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Modern Liquid Chromatography of Macromolecules

by **B.G. BELENKII** and **L.Z. VILENCHIK,** Institute of Macromolecular Compounds, Academy of Sciences of the USSR, Leningrad, USSR

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Researchers, technologists and students in the fields of physics, chemistry, biology and medicine will all find this book of interest. It considers the chromatography of high-molecular-weight compounds and its main theoretical and methodological features.

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Written mainly for the reader who has some practical experience in chromatography, the book also contains much useful information for the beginner.

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ANALYSIS OF THE *cis–trans* ISOMERIZATION KINETICS OF L-ALANYL-L-PROLINE BY THE ELUTION-BAND RELAXATION METHOD

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(First received August 12th, 1986; revised manuscript received January 20th, 1987)

SUMMARY

The "elution-band relaxation method" has been applied to the analysis of the *cis–trans* kinetics isomerization of the proline of L-Ala-L-Pro using reversed-phase liquid chromatography. A procedure suitable for cases where neither isomer can be injected separately is described.

INTRODUCTION

High-performance liquid chromatography (HPLC) is an indispensable tool in biochemistry and biophysics because of its high speed and resolution which cannot be attained by conventional chromatography[1]. Though it is usually used as a separation tool, we have developed methods to use it for conformational and kinetic analyses of molecules and have demonstrated clearly that HPLC has high potential for such applications[2–4]. We also affirmed that this potential can be fully exploited through the use of a micro-computer and mathematics.

We recently described the "elution-band relaxation method" for the analysis of reversible isomerization kinetics where the time constant is comparable to the time of elution[4]. The concept is as follows. Isomerizing molecules give different chromatograms for different flow-rates. When the flow-rate is high, the molecules have little time to isomerize and give a chromatogram consisting almost entirely of peaks due to the injected molecules. In contrast, when the flow-rate is low, the molecules have time to isomerize and give a broad band; this band relaxes and the first moment of the chromatogram shifts depending on the flow-rate. In our elution-band relaxation method the rate constants are obtained by analyzing this shift of the first moment. In the previous study[4] we applied the method to the denaturation–renaturation of protein using the size of the molecule as a probe.

Another approach to the study of isomerization using HPLC was developed by Horváth's group[5,6]. The mathematical basis of their method is quite similar to ours, however, the methods derived are different. They used two procedures to analyze isomerization kinetics: one is to evaluate the plate height contribution due to isomerization, and the other to simulate chromatograms by solving differential equations numerically.

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Separations of isomers of several compounds by reversed-phase liquid chromatography have been reported[7–9]; L-Ala-L-Pro is one such compound. The separation of its *cis* and *trans* isomers was reported by Horváth and co-workers[7], and the kinetics of isomerization was analyzed using the above methods[5,6]. In this study we analyzed this reaction by our elution-band relaxation method with a new procedure appropriate for the present case.

THEORETICAL

The procedures described in the previous study[4] assumed implicitly that each isomer can be injected separately, or that one of the isomers is stabilized under a certain solvent condition. In the present case of the *cis–trans* isomerization, however, this assumption is not valid. Therefore a new procedure must be used as follows.

The reaction under consideration is:

$$A \underset{k'}{\overset{k}{\rightleftarrows}} B$$

The conclusions of our previous study were: (1) in HPLC the diffusion terms have a negligible contribution to the first moment, and (2) the first moment of the chromatogram is expressed in terms of the scaling time, t_S, given by $t_S = l/v$, where l is the column length and v is a velocity or an analogous characteristic of operation, *e.g.*, the flow-rate. The first moment of a chromatogram obtained by injecting isomer A, given as the scaled moment [equal to $M_{1A}(t_S)/t_S$ in ref. 4], applies directly to the first moment of the elution volume when t_S is changed by changing v for a single column

$$M_{1A}(t_S) = \alpha_A[1-\exp(-\beta t_S)]/t_S+\gamma \tag{1}$$

where α_A, β and γ are functions of the velocities of the isomers and the rate constants (see eqns. 18–20 in ref. 4). The same quantity for B is:

$$M_{1B}(t_S) = \alpha_B[1-\exp(-\beta t_S)]/t_S+\gamma \tag{2}$$

When a mixture of the isomers is injected, the first moment is the sum of their contributions

$$M_1(t_S) = \alpha^*[1-\exp(-\beta t_S)]/t_S+\gamma \tag{3}$$

where $\alpha^* = f_A\alpha_A+f_B\alpha_B$ and f_A and f_B are the fractions of A and B injected. By measuring $M_1(t_S)$ for various values of t_S (usually by changing the flow-rate), α^*, β and γ can be determined. The rate constants are determined by β and γ in combination with the peak elution times of the isomers (see eqns. 19 and 20 in ref. 4)

$$k = \frac{\alpha t_{SO}(\gamma t_{SO}-t_A)}{t_A(t_B-t_A)} \tag{4}$$

$$k' = \frac{\alpha t_{SO}(\gamma t_{SO}-t_B)}{t_B(t_A-t_B)} \tag{5}$$

where t_A and t_B are the respective peak times of A and B in the chromatogram the scaling time of which is t_{SO}; t_A and t_B are used as an approximation in order to obtain the unknown velocities of A and B. For this approximation to be valid, t_A and t_B must be obtained from a chromatogram where the reaction extent is so small that two separate peaks are observed.

We can determine also the isomer fraction of the sample by use of the determined parameters:

$$f_A = \frac{(\alpha^*\beta+\gamma)t_{SO}-t_A}{t_B-t_A} \tag{6}$$

EXPERIMENTAL

L-Ala-L-Pro was obtained from Sigma (St. Louis, MO, U.S.A.). A few hours before the experiment it was dissolved in buffer to give a 0.1% solution. An ODS-120T (10 μm) column (7.5 cm × 4.6 mm I.D.) was kindly provided by Toyo Soda (Tokyo, Japan). An HLC-803D pump (Toyo Soda) with a 2.7-μl sample loop was employed. The absorbance at 210 nm was monitored with a detector UV-8 (Toyo Soda), A/D converted by a micro-computer and stored on a flopy disk. Flow-rates were determined from the positions of ghost peaks by taking the value of 0.5 ml/min as standard. Least squares analysis was done by use of the program SALS of the Computer Center of the University of Tokyo[10].

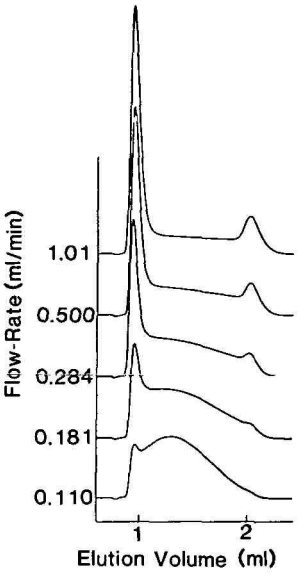
RESULTS AND DISCUSSION

The reaction was analyzed at pH 6.0 and 20°C. The pH was that of the eluent, however, the compound was dissolved in 50 m*M* phosphate, pH 2.4, equilibrated and injected. To obtain a precise value of β, α^* should be as large as possible (see eqn. 3). When the injection mixture is equilibrated in the eluent, α^* is equal to zero; it is necessary to shift the equilibrium towards one of the isomers. Therefore the pH of the sample was made low, because the *trans* isomer of L-Ala-L-Pro was reported to predominate at low pH[7,11].

Fig. 1 shows some of the chromatograms obtained for various flow-rates. Contributions from volumes other than that of the column itself, *e.g.*, connection tubes, detector cell, were subtracted.

These volumes make no contribution to the separation and only increase the first moment by a constant value (25 μl here). Fig. 1 confirms the observations of Melander and co-workers[6,7]: two peaks occur for a high flow-rate, and the lower the flow-rate the greater the chromatogram relaxes. According to their assignment the left peak is due to the *trans* and the right to the *cis* isomer.

The first moment of the elution volume is plotted *versus* the inverse of the flow-rate in Fig. 2, *i.e.*, M_1 (t_S) *vs.* t_S. From the results of the least squares analysis and by use of t_A, t_B and t_{SO} obtained from the chromatogram at the flow-rate of 1.01 ml/min, eqns. 4 and 5 give the rate constants of 0.445 ± 0.049 min^{-1} for the *trans* to *cis*, and 0.290 ± 0.032 min^{-1} for the *cis* to *trans* isomerization. We can also evaluate the isomer fraction of the injected sample by use of eqn. 6; for the *trans* isomer at



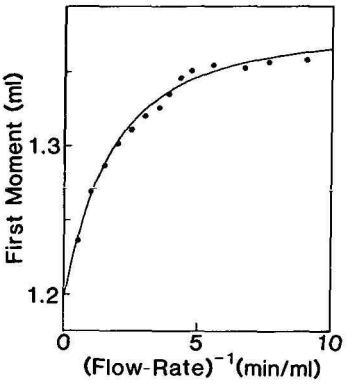


Fig. 1. Examples of chromatograms of L-Ala-L-Pro. Column: ODS-120T (10 μm, 7.5 cm × 4.6 mm I.D.). Eluent: 50 m*M* phosphate, pH 6.0, 20°C. Detection: UV, at 210 nm.

Fig. 2. Plot of first moment *vs.* inverse flow-rate. The curve is the best fit line (see eqn. 3 of text).

pH 2.4 this fraction is calculated as 0.76, *cf.*, 0.89 for the cationic form of L-Ala-L-Pro obtained by NMR spectroscopy[11].

Now we examine the results according to the scheme presented by Melander *et al.*[5]. They considered isomerization in both the mobile and stationary phases. Although our model equations (eqn. 1, 2 in ref. 4) involve only two rate constants, they can be shown to be identical to the "governing differential equations" (eqn. 10 in ref. 5) of Melander *et al.*[5]:

$$c_1 + c_2 = (1 + k_A)c_2 = \rho_A$$

$$c_3 + c_4 = (1 + k_B)c_3 = \rho_B$$

$$u_0/(1 + k_A) = v_A,\ u_0/(1 + k_B) = v_B$$

$$k_2/(1 + k_A) = k,\ k_3/(1 + k_B) = k'$$

The quantities on the left-hand sides are expressed in the notation of Melander *et al.*[5]; k_A and k_B are the capacity factors of molecules A and B. The equilibrium constant in the mobile phase is given by $K_m = k_2/k_3$ (eqn. 12 in ref. 5); in our notation it is:

$$K_m = t_A k / t_B k'$$

With our rate constants, k and k', this relationship yields the fraction 0.41 for the *cis* isomer *cf.*, 0.35 by NMR spectroscopy[11]. With the knowledge of k_A and k_B, and of the rate constants in solution[12], one may discuss the rate constants in the stationary phase just as Jacobson *et al.*[6] have done.

Next, we consider the advantages and disadvantages of our method in comparison with those of other researchers[5,6]. The major advantage of our method is its simplicity. This is attained by focusing on the first moment and by manipulating the equilibrium of the mixture injected. The first moment is easier to measure precisely than the moments of higher orders, and a method using a higher order moment must deal with intrinsic band spreading; the procedure is fairly complex[6]. Without the manipulation of the equilibrium, the first moment would never relax and one would have to resort to another means. Conversely, this is a drawback of our method. There are two other disadvantages. One is the use of t_A and t_B. Because our method employs only the first moment, the velocities of the isomers, or the capacity factors, must be obtained independently. This leads to a restrictive condition mentioned in the Theoretical section: a chromatogram of small reaction extent is indispensable. The other disadvantage is concerned with the different extinction coefficients of the isomers. When the isomers have different extinction coefficients, eqn. 3 is invalid and the expression of the first moment is a little bit complex. The simulation method[6] is free from this problem though it has a trial-and-error character. The important point is that our method can extract as much information as other methods, while at the same time being very simple.

Another method described by Lebl and Gut[8] has one weak point. According to their discussion, the peaks observed correspond to the remainder of the starting compound, and the plateau between them corresponds to the mixture of isomers generated during the time of separation. However, the peaks do contain isomers generated during that time. Under their peak B (see Fig. 1 in ref. 8), for example, not only is there the remainder of species B, but also A generated just before elution, B generated from A just after injection and A and B interconverted several times.

In conclusion, we have demonstrated that the elution-band relaxation method is applicable to reactions whose time constants are approximately 1 min. For the present, the procedure described here together with the two presented previously completes our methodology. Further development of the method may include its application to isomerizations comprising more species, and more rapid separation of isomers with sufficient resolution will enlarge the application time domain of the method.

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CHROM. 19 448

COMPARISON OF OCTADECYL-BONDED SILICA AND STYRENE–DIVINYLBENZENE COPOLYMER SORBENTS FOR TRACE ENRICHMENT PURPOSES

FUNDAMENTAL ASPECTS I. CALIBRATION OF THE STATIONARY PHASES IN PURE WATER

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SUMMARY

An octadecyl-bonded silica (Partisil ODS-3) and a styrene–divinylbenzene copolymer (PRP-1) with comparable particle sizes, average pore diameters and specific pore volumes were investigated for trace-enrichment purposes. The organic resin leads to systematically higher values of the logarithm of the capacity factor, log k', whatever the composition of the methanol–water eluent and whatever the solute investigated. Lower retention differences were observed for two alcohols. Accordingly, the correlation of experimental log k' values obtained for a given eluent composition with calculated hydrophobic log P coefficients leads to one regression line for the C_{18} material and two lines (for hydroxylated and non-hydroxylated solutes respectively) for the organic resin. This also applies to the calibration graphs of log P with the weighted linearly extrapolated log k'_w values in pure water. A weaker affinity of the copolymer for hydroxylated compounds can generally account for all the experimental results.

INTRODUCTION

In the past fifteen years, great progress has been made in both the practice and theory of reversed-phase liquid chromatography (RPLC). Alkyl-bonded silica packings have become the most popular stationary phases for liquid chromatography as

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they allow the investigation of a great variety of compounds covering a wide polarity range. However, these extraordinary developments must not be allowed to obscure the interesting possibilities offered by RPLC as an extraction technique.

This so-called trace-enrichment chromatography takes advantage of the retentive power of non-polar sorbents to extract compounds from dilute aqueous solutions and includes two steps. The first one consists in passing a large volume of water sample through an extraction column. During the second step, the retained compounds are eluted with a small volume of an organic solvent.

Until now, trace-enrichment experiments have been performed almost exclusively in order to study the organic pollution of potable[1,2], river-[3,4] or wastewaters[5,6]. Many papers also reported on the recoveries of test solutes under a given set of experimental conditions and on the influence of some parameters upon the experimental results[7–18]. Most of them deal with organic copolymers of the well known polystyrene–divinylbenzene (PS-DVB) type as extracting sorbents. Until a few years ago, organic resins were available as cheap coarse particles suitable for extraction purposes. On the contrary, alkyl-based stationary phases were less often used and the great majority of published papers concerned analytical investigations of the retention mechanism(s) on those bonded supports. To our knowledge these two kinds of non-polar media have rarely been compared[19,20].

Moreover, as the various studies of trace-enrichment chromatography involved different experimental conditions[14,20–24], no quantitative conclusions can be drawn. Finally, very few authors[25] tackled the problem of the prediction of breakthrough volumes for extraction purposes and the published results apply only to the solutes studied.

The present study is therefore designed to compare both kinds of non-polar stationary phases for the recovery of aroma compounds contained in the effluents from food industries. We also intend to define an "easy-to-apply" calibration procedure for RPLC systems in pure water so that the breakthrough volume of any solute may be estimated.

To achieve this twin goal, one must bear in mind that, when the spreading of the migrating front in the column is neglected, the maximum sample volume that can be passed through a given column before leakage occurs corresponds to the breakthrough volume determined by frontal analysis in pure water. Whether trace-enrichment experiments lead to quantitative recovery of the solutes thus mainly depends on the magnitude of the capacity factor in a totally aqueous eluent, k'_w. As k'_w values are hardly accessible experimentally because of the excessive retention of the solutes in totally aqueous eluents, a reliable method must be employed to estimate them. This generally consists in linear[26–29] or quadratic[30–33] extrapolation of the variations in the logarithm of the capacity factor, $\log k'$, with the mobile phase composition to pure water.

Lastly, one must find a proper parameter such as a physico-chemical constant that can be correlated to the capacity factor of the compounds and enable the *a priori* estimation of k'_w. Among the parameters usually advocated as being useful for the prediction of retention in RPLC[34], the most appropriate is undoubtedly the octanol–water partition coefficient, P (or $\log P$). This constant, which characterizes the molecular behaviour governed by solute distribution between two hydrophilic and hydrophobic regions, is accessible in two main ways. First is the experimental

determination, known as the conventional shake-flask method, which has several disadvantages[35,36]. Second is the *a priori* calculation according to the method developed by Rekker[37] and elaborated from the earlier works of Hansch and co-workers[38–40]. The many reported experiments using alkyl-bonded silica and various organic solvents mixed with water enable the following conclusions to be drawn.

The characteristics of the linear $\log k' = A \log P + B$ correlation may vary with the nature and the composition of the chromatographic eluent as well as the nature of the solutes, but the number of outliers in the regressions are much less numerous when:

calculated log P values are involved rather than those measured by the traditional method[41,42] or even those calculated according to Hansch's method[43];

methanol is used as co-solvent rather than any other organic modifier such as ethanol, acetonitrile or tetrahydrofuran[35,42,44,45];

the water content in the eluent increases[46–49], the highest correlation coefficients being obtained when retentions in pure water, whether estimated or measured, are involved[27–29,35,48].

This first part of the study therefore concerns the retention of model aroma compounds in various methanol–water eluents, the estimation of the corresponding k'_w in pure water and the correlation of all retention values to the hydrophobic octanol–water coefficient.

EXPERIMENTAL

Apparatus

All experiments were carried out using a liquid chromatograph assembled from a Model 6000 A pump (Waters Assoc., Milford, MA, U.S.A.), a Valco six-port sampling valve with a 20-μl injection loop, a Spectromonitor III variable wavelength UV spectrophotometer (LDC, Riviera Beach, FL, U.S.A.) and a R401 differential refractometer (Waters Assoc.). The column, mobile phase and refractometer cells were thermostatted at 25.0 ± 0.1°C using a circulating water-bath.

Solvents and chemicals

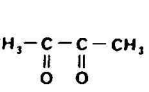
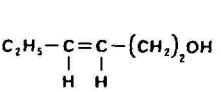
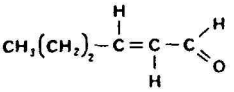
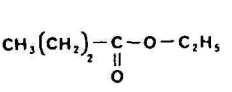
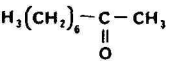
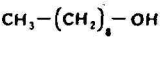
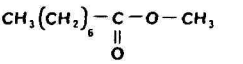
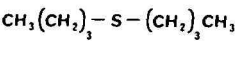
HPLC-grade methanol was obtained from Prolabo (Paris, France). Ultra-pure water was obtained from a purification line consisting of RO-4 and Milli-Q systems (Millipore, Bedford, MA, U.S.A.) mounted in series. Mixtures of methanol and water were degassed by filtration through a 0.45-μm membrane (Millipore) before use.

Chemicals, chosen among hundreds of aroma compounds in order to represent various chemical classes, were tested for purity by gas chromatography. 2,3-Butanedione (> 99.5%), ethyl butanoate (> 99.5%), 2-nonanone (> 99.5%), methyl octanoate (> 99%) were obtained from Fluka (Buchs, Switzerland), *cis*-3-hexen-1-ol (> 99.5%), *trans*-2-hexenal (> 99%), 1-nonanol (> 99.5%) from Interchim (Montluçon, France) and dibutyl sulphide (> 99.5%) from K & K (Plainview, N.Y., U.S.A.). The formulae of these model aroma compounds are shown in Table I.

Samples were prepared as individual solutions from 10^{-5} to 10^{-3} M whenever possible in each mobile phase under investigation or in a water-leaner eluent.

TABLE I

STRUCTURES OF THE AROMA COMPOUNDS STUDIED

			
1	2	3	4
2,3-Butanedione	*cis*-3-Hexen-1-ol	*trans*-2-Hexenal	Ethyl butanoate
			
5	6	7	8
2-Nonanone	1-Nonanol	Methyl octanoate	Dibutyl sulphide

Columns

The stainless-steel columns were 15 cm × 4.1 mm I.D. pre-packed with PRP-1 polystyrene–divinylbenzene copolymer (Hamilton, Reno, NV, U.S.A.) and 15 cm × 4.7 mm I.D. packed with commercially available Partisil ODS-3 octadecyl-bonded silica (Whatman, Clifton, NJ, U.S.A.) according to the well known slurry technique. The Partisil ODS-3 and PRP-1 packings have comparable particle sizes, average pore diameters and specific pore volumes (Table II). The C_{18} silica was bonded with trichlorooctadecylsilane then end-capped with trimethylchlorosilane. As stated by the supplier, this leads to a material of polymeric nature with 10.5% (w/w) carbon.

Chromatographic procedure

Provided that the extracting support is not overloaded, which is *a priori* the case because of the usually very low concentration of aroma compounds in natural products and therefore much lower concentration in food industry effluents, the retention volume determined by the S-shaped curve obtained by frontal analysis exactly corresponds to that of the Gaussian-shaped peak observed in the conventional injection technique. Consequently, the experiments reported in this study were performed according to the latter method.

The methanol–water eluent was pumped isocratically at a flow-rate of 2 ml/min, which was continuously measured. The absorbance of the effluent was monitored at the wavelength of maximum absorbance for each compound.

TABLE II

MAIN CHARACTERISTICS OF THE STATIONARY PHASES STUDIED

Characteristic	*Partisil ODS-3*	*PRP-1*
Nature	Octadecyl silica	PS-DVB copolymer
Particle size (μm)	10 ± 1	10 ± 2
Specific surface area (m^2/g)	350	415
Average pore diameter (Å)	85	75
Specific pore volume (ml/g)	0.85	0.79
pH range	[2; 8]	[1; 13]

Capacity factors were calculated from the retention volume, V_R, of each solute:

$$k' = (V_R - V_0)/V_0$$

The dead volume, V_0, was obtained for each eluent composition and both columns by measuring the elution time of 10^{-2} M potassium nitrate. Each value is the mean of at least three replicate determinations.

RESULTS AND DISCUSSION

Comparison of retention on the two sorbents

Capacity factors of aroma compounds were determined on each stationary phase. The volume content of water in the eluent, x, varied from 0 (pure methanol) to 1 (pure water). Fig. 1 presents the variations in log k' with x for each solute and both sorbents. The graphs are numbered from 1 to 8 according to the elution order of the solutes on the octadecyl bonded silica.

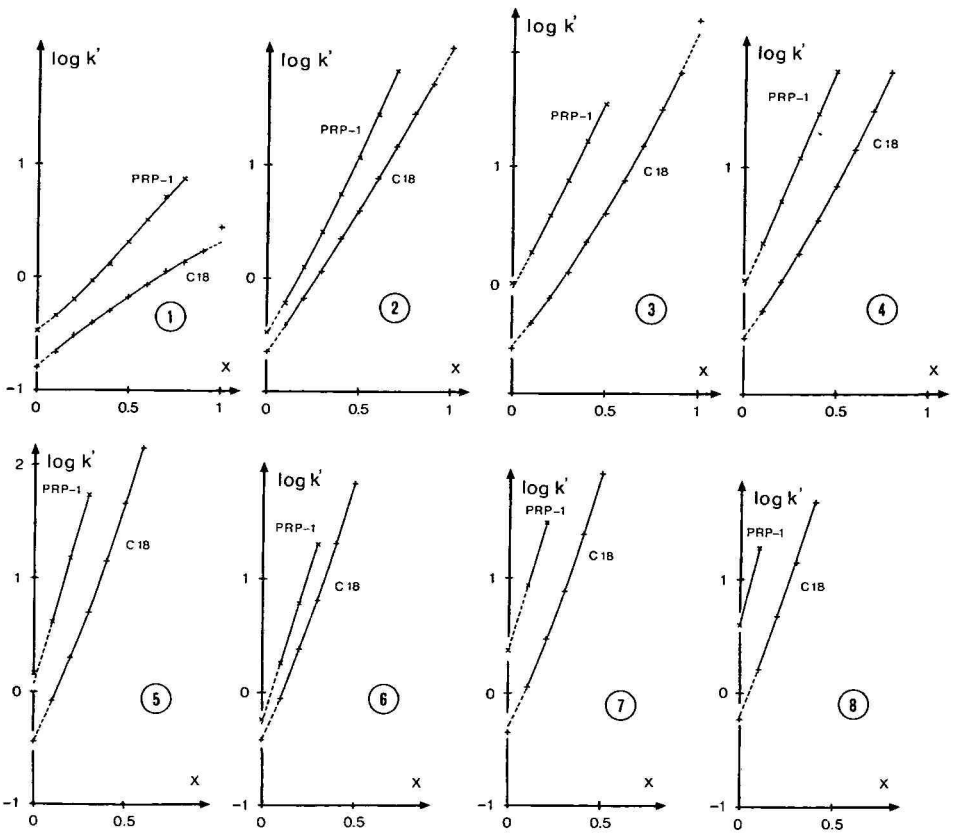


Fig. 1. Change in the capacity factor with increased volume content of water in the eluent, x, for eight model aroma compounds on two hydrophobic sorbents. Columns: (C_{18}) 10-μm Partisil ODS-3 and 10-μm PRP-1. Mobile phase: methanol–water; flow-rate: 2 ml/min. Detectors: UV absorbance and refractive index at 25°C. Volume injected: 20 μl. Solute identification: see Table I.

Experimental retentions on the organic polymer are higher than those on the C_{18} material, whatever the eluent composition and the solute studied:

$$k'_{PRP\text{-}1} > k'_{C_{18}} \quad (1)$$

However, the difference $\Delta k' = k'_{PRP\text{-}1} - k'_{C_{18}}$ is smaller for compounds 2 and 6 in comparison with the other solutes. These two compounds belong to the same chemical class, that of alcohols:

$$\Delta k'(\text{alcohols}) < \Delta k'(\text{non-alcohols}) \quad (2)$$

The first observation clearly shows the superiority of the PRP-1 material for trace-enrichment purposes as larger sample volumes can be treated before breakthrough occurs from columns of comparable dimensions. In other words, a given compound is quantitatively extracted by a smaller quantity of resin compared to bonded silica. This remark is of course valid only for the two stationary phases investigated in this study. The differences in k' would undoubtedly have been smaller if an octadecyl-bonded silica with an higher carbon loading or an organic copolymer with a lower specific surface area had been studied. Nevertheless, we investigated intermediate packings in both classes of reversed-phase media. The specific surface area for PRP-1 is between those of the well known XAD-2 (300 m^2/g) and XAD-4 (780 m^2/g) resins and so is the average pore diameter (75 Å, between 50 and 90 Å respectively). The specific surface area for alkyl-bonded silica usually varies from 150 to 600 m^2/g and carbon loading from 3 to 20%. Partisil ODS-3 is an intermediate packing with 10.5% carbon loading and a specific surface area greater than 350 m^2/g. Besides, (much) higher retentions on PS-DVB resins than on octadecyl-bonded silica with different characteristics than those studied here have already been reported for various compounds and eluents[19,20,25,49–51]. Though it cannot be concluded that PS-DVB copolymers exhibit an higher retentive power than octadecyl-bonded silica as a rule, we are inclined to regard this statement as a general tendency.

Another helpful retention parameter is the selectivity of an LC system, defined for two solutes 1 and 2 by:

$$\alpha_{1,2} = k'_2/k'_1$$

As high retention and high selectivity often go together, higher selectivity is expected, under identical conditions, for the organic resin. Table III lists the selectivities of the two stationary phases, assessed relative to 2,3-butanedione for various water contents in the mobile phase. Except for alcohols, the higher selectivity of PRP-1 is verified for all values of x. For the two alcohols (compounds 2 and 6) PRP-1 exhibits an higher selectivity than C_{18} only at higher water contents ($x > 0.7$ for compound 2 and $x > 0.2$ for compound 6). Fig. 2 compares the selectivities, relative to ethyl butanoate, of both stationary phases for all solutes tested and the eluent with $x = 0.1$. All but the hydroxylated model solutes lie on a straight line. The characteristics of the corresponding lines are almost independent of which compound (except alcohols) is involved as reference in the selectivity calculations, as shown below:

TABLE III

LOGARITHMS OF THE SELECTIVITIES, RELATIVE TO 2,3-BUTANEDIONE, OF BOTH RPLC SYSTEMS FOR VARIOUS WATER CONTENTS IN THE ELUENT, x

	x										
	0.0	*0.1*	*0.2*	*0.3*	*0.4*	*0.5*	*0.6*	*0.7*	*0.8*	*0.9*	*1.0*
Partisil ODS-3											
cis-3-Hexen-1-ol	0.15	0.26	0.34	0.47	0.65	0.78	0.95	1.11	1.32	1.49	1.60
trans-2-Hexenal	0.20	0.29	0.36	0.48	0.64	0.77	0.95	1.13	1.37	1.59	1.84
Ethyl butanoate	0.29	0.40	0.51	0.65	0.83	1.01	1.22	1.44	1.70		
2-Nonanone	0.40	0.59	0.82	1.11	1.45	1.83	2.21				
1-Nonanol	0.38	0.62	0.90	1.22	1.61	2.01					
Methyl octanoate	0.45	0.72	0.99	1.30	1.69	2.10					
Dibutyl sulphide	0.57	0.88	1.20	1.56	1.98						
PRP-1											
cis-3-Hexen-1-ol	0.01	0.12	0.30	0.45	0.64	0.75	0.93	1.12			
trans-2-Hexenal	0.44	0.59	0.77	0.92	1.11	1.24					
Ethyl butanoate	0.47	0.67	0.90	1.12	1.36	1.53					
2-Nonanone	0.63	0.96	1.38	1.77							
1-Nonanol	0.23	0.60	0.99	1.34							
Methyl octanoate	0.84	1.28	1.69								
Dibutyl sulphide	1.07	1.62									

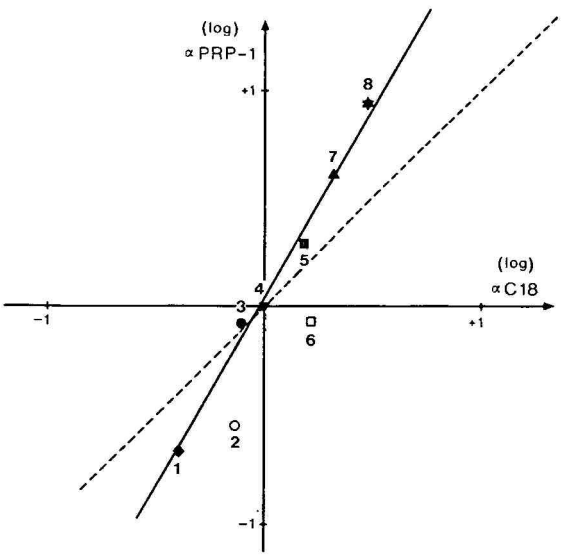


Fig. 2. Comparison of the selectivities of the octadecyl-bonded silica (α C_{18}) and of the PS-DVB copolymer (α PRP-1) relative to ethyl butanoate; co-ordinates are logarithmic. The broken line represents equal selectivity. Mobile phase: methanol–water (90:10, v/v). See Table I for solute identification.

Reference compound	*Slope*	*Intercept*	*Correlation coefficient*
Dibutyl sulphide	1.78	−0.05	0.994
Ethyl butanoate	1.78	0.04	0.994
2,3-Butanedione	1.78	0.00	0.994

Accordingly, the selectivities of both sorbents for methanol–water (90:10, v/v) as the eluent and all but hydroxylated aroma compounds are related according to:

$$\alpha\text{PRP-1} \approx (\alpha C_{18})^{1.8}$$

The high selectivity of a sorbent may not be satisfactory for some particular trace enrichment purpose, *e.g.* when both the quantitative recovery of a given compound and the lowest distorsion in the distribution of the solutes present in the sample before extraction are required. Then the C_{18} material may be preferred. In most cases, however, this shortcoming exhibited by the PRP-1 packing is economically counterbalanced by its higher retentive power.

From the open symbols for solutes 2 and 6 in Fig. 2 it is seen that both alcohols exhibit a particular chromatographic behaviour, *i.e.*, they are too strongly retained by the octadecyl-bonded silica or too weakly by the organic resin. Though the specific interaction of the hydroxyl moiety of these solutes with residual silanol sites of the C_{18} material can never be excluded, it is probably of limited importance, however, as the Partisil ODS-3 is an end-capped packing and the number of such sites is minimized. Furthermore, the silanols are covered with water molecules as soon as the water content in the eluent exceeds a few per cent, which prevents their direct interaction with the alcohols. The behaviour of compounds 2 and 6 must therefore be attributed in large part to the organic resin.

Relationship between log k' and log P

The log *P* coefficients for model aroma compounds were calculated according to Rekker's method (see Table VI). As the hydrophobic contribution of the –CO–CO– fragment is unknown, log *P* for 2,3-butanedione could not be estimated. Accordingly, the regression lines in Fig. 3 correlate the log *P*, log *k'* data points for compounds 2–8 and various mobile phase compositions. The characteristics of the least-squares linear regressions are given in Table IV. The regression coefficients, *r*, are quite satisfactory for retention on the C_{18}-bonded silica. For the PS-DVB copolymer, solutes 2 and 6 are outliers. Indeed, when all compounds are considered in the regressions, the *r* values are very poor ($0.7 < r < 0.8$) but they are satisfactory values when compounds 2 and 6 are excluded. Separate lines, for alcohols (broken lines in Fig. 3b) and non-alcohols (full lines in Fig. 3b) respectively, must therefore be drawn.

The results argue in favour of a weaker interaction of alcohols with the PS-DVB in comparison to the C_{18} material and to other solutes, no particular role being attributed to the mobile phase. We also studied the retention of alcohols having an aliphatic skeleton, and Smith[52] previously reported on aromatic compounds and two PS-DVB resins with methanol–water and acetonitrile–water eluents. Using retention indices based on an homologous series of alkyl aryl ketones, it was clearly that, in contrast to previous studies on silica-based columns, two hydroxylated compounds

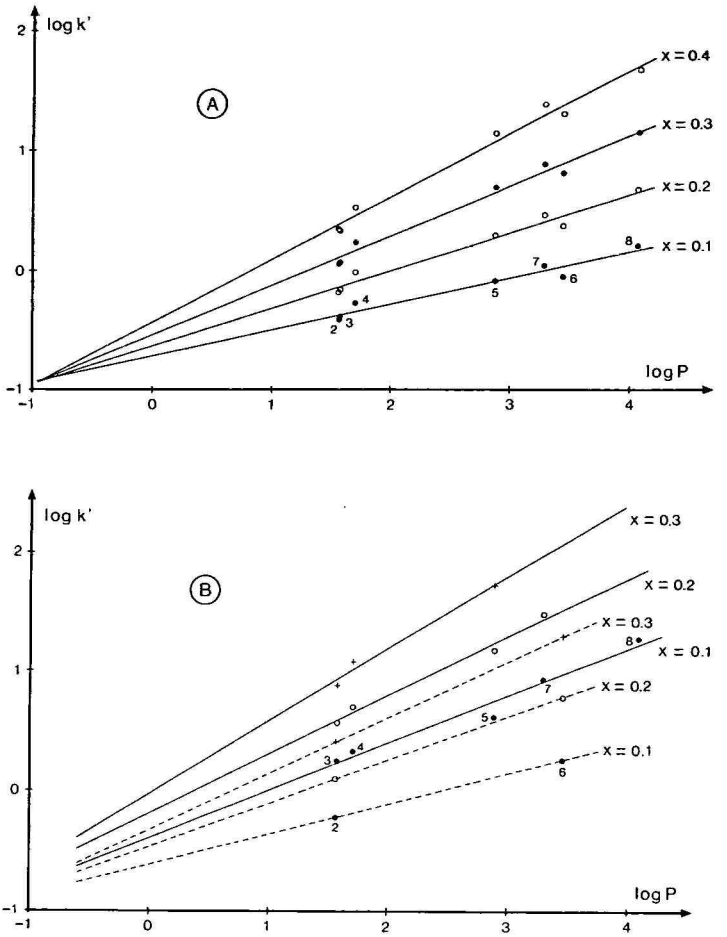


Fig. 3. Correlation between the octanol–water partition coefficient log P and log k' determined on both RPLC systems; (A) Partisil ODS-3; (B) PRP-1. The full lines in (B) correspond to all but hydroxylated solutes and the broken lines to alcohols. See Table I for solute identification.

TABLE IV

RESULTS OF THE LEAST-SQUARES LINEAR REGRESSION log k' = a log P + b FOR BOTH RPLC SYSTEMS AND VARIOUS WATER CONTENTS IN THE ELUENT, x

x	*Partisil ODS-3*				*PRP-1**			
	a	b	N**	r	a	b	N**	r
0.1	0.22	−0.71	7	0.97	0.40	−0.39	5	0.98
0.2	0.32	−0.63	7	0.985	0.50	−0.18	4	0.992
0.3	0.42	−0.54	7	0.990	0.61	−0.02	3	0.991
0.4	0.53	−0.43	7	0.992				

* Alcohols 2 and 6 are excluded.
** Number of data points.

(*p*-cresol and 2-phenylethanol) were less strongly retained on the polymer columns compared to the other solutes. It therefore makes no difference whether the hydrocarbonaceous skeleton of alcohols is aliphatic or aromatic, and the particular chromatographic behaviour of these solutes is due to their hydroxyl moiety. A possible explanation of the observed phenomenon lies in the different nature of the two hydrophobic materials investigated. PS-DVB copolymers are totally hydrocarbonaceous media and thus exhibit an homogeneous chemical structure. Non-polar bonded silica is more complex because alkyl chains as well as ≡Si–O–Si≡, ≡Si–O–C≡ and ≡Si–OH heteroatom-containing moieties co-exist in the material. Though one can assume octadecyl chains to have a somewhat lower solubility parameter ($\delta_{\text{octadecane}} = 8.04$)[53] than PS-DVB resins ($\delta \in [8.5; 9]$)[54]; δ for octadecyl-bonded silica is therefore definitely greater than 8 $cal^{1/2}/cm^{3/2}$ and mixtures of methanol ($\delta = 14.5$) and water ($\delta = 23.4$) solvate the silica-based material better than the copolymer. Accordingly, direct interactions between solutes and the PS-DVB resin are favoured in comparison with the C_{18} packing, which may explain the systematically higher retentions observed for all compounds investigated. If one regards the weaker affinity of the resin for methanol as evidence of a general tendency, the lower retention of hydroxylated solutes may be accounted for as well.

Relationship between extrapolated log k'_w and log P

The major interest in the octanol–water partition coefficient is that it quantifies the hydrophobicity of various compounds. Therefore, log *P* is *a priori* a proper descriptor of the phenomena which govern solute retention in RPLC systems using totally aqueous mobile phases. In the scope of this work, focusing on such a consideration implies that the capacity factors of the solutes in pure water are known and raises the question of their estimation from the log $k' = f(x)$ graphs in Fig. 1.

Each set of experimental x, log k' data was tested for least-squares regressions over the $0.1 \leqslant x \leqslant 0.9$ range. As log k' variations with x (or $1 - x$) are often reported to be linear when using methanol–water eluents with alkyl-bonded materials, the function $\log k' = ax + b$ was first investigated and the results were quite satisfactory ($r \geqslant 0.998$). However, the graphs in Fig. 1 become slightly curved as x increases and the quadratic function $\log k' = ax^2 + bx + c$ fits the experimental data better with $r \geqslant 0.9996$, or at least as well as the linear one. Finally, the variations in retention for 2,3-butanedione on PRP-1 are more accurately fitted by the power equation $\log k' = ax^n + b$ which accounts for the concavity of the somewhat S-shaped graph (curve 1).

Whatever the best mathematical function $\log k' = f(x)$, k'_w may significantly differ from the value predicted by the corresponding equation. For 2,3-butanedione, *cis*-3-hexen-1-ol and *trans*-2-hexenal on the C_{18}-bonded silica, positive differences are noticeable between the experimental k'_w and the quadratically extrapolated value for $x = 1$ (broken lines of graphs 1–3 in Fig. 1). Positive as well as negative differences may generally be observed[31] but neither their sign nor their magnitude can be predicted. The accuracy of the estimation of k'_w by extrapolation of the log $k' = f(x)$ curves may therefore be very poor as shown by several examples in Table V. For some solutes (aniline, biphenyl, dimethyl phthalate), the quadratic functions lead to retention volumes in better agreement with the experimental values than are those obtained by the regression lines. Nevertheless, these quadratic extrapolations may be

TABLE V

COMPARISON OF RETENTION VOLUMES IN PURE WATER

Mobile phase: methanol–(acidified)water. Stationary phase: 5-μm LiChrosorb RP-18. Predicted data which satisfactorily match with the data measured are underlined. From ref. 25 (© American Chemical Society).

Compound	*Retention volume (ml)*		
	*Calculated**		*Experimental***
	Quadratic	*Linear*	
Aniline	0.7	0.5	1.0
Benzophenone	1370	22	62
Biphenyl	365	154	390
Dimethyl phthalate	32	4.3	35
Ethylbenzene	150	43	25
Naphthalene	2320	64	37
Phenol	0.9	0.6	0.4
Toluene	70	14	7.5

* Extrapolated using a quadratic and a linear relationship for ln k' *vs.* eluent composition. Column: 5 cm × 4.6 mm I.D.

** Determined on a 2.2 mm × 4.6 mm I.D. column.

the cause of the most dramatic differences from the experimental retention volumes (predicted volume for benzophenone is 22-fold and that for naphthalene 63-fold greater than the experimental volume). Generally, experimental data for k'_w are unavailable and the choice of an appropriate estimation has to be made. In order to reduce the possible difference between extrapolated and true k'_w values, and as an "easy-to-apply" calibration procedure was sought, we decided to linearly extrapolate the retention observed for the two higher x values experimentally investigated for

TABLE VI

EXTRAPOLATED LOGARITHMS OF CAPACITY FACTORS IN PURE WATER

Compound	*log P*	*x**		*log k'*	
		ODS-3	*PRP-1*	*ODS-3*	*PRP-1*
2,3-Butanedione	0.16**	1.0	0.7–0.8	0.44***	1.19
cis-3-Hexen-1-ol	1.56	1.0	0.6–0.7	2.04***	3.00
trans-2-Hexenal	1.57	1.0	0.4–0.5	2.28***	3.20
Ethyl butanoate	1.70	0.7–0.8	0.4–0.5	2.51	3.69
2-Nonanone	2.88	0.5–0.6	0.2–0.3	4.10	5.58
1-Nonanol	3.45	0.4–0.5	0.2–0.3	4.43	4.87
Methyl octanoate	3.29	0.4–0.5	0.1–0.2	4.57	5.89
Dibutyl sulphide	4.07	0.3–0.4	0.0–0.1	4.86	7.40

* Water content corresponding to the data points involved in the linear extrapolation (see text).

** Mean of seven estimations (see text).

*** Measured data.

each solute. For instance, data points characterized by $x = 0.7$ and 0.8 were considered for ethyl butanoate and $x = 0.3$ and 0.4 for dibutyl sulphide on the Partisil packing. The estimated capacity factors for all model solutes in pure water in both stationary phases are given in Table VI together with the corresponding x values.

This procedure is *a priori* more accurate as the water content in the eluent is higher. As for the correlation between log P and the extrapolated k'_w values, we weighted each log P, log k'_w point according to the highest x value which enabled the estimation of the corresponding k'_w. For the examples given above on the bonded silica, ethyl butanoate was assigned a weight of 8 and dibutyl sulphide one of 4. Whenever available, experimental k'_w values were of course considered in the regression and the highest weight, 10, was assigned to the corresponding points. Finally, 2,3-butanedione was considered as well as other solutes thanks to the octanol–water coefficient determined from the experimental retentions and the various regression characteristics listed in Table IV (mean value : log $P = 0.16$). The correlation lines

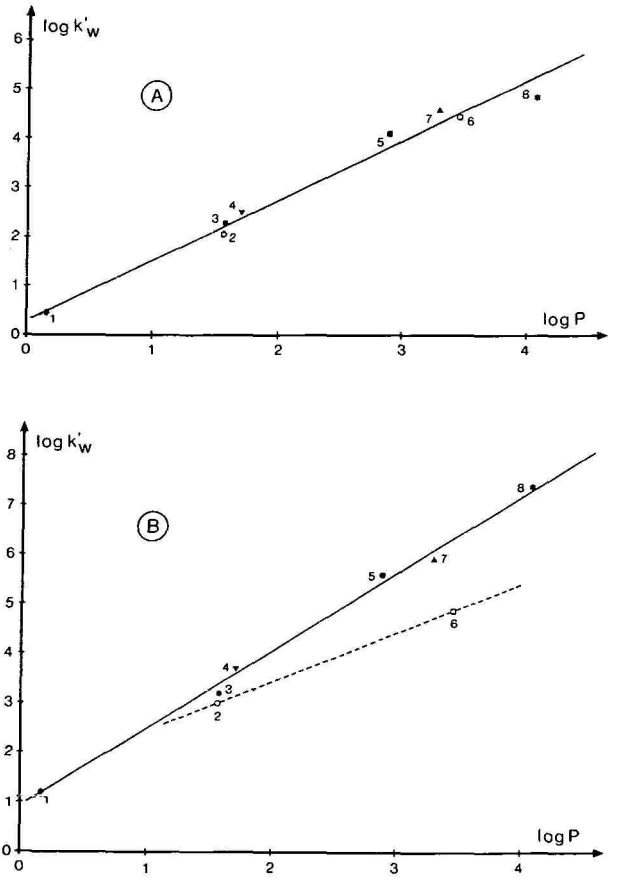


Fig. 4. Calibration graphs for both RPLC systems in pure water; (A) Partisil ODS-3; (B) PRP-1. The full line in (B) corresponds to all but hydroxylated solutes and the broken line to hydroxylated compounds. See Table I for solute identification.

obtained on both non-polar materials are presented in Fig. 4 and can be written as follows:

C_{18}: $\log k'_w = 1.22 \log P + 0.30$ $(r = 0.992)$

PRP-1: non-hydroxylated solutes

$\log k'_w = 1.57 \log P + 0.92$ $(r = 0.998)$

hydroxylated solutes

$\log k'_w = 0.99 \log P + 1.46$ $(r = 1)$

Comparison of the figures in Table IV shows that the correlation coefficient for the octadecyl-bonded packing is as high as those corresponding to lower x values. As for the PRP-1 copolymer, two separate lines (for alcohols and non-alcohols respectively) must be drawn and the correlation coefficient for non-hydroxylated solutes is greater than those obtained for other eluent compositions.

These regression lines calibrate both RP materials in pure water. No additional experiment is needed for the estimation of the retention of any compound. The sole limitation lies in the chemical structure of the solutes considered which must not be too complicated so that the log P calculations be as accurate as possible. The mean error associated with the estimation of k'_w can be calculated by:

$$\bar{e} = \frac{\sum_{i=1}^{N} \frac{k'_{w\ lsr\ i} - k'_{w\ ext\ i}}{k'_{w\ ext\ i}}}{N}$$

$\bar{e}$ is therefore defined as the percentage difference between the $k'_{wlsr\ i}$ value given by the least-squares regression function and the extrapolated $k'_{wext\ i}$ value among the N aroma compounds. It equals 47% for the Partisil material and 30% for non-hydroxylated solutes retained on the PRP-1 resin respectively. It must be emphasized that these $\bar{e}$ values only quantify the mean error associated with the use of the calibration lines for both chromatographic systems in pure water. They do not provide an unequivocal evidence for the accuracy of the k'_w extrapolation method suggested above. Such evidence, which requires (more) experimental k'_w values, will appear later, together with that of the log P, log k'_w ponderal regression, in another article on the trace enrichment of aroma-containing food plant waste-waters[55].

CONCLUSION

Retention experiments performed with methanol–water mobile phases and model aroma compounds showed that the PRP-1 polystyrene–divinylbenzene copolymer exhibits an higher retentive power than the Partisil ODS-3 octadecyl-bonded silica and is therefore better suited for trace-enrichment purposes.

An "easy-to-apply" calibration procedure for both RPLC system has been presented, which correlates the hydrophobic log P coefficient calculated according to Rekker's method with the extrapolated logarithm of the capacity factor for solutes in pure water, log k'_w. This procedure led to one calibration line for the C_{18} material and two lines (for hydroxylated and non-hydroxylated solutes respectively) for the

organic resin. Such a standardization enables the estimation of the retention of any solute in pure water, *i.e.*, the corresponding breakthrough volume as far as dilute samples are concerned. Further investigations which help define the scope of application of such a calibration thanks to frontal analysis experiments as well as its use for the trace enrichment of industrial waste-waters will be presented in forthcoming papers[55,56].

ACKNOWLEDGEMENTS

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REORDERING/RESOLVATION OF SILICA IMMOBILIZED NON-HYDROGEN BONDING LIGANDS USED AS STATIONARY PHASES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The thermally induced reordering/resolvation of ω-haloalkyl modified surfaces are studied as a function of increasing temperature. The thermal on-set, T_0, determined from plots of ln k' (capacity factor) *vs.* $1/T$ varies with chain length and functionality of the immobilized groups. A direct correlation between T_0 and boiling point of corresponding non-immobilized compound is observed. These data are explained by a three term model that accounts for energy differences which occur with reordering/resolvation in terms of: (1) cohesive interaction between the bonded chains; (2) hydrophobic interaction between the bonded chains and the contact solvent, water; and (3) specific interaction resulting from hydrogen bonding between aqueous mobile phase and unreacted surface silanols.

INTRODUCTION

A majority of high-performance liquid chromatographic (HPLC) separations are carried out using chemically attached stationary phases which are prepared by immobilizing various organosilanes to porous silica. A number of techniques have been utilized to investigate the structure and dynamics of such surfaces[1–14]. The alkyl modified surfaces have received the most attention due to their popularity as reversed-phase packing. With a totally aqueous mobile phase the bonded chains assume one of two preferred orientations, an aggregated collapsed state and a resolvated extended state[15–17]. Ligand orientation is dependent on column temperature, chain length[17], silane backbone structure[18,19], and solvent composition[15,17,20].

In a systematic effort to extend earlier work, several monofunctional ω-haloalkyl (chloro- and bromo-) modified surfaces have been synthesized. As in the case of bonded hydrocarbon phases these materials exhibit non-linear changes in plots of ln k' (capacity factor) *vs.* $1/T$. The thermal on-set, T_0, of non-linearity is dependent on chain length of and attached functionality on the immobilized groups. A direct correlation between T_0 and the boiling points of similar non-immobilized compounds exists. These results can be explained by a three term model which accounts for reordering/resolvation of the bonded groups in terms of: (1) cohesive interaction

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between the chains; (2) hydrophobic interaction between the chains and the contact solvent, water; and (3) specific interaction resulting from hydrogen bonding between water and unreacted silanols.

EXPERIMENTAL

Reagents

5-Bromo-1-pentene, 6-bromo-1-hexene, 8-bromo-1-octene, 4-penten-1-ol, 5-hexen-1-ol, allyl bromide, 1,4-dibromobutane and trichlorosilane were purchased from Aldrich. LiChrosorb Si 60 silica (mean particle size 10 μm and surface area 550 m^2/g) was purchased from MCB.

7-Bromo-1-heptane was synthesized from allyl bromide and 1,4-dibromobutane via a Grignard reaction. An amount of 0.1 mol of allyl bromide was added to 0.1 mol of magnesium metal in 50 ml of dry ethyl ether and refluxed 4 h. Excess dibromobutane (0.2 mol) was added to this mixture which was refluxed overnight. The product was purified by distillation.

4-Penten-1-ol and 5-hexen-1-ol were converted respectively to 5-chloro-1-pentene and 6-chloro-1-hexene by reacting with thionyl chloride and pyridine. An amount of 0.1 mol of unsaturated alcohol was added slowly to a mixture of 0.15 mol thionyl chloride and 0.1 mol pyridine in 50 ml of chloroform and refluxed 4 h. The reaction mixture was diluted with 50 ml ethyl ether and washed three times with 5% hydrochloric acid and twice with distilled water. Finally the organic layer was dried over anhydrous sodium sulfate. The product was separated from the mixture by distillation. Following each preparation, the identity and purity of the materials were verified by gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance spectrometry (NMR).

The final coupling step in the synthesis of the trichlorosilane monomers was carried out in a 50-ml stainless-steel reaction vessel which was initially cooled with dry ice–acetone. Ten drops of 0.1 *M* hydrogen hexachloroplatinate in 2-propanol were added to a 1:2.2 mixture of ω-haloalkene–trichlorosilane. The reaction vessel was capped, removed from the dry ice–acetone, and allowed to slowly warm to ambient temperature. Subsequently the vessel was heated to 110°C and allowed to remain at this temperature for two days. The product was purified by vacuum distillation and used to chemically modify LiChrosorb silica as previously described[17].

Column preparation

The modified materials were packed into 25 cm × 2.4 mm I.D. stainless-steel columns using a dynamic procedure[20].

Equipment

An LDC (Riviera Beach, FL, U.S.A.) Model Constametric IIG LC pump with a Model SpectroMonitor III UV detector was used in all studies. Column temperature was controlled in a water bath equipped with a Tempunit (Techne., Princeton, NJ, U.S.A.) Model TU-14 zero cross-over proportional controller and a Neslab (Neslab Instruments, Portsmouth, NH, U.S.A.) Model EN-350 flowthru liquid cooler. The injection system was a Rheodyne (Cotati, CA, U.S.A.) Model 70-10 valve

and a 70-11 loop filter port, which was immersed in the water bath and maintained at the same temperature as the column. To ensure thermal equilibrium of the mobile phase, 6 ft. of 1/16 in. (0.23 mm I.D.) capillary tubing were placed between the injection valve and the column.

Procedure

Before each evaluation cycle, all columns were conditioned with at least 100 ml of acetonitrile followed by 100 ml of water. This procedure was carried out at the lowest temperature studied. Acetonitrile was chosen as the organic conditioning solvent to minimize entrapment problems[21]. All columns were evaluated through a minimum of two complete cycles at a flow-rate of 1.0 ml/min, using water as the mobile phase. Retention measurements were made as a function of increasing temperature from 10°C to 80°C. Each evaluation cycle consisted of three sequential steps. (1) Initially, the retention times of phenol and resorcinol were measured every five degrees over the temperature range studied. These first measurements were always made following conditioning. (2) The column was cooled to the starting temperature and measurements were again made *vs.* increasing temperature. (3) The column was recooled to the starting temperature a second time and a limited set of measurements made over the same temperature range.

RESULTS AND DISCUSSIONS

For linear *n*-alkyl phases thermally induced reordering/resolvation of the immobilized chains have been observed when these surfaces are in contact with water[17]. In the current study this earlier work has been extended to ω-haloalkyl modified silica. The immobilized chains on these latter materials also undergo similar reordering/resolvation. A generalized model has been developed and used to expain previous as well as current data in terms of cohesive, hydrophobic and specific forces arising between the bonded chains, the solvent, and the underlying surface.

In carrying out the current investigation changes in the retention volumes of phenol and resorcinol were studied as a function of increasing temperature. Mean capacity factors, k', were calculated from multiple injections of each solute. ^2H_2O was used to determine void volume. Representative plots of ln k' *vs.* $1/T$ for phenol are shown in Fig. 1. These results were obtained on ω-chloropentyl modified silica. The general shapes and positions of the curves are similar to those previously reported for *n*-alkyl systems[17]. During the initial evaluation step (*i.e.*, line a) a change in slope was noted at higher temperatures. The thermal on-set, T_0, for this change was determined for each of the modified surfaces and is summarized in Table I as a function of the bonded groups' chain length and attached functionality. For comparative purposes, previously reported results for mid-range linear alkyl phases also are included[17]. When each column was cooled to the starting temperature and the experiment rerun, a linear relationship between ln k' and $1/T$ was obtained over the total temperature range but it was offset to lower values along the retention axis. This is illustrated for the ω-chloropentyl surface in Fig. 1 line b. Although not shown, when the column was cooled a second time and again data were collected they were found also to fit line b.

Shown in Fig. 2 are plots of T_0 *vs.* carbon number of the immobilized chains.

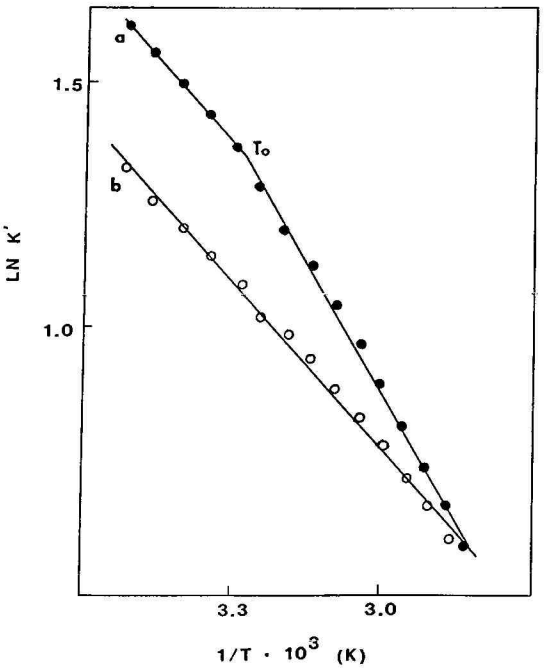

Fig. 1. Graph of ln k' *vs.* $1/T$ for a chloropentyl modified surface. Test solute: phenol. Evaluation cycles: (a) initial run, (b) rerun.

For a given bonded ligand type (*i.e.*, *n*-alkyl, ω-chloroalkyl, or ω-bromoalkyl groups) T_0 increased about 10°C for each methylene added to extend the chain. Likewise, between the group types, the ω-chloroalkyl and ω-bromoalkyl surfaces were shifted respectively about 20°C and 30°C to higher temperatures compared to the normal alkane phases. As noted above, the *n*-alkyl data have been explained in terms of

TABLE I

EFFECT OF CHAIN LENGTH AND FUNCTIONALITY ON CHAIN RESOLVATION

Compound	*On-set temperature (°C)**	*Boiling point (°C)***
n-C_8	41	126
n-C_9	52	151
n-C_{10}	60	174
n-C_5-Cl	35	108
n-C_6-Cl	44	135
n-C_5-Br	43	135
n-C_6-Br	55	155
n-C_7-Br	65	179
n-C_8-Br	none	201

* Determined from linear fits of ln k' *vs.* $1/T$ data labeled point T_0 in Fig. 1 for chloropentyl surface.

** Boiling points of corresponding *n*-alkanes, ω-chloroalkanes or ω-bromoalkanes[22].

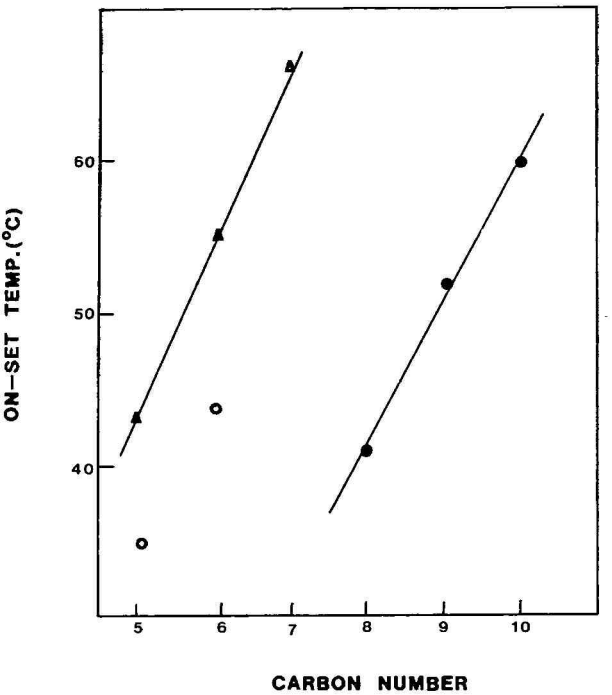

Fig. 2. On-set temperature for reordering/resolvation as a function of chain length of the bonded groups for different ligand types. ● = *n*-Alkanes; ○ = ω-chloroalkanes; △ = ω-bromoalkanes.

changes in the immobilized chains orientation between a collapsed aggregated configuration and a resolvated extended configuration[15–17]. The ω-haloalkyl data are also consistent with this model.

Perhaps, the most interesting and significant feature emerging from the current study is a direct correlation between the thermal on-set of chain reordering/resolvation (T_0) and boiling point of the corresponding non-immobilized compound (*i.e.*, *n*-alkanes, ω-chloroalkanes, and ω-bromoalkanes). This relationship is shown in Fig. 3 where T_0 is plotted against boiling point of the corresponding non-immobilized compound. Changes in T_0 with chain length, shifts in T_0 with functional substitution, and the linear relationship between T_0 and boiling point can be explained qualitatively by a three term model which accounts for energy differences which occur with chain reordering/resolvation in terms of chain–chain (cohesive forces), chain–solvent (hydrophobic forces), and solvent–surface (specific forces) interactions. A fourth term is needed for immobilized ligands containing strongly interacting specific groups (*e.g.*, nitrile, amine, etc.). This is the subject of a forthcoming report in which the experimental work is now in progress for hydrogen bonding ligands.

A generalized expression for the total system energy of non-hydrogen bonding ligands, E_t, is:

$$E_t = E_c + E_h + E_s \tag{1}$$

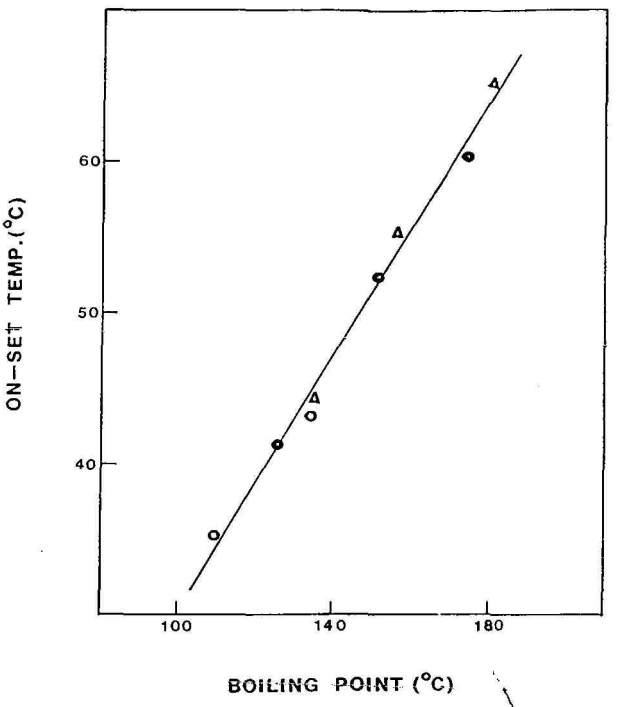

Fig. 3. On-set temperature for reordering/resolvation for the bonded groups *vs.* boiling point of the corresponding *n*-alkanes (●), ω-chloroalkanes (○) or ω-bromoalkanes (△).

where E_c, E_h and E_s are the cohesive, hydrophobic and specific interaction energies respectively. Since the chains can assume one of two preferred orientations (*i.e.* aggregated configuration which is designated state 1, and extended configuration which is designated state 2), the total energy of each state can be written as follows:

$$\text{state 1, } E_{t_1} = E_{c_1} + E_{h_1} + E_{s_1} \tag{2}$$

$$\text{state 2, } E_{t_2} = E_{c_2} + E_{h_2} + E_{s_2} \tag{3}$$

When reordering/resolvation occurs, the change in energy, ΔE, is given by the difference between these two states:

$$\Delta E = E_{t_2} - E_{t_1} = (E_{c_2} + E_{h_2} + E_{s_2}) - (E_{c_1} + E_{h_1} + E_{s_1}) \tag{4}$$

Each of the above individual interaction terms can be expressed as the product of the energy per unit area, E', and a function of the total interaction area, $f(A)$. Thus $E = E'f(A)$. After making this substitution into eqn. 4:

$$\Delta E = [E'_c\, f(A_{c_2}) + E'_h\, f(A_{h_2}) + E'_s\, f(A_{s_2})] - [E'_c\, f(A_{c_1}) + E'_h\, f(A_{h_1}) + E'_s\, f(A_{s_1})] \tag{5}$$

Upon rearrangement,

$$\Delta E = E_c'[f(A_{c_2}) - f(A_{c_1})] + E_h'[f(A_{h_2}) - f(A_{h_1})] + E_s'[f(A_{s_2}) - (A_{s_1})] \quad (6)$$

The first two terms favor the initially formed associated or aggregated state. Since the immobilized moieties are anchored on the silica surface by covalent bonds, they are separated by some average distance. In the collapsed state, the degree of overlap or association between the anchored chains is determined by spacing and chain length. This thus results in an average cohesive interaction energy for a given chain length and spacing (*i.e.*, average reaction density). After resolvation the anchored groups are separated by solvent molecules, reducing the attractive interaction between chains, $f(A_{c_2}) < f(A_{c_1})$. Inversely, the hydrophobic interaction, which is repulsive in nature, increases during resolvation. This is the result of an increase in the solvent–hydrocarbon contact area for extended *vs.* clustered chains. Thus if only cohesive and hydrophobic forces are considered, the collapsed state is energetically favored. However, experimentally this is not the case as noted previously[17] and by the current data. This is illustrated in Fig. 1.

Once the bonded chains undergo thermally induced reordering/resolvation, they remain in the extended state even when the temperature is decreased below T_0. These results imply that the aggregated state is a metastable state. Thus an increase in the specific interactions between the solvent, water, and unreacted silanol groups (*e.g.* third term in eqn. 6) must be the dominant factor in accounting for differences in stability between the initially formed collapsed aggregated state and the resolvated extended state. Under totally aqueous conditions, water hydrogen bonds with unreacted silanol groups on the surface. In the collapsed state, a portion of these silanol groups are shielded from the solvent by the clustered alkyl chains[21]. However, in the extended state, the covered silanols are exposed and are accessible to solvent, which results in an increase in the total number of silanols sterically available for hydrogen bonding. This has been demonstrated by solvent entrapment studies[21]. An increase in hydrogen bonding area after resolvation, $f(A_{s_2}) > f(A_{s_1})$, more than compensates for unfavored cohesive and hydrophobic interactions, E_c and E_h. When the system is recooled below T_0, the chains remain in their more stable extended configuration. The above model is diagrammatically illustrated in Fig. 4. Shown in Fig. 4a is an idealized picture of clustered chains shielding an underlying silanol. Similarly, Fig. 4b shows the resolvated chains and an exposed silanol.

The above model can be used to explain increases in on-set temperature with alkyl chain length and functionality (Fig. 2) as well as correlation between T_0 and boiling point (Fig. 3). In order to simplify the current data set, we assume that the reaction density is within a narrow range for both the hydrocarbons and ω-halohydrocarbons of different chain length. Although this assumption certainly is not valid over a wide range of chain lengths and differences in ligand shape and attachment chemistry, it is reasonable in the current work for immobilized groups of similar size if reaction conditions are carefully controlled. Experimentally this assumption has been found to be reasonable for mid-range linear groups by microcarbon analysis. Therefore, in the current study the total number of unreacted silanols are nearly constant and to a first approximation ΔE_s varies little with small changes in chain length. Conversely, ΔE_c and ΔE_h are chain length dependent. Both are smaller for

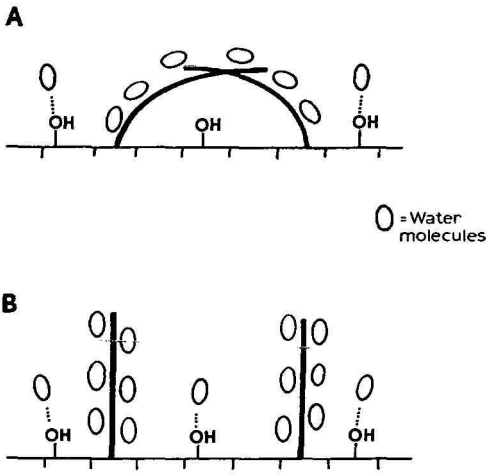

Fig. 4. (A) an aggregated collapsed state. (B) a resolvated extended state. ——— = Immobilized ligands.

shorter chains due to a reduction in the total interaction area and each incrementally increases with addition of a methylene to the chain[23,24]. Thus, ΔE_t which directly reflects a shift to higher temperature, increases with increasing chain length. Similarly, for a given chain length, ΔE_s of *n*-alkyl chains and ΔE_s of ω-haloalkyl chains are about the same, but ΔE_c decreases in the order $-Br > -Cl > -CH_3 > -H$, due to a reduction in van der Waals forces[23]. Although hydrophobic forces decrease in the order of $-CH_3 > -Br > -Cl > -H$, the relative change with functionality is small compared to relative change in cohesive forces with functionality. Thus the shift in T_0 to higher temperatures respectively for the ω-chloroalkyl and ω-bromoalkyl surfaces compared to *n*-alkyl surfaces is due principally to cohesive interactions (eqn. 6).

Since the anchored groups are separated by several chemical bonds, the degree of overlap is less than that for free molecules of equivalent chain length. Thus T_0 should be less than but linearly related to the boiling point of the corresponding free molecule. Additionally, the cohesive interaction arising from the monolayer structure of the immobilized groups is more nearly two dimensional compared to the solution state for free molecules. Hence, the approximately 10°C/methylene incremental increase in T_0 for the immobilized chains (*i.e.*, compared to about a 25°C/methylene increase in boiling point of similar non-immobilized compounds, see Table I) is reasonable based on structural differences between the immobilized groups and free molecules.

CONCLUSION

The thermally induced reordering/resolvation of ω-haloalkyl modified surfaces under totally aqueous conditions exhibit similar behavior to previously studied *n*-alkyl surfaces. A three term model has been proposed to explain observed experimental results for non-hydrogen bonding ligands in the current study. However, in a forth-

coming report, a fourth term is needed for immobilized ligands containing hydrogen bonding groups (*e.g.*, nitrile, amine, etc.).

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TANDEM USE OF CARBOXYPEPTIDASE Y REACTOR AND DISPLACEMENT CHROMATOGRAPH FOR PEPTIDE SYNTHESIS

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SUMMARY

A packed-bed enzyme reactor with immobilized carboxypeptidase Y was used in tandem with a displacement chromatograph for the preparation of N-benzoyl-L-arginyl-L-methioninamide, from N-benzoyl-L-arginine and L-methioninamide. The pumps and valves of the coupled enzyme reactor and displacement chromatograph were controlled by a microprocessor. The enzyme was immobilized on microparticulate amino-silica by glutaraldehyde and packed into a 60 × 4.6 mm I.D. column. The packed-bed reactor was used in the recirculating mode and components of the reaction mixture were subsequently separated by displacement chromatography on a 250 × 4.6 mm octadecyl-silica column using butoxyethoxyethanol as the displacer. Unreacted L-methioninamide was returned to the reaction mixture. Both the progress of the reaction and the extent of separation by displacement chromatography were monitored by high-performance liquid chromatographic analysis. The system was designed so that enzymatic peptide synthesis, separation by displacement chromatography, and column regeneration were carried out simultaneously by using two identical columns in parallel. An amount of 460 mg of N-benzoyl-L-arginyl-L-methioninamide having purity greater than 99% could be obtained in 24 h with this system. The tandem operation of the enzyme reactor and liquid chromatograph operated in the displacement mode offers a means for the synthesis and purification of peptides.

INTRODUCTION

Despite advances in chemical[1,2] and solid phase peptide synthesis[3,4], there is a growing interest in enzymatic peptide synthesis for the preparation of biologically active peptides[5–7] due to the stereospecificity of the enzymatic peptide bond formation.

The serine exopeptidase, carboxypeptidase Y (CPY) from baker's yeast, has been shown to be a general catalyst for peptide synthesis[8–13] and the use of this enzyme in immobilized form (Imm-CPY) has recently been investigated in our lab-

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oratory[14]. In the course of this work we have found that displacement chromatography[15–17] offers significant advantages over elution chromatography in separating the component of the relatively dilute peptide synthesis mixtures[18]. The present work describes the tandem synthesis of N-benzoyl-L-arginyl-L-methioninamide (Bz-Arg-Met-NH_2) by Imm-CPY and product isolation by displacement chromatography.

EXPERIMENTAL

Materials

Carboxypeptidase Y, specific activity of 135 units/mg, was a gift from Carlsberg Biotechnology (Copenhagen, Denmark). Octadecylsilica (10 μm) (lot No. 3223-45A), a gift from Amicon (Danvers, MA, U.S.A.), was packed into 250 × 4.6 mm columns. N-Benzoyl-L-arginine (Bz-Arg) and N-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) were obtained from Sigma (St. Louis, MO, U.S.A.). Butoxyethoxyethanol was purchased from Fisher (Pittsburgh, PA, U.S.A.). The support used for enzyme immobilization was 10-μm Vydac silica having a specific surface area of 100 m^2/g and mean pore diameter of 330 Å (Separation Group, Hesperia, CA, U.S.A.). Z-6050 polyamino-functional silanizing agent was obtained from Dow (Midland, MI, U.S.A.). Glutaraldehyde, 50% (w/w) aqueous solution, was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Sodium borate, methanol, and EDTA were supplied by Fisher. Distilled water was prepared with a Barnstead distilling unit.

Apparatus

Fig. 1 shows a schematic of the tandem reactor–chromatograph system. V_1, V_2, and V_5 are Model 7030 three-way valves (Rheodyne, Cotati, CA, U.S.A.) and V_3 and V_4 are Model 7040 four-way valves (Rheodyne). The operation of these valves is described in Table I. Two recirculating water-baths, Lauda Model K-2/R (Brinkmann, Westbury, NY, U.S.A.) were used independently to control the temper-

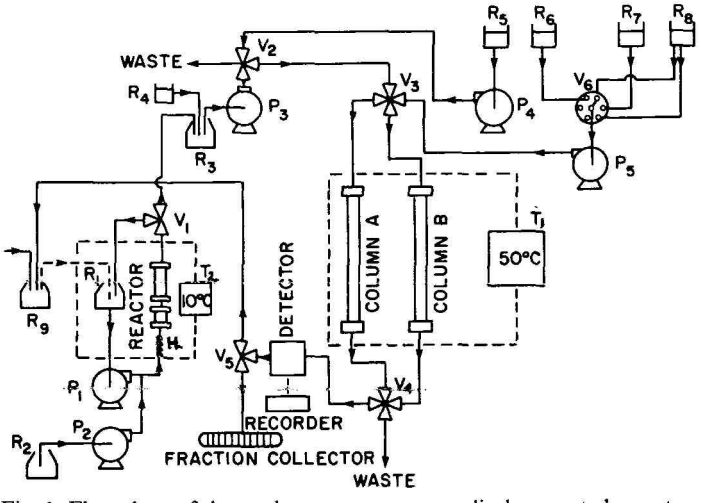

Fig. 1. Flow sheet of the tandem enzyme reactor–displacement chromatograph. The symbols are R, reservoir; P, pump; V, valve; T, thermostated baths; H, heat exchanger; E, enzyme reactor; and G, guard column. See further details in text.

TABLE I

FLOW CONTROL BY VALVE SWITCHING

Valve	*Position*	
	I	*II*
V_1	Reactor effluent to recycle reservoir R_1	Reactor effluent to pH adjustment reservoir R_3
V_2	Displacer to waste: feed to V_3	Feed to waste; Displacer to V_3
V_3	Regenerants to column A; displacer or feed to column B	Displacer or feed to column A; regenerants to column B
V_4	Effluent of column A to detector; effluent of column B to waste	Effluent of column A to waste; effluent of column B to detector
V_5	Effluent from detector to fraction collector	Effluent from detector to Met-NH_2 collection reservoir R_9

ature of the enzyme reactor and the two identical 250 × 4.6 mm columns packed with 10-μm octadecylsilica (Amicon). The temperature of the column undergoing displacement was maintained at 50°C by directing the recirculating water-bath flow into a water-jacket surrounding the column, while circumventing the column undergoing regeneration. The flow through the column water-jackets was manually controlled. The reactor, E, and the reservoir, R_1, were kept at 10°C.

The immobilized enzyme was packed into a 60 × 4.6 mm column and held by two 2-μm stainless-steel fritted disks to obtain the enzyme reactor, E. A 10 × 4.6 mm guard column packed with 5-μm Partisil silica (Whatman), a 200-cm heat exchanger coil of 0.25 mm I.D. and 1.59 mm O.D. No. 316 stainless-steel capillary tubing were placed before the inlet of the reactor as shown in Fig. 1. Reservoir R_1, containing the reaction mixture, was stirred with a Model PC magnetic stirrer (Corning Glass, Corning, NY, U.S.A.).

A Model A-30-S pump (Eldex, Menlo Park, CA, U.S.A.), P_3, was used for the introduction of the 12-ml feed from reservoir R_3. Reservoir R_4 contained 5 ml of 85% phosphoric acid which was used to adjust the pH of the feed as described below. One Model 110A pump (Altex-Beckman, San Ramon, CA, U.S.A.), P_1, was used to recirculate the 15-ml reaction mixture contained in reservoir R_1 and another Model 110A pump, P_4, generated the flow of the displacer solution from the 1-l reservoir R_5. A Model B-100-S pump (Eldex), P_2, was used to pump water from the 100-ml reservoir R_2 through the reactor at the end of the synthesis reaction. A Model 1016/AA-94 dual piston pump (Eldex), P_5, was used to pump water, methanol, and 0.1 *M* phosphoric acid sequentially from the respective reservoirs R_6, R_7 and R_8, each of 1-l volume, through the column during the regeneration step by the use of

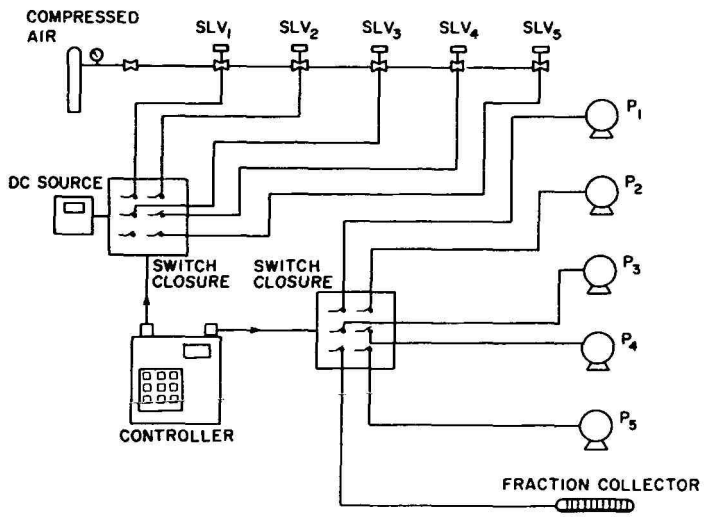

Fig. 2. Schematic of microprocessor control of the valves and pumps employed in the tandem enzyme reactor–displacement chromatograph system. SLV represents pneumatically activated solenoid valve.

a Model II 6-way selector valve (Eldex), V_6. A TriDet detector (Perkin-Elmer, Norwalk, CT, U.S.A.) monitored the column effluent and the signal was recorded with a Model R-100 A strip chart recorder (Perkin-Elmer). An Ultrarac fraction collector (LKB, Bromma, Sweden) was used to collect 150-μl fractions from the column effluent except during the elution of Met-NH_2 which was directed to the 100-ml reservoir R_9 by valve V_5.

The sequencing of all switching valve operations via a Model 70-01 pneumatic actuator (Rheodyne) and the delivery of power to the pumps, detector and fraction collector was controlled by an Eldex Chromat-a-trol as shown in Fig. 2. A Model 7163 solenoid valve kit (Rheodyne) with a switch closure module (Eldex) controlled the operation of the valves as shown in Table I. A power module (Eldex) along with two home made 12-V power supplies were used for the operation of all the equipment.

Procedures

Synthesis of Bz-Arg-Met-NH_2. The preparation of the amino-silica support from 10-μm Vydac wide pore (330 Å) silica gel with Z-6050 polyamino-functional silane, the activation of the support by glutaraldehyde and the immobilization of CPY were carried out as described elsewhere[14]. A 60 × 4.6 mm packed-bed reactor with Imm-CPY was employed for the synthesis of Bz-Arg-Met-NH_2. The 15-ml reaction mixture in reservoir R_1 contained 10 m*M* Bz-Arg-OEt and 50 m*M* Met-NH_2 in 50 m*M* borate buffer, pH 9.0, with 5 m*M* EDTA. It was recirculated through the reactor by pump P_1 at a flow-rate of 4 ml/min and a temperature of 10°C. After 2 h, valve V_1 was set to position II directing the flow of the reaction mixture into reservoir R_3 for pH adjustment. After 12 ml of the reaction mixture accumulated in reservoir R_3, pump P_1 was turned off, leaving 3 ml of the reaction mixture in reservoir R_1 so that pump P_1 remained primed for the subsequent reaction. Pump P_2 was then turned on and water was pumped through the reactor at a flow-rate of 1 ml/min to

transfer the remaining reaction mixture from the reactor into reservoir R_3. After 1 minute the reactor effluent was directed to waste and the reactor was washed with water for an additional 4 min. A volume of 150 μl of 85% phosphoric acid from reservoir R_4 was then added to the content of reservoir R_3 in order to bring the pH to 2.5. A complete reaction cycle took 2 h and 15 min.

Subsequent cycles were initiated by first placing 15 ml of fresh reaction mixture into reservoir R_1, then turning on recirculating pump P_1 to pump the mixture through the reactor. After the first 1 ml of the reactor effluent was directed to waste to eliminate the water present in the reactor, valve V_1 was set to position I to initiate the recirculation of the reaction mixture.

Displacement chromatography. Displacement chromatography was alternately carried out with columns A and B using the system shown in Fig. 1. While one column was separating the other was being regenerated. The same procedure was used for displacement chromatography with both columns. The column was first equilibrated with a 0.1 *M* phosphoric acid solution in water, pH 2.2, used as the carrier. Valve V_2 was then set to position I and the reaction mixture was pumped from reservoir R_3 into the column by pump P_3 at a flow-rate of 0.5 ml/min at 25°C. The feed was directed into column A or B by switching valve V_3 to position II and I, respectively. The effluent of the column was directed to either the detector or to waste by valve V_4 as described in Table I. After 11.5 min of introduction of the feed, the front of Met-NH_2 appeared in the detector and valve V_5 was switched to direct the flow from the detector effluent to reservoir R_9.

After 26 min of introduction of the feed into the column, pump P_3 was turned off and the column temperature was raised to 50°C as described above. The displacer was then introduced into the column at a flow-rate of 0.1 ml/min by pump P_4 upon setting valve V_2 to position II. After 45 min of introduction of the displacer, the Met-NH_2 had eluted from the column and valve V_5 was set to position I directing the flow from the detector effluent to the fraction collector. Following the introduction of the displacer for an additional 40 min, Bz-Arg appeared in the effluent and the collection of 150-μl fractions began. The fractions containing Bz-Arg, Bz-Arg-OEt, and Bz-Arg-Met-NH_2 were collected over the next 25 min until the displacer front emerged from the column and pump P_4 was turned off. The entire separation process took 2 h and 15 min.

Regeneration of the chromatographic column. During the separation process described above, the other column was regenerated by pumping various regenerants in succession at a flow-rate of 2 ml/min using pump P_5 with valves V_3 and V_4 in the appropriate positions given in Table I to direct the regenerating column effluent to waste. Valve V_6 was used to select a given regenerant. The regeneration protocol for removing the displacer from the column called for sequential perfusion with 30 ml of water, 150 ml of methanol, 46 ml of water, and 46 ml of carrier in 2 h and 16 min. During the regeneration process the flow from the recirculating water bath was not directed to the regenerating column and the column was cooled to room temperature by the flow of regenerants.

High-performance liquid chromatographic analysis. For analytical work a Model LC 250/1 pump (Kratos, Westwood, NJ, U.S.A.), a Model 7010 sampling valve with a 20-μl sample loop (Rheodyne) a Model SF 770 UV detector (Kratos) and a Model CI-10 integrator (LDC, Milton-Roy, Riviera Beach, FL, U.S.A.) were

used with a 5-μm C_8 250 × 4.6 mm column (IBM, Danbury, CT, U.S.A.). A 50 m*M* phosphate buffer, pH 3.0, containing 40% (v/v) methanol was used as the eluent at 25°C. The reaction progress at 15-min intervals and the composition of the fractions obtained by displacement development were measured. Samples of 5 μl were taken, diluted with the eluent and 20-μl aliquots were injected. The column effluent was monitored at 254 nm where the Bz protecting group has a strong absorbance. Peak areas were used in quantitative analysis.

RESULTS AND DISCUSSION

The coupling of a packed-bed Imm-CPY recycle reactor[14] and a displacement chromatograph[18] presented here is an extension of the work of El Rassi and Horváth[19]. In that work, a tandem immobilized ribonuclease T_1 reactor–liquid chromatograph system was employed for the preparation of nucleic acid fragments. The present work adapts the microprocessor controlled unit for enzymatic peptide synthesis and employs two chromatographic columns in parallel to enable simultaneous separation and regeneration via column switching.

The flow of reagents throughout the system was directed using a matrix of pneumatically actuated switching valves controlled by the microprocessor. In order to synchronize the individual steps and thus facilitate the control of the overall process, conditions for the peptide synthesis reaction, displacement chromatographic purification, and column regeneration were selected so that their times were approximately the same. This was accomplished using two identical separation columns in parallel, with one undergoing regeneration and the other separating the components of the reaction mixture by displacement chromatography. While the timing of column regeneration was relatively flexible, the timing of displacement purification and enzymatic peptide synthesis were less so as described below. The reaction volume was chosen so that the entire reaction mixture could be used as the feed for a single displacement chromatographic run. While the work presented here is on the preparation of Bz-Arg-Met-NH_2, this system could be used for alternative peptide syntheses[14] by changing the operational parameters of the system.

Bz-Arg-Met-NH_2 synthesis

The CPY catalyzed synthesis of Bz-Arg-Met-NH_2 from Bz-Arg-OEt and Met-NH_2 was selected for this study since both the kinetics and stability of the immobilized enzyme for this reaction have been investigated in conjunction with the enzymatic synthesis of L-methionyl-L-leucyl-L-phenylalanine[14]. Operating conditions were established in that work which retained the enzymatic activity of the reactor after several syntheses[14].

As stated above, the criterion for selecting the volume of reaction mixture was that the volume of a fixed concentration of reactants would match the column capacity in displacement chromatography. Previous experiments had indicated that 15 ml of this reaction mixture could be separated by displacement chromatography with one 25-cm C_{18} column in approximately 2 h using butoxyethoxyethanol as the displacer[18]. Accordingly, a 15-ml reaction volume was selected for this study.

Fig. 3 shows the progress of the reaction in the present system which resulted in a 65% conversion of substrate with 80% selectivity for Bz-Arg-Met-NH_2 in 2 h.

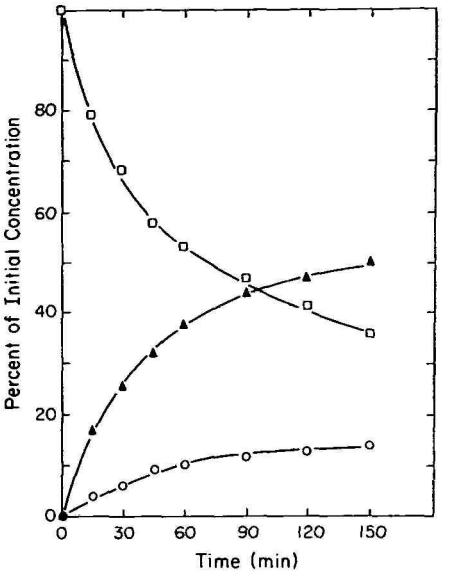

Fig. 3. Time course of Bz-Arg-Met-NH_2 synthesis by the recirculating enzyme reactor. Reaction mixture, 15 ml of 10 m*M* Bz-Arg-OEt and 50 m*M* Met-NH_2 in 50 m*M* borate buffer, pH 9.0, with 5 m*M* EDTA. Reactor, 60 × 4.6 mm packed-bed reactor with Imm-CPY; flow-rate, 4 ml/min; temperature, 10°C. □, Bz-Arg-OEt; ▲, Bz-Arg-Met-NH_2; ○, Bz-Arg.

Product inhibition[14] caused a decrease in the rate of product formation after 2 h. The reaction time was set to 2 h and 10 min to match the time of the displacement run. A water wash of 5 min was added to remove any precipitate that may have formed in the reactor. The peptide synthesis reaction was found to be very reproducible with respect to the yield of product in the several repetitions conducted.

Since the substrate was not completely converted, the unreacted components were also separated by displacement chromatography so that they could be reused in subsequent synthesis reactions. The recycling of the excess Met-NH_2 was controlled in this system. However, unreacted Bz-Arg-OEt could also be recycled by using a similiar set of commands.

Displacement chromatography

The conditions for displacement chromatography of the reaction mixture have been established previously[18] and Fig. 4 shows the separation of the components obtained with the present tandem system. Most of Met-NH_2 eluted during the introduction of the feed and the remainder of the Met-NH_2 eluted within 45 min after the introduction of the displacer. After 85 min of the introduction of the displacer, Bz-Arg emerged as the first band of the displacement train followed by the bands of Bz-Arg-OEt and Bz-Arg-Met-NH_2. The zones were well separated and the displaced components were concentrated during the separation process from feed concentrations of 1.4, 3.6 and 5 m*M* to concentrations of 55, 73, and 104 m*M* of Bz-Arg, Bz-Arg-OEt and Bz-Arg-Met-NH_2, respectively. The recoveries of Met-NH_2, Bz-Arg, Bz-Arg-OEt and Bz-Arg-Met-NH_2, at greater than 99% purity, were 100, 87, 86 and 93%, respectively.

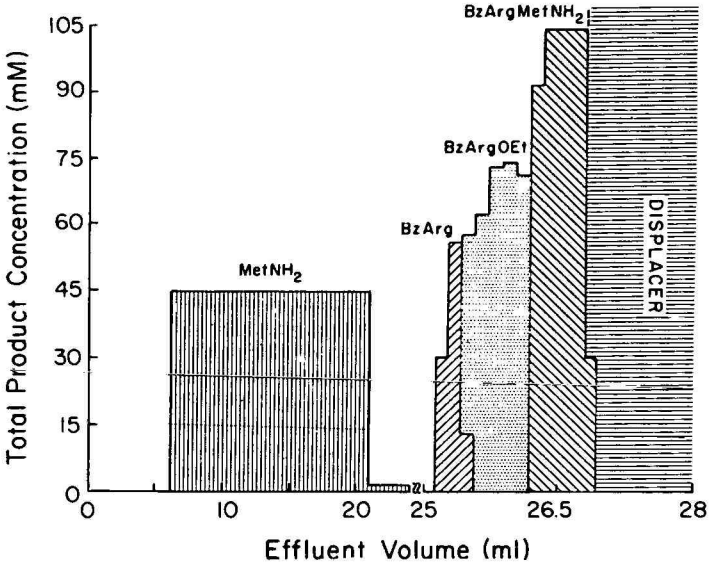

Fig. 4. Displacement chromatogram of the reaction mixture at the end of Bz-Arg-Met-NH_2 synthesis. Column, 10-μm Amicon ODS (250 × 4.6 mm); carrier, 0.1 *M* phosphate buffer, pH 2.2; displacer, 40 g/l butoxyethoxyethanol in the carrier; temperature, 50°C; flow-rate, 0.5 ml/min and 0.1 ml/min for the feed and displacer, respectively. Feed, 13 ml of 5 m*M* Bz-Arg-Met-NH_2, 3.6 m*M* Bz-Arg-OEt, 1.4 m*M* Bz-Arg and 45 m*M* Met-NH_2. Volume of fractions, 150 μl.

In our system, which is based solely on control of the timing of events, the reproducibility of the events is an important criterion. With the two chromatographic columns displacements were very reproducible. The Bz-Arg zone emerged at 85 min ± 10 s when the separation process was repeated six times. In one cycle, the system produced 45 mg of pure Bz-Arg-Met-NH_2 so that 460 mg of pure Bz-Arg-Met-NH_2 was obtained in a 24-h period.

Microprocessor operation files

The tandem reactor–displacement chromatograph system was controlled by the Eldex Chromat-a-trol as described in the Experimental section and the following processes were regulated: synthesis of Bz-Arg-Met-NH_2, introduction of the reaction mixture into reservoir R_3 for pH adjustment, pumping of the feed into the displacement column, separation of the components by displacement chromatography, collection of the purified excess Met-NH_2 in reservoir R_9, collection of the purified reaction products using a fraction collector, and regeneration of the displacement column. Three files were written to control various stages of the simultaneous enzymic reaction, displacement development, and column regeneration. Start-up of the tandem system consisted of a simultaneous synthesis reaction and regeneration of column A using the file shown in Table II to control the timing of each event in these operations. The orientations of the switching valves are listed in Table I and the positions of the regenerant selection valve V_6 for water, methanol, water, and carrier are denoted by $V_{6\text{-}1}$, $V_{6\text{-}2}$, $V_{6\text{-}3}$ and $V_{6\text{-}4}$ respectively.

After the start-up cycle given in file 1, the system was subsequently operated

TABLE II

CONTROLLER FILE 1: PEPTIDE SYNTHESIS AND REGENERATION OF COLUMN A

Time			*Event*	
h	*min*	*s*	*Position*	*Valve/pump*
00	00	00	II	V_4
00	00	00	I	V_3
00	00	00	I	V_6
00	00	00	On	P_5
00	00	00	II	V_1
00	00	00	On	P_1
00	00	15	I	V_1
00	15	00	II	V_6
01	30	00	III	V_6
01	53	00	IV	V_6
02	07	00	II	V_1
02	10	00	Off	P_1
02	10	20	On	P_2
02	15	00	Off	P_2
02	16	00	Off	P_5

according to file 2 which was written to control two subsequent cycles. In the first cycle, peptide synthesis, displacement chromatography using column A and regeneration of column B occurred simultaneously, whereas in the second cycle the role of the two chromatographic columns was reversed. In the course of this work, the two cycles of file 2 were repeated 3 times to test the reproducibility of the system.

The last two cycles of the coupled system were controlled by a third file which was the same as file 2 except without reaction and use of column B during the second cycle. The separation of the reaction mixture from the last synthesis of file 2 and the regeneration of both chromatographic columns were carried out according to the third file.

CONCLUSIONS

The synthesis of Bz-Arg-Met-NH_2 was carried out by an immobilized carboxypeptidase Y reactor coupled to a displacement chromatograph. Two identical chromatographic columns were used in parallel, each alternating between separation and regeneration steps. The system was designed so that the synthesis, separation, and regeneration operations took place simultaneously.

While the tandem reactor–chromatograph was not fully automated, the automation of the remaining manual steps would be relatively straightforward. Since control of the system was based solely on the timing of the events, appropiate feedback controls would be needed to facilitate smooth operation over an extended period of time. Whereas the work presented here demonstrates that the tandem system has found use in the preparation of pure peptides it can also serve as a model for similar instruments suitable for the *ad hoc* preparation or continual supply of complex biochemical substances in the laboratory.

ACKNOWLEDGEMENTS

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STUDIES ON THE NUCLEOTIDE ARRANGEMENT IN DNA

ESTIMATION OF SEQUENCE ISOMERS IN PYRIMIDINE DEOXYRIBO-OLIGONUCLEOTIDES

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SUMMARY

The population of pyrimidine deoxyribo-oligonucleotides obtained from DNA may be separated, first, into fractions comprising identical lengths (isotichs) and, subsequently, into the individual components of an isostich fraction varying in the cytidylic and thymidylic acid composition. At a third level of analysis the proportion of components exhibiting the same composition but different sequence may be determined. Selective removal of cytosine and the subsequent analysis for characteristic thymidine derivatives released during an acid-catalyzed β-elimination reaction allowed estimation of the relative proportion of sequence isomers for pyrimidine isostichs of length two to five.

INTRODUCTION

Hydroxylamine has proven to be a useful reagent in the study of the structure of ribonucleic acid[1] by virtue of its ability to effect a selective degradation of the uracil chromophore at pH 10. The application of this reaction to DNA, following preliminary deamination of the cytosine component to uracil was the subject of previous communications[2,3]. This differential degradation of pyrimidine structures allowed estimation of contiguous thymidylic acid units, T-runs, and was the basis of our conclusion that the longest T-run in T7 bacteriophage DNA has a length of fourteen and occurs, uniquely, once in the molecule[3]. Another application of our technique was demonstrated by Cox and Yanofsky[4] to elucidate distortion of the frequencies of T-runs in the DNA of *Escherichia coli*, mut T1; a strain capable of introducing and accumulating unidirectional transversions (A/T → C/G) into the bacterial genome. These present procedures have purpose in surveying sequence characteristics in DNA where unique-sequence technology[5,6] is not appropriate, and for the analysis of oligonucleotides containing methylcytidylic acid.

Chromatographic procedures[7] and enzymatic approaches[8], are applicable to estimate the proportion of sequence isomers in simple mixtures and have been effec-

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tive for identification of longer sequences[9]. A reasonable and unique separation of sequence isomers for pyrimidine oligonucleotides up to length five by reversed-phase high-performance liquid chromatography (HPLC) has been described by Dizdaroglu and co-workers[10–12] and by Schott and co-workers[13,14].

The selective removal of erstwhile cytosine units from DNA by hydroxylaminolysis can, however, allow subsequent analysis of the proportion of sequence isomers in long pyrimidine tracts. The feasibility of estimating the molar proportions of sequence isomers by this method has previously been demonstrated[2]. In this communication our attention was primarily directed to estimation of the proportion of sequence isomers within pyrimidine nucleotide runs of length 2, 3, 4 and 5 of calf thymus DNA, and to establish the adequacy of the technique for application to oligonucleotides of more complex composition tabulated for plant DNA[15,16].

MATERIALS AND METHODS

Preparation of model pyrimidine deoxyribo-oligonucleotides

Freshly frozen calf thymus was the source of DNA used in this study. The DNA was isolated by the procedure of Kay *et al.*[17]. One gram of purified calf thymus DNA was suspended in 900 ml of 66% formic acid (v/v) containing 2% diphenylamine (w/v) and incubated for 18 h at 30°C, during which time the DNA became completely solubilized and the solution developed the typical purple-blue color for deoxyribose[18,19]. The solution was diluted with two parts water and subjected to continuous liquid–liquid extraction with ether. The clear aqueous phase was evaporated to dryness under reduced pressure at 37°C. The residue was dissolved in 2 l of 0.01 *M* lithium acetate, pH 5.5, and percolated through a column of DEAE-cellulose, Whatman DE-23, prepared from 50 g of cycled exchanger equilibrated with 0.01 *M* lithium acetate buffer. The separation of pyrimidine nucleotide isostich groups by a linear concentration gradient of lithium chloride in 0.01 *M* lithium acetate buffer has been described by Spencer and Chargaff[20]. The total column influent was 5.5 l. Ten-ml fractions were collected.

Each pooled isostich fraction, corresponding to pyrimidine nucleotide runs 1–5 was diluted four-fold with water and re-absorbed into columns prepared from 10 g of DEAE-cellulose. Separation of the isostich components on the basis of composition was achieved by a sodium chloride concentration gradient (0 to 0.4 *M*) in 0.1 *M* formic acid[21,22]. The volume of column influent was 900 ml. Five-ml fractions were collected during each chromatographic separation.

Each fraction described by the general formula $p(dC_n,dT_m)p$ with $n + m = 2, 3, 4$ and 5 was freed of salt by differential adsorption on acid-activated charcoal[23]. The nucleotide material was recovered from the charcoal by elution with 50% ethanol–conc. ammonium hydroxide (99/1, v/v), and evaporated to dryness.

Dephosphorylation and deamination of pyrimidine oligonucleotides

Each oligonucleotide, $p(dC_n,dT_m)p$, was dissolved in 5 ml of 5 m*M* Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 8.0, and dephosphorylated by incubation with 50 μg *E. coli* alkaline phosphatase for 18 h at 37°C. One-fifth of each fraction was removed for subsequent use as chromatographic standards. The remaining material was treated with one volume of 1.3 *M* nitrous acid, pH 3.35, for

TABLE I

SOLVENT PARTITION CHROMATOGRAPHY OF DEAMINATED OLIGONUCLEOTIDES AND THYMIDINE DERIVATIVES

Compound	*Solvent system**		
	n-Propanol–2 N hydrochloric acid	*Ammonium isobutyrate, pH 3.6*	*70% Isopropanol in NH_3 atmosphere*
(UT)p	0.63	0.95	1.75
$(U_2T)p_2$	0.45	0.68	1.21
$(UT_2)p_2$	0.54	0.77	1.40
$(U_3T)p_3$	0.35	0.43	0.58
$(U_2T_2)p_3$	0.37	0.46	0.68
$(UT_3)p_3$	0.40	0.53	0.88
$(U_4T)p_4$	–	0.20	0.23
$(U_3T_3)p_4$	–	0.27	0.36
$(U_2T_3)p_4$	–	0.34	0.40
$(UT_4)p_4$	–	0.36	0.49
pT	1.00	1.00	1.00
Tp	1.13	1.07	1.03
pTp	1.06	0.52	0.55
pTpTp	0.85	0.42	0.41
pTpTpTp	0.63	0.34	0.29
pTpTpTpTp	0.43	0.26	0.22
pTpT	0.76	0.74	0.88
pTpTpT	0.63	0.53	0.83
TpT	0.76	1.09	1.98
TpTpT	0.63	0.85	1.47

* Mobility of components is relative to thymidine 5'-phosphate.

72 h at 37°C in sealed vials. The deaminated, dephosphorylated, pyrimidine components were finally obtained salt-free by differential ammonium bicarbonate elution on small DEAE-cellulose columns[24]. Removal of the ammonium bicarbonate in solution was done under vacuum at 40°C in a rotating evaporator. The salt-free solutions of oligonucleotides were freeze-dried and the residue stored at −20°C. From our previous experience, the recovery of dephosphorylated, deaminated material is approximately 90%[2,19]. Some spectrophotometric and chromatographic characteristics of these deaminated compounds and relevant degradation products are included in Tables I and II. Identification of components relied on these data and upon composition; $T/U/PO_4$.

Hydroxylamine digestion of dephosphorylated, deaminated pyrimidine oligonucleotides

Salt-free hydroxylamine was prepared from hydroxylamine–HCl by the procedure of Verwoerd *et al.*[1]. The hydroxylamine was recovered by vacuum distillation and assayed by titration with sulfuric acid. Hydroxylamine (10 *N*) was stable for over six months when stored at −20°C.

The oligonucleotides (dU,dT), (dU_2,dT), (dU,dT_2), (dU_3, dT), (dU_2,dT_2), (dU,dT_3), (dU_4,dT), (dU_3,dT_2), (dU_2,dT_3) and (dU,dT_4) were individually treated with salt-free hydroxylamine. A shorter period of digestion was used in the present

TABLE II

ABSORBANCE RATIOS OF DEAMINATED PYRIMIDINE OLIGONUCLEOTIDES

Compound	*O.D. 250 nm* / *O.D. 260 nm*	*O.D. 280 nm* / *O.D. 260 nm*	*O.D. 290 nm* / *O.D. 260 nm*
pT	0.64	0.73	0.24
pU	0.74	0.38	0.03
(UT)p	0.69	0.51	0.13
$(U_2T)p_2$	0.81	0.54	0.18
$(UT_2)p_2$	0.74	0.64	0.21
$(U_3T)p_3$	0.77	0.47	0.08
$(U_2T_2)p_3$	0.74	0.52	0.12
$(UT_3)p_3$	0.76	0.68	0.26
$(U_4T)p_4$	0.71	0.47	0.07
$(U_3T_2)p_4$	0.69	0.53	0.11
$(U_2T_3)p_4$	0.68	0.60	0.16
$(UT_4)p_4$	0.66	0.66	0.20

work than was reported previously[2]. Approximately 25 absorbance units (260 nm) of each oligonuleotide were dissolved in 0.5 ml of 7.5 *N* hydroxylamine adjusted to pH 10 with dilute ammonium hydroxide. The reaction tubes were not sealed during 2 h of gentle mixing at 37°C. The contents of each tube were freeze-dried. The residues were solubilized with small portions of water and evaporated to dryness *in vacuo*. This procedure was repeated until all hydroxylamine was removed and a vitreous residue remained. The residues were dissolved in 0.5 ml water and shaken with several drops of benzaldehyde for several hours[25]. Excess benzaldehyde and benzaldoxime were removed by ether extraction. The aqueous phase was evaporated to dryness.

The hydroxylaminolysis degradation products of the (dU_ndT_m) compounds were now hydrolyzed with 0.25 ml of 0.1 *M* sulfuric acid at 100°C for 35 min to catalyze the β-elimination of thymidine derivates[26,27]

Chromatographic analysis for thymidine derivatives

Two dimensional descending solvent partition chromatography on sheets of Whatman 1 filter paper adequately separated the products derived from the series of reactions which we have just described. Preliminary washing of the filter paper (46 cm × 57 cm) sequentially with 1 *N* hydrochloric acid, distilled water, and 95% ethanol was done prior to their use. Portions of a neutralized sulfuric acid digest corresponding to 30–50 μg nucleotide phosphorus were applied to the filter paper and developed with *n*-propanol–2 *N* HCl (3:1) for 18 h. The papers were dried in air, rotated 90° and developed for 18 h with a buffered isobutyrate solvent mixture, pH 3.6, isobutyric acid–0.5 *M* ammonium hydroxide (5:3). Areas of ultraviolet absorption were excised, extracted with standard volumes of 0.01 *N* hydrochloric acid (3–5 ml) and quantitatively estimated spectrophotometrically. (Our results have been calculated using the following molar extinction data: deoxyuridylic acid, $\Delta\varepsilon_{260} - \varepsilon_{290} = 9700$; uracil, $\Delta\varepsilon_{260} - \varepsilon_{290} = 7410$; thymidylic acid, $\Delta\varepsilon_{260} - \varepsilon_{300} = 8620$; thymine, $\Delta\varepsilon_{260} - \varepsilon_{290} = 6730$.)

Analyses of reaction products

Control compounds, UpU and TpT were processed through the hydroxylamine and acid treatment which has been described. Changes in the proportion of organic phosphorus and recovery of chromophores were estimated. The only measurable change for TpT was a 0.8% conversion of organic P to inorganic P. For UpU a 60% decrease in the ultraviolet absorption maximum of deoxyuridine coincided with a 47% conversion of organic P to inorganic P. These values reflect the extent of destruction of the uracil chromophore by hydroxylamine and the subsequent acid-catalyzed β-elimination process which produces inorganic P[2]. Longer periods of exposure to hydroxylamine (3–5 h) cause complete destruction of the uracil chromophore. While the refractory nature of the thymine chromophore is still evident under similar treatment, unacceptable levels of phosphate ester hydrolysis occurs, *i.e.* 10%. Therefore, in the experiments reported here the shorter 2-h exposure to hydroxylamine has been employed. We have also assumed that the hypochromism in pyrimidine oligonucleotides is negligible[8].

RESULTS AND DISCUSSION

The results of our analyses are included in Table III which lists the yields of thymidine derivatives expected from the acid catalyzed β-elimination degradation of the chemically modified (dC_n,dT_m) oligonucleotides. An estimation of the proportion of sequence isomers from each set of data is not difficult (Table IV). For example: The original pyrimidine oligonucleotide (dC_2,dT_2) is composed of six isomers; 1,

TABLE III

THYMIDINE DERIVATIVES RELEASED FROM SEQUENCE ISOMERS

Serial treatment by nitrous acid, hydroxylamine and sulfuric acid are described in Materials and methods.

Gross composition of initial components	*Molar recovery of thymidine derivatives**								*Others*
	pT	*Tp*	*pTp*	*pTpT*	*TpTp*	*pTpTp*	*pTpTpT* + *TpTpTp*		
(dC,dT)	61	39	–**	–	–	–	–	–	
(dC_2,dT)	68	19	12	–	–	–	–	–	
(dC,dT_2)	33	29	–	29	10	–	–	–	
(dC_3,dT)	46	22	32	–	–	–	–	–	
(dC_2,dT_2)	31	17	11	21	12	8	–	–	
(dC,dT_3)	27	16	–	14	23	–	20		
(dC_4,dT)	41	36	23	–	–	–	–	–	
(dC_3,dT_2)	36	21	16		22§	9	–	–	
(dC_2,dT_3)	22	19	9		20§	9	21		pTpTpTp***
(dC,dT_4)	22	12	–	13	11	–	30		pTpTpTpT + TpTpTpTp 13

* Corrected to 100%.

** Indicates not expected and not observed.

*** pTpTpTp expected but not observed.

§ Indicates summation of pTpT and TpTp.

TABLE IV

ESTIMATED PROPORTIONS OF SEQUENCE ISOMERS

Gross composition of initial component	*Percent contribution of each isomer unit*
(dC,dT)	CpT(61), TpC(39)
(dC_2,dT)	CpCpT(68), CpTpC(12), TpCpC(19)
(dC,dT_2)	CpTpT(41), TpCpT(44), TpTpC(14)
(dC_3,dT)	CpCpCpT(46), CpCpTpC + CpTpCpC(32), TpCpCpC(22)
(dC_2,dT_2)	CpCpTpT(30), CpTpCpT(16), TpCpCpT(26), TpCpTpC(10), TpTpCpCp(17), CpTpTpC(11)
(dC,dT_3)	CpTpTpT + TpTpTpC(60), TpCpTpTp(15), TpTpCpT(25)
(dC_4,dT)	CpCpCpCpT(41), CpCpCpTpC + CpCpTpCpC + CpTpCpCpC(23), TpCpCpCp(36)
(dC_3,dT_2)	CpCpCpTpT(22), CpCpTpTpC + CpTpTpCpC(13), TpTpCpCpC(10), CpCpTpCpT + CpTpCpCpT(22), TpCpCpCpT(30), TpCpCpTpC + TpCpTpCpC(2), CpTpCpTpC (0)
(dC_2,dT_3)	CpCpTpTpTp + TpTpTpCpC(37), CpTpCpTpT + TpTpCpTpC(4), CpTpTpCpT + TpCpTpTpC(16), TpCpTpCpT(12), TpCpCpTpT + TpTpCpCpT(31), CpTpTpTpC(0)
(dC,dT_4)	CpTpTpTpT + TpTpTpTpC(22), TpCpTpTpT(20), TpTpTpCpT(37), TpTpCpTpT(20)

CpCpTpT; 2, CpTpCpT; 3, TpCpCpT; 4, TpCpTpC; 5, TpTpCpC; and 6, CpTpTpC. Upon serial dephosphorylation (to remove terminal phosphate groups), deamination (to convert cytosine to uracil), hydroxylaminolysis (to destroy the uracil chromophore) and acid treatment (to catalyze the β-elimination reactions), the folowing thymidine derivatives would be produced: 1, pTpT; 2, pTp = pT; 3, Tp = pT; 4, Tp = pTp; 5, TpTp; and 6, pTpTp, respectively. Isomers 2, CpTpCpT; 3, TpCpCpT; and 4, TpCpTpC have derivatives in common and the proportion of isomers 2, 3 and 4 relies on algebraic solution of the following set of equations

$$31x + 17y = 1$$

$$31(1-x) = 17(1-y)$$

employing the data from table III. Where possible, similar calculations have been made and the proportion of isomers for each structure has been estimated.

For the unit (dC_2,dT_3), the thymidine derivative pTpTpTp would be expected on the basis of a random distribution of linear events. This compound was not observed. Similarly, for the unit (dC,dT_4), components pTpTpTpT and TpTpTpTp were expected but not observed. In several analyses the fragment pairs pTpT + TpTp and pTpTpT + TpTpTp were not chromatographically resolved. The experimental data, therefore, did not always allow a unique solution for the relative frequency by each isomer.

The elegant reversed-phase HPLC separation described by Schott and Eckstein[14] has allowed extensive tabulation of the relative proportion of sequence-isomeric pyrimidine oligonucleotides in herring sperm DNA. There had been a paucity

of data prior to those analyses. Some data, notably that of Peterson and co-workers[7,23] show a CpT to TpC ratio in calf thymus DNA ranging from 1.33 to 1.55. This compares to our ratio of 1.56. Statistically, the frequency ratio of these pyrimidine-sequence isomers should, of course, be unity. A set of data for TpC/CpT from five bacterial DNAs indicated no obvious correlation with the T/C composition of the DNA[23,28]. Another unexpected aspect of these analyses was that the TpC/CpT isomer proportion form calf thymus and herring sperm DNA; having identical nucleotide composition; were not similar. We prefer, therefore, not to compare the data from calf thymus DNA reported in this communication with the unique HPLC data from herring sperm DNA. Those HPLC analyses for units of length two to five demonstrated the presence of most of the pyrimidine sequence isomers in DNA. The pyrimidine run, (dC_4,dT) was not observed on HPLC although its occurrence has been documented by us and others for eukaryote and prokaryote DNA[16,29].

The introduction of 5-methyl deoxycytidylic acid as a third component in pyrimidine oligonucleotide tracts would show a complex HPLC elution profile whose components would be more difficult to fully separate and quantitate. Although animal DNA contains a low proportion of this minor component, plant DNA contains nearly ten nucleotide percent of methylcytidylic acid[30]. Based on our results and on work in progress we believe it is possible to distinguish between cytosine and methylcytosine loci within pyrimidine oligonucleotides by a modification of the procedure employed for chemical deamination of DNA. Under appropriate conditions, 3 *M* sodium bisulfite deaminates cytosine to uracil while methylcytosine, adenine and guanine are refractory to this reagent[31,32]. Incorporation of this slective procedure into the protocol described in this communication would allow an estimation of the proportion of sequence isomers of small pyrimidine oligonucleotides composed of thymine, cytosine and methylcytosine.

We have continued to explore the differential chemical stability of DNA in an effort to obtain maximum information of some features of nucleotide arrangement. Integration of HPLC separation techniques with the chemical manipulations discussed here would extend analytical capabilities for the estimation of sequence isomers of more complex pyrimidine oligonucleotides.

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SEPARATION OF THE ENANTIOMERS OF FLUAZIFOP AND OTHER 2-PHENOXYPROPIONIC ACIDS USING CHIRAL METAL CHELATE ADDITIVES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Most of the work on the use of chiral ligand-exchange systems in reversed-phase liquid chromatography has been focussed on the resolution of the enantiomers of amino acids. This paper describes the novel use of L-prolyl-*n*-octylamide-Ni(II) in the mobile phase for the resolution of the enantiomers of fluazifop and other phenoxypropionic acids.

INTRODUCTION

Several approaches have been used for the separation of enantiomers by high-performance liquid chromatography (HPLC). These include chiral bonded phases on silica, chiral ligand-exchange bonded phases and the addition of chiral selective reagents to the mobile phase. The applicability of these methods for enantiomer resolution depends on the functional groups present on the compound of interest. The chiral bonded phases described by Pirkle are generally applicable to a broader range of compounds than the chiral ligand-exchange systems, where most of the work has been focussed on the resolution of the enantiomers of amino acids.

Using the Pirkle type 1A columns[1,2] we have separated the enantiomers of the selective herbicide fluazifop butyl (I) and its major metabolite fluazifop (II), as its methyl ester. The Pirkle column is an amino propyl derivatised silica modified to give chiral resolution by ionically bonding the chiral acid (D)-N-(3,5-dinitrobenzoyl) phenylglycine. This produces a stable stationary phase as long as relatively non-polar mobile phases are used. However when using this column for the analysis of residues of fluazifop butyl (I) and fluazifop (II) on crop samples, the column performance deteriorated with concomitant loss of chiral selectivity, after 4–5 injections.

The use of chiral selective additives to the mobile phase was considered as an alternative approach. The chiral modification of commercially available reversed-phase columns by the addition of chiral ligands to the mobile phase has been described by Davankov *et al.*[3] and Karger and co-workers[4,5]. The main advantages of

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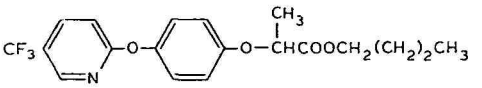
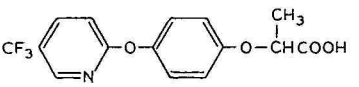


FLUAZIFOP BUTYL
(I)



FLUAZIFOP
(II)

this technique over the use of the Pirkle type 1A column is the higher stability of the chiral phase and its use with polar mobile phases. Karger has used both C_3–C_8 dien-Zn(II) and L-prolyl-*n*-octylamide-Ni(II) as the chiral metal chelate for the separation of amino acids and their dansyl derivatives. The alkyl chain (C_8) of the chiral ligand is adsorbed into the surface layer of the C_8 packing, with the ligand group on the surface available for chelation with metal ions and mobile ligands.

This paper describes the use of L-prolyl-*n*-octylamide-Ni(II) in the mobile phase for the resolution of the enantiomers of fluazifop and other phenoxy propionic acids.

EXPERIMENTAL

Equipment

High-performance liquid chromatography was performed using a Waters Assoc. 6000A pump, a U6-K manual injector and Model 440 absorbance detector. Chromatograms were recorded on a Phillips PM8251 single pen recorder.

An Ultrasphere ODS IP column was used (15 cm × 4.6 mm I.D.) supplied by Altex Scientific. The column temperature was controlled at 35°C using a Waters temperature control unit Model III.

The pH of the mobile phase was measured using an EIL 7050 laboratory pH meter.

Reagents

L-Propyl-*n*-octylamide was synthesised following the method described by Tapuhi *et al.*[5]. HPLC grade acetonitrile and methanol were supplied by Rathburn Chemicals, Walkerburn, U.K. Analar grade acetic acid, ammonia solution and nickel sulphate were supplied by BDH, Poole, U.K. Purified water was obtained through a Milli-Q water purification system supplied by Millipore U.K., Harrow, U.K.

General procedures

Mobile phases were prepared by dissolving L-prolyl-*n*-octylamide in the organic component of the mobile phase and the metal sulphate in the water component. The two components were then combined and the required volume of acetic acid added. Finally the pH was adjusted to the desired value using ammonia solution (sg. 0.880).

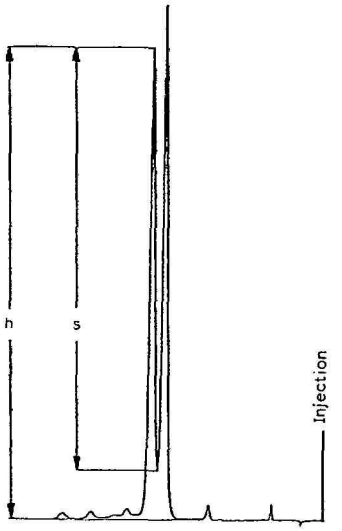


Fig. 1. Calculation of the separation factor. Separation factor = $(s/h) \cdot 100$.

The effects of the following factors were investigated on the separation of the enantiomers of fluazifop (II) as the model compound: pH, temperature, buffer concentration, solvent composition and the concentration of L-prolyl-*n*-octylamide-Ni(II) complex. The effects of other metal ions were also investigated using zinc acetate and copper sulphate as alternative salts to nickel sulphate.

The separation factor was used as a means of displaying the degree of enantiomer separation. A value of 100 indicates that complete separation has been achieved. Although the α-value [the ratio of k'(D) to k'(L)] were more sensitive to the conditions used, they do not indicate whether peak separation to the base-line has been obtained.

$$\text{Separation factor} = \frac{s}{h} \cdot 100$$

where s and h are measured as shown in Fig. 1.

RESULTS AND DISCUSSION

Lindner *et al.*[4] found that the pH of the mobile phase was the major factor affecting complex formation and therefore chiral selectivity with the dansyl derivatives of amino acids. Using C_3–C_8 dien-Zn(II) complex as the chiral additive to the mobile phase, they achieved optimum chiral resolution at pH 9. The same pH was used in work with L-prolyl-*n*-octylamide-Ni(II). Our investigations have also shown that the pH of the mobile phase is the key factor in the separation of the enantiomers of fluazifop using L-prolyl-*n*-octylamide-Ni(II) complex in the mobile phase. However with fluazifop the greatest chiral recognition occurred at pH 7.0–7.5 as shown in Fig. 2. Retention (k') of fluazifop also increased to a maximum at pH 7.0–7.5

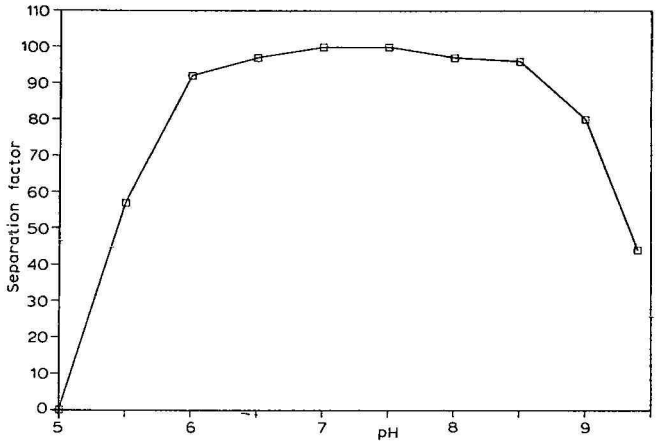


Fig. 2. Change in separation factor as a function of pH. Mobile phase: acetonitrile–methanol–water (35:15:50, v/v/v); L-prolyl-*n*-octylamide-Ni(II), 4 m*M*; ammonium acetate, 88 m*M*.

(Fig. 3). The different mobile phase pHs required for optimum chiral recognition by fluazifop and the dansyl derivatives of amino acids reflect the nature of the interactions when complexation takes place. Lidner *et al.*[4] has postulated that the dansyl derivatives of amino acids act as dianionic species in the basic mobile phase required for chiral recognition with C_3–C_8 dien-Zn(II) chelates. With fluazifop, the attraction between the anion of the carboxylic acid and the nickel cation in the L-prolyl-*n*-octylamide-Ni(II) will be the predominant effect and further coordination is probably between the ether linkage of the 2-phenoxypropionoic acid residue in fluazifop and the nickel in the L-prolyl-*n*-octylamide-Ni(II). As this involves the lone pair of electrons of the oxygens, the association will not be influenced by pH, so that the pH requirements will be governed solely by the needs for optimising the attraction be-

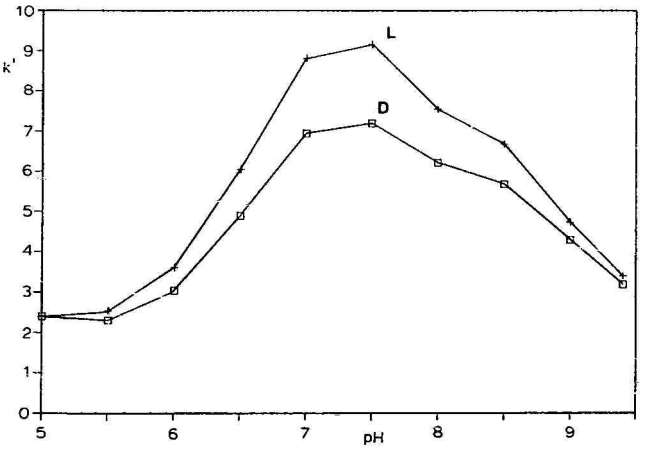
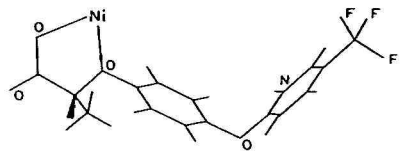
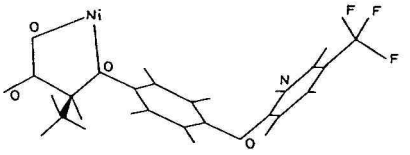


Fig. 3. Change in *k'* as a function of pH. Mobile phase as in Fig. 2.



D-Fluazifop



L-Fluazifop

Fig. 4. Structures of D- and L-fluazifop showing the location of the nickel atom in the L-prolyl-*n*-octylamide-Ni(II) complex.

tween the carboxylic acid group and the nickel in the L-prolyl-*n*-octylamide complex.

Coordination between the ether linkage of the 2-phenoxypropionic acid residue and the nickel of the L-propyl-*n*-octylamide complex allows the formation of a five membered ring (Fig. 4). Lidner *et al.*[4] found that the dansyl derivatives of amino acids that formed five membered ring complexes with C_3–C_8 dien-Zn(II) gave high k' values and greater selectivity due to the higher stability of the complex than those which formed six- or seven-membered ring complexes.

Apart from the pH of the mobile phase, other factors such as temperature, complex and metal ion concentration and organic modifier concentration had less effect on the chiral resolution.

We found that temperature had only a marginal effect on the retention of the enantiomers of fluazifop in contrast to the findings of Lidner *et al.*[4] with C_3–C_8 dien-Zn(II) complex. Over the temperature range 15–45°C k'(D) was reduced by 2% compared to 6% for the L-enantiomer of fluazifop. At 45–70°C the effect was slightly greater with k'(D) reduced by 10% and k'(L) by 11%. Chiral recognition was reduced, the value of $\alpha - 1$ falling by 19% from 15°C to 70°C. However the effect on the separation factor was very slight, the values being 100% at 15°C and 98% at 70°C. We selected 35°C as the optimal temperature for studying the effect of other factors.

Changing the metal ion to copper or zinc had very little effect. The separation of D- and L-fluazifop given by the Ni(II) and Zn(II) complexes of L-prolyl-*n*-octylamide is given in Fig. 5. The Zn(II) complex was less effective than the Ni(II) complex. Lidner *et al.*[4] had found that the elution order of the enantiomers of the dansylated amino acids was reversed on changing the metal from nickel to zinc. In our hands, the elution order for the fluazifop enantiomers was unchanged. The copper

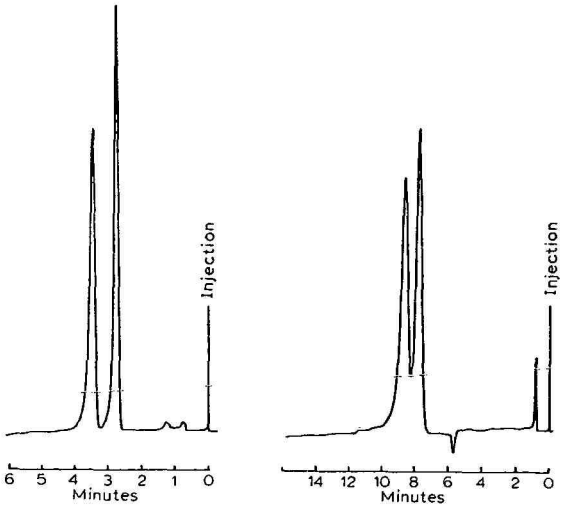


Fig. 5. Separation of the enantiomers of fluazifop (left) with mobile phase containing 4 m*M* L-prolyl-*n*-octylamide-Ni(II) and (right) with mobile phase containing 4 m*M* L-prolyl-*n*-octylamide-Zn(II).

complex gave a high background UV absorbtion, which made its use inappropriate.

Changing the mobile phase composition progressively from methanol–water (50:50, v/v) to acetonitrile–water (50:50, v/v) reduced the retention (k') of the enantiomers and increased the separation factor as shown in Figs. 6 and 7. Davenkov *et al.*[3] noted a similar effect when using N-decyl-L-histidine-Cu(II) for the resolution of α-amino acids.

In view of the improved performance given by acetonitrile–water (50:50, v/v) this mobile phase composition was used in most of the remaining investigations.

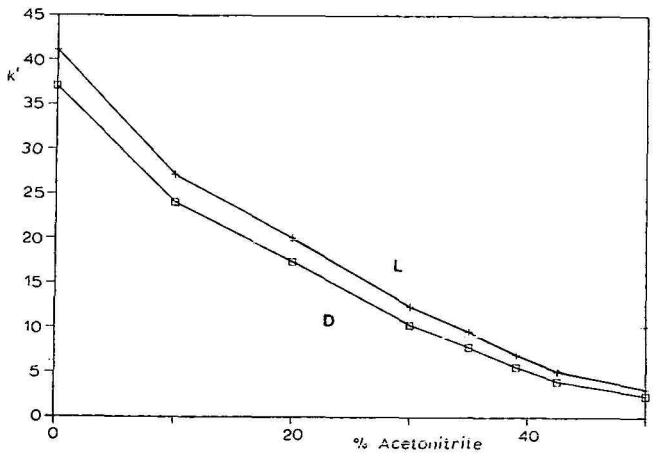


Fig. 6. Change in k' as a function of solvent composition. Mobile phase: water, 50% (v/v); L-prolyl-*n*-octylamide-Ni(II), 4 m*M*; ammonium acetate, 88 m*M*; pH 7.5.

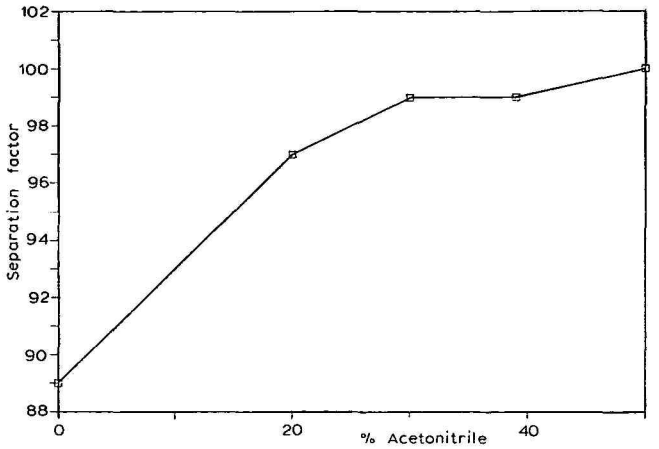


Fig. 7. Change in separation factor as a function of solvent composition. Mobile phase as in Fig. 6.

Increasing the ammonium acetate buffer concentration reduced the retention of the enantiomers of fluazifop as shown in Fig. 8. It is to be expected that the stability of the complex between fluazifop and L-prolyl-*n*-octylamide-Ni(II) would be reduced with increasing concentration of acetate ion.

$$[\text{L-propyl-Ni(II)}\ (OCOCH_3)_2] + [RCOO^-] \rightleftharpoons$$

$$[\text{L-prolyl-Ni(II)}\ RCOO\ CH_3COO] + [CH_3COO^-]$$

Over the range 5–40 m*M* ammonium acetate with the concentration of L-prolyl-*n*-octylamide-Ni(II) and 4 m*M*, the separation factor remained at 100%.

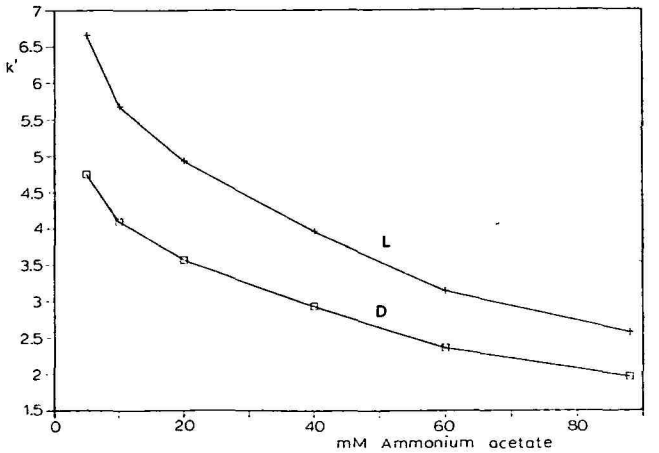


Fig. 8. Change in k' as a function of ammonium acetate concentration. Mobile phase: acetonitrile–water (50:50, v/v); L-prolyl-*n*-octylamide-Ni(II), 4 m*M*; pH 7.0.

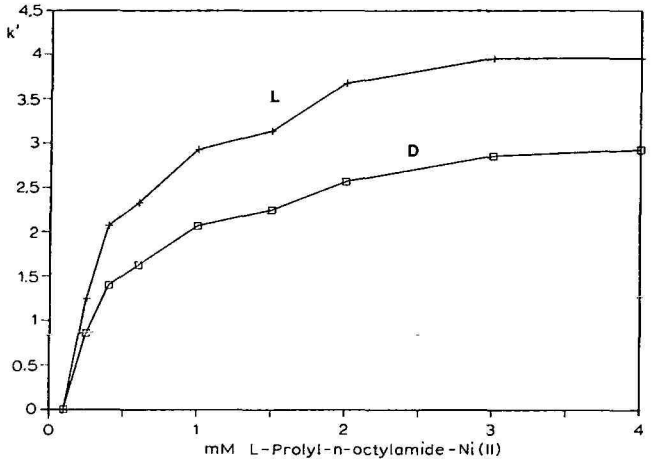


Fig. 9. Change in k' as a function of the concentration of L-prolyl-*n*-octylamide-Ni(II). Mobile phase: acetonitrile–water (50:50, v/v), ammonium acetate at 10 times the concentration of nickel complex, pH 7.0.

The effect of the concentration of L-prolyl-*n*-octylamide-Ni(II) on the retention (k') is given in Fig. 9 and on the separation factor in Fig. 10. Both k' and the separation factor increase as the concentration of the nickel complex is increased, the increase in the separation factor was very rapid over the concentration range 0.1 m*M* (zero) to 0.4 m*M* (97), after which there was a gradual increase to a value of 100 at 2.0 m*M*. When working at low concentrations of the complex (0.25–0.4 m*M*) it was necessary to pump at least 45 column volumes before the system reached equilibrium. The process was more rapid at higher concentrations of the complex.

Residues of D-fluazifop in crops have been determined satisfactorily on a

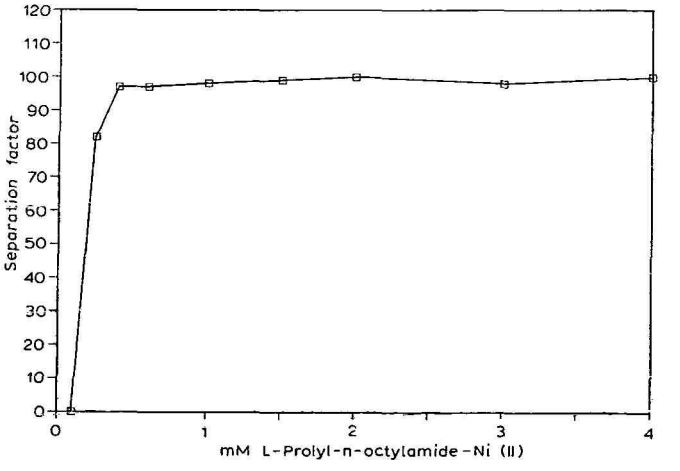


Fig. 10. Change in the separation factor as a function of the concentration of L-prolyl-*n*-octylamide-Ni(II). Conditions as in Fig. 9.

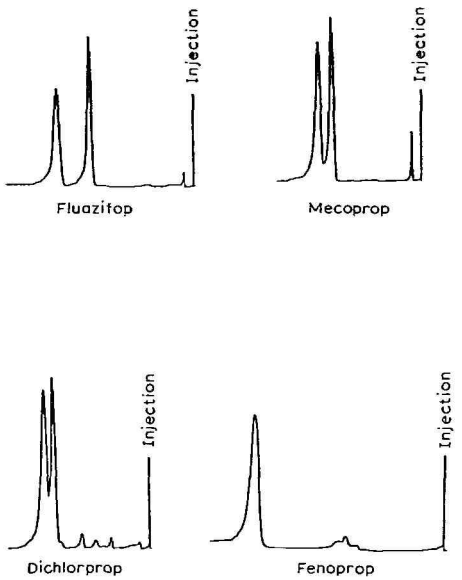


Fig. 11. Enantiomer separation for four 2-phenoxypropionic acid herbicides. Mobile phase: acetonitrile–water (40:60, v/v); L-prolyl-*n*-octylamide-Ni(II), 3.2 m*M*; ammonium acetate, 8 m*M*; pH 7.0.

routine basis by reversed-phase HPLC using L-prolyl-*n*-octylamide-Ni(II) in the mobile phase.

With suitable adjustments to the solvent ratio of the mobile phase, we were able to separate the enantiomers of 2-(4-hydroxyphenoxy)propionic acid, 2-(2-methyl-4-chlorophenoxy)propionic acid (Mecoprop), 2-(2,4-dichlorophenoxy)propionic acid (Dichlorprop), and 2-[4-(4-trifluoromethylphenoxy)phenoxy]propionic acid. We were, however, unable to separate the enantiomers of 2-(2,4,5-trichlorophenoxy)propionic acid (Fenoprop). The separation of the enantiomers of fluazifop, mecoprop and dichlorprop, together with the trace for fenoprop is shown in Fig. 11. The progressive loss of chiral recognition with increasing chlorine substitution of the phenoxy ring is believed to be due to the inductive effect of the chlorine atoms on the ion pair of electrons on the ether oxygen atoms.

ACKNOWLEDGEMENT

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DETECTION OF THE EMBRYONIC ζ CHAIN IN BLOOD FROM NEWBORN BABIES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) using the large-pore Vydac C_4 column has been used to detect and quantitate the embryonic ζ chain in blood samples of normal babies and of newborns with varying degrees of α chain deficiencies. The ζ chain eluted at the end of the chromatogram at about 130 min using a modified and extended gradient. Its identity was confirmed by structural analysis of ζ chain isolated from a blood sample of a fetus without active α globin genes, *i.e.* with hydrops fetalis (– –/– –). The quantity of ζ in normal babies is less than 0.7% [% of (α + ζ)] and is dependent upon the maturity of the baby as it was only present in babies with low levels of β chain or hemoglobin (Hb) A. The presence of a ζ globin gene deletion [A. E. Felice *et al., Hum. Genet.*, 73 (1986) 221; and P. Winichagoon *et al., Nucleic Acids Res.*,10 (1982) 5853] did not affect the level of ζ in the newborn. All babies with an α-thalassemia-2 heterozygosity, *i.e.* with three active α globin genes or –α/αα, had ζ in a range of 0.1–0.9%; again the level showed a negative correlation with that of the β chain. Newborns with an α-thalassemia-2 homozygosity or –α/–α had a varying level of ζ of 0.3–2.3%, which did not correlate with the level of β, suggesting that ζ chain production persists after birth in this condition. Macrochromatographic analyses in combination with RP-HPLC indicated that the ζ chain is present as $\zeta_2\gamma_2$ or Hb Portland-I, as expected.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) has become an important tool in the study of the minor globin chains that may be present in lysates of red blood cells from human newborns and adults. Recently, we identified the $^{M}\gamma$ chain as a newly discovered γ globin chain which resembled the $^{A}\gamma$ chain of the fetal hemoglobin (Hb F) but with a Leu→Met substitution at position γ^{141} (refs. 1 and 2). This $^{M}\gamma$ chain was found, among others, in blood samples from numerous subjects with different forms of hereditary persistence of Hb F (HPFH) and in patients with sickle cell anemia[1,2]. During that study, we observed two minor components (peaks) in extended HPLC chromatograms of newborn red cell lysates. One

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of these was suspected of being the embryonic ζ chain as it occupied a position at the end of the chromatogram, comparable to that reported by others using somewhat different HPLC systems[3–5]. This ζ chain is closely related to the α chain in primary structure (the ζ chain, like the α chain, is 141 amino acids long but differs at 57 positions[6]). It is mainly present during the first three months of fetal life, after which it is replaced by α. Its synthesis is regulated by one ζ globin gene which is located about 3.5 kilobases (kb) 5′ to a nonfunctional pseudo (ψ) ζ globin gene, and about 8 kb 5′ to the two α globin genes on the short arm of chromosome No. 16 (refs. 7 and 8). Hb Portland-I or $\zeta_2\gamma_2$ (ref. 9) has been found in considerable quantities in blood of newborn babies without active α globin genes, *i.e.* hydrops fetalis, and to a lesser extent in newborns with less severe α chain deficiencies.

This communication describes the identification of the minor zone, its occurrence in red cell lysates from newborn babies with and without different types of α chain deficiency, and its quantitative relationship with the level of the β chain in these cell lysates, assuming this to be some measure of fetal maturity[10].

MATERIALS AND METHODS

Blood samples

Cord blood samples were collected in vacutainers with EDTA as anticoagulant, and transported within one week to the laboratory in Augusta, GA, U.S.A. The samples were from normal babies without a detectable hemoglobinopathy except for the presence of variable quantities of Hb Bart's (γ_4). Most babies were born in local hospitals or in hospitals within the State of Georgia. A few samples were received from institutions in cities on the west coast of the United States, and from Nanning, People's Republic of China. Most babies were Black, some were Caucasian, and a few were Chinese or Vietnamese. Consent to collect the samples was obtained.

Hb analyses

All samples were analyzed by cellulose acetate electrophoresis at alkaline pH; samples with an α or β or γ chain variant were not included in the study. The presence of Hb Bart's (γ_4) was indicated for each sample as negative or undetectable, as small (less than *ca.* 5%), as large (5–10%), and high (over 10%). Quantitation of Hb Bart's was by microcolumn chromatography, as described before[11].

RP-HPLC

The method of Shelton *et al.*[12] was used for the separation of the globin chains. About 50 μg Hb was applied onto a 250 × 4.1 mm I.D. Vydac C_4 column, and the chromatogram was developed with a gradient between solution A [acetonitrile–water (60:40) with 0.1% trifluoroacetic acid (TFA) in final concentration] and solution B [acetonitrile–water (20:80) with 0.1% TFA]. The first gradient was 50% A to 60% A in 80 min, and the second was 60% A to 78% A in 60 min at a flow-rate of 1 ml/min.

Preparative RP-HPLC made use of a Bio-Sil TSK-ODS-120T (C_{18}) column (300 × 21.5 mm I.D.). The amount of Hb applied varied from 6 to 8 mg. The developers were developer A or acetonitrile–water (52:48) (0.1% TFA), developer B or acetonitrile–water (35:65) (0.1% TFA) and developer C or acetonitrile–water

(60:40) (0.1% TFA). The gradients applied were 57% A (+ 43% B) to 69% A (+ 31% B) in 150 min, followed by 69% A (+ 31% B) to 85% A (+ 15% B) in 70 min, and 74% C (+ 26% B) to 78% C (+ 22% B) in the last 80 min. The flow-rate was maintained at 6 ml/min.

Isolated ζ chain was digested with trypsin (TPCK Trypsin, Worthington, U.K.) overnight at pH 8.9 and at 37°C. The resulting soluble peptides were separated by RP-HPLC[13] and their amino acid compositions determined with an automated Beckman Spinco Model 121M amino acid analyzer. Data obtained were compared with compositions of peptides as provided in refs. 6 and 14.

In some experiments, the Hbs in red cell lysates were chromatographed on 30 × 2 cm I.D. DEAE-cellulose columns as described before[15]. The various Hb components (Hb A, Hb F, and the minor Hb components which eluted behind Hb F) were concentrated by filtration under pressure, and next analyzed by starch gel electrophoresis[16], and by RP-HPLC on the Vydac C_4 column (see above).

DNA analyses

DNA was isolated from white blood cells using the method of Poncz *et al.*[17]. The number of α and ζ globin genes was determined as described before[18,19]. In these gene mapping procedures, use was made of the following restriction enzymes: Eco RI, Bam HI, Xba I, Bgl II, and Hind III, and of the following two probes: 1.5 kb Pst I fragment pRBα1 and a 0.5 kb Pst I fragment pHζcDNA. A normal condition with four α globin genes will be indicated as αα/αα; any α-thal-2 trait as −α/αα; any α-thal-2 homozygosity as −α/−α (including double heterozygosity for the two different types of α-thal-2, −4.2 kb and −3.7 kb); any α-thal-1 heterozygosity as − −/αα; an Hb H disease as − −/−α; and hydrops fetalis or α-thal-1 homozygosity as − −/− −. Abnormalities in ζ globin genes were rare; when observed, these will be mentioned for each individual case.

RESULTS

Chromatographic detection of the ζ chain

Fig. 1 compares five chromatograms of red cell lysates from newborn babies without and with an increasingly severe α globin gene deficiency, as indicated. All five chromatograms have a (minor) peak, identified as ζ, which elutes between 125–130 min. The ζ zone is preceded by an unknown component, marked X in Fig. 1. The quantity of ζ varies considerably; when expressed as % of (α + ζ) it is present from 0.6 to 100% in the baby with hydrops fetalis (additional quantitative data are given later). The presence of the ζ chain is particularly marked in the babies with only two (or less) active α globin genes. Baby No. 9279, with an α-thal-2 homozygosity (−α/−α), had 1.74% ζ [as % of (α + ζ)] and 9.1% Hb Bart's or γ_4 (as % of total Hb); baby No. 9453 with a Hb H disease (− −/−α) had 4.36% ζ and 15% Hb Bart's; and the hydrops fetalis baby HF, without any active α globin genes (− −/− −), had 100% ζ and nearly 80% Hb Bart's. Thus, the level of the ζ chain, like that of the α chain lacking Hb Bart's[20], is a measure of the severity of the α chain deficiency. The presence of a notable quantity of ζ in red cells of normal baby No. 10903 (αα/αα) and baby No. 11655 with an α-thal-2 trait (−α/αα) is of interest; data to be presented below will show that the ζ chain is detectable in about 50% of all normal newborns, and in all babies with the α-thal-2 heterozygosity.

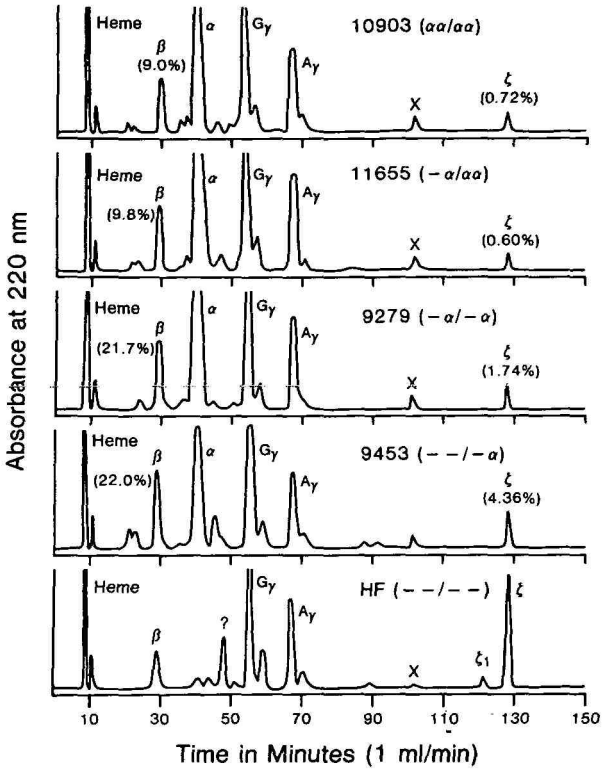

Fig. 1. Separation of globin chains by RP-HPLC using a large-pore Vydac C_4 column. All five samples are from newborn babies with the stated α globin gene deficiencies (HF = baby with hydrops fetalis). Several known chains are identified as α, ζ, β, or $^{G}\gamma$ and $^{A}\gamma$ chains. The %β is calculated as % of (β + γ); the %ζ is also calculated as % of (α + ζ).

Identification of the ζ chain

A larger quantity of the ζ chain was isolated from the red cell lysate of the hydrops fetalis baby HF (– –/– –) using the larger Bio-Sil TSK-ODS-120T (C_{18}) column. Fig. 2 illustrates the separation that was obtained; material from the ζ peak (protein zone No. 10) of four such chromatograms was combined for use in the structural analyses. Peptides from a tryptic digest were separated and isolated by RP-HPLC (data not shown), and their amino acid compositions were determined. These data, summarized in Table I, offer convincing evidence to conclude that the protein present in the ζ peak is indeed the embryonic ζ globin chain. It is, therefore, assumed that the components observed in identical position in HPLC chromatograms of other red cell lysates are the same as this embryonic chain.

Isolation of the ζ chain as Hb Portland-I

Both CM-cellulose and DEAE-cellulose macrochromatography were used for this purpose; data from DEAE-cellulose chromatograms were most informative. One such chromatogram is shown in Fig. 3. The sample used was cord blood red cell lysate from a baby with an α-thal-2 homozygosity (–α/–α), which contained 9.1% Hb

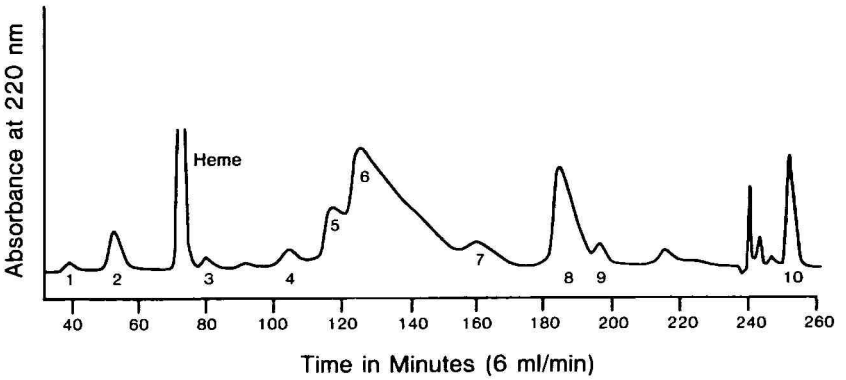

Fig. 2. Separation of globin chains on a preparative RP-HPLC column (C_{18}). Approximately 9 mg Hb from baby HF was applied. Several protein zones (Nos. 1, 2, 3, 4, and 5) remain unidentified. The chromatogram should be compared with that obtained on an analytical C_4 column given in Fig. 1. Material from protein zone No. 10 was used for structural analysis.

Bart's or γ_4. Besides the expected Hb zones A and F_0, three minor zones (labelled 1, 2 and 3) were isolated, which were assumed to contain Hb F_1 or fetal Hb with acetylated γ chains[15] and the α chain lacking Hb Bart's or γ_4 (ref. 20). Starch gel electrophoresis (insert of Fig. 3) identified component No. 1 (present for 9.7%) as being mainly Hb A like (Hb F_1 has a mobility similar to Hb A in this system), and component No. 3 (present for 6.6%) as being primarily the fast-moving Hb Bart's.

TABLE I

AMINO ACID COMPOSITION OF SOLUBLE TRYPTIC PEPTIDES OF THE ζ CHAIN*

Amino acid	*T-2*	*T-4*	*T-5*	*T-6*	*T-7*	*T-8***	*T-9*	*T-15*
Aspartic acid		1.00(1)		1.14(1)		1.63(1)	2.22(2)	
Threonine	0.83(1)	3.22(4)	1.01(1)	0.97(1)				
Serine		0.95(1)	0.86(1)	1.28(1)	0.88(1)		1.95(2)	
Glutamic acid	1.03(1)	2.60(3)	1.12(1)	1.00(1)				
Proline			0.99(1)	2.03(2)				
Glycine		1.00(1)		1.14(1)	1.09(1)	1.24(1)	1.78(2)	
Alanine		1.00(1)		0.91(1)	0.93(1)	3.08(3)	1.03(1)	
Valine						3.03(4)		
Isoleucine		1.71(2)					1.93(2)	
Leucine		0.94(1)	2.06(2)	2.10(2)			1.03(1)	
Tyrosine				0.98(1)				0.98(1)
Phenylalanine			0.95(1)	1.71(2)				
Histidine			0.89(1)	2.27(2)	0.81(1)			
Lysine			1.00(1)		1.29(1)	1.00(1)	1.07(1)	
Arginine	1.17(1)	1.00(1)		0.90(1)				1.02(1)
Residues in ζ Chain	5–7	17–31	32–40	41–56	57–61	62–71	72–82	140–141

* In moles/peptides. Peptides 1, 3 and 13 were not isolated in pure form while peptides T-10, T-11, T-12, and T-14 were not observed in the HPLC chromatogram. Values between parentheses are expected numbers of residues from refs. 6 and 14.

** Rather impure; valine value is low due to Val–Val bond.

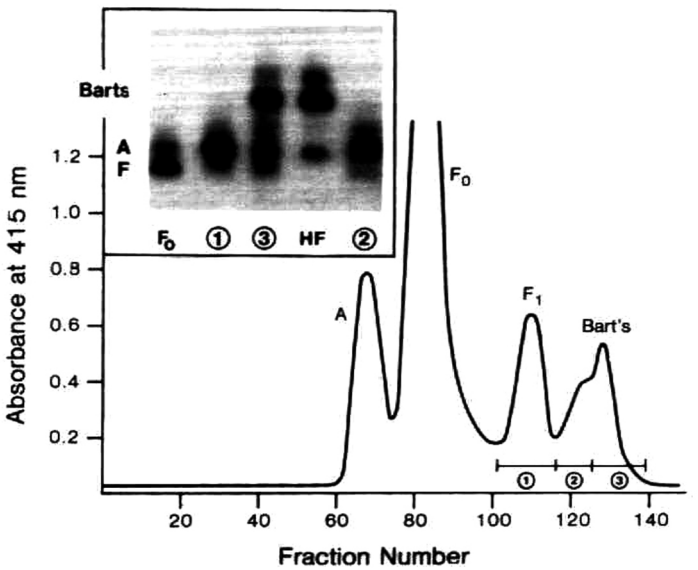

Fig. 3. Separation of Hb components by DEAE-cellulose macrochromatography. About 60 mg Hb was applied on a 30 × 2 cm I.D. column. The sample was from baby No. 9279 with an α-thal-2 homozygosity (−α/−α) and 9.1% Hb Bart's. Zones A, F_0, No. 1, No. 2, and No. 3 were isolated, concentrated, and analyzed by starch gel electrophoresis (insert), and by RP-HPLC (Fig. 4).

Component No. 2 (present for 4.4%) eluted as a shoulder, slightly ahead of component No. 3; its major Hb component had a mobility slightly faster than Hb A (insert of Fig. 3).

Considerably more information was obtained from the RP-HPLC chromatograms of the isolated zones (Fig. 4). Hb A (first chromatogram) was highly impure and contained both Hb A ($\alpha + \beta$) and Hb F ($\alpha + {}^{G}\gamma + {}^{A}\gamma$). The isolated Hb F_0 zone, however, was nearly pure; it contained nearly no β chain (second chromatogram). The ζ chain was absent. The Hb of zone No. 1 (third chromatogram) was a complex mixture; besides α chain it contained primarily modified β chain [β_1 is presumably glycosylated β (ref. 15)] and modified γ chains ($^{G}\gamma_1$ and $^{A}\gamma_1$ are presumably the acetylated $^{G}\gamma$ and $^{A}\gamma$ chains). Normal β, $^{G}\gamma$, and $^{A}\gamma$ were also present, as was a small quantity of ζ [1.3% as % of ($\zeta + \alpha$)]. Zone No. 2 (fourth chromatogram) contained nearly no modified β and γ chains but α and ζ [ζ was about 1/3 of ($\alpha + \zeta$)] and $^{G}\gamma$ and $^{A}\gamma$, suggesting the presence of Hb F ($\alpha_2\gamma_2$), of Hb Portland-I ($\zeta_2\gamma_2$), and perhaps of a hybrid Hb ($\alpha\zeta\gamma_2$). Zone No. 3 (fifth chromatogram) contained primarily $^{G}\gamma$ and $^{A}\gamma$ (presumably in the form of Hb Bart's) with only small amounts of α and ζ chains. Based on the relative quantities of the Hb zones Nos. 1, 2 and 3, and the ζ chain percentages in these zones, it was possible to calculate the quantities of the ζ chain in each zone and in the total red cell lysate; the latter value [as mg ζ/100 mg ($\alpha + \zeta$)] was 1.65 mg, which is quite similar to the 1.74% observed in the whole red cell lysate of this newborn (third chromatogram of Fig. 1). It is worth noting that component X (Fig. 1) did not appear in the chromatograms of the isolated Hb fractions, although some material eluted at 90–95 min in some of the chromatograms (Fig. 4).

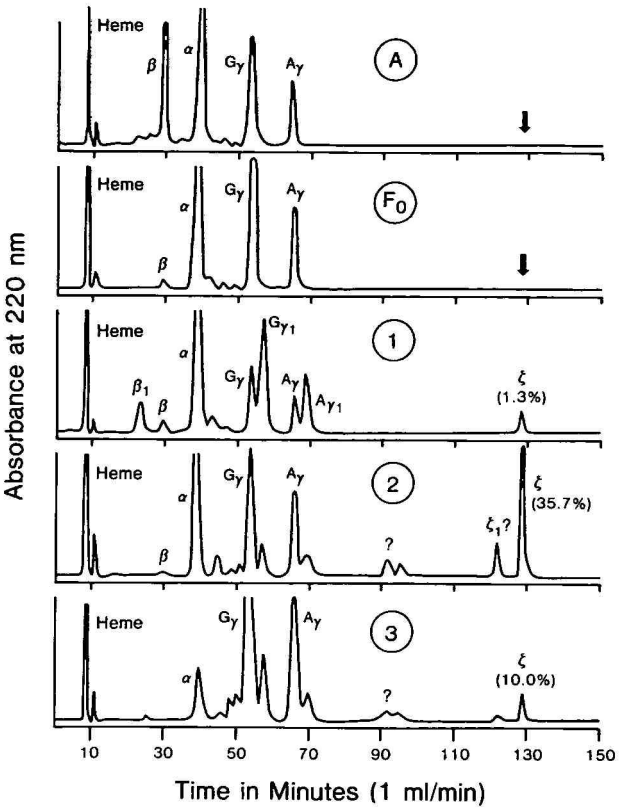

Fig. 4. Separation of globin chains by RP-HPLC using a large-pore Vydac C_4 column. The five samples are isolated Hb fractions from the DEAE-cellulose chromatogram shown in Fig. 3. Known chains are appropriately identified. The %ζ chain is calculated as % of (α + ζ).

TABLE II

RECOVERY OF THE ζ CHAIN FROM MINOR HEMOGLOBIN FRACTIONS ISOLATED BY DEAE-CELLULOSE CHROMATOGRAPHY

Case	*Condition**	*Bart's***	*%ζ Chain****			
			Fraction			*Hemolysate*
			1	*2*	*3*	
10395	−α/αα	1.7	0.4	4.9	2.5	0.61
11265	−α/αα	2.5	0	0.9	0	0
11244	−α/−α	5.6	0.2	11.2	3.5	0.46
8476	−α/−α	5.6	1.0	12.6	10.2	0.76
8550	−α/−α	8.9	0.3	7.6	2.2	0.98
9279	−α/−α	9.1	1.3	35.7	10.0	1.74
9453	− −/−α	15.0	4.4	25.4	12.5	4.36

* See text.
** By microcolumn chromatography.
*** $\%\zeta = 100 \cdot \zeta/[\alpha+\zeta]$.

Similar experiments were conducted for six additional cord blood samples (two from babies with $-\alpha/\alpha\alpha$; three from babies with $-\alpha/-\alpha$; one from a newborn with Hb H disease or $--/-\alpha$). The data, summarized in Table II, are similar to those obtained for baby No. 9279. Fraction No. 2 contained the ζ chain, but incomplete separation on the DEAE-cellulose column often resulted in a considerable overlap with the other minor Hb zones.

Quantitative aspects

Quantitative data were collected for 31 newborns with four active α globin genes ($\alpha\alpha/\alpha\alpha$), 19 with an α-thal-2 trait ($-\alpha/\alpha\alpha$), and 19 with an α-thal-2 homozygosity ($-\alpha/-\alpha$). All these babies were Black and lived in various communities in Georgia (U.S.A.). Eight of the 31 normal newborns had an additional ζ gene deletion. This genetic anomaly concerns a deletion of an 11 kb DNA fragment between the ζ and $\psi\zeta$ genes; the resulting chromosome carries one ζ globin gene with its 5′ end derived from the ζ gene and its 3′ end from the $\psi\zeta$ gene[19,21]. All babies with the α-thal-2 heterozygosity or homozygosity had the normal ζ globin gene arrangement. DNA data to support these results will not be presented here but will be published at a later time.

Fig. 5 compares the $\%\zeta$ with the level of Hb Bart's for all 69 newborns. Eleven of the 23 normal babies ($\alpha\alpha/\alpha\alpha$; no ζ deletion) had no detectable ζ chain, in four this level was 0.1% (*i.e.* the lower limit of detection), while an average value of 0.35% (range 0.2–0.7%) was observed for the remaining eight babies. All eight normal babies with a ζ globin gene deletion had a detectable ζ chain peak; its quantity averaged 0.3% (range 0.1–0.5%). These values were slightly higher for the 19 babies with the

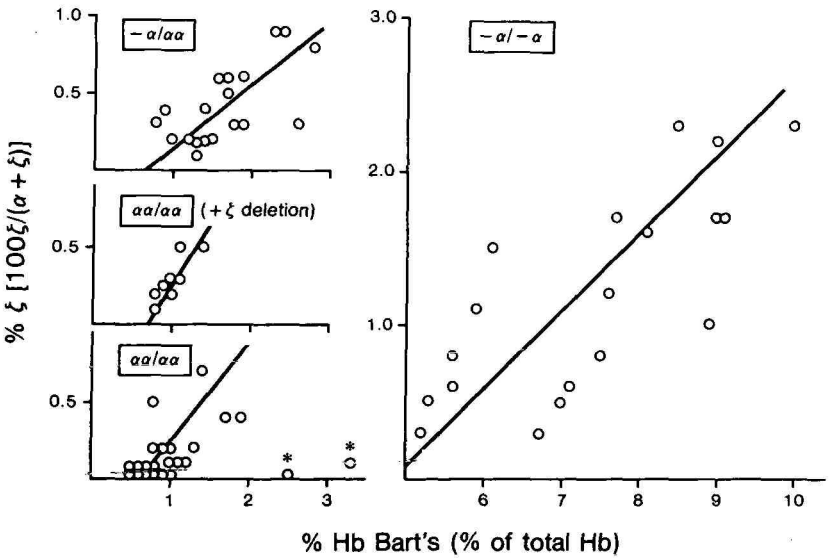

Fig. 5. The relationship between the levels of Hb Bart's (γ_4) and the ζ chain in blood from normal newborn babies, normal newborn babies with a ζ globin gene deletion, and newborns with an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$) or homozygosity ($-\alpha/-\alpha$). The samples marked with an asterisk (*) were stored for two months prior to the analyses; the "Hb Bart's" level is probably too high, due to contaminants formed during storage.

α-thal-2 heterozygosity (–α/αα); the average value was 0.42% with a range of 0.1–0.9%. Considerably larger percentages were observed for the 19 babies with the α-thal-2 homozygosity (–α/–α) as their ζ level averaged 1.20% with a range of 0.3–2.3%. A direct correlation was present between the %ζ and the % Hb Bart's for all four groups of babies. This is not surprising because the Hb Bart's fraction, isolated by microchromatography, is a mixture of several proteins including Hb Bart's or γ_4, Hb Portland-I or $\zeta_2\gamma_2$, and some non-Hb proteins (the latter are responsible for about 0.5–0.8% of the "Hb Bart's" level).

Fig. 6 illustrates a possible relationship between the percentages of ζ chain [as % of (α + ζ)] and β chain [as % of (β + γ)] in the cord blood samples. The % β chain was readily calculated from the data provided by the RP-HPLC chromatogram (Fig. 1) and is considered to be a measure of fetal maturity. The data show higher ζ percentages at lower levels of β chain. Normal babies (αα/αα) with 15–20% β chain or higher, appear not to have ζ chain, while this value is slightly higher (25–30%) for babies with an α-thal-2 heterozygosity (–α/αα). Such a relationship was not evident for the babies with an α-thal-2 homozygosity (–α/–α).

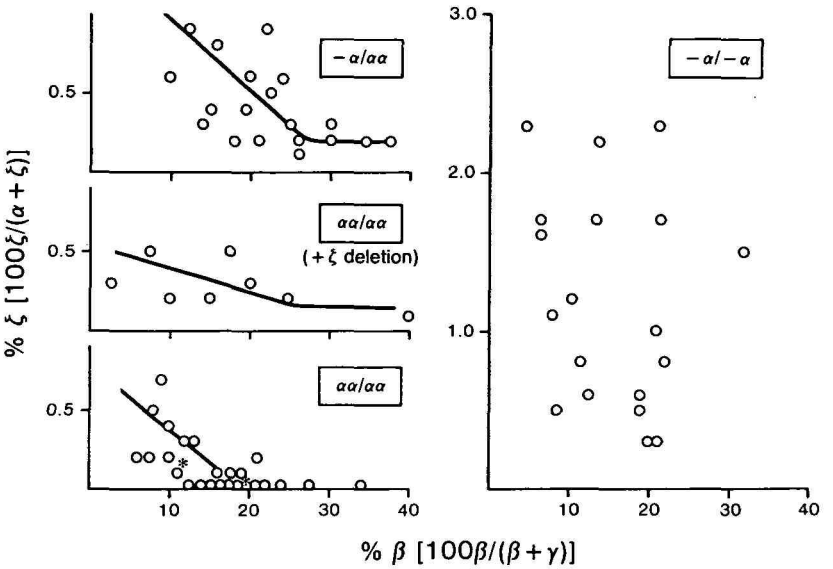

Fig. 6. The relationship between the levels of β chain and ζ chain in blood from normal newborn babies, normal newborn babies with a ζ globin gene deletion, and newborns with an α-thal-2 heterozygosity (–α/αα) or homozygosity (–α/–α). For the two samples with an asterisk (*) see legend of Fig. 5.

DISCUSSION

This study has offered structural and chromatographic evidence for the presence of variable quantities of the embryonic ζ chain in cord blood samples from all newborn babies with a mild to severe α chain deficiency and in many normal newborns. Its position in the RP-HPLC chromatogram is unique as it elutes very late, confirming earlier observations made with a different form of HPLC[3–5]. This ζ chain

is probably present in newborn red cells as Hb Portland-I or $\zeta_2\gamma_2$, which has specific chromatographic and electrophoretic properties (Fig. 3). The presence of this Hb in some minor Hb fractions, isolated from cord blood lysates by macrochromatography, has been difficult to evaluate because of its low quantity; identification of the globin chain composition by RP-HPLC, however, readily located the minute Hb Portland-I component. Theoretically, one can also expect the presence of Hb Portland-II or $\zeta_2\beta_2$ (ref. 4); its quantity in many cord blood red cell lysates, however, will be too low to allow its detection. A second peak (labelled X in Fig. 1) which elutes about 25 min ahead of the ζ chain was not detected in the Hb fractions isolated by DEAE-cellulose chromatography (Fig. 4). It may well be that it represents one of the many non-Hb proteins present in red cell lysates; it appears to be present in cord blood samples as well as in various lysates of adult red blood cells (data not shown).

Variable amounts of Hb Bart's (γ_4) are found in red cells of babies with the different forms of α-thalassemia, and microcolumn chromatography has been applied for its quantitation[11]. The fast-moving Hb zone, isolated by this method, which is comparable to the peaks 2 and 3 isolated by macrocolumn DEAE-cellulose chromatography (Fig. 3), contains besides Hb Bart's notable quantities of Hb Portland-I ($\zeta_2\gamma_2$), as evidenced from the levels of ζ chain in these fractions (Fig. 4). For instance, an increase in the quantity of the Hb Bart's fraction above 5% in red cell lysates of babies with an α-thal-2 homozygosity ($-\alpha/-\alpha$), appears to be primarily due to an increase in the production of the ζ chain (Fig. 5) which combines with γ to form Hb Portland-I. The slight increases in the level of "Hb Bart's", *i.e.* above the baseline value of 0.5–0.8%, in normal babies ($\alpha\alpha/\alpha\alpha$) and in babies with an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$), are also caused by the presence of the ζ chain containing Hb Portland-I (Fig. 5).

Recently, Chui *et al.*[22] used a highly sensitive and specific radioimmunoassay (RIA) to detect ζ globin chains in blood from normal and α-thalassemic newborns. They observed an average value of 0.15% ζ in normal ($\alpha\alpha/\alpha\alpha$) babies (20% had no detectable ζ chain) and higher values in babies with notable quantities of Hb Bart's or γ_4. These investigators also found that the ζ chain disappears soon after birth in normal babies. These results are in close agreement with ours. We observed the ζ chain in 16 of the 31 normal babies tested; the lowest level of detection was 0.1% [expressed as % of ($\alpha + \zeta$)]. Among these 16 newborns were eight with a ζ globin gene deletion on one chromosome. As the RP-HPLC analysis also offers a satisfactory quantitation of the β chain, a comparison between the relative quantities of β and ζ chains made it possible to evaluate the existence of a negative correlation between these two parameters. Such a correlation was observed for normal babies ($\alpha\alpha/\alpha\alpha$) and babies with an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$) (Fig. 6). In both instances, the ζ chain level decreases with increasing β chain level, suggesting a direct relationship with fetal maturity. It is interesting to note that the disappearance of the ζ chain takes somewhat longer in the α-thal-2 baby than in the normal newborn; the ζ chain is not detectable in the normal baby when the β chain level has reached a level of 15–20%, and in the α-thal-2 heterozygous baby when this level approaches 25–30%. No such relationship was detected for the α-thal-2 homozygous ($-\alpha/-\alpha$) newborn baby (Fig. 6), suggesting a continuous synthesis of ζ, perhaps even into adult life.

Our limited data for babies with a ζ globin gene deletion, suggest functional ζ genes on both chromosomes because the ζ chain level in these babies at birth, was the same as that for normal babies without such a deletion (Fig. 6).

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IDENTIFICATION OF COSMETIC DYES BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method based on ion-pair reversed-phase high-performance liquid chromatography with detection at four wavelengths between 400 and 600 nm is reported for the separation and identification of the most common synthetic colour additives in cosmetic products. All the dyes generally employed in the U.S.A. and almost all those in current use in cosmetics in the European Community have been taken into account. The chromatography was performed on a C_8 bonded silica packed column, with a 60-min gradient changing from 10 to 95% acetonitrile in water containing 10^{-2} *M* sodium perchlorate (pH 3.0) as mobile phase (flow-rate 2.5 ml/min). Detection limits are in the range 20–100 ng for all dyes investigated. The method has been applied to the analysis of commercial lipsticks.

INTRODUCTION

In recent years concern has arisen over the extensive use of synthetic colour additives, especially in food and cosmetics. Toxicological investigations of cosmetic colours are undertaken in many countries, and the resulting data have led to repeated revisions of the number of permitted food or cosmetic dyes.

In view of the desire to restrict the indiscriminate usage of dyestuffs, likely to come into contact with the skin or mouth, the Council of the European Economic Community (EEC) has enacted Directive 76/768, modified by Amendment Directive 82/368, which lists 256 organic colouring agents permitted in cosmetic products but imposes maximum allowed concentrations only on a few of them. Unfortunately, different countries have different legislations and cosmetic products may thus be imported into a country which forbids the use of the colouring matter present in them.

Consequently, the control of cosmetics containing synthetic dyes requires methods for detection, identification and quantitation of the most of them.

Market indications suggest that at most 30–50 colouring agents are in current

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use in cosmetics in the European Community. Because lipsticks usually contain a more complex mixture of dyes than other cosmetics, their separation and identification have been the subject of much methodological development work, undertaken on the basis of the results already obtained in the closely related field of food-dye analysis. Previous qualitative methods for the identification of food colours were mostly based on paper[1,2] and thin-layer chromatography (TLC)[1,3–9]. Quantitative analysis has received comparatively little attention. After the initial extraction of the dyes, quantitation is carried out by spectrophotometry[10,11], titration with titanium(III) chloride solution[12,13] or electrophoresis on polyacrylamide[14].

In recent years, high-performance liquid chromatography (HPLC) has been shown to be more powerful for synthetic food-colour analysis, mostly for the detection of impurities in single dyes[15,16], but also for the separation of dye mixtures. Anion-exchange columns have been used for this purpose[17], but ion-pair reversed-phase HPLC has been found particularly convenient for the separation of a large number of food colours[18–24].

A recent paper[25] demonstrated the suitability of this technique, in combination with rapid-scanning visible-region spectrophotometry, for the separation and determination of 21 representative dyes used in lip cosmetics. However, TLC techniques are still widely employed in the analysis of cosmetic dyes because a good separation of all colours can often be obtained by direct application to the plate, *i.e.* without requiring a preliminary clean-up[4,26,27]. On the other hand, electrochemical techniques have been successfully applied to the determination of colouring agents, particularly for studying their degradation[28]. However, these electrochemical techniques are very useful when simple mixtures of dyes are to be analyzed and very low concentrations are to be evaluated.

We think that HPLC is a suitable way to screen complicated mixtures of synthetic dyes, particularly when the extraction of the colouring matter from the cosmetic product is complete. We report here the use of ion-pairing reversed-phase HPLC for the separation of 75 organic colours commonly used in the cosmetic industry, and its application to the analysis of two commercial lipsticks.

EXPERIMENTAL

Materials

All dyes in this study were commercial samples used as received. Throughout this paper they are referred to by their CI reference numbers[29]. We have investigated 87 dyes, 75 of which are listed in Table I; the remaining twelve dyes are not reported since they are insoluble in the mobile phase. (CI 12490, CI 51319, CI 69800, CI 69825, CI 71105, CI 73000, CI 73360, CI 73385, CI 74100, CI 74160, CI 74260 and CI 75810).

Standard solutions of individual dyes and of mixtures were prepared in dimethylformamide (DMF) containing 5% (v/v) orthophosphoric acid to give a concentration of 1 mg/10 ml or 0.4 mg/ml for each dye. These solutions were stored under subdued lighting.

All chemicals used were of analytical grade obtained from Farmitalia-Carlo Erba (Milan, Italy). Water was deionized and doubly distilled from glass apparatus. Acetonitrile was of HPLC grade. All solvents and solutions for HPLC analysis were

filtered through a filter (Millipore, Bedford, MA, U.S.A.), pore size 0.5 μm, and vacuum degassed by sonication before use. The samples of lipsticks were obtained from perfume shops.

Apparatus

A Model 5000 liquid chromatograph (Varian, Zug, Switzerland) equipped with a variable-wavelength UV–VIS detector (Varichrom UV 50), a Valco AH 60 injection valve and a Model 730 integrator recorder (Waters Assoc., Milford, MA, U.S.A.) were used. The stationary phase was octylsilane bonded to silica (silica gel 60 HPLC, C_8, 10 μm) obtained from Riedel De Haen (Seelze-Hannover, F.R.G.). This was packed into a 300 mm × 5 mm I.D. stainless-steel column at a pressure of $5.1 \cdot 10^7$ Pa, using glycerol–water (1:1, v/v) as a solvent.

HPLC conditions

The chromatographic conditions were as follows: mobile phase, acetonitrile–water containing 0.01 *M* sodium perchlorate (pH 3.0 adjusted with perchloric acid) with a linear gradient from 10 to 95% acetonitrile in 60 min; flow-rate 2.5 ml/min; column temperature, 25°C; injection volume, 10 μl; detection wavelengths, 400, 475, 525 and 600 nm; detector sensitivity, 0.64 or 0.16 a.u.f.s.; chart speed, 0.5 cm/min.

Assay of dyes in lip cosmetics

A 100-mg amount of the lipstick sample was accurately weighed and dissolved in 2 ml of a solution of orthophosphoric acid (5%, v/v) in DMF. In order to extract any fatty material, the mixture was treated five times with a few millilitres of hexane. If the combined hexane extracts were coloured, a back-extraction with 2 ml of DMF–orthophosphoric acid was needed and this extract was added to the DMF extract. The resulting solution was diluted in the mobile phase until a suitable extinction was reached. Aliquots (10 μl) of this solution were injected into the chromatograph.

RESULTS AND DISCUSSION

The chromatographic behaviour of the cosmetic dyes examined and their relative absorptions at the four detection wavelengths are summarized in Table I. The retention times are reproducible under the experimental conditions used. The mobile phase employed enables good column performance for long periods of time.

Chromatograms of some individual dye samples (not shown) clearly demonstrated the presence of colour impurities whose nature is unknown. In such cases we have put the symbols (I), (II), (III), etc., next to the CI number.

The separation obtained for a standard mixture of 20 dyes is illustrated in Fig. 1. The identification of two dyes with similar or identical retention times is aided by the fact that generally they have absorption maxima at different wavelengths in the visible region. Spectra of the dyes investigated were recorded by use of the spectrophotometer, but most of the spectra are available in the literature.

For example, the dyes CI 10020 and CI 19140 were found to have the same retention times but they can easily be distinguished, since the former is detectable at 525 or 600 nm where the latter does not absorb. The same holds true for another

TABLE I

RETENTION TIMES AND RELATIVE ABSORPTIONS, A_λ, OF THE DYES AT THE FOUR DETECTION WAVELENGTHS

Colour index No.	*Retention time (min)*	A_{400}	A_{475}	A_{525}	A_{600}
10020	1.07	100	35	15	45
10316	3.85	100	25	0	0
12075	31.30	50	100	25	5
12085	34.23	45	100	35	0
12150	30.87	35	70	100	0
13015	1.30	100	20	0	0
13065	16.91	100	65	5	0
14700	7.20	35	80	100	0
14720	6.77	25	60	100	5
15510	11.90	45	100	60	0
15585	19.40	45	100	55	0
15630	18.50	35	100	85	0
15850	15.74	50	100	100	0
15985(I)	1.35	50	100	55	0
15985(II)	2.95				
17200(I)	1.35	20	40	100	10
17200(II)	3.35				
18965	4.67	100	25	0	0
19140	1.07	100	60	0	0
26100	40.00	50	85	100	0
27755	1.16	30	60	65	100
42051(I)	12.38	20	0	5	100
42051(II)	15.74				
42053	9.98	15	5	5	100
42090(I)	10.45	20	0	5	100
42090(II)	21.08				
44045	38.80	15	0	15	100
45170	32.31	10	15	100	0
45350	19.40	15	100	5	0
45370(I)	24.71	5	60	100	0
45370(II)	25.67				
45380	25.67	5	15	100	0
45396	22.64	15	100	5	0
45410(I)	29.36	5	20	100	5
45410(II)	34.78				
47005	7.95	100	0	0	0
59040	1.03	100	0	0	0
60725	37.75	10	30	85	100
61565	44.06	45	35	55	100
75300	24.67	100	40	0	0
11920	23.55	100	20	5	0
14270	5.54	100	45	5	0
14815	5.59	40	100	10	5
15525	8.87	50	100	40	0
15800	17.95	20	80	100	0
15865(I)	18.88				
15865(II)	11.71	40	70	100	5
15865(III)	21.58		--		
15880(I)	17.8	40	50	100	10
15880(II)	19.05				
15980	3.95	60	100	30	0

TABLE I (*continued*)

Colour index No.	*Retention time (min)*	A_{400}	A_{475}	A_{525}	A_{600}
16035(I)	4.42	0	75	100	0
16035(II)	1.38				
16185	1.13	25	60	100	0
16230(I)	3.28	45	100	25	5
16230(II)	4.06				
16255(I)	1.39	25	80	100	10
16255(II)	2.23				
16290	0.96	20	65	100	40
20170(I)	11.51	100	60	10	0
20170(II)	4.38				
20470	11.71	20	20	35	100
21230	61.61	100	25	15	0
26105(I)	43.62	60	85	100	5
26105(II)	33.06				
28440(I)	1.44				
28440(II)	3.45	30	20	65	100
28440(III)	3.86				
42040(I)	38.36	20	0	10	100
42040(II)	34.71				
42045(I)	12.96	20	0	5	100
42045(II)	10.36				
42520(I)	23.26	5	40	100	5
42520(II)	22.33				
42555	33.58	0	5	55	100
42563(I)	40.70				
42563(II)	39.23	5	5	45	100
42563(III)	42.66				
42563(IV)	29.01				
42775(I)	6.79				
42775(II)	8.52	5	5	35	100
42775(III)	1.40				
42775(IV)	4.37				
44090(I)	8.15	0	0	5	100
44090(II)	6.90				
45425(I)	25.32	45	60	100	0
45425(II)	23.60				
45430	26.20	15	30	100	0
47000	22.29	100	0	0	0
52015(I)	16.63	0	5	10	100
52015(II)	14.98				
58000	17.88	100	50	5	0
60724	34.28	25	40	90	100
60730(I)	17.82	15	25	80	100
60730(II)	14.82				
61570(I)	14.17	65	10	30	100
61570(II)	12.60				
62045	18.66	10	35	50	100
62560(I)	13.31				
62560(II)	11.80	70	25	30	100
62560(III)	11.55				
73015(I)	1.43	5	10	20	100
73015(II)	2.57				
75470	4.95	20	70	100	5
Bromothymol blue	23.08	100	30	5	0
Bromocresol green	19.50	100	45	5	5
Bromocresol purple	16.27	100	30	0	0
Lactoflavin	4.43	75	100	0	0

pair of dyes, CI 15585 and CI 45350. The former is detectable at 525 nm where the latter absorbs very weakly. Therefore, when two dyes are not completely resolved, they can be identified and also quantitated by appropriate wavelength selection if they absorb at different wavelengths.

There are, however, some cases where dyes have coincident HPLC peaks and also exhibit very similar absorption spectra, *e.g.*, CI 45370 and CI 45380. The problem can be overcome by means of a numerical method using visible spectra and multiple regression analysis as proposed by Wegener *et al.*[25]. In the example cited, an unresolved HPLC fraction containing either CI 45370 or CI 54380 should be collected and its visible spectrum recorded and compared to the reference spectra for both dyes, recorded under similar conditions.

The applicability of this HPLC method has been demonstrated on two commercial lipstick samples of unknown composition, I and II. Fig. 2A shows the chromatogram, recorded at 475 nm obtained by injecting an extract from sample I. Similar chromatograms were obtained at 400 and 525 nm, while at 600 nm there was no appreciable absorbance. The results indicate the presence of five dyes, all fully iden-

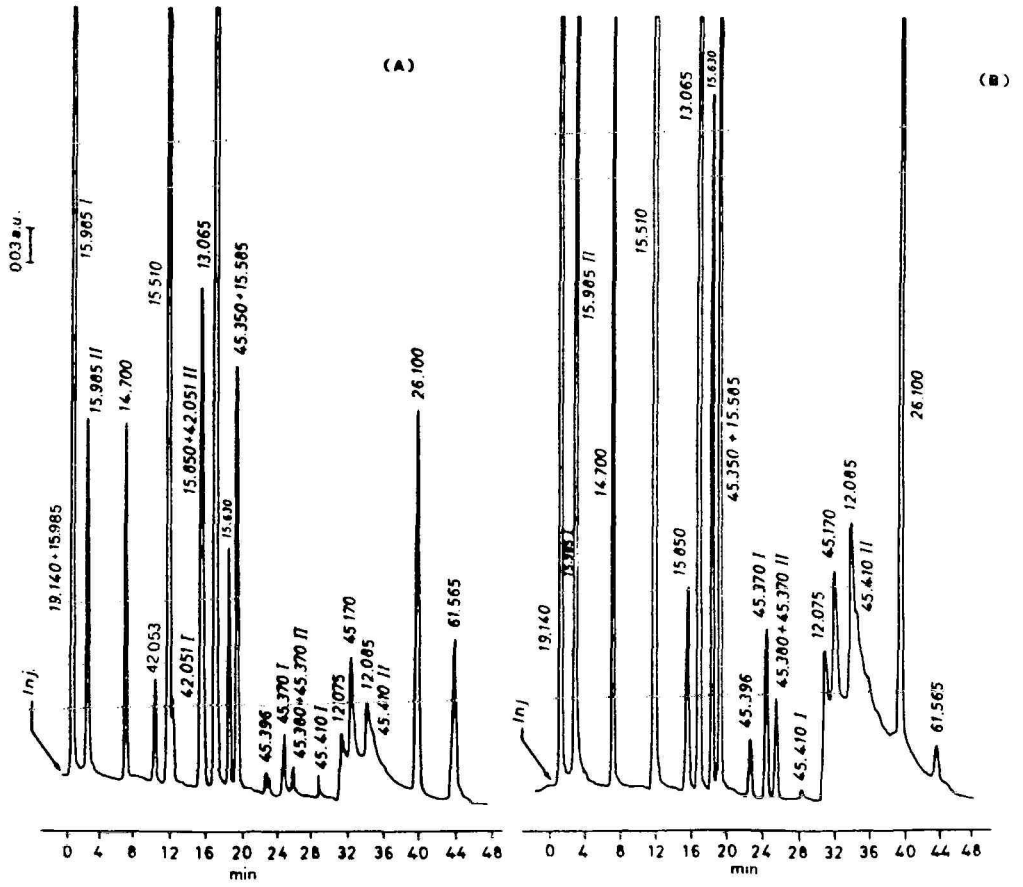

Fig. 1.

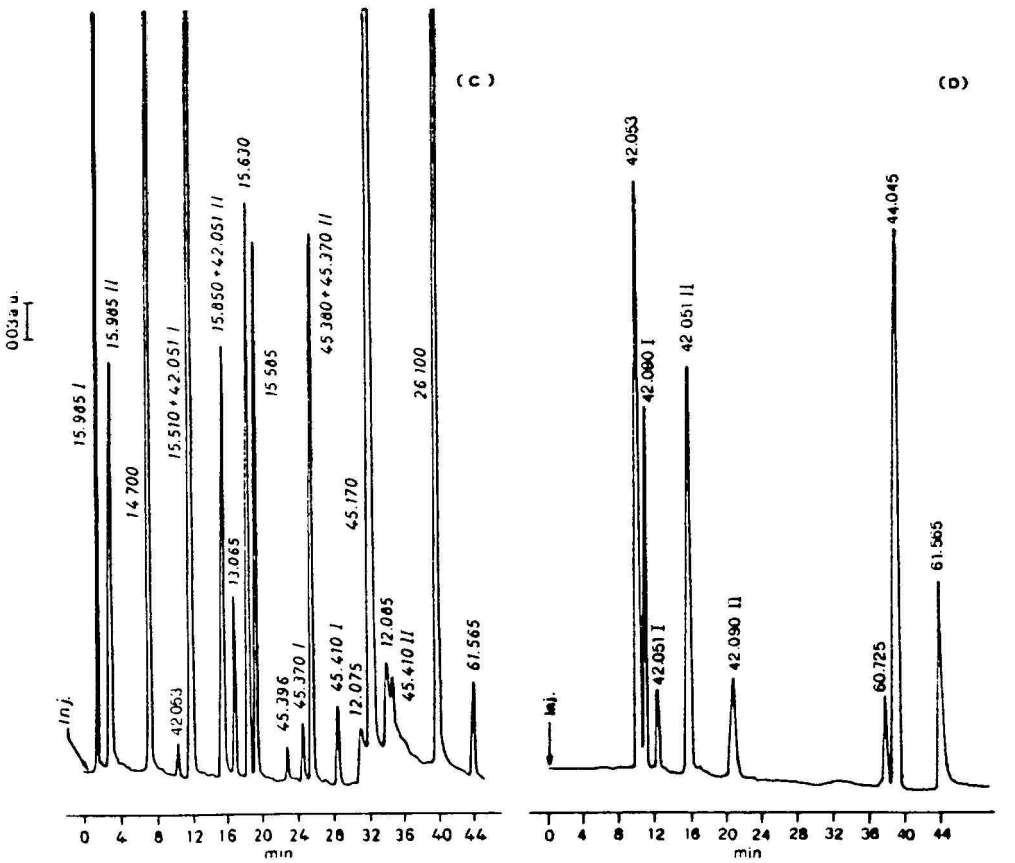

Fig. 1. Typical chromatograms of a standard mixture of 20 dyes (numbered as in Table I). Detection at 400 (A), 475 (B), 500 (C), and 600 nm (D), respectively. Chromatographic conditions as in the text.

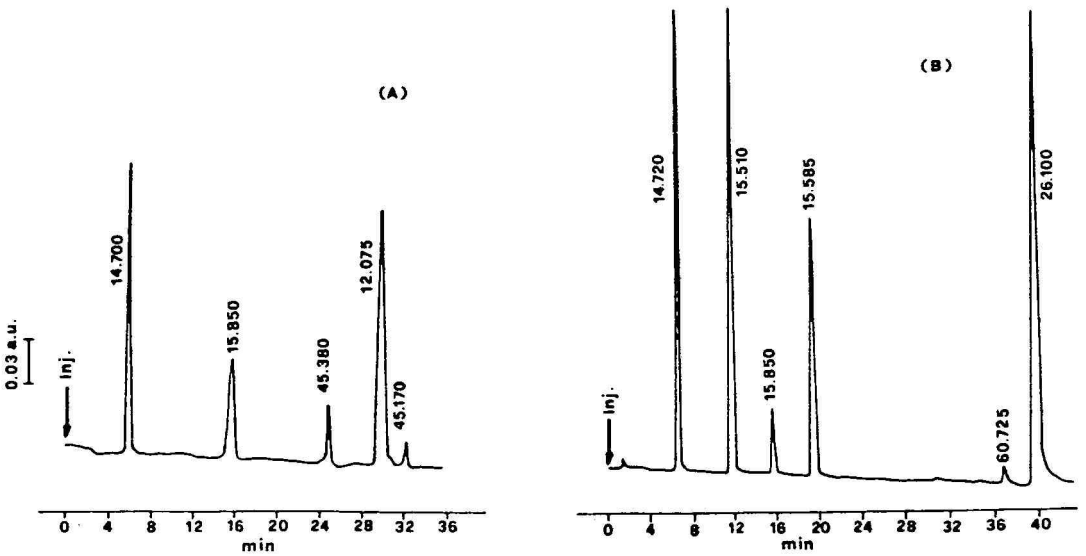

Fig. 2. Chromatograms recorded at 475 nm for an extract from lipstick samples I (A) and II (B), respectively.

tified. Similar successful results were obtained for an extract from sample II, whose chromatogram is shown in Fig. 2B. In this case, it is obvious that at least six dyes must be present, all detectable at the same wavelength.

In conclusion, the described HPLC method enables the identification, by a relatively simple procedure, of 75 representative cosmetic dyes and allows a good separation of at least 50 of them. Its application to the analysis of lipsticks has demonstrated the potential of the method for rapid screening of samples for non-permitted colours. The detection limits are of the order of 20–100 ng of pure dye in an injection volume of 10 μl and they have been estimated for a full scale absorbance.

At present the EEC's legislation on dyes in cosmetics does not generally prescribe the maximum amounts permitted; however, were this to be the case, the proposed method could be also applied to the quantitation of permitted dyes.

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QUANTITATIVE DETERMINATION OF PROSTAGLANDINS E_1, E_2 AND E_3 IN FROG TISSUE

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SUMMARY

A method was developed for quantitative determination of endogenous production of prostaglandin (PG)E_1, PGE_2 and PGE_3 by *Rana temporaria* lung, heart and urinary bladder homogenates, since these tissues contain the precursors, 8,11,14-eicosatrienoic, arachidonic and 5,8,11,14,17-eicosapentaenoic acids. Following homogenization and shaking at 22°C for 30 min, media were extracted by XAD-2, treated with sodium hydroxide in order to convert PGE compounds into PGB compounds, purified by thin-layer chromatography, and analyzed by high-performance liquid chromatography with homo-PGE_1 as an internal standard. The ratio of prostaglandins E_1, E_2 and E_3 compared to the ratio of fatty acid precursors in tissue suggested that the tissue content of precursor is not the only factor determining the type of prostaglandin synthesized.

INTRODUCTION

Prostaglandin synthesis from endogenous and exogenous precursors has been extensively studied in mammalian tissues[1–4], but little is known of endogenous prostaglandin synthesis in non-mammalian vertebrates. While mammals produce dienoic prostaglandins utilizing arachidonic acid ($C_{20:4}$, $\omega - 6$) as substrate[1,2,4], studies of the fatty acid composition of tissue lipids in frogs show that, unlike mammals, membranes contain not only arachidonic acid, but also eicosapentaenoic acid ($C_{20:5}$, $\omega - 3$), and eicosatrienoic acid ($C_{20:3}$, $\omega - 6$), the precursors for the trienoic and monoenoic prostaglandins, respectively[5,6]. Therefore, the potential exists in frogs for production of prostaglandins from different fatty acid precursors.

Exogenous fatty acid precursors have been shown to have effects in amphibians[6–10], presumably due to their conversion to biologically active products. *In vitro* studies using *Rana pipiens* urinary bladder showed opposite effects of arachidonic and eicosapentaenoic acid derived metabolites on water permeability[6,7]. Exogenous PGE_2 and arachidonic acid both inhibited water flow, while PGE_3 and eicosapen-

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taenoic acid stimulated water flow. The effects of the exogenous precursors could be attenuated with indomethacin. *In vivo* studies using cannulated *Rana catesbeiana* showed that infused PGE_1, PGE_2 and PGE_3 or their precursors, eicosatrienoic, arachidonic and eicosapentaenoic acids respectively all resulted in hypotension and the effect of the precursors was attenuated with indomethacin[8–10].

Prostaglandin synthesis studies in non-mammalian vertebrates have been carried out using exogenous substrates[11–14] or tissue extracts[15]. In studies examining the conversion of [1-^{14}C]arachidonic acid by several invertebrate and vertebrate species, the vertebrates produced more PGE_2 as measured by thin-layer chromatography (TLC)[11]. Tissues from several species were examined for their ability to convert [^{3}H]eicosatrienoic acid (10 μg) into PGE_1 and $PGF_{1\alpha}$[12]. Frog (Rana) urinary bladder converted 17% of the eicosatrienoic acid into PGE_1 measured as PGB_1 at 278 nm following alkaline treatment while conversion by frog lung was 10%[12]. Toad urinary bladder (*Bufo marinus*) converted [1-^{14}C]arachidonic acid to PGE_2 and $PGF_{2\alpha}$ as measured by TLC[13]. Isolated, perfused frog hearts (*Rana esculenta*) produced basal quantities of PGE_2, TXB_2 and 6-keto $PGF_{1\alpha}$ as detected by radioimmunoassay, and the production was stimulated with exogenous unlabelled arachidonic acid[14]. Tissue contents of PGE_2 from frog (*Rana nigromaculata*) tissues was greatest in the gastrointestinal tract and lowest in liver of the tissues examined, as measured by bioassay[15].

Studies with exogenous substrate may not accurately reflect endogenous product formation. Analysis of monoenoic, dienoic and trienoic prostaglandins has been difficult due to the low amounts of compounds that are produced and the lack of discrimination of current radioimmunoassays for the prostaglandins produced from different 20 carbon precursors. The purpose of this study was to develop a method which would allow the quantitation of PGE_1, PGE_2 and PGE_3 produced by frog tissues. The representative tissues chosen for analysis were lung, heart, and urinary bladder.

EXPERIMENTAL

Animals

Rana temporaria were obtained locally or from the Stockholm Biological Laboratory, Stockholm, Sweden. The frogs were housed in large tanks with water (22°C) under controlled light conditions (12 h light/12 h dark). They were force-fed pig heart three times a week.

Materials

PGE_3 was a kind gift from Ulf Diczfalusy, Department of Clinical Chemistry, Huddinge University Hospital, Huddinge, Sweden. PGE_1, PGE_2 and XAD-2 were purchased from Sigma, St. Louis, MO, U.S.A. BSTFA [bis(trimethylsilyl)trifluoroacetamide], polyunsaturated fatty acid mixtures, boron trifluoride in methanol, and SP-2330 (10%) on Supelcoport (100–120 mesh) were obtained from Supelco, Bellafonte, PA, U.S.A. [1-^{14}C]Arachidonic acid (59.3 mCi/mmol), [1-^{14}C]-5,8,11,14,17-eicosapentaenoic acid (56.9 mCi/mmol), [1-^{14}C] 8,11,14-eicosatrienoic acid (56.0 mCi/mmol) and [5,6,8,11,12,14,15-^{3}H]prostaglandin E_2 (200 Ci/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. Sep-Pak C_{18} car-

tridges were obtained from Waters Assoc., Milford, MA, U.S.A. Silica gel G TLC plates (250 μm, without fluorescent indicator) were purchased from Merck, Darmstadt, F.R.G.

Analysis of tissue fatty acids

Pooled frog lung, heart and urinary bladder tissues (approximately 200 mg per sample) were homogenized with a polytron and saponified in potassium hydroxide and methanol as previously described[16]. Following reflux for 1 h, samples were acidified to pH 3.0, extracted twice with diethyl ether, and evaporated to dryness with nitrogen. Samples were methylated with boron trifluoride in methanol[10]. The analysis for fatty acid composition was made using a Hewlett-Packard gas chromatograph, Model 5710A equipped with a splitless inlet, a flame ionization detector, and a 2.5 m × 2 mm I.D. glass column containing 1.4 g of SP-2330 (10%) on Supelcoport (100–200 mesh). Gas chromatographic conditions were as follows: oven temperature 175°C (4 min), then increased to 220°C at the rate of 8°C/min. Detector and injector port temperatures were 250°C, and the nitrogen flow-rate was 30 ml/min. Standard mixtures of methylated polyunsaturated fatty acid standards were used and calculation of the percent fatty acid in the total mixture was made by integration of peaks in the chromatogram using a Hewlett-Packard 3390A integrator. The data represent mean ± S.E.M. of 4 determinations.

Two-dimensional TLC

Frog lung, heart and urinary bladder (250 mg/ml 0.1 *M* phosphate buffer, pH 7.4) were homogenized with a polytron and incubated with [1-^{14}C]arachidonic acid (100 000 cpm) at 22°C for 30 min. The media were adjusted to pH 3.0 and the samples were purified with Sep-Pak C_{18} cartridges[17]. The methyl formate fraction was analyzed by TLC on silica gel plates using diethyl ether–acetic acid (100:2) in the first dimension and chloroform–methanol–acetic acid (90:10:2) in the second dimension. Prostaglandin standards were visualized with phosphomolybdate spray reagent and heating. Radioactive spots were visualized by autoradiography[18].

One-dimensional TLC

Lung tissue was homogenized using a polytron (250 mg/ml 0.1 *M* phosphate buffer, pH 7.4) and incubated with [1-^{14}C]arachidonic acid (2 000 000 cpm) at 22°C for 30 min. Medium was adjusted to pH 3.0 and extracted twice with diethyl ether. Samples were esterified by reaction with an excess of diazomethane in diethyl ether for 2 min at room temperature and analyzed by TLC using silica gel plates and diethyl ether–methanol (98:2) as solvent. Control incubations and those containing indomethacin (0.1 m*M*) were compared. The zone corresponding to the authentic methyl PGE_2 standard in the control incubation was scraped from the plate and the silica gel eluted with 1 ml methanol. The sample was then treated with 15 mg sodium borohydride for 30 min at room temperature and subjected to re-chromatography on silica gel plates using diethyl ether–methanol (95:5) as solvent to confirm the identity of the radioactive zone as methyl PGE_2 by its conversion to methyl $PGF_{2\alpha}$ and methyl $PGF_{2\beta}$.

Synthesis of homo-prostaglandin E_1

Homo-prostaglandin E_1 for use as an internal standard was prepared as previously described by (i) one-carbon elongation of 8,11,14-eicosatrienoic acid into 9,12,15-heneicosatrienoic acid followed by (ii) incubation of 9,12,15-heneicosatrienoic acid with the microsomal fraction of homogenates of the sheep vesicular gland[19]. The homo-PGE_1 was purified by silicic acid chromatography[19] and high-performance liquid chromatography (HPLC) using a 200 × 4.6 mm reversed-phase C_{18} column (Nucleosil 5, Alltech) monitored at 205 nm with a mobile phase of acetonitrile–water [36:64 (v/v) containing 0.1% acetic acid to give an apparant pH of 3.7]. The injection volume was 100 µl containing 100 µg homo-PGE_1. The flow-rate was 0.5 ml/min and the retention time for homo-PGE_1 was 19 min.

Quantitation of PGE compounds by HPLC

Frog lung, heart and urinary bladder tissues were homogenized with a polytron (250 mg/ml in 0.1 *M* phosphate buffer, pH 7.4) and incubated at 22°C for 30 min. Following the incubation, homo-prostaglandin E_1 (1275 ng) was added as an internal standard. The incubation medium was diluted to 10 ml, centrifuged at 750 *g*, the pH adjusted to 3.0, and applied to a 25 × 2 cm glass column containing 25 ml XAD-2 with a bed height of 9.5 cm. The column was washed with 90 ml distilled water, and then eluted with 80 ml ethanol. The ethanol fraction containing the PGE compounds was evaporated, redisolved in 1 ml ethanol, and subjected to treatment with 1 ml 0.5 *M* sodium hydroxide for 20 min. The reaction mixture was acidified to pH 3.0 and extracted twice with diethyl ether. The diethyl ether was evaporated and the samples disolved in ethyl acetate and purified by preparative TLC on silica gel plates using diethyl ether–methanol–acetic acid (98:2:0.5) as solvent and [^{3}H]PGB_2 (9000 cpm) as standard. The zone corresponding to PGB was determined by scanning the plate to determine the location of the standard. The zone was scraped and the silica gel eluted with 5 washes of 10 ml methanol containing 1% acetic acid. The solvent was centrifuged at 750 *g* to remove the silica gel, evaporated, and redisolved in the mobile phase (200 µl) for HPLC. Analysis was carried out using a 200 × 4.6 mm reversed-phase C_{18} column (Nucleosil 5, Alltech) monitored at 280 nm and a mobile phase of acetonitrile–water (40:60, v/v) containing 1% acetic acid, to give an apparent pH of 3.7[20]. The typical injection volume ranged between 10 and 100 µl. Authentic PGB_1, PGB_2 and PGB_3 standards were prepared from PGE_1, PGE_2 and PGE_3, respectively, by treatment with sodium hydroxide as described above for determination of retention times. [^{3}H]PGB_2 was prepared from [^{3}H]PGE_2 for TLC standard. The structures of the PGB standards were verified by mass spectrometry (MS).

MS of compounds from HPLC

Samples were collected from the HPLC, esterified by treatment with an excess of diazomethane in diethyl ether for 2 min, and further purified by preparative TLC on silica gel plates, using diethyl ether–methanol (98:2) as a solvent. They were eluted from the plate with ethyl acetate, and the trimethylsilyl derivative prepared using BSTFA (25 µl) and pyridine (10 µl). The samples were evaporated and dissolved in hexane. Mass spectrometry was performed on a 25 m × 0.32 mm I.D. (SPB-1, Supelco) capillary column on a Finnigan Model 4500 mass spectrometer. The oven temperature was 250°C and the ion source temperature was 190–200°C. The energy

of the ionization beam was maintained at 70 eV. A standard fatty acid methyl ester mixture was used for the conversion of retention times to *C* values.

RESULTS

Table I shows the fatty acid profile for lung, heart and urinary bladder tissues. The principle prostaglandin precursor found in all three frog tissues was arachidonic acid (6.5 ± 1.0 to 9.4 ± 0.9% of the total fatty acid composition). Eicosapentaenoic acid ranged from 1.7 ± 0.1 to 4.5 ± 1.0%, and eicosatrienoic acid was the least abundant precursor (0.6 ± 0.1 to 1.6 ± 0.3%).

Studies using [1-^{14}C]arachidonic acid analyzed by two-dimensional TLC and autoradiography demonstrated that the primary product of cyclooxygenase metabolism was a compound migrating with the authentic PGE_2 standard. Fig. 1 shows a tracing of the prostaglandin standards on the TLC plate with the X-ray film placed over the TLC plate. The figure shows results from an incubation of frog lung, however heart and urinary bladder demonstrated identical profiles. In all cases, only one radioactive spot was observed and it co-migrated with the authentic PGE_2 standard. Likewise, in other experiments when [1-^{14}C]eicosatrienoic or [1-^{14}C]eicosapentaenoic acids (100 000 cpm) were incubated with these tissues, one major radioactive spot was observed, co-migrating with PGE_2 standard (data not shown).

When lung tissue was incubated with [1-^{14}C]arachidonic acid followed by methylation of the compounds in the reaction mixture and analysis by one-dimensional TLC using a different solvent system, one principal radioactive product was produced which co-migrated with the authentic methyl PGE_2 standard (Fig. 2a). This peak could be abolished by the inclusion of 0.1 m*M* indomethacin in the in-

TABLE I

FATTY ACID DISTRIBUTION IN LIPIDS OF FROG TISSUES

Rana temporaria tissues (approximately 200 mg) were extracted and fatty acid composition was determined by gas chromatography as described in Experimental. (n = 4).

Carbon No.	*Mean percent of total fatty acid ± S.E.M.**		
	Lung	*Heart*	*Urinary bladder*
14:0	2.0 ± 0.3	2.1 ± 0.2	1.1 ± 0.5
16:0	16.2 ± 2.0	12.3 ± 0.3	16.7 ± 4.2
16:1	10.3 ± 1.9	12.2 ± 4.0	8.8 ± 0.9
18:0	5.8 ± 1.3	3.5 ± 0.6	5.2 ± 2.5
18:1	22.1 ± 1.8	20.7 ± 3.3	24.7 ± 1.9
18:2	9.7 ± 1.0	10.1 ± 1.7	8.9 ± 0.8
20:3	1.6 ± 0.3	0.7 ± 0.1	0.6 ± 0.1
20:4	9.4 ± 1.0	6.5 ± 1.0	8.7 ± 0.2
20:5	4.5 ± 1.0	2.0 ± 0.4	1.7 ± 0.1
22:4	1.4 ± 0.4	2.0 ± 0.1	3.9 ± 1.9
22:5	1.0 ± 0.4	0.7 ± 0.2	0.9 ± 0.3
22:6	0.8 ± 0.1	1.7 ± 0.1	0.9 ± 0.2

* Fatty acid percentages do not sum to 100%. The differences represent minor or unidentified peaks.

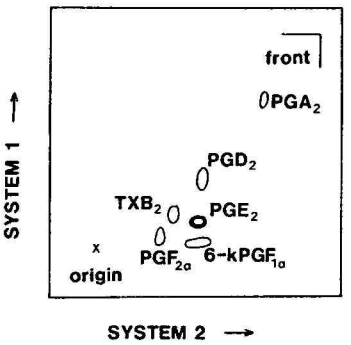
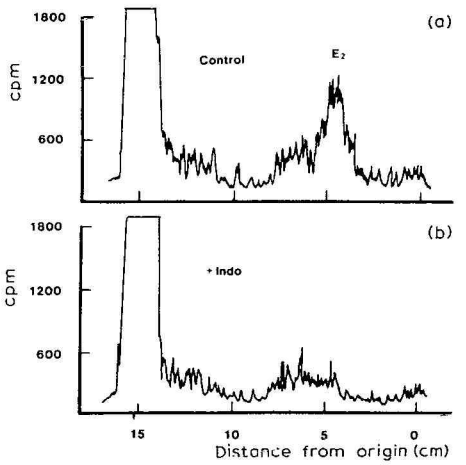


Fig. 1. Frog lung incubation with [1-^{14}C]arachidonic acid with results shown on two-dimensional TLC. Authentic prostaglandin standards were visualized with phosphomolybdate spray reagent and heating and are shown as open circles. Overlay of X-ray film showed one major radioactive spot which co-migrated with the PGE_2 standard (dark circle).



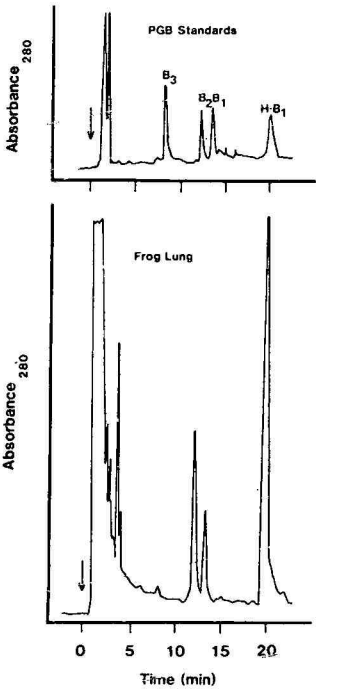


Fig. 2. Frog lung incubation with [1-^{14}C]arachidonic acid with results shown on one-dimensional thin-layer radiochromatography. (a) One major radioactive peak co-migrated with the authentic PGE_2 standard, visualized with phosphomolybdate spray and heating. (b) Absence of radiolabelled peak in the presence of 0.1 m*M* indomethacin.

Fig. 3. Quantitation of PGE compounds in frog lung by HPLC on Nucleosil 5-C_{18}. Other conditions: see text. PGE compounds were treated with 0.5 *M* sodium hydroxide and analyzed as PGBs. Retention times of standards PGB_3, PGB_2, PGB_1 and homo-PGB_1 were 8.3, 12.2, 13.8 and 20.0 min, respectively.

TABLE II

QUANTITATION OF PGE_1, PGE_2 AND PGE_3 IN FROG TISSUES

Rana temporaria tissues (250 mg/ml) were homogenized and incubated for 30 min at 22°C. Homo-PGE_1 (1275 ng) was added as an internal standard. Incubation media was extracted by XAD-2 and treated with 0.5 *M* sodium hydroxide to convert E to B compounds. The media were purified by preparative TLC and PGB compounds quantitated by HPLC as described in Experimental. Results represent mean ±S.E.M. of 4 determinations.

	pg prostaglandin/mg wet weight/30 min		
	PGE_1	*PGE_2*	*PGE_3*
Lung	1731.0 ± 101.6	3619.4 ± 288.9	192.0 ± 24.8
Heart	723.0 ± 150.0	1515.5 ± 225.7	47.3 ± 1.0
Urinary bladder	772.0 ± 70.0	7444.0 ± 840.0	263.0 ± 20.1

cubation media (Fig. 2b). Furthermore, the radioactive peak eluted from the TLC plate, when reduced with sodium borohydride resulted in the generation of two peaks which co-migrated with methyl $PGF_{2\alpha}$ and methyl $PGF_{2\beta}$.

Fig. 3 shows a representative tracing of a lung tissue analysis in which the principle E prostaglandin detected as PGB is PGE_2. The amount of PGE_1 was approximately half of the PGE_2. Smaller quantities of PGE_3 were detected. The proportions of PGE_1, PGE_2 and PGE_3 measured were dependent on the tissue, and results for lung, heart and urinary bladder are presented in Table II. However, PGE_2 was always the major E prostaglandin produced. PGE_2 and PGE_3 production was greatest in urinary bladder while the lung produced the greatest quantity of PGE_1. When urinary bladder was incubated whole, prostaglandins produced were approximately half of those shown for the homogenized tissue. In separate studies using the same tissues from *Xenopus laevis*, PGE_2 was also the principal PGE compound produced (data not shown).

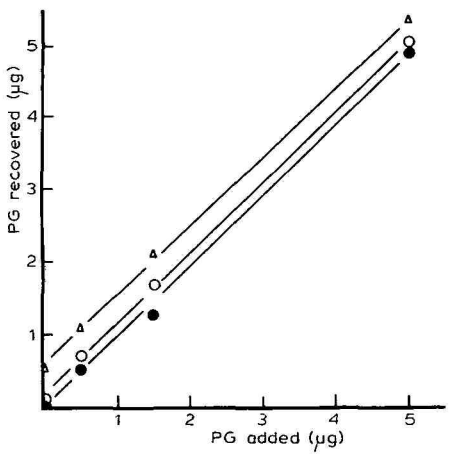


Fig. 4. Recovery of added prostaglandin E_1 (○), E_2 (△) and E_3 (●). Pooled frog lung was divided into samples containing 130 mg and incubated at 22°C for 30 min. PGE_1, PGE_2 and PGE_3 were added in quantities of 0.5, 1.5 and 5.0 µg following the incubation and samples were analyzed as described in Experimental.

To test the recovery of added prostaglandins, a large pool of frog lung was homogenized and equally subdivided to contain 130 mg of lung tissue in each sample. Following the 30-min incubation, PGE_1, PGE_2 and PGE_3 at concentrations of 0.5, 1.5 and 5.0 μg were added. Fig. 4 shows the results of this recovery experiment. Recovered amounts of PGE_1, PGE_2 and PGE_3 were the same as added amounts of prostaglandins plus endogenous levels. Without addition, lung tissue contained 225.0 ± 12.4, 470.5 ± 34.5 and 25.0 ± 3.2 ng of PGE_1, PGE_2 and PGE_3, respectively (n = 4, mean ± S.E.M.).

Fig. 5a shows the mass spectrum of the trimethylsilyl derivative (C=24.0) of the compound from lung tissue co-eluting with the PGB_2 standard on HPLC. The mass spectrum showed ions of high intensity at m/e 420 (M), 349 (M − 71; loss of $\cdot C_5H_{11}$), 321 (M − 99: loss of $\cdot C_5H_{11}$ plus CO from the five-membered ring), 299 [M − (90 + 31); loss of $(CH_3)_3SiOH$ plus $\cdot OCH_3$], 279 [M − 141; loss of $\cdot CH_2{-}CH{=}CH{-}(CH_2)_3{-}COOCH_3$] and 247 [M − 173, loss of $(CH_3)_3SiO{=}CH{-}(CH_2)_4{-}CH_3$]. An identical mass spectrum was obtained from the peak from urinary bladder which co-eluted with the PGB_2 standard.

Fig. 5b shows the mass spectrum of the trimethylsilyl derivative (C=24.0) of the compound from lung tissue co-eluting with the PGB_1 standard on HPLC. The mass spectrum showed ions of high intensity at m/e 422 (M), 351 (M − 71; loss of $\cdot C_5H_{11}$), 323 (M − 99; loss of $\cdot C_5H_{11}$ plus CO from the five-membered ring), 301 [M − (90 + 31); loss of $(CH_3)_3SiOH$ plus $\cdot OCH_3$] and 249 [M − 173; loss of $(CH_3)_3$ $SiO{=}CH{-}(CH_2)_4{-}CH_3$].

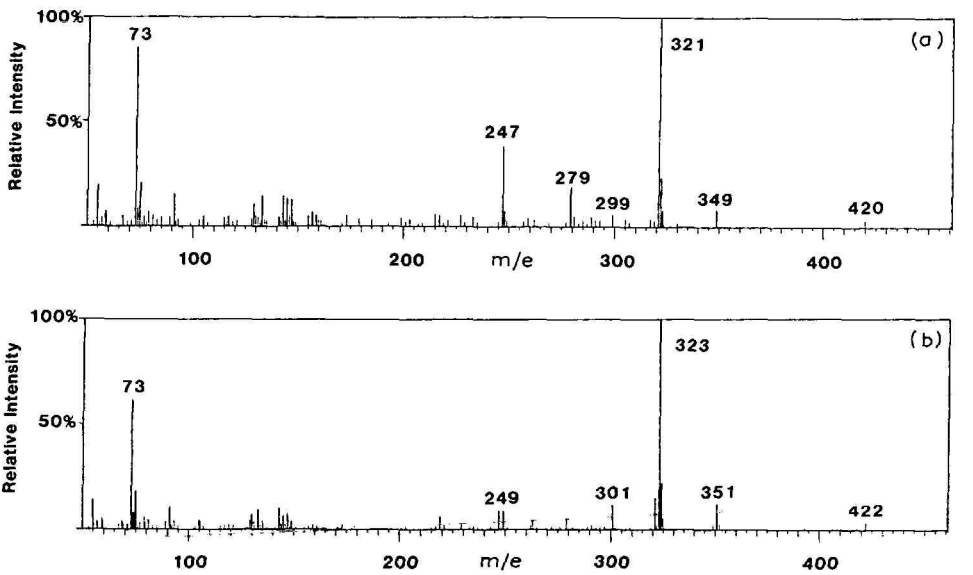


Fig. 5. Mass spectrum of (a) PGB_2 and (b) PGB_1 from frog lung. Purified fractions from the HPLC purification step were methylated with diazomethane and further purified by TLC (diethyl ether–methanol, 98:2). Trimethylsilyl derivatives were prepared using BSTFA (25 μl) and pyridine (10 μl) and analyzed as described in Experimental.

DISCUSSION

The gas chromatographic analysis of the fatty acid composition of *Rana temporaria* lung, heart and urinary bladder tissues showed that in this species, arachidonic acid was the principal 20 carbon fatty acid substrate for prostaglandin synthesis. When the tissues were compared, the heart had the lowest percent of arachidonic acid, while the lung had the highest. The lung also had higher percentages of eicosapentaenoic and eicosatrienoic acids than did the other tissues. The natural abundance for all tissues in decreasing order was arachidonic acid, eicosapentaenoic acid, and eicosatrienoic acid. These results are similar to analysis of these tissues in *Rana pipens*[5,6] and *Rana catesbeiana*[5,10].

Studies with exogenous [1-^{14}C]arachidonic acid suggested that the principal product formed by these frog tissues was a compound with the mobility of PGE_2 on both one and two-dimensional TLC. The inhibition of the radioactive peak in the presence of indomethacin added further support that the product was derived via cyclooxygenase. The conversion of radioactive eicosapentaenoic and eicosatrienoic acids to compounds migrating with PGE_2 suggested PGE compounds may be formed from them as well as arachidonic acid. However, the two-dimensional TLC method used in this study does not discriminate between monoenoic, dienoic, and trienoic prostaglandins.

The development of a method to separate and quantitate PGE_1, PGE_2 and PGE_3 as PGBs allowed evaluation of production of these products by frog tissues. The primary product of *Rana temporaria* lung, heart, and urinary bladder was PGE_2, measured as PGB_2. The structure was verified by mass spectrometry. The largest production of PGE_2 was by urinary bladder. While the studies reported here are for tissue homogenates and would therefore be expected to be considerably higher than baseline values, pilot studies with whole urinary bladder demonstrate that homogenization is not necessary for quantitation of prostaglandins by this method. Values for whole urinary bladder were approximately half those reported here. Heart produced the least PGE_2 of the three homogenized tissues examined. The production of primarily PGE_2 by frog tissues correlates with the fact that arachidonic acid is the most abundant substrate in all three tissues. In additional studies, PGE_2 also appears to be the most abundant E prostaglandin in tissues of *Xenopus laevis*.

Frog lung contained significantly more PGE_1 as verified by MS than did heart or urinary bladder. This may be due to differences in abundance of eicosatrienoic acid in the three tissues. However, eicosatrienoic acid in lung was approximately one-fifth that of arachidonic acid, so the ratio of PGE_1 to PGE_2 is significantly greater than one would predict based on substrate concentration alone. Release of eicosatrienoic acid from membrane phospholipids and/or suitability for the cyclooxygenase may account for its greater than expected conversion to PGE_1. Other reports show a greater production of PGE_1, measured as PGB_1, from exogenous eicosatrienoic acid from urinary bladder than from lung, although the species of *Rana* is not given[11].

The production of PGE_3 in all three tissues was significantly less than that of PGE_1, despite the fact that eicosapentaenoic acid had a greater abundance than eicosatrienoic acid. Eicosapentaenoic acid has been shown to be a poor substrate for mammalian cyclooxygenase and to inhibit arachidonate metabolism[21]. The relative

production of PGE_3 does not appear dependent on relative precursor concentration since the urinary bladder produced the greatest amount of PGE_3 while the lung contained the greatest proportion of its precursor. In this study and others[6,7,9], exogenous eicosapentaenoic acid is efficiently converted into products. This contrast with the low endogenous production of PGE_3 found in the current study. Endogenous release of eicosapentaenoic acid and its subsequent conversion may depend on other regulatory factors not examined in this study.

The tissue content of precursor is clearly not the only factor determining which type of prostaglandins will be synthesized in frogs. As prostaglandins which are structurally related can have different physiological effects in frogs[6,7], the development of this method to quantitate prostaglandins in a species containing several potential precursors is essential to understanding the physiological roles of these compounds in amphibians. In addition, this method can be used to quantitate E prostaglandins in systems which have the potential of utilizing different substrates for prostaglandin production.

ACKNOWLEDGEMENTS

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CHROM. 19 447

Note

Factors affecting the relationship between the plate height and the linear mobile phase velocity in gel filtration chromatography of proteins

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Gel filtration chromatography (GFC) has been widely used for the separation and purification of proteins. However, the number of components resolvable in GFC is strongly dependent on the column efficiency[1]. Therefore, GFC with smaller than conventional gel particles, known as medium- or high-performance GFC (MPGFC, HPGFC), has recently been employed to increase the resolution.

We have reported the heights equivalent to a theoretical plate (HETPs) for proteins on soft dextran gels[2] and in MPGFC[3]. In the latter study the effect of the column dimensions, sample volume and sample concentration on HETP were also investigated, and the effect of the column dimensions was found to be negligible for columns between 30.0 cm × 1.0 cm and 90 cm × 9.0 cm (ref. 3).

In this study, HETP values for proteins in MPGFC and HPGFC were measured as a function of the linear mobile phase velocity, u. The effects of the particle diameter, d_p, the gel type, the type of proteins and the temperature were examined on the basis of plots of the reduced HETP, $h(=\mathrm{HETP}/d_p)$ *vs.* the reduced flow-rate, $v(=ud_p/D_m)$[4,5], where D_m is the molecular diffusion coefficient.

EXPERIMENTAL

The apparatus and the method were essentially the same as in our previous study[2,3].

The MPGFC gels from Toyo Soda (Japan) employed in this study included Toyopearl (TSK gel) HW40F ($d_p = 44$ μm), HW55SF ($d_p = 35$ μm), HW55F ($d_p = 44$ μm) and HW55C ($d_p = 75$ μm).

In the case of a packed HPGFC column (TSK gel G3000SW, $d_p = 11$ μm, 30 cm × 0.75 cm), the sample was introduced by means of a Rheodyne 7120 injection valve connected to a Model 100 pump (Altex, U.S.A.).

Myoglobin (Mb) (Cat. No. M0630) from Sigma (St. Louis, MO, U.S.A.) and ovalbumin (OA) (five times crystallized) from Seikagakukogyo (Japan) were employed without further purification. The same purified fraction of bovine serum albumin (BSA) as that in our previous study[6] was used. All other reagents were analytical grade.

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RESULTS AND DISCUSSION

Factors affecting HETP

As shown in Fig. 1, the HETP values for Mb, OA, BSA and vitamin B_{12} (B_{12}) on an HW55F gel column were found to be a linear function of u. As the molecular weight increased, the slope of the line increased while the HETP value extrapolated to $u=0$ from the experimental results did not change appreciably. HETP values for proteins on HW40F were almost parallel to the u-axis and the HETP value extrapolated to $u=0$ was almost the same as that on the HW55F column. Similar experimental results were found previously[2,3,8–10].

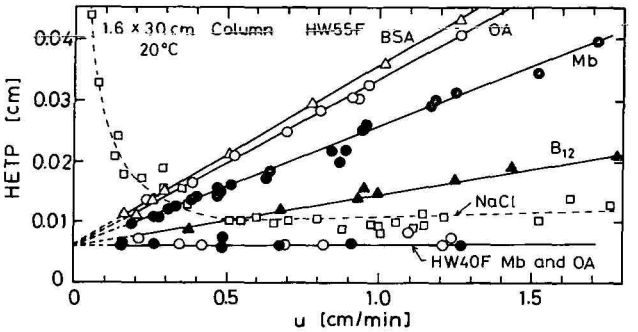

Fig. 1. Plots of HETP *vs.* u with MPGFC columns. The distribution coefficient, K, was measured from the peak elution volume, V_e, according to the relationship $K = (V_e - V_o)/(V_t - V_o)$ (ref. 7) where V_o = void volume and V_t = total column volume. $K=0.30$ for BSA, 0.34 for OA, 0.41 for Mb, 0.74 for sodium chloride and 0.9 for B_{12} on HW55F; $K=0$ for Mb and OA on HW40F. Sample volume: 0.5 ml. Sample concentrations: 0.3–0.5% for Mb, OA and BSA; 0.5 M for sodium chloride and 0.1% for B_{12}. Vitamin B_{12} was weakly adsorbed to the HW55F gel, although the exact mechanism of the adsorption is unknown. This is the reason why K for B_{12} is higher than that for sodium chloride. Note that the values of HETP at $u=0$ extrapolated from the experimental results are very similar except that for sodium chloride.

These results can be interpreted on the basis of the equation[2,10–12]

$$\text{HETP} = 2(D_L/u) + R(1-R)ud_p^2/(30D_s) = A + Cu \quad (1)$$

where D_L is the axial dispersion coefficient, D_s the gel phase (intraparticle) diffusion coefficient and R is defined as the equilibrium fraction of solute in the mobile phase[4,12].

The first term on the right-hand side of eqn. 1 expresses the contribution from axial dispersion, which can be assumed to be constant under the usual conditions in GFC of proteins[2,10–13]. The A value is therefore obtained from the HETP value extrapolated to $u=0$. The second term is for the gel phase (intraparticle) diffusion and the C value can be obtained from the slope of a plot of HETP *vs.* u. Since the distribution coefficient, K, of proteins was 0 for the HW40F column, eqn. 1 simplifies to HETP $= 2D_L/u = A =$ constant.

The HETP for sodium chloride on the HW55F column showed a minimum at

around $u=0.5$ cm/min and below this value it increased sharply. This is due to the contribution from the molecular diffusion coefficient, D_m, to D_L. If we split D_L into $\lambda d_p u$ and $\gamma_m D_m$ where λ is the packing characterization factor and γ_m the tortuosity factor in interparticle space as shown by Van Deemter *et al.*[14], eqn. 1 becomes[11,12]:

$$\text{HETP} = 2\lambda d_p + 2\gamma_m D_m/u + R(1-R)ud_p^2/(30D_s) = A + B/u + Cu \quad (2)$$

This equation is quite similar to the Van Deemter equation[14].

When $A=2\lambda d_p=66$ μm (the HETP value extrapolated to $u=0$ in Fig. 1), $D_m=1.5\cdot 10^{-5}$ cm^2/s (value for sodium chloride[15]) and $\gamma_m=0.6$[4,14] are inserted to eqn. 2, the second term becomes more than half of the total HETP at $u=0.1$ cm/min.

On the other hand, in the case of Mb, D_m is $1.1\cdot 10^{-6}$ cm^2/s (ref. 16), which is 14 times lower than that of sodium chloride. With this D_m, $2\lambda d_p=66$ μm and $\gamma_m=0.6$[4,14], the second term does not contribute to the total HETP even at $u=0.1$ cm/min. Therefore, there will be a minimum in the HETP *vs.* u relationship only at extremely low flow-rates[17]. This minimum will be discussed later in terms of the reduced velocity.

GFC is often performed at subambient temperature for the separation of unstable substances such as proteins and at high temperature for highly viscous samples such as sugars. As in Fig. 2, the lower the temperature the higher is the HETP. It is interesting that the HETP value extrapolated to $u=0$ (the A value) is affected little by temperature whereas the slope changes markedly. Since the K value ($=0.41$) did not vary with temperature, this change in the slope is due to the variation of D_s.

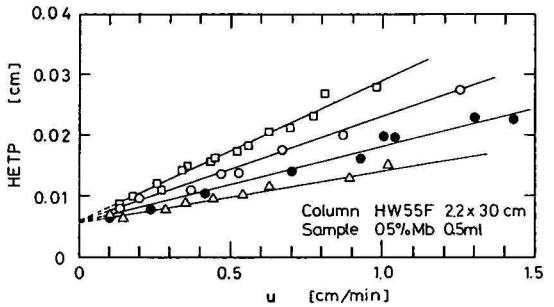

Fig. 2. Effect of temperature on HETP. Note that the values of HETP at $u=0$ extrapolated from the experimental results for different temperatures are very similar, although the slopes are different. □ = 10°C; ○ = 20°C; ● = 30°C; △ = 40°C.

Reduced HETP

As already suggested by several researchers[4,5], a plot of the reduced HETP, h, *vs.* the reduced velocity, v, is useful for examining the influence of various factors such as d_P and temperature, since the HETP values over a wide range of experimental conditions can be compared in the same diagram. Egn. 1 is rewritten as [12]

$$h = (2/Pe) + R(1-R)v(30\gamma_{sm}) \quad (3)$$

where Pe is the Peclet number (ud_p/D_L) and $\gamma_{sm} = D_s/D_m$.

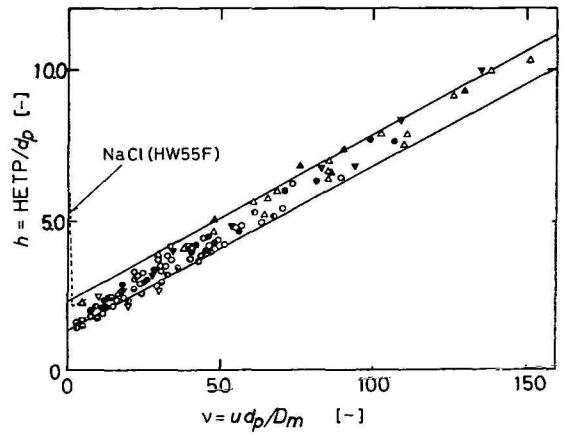

Fig. 3. Plots of reduced HETP *vs.* reduced velocity. The experimental points are cited from Figs. 1 and 2 except those for Mb on HW55SF and HW55C columns (30 cm × 1.6 cm). The data for sodium chloride were calculated from the broken curve in Fig. 1. The D_m values were from refs. 15, 16 and 18 and its temperature dependence was estimated according to the relationship $D_m\eta/T$ = constant[18], where η is the solvent viscosity. The two straight lines indicate the region of the experimental points and are also shown in Fig. 4. ○ = Sample Mb, HW55F gel (d_p = 44 μm), 10°C; ● = Mb, HW55F, 20°C; ◐ = Mb, HW55F, 30°C; ◒ = Mb, HW55F, 40°C; △ = Mb, HW55C gel (d_p = 75 μm), 20°C; ▽ = MB, HW55SF gel (d_p = 35 μm), 20°C; ▲ = sample OA, HW55F, 20°C; ▼ = sample BSA, HW55F, 20°C.

Fig. 3 shows plots of h *vs.* v for the present results with MPGFC columns. All the results are gathered in a very narrow range encompossed by the two lines. This implies the following. (1) Since the plots of HETP *vs.* u for different particle diameters are reduced to a single h *vs.* v relationship, the first and the second terms in eqn. 3 are independent of the particle diameter. (2) The D_L/u ($=A/2=\lambda d_p$) value is hardly dependent on temperature, the type of proteins or d_p, and is approximated to 0.8–

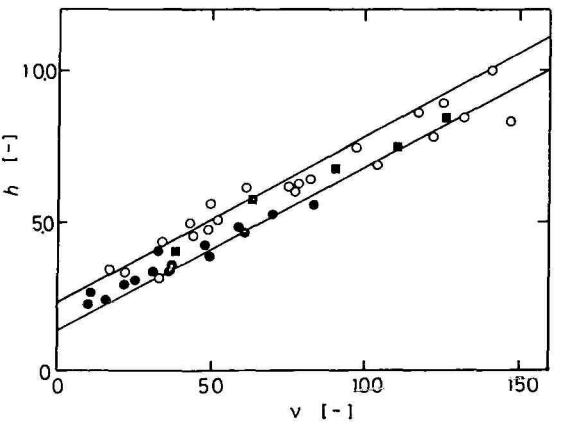

Fig. 4. Plots of h *vs.* v for low-pressure GFC and HPGFC. The two straight lines are the same as those in Fig. 3 and are given for comparison. Experimental conditions for G3000SW HPGFC: sample, 0.4% Mb, 20 μl; 0.1% OA, 100 μl; 20°C. The data for Mb on a Sephadex G-150 column are from ref. 2. ○ = sample Mb, G150 gel (d_p = 201 μm), 13 cm × 1.5 cm column; ● = Mb, G3000SW gel (d_p = 11 μm), 30 cm × 0.75 cm column; □ = sample OA, G3000 SW, 30 cm × 0.75 cm column.

$1.5 \cdot d_p$, *i.e.*, $Pe = 0.7$–1.3. This Pe value is similar to that reported by Cluff and Hawkes[13] for the range of v employed in this study, but slightly higher than that calculated from the data reported by Katz *et al.*[17]. (3) The h *vs.* v plots for different temperatures are very similar as shown in Fig. 3. According to eqn. 3, this implies that the temperature dependence of D_s is similar to that of D_m (4). When the HETP *vs.* u plots for different proteins shown in Fig. 1 are expressed in the reduced variables h and v, they are similar. This is because v increases with molecular weight at certain u and d_p due to the decrease in D_m with molecular weight[18]. (5) h shows its minimum at v less than 5. This indicates that the HETP for proteins will not show a minimum with the flow-rates normally used and it can be regarded as a linear function of u due to the low D_m of proteins (less than $1 \cdot 10^{-6}$ cm^2/s at 20°C[18]).

In Fig. 4 the above results are compared with those obtained by conventional low-pressure GFC and HPGFC. It is interesting that the results also fall in a very narrow range. It should be noted that the flow-rate in GFC is usually low with larger particle diameters and high with smaller ones. Therefore, v ranges between 10 and 150 under the usual operating conditions.

The present study has shown that the HETP for proteins in GFC can be described by eqn. 1 and that a plot of the reduced HETP *vs.* the reduced velocity is useful for assessment of the column efficiency.

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Note

Mobility of various buffers in reversed-phase thin-layer chromatography

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The application of buffers in planar chromatography is as old as paper chromatography (PC) and thin-layer chromatography (TLC) themselves. Buffered systems were used to chromatograph dissociable compounds (salts of weak acids or bases) under definite acid–base conditions[1], and were applied either to impregnate the paper[2] or the thin-layer support[3,4] or as a component of the mobile phase.

In the impregnation technique it was accepted that the buffering effect is evenly distributed over the whole plate surface and the latter was suposed to show the same pH value as that of the buffer. The effect of the pH of the buffer on the support (silica, cellulose) was not taken into account. To prevent a change in buffer composition by elution of its components during the flow of the mobile phase, most authors saturated the latter with the buffer solution prior to development.

The use of buffer solutions as mobile phases in PC can be demonstrated by salting-out chromatography[5] and by pH chromatography[6,7]. Buffered mobile phases have frequently been applied in TLC and in reversed-phase (RP)TLC systems for analytical separation[8] or for lipophilicity determination[9–11]. The application of buffered mobile phases is also very common in ion-exchange TLC[12–15].

It is well known both in PC and TLC that the composition of multi-component mobile phases is changed during their flow through the suport. This is the case not only with solvent mixtures[16–19] but also with salt solutions[5]. Thus Hagdahl and Tiselius[5] using 1 *M* disodium hydrogenphosphate and 2 *M* sodium dihydrogenphosphate as mobile phases in so-called salting-out PC stated that a concentration gradient with the maximum at the front should be established on the chromatogram. Therefore, during the flow of the mobile phase through the support, its composition and consequently its pH value is expected to change. The type of support and its acid–base properties should, however, also be taken into consideration. These phenomena usually do not decrease the performance of the chromatographic system, on the contrary, the established pH gradient can work positively and improve the separation.

In quantitative structure–activity relationship studies the use of buffer solutions as mobile phases in both normal-phase and RP systems is a problem in that

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the expected pH change of the support due to the mobile phase decomposition and the effect of the acid–base properties of the support could lead to false results. Here, buffer solutions are used as mobile phases on untreated[11] layers or on layers impregnated with apolar stationary phases (liquid paraffin, silicone oil, etc.)[9,10] and the R_M values determined are used as the basis of calculations to correlate the lipophilicity of analytes with their biological activities and to design new bioactive compounds.

Recent research indicates that the retention of analytes is also influenced by the quality of the buffer cations[20], and the actual pH values depend considerably on the quantity of organic modifier in the eluent[21]. It was found[22] that by using a veronal buffer of pH 8.8 as mobile phase on silica layers the pH was 8 at the starting line and decreased successively to pH 4 at the solvent front, at a distance of 10 cm from the start. The pH value of undeveloped silica was also 4. This difference on cellulose layers under identical conditions was only 1 pH unit.

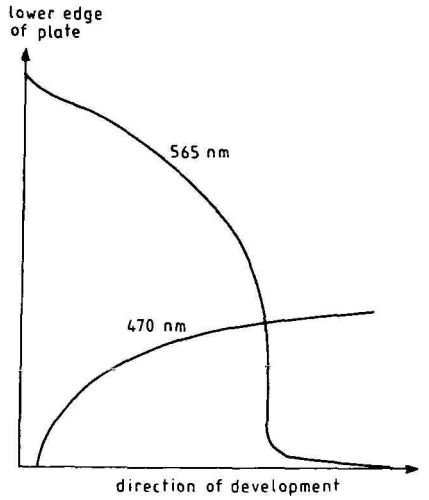
The objectives of our work were to study in more detail the retention of various buffering ions by the layer and to determine their buffering capacity.

EXPERIMENTAL

Polygram Sil G plates (Macherey-Nagel, F.R.G.) were impregnated with paraffin oil as described[23]. The plates were developed with water and water–methanol mixturès (4:1, 3:2, 2:3 and 1:4, v/v) containing final buffer conentrations of 0.1, 0.2, 0.3 and 0.4 *M*. The pH value of the buffers (sodium phosphate, sodium acetate and sodium diethylbarbiturate) was set to 8.50. After development the movement of the alkalinity front was detected by spraying half the plate with a 0.1% solution of phenol red indicator (red at pH 8.50 and yellow at the pH value of the impregnated silica). The evaluation was carried out by a Shimadzu dual-wavelength TLC scanner CS-930 at 565 (red) and 470 nm (yellow). In case of veronal buffers the movement of diethylbarbituric acid was detected on the other half of the plate at 250 nm. Phosphate ions were detected by use of the ammonium molybdate–tin(II) chloride reagent[24] and then evaluated at 600 nm.

As the mobility of the alkalinity front generated by sodium acetate was high it was unnecessary to detect the acetate ions separately. Each experiment was performed in quadruplicate.

We assumed that the hypothetical spots were placed on the plates at 15 mm above the eluent level and calculated the R_F value of the alkalinity front accordingly. As these R_F values indicated that not only the water–methanol ratio but also the final concentration of buffer influences the mobility and the exact type of correlation (linear or logarithmic) between independent (methanol and buffer concentration) and dependent variables (R_F value of alkalinity front) was not previously established we used stepwise regression analysis to elucidate this problem[25]. The R_F values were taken as dependent, the linear and logarithmic forms of methanol (%) and buffer concentrations (M) as independent variables. The number of accepted variables was not limited, the partial F value of the variables being set to $F = 1$. The calculations were carried out for sodium acetate and sodium phosphate separately. The low mobility of veronal buffers makes these data irrelevant from a practical point of view; therefore they were omitted from the calculations.



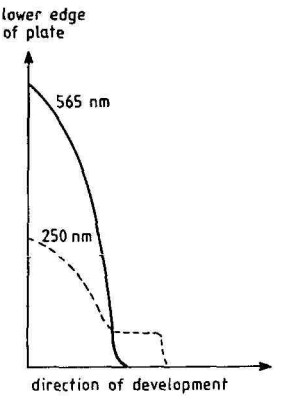


Fig. 1. Movement of the alkalinity front detected at two wavelengths. R_F of alkalinity front: 0.50. Final concentration of phosphate buffer: 0.4 *M*. Eluent: water.

Fig. 2. Movement of veronal buffer detected at two wavelengths. R_F of alkalinity front: 0.12. Final concentration of veronal buffer: 0.4 *M*. Eluent: water.

As it was previously reported that the extent of impregnation exerts a considerable effect on the retention behaviour of RPTLC layers[26], Polygram plates impregnated with 1, 2.5, 5 and 10% paraffin oil were developed in 0.4 *M* buffer solutions in water as eluent and then evaluated as described above. Unimpregnated plates served as controls.

RESULTS AND DISCUSSION

A typical chromatogram evaluated at 565 and 470 nm is shown in Fig. 1. As the alkaline form of phenol red also shows a marked absorption at 470 nm the change in pH of the plate surface cannot be adequately followed at this wavelength. Consequently the determination of the alkalinity front was carried out at 565 nm. The lower edge of the plates indicates in each case the first point of the plate above the eluent level. Veronal buffers exhibited poor mobilities in each eluent (Fig. 2), however, the diethylbarbituric acid moved further on the plates than the alkalinity. This somewhat surprising observation can be explained by the assumption that free silanol groups exhibit higher affinity to sodium ion than to diethylbarbituric acid. The adsorption of sodium ions makes the silica surface alkaline and the veronal buffer depleted of sodium ions moves alone. The R_F value of the alkalinity front decreased with increasing methanol concentration. This is caused by the fact that the decreasing dielectric constant of the eluent (higher methanol content) suppresses the dissociation of veronal. The undissociated form is more lipophilic and is therefore more strongly retained by the reversed phase.

The separation of the anionic and cationic parts of the buffer was also observed in sodium phosphate buffers (Fig. 3) and can be explained as in the case of veronal buffers. We have, however, to take into consideration that sodium ions can be re-

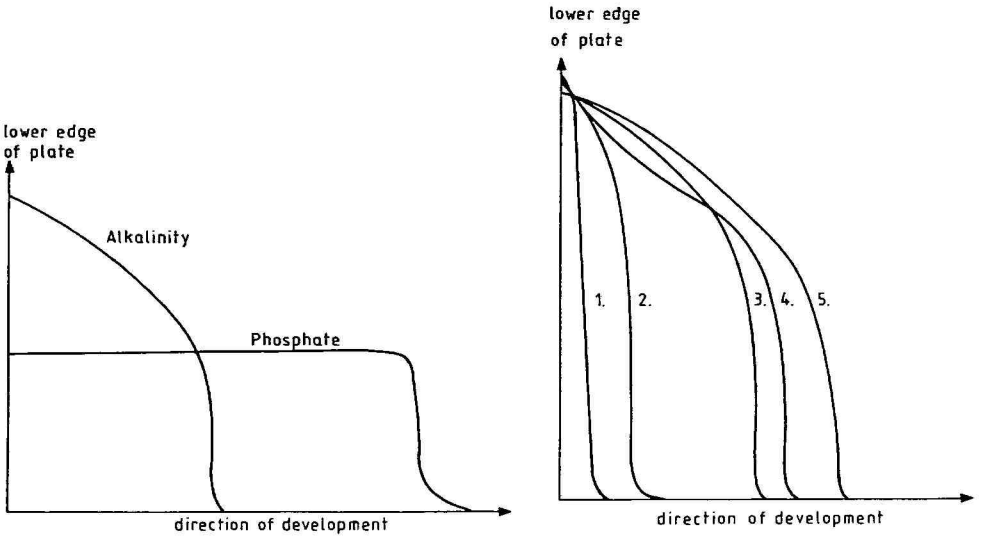


Fig. 3. Movement of alkalinity and phosphate fronts. R_F of alkalinity and phosphate front: 0.49 and 0.92 respectively. Final concentration of phosphate buffer: 0.3 *M*. Eluent: water.

Fig. 4. Effect of the eluent composition on the position of the alkalinity front. Final concentration of phosphate buffer: 0.3 *M*. Eluent: 1, water–methanol (1:4); 2, water–methanol (2:3); 3, water–methanol (3:2); 4, water–methanol (4:1); 5, water.

tained not only by the free silanol groups uncovered by paraffin oil but also by the paraffin oil itself resulting in the same dissociation. Our other investigations showed that, under similar RPTLC conditions, monovalent cations exhibit high apparent "lipophilicity". The position of the alkalinity front depends also in this case on the methanol content, decreasing with increasing methanol concentration (Fig. 4). The distribution of pH is uneven; it decreases very slowly near to the lower edge of the plate, but ends with a well defined, abrupt change. Sodium acetate buffers move readily with the eluent, and are hardly influenced by the methanol concentration of

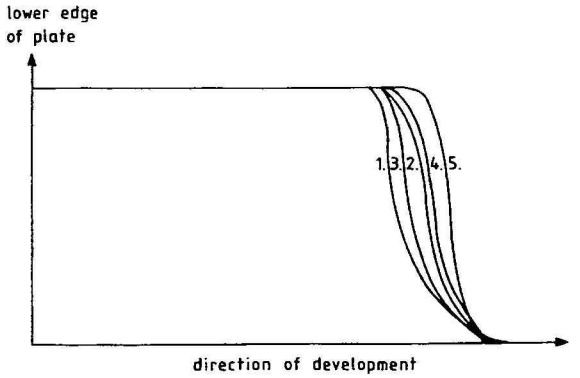


Fig. 5. Effect of the eluent composition on the position of the alkalinity front. Final concentration of acetate buffer: 0.3 *M*. Eluents as in Fig. 4.

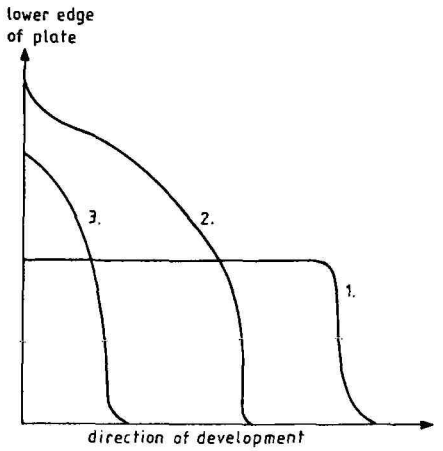


Fig. 6. Positions of the alkalinity fronts generated by various buffers. Final concentration of buffers: 0.4 *M*. Eluent: water. Buffers: 1, acetate; 2, phosphate; 3, veronal.

the eluent (Fig. 5). The distribution of pH is even; the change is very abrupt and it is near to the eluent front.

The behaviour of the three buffers is compared in Fig. 6. The low mobility of veronal buffers (highest R_F value observed: 0.12) makes them unsuitable to buffer similar RPTLC systems. Phosphate has an higher mobility (up to $R_F = 0.50$), how-

TABLE I

DEPENDENCE OF THE POSITION OF THE ALKALINITY FRONT IN PHOSPHATE, y_1, AND ACETATE, y_2, BUFFERS ON THE METHANOL, x_1, AND BUFFER, x_2, CONCENTRATION OF THE ELUENT

Results of stepwise regression analysis.

$y_1 = a + b_1x_1 + b_2x_2$ (A)

$y_2 = a + b_1x_1 + b_2 \log x_2$ (B)

*Parameter**	*Eqn. A*	*Eqn. B*
a	0.31	0.83
b_1	$-5.10 \cdot 10^{-3}$	$-1.27 \cdot 10^{-3}$
b_2	0.42	$5.21 \cdot 10^{-2}$
r^2	0.8753	0.7386
b'_1 (%)	75.53	75.37
b'_2 (%)	24.47	24.63
s	$6.71 \cdot 10^{-2}$	$2.45 \cdot 10^{-2}$
s_{b1}	$5.30 \cdot 10^{-4}$	$1.91 \cdot 10^{-4}$
s_{b2}	0.13	$2.42 \cdot 10^{-2}$
F	51.07	24.01
t_1	9.61	6.59
t_2	3.11	2.15

$n = 20$, $F_{99.9\%} = 10.66$, $t_{95\%} = 2.11$, $t_{99\%} = 2.90$, $t_{99.9\%} = 3.97$

* b'_1 and b'_2 = Contribution of independent variables x_1 and x_2 to the position of the alkalinity front (so-called path coefficients); s, s_{b_1} and s_{b_2} = standard deviations of y_1, x_1 and x_2; t_1 and t_2 = indicators of the significance level of b_1 and b_2.

ever, its mobility depends heavily on the organic phase concentration. We suggest that before applying phosphate buffers it is advisable to check that the compounds to be separated are in the buffered zone. Our data clearly show that acetate buffers are the most suitable in RPTLC to produce a buffering effect which is evenly distributed along the plates. The results of stepwise regression analysis are compiled in Table I. The calculations entirely support our qualitative conclusions based on the chromatograms.

In both cases the independent variables (methanol and buffer concentrations had a significant) influence (significance level over 99.9%) on the R_F value of the alkalinity front. The r^2 values show that the change in the independent variables is responsible for about 80% of the change in R_F value. The R_F value decreased with increasing methanol concentration (negative b_1 values) and increased with increasing buffer concentration (positive b_2 values), however, these effects were higher in the case of phosphate buffer (b_1 and b_2 values of eqn. A are higher than the corresponding values of eqn. B). The difference in the intercept value (position of alkalinity front at zero methanol and buffer concentrations), a, shows that acetate buffer ($a = 0.81$) has a higher mobility than the phosphate one ($a = 0.31$). The significant t values indicate that both variables have a significant effect on the R_F value of the alkalinity front, however, the impact of the methanol concentration is about three times higher than that of the buffer concentration (see path coefficients b'_1 and b'_2). The movement of phosphate ions depended only on the methanol concentration:

$$R_{F\text{phosphate}} = 0.92 - (3.69 \pm 0.30) \cdot 10^{-3} \cdot \text{methanol concentration}$$
$$r_{\text{calc.}} = 0.9458 \qquad r_{99.9\%} = 0.6787$$

Summarizing our results, we established that the buffering capacity of veronal, phosphate and acetate buffers is different in RPTLC. The movement of the alkalinity front depends strongly on the organic phase ratio and to a lesser extent on the buffer concentration of the eluent. The buffering capacity increased in the order veronal < phosphate < acetate. Our results concerning the effect of the extent of impregnation on the retention of buffer ions are compiled in Table II. The data show that the retention increases in each case with increasing extent of impregnation. This finding is in good accordance with the fact that the charge in the ratio of the parti-

TABLE II

R_F VALUES OF BUFFER ION FRONTS ON RPTLC PLATES IMPREGNATED TO DIFFERENT EXTENTS

	Paraffin oil concentration (%)				
	0	*1*	*2.5*	*5*	*10*
Phosphate anion	1.00	0.98	0.99	0.96	0.94
Alkalinity caused by phosphate	0.76	0.73	0.71	0.64	0.61
Veronal anion	1.00	0.58	0.37	0.29	0.20
Alkalinity caused by veronal	0.12	0.10	0.07	0.07	0.05
Alkalinity caused by acetate	0.90	0.89	0.85	0.85	0.82

tioning phases (the mobile water phase is identical, the quantity of adsorbed paraffin oil increases) modifies the partition in favour of the phase present in higher quantity. However, this effect is fairly low and it does not change the capacity order of the buffers established above.

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Note

Argentation liquid chromatography of polynuclear aromatic hydrocarbons on a silver(I)-loaded mercaptopropyl silica gel stationary phase

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One of the more intensively investigated areas of high-performance liquid chromatography (HPLC) today involves the development of selective stationary phases to complement the general nature of non-polar reversed-phase materials. One facet of this effort has employed secondary chemical equilibria involving metal ions to effect greater selectivity in the separation of analyte ligands. The metal ion can be included in the mobile phase[1] or immobilized on the stationary phase[2,3]. This approach is generally called ligand-exchange chromatography (LEC), as an analyte ligand of interest is exchanged for a mobile phase solvent or buffer ligand in the coordination sphere of the metal ion[4].

An interesting and useful subset of LEC is argentation chromatography, where silver(I) is the metal ion emplolyed[5]. The selective interaction of silver ion with nitrogen- and sulfur-containing species and unsaturated compounds is exploited in this approach. The relative merits of incorporating the silver ion in the stationary phase *versus* including it in the mobile phase have been discussed[1]. The major disadvantages of the mobile phase approach seem to be possible column deterioration and the build-up of metallic silver on the chromatographic system. Silver nitrate has been incorporated in the silica gel matrix, but bleeding of the metal from the column is a severe problem. Frei and co-workers[6] deposited insoluble silver halides onto silica gel and obtained some good separation of nitrogen-containing species, but interaction with unsaturated species was too weak to be useful due to the low coverage by the silver halide. Furthermore, with this approach, uneven deposition may leave residual silanols exposed and lead to mixed chromatographic mechanisms and poor efficiency. The use of high-capacity strong cation-exchange resins to bind the silver ion tightly minimizes the low coverage and bleeding problems, but these phases are not useable in the presence of buffers or other ion-containing eluents as silver will be lost by exchange with mobile phase cations. Walton[7] has discussed the use of ion-exchange materials as LEC supports.

This paper reports our initial results with mercaptopropyl silica gel (MPSG) as a stationary phase for argentation chromatography. This material appears to solve the bleeding and low-capacity problems which have been annoyances in the past use of this interesting and selective LC mode. As shown below, silver(I) is very tightly held to the sulfur site and thus provides a very stable stationary phase. Also, as coverage of the silica by the mercaptopropyl group can be made relatively high,

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sufficient silver ion can be loaded to provide strong interaction with unsaturated compounds. Several polynuclear aromatic hydrocarbon (PAH) compounds were used for the initial chromatographic characterization of this stationary phase.

EXPERIMENTAL

Apparatus

Two chromatographic systems were used, a Beckman Model 324 consisting of two Model 100-A pumps and a Hitachi Model 100-10 detector, or a Beckman Model 110-A pump and Model-153 detector. Both systems employed 20-μl sample loops and a 254-nm detector wavelength. All columns were 25 cm × 4.0 mm I.D. glass-lined stainless steel (SGE, Austin, TX, U.S.A.) and were slurry packed using a Model-26980-4 pneumatic pump (Haskel, Burbank, CA, U.S.A.) according to the method of Manius and Tscherne[8]. An isopropanol–methanol (70:30) mixture was used as the slurrying medium, and methanol as the packing liquid. Columns were packed at 6000 p.s.i. for about 45 min.

Reagents and solutions

Water used for solutions was purified with a 3-cartridge system consisting of two deionizers and a charcoal filter (Milli-Q, Millipore, Bedford, MA, U.S.A.) or with a doubly deionized-distilled water system constructed in our laboratory[9]. The PAH test samples were about 10^{-7} *M* and dissolved in either methanol or hexane. All solvents were either ACS Reagent or HPLC grade. Several silica gels were used in this study: a TLC-grade, 10–50-μm, 60-Å pore, 550-m^2/g material (type HR, E. Merck) for metal extraction and stoichiometry studies; Adsorbosil, a 10 μm, preparative grade silica with 70-Å pores and a 480 m^2/g surface area (Applied Science, Deerfield, IL, U.S.A.); 5 μm LiChrosorb Si 100 silica with 100-Å pores and a 300-m^2/g surface area (E. M. Science); and 5 μm Hypersil WP-300 (Shandon Southern, Sewickley, PA, U.S.A.) with 300-Å pores and a 60-m^2/g surface area. The 3-mercaptopropyltrimethoxysilane was obtained from either Petrarch Systems (Bristol, PA, U.S.A.) or Dow-Corning (Midland, MI, U.S.A.). It was stored in a refrigerator and used as received. Toluene used in the syntheses was dried over 4-Å molecular sieves.

Syntheses

The MPSGs were prepared by first heating the silica at about 120°C to remove adsorbed water; cooling under vacuum; refluxing with a toluene solution of 3-mercaptopropyltrimethoxysilane; filtering off the MPSG; washing it with toluene, methanol and/or acetone; and curing the solid at about 80°C under vacuum for several hours. Specifics for each preparation are given in Table I. Approximately a two-fold excess of silane to available silica –OH groups was used for the Adsorbosil material, nearly a twenty-fold excess for the LiChrosorb, and a 180-fold excess for the Hypersil. No real difference in surface coverage was observed for the Adsorbosil and LiChrosorb materials, these values being about 1.4 and 1.5 μmol/m^2 respectively. The coverage of the Hypersil-based MSPG, however, was about 2.9 μmol/m^2. Some of the fines were removed from the Adsorbosil-based MPSG by stirring up the material in water and pouring off the liquid after 10 min. This was repeated twice. All the MPSG materials were white or slightly off-white. If the MPSG-Ag materials were left out

TABLE I

SYNTHESIS CONDITIONS AND FINAL METAL-UPTAKE CAPACITY FOR MPSG PHASES

Substrate material	*Mass (g)*	*Volume toluene, silane (ml)*	*Reflux time (h)*	*Metal uptake (μmol/g)*	*Ligand coverage*** *(μmol/m²)*
TLC silica	50	200, 5	2	400*	0.7
Adsorbosil	20	175, 10	2.5	650**	1.4
LiChrosorb	6	80, 20	5.5	440**	1.5
Hypersil	4	80, 14	5	175**	2.9

* Based on mercury(II) extraction; carbon determination gave 383 μmol/g[1].
** Based on silver(I) extraction.
*** Assuming ligand: metal stoichiometry (1:1).

in the light for several months they turned tan or dirty yellow, but MPSG itself remained white.

Capacity determination

Generally a 100–200-mg sample of MPSG was mixed with a 20–50 fold excess of silver nitrate solution and stirred for 30 min. The solid was filtered off and washed with water. Washings and filtrate were combined and unextracted silver ion was determined by atomic absorption spectrometry (AAS). The capacity of the TLC silica-based MPSG was obtained by extraction of mercury(II). Unextracted mercury was determined by displacement titration with Mg-EDTA and Erichrome Black T indicator.

In some cases an acid digestion of the silver-loaded MPSG was performed. Three 5-ml aliquots of concentrated nitric acid were added to 100–200 mg of the MPSG-Ag and the mixture was heated for several min after each addition to free the silver ion, which was determined by AAS.

Loading of silver(I) on MPSG

To about 4 g of MPSG 6 ml of 1 *M* silver nitrate solution and 10–20 ml of water were added. The mixture was stirred for about 20 min. The solid was filtered off and washed with water and then methanol.

Extraction studies

About 200 mg of MPSG were placed in a 100-ml beaker and 30 ml of water and 0.04 mmol of the desired metal ion were added. Dilute hydrochrloric acid or sodium hydroxide were used to adjust the pH to that desired. Stirring was continued for 15 min and the final pH reading was made. The solid was filtered off, washed with water, and washings and filtrate combined. The unextracted cadmium(II) and zinc(II) were determined by direct EDTA titration; mercury(II) and lead(II) by displacement titration with Mg-EDTA; and silver(I) by the Volhard method.

General chromatographic conditions

All columns were first equilibrated with 20–30 ml of mobile phase. Most runs used a flow-rate of 1 ml/min. All injection volumes were 20 μl, and detector wave-

length was 254 nm. Mobile phases were helium-degassed for 5 min before use. All runs were at ambient temperature.

RESULTS AND DISCUSSION

Stationary phase characteristics

While it is well known that silver(I) has a high affinity for sulfur-containing compounds and sulfide ion (*e.g.* pK_{sp} for silver(I) sulfide is *ca.* 50) it was desirable to test the interaction of silver and several other ions with MPSG. As seen in Fig. 1 silver(I) [and mercury(II)] are completely extracted by MPSG even at pH 0. In fact, only about 75% recovery of extracted silver ion was obtained with 6 *M* nitric acid. These extraction characteristics imply possible utility for MPSG as a silver-scavanging material. Efforts are underway in our laboratory to develop methods to recover the metal ion from the MPSG without destroying the solid phase.

Another implication of the extraction data is that silver-loaded MPSG should be an extremely stable HPLC stationary phase, even in acidic eluents. Indeed, column effluents were periodically monitored for silver(I) during this work by AAS and no significant loss of silver was observed. In addition, several columns were emptied after considerable use, the packing was acid-digested, and silver ion was determined by AAS. The initial silver-loading values compared favorably with the acid-digestion results, within experimental error [*e.g.* 650 μmol/g silver(I) for fresh MPSG-Ag, *versus* 670 μmol/g after 90 h of use for an Adsorbosil-based column].

While aminopropyl silica gel (APSG) has been shown to exhibit 2:1 ligand-to-metal stoichiometry with copper(II)[10], mercaptopropyl silica gel exhibits 1:1 stoichiometry with mercury(II) and silver(I). Extraction of mercury(II) (large excess of metal ion) with one batch of MPSG yielded a metal uptake value of 400 μmol/g while elemental carbon analysis gave a ligand surface coverage value of 383 μmol/g. Another batch of MPSG had a silver-uptake value of about 350 μmol/g and an elemental

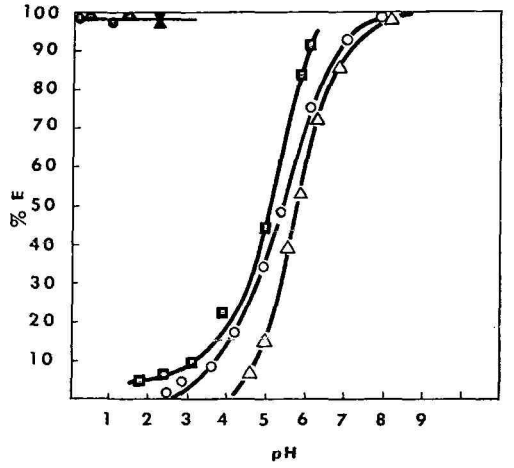


Fig. 1. Percent metal ion extracted (%E) by MPSG as a function of pH. Metal-uptake capacity was about 400 μmol/g. ● = mercury(II); ▲ = silver(I); ■ = lead(II); ○ = cadmium(II); △ = zinc(II).

TABLE II

RETENTION ON MPSG AND MPSG-Ag IN METHANOL–WATER MIXTURES

Both phases were from the same batch of Adsorbosil-based material, 680 μmol/g.

Compound	*Retention time (min)*			
	MPSG		*MPSG-Ag*	
	70:30	*50:50*	*70:30*	*50:50*
Naphthalene	2.9	3.6	4.4	–
Acenaphthene	3.0	6.2	6.2	16.3
Anthracene	3.0	9.3	9.6	36.5

sulfur analysis of 367 μmol/g. The implication of these results is that one of the two silver(I) coordination sites is available for interaction with analyte ligands.

With regard to the reactivity of these phases we have indirect evidence that the –SH groups of MPSG columns can be gradually converted to disulfides by certain mobile phases or samples. However, in the silver-loaded form, this sort of column degradation is minimized. Furthermore, protection of the –SH group by the silver ion permits selective end capping of residual silanols, while the silver content of the MPSG-Ag is not substantially altered, even in refluxing toluene-trimethylchlorosilane. One batch of MPSG-Ag had silver contents of 136 and 133 μmol/g before and after end capping, respectively.

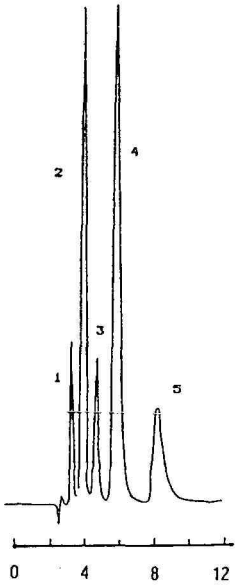
Chromatographic characterization

As silver ion is expected to interact less strongly with unsaturated compounds than with nitrogen or sulfur bases, the PAHs were chosen as initial test probes for the MPSG-Ag phases. We first compared a MPSG column with a MPSG-Ag column to see what effect the silver ion had on retention of the PAHs. It is clear from Table II that there is significantly less interaction of the PAHs with the mercaptopropyl group itself than with the silver-loaded phase. Indeed, at high methanol content there is practically no interaction of the smaller PAHs, whereas interaction with MPSG-Ag is extensive.

It would appear that, for aromatic compounds at low water content, essentially a single solute–stationary phase mechanisms obtains for MPSG-Ag columns, namely the interaction with the silver ion.

A second portion of the same batch of Adsorbosil-based MPSG was loaded to a lower silver content, 400 μmol/g as compared to 680 μmol/g for the first batch, and several PAHs run in methanol–water (70:30) and compared to the same system on the more highly-loaded column. Not unexpectedly, shorter retention times were observed with lower silver loading. For example, retention times for anthracene were 9.6 min on the more heavily loaded and 6.2 min on the less heavily loaded column.

MPSG-Ag was used for the separation of mixtures of several PAHs in both normal- and reversed-phase eluents. In Fig. 2 is shown the chromatogram for five compounds in isooctane–isopropanol (70:30) on Adsorbosil-based MPSG-Ag. Good resolution is achieved in about 8 min.



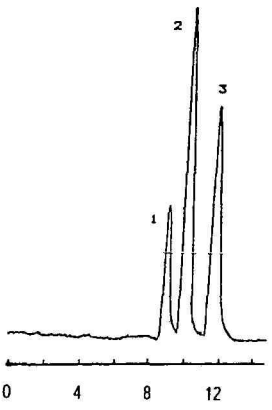


Fig. 2. Separation of five PAHs on 10 μm, Adsorbosil-based MPSG-Ag, uptake capacity 650 μmol/g, in isooctane–isopropanol (70:30, v/v). 1 = acenapthene; 2 = anthracene; 3 = pyrene; 4 = chrysene; 5 = perylene.

Fig. 3. Separation of three PAHs on 5 μm, LiChrosorb Si 100-based MPSG-Ag, uptake capacity 440 μmol/g, in 100% methanol. 1 = benzo[*b*]fluoranthene; 2 = benzo[*k*]fluoranthene; 3 = benzo[*a*]pyrene.

Some work was also done with 5-μm diameter phases. In Fig. 3 is shown the separation of three 5-ring compounds in 100% methanol on 5-μm LiChrosorb Si 100 based MPSG-Ag. Extensive interaction of these aromatic compounds evidently occurs, as even in this strong mobile phase significant retention is observed.

A finding which suggests that further investigation is required, however, is the significant drop in efficiency on going from 3-ring compounds to larger ones, as exhibited by broader peaks with noticeable tailing. For example, the number of theoretical plates for the Adsorbosil-based 70-Å MPSG-Ag column was 3200 for acenapthene, 2700 for naphthalene, but dropped to 1000 for chrysene (four rings) and to 500 for perylene (five rings) in isooctane–isopropanol (92:8). Similar effects were found for other MPSG-Ag columns and other mobile phases. Anthracene exhibited a plate count of 2500 on a LiChrosorb Si 100 based MPSG-Ag column in methanol–water (75:25), while the number of plates for pyrene (four rings) was just 1300.

It is possible that these effects are due to increasingly poor mass transfer in the relatively small-pore structure of the 70- and 100-Å pore materials. Sander and Wise[11] showed that larger ring compounds are more efficiently separated on larger pore silicas, and so we performed some preliminary studies on 300-Å Hypersil silica. A trade off is involved, however, as the larger pore materials have lower surface areas. Indeed, the capacity of the Hypersil-based MPSG was only about 175 μmol/g,

although this coverage, 2.9 μmol of ligand per m^2 of original surface, is more complete than that of the smaller pore materials described above. In any case, our preliminary qualitative results do indicate that higher efficiency is obtained for the larger ring compounds on this large-pore material. More intensive investigation is required, however, before we can be more definitive about this question.

It is also possible that some of the tailing is due to interaction of the larger, more polarizable PAHs with residual silanol sites. The fact that poor peak shape and long retention times were observed in 100% hexane or isooctane but that only a fraction of a percent of isopropanol sharpended up the peaks and dramatically reduced retention lends some credence to this suggestion. The polar co-solvent apparently attenuates the effect of the silanols. We are presently studying the effect of end capping the MPSG-Ag phases to determine if better efficiencies are obtained as compared to those on the uncapped material described here.

While the PAHs can be separated quite well on reversed-phase non-polar columns, the significant interaction of these test compounds with the MPSG-Ag columns, and the stability of the phases under use, imply that these materials can be employed for more difficult separations requiring the greater selectivity that the silver(I) ion affords. Intensive investigation is underway in our laboratory with several classes of nitrogen- and sulfur-containing analytes on these new materials.

ACKNOWLEDGEMENTS

We thank the UNCG Research Council, the Research Corporation, and the North Carolina Board of Science and Technology for financial assistance with this work. We also thank E. M. Science for providing some of the LiChrosorb materials and Shandon Southern for the Hypersil silica.

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CHROM. 19 444

Note

Reversed-phase high-performance liquid chromatography of metronidazole benzoate in suspension dosage form

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Metronidazole is a drug widely used in the treatment of anaerobic infections but with restricted application to paediatrics because of its bitter taste. Therefore, its derivative metronidazole benzoate is used for these purposes as a suspension dosage form. The most probable degradation products of metronidazole benzoate in such a drug formulation are benzoic acid and metronidazole. Hence, their presence can serve as evidence of instability of the drug.

Spectrophotometry[1] and differential-pulse polarography[2] have been utilized for the determination of metronidazole benzoate in suspension forms, but these methods are time-consuming and non-specific. Metronidazole alone has been determined by gas chromatography, while benzoic acid was separately evaluated by non-aqueous titration of the drug[3]. These methods are not directly applicable to analysis of the final suspension dosage form. Previously[4] it was shown that the determination of both the impurities could be effected on a reversed-phase column. In spite of the high selectivity, the elution of the strongly retained metronidazole benzoate was not possible and regeneration of the column had to be performed after each analysis.

Reversed-phase high-performance liquid chromatography (RP-HPLC) for monitoring of metronidazole either in biological fluids or in dosage forms has been extensively used (see, *e.g.*, refs. 5 and 6) with buffered aqueous mobile phases containing a small amount of organic modifier. The acid–base properties of metronidazole were not interpreted in these works and the chromatographic systems proposed were not suitable for simultaneous analysis of metronidazole benzoate and metronidazole.

In the present study two reversed-phase chromatographic systems were developed on acid-base considerations for simultaneous analysis of metronidazole benzoate, admixtures of benzoic acid and metronidazole and a pair of preservatives (methyl- and propylparaben), being the most important components of a metronidazole benzoate suspension dosage form.

EXPERIMENTAL

Instrumentation

A Perkin-Elmer Model Series 4 liquid chromatograph with a Rheodyne Model 7125-075 syringe-loading sample injector and a Perkin-Elmer Model LC-85B vari-

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able-wavelength detector were used. The detector was operated at 254 nm. A Shimadzu computing integrator Chromatopac Model C-R3A was employed with a BASIC program for statistical evaluation of the chromatographic constants.

The potentiometric titrations and pc_H^* measurements were performed as previously described[7] using a Radiometer PHM 64 digital pH meter at about 22–25°C.

Columns

The chromatographic experiments were carried out with a C_{18} reversed-phase Perkin-Elmer analytical column (250 mm × 4 mm I.D.), mean particle size 10 μm, under isocratic elution conditions. A guard column (50 mm × 2 mm I.D.) filled with 30–38 μm Co:Pell ODS (Whatman, U.K.) was attached to the main column.

Reagents and sample preparation

Methanol and acetonitrile (Merck, F.R.G.) were used without further purification for aqueous–organic solvents. The buffer salts, dipotassium hydrogenphosphate, sodium formate, sodium acetate, orthophosphoric acid, acetic acid, nitric acid and potassium nitrate were of analytical reagent grade. Sodium dodecyl hydrogensulphate was from Merck (F.R.G.). The standard solutions were prepared from analytes of pharmacopoeial purity, except for metronidazole benzoate which was in correspondence to an analytical certificate (Trans-Medica, F.R.G.).

About 2.0 g (accurately weighed) of the suspension sample ("Metronidazole suspension", Bulgaria)* containing 3.5% (w/w) metronidazole benzoate were transferred to a 100.0-ml volumetric flask. An 80-ml volume of methanol–water (80:20) was added. The contents were ultrasonicated for a few minutes then made up to the mark with the solvent. A portion of the mixture obtained was centrifuged at *ca.* 900 *g* for 10 min. Aliquots (10 μl) from the upper clear layer were chromatographed.

A standard solution containing 0.5% (w/w) of both metronidazole and benzoic acid was prepared, and quantitation by the external standard method was performed.

Chromatographic procedures

Solutions of 0.005 *M* buffer salts and 0.05 *M* potassium nitrate were prepared in methanol–water (60:40, v/v). Solutions of *X M* potassium nitrate and *Y M* sodium dodecyl hydrogensulfate, where $X + Y = 0.04$ *M*, and 0.01 *M* nitric acid at a constant ionic strength of 0.05 *M* and $pc_H^* = 2.0$ were prepared in methanol–water (60:40) or in acetonitrile–water (35:65) for ion-pair chromatographic experiments.

The mobile phase hold-up time was determined as described[7].

RESULTS AND DISCUSSION

The dependence of the capacity factors, k', on the composition of either water–methanol (Fig. 1A) or water–acetonitrile (Fig. 1B) mixtures has been determined for all the substances investigated: metronidazole, benzoic acid, methylparaben, propylparaben and metronidazole benzoate. It was concluded that there is no isocratic mobile phase composition at which a good retention for the most polar analytes (metronidazole and benzoic acid) and an adequate separation of all the

* "Klion-suspension" (Hungary) is an identical dosage form.

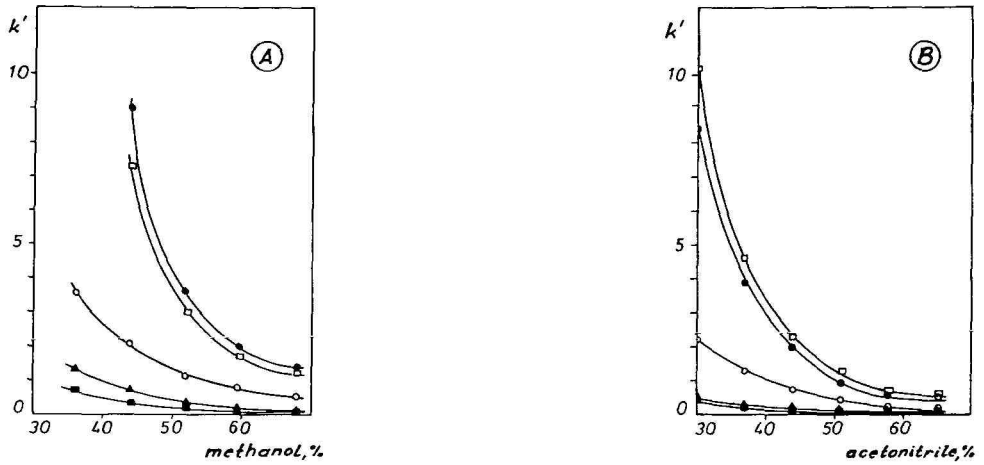

Fig. 1. Dependences of the capacity factors, k', on solvent composition: (A) methanol–water, (B) acetonitrile–water mixtures. Solutes: metronidazole (■); benzoic acid (▲); methylparaben (○); metronidazole benzoate (□) and propylparaben (●).

components could be achieved. Furthermore, a poor peak shape for benzoic acid was observed with the methanolic mobile phases. The resolution of the pair benzoic acid/metronidazole in acetonitrilic eluents was not satisfactory, and that of the pair metronidazole benzoate/propylparaben was not sufficient for quantitation purposes because of the high mass ratio for these components in the dosage form.

Three of the above mentioned substances show acid–base properties: metronidazole and metronidazole benzoate are protonated acids, BH^+, and benzoic acid is an uncharged acid, HA. Thus, the following approaches were preferred.

A. A simple pH-control approach

Fig. 2 shows the dependence of k' on pc_H^* in methanolic mobile phases. The retention of the fully protonated acids, BH^+, is not possible because of the instability of the silica-based columns when the mobile phase pH is lower than 2. With the computer program, the dissociation constants pK_a^* and one of the capacity factors of

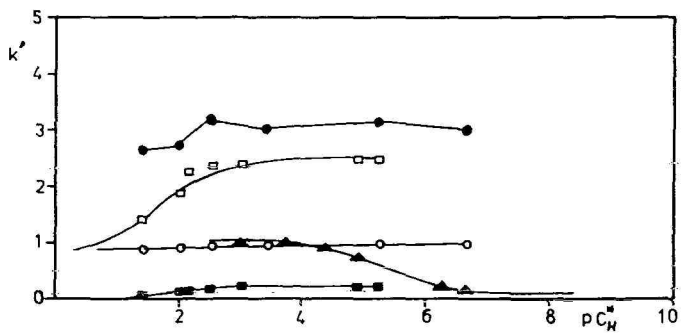

Fig. 2. Plots of k' *vs.* pc_H^*. Mobile phases: 0.005 *M* buffers and 0.05 *M* potassium nitrate in methanol–water (60:40). Solutes as in Fig. 1.

TABLE I

CAPACITY FACTORS AND pK_a^* VALUES OF BENZOIC ACID, METRONIDAZOLE AND METRONIDAZOLE BENZOATE

Mobile phases: 0.005 *M* buffers and 0.05 *M* potassium nitrate in methanol–water (60:40).

Compound	*Experimental values*		*Calculated values*			pK_a^*
	k'_0	k'_{-1}	k'_0	k'_1	pK_a^*	
Metronidazole	0.23	–	–	0.0 (±0.03)**	2.11 (±0.12)	2.55* (ref. 9)
Metronidazole benzoate	2.47	–	–	0.80 (±0.07)	1.59 (±0.06)	–
Benzoic acid	–	0.05	0.92 (±0.06)	–	5.49 (±0.05) 5.27§ (±0.01)	5.54*** (ref. 10)

* Determined spectrophotometrically in water.
** Confidence interval (p = 0.95).
*** Determined potentiometrically in methanol–water (60:40).
§ Determined potentiometrically in this work.

the respective species were calculated* and the theoretical curves were drawn. A good agreement between experimental and theoretical data was observed, hence, the retention change of the monoprotolytes in such buffered eluents is as predicted by the theory[7,8]. The chromatographic constants are presented in Table I. It is seen that the pK_a^* values obtained and those cited are in good agreement.

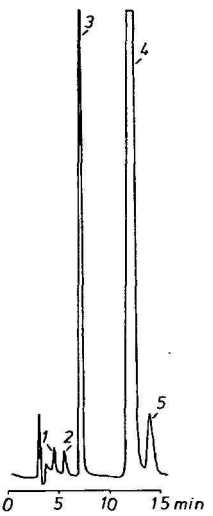

Fig. 3. Sample chromatogram ("Metronidazole suspension"). Mobile phase: 0.005 *M* acetate buffer and 0.05 *M* potassium nitrate in methanol–water (60:40), pc_H^* = 5.2, flow-rate 1 ml/min. Peaks: 1 = metronidazole; 2 = benzoic acid; 3 = methylparaben; 4 = metronidazole benzoate and 5 = propylparaben.

* The program required one of the two constants, k'_0 for uncharged species B and HA or k'_1 for BH^+, k'_{-1} for A^-, respectively, to be determined experimentally.

Based on the constants and capacity factors determined for all substances from Fig. 2, a mobile phase having pc_H^* about 5.5 was chosen as the most appropriate one. The separation of the sample solution using such an eluent is shown in Fig. 3.

*B. An ion-pair liquid chromatographic approach**

The results obtained by approach A show that metronidazole and metronidazole benzoate are relatively strong acids, being of the type BH^+. Therefore, one can use an ion-pair reagent to enhance the retention of the poorly retained metronidazole. The dependence of k' on the counter ion (dodecyl hydrogensulphate) concentration in both the methanol- and acetonitrile-containing mobile phases is presented in Fig. 4. The counter ion concentration was varied at a constant pH ($pc_H^* = 2.0$). With methanolic mobile phases (Fig. 4A), metronidazole is more strongly retained but benzoic acid and methylparaben are not resolved —the well known drawback of this chromatographic technique. A change in the elution order of propylparaben and metronidazole benzoate was observed at about 4 mM dodecyl hydrogensulphate.

For better selectivity towards these two pairs, acetonitrile (being more selective towards parabens[11] was used instead of methanol as an organic modifier (Fig. 4B). A good retention for metronidazole ($k' \approx 1$) and a separation of the pair methylparaben/benzoic acid (resolution, $R_s \approx 1$) for quantitation purposes was achieved at a concentration 4 mM dodecyl hydrogensulphate, although the capacity factors (and analysis time) were increased two-fold. The separation of all substances was possible over a wide range of a counter-ion concentrations. The chromatographic system is more selective towards BH^+ type compounds and can be used when any doubts exist about peak purity or identity for metronidazole.

A statistical evaluation by the least-squares method (multiple standard mode) in the region of 80–120% (w/w) of each compound (declared content) was performed by means of the two chromatographic methods. Linear detector responses were observed and the sensitivity of both methods was estimated as equivalent to three times the baseline noise, *i.e., ca.* 0.1% for both benzoic acid and metronidazole. The relative standard deviation did not exceed 2% in the course of a study of drug stability and the contents of metronidazole and benzoic acid were less than 0.5% (usually 0.3%).

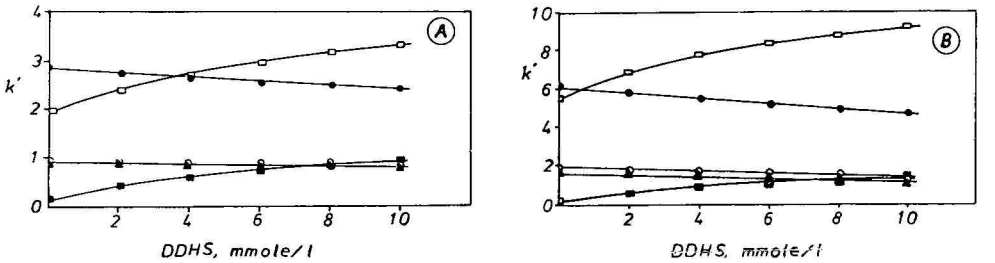

Fig. 4. Ion-pair chromatography. Plots of k' *vs.* dodecyl hydrogensulphate (DDHS) concentration. Mobile phases: 0.01 M nitric acid, potassium nitrate and DDHS at constant ionic strength $I = 0.05$ M and $pc_H^* = 2.0$ in (A) methanol–water (60:40), (B) acetonitrile–water (35:65). Solutes as in Fig. 1.

* More precisely, this is ion-pair liquid chromatography with pH control. Some ionogenic substances which do not possess acid–base properties can form ion-pairs, too, and no pH control is needed.

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Note

Determination of enantiomeric purity of *Z*-oxylysine by capillary gas chromatography

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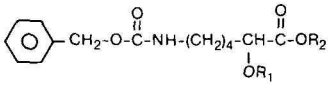
The L enantiomer of *Z*-oxylysine (compound I, Table I) is a key intermediate in the synthesis of a new angiotensin converting enzyme (ACE) inhibitor used in the treatment of hypertension[1,2]. An optical purity method was required to determine the optical purity of the desired L enantiomer. The enantiomeric separation by gas chromatography (GC) employs chiral reagent–achiral GC column[3] or achiral reagent–chiral GC column combination[4–7]. While the latter approach is preferred, the direct separation of the enantiomers of *Z*-oxylysine with commercially available chiral column was not feasible. A GC method using a chiral reagent for the diastereomer formation and a capillary achiral column for chromatography is described. As little as 0.2% of the D enantiomer in a sample of L-*Z*-oxylysine can be determined.

TABLE I

CHEMICAL STRUCTURES OF *Z*-OXYLYSINE AND ITS DERIVATIVES



Compound	R_1	R_2
I (*Z*-oxylysine)	H	H
II	H	-CH-(CH$_2$)$_5$-CH$_3$ (CH$_3$ branch)
III	H	-CH-CH$_2$-CH$_3$ (butyl) (CH$_3$ branch)
IV	-C(=O)-CF$_2$CF$_3$	butyl
V	-Si(CH$_3$)$_3$	butyl
VI	-Si(CH$_3$)$_2$ C(CH$_3$)$_3$	butyl
VII	-C(=O)-NH-CH(CH$_3$)$_2$	butyl

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EXPERIMENTAL

Gas chromatography

A Hewlett-Packard 5840 gas chromatograph equipped with a flame ionization detector and an autosampler was used. Chromatography was performed on a DB-17+, 15 m × 0.32 mm I.D. with a 0.5 μm stationary phase film thickness (J&W). The inlet pressure of the helium carrier gas was 90 KPa (13 p.s.i.g.) and the flow-rate of the helium make-up gas for the flame ionization detector was 25 ml/min. Injections were made in the split mode at a split flow-rate of 50 ml/min, using an empty glass insert (Hewlett-Packard). The oven temperature was held isothermally at 240°C. The injector and detector temperatures were maintained at 280°C.

Reagents and chemicals

The L and D enantiomers of *Z*-oxylysine were characterized reference materials obtained from the Department of Chemical Process Technology (E. R. Squibb & Sons). Thionyl chloride, *l*-2-octanol and *d*-2-octanol were obtained from Aldrich. A thionyl chloride solution was prepared by dissolving 2.5 ml of thionyl chloride and 2.5 μl of dimethylformamide in 25 ml of *n*-hexane. The solution of *l*-2-octanol was prepared by dissolving 2.5 ml of *l*-2-octanol in 25 ml of methylene chloride.

Sample preparation

To approximately 6 mg of L-*Z*-oxylysine sample, weighed directly into an autosampler vial, 1.0 ml of thionyl chloride solution was added. The vial contents, sealed with a PTFE-lined cap, were vortexed and kept at room temperature for 30 min. The vial was then uncapped and the reagent was removed by evaporation at 50°C under a stream of nitrogen. To the dried residue, 0.3 ml of *l*-2-octanol solution was added to the vial. After sealing and vortexing, the solution was heated at 60°C for 30 min. The cooled vial was then uncapped and the reagent was removed by evaporation at 50°C, under nitrogen. The samples, reconstituted with 0.5 ml of methylene chloride, were placed into the autosampler after recapping the vials. A 1.0-μl aliquot was then injected.

Quantitation

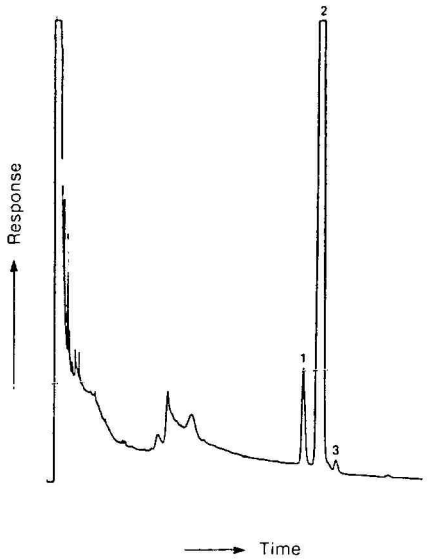
The percentage of the L enantiomer in the L-*Z*-oxylysine sample was calculated using the formula:

$$L = \frac{P(A_1 + A_2) - A_1}{(A_1 + A_2)(2P - 1)} \cdot 100$$

where L = percentage of the L enantiomer of *Z*-oxylysine; P = percentage of the *l* enantiomer of 2-octanol divided by 100; A_1 = area of peak 1 (Fig. 1 and Appendix); A_2 = area of peak 2 (Fig. 1 and Appendix)

RESULTS AND DISCUSSION

The method utilizes a chiral reagent for derivatization of *Z*-oxylysine (compound I, Table I) and achiral fused-silica capillary column for chromatographic sep-



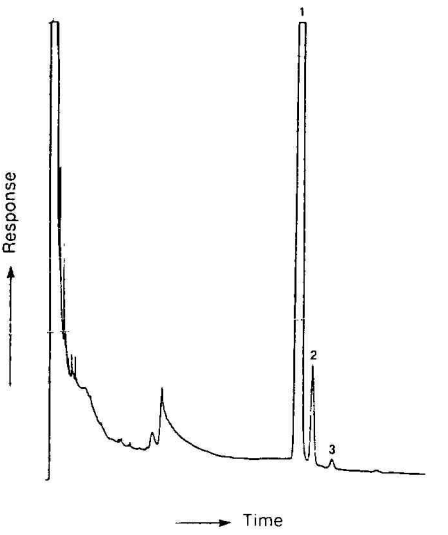


Fig. 1. Chromatogram of L-*Z*-oxylysine sample spiked with 3.0% of D-*Z*-oxylysine, after esterification with *l*-2-octanol. Peak 1 = D-*Z*-oxylysine (16.3 min); peak 2 = L-*Z*-oxylysine (17.5 min); peak 3 = unknown.

Fig. 2. Chromatogram of L-*Z*-oxylysine sample spiked with 3.0% D-*Z*-oxylysine, after esterification with *d*-2-octanol. Peak 1 = L-*Z*-oxylysine (16.3 min); peak 2 = D-*Z*-oxylysine (17.5 min); peak 3 = unknown.

aration. Compound I is first converted into the acid chloride with thionyl chloride which is then reacted with the chiral *l*-2-octanol to form diastereomeric esters (compound II, Table I). GC using a DB-17+ achiral capillary column is used to resolve the octyl diastereomeric esters (Figs. 1 and 3).

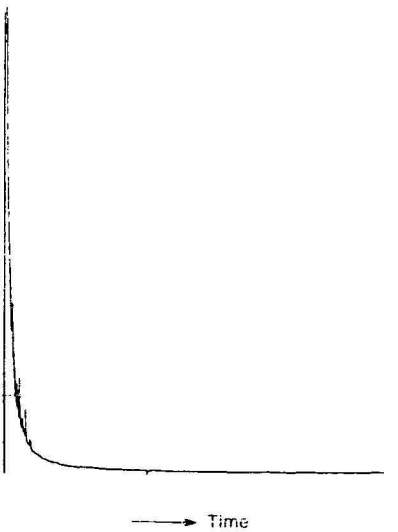


Fig. 3. Chromatogram of a reagent blank corresponding to Fig. 1.

TABLE II

RECOVERY OF D-*Z*-OXYLYSINE ADDED TO L-*Z*-OXYLYSINE

The values shown in parentheses are obtained after correction for concentration from the L enantiomer sample.

Added (%)	*Recovered (%)*
0	0.53 (0)
0.50	1.03 (0.50)
1.49	2.08 (1.55)
4.98	5.59 (5.06)

By reacting L-*Z*-oxylysine with *l*-2-octanol and *d*-2-octanol, separately, it was established that the L*l* and L*d* diastereomers are chromatographically separable and that L*l* elutes after L*d* (Figs. 1 and 2). By reacting D-*Z*-oxylysine with *l*-2-octanol and *d*-2-octanol, it was also shown that D*d* elutes after D*l*. As expected for a non-chiral column, L*l* co-eluted with D*d* and L*d* co-eluted with D*l*. For the determination of a trace amount of the D enantiomer in L-*Z*-oxylysine sample, *l*-2-octanol was used as the reagent, since the minor peak will elute before the major peak to yield a more accurate measurement.

The two reactions used for sample preparations, acid chloride formation and octyl ester formation, were studied by varying reaction times. No difference was seen in % D of L-*Z*-oxylysine when each reaction time was increased up to four hours. Consequently, no racemization occurs.

The accuracy of the method was established by analyzing L-*Z*-oxylysine samples spiked with varying amounts of D-*Z*-oxylysine. As shown in Table II, added D-*Z*-oxylysine was quantitatively recovered. Excellent precision was obtained for replicate sample preparations of a batch of L-*Z*-oxylysine (Table III). The limit of quantitation was estimated to be 0.2% of the D enantiomer.

Various derivatization reactions were investigated. Esterification with *d*-2-butanol following acid chloride formation with thionyl chloride gave compound III (Table I), but there was no separation between the butyl ester diastereomers on the achiral column. Compound IV, formed by acylation of III with pentafluoropropionic

TABLE III

REPRODUCIBILITY OF REPLICATE SAMPLE PREPARATIONS OF A BATCH OF L-*Z*-OXYLYSINE

R.S.D. = Relative standard deviation.

Replicate No.	*% D*
1	0.63
2	0.68
3	0.67
4	0.63
5	0.69
Average	0.66
R.S.D.	4.5%

anhydride (PFPA), eluted earlier than III, but the diastereomers could not be resolved. Silylation of III with trimethylsilylimidazole in pyridine (Tri-Sil Z) or N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) to form V and VI, respectively, did not achieve the desired separation. Carbamate formation reaction[4,7] of III with 0.5 ml of isopropyl isocyanate was nearly complete with triethylamine, 10 μl, used as a catalyst, at 60°C, 30 min. The diastereomers (VII) were not resolved, however.

There was no resolution between the diastereomers of II when the alcoholic group was acylated or silylated, with PFPA, Tri-Sil Z or MTBSTFA. The carbamate product formed when II was reacted with isopropyl isocyanate and triethylamine eluted at a temperature higher than that of II. The resolution between the carbamate diastereomers was, however, not as good as the diastereomers of II. There was no resolution for the diastereomers of the ethyl isocyanate product.

The advantages of determining optical purity of a chiral compound by using a chiral column is well documented[4–7]. Thus, the use of Chirasil-Val III, a chiral fused-silica capillary column (Alltech), was investigated. The direct approach, without diastereomer formation, via the methyl ester of I, failed to achieve separation between the enantiomers. Acylation or silylation of the methyl ester did not produce resolution of the enantiomers. The diastereomers of the butyl ester, III, and its acyl or silyl derivatives were not resolved on the chiral column. The use of the octyl ester (II), and its acyl or silyl derivatives, has no practical application because of the prolonged retention on the chiral column, operated near the maximum allowable temperature.

ACKNOWLEDGEMENT

The authors express their gratitude to Mr. R. Mark for his technical assistance.

APPENDIX

Derivation of the formula used for the determination of the L *isomer of* L*-Z-oxylysine*

The following symbols are used: D = the D isomer of *Z*-oxylysine; L = the L isomer of *Z*-oxylysine; d = the d isomer of 2-octanol; l = the l isomer of 2-octanol; p = the fraction of l in 2-octanol; q = the fraction of L in *Z*-oxylysine.

When *Z*-oxylysine reacts with 2-octanol, four stereoisomers are formed. They are: Dd, Dl, Ld and Ll. Dd and Ll are a pair of enantiomers. Dl and Ld are a second pair of enantiomers. Either Dd or Ll is a diastereomer of either Dl or Ld. Let the total quantity of the four stereoisomers = T. Then, the quantity of each stereoisomer can be computed as follows:

$$\text{L}l = qpT \quad (1)$$

$$\text{L}d = q(1 - p)T \quad (2)$$

$$\text{D}l = (1 - q)pT \quad (3)$$

$$\text{D}d = (1 - q)(1 - p)T \quad (4)$$

Note that when the four equations are added, Dd + Dl + Ld + Ll = T, as expected. Note also that the symbols are used to denote the stereoisomers and their respective quantities.

By rearranging eqn. 1 and solving for q:

$$q = \frac{\text{L}l}{pT} \tag{5}$$

In the achiral chromatographic system used, Dd and Ll stereoisomers (enantiomers) are not separated from each other, but as an unresolved peak are separated from the Dl, Ld combination. Thus, the above equation cannot be used as such since one cannot obtain the amount of Ll directly.

Referring to Fig. 1, let area of peak 1 = A_1; area of peak 2 = A_2. When *l*-2-octanol is reacted with L-*Z*-oxylysine as described under Experimental, peak 2 is the sole or major peak, depending on the optical purity of the *l*-2-octanol or the L-*Z*-oxylysine. Thus, A_1 is the response due to the stereoisomers Dl and Ld. A_2 is the response due to Dd and Ll. Thus, assuming that area is proportional to the quantities of the stereoisomers and the response factors (response/quantity) of the stereoisomers are identical.

$$A_2 = \text{D}d + \text{L}l \tag{6}$$

Note that the proportionality constant that relates area to quantity is assumed to be one for simplicity. Thus,

$$\text{L}l = A_2 - \text{D}d \tag{7}$$

Also,

$$T = A_1 + A_2 \tag{8}$$

Eqn. 5 can now be rewritten as:

$$q = \frac{A_2 - \text{D}d}{p(A_1 + A_2)} \tag{9}$$

The numerator of eqn. 9 can be worked out as follows:

$$\begin{aligned} \text{D}d &= (1 - q)(1 - p)T \\ &= (1 - q)(1 - p)(A_1 + A_2) \end{aligned} \tag{10}$$

Thus $A_2 - \text{D}d$ can be rewritten as:

$$A_2 - [(1 - q)(1 - p)(A_1 + A_2)]$$

i.e.,

$$A_2 - [(1 - p - q + qp)(A_1 + A_2)]$$

i.e.,

$$A_2 - (A_1 + A_2 - pA_1 - pA_2 - qA_1 - qA_2 + qpA_1 + qpA_2)$$

i.e.,

$$A_2 - A_1 - \text{A}_2 + pA_1 + pA_2 + qA_1 + qA_2 - qpA_1 - qpA_2$$

i.e.,

$$pA_1 + pA_2 + qA_1 + qA_2 - qpA_1 - qpA_2 - A_1 \tag{11}$$

The product of the quotient and the denominator of eqn. 9 can be worked out as follows (cross multiplication):

$$qp(A_1 + A_2)$$

i.e.,

$$qpA_1 + qpA_2 \tag{12}$$

Therefore, equating the numerator to the product of the quotient and the denominator,

$$qpA_1 + qpA_2 = pA_1 + pA_2 + qA_1 + qA_2 - qpA_1 - qpA_2 - A_1$$

i.e.,

$$2qpA_1 + 2qpA_2 - qA_1 - qA_2 = pA_1 + pA_2 - A_1$$

i.e.,

$$2qp(A_1 + A_2) - q(A_1 + A_2) = pA_1 + pA_2 - A_1$$

i.e.,

$$(A_1 + A_2)(2qp - q) = pA_1 + pA_2 - A_1$$

i.e.,

$$q(A_1 + A_2)(2p - 1) = p(A_1 + A_2) - A_1 \tag{13}$$

Therefore,

$$q = \frac{p(A_1 + A_2) - A_1}{(A_1 + A_2)(2p - 1)}$$

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CHROM. 19 518

Note

High-performance liquid chromatography assay for the measurement of benzydamine hydrochloride in topical pharmaceutical preparations

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Benzydamine or N,N-dimethyl-3-{[1-(phenylmethyl)-1H-indazol-3-yl]oxy}-1-propanamine is a non-steroidal anti-inflammatory drug. Available data suggest that benzydamine interferes selectively with the localized phenomena of inflammation by stabilizing the cellular membrane and selectively inhibiting prostaglandin systems[1]. Benzydamine has been shown to be active after both topical[2,3] and systemic administration[4], and to be relatively devoid of both local and systemic side effects[5]. It has been reported that a high concentration of benzydamine is maintained in inflamed tissues for a much longer period of time when the drug is administered topically as opposed to orally[6]. In one study it was shown that 6 h after topical application, the concentration of benzydamine in inflamed tissues was approximately four times higher than the concentration observed 6 h after oral administration[6].

Due to these properties, benzydamine is widely used in the form of Difflam® cream [3% (w/w) benzydamine hydrochloride] for the relief of symptoms associated with painful inflammatory conditions of the musculo-skeletal system. A gel preparation, containing 3% benzydamine hydrochloride has also been formulated for specific purposes (*e.g.* use with ultrasound), but is not yet commercially available.

Few methods have been described for the measurement of benzydamine. Assays reported involve ^{14}C-labelled drug[3], fluorescence[7] and high-performance liquid chromatography (HPLC) with fluorescence detection[8].

In order to determine benzydamine concentration, within both the gel and cream formulations, a new analytical method based on HPLC with UV detection was devised. This procedure provides a simple, quick, reproducible method of quantification of benzydamine.

EXPERIMENTAL

Apparatus

The HPLC equipment used consisted of an Altex 110A pump and Kontron MS1660 Autosampler with a Spectroflow 773 variable-wavelength UV detector set to 305 nm (the measured λ_{max} of benzydamine hydrochloride in mobile phase). Chromatograms and peak area values were recorded on a Shimadzu Chromatopac C-R3A recording data processor. The column used was a Novapak C_{18} (5-μm particle size,

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150 × 3.9 mm I.D.; Waters). Both the analytical column and the guard column (5 cm) packed with Pellicular ODS C_{18} (Millipore) were enclosed in a block heater (Anachem) and maintained at 30°C.

Mobile phase

The mobile phase was acetonitrile–water–acetic acid (62:37.5:0.5, v/v) and contained 5 m*M* sodium dodecylsulphate. Incorporation of the acetic acid maintained the mobile phase pH at 4.00. The mobile phase was filtered through a 0.45-μm HA membrane filter (Millipore) and thoroughly degassed in an ultrasonic bath prior to use. The flow-rate of the mobile phase was 0.9 ml/min.

Internal standard

Indomethacin was used as the internal standard for the assay and peak area ratios were utilised in preparation of calibration curves and in determination of unknown benzydamine concentrations.

Sample preparation

Dissolution of benzydamine gel samples was carried out using a solvent mixture composed of acetonitrile–water (1:1, v/v). Aliquots of gel were dissolved in 10 ml of the solvent by vortexing for 30 s, and injected directly onto the column. Indomethacin solution (0.1 ml of 1 mg/ml) was added to each of the 10-ml samples prior to vortexing.

Dissolution of Difflam cream was carried out in a solvent mixture consisting of tetrahydrofuran–isopropanol (30:60). Difflam cream samples were weighed into centrifuge tubes, and dissolved in 10 ml of the solvent mixture by vortexing for 5 min. All samples were centrifuged (1100 *g*, 15 min) prior to injection directly onto the column. Indomethacin solution (0.1 ml of 1 mg/ml) was added to each of the 10-ml samples prior to vortexing. An injection volume of 20 μl was used at all times.

Calibration graph

The normal quantity of gel or cream used in preparing the 10 ml of solvent mixture for injection on to the HPLC system was 200 mg. Since a range of gels or creams containing different concentrations of benzydamine were not available and since no blank gel or cream was available, calibration graphs were prepared after dissolving a range of amounts of the product in 10 ml of the appropriate solvent mixture.

RESULTS AND DISCUSSION

Reversed-phase HPLC with UV detection was an effective method for quantifying benzydamine in both cream and gel formulations. Typical chromatograms obtained from gel formulation and cream formulation are shown in Figs. 1 and 2 respectively.

Under the assay conditions described, the indomethacin internal standard and benzydamine had elution times of 3.2 and 5.0 min respectively.

A calibration curve was prepared for benzydamine gel using data points over a concentration range of 0.625 to 20 mg gel/ml of solvent (equivalent to 18.75–600

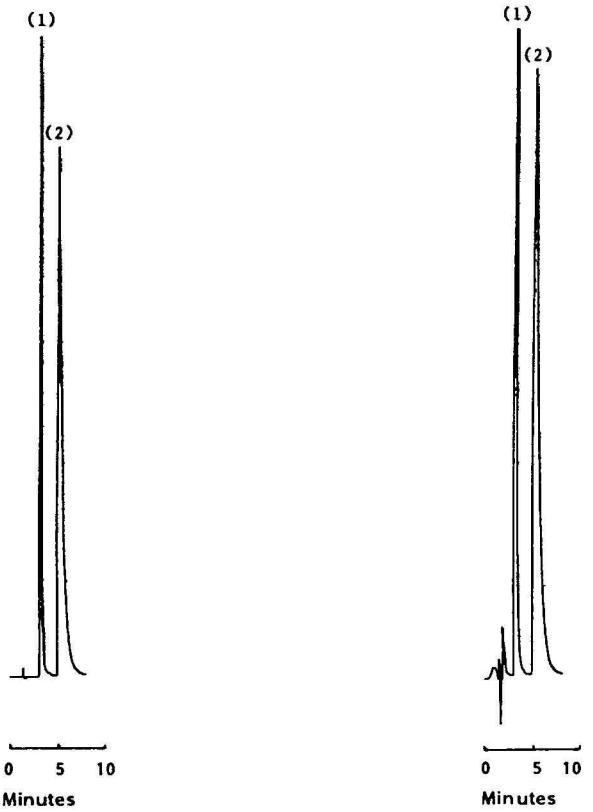

Fig. 1. Chromatogram of benzydamine gel: indomethacin internal standard (1) and benzydamine (2). Correlation coefficient = 0.999 (concentration range 0.625–20 mg gel per ml of solvent equivalent to 18.75–600 μg benzydamine hydrochloride per ml of solvent). Coefficient of variation = 1.15% (ten injections of 20 mg gel per ml of solvent equivalent to 600 μg benzydamine hydrochloride per ml of solvent).

Fig. 2. Chromatogram of Difflam cream: indomethacin internal standard (1) and benzydamine (2). Correlation coefficient = 0.999 (concentration range 0.625–20 mg cream per ml of solvent equivalent to 18.75–600 μg benzydamine hydrochloride per ml of solvent. Coefficient of variation = 1.60% (ten injections of 20 mg cream per ml of solvent equivalent to 600 μg benzydamine hydrochloride per ml of solvent).

μg/ml benzydamine hydrochloride). The correlation coefficient for the curve was 0.999. The coefficient of variation for ten sample replicates (20 mg gel/ml of solvent equivalent to 600 μg benzydamine hydrochloride per ml of solvent) assayed as above was 1.15%.

A calibration curve was also prepared for Difflam cream using data points over a concentration range of 0.625 to 20 mg cream/ml of solvent (equivalent to 18.75–600 /ml benzydamine hydrochloride). The correlation coefficient for the curve was 0.999. The coefficient of variation for ten sample replicates (20 mg cream/ml of solvent equivalent to 600 μg benzydamine hydrochloride per ml of solvent) assayed as above was 1.60%.

Using this method we have analysed the percutaneous absorption of benzyd-

amine from the gel preparation, in order to investigate the effectiveness of phonophoresis *i.e.* movement of drugs through intact skin into soft tissue by ultrasonic perturbation[9], in a double-blind placebo controlled clinical trial in ten healthy volunteers. The surface recovery method, which involves determination of the loss of drug from the vehicle as it penetrates into the skin, was used. The results obtained for percutaneous absorption of benzydamine (expressed as a percentage of the amount applied) following treatment with a range of continuous ultrasound frequencies (0.75 MHz, 1.5 MHz and 3.0 MHz) at an intensity of 1.5 W cm^{-2} for 5 min are given in Table I. A placebo control involving massage of the applied benzydamine gel without ultrasound for 5 min was included in the protocol. Statistical comparison of the results (analysis of variance) showed that there were no significant differences ($P > 0.05$) in absorption between control data and those using ultrasound at the frequency–intensity combinations noted.

TABLE I

COMPARISON OF BENZYDAMINE ABSORBED FOLLOWING TREATMENT WITH CONTINUOUS OUTPUT ULTRASOUND (INTENSITY 1.5 W cm^{-2} AT DIFFERENT FREQUENCIES)

S.E.M = Standard error of the mean. Ten determinations.

Ultrasound frequency (MHz)	*Benzydamine absorbed (%) ± S.E.M.*
0	24.33 ± 1.75
0.75	23.69 ± 1.94
1.5	24.97 ± 2.05
3.0	24.60 ± 1.25

CONCLUSION

A simple and rapid technique for the determination of benzydamine hydrochloride in topical formulations has been described. The use of direct injection and UV detection enables a limit of detection of 0.1 mg gel/ml of solvent (equivalent to 3 μg benzydamine hydrochloride per ml of solvent) and 0.26 mg cream/ml of solvent (equivalent to 7.8 μg benzydamine hydrochloride per ml of solvent). The method would be suitable for quality control of benzydamine content of topical formulations and also for studies on the percutaneous absorption of the drug. We are currently using this assay for further investigation of the influence of ultrasound on the percutaneous absorption of benzydamine both *in vitro* and *in vivo*.

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Note

High-performance liquid chromatographic determination of antioxidants in fats and oils

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Numerous methods are available for the determination of one or more antioxidants in fats and oils, including thin-layer chromatography[1,2], colorimetry[3], gel chromatography[4], gas–liquid chromatography (GLC)[5–9] and high-performance liquid chromatography (HPLC)[10,11]. All these methods require extraction and, in the case of the GLC methods, derivatization of the antioxidants prior to quantification, increasing the analysis time and the likelihood of losses during analysis. Min *et al.*[12] and Yu *et al.*[13] determined *tert.*-butylhydroquinone (TBHQ) directly in oils using GLC with flame ionization detection. Van Niekerk and Du Plessis[14] and Indyk and Woollard[15] determined TBHQ directly in oils using normal-phase HPLC with fluorescence and UV detection respectively.

This paper reports a simple HPLC procedure which requires neither extraction nor derivatization for the direct determination of six antioxidants, *viz.*, BHA (3-*tert.*-butyl-4-hydroxyanisole), PG (propyl gallate), OG (octyl gallate), DG (dodecyl gallate), TBHQ and NDGA (nordihydroguaiaretic acid), in edible oils and fats.

EXPERIMENTAL

Instrumentation

A Model 5000 liquid chromatograph (Varian Associates, Palo Alto, CA, U.S.A.), a Valco loop injector (capacity 50 μl), a Model III UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) were employed. Separations were achieved using a 25 cm × 0.46 cm column packed with 5-μm LiChrosorb DIOL (Merck, Darmstadt, F.R.G.) at ambient temperature (23 ± 1°C).

Reagents

Hexane and 1,4-dioxane were obtained from Merck. Acetonitrile was redistilled before use. The mobile phase, hexane–1,4-dioxane–acetonitrile (62:28:10), was filtered through a 0.45-μm membrane filter (Millipore Corporation, Bedford, MA, U.S.A.) and degassed before use. The antioxidants were obtained from a number of commercial sources.

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Procedure

Stock solutions of the six antioxidants were freshly prepared in mobile phase at concentrations up to 100 μg/ml. Approximately 1 g of an oil or fat sample was accurately weighed into a 10-ml volumetric flask, dissolved, with the aid of an ultrasonic bath if necessary, in 4–6 ml of mobile phase and diluted to volume in the same solvent.

RESULTS

The chromatogram obtained for a synthetic standard solution is reproduced in Fig. 1, showing the excellent resolution of the six components. A chromatogram of a sample of olive oil spiked with these antioxidants is shown in Fig. 2. A blank oil sample showed no peaks at the corresponding retention volumes. The peak identities were confirmed by co-chromatography, and linear calibration plots were obtained for all six antioxidants over the concentration range 0–200 mg/kg. The coefficients of variation for replicate (n = 6) injections were 0.99 (BHA), 1.63 (DG), 1.22 (TBHQ), 1.53 (OG), 1.56 (PG) and 1.93% (NDGA). Limits of detection (signal-to-noise ratio = 3) were 25 (BHA, DG, TBHQ, OG), 35 (PG) and 100 ng (NDGA).

The procedure may be applied to the analysis of peanut, soya, marula, sunflower, safflower and rapeseed oils, and beef fat, for which chromatograms similar to Fig. 3 are obtained. Interference from co-eluting material restricts the application of the procedure to the determination of PG and NDGA in maize oil and only to NDGA in cottonseed oil.

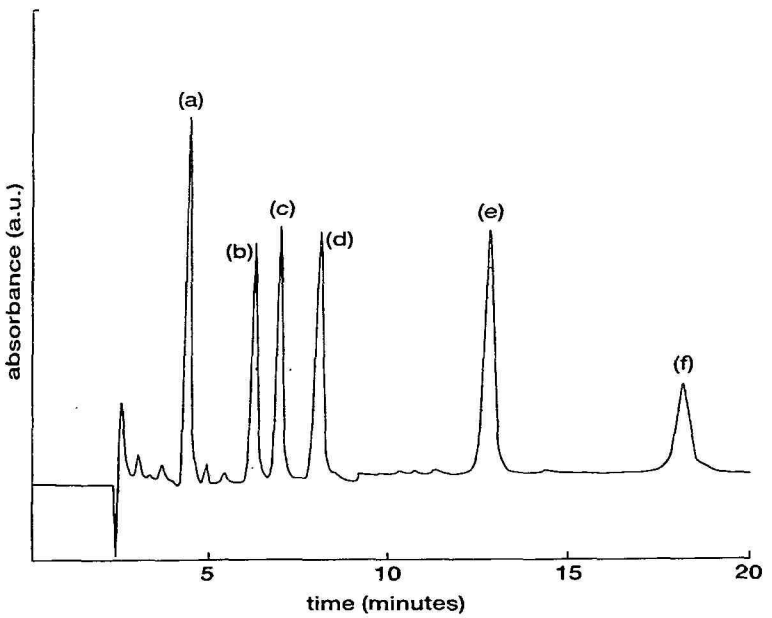

Fig. 1. Chromatogram of antioxidant standards, each approximately 5 μg/ml. Conditions: 25 cm × 0.46 cm LiChrosorb DIOL (5 μm) column; mobile phase, hexane–1,4-dioxane–acetonitrile (62:28:10) at 1.0 ml/min; detection, 0.016 a.u.f.s. at 280 nm; injection 50 μl. Peaks: a = BHA; b = DG; c = TBHQ; d = OG; e = PG; f = NDGA.

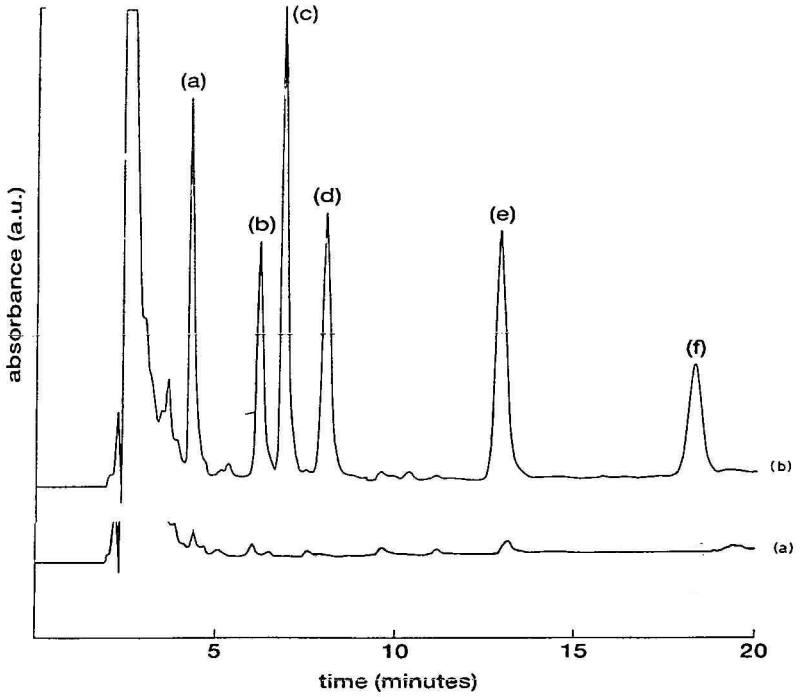

Fig. 2. Curve (a) shows a commercial sample of olive oil (approximately 10%, w/v) and curve (b) the oil spiked with BHA (116), DG (106), TBHQ (206), OG (104), PG (139) and NDGA (144 mg/kg). Conditions and peak identities as in Fig. 1.

Under the HPLC conditions described, BHT (3,5-di-*tert.*-butyl-4-hydroxytoluene), another widely used antioxidant, is not resolved from the neutral lipids in the sample. The method is useful for the rapid determination of BHA, DG, TBHQ, OG, PG and NDGA at relatively low levels in numerous types of oils and fat.

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CHROM. 19 483

Note

Determination of thyreostatics in meat by reversed-phase liquid chromatography with ultraviolet and electrochemical detection

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Thyreostatic "anti-hormones" can be used to increase the weight of animals prior to slaughter. The weight increase is due to the increased water content of the gastrointestinal tract and the retention of water in tissue[1]. Meat from animals treated with thyreostatics is subject to exudation and is of inferior quality. Regulations in The Netherlands prohibit the use of anti-hormones in the fattening of cattle[2].

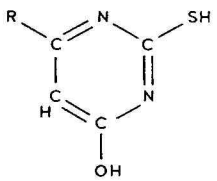
Various techniques have been used for the analysis of thyreostatics such as high-performance thin-layer chromatography[1,3], high-performance liquid chromatography (HPLC) with UV detection[4–7] and histological techniques[8,9]. The use of liquid chromatography with electrochemical detection (LC–ED) of anti-hormones has not yet been reported. An advantage of electrochemical detection is that it is more selective than UV detection in analyses of complex matrices and the signal-to-noise ratio is higher than for UV detection[10–14].

We present a method for the determination of thiouracil (TU) and methylthiouracil (MTU) in meat at the mass fraction level of 10^{-9} (= ppb or μg/kg) by HPLC with UV detection and oxidative electrochemical detection.

EXPERIMENTAL

Chemicals and reagents

All reagents used were analytical grade products. Thiouracil (4-hydroxy-2-mercaptopyrimidine) and methylthiouracil (4-hydroxy-2-mercapto-6-methylpyridimide) were obtained from Fluka.



R = H: thiouracil
R = CH_3: methylthiouracil

Apparatus

The solvent-delivery system (Waters Model 6000 A) was equipped with a pressure gauge as pulse damper. The original pump heads were replaced by Swip pump

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heads (Saphirwerk Industrieprodukte). The advantage of these pump heads is that the plungers can be washed with water to dissolve salts and that the eluent flow is more stable. A Waters Intelligent Sample Processor (WISP) was used as autosampler. A LiChrosorb RP-18 pre-column (10 μm, 30 mm × 3.2 mm I.D., Brownlee) and a Shandon Hypersil ODS analytical column (5 μm, 250 mm × 4.6 mm I.D., Chrompack) were used. The mobile phase, 0.01 *M* potassium dihydrogenphosphate–methanol (90:10) with 2 g tetrabutylammonium chloride per litre, was filtered through a 0.45-μm membrane filter with the aid of a solvent clarification kit (Millipore). The flow-rate was adjusted to 1.0 ml/min. All separations were performed at 40°C. The injection volume was 5–200 μl.

The wavelength of the UV detector (Waters Model 450) was 280 nm. Electrochemical detection was achieved with a Metrohm 656 electrochemical detector equipped with a glassy carbon working electrode, a glassy carbon auxilliary electrode and a silver–silver chloride–lithium chloride (3 *M* in water) electrode as reference electrode. The potentiostat was a Metrohm Model VA 641. The potential applied was +1.25 V *vs.* the reference electrode. Chromatographic recordings were made on a dual-pen recorder (Kipp Model BD 41); UV 10 mV, ED 1 V.

Sample pretreatment

A 5-g amount of minced meat was mixed thoroughly with 10 ml of ethyl acetate in a 25-ml test-tube using a Whirlmix. The homogenate was centrifuged for 10 min at a rotational frequency of 1500 min^{-1} (200 *g*). The supernatant was decanted into another test-tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 2.5 ml methylene chloride and transferred to a Baker silica gel (6 ml) cartridge which had been washed with 5 ml acetonitrile–water (20:80), 5 ml acetonitrile and 5 ml methylene chloride. The tube was rinsed with 2.5 ml methylene chloride and the solution was added to the cartridge. The cartridge was washed with 10 ml methylene chloride and after drying it was eluted with 5 ml of acetonitrile–water (20:80). About 10 ml of acetonitrile were added to the eluate and the eluate was evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 500 μl eluent for liquid chromatography.

RESULTS AND DISCUSSION

Electrochemical operations

The electrochemical pretreatment of the glassy carbon electrode was adapted from that of Engstrom and Strasser[15]. The glassy carbon electrode was pretreated at +1.75 V for 5 min and then at −1.0 V for 1 min. The pretreatment was carried out on-line in the eluent. This pretreatment gave a stable baseline more rapidly in comparison with other methods, like polishing with Alox powder.

An hydrodynamic voltammogram was measured by injecting a solution containing 100 ng of TU and 100 ng MTU. The hydrodynamic voltammogram of TU shows an oxidative wave in the range 600–1300 mV *vs.* the reference electrode (Fig. 1). The hydrodynamic voltammogram of MTU gives almost the same wave. We chose a potential of +1.25 V because this gives the best signal-to-noise ratio.

Fig. 2 shows the chromatograms of a standard solution of 40 ng TU and 40 ng MTU. The electrochemical detector was set to 0.5 μA full scale and the UV

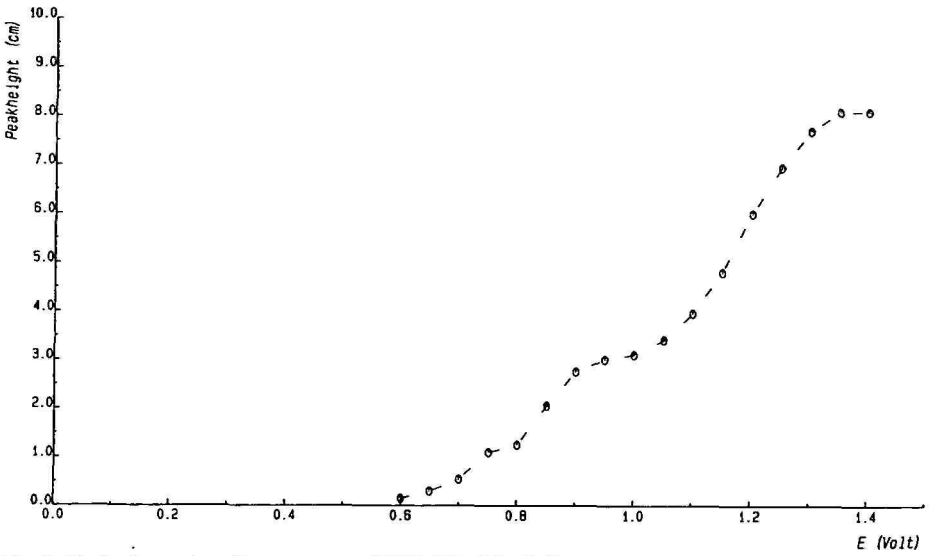

Fig. 1. Hydrodynamic voltammogram of TU. ED: 0.5 μA f.s.

detector operated at the highest sensitivity (0.01 a.u.f.s). In Fig. 3 the results are given for a real sample which contained MTU. The mass fraction of MTU was $8 \cdot 10^{-6}$, *i.e.*, 8 mg/kg or ppm.

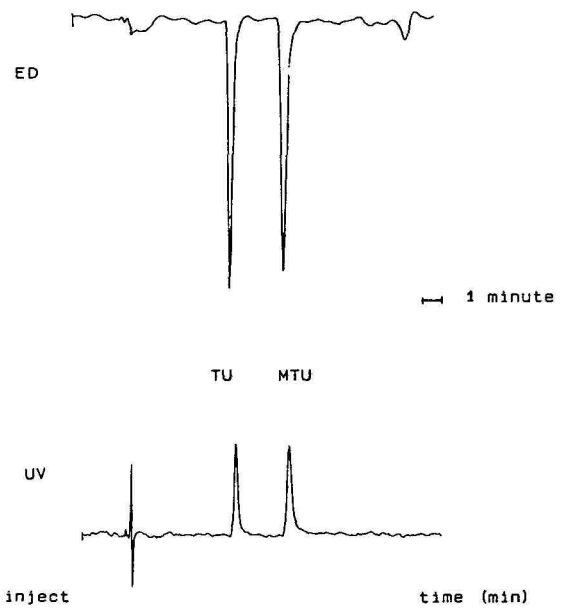

Fig. 2. Chromatogram of a standard solution of 40 ng TU and 40 ng MTU. ED: 0.5 μA f.s. UV: 0.01 a.u.f.s.

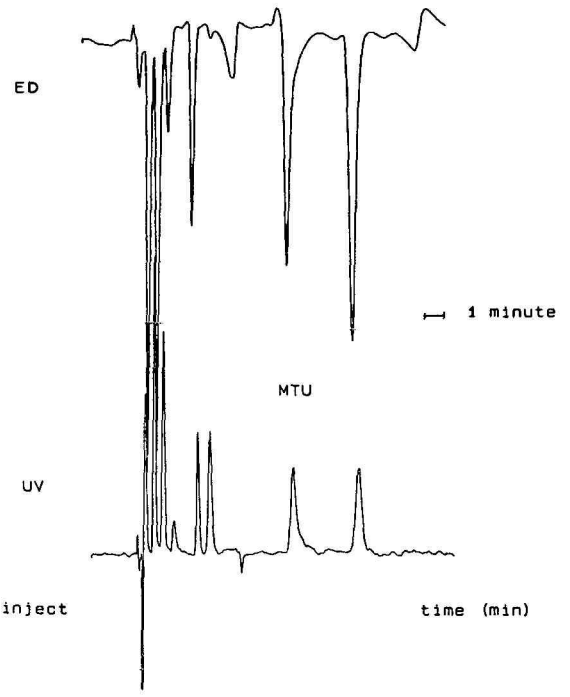

Fig. 3. Chromatogram of a meat sample, containing about 8 μg MTU/g. ED: 5 μA f.s. UV: 0.02 a.u.f.s.

Linear studies

The linearity was evaluated with standard solutions containing various amounts of TU and MTU in the range 4–150 ng for ED and 20–150 ng for UV detection. In the first case the peak heights gave linear relationhips with correlation coefficients of 0.9991 for TU (slope 2.97, *y*-intercept −12.3) and 0.9996 for MTU (slope 3.59, *y*-intercept −11.4). For UV detection the correlation coefficients were 0.997 for TU (slope 0.277, *y*-intercept −0.255) and 0.999 for MTU (slope 0.574, *y*-intercept 0.903).

Recovery experiments

Recovery experiments were performed by adding TU and MTU to meat at concentrations from 100 to 2000 ng/g. The mean recovery (± S.D.) for TU was 66 ± 8% and for MTU was 70 ± 11% (n = 5).

CONCLUSIONS

In analyses of prohibited drugs there is always a risk of false negative or false positive results. To prevent such false positive results, it is advisable to apply two detection methods which are based on different physico-chemical principles. The described combination of ultraviolet detection and electrochemical detection after HPLC separation can fulfil this requirement.

We applied this method to determine MTU in meat samples. Both UV detection as ED gave positive results comparable with the thin-layer chromatography method described by De Brabander[1]. Starting with a portion of 5 g of meat, the limit of detection can be estimated as a mass fraction of $2.5 \cdot 10^{-8}$ (25 μg/kg) for UV detection and of 10^{-8} (10 μg/kg) for ED.

The very simple sample pretreatment using commercial silica gel columns was a sufficient clean-up procedure.

With the proposed method it is possible to determine TU and MTU in meat.

ACKNOWLEDGEMENT

We thank Inacom B.V., Veenendaal, The Netherlands, for the gift of the Swip pump heads.

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CHROM. 19 517

Note

Determination of uracil, uridine and formic acid in egg products by high-performance liquid chromatography

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Uracil is a potentially useful indicator of deterioration in egg products and uridine appears to be one of its precursors[1]. No method has been reported for determination of uracil or uridine in such products. High-performance liquid chromatography (HPLC) in the reversed-phase mode has become the method of choice for determining nucleosides and their bases in biological matrices[2]. Uracil is so weakly retained on reversed-phase columns, however, that it has been recommended as a void-volume marker for reversed-phase HPLC under a variety of conditions[3]. Thus, reversed-phase analysis of biological materials may result in coelution of uracil with other weakly retained components[4].

The contents of formic, acetic, lactic and succinic acids in eggs have been found to be related to odor and bacterial counts and have been suggested as chemical criteria for egg decomposition[5]. For this reason, AOAC methods have been developed for determination of these acids in eggs using gas chromatography[6]. Different methods are required for the volatile acids and the non-volatile ones, however, and both methods require relatively complex sample preparation. The availability of commercial columns for separation of organic acids by ion exclusion and partition chromatography on a strong cation-exchange resin, a technique often referred to as ion-moderated partition chromatography, prompted evaluation of one such column for simultaneously determining volatile and non-volatile acids in eggs. It was observed that the pH of the mobile phase was such that most nucleotides, nucleosides, and nucleobases were protonated and therefore retained on a guard column, but uracil, thymine and their derivatives were eluted[7]. An application of this HPLC technique for analysis of goats' milk for uridine and uracil was reported during the course of this investigation[8].

This paper describes a selective method for the simultaneous determination of uracil and uridine in egg products by use of a column marketed for HPLC of organic acids. Using the same method with UV absorbance detection at 210 nm allows simultaneous quantitation of uracil and formic acid.

EXPERIMENTAL*

Materials

The reagents used were: uracil (Sigma, St. Louis, MO, U.S.A.), uridine (Sigma), sodium formate (analytical reagent, Mallinckrodt, St. Louis, MO, U.S.A.), sulfuric acid (ULTREX, J. T. Baker, Phillipsburg, NJ, U.S.A.), perchloric acid (Baker Analyzed, J. T. Baker), and acetonitrile (UV, American Burdick & Jackson, Muskegon, MI, U.S.A.). Deionized water was further purified by passing it through a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.).

The eluent consisted of 5% (v/v) acetonitrile in 0.01 *N* sulfuric acid. It was filtered through a 0.45-μm Nylon-66 membrane and degassed by sonication for 20 min.

Samples of liquid whole egg obtained from processing plants and allowed to deteriorate for various times were supplied by the Agricultural Marketing Service, U.S. Department of Agriculture. They were kept at temperatures no higher than -20°C.

Sample preparation

A portion of the frozen egg sample was obtained with the aid of an electric drill. About 0.7 g of egg, 0.7 g of water, 1.12 g of 6% perchloric acid, and enough acetonitrile to make its concentration in the final solution 5% (w/w) were weighed into a stoppered Tefzel ETFE (fluorocarbon) centrifuge tube and the mixture was stirred for 1 min. After standing for at least 15 min, the mixture was shaken for 10 s and centrifuged for 10 min at 12000 *g*. The supernatant was filtered through a 0.45-μm Durapore membrane (Millipore). An aliquot was analyzed on the same day the solution was prepared. In calculating contents of analytes in the egg, the weight of egg in the solution was corrected by subtracting 23.29% of the egg weight[9], the estimated weight of the precipitated solids.

In recovery studies, a portion of one of the standard solutions used to generate calibration curves was substituted for all or part of the added water.

Chromatography

The HPLC system (Waters Chromatography Division, Millipore) included a Model 510 pump, a Model U6K injector, and a Model 481 variable-wavelength UV detector set at 210 or 254 nm. A 300 × 7.8 mm Aminex HPX-87H cation-exchange column protected by a Micro-Guard ion exclusion cartridge (Bio-Rad Labs.) was used. Data were analyzed using Computer Automated Laboratory System (Beckman) software, implemented on a Hewlett-Packard 1000 computer.

The operating conditions were: column temperature, ambient; flow-rate, 0.5–0.6 ml/min; and volume injected, 10 μl. The lower flow-rate was preferable because it minimized problems with increasing back pressure.

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* Reference to a company or product name does not imply approval or recommendation by the United States Department of Agriculture.

Quantitation

Quantitation was effected by an external standard method. Five standard solutions in 5% (v/v) acetonitrile were chromatographed in duplicate for each detector setting. The resulting peak areas (for uridine and uracil) or heights (for formic acid) were subjected to linear regression analysis.

RESULTS AND DISCUSSION

Several precipitants reported to be effective for deproteinizing plasma[10] were tried for precipitating the protein and lipid in eggs. Of those tested, perchloric acid was found most suitable because it was reasonably effective and produced minimal interference with peaks in the chromatograms. A more effective precipitant was perchloric acid plus a small amount of acetonitrile, as specified in the section on sample preparation. A chromatogram for a blank prepared using this combination of agents contained no peaks past the solvent front region when detection was at 254 nm; with detection at 210 nm, small blank corrections were required for the formic acid and uracil peaks.

Typical chromatograms obtained using UV detection at 210 nm for an acceptable product and one that had developed spoilage odors after longer storage are compared in Fig. 1. Peaks were tentatively identified by comparing relative retention times with those of standards, and for those of interest supporting evidence was obtained by dual-column chromatography as described previously[7]. For samples with relatively low contents of formic acid, its peak appeared as a shoulder on the much larger uric acid peak; therefore, formic acid concentrations were calculated by comparison of peak heights rather than peak areas, which are more influenced by overlapping peaks[11]. Injection of a solution with an acetonitrile content substantially different from that of the eluent resulted in a small system peak that interfered with the quantitation of uracil. Uridine and succinic acid coeluted under the conditions used, but detection at 254 nm made determination of uridine possible. When the detector was set at 254 nm, the only major peaks after the solvent front region were those for uridine, uric acid, and uracil (Fig. 2).

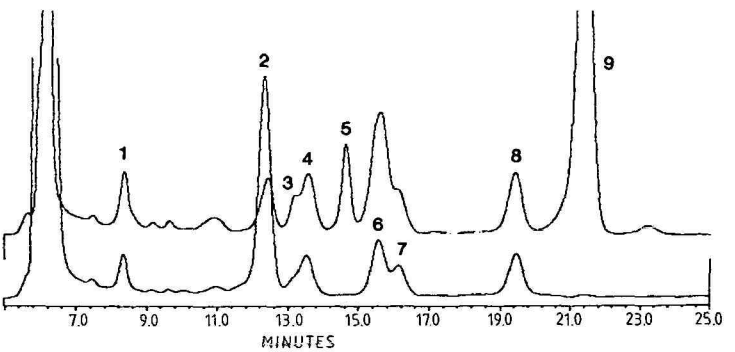

Fig. 1. Chromatograms for liquid whole egg stored for 24 h (lower) and 50 h (upper). Detection, UV absorption at 210 nm; flow-rate, 0.6 ml/min. Retention times (min) are given in parentheses. Peaks: 1 = citric acid? (8.38); 2 = uridine (+ succinic acid?) (12.34); 3 = lactic acid? (13.22); 4 = unknown (13.57); 5 = formic acid (14.64); 6 = uric acid (+ acetic acid?) (15.56); 7 = fumaric acid (16.14); 8 = pyroglutamic acid (19.42); 9 = uracil (21.37).

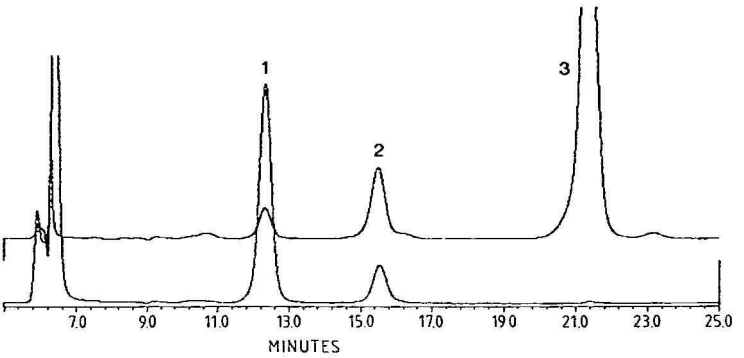

Fig. 2. Chromatograms correspond to those in Fig. 1 except detection was at 254 nm. Peaks: 1 = uridine; 2 = uric acid; 3 = uracil.

With detection at 254 nm, calibration curves were linear in the range 0.25–400 ng for uridine (r = 0.9999) and in the range 0.1–120 ng for uracil (r = 0.9999). With detection at 210 nm, linear response was established in the range 0.2–60 ng for uracil (r = 0.9987) and in the range 13 ng to 2.6 μg for formic acid (r = 0.9987). Approximate detection limits for standard solutions with detection at the specified wavelengths and the corresponding concentrations in egg analyzed by this method (assuming 100% recovery) are listed in Table I. The repeatability of response was tested by repeated injection of the same standard solutions. The coefficients of variation (C.V.) obtained ranged from 1.9 to 4.4% (Table I).

The precision of the method was evaluated by the analysis of solutions individually prepared from the same egg sample. The data presented in Table II for three independent determinations of uridine and uracil in a single portion of the sample indicate that the method is highly reproducible. The reproducibility of results of formic acid determinations also was satisfactory when its content in the egg was relatively high, but was unsatisfactory at a lower concentration, when the formic acid

TABLE I

DETECTION LIMITS AND REPEATABILITY FOR ANALYSES OF STANDARD SOLUTIONS

Repeatability data are for five consecutive injections of the same solution.

	Uridine	*Uracil*		*Formic acid*
	254 nm	*254 nm*	*210 nm*	*210 nm*
*Detection limit**				
ng	0.24	0.10	0.20	13
μg/g egg	0.09	0.04	0.07	4
Calculated concentration				
Mean (μg/ml)	1.092	0.3258	0.5963	24.89
Standard deviation	0.021	0.0084	0.0211	1.09
C.V. (%)	1.9	2.6	3.5	4.4

* Signal-to-noise ratio = 2.

TABLE II

REPRODUCIBILITY DATA FOR ANALYSES OF EGG

Either 5 separate portions of a frozen sample were analyzed, or a single portion was allowed to thaw and stirred before being divided into 3 portions for analysis.

	Uridine 254 nm	*Uracil*		*Formic acid* 210 nm	
		254 nm	210 nm		
Single portion (n = 3)					
Mean (μg/g egg)	30.11	19.16	20.40	12.49	88.58
Standard deviation	0.10	0.15	0.63	9.53	2.84
C.V. (%)	0.3	0.8	3.1	73.6	3.2
Separate portions (n = 5)					
Mean (μg/g egg)	30.23	18.12	19.52	–	87.06
Standard deviation	2.98	1.34	1.36	–	6.42
C.V. (%)	9.9	7.4	7.0	–	7.4

peak appeared as a shoulder on a much larger peak. The relatively large coefficients of variation resulting from analysis of separate portions of the sample appear to have been due to sample inhomogeneity. The accuracy of the determinations was established by spiking egg samples with standard solutions to approximately double their contents of the compound determined; recovery data are presented in Table III.

TABLE III

RECOVERY FROM SPIKED EGG SAMPLES

	Uridine 254 nm	*Uracil*		*Formic acid* 210 nm
		254 nm	210 nm	
Concentration before spiking (μg/g egg)	20.2	8.9	6.0	39.9
n	3	3	3	4
Recovery (%)	97.0	95.8	96.5	98.6
Standard deviation	1.9	1.5	2.0	7.5
C.V. (%)	2.0	1.6	2.1	7.6

The results show that chromatography on a column of cation-exchange resin with an acidic eluent and UV detection at 254 nm provided a selective, reproducible, and accurate method for determination of uracil and uridine in egg products. With detection at 210 nm, uracil and formic acid could be determined in eggs, but determination of formic acid at lower concentrations was adversely affected by incomplete resolution under the conditions used.

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Book Review

Selective gas chromatographic detectors (*Journal of Chromatography Library*, Vol. 36), by M. Dressler, Elsevier, Amsterdam, Oxford, New York, 1986, xiv + 319 pp., price US$ 72.25, Dfl. 195.00, ISBN 0-444-42488-1.

This is not a general book of detectors for gas chromatography. The word "selective" in the title is meant to indicate to the reader that only those devices capable of discriminating between solutes based on some function of their structure or composition are discussed. Thus, for example, the flame-ionization detector receives about one page of text and the thermal conductivity detector is not even mentioned. The layout of the book is rather conventional with each of the common selective detectors given an individual chapter, excepting the alkali flame-ionization detector, which gets two chapters. The first of these presents information largely of historic interest on the poorly reproducible salt-in-flame type detectors. As this version of the detector is almost unused these days, one could argue that 46 pages of text is a little excessive since the flameless alkali-sensitized detector, the version in current use, receives only 27 pages of text. Equally surprising, given the nature and emphasis of the book, mass spectrometric and infrared spectroscopic detectors are dismissed in a few pages in a final chapter headed "Miscellaneous Detectors". The treatment given to the photoionization, flame photometric, chemiluminescence (thermal energy analyzer), electrolytic conductivity, coulometric, and electron-capture detectors is fairly even and covers most of the important characteristics of these devices. The style of each chapter is rather accumulative with respect to information rather than providing an insightful interpretation of the data available. To a large extent the book tends to be historically correct rather than current in presenting details of the devices likely to be used with modern instruments. This is also reflected in the bibliography of references which contains a few entries for 1984 and 1983, but generally presents information from pre-1980s papers.

Overall, this is a useful book without being a very good book. It contains few errors of fact or omission beyond those noted earlier. It is a sound reference work and should be purchased by technical libraries.

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Book Review

Ion chromatography (*Chromatographic Science Series*, Vol. 37) edited by J. G. Tarter, Marcel Dekker, New York, Basel, 1987, 448 pp., price US$ 79.75 (U.S.A. and Canada), US$ 95.50 (rest of world), ISBN 0-8247-7634-8.

This is a puzzling book and, although it contains chapters by excellent chromatographers, one wonders what the purpose of such a book is. The first problem is how one understands the term "ion chromatography". The definition given only recently by J. S. Fritz *et al.* in their book *Ion Chromatography* (Hüthig, 1982) obviously does not hold any more, and a wide range of separations is now either termed ion chromatography or high-performance liquid chromatography (HPLC) indiscriminately. There are some attempts to limit the term to the chromatography of ionised substances. The "definition", however, does not consider, for example, the paper chromatographic systems by Macheboeuf *et al.* for alkaloids in which the alkaloids are chromatographed in solvents containing hydrochloric acid. I would say the many new terms used around ion chromatography, such as "eluent suppressed", "single column", "ion chromatography exclusion", would all require rethinking or preferably abolishing for lack of consistency.

Then this book excels itself; Chapter 6 which is almost half of the book, finds the term "ion chromatography" unsatisfactory and proposes a new one, "ionic chromatography", which on some reflection is just as unsatisfactory. So even within the volume there is dissidence.

It is difficult to say whether the various chapters cover their topic adequately as in many types of separations, *e.g.* sugars or organic acids, the authors consider only some papers while others (presumably because they have HPLC in the title) are not considered. The chapter on Detection Methods, by Haddad and Jandik, is worth reading in spite of the fact that the authors avoid discussing the early work (such as the papers of Kemula) for most methods.

In addition to the confusion in terminology there is a profusion of abbreviations: ICE, IEC, IEP, DEC, IMP ... Phrases like "The coupled ICE/IC system..." or a subheading "Unusual PCR Systems" will surely be enjoyed by beginners. In the chapter on the "Bibliographic Review of Ion Chromatography" there is a subheading "Non-English" for the section "Specific Reviews". I suppose non-readers and non-chromatographers can skip this one.

The book contains a lot of material, some of it good, but as the authors decided on arbitrary divisions none of the chapters can give complete information on a topic. It would have been wiser to cover topics such as analysis for metal ions or for inorganic anions and permit the reader then to decide which technique is best suited for his problem and within the range of his apparatus and skills.

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journal of chromatography news section

NEW BOOKS

Gas chromatography in essential oil analysis, edited by P. Sandra and C. Bicchi, Hüthig, Heidelberg, 1987, price DM 98.00, US$ 47.00, ISBN 3-7785-0860-1.

Gas chromatography, by J.E. Willett, ACOL, London/Wiley, Chichester, New York, 1987, XVIII + 283 pp., price £ 9.95 (paperback), £ 28.00, US$ 47.50 (cloth), ISBN 0-471-91332-4 (paperback), 0-471-91331-6 (cloth).

Detectors for liquid chromatography, edited by E.S. Yeung, Wiley, New York, Chichester, 1986, XII + 366 pp., price £ 52.75, ISBN 0-471-82169-1.

Microbiological applications of high-performance liquid chromatography, by D.B. Drucker, Cambridge University Press, Cambridge, London, New York, 1987, XII + 354 pp., price £ 40.00, US$ 59.50, ISBN 0-521-30491-1.

Quantitative analysis using chromatographic techniques, edited by E. Katz, Wiley, Chichester, New York, 1987, XVIII + 427 pp., price £37.50, ISBN 0-471-91406-1.

Ion chromatography, by D.T. Gjerde and J.S. Fritz, Hüthig, Heidelberg, 2nd ed., 1987, XI + 283 pp., price DM 86.00, US$ 45.00, ISBN 3-7785-1207-2.

Electrophoresis, by M. Melvin, Wiley, Chichester, New York, ca. 152 pp., price US$ 47.80 (hardback), 17.00 (paperback), ISBN 0471 91374 X (hardback), 0471 91375 8 (paperback).

Methods of biochemical analysis, Vol. 32, edited by D. Glick, Wiley, Chichester, New York, ca. 416 pp., price ca. US$ 69.90, ISBN 0471 82195 0.

Methods of protein and nucleic acid research, *Vol. 3, Chromatography*, L.A. Osterman, Springer, Berlin, Heidelberg, New York, 1986, XII + 505 pp., price DM 248.00, ISBN 3-540-16855-9.

Organic pollutants in water, edited by I.H. Suffet and M. Malaiyandi, American Chemical Society, Washington, DC, 1987, XV + 797 pp., price US$ 109.95 (U.S.A. and Canada), US$ 131.95 (rest of world), ISBN 0-8412-0951-0.

Personal computers for scientists, by G.I. Ouchi, American Chemical Society, Washington, DC, 1986, X + 250 pp., price: hardbound, US$ 34.95 (U.S.A. and Canada), US$ 41.95 (rest of world); paperbound, US$ 22.95 (U.S.A. and Canada), US$ 27.95 (rest of world), ISBN 0-8412-1000-4 (hardbound), 0-8412-1000-2 (paperbound).

ANNOUNCEMENTS OF MEETINGS

GORDON RESEARCH CONFERENCES, "FRONTIERS OF SCIENCE"

The Gordon Research Conferences for the summer of 1987 will be held in New Hampshire and Rhode Island. The object and exclusive purpose of the Gordon Research Conferences is to foster and promote education and science by organizing and operating meetings of research scientists with common interests in the fields of chemistry or related sciences for the purpose of discussions and the free exchange of ideas, thereby stimulating advanced thinking in research at universities, research foundations, and industrial laboratories. This type of meeting is a valuable means of disseminating information and ideas to an extent that could not be achieved through the usual channels of publication and presentation at scientific meetings. It is hoped that each Conference will extend the Frontiers of Science by fostering a free and informal exchange of ideas among persons actively interested in the subject under discussion.

Some meetings in areas related to this journal are: Reactive Polymers, Ion Exchangers and Adsorbents (Newport, RI, U.S.A., August 3–7, 1987); Separation and Purification (New London, NH, U.S.A., August 10–14, 1987); Analytical Chemistry (New Hampton, NH, U.S.A., August 10–14, 1987).

The complete programme for the 1987 Gordon Research Conferences is published in *Science (Washington, D.C.)*, March 6, 1987. Reprints are available on request.

Requests for applications to the conferences, or for additional information, should be addressed to: Dr. Alexander M. Cruickshank, Gordon Research Conferences, Gordon Research Center, University of Rhode Island, Kingston, RI 02881-0801, U.S.A. Tel.: (401) 783-4011 or (401) 783-3372.

PETRO ANALYSIS '87, INTERNATIONAL SYMPOSIUM ON ADVANCES IN THE ANALYSIS OF PETROLEUM AND ITS PRODUCTS, LANCASTER, U.K., JUNE 30–JULY 3, 1987

The above-mentioned conference will be organised by the Petroanalysis '87 Trust on behalf of the Institute of Petroleum and the North-West Region of the Analytical Division of the Royal Society of Chemistry.

The scientific programme will consist of plenary lectures by Professor D. Betteridge, "Tough problems — novel solutions"; Professor L.S. Bark, "Petroleum products analysis — the future"; Professor C.A. Cramers, "Developments in the analysis of petroleum products by GC". These will be followed by contributed lectures; there will also be time set aside for poster sessions.

There will be an exhibition of equipment, products and literature concerned with the analysis of petroleum and its products over the Wednesday and Thursday of the conference. Companies considering participating should contact the secretariat as soon as possible for fuller information.

The conference is held on the campus of the University of Lancaster. Rooms (mostly single bedded) are available on the campus very close to the conference and exhibition locations. In addition to the evening social programme for all participants there will also be a range of daytime excursions for accompanying persons.

Registration fees are expected to be about £115.00 for the whole conference and £45.00 for a single day. Concessionary rates for students and retired persons should be about £40.00 (whole meeting) and £20.00 (1 day). Bed, breakfast and evening meal at the University campus is about £20.00 per person per night (single room — there are only a few double rooms available.)

For full details of fees, including for the social programme contact: PetroAnalysis '87 Secretariat, c/o Dr. C.J. Peacock, Department of Chemistry, The University, Lancaster LA1 4YA, U.K.

ENVIROCHEM '87, SYMPOSIUM ON ANALYTICAL CHEMISTRY AND POLLUTION MONITORING, CAMBRIDGE, U.K., JULY 21–22, 1987

A two-day symposium focussing on environmental analytical chemistry and pollution monitoring is to be held at Robinson college, Cambridge, U.K., July 21–22, 1987.

Part of the U.K. contribution to the European Year of the Environment, Envirochem '87 will consist of four separate sessions, each following a separate theme. All major aspects of environmental pollution — including atmospheric, fresh water/lacustrine, terrestrial and marine/estuarine — will be covered.

For further information on Envirochem '87 — including details of poster contributions, registration and residential accommodation — please contact Linda Doggett at Philips Analytical, York Street, Cambridge, U.K. Tel.: (0223) 358866.

ANABIOTEC '88, 2nd INTERNATIONAL SYMPOSIUM ON ANALYTICAL METHODS AND PROBLEMS IN BIOTECHNOLOGY, NOORDWIJKERHOUT, THE NETHERLANDS, MARCH 29–31, 1988

Following the successful first ANABIOTEC symposium, held in 1984, the Second International Symposium on Analytical Methods and Problems in Biotechnology, ANABIOTEC '88, will be held in Noordwijkerhout, The Netherlands, March 29–31, 1988.

The following considerations have led to the organization of this symposium:

— Analytical methods and systems for biotechnological applications are becoming increasingly important. The developments of these methods and systems requires a growing interdisciplinary approach.
— The use of analytical methods in daily practice in biotechnological research, development and industrial production has come to be seen more and more as essential for progress in biotechnology in general.
— Close cooperation is needed between experts in analytical methodology, system development, and biotechnology.

Topics covered will include:

— State of the art analytical techniques already successfully applied in biotechnology.
— Strategies for the selection of analytical procedures with regard to optimum process control in industrial biotechnology, environmental biotechnology, and fundamental and developmental research.
— Development of new analytical techniques for the above-mentioned areas.

The scientific programme will consist of invited plenary lectures, invited and submitted papers (both oral and poster presentations) and discussion sessions. The symposium language will be English. Sessions are planned on: sampling strategies, biosensors, mass spectrometry in process control, application of computers in analysis and process control, prospects for practical application of new analytical techniques, analytical problems in biotechnology.

Participants wishing to present a paper should submit an abstract of about 250 words to the Symposium Secretariat before 15 October 1987.

An exhibition of scientific products and equipment will be held in conjunction with the symposium.

Information on ANABIOTEC '88 can be obtained from: Symposium Secretariat ANABIOTEC '88, c/o QLT Convention Services, Keizersgracht 792, 1017 EC Amsterdam, The Netherlands. Tel.: (20) 261372; telex 31578 INTER NL attn. QLT.

17th INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY, VIENNA, AUSTRIA, SEPTEMBER 18–23, 1988

The 17th International Symposium on Chromatography, organized by the Österreichische Gesellschaft für Mikrochemie und Analytische Chemie and Arbeitskreis Chromatographie of Fachgruppe Analytische Chemie of Gesellschaft Deutscher Chemiker in association with Chromatographic Society (London) and G.A.M.S., will be held in the Hofburg, Vienna, Austria.

The scientific programme will comprise lectures, poster presentations, and discussion sessions dealing with all aspects of chromatography and related techniques. The symposium chairman is Professor J.F.K. Huber.

An exhibition of chromatographic equipment and literature will be held adjacent to the meeting. Companies interested in acquiring exhibition space should send their enquiries to the address given below.

Persons interested in attending the symposium should write to the Gesellschaft Deutscher Chemiker (address given below). They will then receive the second cicular which will be the call for papers about September 1987 and the 3rd circular which includes the programme and details of hotel reservations. This is scheduled for about May 1988.

An attractive social programme and a special programme for accompanying persons, including tours and visits are in preparation.

Correspondence concerning the scientific programme should be addressed to: Priv.-Doz. Dr. Gerhard Schomburg, Max-Planck-Institut für Kohlenforschung, Postfach 10 13 53, D-4330 Mülheim-/Ruhr, F.R.G. Tel.: (02 08) 30 64 30. All correspondence concerning organization, exhibition etc. should be addressed to: Gesellschaft Deutscher Chemiker, Abt. Tagungen, Postfach 90 04 40, D-6000 Frankfurt/Main 90, F.R.G. Tel.: (069) 79 17-360/366.

PUBLICATION SCHEDULE FOR 1987

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	384 385 386 387	388/1 388/2 389/1	389/2 390/1 390/2 391/1	391/2 392 393/1 393/2	393/3 394/1 394/2 394/3	395 396 397		The publication schedule for further issues will be published later.				
Bibliography Section		412/1		412/2		412/3						
Cumulative Indexes, Vols. 351–400												
Biomedical Applications	413	414/1	414/2 415/1	415/2 416/1	416/2	417/1	417/2 418	419	420/1 420/2	421/1 421/2	422	423

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, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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