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REVIEW

FUNDAMENTALS OF THE THEORY AND PRACTICE OF POLYMER GEL-PERMEATION CHROMATOGRAPHY AS A METHOD OF CHROMATOGRAPHIC POROSIMETRY

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CONTENTS

1. INTRODUCTION

The idea of using chromatography as a porosimetric method has been actively developed by many workers since the 1960s[1–25]. Compared with other methods used for investigating porous structures (mercury porosimetry, electron microscopy, low-angle X-ray scattering, gas and vapour adsorption and desorption methods), chromatographic porosimetry holds a number of advantages and is particularly attractive as a method for studying sorbent structures.

Porosimetry based on gel-permeation chromatography (GPC) is inexpensive and generally accessible, being based on the use of normal chromatographic equipment. There is no need to use high pressure or low temperatures, no call for the

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test specimens to be subjected to special preparation, nor is there any influence on the sorbent structures and properties. Chromatographic porosimetry permits sorbent investigations under conditions identical with, or close to, conditions of practical usage, which is of particular importance for swellable polymeric sorbents. Also, of the above methods, GPC porosimetry is the only one suitable for certifying commercial packed columns for liquid chromatography.

The theoretical fundamentals of polymer GPC have been laid down by Casassa and co-workers[26–28]. The results predicted by Casassa's theory have been corroborated experimentally, have gained recognition and are being widely used by those specializing in the field of polymer analysis. For all that, these ideas do not appear to be generally considered as eventually forming a theoretical basis for a GPC porosimetric method. As a result, some work on chromatographic porosimetry has been found to contain incorrect interpretations of experimental results and, sometimes, incorrect results.

It is for this reason that we consider it necessary once again to discuss the principal conclusions from macromolecular GPC theory, paying particular attention to those questions which have previously been little dwelt upon but which are essential to the understanding of the problems involved in GPC porosimetry, specifically questions of calibration and of the meaning of porous structure characteristics obtainable with this method.

This review surveys current ideas concerning macromolecular GPC using polydisperse sorbents, ideas which are used as a basis for analysing in detail the principal methods employed for interpreting experiments in chromatographic porosimetry. The conditions that are necessary for the GPC porosimetric method to be realized in practice are discussed.

2. FUNDAMENTALS OF THE THEORY OF MACROMOLECULAR GPC

The basic quantity measured in chromatography is the retention volume, V_e:

$$V_e = V_0 + V_p K \tag{1}$$

where V_0 and V_p are the volumes of the mobile and stationary phases, respectively, and K is a distribution coefficient related to the sizes and types of the molecules being chromatographed, the sizes and forms of the sorbent pores and the molecule-to-sorbent interaction conditions.

In gel-permeation (size-exclusion) chromatography realizable in the absence of adsorption interactions, the distribution coefficient depends on the molecule-to-pore size ratio, and for this reason GPC is a suitable method for determining both molecule size and pore size.

The theory of macromolecular GPC was essentially formulated by Casassa and co-workers[26–28]. The theory is based on the calculation of changes in the entropy of a macromolecule as it penetrates from the mobile phase (solution) into a sorbent pore, and makes use of a model of a flexible-chain macromolecule in a thermodynamically ideal solvent, assuming a low polymer concentration in the solution and a quasi-equilibrium nature of the chromatographic process.

2.1. Universal distribution coefficient versus molecule-to-pore size ratio relationship

The chief result of the theoretical studies[26–28] was an universal relationship between the distribution coefficient, K, and the ratio of the radius of gyration of the macromolecule, r, to the pore radius, R. For a model of a slit-like pore of width $2R$, this relationship has the following form[26]:

$$K = \frac{8}{\pi^2} \sum_{m=1}^{\infty} m^{-2} \cdot \exp\left[- \left(\frac{\pi m}{2} \cdot \frac{r}{R} \right)^2 \right] \tag{2}$$

where the summation is performed for odd values of m. In the limiting wide-pore and narrow-pore cases, the K *vs.* r/R relationship acquires simpler forms:

$$K \approx 1 - \frac{2}{\sqrt{\pi}} \cdot \frac{r}{R}; \qquad r \ll R \tag{3}$$

$$K \approx \frac{8}{\pi^2} \cdot \exp\left[- \left(\frac{\pi r}{2R} \right)^2 \right]; \qquad r \gg R \tag{4}$$

Fig. 1 shows the precise relationship of eqn. 2 as a solid line and the asymptotic eqns. 3 and 4 as dashed and dotted lines. It can be seen that the precise K *vs.* r/R function is well approximated by the set of asymptotes for all macromolecule-to-pore size ratios.

It follows from eqn. 4 that with large macromolecules and narrow pores, at $r > R$, the distribution coefficient is not equal to zero. This signifies that a number of large macromolecules penetrate into narrow pores, assuming elongated conformations that are different from the equilibrium conformations of macromolecules in solution (Fig. 2a and b). Penetration of large molecules into narrow pores is a specific feature of polymer chromatography, which Casassa[26] was the first to recognize and which was not accounted for in other GPC theories[29,30].

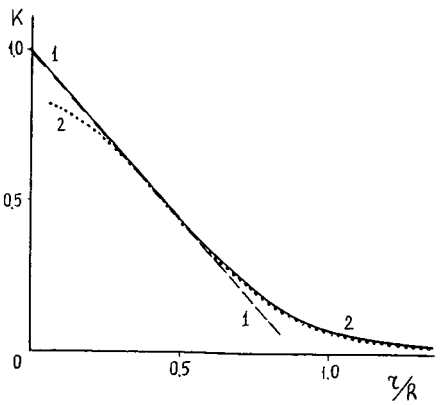

Fig. 1. Distribution coefficient K *versus* the ratio of the radius of gyration, r, to the slit-like pore half-width R (based on the theory[26–28]). Solid line, eqn. 2; dashed line (1), eqn. 3; dotted line (2), eqn. 4.

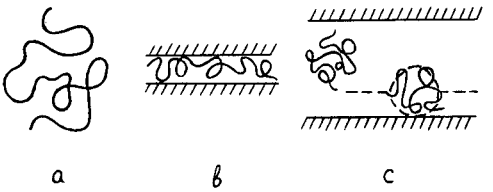

Fig. 2. Typical macromolecular conformations: (a) in a solution; (b) in narrow pores; (c) in wide pores.

The linear dependence of K on r/R in wide pores is of a fairly general nature[28,29]. It reflects a decreasing effective volume available for accommodation of a macromolecule in a pore. In wide pores, macromolecules have approximately the same conformations as in an unrestricted volume (Fig. 2c) and behave in chromatography like spherical solid particles with an equivalent radius. In fact, eqn. 3 is a definition of the effective chromatographic radius of the macromolecule.

2.2. Chromatographic radius of macromolecules

It can be seen from comparing eqn. 3 with the expression for the distribution coefficient of a spherical particle of radius ρ in a slit-like pore at $\rho < R$[29],

$$K = 1 - \frac{\rho}{R} \tag{5}$$

that in the process of chromatography in wide pores the polymer chain is like a spherical particle of an equivalent radius

$$S = \rho_{\text{equiv.}} = \frac{2}{\sqrt{\pi}} \cdot r \tag{6}$$

Comparing the results of GPC theory for various forms of molecules, Casassa[28] drew a general conclusion to the effect that the equivalent chromatographic radius of an arbitrary particle is equal to half its mean span (the mean span being the greatest projection of the molecule on to the axis selected, averaged for all possible molecular orientations and conformations).

The effective chromatographic radius for molecules of all types studied is proportional to the radius of gyration, but the coefficients of proportionality have proved to be different both for various forms of rigid particles[29,31], *viz.*, spherical, ellipsoidal and rod-like, and for polymer molecules of various topology, *i.e.*, linear, branched and ring[27,32].

It has thus been shown that the radius of gyration is not strictly a universal chromatographic characteristic for macromolecules. Other characteristic dimensions, *e.g.*, the Stokes radius determinable from the friction coefficient in diffusion and sedimentation processes and the hydrodynamic radius associated with Benoit's "universal calibration" dependence parameter[33], have also been denied the status of universal chromatographic characteristics, which they are not, in fact[28]. However, in a series of molecules of a certain type, any of these dimensions may be used as a characteristic chromatographic radius.

2.3. Various pore forms. Universal chromatographic porous medium characteristics

In addition to the simpler slit-like pore model, Casassa and co-workers[26–28] also considered cylindrical- and spherical-form pore models. They postulated that the expression for the distribution coefficient of a flexible-chain macromolecule, common to all three models, has the following form:

$$K^{(\alpha)} = \sum_{m=1}^{\infty} \frac{2\alpha}{[\beta_m^{(\alpha)}]^2} \cdot \exp\left[-\left(\beta_m^{(\alpha)} \cdot \frac{r}{R}\right)^2\right] \tag{7}$$

For slit-like pores, $\alpha = 1$, $\beta_m^{(\alpha=1)} = \pi\,(m - \frac{1}{2})$; for cylindrical pores, $\alpha = 2$ and $\beta_m^{(\alpha=2)}$ is the mth root of the Bessel function $J_0(\beta)$; for spherical pores, $\alpha = 3$, $\beta_m^{(\alpha=3)} = \pi m$. In narrow pores, at $r \gg R$,

$$K^{(\alpha)} \approx \frac{2\alpha}{[\beta_1^{(\alpha)}]^2} \cdot \exp\left[-\left(\beta_1^{(\alpha)} \cdot \frac{r}{R}\right)^2\right] \tag{8}$$

In the other limiting case, that of wide pores, at $r \ll R$, it follows from eqn. 7[27] that

$$K^{(\alpha)} \approx 1 - \frac{2\alpha}{\sqrt{\pi}} \cdot \frac{r}{R} \tag{9}$$

Eqn. 9 for the distribution coefficient in wide pores acquires the most universal and model-independent form if a transition is made from the radius of gyration of the macromolecule, r, to its effective chromatographic radius $s = \frac{2}{\sqrt{\pi}} \cdot r$ and the parameter $\Sigma = \alpha/R = S_p/V_p$ = the ratio of pore surface S_p to pore volume V_p is introduced in place of pore radius R:

$$K \approx 1 - s\Sigma \tag{10}$$

The $K^{(\alpha)}$ *versus* $g = s\Sigma$ relationships calculated from eqn. 7 are shown in Fig. 3. It can be seen that the curves plotted in these coordinates for various pore models are close to one another and at $g < 0.4$ they almost coincide. Hence the pore form is of little effect as regards the laws governing macromolecular GPC. Consequently, chromatographic measurements cannot be used to obtain reliable information about the pore form of a sorbent.

In contrast to the radius R, the pore specific surface area, Σ, is a model-independent characteristic, of significance for pores of any geometry. Σ can therefore be considered as a universal sorbent pore characteristic; it can be determined by experiment, without making any assumptions regarding the sorbent pore form. Although the pore form of a real sorbent is generally not known (it may be complicated), knowing the value of Σ one can always obtain the effective radius, $R_{\text{equiv.}} = \alpha\Sigma^{-1}$, of the equivalent pores of a standard form (*e.g.*, cylindrical or slit-like) and use it as a model characteristic of the porous structure.

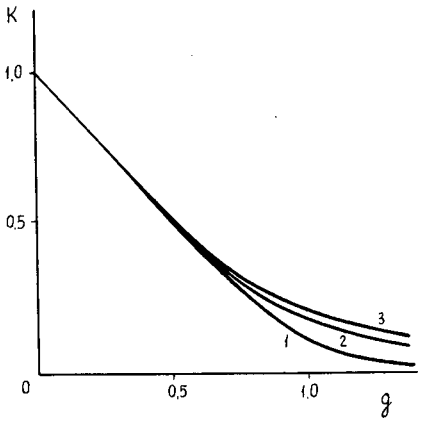

Fig. 3. Distribution coefficient K *versus* $g = s\Sigma$ for (1) slit-like pore model, (2) cylindrical pore model, and (3) spherical pore model. The curves plotted are based on eqn. 7.

2.4. Comparison of theory and experiment

The existence of a universal relationship between distribution coefficient and macromolecule-to-pore size ratio under GPC conditions has been corroborated by many experimental studies and may now be considered as firmly established. Similar relationships have been quoted for polystyrenes[26,34,35], and similar data are also available for water-soluble polymers, *viz.*, dextrans and polyethylene glycols[36,37]. As an example, Fig. 4 shows the K *vs.* r/R relationship plotted in ref. 17 based on data from ref. 38 pertaining to the GPC of dextrans on controlled pore glasses having pore radii varying from 4 to 26 nm. It can be seen that the experimental points related to different pore sizes fit the same curve, fully in agreement with theory.

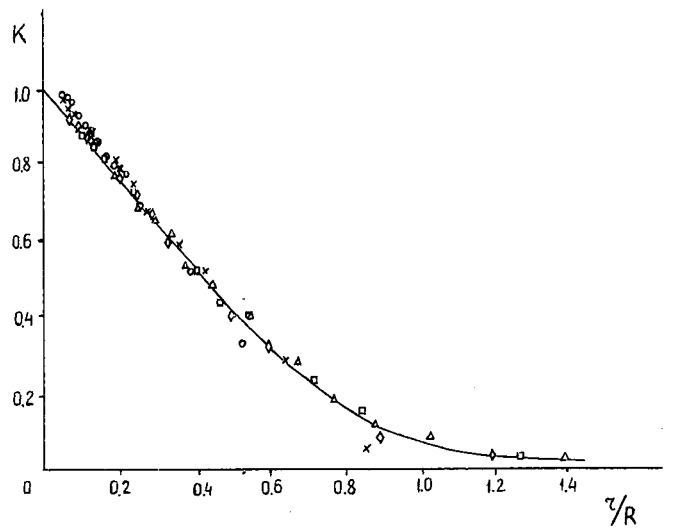

Fig. 4. Distribution coefficient K *versus* r/R for GPC of dextrans on narrow size range porous glasses with pore radii R = 4 (△), 8 (□), 11.5 (◇), 15.5 (×) and 26 (○) nm. Data from ref. 38.

Hence there is complete qualitative agreement between Casassa's theory and experimental data on macromolecular GPC. At the same time, discussions concerning the problem of the quantitative coincidence between theory and experiment have been going on for about 20 years. The reason is that independent methods must be used to measure macromolecule and pore sizes in order to elucidate this problem. However, there are a number of difficulties to be faced. First, there are no independent methods for determining the chromatographic radius of macromolecules, so the radius of gyration, the Stokes radius or some other characteristic molecular dimensions are used for this purpose. Then there also arise problems associated with independent sorbent pore size measurements. Generally, pore sizes are determined using mercury porosimetry[39], electron microscopy[40] or adsorption methods (BET method)[41], and there are limitations for each of these methods. Thus, mercury porosimetry requires the use of high pressures when narrow-pore sorbents are to be investigated (over 1000 atm at $R < 7.5$ nm)[1,5,39]. The nitrogen BET method requires the use of low temperatures (-183°C) and is known to become inaccurate at $R > 20$ nm[5,21]. Additional problems are associated with the fact that different methods provide differently averaged pore sizes. Thus, in mercury porosimetry, the actual quantity measured is the mean radius of curvature of the pore inlet openings[39], whereas the polymer GPC method is also sensitive to inner pore expansions. This is probably the cause of the qualitative variance observed by several workers between mercury porosimetry data and chromatographic data[34]. Difficulties also arise in the interpretation of electron microscopy data when identifying image details with sorbent pores[40].

Discussions on the problem of conformity between GPC data, on the one hand, and theory and data obtainable by other methods, on the other, are of importance for calibrating GPC as a porosimetric method. Two calibration methods are possible, one using GPC theory and the other using other porosimetric methods. The use of GPC theory for calibration purposes would appear preferable, in our opinion, as it makes the method independent of limitations and errors introduced by other porosimetric methods and the pore size measuring accuracy by the GPC method comparable to the test macromolecule measuring accuracy.

3. MACROMOLECULAR GPC USING POLYDISPERSE SORBENTS

Studies aimed at ascertaining the principles of macromolecular GPC using inhomogeneous sorbents are generally undertaken with a view to optimizing polymer separation and analysis conditions[34]. At the same time, these studies form an indispensable theoretical and methodological basis for the GPC analysis of sorbent structures. We may briefly consider the main results of theoretical studies[10,17,42] dealing with macromolecular GPC using polydisperse sorbents.

3.1. Distribution functions, mean sizes and pore size inhomogeneity characteristics

Real sorbents are mostly polydisperse, *i.e.*, inhomogeneous in pore size. To describe such sorbents, Gorbunov *et al.*[17] introduced differential functions for the pore volume distribution, $f_v(R)$, and for the pore surface distribution, $f_s(R)$, normalized for the total pore volume, V_p, and for the total pore surface area, S_p. The

mean sizes R_v and R_s corresponding to these functions are defined by the following relationships:

$$R_s = S_p^{-1} \int_0^\infty R f_s(R)\, dR = V_p \left[\int_0^\infty R^{-1} f_v(R)\, dR \right]^{-1} = \alpha \cdot \frac{V_p}{S_p} \tag{11}$$

$$R_v = V_p^{-1} \int_0^\infty R f_v(R)\, dR = R_s^{-1} S_p^{-1} \int_0^\infty R^2 f_s(R)\, dR \tag{12}$$

Pore size inhomogeneity (polydispersity) can be characterized by the standard width σ or dispersion σ^2:

$$\sigma^2 = S_p^{-1} \int_0^\infty R^2 f_s(R)\, dR - R_s^2 \tag{13}$$

More convenient than polydispersity characteristics are the dimensionless parameters relative distribution function width,

$$\gamma = \frac{\sigma}{R_s} \tag{14}$$

and polydispersity,

$$U = 1 + \gamma^2 \tag{15}$$

It follows from eqns. 11–15 that U is equal to the ratio of the two different mean pore sizes:

$$U = R_v/R_s \tag{16}$$

and resembles, from the standpoint of meaning, the well known parameter M_w/M_n which is normally used to characterize the molecular weight inhomogeneity of polymers. For a sorbent with all pores identical, $\gamma = 0$ ($U = 1$), and both γ and U increase with increasing polydispersity.

3.2. Theory of polymer GPC using polydisperse sorbents

So far as we know, the first model that can be regarded as a model of macromolecular GPC using a polydisperse sorbent was considered by Doi[42], who discussed an equilibrium distribution of a flexible-chain macromolecule of radius of gyration r between the solution phase and the stationary phase, with a space randomly filled with impermeable spherical elements of radius a used as a model of the latter. In the free volume, V_p, of the stationary phase there were n spherical elements having

a concentration $c = n/V_p$. For this model, Doi obtained an approximate equation for the distribution coefficient:

$$K \approx \exp\left(-8\sqrt{\pi}\, ca^2 r - 4\pi car^2\right) \tag{17}$$

This model is inconvenient, however, for analysing the principles of macromolecular GPC using polydisperse sorbents, as the pore form, pore sizes and pore size distribution are not specified explicitly.

An alternative approach based on the introduction of a model pore size distribution function, $f_v(R)$, was developed[4,10,17]. As the retention volume, V_e, is the overall statistical sum of the macromolecule in the mobile and stationary phases, then in calculating V_e for a polydisperse sorbent the summation should be performed for all the elements of the stationary phase, which results in

$$V_e = V_0 + \int_0^\infty f_v(R) K\left(\frac{r}{R}\right) dR \tag{18}$$

where $K(r/R)$ is the distribution coefficient for a monodisperse sorbent having pores of radius R. Eqn. 18 can be written in the usual form as

$$V_e = V_0 + V_p \bar{K} \tag{19}$$

in which case the distribution coefficient for a polydisperse sorbent, $\bar{K}$, will be defined by the following equation[10,17]:

$$\bar{K}(r) = V_p^{-1} \int_0^\infty K(r/R) \cdot f_v(R)\, dR \tag{20}$$

A detailed analysis of the theoretical $\bar{K}(r)$ relationships for polydisperse sorbents was given by Gorbunov *et al.*[17]. It was established,in particular, that with polydisperse sorbents also the initial course of the $K(r)$ relationship is described by a simple universal equation

$$\bar{K} \approx 1 - \frac{2\alpha}{\sqrt{\pi}} \cdot \frac{r}{R_s} = 1 - s\Sigma; \qquad r \ll R_s \tag{21}$$

with $\Sigma = S_p/V_p = \alpha/R_s$ now having the meaning of the ratio of the total surface area of all pores to their total volume.

It will be noted that for the random sphere model in the limiting case of $ca^2 r \ll 1$, eqn. 17 also leads to eqn. 21, because for this model $\Sigma = S_p/V_p = 4\pi ca^2$ (ref. 42).

Hence the initial slope of the $\bar{K}(r)$ relationship depends only on the value of the sorbent's specific surface area, Σ (mean pore radius R_s), and is independent of the width and type of the pore size distribution function.

When the macromolecule sizes are comparable to the mean pore radius R_s, the distribution coefficient becomes dependent on the width and type of the function $f_v(R)$. Eqns. 20 and 7 enable the $\bar{K}(r)$ relationship to be calculated for sorbents having model distribution functions $f_v(R)$. An example of such a calculation is given in Fig. 5. Monodisperse sorbents are considered with R = (1) 5 nm and (2) 15 nm, in addition to inhomogeneous model sorbents with (3) a unimodal and (4) a bimodal distribution function. Case (3) corresponds to a logarithmically normal distribution of the type

$$f_v(R) = \frac{V_p}{R\sqrt{2\pi \ln U}} \cdot \exp\left\{ -\frac{\left[\ln\left(\frac{R}{R_s\sqrt{U}}\right)\right]^2}{2 \ln U} \right\} \tag{22}$$

with parameters R_s = 15 nm and U = 2. Case (4) is a bimodal distribution with the same values of R_s and U (two maxima at R_1 = 8.8 nm and R_2 = 51.2 nm). With bimodal distributions with greatly (by an order of magnitude or more) differing pore sizes, two linear segments can be observed in the $\bar{K}(r)$ curve. The initial segment contains the information regarding the mean pore size R_s, just as in the general case, whereas the slope and intercept of the second linear segment depend on the large pore size and volume fraction[17].

The analysis carried out previously[17] showed that the type of $\bar{K}$ *versus* r relationship depends chiefly on two parameters, the mean pore radius R_s and the pore

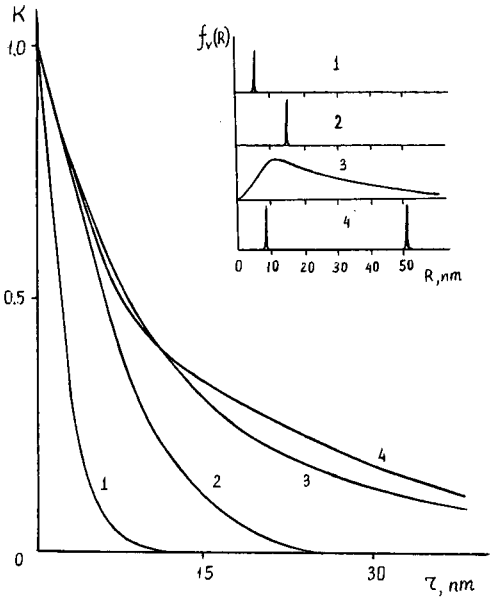

Fig. 5. Theoretical dependences of the distribution coefficient K on the radius of gyration of the macromolecules chromatographed for two monodisperse sorbents (1 and 2) and for inhomogeneous model sorbents having (3) a unimodal and (4) a bimodal pore size distribution. Mean pore radius R_s = (1) 5 and (2–4) 15 nm; polydispersity parameter U = (1 and 2) 1 and (3 and 4) 2. Shown at top are the types of the respective distributions.

size distribution function width. The type of distribution function $f_v(R)$ has little effect on the macromolecular GPC principles.

3.3. Experimental data on macromolecular GPC using polydisperse sorbents

Inhomogeneously porous sorbents are used in chromatographic practice for optimizing methods used for analysing polymer molecular weights and molecular weight distributions (MWDs). It is known, for instance, that the use of a set of columns containing sorbents of varying pore sizes or mixtures of such sorbents permits the working range of molecular weights in the GPC analysis of polymers to be expanded. Yau *et al.*[34] and Vilenchik *et al.*[43] described specially selected sorbent mixtures ensuring linearity of the calibration graph (coordinates K *versus* ln M) over a wide range of molecular weights, the latter group making use of the theoretical eqn. 21 for designing such sorbents. Experimental data have also been published on molecular GPC based on the use of biporous sorbents[10,15,18,21,34]. Most of these studies, however, were not aimed at comparing experimental data and GPC theory based on the use of polydisperse sorbents.

The sensitivity of the chromatographic method to pore size polydispersity was demonstrated experimentally[17]. Based on four sorbents of narrow size range (modified porous glasses and silica gel) with pore sizes R = 2.3 (I), 7.2 (II), 11.7 (III) and 31.3 nm (IV), two model specimens were prepared: a two-component mixture of sorbents II and III (61:39, v/v), and a four-component mixture of sorbents I–IV (10:22:31:37, v/v). The compositions of these mixtures were specially selected so that while having identical mean pore radii, the two- and four-component sorbents would have different polydispersities. In accordance with theory, one would expect for such sorbents $\bar{K}(r)$ relationships similar to curves 2 and 3 in Fig. 5, *i.e.*, coinciding initially but diverging at large values of r. The $\bar{K}(r)$ relationships obtained experimentally for the two- and four-component sorbents (Fig. 6) are in fact as would be expected, which is evidence that the theoretical views are correct.

Careful measurements of the distribution coefficients of dextrans using as the sorbent a mixture of CPG-10 porous glasses with various pore sizes were performed by

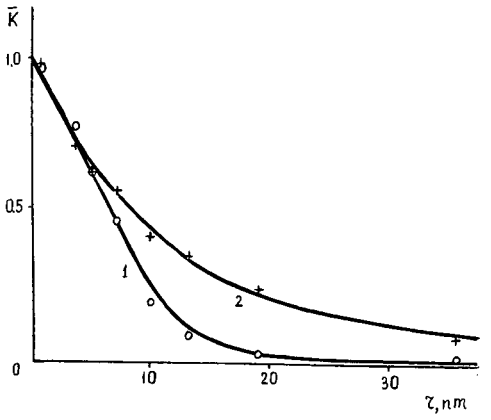

Fig. 6. Experimental $\bar{K}(r)$ relationships for dextran molecules on mixed sorbents. 1, Two-component sorbent; 2, four-component sorbent. Data from ref. 17.

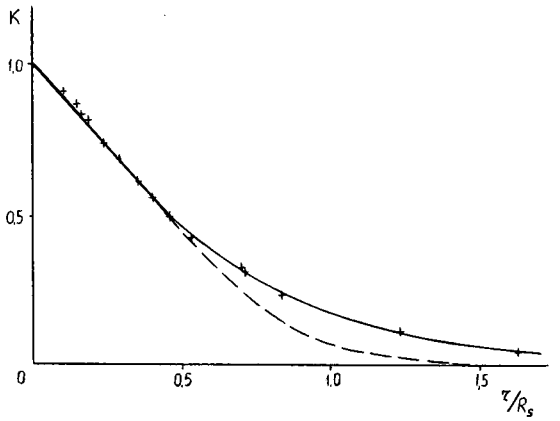

Fig. 7. Distribution coefficient K *versus* r/R_s for chromatography of dextrans on mixed sorbent CPG-10. Experimental data from ref. 44; dashed line as per theory, ref. 26; solid line as per theory, ref. 17, at $U = 1.22$.

Basedow *et al.*[44]. All the glasses used for preparing the mixed sorbent had a high degree of pore size homogeneity. The data on the GPC of dextrans on these monodisperse glasses[38] fitted the same common curve in the coordinates of K *versus* r/R, coinciding with the theoretical relationship (eqn. 2) for monodisperse sorbents (Fig. 4). The results for the mixed sorbent[44] are shown in the same coordinates in Fig. 7. The experimental points for high-molecular-weight dextrans can be seen to deviate noticeably from the $K(r/R)$ relationship for monodisperse sorbents, shown by the dashed line. The solid line in Fig. 7, passing through the experimental points, was calculated from eqns. 20, 22 and 2 of the polydisperse sorbent GPC theory and corresponds to a value of the polydispersity parameter $U = 1.22$.

Hence the available experimental data are in good agreement with the theory[17] of macromolecular GPC based on the use of polydisperse sorbents.

4. CHROMATOGRAPHIC POROSIMETRY OF SORBENTS

The establishment of the basic principles of macromolecular GPC using inhomogeneously porous sorbents permits the solution of a practically important inverse problem, *viz.*, finding the porous structure characteristics of sorbents based on macromolecular GPC data.

Various methods have been proposed, and have gained acceptance in practical work, for the chromatographic determination of pore sizes, polydispersities and pore size distribution functions. However, there have been no detailed discussions until now of these methods, based on common theoretical grounds, which makes it difficult to assess and compare experimental results obtained by different workers.

We shall analyse the principal approaches used to interpret experiments in GPC porosimetry and discuss the conditions required for the correct practical realization of the method.

4.1. Determination of sorbent pore volume, surface area and size

Pore volume is an important sorbent characteristic as it defines the maximum

range of V_e values in which the size separation of macromolecules is possible. The pore volume is required for calculating the distribution coefficients K in eqn. 1. Normally, the pore volume, V_p, is determined by measuring the retention volume difference, $V_t - V_0$, for small molecules capable of penetrating into every pore and large particles that are known *a priori* to be incapable of entering the pores. Sorbent porosity can be conveniently characterized as the pore volume fraction, $x = V_p/(V_0 + V_p)$.

The GPC method likewise readily allows the sorbent pore surface area, S_p, to be assessed. By combining eqns. 1 and 21 we obtain

$$V_e \approx V_0 + V_p - \frac{2}{\sqrt{\pi}} \cdot rS_p \tag{23}$$

Eqn. 23 is valid only when the size of the molecule is smaller than the pore size, as for eqn. 21. According to eqn. 23, the value of S_p can be determined from the initial slope of the curve of V_e *versus* r.

Hence S_p and V_p can easily be measured, the specific surface area $\Sigma = S_p/V_p$ determined and the mean pore size $R_s \approx \Sigma^{-1}$ assessed. However, this method will not be accurate for polydisperse sorbents as the linear region of the $\bar{K}(r)$ curve will decrease with increasing polydispersity, leading to large errors in the determination of the initial slope.

Other simple methods are also known for assessing pore sizes from GPC data, based on the empirically determined principles of macromolecule retention.

4.2. Empirical methods for pore size assessment

One such method is associated with determining the "critical" size of macromolecules which are still capable of penetrating into pores[45]. This method is based on a linear relationship between $\ln(1 - K)$ and $\ln M$, discovered empirically by Haller *et al.*[38,46]. The "critical" radius of a macromolecule, r_0, can be determined from this relationship by extrapolation to $K \to 0$.

Let us consider this method from the standpoint of the theory of macromolecular chromatography. It follows from eqn. 9 that under conditions where macromolecule sizes are substantially smaller than pore sizes, a linear relationship between $\ln(1 - K)$ and $\ln M$ must indeed occur while the "critical" radius r_0 extrapolated to $K \to 0$ must be related to $\Sigma = S_p/V_p$ by

$$r_0 \approx \frac{\sqrt{\pi}}{2} \cdot \Sigma^{-1} \tag{24}$$

However, with comparable macromolecule and pore sizes, theory predicts a different functional $K(r)$ relationship (eqn. 8) which fails to give a straight line in the coordinates of $\ln(1 - K)$ *versus* $\ln M$. Consequently, within the region of small K values, the method of size extrapolation to $K \to 0$, suggested by Basedow *et al.*[45], becomes incorrect. A rigorous theoretical relationship (eqn. 7) in the coordinates of $\ln(1 - K)$ *versus* $\ln(r\Sigma)$ is illustrated in Fig. 8. Fig. 8 also illustrates dependences calculated for polydisperse model sorbents. Polydispersity can be seen to lead to expanding non-linear regions in the curves and hence to incorrect, *i.e.*, too high, pore

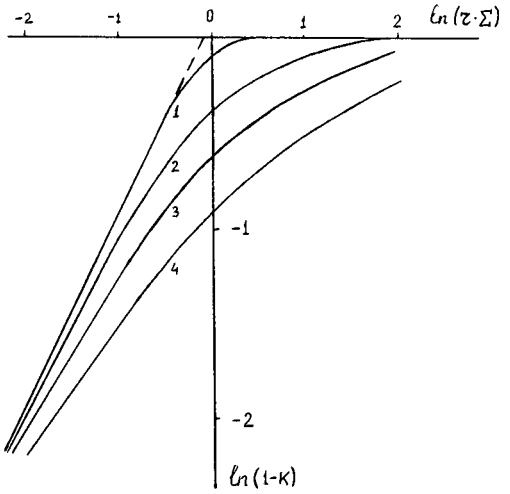

Fig. 8. Theoretical ln ($r\Sigma$) dependence of ln ($1 - K$) for a monodisperse sorbent of U = (1) 1 and for polydisperse sorbents of U = (2) 2, (3) 5 and (4) 20.

size values obtained by the use of the above procedure. It will be noted that any procedures for determining a "critical" size are based on the concepts of rigid non-deformable test particles and are therefore inapplicable where flexible-chain molecules are used as chromatographic standards.

Another technique currently in use is the "median" method of pore size determination, which consists in determining experimentally the macromolecular radius $r_{1/2}$ corresponding to a distribution coefficient $K = 1/2$. This dimension is identified with the mean pore radius, various correction factors being introduced, as a rule[21,23].

It follows, in fact, from macromolecular GPC theory that $r_{1/2} \approx R$. Using the approximate eqn. 3 and assuming $K = 0.5$, we obtain

$$r_{1/2} = \frac{\sqrt{\pi}}{4} \cdot \Sigma^{-1} \tag{25}$$

whence, considering the connection between Σ and the radii of equivalent pores of regular geometry, a transition can be made to equivalent model pore sizes. To give more precise relationships based on the use of the rigorous eqn. 7:

$$r_{1/2} = 0.444R_{\text{equiv.slit}} = 0.251R_{\text{equiv.cyl.}} = 0.175R_{\text{equiv.sphere}} \tag{26}$$

Thus, an uncorrected "median" method will give too low pore size values, yet, by using eqn. 26, this method can be employed to obtain correct results for sorbents with identical pores.

Let us now consider how the value of the "median radius" $r_{1/2}$ is affected by pore size inhomogeneity. Specifying a logarithmically normal pore size distribution function (eqn. 22), we calculated the dependence of $\bar{K}$ on $r\Sigma$ for various values of the

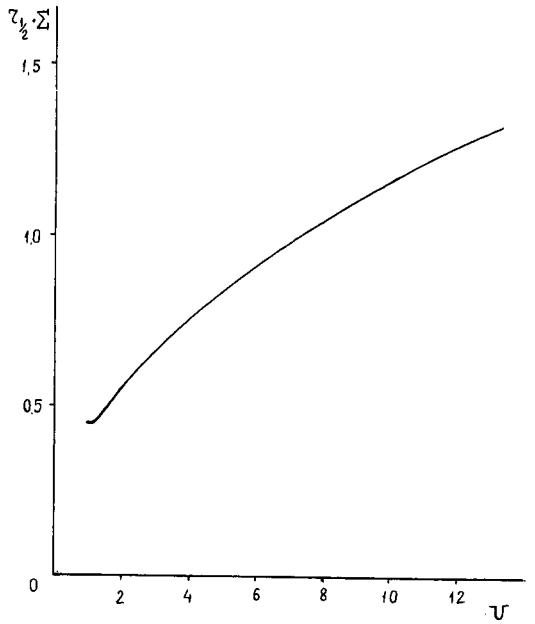

Fig. 9. $r_{1/2}\Sigma$ *versus* sorbent pore polydispersity parameter U.

polydispersity parameter U, and used these data to plot $r_{1/2} \cdot \Sigma$ *versus* U (Fig. 9). It can be seen that in the general case of polydisperse sorbents there is no definite relationship between $r_{1/2}$ and Σ (pore sizes), which means that the median method produces distorted results in this instance.

To summarize, all of the simple methods discussed above produce inaccurate results for sorbents that are inhomogeneous in pore size. More complicated methods are required in this instance, accounting not only for mean sizes, but also for pore size distribution.

4.3. Methods for calculating pore size distribution functions

Halász and Martin[2,5] suggested a method for the chromatographic determination of pore size distribution functions, which has gained wide acceptance owing to its simplicity. This method was based on the assumption that $K = 1$ for all macromolecules of a size smaller than the pore size and $K = 0$ if the molecule size exceeds the pore size. Identified with the sorbent pore diameter was the "exclusion value" of the molecule diameter φ, which was selected by the trial-and-error method such that the mean pore sizes obtained in the process would agree with the results obtained by the "classical" methods[5]. As a consequence of the assumption made[2,5], the ln φ dependence of $-\mathrm{d}K/\mathrm{d}(\ln \varphi)$ was interpreted as a differential pore size distribution function.

Unfortunately, the method described above and all of the results obtained therewith are not correct, as first noted by Knox and Scott[16]. The assumption that $K = 1$ for all macromolecules capable of penetrating into the pores is in contradiction with GPC theories[26–29]; also, it does not agree with the available experimental results. Owing to the incorrect conceptions of the GPC mechanism, which Halász and Martin

used as the basis for their method, this method will give pore size distribution functions that are far too wide, with gross errors for narrow size range and monodisperse sorbents. This may be demonstrated by using the example of a monodisperse model sorbent with identical slit-like pores of width $2R_0$. The theoretical $K(r)$ relationship for this sorbent is obtainable from eqn. 7 at $\alpha = 1$. Considering this relationship as ideal for an experimental $K(r)$ function, free from any measuring errors, a differentiation procedure may be applied, such as proposed by Halász and Martin[2,5]. Fig. 10 shows the results of such differentiation (curve 2) compared with the specified pore size distribution in the form of a delta function, $f(R) = \delta(R - R_0)$ (curve 1). It can be seen that curve 2 is shifted towards lower R values relative to the initial distribution function, and is considerably broader. Calculation of the moments of function 2 gives a mean pore size $\bar{R} = 0.48\ R_0$, and a relative width for this function $\gamma = \sigma_R/\bar{R} \approx 0.67$ ($U = 1.44$), while the true distribution is characterized by $\bar{R} = R_0$ and $\gamma = 0$ ($U = 1$). Of course, by choosing a correction factor for the relationship between r and φ, as recommended[5], curve 2 can be shifted parallel to the right until its maximum coincides with the value of $R = R_0$. Such a correction will not improve the assessment of relative width γ and polydispersity U, however.

Curve 3 in Fig. 10 was plotted in a similar manner, using eqn. 7 at $\alpha = 2$. This curve illustrates the use of the method[5] with a monodisperse sorbent with cylindrical pores. Curve 3 is characterized by the parameters $\bar{R} = 0.29\ R_0$ and $\gamma = 0.72$ ($U = 1.52$). Hence the method advanced by Halász and Martin produces unsatisfactory results for cylindrical pore sorbents also.

Another method for calculating pore size distribution functions was suggested by Knox and Scott[16] for cylindrically shaped pores and further developed by

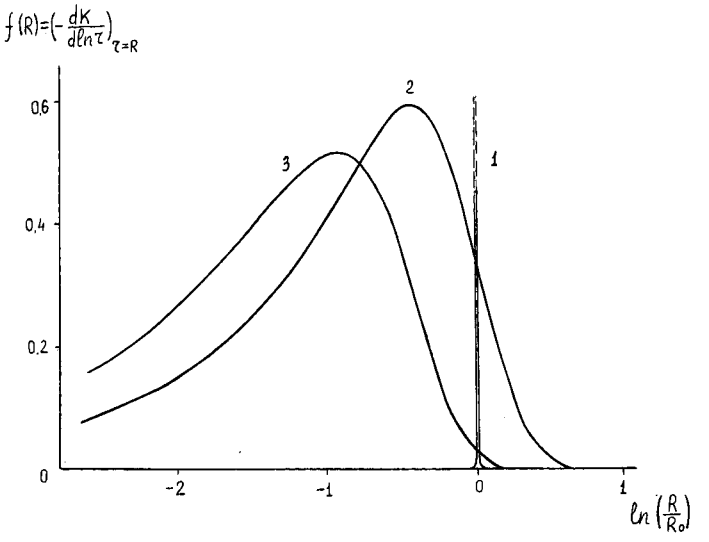

Fig. 10. $\left(-\frac{\mathrm{d}K}{\mathrm{d}\ln r}\right)_{r=R}$ versus $\ln (R/R_0)$ as calculated from eqn. 7 for monodisperse model sorbents with slit-like (curve 2) and cylindrical (curve 3) pores. Curve 1 represents the true pore size distribution.

Nikolov[24] for pores of other geometries. Some studies[16,20,24,25] were based on the equations of GPC theory for rigid spherical particles[29]:

$$K^{(\alpha)}\left(\frac{\rho}{R}\right) = \begin{cases} \left(1 - \frac{\rho}{R}\right)^{\alpha}; & \rho < R \\ 0; & \rho > R \end{cases} \tag{27}$$

where $\alpha = 1$ for slit-like pores, $\alpha = 2$ for cylindrical pores and $\alpha = 3$ for spherical pores.

By substitution of eqn. 27 into eqn. 20 and subsequent differentiation, equations were obtained[16,24] for determining pore size distribution functions for pores of different shapes. These equations can be conveniently written in the following general form:

$$f^{(\alpha)}(R) = (-1)^{(\alpha+1)} \cdot \frac{R^{\alpha}}{\alpha!}\left[\frac{d^{(\alpha+1)}K}{d\rho^{(\alpha+1)}}\right]_{\rho=R} \tag{28}$$

In our opinion, this method of calculating pore size distribution should be correct if non-deformable spherical particles are used as standards, such as a series of proteins of approximately spherical form and known size.

However, in the original works[16,24,25] flexible-chain macromolecules, polystyrenes, were used. At the same time, the theory based on a rigid solid particle model is not fully usable for describing the principles of flexible-chain macromolecular GPC (particularly where the molecule and pore sizes are comparable), as it does not account for changes in the conformational entropy of macromolecules as they enter the pores. It is for this reason that the method advanced by Knox and Scott, which uses macromolecules as test particles, will produce distorted results.

Fig. 11 demonstrates the use of eqn. 28 for monodisperse model sorbents of slit-like (curve 2) and cylindrical (curve 3) forms. In calculating these functions as "experimental" $K(r)$ relationships, use was made, as previously, of eqn. 7 from Casassa's theory of macromolecular GPC[26].

Comparing Figs. 10 and 11, one can see that, in general, the Knox and Scott method is better than that of Halász and Martin. The functions in Fig. 11 give near-correct mean pore sizes ($\bar{R} = 0.96R_0$ for slit-like pores and $\bar{R} = 0.86R_0$ for cylindrical pores). Consequently, the Knox and Scott method needs almost no correction factors. However, the function width obtainable by this method is still found to be excessive, *e.g.*, $\gamma = \sigma_R/\bar{R} \approx 0.28$ instead of $\gamma = 0$, for slit-like pores. In addition, as Fig. 11 shows, the use of eqn. 28, with $\alpha = 2$ (for the cylindrical pore model), leads to an artefact, giving negative function values in the smaller R region.

In our opinion, a more accurate method for calculating pore size distribution functions is that proposed by Vilenchik and co-workers[10,14]; this method consists in solving directly the integral eqn. 20 having the experimentally determined function $K(r/R)$ for its kernel.

One difficulty should be noted, however, as a matter of principle, this difficulty

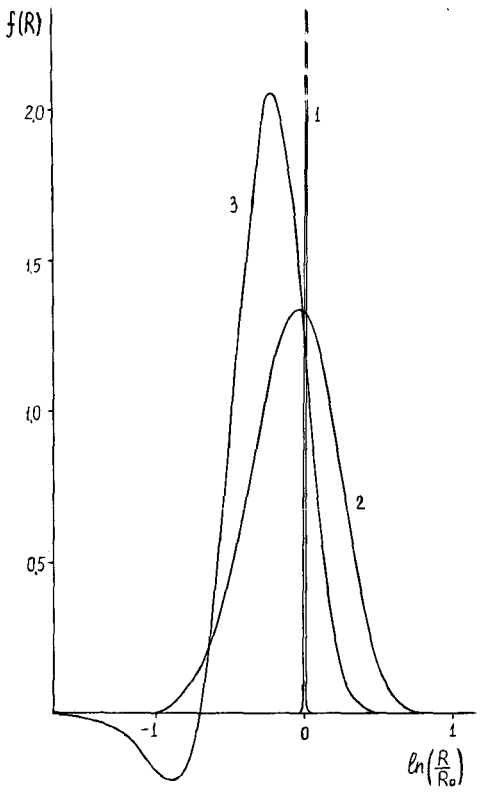

Fig. 11. "Pore size distributions" as calculated from eqns. 28 and 7 for monodisperse model sorbents with slit-like (curve 2) and cylindrical (curve 3) pores. Curve 1 represents the true pore size distribution.

arising in all instances where a pore size distribution function is derived from GPC data: eqn. 20 is a first-kind Fredholm equation, and the problem of using this equation to find an unknown $f_v(R)$ function is classed among "ill-posed" mathematical problems. For all practical purposes, it implies that minor errors in the initial data will have a considerable effect on the calculation results. In fact, the Knox and Scott method[16], whose only difference from the method advanced by Vilenchik *et al.*[10] is in the $K(r/R)$ function as the kernel type, also comes down to solving eqn. 20 numerically. In this method there are great uncertainties arising in calculating high-order derivatives of the experimental $\bar{K}(r)$ function.

In this connection, the question had arisen as to how reliably a distribution function and its moments could be derived from experimental GPC data. In other words, it was thought necessary to establish how sensitive the GPC porosimetric method was to the mean pore size, and also to the distribution function width and type.

4.4. Analysing the sensitivity of GPC porosimetry to various porous structure characteristics

The possibility of reliably determining various characteristics of porous structures by using the GPC method was treated theoretically by Gorbunov *et al.*[17].

The principal conclusions are evident from Fig. 5. Comparing curves 1 and 2–4 in Fig. 5 shows that the mean pore size has a pronounced effect on the type of $\bar{K}(r)$ relationship and, as a result, can be reliably determined from GPC experiments. It can also be seen that curves 3 and 4 for inhomogeneous model sorbents deviate from curve 2 at large r values. Analysis shows that the difference increases with increasing polydispersity parameters γ and U. Consequently, chromatographic measurements can also be used for quantitatively assessing the distribution function width and the polydispersity characteristics γ and U related thereto.

Now let us discuss the question of the sensitivity of the chromatographic method to the type of distribution function. Consider curves 3 and 4 in Fig. 5. They are close to each other although calculated for distributions that differ greatly in type. This signifies that the chromatographic method is only slightly sensitive to the type of pore size distribution function. Therefore, attempts at calculating in detail the type of distribution function from chromatographic data appear to us to be of little promise at present. Evidently, it is only in certain special instances, namely those of bimodal distributions with greatly differing (by an order of magnitude or more) pore sizes, that a reliable conclusion can be drawn from the type of $\bar{K}(r)$ relationship regarding the distribution function form.

We are hopeful, nevertheless, that in the future, when the accuracy and reproducibility of chromatographic measurements have been improved as a result of improvements in chromatographic equipment, chromatography will also be capable of providing information about details of porous structures.

For the present, however, we suggest that chromatographic porosimetry be considered as a method for determining the basic porous structure characteristics of sorbents, *viz.*, the mean pore size R_s (or specific surface area Σ) and the polydispersity parameter γ or U related to the pore size distribution function width. The knowledge of these characteristics has been shown to be sufficient for predicting GPC principles.

4.5. Determination of the mean sizes and polydispersities of sorbent pores

To calculate the mean pore size and polydispersity, a method was proposed[17] based on approximating the experimental $K^{(i)}(r_i)$ relationship obtained by using a series of polymer homologues of known radii r_i, by means of the theoretical eqn. 20. The kernel substituted here into eqn. 20 is the $K(r/R)$ function of the form of eqn. 7, while the desired distribution $f_v(R)$ is specified by a logarithmically normal law (eqn. 22) with varying parameters R_s and U. The problem of selecting a distribution function $f_v(R)$ model is not a matter of principle, as the GPC porosimetric method has a low sensitivity to the pore size distribution function form, as shown previously.

A computer is used for calculations by the least-squares method, using a BASIC program specially developed for the purpose. A set of varied values of parameters (R_s, U) is searched to find those which minimize the function

$$T(R_s, U) = \sum_i (K_i^{exp} - K_i)^2 \tag{29}$$

where K_i are the distribution coefficients for the ith experimental point, calculated from eqns. 20, 7 and 22.

TABLE 1

MIXED-SORBENT POROUS STRUCTURE CHARACTERISTICS CALCULATED AND MEASURED BY THE GPC POROSIMETRIC METHOD

Sorbent	*Mean pore radius, R_s (nm)*		*Polydispersity parameter, U*	
	Calculated	*Experimental*	*Calculated*	*Experimental*
Two-component mixture	8.5	7.8	1.06	1.04
Four-component mixture	8.5	7.7	2.2	2.1

The solid lines in Fig. 6 show the results of the best approximation by eqns. 20, 7 and 22 of the experimental data for the example discussed above of mixed two- and four-component sorbents. Table 1 gives quantitative data on the pore sizes and degrees of inhomogeneity of the mixed sorbents. The values of R_s and U in Table 1 were obtained in two ways: (a) calculated from eqns. 11, 12 and 15 on the basis of the model mixture preparation method; and (b) determined by the GPC porosimetric method under discussion, using the experimental chromatographic data shown in Fig. 6. Comparison of the theoretical and experimental values in Table 1 shows that the GPC method provides correct quantitative estimates of the polydispersities and pore sizes of inhomogeneous sorbents.

We are using GPC porosimetry as the basic method for studying sorbent characteristics. To give an example, Table 2 gives data relating to the porous structure characteristics of several sorbents, *viz.*, Ultragel AcA (LKB), TSK-gel Toyopearl HW (Toyo Soda), hydrophobic Octyl-Sepharose CL-4B and a series of hydrophobic ion-exchange sorbents, SOLOZA K, with variable amounts of hydrophobic component[37].

TABLE 2

CHROMATOGRAPHIC POROSIMETRIC RESULTS FOR SOME SORBENTS INTENDED FOR USE IN GPC AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Sorbent	x^*	Σ^{**}	R_s^{***}	U
Toyopearl HW-65 F	0.33	83	24	1.6
Toyopearl HW-60 F	0.42	100	20	1.1
Toyopearl HW-55 F	0.49	160	12.5	1.9
Octyl-Sepharose CL-4B	0.53	55	36	1.1
Ultragel AcA-54	0.56	83	24	1.06
Ultragel AcA-34	0.60	49	41	1.07
Ultragel AcA-22	0.65	38	52	1.1
SOLOZA K-0	0.43	87	23	1.1
SOLOZA K-10	0.42	95	21	1.0
SOLOZA K-20	0.47	100	20	1.3
SOLOZA K-30	0.46	77	26	2.0
SOLOZA K-40	0.49	59	34	2.1

* $x = V_p/(V_0 + V_p)$ = sorbent pore volume fraction.
** Σ = Specific pore surface area (m^2/ml of pore volume).
*** $R_s = 2\Sigma^{-1}$ = mean radius of equivalent cylindrical pores (nm).

4.6. Mean size and polydispersity parameter errors

It is difficult to determine the overall error in absolute pore size values determined by the GPC porosimetric method. Apart from the chromatographic measurements as such, the overall error is contributed to by the errors involved in the determination of test macromolecule sizes, and also by the inaccuracies that may be due to the real-life systems and their theoretical models not being completely compatible with each other. The above types of error may be regarded as systematic errors involved in the method.

When carrying out comparative sorbent studies in which the important point is to establish the equivalence of, or difference between, the structures and chromatographic characteristics of the sorbents, it is convenient to make use of accidental error estimates based on the scatter of experimental points. In the case of two parameters being determined simultaneously by the non-linear least-squares method, the perception of the scattering errors will define the confidence region corresponding to the reliability level specified, normally 90 or 95%[47]. (Using confidence intervals for each of the parameters taken separately is allowable only when the parameter estimates are not correlated, and thus when the parameters are obtained by independent methods.)

In the proposed method, the parameters to be determined simultaneously are the mean pore size R_s and the polydispersity U, the confidence region in this case being a set of $\{R_s, U\}$ values, including the point corresponding to the best estimate. The values of R_s^* and U^* corresponding to the confidence region boundary are determined from the following equation[47]:

$$T(R_s^*, U^*) = T_{\min}\left[1 + \frac{2}{N-2} \cdot F_{95}(2, N-2)\right] \tag{30}$$

where N is the number of experimental points, $T_{\min}$ is the minimal value of function T (eqn. 29) and $F_{95}(2, N-2)$ is the Fischer number for a probability of 95% and 2 and $N-2$ degrees of freedom[47].

As an example, Fig. 12 illustrates 95% confidence regions calculated for the two- and four-component mixed sorbents, for which the data can be seen in Fig. 6 and Table 1. The central points of these confidence regions correspond to the most probable values of R_s and U for the sorbents. Generally, confidence regions will have an asymmetric form. For inhomogeneous sorbents these regions are usually more elongated along the U axis. This signifies that the GPC porosimetric method determines polydispersity to a lower accuracy than the mean pore size.

To reduce the scattering errors, it may be convenient to increase the number of experimental points or re-measure the distribution coefficients (two to four times for each polymer standard involved).

4.7. Conditions for realizing the GPC porosimetry method

Suitable systems to use for GPC porosimetry are polystyrenes in organic solvents[5,10,16] and dextrans and polyethylene glycols as aqueous solutions[15,17,34]. The polymer homologue series used should preferably have a wide range of molecular weights (molecular sizes), and narrow molecular weight distributions are desirable for all polymer standards.

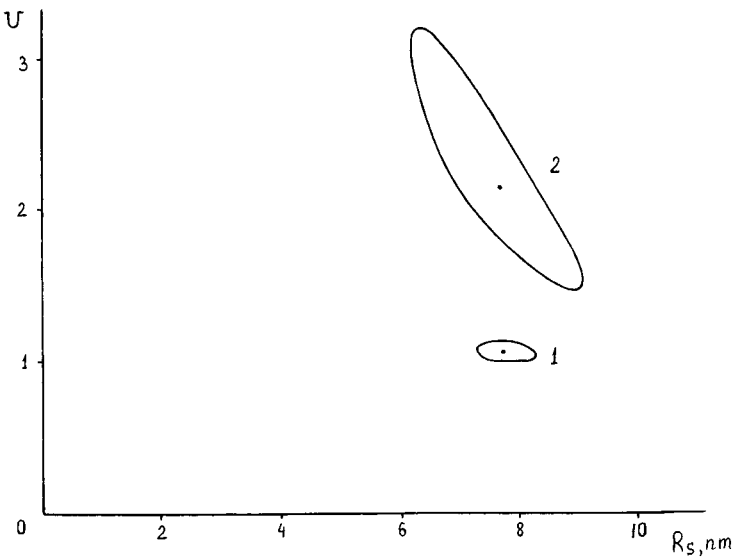

Fig. 12. 95% confidence regions obtained for (1) two- and (2) four-component sorbents.

The requirements that experiments in GPC porosimetry should meet have been discussed[5,17,22]. For porous structure characteristics to be estimated reliably in such experiments, the following basic conditions should be satisfied: (i) no adsorption interactions between polymer and porous material: (ii) chromatography to be carried out in a quasi-equilibrium mode; and (iii) no intermolecular interactions in the solution or in the stationary phase. Provisions to satisfy these conditions include selection of a suitable polymer-solvent system, low polymer concentration in the sample and a sufficiently low elution rate. The criteria that will signify the correct selection of the conditions are the chromatographic results being independent of temperature, solvent composition, concentration and flow-rate, with the conditions selected varying only slightly, and also no double or asymmetric peaks present in the chromatograms.

The condition that is most difficult to satisfy but of extreme importance at the same time is the first one, *i.e.*, that of guaranteeing a macromolecular GPC mode free from adsorption effects. Adsorption effects, coupled with a size-exclusion mechanism, are frequently encountered in polymer chromatography[4,22,35,48–55], and they may seriously distort chromatographic porosimetric results. One must be able to recognize such events and prevent them by selecting suitable polymer-solvent systems, temperatures, pH values and other experimental conditions.

The conformational properties of macromolecules adsorbed within pores and the adsorption effects in polymer chromatography have been investigated theoretically[56–64]. Recently, a general theory of macromolecular chromatography has been developed[65–67] that agrees well with experiment, holds good no matter what adsorption interactions may be present and incorporates Casassa's theory of GPC as a specific case.

This theory[65] implies that several different modes of polymer chromatography may be realized depending on the energy of interaction between the polymer and the

sorbent. Strong interactions lead to an adsorption chromatographic mode that is characterized by a different order of chromatography of macromolecules than in GPC. A "critical" chromatographic mode is also possible when K is independent of molecular weight. Generally, such modes are easily recognizable in experiments.

At the same time, for weak adsorption interactions the theory predicts a "subcritical" GPC-like mode, in which the $K(r)$ relationship is similar to its GPC counterpart in quality, but differs from it quantitatively. Specifically, in place of eqn. 4 the general theory[65] yields for the GPC-like mode the following expression:

$$K \approx \frac{8}{\pi^2} \cdot \exp\left\{-\left[\frac{\pi r}{2\,(R + |H|)}\right]^2\right\} \tag{31}$$

where $|H|$ is a length-dimensioned parameter which depends on the adsorption interaction energy and is referred to as the "correlation length of adsorption". Corresponding to GPC conditions $|H| = 0$; as adsorption effects come into play, $|H|$ varies on a small scale initially, but then starts to increase sharply.

It follows from eqns. 4 and 31 that the apparent pore radius $\tilde{R}$ measured under such conditions will be greater than the true radius R by the value of $|H|$:

$$\tilde{R} = R + |H| \tag{32}$$

The theoretical dependence of $\tilde{R}$ on the adsorption energy for a macromolecule in a cylindrical pore was calculated by Gorbunov *et al.*[61]. Experimentally, variation of adsorption interaction energy (and $|H|$) is usually achieved by varying the mixed solvent composition or the temperature. Nefyodov and co-workers[35,68] gave experimental data on the chromatography of polystyrenes with a molecular weight range between 200 and 400 000. Silica gel KSK was used as the sorbent. The parameters varied were temperature and the composition of the mixed carbon tetrachloride–

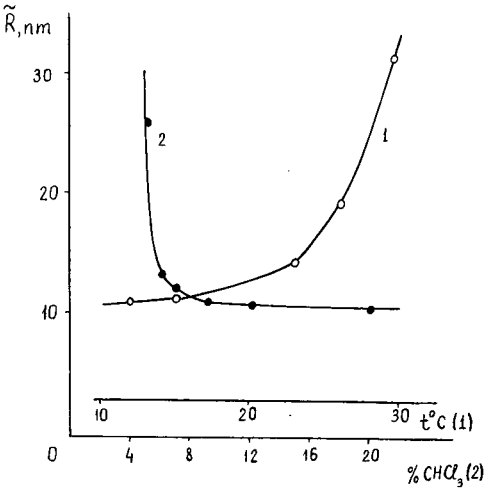

Fig. 13. Apparent pore size $\tilde{R}$ *versus* (1) temperature and (2) mixed solvent composition for GPC-like chromatographic modes. Data from refs. 35, 67 and 68.

chloroform solvent. All of the characteristic chromatographic modes were observable, including the GPC-like mode. Using GLC-like mode data[35,67,68], we have calculated apparent pore sizes in relation to temperature and mixed solvent composition (Fig. 13). The GPC mode is seen to be attained as the temperature drops to 12°C or as the chloroform content in the mixed solvent rises to 20%, both of these methods of eliminating adsorption leading, within the limits of error, to an identical pore radius R of 11 nm. Similar results were obtained in a study[37] of the chromatography of polyethylene glycols on SOLOZA hydrophobic ion-exchange sorbents in aqueous buffer solutions. It was shown[37] that the GPC mode may be achieved in various ways, by temperature or pH variations or by adding agents to reduce adsorption interactions (Triton X-100[37]).

Hence the GPC porosimetric method can be used to analyse not only the structures of neutral porous materials, but also those of sorbents for use in adsorption (hydrophobic, ion-exchange, bioaffinity) chromatography.

5. CONCLUSIONS

The macromolecular GPC method permits direct sorbent pore surface and volume measurements. The pore surface-to-volume ratio is an universal model-independent sorbent pore characteristic which is meaningful for practical sorbents with irregularly shaped and variously sized pores. Together with this characteristic, use can be made of the more readily visualizable such as those of equivalent model pore sizes.

The known simple methods of pore size determination (the "critical molecular weight" and "median" methods) require the use of correction factors. No correction is needed for the method using the initial slope of the $K(r)$ plot for pore size assessment. All of these methods, however, become inaccurate with polydisperse sorbents.

The pore size distribution function calculation method proposed by Halász and Martin is essentially incorrect. The mean pore sizes as determined by this method can be corrected, but the function width is far too large. The method advanced by Knox and Scott does not require mean size corrections, but will give too high estimates for polydispersity as it fails to account for the specific behaviour of polymeric molecules in the process of chromatography. A more accurate method is that advanced by Vilenchik and co-workers. It will be noted, however, that in most instances the type of pore size distribution function cannot be determined reliably from GPC porosimetric data, nor can chromatographic data be used to determine the pore form.

It has been shown that reliable measurements are possible for the mean pore size and for the polydispersity parameter that characterizes the pore size distribution function width. An algorithm has been developed for determining these parameters from data obtainable by GPC porosimetry based on the theory of macromolecular GPC using inhomogeneous sorbents.

Conditions have been discussed to permit the proper realization of chromatographic porosimetry. It has been shown that adsorption effects may lead to incorrect, *i.e.*, too high, pore size estimates and, for this reason, precautions must be taken to guard against such non-exclusion effects while undertaking the practical realization of the GPC porosimetric method.

The theoretical fundamentals of the GPC porosimetric method have by now

been well developed, and the possibilities offered by this method, as also its application potential, are recognized. We believe that, if the necessary conditions are duly observed and the resulting data are correctly interpreted, chromatographic porosimetry will become one of the best methods for investigating the porous structures of sorbents.

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7. SUMMARY

Considered in this work are the basic results from the theory of, and experiments in, macromolecular GPC using porous sorbents, which are essential for understanding the problems involved in GPC porosimetry. Current views on macromolecular GPC based on the use of inhomogeneous sorbents are presented. The known methods of interpreting experiments on GPC porosimetry are analysed on the basis of common theoretical grounds. The necessary conditions for proper realization of the GPC porosimetric method are discussed.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIS[1,2-BIS-(DIPHENYLPHOSPHINO)ETHANE]GOLD(I) CHLORIDE, A POTENTIAL ANTINEOPLASTIC AGENT

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SUMMARY

The chromatography of $[Au(dppe)_2]^+$ (I), a potential antineoplastic drug, was studied on a variety of stationary phases (ODS Hypersil, PLRP-S, Partisil SAX and Partisil SCX) using aqueous mobile phases containing 60% acetonitrile, 15% tetrahydrofuran and various electrolytes. The effects of both the concentration (0–20 m*M*) and the nature of the electrolytes, added to the mobile phase, on the chromatography of I were investigated. A wide variety of electrolytes were investigated in which the hydrophobicity of both the anion and the cation were varied. The analyte of interest was found to be unretained by the like-charged Partisil SAX column. On the other hand, I was retained on the Partisil SCX by an ion-exchange mechanism and retention could be controlled by manipulating the electrolyte composition of the mobile phase. I was retained on the two reversed-phase materials by a mixture of solvophobic and electrostatic interactions but, under the conditions studied, the latter mechanism was the dominant one. The retention of I on the two reversed-phase materials was influenced much more by the nature and concentration of the cation added to the mobile phase than it was by the nature and concentration of the anion. Therefore, manipulation of the nature and concentration of the cationic species in the mobile phase appears to afford the most useful means of manipulating the retention of I, and presumably its analogues, on reversed-phase columns.

INTRODUCTION

Bis[1,2-bis(diphenylphosphino)ethane]gold(I) ($[Au(dppe)_2]^+$) is a cationic, organometallic, complex of gold(I). This complex is an experimental anticancer drug, having been synthesized as an analogue of auranofin which itself has only weak antineoplastic activity *in vitro* and *in vivo*[1]. The complex has been formulated as an aqueous solution of the chloride salt ($[Au(dppe)_2]Cl$, I, Fig. 1). The preformulation and prepharmacokinetic studies on I required the development of high-performance liquid chromatographic (HPLC) methods for the determination of the complex in aqueous formulations and biological fluids, respectively.

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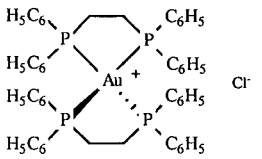

[Au(pdde)$_2$].Cl, I

Fig. 1. The structure of bis[1,2-bis(diphenylphosphino)ethane]gold(I) ([Au(dppe)$_2$]Cl, I).

The reversed-phase HPLC of organometallic complexes, inorganic metal complexes and metal ions have been studied by a number of workers[2–4] and the whole subject has been reviewed by Willeford and Veening[2]. In general, ion-pair chromatography is the method of choice for the separation of metal ions or ionic metal complexes[2–4]. For example, Valenty and Behnken[3] separated several derivatives of tris(2,2′-bipyridyl)ruthenium(II) on a C_{18} bonded phase by the addition of alkylsulfonates to the mobile phase. Similarly, Buckingham *et al.*[4] found that adding *p*-toluenesulfonate or hexanesulfonate to the mobile phase facilitated the separation of Co^{3+} complexed with various amino acids and ethylenediamine.

Despite being a positively charged species, initial attempts to elute I from silica-based or polymeric reversed-phase columns were unsuccessful. Consequently, a thorough investigation of the chromatographic properties of I was initiated so that the retention mechanism could be characterized and the factors which allow manipulation of the retention could be identified. In addition, the results obtained should be applicable to the assay development of pharmaceutically relevant analogues of I.

EXPERIMENTAL

Chemicals and reagents

Crystalline I was kindly provided by Smith Kline and French, Philadelphia, PA, U.S.A. HPLC-grade acetonitrile and Spectranalyzed tetrahydrofuran (THF) were obtained from Fisher Scientific. All the tetraalkylammonium salts were obtained from Fluka, Ronkonkoma, NY, U.S.A., with the exception of tetrabutylammonium hydrogensulfate (TBA · HS) which was obtained from Aldrich, Milwaukee, WI, U.S.A. The following tetraalkylammonium salts were used: tetramethylammonium hydrogensulfate (TMA · HS) and bromide (TMA · Br), tetraethylammonium hydrogensulfate (TEA · HS) and bromide (TEA · Br), tetrabutylammonium hydrogensulfate (TBA · HS), bromide (TBA · Br), nitrate (TBA · N.), methanesulfonate (TBA · Ms), hydrogenphosphate (TBA · Ph), perchlorate (TBA · Pe), *p*-toluenesulfonate (TBA · Ts) and tetraphenylborate (TBA · TPB), tetrapentylammonium bromide (TPA · Br), tetrahexylammonium bromide (THxA · Br), tetraheptylammonium bromide (THpA · Br) and tetraoctylammonium bromide (TOA · Br). The sodium ethanesulfonate (SES) was obtained from American Tokyo Kasei, Harborgate, OR, U.S.A. The sodium pentanesulfonate (SPS) and the sodium octanesulfonate (SOS) were obtained from Eastman Kodak, Rochester, NY, U.S.A. The other chemicals were at least reagent grade and were obtained from various sources.

Equipment

The chromatography was performed on a modular system consisting of an Altex 110A pump (Beckman Instruments, Chicago, IL, U.S.A.), an Altex 210 injector fitted with a 20-μl loop and either a Beckman 153 (254 nm) or a Waters 450 (280 nm) detector (Waters Assoc., Milford, MA, U.S.A.). The chromatograms were recorded on a Spectra-Physics 4290 integrator (Spectra-Physics, San Francisco, CA, U.S.A.). The following HPLC columns were obtained from their respective manufacturers: PLRP-S (5 μm, 15 cm $\times$ 4.6 mm I.D., Polymer Labs., Amherst, MA, U.S.A.), Partisil SAX (10 μm, 25 cm $\times$ 4.6 mm I.D. Whatman, Nutley, NJ, U.S.A.), Partisil SCX (10 μm, 25 cm $\times$ 4.6 mm I.D.). ODS Hypersil (5 μm, 15 cm $\times$ 4.6 mm I.D., Keystone Scientific, State College, PA, U.S.A.) was packed in the upward direction at 48 MPa (7000 p.s.i.) using chloroform as the slurry solvent and methanol as the packing solvent.

Procedures

Measurements of the chromatographic parameters were made at least in duplicate by injecting (20 μl) solutions of I dissolved in acetonitrile. The effects of adding various salts to a mobile phase of acetonitrile–THF–water (60:15:25) on the chromatography of I were investigated. After each experiment the column was flushed with 10–15 column volumes of water. The columns were equilibrated until constant retention times were achieved with fresh mobile phase prior to recording of the results. This was generally after 10–15 column volumes.

RESULTS AND DISCUSSION

In the absence of added electrolytes, I (Fig. 1) was completely retained by both a silica-based stationary phase (ODS Hypersil) and a macroporous copolymer of poly(styrene–divinylbenzene) (PLRP-S), using hydro-organic mobile phases containing either acetonitrile (0–100%) or THF (0–100%). The strong retention of I may be attributed, at least in part, to solvophobic interactions[5,6] between the phenyl groups of the analyte and the hydrophobic regions of the stationary phases. However, the presence of a positive charge on I suggested that interactions with the negatively charged silanol groups on the silica backbone might also be involved in the retention process[7–9]. This was supported by the results of preliminary experiments which showed that I could be displaced from both types of reversed-phase column by the addition of electrolytes, such as KBr or TBA · Br, to the mobile phase.

Following these initial observations, a series of experiments were conducted to characterize the retention behavior of I on reversed-phase columns. For comparison and to characterize the chromatography further, the retention behavior of I on a strong cation (Partisil SCX) and a strong anion (Partisil SAX) exchanger was also studied. Throughout these experiments the organic modifier composition of the mobile phase was maintained constant (acetonitrile–THF–water; 60:15:25) and the effects of various electrolytes were investigated.

Initially, the retention behavior of I on ODS Hypersil was studied by investigating the effects of adding KBr, TBA · Br and TBA · HS (0–20 m*M*) to the mobile phase. It can be seen from Table I and Fig. 2, that in all cases the retention of I decreased with increasing electrolyte concentration. There was little difference in the effects of TBA · Br and TBA · HS on the retention of I; KBr however was much less

TABLE I

THE EFFECTS OF ADDING TBA · Br, TBA · HS AND KBr TO THE MOBILE PHASE ON THE RETENTION OF I ON ODS HYPERSIL

Temperature: ambient. Mobile phase: acetonitrile–THF–water (60:15:25) containing TBA · Br, TBA · HS or KBr.

[TBA · Br] (mM)	*k'*		*[TBA · HS] (mM)*	*k'*		*[KBr] (mM)*	*k'*	
	Obs.	*Calc.**		*Obs.*	*Calc.**		*Obs.*	*Calc.**
5.0	10.7	10.6	1.0	35.0	37.0	5.0	38.0	33.0
7.5	7.44	7.88	5.0	8.50	8.77	10.0	22.0	21.8
10.0	6.47	6.38	10.0	5.10	5.14	20.0	13.0	13.2
12.5	5.47	5.45	20.0	3.26	3.14			
20.0	4.00	4.01						

* Calculated from eqn. 3 and the constants in Table II.

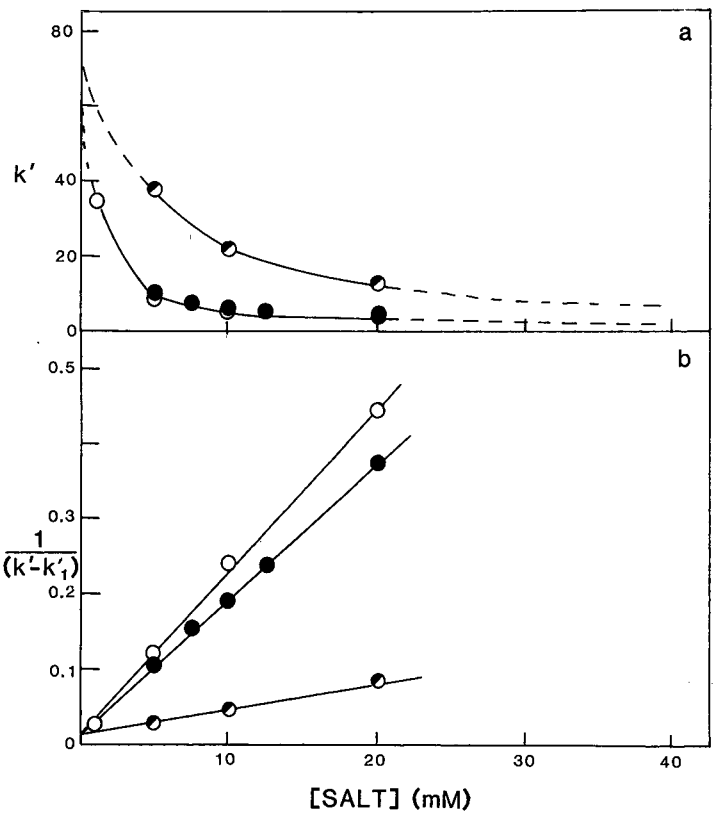

Fig. 2. The relationships between the capacity ratio (k') of I on ODS Hypersil and the concentrations of KBr (◐), TBA · Br (●) and TBA · HS (○) added to the mobile phase. The data in the upper section (a) have been replotted in the lower section (b) according to eqn. 3. The points are experimental and the lines have been drawn using eqn. 3 and the constants given in Table II. Temperature: ambient. Mobile phase: acetonitrile–THF–water (60:15:25).

effective in displacing I from ODS Hypersil. This indicates that the nature of the cation plays a more important role than the nature of the anion in determining the retention of I on this type of column. The results presented so far are consistent with a mixed mechanism of solvophobic and silanophilic interactions as described by Horváth and coworkers[7–9]. The latter is presumed to arise from an electrostatic attraction between the residual silanols on the surface of the stationary phase and the cationic solute. Thus the effects of adding electrolytes may be explained in terms of competition between the cation added to the mobile phase and the analyte for the oppositely charged stationary phase. This mixed mechanism of retention has been described, phenomenologically[7–9], by the following equation:

$$k' = k'_1 + k'_2/(1 + K_A/[A^+]) \quad (1)$$

where k'_1 and k'_2 are the incremental solvophobic and silanophilic retention factors[7], respectively, and $[A^+]$ is the concentration of the competing cation added to the mobile phase. The reversible interaction of the competing cation, A^+, with the stationary phase is given by[7]:

$$K_A = [SiO \cdot A]/([SiO^-] \cdot [A^+]) \quad (2)$$

To test the appropriateness of this model to the present study, the data (Table I, Fig. 2a) relating retention to the concentrations of competing ions were fit by least squares linear regression to eqn. 3, which is a linearized form of eqn. 1, to obtain the values of k'_1, k'_2 and K_A.

$$1/(k' - k'_1) = 1/k'_2 + (K_A/k'_2) \cdot [A^+] \quad (3)$$

The results of this analysis are shown in Table II. In addition, the values of the constants (k'_1, k'_2 and K_A) were used to recalculate the values of k' and it can be seen that agreement between the observed and the predicted values is excellent (Fig. 2 and Table I). By definition (eqn. 2), K_A is dependent on the natures of the stationary phase and the competing ion and is independent of the natures of the analyte and the

TABLE II

VALUES OF THE FORMATION CONSTANTS, K_A, FOR THE ASSOCIATION OF TBA IONS (AS THE BROMIDE AND HYDROGENSULFATE SALTS) AND POTASSIUM IONS WITH ODS HYPERSIL

The values of k'_1, k'_2 and the recommended concentrations of the salts, C_A, to be added to the mobile phase, are also provided. Temperature: ambient. Stationary phase: ODS Hypersil. Mobile phase: see Table I. The parameters were taken from the data in Table I and Fig. 2, using eqn. 3.

Salt	$K_A \cdot 10^{-3}$ (M^{-1})	C_A* *(mM)*	k'_1	k'_2
TBA · Br	1.08	9.3	1.37	59.2
TBA · HS	1.43	7.0	1.00	63.3
KBr	0.25	40.0	1.37	71.4

* C_A is defined as $10/K_A$.

counterion to the competing ion. This is confirmed by the values of K_A which were found to be $1.1 \cdot 10^3\ M^{-1}$ and $1.4 \cdot 10^3\ M^{-1}$ for TBA · Br and TBA · HS and only $2.5 \cdot 10^2\ M^{-1}$ for KBr. Bij *et al.*[7] have suggested that 90% of the silanophilic interactions are abolished if the concentration of competing ions in the mobile phase, C_A, is equal to $10/K_A$. Accordingly, Table II gives the recommended values for the concentrations (C_A) of KBr, TBA · Br and TBA · HS which would satisfy this condition. It is clear from Table II that the concentration of KBr ($\approx$ 40 mM) required to abolish 90% of the silanophilic interactions is much greater than that for the two TBA salts ($\approx$ 10 mM), reflecting the difference in the affinities of K^+ and TBA^+ for the stationary phase (Table II).

The results obtained with K^+ and TBA^+ salts (Table II) suggest that while the driving force for the interaction of competing ion and the silanol groups may be electrostatic, there is clearly a hydrophobic (or solvophobic) component to this interaction as described previously by Bij *et al.*[7]. To probe this phenomenon further, the effect of the hydrophobicity (total carbon number) of the tetraalkylammonium ions, added to the mobile phase, on the retention of I was investigated and the results are shown in Fig. 3. It was found that the retention of I decreased with increasing hydrophobicity of the competing ion and this could be described by the empirical relationship

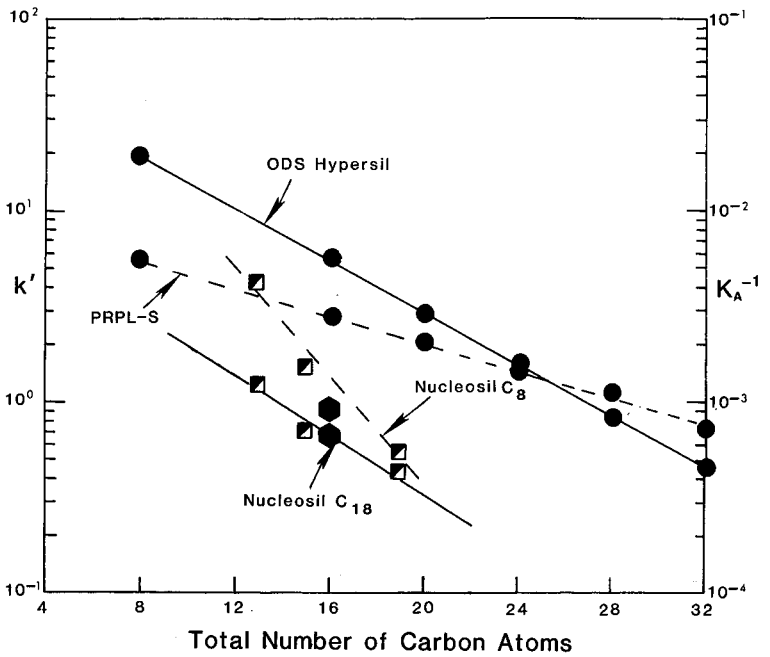

Fig. 3. The relationships between the capacity ratio (k') of I and the total number of carbon atoms in the tetraalkylammonium bromides, added to the mobile phase (circles), and the relationship between the reciprocal of the association constant (K_A^{-1}) of and the total number of carbon atoms in the trimethylalkylammonium ions, added to the mobile phase (squares). The retention data were obtained in this study using the chromatographic conditions given in Fig. 2. The values of K_A were taken from the work of Bij *et al.*[7]. For comparison the values of K_A^{-1} obtained in this study using TBA · Br and TBA · HS are also shown (●).

$$\log k' = -0.069n + 1.85 \quad (4)$$
$$r = 0.9994$$

These results indicate that although the effect of adsorption of competing cations onto the surface of silica-based stationary phases is to mask the residual silanol groups, there is a significant solvophobic contribution to this interaction. These observations are consistent with those of Bij *et al.*[7] who found that the value of the association constant for the interaction of alkyltrimethylammonium ions with Nucleosil C_8 and Nucleosil C_{18} increased with increasing length of the alkyl chain (Fig. 3). The data obtained in this study are compared with those obtained previously[7] in Fig. 3. It is interesting to note that the values of K_A obtained by Bij *et al.*[7] exhibit a stronger dependency on hydrophobicity in the interaction of the alkyltrimethylammonium ions with Nucleosil C_8 compared with the interaction with Nucleosil C_{18}. What is of more interest, however, is that the values of K_A obtained here with tetraalkylammonium ions are consistent with those obtained previously[7] with the alkyltrimethylammonium ions. This may suggest that the ability of an ammonium ion to mask residual silanols is more dependent on its hydrophobicity than the distance separating the charge centers.

The phenomenological model described by eqn. 1 assumes that the cations added to the mobile phase compete with the analyte for one of the two sites of retention, presumed here to be the silanol groups. Adsorption of a hydrophobic cation onto the surface of the ODS Hypersil could also have the effect of masking the solvophobic sites of interaction and these two models would be mathematically equivalent since k'_1 and k'_2 (eqn. 1) would simply be reversed. However, if this alternative were true then it is to be expected that k'_1 (eqn. 1) would be significantly effected by the nature of the electrolytes, which appears not to be the case (Table I and Fig. 2).

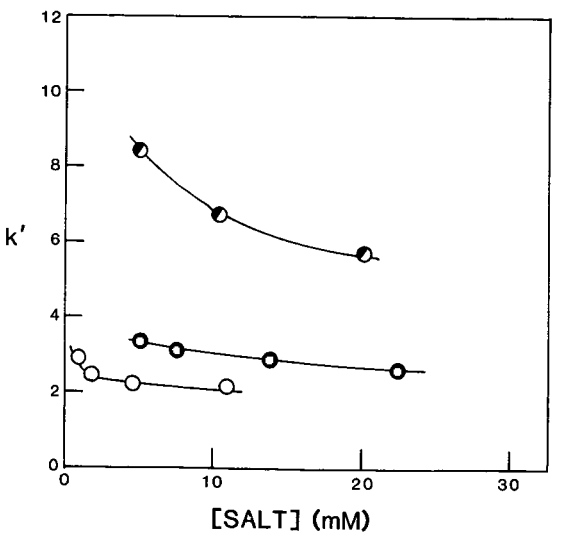

Fig. 4. The relationships between the capacity ratio (k') of I on PLRP-S and the concentrations of KBr (◐), TBA · Br (●) and TBA · HS (○) added to the mobile phase. Other conditions as Fig. 2.

It has been well established[10,11] that ions are strongly adsorbed onto the surface of hydrophobic polymers such as PLRP-S. Fig. 4 shows that the retention of I decreased with increasing concentration of KBr, TBA · Br and TBA · HS, consistent with the previous observations of Cantwell and Puon[10]. These workers have shown that the retention of such analytes may be described by the Stern–Gouy–Chapman theory of double layer adsorption and they are displaced from the column by the addition of like-charged molecules to the mobile phase. This phenomenon is similar to that seen for the retention behavior of I on the silica-based reversed-phase material, however it was experimentally impossible to quantify these observations in the same fashion. Very low concentrations (1 m*M*) of electrolytes, particularly the tetraalkylammonium ions, were extremely effective at displacing I from the column (Fig. 4) and it was not possible to obtain an adequate range of k' values to fit the data to a mathematical model.

It was expected that the retention of I on the PLRP-S column would be dependent on the nature (hydrophobicity) of the competing cation added to the mobile phase. This was supported by the data in Fig. 4 in which the effects of KBr and TBA · Br are compared and confirmed more substantially by the results in Fig. 3. Fig. 3 shows that the retention of I decreased with increasing hydrophobicity of the tetraalkylammonium ions added to the mobile phase at a concentration of 10 m*M*. Again this could be described empirically by a logarithmic relationship between k' and the total number of carbon atoms in the competing ions, n:

$$\log k' = -0.0355n + 1.01 \quad (5)$$
$$r = 0.9974$$

It is interesting to note that the hydrophobic contribution to the interaction is less (slope = −0.0355) in the case of the PLRP-S column compared with the silica based ODS Hypersil column (slope = −0.069).

TABLE III

THE EFFECTS OF THE NATURE OF ANION ON THE RETENTION OF I ON ODS HYPERSIL AND PLRP-S COLUMNS ELUTED WITH MOBILE PHASES CONTAINING VARIOUS TETRABUTYLAMMONIUM SALTS (TBA · X)

Temperature: ambient. Mobile phase: acetonitrile–THF–water (60:15:25) containing TBA · X (10 m*M*).

Anion (X^-)	k'	
	ODS Hypersil	*PLRP-S*
$(C_6H_5)_4B^-$	11.3	26.8
ClO_4^-	6.65	3.46
$CH_3SO_3^-$	6.37	2.40
Br^-	6.25	2.43
p-TsO^-	6.12	2.64
NO_3^-	5.85	2.52
$H_2PO_4^-$	5.63	2.06
HSO_4^-	5.14	2.21

TABLE IV

THE EFFECTS OF ADDING SODIUM ALKYLSULFONATES (SES, SPS AND SOS) TO THE MOBILE PHASE ON THE RETENTION OF I ON THE PLRP-S COLUMN

Temperature: ambient. Mobile phase: acetonitrile–THF–water (60:15:25) containing SES, SPS or SOS.

Alkylsulfonate	k'
SES (5 m*M*)	4.76
SPS (5 m*M*)	4.86
SOS (1 m*M*)	6.71
SOS (5 m*M*)	5.70
SOS (10 m*M*)	5.83
SOS (15 m*M*)	6.08

The ratio k'_2/k'_1 reflects the relative contributions of the two interactions in a dual retention process in the absence of a masking agent[7–9]. It can be seen (Table II and Fig. 2) that, under the conditions studied here, the solvophobic contribution to retention on the ODS Hypersil column is between 1 and 2% of the total. This can be increased to about 20% if the contribution of the silanophilic interactions is reduced substantially by the addition of 10 m*M* TBA to the mobile phase (Table III). Under these conditions it is reasonable to assume that the retention of I might be somewhat influenced by the nature of the counter ion to TBA, due to the effects of ion-pairing[12,13]. Table III shows the effect of various salt forms of TBA on the retention of I on ODS Hypersil. For comparison, the retention of I on the PLRP-S column with various salt forms of TBA added to the mobile phase is also shown. In addition, Table IV shows the effect of the chain length of sodium alkylsulfonates on the retention of I on the PLRP-S column. In general, it may be concluded from the results of these experiments (Table III and IV) that the nature of the anion added to the mobile phase is relatively unimportant compared with the nature of the cation. The exception to this is the tetraphenylborate ion which is expected to form strong solvent reinforced ion pairs[13] with I and hence resulted in a significant enhancement of retention.

To complete the chromatographic characterization of I, its retention was investigated on Partisil SAX and Partisil SCX using aqueous mobile phases containing 60% acetonitrile and 15% THF. Not unexpectedly, I was completely unretained by the like-charged Partisil SAX and completely retained by the oppositely-charged Partisil SCX, in the absence of added electrolyte. No further experiments on Partisil SAX were conducted. It was found that I could be displaced from the Partisil SCX column by the addition of TBA · Br to the mobile phase and that the capacity ratio of I was linearly related to the reciprocal of the concentration of the added electrolyte with a negligible intercept, according to the relationship:

$$k' = 0.034/[\text{TBA}] + (0.113) \qquad r = 0.999 \qquad (6)$$

This result is consistent with a single mechanism of ion exchange for I on Partisil SCX. The effect of the size of competing ion on the retention of I on Partisil SCX was also studied, by adding tetraalkylammonium (TMA, TEA, TBA, TPA, THxA, THp and TOA) bromides to the mobile phase at a concentration of 10 m*M*. It was found that, in

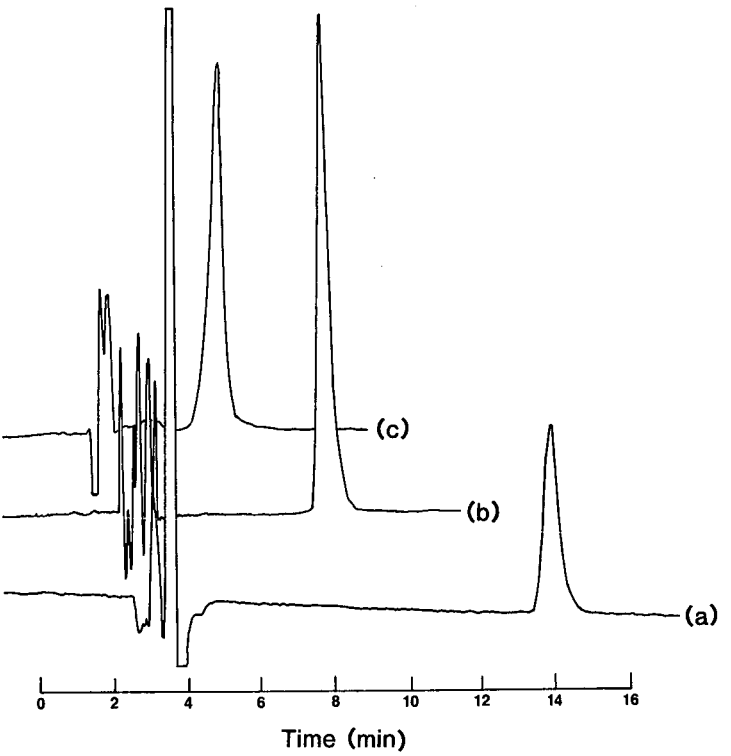

Fig. 5. Chromatograms of I on (a) ODS Hypersil, (b) Partisil SCX and (c) PLRP-S. Mobile phase: acetonitrile–THF–water (60:15:25) containing (a and c) 16 m*M* TBA · HS, (b) 15 m*M* TBA · Br. Flow-rate: 1.0 ml/min. Detection: 254 nm. Injection volume: 20 μl. Solute concentrations: (a and b) 12 μg/ml, (c) 36 μg/ml.

contrast with the reversed-phase materials (ODS Hypersil and PLRP-S), the retention of I increased with increasing size of the competing ion, reflecting the importance of electrostatic interactions and charge separation on the retention of cations by Partisil SCX. Although the retention of I on Partisil SCX increased with increasing size of the tetraalkylammonium bromides added to the mobile phase, no mathematical relationship describing this effect could be developed.

In conclusion, the cationic gold complex, I, was strongly adsorbed onto both silica-based and polymeric reversed-phase materials, apparently by mixed mechanisms of retention. However, the affinity of the analyte for these columns and also for the strong cation exchanger (Partisil SCX) could be substantially reduced by the addition of electrolytes to a mobile phase containing acetonitrile–THF–water (60:15:25). By appropriate choice of the electrolyte and its concentration in the mobile phase, good chromatography with almost symmetrical peaks and efficiencies between 7000 and 20 000 plates/m could be obtained on each of the columns (Fig. 5).

ACKNOWLEDGEMENTS

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PLANAR CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS AND DIASTEREOMERS WITH CYCLODEXTRIN MOBILE PHASE ADDITIVES

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SUMMARY

A variety of racemic compounds were resolved using reversed-phase thin-layer chromatography (TLC) with mobile phases containing highly concentrated solutions of β-cyclodextrin (β-CD). These include the drugs labetalol and mephenytoin, metallocenes, crown ethers, methyl-*p*-toluenesulfinate, nornicotine derivatives and several dansyl and β-naphthylamide substituted amino acids. It was possible to resolve some racemates that could not be separated on β-CD bonded phase liquid chromatography (LC) columns with this technique. Likewise there were some compounds that could be resolved with the LC approach that failed to separate with the present TLC method. In cases of racemates that could be resolved by either approach, it was found that the retention order was exactly opposite for the two methods. Enantiomeric resolution is highly dependent on mobile phase composition. In particular, the type and amount of organic modifier as well as the concentration of β-CD affect the observed resolution. Possible reasons for the chromatographic behavior are discussed. Several diastereoisomeric compounds were separated as well, including steroid epimers and pharmaceutical compounds.

INTRODUCTION

Reports on the liquid chromatographic (LC) separation of enantiomers have increased substantially in the last few years. Several new chiral stationary phases (CSPs) have been proposed and evaluated[1–16]. In addition, a wide variety of chiral mobile phase additives have been shown to resolve certain racemates[17–24]. Unfortunately planar chromatographic methods [such as thin-layer chromatography (TLC) and paper chromatography] have lagged far behind their LC counterparts. There have been a few isolated reports on the TLC of a limited number of compounds. For example, Yuasa *et al.*[25] reported the partial separation of DL-tryptophan on a crystalline cellulose coated plate. Wainer *et al.*[26] separated racemic 2,2,2-trifluoro-1-(9-anthryl) ethanol on a chiral dinitrobenzoylphenylglycine bonded phase. Weinstein[27], Grinberg and Weinstein[28], and Gunther *et al.*[29] separated several racemic dansyl amino acids on reversed-phase plates impregnated with copper(II) complexes of chiral alkyl α-amino acid derivatives. Alak and Armstrong[30] reported

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the separation of several racemic amino acid and ferrocene derivatives on β-cyclodextrin (β-CD) bonded phase TLC plates. With the possible exception of the ternary complex–ligand exchange plates, none of the aforementioned CSPs are available commercially in a planar format. Consequently, the most readily available approach for the TLC separation of different enantiomers remains the use of chiral mobile phase additives. Unfortunately even less has been published on this subject than on TLC with CSPs.

Cyclodextrins were first used as mobile phase additives for chromatography in 1980 to separate a series of structural isomers[31,32]. In 1982, Sybilska and co-workers[20,22] used cyclodextrins as mobile phase additives in high-performance liquid chromatography (HPLC) to effect the resolution of racemic mandelic acid and its derivatives. Since this time, there have been a few additional reports on the use of cyclodextrin mobile phase additives in HPLC[23,24]. However, no racemate has been resolved by TLC using a cyclodextrin mobile phase additive to our knowledge. One of the reasons for this is the limited solubility of cyclodextrins (particularly β-CD) in hydro-organic solvents. Indeed, a saturated solution of β-CD in pure water is approximately 0.017 *M*, which is insufficient for the TLC separation of most enantiomers.

In this work we report the resolution of 21 racemates by reversed-phase TLC using a β-CD mobile phase additive. Resolution is achieved only when the concentration of β-CD is increased to levels exceeding its solubility in pure water. Types of racemates resolved include drugs, nicotinoids, amino acid derivatives, sulfinates, metallocenes and crown ethers. The TLC resolutions (R_s) of some of the racemates was equivalent to or better than analogous HPLC separations on CSPs. As expected, this planar method easily separated a number of diastereomeric compounds as well.

EXPERIMENTAL

Materials

Chemically bonded octadecylsilane reversed phase TLC plates, KC18F (200 μm layer thickness, 5 × 20 cm and 20 × 20 cm) were obtained from Whatman (Clifton, NJ, U.S.A.). All dansyl amino acids, cinchonine, cinchonidine, quinine, quinidine, 17α,20α-dihydroxy-4-pregnen-3-one; 17α,20β-dihydroxy-4-pregnen-3-one; 17α,20α, 21-trihydroxy-4-pregnene-3,11-dione; 17α,20β,21-trihydroxy-4-pregnene-3,11-dione; 20-hydroxy-4-pregnen-3-one and 20β-hydroxy-4-pregnen-3-one were obtained from Sigma (St. Louis, MO, U.S.A.). (±)2-Chloro-2-phenylacetyl chloride, DL-alanine-2-naphthylamide hydrochloride, (1*R*,2*S*,5*R*)-(−)-menthyl-(*S*)-*p*-toluenesulfonate, (1*S*, 2*R*,5*S*)-(+)-menthyl-(*R*)-*p*-toluenesulfinate and α-ethyltryptamine acetate were obtained from Aldrich (Milwaukee, WI, U.S.A.). Urea and sodium chloride were obtained from MCB (Cincinnati, OH, U.S.A.). β-Cyclodextrin was obtained from Advanced Separation Technologies (Whippany, NJ, U.S.A.) and Ensuiko Sugar Refining. HPLC-grade water, acetonitrile, triethylamine, hydrochloric acid and methanol were obtained from Fisher Scientific (Plano, TX, U.S.A.). The (±)2-chloro-2-phenylacetyl chloride was hydrolyzed to the free acid before use. All other chemicals were used as received.

Ferrocene enantiomers [(±)*S*-(1-ferrocenyl-2-methylpropyl)thioethanol, (±)*S*-

(1-ferrocenylethyl)thiophenol], nicotine enantiomers [N′-benzylnornicotine, N′-(2-naphthylmethyl)nornicotine], N′-(methoxycarbonyl)-anabasine, N′-(menthoxycarbonyl)-3-pyridyl-1-aminoethane and crown ether enantiomers [(±)2,2-binaphthyldiyl-N-benzyl-monoaza-16-crown-5] were produced as previously reported[24,33–35]. Mephenytoin and labetalol were obtained from R. D. Armstrong of the La Jolla Cancer Research Foundation.

Methods

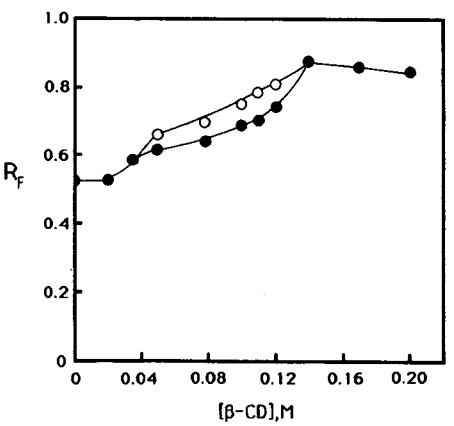
The solubility of β-CD in water is $1.67 \cdot 10^{-2}$ *M* at 25°C; however, when urea is added, one can increase the solubility of β-CD. In this study, saturated solutions of urea were used. 0.6 *M* Sodium chloride also was added to the mobile phase to stabilize the binder of the reversed-phase plates. Without this salt, mobile phases containing more than 50% water tend to dissolve the binder of Whatman reversed-phase plates, thereby resulting in the separation of the stationary phase from the glass support during development.

It took approximately 6–8 h to develop completely a 20 × 20 cm and a 5 × 20 cm TLC plate with a cyclodextrin mobile phase. All developments were done at room temperature (20°C) in 23 × 6 cm I.D. cylindrical glass chambers and a 28.5 × 9.5 × 27.0 cm glass chamber.

Spot visualization was done by use of a fixed-wavelength (254 nm) UV lamp. A Shimadzu dual-wavelength TLC scanner (CS-910) was used to measure resolution. A single-wavelength, reflection mode and linear scanning were used. The wavelength selected corresponded to that of maximum absorbance for each compound.

RESULTS AND DISCUSSION

The solubility of β-CD in neat water and hydro-organic solvent mixtures can be



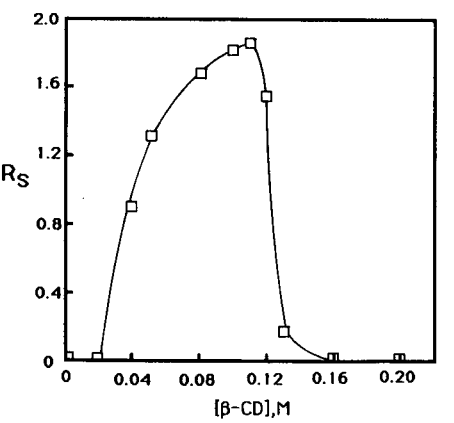


Fig. 1. Plot showing the effect of β-cyclodextrin concentration in the mobile phase on the R_F values of dansyl-D-glutamic acid (○) and dansyl-L-glutamic acid (●). In addition to the indicated levels of β-CD the mobile phase consisted of acetonitrile–water (30:70) (saturated with urea).

Fig. 2. Plot showing the effect β-cyclodextrin concentration on the TLC resolution (R_s) of dansyl-DL-glutamic acid. Other conditions are the same as in Fig. 1.

increased by over an order of magnitude using various additives. Both urea and sodium hydroxide tend to enhance the solubility of β-CD in the aforementioned solvents. In this study, aqueous solutions saturated with urea (see Experimental section) proved to be most effective. The significance of this in planar chromatography is that one can resolve many racemates by reversed-phase TLC using these "enhanced concentration" cyclodextrin solutions. This is illustrated in Fig. 1. Significant resolution of dansyl DL-glutamic acid occurs when the mobile phase contains more than 0.04 *M* β-CD. Optimum enantiomeric resolution occurs between approximately 0.08 and 0.12 *M* β-CD (Fig. 2). This range varies slightly with the compound studied and more substantially with the amount of organic modifier present. The resolution deteriorates at very high β-CD concentrations as the spots blend together near the solvent front (Fig. 2).

Both the concentration and type of organic modifier affect enantiomeric resolution in this technique. The results illustrated in Fig. 3 are typical for all of the solutes in this study. Enantiomeric resolution occurs over a narrow range of organic modifier concentrations but not outside that range. Optimum resolution occurs over a range of 10 to 15% modifier and the R_F values of solutes tend not to change appreciably in this region (see Fig. 3 between 20 and 30% acetonitrile). An analogous curve to that shown in Fig. 3 was generated using methanol as the modifier. The only difference was that the "plateau of optimum resolution" was shifted to 10% higher concentration of modifier (*i.e.*, to 30–40% methanol) and to slightly higher R_F values (*i.e.*, 0.5–0.6). The general effect of organic modifier type on enantiomeric resolution is shown in Fig. 4. Again it is apparent that resolution occurs over a relatively narrow range of mobile phase compositions. Also, the range for methanol is slightly greater than that for acetonitrile. The point of maximum resolution occurs at a lower modifier percentage in the acetonitrile case (Fig. 4). This was true for all of the solutes in this

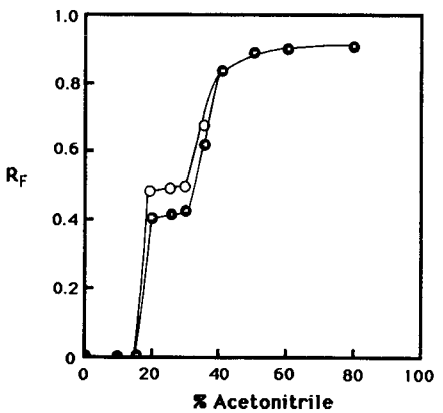


Fig. 3. Plot showing the effect of %acetonitrile in the mobile phase on the TLC separation of dansyl-D-serine (○) from dansyl-L-serine (●). The concentration of β-cyclodextrin is 0.106 *M*.

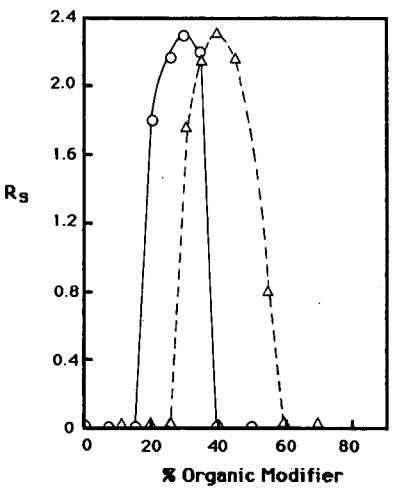


Fig. 4. Plots showing the difference in acetonitrile (○) *versus* methanol (△) phase modifiers in the TLC resolution of dansyl-DL-threonine with β-CD additives. The concentration of β-cyclodextrin is 0.106 *M*.

study. Although Fig. 4 shows that the maximum obtainable resolution was the same for both acetonitrile and methanol modifiers ($R_s \approx 2.3$) this was not true for all solutes in this study. Comparison plots, such as those in Fig. 4, could vary as to the height of their respective maxima as well as baseline range.

Table I gives the separation data and conditions for the resolution of twelve dansyl amino acids. Table II gives equivalent data for ten other racemates that are not related structually to the dansyl amino acids or to one another. In all cases the solubility of the β-CD mobile phase modifier had to be enhanced with urea before enantiomeric resolution was possible. Fig. 5 is a TLC chromatogram showing the baseline resolution of several dansyl amino acid racemates. Note that the D-enantiomer always elutes ahead of the L isomer. This retention behavior is opposite to that observed for the β-CD bonded phase.

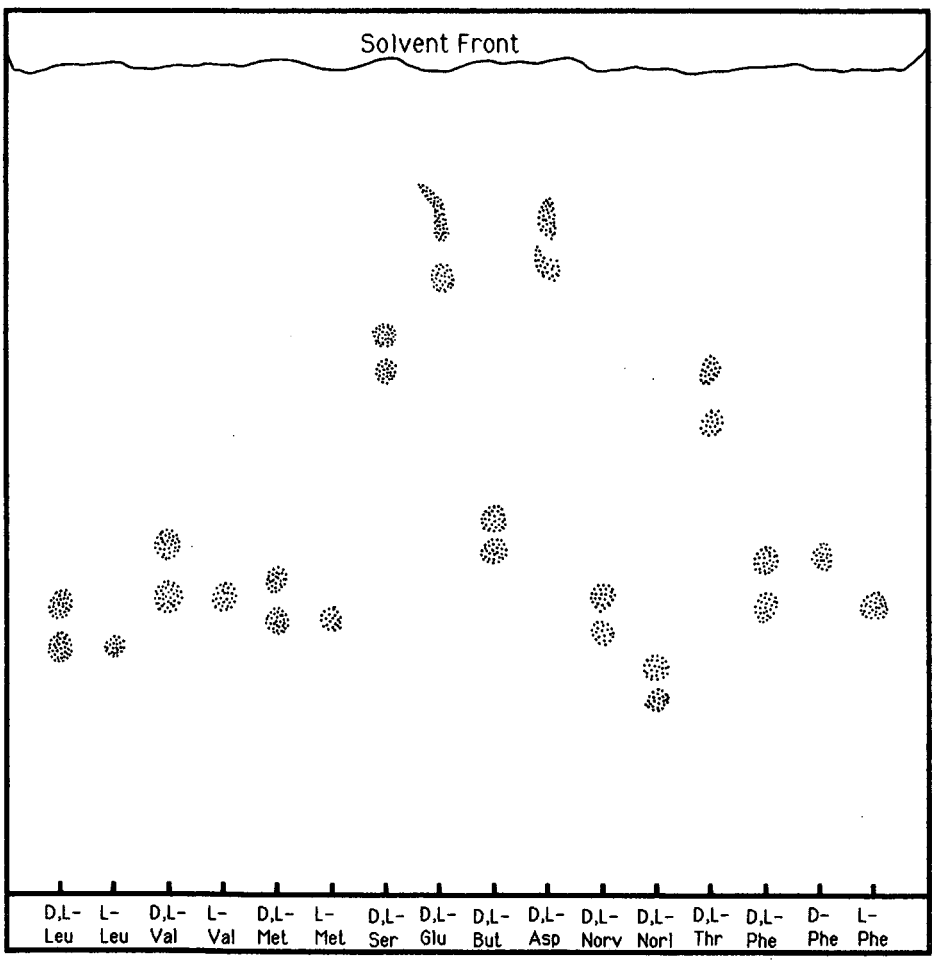


Fig. 5. RP-TLC chromatogram showing the resolution of the racemates: dansyl-DL-leucine; dansyl-DL-valine; dansyl-DL-methionine; dansyl-DL-serine; dansyl-DL-glutamic acid; dansyl-DL-α-amino-*n*-butyric acid; dansyl-DL-aspartic acid; dansyl-DL-norvaline; dansyl-DL-threonine and dansyl-DL-phenylalanine. The mobile phase consisted of acetonitrile–0.10 *M* β-CD (aq.) (30:70, v/v) (see Experimental section).

TABLE I

SEPARATION DATA FOR DANSYL AMINO ACIDS

A UV lamp (254 nm) was used for detection of fluorescence spots.

Compounds	R_{F_1}*	R_{F_2}	α	R_s	*Mobile phase***
Dansyl-DL-leucine	0.30	0.35	1.17	2.0	Acetonitrile–0.151 *M* β-CD (30:70)
Dansyl-DL-valine	0.36	0.43	1.19	2.5	Acetonitrile–0.151 *M* β-CD (30:70)
Dansyl-DL-methionine	0.34	0.38	1.12	2.1	Acetonitrile–0.151 *M* β-CD (30:70)
Dansyl-DL-glutamic acid	0.65	0.72	1.11	2.0	Methanol–0.163 *M* β-CD (35:65)
Dansyl-DL-α-amino-*n*-butyric acid	0.42	0.47	1.12	1.5	Acetonitrile–0.151 *M* β-CD (30:70)
Dansyl-DL-norvaline	0.32	0.34	1.06	1.4	Acetonitrile–0.151 *M* β-CD (30:70)
Dansyl-DL-norleucine	0.24	0.28	1.17	1.6	Acetonitrile–0.151 *M* β-CD (30;70)
Dansyl-DL-phenylalanine	0.35	0.39	1.11	1.4	Acetonitrile–0.151 *M* β-CD (30:70)
Dansyl-DL-serine	0.41	0.47	1.15	1.5	Acetonitrile–0.133 *M* β-CD (20:80)
Dansyl-DL-aspartic acid	0.64	0.70	1.09	1.8	Acetonitrile–0.133 *M* β-CD (25:75)
Dansyl-DL-tryptophan	0.43	0.45	1.05	0.8	Acetonitrile–0.231 *M* β-CD (35:65)
Dansyl-DL-threonine	0.42	0.51	1.24	2.0	Methanol–0.151 *M* β-CD (30:70)

* D-Isomer was eluted first.

** Solutions also contained urea and sodium chloride (see Experimental section).

TABLE II

SEPARATION DATA FOR ENANTIOMERIC COMPOUNDS

Compounds	R_{F1}	R_{F_2}	α	R_s	*Mobile phase**	*Detection method*
Mephenytoin	0.32	0.38	1.19	1.5	Methanol–0.308 *M* β-CD (35:65)	UV (254 nm)
(±)*S*-(1-Ferrocenyl-2-methylpropyl)-thioethanol	0.42	0.51	1.21	2.0	Acetonitrile–0.125 *M* β-CD (15:85)	TLC scanner**
(±)*S*-(1-Ferrocenylethyl)thiophenol	0.38	0.42	1.07	0.5	Acetonitrile–0.151 *M* β-CD (30:70)	TLC scanner**
N′-Benzylnornicotine	0.29	0.34	1.17	1.7	Methanol–0.200 *M* β-CD (60:40)***	UV (254 nm)
N′-(2-Naphthylmethyl)nornicotine	0.19	0.24	1.26	1.7	Methanol–0.200 *M* β-CD (60:40)***	UV (254 nm)
(±)2-Chloro-2-phenylacetyl chloride	0.02	0.07	3.5	0.55	Acetonitrile–0.151 *M* β-CD (30:70)	TLC scanner**
DL-Alanine-2-naphthylamide hydrochloride	0.59	0.66	1.12	1.2	Methanol–0.163 *M* β-CD (35:65)	TLC scanner**
(1*R*,2*S*,5*R*)-(−)-Menthyl-(*S*)- and (1*S*,2*R*,5*S*)-(+)-menthyl-(*R*)-*p*-toluenesulfinate	0.06	0.08	1.33	0.6	Acetonitrile–0.151 *M* β-CD (30:70)	TLC scanner**
(±)2,2′-Binaphthyldiyl-N-benzylmonoaza-16-crown-5	0.05	0.08	1.60	0.6	Methanol–0.265 *M* β-CD (60:40)	TLC scanner**

* Solutions also contained urea and sodium chloride (see Experimental section).

** Wavelengths for detection were 254, 280 and 230 nm.

*** 1% Aqueous triethyl ammonium acetate (pH 7.1).

Another interesting aspect of this technique is that sometimes there seems to be no relation between the ease of separation with the β-CD mobile phase modifier *versus* the β-CD bonded phase. Many compounds are separated equally well by both techniques with the expected reversal in retention order as the main difference. However, some racemates can be resolved by one method but not the other. For example, and menthyl-*p*-toluenesulfinate (Table II) and dansyl glutamic acid and aspartic acid (Table I) are difficult to separate on β-CD bonded phase LC columns. Conversely, some chiral crown ethers[34] and racemic metallocenes[35] could not be resolved with β-CD mobile phase additives and RP-TLC even though they were easily resolved by HPLC with a β-CD bonded phase. This is interesting because the chiral resolving agent (β-CD) is the same in both cases. Clearly, the mechanism of chiral recognition and resolution is not always analogous in the two related methods. While the reasons for these differences in chiral selectivity are not yet clear, there are a number of possible factors. In the case of the bonded phase, the cyclodextrin is linked to the silica gel via one to three, eight atom spacer arms. The spacer arms can restrict the motion of the cyclodextrin and provide an additional possible interaction site for a complexed molecule. Also, the surface density and configuration of the cyclodextrin on the bonded phase media may be more fixed than when using the cyclodextrins as mobile phase modifiers. As a mobile phase additive, the cyclodextrin is readily

TABLE III

SEPARATION DATA FOR DIASTEREOMERIC COMPOUNDS

UV lamp (254 nm) was used for detection of spots.

Compounds	R_F	α	R_s	*Mobile phase**
Labetalol	0.49 0.53	1.08	0.7	Methanol–0.262 *M* β-CD (35:65)
Cinchonine Cinchonidine	0.21 0.18	1.16	0.9	Acetonitrile–0.133 *M* β-CD (20:80)
Quinine Quinidine	0.19 0.25	1.32	1.5	Methanol–0.250 *M* β-CD (40:60)
N′-(Menthoxycarbonyl)-anabasine	0.04 0.07	1.75	2.0	Methanol–0.200 *M* β-CD (60:40)**
N′-(menthoxycarbonyl)-3-pyridyl-1-aminoethane	0.24 0.30	1.25	3.1	Methanol–0.200 *M* β-CD (60:40)**
17α,20α-Dihydroxy-4-pregnen-3-one 17α,20β-Dihydroxy-4-pregnen-3-one	0.56 0.48	1.17	2.7	Acetonitrile–0.151 *M* β-CD (30:70)
17α,20α,21-Trihydroxy-4-pregnene-3,11-dione 17α,20β,21-Trihydroxy-4-pregnene-3,11-dione	0.82 0.68	1.20	1.5	Methanol–0.151 *M* β-CD (30:70)
20α-Hydroxy-4-pregnen-3-one 20β-Hydroxy-4-pregnen-3-one	0.59 0.33	1.79	5.6	Methanol–0.151 *M* β-CD (30:70)

* Solutions also contained urea and sodium chloride (see Experimental section).

** 1% aqueous triethylammonium-acetate (pH 7.1).

available for multiple complexation[36]. Currently the effect of multiple complexation and equilibria on chiral recognition is unknown. When using cyclodextrins as mobile phase modifiers they are both adsorbed on the achiral stationary phase and present as carriers in solution. If solution demixing occurs during development and a racemate travels ahead of the cyclodextrin solvent front, it would not be expected to resolve. Indeed, this may be occuring at the higher organic modifier concentrations. However, this cannot explain why some racemates resolve via the mobile phase additive method but not on the chiral stationary phase.

It is not surprising that a number of diastereomeric compounds are more easily separated by "chiral LC" technique than by conventional normal and reversed-phase methods. Likewise, RP-TLC with chiral cyclodextrin mobile phase additives effectively separates a variety of these isomers. Table III gives a few typical examples including steroid epimers and alkaloids. Coupling the differential affinity of cyclodextrins for many different isomers with planar chromatography allows one to evaluate many different solvent systems and isomeric mixtures simultaneously and inexpensively.

CONCLUSIONS

Given the paucity of readily available planar chromatographic techniques for resolving enantiomers, the "cyclodextrin mobile phase approach" is particularly facile and attractive. It also seems that this method can be more than a simple alternative for LC on cyclodextrin bonded phases. The fact that racemates can be resolved via the mobile phase additive approach which cannot be resolved on the analogous CSP and *vice versa* raises a number of mechanistic questions involving chiral recognition. The TLC resolution of enantiomers occurs only under a fairly narrow range of mobile phase conditions. As such, some knowledge as to the effect of organic modifier and cyclodextrin concentration is essential for the successful utilization of this method.

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CARBOHYDRATE–BORATE ELUENTS FOR ANION CHROMATOGRAPHY

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SUMMARY

The chromatographic efficiencies of four different carbohydrate–borate eluents at pH values between 8.0 and 9.5 were compared. The carbohydrates studied were gluconate, mannonic acid, glucose and mannitol. The mannonic acid–borate eluent was as efficient as the original gluconate–borate eluent but the glucose–borate and mannitol–borate eluents gave poor results. For each eluent several carbohydrate borate complexes were responsible for the elution of anions.

INTRODUCTION

Single column, or non-suppressed, ion chromatography is now a well established technique for anion analysis[1]. The use of a dilute low conductance eluent, together with a low-capacity anion-exchange column, makes it possible to detect anions conductometrically without the need to suppress eluent conductivity. Eluents that have been used in this technique include phthalic acid and other aromatic acids[1–4]. Single column chromatography, however, does not provide the sensitivity that can be achieved with the two-column suppressed system[5].

In 1983 a new eluent for single column anion chromatography was introduced that was very different from those previously used[6]. It consisted of a gluconate–borate mixture that, when used with a Toyo Soda polymethacrylate column, offered increased sensitivity with excellent resolution. The chemistry and elution mechanism of this useful eluent have not been fully explained but Schmuckler *et al.*[7] concluded that since both gluconate and borate when used separately as eluents gave very poor results, the efficiency of the eluent depended on the formation of a gluconate–borate complex.

The present work was undertaken for two purposes: (1) to atempt to elucidate further the nature of the gluconate–borate complex and determine the driving force, or developing ion, of the eluent and (2) to determine if carbohydrates other than gluconate, when mixed with borate, would form efficient eluents. The gluconate that was used in the original eluent was replaced in turn by three other polyhydroxy compounds (POHC) —mannonic acid, mannitol and glucose— and each new eluent was tested for chromatographic efficiency at several pH values and several different

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borate–carbohydrate concentrations. ^{13}C NMR spectra of the carbohydrates were taken before and after the addition of boric acid and borate.

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Waters M-45 pump used at a constant flow-rate of 1.2 ml/min, a Rheodyne 7010 injector with a 100-μl sample loop, a Waters IC-PAK anion column (50 × 4.6 mm I.D., 10 μm particle diameter, 0.03 mequiv./ml capacity) and a Wescan 213 (10 mV output) conductivity detector coupled to a Hewlett-Packard 3390A integrator.

Reagents

All reagents used were analytical grade and solutions were prepared in Milli-Q water. Borate buffer eluents were prepared with (a) D-gluconic acid, potassium salt, (b) α-D-glucose, (c) D-mannitol, and (d) L-mannonic acid (γ-lactone). The composition of the initial eluents was as follows: carbohydrate 1.48 m*M*, boric acid 5.82 m*M*, sodium tetraborate decahydrate 1.30 m*M*, acetonitrile 12% (v/v), glycerol 0.25% (v/v). The pH was adjusted with 0.1 *M* potassium hydroxide or 0.1 *M* hydrochloric acid. For the more dilute eluents, carbohydrate, boric acid and borate concentrations were made (a) 2/3 and (b) 1/2 of their initial concentrations, concentrations of acetonitrile and glycerol were not changed. Standard anion solutions were prepared from the sodium salts.

NMR measurements

^{13}C NMR spectra were obtained in $^{2}H_2O$ with 1,4-dioxane as internal reference, using a Bruker WP-80 FT spectrometer (20.1 MHz, broad band decoupled).

RESULTS AND DISCUSSION

Typical chromatograms obtained using the four different carbohydrate–borate eluents, at pH 8.5, for the separation of a group of anions are shown in Fig. 1. Based on previous results with gluconate–borate eluent[7], and ^{13}C NMR studies that are described later, it is concluded that in each case a carbohydrate–borate complex was responsible for the elution of the anions. With mannonic acid–borate eluent, retention times were slightly shorter than with the original gluconate–borate eluent and resolution was equally good. When mannitol–borate or glucose–borate eluents were used, however, retention times increased considerably, particularly for the glucose–borate eluent. These findings suggest that a carboxyl group in the carbohydrate increases the eluting power of the carbohydrate–borate eluent.

Boric acid and borate complexation with POHC has been studied for many years using a variety of techniques including conductivity, pH, optical rotation, and refractive index measurements, ionophoresis, potentiometric titrations and, more recently, ^{1}H, ^{13}C and ^{11}B NMR[8–23]. Equilibria between boric acid (B^0), borate (B^-), and diol functions (L), are summarized in Fig. 2 (eqns. 1–4). In addition to complexes of type B^0L, B^-L and B^-L_2, many POHCs, including gluconate, mannonate, glucose and mannitol, can, depending on conditions, also form complexes of type $(B^-)_2L$ (Fig.

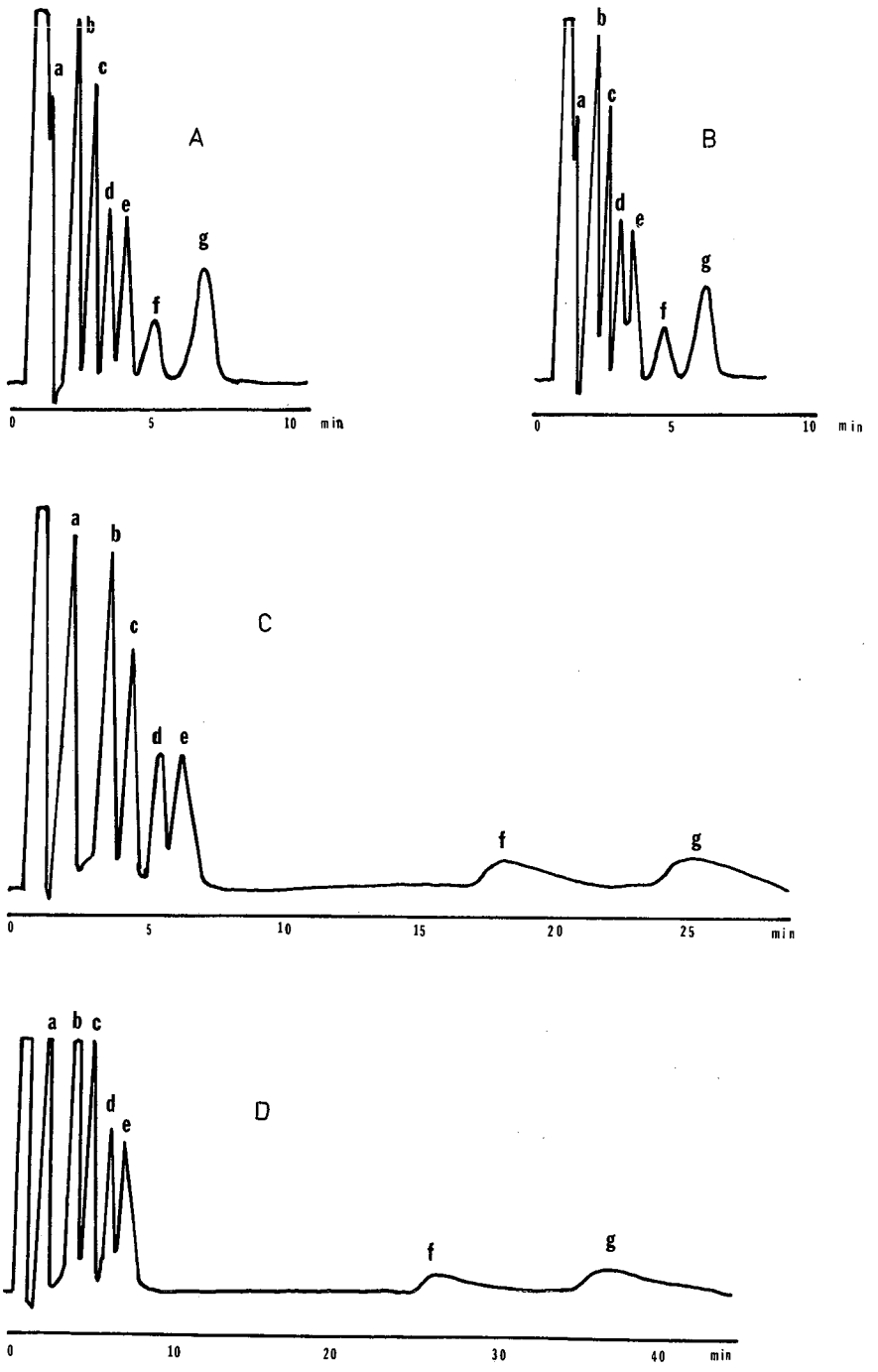

Fig. 1. Separation of seven anions with (A) gluconate–borate, (B) mannonic acid–borate, (C) glucose–borate and (D) mannitol–borate eluents. Flow-rate, 1.2 ml/min; sample concentration, 50 ppm of each anion. Peaks: a = F^-, b = Cl^-, c = NO_2^-, d = Br^-, e = NO_3^-, f = HPO_4^{2-}, g = SO_4^{2-}.

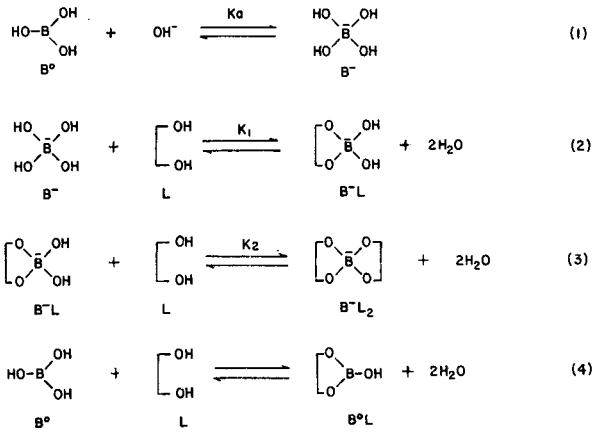

Fig. 2. Equilibria between boric acid (B^0), borate (B^-) and a diol function (L).

3, eqns. 5 and 6)[20–23]. In strongly alkaline conditions very little complex of typ B^0L would be present (Fig. 2, eqn. 4). Tridendate complexes also exist but require polyols with very specific configurations[21,24]. 6-Polyhydroxy compounds because of their many diol functions can, theoretically, form a large number of different borate esters. Polyhydroxycarboxylic acids can form both diol and α-hydroxycarboxylic acid esters with borate[20]. In aqueous solution at pH $>pK_a$ polyhydroxycarboxylic acid but $<pK_a$ boric acid ($pK_a = 9.07$), B^-L_A type esters are formed according to eqn. 7 in Fig. 4. At pH above 9 more B^- is formed and the equilibrium can be written as shown in eqn. 8. With increasing pH the B^-L_A esters dissociate and formation of diol esters, B^-L^-, is favored (eqn. 9).

Experiments in which the eluent pH was changed from 8.0 to 9.5 showed that, for each of the four carbohydrate–borate eluents studied, anion retention times were pH dependent and decreased as pH increased (Table I). It has been shown, using model dihydroxy compounds, that the amount of borate esterified increases with increasing pH[20,22]: the equilibria in eqns. 1, 2 and 3 (Fig. 2) lie increasingly to the right thus increasing the concentration of charged species in the eluent. This would be expected to increase the efficiency of the eluent in displacing anions from their sites on the ion exchange resin, as was found experimentally. In the carbohydrate–borate eluents that were used total boron concentration always exceeded carbohydrate concentration (molar ratio B/carbohydrate = 7.4) and, therefore, monoesters (B^-L) rather than the diesters (B^-L_2) would be the main species formed.

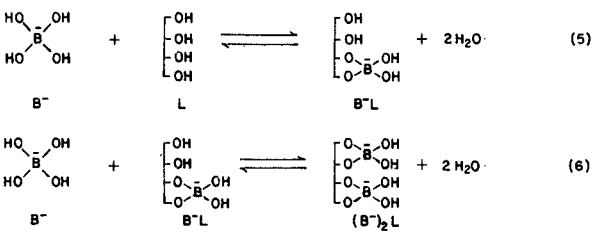

Fig. 3. Equilibria between borate and a polyol function: borate in excess.

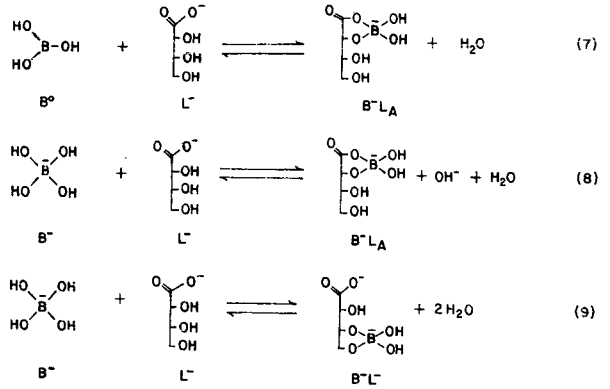

Fig. 4. Borate ester formation by polyhydroxy carboxylates.

It is of interest to note that at pH 8.0 anion retention times obtained with the mannonic acid–borate eluent were considerably longer than those obtained with the gluconate–borate eluent, whereas at higher pH the mannonic acid–borate retention times were nearly all slightly shorter than the corresponding gluconate–borate retention times (Table I). This may be due to the very complex equilibria that exist in the eluents, particularly at pH below 9, and the fact that with increasing pH the relative concentrations of the various charged species in solution will not necessarily change at

TABLE I

ADJUSTED RETENTION TIMES (min) OF ANIONS SEPARATED AT DIFFERENT pH VALUES BY THE FOUR CARBOHYDRATE–BORATE ELUENTS

Anion concentrations: 50 ppm.

Anion injected	*Carbohydrate in eluent*	*pH*			
		8.0	*8.5*	*9.0*	*9.5*
Cl^-	Gluconate	2.15	1.45	0.94	0.75
	Mannonic acid	2.60	1.24	0.89	0.70
	Mannitol	–	2.60	1.45	1.18
	Glucose	–	2.95	1.74	1.07
NO_3^-	Gluconate	3.92	3.20	1.98	1.61
	Mannonic acid	5.93	2.73	1.94	1.53
	Mannitol	–	5.19	2.91	2.37
	Glucose	–	5.75	3.36	2.20
HPO_4^{2-}	Gluconate	5.66	4.22	2.16	1.65
	Mannonic acid	11.70	3.87	2.33	1.61
	Mannitol	–	17.01	5.78	4.25
	Glucose	–	22.91	8.12	3.65
SO_4^{2-}	Gluconate	8.51	5.98	3.03	2.19
	Mannonic acid	18.09	5.31	3.11	2.10
	Mannitol	–	24.06	7.85	5.66
	Glucose	–	32.04	10.73	4.69

the same rate for both gluconate and mannonate. As will be discussed later, the association constants, k_1 and k_2 (Fig. 2), for gluconate and mannonate differ considerably[21].

It can also be seen in Table I that at all pH values tested, gluconate–borate and mannonic acid–borate were stronger eluents than glucose–borate and mannitol–borate eluents and that this difference in elution strength became less marked as pH was increased. This again suggests that the carboxylate group is a factor in determining eluent strength.

The association constants, k_1 and k_2 (Fig. 2), have been calculated for a wide variety of diols and POHC using refractive index/optical rotation data[10] and, more recently, ^{11}B NMR measurements[17,20–23]. ^{11}B and ^{13}C NMR[14–23] studies of boric acid–borate complexation with a variety of POHC provided direct evidence for the formation of both B^-L and B^-L_2 type complexes. Association constants have been found to vary greatly with the nature of the POHC[17,20–22]. Steric hindrance, stabilization as a result of substitution in the parent POHC, relative positions of the hydroxyl groups in the molecule, and possibilities for hydrogen bonding between the POHC and borate, were all found to be determining factors. Van Duin *et al.*[21] determined that the stability of the borate–carbohydrate complexes increased as the number of hydroxyl groups in the molecule increased, and that *threo* complexes were more stable than the corresponding *erythro* complexes. They observed that the introduction of a negatively charged carboxylate group into the carbohydrate generally decreased the stability of the complex as a result of electronic repulsion between the carboxylate group and borate (B^-).

^{11}B NMR studies by Van Duin *et al*[21] showed that for both gluconate and mannonate in strongly alkaline solution, the main borate complex formed was a B^-L^- *threo*-complex. Association constants, measured at pH 11, were 240 and 1200 l/mol, respectively. Smaller amounts of *erythro* (association constants 72 and 140 l/mol, respectively) and α,γ-complexes (association constants 19 and 54 l/mol, respectively) were also formed. Only a 3,4-*threo* complex can be formed by mannonate but for gluconate a 2,3-*threo* complex is also possible. ^{13}C NMR[23] showed that the 2,3-*threo* complex for gluconate made up only 10% of the total *threo* signal, probably because in this position repulsion between the borate and carboxylate ions would be stronger and this would decrease the stability of the complex. It is evident from the values of the association constants that the mannonate–borate complexes were more stable than the corresponding gluconate–borate complexes. ^{11}B NMR is unable to distinguish between borate esters of type B^-L, $(B^-)_2L$ and $(B^-)_3L$ (where L may be charged or uncharged), all of which can be formed, but with ^{13}C NMR it was possible to show that, at pH 11, $(B^-)_2L^-$ complexes as well as B^-L^- complexes, were formed by both gluconate and mannonate[23]. Association constants were also determined[21] for the diesters, $B^-(L^-)_2$, and were reported to be 31 and 48 l/mol, respectively.

As already reported[7], ^{13}C NMR confirmed that, on adding boric acid and borate to potassium gluconate in the same proportions as in the initial eluent, a gluconate–borate complex was formed. A similar result was obtained for mannonate. In both cases, the main change in the spectrum on adding boric acid and borate was a broadening and overlapping of the C-2, C-3, C-4 and C-5 signals so that one broad resonance (approximately 10 ppm wide) with two unresolved peaks was observed. Based on the work of Van Duin *et al.*[21,23] it can be concluded that the two

unresolved peaks represent the 3,4-*threo* complex. In both the gluconate–borate and mannonate–borate spectra slight broadening of the C-1 signal was observed, suggesting the formation of an α-hydroxycarboxylic acid ester with formula B^-L_A. The absence of a peak at approximately 80 ppm, and the similarity of the mannonic acid–borate spectrum to the gluconate–borate spectrum, indicated that in its borate complexes mannonic acid was in the straight chain form[25].

Makkee *et al.*[22] used ^{11}B and ^{13}C NMR spectroscopy to investigate glucose–borate complexes in aqueous solution at pH 6–12. They found that when excess borate was present several borate monoesters were formed. When the B/L ratio was 10, the main complex formed at pH 12 was of type $(B^-)_2L$ and at this pH complexation reached a maximum. They concluded that an equilibrium was formed between α-D-glucofuranose 1,2:3,5-diborate (5- and 6-membered rings) and α-D-glucofuranose 1,2:5,6-diborate (two 5-membered rings). D-Glucose on forming borate complexes was transformed from the pyranose to the furanose form.

The ^{13}C NMR glucose–borate spectrum that we obtained was complex and difficult to interpret but clearly showed that complexation had occurred. Marked downfield shifts in the C-1 and C-6 resonances and the appearance of peaks between 80 and 90 ppm indicated that at least one furanoid complex was formed[25]. However, as a result of line broadening it was not possible to make definite assignments.

^{11}B and ^{13}C NMR spectra of mannitol–borate complexes obtained by Makkee *et al.*[22] showed that, as was the case for glucose when excess borate was present, in addition to B^-L complexes, $(B^-)_2L$ and possibly $(B^-)_3L$ complexes, were also formed, complexation increasing as pH was raised. The preferred position for complex formation was at the 3- and 4-hydroxyl groups. Mannitol very readily forms a B^-L_2 complex but, as already mentioned, at the B/L ratios used in our study this complex would not be expected to be the main product. However, the ^{13}C NMR spectra that we obtained on adding boric acid and borate to mannitol showed a considerable broadening of both the C-2/C-5 and C-3/C-4 signals with a downfield shift of 2 ppm for both signals which suggests formation of a B^-L_2 complex. Makkee *et al.*[22] reported that association constants for borate complexes with mannitol were approximately two orders of magnitude higher than those for glucose.

Our results and the work of Van Duin *et al.*[20,21,23] and Makkee *et al.*[22] suggest that, under the conditions that we used, the main borate complex formed by gluconate and mannonic acid was a diol ester of type B^-L^-. Smaller concentrations of a $(B^-)_2L$ complex and the α-hydroxycarboxylic acid ester, B^-L_A, also appear to be formed. For glucose and mannitol the complexes formed would be of types B^-L and $(B^-)_2L$, with possibly some formation of B^-L_2 in the case of mannitol. The superior performance of the gluconate–borate and mannonic acid–borate eluents, as compared to the glucose–borate and mannitol–borate eluents, would at first appear to be due to the charge contributed by the carboxylate group. However, as shown by the experiment described in the following section, this cannot be the complete explanation.

For many anions there is a linear relationship between the logarithm of their retention times and the logarithm of the eluent concentration[26,27]. If a monovalent eluent is used, the slope of the line will be equal to one for a monovalent anion and two for a divalent anion. Similarly, if a divalent eluent is used, the slopes will be 0.5 and one, for monovalent and divalent anions, respectively. Therefore, theoretically, if anions of known charge are separated at different eluent concentrations, it should be possible to deduce the charge on the eluent.

TABLE II

ELUENT CHARGE VALUES CALCULATED FROM THE SLOPES OF THE PLOTS OF LOG RETENTION TIME *VERSUS* LOG ELUENT CONCENTRATION[26,27]

Three different eluent concentrations were used to determine the slopes. Correlation coefficients were 0.99 or greater except where noted.

	Cl^-	NO_2^-	Br^-	NO_3^-	HPO_4^{2-}	SO_4^{2-}	*Average*
Gluconate–borate	1.30	1.37	1.43	1.51	1.39	1.36	1.39
Mannonic acid–borate	1.08**	1.02*	1.05*	1.13**	1.16	1.18*	1.10
Glucose–borate	1.25	1.25	1.25	1.20	1.14	1.09	1.20
Mannitol–borate	1.32	1.37	1.37	1.32	1.29	1.29	1.33

* Correlation coefficient, 0.98.
** Correlation coefficient, 0.97.

The relationship between eluent concentration and retention times for six anions was determined for the four carbohydrate–borate eluents at three different concentrations: the initial concentration and two lower concentrations. In each case, as the carbohydrate concentration was changed the ratio of carbohydrate concentration to buffer (boric acid and borate) concentration was kept constant. From the slopes of the plots the charge on the eluent was calculated (Table II) and found to range from 1.02 to 1.51, with the mannonic acid–borate data giving charge values that were closest to one. Charge values that deviated furthest from one were obtained from the gluconate–borate data. The difference between the eluent charge as calculated from the gluconate–borate data and from the mannonic acid–borate data is surprising since the elution properties of these two eluents have been shown to be very similar. Assuming that the relationship between eluent concentration and anion retention times holds true for the complex eluents being studied, the difference between the charge found for the gluconate–borate eluent and the charge found for the mannonic acid–borate eluent must be due to a difference in the ratio of mono- to divalent complexes in the two eluents. For the gluconate–borate eluent, presumably doubly charged B^-L^- is the main driving ion with sufficient contribution from singly charged B^-L_A to give an eluent charge of approximately 1.4. Erkelens *et al.*[28] have indicated that for this eluent triply charged $B^-(L^-)_2$ acts as a driving ion but, as already mentioned, formation of this complex appears unlikely when borate is present in excess. The results obtained for the mannonic acid–borate eluent suggests that in this case the B^-L_A complex is the main driving ion. The comparable efficiency of the gluconate–borate and mannonic acid–borate eluents may depend on the fact that mannonic acid complexes more readily with boric acid and borate than does gluconate, and the higher concentration of the singly charged mannonic acid–borate complex in the eluent may outweigh the advantage of the greater charge on the gluconate–borate complex.

For glucose and mannitol it can be concluded that the B^-L complex is the main driving ion, with some contribution from the $(B^-)_2L$ complex to give the observed values for the eluent charge. With mannitol B^-L_2 may also be a factor. The low efficiency of the glucose–borate and mannitol–borate eluents may be due, in part, to structural considerations. The ring structure of glucose in its borate complexes may prevent the glucose–borate eluent from being as efficient as those eluents in which the

carbohydrate is in the straight chain form. Large spcies such as $(B^-)_2L$ and B^-L_2 would further decrease eluent efficiency.

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CHROM. 20 645

COMPARATIVE STUDY OF ALKYL AND FLUOROALKYL N-(2-HYDROXY-ETHOXYETHYL)-AMIDES AS REVERSED-PHASE LIQUID CHROMATOGRAPHIC MODIFIERS

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SUMMARY

The use of the non-ionic surfactant, N-(2-hydroxy-ethoxyethyl)-2,2,3,3,4,4,4-heptafluorobutanamide, as a liquid chromatographic mobile phase modifier has been studied. Comparisons between this fluorinated compound and two similar hydrocarbon surfactants, N-(2-hydroxyethoxyethyl)-hexanamide and N-(2-hydroxyethoxyethyl)-heptanamide, have been made. Although surface tension data were similar for all three surfactants, the fluoroalkyl compound was found to have a larger influence on retention. Likewise, the positional isomers of cresol and toluidine were resolvable using the fluorinated surfactant and were not with the equivalent alkyl surfactant with a similar hydrophilic–lipophilic balance.

INTRODUCTION

Although ionic surfactants have found wide-spread acceptance as mobile phase modifiers in reversed-phase liquid chromatography (RPLC), non-ionic surfactants have not been utilized to the same extent[1–3]. Typically, when non-ionic compounds have been used, they have been either alkylpolyoxyethylene ethers, esters, or amides. In general, except for a few fluorinated alcohols[4,5], fluorine-containing compounds have not been employed as modifiers in RPLC.

The potential of fluoroalkyl surfactants to effect solute retention may be inferred from the chromatographic properties of fluoroalkyl modified silica[6–8]. Under equivalent reversed-phase conditions these surfaces have been found to retain solutes to a lesser degree than the corresponding alkyl modified materials. This coupled with the unique physical properties of fluorine-containing compounds make fluorosurfactants potentially interesting as reversed-phase modifiers.

A major source of interest in non-ionic fluorinated surfactants arise from their potential use in the preparation of blood substitutes[9–11]. In conjunction with this bioengineering work, various physical properties of these compounds such as their critical micelle concentration (CMC), phase inversion temperature, and wetting characteristics have been compared to corresponding alkyl surfactants[10,11]. For a given head group the surface active properties of fluoroalkyl surfactants are

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equivalent to those of structurally similar alkyl surfactant having 1.5 times more carbon in hydrophobic end of the molecule.

In the current study, the properties of N-(2-hydroxy-ethoxyethyl)-2,2,3,3,4,4,4-heptafluorobutanamide (I) as a mobile phase modifier have been investigated. The ability of I to affect chromatographic selectivity has been compared to that of two similar hydrocarbon surfactants, N-(2-hydroxyethoxyethyl)-hexanamide (II), and N-(2-hydroxyethoxyethyl)-heptanamide (III), which has a hydrophilic–lipophilic balance similar to that of I. Significant differences in chromatographic selectivity in the presence of the two classes of surfactants have been observed.

EXPERIMENTAL

Apparatus

Chromatographic experiments were carried out with an IBM Instruments (Danbury, CT, U.S.A.) Model LC/9533 ternary gradient liquid chromatograph equipped with UV and refractive index detectors. Retention data were recorded and processed on an IBM Instrument Model 9000 data system. The octadecyl column was (15 cm × 4.6 mm I.D.) also from IBM Instruments. Surface tension measurements were made using a DuNouy interfacial tensiometer (Central Scientific, Chicago, IL, U.S.A.). All experiments were performed at ambient temperature.

Reagents and procedures

The mobile phases were prepared from high-performance liquid chromatographic (HPLC)-grade methanol (Fisher Scientific, Pittsburgh, PA, U.S.A.) and deionized water which was purified using a Milli-Q reagent water system (Bedford, MA, U.S.A.). Hexanoic acid, heptanoic acid, and methyl heptafluorobutyrate were obtained from Matheson (Houston, TX, U.S.A.), Eastman Kodak (Rochester, NY, U.S.A.), and Aldrich (Milwaukee, WI, U.S.A.), respectively.

Hexanoic acid and heptanoic acid were converted to their methyl esters by treatment with methanol in the presence of concentrated sulfuric acid. Subsequently, these esters as well as methyl heptafluorobutyrate were reacted with excess 2-(2-amino-ethoxy)ethanol in the presence of zinc oxide to yield the corresponding surfactants[12]. The final products were vacuum distilled and characterized by nuclear magnetic resonance and infrared spectrometry.

Prior to use the chromatographic column was rinsed with about 100 ml each of water, methanol, and water and then it was conditioned with a minimum of 100 ml of the mobile phase. This same procedure was used with each new mobile phase. The column void volume was determined using ^2H_2O. All retention and surface tension measurements were made at least twice and in most cases in triplicate.

RESULTS AND DISCUSSION

Shown in Fig. 1 are plots of surface tension *vs.* ln concentration for the three surfactants studied. The linear dependencies of these plots are consistent with trends reported for other non-ionic surfactants[13,14] and indicate that the concentration range studied was below the CMC for I, II and III. The slopes of the plots in Fig. 1 are related to the surface excess concentration of the surfactants according to the Gibbs

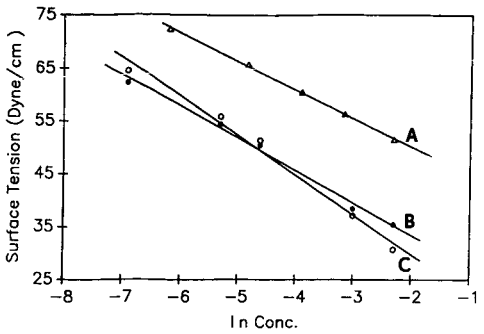

Fig. 1. Surface tension *vs.* ln surfactant concentration. Surfactants: (A) II, (B) III and (C) I.

equation[14]. Similar values were obtained for the two hydrocarbon surfactants and only a slightly larger slope was observed for I. Additionally, the data for I and III (Fig. 1, curves B and C) are nearly superimposable. The above results indicate that there is a relatively small difference between the three surfactants based on surface tension measurements and that I and III have a similar hydrophilic–lipophilic balance.

The ln–ln plots of the capacity factors for *o*-nitroaniline, phenol, and resorcinol *vs.* surfactant concentration are shown in Fig. 2. Linearity in such plots (*i.e.*, ln *k'* *vs.* ln additive concentration) also have been reported for other reversed-phase systems[15,16]. For a given modifier, simple hydrophobic theory predicts straight line plots of similar slope but different intercepts for solutes which have nearly the same properties[15].

The slopes of the linear fits to the data in Fig. 2 are summarized in Table I. Similar values were observed for both alkyl modifiers for a given solute. However, an approximately three to five-fold increase was noted with the fluoroalkyl modifier. The above trends demonstrate that I has a more pronounced effect on retention than the corresponding hydrocarbon analogue, III. The reductions in solute retention are likely due to unfavorable interactions between solute and surfactant sorbed into the bonded layer with all or part of its fluoroalkyl end exposed. The proposed sorption model is consistent with published chromatographic data obtained on bonded fluoroalkyl phases[6–8]. In this latter instance, reduced retentions have also been explained in terms of unfavorable interactions between the immobilized fluorocarbon chains and the solutes.

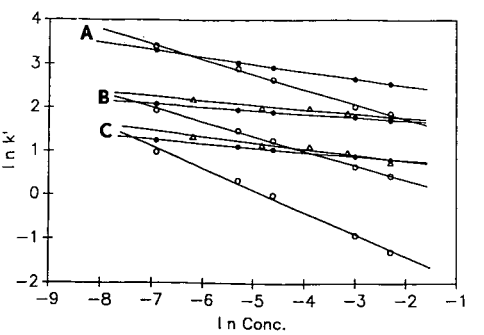

Fig. 2. ln *k'* *vs.* ln surfactant concentration. Solutes: (A) *o*-nitroaniline, (B) phenol, (C) resorcinol. Surfactants: (○) I, (△) II, and (●) III.

TABLE I

SLOPES OF ln k' *vs.* ln SURFACTANT CONCENTRATION

Solutes	*Surfactant*		
	I	*II*	*III*
Resorcinol	0.54	0.14	0.10
Phenol	0.50	0.10	0.08
o-Nitroaniline	0.41	–	0.17

In order to further evaluate the properties of I, relative changes in k' were measured as a function of increasing carbons for a homologous series of ethyl alkanoate esters (*i.e.*, acetate, propionate, butyrate and valerate). Measurements were made as a function of concentration for the aqueous mobile phase prepared from either I or III. The methylene selectivity for a given mobile phase was determined from a plot of ln k' *vs.* carbon number (Fig. 3). The data from this set of experiments are summarized in Table II. Likewise, for comparative purposes, methylene selectivity was also determined for binary combinations of methanol and water which did not contain surfactant (Fig. 3 and Table II). The methylene selectivities obtained in methanol and water are comparable to previously reported values obtained under similar condi-

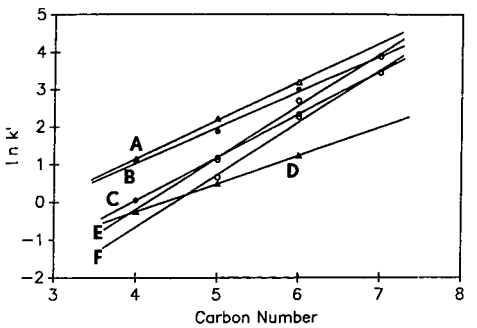

Fig. 3. ln k' *vs.* carbon number for ethyl alkanoate esters. Mobile phase: (A) methanol–water (20:80), (B) 0.02 *M* III in water, (C) 0.10 *M* III in water , (D) methanol–water (50:50), (E) 0.02 *M* I in water, (F) 0.08 *M* I in water.

TABLE II

SLOPES OF ln k' *vs.* CARBON NUMBER ETHYL ALKANOATE ESTERS

Concentration	*Slope*		
	I	*III*	*Methanol–water*
0.02	1.39	0.95	1.08 (20% Methanol)
0.05	1.40	1.07	1.00 (30% Methanol)
0.08	1.38	–	0.85 (40% Methanol)
0.10	–	1.15	0.75 (50% Methanol)

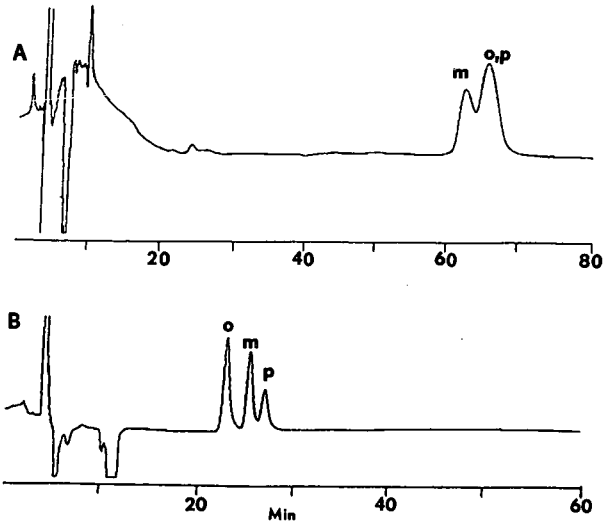

Fig. 4. Chromatograms of *o*-, *m*- and *p*-cresol. Mobile phase: (A) 0.10 *M* III and (B) 0.05 *M* I.

tions[17–19]. Of particular significance in the current study is the 30–50% higher methylene selectivity with the fluoroalkyl surfactant compared to either the corresponding hydrocarbon surfactant, III, or binary combinations of methanol and water.

Fig. 4 and 5 show differences in the separation of positional isomers of cresol and toluidine using I and III as mobile phase additives. Resolution was significantly enhanced for both sets of isomers with the fluoroalkyl surfactant added to the mobile phase. The overall retention times of the total chromatogram were equal or less than that obtained with the alkyl surfactant using only half the concentration of I compared to III. Also, a significant improvement in peak shape was observed for the toluidine isomers when the fluorinated surfactant was used. This is also further evidence for the

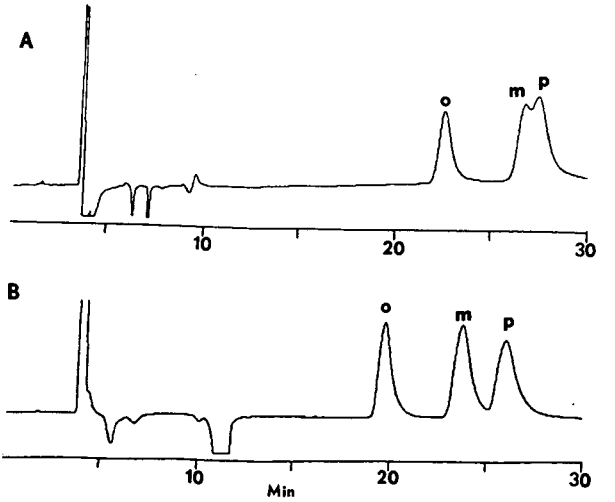

Fig. 5. Chromatograms of *o*, *m* and *p*-toluidine. Mobile phase: (A) 0.10 *M* III and (B) 0.05 *M* I.

presence of sorbed surfactant which blocks residual silanols. This behavior is similar to that obtained with nitrogen containing organic modifiers used to reduce peak tailing in reversed-phase chromatography.

The current results demonstrate the potential usefulness of non-ionic fluorinated surfactants in liquid chromatography. Similar studies, with other fluorinated surfactants are now in progress.

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SEPARATION OF TUBULIN SUBUNITS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

When properly solubilized with trifluoroacetic acid (TFA), α- and β-tubulin subunits from a variety of sources may be resolved at high yield by reversed-phase high-performance liquid chromatography (HPLC), using a Waters μBondapak C_{18} column and simple linear aqueous acetonitrile gradients containing TFA. The tubulin subunits are typically the most non-polar proteins present, with the β-tubulin subunit eluting before the α. Column temperatures above ambient improve both the resolution and the yield; less polar solvent systems do not. Tubulins not freely soluble in aqueous TFA may be solubilized in 6 *M* guanidine–hydrochloric acid with no change in retention time. Other columns with shorter carbon chain lengths and larger pore size produce a single, unresolved tubulin peak. Reversed-phase HPLC analysis provides an independent comparative evaluation of organelle-specific tubulins, with characteristic retention time differences observed between homologous ciliary and flagellar outer doublet tubulin subunits and also between them and their cytoplasmic counterparts.

INTRODUCTION

Tubulin, the major structural protein that forms microtubules, is a heterodimer of two distinct but related subunits[1–3]. Named originally with reference to their relative electrophoretic migration on sodium dodecylsulfate (SDS)-polyacrylamide gels, α- and β-tubulin have essentially the same molecular weight but bind SDS differentially. The relative migration rates are a function of the alkyl chain composition of commercial SDS and also depend on whether urea is present during electrophoresis[4,5]. Some striking examples of tubulin variants detectable by SDS-polyacrylamide gel electrophoresis (PAGE) have been reported, for example a testis-specific β-tubulin has been detected in *Drosophila*[6] while a chick erythrocyte β-tubulin is easily separable from its brain tubulin counterpart[7]. In both cases, these represent distinct gene products. Numerous isoelectric variants also have been reported, for example seventeen distinct brain tubulins can be resolved[8] and the specific acetylation of cytoplasmic α-tubulin, leading to a distinct isotype, occurs before incorporation of the tubulin into *Chlamydomonas* flagella[9]. Differences in hydrophobicity between the two tubulin subunits serve as a basis for their separation in Triton–acid–urea gels while

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charge differences allow further isotype separation[10]. On the other hand, homologous tubulin subunits isolated from sea urchin sperm flagella, embryonic cilia, and mitotic cytoplasm are not distinguishable by SDS-PAGE, isoelectric focussing, or Triton–acid–urea gels. However, two-dimensional peptide mapping of these same tubulin subunits, obtained by morphologic fractionation and electrophoretic purification, shows major organelle-specific differences, not easily interpretable in terms of single post-translational modifications[11]. Supporting this observation of organelle-specific tubulins in sea urchin is the fact that nearly a dozen genes for each tubulin subunit have been documented[12].

The presence of local regions of difference among various organellar tubulin subunits, mainly in the form of highly conserved hydrophobic substitutions[11], led to the present study in which reversed-phase high-performance liquid chromatography (RP-HPLC) was explored as a method for resolving α- and β-tubulin quickly and in high yield, with the additional hope of detecting organelle-specific chain types. The general methodology was suggested by the successful resolution of a hemoglobin variant containing the silent, neutral substitution of alanine for valine[13]. A brief report of this approach applied to sea urchin tubulins has appeared in abstract form[14].

EXPERIMENTAL

Protein fractions

Sea urchin sperm flagella and the outer doublet microtubule and central pair fractions thereof, embryonic cilia, and egg cytoplasmic tubulin–vinblastine crystals were prepared as described previously[11,15], using *Strongylocentrotus droebachiensis* (Maine) and *Tripneustes gratilla* (Hawaii). Scallop sperm flagella, the outer doublet and central pair fractions thereof, and gill cilia were prepared by the methods of Linck[16] and Stephens[17], using *Aequipecten irradians* (Cape Cod) and *Placopecten magellanicus* (Maine). The flagellar and ciliary axonemes, fractions thereof, or tubulin-vinblastine crystals were either used directly or stored at −20°C in glycerol–water (50:50) containing 1.5 m*M* magnesium chloride, and 15 m*M* Tris–HCl (pH 8.0).

Sea urchin cytoplasmic tubulin was also prepared from unfertilized eggs of *S. droebachiensis* by two cycles of *in vitro* polymerization, using the controlled pH and 0°–24°C temperature method of Suprenant and Marsh[18]. Elasmobranch tubulin was prepared by two cycles of *in vitro* polymerization from brains of the skate *Raja erinacea* using the method of Langford[19]. Recycled bovine brain tubulin was obtained from Dr. Mitchison. Egg and brain tubulins were frozen in liquid nitrogen as high speed pellets and stored at −80°C until use.

Before use, axonemes in glycerol were recovered by centrifugation after 1:10 dilution with 10 m*M* Tris–HCl (pH 8.0). In some cases, glycerol was dialysed away from glycerinated fractions using 1 m*M* Tris–HCl (pH 8.0), 0.1 m*M* EDTA, and 0.25% mercaptoethanol. Freshly isolated axoneme and outer doublet microtubule pellets or fresh or frozen cytoplasmic tubulin pellets were suspended in distilled water. In all cases, trifluoroacetic acid (TFA) was added to a final concentration of 0.2–0.3%. Some samples were solubilized by dialysis against or suspension in 6 *M* guanidine–HCl, 10 m*M* dithiothreitol (DTT), and 0.2% TFA. The samples were centrifuged at 45 000 *g* for 15 min before use. Additional details concerning solubilization are found in the Discussion section.

Reversed-phase HPLC

A Waters Assoc. high-performance liquid chromatography system consisted of two Model 510 pumps, U6K manual sample injector, Model 721 programmable system controller, Model 441 UV (215 nm) monitor, Model 730 data module, and a column heater. The major column used was a Waters μBondapak C_{18} (30 × 0.39 cm; 125 Å pore size; 10 μm particle size) equipped with a guard column of the same material. Also used were Beckman Ultrasphere Octyl (C_8, 25 × 0.46 cm, 80 Å pore size, 5 μm particle size) and Ultrapore RPSC (C_3, 7.5 × 0.46 cm, 300 Å pore size, 5 μm particle size) columns. The solvents routinely used were 0.1% aq. TFA–acetonitrile (70:30) (solvent A) and 0.1% aq. TFA–acetonitrile (40:60) (solvent B). Runs were generally done at 40°C. To dissociate aggregated material from the column, 1.0 ml of dimethylsulfoxide (DMSO) was injected onto the column[20], followed by an acetonitrile gradient. Columns were considered clean when no "ghost" bands were detected. Fractions were collected at 1- or 2-min intervals and then evaporated to dryness in a Savant Instruments "Speed-Vac" vacuum centrifuge concentrator equipped with both a water aspirator unit and a high vacuum cold trap.

SDS-PAGE

The discontinuous, SDS-containing system of Laemmli[21] was used with 1.5 mm thick × 10 cm long slab gels having a 5 to 15% acrylamide gradient. The SDS was obtained from Sigma and was chosen for its ability to resolve tubulin subunits maximally. Gels were equilibrium-stained with Coomassie Blue (Serva) by the method of Fairbanks *et al.*[22].

RESULTS

Systematic variations

Primary separation conditions. Sea urchin sperm flagellar tubulin, representing approximately two-thirds of the protein in the organelle, is freely soluble below pH 2.5[23]. When sea urchin axonemes are suspended in 0.2–0.3% aq. TFA, more than 85% of the total protein is rapidly solubilized; higher TFA generally decreases the degree of solubilization. Observations in the analytical ultracentrifuge indicate a major peak sedimenting at 2–3 S, characteristic of monomeric tubulin (data not shown).

With these facts in mind, the total protein from *S. droebachiensis* flagellar 9 + 2 axonemes, solubilized in 0.3% aq. TFA, was injected onto a C_{18} column and eluted with a linear gradient of 0 to 60% acetonitrile in 0.2% aq. TFA. Fig. 1 represents an initial survey to effect an RP-HPLC separation of the tubulin subunits. Minor proteins begin to elute when the solvent composition exceeds 30% acetonitrile. Near the end of the gradient (>45% acetonitrile), two major equimolar peaks elute at 45.2 and 46.9 min. Using raw figures from the integrator, these together account for 71% of the total, almost exactly as expected for tubulin. SDS-PAGE identifies these peaks as tubulin, the first being the β and the second the α subunit (see below). In the example shown, direct protein measurement demonstrates that 78% of the injected protein is recovered in the eluate.

Optimization and solvent system variations. By stepping the gradient up to 30% acetonitrile in 10 min, creating a more shallow linear gradient for protein elution, the tubulin subunits are further separated, eluting at 40.8 and 43.7 min. Using such

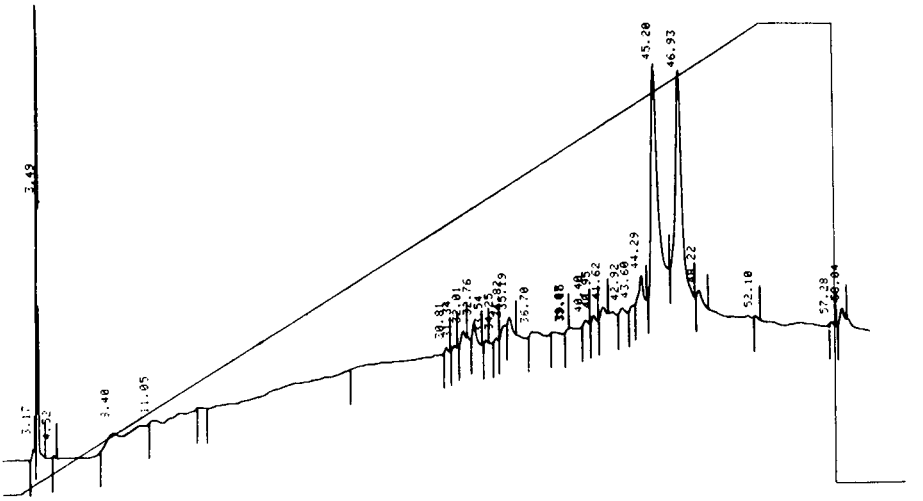

Fig. 1. Elution of sea urchin (*S. droebachiensis*) flagellar tubulin on a μBondapak C_{18} column using a linear 0 to 60% acetonitrile in 0.2% aq. TFA gradient in 50 min, constant 60% acetonitrile for 10 min, 1.0 ml/min, 40°C. Full-scale = 0.2 optical density. The two equal peaks eluting at 45.2 and 46.9 min are, respectively, β- and α-tubulin (25 μg total protein).

a gradient profile as a standard for comparison, the effects of ternary solvent composition on subunit separation and yield may be evaluated. For example, Power *et al.*[20] explored continuously-variable selective elution, based on acetonitrile–propanol ratios, to improve the resolution of subunits of yeast cytochrome *c* oxidase. Similar to the results of Power *et al.*[20], the presence of 2-propanol initially results in longer protein retention on the column, the tubulins eluting in 44.1 and 46.1 min with 25% 2-propanol. As the amount of 2-propanol is increased further, retention times become earlier than with pure acetonitrile (39.7 and 41.3 min at 50%; 32.0 and 33.3 min at 75%). Although the yield of tubulin approaches 100% with 75% 2-propanol, the resolution is decreased. With pure 2-propanol, the subunits are barely resolved at 27.3 and 27.9 min. Since possible ion-pair binding differences between the tubulin subunits might influence retention differentially, a 1:1 mixture of triethylamine (TEA) and TFA, at 0.1% each, was also tested (*cf.* ref. 20). This modification results in even less separation between the tubulin subunits, although tubulin yield is typically >90% over the entire acetonitrile–2-propanol range.

Various minor proteins shift systematically with respect to the tubulin subunits and hence their resolution can be improved with these variations. However, it is evident that the simple water–acetonitrile–TFA system can produce optimal separation of tubulin subunits. All further comparisons were made using it, with the trivial modifications that the TFA concentration was reduced to 0.1% to minimize solvent absorbance and the gradient was stepped rapidly from either 0 or 30% acetonitrile to various intermediate levels to generate final linear elution profiles of variable slope. As an example of these various principles, Fig. 2A illustrates a shallow, stepped linear gradient, designed both to separate the tubulins from one another and to maximize their separation from the minor proteins associated with the sperm flagellar axoneme.

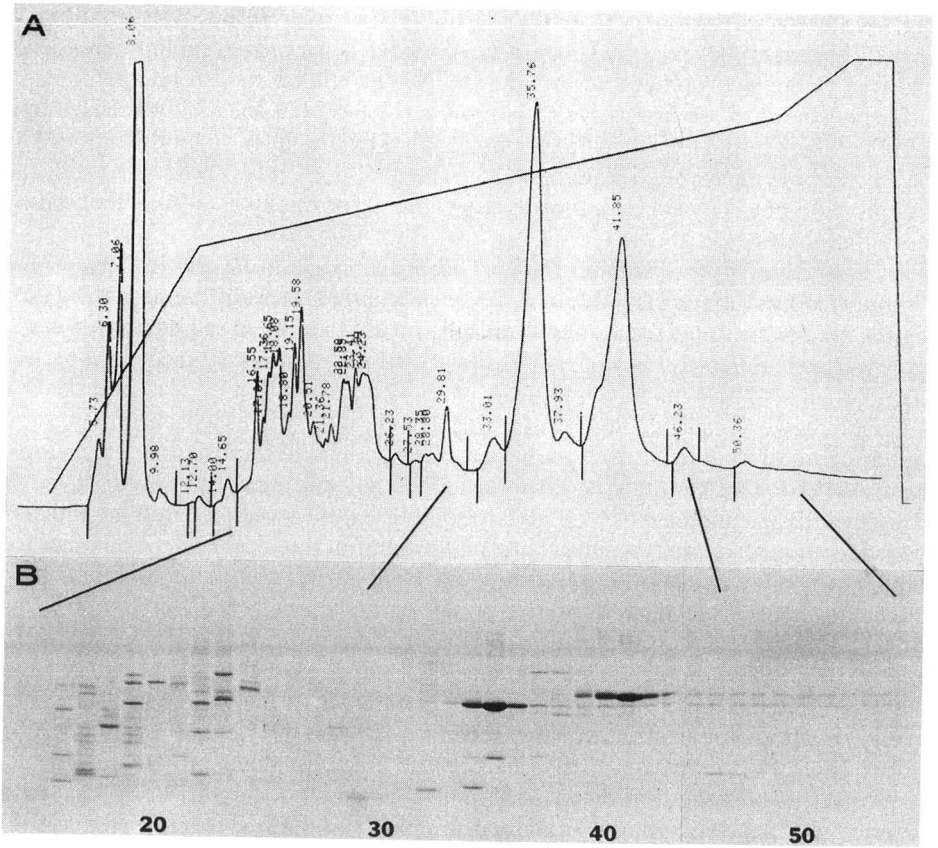

Fig. 2. High-resolution separation of sea urchin outer doublet tubulin. (A) HPLC profile: solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA; gradient: 0 to 55% B in 10 min, 55 to 85% B in 40 min, 85 to 100% B in 8 min, 100% B for 3 min, 1.0 ml/min. Full-scale = 0.5 O.D. (B) SDS-PAGE analysis: forty 1-min fractions from 15 to 55 min.

SDS-PAGE (Fig. 2B) indicates that the tubulin subunits, separated by over 6 min, each can be obtained in 1-ml fractions at >90% purity, >60% yield, all within 45 min. In fact, one can halve the running time, at the same flow-rate, with only a slight increase in tubulin trailing but a yield of >90%.

Effects of reduction and alkylation. Alkylation of the cysteines of sea urchin flagellar tubulin subunits (7 in β-, 9 in α-tubulin[11]) by reaction with iodoacetic acid generates the carboxymethyl derivative which interacts even more anomalously with SDS to produce striking subunit separations. To determine if carboxymethylation of tubulin would improve subunit resolution by HPLC, axonemal proteins were reduced and alkylated, dissolved in 0.2% aq. TFA, and analyzed in parallel with underivatized samples. After reduction and alkylation, both subunits elute earlier; the β subunit by 14.6% and the α by 6.8%. The consequence of this is that the time interval between the two peaks increases by nearly 50%. In parallel, the tubulins from scallop (*A. irradians*) cilia were similarly derivatized. In this case the β- and α-tubulin subunits (which

average eleven cysteines apiece[17]) elute 12.7 and 4.3% earlier, respectively, resulting in an 89% increase in peak-to-peak separation time. Each derivatized subunit is rendered relatively more hydrophilic but not by an amount related to its cysteine content. Offsetting the increase in relative resolution is the fact that the yield of derivatized tubulins in aqueous TFA is generally compromised by the chemical modification. The yield of carboxy-methylated sea urchin flagellar tubulins is relatively unaffected by the modification but the yield of scallop ciliary tubulins, normally $>80\%$, is reduced by more than half.

Solubilization in guanidine–HCl. The reduction and alkylation procedure typically renders tubulin insoluble in water since denaturing conditions need to be used for the reduction step. Total solubilization in 0.2% aq. TFA is not always easily accomplished. Similarly, although the underivatized tubulins studied here as test examples are soluble in aqueous TFA, not all native tubulins are (see below). A number of workers have found that highly associating proteins will chromatograph well after solubilization in 6 *M* guanidine–HCl, perhaps the most striking example being the RP-HPLC analysis of total ribosomal proteins by Ferris *et al.*[24]. Consequently, guanidine–HCl was used to solubilize total axonemal proteins and also the reduced and alkylated tubulins. Under all conditions tested, the retention times for tubulin subunits dissolved in 6 *M* guanidine–HCl, derivatized or unmodified, were indistinguishable from their respective counterparts solubilized directly with TFA. Most minor proteins were also unaffected both in terms of retention times and relative amount (Fig. 3). Solubilization in guanidine–HCl will increase the yield of reduced and alkylated tubulins when these are poorly soluble in aqueous TFA alone, but guanidine–HCl solubilization will also generally decrease the yield of the unmodified protein such that comparable final yields are obtained for both, probably reflecting simple irreversible denaturation on the column irrespective of derivatization. The yield of sea urchin flagellar tubulin solubilized in guanidine–HCl is generally reduced to $<50\%$ while that of scallop ciliary tubulin is relatively unaffected, *i.e.* the converse of the carboxymethylation results noted above.

Temperature effects. To explore the effects of column temperature on tubulin subunit resolution and yield, the relatively temperature-sensitive flagellar tubulin from the sea urchin *S. droebachiensis* was compared with the more stable ciliary tubulin from the bay scallop *A. irradians*. The results are given in Table I. Increased temperature results in shorter overall retention times but an increase in both resolution and yield up to 50°C in the case of sea urchin tubulin and 60°C in bay scallop, above which both resolution and yield deteriorate and trailing increases. These apparent temperature optima are each 25–30°C above the maximum temperature at which these organisms can survive and approximate the temperature above which the respective tubulins will denature at neutrality.

Column variations. Limited by cost, a systematic study of various columns was not attempted but two frequently used and suggested columns were compared with the Waters C_{18} μBondapak. A popular shorter chain length C_8 column, the Beckman Ultrasphere Octyl, was expected to be less retentive. Both columns were run at 25°C, with a 0 to 60% gradient over 1 h. The C_8 column retained flagellar tubulin 27% longer than the μBondapak, the two subunits were not resolved, the single tubulin peak trailed badly, and earlier eluting peaks were less sharp (data not shown). No attempt was made to further optimize conditions. An Altex RPSC column was used to

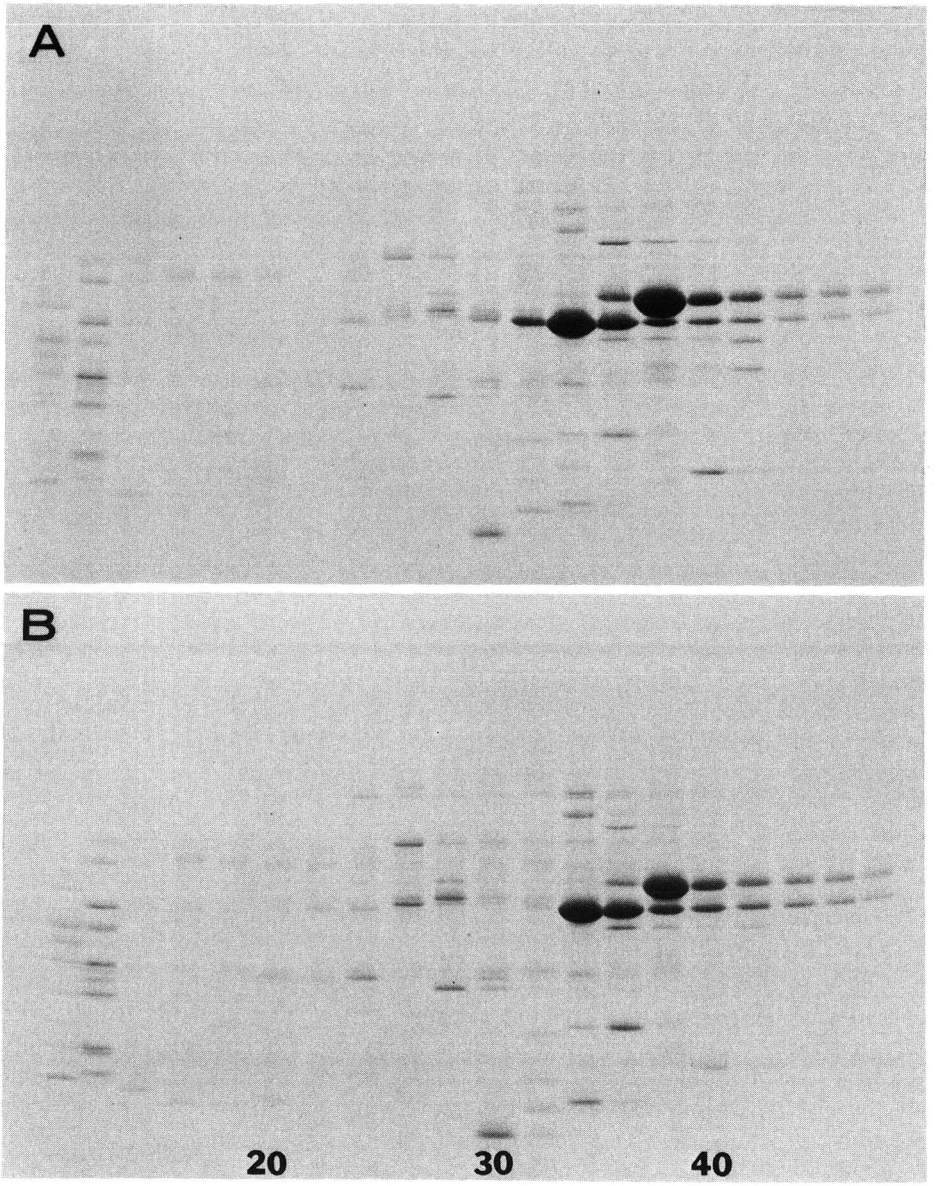

Fig. 3. The effect of guanidine–HCl solubilization on retention and yield. (A) Sea urchin flagellar proteins solubilized with 0.2% aq. TFA alone. Solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA. Gradient: 0 to 20% solvent B in 5 min, 20 to 100% B in 45 min, constant solvent B for 10 min. SDS-PAGE analysis of 2-ml fractions from 10–50 min. (B) Same, but solubilized in an equivalent amount of 6 *M* guanidine–HCl, 10 m*M* dithiothreitol, 0.2% TFA.

advantage over other columns, including the μBondapak, by Ferris *et al.*[24] in their ribosomal protein study cited above. The Ultrapore RPSC column has a substantially shorter chain length (C_3) and larger pore size (300 Å) than the μBondapak. Gradient

TABLE I

EFFECT OF TEMPERATURE ON TUBULIN SUBUNIT RESOLUTION

Solvent A = 30% acetonitrile, solvent B = 60% acetonitrile. Gradient: 0 to 20% B in 5 min, 20 to 100% B in 45 min, constant solvent B for 10 min.

Temperature (°C)	*Retention time*			*Relative tubulin yield (%)*
	α	β	*Difference*	
S. droebachiensis flagellar axonemes				
30	36.61	39.83	3.22	83
40	35.36	38.71	3.35	95
50	33.81	37.16	3.35	100
60	32.16	35.46	3.30	99
70	30.71	33.91	3.20	90
A. irradians ciliary axonemes				
30	37.05	39.33	2.28	56
40	36.00	38.40	2.40	71
50	34.36	36.98	2.62	86
60	32.20	34.93	2.73	100
70	30.30	32.88	2.58	85

and flow conditions were designed so that the two columns could be compared directly (Fig. 4). The Ultrapore RPSC yields a very sharp tubulin peak eluting well away from its nearest neighbors but the two subunits are not resolved. In comparison with the μBondapak, there is essentially no trailing. In the example shown, the relative yield of tubulin from the RPSC column operating at 25°C approached 100% while that of the μBondapak was 57% at 25°C and 69% at 40°C.

Separation of characteristic α and β chains in organelle fractions

Flagellar, ciliary, and cytoplasmic tubulins. Using a common gradient profile, the flagellar, ciliary, and cytoplasmic tubulins from two species of sea urchin and the flagellar and ciliary tubulins from two species of scallop were compared. These were chosen since the pairs exist at relative extremes of the environmental temperature range and well-established methods already exist for tubulin isolation and subfractionation. In addition, the subunits of *S. droebachiensis* and *A. irradians* tubulins have been analyzed in terms of peptide maps and amino acid composition[11,17] while equimolar β chains have been identified in flagella of *T. gratilla* and cilia of *A. irradians*[10].

Central pair tubulin is solubilized by low ionic strength dialysis of flagellar axonemes but dialysis of cilia will solubilize the B-subfibers and one central pair member[11]. In the case of the flagella from the two sea urchin species and the one molluscan species tested, the central pair fraction appears unique in that it yields an early-eluting β subunit and little or no β-tubulin corresponding in elution time to that of the outer doublet. An SDS-PAGE analysis of this is shown in Fig. 5 where the total dialysed sample (A: outer doublet plus central pair) is compared with the central pair fraction (B). Similar results are obtained for the ciliary B-subfiber fraction from both sea urchin species but not for that from scallop cilia.

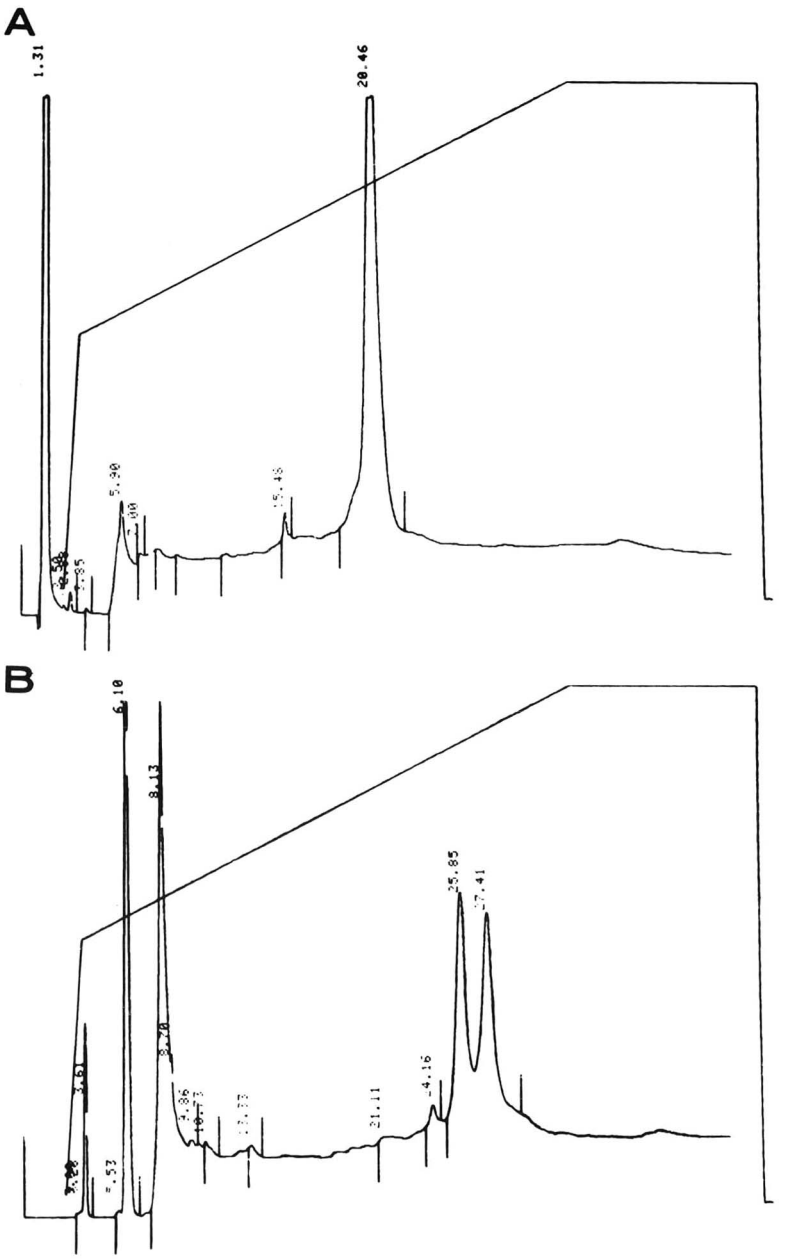

Fig. 4. Direct comparison of Ultrapore RPSC C_3 and μBondapak C_{18} columns. (A) Sea urchin outer doublet tubulin on an Altex Ultrapore RPSC C_3 column. Solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA. Gradient: 0 to 50% solvent B in 1 min, 50 to 100% solvent B in 30 min, constant solvent B for 10 min, 1.0 ml/min, 25°C. Full-scale = 0.2 O.D. (B) Same, but chromatography on a Waters μBondapak C_{18} column, 40°C.

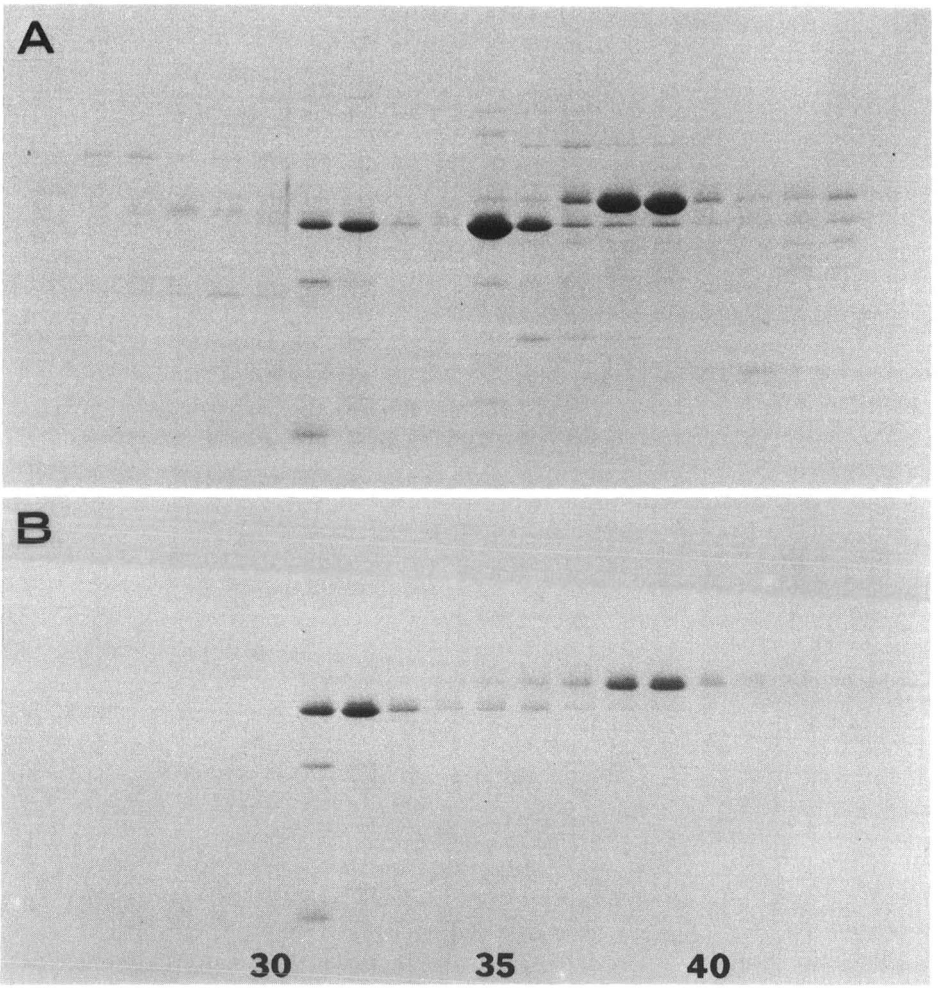

Fig. 5. SDS-PAGE analysis of sea urchin (*S. droebachiensis*) flagellar central pair tubulin fractionation. Gradient as defined in Fig. 3 and Table II. (A) Total axonemal proteins after 48 h dialysis against 1 m*M* Tris–HCl (pH 8), 0.1 m*M* EDTA; injected in aqueous TFA; fractions from 25 to 45 min. (B) The solubilized central pair fraction obtained by pelleting out the outer doublet microtubules from (A); the major β-tubulin elutes at 32 min.

The early-eluting β-tubulin is evidently a conformational variant that results from EDTA dialysis, based upon three independent arguments. First, no comparable amount of an early-eluting β-tubulin is detectable when undialyzed flagellar or ciliary axonemes are analyzed (*cf.* Fig. 3 or Fig. 6A). Second, when sea urchin flagellar central pair tubulin is prepared by direct high-salt extraction[23] or if ciliary B-subfiber is prepared by thermal fractionation[25], the β-tubulin elutes at the same retention time as that from the outer doublet or the A-subfiber, respectively. Finally, when either the central pair or B-subfiber fraction (containing the early-eluting β-tubulin) is injected in 6 *M* guanidine–HCl, only a normally-eluting β chain is seen after renaturation.

Nevertheless, such a variant still has some usefulness for comparative purposes.

Table II summarizes the relative retention times for the major and minor β and α chains for these various invertebrate tubulins and for brain tubulin as well. In addition, selected examples of the basic HPLC data illustrating important variations are shown in Fig. 6. In spite of the wide range of environmental temperatures that these organisms span, there is no clear relationship between growth temperature and tubulin HPLC elution time. The sperm flagella α chains of the cold-water sea urchin or scallop are more hydrophobic (elute later) than their warm-water counterparts but the reverse is true when one compares ciliary α chains. The two sea urchins have nearly identically eluting ciliary or flagellar β chain counterparts while the scallops do not.

In cases where cilia and flagella are compared directly, reproducible organelle-

TABLE II

COMPARATIVE RETENTION TIMES FOR TUBULINS FROM VARIOUS SOURCES

All runs at 40°C, solvent and gradient as in Table I, all intraspecies comparisons were made on the same column with the same solvent batch, values rounded to nearest 0.1 min, replicates reproducible within a S.D. of ± 0.04 min. Numbers in parentheses represent minor components.

	β	β	α
Sea urchin (S. droebachiensis)			
Sperm flagella	–	35.4	38.7
Outer doublet fraction	–	35.4	38.7
Central pair fraction	31.8	(35.4)	38.7
Embryonic cilia	–	35.7/36.2	37.3
A-tubule fraction	–	35.7/36.2	37.3
B-subfiber fraction	31.8	(35.7/36.2)	37.3
Egg cytoplasm	–	36.6	37.5
Sea urchin (T. gratilla)			
Sperm flagella	–	35.4	38.4
Outer doublet fraction	–	35.4	38.4
Central pair fraction	32.0	(35.3)	38.4
Embryonic cilia	–	35.4/36.2	38.3
A-tubule fraction	–	35.4/36.2	38.3
B-subfiber fraction	32.1	(35.5/36.2)	38.3
Egg cytoplasm	–	36.4	38.2
Scallop (P. magellanicus)			
Sperm flagella	–	36.8	39.4
Outer doublet fraction	–	36.7	39.4
Central pair fraction	32.7	(36.6)	39.4
Gill cilia	–	37.3	39.6
A-tubule fraction	–	37.3	39.5
B-tubule fraction	–	37.1	39.6
Scallop (A. irradians)			
Gill cilia	–	36.0	38.8
A-tubule fraction	–	36.1	38.9
B-subfiber fraction	–	36.1	38.8
Skate (*R. erinacea*) brain	–	36.9	37.9

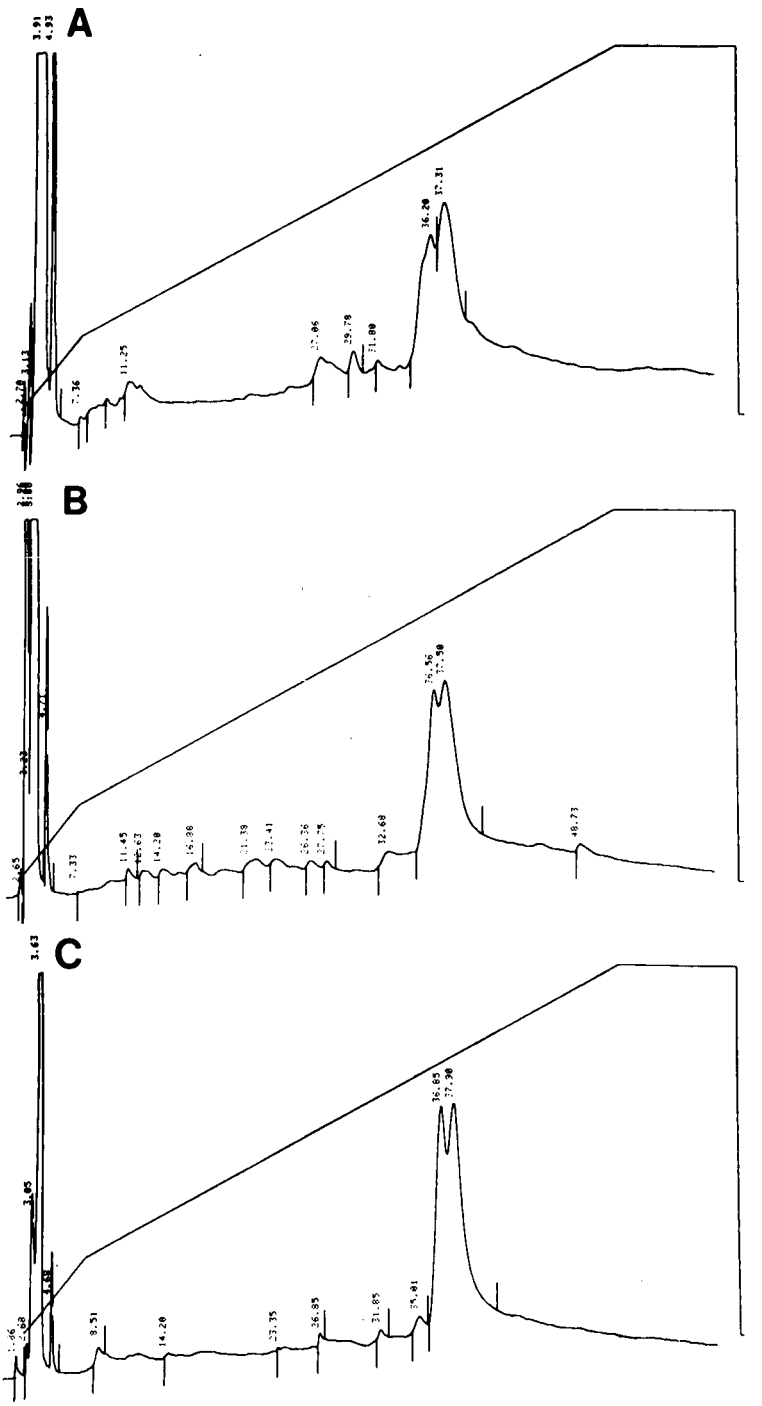

Fig. 6. HPLC profiles of tubulins from ciliary and cytoplasmic microtubules. Gradient as defined in Fig. 3 and Table II. (A) Sea urchin (*S. droebachiensis*) embryonic ciliary tubulin, showing partial resolution of two approximately equimolar β-subunits. Full-scale = 0.1 O.D. (B) Sea urchin (*S. droebachiensis*) egg cytoplasmic tubulin, having closely-eluting β and α subunits (solubilized in 6 *M* guanidine–HCl). Full-scale = 0.2 O.D. (C) Skate (*Raja erinacea*) brain tubulin, also showing closely-eluting β and α subunits. Full-scale = 0.2 O.D.

specific differences are evident. The most striking is seen in the α chains of *S. droebachiensis*. The ciliary β-tubulin in both sea urchin species is evidently comprised of two equimolar subspecies (Fig. 6A), both of which have slightly greater retention times than their single flagellar counterpart. (These cannot correspond to the equimolar electrophoretic β chains described earlier[17] since neither scallop cilia nor *T. gratilla* flagella show the two HPLC β chains whereas they, but not *S. droebachiensis* cilia, have two equimolar electrophoretic β chains). In *P. megellanicus*, the (single) ciliary β chain has a significantly greater retention time than its flagellar counterpart. The early-eluting β chains, discussed above, show no significant differences in retention times when cilia are compared with flagella.

The β chain of sea urchin egg cytoplasmic tubulin elutes later than either of the 9 + 2 counterparts while the α chain elutes earlier than the flagellar subunits and in *T. gratilla* even earlier than the ciliary α chain. Consequently the cytoplasmic tubulins are not well resolved (Fig. 6B). One interesting further difference is that *S. droebachiensis* egg cytoplasmic tubulin is nearly insoluble in 0.2% aq. TFA alone, requiring 6 *M* guanidine–HCl solubilization, whereas the *S. droebachiensis* 9 + 2 tubulins are freely soluble, as is egg cytoplasmic tubulin from *T. gratilla*.

Elasmobranch and mammalian brain tubulins. Skate brain tubulin was found to be very freely soluble in aqueous TFA and served as an early test material for this study. Like the egg cytoplasmic tubulin described above, the tubulin subunits were barely resolved and had retention times comparable to but later than *S. droebachiensis* egg tubulin (Fig. 6C). After publication of an abstract of this basic methodology[14], a number of workers attempted to apply it to the separation of mammalian brain tubulin subunits but with little reported success. The major problem was mainly one protein solubility.

Fig. 7 illustrates the rapid resolution of bovine brain tubulin into single fractions each containing the β and α subunits at better than 80% purity, as judged by SDS-PAGE gel densitometry. Unlike skate brain tubulins, which elute at 18.6 and 19.7 min on this gradient, these comparatively more hydrophilic subunits are well resolved at 14.6 and 17.1 min, with relatively little trailing, considering the load and the speed of the run. This particular sample was prepared by diluting one part of the protein contained in a 0.1 *M* piperazine-N,N′-bis(2-ethane sulfonic acid) repolymerization buffer (at 15 mg/ml) with nine parts of 0.5% aq. TFA. Direct addition of TFA to the highly buffered salt solution results in the irreversible precipitation of tubulin, as does direct dialysis against 0.2% aq. TFA. Unlike the sea urchin cytoplasmic and 9 + 2 tubulins, vertebrate brain tubulin subunits do not chromatograph well after guanidine–HCl solubilization.

DISCUSSION

The RP-HPLC methods presented here are generally useful for rapid separation of the major α- and β-tubulin subunit classes in relatively high yield, although these may co-elute with some minor proteins. In the species studied thus far, β-tubulin always elutes before α-tubulin. The β/α subunit separation is species-specific, particularly evident with brain tubulins, and it is also organelle-specific, notably in cytoplasmic *versus* ciliary *versus* flagellar tubulins, where the degree of separation increases in the order given.

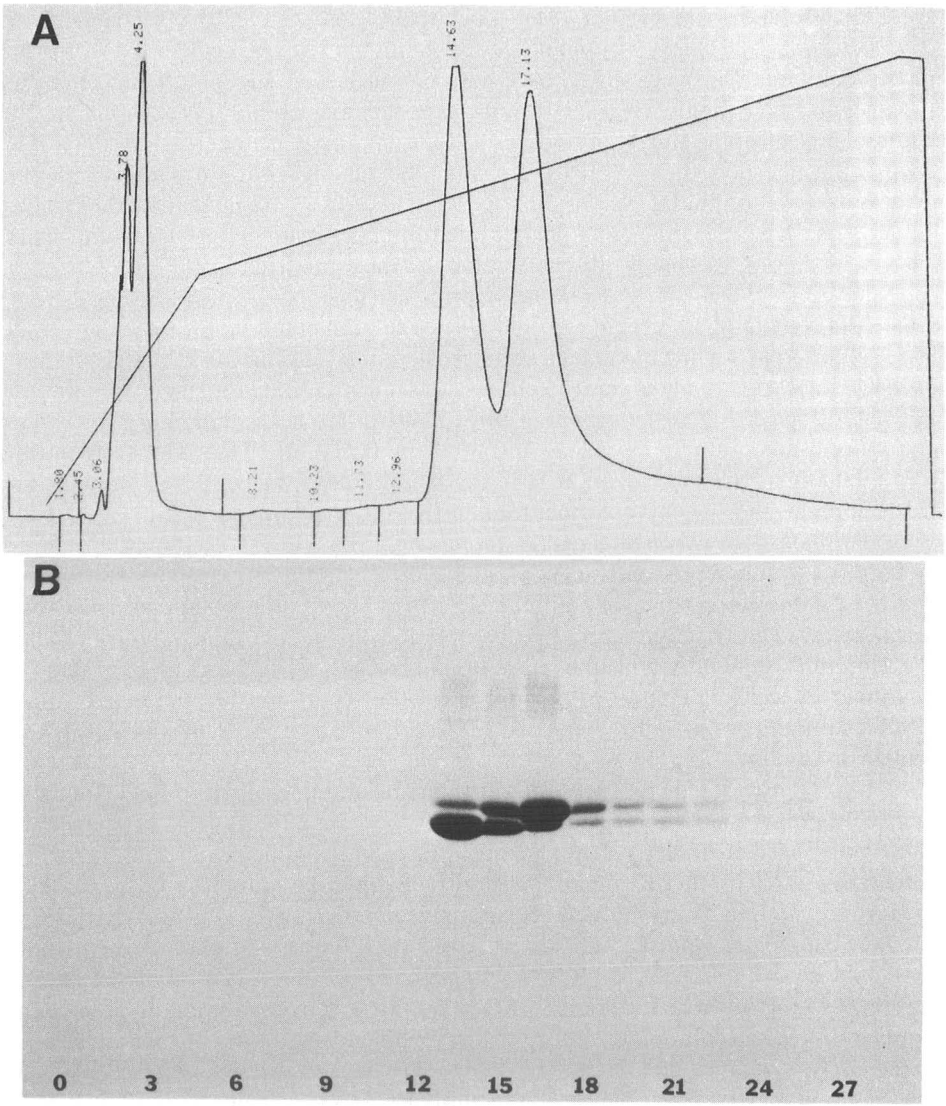

Fig. 7. Rapid elution of bovine brain tubulin subunits. (A) HPLC profile. Solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA. Gradient: 0 to 50% in 5 min, 50 to 100% in 25 min, 1.0 ml/min. Full-scale = 1.0 O.D.; sample = 0.4 mg total protein. (B) SDS-PAGE analysis of the entire gradient, 1.5-ml fractions.

An additional aspect of this approach is that it produces the equivalent of a two-dimensional analysis of ciliary or flagella proteins, basically in accord with increasing relative hydrophobicity. The examples here used 20–40 fractions but obviously this can be increased almost without limit. Such an analysis has other limitations, however, since proteins with molecular weights in excess of about 100 000

are either voided from or irreversibly retained by the column. Other proteins may simply be insoluble under the conditions employed.

The key to good resolution and high yield is the initial solubilization to produce fully dissociated tubulin subunits. This is dependent upon complete reduction of –S–S– bridges, the absence of salt and divalent cations, and, of course, the tubulin in question must be soluble in the pH range of 2–2.5. The first conditions are easily met by dialysis of the protein into a weakly Tris-buffered solution (maximum 10 m*M*, pH 8) containing 10 m*M* DTT or 0.1 *M* mercaptoethanol, followed by acidification with TFA. Surprisingly, most 9 + 2 axonemes studied thus far dissolve almost totally when suspended in 0.2–0.3% aq. TFA. On the other hand, some tubulins are simply insoluble in aqueous TFA even if they are initially water soluble, *e.g.* one urchin cytoplasmic tubulin but not the other. Guanidine–HCl solubilization is required in such cases, generally accompanied by some loss of protein on the column.

After HPLC resolution, some tubulins, when pure, self-associate or simply denature and may be quite insoluble after evaporation of TFA–acetonitrile. This problem may be circumvented in many cases by drawing off most of the acetonitrile under aspirator vacuum and then dialyzing the aqueous TFA solution against the desired final buffer or renaturation solution. Alternatively, the dried sample may be dissolved in and renatured from urea or guanidine.

A good example of the application of these facts learned from tubulin separation, after the initial publication of the basic method[14], has been the successful resolution of the tektins, outer doublet microtubule-associated proteins which are extremely insoluble[26]. Derived from sea urchin (*S. purpuratus*) sperm flagella, these require solubilization in 6 *M* guanidine–HCl and DTT prior to HPLC analysis. In this case, > 80% yields and essentially complete separation of these three extremely similar proteins is accomplished on a C_{18} column with a shallow, concave gradient of acetonitrile. Attempts to apply this same approach to the tektins from the closely-related sea urchin *S. droebachiensis* studied here requires 9 *M* guanidine–HCl for solubilization and yields poorly-resolved tektins at comparatively low yield. Conversely, the tubulins from *S. purpuratus*, although resolving as well as those from *S. droebachiensis*, chromatograph at considerably lower yields.

Columns which theoretically should bind tubulin less tightly, produce better resolution, and give higher yields, at best do only the latter. O'Hare *et al.*[27] and Pearson and Regnier[28] present arguments for increased resolution and recoveries on shorter chain length, larger pore-size columns. In basic agreement with our own results, Steffensen and Anderson[29] recently compared the relevant column chemistries directly, using water soluble, monomeric proteins both larger and smaller than tubulin subunits, and found that properly end-capped C_{18} columns produced separations and yields comparable to shorter chain length columns, while larger silica pore size increased yield only slightly. The μBondapak C_{18} medium is end-capped and bears a relatively light carbon load. Evidently the unique efficacy of this column is a function of some differential interaction of the C_{18} groups and/or the silica surface with very specific and distinctive hydrophobic surface regions characteristic of the two tubulin subunits.

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Note

Checking the capacity of a splitless injector — a simple test

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This paper deals with an old problem that has still not been adequately solved. In conventional splitless injection, sample vapour generated on introduction into the hot injector must be "stored" within the vaporization chamber until transferred into the column[1]. However, a rapid calculation immediately reveals that many vaporizing injectors built into current gas chromatographic (GC) equipment are simply too small to house the sample vapour.

REQUIRED INJECTOR VOLUME

On injecting a sample volume that corresponds to 1 μl read on the barrel of a standard 10-μl syringe, usually about 2 μl of liquid are introduced into the injector, as the needle is likely to be emptied by evaporation of the sample matrix (*e.g.*, if the latter consists of one of the commonly used solvents). If hexane is the sample solvent, these 2 μl of liquid form a vapour about 600 μl in volume, depending on the carrier gas inlet pressure and the temperature of the injector. If methanol is the solvent, the vapour has the large volume of about 1.5 ml. Further, it must be considered that one cannot prevent that sample vapour being diluted with carrier gas. In fact, the vapour clouds are easily 50–100% larger than calculated above. A vaporization chamber of, *e.g.*, 8 cm length and 2 mm I.D. has a volume of 250 μl, thus is far too small to retain the sample vapour in splitless injection, and we should consider what happens to these vapours then. The explosion-like evaporation of the sample increases the pressure in the vaporization chamber, pushing the vapour into all accessible cavities. The easiest means of expansion usually involves returning into the carrier gas supply line. When pressure decreases again, the vapour returns. However, a substantial proportion of high-boiling and adsorptive solute material remains within this line as only a short section (if any) of the latter is heated. Solute material may slowly return when the split exit is opened again, then being split, with the effect that most material is lost for the analysis. Often solutes of intermediate volatility return during subsequent runs, creating "memory" effects. It is obvious that no optimal quantitation is possible under such conditions.

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CONCEPT OF THE TEST

The problem of too small vaporization injectors was recognized a long time ago, and it is difficult to understand why many instrument manufacturers have not solved the problem in the meantime. Some appear to hope that the pressure increase in the injector will keep the vapour together and others rely on the recondensation of the solvent in the column inlet, accelerating the vapour transfer. To our knowledge, neither of these theories has been checked experimentally, and in our experience their effects are too weak to prevent injector overflow. Rather than believing or not believing, the operator should check himself whether the injector has a sufficient capacity for housing the sample for the volume injected and using a particular (possibly too short) syringe needle. The test is simple, taking hardly 5 min. It is based on the detection of solvent vapour leaving the septum purge exit during splitless injection. The gas flow through the septum purge line passes the top end of the injector insert and carries away sample vapour if the insert overflows. As this gas flow does not pass through the vaporization chamber, vapour stored within the glass insert of this chamber are not affected.

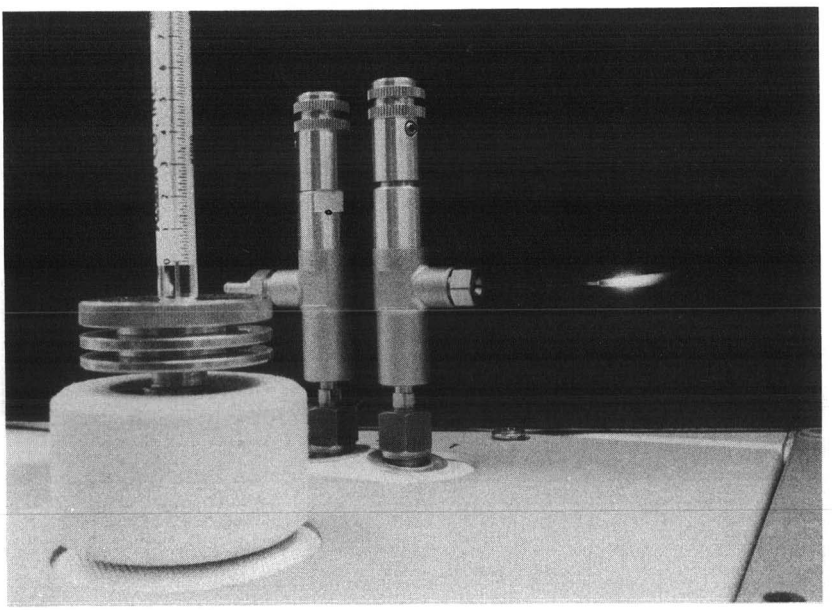

Fig. 1. Flame on the septum purge exit indicating back-flushing of sample vapour. Conventional vaporizing injector (Carlo Erba) on the left. Behind are the two needle valves of the split line (left) and the septum purge (right). The short fused-silica capillary mounted in the exit of the septum purge needle valve is not visible, but the glowing tip of the latter, free of polyimide coating, can be seen. The flame was photographed about 1 s after injecting 3 μl (reading 2 μl on the barrel of the syringe) of diethyl ether into the large (1 ml) injector insert, using a 70 mm long syringe needle. Injection of a 2-μl volume produced only a small, weakly yellow flame, indicating a small loss by back-flow. This allows the conclusion that (under the conditions applied) the injector had the capacity to take 2 μl of diethyl ether.

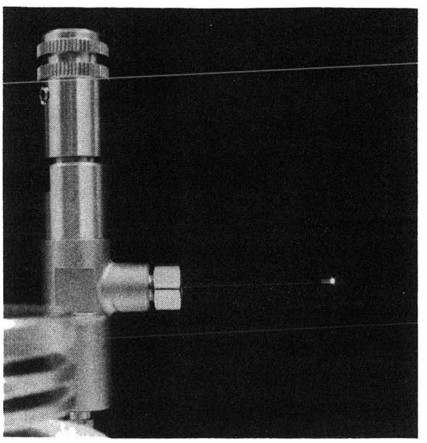

Fig. 2. Glowing of the fused-silica capillary tip in the small flame photographed *ca.* 15 s after an injection as shown in Fig. 1. The flame burns sample vapour backwards leaving the injector insert by diffusion. After opening the split exit, purging the injector, a flame is no longer visible.

EXPERIMENTAL

The GC carrier gas must be hydrogen. The septum purge exit is equipped with a short piece of 0.25–0.32 mm I.D. fused-silica capillary, as shown in Fig. 1. The septum purge flow-rate is adjusted to *ca.* 10–30 ml/min and the hydrogen at the tip of the fused-silica line is lit. As long as pure hydrogen leaves this exit, the flame is colourless, visible only as a weak glow at the tip of the fused-silica capillary. However, the flame turns yellow as soon as hydrocarbons are mixed with the hydrogen. Large dead volumes within the septum purge exit line disturb the experiment, as do needle valves and lines kept far below the boiling point of the solvent (the solvent recondenses and re-evaporates over extended periods of time, giving a wrong picture of the duration of back-flushing of sample vapour).

We assume that an injection is carried out by the "hot needle" technique, involving withdrawal of the sample liquid into the barrel when introducing the needle. During the introduction of the first part of the needle, the flame usually turns slightly yellow for a short period owing to solvent eluted from the tip of the syringe needle into the septum purge zone of the injector (vapour of sample liquid coating the needle wall). When the tip of the needle enters the vaporization chamber, eluted vapour is flushed towards the column and the flame becomes colourless again. Injection of the sample, and here we assume an excessively large volume, causes the flame immediately to turn yellow (Fig. 1), indicating back-flushing due to the pressure wave. The flame rapidly returns to a weak yellow colour, persisting for several tens of seconds. This is due to solvent (and solute material?) slowly diffusing backwards out of the injector insert. Sometimes these small amounts of solvent are only observed by a more intense glow of the fused silica (Fig. 2), caused by the higher temperature of the flame. On opening the split exit and flushing the injector chamber, the flame becomes colourless again and the glow is the same as before the injection.

The results of the test depend on many factors. Of course, losses are reduced by

reducing the sample volume injected. Losses through the septum purge also depend strongly on the length of the syringe needle, determining whether or not the available injector volume is fully used. Further, they depend on the sample solvent, the column inlet pressure, the column flow-rate and the column temperature (recondensation effect).

The experiment fails with solvents that do not create yellow flames, *e.g.*, methanol. Aromatic solvents produce the most intensely bright flame and allow the most sensitive detection of the back-flow. However, as solvents of smaller molecular size and higher densities form considerably larger vapour clouds per unit volume of liquid injected, they do not represent "tough" cases.

CONCLUSIONS

Back-flushing of sample vapour as detected by the above test causes immediate loss of sample material if the septum purge remains open during splitless injection. Closure of the septum purge exit prevents immediate loss, but does not represent a convincing solution to the problem, as overflowing vapour is forced into the carrier gas supply system, from where it returns incompletely and selectively, causing losses and "memory effects". Therefore, the injection volume should be reduced to a level that keeps the losses by back-flow small. Unfortunately, many analysts will find that on their equipment even the smallest sample volume that they can inject produce a considerable back-flow. The internal volume of the injector should be around 1 ml[1]; larger volumes make sample transfer from the injector into the column difficult[2]. Usually the possibilities of enlarging the vaporization chamber of conventional split–splitless injectors are limited, mostly being restricted to the use of injector glass inserts with thin walls, which allow the injector cavity to be exploited as efficiently as possible.

A more quantitative study on losses through back-flow and a more detailed discussion of the consequences will be published elsewhere.

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Note

Cyclodextrin–ligand interaction as a simplified model of biospecific affinity chromatography

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Cyclodextrins (CDs) are generally considered to form inclusion complexes which mimic biological interactions, *e.g.*, the complex between an enzyme and substrate[1]. These molecular adducts of CDs have been extensively studied in solution[2] and by X-ray crystallography[3–5]. Matrix-bound CDs have manifold chromatographic applications in the purification of low-molecular-weight substances[6]. In contrast, the chromatography of CDs on chemically modified supports has not been studied. Formally this system closely resembles affinity chromatography and hence could serve as a model of it. Since CDs are small, non-ionic, commercially available pure substances, whose concentration can be varied over large ranges, experiments which cannot be carried out with other biomolecules are possible. The existence of three homologous forms of CDs, α-, β- and γ-, with variable complexing properties further facilitates analysis of this model.

We describe here an affinity system for CDs involving naphthoxyacetic acid bound to aminated Bio-Gel P-6 or to cellulose. These supports separated the three CD-forms. The effects of concentration, ligand, temperature and binding capacity were studied. Interactions of CDs between free and support-bound ligands are discussed.

EXPERIMENTAL

Preparation of the modified sorbents

Bio-Gel P-6 (200–400 mesh; Bio-Rad Labs., Richmond, CA, U.S.A.) was aminated and the concentration of the amino groups determined by normal acid–base titration[7]. (2-Naphthoxy)acetic acid (1–5 g; Aldrich, Milwaukee, WI, U.S.A.) was dissolved in aqueous 20% dimethyl sulphoxide and then 50 ml (a settled volume) of aqueous aminated Bio-Gel P-6 were added and the pH was adjusted to 4.7 with 1 *M* sodium hydroxide. The suspension was stirred at 20°C while N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (4 g in 20 ml of water at 0°C; Sigma, St. Louis, MO, U.S.A.) was added dropwise during 30 min while the pH was maintained at 4.7. Gentle stirring was continued for 4 h. The gel was washed with water, 50% ethanol, ethanol, 50% ethanol, water, 0.5 *M* sodium chloride and water, 500 ml of each.

A 10-g amount of cellulose MN 300 (Sigma) was suspended in water and swelled overnight. It was washed with 50 ml of 2 *M* sodium hydroxide at 0–5°C on a glass filter

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and then with 100 ml of acetone at −20°C. The solvent was filtered off and the powder immediately transferred to a vial containing 3 g of cyanuric chloride (Aldrich) in 50 ml of acetone at −20°C. The suspension was continuously stirred while 50 ml of ice-cold water were added. Then the solution was allowed to warm to 20°C within about 10 min. The pH should then be 1–2. The derivative was washed on a glass filter with ice-cold acetic acid (20% in water), and acetone (each 200 ml). A solution of bis(aminopropyl)amine (0.5 *M* 250 ml) was adjusted to pH 10 with hydrochloric acid and cooled in an ice-bath. The triazine-activated cellulose was added and the pH was maintained at 10 with 5 *M* sodium hydroxide with stirring. When the consumption of the base had ceased, the aminated cellulose was washed with water, ethanol, acetone and water (each 500 ml). (2-Naphthoxy)acetic acid was coupled to the aminated cellulose as above.

Equipment

The chromatographic system usually consisted of a gel column (450 mm × 6 mm), a peristaltic pump (P-3; Pharmacia, Uppsala, Sweden) and a refractive index detector (Model 2142; LKB, Stockholm, Sweden). In typical experiments, 0.2 ml of 5 m*M* CD solutions (Sigma) were applied. The elution was carried out with distilled water.

In the studies at different temperatures, a jacketted column (118 mm × 10 mm; Pharmacia) and an high-precision, medium-pressure pump (of an amino acid analyzer Perkin-Elmer KLA-2, Japan) were used. The samples (0.2 ml; containing 3.33 m*M* of each CD) were applied with an applicator described earlier[8].

Determination of the ligand concentration

(2-Naphthoxy)acetic acid absorbs light at 326 nm in acidic aqueous dimethyl sulphoxide and the absorption is linearly proportional to the concentration of (2-naphthoxy)acetate up to 9 m*M*. The gel was brought into solutions as follows: 0.2 g of suction-dry moist gel were suspended in a mixture of 6 *M* hydrochloric acid (5 ml) and dimethyl sulphoxide (1 ml), and the suspension was kept in a bath of boiling water for 1 h. Occasional slight turbidity was removed by centrifugation (10 000 rpm, an Eppendorf centrifuge) and the absorbance of the solution was measured. (2-Naphthoxy)acetic acid (1–20 mg) served as the standard for aminated Bio-Gel P-6, while the mixture was treated as above. This method produced similar results to the acid–base titration of the unmasked amino groups.

Determination of the binding capacity

The binding capacity of a gel containing 23 g naphthoxy ligands per litre of Bio-Gel P-6 was determined by using frontal analysis[9] of 5 m*M* CD solutions at 22°C with a flow-rate of 24 ml/h (gel column 450 mm × 6 mm).

RESULTS

The elution of α-, β-, and γ-CDs on naphtyl-derivatized Bio-Gel P-6 is illustrated in Fig. 1. The separation of pure CDs was complete on this gel, but on the naphthyl derivative of cellulose α- and β-CDs were not completely separated. This was probably due to an adsorption of α-CD on the non-modified amino ligands. The results on naphthyl-Bio-Gel are mainly dealt with here.

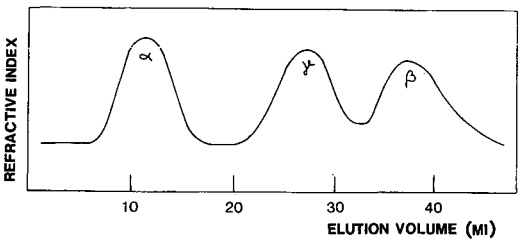

Fig. 1. Separation of CDs on (2-naphthoxy)acetate-liganded Bio-Gel P-6 (300 × 6 mm; 8 ml) with a flow-rate of 24 ml/h water. The ligand concentration of the gel was 12 g/l. The sample (0.2 ml) contained 5 mmol/l of each CD form. The elution volume of α-CD was 11.5 ml and those of glucose and ^2H_2O were 13.1 and 13.4 ml, respectively.

The elution volume of α-CD (Fig. 1) was less than that of glucose or ^2H_2O and hence α-CD was slightly excluded by the gel. The general appearance of the chromatogram shows broad peaks. A tailing of the peak of β-CD is also evident. The flow-rate of the mobile phase did not affect the elution volumes within the range applicable. While at high concentrations of CDs the elution volumes of β- and γ-CDs were shortened, at 5 m*M* they were eluted equally when chromatographed separately or as the mixture.

Effects of the substitution degree

Variation of the ligand concentration over a quite narrow range compared to the total content of the amino functions in the gel has dramatic effects on the elution behaviour of the CDs (Table I). While the elution of α-CD is independent of the ligand concentration, the elutions of β- and γ-CDs are strongly dependent on it. The limit of non-elution was met abruptly (Table I).

TABLE I

ELUTION VOLUMES OF CYCLODEXTRINS ON NAPHTHOYLATED BIO-GEL P-6

The aminated Bio-Gel P-6 contained 0.57 mol amino groups per litre of gel. The concentration of naphthyl functions is indicated. The column volume was about 13 ml and the sample volume was 0.2 ml. The CDs were chromatographed at 22°C with distilled water as the mobile phase. The flow-rate was measured by collecting the eluent in a graduated cylinder.

Ligand concentration (g/l of gel)	*Elution volume (ml)*			*Flow-rate (ml/h)*
	α-CD	*β-CD*	*γ-CD*	
4	15.2*	28.6*	22.6*	32.6*
	14.7	28.2	22.2	29.0–30.2
12	15.3*	36.0*	27.0*	13.1*
	14.8	36.8	26.0	12.6–17.0
20	15.3*	52.1*	36.6*	13.0*
	15.0	50.3	38.0	13.1–17.8
23	14.8*	**	**	14.0

* Chromatographed as the mixture of the CDs.
** Bound irreversibly.

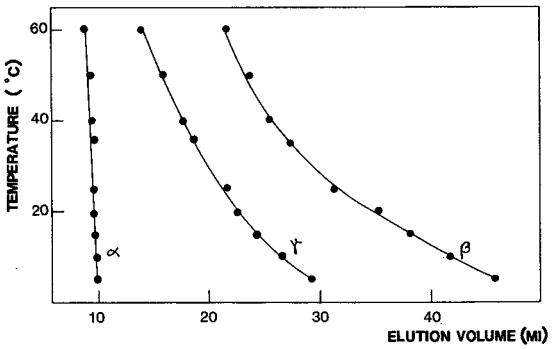

Fig. 2. Temperature dependence of the retention of CDs on (2-naphthoxy)acetic acid-derivatized Bio-Gel P-6 (jacketted column, 118 mm × 10 mm; Pharmacia) with a flow-rate of 30 ml/h. The gel contained 4 g naphthoxy ligands per litre. Samples (0.2 ml) contained 3.33 mmol/l of individual CDs and the elution was isocratic with water.

Operational capacity of the sorbent

The operational CD-binding capacities of naphthoylated supports were determined by using a frontal analysis method[9] with separate 5 m*M* CD solutions. For naphthyl-cellulose the capacities were 0.3, 1.5 and 0.5 g/l of packed support with α-, β- and γ-CD, respectively. With the Bio-Gel derivative the capacity was negligible for α-CD, whereas with β-CD it was 14.1 g/l of packed gel. The capacity was not studied for γ-CD. With a benzoylated Bio-Gel[7] the effect of temperature was also tested for β-CD. Surprisingly, the capacity was higher at 22°C (8.3 g/l) than at 0°C (7.3 g/l).

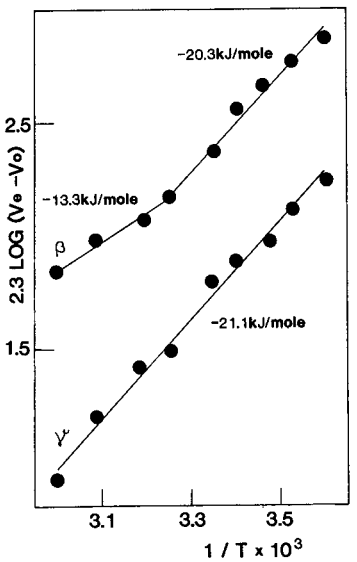

Fig. 3. The Van 't Hoff plots obtained from the data in Fig. 2 for the interaction of β- and γ-CDs with naphthoxylated Bio-Gel P-6. The enthalpy values are shown in the figure.

Effects of the temperature

Fig. 2 shows the elution volumes of separately chromatographed α-, β- and γ-CDs on the naphthyl-Bio-Gel at different temperatures. The elution of α-CD slightly decreases at higher temperatures and the dependence is linear whereas the elutions of β- and γ-CDs are logarithmic functions. Their resolution shows little improvement at low temperatures even though the separation from α-CD considerably increases.

Fig. 2 much resembles the one obtained previously by us for affinity chromatography of alanine aminotransferase[10]. The linear dependence of α-CD on the temperature can be ascribed to a slight effect of temperature on the size-exclusion term of the separation with a close analogy to the behaviour of the non-binding reference protein described previously[10]. Although β- and γ-CDs have slightly higher molecular weights than that of α-CD, their elution in the absence of specific ligands should be nearly equal to that of α-CD. Evidence in supports of this was provided by results obtained on chemically derivatized gels which did not separate the CDs (data not shown). On this basis the difference between the line for α-CD and the curve for β- and γ-CDs (Fig. 2) reflects the term due to specific adsorption. Therefore the heat of adsorption is obtained from this difference. Fig. 3 shows that the Van 't Hoff plots for

TABLE II

INTERACTION OF α-, β- AND γ-CDs WITH NAPHTHYL LIGANDS IN SOLUTION

Compound	*CD form*	*K (M^{-1})*	*Conditions*	*Refs.*
Naphthalene*	β	685	Fluorescence, 25°C, water	16
2-Naphthol	α	127	Fluorescence, water	12
	β	625		
	γ	53		
	β	32	Spectrophotometry, potassium chloride–hydrochloric acid, I = 0.06, pH 2.2, 25°C	17
1-Bromonaphthalene**	β	≈10 000	Phosphorescence, water, 15°C	18
2-Methoxynaphthalene***	β	630	Fluorescence, water, 25°C	16
1-Naphthoylacetic acid	β	714	Fluorescence, 0.05 M borate, pH 10, 25°C	19
2-Naphthoyltrifluoroacetone§	α	73	Gel chromatography, 10 mM sodium acetate, pH 4.3, 25°C	13
	β	1320		
	γ	142		
1-Anilino-8-naphthalene-sulphonate	β	77	Fluorescence, 0.05 M borate, pH 11.0, 25°C	19
2-*p*-Toluidinonaphthalene-6-sulphonate	β	4000	Fluorescence, 0.1 M phosphate, pH 5.9, 25°C	20
	α	18	Fluorescence, 0.08 M sodium acetate, pH 5.3, 25°C	21
	β	1538		
	γ	1515		
1,3-Di-α-naphthylpropane	α	≈0	Solubility, water, 25°C	15
	β	840		
	γ	5800		

* $\Delta H^0 = -19$ kJ/mol, $\Delta S^0 = -10$ J/mol · K.
** $\Delta H^0 = -96$ kJ/mol, $\Delta S^0 = -60$ J/mol · K.
*** $\Delta H^0 = -17$ kJ/mol, $\Delta S^0 = -5$ J/mol · K.
§ $\Delta H^0 = -38$ kJ/mol, $\Delta S^0 = -67$ J/mol · K.

the CDs are reasonably linear for the calculation of adsorption enthalpy. However, the line for β-CD has an inflection point which may indicate a change of the complexation mode (Fig. 3).

DISCUSSION

The order of elution of the individual CD forms is in reasonable accord with the equilibrium constants between them and the naphthyl group in solution (Table II). However, an exact correlation is difficult because the various methods for measuring the equilibria show considerable deviation[11]. On the other hand, even small differences in the substituents of naphthalene may affect the equilibria; the situation is related to the properties of the spacer arm in affinity chromatography.

In some studies (Table II), 2-substituted naphthalene has been found to form a complex with α-CD[12,13]. The present results clearly show that α-CD does not bind with the naphthyl group, at least in the absence of a polar "introductory" function (Table I, Fig. 1). This is probably not due to any special property of the support-bound naphthyl group since the complex of β- and γ-CDs forms normally and rapidly on the time-scale of chromatography. Therefore the naphthyl ring can, at most, penetrate α-CD to only a limited extent.

The samples (0.2 ml) for the chromatographic experiments were usually 5 m*M*, while the concentations of naphthyl groups were from 17 to 115 m*M* (Table I). Supposing a "plate volume" of 0.2 ml, the maximum molar amount of the solute in the stationary phase was 4–30% of that of the ligands. On the other hand, by neglecting the volume taken by the gel and considering the operational capacity of the affinity gel for β-CD of 14 g/l (with the gel containing 89 m*M* ligand), at breakthrough conditions the column contains 12 m*M* solute in the stationary phase (5 m*M* in the mobile phase) which means a 13% complexation of the ligands. Supposing an even distribution of the ligands in the column volume, and a 1:1 complexation mode, an equilibrium constant of 31 M^{-1} is calculable. Compared with the results of solutions studies, this value is at least one order of magnitude too small. More exact analysis will readily be achieved by frontal analysis of CDs at different concentrations[14].

In solution, the complexation degree of CDs is strongly dependent on the temperature[11]. While the elution volumes indicate the same behaviour on the naphthyl gel (Fig. 2), the binding capacity of 5 m*M* β-CD was slightly less at 0 than at 22°C with the benzoylated Bio-Gel. Thus the supports should have ligands whose availability is independent of the temperature and these ligands interact with CDs as in solution. Possibly a considerable heterogeneity of the ligands exists and some clusters of them are dissolved at higher temperatures or at higher concentrations of CDs. A consequence of this is that the capacity for zone elution may be relatively high from more concentrated solutions which allows practical preparation of analytical grade CDs by affinity chromatography. Preliminary evidence for this has been obtained.

The strong and relatively abrupt increase in the binding strength as a function of ligand concentration in the case of γ-CD compared with β-CD suggests that when the average distance between naphthyl groups reaches a certain value on the matrix, the binding mode changes from 1:1 to 1:2 (CD:ligand). Evidence in support of this is provided by the very strong forces between 1,3-di-α-naphthylpropane and γ-cyclodextrin (Table II, ref. 15).

Despite relatively complex ligand–ligand or ligand–gel interactions probably occur in the systems studied, the interactions should be equal for β- and γ-CDs. Hence the two-phase Hill plot (Fig. 3) for β-CD may not originate from the support itself. The ΔH^0 values for β- and γ-CDs are in a good accord with previous values in solution (see footnotes to Table II).

Although this study is far from a detailed analysis of the system, it shows many chromatographically interesting aspects which can be illustrative in the context of affinity chromatography. The benefit of the present model lies in the fact that cross-studies of zone and broad-zone elution concomitantly with batch studies can be carried out to obtain thermodynamic and other parameters through independent routes. Theoretical development of the affinity method is especially desirable to improve its predictability.

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Note

Separation of tocopherols on various chemically bonded phases in microcolumn liquid chromatography

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Vitamin E, as it occurs naturally, consists of eight compounds which belong to two series of methyl-substituted chromanols with either a saturated (the tocopherols) or unsaturated (the tocotrienols) side chain in the 2-position. The four naturally occurring tocopherols, *i.e.*, *d*-α, *d*-β, *d*-γ and *d*-δ tocopherols, differ in the number and position of the methyl groups on the chromanol ring. They show pronounced quantitative differences in biological activity and, hence, contribute to different degrees to the overall vitamin E supply. They also differ in their ability to protect natural products, *e.g.*, vegetable oils, from oxidative degradation (antioxidant properties)[1].

Tsen[2] reported an improved spectrometric method for the determination of tocopherols, using 4,7-diphenyl-1,10-phenanthroline. A simultaneous determination of α-tocopherol and retinol (vitamin A) is meaningful in view of the recently suggested biological interaction between the two vitamins, and several have been established for their simultaneous in biological materials, especially in human serum using high-performance liquid chromatography (HPLC)[3–5]. Cort *et al.*[6] reported the separation and identification of vitamin E isomers using a normal-phase Chromegasphere SI 60 column with 2.5% tetrahydrofuran (THF) in isooctane as the mobile phase and fluorescence detection.

Normal-phase chromatography on a polar stationary phase allows rapid and easy differentiation of positional vitamin E isomers, in agreement with the well known stereochemical selectivity of silicic acid for isomers[1]. Generally, the separation of tocopherols is achieved using normal-phase silica columns[7–10] and also amino bonded columns[11]. Separation of the isomers using reversed-phase LC was tried[10,11], and the tocopherols were easily separated into α-, β- plus γ- and δ-forms. Separation of β- and γ-tocopherols could not be achieved under a variety of experimental conditions, however.

Developments in microcolumn LC have recently led to high resolution and higher sensitivity[12]. In this investigation, separations of tocopherols using micro-column LC have been performed on various chemically bonded phases in order to find a good system to separate β- and γ-tocopherols.

EXPERIMENTAL

The *d*-α-, *d*-β-, *d*-γ- and *d*-δ-tocopherols (Ezai, Tokyo, Japan) were dissolved in *n*-hexane (Wako, Osaka, Japan) and injected directly. Hexafluoroisopropanol (FIPA)

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TABLE I

PACKING MATERIALS USED

No.	*Stationary phase*	*Particle diameter (μm)*	*Pore size (Å)*
1	Naphthylethyl (N. Tanaka, Kyoto Institute of Technology, Kyoto, Japan)	5	147
2	Phenyl (M. Okamoto, Tajimi Hospital, Tajimi, Japan)	5	150
3	Diphenyl (M. Okamoto)	5	150
4	Triphenyl (M. Okamoto)	5	180
5	Pyrenylethyl (N. Tanaka)	5	147
6	Polymer-based C_{18} (1) (TSK Gel octadecyl PW4; Tosoh, Nanyo, Japan)	7	110–120
7	Polymer-based C_{18} (11) (Asahi pak ODP; Asahi Kasei, Kawasaki, Japan)	5	*
8	Polymeric C_{18} (Vydac 201TPB5; Separation Group, Hesperia, CA, U.S.A.)	5	300
9	Monomeric C_{18} (N. Tanaka)	5	110
10	Polymer-coated C_{18} (Capcell pak; Shisheido, Tokyo, Japan)	5	120

* Informations not available.

(Showa Denko, Tokyo, Japan) was used as a mobile phase modifier for the normal-phase system. All other chemicals such as methanol, acetonitrile and isopropanol (IPA) were commercially available.

The microcolumn LC system was comprised of a MF-2 microfeeder (Azuma Electric, Tokyo, Japan) and an Uvidec 100 detector (Jasco, Tokyo, Japan) set at 295 nm, the cell volume being 0.65 μl. A microloop injector Jasco ML-422 (0.1 μl) was used for sample introduction. The columns used were fused-silica capillaries (Quadrex; Kasei Kogyo, Tokyo, Japan), 500–1000 mm × 0.53 mm I.D., packed with various bonded phases by the conventional slurry technique. Details of the packing materials utilized are summarized in Table I.

RESULTS AND DISCUSSION

LC is very suitable for the analysis of tocopherol isomers, especially since specific detection methods can be employed and exposure of the tocopherols to air can be minimized. LC analysis of tocopherols has mainly been performed with silica columns, *e.g.*, Corasil[10]. Although chemically bonded polar phases have been little used, Westerberg *et al.*[11] resolved all tocopherols using a μBondapak-NH_2 column with *n*-hexane–ethanol (99.2:0.8) as the mobile phase. In this work, microcolumns packed with various chemically bonded phases were tried for tocopherol separation in both normal- and reversed-phase separation systems, in order to find a better separation system for vitamin E isomers.

For the normal-phase system, bonded phases such as naphthylethyl, pyrenylethyl, phenyl, diphenyl and triphenyl were evaluated. First, 100% *n*-hexane was used as the mobile phase and the relative retention times for all tocopherols are listed in Table II. It is apparent that the pyrenylethyl, phenyl, diphenyl and triphenyl bonded phases did not give sufficient retention to enable separation of the four tocopherols, especially β- and γ-tocopherols. However, with the naphthylethyl bonded phase column, the tocopherols seem to be completely separated, and an unique elution order was observed. Generally, the elution order to tocopherols in a normal-phase system is

TABLE II

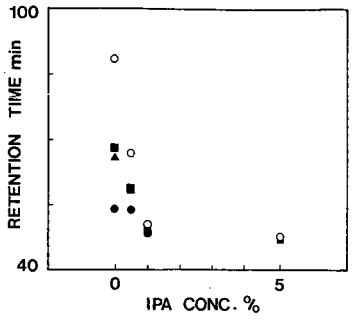
RELATIVE RETENTION TIMES OF TOCOPHEROLS IN THE NORMAL-PHASE SYSTEM USING 100% HEXANE AS THE MOBILE PHASE

Stationary phases are numbered as in Table I.

Stationary phase	*Relative retention time*		
	β/α	γ/α	δ/α
1	1.258	1.218	1.635
2	1.145	1.156	1.447
3	1.212	1.215	1.433
4	1.112	1.125	1.224
5	1.008	1.012	1.020

α-, β-, γ- and δ-tocopherols, respectively[6–11]. However, on the naphthylethyl bonded phase column the order is α-, γ-, β- and δ-tocopherols. This indicates the existance of some effect of the naphthylethyl surface bonded phase on the retention, because the shape of the aromatic moieties on the stationary phase surface is planar and they are readily polarizable; thus some π–π interactions are expected. Consequently, the naphthylethyl bonded phase should be more retentive for aromatic substances, particularly in certain biochemical separations and for molecules having rigid three-dimensional structures, *e.g.*, tocopherols. Finally, it appears that, on the naphthylethyl bonded phase column, γ-tocopherol seems to be more bulky or planar than β-tocopherol, although in the normal-phase system generally the elution order is that of decreasing sample size. However, the γ- and β- order as seen on the naphthylethyl phase is the same as that found by Lin and Horning[13] in gas chromatography using an open-tubular glass capillary column, coated with a polar phase PZ-176, on which the tocopherols were separated as their trimethylsilyl ether derivatives.

Further evaluation of the separation of tocopherols in the normal-phase system was carried out by using a naphthylethyl bonded phase column (750 mm × 0.53 mm I.D.), because this is very promising for the separation. The effect of the mobile phase composition on the resolution was evaluated with various concentration rates of isopropanol in *n*-hexane. The results are shown in Fig. 1, where the relationship between the retention times of tocopherols and the isopropanol concentration in the mobile phase is illustrated. These results indicated that isopropanol is not suitable as a modifier for tocopherol separation on the naphthylethyl bonded phase column. Therefore, the separation of tocopherols was then performed using hexafluoroisopropanol in *n*-hexane as the mobile phase. The relationship between the retention times of the tocopherols and the hexafluoroisopropanol concentration in the mobile phase is shown in Fig. 2. It is seen that good separation should be achieved at 0, 0.05 and 0.1% hexafluoroisopropanol in the mobile phase. The time required for the analysis at 0.1% hexafluoroisopropanol is shorter than that at 0 or 0.05% hexafluoroisopropanol. In addition, at 0 and 0.05% hexafluoroisopropanol the tocopherols peaks show some tailing. It is concluded that 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase gives the best separation of tocopherols on the naphthylethyl bonded phase column.



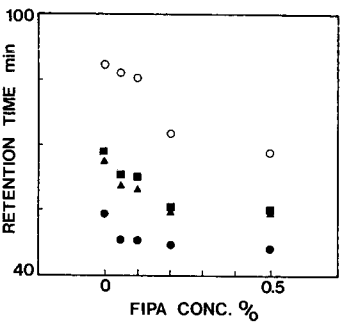


Fig. 1. Relationship between the retention times of tocopherols and the isopropanol (IPA) concentration in *n*-hexane as the mobile phase on a naphthylethyl bonded phase column (750 mm × 0.53 mm I.D.). ●, α-; ■, β-; ▲, γ- and ○, δ-tocopherol.

Fig. 2. Relationship between the retention times of tocopherols and the hexafluoroisopropanol (FIPA) concentration in *n*-hexane on a naphthylethyl bonded phase column. Details as in Fig. 1.

A longer column of the naphthylethyl bonded phase (1000 mm × 0.53 mm I.D.) was then tested using 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase. Fig. 3 shows the resulting chromatogram of the separation of tocopherols.

Although the reversed-phase system offers some practical advantages, *e.g.*, column stability, reproducibility of retention times and rapid equilibration, the retention on this type of support is essentially governed by the number of carbon atoms in a molecule. Therefore, this system generally fails to resolve some positional isomers[1]. Nevertheless, the separation of tocopherols using reversed-phase systems has been attempted[10,11] and the tocopherols were easily separated into α-, β- plus γ- and δ-forms.

In this work, the separation of tocopherols using a reversed-phase system has been atempted using various stationary phases. We sought the optimum separation system for β- and γ-tocopherols. No separations have been observed on bonded phases such as phenyl, diphenyl, triphenyl, naphthylethyl and pyrenylethyl with various mobile phase systems. Therefore, C_{18} phases were evaluated, namely two polymer-based C_{18}, polymeric C_{18}, monomeric C_{18} and polymer-coated C_{18} phases. First, 100% methanol as the mobile phase was tried and the relative retention times of the tocopherols are summarized in Table III. From these results it appears that β- and γ-tocopherols do not have sufficient difference in retention for their good separation. 100% Acetonitrile was then tried as the mobile phase and the relative retention times of the tocopherols are listed in Table IV. It is apparent that only the polymeric C_{18} column gave a reasonable separation, although the tocopherols peaks showed tailing.

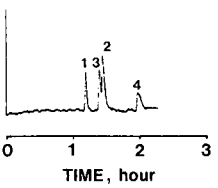


Fig. 3. Chromatogram of tocopherols on a naphthylethyl bonded phase column with 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase. Peak assignment: 1 = α-; 2 = β-; 3 = γ- and 4 = δ-tocopherol.

TABLE III

RELATIVE RETENTION TIMES OF TOCOPHEROLS IN THE REVERSED-PHASE SYSTEM USING 100% METHANOL AS THE MOBILE PHASE

Stationary phases numbered as in Table I.

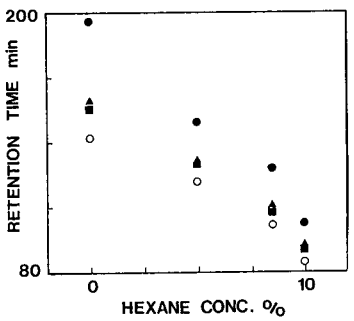
Stationary phase	*Relative retention time*		
	β/α	γ/α	δ/α
6	0.926	0.932	0.840
7	0.878	0.872	0.760
8	0.856	0.875	0.815
9	0.860	0.850	0.732
10	0.878	0.871	0.760

TABLE IV

RELATIVE RETENTION TIMES OF TOCOPHEROLS IN THE REVERSED-PHASE SYSTEM WITH 100% ACETONITRILE AS THE MOBILE PHASE

Stationary phases numbered as in Table I.

Stationary phase	*Relative retention time*		
	β/α	γ/α	δ/α
6	0.980	0.986	0.978
7	0.969	0.964	0.890
8	0.773	0.790	0.708
9	0.946	0.943	0.774
10	0.844	0.842	0.710



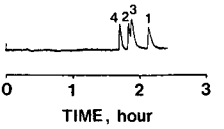


Fig. 4. Relationship between the retention times of tocopherols and the *n*-hexane concentration in acetonitrile as the mobile phase on a polymeric C_{18} column (750 mm × 0.53 mm I.D.). Other details as in Fig. 1.

Fig. 5. Chromatogram of tocopherols on the polymeric C_{18} column with 8.5% *n*-hexane in acetonitrile as the mobile phase. Peaks numbered as in Fig. 3.

From these results, the separation of four tocopherols on the polymeric C_{18} column was investigated using various mobile phase compositions, *e.g.*, acetonitrile–methanol, acetonitrile–THF and acetonitrile–hexane. Acetonitrile–methanol and acetonitrile–THF could not separate β- and γ-tocopherols, although there was no peak tailing. Acetonitrile–hexane gave a good separation of tocopherols.

The longer polymeric C_{18} column (length 750 mm) was employed to improve the resolution, using various concentration ratios of *n*-hexane in acetonitrile as the mobile phase. The result is shown in Fig. 4, where the relationship between the retention of tocopherols and the *n*-hexane concentration in the mobile phase is demonstrated. These results indicate that the best separation should be achieved with a mobile phase of 8.5% *n*-hexane in acetonitrile. A chromatogram of the separation of tocopherols in the reversed-phase system using the polymeric C_{18} phase is presented in Fig. 5.

CONCLUSION

The separation of tocopherols on various chemically bonded phases in microcolumn liquid chromatography has been investigated. For the normal-phase system, a naphthylethyl bonded phase column with 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase gave the best separation. The elution order of the tocopherols was unique, namely α-, γ-, β- and δ-tocopherol. A polymeric C_{18} column with 8.5% *n*-hexane in acetonitrile as the mobile phase gave the best results in various reversed-phase systems, the four tocopherols being well separated. Based on the results, a reversed-phase separation of tocopherol isomers is proposed.

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Note

Simplified protein hydrolysis with methanesulphonic acid at elevated temperature for the complete amino acid analysis of proteins

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(First received March 25th, 1988; revised manuscript received May 23rd, 1988)

The importance of an accurate and convenient method of amino acid analysis for proteins and peptides is becoming increasingly obvious with the recent developments in biotechnology and protein engineering. The amino acid analyzer based on ion-exchange chromatography developed by Moore and Stein[1,2] has remained the method of choice. The most commonly used conventional protocol of Hirs *et al.*[3] utilizes 6 *M* hydrochloric acid and 110–120°C/24 h for hydrolysis of most proteins. A time-course study of hydrolysis extended to 72 h is always needed to ensure a complete hydrolysis of peptide bonds next to some hydrophobic amino acids such as valine or isoleucine, in addition to correction for losses of some labile amino acids such as cystine, tyrosine, serine and threonine residues in the protein samples. The rate-determining step in a successful determination of amino acid compositions is thus dependent on the careful anaerobic preparation of protein hydrolysates and time-consuming calibration and extrapolation of the data from different time courses of hydrolysis for these amino acids.

The determination of tryptophan by amino acid analysis is non-trivial. More than a decade ago, Simpson *et al.*[4] introduced the non-volatile solvent, 4 *M* methanesulphonic acid containing 3-(2-aminoethyl)indole in place of hydrochloric acid for complete amino acid analysis from a single protein hydrolysate. However, it has not become a standard method compared to the 6 *M* hydrochloric acid/110°C protocol. Recently we have introduced rapid heating for protein hydrolysis using microwave irradiation[5]. During the refinement and application of this new technique, we have determined the temperature inside the microwave oven to be higher than 150°C. This high temperature offers the advantage of shortening the hydrolysis time from 24 h to several minutes. With this finding in mind, we feel that it is important to study the effect of temperatures higher than the conventional 110°C for hydrolysis upon the accuracy of amino acid analyses for some standard proteins. Previous reports on the application of higher temperatures (> 150°C) in peptide and protein hydrolysis have emphasized the use of mixed-acid solvents such as hydrochloric acid–trifluoroacetic acid or –propionic acid[6,7]. In this report the use of a high temperature and shorter time has been applied to the amino acid analysis of proteins with a single non-volatile acid, 4 *M* methanesulphonic acid, which yields amino acid composition data including tryptophan and half-cystine. Some simplification and increase in speed relative to the conventional protocol of employing vacuum-sealed tubes in the 6 *M* hydrochloric

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acid/110°C hydrolysis can be achieved without loss of accuracy and performance in amino acid analyses of proteins and peptides.

EXPERIMENTAL

Materials and equipment

The Pyrex reusable hydrolysis tubes (4 mm I.D.) were ordered from the local glass shop according to the design shown in Fig. 1. Each tube can contain up to 5.0 ml of solution without contact with the tip of the poly(tetrafluoroethylene) (PTFE) sealing cap. In practice, less than 1 ml of hydrolysis solvent is added to the tube for the preparation of the protein or peptide hydrolysate. Each PTFE cap contains three sealing O-rings to ensure complete leak-free operation during high-temperature heating. Our design differs from the commercially available reusable hydrolysis tubes (Pierce Chemical Company, Rockford, IL, U.S.A.) in that it is based on the inert-gas flushing instead of the vacuum-sealing procedure for the removal of oxygen inside the tubes. Therefore one long arm of the Pyrex tube with a wide bore is used as the inlet of the flushing gas and the short arm opening with a small bore as the outlet of the inert gas (Fig. 1).

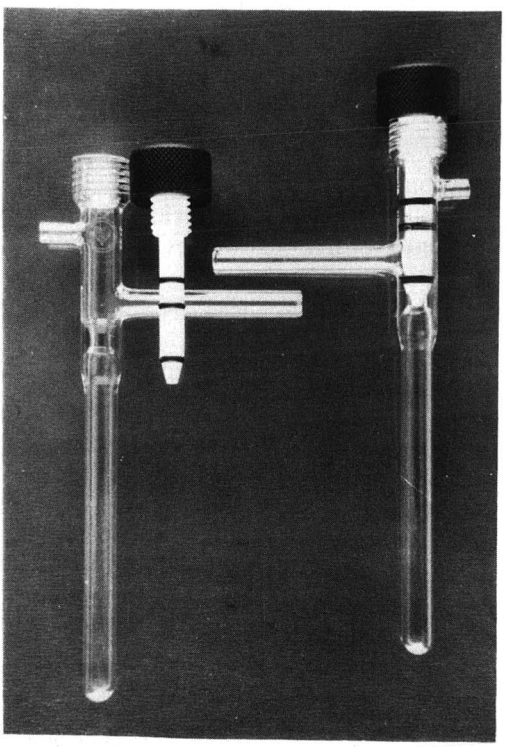

Fig. 1. The design of reusable Pyrex hydrolysis tubes used for the dry heating block. The PTFE cap contains three sealing O-rings to ensure leak-free operation during high-temperature heating. The long-arm opening is the inlet used for flushing the tube with inert gas before heating. The short arm opening with a small bore is the outlet for the flushing gas.

A Reacti-Therm dry block heating system (Pierce) was used as the heat source and was capable of reaching up to 165°C at maximum setting. The deviation of the temperature caused by the surrounding air current could be controlled to 0.5–1°C by wrapping the whole block with aluminium foil.

4 *M* Methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole and 3 *M* mercaptoethanesulphonic acid in 1-ml ampoules were obtained from Pierce. Native chicken egg white lysozyme and porcine heart lactate dehydrogenase were from Sigma (St. Louis, MO, U.S.A.). Cardiotoxin from the venom of *Naja naja siamensis* (Thailand cobra) was isolated, purified and sequenced in this laboratory[8].

Sample preparation and hydrolysis protocol

Hydrolyses were carried out in the acid-cleaned and dry reusable hydrolysis tubes. Protein samples (0.2–0.5 mg) were dissolved in 0.2–0.5 ml methanesulphonic acid. The tubes with samples were each flushed with pure nitrogen gas for 1 min with gentle shaking. Then the outlet of the tube was closed with a finger and sealed by screwing down the PTFE cap. The tubes were put in the dry heating block at the pre-set temperature of 160°C for 45 min unless otherwise stated. At the end of heating, the acid-digested mixtures were pipetted into acid-cleaned vials and partially neutralized with 8 *M* sodium hydroxide (about 20–30 μl of base per 200 μl of methanesulphonic acid); the final mixture was checked with pH-indicator paper to the colour range corresponding to pH 2. The reaction mixture becomes cloudy when it is basic. The pH-adjusted hydrolysate was diluted in the citrate buffer (pH 2.2), filtered and applied to the LKB-4150 amino acid analyzer using a single-column system.

Conditions for amino acid separation on an LKB-4150 analyzer

The buffer formulations and the three buffers of successively higher pH were similar to those employed in the conventional protocol for the hydrochloric acid hydrolysate except that the sodium borate buffer of pH 10.0 was used as the third buffer instead of the citrate buffer of pH 6.45 (LKB-4150 elution program No. 10). Tryptophan was eluted between the peaks of histidine and lysine. The quantitation of tryptophan and other amino acids was precalibrated with a standard mixture of seventeen amino acids plus reagent-grade tryptophan. The instrument was equipped with an automated peak integrator. The correction factor for the colour constant of tryptophan was estimated as 72% of that for lysine based on a previous calibration of the 570-nm detector. The amino acid composition data including half-cystine and tryptophan were compared with the theoretical amino acid residue numbers reported for these proteins based on their sequences.

RESULTS AND DISCUSSION

Although conventional anaerobic hydrolysis of purified proteins with 6 *M* hydrochloric at 110°C for 24 h has been widely used for more than 30 years, a time-course study of the hydrolysis must be performed to obtain an accurate estimation of labile amino acids plus other stable amino acids such as isoleucine, valine and leucine[9,10]. Ideally, the less amount of sample preparation and hydrolysis time required, the lower is the risk of amino acid degradation during hydrolysis[11]. Therefore we have taken a systematic approach of a high temperature and

TABLE I

AMINO ACID ANALYSIS OF LYSOZYME UNDER DIFFERENT CONDITIONS

Data are expressed as the number of residues per molecule of protein using alanine as the reference. Values represent the mean of triplicate determinations. Hydrolysis conditions: 4 *M* methanesulphonic acid, 110°C/24 h or 160°C for different times using Pyrex reusable tubes flushed with nitrogen and dry heating block. The values in the parentheses are the theoretical residue numbers of various amino acids predicted from the protein sequence.

Amino acid	*110°C/24 h*	*160°C for*			
		15	*30*	*45*	*90 min*
1/2Cys	6.6 (8)	5.7	6.2	7.7	7.0
Asx	22.2 (21)	19.6	20.3	21.9	22.4
Thr	6.2 (7)	6.0	6.3	6.8	6.1
Ser	8.1 (10)	8.6	8.5	8.9	8.2
Glx	5.2 (5)	4.5	5.0	5.1	5.3
Pro	2.4 (2)	1.3	1.7	2.4	2.3
Gly	12.5 (12)	10.7	11.2	12.2	11.9
Ala	12	12	12	12	12
Val	5.3 (6)	4.2	4.7	5.8	6.1
Met	1.8 (2)	1.7	1.8	1.9	1.9
Ile	5.0 (6)	4.6	5.0	5.2	5.9
Leu	8.3 (8)	6.7	7.3	7.9	7.9
Tyr	3.5 (3)	2.6	2.7	3.5	2.7
Phe	2.8 (3)	1.8	2.3	3.3	3.0
His	0.8 (1)	0.4	0.6	0.6	0.8
Lys	5.3 (6)	4.2	4.8	6.1	5.8
Arg	11.6 (11)	9.5	9.9	11.5	10.7
Trp	5.8 (6)	5.4	5.6	5.6	5.4

improvements in the pre-hydrolysis sample preparation to achieve accurate and comparable results to those of the conventional protocol[12]. Unexpectedly, we found that, by employing a higher temperature and a shorter time in the hydrolysis of proteins with methanesulphonic acid as originally proposed by Simpson *et al.*[4], accurate results for all the amino acids, including tryptophan and half-cystine, were obtained from a single hydrolysate.

Fig. 1 shows the reusable hydrolysis tubes which are designed for the purpose of inert-gas flushing and high-temperature hydrolysis using a dry heating block. Previous experience has shown the applicability of this convenient alternative to that of the conventional one using disposable vacuum-sealed tubes and an oven at 110°C[12]. The reason for adopting 160°C and 45 min instead of 110°C and 24 h is that in general the hydrolysis rate doubles for every 10°C increase in temperature according to the theory of the temperature dependence of reaction rates[13]. For example, the time required for the heating at 160°C will be 24 h/2^5 = 0.75 h to ensure a similar peptide-bond hydrolysis at 110°C and 24 h.

In this study the recoveries of most amino acids at the higher temperature and shorter time were similar to those obtained from the conventional protocol (Table I), in accordance with the correlation of temperature and hydrolysis rate. Table I also shows that the minimum heating time for lysozyme hydrolysis using 4 *M* methanesulphonic acid at 160°C is more than 30 min since the hydrolyses for 15 and 30 min

clearly indicated incomplete recoveries of several amino acids. In general the recoveries of the amino acids fall within a constant range of 97–102% at 160°C for 45 min using this non-volatile solvent, with the exception of hydrophobic amino acids such as valine and isoleucine. The lower yields of these two amino acids in the conventional 6 *M* hydrochloric acid/24 h procedure necessitate additional hydrolyses for longer times, which also holds true for the present protocol. Hydrolysis at 160°C for 90 min improves the yields for these two amino acids at the expense of good recoveries for cysteine and tryptophan. However the results from methanesulphonic acid hydrolysis are not inferior to those of the conventional protocol, and methanesulphonic acid offers the additional advantage of a reliable estimation of cysteine and tryptophan. Another striking feature of high temperature hydrolysis for a shorter time is the prevention of serine, threonine and tyrosine from destruction which occurs during prolonged hydrolysis (Table I).

In the original protocol for methanesulphonic acid hydrolysis proposed by Simpson *et al.*[4], proteins were hydrolyzed *in vacuo* at 115°C for 22, 48 and 72 h similar to the conditions of conventional 6 *M* hydrochloric acid hydrolysis. In this report we show that the adoption of a higher temperature and proportionally less time gives data similar to those from the old protocol. In addition we found that cysteine could also be estimated without the chemical modification of sulphydryl groups by carboxymethylation[4] or performic acid oxidation[3]. The method described here is also superior to that of Penke *et al.*[14]. who used 3 *M* mercaptoethanesulphonic acid for the determination of tryptophan in proteins. Fig. 2 shows the elution patterns for lysozyme analyses with these two different solvents. About 90% of cysteine residues were recovered after hydrolysis at 160°C for 45 min with 4 *M* methanesulphonic acid, whereas these residues were completely destroyed under the same conditions of hydrolysis with 3 *M* mercaptoethanesulphonic acid. A recent report by Ng *et al.*[15] on

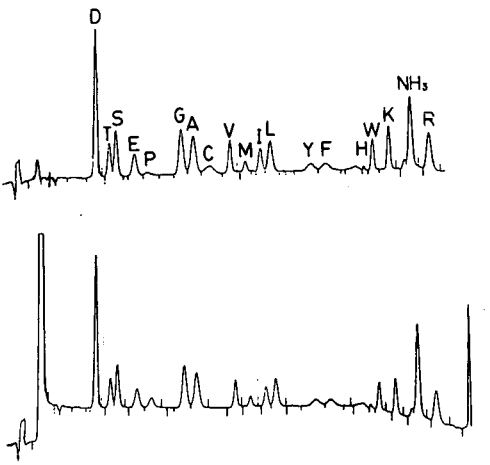

Fig. 2. Amino acid separation patterns of the lysozyme hydrolysates on an LKB-4150 analyzer. The buffer formulation and conditions are as described in Experimental. (Top) Amino acid analysis of lysozyme hydrolyzed with 4 *M* methanesulphonic acid. (Bottom) Amino acid analysis of lysozyme hydrolyzed with 3 *M* mercaptoethanesulphonic acid. The peaks of the various amino acids eluted are denoted with one-letter symbols. Note the complete absence of the cystine peak from the lower figure.

TABLE II

AMINO ACID ANALYSIS OF LACTATE DEHYDROGENASE AND CARDIOTOXIN

Data are expressed as the number of residues per molecule of protein based on the total residues of 333 and 60 for lactate dehydrogenase and cardiotoxin, respectively. Values represent the mean of triplicate determinations. Hydrolysis conditions: 160°C for 45 min using 4 *M* methanesulphonic acid. The values in the parentheses are the theoretical residue numbers of amino acids predicted from the protein sequences.

Amino acid	*Lactate dehydrogenase*	*Cardiotoxin*
1/2Cys	4.8 (5)	7.7 (8)
Asx	36.4 (36)	8.1 (8)
Thr	13.4 (14)	2.8 (3)
Ser	24.8 (26)	2.7 (3)
Glx	33.7 (33)	0.2 (0)
Pro	10.7 (11)	3.9 (4)
Gly	24.6 (24)	1.9 (2)
Ala	21.2 (20)	2.1 (2)
Val	39.1 (39)	3.9 (4)
Met	8.7 (9)	3.3 (3)
Ile	22.1 (23)	3.6 (4)
Leu	36.7 (36)	5.9 (6)
Tyr	6.2 (7)	2.0 (2)
Phe	4.9 (5)	1.0 (1)
His	6.8 (7)	0.3 (0)
Lys	24.6 (24)	8.1 (8)
Arg	8.1 (8)	1.9 (2)
Trp	6.1 (6)	0.1 (0)

the use of hydrochloric acid hydrolysis of proteins in the presence of 0.4% 2-mercaptoethanol for the determination of tryptophan by reversed-phase high-performance liquid chromatography (HPLC) also encountered the problem of destruction of cysteine residues. The presence of sulphydryl reducing groups in the hydrolysis solvent probably also acts as a catalyst in the oxidation of cysteine and other labile amino acids during the process of high temperature hydrolysis.

In conclusion, high temperature hydrolysis with 4 *M* methanesulphonic acid has been successfully applied to the amino acid analysis of peptides and proteins. The salient feature of this protocol is that the complete amino acid content including the determination of cysteine and tryptophan is obtained with a single protein hydrolysate without previous chemical modification of the proteins. The hydrolysis time has been shortened to 45 min from 24 h without loss of accuracy and performance. The method should prove to be of great value in the analysis of other large and small proteins as shown by our analysis of porcine lactate dehydrogenase (140 000 daltons) and snake venom toxin (7000 daltons) (Table II). With the recent advancement of instrumentation for amino acid analysis or HPLC there is a great advantage to shortening the pre-analysis hydrolysis process with the potential of on-line automation of the complete process of amino acid analysis starting from the preparation of protein hydrolysates.

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Note

High-performance liquid chromatography of chloroplast pigments

One-step separation of carotene and xanthophyll isomers, chlorophylls and pheophytins

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Owing to their light-harvesting and protective functions[1], the chlorophylls and carotenoids of the photosynthetic membrane are of key interest in studies on thylakoid architecture[2], chloroplast development[3] and senescence[4], adaptation to environmental conditions[5] and effects of pollution-induced stress[6]. In higher plants, the pattern of plastid pigments is highly conserved. In addition to the porphyrin-derived pigments chlorophyll *a*, chlorophyll *b* and pheophytin *a*[7], the chloroplasts generally contain β-carotene, lutein, violaxanthin and neoxanthin as major and lutein epoxide and antheraxanthin as minor carotenoids[8]. Zeaxanthin, formed from violaxanthin via antheraxanthin under light[9,10] may be present at different levels depending on the light exposure of the plant. Only a few deviations from this general pattern have been observed; spruce needles, for example, further contain α-carotene[11] and *Lactuca* species and some closely related genera synthesize lactucaxanthin[12]. Thus, the plastid pigments include groups of isomeric carotenoids with either β–β-, or β–ε- or ε–ε-end-groups (*i.e.*, β- or α-carotene, zeaxanthin or lutein or lactucaxanthin, antheraxanthin or lutein epoxide) and cover a wide range of polarity.

In recent years, high-performance liquid chromatographic (HPLC) techniques have facilitated the separation and quantification of plastid pigments, but fast and efficient one-step procedures for higher plants have not yet been fully developed. Clearly, separation procedures developed for carotenoids of similar polarity[13,14] are of little use for mixtures of plastid pigments. The HPLC systems in refs. 15–21 allowed the major groups of plastid pigments to be separated but were either not successful in or not tested for the separation of carotenoid isomers. With other procedures[22,23] carotenoid isomers could be separated, but the pigment extracts examined contained no chlorophylls. In one study[24] more than 40 chlorophyll and carotenoid pigments from phytoplankton species were separated. This procedure covered an extended polarity range and even resolved the highly polar algal pigments chlorophyll *c*, peridinin and fucoxanthin. Lutein and zeaxanthin were partially resolved, but the resolution of α- and β-carotene was not satisfactory.

In this paper a fast one-step separation of chlorophylls and carotenoids from higher plant chloroplast using HPLC is described. By modifying the HPLC system of

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Krinsky and Welankiwar[22] we improved the separation efficiency for chlorophylls *a* and *b* and isomeric carotenoids. Also, we improved the stability of H^+-sensitive pigments during separation. Pigment extracts from plants differing in their carotenoid isomer content were used to demonstrate the separation power of the system.

EXPERIMENTAL

Plant material

Endive (*Cichorium endivia*) and lettuce (*Lactuca sativa*) were obtained from local markets and spruce needles (*Picea abies*) from the shaded lower branches of a 15-year-old tree growing near the Institute. The needle generation of 1987, harvested in October 1987, was examined.

Chloroplasts of *Lactuca sativa* with low or high zeaxanthin content were prepared as follows. *Lactuca sativa* plants were kept in the dark at 4°C overnight to allow for conversion of zeaxanthin into violaxanthin[10], and chloroplasts were prepared from this material as in ref. 25. The chloroplasts were resuspended in 50 m*M* citrate–sodium hydroxide buffer (pH 5.2) and aliquots were incubated for 15 min at room temperature in the dark with or without 15 m*M* ascorbic acid. The former but not the latter condition induced the enzymatic de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin[26,27]. The incubated chloroplasts were sedimented by centrifugation and washed twice with 50 m*M* HEPES–sodium hydroxide buffer (pH 7.5).

Pigment standards for HPLC

Lutein, violaxanthin, neoxanthin and β-carotene, extracted from *Cichorium endivia* leaves, were separated on Kieselgel G plates with hexane–isopropanol–water (100:10:0.25). Commercially available α- and β-carotene were also used.

Chemicals

Acetonitrile, hexane and methanol (Promochem, Wesel, F.R.G.) were of Chrom AR grade. For HPLC, acetonitrile and methanol were filtered through HULP-type and hexane through HAWP-type Millipore filters (pore size 0.45 μm), degassed at reduced pressure and stored under helium. Kieselgel G was purchased from Merck (Darmstadt, F.R.G.) and α- and β-carotene from Sigma (St. Louis, MO, U.S.A.).

Pigment extraction

Pellets of pre-treated and washed lettuce chloroplasts were quantitatively extracted with acetone and the extract was clarified by centrifugation and used immediately for chlorophyll determination[28] and HPLC analysis. Green leaves of *Cichorium endiva* (1 g) were homogenized together with 1 g of calcium carbonate in an ice-cold mortar, suspended in acetone at room temperature and the mixture was quantitatively extracted in an Allihn's glass filter tube (pore size 10–16 μm) (Schott, Mainz, F.R.G.) under gentle suction. Spruce needles (100 mg) were frozen in liquid nitrogen, mixed with 100 mg of calcium carbonate and homogenized for 1 min in the 5-ml cuvette of a dismembrator (Type II) (Bachofer, Reutlingen, F.R.G.). The still frozen powder was suspended in acetone and the mixture was quantitatively extracted and filtered as described above. The pigment extracts (adjusted to 10–20 μg/ml of

chlorophyll *a*) were either used immediately for HPLC analysis or were stored under nitrogen at −20°C in the dark for a few days without pigment alteration.

Liquid chromatographic system

The chromatograph (all components from Waters Millipore, Eschborn, F.R.G.) consisted of two Model 510 pumps, a U6K universal liquid chromatograph injector, a Model 680 automated gradient controller, a Model 490 programmable multi-wavelength detector and Model 740 data module.

Liquid chromatographic conditions

Pigment separations were performed at room temperature on a Waters Nova-Pak C_{18} Radial-Pak cartridge (dimensions 8 × 100 mm, filled with 4-μm spherical particles, end-capped) combined with a Waters RCM-100 radial compression separation system. The cartridge was protected with a Guard-Pak precolumn insert of μBondapak C_{18} (end-capped). The following solvent mixtures were used: (A) acetonitrile–methanol–0.2 *M* Tris–HCl buffer (pH 8.0) (74:6:1); (B_1) methanol–hexane (5:1) or (B_2) methanol–hexane (7:1). For pigment separation, isocratic chromatography with 100% A (from 0 to 4 min) was followed by a linear gradient from 100% A to 100% B (from 4 to 9 min), isocratic chromatography with 100% B (from 9 to 18 min) and a linear gradient from 100% B to 100% A from 18 to 20 min). The flow-rate was 2 ml/min. Pigment samples in acetone (10–20 μl containing chlorophyll *a*, chlorophyll *b* and total carotenoids in approximate amounts of 200, 60 and 60 pmol, respectively) were injected. The back-pressure increased during the runs from initial values of 150–200 p.s.i. ($1 \cdot 10^6$–$1.4 \cdot 10^6$ N m^{-2}) to final values of 300–400 p.s.i. The principal absorbance detector wavelength was 440 nm, which suits both chlorophylls and carotenoids, but 400 nm (to detect pheophytin *a* and acid-treated epoxy-carotenoids) and 280 nm were also routinely checked.

Absorbance spectroscopy

Absorbance spectra were monitored with a DW-2000 spectrophotometer (SLM Instruments, Urbana, IL, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC separation of pigments from three plants selected for differences in their pigment patterns, *i.e.*, *Cichorium endivia*, *Picea abies* and *Lactuca sativa*. The major peaks, *i.e.*, peaks 1, 2, 7, 10, 11 and 13, observed for all three plants, are assigned to neoxanthin, violaxanthin, lutein, chlorophyll *b*, chlorophyll *a* and β-carotene, respectively. Their retention times agreed with those of the purified standards, and their elution sequence corresponded to that observed with various reversed-phase HPLC systems[16,17,18–21]. Our neoxanthin standard (λ_{max} in ethanol 466, 438, 414 and 330 nm) exhibited a well defined fine structure in its visible spectrum but no strong *cis* peak in its UV absorbance spectrum, as reported for 9′-*cis*-neoxanthin[21,29]. The neoxanthin isolated from our pigment extracts by HPLC appears to have the same configuration. This assumption, based on identical retention times, is supported by the well established occurrence of 9′-*cis*-neoxanthin in chloroplasts[21]. A small peak occurring just before neoxanthin for both the neoxanthin

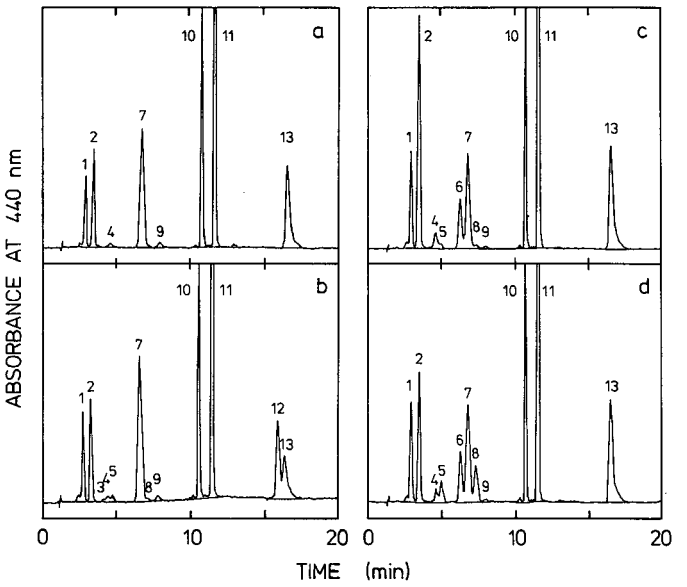

Fig. 1. HPLC separation of pigments extracted from leaves of *Cichorium endivia* (a), needles of *Picea abies* (b) and chloroplasts of *Lactuca sativa* before (c) and after (d) partial de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin. Solvent systems A and B_1 (see Experimental) were used. Peaks: 1 = neoxanthin, 2 = violaxanthin, 3 and 4 = lutein epoxides (?), 5 = antheraxanthin, 6 = lactucaxanthin, 7 = lutein, 8 = zeaxanthin, 9 = ?, 10 = chlorophyll b, 11 = chlorophyll a, 12 = α-carotene, 13 = β-carotene.

standard and plant pigment extracts (Fig. 1) could be strongly enhanced during standard preparation by prolonged drying of the developed TLC plate. Therefore, this compound appears to be, at least in part, an isomerization artifact.

In addition to the generally occurring pigments, α-carotene was resolved from the needles of *Picea abies* (Fig. 1b, peak 12). The ratio of α- to β-carotene is known to vary and to increase in shade-adapted needles[30], as in the material we used. Lactucaxanthin, observed in significant amounts in *Lactuca sativa* chloroplasts[31] was clearly separated from lutein, as shown in Fig. 1c and d (peak 6). Subjection of *Lactuca sativa* chloroplasts to violaxanthin de-epoxidation (Fig. 1d) allowed peak 5 to be assigned to antheraxanthin, the intermediate, and peak 8 to zeaxanthin, the final product of the de-epoxidation sequence[32]. Owing to their retention times, the minor peaks 3 and 4 might tentatively be assigned to lutein epoxides[21], but their identification has not been attempted.

Short-term treatment of pigment extract with 2 m*M* hydrochloric acid caused 99% of the chlorophyll *a*, 13% of the chlorophyll *b*, 81% of the violaxanthin, 40% of the pigments of peaks 3–5 and 9% of the neoxanthin to be converted into pheophytins or 5,8-epoxy-carotenoids[33], respectively, whereas lutein, zeaxanthin and α- and β-carotene were not affected. Fig. 2 shows the acid-induced pigment change and the occurrence of pheophytin b (peak $\overline{10}$) and pheophytin a (peak $\overline{11}$). Two further peaks also detected after acid treatment (Fig. 2b, peaks x_1 and x_2) are assumed to represent 5,8-epoxy-carotenoids.

To obtain the described pigment separations, the original procedure of Krinsky and Welankiwar[22] was subjected to several modifications. We added 1.25% of 0.2 *M* aqueous Tris buffer (pH 8) to solvent system A to remove traces of acid (brought in by acetonitrile) and to protect the highly acid-labile pigments chlorophyll *a* and violaxanthin from conversion. This modification was a prerequisite for reproducible quantification of the pigments (S.D. < 2%). Also, we changed the step gradient in ref. 22 to a 5-min linear gradient, which allowed the time interval between the elution of chlorophyll *b* and chlorophyll *a* to be trebled. Finally, we lowered the hexane content in solvent system B, thus improving the lifetime of the cartridge and also the separation efficiency of the system for chlorophylls and carotenes. Comparison of Figs. 1b and 2a demonstrates the latter effect for identical pigment extracts. On lowering the hexane content in solvent system B further (methanol–hexane: 5:1 for Fig. 1 and 7:1 for Fig. 2) each of the chlorophyll peaks was resolved into a major and a minor peak, comparable to those observed in ref. 21. While the retention times of α- and β-carotene increased, their separation was also improved.

While searching for separation procedures for higher plant pigments, we chose to optimize the HPLC system in ref. 22 rather than that in ref. 24 because it worked with one rather than two columns, with solvent systems of relatively low viscosity and, as a consequence, with back-pressures far below the rated limit of the RCM columns.

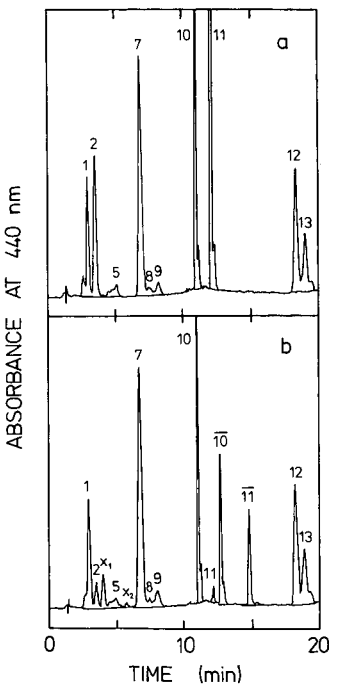

Fig. 2. HPLC separation of a pigment extract from *Picea abies* before (a) and after (b) acid treatment. Solvent systems A and B_2 (see Experimental) were used. For acid treatment, the pigment solution in acetone (0.96 ml) was mixed with 0.1 *M* hydrochloric acid (0.02 ml) and immediately neutralized with 0.2 *M* Tris–HCl buffer (pH 8.0) (0.02 ml). Peaks 1–13 as in Fig. 1; 10 = pheophytin *b*, 11 = pheophytin *a*, x_1 = ?, x_2 = ?

In spite of the simpler experimental setting up, an improved resolution of the critical isomer pairs lutein–zeaxanthin and α-–β-carotene was obtained here.

The described HPLC procedure for the one-step separation of chloroplast pigments, requires less than 20 min and should help in the elucidation of several questions of physiological significance, such as pheophytin formation, the activity of the violaxanthin de-epoxidation and zeaxanthin epoxidation reactions and the regulation of α- and β-carotene levels in α-carotene-containing plants.

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Note

Isocratic reversed-phase high-performance liquid chromatographic analysis of pigments in Norway spruce

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The analysis of plant pigments has been one of the major subjects of research in plant science, especially plant physiology and biochemistry, for many years. The degradation of plant pigments due to pollution has been investigated by many groups. Discoloration of leaves, especially yellowing on the sunny upper sides of leaves, has been found to occur in forest trees in various parts of West Germany, most prevalent in Bavarian Forests at high altitudes. The mechanisms of yellowing are still unclear. It has been suggested that the yellowing of needles or leaves may be due to magnesium deficiency[1], photo-oxidation by ozone[2] or the accumulation of UV-light-absorbing growth-inhibiting substances such as *p*-hydroxyacetophenone (*p*-HAP) and its β-D-glucopyranoside (*p*-HAPG), especially in the needle epidermis[3–5]. In numerous physiological and biochemical investigations, improved methods of analysis of plant pigments, especially for forest trees, have been urgently required to handle a large number of samples.

Until now, plant pigment analysis has been mainly carried out by thin-layer chromatography and/or high-performance adsorption or high-performance gradient reversed-phase liquid chromatography (HPLC)[6–8]. Although high-performance adsorption chromatography gives good separations of plant pigments, losses of samples due to irreversible adsorption and/or frequent cleaning of the adsorption columns or re-packing of the columns with new adsorption material are major obstacles to its efficient and reliable use. Gradient reversed-phase HPLC suffers from problems due to baseline drift and longer durations of equilibrium in comparison with isocratic reversed-phase HPLC. Therefore, a quantitative method based on isocratic reversed-phase HPLC for plant pigment analysis was investigated.

This paper describes the isocratic reversed-phase HPLC determination of various pigments in needles of Norway spruce plants exposed to ozone, and treated with *p*-HAP + UV light in the nanogram range of pigment analysis.

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EXPERIMENTAL

Plant materials

Needles were detached from current year shoots of Norway spruce plants, *Picea abies* (L.) Karst. (experiment 1, 3.5 years old, clone no. 1027, supplied by Pflanzgarten Laufen, harvest September 1986; experiment 2, 4 years old, clone no. 14, supplied by Pflanzgarten Laufen, fumigated with different concentrations of ozone, harvest April and December 1986).

Authentic samples

Authentic samples of β-carotene (HPLC purity, 86.67%), chlorophyll *a* (HPLC purity, 100%), chlorophyll *b* (HPLC purity, 84.29%), lutein (HPLC purity, 97.49%), phaeophytins *a* and *b* and zeaxanthin (HPLC purity, 70.15%) were obtained from C. Roth (Karlsruhe, F.R.G.) and Serva (Heidelberg, F.R.G.).

Treatment of plants with p-HAP + UV light and long-term low-level ozone

p-HAP + UV light. The current-year shoots of 3-year-old healthy Norway spruce plants were cut (three shoots per treatment), 20 mg of *p*-HAP per shoot were dissolved in water and the dissolved *p*-HAP was fed to each shoot through the cut surfaces for *ca.* 7 h (40 klux, 30°C, relative humidity 45%). The amount of substance left after feeding was dissolved in 200 μl of ethanol and injected into the cut surfaces of each shoot. The control shoots were fed simultaneously only with water and subsequently injected with 200 μl of ethanol. The shoots treated with *p*-HAP were simultaneously exposed to UV light (λ = 254 nm, 8 W) for *ca.* 69 h.

Long-term low-level ozone exposure. Long-term low-level ozone exposure of Norway spruce plants was performed in the continuous fumigation chambers of the MAGL Project, GSF, Munich, F.R.G.

High-performance liquid chromatography with ultraviolet detection

The analysis was performed on a Kontron HPLC system (T-414 HPLC pump, Uvikon 722LC detector) and a Beckman HPLC system (114M HPLC pump solvent delivery module, absorbance detector).

For the separation of Norway spruce pigments using the Kontron HPLC system two Kontron Spherisorb ODS-2 columns connected in series (each 250 mm × 5 mm I.D., 5 μm particle size) were connected with a Vydac 201 SC precolumn (50 mm × 5 mm I.D., 30–40μm particle size) and the absorbance was monitored at 400 nm (experiment 1). Under these conditions, the detection limit of chlorophyll *a* and *b* was *ca.* 30 ng.

Norway spruce pigments were separated further using the Beckman HPLC system with two Altex Ultrasphere ODS columns (each 250 mm × 4.6 mm I.D., 5 μm particle size) connected with an MN cartridge (50 mm × 5 mm I.D., 30–40 μm particle size) and the absorbance was monitored at 430 nm (experiment 2). Under these conditions, the detection limit of chlorophyll *a* and *b* was *ca.* 10 ng. The data were acquired and processed by a Basic-programmable Shimadzu integrator Chromatopack C-R3A). The chromatographic conditions are given in the figure legends.

Preparation of plant samples for measurement by HPLC

The plant samples (0.2–0.5 g, needles from current year's shoots, experiment 1) or 9–10 needles (0.007–0.03 g, needles from 1-year-old main shoot, experiment 2) were finely homogenized in water and extracted with cold (−18°C) 100% acetone [+ 100 ppm butylated hydroxytoluene (BHT) as antioxidant, experiment 1] or homogenized in 100 µl of eluting solvent using a micro-grinder at room temperature each time prior to injection (experiment 2). The extracts were filtered, dried *in vacuo* at −18°C (experiment 1) or dried under a stream of nitrogen at room temperature (experiment 2). The prepared samples were diluted in the ratio 1:200 to 1:100, equivalent to *ca.* 1–5 mg fresh weight of needles (experiment 1), or in the ratio 1:15 to 1:10, equivalent to *ca.* 0.5–3 mg fresh weight of needles (experiment 2), isooctane being added to the dissolving eluting solvent in the ratio 1:3. Further, 10 µl of isooctane were added to the sample for injection in order to ensure a rapid on-line delivery of non-polar pigment samples from the precolumns (experiment 2). No influence of BHT could be detected in the analysis under the conditions described (experiment 1).

RESULTS AND DISCUSSION

β-Carotene, chlorophyll *a* and *b*, lutein and zeaxanthin could be detected at 400 (experiment 1) and 430 nm (experiment 2). Owing to the non-availability of an absorbance detector system for 445 nm in our laboratory, we used the above wavelengths for the quantification of various pigments. In our work on the levels of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin, we observed no significant

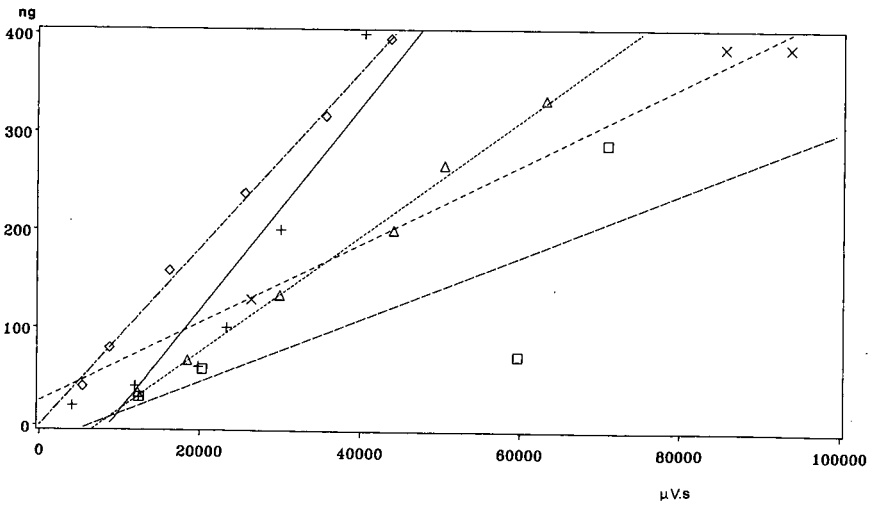

Fig. 1. Test of linearity of the measurement of β-carotene (×), chlorophyll *a* (+), chlorophyll *b* (△), lutein (□) and zeaxanthin (◇) by isocratic HPLC [flow-rate, 1 ml/min; λ = 430 nm; t = 0.2 s; mobile phase, methanol–isooctane–acetonitrile (9:1:0.05)]. The linearity of the measurements of β-carotene, chlorophyll *a*, chlorophyll *b*, lutein and zeaxanthin can be described by the regression lines $y = 24.76 + 0.0040224x$ (r^2 = 0.99), $y = -84.97 + 0.0102688x$ (r^2 = 0.85), $y = -42.97 + 0.0059128x$ (r^2 = 0.99), $y = -22.03 + 0.0032962x$ (r^2 = 0.63), and $y = 0.03 + 0.0090737x$ (r^2 = 1.0), respectively, where y = ng of substance and x = µV s.

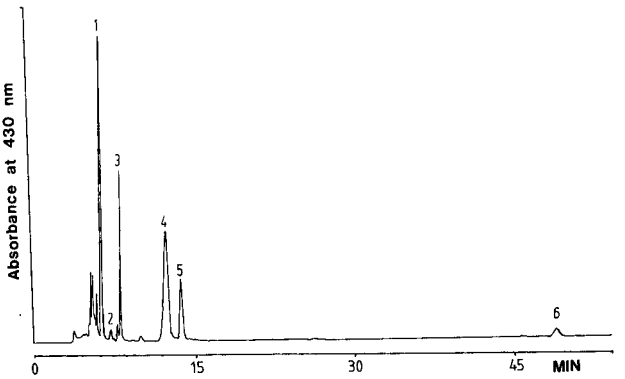

Fig. 2. Separation of authentic samples of lutein (1; 301 ng), zeaxanthin (2; 119 ng), chlorophyll *b* (3; 139 ng), chlorophyll *a* (4; 8 ng), phaeophytins (5; 3.84 μg) and β-carotene (6; 386 ng).

TABLE I

RETENTION TIMES OF PIGMENTS

Compound	*Retention time (min)*
Lutein	5.9
Zeaxanthin	6.7
Chlorophyll *b*	7.7
Chlorophyll *a*	13.5
β-Carotene	47.7

TABLE II

PRECISION AND ACCURACY OF THE HPLC MEASUREMENT OF β-CAROTENE, CHLOROPHYLL *a* AND *b*, LUTEIN AND ZEAXANTHIN BY HPLC IN NEEDLES OF NORWAY SPRUCE

Sample	*Sample size (n)*	*Substance*	*Average ($\bar{x}$) (μg/g fresh weight)*§	*Standard deviation (μg/g fresh weight)*	*Precision** *(%)*	*Accuracy*** *(%)*
Authentic samples	3	β-Carotene	128.79	0.08	0.06	0.18
	4	Chlorophyll *a*	100.00	2.43	2.43	4.47
	5	Chlorophyll *b*	112.38	11.71	10.42	14.46
	5	Lutein	100.22	0.81	0.80	1.11
	5	Zeaxanthin	39.63	0.92	2.32	3.22
Plant samples***	3	β-Carotene	200	10	0.05	0.15
	4	Chlorophyll *a*	740	30	3.53	6.49
	4	Chlorophyll *b*	390	20	4.65	8.55
	5	Lutein	6650	10	0.12	0.17
	4	Zeaxanthin	50	10	21.28	39.14

* Precision = coefficient of variation (C.V., %) = standard deviation × 100)/$\bar{x}$.

** Accuracy ($S\bar{x}$, %) = (C.V. × t)/$(n-1)^{\frac{1}{2}}$, where t = t-value of two-tailed Student's t-test at p = 0.05 and $n-1$ degrees of freedom.

*** Samples from experiment 2 (fumigated with 40 ppb of ozone).

§ Authentic samples (in μg).

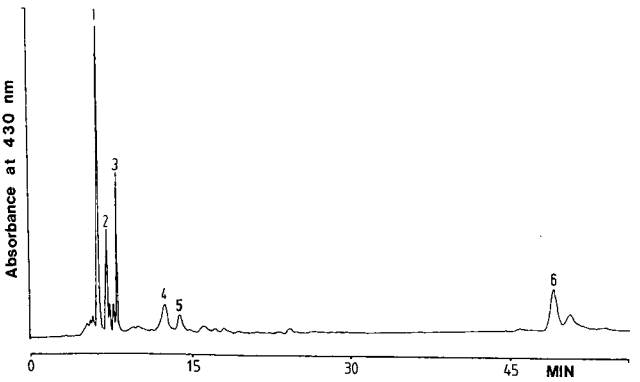

Fig. 3. Analysis of lutein (1), zeaxanthin (2), chlorophyll *b* (3), chlorophyll *a* (4), phaeophytins (5) and β-carotene (6). An aliquot of the diluted extract equivalent to 1.45 mg fresh weight was injected for measurement. The chromatogram shown was obtained from needles of an ozone-exposed Norway spruce plant (fumigated with 40 ppb of ozone for 118 days).

interferences at these wavelengths from contaminants in the 100% acetone or eluting solvent extracts of Norway spruce needles.

Fig. 1 shows the linearity of the measurements of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin by HPLC and Fig. 2 illustrates the separation of the authentic compounds. The separation of these substances from plant material is depicted in Fig. 3. The pigments were eluted in the order lutein, zeaxanthin, chlorophyll *b*, chlorophyll *a*, β-carotene (Table I).

Quantification was performed using the external standard method and the results are summarized in Tables I–III. The precision and accuracy of the HPLC measurements shown in Table II demonstrate the good reliability of the method. Table III illustrates the changes in contents of chlorophyll *a* and *b* in needles of current year's shoots in response to treatment with *p*-HAP + UV light (experiment 1) and the changes in the contents of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin in the needles in relation to low-level ozone exposure (experiment 2).

CONCLUSIONS

The results show that the determination of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin can be conveniently carried out by isocratic reversed-phase HPLC. The merits of the method are convenient, rapid measurement (8–10 samples per day), stable baseline, rapid cleaning of the column system and short equilibrium period, sensitive measurement in the nanogram range, low cost per measurement and measurement of different pigments in the same run. Re-filling of precolumns is usually required after about 40 measurements in order to ensure high reproducibility and reliability. A further increase in sensitivity for the quantitative measurement of plant pigments could be achieved by using an absorbance detector system at 445 nm.

TABLE III

CONTENTS OF LUTEIN, ZEAXANTHIN, CHLOROPHYLL *a* AND *b* AND β-CAROTENE IN NEEDLES DETERMINED BY HPLC

Treatment	*Concentration (ppb)*	*Exposure (days)*	*Plant No.*	*Units**	*Lutein*	*Zeaxanthin*	*Chlorophyll a*	*Chlorophyll b*	*β-Carotene*
Ozone	20	118	1	a	237.44	23.65	970.33	19.45	0
	20		1	b	0.41	0.04	1.64	0.03	0
	20		2	a	166.23	15.25	938.14	22.14	8.58
	20		2	b	0.56	0.05	3.16	0.07	0.03
	40		3	a	39.10	0	633.72	82.29	–
	40		3	b	0.06	0	0.98	0.13	–
	40		4	a	29.35	13.25	374.98	291.77	–
	40		4	b	0.73	0.03	0.93	7.21	0.03
	20	238	1	a	321.47	17.45	455.76	267.70	14.60
	20		1	b	0.29	12.24	0.42	0.24	0.01
	20		2	a	146.84	11.71	103.51	122.51	89.71
	20		2	b	0.24	0.02	0.17	0.20	0.15
	40		3	a	191.65	1.82	249.96	179.57	89.72
	40		3	b	0.24	0.002	0.32	0.23	0.11
	40		4	a	192.55	9.62	241.88	154.64	34.57
	40		4	b	0.34	0.02	0.43	0.27	0.06
p-HAP + UV light	0**		1	a			161.67	16.90	
			2	a			19.17	0.51	
			3	a			17.43	–	
	20**		4	a			42.42	1.93	
			5	a			34.68	0.19	
			6	a			20.15	0.38	

* a, μg/g fresh weight; b, μg per needle.
** mg *p*-HAP per current year's shoot.

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Note

Simultaneous detection of trichothecenes and rosenonolactone in grape juice and wine by capillary gas chromatography

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The fungus *Trichothecium roseum* Link ex Fr. produces several mycotoxins of the trichothecene group, trichothecin and trichothecolone being the major toxins[1,2]. Both are toxic against fungi, some bacteria, viruses, eucaryotic cell cultures and mammals[1,3–6]. In addition *T. roseum* produces some diterpenelactones, of which rosenonolactone is the main component[6]. The biological activity of this metabolite has been reported recently[7].

Because of the increasing occurrence of *T. roseum* on graphes during the last few years it was necessary to examine the contamination of grape juices and wines with these mycotoxins. Trichothecin inhibits the alcoholic fermentation. It is stable during the usual treatment or storage of wine[8]. A method for simultaneous detection of trichothecene and rosenonolactone contaminations in grape juice and wine was developed.

EXPERIMENTAL

Materials

Trichothecin and rosenonolactone were extracted from culture filtrates of *Trichothecium roseum* Link ex Fr. (isolate 5388) and purified by liquid chromatography on silica gel. Trichothecolone was prepared from trichothecin by alkaline hydrolysis with 1 *M* potassium hydroxide. The substances were identified by mass spectroscopy and ^{1}H NMR spectroscopy.

All chemicals (analytical grade), silica gel (40 μm), octadecylsilane bonded silica gel disposable columns (SPE, volume 6 ml) and the vacuum extraction system were obtained from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

The capillary gas chromatography was carried out with a Model 8320 instrument (Perkin-Elmer, Überlingen, F.R.G.) equipped with a capillary column (CPMS, OV-225, 0.32 mm I.D.) and flame ionization detection (FID). Hydrogen served as the carrier gas (150 kPa) and the split mode of injection (20 ml/min) was used. The column temperature was increased from 140 constant (for 5 min) to 240°C constant (for 15 min) at 10°C/min, then to 280°C (constant for 1 min). The temperature of the injector and the determination zone were 230 and 300°C, respectively. For the peak integration, a Perkin-Elmer Sigma 10B data system was used.

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Extraction

An octadecyl extraction column was conditioned with methanol and water according to the instructions of the supplier. A sample of 25 ml grape juice or wine was aspirated through the column using the vacuum extraction system. The lipophilic toxins were eluted from the dry column with 1.5 ml chloroform into a graduated vial, concentrated by evaporation of the chloroform and redissolved in methanol to a final volume of 200 μl. These extracts were used for all analyses without further clean-up. For the quantitative analysis of trichothecolone, samples of 25 ml were extracted two times each with 10 ml chloroform in a separation funnel and the methanol extract was obtained as described before. Recovery experiments were carried out by adding 113–2800 μg/l trichothecin, 106–158 μg/l trichothecolone and 100–500 μg/l rosenonolactone to untreated grape juice and wine. To investigate the stability of the toxins, methanolic extracts of different toxin concentrations were stored at 4°C for 2 weeks.

Analysis

A linear calibration graph was obtained by measuring a number of toxin standards with an increasing concentration from 10 to 300 μg/ml. The toxins in the methanolic extracts of grape juice and wine (5 μl injected) could be quantified in a range of 50–2600 μg/l for trichothecin, 50–800 μg/l for trichothecolone and 100–480 μg/l for rosenonolactone by peak area integration. The limits of the detectable amounts of toxins were found by adding 100, 50 and 20 μg/l of each toxin to grape juice and wine. To detect substances interfering with the toxins in the analysis, extracts from 20 wine varieties, *e.g.*, Riesling, Silvaner were analysed.

RESULTS AND DISCUSSION

The rapid method using reversed-phase silica gel for the extraction of the liphophilic toxins from grape juice and wine was suitable for quantitative separation of trichothecin and rosenonolactone from the matrix. To detect trichothecolone, which was recovered in a range between 30 and 40%, the method is only suitable for qualitative analysis. The quantitative extraction of trichothecolone was accomplished by means of a separation funnel (Table I).

The gas chromatographic method allowed the separation of the toxins from the multi-component matrix without any derivatization of the extracts. The reproducibility was measured by injecting ten times the same extract with the toxins at about

TABLE I

RECOVERY (%) OF THE TOXINS FROM GRAPE-JUICE AND WINE

Means and standard deviations from twelve eperiments.

	Grape juice	*Wine*
Trichothecin*	77.0 (8.9)	93.2 (5.6)
Trichothecolone**	64.7 (9.1)	75.4 (11.7)
Rosenonolactone*	82.5 (7.8)	93.7 (3.2)

* Column extraction (C_{18}).

** Separation funnel method.

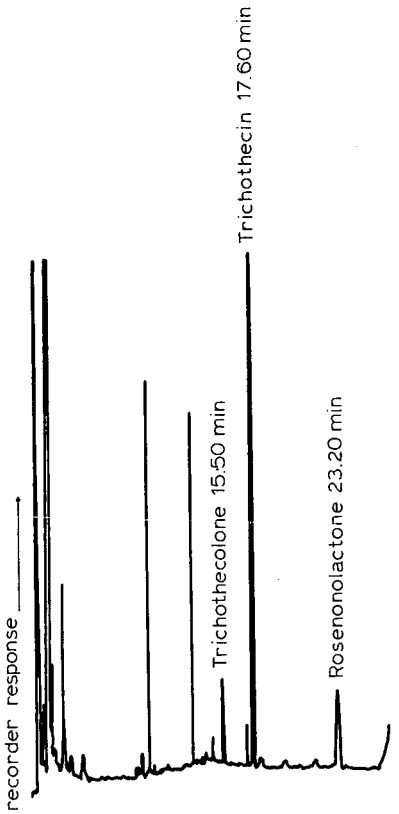

Fig. 1. Capillary gas chromatogram of wine extract (Ruländer), contaminated with mycotoxins.

200 μg/ml, which resulted in analysis differences of less than 7%. The detection limit was 50 μg/l. The analysis of every wine extract was done twice. The retention times were 15.50 min for trichothecolone, 17.60 min for trichothecin and 23.30 min for rosenonolactone (Fig. 1).

All toxins were stable in methanolic extracts for 2 weeks (differences less than 5%), which was important for storage in laboratory routine analysis.

In the extracts from the twenty wine varieties, no substances were found to interfere with the toxins.

Now we are preparing an extensive wine research programme, in which the C_{18} extraction and the gas chromatographic method will be used to search for trichothecin and rosenonolactone in wines from the German wine growing region "Rheinpfalz".

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Note

Gas chromatographic analysis of underivatized resin acids

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Resin acids are diterpenes of the abietane, pimarane or labdane types. They are characteristic compounds of conifers and often comprise the major portion of the lipophilic extractives.

Gas–liquid chromatography (GLC) is the prevailing technique for the analysis of diterpene resin acids. Usually they are converted into their corresponding methyl esters prior to analysis. Holmbom[1,2], Nestler and Zinkel[3] and Foster and Zinkel[4] have reported retention data for resin acid methyl esters on non-polar and polar packed or wall-coated open-tubular capillary columns. Zinkel *et al.*[5] have reported on the GLC of trimethylsilyl (TMS) esters of resin acids and conclude that such esters are rapidly hydrolyzed on most polar liquid phases.

This paper describes the GLC of underivatized resin acids on a non-polar fused-silica capillary column.

EXPERIMENTAL

Chemicals

The following pure resin acid standards were analysed: abietic, dehydroabietic, isopimaric, levopimaric, manoyloxid and pinifolic acids. A Portugese gum rosin was also analysed.

Heptadecanoic acid (10 mg) was added to each sample as an internal standard. A portion of each standard was converted into the corresponding methyl ester with freshly prepared diazomethane in diethyl ether–methanol (90:10, v/v). Prior to the GC analyses the samples were dissolved in hexane–ethyl acetate (80:20, v/v).

Gas–liquid chromatography

A Varian 3700 gas chromatograph equipped with a split/splitless capillary injector and flame ionization detection (FID) was used. The injector was equipped with a glass precolumn (70 mm × 2 mm I.D.) packed with 1.5% SE-30 on Chromosorb W HP (80–100 mesh). A fused-silica capillary column (15 m × 0.25 mm I.D.) with a 0.25-μm film of DB-1 (J & W Scientific) was used. The hydrogen carrier gas flow-rate was 1.60 ml/min (56 cm/s). Analyses were done with a 20:1 splitting ratio. The chromatograph was operated isothermally at 210 or 230°C. The injector and detector temperatures were kept at 300°C. Peak aras relative to the internal standard and retention times were measured with a Varian CDS 111 integrator.

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RESULTS AND DISCUSSION

Typical gas chromatograms obtained for the gum rosin are shown in Fig. 1 and the relative retention times of the resin acids and their methyl esters are given in Table I. The monocarboxylic resin acids were almost equally well resolved underivatized at 230°C as their corresponding methyl esters at 210°C. Although underivatized pimaric–sandaracopimaric and isopimaric/levopimaric/palustric–dehydroabietic acids did not show baseline separation, the resolutions were acceptable. Also the overall analysis time was short, being completed in 5 min. At 230°C the methylated acids were subject to some overlapping. The non-polar DB-1 column did not resolve

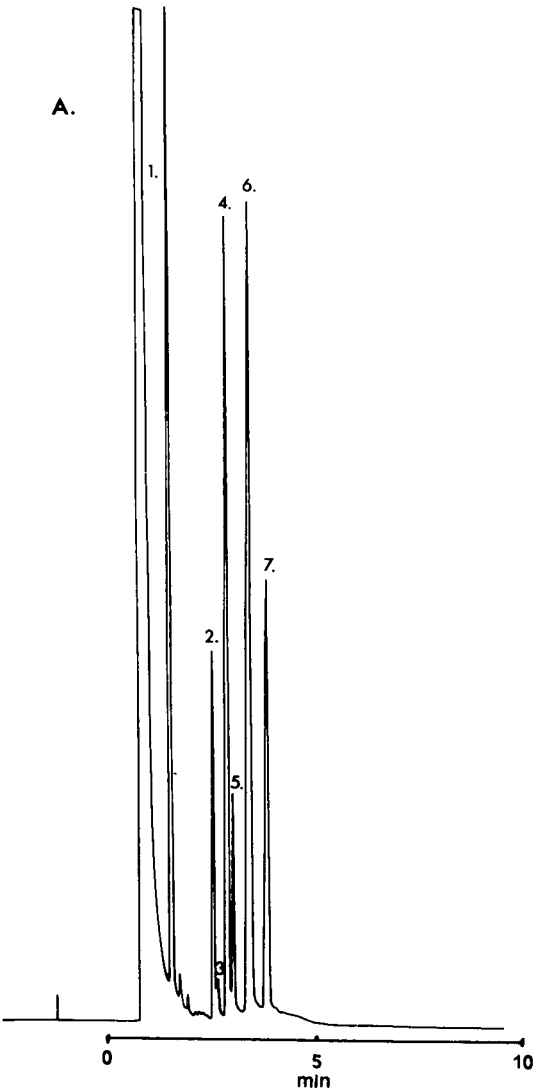

Fig. 1.

(Continued on p. 430)

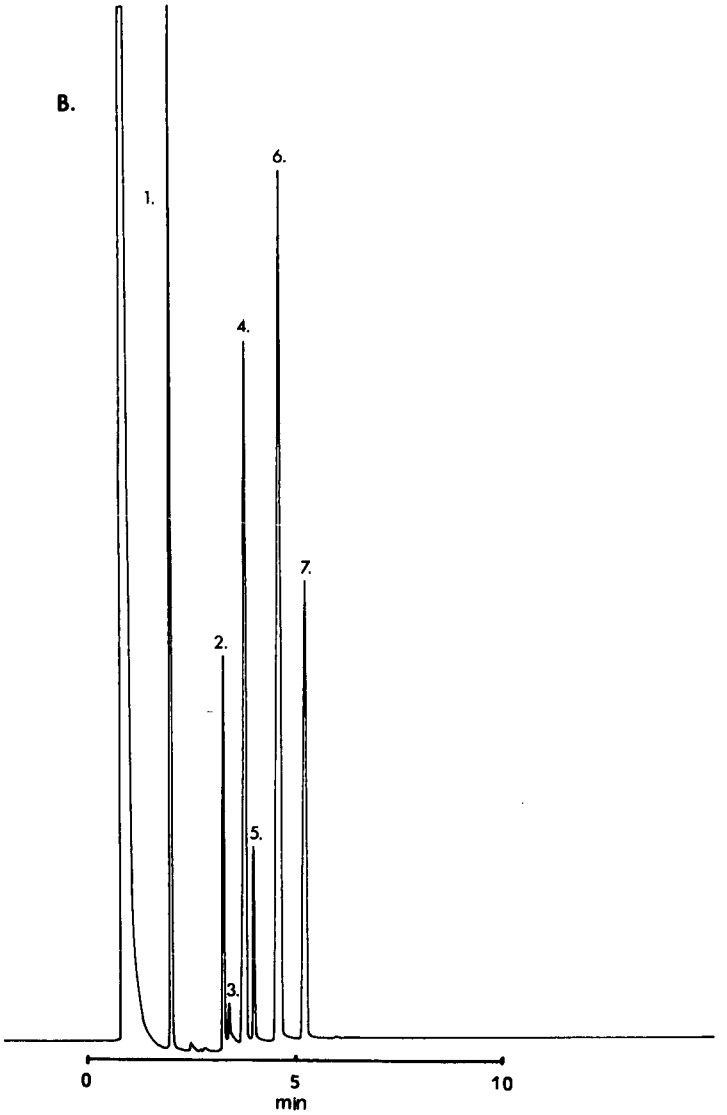

Fig. 1. GC analyses of underivatized resin acids (A) at 230°C and of methylated resin acids (B) at 210°C in Portugese gum rosin. Column: DB-1, 15 m × 0.25 mm I.D. Carrier gas: hydrogen, 1.60 ml/min. Peaks: 1 = heptadecanoic acid (internal standard); 2 = primaric; 3 = sandaracopimaric; 4 = levopimaric + palustric + isopimaric; 5 = dehydroabietic; 6 = abietic; 7 = neoabietic.

isopimaric, levopimaric and palustric acids, underivatized or as their methyl esters. The dicarboxylic pinifolic acid was not eluted unmethylated, even at 300°C.

Tests with the DB-1 column at 230°C gave theoretical plate values for underivatized resin acids of 1700–2100/m and for their corresponding methyl esters at 210°C of 3500–4900/m.

Heptadecanoic acid was shown to be a suitable internal standard for the GC

TABLE I

RELATIVE RETENTION TIMES OF RESIN ACIDS AND THEIR CORRESPONDING METHYL ESTERS ON A DB-1 FUSED-SILICA CAPILLARY COLUMN

Retentions relative to heptadecanoic acid or methyl heptadecanoate

	Relative retention time		
	Resin acid column temp. 230°C	*Resin acid methyl ester column temp.*	
		230°C	*210°C*
Pimaric	1.86	1.64	1.78
Sandaracopimaric	1.92	1.9	1.85
Isopimaric + levopimaric + palastric	2.16	1.86	2.11
Dydroabietic	2.26	1.95	2.23
Abietic	2.63	2.25	2.63
Neoabietic	2.96	2.53	3.01
Manoyloxid	1.88	1.66	1.82
Pinifolic	–	2.83	3.53

analyses of underivatized resin acids because it was completely separated from other compounds of interest in the samples. The quantitative behaviour of the underivatized resin acids and their methyl esters in separate 30-mg samples of Portugese gum rosin is shown in Table II. The values are uncorrected for differences in detector responses. As is seen, the values are in acceptable accordance with each other and some of the divergences must be attributed to poor performance of the split injector in quantitative analyses.

The monobasic resin acids are readily analysed without derivatization by gas chromatography with a non-polar DB-1 fused-silica capillary column using hydrogen as the carrier gas. A column temperature of 230°C seems to be optimal giving acceptable resolution of the most common resin acids (with the exception of isopimaric, levopimaric and palustric acids).

TABLE II

COMPARISON OF QUANTITATIVE BEHAVIOUR OF UNDERIVATIZED AND METHYLATED RESIN ACIDS FROM GUM ROSIN ON A NON-POLAR DB-1 FUSED-SILICA CAPILLARY COLUMN

Values (mean and S.D.) are means from five analyses.

	Composition (%, w/w)	
	Resin acid column temp. 230°C	*Resin acid methyl ester column temp. 210°C*
Pimaric	8.12 (0.08)	8.31 (0.07)
Sandaracopimaric	1.96 (0.02)	1.91 (0.01)
Isopimaric + levopimaric + palustric	24.23 (0.40)	24.99 (0.27)
Dehydroabietic	5.73 (0.10)	4.72 (0.08)
Abietic	39.53 (0.70)	40.58 (0.50)
Neoabietic	20.42 (0.6)	19.50 (0.26)

The use of an appropriate internal standard like heptadecanoic acid gives comparable results for the composition and standard deviation to those obtained by GC of methylated samples of resin acids in, *e.g.*, rosins and oleoresin. A direct method for analysis of underivatized resin acids would be useful for on-line quality control in rosin/tall oil distillation. The major advantage of the method is a decrease in both the preparation and analysis time which minimizes losses and alterations of the samples. The durability of the column has been found not to be affected by injections of underivatized resin acids.

ACKNOWLEDGEMENT

The author is greatful to Professor B. Holmbom for helpful discussions and a gift of the reference sample.

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Note

Assay of methoxyacetic acid in body fluids and tissues by gas chromatography–mass spectrometry following *tert.*-butyldimethylsilylation

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2-Methoxyethanol is a glycol ether widely used in surface coatings and removers, as an anti-icing additive in aviation fuels and in many consumer products. Occupational exposure to this chemical has recently become of great concern because of its teratogenicity and reproductive toxicity in mice, rats and rabbits[1–6]. Evidence has accumulated that the tetragenicity of 2-methoxyethanol is mediated via its metabolite 2-methoxyacetic acid (MAA)[6–9]. Detailed pharmacokinetic studies are necessary for an evaluation of the experimental findings and their significance with regard to the teratogenic risk of exposure of humans to 2-methoxyethanol.

Smallwood *et al.*[10] described a gas chromatographic (GC) method for the assay of MAA using dichloromethane extraction, perfluorobenzylation and flame ionization detection. Although large samples were used (1 ml), the detection limit was 11 μg/ml, which is not sufficiently low for measurements in small samples or for the kinetic analysis of lower concentrations. The GC method by Groeseneken *et al.*[11] is more sensitive, but poor recoveries were obtained.

We have developed an assay method for MAA which employs ethyl acetate extraction of the samples, *tert.*-butyldimethylsilylation of the concentrated extracts and capillary gas chromatographic–mass spectrometric (GC–MS) analysis. The high sensitivity and selectivity of the assay allowed measurements of MAA in small samples of body fluids and tissues.

EXPERIMENTAL

Chemicals and reagents

MAA and the internal standard (*n*-valeric acid) were obtained from EGA-Chemie (Steinheim, F.R.G.), acetonitrile and ethyl acetate (Nanograde) from Promochem (Weser, F.R.G.) and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) from Regis (Günther Karl OHG, Geisenheim, F.R.G.).

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Sample preparation

Serum samples (20–50 μl, depending on the concentrations or sample amounts available) were pipetted into 1.5-ml disposable microtubes. Embryo and other tissue homogenates were prepared by adding 200 μl of distilled water and four small glass

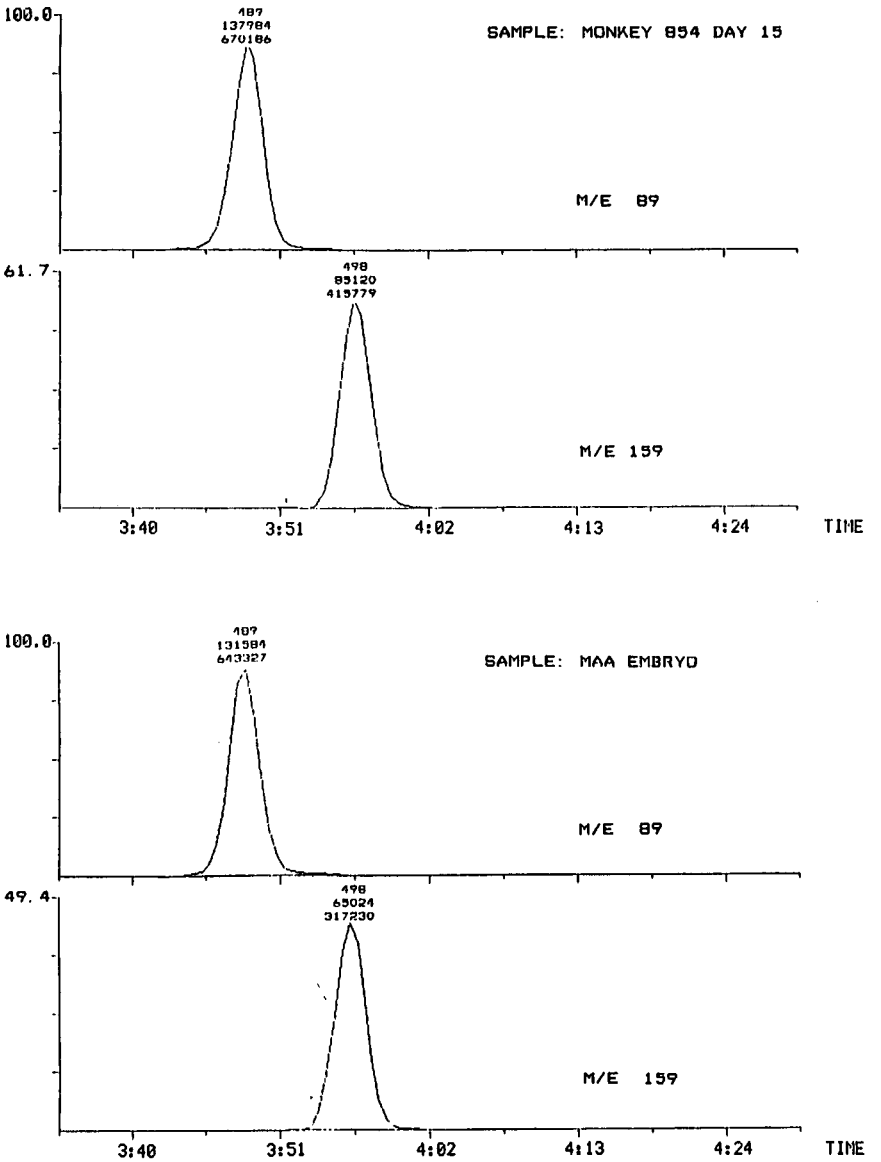

Fig. 1. Selected ion monitoring (*m/z* 89 for MAA; *m/z* 159 for the internal standard, *n*-valeric acid) of extracted and tert.-butyldimethylsilylated samples from (A) monkey treated orally with 0.47 mmol of MAA per kg body weight (50 μl of plasma taken 2 h after drug application containing 115.6 μg·ml/of MAA); (B) monkey embryo following maternal treatment with 0.47 mmol MAA per kg body weight (43 mg wet weight taken 4 h after drug application containing 156 μg MAA per gram of embryo).

pearls (2 mm diameter) to the microtube containing one embryo or a small amount of tissues. The samples were ultrasonicated for 15–30 min at 4°C in an ultrasonication bath to obtain an opaque suspension.

To the serum or homogenates, 50 μl of 1 *N* NaH_2PO_4 buffer (adjusted to pH 5.0), 10 μl of 1 *N* hydrochloric acid and 1.0 ml of ethyl acetate (via a Dispensette from Brand) containing the internal standard (*n*-valeric acid) were added. The tubes were shaken for 20 min and then centrifuged for 2 min at 500 *g* in a Model 5012 Eppendorf centrifuge. An 800-μl portion of the supernatant organic phase was transferred into a 1-ml glass reaction vial and preconcentrated to about 100 μl with a stream of nitrogen, followed by the addition of 100 μl of acetonitrile. The extraction was repeated using 1 ml of ethyl acetate. The combined extracts were evaporated at 30°C under a stream of nitrogen to a final volume of 10–20 μl. The samples were *tert.*-butyldimethylsilylated by adding 20 μl of acetonitrile and 30 μl of MTBSTFA. The samples were allowed to react at room temperature for 20 min or longer prior to analysis by GC–MS. The derivatized compounds were stable for several days in the capped glass vials.

GC–MS analysis

Samples of 1 μl were injected splitless into the GC–MS system (Finnigan-MAT 4600 operated by a 2100D Superincos). The GC separations were achieved using a 30 m × 0.25 mm I.D. bonded phase DB 17 (0.25 μm film thickness) fused-silica capillary column (from ict Handelsgesellschaft, Frankfurt, F.R.G.) with helium as carrier gas (40 cm/s). The initial temperature of 80°C was held for 1 min, then raised at 5°C min^{-1} to 130°C. The injector temperature was 200°C.

The quadrupole mass spectrometer (electron impact, 80 eV electron energy) was operated in the multi-ion detection mode, switching between *m/z* 89 (MAA) and *m/z* 159 (internal standard).

Quantification

Standard samples were prepared by spiking serum from untreated animals directly with MAA at the highest concentration (100 μg/ml). Lower concentrations were prepared by diluting this highest concentration sample with serum. The concentration range was 0.5–100 μg/ml. Calibration graphs were constructed from the data system by plotting the ratios of the peak area of MAA to that of the internal standard.

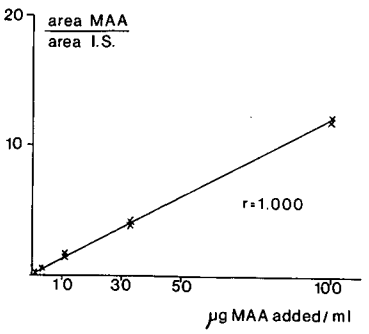

Fig. 2. Calibration graph for determination of MAA.

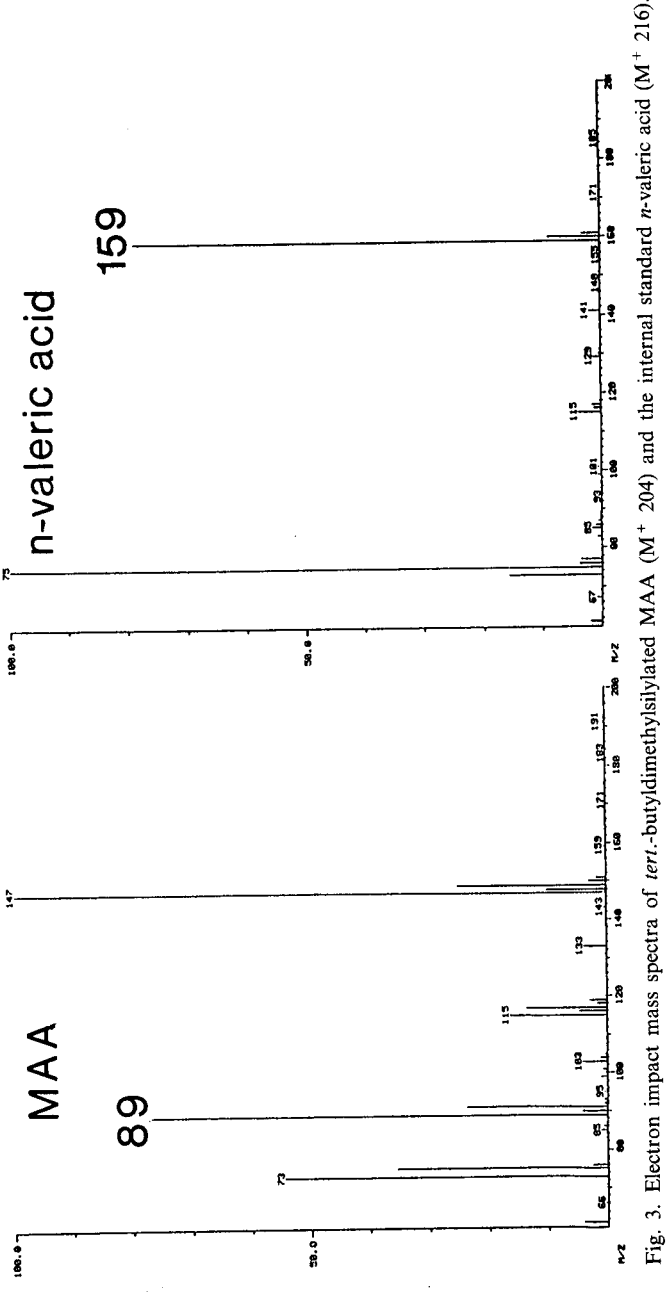

Fig. 3. Electron impact mass spectra of *tert.*-butyldimethylsilylated MAA (M^+ 204) and the internal standard *n*-valeric acid (M^+ 216).

RESULTS

Representative ion chromatograms obtained from the analysis of a monkey serum sample and a monkey embryo homogenate are shown in Fig. 1. The precision of the determination was established by repeated analysis ($n = 10$) of one extracted serum samples all of which were processed through the extraction, derivatization and analysis procedure (<4.3%). The slope of the plot (peak-area ratio *vs.* amount of MAA added) indicated a linear dependence in the concentration range 0.5–100 μg/ml (Fig. 2). The detection limit, using 50-μl samples, was 50 ng/ml. The recovery of the extraction procedure was 90% for serum samples.

DISCUSSION

The derivatives of both MAA and the internal standard exhibited simple mass spectra with few but intense ions, including the M – 57 ions (elimination of *tert.*-butyl group) on electron impact (*m/z* 147 for MAA; *m/z* 159 for *n*-valeric acid) (Fig. 3). We used this M – 57 ion for the measurement of the internal standard (*m/z* 159), but not for MAA, because the ion of *m/z* 147 did not yield an acceptable baseline. Therefore, the ion of *m/z* 89 was chosen for the measurement of MAA because of the very low and stable base values.

The method proved simple to perform, the derivatized samples were very stable and the reproducibility was good. Of great importance is the high sensitivity of the assay: concentrations of 50 ng/ml could be measured with relatively small sample sizes (50 μl).

This assay was applied to the study of the pharmacokinetics of MAA following administration of 2-methoxyethanol to the cynomolgus monkey[12]. Surprisingly, the half-life of the drug was relatively long in this species (around 18–25 h); daily

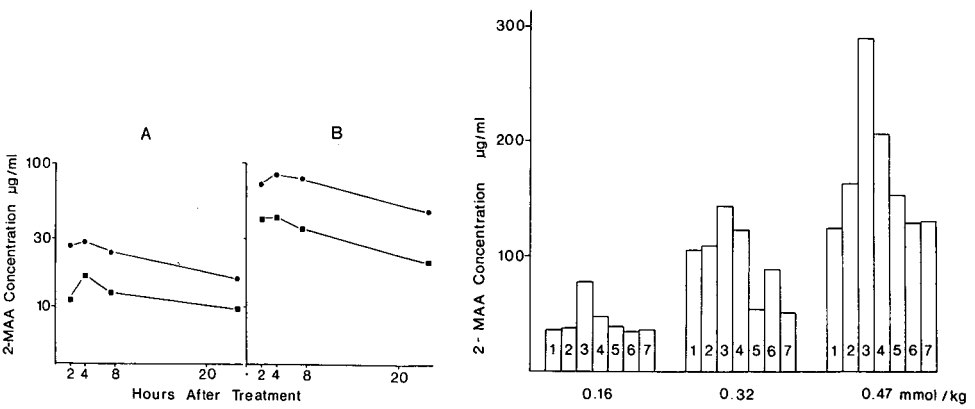

Fig. 4. MAA concentrations in monkey plasma following the first application (■) and after eight daily treatments (●) at two dose levels: (A) 0.16 mmol/kg; (B) 0.32 mmol/kh.

Fig. 5. MAA concentrations in maternal plasma and various gestational tissues and fluids following different doses. Key: 1 = serum maternal; 2 = embryo; 3 = yolk sac; 4 = amniotic fluid; 5 = chorionoc fluid; 6 = placenta secondary; 7 = placenta primary.

administration therefore resulted in considerable drug accumulation (Fig. 4). This finding may be important with regard to human occupational exposure. MAA was also found to cross the placenta in the monkey (Fig. 5). The concentrations in most of the gestational compartments, including the embryo, were similar to those in maternal plasma; the yolk sac exhibited higher concentrations (Fig. 5).

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Note

Separation of pyridone carboxylic acid enantiomers by high-performance liquid chromatography using copper(II)–L-amino acid as the eluent

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Pyridone carboxylic acids such as ofloxacin (OFLX)[1] are important substances in the field of medical treatment. Related compounds are widely used as chemotherapeutic agents in clinical medicine, and OFLX, for instance, is administered clinically as a racemic mixture[2]. When the pharmacological properties of enantiomers differ from each other, there is considerable interest in the chromatographic resolution of the enantiomers for analytical and preparative purposes. A variety of approaches have been used for the resolution of enantiomers by high-performance liquid chromatography (HPLC), which is of especial pharmaceutical interest, but no studies on the chromatographic resolution of the enantiomers of pyridone carboxylic acids have yet been reported. A useful method for the analysis of these isomers is required for the determination of optical purity in pharmaceutical preparations and in biological fluids for pharmacokinetic purposes.

Various methods have been reported for the separation of enantiomeric mixtures[3]. These involves derivatization with chiral reagents[4,5], the use of chiral stationary phases[6] and of chiral eluents[7–9]. Chiral mobile phase methods are particularly simple and can be used with conventional HPLC columns, but their applications have been limited to the separation of amino acids, hydroxy carboxylic acids and their derivative compounds[10].

In this paper, we describe a new, simple and convenient HPLC method for the resolution of the enantiomers of pyridone carboxylic acids using a chiral mobile phase.

EXPERIMENTAL

Reagents

The ofloxacin enantiomers (+)- and (−)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-pyrido(1,2,3-*de*)-1,4-benzoxazine-6-carboxylic acid and their derivatives were kindly donated by the Chemical Research Center of Daiichi Seiyaku.

The amino acids were from Kishida Kagaku (Osaka, Japan). All other chemicals and reagents used were obtained from the usual commercial sources. Water (HPLC grade) was obtained by purification through a Milli Q Purification System (Millipore, Bedford, MA, U.S.A.).

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TABLE I

RESOLUTION OF OFLX ENANTIOMERS WITH VARIOUS LIGANDS

Copper(II) concentration: 1/2 mol of ligand concentration. Methanol concentration: 15%. The pH was not adjusted.

Ligand	*Concentration (mM)*	*Separation coefficient, α*
L-Phenylalanine	6	1.26
L-Isoleucine	6	1.24
L-Leucine	6	1.16
Aspartame*	2	1.14
N-Methyl-L-phenylalanine	2	1.13
L-Valine	6	1.10
L-Tryptophan	6	1.07
L-Methionine	6	1.04
L-Tyrosine	6	1.06
D-Phenylglycine	6	1.00**
Toluenesulphonyl-L-phenylalanine	1	1.00**

* Aspartame is N-L-α-aspartyl-L-phenylalanine 1-methyl ester.
** Not separated.

Chromatographic conditions

The mobile phase contained between 15 and 40% of methanol in water or a 10 m*M* phosphate buffer containing one of nine amino acids (see Table I) and copper sulphate, the concentration of which was 1/2 mol of amino acid (these concentrations are indicated in Table I and the figures). The buffer was adjusted to various pH with phosphoric acid. The flow-rate was 1.0 ml/min and the detection wavelength was 300 nm (UV), or 330 (excitation) and 505 nm (emission) (fluorescence), respectively.

Instrumentation

The chromatographic system consisted of an high-pressure pump (Oriental Motor Model RLD-150) equipped with a Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.), a YMC AM-312 ODS column (15 cm × 6 mm I.D., particle size 5 μm; Yamamura Chemical, Kyoto, Japan), an UVIDEC-100-II spectrophotometric detector (Nihon Bunko, Tokyo, Japan) and a F-1100 fluorescence detector (Hitachi Seisakushyo).

RESULTS

The enantiomers of OFLX and their derivatives were resolved as mixed complexes of Cu(II) and amino acids on a reversed-phase column. The chromatograms are shown with the (+)-(*R*)-isomers more strongly retained. Table I shows the correlation between the ligands added and the separation coefficients, α, of the OFLX enantiomers. Good separation was obtained with L-phenylalanine, but not with phenylglycine and toluenesulphonyl-L-phenylalanine, which have been used for enantiomer analysis[11].

Fig. 1 shows the fluorescence spectra of OFLX in the L-phenylalanine chiral mobile phase (maximum excitation and emission wavelengths were about 330 and 505 nm).

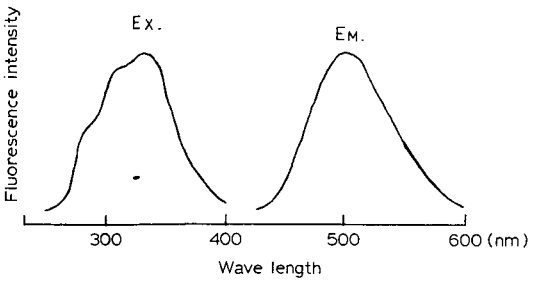

Fig. 1. Fluorescence spectra of OFLX in the mobile phase: 6 m*M* L-Phe, 3 m*M* copper sulphate; the pH level was not adjusted.

The retention and separation behaviour are dependent on the ligand concentration and mobile phase pH (Figs. 2 and 3). Above pH 4.5, copper was deposited as a milky precipitate, and at high ligand concentrations the column was easily damaged. However at higher pH values and ligand concentrations, better resolution was obtained. As in other reversed-phase systems, lowering of the methanol concentration usually resulted in longer retention of the solutes and slightly better resolution of the optical isomers when the percentage of methanol was varied from 12.0 to 18.0%. We therefore decided that the optimum HPLC conditions were as shown in Fig. 4. The detection limit of the optical purity was about 0.1%.

The derivatives of OFLX can also be separated by this method (Fig. 5).

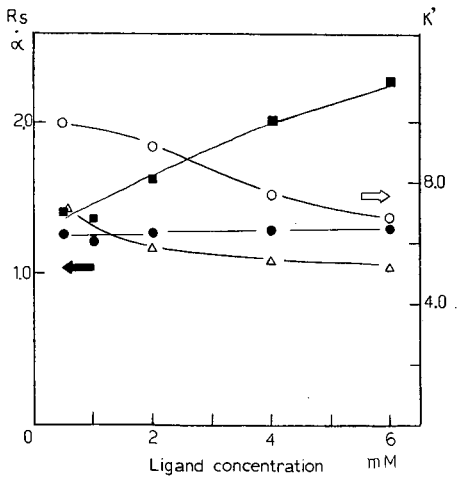

Fig. 2. Effects of the ligand concentration (L-Phe) on the resolution, R_s, separation coefficient, α, and capacity factor, k'. The pH levels were not adjusted and the copper(II) concentration was changed in proportion to the increasing ligand concentration. ■ and ●, R_s and α; ○ and △, (+)-(*R*)- and (−)-(*S*)-isomer.

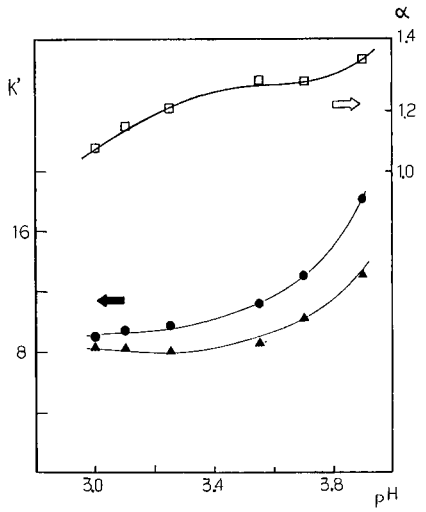

Fig. 3. Effect of the pH of the mobile phase on α and k' (6 m*M* L-Phe, 3 m*M* copper sulphate). □, α; ● and ▲, (+)-(*R*)- and (−)-(*S*)-isomer.

DISCUSSION

It has long been known that the carboxylate and nitrogen of an amino acid can chelate copper(II) ions in a bidentate manner to form bis(amino acidato) complexes; this technique for the isomeric resolution of amino acids is also applicable to keto-carboxylic acids. Table I shows the results of resolution of OFLX enantiomers with various ligands. Among the chiral ligands we studied, both aromatic amino acids

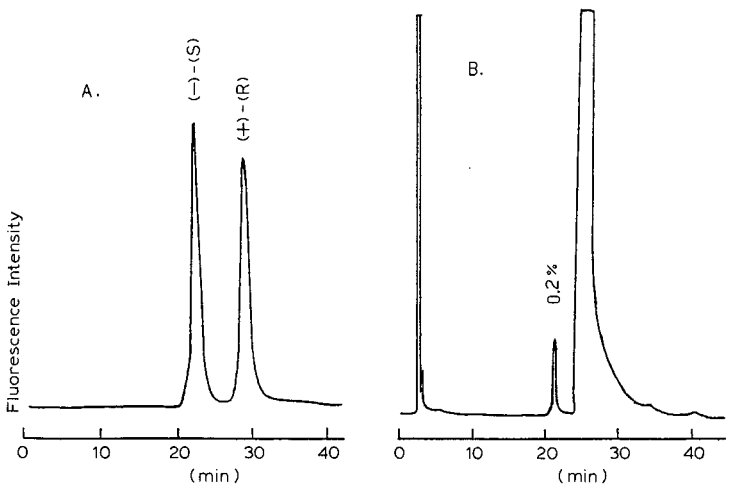

Fig. 4. Typical chromatograms of OFLX enantiomers under the optimum HPLC conditions. Mobile phase: 6 m*M* L-Phe, 3 m*M* copper sulphate (pH 3.5). Detection: fluorescence. Sample amount: (A) about 200 ng as a mixture; (B) about 3 μg as the (+)-enantiomer containing 0.2% (−)-enantiomer.

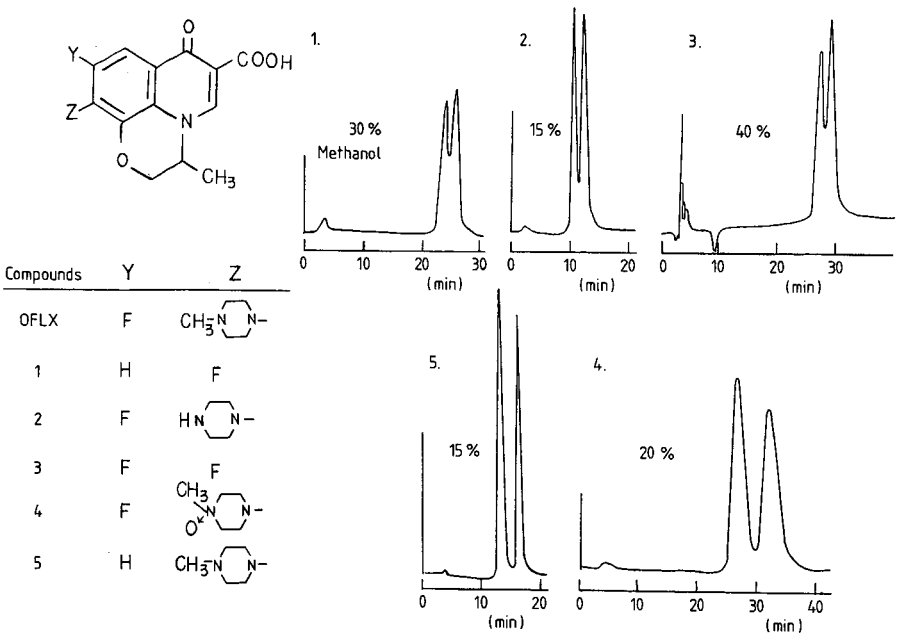

Fig. 5. Chromatograms of OFLX enantiomer derivatives. HPLC conditions: 6 m*M* L-Phe, 3 m*M* copper sulphate; the pH was not adjusted. Methanol concentrations as indicated in the figure. Sample amount: about 200 ng as a mixture.

(*e.g.*, L-Phe) and aliphatic amino acids (*e.g.*, L-Ile) exhibited good separation. With the D-phenylglycine and toluenesulphonyl-L-phenylalanine system, however, less stereoselectivity is observed with the isomeric pairs of OFLX. This seems to be the result of steric hindrance between these ligands and OFLX, which possesses a tricyclic structure. We suggest that ternary complexes of amino acids and OFLX isomers with Cu(II) possess simple structures, as shown in Fig. 6.

OFLX derivatives, which have an asymmetric carbon in the position C-3, are also separated with L-Phe. The substituents of these derivatives do not affect the resolution of the optical isomers.

Since the stereoselectivity is dependent on the stability and the hydrophobicity of the diastereomeric metal complex in the ODS stationary phase, the pH value (Fig. 3), ligand concentration (Fig. 2) and methanol concentration would thus affect both the

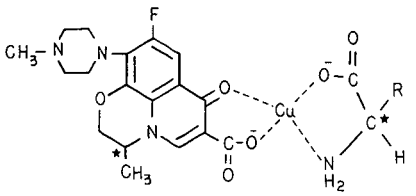

Fig. 6. Proposed structure of the ternary complex of OFLX and amino acid with Cu(II).

capacity factor, k', and selectivity, α, R_s. The extent of complex formation is increased and the resolution of the optical isomers is improved by raising the pH value and ligand concentration. On the other hand, lowering the methanol content in the mobile phase results in longer retention and slightly improved resolution.

In conclusion, a procedure has been described for the separation of pyridone carboxylic acid enantiomers by ligand-exchange chromatography. The compounds of pyridone carboxylic acid enantiomers are directly detectable with UV or fluorescence spectroscopy. This method will be useful and convenient for the simple determination of such enantiomers, for example OFLX, in pharmaceutical preparations and biological fluids.

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CHROM. 20 692

Note

High-performance liquid chromatographic analysis of major carotenoids from *Rhodotorula glutinis*

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A potent intracellular lipid-producing yeast, *Rhodotorula glutinis*, produces carotenoid pigments mainly during the stationary phase of its growth and the characteristics of the carotenoid pigments have been well established and four major carotenoids, *i.e.*, torulene, torularhodin, β-carotene and γ-carotene were identified[1–3].

Until recently, column chromatography has been mainly used for the microbial carotenoids including this organism[2–5]. However, this technique has its limitations such as the large quantity of sample required, poor resolution, incomplete recovery and artifact production[6]. On the other hand, the use of high-performance liquid chromatography (HPLC) offers several advantages over classical chromatography including high effiency, selectivity, speed and mild conditions[7].

Although both plant carotenoids[8–12] and plasma carotenoids[13–15] were analyzed by HPLC, there are few reports on the HPLC analysis of microbial carotenoids except for a few examples[16]. Moreover, to our knowledge, no published methods can quantify all major carotenoids of *Rhodotorula glutinis* simultaneously in one HPLC step.

The purpose of this study was to develop a simple and rapid procedure for the analysis of major carotenoids from *Rhodotorula glutinis*.

EXPERIMENTAL

Microorganism

A strain of the yeast, *Rhodotorula glutinis* NRRL Y-1091, was used. It was maintained and cultured as described by Yoon and Rhee[1].

Samples and reagents

Pigment mixtures from *Rhodotorula glutinis* were prepared as described by Simpson *et al.*[17]. β-Carotene was obtained from Sigma. Other carotenoid standards were prepared by the procedure of Simpson *et al.*[17]. Each standard compound was identified by its position on the column, and by their light absorption curve[17]. Acetonitrile and tetrahydrofuran were of HPLC grade (Merck). All other reagents were of analytical grade (J. T. Baker).

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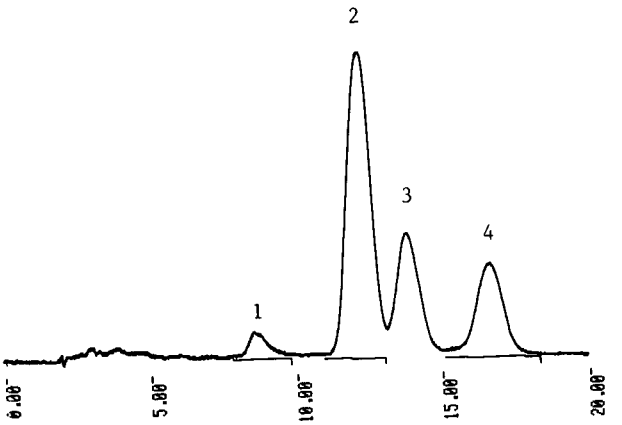

Fig. 1. HPLC chromatogram of carotenoid pigments from *Rhodotorula glutinis*. Conditions: Column, C_{18} Z-module; flow-rate, 2.00 ml/min; eluent, acetonitrile–tetrahydrofuran–water (50:38.5:11.5, v/v/v); detection, 436 nm; sensitivity, 0.05 a.u.f.s. Peaks: 1 = torularhodin; 2 = torulene; 3 = γ-carotene; 4 = β-carotene.

Chromatography

The carotenoid analyses were performed with a Waters Assoc. chromatograph which was equipped with a Waters Model 510 pump and connected to a Waters 740 data module. The column used was Z-module C_{18} and the operating conditions were as follows; elution solvent, acetonitrile–tetrahydrofuran–water 50:38.5:11.5, v/v/v); sample solvent, light petroleum (b.p. 30–70°C); flow-rate, 2.00 ml/min.

The absorption of the carotenoids was measured at 436 nm by a Waters Model 440 absorbance detector. The quantitative analysis was performed with the single-point calibration method using an external standard, the concentration of which was confirmed by a spectrophotometric measurement[17].

RESULTS AND DISCUSSION

Classical column chromatography for the separation of carotenoid components was accompanied by a time-consuming procedure involving saponification, washing, column separation, phase separation, drying and spectrophotometric measure-

TABLE I

AMOUNTS OF CAROTENOID COMPONENTS IN *R. GLUTINIS*

NA = Not available.

No. of injection	*μg Carotenoid component per g cell dry weight*			
	Torularhodin	*Torulene*	*γ-Carotene*	*β-Carotene*
1	1.13	40.73	13.91	21.34
2	1.15	43.70	14.74	21.46
3	0.91	44.01	14.56	NA

ment[2–5]. Compared to this method, pigment mixtures on the HPLC column could be satisfactorily separated in less than 20 min, as shown in Fig. 1. Since a reversed-phase column (C_{18}-Z-module) was used, the compounds were eluted in the order of decreasing polarity: torularhodin, torulene, γ-carotene, β-carotene. Each peak was identified by comparison of its retention time with that of the standard compound, and confirmed by the corresponding peak area, when co-injected with each standard compound. The purity of each standard compound was >95% as determined from the HPLC peak area. The chromatogram of pigment mixtures was reproducible. The reproducibility of the quantification of the carotenoid components is shown in Table I.

In conclusion, HPLC provides an efficient method for the separation and quantification of carotenoid pigments from *Rhodotorula glutinis*. Furthermore, the method may be extended for preparative separation.

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CHROM. 20 710

Note

High-performance liquid chromatography of phytoalexins in stem callus tissues of rapeseed

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(Received May 25th, 1988)

Phytoalexins are defined as low-molecular-weight antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms[1]. Phytoalexins have been isolated and characterized from a number of plant species grown at tissue cultures, among them cajanol from *Cajanus cajan*[2], phaseolin from *Phaseolus vulgaris*[3], glyceolin from *Glycine max*[4] and pisatin from *Pisum sativum*[5]. The ability to synthesize and accumulate phytoalexins is considered to be one mechanism of disease resistance in plants. Inducing resistance in plants by adding non-pathogenic fungi or elicitors could be an economically and environmentally acceptable method for disease control[6]. Methoxybrassinin and cyclobrassinin are two recently reported sulphur-containing phytoalexins from rapeseed tissues challenged with *Pseudomonas cichorii*[7,8], *Alternaria brassicae*[9] and *Leptosphaeria maculans*[10]. Up to now their isolation and quantification has been carried out by thin-layer chromatography (TLC). This report describes a method for rapid purification and quantification of methoxybrassinin and cyclobrassinin by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Seed

Rapeseed (*Brassica juncea*) cv. Cutlass used in the present study was obtained from Agriculture Canada, Research Station, Beaverlodge, Canada.

Fungal isolate

Fungal isolates (*Leptosphaeria maculans*) used in the present studies were isolated from infected rapeseed plants from the Blackleg Nursery maintained near Elgin, Manitoba, Canada.

Elicitation of phytoalexins

Callus tissues were induced from the stem (*Brassica juncea* cv. Cutlass) by standard procedures on Linsmaier-Skoog agar medium, containing 3 mg indole acetic

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acid, 3 mg naphthalene acetic acid and 0.1 mg kinetin per l. Callus tissues were subcultured every 4 weeks on 25 ml medium six times before use.

Leptosphaeria maculans was grown on V8 agar medium for 25–30 days at 20°C. Inoculums were prepared by suspending pycnidiospores in sterile distilled water ($5 \cdot 10^6$ pycnidiospore/ml).

Well grown callus tissues (20 g fresh wt./flask) were infiltrated with 1 ml/flask of the pycnidiospore suspension. The infected callus exhibited a cellular browning response within 8 days of infiltration. The callus tissues, incubated for 16 days after infiltration of *L. maculans* were harvested and freeze dried. The dried material (8 g dry wt.) was extracted with ethyl acetate. The extract was evaporated to dryness *in vacuo* to give 56.8 mg of yellow viscous oil. The oil was dissolved in acetonitrile–distilled water (70;30, v/v) and analysed by HPLC. Prior to analysis, 100 μl isoprenylated genistein (1 mg/ml) was added to each sample as an internal standard.

Abiotic elicitor

A freshly prepared solution of silver nitrate (10^{-3} *M*) was used as an abiotic elicitor of methoxybrassinin and cyclobrassinin. It was applied to stem callus tissues in the same way as pycnidiospore suspensions.

Time course experiment

A time course experiment was conducted to study at what stage phytoalexin accumulation starts. Samples in triplicate were taken out at an interval of 2 days up to 16 days, the tissues were macerated in 70% (v/v) methanol, filtered, the filtrate was dried *in vacuo* at 40°C and the residue was redissolved in acetonitrile–water (70:30, v/v). The experiment was repeated twice. Quantification of the phytoalexins was done by analytical HPLC using the analytical column (Ultramax 5 C_{18}, 25 × 0.4 cm I.D.) with a flow-rate of 1.5 ml/min, and isoprenylated genistein as the internal standard.

Phytoalexin standards

Authentic methoxybrassinin and cyclobrassinin was supplied by Dr. Mitsuo Takasugi, Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, Japan. The internal standard isoprenlated genistein was synthesized by the procedure described elsewhere[11].

Chromatography

The HPLC equipment (Beckman Model 420) was supplied by Beckman Instruments, Toronto, Canada and consisted of an Altex pump (Model 110A) and injection valve. The LC–UV detector was set at 267 nm. A Hewlett-Packard 3390A integrator was used for measuring peak areas. The semipreparative column used was Ultramax 5 C_{18} (25 × 1.0 cm I.D., Terrochem, Edmonton, Canada) and the elution was made at 20°C with acetonitrile–water (70:30, v/v). The flow-rate was 3.0 ml/min. A guard column (4 × 0.4 cm I.D.) packed with LiChroprep RP-18 (35–50 μm particle size) was used to prevent deterioration of the main column. Solvents used were of HPLC grade purchased from Fisher Scientific, Winnipeg, Canada.

Active fractions were defined originally by their ability to inhibit *Cladosporium cucumerinum* in the TLC assay[12] (Fig. 2) and subsequently by their retention time and absorption of light at λ267 nm. Identification of the active fractions was established

through UV, mass (MS) and nuclear magnetic resonance (NMR) spectral analysis reported elsewhere[7,8,10].

RESULTS

Fig. 1 illustrates the HPLC chromatogram of the phytoalexin analysis from crude extract of stem callus tissue infiltrated with the pycnidiospore suspension of *Leptosphaeria maculans*. Two fractions with HPLC retention times of 5.2 and 7.5 min were found to be fungitoxic when bioassayed for antifungal activity using *Cladosporium* Si-gel TLC bioassay[12] (Fig.2). These fractions were identified and characterized as methoxybrassinin and cyclobrassinin (Fig. 3) based on UV, MS and NMR spectral data reported elsewhere[7,8,10].

A time course study of phytoalexin accumulation was performed using *B. juncea* cv. Cutlass stem callus tissues. No phytoalexin was found to accumulate in control tissues treated with sterile water only. Methoxybrassinin synthesis started after 4 days of incubation and the level of methoxybrassinin increased up to 12 days. After that, no further increase was recorded. Cyclobrassinin was detected only after 8 days of incubation and its level increased up to 12 days followed by a decline (Fig. 4). Decline in the level of cyclobrassinin implies metabolism of the compound by host tissues.

Accumulation of both phytoalexins was more in the callus tissues treated with silver nitrate than with fungal infection (Fig. 5). Both phytoalexins were found to accumulate in response to a non-aggressive isolate of *Leptosphaeria maculans* whereas only cyclobrassinin was found to accumulate in response to an aggressive isolate. Phytoalexin accumulation was significantly reduced when a mixture of both aggressive as well as non-aggressive isolates of *L. maculans* was used as inoculum for phytoalexin elicitation implying that a suppressor of phytoalexin synthesis might be secreted by the aggressive isolate.

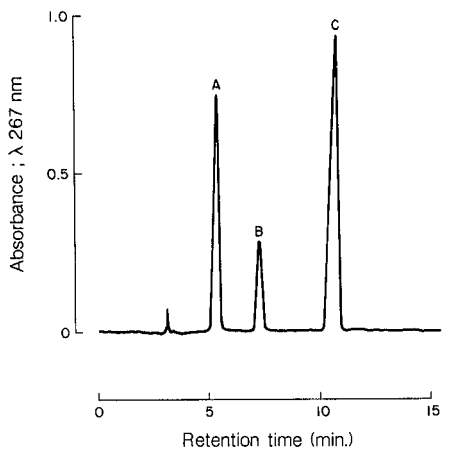


Fig. 1. HPLC trace of phytoalexins (crude extract from stem callus tissues of *B. juncea* cv. Cutlass infected with *Leptosphaeria maculans*). Peaks: A = methoxybrassinin; B = cyclobrassinin; C = isoprenylated genistein (internal standard). HPLC solvent system: acetonitrile–water (70:30, v/v). Flow-rate: 30 ml/min. Column: Ultramax 5 C_{18} (25 × 1.0 cm I.D.).

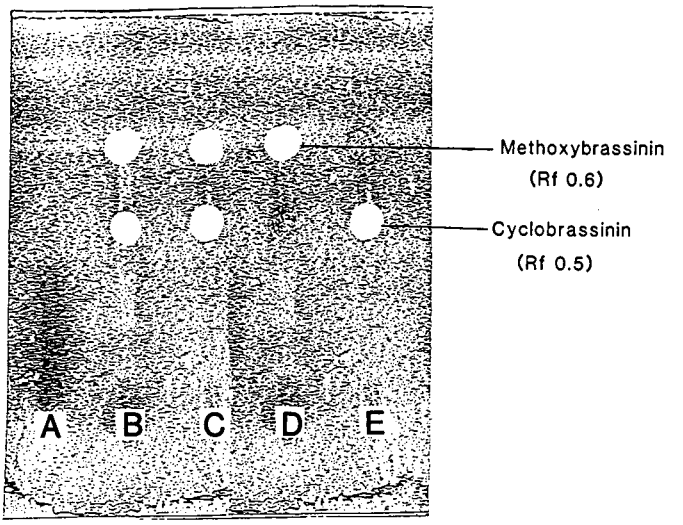
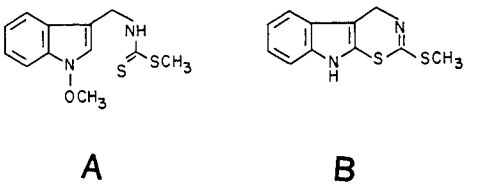


Fig. 2. *Cladosporium* silica gel TLC bioassay. Solvent used: chloroform–methanol (95:5 v/v). A = control tissues extract; B = tissues extract (infected with *L. maculans*); C = tissues extract (treated with silver nitrate); D = methoxybrassinin (HPLC fraction with retention time 5.2 min); E = cyclobrassinin (HPLC fraction with retention time 7.5 min).



A B

Fig. 3. Chemical structures of rapeseed phytoalexins. A: methoxybrassinin; B: cyclobrassinin.

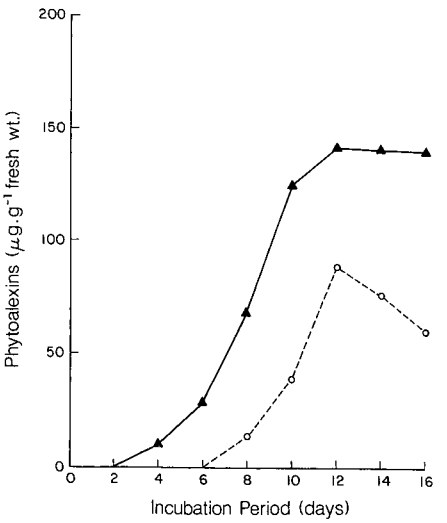


Fig. 4. Time course experiment. (▲—▲) Methoxybrassinin; (○----○) cyclobrassinin.

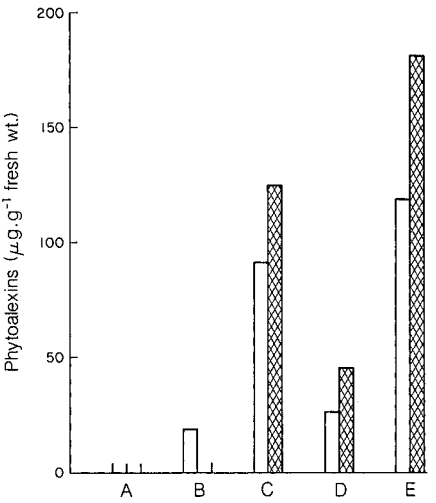


Fig. 5. Phytoalexin accumulation in stem callus tissues of *Brassica juncea* cv. Cutlass in response to fungal infection and treatment with silver nitrate (10^{-3} *M*). Hatched bars, methoxybrassinin; open bars, cyclobrassinin. A = Control tissues (sterile water treatment); B = callus tissues infected with an aggressive isolate of *Leptosphaeria maculans*; C = callus tissues infected with a non-aggressive isolate of *L. maculans*; D = callus tissues infected with a mixture of aggressive and non-aggressive isolates of *L. maculans*; E = callus tissues treated with silver nitrate solution.

DISCUSSION

Since methoxybrassinin and cyclobrassinin are both antimicrobial and are synthesized by and accumulated in *Brassica juncea* tissues after exposure to certain micro-organisms, they qualify as phytoalexins according to the revised definition[1].

Prior to this investigation, the only compounds described as phytoalexins in the cruciferae were spirobrassinin from Japanese radish (*Raphanus sativus*) and methoxybrassinin, brassinin and cyclobrassinin from leaves of Chinese cabbage (*B. campestris* ssp. *pekinensis*) inoculated with *Pseudomonas cichorii*[7,8]. Accumulation of methoxybrassinin and cyclobrassinin in certain crucifers in response to *Alternaria brassicae*[9] and *Leptosphaeria maculans*[10] has been reported recently. These compounds do not seem to be hydrolysed products of indole glucosinolate since none of these compounds was detected in the extract following enzymic hydrolysis[7].

It is obvious from the HPLC chromatogram that the two phytoalexins were satisfactorily resolved. The position of isoprenylated genistein was of special importance with regard to its use as an internal standard. It eluted long enough after the phytoalexins implying no interference with the separation of the compounds of interest. Because of its phytoalexin property[13], it could be used as an internal standard to calculate extraction efficiency.

This HPLC procedure makes large scale TLC separations unnecessary. It is much faster and lowers the phytoalexin detection limit to 0.1 μg. With isoprenylated genistein as an internal standard, the method is accurate and useful for routine analysis and study of their role in disease resistance.

ACKNOWLEDGEMENTS

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CHROM. 20 678

Note

Chromatographic resolution

XXI*. Direct optical resolution of abscisic acid by high-performance liquid chromatography on cellulose tris(3,5-dimethylphenylcarbamate)

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Abscisic acid (ABA) is an important plant hormone which appears to be involved in plant growth and development[1]. Enantiomers of ABA are catabolized in different manners in plants, and naturally occurring ABA is the (+)-(*S*)-isomer. Therefore, the ready availability of this isomer is highly desirable for biological studies.

Optical resolution of commercially available racemic ABA and its methyl ester by liquid chromatography has been attempted by several groups[2–6], and efficient complete resolution of the ester[6] has recently been achieved by high-performance liquid chromatography (HPLC) using cellulose tris(3,5-dimethylphenylcarbamate) as a chiral stationary phase, which we developed[7].

Quite recently, we found that direct optical resolution of racemic carboxylic acids is possible on the above chiral column using hexane–2-propanol containing a small amount of a strong acid like formic acid, trichloroacetic acid or trifluoroacetic acid[8]. In this note, we report a very efficient direct optical resolution of ABA on cellulose tris(3,5-dimethylphenylcarbamate). Direct resolution is more valuable than the resolution of ABA esters in several respects.

EXPERIMENTAL

Cellulose tris(3,5-dimethylphenylcarbamate), prepared by the reaction of cellulose and 3,5-dimethylphenylisocyanate, was coated on the macroporous silica gel LiChrospher SI 4000 treated with 3-aminopropyltriethoxysilane[7]. This packing material was packed in a column (25 cm × 0.46 cm I.D.) by a slurry method. Chromatographic resolution was performed on a Jasco Trirotar-II chromatograph equipped with UV (240 nm) and polarimetric detection (mercury, full lamp).

* For Part XX, see Y. Okamoto, R. Aburatani, K. Hatano and K. Hatada, *J. Liq. Chromatogr.*, in press.

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RESULTS

Fig. 1 shows the optical resolution of (±)-ABA on a cellulose tris (3,5-dimethylphenylcarbamate) column. Complete direct optical resolution was achieved by using hexane–2-propanol containing 1% trifluoroacetic acid. The polarimetric detector showed that the (+)- and (−)-isomers were eluted at about 9 and 13 min, respectively. Without trifluoroacetic acid in the eluting system, ABA was not eluted from the column, like other racemic acids[8]. The elution order of ABA seems to be the same as that of ABA methyl ester[6]. The separation coefficient α, was 2.14, comparable to that of the methyl ester. Preparative separation was also possible. On the present analytical column, about 1 mg of ABA was completely resolved in one injection.

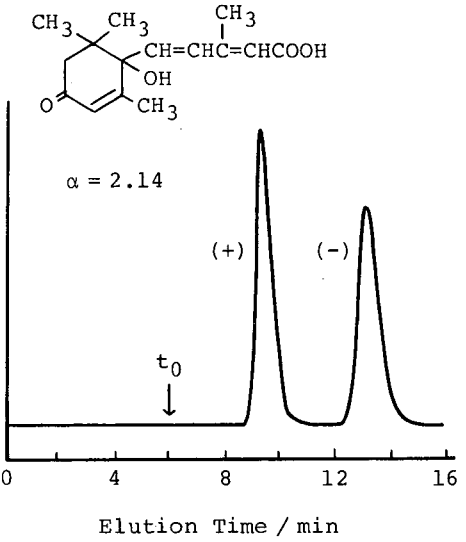

Fig. 1. Optical resolution of abscisic acid on a cellulose tris(3,5-dimethylphenylcarbamate) column with hexane–2-propanol–trifluoroacetic acid (80:20:1), at 0.5 ml min^{-1}.

By the use of the cellulose tris(3,5-dimethylphenylcarbamate) and hexane–2-propanol–trifluoroacetic acid (80:20:1), ABA was directly separated. This method may be useful for obtaining optical isomers of ABA as well as for determining the optical purity of ABA.

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CHROM. 20 650

Letter to the Editor

Flow velocity profiles in rectangular channels containing two liquid layers of different densities and viscosities for sedimentation field flow fractionation

Sir,

A numerical method for calculation of the flow velocity profiles in channels containing several liquid layers of different densities and viscosities has recently been presented by Janča[1]. Unfortunately, both the approach to the problem and the results seem incorrect. The failure of the proposed solution is manifested in the discontinuous velocity profiles at the interface between two moving liquids of different viscosities. This would mean no viscosity or no momentum transport at the interface.

To clarify the problem, let us consider a rectangular channel possessing a sufficiently high aspect ratio, $A = w/b$, w being the channel width and b its thickness. The lower part of the channel between 0 and x_0 is occupied by a heavier liquid (viscosity, η_1), the upper part between x_0 and b by a lighter one (viscosity, η_2).

Using the one-dimensional approximation, commonly used in field flow fractionation (FFF), we may write the steady flow equations

$$\frac{d^2u_i}{dx^2} = -\frac{\Delta P}{\eta_i L} = -K_i \qquad i = 1, 2 \tag{1}$$

where u_i and η_i are the fluid velocity and viscosity, respectively, and, ΔP is the pressure drop across the channel of length L.

The boundary problem can be formulated as follows:

$$u_1(0) = u_2(b) = 0 \tag{2}$$

$$u_1(x_0) = u_2(x_0) \tag{3}$$

$$\eta_1 \cdot \frac{du_1(x_0)}{dx} = \eta_2 \cdot \frac{du_2(x_0)}{dx} \tag{4}$$

Conditions 2 are classical in problems dealing with the laminar flow. Eqn. 4 expresses the balance of friction forces acting at the interface.

By solving the system 1–4, we obtain

$$u_1 = \frac{K_1}{2} \cdot x(x_0 - x) + \frac{K_2 x}{2\,x_0}(b - x_0)[(b + x_0) - f(\eta)] \tag{5}$$

and

$$u_2 = \frac{K_2}{2}(b - x)[(b + x) - f(\eta)] \tag{6}$$

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where

$$f(\eta) = \frac{\eta_1(b^2 - x_0^2) + \eta_2 x_0^2}{\eta_1(b - x_0) + \eta_2 x_0}$$

The velocity profiles 5 and 6 do not display any discontinuity; the flow of the more dense liquid is influenced by the flow of the less dense one, and *vice versa.*

Denoting the volumetric flow-rates of the liquids in the channel as q_i, we may write

$$q_1 = w \int_0^{x_0} u_1 dx \qquad q_2 = w \int_{x_0}^{b} u_2 dx$$

or

$$\frac{q_1}{w} = \frac{K_1}{12} \cdot x_0^3 + \frac{K_2}{4} \cdot x_0(b - x_0)[(b + x_0) - f(\eta)] \tag{7}$$

and

$$\frac{q_2}{w} = \frac{K_2}{2}(b - x_0)\left[b^2 - \frac{b^3 - x_0^3}{3(b - x_0)} - \frac{b - x_0}{2} \cdot f(\eta)\right] \tag{8}$$

Given q_1 or q_2, the interface position, x_0, can be calculated from eqn. 7 or 8, and the corresponding velocity profiles are determined by eqn. 5 and 6.

If merely the less dense liquid is pumped into the channel ($q_1 = 0$), then, according to eqn. 7, $x_0 = 0$ and from eqn. 8 we arrive at the familiar formula:

$$\frac{q^2}{wb} = \frac{K_2}{12} \cdot b^2$$

Similarly, if $q_2 = 0$, then $x_0 = b$ and

$$\frac{q_1}{wb} = \frac{K_1}{12} \cdot b^2$$

If both liquids display the same viscosity, then $K = K_1 = K_2$, $f(\eta) = b$ and

$$u_1 = u_2 = \frac{K}{2} \cdot x(b - x)$$

as expected.

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STANISLAV WIČAR

1 J. Janča, *J. Chromatogr.*, 404 (1987) 23–32.

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Errata

J. Chromatogr., 405 (1987) 107–116

Page 114, line 9, "0.2" should read "3.6" and "compound number 4" should read "compound number 7".

Page 114, line 10, "−13.2" should read "−14.3".

Page 114, line 15, "Compounds 1, 2, 3, 4, 7, 8, 9, and 14" should read "Compounds 1, 3, 7, and 8".

Page 114, line 16/17, "compounds 5, 6, 10, 11, 12, and 13" should read "compounds 2, 4, 5, 6, 9, 10, 11, 12, and 13".

Page 114, line 28, "0" should read "−0.8", "1.5" should read "1.0" and "compounds 2, 9, and 14" should read "2, 8, 9, and 14".

Page 114, Table V, the "Calc. A_s" and "ΔA_s" values should read as follows:

Compound	*Calc.* A_s	ΔA_s
1	27.4	0.1
2	22.6	−0.2
3	25.0	1.8
4	24.8	−1.2
5	18.2	−3.1
6	29.9	−14.3
7	19.4	3.6
8	18.4	0.9
10	17.7	−3.9
11	18.0	−3.7
12	18.2	−1.9
13	29.8	−9.3
14	20.7	0.0

Page 115, line 1, "Compounds 3, 7, and 8" should read "Compounds 3 and 7".

Page 115, line 4, "positive ΔA_s values" should read "ΔA_s values greater than −0.8".

Page 115, line 5, "ΔA_s values" should read "ΔA_s values below −0.8".

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journal of chromatography news section

NEW BOOKS

Ion exchange for industry; Development and use, edited by M. Streat, Wiley, Chichester, New York, 1988, *ca.* 690 pp., price *ca.* US$ 65.00, ISBN 0471-91592-0.

Practical statistics for the physical sciences, edited by L.L. Havlicek and R.D. Crain, American Chemical Society, Washington, DC, 1988, XVI + 489 pp., price US$ 59.95 (U.S.A. and Canada), US$ 71.95 (rest of world), ISBN 0-8412-1453-0.

Poison detection in human organs, *4th edition*, by A.S. Curry, Charles C. Thomas Publisher, Springfield, IL, 1988, XXII + 336 pp., price US$ 54.50, ISBN 0-39-05425-8.

Frontiers of flavor, Proceedings of the 5th International Flavor Conference, Portas Karras, Chalkidiki, Greece, 1–3 July, 1987 (*Developments in Food Science*, Vol. 17), edited by G. Charalambous, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, 836 pp., price Dfl. 470.00, US$ 247.25, ISBN 0-444-42940-9.

AWARDS

1988 TSWETT CHROMATOGRAPHY MEDALS

The M.S. Tswett Chromatography Medals for 1988 were awarded to Phyllis Brown, Fabrizio Bruner and Tsuneo Okuyama at the 25th Anniversary International Symposium on Advances in Chromatography which took place August 29–September 1, 1988 in Minneapolis, MN, U.S.A. These scientists were recognized for their contributions to the development of chromatography.

ANNOUNCEMENTS OF MEETINGS

5th INTERNATIONAL SYMPOSIUM ON SEPARATION SCIENCE AND BIOTECHNOLOGY, FORT LAUDERDALE, FL, U.S.A., JANUARY 17–19, 1989

Scientific and Economic aspects of the role played by separation principles in biotechnology will be examined by recognized experts and discussed with participants at the 5th International Symposium on Separation Science and Biotechnology, presented by the Washington Chromatography Discussion Group, on January 17–19, 1989, at the Bahia Mar, 801 Seabreeze Blvd., in Fort Lauderdale, FL, U.S.A.

The intention of this symposium is to bring together experts from the areas of separation science, biotechnological processing, the regulatory arena and the marketing sector to examine the practical application of separation science to solving problems in biotechnology. In addition to analytical and preparative chromatographic techniques, other state-of-the-art approaches such as field flow fractionation, capillary to preparative gel electrophoresis and other biomacromolecular and particle separation techniques will be included. Recent advances in applying separation science and engineering to biotechnological processes will be highlighted in this symposium. The economics of bioprocessing with regard to the development of cost-efficient systems with enhanced yields; and to ensuring regulatory compliance as well as market prospectives will also be treated by leading authorities. All attendees will have ample opportunity to interact with the experts, thus will gain valuable insights to solve problems of their particular interest. Successful interaction is ensured by limiting the size of this symposium to 80 delegates.
All correspondence should be sent to: Mrs. Janet Cunningham, BIOSEP-89 Symposium Manager, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.

13th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY, STOCKHOLM, SWEDEN, JUNE 25–30, 1989

The 13th Symposium on Column Liquid Chromatography (CLC '89), organized in cooperation with the Swedish Academy of Pharmaceutical Sciences, will be held at the Folkets Hus in Stockholm, Sweden, June 25–30, 1989.

The symposium aims to cover essential trends in modern column liquid chromatography (CLC). Areas closely related to CLC, including supercritical fluid chromatography, capillary electrophoresis and field flow fractionation, will also be treated. The programme will consist of review, keynote and regular lectures, posters and discussion sessions. The opening lecture will be delivered by Professor John Knox, Edinburgh (Future Directions in Separation Science). Internationally recognized scientists will be invited to give the review and keynote lectures, and to chair and initiate the discussion sessions. In addition the programme will contain regular lectures in two to three parallel sessions, but the majority of contributed papers will be displayed as posters.

Papers describing original unpublished work regarding all aspects of CLC and related areas are requested. Scientists intending to submit a paper are requested to send an abstract of 200–400 words with the names of author(s) and the title. The abstract must reach the secretariat not later than November 1, 1988. Authors are requested to indicate their preference for oral poster presentations. However, the final decision regarding acceptance and form of presentation will be made by the organizing committee. Authors will be informed on the decisions made before February 1, 1989. Authors of accepted papers will then receive special typing paper for submission of definitive abstracts, to be returned prior to February 15, these being intended for publication in the symposium book of abstracts.

Authors are also requested to submit their contributions (in triplicate) at the symposium for publication in the proceedings. These papers will be subjected to regular refereeing procedures and acceptance will be decided by the Editor of the *Journal of Chromatography*. The proceedings are included in the symposium fee, and will be delivered by mail to each participant after the meeting.

All manufacturers of importance in liquid chromatography and related areas are invited to participate in the exhibition. Companies interested in this exhibition are requested to direct their inquiries to the Exhibition Manager: Mr. Björn Wengse at the address given below.

The symposium programme includes a welcome party, a reception and various excursions. The tentative fees for full participation in the symposium will be SEK 2800, for Ph.D.-students SEK 1400 and for accompanying persons SEK 700, provided payment is received before May 1, 1989. After this date the fees will be raised by about 20%.

For further information contact: Conference secretariat, Stockholm Convention Bureau, CLC '89, Box 6911, S-102 39 Stockholm, Sweden. Tel.: (46) 8 230990; telex: S-11556; telefax: (46) 8 348441.

INTERNATIONAL SYMPOSIUM ON THE ANALYSIS OF NUCLEOSIDE, NUCLEOTIDE AND OLIGONUCLEOTIDE COMPOUNDS, ANTWERP, BELGIUM, SEPTEMBER 19–22, 1989

The International Symposium on the Analysis of Nucleoside, Nucleotide and Oligonucleotide Compounds will be held at the University of Antwerp, Antwerp, Belgium, September 19–22, 1989.

The chemistry of nucleosides, nucleotides, oligonucleotides and related compounds has always been an exciting area of research. The growing interest in this field has certainly been stimulated by the progress made in genetic engineering, in the synthesis of DNA-probes, in the synthesis of more potent antiviral and anticancer agents, in the study of their metabolites and by the progress made in many other related topics. However, these results would not have been possible without an equal development of more powerful, supporting analytical techniques.

In this symposium we would like to invite all scientists who are interested in presenting and discussing recent results made in the analysis of the above mentioned compounds by using techniques such as mass spectrometry, NMR, HPLC, SFC, GC, X-ray and other analytical techniques. This symposium is interesting to scientists from medicinal, pharmaceutical or chemical laboratories working on the synthesis, the analysis and bioassay in this particular field of research.

The registration fee will be BF 8000 (BF 4000 for Ph.D. Students).

Before and after the Symposium all correspondence should be sent to: J. Schrooten, Laboratory of Organic Chemistry, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium. Tel.: (03)-2180233, telex: RUCABI 33362; telefax: (03) 2180217.

SYMPOSIUM ON DETECTION IN FLOW INJECTION ANALYSIS AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, CORDOBA, SPAIN, SEPTEMBER 20–22, 1989

Detection in flowing liquid streams as encountered in flow injection analysis (FIA) and high-performance liquid chromatography (HPLC) can often be considered a "weak-point" in such analytical systems. Nevertheless, considerable advances have been made in the past years and a lot of imagination has been invested in improving detection systems and in introducing new detection models. Much research has also been carried out in the direction of adapting chemical derivatization principles to improve detection characteristics and hence the selectivity and sensitivity of the total analytical system. Further efforts have also been made to render such techniques suitable for automation and to adapt detection processes to miniaturized systems.

All these aspects shall be presented and discussed at this symposium and the state-of-art and shortcomings critically assessed. As a novel feature experts from two areas, FIA and HPLC, are called to participate since detection problems are often similar in both fields and hence a stimulating cross-fertilization is attempted and expected by combining the joint, often complementary know-how of these two groups of specialists and users.

Proceedings are planned in the *Journal of Chromatography* and *Analytica Chimica Acta*. The symposium language is English, no simultaneous translation will be given. The symposium coordinators are M. Valcarcel (Chairman), Spain; R.W. Frei, The Netherlands; K.P. Hupe (Co-chairman), F.R.G.; J. Blanco, Spain; and W. van der Linden, The Netherlands. Sponsors are International Association of Environmental and Analytical Chemistry; Cordoba University; Hewlett-Packard; Elsevier; and National Organisations.

For further information, please contact: Workshop Office IAEAC, Ms. M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland.

8th INTERNATIONAL SYMPOSIUM ON AFFINITY CHROMATOGRAPHY AND BIOLOGICAL RECOGNITION, REHOVOT, ISRAEL, OCTOBER 29–NOVEMBER 3, 1989

The 8th International Symposium on Affinity Chromatography and Biological Recognition will take place in Israel in the fall of 1989.

The symposium will focus on the recent developments in the field of affinity chromatography and its application to biotechnology. Other soild-phase, affinity-based methods will also be discussed. The

meeting will consist of plenary lectures, contributed papers, and poster sessions. Among the topics to be covered will be: new advances in affinity chromatography in research and industry; high-performance affinity chromatography; advances in hydrophobic (reversed-phase) chromatography; avidin-biotin technology; solid-phase affinity-based techniques; immunoaffinity techniques; and general subjects related to biorecognition.

For further information, contact: E.A. Bayer and F. Kohen, AC&BR Secretariat, The Weizmann Institute of Science, Rehovot 76100, Israel.

ANABIOTEC '90, 3rd INTERNATIONAL SYMPOSIUM ON ANALYTICAL METHODS IN BIOTECHNOLOGY, SAN FRANCISCO, CA, U.S.A., OCTOBER 28–31, 1990

The purpose of this 3rd ANABIOTEC symposium, under the Chairmanship of Dr. Ronald E. Majors, is to outline the progress already made as well as to deal with the future directions of the analytical methodology required. Because it is being held for the first time in the U.S.A., ANABIOTEC '90 should give American scientists the opportunity to contribute and benefit more strongly and should also encourage more Japanese and Far Eastern scientists to attend.

The symposium will cover a wide and representative range of current research activities on all aspects of analytical chemistry related to biotechnology. The programme will consist of invited plenary lectures, submitted and invited papers (both oral and posters), information discussion sessions, and scientific roundtables where very focussed research topics can be explored in detail. ANABIOTEC '90 will be sponsored by chemical and biotechnology societies in the U.S.A., Europe and Japan, making it truly international in scope. Among the topics being planned for scientific sessions are: biosensors; antibody/antigen interactions; DNA probes; fermentation monitoring; animal/plant cells; environmental control and regulatory aspects; liquid chromatography including affinity; capillary zone electrophoresis; spectroscopic structure determinations (NMR, mass spectroscopy, etc.); flow injection analysis; robotics; and analytical aspects of protein engineering. Other topics of interest will be added as the symposium develops.

In conjunction with ANABIOTEC '90, an exhibition of the latest instrumentation, accessories, chemicals, and supplies is planned. Companies interested in participating in this Exhibition should direct their enquiries to the Symposium Management. Booth space will be limited and early response is advised. San Francisco and the Bay Area has no shortage of entertainment and culinary opportunities and they will be used to full advantage for attendees and guests.

For further details, contact: Symposium Management, Shirley Schlessinger, ANABIOTEC '90, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011.

CALENDAR OF FORTHCOMING MEETINGS

Oct. 17–19, 1988 Nice, France	**International Symposium on Supercritical Fluids: Properties and Applications** Contact: M. Perut, E.N.S.I.C., 1 rue Grandville, F-54042 Nancy Cédex, France. (Further details published in Vol. 404, No. 2.)
Oct. 18–20, 1988 Buenos Aires, Argentina	**2nd Latin-American Congress on Chromatography** Contact: Dr. Fernando M. Lanças, University of São Paulo, Institute of Physics and Chemistry of São Carlos, 13560 São Carlos (SP), Brazil. Tel.: (0162) 726222–275, telex: (16) 2374. (Further details published in Vol. 445, No. 1.)
Oct. 26–27, 1988 Uppsala, Sweden	**Coupled Column Separations** Contact: The Swedish Chemical Society, The Analytical Section, Upplandsgatan 6A, S-111 23 Stockholm, Sweden. (Further details published in Vol. 437, No. 1.)

Oct. 30–Nov. 4, 1988 Boston, MA, U.S.A.	**FACSS XV, Federation of Analytical Chemistry and Spectroscopic Societies 1988 Meeting** Contact: Roger Gilpin, Kent State University, Department of Chemistry, Kent, OH 44242, U.S.A. Tel.: (216) 672-2032. (Further details published in Vol. 437, No. 1.)
Oct. 31–Nov. 2, 1988 Copenhagen, Denmark	**8th International Symposium on HPLC of Proteins, Peptides and Polynucleotides** Contact: The Symposium Organizer, DIS Conference Service, Linde Alle 38, Copenhagen DK-2720, Denmark. (Further details published in Vol. 447, No. 2.)
Nov. 2–4, 1988 Freiburg, F.R.G.	**5th (Montreux) Symposium on Liquid Chromatography–Mass Spectroscopy** Contact: Professor R.W. Frei, Department of Analytical Chemistry, De Boelelaan 1083, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 407.)
Nov. 8–11, 1988 Vienna, Austria	**Symposium on Two-Dimensional Electrophoresis** Contact: Dr. A.T. Endler, Kaiserin Elizabeth Hospital, Hüglgasse 1–3, A-1150 Vienna, Austria.
Nov. 13–17, 1988 Moscow, U.S.S.R.	**International Symposium on Surface Chemistry, Adsorption and Chromatography** Contact: Dr. L.N. Kolomiets, Institute of Physical Chemistry, Academy of Sciences of the U.S.S.R., Leninsky Prospekt 31, Moscow 117915, U.S.S.R. (Further details published in Vol. 445, No. 1.)
Nov. 14–17, 1988 Rehovot, Israel	**International Symposium on Chromatography** Contact: Miss R. Assayag, Secretariat Chromatography 1988, Peltours Conventions Division, P.O. Box 394, Tel Aviv 61003, Israel. Tel.: (03) 650-862, telex: 33803 35819 peltg il, FAX: Tel Aviv 660060. (Further details published in Vol. 411.)
Nov. 19–23, 1988 San Diego, CA, U.S.A.	**14th Biennial Polymer Symposium — International Symposium on Multiphase Macromolecular Systems** Contact: B. Culbertson, Ashland Chem. Co., P.O. Box 2219, Columbus, OH 43216, U.S.A.
Nov. 20–24, 1988 Leipzig, G.D.R.	**1st Symposium on Analytics in Agricultural Research, including Ecology and Toxicology** Contact: Akademie der Landwirtschaftswissenschaften der DDR, Institut für Futterproduktion Paulinenaue, Dr. sc. W. Seyfarth, Paulinenaue, G.D.R.-1551. (Further details published in Vol. 437, No. 1.)
Dec. 12–13, 1988 Iselin, NJ, U.S.A.	**High-Performance Liquid Chromatography of Biopolymers** Contact: Janet Cunningham, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.
Jan. 17–19, 1989 Fort Lauderdale, FL, U.S.A.	**5th International Symposium on Separation Science and Biotechnology** Contact: Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.

Feb. 5–9, 1989 Perth, Australia	**Advances in Biopolymers** Contact: Mr. G. Ferguson, WA Govt. Chemical Labs., 125 Hay Street, Perth, Australia.
Feb. 21–24, 1989 Brighton, U.K.	**Joint Meeting of the 2nd International Symposium on Thin-Layer Chromatography and the 5th International Symposium on Instrumental and High-Performance Thin-Layer Chromatography (Planar Chromatography)** Contact: Dr. I.D. Wilson, ICI Pharm. Division, Mereside Alderley Park, Drug Metabolism Department, Macclesfield, Cheshire SK10 4TG, U.K. (Further details published in Vol. 447, No. 2.)
March 6–10, 1989 Atlanta, GA, U.S.A.	**40th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Alma Johnson, Program Secretary, 12 Federal Drive, Suite 322, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 445, No. 1.)
April 9–12, 1989 Sils-Maria, Switzerland	**6th Symposium on Chromatography** Contact: Workshop Office IAEAC, Ms. M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland. (Further details published in Vol. 447, No. 2.)
April 10–12, 1989 Boston, MA, U.S.A.	**1st International Symposium on High Performance Capillary Electrophoresis** Contact: Shirley E. Schlessinger, Symposium Manager, HPCE '89, 400 East Randolph Drive, Suite 1015, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011. (Further details published in Vol. 447, No. 2.)
May 7–12, 1989 Nürtingen, F.R.G.	**3rd International Conference on Fundamentals of Adsorption** Contact: DECHEMA, Abteilung Tagungen, P.O. Box 970146, Theodor-Heuss-Allee 25, D-6000 Frankfurt am Main, F.R.G.
May 8–10, 1989 Washington, DC, U.S.A.	**6th International Symposium on Preparative Chromatography** Contact: Mrs. Janet Cunningham, Prep-89 Symposium Manager,Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 447, No. 2.)
May 16–19, 1989 Antwerp, Belgium	**3rd International Symposium on Drug Analysis** Contact: Dr. Apr. G. Laekeman, 3rd International Symposium on Drug Analysis, Universitaire Instelling Antwerpen, Departement Farmaceutische Wetenschappen, Universiteitsplein 1, B-2610 Wilrijk, Belgium. (Further details published in Vol. 438, No. 2.)
May 22–25, 1989 Baltimore, MD, U.S.A.	**3rd Annual Seminar on Analytical Biotechnology** Contact: Barr Enterprices, P.O. Box 279, Wallkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.
May 23–26, 1989 Ghent, Belgium	**3rd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. Willy R.G. Baeyens, Symposium Chairman, State University of Ghent, Pharmaceutical Institute, Laboratory of Pharmaceutical Chemistry and Drug Quality Control, Harelbekestraat 72, B-9000 Ghent, Belgium. (Further details published in Vol. 447, No. 2.)

June 10–14, 1989 Amsterdam, The Netherlands	**3rd Amsterdam High-Performance Liquid Chromatography Summer course** Contact: Dr. J.C. Kraak, Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands.
June 13–15, 1989 Snowbird, UT, U.S.A.	**1989 Workshop on Supercritical Fluid Chromatography** Contact: Dr. Milton L. Lee, Department of Chemistry, Brigham Young University, Provo, UT 84602, U.S.A. Tel.: (801) 378-2135. (Further details published in Vol. 447, No. 2.)
June 14–16, 1989 Salt Lake City, UT, U.S.A.	**1st International Symposium on Field-Flow Fractionation and FFF Workshop** Contact: Julie Westwood, Department of Chemistry, University of Utah, Salt Lake City, UT 84112, U.S.A. Tel.: (801) 581-5419. (Further details published in Vol. 447, No. 2.)
June 25–30, 1989 Montreux, Switzerland	**Transducers '89, 5th International Conference on Solid-State Sensors and Actuators & Eurosensors III** Contact: COMST S.A., Conference Organizers in Medicine, Science and Technology, P.O. Box 415, 1001 Lausanne 1, Switzerland. Tel.: (021) 234 886, Telefax: (021) 234 972. (Further details published in Vol. 445, No. 1.)
June 25–30, 1989 Stockholm, Sweden	**13th International Symposium on Column Liquid Chromatography** Contact: 13th International Symposium on Column Liquid Chromatography, The Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, S-111 81 Stockholm, Sweden. Tel.: (468) 24 50 85. (Further details published in Vol. 404, No. 2.)
July 2–7, 1989 Bratislava, Czechoslovakia	**8th International Symposium on Advances and Application of Chromatography in Industry** Contact: Department of Analytical Chemistry, "Symposium on Chromatography", Faculty of Chemical Technology, Radlinského 9, 812 37 Bratislava, Czechoslovakia. (Further details published in Vol. 438, No. 2.)
July 2–9, 1989 Sofia, Bulgaria	**XXVI Colloquium Spectroscopium Internationale** Contact: XXVI CSI '89, Sofia University, Faculty of Physics, Department of Optics and Spectroscopy, 5, A. Ivanov Blvd., 1126-30 Sofia, Bulgaria. Tel.: (3592) 627475, Telex: SUKO 23296 R BG. (Further details published in Vol. 445, No. 1.)
July 30–August 5, 1989 Cambridge, U.K.	**SAC 89, International Conference on Analytical Chemistry** Contact: SAC 89, Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, U.K. Tel.: (01) 437-8656. (Further details published in Vol. 407.)
Aug. 2–7, 1989 Lund, Sweden	**32nd IUPAC Congress** Contact: IUPAC, c/o Stockholm Convention Bureau, P.O. Box 6911, S-102 39 Stockholm, Sweden. Tel.: (46) 8230990, telex: 11556, FAX: 46 8 34 84 41.

Aug. 21–25, 1989 Leipzig, G.D.R.	**7th Danube Symposium on Chromatography** Contact: 7th Danube Symposium on Chromatography, Karl-Marx-Universität Leipzig, Sektion Chemie, Talstrasse 35, Leipzig, G.D.R. (Further details published in Vol. 411.)
Aug. 21–25, 1989 Amsterdam, The Netherlands	**5th International Conference on Particle Induced X-Ray Emission and its Analytical Applications** Contact: 5th PIXE Conference, Department of Physics and Astronomy, Free University, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5486224. (Further details published in Vol. 445, No. 1.)
Aug. 28–Sept. 1, 1989 Wiesbaden, F.R.G.	**11th International Symposium on Microchemical Techniques** Contact: Gesellschaft Deutscher Chemiker, Abt. Tagungen, P.O. Box 900440, D-6000 Frankfurt/Main 90, F.R.G. Tel.: (069) 79 17-366/360, telex: 4170497 gdch d.
Sept. 1–3, 1989 Leiden, The Netherlands	**2nd International Symposium on Disposition and Delivery of Peptide Drugs** Contact: Dr. J. Verhoef, Center for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden, The Netherlands.
Sept. 4–8, 1989 Colymbari, Crete Greece	**Pesticides and Alternatives, International Conference on Innovative Chemical and Biological Approaches to Pest Control** Contact: Professor John Casida, Department of Entomological Sciences, University of California, Berkeley, CA 94720, U.S.A. Tel.: (415) 642-5424.
Sept. 10–15, 1989 Antwerp, Belgium	**International Symposium on Gas Separation Technology** Contact: Dr. R. Dewolfs, University of Antwerp, Department of Chemistry, Universiteitsplein 1, B-2610 Antwerp-Wilrijk, Belgium. Tel.: (32) 3-828 25 28, ext. 204 or 215; telex: 336 46 UIA B; telefax: (32) 3-827 08 74. (Further details published in Vol. 438, No. 2.)
Sept. 19–21, 1989 Birmingham, U.K.	**5th BOC Priestly Conference** Contact: Dr. B.D. Crittenden, School of Chemical Engineering, Claverton Down, Bath BA2 7AY, U.K. Tel.: (0225) 826826, telex: 449097.
Sept. 19–22, 1989 Antwerp, Belgium	**International Symposium on the Analysis of Nucleoside, Nucleotide and Oligonucleotide Compounds** Contact: Dr. E.L. Esmans or Mr. J. Schrooten, University of Antwerp (R.U.C.A.), Laboratory for Organic Chemistry, Groenenborgerlaan 171, B-2020 Antwerp, Belgium. Tel.: (03) 2180233 or (03) 2180496, telex: 33362 rucabi, Fax: (03) 2180217.
Sept. 20–22, 1989 Cordoba, Spain	**Symposium on Detection in Flow Injection Analysis and High-Performance Liquid Chromatography** Contact: Workshop Office IAEAC, Ms. M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland.

Sept. 24–29, 1989 New York, NY, U.S.A.	**28th Eastern Analytical Symposium** Contact: David S. Klein, 642 Cranbury Cross Road, North Brunswick, NJ 08902, U.S.A.
Sept. 25–28, 1989 St. Louis, MO, U.S.A.	**103rd AOAC International Meeting and Exposition** Contact: Margaret Ridgell, AOAC, 1111th North 19th Street, Suite 210, Arlington, VA 22209, U.S.A. Tel.: (703) 522-3032.
Oct. 1–4, 1989 Hamilton, Canada	**2nd International Conference on Separation Science and Technology** Contact: V. Lakshmanan, Ontario Research Foundation, Mississauga, Ontario, L5K 1B6 Canada.
Oct. 29–Nov. 3, 1989 Rehovot, Israel	**8th International Symposium on Affinity Chromatography and Biological Recognition** Contact: E.A. Bayer and F. Kohen, AC&BR Secretariat, The Weizmann Institute, Institute of Science, Rehovot 76100, Israel.
Aug. 26–31, 1990 Vienna, Austria	**Euroanalysis VII, 7th European Conference on Analytical Chemistry** Contact: Professor Dr. M. Grasserbauer, c/o Interconvention, Austria Center Vienna, A-1450 Vienna, Austria. Tel.: (43) 222-2369/647; telex: 111803 icos a, Fax: (43) 222-2369/648.
Sept. 24–28, 1990 Amsterdam, The Netherlands	**18th International Symposium on Chromatography** Contact: Professor Dr. U.A.Th. Brinkman, Free University, Department of General and Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5484773.
Oct. 28–31, 1990 San Francisco, CA, U.S.A.	**ANABIOTEC '90, 3rd International Symposium on Analytical Methods in Biotechnology** Contact: Shirley Schlessinger, ANABIOTEC '90, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A.

MEETING REPORT

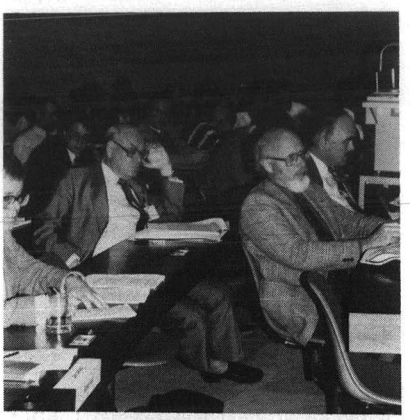
4th SYMPOSIUM ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY, BASEL, SWITZERLAND, APRIL 27–29, 1988

This symposium was the fourth in a series of successful meetings organized by the International Association of Environmental Analytical Chemistry. The previous symposia were held in Lausanne, Freiburg and Mallorca.

The 4th Symposium took place in the Auditorium of Sandoz Ltd. (Basel) and the facilities were really excellent. It was attended by about 200 participants, demonstrating again that there is much interest in the theme of handling complex samples (Figs. 1–3). Many good lectures and posters were presented and the atmosphere of the symposium was stimulating and informal. It seems that demands placed on the trace analysis of environmental and biological samples are still increasing. Moreover, the number of samples is also increased and, therefore, automation of the total analytical procedure is very important. This automation aspect was one of the central subjects of this symposium and

Fig. 1. Dr. F. Erni in action.

special attention was paid to the role of robotics in sample handling. Another important aspect was the use of more selective stationary phases for solid–liquid extraction and the reproducibility of these phases. Generally, it seems that solid–liquid extractions have now superseded liquid–liquid extractions in many laboratories. New promising developments for sample handling such as dialysis, zone electrophoresis and micellar systems already appear on the horizon and perhaps the next symposium will show the breakthrough of these techniques.



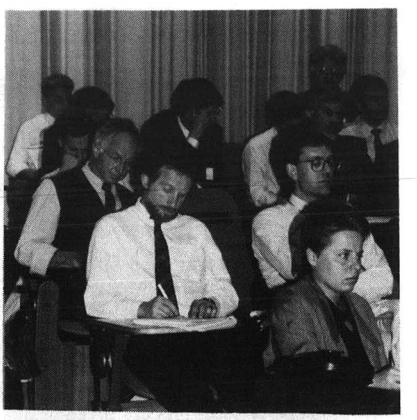


Fig. 2. The lectures were enthusiastically received by the audience. On the left-hand photograph, four members of the Coordinating Committee can be seen on the foreground: Professor R.W. Frei, Dr. K. Zech (first row), Professor D. Westerlund and Dr. E. Merian (second row).

Fig. 3. Also outside the lecture halls samples of various origin were adequately handled.

Automation

The possibilities of robotics were discussed in some papers and in an informative discussion at the end of the first day. During this discussion it was stressed that there is no clear definition of robotics and that robots are worthwhile only for a special segment of the automation problem in the laboratory. In general, robotics seem attractive for simple handling steps which must be repeated many times over a long period. Franzen (Zymark, F.R.G.) and Naundorf (Perkin-Elmer, F.R.G.) showed that various commercial modules are now available, *e.g.*, for solid–liquid extraction. Interesting examples of the application of robotics were presented. Nielen (TNO, The Netherlands) has developed a robotic system for the analysis of Tenax adsorption tubes, including identification and de-capping of the tubes, transport to the desorption oven, starting of the thermodesorption on-line with gas chromatography (GC) and removal of the tubes. Trouble-shooting of the tubes and the apparatus was also covered. De Jong and co-workers (Servier, France) demonstrated in a poster the usefullness of robotics for pharmaceutical and biomedical analysis, not only for routine analysis but also for optimization of sample handling procedures. The system presented by Cooper (Coventry and Warwickshire Hospital, U.K.), including robotics, dialysis of biological samples with a membrane and on-line trace enrichment of the dialysates, seems very promising. Impressive examples were shown and the possibilities for continuous monitoring of patients were mentioned. This system (ASTED) is commercially available from Gilson (France).

The use of apparatus for automated sample preparation with solid–liquid extraction was demonstrated in some lectures and posters. Lai (Varian) discussed the possibilities of method development with the advanced automated sample processor (AASP). The purge pump for purging the cartridges with small amounts of solvents makes the clean-up of the samples more flexible. Doyle (Smith Kline and French, U.S.A.) discussed a totally automated system for the analysis of biological fluids. The samples (plasma or urine) are injected directly onto a precolumn by an autosampler or robot. After separation by liquid chromatography (LC), the chromatograms are collected by a microcomputer and transferred to a larger computer for processing and reporting. Also, in two posters (Grossi, Rouan) the possibilities of the combination of a robotic system with the AASP were clearly illustrated.

A general overview of the automation of precolumn sample preparation steps was given by Goewie (RIVM, The Netherlands). A computer program was developed for establishing the best solvent conditions for certain pre-separations in a column switching system. A systematic review of column switching approaches for automated sample handling was also presented by Ramsteiner (Ciba-Geigy, Basel). The various functions of precolumns were critically assessed and various sample transfer techniques outlined. Sample handling and detection in chromatography cannot also be judged independently.

Sufficient sensitivity and/or selectivity can also be obtained by the right detection mode, as was shown in many lectures and posters. For many compounds derivatization can make the detection much more sensitive and/or selective. Rozing (Hewlett-Packard, F.R.G.) demonstrated the possibilities for on-line automated precolumn derivatization. Methods were shown for amines, aldehydes, ketones, alcohols and acids. This combination of chemistry and automation seems very promising. This was also concluded by Widmer (Ciba Geigy, Basel) in his lecture, in which he described flow injection analysis (FIA) for sample handling. In this fast and flexible method, a lot of chemistry can be used in order to obtain sufficient selectivity. An interesting point of discussion and controversy was that many robotics operations could be taken over by FIA systems at higher sample throughput and lower cost.

Solid–liquid extraction

The use of small precolumns or cartridges (on-line or off-line) for sample handling is now routine in various laboratories. Therefore, just as for high-performance liquid chromatographic (HPLC) stationary phases, the reproducibility of the materials in the precolumns becomes more and more important. Nearly always hydrophobic materials such as C_8- or C_{18}-bonded silica are applied. Also in this symposium some examples of this were shown. Keller (Sandoz, Basel) discussed a number of methods for the determination of cyclosporins in blood and demonstrated that column switching methods have the highest potential. This lecture gave a good insight into the prblems inherent in the determination of low concentrations of drugs in blood, *e.g.*, protein binding, accuracy and precision. Wyss (Hoffman-La Roche, Basel) showed that for highly protein bound drugs the recovery is not complete after preconcentration of plasma on a hydrophobic stationary phase. For retinoids and metabolites this was solved by deproteination before injection or in other instances by addition of a small amount of ethanol to the sample.

Schmid (Physiatric Hospital of Vienna, Austria) discussed in a very informative lecture the complex retention mechanism for basic compounds on hydrophobic materials with residual silanol groups. This can influence the capacity of the precolumn for such compounds and the recovery. The proposal was to use the mixed mechanism in two separate steps: a C_{18}-bonded silica precolumn wich is loaded, *e.g.*, with an ion-pair reagent in series with a silica precolumn. This design with two different precolumns was applied for the analysis of benzodiazepines. Musch (Free University, Brussels) investigated the role of silanol groups on CN-bonded silica for different types of compounds and found that for basic compounds sometimes a high percentage of modifier can be used during the preconcentration step because secondary interactions (silanol groups) dominate.

The possibility of large volume sampling on various precolumns in series, described by Hennion (Ecole de physique et chimie, Paris), was used for drinking water control. Models were proposed to predict breakthrough volumes and enrichment factors for several selected organic priority pollutants in the sub-ppb concentration range.

There is a clear trend towards the use of more selective stationary phases for solid–liquid extraction. Size-exclusion chromatography (SEC) can be very useful for clean-up and trace enrichment, as was demonstrated by Barcelo (CID-CSIS, Barcelona) for the determination of PCBs and PAHs in environmental matrices, and by Shepherd (Ministry of Agriculture Fisheries and Food, U.K.) for the determination of contaminants and additives in food. In the latter instance special attention was paid to the coupling of non-aqueous SEC to reversed-phase HPLC.

Boos (University of Paderborn, F.R.G.) discussed the use of a stationary phase for *cis*-diol-containing biomolecules (*e.g.*, catecholamines and ribonucleosides). The material is a semi-rigid polyvinyl polymer (30 μm) on which phenylboronic acid has been immobilized. In this way SEC for the removal of proteins is combined with the selective formation of boronate esters with the analytes. In total, more than 250 ml of plasma have been injected directly without problems. For detection in the

low-picogram range post-column derivatization, which generates highly fluorescent trihydroxyindole derivatives, was used. Härdi (University of Geneva, Switzerland) employed 8-quinolinol-silica gel-iron(III) for preconcentration of catecholamines and related compounds by complexation with iron(III). Desorption can be carried out by a pH switch.

Farjam (Free University, Amsterdam) illustrated the high selectivity of a precolumn with an immobilized antibody for the determination of B19-nortestosterone in calf urine. Desorption was achieved by an excess of a cross-reacting steroid. It seems that in the future only short LC columns will be necessary after such a selective preconcentration and clean-up step.

Reust (Sandoz, Basel) showed the high potential of selective sample pretreatment and/or detection (electrochemical) in ion chromatography.

Special techniques

New techniques for sample handling and detection were presented in various lectures and posters. Micellar systems seem very suitable for the direct injection of biological samples (Weinberger, Applied Biosystems, U.S.A.). Surfactant molecules can bind strongly to proteins and hence protect the clean-up and trace-enrichment column. Micellar systems can also enhance derivatization possibilities (v.d. Horst, University of Utrecht, The Netherlands). Reactions that are normally incompatible with water can be carried out in aqueous micellar solutions or even directly in a biological matrix. Micelles were also found to have catalytic properties. Derivatization of carboxylic acids with 7-bromomethyl-7-methoxycoumarin was one of the examples that was extensively discussed in this presentation. Westerlund (University of Uppsala, Sweden) showed that micelles are also useful as a modifier for a chiral column that loses its stereoselectivity with conventional modifiers. The use of micelles also made it possible to inject incubates of liver microsomes directly onto a precolumn. In the same lecture Westerlund discussed the sample possibilities for the synthesis of glucuronides of drugs via enzymatic pathways. Further optimization of this method is still necessary.

Kok (University of Amsterdam, The Netherlands) presented an original clean-up method for the determination of ionogenic compounds in biological samples involving zone electrophoresis coupled on-line to HPLC. Many parameters, *e.g.*, electric field strength, diameter of the capillary and buffer composition, have been investigated. The removal of proteins and other interfering compounds is very efficient, as was illustrated for the determination of salicylic acid in plasma and amphetamines in urine.

De Jong (Free University, Amsterdam) outlined the principles and some applications of the on-line coupling of LC and GC. LC can be used for trace enrichment and/or clean-up (heart-cutting technique) of environmental and biological samples. Verzele (University of Gent, Belgium) stated that micro-LC with packed capillary columns (now provided with an 'inner wall elastic coating') has many advantages over the use of conventional columns, *e.g.*, higher permeability, better inertness and easier coupling with mass spectrometric, GC detectors and GC. However, the high mass sensitivity will be an advantage only if good systems for on-line trace enrichment can be developed. In another somewhat exceptional lecture, Klockow (University of Dortmund, F.R.G.) gave an overview of sensors for the detection of air pollutants. Chemical sensors were especially emphasized. Different principles, *e.g.*, electrochemical and chemiluminescence, are used for the detection of various compounds. The role of optical fibres in these systems is also very interesting. Chemical sensors were compared with receptors, but generally sensors need more selectivity to make this comparison really valid.

More specifically on the air sampling side, in connection with air pollution problems, Canela (Sandoz, Basel) discussed a comprehensive approach for the measurement of organic emissions from a multi-purpose production facility. With varying adsorbents a wide range of compounds were sampled and analysed by headspace chromatography after elution. Another 'real world' example was presented by Thöne (Sandoz, Basel) who discussed the problems and possibilities of thermal desorption/capillary GC for the control of workplace atmospheres. Dimethyl and diethyl sulphate and ethylene oxide were discussed as model compounds with Tenax and Carbosieve S III as sorbents.

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PUBLICATION SCHEDULE FOR 1988

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

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