EI ionization[17–20]. Holmstead and Casida[21] obtained significantly greater abundances of [M + 1] and [M − 1] ions in CI as compared to EI ionization. We observed that the [M + 1] ion was the most intense ion in the spectra of DVVP, Phorate, Cygon, Diazinon, and Co-ral. The abundances of [M + 1] ions were 50–75% in the spectra of Phosdrin, Di syston, Malathion and Ethion. The spectra of these insecticides also indicated that the ion which was present in highest abundance was M − 31 (loss of $-OCH_3$ group) for Phosdrin, M − 61 for Di-syston, and M − 185 [loss of $-SP(S)(OC_2H_5)_2$] for Ethion. The abundances of the [M + 1] ion for Naled, Guthion, and Zolone was less than 10%. The *m/z* 160 ion was present in highest abundance in the spectra of Guthion, this represents the alkyl tail of the molecule. Ions at *m/z* 221 and 223 for Naled were formed by the loss of Br, and the ion at *m/z* 184 for Zolone was formed by the loss of $^+CH_3$–S–C_2H_5 from the molecule. Diazinon and Ronell produced the [M + 29] ion which represent the addition of C_2H_5 ion to the insecticide molecule.

Selection of ions for the analysis of organophosphorus insecticides

The EI and CI ions which are present in high abundance for the thirteen insecticides are listed in Table I. In the case of EI, the ions selected for monitoring were characteristic of the particular family of the insecticide, unique to the insecticide,

TABLE I

IONS WHICH ARE PRESENT IN HIGH ABUNDANCE FOR EACH INSECTICIDE

M = molecular ions.

	m/z (relative abundance)	
	EI	*CH_4CI*
DVVP	109(100), 185(11)	221(100) M + 1, 223(64)
Phosdrin	127(100), 109(24)	192(100), 225(75) M + 1
Naled	109(100), 185(18)	221(100), 223(65)
Phorate	75(100), 97(28), 153(5)	199(28), 261(100) M + 1
Cygon	87(100), 125(55)	199(54), 230(100) M + 1
Guthion	77(100), 160(94)	160(100), 318(4) M + 1
Diazinon	179(100), 137(85), 153(35)	305(100) M + 1, 333(16) M + 29
Di-syston	88(100), 97(36), 153(20)	213(100), 275(51 M + 1
Ronnel	285(5), 125(97), 97(18)	323(100) M + 1, 349(21) M + 29
Malathion	173(70), 125(100)	129(100), 331(50) M + 1
Ethion	97(100), 231(93), 153(69)	199(100), 385(75) M + 1
Zolone	97(39), 153(15)	184(100)
Co-ral	97(100), 362(60), 125(46)	363(100) M + 1

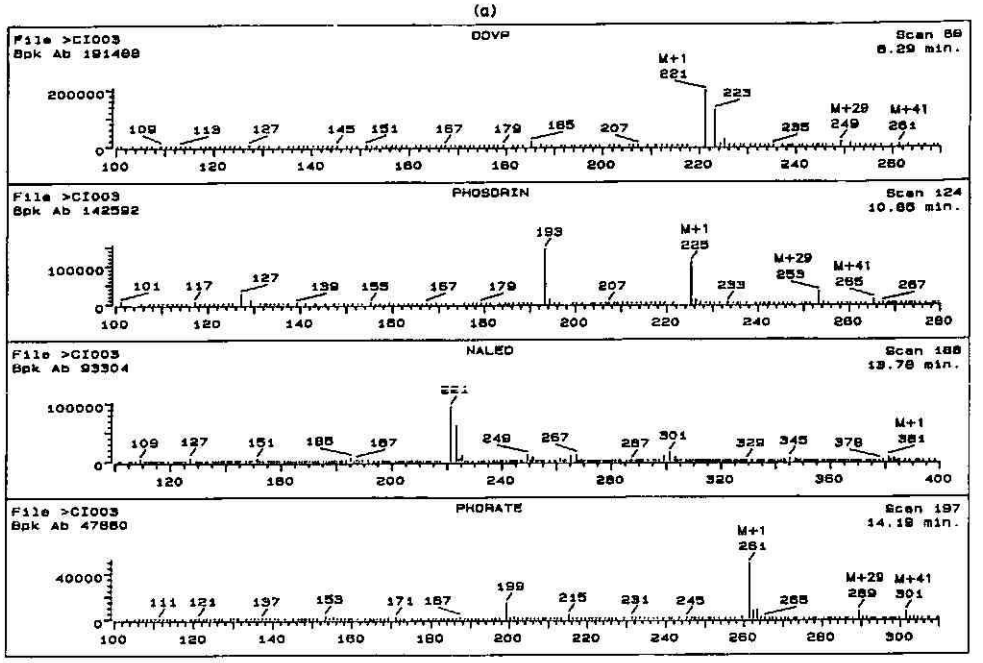
TABLE II

PROGRAMMING OF THE GC–MS SYSTEM TO MONITOR SELECTED IONS FOR THE ANALYSIS OF ORGANOPHOSPHORUS INSECTICIDES

SIM Program	*Group No.*	*Group start time (min)*	*Group run time (min)*	*No. of ions in each group*		*Ions monitored (m/z)*	
				EI	*CI*	*EI*	*CI*
1	1	7	20	17	17	109, 185, 127, 185, 75, 77, 87, 88, 97, 125, 137, 160,153, 173, 231, 153, 362	221, 223, 192, 225, 160, 199, 230, 261, 213, 275, 305, 323, 349, 129, 331, 184, 363
2	1	7	3	2	2	109, 185	221, 223
	2	10	3	2	2	127, 109	191, 225
	3	13	2	2	2	109, 185	221, 223
	4	15	2	9	9	75, 77, 87, 88, 97, 153, 137, 160, 125	160, 199, 230, 261, 305, 318, 333, 213, 275
	5	17	2	4	4	125, 97, 173, 125	323, 349, 129, 331
	6	19	3	3	3	97, 231, 153	199, 331
	7	22	5	5	2	97, 153, 125, 362	184, 363

or present in the highest abundances. The CI ions were selected only on the basis of their abundances. Other low-abundance ions could be included in ion monitoring. However, in our experience, increasing the number of low-abundance ions monitored decreased the sensitivity of the assay. Two different SIM programs were used to monitor the organophosphorus insecticides (Table II). In program 1, all 17 ions were monitored in one group. Whereas in program 2, ions were divided into seven groups (Table II). Each group had specified group-start and group-run times, and ions monitored (Table II). Although program 1 was simple to use, results of this study indicated that program 2 was several times more sensitive than program 1 since the number of ions monitored for each insecticide could be increased in the program 2. The urine, saline and plasma data reported in this study were obtained by using SIM program 1.

Our capillary column provided excellent separation of all 13 pesticides in saline, urine and plasma samples. Fig. 4 and 5 show the separation of 13 pesticides in CI and EI modes respectively. Fig. 6 shows the separation of organophosphorus insecticides added to plasma and analyzed in EI mode. Sass and Fisher[22] have reported that methane CI was at least 10 times more sensitive than EI. This study demonstrated that the CI method was more sensitive for Naled, Cygon, Guthion, Zolone and Diazinon; the EI method was relatively sensitive for Di-syston, Ronell, Malathion, Ethion and Co-ral; and the EI and CI methods were equally sensitive for DVVP, Phosdrin and Phorate. Since CI produced higher-mass ions than EI, and since significant abundances of M + 1, M − 1, M − 31, and M − 29 ions were produced by CI; it is proposed that the CI method might be more specific for these insecticides than the EI method.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DDVP
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M+1
221
223
M+29
249
M+41
261
File >CI003
Bpk Ab 142592
PHOSDRIN
Scan 124
10.66 min.
193
M+1
225
M+29
253
M+41
265
267
File >CI003
Bpk Ab 93304
NALED
Scan 166
13.78 min.
M+1
381
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Bpk Ab 47880
PHORATE
Scan 197
14.19 min.
M+1
261
M+29
289
M+41
301

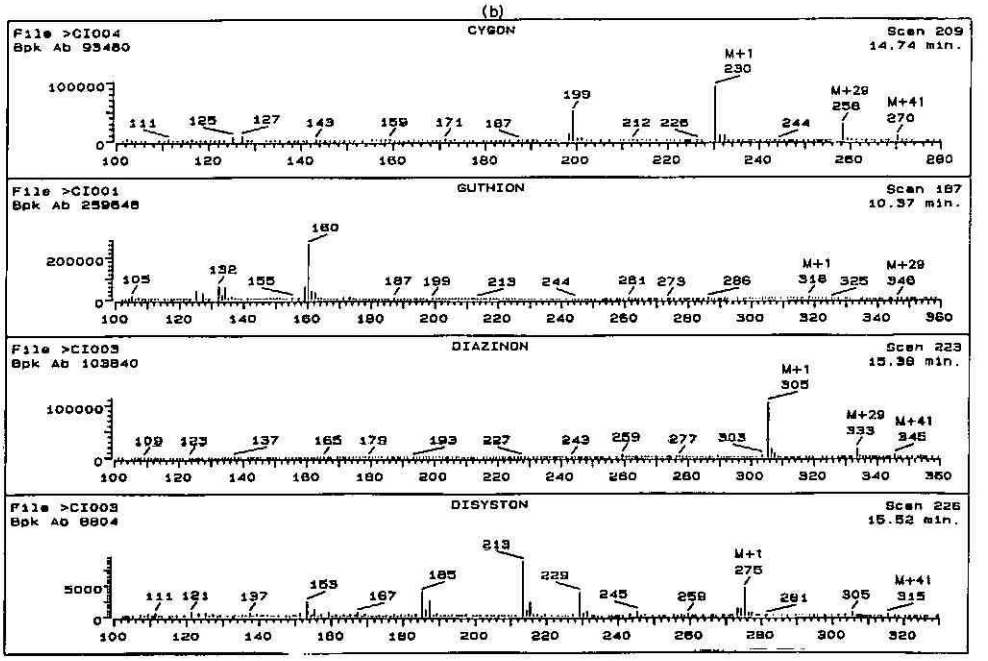
(b)
File >CI004
Bpk Ab 93480
CYGON
Scan 209
14.74 min.
M+1
230
M+29
258
M+41
270
File >CI001
Bpk Ab 259648
GUTHION
Scan 187
10.37 min.
160
M+1
318
M+29
346
File >CI003
Bpk Ab 103840
DIAZINON
Scan 223
15.38 min.
M+1
305
M+29
333
M+41
345
File >CI003
Bpk Ab 8804
DISYSTON
Scan 226
15.52 min.
213
M+1
275
M+41
315

Fig. 3.

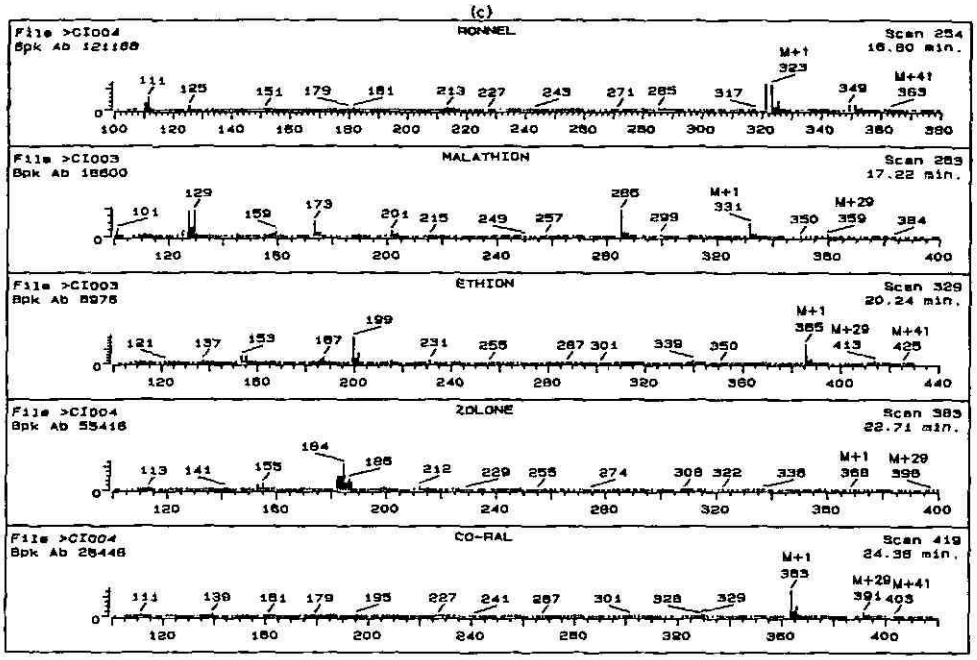

Fig. 3. CI spectra of (a) DVVP, Phosdrin, Naled and Phorate; (b) Cygon, Guthion, Diazinon and Disyston; (c) Ronell, Malathion, Ethion, Zolone and Co-ral.

Extraction efficiency and recovery of insecticides from saline, urine and plasma

The recovery of organophosphorus insecticides from urine, saline and plasma is shown in Fig. 7a and b. A linear relationship was observed between the amount of insecticides added and the amount recovered from both urine and saline. The extraction efficiency from urine or saline was 75–80% for each insecticide.

Although a similar procedure was utilized for the extraction of insecticides added to plasma or saline, the overall recovery was less in plasma than saline (Figs. 7a and b, and 8). Since a proper internal standard is not known for organophosphorus insecticides, the extraction efficiency of free, extractable insecticides from plasma was determined by using a double extraction method described by Singh *et al.*[4]. It was observed that approximately 80% of the free extractable insecticides present in the plasma was extracted by using the extraction procedure described above. Therefore, low recovery of insecticides from plasma might be the result of binding of insecticides to plasma esterases and other proteins. Sterri *et al.*[2], Fonnum and Sterri[3] and Singh *et al.*[4] have reported that mammalian blood contains several enzymes which bind with free organophosphorus compounds and reduce the level. Several other extraction procedures, such as deproteinization of plasma with perchloric acid before extraction, extraction of plasma samples in the presence of salt, and extraction of plasma samples by acetonitrile, were tried but did not improve the recovery of organophosphorus insecticides from plasma (data not shown). Perchloric acid which is known to hydrolyze organophosphorus compounds[4], caused a time-dependent decay in the concentration of organophosphorus insecticides.

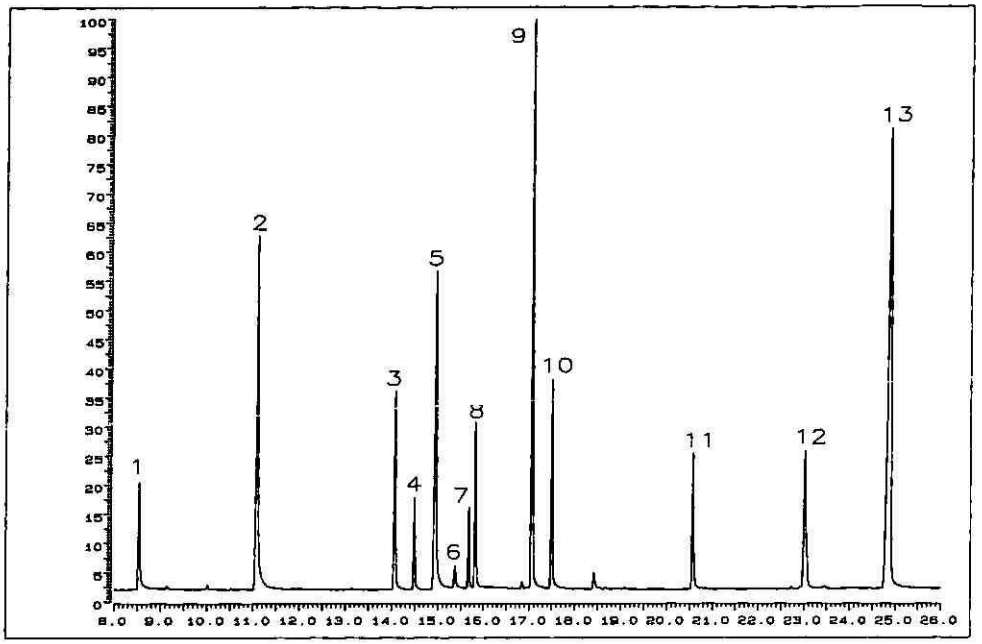

Fig. 4. Chromatographic separation of a mixture containing 0.1 ng/μl of 13 organophosphorus insecticides by EI-SIM, program 1. Ions monitored are listed in Table II. Peaks: 1 = DVVP; 2 = Phosdrin; 3 = Naled; 4 = Phorate; 5 = Cygon; 6 = Guthion; 7 = Diazinon; 8 = Di-syston; 9 = Ronnel; 10 = Malathion; 11 = Ethion; 12 = Zolone; 13 = Co-ral.

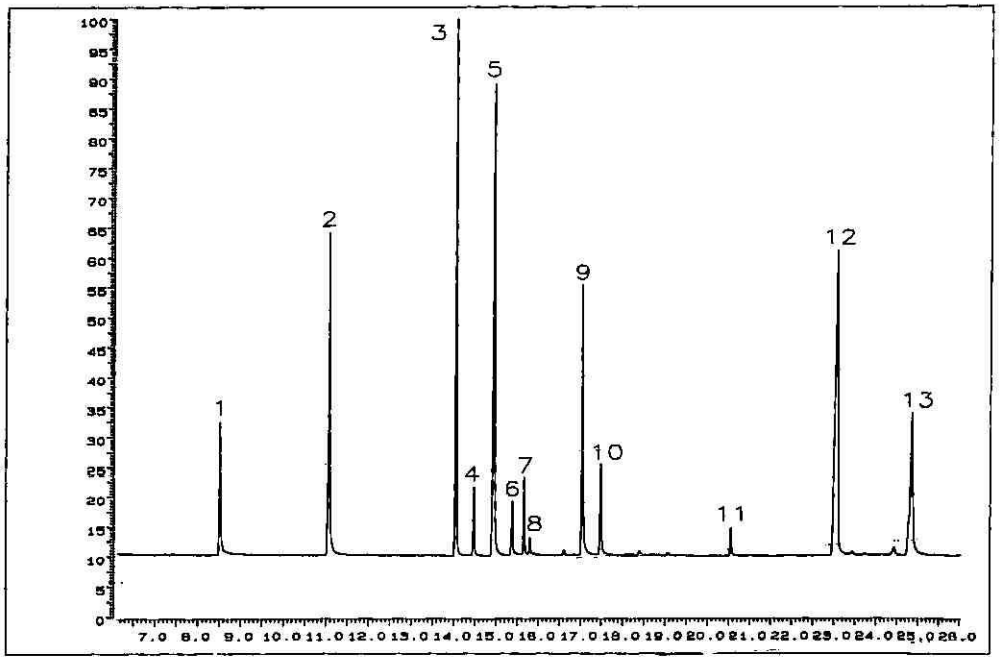

Fig. 5. Chromatographic separation of a mixture containing 0.1 ng/μl of 13 organophosphorus insecticides by CI-SIM, program 1. Ions monitored are listed in Table II. Peaks as in Fig. 4.

The results of this study indicate an interesting relationship between the LD_{50} value of an insecticide and its recovery from plasma. The recovery of Phorate, Phosedrin, Di-syston and Guthion (LD_{50} 2.5, 6.7 and 11 mg/kg respectively) was less than 10%; the recovery of Co-ral, Ethion, DVVP, and Zolone (LD_{50} 40, 65, 80 and 100 mg/kg respectively) was 15–30%; and the recovery of Cygon, Diazinon, Malathion and Ronel (LD_{50} 250, 500, 1000 and 1225 mg/kg respectively) was > 40% when 50 ng of each insecticide was added to 1 ml of plasma (Fig. 8). Increasing the dose resulted in a significant increase in extractable insecticides in the plasma. At a dose of 200 ng insecticides/ml plasma, 50–70% of the insecticides were recovered. However, the insecticides which had lower LD_{50} values showed lower recovery (Fig. 8). These observations suggest that at a lower dose most of the organophosphates added to plasma were present in an enzyme- (or protein-) bound form. Compounds which had a lower LD_{50} value exhibited a relatively higher reactivity to plasma proteins than the compounds which had relatively high LD_{50} values. The results find support with our previous observation[4] that the recovery of Sarin (an organophosphorous compound with LD_{50} 0.25 mg/kg) from plasma was less than the recovery observed in this study for Phorate (LD_{50} 2.6 mg/kg). As the dose was increased, the amount of free extractable insecticides increased in the plasma, which might be the result of the saturation of organophosphate-binding proteins.

From the results of this study it is concluded that (i) both EI and CI-selected ion monitoring provided a simple and sensitive method for measuring organophosphorus insecticides, (ii) CI produced higher-mass ions which might increase the se-

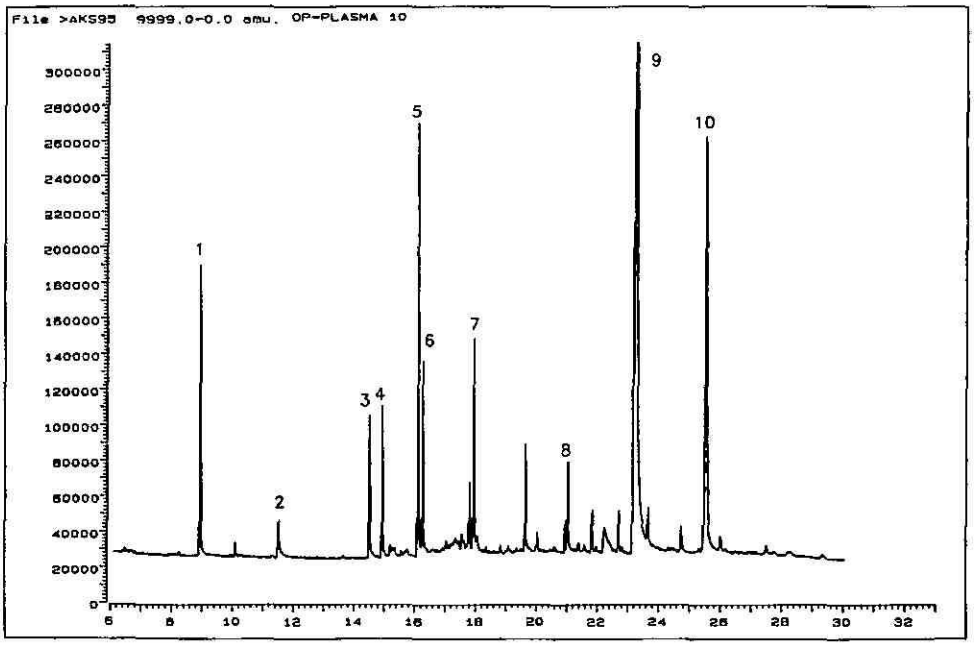

Fig. 6. Chromatographic separation of a mixture of organophosphorus insecticides added to plasma. The insecticides were extracted by a method described earlier. One μl of ethyl acetate extract was injected into the GC–MS system. Peaks: 1 = DDVP; 2 = Phosdrin; 3 = Naled; 4 = Phorate; 5 = Diazinon; 6 = Di-syston; 7 = Malathion; 8 = Ethion; 9 = Zolone; 10 = Co-ral.

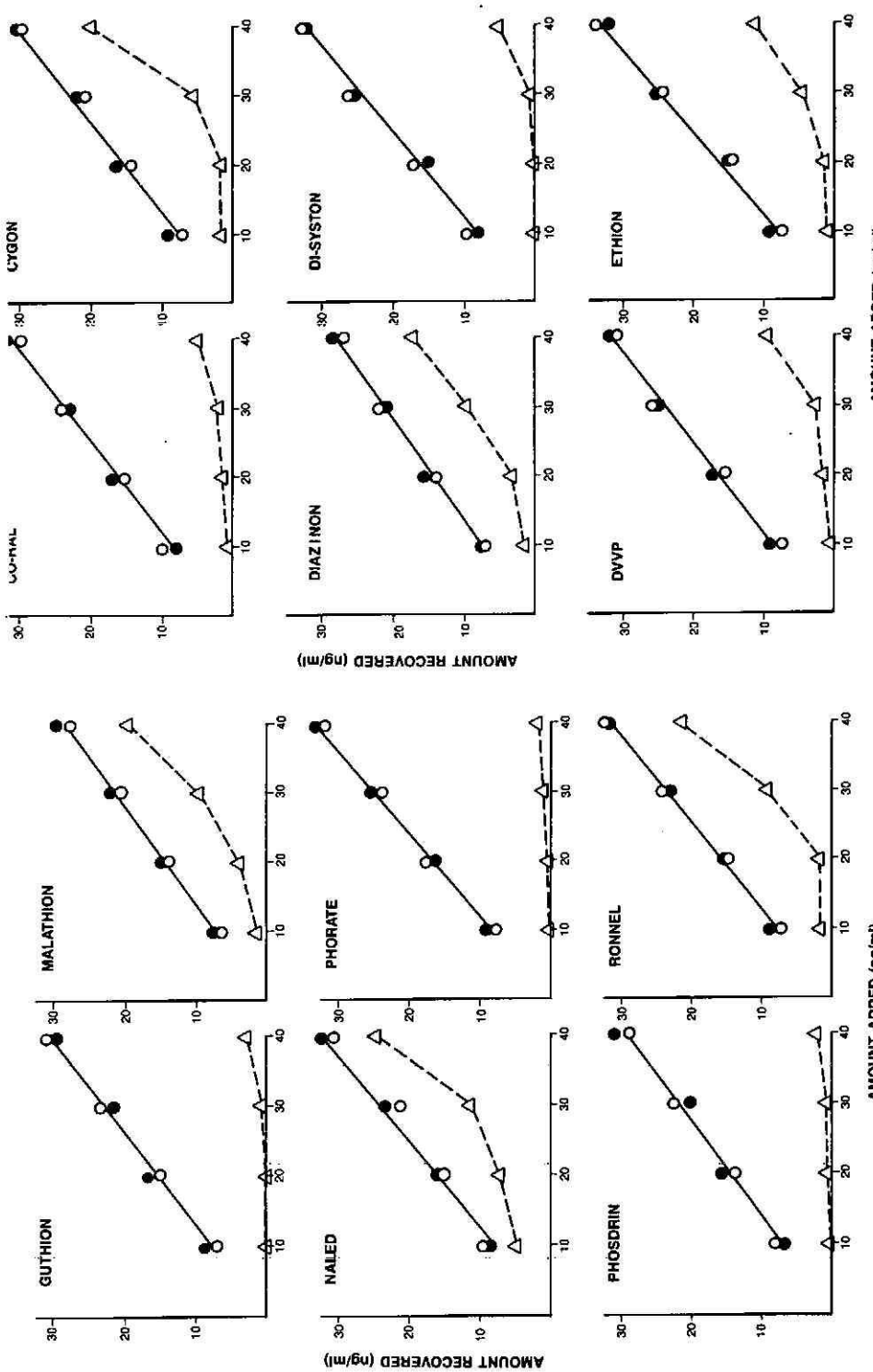

Fig. 7. Recovery of free organophosphorus insecticides from saline, urine and plasma at 10, 20, 30 and 40 ng/ml dose. Values are mean ± S.D. ○ = saline, ● = urine and △ = plasma.

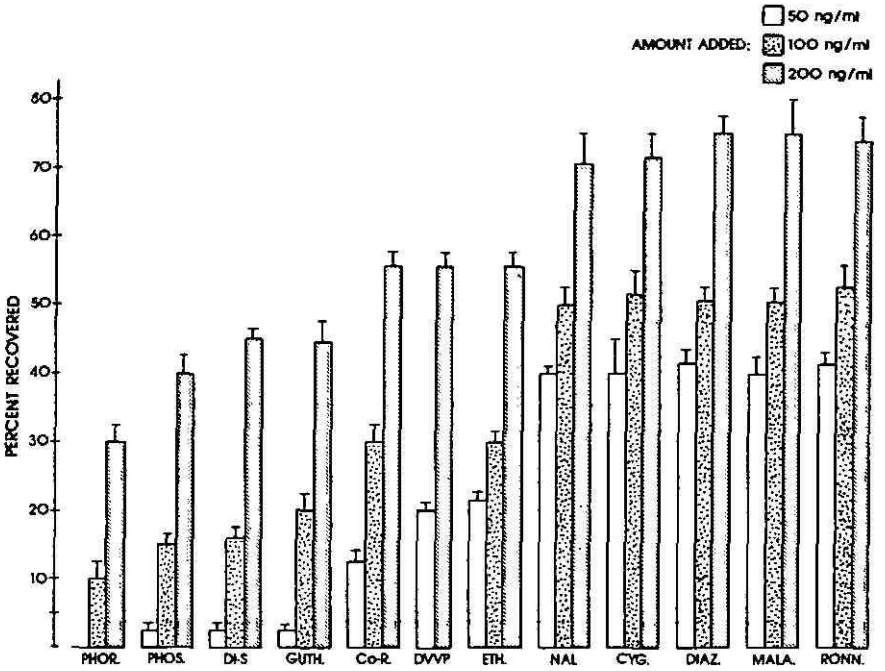

Fig. 8. Recovery of free organophosphorus insecticides from plasma when 50, 100 and 200 ng were added into 1.0 ml of plasma. Values are mean ± S.D.

lectivity and sensitivity (for certain insecticides) of the assay, (iii) the recovery of organophosphates from plasma was less than saline or urine because of the binding to plasma enzymes and proteins, and (iv) the organophosphates with lower LD_{50} values exhibited lower recovery from plasma than the organophosphates with higher LD_{50} values.

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GAS–LIQUID CHROMATOGRAPHY OF 3-CHLOROPROPANEDIOL

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SUMMARY

To improve the gas chromatographic properties of 3-chloropropanediol, a phenylboronic acid derivative was prepared. This method appears to be suitable for trace analysis of the title compound. Epichlorohydrin, 1,2-propanediol, and 1,2-dichloropropanol were among the structurally related compounds shown not to interfere. The structure of the derivative was confirmed by gas chromatography–Fourier transform infrared spectroscopy utilizing a matrix isolation interface and gas chromatography–mass spectrometry.

INTRODUCTION

3-Chloropropanediol (3CPD) is a post-testicular male anti-fertility agent[1,2], which has been shown to be mutagenic in bacterial assays[3]. Animal testing of its carcinogenicity has given equivocable results[4]. In addition, 3CPD is nephrotoxic[5]. 3CPD is a hydrolysis product of epichlorohydrin[6], a widely used commercial chemical[7]. 3CPD, as well as several of its fatty acid esters, has been reported to be present in food[8–12].

The gas chromatography of underivatized 3CPD has been reported in several papers, but the reproducibility and linearity of response were not reported[9]. A variety of gas chromatographic columns have been utilized for the determination of 3CPD and other short chain dihydroxy compounds. Recommended columns for ethylene glycol, 1,2-propanediol, and 1,3-propanediol include 0.8% Theed on 80–100 mesh Carbopack C[13], 3% Carbowax 20M on Chromosorb 101[14], Chromosorb 101, 100–120 mesh or Non-Pakd Superox[15].

Although good separation, linearity, and a precision of 4% were reported using 0.8% Theed[13], in our hands the use of this packing material for the chromatography of 3CPD yielded poor reproducibility and chromatography. Maximum temperature before deterioration of column packing (for 0.8% Theed) is 125°C. Because of this low temperature, tailing of 3CPD on this column was a major problem.

Unsatisfactory results for the chromatography of ethylene glycol were demonstrated[14] on uncoated Chromosorb 101, 102, and Chromosorb 102 coated with Carbowax 20M. Super-pak 20M coated with Carbowax 20M gave marginal results, while

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better results were obtained using 3% or 6% Carbowax 20M on Chromosorb 101. Peak tailing, column bleed, ghost peaks, and the poor reproducibility with extended usage of these columns makes these columns undesirable for analysis of diol compounds.

Because of the lack of a satisfactory packed column for 3CPD, derivatization to facilitate gas chromatography was explored. N-Butylboronate has been used for gas chromatographic separation of the enantiomers of 3CPD[16]. The well-characterized reaction between phenylboronic acid and dihydroxy compounds[17,18] was utilized here to prepare a derivative for gas chromatographic determination of 3-chloropropanediol.

EXPERIMENTAL

Reagents and standards

Standards of 3CPD, epichlorohydrin, 1,3-dichloropropanol, 3-bromopropanediol, 3-chloro-2-propanol, 1,3-propanediol, 1,2-propanediol, 3-chloro-1-propanol, and ethylene glycol were obtained from the Aldrich (Milwaukee, WI, U.S.A.) and used as received. Phenylboronic acid was obtained from the Sigma (St. Louis, MO, U.S.A.). HPLC grade 2,2-dimethoxypropane was obtained from Fisher Scientific. The derivatization reagent consisted of 40 mmol/l phenylboronic acid in 2,2-dimethoxypropane. 2,2-Dimethoxypropane was added as a scavenger for water[19].

Derivatization procedure

A volume of 50 μl of sample solution in acetonitrile was added to 100 μl of phenylboronic acid derivatizing reagent. The solution was vortex mixed and a 1-μl aliquot was injected into the gas chromatograph.

Instrumentation

Gas chromatography–matrix isolation-Fourier transform infrared spectroscopy (GC–MI-FT-IR) was performed with a Varian 3700 gas chromatograph operating in the split mode interfaced to a Sirius 100 FT-IR spectrophotometer (Mattson Instruments). A matrix isolation interface (Cryolect, Mattson Instruments) was employed. With this instrumentation the effluent from a capillary gas chromatograph is split between a flame ionization detector (20%) and the matrix isolation device (80%). The effluent from the gas chromatograph which goes to the matrix isolation instrument is trapped in a frozen argon matrix on a rotating gold plated drum. The drum is kept under vacuum at 12 K. At the end of the chromatographic run, the FT-IR spectrophotometer is used to obtain spectra of the argon embedded GC effluent. The spectra of the peaks corresponding to the flame ionization detector response can readily be obtained. The flame ionization detector response is sent to the computer which controls the FT-IR spectrophotometer and the matrix isolation interface. The computer can then identify the area of matrix isolated effluent corresponding to a particular time in the GC run as characterized by the flame ionization detector. IR spectra were generated by co-addition of 100 scans with 4 cm^{-1} resolution.

GC–electron impact mass spectroscopy was performed using a Finnigan Model 4500 with a source potential of 70 eV. The gas chromatograph was operated with an initial temperature of 100°C for 4 min, programmed to 200°C at 20°C/min

and held at 200°C for 4 min. A 10M methylsilicone wide-bore capillary column (Alltech; RSL 150, 0.53 mm I.D., film thickness, 1.2 μm) was used.

RESULTS AND DISCUSSION

Direct chromatography without derivatization is attractive because of its simplicity. However, with 0.8% Theed on Carbopack C, 5% Carbowax 20M on Chromosorb W HP, Super Q (Alltech), and Tenax-GC, peak broadening, difficulties with low sensitivity and ghost peaks were observed with the chromatography of 3CPD. These results suggest that substantial retention of these compounds may occur on the chromatographic column. To overcome the undesirable chromatographic properties, a derivatization procedure was utilized. The chromatography of the phenylboronic acid derivative of 3CPD on two different types of columns is shown in Figs. 1 and 2.

Under the described GC conditions (Fig. 1), concentrations of 3CPD-phen-

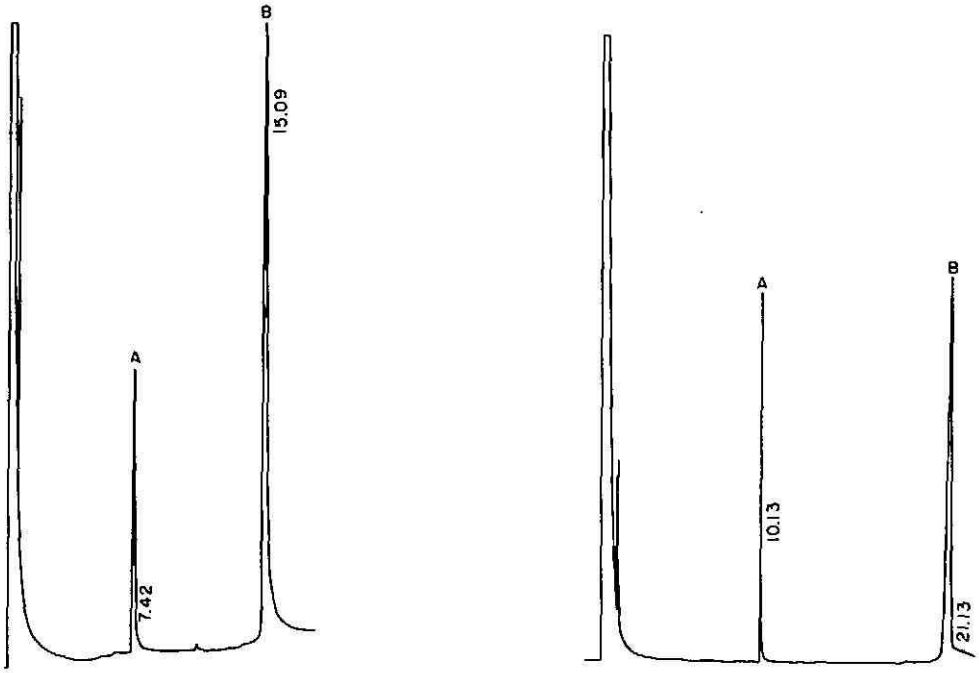

Fig. 1. Chromatograph of 3CPD using a 3% SP 2100 on 100–120 mesh Supelcoport column. Conditions: He = 40 ml/min, air = 300 ml/min, H_2 = 30 ml/min, FID sens. = 10^{-11} (ATT = 1), inj. temp. = 250°C, det. temp. = 300°C, chart speed = 0.5 cm/min; ATT (integrator attenuation) = 64, program run = 100°C (4 min), program of 15°C/min to 280°C. Indicated retention times are in min. A = 3CPD-phenylboronate (98 ng); B = phenylboronic acid.

Fig. 2. Chromatograph of 3CPD on a DB5-30M (0.25 μ) fused-silica capillary column. Condition: He = 98.5% + Argon = 1.5% carrier, flow = 1.0 ml/min, make-up = 29 ml/min, split ratio = 1:10, air = 300 ml/min, H_2 = 30 ml/min, FID sens. = 10^{-11} (ATT = 2), inj. temp. = 250°C, det. temp. = 300°C, program run = 100°C (4 min), program of 10°C/min to 280°C, chart speed = 0.5 cm/min. ATT = 16. Indicated retention times are in minutes. A = 3CPD-phenylboronate (2 ng); B = phenylboronic acid.

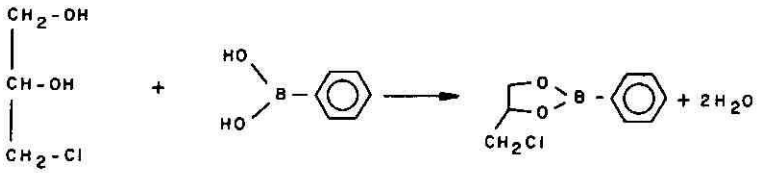

Fig. 3. 3-Chloropropanediol reaction with phenylboronic acid.

ylboronate ranging from 2.6 ng/μl to 133 ng/μl were analyzed for reproducibility and linearity. Concentrations of 17 (n = 2), 33 (n = 2), 66 (n = 3), and 133 (n = 4) ng/μl, using 1-μl injections, showed coefficients of variation (C.V.) of less than 2.5%. A C.V. of 11% was observed with injections at a concentration of 133 ng/μl over 2 days. Even with the large C.V. seen with injections made over a period of days, injections of 2.6–133 ng/μl over three days were linear (r=0.99). Linearity and reproducibility for the derivative of 3CPD were not tested on the DB-5 30M fused-silica capillary column, although a sizeable response was seen for the 2 ng/μl on-column concentration seen in Fig. 2.

The proposed structure of the 3CPD derivative is shown in Fig. 3. This type of derivatization has been utilized for determination of ethylene glycol[20], and is shown to be a general reaction for diols[19,17]. It is felt that the derivative is formed in the gas chromatographic injection port, although this has not been documented. Derivatization using the phenylboronic acid reagent gave distinct peaks for 3-bromopropanediol, 1,3-propanediol, 1,2-propanediol, and ethylene glycol which did not interfer with 3CPD. Compounds which were found to give no peaks with derivitization were 1,2-dichloropropanol, 1,3-dichloropropanol, 2-propanol, methanol, and epichlorohydrin. Although it is likely that reaction occurs with these compounds, either the conversion is too poor for these compounds to be detected, or they do not appear with the chromatographic conditions utilized. No interference occurred with the solvent blank (acetonitrile).

The IR spectra of the phenylboronic acid derivatives of 1,2-propanediol and 3-chloropropanediol are shown in Figs. 4 and 5 respectively. Both IR spectra dem-

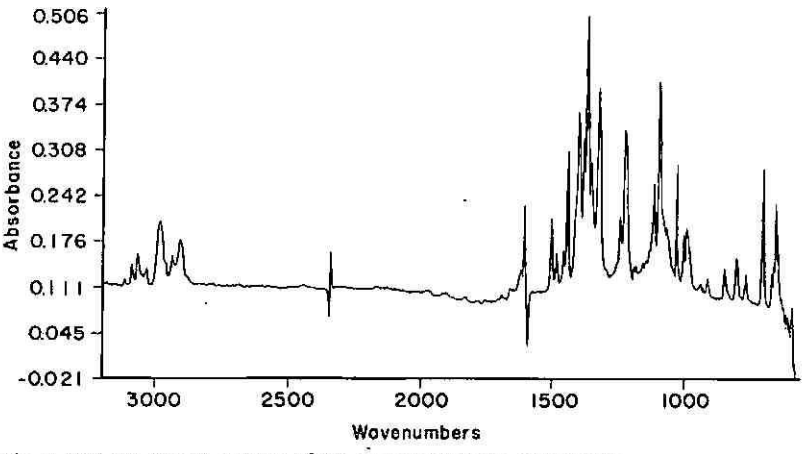

Fig. 4. GC–MI-FT-IR spectra of 1,2-propanediol phenylboronate.

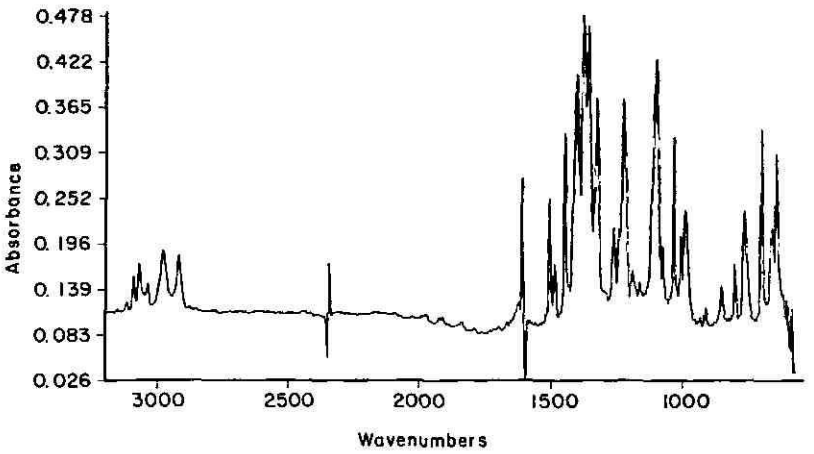

Fig. 5. GC–MI-FT-IR spectra of 3-chloropropanediol phenylboronate.

onstrate similar aromatic CH stretches above 3000 cm^{-1}. The methyl CH bending absorbances near 1370 cm^{-1} and 1440 cm^{-1} are nearly identical for the two compounds as are the methylene C–H stretch absorbances near 2940 cm^{-1}. The aromatic disubstituted ring bending occurs at 764 cm^{-1} for 1,2-propanediol derivative. For the 3CPD derivative this is obscured by the strong C–Cl absorbance at 763 cm^{-1}.

The mass spectra of the phenylboronic acid derivatives of 1,2-propanediol and 3CPD are shown in Figs. 6 and 7 respectively. In each mass spectrum we see a small

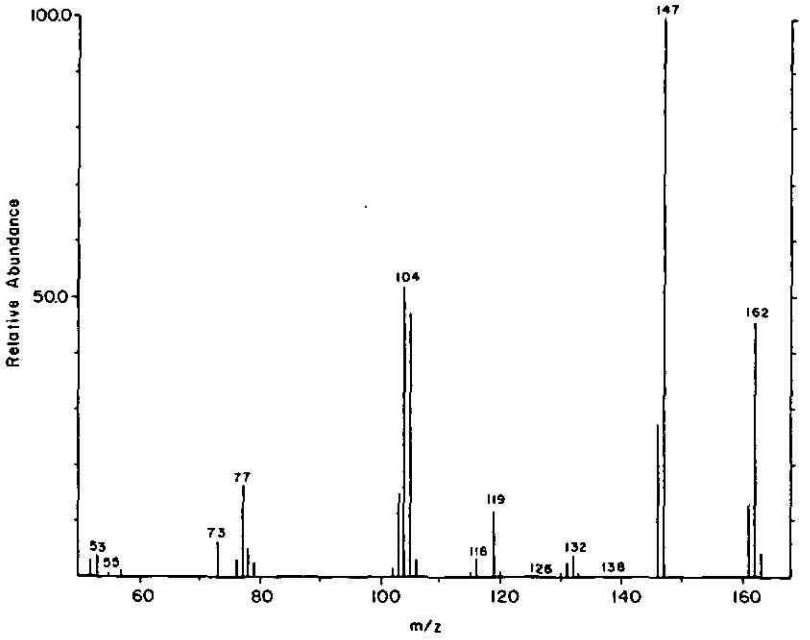

Fig. 6. Mass spectra of 1,2-propanediol phenylboronate.

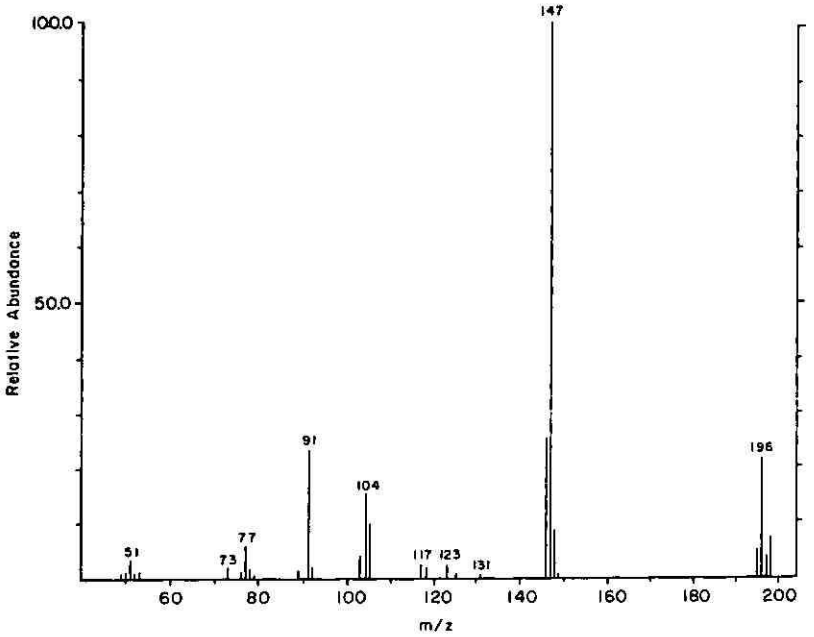

Fig. 7. Mass spectra of 3-chloropropanediol phenylboronate.

molecular ion. For 1,2-propanediol the base peak is the loss of CH_3 peak. Similarly the base peak for 3CPD is the peak corresponding to the loss of CH_2Cl. Both spectra demonstrate a $C_6H_5BO^+$ peak.

Because of the sensitivity and reproducibility, this procedure is suitable for use in applications where trace determination of 3CPD is necessary.

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DERIVATIZATION OF PRIMARY AMINES BY 2-NAPHTHALENESULFONYL CHLORIDE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF NEOMYCIN SULFATE

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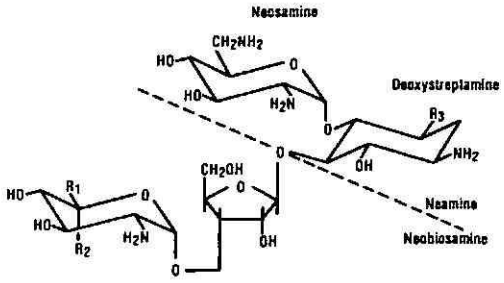
SUMMARY

A normal phase high-performance liquid chromatographic (HPLC) method has been developed for the assay of neomycin sulfate. The method involves pre-column derivatization with 2-naphthalenesulfonyl chloride (NSCl) followed by extraction in chloroform and chromatography using a normal phase silica column with detection at 254 nm. The standard curve for the HPLC assay of neomycin sulfate is linear with a correlation coefficient of 0.9996 over the range of 0.02 to 0.4 mg/ml. Neomycins B, and C, and neamine can be separated and quantified isocratically with relative standard deviations of 0.92% and 1.4% for neomycin (B + 1/2C) and neamine, respectively. Prednisolone is used as an internal standard to aid in quantification. Mass spectrometric data confirms that neomycin is derivatized at all the six primary amines on the neomycin molecule. Eight lots of neomycin sulfate were used to compare the HPLC [NSCl and 1-fluoro-2,4-dinitrobenzene (DNFB)], gas–liquid chromatographic and microbiological assay methods. The average results of the NSCl–HPLC method fell between those of the microbiological and DNFB–HPLC methods. Also, good correlation of the neomycin C contents in neomycin were obtained between the NSCl–HPLC and DNFB–HPLC methods.

INTRODUCTION

Neomycin sulfate is a medium to broad spectrum aminoglucoside antibiotic used mostly in topical preparations and agricultural products. Neomycin is composed of a mixture of two stereoisomers, neomycins B and C (Fig. 1). Cleavage of the ring structure at the ether linkage results in the formation of neobiosamine and neamine. Neobiosamine further degrades to neosamine and ribose. Neomycin, its process intermediates, and degradation compounds all lack UV absorbing chromophore, thereby posing difficulties in the development of a high-performance liquid chromatographic (HPLC) assay method for quantitation of the antibiotic.

Both gas–liquid chromatographic (GLC) and HPLC methods have been reported for the separation and quantification of neomycins B and C. The GLC method



Neomycin B	$R_1 = H$	$R_2 = CH_2NH_2$	$R_3 = NH_2$
Neomycin C	$R_1 = CH_2NH_2$	$R_2 = H$	$R_3 = NH_2$

Fig. 1. Structure of Neomycin.

is difficult to use, as it requires frequent bracketing of samples due to the instability of the silylated derivative[1–3].

HPLC methods for the assay of neomycin may be categorized into the following two classes: (a) reversed-phase ion-pair chromatography with post-column derivatization[4–6] and (b) pre-column derivatization followed with normal phase chromatography[7–10]. Myers and Rindler[4] adapted the paired ion, *o*-phthalaldehyde method for gentamicin[11] to monitor neomycin in fermentation broth. This method requires neomycin sulfate to be converted to the base form by ion-exchange or calcium carbonate treatments prior to chromatography. The pre-column derivatization method with 1-fluoro-2,4-dinitrobenzene (DNFB)[7] requires frequent re-injection of samples due to absence of an adequate internal standard for the method and is beset by the use of a hazardous, DNFB derivatization reagent.

The HPLC method reported in this paper utilizes the 2-naphthalenesulfonyl chloride (NSCl) derivatization method developed for derivatization of secondary amines of spectinomycin[12] and forms a sulfonyl derivative of neomycin for normal phase chromatographic separation and quantification at 254 nm.

EXPERIMENTAL

Instruments

A Perkin-Elmer Series 4 solvent delivery system (Perkin-Elmer, Norwalk, CT, U.S.A.), a Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) or an LDC minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to pump the mobile phase at flow-rates of 1–2 ml/min. Analysis was performed using a 5-μm particle size high-speed silica column (Part No. 0258-1000, Perkin-Elmer P-E HS-5 silica, 125 × 4.6 mm I.D.) or conventional 5-μm irregular particle silica columns (Spheri-5, Brownlee Labs., Santa Clara, CA, U.S.A. or LiChrosorb Si 60, E. Merck, Darmstadt, F.R.G., both 250 × 4.6 mm I.D.) at ambient temperature. A 50-μl sample of derivatized neomycin was injected quantitatively onto a column utilizing a Waters WISP 710B injector. The column effluent was monitored at 254 nm using either a LDC Spectromonitor D variable-wavelength detector with an attenuation setting of 0.1 absorbance units full scale (a.u.f.s.) or with a LDC UV III

Monitor fixed-wavelength detector (Model 1203, LDC) at an attenuation setting of 0.064 a.u.f.s. Quantitation of peak area responses was performed by a VAX computer system (Digital Equipment, Maynard, MA, U.S.A.), and/or a Hewlett-Packard 3390A integrator (Hewlett-Packard, San Diego, CA, U.S.A.). A Cahn electronic balance (Model 21, Cahn, Division of Ventron Corporation, Cerritos, CA, U.S.A.) was used to weigh neomycin powders. A Labline oil bath (Catalogue No. 3005-7, Labline, Chicago, IL, U.S.A.) or a Thermolyne Dri Bath (Model No. D816525, Thermolyne, Division of Sybron, Dubuque, IA, U.S.A.) equipped with a heating block was used for derivatization of samples.

Reagents

Chloroform and methanol (both acetone and ethanol free), UV-grade, distilled in glass, were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Acetic acid was analytical reagent grade from Mallinckrodt and 2-naphthalenesulfonyl chloride (NSCl) was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Analytical reagent grade anhydrous dibasic sodium phosphate and monobasic sodium phosphate (Mallinckrodt) were used to prepare a phosphate buffer solution. The phosphate buffer solution was prepared by adding 475 ml of 0.2 *M* dibasic sodium phosphate and 25 ml of 0.2 *M* monobasic sodium phosphate into a 1-l flask and diluting to volume with double distilled water to obtain a 0.1 *M* solution with a pH of approximately 8.0.

The derivatization reagent was prepared by dissolving the NSCl in acetonitrile at a concentration of 40 mg/ml. The reagent was prepared fresh daily prior to derivatization.

The extraction solution was prepared by dissolving the internal standard, prednisolone, in a small amount of tetrahydrofuran (THF) to obtain a final concentration of 2.0 mg per 100 ml of chloroform. It is critical that the internal standard be first dissolved in THF to assure complete dissolution.

The mobile phase was prepared by combining 950 ml of chloroform, 23 ml of methanol, and 25 ml of acetic acid. The amount of methanol was adjusted to obtain optimum chromatographic performance.

Reference standard and sample preparation

Neomycin reference standard solution. Approximately 20 mg of neomycin sulfate reference standard (USP issue K, 765 μg base per mg sulfate) was placed in a Cahn balance aluminum weighing cup. This cup containing the neomycin reference standard was dried for 3 h under less than 5 mmHg pressure at 60°C. After drying, the standard in the aluminum cup was immediately weighed and the contents placed in a 100-ml volumetric flask and diluted to volume with 0.1 *M* sodium phosphate buffer (pH 8.0).

Neamine reference standard solution. Approximately 10 mg of neamine in-house reference standard was dried at 60°C in a vacuum oven for 3 h under less than 5 mmHg pressure and immediately weighed into a 100-ml volumetric flask and filled to the volume with 0.1 *M* phosphate buffer. The solution was further diluted by transferring 3.0 ml to another 100-ml volumetric flask and filled to volume with 0.1 *M* phosphate buffer to obtain a 0.3 mg/100 ml neamine reference standard solution. The neamine concentration, after derivatization, was equivalent to *ca.* 1.5% by weight of neomycin preparation.

Neomycin sulfate sample preparation. Approximately 20 mg of neomycin sulfate bulk powder were accurately weighed "as is" into a 100-ml volumetric flask. A 0.1 *M* sodium phosphate buffer solution (pH 8.0) was then added to volume.

Derivatization

Volumes of 10 ml each of the neomycin reference standard solution, neamine reference standard solution or sample solutions were quantitatively transferred into 40-ml disposable screw cap vials. A 10-ml aliquot of the derivatization solution was added to the vial using a repipet dispenser. The vial was capped tightly to avoid sample loss during derivatization, shaken briefly and placed in a 100°C silicone oil bath or 105°C heating block for 10 min to form the derivative. The amount of NSCl is more than 90 times in excess of the amount required for derivatization. The flask was then cooled to room temperature and approximately 15 ml of the extraction solution was added to each vial for quantitative extraction. The vial was shaken vigorously for 10 min, and centrifuged, if necessary, at low speed (< 300 *g*) for 3–5 min to obtain a clear lower organic layer. Portions of the lower organic layer were then chromatographed.

RESULTS AND DISCUSSION

Derivatization of neomycin

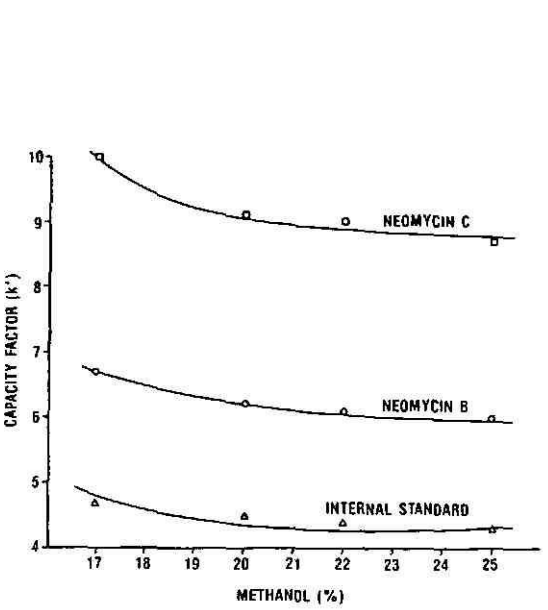
The lack of UV absorbing chromophore of neomycin presented a problem for easy detection by UV. Since there are six primary amines and seven hydroxyl groups on the neomycin molecules, derivatization of neomycin with NSCl[12], which was used to successfully form derivatives of the secondary amines of spectinomycin, was attempted. Neomycin sulfate was reacted with the NSCl reagent by heating at 100°C for 10 min. After cooling, samples were extracted in chloroform and then spotted on thin-layer chromatographic (TLC) silica plates. Plates were developed using a chloroform–tetrahydrofuran (60:40) mixture. Upon drying, the plates were examined by use of a short-wave UV lamp. Formation of a derivative was observed as a spot on TLC plates.

Development of chromatographic condition

The following columns were evaluated: 5-μm LiChrosorb Si 60, 5-μm Spheri-5, Zorbax SIL 5-μm (Du Pont, Wilmington, DE, U.S.A.) and P-E HS-5 silica columns.

Initial attempts to chromatograph NSCl derivatized neomycin utilized the LiChrosorb Si 60 silica column with a mobile phase composition of 70% chloroform and 30% acetonitrile–acetic acid (7:1). A large frontal peak eluted at approximately 5 min followed by a larger peak at about 11.6 min; however, no other peak was eluted. Further work revealed that inclusion of methanol (*ca.* 3%) in the mobile phase was essential for elution of the neomycin C peak.

The P-E HS-5 silica column showed the best separation of the major peaks with short chromatographic run times and high theoretical plates among the columns examined. The capacity factor (k') for the internal standard prednisolone, neomycin B and neomycin C on the column indicated adequate separation of the three components over a wide range of mobile phase compositions, thus suggesting ruggedness of the chromatographic system (Fig. 2). Although the optimum mobile phase com-



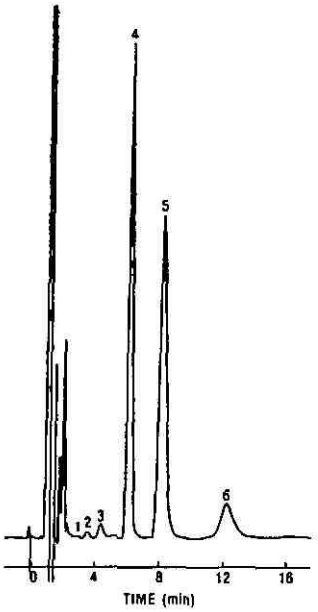


Fig. 2. Effect of methanol (%) in the mobile phase on capacity factor (k').

Fig. 3. Typical HPLC analysis of neomycin. Peak identification: 1 = neamine; 2 and 3 = unidentified; 4 = internal standard; 5 = neomycin B; 6 = neomycin C. Mobile phase, chloroform–methanol–acetic acid (950:23:25). Column: P-E HS-5 Silica, 5-μm, 125 × 4.6 mm I.D.

position was determined to be chloroform–methanol–acetic acid (950:23:25), the amount of methanol may be slightly modified to compensate for changes in column conditions. This chromatographic condition resulted in isocratic elution of neamine, the internal standard, neomycin B and neomycin C at 2.5, 6.2, 8.3 and 12.3 min, respectively (Fig. 3). The use of the high-speed column required a flow-rate of 1.7 ml/min for optimum performance.

For the LiChrosorb Si 60 column the optimum mobile phase composition was determined to be 85% chloroform, 12% acetonitrile and 3% methanol–acetic acid (10:1). Neomycin B, the internal standard, prednisolone, and neomycin C eluted at 8.7, 11.0, and 20.6 min, respectively.

For the Zorbax SIL column, the optimum mobile phase composition was chloroform–methanol–acetic acid (96:2:2). The internal standard, neomycin B, and neomycin C eluted at 21.1, 26.0 and 31.2 min, respectively.

For the Spheri-5 column an optimum mobile phase composition was determined to be chloroform–methanol–acetic acid (95:2.5:2.5). The internal standard, neomycin B and neomycin C eluted at 13.7, 16.2 and 24.2 min, respectively. However, both the Zorbax and Spheri-5 columns necessitated long chromatographic run times (25 min or more) to achieve adequate resolution of neomycin peaks.

Derivatization

Selection of buffer. The optimum pH for the NSCl derivatization of amines was shown to be between pH 8.0 and 9.0 in either 0.1 *M* phosphate or 0.05 *M*

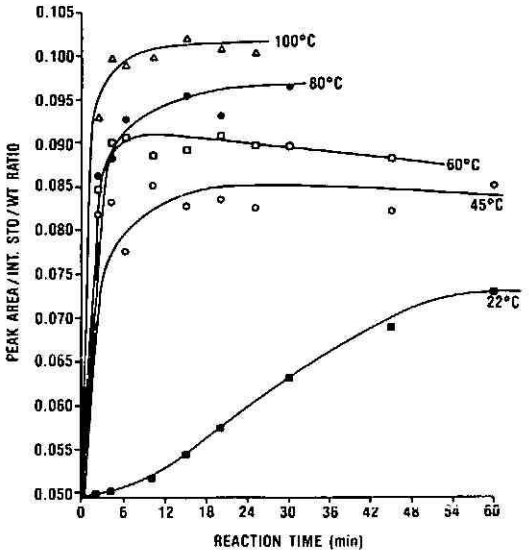


Fig. 4. Derivatization time–temperature for the formation of naphthalenesulfonyl neomycin.

bicarbonate buffer[12]. These two phosphate and bicarbonate buffers were examined for the derivatization of neomycin. The peak area of neomycin derivatized in phosphate buffer was larger (*ca.* 8%) than that of the bicarbonate buffer. Thus, the phosphate buffer (0.1 *M*) was utilized for further work.

Amount of derivatization reagent. The NSCl concentrations of 5, 25, 50, 75, 100, 200 and 400 mg/10 ml were examined for derivatization of neomycin when reacted at 100°C for 10 min. At each concentration of NSCl the peak area ratio of neomycin B and C to internal standard were examined. The data indicated that the peak area ratio increased rapidly to NSCl concentration of 200 mg/10 ml and reached the maximum thereafter while the peak area of neomycin C reached maximum at NSCl concentration of 100 mg/10 ml. To assure complete derivatization, the concentration of 300 mg/10 ml was chosen for time–temperature study. The NSCl concentration of 300 mg/10 ml is approximately a 90-fold excess for derivatization of neomycin; therefore, a minor deviation in the concentration of NSCl would have no undue effect on the quantification of neomycin.

Derivatization time and temperature. A reaction time–temperature study was performed to determine the optimum derivatization conditions for complete derivatization of neomycin. Samples of neomycin sulfate bulk powder were dissolved in the buffer solution and derivatized for 2, 4, 6, 10, 15, 20, 25, 30, 45 and 60 min at each of 22, 45, 60, 80 and 100°C. The results, displayed in Fig. 4, indicated that the peak area/internal standard/weight ratio for derivatization of neomycin was highest when reacted at 100°C and that the reaction reached maximum at 7 min and remained at a plateau from 7 to 30 min. Thus, the derivatization condition of 100°C for 10 min were utilized for further study.

Catalysts, such as 1-methylpyrrole and 2-acetylpyrrole, found essential for the sulfonylation of secondary amines[12] were then examined for the NSCl derivatization of neomycin. The catalysts showed no effects on the rate nor quantity of the deri-

vatization of primary amines nor the relative standard deviation (R.S.D.) of the neomycin assay method.

Extraction of derivatives. After derivatization, the sulfonyl neomycin is extracted in an organic phase to minimize the excess derivatization reagent and excipients for ease of chromatography. Extraction with chloroform yielded the best recovery and ease of operation. The derivatized neomycin is extracted in the lower, organic layer and the commonality of extraction solution and mobile phase yields an uncluttered chromatographic result. The refractive index negative peak found just after the major solvent front peak is due to the acetonitrile used to dissolve the derivatization reagent (Fig. 3). The acetonitrile is not common to the mobile phase.

Internal standard

The lack of an internal standard in the DNFB–HPLC assay method for neomycin was a major disadvantage of the method[7]. The problem encountered for selection of an internal standard was finding a compound that elutes fast enough not to increase the chromatographic time and yet not to interfere with the elution of neomycins B and C, process intermediates, and degradation compounds. Steroids such as prednisolone, triamcinolone and 25 other various steroids (*e.g.*, 6α-fluoroprednisolone-21-hydrogen succinate; 11β,17α,21,21-tetrahydroxy-1,4-pregnadiene-3,20-dione; prednisolone-21-hydrogen succinate) with polar characteristics were evaluated for elution time and peak resolution.

Triamcinolone (6.8 min) eluted later than prednisolone (5.5 min) but was characterized by a broad peak. The other steroids examined were unsatisfactory; they either eluted too early or were characterized by broad peaks. Prednisolone was therefore chosen due to its availability and sharp resolution, although it eluted approximately 5 min after the solvent front (Fig. 3). In order to ensure optimum resolution of the internal standard peak from the neomycin B peak, a slight adjustment of the mobile phase with methanol may be required.

Incorporation of the internal standard in the derivatization reagent was also attempted to improve assay efficiency. However, presence of the internal standard in the derivatization solution resulted in high R.S.D. (2–3%), while the internal standard added in the extraction solution resulted in R.S.D. of less than 1%.

Chromatography

Fig. 3 is a typical chromatogram of neomycin sulfate with the internal standard, prednisolone. Retention times for the internal standard, neomycin B, and neomycin C were 6.2, 8.3 and 12.3 min, respectively.

One of the goals of this assay development was to achieve isocratic assay of neamine, a degradation compound, along with neomycins. This objective was accomplished using the mobile phase composed mainly of chloroform (Fig. 3). Neamine eluted at 2.5 min.

The reagent blank eluted no compound which interfered with chromatography of the neomycins.

Linearity and precision

Neomycin. The linearity of the HPLC method for the assay of neomycin B was determined by analyzing seven different quantities of the neomycin reference stan-

TABLE I

PRECISION OF NSCl–HPLC NEOMYCIN ASSAY METHOD

Sample	*Weight (mg)*	*Peak area*		
		Neomycin (B+1/2C)	*Int. Std.*	*Neomycin/Int. Std/wt.*
1	20.56	4861000	3136000	0.07541
2	20.29	4939000	3137000	0.07758
3	20.72	4904000	3099000	0.07638
4	20.21	4774000	3078000	0.07675
5	20.50	5022000	3184000	0.07693
6	20.27	5020000	3203000	0.07732
7	20.36	4842000	3111000	0.07645
			Average	0.07669
			R.S.D.	0.92%

dard ranging from 12.5% to 200% of the mid-point of the standard curve (20 mg/100 ml). The plot of amount recovered *versus* amount assayed was linear with a correlation coefficient of 0.9996 and an intercept not significantly different from zero.

The precision of the assay was determined by the use of seven individually weighed and derivatized neomycin sulfate powders. The relative standard deviation of the assay for the determination of neomycin sulfate was 0.92% (Table I).

Neamine. The linearity for the assay of neamine was determined by analyzing six different quantities of neamine reference standard ranging from 16.6% to 333% of the mid-point of the neamine standard curve (0.3 mg/100 ml or equivalent to 1.5% of the neomycin). The plot of amount recovered *versus* amount analyzed was linear with a correlation coefficient of 0.9977 and an intercept not significantly different from zero.

TABLE II

BIO-EQUIVALENT POTENCIES (NEOMYCINS B + 1/2C) OF NEOMYCIN SULFATE BULK DRUG (μg/mg); COMPARISON BETWEEN HPLC (NSCl AND DNFB), GLC, AND MICROBIOLOGICAL ASSAY METHOD

Lot No.	*HPLC*		*GLC*	*Microbiological*
	NSCl	*DNFB*		
A	622	636	625	602
B	625	638	632	621
C	629	645	655	604
D	654	666	670	592
E	632	638	647	590
F	649	657	681	593
G	644	655	654	596
H	659	673	688	615
Average	639	651	657	602

The precision of the neamine assay was determined by individually derivatizing seven samples of a solution containing 0.3099 mg/100 ml of neamine reference standard. This neamine concentration corresponds to 1.5% by weight in a neomycin sample. The relative standard deviation of the assay was 1.4%.

Neomycin sulfate powder assay

Neomycin potency. Eight current lots of neomycin sulfate bulk drug were assayed by the NSCl–HPLC, DNFB–HPLC, GLC, and microbiological assay methods and the results, expressed as biological equivalencies (neomycins B + 1/2 C)[13], are summarized in Table II. The average results of the NSCl–HPLC method was 639 μg/mg, while those of the DNFB–HPLC, GLC, and microbiological methods were 651, 657, and 602 μg/mg, respectively.

The paired *t*-test indicated that there is a statistically significant difference at the 95% confidence level between the methods compared. The difference between the NSCl–HPLC and microbiological methods represent a 5.8% difference. This significant difference may be rationalized given the high variability associated with the microbiological method. It is interesting to note that the results of the NSCl–HPLC method falls between those of the microbiological and DNFB–HPLC methods. The average potency determined by the NSCl–HPLC and DNFB–HPLC methods (639 and 651 μg/mg) represented a 1.8% difference. The cause of this minor, 1.8% difference between the two HPLC methods has been traced to the difference in the preparation (weighing after drying) of the reference standard as practiced in two laboratories performing the two different assays; it is extremely difficult to precisely weigh neomycin reference standard since the neomycin standard absorbs 8% moisture in 5 min.

The average percentage neomycin C in neomycin was determined by the NSCl–HPLC method to be 13.8%, while those by the DNFB–HPLC and GLC methods was 14.0% and 20.1%, respectively (Table III). Inadequate separation of neomycin C peak from the neomycin B peak by the GLC method could be the cause

TABLE III

ASSAY OF NEOMYCIN C (%) IN NEOMYCIN SULFATE BULK DRUG; COMPARISON BETWEEN HPLC (NSCl AND DNFB) AND GLC ASSAY METHOD

Lot No.	*HPLC*		*GLC*
	NSCl	*DNFB*	
1	16.2	15.8	22.0
2	16.1	16.2	22.0
3	13.0	13.5	19.7
4	12.3	12.4	18.3
5	13.4	13.1	19.1
6	14.5	15.4	21.1
7	14.5	15.2	21.6
8	10.2	10.1	16.9
Average	13.8	14.0	20.1

TABLE IV

ASSAY OF NEAMINE (%) IN NEOMYCIN SULFATE BULK DRUG

Lot No.	*Day 1*	*Day 2*	*Average*
Current lot			
A	0.107	0.101	0.104
B	0.133	0.117	0.125
C	0.084	0.096	0.090
Old lot			
D	0.353	0.378	0.370
E	0.324	0.328	0.326
F	0.387	0.389	0.388

of the difference obtained by the NSCl–HPLC and GLC methods. The percentage of neomycin C content determined by the two HPLC methods showed no significant difference indicating that the two methods are compatible for the detection and qualification of both neomycin B and neomycin C.

Neamine. Three current and three old neomycin sulfate lots were assayed for neamine content by the NSCl–HPLC method (Table IV). The percentage of neamine in three current lots examined were *ca.* 0.1% while the old lots were *ca.* 0.3%.

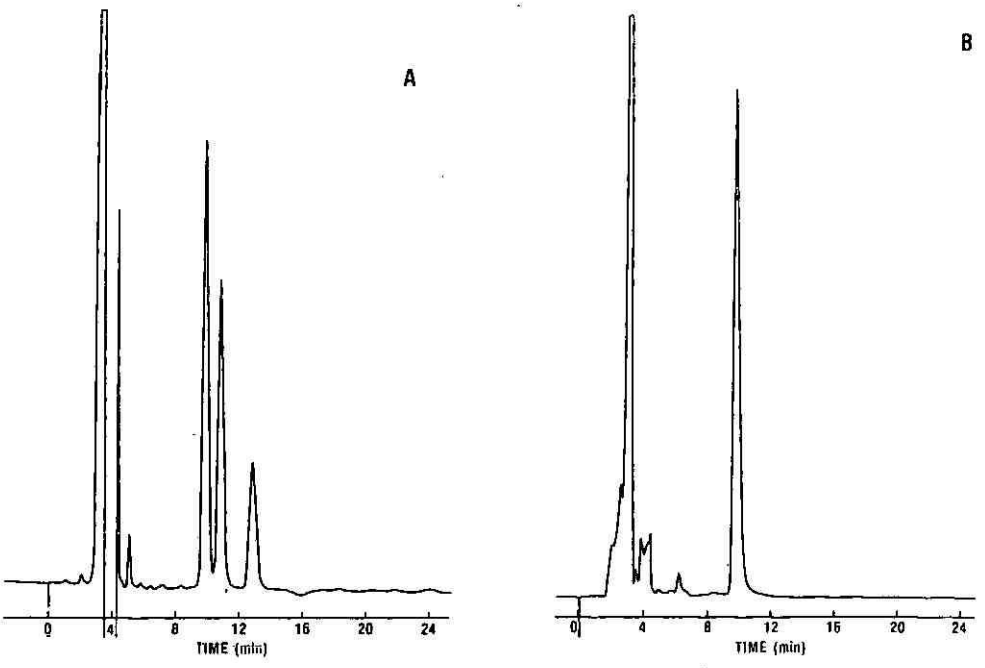


Fig. 5. HPLC analysis of naphthalenesulfonyl derivatives of aminoglucoside antibiotics (A) gentamicin and (B) kanamycin using a silica column. Mobile phase, chloroform–methanol–acetic acid: (A) 700:10:7; (B) 560:400:40.

Mass spectrometric identification of sulfonyl-neomycin

NSCl derivatized neomycin B, neomycin C and neamine were concentrated by extraction and evaporation under a stream of nitrogen for analysis by fast atom bombardment mass spectrometry (FAB-MS). Column fractions corresponding to derivatized neomycins were also collected and analyzed by MS.

Examination of the mass spectrum of neamine indicated the presence of a relatively strong molecular ion plus $2H^+$ at *m/e* 1083, indicating that all four primary amines in the molecule were derivatized. A weak ion observed at *m/e* 893 is the three substituted neamine plus $2H^+$ and the ion at *m/e* 543 represents neosamine and/or deoxystreptamine plus $2H^+$.

The mass spectrum of neomycin C showed a relatively strong molecular ion plus Na^+ at *m/e* 1777 indicating a 6 substituted molecule. The *m/e* peaks at 1566, 1376 and 1185 indicated 5, 4 and 3 substitutions. Also the *m/e* peaks of 1105, 893 and 543, contributed from the neamine portion of the neomycin molecule, were observed. MS of neomycin B and the HPLC column fractions were essentially identical with those of the solvent extracted samples described above.

Potential application to analyze other aminoglucoside antibiotics

The HPLC method developed may be used to analyze varieties of other aminoglucoside antibiotics. Chromatogram of gentamicin and kanamycin illustrating such an application are shown in Fig. 5. Sensitivity for the detection of these antibiotics improves when the column effluent is monitored at 227 nm ($\log \varepsilon = 4.98$).

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DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS WITH MORE THAN FOUR RINGS IN CRUDE OILS ON A RP-18 COLUMN

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SUMMARY

The hydrocarbon fraction in crude oils has been separated from Asphaltenes on a RP-18 column. The hydrocarbons were subsequently fractionated on an aluminium oxide micro-column, yielding an homogeneous fraction of polycyclic aromatic hydrocarbons with more than four rings. The method was applied to crude oils from different geographical areas. The limits of application of the method, related to the percentage of Asphaltenes in crude oils, are discussed.

INTRODUCTION

The identification and determination of primary pollutants are the main aims in the analysis of complex organic matrices, such as crude oils, fuel oils, etc. Polycyclic aromatic hydrocarbons (PAHs) with more than four rings are an important part of this group[1–5].

In the analysis of such compounds it is necessary to use pre-separation procedures to obtain homogeneous hydrocarbon fractions separated from non-hydrocarbon compounds, which such as Asphaltenes[6]. The presence of Asphaltenes may cause problems, such as saturation of the stationary phase of the chromatographic column, changing its physical properties. In this way, absorption problems and/or non-reproducibility of the results may occur, as well as contamination of the hydrocarbon fraction.

This separation is quite difficult, because Asphaltenes are not a homogeneous class, but are identified by their insolubility in aliphatic solvents and their solubility in aromatic ones. Their chemical composition is very complex, as they contain organic compounds with condensed rings[7,8], short aliphatic chains[9–11], heteroatoms[12–16] and metals[17,18]. There are several methods for the elimination of Asphaltenes, such as extraction with organic solvents (ASTM D893,BS 200 part. 143/85, IP 143/84) and chromatographic techniques (API-60, SARA)[12,19–21]. All of these procedures have some disadvantages[21]: the amount of solvents used, lengthy analysis time and difficulties in obtaining reproducible fractions for the subsequent separation into homogeneous classes[12,21–23].

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In this work we studied the possibility of separating hydrocarbons from Asphaltenes in crude oils, and of obtaining an homogeneous fraction of PAHs having more than four rings, by using a RP-18 micro-column.

EXPERIMENTAL

Reagents and materials

Crude oils from the Middle East, Africa, Russia and South America were used: Arabian Light (LA) (Saudi Arabia), Dubay (DU) (Dubay), Qatar Marine off-shore (QM) (Qatar), Emeraude (EM) (Zaire), Mandji (MA) (Gabon), Es Sider Oasis (LI) (Libya), Ural (RU) (Russia), Tia Juana (TJ) (Venezuela). The samples were stored in the dark at 5°C.

The stationary phases for column chromatography were LiChroprep RP-18 (Merck) and aluminium oxide Type E (activity I) (Merck). The latter was maintained at 130°C for 12 h before use. These supports were dry-packed in glass columns.

n-Pentane (spectroscopy), *n*-hexane (pesticide), toluene (Lichrosolv), carbon tetrachloride (analysis), all purchased from Merck, and dichloromethane (Chromasolv, Riedel) were used.

Fractionation of crude oils on RP-18 micro-column

A 0.1-ml volume of a solution containing 500 mg of crude oil per millilitre of carbon tetrachloride was fractionated on an RP-18 micro-column (9 cm × 0.6 cm) filled with 1.3 g of stationary phase after treatment of the with 20 ml of *n*-hexane. The elution was carried out with 3.5 ml of *n*-hexane and then with 10 ml of toluene, after drying the column under nitrogen.

Fractionation of standard hydrocarbons on RP-18 micro-column

The RP-18 column employed was the same as described above. The standard hydrocarbons were dissolved in carbon tetrachloride (concentration 1 mg/ml) and the solutions stored in the dark. Part of the solution (30 μl) was placed onto the column which was then eluted with *n*-hexane, 100-μl fractions being collected for gas chromatographic (GC) analysis.

Isolation of PAHs with more than four rings

The same RP-18 micro-column and an aluminium oxide one (11 cm × 0.6 cm) treated with 20 ml of *n*-pentane were used for a two-step fractionation. The elution scheme is shown in Fig. 1. Eluents: (a) 2.5 ml of *n*-hexane; (b) 2.5 ml of *n*-hexane; (c) 7 ml of *n*-pentane–dichloromethane (6:4, v/v); (d) 3 ml of dichloromethane. The PAH fraction was evaporated to 0.1 ml under nitrogen[24].

Extraction

A 500-mg sample was directly weighed in a flask and refluxed with 100 ml of *n*-hexane for 1 h. After cooling, the suspension was filtered on Millipore 0.45-μm filters, and the residue dried in a vacuum. The solution was then analyzed by UV spectrophotometry and the dry residue weighed. The residue was then dissolved in 100 ml of toluene and the solution analyzed by UV spectrophotometry.

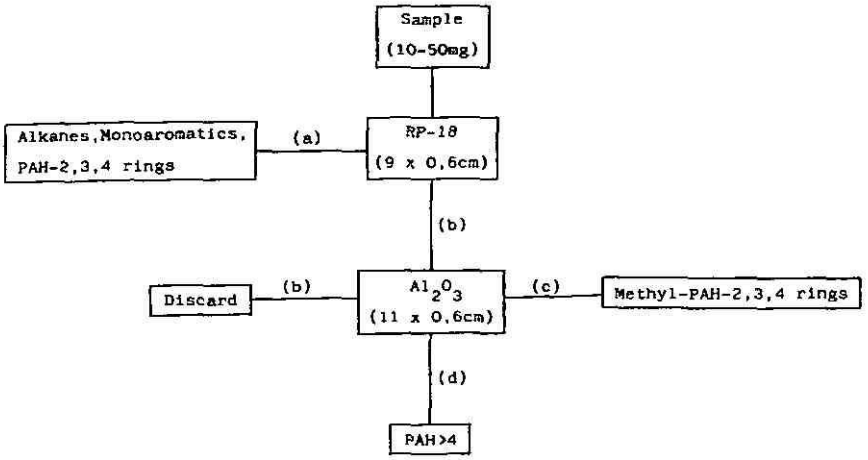

Fig. 1. Separation scheme. Eluents: (a) 2.5 ml of *n*-hexane; (b) 2.5 ml of *n*-hexane; (c) 7 ml of *n*-pentane–dichloromethane (6:4); (d) 9 ml of dichloromethane.

Apparatus

A UV–VIS spectrometer Model 552 S (Perkin-Elmer) was used. The spectra were registered in the 200–400 nm region.

An HRGC-5160 Mega series gas chromatograph (Carlo Erba) with flame ionization detection (FID) was used, together with a computer system Mega-2 (Shimadzu). Standards were determined with the "on-column" technique, by using a glass capillary column with a "retention gap" (25 m × 0.32 mm I.D., film thickness 0.15 μm) (Mega-Carlo Erba). Chromatographic conditions: 50°C for 1 min, linear increase of 8°C/min to 300°C; carrier gas hydrogen. Crude oil fractions were analyzed by the "splitless" technique, with a 20 ml/min head flow and silica capillary column SPB-5 (30 m × 0.25 mm I.D. film thickness 0.25 μm) (Supelco). Chromatographic conditions: injector temperature 270°C; initial temperature 50°C, then increase at 5°C/min to 300°C; carrier gas, hydrogen.

Gas chromatographic–mass spectrometric (GC–MS) analyses were performed with a spectrometer Model VG 7070 EQ (VG Analytical), under the following conditions: acceleration voltage 6 kV; source current 100 μA; ionizing voltage 70 eV.

RESULTS AND DISCUSSION

Isolation and fractionation of hydrocarbons in crude oils on RP-18 micro-column

Crude oils from different geographical (see Experimental) areas were examined to determine the reliability of results for different samples. Small amounts of crude oil (50 mg) were applied to a RP-18 micro-column to obtain a fast and reproducible fractionation with small volumes of eluents. For the elution of the hydrocarbon fraction 3.5 ml of *n*-hexane were used. Tests on a standard hydrocarbon mixture on the same micro-column showed that all hydrocarbons examined (*n*-paraffins, alkylbenzenes, PAHs) are eluted with this solvent volume (see Fig. 2). PAHs were eluted differently on the basis of their aromatic rings; those with the highest number of rings having the highest retention volumes. In particular, it was possible to obtain an

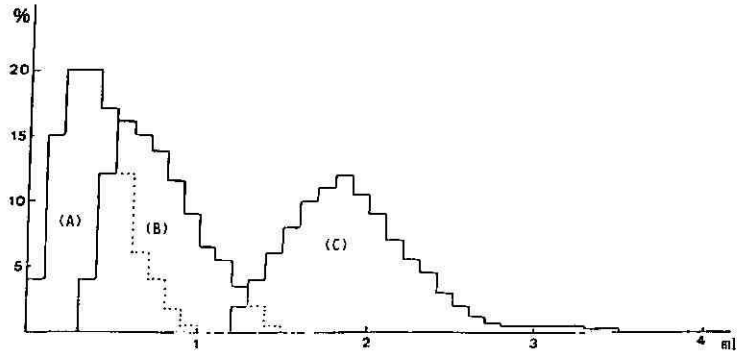

Fig. 2. Fractionation of a hydrocarbon standard mixture with 3.5 ml of *n*-hexane. Column: 9 cm × 0.6 cm I.D. packed with 1.3 g of LiChroprep RP-18. Abscissa: elution volumes of *n*-hexane from the RP-18 micro-column. Ordinate: percentage of elution for (A) *n*-paraffins, alkylbenzenes, PAH with two rings; (B) PAHs with three or four rings; (C) PAHs with greater then four rings.

homogeneous fraction of PAHs having greater than four rings without *n*-paraffins, monoaromatic and other polycyclic hydrocarbons, which constitute the main part of the hydrocarbon fraction of crude oils, and which are eluted in the first millilitre of solvent. Such a separation, in a short time (5 min) and in small volumes of solvent, was difficult to obtain on silica[25]. The recovery for all hydrocarbons was practically quantitative, as shown in Table I, where the percentage recovery and the standard deviation relative to five measurements are reported.

It is important to note that the elution profile of Fig. 2 was maintained in crude oil fractionation. The only difference concerned the composition of fraction C, which contained some residual methylnaphthalenes, methyl-substituted chrysenes and yellow-brown compounds (resins) strongly retained on silica and aluminium oxide columns[26]. Fraction C did not contain aliphatic, monoaromatic compounds and the greater part of polycyclic compounds with two to four rings. Furthermore, *n*-hexane-insoluble compounds remained on the column.

As regards the retention mechanism on this column, it is important to note the increase in retention volumes from *n*-decylbenzenes to toluene, as shown in Table I, where alkylbenzenes are reported in their order of elution. Xylenes are the most strongly retained compounds, owing to their higher electron density. Analogous behaviour has been reported for the same compounds on silica columns[27], and shows that, under the present experimental conditions, RP-18 behaves as a polar stationary phase. The retention of hydrocarbons is therefore due to the free silanol groups on the surface of the silanized silica[28–30].

Isolation of PAHs with more than four rings

This hydrocarbon fraction, which has already been used in "fingerprinting" of crude oils[25], is one of the most important from an analytical and environmental point of view. As stated above, RP-18 is suitable for a first isolation step, even if it is not possible to obtain a sufficiently homogeneous PAH fraction for analysis. This can be achieved by a two-step fractionation (see Fig. 1), using a second micro-column of aluminium oxide. The choice of this stationary phase is based upon its class frac-

TABLE I

RECOVERY OF HYDROCARBONS FROM THE RP-18 MICRO-COLUMN

Hydrocarbon	*Recovery (%)*	*S.D. (%)*
n-C_8	93	4
n-C_9	93	6
n-C_{10}	92	6
n-C_{12}	92	5
n-C_{14}	98	7
n-C_{16}	95	5
n-C_{20}	95	6
n-C_{22}	93	6
n-C_{24}	93	7
n-C_{28}	95	5
n-C_{30}	96	5
n-C_{32}	95	6
n-C_{36}	93	5
Decylbenzene	91	6
Octylbenzene	95	5
p-Cymene	92	6
Hexylbenzene	93	6
Mesitylene	91	6
Butylbenzene	95	4
Cumene	97	4
Propylbenzene	96	5
Ethylbenzene	95	4
o-Xylene	93	4
m-/*p*-Xylene	95	4
Toluene	92	6
Naphthalene	93	6
1-Methylnaphthalene	94	7
Acenaphthylene	93	5
Acenaphthene	91	6
Fluorene	92	6
Phenanthrene	90	6
Anthracene	90	7
Fluoranthene	92	7
Pyrene	93	5
Chrysene	95	6
Benzo[*a*]pyrene	92	5
Perylene	97	5
Dibenz[*ah*]anthracene	95	5
Benzo[*ghi*]perylene	97	5
1,2-4,5-Dibenzopyrene	93	5
3,4-8,9-Dibenzopyrene	95	5

tionation power for PAH, with eluents of increasing and definite ε° (the solvent strength parameter in liquid–solid chromatography)[24,26,31–33]. The second 2.5 ml portion of *n*-hexane (b) used for the elution of the PAH fraction on RP-18 was passed through an aluminium oxide column and then discarded; in the next 7 ml of *n*-pentane–dichloromethane (6:4) the majority of methyl-substituted PAHs with two

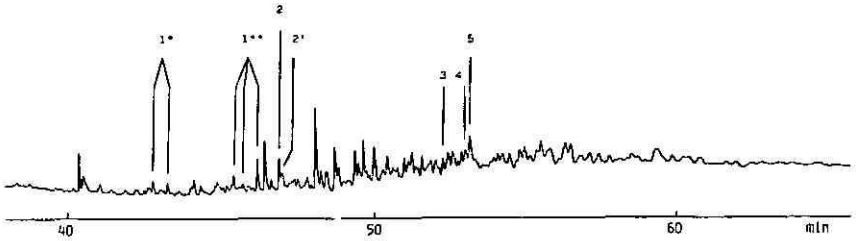

Fig. 3. Gas chromatogram of PAH fraction having greater then four rings for LA crude. Peaks: 1* and 1** = methylchrysenes; 2 = benzo[*e*]pyrene; 2' = benzo[*a*]pyrene; 3 = indeno[1,2,3-*cd*]pyrene; 4 = picene; 5 = benzo[*ghi*]perylene.

to four rings were also eliminated, and in 3 ml of dichloromethane it was possible to obtain an homogeneous greater than four-ring fraction, containing only traces of residual methyl-PAHs with four rings.

GC[34]/GC–MS[35,36] analysis confirms the homogeneity of this fraction (see Fig. 3); the very low concentration of methylchrysenes (1*, 1**) is of particular interest. Some other components of the mixtures were identified as benzopyrenes, indenopyrenes, picenes and corresponding methyl derivatives, together with some polycyclic compounds containing sulphur. Fig. 3 shows the gas chromatogram of some compounds whose identification is important for their carcinogenic properties: benzo[*e*]pyrene (2); benzo[*a*]pyrene (2'); indeno[1,2,3-*cd*]pyrene (3); picene (4); benzo[*ghi*]perylene (5). It should be noted that with this new fractionation method it is possible to enrich the PAH fraction. The maximum capacity of the method is normally 50 mg of crude, but when the percentage of Asphaltenes is low it is possible to analyze up to 100 mg of sample without altering the PAH fraction. In this way the sensitivity of the method is increased 5–10 times with respect to other methods that do not use deasphaltening procedures.

These results are of great analytical interest, because with the micro-fractionation method it is possible to save time by eliminating tedious extraction methods and also the formation of micelles[13], artefacts and loss of volatile hydrocarbons such as methyl-substituted benzenes and naphthalenes, due to heating. It is important to note that there was a high recovery, as shown in Table II, where the percentage of PAHs with greater then four rings is given for the most critical conditions (evaporation to 0.05 ml).

TABLE II

RECOVERY OF PAHs WITH MORE THAN FOUR RINGS

The recoveries are average values from five determinations.

Hydrocarbon	*Recovery (%)*	*S.D. (%)*
Benzo[*a*]pyrene	90	5
Perylene	97	5
Dibenz[*ah*]anthracene	93	6
Benzo[*ghi*]perylene	97	6
Coronene	93	6
1,2-4,5-Dibenzopyrene	90	5
3,4-8,9-Dibenzopyrene	90	6

TABLE III

COMPARISON BETWEEN CHROMATOGRAPHIC AND EXTRACTION METHODS

Crude oil	*Asphaltenes (%)*	
	*Chromatography**	*Extraction*
TJ	5.7	6.8
LA	3	2.8
EM	2.9	3.2
RU	2	1.6

* The measurements were performed on 500 mg of sample by using a column (11 cm × 1 cm) filled with 5.7 g of RP-18.

This important family of environmental compounds contains highly oxidizable components[37–39], such as benzo- and dibenzopyrenes. With this method is it possible to identify such compounds in complex mixtures, without changing their composition, even when they are present at trace levels.

Fractionation of the crude oil portion remaining on the column after elution with n-hexane

Elution of the crude oil portion insoluble in *n*-hexane was achieved with toluene. The content of this fraction was analyzed by UV spectroscopy and gravimetric measurements on the residue obtained by evaporating the solvent. The results were compared with those obtained with the usual extraction methods (see Experimental) for the following samples: TJ (South America), LA (Middle East), EM (Africa), RU (Russia).

As shown in Table III there is good agreement between the two series of results. Furthermore the UV spectra of the toluene fractions, obtained with the two above-mentioned techniques, were similar, with differences only in the maximum of absorption at 287 nm. Such data confirm the presence of Asphaltenes in these fractions. Finally, there is a direct relationship between the absorbance values and the percentages of Asphaltenes determined gravimetrically.

Loading experiments carried out on RP-18 columns containing different amounts of stationary phase showed that the maximum operating limit was 3 mg of Asphaltenes per gram of RP-18. Above this limit the hydrocarbon fraction was contaminated by Asphaltenes. In the case of TJ crude oil, which has a high percentage of Asphaltenes (see Table III), it would be necessary to use an RP-18 column with a larger amount of stationary phase. By using the above-mentioned micro-column, the *n*-hexane fraction contains some Asphaltenes, and it was necessary to pass it through 0.45-μm filters to eliminate those compounds.

In conclusion, the measurements of the absorption maximum at 287 nm of the toluene fraction obtained from the RP-18 micro-column indicate the percentage of Asphaltenes in a crude oil. It is thus possible to evaluate the maximum quantity of sample for chromatographic separations, or for its characterization for industrial processing[36,40].

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF DEUTERATED PHOTOSYNTHETIC PIGMENTS FROM THEIR PROTIO ANALOGUES

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SUMMARY

A high-performance liquid chromatographic method with a visible method of detection is described for the facile baseline separation of deuterated chlorophylls from their protio analogues. A quaternary solvent system comprising of water–organic phase and an octadecyl-silica (C_{18}) column were used. In every instance the deuterio compound eluted ahead of its protio analogue indicating that Van der Waals forces are operational during the separation process. Specificity, sensitivity, and reproducibility make these methods particularly suitable in plant chemistry for semi-preparative purification processes and methodologies.

INTRODUCTION

Many experiments in photosynthesis require pure chlorophylls. The role of chromatography in the studies of chlorophylls is apparent from many investigations[1]. Studies of the leaf pigments lead not only to Tswett's perfection of column chromatographic procedures but also to the discovery of additional chlorophylls. Most active adsorbents like alumina, magnesia, and charcoal alter the chlorophylls so that they cannot be eluted and recovered. Polysaccharides such as starch, and cellulose have been used extensively since they do not alter the chlorophylls and separation of the chlorophylls from one another is achieved. A recent investigation showed Sepharose CL-6B to be the best adsorbent for the separation of chlorophylls amongst four adsorbents studied[2]. Still the most frequently used separation method for extracts of chloroplast pigments is adsorption chromatography on sugar–starch mixtures as the stationary phase[3]. In the last few years high-performance liquid chromatography (HPLC) has served as the most popular method for the separation of chloroplast pigments[4–15].

A clear understanding of the biological effects of deuterium can be obtained by studying the effect of isotopic substitution at the molecular level[16]. Interest in

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conducting studies related to the biological effects of deuterium started early. In 1959 it was demonstrated that it was possible to grow organisms under fully deuterated conditions[17]. In other words prior to this period it was believed that the replacement of hydrogen by deuterium was incompatible with life. Not so widely recognized is that many fully deuterated compounds of biological importance, whose syntheses are quite beyond the resources of modern synthetic organic chemistry, can be readily obtained by biosynthesis. Many species of microorganisms have been cultivated in fully deuterated form, and some species of higher plants and mammals have been partially deuterated. The first successful cultivation of a deuterated living system was accomplished in 1960 when two species of green algae, *Chlorella vulgaris* and *Scenedesmus obliquus* were grown in media containing 99.7% heavy water[18]. The chloroplast pigments contained deuterium instead of hydrogen. Many other species of algae have been grown since that time. As facilities now exist, many deuterated compounds of great biological interest are now available for chemical and biological investigation[19–21]. The effect of deuterium substitution on the chromatographic behaviour of numerous compounds (carbohydrates, steroids) as well as deuterium isotope effects is accounted for in a review[22]. Fully deuterated pigment were not separated from ordinary hydrogen containing pigments on columns of powdered sugar.

Interest in the fractionation of compounds containing tracer ^{2}H, ^{3}H, and ^{14}C soon led to the discovery that complete resolution of mixtures of ^{1}H and ^{2}H compounds could be achieved by gas–liquid chromatography. The first total resolution of a fully deuterated compound from its ^{1}H analogue was demonstrated for ^{1}H and ^{2}H labeled β-carotene by open column liquid chromatography on activated magnesia[23]. Subsequently, a number of ^{1}H and ^{2}H labeled aromatic hydrocarbons and fatty acids were separated by reversed-phase (RP) HPLC[24]. This study extends to the development of the separation of ^{1}H and ^{2}H chlorophylls, and related compounds. There is clear indication that RP-HPLC may become a general procedure for the separation of mixtures of photosynthetic compounds of differing hydrogen isotopic composition.

EXPERIMENTAL

Materials

Samples of protio and fully and partially deuterio chlorophyll *a*, chlorophyll *b*, bacteriochlorophyll *a* (from cultures of *Rhodospirillum rubrum* and *Rhodospirillum spheroides*) and pyrochlorophyll *a* were obtained according to procedures reported earlier[1].

Various solvents were used in the chlorophyll studies; all solvents were distilled in glass (HPLC grade) and were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetonitrile, tetrahydrofuran, methanol, and acetone were employed in experiments with chlorophylls. Water was incorporated in some separation systems and was prepared by a commercial deionization system. All samples and solvents were used without further purification.

In all chlorophyll studies, 1 mg of the sample was accurately weighed and dissolved in 2 ml of HPLC grade tetrahydrofuran. This was equivalent to a concentration of approximately 0.5 mg/ml. All samples were vortexed and were syringe filtered using a Waters organic sample clarification kit (No. 26870). A volume of 90

μl of a solution of one isotopic form was mixed with 100 μl of the solution of the other isotopic form. Mixing unequal volumes of isotopic forms further facilitated the identification of peaks in the chromatogram. A volume of 2 μl of the sample mixture representing approximately 500 ng of each isotopic form was injected into the chromatograph. Chlorophyll *a* studies were monitored at 663 nm, chlorophyll *b* at 642 nm, pyrochlorophyll *a* at 663 nm, and bacteriochlorophyll *a* at 773 nm. Chart speed was kept at 1 cm/min and the flow-rate was set at 1 ml/min.

Chromatography conditions

All of the RP-HPLC separations were carried out on a Beckman Model 332 liquid chromatograph system equipped with two Model 110A metering pumps under the control of a No. 421 microprocessor. A Hitachi UV–VIS variable-wavelength spectrophotometer (Model 155-40) was used as the detector. Samples were introduced by a Model 210 syringe loading injector. Ultrasphere C_{18} ODS (Beckman), 5 μm, 250 mm × 4.6 mm columns were used. A 0.5-μm pre-filter was always inserted before the column and careful attention was directed to the removal of particulate matter from the samples and solvents.

Mobile phases consisted of mixtures of HPLC-grade acetonitrile, methanol, tetrahydrofuran, and water. Experiments were conducted to optimize the eluent composition.

RESULTS

The deuterio and the protio analogues of chlorophyll (chl) *a* were baseline separated in 22 min (Fig. 1). Two very small peaks for the stereoisomers were seen at 18.2 min ($k' = 5.7$) for deuterated chl *a'* and at 20.6 min ($k' = 6.6$) for the protio analogue. The isotopic forms of chlorophyll *b* were resolved in 16 min (Table I) with the deuterio compound eluting in 12.1 min ($k' = 3.2$) followed by the protio analogue in 13.3 min ($k' = 3.7$). Bacteriochlorophyll (Bchl) *a* and pyrochlorophyll *a* studies were also conducted with the mixture but with slightly different composition for A:B. In each instance baseline separation was accomplished; retention times were noted and the capacity factors calculated.

Partially deuterated chlorophylls were obtained from green algae grown in media containing 50% ^2H_2O, 80% ^2H_2O, and 90% ^2H_2O. Stated another way, deuterated chlorophyll compounds were isolated from organisms grown in media con-

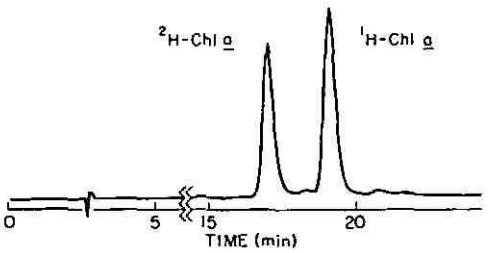

Fig. 1. Reversed-phase separation of deuterated chlorophyll *a* and protio chlorophyll *a* on a 25 cm × 4.6 mm I.D. C_{18} Ultrasphere ODS column. The mobile phase consited of water–methanol–acetonitrile–tetrahydrofuran (5:28:38:23, v/v). Flow-rate was 1 ml/min and detection set at 663 nm.

TABLE I

RETENTION TIME AND CAPACITY FACTOR CHARACTERISTICS FOR VARIOUS DEUTERATED CHLOROPHYLLS AND THEIR PROTIO ANALOGUES

Compound	*Mobile phase (A:B)**	*Retention time (min)*		*Capacity factor (k')*	
		Deuterio	*Protio*	*Deuterio*	*Protio*
Chlorophyll *a*	5:95	17.0	19.0	5.3	6.0
Chlorophyll *b*	5:95	12.1	13.3	3.2	3.7
Bacteriochlorophyll *a* (geranylgeranyl chain)	10:90	16.6	18.6	4.7	5.4
Bacteriochlorophyll *a* (phytyl chain)	7:93	17.4	19.2	5.1	5.7
Pyrochlorophyll *a*	0:100	8.2	8.9	1.8	2.1

* A = Water; B = [methanol–acetonitrile–tetrahydrofuran (30:40.5:24.5, v/v)].

taining different amounts of ^2H_2O. (As there is discrimination against ^2H in biosynthetic processes, the resultant chlorophylls contain somewhat less ^2H than the $^2H/^1H$ ratio that would be obtained in the absence of isotopic fractionation effects.) All of the partially deuterated chlorophylls were run individually against ^1H-chl *a* and in each case baseline separation was achieved. The system developed for chl *a* was employed in separating ^1H-chl *a* from 90% ^2H-chl *a* (Fig. 2). Baseline separation was accomplished in 24 min with retention times as follows: 90% ^2H-chl *a*, 19 min ($k' = 5.6$) and chl *a*, 21 min ($k' = 7.4$). Similarly, 80% ^2H-chl *a* eluted in 19.6 min and 50% ^2H-chl *a* had a t_R of 20.4 min when paired separately with chl *a* ($t_R = 21.4$ min).

A mixture of ^1H-chl *a*, 50% ^2H-chl *a* and ^2H-chl *a* was resolved along with their respective stereoisomers (Fig. 3). For a separate mixture consisting of 100% ^2H-chl *a*, 90% ^2H-chl *a*, 80% ^2H chl *a*, 50% ^2H-chl *a* and ^1H-chl *a* the solvent system provided a separation for three peaks, *viz.*, one for ^1H-chl *a*, the other for 50% ^2H-chl *a*, and the third was an asymmetric peak composed of 100% ^2H-chl *a*, 90% ^2H-chl *a* and 80% ^2H-chl *a*. Baseline separation was not accomplished. The asymmetric peak eluted first with a retention time of 20.8 min ($k' = 6.3$), followed by

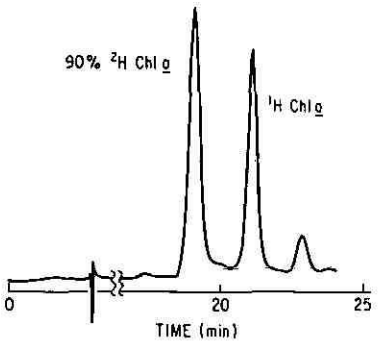

Fig. 2. Separation of 90% deuterated chlorophyll *a* from protio chlorophyll *a*. Experimental conditions were as in Fig. 1.

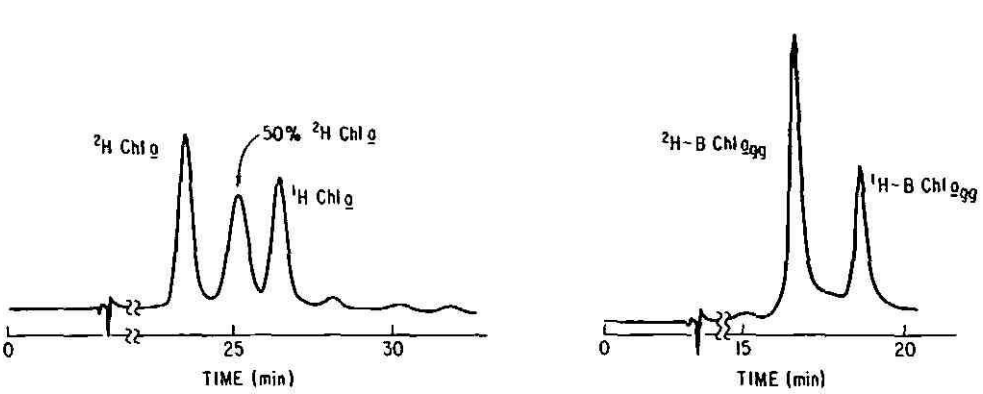

Fig. 3. Baseline separation of deuterated chlorophyll *a*, 50% deuterated chlorophyll *a* and protio chlorophyll *a* using a reversed-phase C_{18} column. Experimental conditions were as in Fig. 1.

Fig. 4. Reversed-phase HPLC separation of deuterio bacteriochlorophyll *a* (geranylgeranyl chain) from its protio analogue. The mobile phase consisted of water–methanol–acetonitrile–tetrahydrofuran (10:27:36:21, v/v). Flow-rate was 1 ml/min and detection set at 773 nm.

50% ^{2}H-chl *a* (t_R = 22.1 min, k' = 6.7), and finally by ^{1}H-chl *a* (t_R = 23.2 minutes, k' = 7.1). A solvent system water–organic (10:90) separated the three peaks at baseline level but the run was extended (approximately 100 min). Again the asymmetric peak failed to resolve further.

Fig. 4 shows the separation of ^{2}H-Bchl $a_{\text{geranylgeranyl (gg)}}$ from ^{1}H-Bchl a_{gg}. A similar separation was possible for the isotopic forms of Bchl a_{phytyl} (Table I). It was also possible to baseline separate protio Bchl a_{gg} (t_R = 7.5 min, k' = 1.6) from protio Bchl a_{phytyl} (t_R = 13.6 min, k' = 3.8) (Fig. 5). Similarly, deuterio Bchl a_{gg} when

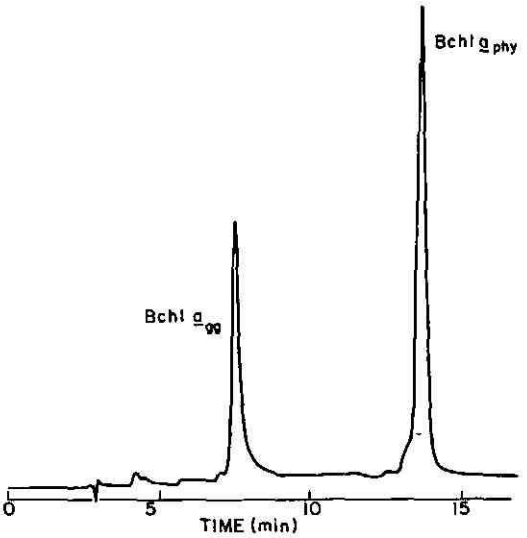

Fig. 5. Separation of protio bacteriochlorophyll *a* (geranylgeranyl chain) from protio bacteriochlorophyll *a* (phytyl chain). The geranylgeranyl chain has three double bonds more (*i.e.*, six hydrogen atoms less) than the phytyl chain.

paired and run with deuterio Bchl a_{phytyl} had a retention time of 7 min ($k' = 1.5$), and the latter eluted in 12.5 min ($k' = 3.4$). The deuterio isotopes remained on the column for a shorter period of time than their respective protio analogues. These studies were designed to observe the effects of the two kinds of tail chains present.

Chlorophylls lose the carbomethoxy group at the C-10 position when heated in pyridine solution to yield green "pyro" derivatives. The spectral properties are similar to those of the parent chlorophyll, With the quaternary solvent system, deuteriopyrochlorophyll *a* emerged in 8.2 min followed by protio pyrochlorophyll *a* in 8.9 min.

DISCUSSION

In all chlorophyll studies the deuterated compound eluted ahead of its protio analogue. When examined in pairs, *i.e.*, deuterio compound versus 100% ^{1}H-chl *a*, baseline separation was observed. There is evidence on the basis of t_R values that the greater the percentage of deuterium in the compound, the faster it will move through the column. In other words the elution pattern is related to the percent ^{2}H character. Fully deuterated chl *a*, partially deuterated chl *a*'s (90% ^{2}H-chl *a*, 80% ^{2}H-chl *a* and 50% ^{2}H-chl *a*) and ordinary chl *a* were mixed and run together. It was observed that it was not possible to separate 100% ^{2}H-chl *a*, 90% ^{2}H-chl *a* and 80% ^{2}H-chl *a* from one another in the mixture. Fifty percent deuterated chl *a* and 100% ^{1}H-chl *a* did separate as individual peaks from the mixture. In another study, 100% ^{2}H-chl *a* and 100% ^{1}H-chl *a* were mixed and chromatographed together. In this instance a clear baseline separation was observed for the compounds. Thus, while it was not possible to achieve baseline separation in a mixture of all five components, a good baseline separation in a mixture of 100% ^{2}H-chl *a*, 50% ^{2}H-chl *a* and 100% ^{1}H-chl *a* can be obtained.

Bacteriochlorophyll *a* obtained from the culture *Rhodospirillum rubrum* possesses a geranylgeranyl$_{(gg)}$ tail chain whereas Bchl *a* from the organism *Rhodospirillum spheroides* has the phytyl tail chain. The geranylgeranyl chain has three double bonds more than the phytyl chain, *i.e.*, six hydrogen atoms less. In a separation study involving Bchl a_{gg} and Bchl a_{phytyl}, the former eluted first. This should suggest then that not only does the tail chain participate in the chromatographic process, but that at the atomic level, the number of hydrogens present also influences Van der Waals attractive forces between the solute and the adsorbent. Therefore, Bchl *a* from *R. spheroides*, which possesses the phytyl chain, adhered strongly compared to Bchl *a* obtained from *R. rubrum* which has the geranylgeranyl chain.

At the outset it appears that such chromatographic behaviour is independent of the size of the molecule. It has been mentioned that the entire molecule need not be considered when dealing with isotope effects[22]. The major contribution for such effects is essentially from isotopically participating bonds and not from vicinal bonds. In other words, the chromatographic behaviour at the fundamental level is the interaction between the C–H bond (for the protio compound) or the C–^{2}H bond (for the deuterated compound), and the stationary phase. The elution of the ^{2}H-isotopic form before its protio analogue suggests then that Van der Waals forces are operational. A C–H bond has a higher oscillation frequency (3300 cm^{-1}) than the C–^{2}H bond (2333.8 cm^{-1}). With the oscillation frequency an electromagnetic field is created

and the electrons in the C–H bond are subject to this field. In turn, this electromagnetic field for a C–H bond creates a large induced field of opposite charge in other molecules around it. The C–H bond induces greater forces of attraction between itself and the stationary phase. The C–^{2}H bond, on the other hand, has a lower oscillation frequency, a less electromagnetic field is created, and the forces of attraction that develop between the C–^{2}H bond and the stationary phase are weaker. Therefore, the C–^{2}H bond with a lower Van der Waals force of attraction elutes ahead of its protio analogue.

RP-HPLC has been shown to be a versatile tool for separating isotopically analogous compounds. In this study the compounds were identical in all respects except the C–H and the C–^{2}H bond which differ in frequency of vibration. It appears that the larger the aliphatic C–H component in the compound, the easier will separation be effected. With the increased popularity of HPLC and the availability of more efficient columns isolation, purification, and quantification studies can now be performed. Thus, RP-HPLC has the very strong potential of becoming the standard analytical method for the separation of mixtures of photosynthetic compounds of differing hydrogen isotopic composition.

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SEPARATION OF GUINEA-PIG PANCREATIC JUICE PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Using a Protesil 300 octyl reversed-phase column with a multistage water–acetonitrile solvent gradient system, it was possible to obtain a well-resolved separation of the nine major proteins present in guinea-pig pancreatic juice. The protein present in each peak of the pancreatic juice chromatogram could only be identified by molecular weight analysis as the acetonitrile denaturated the enzymes and altered their isoelectric points. However, using sodium dodecyl sulphate gel electrophoresis the protein fractions obtained by high-performance liquid chromatography were characterised. Preliminary work has indicated that this system may be capable of separating other complex biological protein mixtures, *i.e.*, saliva.

INTRODUCTION

Hitherto, separation and quantitation of the proteins present in pancreatic juice has been achieved using electrophoretic techniques. Scheele[1] used two-dimensional gel electrophoresis to separate ^{14}C-labelled proteins secreted by isolated guinea-pig pancreatic lobules. Isoelectric focussing was used in the first dimension and gradient polyacrylamide gel electrophoresis in the second. After electrophoresis and staining, each protein spot was excised from the gel and its radioactivity counted, allowing the mass of protein present in each band to be determined. Dagorn[2] simplified the system by subjecting the pancreatic juice only to isoelectric focussing and by relating the degree of Coomassie blue staining of each band to the mass of protein present. The staining pattern of the gels was measured using a scanning UV spectrophotometer, the quantity of protein present in each band being directly related to its absorbance.

Though the techniques developed by Scheele and by Dargorn do give a sep-

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aration of the major proteins present in pancreatic juice, they have one major drawback. The preparation, running and staining of electrophoretic gels requires skill and is both tedious and time consuming. These techniques are therefore not ideally suited for the analysis of large numbers of samples.

In this paper we describe a separation technique for guinea-pig pancreatic juice proteins based on reversed-phase high-performance liquid chromatography (HPLC). Using one of the relatively new, wide-pore (300 Å), reversed-phase columns specifically designed for protein separation it is possible to separate pancreatic secretory proteins. Coupled with UV spectrophotometric analysis of the eluted proteins, this allows simple, accurate and reproducible quantitation of the proteins present in pancreatic juice.

A report of the initial work was given at the 1984 meeting of the European Pancreatic Club[3].

EXPERIMENTAL

Analytical HPLC

Equipment. All chromatography was performed on a Varian (Palo Alto, CA, U.S.A.) 5020 liquid chromatography system. The eluted proteins were detected by a Varian UV50, UV–visible, variable-wavelength detector and the resulting chromatograms were printed out either by a Varian 9176 chart recorder or a Varian Vista 402 integrator system.

Column. A Protesil 300 octyl, 250 × 4.8 mm reversed-phase column was used (Whatman, NJ, U.S.A.).

Solvent system. Water and acetonitrile, each containing 0.1% (v/v) trifluoroacetic acid (TFA), were run as a gradient system, the details of which, with their flow-rates, are given in the legends to the figures.

Preparation of samples. The samples of pancreatic juice were untreated. However, their protein concentrations were determined and adjusted to 1.0 to 1.5 mg/ml by addition of deionized water. Samples were loaded onto the column via a fixed-volume (10 μl) loop injector.

Preparative HPLC

Equipment. The chromatography system, detector and integrator were as stated above. Eluted fractions from the column were collected in 0.2-min timed volumes using an LKB Ultrorac 7000 fraction collector. The void volume between the flow cell of the detector and the fraction collector was approximately 50 μl.

Column. A Protesil 300 octyl, 250 × 9 mm reversed-phase column was used (Whatman).

Solvent system. Water and acetonitrile, each containing 0.1% (v/v) TFA, were run in a gradient consisting of five steps. Flow was initiated at 0% acetonitrile, 100% water. The concentration of acetonitrile was increased at a rate of 12%/min for 3 min. After 3 min, the rate of acetonitrile addition was changed to 0.5%/min for 8 min, 0%/min for 4 min, 0.5%/min for 18 min and finally to 2%/min for 12 min. This gradient is identical to the one found to produce the best separation on the analytical Protesil column (see Results). However, the flow-rate was increased from 1.5 to 6.2 ml/min to compensate for the larger volume of the column.

Preparation of samples. Pancreatic juice samples for the preparative runs were obtained by freeze drying bulk volumes of guinea-pig pancreatic juice. The dried protein was redissolved in deionized water to give a final protein concentration of 50 mg/ml and 500 μl of this solution were used for each run.

Storage of collected fractions for electrophoresis. After collection the fractions were freeze dried and stored at −80°C.

Collection of pancreatic juice. Male guinea-pigs weighing 400 to 500 g which had been denied food overnight were anaesthetized with sodium pentobarbitone (30 mg/kg, i.p.). Through an abdominal incision the main pancreatic duct was then cannulated and the pylorus ligated to prevent gastric contents entering the duodenum. Pancreatic secretion was stimulated by a constant infusion of synthetic porcine secretin (2.0 μg/kg · h) via a catheter in the jugular vein. Pancreatic juice was collected for 20 consecutive 5-min periods and stored at −4°C.

Sodium dodecyl sulphate (SDS) gel electrophoresis

SDS gel electrophoresis was carried out according to the method of Laemmli[4]. The running gel contained 0.1% (v/v) SDS, 0.375 *M* Tris–HCl, pH 8.8, and either 10 or 15% (v/v) acrylamide. Polymerization was initiated by the addition of 0.025% (v/v) tetramethylethylenediamine (TEMED) and was complete within 1 h. The gels were formed in a Perspex mould (dimensions 90 × 120 × 2 mm).

Preparation of protein samples. The protein samples (1.0 mg/ml concentration) were boiled for 15 min with an equal volume of buffer containing 2.5 m*M* Tris–HCl, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (v/v) bromophenol blue. Samples were delivered to the gel slots in 40-μl quantities. The loaded gel was run with a current of approximately 5 mA, the current being adjusted occasionally to allow the gels to run within 3 h.

Staining procedure. Gels were stained by immersing them for 1 h in an aqueous solution of 5% (v/v) sulphosalicyclic acid, 5% (v/v) trichloroacetic acid (TCA), 30% (v/v) isopropanol and 0.12% (v/v) Coomassie blue. The stained gels were then destained by placing them in an aqueous solution containing 30% (v/v) isopropanol and 10% (v/v) acetic acid. After destaining the gels were stored under water at 22°C.

Materials

All the chemicals used for the electrophoresis were obtained from Sigma, Poole, U.K. Synthetic secretin used was supplied by Cambridge Research Biochemicals, Marston, U.K. The solvents used for the HPLC were purchased from Rathburn, Walkerburn, U.K.

RESULTS

Initially a very crude separation of guinea-pig pancreatic juice proteins was obtained using a relatively steep elution gradient (4%/min) (Fig. 1). By adjusting the rate of addition of the acetonitrile and changing the solvent flow-rate, the preliminary separation was improved to that shown in Fig. 2. The gradient alterations consisted of slowing to different degrees various segments of the original gradient. To combat the peak-broadening effect of slowing the addition of acetonitrile, the eluent flow-rate was increased from 1.0 to 1.5 ml/min.

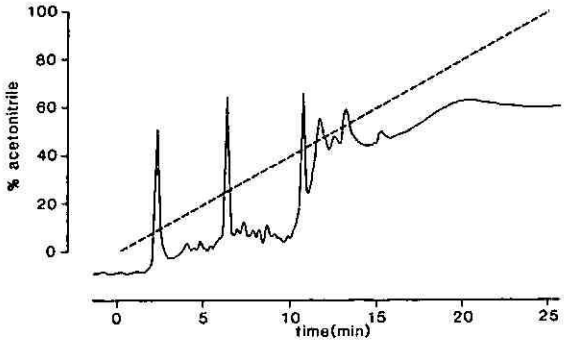

Fig. 1. The separation obtained on placing 10 μl of secretin-stimulated guinea-pig pancreatic juice on the Protesil 300 octyl (250 × 4.8 mm) reversed-phase column. Conditions: flow-rate 1.0 ml/min; solvent, water–acetonitrile; gradient, acetonitrile increasing linearly at the rate of 4%/min (denoted by the broken line); detection wavelength, 206 nm.

After the system had been optimized for the separation of guinea-pig pancreatic juice proteins, each of the eluted peaks was identified. Characterization of the eluted proteins by their biological activity or isoelectric point proved impossible because the strong denaturing effect of acetonitrile destroyed the enzymatic activity and altered the isoelectric point of the pancreatic enzymes[5]. One parameter which was not affected by the eluting solvent was protein molecular weight. Consequently the protein constituent of each of the eluted peaks was identified on the basis of its molecular weight using SDS gel electrophoresis.

Pure samples of each of the chromatographic peaks were obtained using a preparative form of the analytical column. The chromatogram obtained from the larger column is shown in Fig. 3. To minimize the problem of contamination from adjacent peaks, only mid-peak fractions were collected for analysis. Initially all the peak fractions were run on 10% SDS gels. From these it was possible to observe

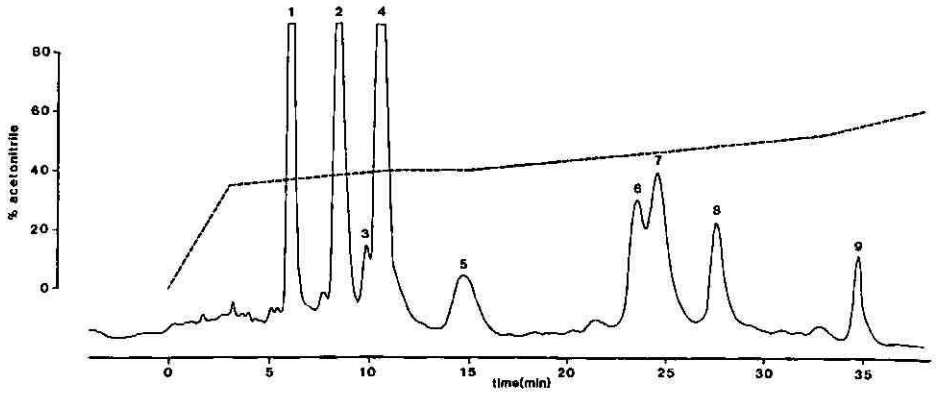

Fig. 2. The optimal separation obtained on placing 10 μl of secretin-stimulated, guinea-pig pancreatic juice on the Protesil 300 octyl (250 × 4.8 mm) reversed-phase column. Conditions: flow-rate, 1.5 ml/min; solvent, water–acetonitrile; gradient, 5 steps, denoted by the broken line; detection wavelength, 206 nm. The sloping baseline was eliminated by the use of the Vista 402 integrator. For peak identification, see Table I.

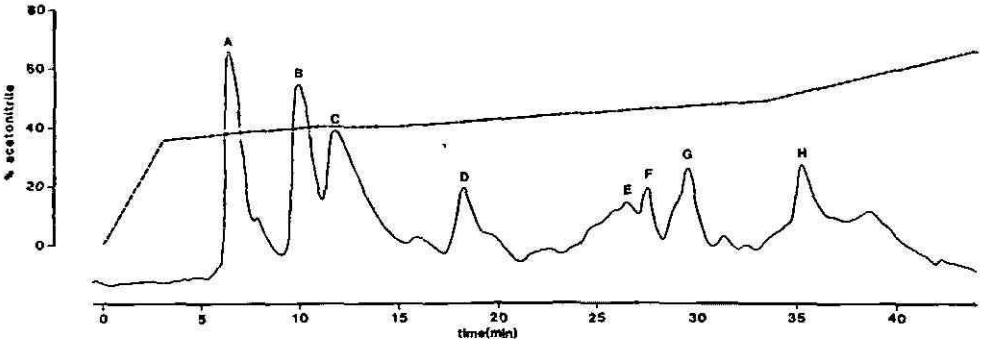

Fig. 3. The separation obtained from 500 μl of guinea-pig pancreatic juice placed on the Protesil 300 octyl (250 × 9 mm) reversed-phase column. Conditions: flow-rate, 6.2 ml/min; solvent, water–acetonitrile; gradient, 5 steps, denoted by the broken line; detection wavelength, 206 nm. The sloping baseline was eliminated by the use of the Vista 402 integrator. For peak identification, see Table I.

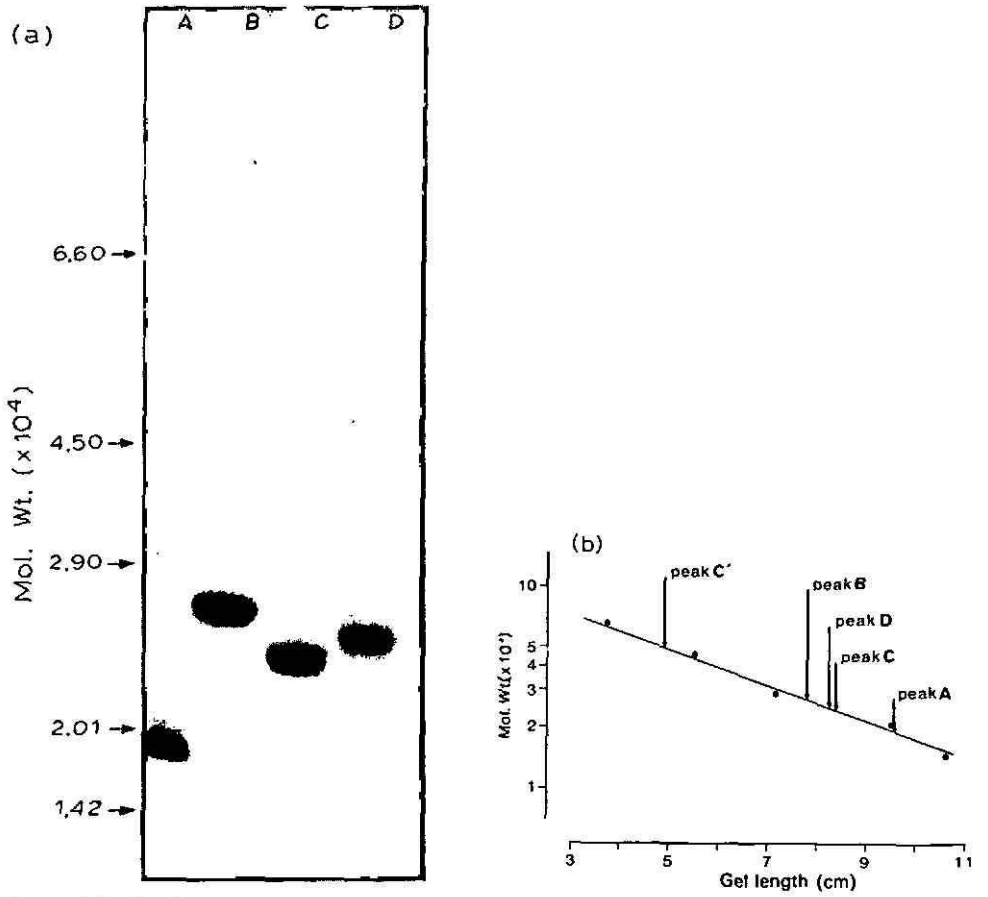

Fig. 4. (a) Sodium dodecyl sulphate electrophoresis on a 15% polyacrylamide gel of the unreduced isolated fractions from peaks A, B, C and D from the preparative HPLC run shown in Fig. 3. Running time 3 h; protein load, 20 μg per track. The positions of the molecular weight standards are represented on the left hand edge of the gel. (b) Semi-log plot of molecular weight *versus* gel length for the non-reduced molecular weight standards.

which of the samples contained low-molecular-weight proteins (15 000 to 30 000) and which contained the larger proteins (30 000 to 70 000). This was important because the 10% SDS gels did not have the required sensitivity to analyse the lower-molecular-weight proteins. Therefore the peak samples containing the lower-molecular-weight fractions were re-run on 15% SDS gels, and the larger proteins were run again on 10% SDS gels (Figs. 4 and 5).

From the gels it was apparent that, even though mid-peak fractions were collected, contamination from adjacent peaks still occurred. In general the minor bands resulting from this cross-contamination were ignored and the main band used for molecular weight determination. However, there was one exception. In the case of track C (Fig. 4), in addition to the usual contamination, there was also an extra

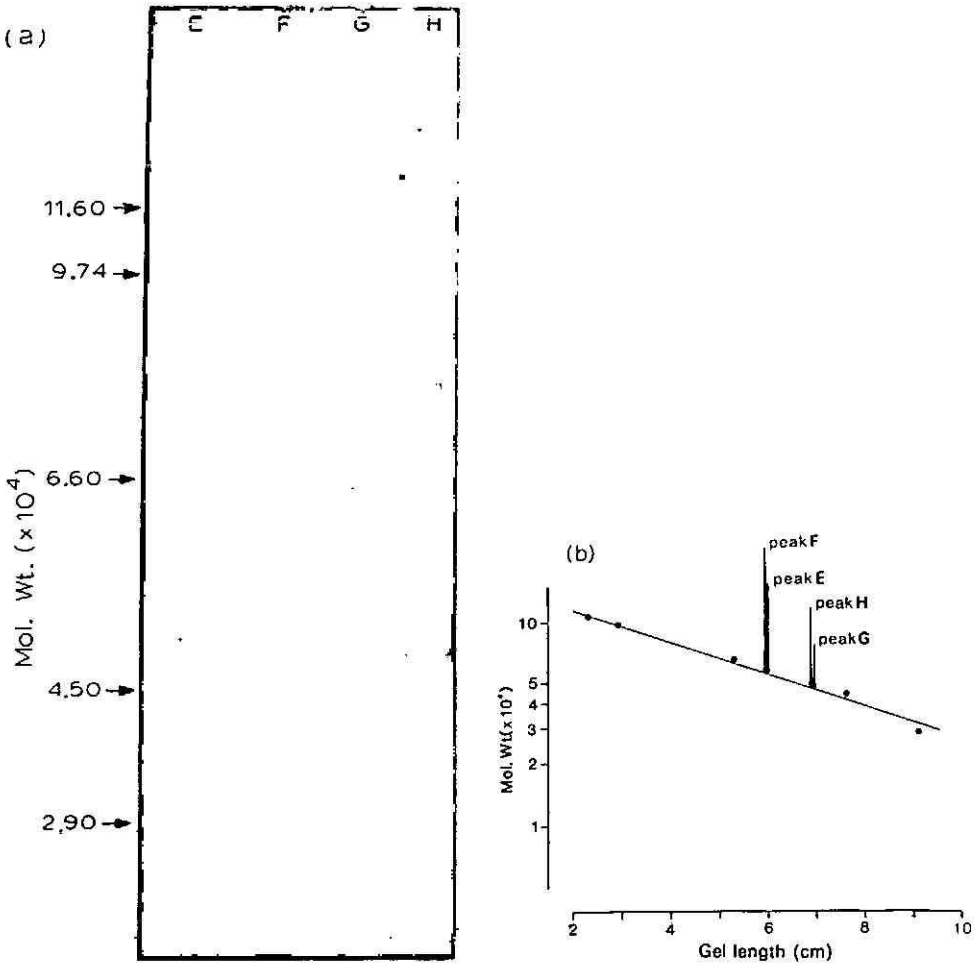

Fig. 5. (a) Sodium dodecyl sulphate electrophoresis on a 10% polyacrylamide gel of the unreduced isolated fractions from peaks E, F, G and H from the preparative HPLC run. Running time 3 h; protein load, 20 μg per track. The positions of the molecular weight standards are represented on the left-hand edge of the gel. (b) Semi-log plot of molecular weight *versus* gel length for the non-reduced molecular weight standards.

TABLE I

MOLECULAR WEIGHTS (OBTAINED BY SDS ELECTROPHORESIS) OF THE PEAK FRACTIONS COLLECTED FROM THE PREPARATIVE HPLC COLUMN

For identification the molecular weight values obtained are compared with the values obtained by Scheele[1] for the guinea-pig pancreatic enzymes.

Peak No.		*Molecular weight*		*Identity*
Analytical HPLC	*Preparative HPLC*	*This study*	*Scheele*	
1	A	19 000	18 750	Trypsinogen
2	B	28 300	28 200 and 28 700	Proelastase
3	C'	47 200	47 600	Lipase
4	C	24 600	25 000	Chymotrypsinogen 1
5	D	25 200	25 000	Chymotrypsinogen 2
6	E	52 000	51 000	Amylase
7	F	54 300	54 000	Amylase
8	G	45 200	45 100 and 45 300	Procarboxypeptidase A
9	H	46 000	46 000	Procarboxypeptidase B

protein band (C') with a molecular weight of 47 200. This may represent peak 3 on the analytical chromatogram (Fig. 2) which was not resolved in the preparative run (Fig. 3), probably because it was masked by the much larger B and C peaks. The molecular weight of each of the isolated peak fractions are listed in Table I.

The chromatogram from the preparative column (Fig. 3) was different from that obtained using the analytical column (Fig. 2) due to inter-column variance and the size difference between the columns. Overall the elution profiles were similar but the elution times were altered. Therefore, to match each peak on the preparative chromatogram with its associate on the analytical chromatogram, a sample of each of the collected fractions from the preparative column was loaded, in turn, onto the smaller protesil column. The resulting chromatograms were compared with a standard separation of whole guinea-pig pancreatic juice. The main peak from each preparative fraction could then be directly related to a peak in the analytical chromatogram (Fig. 6). Using this procedure each of the isolated peak fractions was assigned to a peak on the analytical chromatogram (see Table I). Close examination of the trace obtained from fraction C shows it to contain a peak which is comparable to peak 3 on the analytical chromatogram. This would appear to confirm the suggestion that the extra protein band observed in track C of the 15% SDS gel is due to this peak.

The above maneouvres allowed the molecular weights of the protein present in each of the peaks eluted from the analytical column to be obtained. To identify these proteins their molecular weights were compared with those of known guinea-pig pancreatic juice proteins[1]. This comparison is shown in Table I.

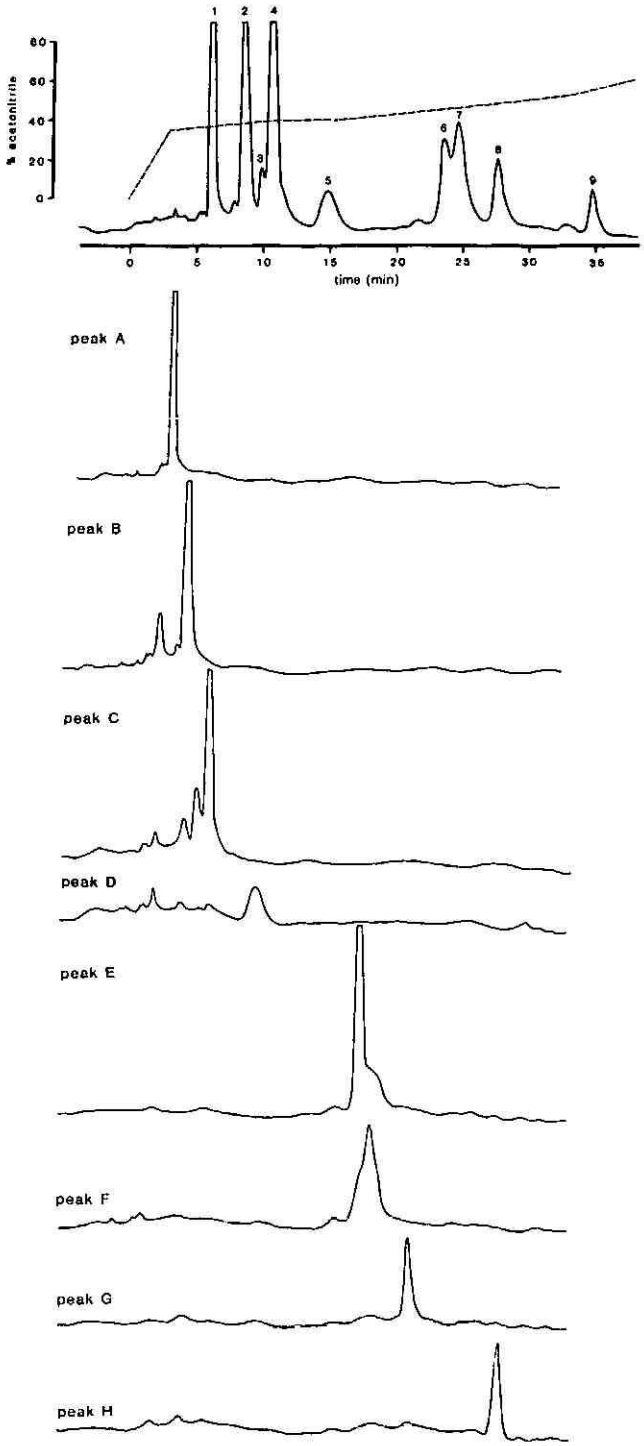

Fig. 6. Comparison of the chromatograms produced from 10-μl samples of whole guinea-pig pancreatic juice and the mid-peak fractions collected during preparative HPLC. All separations were obtained using the Protesil 300 octyl (250 × 4.8 mm) reversed-phase column. Conditions: flow-rate, 1.5 ml/min; solvent, water–acetonitrile; gradient 5 steps, denoted by the broken line on the top chromatogram; detection wavelength 206 nm.

DISCUSSION

Initially we determined the feasibility of using a gel permeation system to obtain a separation of pancreatic juice proteins. However, the columns tested did not have the resolution required to separate the individual secretory enzymes. Consequently we experimented with reversed-phase HPLC. Using a wide-pore (300 Å) C_8 reversed-phase column with a water–acetonitrile gradient elution system it is possible to obtain a clear separation of the main protein constituents in guinea-pig pancreatic juice. Other solvent pairs were tested (water–methanol, triethylamine acetate–propanol) but these did not produce any clear separations.

Though the reversed-phase mode of HPLC does have a high power of resolution, it has one severe drawback: the solvents used to produce the separations often have deleterious effects on the proteins they elute, making characterization of the separated proteins difficult. Unfortunately this was true of acetonitrile: its strong hydrophobic nature and low pH altered the isoelectric points and destroyed biological activity of the pancreatic juice enzymes. However, the solvent does not appear to change a protein's molecular weight. Therefore, using SDS electrophoresis, it was possible to determine the molecular weight of each of the major protein peaks present in the analytical chromatogram. By comparing these values with the known[1] molecular weights of the guinea-pig pancreatic juice enzymes, it was possible to identify each of the peaks observed in Fig. 2.

As the molecular weights obtained for the eluted protein peaks were very close to values determined by Scheele[1], identification of each of the peaks could be made with some certainty. A second method of characterizing the identity of these peaks is needed to confirm this study. At the present time we believe this study is of value in its own right since it can be applied to other protein systems equally well. It has the big advantage that no biological property of the proteins is being assayed, since fractionation and characterization depends solely on the physical properties of individual proteins.

In conclusion, the reversed-phase HPLC method we describe allows rapid, accurate separation of the major constituents of guinea-pig pancreatic juice. Preliminary observations suggest that this method is applicable to pancreatic juice from human, cat, rat and hamster and to saliva from rabbit mandibular gland, although the system must be optimized for each of these examples.

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ISOLATION OF HUMAN HAEMOGLOBIN VARIANTS WITH ALTERED BOHR EFFECT

APPLICATION TO HAEMOGLOBIN RAINIER

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SUMMARY

Isoelectric focusing on polyacrylamide gel in the absence of haem ligands represents a useful, convenient and rapid procedure to isolate silent Hb variants in their native forms, provided that they exhibit an abnormal Bohr effect. The amount of material which is eluted is sufficient for both a limited functional study and a structural determination using microscale high-performance liquid chromatography. This is exemplified by the isolation and the study of Hb Rainier.

INTRODUCTION

Almost 500 human haemoglobin (Hb) variants have been described[1] but the isolation of those with electrophoretic mobility identical to that of HbA remains technically difficult.

The probability of detecting these neutral Hb variants is very low when they are not associated with haematological disorders. Abnormal Hbs are mainly discovered by systematic electrophoretic screening of blood samples during population studies, and neutral substitutions are therefore not detected. The discovery of these neutral substitutions may be of interest for genetic reasons, as is the case for HbF Sardinia which is linked to specific molecular forms of β thalassemia[2]. In populations in which other Hb disorders such as sickle cell anaemia are common, it may be also important to know whether some silent variant is interacting with the more frequent ones modifying the course of the disease.

By contrast, in patients suffering from haemolytic anaemia, erythrocytosis or cyanosis, a haemoglobin abnormality is carefully looked for, even if the standard electrophoretic study exhibits a normal pattern. Additional electrophoretic methods are necessary, easy ones such as citrate agar gel electrophoresis, or more discrimi-

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native ones, such as isoelectric focusing (IEF) on polyacrylamide gels[3] or, even, immobilized pH gradient IEF[4]. When the abnormal component is separated from the normal one by any of these techniques, this difference in electrophoretic behaviour, small as it is, can form the basis of a preparative separation of the mutant Hb[5].

Other abnormal properties, like a decrease in stability or solubility, may reveal a neutral Hb variant. These properties may be used for purifying the variant. However, this results in the isolation of a non-functional Hb.

In some cases the abnormality is demonstrated through a study of the lysate by reversed-phase high-performance liquid chromatography (RP-HPLC). Normal and abnormal subunits will be observed in the elution pattern[6]. Nevertheless, the separation of the tetrameric native form cannot be achieved by this method.

Oxygen equilibrium curves for red blood cells may also indicate the presence of a variant Hb. An alteration in the oxygen binding properties, when it modifies the exposure of the Cys β 93 SH group, has been used for isolating Hb Creteil[7] after selective binding of cystamine.

The isoelectric point differs between the two quaternary conformations of the Hb molecule in accord with the alkaline Bohr effect[8]. Therefore when an electrophoretically silent Hb variant displays an increased oxygen affinity and a decreased Bohr effect, its purification by IEF in the deoxy form is expected.

In this paper we demonstrate the use of this property for isolating a variant having high oxygen affinity, Hb Rainier, α_2 β_2 145 (HC2) Tyr → Cys[9]. Another variant possessing high oxygen affinity, but with a normal Bohr effect, Hb San Diego, α_2 β_2 109 (G11) Val → Met, the preparation of which by immobilized pH gradient IEF we have described previously[5], could not be distinguished by this procedure.

MATERIALS AND METHODS

Haematological studies

Blood was collected on EDTA. Haematological studies were performed according to standard procedures.

2,3-Diphosphoglycerate (2,3-DPG) in the red blood cells (RBCs) was measured by the phosphoglycerate method (Boehringer Kit, Mannheim, F.R.G.).

Haemoglobin studies and analytical electrophoreses

RBCs were lysed with water, extracted with toluene and centrifuged at 12000 *g* for 45 min.

Standard electrophoreses were performed at pH 8.6 in Tris–EDTA–borate buffer on cellulose acetate plates (Helena Labs., Beaumont, TX, U.S.A.). Citrate agar gel electrophoreses were done at pH 6.3 (Corning, Palo Alto, CA, U.S.A.).

Isoelectric focusing studies were carried out on a 0.2-mm polyacrylamide gel containing a mixture of Ampholines pH 3.5–10, 6–8, 7–9 (LKB, Bromma, Sweden) and of separators (6-aminocaproic acid and alanine) as described by Cossu *et al.*[10].

The alkali denaturation was tested with 0.083 *M* sodium hydroxide as described by Singer *et al.*[11]. A kinetic study was performed on the patient's haemolysate in comparison with a normal one.

Preparative IEF in the deoxy form

The Hb solutions were deoxygenated in a glass tonometer under pure

argon until the ratio of absorbances at 554.5 and 540 nm was at least equal to 1.25. To complete the deoxygenation of the high affinity variant, a two-fold molar excess of dithionite was added. IEF was performed in cylinders of polyacrylamide gel using the technique of Bunn[12] as modified by Poyart *et al.*[8]. To each tube, 40–50 μl (corresponding to about 200–250 μg Hb) were applied. At the end of the experiment the bands were cut and the Hb was eluted from the gel in 50 m*M* bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane (bis-tris), pH 7.2 buffer.

Functional studies

Oxygen equilibrium curves for red blood cell suspensions were determined with an Hemox-Analyzer (T.C.S., Southampton, PA, U.S.A.) as described[13]. For the isolated abnormal component, such curves were obtained by the discontinuous equilibrium technique[14] using a Cary 219 spectrophotometer, in 0.1 *M* sodium chloride, 50 m*M* bis-tris, pH 7.2 buffer at 25°C. The amount of metHb present in the sample was measured from the ratio of absorbances at 576 and 500 nm under an atmosphere of pure oxygen.

The Bohr effect was estimated by measuring the difference in isoelectric points between the deoxy form and the fully liganded carbonyl form.

Structural characterization

The pure Hb fractions isolated as described above were concentrated under vacuum. The subunits were separated by RP-HPLC on an Aquapore RP 300 column (C_8, porosity 300 Å). A 1-mg amount of globin was chromatographed using a gradient of acetonitrile–trifluoroacetic acid–methanol as described by Baudin and Wajcman[15]. The fractions were collected manually and dried. The chains were then hydrolyzed with trypsin.

The tryptic digest (0.2–0.5 mg) was dissolved in 10% acetic acid and applied on a RP-HPLC column. In order to obtain a good resolution of all the peptides, including the small ones, a Microbondapak column (C_{18}, porosity 100 Å; Waters Assoc.) was selected and the elution performed as described previously[16].

In parallel, a structural study was undertaken using more conventional methods including globin precipitation with acid acetone, subunit separation by CM-cellulose chromatography in 8 *M* urea, aminoethylation and peptide analysis as described[17].

The amino acid composition of the peptides was determined by HPLC according to a modification of the procedure described by Heinrikson and Meredith[18]. The retention time of phenylthiocarbamoyl(aminoethyl)cysteine (PTC-AECys) was determined by the analysis of normal Cys-containing peptides after aminoethylation. PTC-AECys is slightly more strongly retained than PTC-Lys.

RESULTS

Case report

The patient, 56 years old, was referred to the hospital as exhibiting polycythaemia. The haematological parameters were as follows: RBC, $6.46 \cdot 10^{12}$ l^{-1}; Hb, 20.9 g/dl; haemocrit, 0.59. The leukocyte and platelet counts were normal. This polycythaemia had been found in several other members of the patient's family. The

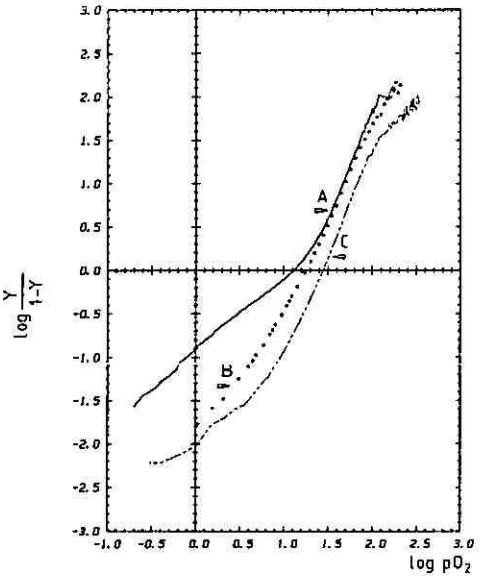

Fig. 1. Oxygen equilibrium curves obtained for red blood cell suspensions. Experimental conditions: 50 m*M* bis-tris, 140 m*M* sodium chloride 10 m*M* glucose, pH 7.4, 37°C. (A) Heterozygote HbA/Hb Rainier; (B) heterozygote HbA/Hb San Diego; (C) normal control. Y represents the fractional saturation of haemoglobin with oxygen.

erythrocytic 2,3-DPG level was normal (0.9 mmol per Hb tetramer, normally 0.85 ± 0.05). The oxygen affinity of red blood cell suspensions was increased two-fold [P50 (the partial pressure of oxygen at half saturation in mmHg) = 13 mmHg, normally = 26 ± 1 mmHg at pH 7.4 and 37°C]. These facts argued for the presence of an abnormal haemoglobin.

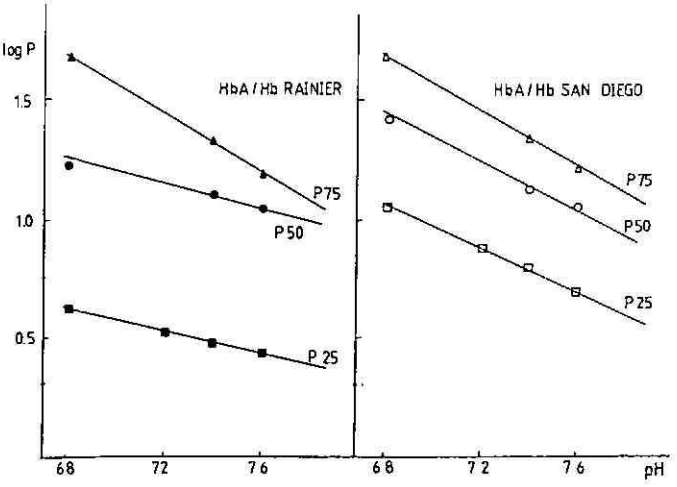

Fig. 2. Variation of log pO_2 with pH at different oxygen saturation levels. A decreased alkaline Bohr effect is observed in the bottom of the oxygen equilibrium curve of the HbA/Hb Rainier red blood cell suspension.

Haemoglobin studies

(a) Electrophoreses. No abnormal component nor HbF could be detected by alkaline pH electrophoresis of the lysate and the stability of the lysate was found to be normal. Citrate agar electrophoresis showed an abnormal Hb migrating like HbF. The resistance to alkali denaturation was increased (9.6% in 2 min, *versus* 0.8% in the control), suggesting the presence of Hb Rainier.

Standard IEF of the propositus haemolysate showed no abnormal band.

(b) Blood oxygen binding properties. Fig. 1 illustrates the oxygen binding curve for the propositus red blood cell suspension compared to that of a patient heterozygous for Hb San Diego and to that of a normal individual. For both abnormal red blood cells the oxygen affinity was increased. A biphasic Hill plot and a lower cooperativity at P50 were found in the red cells of the propositus.

The variation of $\log pO_2$ with pH, at different oxygen saturation levels, showed a decreased alkaline Bohr effect in the bottom portion of the oxygen equilibrium curve. By contrast, a constant and normal value was observed for the red cells of the patient heterozygous for Hb San Diego (Fig. 2).

On this basis the separation of the abnormal fraction was attempted by isoelectric focusing in the deoxy form of the haemolysate.

IEF of the deoxy form (Fig. 3)

The IEF pattern of the deoxygenated lysate demonstrated the presence of a component with a slightly lower isoelectric point than deoxyHbA (7.04 *versus* 7.12 for the control) and a content of approximately 30%. The difference in isoelectric point (0.09 against 0.17 pH units for the normal case) suggests a 40% reduction in the alkaline bohr effect.

When the lysate was fully liganded with CO, only one band could be seen with a p*I* of 6.95.

Hb San Diego, which exhibits a normal Bohr effect[19], showed a similar difference in isoelectric points, between the deoxy and the liganded forms as compared to HbA.

From eleven tubes, 1 mg of abnormal component was recovered.

Oxygen binding properties of the isolated fractions

Only one experiment could be performed due to the limited amount of Hb recovered from the IEF gels.

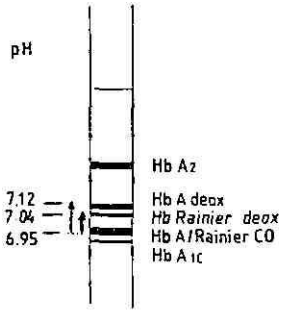

Fig. 3. *Isoelectric focusing on polyacrylamide gel. The lysate was applied both in the deoxy and in the carbonyl form; the decrease in Δp*I *upon deoxygenation of Hb Rainier is clear (0.09 instead of 0.17 pH units).*

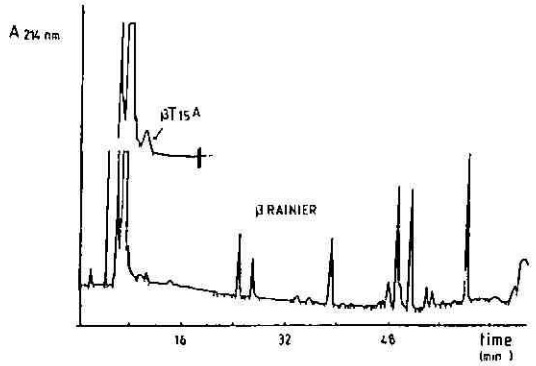

Fig. 4. RP-HPLC analysis of tryptic peptides. In the β chain of the isolated abnormal component, the peptide T15 is missing.

The visible spectrum of the oxygenated abnormal component was identical to that of HbA.

The P50 was 0.2 mmHg and the n50 (the slope of the Hill plot at half ligand salination) = 0.8 (0.1 *M* sodium chloride, 50 m*M* bis-tris buffer at 25°C and pH 7.2, 20 μg/ml catalase, 60 μM haem). Under identical experimental conditions, the oxygen binding parameters for HbA, eluted from the gels, were P50 = 3 mmHg and n50 = 2.4. In both samples, 6–8% metHb was present at the end of the experiments.

The abnormal functional properties for Hb Rainier have been previously studied in detail by Imai[20]. The values of P50, haem–haem interaction and Bohr effect reported in the present study correspond closely to those given by this author.

Structural determination

The abnormal subunit could not be isolated from the normal one by CM-cellulose chromatography in 8 *M* urea, and therefore a preliminary study was performed on a mixture containing the normal and abnormal β chains. After aminoethylation and digestion with trypsin, the RP-HPLC elution pattern on an Aquapore RP 300 column showed no abnormal peptide.

In agreement with the hypothesis of Hb Rainier heterozygoty, a peak containing only His and AE-Cys and another one corresponding to normal T15 were found in the front of the chromatogram using a Microbondapak column.

A study of the IEF-purified haemoglobin components showed no difference in the RP-HPLC elution pattern of the subunits between the normal and abnormal haemoglobins. After digestion with trypsin the peptides were analyzed by HPLC (Fig. 4). The β T15 peptide was missing from the abnormal sample, identifying the variant as Hb Rainier.

DISCUSSION

In many cases the size or charge properties of a protein do not allow its isolation from a mixture. Therefore separation methods based on biological properties have to be used. We can distinguish the purification of a protein either by true biospecific affinity, by non-biospecific affinity methods or by conformational specificities.

In the first case, affinity chromatography methods are widely used; a ligand which may be a substrate, a cofactor, a product or a stereospecific receptor is bound to the matrix. Immunoreactive properties may also be used. In the second case the ligand is a synthetic or a natural molecule displaying a less specific interaction; examples have been reported of the use of hydrophobic chromatography, boronate chromatography or agar gel electrophoreis for haemoglobin separation[21].

In this paper we have demonstrated that Hb molecules can be separated on the basis of their oxygen binding properties. It is known that there is a difference in the surface charges between the oxy and the deoxy forms. In variants with altered Bohr effects this difference will be reduced. Isoelectric focusing, being one of the most sensitive methods for separating Hbs on both analytical and preparative scales, can be applied to this problem.

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ION-EXCHANGE CHROMATOGRAPHY WITH POST-COLUMN REACTION FOR THE ANALYSIS OF PHOSPHONOFORMATE, PHOSPHITE AND PHOSPHATE

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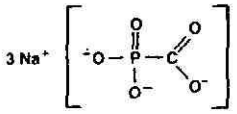
SUMMARY

Phosphonoformate, phosphite and phosphate are baseline separated within 6 min on a silica-based DEAE anion exchanger. The mobile phase consists of acetic acid, sulphate and a small amount of citric acid. Without citric acid, phosphonoformate is eluted with severe tailing. The presence of citric acid sharpens the peak dramatically. The detection system comprises an initial oxidation to phosphate by post-column addition of bromine. Thereafter a molybdovanadate reagent is added. Finally sulphite is added to reduce the excess of bromine which otherwise would disturb the subsequent detection at 340 nm. The detection limit for phosphate and phosphite is about 4 ng calculated as phosphorus, and for phosphonoformate is about 12 ng. The relative standard deviation for the peak area of 3.3 m*M* phosphonoformate is about 0.4%.

INTRODUCTION

Phosphonoformate (PFA) (I) is a potent antivirus substance with activity against several types of viruses[1,2]. Its use in different drug formulations requires methods of analysis of PFA and of its degradation products phosphite and phosphate. An ion-pair chromatography system with amperometric detection for PFA has been presented[3]. In that work a phosphate buffer was used. Furthermore, pyrophosphate was added to the mobile phase in order to obtain sharp peaks for PFA, which otherwise gave tailing peaks. However, this system is unable to determine phosphate and phosphite. Detection of these two ions in chromatography is complicated due to the lack of physical properties utilizable by, *e.g.*, UV and amperometric detectors. A conductometric detector could of course be employed. However,



(I)

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the choice of mobile phase is limited with this detector. Only phases with a sufficiently low background conductivity could be employed. Consequently, there is little possibility of finding a chromatographic system for the three title compounds which has sufficiently high stability, efficiency and resolution for drug formulation analysis.

In the present work, ion-exchange chromatography is explored for the separation of these three components. The column used is a silica-based DEAE anion exchanger. Detection is accomplished by employing a post-column reaction system based on the reaction between phosphate and a molybdovanadate reagent to form a yellow product[4,5]. PFA and phosphite are initially oxidized to phosphate by addition of bromine. A post-column reaction system for phosphate, phosphite and hypophosphite, based on oxidation by sulphite to phosphate and a subsequent reaction with molybdenum, has been presented[6]. However, it requires a reaction coil with a hold-up time of 2.5 min, and heating to 140°C. The present system is rapid and does not require elevated temperature.

The mobile phase used must be compatible with the detection system. The eluting ion employed here is sulphate. Citric acid is also added, otherwise PFA shows severe tailing. However, citric acid interferes in the detection system. The optimization of the citric acid content in the mobile phase, as well as other factors influencing the chromatography and detection, are discussed.

EXPERIMENTAL

The chromatographic system

An LKB 2150 high-pressure pump (LKB, Bromma, Sweden) was used with a flow-rate of 1.0 ml/min. The column contained DEAE Si100 Polyol, 3 μm (125 mm $\times$ 4.6 mm) (Serva, Heidelberg). Injections of 20 μl sample were performed with a Waters WISP autoinjector. Peak evaluations were made with a Spectra-Physics 4270 integrator.

The mobile phase consisted of 0.1 *M* acetic acid, 1 m*M* citric acid and 0.015 *M* sulphate. The sulphate was added as a mixture of sodium sulphate and sodium hydrogensulphate so that the final pH of the mobile phase was 2.7. When the effect of pH on retention was investigated, pH values below 3 were adjusted with appropriate amounts of the sulphate salts; above pH 3, 0.1 *M* sodium acetate was employed. When the effect of the sulphate concentration was investigated, the pH was kept constant at 2.9.

Previously unused columns were pre-conditioned at a flow-rate of 1 ml/min for 1–2 days with a mobile phase consisting of 8 m*M* citric acid, 0.015 *M* sulphate and 0.1 *M* acetic acid, pH 2.7.

The post-column detection system

Fig. 1 shows the detection system. First the bromine solution is added to the column effluent, the PFA and phosphite being oxidized to phosphate. Secondly, the molybdovanadate reagent and finally the sulphite solutions are added. The sulphite reduces the excess of bromine which otherwise would give rise to a background absorbance in the final detection. The detector was an LDC Spectromonitor III used at a wavelength of 340 nm. The reference cell of the detector was used due to its slightly larger dimensions and thus lower back-pressure. This was found advanta-

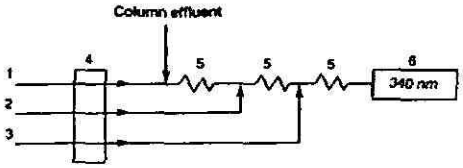


Fig. 1. The post-column detection system. 1 = Bromine; 2 = molybdovanadate reagent; 3 = sodium sulphite; 4 = peristaltic pump; 5 = PTFE mixing coil; 6 = detector.

geous for minimizing the risk of leakage. The mixing block was a Bifok Chemifold Type III (Tecator, Sweden) and the mixing coils were PTFE tubings (600 mm × 0.5 mm I.D.). The peristaltic pump used was an Alitea C-4V (Ventur AB, Uttran, Sweden). The success of the detection system was found to depend largely on the choice of peristaltic pump. Other pumps examined gave poor results due to large pulsations in the flow. The pump speed and choice of pump tubings were adjusted so that the bromine and sulphite solutions were added at 0.4 ml/min, and the molybdovanadate reagent at 0.8 ml/min.

Reagent and standard solutions

The bromine was prepared by the reaction between bromide and bromate in acidic solution. A 2.9-g amount of potassium bromide and 0.64 g of sodium bromate were dissolved in 25 ml of 1 *M* hydrochloric acid. The reaction was allowed to proceed for at least 2 h. This solution was then diluted to 500 ml in 0.1 *M* sodium acetate in order to obtain a slightly alkaline pH. Prior to use, the stock solution was diluted four times in deaerated 0.1 *M* sodium acetate. The concentration of the bromine solution was determined by titration with sodium sulphite to decolouration.

The molybdovanadate solution consisted of 1.6 m*M* ammonium molybdate and ammonium monovanadate in 0.15 *M* deaerated hydrochloric acid.

The sodium sulphite solution was made 0.03 *M* in deaerated water. This solution was freshly prepared each day.

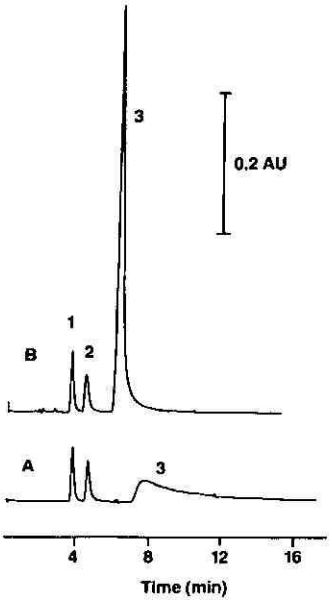
Trisodium phosphonoformate hexahydrate (Astra, Södertälje, Sweden), sodium dihydrogenphosphate (Merck) and sodium phosphite pentahydrate (Riedel-DeHaen) were used for preparation of standard solutions in water. All chemicals used were of analytical grade.

RESULTS AND DISCUSSION

Influence of citric acid

The dramatic influence of citric acid in the mobile phase on the PFA peak shape is illustrated in Fig. 2. Without citric acid, PFA is eluted with severe tailing. A pronounced sharpening of the peak is obtained at a citric acid concentration of 0.5 m*M*. With increasing concentration of citric acid, the peak tailing is reduced, though only minute changes can be seen by increasing the concentration from 1 to 5 m*M*.

Citric acid does however interfere in the detection system, and causes a decrease in response when the concentration reaches a critical level compared to the concentration of molybdovanadate reagent added. Under the conditions stated in



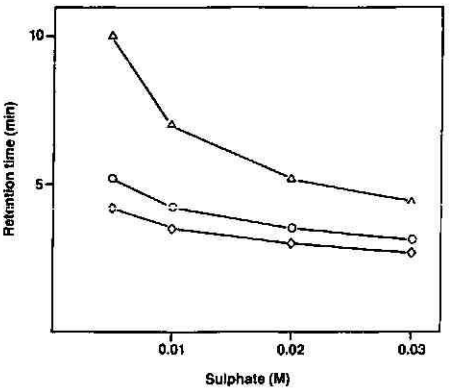


Fig. 2. Influence of citric acid on the peak shape of PFA. Sample: 0.23 m*M* phosphate (1), 0.23 m*M* phosphite (2) and 3.3 m*M* PFA (3). (A) Mobile phase: 0.1 *M* acetic acid and 0.01 *M* sulphate, pH 2.9. (B) As (A) but 1 m*M* citric acid added.

Fig. 3. Retention time at different sulphate concentrations. Mobile phase: 0.1 *M* acetic acid and 1 m*M* citric acid, pH 2.9. △—△, 3.3 m*M* PFA; ◇—◇, 0.25 m*M* phosphate; ○—○, 0.25 m*M* phosphite.

the Experimental section, the response decreases rapidly when the citric acid concentration exceeds 5 m*M*. In order to minimize this effect a citric acid concentration of 1 m*M* was chosen.

The pre-conditioning of new columns (see Experimental) with 8 m*M* citric acid was found necessary for good chromatography of PFA. Without this pre-treatment, the columns gave broad and asymmetrical PFA peaks. It should be noted that the investigation of the influence of citric acid concentration on the peak shape, as shown in Fig. 2, was performed on a column pre-conditioned as described. Shorter conditioning times than the one stated in the Experimental section were not tested, but may well be sufficient.

Effect of sulphate and pH

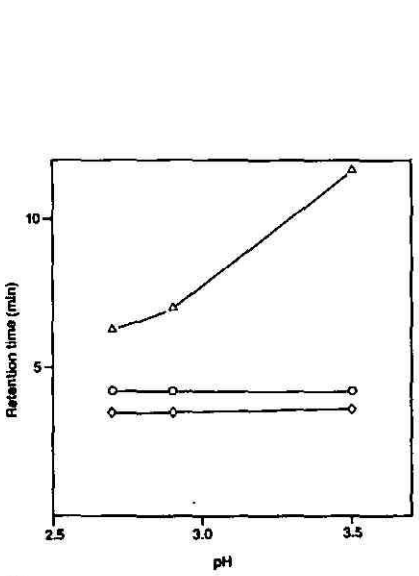
The effect of the sulphate concentration on the elution of the three ions is illustrated in Fig. 3. PFA shows the most pronounced reduction in retention time with increasing sulphate concentration. With the sample concentrations used (see Fig. 3), baseline separation between PFA and phosphite was achieved at all sulphate concentrations examined. Phosphate and phosphite were baseline separated at sulphate concentrations up to 0.02 *M*.

The retention of PFA is highly dependent on the pH of the mobile phase (Fig. 4). Phosphite and phosphate are less influenced, though the resolution between them increases slightly with decreasing pH. A representative chromatogram obtained with the mobile phase finally chosen (see Experimental) is shown in Fig. 5.

The detection system

The detection system consists of two consecutive reactions. First, the oxidation of PFA and phosphite to phosphate by bromine. Secondly, the reaction between phosphate and the molybdovanadate reagent. It is desirable that the oxidation to phosphate proceeds to completion, though this is not a requirement. The effectiveness of the oxidation was investigated by measuring the peak areas of PFA and phosphite compared to that of phosphate. Since PFA and phosphite are converted into phosphate prior to detection, all three components should give the same detector response calculated on a molar basis. By injecting three solutions containing 3.333 m*M* PFA, 1.638 m*M* phosphite and 1.699 m*M* phosphate respectively, the following responses were obtained: PFA, 255 area units/m*M*; phosphite, 254 area units/m*M*; phosphate, 258 area units/m*M*. It can thus be concluded that the oxidation is approximately complete. The responses of PFA and phosphite were constant when the bromine concentration was varied between 2 and 8 m*M*. However, below 2 m*M*, the responses decreased due to incomplete oxidation. The bromine concentration used in the response test above, as well as in all subsequent work, was about 4 m*M*.

The influence of different molybdovanadate concentrations on the response of PFA is shown in Fig. 6. Between 1.6 and 2.7 m*M* the response differs only by 1.5%. With lower concentrations, however, the response decreases rapidly. It should be noted that the citric acid concentration in the mobile phase will influence the relationship between the reagent concentration and response due to the interference discussed above. If the citric acid concentration is higher than 1 m*M*, the response will decrease at a higher molybdovanadate concentration compared to that shown in Fig. 6. Too



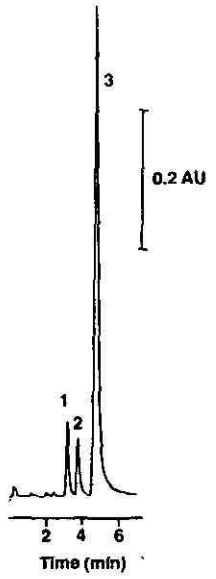


Fig. 4. Influence of pH on retention time. Mobile phase: 0.1 *M* acetic acid, 0.01 *M* sulphate and 1 m*M* citric acid. Sample concentrations and symbols as in Fig. 3.

Fig. 5. Chromatogram of 0.26 m*M* phosphate (*1*), 0.26 m*M* phosphite (*2*) and 3.3 m*M* PFA (*3*). Mobile phase: 0.1 *M* acetic acid, 0.015 *M* sulphate and 1 m*M* citric acid, pH 2.7.

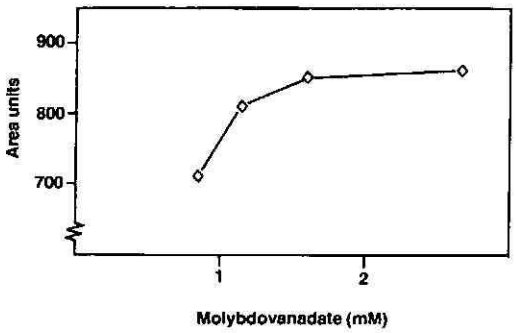


Fig. 6. Influence of molybdovanadate reagent concentration on the peak area of 3.3 m*M* PFA. Mobile phase as in Fig. 5.

high reagent concentrations are unfavorouble due to the generation of a high background absorbance and thus increased noise. The reagent concentration chosen for subsequent work with 1 m*M* citric acid was 1.6 m*M*.

The final reduction of the excess of bromine by addition of sulphite is not critical, and requires only an excess of sulphite. Since bromine is eliminated before the final detection, only the molybdovanadate reagent contributes to the background absorbance. It was found that a good quality peristaltic pump must be used for the reagent delivery or else pulsation in combination with the reagent background gave a noisy baseline.

The total residence time in the reaction detection system is only about 18 s. PFA shows UV absorbance at 230 nm, and can thus be detected directly without the reaction detection system. By comparing the PFA peak obtained by direct UV detection and that obtained with the reagent detection system, the band broadening in the detection system was found to be too small to be measurable.

Linearity

The peak areas of phosphate and phosphite are linearly related to concentration at least between $3 \cdot 10^{-5}$ and $2.7 \cdot 10^{-3}$ *M*; $r > 0.9995$. The peak height of phosphite is linear within the same interval, while the phosphate peak height is only linear up to about 1.5 m*M*. The PFA peak area is linear with concentration within the interval investigated, 1–4 m*M*; $r > 0.9998$. A plot of peak height against concentration, however, is slightly concave at low concentrations. This is probably due to the tailing tendency of PFA, which may result in relatively broader peaks at lower concentrations.

A typical value for the relative standard deviation (R.S.D.) of the peak area for ten injections of 3.3 m*M* PFA is 0.4%. For $1.5 \cdot 10^{-4}$ *M* phosphate and phosphite, the R.S.D. was 2%.

Detection limits

The detection limit was calculated as the amount giving a peak height three times the amplitude of the background noise. This peak height was found for phosphite and phosphate after injecting 20 µl of a $6 \cdot 10^{-6}$ *M* solution of the two components. The detection limit is then about 4 ng calculated as phosphorus, for both

substances. For PFA, 20 μl of a $2 \cdot 10^{-5}$ M solution, and thus about 12 ng phosphorus, could be detected.

CONCLUSIONS

The chromatography and detection system presented shows sufficient reproducibility, separation efficiency and sensitivity for drug formulation analysis of the three phosphorus compounds discussed. So far, three columns of the same packing material, but of different batches, have been tested, with similar results concerning the peak shape and retention times. One column has been in continuous use for about 6 months, without any changes in chromatographic behaviour. Undisturbed performance of the detection system can be achieved, provided pulsations in the delivery of the molybdovanadate reagent are kept low, and deaerated solutions are used to avoid air-bubbles in the detection system.

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PURIFICATION OF SPECTRIN AND ITS SUBUNITS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Technological and methodological advances in the techniques of structural and biological studies of proteins have reduced the required amount of sample. In conjunction with these advances, high-performance liquid chromatography (HPLC) has emerged as a technique of high utility for the purification of complex molecules. Using a combination of size-exclusion and reversed-phase HPLC and ionic buffers containing sodium dodecyl sulfate, the red cell membrane-associated high-molecular-weight polypeptide spectrin and its subunits have been purified. The system described in this paper is fast, reproducible and quantitative.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been extensively employed for the separation of polypeptides and their fragments. However, not many reports have been published for the purification of high-molecular-weight proteins, particularly those which are associated with the plasma membranes. The red cell has attracted much interest as a model of biological membranes. The major proteins of these membranes have been well characterized[1]. On the cytoplasmic surface of red cell membranes is a network of structural proteins, known as the cytoskeleton, of which the major component is spectrin[2,3]. Human erythrocyte spectrin is composed of two high-molecular-weight polypeptide chains: α (240 000 daltons) and β (220 000 daltons). Conventional procedures for the purification of these subunits include preparation of ghosts, low salt extraction and open-column gravity-feed, size-exclusion and ion-exchange chromatography[4,5]. The procedures give good yields but are time-consuming. We have developed an HPLC system for the rapid isolation of pure spectrin and its α-subunit. The procedural details and results are presented in this communication.

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MATERIALS AND METHODS

Chemicals

HPLC chemicals were purchased from EM Science (Cherry Hill, NJ, U.S.A.). Ultrapure Tris[tris(hydroxymethyl)aminomethane; Cat. No. 604205] with no protease activity was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). All other chemicals were of highest purity grade obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.), Pierce (Rockford, IL, U.S.A.) and Fischer Scientific (Fairlawn, NJ, U.S.A.). All solvents for HPLC were constituted in water purified through a Milli-Q system and filtered through a 0.2-μm filter before use.

Preparation of red cell ghosts

Normal human red cells were washed three times with physiological saline and lysed in 30–40 times their volume of lysis buffer (10 m*M* sodium phosphate pH 7.0). The membranes were pelleted by centrifugation at 30 000 *g* for 30 min at 4–5°C and repeatedly washed with cold lysis buffer, until white or light pink.

Extraction of spectrin

Spectrin along with some low-molecular-weight proteins was extracted from ghost membranes prepared from freshly drawn normal human blood by osmotic lysis in dilute phosphate buffer[6]. Ghost membranes were suspended in ten volumes of 0.1 m*M* EDTA pH 8.0 containing 0.4 m*M* phenylmethyl sulfonyl fluoride (PMSF) and incubated at 37°C for 30 min as described by Marchesi[6]. Spectrin which was released in the medium was separated from membrane fragments by centrifugation at approximately 30 000 *g* for 45 min at 4°C, and lyophilized.

Purification of spectrin by size-exclusion HPLC

Spectrin was purified free of small-molecular-weight proteins which co-extract with spectrin by gel-permeation HPLC.

The size-exclusion TSK-4000-SW column (60 × 0.76 cm I.D.) was purchased from Sci-Con (Winter Park, FL, U.S.A.) while the TSK-4000-PW column (60 × 0.76 cm I.D.) was a gift from Dr. Yoshio Kato (Toyo Soda, Japan).

The columns were developed at a flow-rate of 0.2 or 0.4 ml/min with 10 m*M* Tris pH 6.5 for SW and pH 8.5 for PW columns containing per litre 168 mg EDTA, 0.2 g sodium azide, 87 mg PMSF, 1.0 g sodium dodecyl sulfate (SDS) and 50 μl mercaptoethanol. The effluent was monitored at 280 nm in an LKB 2138 Uvicord S detector. A Waters Model 6000 A pump was employed as a solvent delivery system and a Model 7125 Rheodyne injector was used for sample loading.

Lyophilized extract was dissolved in HPLC eluting buffer (2 mg/ml) and up to 1 mg fractionated on a TSK-4000 column as described above. Peaks were collected manually, lyophilized and analyzed for purity on SDS-polyacrylamide (gradient 5–20%) slab gel electrophoresis (SDS-PAGE).

Purification of subunits by reversed-phase HPLC

Pure spectrin obtained as above was desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) in 0.1% SDS containing 0.4 m*M* PMSF, and lyophilized. Its α- and β-subunits were separated on a reversed-phase PLRP-S 300 Å, 8-μm column

(150 × 4.6 mm I.D.) manufactured by Polymer Labs. (Shropshire, U.K.) and marketed by Polymer Labs. (Amherst, MA, U.S.A.). Buffer A was 0.1% SDS in 0.5% trifluoroacetic acid (TFA) and buffer B was acetonitrile containing 0.5% TFA.

Separation of subunits was performed on a Waters HPLC system consisting of two 6000A solvent delivery pumps, a U6K Universal septumless injector, Model 440 dual-channel absorbance detector and Model 720 system controller. The column was developed for 5 min at initial condition (100% A) followed by a 5-min linear gradient to 50% A + 50% B, then to final condition of 32% A + 68% B in 35 min and held at final condition for 5 min at a flow-rate of 1.0 ml/min. The effluent was monitored at 280 nm and recorded on a Fisher Recordall Series 5000 recorder using a chart speed of 1.0 cm/min. The peaks as they eluted were collected manually. A 10–15 μl volume of 10 m*M* Tris buffer used for gel-filtration HPLC was added to each fraction, lyophilized and analyzed on SDS-PAGE.

Gel electrophoresis

The separated products were analyzed on a slab of polyacrylamide gel (gradient 5–20%), 0.7 mm thick, as described by Laemmli[7]. Polypeptides were located by staining with Coomassie blue.

RESULTS AND DISCUSSION

The most pragmatic approach for biochemical characterization of a protein molecule capitalizes on the efficiency of techniques available to obtain the protein molecule in (i) high yields, (ii) a highly purified form and (iii) a short period of time.

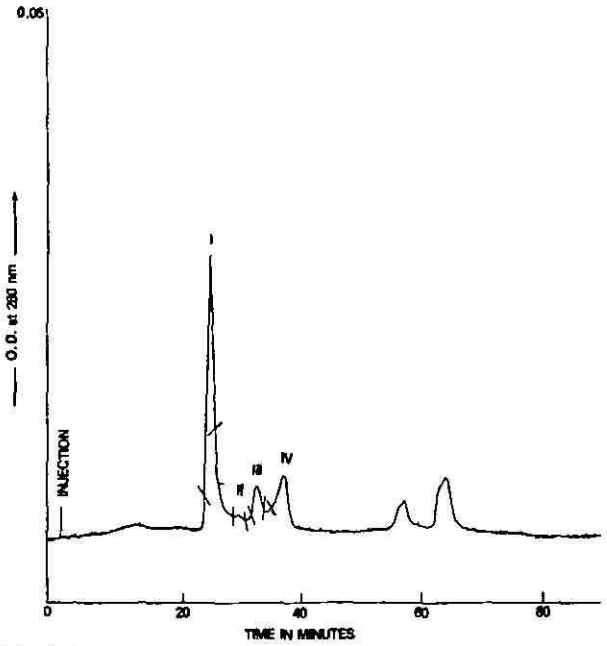

Fig. 1. Elution profile of low salt extract when injected on a TSK-4000-PW column (60 × 0.76 cm I.D.).

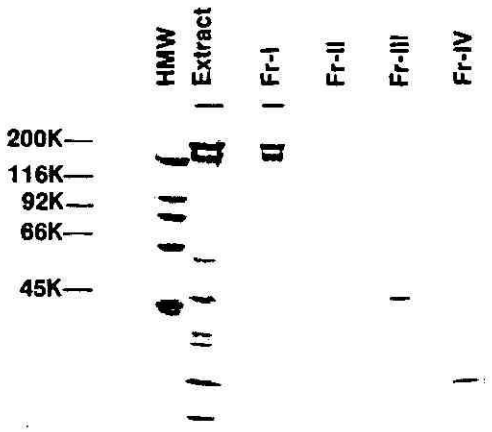

Fig. 2. SDS-PAGE analysis of the four fractions collected from a TSK-4000-PW column. HMW = high-molecular-weight protein standard; Fr = fraction.

Previously, available techniques for the isolation of spectrin and its subunits were time-consuming and required large amounts of starting material.

Fig. 1 shows the protein elution profile of human red cell ghost extract obtained on a TSK-4000-PW column. Each fraction was analyzed on SDS-PAGE and the results are presented in Fig. 2. As expected on the basis of molecular weight, peak I [Fraction I (Fr-I)] contains pure spectrin, while peaks II, III and IV contain low-molecular-weight proteins (Fig. 2). Up to 1 mg of total protein was injected without any loss in resolution. The time between injections was about 60 min. It is interesting to note that the spectrin peak elutes within 25–30 min, which is much faster than classical chromatography.

Wide-bore (7.6 mm) columns have an advantage that large sample volumes (1

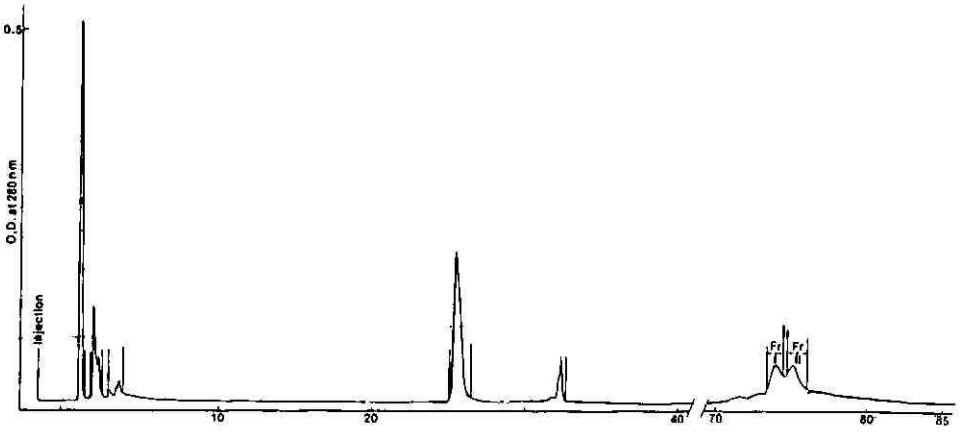

Fig. 3. Elution profile of spectrin (Fr-I; Fig. 1) on a reversed-phase column (15 × 0.46 cm I.D.). For details see text.

TABLE I

PARAMETERS FOR RP-HPLC SEPARATION OF SPECTRIN SUBUNITS

% B		Time (min)
From	To	
0	0	5
0	50	5
50	68	35
68	68	5
68	0	5

ml) can be injected to make them semi-preparative columns, but a slow flow-rate (< 0.5 ml/min) must be maintained in order to avoid the column compression creating a void at the column inlet which is a major drawback of these types of columns. We have been using these columns for more than a year without any loss in the resolution.

Fig. 3 shows the separation profile when pure spectrin is injected in a reversed-phase column and column developed as shown in Table I. Fr-I was found to be pure α-chain while Fr-II contains β-chain with 10–15% α-chain when analyzed on SDS-PAGE (Fig. 4). Previously the subunits of spectrin had been separated by treatment with high urea followed by classical chromatography on hydroxylapatite[8], DEAE-cellulose[5] or gel filtration[4]. However, use of 4 *M* urea in our hands produced little or no success under the conditions used in this study. In the present report we describe

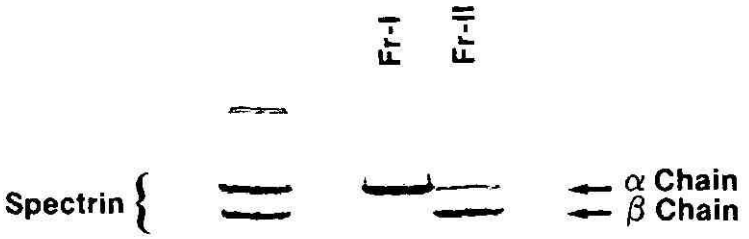

Fig. 4. SDS-PAGE analysis of Fr-I and Fr-II (Fig. 3) obtained on reversed-phase HPLC of spectrin.

for the first time an HPLC procedure for the separation of spectrin and its subunits, which incorporates all the advantages of an HPLC system, such as speed and reproducibility. In addition, both the HPLC systems employed in this study are quantitative. Based on the assay of Lowry *et al.*[9] the yields of pure spectrin and α-subunits have been 80–90%. We have observed that α- and β-subunits, when lyophilized, irreversibly stick to the walls of the test tubes. However, addition of 100–200 μl of 10 m*M* Tris buffer (employed for gel-filtration HPLC) to these fractions before lyophilization helps to avoid this problem.

Due to refinements in the techniques of structural and biological studies of protein molecules, the amount of sample needed is decreasing. In view of this, it is our belief that the HPLC method and buffer system presented in this communication will be a helpful aid for purification and structural and biological studies of this and other high-moecular-weight proteins, particularly in phylogenetic studies of spectrin from related and different species. In addition, we have further established that the use of ionic buffers containing SDS, in conjunction with or without organic modifers, has no detrimental consequences on the column life and instrument performance. The procedure has been successfully employed to obtain these molecules from erythrocyte membranes of other species.

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CHROM. 18 900

SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANTAZOLINE PHOSPHATE AND TETRAHYDROZOLINE HYDROCHLORIDE IN OPHTHALMIC SOLUTION

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SUMMARY

A method for the determination of 2-[(N-phenyl)benzylaminomethyl]-2-imidazoline · H_3PO_4 (antazoline phosphate) and 2-(1,2,3,4-tetrahydro-1-naphthyl)-2-imidazoline · HCl (tetrahydrozoline hydrochloride) in ophthalmic solution is described. The pharmaceutical preparation is analysed directly by reversed-phase ion-pair high-performance liquid chromatography and the method is very rapid, selective and simple.

INTRODUCTION

Antazoline {2-[(N-phenyl)benzylaminomethyl]-2-imidazoline} (I) is an H_1 receptor antagonist of histamine that has been used as a topical agent in the treatment of ocular disorders[1,2]. This drug is much less irritating to ocular tissues than are other members of the histamine antagonizing group and it is used in the treatment of ocular allergy, usually in combination with decongestants.

Among them, tetrahydrozoline [2-(1,2,3,4-tetrahydro-1-naphthyl)-2-imidazoline] (II), a sympathomimetic agent with marked α-adrenergic activity[3], has recently been introduced in some ophthalmic solutions to replace the more widely used naphazoline.

Although several analytical procedures have been developed for the determination of either I or II in pharmaceutical preparations, no method has been reported to achieve the simultaneous quantification of the two compounds.

Colorimetric or spectrophotometric methods previously reported for the quantification of imidazolines[4–8] can be tedious and most are not applicable to formulations containing mixtures of these compounds. A gas chromatographic approach[9] for the determination of a number of imidazolines has been also described but it does

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not seem to be reliable as it requires a difficult preliminary extraction of these strong bases from an aqueous phase into organic solvents and yield peaks with heavy tailing; in addition, II was not taken into account in that work.

In recent years, high-performance liquid chromatography (HPLC) has appeared to be the method of choice in developing rapid and specific analyses for imidazoline derivatives. A number of imidazolines have been analysed by HPLC using a strong cation-exchange column[10], but no separation has been achieved for I and II, whose peaks totally overlapped. An ion-pair HPLC assay of decongestants and antihistamines[11] has also been described; the chromatographic behaviour of I and naphazoline was reported, but not that of II. Finally, the only published procedure for determining tetrahydrozoline alone in pharmaceutical preparations involved reversed-phase HPLC on a C_{18} column[12].

This paper describes an HPLC method that can be routinely used to assay I and II simultaneously in ophthalmic solution; the method is simple and combines specificity and sensitivity that are not attainable by previously described methods.

EXPERIMENTAL

Materials

Disodium phosphate, hydrochloric acid and 4-dimethylaminobenzaldehyde were purchased from Carlo Erba (Italy). 1-Octanesulphonic acid sodium salt was a product of Fluka (Switzerland). Antazoline phosphate and tetrahydrozoline hydrochloride were obtained from Sigma (U.S.A.). These chemicals were all of analytical-reagent grade and were used as received. Water, acetonitrile and methanol were of HPLC grade (Waters Assoc., U.S.A.).

Ophthalmic solutions

Ophthalmic solutions A, B and C were prepared containing different amounts of each drug as reported in Table II. Solution A contained sodium ethylmercurithiosalicylate as a preservative and benzalkonium chloride was present in B and C.

Mobile phase

The solvent systems used were (A) acetonitrile–methanol (1:1) and (B) 0.005 *M* octanesulphonic acid sodium salt in aqueous 0.005 *M* disodium phosphate (adjusted to pH 7 with hydrochloric acid). The mobile phase contained 10% of B in A and was filtered through 0.45-μm microporous PTFE membrane filters (Millipore, U.S.A.) before use. It was pumped through the column at a flow-rate of 1.5 ml/min.

Instrumentation

A Spectra-Physics Model SP 8700-8750 high-performance liquid chromatograph equipped with a 10-μl loop injector and fitted with a variable-wavelength UV detector (Polychrom 9060) set at 222 nm was used with a Hypersil C_8 column (150 mm × 4.5 mm I.D.) packed with a spherical silica particulate (dimethyloctylsilane).

Spectroscopic analysis

UV spectra of aqueous solutions of antazoline phosphate (0.01 mg/ml) and tetrahydrozoline hydrochloride (0.01 mg/ml) were recorded on a Perkin-Elmer Model 330 double-beam spectrophotometer fitted with a UV data station.

Standards

Separate stock solutions of I and II were prepared in the mobile phase and contained 2 mg/ml. These solutions could be stored in a refrigerator for at least 2 weeks without deterioration. Working standards were prepared daily using the mobile phase to make the desired dilutions.

The internal standard solution was prepared by dissolving dimethylaminobenzaldehyde (0.1 mg/ml) in the mobile phase. Accurately measured volumes of this solution were added to standard or sample solutions to obtain a final concentration of 0.05 mg/ml.

Preparation of ophthalmic aqueous solutions

Each opthalmic solution (1 ml) was diluted with the mobile phase to a final concentration of near 0.1 mg/ml for I and 0.05 mg/ml for II. A portion of this solution was filtered and diluted 1:1 with internal standard solution before injection into the chromatograph.

Assay procedure and quantitation

Using the chromatographic conditions described, injections of the sample and standard solutions were made. Results were calculated from the linear regression of five standards of I and five standards of II relating peak-area ratio (standard-to-internal standard) and concentration.

Degraded solutions of I and II

According to Andermann and Richard[12], tetrahydrozoline hydrochloride solutions were treated with 10 *M* sodium hydroxide solution at 50°C for 24 h. The pH was then adjusted to 7 with concentrated hydrochloric acid and the solution was injected directly into the chromatograph. Accelerated degradation of antazoline phosphate solutions was achieved with the same procedure.

RESULTS AND DISCUSSION

Preliminary studies involved trying several C_8 and C_{18} reversed-phase columns from various manufacturers and testing several mobile phase compositions for the effective separation of the sample compounds and for minimizing peak tailing. A C_8 column (5 μm spherical silica particles; 25 mm × 4.6 mm I.D.) eluted with a mobile phase containing sodium octylsulphonate in aqueous phosphate mixed with acetonitrile and methanol, as described under Experimental, afforded the best separation of I, II and the internal standard and provided better separations of the active compounds from excipients such as benzalkonium chloride and sodium ethylmercurithiosalicylate, used as preservatives in the ophthalmic solutions tested.

UV spectra of aqueous solutions containing I and II at the same concentration were also preliminarly studied (Fig. 1a and b) in order to select the wavelength at which the UV detector had to be operated.

These spectra showed the same absorption value at 222 nm and this wavelength was chosen for the UV detection. A representative chromatogram of a standard mixture of I, II and the internal standard is given in Fig. 2; the overall chromatographic time of 10 min provides efficient assay capability.

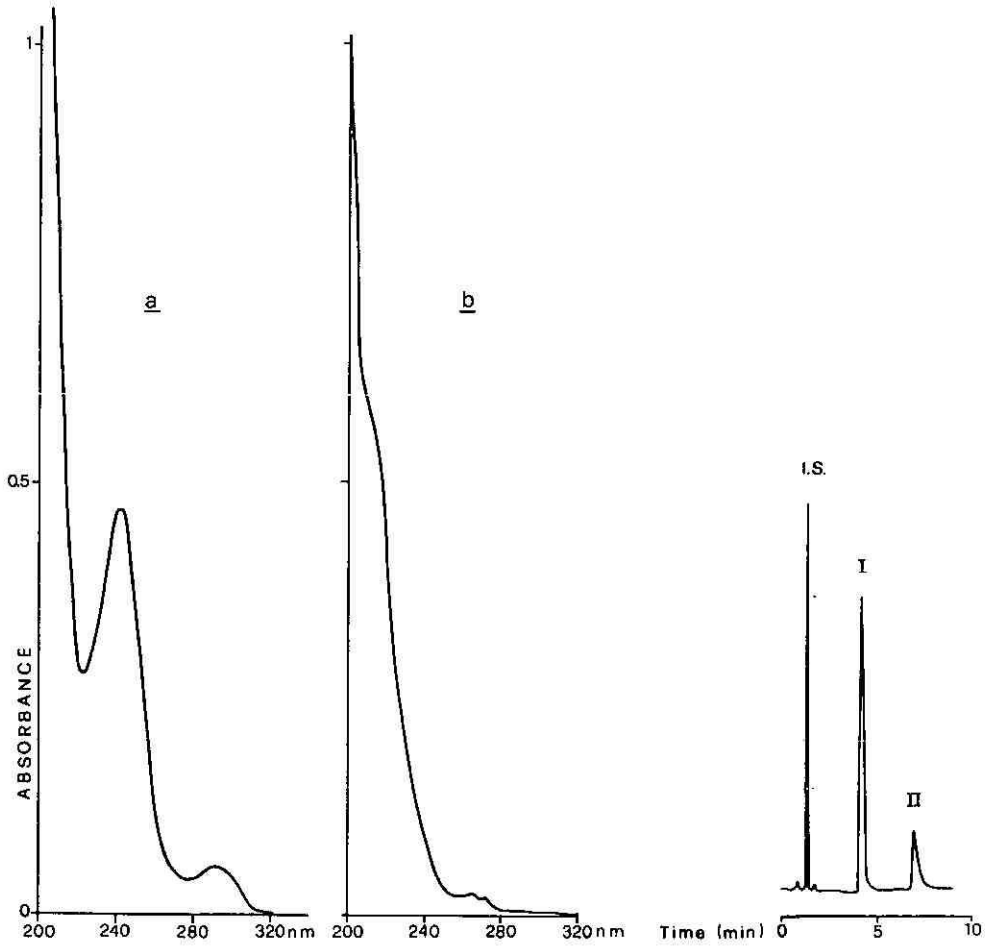

Fig. 1 UV spectra of 0.01 mg/ml solutions of (a) antazoline phosphate and (b) tetrahydrozoline hydrochloride.

Fig. 2. Typical chromatographic separation of I, II and the internal standard (I.S.).

The precision of the assay was determined by independent analyses of six aliquots of the same sample, using solution C; the method was shown to be reproducible with relative standard deviations (R.S.D.) of 0.36 and 0.42% for I and II, respectively (Table I).

The precision was evaluated by making several injections of the same standard solution containing I, II and the internal standard. The R.S.D. values of the response ratios for I and II were 0.35 and 0.36%, respectively.

The linearity of the method was also studied; five standards containing concentrations of I and II spanning 50–200% of the expected working range were analysed by the method. The calibration graph thus obtained for antazoline showed a correlation coefficient of 0.9996 in the range 0.4–10 μg injected. The equation of the linear regression line was $y = 27.096x - 0.082$ where y is the peak-area ratio (an-

TABLE I

PRECISION OF THE ASSAY OF I AND II IN OPHTHALMIC SOLUTION

*Sample No.**	*Assay value (mg/ml)*	
	Compound I	*Compound II*
1	1.038	0.5029
2	1.032	0.5021
3	1.031	0.4989
4	1.039	0.5030
5	1.038	0.4985
6	1.032	0.4994
Mean	1.035	0.5008
R.S.D.	0.36	0.42

* Aliquots of ophthalmic solution C.

tazoline to internal standard) at 222 nm and x the concentration (mg/ml) of antazoline. The equation of the linear regression line for tetrahydrozoline was $y = 29.324x - 0.232$ with a correlation coefficient of 0.9992 in the range 0.25–1 μg injected. These statistics indicate that HPLC analysis gives a linear response, and a single point standard may be used.

Three different ophthalmic solutions (see under Experimental) were analysed for I and II content by the method and the results are summarized in Table II. All the chromatograms obtained were essentially devoid of any interfering peaks due to excipients.

The chromatographic behaviour of potential degradation products from I and II was also studied. Solutions of tetrahydrozoline hydrochloride subjected to accellerated degradation by alkali[12] were chromatographed and a typical chromatogram is given in Fig. 3. The purity of the peak of the undegraded tetrahydrozoline II which is present in this chromatogram was confirmed by acquiring several UV spectra during its elution. Solutions of antazoline phosphate, treated in the same way with alkali, gave a typical chromatogram as shown in Fig. 4. These results show that the method is capable of the separation of the potential degradation products.

The analysis of an aged (21 months) sample of ophthalmic solution C gave the

TABLE II

RESULTS OF HPLC ASSAY OF I AND II IN OPHTHALMIC SAMPLES

Ophthalmic solution	*Formulated (mg/ml)*		*Determined by HPLC (mg/ml)*	
	I	*II*	*I**	*II**
A	1.553	0.502	1.572 (0.46)**	0.506 (0.50)**
B	5.176	0.412	5.179 (0.62)	0.419 (0.46)
C	1.037	0.502	1.039 (0.45)	0.499 (0.51)

* Each value is the mean of three determinations.

** R.S.D. (%) in parentheses.

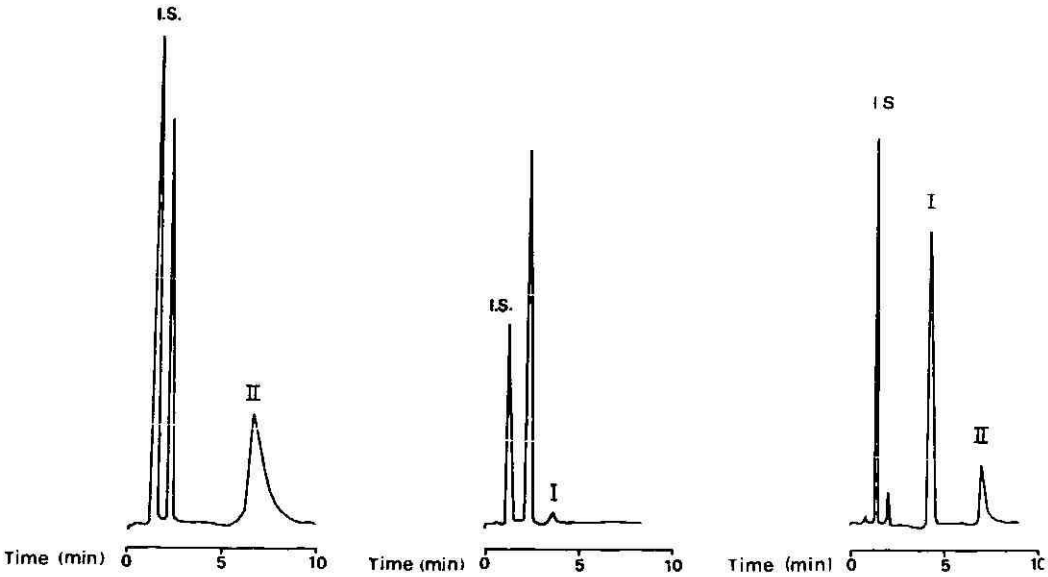

Fig. 3. Typical chromatogram of a degraded tetrahydrozoline hydrochloride solution.

Fig. 4. Typical chromatogram of a degraded antazoline phosphate solution.

Fig. 5. Typical chromatogram the ophthalmic solution C stored at room temperature for 21 months.

chromatogram in Fig. 5, which shows that little decomposition of the two active compounds had occurred.

The HPLC assay method for I and II described here is simple, rapid and precise and provides a sufficiently sensitive method for the determination of these two drugs in ophthalmic solution.

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Note

Portable computer-based temperature programmer

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This project was initiated by the need for a temperature programmer to replace the electro-mechanical unit in an older gas chromatograph. The original unit needed replacement parts that were no longer available. Modern stand-alone controllers with linear temperature programming capability suitable for gas chromatography (GC) are not readily available. In this note we describe our approach to the problem using readily available components.

HARDWARE

A lap type computer (Radio Shack TRS80 Model 100) was chosen as a trade-off between cost and development time. It also offered easy modifications of the control program if required at a later time.

An overview of the circuit is shown in Fig. 1. The thermocouple voltage is amplified (nominal 10 mV/°C) and cold junction compensated by the AD595. This voltage is then digitised by the 7109 12-bit A/D converter. The digital output is sent via the 6403 UART to the computer's RS232C serial port as two 8-bit bytes. Since the 7109 and the 6403 were designed to work together this transfer is easily accomplished using the handshake lines provided.

An 8-bit data word (0-255), calculated according to the algorithm discussed below, controlled the mark/space ratio of the 7240 (programmable timer), which in turn controlled the column oven triac. At the end of each cycle the data input of the 7240 is reset to zero.

The timing for the 7109, the 6403 and the 7240 was derived from a 2.4576-MHz crystal connected to the UART. A divider chain was used to derive the 51.2-kHz signal required by the A/D converter and the 1-Hz signal for triggering the A/D converter, resetting the programmable timer and initiating data transmission to the computer at 9600 baud.

The design freed the computer from any time keeping requirements. This greatly simplified the software and also made it fail-safe if the computer is inadvertently turned off.

The cost of parts for the interface was approximately NZ$ 250. The time for design and construction was less than three weeks.

The best control, as determined by the reproducibility of the chromatogram of a test sample, was obtained with a bare thermocouple suspended in air near the column.

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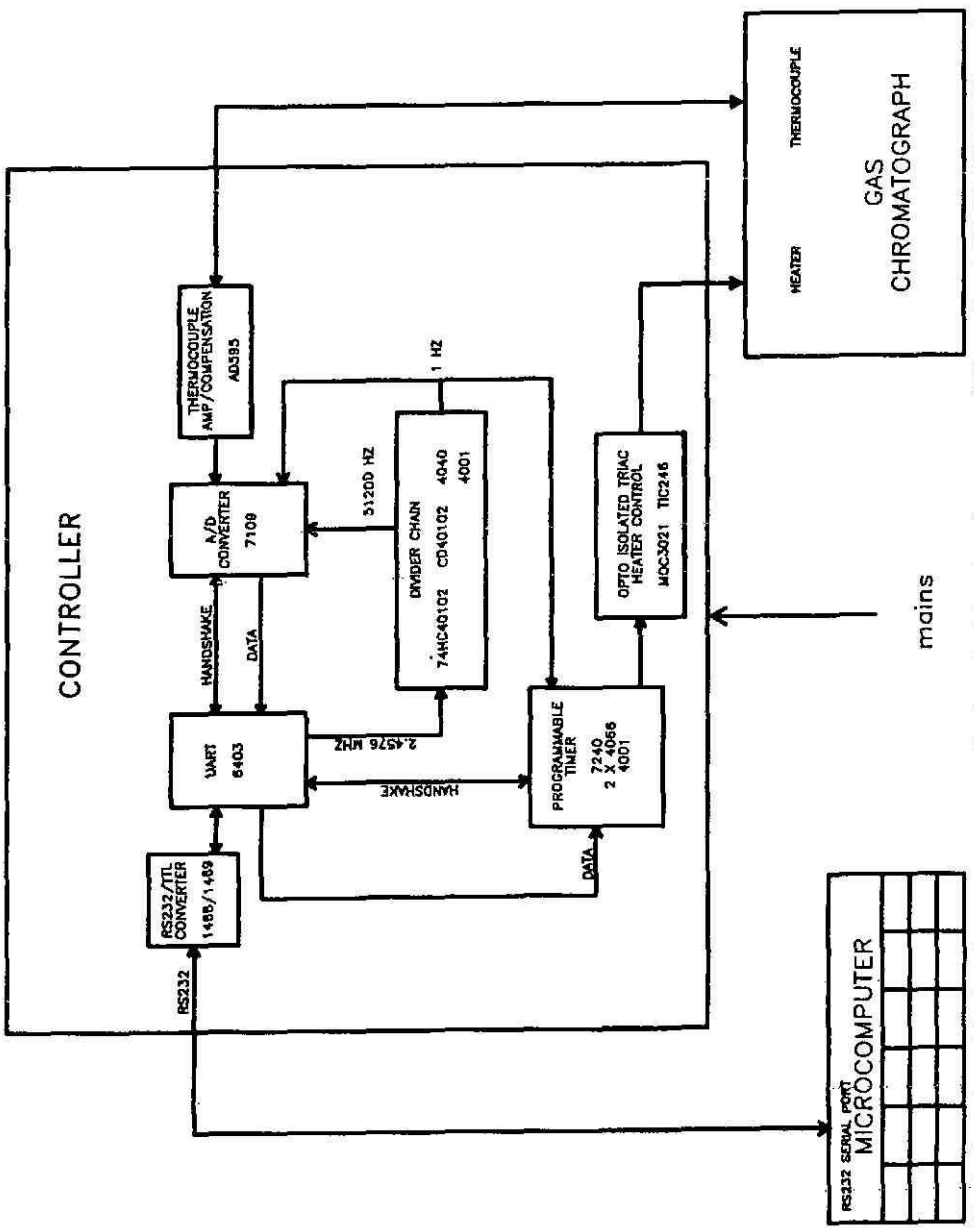

Fig. 1. Overview of the interface. The programmable timer (ICM7240), A/D converter (ICL 7109) and UART (ICM6403) are from Intersil (Cupertino, CA, U.S.A.), the compensated thermocouple amplifier (AD595) is from Analog Devices (Norwood, MA, U.S.A.). The computer used was a Radio Shack TRS80 Model 100.

SOFTWARE

The program, written in BASIC, included a machine code subroutine in order to bypass a feature of the Model 100's BASIC[1]. The input of data equivalent to decimal 26 on the RS232 port is interpreted as an end-of-file. A short machine code subroutine called from BASIC overcame this problem. We found that the ROM interrupt that reads the RS232 port into the internal buffer in real time and the routine that reads this buffer do not check for end-of-file. The machine code subroutine calls the internal ROM routine to read the RS232 buffer 2 bytes at a time (representing the high and low order bytes of the A/D converter data word) and places them in a BASIC integer (16-bit) variable for use by the BASIC program.

The Model 100's BASIC interrupt system is used to determine when temperature data are available for processing. This is not a real-time interrupt, it appears that the RS232 buffer is polled at the end of a BASIC line. With a long BASIC line involving slow functions, *e.g.* printing to the screen, the delay could run into hundreds of milliseconds. This variation in response time could cause data to be supplied to the 7240 timer before the previous cycle has finished. If this happened, the new data would be ignored and the cycle missed.

Temperature control is based on the proportional plus integral plus rate (PID) algorithm[2]. The proportional, integral and differential gains were set empirically. The algorithm calculates the required power level, which is then converted to a number proportional to the power required to maintain the temperature at the set point. The number is limited to range from 0 to 253 representing 0 to 99% of maximum power. Although theoretically the input to the 7240 could range from 0 to 255 (0 to 100%), we have limited the output to a maximum of 253 because of the "interrupt" latency mentioned above. With the GC instruments (Varian Models 1520 and 2700) used this is immaterial since the power demand at equilibrium is less than 50% of maximum, even at 300°C and at 10°C/min. In fact the high wattage of the heater (1.5 kW) necessitated some modification of the PID algorithm to minimise the overshoot during the initial heating up. When the temperature is within 5°C of the set-point power is only applied when the rate of rise of temperature is less than or equal to zero. Once the set-point has been reached normal PID action is resumed.

The program as implemented allows for two temperature ramps and isothermal periods at the start, between ramps, and at the end of temperature programming. The temperature programming parameters may be changed at any time during the run. Status information, such as run time, set point and actual temperature, is displayed on the screen. It would be a simple task to incorporate additional ramps and other control features if desired.

CONCLUSION

The circuit described here presents a cost effective approach to upgrading an older GC instrument in which the electro-mechanical analogue temperature controller has become unreliable or inadequate. Since the program is written in BASIC for a microcomputer, custon features, *e.g.* starting of integrators and valve switching, could be easily incorporated.

During 12 months of use with capillary columns the reproducibility of the

retention times for various samples was found to be similar to that obtained on a HP5790 gas chromatograph (± 0.1 min).

The circuit diagram and a program listing are available from the authors.

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Note

Generation of extreme selectivity in chiral recognition

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In previous papers, we have described rational approaches to the design of chiral stationary phases (CSPs) for the liquid chromatographic separation of the enantiomers of a large and diverse array of chiral molecules[1–3]. Efforts have been focused on attaining an understanding of the mechanistic origins of chiral recognition, for such understanding can be used to design CSPs of enhanced selectivity and expanded scope. In this paper, we describe an approach to analyte modification which can sometimes be used to confer an extreme degree of selectivity. Although illustrated by the separation of enantiomers on a chiral column, the approach is more general and is not restricted just to the separation of enantiomers.

Separation of enantiomers on a CSP requires that the diastereomeric adsorbates formed have non-identical (*i.e.* $\Delta\Delta G \neq 0$) free energies, ΔG, of association with the CSP. The extent of selectivity, α, is related to $\Delta\Delta G$ by the expression: $\Delta\Delta G = -RT \ln \alpha$. If one could double $\Delta\Delta G$, one would increase α to the square of its original value. Many compounds are amenable to selectivity-enhancing modification. For example, when analytes such as methyl or ethyl esters, acetates or propionates, or amides of simple amines are encountered, one may have the option of making bis-esters or bis-amides from the chiral constituent of interest and achiral diols, diacids or diamines. If the spacing between the ends of the bis-derivative permits the chiral moieties to interact independently and simultaneously with the CSP, one expects, on *simplistic* grounds, that the $\Delta\Delta G$ observed for the enantiomers of the bis-analyte will be roughly twice that observed for the enantiomers of the corresponding mono-analyte. Hence, the α noted for the enantiomers of the bis-analyte should be *roughly* the square of the α observed for the enantiomers of the mono-analyte. We herein report the use of CSP 1 to compare the chromatographic behavior of the enantiomers of mono-analyte 2 with those of the corresponding bis-analyte 3.

EXPERIMENTAL

Chromatography was performed isocratically using a Rainin Rabbit HPX pump, an LDC/Milton Ray UV Monitor D fixed-wavelength (254 nm) detector, a Kipp-Zonen BD-41 recorder, a Reodyne injector and a (*S*)-N-(2-naphthyl)alanine column (Regis, Morton Grove, IL, U.S.A.).

The mono- and bis-amides of N-(3,5-dinitrobenzoyl)leucine[4] were prepared by activation of the carboxyl group with 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroqui-

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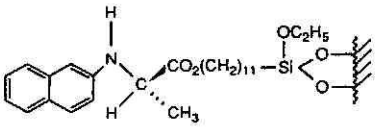

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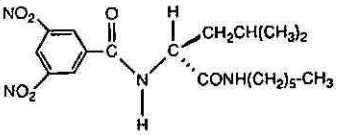

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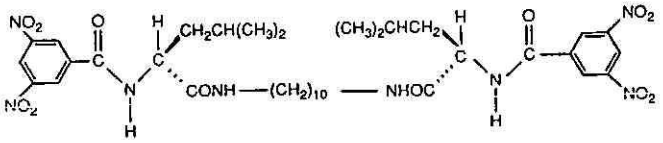

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noline (EEDQ) (Aldrich) in methylene chloride and subsequent slow addition of dilute methylene chloride solutions of either *n*-hexylamine or 1,10-diaminodecane. These solutions were washed sequentially with 1 *M* sodium hydroxide, 1 *M* hydrochloric acid, water and brine, then dried over anhydrous sodium sulfate. Filtration afforded analyte solutions which can be used directly. Crystallization of bis-amide 3 was avoided so as to not alter the ratio of diastereomers. Derivatives of both racemic and (*S*)-N-(3,5-dinitrobenzoyl)leucine were made and chromatographed to establish rigorously elution orders. On a racemic Regis N-(2-naphthyl)alanine column, the enantiomers are coeluted and, in the case of the bis-amide, 3, they are coeluted with the meso diastereomer.

RESULTS AND DISCUSSION

The recently described and now from Regis available CSP 1, derived from N-(2-naphthyl)alanine, shows a high degree of chiral recognition toward the enantiomers of the N-3,5-dinitrobenzoyl derivatives of amino acids and achiral amines[3]. For example, the enantiomers of N-(3,5-dinitrobenzoyl)leucine *n*-hexylamide, 2, show separation factors of 2.35 and 10.5, respectively at 25°C in mobile phases of methanol or 30% 2-propanol in hexane, the (*R*)-enantiomer being eluted before the (*S*)-enantiomer. Activation of the carboxyl of racemic N-(3,5-dinitrobenzoyl)leucine with EEDQ, followed by reaction with 1,10-diaminodecane, affords a 1:2:1 ratio of the (*R,R*)-, (*R,S*)- and (*S,S*)-bis-derivatives, 3. Chromatography on CSP (*S*)-1 cleanly separates the three stereoisomers, the *meso* (*R,S*)-isomer eluting after the (*R,R*)- but before the (*S,S*)-enantiomer, *as expected.* The separation factors observed for the enantiomers in methanol and 30% 2-propanol in hexane are 10.0 and 121.0, respectively! In the second instance, the (*R,R*)-enantiomer eluted after 8 min, the *meso* after 96 min, and the (*S,S*) after 590 min, almost 10 h after its enantiomer. Even so, the resolution factor for the enantiomers exceeds eighty since good peak shapes are retained despite the strong retention.

It should be evident that, by making tris, tetrakis, etc. derivatives, selectivity can be elevated to rather large values. For example, the enantiomers of a tetrakis-analogue of compound 3 would be expected to show a separation factor in excess of 10^4 on CSP 1. While such selectivity is unnecessary (even highly undesirable) for analytical work, it would trivialize the preparative separation of enantiomers, reduc-

ing the process to filtration (à la affinity chromatography) rather than chromatography. However, the reduced solubility to be anticipated for such analytes and the number of stereoisomers expected to arise from *non-selective* derivatization are disadvantages that would be encountered in practice.

The simplistic doubling of $\Delta\Delta G$ seemingly underestimates the consequences of progressing from a mono-analyte to a bis-analyte. Our initial expectations were that, for entropic reasons, the mono-analytes would not be an accurate "half-model" for the bis-analytes. We expected that the $\Delta\Delta H$ contribution from the bis-analyte would be somewhat less than twice that of the mono-analyte since simultaneous dual interaction might not always be achieved. The bis-analyte was expected to show a significantly larger $\Delta\Delta S$, owing to its greater restriction of freedom while adsorbed. The entropic considerations here are similar to the well-known "chelation effect" in coordination chemistry[5]. To obtain values of $\Delta\Delta H$ and $\Delta\Delta S$, the temperature dependence of α was determined for analytes 2 and 3 on CSP 1. Methanol was used as a mobile phase to avoid runs of multi-hour duration. The α values observed for compounds 2 and 3 ranged from 3.31 and 29.5 at 0°C to 1.93 and 4.15 at 50°C. Plots of ln α *vs.* $1/T$ are linear and afford values for $\Delta\Delta H$ and $\Delta\Delta S$ of -0.52 ± 0.01 kcal mol^{-1} and -1.32 eu for compound 2 and -1.73 ± 0.1 kcal mol^{-1} and -4.65 eu for compound 3. While we believe that compounds 2 and 3 have basically the same chiral recognition processes available to them, the question as to why the $\Delta\Delta H$ value observed for compound 3 is so much larger than anticipated is fascinating and presently lacks a detailed answer. Analytes are retained by a blend of interactions. Clearly, the blend is different for analytes 2 and 3, but different in what way? If the answer were known, one might employ this insight in designing improved CSPs.

We are engaged in a general study of bis-analytes so as to refine our understanding of how to best employ this approach to the amplification of chiral recognition. Such studies may also provide a probe for estimation of average interstrand spacing and give insight into types of retention processes which might otherwise be difficult to study.

CONCLUSION

The use of multidentate analytes has been shown to amplify vastly chromatographic selectivity owing to the additivity of the binding effects. The ramifications of the approach are apt to be of greater preparative than analytical significance.

ACKNOWLEDGEMENT

This work has been supported by grants from the National Science Foundation and from Eli Lilly and Co.

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Note

Omission of aspartate values from amino acid analysis reports

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It has been observed that, when using any chromatographic procedure which separates amino acids in a short period of time, even small variations in mobile phase pH will shift the relative retention time[1]. With the earlier models of Beckman amino acid analyzers, such as the 120B and 119CL, aspartate was retained on the ion-exchange column long enough so as to rid the sample of minor variations in pH due to acid contamination merely by the pumping of elution buffer. After continuous pumping of buffer, aspartate was eluted from the resin in 40 min with the Model 120B[2], and in 13 min with the 119CL analyzer[3,4]. The Beckman System 6300 analyzer, however, can elute aspartate in *ca.* 5–6 min, depending on buffer pumping rate, and this faster rate of analysis does not permit any variation in pH of the applied amino acid solutions.

This communication describes changes in procedures so as to overcome the problem of prolonged retention times for aspartate when using fast amino acid analyzers. It also discusses deficiences in the model of integrator recommended to be used with the analyzer.

EXPERIMENTAL

All analytical determinations were carried out with a Beckman System 6300 Analyzer, the analog output of which was monitored by a Hewlett-Packard 3390A reporting integrator. The amino acid analyzer used reagents supplied by Beckman (Palo Alto, CA, U.S.A.). Amino acids were reacted with ninhydrin reagent to develop colour and the total analysis time, including column regeneration, was 50 min. The instrument was calibrated with an amino acid concentration of 2.5 nmol. Taurine and γ-aminobutyric acid were added to the standard calibration mixture (Beckman P/N 338088) and retention times for taurine, aspartate, glutamate, glycine, alanine and γ-aminobutyric acid were determined with the Hewlett-Packard reporting integrator. The sample loop injection volume was 50 μl.

RESULTS AND DISCUSSION

Retention time delay for aspartate was first experienced after using a reversed-phase column to separate a mixture of peptides that had been obtained by cyanogen

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bromide cleavage of a protein. The peptides were separated by using a gradient beginning with 1% orthophosphoric acid solution against acetonitrile, on a Varian 5000 liquid chromatograph[3,4]. After hydrolysis of the collected peptides, the amino acid mixtures were taken up in Beckman Na-S dilution buffer and analysed. Some of the mixtures containing aspartic were found to contain residual orthophosphoric acid. Addition of a droplet of concentrated orthophosphoric acid to the standard calibration mixture used for re-calibrating the amino acid analyzer was sufficient to simulate the necessary delay in retention time for aspartate so that no further omission of aspartate value occurred when the hydrolyzed peptides were re-analysed.

When the integrator is initially calibrated it sets a time interval or window for each retention time that represents the presence of an amino acid. These windows are opened and closed automatically during the course of an analysis in a programmed sliding scale controlled by the integrator; narrow window openings at the start of an analysis where sharp peaks occur, and longer openings at the end of the chromatogram trace where wide peaks occur. Aspartate is eluted from the ion-exchange column near the start of the analysis and is covered by one of the shortest windows, 9.6 s, thus, any moderate shift in retention time can easily position the peak outside the window.

When the analysis of a series of physiological fluid extracts was attempted, it was discovered that the extracts contained varying amounts of perchloric acid which had been used to precipitate unwanted proteins during preparation of the samples for analysis. Upon analysis a wide variation in the retention time for aspartate was encountered; some samples provided retention times within the window, others provided retention times before the window opened and others after it had closed.

In Fig. 1 the retention time for aspartate is listed as 7.04 min. When the analyzer had been calibrated (with perchloric acid added to the calibration mixture), a retention time of 7.38 min had been established for aspartate. Thus, the small deviation from the ideal retention time proved to be of no consequence. In Fig. 2, which shows another chromatogram from the same series, the retention time for aspartate is recorded at 6.43 min. This retention time proved to be recorded before the window opened and although the peak was given a retention time, quantitation of the peak

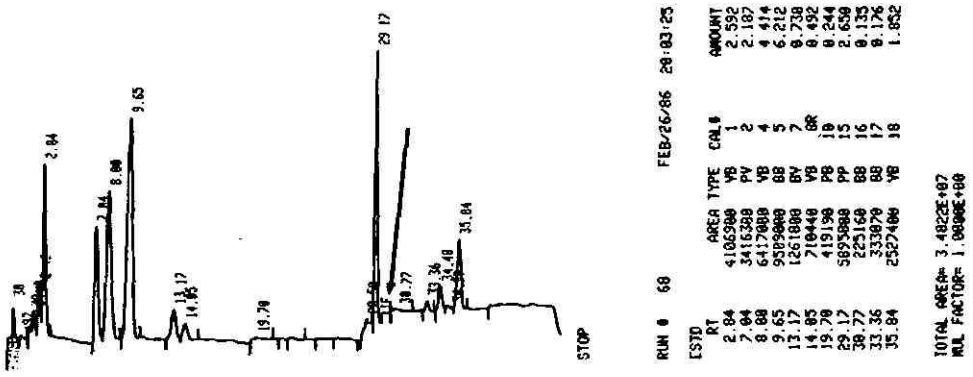

Fig. 1. Chromatogram of deproteinised nervous tissue extract showing the inclusion of aspartate in analysis report.

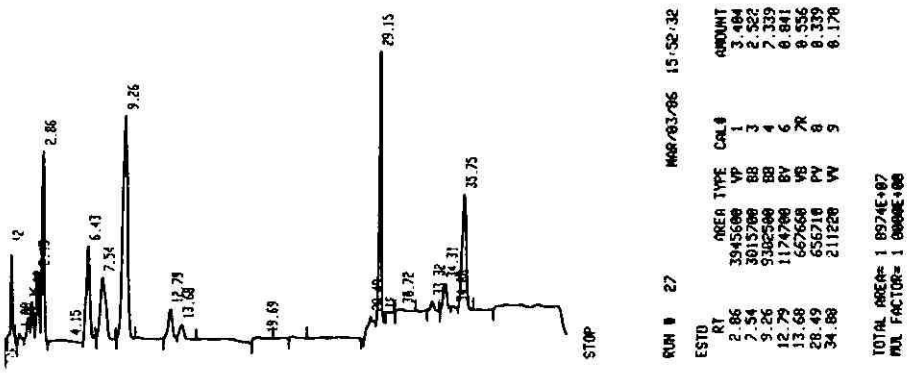

Fig. 2. Chromatogram of deproteinised nervous tissue extract showing the omission of aspartate from the analysis report.

did not take place. For those extracts in which aspartate had been delayed beyond the duration of the window a simple procedure was adopted; one of the reagents supplied with the System 6300 Analyzer was an alkali solution labelled Na-R, and a droplet of this reagent was added to the extracts before re-analysis. Addition of the alkali permitted the retention time to be shortened and the aspartate peak was now within the window. However, for those extracts in which aspartate had been prematurely eluted from the column, *i.e.*, before the window opened, the procedure initially adopted of adding further amounts of perchloric acid to the extract (so as to prolong the retention time of aspartate) proved to be unreliable. Thus, it was decided to utilize the integrator's capacity to store methodologies; the amino acid analyzer was recalibrated with standard mixtures having a variation in pH and each stored as a separate methodology. These methodologies provided an overlap of the aspartate window and extended the open time duration in both directions. If the initial analysis did not provide a value for aspartate, the alternative methodology was used for a repeat analysis of the extract.

In the cases where minute additions of reagents had been used to adjust the pH of the samples, no significant effect on the accuracy of the levels of amino acid amounts reported was discovered. The presence of varying amounts of perchloric acid in the extract did not have any significant effect on the retention times for taurine. Although taurine is eluted before aspartate, at 2.84 min in the chromatogram shown in Fig. 1 and at 2.86 min in Fig. 2, no omission from the final reports was observed for this amino acid. It is concluded, therefore, that taurine is unimpeded in its passage through the ion-exchange column.

The omission of the amount of amino acid from a final report can occur even when a peak is accurately located with the window allocated to it. Interference to the integrator's monitoring of a peak can occur if a forced baseline correction has been programmed to take place as the integrator is about to print out a retention time for a peak. Such is the case in Fig. 2 where the γ-aminobutyric acid value has been left out although a retention time of 29.15 min has been assigned to the peak. The occurrence of an automatic baseline correction has been marked by an arrow in Fig. 1. The purpose of this function is to compensate for the entry of a more concentrated buffer into the colorimeter of the analyzer.

The linking of the Hewlett-Packard Model 3390A reporting integrator to the Beckman System 6300 analyzer has proved to be successful for the routine, rapid analysis of proteins. It is when analytical samples have been specially treated during their preparation for analysis that the integrator's deficiency becomes demonstrable. The rigid fixed range of window openings utilized prevents modest adjustments to a methodology to be made, and once a methodology has been prepared, the storing of this methodology into the memory of the integrator precludes any further attempts to change any parameter contained in that methodology. To make even a minor change in operating conditions the entire methodology has to be deleted and the analyzer recalibrated.

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Note

Gas chromatography on porous polymers

III*. Anomalies in the behaviour of *n*-alkanes chromatographed on copolymers of 1,4-di(methacryloyloxymethyl)naphthalene and 1,4-divinylbenzene

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In Part II[1], we reported the chromatographic behaviour of *n*-alkanes and aromatic compounds on porous polymers obtained by copolymerization of 1,4-di(methacryloyloxymethyl)naphthalene (1,4-DMN) and 1,4-divinylbenzene (1,4-DVB). It was shown[1,2] that by changing the molar ratio of 1,4-DMN to 1,4-DVB, polymers of varying polarity could be obtained. On the other hand, the parameters characterizing the properties of sorbents, such as the specific surface area, total porosity, the glass transition temperature and the initial temperature of decomposition, appeared to be only slightly dependent on the relative amounts of monomers.

The previous study[1] also indicated that for various aromatic compounds (methylbenzenes, chlorobenzenes, methylphenols and chlorophenols) the retention indices exhibited a nearly linear dependence on the polarity of the sorbent, expressed in terms of total selectivity determined by the McReynolds test. On the other hand, the behaviour of *n*-alkanes showed some peculiarities. The most puzzling was their behaviour on the most polar sorbent used, which corresponded to the highest molar ratio of 1,4-DMN to 1,4-DVB of 0.8.

The purpose of this work was to carry out a more detailed study of the chromatographic behaviour of *n*-alkanes on this sorbent.

EXPERIMENTAL

Chromatographic measurements were carried out on a GCHF-18.3 (Chromatron, Berlin, G.D.R.) gas chromatograph with a stainless-steel column (100 cm × 4 mm I.D.), equipped with a thermal conductivity detector. Hydrogen at a flow-rate of 50 ml/min was used as the carier gas.

The measurements of the adjusted retention times of *n*-alkanes were performed at 5°C intervals in the range 190–250°C. The samples were injected by means of a 1-μl syringe (Scientific Glass Engineering, North Melbourne, Australia). Mixtures of C_5–C_{18} *n*-alkanes were injected.

* For Part II, see ref. 1.

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RESULTS AND DISCUSSION

Fig. 1 shows plots of log [adjusted retention time, $t'_{R(n)}$] *versus* number of carbon atoms in the *n*-alkanes, n_C. The dependence can be expressed in the form

$$\log t'_{R(n)} = An_C + B \quad (1)$$

It can be seen that for $n_C \leqslant 9$ the slopes A are slightly different than those for $n_C \geqslant 10$ at temperatures higher than 215°C. As we have shown previously[1], the parameters A and B are closely related to the enthalpy, ΔH_n, and the entropy, ΔS_n, of sorption

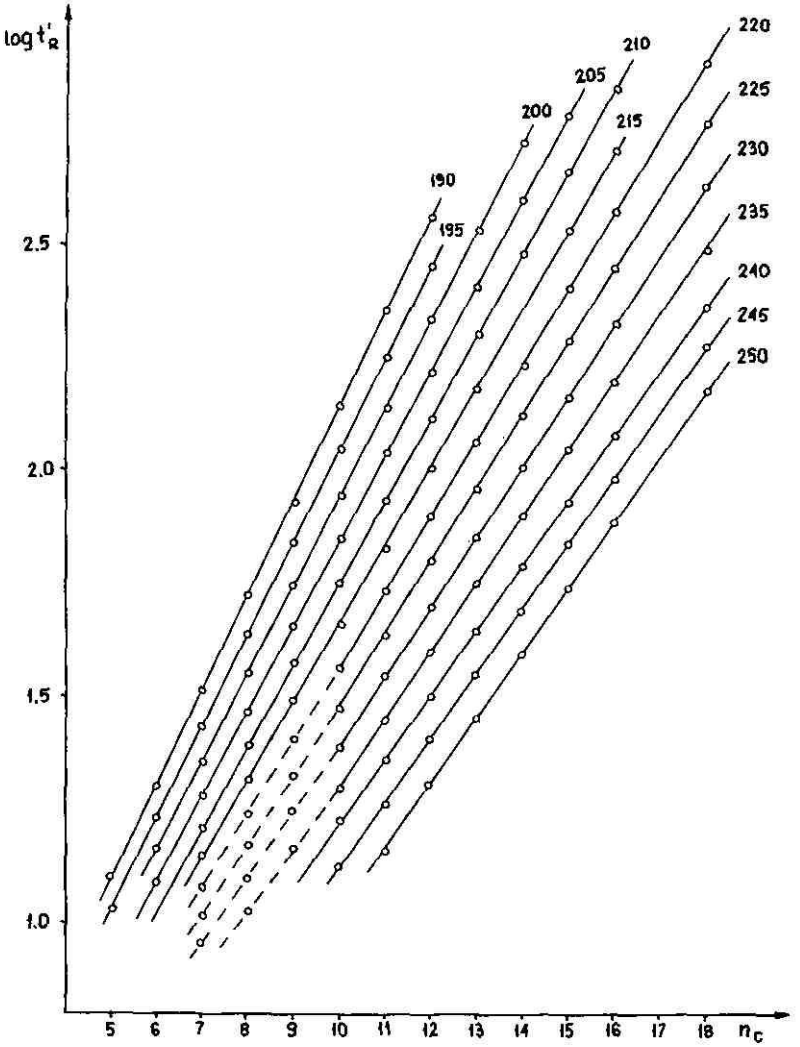

Fig. 1. Dependence of log (adjusted retention time, log t'_R) on the number of carbon atoms in *n*-alkanes, n_C.

via the following relationships:

$$A = \frac{1}{2.3}\left(-\frac{\Delta H_1}{RT} + C_1\right) \tag{2}$$

and

$$B = \frac{1}{2.3}\left(-\frac{\Delta H_0}{RT} + C_0\right) + \log t_0 \tag{3}$$

where

$$\Delta H_n = \Delta H_1 n_C + H_0 \tag{4}$$

$$C_n = C_1 n_C + C_0; \quad \Delta S_n \sim C_n \tag{5}$$

and t_0 is the retention dead time.

From eqns. 2 and 3 it follows that we can determine ΔH_1, C_1, ΔH_0 and C_0 from the plots of A and B against $1/T$. Fig. 2 shows the dependences obtained. It can be seen that at 240°C and above A is independent of temperature as observed in our earlier study[1]. This implies that $\Delta H_1 = 0$ and hence the enthalpy of sorption does not depend on the number of carbon atoms in the n-alkane molecule (see Fig. 3). Below 240°C two linear dependences between A and $1/T$ are observed one corresponding to n-alkanes containing more then nine carbon atoms (open circles in Fig. 2) and the other corresponding to lower alkanes (crosses in Fig. 2). However, both lines meet at about 210°C, and at lower temperatures only one line is observed (squares in Fig. 2a). Hence we have obtained clear evidence that at a temperature

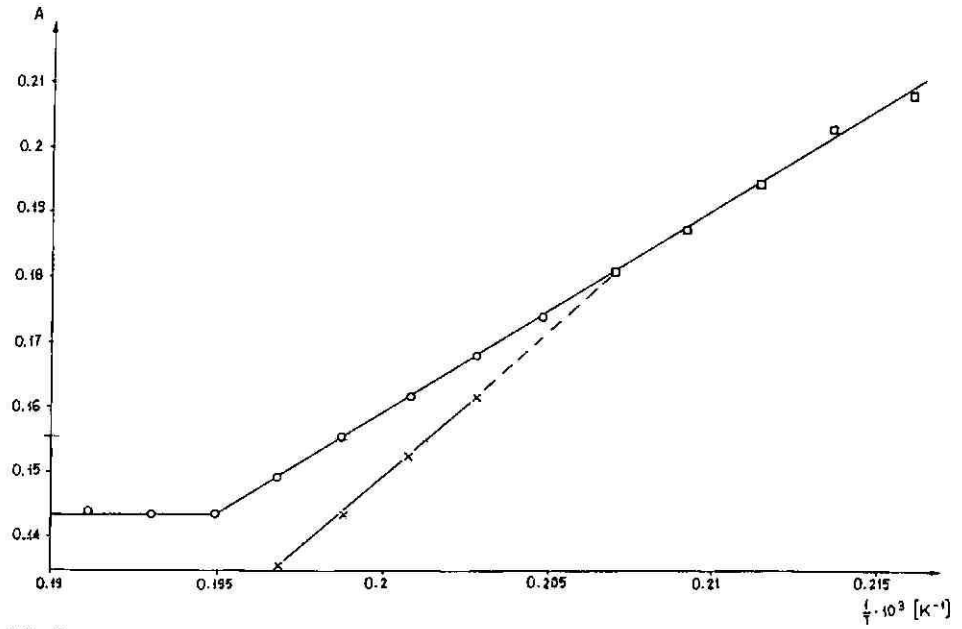

Fig. 2.

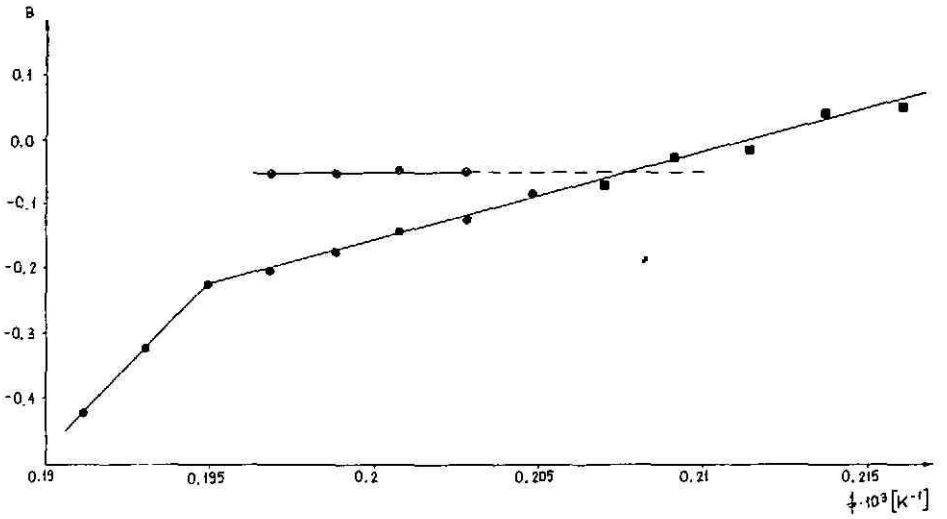

Fig. 2. Dependence of A (eqn. 2) on the reciprocal of absolute temperature, $1/T$. ○, $n_C > 9$; ×, $n_C \leqslant 9$; □, any n-alkanes with $n_C \leqslant 9$ as well as with $n_C > 9$; and dependence of B (eqn. 3) on the reciprocal of absolute temperature, $1/T$. ●, $n_C > 9$; ⊗, $n_C \leqslant 9$; ■, any n-alkanes with $n_C \leqslant 9$ as well as with $n_C > 9$.

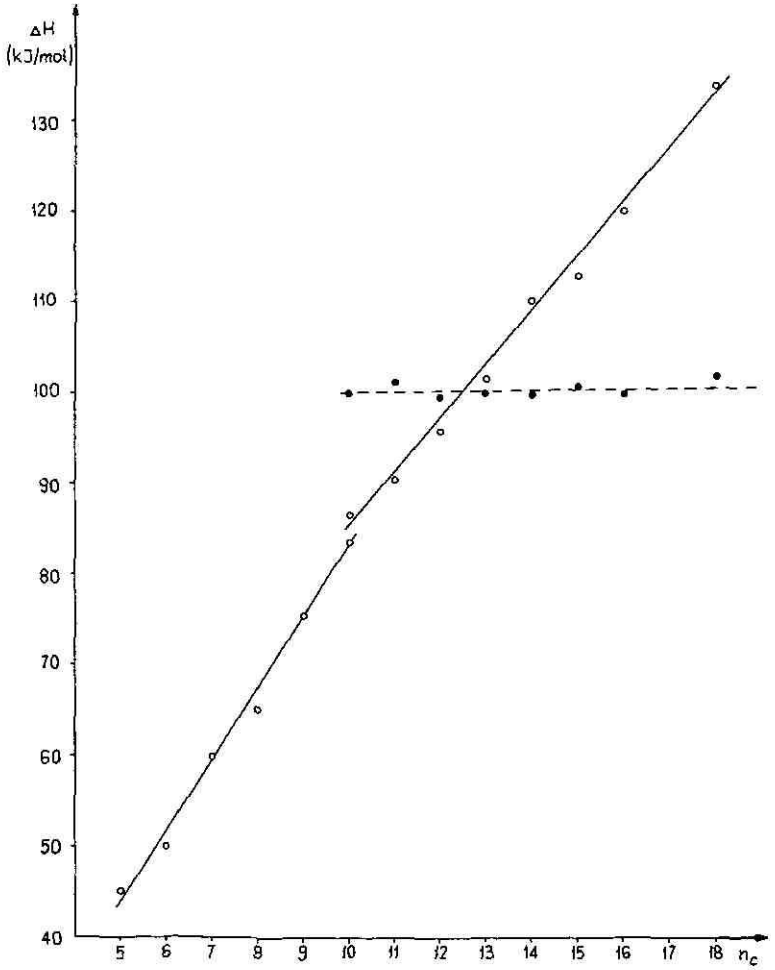

Fig. 3. Relationships between enthalpy of sorption, ΔH, and number of carbon atoms in n-alkanes, n_C. ○, At temperatures below 240°C; ●, at temperatures above 240°C.

close to 210°C the sorbent probably undergoes some structural change. The glass transition temperature (T_g) for this sorbent, determined by means of thermal analysis of a block polymer, is *ca.* 260°C. On the other hand, the value of T_g obtained by a chromatographic method[3] appears to be about 230–250°C. This difference in T_g is probably due to the different physical state of the porous polymer used in the chromatographic measurements compared with a block polymer. Hence the difference in the behaviour of *n*-alkanes at low (< 240°C) and high (≥ 240°C) temperatures can be atributed to the different sorption conditions. However, we are not able to give any plausible explanation of the unusual behaviour of *n*-alkanes observed at high temperatures other than that suggested previously[1].

Fig. 2 shows that for small *n*-alkane molecules ($n_C \leqslant 9$), the parameter B is independent of temperature above 210°C, implying that $\Delta H_0 = 0$. Also, from the temperature dependence of A the contribution to ΔH of one CH_2 group is $\Delta H_1 \approx 8$.

Unfortunately, we have not been able to obtain any reliable results for *n*-alkanes with $n_C \leqslant 9$ at temperatures above 235°C, as the retention times were very short. Nevertheles, the observed differences in the behaviour of *n*-alkanes with $n_C \leqslant 9$ and with $n_C \geqslant 10$ support, at least partially, our hypothesis[1] that small molecules should interact more strongly with the sorbent.

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Note

Reversed-phase high-performance liquid chromatographic determination of gadolinium–diethylenetriaminepentaacetic acid complex

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Recent investigations indicate that paramagnetic compounds may be extremely useful in enhancement of contrast in magnetic resonance imaging[1–10]. Of all compounds investigated, gadolinium–diethylenetriaminepentaacetic acid complex (Gd–DTPA) possesses the most suitable characteristics of high magnetic moment of the gadolinium atom, high stability (stability constant, 10^{23})[7] and low toxicity[3]. However, one of the major concerns for use in humans is the acute toxicity of the free gadolinium ion which may potentially result from dissociation of the complex *in vivo*[3].

Several investigators have applied ion-pair chromatography in normal- or in reversed-phase mode in analysis of metal complexes[11–16]. Recently, high-performance liquid chromatographic (HPLC) analyses of amine-N-carboxylic acid complexes of Fe(III) and Cu(II) have been reported[11,12,16]. An HPLC method was employed for determination of Gd–DTPA by Weinmann *et al.*[4], but details of the method development have not been reported.

We have developed an HPLC method which can be used in the determination of not only Gd–DTPA, but also free gadolinium ion (Gd^{3+}) and free DTPA ligand. We investigated the effect of various mobile phase variables, such as molarity and pH of the buffer, organic modifier, and ion-pairing agents on the capacity factor of Gd–DTPA, DTPA and Gd^{3+}, which led to improved separation and peak shapes. A UV detector operated at 200 nm was used in detection of Gd–DTPA and DTPA. In order to facilitate the detection of Gd^{3+} and Gd–DTPA, radioactive ^{153}Gd ($t_{\frac{1}{2}}$ = 241.6 d) was used.

In this paper we report our findings related to the retention characteristics of Gd-DTPA, DTPA and Gd^{3+} on a reversed-phase column with on-line UV and radioactivity detectors.

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EXPERIMENTAL

Equipment

The chromatographic system used in this investigation consisted of an Altex Model 110A pump, an Altex 155-40 variable-wavelength detector operated at 200 nm, and an Omniscribe Series D5000 recorder. The Altex injector was fitted with a 20-μl loop. Radioactivity was detected by using an on-line NaI(Tl) well counter coupled to a Nuclear Data Model 60A analyzer operated in multichannel scaling mode. The column used for the chromatographic analysis was an Alltech C_{18} column (10 μm, 25 cm × 4.6 mm I.D.). The column was operated at ambient temperature and all injections were 20 μl (full loop). A guard column (2 cm × 2 mm I.D.) filled with C_{18} Analytichem material (40 μm) was used for protection of the analytical column. Another guard column (5 cm × 4.7 mm I.D.) filled with silica (40 μm) was placed in the flow stream before the injector to saturate the mobile phase with silica.

Mobile phase

Primarily, the mobile phase consisted of 5 m*M* potassium dihydrogen phosphate–acetonitrile (90:10), with varying amount of ion-pairing agent (octylamine, tetrabutylammonium bromide or tetraethylammonium bromide). The pH of the mobile phase was adjusted to a desired value with phosphoric acid (2 *M*) or potassium hydroxide (2 *M*). The mobile phase was filtered through a 0.5-μm filter and degassed ultrasonically under reduced pressure.

Materials

No-carrier-added ^{153}Gd ($t_{\frac{1}{2}}$ = 241.6 d) was cyclotron-produced by irradiation of a gadolinium metal foil which produces mostly the terbium isotopes (^{153}Tb–^{160}Tb). The Tb radioisotopes are separated from the gadolinium target material by ion exchange. The ^{153}Tb ($t_{\frac{1}{2}}$ = 2.34 d) decays to ^{153}Gd, which is purified and separated from the remaining Tb radioisotopes[17].

Gd–DTPA and ^{153}Gd–DTPA (as disodium or dimethylglumine salts) were prepared according to the literature procedures[3]. All samples were prepared in the mobile phase and adjusted to appropriate concentrations. To facilitate the detection of Gd^{3+} and Gd–DTPA, the samples were spiked with radioactive $^{153}Gd^{3+}$ and ^{153}Gd–DTPA, respectively. For quantitation, stock solutions of Gd–DTPA and DTPA in mobile phase (10 mg/100 ml) were prepared. Appropriate dilutions were made to construct standard curves of peak height versus concentration.

The time required for the first unretained peak was used in the calculations of the capacity factors.

RESULTS AND DISCUSSION

Fig. 1 represents a typical chromatogram of a mixture of Gd–DTPA and DTPA. Gd^{3+} eluted at the solvent front in all chromatographic conditions investigated. The retention time of each analyte was established individually. It was also confirmed with the use of radioactive Gd–DTPA that there was no dissociation of the complex into free Gd^{3+} and DTPA under the chromatographic conditions used. Initially, 5 m*M* phosphate buffer (pH 4.5) was used as the mobile phase without any

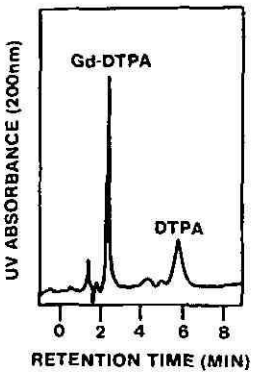

Fig. 1. Simultaneous HPLC analysis of Gd–DTPA and DTPA. Column: Alltech C_{18} (10 μm, 25 cm × 4.6 mm I.D.); mobile phase: potassium dihydrogen phosphate–acetonitrile (85:15) containing 2.5 m*M* octylamine; pH 7.0; flow-rate: 2 ml/min.

added organic modifier. All three compounds were completely unretained and eluted with the solvent front. Upon changing the pH of the buffer to 2–3, there was a slight retention of Gd–DTPA and DTPA. This is expected as ion-suppression at lower pH causes the unionized Gd–DTPA and GTPA to become more hydrophobic and therefore have greater interaction with the lipophilic column bed. Also, Gd–DTPA was retained longer than DTPA, perhaps because the Gd–DTPA has fewer ionizable carboxylic groups than the uncomplexed DTPA.

Upon addition of an ion-pairing agent, *n*-octylamine or tetrabutylammonium bromide, both Gd–DTPA and DTPA were retained. However, Gd^{3+} was not retained under these conditions and eluted at the solvent front. An organic solvent was added to the mobile phase to obtain reasonable capacity factors. Acetonitrile was chosen as the organic modifier because of its lower UV cut-off wavelength.

As generally occurs in reversed-phase chromatography, retention of Gd–DTPA and DTPA on the column decreased with increased percentage of acetonitrile in the mobile phase[18,19]. Similarly, capacity factors of Gd–DTPA and DTPA decreased upon increase in molarity of the phosphate buffer. This is expected since the increased ionic strength reduces the formation of ion pairs[18,19].

Ion pairing occurring only with ion-pairing agents such as *n*-octylamine and tetraalkylammonium bromide. No retention was observed with sulfonic acid ion-pairing agents, indicating that the ion pairing occurs at the carboxylic moieties and not at the amine moieties in Gd–DTPA and DTPA. In the Gd–DTPA complex, metal–ligand bonds are formed and broken constantly, resulting in at least one, if not two, carboxylic moiety which is not complexed with the central Gd atom and therefore available for ion-pair formation[20]. The order of elution of Gd–DTPA and DTPA is reversed as compared to that in the ion-suppression mode in the absence of an ion-pairing agent. This can be explained by consideration of the ion pairing at carboxylic moieties. DTPA ligand, having more carboxylic groups available for ion pairing than Gd–DTPA, is retained longer due to greater ion pairing.

The retention of Gd–DTPA (Fig. 2) increased rapidly upon increase in the concentration of the ion-pairing agent, while maintaining constant pH, and then reached a plateau. This is in accordance with the theoretical considerations of the

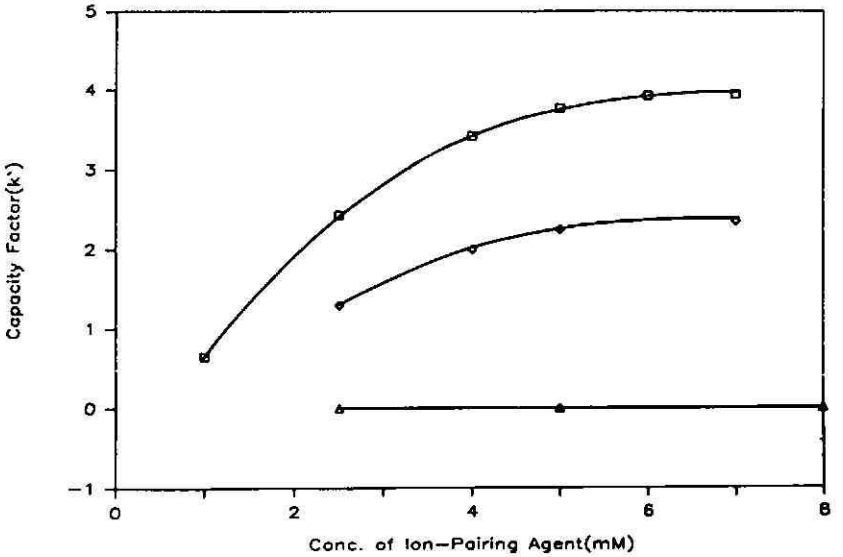

Fig. 2. Effect of concentration of ion-pairing agent in the mobile phase on capacity factor of Gd–DTPA. Mobile phase: 5 m*M* potassium dihydrogen phosphate–acetonitrile (90:10) with a variable amount of ion-pairing agent, pH 7.0. Flow-rate: 2 ml/min. □, Octylamine; ◇, tetrabutylammonium bromide; △, tetraethylammonium bromide.

ion-pair reversed-phase chromatography of weak acids[18,19]. Of interest is that *n*-octylamine and tetrabutylammonium bromide caused retention of Gd–DTPA and DTPA; however, as much as 10 m*M* concentration of tetraethylammonium bromide had no retaining effect. This is perhaps related to lower lipophilicity of tetraethylammonium bromide compared to tetrabutylammonium bromide. The mobile phase containing tetrabutylammonium bromide had UV absorption at 200 nm due to tetrabutylammonium bromide itself and consequently could not be used in conjunction with the UV detector at this wavelength. In this situation, retention time of Gd–DTPA was assessed with the radioactivity detector only. All retention times were corrected for the time-lapse between the two detectors. For routine analysis, the mobile phase containing *n*-octylamine was employed.

Capacity factors of the analytes are greatly affected in ion-pair chromatography by a change in pH of the mobile phase[18,19]. This mode of mobile phase control is often a powerful tool in ion-pair chromatography for regulating the separation selectivity of the analytes of interest. Although weak acids are generally retained longer upon increase in pH of the mobile phase due to the increased ion pairing, retention times of Gd–DTPA and DTPA decreased rapidly upon increasing pH. Fig. 3 shows the effect of pH on capacity factor in the presence of octylamine. Similar behavior was observed when tetrabutylammonium bromide was used as the ion-pairing agent. This seemingly anomalous behaviour has been well documented in literature[21,22].

Relative separation (selectivity, α) between Gd–DTPA and DTPA can be optimized by a combination of pH of the mobile phase and the concentration of the ion-pairing agent. Although the retention times of Gd–DTPA and DTPA decreased with increase in pH, increasing the amount of the ion-pairing agent increased the

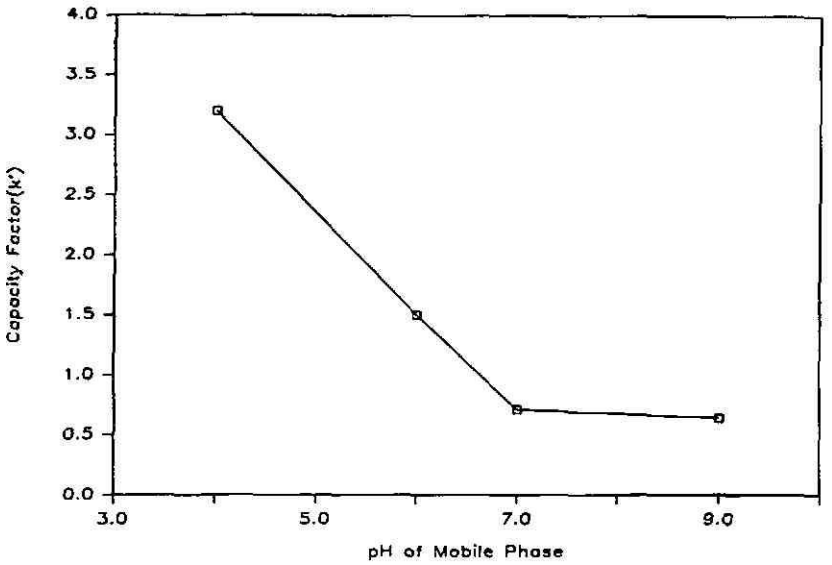

Fig. 3. Effect of pH of the mobile phase on capacity factor of Gd–DTPA in presence of octylamine. Mobile phase: potassium dihydrogen phosphate–acetonitrile (85:15) containing 2.5 m*M* octylamine; pH 7.0; flow-rate: 2 ml/min.

selectivity since DTPA by the virtue of having greater number of free carboxylic moieties is more affected than Gd–DTPA. There is an additional advantage in performing the analysis at higher pH ($\geqslant$ 7). The peaks were noticeably sharper due to greater and complete ion pairing at higher pH as compared to incomplete ion pairing at lower pH, leading to split or broadened peaks[23]. This is particularly true of DTPA. At lower pH, not only was the retention of DTPA too high, multiple peaks were observed due to different degrees of ionization of the five carboxylic moieties and consequently incomplete ion-pairing.

A linear relation between the peak height and concentration was observed for both Gd–DTPA and DTPA within the range of concentration investigated (100 ng–2 μg). The limit of detection was established at 100 ng (20 μl injection) with a signal-to-noise ratio of four.

CONCLUSION

A reversed-phase ion-pair chromatographic method has been developed for determination of Gd–DTPA, which can be extended to analyze free Gd^{3+} (with radioactivity detector only) and free DTPA which may result from dissociation of Gd–DTPA. It is shown that the regulation of mobile phase variables, such as pH, ion-pairing agent, organic modifier, and ionic strength leads to improved peak shapes and separation selectivity for Gd–DTPA and DTPA. This method may be applicable in quality control of samples containing Gd–DTPA and possible impurities of Gd^{3+} ion and DTPA ligand. Biological samples may be analyzed with the use of radioactive Gd–DTPA in conjunction with a radioactive detector.

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Note

Method for the analysis of ethyl carbamate in alcoholic beverages by capillary gas chromatography

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A number of methods have been described in the literature for the analysis of ethyl carbamate in alcoholic beverages. Walker *et al.*[1] described a suitable method of analysis for wine. After sample clean-up, packed column gas chromatography was used and a number of different detectors evaluated. Flame ionisation (FID), alkali flame ionisation (AFID) and electron capture were tried but only the Coulson electrolytic conductivity detector was considered suitable. With this technique it was possible to quantify ethyl carbamate in wine at a level of 100 μg/l. Mass spectrometry (MS) was used for identity confirmation but because of interferences it was necessary to analyse the ethyl carbamate as the trifluoroacetyl derivative. Ough[2] essentially followed Walker's procedure for his analyses of fermented beverages and foods. Joe *et al.*[3] were able to improve on Walker's original method. By further concentrating the sample and adopting a more rigorous clean-up regime they were able to use FID or AFID for levels of ethyl carbamate down to 10 μg/l although MS confirmation was still required.

A renewal of interest in this area of analysis, prompted by the Canadian Government decision to impose limits on the ethyl carbamate content of imported beverages[4], has led us to apply a number of new techniques to this problem. We have used solid-phase extraction and clean-up techniques to streamline the sample work-up procedures and employed capillary chromatography in place of the packed columns previously adopted. The performance of three different detectors has also been investigated in order to increase the sensitivity and selectivity of the analysis.

EXPERIMENTAL

Each sample of alcoholic beverage was analysed in duplicate. Recovery was

estimated by spiking a third aliquot of the sample with ethyl carbamate (5 μg in 50 μl ethanol) and taking this through the procedure.

Extraction and clean-up procedure

Samples of alcoholic beverage were taken and diluted to 50 ml so that the final alcohol content was below 5%. This solution was then absorbed on a CT 2050 Chemtube (Analytichem) (or Extrelut, Merck 42 g) and eluted with dichloromethane (3 × 50 ml). The eluent was passed through a short column containing anhydrous sodium sulphate (10 g) into a Kuderna-Danish concentrator. After rinsing the sodium sulphate with dichloromethane (5 ml) the combined extract was reduced to 4 ml on a waterbath at 55°C. The apparatus was then washed with dichloromethane (1 ml) and the concentrate applied to a Florisil Sep-Pak (Waters) (pre-rinsed with dichloromethane, 10 ml). A further 5 ml of dichloromethane was used to rinse the apparatus and applied to the Sep-Pak and the entire contents to this stage discarded. The fraction containing ethyl carbamate was eluted with 7% methanol in dichloromethane (5 ml) and the eluent was reduced in a micro Kuderna-Danish concentrator to about 0.7 ml. The apparatus was rinsed with dichloromethane (0.3 ml) and the final volume was measured with a syringe.

Gas chromatographic measurement

Three different detectors were evaluated under the following operating conditions:

(i) A thermal energy analyser Model 610 (Thermedics) was used in the nitrogen mode with an interface temperature of 200°C and a pyrolyser temperature of 800°C. The reaction chamber was operated at 2 mm mercury and no make-up gas was employed.

(ii) A Hall 700A electrolytic conductivity detector (Tracor) was used in the nitrogen mode without a scrubbing system. Furnace temperature was 840°C, the electrolyte was isopropanol–water (50:50) at 0.4 ml/min with manual venting and hydrogen fuel gas flow was 40 ml/min.

(iii) A VG 7070 H mass spectrometer was operated with electron impact ionisation (70 eV, 200 μA trap current, 200°C) and with the interface heater set to 200°C. Selected ion monitoring was employed, controlled by a VG 11/250 data system, at ion currents m/z 61, 62, 74.

Slightly different gas chromatographic conditions were employed for each detector to gain optimum performance from each instrument however the following, used for the thermal energy analyser (TEA) detector, was typical. A CP Wax 52 CB column (Chrompack), 25 × 0.31 mm I.D., 0.21 μm film thickness, was used with a temperature programme of 65°C (for 1 min) then 10°C/min to 100°C and 20°C/min to 200°C (for 10 min). On-column injection (2 μl) with secondary cooling was employed with helium carrier gas (0.6 bar).

RESULTS AND DISCUSSION

Extraction and clean-up procedure

The use of Chemtube or Extrelut materials greatly facilitates the dichloromethane extraction of the beverages since no emulsions are formed using this tech-

nique. It is not then necessary to clear the emulsions by centrifugation[3] and hence considerable savings in time are experienced. Similarly Florisil Sep-Paks can be used to clean-up the sample in about 1 min whereas the conventional column techniques previously used would require about 30 minutes. While this clean-up may not be essential where the use of the TEA detector is planned, we have found that it is necessary if a full mass spectrum is required for confirmatory purposes. It also leads to a significant extension of capillary column life.

GC detector comparison

Two chromatograms of Scotch whisky samples obtained using the Hall and TEA detectors are shown in Figs. 1 and 2. The level of ethyl carbamate corresponds to 43 μg/l for the TEA analysis and 32 μg/l for the Hall analysis. The retention times for ethyl carbamate (peaks shadowed) varies for the different instruments because of minor differences in the columns and temperature programmes used. Both the TEA and Hall detectors show relatively few peaks indicating the selectivity of these instruments in the nitrogen mode. Some problems were initially experienced with the use of the Hall detector. Dichloromethane is not a good solvent to use for samples when using this instrument since it produces hydrochloric acid on reduction and this leads to a high background signal. This can be overcome by exchanging dichloromethane for a different solvent (*e.g.* ethyl acetate) during the sample work-up procedure. However, manual venting of the detector until just before the ethyl carbamate elutes is equally effective and more convenient. This procedure does however lead to a marked disturbance of the baseline as can be seen from the chromatogram. The Hall detector possesses a scrubbing system which is designed to remove traces of acidic gases from the GC eluent. In fact this system leads to significant band spreading of the chromatographic peaks and its removal considerably improved chromatographic performance without causing any increase in background noise.

The electron impact mass spectrum of ethyl carbamate (Fig. 3) was dominated by fragment ions of *m/z* 44, 45 and 62. The first two ions and the molecular ion (*m/z* 89) were either not sufficiently specific or not intense enough to be useful in

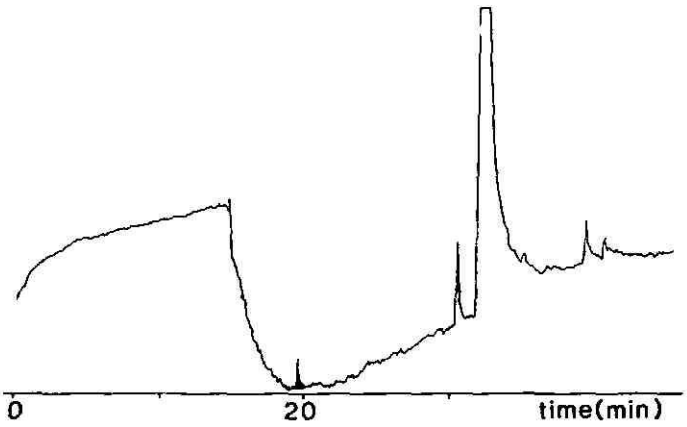

Fig. 1. Capillary GC analysis of whisky sample for ethyl carbamate (shadowed, 32 μg/l) using Hall 700A electrolytic conductivity detector.

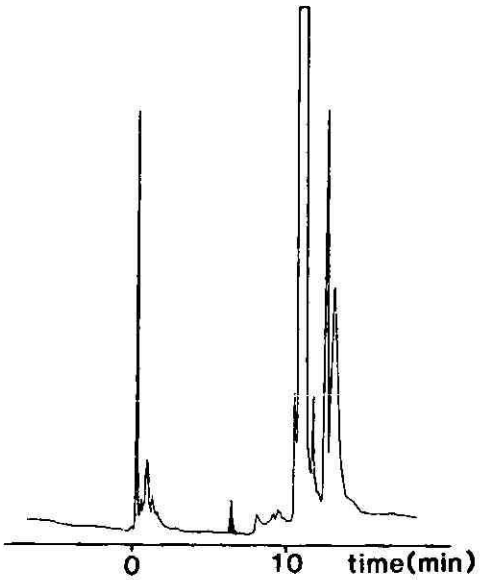

Fig. 2. Capillary GC analysis of whisky sample for ethyl carbamate (shadowed, 43 μg/l) using thermal energy analyser in the nitrogen mode.

trace analysis. However, as illustrated in Fig. 4, *m/z* 62 provided high selectivity and allowed a detection limit of 1 μg/l. The two additional ions chosen for selected ion monitoring (*m/z* 61 and *74*) were of low relative abundance and thus selectivity, but gave adequately resolved and measurable peaks for samples containing in excess of 30 μg/l. By ensuring that the ratios of all three ions were consistent with that of the ethyl carbamate standard, the identity of this component in the chromatogram could be ascertained with confidence.

No attempt was made to use the trifluoroacetylation recommended by Walker *et al.*[1]. The use of trimethylsilyl derivatives was examined but the EI spectra were dominated by non-specific fragments originating from the derivatising group. Chem-

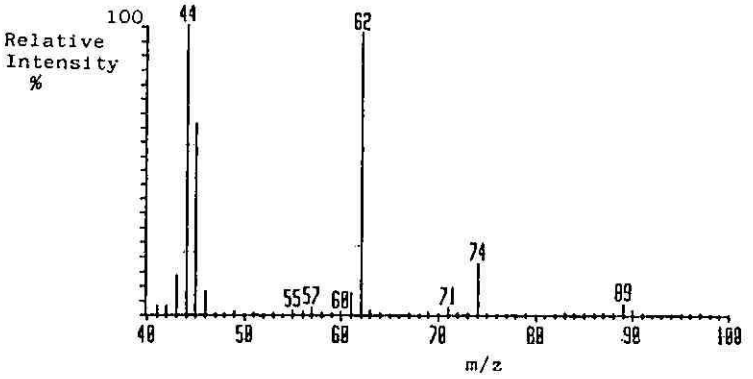

Fig. 3. Electron impact mass spectrum of ethyl carbamate.

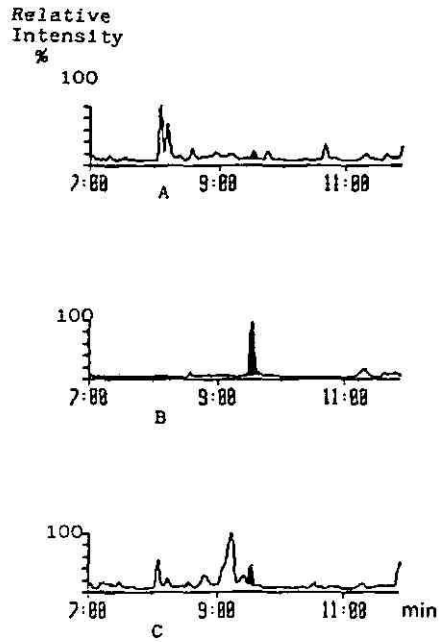

Fig. 4. Capillary GC analysis of North American whisky sample for ethyl carbamate (shadowed, 124 μg/l) using selected ion monitoring at *m/z* 61 (A), 62 (B) and 74 (C).

ical ionisation did not offer any advantage in terms of sensitivity compared with the monitoring of *m/z* 62.

Each of the detectors studied had similar detection limits for wine (defined as 3 × noise level) with the TEA (1 μg/l) and Hall (2–5 μg/l) detectors having comparable sensitivity to the mass spectrometer (1 μg/l). The results obtained from the three detectors (Table I) showed remarkable consistency between the different instruments

TABLE I

COMPARISON OF RESULTS FOR ETHYL CARBAMATE (μg/l) OBTAINED FROM DIFFERENT DETECTORS

Sample	*Mass spectrometer (m/z 62)*	*TEA detector*	*Hall detector*
Bourbon whisky a*	216**	204	176
b	212**	208	184
Scotch whisky a	75	80	72
b	77	99	84
Red wine a	22	16	13
b	Not analysed	13	10

* Each sample was extracted and concentrated in duplicate (a and b). Each concentrate was then analysed by the different detectors.

** Confirmed from *m/z* 61, 74.

given that some weeks elapsed between individual analyses. This serves further to confirm the identity of the contaminant measured as ethyl carbamate and indicates that any of these detectors might reasonably be used for monitoring the levels of ethyl carbamate in alcoholic beverages. In practice the TEA detector is used on a routine basis for this analysis since it proved consistently reliable and also permitted better detection limits than the Hall detector.

Analytical precision

On a day-to-day basis the sample clean-up and GC–TEA technique works very reliably. Blanks were consistently below the detection limit of the method and an average recovery of 84% (minimum 75%) was obtained for 12 whisky and wine samples. Duplicate analyses also proved very reproducible with the range normally within ± 5% of the mean. The repeatability shown by replicate analyses on three separate days proved consistent with a mean of 303 μg/l (coefficient of variation 6.34%) obtained for six analyses of a bourbon whisky.

In a limited study of the application of the methodology, ethyl carbamate levels in various alcoholic beverages showed a wide range of concentrations. Wines averaged 5 μg/l with a range of < 1 to 18 μg/l (13 samples), whilst the mean for 6 samples of sherry was 28 μg/l, range < 1 to 60 μg/l. The level in gin (2 samples) and vodka (2 samples) was below the 1 μg/l detection limit. In the case of whiskies concentrations in the range 20–90 μg/l were detected in 11 samples of Scotch whisky and in the range < 1 up to 230 μg/l for six imported whiskies.

CONCLUSION

The procedures described in this study are suitable for routine analysis of ethyl carbamate in alcoholic beverages at levels of down to 1 μg/l. The use of the Chemtube or Extrelut materials improves the sample analysis time by avoiding the use of liquid-liquid extraction and time consuming centrifugation of resultant emulsions. Additionally the Florisil clean-up stage provides a sample extract that is sufficiently clean that little deterioration in performance of the gas chromatographic analysis was noticeable even after several hundred injections. Studies are currently in progress to identify the reasons for the presence of ethyl carbamate in some alcoholic beverages. These findings will be reported elsewhere as will an investigation into the possible presence of ethyl carbamate in other fermented foodstuff.

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Note

Determination of ethyl carbamate in alcoholic beverages by methylation and gas chromatography with nitrogen–phosphorus thermionic detection

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Ethyl carbamate has recently become of concern because it has been found in certain alcoholic beverages at levels much higher than would be expected as a result of the fermentation process. This carcinogenic substance[1] has appeared in alcoholic beverages in the past as a result of the use of diethyl pyrocarbonate[2] (an antimicrobial agent which, in Canada, is no longer permitted). It is also possible that ethyl carbamate could form from the use of urea as a yeast food. Urea is known to react with ethanol to produce ethyl carbamate[3] and its use is presently being reviewed.

Gas chromatography (GC) has proven to be one of the most useful techniques for the determination of ethyl carbamate in alcoholic beverages[4–6]. Detection of the compound has been achieved by Coulson electrolytic conductivity[4,5], flame ionization[6], alkali flame ionization[6] or by mass spectrometry[6]. Ethyl carbamate has been treated with trifluoroacetic anhydride and determined as the N-trifluoroacetyl derivative by GC with electron-capture or alkali-flame ionization detection[4]. Of these methods, electrolytic conductivity (Coulson or, more recently, Hall) because of its selectivity appears to be the most useful for routine analysis of alcoholic beverages at ethyl carbamate concentrations in the low μg/kg range. However, few laboratories are equipped with this type of detector. It is the purpose of this work to demonstrate the use of the nitrogen–phosphorus (NP) thermionic detector for the determination of low levels of ethyl carbamate in alcoholic beverages. Thermionic detection is selective; however, it is not sensitive enough to be useful for direct determination of ethyl carbamate at low μg/kg levels in many alcoholic beverages. By forming the dimethyl derivative the detector response is increased considerably enabling improved detection limits.

EXPERIMENTAL

Reagents

All chemicals and solvents were reagent grade materials. Sodium hydride was obtained as a 50% oil mixture (J. T. Baker).

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Gas chromatography

A Hewlett-Packard 5830 gas chromatograph equipped with a DBWAX-30W (0.25 μm) (J&W Scientific) capillary column and a Hewlett-Packard NP (thermionic) detector was employed for the analyses. The conditions were as follows: carrier gas, helium at *ca.* 1 ml/min; injector, 250°C; detector, 280°C. The temperature program was: 60°C hold 3 min, 10°C/min to 240°C, hold 15 min. Retention times were 10.3 min for ethyl carbamate and 4.6 min for ethyl N,N-dimethylcarbamate.

Sample extraction

Sample preparation and cleanup were carried out essentially as described elsewhere[7]. Briefly, alcoholic beverages were diluted where necessary to yield about a 10% concentration of ethanol. A 50-g sample of diluted beverage was mixed with 30 g of potassium chloride and the mixture extracted with 3 × 100 ml of methylene chloride. A 3-ml volume of toluene was added to the combined methylene chloride extracts and the solution evaporated (rotary evaporator, 28°C) to *ca.* 2 ml and then diluted accurately with toluene to 5.0 ml for derivatization or direct analysis by GC. Normally, 2 μl of solution were injected.

Alkylation

A 1-ml aliquot of the toluene extract from above was transferred to a 15-ml graduated centrifuge tube with PTFE-lined screw cap. To this were added 0.5 ml of dimethyl sulfoxide and 0.5 ml of methyl iodide and the contents mixed gently. About 20–50 mg of hexane-washed sodium hydride were added to the tube which was then capped and shaken gently on a wrist-action shaker for 15 min. After this, 3 ml of hexane were added followed by the careful dropwise addition of water until the evolution of hydrogen ceased. (Sodium hydride reacts rapidly with water to evolve hydrogen.) Additional water, to a total of 10 ml, was then added and the mixture shaken vigorously for 1 min. After the phases separated, the organic layer was removed to a second centrifuge tube for GC analysis. If the layer was cloudy, a small quantity of anhydrous sodium sulfate was added to remove moisture.

RESULTS AND DISCUSSION

In a comparison of the Hewlett-Packard NP detector with the Hall electrolytic conductivity detector it was found that the former was less sensitive by a factor of at least 10 for ethyl carbamate (0.4 cm/ng and 5 cm/ng, respectively). However, upon the addition of two methyl substituents to the nitrogen atom of the molecule, the NP thermionic response became equal to that of the Hall for ethyl carbamate. The Hall response, however, decreased by a factor of about 10 for the derivative. These results are expected since the detection principle with thermionic detectors is known to involve a nitrogen–carbon species. Therefore the addition of two carbon atoms to the nitrogen of ethyl carbamate would be expected to increase the abundance of the species in the plasma. On the other hand, the principle of the Hall detector involves pyrolysis of nitrogen compounds to yield ammonia. Thus the additional carbons in the methylated derivative would be expected to hinder the formation of ammonia compared to the $-NH_2$ moiety of the underivatized ethyl carbamate.

Under the chromatography conditions used, the ethyl N,N-dimethylcarbamate

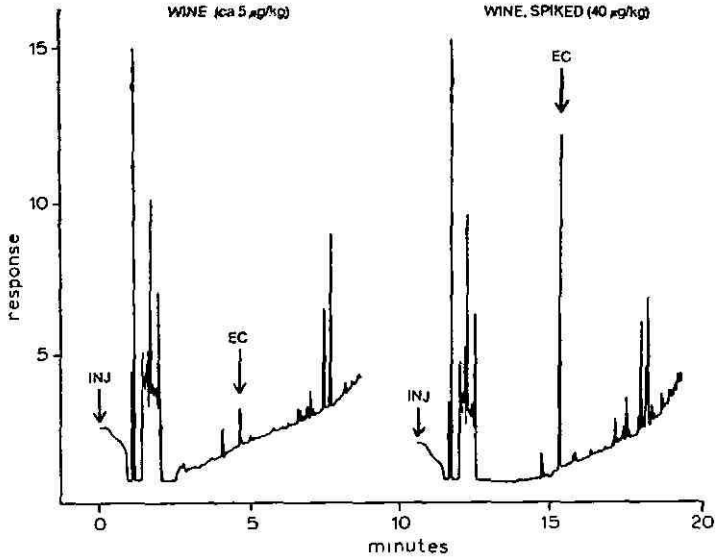

Fig. 1. Chromatograms of a wine sample containing *ca.* 5 μg/kg ethyl carbamate and the same sample spiked with 40 μg/kg ethyl carbamate. Chromatographic conditions are described in the text. EC = Ethyl carbamate.

was eluted at about 4.6 min compared to 10.3 min for the parent compound.

Fig. 1 shows typical results obtained for a wine sample containing *ca.* 5 μg/kg along with a similar sample spiked at 40 μg/kg. The ethyl carbamate derivative can be easily seen in both cases. Fig. 2 shows chromatograms of two samples (wine and

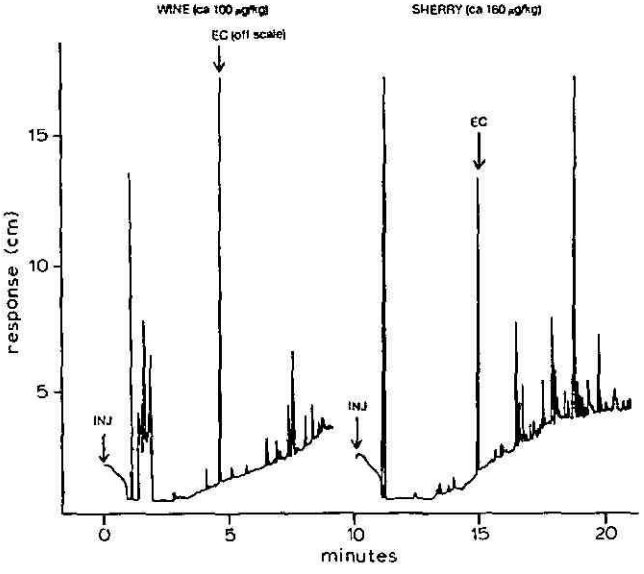

Fig. 2. Chromatograms of wine (*ca.* 100 μg/kg ethyl carbamate) using the DBWAX column and sherry (*ca.* 160 μg/kg ethyl carbamate) using a DB210 column. The sherry sample was diluted two fold before analysis. EC = Ethyl carbamate.

sherry) found to contain ethyl carbamate in excess of 100 μg/kg. The sherry sample was analyzed on a DB210 column under the same chromatographic conditions as described for the DBWAX column. Although the derivative was eluted in less than 5 min, it was necessary to continue temperature programming with both columns to 240°C and hold for about 15 min in order to remove other late eluting coextractives.

The recoveries of ethyl carbamate added to sherry at levels of 80 and 120 μg/kg were 91 and 95%, respectively, and agree well with values obtained by direct analysis with the Hall detector (92 and 87%, respectively). The repeatability of the derivatization reaction was tested by replicate derivatizations of 1-μg and 400-ng quantities of ethyl carbamate in toluene. The coefficients of variation were 3.3% and 2.5%, respectively. The reaction also was tested over a range of ethyl carbamate concentrations from 200 ng to 1.2 μg and found to yield linear results over the whole range. Solutions of the derivative were found to be stable for at least several weeks.

The identity of the alkylation product was determined by GC–mass spectrometry (VG 7070E at 4000 resolution) and found to have a molecular ion of 117 indicating the addition of two methyl groups to the nitrogen atom of ethyl carbamate.

The alkylation procedure described herein enables the use of the NP thermionic detector and thus provides an alternative to the Hall detector for the determination of ethyl carbamate in alcoholic beverages at levels in the low μg/kg range. This method has been successfully applied to a variety of beverages and can act as a confirmation of results obtained by direct analysis using the Hall conductivity detector.

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Note

Gas chromatographic separation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from polychlorinated biphenyls and tetrachlorodibenzo-*p*-dioxin isomers using a polymeric liquid crystal capillary column

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The extraordinary toxicities of the polychlorinated dibenzo-*p*-dioxins (PCDD) have been demonstrated by animal tests and, to some extent, by accidental exposure of human to these compounds. This has prompted extensive efforts to separate and identify these compounds in various chemical, biological and environmental media[1]. The various experiments have indicated that there is a pronounced difference in toxic and biological effects among different PCDD isomers. Based on the results of whole animal studies and *in vitro* assays, the symmetrical substituted 2,3,7,8-tetrachloridibenzo-*p*-dioxin (2,3,7,8-tetra-CDD) isomer appears to be the most toxic. A factor of 1000 to 10 000 difference in toxicity can be found for the closely related 2,3,7,8- and 1,2,3,8-tetra-CDD isomers[2,3].

The isomer specific separation and isolation of 2,3,7,8-tetra-CDD using reversed-phase and normal-phase high-performance liquid chromatography has been reported previously. The method appears to be too labor-intensive for use as a routine analytical technique; moreover further analysis is needed by ultra-sensitive gas chromatography–mass spectrometry (GC–MS)[4]. The high-resolution GC–MS has shown high potential for the separation and quantitative identification of 2,3,7,8-tetra-CDD in very complex samples. The U.S. Environmental Protection Agency (EPA) has developed a method for the separation and quantification of 2,3,7,8-tetra-CDD where the application of very long capillary columns (60 m) is recommended[5]. The success of the separation and quantification of PCDD depends on the concentration of the interfering compounds; the separation and positive identification of 2,3,7,8-tetra-CDD in sample matrices that contain million fold higher levels of other naturally occurring compounds or other chlorinated industrial pollutants has presented a challenging task for analytical chemists. It is especially difficult to analyse for the 2,3,7,8-tetra-CDD in presence of polychlorinated biphenyls (PCBs) because of interferences in GC and MS. The complex sample clean-up procedures used for PCB separation prior to analysis of 2,3,7,8-tetra-CDD and other PCDD in samples from fires in transformers filled with PCB are partially effective[5].

Liquid crystals have been used as selective stationary phases in GC for the separation of close boiling isomeric organic compounds (6–8). Recently polymeric liquid crystals have been developed and used in capillary column GC (9–14) for separation of polyaromatic compounds. But to date, polymeric liquid crystal columns

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have not been used for the separation of PCDD. In this paper we report, for the first time, the application of polymeric liquid crystals to complete separation of the most toxic 2,3,7,8-CDD from all tetra-CDD isomers present in flyash extrct, and the separation of this isomer from all PCB isomers.

EXPERIMENTAL

The gas chromatograph used was a Hewlett-Packard 5880 equipped with an electron-capture detector and a cool on-column injector. The GC–MS system used was a Hewlett-Packard 5987A with an HP 1000 data system. The Aroclors (1216, 1242, 1248, 1254) were purchased from Ultra Scientific (RI, U.S.A.). The labelled [$^{13}C_{12}$]-2,3,7,8-tetra-CDD was obtained from Cambridge Isotope Labs. (Cambridge, MA, U.S.A.). A fused-silica capillary column coated with polymeric liquid crystal and reported previously for the separation of polyaromatic compounds was used[14]. The Ontario flyash was extracted as reported previously[15]. The extract was spiked with labelled [$^{13}C_{12}$]-2,3,7,8-tetra-CDD and was analysed by GC–MS using the selected ion monitoring mode. The tetra-CDD isomers were confirmed by proper intensities of M, M + 2, M + 4 (320, 322, 324, respectively) ions. In addition 2,3,7,8-tetra-CDD was confirmed by retention time of standard labelled [$^{13}C_{12}$]-2,3,7,8-tetra-CDD.

RESULTS AND DISCUSSION

The complete GC separation of 2,3,7,8-tetra-CDD from PCB isomers using fused-silica capillary column coated with polymeric liquid crystal is shown in Fig. 1. The PCB mixture containing many different isomers was prepared by mixing the commercial Aroclors (1216, 1242, 1248, 1254). Because of the high toxicity of PCBs as well as of 2,3,7,8-tetra-CDD, a very small sample size and the very high sensitive ECD were used in the separation studies. For the positive identification of PCDDs in complex samples by GC–MS in the selected ion monitoring mode various criteria have to be satisfied. It is possible to identify and quantify the PCDDs if the concentrations of the interfering compounds are low. However, in samples such as those obtained from PCB fire, the interfering compounds are million times more concentrated than the PCDDs and it is therefore extremely difficult to satisfy all the criteria for positive identification. The pentachlorobiphenyls have a molecular ion (M = 324) equal to the molecular isotope ion (M + 4) of tetra-CDD, hence the use of criteria such as correct isotopic abundances is very difficult or impossible. It could be possible to identify selectively the 2,3,7,8-tetra-CDD if it could be separated from interfering compounds. With capillary columns such as those coated with SE-30 or DB-5 such separation cannot be obtained presumably because of close volatilities of pentachlorobiphenyls and tetra-CDDs. Liquid crystals are selective stationary phases used in GC, where separation is based on the structural differences of the solutes along with their volatilities. Based on the separation mechanism of liquid crystal stationary phases, for the compounds with equal volatility the linear and symmetrical molecules will be retained longer than the bulkier molecules because of their favorable geometry in retention to that of liquid crystal matrix. Fig. 2 shows that the PCB isomers are flexibil because of the single bond connecting two benzene rings while

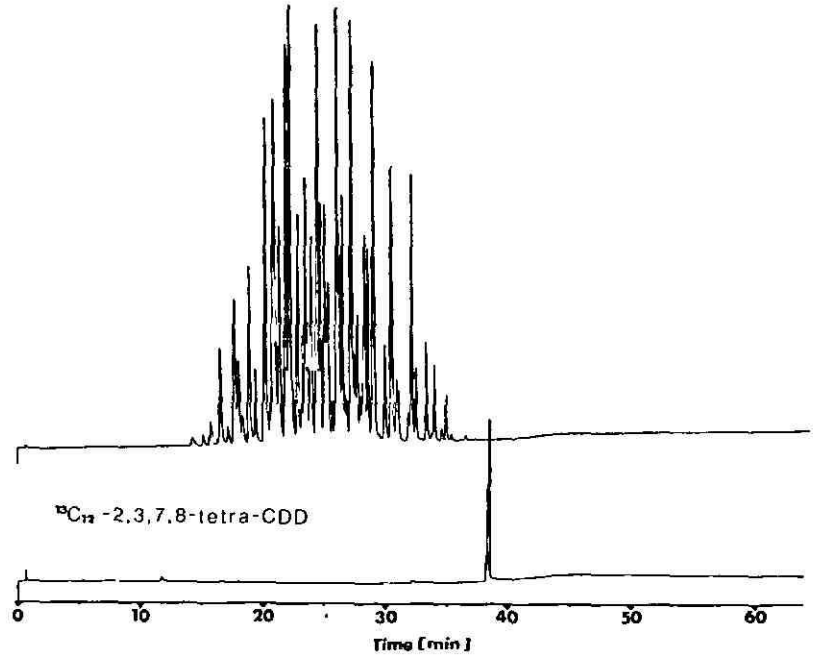

Fig. 1. Gas chromatogram of PCBs (Aroclor mixture) and 2,3,7,8-tetra-CDD. Chromatographic conditions: 20 m × 0.25 mm I.D. liquid crystalline polysiloxane coated fused-silica column; temperature at 100°C for 1 min, programmed to 270°C at 3°C/min.

the PCDD structure is rigid. This structural difference results in longer retention times for PCDDs than for PCBs. In particular the 2,3,7,8-tetra-CDD isomer is the most liner and symmetrical hence it was eluted after all PCB isomers (Fig. 1).

Considering the structural differences of the tetra-CDD isomers it can be seen that 2,3,7,8-tetra-CDD is the most symmetrical isomer. The elution order and separation of 2,3,7,8-tetra-CDD as compared to all other tetra-CDD isomers present in an extract of organic compounds obtained from municipal incinerator flyash was confirmed by GC–MS analysis of a flyash extract spiked by standard [$^{13}C_{12}$]-2,3,7,8-tetra-CDD. There is a very small difference of about two scans in GC–MS analysis of labelled [$^{13}C_{12}$]-2,3,7,8-tetra-CDD and unlabelled 2,3,7,8-tetra-CDD isomers[16]. Hence, the 2,3,7,8-tetra-CDD isomer in flyash extract was considered to be eluted at the same time as labelled 2,3,7,8-tetra-CDD was eluted (Fig. 3). The GC–

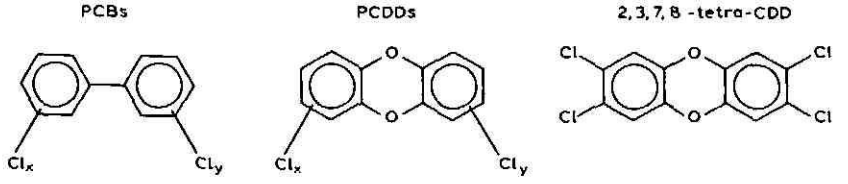

Fig. 2. Structures of PCBs, PCDDs and 2,3,7,8-tetra-CDD.

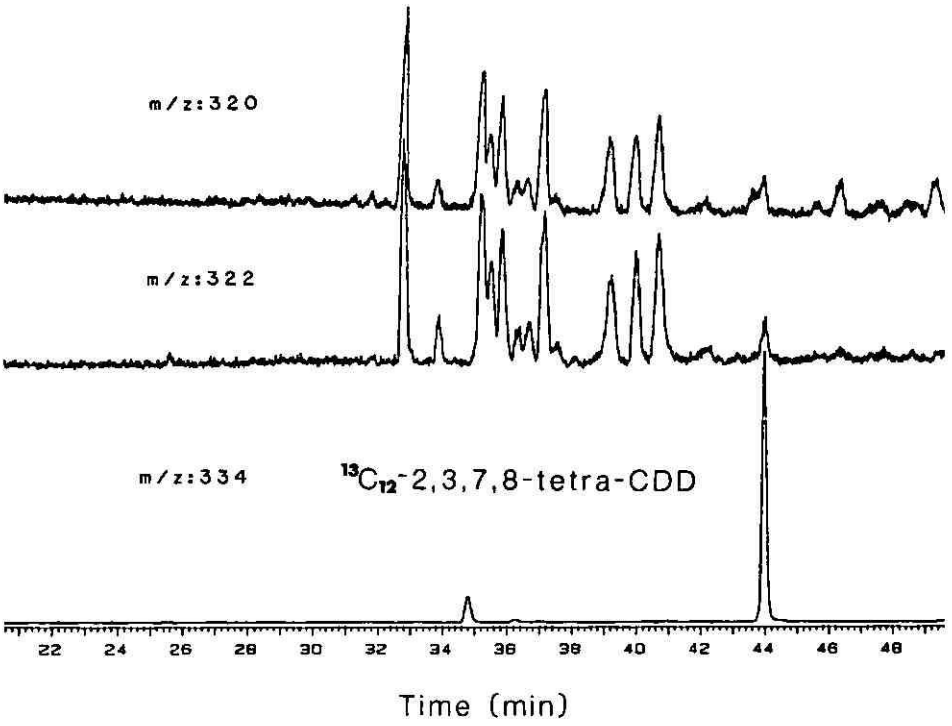

Fig. 3. Separation of 2,3,7,8-tetra-CDD from other tetra-CDD isomers in flyash extract, mass chromatogram of m/z = 320 (M), m/z = 322 (M + 2) (for tetra-CDD isomers) and m/z = 334 (M + 2) (for [$^{13}C_{12}$]-2,3,7,8-tetra-CDD) isomer. Chromatographic conditions: temperature program rate 4°C/min, all other conditions as in Fig. 1.

MS system, was operated in the electron-impact selected ion monitoring mode where m/z = 320, 322, 324 (M, M + 2, M + 4) ions for tetra-CDD and m/z = 332, 334, 336 for labelled 2,3,7,8-CDD were monitored. Flyash extract used in this study was not obtained for the quantation purpose. The concentration of 2,3,7,8-tetra-CDD can be estimated roughly to be 2–3 ng per gram of flyash. In Fig. 3 all peaks present both in M and M + 2 (m/z = 320, 322) ions are the tetra-CDD isomers according to the criteria for positive identification of chlorinated dioxins. The small peak present in M + 2 ion trace of [$^{13}C_{12}$]-2,3,7,8-tetra-CDD isomer is from an unknown compound present in flyash extract. This peak was not observed when only [$^{13}C_{12}$]-2,3,7,8-tetra-CDD was injected. There is a correlation between the chemical structures and the selectivity of the liquid crystal stationary phases[17]. It should be possible to synthesize specific liquid crystalline polymer stationary phases which can separate all PCDDs from PCBs.

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Note

Molecular characterization of fructans by high-performance gel chromatography

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Linear β-2,1-linked fructans (inulins) occur as reserve carbohydrates in Compositae and Campanulaceae[1]. Fructans with a linear β-2,1 backbone, branched β-2,6, are deposed by certain Liliaceae in their perennial rhizomes[2]. As is well known from the glucan series, it might be expected that these two different fructan types would differ considerably in their physico-chemical behaviour. The linear inulin, assumed to have a helical structure[3], is soluble only in hot water and can be crystallized from it by cooling or precipitation with alcohol. The highly branched Liliaceae fructans, in this work commercially available sinistrin from red squill and a laboratory-prepared fructan from *Lilium bulbiferum*, are amorphous, glassy and hygroscopic substances that are very soluble in cold water and cannot be precipitated with even 80% methanol.

The molecular characterization of fructans by modern chromatographic methods has been achieved using either high-performance liquid chromatography (HPLC)[4,5] or low-pressure gel permeation chromatography (GPC)[6–8]. The more time-consuming GPC has the advantage that the average molecular weights $\overline{MW}_w$ and $\overline{MW}_n$, the average chain lengths $\bar{P}_w$ and $\bar{P}_n$ and the dispersity factor $\bar{P}_w/\bar{P}_n$ can be calculated easily using the integral mass-distribution function $I(P)$[6]. The HPLC methods offer the possibility of determining the concentration of single sugar oligomers, but the molecular parameters that are necessary for characterizing the disperse distribution of polymers cannot be calculated.

A high-performance GPC method is described that reduces the inconveniently long analysis time of low-pressure GPC to that of the silica HPLC method. Similarly to low-pressure GPC, this new method allows the molecular parameters of fructans to be calculated after appropriate calibration.

EXPERIMENTAL

Commercially available inulin (chickory) and sinistrin (red squill) were gifts from the Laevosan Gesellschaft (Linz, Austria). Native and purified inulin from Jerusalem artichoke tubers and chickory roots were isolated as described earlier[6]. Fructan from *Lilium bulbiferum* was prepared analogously to the inulins.

A 20-μl volume of a 5% fructan solution was applied with a Rheodyne 7125

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loop valve to a Superose 12 column (Pharmacia) (300 × 10 mm I.D.); solvent deaerated distilled water was used (LKB 2150 pump). Detection was effected with a Waters Assoc. RI 401 detector and monitoring with an LKB 2210 flat-bed recorder. The separation was carried out at room temperature. Calculation and calibration of the chromatographic system were carried out analogously to low-pressure GPC as described by Praznik and Beck[6]. The void volume was determined with dextran 5000 (a gift from R. W. Klingler). The total volume of the system was determined with deuterium oxide (Merck, Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

It is well known that the migration behaviour in GPC is influenced by the structure of polysaccharides. It has been shown that the higher the molecular weight of a polysaccharide, the more its migration behaviour differs[9]. In the low-molecular-weight region of polysaccharides (less than 20 000 daltons) the structure was found not to influence the migration behaviour significantly on low-pressure gel columns. A narrow packed, high-pressure packing material, however, is not only expected to act as a simple gel matrix as described above, but also, because of the counter-pressure and pressure decrease in the column, the migration behaviour might be influenced by the intrinsic viscosity of samples[10]. Shearing and other effects should also not be neglected in this context. If this hypothesis were true, a slight difference in the intrinsic viscosity of the samples would distinctly affect the migration behaviour in high-pressure GPC systems.

As can be seen from Fig. 1, the calibration graph of dextrans differs significantly from that of the fructans. This indicates that the structural construction of the monosaccharide subunits and the different polymeric structure, resulting in a different intrinsic viscosity $[\eta]$ and therefore in a different hydrodynamic volume $[\eta] \cdot MW$, affects the migration behaviour on narrow packed gel columns even in a relatively low-molecular-weight region[10].

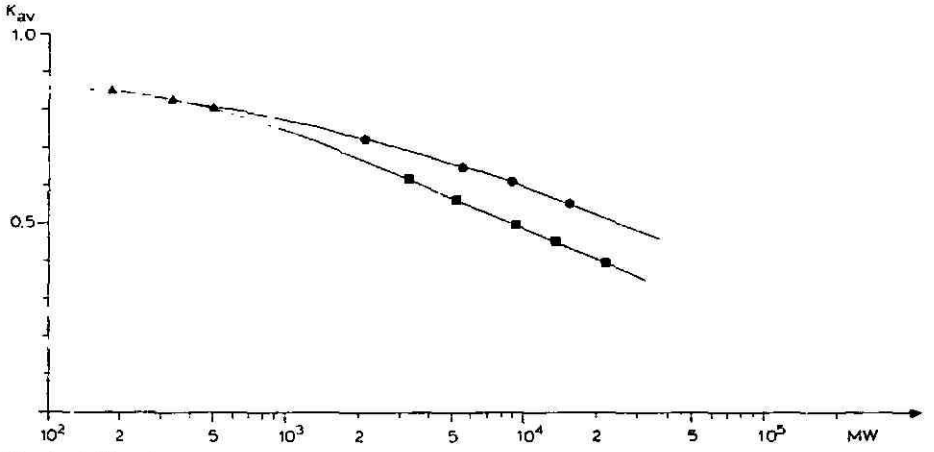

Fig. 1. Calibration graph for the Superose 12 column: relationship between the distribution coefficient, K_{av}, and the molecular weight. ■, Dextran T 10, T 40; ▲, fructose, sucrose, isokestose; ●, molecular defined (osmometry, light scattering) inulins.

Figs. 2 and 3 show the separation of linear and branched fructans. Possible impurities such as proteins or pectins occurring in biological materials are totally separated from the fructans and do not disturb the calculation. This means that the samples need not be highly purified before being applied to the column, which is desirable in low-pressure GPC[6] and necessary in HPLC[4]. This is especially important for fructans, because of their acid lability. Even on passing through an acidic ion exchanger hydrolysis can occur.

The purified fructans are narrowly distributed, as expected. The native fructans, however, have a dispersity $\bar{P}_w/\bar{P}_n \approx 2.0$, which corresponds well with the idea that linear unbranched polysaccharides should be binomially distributed[11]. Table I lists the molecular parameters of the fructans shown in Figs. 2 and 3.

It is remarkable that the branched fructans have a dispersity factor under 1.40. Especially the native fructan from *Lilium bulbiferum* is remarkably uniform compared with the native inulins. This uniformity could lead to the notion that some "repeating

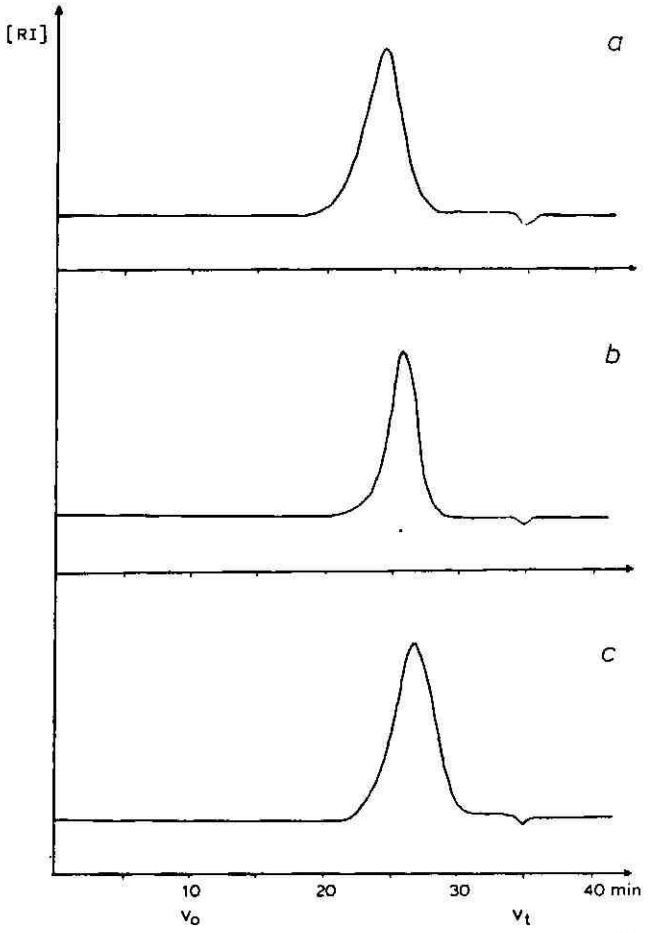

Fig. 2. High-performance GPC separation on Superose 12 of purified (a) Jerusalem artichoke inulin, (b) chickory inulin and (c) sinistrin (red squill).

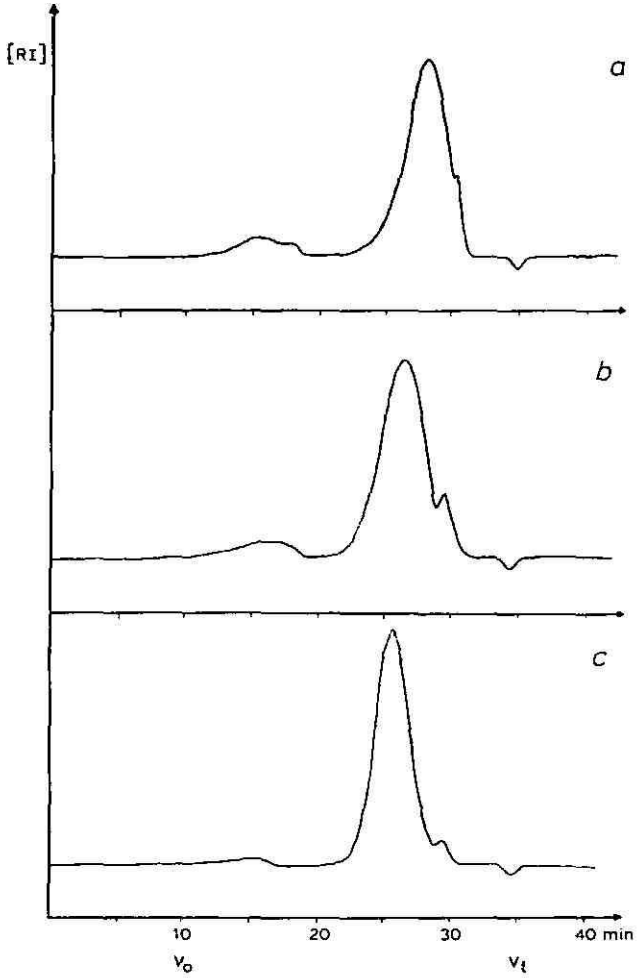

Fig. 3. High-performance GPC separation on Superose 12 of native (a) Jerusalem artichoke inulin, (b) chickory inulin and (c) fructan from *Lilium bulbiferum*.

TABLE I

NUMBER- AND WEIGHT-AVERAGE MOLECULAR WEIGHTS AND CHAIN LENGTHS AND DISPERSITY FACTORS OF CERTAIN FRUCTANS

State	*Fructan*	$\overline{MW}_w$	$\overline{MW}_n$	$\overline{P}_w$	$\overline{P}_n$	$\overline{P}_w/\overline{P}_n$
Purified	Inulin (Jerusalem artichoke)	10 500	8800	65	54	1.20
	Inulin (chickory)	6450	5300	40	33	1.22
	Sinistrin (red squill)	7000	5070	43	31	1.38
Native	Inulin (Jerusalem artichoke)	2900	1300	18	8	2.22
	Inulin (chickory)	4450	2350	28	15	1.88
	Fructan (*Lilium bulbiferum*)	3180	2430	20	15	1.31

blocks" of oligosaccharide sub-chains are used for the synthesis of this polysaccharide.

ACKNOWLEDGEMENTS

The authors thank Laevosan Gesellschaft for providing chickory inulin and sinistrin and Pharmacia for generously providing the Superose 12 column. This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung.

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Note

British Pharmacopoeial gentamicin sulphate component ratio test by high-performance liquid chromatography

The effect of derivative breakdown on final result

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Gentamicin sulphate is an aminoglycoside antibiotic complex, consisting of four major components, designated C1, C1a, C2 and C2a[1–5]. The structures of these components have been determined spectroscopically[6]. Since the C-complex components may differ in antibiotic activity and in toxicity, pharmacopoeial monographs on gentamicin contain tests which limit their relative ratios. In the United States Pharmacopoeia (U.S.P.) procedure[7], three components (C1, C1a and C2) are separated by paper chromatography and are determined by microbiological assay. Until recently, the British Pharmacopoeia (B.P.)[8] utilised an NMR method. The 1983 B.P. Addendum[9] introduced a reversed-phase high-performance liquid chromatographic (HPLC) method[10] in which limits are applied to the relative ratio of the normalised C1, C1a, and combined C2 + C2a peak areas.

The B.P. procedure requires that gentamicin is derivatised prior to HPLC. Primary amine groups of the gentamicin components are reacted with *o*-phthalaldehyde (OPA) and mercaptoacetic acid in borate buffer at pH 10.4 to yield 1-alkylthio-2-alkylisoindole derivatives. These provide strong chromophores for UV detection at 330 nm. Although derivatisation occurs under alkaline conditions, the derivatised components are chromatographed under acidic conditions (pH 4–5). Since isoindoles are generally highly reactive compounds[11–13], concern was voiced as to the stability of these derivatives during the HPLC procedure.

In this study, the stability of gentamicin derivatives was determined under the alkaline conditions required for derivatisation and under the acidic conditions used for chromatography. It was envisaged that any derivative breakdown during chromatography would be time-dependent and that component derivatives with long retention times (C2 and C2a) would be particularly affected by degradation prior to detection. Since this could influence the peak area ratios obtained by this method,

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the effect on the final result of changing the HPLC analysis time was investigated by varying the flow-rate of the mobile phase.

EXPERIMENTAL

Apparatus

A Gilson 302 pump, a Cecil CE2112 variable-wavelength UV detector and a Rheodyne 7125 loop-valve (20 μl) were used. Peak area data was obtained with a Trilab computer (Trivector Scientific).

Chemicals

Gentamicin sulphate BP was from Roussel Laboratories Ltd. All other chemicals and solvents were obtained from BDH (Poole, U.K.).

Stability of derivatives diluted with borate buffer, pH 10.4, and with mobile phase

A 0.0624% (w/v) solution of gentamicin sulphate was derivatised with OPA in accordance with the B.P. method[9]. The derivative solution was then diluted 1:5 with (a) 0.04 *M* borate buffer, pH 10.4, or (b) mobile phase used in the HPLC procedure, 0.02 *M* sodium heptanesulphonate in water–glacial acetic acid–methanol (25:5:70, v/v) (final pH of this dilution = 4.57). Both dilutions were protected from light and maintained at ambient temperature (22°C). At intervals of 0, 30 and 60 min after preparation, samples of both dilutions were analysed by the HPLC procedure of the B.P.[9], using a 12.5 cm × 5 mm I.D. column packed with Hypersil C_{18} (5 μm particle size) and UV detection at 330 nm.

Effect of HPLC analysis time on normalised peak area ratio

Solutions of gentamicin sulphate (0.0624%, w/v) were derivatised in accordance with the B.P. procedure[9]. Freshly prepared derivatives were immediately chromatographed under B.P. conditions, but using a 25.0 cm × 5 mm I.D. column (packed with 5-μm Spherisorb ODS) to increase resolution at high flow-rates. The flow-rate was varied over the range 0.25–3.0 ml min^{-1} for successive injections of freshly prepared derivatives.

RESULTS AND DISCUSSION

Stability of derivatives diluted with borate buffer, pH 10.4, and with mobile phase

Gentamicin derivatives diluted with borate buffer, pH 10.4, for up to 60 min prior to HPLC analysis showed no significant decrease in peak area (Table Ia). Under the alkaline conditions used for derivatisation, all four gentamicin component derivatives are therefore stable. When diluted with acidic mobile phase, the peak area of all four component derivatives decreased with time. Changes in peak area over the time intervals studied are expressed as a percentage in Table I. Decreases in peak area varied between components, and over a 60-min period ranged from 16.7% in the case of the C1 derivative peak area to 43.7% with the C1a derivative peak (Table Ib).

Since the on-column situation would not be one in which excess derivatisation reagent and all gentamicin components would be intimately mixed, simple dilution

TABLE I

HPLC PEAK AREAS AND PERCENTAGE CHANGE IN PEAK AREAS OF OPA-GENTAMICIN DERIVATIVE SOLUTION DILUTED IN 0.04 *M* BORATE BUFFER, pH 10.4 AND MOBILE PHASE, ANALYSED AT INTERVALS OF 0, 30 AND 60 min FROM TIME OF DILUTION

Peak area is expressed in arbitrary units.

Time after dilution (min)	*Peak area (percentage change in peak area)*				
	C_1	C_{1a}	C_{2a}	C_2	*Total area*
Borate buffer, pH 10.4					
0	6321	5031	2378	6576	20 306
30	6334 (+0.2)	5052 (−0.4)	2436 (+2.4)	6770 (+3.0)	20 592 (+1.4)
60	6387 (−0.5)	5027 (−0.1)	2425 (+2.0)	6804 (+3.5)	20 543 (+1.2)
Mobile phase, pH 4.57					
0	6526	4380	2249	6184	19 339
30	5890 (−9.8)	3361 (−23.3)	2048 (−8.9)	5646 (−8.7)	16 945 (−12.4)
60	5439 (−16.7)	2466 (−43.7)	1756 (−21.9)	5141 (−16.9)	14 802 (−23.5)

experiments of the type described could not be used to determine exact rates of derivative breakdown during chromatography. Further experiments were required in which the retention time of the component derivatives on the column was varied by altering the flow-rate of the mobile phase.

Effect of HPLC analysis time on derivative breakdown and normalised peak area ratios

Variation of the mobile phase flow-rate between 0.25 and 3.0 ml min^{-1} produced HPLC analysis times ranging from approximately 10 to 100 min. The retention times and corresponding normalised peak areas of gentamicin derivatives, obtained at different flow-rates are shown in Table II. These data are presented graphically in

TABLE II

EFFECT OF CHROMATOGRAPHIC ANALYSIS TIME ON GENTAMICIN COMPONENT RATIOS OBTAINED BY THE B.P. PROCEDURE

Flow-rate (ml min^{-1})	*Component retention time (t_R)*				*Normalised peak areas (%)*			
	C_1	C_{1a}	C_{2a}	C_2	C_1	C_{1a}	C_{2a}	C_2
3.0	2.5	6.4	8.5	9.6	29.6	22.0	14.3	34.1
2.0	3.7	9.5	12.5	14.2	29.7	22.7	15.0	32.6
1.5	4.8	12.0	15.8	17.9	30.5	22.1	14.1	33.2
1.0	7.5	18.9	24.9	28.2	30.3	23.7	13.9	32.1
0.5	14.9	37.0	48.6	54.9	32.3	20.9	15.0	31.8
0.25	28.5	69.1	90.2	101.7	33.3	20.6	13.2	30.8
		Extrapolated to t_R = 0:			29.4	22.9	14.6	33.1
		B.P. limits:			25–50	15–40	20–50	

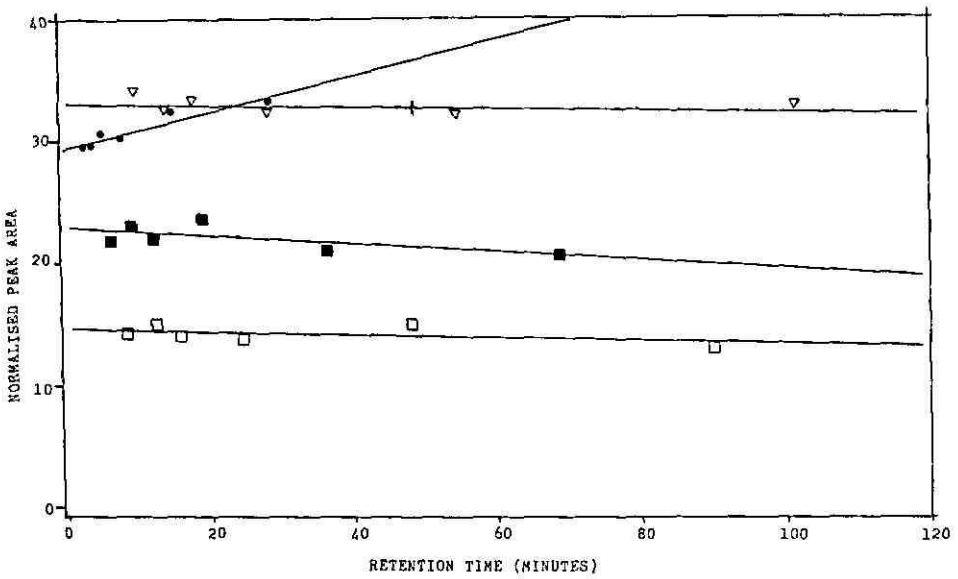

Fig. 1. Plot of normalised peak area ratio for gentamicin C-Complex components against HPLC retention time. ●, Component C_1; ■, component C_{1a}; □, component C_{2a}; ▽, component C_2.

Fig. 1, where extrapolation to zero-time yields the true value of normalised peak area ratios without any contribution from derivative breakdown. The extrapolated "zero-time" peak area ratios are included in Table II together with the B.P.[9] limits.

The normalised peak areas obtained at different flow-rates (Table II) were used to calculate the effect of HPLC analysis time on the accuracy of normalised peak areas. As the HPLC retention time increased, the normalized peak areas of the C1a, C2a and C2 components decreased while that of the C1 component increased. These observations were attributed to derivative breakdown during chromatography. The error associated with the normalised peak areas could be decreased by reducing the HPLC analysis time. In our experience with the B.P.[9] method, adequate resolution has been achieved with analysis times of 20–30 min. In this study an analysis time of 30 min (flow-rate = 1 ml min^{-1}) gave errors of +3.1%, +3.5% and −3.6% for the respective C1, C1a and C2a + C2 normalised peak areas. These errors would be insignificant except in the most borderline of cases.

This study highlights potential difficulties in the HPLC analysis of complex drug compounds requiring pre-column derivatisation. In the case of gentamicin, it is possible to circumvent these problems by limiting the HPLC analysis time. Alternatively, the use of normalised peak areas could be eliminated from this method by the use of a gentamicin reference compound, the composition of which would be determined by pharmacopoeial laboratories using the graphical approach described in this report. It is our opinion that one of these simple modifications should be incorporated into the B.P.[9] procedure, which in all other respects is a valuable step forward in the quality control of gentamicin for pharmaceutical use.

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Note

Thin-layer and high-performance liquid chromatography of biologically active agents

II. Semicarbazones

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In this paper we describe a convenient thin-layer chromatographic (TLC) method for the separation and identification of semicarbazones, which is also suitable for testing thiosemicarbazones[1], and an high-performance liquid chromatographic (HPLC) separation of some semicarbazones.

Di Modica and Spriano[2] separated aliphatic semicarbazones by partition chromatography. Priel and Fisher[3] used semicarbazones as reagents in order to separate aromatic aldehydes. Camp and O'Brien[4] separated the semicarbazones of some common aldehydes by TLC. Tumlinson *et al.*[5] reported a gas–liquid/thin layer chromatographic technique for the identification of carbonyl compounds which involves formation of 2,4-dinitrophenylsemicarbazone derivatives.

The semicarbazones are of interest because of their use as herbicides and in the identification of aldehydes[6] and ketones. Pilgram[7] demonstrated that several semicarbazones obtained from aldehydes are effective as herbicides. Ogura *et al.*[8] reported UV data for semicarbazones and thiosemicarbazones with aliphatic aldehydes, which act as antibiotics.

MATERIALS AND METHODS

The semicarbazones were synthesized according to the method of Shriner and Turner[6].

TLC

Thin-layer chromatographic plates were prepared from silica gel G (SMI, octadecyl silane, 20 cm × 20 cm, thickness 0.25 mm) according to the manufacturer's instructions. The silica gel G was air-dried, activated at 110°C for 3 h and stored in a desiccator. The solvent systems comprised benzene–chloroform–methanol (9:3:2) and chloroform–methanol (3:1).

* Deceased on August 21st, 1985.

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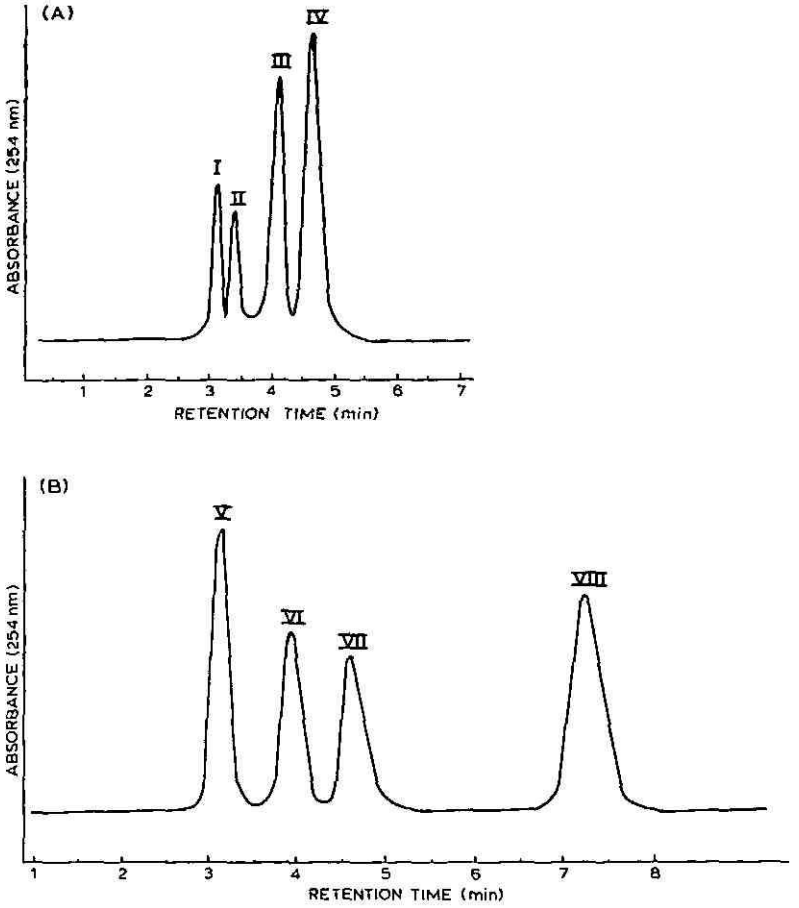

Fig. 1. Chromatograms of some semicarbazones. HPLC conditions as described in the Experimental section. Peaks: (A) I = Sc *p*-HO-Bz; II = Sc Fur; III = Sc Bz; IV = Sc Acetophenone; (B) V = Sc *m*-HO-Bz; VI = Sc 2-Butanone; VII = Sc Cyclohexanone; VIII = Sc *o*-HO-Bz.

A 1% methanolic solution of each compound was prepared and 1 μl (corresponding to 10 μg of each compound) was spotted 2.0 cm from the edge of the plate. The solvent was allowed to migrate 15 cm from the starting line. The chromatograms were developed at room temperature (20 ± 3°C) in a normal chromatographic chamber presaturated with the solvents for at least 30 min. Two different spray reagents were used. One was ferric chloride in butanol (1%, w/v); in this case, heating at 110°C was required. The other was a sensitive reagent, 2,4-dinitrophenylhydrazine dissolved in ethanol (3%, w/v).

The reversed-phase (RP) TLC solvent systems comprised water–acetonitrile, water–methanol and water–tetrahydrofuran (10:90, 25:75, 50:50).

HPLC

HPLC was carried out with a MCHS Varian liquid chromatograph equipped

TABLE I

COLOUR REACTIONS AND ULTRAVIOLET SPECTRA OF SEMICARBAZONES

Sc = Semicarbazone; Bz = benzaldehyde; Thph = thiophenecarbaldehyde; Fur = furaldehyde; AcNaph = acetylnaphthalene. Solvents: A = benzene–chloroform–methanol (9:3:2); B = chloroform–methanol (3:1). D_1 = 2,4-dinitrophenylhydrazine; D_2 = ferric chloride. Colours: Y = yellow; Br = brown; R = red; P = pink; Or = orange; L = light. Each $R_F \cdot 100$ value represents the mean of five determinations. Absorbent: silica gel G.

Semicarbazone	*$R_F \cdot 100$ in solvent*		*Colour reaction*		*Ultraviolet spectra*	
	A	*B*	*D_1*	*D_2*	*$\lambda_{max.}$*	*log ε*
Sc Bz	54	–	Y	Y	282	4.29
Sc *o*-OH-Bz	40	–	Y	Y	310/286/278	4.12/4.26/4.32
Sc *m*-OH-Bz	35	–	Y	Y	280	3.96
Sc *p*-OH-Bz	31	–	Br-R	Br-R	320	4.45
Sc 2,3-diOH-Bz	–	68	Or	Or	314/288	4.38/4.12
Sc 3,4-diOH-Bz	–	82	Y	Or	286	4.36
Sc 2,4-diOH-Bz	–	88	Y	Or	312/284/236	4.24/4.22/4.08
Sc 3,4-diOH-5-OCH_3-Bz	20	–	Or	Or	306/238	4.24/4.13
Sc Fur	37	–	R	R	292	4.39
Sc 2-Thph	51	–	Or	Or	304	4.21
Sc Acetophenone	79	–	Or	LY	268	4.12
Sc 5-NO_2-2-Fur	37	–	Y	Y	360/259	4.01/3.98
Sc Cyclohexanone	78	–	Y	LBr	240	3.59
Sc 9-Anthraldehyde	75	–	Br-R	Br-R	383/364/348/254	3.86/3.84/3.65/4.72
Sc AcNaph	62	–	Y-R	LY	304/290/260/238	4.35/4.37/4.41/4.34
Sc Acetone	71	–	Y	LBr	236	3.39
Sc 2-Pentanone	64	–	Y-R	LP	238	3.45
Sc Benzophenone	87	–	Y	Y	278	4.06
Sc Acetaldehyde	52	–	P	P	244	2.54
Sc 2-Butanone	62	–	Y	LBr	236	3.42
Sc Methyl isopropyl ketone	68	–	Y-R	LP	238	3.40

with a variable-wavelength UV detector. An octadecyl silane reversed-phase column with 5-μm particles was used (30 cm × 0.4 cm). The mobile phase was water–acetonitrile (45:55) at a flow-rate of 1 ml/min (3000 p.s.i.). All operations were carried out at 35°C. The working solutions contained 1–5 mg of each substance in 2 ml of acetonitrile. Samples of 50 μl were injected and the peaks were detected at 254 nm.

RESULTS AND DISCUSSION

The chromatographic results on silica gel G are shown in Table I. Each $R_F \cdot 100$ value represents the mean of five determinations. Each series of determinations showed only slight variations within the limits of experimental error.

The detection limit was found to be *ca.* 1 μg for each compound. The spray, 2,4-dinitrophenylhydrazine, located the semicarbazone spots unequivocally and did not require heating.

The convenience of polyamide as an adsorbent was examined but incomplete separations were obtained. Sharp spots free from tailing were found only on silica gel G.

The R_F values obtained in TLC on silica gel G were adequate for the separation and identification of these compounds.

RP-TLC using octadecyl silica as stationary phase was not an adequate method because the R_F values were very similar.

Fig. 1 shows a typical example of the HPLC elution pattern for some semicarbazones.

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Note

Spectrofluorodensitometric estimation in thin-layer chromatography of gibberellic acid produced by solid-state fermentation

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Gibberellic acid, an important plant growth regulator, is used extensively in agriculture, nurseries, green houses, viticulture, tea-gardens, etc.[1]. It is traditionally produced by submerged fermentation by employing *Gibberella fujikuroi* or *Fusarium monoliforme*[2]. Spectrophotometric, fluorometric and bioassay methods are routinely used for the estimation of gibberellic acid in fermentation broths or in crude forms[3–5], though a variety of other methods are available for its estimation[6–10]. Recently, the potential of solid-state fermentation for production of gibberellic acid was established[11]. Preliminary studies indicated that the above routine analytical methods give poor responses unless the crude extract of mouldy bran obtained by solid-state fermentation is extensively purified. Since microbial products, such as mycotoxins[12–18], can now be estimated by spectrofluorodensitometry with greater accuracy[19] without resorting to extensive purification, we studied the application of this technique to the estimation of gibberellic acid in crude extracts of mouldy bran obtained by solid-state fermentation.

EXPERIMENTAL

Fermentation

The methodology adopted for solid-state fermentation was as described by Sreekantiah[20]. The dry mouldy bran was extracted with ethyl acetate (1:6) at 30°C for 30 min. In case of submerged fermentation, Czapek Dox liquid medium was used and the product was extracted with ethyl acetate at pH 2.5. The extracts were concentrated under vacuum.

Spectrofluorodensitometry

Thin-layer chromatographic (TLC) plates, coated with silica gel G, (46% suspension in water, Glaxo Labs.) to a thickness of 300 μm, were activated at 110°C for 1 h. A 10-μl volume of concentrated extract and of standard gibberellic acid solutions (Sigma) were spotted. The plates were eluted with chloroform–ethyl acetate–acetic acid (5:4:1), then sprayed with concentrated sulphuric acid containing 5% ethanol, heated at 100°C for 30 min and finally observed under UV light at 254 nm. The intensity of the fluorescence of the spots was measured by an automatic recording spectrofluorodensitometer (Model SD 3000; Schoeffel, F.R.G.).

Comparative accuracy

Standard solutions of gibberellic acid and concentrated crude extracts obtained by submerged and solid-state fermentations were analyzed by spectrophotometric[3], fluorometric[4], bioassay[5] and spectrofluorodensitometric methods. Gibberellic acid, eluted from a TLC plate spotted with crude extract, was also analyzed by these methods. Two-dimensional TLC was also carried out using ethyl acetate–chloroform–acetic acid (4:5:1) followed by benzene–acetone–acetic acid (13:6:1).

RESULTS AND DISCUSSION

Characteristics of crude extract

Among the 5–7 distinct spots having different R_F values on the chromatogram of the crude extract from solid-state fermentation, 2–3 spots emit fluorescence at the wavelength used for estimation of gibberellic acid, but only one of these corresponds to authentic gibberellic acid. The particular strain employed is also known to co-produce plant growth regulators other than gibberellic acid[21]. In addition, the constituents of wheat bran, a substrate used in solid-state fermentation, are also co-extracted by the solvent. These compounds interfere with the accurate estimation of gibberellic acid when methods based on fluorescence or plant-growth-promoting activity are used. They also have similar solubilities to gibberellic acid in solvents used for its extraction from mouldy bran. The complete separaton of gibberellic acid from other constituents was not possible with various solvent systems such as chloroform–methanol–acetic acid–water (70:20:3:2) and ethyl acetate–chloroform–acetic acid (15:5:1)[22,23].

Reliability of spectrofluorodensitometry

The spectrofluorodensitometric analysis of standard solutions of gibberellic acid, varying in concentration from 2 to 10 μg per 10 μl, gave a linear relationship: $Y = 0.998X$. The sum of the squares of the deviation from this regression equation[24] was 0.002, while the standard error was 0.0265. The sensitive range for gibberellic acid lies between 2 and 10 μg per 10-μl spot at 0.1 absorbance unit of the spectrofluorodensitometer and it can be enhanced to 5–40 μg per 10-μl spot, without affecting the reliability, if 2.0 absorbance units are used. The percentage error is less than $\pm$ 2 in both cases.

TABLE I

CO-SPECTROFLUORODENSITOMETRIC RESPONSE

Sample spotted	*Gibberellic acid (ppm)*	
	Spectrofluoro-densitometry	*Probable range**
Standard solution (1000 ppm)	1000	980–1020
Unknown sample	393	401–385
Unknown sample + standard solution (1000 ppm)	1379	1351–1407

* Based on error of $\pm$ 2%.

TABLE II

RESULTS OF THE ANALYSIS OF A CRUDE EXTRACT OF DRY MOULDY BRAN BY SPECTROFLUORODENSITOMETRY OF FOUR REPLICATES

S.D. = Standard deviation; C.V. = coefficient of variation.

Experiment	*Gibberellic acid (ppm) after incubation for period*		
	5 days	*6 days*	*7 days*
1	139.5	247.0	355.0
2	146.2	251.6	352.0
3	145.4	237.4	348.0
4	148.4	248.0	364.0
Mean ± S.D.	144.9 ± 3.80	246.0 ± 6.06	353.5 ± 8.66
C.V. (%)	2.62	2.46	2.45
Mean ± 2 S.D.	137.3–152.5	233.9–258.1	336.2–370.8

The results of the co-spectrofluorodensitometric analysis of a mixture of a crude extract of unknown concentration and of standard gibberellic acid solution were similar to those of individual spectrofluorodensitometric analyses of these samples (Table I). Even two-dimensional TLC showed a single spot corresponding to gibberellic acid, thereby confirming the purity of the spot. The results and corresponding statistical analysis for the crude extract of mouldy bran, performed as four

TABLE III

COMPARATIVE RESPONSES OF VARIOUS METHODS FOR ASSAYING PURE GIBBERELLIC ACID IN STANDARD SOLUTIONS

Figures in parentheses are the per cent errors.

Pure gibberellic acid concentration (ppm)	*Estimated value (ppm)*			
	Spectrofluorodensitometry	*Fluorometry*	*Spectrophotometry*	*Bioassay*
1	–	1.02 (2.0)	–	–
2	1.96 (2.0)	1.99 (0.5)	–	1.88 (6.0)
4	3.96 (1.0)	3.98 (0.5)	–	–
5	5.00 (0)	4.95 (1.0)	–	4.89 (2.2)
6	5.97 (0.5)	–	–	–
8	7.93 (0.9)	–	–	–
10	10.14 (1.4)	–	–	10.80 (8.0)
20	20.19 (1.0)	–	–	–
25	–	–	–	25.63 (2.5)
30	30.14 (0.5)	–	–	–
40	40.09 (0.2)	–	–	–
50	–	–	51.25 (2.5)	–
100	–	–	102.50 (2.5)	–
200	–	–	209.10 (4.6)	–
400	–	–	403.90 (1.0)	–
600	–	–	590.40 (1.6)	–

TABLE IV

COMPARATIVE RESPONSES OF CHEMICAL AND BIOLOGICAL ASSAYS OF GIBBERELLIC ACID IN CRUDE EXTRACTS FROM SOLID-STATE AND SUBMERGED FERMENTATIONS

Fermentation technique	*Volume of crude extract processed for spotting (l)*	*Estimated value (mg) in crude extract processed*				
		Spectro-fluoro-densitom-etry	*Fluoro-metry*	*Bio-assay*	*Spectro-photom-etry*	*Probable range (mg)*
Solid state	0.005	1.29	1.86	2.81	–	1.26–1.32
	0.010	2.60	3.77	5.99	–	2.55–2.65
	0.015	3.87	5.70	8.49	–	3.79–3.95
Submerged	1.0 (5-day incubation)	11.50	–	31.00	17.00	11.27–11.73
	1.0 (6-day incubation)	29.20	–	76.00	44.50	28.62–29.78
	1.0 (7-day incubation)	44.50	–	92.00	60.00	43.61–45.39

* Based on error of ± 2% for spectrofluorodensitometric method.

replicates, are presented in Table II. The estimated values are within the limits of the mean ± two standard deviations.

Comparison of methods

The results of the estimation of gibberellic acid in standard solutions and crude extracts by different chemical and biological methods are compared to those from the spectrofluorodensitometric method in Tables III and IV. The per cent error was smaller (less than 2%) for the spectrofluorodensitometric and fluorometric methods. However, the estimated values varied widely when a crude extract of dry mouldy bran was analyzed (Table IV). The spectrofluorodensitometric method gave the lowest values. The higher values for gibberellic acid obtained by spectrophotometric, fluorometric and bioassay methods is due to the presence of fluorescent compounds other than gibberellic acid in the crude extracts. These methods are unable to distinguish gibberellic acid from these interfering compounds. On the other hand, the spectrofluorodensitometric method is specific for gibberellic acid as the interfering compounds are separated from the spot of gibberellic acid on TLC plates.

The results of the bioassay of the gibberellic acid spot from a crude extract of mouldy bran confirm the reliability of the spectrofluorodensitometric method. It gave about 80% of the value obtained for the original crude extract by spectrofluorodensitometry. The difference of 20% is due to losses of gibberellic acid[25] during scraping of the spot from the plate, extraction and transfers. On the other hand, the value obtained by bioassay was about one-half and one-fourth of those estimated by fluorometry and bioassay respectively in the original crude extract. Such large differences

are due mainly to the complete elimination of plant-growth regulatory and fluorescent compounds other than gibberellic acid in the eluted sample.

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Note

Characterization of nanogram levels of metalloporphyrins with thin-layer chromatography–resonance Raman spectroscopy

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Several useful techniques exist for detecting compounds adsorbed on various solid supports and matrices. Of these the UV–VIS[1,2] and luminescent[3–6] methods are the most popular and straightforward. However, in many cases more extensive structural information is needed to evaluate thoroughly a substance. Vibrational spectra, in particular, contain the type of information needed to characterize a compound. Ideally one would like a technique that provides the information of vibrational spectra and that also has the sensitivity of the aforementioned UV or luminescence methods. Until recently this combination was impossible. For example, it is generally difficult to obtain quality infrared spectra from low levels of compounds adsorbed on cellulose or silica gel based matrices. Although diffuse reflectance infrared Fourier transform (DRIFT) spectrometry can be used, it is relatively insensitive, costly and time consuming[7]. The use of Raman scattering eliminates problems due to moisture and background adsorption which plague the infrared based techniques. However, early attempts to use Raman spectrometry as a detection method for thin-layer chromatography (TLC) suffered from poor sensitivity, sample heating and decomposition[8,9]. Hence the technique was not widely utilized.

Recently, an important advance in methodology was made by Tran[10,11]. It was shown that excellent Raman spectra could be obtained for nanogram to subnanogram levels of several dyes adsorbed on filter paper or paper chromatographic supports by using surface enhanced Raman scattering (SERS). The technique involved impregnating the cellulose matrix with a colloidal silver sol and taking a Raman spectra of the wet spot[10,11]. Both the sensitivity and vibrational information obtained with this technique were impressive.

In this work, resonance Raman spectroscopy in conjunction with TLC is used to separate, detect and characterize trace levels of porphyrins. The Raman spectra obtained from various planar surfaces are compared to traditional solution spectra. There are a number of important advantages to this technique. For example, most common TLC matrices can be used with little interference. Spectra can be taken from wet or dry plates and are not appreciably affected by most impurities or sampling methods. There is no need to prepare a stabilized colloid and treat the plate with the

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preparation. One disadvantage of this technique is that fluorescence can be a major source of interference for some compounds. However, using micellar mediated resonance Raman spectroscopy[12], this problem can be circumvented.

EXPERIMENTAL

All porphyrins were obtained from Porphyrin Products and used as received. Silica gel TLC plates (K5) were obtained from Whatman. Spectroscopically pure solvents were used in all experiments. The continuous wave (CW) spectra were recorded using a Coherent Innova Argon ion laser and a SPEX Triplemate monochromator with 1200 gratings/mm. A PAR (Model 1420) optical multichannel analyzer with grated intensifier was used for signal detection. The optical multichannel analyzer was controlled with the PAR Model 1215 optical multichannel analyzer controller. A backscattering geometry and an unfocused laser beam were used for the samples deposited on TLC plates. A 90° geometry was used for the solution spectra. The concentration of porphyrin solutions was 100 μM. The amount of material spotted on the TLC plate was varied between 50 ng and 20 μg. The sensitivity was very dependent on spot dispersion. The laser power for the TLC spectra was 200 mW while that for the solution spectra was 400 mW. The background (presumably light reflected from the TLC plate) was diminished considerably if the plate was skewed 3–10° from perpendicular to the laser beam. Further experimental details are given with each figure.

RESULTS AND DISCUSSIONS

Porphyrin and metalloporphyrin research is an important and rapidly expanding field[13,14]. Studies involving vibrational spectra are essential in the characterization and understanding of these molecules[15,16]. Consequently TLC–resonance Raman spectroscopy was developed to evaluate the purity and to characterize trace levels of these and other compounds. Fig. 1 shows the TLC chromatograms for nickel uroporphyrin (NiUroP) and nickel protoporphyrin dimethyl ester (NiPPDME). Chromatographic analysis revealed the presence of three to four compounds in all of the supposedly pure metallouroporphyrin samples. A single spot was found for

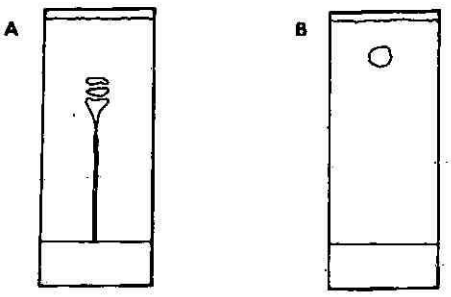

Fig. 1. TLC plates of: (A) nickel uroporphyrin developed with ethylene glycol–isopropanol (6:4, v/v) and (B) nickel protoporphyrin dimethyl ester developed with toluene–ethyl acetate (6:4, v/v). Silica gel plates were used in both cases.

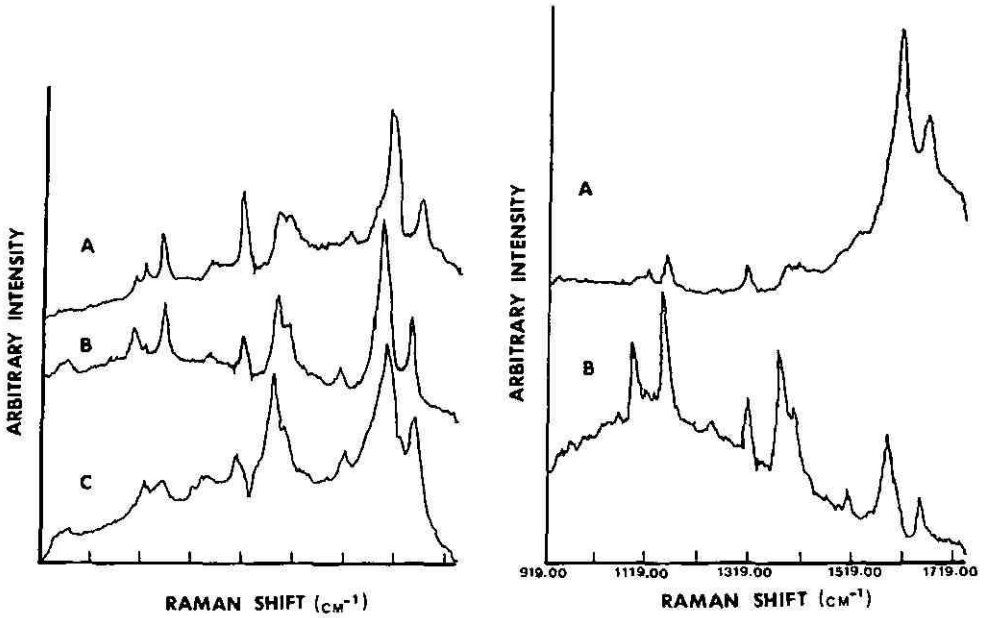

Fig. 2. Resonance Raman spectra of: (A) nickel uroporphyrin, (B) copper uroporphyrin, and (C) nickel protoporphyrin dimethyl ester taken from dried silica gel TLC plates. Excitation wavelength was 514.5 nm. The Raman shift scale is identical to that in Fig. 3.

Fig. 3. Solution resonance Raman spectra of: (A) nickel uroporphyrin and (B) nickel protoporphyrin dimethyl ester. The concentration of metalloporphyrin in both solutions was 100 μM. Excitation wavelength was 514.5 nm.

NiPPDME. Resonance Raman analysis was done on all TLC samples and on solutions made from the same unpurified samples. Typical resonance Raman spectra taken from the TLC plates are shown in Fig. 2 and the corresponding solution spectra for NiUroP and NiPPDME are shown in Fig. 3. A number of things are evident from this data. First, the quality of the resonance Raman spectra obtained from the surface of chromatographic media often is as good or better than that obtained from analogous solution samples. This is true even though considerably less sample is needed for the surface technique. Also, there are significant changes in the relative peak intensities and small shifts ($< 5\ cm^{-1}$) in the peak positions. Note for example, the ν_2 bands between 1582 and 1603 cm^{-1}. This could be indicative of changes in certain vibrational modes of freedom in the adsorbed *versus* solution state. Interestingly there were no significant differences, within the experimental limits of this equipment, in the resonance Raman spectra of the various spots obtained from the TLC of the uroporphyrins (Fig. 1A). Vibrational modes in the high-frequency region ($> 1000\ cm^{-1}$) involve in-plane motion of the conjugated bonds of the porphyrin macrocycle[17] and are largely insensitive to the ordering of peripheral substituents. Thus, the different TLC spots most likely represent isomers of the uroporphyrins which occur as a result of differences in the ordering of these substituents. As was also noted by Tran[10], significantly increasing the laser power and focusing the laser beam increases the chance of local heating effects and photodecomposition of the sample.

It is apparent that the combination of planar chromatographic methods with specialized Raman detection is a potentially powerful technique for the evaluation and characterization of trace levels of organic compounds. The future possibility of a hyphenated micellar liquid chromatography–resonance Raman spectroscopic technique is even more intriguing, as this method would not suffer from the solvent background problems currently plaguing liquid chromatography–Fourier transform infrared spectroscopy. Experiments which will lead to the development of this technique are now in progress in our laboratory.

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Note

Facile gas chromatographic method for the determination of residues of Bidrin in pecan*

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Bidrin or dicrotophos[1,2], the chemical name of which is dimethyl-(*E*)-2-dimethylcarbamoyl-1-methylvinyl phosphate according to IUPAC or (*E*)-3-(dimethylamino)-1-methyl-3-oxo-1-propenyl dimethyl phosphate according to *C.A.* nomenclature, is a contact and systemic insecticide approved in the U.S. for registered use primarily to control several insecticidal and acaricidal adversaries in cotton. It is also effective in control of elm bark beetle and coffee borer. Like other agrichemicals, the organophosphorus insecticide is highly beneficial to crops, but is also extremely toxic[3,4] to fish, birds and other wild life. Organophosphate poisoning resulting in death of a wide population of birds due to misuse of dicrotophos (and monocrotophos) in Texas in 1982 were reported[5]. Organophosphates inhibit acetylcholinesterase in the nervous system whereby synaptic transmission of nerve impulses are disrupted. Death usually occurs from asphyxiation because of failure of the respiratory center of the brain[6]. The gastrointestinal tract of the dead birds contained residues[5] of dicrotophos at a level of 5.6–14 mg/kg.

Recently there has been great interest in Bidrin for use in pecan cultivation to control pecan aphid bud moth, phylloxera and webworms. It is, therefore, desirable to conduct experimental field trials representative of different geographical areas of the U.S. to generate data for tolerance and registration purposes. Consequently, a need exists for validation and/or development of an adequate analytical methodology for quantitative assessment of the insecticide residues in this particular commodity.

Residue analysis of Bidrin in several substrates employing gas–liquid chromatography (GLC) with flame photometric detection is known[7–11]. However, Bidrin residue analysis in pecans is relatively unknown, and hence the present research was undertaken to develop an analytical method to determine Bidrin in pecan at various stages of treatment and/or use pattern. It is through modification and refinement of an earlier technique[11] that the procedure reported herein has been developed.

MATERIALS AND METHODS

Field trial and Bidrin application

An experimental protocol was developed by IR-4, the U.S. National Agricul-

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tural Program for clearance of pesticides, animal drugs, microbials and biochemicals for minor or special uses. It consists in employing 2 ml of the formulation Shell Bidrin 8 water miscible insecticide (EPA Reg. No. 201-274; contains 82% active ingredient by weight, or 2 lbs. of active ingredient per quart) diluted to 2 l with water. The pecan trees were grown on plots adequate to reflect actual commercial use conditions, each plot having four replicates. The insecticide was applied by low-pressure trunk injection of 2 ml of the diluted material prepared as above per 6 in. trunk circumference of the pecan trees. Applications were restricted to a minimum of one and a maximum of two per season beginning at early bud break stage and/or at half leaf maturity. In addition, the insecticide was not applied within 120 days of harvest. Samples for residue analysis were collected at harvest from trees receiving one application at early bud break, and from those receiving one application at half leaf maturity, and also from those receiving applications at both early bud break stage and half leaf maturity.

Preparation, extraction and cleanup of samples

Soon after harvest the pecans were shelled, and the nuts and shucks were stored at −10°C. Before analysis, they were thawed and chopped. Representative 25-g samples of the crop (nuts and shucks) were extracted with 100 ml methanol in a Polytron Homogenizer for 5 min at medium speed. The contents were then centrifuged. Aliquots of 10 ml of the supernatant representing 2.5 g crop were transferred to 120 × 10 mm PTFE-lined, screw-capped test tubes followed by addition of 0.5 ml water. After reducing the volume of the mixture to *ca.* 0.7 ml by a gentle stream of nitrogen, 4.5 ml water and 5 ml hexane were added to the concentrated aliquot. The contents were thoroughly shaken and centrifuged for complete separation of aqueous and organic layers. The hexane phase was discarded, and the washing of the aqueous phase was repeated with another volume of 5 ml hexane. The layer was again discarded, and the aqueous layer was partitioned thrice with 5 ml chloroform each time. The chloroform layers were pooled while the aqueous layer was finally discarded. The chloroform extract thus obtained was concentrated almost to dryness using a gentle stream of nitrogen, and the residue was dissolved in 1 ml ethyl acetate. Aliquots of 5 μl were used for GLC.

Reagents

All organic solvents were pesticide grade or HPLC grade. Bidrin of 99.0% purity was obtained from the Environmental Protection Agency, U.S.A.

Gas–liquid chromatography

A Hewlett Packard 5730A gas chromatograph equipped with a flame photometric detector (phosphorus mode) and a 1.2 m × 4 mm I.D. glass column packed with 1% Reoplex 400 on Gas-Chrom Q, 100–120 mesh, were used. The operating parameters were: injector port, 200°C; column, 170°C; and detector, 200°C. The column flow was 60 ml/min of nitrogen, and the air, hydrogen and oxygen flow-rates to the detector were regulated at 80 ml/min, 200 ml/min and 30 ml/min, respectively.

Fortification procedure

Representative 25-g samples of finely chopped crop demonstrated not to con-

tain any interferences or residues of Bidrin were fortified with known volumes of working standard solutions to give 0.01–0.5 mg/kg level of Bidrin. The fortified samples were mechanically shaken, and allowed to stand for *ca.* 1 h at room temperature before extraction. The entire constants were extracted without any subsampling.

RESULTS AND DISCUSSION

Bidrin was found to be eluted at 2.38 min in GLC, and hence the determinative step did not take more than 5 min per sample. The limit of detection was found to be 0.01 mg/kg. Typical chromatograms obtained from standard, control and fortified pecan are shown in Fig. 1. Recoveries ranged from 92 to 98% at fortification levels of 0.01–0.5 mg/kg (Table I). No residues of Bidrin were found in pecan harvested 120 days or longer after Bidrin treatment.

Fresh, untreated, finely chopped samples were fortified with standard solution of Bidrin, and subjected to the same treatment as the samples as a check on the analytical procedure, and any apparent loss or degradation during storage. No conceivable breakdown was observed under the conditions employed.

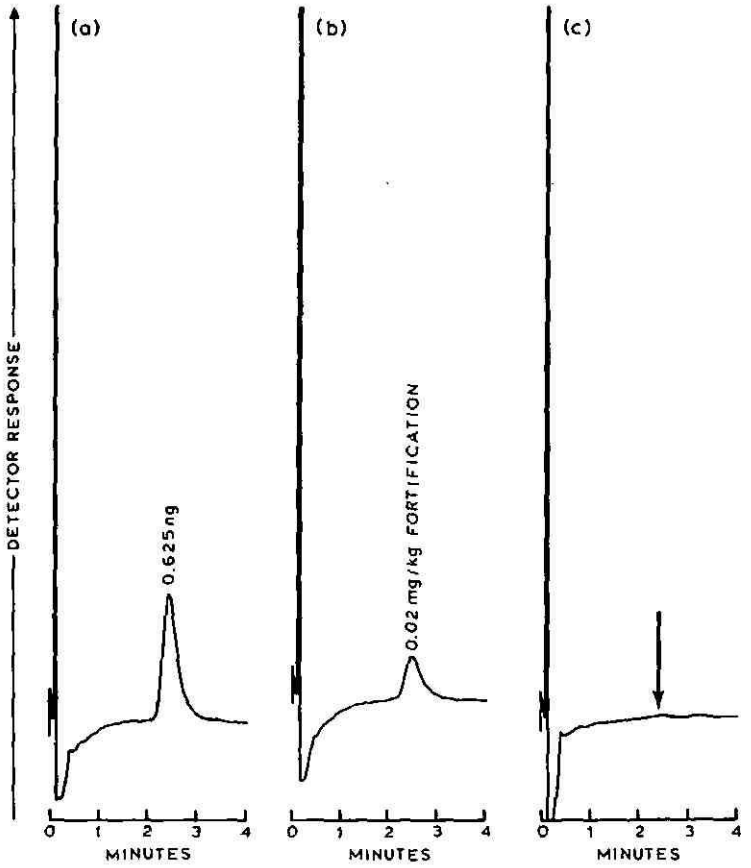

Fig. 1. (a) Chromatograms of (a) standard Bidrin (0.625 ng), (b) pecan control sample fortified with Bidrin (0.02 mg/kg) and (c) pecan control sample. See text for chromatographic conditions.

TABLE I

RECOVERY OF BIDRIN FROM PECAN

Values represent the average of three replicate determinations with standard deviations.

Fortification level (mg/kg)	*Recovery (%)*
0.01	96 ± 5.2
0.02	94 ± 4.1
0.05	98 ± 5.3
0.10	98 ± 4.8
0.50	93 ± 5.6

According to these results, residues of Bidrin in pecan at harvest 120 days or longer following treatment with the insecticide at the given rate would be less than 0.01 mg/kg. In addition, translocation from the treatment sites would be so low as to be undetectable. Because of its low application rate and residue contents in pecan, Bidrin should cause minimal environmental and food contamination. However, residue analysis in soil and run-off water, and studies concerning any possible degradation of Bidrin to other compounds are needed for proper environmental impact and food toxicity assessment.

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Note

Rapid purification of glucokinase and glycerokinase from *Bacillus stearothermophilus* by hydrophobic interaction chromatography

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(Received August 4th, 1986)

Glucokinase (ATP:α-D-glucose-6-phosphotransferase, E.C. 2.7.1.2) and glycerokinase (ATP:glycerol-3-phosphotransferase, E.C. 2.7.1.30) from *Bacillus stearothermophilus* have been previously purified by triazine dye affinity chromatography. Dye affinity chromatography is a rapid and inexpensive step giving far greater binding capacities than affinity chromatography with an immobilised ligand[1–3].

In the course of an investigation into the association and dissociation of glucokinase subunits by fluorescence polarisation spectroscopy it was observed that the enzyme fluoresced even in the absence of covalently bound fluorescent label. This fluorescence was found to be caused by breakdown-products of the dye, Procion Brown H-3R, used as a ligand when the enzyme was purified by dye affinity chromatography. Because this contamination could not be removed by any mild technique and its presence could interfere in any structural studies to be carried out on this enzyme, an alternative purification was developed.

This paper describes a purification method suitable for gram quantities of glucokinase. Triazine dyes and conventional affinity chromatography were avoided by using hydrophobic interaction chromatography. Glycerokinase was also found to bind to the hydrophobic matrix and could be completely separated from the glucokinase. The two enzymes previously proved difficult to separate by methods which did not include the use of triazine dyes and even then low level contamination (0.1%) was evident, reducing the value of the enzyme for diagnostic purposes. Pure glycerokinase and glucokinase were obtained by a procedure which included a total of three pseudo-affinity chromatography steps following partial separation by ion-exchange chromatography[2,3].

Glucokinase and glycerokinase of diagnostic grade were obtained free of each other and free of contaminating dyes by one step on Phenyl-Sepharose. The procedure is simple and involves commercially available chromatography matrices.

MATERIALS AND METHODS

N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (Hepes), bovine serum albumin, dithiothreitol and phenylmethanesulphonyl fluoride were obtained from Sigma (London). Phosphate buffers, deoxyribonuclease and 2-mercaptoethanol were

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obtained from BDH. All enzyme assay reagents were obtained from Boehringer. DEAE-Sepharose CL-6B and Phenyl-Sepharose CL-4B were obtained from Pharmacia. Ultrogel AcA 34 was obtained from LKB.

Assay methods

The assay mixture for glucokinase contained 92 m*M* triethanolamine–HCl (pH 7.6), 1.9 m*M*ATP, 5.4 m*M* magnesium chloride, 1.8 m*M* potassium chloride, 0.32 m*M* NADP, 2.55 m*M* α-D-glucose and 3.5 E.C. units of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) in a final volume of 1 ml. The reaction was initiated with 1–10 μl of glucokinase solution.

Glycerokinase was assayed the method described by Garland and Randle[4]. The assay mixture contained 75 m*M* potassium chloride , 6.5 m*M* phosphoenolpyruvate, 0.3 m*M* $NADH_2$, 2.4 m*M* glycerol and 8 E.C. units of pyruvate kinase (E.C. 2.7.1.40)/lactate dehydrogenase (E.C. 1.1.1.27) in a final volume of 1 ml. The reaction was initiated with 1–10 μl of glycerokinase solution.

One E.C. unit is defined as the amount of enzyme which catalyses the conversion of 1 μmol substrate per min under these conditions at 30°C.

Protein was estimated by the Coomassie Brilliant Blue binding method of Bradford[5] with bovine serum albumin as standard. Column eluates were monitored by absorbance at 280 nm.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out with 10% slab gels by the method of Laemmli[6].

ENZYME PURIFICATION

Enzyme purification was carried out at 4°C. All buffers contained 0.1 m*M* phenylmethanesulphonyl fluoride and 10 m*M* 2-mercaptoethanol.

Frozen cell paste (1 kg) was suspended in 2 l of 100 m*M* potassium phosphate buffer, pH 8.0, containing 0.5 μg/ml deoxyribonuclease to reduce viscosity caused by nucleic acids[7]. The thawed cell suspension was disrupted with a 15M-8BA Type Manton-Gaulin homogeniser operated at 550 kg/cm^2. The homogenate was centrifuged at 1000 *g* for 1 h and the supernatant filtered through glass wool to remove cell debris and lipid. The supernatant was mixed with an approximately equal volume of 100 m*M* potassium dihydrogen phosphate and the pH adjusted to 5.6 with 3.2 *M* orthophosphoric acid. The raised ionic strength prevented adsorption of the enzyme on to the precipitate. The precipitate was removed by centrifugation at 1000 *g* for 1 h. The pH of the supernatant was raised to 8.0 with 10 *M* potassium hydroxide.

Ion-exchange chromatography on DEAE-Sepharose CL-6B

The supernatant was loaded onto a 3.5-l DEAE-Sepharose CL-6B column (22 cm × 14 cm I.D.) equilibrated with 100 m*M* potassium phosphate buffer, pH 8.0. Enzyme was eluted at 600 ml/h with a 20-l linear gradient from 100 m*M* to 400 m*M* potassium phosphate at pH 8.0, collected in fractions of 500 ml.

Hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B

Active glucokinase and glycerokinase fractions were pooled and concentrated to 500 ml with an Amicon CH4 ultrafiltration unit fitted with an H10P10 hollow-

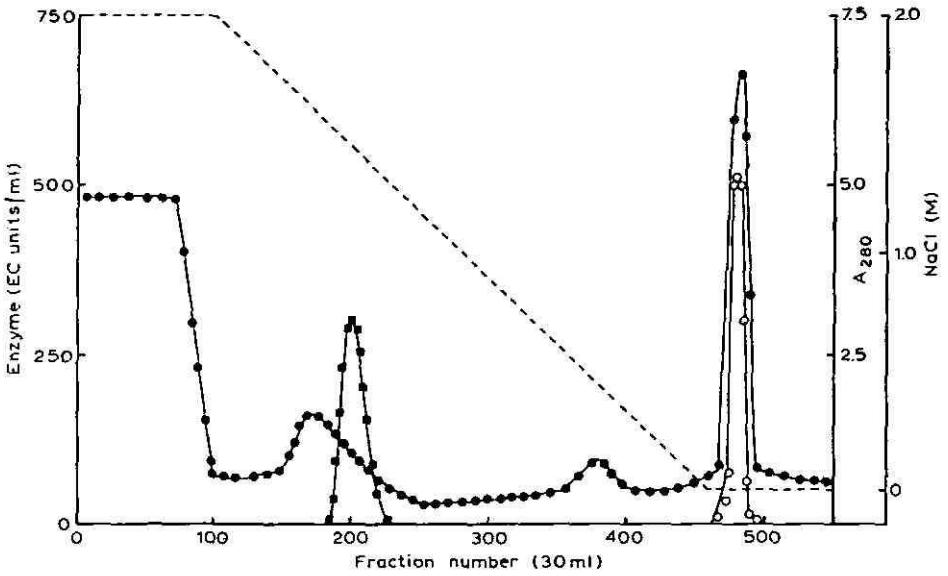

Fig. 1. Chromatography of glucokinase (■) and glycerokinase (○) on Phenyl-Sepharose. Conditions are given in the text. The broken line indicates the sodium chloride content of the elution buffer. Absorbance at 280 nm (●).

fibre cartridge. The enzyme pool was made 2 *M* in sodium chloride and chromatographed on a 1-l Phenyl-Sepharose column (16 cm × 9 cm I.D.) equilibrated with 10 m*M* Hepes, pH 7.0, containing 2 *M* sodium chloride. It was not necessary to change the buffer component of the enzyme solution prior to loading the column. The column was washed with 3 l of equilibration buffer and enzyme eluted with an 11-l linear gradient from 2 *M* to 0 *M* sodium chloride in 10 m*M* Hepes, pH 7.0, at 1500 ml/h, collected in fractions of 30 ml.

Elution was continued with 10 m*M* Hepes, pH 7.0, after completion of the gradient. Glucokinase eluted at about 1.5 *M* sodium chloride while glycerokinase eluted only in the absence of sodium chloride (Fig. 1).

Gel filtration chromatography on Ultrogel AcA34

Active glucokinase fractions were concentrated to 10 ml with Amicon PM10 membranes. The concentrate was adjusted to pH 7.6 and loaded on to a 1.75-l column of Ultrogel AcA34 (90 cm × 5 cm I.D.), equilibrated with 50 m*M* potassium phosphate at pH 7.6. Fractions of 10 ml were collected at 60 ml/h. Active glycerokinase fractions were concentrated to 15 ml and chromatographed as described for glucokinase. Enzyme fractions were pooled on the basis of activity and electrophoretic homogeneity.

Glucokinase and glycerokinase pools were concentrated to a protein concentration of 100 mg/ml using Amicon PM10 membranes and preserved by addition of 0.1 m*M* dithiothreitol and 0.02% sodium azide. The results of this purification are summarised in Tables I and II. Both enzymes were shown to give single protein bands by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

TABLE I

PURIFICATION OF GLUCOKINASE FROM 1 kg *B. STEAROTHERMOPHILUS*

Purification step	*Volume (ml)*	*Amount of protein (g)*	*Enzyme activity (E.C. units)*	*Specific activity (E.C. units/mg protein)*	*Activity recovered (%)*
Homogenate	2000	60	81 600	1.4	100
pH 5.6 supernatant	3600	61	75 200	1.2	92
DEAE-Sepharose	4000	5.6	55 600	9.9	68
Phenyl-Sepharose	330	0.27	50 400	187.0	62
Ultrogel AcA 34	100	0.12	40 800	340.0	50

TABLE II

PURIFICATION OF GLYCEROKINASE FROM 1 kg *B. STEAROTHERMOPHILUS*

Purification step	*Volume (ml)*	*Amount of protein (g)*	*Enzyme activity (E.C. units)*	*Specific activity (E.C. units/mg protein)*	*Activity recovered (%)*
Homogenate	2000	60	95 500	1.6	100
pH 5.6 supernatant	3600	61	86 400	1.4	90
DEAE-Sepharose	4000	5.6	74 400	13.3	78
Phenyl-Sepharose	180	0.7	64 800	92.8	68
Ultrogel AcA 34	140	0.5	58 400	117.0	61

DISCUSSION

Hydrophobic interaction chromatography can be applied to the large-scale purification of glucokinse and glycerokinase as an alternative to pseudo-affinity chromatography with triazine dyes. This paper describes purification of the enzymes from 1 kg of bacterial cells but the procedure has been applied to extraction from 25 kg of cells[8]. Advantages of hydrophobic interaction chromatography are high flow-rates, excellent recovery of diagnostic-grade enzyme from a commercially available matrix, enzyme free of contamination from triazine dye and low cost relative to conventional affinity chromatography. Phenyl-Sepharose also has a high binding capacity; more than 6 mg of glucokinase and 3 mg of glycerokinase have been shown to bind to 1 ml of matrix. Triazine dye affinity matrices used in the purification of both enzymes bind about 3 mg of glucokinase and 1.5 mg of glycerokinase per ml of column[2,3]. It should be noted, however, that gel filtration after pseudo-affinity chromatography is unnecessary for glycerokinase. The hydrophobic interaction chromatography profile shows that complete separation of glucokinase from glycerokinase is possible from a single elution.

ACKNOWLEDGEMENT

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Note

Determination of inulase isoenzymes on polyacrylamide gels by activity staining

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Fructofuranosidases, which are able to hydrolyze inulin, are commonly found in bacteria[1–5], yeast[6–11], moulds[12–15] and plants[16–20]. Three principal patterns of action of inulases have been described. The most widespread is that of the *exo*-fructan-fructano hydrolase, *exo*-inulase, which liberates single fructose molecules from the polysaccharide chain. It is found in all the sources mentioned above. The second type are the *endo*-fructan hydrolases, which hydrolyze inulin to reducing fructo-oligomers, the inulo-oligooses. The occurrence of this enzyme type has been described only in moulds[15]. The third type, a fructotransferase, found only in bacteria and plants[3–5], is quite different from the usual catabolic carbohydratases. It splits difructose anhydride from the inulin chain and anhydratizes the two fructoses, leading to a difructose dianhydride.

The common analytical procedures for the characterization of inulase isoenzymes confirm only the affinity towards DEAE-cellulose or other ion exchangers[8–11,16–18]. Rocket immunoelectrophoresis[15] was able to detect two isoenzymes, one *endo*- and one *exo*-type enzyme, in a commercial inulase preparation from an Aspergillus species.

In this article we describe a non-denaturating separation of inulase isoenzymes by polyacrylamide gel electrophoresis (PAGE) and visualization by an activity-staining procedure. Further characterization of the type of action of the isolated isoenzymes was done by high-performance thin-layer chromatography (HPTLC).

MATERIALS AND METHODS

PAGE was carried out as described elsewhere[21]: in a block gel apparatus, a separation gel with 10% acrylamide (Merck, Darmstadt, F.R.G.) cross-linked 1:100 with bismethyleneacrylamide (Merck), pH 8.9, containing 6% inulin (Laevosan Gesellschaft, Linz, Austria) was used. The electrophoresis buffer was a Tris–glycine system. For an experiment lasting 2 h, a voltage of 300 V and a starting current of 120 mA were used. The apparatus was cooled with a flow of tap-water.

A 5-μl volume of crude enzyme preparation (Novo Industri, Bagsvaerd, Denmark) was separated. To visualize the zones of enzymatic activity, the gels were immersed in 100 ml of a 0.1 *M* acetate buffer (pH 5.0) and kept at 37°C for 1 h. Then

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the gels were incubated overnight in 100 ml of methanol–acetic acid–water (5:1:5) in a refrigerator. The inulin in the gel precipitates and the zones of enzymatic activity appear as transparent bands.

For the inhibition tests, the gels were incubated in 10^{-3} *M* solutions of Fe^{3+}, Mn^{2+}, EDTA, I_2 or aniline in acetate buffer pH 5.0 for 1 h.

HPTLC was performed on Whatman precoated silica plates, 200 μm, (LHP-KF). The solvent was *n*-butanol–*n*-propanol–ethanol (96%)–water (40:10:30:20). Thymol–sulphuric acid spray reagent was used to visualize the carbohydrates[22]. As a reference, non-reducing inulo-oligosaccharides were isolated from Jerusalem artichoke tubers[23].

RESULTS AND DISCUSSION

As shown in Fig. 1, four enzymes able to hydrolyze inulin are detectable with this electrophoretic method. The three more anodic enzymes (A,B,C) are inhibited with 10^{-3} *M* I_2 (lane 2). The more cathodic, very active enzyme band (D), however, is not inhibited by 10^{-3} *M* I_2. On testing the inulase inhibitors described by Zittan[15], *i.e.*, Fe^{3+}, Mn^{2+}, and EDTA, no inhibition of enzymatic activity was detected.

In one of the first articles about inulases, Schlubach and Grehn[24] describes aniline as a potential inhibitor of fructofuranosidases, both invertases and inulases. As seen in Fig. 1, lane 3, the activity of the more cathodic enzyme band (D) is clearly reduced by 10^{-3} *M* aniline. Regarding the results of Schlubach and Grehn[24], the more

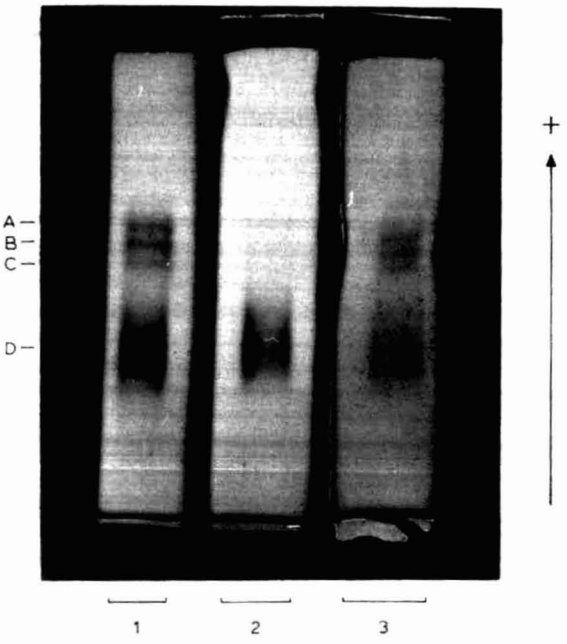
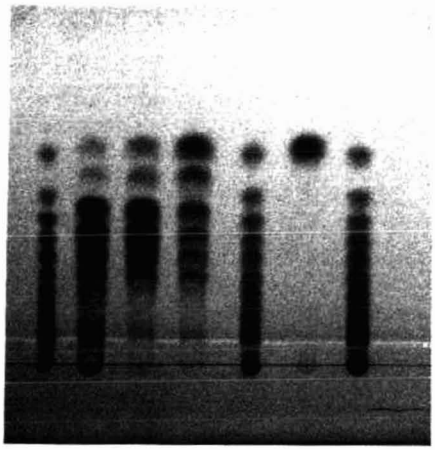


Fig. 1. Zymogram of Aspergillus inulase. 1, 0.1 *M* Acetate buffer pH 5.0, 37°C, 1 h; 2, 0.1 *M* acetate buffer pH 5.0, 10^{-3} *M* I_2, 37°C, 1 h; 3, 0.1 *M* acetate buffer pH 5.0, 10^{-3} *M* aniline, 37°C, 1 h. Each lane contains 5 μl of crude Aspergillus inulase. For staining procedure see Materials and methods.



I A B C I D I

Fig. 2. HPTLC of the digestion products. I = Inulo-oligosaccharides isolated from Jerusalem artichoke tubers; A = digestion products from enzyme A on inulin; B = digestion products from enzyme B on inulin; C = digestion products from enzyme C on inulin; D = digestion products of enzyme D on inulin; after spraying with thymol–sulphuric acid, the plate was heated at 105°C for 5 min. For chromatographic conditions see Materials and methods.

cathodic enzyme should be a fructofuranosidase able to hydrolyze inulin, and therefore be an *exo*-inulase.

To identify and distinguish between the type of action exhibited by the four different inulases, a gel strip was cut into sufficiently small pieces each containing only one isoenzyme band. The gel pieces were then incubated in acetate buffer (pH 5.0) at 37°C for 1 h. The products of the enzyme digestion were analyzed by HPTLC. Fig. 2 shows the chromatogram of the digestion products. The cathodic enzyme (D) produces only monosaccharides from inulin. The three more anodic enzymes (A–C) hydrolyze inulin into reducing fructo-oligosaccharides. Even at very long incubation times, more than 24 h, the composition of the degradation products does not change. The three enzymes A–C are therefore identified as *endo*-inulase isoenzymes.

The migration behaviour of the enzymatically produced fructo-oligosaccharides differs from that of the non-reducing oligosaccharides of the inulin series because of their lack of a terminal glucose.

This new method of electrophoretic identification of inulase isoenzymes is easier and more specific than the time-consuming immunoelectrophoresis.

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Note

Chromatographic evaluation of the binding of haemoglobin to polyanionic polymers

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The interactions between haemoglobin and polyanionic compounds have been extensively investigated and several methods have been proposed for determining the stoichiometry of the complexes and their binding constants. However, these methods are generally suitable only for low-molecular-weight ligands, since they are often based on a good separation between macromolecules and small compounds[1,2].

It is now known that some polyanionic polymers, by interaction with haemoglobin, cause a decrease in its affinity for oxygen[3–5] and that the extent of this effect depends on various parameters such as the nature of the ionic groups, their concentration on the polymers, etc. These charged polymers can then be regarded as macromolecular allosteric effectors, whose association with haemoglobin, by giving rise to high-molecular-weight complexes with low affinity for oxygen, could be utilized in the field of blood substitutes[6].

Such complexes between haemoglobin and macromolecular polyanions have already been studied by methods such as sedimentation[3,4] and X-ray scattering[3], but this type of investigation is rather complicated as it requires special equipment. On the other hand, the information obtained from oxygen-binding determinations[4] is not easy to interpret as, during the oxygen equilibrium experiments, the conformation of haemoglobin changes from the deoxy to the oxy form.

In this paper, we report the results of an high-performance liquid chromatographic (HPLC) study of the complexation of polyanionic polysaccharides with oxyhaemoglobin, by the Hummel and Dreyer method[7]. This report essentially deals with polysaccharide sulphates such as dextran sulphate, whose interactions with haemoglobin were recently studied by a potentiometric technique[5], and λ carrageenan, but the same method could be applied to any other charged polymer.

EXPERIMENTAL

Materials

Human haemoglobin was prepared from outdated blood, according to the usual method[8]. It was deionized and freed from organic phosphates by passage through a column of Ultrogel AcA-202 (IBF, France; linear fractionation range 1000–15 000; exclusion limit 22 000) at pH 8.9, 0.1 *M* sodium chloride.

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Dextran sulphate was synthesized from dextran T40 (Pharmacia, Sweden; $\bar{M}_w \approx 40000$, $\bar{M}_n \approx 26000$), as described by Ricketts[9]. It contained 17% sulphur which corresponds to about 1.9 sulphate groups per glucose unit; it was in the sodium salt form and its $\bar{M}_n$ was evaluated as 54 500.

λ carrageenan was obtained from Sigma (St. Louis, MO, U.S.A.). It was essentially in the calcium salt form and contained 10.6% sulphur, which corresponds to 0.88 sulphate groups per glucoside unit. Its molecular weight was not known.

All products were dissolved in 0.05 *M* Tris buffer.

Equipment

A Waters Assoc. Model ALC 200 liquid chromatograph equipped with a M 600 A pump, a M 440 UV detector and an U6K injector was used. The size-exclusion column (60 cm × 0.75 cm I.D.), Ultropac TSK G3000 SW, (linear fractionation range: 1000–300 000) was obtained from LKB (Sweden).

Conditions

For the determination of the stoichiometries of polymer–haemoglobin complexes, we used the Hummel and Dreyer method, as already described for the study of drug–protein interactions[10]; thus, we equilibrated the size-exclusion column with a solution of haemoglobin considered as the ligand and injected small amounts of polymer solutions.

The concentration of haemoglobin in the eluent (0.05 *M* Tris buffer) being fixed at a defined value, mixtures in the same eluent of polysaccharide sulphate at a constant concentration and of haemoglobin at increasing concentrations were injected (100 μl). The chromatograms thus obtained showed the presence of protein–polymer complex in the void volume, followed by a negative or positive peak at the retention volume of haemoglobin, which represents the amount of protein bound to the polymer. By plotting the area of this peak *versus* the excess (relative to the eluent concentration) of haemoglobin, and by interpolating to zero, the excess of haemoglobin corresponding to the exact amount of protein bound to the polymer could be determined (internal calibration[6]). By increasing the haemoglobin concentration in the eluent and keeping constant that of the polymer in the injection, the average stoichiometry of the haemoglobin–polymer complex at saturation was determined. The concentration of dextran sulphate in the injections was 0.27 g/l which corresponds to $5 \cdot 10^{-6}$ *M* (with $\bar{M}_n = 54500$), and that of λ carrageenan was 0.56 g/l. The concentration of haemoglobin in the eluent, determined by the Drabkin method[11], was between 0.5 and 15 μM, *i.e.*, between 0.32 and 0.97 g/l. Detection was made at 254 nm, and the flow-rate was 1 ml/min. The experiments were carried out at room temperature.

RESULTS AND DISCUSSION

Fig. 1 shows the internal calibration curve obtained for the interaction of haemoglobin with dextran sulphate, for an haemoglobin concentration in the eluent of 1.8 μM at pH 7. The interpolation at 0 gives the amount of haemoglobin, n_{Hb}, bound to the amount of dextran sulphate injected, $n_{DS} = 5 \cdot 10^{-10}$ mol, and leads to the

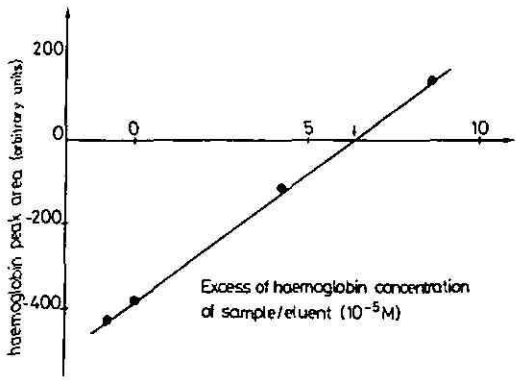

Fig. 1. Internal calibration for binding of oxyhaemoglobin to dextran sulphate. Eluent. 1.8 μM of haemoglobin in 0.05 M Tris buffer pH 7. Injection: 100 μl of mixtures of dextran sulphate (0.27 g/l) and haemoglobin at increasing concentrations in 0.05 M Tris buffer pH 7, 25°C.

mean molar ratio:

$$\bar{r} = \frac{n_{Hb}}{n_{DS}} = \frac{6.4 \cdot 10^{-9}}{5 \cdot 10^{-10}} = 12.8$$

$\bar{r}$ corresponds to the average number of haemoglobin molecules bound to one molecule of dextran sulphate under the concentration conditions used. The same internal calibration was performed for various haemoglobin concentrations in the eluent, and by plotting the different values calculated for $\bar{r}$ as a function of the haemoglobin concentration in the eluent a curve (Fig. 2) was obtained from which the average molar stoichiometry, $\bar{s}$, of the saturated haemoglobin–dextran sulphate complex was calculated, *i.e.*, $\bar{s} = 14$ (haemoglobin tetramer per dextran sulphate).

To appreciate the effect of pH on the ability of dextran sulphate and haemoglobin to form a complex, the same experiments were performed at various pH val-

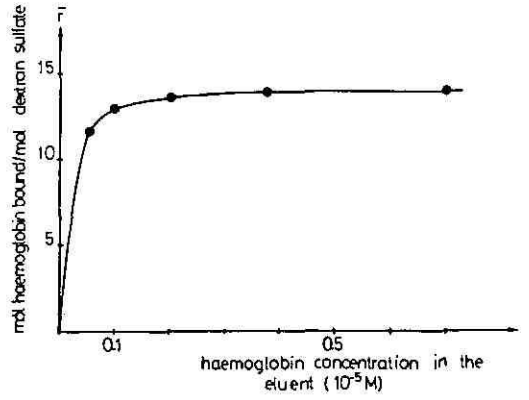

Fig. 2. Binding of oxyhaemoglobin to dextran sulphate as a function of the haemoglobin concentration in the eluent (0.05 M Tris buffer pH 7). Conditions as in Fig. 1.

ues. The average molar stoichiometries of the saturated complexes are 14, 13.4 and 8.75 at pH 7, 7.2 and 7.5, respectively. The results show that, as expected, when the pH increases, the average amount of haemoglobin molecules complexed with one molecule of dextran sulphate decreases, which corresponds to the deprotonation of some of the haemoglobin amino groups involved in the ionic interactions.

In order to compare the relative capacity of different polyanionic polysaccharides to form a complex with haemoglobin, a new parameter, $\bar{s}'$, was calculated, defined as the average amount of haemoglobin molecules bound to one molecule of sulphated glucoside monomer in the saturated complex. Thus, for dextran sulphate (17% sulphur) at pH 7, $\bar{s}'$ is equal to $9 \cdot 10^{-2}$, which means that, at saturation, the complex is formed on average by the association of 9 molecules of haemoglobin with 100 sulphated glucoside units. In the case of λ carrageenan (10.6% sulphur), at pH 7, $\bar{s}' = 6 \cdot 10^{-2}$, which shows that when the concentration of sulphate ions linked to the polymer decreases its capacity to form a complex with haemoglobin is reduced.

Other similar quantifications are now being carried out with dextran carboxylates and phosphates. The results of these studies should lead us to define the best polyanionic polysaccharide to be used in association with haemoglobin in the field of blood substitutes[6], the aim being to find a polymer with as low a charge as possible, but able to lead to a complex with haemoglobin as stable as possible under the conditions (pH and ionic strength) of the physiological media.

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Note

Determination of organic acids in highly alkaline solutions

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Since its introduction by Small *et al.*[1], ion chromatography has been developed into a rapid and sensitive means for determining ions in aqueous solution, especially inorganic anions. Ion chromatography differs from conventional ion-exchange chromatography in that a low-capacity ion-exchange column, eluents of low concentration and conductivity detection are used. Although the method is effective for the determination of anions in relatively pure solutions such as river waters, complicated pre-treatment operations are necessary when analysing anions in industrial solutions such as brines and sodium hydroxide solutions, because high salt solutions disturb the equilibrium in the low-capacity column, and large salt peaks sometimes interfere in the separation[2].

Many reports on the separation of organic anions by conventional ion-exchange chromatography with spectrophotomeric detection have been published[3–6]. One of the features of the method is that it is applicable to various industrial samples because of the stable equilibrium in the column. However, there have been few reports on the determination of anions in highly alkaline solutions with direct injection of the samples.

In this paper a liquid chromatographic procedure suitable for the determination of organic anions in highly alkaline solutions is described. First the injected sample is introduced into the pre-treatment column, which is packed with a strong sulphonic acid-type cation-exchange resin (H-type) where sodium or alkali metal cations are exchanged. Subsequently, the exchanged neutral sample is introduced into the separation column and the organic anions are eluted. The eluting anions are detected by monitoring at 210 nm. In this work the gel used in both the pre-treatment and the separation columns was the same. In spite of the direct injection of highly alkaline solutions, the difficulties associated with that of high salt solutions were not observed. This liquid chromatographic procedure is applicable to determinations of other compounds, for example, cations in highly acidic solutions.

EXPERIMENTAL

All chemicals and reagents were of analytical-reagent grade and were used without further purification. Deionized water was used throughout.

A flow diagram of the procedure is shown in Fig. 1. Liquid chromatographic

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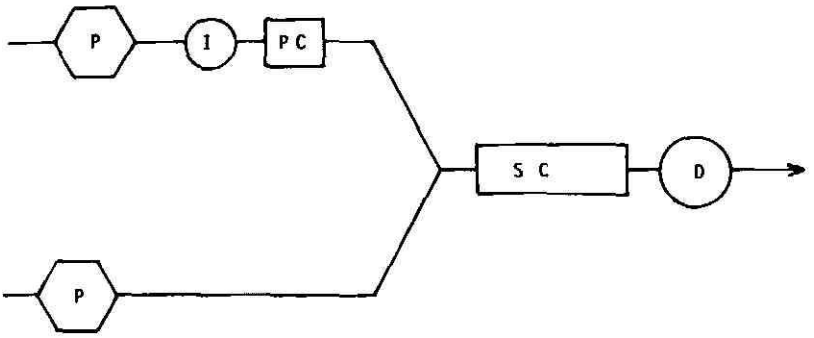

Fig. 1. Schematic flow diagram of on-line pre-treatment system. P = pump; PC = pre-treatment column; SC = separation column; D = detector; I = injector.

separation was performed on a system consisting of a CCPM pump (two heads, metal-free; Toyo Soda), a Rheodyne 7125 injector with a 100-μl loop and a UV-8000 variable-wavelength detector (Toyo Soda). A detection wavelength of 210 nm was employed and peak integrations were carried out using a CP-8000 data station (Toyo Soda). The separation column was 10 cm × 8 mm I.D. and the pre-treatment column was a 1 cm × 8 mm I.D. glass column. The gel packed in these columns was TSK-gel SCX (10 μm) and the cation-exchange capacity was almost 4.2 mequiv./g. After breakthrough of the alkaline material in the pre-treatment column, it was washed with 0.2 vol.-% phosphoric acid solution for 2 h at a flow-rate of 1 ml/min.

Under the usual chromatographic conditions, 0.2 vol.-% phosphoric acid solution was pumped at a flow-rate of 0.2 ml/min as the mobile phase. Deionized water was also pumped to the injector at a flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

TSK-gel SCX is a strong cation-exchange resin consisting of totally porous sub-10 μm particles. Two retention mechanisms, ion exclusion and reversed-phase adsorption, are reported primarily to influence the retention behaviour of organic acids on cation-exchange resins[7–10]. Organic acids with low pK_a values are separated on this type of column principally by an ion-exclusion mechanism, whereas organic acids with higher pK_a values such as acetic acid, which exists predominantly in the undissociated form at the mobile phase pH, are separated by adsorption on the relatively non-polar stylene–divinylbenzene resin backbone[5].

A typical chromatogram of a standard sample containing 50 ppm each of formic acid acetic acid and 100 ppm of propionic acid in deionized water obtained with the present system is shown in Fig. 2. These three acids could be separated and determined in 7 min. Inorganic anions such as nitrate and sulphate were eluted rapidly and the first large solvent front peak corresponded to these inorganic anions. The separation of the acids was clear in spite of the use of the two-column system. The small volume of pre-treatment column and the efficient mixing of the carrier and the mobile phase enabled effective separations to be achieved.

The linearity of calibration graphs was examined. Linear graphs with intercept

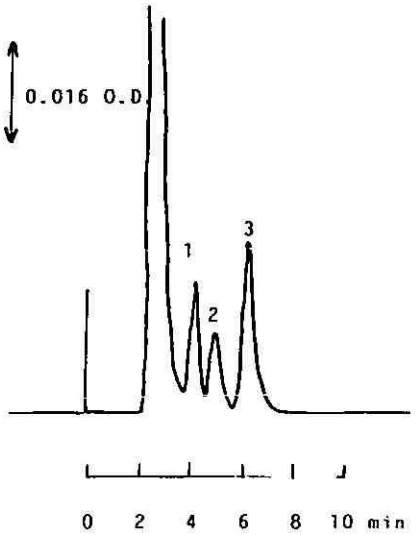

Fig. 2. Separation of acids in pure water. 1, Formic acid (50 ppm); 2, acetic acid (50 ppm); 3, propionic acid (100 ppm). Flow-rate of carrier, 0.8 ml/min; flow-rate of eluent, 0.2 ml/min; injection volume, 100 μl.

close to zero were obtained throughout, as is usual with a one-column system. The dynamic range was 0–200 ppm for the above acids, which would be adequate for their determination in industrial samples.

The precision of the liquid chromatographic procedure was evaluated by running five repeated analyses of a series of standard samples (in deionized water). Table I summarizes the results. The relative standard deviations were 0.5–1.7%, which are satisfactory. These good results may be due the high quality of the pump delivery system.

A typical chromatogram of the acids in 1 *M* sodium hydroxide solution is shown in Fig. 3a. In spite of direct injection of a highly alkaline solution, these acids could be separated and determined as in Fig. 2. Much of the sodium could be eliminated with high efficiency in the pre-treatment column. A typical chromatogram when the pre-treatment column was omitted is shown in Fig. 4. A suitable separation could not be achieved and it took too long to obtain a stable baseline again. This result suggests that the two-column system may be favourable for the determination of the acids in industrial samples.

TABLE I

REPRODUCIBILITY OF THE INJECTION OF ACIDS IN PURE WATER

Acid	*Peak area*	*Relative standard deviation (%)*	*Peak height*	*Relative standard deviation (%)*
Formic	1.58	1.13	7.56	1.35
Acetic	1.12	1.64	4.60	1.37
Propionic	3.40	1.26	10.94	0.45

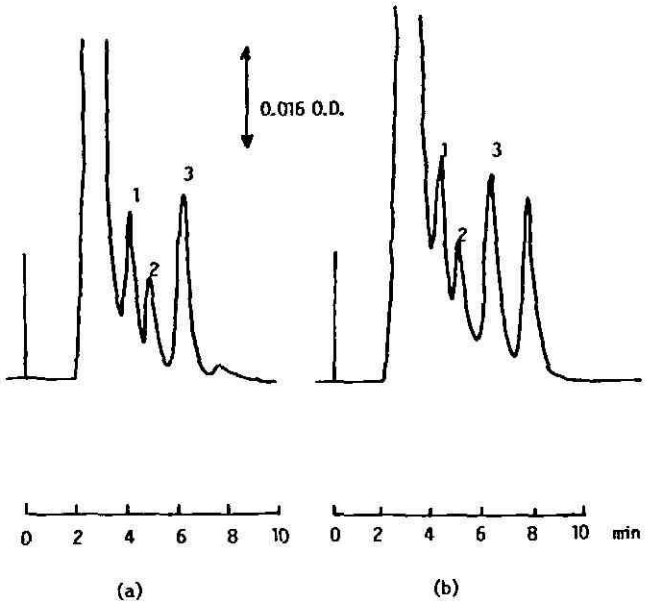

Fig. 3. Separation of acids in 1 *M* NaOH solution: (a) with the fresh pre-treatment column; (b) with the "breakthrough" pre-treatment column. 1, Formic acid (50 ppm); 2, acetic acid (50 ppm); 3, propionic acid (100 ppm). Conditions as in Fig. 2.

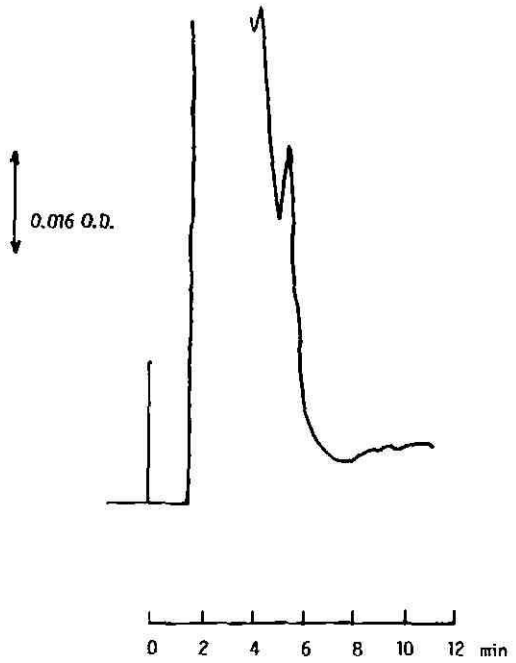

Fig. 4. Chromatogram of the acids in 1 *M* NaOH solution without the pre-treatment column. Conditions as in Fig. 2.

TABLE II

REPRODUCIBILITY OF THE INJECTION OF ACIDS IN 1 *M* SODIUM HYDROXIDE SOLUTION

Acid	*Peak area*	*Relative standard deviation (%)*	*Peak height*	*Relative standard deviation (%)*
Formic	1.51	2.66	7.45	0.67
Acetic	1.15	0.97	4.85	1.03
Propionic	3.56	1.02	11.35	2.37

Table II summarizes the results of reproducibility experiments on the highly alkaline solution. The sample solution contained 50 ppm each of formic acid and acetic acid and 100 ppm of propionic acid in 1 *M* sodium hydroxide solution. The peak height and area were almost the same as those in the former instance. This result suggests that the on-line pre-treatment in the flow stream was effective and neutralization of sodium in the pre-treatment column did not affect the separation. The relative standard deviations were as low as in the former instance.

One of the major problems of this system is "breakthrough" of the pre-treatment column on passing through highly alkaline sample solutions. Before injection of alkaline samples the column is totally in the $-SO_3H$ form, but once the samples have been injected it is partly exchanged to the $-SO_3Na$ form and the exchange capacity for alkaline solutions is diminished. When 100 μl of 1 *M* sodium hydroxide solution were injected five times, the capacity of the pre-treatment column was reduced and a clear separation could not be achieved. The "fifth" chromatogram is shown in Fig. 3b. The front peak became larger and the unknown peak after propionic acid also became larger. Hence formic and acetic acids could not be determined. The unknown peak is currently under investigation.

CONCLUSION

With deionized water as the carrier and phosphoric acid solution as the mobile phase, organic acids in highly alkaline solutions could be determined without any pre-treatment. Sodium could be easily excluded using the cation-exchange gel column. Although the system consisted of two pumps and two columns, very rapid and precise separations could be achieved. The results make the system particularly suitable for the determination of organic acids in industrial sample solutions.

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Note

Liquid chromatographic purification of tissue samples prior to chemiluminescence immunoassay of 19-nortestosterone

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Several anabolics are reported to be used mostly illegally in livestock breeding. Almost no reference was made to the androgen 19-nortestosterone (nandrolone) (19-NT) until its intensive use in The Netherlands was reported[1,2]. It seems that nowadays 19-NT is the most frequently used anabolic steroid in cattle fattening in The Netherlands and probably in Belgium as well.

Almost no relevant methods for detection and quantitative assay are available. The multi-residue method of Verbeke[3] for detection of anabolics in urine and in tissues includes 19-NT. Jansen *et al.* have developed an immunoassay for urine, employing either radioactive[4] or chemiluminescent labels[2]. Its combination with high-performance liquid chromatography (HPLC) was shown to be necessary in order to avoid false positive results[5,6].

As far as I know, no methods for detection and quantitative assay in muscle tissue have yet been described. From a practical point of view, screening of urine samples is much easier to perform, however meat is virtually the only sample type which is available from slaughtered animals or the retail trade.

The present paper describes the extraction and clean-up of tissue samples to such a degree of purity that the chemiluminescent immunoassay for 19-NT described by Jansen *et al.*[2] can be successfully applied.

EXPERIMENTAL

Reagents and instrumentation

All solvents were reagent grade and used as such, except diethyl ether which was shaken with 25% ferrous sulphate solution and washed three times with distilled water and subsequently distilled. 19-Nortestosterone was from Serva (F.R.G.). Subtilisin A (dialyzed and lyophilized, 29.3 Anson units/g) was from Novo Industri (Denmark). Lipidex-5000 was obtained from Pharmacia (Sweden).

The chemiluminescent label, *i.e.*, the N-(4-aminobutyl) N-ethylisoluminol conjugate of 19-NT, and the antiserum against 19-NT were kindly supplied by Drs. E. H. J. M. Jansen and R. W. Stephany, Laboratory for Endocrinology, National Institute for Public Health and Environmental Hygiene, Bilthoven, The Netherlands.

The HPLC equipment (Fig. 1) included a Waters pump and an automatic injector Wisp 710 B (Waters). UV absorption was monitored at 240 nm with a vari-

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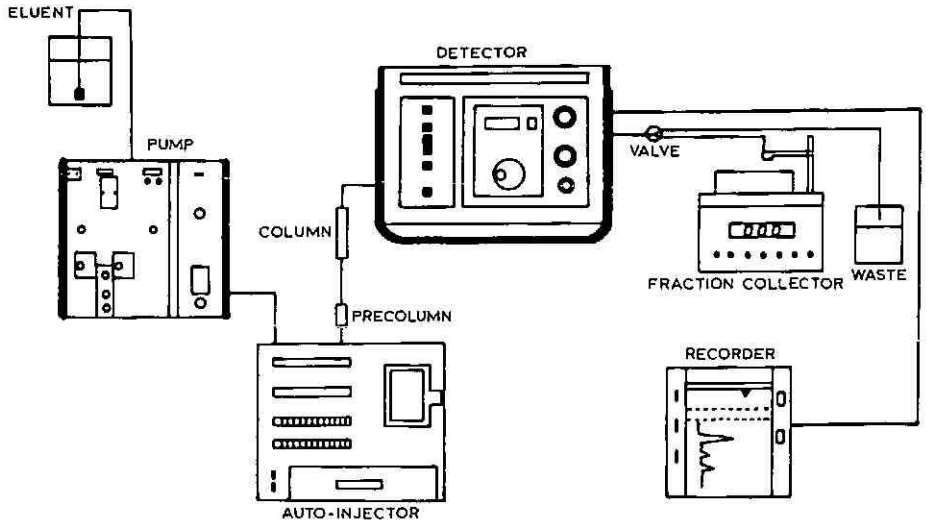

Fig. 1. Diagram of the instrumentation used in the HPLC purification step.

able wavelength detector (Pye Unicam, Model 4020). The HPLC column (250 mm × 4.6 mm) was packed with LiChrosorb RP-18 (5 μm, Merck) and was protected by a guard column (75 mm × 2.1 mm; Chrompack, Cat. No. 28603); it was operated at ambient room temperature. The eluent was methanol–water (65:35, v/v) at a flow-rate of 1.5 ml/min. The fraction collecting system consisted of a LKB Model 2212 Helirac fraction collector.

Luminescence was measured in a Lumac 3M Biocounter M 2010 A. Quantitative results were calculated with a Tulip PC after logit/log transformation.

Isolation of 19-NT from tissue samples

A 1.0-g sample of minced meat was deproteinated enzymatically with 1 mg of Subtilisin A in 4 ml of 0.1 *M* Tris solution (pH 9.5) for at least 2 h at 60°C in a water-bath. Overnight digestion does not affect the quality of the digestate. The liquid suspension was chilled to room temperature and extracted twice with 5-ml portions of diethylether. The combined layers were evaporated to dryness in a stream of nitrogen at 35°C, yielding the crude extract.

Column chromatography on Lipidex-5000

The crude extract was taken up in 0.2 ml of hexane–dichloromethane (85:15, v/v) and applied on top of a small glass column (145 mm × 6 mm), plugged at the bottom with glass wool and filled with 6 cm of Lipidex-5000, swollen and conditioned with the same solvent. The column was then eluted with the same mixture. The first 2.5 ml were discarded; the next 5 ml were collected and evaporated to dryness at 35°C in a stream of nitrogen.

HPLC

The residue obtained after evaporation of the Lipidex-5000 fraction was taken up in 100 μl of methanol and transferred to a vial suited for the Wisp. A diagram of

the instrumentation is given in Fig. 1. The eluate between 5.30 and 7.15 min is automatically collected. The total elution time per sample was 15 min. The 19-NT-containing fraction was evaporated to dryness at 45°C under nitrogen, yielding the final extract. The latter was taken up in 500 μl of buffer pH 7.0.

Chemiluminescence immunoassay (CLIA)

Duplicate 200-μl aliquots of the final extract were subjected to CLIA as described by Jansen *et al.*[2]. A standard curve covering the range 62.5–2000 pg was established by means of methanolic 19-NT solutions. Concentrations were calculated from the logit/log transformation of the experimental values.

RESULTS AND DISCUSSION

Enzymatic digestion or deproteination of tissue samples has been reported to be succesful in toxicological analyses for benzodiazepines, barbiturates, salicylic acid and other acidic compounds[7,8]. This approach yielded far higher recoveries than conventional extraction methods and the subsequent chromatographic analysis allowed rapid separation and detection of nanogram quantities of drugs without elaborate preliminary purification. It was not possible in the case of 19-NT to compare recoveries with those obtained by other methods, due to a lack of such methods. No radioactive tracer was available to measure them by radioactivity counting. Yet the enzymatic deproteination and extraction offers the considerable advantage that a large number of samples can be left to digest overnight.

Although the cornerstone of the purification step is the HPLC fractionation, the preliminary Lipidex column remains indispensable in view of the lifetime of the guard column and even of the analytical column. When fatty meat or sausage meat is extracted, a considerable amount of lipids is present in the crude extract and as much as possible should be removed. Six crude extracts can easily be handled simultaneously by one analyst in about half an hour, excluding column preparation time.

The HPLC phase allows automation of injection and of fraction collection, so that this step can be carried out overnight after the first analysis day. One cycle, including the preparation of the injection, the injection itself and the chromatographic analysis, is completed in about 16 min.

Fig. 2 shows a typical chromatogram of a certified blank meat sample (left) and of a meat sample of an experimentally treated animal (right). The concentration of 19-NT in the latter, as assayed by CLIA, was 54 ppb*. Highly positive samples show a peak with a retention time of *ca.* 6.15 min, corresponding to the retention time of 19-NT. The collection window, 1.45 min broad, indicated by the shaded zone on the chromatogram, is determined from the absorption at 240 nm of a 19-NT standard (100 ng). Special attention should be paid to possible contamination of the injection system by this relatively massive dosage.

According to information from the supplier, the antiserum against 19-NT shows a cross-reactivity of 5.1% for 17β-trenbolone and of 3.6% for 17β-testoster-

* Throughout the article the American billion (10^9) is meant.

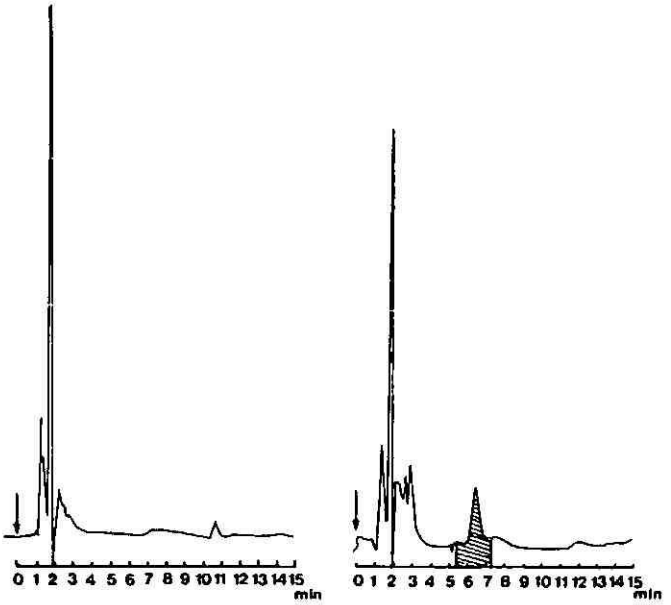

Fig. 2. Typical chromatogram of a blank meat sample (left) and of a sample from an animal experimentally treated with 19-NT (right).

one. High concentrations of the latter naturally occurring but also exogenously applied steroid could cause positive responses. It is obvious that the HPLC separation largely contributes to an increased specificity of the method which cannot be guaranteed completely by use of the antiserum alone.

It was not possible, as already mentioned, to standardize the developed method against existing methods or against a radioimmunoassay (RIA) method in particular. The main objective however of the method is to detect unwanted and illegal 19-NT residues in meat for human consumption. This means that, for forensic investigations, priority should be given to reliability rather than to accuracy. If the method is used for screening of 19-NT residues, it should be sensitive enough to detect all positive samples and it should be specific enough to avoid false positives. In other words, if the method is correctly applied, all negative samples should show negative signals and all positive samples should give a positive signal. In practice it is not always easy to distinguish between positive and negative responses. When certified blank meat samples, obtained from a *bona fide* breeder, are analysed, luminescence signals are obtained which clearly differ from the zero standard, showing the effect of quenching. Chemiluminescence suffers from the same disadvantages as β-scintillation counting[9]. The luminescence of a series of blank samples was significantly different ($p \leqslant 0.001$) from that of the 125-pg standard but not from that of the 62.5-pg standard. It was concluded that blank meat samples contain a certain amount of "background 19-NT" which is indistinguishable from 62.5 pg of 19-NT. This means that the limit of quantification is 125 pg per reagent tube or, expressed in terms of the concentration in muscle tissue, *ca.* 0.6 ppb. Therefore samples showing an apparent 19-NT content of less than 125 pg per reagent tube or 0.6 ppb in the tissue are considered negative.

The limit of quantification should not be mistaken for the limit of detection. The latter is the minimum quantity of analyte which can be detected on the standard curve. It depends on the slope of that curve but equally on the variance of the 0-pg standard. The limit of detection, which for the present method is 12 ± 8 pg ($n=8$), is determined as the quantity of analyte which yields a luminescence signal three standard deviations above (under) that of the 0-pg standard.

In each analysis series a certified negative sample and a spiked (1-ppb) sample are used as controls. Results are rejected when the negative control has an apparent 19-NT concentration higher than 0.6 ppb or when the positive control shows unexpected results, *i.e.*, an abnormally high response or a response lower than 0.6 ppb.

Despite the inherent but limited specificity of the antiserum and the additional specificity from the HPLC separation by which a number of possible interferences are excluded, immunoassays remain screening methods. This means that if the method is correctly applied, negatives may be considered as real negatives but that positives may be caused by nonspecific interferences. In other words, in forensic investigations, a confirmatory test by an independent technique is required. The sensitivity of the immunochemical methods can only be matched by chromatographic methods with specific or selective detection systems. Work is in progress in our laboratory to confirm positive CLIA results by gas chromatography–mass spectrometry. Several derivatives (heptafluorobutyrates, *tert.*-butyldimethylsilyl ethers) show excellent chromatographic and mass spectrometric properties which should enable unambiguous identification of the parent compound in the extract.

ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatographic separation of carbazole alkaloids

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Recently we reported[1] the gas–liquid chromatographic (GLC) separation of carbazole alkaloids. The GLC method requires high temperatures for some alkaloids. It is therefore of interest to find an effective method of separation of this group of alkaloids using milder conditions. We report here the separation of C_{13}, C_{18} and C_{23} carbazole alkaloids at room temperature by high-performance liquid chromatography (HPLC). The method has been found to be useful for the detection of these alkaloids in plant extracts.

EXPERIMENTAL

Samples and reagents

Carbazole (I) and seven carbazole alkaloids[2] (II–VIII) were used.

Analytical grade reagents were used. Methanol and chloroform were distilled before use. Hexane was treated with conc. sulphuric acid and distilled. The solvents were prefiltered through a Millipore Type F-H 0.5-μm filter and degassed.

A mixture containing 1 mg of each sample was dissolved in methanol (30 ml) and aliquots (10 μl) were injected into the HPLC system.

Apparatus and conditions

HPLC was conducted on a Waters Millipore 2504 liquid chromatograph with a μPorasil Radial-Pak cartridge (particle size 10 μm, 10 cm × 8 mm I.D.). The column effluent was monitored at 254 nm using a Waters Model-441 absorbance detector. The mobile phase flow-rate was 0.7 ml/min.

RESULTS AND DISCUSSION

Preliminary studies indicated that the carbazole alkaloids (I–VIII) could not be resolved satisfactorily on a μBondapak C_{18} Radial-Pak cartridge using methanol–water (8:2 or 7:3). However, resolution could be achieved on a μPorasil Radial-Pak cartridge using a mobile phase of hexane–chloroform (7.5:2.5). The sep-

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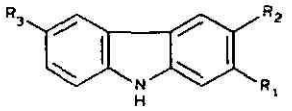
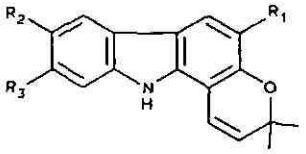


Carbazole (I) $R_1 = R_2 = R_3 = H$

3 Methylcarbazole (II) $R_1 = R_3 = H$; $R_2 = CH_3$

Glycozoline (III) $R_1 = H$; $R_2 = CH_3$; $R_3 = OCH_3$

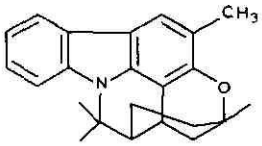
Glycozolidine (IV) $R_1 = R_3 = OCH_3$; $R_2 = CH_3$



Heptazolidine (V) $R_1 = OCH_3$; $R_2 = CH_3$; $R_3 = H$

Koenimbine (VI) $R_1 = CH_3$; $R_2 = OCH_3$; $R_3 = H$

Koenidine (VII) $R_1 = CH_3$; $R_2 = R_3 = OCH_3$



Murrayazoline (VIII)

aration is shown in Fig. 1. Better resolution of carbazole (I) and 3-methylcarbazole (II) could be obtained by decreasing the percentage of chloroform, but koenidine (VIII) took much longer to elute. For example, koenidine was eluted from the column in 1 h as a broad tailing peak when 20% chloroform in hexane was used as the mobile phase. It may be mentioned here that the order of elution of these alkaloids in HPLC was different from that obtained in gas chromatography[1].

The detection limits for these alkaloids were determined at 0.005 a.u.f.s. The limit for carbazole, 3-methylcarbazole, heptazolidine, koenimbine and koenidine was found to be 1–2 ng. This method can detect less than 1 ng of glycozoline, glycozolidine and murrayazoline.

In order to investigate the applicability of this method to the identification of carbazole alkaloids in plant extracts, the neutral fraction of a light petroleum (b.p. 40–60°C) extract of the root bark of *Glycosmis pentaphylla* (Retz) DC was examined using the present technique. It was observed that, besides glycozoline (III) and glycozolidine (IV), the two major carbazole alkaloids present in the plant, carbazole (I) and 3-methylcarbazole (II) could also be detected in the chromatograms. This is the first report of the detection of carbazole in a plant source. In addition, 3-methylcarbazole was detected for the first time in *Glycosmis pentaphylla*.

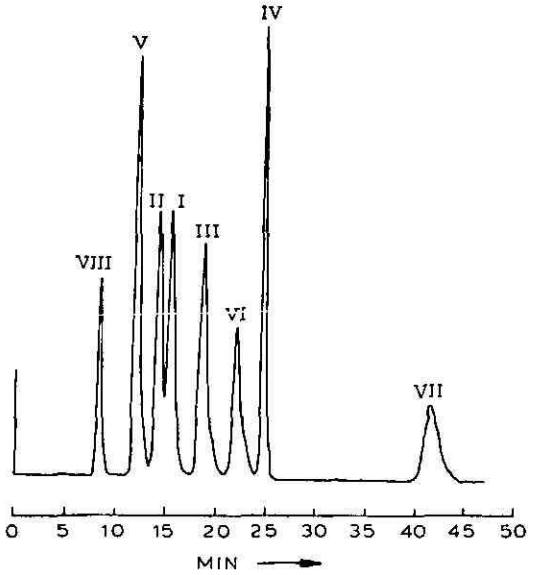


Fig. 1. HPLC chromatogram of carbazole (I) and seven carbazole alkaloids (II–VIII) on a μPorasil Radial-Pak cartridge using 25% chloroform in hexane; flow-rate 0.7 ml/min. Detection 254 nm; sensitivity 0.1 a.u.f.s.

In conclusion, HPLC provides a method for the resolution and identification of complex mixtures of carbazole alkaloids. The method may be extended for preparative or semipreparative separations.

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Note

Determination of sennosides A and B in Senna extracts by high-performance liquid chromatography

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Senna leaves and pods have long been used as laxatives and purgatives[1], and numerous studies have been made to determine their principal constituents: anthracenic derivatives (anthronic, dianthronic and anthraquinonic forms) and flavonic derivatives. Sennosides A and B[2] (dianthronic glucoside derivatives) have proved to be the most active Senna constituents[3].

Several different methods, such as thin-layer chromatography (TLC)[4–11] and high-performance liquid chromatography (HPLC)[11–18], have been proposed for the specific evaluation of sennosides A and B. However, only a few of them result in simple, rapid and accurate quantitation, and are applicable to Senna extracts. A method was thus developed for the qualitative and quantitative assay of sennosides A and B in Senna extracts by ion-pairing HPLC.

MATERIALS AND METHODS

Reagents

Sennosides A and B, Standard quality (Sarsyntex, Mérignac, France) were used as reference samples. Methanol and ethanol, analytical-reagent grade were obtained from (Merck, Darmstadt, F.R.G.), phosphoric acid RP Normapur and ammonia solution RP Normapur from Prolabo (Paris, France). The ion-pairing reagent was obtained from a PIC A vial (Millipore-Waters) containing 5 m*M* tetrabutylammonium phosphate.

Sep-Pak C_{18} cartridges (Millipore-Waters) were employed. The mobile phase consisted of methanol–water plus PIC A (36.5:63.5); if necessary, the pH was adjusted to 7.5 with a few drops of phosphoric acid. It was filtered before use, through a 0.45-μm HAWP filter (Millipore), then degassed using helium. The flow-rate was 0.6 ml/min.

Apparatus

The liquid chromatograph consisted of a Spectra-Physics 8700 solvent-delivery system, a Rheodyne injection valve (sample loop of 10 μl) and a variable wavelength UV detector (UV/Visible SF 770 Schoeffel detector) operating at 215 or 270 nm, the absorbance maxima of sennosides A and B. A Hibar LiChrosorb RP-8 column (250 mm × 4 mm, 10 μm) (Merck) fitted with a pre-column packed with Corasil C_{18} (50 μm) (Millipore-Waters), was used.

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Sample preparation

Standard solutions of sennosides A and B. Accurately weighed quantities of sennosides A and B were diluted to known concentrations in the mobile phase. Where necessary, a trace of ammoniac solution can be added to effect complete dissolution of the sennosides.

Extracts of Cassia angustifolia leaves. Different extraction methods for sennosides A and B were tested on different batches of Senna leaves. Two of these processes were found to be efficient: aqueous extraction with heating under a reflux condenser for 45 min; 70% ethanol extraction at room temperature[3,4]. Where necessary, Senna extracts obtained using these procedures were diluted to 25 μg/ml in water or 70% ethanol respectively, then injected for HPLC.

Pre-treatment on Sep-Pak cartridge. A Sep-Pak C_{18} cartridge was conditioned with 5 ml methanol, then 5 ml water. The Senna extract (0.5 ml) was loaded on the cartridge. A 0.5-ml volume of methanol–water (10:90) and 1.0 ml methanol–water (30:70) were passed successively through the cartridge, and discarded. Then two 0.5-ml volumes of methanol–water (70:30) were passed, the fractions containing sennosides (fractions 1 and 2) were collected and examined by HPLC.

RESULTS AND DISCUSSION

Several methods using reversed-phase chromatography, ion pairing or ionic suppression techniques were tested.

The acidic character of sennosides A and B was used to form an ion pair between their carboxylic functional groups and the quaternary ammonium compounds. Then the separation method for standard sennosides was optimized by preparing different mobile phases (Table I). These tests demonstrated that sennosides A and B could easily be separated. Not all mobile phases, however, allowed the study of Senna extracts, because certain components were eluted with sennoside A and/or B. For all the Senna extracts tested, only methanol–water plus PIC A (36.5:63.5), flow-rate 0.6 ml/min, λ = 270 or 215 nm, at room temperature, allowed the separation of the peaks of sennosides A and B from the peaks of the other constituents, in a reasonable time (Fig. 1). We compared the identity of the sennoside peaks with those of standard substances: the absorbance ratio was found to be similar at two

TABLE I

SEPARATION OF SENNOSIDES A AND B WITH VARIOUS MOBILE PHASES

Mobile phase	*k'*	
	Sennoside A	*Sennoside B*
Methanol–water + PIC A (30:70)	10.6	5.4
Methanol–water + PIC A (35:65)	5.8	3.4
Methanol–water + PIC A (36.5:63.5)	4.1	2.3
Methanol–water + PIC A (38:62)	3.4	1.9
Methanol–water + PIC A (40:60)	2.0	1.3
Methanol–water + PIC A (45:55)	0.9	0.6

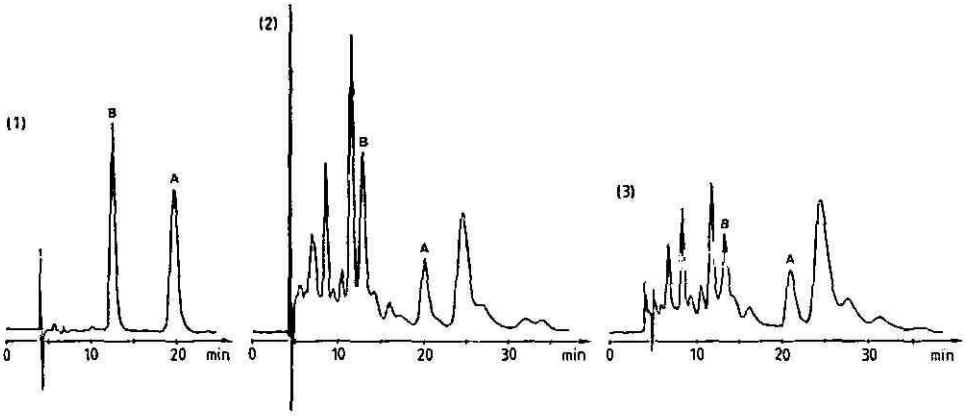

Fig. 1. HPLC chromatograms of Senna extracts. Column: LiChrosorb RP-8. Mobile phase: methanol–water *plus* PIC A (36.5 : 63.5); flow-rate 0.6 ml/min. Detection: UV, 270 nm. (1) Standard sennosides A and B; (2) aqueous extraction; (3) 70% ethanol extraction. Peaks: A = sennoside A; B = sennoside B.

wavelengths, 270 and 215 nm; the absorbance ratio for standard sennosides A and B is 3.11.

This analysis method can be used for a quantitation of sennosides A and B. Its linearity was tested for sennoside A or B concentrations up to 30 μg/ml, which corresponded with the areas for the concentrations under study.

The constitution of Senna extracts is very complex (Fig. 1). It can be modified according to the Senna part employed (leaves or pods) or by drying of the leaves (which has a great influence on the formation of sennosides)[19]. The pre-treatment on a Sep-Pak C_{18} cartridge that we propose allows chromatogram clarification and confirms the presence of sennosides in the sample, especially when they are present in low concentrations. However, such pre-treatment of Senna extracts has not often been described[18].

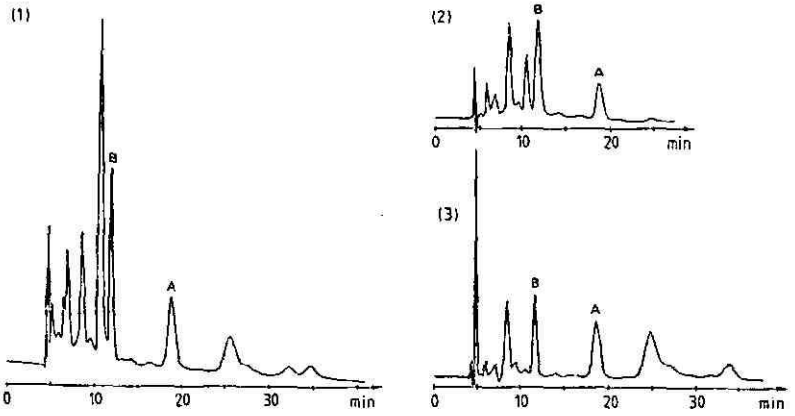

Fig. 2. HPLC chromatograms of Senna extracts. Conditions as in Fig. 1. (1) Without pre-treatment; (2) fraction 1 after pre-treatment with Waters Sep-Pak C_{18} cartridge; (3) fraction 2 after pre-treatment with Waters Sep-Pak C_{18} cartridge. Peaks: A = sennoside A; B = sennoside B.

Sennosides A and B were present in fractions 1 and 2 (Fig. 2). When these fractions were concentrated, traces of sennosides A and/or B could be detected after such simple and rapid pre-treatment.

Satisfactory results were obtained by the present procedure for the qualitative and quantitative determination of sennosides A and B in Senna extracts.

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CHROM. 19 000

Letter to the Editor

Comment on the observation of enhanced peak responses due to solvent interactions in high-performance liquid chromatography

Sir,

Perlman and Kirschbaum[1] have reported recently that ultraviolet detector response in liquid chromatography is dependent upon the solvent used for the analyte and the analyte's ability to form intramolecular hydrogen bonds. However, a number of points arise from their discussion that require comment.

(1) It is unfortunate that the paper does not supply sufficient experimental detail to facilitate easy repetition of their work. Thus no information is provided concerning the actual columns used, their dimensions or the specific packing and its particle size. Additionally, the details on solution concentrations are not fully explained.

(2) There appear to be more errors in the text than are consistent with simple typographical mistakes. Reference is made to the increase in areas *and* heights that occurs as solvent polarity increases yet the order quoted (water to methanol to ethanol) represents a decrease in polarity. Furthermore, Fig. 1 shows a decrease in height for the UV detected peaks going from water to ethanol and, if the area units are correct on the figure, a decrease in area. Simple mensuration of the peaks, assuming that they have been plotted at the same sensitivity, clearly shows that, in contrast to the published figure, areas are increasing. It is extremely difficult to judge the refractive index detector data since the positive going peak due to the solute suffers from interference from a negative going (solvent?) peak. However, the published figures on the diagram indicate a simultaneous reduction in both height and area.

(3) The proposition that a peak detected at 270 nm is directly due to ethanol is surely worthy of a much more detailed investigation than is evident in this paper.

(4) Fig. 4, which shows the responses for the disulphonamide of bendroflumethazide, surely contradicts the hypothesis presented. Assuming identical weights were used, the areas are sufficiently close (a 9% difference) that the difference could be accounted for by the difficulty in accurately integrating such a broad peak: no mention is made of how peak mensuration was carried out. The peak areas are thus approximately constant and height response has dropped, consistent with reduced chromatographic efficiency.

(5) The comment that peak retention times "often are identical" is too imprecise in a study of this nature to be valuable and warrents a more detailed investigation. Three of the solutes described (captopril, nadalol and methyl *para*-hydroxybenzoate) have thus been re-investigated since, if the hypothesis presented can be substantiated, it follows that a method of probing intramolecular hydrogen bonding becomes available. All separations were carried out using a Hewlett-Packard Model 1090A liquid chromatograph, equipped with a diode-array detector and on-board

TABLE I

DETECTOR RESPONSES FOR CAPTOPRIL, NADALOL AND METHYL *para*-HYDROXYBENZOATE SOLUTIONS

Conditions: 11 cm × 4.6 mm I.D. Partisphere C_{18} column eluted with methanol–water–orthophosphoric acid (50:50:1); 5 μl was injected.

Solute	*Solvent*	*Retention time (min)*	*k'*	*Detection (nm)*	*Area (ma.u. s)*	*Height (ma.u.)*
Captopril	Water	1.088	0.96	220 ± 10	37.35 C.V. 0.6%	10.10 C.V. 0.6%
	Methanol	1.078	0.94		38.24 C.V. 1.2%	9.41 C.V. 0.7%
Nadalol	Water	2.851	4.13	270 ± 10	11.44 C.V. 0.5%	1.007 C.V. 0.6%
	Methanol	2.831	4.09		12.03 C.V. 1.7%	1.013 C.V. 1.2%
Methyl *p*-hydroxy-benzoate	Water	1.338	1.41	270 ± 10	285.2 C.V. 0.5%	89.2 C.V. 0.6%
	Methanol	1.329	1.39		299.7 C.V. 2.0%	83.1 C.V. 1.1%

DPU integrator, at 40°C. Two columns were used, an 11 cm × 4.6 mm I.D. stainless-steel column packed with 5-μm Partisphere C_{18} (Whatman) and a 4.5 cm × 4.6 mm I.D. stainless-steel column containing 5-μm Ultrasphere ODS (Beckman). Mobile phases comprised methanol and water with 1% added orthophosphoric acid.

Table I shows the chromatographic data obtained with 5-μl injections of solutions of the three solutes in either methanol or water solution. The mobile phase was 50% methanol in water with 1% orthophosphoric acid and the flow-rate was 1.5 ml min^{-1}. Peak areas and heights are quoted in ma.u. s or ma.u., respectively, and have been adjusted to account for the actual weight of solute in solution (0.5 to 0.8 mg ml^{-1}). The chromatographic system was equilibrated until retention time coefficients of variation were below 0.1%. In contrast to the results presented earlier[1], it is seen that, for solutes dissolved in water, retention times are significantly increased compared to those in methanol and, whilst heights are slightly increased, areas stay constant or decrease. While the absolute changes in areas and heights are within experimental error, the area–height relationship changes are significant —particularly for captopril and methyl *para*-hydroxybenzoate. These solutes represent one each from the hydrogen bonding and non-hydrogen bonding groups.

The peak response phenomena could also be produced with the shorter Ultrasphere column. A mobile phase of methanol–water–orthophosphoric acid (40:60:1) was used with a flow-rate of 1 ml min^{-1}. As before, 5-μl injections of the same solutions of captopril were made. In water retention times, areas and heights for captopril solutions were 1.255 min, 50.9 ma.u. s (C.V. 1.0%) and 5.98 ma.u. (C.V. 1.7%), respectively. In methanol values were 1.230 min, 50.5 ma.u. s (C.V. 1.5%) and 6.18 ma.u. (C.V. 0.5%), respectively. There are no significant differences in peak areas or heights although retention times have changed as before.

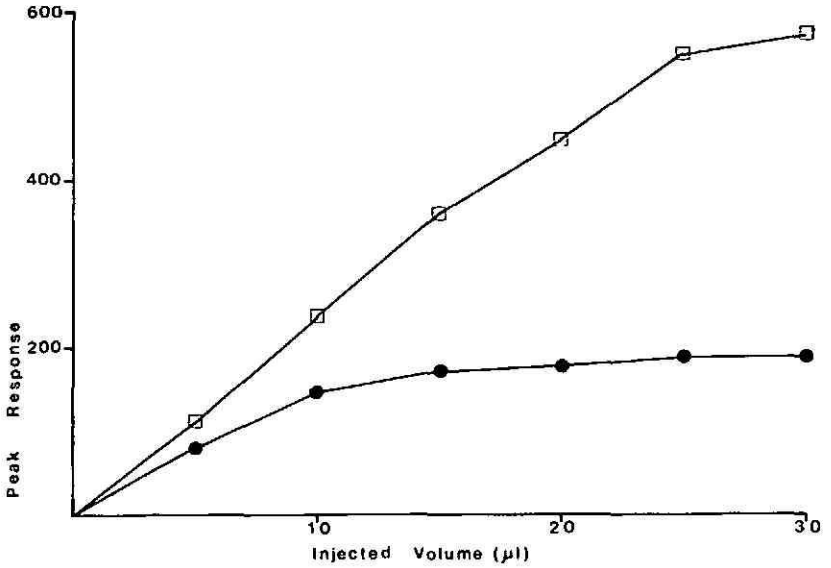

Fig. 1. Peak area and height responses for solution of captopril in methanol (0.8 mg ml^{-1}). A 4.5 cm × 4.6 mm Ultrasphere ODS column was eluted with methanol–water–orthophosphoric acid (40:60:1) at 1 ml min^{-1}. (□) Area, ma.u. s × 0.1; (●) height, ma.u.

To investigate the effect of mass of compound injected, injections of 5 to 30 μl were made using captopril in methanol. Linearity is lost above 10 μl and detector response now starts to follow the pattern described by Perlman and Kirschbaum[1]. Fig. 1 shows peak response as a function of injected volume while Fig. 2 compares a 25-μl injection of a solution of captopril in water with a 25-μl injection using methanol as solvent. The areas of the peaks are essentially identical.

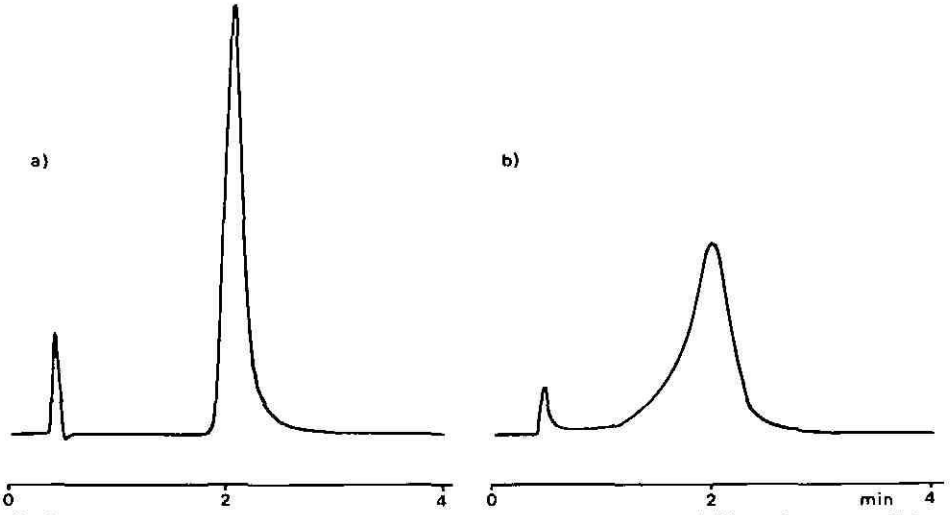

Fig. 2. Comparison of 25-μl injections of captopril solution in (a) water and (b) methanol. Conditions as for Fig. 1.

In conclusion, the proposition that intramolecular hydrogen bonding can affect peak response is a hypothesis that should be of interest to all chromatographers. Unfortunately, the original data presented cannot be considered to substantiate the hypothesis and it has proved impossible to reproduce the phenomena. May I suggest that this a subject for a more carefully controlled study which must include greater experimental detail and an investigation to ensure that instrumental artefacts are not influencing the conclusions?

ACKNOWLEDGEMENT

The interest of Mr. P. F. Wadsworth and Dr. D. V. Bowen of this department in this work is gratefully acknowledged.

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CHROM. 19 001

Letter to the Editor

Reply to comment on the observation of enhanced peak responses due to solvent interactions

Sir,

The gist of Dr. J. C. Berridge's letter[1] is that the phenomenon of solvent dependent peak responses of some compounds[2] in high-performance liquid chromatography (HPLC) could not be reproduced by him and that he suggests an instrumental artifact may be responsible for our data.

Most of our studies involved captopril and related compounds chromatographed on Whatman ODS-2 or Waters μBondapak octadecylsilane columns with a mobile phase of methanol–water–phosphoric acid (50:50:0.05) flowing at 1–2 ml/min. Absorbances were determined at 214 nm using Kratos Models 770 or 773 or Perkin-Elmer Model LC 85 detectors. Solutions were usually at a concentration of 0.1 mg/ml. Linearity was found from 0.05 mg/ml to 1 mg/ml. Since many compounds related to captopril, such as the disulfide and the S-acetyl derivative, do not show the peak response effect that captopril does, an instrument artifact is not likely.

Fortunately, this solvent-dependent peak response effect was observed in another HPLC laboratory. Fig. 1 shows chromatograms of 300 μg of the synthetic antimicrobial agent aztreonam and arginine dissolved in 1 ml of either aqueous phosphate buffer mobile phase or water. A silica column was used with detection at 206

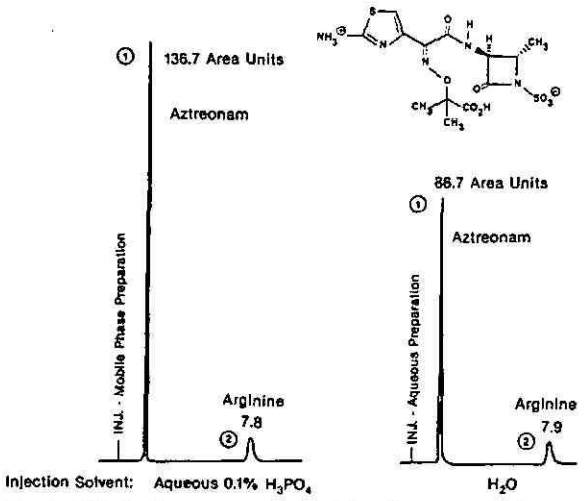

Fig. 1. HPLC of aztreonam and arginine dissolved in either aqueous 0.1% phosphoric acid (mobile phase) or water and chromatographed on a silica (μBondapak) column. Detection was at 206 nm.

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nm. The retention times and the response factors for arginine (peak height or area divided by concentration) were similar. However, the aztreonam peak areas and heights are different, with a response factor in water of 3.5 and in mobile phase of 5.4. When identical solvents were used, the responses for aztreonam were equivalent, thus providing independent confirmation of this effect.

The Squibb Institute for Medical Research, New Brunswick, NJ 08903 (U.S.A.)

J. J. KIRSCHBAUM
S. PERLMAN

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Book Review

Experiments in molecular biology, edited by R. J. Slater, Humana Press, Clifton, NJ, 1986, 416 pp., price US$ 27.50, ISBN 0-89603-082-2.

This book describes 25 well worked out experiments in molecular biology. The author deals with modern DNA and RNA handling techniques as well as with well established biochemical methods of protein and peptide analysis. Each of the 25 chapters starts with a discussion of theory followed by a detailed experimental procedure, including a list of the items that should be available before the experimental work begins. The theoretical part is sometimes (see, for example, the description of gel filtration) too simplified, but as the book is primarily aimed at the practical worker, this is not a real drawback.

In the first part of the book the handling of DNA is described. Chapter 1 describes an introductory, easily carried-out experiment of DNA isolation and transformation in *E. coli*. This is followed by an isolation procedure for plasmid DNA. Other techniques dealt with are: restriction endonuclease digestion and agarose gel electrophoresis of DNA, restriction site mapping, Southern blotting, extraction of nucleic acids from gels after preparative electrophoresis, microisolation and microanalysis of plasmid DNA.

In Chapters 12 and 13 the handling of RNA (*e.g.* extraction and affinity chromatography of mRNA) is described.

In the third part of the book, biochemical methods of protein and peptide analysis are dealt with (*e.g.* sodium dodecyl sulphate-polyacrylamide gel electrophoresis, isoelectric focusing in thin-layer polyacrylamide gels, thin-layer gel filtration and two-dimensional immunoelectrophoresis).

In conclusion, this is an excellent experimental guide to molecular biology, which can be recommended to everyone beginning in this field.

Sils-Maria (Switzerland) ALFRED KUHN

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CHROM. 19 038

Book Review

The chemist's English, by R. Schoenfeld, VCH, Weinheim, 1985, xii + 173 pp., price DM 42.00, US$ 17.95, ISBN 3-527-26309-8.

This book really was fun to read. That it is timely there can be no doubt as most editors of scientific journals regularly appeal to their authors to improve their literary skill. However, far from hearing these appeals, most authors dont even seem to read them, nor does the perusal of the instructions to authors make any difference, perhaps because these also are not read? Could it be that the main trouble is not the writing but the reading?

Now *if* you can induce scientists to read this book, they, their editors and the readers will undoubtedly benefit, but without wanting to cavill, one should remark that most of it would be incomprehensible to readers outside the English-speaking countries, though the sidelights it provides on the Aussie scientific culture are of definite importance to people not from New Zealand or Australia.

This reviewer is of the opinion that this small volume is an exhilarating addition to anyone's scientific library and should not be missed.

PUBLICATION SCHEDULE FOR 1986

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

MONTH	1985	J 1986	F	M	A	M	J	J	A	S	O	N	D
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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 362, No. 3, pp. 461–464. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

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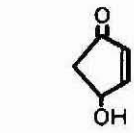

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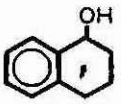
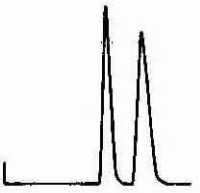
Detection: UV 210nm

CHIRALCEL OB





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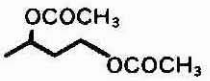
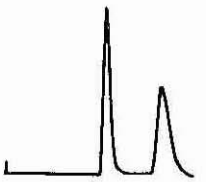
Detection: UV 210nm

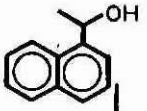
CHIRALCEL OB

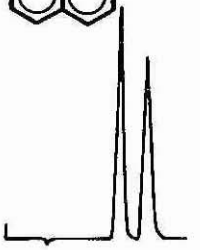




Eluent: hexane-2-propanol (9/1)
Flow rate: 0.5ml/min.
Detection: UV 210nm

CHIRALCEL OC





Eluent: hexane-2-propanol (9/1)
Flow rate: 0.5ml/min.
Detection: UV 254nm

For more details about HPLC columns, please contact:

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