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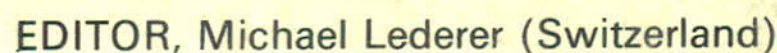

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edited by **P.L. DUBIN,** *Indiana-Purdue University*

(Journal of Chromatography Library, 40)

The rapid development of new packings for aqueous size-exclusion chromatography has revolutionized this field. High resolution non-adsorptive columns now make possible the efficient separation of proteins and the rapid and precise determination of the molecular weight distribution of synthetic polymers. This technology is also being applied to the separation of small ions, the characterization of associating systems, and the measurement of branching. At the same time, fundamental studies are elucidating the mechanisms of the various chromatographic processes.

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- Protein chromatography is dealt with in both dedicated sections and throughout the book as a whole.

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EXTENSION OF THE ELECTROSTATIC THEORY OF REVERSED-PHASE ION-PAIR CHROMATOGRAPHY FOR HIGH SURFACE CONCENTRATIONS OF THE ADSORBING AMPHIPHILIC ION

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(First received May 25th, 1988; revised manuscript received September 1st, 1988)

SUMMARY

To understand the mechanism of ion-pair chromatography, a correct description of the adsorption isotherm of the amphiphilic modifier is important. The adsorption isotherms of butyl-, hexyl- and octylsulphonate were investigated according to the concepts of the electrostatic theory of ion-pair chromatography. This theory was also extended to include the simultaneous effect of surface potential and competition between the amphiphile and the analyte for the available surface area. It was found that there is good agreement between the theory and experimental results.

INTRODUCTION

Liquid chromatographic analysis of organic molecular ions is usually performed with a reversed stationary phase (RP) and a polar mobile phase containing an amphiphilic ion as modifier. The capacity factor of the ionic analytes is regulated by varying the concentration of the amphiphile in the mobile phase. A number of different names have been proposed for this chromatographic technique[1], and this reflects the uncertainty that exists about the physical mechanism that regulates the capacity factor of the analytes. The name ion-pair chromatography seems to be most widely used and will therefore be used in this paper.

In recent years a number of papers have been published by Bartha, Vigh and co-workers that contain experimental data[2–4] which can be used to test the existing theories of ion-pair chromatography. An important aspect of these studies was to test if the most frequently proposed isotherms, *i.e.*, the Langmuir and Freundlich isotherms, agree with the isotherm experimentally obtained. It is generally found that

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the experimental isotherm does not fit either of these two theoretical isotherms when the mobile phase concentration of the amphiphilic ion is varied over wide, but chromatographically meaningful, concentrations. As the isotherm of the amphiphile is fundamental to all theories of ion-pair chromatography, a correct description is needed in order to understand its mechanism.

It is not surprising, from a theoretical point of view, that isotherms that do not consider an electrostatic surface potential disagree with what is found experimentally. A surface potential exists whenever there is an excess of charged species of one type of sign over species of the opposite sign on the surface. In ion-pair chromatography, the affinity of the amphiphile for the surface is higher than that of the electrolytic counter ions. This creates a surface potential, *i.e.*, there is a higher concentration of amphiphilic ions than of electrolytic counter ions in the hydrophobic surface layer. It can therefore be concluded that the theoretical expression for the isotherm must include the effect of the surface potential. This conclusion also holds for the mechanism of ion-pair chromatography, *i.e.*, a physically realistic theory must consider the effect of the surface potential. A theory has been developed which assumes that the surface potential is responsible for the changes in capacity factor when an amphiphile is added to the mobile phase[5]. It has been found that this theory of ion-pair chromatography agrees well with experimental results[5–8].

In a recent paper a quantitative evaluation of the adsorption isotherm of tetrabutylammonium ion on an RP-18 surface was reported[8]. It was found that the isotherm, when expressed in terms of the electrostatic theory, is independent of ionic strength and type of counter ion. It was also found that the experimental isotherm agrees with a Langmuir type of isotherm modified with a term for the surface potential. A similar approach was used in this work and it was found that a number of different alkylsulphonates also follow this isotherm.

The electrostatic theory of ion-pair chromatography assumes that the relative changes in capacity factors are due to changes in the surface potential of the stationary phase. When the surface concentration of amphiphilic modifier is high, the amphiphilic modifier competes with the analyte ions for the limited surface area available. In this paper the electrostatic theory is extended to include this effect on the relationship between the capacity factor and the concentration of amphiphile in the mobile phase. A comparison of the theory with experimental results indicates that this extension of the theory is correct and necessary at high surface coverage of amphiphile.

THEORY

In the electrostatic theory of ion-pair chromatography, it is assumed that the relative change in the capacity factor of an ionic analyte is determined only by changes in the electrostatic surface potential of the stationary phase. This assumption gives the following relationship[6] between the capacity factor for an analyte B, k'_{cB}, its charge, z_B, and the electrostatic surface potential, ψ_0:

$$k'_{cB} = k'_{0B} \exp(-z_B F \psi_0 / RT) \tag{1}$$

where k'_{0B} is the capacity factor for B at a reference composition of the mobile phase for which the surface potential is set to zero, *e.g.*, when no amphiphilic modifier is added to the mobile phase.

The theory is extended here to include the effect of competition between the amphiphilic modifier and the analyte for the limited surface area available in the column. This is done by equating the electrochemical potential of the different species i, μ_i, in the mobile phase, m, and stationary phase, s. The electrochemical potentials for an amphiphilic modifier, A, of charge z_A and an analyte, B, of charge z_B are expressed by the following equations:

$$\mu_{Am} = \mu^0_{Am} + RT \ln c_A \quad (2)$$

$$\mu_{As} = \mu^0_{As} + RT \ln X_{As} + z_A F \psi_0 \quad (3)$$

$$\mu_{Bm} = \mu^0_{Bm} + RT \ln c_B \quad (4)$$

$$\mu_{Bs} = \mu^0_{Bs} + RT \ln X_{Bs} + z_B F \psi_0 \quad (5)$$

The electrochemical potential of a surface site is expressed by the following equation:

$$\mu_s = \mu^0_s + RT \ln X_s \quad (6)$$

In eqns. 3 and 5, X_{is} (i = A or B) is the fraction of the total surface area occupied by A or B molecular ions and X_s in eqn. 6 is the fraction that is unoccupied. Assuming that the analyte ion 'and the amphiphilic ion require the same surface area, the following relationship holds:

$$X_{As} + X_{Bs} + X_s = 1 \quad (7)$$

The conditions of equilibrium are

$$\mu_{As} = \mu_{Am} + \mu_s \quad (8))$$

and

$$\mu_{Bs} = \mu_{Bm} + \mu_s \quad (9)$$

Combination of eqns. 2, 3 and 8 gives

$$\frac{X_{As}}{c_A X_s} = K_{As} \exp(-z_A F \psi_0 / RT) \quad (10)$$

where $K_{As} = \exp[-(\mu^0_{As} - \mu^0_s - \mu^0_{Am})]$. From eqns. 4, 5 and 9 we obtain

$$\frac{X_{Bs}}{c_B X_s} = K_{Bs} \exp(-z_B F \psi_0 / RT) \quad (11)$$

where $K_{Bs} = \exp[-(\mu^0_{Bs} - \mu^0_s - \mu^0_{Bm})]$

Solving eqn. 10 for X_s and inserting in eqn. 11 gives

$$\frac{X_{Bs}c_A}{c_B X_{As}} = \frac{K_{Bs}\exp(-z_B F\psi_0/RT)}{K_{As}\exp(-z_A F\psi_0/RT)} \tag{12}$$

where $X_{is} = n_i/n_{0i}$, n_{0i} is the monolayer capacity of the surface and n_i is the surface concentration of species i on the surface. Eqn. 12 can therefore be reformulated as

$$\frac{n_B}{c_B} = \frac{n_A}{c_A}\cdot\frac{K_{Bs}\exp(-z_B F\psi_0/RT)}{K_{As}\exp(-z_A F\psi_0/RT)}\cdot\frac{n_{0B}}{n_{0A}} \tag{13}$$

Assuming that the surface concentration of analyte ions is negligible in relation to the surface concentration of amphiphilic modifier, *i.e.*, $X_{Bs} \to 0$ in eqn. 7, the adsorption isotherm of the amphiphile is then found to be eqn. 14 (see ref. 8). This means that the adsorption isotherm for A is independent of the presence of B.

$$n_A = \frac{n_{0A}K_{As}c_A\exp(-z_A F\psi_0/RT)}{1 + K_{As}c_A\exp(-z_A F\psi_0/RT)} \tag{14}$$

Inserting eqn. 14 in eqn. 13 and introducing the capacity factor gives

$$k'_{cB} = \varphi\cdot\frac{n_{0B}K_{Bs}\exp(-z_B F\psi_0/RT)}{1 + K_{As}c_A\exp(-z_A F\psi_0/RT)} \tag{15}$$

where φ is the phase ratio.

In the absence of an amphiphilic modifier the capacity factor of the analyte is

$$k'_{0B} = \varphi n_{0B}K_{Bs} \tag{16}$$

which gives

$$k'_{cB} = k'_{0B}\exp(-z_B F\psi_0/RT)\cdot\frac{1}{1 + K_{As}c_A\exp(-z_A F\psi_0/RT)} \tag{17}$$

In order to test this equation experimentally it is combined with eqn. 14 to give

$$n_A = \left(\frac{k'_{cB}}{k'_{0B}}\right)\exp(z_B F\psi_0/RT)\,K_{As}c_A\exp(-z_A F\psi_0/RT) \tag{18}$$

When $z_B = z_A$ this equation is reduced to a form that is easily tested, *i.e.*,

$$n_A = \left(\frac{k'_{cB}}{k'_{0B}}\right)\cdot K_{As}c_A \tag{19}$$

Another way to test this equation will also be used in this paper. Consider two analytes,

B_1 and B_2, with charge of different sign but with the same magnitude. Using eqn. 17 for the capacity factor of the two analytes it is found that the ratio is

$$\frac{k'_{cB_1}}{k'_{cB_2}} = \frac{k'_{OB_1}\exp(-z_{B_1}F\psi_0/RT)}{k'_{OB_2}\exp(-z_{B_2}F\psi_0/RT)} \tag{20}$$

By considering that $z_{B_1} = -z_{B_2}$, the equation can be rewritten as

$$\psi_0 = -\frac{RT}{2z_{B_2}F}\ln\left[\left(\frac{k'_{OB_1}}{k'_{cB_1}}\right)\left(\frac{k'_{cB_2}}{k'_{OB_2}}\right)\right] \tag{21}$$

The value of the electrostatic potential calculated from this equation can be compared with that obtained from the Gouy–Chapman equation. It can also be used in eqn. 14 to calculate the adsorption isotherm of the amphiphilic modifier.

EXPERIMENTAL

The solutes were purchased from Janssen Chimica (Beerse, Belgium). The ion-pairing reagents [sodium butylsulphonate ($BuSO_3$), sodium hexylsulphonate ($HexSO_3$) and sodium octylsulphonate ($OctSO_3$)] and the buffer components were obtained from Merck (Darmstadt, F.R.G.). Mobile phases were prepared from deionized water and contained 25 mM phosphoric acid, 25 mM sodium dihydrogenphosphate (pH 2.1), varying amounts of sodium bromide and/or alkylsulphonate pairing ion. In some series of experiments the eluents also contained 10% (v/v) of methanol. ODS-Hypersil (5 μm) with a BET surface area of 173 m^3/g and a nominal carbon content of 8.8% (w/w) (according to the manufacturer) was used as the stationary phase (Shandon Southern Products, London, U.K.).

The equipment and the experimental technique used were as described previously[2,3] and allowed for the simultaneous determination of both the adsorption isotherm (by using the breakthrough method) and retention data for the solutes[2]. All measurements were performed at 25 $\pm$ 0.1°C.

RESULTS AND DISCUSSION

In Fig. 1 two different adsorption isotherms of $BuSO_3^-$ on RP-18 stationary phase are shown. The experimental difference between the two curves is due to the difference in ionic strength. One curve represents the adsorption isotherm with a constant ionic strength ($\times$), where the pairing ion variation is compensated by sodium bromide, whereas the other adsorption isotherm is obtained with a varying ionic strength ($\bigcirc$). The variation in ionic strength is accomplished by starting with 25 mM sodium dihydrogen phosphate and then adding $NaBuSO_3$ to the mobile phase. As the concentration of $BuSO_3^-$ varies between each experiment, each point represents different ionic strengths. The adsorption isotherm for $HexSO_3^-$ on RP-18 from a buffer–methanol mixture (90:10, v/v) at constant ionic strength is also shown in Fig. 1. According to the electrostatic theory of ion-pair chromatography, the adsorption isotherm should follow eqn. 14. To use this equation the surface potential must be

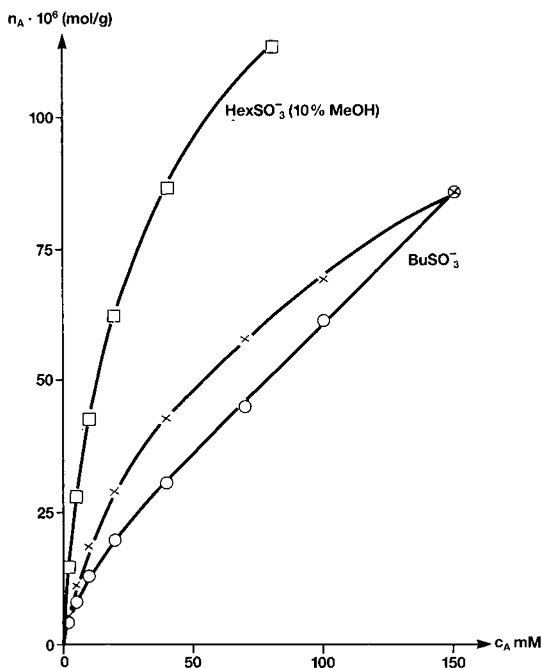

Fig. 1. Adsorption isotherm of $BuSO_3^-$ from a phosphate buffer (pH 2.1) on to an RP-18 stationary phase at constant (×) (175 mM Na^+) and varying (○) ionic strength (25–175 mM Na^+). Also shown is the adsorption isotherm of $HexSO_3^-$ (□) from phosphate buffer–methanol (90:10, v/v) (pH 2.1; $[Na^+]$ = 175 mM).

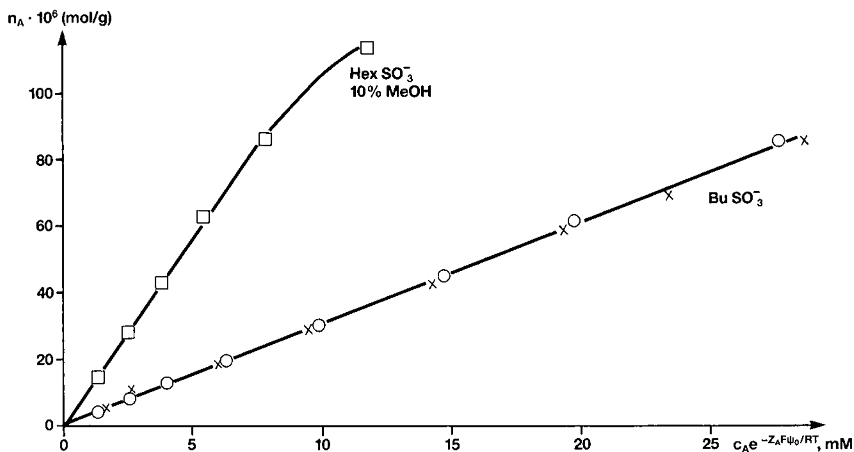

Fig. 2. Adsorption isotherms shown in Fig. 1 after correction for the surface potential. Symbols as in Fig. 1.

known. For all isotherms in this paper the surface potential was calculated using eqn. 21. Solute B_1 is *p*-toluensesulphonate in the buffer systems and naphthalene-sulphonate in the buffer–methanol eluents, and solute B_2 is adrenaline in all instances. All the experimental data for the $BuSO_3^-$ system can be found in ref. 3. Capacity factors below 0.4 are neglected because of the uncertainties in the determination of the column dead volume.

In Fig. 2 are shown the adsorption isotherms from Fig. 1 after appropriate correction for the surface potential. It can be seen that the two different adsorption isotherms for $BuSO_3^-$ now coincide. This is in agreement with the electrostatic theory and illustrates how isotherms can be rationalized by including the surface potential. It can also be seen that the surface potential-modified isotherm is linear, *i.e.*, the value of the denominator in eqn. 1 is close to unity in this low concentration range. The corresponding isotherm for $HexSO_3^-$ is also linear up to $100 \cdot 10^{-6}$ mol/g surface concentrations, where non-linearity begins.

It can be concluded that the properties of these three systems are entirely in agreement with the electrostatic theory as formulated in refs. 5 and 8. However, when the surface concentration of the amphiphile increases, systematic deviations from this simple theory occur. This is seen in the disappearance of the symmetrical behaviour of the capacity factor for analytes of opposite charge as a function of amphiphile concentration in the mobile phase. This is illustrated in Fig. 3, where the relative changes in capacity factors for *p*-toluenesulphonate and adrenaline are plotted as

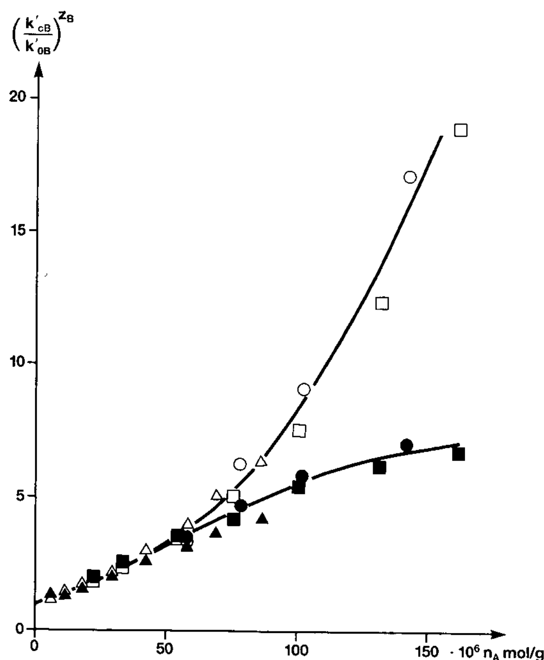

Fig. 3. $(k'_{cB}/k'_{0B})^{z_B}$ for *p*-toluenesulphonate (open symbols) and adrenaline (closed symbols) as a function of surface concentration of $BuSO_3^-$ (△, ▲), $HexSO_3^-$ (□, ■) and $OctSO_3^-$ (○, ●). Mobile phase, phosphate buffer (pH 2.1; $[Na^+]$ = 175 m*M*) for all points.

a function of surface concentration of various alkylsulphonates in a phosphate buffer eluent. A corresponding plot for naphthalenesulphonate and adrenaline in a buffer–methanol (90:10, v/v) eluent is shown in Fig. 4.

From Fig. 3 it can be seen that large discrepancies in the retention behaviour of the two ions start at about $100 \cdot 10^{-6}$ mol/g. The plots in Fig. 4 show the same behaviour but shifted to higher surface concentrations. The hypothesis made in this paper is that this deviation from the simple theory is due to the decrease in available surface area for the analytes, *i.e.*, it is connected with the Langmuir behaviour of the adsorption isotherm of the amphiphile (see eqn. 14). This hypothesis was tested in two ways: (i) by testing eqn. 19 and (ii) by examining the isotherms according to eqn. 14.

In Figs. 5 and 6 the experimentally obtained data are presented according to eqn. 19. The data in Fig. 5 are for the amphiphile $HexSO_3^-$ with phosphate buffer as mobile phase and for $OctSO_3^-$ with buffer–methanol (90:10, v/v) as mobile phase. The corresponding values for the capacity factors were obtained with the analytes *p*-toluenesulphonate and naphthalenesulphonate, respectively. In Fig. 6 the experimental findings are shown for $OctSO_3^-$ as amphiphile in buffer solution and with *p*-toluenesulphonate as analyte.

The results are in good agreement with the extended theory as presented in this paper, *i.e.*, the relationships are linear and pass through the origin. However, in Fig. 5 and at the highest surface concentrations there is a slight tendency for the analyte ion to overcompensate the non-linearity of the isotherms. More experimental data and a refined theory are needed in order to understand the origin of this behaviour.

The agreement between the presented theory and experimental results indicates that it is possible to calculate the surface potential from eqn. 21, even when analytes of opposite charge show asymmetric behaviour. According to the presented theory it is

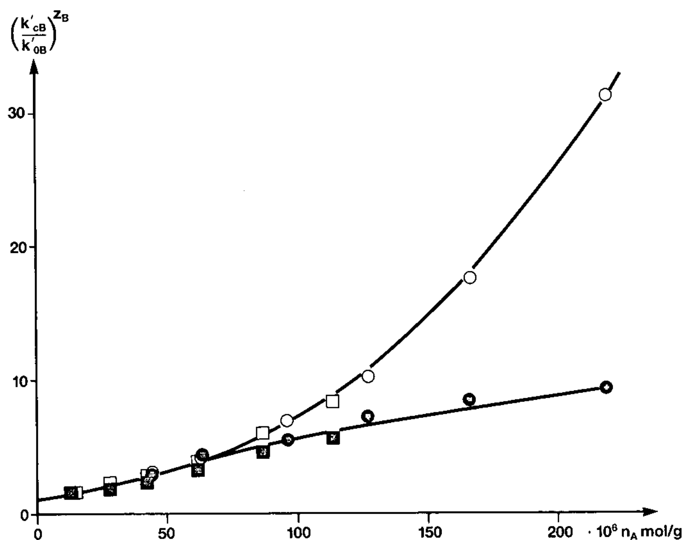

Fig. 4. $(k'_{cB}/k'_{0B})^{z_B}$ for naphthalenesulphonate (open symbols) and adrenaline (closed symbols) as a function of surface concentration of $HexSO_3^-$ (□, ■) and $OctSO_3^-$ (○, ●). Mobile phase, phosphate buffer–methanol (90:10, v/v) (pH 2.1; $[Na^+]$ = 175 mM) for all points.

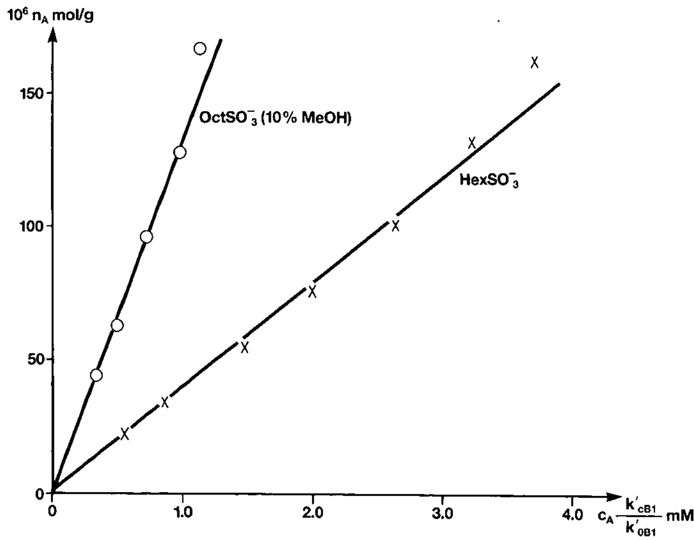

Fig. 5. Surface concentration of $HexSO_3^-$ (×) and $OctSO_3^-$ (○) as a function of $c_A(k'_{cB}/k'_{0B})$ (see eqn. 19). B is *p*-toluenesulphonate when $HexSO_3^-$ is used and naphthalenesulphonate when $OctSO_3^-$ is used. Mobile phase: $HexSO_3^-$, phosphate buffer (pH 2.1; [Na^+] = 175 m*M*); $OctSO_3^-$, phosphate buffer–methanol (90:10, v/v) (pH 2.1; [Na^+] = 175 m*M*).

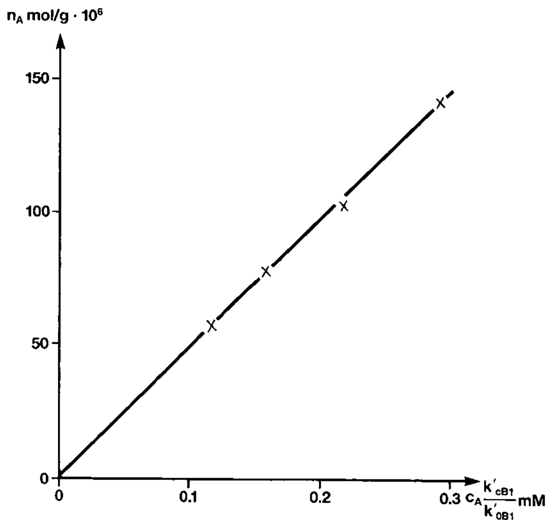

Fig. 6. Surface concentration of $OctSO_3^-$ as a function of $c_A(k'_{cB}/k'_{0B})$ (see eqn. 19). B is *p*-toluenesulphonate: Mobile phase, phosphate buffer (pH 2.1; [Na^+] 175 m*M*).

therefore possible to calculate the surface potential by using eqn. 21. This conclusion can be tested by inserting the value obtained for the surface potential in the equation for the isotherm (eqn. 14) and evaluating the result. To simplify the evaluation, eqn. 14 is rewritten in a reciprocal form:

$$\frac{c_A \exp(-z_A F\psi_0/RT)}{n_A} = \frac{1}{n_{0A}K_{As}} + \frac{c_A \exp(-z_A F\psi_0/RT)}{n_{0A}} \tag{22}$$

If the obtained potential is correct, the left-hand side of eqn. 22 will be a linear function of $c_A\exp(-z_A F\psi_0/RT)$ and from the slope n_0 can be calculated. The experimental results are plotted in Figs. 7–9, from which it is seen that in all instances, and over the entire concentration range, the adsorption isotherms follow eqn. 22. It can therefore be concluded that the obtained surface potential is correctly estimated over the whole concentration range.

It is also of interest to compare the experimentally obtained relationship between the surface potential and surface charge concentration with the corresponding relationship obtained by solving the Poisson–Boltzmann equation. The solution of the Poisson–Boltzmann equation is, however, dependent of the geometry of the system. As the Debye length (7.3 Å) is small, compared with the pore radius of the particles (60–100 Å), it is possible to use the Gouy–Chapman equation for a plane surface in these instances. This equation expresses the relationship between the surface potential and the concentration of the charges expressed in mol/m^2. The difficulty is then how to relate the amount of adsorbed amphiphile per gram of stationary phase to the concentration in mol/m^2. This is formally done by using the surface area as a conversion factor. The surface area reported by the manufacturer is 173 m^2/g. Using

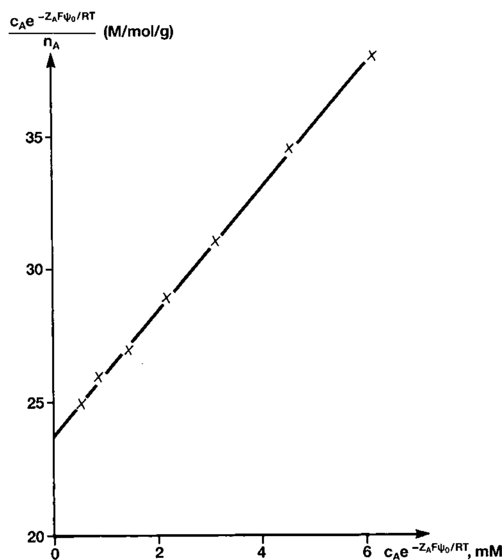

Fig. 7. Surface potential-modified Langmuir isotherm for $HexSO_3^-$ (see eqn. 22). Mobile phase, phosphate buffer (pH 2.1; [Na^+] = 175 m*M*).

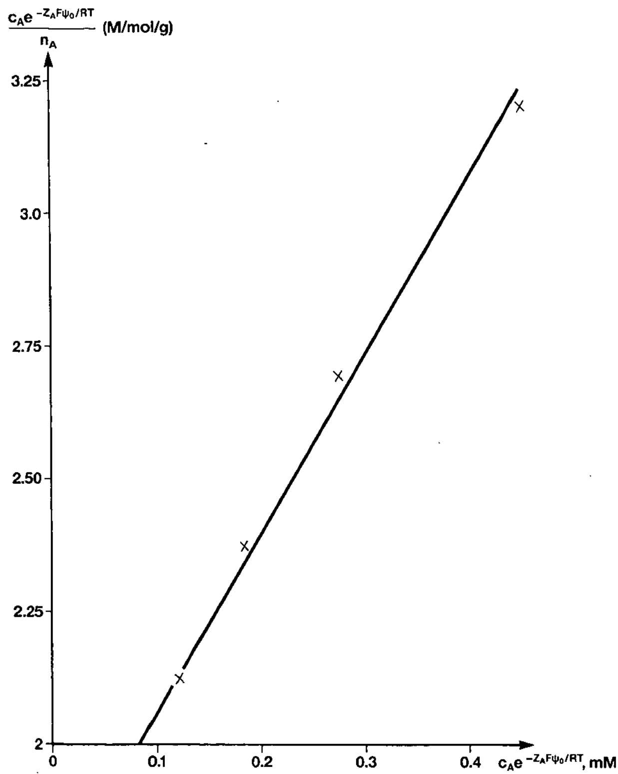

Fig. 8. Surface potential-modified Langmuir isotherm for $OctSO_3^-$ (see eqn. 22). Mobile phase, phosphate buffer (pH 2.1; $[Na^+]$ = 175 m*M*).

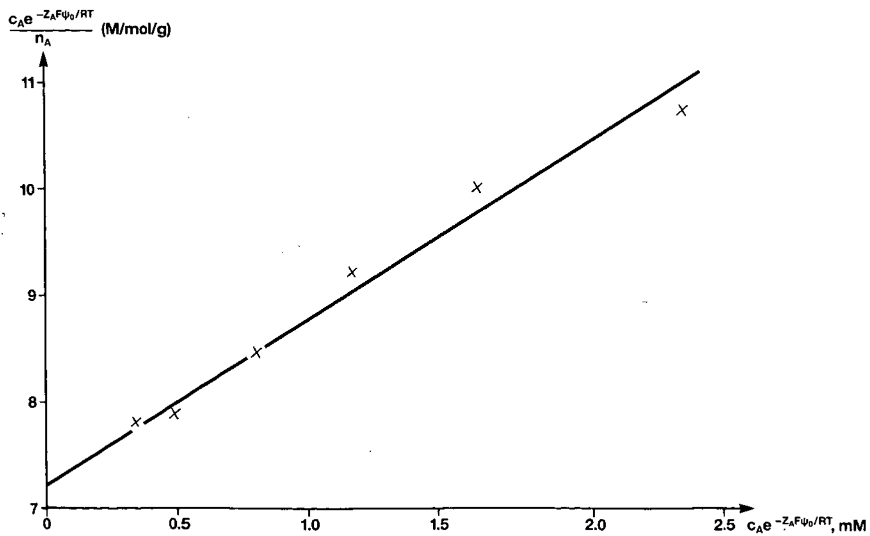

Fig. 9. Surface potential-modified Langmuir isotherm for $OctSO_3^-$ (see eqn. 22). Mobile phase, phosphate buffer–methanol (90:10, v/v) (pH 2.1; $[Na^+]$ = 175 m*M*).

this value for the surface area in combination with the Gouy–Chapman theory, the full line in Fig. 10 is obtained. Fig. 10 also shows the experimental values of the surface potential for all the studied amphiphiles in the buffer and in the buffer–methanol mixture. It is seen that the relationship between surface potential and surface concentration is independent of the chain length of the alkylsulphonate and that the same relationship holds when the mobile phase contains 10% of methanol. Both of these observations are in agreement with the concept of the electrostatic theory. Bartha and Vigh[9] have shown that retentions of ionic solutes form a common retention surface when plotted against the surface concentration of adsorbed alkylsulphonates, irrespective of the length of the alkyl chain, at constant inorganic counter ion concentration. As shown above, surface concentration and ionic strength are two of the parameters that determine the surface potential and consequently the solute retention, providing a theoretical explanation of the empirically found behaviour.

From the slope of the plots in Figs. 7–9 in combination with the surface area, it is possible to calculate the area that the ions occupy at the surface. The results are 44 $Å^2$ for $OctSO_3^-$ (10% methanol), 68 $Å^2$ for $HexSO_3^-$ and 92 $Å^2$ for $OctSO_3^-$. The results can be compared with the cross-sectional areas for straight-chain acids at a water–oil interface (45–60 $Å^2$). It is concluded that the values found on this stationary phase are of the correct order of magnitude except for that of $OctSO_3^-$ in buffer, which is higher than expected. It is interesting that the results indicate that the cross-sectional area for octylsulphonate is smaller at the methanol–buffer/RP-18 interface than at the buffer/RP-18 interface. The same trend is found when Figs. 3 and 4 are compared, *i.e.*, the asymmetric behaviour is shifted to higher surface concentration for the buffer–methanol mixture.

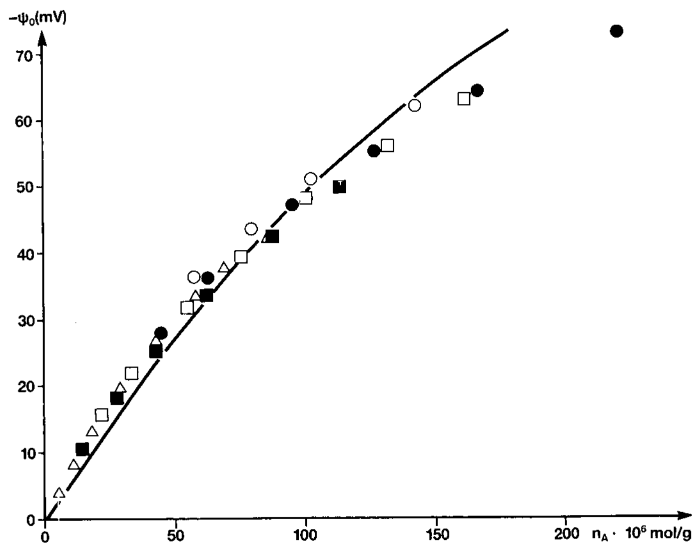

Fig. 10. Surface potential as a function of surface concentration of $BuSO_3^-$ (△), $HexSO_3^-$ (□, ■) and $OctSO_3^-$ (○, ●). The full line is the theoretical function according to the Gouy–Chapman theory with surface area 173 m^2/g. Mobile phase for open symbols, phosphate buffer (pH 2.1; $[Na^+]$ = 175 m*M*). Mobile phase for closed symbols, phosphate buffer–methanol (90:10, v/v) (pH 2.1; $[Na^+]$ = 175 m*M*).

It is appropriate in this context to comment on the determination of surface area for irregular surfaces. The surface area is usually measured with the BET method using nitrogen as a probe molecule. For irregular materials the surface accessibility may depend on the size of the probe molecule, which is due to the inability of a large adsorbate molecule to follow the irregularity of the surface[10]. The value obtained from BET measurements can therefore *a priori* not be used. In this particular instance there is, however, relatively good agreement between the values from the BET method and the Gouy–Chapman theory. The use of this value for the surface area in the determination of the cross-sectional areas above is therefore justified. The relationship between the geometric structure of the surface, the adsorption conformation of the molecules and the electrostatic laws is a complicated topic on which further studies are needed.

It is important to note that a number of secondary effects are neglected in this theory, *e.g.*, specific interactions between the ions, monopole–dipole interactions, changes in the properties of the surface layer and mobile phase when varying the concentration of amphiphile, image forces, etc.

CONCLUSIONS

Several important conclusions regarding the mechanism of ion-pair chromatography can be drawn from this work. Differences in the isotherms of $BuSO_3^-$ can be rationalized by the different surface potential at different inorganic counter ion concentrations. The isotherms coincide and become linear when the effect of the surface potential is taken into account. The variation of the surface potential with the ionic strength is also in agreement with the electrostatic theory. Differences in adsorption isotherms have previously been shown to be caused by pure electrostatic effects also for tetrabutylammonium ion as amphiphile[8]. When the surface concentration of the amphiphile is high, the simultaneous effect of competition for surface area and the effect of surface potential must be considered. This simultaneous effect can be treated quantitatively by the extended electrostatic theory presented in this paper. Good agreement is found between the theoretically expected and experimentally obtained adsorption isotherms of alkylsulphonate amphiphilic ions in both aqueous and methanol (10%, v/v) containing mobile phases. The consistency of the theory used is also illustrated in the agreement between the theoretical and experimental surface potential–surface charge density relationship (*cf.*, Fig. 10).

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RETENTION AND SEPARATION OF INORGANIC ANIONS BY REVERSED-PHASE ION-INTERACTION CHROMATOGRAPHY ON OCTADECYL SILICA

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SUMMARY

The retention behaviour of chloride, nitrite, bromide, nitrate and sulphate was investigated using Partisil 10 ODS-3 as the stationary phase and a solution of tetrabutylammonium in aqueous phthalate or phosphate buffer as the eluent. The quaternary ammonium ion adsorption behaviour and the influence of the quaternary ammonium concentration, the pH and the buffer concentration on the retention of the inorganic anions is discussed. The major adsorption and ion-exchange equilibria in the retention process were studied and used to develop a retention model for the different analytes. This retention model was verified experimentally. Ion-interaction reversed-phase chromatography with an octadecyl-bonded silica stationary phase and tetra-butylammonium iodide–phthalate eluent was found to be a successful system for the separation and determination of inorganic anions and when conductivity detection is used.

INTRODUCTION

Inorganic anions have frequently been separated and determined by means of ion chromatography (IC) using an anion-exchange resin. Reflecting the rapid developments in recent years in the IC technique, a number of remarkable new anion exchangers and instrumentation have appeared on the market[1–3], which allow the efficient separation of common anions such as F^-, Cl^-, NO_2^-, Br^-, NO_3^-, PO_4^{3-} and SO_4^{2-} with detection limits at the sub-ppm level.

As an alternative to ion chromatography, several approaches based on reversed-phase chromatography (RPC) have been used to separate the common inorganic anions[4–6]. Among these, ion-pair chromatography (IPC) or ion-interaction chromatography (IIC) using an octadecyl-bonded silica as a nonpolar stationary phase has developed considerably. In this technique, a buffered[7] or unbuffered[8] aqueous eluent containing a hydrophobic positively charged ion is used. The major advantages of this technique are (a) the system is nearly completely nonspecific, (b) the separation system takes advantage of highly efficient reversed-phase columns and (c) it

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can be readily modified to meet new analytical conditions and hence shows a much greater degree of flexibility than do conventional "fixed-site" ion exchangers. In addition, no special equipment is required to perform ion analyses by this technique; only conventional high-performance liquid chromatographic (HPLC) pumps and detectors are required[9].

It is well known that retention and separation occur when a hydrophobic ion-interaction reagent is added to the mobile phase of a reversed-phase system. Several models of the retention mechanism of organic ionized solutes have been proposed for reversed-phase IPC, the most important being (a) ion-pair adsorption[10,11], (b) dynamic ion-exchange and a similar ion-interaction model[5,9,12–15] and (c) the Stern–Gouy–Chapman theory of the electrical double layer and more recently a combination of the Gouy–Chapman theory and the Langmuir isotherm[16,17].

The above retention models have been extensively discussed in the recent literature but their application in reversed-phase inorganic separations is still not clear. It was also often observed that the separation abilities of reversed-phase packings differ considerably. The frequently employed reversed-phase columns for the separation of inorganic or organic anions are μBondapak C_{18}, LiChrosorb RP-18, Zorbax ODS and ODS Hypersil. Skelly[4] used a Partisil 10 ODS-3 packing for the separation of a mixture of IO_3^-, Br^-, NO_2^-, NO_3^- and I^-, and Cassidy and Elchuk[6] used it for the separation of IO_3^-, $S_2O_3^{2-}$, NO_2^-, NO_3^- and I^-. In both investigations, a phosphate buffer and UV detection at low wavelength were used. Important anions such as Cl^- and SO_4^{2-} were not included in the separation. In addition, degradation of the column performance much shorter column lifetimes occurred with a phosphate buffer at higher pH[6].

We therefore studied the retention behaviour of common inorganic anions on a reversed-phase ion-interaction system with an octadecyl bonded stationary phase (Partisil 10 ODS-3) and a mobile phase consisting of a hydrophobic quaternary ammonium salt dissolved in a phosphate or phthalate buffer. The adsorption characteristics of the quaternary ammonium salt on the solid phase, the factors that control retention of sample anions and the differences in the column performance with different buffers present in the eluent are discussed. We found that using a phthalate buffer together with an ion-pair or ion-interaction reagent increases the column performance and its lifetime significantly in comparison with the conventional phosphate-buffered ion-pair chromatographic system. It also allows an interesting comparison with anion-exchange chromatography, where a simple phthalate eluent is used[18–20]. The similar separation characteristics with these two techniques are reflected in very close or identical anion separation sequences. This is useful for the understanding of the separation mechanism of ion-pair or ion-interaction reversed-phase chromatography, which may result in an important routine anion analysis method, more advantageous than the ion-exchange mode.

EXPERIMENTAL

Apparatus and reagents

The chromatographic system consisted of (1) a DuPont 870 HPLC pump; (2) a Valco injection valve with a 100- or 50-μl loop volume; (3) a Partisil 10 ODS-3 RP column (250 × 4.6 mm I.D.) (Whatman) acting as a separation column and protected

with a 60 × 2.1 mm guard column with the same packing; (4) a Perkin-Elmer LC-21 conductivity detector and (5) an Omniscribe B5 217-5 strip-chart recorder. The separation column, guard column and detector cell were thermally isolated in a wooden box to minimize short-term temperature variations. All separations were carried out at room temperature and a mobile phase flow-rate of 2 ml/min unless stated otherwise.

Analytical-reagent grade chemicals were used unless stated otherwise. Water was deionized and passed through a Millipore (Bedford, MA, U.S.A.) Milli-Q water purification system. Tetrabutylammonium iodide (TBAI, 98% for synthesis), tetrabutylammonium hydroxide (TBAOH), potassium dihydrogen phosphate and phthalic acid (H_2P) were obtained from Merck (Darmstadt, F.R.G.), potassium hydrogen phthalate (KHP), from RCB (Belgium) and sodium monohydrogenphosphate from Baker (Deventer, The Netherlands). Standard solutions (1000 ppm) of inorganic anions (chloride, nitrite, bromide, nitrate, sulphate and dichromate) were prepared by dissolving appropriate amounts of the corresponding potassium salt in pure water. These solutions were diluted daily to give the trace required.

Mobile phases

The eluents used contained (1) TBAI or TBAOH with KHP or H_2P at pH between 4 and 6.5 or (2) TBAI with phosphate buffer (KH_2PO_4–Na_2HPO_4) at pH between 5.8 and 7.3. The concentrations of TBAI ranged from 0.5 to 16 mM. The concentrations of KHP and phosphate buffer are stated for each individual experiment. The pH of all mobile phases was adjusted by adding to a solution, containing a weighed amount of the salt, potassium hydroxide or acetic acid followed by dilution to volume. The effect of these ions on the separation behaviour of anions (see Results and Discussion) is included in the global effect of ionic strength or buffer anion. These eluents were prepared daily, filtered through a 0.45-μm porosity membrane filter (Millipore HSWP 04700) and degassed before use.

Column preparation

The columns were packed in the laboratory by a slurry technique using 2-propanol as a suspension medium under a pressure of 6000–6500 p.s.i. They were washed successively with 100 ml of methanol and 60 ml water before use. Column testing was carried out with the mobile phase. A sample containing a mixture of chloride, nitrite, bromide, nitrate and sulphate was prepared giving a capacity factor (k') of about 1.3–7. Columns that gave theoretical plate heights of more than 15 000 were accepted for use.

Breakthrough method

In order to determine the amount of TBAI adsorbed on the stationary phase, and the way it is affected by TBAI concentration, phthalate concentration and pH of the eluent, a series of experiments in which the latter variables were varied, was carried out. Each experiment was followed by column washing with methanol and water, equilibrating first the guard column then the analytical column (by disconnecting the analytical column from the guard column and reconnecting them later).

The amount of TBAI adsorbed by the stationary phase (Q_s, mol/g) was determined by use of the breakthrough volume (V_R):

$$Q_s = (V_R - V_m)Q_m/W_s$$

where Q_m is the concentration of TBAI in the eluent (mol/l), W_s is the amount of solid phase in the column (g) and $V_R - V_m$ is the net retention volume.

RESULTS AND DISCUSSION

Choice of hydrophobic quaternary ammonium salt

Initially, a number of hydrophobic quaternary ammonium salts such as tetrabutylammonium salt (C_{16}), trioctylmethylammonium salt (C_{25}) and tridodecylmethylammonium salt (C_{37}) were used as ion-interaction reagents. Their adsorption behaviour on Partisil ODS-3 stationary phase differed. C_{25} and C_{37} were permanently coated on the stationary phase such that the aqueous mobile phase used did not contain any of the quaternary ammonium salt. The C_{16} salt, however, was not permanently adsorbed on the solid phase and a dynamic equilibrium existed between the solid phase and the aqueous mobile phase which must contain a certain amount of the salt. Because the reproducibility of the retention time and the peak height of the sample anion was not very good with the permanently coated C_{25} and C_{37} salts, the C_{16} salt (TBAI or TBAOH) was selected as the hydrophobic ion-interaction reagent and was used throughout.

Hydrophobic ion-adsorption behaviour on Partisil 10 ODS-3 stationary phase

Tests have shown that, when no quaternary ammonium salt is precoated on the reversed-phase column packing or is present in the eluent, no anion retention will occur. The retention of inorganic anions depends primarily on the amount of quaternary ammonium ion adsorbed on the stationary phase.

The adsorption isotherms of TBAI are shown in Fig. 1. They were obtained by the breakthrough method with and without KHP at different pH values. An initial increase in TBA concentration results in an increase in the fraction of the TBA ion adsorbed on the column packing surface. The isotherms indicate that with a further increase in TBA concentration in the eluent, the amount of TBAI adsorbed approaches a plateau. In other words, the surface area of the modified silica gel that can be covered by TBA is limited. Further increases in the TBAI concentration in the mobile phase do not result in further increases in the TBA adsorbed.

Data resulting from the breakthrough method given in Table I and Fig. 2 indicate that the absence or presence of KHP and higher or lower pH values have little influence on the amount of TBA ion adsorbed. They suggest that the competing adsorption of KHP with the TBA adsorption on the stationary phase can be neglected.

This observation, however, is different from the results of other workers. Melin *et al.*[10] studied the retention behaviour of some acids and bases using μBondapak C_{18} as the stationary phase and a solution of tetrabutylammonium in acetonitrile and phosphate buffer as the mobile phase. More recently, Sokolowski[11] studied the adsorption of organic ions on μBondapak from a water-based mobile phase. They concluded that all ions in the eluent may affect the adsorption and retention of the quaternary ammonium ion. The adsorption of the quaternary ammonium ion increases when a second organic ion with opposite charge is added, or when the pH of the mobile phase is increased. It should pointed out that under our chromatographic

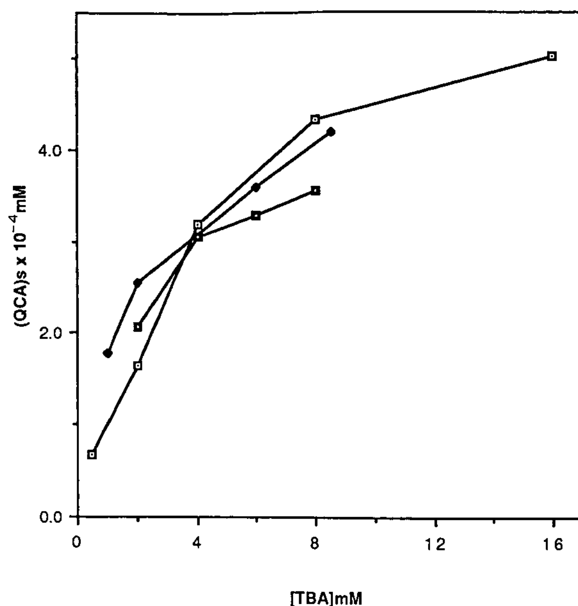


Fig. 1. Experimentally determined adsorption isotherms of tetrabutylammonium iodide on Partisil 10 ODS-3 column at 25°C from different eluent series. KHP concentration: (□) 0 m*M*, pH 4; (◆) 1.5 m*M*, pH 5; (■) 0.5 m*M*, pH 6.

TABLE I

INFLUENCE OF KHP CONCENTRATION AND pH ON THE AMOUNT OF TBAI ADSORBED ON ODS-3 STATIONARY PHASE

Eluent	*KHP (mM)*	*Amount of TBAI adsorbed* ($\times 10^{-4}$ *mM*)	
		Per column	*Per gram*
4 m*M*, TBAI	0.00	3.25	1.12
pH 6	0.50	3.13	1.08
	1.00	3.41	1.18
	2.00	3.27	1.13
6 m*M*, TBAI	0.88	3.25	1.12
pH 5	1.50	3.35	1.16
	2.13	3.31	1.16
Eluent	*pH*	*Amount of TBAI adsorbed* (*mol* $\times 10^{-4}$)	
		Per column	*Per gram*
6 m*M* TBAI–	3.8	3.00	1.03
1.5 m*M* KHP	5.0	3.35	1.16
	6.3	3.38	1.17

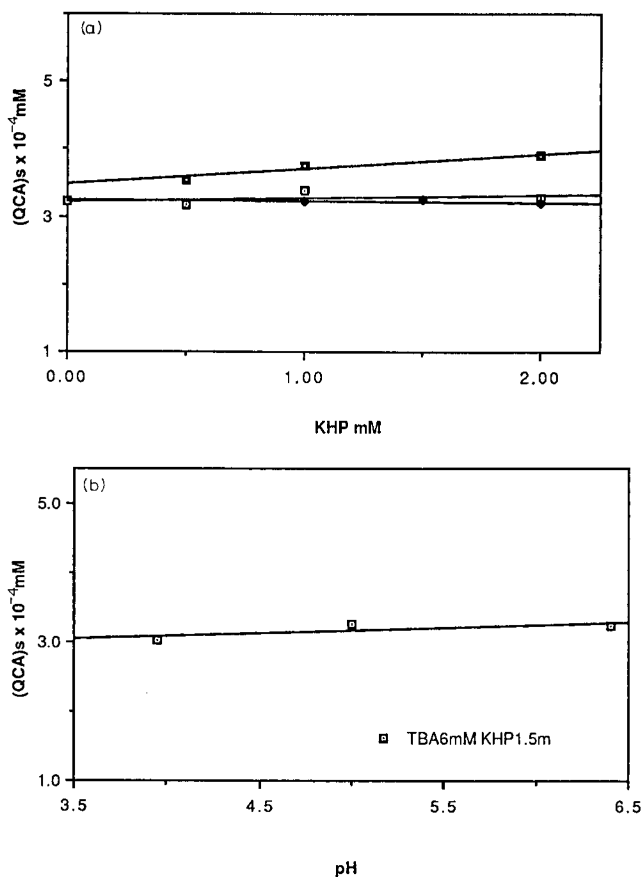


Fig. 2. (a) Influence of KHP concentration on the amount of TBAI adsorbed on Partisil 10 ODS-3 column. TBAI concentration: (⊡) 4 m*M*, pH 6; (◆) 6 m*M*, pH 5; (■) 8 m*M*, pH 6. (b) Influence of pH on the amount of TBAI adsorbed on Partisil 10 ODS-3 column. TBAI, 6 m*M*; KHP, 1.5 m*M*.

conditions, the amounts of the second organic ion (HP^-) added are relatively small; in addition, the natures of the two organic compounds present are very different. Adsorption of TBA ion on the ODS stationary phase is due to a strong hydrophobic interaction force whereas KHP lacks such an interaction force with the stationary phase.

Our results are in agreement with those of Barber and Carr[9], who studied the retention and UV detection of inorganic anions by reversed-phase ion-interaction chromatography. They observed that the buffer ions were unable to cause a significant change in the surface concentration of ion-interaction reagent. The buffer ions used in their work were either inorganic (phosphate) or small organic ions such as acetate, which are relatively hydrophilic and have relatively small extraction constants compared with long-chain organic sulphonates or sulphates. For example, higher "breakthrough" volumes are obtained on equilibration of a column when the eluent contains an alkanesulphonate.

Factors that affect the inorganic anion retention

The discussion will focus on the three most important factors that influence the retention of sample anions: concentration of the quaternary ammonium salt, concentration of the buffer and pH of the eluent. In addition, no organic modifier must be added to the mobile phase, as we found that the stationary phase with a Partisil 10 ODS-3 packing is very weak, considering the retention of inorganic anions, even when quaternary ammonium salts are present.

TBAI concentration. Figs. 3 and 4 show the dependence of the capacity factor of five anions on the TBAI concentration in the eluent. In Fig. 3 the k' values of the anions increase with increasing TBAI concentration, reach a maximum and then decrease with further increase in TBAI concentration. Although all k' *versus* TBA concentration plots have a similar convex shape, the degree of convexity differs. The plot for SO_4^{2-} has the most pronounced convex shape and Cl^- has the smoothest curve. At low TBA ion concentrations, the magnitude of the retention increase with increasing TBA ion concentration follows the order $SO_4^{2-} > NO_3^- > Br^- > NO_2^- > Cl^-$. After each sample anion has passed the maximum k' value, the retention decreases with further increase in TBA ion concentration in the order $SO_4^{2-} > NO_3^- > Cl^- \approx NO_2^- \approx Br^-$. The relationship between k' and TBAI concentration in Fig. 4 is similar to that in Fig. 3. However, we observe only the initial part of the curve in Fig. 4, as the concentration range of TBAI tested was narrow.

Phosphate or phthalate concentration and pH of the eluent. Figs. 5–8 show partial linear relationships between $1/k'$ and the mobile phase pH (except at high phosphate buffer concentration in Fig. 8) or ionic strength (KH_2PO_4–Na_2HPO_4 buffer or KHP

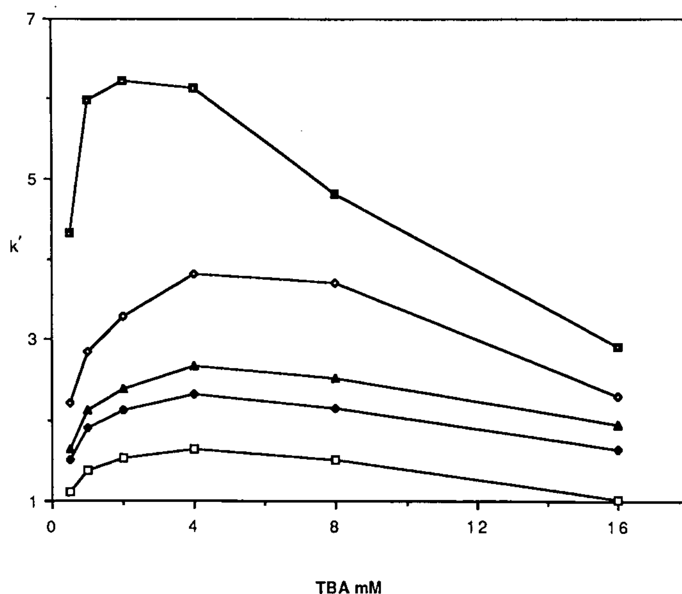


Fig. 3. Dependence of the capacity factor of inorganic anions on the concentration of TBAI in an aqueous phthalate eluent. Chromatographic conditions: flow-rate, 2 ml/min; Partisil 10 ODS-3 column; conductivity detector. Anions: □, Cl^-; ◆, NO_2^-; ▲, Br^-; ◇, NO_3^-; ◘, SO_4^{2-}.

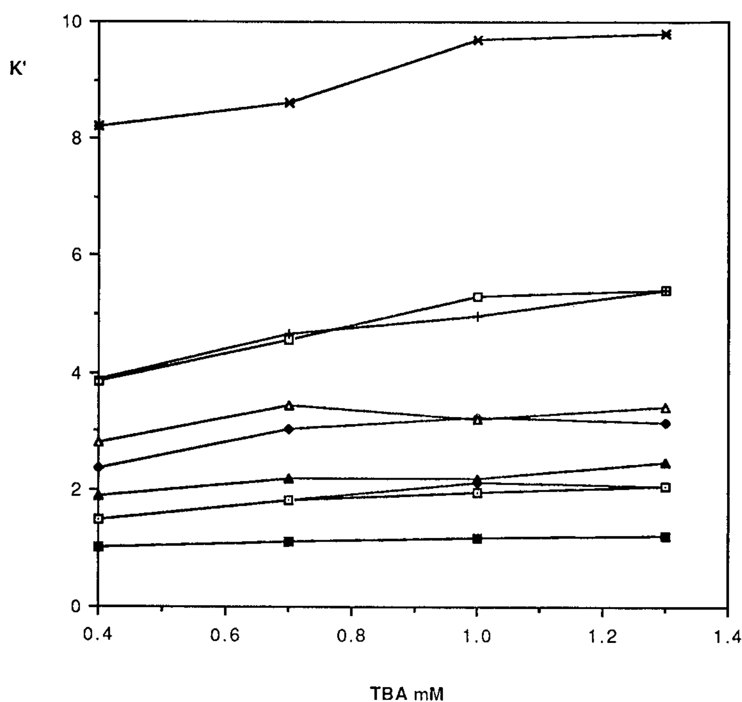


Fig. 4. Dependence of the capacity factor of inorganic anions on the concentration of TBAI in aqueous phosphate eluent of pH 6 and 7.15. Chromatographic conditions as in Fig. 3. pH 6: ◇, Cl^-; ◆, NO_2^-, Br^-; □, NO_3^-; ×, SO_4^{2-}. pH 7.15: ■, Cl^-; ⊡, NO_2^-; ▲, Br^-; △, NO_3^-; +, SO_4^{2-}.

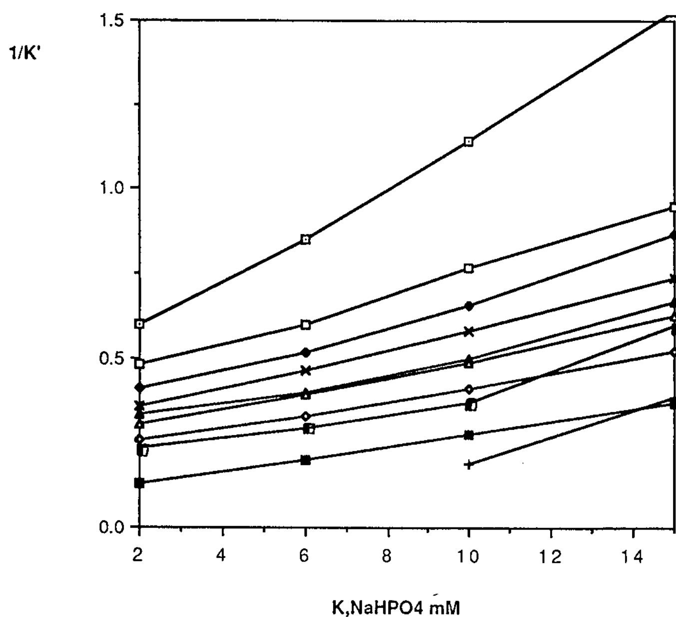


Fig. 5. Effect of phosphate concentration on anion retention. Eluent: 1 m*M* TBAI at pH 6.4 and 7.15. Chromatographic conditions as in Fig. 3. pH 6.4: □, Cl^-; ▲, NO_2^-; △, Br^-; ▮, NO_3^-; +, SO_4^{2-}. pH 7.15: ⊡, Cl^-; ◆, NO_2^-; ×, Br^-; ◇, NO_3^-; ■, SO_4^{2-}.

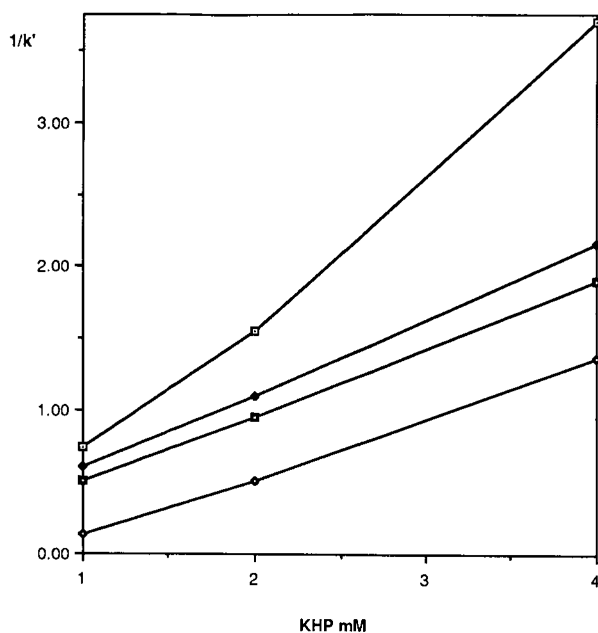


Fig. 6. Effect of phthalate concentration on anion retention. Eluent: 1 m*M* TBAI at pH 4.05. Chromatographic conditions as in Fig. 3. □, Cl^-; ◆, NO_2^-, Br^-; ■, NO_3^-; ◇, SO_4^{2-}.

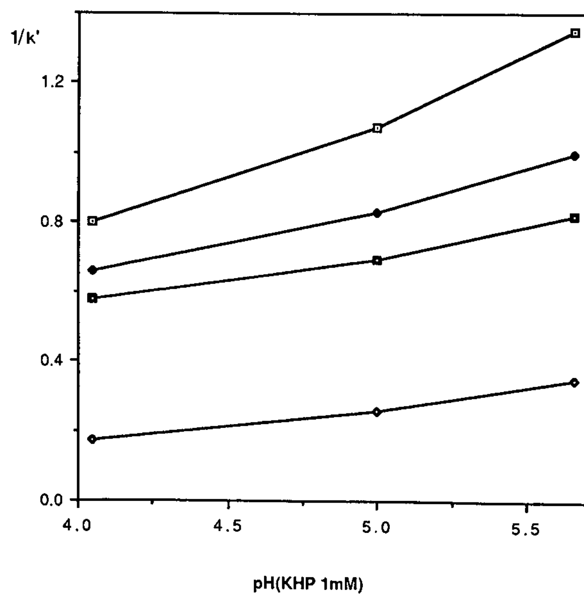


Fig. 7. Dependence of anion retention on pH with phthalate eluent containing 1 m*M* KHP and 1 m*M* TBAI. Chromatographic conditions as in Fig. 3. □, Cl^-; ◆, NO_2^-, Br^-; ■, NO_3^-; ◇, SO_4^{2-}.

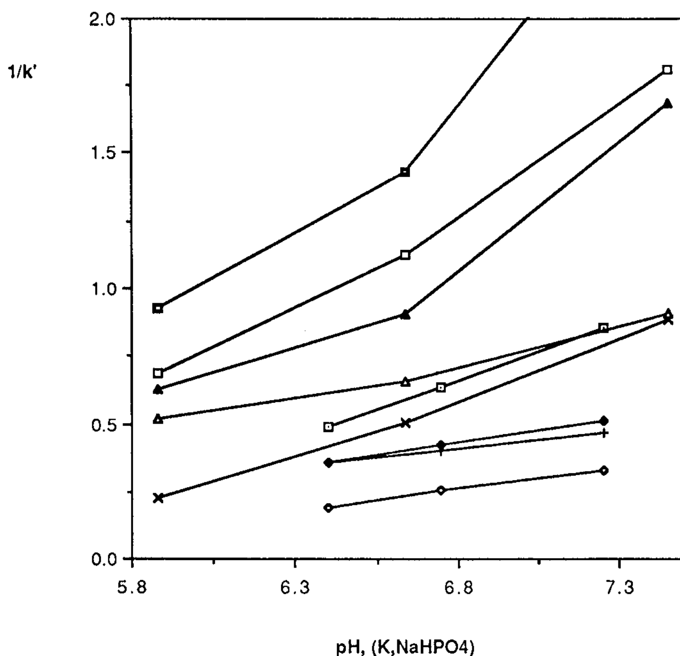


Fig. 8. Dependence of anion retention on pH with phosphate-buffered eluent. Eluent: TBAI concentration, 1.0 m*M*; phosphate buffer concentration, 6 and 22.5 m*M*. Chromatographic conditions as in Fig. 3. 6 m*M*: ⊡, Cl^-; ◆, NO_2^-; +, Br^-; ◇, NO_3^-. 22 m*M*: ◘, Cl^-; □, NO_2^-; ▲, Br^-; △, NO_3^-; ×, SO_4^{2-}.

buffer). An increase in the buffer concentration results in a decrease in the capacity factor, as shown in Fig. 5 (phosphate buffer) and Fig. 6 (phthalate buffer). pH has a similar effect on the capacity factor, as an increase in pH shifts the equilibria of phthalate to HP^- and P^{2-}, of phosphate to H_2P^-, HP^{2-} and P^{3-} or, generally, to B^- and B^{2-}. A higher B^- concentration yields a lower capacity factor, as shown in Fig. 7 (with phthalate buffer) and Fig. 8 (with phosphate buffer). The basis for this observation may be two-fold[9]. First, a higher buffer concentration means that the ionic strength of the eluent increases, which will tend to shield the sample ions from the ion-interaction reagent ions on the surface, thus decreasing its retention. Second, the buffer ions, which would normally be less strongly retained than the sample, will compete more effectively with the sample ions for retention on the surface at high buffer concentration.

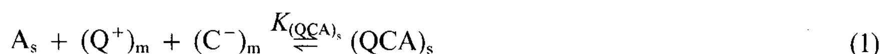
In order to understand these results better, we shall use a mathematical model describing the relationship between k' and those factors which influence the anion retention.

Retention model. Retention of inorganic anions on a Partisil octadecyl-bonded stationary phase can be considered as a result of two processes: (a) the formation of an electrical double layer at the boundary of adsorbed TBA and (b) an exchange process. The quaternary ammonium salt (Q^+C^-) was pre-adsorbed on the surface of the stationary phase based on hydrophobic interactions and led to a dynamic adsorption equilibrium of Q^+ between the solid phase and mobile phase. The co-anion C^- of

R_4N^+ formed a secondary layer on the adsorbed Q^+; it is here that the ion-exchange process takes place. In our system three significant ion-exchange equilibria have to be considered: ion exchange between (1) the co-anion of R_4N^+ and the anion of additional ionic strength (B^-) (hydrogenphosphate or hydrogenphthalate), (2) the co-anion of R_4N^+ and the analyte anions (X^-) and (3) the anion of ionic strength salt that replaced the co-anion of adsorbed R_4N^+ and the analyte anion.

Concerning the buffer anions, the following predominant ionic forms are calculated. For phthalate buffer at pH 5 (a typical experimental condition), and using K_{a_1} and K_{a_2} values of $1.3 \cdot 10^{-3}$ and $3.9 \cdot 10^{-6}$, respectively, the ratio $HP^-/H_2P = 130$ and the ratio $HP^-/P^{2-} = 2.6$. HP^- is therefore the predominant form, but P^{2-} is not negligible. For phosphate buffer at pH 6 (also a typical experimental condition), and using K_{a_1}, K_{a_2} and K_{a_3} values of $7.52 \cdot 10^{-3}$, $6.23 \cdot 10^{-8}$ and $2.2 \cdot 10^{-13}$, respectively, the ratio $H_2PO_4^-/HPO_4^{2-} = 16$ and the ratio $H_2PO_4^-/H_3PO_4 = 7520$. $H_2PO_4^-$ is therefore the predominant form. In the model we shall only consider monovalent buffer or additive anions. We also asume that the adsorption on the stationary phase of an organic ion other than quaternary ammonium ion is negligible. The amount of quaternary ammonium salt and the ionic strength salt (or buffer components) added to the mixed eluent are approximately equal. The major equilibria in the retention processes can be expressed as follows:

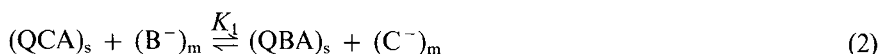
(a) The adsorption of quaternary ammonium by the stationary phase:



$$A_s + (Q^+)_m + (C^-)_m \overset{K_{(QCA)_s}}{\rightleftharpoons} (QCA)_s \quad (1)$$

where A_s is the number of free sites on the stationary phase, $(Q^+)_m$ and $(C^-)_m$ are the quaternary ammonium ion and the co-anion in the mobile phase, respectively, and $K_{(QCA)s}$ is the equilibrium constant.

(b) The ion-exchange equilibria:



$$(QCA)_s + (B^-)_m \overset{K_1}{\rightleftharpoons} (QBA)_s + (C^-)_m \quad (2)$$

$$(QCA)_s + (QBA)_s + 2(X^-)_m \overset{K_2}{\rightleftharpoons} 2(QXA)_s + (C^-)_m + (B^-)_m \quad (3)$$

where $(B^-)_m$ and $(X^-)_m$ are the buffer anion and sample anion, respectively. K_1 and K_2 are the equilibria constants of eqns. 2 and 3, respectively.

(c) The sorption capacity (K_0):

$$K_0 = A_s + (QXA)_s + (QCA)_s + (QBA)_s \quad (4)$$

where K_0 is the sorption capacity of the stationary phase and is a measure of the total number of sites that can be occupied in the retention process (moles sorbed per gram of sorbent).

(d) The capacity factor k'_x, given by

$$k'_x = q(QXA)_s/(X^-)_m \quad (5)$$

where q is the phase ratio.

Combining eqns 1–4 with elimination of A_s, $(QCA)_s$ and $(QBA)_s$ and substituting the solution of $(QXA)_s$ into eqn. 5 gives

$$1/k'_x = 1/qK_0[(C^-)_m/K_1^{1/2}K_2^{1/2} + K_1^{1/2}(B^-)_m/K_2^{1/2} + 1/K_{(QCA)s}\,K_1^{1/2}K_2^{1/2}(Q^+)_m + (X^-)_m] \quad (6)$$

$$1/k'_x = 1/qK_0K_1^{1/2}K_2^{1/2}[(C^-)_m + 1/K_{(QCA)s}(Q^+)_m + K_1(B^-)_m + K_1^{1/2}K_2^{1/2}(X^-)_m] \quad (7)$$

Interpretation of the experimental results. Eqn. 7 describes a linearship between the reciprocal capacity factor, $(k')^{-1}$, and the concentrations in the mobile phase of the buffer anion or more generally the ionic strength of the mobile phase (B^-), the anion of the quaternary ammonium salt (C^-), the reciprocal quaternary ammonium ion (Q^+) and the sample anion (X^-). The linear relationship between the reciprocal capacity factor and the buffer anion concentration (eqn. 7) was demonstrated experimentally (Figs. 5 and 6). pH has a similar effect on the capacity factor. An increase in pH increases the concentration of the phthalate or phosphate ions, thus increasing B^- and B^{2-} (Figs. 7 and 8). As the ratio B^-/B^{2-} may change substantially when the pH is varied over a relatively broad range, the relationship between pH and $(k')^{-1}$ may no longer be linear (see, for example, Fig. 8). The relationship between the capacity factor and the quaternary ammonium salt concentration is more complicated. Indeed, an increase in the latter results in an increase of both ions (C^- and Q^+) in the mobile phase, but these ions, according to eqn. 7, have opposing effects on the capacity factor.

Bartha and Vigh[21] emphasized the competing effects on the capacity factor of sample ions of an increased surface concentration of the hydrophobic ion (Q^+) and an increased mobile phase concentration of the counter ion (C^-). We found that an increased concentration of TBA in the mobile phase results in increased adsorption on the hydrophobic surface, as indicated by the adsorption isotherm, and hence increased retention of the sample anions. Unless the counter ion, C^-, is a long-chain compound, which was not the case in our experiments, counter ions are not expected to have a large effect on the adsorption of the quaternary ammonium salt. The quaternary ammonium counter ion (C^-) can, however, compete with the sample anions for retention on the surface, especially at high mobile phase concentrations of C^-. The retention of sample anions may then decrease.

To explain the results in Figs. 3 and 4 with our model, we have rewritten eqn. 7 using eqn. 1:

$$1/k'_x = 1/qK_0K_1^{1/2}K_2^{1/2}\{[(QCA)_s + A_s]/K_{(QCA)s}(Q^+)_mA_s + K_1(B^-)_m + K_1^{1/2}K_2^{1/2}(X^-)_m\} \quad (8)$$

or

$$1/k'_x \propto [1/(Q^+)_m] \cdot [(QCA)_s + A_s]/A_s$$

where $(Q^+)_m$ is the amount of quaternary ammonium ion not adsorbed at equilibrium, $(QCA)_s$ the amount of quaternary ammonium salt QC adsorbed at equilibrium and A_s the number of free sites at equilibrium.

We shall now consider four situations to explain the experimental results shown in Figs. 3 and 4.

(1) At low quaternary ammonium salt concentration $(Q^+)_m$ is low, A_s is high and $(QCA)_s$ is small compared with A_s. This means that

$$1/k'_x \propto 1/(Q^+)_m$$

or

$$k'_x \propto (Q^+)_m$$

The initial part of the curve shows the greatest increase in k'_x for a given increase in TBA concentration.

(2) When half of the initially available adsorption sites on the resin are occupied, $A_s = (QCA)_s$. This means that

$$1/k'_x \propto 2/(Q^+)_m$$

or

$$k'_x \propto (Q^+)_m/2$$

and the increase in k'_x with increasing TBA concentration is lower than in the first instance; the curve has a slower slope.

(3) Still increasing the quaternary ammonium salt concentration leads to a maximum of the k'_x *versus* $(Q^+)_m$ curve; A_s is very low and $(QCA)_s \propto K_0$ is much larger than A_s. This means that

$$1/k'_x \propto [(QCA)_s/A_s]\,[1/(Q^+)_m] \propto (K_0/A_s)\,[1/(Q^+)_m]$$

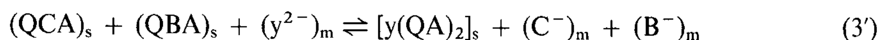
or

$$k'_x \propto (A_s/K_0)\,(Q^+)_m$$

As A_s/K_0 is very small, the increase in k'_x with TBA also becomes very small or even zero.

(4) A further increase in the quaternary ammonium salt concentration reduces the capacity factor ($A_s \rightarrow 0$) when all free sites are occupied. The term A_s/K_0, independently of the magnitude of $(Q^+)_m$, will tend to zero if A_s tends to zero. In this TBA concentration range, k'_x decreases for a given increase in TBA concentration. This mathematical result means in fact that the maximum amount of TBA that can be adsorbed is reached (saturation of the surface of the solid material), and that increasing amounts of TBA in the mobile phase result in increased competition between the counter ions and sample ions. Although under our experimental conditions the possibility of micelle formation is very small, this effect cannot be totally excluded as an alternative explanation for the reduction in the capacity factor at high TBA concentration.

Corresponding to eqn. 3, the ion exchange of a divalent anion y^{2-} becomes



$$(QCA)_s + (QBA)_s + (y^{2-})_m \rightleftharpoons [y(QA)_2]_s + (C^-)_m + (B^-)_m \qquad (3')$$

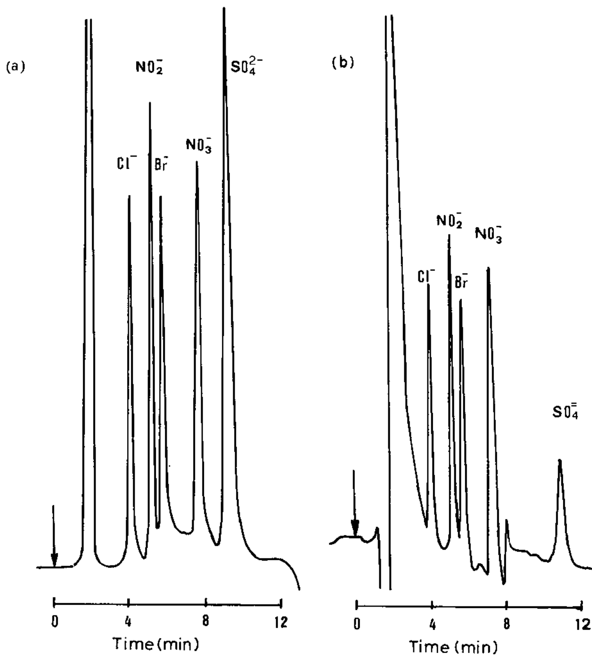


Fig. 9. Separation of sample anions with TBAI–phthalate and TBAI–phosphate eluents. Chromatographic conditions: Partisil 10 ODS-3 column (250 × 4.6 mm I.D.); flow-rate, 2 ml/min; sample injection volume, 25 μl; conductivity detection at room temperature. Eluent: (a) TBAI concentration 8 m*M*, phthalate concentration 1.0 m*M*, pH 6; (b) TBAI concentration 0.5 m*M*, phosphate concentration 4 m*M*, pH 7.3.

TABLE II

PERFORMANCE CHARACTERISTICS OF COLUMNS FOR THE SEPARATION OF A STANDARD ANION MIXTURE

Column	*Eluent*	*pH*	*Retention time (s)*				
			Cl^-	NO_2^-	Br^-	NO_3^-	SO_4^{2-}
ODS-3	TBAI (0.5 m*M*)–K,NaHPO$_4$ (4 m*M*)	7.3	240	315	338	425	645
ODS-3	TBAI (0.5 m*M*)–KHP (1 m*M*)	5.0	210	250	268	324	540
ODS-3	TBAI (8 m*M*)–KHP (1 m*M*)	6.0	229	302	342	462	528
Hamilton PRP-X100	KHP (1 m*M*)	5.5	128	148	169	195	490
Hamilton PRP-X100	*p*-Hydroxybenzoic acid (4 m*M*)	8.5	160	186	242	300	750
Vydac 302 IC*	KHP (2 *M*)	5.0	–	–	–	–	–

* Efficiency data supplied by manufacturer.

Using a similar treatment as above to eqn. 3′ results in a quadratic equation. The solution of this equation is too complex a function of (B^-), (C^-), (X^-) and (Q^+) to be useful for interpretation of the experimental results. The plot of SO_4^{2-} has a more pronounced convex shape than the plots for monovalent anions (Fig. 3).

Partisil 10 ODS-3 column performance characteristics

The efficiency of nonpolar columns is usually assessed by chromatographing aromatic solutes with methanol–water as the mobile phase. However, such an evaluation of the performance of a nonpolar packing material has no meaning when it is used in an ion-pair or ion-interaction chromatographic rather than in a reversed-phase system[8]. Moreover, a realistic test for an IPC or IIC system must be carried out. The ODS-3 columns used for this performance study were all freshly slurry packed in the laboratory. A standard mixture containing 20 ppm of chloride, 40 ppm of nitrite and sulphate and 50 ppm of bromide and nitrate was used for the evaluation of the column performance. Sample injection volumes were 25 μl. The chromatograms obtained are shown in Fig. 9. The chromatograms obtained with the above standard mixture using TBAI phthalate (pH 6) and TBAI phosphate (pH 7.3) were used to calculate the column efficiency and the resolution of a critical peak pair. The results are given in Table II and show that an eluent containing tetrabutylammonium iodide and phthalate buffer has more advantages than using a phosphate buffer. The ODS-3 column gives the highest theoretical plate number when an eluent containing 0.5 m*M* TBAI and 1 m*M* KHP was used. Under these conditions, the resolution of the nitrite–bromide peak pair and the peak heights of chloride, nitrate and sulphate, however, were low. When a more concentrated TBAI (8 m*M*) eluent was used at a higher pH (6), the column gave a lower theoretical plate number, but an adequate resolution for the nitrite–bromide peak pair. In addition, we obtained the highest conductance (peak heights) for chloride, nitrate and sulphate, and also the shortest overall retention time.

Efficiency, theoretical plates (k′ = 4)		*Resolution of critical peak pair*		*Peak height (cm) (conductance measurement)*		
Per column	*Per metre*	NO_2^-–Br^-	NO_3^-–SO_4^{2-}	Cl^-	NO_3^-	SO_4^{2-}
3688	14 752	1.14	6.00	6.4	8.1	3.2
4996	19 984	0.95	7.20	6.8	6.4	4.1
3780	15 120	1.38	1.52	10.2	11.7	19.4
2158 (Br^-) (150 cm column length)	14 387	1.40	1.33 (Cl^-–NO_2^-)	6.7	2.9	1.2
1442 (150 cm column length)	9613	1.70	0.60 (F^-–CO_3^{2-})	9.2	2.0	2.5
4936 (NO_3^-)	19 744	–	–	–	–	–

TABLE III

COMPARISON OF COLUMN PERFORMANCE DEGRADATION WITH TBAI–KHP AND TBAI–PHOSPHATE ELUENTS

Eluent	*Retention time (s)*					*Resolution* (R_s), NO_2^-–Br^-	*Theoretical plates per column*	*Peak height (cm)*			*Time period*	*No. of injections*
	Cl^-	NO_2^-	Br^-	NO_3^-	SO_4^{2-}			Cl^-	NO_3^-	SO_4^{2-}		
8 m*M* TBAI–	230	295	328	440	527	1.20	4190	8.80	8.20	12.5	3–8/1/86	56
1 m*M* KHP, pH 5	220	290	325	445	530	1.06	2943	8.30	9.00	17.0	9–16/1/86	38
	235	298	325	429	545	0.90	2824	11.1	12.0	19.9	16/1–4/2/86	100
	238	305	325	425	565	0.59	2772	11.0	10.2	21.0	5/2–6/5/86	85
0.5 m*M* TBAI–				440	640	1.54		5.3	6.5	3.6	12/10/85–12/11/85	80
4 m*M* K,NaHPO$_4$,				325	405	0.93		5.1	4.7	5.9		
pH 7.3				320	360	0.73		5.9	5.2	6.1		
8 m*M* TBAI– 1 m*M* KHP, pH 6.0	184	238	269	378	434	0.94	2594	8.0	7.9	14.4	Repacked column	–

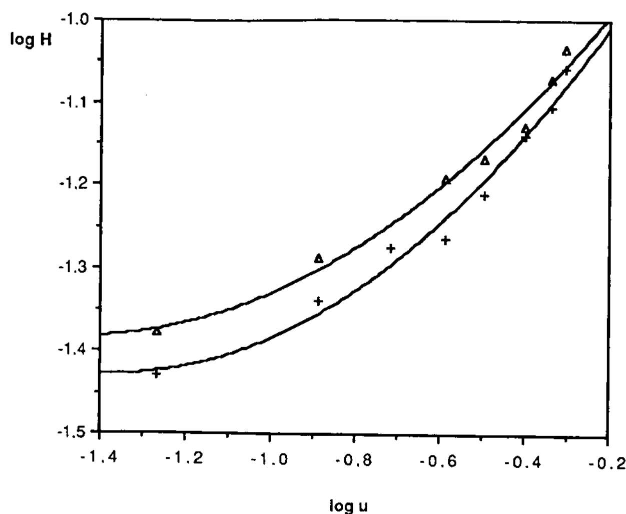


Fig. 10. Plate height *versus* mobile phase flow-rate plots for a laboratory-packed Partisil 10 ODS-3 column. Eluent: TBAI concentration 0.5 m*M*, KHP concentration 1 m*M*, pH 5. Chromatographic conditions as in Fig. 3. +, Nitrate; △, sulphate.

The performance of the ODS-3 column was also compared with those of Hamilton PRP-X100 and Vydac 302 IC low-capacity pre-packed ion-exchange columns. The separation of anions with these ion-exchange columns was carried out with a simple phthalate eluent. The plate number and resolution values given in Table II show that the efficiency of the ODS-3 column when used with a TBAI–phthalate eluent is as good as that of the commercially pre-packed columns. The peak heights of nitrate and sulphate obtained with the ODS-3 column using TBAI–phthalate eluent were several times higher than those obtained with the PRP-X100 column. This indicates that the TBAI–phthalate eluent together with an ODS-3 column provides a sensitive means of separation and detection of common inorganic anions. In addition, the same order of elution of the analytes as with the different ion-exchange columns listed in Table II was obtained, indicating that an ion-exchange mechanism is involved in the separation of anions when using a reversed-phase ODS-3 packing.

The plate height (H) is also a measure of the column packing according to Snyder and Kirkland[22]. The best packed column will give a value of H between 0.01 and 0.03 mm. The relationship between plate height and mobile phase velocity was studied (Fig. 10). The plate heights for nitrate were 0.037 mm at a mobile phase flow-rate of 0.4 ml/min and 0.053 mm at 2 ml/min and those for sulphate were 0.042 mm at 0.4 ml/min and 0.064 mm at 2 ml/min. These values are close to those given by Snyder and Kirkland[22].

pH limitation and column degradation

The basic material of the Partisil ODS-3 packing consisted of silica gel. This material provides excellent rigidity of the packing and hence tolerance to high back-pressures and flow-rates, but creates problems when eluents of high or low pH are used. In addition, the tendency to adsorb fluoride makes it difficult to determine this ion.

The pH limitation towards alkaline eluents is imposed by the solubility of the silica material and represents a serious drawback to the use of the ODS-3 column. In addition, phosphate eluents are relatively aggressive and attack the silica packing[22], especially at higher pH. Therefore, it was necessary to seek other eluents to replace the TBAI–phosphate eluent.

Phthalate is usually a powerful eluent, often used in low-capacity single-column anion-exchange chromatography[19]. The equivalent conductance of phthalate is relatively low compared with other inorganic ions, *e.g.*, the limiting equivalent conductance of phthalate is 38 whereas those of phosphate, potassium and sodium ions are 67, 74 and 50, respectively. A low conductance is an important factor when using a conductivity detector. We found that for anion separations using a reversed-phase ODS-3 packing, phthalate is the most suitable additive when a quaternary ammonium salt is present. The advantages of using phthalate as an additive to the TBAI eluent are (1) better anion separations can take place even when the pH is below 6, (2) there is a wider pH range (4–6.5) in which the variation of the chromatograms is relatively small, (3) a relatively low background conductance increased the detection response when using conductivity detection and (4) reduced column degradation and longer column lifetime were obtained compared with the use of a TBAI–phosphate eluent.

Table III shows the degradation data when TBAI–phosphate and TBAI–phthalate eluents were used. The lifetime of the ODS-3 column was approximately three times longer when using the TBAI–phthalate eluent. For further prolongation of the column lifetime a regeneration technique (repacking the column) can be used[23]. In our work, we tested a simple repacking procedure in which no fresh column material was added, *i.e.*, the packing was washed with methanol and then suspended in de-gassed 2-propanol. Using the common slurry method for repacking, gave the results shown in Table III. The repacked columns are good enough for a new series of analyses.

CONCLUSION

Ion-interaction or ion-pair reversed-phase chromatography carried out with an aqueous eluent containing a quaternary ammonium salt and a phthalate buffer was found to be an efficient system for the separation and determination of inorganic anions. The presence of phthalate and variation of the pH in the eluent have a minor influence on the adsorption of tetrabutylammonium iodide on an octadecyl-bonded stationary phase. A model developed to explain the retention of inorganic anions has been verified by the experimental results. Ion-exchange-based anion retention is primarily decided by the adsorption of a quaternary ammonium salt on the stationary phase and its amount in the mobile phase; second, it is influenced by the type and amount of buffer and the pH of the eluent.

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ELECTROSORPTIVE DETECTION OF SIMPLE ORGANIC COMPOUNDS IN LIQUID CHROMATOGRAPHY

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SUMMARY

The detection of a range of neutral organic compounds in liquid chromatography based on decreases in differential double-layer capacitance, ΔC_d, at a mercury–aqueous interface caused by analyte specific adsorption is described. The arrangement employs alternating current phase-selective measurements using a large-volume wall-jet configuration. The low-molecular-weight organic solutes examined include aliphatic alcohols, diols, mono- and dicarboxylic acids, amines and alkanolamines. The differential capacitance measurements were conducted close to the potential of zero charge, where adsorption of such species is most extensive. Plots of ΔC_d *versus* analyte concentration were generally sigmoidal, in accordance with expectations from the Frumkin adsorption isotherm. A potential-step coulometric method, where variations in the non-faradaic charge, $\Delta(\Delta q)$, are measured, was found to be a useful alternative detection scheme. Methods based on measuring transient capacitive currents associated with tensammetric adsorption–desorption behavior were also briefly investigated. A virtue of these double-layer capacitance detection schemes is that the magnitude of the ΔC_d or $\Delta(\Delta q)$ response exhibits a clear sensitivity to the organic molecular structure, as anticipated in view of the known dependence of the adsorption thermodynamic upon solute hydrophobicity.

INTRODUCTION

Many batch electrochemical procedures based on electrosorption (ES), or specific adsorption at an electrode, have been published for the determination of surface-active (surfactant) organic compounds. These procedures have been developed almost exclusively for mercury as the electrode material, and fall under the general heading tensammetry. Early work in electrosorption analysis is reviewed in an ACS monograph[1] and in Jehring's comprehensive text[2]. Most of the early methods were based on alternating current (a.c.) impedance involving the measurement of decreases in capacitive current induced by specific adsorption. These methods continue to be employed[3–12]. Recent work has focussed on overcoming the limitation of such batch methods in distinguishing between components in mixtures. To this end, several recent studies have utilized instead various pulse techniques, taking advantage

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of the sharp capacitance-potential peaks associated with adsorption–desorption (so-called "tensammetric peaks") that are commonly found for organic adsorbates at aqueous interface. These methods include galvanostatic pulses[13] as well as multiple potential-step perturbations analogous to normal[14–16] or differential pulse polarographic techniques[17–21].

Despite the gains made in discrimination through the application of pulse and other techniques, the resolving power for complex mixtures continues to be lacking. An obvious approach to dealing with this shortcoming is to couple electrosorption measurements to a chromatographic effluent. We shall label this general approach as LC–ES (liquid chromatography–electrosorptive detection). Kemula *et al.*[22] and Borkowska *et al.*[23] applied an a.c. method based on changes in double-layer capacitance (DLC) to LC detection. Lankelma and Poppe[24] investigated the use of electrosorptive detection for the determination of several non-ionic as well as ionic surfactants in an LC couple. They measured the diminution in a.c. current at a fixed phase angle. DeJong *et al.*[25] took a similar approach to the determination of a steroid hormone and several cardiac glycosides. Kemula and co-workers[26,27] employed a variation on this technique, presenting the data as a reciprocal capacity. The latter authors argued that this approach is better suited for measurements in solutions of low conductivity. Bond and co-workers[28,29] developed microprocessor-based instrumentation with an assortment of excitation profiles from which to choose —alternating-current, square-wave, differential-pulse and normal-pulse— for use in LC–ES. After comparing these, they recommended the use of the normal pulse format with a complete series of potential steps applied to each mercury drop.

Batch electrosorptive analysis has undergone more study than has LC-ES, but the limitations of the former, particularly with regard to mixtures, are clear. Prior separation, as can be provided by LC, is essential in most practical situations. However, the few LC–ES studies noted above have not demonstrated great promise. A primary reason, in our opinion, has been the lack of suitable chromatography. All separations save one[24] were conducted by reversed-phase chromatography, and employed significant amounts of organic modifier in the mobile phase. This has the unfortunate consequence of diminishing the extent of analyte adsorption by competition with the organic modifier.

We have, therefore, sought in our work to demonstrate the potential of LC–ES by utilizing recent developments in column-packing technology that allow the use of strictly aqueous eluents in the separation of several classes of low-molecular-weight, sparingly water-soluble organic compounds known to electrosorb[30]. These include alcohols, diols, mono- and dicarboxylic acids, amines and alkanolamines, all detected in the neutral form. Several of these compound classes are not readily detected by other means.

The primary means of detection utilized in this work is based on changes in the differential double-layer capacitance, ΔC_{d}, induced at a suitable potential by analyte specific adsorption. We have recently applied this approach to the determination of inorganic anions at both mercury[31] and silver[32] in ion chromatography. We also employ here the use of a related detection scheme based on potential-pulse coulometry (PPC)[33,34]. Additionally, two techniques based on "tensammetric peak" detection were briefly investigated. The first involves the measurement of non-faradaic current transients induced upon application of a potential step through the adsorption/

desorption peak, while in the second the corresponding charge is measured. These techniques are complementary to DLC detection.

EXPERIMENTAL

Instrumentation/measurement techniques

Differential capacitance measurements were made utilizing a.c. impedance methodology, as detailed in ref. 31. The experimental arrangement is shown in Fig. 1. In this scheme the time-dependent capacitance change, ΔC_d, resulting from analyte adsorption is obtained by means of phase-selective detection, and is calculated in real time by a computer using:

$$C_d = (\omega E)^{-1} [(I_{out}^2 + I_{in}^2)/I_{out}] \quad (1)$$

In this expression, I_{in} is the in-phase and I_{out} the quadrature cell current from the lock-in analyzer, E is the magnitude of the superimposed sinusoid and ω is its frequency in rad s^{-1}. Eqn. 1 will only be valid in the absence of reversible faradaic currents. We therefore included in the measurement scheme an amperometric monitor, which is the low-pass filter/amplifier shown in Fig. 1. Ordinarily we operated at a.c. frequencies in the range 45–150 Hz. Capacitance measurements were made in the vicinity of the potential of zero charge (pzc), with the exact potential chosen to maximize the ΔC_d response.

In the PPC technique the double-layer charging current that flows in response to a potential step is integrated[33]. We have applied PPC previously to the determination of inorganic ions at a silver electrode[33]. The only change required to adapt the arrangement used there to mercury is the inclusion of a synchronized drop dislodgement pulse.

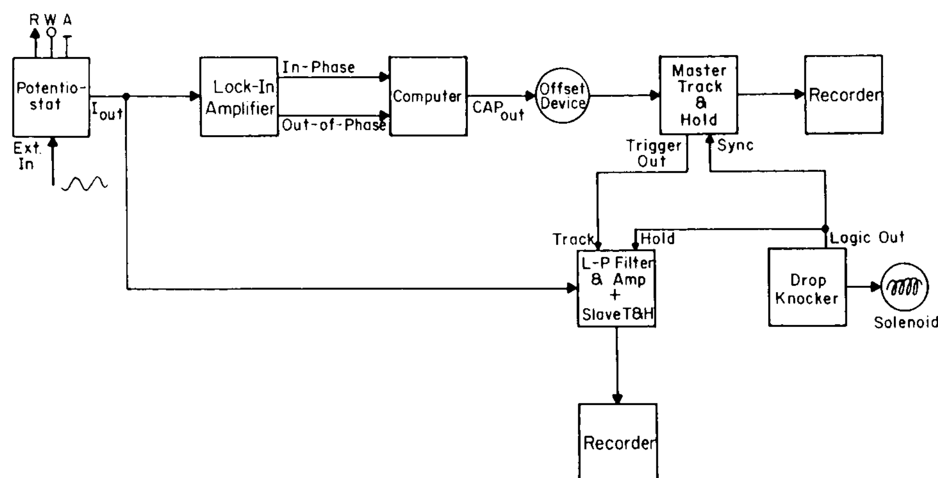

Fig. 1. Block diagram of the instrumental arrangement for double-layer capacitance detection at the DME with simultaneous faradäic electrochemical detection.

The primary means of tensammetric peak detection involved stepping or sweeping rapidly the potential in a negative direction through the adsorption/desorption peak and processing the resulting current by computer. The current transient that derives from the adsorption/desorption peak is identified as the largest value stored in memory, and is then output to the strip chart recorder. The excitation pulse or sweep used in tensammetric peak detection must account for the variation in the tensammetric peak potential with concentration[29]. With each of these transient techniques the measurement was repeated about once a second, or as the chromatography dictated.

To support this work, several electronic modules were built in-house. These include a mercury drop knocker, a sample and hold amplifier, a low-pass filter/

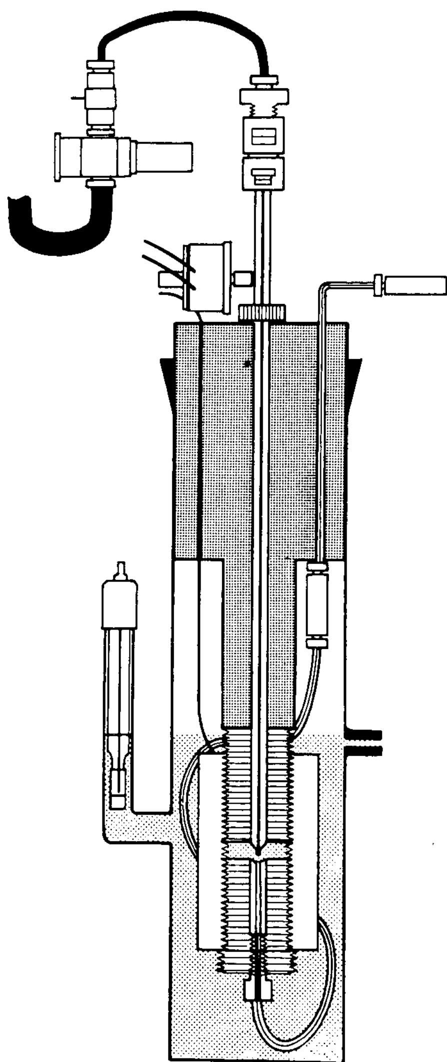

Fig. 2. Large-volume wall-jet cell/electrode assembly with drop knocker for the DME.

amplifier/(slave) sample and hold, a "computer interface" with a wideband amplifier, a synchronized analog coulometer and a drop dislodgement detector. A detailed description of these modules and their incorporation into various electrosorptive detection schemes is given elsewhere[34]. A PAR 5204 lock-in analyzer was also used along with a PAR 173 or 273 potentiostat and a DEC PDP-11/23 microcomputer.

Cell/electrode

A cell based on the large-volume wall-jet (LVWJ) concept[31,35,36] was used in most of the work reported here. The arrangement used with a dropping mercury electrode (DME) is shown in Fig. 2. During use, the cell was tilted at about 15° from the vertical to prevent mercury from clogging the lower capillary through which eluent was delivered. The attributes of the LVWJ used in the present study have been enumerated previously[31]. An important feature is that a high-concentration make-up stream can be conveniently added to maintain virtually any desired overall electrolyte concentration. DME capillaries used were 8 in. × 0.25 in. O.D. × either 0.004 or 0.005 in. I.D. (Wilmad Glass). For the most part, flat-bottom capillaries were used. A drop time of *ca.* 1 s was generally employed, either forced or natural. In the latter case, which was only used in DLC detection, a drop dislodgement detector served to synchronize the measurement to drop disengagement. A coiled platinum wire served as the auxiliary electrode, and an silver–silver chloride (3 *M* sodium chloride) (Model RE-1, Bioanalytical Systems) as the reference electrode.

LC methods

Several related chromatographies were employed in our studies. The aqueous separations were accomplished using mixed interaction chromatography (MIC)[37], known variously as ion exclusion, ion-moderated partition and Donnan exclusion chromatography[37]. As MIC is commonly practiced, it makes use of cation exchange packings based on macroreticular resins composed of polymerized styrene–divinylbenzene derivatized with sulfonic acid groups. Generally the mobile phase consists only of dilute mineral acid. Sulfuric (and nitric) are acceptable choices from the standpoint of DLC detection since both are only weakly specifically adsorbing[38].

To separate alcohols, diols, mono- and dicarboxylic acids, we ordinarily used a Brownlee Polypore H cartridge (either 10 cm × 4.6 mm or 25 cm × 4.6 mm), generally at elevated temperature to improve column efficiency; either a Bio-Rad Cation H^+ or Polypore H was used as the guard (3 cm × 4.6 mm cartridge). Elevated temperature capability was provided by an Alltech HPLC water jacket (Cat. No. 95024) in conjunction with a Haake (Type FJ) water-bath. For ion exclusion separations of amines and alkanolamines, a Bio-Rad Anion-OH guard was used alone with dilute, high-purity potassium hydroxide (Alfa) as eluent. Completing the LC system were a Waters 6000A pump and a Rheodyne 710 injector with either a 10- or 20-μl injection loop. To guard against background noise derived from dissolved oxygen, the eluent was heated and deaerated and the cell contents blanketed with nitrogen throughout an experiment. Most chemicals were purchased from Aldrich, although diols and glycols were obtained from Wilmad Glass (Cat. No. 213-12) and acetonitrile and methanol from Burdick & Jackson. The water was "Milli Q" purified water (Millipore).

BACKGROUND

The thermodynamic basis of DLC detection has been discussed earlier for inorganic anions[31]. In that case the differential capacitance, C_d, at a given potential is increased progressively by specific adsorption to an extent determined by the anion surface concentration, Γ, and hence the bulk concentration, c_x. It was shown that the relationship between ΔC_d and c_x is approximately linear as long as Γ is proportional to c_x, *i.e.*, Henry's Law applies.

In contrast to anions, adsorption of neutral organic species usually leads to marked decreases in C_d, a least in the vicinity of the pzc. This behavioral difference can be understood as follows. For anionic adsorbates, the extent of adsorption is typically strongly potential dependent, and yields substantial increases in the electrode charge at a given potential. These two coupled effects, which constitute the so-called "adsorption capacity"[39], necessarily yield large increases in C_d upon adsorption. For neutral organic molecules, on the other hand, the potential dependence of the adsorption in the vicinity of the pzc is commonly mild and non-monotonic, so that the effect of the adsorption capacity is unimportant. However, in contrast to inorganic anions, the dielectric properties of the inner layer can be altered substantially upon organic adsorption; usually C_d will exhibit significant decreases since replacement of interfacial water by organic molecules will both decrease the polarity and increase the thickness of the inner-layer region.

The relationship between the capacitance at a given electrode potential and the fractional coverage of the organic adsorbate, θ, can be given approximately under these conditions as[39]

$$C_d = C_d^0(1 - \theta) + C_d^1 \theta \tag{2}$$

where C_d^0 is the capacitance in the absence of the adsorbate, and C_d^1 is the value for monolayer coverage, *i.e.*, at saturation. From eqn. 2 it is simple to deduce that the decrease in capacitance, ΔC_d, brought about by adsorption will be proportional to the surface concentration, Γ, for $\theta \ll 1$. Additionally, if Henry's Law applies, *i.e.*, $\Gamma \alpha c_x$, then ΔC_d will be proportional to the bulk concentration; such a simple linear ΔC_d–c_x relation is clearly desirable for analytical purposes.

For higher coverages, however, more complex ΔC_d–c_x relations are anticipated, dependening on the adsorption isotherm encountered. A convenient and much-used form for data analysis and interpretation is the Frumkin isotherm[40], which can be expressed as

$$Bc_x = [\theta/(1 - \theta)] \exp(-2g\theta) \tag{3}$$

where B is the adsorption coefficient, and g is a parameter describing the adsorbate–adsorbate interactions, having a positive or negative sign for systems displaying attractive or repulsive interactions, respectively. This relation is employed below.

RESULTS AND DISCUSSION

In this section we present our results for aqueous DLC detection applied to several classes of organic compounds —to alcohols, diols, mono- and dicarboxylic

acids, amines and alkanolamines. Sample chromatograms are shown for each class. The response–concentration behavior typifying these systems is discussed. While most application is to DLC detection, PPC and tensammetric peak detection are also briefly considered.

Alcohols

Alcohols were separated by mixed interaction chromatography utilizing dilute mineral acid as eluent; sulfuric and nitric acids gave roughly comparable performance. Capacitance–potential (C_d–E) curves under hydrodynamic flow were generated for nitrate and sulfate to establish an acceptable lower limit for the supporting electrolyte concentration. A concentration in excess of 0.05 M was found to be desirable. An optimization experiment that, in principle, should be performed at the start of each LC-DLC experiment is the determination of the potential at which the capacitance depression, ΔC_d, is a maximum for a representative substrate. While the surface excess, Γ, will be a maximum near the pzc, it does not necessarily follow that $|\Delta C_d|$ will be largest there. A potential in the range -0.50 to -0.525 V was found optimal for alcohols, diols, mono- and dicarboxylic acids. A slightly more positive potential was most effective for amines and alkanolamines.

Two illustrative separations of alcohols with double-layer capacitance detection at the DME are shown in Fig. 3. Note that ΔC_d is negative. These separations were

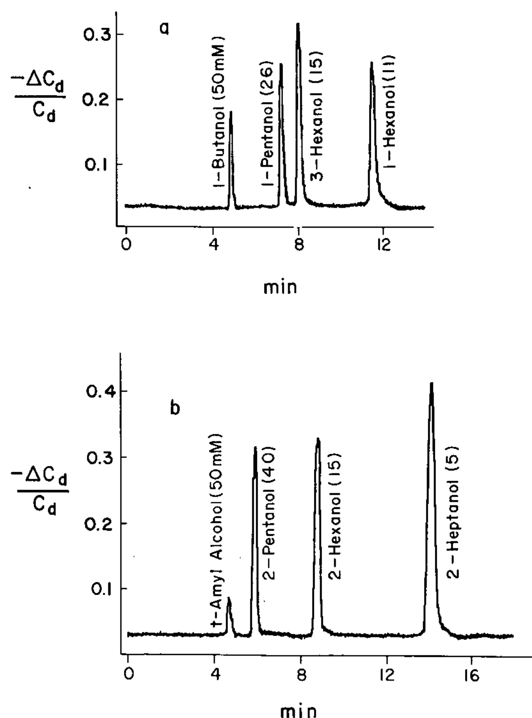

Fig. 3. Two separations of alcohols with DLC detection. Conditions: column, 3 cm + 10 cm Polypore H operated at *ca.* 55°C; eluent, 0.02 N sulfuric acid pumped at 0.7 cm^3/min; initially in large-volume wall-jet cell, 0.1 M sodium perchlorate; make-up, 0.53 M sodium perchlorate; sample loop size, 10 μl; DME, 0.004 in. I.D.; forced drop time, 1 s; f = 145 Hz; E = -0.525 V.

achieved on a (3 cm + 10 cm) Polypore H column operated at 55°C. The eluent consisted of 0.02 *N* sulfuric acid delivered at 0.7 cm^3/min. Tenth molar sodium perchlorate was initially placed in the large-volume wall-jet cell, with a 0.5 *M* sodium perchlorate make-up added at 0.13 cm^3/min. The use of both nitric and sulfuric acids was investigated at concentrations ranging from 0.01 to 0.05 *N*. Considering chromatographic resolution, baseline noise and the form of the ΔC_d–concn. response curves, 0.02 *N* sulfuric acid was adjudged optimal.

It is well recognized that the extent of adsorption of an aliphatic organic compound at the mercury–aqueous interface depends on its alkyl content[2], *i.e.*, on the degree of hydrophobicity. This effect is apparent in the chromatograms in Fig. 3. Thus 1-hexanol is more readily detected than is 1-butanol (Fig. 3a), as is 2-heptanol relative to *tert.*-amyl alcohol [Fig. 3b; the latter is barely detectable even at 50 m*M* (10-μl loop)]. Alcohols containing fewer than four carbons are not readily detected by this technique.

A comprehensive comparison of relative capacitance depression at mercury was made for alcohols using flow injection analysis (FIA), *i.e.*, in the absence of a preceding chromatographic column. The FIA stream consisted of 0.02 *N* sulfuric acid + 0.07 *M* sodium perchlorate, which approximates the solution composition in the cell under chromatographic conditions (*vide supra*). Twenty microliter injections of 10 m*M* concentration were made for each alcohol tested, and the capacitance depression monitored at −0.50 V. The results, given in Table I, are presented as dimensionless $\Delta C_d/C_d$ values *i.e.*, with respect to the baseline C_d value and relative to the

TABLE I

RELATIVE CAPACITANCE DEPRESSION OF ALCOHOLS BY DLC, NORMALIZED TO 1-BUTANOL

For 20-μl injections of 10 m*M* concentration, $E = -0.50$ V.

Compound	*Relative* $\Delta C_d/C_d$
2-Propanol	—
1-Propanol	—
Isobutyl alcohol	0.62
sec.-Butyl alcohol	0.62
tert.-Butyl alcohol	—
1-Butanol	1.00
tert.-Amyl alcohol	1.19
3-Methyl-1-butanol	2.19
2-Methyl-1-butanol	2.43
3-Pentanol	2.52
2-Pentanol	2.67
1-Pentanol	4.14
3-Hexanol	22.4
2-Hexanol	27.0
1-Hexanol	45.3
(±)-2-Heptanol*	115
1-Heptanol*	220

* Lower concentrations run and the values extrapolated upward to 10 m*M* (not rigorously correct because of the non-linear Frumkin isotherm that applies).

corresponding value (0.011) for 1-butanol, which is the shortest-chain alcohol that is readily detected by DLC. Equivalent measurements made in the absence of sulfuric acid revealed slightly lower $\Delta C_d/C_d$ values for C_5 and higher alcohols, probably due to a "salting-out" effect. For more hydrophilic alcohols (C_3 and C_4), slightly lower $\Delta C_d/C_d$ values were observed with added acid.

The listed values clearly show the correlation between the magnitude of $\Delta C_d/C_d$ and hydrophobicity, *i.e.*, the number of alkyl carbons. It is noteworthy that in the chromatographic experiments, adsorbability for the higher members was also observed to increase as the acid concentration was raised. However, acid concentrations could not be made higher than about 0.05 *N*, since the chromatography was then degraded somewhat.

Plots of ΔC_d *versus* bulk adsorbate concentration were generated for 1-pentanol and 3-hexanol under varying conditions of acid and electrolyte concentration. While not every curve was subjected to rigorous analysis, each was suggestive of underlying obeyance to the Frumkin adsorption isotherm. The $\Delta C_d/C_d$–concentration curve for 3-hexanol, shown in Fig. 4, is representative. This curve was analyzed in terms of eqn. 3, with θ being obtained from the ΔC_d values using eqn. 2. This analysis yielded $g = 1.40 \pm 0.02$ for $\theta \leqslant 0.7$, and $B = 29.8 \pm 2.0\ M^{-1}$. These values are consistent with literature values for similar alcohols obtained under conventional conditions, *i.e.*, in stationary electrolytes[41,42]. More significantly, the sigmoidal shape of the ΔC_d–concentration curve, as exemplified in Fig. 4, can readily be accounted for by the form of the Frumkin isotherm (eqn. 3). The non-linearity, allthough not severe below the plateau, can be attributed to the site occupancy, $(1 - \theta)$, and interaction parameter terms, $g\theta$, in eqn. 3. In particular, the approach to a plateau denotes the onset of monolayer formation; above this point no further change in $\Delta C_d/C_d$ will occur with increasing concentration, signaling the upper limit of the dynamic range for the system under consideration.

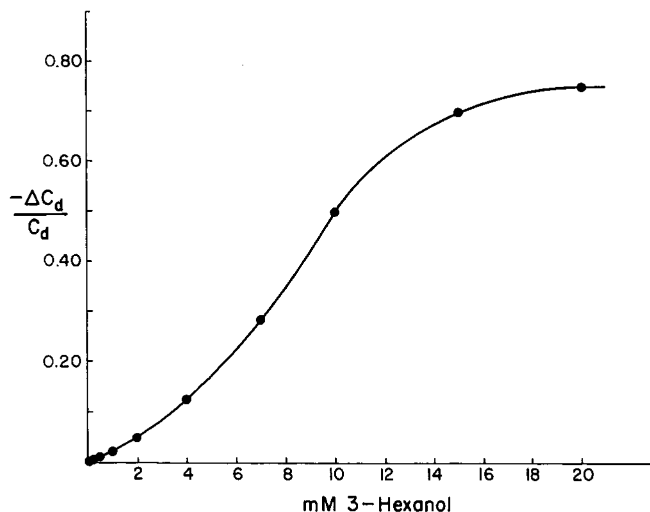

Fig. 4. ΔC_d–concn. response curve obtained at mercury for 3-hexanol using the conditions of Fig. 3 except for a 20-μl loop size and $E = -0.55$ V.

To note the effect, if any, of varying the concentration of mineral acid on the shape of the ΔC_d–concn. response curves, 1,7-heptanediol was chromatographed on Polypore H with 0.002 N sulfuric acid as eluent (10 times lower than the usual concentration, *vide supra*). To achieve a sufficient ionic strength for DLC detection, a make-up stream was teed-in post-column. Several effects were apparent: for the moderately hydrophobic 1,7-heptanediol, the detection limit was lowered for smaller acid concentrations. Also, the ΔC_d–concn. curve was observed to be only slightly non-linear. Hence, depending on the molecular system and on experimental conditions, the shape of the ΔC_d–concn. response curve may vary somewhat.

Diols

Short-chain diols are also separable by mixed interaction chromatography using dilute sulfuric acid in the eluent, as for low-molecular-weight alcohols. An example with DLC detection is shown in Fig. 5. Again, as for the alcohols, the dependence of the ΔC_d response on hydrophobicity is apparent: the detection limits are lower the longer the chain. An example of this dependence is provided by the response for 1,5-pentanediol relative to that for 2,4-pentanediol. The three contiguous methylene groups in the former as opposed to isolated groups in the latter lend greater detectability to 1,5-pentanediol. This enhancing effect of contiguous carbons is also evident from a comparison of the ΔC_d responses for 1,5-pentane- and 2,5-hexanediol. It is of interest to note that the strongly hydrophilic triol glycerol was not detectable under the conditions used here, at least at a concentration of 25 mM.

While the C_d depression is greater for 1,8-octanediol than for 1,7-heptanediol, an even larger effect is observed in going from 1,8-octanediol to 1,9-nonanediol (not shown). However, the attraction to the column packing is also greater for this more hydrophobic compound, resulting in a significantly longer retention time; therefore the peak is broad. To counter this, some organic modifier could be added, but of course this would diminish the electrosorption.

It is apparent that there are conflicting requirements to successful application of

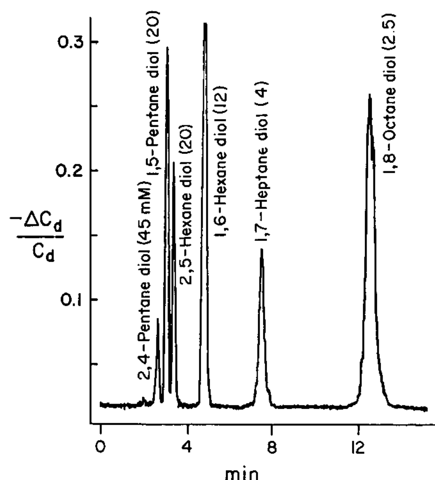

Fig. 5. Separation of six diols with DLC detection; conditions as in Fig. 3.

electrosorptive detection for such hydrophobic organic species. On the one hand we desire a highly surface-active substance. However, such a species will usually also exhibit a high preference for a column packing, thereby necessitating the addition of organic modifier to achieve reasonable k' (capacity factor) values. Hence, in general, it may not be possible in LC–ES to achieve the same low detection limits for surface-active compounds as are possible in batch electrochemical experiments.

Relative capacitance response data such as those in Table I were not generated for the diols. Nevertheless, comparison of Figs. 3 and 5 allows an assessment of the relative sensitivities of the DLC technique to alcohols and diols. Clearly, for both classes of compounds, the response is largely determined by the number and arrangement of $-CH_n$ groups. A welcome feature of analytical consequence for diols relative to alcohols is that the greater water solubility lent the former through the additional alcoholic group expands the number of compounds determinable by DLC.

Carboxylic acids

The relative $\Delta C_d/C_d$ response of aliphatic carboxylic acids were also determined by FIA; the resulting data are shown in Table II. These were obtained at $E = -0.50$ V with 0.02 N sulfuric acid/0.075 M sodium perchlorate as the FIA stream, which approximates closely the composition of the mobile phase used in the present LC-ES experiments. The acidic stream assured that the carboxylates were in the protonated form. As in Table I, the values are listed relative to the normalized response for the n-C_4 member of the group (butyric acid, for which $\Delta C_d/C_d = 0.018$). Again, it is apparent that detectability improves significantly with increased chain length and hydrophobicity. Comparison of the values in Table II with those in Table I reveals that somewhat smaller capacitance depressions are obtained for carboxylic acids than for the corresponding alcohols. This probably stems from the lower water solubility of the latter.

A separation of five aliphatic acids with DLC detection is shown in Fig. 6. Consistent with the data given in Table II and with the results obtained for both

TABLE II

RELATIVE CAPACITANCE DEPRESSION OF CARBOXYLIC ACIDS BY DLC, NORMALIZED TO BUTYRIC ACID

For 20-μl injections of 10 mM concentration, $E = -0.50$ V.

Compound	*Relative $\Delta C_d/C_d$*
Acetic acid	–
Propionic acid	–
Butyric acid	1.00
(±)-2-Methylbutyric acid	1.67
Isovaleric acid	1.58
Valeric acid	3.74
Hexanoic acid	14.4
Heptanoic acid*	77
Octanoic acid*	330

* 2 mM and 1 mM concentrations used for heptanoic and octanoic acids, respectively, and the responses extrapolated upward.

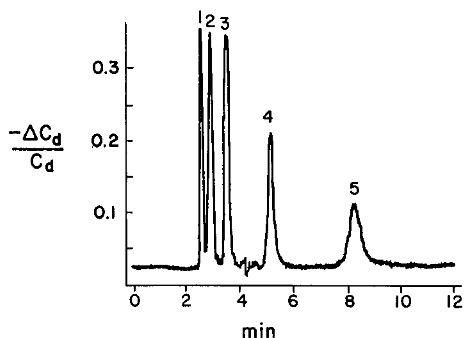

Fig. 6. Separation of five carboxylic acids with DLC detection; conditions as in Fig. 3 except that $t_{col} \approx 60°C$. Peaks: 1 = butyric (50); 2 = isovaleric (40); 3 = valeric (22); 4 = hexanoic (8.5); 5 = heptanoic acid (4 m*M*).

alcohols and diols, the detectability clearly increases as we progress from butyric acid, which is miscible with water, through heptanoic acid, which is only sparingly soluble. Octanoic acid (not shown) is strongly retained on the column, with a full width at half maximum (fwhm) of 1.4 min. Some organic modifier would be necessary to yield even moderate column efficiency for this strongly retained acid.

Calibration (ΔC_d–concn.) curves for the acids were not subjected to rigorous analysis. However, their shape is suggestive of Frumkin behavior with strong, attractive interadsorbate interactions. Thus, for some acids there was a very sharp rise in the ΔC_d–concn. curve, reflecting rapid increases in coverage with increasing analyte concentration. A few also exhibit an apparent "threshold effect", in that no change in capacitance is apparent until a certain bulk concentration is reached, whereupon easily measurable ΔC_d values were obtained. This behavior also stems from the presence of large attractive interadsorbate interactions[27].

Dicarboxylic acids

Relative responses for several dicarboxylic acids were determined by measuring the areas under peaks obtained using a 10-cm Polypore H column with 0.02 *N* sulfuric acid as eluent. The results are compiled in Table III. In this case the capacitance

TABLE III

RELATIVE CAPACITANCE DEPRESSION OF DICARBOXYLIC ACIDS BY DLC, NORMALIZED TO GLUTARIC ACID

For 20-μl injections of 10 m*M* concentration, $E = -0.525$ V.

Compound	*Relative* $\Delta C_d/C_d$
Succinic (C_4)*	0.26
Glutaric (C_5)	1
Adipic (C_6)	2.91
Pimelic (C_7)	5.44
Suberic (C_8)	11.6
Azelaic (C_9)*	37
Sebacic (C_{10})*	97

* Higher (succinic) and lower (azelaic, sebacic) concentrations, respectively, run and the values extrapolated to 10 m*M*.

changes are listed relative to glutaric acid, which contains five carbons. This was done because succinic acid is only marginally determinable by DLC due to its high water solubility. Improved detectability with increasing molecular hydrophobicity is as previously noted for alcohols, diols and monocarboxylic acids.

A separation of six dicarboxylic acids with DLC detection is shown in Fig. 7. In order to achieve baseline resolution of early-eluting compounds, a 25-cm Polypore H column was used in place of the earlier-used 10-cm cartridge. Note that there is some "noise" in the azelaic acid peak. Similar noise was also noted for other highly hydrophobic/surface-active compounds.

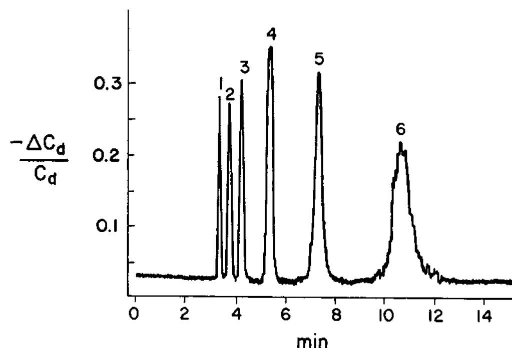

Fig. 7. Separation of six dicarboxylic acids with DLC detection; conditions as in Fig. 3 except that t_{col} = 65°C and the Polypore H column was 25 cm rather than 10 cm long. Peaks: 1 = succinic (40); 2 = glutaric (12.5); 3 = adipic (5); 4 = pimelic (2.5); 5 = suberic (1.2); 6 = azelaic acid (1 m*M*).

Amines

Aliphatic amines may be separated by ion-moderated partition chromatography using dilute hydroxide as the eluent[43]. Although a preferred column is the Bio-Rad HPX-72-0, we used the less expensive, recommended guard alone, the Bio-Rad Anion-OH. Values of $\Delta C_d/C_d$ were measured for the aliphatic amines, again by FIA, at −0.50 V in a stream consisting of 0.02 *M* sodium hydroxide + 0.075 *M* sodium perchlorate. As the hydroxyl ion is very weakly adsorbing[44], it interferes only minimally with solute specific adsorption. The $\Delta C_d/C_d$ responses obtained for the amines normalized to that for butylamine (0.020) are listed in Table IV. Comparison with Tables I and II reveals that the capacitance depression of amines increases to a slightly greater extent with chain length than for alcohols and decidedly more than for acids. Also, the absolute $\Delta C_d/C_d$ values are greater for amines than for either alcohols or acids. This is made apparent in Table V, which compares absolute $\Delta C_d/C_d$ values for these three classes of compounds. Table V consists of data selected from those used to generate the relative values given in Tables I, II and IV.

A separation of four amines on the Anion-OH guard (3 cm × 4.6 mm) with DLC detection is shown in Fig. 8. Maximal response for the amines was found in the region −0.425 to −0.45 V. The dynamic range for the higher amines using the guard column proved quite limited, symptomatic of strong, attractive interadsorbate interactions. A "threshold effect", previously mentioned with respect to acids, was also observed for

TABLE IV

RELATIVE CAPACITANCE DEPRESSION OF AMINES BY DLC, NORMALIZED TO BUTYLAMINE

For 20-μl injections of 10 m*M* concentration, $E = -0.50$ V.

Compound	*Relative* $\Delta C_d/C_d$
Isopropylamine	–
Propylamine	–
sec.-Butylamine	0.70
Butylamine	1.00
tert.-Amylamine	1.20
Isoamylamine	4.35
Amylamine	9.0
Hexylamine*	48
Heptylamine*	230
Octylamine*	720

* Lower concentrations injected, and the responses extrapolated to 10 m*M*.

some higher amines. The clipped peaks for hexyl- and heptylamine seen in Fig. 8 are due to saturation capacitance depression, associated with the onset of monolayer formation.

Baseline noise tended to be higher with the alkaline eluent (high-purity

TABLE V

CAPACITANCE DEPRESSION OF ALCOHOLS, ACIDS AND AMINES BY DLC*

Compound	$\Delta C_d/C_d$**
1-Butanol	0.011
Butylamine	0.020
Butyric acid	0.018
3-Methyl-1-butanol	0.026
Isoamylamine	0.087
Isovaleric acid	0.028
tert.-Amyl alcohol	0.014
tert.-Amylamine	0.024
1-Pentanol	0.048
Amylamine	0.180
Valeric acid	0.067
1-Hexanol	0.527
Hexylamine	0.97
Hexanoic acid	0.260
1-Heptanol	2.54
Heptylamine	4.58
Heptanoic acid	1.38
Octylamine	14.4
Octanoic acid	6.01

* For 20-μl injections of 10 m*M* concentrations, except as noted below, utilizing the conditions of Tables I, II and IV. For the C_6's, 5 m*M* concentrations were used, for the C_7's, 2 m*M* and for the C_8's, 1 m*M*; the values obtained were then extrapolated to 10 m*M*.

** The $\Delta C_d/C_d$ values are the actual relative change in capacitance measured.

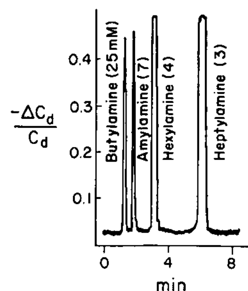

Fig. 8. A separation of four aliphatic amines with DLC detection at $E = -0.45$ V. Conditions: column, Bio-Rad Anion-OH guard, 3 cm × 4.6 mm; eluent, 0.015 M potassium hydroxide (high-purity, Alfa).

potassium hydroxide used), when monitoring near the pzc, compared to an acidic eluent of equal concentration. As a final note, the amines are not only detectable as neutrals in basic solution, but also as the protonated, cationic partner of an ion-pair. The DLC detection of this and other charged organic species will be discussed elsewhere.

Alkanolamines

Alkanolamines were also separated on the Anion-OH guard and detected as neutrals by DLC, although this short column did not yield much resolution under the conditions investigated. A two-component separation with DLC detection is shown in Fig. 9. As for the amines in alkaline solution, baseline noise tended to be high.

Comparison of DLC to conventional means of detection

There is not obvious conventional method of choice for determining several of the classes of compounds examined here. Hence, the application of DLC detection to these compounds is of more than passing interest.

Refractive index (RI) has traditionally been used to detect alcohols and diols[45,46], although other methods, including indirect photometric detection[47] and

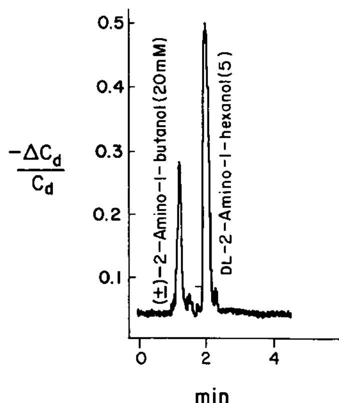

Fig. 9. Separation of two alkanolamines with DLC detection; conditions as in Fig. 8.

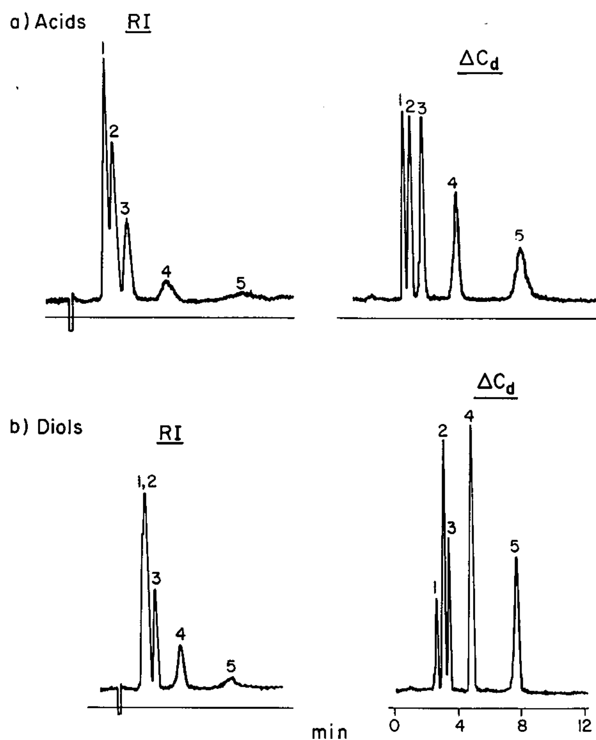

Fig. 10. Comparison of DLC and RI detection for (a) acids and (b) diols; conditions are as in Figs. 6 and 5, respectively. Peaks: (a) 1 = butyric (50); 2 = isovaleric (40); 3 = valeric (22.5); 4 = hexanoic (8.5); 5 = heptanoic acid (4); (b) 1 = 2,4-pentanediol (45); 2 = 1,5-pentanediol (20); 3 = 2,5-hexanediol (20); 4 = 1,6-hexanediol (12); 5 = 1,2-heptanediol (4 m*M*).

post-column derivatization followed by UV–VIS measurement[48], have been employed. RI may also be used to detect carboxylic acids[49]. A more selective method for aliphatic acids utilizes conductivity[50,51], but this is not a sensitive measure for weak acids. Yet another, UV detection, is a more restricted approach, largely confined to conjugated acids[52].

We compared the present DLC approach with RI detection for the determination of several acids and diols, using a Waters Differential RI Detector. The comparisons, shown in Fig. 10, were made using a Polypore H column with 0.02 *N* sulfuric acid as the eluent. It can be seen that these two techniques are comparable for moderately surface-active compounds, RI is superior for weakly surface-active materials, and DLC superior for the more strongly surface-active substances. The more selective nature of DLC detection is apparent along with greater band resolution, although the latter factor presumably depends upon the particular RI detector employed.

Potential-pulse coulometric detection

An alternate means of electrosorptive detection, closely related to DLC, is provided by potential-pulse coulometry (PPC)[33]. Rather then evaluating the dif-

ferential capacitance at a given potential by a.c. impedance, the PPC method as configured for the present systems involves measuring the change in electrode charge, Δq, produced by a small (*ca.* 0.1 V) negative potential step in the vicinity of the pzc. Since the capacitance is usually approximately independent of potential in this region, Δq is essentially proportional to C_d ($\Delta q \approx C_d \cdot \Delta E$, where ΔE is the amplitude of the potential step). The measurement in the PPC method is typically complete within 0.2–0.3 ms.

Analogously to conventional DLC, detection by PPC involves measurement of $\Delta(\Delta q)$ [or $\Delta(\Delta q)/\Delta q$] as the chromatographic elution proceeds. The signal-to-noise evident in the resultant chromatograms was typically about one-half that obtained by DLC detection for all compound types investigated here. As expected, calibration curves obtained by small-potential-step coulometry exhibit the same shape as those obtained by DLC.

Tensammetric (adsorption/desorption) peak detection

As noted above, an inevitable limitation on the quantitative utility of conventional DLC detection methods is that ΔC_d will respond to varying analyte concentration only within the region where the adsorbate coverage is below a monolayer. This can provide a significant limitation for the determination of the more strongly surface-active compounds. However, even under these conditions the position and amplitude of the "tensammetric" C_d–E peaks, signaling where adsorption–desorption occurs on either side of the pzc, are still dependent on the analyte concentration. This suggests the suitability of detection methods that sense these properties, rather than C_d close to the pzc.

We have employed a scheme utilizing either a negative-going pulse or rapid potential sweep applied near the end of drop lifetime, arranged so to span one of the adsorption–desorption regions. The resulting transient capacitive current, i_c, is sampled at the computer's maximum sampling rate ($\approx 2.5 \cdot 10^4$ s^{-1}) and stored. The stored values are then scanned and the maximum value, $(i_c)_{max}$, output to a recorder. This $(i_c)_{max}$ value will reflect the amplitude of the C_d–E adsorption–desorption peak, which is dependent upon the analyte concentration. At lower concentrations $(i_c)_{max}$ is usually observed to vary roughly linearly with concentration, while at higher values a change in proportion to the logarithm of concentration is ordinarily observed[53]. Although this scheme has less general value than conventional DLC detection, it is in a sense complementary in view of its utility at higher analyte concentrations and/or adsorbabilities.

A cursory examination was also made of the utility of applying a potential step through the tensammetric peak and integrating the current. However, this is complicated by the difference in sign of the capacitive current in the depression and adsorption/desorption regions; the result can be a transient partial cancelation of charge. This approach should be more useful when restricted to capacitive current of only one sign, *i.e.*, to only the current associated with the adsorption/desorption peak.

CONCLUSIONS

We have demonstrated the applicability of electrosorptive detection in liquid chromatography utilizing aqueous eluents to the determination of several classes of

low-molecular-weight, neutral organic compounds, specifically alcohols, diols, mono- and dicarboxylic acids, amines and alkanolamines. The primary and most useful means of detection is differential double-layer capacitance depression, monitored at a potential in the vicinity of the pcz, although small potential-step coulometry provides an alternate, closely related approach. The magnitude of the response, either ΔC_d or $\Delta(\Delta q)$ for these two techniques, shows a clear dependence on carbon number and structure —*i.e.*, on the extent of hydrophobicity. The ΔC_d–concn. behavior evident in the response curves may be accounted for in terms of underlying Frumkin behavior. Detection based on tensammetric peak height may also be employed, but was found to be only marginally useful for the weakly-to-moderately-adsorbing molecular systems investigated in this work.

As stated, compounds exmined in this work may be classified as weakly-to-moderately specifically adsorbing. Hence, these compounds do not serve to fully realize the potential of LC–ES. To this end, non-iogenic surfactants, water-soluble polymers and, possibly, larger biomolecules may be logical candidates. With regard to surfactants, complications may arise from the need to incorporate high concentrations of organic modifier in the mobile phase. Interestingly, this appears not to be the case for ionic surfactants, since these compounds may be detected at potentials far removed from the pzc[54]. The sorptive properties of many water-soluble polymers have been characterized[55]; for these large molecules, changes in double-layer capacitance may be substantial. As to the last of these molecule types, proteins and other biomolecules often contain significant hydrophobic portions, which are highly surface active[42]. Hydrophobic interaction chromatography, which capitalizes on this property, and which has experienced explosive growth in recent years[56], may well be suitable for coupling to electrosorptive detection, since DLC exhibits considerable tolerance to concentration gradients[31].

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ELECTROSORPTIVE DETECTION BASED ON DOUBLE-LAYER CAPACITANCE FOR SELECTED ION MONITORING IN ION CHROMATOGRAPHY

II. SILVER

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SUMMARY

A scheme is presented for the detection of selected anions in ion chromatography based on changes in differential double-layer capacitance, ΔC_d, induced by specific anion adsorption on a solid metal electrode. The detector in the liquid chromatography–double-layer capacitance (LC–DLC) arrangement described here is a silver electrode configured in a large-volume wall-jet geometry. The aqueous-phase anions most readily determined by this approach are chloride, bromide, iodide, azide and thiocyanate; additional ions, including iodate, periodate, bromate, thiosulfate and sulfide, are also determinable. Detection limits are sub ppm in most cases. The measurement scheme developed includes provision for faradaic current measurement, thereby providing for simultaneous capacitive and amperometric detection. The benefits of electrode rotation in capacitance detection are also discussed. It is shown that DLC detection is suitable for use with either low-capacity ion chromatography columns or conventional ion exchange columns.

INTRODUCTION

In a previous paper we described the application of differential double-layer capacitance (DLC) detection at mercury – an example of electrosorptive (ES) detection – to the determination of selected anions in ion chromatography (IC)[1]. In this technique, based on a.c. impedance measurements, the analyte is detected by means of changes in the differential capacitance, C_d, for an electrode brought about by specific adsorption. Several previous reports have dealt with the coupling of liquid chromatography and electrosorptive detection (LC–ES) for the determination of organic substances on mercury, but our report[1] appears to be the first that treats the determination of ions in an IC mode. Capacitance detection using a solid metal electrode may offer an advantage relative to mercury for the determination of low concentrations of weakly adsorbing ions such as chloride and azide due to the strong adsorption often observed on silver. Another motivation for extending ES detection to a solid electrode is that some analysts find the use of mercury inimical.

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Although platinum has been most employed as a solid electrode substrate in fundamental studies[2], meaningful capacitance measurements cannot be made on this surface for a variety of reasons. Both gold and silver are more suitable substrates and rival each other in terms of the range of anions known to adsorb specifically and in the strength of adsorption[3–6]. A dissimilarity between silver and gold, though, is that the potential regions over which adsorption occurs are markedly different, associated with their disparate potentials of zero charge (pzc)[3]. A potential drawback of gold is that irreversible adsorption effects are often observed[5], along with capacitance frequency dispersion[6]. Silver does not suffer from these difficulties; overall, it appears to be the "best behaved" of commonly available solid electrode materials. Silver also possesses a wide polarizable potential window, and a proven electrochemical pretreatment procedure is available[7].

Anions whose adsorption has been studied previously at either single-crystal or polycrystalline silver by means of capacitance measurements include OH^-, ClO_4^-, SO_4^{2-}, Cl^-, Br^-, I^-, N_3^- and SCN^-[3,4,8–12]. These studies have shown the last five of these to be strongly adsorbed. Given the desirable characteristics of silver coupled with our previous experience with capacitance measurements at this metal[8,9], we undertook a detailed study of the application of silver to DLC detection of anions. (Adsorption of common *cations* is far less pronounced[13], and hence DLC detection is less applicable to this class of ions). The results of this work are described here. Besides the examination of inorganic anions using DLC at mercury, as already noted[1], we also have reported elsewhere the application of this technique to the detection of simple charged organic species[14].

BACKGROUND

Since a detailed discussion of the principles underlying DLC detection was given in ref. 1, only the salient points will be noted here. Fundamentally, the technique is based on the variations in differential double-layer capacitance, ΔC_d, caused at an electrode poised at a suitable potential by specific adsorption of analyte as it flows past the electrode detector. In contrast to conventional faradaic electrochemical detection, then, this scheme relies on sensing changes in *non-faradaic* currents, most simply obtained from a.c. impedance measurements. Such a detector will provide a reversible response provided that the adsorption–desorption kinetics are suitably rapid.

Of obvious importance is the relationship between the measured ΔC_d values and the analyte concentration, c_x. If the adsorbate surface concentration, Γ_x (mol cm^{-2}), is sufficiently small at a given electrode potential so that Γ_x is proportional to c_x, *i.e.*, Henry's Law applies. As shown in ref. 1, we anticipate that ΔC_d will be approximately proportional to c_x. At higher adsorbate concentrations, the effects of increasing anion–anion repulsion are expected to decrease progressively the ΔC_d–c_x slope so that these plots are generally expected to be non-linear. This behavior was confirmed for a number of anionic adsorbates at mercury[1]. Fig. 1. gives an example of such a ΔC_d–c_x plot obtained for bromide adsorbed on polycrystalline silver at -0.90 V, taken from ref. 8. The shape of this plot is typically found for other electrode potentials and adsorbates. While the plot does clearly exhibit non-linearity in the manner anticipated above, it also indicates that approximate concordance to a

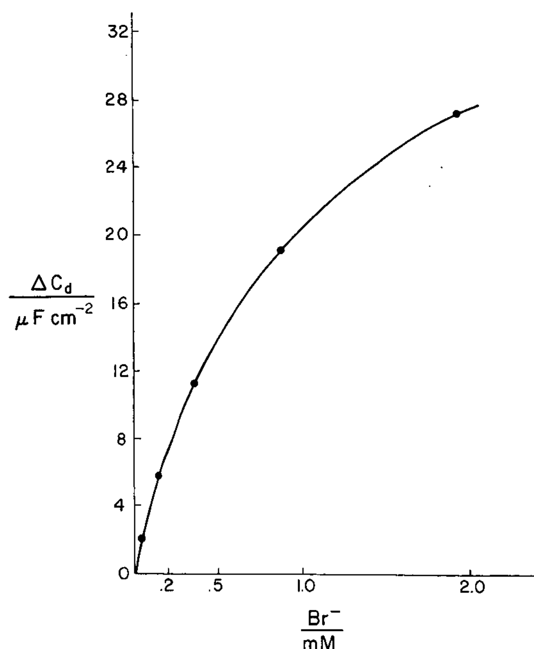

Fig. 1. ΔC_d–c_x curve for Br^- for polycrystalline silver at −0.90 V *vs.* saturated calomel electrode (SCE); from data given in Fig. 8 of ref. 8. Electrolyte is $(500 - c_x)$ m*M* sodium fluoride + c_x m*M* sodium bromide.

linear ΔC_d–c_x relationship can be obtained for relatively small ΔC_d values ($\leq$ 10 μF cm^{-2}, *cf.*, ref. 1).

EXPERIMENTAL

Differential double-layer capacitance (DLC) at the silver–aqueous interface was measured using the methodology of phase-sensitive a.c. voltammetry. The instrumental arrangement used is shown in Fig. 2. A 10-mV peak-to-peak sinusoid in the approximate frequency range 50–150 Hz modulates the potential set by a Prince-

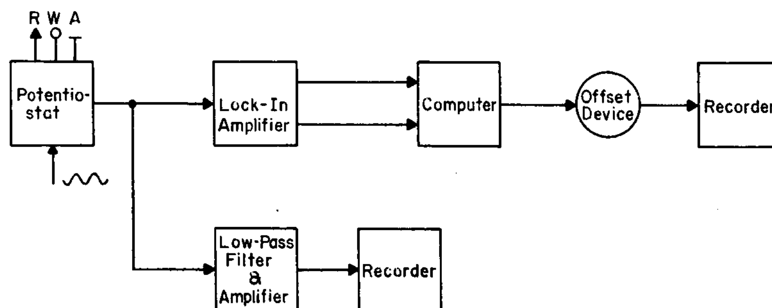

Fig. 2. Block diagram of the instrumental arrangement for the fixed-potential monitoring of differential double-layer capacitance (DLC) at a silver electrode with simultaneous faradaic electrochemical detection. R = Reference electrode; W = working electrode; A = auxiliary electrode.

ton Applied Research Corp. (PAR) 173 or 273 potentiostat. The alternating cell current is separated into its in-phase and out-of-phase components by a PAR 5204 lock-in analyzer. These in turn are directed to a PDP-11/23 computer, which calculates the capacitance according to $C_d = (\omega E)^{-1} [(I^2_{out} + I^2_{in}) / I_{out}]$, where ω is the frequency in rad s^{-1}, E is the magnitude of the sinusoid and I_{in} and I_{out} are the magnitudes of the in-phase and out-phase components, respectively, of the cell current. Changes in the computer output, ΔC_d, are registered by applying the offset output to a strip chart recorder. The low-pass filter/amplifier shown as a parallel branch in Fig. 2 provides for a simultaneous record of the presence of faradaic current. In the event of a sizable faradaic component, the capacitance calculation may be significantly in error. Additional details regarding instrumentation are available elsewhere[1,15].

The electrochemical cell used in most experiments is shown in Fig. 3. It is of the large-volume wall-jet type (LVWJ)[1,16], its virtues in conjunction with a mercury

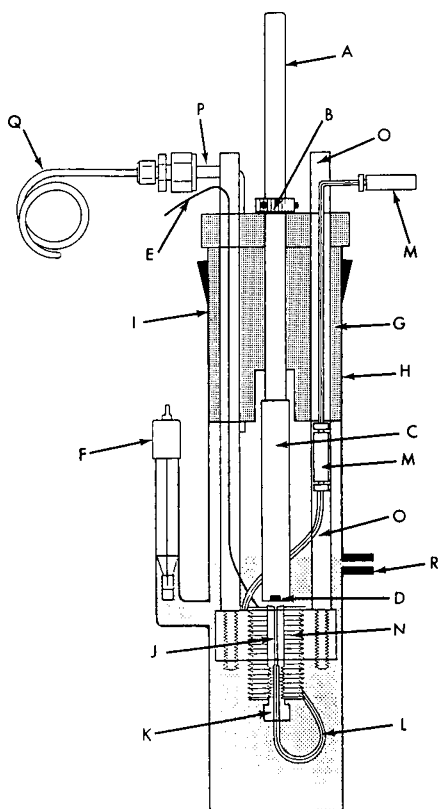

Fig. 3. Large-volume cell with RDE in wall-jet configuration. A = Electrode shank; B = positioning collar; C = PTFE shroud; D = silver working electrode; E = platinum auxiliary electrode; F = silver–silver chloride (3 *M* sodium chloride) reference electrode; G = PTFE holder; H = cell tube, either 51 or 54 mm; I = 45/50 ground glass joint; J = eluent-delivery capillary (nozzle); K = Upchurch Kel-F male nut; L = 1/16 in. × 0.3 mm PTFE tubing; M = Swagelok union; N = Kel-F capillary bushing; O = Delrin holder rods for nozzle assembly; P = gas exit tube; Q = capillary pigtail; R = overflow port.

electrode for DLC application are described in ref. 1. An important attribute of the LVWJ design for our application is that virtually any desired supporting electrolyte concentration can be maintained by introducing a high-concentration make-up stream via a port (not shown in Fig. 3). One additional benefit observed for a solid electrode is that when bathed in a non-confining volume, the electrode surface suffers less fouling than when confined to a tiny volume, as in a thin-layer channel. This is an important consideration for a technique whose efficacy depends on the state of the surface. The electrode shown in Fig. 3 is a rotating disk electrode (RDE, Pine Instrument Co.). With this design the electrode can be readily coupled to a rotator, if desired. Our early work on silver, conducted prior to arriving at a final design, made use of a Bioanalytical Systems (BAS) silver detector block (TL-11A cell half), mounted, not in its customary thin-layer configuration, but rather adapted to a wall-jet geometry in the large-volume cell.

Any desired separation between the delivery nozzle and the electrode in Fig. 3 is readily achieved by adjusting the position of the retaining collar (B) on the electrode shank and/or by sliding the eluent capillary delivery assembly up or down via the holder rods (O). When using a rotator (Pine Instrument Co.), additional control is afforded by the rotator height adjustment knob. Use of the rotator required an electrode shank length of $\approx$ 13 cm, this in addition to a PTFE shroud length of 6.35 cm. The electrode diameter was 2–4 mm, shroud diameters 9–12 mm. A coiled platinum wire served as the auxiliary electrode, and the reference electrode was a BAS silver–silver chloride (3 *M* sodium chloride) Model RE-1 with Vycor membrane.

From an operational standpoint the LVWJ design used here is particularly well suited for rotating an electrode, obviating the problems (engineering, reliability) associated with incorporating an RDE into a miniature (thin-layer) wall-jet design[17–19]. Rotation of the electrode has a salutary effect on DLC detection in that it sets up a flow steady state. The Schlieren lines, which are otherwise visible in the vicinity of the electrode, vanish as reactants/adsorbates are swept out into solution. The net result is a quieter, straighter C_d–time baseline. A lowered susceptibility to flow variations is a further attribute[17].

Exclusion of oxygen is critical to the performance of silver in DLC detection. A signal arising from the reduction of oxygen can manifest itself in two ways: (1) as a small a.c. component[20]; and (2) as a larger d.c. signal due to the reduction of oxygen, which can lead to excessive baseline noise. Hence, the entire system – mobile phase reservoir + electrochemical cell – was blanketed under nitrogen. The pigtail shown in Fig. 3. was included to prevent back diffusion of air via the gas exit port.

The silver electrode was mechanically polished, sequentially, with 1.0-, 0.3- and 0.05-μm alumina, either on a wheel or by hand using a specially fabricated PTFE holder; ultrasonication was used to remove residual alumina grit between steps. Mechanical polishing was always followed by an electrochemical polishing sequence[7]. Electrochemical polishing is essential, as without it, both the background capacitance and baseline noise are high and sensitivity (ΔC_d response) prohibitively poor. Subsequent transfer to the working cell was as rapid as possible and was carried out with the working electrode covered with a water meniscus to avoid exposure to air.

All chromatography was conducted with aqueous eluents, prepared in "Milli-Q" water (Millipore). The mobile phase usually consisted of solutions of sodium perchlorate as displacer at concentrations of $2 \cdot 10^{-2}$ *M* and below. Where a higher

concentration of electrolyte was desired, the more weakly displacing NO_3^- was used. Acidic or basic eluents were prepared by adding (usually) perchloric acid or sodium hydroxide, respectively. Similarly to perchlorate[21], both H^+ and OH^- exhibit negligible er weak specific adsorption at silver[22]; this property is desirable for DLC[1].

Prior to introducing column effluent to the cell, the cell was filled with 0.1 *M* sodium perchlorate at an appropriate pH. Throughout an experiment a make-up stream of approximately 0.7 *M* sodium perchlorate was added to the cell at a flow-rate of 0.13 cm^3/min in order to maintain an overall cell electrolyte concentration of approximately 0.1 *M*. This is done to offset the dilution that would be caused by continuous delivery of the low-ionic-strength carrier solution; a low overall (sensed) electrolyte concentration leads to increased baseline noise and to consequent higher detection limits. The mobile phase was deoxygenated by nitrogen purging prior to use, and kept under a nitrogen blanket throughout.

A Waters Model 6000A pump was used in conjunction with either a Vydac IC column (silica backbone, No. 302-IC46, The Separations Group), or a Hamilton PRP-X100 IC column (resin based) for most of this work; both columns measured 25 cm long. The guard consisted of Vydac SC pellicular packing with the former and Hamilton PRP-1 with the latter. Receiving less use was a conventional (higher exchange capacity) anion exchange column, a Nucleosil 5SB, also 25 cm long. The injector was a Rheodyne Model 710, usually fitted with a 50-μl loop.

Capacitance–potential (C_d–E) curves were generated in a stationary electrolyte using conventional electrochemical cells for candidate adsorbate ions. These curves allow an assessment of the potential region over which specific adsorption occurs, in order to select suitable conditions at which to conduct the LC–DLC measurements. The curves were obtained using the set-up of Fig. 2 save for the substitution of an X-Y recorder (HP 7045) for the strip chart recorder, and the addition of a ramp driver (PAR 175) to sweep the potentiostat (usually at 5 mV s^{-1}). By keeping the low-pass filter/d.c. amplifier of Fig. 2 in the circuit, the true extent of the polarized window for any analyte could be ascertained simultaneously.

RESULTS AND DISCUSSION

We present here our findings on the utility of electrosorptive detection at silver coupled to ion chromatography. Those anions possessing sufficiently strong adsorptivities to be detectable at analytically useful levels by differential double-layer capacitance are detailed, and their ΔC_d–E and ΔC_d–c_x behavior presented. A discussion is given of the benefits afforded by simultaneous DLC and faradaic electrochemical (FED, amperometric) detection, and of RDE operation. We also describe an extension of DLC detection from low-capacity IC columns to conventional ion exchange columns. All results given pertain to non-stationary, *i.e.*, flowing electrolytes, unless otherwise noted.

Identification of anions suitable for IC–DLC detection

As noted above, many anions specifically adsorb at silver, as at mercury[1]. However, we may anticipate that relatively few possess specific adsorbabilities sufficiently large to render them analytically viable in an IC–ES format. We tested virtually all anions of common interest in IC. Of these, five are readily determinable at

concentrations competitive with conductometric detection: Cl^-, N_3^-, Br^-, I^- and SCN^-. Cyanide, which is known to exhibit strong adsorption at silver, presumably did not elute as a sharp band under the chromatographic conditions employed. Several more anions yield "apparent capacitive" responses, which may arise at least partly from anion electroreduction or oxidation. (Note that the capacitance analysis embodied in eqn. 1 is vitiated in the presence of such faradaic processes.) Included among these ions are IO_4^-, IO_3^-, BrO_3^-, S^{2-} and $S_2O_3^{2-}$. These ions are further characterized by strong adsorption with, usually, accompanying chromatographic tailing at readily accessible negative potentials. Even if an electrode reaction does occur simultaneously with adsorption, though, there will be virtually no response by a.c. voltammetry if the electrode reaction is irreversible[23], as is the case for these ions, *e.g.*, refs. 24, and 25. Hence, they are also determinable by DLC detection. A further, utilitarian requirement that emerged at silver was that a usable analytical signal manifest itself at a potential no more positive than about -0.5 V: at more positive potentials chromatograms are often subject to baseline disturbances from widespread adsorption. The consequence of this is that surface deactivation can occur quite rapidly.

Plots of relative ΔC_d *vs.* E are shown for the five adsorptioactive ions listed above in Fig. 4. These curves were obtained by injecting a given concentration of adsorbate into the ion chromatograph over a range of potentials. They can be used to select appropriate potentials for analytical determinations by DLC. An example of a separation obtained for these five ions using specific adsorption detection is given in

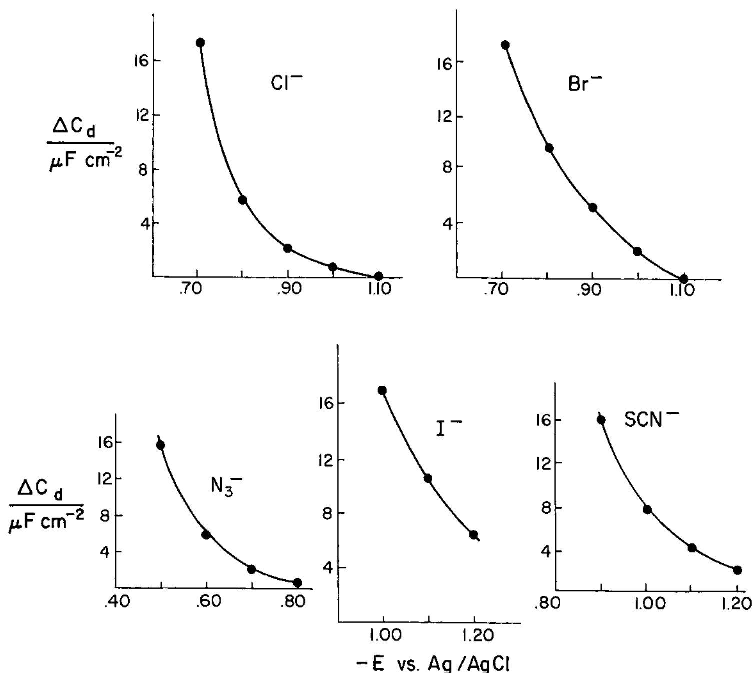

Fig. 4. Relative change in capacitance determined by LC–DLC for five adsorptioactive ions as a function of potential. Concentrations (ppm): Cl^-, 40; Br^-, 10; N_3^-, 50; I^-, 5; SCN^-, 10.

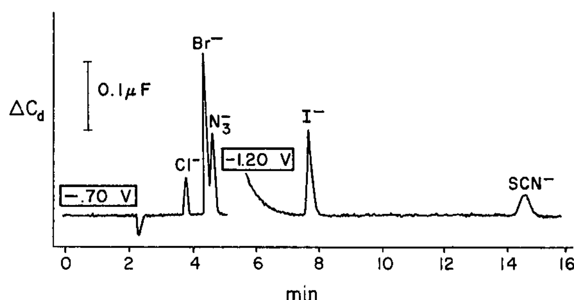

Fig. 5. A separation of five ions with specific adsorption (DLC) detection at silver. Conditions: column, Hamilton PRP–X100; eluent, $1.8 \cdot 10^{-2}$ F sodium perchlorate, pH 10.1 with sodium hydroxide; flow-rate, 0.9 cm^3/min; initially in cell, 0.1 F sodium perchlorate, pH 10.1; make-up, 0.74 F sodium perchlorate, pH 10.1, pumped at 0.13 cm^3/min; sample composition: 20 ppm Cl^-, 5 ppm Br^-, 25 ppm N_3^-, 10 ppm CN^- (not detected), 5 ppm I^- and 25 ppm SCN^-.

Fig. 5. A difference evident here compared to corresponding chromatograms obtained for mercury[1] is the potential region over which specific adsorption occurs; this results from the more negative pzc of silver (*ca.* −0.95 V, ref. 8) relative to mercury (−0.43 V, ref. 26). (These values refer to non-specifically adsorbing electrolytes). Note that two applied potentials (−0.70 and −1.20 V) were used to produce the chromatogram in Fig. 5. Other potentials could be chosen as well. Hence, considerable control over selectivity is available.

Similar to the situation at mercury[1], several of these "cleanly" adsorptioactive anions – Br^-, I^-, SCN^- (plus CN^-) – are also detectable amperometrically at silver, albeit at considerably more positive potentials[27,28] than those required for specific adsorption detection. Detection limits by amperometry are generally lower, by an order of magnitude[29], than those achieved by DLC. However, detection at the more positive potentials required for electrooxidation can lead to irreversible changes in the metal surface. A case in point is provided by the detection of halides, in which the product of the electrochemical reaction is the poorly soluble AgX, where X is a halide. An advantageous feature of the DLC method is that detection occurs at more negative potentials, within the ideally polarized region. A distinct advantage of silver relative to mercury for specific adsorption detection is that all specifically adsorbing anions can be determined at potentials well negative of where metal dissolution occurs.

Detection limits obtained at silver by DCL detection are slightly lower than those obtained at mercury, ≈ 0.2 *vs.* 0.5 ppm (50-μl loop). In general, for any concentration of analyte, the signal-to-noise is somewhat superior with silver. Obviously, where lower detection limits are required, either a preconcentration step could be used, preferably on-line and automated[30], or larger volumes could be injected[31,32]. The detection limits realized at silver are comparable to those generally cited – 0.2–1.0 ppm – for both conductometry and indirect photometry[31], the two most popular forms of detection in IC.

Capacitance–concentration (ΔC_d–c_x) calibration curves were generated by IC–DLC for Cl^-, N_3^-, Br^- and I^- at silver. The first three were obtained at a stationary electrode (BAS half-cell operated as a wall-jet), the last at an RDE rotated at 400

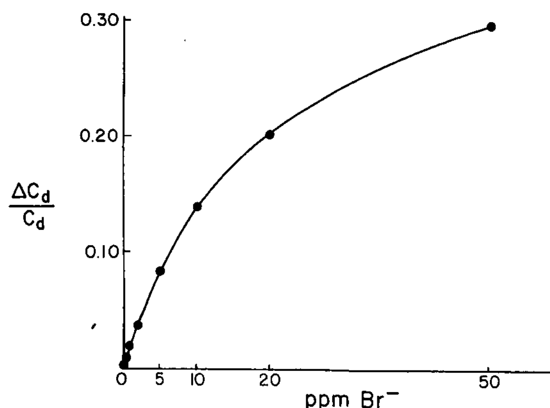

Fig. 6. DLC calibration curve for Br^- at -0.80 V and at a frequency f = 145 Hz, obtained using essentially the chromatographic/electrolyte conditions of Fig. 5 save for the substitution of the Vydac column for the Hamilton; 1 ppm Br^- corresponds to $1.25 \cdot 10^{-2}$ mM.

rpm. The curve in Fig. 6, which is a plot of $\Delta C_d/C_d$ *vs.* concentration of Br^- from the limit of detection, 0.2 ppm (= $2.5 \cdot 10^{-3}$ mM), to 50 ppm (= 0.62 mM) (50-μl loop), with $E = -0.80$ V, is representative of those obtained. Note that the nature of the response agrees with that expected, *cf.*, Figs. 1 and 6. While the curvature over the tested concentration range is somewhat greater for silver than for mercury[1], the relative change in capacitance is also greater, a reflection of stronger adsorption on silver. Calibration plots for Cl^- and N_3^- at -0.70 V (not shown) show less curvature than that for Br^- at -0.80 V, which is consistent with their lower adsorptivities, whereas at -0.60 V the plot curvature for these two ions is comparable to that for bromide at -0.80 V (Fig. 6). This again is in agreement with the anticipated greater degree of ΔC_d–c_x curvature at more positive potentials since more extensive adsorption, and hence larger ΔC_d values, occur under these conditions. Generally speaking, the closest approach to linearity is observed at more negative potentials. This is exemplified in a plot of $\Delta C_d/C_d$ *vs.* c_x for I^- at -1.20 V shown in Fig. 7; less curvature

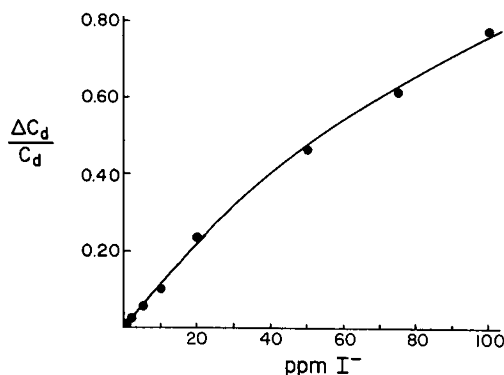

Fig. 7. DLC calibration curve for I^- at -1.20 V and at a frequency f = 45 Hz, obtained using the conditions of Fig. 5; 1 ppm I^- corresponds to $7.9 \cdot 10^{-3}$ mM.

is seen in Fig. 7 compared to Fig. 6 even though the former spans a larger range of $\Delta C_d/C_d$ values. It is noteworthy that chromatographic peak tailing was minimal or non-existent for each of these ions at the potentials used in generating the calibration curves. The chloride, azide and bromide experiments employed the Vydac column, using either sodium nitrate or sodium perchlorate as displacer, whereas iodide was examined with the Hamilton PRP-X100 using sodium perchlorate.

Detector optimization

The LVWJ concept has been described in detail[1,15,16]. A revolutionary aspect of the LVWJ design is that the separation between the effluent delivery nozzle and the working electrode may be varied over a considerable distance, depending on conditions, with no increase in chromatographic band dispersion. Hence, there is no one optimum electrode–nozzle separation. At small separations for the low electrolyte concentrations commonly employed in IC, the in-phase current grows greatly at the expense of the quadrature (out-of-phase) component, *i.e.*, the effective solution resistance increases. This happens because, as the separation becomes smaller, the electrode senses more completely the low-ionic-strength effluent stream and less of the higher-concentration batching solution. The consequence of this is dramatically increased noise, higher detection limits and generally unreliable performance. The maximum allowable electrode-nozzle separation is dictated by the onset of peak broadening, although well before this occurs the response declines to an unacceptably low level. We generally observed best overall performance at a separation of $\approx$ 1.2–1.6 mm.

A potential pitfall attendant to DLC detection at silver is that the ΔC_d response can diminish rapidly with time, presumably due to the accumulation of adsorbed matter. However, this occurrence can be avoided through vigilant attention to detail. The major precaution to be taken is the avoidance of overly positive monitoring potentials. In most instances a suitable compromise between sensitivity and longevity is provided by a potential setting 0.2–0.3 V negative of the maximum in the C_d–E or ΔC_d–E curve. With due care we are able to run for an entire day incurring only minimal losses in sensitivity. However, in the event of reduced response, virtual full restoration of the sensitivity can usually be achieved by stepping to a sufficiently negative potential for a short period. Consequently, an alternate approach is to employ a measurement scheme that incorporates a periodic cleaning pulse. We have devised two: (1) a differential DLC measurement that includes a negative-going desorption step, and (2) a pulse coulometric measurement scheme that utilizes positive-going potential steps from suitably negative values. A detailed description of these two techniques is given in a separate report[14].

Simultaneous faradaic detection and RDE operation

As previously mentioned, the cell/electrode configuration used here made for ready coupling of an RDE to a rotator. As also stated, a rotating electrode has a beneficial effect on DLC detection by creating a steady state flow condition which leads to a straighter baseline. Presumably, by sweeping material away from the electrode and out into solution, the electrode is less susceptible to accumulation of impurities. The net result is that there is less drift in the in-phase and out-of-phase current components. Use of a rotator does not introduce significant noise until a rotation

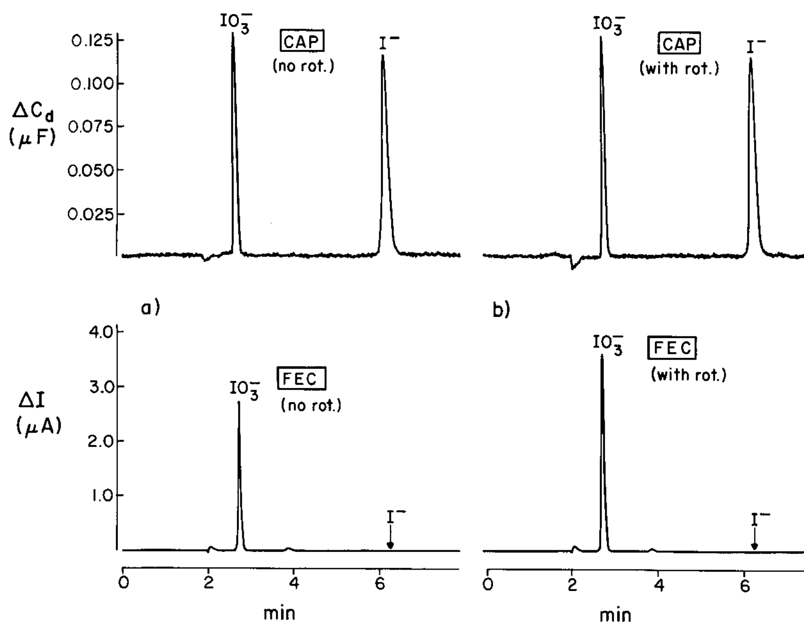

Fig. 8. Demonstration of the utility of electrode rotation in conjunction with combined DLC and FED detection; sinusoidal frequency, f = 45 Hz; rotational frequency, ω (a) 0, (b) 2150 rpm; potential, −1.20 V; sample composition: 5 ppm IO_3^-, ≈ 5 ppm I^-.

speed of about 750 rpm is reached. Of course, quite apart from its use in capacitance detection, the cell/electrode/rotator configuration used here is also suitable for faradaic ED (FED) measurements by providing well defined and reproducible mass transport of the electroactive species to and from the electrode surface.

An example of the utility afforded by use of a rotator is evident in Fig. 8 which shows simultaneous DLC and FED chromatograms for IO_3^- and I^- with (a) no electrode rotation, and (b) at a rotation speed of 400 rpm. As expected, the purely capacitive response for I^- is unaffected by rotation, whereas the d.c. FED response for IO_3^- shows the expected effect. As attested to by the invariant "apparent capacitive" response for IO_3^-, its response is also essentially purely capacitive. This is because the reduction of iodate is irreversible[24], and hence, virtually no faradaic signal is obtained by a.c. voltammetry[23].

A simultaneous amperometric signal, as obtained above, serves both to complement DLC detection and to act as a sentry toward it. Fig. 8 above provides an example in conjunction with electrode rotation. Another example, but without rotation, is presented in Fig. 9. As seen in Fig. 9a, the DLC response at −1.20 V to $S_2O_3^{2-}$ is very weak, but the d.c. faradaic, reductive signal is strong, and *vice versa* for I^-. Hence, one species is detected readily by DLC (I^-) and the other by amperometry ($S_2O_3^{2-}$) at this potential. Fig. 9b shows a separation of IO_4^- and I^-. The d.c. response for the reduction of IO_4^- signals an alert that the corresponding "capacitive" trace may not be entirely valid, although again in this case the apparent capacitance is essentially a true value since the reduction of IO_4^- is also irreversible[24]. Of

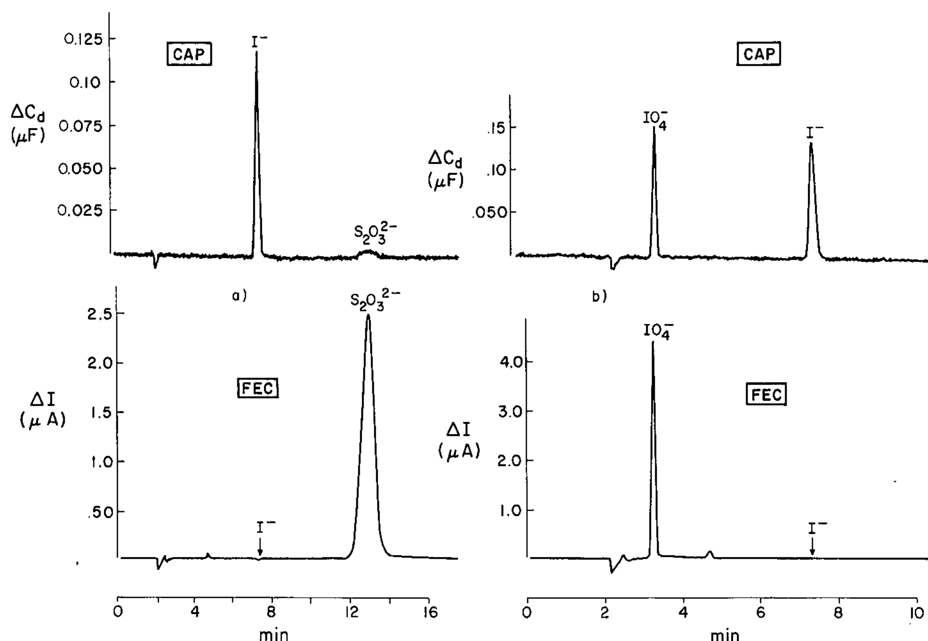

Fig. 9. Dual and FED traces for (a) a 5 ppm I^-/5 ppm $S_2O_3^{2-}$ mixture, and (b) a 10 ppm IO_4^-/5 ppm I^- mixture; both separations conducted with $E = -1.20$ V.

course, with simultaneous amperometric detection, an invalid capacitance output may be viewed as immaterial, since in that case, the FED trace would still furnish the requisite quantitation. The double-layer response to I^- in Fig. 9b is valid, since I^- is electroinactive at -1.20 V.

Extension of DLC detection to conventional ion exchange columns

An inconvenience to the use of standard IC columns in DLC detection is that a make-up stream is required to produce a quiet baseline and reliable operation. The relatively small ion exchange capacities of standard IC columns necessitates the use of displacer ion (electrolyte) concentrations on the order of (1–2) · 10^{-2} *M* and lower (often just a few millimolar with the lightest loaded packings). Such low electrolyte concentrations mitigate against DLC detection. The problem is illustrated in Fig. 10 in which noise and signal levels are compared under different conditions of operation. Fig. 10a shows the 10-mV peak-to-peak modulating sinewave, $f = 145$ Hz, applied atop the d.c. potential. In Fig. 10b we see the alternating portion of the cell current obtained for quiescent operation (no flow) in the LVWJ cell in 0.1 *M* electrolyte. Upon introduction of eluent and make-up flow concentrations of 2.5 · 10^{-3} and 0.64 *M*, respectively (the intent being to maintain an overall electrolyte concentration of 0.1 *M*), the a.c. signal in Fig. 10c was obtained. Under the conditions of c, chromatogram d was obtained for a 10-ppm injection of Cl^-. The same experiment performed in the absence of make-up produced the alternating cell current in e and the chromatogram in f. The deterioration in performance is obvious, the result of a

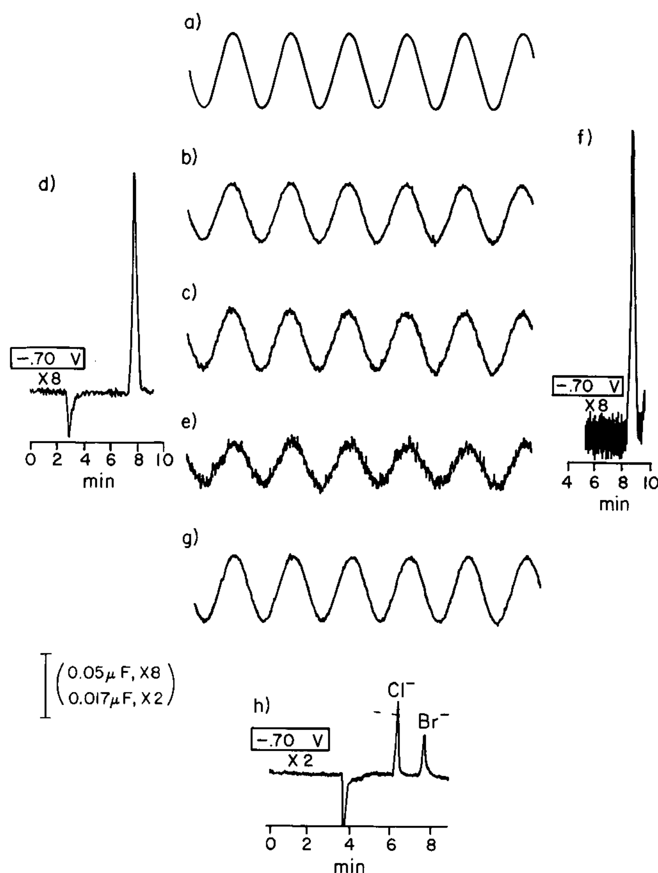

Fig. 10. Comparison of the a.c. current and corresponding chromatograms obtained under varying conditions of operation. See text for details.

substantial solution resistance. Hence, clearly, sensitive capacitance detection under these conditions, *i.e.*, in the absence of make-up is precluded.

Double-layer capacitance detection without a make-up can be achieved, though, by utilizing a conventional anion exchange packing in place of the low-capacity IC packing. This is illustrated in Fig. 10g and h. A Nucleosil 5SB anion exchanger (exchange capacity of 1.0 mequiv./g) with a 0.078 *M* sodium perchlorate eluent (the cell was initially charged with same), produced the a.c. current shown in g and the chromatogram seen in h for an injection of a mixture of 1 ppm Cl^- and 0.5 ppm Br^-. All of the adsorptioactive ions may be similarly determined on the Nucleosil column. While it would be folly to attempt conductometric detection on a conventional ion exchange column, a similar tact has also proven workable in indirect photometric detection[33,34].

Operation at higher electrolyte strengths would seem to auger well for double-layer detection using a thin-layer cell, as is commonly used in amperometric detection. Use of a thin-layer device with a low-capacity IC column would require a second

LC pump and post-column addition of a make-up solution. The need for these would be obviated for a thin-layer cell coupled to a high-capacity ion exchange column.

CONCLUSIONS

We have introduced here electrosorption at silver as manifested in changes in double-layer capacitance as a basis of detection in IC. To our knowledge, this is the first application of specific adsorption at a solid electrode to the detection of either inorganic or organic species in liquid chromatography. We have applied the technique at silver to the unequivocal determination of Cl^-, Br^-, N_3^-, I^- and SCN^- at concentrations of interest in contemporary IC. Additional anions, including IO_3^-, IO_4^-, BrO_3^-, S^{2-} and $S_2O_3^{2-}$, are also determinable by differential DLC, despite the fact that they undergo electroreaction at the electrosorptive potentials. Presumably, if concentrations higher than those typically determined in modern IC were additionally of interest, the technique could be extended to many other ions.

The ΔC_d–c_x calibration curves obtained for silver exhibit more non-linearity than those for mercury, but, nevertheless, conform to the shape expected on fundamental grounds. The greater curvature is in part due to stronger adsorption at silver. Detection limits are comparable to those generally claimed for conductivity and indirect photometry, and are marginally better than those achieved using the same procedure at mercury. While some loss of surface activity is unavoidable with time in this technique, careful attention to sample composition/cleanliness and to electrode potential settings allow for extended operation.

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DETERMINATION OF BINDING AFFINITY OF ENANTIOMERS TO ALBUMIN BY LIQUID CHROMATOGRAPHY

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SUMMARY

The principles of the determination of the binding affinity constants of small molecules to albumin by liquid chromatography, using albumin as a mobile phase additive, are outlined. Chromatographic conditions for determinations of constants are presented and applied to enantiomers of tryptophan and omeprazole. The influence of albumin on the retaining properties of LiChrosorb RP-8, Phenyl Hypersil and LiChrosorb Diol was studied.

INTRODUCTION

A knowledge of protein binding is of fundamental importance when studying the biological activities of drugs, *e.g.*, in pharmacodynamic and pharmacokinetic studies[1]. The main techniques for determining the interaction between proteins and small ligands are equilibrium dialysis and ultrafiltration, but liquid chromatography can also be used[2]. The chromatographic techniques are fast and generally easy to perform as ordinary high-performance liquid chromatographic (HPLC) equipment is sufficient. The chromatographic technique of Hummel and Dreyer[3] has been used to study the binding of L-tryptophan[4] and warfarin[5]. The affinities of warfarin, furosemide and phenylbutazone[6] and also L-tryptophan[7] to albumin have been studied by adding the protein to the eluent. An equilibrium saturation method[8] has been used to study warfarin–albumin binding and the influence of free fatty acids and sodium dodecyl sulphate on this interaction.

Chromatographic systems with proteins in the mobile or the stationary phase have been utilized for the separation of compounds with different degrees of protein binding. Albumin and α_1-acid glycoprotein are known to bind enantiomers to different extents and can be used as chiral selectors immobilized on a solid phase for the separation of racemates[9–11].

A previous study showed that enantiomeric carboxylic acids can be separated by addition of albumin to the mobile phase[12]. The retention of the enantiomers is regulated

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by the concentration of albumin, pH and type of solid phase. A retention model was presented. In this study the model was applied to the determination of the binding affinities of omeprazole and tryptophan to albumin. The principles for the choice of experimental conditions, *e.g.*, the solute and the albumin concentration, are presented.

Proteins interact strongly with certain silica-based solid phases[13] and it has been found that bovine serum albumin affects the retaining properties of an ODS column[14]. The adsorption of albumin to the solid phases (LiChrosorb Diol, LiChrosorb RP-8 and Phenyl Hypersil) used in the binding studies was investigated.

EXPERIMENTAL

Apparatus

The pump was an Altex Model 110 A solvent metering pump (Beckman, Berkeley, CA, U.S.A.) and the stainless-steel frit in the outlet check valve Kel-F washer was replaced with a PTFE supporting net (from a HibarLiChroCart unit; E. Merck, Darmstadt, F.R.G.). The pump was equipped with a pulse damper (Touzard et Matignon, Vitry, France). The UV detector was a SpectroMonitor D variable-wavelength detector (LDC, Riviera Beach, FL, U.S.A.). The injector was a Rheodyne Model 7120 (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop.

The separation columns (100 × 4.6 mm I.D.) were made of stainless-steel with a polished inner surface, equipped with modified Swagelok® connectors. A precolumn, identical with the separation column and packed with LiChroprep RP-18 (25–40 μm), was inserted before the injector to protect the analytical column from impurities in the mobile phase unless stated otherwise. The precolumn was equipped with stainless-steel frits (2-μm porosity) and the separation columns were equipped with stainless-steel sieve filters (2- or 3-μm porosity) with spreaders.

The chromatographic system was thermostated by a HETO water-bath (Birkerød, Denmark) Type 02 PT 923.

Chemicals

Phenyl Hypersil (5 μm, 120 Å pore diameter, basic silica) was obtained from Shandon (London, U.K.) and LiChrosorb Diol and LiChrosorb RP-8 (5- and 7-μm, respectively, 100 Å pore diameter, basic silica) were obtained from E. Merck.

Human serum albumin (HSA) fraction V, essentially fatty acid free (A-1887), and D- and L-tryptophan were obtained from Sigma (St. Louis, MO, U.S.A.). Racemic 2-(*p*-chlorophenoxy)propionic acid was obtained from Janssen (Beerse, Belgium) and (−)-2-(*p*-chlorophenoxy)propionic acid was kindly supplied by the Department of Organic Pharmaceutical Chemistry, University of Uppsala, Sweden. Racemic omeprazole was a gift from Hässle (Mölndal, Sweden) and N,N-dimethyl-N-octylamine (DMOA) was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.).

All other substances and solvents were of analytical-reagent or reagent grade and used without further purification.

Chromatographic technique

Column packing and column testing were performed as described previously[12]. In the studies, the packings of the analytical columns were fixed with sieve filters, as frits sometimes give rise to sudden pressure increases. Eluents containing albumin gave

rise to slow pressure increases. During 1 week the back-pressure increased from 400–500 to 800 p.s.i. for the LiChrosorb Diol and the Phenyl Hypersil columns and from 450–600 to 1000 p.s.i. for the LiChrosorb RP-8 column.

The mobile phase, the columns and the injector were thermostated at 20.0 ± 0.1°C. No solvent inlet filter was used and the mobile phases were not recirculated. The flow-rate was 1 ml/min in the tryptophan study and 0.5 ml/min in the omeprazole study. The reference cuvette of the UV detector was filled with mobile phase when albumin was used at concentrations $\geqslant 30\ \mu M$.

The mobile phases were aqueous phosphate buffers with different additives such as albumin, DMOA or inorganic salts. The solutes were dissolved in the mobile phase. Sample solutions and stock solutions of albumin were stored in a refrigerator at 6°C for a maximum of 1 week.

The capacity factor, k', was calculated from the solute retention volume, V_R, and the elution volume of albumin, V_a, from $k' = (V_R - V_a)/V_a$. V_a represents the interparticle volume of the column once it has been equilibrated with an albumin-containing mobile phase. V_a was determined by injection of an excess of albumin or pure phosphate buffer. The total porosity, ε_m, was obtained by injection of sodium nitrate, which was assumed to be unretained.

RESULTS AND DISCUSSION

Secondary equilibria in the mobile phase, *e.g.*, acid–base reactions and complexations, are frequently used to regulate retention and selectivity in liquid chromatography[15]. The chromatographic technique can also be used to evaluate complexation constants[16]. Addition of albumin as a complexing agent to the mobile phase can affect the retention and selectivity of solutes due to protein binding:

$$X + P_i \overset{K_{XP(i)}}{\rightleftharpoons} XP_i \quad (1)$$

where $K_{XP(i)}$ is the equilibrium constant for binding of X to a binding site (i) on the protein P. This protein–solute interaction has been used to obtain highly selective chromatographic separations, *e.g.*, the resolution of enantiomers[12,17]. An application of the technique with albumin as the chiral selector in the mobile phase is shown in Fig. 1. The two enantiomers of omeprazole are completely resolved ($R_s = 1.7$) within 18 min. The binding affinity of ligands to albumin can be determined from retention data in systems with different albumin concentrations in the mobile phase.

Retention model

The binding affinities of omeprazole ($pK_1 = 4.0$, $pK_2 = 8.7$) and tryptophan ($pK_1 = 2.4$, $pK_2 = 9.4$) were studied at pH 7.4, where omeprazole is mainly uncharged whereas tryptophan has a zero net charge. In the retention model previously derived[12] it was assumed that negatively charged solutes were retained as ion pairs with a counter ion to the stationary phase. Uncharged solutes are retained according to

$$X_m + A_s \overset{K_{XA}}{\rightleftharpoons} XA_s \quad (2)$$

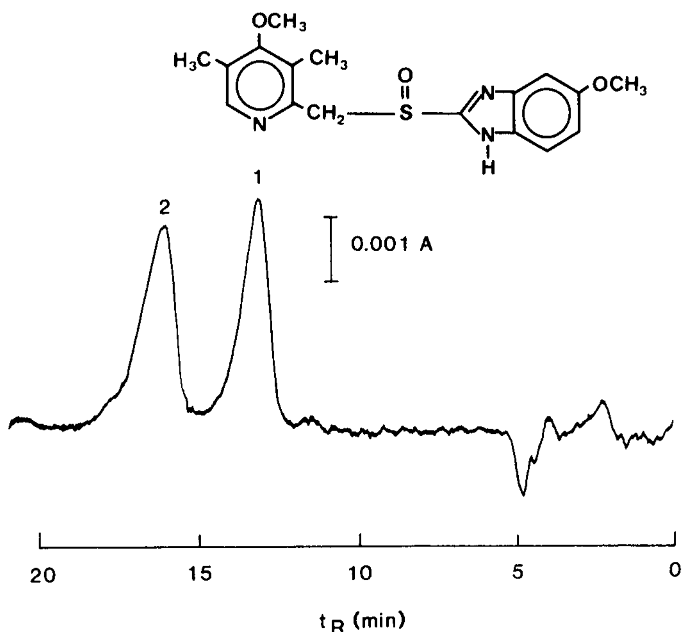

Fig. 1. Resolution of the enantiomers of omeprazole. Solid phase: LiChrosorb Diol. Mobile phase: 50 μM HSA and 0.010 M DMOA in phosphate buffer (pH 6.5, μ = 0.1). Analytical columns: two 100 × 4.6 mm I.D. UV detection: 302 nm. Flow-rate: 0.33 ml/min. The order between the two enantiomers has not been determined.

where K_{XA} is the equilibrium constant for the adsorption of the solute X to the adsorption site A on the solid phase. The monolayer capacity of the adsorbing stationary phase, K° (mol/g), is defined as

$$K^\circ = [A]_s + [XA]_s \tag{3}$$

and the binding of buffer components to the solid phase is assumed to be negligible. The assumption was also made[12] that binding to one site (P_1) on the protein is dominating and that the solute is applied in such a low concentration that the binding isotherms to both the solid phase and the protein are linear. The following equation for the capacity factor, k', was derived:

$$k'_X = \frac{W_s K^\circ K_{XA} + V_{is}}{V_a[1 + n_1 \; K_{XP(1)} \; C_p]} \tag{4}$$

where W_s is the weight of the solid phase (g), n_1 is the number of binding sites P_1 of 1 mol of protein, $K_{XP(1)}$ is the equilibrium constant of the binding of the solute X to binding site P_1 on the protein $(mol/l)^{-1}$ (see eqn. 1) and C_p is the total concentration of albumin (mol/l). The stationary phase is defined here as the sum of the phase in the pores not accessible to albumin, V_{is} (ml), and the solid phase. The aqueous phase outside the pores containing albumin, V_a (ml), is regarded as the mobile phase. In eqn.

4 it is assumed that albumin is not affecting the retaining properties of the solid phase. However, the model has to be modified if albumin interacts with the solid phase and thus changes its retaining properties.

Adsorption of albumin

The adsorption of albumin to LiChrosorb RP-8, Phenyl Hypersil and LiChrosorb Diol was studied by frontal analysis. The column was equilibrated with phosphate buffer (pH 6.5, μ = 0.1). A mobile phase containing 30 μM of albumin was then applied and the breakthrough of the protein was registered, as demonstrated in Fig. 2, by the front boundary (1) of the respective solid phase. The amount of albumin adsorbed on the column was calculated by integration between V_m, obtained from the retention of sodium nitrate before the adsorption of albumin, and the breakthrough volume of albumin as shown by the shaded areas in Fig. 2. The amounts adsorbed were 0.86 μmol/g on LiChrosorb RP-8 and 0.24 μmol/g on Phenyl Hypersil. The adsorption to LiChrosorb Diol was too low to be measured with acceptable precision. The amount of albumin adsorbed corresponds to a coverage of about 12% of the total surface area

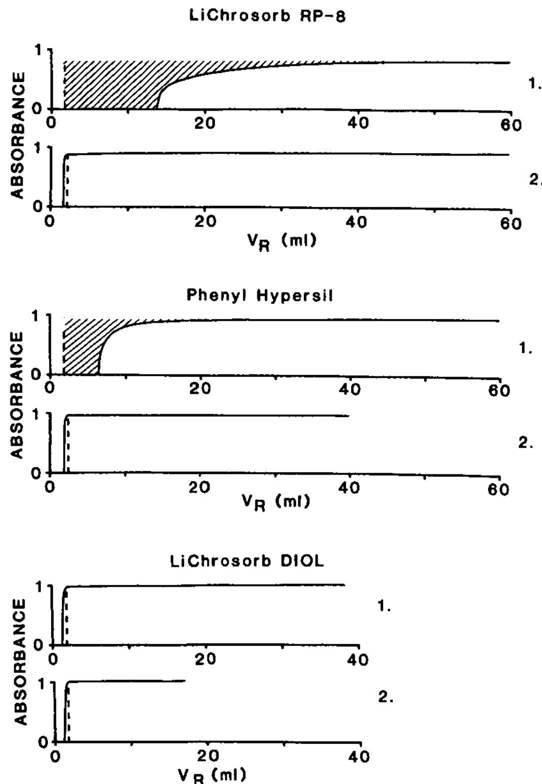

Fig. 2. Frontal analysis of albumin. Mobile phase: 30.5 μM HSA in phosphate buffer (pH 6.50, μ = 0.10). Column: 100 × 4.6 mm I.D. (no precolumn used). UV detection: 280 nm (LiChrosorb RP-8), 276 nm (Phenyl Hypersil and LiChrosorb Diol). Broken lines, V_m. The mobile phase was applied twice, corresponding to front boundaries (1) and (2). Columns were washed with 400 ml of buffer between the applications. The amount of albumin adsorbed was calculated from the shaded areas.

of LiChrosorb RP-8 and 5% of Phenyl Hypersil, assuming a monolayer adsorption of albumin (elliptically shaped, 38 × 150 Å[18]) and using the specific surface areas of the solid phases given by the manufacturers.

The reversibility of the albumin binding was studied by passing 400 ml of the buffer through the column, followed by a new application of the albumin-containing mobile phase. The breakthrough of the albumin now occurred at a volume lower than the V_m obtained with sodium nitrate [see front boundary (2) of the respective solid phase in Fig. 2]. This indicates a high degree of irreversibility for the albumin adsorption, and is in agreement with the observations of Barford and Sliwinski[19] that bovine serum albumin is irreversibly bound to C_8 and C_{18} alkylsilicas from phosphate buffer at pH 2 and 7.

The changes in the binding properties of the columns after equilibration with an albumin containing phase are further illustrated by the retention studies presented in Tables I and II. These were made in connection with the frontal analysis studies. Samples of albumin, sodium nitrate and tryptophan were injected before the application of the albumin-containing phase and albumin and sodium nitrate were injected after the application. Injections of all three samples were made after washing the columns with buffer. After a new application of albumin, the protein and sodium nitrate were injected again. Finally, after washing with buffer, albumin, sodium nitrate and tryptophan were injected. The frontal analysis studies, and also the binding studies presented below, were performed over a 5–7 day period, with the exception of the frontal analysis study with the Diol phase. The decrease in retention volume due to the influence of pure buffer on the solid phase was checked using the Diol phase. Phosphate buffer was recirculated through the column during 19 days after the albumin-containing phase had been washed off, after the first application. After this period a 4% decrease in the retention volume of albumin, sodium nitrate and tryptophan were observed (Tables I and II).

TABLE I

RETENTION VOLUME OF ALBUMIN (V_a) AND SODIUM NITRATE (V_m) AND POROSITY, ε_a, CALCULATED FROM V_a

Mobile phase solvent: phosphate buffer (pH 6.5, μ = 0.1).

*Mobile phase**	*LiChrosorb RP-8*			*Phenyl Hypersil*			*LiChrosorb Diol*		
	$V_a(ml)$	ε_a	$V_m(ml)$	$V_a(ml)$	ε_a	$V_m(ml)$	$V_a(ml)$	ε_a	$V_m(ml)$
Solvent	1.04	0.62	1.21	0.99	0.60	1.11	0.73	0.44	1.10
30 μM HSA** in solvent	0.73	0.44	1.19	0.66	0.40	1.14	0.68	0.41	1.06
Solvent***	0.70	0.42	1.21	0.67	0.40	1.12	0.70 0.67§	0.42 0.40§	1.07 1.04§
30 μM HSA** in solvent	0.68	0.41	1.18	0.64	0.38	1.10	0.66	0.40	1.02
Solvent***	0.68	0.41	1.17	0.67	0.40	1.14	0.66	0.40	1.00

* Applied to an unused column in the order indicated.

** 200 ml passed before injection.

*** 400 ml passed before injection.

§ After 19 days of recirculating mobile phase.

TABLE II

RETENTION VOLUME OF D- AND L-TRYPTOPHAN BEFORE AND AFTER HSA HAS BEEN USED AS A MOBILE PHASE COMPONENT

Mobile phase: phosphate buffer (pH 6.5, μ = 0.1).

Treatment of column	*Enantiomer*	*LiChrosorb RP-8**		*Phenyl Hypersil**		*LiChrosorb Diol**	
		$V_R \pm s(ml)$	*n*	$V_R \pm s(ml)$	*n*	$V_R \pm s(ml)$	*n*
Before application of HSA		12.5 ± 0.03	3	5.17 ± 0.008	6	1.47 ± 0.02	3
After 1st application	D	9.73 ± 0.04	3	4.56 ± 0.005	4	1.42 ± 0.001	3
of 30 μM HSA	L	9.78 ± 0.02	3	4.62 ± 0.03	7	1.40 ± 0.005	3
	D					1.36 ± 0.004**	3
	L					1.36 ± 0.003**	4
After 2nd application	D	9.18 ± 0.06	3	4.41 ± 0.002	4	1.33 ± 0.004	3
of 30 μM HSA	L	9.23 ± 0.02	3	4.50 ± 0.01	4	1.30 ± 0.003	3

* s = standard deviation; n = number of repeated injections.

** After 19 days of recirculating mobile phase.

The adsorption of albumin to the LiChrosorb RP-8 and Phenyl Hypersil phases resulted in a significant decrease in the albumin retention volume (V_a) and of ε_a (Table I). The porosity was calculated from the retention volume of albumin, $\varepsilon_a = V_a/V_0$ (where V_0 is the volume of the empty column tube). ε_a of the phases is close to the value given for a totally excluded solute, $\varepsilon_0 = 0.4$ (the interparticle porosity)[20]. The retention of sodium nitrate (V_m) and, consequently, the total porosity ε_m was almost unchanged.

The results in Table I clearly indicate that part of the aqueous phase in the column is not available to albumin. A further decrease in the retention volume of albumin on LiChrosorb RP-8 and Phenyl Hypersil was obtained after the equilibration with an albumin-containing phase. This decrease remained after changing to an albumin-free mobile phase indicating that the retention change was due to an irreversible process.

On the LiChrosorb Diol phase the retention volume of albumin was almost the same before and after the introduction of albumin in the mobile phase. It is interesting that the adsorption of albumin on LiChrosorb RP-8 and Phenyl Hypersil has such an effect on the binding properties that the albumin retention volume became the same as on LiChrosorb Diol. The reason might be that hydrophobic parts of the albumin molecule are sorbed at the hydrophobic moieties on the surface of LiChrosorb RP-8 and Phenyl Hypersil and the remaining retention is mainly due to interactions with the hydrophilic parts of the three phases.

The effect of albumin adsorption on the retention of tryptophan on the three solid phases is shown in Table II. The adsorption of albumin gave rise to a decrease in the retention, which was 4% on LiChrosorb Diol but 21% and 11% on LiChrosorb RP-8 and Phenyl Hypersil, respectively. The subsequent application of albumin gave a minor decrease in the retention of about 4% on all the supports. As shown in Table II, there is a small difference in the retention volume for D- and L-tryptophan. This indicates that the adsorbed albumin has a low capacity and/or a change in the binding properties of albumin occurs when it is immobilized on the solid phase.

The retention model shown above (eqn. 4) assumes that albumin is excluded

from the pores and that the binding properties of the solid phase are independent of the albumin concentration in the mobile phase. The albumin adsorption studies presented above show that the model is valid when using the LiChrosorb Diol phase. However, when applying the more hydrophobic phases, *e.g.*, LiChrosorb RP-8, the effect of adsorbed albumin has to be taken into consideration. The solid phase can be regarded as a heterogeneous surface with two kinds of binding sites (A, A*) with limited binding capacities[21]. The unmodified solid phase (sites A) has the capacity $K^{\circ\prime}$; $K^{\circ\prime}$ is less than K° (eqn. 3) owing to adsorption of albumin. The capacity of the immobilized albumin, $K^{\circ *}$, is given by

$$K^{\circ *} = [A^*]_s + [XA^*]_s \tag{5}$$

where A* is an adsorption site of the immobilized albumin. Assuming linear binding isotherms to both sites gives the following expression for the capacity factor:

$$k'_x = \frac{W_s\ (K^{\circ\prime}K_{XA} + K^{\circ *}K^*_{XA}) + V_{is}}{V_a\ [1 + n_1 K_{XP(1)} C_P]} \tag{6}$$

where K^*_{XA} is the equilibrium constant of the adsorption of solute X to the site of adsorbed albumin. When determining the binding affinity for solutes it is important to use a column that previously has been exposed to albumin, as the retaining properties of the solid phase might be affected.

Influence of albumin binding on chromatographic behaviour

A protein, P, present in the mobile phase, can affect the retention of a solute, X, by formation of a complex, XP, in the mobile phase. If one binding site of the protein (P_1) is dominant, the stability constant of the complex is defined by

$$\frac{[XP_1]}{[X][P]} = K_{XP(1)} \tag{7}$$

The complexation has a constant influence on the chromatographic retention if the conditions are such that the binding ratio $[XP_1]/[X]$ is unchanged during the elution.

The binding ratio depends on the total concentration of the protein and the magnitude of the binding affinity, as shown by eqn. 8, and also on the total concentration of the solute[22]:

$$\frac{[XP_1]}{[X]} = \frac{n_1\ K_{XP(1)} C_P}{1 + K_{XP(1)}[X]} \tag{8}$$

where C_P is the protein concentration in the mobile phase, and the binding ratio will assume a constant value when $K_{XP(1)}[X] \ll 1$.

The influence on the binding ratio of the magnitude of the three parameters stability constant, protein concentration and solute concentration is illustrated in Figs. 3 and 4. The computations are based on eqn. 8 assuming $n_1 = 1$. Fig. 3 is valid for

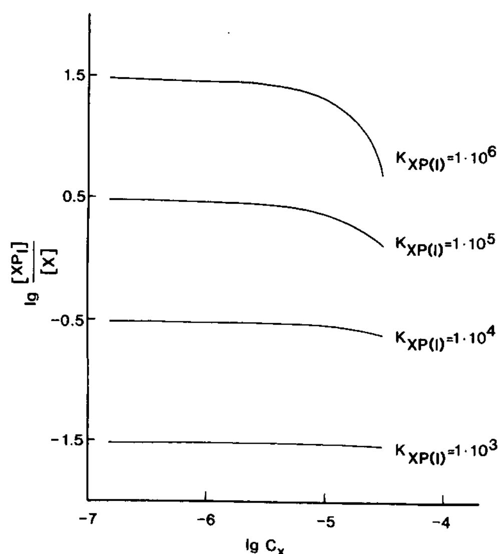

Fig. 3. Calculated solute–protein binding ratios at constant protein concentration (C_p = 30 μM).

a constant protein concentration of 30 μM. It shows that the maximum solute concentration giving a constant binding ratio decreases strongly with increasing $K_{XP(1)}$. Fig. 4 shows the influence of the protein concentration when $K_{XP(1)} = 10^5$. The limiting sample concentration for a constant binding ratio increases with increasing C_p.

When applying the results of the calculations to chromatographic conditions, it must be kept in mind that the calculations are valid for the solute concentrations in the mobile phase. The distribution of the solute to the stationary phase decreases its concentration in the mobile phase and the limiting concentration of the solute in the injected sample will increase with increasing retention. The relationship between the initial solute concentration, C_X°, and the actual concentration in the mobile phase in the

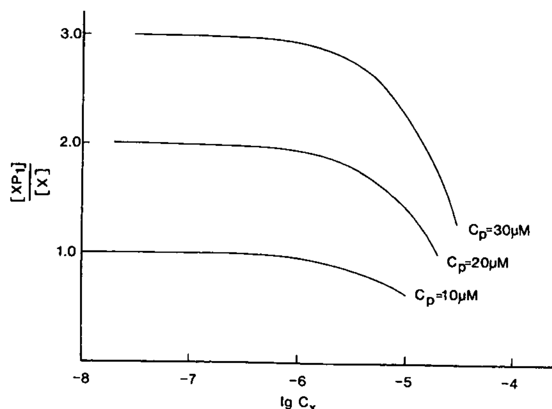

Fig. 4. Calculated solute–protein binding ratios with a binding constant $K_{XP(1)} = 1 \cdot 10^5$.

injected zone, C_X, is given by

$$C_X = \frac{C_X^\circ}{1 + k'_X} \tag{9}$$

where

$$C_X = [X] + [XP_1] \tag{10}$$

It is of vital importance for the chromatographic performance to work in a solute concentration range where the binding ratio $[XP_1]/[X]$ is constant. The maximum concentration of the solute decreases during the chromatographic migration and if the concentration change is accompanied by a change in the binding ratio, it will result in a peak deformation and a concentration-dependent capacity ratio[12].

Evaluation of binding affinity

When the retaining phase is an adsorbent, the capacity ratio can also be influenced by the competition of the solute molecules for its limited binding capacity. However, eqn. 6 is only valid for linear adsorption isotherms, *i.e.*, when the competition for the binding capacity of the adsorbent is negligible.

When the binding ratio is constant and the adsorption isotherm is linear, the binding affinity of the solute to the protein can be determined from a reciprocal plot based on eqn. 6. For an uncharged solute, X, it has the following form:

$$\frac{1}{k'_X} = \frac{1}{q(a + b)} + \frac{n_1 K_{XP(1)} C_P}{q(a + b)} \tag{11}$$

where $q = W_s/V_a$, $a = (K^{\circ\prime} K_{XA} + K^{\circ *} K^*_{XA})$, $b = V_{is}/W_s$, $K_{XA} = [XA]_s/[X]_m [A]_s$ and $K^*_{XA} = [XA^*]_s/[X]_m [A^*]_s$.

A plot of $1/k'_X$ *versus* C_p should be linear and the affinity constant, $n_1 K_{XP(1)}$, is obtained from the ratio of the slope to the intercept. Thus, modification of $K^\circ K_{XA}$ due to albumin adsorption has no importance in the evaluation of the binding affinity, since this term is eliminated in the calculation of $K_{XP(1)}$.

To obtain estimates of $n_1 K_{XP(1)}$ of acceptable accuracy, the intercept should preferably not be lower than 0.1, which means that the capacity ratio of the solute in the absence of protein should not exceed 10; k'_X lower than 1 should be avoided since it is usually determined with too low precision. The choice of the solid phase then has to be based on the properties of the solute, giving a suitable retention.

The protein concentration in the mobile phase should, if possible, be varied to such an extent that the ratio between the limiting k'_X values is at least 2. The choice of albumin concentration is, however, limited by its UV absorbance. For this reason it is hardly possible to determine binding affinities below 10^3 with this technique. Using UV detection below 300 nm the concentration of albumin is limited to about 80 μM. A higher concentration can be used if the solute or an added UV-absorbing probe (indirect detection) has absorptivity above 300 nm[12].

Determination of high binding affinities requires low protein concentrations. To obtain a sufficient excess of the protein, the solute concentration must be infinitesimal and hence problems with the detectability will occur. In practice the highest binding affinity that can be determined is about 10^5.

The application of these principles to the determination of the binding affinities of omeprazole and tryptophan is demonstrated in Figs. 5 and 6. Both compounds can appear in two enantiomeric forms with different binding affinities.

Omeprazole is highly hydrophobic and a suitable retention was obtained with LiChrosorb Diol as solid phase. The peaks showed strong tailing, indicating non-linear binding to the adsorbent. However, it has been shown[21] that such effects can be eliminated by a competing ion pair, and good peak symmetry and concentration-independent retention were obtained when 0.01 *M* DMOA was present in the mobile phase.

The plotting of the results in accordance with eqn. 11 is shown in Fig. 5. Several injections were made at each albumin concentration and the $1/k'$ range is indicated by bars. The resolution of the enantiomers in the presence of albumin was never less than 0.8 and the overlapping of the two peaks did not affect the k' values. The experiments were performed with successively increasing albumin concentrations in the mobile phase. The resulting affinity constants are given in Table III; the standard deviations were obtained by combining the standard deviations of the slopes with that of the intercept. The constants found are of the same magnitude as those obtained by equilibrium dialysis[23]. The latter study did not give the different affinity constants of the enantiomer as a racemic solute mixture was used.

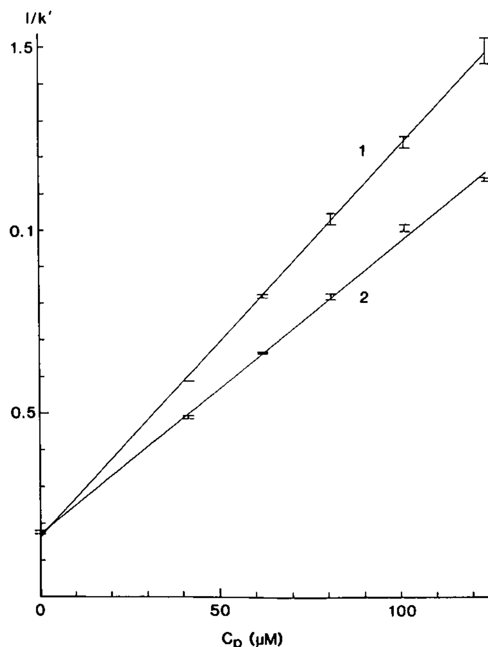

Fig. 5. Evaluation of binding affinities of the omeprazole enantiomers. Solid phase: LiChrosorb Diol. Mobile phase: HSA and 0.010 *M* DMOA in 0.066 *M* phosphate buffer (pH 7.35, μ = 0.17). Analytical columns: two 100 × 4.6 mm I.D. Solute concentration: 6.8 μM of racemate. UV detection: 302 nm.

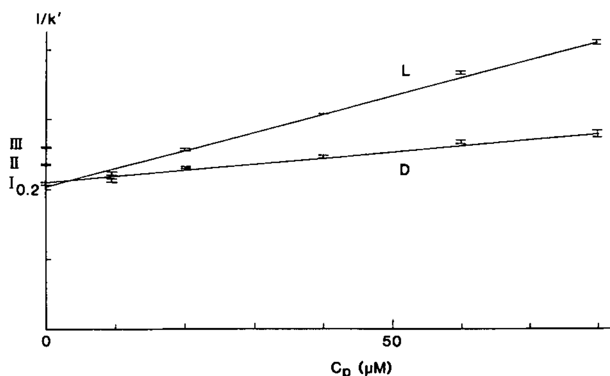

Fig. 6. Evaluation of binding affinities of L- and D-tryptophan. Solid phase: Phenyl Hypersil. Mobile phase: HSA, 0.151 M sodium chloride and 0.02% (w/w) sodium azide in 0.040 M phosphate buffer (pH 7.40, μ= 0.25). Analytical column: 100 × 4.6 mm I.D. Solute concentration: 10.2 μM of each enantiomer injected separately, except at C_p = 9.60 μM, where the concentration was 2.6 μM. UV detection: 280 nm. $1/k'$ at C_p = 0 obtained after equilibrating the column with albumin of concentration (I) 9.60, (II) 40.0 and (III) 80.0 μM.

Tryptophan is fairly hydrophilic and a suitable retention was obtained with Phenyl Hypersil as the adsorbent; 0.02% (w/w) sodium azide was added to the mobile phase as a bacteriostat. The runs were made with successively increasing concentrations of albumin and the results are plotted in Fig. 6. The $1/k'$ range obtained by repeated injections and the affinity constants found are given in Table III. The affinity constant found for the L-form is in very good agreement with results obtained by affinity chromatography, whereas there is larger difference in the constants for the D-form[24].

Experiments were also carried out to determine the binding affinities of the enantiomers of 2-(p-chlorophenoxy)propionic acid to albumin in phosphate buffer (pH 7.4, μ = 0.25). Owing to the high binding affinity of the enantiomers to albumin $[n_1 K_{XP(1)} \rangle 10^5]$, a strongly retaining solid phase (LiChrosorb RP-8) had to be used. Good resolution of the enantiomers was obtained but the resulting intercept was too small to enable the affinity constants to be measured with acceptable precision.

Repeated tests of the properties of the solid phases were made during the experimental series by runs at C_p = 0 after washing the columns with 400 ml of an albumin-free mobile phase. On the LiChrosorb Diol phase such test runs were made after the use of 41.4, 81.2 and 124 μM albumin. The k' values decreased successively but the total decrease after the use of 124 μM albumin was less than 2%. The test with the Phenyl column was made after the use of 9.60, 40.0 and 80.0 μM albumin. The k' values at C_p = 0 decreased when exposed to increasing albumin concentration, as indicated by the $1/k'$ values shown in Fig. 6. The first k' value fits the lines in Fig. 6 well. An explanation might be that there is a change in the retention characteristics of the solid phase due to the unfavourable high pH used and/or a rearrangement of adsorbed albumin with time exposing adsorbing sites with different adsorptive properties.

TABLE III

BINDING AFFINITY OF OMEPRAZOLE AND TRYPTOPHAN

Chromatographic conditions as in Figs. 5 and 6.

Compound	$n_1K_{XP(1)} \pm s$*	
	*Found***	*Literature*
Omeprazole:		
Enantiomer 1	$(6.6 \pm 0.4) \cdot 10^4$ $(n=15)$	$2 \cdot 10^4$***
Enantiomer 2	$(4.6 \pm 0.2) \cdot 10^4$ $(n=14)$	
L-Tryptophan	$(1.29 \pm 0.02) \cdot 10^4 (n=23)$	$1.1 \cdot 10^4$§
D-Tryptophan	$(4.4 \pm 0.1) \cdot 10^3$ $(n=23)$	$1.3 \cdot 10^3$§

* s = standard deviation; n = total number of injections.

** Evaluated from one experimental series of successively increasing HSA concentration in the mobile phase.

*** Equilibrium dialysis[23] using racemate. $n_1K_{XP(1)}$ calculated from degree of binding assuming $n_1 = 1$. Buffer composition: phosphate buffer (pH 7.35, μ = 0.17).

§ Affinity chromatography[24]. Mobile phase: phosphate buffer (0.04 *M*, pH 7.4), containing 0.15 *M* sodium chloride and 0.02% (w/w) sodium azide.

CONCLUSIONS

The binding affinity of small molecules to albumin can be determined using the protein as a complexing agent in the mobile phase. The technique is generally applicable to affinity constants $[n_1K_{XP(1)}]$ in the range 10^3–10^5. Silica-based surface-derivatized solid phases with mean pore diameters of approximately 100 Å can be used, provided that the solid phases are exposed to an albumin solution prior to the binding study. The choice of the solid phase must be based on the solute properties in each instance. The albumin concentration range is limited by its inherent UV absorbance. The method has been applied to the determination of affinity constants of the enantiomers of tryptophan and omeprazole.

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SYNTHESIS OF BONDED LAYERS FROM CYCLIC ORGANOSILICONS IN THE GAS PHASE

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SUMMARY

As a sequel to an earlier study of modifying silica gel 62 by reacting it with cyclic organosiloxanes or silazanes in a liquid-phase reaction, a similar formation of bonded layers has been attempted under a wide variety of conditions in a gas-phase type reaction. This attempt was successful and the resulting products were in many aspects similar. They often matched (however, they never surpassed) the earlier materials in gas chromatographic performance.

INTRODUCTION

Some time ago, our group described the syntheses of bonded phases from organosiloxane and organosilazane rings on typical silicic supports, and the performance of these materials in packed-column gas (GC) and liquid chromatography (LC)[1]. The bonding process used a non-polar, inert, high-boiling solvent under reflux, similar to a technique developed earlier for bonding polyether layers[2].

In either case it seemed likely that the conditions characteristic of this type of synthesis had favored a sterically defined approach of the monomer/polymer onto the solid surface. In particular, the monomer/polymer was only sparingly soluble in the high-boiling alkane and, of course, there existed a large difference in polarity between the silanol-covered support and the hydrocarbon solvent. Furthermore, the high temperature promoted both chemical reactions and optimal chain orientation on the surface.

Thus it appeared that the condition of a solid–liquid interface may have exerted a decisive influence on the outcome of the synthesis. The question therefore arose whether a solid–gas interface would have resulted in a significantly different product. Could efficient chromatographic phases be obtained when the monomer/oligomer approached the solid support from the gas phase? Could the easier variation of temperature in a gas-phase reaction (as opposed to a liquid-phase reaction where the choice of solvent is limited to high-boiling alkanes and the temperature thus

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determined by their boiling points) bring significant benefits? Is there a fundamental difference between the two approaches?

This study was designed to answer precisely these questions, as well as to represent a direct sequel to the earlier report involving cyclic siloxanes and silazanes at the solid–liquid interface. Before describing the experiments, however, we should take note of the vast literature that, in one form or another, impinges on the question.

Silicic surfaces and organosilicon compounds —as well as the interactions between the two— have been extensively investigated (see, for instance, refs. 3–5); they serve a wide variety of purposes and are found in a wide variety of products. Even in the much more narrow chromatographic context, individual studies of silica and silicones are far too numerous to cite. Such studies cover primarily the deactivation of active surfaces and the formation of bonded layers. Similarly, the use of high temperatures —whether in column conditioning, deactivation of the inside of quartz capillaries, crosslinking of thicker films, or simply in day-to-day GC analysis— is commonplace.

The deliberate use of gaseous reagents, however, is far less common. GC columns can be brought back to life that way, and one could also argue that bleed from GC phases interacts with nearby surfaces. Deactivation and hydrophobization have been occasionally attempted with vapors of such compounds as dimethyldichlorosilane or hexamethyldisilazane. Nigh exclusively, though, conventional synthesis of bonded layers, whether destined for LC or for GC, is based on solution chemistry, *i.e.* on the solid–liquid interface.

In our own group, we have had some experience in using the gas phase for cleaning surfaces[6], for coating materials with inorganic layers[7], for transporting reagent to a polymerizing silicone[8], or for collecting bleed constituents[9]. We have also had some experience with cyclic organosilicon compounds[1], which are rarely if ever used for conventional phase synthesis. (They figure prominently, however, as products of GC liquid-phase degradation. Furthermore, there are some reports in the non-chromatographic literature on their use as silica modifiers applied via the gas phase, *e.g.* ref. 10.)

Hence, despite the odds against producing a new, high-efficiency phase, we considered it worthwhile to use the cyclics for a synthetic comparison of solid–liquid *vs.* solid–gas interfacial systems. That the two could be very different appears obvious in light of their very different sorption and mass transfer characteristics, as well as their possible differences in the establishment of equilibria, the disposal of side-products, and the chances for reagent transmutation.

Of the various possibilities for bringing a support in contact with monomer vapor, we choose one of the simplest to control: we would expose loose support particles to diluted monomer vapor at different temperatures. The resulting materials would then be exhaustively extracted and characterized by carbon content and chromatographic behavior. Wide pore silica gel (neutral or acidified) would be used as the only support, and chromatographic tests restricted to gas chromatography. Besides the variation of temperature, inert gas (diluent) flow could provide different reagent concentrations/contact times and also allow the fluidization of the silica particles. A series of commercially available cyclic siloxanes, and one cyclic silazane, were to serve as silanizing reagents.

EXPERIMENTAL

Materials

Hexamethylcyclotrisiloxane, hexaethylcyclotrisiloxane, octamethylcyclotetrasiloxane, decamethylcyclopentasiloxane, octaphenylcyclotetrasiloxane, and octamethylcyclotetrasilazane were purchased from Petrarch Systems and used without further purification. Davison silica gel grade 62 was obtained from Grace Chemical and sieved to retrieve the 60–80 mesh fraction. This fraction was then extracted with azeotropic hydrochloric acid for four days in a Soxhlet, rinsed with distilled water to constant pH, and dried at 130°C and water-aspirator vacuum overnight ("neutral silica"). Some of the material was re-immersed in concentrated hydrochloric acid for 2 h and similarly dried ("acidic silica").

Layer synthesis

Various models of simple reaction flasks were constructed from glass. The principal *modus operandi* remained the same, but the flasks differed in size and whether or not they allowed preheating of reagent vapors and collection of non-reacted cyclics to take place. Fig. 1 shows the latest and most "complex" of these flasks (and,

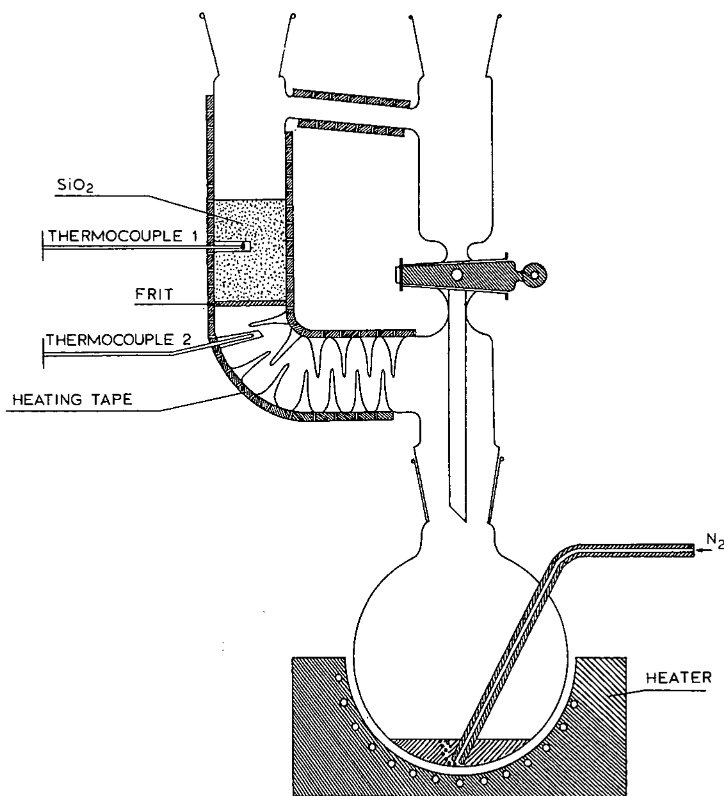

Fig. 1. Schematic of reaction flask. The top ground glass joints connect to a reflux condensor, an exhaust tube, and/or a stopper as required.

incidentally, the one that was used for most experiments). Silica is kept on a coarse frit in a separately heated section of the side arm and, depending on the nitrogen flow, awaits the arrival of reagent in either a fixed or a fluidized bed. The concentration of cyclic reagent in nitrogen gas is regulated by the temperature of the round-bottom flask. The carrier–reagent stream can be preheated by a separate section of heating tape. The combination of nitrogen flow, of volatility and temperature (as well as sorptivity on silica gel) of the cyclic, and of temperature in the reaction zone, determines the contact time. The effluents from the reaction can either be directly exhausted or stripped of most of the unreacted reagent by a condensor. Obviously, the apparatus can also work in a reflux mode. Except for some marginal experiments, however, care was taken to obey the gas-phase criterion to the extent that liquid reagent was not allowed to touch the silica gel and the silica gel was not allowed to look "wet".

The synthesis conditions varied to some degree with the nature of the cyclic reagent, but almost all procedures involved the following typical steps. About 2 g of cyclosiloxane or cyclosilazane were placed into the round-bottom flask and about 7 g of silica gel were poured onto the frit. The apparatus was closed and the silica (but not the cyclic) heated to reaction temperature with a very slow flow of nitrogen passing through to dispose of removable moisture. To start the reaction proper, the round-bottom flask was heated and/or a fast flow of nitrogen was turned on. The reaction was considered finished when all of the cyclic had vanished from the flask. After allowing the modified silica gel to cool down in the nitrogen stream, it was exhaustively extracted (usually for 10 h) in a continuous-flow apparatus[11] at the boiling point of the solvent (usually toluene).

Tests

The resulting materials were characterized by carbon analysis (Guelph Chemical Labs.), and by some standardized chromatographic runs. For the latter, the phases were filled into 1 m × 2 mm I.D. glass columns, conditioned at 230°C overnight (250°C for silica modified with octaphenylcyclotetrasiloxane), and tested with a variety of analyte mixtures (alkanes, alkenes, aromatics, oxygenates) in a standard temperature program starting at 40°C and rising at 8°/min. Nitrogen served as carrier gas (18–19 ml/min at 40°C).

RESULTS AND DISCUSSION

Effect of reaction conditions

Criteria. The effect of reaction conditions on the resulting product were judged by two criteria: carbon content (modifier load) and GC efficiency. As shall be seen later, the two appear related, but the precise reasons for this relationship are by no means obvious. Therefore they shall be treated separately in the following paragraphs. Furthermore, it should be understood that trends in behavior, as is not suprising in a complex system, will hold true for most, but not for each and every one of the experimental sequences.

Acidity. The reason for testing "acidified" *versus* "neutral" silica gel surfaces was the often-mentioned role of acids in the scission of siloxane bonds[12]. All cyclics were reacted with "neutral" silica gel, while only octamethylcyclotetrasiloxane and

octamethylcyclotetrasilazane were reacted with the "acidified" surface. As judged by carbon content, the difference between the two surfaces approaches experimental variability. Chromatographic performance, again, was not significantly different for the two surfaces. (Silica gel itself is, of course, considered "acidic" in its pure form, and we did not treat its surface with alkali just for the sake of comparison.)

Temperature of reaction. This is the temperature measured in the thermocouple well protruding into the silica gel bed (Fig. 1). It is to some degree dependent on the flow-rate and the preheating of the diluent–reagent mixture and gives only a rough indication of the true reaction temperature.

Reaction temperatures were varied in the 200–400°C range with all cyclic reagents, depending, to some extent, on the vapour pressure of the cyclic. It should be realized in this context that two different reagents, even when present in the carrier stream at equal concentration, flow-rate and temperature, will not experience the same contact time with silica gel owing to different retention (adsorption) characteristics. And, of course, the rate of reaction increases, while the extent of adsorption decreases, with temperature.

In general, and not unexpectedly in light of the preceding comments and the limited support area, carbon content first increases strongly and then levels off as temperature is increased. At what temperature the levelling off (and perhaps slight downturn) occurs, depends on the nature of the cyclic as well as on the particular reaction conditions.

To mention some typical series: carbon contents of 0.26, 0.50, 0.75 and 1.42% were measured for octamethylcyclotetrasiloxane at reaction temperatures of 230, 300, 350 and 400°C, respectively, under fluidized-bed conditions and with preheating of reagent vapors. Hexaethylcyclotrisiloxane gave values of 1.40, 3.08 and 3.10 %C at 230, 300 and 350°C, while the values for hexamethylcyclotrisilazane were 3.20, 3.00 and 2.95 %C under similar and respective circumstances.

The fact that the silazane, in contrast to the siloxane, did not show a pronounced temperature dependence in that range, is simply a consequence of the much higher reactivity of the Si–N *vs.* the Si–O bond: the lowest temperature was already high enough to saturate the available surface. As a rule-of-thumb for the more extensively investigated cyclosiloxanes, the optimum temperature is generally in the range of 300 to 350°C.

Nitrogen flow. The flow of the carrier gas nitrogen serves to transport cyclic reagent to, and to remove unreacted reagent plus side products from, the silica gel. When the flow is large, it can also serve to fluidize the silica gel bed and thereby remove any spatial discrimination (as may occur in a fixed bed depending on whether a particular particle is situated at the entrance or the exit of the reagent gas stream, and in what position/contact it finds itself in regard to its neighbors).

Almost all of the fifty-odd experiments that were carried out needed between 3 and 5 h to come to "completion", *i.e.* to reach the stage where all of the cyclic reagent had evaporated from the round-bottom flask. However, this overall reaction time says little about the actual contact time between silica gel and cyclic reagent. Clearly, the faster the flow under otherwise similar conditions, the shorter the contact time. Of particular relevance here is the very fast flow necessary to fluidize the solid bed. Thus, experiments carried out under these conditions, some thirty-odd runs, produced in general lower carbon contents. However, these runs often (particularly with phases

made from octamethylcyclotetrasiloxane) lead to somewhat better-looking chromatograms (less tailing, sharper peaks). It is not immediately obvious whether this was caused primarily by spatial factors (the fluidized *vs.* the fixed bed) or by surface chemistry (the type and frequency of retention centers, removal of reaction products, etc.) or both. For instance, one could argue that the improved access to all surface areas, which is characteristic of the fluidized bed, allowed a more uniform coating and favored the reaction of the most active types of silanols (*c.f.* ref. 13), thereby creating, in terms of activity and space, a chromatographically more homogeneous surface.

GC retention

It is reasonable to expect carbon content and chromatographic retention to correlate in some way. Whether retention increases or decreases with carbon content depends generally on the chemical nature of the support and the liquid phase (or bonded layer), on the amount of liquid phase present, and on the chemical nature of the solute probe. Most often, one speaks of gas–liquid chromatography when retention increases, and of gas–solid chromatography when retention decreases, with an increase in carbon load.

In this study, carbon loads varied from very small values to about 3% for the methyl phases, 5% for the ethyl phase and 9% for the phenyl phase (no deliberate attempts were made to drive carbon contents to their upper limits). It is instructive to calculate the nominal layer thickness and the molar coverage that corresponds to the largest values. If, purely for purpose of estimate, the organic material is assumed (1) to cover evenly the total BET surface (which is treated as flat), (2) to have unit density, and (3) to consist of silyl units (*i.e.* R_2Si), then the nominal layer thickness d is given in Ångstroms by

$$d = \frac{\%\text{org} \times 10^4}{(100 - \%\text{org}) \times S} \text{ Å}$$

where %org is percent organic matter, calculated for silyl units from %C data without correction for blank values, and S is the BET surface of silica gel 62 (about 300 m^2/g with nitrogen). The estimate considers the pore radius large enough to be neglected at low loadings. Similarly, the micromoles of silyl groups per square meter of support can be estimated by

$$\alpha_{\text{exp}} = \frac{\%\text{org} \times 10^6}{\text{MW}_{\text{org}} \times S \times (100 - \%\text{org})} \; \mu\text{mol/m}^2$$

where MW_{org} is the gram molecular weight of the silyl unit R_2Si.

When this is done, 3% carbon from dimethylsilyl translates to about 2.6 Å and 4.5 $\mu mol/m^2$, 5% carbon from diethylsilyl to 3.3 Å and 3.8 $\mu mol/m^2$, and 9% carbon from diphenylsilyl to 4.3 Å and 2.4 $\mu mol/m^2$.

In comparison, a liquid phase synthesis in refluxing hexadecane yielded a value of 3.1 Å for diphenylsilyl from diphenyldiethoxysilane[14]. Values of that magnitude are quite comparable to literature values of good bonded phases, particularly when the correlation of pore size and nominal layer thickness, and the different calculation methods employed by different researchers, are taken into account[14].

Gas chromatographically speaking, maximum nominal thicknesses for the organic layer between 2 and 5 Å suggest that the materials best be designated modified solids, not bonded liquids (if such a distinction, indeed, makes much sense under the circumstances).

As an aside, this does not mean that systems could not be found where similar reactions would lead, directly or indirectly, to what might be considered typical "bonded liquids". For instance, it appears possible to "bond" silicones to the surface of Chromosorb by a simple heat treatment[15], a reaction that found some application in the commercial production of capillary columns. It is well known that thermal degradation of silicones leads to cyclics and it is tempting to speculate that it are these cyclics that are involved in some of the necessary surface bonding/crosslinking that stabilizes the liquid layer and makes it resistant to extraction. Cyclics have also been used directly for deactivating quartz capillaries[16].

To return to the problem at hand: if the obtained phases are to be characterized as "modified solids", retention should decrease with carbon content. That this is indeed so is shown in Fig. 2 for all retention data obtained for hexylbenzene on silica gel modified with octamethylcyclotetrasiloxane (the most frequently used cyclic). It should be borne in mind that this graph combines data from materials obtained in different vessels, at different reaction temperatures, at different flow-rates, etc. Given this fact, the correlation with carbon content appears as good as can be expected. Clearly, retention is based, exclusively or predominantly, on inorganic (not organic) surface features. Similar dependencies are found for all other test compounds.

The star in Fig. 2 marks a phase synthesized in a solid–liquid system, *i.e.* by using octamethylcyclotetrasiloxane in refluxing *n*-hexadecane[1]. Retention appears slightly higher but is still considered within experimental variability. A similar situation was found with materials derived from hexaethylcyclotrisiloxane. It also appeared that, for equal carbon load, the peak-to-peak differences in retention temperatures (*e.g.* between adjacent *n*-alkanes) were slightly larger for the solid–liquid than for the solid–gas phase syntheses. However, the differences could have conceivably arisen from experimental variability, and a more detailed and closer controlled study of this minor aspect was not considered necessary in the present context.

A comparison of the retention data for all synthesized materials showed that the polarity of the retention medium, as expressed in the difference of retention temperatures between alkanes on one hand and alkenes or alkylbenzenes on the other,

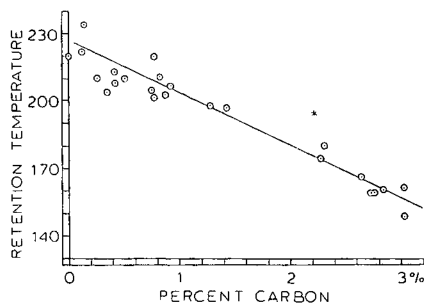

Fig. 2. Retention temperature of *n*-hexylbenzene in a standard temperature program on various octamethylcyclotetrasiloxane-modified silica gels *vs.* their carbon contents. The star denotes a similar phase synthesized in refluxing hexadecane[1].

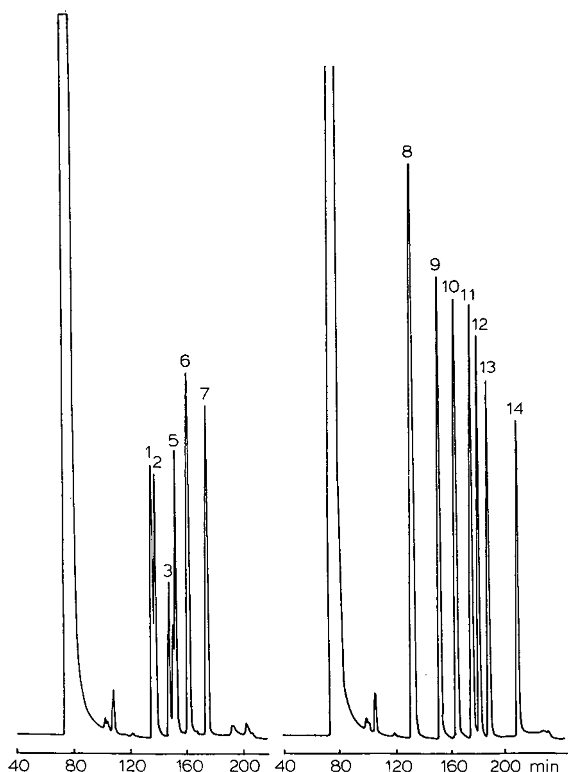

Fig. 3. Temperature-programmed GC of mixtures of olefins and alkylbenzenes on silica gel 62, 60–80 mesh, modified by hexaethylcyclotrisiloxane at 350°C in a fluidized bed. Compounds: 1 = *trans*-2-heptene, 2 = *cis*-2-heptene, 3 = 1-octene, 4 = *trans*-2-octene, 5 = *cis*-2-octene, 6 = 1-nonene, 7 = 1-decene; 8 = benzene, 9 = toluene, 10 = ethylbenzene, 11 = *n*-propylbenzene, 12 = mesitylene, 13 = *n*-butylbenzene, 14 = *n*-hexylbenzene.

did not vary to a great extent. This is to be expected if the silica gel surface (as opposed to the modifying groups) provides most or all of the retention.

The comparison of liquid- and gas-phase syntheses thus shows little differences in the retention of solutes of low polarity, and the peaks are of similar width and symmetry. On the other hand, highly polar, hydrogen-donor type materials such as alcohols had better peak shapes on the materials derived from a liquid-phase synthesis and hence were not used for testing purposes in this study.

Two sample chromatograms, of alkenes and alkylbenzenes on a silica gel modified in fluidized bed with hexaethylcyclotrisiloxane at 300°C (3.1% C), are shown in Fig. 3. They are comparable to chromatograms from a corresponding material (*i.e.* same cyclic, similar load) synthesized in boiling hexadecane, except that the latter did not resolve *n*-propylbenzene and mesitylene.

Taking into account all synthesized materials, which represent a wide range of reaction conditions, the following conclusions can be drawn: (1) It is possible to produce modified silica gel surfaces of chromatographically acceptable quality from organocyclosiloxanes and -silazanes in a gas-phase type synthesis. (2) Conditions for

the synthesis of these materials are not overly critical: most products of this study yielded reasonably looking chromatograms. (3) However, while the materials obtained by gas-phase synthesis have often come close to, and sometimes matched, those obtained by liquid-phase synthesis, they have never clearly surpassed the latter in terms of general GC performance.

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Note

Effect of the composition of coordinated β-diketonato ligands on R_F values of trasition metal complexes obtained by thin-layer chromatography on silica gel

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The effect of the composition of coordinated β-diketonato ligands on R_F values of transition metal complexes, obtained by thin-layer chromatography (TLC) on silica gel, has already been investigated.

Dilli and Robards[1] have undertaken an examination of a number of aryl β-diketones and their chelates in an effort to highlight the effect of certain structural factors during TLC and to compare these with the known gas chromatographic (GC) behaviour. For TLC, air-dried silica gel plates were used and the development was performed by means of solvent systems benzene and benzene–methanol (92:8, v/v). On the basis of the results obtained, the authors concluded that "the trend in R_F values varied considerably for the different metal ions and a parallel between the TLC an GC behaviour of the chelates is immediately apparent".

Timerbaev and Petrukin[2] performed chromatographic separations by using only two single component solvent systems (chloroform and diisopropyl ether) keeping a constant humidity in the cylinder in the course of development. Thereby they established that the R_F values of the complexes investigated increase in the following order of the ligands*: acac $<$ bzac $<$ dibzac $<$ ttfac $<$ tfac $<$ hfac. These results were ascribed to the weakening of the strength of adsorption of the complexes to the adsorbent, caused by decreasing oxygen atom electron density. This is due to the effect of substituents attached to the 2,4-pentanedionato ligand.

However, in our earlier investigations on the effect of the composition and structure of metal complexes on their R_F values[3–5] we have established that the behaviour of complexes may be quite different when, instead of single component solvents, polycomponent solvent systems are used. The reason is that in the latter case, in addition to the adsorption mechanism, a partition separation mechanism may also be involved. This aspect is not well defined in the previously mentioned papers[1,2]. In the first study the authors applied only one single component solvent system and air-dried silica gel plates, which means that their silica gel contained adsorbed water[6].

* acac = 2,4-Pentanedionato ion; bzac = 1-phenyl-1,3-butanedionato ion; dibzac = 1,3-diphenyl-1,3-propanedionato ion; tfac = 1,1,1-trifluoro-2,4-pentanedionato ion; tfacbzac = 1-phenyl-4,4,4-trifluoro-1,3-butanedionato ion; hfac = 1,1,1,6,6,6-hexafluoro-2,4-pentanedionato ion; ttfac = 1-(2-thienyl)-4,4,4-trifluoro-1,3-butanedionato ion.

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In the second study the authors performed chromatographic separations with single component solvent systems in an humid atmosphere. That is why we wanted to investigate the aforementioned effect with a greater number of transition metal complexes, using dry single component solvent systems and silica gel. In addition, we wanted to establish whether the previously found regularities hold also when polycomponent solvent systems are applied.

EXPERIMENTAL

The complexes investigated were synthesized according to procedures reported in the literature (Table I). Chromatographic separations on thin layers of silica gel (DG; Riedel, Hannover, F.R.G.), as well as the drying of the solvent used, are described in our previous papers[3,7].

RESULTS AND DISCUSSION

As seen from Tables I and II, eighteen complexes of Co(III), Cr(III), Ru(III) and Rh(III) of the non-electrolyte type, containing some of the ligands acac, bzac, dibzac,

TABLE I

R_F VALUES OF COMPLEXES OBTAINED BY TLC ON SILICA GEL USING SINGLE COMPONENT SOLVENT SYSTEMS

No.	*Complex*	*Ref.*	$R_F \cdot 100$*							
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	[Co(acac)$_3$]	8	1	23	2	7	2	10	74	2
2	[Co(bzac)$_3$]**	9	13	46	4	13	13	30	87	9
			–	–	–	–	–	51	–	–
3	[Co(dibzac)$_3$]	9	64	83	33	–	64	86	90	55
4	[Co(tfac)$_3$]**	10	68	84	36	36	68	88	96	84
			78	–	41	50	78	–	–	–
5	[Co(tfacbzac)$_3$]	11	87	85	78	70	88	90	–	93
6	[Co(hfac)$_3$]	12	–	96	–	–	–	99	98	98
7	[Cr(acac)$_3$]	13	6	21	3	9	8	16	75	3
8	[Cr(bzac)$_3$]**	9	17	55	11	14	17	39	87	3
			28	67	25	49	28	64	–	–
9	[Cr(dibzac)$_3$]	9	75	89	48	75	76	92	90	67
10	[Cr(tfac)$_3$]**	10	77	94	48	–	78	–	92	67
			85	–	–	–	86	–	–	81
11	[Cr(tfacbzac)$_3$]	11	93	95	88	–	93	–	94	67
12	[Cr(hfac)$_3$]	14	–	96	–	–	–	99	98	89
13	[Ru(acac)$_3$]	15	6	5	–	–	–	11	–	4
14	[Ru(tfac)$_3$]	12	81	82	–	–	–	90	–	53
15	[Ru(hfac)$_3$]	12	86	88	–	–	–	93	–	87
16	[Rh(acac)$_3$]	16	3	13	–	–	–	5	–	1
17	[Rh(bzac)$_3$]**	9	14	15	–	–	–	11	–	10
			28	30	–	–	–	40	–	22
18	[Rh(tfac)$_3$]	10	78	72	–	–	–	88	–	68

* The compositions of the solvent systems are given in Table III.
** The R_F values correspond to mixture of the facial and meridional isomers.

TABLE II

R_F VALUES OF COMPLEXES OBTAINED BY TLC ON SILICA GEL USING POLYCOMPONENT SOLVENT SYSTEMS

No.	Complex	$R_F \cdot 100$*							
		9	10	11	12	13	14	15	16
1	[Co(acac)$_3$]	5	10	6	3	27	25	21	92
2	[Co(bzac)$_3$]**	14	22	20	5	38	26	32	64***
		26	–	36	–	59	–	46	–
3	[Co(dibzac)$_3$]	75	61	86	6	93	49	88	0
4	[Co(tfac)$_3$]**	81	79	83	8	94	61	89	56***
		–	83	–	–	–	73	–	–
5	[Co(tfacbzac)$_3$]	88	90	–	16	95	89	–	–
6	[Co(hfac)$_3$]	89	96	95	17	96	–	94	–
7	[Cr(acac)$_3$]	11	15	16	3	32	5	28	89
8	[Cr(bzac)$_3$]**	13	45	31	5	45	13	34	52***
		30	63	54	–	69	34	52	–
9	[Cr(dibzac)$_3$]	71	77	87	13	92	59	85	0
10	[Cr(tfac)$_3$]**	79	80	87	13	92	62	85	50***
		–	–	–	–	–	75	–	–
11	[Cr(tfacbzac)$_3$]	84	91	97	20	–	96	93	42***
12	[Cr(hfac)$_3$]	93	95	95	21	91	–	96	–
13	[Ru(acac)$_3$]	2	32	10	–	29	3	–	–
14	[Ru(tfac)$_3$]	89	82	86	–	92	73	–	–
15	[Ru(hfac)$_3$]	95	–	–	–	–	90	–	–
16	[Rh(acac)$_3$]	2	31	14	–	29	2	–	–
17	[Rh(bzac)$_3$]	7	44	30	–	49	7	–	–
		20	55	49	–	63	–	–	–
18	[Rh(tfac)$_3$]	85	80	82	–	87	21	–	–

* The compositions of the solvent systems are given in Table III.

** The R_F values correspond to mixture of the facial and meridional isomers.

*** Small degree of fronting.

tfac, hfac or tfacbzac, were chromatographed. The compositions of the eight single component and eight polycomponent solvent systems used are presented in Table III.

From Table I it is seen that in all dry single component solvent systems applied the R_F values increased in the same order of ligands as established by Timerbaev and Petrukin[2], who had carried out chromatographic separations with two single component solvent systems but in an humid atmosphere. The same order of complexes was obtained with all polycomponent solvent systems used, except when acetone–water (60:40, v/v) was used (Table II).

On the basis of our results, obtained by the application of dry single component solvent systems and silica gel, it may be concluded that in all cases investigated the complexes are separated by an adsorption mechanism. As to the mechanism operating during the chromatographic development of the complexes by polycomponent solvent systems on dry silicagel, or by single component solvent systems on silica gel containing adsorbed water, nothing can be said with certainty. The reason is that in this case both adsorption and partition are possible. This is in accordance with the fact that the order of the complexes when seven multicomponent solvent systems with

TABLE III
SOLVENT SYSTEMS USED

No.	*Composition*	*Proportion (v/v)*	*Time of development (min)*
1	Benzene		7
2	Chloroform		7
3	1,2-Dichlorobenzene		17
4	*n*-Hexane		14
5	Carbon tetrachloride		15
6	Dichloromethane		6
7	Ethyl acetate		8
8	Toluene		8
9	Benzene–dichloromethane	50:50	5
10	Chloroform–carbon tetrachloride	70:30	12
11	Dichloromethane–toluene	70:30	8
12	*n*-Hexane–carbon tetrachloride	20:80	15
13	Chloroform–dichloromethane	70:30	10
14	Benzene–toluene	50:50	10
15	Chloroform–benzene–dichloromethane	60:20:20	10
16	Acetone–water	60:40	20

small dielectric constants are used is the same as that obtained with single component solvent systems. Contrary to this, using the polycomponent solvent system 16, which contains, besides acetone, 40% (v/v) of water, the above mentioned order disappeared (see Table II). In fact, in the former case, substituents with a more negative inductive effect decrease the electron density on the ligator (oxygen atom), which causes a weakening of the hydrogen bonding to the silanol groups of silica gel, and an increased R_F value.

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Note

Gas chromatography of organic compounds using inorganic salts as components of the stationary liquid phase and steam as a carrier gas*

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In gas chromatography (GC) the separation of complex mixtures of organic compounds is largely governed by the choice of the selective stationary liquid phase (SLP). Therefore, although the total number of commercially available SLPs used in GC exceeds several hundred[1], the search for new types is continuing.

Previously we have shown the possibility of using a wide range of inorganic salts as components of SLPs stable in the flow of a carrier gas containing steam[2–6]. Of interest is the use of crystal hydrates of inorganic salts, the melting point of which is lower than the temperature at which separation takes place[2,5,6].

When using pure steam as a carrier gas at temperatures above 100°C, not merely the crystal hydrates are in a liquid state but also other salts [6,7].

Poole *et al.*[8] showed that the use of hydrated melts, such as calcium nitrate tetrahydrate, either prevents elution of compounds more polar than halobenzenes at the maximum operating temperature or leads to long retention times and unacceptable peak shape. As the chromatographic process involves the use of an inert gas as the carrier gas, this behaviour is connected with the loss of water by the salt. Phifer and Plummer[9] pointed out the extraordinary selectivity of water as an SLP, which causes the elution order of C_1–C_5 alcohols to be governed by the polarity of the alcohols rather than by their molecular masses. However, they also indicated the instability of water as an SLP. On the other hand, the use of inorganic salts, as adsorbents modified with water vapours does not result in the selectivity usual for water[10].

It is of interest to study the chromatographic characteristics of sorbents containing inorganic salts, *e.g.*, alkali metal salts, capable of stabilizing water in equilibrium with aqueous vapours as a mobile phase at temperatures above 100°C.

EXPERIMENTAL

Use was made of a chromatographic system securing a constant steam content in the mobile phase (Fig. 1) and similar to those used earlier[6]. This system was used in combination with a gas chromatograph LHM-8MD (Khromatograf" works, Moscow, U.S.S.R.), equipped with a flame ionization detector.

* Dedicated to Dr. E. Heftmann on the occasion of his 70th birthday.

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TABLE I

RELATIVE RETENTION VOLUMES OF *n*-ALCOHOLS (STANDARD, PENTANOL) AND QUANTITY OF WATER ABSORBED (P IS % WATER RELATIVELY TO SALT WEIGHT) FOR THE SORBENT WITH ALKALI METAL SALTS

Temperature = 110°C; pressure at the column entrance is close to the saturated water vapour pressure; the inlet pressure is atmospheric.

Alcohol	*LiCl*	*NaCl*	$NaNO_2$	$LiNO_3$	$NaNO_3$	*KCl*	KNO_3	Na_2CO_3	Na_2SO_4	$NaHCO_3$	Na_3PO_4
Methanol	12.5	7.6	7.5	5.5	4.6	4.45	3.6	0.45	0.15	1.7	0.08
Ethanol	5.7	5.1	3.2	2.9	2.1	2.2	1.8	0.31	0.22	0.8	0.18
Propanol	3.1	3.1	1.7	1.8	1.25	1.25	1.1	0.48	0.45	1.05	0.11
Butanol	1.9	1.7	1.3	1.2	1.0	1.05	1.0	0.65	0.64	1.15	0.48
P_{H_2O} (%)	379	210	210	215	169	130	86	50	6	–	–

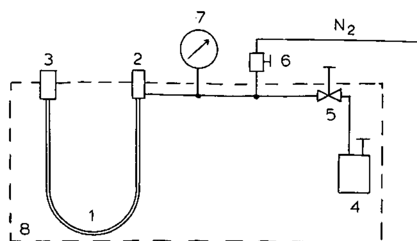


Fig. 1. Gas chromatograph for use with water vapour as the mobile phase. 1 = Column; 2 = sample injector; 3 = flame ionization detector; 4 = water vessel; 5 = fine adjustment valve; 6 = stop valve; 7 = pressure gauge; 8 = thermostat.

The solid support was Celite C-22 (60–80 mesh) (Ferak, Berlin) or Inerton N-AW (Lachema, Brno, Czechoslovakia), 0.125–0.16 mm. The sorbent was prepared by deposition of an inorganic salt from its aqueous or ethanol solution in amounts up to 20% (w/w) of the support. The column dimensions were 1–2 m × 2–3 mm I.D. The column temperatures were 110 and 120°C. Water vapour was used as the carrier gas; water consumption rate 3 ml/h. The quantity of water sorbed by the salt was measured by weighing the column after equilibration in the water–steam flow.

RESULTS AND DISCUSSION

A direct relationship was found between the ability of the salt to absorb water and its selectivity (Table I). The ability of salts to retain water and correspondingly to elute alcohols depended on the nature of both the anion and the cation. Thus, the selectivity of sodium salts with respect to alcohols declined in the series NaCl, $NaNO_3$, $NaHCO_3$, Na_2CO_3, Na_2SO_4, Na_3PO_4. Sodium salts had a greater capacity for inversion than potassium salts and a lower capacity than lithium salts. The selectivity decrease is revealed first in a transition from an inversed order of alcohol elution (from pentanol to methanol, columns with LiCl, NaCl, $NaNO_2$, $LiNO_3$, $NaNO_3$, KCl, KNO_3), through partial inversial (on $NaHCO_3$) ethanol first, methanol last, C_3–C_4 alcohols intermediate; on Na_2CO_3, ethanol first, methanol second, followed by C_3–C_4 alcohols to a "normal"order (from methanol to pentanol on columns with Na_2SO_4 and Na_3PO_4). This behaviour is connected with the water content of the sorbents and accordingly with the transition from gas–liquid to adsorption chromatography. Secondly the decrease in selectivity leads to a diminishing distance between the peaks of C_1–C_5 alcohols with decreasing water content in the sorbents. The alcohol peaks were symmetrical for all sorbents during chromatography with steam.

It is interesting that the ability of salts to retain water during the chromatography is not correlated with their ability to form salt crystal hydrates under normal conditions. For example (as is shown in Table I), sodium chloride absorbs much more water than sodium sulphate capable of forming a decahydrate, or sodium phosphate which can form a dodecahydrate. In addition to alkali metal salts, nitrates of other metals, such as aluminium, magnesium and zinc can selectively retain acohols[6].

The dependence of the retention of alcohols on the carbon number is shown in Fig. 2. For sodiumsulphate (curve 1) this reflects the increasing retention in

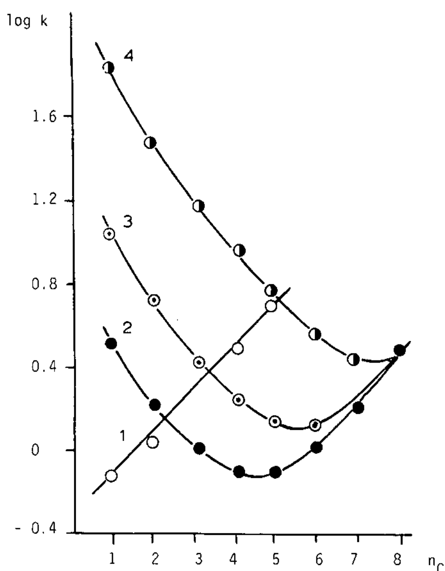


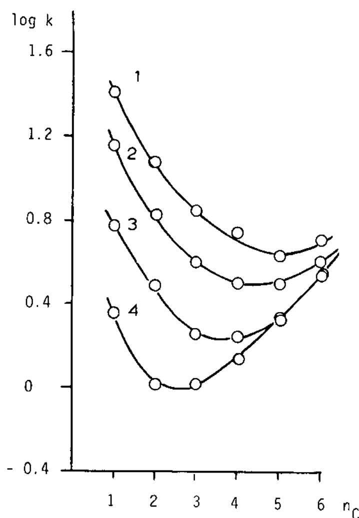
Fig. 2. Logarithmic dependence of the capacity factors (log k) of n-alkanols on carbon number, n_c, for the sorbents with alkali metal salts; 110°C, inlet pressure about 1.4 atm. 1, 20% Na_2SO_4 on Celite C-22; 2, 10% KCl on Inerton N-AW; 3, 20% NaCl on Celite C-22; 4, 20% LiCl on Celite C-22.

homologous series subject to adsorption chromatography. The different behaviour of chloride salt is manifested in curves 2–4. This may be explained by a lower influence of adsorption on the interfaces of the SLP. The left branches of curves 2–4 reflect an increase in solubility with increasing polarity of the compounds chromatographed. This is in accordance with the data of Chatterjee *et al.*[11] which showed for a water layer on a solid support that with increasing compound polarity the solubility increases but adsorption decreases

As is shown in Fig. 2 for the sorbents containing potassium chloride, sodium chloride and lithium chloride at 110°C, the first compounds eluted are pentanol, hexanol and heptanol respectively. On the column with lithium chloride the C_2–C_9 alcohols are eluted before methanol. So some salts reveal the same or greater selectivity towards polar compounds than does pure water as an SLP.

The ability to retain water and accordingly the selectivity towards alcohols decreases at decreasing water vapour pressure at constant temperature (Table II) and at increasing temperature (Fig. 3).

Fig. 4 shows an example of the analysis of a water layer containing the reaction products of catalytic hydrolysis on a sorbent coated with lithium chloride. This column exhibits the following possibilities. (1) Analysis of pollutants in methanol, since before methanol, compounds both with lower and with higher boiling points are eluted: C_2–C_9 n- and *iso*-alcohols, ketones, ethers, ester, C_1–C_{17} hydrocarbons. Relative retention volumes are 1.36 for acetone and 0.39 for pentanone-2, 0.29 for ethyl formate and 0.06 for isoamyl acetate (the standard n-pentanol). (2) Analysis of organic pollutants in ethanol, since C_3–C_9 n- and *iso*-alcohols and other pollutants are eluted before ethanol and what's more the number of them less than at analysis on impurities in



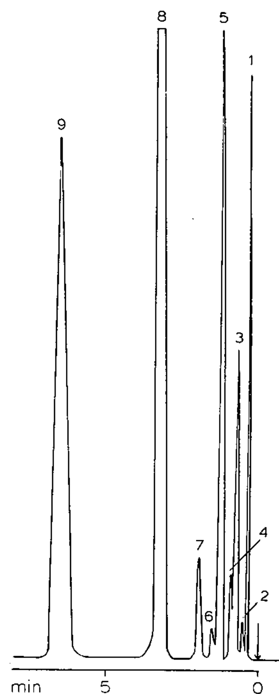


Fig. 3. Logarithmic dependence of the capacity factors of *n*-alkanols (log *k*) on carbon number, n_c, at different temperatures, inlet pressure close to the saturated water vapour pressure. Temperatures: 1, 107°C; 2, 110°C; 3, 114°C; 4, 127°C.

Fig. 4. Chromatogram of polar organic compounds contained in the water layer of hydrolysis reaction products. Column: 190 cm × 2 mm I.D. Sorbent: 7% lithium chloride on Inerton N-AW (0.125–0.16 mm). Temperature: 119°C. Inlet pressure: about 1.9 atm. Carrier gas: water vapour. Peaks: 1 = diethyl ether; 2 = ethyl acetate; 3 = methyl propyl ketone; 4 = methyl ethyl ketone; 5 = acetone and *n*-butanol; 6 = isobutanol; 7 = *n*-propanol; 8 = ethanol; 9 = methanol.

methanol. (3) Selectivity regulation by a small alteration in column temperature; when decreasing the temperature from 119 to 116°C in the analysis of reaction products, the peaks of fourteen rather than nine compounds were revealed. (4) Analysis of aqueous solutions as water is the basis of steam and the SLP. (5) High efficiency: the height

TABLE II

CAPACITY FACTOR, *k*, AND RELATIVE VOLUME, V_{rel}, OF THE LITHIUM CHLORIDE UNDER DIFFERENT INLET PRESSURES, *P*, AT 110°C

10% lithium chloride on Celite C-22 (60–80 mesh)

Solute	*k*			
	P = 1.125 atm	*P = 1.225 atm*	*P = 1.562*	*P = 1.70 atm*
Methanol	24.57	25.19	28.60	33.27
Pentanol	3.07	3.09	2.78	2.68
V_{rel}	8.00	8.15	10.0	12.40

equivalent to a theoretical plate (HETP) on methanol is near 1 mm. (6) Symmetrical peaks, obviously the inorganic salt solution may decrease the adsorptive activity of the support. (7) Instead of lithium chloride other inexpensive inorganic salts and hydrates of salts can be used at temperatures from 105 to 150°C. Not only alkali metal salts but also other salts are useful. For example, the retention volumes of methanol relative to pentanol are: 7.4 for $Cu(NO_3)_2$, 7.2 for $Cd(NO_3)_2$, 3.5 for $Zn(NO_3)_2$, 2.25 for $CuSO_4$ and 1.65 for $CoCl_2$.

Sufficiently high efficiency can be obtained and under a high mobile phase flow-rate. So with increasing oven temperature up to 127°C, the mobile phase flow-rate through the packed column increased to 20 cm/s, *i.e.*, about five times as that deemed, as a rule, to be optimal for a packed column (3–4 cm/s). In this case, for the column of 2 mm I.D. with sodium chloride the HETP increases only to 2 mm for methanol, propanol and pentanol. This can be associated with a low molecular mass transfer rate.

Thus, for a selective GC separation of polar and non-polar organic compounds it appears practicable to use the system water–inorganic salts as an SLP with steam as a mobile phase. The use of inexpensive and accessible inorganic salts allows selective and stable resolution of complex organic mixtures which can be obtained as a rule only by use of high efficiency capillary columns.

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CHROM. 20 897

Note

Determination of amino acids on Merrifield resin by microwave hydrolysis

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(First received May 18th, 1988; revised manuscript received August 16th, 1988)

An accurate and convenient method for the amino acid analysis of proteins and peptides is becoming increasingly important with the recent developments in molecular biology and biotechnology. The classical method of Hirs *et al.*[1] utilizing 6 *M* hydrochloric acid at 110–120°C coupled with the ion-exchange separation of the hydrolysates of proteins using the method developed by Moore *et al.*[2] is still the most commonly used and unambiguous methodology for the amino acid analysis of most proteins and peptides. Amino acid analysis is of special importance for researchers carrying out routine peptide synthesis. The cleavage of single amino acids from Merrifield solid-phase resin and the determination of the first amino acid coupled to the chloromethylated resin are prime requirements for the determination of the exact amounts of blocked amino acids needed for later steps of solid-phase peptide synthesis. Westall and co-workers[3,4] employed a mixed-acid solvent [propionic acid–12 *M* hydrochloric acid (1:1)] at 130°C for 2 h for the hydrolysis of peptide fragments generated in solid-phase peptide synthesis.

In this paper we describe a novel and faster alternative to the conventional hydrolysis protocol using a rapid microwave oven-based technique[5] that yields a quantitative and reproducible recovery of amino acids from solid-phase resin in as little as 6–8 min. The technique has important implications for the total automation of the amino acid analysis of peptides and proteins starting from the pre-analysis sample preparation.

EXPERIMENTAL

Equipment

Custom-made thick-walled PTFE vials (6 mm thick) were fabricated locally according to the design shown in Fig. 1. Previous experience with Pyrex glass tubes commonly used for the hydrolysis of proteins with 6 *M* hydrochloric acid indicated that the high pressure and temperature readily induced in the sealed hydrolysis tubes by the microwaves easily caused explosion of the reaction tubes inside the microwave oven.

Each PTFE vial can contain up to 2 ml of solution and is equipped with a silicone-rubber septum and a cap made of the same Teflon material. For safety

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Fig. 1. PTFE vials and container shown inside a commercial microwave oven. The thicknesses of the two PTFE vials (right) and the container (left) are 6 and 5 mm, respectively. The container is 7.5 cm high with I.D. 7.5 cm and the vials are 5.5 cm high and with O.D. 3.0 and I.D. 1.8 cm. At the top of the small vial, a sealing septum of silicone-rubber was attached (not shown) for insertion of a needle to flush the vial with inert gas (*e.g.*, nitrogen) before the microwave heating. The container can contain 1–5 vials at one time. It is preferably placed at the same location to ensure constant and reproducible heating.

precautions, a large Teflon container with a thinner wall (5 mm thick, Fig. 1) was used to hold all hydrolysis vials during the microwave heating process. The microwave oven used was a commercially available cooking apparatus (compact Model MW3500XM, Whirlpool, MI, U.S.A.) without any modification. The total power of the microwaves was 1.2 kW. There is no temperature indicator on this model, hence the exact temperatures for different settings need to be measured separately using several compounds with known melting points[5].

Reagents and amino acid-attached resin samples

Propionic acid and concentrated hydrochloric acid (30%, Suprapur) for the preparation of the mixed-acid solvent were obtained from Merck (Darmstadt, F.R.G.). Propionic acid–12 *M* hydrochloric acid (1:1, v/v) in 1-ml vials can also be obtained from Pierce (Rockford, IL, U.S.A.). N-*tert*.-Butoxycarbonyl (N-t-Boc) amino acid resin esters for peptide synthesis by the Merrifield method were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals and buffers used for the amino acid analyser were of the highest grade commercially available.

Procedures

Hydrolyses of N-t-Boc amino acid resins were carried out in thick-walled Teflon vials as described above. Samples (5–7 mg) were dissolved in 0.2–0.8 ml of propionic acid–12 *M* hydrochloric acid mixed-acid solvent. The vials were covered with tight silicone-rubber septa and flushed with nitrogen through two small-sized needles (one for gas inlet and the other outlet). They were then screw-fastened with the Teflon caps and placed in a large Teflon container. The container was placed at the centre of the

bottom plate in the microwave oven (Fig. 1). We set the "microwave power" on the command pads to 70% of full power (*i.e.*, 0.84 kW) and varied the time from 1 to 7 min.

As the exact pressure and temperature induced inside commercial microwave ovens are difficult to measure, it is advisable to make a preliminary calibration of the microwave oven using a series of compounds with known melting points in order to find the approximate temperature ranges obtained at specified settings.

At the end of rapid heating (less than 10 min), the acid-digested mixtures were centrifuged on a microcentrifuge to remove the undigested resins and the supernatants were pipetted into acid-cleaned vials and evaporated to dryness using a Speed Vac Concentrator (Savant, U.S.A.) with a refrigerated condensation trap. The amino acid compositions of the digested samples were determined with an LKB-4150 amino acid analyser based on an ion-exchange method using a single-column system.

The samples were also hydrolysed at 140°C for 3 h by using a modified protocol of Westall and co-workers[3,4] with the same mixed-acid solvent. The amino acid resins were dissolved in 0.6 ml of propionic acid–12 *M* hydrochloric acid contained in Pierce re-usable vacuum hydrolysis tubes designed for a dry heating block. The sample tubes were freeze–thawed several times in liquid nitrogen, evacuated with an oil pump and sealed by screwing down the cap. The hydrolysis were conducted in a heating block set at 140°C for 3 h. Thereafter the sample analyses with the amino acid analyser were the same as those described for the microwave method. The data from the different heating procedures were compared with the reported values of millimoles of N-t-Boc-amino acid per gram of resin given by the manufacturer (Sigma).

RESULTS AND DISCUSSION

The conventional anaerobic hydrolyses of synthesized peptides and purified proteins with 6 *M* hydrochloric acid and 110°C for 24 h or more have been widely used for more than 30 years[1,2]. Although the conventional method is in general satisfactory for the purpose of obtaining amino acid contents of peptides and proteins, it is always time consuming for a skilled researcher to obtain accurate values for some labile amino acids such as serine, threonine, tyrosine, cysteine and methionine. Therefore, we have attempted to devise a novel approach of utilizing microwave technology for the rapid hydrolysis of peptide bonds with the aim of shortening the analysis time and obtaining data as reliable as those given by the conventional method[5]. We have extended the previous microwave heating protocol for the cleavage of N-t-Boc amino acid derivatives attached to Merrifield resins.

The designs of the PTFE vials and accessories for the purpose of conducting hydrolysis are shown in Fig. 1 together with the microwave oven. It should be emphasized that the high pressure and temperature developed under the microwave oven conditions often result in explosion of the sealed Pyrex tubes even with short heating times (less than 5 min). Therefore, the use of PTFE vials is strongly recommended. It is known that the nature of microwave heating precludes the conventional means of temperature determination. The preliminary calibration of the approximate temperature inside the microwave oven using thermocouples in conjunction with a digital thermometer failed owing to strong arcing. We therefore conducted a preliminary calibration of the temperature inside the microwave oven by use of several organic compounds with known melting points. The setting of 70% power and

7 min on the control pads corresponded to the temperature range between the melting points of semicarbazide hydrochloride (178°C) and *p*-anisic acid (186°C). The temperature of our microwave hydrolysis is tentatively concluded to be about 180°C. The pressure factor inside the hydrolysis vial remains to be determined. Nevertheless, reproducible data can always be obtained by seting the microwave at a specified "power" and "time".

Scotchler *et al.*[6] first improved the procedure of Merrifield[7] and Gutte and Merrifield[8] by substituting one protocol of propionic acid–hydrochloric acid hydrolysis for the two-step (first with anhydrous hydrofluoric acid–anisole treatment followed by hydrolysis with constant-boiling hydrochloric acid) removal of the resin and determination of amino acid residues in the initial stage of the solid-phase peptide synthesis. We performed experiments to optimize the conditions for the amino acid analysis of attached amino acid resin esters using the microwave method. In our experience, insufficient solvation of the Merrifield resin often adversely affected the analytical data owing to some resin samples being charred by the high temperature induced inside the microwave. Fig. 2 shows the effect of solvent volume on the recovery of alanine from the resin. Clearly the minimum volume needed for the complete hydrolysis of resin samples (5–7 mg) is about 0.6 ml. Hence 0.6 ml of the mixed-acid solvent [propionic acid–12 *M* hydrochloric acid (1:1, v/v)] were used for all hydrolyses of resin samples measured accurately with an analytical balance in the range 5–7 mg. N-t-Boc-alanine resin was also used to calibrate the hydrolysis time for the complete recovery of amino acids from the Merrifield resin. The recovery curve reached a plateau at times longer than 6 min (Fig. 3). We chose 7 min for safety reasons as heating times longer than 10 min at the specified power caused leakage of the reaction mixtures from the Teflon vials, probably due to the release of isobutylene gas generated from the butoxycarbonyl blocking group by acid hydrolysis.

Table I gives the results for 18 amino acid resin esters (histidine and tryptophan resin derivatives are not available from Sigma) obtained by the microwave technique using the above mixed-acid solvent with a pre-set power of 70% of full power and a heating time of 7 min. It is evident that this fast microwave heating yielded reproducible recoveries with a standard deviation of less than 5% for most samples. It

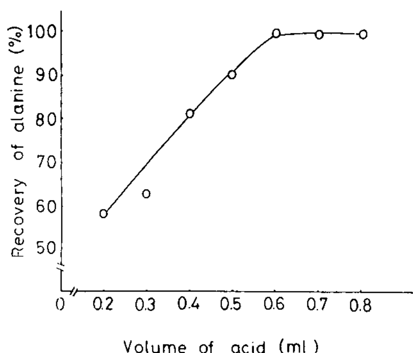

Fig. 2. Effect of solvent volume on the recovery of alanine from N-t-Boc-Ala resin for microwave hydrolysis. Resin samples of about 5–7 mg were used and different amounts of the mixed-acid solvent were added to the vials for microwave hydrolysis with a setting of 70% power for 7 min.

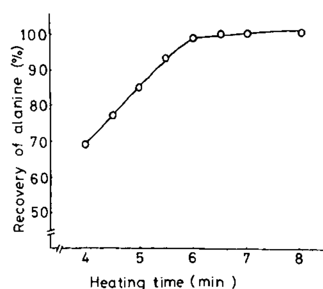

Fig. 3. Determination of heating time for the maximum recovery of alanine from N-t-Boc-Ala resin for microwave hydrolysis. Resin samples of 5–7 mg were used and 0.6 ml of the mixed-acid solvent were added to each vial for microwave hydrolysis with a setting of 70% power and different heating times.

compared favourably with those obtained by the conventional method[6] with the exception that our data for serine and threonine amino acid derivatives were much higher than those obtained by the old heating method. Using aerobic hydrolysis with propionic acid–hydrochloric acid, Westall and Hesser[4] claimed complete hydrolysis of peptides in 15 min at 150–160°C. In our experience with various peptides, application

TABLE I

RECOVERY OF AMINO ACIDS AFTER MICROWAVE HYDROLYSIS

*Boc-amino acid resin**	*Stated concentration (mmol/g resin)***	*Found concentration (mmol/g resin)****	*Recovery (%)*§
N-t-Boc-Ala	0.61	0.64 ± 0.03	103.0 ± 2.2
N-t-Boc-NO_2-Arg	0.17	0.10 ± 0.01	100.3 ± 3.1
N-t-Boc-Asn	0.40	0.33 ± 0.02	104.9 ± 0.9
N-t-Boc-Bzl-Asp	0.38	0.44 ± 0.02	99.4 ± 1.4
N-t-Boc-(S-Bzl)-Cys	0.29	0.33 ± 0.08	105.2 ± 1.7
N-t-Boc-Gln	0.35	0.41 ± 0.04	102.2 ± 3.2
N-t-Boc-Bzl-Glu	0.13	0.32 ± 0.06	102.1 ± 4.7
N-t-Boc-Gly	0.44	0.36 ± 0.03	102.4 ± 3.7
N-t-Boc-Ile	0.29	0.34 ± 0.05	117.2 ± 1.6
N-t-Boc-Leu	0.24	0.36 ± 0.03	97.6 ± 4.4
N-t-Boc-Met	0.27	0.50 ± 0.06	102.2 ± 5.1
N-t-Boc-Phe	0.70	0.78 ± 0.05	101.0 ± 4.6
N-t-Boc-Pro	0.38	0.44 ± 0.07	103.1 ± 2.5
N-t-Boc-(O-Bzl)-Ser	0.20	0.13 ± 0.04	343.7 ± 3.6
N-t-Boc-(O-Bzl)-Thr	0.37	0.30 ± 0.03	113.8 ± 2.7
N-t-Boc-Cbz-Lys	0.46	0.23 ± 0.02	103.8 ± 3.7
N-t-Boc-(O-Bzl)-Tyr	0.05	0.31 ± 0.04	107.0 ± 2.8
N-t-Boc-Val	0.13	0.40 ± 0.03	102.0 ± 3.6

* All N-t-Boc amino acid resin esters were obtained from Sigma, except that of alanine, which was synthesized in our laboratory. NO_2 = nitro; Bzl = benzyl; Cbz = chlorobenzyloxycarbonyl.

** Analytical data on the labels supplied by Sigma.

*** Data (mean ± S.D., n = 3) obtained by hydrolyses of the amino acid resin esters using propionic acid–12 M hydrochloric acid at 140°C for 3 h.

§ Recovery (mean ± S.D., n = 3) by the method of microwave heating with the mixed acid assuming the corresponding data in the previous column to represent 100%.

of the same solvent at 140°C for 3 h under anaerobic conditions is more suitable for achieving a higher yield and reproducible cleavage of peptides from the resin in the final step of solid-phase synthesis[9]. Therefore, we calculated the recovery data for the corresponding amino acid resin esters obtained by the microwave method on the basis of data obtained by hydrolysis at 140°C for 3 h. It was also shown previously[5] that microwave heating yielded much better recoveries of serine and threonine than those obtained by traditional hydrolysis at 110°C for 24 h. We believe that the more than 3-fold recovery of serine obtained here with microwave heating probably indicates a great improvement in the analysis of labile amino acids by this fast method over the previous methods.

The reason for the low concentrations of Boc-Glu, Boc-Tyr and Boc-Val in the resins reported by the manufacturer, in contrast to the much higher concentrations obtained by our analyses, is not clear at present. However, it is well known that determinations of the concentrations of amino acid resin esters often vary greatly among different manufacturers using different methods of analysis. It is noteworthy that the extra benzyl groups introduced in Glu and Tyr resin esters probably account for the low recoveries of these amino acid derivatives. The hydrophobic nature of valine in Boc-Val is also known to yield a low recovery in the regular amino acid analysis of proteins and peptides[10,11].

CONCLUSION

Microwave hydrolysis has been applied with success for the first time to the cleavage of amino acids from Merrifield solid-phase resins. The fast and accurate analysis of the first amino acid attached to the solid-phase resin is an essential step in peptide synthesis. The analysis time of the rate-determining step in the initial stage of solid-phase peptide synthesis can be shortened by a factor of 2–5 by using the mixed-acid solvent and microwave heating. Improvements in the design of commercial microwave ovens should prove valuable in the important application of amino acid analysis of peptides and proteins. Currently we are in the process of refining the microwave-heating step in order to interface it with the amino acid analyser for the on-line automation of protein hydrolysis and analysis.

ACKNOWLEDGEMENTS

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CHROM. 20 929

Note

Device to transform the liquid chromatographic effluent to a continuous solid plug: a new approach to direct liquid introduction for liquid chromatography–mass spectrometry*

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(First received March 1st, 1988; revised manuscript received August 23rd, 1988)

The effluent from a liquid chromatographic column is generally led into a detector and/or fraction collector. For flame photometric, mass spectrometric and other detection methods used for gas chromatography, the liquid effluent is changed into the vapour state. In the present paper we propose a process in which a liquid effluent is changed into a solid plug continuously.

In the direct liquid introduction interface (DLI) for liquid chromatography–mass spectrometry (LC–MS) it is essential to heat up a nozzle of a capillary tubing from which a liquid jet vapour is sprayed into a vacuum chamber[1–3]. A thermal supply at least equal to the amount of heat required for evaporation of the liquid is needed. Without such a thermal supply at the nozzle, the liquid becomes solidified at the tip of the DLI interface due to cooling of the liquid below its melting point.

Arpino *et al.*[4] observed that acetonitrile froze and appeared as a solid mass. We found that the solidification can be controlled by adjusting the thermal supply to the tip of the DLI interface. Thus, the eluent is solidified at the head of the capillary tubing, and the solid is continuously extruded into a vacuum chamber. We call the above process "solidified introduction" and have studied the basic phenomena involved under simulated LC–MS conditions. This process may be useful for sample storage or as an alternative method of DLI LC–MS.

EXPERIMENTAL

An experimental set up for observing the phenomena of solidified introduction under simulated LC–MS conditions is shown in Fig. 1. Instruments used were as follows: injector, Rheodyne Model 7520, 0.5 μl (Rheodyne, Cotati, CA, U.S.A.); fused-silica capillary tubing, 50 μm I.D. (S.G.E., Melbourne, Australia); open-tubular capillary tubing (Pyrex glass, 20–200 μm I.D.); video camera, Victor GX-N4CH (Japan Victor, Tokyo, Japan); microscope, Olympus BH-2 (Olympus Optical, Tokyo,

* Part of this paper was presented at the *4th Symposium on LC–MS and MS–MS, Montreux, October 22–24, 1986*. The majority of papers presented at this symposium has been published in *J. Chromatogr.*, Vol. 394, No. 1, 1987.

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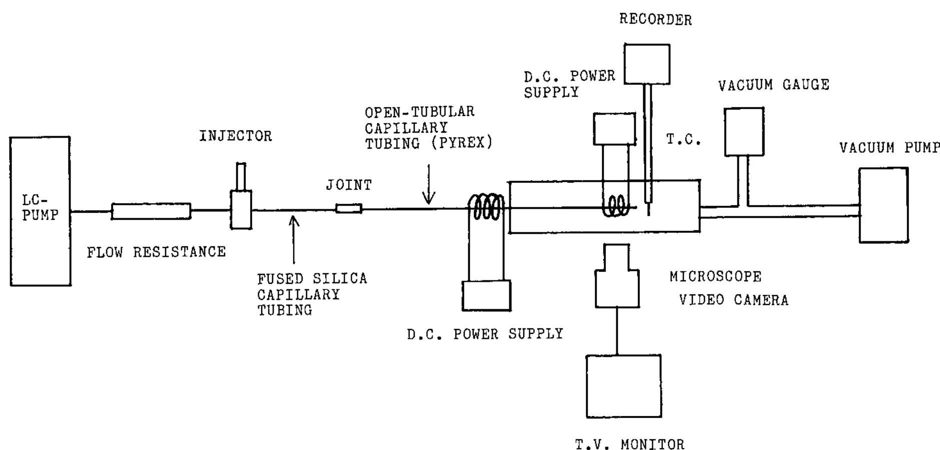

Fig. 1. Diagram of apparatus.

Japan); vacuum gauge, Macleod gauge (Okano, Osaka, Japan); rotary pump (Tokuda, Osaka, Japan) and thermocouple (alumel–chromel wire, 0.1 mm O.D.; The Japan Lamp Ind., Tokyo, Japan).

The extent of magnification obtained by means of the TV–microscope system was a factor of 118 on the 14-in. screen. A vacuum chamber, in which the head of the capillary tubing was set, was formed by a quartz tube (50 mm × 9 mm I.D. × 12 mm O.D.). Both ends of the quartz tube were sealed with silicone rubber. The pressure in the vacuum chamber was 0.01–0.3 Torr. A kanthal wire (1 mm O.D.; The Japan Lamp Ind.) with windings at the capillary head was used, as shown in Fig. 1. The temperature was adjusted by the application of a voltage from a d.c. power supply to the kanthal wire.

The inner diameters of open-tubular capillary tubings (Pyrex glass) used for the interface were between 20 and 200 μm. The glass capillary tubing was made by using a drawing machine (GDM-1; Shimadzu, Kyoto, Japan). Most experiments were done with 30–40 μm I.D. capillary tubing. For effective heating of the capillary head, it was necessary to keep an appropriate distance between the capillary tubing and the kanthal wire. For the above experimental conditions it was easy to use capillary tubing with larger outside diameters. Therefore, Pyrex glass capillary tubings were selected so as to produce capillary tubings with outside diameters such as 0.6 mm.

The flow-rate of the eluent was 0.3–8.0 μl/min. Coloured solutes, rhodamine 6G and 1-aminoanthraquinone, were used. Each was dissolved in the eluent used.

RESULTS AND DISCUSSION

The process of solidified introduction is shown in Fig. 2. An eluent is passed into a capillary tubing of the DLI interface at L_a, with a linear flow velocity, u, and a temperature at T_a (°C). The liquid is gradually cooled, and it starts to solidify and becomes a solid at L_s. Then, the solid is smoothly extruded into a vacuum chamber at L_o. The temperature of the solid at L_o is T^*. At L_s, the heat of solidification, Q_s, is evolved. Electrical heating, YQ_e, is supplied at the head of the capillary tubing, where

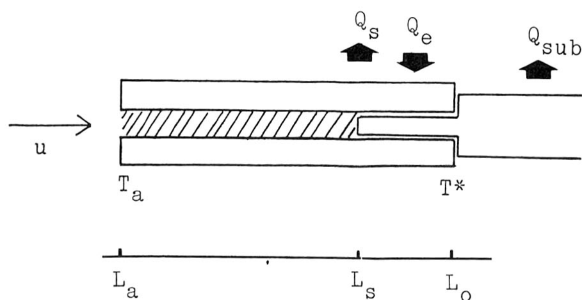

Fig. 2. The process of solidified introduction. See text for definitions.

Y is the efficiency believed to be around a few % or less. After the solid is extruded into the vacuum chamber, the heat of sublimation, Q_{sub}, is lost from the solid. The energy balance in the process of solidified introduction will be discussed elsewhere.

The liquid in the capillary tubing of the DLI interface is solidified, and then the solid is extruded into the vacuum chamber continuously. When the solute reaches L_s it is co-solidified with its solvent, and is carried into the vacuum chamber. These processes were observed by the present TV–microscope system.

The transfer of the solute by solidified introduction is shown in Fig. 3. The processes 1–5 are illustrated by sketches made from the TV screen. The experimental conditions were as follows: pressure of the vacuum chamber, 0.1 Torr; intensive heating at the head of capillary tubing (30 μm I.D.). Water, *ca.* 6 μl/min, and rhodamine 6G ($2 \cdot 10^{-3}$ *M* aqueous solution) were used as the eluent solute, respectively. The amount of rhodamine 6G injected was 0.5 μl.

The solid extruded into the vacuum chamber was at first colourless (1 in Fig. 3), and then started to show a faint red colour (2 in Fig. 3). The colour became brighter

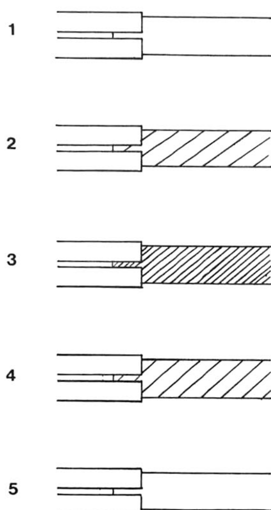

Fig. 3. The process of solute transfer. The solute and eluent were rhodamine 6G and water, respectively. The times for stages 1, 2, 3, 4 and 5 were 0, 6, 9, 15 and 18 s after injection, respectively.

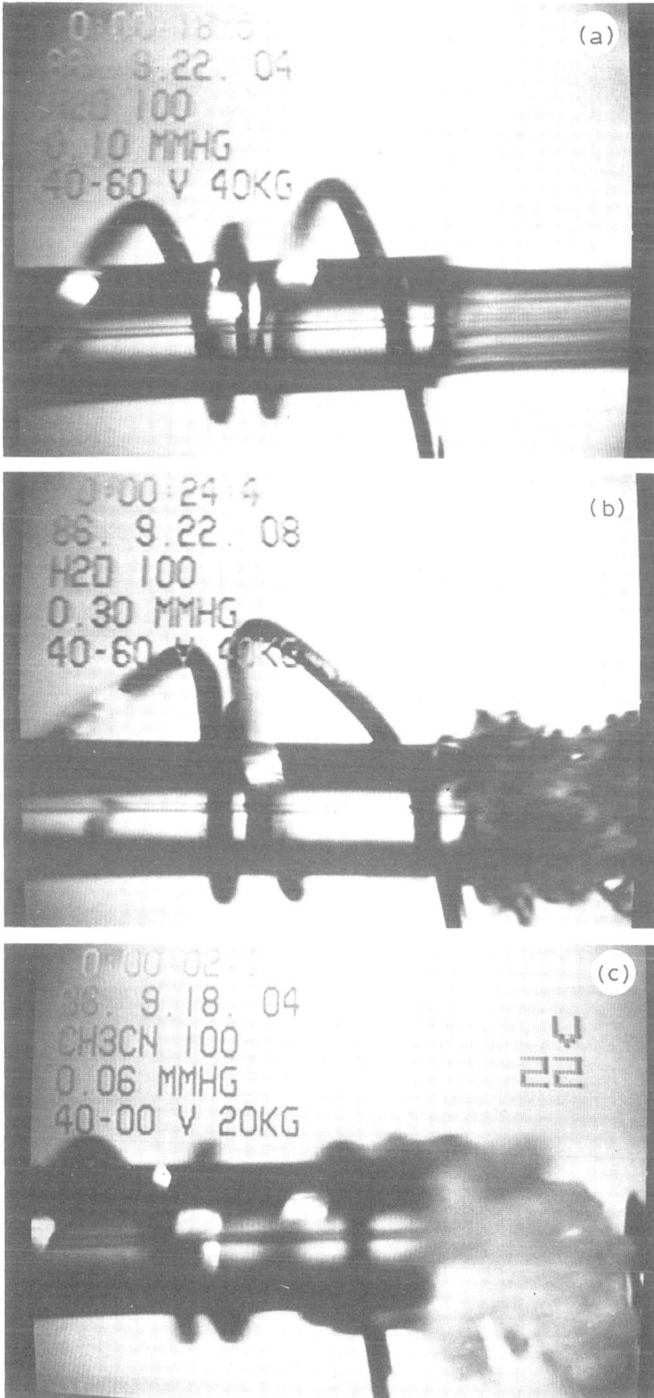

Fig. 4.

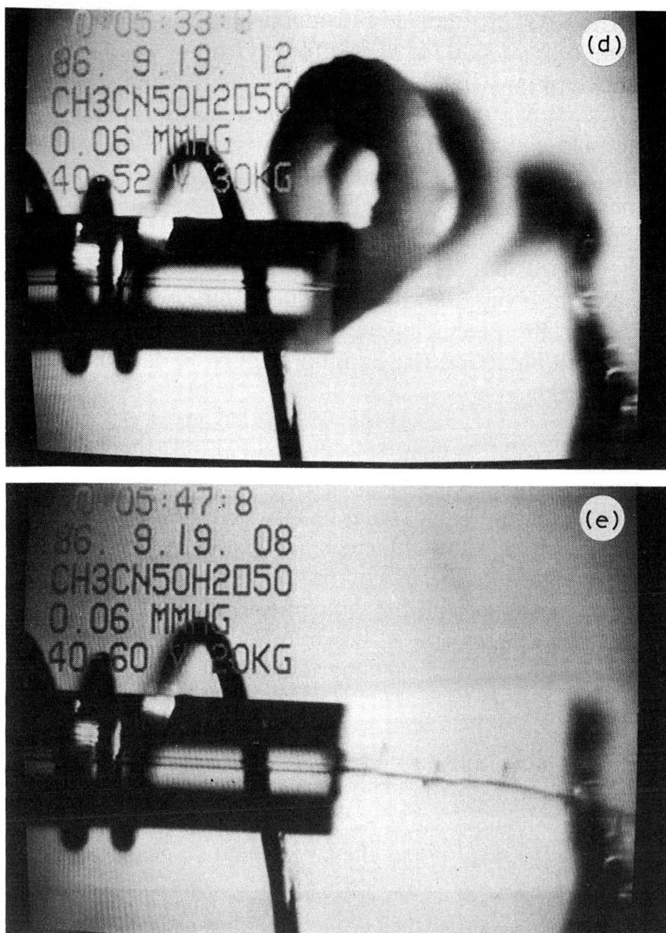

Fig. 4. Different types of patterns in solidified introduction. Each plate shows a glass capillary tubing, 30–40 μm I.D. and 0.5–0.6 mm O.D., a kanthal wire for heating and a solidified bar. In d and e, a thermocouple is seen at the right-hand side. Eluents: water (a and b); acetonitrile (c) and acetonitrile–water (50:50) (d and e). Pressures of the vacuum chamber: 0.10 (a), 0.30 (b) and 0.06 Torr (c, d and e). Other conditions as in the text.

(3 in Fig. 3) and then gradually faded; finally (5 in Fig. 3) the solid became colourless again. The duration of the red colour was about 10 s. Its band width was about 1.5 times that of the original one upon injection, which was simply estimated from the eluent flow-rate and the band width of the solute upon injection. In the present system, the solute in the eluent is smoothly solidified in a very small capillary tubing, and then extruded into the vacuum chamber without much band broadening. After the solidification at L_s, the phase surrounding the solid bar, which is in contact with the inner wall of the capillary tubing, may partly consist of liquid due to the electrical heating.

Five different types of patterns in solidified introduction are shown in Fig. 4. The flow-rates were 4–6 μl/min for a–d, and 0.5 μl/min for e. Heating was effected at the head of capillary tubing except in c. In a a smooth solidified bar appeared. In b and c, this bar had a rough surface. The surface of the solidified bar in d was smooth, but the bar was bent and formed a circular rod. As the flow-rate in e was only 0.5 μl/min, a very narrow solidified bar appeared. The outside diameters of the solidified bars in a–d were several times larger than the inner diameter of the capillary tubing. In the case of e, these two diameters were nearly equal due to the low eluent flow-rate. Usually a straight bar was obtained, but sometimes a bent one. Although it is not clear why the solidified bar becomes bent, this might be due to the surface condition of the capillary head and/or variations of temperature at the capillary head. The extrusion was continued up to 10 min. Cases a, b and e seem to be favourable for solidified introduction.

A thin thermocouple (T.C. in Fig. 1) was mounted just in front of the capillary head so as to be in contact with the solidified bar. It is also seen at the right in d and e in Fig. 4. The distance between the thermocouple and the head of the capillary (L_o in Fig. 2) was about 1 mm. The temperature of ice–water in a vacuum bottle was used as a reference in the thermocouple system. The solid which had just been extruded from the capillary head was contacted with the thermocouple. The output of the thermocouple was recorded with a potentiometer and the temperature of the solid bar thus calculated. A typical temperature measurement is shown in Fig. 5. The experimental conditions were as follows: eluent 6, μl/min; vacuum at capillary head, 0.06 Torr; inner diameter of capillary tubing, 30 μm.

In Fig. 5 a large negative peak with a flat shape and several sharp peaks are observed. These differences in thermocouple responses were due to the different durations of contact of the thermocouple with the solidified bar. The contact duration should be sufficient to enable the real temperature of the solidified bar to be recorded. This temperature, estimated from the response of the large negative peaks in Fig. 5,

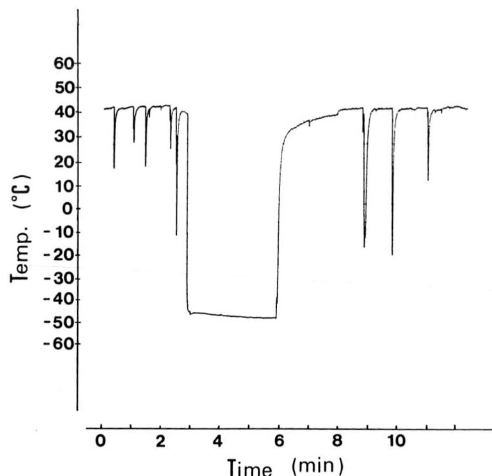

Fig. 5. Temperature of solidified bar, estimated from the output of the thermocouple. Time means the time of recording of the output. The peak widths correspond to the durations for which the solid bar was in contact with the thermocouple.

was about −47°C. Solid acetonitrile was cooled slightly below its melting point, −45.7°C at atmospheric pressure. The sharp peaks in Fig. 4 were recorded when the solid was contacted with the thermocouple for just a few seconds.

If strong heating was applied at the capillary head, the solidified bar melted and liquid was observed in the capillary tubing, which was sprayed into a vacuum room. This case is very similar to the behaviour of the DLI interface in LC–MS (DLI mode). It is possible to select solidified introduction or the DLI mode by adjusting the heating of the capillary head. Eluents such as water, acetonitrile, acetonitrile–water (95:5 or 50:50) and acetonitrile–0.1 *M* phosphate buffer (50:50, pH 7) were examined and found to be applicable for both modes. With pure water as the eluent, intensive heating was needed to change the solidified introduction to the DLI mode. The use of intensive heating means that the temperature of the kanthal wire itself was around 600°C. With acetonitrile or a mixture containing it, heating at the capillary head was moderate or, in some cases, not necessary for solidified introduction. Eluents containing methanol or ethanol were always in the liquid state (DLI mode) under the present experimental conditions. If the capillary interface is cooled with a coolant, these eluents may be solidified at the capillary head.

If the present process of solidified introduction is utilized for LC–MS, it may be more gentle to a solute than the ordinary process of DLI, because the latter needs at least a thermal supply equal to the heat of vaporization of the liquid used. In the process of solidification, the temperature of the eluent and solid bar is always decreasing toward that of the vacuum room. Therefore, the solidification process is milder to the solute. Thus the present system is suitable for thermally labile and high-molecular-mass substrates.

ACKNOWLEDGEMENT

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Note

Use of a simultaneous conductivity and amperometric detector with a manganin electrode in the microcolumn liquid chromatography of dicarboxylic acids

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The determination of aliphatic dicarboxylic acids by liquid chromatography is an important method for the analysis of biological samples. Owing to the complexity of the samples and the lack of a strong chromophore in the most common aliphatic dicarboxylic acids, the use of UV detection[1–5] suffers from interference problems and poor detection limits. Conductivity detection with post-column suppression[6–8] takes advantage of the ionic nature of acids. Selectivity for complexing acids can be achieved by amperometric[9] and potentiometric[10] detection on a copper working electrode. However, the use of the copper electrode has the disadvantage of a lower variability of pH and nature of the co-ion in the mobile phase, which decreases the possibility of optimizing acid separations. Further, the non-complexing dicarboxylic acids remain undetected. Simultaneous conductivity and amperometric detection can bring improvements, *e.g.*, the simultaneous detection of complexing and non-complexing inorganic anions was achieved by connecting two detectors in series[11].

Recently, the sensitive amperometric detection of amino acids on a manganin working electrode was reported[12]. On the basis of the described amperometric[9] and potentiometric[10] detection of dicarboxylic acids on a copper electrode, a manganin electrode was tried in this work for the amperometric detection of dicarboxylic acids. Further, the advantage of the use of a simultaneous conductivity and amperometric detector with a single electrochemical cell[13] was applied to the simultaneous detection of both complexing and non-complexing organic acids. A complexing cation, Zn^{2+}, was added to the mobile phase to control the retention and separation of acids. This approach is similar to the regulation of the retention of amino acids by adjusting the content of Cu^{2+} ion in the mobile phase[14]. In order to keep the number of system peaks small, the number of ionic components in the mobile phase was minimized. The previously developed instrumentation[12,13] allowed us to carry out experiments using microcolumn liquid chromatography.

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EXPERIMENTAL

Apparatus

The microcolumn reversed-phase ion-pair chromatography of dicarboxylic acids was performed on a laboratory-built chromatograph. The mobile phase was pumped by an HPP 5001 syringe pump (Laboratory Instruments, Prague, Czechoslovakia) modified for microcolumn liquid chromatography. A 1-μl volume of an acid solution was introduced on to the microcolumn by a laboratory-made four-port injection valve described previously[15]. A CGC glass microcolumn (150 $\times$ 1 mm I.D.) (Tessek, Prague, Czechoslovakia) was packed with 7.5-μm Silasorb SPH C_{18} (Lachema, Brno, Czechoslovakia) by the viscosity packing technique[16]. A simultaneous conductivity and amperometric detector[13] was equipped with a manganin working electrode[12]. The detector had a flow cell of volume 20 nl and cell constant 1.5 cm^{-1}. The d.c. polarizing voltage of the working electrode was +0.3 V. The chromatograms were recorded with an A-25 two pen recorder (Varian, Walnut Creek, CA, U.S.A.).

Chemicals

The mobile phase was prepared from 10 m*M* stock solutions of its components. They were prepared by dissolution of tetrabutylammonium (TBA^+) hydroxide (Aldrich, Milwaukee, WI, U.S.A.) phthalic (P^{2-}) acid (Lachema), sodium hydroxide (Lachema) and zinc hydrogen phthalate. The solution of zinc hydrogen phthalate was obtained by dissolution of zinc oxide (Lachema) and 2 equiv. of phthalic acid in a small volume of warm water and by subsequent dilution to a 10 m*M* concentration of Zn^{2+}.

Stock solutions of TBA^+ hydroxide, phthalic acid, zinc phthalate and sodium hydroxide were mixed and diluted with distilled water to obtain the required mobile phase composition. Its final pH was adjusted with sodium hydroxide solution using an OP-208/1 pH meter (Radelkis, Budapest, Hungary). The conductivity of the mobile phase was measured with an OK 102/1 batch conductimeter (Radelkis). Oxalic (Ox), malonic (Mo), malic (Mi), maleic (Me), fumaric (Fu), tartaric (Ta), succinic (Su), glutaric (Gl), adipic (Ad) and citric acids (Ci) were supplied by Lachema.

RESULTS AND DISCUSSION

The dependence of the capacity factors, *k*, of the acids studied on the content of Zn^{2+} ions in the mobile phase are summarized in Fig. 1. In agreement with previous results[9], a mobile phase containing only TBA^+ phthalate (*i.e.*, without Zn^{2+} ions) (see Fig. 1) cannot separate dicarboxylic acids effectively. Neither the substitution of phthalate ions by other co-ions, *e.g.*, benzoate or sulphate, nor the variation of the TBA^+ ion concentration in the mobile phase improved the separation of dicarboxylic acids. Addition of Zn^{2+} ion to the mobile phase led to a decrease in solute retention, depending on its ability to complex with the metal cation. For instance, the retentions of oxalate and citrate anions are the most susceptible to variations in Zn^{2+} concentration (see Fig. 1). The decrease in retention is due to the formation of uncharged complexes of acids with Zn^{2+} ion, which decreases the interaction of acids with TBA^+ ion adsorbed on the hydrophobic stationary phase.

An example of the separation of some dicarboxylic acids is shown in Fig. 2. In

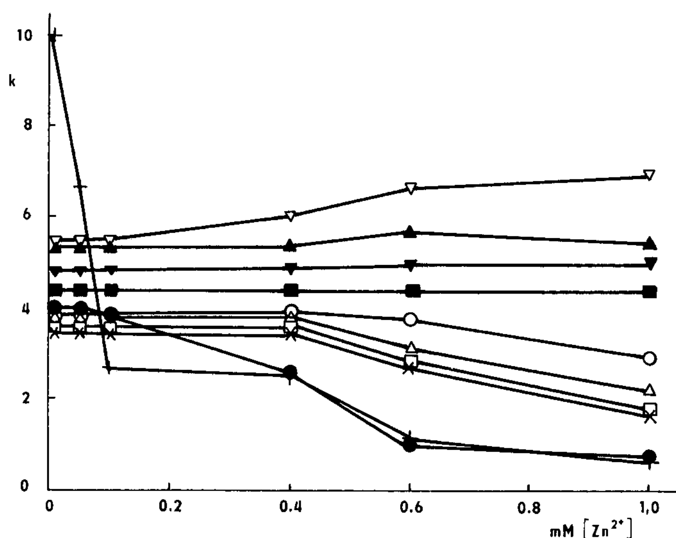

Fig. 1. Dependence of acid retention on Zn^{2+} concentration. Mobile phase, 1 mM TBA^{+}–2mM P^{2-} with the indicated concentration of Zn^{2+}; pH = 6.0, adjusted with sodium hydroxide. Flow-rate, 40 μl/min. Column: glass CGC (150 × 1 mm I.D.), packed with Separon SPH C_{18} (7.5 μm). Solutes: ● = oxalate; × = malate; □ = malonate; △ = tartrate; + = citrate; ○ = succinate; ■ = glutarate; ▼ = fumarate; ▽ = adipate; ▲ = maleate.

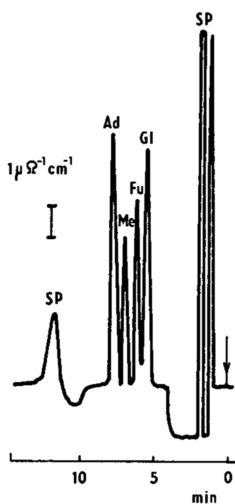

Fig. 2. Chromatogram of a mixture of glutarate (Gl), fumarate (Fu), maleate (Me) and adipate (Ad), each 3 nmol. SP = system peaks. Mobile phase: 1 mM TBA^{+}–2 mM P^{2-}–0.6 mM Zn^{2+}–1.8 mM Na^{+}; pH = 6.1. Conductivity, 460 μohm^{-1} cm^{-1}. Column as in Fig. 1. Detection: conductivity output of the detector described under Experimental.

spite of their low complexing ability, their separation can be improved by the presence of Zn^{2+} ion in the mobile phase. On the other hand, the low complexing ability suggests the use of conductivity detection.

Further optimization of acid separations can be achieved by the simultaneous reduction of the concentration of all the mobile phase components. Similarly to the retention of inorganic anions eluted by tetrabutylammonium phthalate mobile phase[17], the retentions of organic anions increase with increasing dilution of the mobile phase. A lower ionic concentration in the mobile phase is advantageous also for the improvement of conductivity detection.

The application of a mobile phase with decreased concentrations of ionic components to the simultaneous conductivity and amperometric detection of dicarboxylic acids is shown in Fig. 3. The chromatograms indicate that complexing acids, *e.g.*, tartaric and malic acid, can be detected amperometrically by the proposed device with sufficient sensitivity. In spite of giving a high amperometric response, citrate and oxalate are not shown in this example, as they are co-eluted with system peaks close to the dead volume. Tartrate and malate are almost undetected conductrimetrically (see Fig. 3), which indicates that they are present in the mobile phase mainly in the uncharged form of the Zn^{2+} complex. On the other hand, Fig. 3 demonstrates that weakly complexing anions, *e.g.*, adipate, glutarate and fumarate, are better detected conductimetrically than amperometrically.

The detection limits can be derived with the help of the peak-to-peak noise

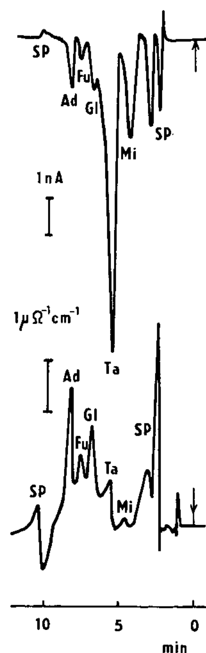

Fig. 3. Chromatogram of dicarboxylic acids detected with simultaneous conductivity (lower trace) and amperometric (upper trace) detector. Sample: malic (Mi), tartaric (Ta), glutaric (Gl), fumaric (Fu) and adipic (Ad) acids, each 1 nmol. Mobile phase: 0.9 mM TBA^+–0.45 mM Zn^{2+}–0.9 mM P^{2-}; pH = 6.6. Flow-rate, 40 μl/min. Column as in Fig. 1.

measured under the chromatographic conditions for both detection modes. Based on the amount of the acid injected and the experimental conditions, the calculated amperometric response to tartrate is 0.2 mA l mol^{-1}. Together with the noise of 40 pA this gives a detection limit of 0.4 μM in the detector or 1 ng or 1 mg/l in the sample for a peak height of twice the peak-to-peak noise. For comparison, detection limits of 5 ng for oxalate and 30 ng for malonate were found[9] for amperometric detection on the copper electrode and a 100 × 4.6 mm I.D. separation column. Simultaneously, a conductimetric noise equivalent to 0.03 μohm^{-1} cm^{-1} was found with a mobile phase of conductivity 460 μohm^{-1} cm^{-1}. The conductimetric noise then corresponds to 1/15000 of the mobile phase conductivity. Based on the parameters of the adipate peak, its detection limit in the sample can be calculated to be about 2 mg/l.

CONCLUSION

The simultaneous conductivity and amperometric detector with a single electrochemical micro-flow cell can work efficiently with a manganin working electrode. Such a device detects sensitively both complexing and non-complexing dicarboxylic acids separated by reversed-phase ion-pair microcolumn liquid chromatography. The control of solute retention by the content of Zn^{2+} cation in the mobile phase is compatible with both detection modes. The retention and selectivity control together with simultaneous conductivity and amperometric detection can help to identify sample components.

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Note

Post-column detection for capillary zone electrophoresis*

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Ultraviolet absorption[1], fluorescence[2], potential gradient[3–5], conductivity[6], mass spectrometric[7] and carbon fibre electrochemical[8] detectors have been used in capillary zone electrophoresis. Post-column detection is widely used in liquid chromatography because it is highly selective, and if it could be used in capillary zone electrophoresis it would be particularly useful in the medical and pharmaceutical fields. In this paper, we propose a post-column detection method for this purpose. As a certain pressure is generated at the outlet side of the capillary column due to the mixing of reagents with the effluent, a closed system for capillary electrophoresis is proposed that uses three pumps and two mixing parts for post-column detection.

EXPERIMENTAL

A schematic diagram is shown in Fig. 1. Fluoroethylene–propylene (FEP) tubing was supplied by Shimadzu (Kyoto, Japan). A pyrex glass capillary tube was drawn with a glass drawing machine (GDM-1; Shimadzu). These tubes were used as columns for capillary electrophoresis. Column media were supplied by pump 1 (Microfeeder, MF-2; Azuma Denki Kogyo, Tokyo, Japan) at a very low flow-rate of 0.1–2 μl/min. For post-column reaction, buffer and fluorescent reagent were supplied by pump 2 (FLC-A700; JASCO, Tokyo, Japan) and pump 3 (MF-2), respectively. A mixture of 0.1 *M* disodium hydrogen phosphate and sodium hydroxide solution (pH 11) containing 0.5% ethylene glycol was used as a buffer at a flow-rate of 0.15–0.40 ml/min.

The design of the four-way tetrafluoroethylene connector (Tuff connector; Gasukuro Kogyo, Tokyo, Japan) is shown in Fig. 2A. The end of the capillary column was inserted about 5 mm into a tetrafluoroethylene tube (0.5 mm I.D.). As the outside

* Part of this work was presented at the *6th Symposium on Capillary and Isotachophoresis, December 11–12th, 1986, Nagoya, Japan.*

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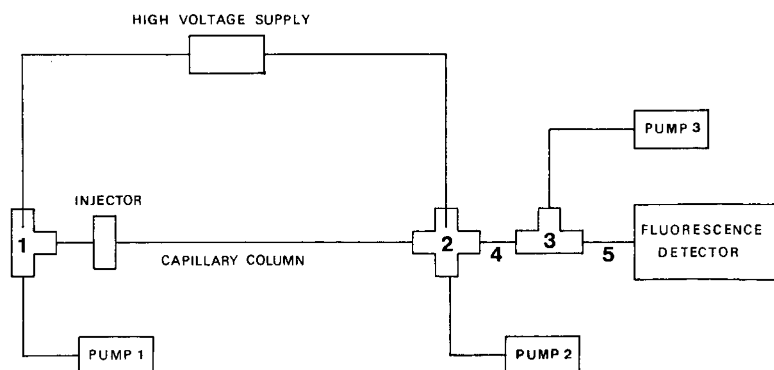

Fig. 1. Schematic diagram of post-column detection for detection in capillary electrophoresis. 1 = Positive terminal; 2 = four-way connector for earth terminal and mixing of column media and buffer; 3 = three-way connector for mixing with fluorescent reagent; 4 and 5 = PTFE tubes (0.5 mm I.D.) of length 5 and 70 cm, respectively. Column medium, alkaline buffer solution and fluorescent reagent were supplied by pumps 1, 2 and 3, respectively.

diameter of the FEP tubing used as the column was more than 0.5 mm, a fused-silica capillary (*ca.* 15 mm × 250 μm I.D. × 340 μm O.D.) (Scientific Glass Engineering, North Melbourne, Australia) was inserted in the end of the column, as shown in Fig. 2A. Detailed designs of the positive terminal and three-way connector are shown in Fig. 2B.

A $2 \cdot 10^{-4}$ *M* fluorescamine–dioxane solution was mixed with the mixture of effluent and buffer in a T-type three-way connector (1 mm hole) (Gasukuro Kogyo). For fluorescence development, the mixed solution was passed through a coil of PTFE tubing (70 cm × 0.5 mm I.D.). The PTFE tubing was of 1/16 in. O.D. except where stated otherwise. A fluorescence detector (RF-530; Shimadzu) with a 12-μl cell was used without modification. A high-voltage power supply (50 kV and 500 μA) (Matsusada Precision, Kusatsu, Shiga, Japan) and a rotary-type injector[9] were used.

Operational procedure

First pump 1 was started, then pump 2 and finally pump 3. After starting the three pumps, sample was injected with a rotary injector.

To avoid flow backwards into the capillary column, the order of starting the three pumps is important. If alkaline buffer solution does flow into the capillary column, the column should be washed with column medium. If fluorescamine–dioxane solution flows into the capillary column, the column should be washed with an organic solvent such as dichloromethane.

As the fluorescamine–dioxane reagent should be kept dry, it is preferable to fit a stop valve in the line between pump 3 and the three-way connector. After starting pump 3, the stop valve is opened.

RESULTS AND DISCUSSION

The apparatus used consists of three pumps and two mixing parts. To avoid pulsing noise, all the pumps used were of the syringe type. Special care was taken in

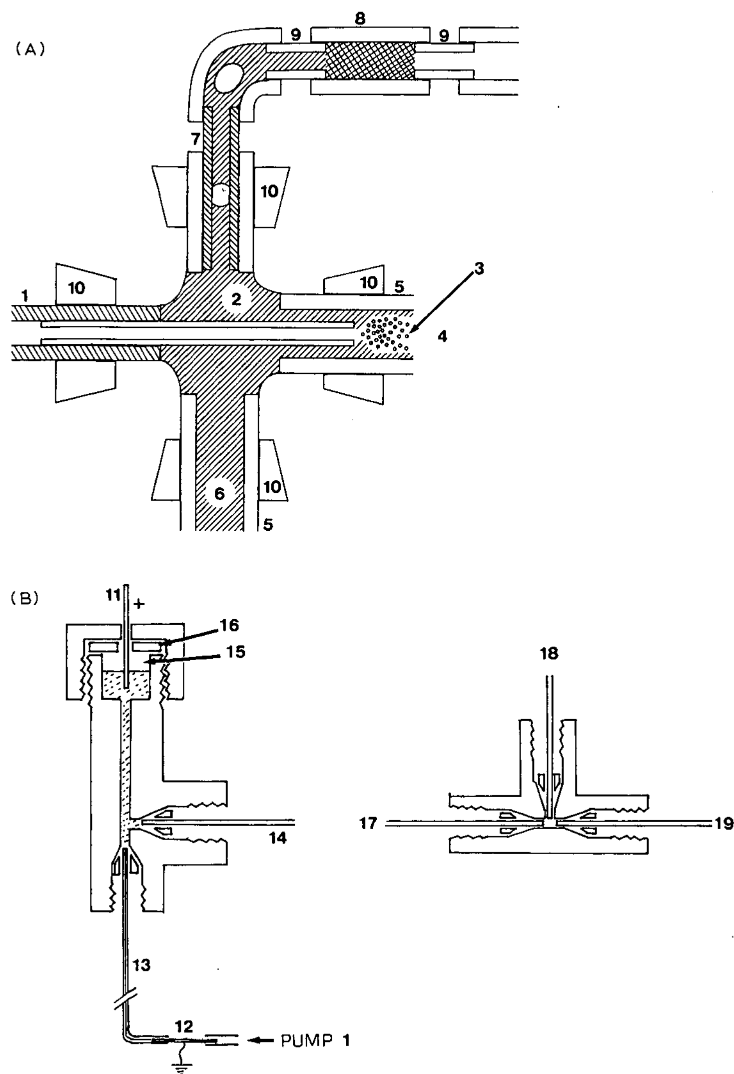

Fig. 2. (A) Mixing part for column medium and buffer at four-way connector. This part corresponds to 2 in Fig. 1. 1 = FEP capillary column; 2 = fused-silica capillary, inserted into the end of the FEP column; 3 = mixing zone; 4 = to fluorescence detector; 5 = PTFE tube (0.5 mm I.D.); 6 = alkaline buffer; 7 = earth terminal (platinum tubing); 8 = flow resistance, PTFE tubing packed with polymer beads; 9 = stainless-steel tubing; 10 = PTFE ferrule. (B) Detailed diagrams of positive terminal (left) and three-way connector (right), corresponding to 1 and 3, respectively, in Fig. 1. 11 = Positive terminal, platinum wire; 12 = platinum tubing (3 cm × 0.3 mm I.D. × 0.7 mm O.D.) connected to earth; 13 = PTFE tubing (2 m × 0.1 mm I.D. × 2 mm O.D.) as an electric resistance; 14 = FEP tubing connecting positive terminal and injector; 15 = space for bubbles; 16 = silicone-rubber septum. The mixtures of column medium and alkaline solution (17) and fluorescent reagent (18) were mixed in the three-way connector, and were led into a coil (19).

in constructing the four-way connector (Fig. 2A). The flow-rate of the column medium supplied by pump 1 was less than a few microlitres per minute. This column medium was mixed with alkaline buffer at the mixing zone (3 in Fig. 2A) just after it flowed out from the fused-silica capillary tubing. Although the alkaline buffer has a relatively high flow-rate (*e.g.*, 300 μl/min) compared with that of the column medium, it is essential to insert the column end into the 0.5 mm I.D. PTFE tube to avoid band broadening, as shown in Fig. 2A. The bubbles that were generated at the inner wall of the earth terminal were led to the upper part and carried outside after passing through 8 and 9 in Fig. 2A. Only a small fraction of the flow, *ca.* 10 μl/min, consistently came out. This device was also effective in excluding bubbles that were unexpectedly present in the alkaline solution. This device was essential for avoiding detector noise due to bubbles. Bubbles generated at the positive terminal and/or existing in the column medium were also collected (16 in Fig. 2B). To protect pump 1 from damage due to high voltage, a long PTFE tube (13 in Fig. 2B) was used as an electrical resistance. Between pump 1 and the tubing an earthed platinum tube (12 in Fig. 2B) was connected. Column medium supplied by pump 1 flowed through the tubing to the capillary column. There was a small current between 11 and 12 in Fig. 2B. Fluorescence reagent was mixed with the mixture of column medium and alkaline solution inside a simple three-way connector, as shown in Fig. 2B.

A certain pressure was generated at the column end owing to the mixing of reagent and buffer and to the long path from the end of the capillary column to the outlet of the flow cell. Therefore, it was necessary to impose at least an equal pressure at the side of the injector to prevent reverse flow from the column end. For this purpose, a pressurized flow was created by pump 1. Thus the solute was conveyed

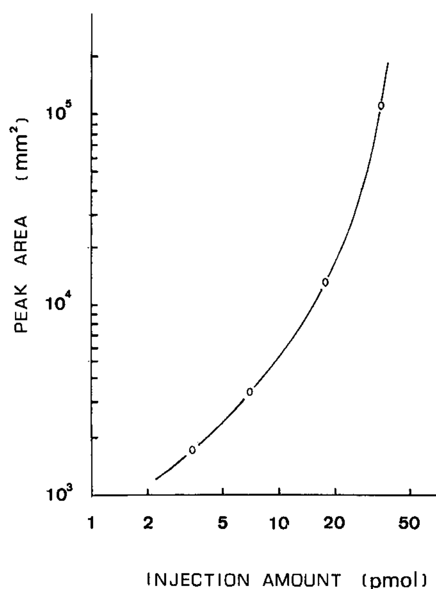

Fig. 3. Calibration graph for putrescine. Column, Pyrex glass capillary tube, (110 cm × 72 μm I.D.); column medium, 0.2 *N* lactic acid–0.5% ethylene glycol–0.1% sodium dodecyl sulphonate; applied voltage, 20 kV; flow-rates of pumps 1, 2 and 3, 0.52, 250 and 42 μl/min, respectively.

under a pressurized flow, v(pres), its mobility and electroosmotic flow. The experimental conditions were as in Fig. 3 except for alkaline buffer, 0.40 ml/min, sample 10 pmol putrescine in 0.1 *M* hydrochloric acid and pressurized flow. When the pressurized flow was varied, *e.g.*, 0.33, 0.52 and 1.04 μl/min, the number of theoretical plates, N, of the solute was 3010, 1020 and 360, respectively. N had the following relationship with v(pres):

$$\log N = A - B \log v(\text{pres})$$

where A and B are constants. Hence v(pres) should be kept as low as possible.

We examined the effect on N of changing the flow-rate of alkaline buffer from 0.15 to 0.40 ml/min. At more than 0.30 ml/min, N was almost constant. The solute leaving the capillary column took about 20 s to reach the cell of the detector at an alkaline buffer flow-rate of 0.4 ml/min. 20 s was sufficient to complete the reaction between solute and fluorescamine.

A calibration graph for the fluorescamine derivative of putrescine is shown in Fig. 3. The calibration graph is slightly curved, possibly because a certain amount of putrescine had been adsorbed on the inner wall of the capillary column. The detection limit was at the picomole level.

Chromatograms with different pressurized flows are shown in Fig. 4. Both peaks have the same area, although the pressurized flow-rate of in Fig. 2B (2.08 μl/min) was four times that in Fig. 2A. Variation of the pressurized flow in the range 0.33–3 μl/min had no effect on the peak area because the proportion of pressurized flow in the total flow-rate supplied by pumps 1, 2 and 3 was less than 1.5%. The calibration graph was independent on the flow-rate of the column medium and is valid for the experiment using FEP columns.

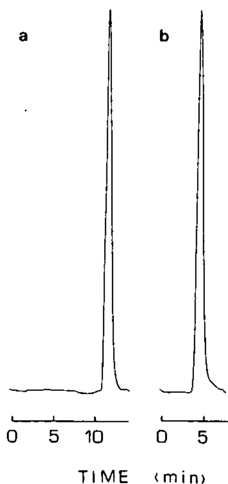

Fig. 4. Chromatograms of putrescine with different pressurized flows: (a) 0.52 and (b) 2.08 μl/min. Sample amount, 3.5 pmol. Other experimental conditions as in Fig. 3.

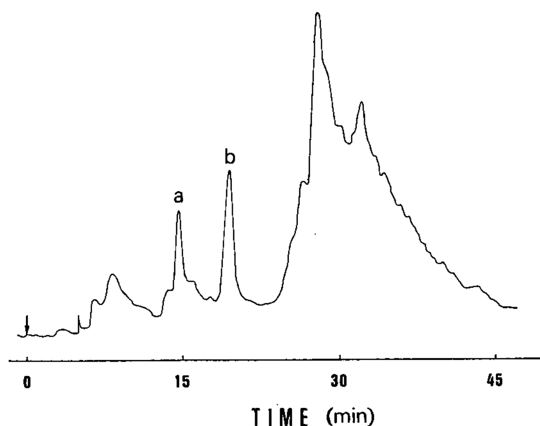

Fig. 5. Separation of groups of (a) free and (b) acetylpolyamines in human urine. Column, FEP (1.5 m × 0.3 mm I.D.); column medium, formic acid–0.01% ethylene cyanohydrin–5% ethylene glycol; applied voltage, 25 kV; current, 147 μA; flow-rates of pumps 1, 2 and 3, 2, 400 and 42 μl/min, respectively.

Application

The method was applied to the determination of polyamines in human urine and organs in rats. A typical example is shown in Fig. 5. The polyamines in human urine are almost always acetylpolyamines[10]. The urine sample was partially hydrolysed with 20% (v/v) hydrochloric acid. Two peaks were obtained, total free polyamines (16 μmol) and total acetyl polyamines (28 μmol). The concentration of total polyamines excreted in human urine in Fig. 5 was 66 μmol/day. Peak a in Fig. 5 belongs to the group of polyamines that have two charges per molecule and peak b one per molecule.

One of the advantages of capillary electrophoresis is that group separation is possible. The relationship between the amount of polyamines in human body fluids and cancer is well established[10–12]. Previous methods for the determination of polyamines have included enzymatic assay and liquid chromatography[12]. The present results indicate that capillary electrophoresis may be a good method for the determination of polyamines.

ACKNOWLEDGEMENT

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Note

Simultaneous XAD-2 resin extraction and high-resolution electron-capture gas chromatography of chlorine-containing herbicides in water samples

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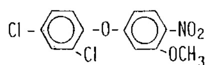
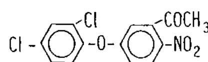
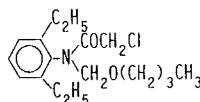
Environmental pollution by organochlorine pesticides is a serious problem with regard to public health. The agricultural use of organochlorine insecticides (OCIs) such as 1,2,3,4,5,6-hexachlorocyclohexane (HCH) and DDT, aldrin, dieldrin and endrin have been banned in Japan since 1971. However, residues have subsequently been found in soil[1] and water samples[2–4]. Also large amounts of chlorine-containing herbicides (CCHs) such as oxadiazon [5-*tert.*-butyl-3- (2,4-dichloro-5-isopropoxy-phenyl) -1,3,4-oxadiazolin-2-one], CNP (*p*-nitrophenyl 2,4,6-trichlorophenyl ether), butachlor [2-chloro-2′,6′-diethyl-N-(butoxymethyl) acetanilide], chlormethoxynil (2,4-dichlorophenyl-3-methoxy-4-nitrophenyl ether) and bifenox [methyl 5-(2,4-di-chlorophenoxy)-2-nitrobenzoate] have recently been used as pre- and post-emergence herbicides in paddy fields in Japan (Fig. 1)[5]. Monitoring of CCHs in river water and



CHLORMETHOXYNIL



BIFENOX



BUTACHLOR

Fig. 1. Structures of chlorine-containing herbicides.

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tap water samples is important for assessing environmental pollution by these CCHs, but as yet little information has been obtained on the simultaneous determination of CCHs.

When determining these CCHs at trace levels, conventional liquid–liquid extraction has required large volumes of samples and solvent. However, the adsorption of a variety of trace organics in water on XAD-2 resin has been one of the best choices for the preparation of extracts[6], because this method is simpler and needs less solvent than the liquid–liquid extraction. In previous studies, an XAD-2 resin column showed excellent retention and desorption for HCH, CNP and oxadiazon[2,7,8]. However, little information has been obtained on the extraction of butachlor, chlormethoxynil and bifenox with this resin. In this paper, the extraction of these herbicides with XAD-2 resin is described.

Gas chromatography with electron-capture detection (GC–ECD) and with a packed column has commonly been employed for the determination of residue levels of OCIs or CCHs. Because low-resolution GC with packed columns could not resolve each component of the CCHs from the co-extractives derived from environmetal samples, a clean-up procedure such as column chromatography has generally been utilized. However, the polarities of CCHs themselves are different, so that it is necessary to elute them from the column with various solvents whose polarities are also different. Therefore, the simultaneous determination of CCHs residues is extremely difficult. Up to now, each component of the OCIs or CCHs has been determined individually[2,7,8]. However, it is most desirable with regard to time and expense for a number of CCHs to be analysed simultaneously, with convenient data evaluation. Three chemically bonded fused-silica capillary columns (CB-FSCs), OV-17, OV-1701 and SE-52, were tested for the separability of 28 OCIs and related compounds, and excellent resolution was achieved, especially with OV-17 CB-FSC[1]. This paper also shows that the simultaneous determination of CCHs is possible using GC–ECD with CB-FSCs, which can resolve CCHs from the co-extractives without any clean-up.

EXPERIMENTAL

Materials

Hexane was redistilled as desribed previously[2]. Diethyl ether and methanol were commercial reagents of pesticide-residue grade and used without further purification. Liquid chromatographic grade isooctane was redistilled. Anhydrous sodium sulphate was heated at 625°C for 2 h to eliminate interferences. Amberlite XAD-2 resin was screened (20–60 mesh) and subsequently cleaned up with methanol, acetonitrile and diethyl ether in a Soxhlet extractor for 8 h for each solvent and immersed in methanol to maintain its activity until use. Pesticide-free water was prepared by distillation of tap water with an all-glass distiller and then passing the distillate through a column of XAD-2 resin (20 × 3 cm I.D.). All glassware employed was rinsed twice thoroughly with acetone and hexane before use.

Recovery experiments

The extraction methodology with XAD-2 resin was developed for butachlor, chlormethoxynil and bifenox. The extraction system has been described previously[2]. For recovery experiments, standard solutions containing butachlor, chlormethoxynil

and bifenox in acetone were prepared. Acetone standard solutions (0.50 ml) were spiked into 2 l of pesticide-free water to give concentrations of 25, 50, 250 and 500 ng/l for chlormethoxynil and 50, 100, 500 and 1000 ng/l for butachlor and bifenox. Then the sample was passed through the XAD-2 column (10 cm × 1 cm I.D.) at a flow-rate of *ca.* 50 ml/min under gentle suction. The herbicide standards adsorbed on the resin were desorbed with 60 ml of diethyl ether after standing for 15 min to equilibrate. Then the extractant and an additional 40 ml of diethyl ether eluent were drained into a separating funnel, to which 50 ml of hexane were added to remove the aqueous layer. Following the removal of the aqueous layer, the organic layer was dried by passing it through a column of anhydrous sodium sulphate and concentrated with a Kuderna–Danish evaporative concentrator. The final concentration was accomplished by passage of a stream of nitrogen and the residue was dissolved into 1 ml of isooctane and subjected to GC–ECD on a CB-FSC.

Gas chromatography

A Shimadzu GC-9APFE gas chromatograph equipped with an electron-capture detector and an OV-17 CB-FSC (25 m × 0.32 mm I.D., 0.25 μm film) was employed. A Shimadzu C-R2AX integrator performed data processing. Aliquots of standards and sample extracts, normally 1 μl, were automatically introduced through a Shimadzu AOC-9 autosampler by splitless injection. The temperature programme was as follows: 90°C for 3 min, then increased to 170°C at 20°C/min and further to 275°C at 3°C/min, the final temperature being maintained for 8 min. The injector temperature was 290°C throughout the experiment. The flow-rate of make-up gas was 70 ml/min. Identification of CCHs was performed by the internal standard method, comparing relative retention times (RRTs) with respect to heptachlor epoxide. Other CB-FSCs, OV-1701 (25 m × 0.25 mm I.D., 0.25 μm film) and SE-52 (25 m × 0.25 mm I.D., 0.30 μm film) were used to confirm identification. Calibration graphs for each CCH were prepared, using the internal standard method. Quantification was performed by peak-height ratio measurements of each CCH relative to the internal standard.

Field experiments

Water samples were taken at the sampling sites shown Fig. 2. Sampling site 1 was by the Onga River, which is one of the longest rivers in Kyushu Island and its total catchment is 1032 km^2. Sampling sites 2, 3, 5 and 6 were located in argricultural areas, the streams being used for agricultural drainage. Sampling site 4 was by the Murasaki River, which is located in an urban area of Kitakyushu City. Sampling site 7 was laboratory tap water which is supplied after conventional treatment of Onga River water. The water samples were taken once a month, provided that no rain had fallen for three days before sampling. Grab water was taken in a 3-l glass vessel and rapidly transferred to the laboratory for analysis. The samples were filtered through a G4 Büchner filter (150–200 mesh) to remove sediments. The filtered water sample (2 l) was drained into another glass vessel and stirred for 15 min to achieve thorough mixing after addition of an acetone solution of heptachlor epoxide (0.015 μg) as an internal standard. The subsequent procedures were the same as in the recovery experiments.

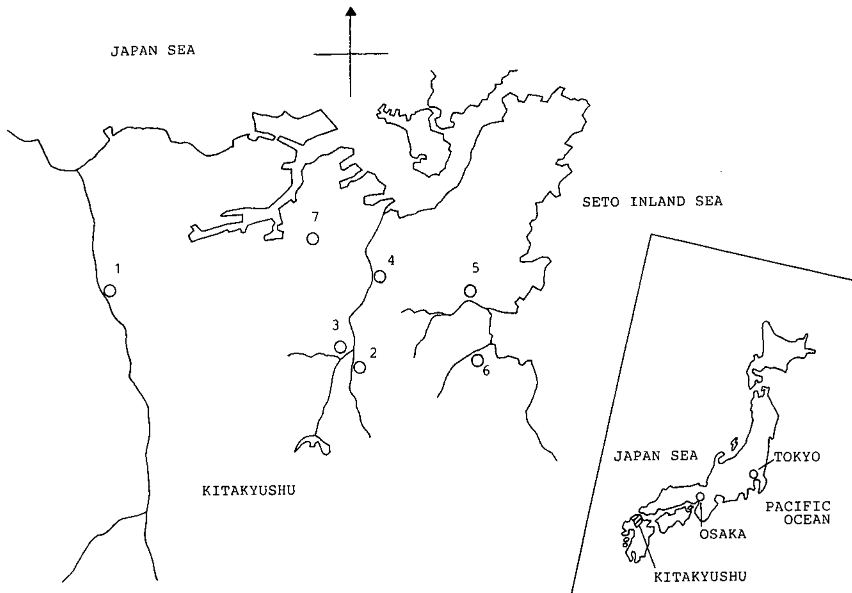

Fig. 2. Sampling sites: 1, Onga River; 2, Higashitani River; 3, Nishitani River; 4, Murasaki River; 5, Chikuma River; 6, Nuki River; 7, tap water.

RESULTS AND DISCUSSION

Recovery experiments

It is known that polar compounds are poorly adsorbed on XAD-2, resulting in low recoveries[6,9]. As butachlor, chlormethoxynil and bifenox have some polarity, their recovery using the method was examined. Recoveries from water samples at four fortification levels are given in Tabel I. All recoveries exceeded 90% and the

TABLE I

RECOVERIES OF BUTACHLOR, CHLORMETHOXYNIL AND BIFENOX FROM FORTIFIED SAMPLES USING THE XAD-2 EXTRACTION METHOD

Compound	*Fortification level (μg/l)*	*Recovery ± C.V.* (%)*
Butachlor	0.05	96.7 ± 3.1
	0.10	96.3 ± 4.0
	0.50	95.5 ± 8.2
	1.00	94.3 ± 6.2
Chlormethoxynil	0.025	102.0 ± 5.3
	0.05	103.7 ± 9.0
	0.25	100.3 ± 8.4
	0.50	102.8 ± 0.4
Bifenox	0.05	93.7 ±4.9
	0.10	103.0 ± 9.2
	0.50	93.7 ± 3.2
	1.00	102.9 ± 2.5

* C.V. = coefficient of variation (n = 3).

standard deviations were less than 10%. Only the mean recoveries of chlormethoxynil were over 100% at all levels spiked. Although some fairly large deviations of the recoveries can be seen in Table I, they are within the ranges of deviations for GC determinations. Therefore, almost quantitative adsorption–desorption kinetics of these pesticides from XAD-2 resin at all the fortified levels was demonstrated.

These results were considered to be satisfactory for determining the three CCHs in a water matrix. Also, these CCHs could be simultaneously and rapidly extracted from water samples together with HCHs, CNP and oxadiazon, as in previous studies[2,7,8]. XAD-2 resin adsorbed less co-extractives such as organic materials with high molecular mass which cause more serious problems with GC–ECD than liquid–liquid extraction[9]. In many instances the extracts could be directly applied to GC–ECD without any clean-up. High blank levels derived from impurities in the resin have been one of the disadvantages of the method, but few interfering peaks were seen in the chromatogram of the extracts from pesticide-free water using the GC–ECD method (Fig. 3a). Further, the resin could be used repeatedly after washing with an appropriate amount of methanol.

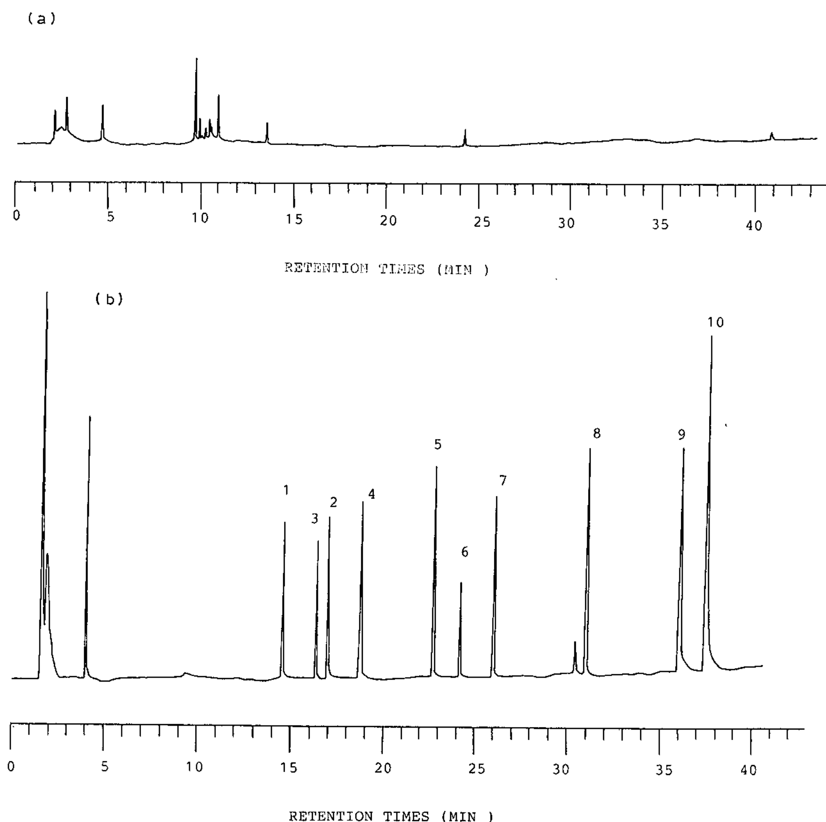

Fig. 3. (a) GC–ECD trace of blank run on an OV-17 CB-FSC. A = Diethyl ether extract was obtained from the XAD-2 resin column after passing pesticide-free water. (b) GC–ECD trace of standards of chlorine-containing pesticides on an OV-17 CB-FSC column. Peaks: 1, α-HCH; 2, β-HCH; 3, γ-HCH; 4, δ-HCH; 5, heptachlor epoxide (internal standard); 6, butachlor; 7, oxadiazon; 8, CNP; 9, chlormethoxynil; 10, bifenox.

High-resolution GC–ECD

A chromatogram of standards of HCH isomers, butachlor, oxadiazon, CNP, chlormethoxynil and bifenox on the OV-17 CB-FSC is shown in Fig. 3b. Under the GC conditions applied, the peaks of each component were clearly resolved from each other. The internal standard method was employed to avoid misunderstandings or false identification due to fluctuations in retention times and to minimize the deviation of the recovery during the procedure. The peaks derived from pesticide components could be identified by comparison of the RRTs with respect to the internal standard, heptachlor epoxide. The retention times fluctuated periodically owing to changes in room temperature, whereas the RRTs were almost constant (the coeffi-

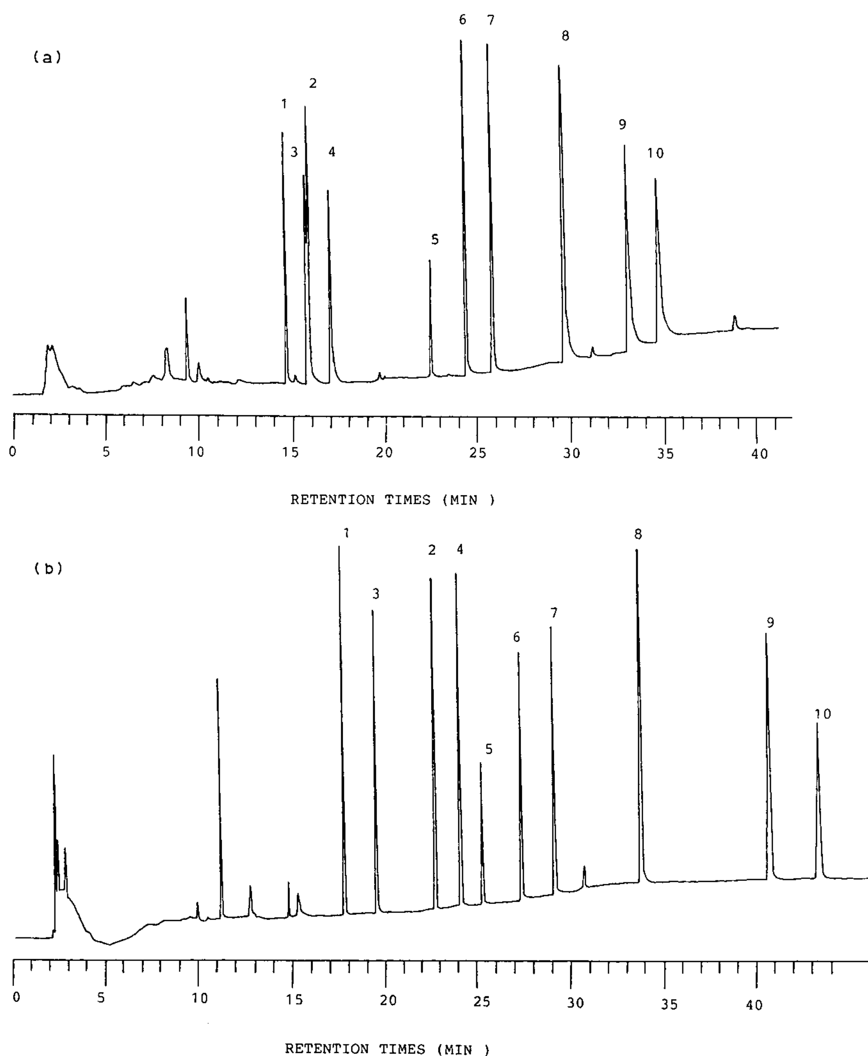

Fig. 4. GC–ECD traces of standards of chlorine-containing pesticides on (a) SE-52 and (b) OV-1701 CB-FSC. Peaks as in Fig. 3b.

cients of variation of the RRTs with ten replicate injections of CCHs were less than 0.1%). Therefore, comparison of RRTs was adequate for the identification of CCHs in river water samples.

For further identification, it is desirable to use a column of different polarity, *i.e.*, a non-polar column for confirmation of the identification, but chlormethoxynil and bifenox, which are slightly polar, could not be resolved satisfactorily, and they were absorbed on the column readily. In such instances an SE-52 or OV-1701 CB-FSC could be used (Fig. 4). With the SE-52 CB-FSC, β-HCH and γ-HCH could not be resolved, which was a disadvantage as HCH isomers have to be monitored simultaneously and they have often been detected in water samples. The OV-1701 CB-FSC was more suitable for confirmation of the identification.

Quantification was performed by peak-height ratio measurements relative to the internal standard. Calibration graphs were obtained with an amount of internal standard of 0.015 ng and amounts of chlormethoxynil, bifenox, CNP and oxadiazon in the range 0.01–0.15 ng and of butachlor in the range 0.04–0.60 ng. Linear graphs were obtained between 0 and 0.25 ng for chlormethoxynil, bifenox, oxadiazon and CNP and between 0 and 1 ng for butachlor. The minimum detectable amounts were 0.01 ng for chlormethoxynil and bifenox and 0.02 ng for butachlor, which corresponded to 0.005 and 0.01 μg/l respectively, with concentration of the water samples under the experimental conditions.

On extended use, late-eluting CCHs, *i.e.*, chlormethoxynil and bifenox, tended to be adsorbed on the column wall, resulting in diminution of the peak heights. This might be due to the discrimination[10]. Some measurements were made by determination of the peak-height ratio of the CCHs relative to the internal standard. The discrimination was improved by injection of a silylating reagent or by cutting a part of the column of the injector side.

Field experiments

CCH residues in river surface water could be identified and determined using the present methodology. Fig. 5a showed a typical gas chromatogram of CCH resi-

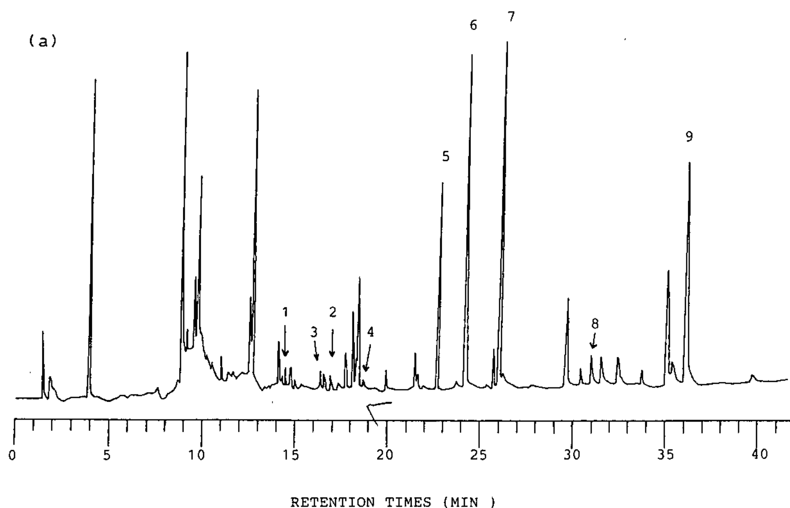

Fig. 5.

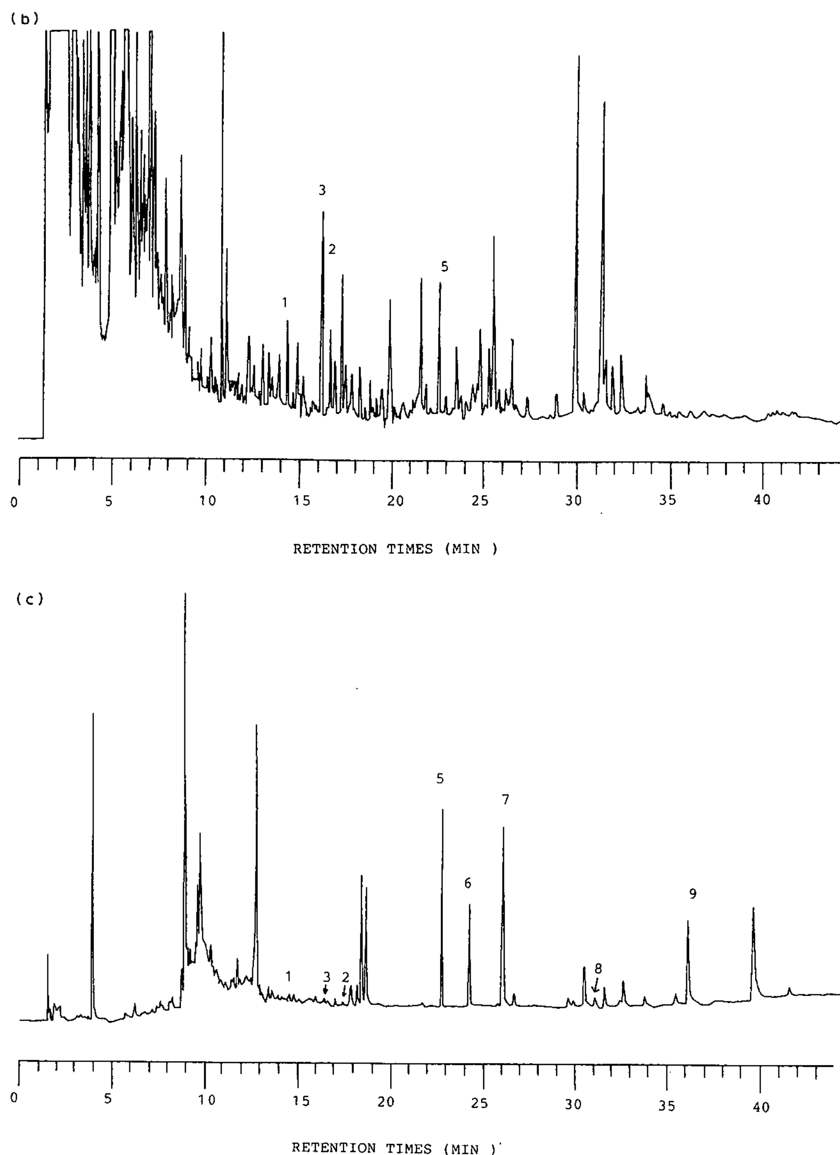

Fig. 5. Typical GC–ECD traces on an OV-17 CB-FSC column of samples taken from the Onga River in (a) June 1987 and (b) April 1987, and (c) tap water sample taken in June, 1987. The sample (2 l) was concentrated to 10 ml.

dues in a sample taken from the Onga River in June 1987, when high concentrations of herbicide residues were to be expected. The extract was concentrated to 10 ml. Many peaks derived from co-extractives were recorded. Overlapping of peaks with similar retention times commonly occurred when GC–ECD with a packed column without clean-up of the sample extracts was performed, but excellent separations of CCHs and other co-extractives were obtained with the OV-17 CB-FSC. In the surface

TABLE II

RESIDUE CONCENTRATIONS (μg/ml) OF BUTACHLOR, CHLORMETHOXYNIL AND BIFENOX IN SURFACE WATER AND TAP WATER IN 1987

ND is below the detection limit, which is 0.01 μg/l for butachlor and 0.005 μg/l for chlormethoxynil and bifenox.

Compound	*Sample*	*22.1.87*	*19.3.87*	*17.4.87*	*21.5.87*	*19.6.87*	*28.7.87*	*21.8.87*	*22.9.87*	*21.10.87*
Butachlor	Onga R.	ND	ND	ND	0.01	2.26	0.15	0.07	ND	ND
	Higashitani R.	ND	ND	0.05	ND	3.44	0.08	0.03	ND	ND
	Nishitani R.	ND	ND	ND	ND	1.32	0.02	ND	ND	ND
	Murasaki R.	ND	ND	ND	ND	2.10	0.04	0.04	ND	ND
	Chikuma R.	ND	ND	ND	ND	1.28	0.07	0.06	ND	ND
	Nuki R.	ND	ND	ND	ND	0.75	0.01	ND	ND	ND
	Tap water	ND	ND	ND	ND	0.59	0.04	0.01	ND	ND
Chlormethoxynil	Onga R.	ND	0.010	ND	ND	0.254	0.017	ND	ND	ND
	Higashitani R.	ND	ND	ND	ND	0.049	ND	ND	ND	ND
	Nishitani R.	ND	ND	ND	ND	0.027	ND	ND	ND	ND
	Murasaki R.	ND	ND	ND	ND	0.020	ND	ND	ND	ND
	Chikuma R.	ND	0.007	ND	ND	0.012	ND	ND	ND	ND
	Nuki R.	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Tap water	ND	ND	ND	ND	0.095	0.006	ND	0.009	ND
Bifenox	Onga R.	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Higashitani R.	ND	ND	ND	ND	0.019	ND	ND	ND	ND
	Nishitani R.	ND	ND	ND	ND	0.009	ND	ND	ND	ND
	Murasaki R.	ND	ND	ND	ND	0.006	ND	ND	ND	ND
	Chikuma R.	ND	ND	ND	ND	0.064	ND	ND	ND	ND
	Nuki R.	ND	ND	ND	ND	0.032	ND	ND	ND	ND
	Tap water	ND	ND	ND	ND	ND	ND	ND	ND	ND

water samples residues of butachlor and chlormethoxynil could be detected in addition to HCH isomers, oxadiazon and CNP. The RRTs of the pesticides with respect to heptachlor epoxide were in fair agreement with those of standards. The residue concentrations of butachlor, oxadiazon, CNP and chlormethoxynil were 2.26, 0.66, 0.02 and 0.25 μg/l, respectively; α-, β-, γ- and δ-HCH isomers were detected at 0.005, 0.013, 0.006 and 0.002 μg/l, respectively.

Fig. 5b shows a typical chromatogram of a sample taken from the Onga River in April 1987, when the concentrations of herbicides were expected to be very low. The extract was concentrated to 1.0 ml. Although more peaks were monitored than in Fig. 5a, separation of most of them was accomplished. HCH isomers except δ-HCH were detected. However, none of the peaks derived from herbicides, butachlor, oxadiazon, CNP, chlormethoxynil or bifenox could be detected. The three large peaks eluting between 25 and 35 min were not identified. They do not seem to be CCHs because they appeared without regard to changes of season.

Fig. 5c shows a chromatogram of a sample of tap water taken in our laboratory in June 1987. The extract was concentrated to 10 ml. The chromatogram was similar to that in Fig. 5a, although the levels of CCHs were slightly low. The residue concentrations of butachlor, oxadiazon, CNP and chlormethoxynil were 0.59, 0.28, 0.01 and 0.095 μg/l, respectively. These results indicate that the conventional water purification process had not removed these compounds.

Table II gives the residue levels of butachlor, chlormethoxynil and bifenox in river surface water and tap water in 1987. Residues of butachlor and chlormethoxynil were frequently detected from June to August and occasionally from March to September. However, residues of bifenox could be measured only in June. Of the three CCHs, butachlor was most often detected and its residual concentrations were the highest. In the Kitakyushu District, herbicide formulations containing these components are applied to control the weeds in flooded paddy fields in mid-June after rice-seedling transplantation. Herbicide application is terminated after the rice-plants have grown to a moderate height, and residues then could not be detected. These results indicate that the herbicides under study are not persistent in the aquatic environment.

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Note

Determination of flavonol glycosides in Epimedii Herba by high-performance liquid chromatography

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(Received July 27th, 1988)

Epimedii Herba, the dried aerial parts of plants of the genus *Epimedium* (Berberidaceae), is an important crude drug used as a tonic. More than 40 species of the plant grow wild in China, Korea and Japan. It has been reported that icariin[1–4], a flavonol glycoside, is the principle in this crude drug (aerial parts), whereas icariin and epimedoside A–E[5–9] occur contained in the underground parts. Recently, a number of glycosides[10–18] have been also isolated.

For the evaluation of Epimedii Herba, a coulometric method for icariin has been reported[19], but this method is complicated and inaccurate because it involves pre-treatment by preparative thin-layer chromatography. Therefore, a simple method is required to evaluate the quality of this crude drug.

During out studies on Epimedii Herba[20–22], three new flavonol glycosides, 1, 2 and 3 [4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-glucosyl(1→2)-rhamnoside-7-glucoside, 3-xylosyl(1→2)rhamnoside-7-glucoside and 3-rhamnosyl-(1→2)rhamnoside-7-glucoside] were isolated from *Epimedium koreanum*, together with icariin (Fig. 1). Their contents were comparatively high and these glycosides were considered as indicative compounds for the evaluation.

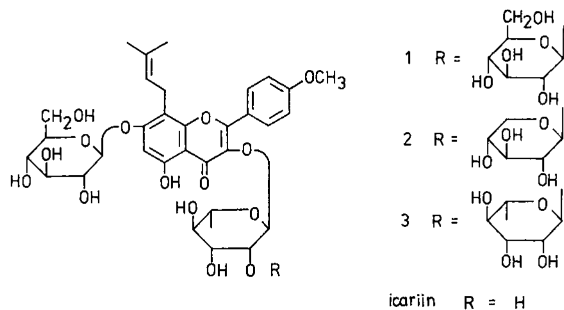

Fig. 1. Structures of four flavonol glycosides. 1 = 4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-O-β-D-glucopyranosyl (1→2)-α-L-rhamnopyranoside-7-O-β-D-glucopyranoside; 2 = 4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-O-β-D-xylopyranosyl (1→2)-α-L-rhamnopyranoside-7-O-β-D-glucopyranoside; 3 = 4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-O-α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranoside-7-O-β-D-glucopyranoside.

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In this paper, the application of a high-performance liquid chromatographic (HPLC) method to the separation and determination of these four flavonol glycosides, is demonstrated and the analytical results for 20 plant materials are presented.

EXPERIMENTAL

Plant materials

Plant materials were supplied by Showa University, Kyoto College of Pharmacy, Hiroshima University, Hokuriku University and Kyushu University, and were cultivated in our laboratory.

Reagents

Flavonol glycosides, 1, 2, 3 and icariin were isolated from *E. koreanum* and purified by preparative HPLC. The acetonitrile used for the chromatography was of HPLC grade. Deionized water was further purified using a Millipore filter.

Apparatus

A Tosoh Model CCPM liquid chromatograph equipped with a UV-8000 UV spectrophotometer and a stainless-steel column (150 × 4 mm I.D.) packed with chemically bonded ODS silica gel (TSK gel ODS-120A, 5 μm; Tosoh) was used.

Procedure

About 0.5 g of dried, powdered crude drug was weighed accurately, placed in 25 ml of the mobile phase and refluxed on a water-bath at 85°C for 30 min. After cooling, the solution was centrifuged and decanted. The residue was washed twice with 10-ml portions of the mobile phase. The extract and washings were placed in a 50-ml volumetric flask together with 5 ml of internal standard solution (1 mg/ml of benzoin in ethanol) and diluted to 50 ml with the mobile phase. A 10-μl volume of the solution was injected into the HPLC system. The content of each flavonol glycoside was calculated from the ratio of its peak area to the peak area of the internal standard.

HPLC conditions

Water–acetonitrile (73:27) was used as the mobile phase at a flow-rate of 1.0 ml/min. The temperature of the column was maintained at 50°C. The substances eluted were detected with a UV detector at 270 nm.

Calibration graphs and detection limits

Calibration graphs for 1, 2, 3 and icariin were obtained for the concentration ranges 2.5–25.0, 5.0–50.0, 5.0–50.0 and 12.5–125.2 μg/ml, respectively. The corresponding regression equations were as follows: $y = 0.01955x + 0.0008$ ($r = 0.999$); $y = 0.02152x + 0.0022$ ($r = 0.999$); $y = 0.01883x + 0.0102$ ($r = 0.999$) and $y = 0.02823x - 0.0051$ ($r = 0.999$). The detection limits were 2.5, 3.0, 5.0 and 1.3 ng, respectively, at a signal-to-noise ratio of 3:1 for the peak heights.

RESULTS AND DISCUSSION

HPLC conditions

The acetonitrile concentrations of the mobile phase and the column temperature were varied in order to find the optimal elution conditions on chemically bonded ODS silica gel. The acetonitrile concentration was varied from 25 to 29% and 27% was selected for subsequent work, based on the resolution and retention times (Fig. 2). Column temperatures of 30, 40 and 50°C were tried and were found to affect the retention times slightly (Fig. 3); a temperature of 50°C was subsequently adopted.

Extraction solvent for flavonol glycosides

The mobile phase, methanol, methanol–water (50:50), ethanol–water (70:30), ethanol–water (50:50) and water were tried for the extraction of four flavonol glycosides. The temperature of the water-bath and the extraction time were maintained at 85°C and 30 min, respectively. The mobile phase gave the best extraction efficiency (Table I) and was selected for further work.

Determination of flavonol glycosides

The chromatogram of flavonol glycosides in Epimedii Herba is shown in Fig. 4. Table II gives the analytical results for 20 plant materials.

The contents of 1, 2, 3 and icariin varied from 0.05 to 0.98%, from 0.03 to 1.18%, from 0.01 to 0.78% and from 0.02 to 0.76%, respectively, except for *E. sagittatum*. Icariin has been thought to be the main component of Epimedii Herba. However, none of the plant materials analysed in our study contained icariin as the main component.

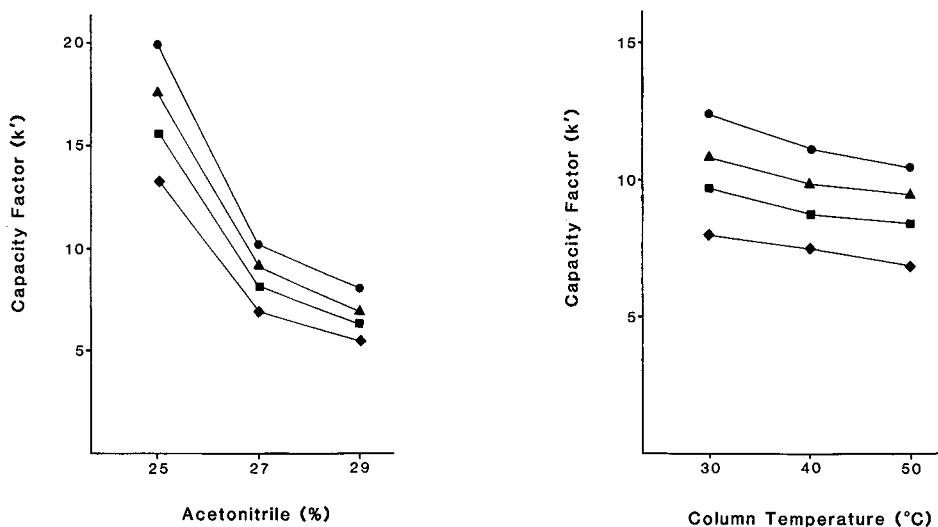

Fig. 2. Effect of acetonitrile concentration on the capacity factors of 1 (◆), 2 (■), 3 (▲) and icariin (●). Mobile phase, water–acetonitrile. Flow-rate, 1.0 ml/min. Column temperature, 50°C.

Fig. 3. Effect of column temperature on the capacity factors of 1 (◆), 2 (■), 3 (▲) and icariin (●). Mobile phase, water–acetonitrile (73:27). Flow-rate, 1.0 ml/min.

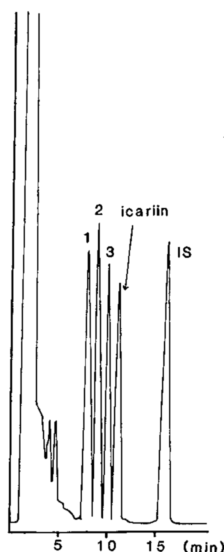

Fig. 4. Chromatogram of Epimedii Herba. Column, TSK gel ODS-120A (5 μm, 150 × 4 mm I.D.). Mobile phase, water–acetonitrile (73:27). Flow-rate, 1.0 ml/min. Column temperature, 50°C.

E. koreanum showed the highest total amount of the four glycosides. These glycosides were not detected in *E. sagittatum*. *E. sagittatum* grows naturally only in China, although the plant materials analysed in this study were all cultivated in Japan. As there are few channels for the importation of *E. sagittatum* from China to Japan, it is concluded that the *E. sagittatum* used in this experiment were grown from the same original plant, which unexpectedly contained few flavonol glycosides.

In conclusion, the proposed HPLC method is simple, rapid and precise, and seems to be useful for the quality control of Epimedii Herba.

TABLE I

EFFECT OF SOLVENTS ON EXTRACTION EFFICIENCY

Solvent	*Extraction (%)*			
	1	*2*	*3*	*Icariin*
Mobile phase	100.0	100.0	100.0	100.0
Methanol	101.1	96.7	94.0	100.5
Methanol–water (50:50)	99.7	99.3	97.9	96.4
Ethanol–water (70:30)	92.6	90.3	89.1	93.5
Ethanol–water (50:50)	99.3	99.0	93.7	100.3
Water	81.4	74.7	75.3	76.8

TABLE II

CONTENTS OF FLAVONOL GLYCOSIDES (%)

Plant material	*1*	*2*	*3*	*Icariin*	*Total (1 + 2 + 3 + icariin)*
E. sagittatum (Sieb. et Zucc.):					
(1) Cultivated in Saitama	Trace	Trace	Trace	Trace	–
(2) Cultivated in Saitama	Trace	Trace	Trace	Trace	–
(3) Cultivated in Kyoto	Trace	Trace	Trace	Trace	–
(4) Cultivated in Yamanashi	Trace	Trace	Trace	Trace	–
(5) Cultivated in Fukuoka	Trace	Trace	Trace	Trace	–
E. sempervirens Nakai:					
(1) Cultivated in Saitama	0.34	0.58	0.25	0.50	1.67
(2) Cultivated in Kyoto	0.05	0.03	0.16	0.05	0.29
(3) Native in Ishikawa	0.11	0.08	0.01	0.02	0.22
(4) Native in Ishikawa	0.08	0.06	0.02	0.01	0.17
E. sempervirens Nakai var. *hypoglaucum* (Makino) Ohwi:					
(1) Cultivated in Hiroshima	0.41	0.40	0.78	0.21	1.80
E. koreanum Nakai:					
(1) Cultivated in Saitama	0.54	0.78	0.46	0.76	2.54
(2) Cultivated in Kyoto	0.71	1.18	0.33	0.53	2.75
E. macranthum Morr. et Decne.:					
(1) Cultivated in Saitama	0.36	0.46	0.68	0.61	2.11
E. diphyllum (Morr. et Decne.) Lodd.:					
(1) Cultivated in Kyoto	0.28	0.27	0.31	0.15	1.01
E. grandiflorum Morr. var. *thunbergianum* (Miq.) Nakai:					
(1) Cultivated in Saitama	0.98	0.60	0.17	0.56	2.31
(2) Cultivated in Saitama	0.66	0.48	0.05	0.15	1.34
(3) Cultivated in Kyoto	0.60	0.64	0.45	0.29	1.98
E. grandiflorum var. *higoense* T. Shimizu:					
(1) Cultivated in Hiroshima	0.31	0.56	0.16	0.49	1.52
E. setosum Koidz.:					
(1) Cultivated in Hiroshima	0.40	0.43	0.65	0.14	1.62
E. setosum Koidz. nm. Sasakii Sugimoto:					
(1) Cultivated in Hiroshima	0.31	0.29	0.38	0.19	1.17

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Note

Improved high-performance liquid chromatographic method with fluorimetric detection for the determination of glycerol using an immobilized enzyme column reactor

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On-line post-column derivatization techniques have greatly extended the usefulness of high-performance liquid chromatography (HPLC). Post-column reactors are employed to improve the analytical sensitivity and/or detection selectivity. One of the disadvantages of the post-column techniques is band broadening due to post-column addition of reagents. To overcome this disadvantage, attempts have been made to develop pumpless reaction units[1].

We have previously reported an HPLC method for the determination of glycerol using a post-column reactor containing immobilized glycerol dehydrogenase (E.C. 1.1.1.6)[2]. Polyhydric alcohols were separated on a reversed-phase ODS column, followed by an enzymatic reaction of glycerol with nicotinamide adenine dinucleotide (NAD) and the resulting reduced form of NAD (NADH) was monitored with a fluorescence detector. Three pumps were used in the system, that is, a mobile phase pump, a buffer solution pump and an NAD solution pump. This paper describes a one-pump system for the determination of glycerol using immobilized glycerol dehydrogenase (GDH). Aminobutylpolystyrene beads were used as a support for covalent attachment of GDH.

GDH has been immobilized on the inner surface of nylon tubing and used as a reactor in a continuous-flow system[3,4]. However, an open-tubular reactor is unsuitable as a post-column reactor[5]. In this work the separation of polyhydric alcohols is effected by using a cation exchanger with carbonate buffer solution (pH 10.0) as the mobile phase. The analytical sensitivity and resolution are improved by changing the three- to the one-pump system. The method was applied to the determination of triglycerides in serum.

EXPERIMENTAL

Chemicals

Polystyrene beads (Bio-Beads SX-8, 55 $\pm$ 20 μm) were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.), NAD (grade II) from Boehringer (Mannheim, F.R.G.), GDH (66.6 U/mg of solid, from *Cellulomonas* sp.) from Toyobo (Osaka, Japan) and lipoprotein lipase (1070 U/mg of solid, from *Pseudomonas* sp.) from Amano Pharmaceutrical (Nagoya, Japan). Anhydrous aluminium chloride, 1,4-

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butanediamine and 1,1′, 2,2′-tetrachloroethane were used without further purification. Chloromethyl methyl ether, purchased from Wako (Osaka, Japan), was purified by distillation.

Preparation of the immobilized enzyme column reactor

Polystyrene beads (10 g) were allowed to swell in a mixture of 70 ml of chloromethyl methyl ether and 10 ml of 1,1′, 2,2′-tetrachloroethane for 6 h. Anhydrous aluminium chloride (15 g) was added and the mixture was stirred at 0°C for 1 h. After filtration, the beads were washed successively with tetrachloroethane, methanol, water and methanol and dried at 40°C under vacuum. The chloromethylated beads (10 g) were left to swell in 10 ml of benzene and then 20 ml of 1,4-butanediamine were added. The mixture was refluxed for 10 h.

The beads were removed by filtration and washed successively with benzene, methanol and water. The attached amine was measured by the Kjeldahl method[6] and amounted to 0.81 mequiv. per gram of dry beads.

The butylaminated beads (4 g) were slurried with 50 ml of methanol–water (1:1) and poured into a packing column, which was fitted with a stainless-steel column (150 mm × 4.0 mm I.D.). The methanol–water mixture was pumped through the column for 30 min, maintaining a pressure of 50 kg/cm^2 at room temperature. The stainless-steel column was disconnected from the packing column and connected to the HPLC pump. Glutaraldehyde (2.5%) in phosphate bufer (0.01 *M*, pH 7.0) was pumped through the column for 2 h at a flow-rate of 0.5 ml/min at room temperature, followed by deaerated water for 3 h at a flow-rate of 0.5 ml/min. Enzyme solution (10 ml) containing 10 mg of GDH in phosphate buffer (0.05 *M*, pH 7.0) was circulated through the column for 6 h at a flow-rate of 0.5 ml/min at room temperature. The enzyme solution was kept at 0–4°C in an ice-box throughout the immobilization procedure.

Apparatus

A schematic diagram of the system is shown in Fig. 1. The mobile phase was a mixture of carbonate buffer (sodium hydrogencarbonate–potassium carbonate, 0.4 *M*, pH 10.0) and NAD solution [10 m*M* in the phosphate buffer (0.05 *M*, pH 7.0)], both at flow-rates of 0.35 ml/min. The buffer and NAD solution were pumped by a double-plunger pump (Model KHU-W-52; Kyowa Seimitsu) through an injector (Model KHP-UI-130A; Kyowa Seimitsu) with a 100 μl loop, to a column (300 mm

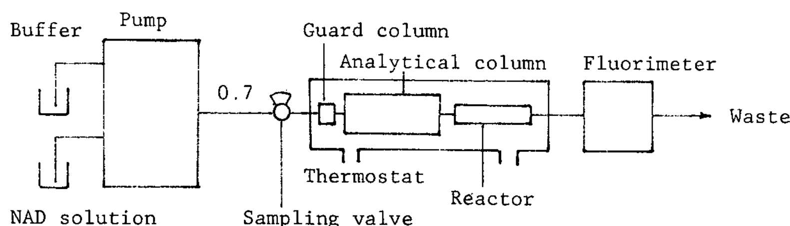

Fig. 1. Schematic diagram of the system. The number on the line is the flow-rate in ml/min. Buffer: sodium hydrogencarbonate–potassium carbonate (0.4 *M*, pH 10.0). NAD solution, 10 m*M* in phosphate buffer (0.05 *M*, pH 7.0). See text for details.

× 7.8 mm I.D.) of TSK gel SCX (5 μm) fitted with a guard column of Shodex Ionpak KS800P (50 mm × 6 mm I.D.). The solution then passed into the stainless-steel column reactor (150 mm × 4.0 mm I.D.). Both the analytical column and the reactor were thermostated at 40 ± 0.2°C. A Hitachi Model 650-10s fluorescence spectrophotometer fitted with a flow cell (18 μl) was operated at λ_{ex} = 340 nm and λ_{em} = 465 nm and the response was monitored on a Hitachi Model 056 recorder.

RESULTS AND DISCUSSION

Choice of support

For an immobilized enzyme column reactor, as the flow-rate is lowered the enzymatic reaction proceeds to completion. However, in general, in the column the peaks become broader at lower flow-rates because the elution times are longer and there is more time for diffusion to spread the peaks. Therefore, one should select a support the grain size of which is as small as possible in order to obtain a higher activity of immobilized enzyme and to minimize excessive peak broadening.

Another phenomenon that affects the shape of the peak in the column reactor is the adsorption of the reaction product on the surface of the support. As small beads have a large surface area, their adsorption capacity is high. TSK gel styrene-250 (grain size 10 μm) was examined as a support for covalent attachment of the enzyme. The column reactor (100 mm × 4.0 mm I.D.) which was packed with the beads gave pronounced peak tailing because of the adsorption of NADH, while the yield of the enzymatic reaction was about 100%.

The degree of cross-linking is also important. At low degrees of cross-linking the beads were crushed in the column reactor at high flow-rates and a gap was produced at the top of the reactor. On the other hand, the amounts of attached amino groups for beads with 2% cross-linking was 4.0 mequiv./g and with 12% cross-linking 0.3 mequiv./g. Bio-Beads (grain size 55 ± 20 μm, degree of cross-linking 8%) were chosen as a compromise. During the use of the column reactor which was packed with the beads, peak broadening was not observed even after 600 samples had been injected.

Evaluation and optimization of the enzyme column reactor

Effect of temperature. The reactor was placed in a water-bath and the temperature was varied between 30 and 50°C to determine the effect of temperature on GDH activity. A standard solution of glycerol (0.1 m*M*) was injected at each temperature. The reactor showed an increase in activity as the temperature was increased (Fig. 2). Although the reactor exhibited the highest activity at 50°C, this temperature reduced the lifetime of the reactor. For routine analysis, the reactor was operated at 40°C.

Effect of pH. The effect of pH on the activity of the immobilized enzyme was examined in the pH range 9.5–11.0. The results indicated a constant activity above pH 10.0 (Fig. 3). pH 10.0 was chosen for the mobile phase buffer. The concentration of carbonate buffer did not influence the peak height in the range 0.01–0.3 *M*.

Effect of concentration of NAD. Under the conditions used the Michaelis constant of the immobilized enzyme for NAD was $2 \cdot 10^{-4}$ *M*. With an excess of NAD (above $2 \cdot 10^{-3}$ *M*), the rate of the enzymatic reaction was independent of the concentration of NAD. We used an NAD concentration of 5 m*M* to shift the

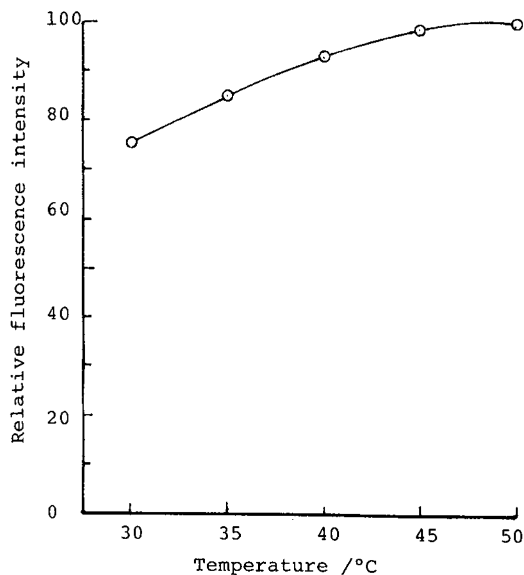

Fig. 2. Effect of temperature on the activity of immobilized glycerol dehydrogenase.

equilibrium of the reaction to the right. A higher concentration of NAD was undesirable for the chromatographic separation of polyhydric alcohols.

Size of reactor. In post-column HPLC systems using immobilized enzymes in a post-column reactor, it is difficult to optimize the size of the reactor. Generally, the selection of the reactor involves a compromise between obtaining an adequate

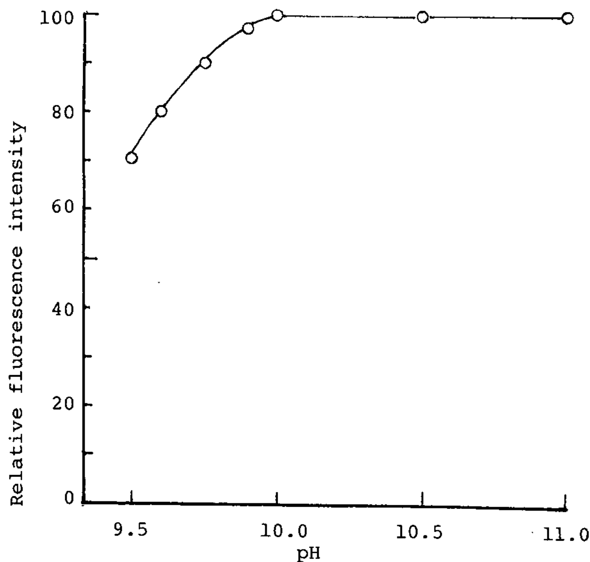

Fig. 3. Effect of pH on the activity of immobilized glycerol dehydrogenase.

sensitivity and avoiding peak broadening. As pointed out by Shih and Carr[7], a fast reaction in the reactor gives the negligible peak broadening. As the rate of enzymatic reactions is directly proportional to the activity of the enzyme, a decrease in activity causes an increase in peak width. To obtain immobilized GDH with higher activity levels and to use it for long periods in practical analyses, we selected a column of length 15 cm and I.D. 4 mm. By using this column reactor, peak broadening was controlled by the chromatographic column, and the contribution of the reactor to the peak broadening was about 10% of the peak width. The reactor could be used for 2 months without pronounced peak broadening. A column of length 5 cm could be used for about only 2 weeks in a similar manner to the above; this reactor was not suitable for practical use because of its short lifetime.

The specificity and storage stability of immobilized GDH were similar to those reported previously[2].

Separation

GDH oxidizes not only glycerol but also ethylene glycol, propylene glycol and 1,2-butanediol[2]. In certain instances, samples contain compounds that fluoresce at the monitored wavelength. Alcohols and other such compounds must be separated from glycerol. Ion exchangers were screened for their ability to separate polyhydric alcohols at higher pH (about 10). A good separation was achieved with the anion exchanger TSK gel SAX (5 μm) (150 mm × 6 mm I.D. column) with borate buffer (0.1 M, pH 9.5) as the mobile phase. The retention times of ethylene glycol, glycerol, propylene glycol and 1,2-butanediol were 7.1, 8.5, 12.0 and 14.3 min, respctively. However, the borate complexes of the alcohols were too stable to act as substrates of the enzyme.

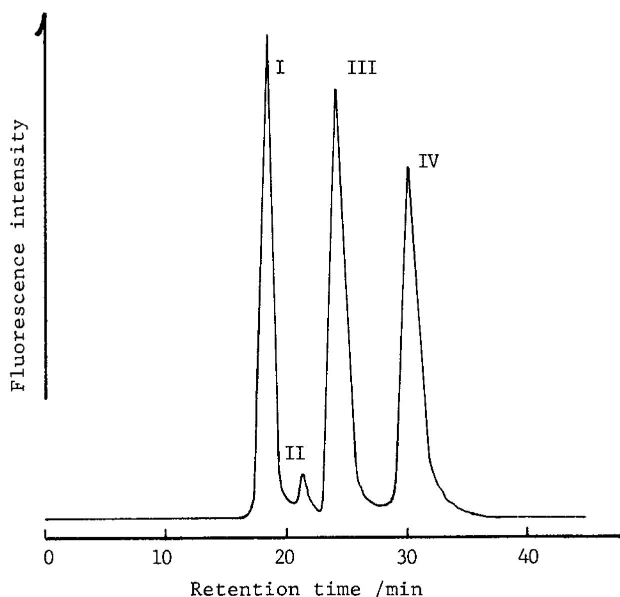

Fig. 4. Chromatogram of (I) glycerol, (II) ethylene glycol, (III) propylene glycol and (IV) 1,2-butanediol. Sample, 50 nmol of each in 100 μl. Mobile phase, 0.2 M carbonate–5 mM NAD (pH 10). Flow-rate, 0.7 ml/min. Column, TSK gel SCX (5 μm) (300 × 7.8 mm I.D.) at 40°C. Fluorescence monitored at 340/465 nm.

TABLE I

SEPARATION PARAMETERS FOR POLYHYDRIC ALCOHOLS

Alcohol	*Retention time (min)*	*Capacity factor*	*Resolution*	*Separation factor*
Glycerol	18.4	0.28		
			1.27	1.70
Ethylene glycol	21.2	0.37		
			1.08	1.41
Propylene glycol	24.0	0.67		
			1.89	1.63
1,2-Butanediol	30.0	1.08		

The separation of a mixture of the polyhydric alcohols into its components was effected by the cation exchanger TSK gel SCX (5 μm) (300 mm × 7.8 mm I.D. column) with carbonate buffer (0.2 *M*, pH 10.0) as the mobile phase, as shown in Fig. 4. The separation parameters of the alcohols are given in Table I.

The resolution was not influenced by the presence of NAD at concentrations below 5 m*M* in the carbonate buffer. The carbonate buffer (0.4 *M*, pH 10.0) and NAD solution [10 m*M* in 0.05 *M* phosphate buffer (pH 7.0)] were applied at equal flow-rates and were combined before the injector because NAD is unstable in a basic solution. The optimal flow-rate for good sensitivity and reasonable resolution was 0.70 ml/min.

The chromatographic column was thermostated at 40°C. At a column temperature of 60°C the chromatographic peak was sharper than at 40°C. However, NAD was decomposed to some extent, leading to a smaller analytical peak. On the other hand, at 20°C, the resolution was inferior to that at 40°C.

There is a linear relationship between the peak height and the concentration of glycerol and calibration graphs were prepared for the range 0.005–0.5 m*M*. The detection limit was 0.001 m*M* of glycerol.

Application

Table II gives results for the determination of triglycerides in serum (Precilip; Boehringer). The serum (100 μl) was added to 1.0 ml of phosphate buffer (0.1 *M*, pH 7.0) containing lipoprotein lipase (1000 U) and incubated for 15 min at 35°C. The supernatant liquid was withdrawn from the suspension with a syringe through a guard column (pore size 0.45 μm). An aliquot (100 μl) of the solution was injected into the

TABLE II

RESULTS FOR TRIGLYCERIDES IN CONTROL SERA

Serum sample	*Triglycerides found (mM)*	*Relative standard deviation (n = 10) (%)*		*Certified value (mM)*
		Within day	*Between-day*	
Lot 1-375	1.52	3.2	3.6	1.54
Lot 151 662	2.11	3.0	3.6	2.10

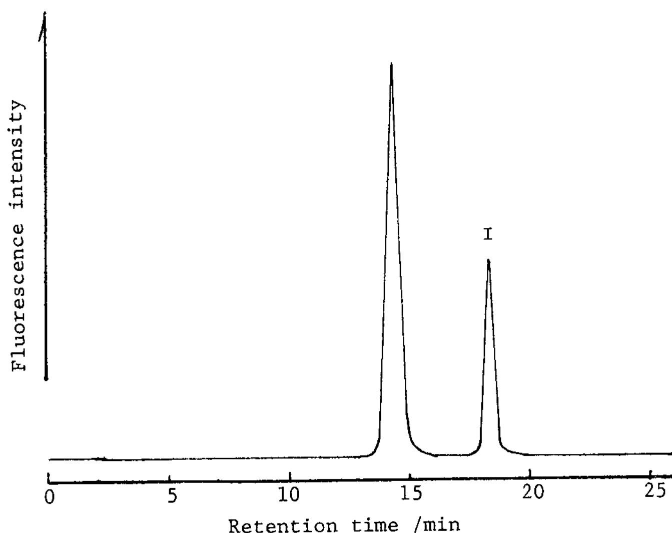

Fig. 5. Chromatogram of serum. Peak I corresponds to glycerol. Analytical conditions as in Fig. 4.

column. The concentration of triglycerides was calculated from the calibration graphs for glycerol. A typical chromatogram for the serum is shown in Fig. 5.

CONCLUSION

The HPLC system with an immobilized GDH column reactor was highly selective for the determination of glycerol. Glycerol has previously been determined fluorimetrically by reversed-phase chromatography using a post-column immobilized reactor[2] and the detection limit was 0.01 m*M*. The use of the pumpless reagent unit (one-pump system) provided a 10-fold increase in sensitivity.

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Note

Determination of L-canavanine and L-canaline in plant tissues by high-performance liquid chromatography

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The amino acid L-canavanine, an analogue of L-arginine, is accumulated in the seeds of several legumes[1]. In some species the L-canavanine content is over 12% of the seed dry weight, representing more than 95% of the free amino acid nitrogen[2]. This amino acid can be translocated from the cotyledons to the growing seedling tissues[3] and metabolized by arginase to L-canaline and urea[4]. Both L- canavanine and L-canaline are potent antimetabolites, toxic to different types of organisms[5], and accordingly roles have been proposed for L-canavanine as a nitrogen-storing metabolite and as a defence against predator insects[5].

In most studies L-canavanine has been determined using the method reported by Fearon and Bell[6]. However, this method lacks specificity and is relatively insensitive. A method with improved sensitivity has been reported[7], based on the formation of a fluorescent ring system between guanidino compounds and phenanthrenequinone. However, interference by L-arginine cannot be discounted. This method was further improved by the separation of the amino acids using ion-exchange chromatography and post-column derivatization[8].

An indirect spectrophotometric method has been reported for the determination of L-canaline[9], but to our knowledge no data obtained by this method have been published. Indeed, very little information is available concerning L-canaline in plants.

In order to understand the importance of L-canavanine and its metabolites in plants, an appropriate method for their determination is required. This paper describes a method for the simultaneous determination of L-canavanine and L-canaline in samples containing other amino acids.

EXPERIMENTAL

Instrumentation

The equipment consisted of a Varian Model 5060 single-pump liquid chromatograph capable of gradient formation, a Varian Fluorichrom detector set with Corning filters 7-60 and 7-54 (excitation) and 4-76 and 3-71 (emission) and a Varian Vista 401 chromatography data system.

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Chemicals

Methanol (HPLC grade), acetone and acetonitrile (analytical-reagent grade) were obtained from Merck (Mexico). Dansylated and underivatized amino acids and dansyl chloride were obtained from Sigma (St. Louis, MQ, U.S.A.). All other chemicals were obtained from J. T. Baker (Mexico). Water was distilled twice before use.

Columns

Two reversed-phase (C_{18}) columns were tested: a MicroPak MCH-10, particle size 10 μm (30 cm × 4 mm I.D.), from Varian and a Hibar C_{18}, particle size 5 μm (25 cm × 4 mm I.D.), from Merck.

Derivatization

An aliquot of standard amino acid solution or plant extract (usually 20 μl) was mixed with 0.1 *M* sodium hydrogencarbonate (pH 10.5) to give a final volume of 100 μl, then 50 μl of a 1.5 mg/ml solution of dansyl chloride in acetone were added and the mixture was shaken vigorously and incubated for 90 min at room temperature. A volume of 30 μl was loaded into the 10-μl loop injector.

Extraction

Canavalia ensiformis (L.) DC. seed parts were finely ground and then shaken for 10 min in water (10 ml/g). The extracts were centrifuged (2000 *g*) for 10 min and the resulting supernatant was extracted with an equal volume of chloroform and centrifuged (2000 *g*) for 10 min. The chloroform extraction was repeated and the aqueous phase was recovered and filtered through a Millipore HAWP filter (0.45 μm). All operations were carried out at 4°C. The extracts were then analysed immediately or stored at −20°C.

Elution gradient

Mobile phase A was methanol in 0.01 *M* Tris–HCl (pH 7.7) (5:95) and mobile phase B was methanol. The gradient used was from 0 to 47% B in 70 min and then from 47 to 100% B in 10 min. Re-equilibration to the initial gradient conditions took 30 min.

RESULTS AND DISCUSSION

There are no previous reports on the dansylation of L-canavanine or L-canaline. The initial protocol used dansyl chloride dissolved in acetonitrile. A dansylated derivative was obtained with L-canavanine but not with L-canaline. However when acetone was used as the solvent, dansylated derivatives of both amino acids were obtained (Fig. 1).

The separation of a mixture of 23 dansyl amino acids, including dansyl-L-canavanine and dansyl-L-canaline, was compared using two different columns, Hibar C_{18} and MicroPak MCH-10. Preliminary tests showed that the resolution was better with the Hibar C_{18} column (data not shown) and all subsequent work was carried out with this column.

The separation of a mixture of dansyl-L-canavanine and dansyl-L-canaline was easily achieved (Fig. 1). However, in a mixture containing the dansyl amino acids

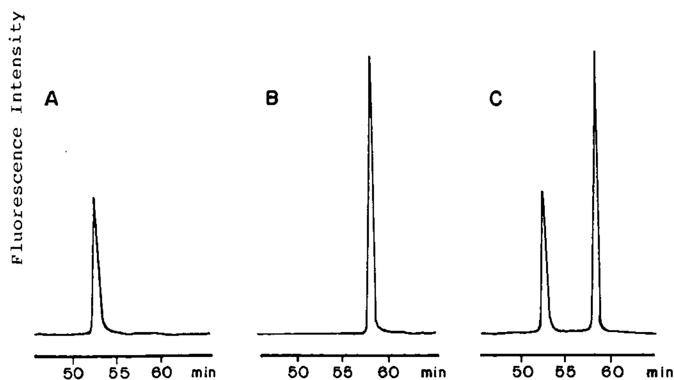

Fig. 1. Reversed-phase HPLC of (A) dansyl-L-canavanine, (B) dansyl-L-canaline and (C) dansyl-L-canavanine with dansyl-L-canaline.

listed in Table I, the best separation was obtained with a gradient from 0% to 47% of solvent B in 70 min and from 47% to 100% in the following 10 min. A typical chromatogram is shown in Fig. 2 with the corresponding retention times listed in Table I. The elution time is long and one sample can be injected every 110 min, but shorter elution times, using steeper gradients, or starting with a higher proportion of

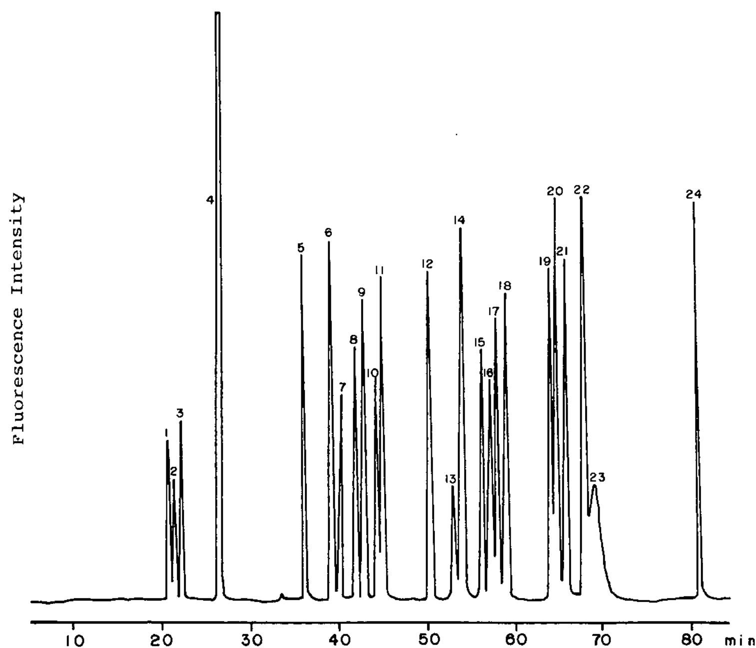

Fig. 2. Gradient separation of dansyl amino acids by reversed-phase HPLC. (1) Dns-Cys; (2) Dns-Asp; (3) Dns-Glu; (4) Dns-OH; (5) Dns-Asn; (6) Dns-Gln; (7) Dns-Ser; (8) Dns-Gly; (9) Dns-Thr; (10) Dns-His; (11) Dns-Ala; (12) Dns-GABA; (13) Dns-canavanine; (14) Dns-Pro; (15) Dns-Val; (16) Dns-Arg; (17) Dns-Met; (18) Dns-canaline; (19) Dns-Ile; (20) Dns-Try; (21) Dns-Leu; (22) Dns-Phe; (23) Dns-Lys; (24) DNS-Tyr.

TABLE I

RETENTION TIMES OF DANSYL AMINO ACID STANDARDS

Amino acid	*Retention time (min)*	*Amino acid*	*Retention time (min)*
Cysteine	20.9	Proline	54.0
Aspartic acid	21.5	Valine	56.5
Glutamic acid	22.5	Arginine	57.3
Asparagine	36.2	Methionine	58.0
Glutamine	39.3	Canaline	58.9
Serine	40.2	Isoleucine	64.0
Glycine	42.4	Tryptophan	64.7
Threonine	43.0	Leucine	65.7
Histidine	44.6	Phenylalanine	67.9
Alanine	45.0	Lysine	69.0
δ-Aminobutyric acid	50.4	Tyrosine	69.0
Canavanine	52.8		

solvent B, always gave a poorer separation of certain derivatives, including dansyl-L-canaline.

The retention times were highly reproducible: 52.8 ± 0.15 min for dansyl-L-canavanine and 58.5 ± 0.18 min for dansyl-L-canaline (data calculated from nine consecutive chromatograms performed within 48 h). Calibration graphs were prepared for L-canavanine and L-canaline. A linear response was obtained wihtin the ranges 0.15–5 and 0.15–3.6 nmol for dansyl-L-canavanine and dansyl-L-canaline, respectively. The detection limits (signal-to-noise ratio = 2) were 50 pmol for dansyl-L-canavanine and 10 pmol for dansyl-L-canaline.

This method was applied to the determination of L-canavanine and L-canaline in seed parts of *C. ensiformis* (Table II). L-Canaline was not detected in any of the parts studied, in contrast to results reported by Rosenthal[10].

Using a paper chromatographic method, Rosenthal detected L-canaline in jack bean cotyledons 24 h after the start of imbibition. The absence of L-canaline in our

TABLE II

CONTENT OF L-CANAVANINE AND L-CANALINE IN SEED PARTS OF *CANAVALIA ENSIFORMIS*

The results shown are averages of analyses of three different samples. Figures in parentheses correspond to mg per pair of cotyledons or mg per embryo.

Part	*Concentration (mg/g fresh weight)*	
	L-canavaline	*L-canaline**
Dry cotyledon	27.0 (48.6)	N.d.
Dry embryo	32.0 (0.45)	N.d.
Hydrated cotyledon**	17.5 (45.5)	N.d.
Hydrated embryo**	7.6 (0.45)	N.d.

* N.d. = not detected.
** After 24 h imbibition.

extracts cannot be explained by an insufficient recovery of L-canaline by the extraction method used, as the recovery of authentic L-canaline added to tissue samples before extraction was 95% (average of two results). On the other hand, we observed that if samples were not adequately stored the L-canavanine peak decreased with a concomitant appearance of an L-canaline peak.

The content of L-canavanine in dry seeds has been previously reported[6,11,12] to range from 25 to 31 mg/g. The analysis reported here gave a content of 27 mg/g. With the dry embryo there have been no previous reports; the L-canavanine content was found to be similar to that of dry cotyledons (Table II). This seems logical, assuming a defence role for L-canavanine, as reduced levels of this amino acid would make the embryo more susceptible than cotyledons to insect attack. The content is nevertheless very high and the total amount present in the embryo after imbibition remains constant for at least 24 h. It will be interesting to study how embryo tissues deal with this highly toxic amino acid. The content of L-canavanine in cotyledons also showed very little change after 24 h of imbibition.

CONCLUSION

A method has been developed that can determine L-canavanine and L-canaline simultaneously in mixtures containing all the common amino acids. Further, it has potential for the determination of each of the common amino acids.

ACKNOWLEDGEMENT

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Note

Determination of carboxylic acids by liquid chromatography after phase-transfer-catalysed fluorogenic labelling

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Owing to the biological importance of carboxylic acids there is a great need for sensitive methods for their determination. For liquid chromatography a number of methods of preparing fluorescent ester derivatives have been reported[1], many based on reactions either with aryldiazomethanes[2–4] or with activated halogen compounds[5–13]. In the latter instance the analyte has to be present in ionized form, which means that solubilization of the carboxylate and the reagent has to involve either a mixed solvent system or a two-phase system incorporating a phase-transfer agent such as a crown ether or tetraalkylammonium ion. Although extractive alkylations of the latter type have been used for preparative purposes, investigations on their application to the analytical-scale derivatization of carboxyl groups seem to be limited to the fundamental studies published by Gustavii and Furängen[14–18]. A paper describing the dansylation of phenolic compounds via phase transfer of this kind has also recently appeared[19].

In general, however, very little use has been made of this type of pre-column derivatization, despite great progress in the synthesis of new fluorogenic reagents. The method of choice is the use of a solid potassium carbonate–crown ether–acetonitrile system[4,7,9–13].

This paper describes how the tetrabutylammonium-mediated transfer of the analyte as an anion into ethylene dichloride containing the fluorogenic reagent can be applied for pre-concentration with simultaneous derivatization.

EXPERIMENTAL

Reagents and chemicals

Alkanoic acids, tetrabutylammonium hydrogen sulphate (TBA) and 2-naphthacyl bromide (NABr) were obtained from Fluka (Buchs, Switzerland). 2-(Bromoacetyl) fluorene was prepared by direct bromoacetylation of fluorene in the presence of aluminium chloride in carbon disulphide at $-5°C$ according to Sargent and Small[20]. The crude product was repeatedly recrystallized from acetone; thin-layer chromatography [silica gel plate; toluene–ethyl acetate (70:30)] gave a single spot; m.p. 142–143°C (lit.[20] m.p., 147–149°C).

The following reagent solutions were prepared in order to study the derivatization kinetics. Alkanoic acids were used as solutions in 50 m*M* phosphate buffer

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(pH 8.1) of varying concentrations. TBA was used as a 10 mM solution in water. NABr and 2-(bromoacetyl) fluorene were used as 10 mM solutions in ethylene dichloride (b.p. 84°C).

Procedure for extractive derivatization with formation of 2-naphthacyl esters

The kinetics of the 2-naphthacyl ester formation were determined by the use of 10-ml screw-capped vials to each of which were added 2 ml of a solution of the alkanoic acid(s) (50μM–5 mM) in 50 mM buffer, 1 ml of TBA solution and 2 ml of NABr solution. The vials were stirred vigorously at the reaction temperature chosen and taken for further processing at different times. After rapid cooling, the vial was centrifuged to separate the phases and 10 μl of the clear organic phase were diluted with acetonitrile to 0.1–1.0 ml prior to injection into the liquid chromatographic (LC) system. The degree of dilution was dependent on the initial alkanoic acid concentration.

The concentration experiments were performed by using varying concentrations of 10 μmol of alkanoic acid in each derivatization. The initial concentrations used were 0.1–5.0 mM, corresponding to volumes between 100 and 2 ml. These reactions were performed at 60°C for 1 h.

Procedure used to obtain other ester derivatives

By the use of the same technique of extractive alkylation, derivatization with two other aracyl bromides was tried, *viz.*, with 9-anthracyl bromide and 2-(α-bromoacetyl) fluorene. Derivatives were readily obtained only with the latter reagent.

Reduction of the aracyl esters obtained in the organic phase was performed by shaking the sample with an excess of solid sodium borohydride for a few minutes at room temperature prior to dilution.

Liquid chromatography

The LC apparatus was composed of an LKB 2150 high-pressure pump, a Rheodyne 7120 injection valve equipped with a 10-μl loop, a 100 × 2.6 mm I.D. stainless-steel column packed with Nucleosil-100 C_{18} (5 μm), an ISCO V^4 variable-wavelength UV detector and a Waters 740 electronic integrator. Fluorimetric detection was carried out by means of a Shimadzu RF-510 LC spectrofluorimeter equipped with a 10-μl flow cell. In this instance, the mobile phase was delivered by a Waters Model M 45 high-pressure pump.

The mobile phase was acetonitrile (HPLC grade, Rathburn Chemicals, Walkerburn, U.K.)–water (9:1) at a flow-rate of 1.5 ml/min, unless indicated otherwise.

Removal of excess of reagent by thiolate–silica

Preparation of the sorbent. A modification of the method of Pirkle and House[21] was used. Silica (Polygosil 60Å, 63–100 μm; Macherey, Nagel & Co., Düren, F.R.G.) was first treated in boiling toluene for azeotropic removal of water. Then, an excess of 3-mercaptopropyltrimethoxysilane (Serva, Heidelberg, F.R.G.) was added and the mixture kept at *ca.* 100°C for 5 h with stirring. After cooling the thiol–silica was isolated by filtration, washed carefully with toluene and diethyl ether and dried. To activate the sorbent, *i.e.*, to convert it into the sodium thiolate form, it was treated with an excess of saturated sodium carbonate (pH 10.4), filtered and washed succes-

sively with water, acetone and diethyl ether. The capacity of the dry sorbent was *ca.* 300 μmol/g.

Elimination of reagent. Solutions containing different concentrations of bromomethyl reagent and ester in ethylene dichloride were shaken with an excess of the thiolate–silica for 5 min at 50°C. The concentration changes were followed by determination of peak areas after injection of the supernatant into the LC system.

RESULTS AND DISCUSSION

Effect of pH, phase-transfer reagent concentration and temperature on the yield of labelled product

As preliminary experiments showed that the extractive labelling was preferably carried out in a buffer–ethylene dichloride two-phase system, the effect of buffer pH was then investigated. Under the conditions used it was found that the rate of ester formation increased with increasing pH, and that phosphate or borate buffers of pH between 8 and 9.5 were suitable. Control experiments without a phase-transfer reagent yielded almost no ester.

As expected, the rate of the overall reaction increased with increasing concentration of the phase-transfer reagent. However, at a reaction temperature of 80°C and a buffer pH of 8.05, complete derivatization of 10μmol of palmitic acid was obtained after 10 min even when the molar ratio of phase-transfer reagent to acid was reduced from 1:1 to 1:5.

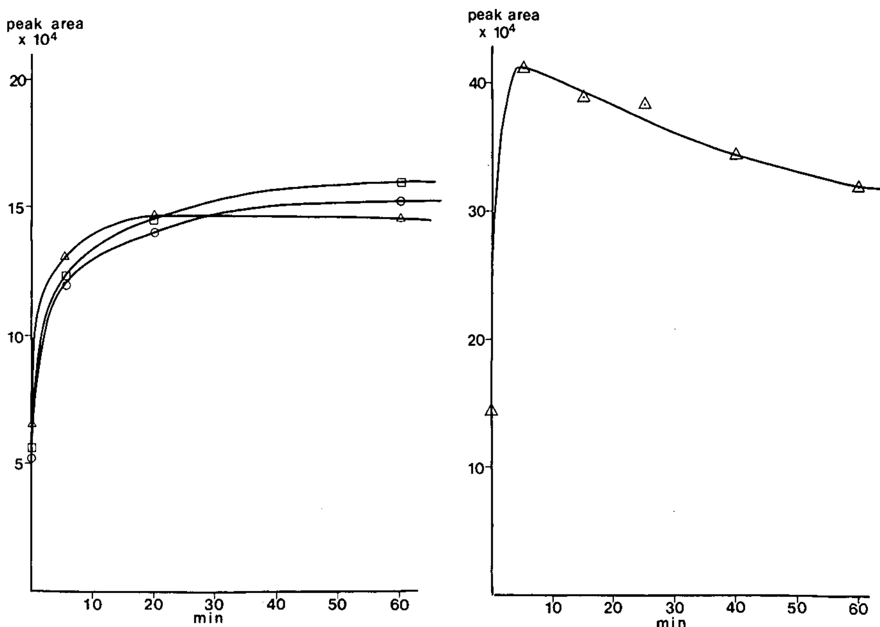


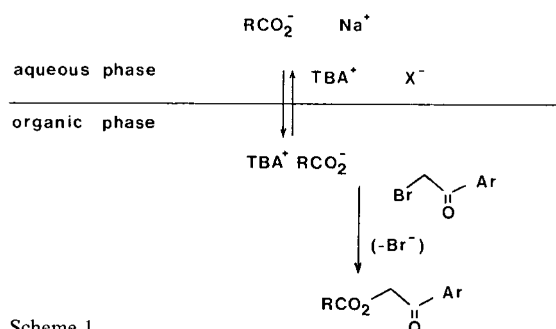
Fig. 1. Rate of 2-naphthacyl ester formation at different temperatures. Left: 60°C, (△) C_{12}, (□) C_{14}, (○) C_{16} acids, equimolar mixture of total concentrations 5.0 m*M*. Right: 100°C, C_{16} acid, 5.0 m*M*. The aqueous to organic phase volume ratio was 1.5 (see Experimental) and a 100-fold dilution with acetonitrile prior to injection was used.

Kinetic studies of the ester formation at 60 and 100°C showed that the reaction was complete after 60 and 5 min, respectively (Fig. 1). However, a slight decrease in ester concentration is observed on prolonged heating at 100°C. Therefore, a reaction temperature of 80°C was considered optimal and the derivatization was best carried out in a closed reaction vial with efficient mixing by warming for 15 min on a water-bath kept at 80°C.

The concentration decrease found at 100°C is probably due to a competing hydrolysis reaction caused by the alkaline medium. Compared with the rate of esterification, however, this hydrolysis rate is slow enough to be tolerated under the derivatization conditions suggested here.

Extractive concentration of the analyte from dilute solution

When a series of palmitic acid solutions of decreasing concentration was studied with respect to the formation of 2-naphthacyl esters using the technique described, it was found that quantitative yields of ester were obtained even at phase ratios of buffer to ethylene dichloride as high as 100 or more. Therefore, provided that adequate mixing between the phases is ensured, a substantial concentration effect can be achieved. It is obvious that the reaction takes place in the organic phase, where it is highly accelerated owing to the desolvation of the carboxylate anion. The total process is outlined in Scheme 1. Owing to the preference of both the reagent and the ester for the organic phase, the carboxylate anion will be continuously removed from its equilibrium between the phases by its fast reaction in the organic phase.



Scheme 1.

The reaction in the organic phase is kinetically of second order and therefore dependent on the actual concentration of the anion (as the TBA ion pair). This is determined by the partition coefficient of the ion pair which, in turn, depends on the lipophilicity of the anion part. Consequently, the derivatization should be slower for the lower homologues in a series. However, from the applications point of view, this effect will be unimportant as long as the reaction conditions are chosen such as to ensure completeness of the reaction.

Thorough investigations of the kinetics of the tetraalkylammonium-mediated alkylation of carboxylic acids in two-phase systems at 25°C have been published[16,18].

Certain other advantages are inherent in the technique. Often, when derivatizations of protolytes are carried out in water or mixed aqueous solvents, there is a strong competition between the analyte and hydroxide ions for the reagent. At low analyte concentrations this may lead to substantial by-product formation even if a

pH is chosen to minimize solvolysis of the reagent. Owing to the low extractability of hydroxide ions even as TBA ion pairs into an organic phase, such by-product formation is negligible with the present method.

Elimination of excess of derivatization reagent

Kinetic studies of the nucleophilic substitution reaction between the solid-phase reagent, thiolate–silica, and the derivatization reagent showed the reaction to be fast enough to be useful for convenient and complete removal of the excess of the latter. This very simple procedure consisted in adding an excess of the thiolate–silica to the ethylene dichloride phase after completion of the derivatization reaction, followed by stirring at 50°C for 5 min. Dilution of the supernatant with acetonitrile and injection on to the column gave a reagent-free chromatogram with no loss of ester product.

Because in our system the reagent peak did not interfere with the analytes, reagent peak elimination was not essential. The technique should be most useful, however, when dealing with less retained analytes.

Fluorogenic labelling and sensitivity

The 2-naphthacyl esters possess excellent UV-absorbing properties ($\varepsilon = 1.2 \cdot 10^4$ at 248 nm)[22], but give no useful fluorescence. The sensitivity achieved by UV detection is illustrated in Fig. 2, which shows a chromatogram of the C_{12}, C_{14} and C_{16} acids obtained by the technique described. The detection limit (signal-to-noise ratio = 2) was calculated to be 1–3 pmol. However, for many applications fluorogenic ester derivatives are desirable because of the gain in both sensitivity and selectivity. On attempting to extend the procedure to the use of other aryl bromomethyl ketones

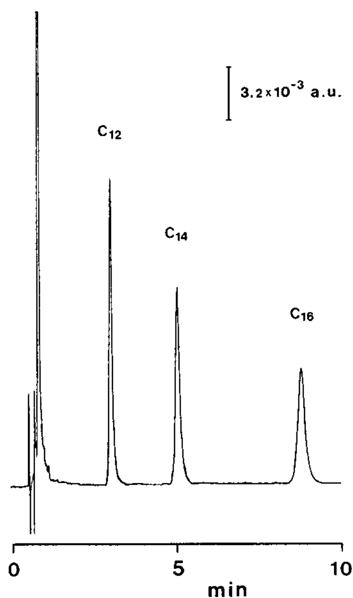


Fig. 2. Chromatogram showing the 2-naphthacyl esters of an equimolar mixture of C_{12}, C_{14} and C_{16} acids. UV detection 235 nm. Each peak represents 167 pmol. Mobile phase: acetonitrile–water (9:1).

as reagents, we found that 9-anthracyl bromide and 2-(α-bromoacetyl) fluorene, both of which are relatively readily available, could not be used with success. Application of the first of these gave only traces of ester derivative. This might be due to a too low reactivity, possibly because of steric hindrance. This assumption is supported by earlier results, showing the failure of this compound to react with amine nucleophiles[23]. Application of the second reagent gave a quantitative yield of the corresponding ester under the conditions given. The fluorescence yield of this product was low, however, owing to the perturbation of the fluorene π-electron system by the conjugated carbonyl group. This effect was demonstrated by reduction of the carbonyl group with sodium borohydride. The ester alcohol produced had excellent fluorescence properties but, unfortunately, two products were obtained from each non-reduced species, thus giving two peaks for each analyte. The result is shown in Fig. 3. The reason for this behaviour has not yet been elucidated.

In order to make efficient use of aracyl esters of the type described, a larger aromatic π-system is desirable. Therefore, the recently described use of 1-bromoacetylpyrene as reagent[11] is of great interest as strong fluorescence (excitation at 360 nm, emission at 450 nm) has been reported for these ester derivatives.

It also seems very reasonable that substituted bromomethylcoumarins[10] and related reagents[13] can be successfully used in this phase-transfer reagent mediated derivatization procedure. Work is in progress to sustantiate this point.

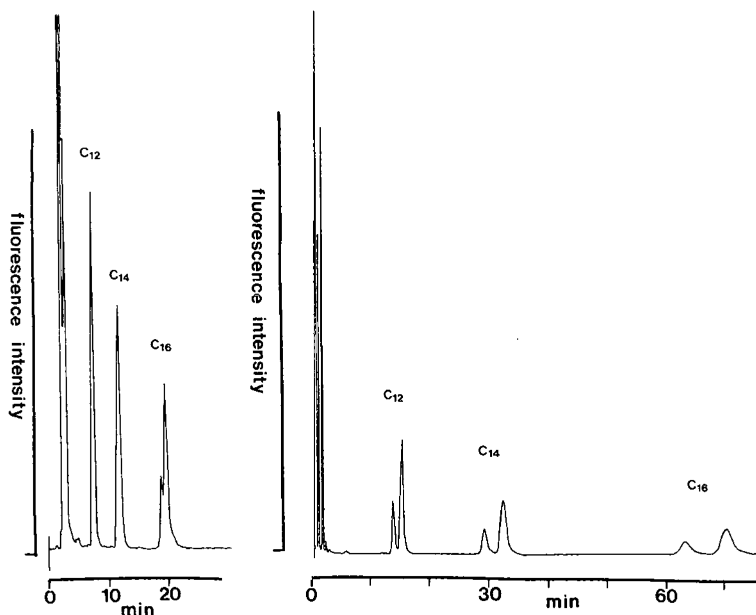


Fig. 3. Chromatograms of an equimolar mixture of C_{12}, C_{14} and C_{16} acids after derivatization with 2-(bromoacetyl)fluorene and reduction with sodium borohydride. Mobile phase: left, acetonitrile–water (9:1); right, acetonitrile–water (7:3). $\lambda_{ex.}$ and $\lambda_{em.}$ = 271 and 313 nm, respectively.

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Note

Einfache Probenvorbereitung zur quantitativen "Multiple-Headspace"-Bestimmung flüchtiger Komponenten mit Hilfe von Adsorptionspatronen

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(Eingegangen am 24. Juni 1988; geänderte Fassung eingegangen am 7. September 1988)

Die für die kapillare Gaschromatographie mit Thermodesorption arbeitenden neu eingeführten Probengeber wie Microwave Sampler MW-1 (J. Rektorik, Genève, Schweiz), Thermal Desorption AutoSampler TDAS (Carlo Erba Strumentazione/Brechbuehler, Schlieren, Schweiz) und Automatic Thermal Desorption System ATD 50 (Perkin-Elmer) bieten gegenüber herkömmlichen Methoden zahlreiche Vorteile: keine Verwendung organischer Lösungsmittel, tiefere Nachweisgrenze, beträchtliche Zeit- und Arbeitseinsparung bei der Probenvorbereitung, einfache und ungefährliche Handhabung, Ortsunabhängigkeit bei der Probenahme usw.[1–6]. Für die halbquantitative und quantitative Bestimmung von flüchtigen Stoffen müssen jedoch genau kontrollierbare und reproduzierbare Arbeitsbedingungen wie z.B. Aufladezeit der Patrone, Trägergasdurchfluss und -volumen, Temperatur und Druck streng eingehalten werden. Die entwickelte Apparatur ermöglicht es, auch in kleinen Laboratorien solche Arbeitsbedingungen zu schaffen.

APPARATUR

Die Apparatur besteht aus folgenden Teilen (siehe Fig. 1): Pos. 1: thermostatisierbares Gefäss aus Klarglas (ähnlich wie Titriergefäss Typ 6.1418.250 von Metrohm, Herisau, Schweiz). Pos. 2: Gefässoberteil mit 3 oder 4 Hälsen NS 14/23 aus Klarglas (Gesamtvolumen: z.B. 285 ml). Pos. 3: Dichtungsring aus Silikonkautschuk mit Metall-Schnellverschluss. Pos. 4: Magnetrührwerk mit -rührstäbchen. Pos. 5: Glaswollebausch aus silanisiertem Material. Pos. 6: Adsorptionspatrone: (a) leer für die Sättigungsstufe des Dampfraums; (b) mit Aktivkohle, Graphitpulver oder Tenax[R] für die Analyse (je nach Hersteller des verwendenten Thermodesorptionsofens). Pos. 7: Tauchrohr. Pos. 8: Kontrollmanometer mit Wassersäule (U-Rohr). Pos. 9: umgebaute automatische Titrierbürette (z.B. Dosimat E 535, Metrohm): Sie saugt mit einstellbarer Geschwindigkeit und schaltet bei den Anschlägen nicht aus. Bürettenvolumen: z.B. 50 ml für offenen Kreislauf oder 10 ml für geschlossen Kreislauf. Pos. 10: Kolbenhubzählwerk. Pos. 11: Thermostat mit Umwälzpumpe (Temperatur = T_1), Arbeitstemperaturbereich: *ca.* 20–60°C. Pos. 12: 4-Weghahn aus PTFE: (o) positioniert im offenen Kreislauf, (g) positioniert im geschlossenem Kreislauf. Pos. 13: Stickstoff-Einleitungsrohr mit Druckausgleich. Pos. 14: Wärmeschrank (Temperatur = $T_2 > T_1$: z.B. $T_2 = T_1 + 5$°C).

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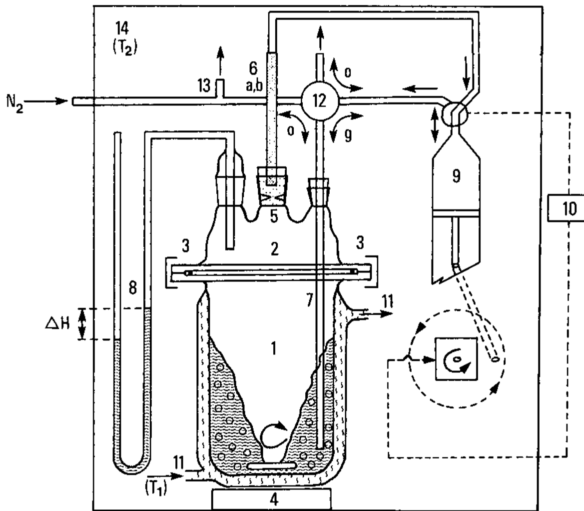


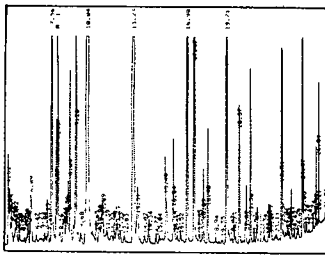
Fig. 1. Apparatur für die Isolierung und Konzentrierung flüchtiger Komponenten mittels Adsorptionspatronen.

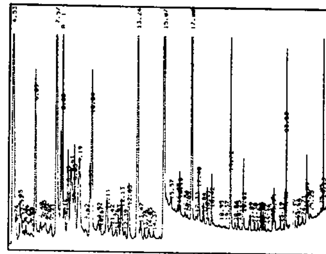
ARBEITSPRINZIP

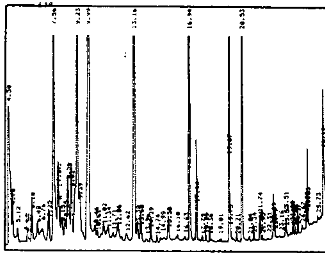
Der Dampfraum eines gasdichten (Pos. 3), thermostatisierten (Pos. 11) und unter Atmosphärendruck stehenden Glasgefässes (Pos. 1) wird zuerst mit den flüchtigen Komponenten einer flüssigen oder in Wasser fein dispergierten Probe gesättigt (Gleichgewicht Dampf- und Probephase). Dazu wird langsam und regelmässig das 1–2-fache Gasvolumen des Dampfraumes mit Hilfe einer umgebauten Titrierbürette (Pos. 9) durch eine leere Adsorptionspatrone (Pos. 6a) gesaugt. Arbeitet das System im offenen Kreislauf (Pos. 12/o), wird dabei ein äquivalentes, auf Atmosphärendruck eingestelltes Ausgleichsvolumen von reinem Stickstoff (Pos. 13) in das Gefäss durch das Tauchrohr (Pos. 7), unter sorgfältigem Rühren (Pos. 4) des Messgutes angesaugt. Das durch die Bürette aufgesaugte Dampfraumvolumen wird kurz nachher in die Atmosphäre ausgestossen. Arbeitet das System im geschlossenen Kreislauf (Pos. 12/g), wird dieses Volumen durch dasselbe Tauchrohr wieder in das Probengefäss zurückgeführt. Für die Analyse wird dann die leere Adsorptionspatrone (Pos. 6a) gegen eine gefüllte (Pos. 6b) ausgetauscht (Sovirel Schraubdeckel/Durchführung SVL 15 mit PTFE-Dichtung). In gleicher Weise wird diese mit einem genau bestimmten Dampfraumvolumen (d.h. Pos. 9 × Pos. 10) im offenen, resp. geschlossenen Kreislauf aufgeladen. Schliesslich wird die geladene Patrone herausgenommen, in den entsprechenden GC-Probengeber (MW-1, TDAS 5000 oder ATD 50) eingeführt, desorbiert und analysiert.

Mit dem mit Wasser gefüllten Kontrollmanometer (Pos. 8) lässt sich gleichzeitig die Dichtigkeit der Apparatur überprüfen sowie das Tauchrohr (Pos. 7) auf Verstopfung kontrollieren. Um zu grosse Druckschwankungen im System bei der Aufsaugphase (nur im geschlossenen Kreislauf) zu vermeiden, soll das Volumenverhältnis Bürette/Dampfraum klein (z.B. $< 5:100$) gehalten werden. Der

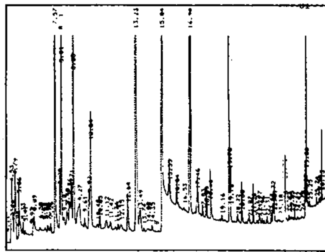
Glaswollebausch (Pos. 5) und ein geringer Antischaummittelzusatz in der Probephase (z.B. Stearinsäure à 0.1%) verhindern meistens die Kontamination der Adsorptionspatrone mit Schaum oder Spritzer. Durch Erwärmung des Gefässes mit einem Umwälzthermostaten (Pos. 11: Temperatur = T_1) kann die Konzentration der zu analysierenden Komponenten im Dampfraum erhöht werden. Eine Erwärmung der gesamten Vorrichtung mit Hilfe eines Wärmeschrankes (Pos. 14: Temperatur $T_2 > T_1$) verhindert zudem Kondenswasserbildung im System, d.h. unkontrollierte Verluste von flüchtigen Komponenten sowie unreproduzierbare Adsorption in der Patrone. Für die Verbindungen wurden PTFE-Schläuche gewählt, da dieser Kunststoff sehr wenige Fremdkomponenten abgibt und keine der extrahierten Stoffe adsorbiert. Ein Blindwert ohne Probe (aber mit dem entsprechenden Lösungsmittel im Gefäss) soll immer ermittelt werden, um die unvermeidbaren Verunreinigungen und Artefacte zu


Sbrinz


Appenzeller


Emmentaler

Tilsiter


Greyerzer

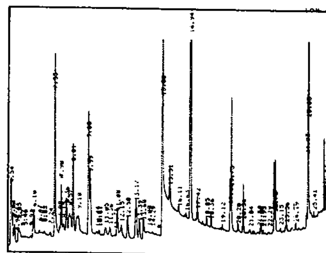

Raclette

Fig. 2. "Fingerprints" der flüchtigen neutralen Aromakomponenten von 6 typischen Schweizerischen Käsesorten.

erkennen. Für die Streuung der Bestimmung ist die Qualität der Adsoptionspatrone meist massgebend.

Der Hauptvorteil des geschlossenen Kreislaufs ist die hohe Ausbeute der Extraktion, mit der jegliche Verluste von flüchtigen Komponenten und Verdünnung der Dampfphase vermieden werden können. Die Verwendung dieses Systems ist jedoch durch die Temperatur des Wärmeschranks beschränkt, in dem sich die Bürette befinden sollte. Für Temperaturen gleich oder grösser als ca. 45°C soll nur bei offenem Kreislauf gearbeitet werden, wobei die Bürette extern, d.h. bei Raumtemperatur eingesetzt wird.

Fig. 2 zeigt eine praktische Verwendung der Apparatur für die Bestimmung flüchtiger neutraler Aromakomponenten ("Fingerprints") von einigen typischen Schweizerischen Käsesorten. Die Arbeitsbedingungen sind die im folgenden.

PROBEVORBEREITUNG

Probe: 20 g feingeriebener Käse (Emmentaler, Greyerzer, Tilsiter, Appenzeller, Sbrinz und Raclette) in 50 ml frisch bidestilliertem Wasser dispergieren; mit 5 mol/l NaOH auf pH 7.5 einstellen (um die vorwiegend flüchtigen Fettsäuren zu neutralisieren). System: offener Kreislauf. Temperatur: T_1 = 40°C; T_2 = 50°C. Angesaugtes Dampfvolumen: 10 × 50 ml. Durchfluss durch die Patrone: 21 ml/min. Adsorptionspatrone: Quarzrohr gefüllt mit *ca.* 100 mg Graphitpulver (Bestell-Nr. 1-1010 R, J. Rektorik).

ANALYSENBEDINGUNGEN

Desorptionsgerät: MW-1 Sampler (J. Rektorik). GC-Gerät: Sigma 1 (Perkin-Elmer) ausgerüstet mit FID und DB-Wax-Säule (60 m × 0.25 mm × 0.25 μm); Temperaturprogramm: 13 min bei 45°C, 10°C/min bis 120°C, 15°C/min bis 220°C, 15 min bei 220°C. Weitere Details werden demnächst in einer separaten Arbeit veröffentlicht[7].

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Note

Liquid chromatographic determination of biotin by using 1-pyrenyldiazomethane as a pre-column fluorescent labelling reagent

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(First received June 9th, 1988; revised manuscript received August 15th, 1988)

Biotin is a coenzyme essential in amino acid or carbohydrate metabolism, and it plays an important rôle in the growth of animals and plants.

Biological methods have generally been used for the determination of biotin[1–4]. However, these methods require culture of the tissue and preservation of its strain. Furthermore, the procedure for sample preparation is tedious and the incubation time-consuming. On the other hand, many workers have reported spectrophotometric methods, based on the binding of a dye to the avidin–biotin complex[5–10], oxidation with potassium iodate[11] or reaction with 4-dimethylaminocinnamaldehyde[12]. However, the selectivities and sensitivities were insufficient for the microdetermination of biotin either in multi-vitamin pharmaceutical preparations or in biological fluids.

Recently, attempts have been made to improve the selectivity by employing high-performance liquid chromatography (HPLC) accompanied by fluorometric detection using 4-bromomethylmethoxycoumarin (Br-Mmc)[13] and 9-anthryldiazomethane (ADAM)[14,15] as the precolumn labelling reagents. However, the use of Br-Mmc involved tedious pretreatment, and the ADAM reagent was unstable.

In the present study, fluorescent labelling of biotin with 1-pyrenyldiazomethane (PDAM), which has recently been developed in our laboratory, was examined. PDAM readily reacted with the carboxylic group of biotin without any catalyst at room temperature, and the ester derivative was separable on a reversed-phase column. The HPLC method was applied to pharmaceutical preparations containing biotin and other vitamins, and to a control serum spiked with biotin.

EXPERIMENTAL

Materials

Biotin was obtained from Sumitomo (Osaka, Japan). Methanol and hexane were HPLC grade (Wako, Osaka, Japan). Other solvents were from Kokusan Kagaku (Tokyo, Japan). All reagents and solvents were of analytical grade. Water was

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purified with a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.) before use. A control serum, Consera, was obtained from Nissui Seiyaku (Tokyo, Japan). 1-Pyrenecarbaldehyde was from Aldrich (Milwaukee, WI, U.S.A.). Sep-Pak C_{18} and Si were from Waters Assoc. (Milford, MA, U.S.A.).

A standard solution was prepared by dissolving 100 μg of biotin per 1 ml of methanol and it was diluted in methanol before use.

Derivatization procedure

PDAM was synthesized as described previously[16]. To 100 μl of a sample solution in methanol were added 100 μl of a 1 mg/ml solution of PDAM in ethyl acetate. The mixture was allowed to stand for 60 min at 40°C and then cooled to room temperature. To the reaction mixture were added 800 μl of methanol and an aliquot (5 μl) of the resulting mixture was directly injected into the chromatograph.

On the other hand, an aliquot (50 μl) of the above mixture was injected for normal phase HPLC and the peak fraction of the PDAM derivative of biotin was collected. The fraction was evaporated to dryness and the residue was examined using a mass analyzer Model RKB-9000 (Shimadzu Seisakusho, Kyoto, Japan).

Chromatography

The HPLC system consisted of a Model TRIROTAR-VI (Japan Spectroscopic, Tokyo, Japan) with a degasser Model DG-3510 (Japan Spectroscopic) and an ultraviolet spectrophotometer Model UVIDEC-100-VI (Japan Spectroscopic) or a Model F-1000 spectrofluoromonitor (Hitachi, Japan).

The effluent was measured spectrophotometrically at 240 nm or fluorometrically at the excitation and fluorescence wavelengths of 340 and 395 nm.

The chromatographic separations were performed on a normal phase column packed with LiChrosorb Si 60 (particle size 5 μm, Merck) and a reversed-phase column packed with TSK-gel 80 TM (particle size 5 μm; TOSOH, Tokyo, Japan). Both columns were the same size, 150 mm × 4 mm I.D. In the normal phase mode, the separation was carried out at ambient temperature by using hexane–isopropanol–water (80:20:1) as the mobile phase at a constant flow-rate of 1.0 ml/min. In the reversed-phase mode, the biotin derivative was separated on the ODS column kept at 50°C by using water–acetonitrile (43:57) at a constant flow-rate of 1.0 ml/min.

Application to the biotin preparation

Tablets of a biotin preparation (250 μg per tablet) were finely powdered, and then to the powder containing 500 μg of biotin were added 30 ml of methanol. The mixture was extracted on a Branson Model B-521 ultrasonic cleaner for 10 min and then shaken for 10 min. The mixture was then centrifuged at 3000 rpm for 5 min. The supernatant was transferred to an 100-ml volumetric flask. The above extraction procedure was repeated three times and the supernatants were pooled and made up to 100 ml with methanol. To 200 μl of this solution were added 200 μl of 5 mg/ml PDAM solution in ethyl acetate. The mixture was allowed to stand for 60 min at 40°C and then cooled to room temperature. The resulting mixture was applied to Sep-Pak Si. After washing the cartridge column with 5 ml of hexane, it was eluted with 10 ml of methanol and the effluent was evaporated. The residue was redissolved in 1 ml of methanol and a 5-μl aliquot of the mixture was subjected to HPLC as described above.

Application to biotin-spiked serum

To 1 ml of Consera were added 10 ng/ml biotin solution and mixed. To the resulting mixture was added 1 ml of 10% trichloroacetic acid, and then the mixture was centrifuged at 2000 *g* for 5 min. The supernatant (1.5 ml) was applied to Sep-Pak C_{18}, which had previously been washed with 10 ml of methanol, 10 ml of water and 5 ml of 1% aqueous acetic acid. After washing the Sep-Pak with 10 ml of 1% acetic acid and 1 ml of water, the biotin was eluted with 10 ml of methanol and the effluent was evaporated. The residue was redissolved in 100 μl of methanol on a ultrasonic cleaner. To 100 μl of this solution were added 100 μl of 1 mg/ml PDAM solution and then the mixture was allowed to stand for 60 min at 40°C. After cooling to room temperature, 300 μl of methanol were added and a 10-μl aliquot of the mixture was directly injected in the chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows the reaction of biotin with PDAM. It was carried out in ethyl acetate at room temperature without a catalyst, reached a plateau in 1.5–2 h and was accelerated by heating. Fig. 2 shows the yield of the reaction as a function of the reaction time at room temperature and at 40°C. The derivatization reaction required 120 min at room temperature or 60 min at 40°C for completion, so the latter condition was adopted in the standard procedure.

The PDAM derivative of biotin was purified by HPLC fractionation and subjected to mass spectrometry to comfirm its structure. The mass spectrum showed the molecular ion peak of the biotin ester at *m/z* 458, and fragment ions at *m/z* 215 and 232 formed by ester cleavage.

The excitation and fluorescence spectra of the purified ester were approximately the same in any solvent system used as the mobile phase, showing maxima at 340 and 395 nm.

The separation of the biotin ester was examined by reversed-phase HPLC. Fig. 3 shows a chromatogram of the biotin ester on an octadecylsilyl silica gel column eluted with acetonitrile–water. The biotin ester was clearly separated from the degradation products of PDAM, and it was sensitively detected at the sub-pmol level without interferences from contaminants. Since some contaminants moved far more slowly than the biotin ester under these conditions, it took 80 min to complete the chromatography. The time for separation could, however, be shortened to 40 min by eluting the column with acetonitrile alone immediately after the elution of the biotin ester.

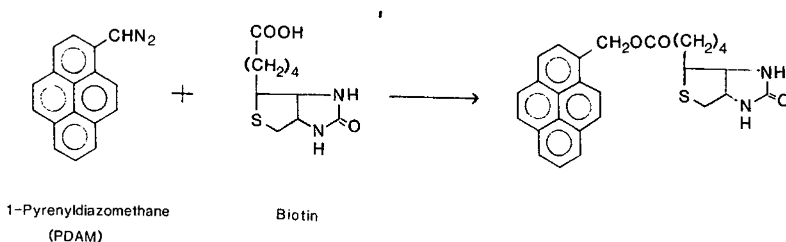

Fig. 1. Reaction course of PDAM with biotin.

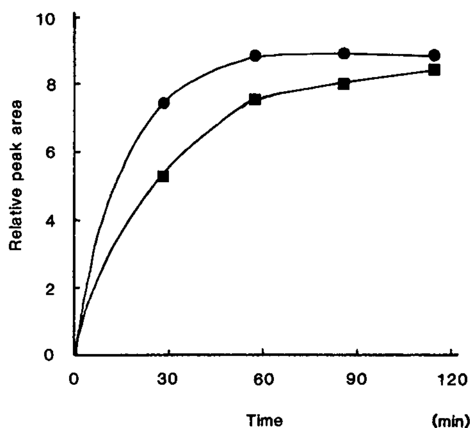

Fig. 2. Yield of the PDAM derivative of biotin as a function of reaction time at room temperature (■) and at 40°C (●).

The standard graph for biotin showed excellent linearity in the range from 200 fmol to 200 pmol per injection ($y = 1.101x + 0.022$, $r = 0.999$). The reproducibility of this procedure was also sufficient, the coefficient of variation for 10 pmol of biotin being 1.1% ($n = 5$). The detection limit of biotin under this condition was about 100 fmol per injection (signal-to-noise ratio, S/N = 3).

The present standard procedure was applied to the determination of biotin in a vitamin preparation. Both the derivatization and chromatographic separation were not influenced by other components in the preparation. Fig. 4A shows a chromatogram of the PDAM derivative of biotin extracted from the preparation. The recov-

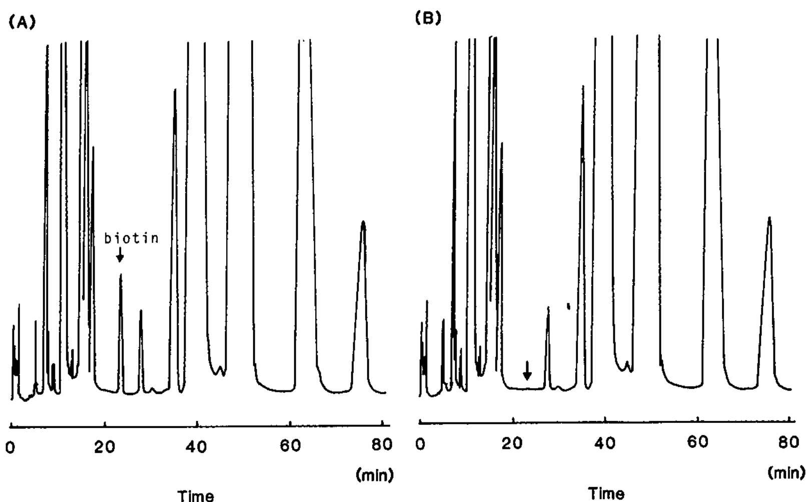

Fig. 3. Reversed-phase liquid chromatographic profiles of the PDAM derivative of standard biotin (A) and a reagent blank (B). Mobile phase, acetonitrile–water (43:57); flow-rate, 1.0 ml/min. The peak corresponds to about 10 pmol of biotin.

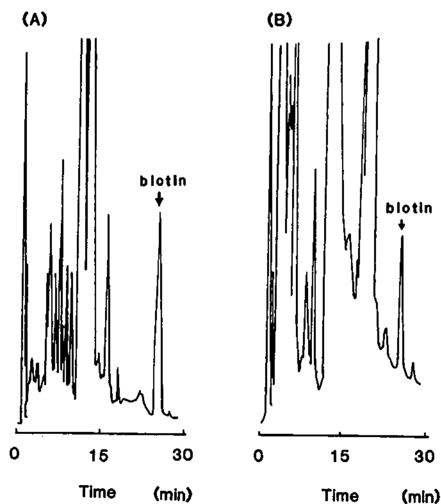

Fig. 4. Reversed-phase liquid chromatographic profiles of the PDAM derivative of biotin extracted from a preparation (A) and from serum (B). Mobile phase as in Fig. 3.

ery of biotin was 98.8% and the coefficient of variation for the reproducibility of this procedure was 1.2% (n = 4).

The determination of biotin spiked in serum was also examined by the present method. Extraction of biotin from serum has previously been carried out by using active charcoal[15], but the recovery and reproducibility were poor. We investigated a more efficient extraction method by using a cartridge type extraction column packed with ODS silica gel. The recoveries of biotin from serum at the various concentrations

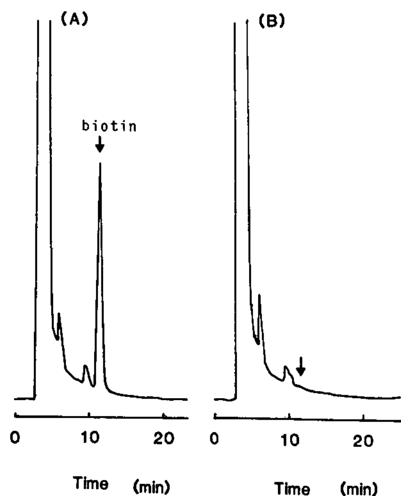

Fig. 5. Normal phase liquid chromatographic profiles of the PDAM derivative of biotin (A) and a reagent blank (B). Mobile phase, hexane–isopropanol–water (80:20:1); flow-rate, 1.0 ml/min. The peak corresponds to about 100 pmol of biotin.

were 90.1 to 104.2%. Fig. 4B shows a chromatogram of the PDAM derivative of biotin extracted from serum.

Furthermore, normal phase HPLC separation of the PDAM derivative of biotin was examined in order to simplify the determination procedure. Fig. 5 shows chromatograms of the derivatization mixture of PDAM and standard biotin, and the reagent blank, directly injected on a silica gel column and eluted with hexane–isopropanol–water (80:20:1). Most of the degradation products of the reagent were eluted at the solvent front, and the PDAM derivative of biotin was clearly separated from these contaminants. However, the sensitivity for the PDAM derivative under this chromatographic condition was about ten times lower than that under reversed-phase conditions, because the relative fluorescence intensities of PDAM derivatives in aqueous media and organic solvents greatly differ. Consequently, the chromatographic conditions should be chosen according to each application.

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Note

High-performance liquid chromatographic analysis of the by-products of the synthesis of ethynylestradiol, mestranol, 17α-hydroxyprogesterone caproate and 17α-hydroxy-6-dehydroprogesterone acetate*

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(Received August 4th, 1988)

Purity control of drugs is of great importance because the presence of by-products may induce undesirable and sometimes serious complications[1–3]. Usually the acceptable level of impurities in drugs varies from 0.5 to 1.5%. For the detection of minor components and their correct determination it is necessary to have them available in the pure state.

They can be obtained either by synthesis or by isolation from mother liquors during hormone purification. For the isolation, identification and accumulation of minor components from mother liquors we have used different variants of liquid chromatography. The preliminary determination of the components was carried out by thin-layer chromatography (TLC) and the final determination by liquid chromatography.

Preparative high-performance liquid chromatography (HPLC) represents a fast and simple method for the separation and isolation of impurities. Approximately 20 min are required for a 5–10-g sample of a mother liquor, but as the concentration of minor compounds in the mother liquor of drugs is less than 1%, their accumulation requires repetition of the process. After confirmation of the structures of all isolated minor components by physico-chemical methods (mainly by mass, NMR, IR and UV spectrometry), the determination of impurities in the drugs was carried out.

EXPERIMENTAL

As standards of estrogen drugs we used samples specified as "chemical reference substances" in the *Pharmacopoeia of the G.D.R.* (6th edition), the *Compendium*

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Medicamentorum CMEA and the *International Pharmacopoeia* of the World Health Organization (3rd edition), and also the samples from laboratory preparations[4].

A Waters Model Prep 500 liquid chromatograph equipped with a Prep Pak silica column (300 × 57 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) and a refractometric detector was used. Samples were injected onto the column with a 10-ml syringe. Also, a DuPont Model 830 liquid chromatograph equipped with a Model 837 spectrophotometer and a column with either Zorbax-Sil, Silasorb 600, LiChrosorb Si 60 (250 × 22.7 mm I.D.), Zorbax-Sil or Zorbax CN (250 × 4.6 mm I.D.) was used. Samples were injected onto the column using a Model 7120 syringe-loading injector (0.5 μl) (Rheodyne, Cotati, CA, U.S.A.) with a sample loop of 20–200 μl. For calculations we used a Model 8830 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

All reagents were of analytical-reagent grade and solvents were purified by distillation. Under the above conditions we examined a series of steroid drugs, namely ethynylestradiol, mestranol, 17α-hydroxyprogesterone caproate and 17α-hydroxy-6-dehydroprogesterone acetate (sources of these drugs were given previously[5,6]).

The purification and isolation of the contaminants in the steroid drugs were carried out as follows. First, the content of steroids in a mother liquid or sample was determined by TLC on Silufol UV-254 plates with *n*-hexane–acetone (7:3) as mobile phase[4].

The substance to be investigated was dissolved in the mobile phase used and subjected to preparative or semi-preparative HPLC. From ethynylestradiol (mother liquor, 6.5 g) we isolated seven fractions by preparative HPLC on a Waters Prep 500 chromatograph with ethyl acetate–chloroform (5:95) as mobile phase at a flow-rate of 200 ml/min and IR detection (see Fig. 1): I, 0.10 g; II, 4.11 g; III, 1.23 g; IV, 0.11 g; V, 0.14 g; VI, 0.18 g; VII, 0.36 g.

The semi-preparative HPLC separation of these fractions in different solvents, independent of the polarity, we isolated ten compounds, which physico-chemical analysis showed to have a steroidal structure. Other samples were examined using this procedure[5,6].

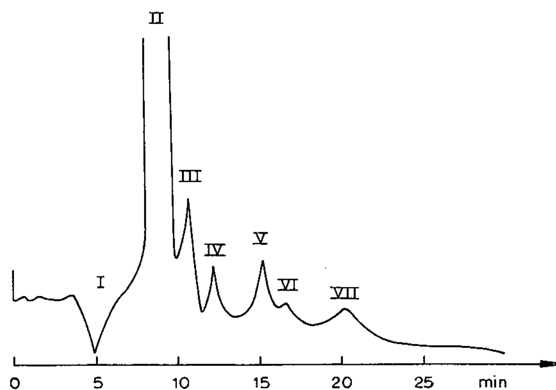

Fig. 1. Preparative separation of a mother liquor sample of ethynylestradiol on a Waters Prep 500 chromatograph with a Prep Pak silica column (300 × 57 mm I.D.) with ethyl acetate–chloroform (5:95) at a flow-rate of 200 ml min and IR detection.

TABLE I

IMPURITIES ISOLATED FROM MOTHER LIQUOR OF ETHYNYLESTRADIOL

No.	*Compound*	*R_F in n-hexane–acetone (7:3)*	*Mol. weight*	*UV maximum (nm)*	*Approximate concentration (%)*
1	17α-Isobutylestradiol	0.12	328	279	0.67
2	Steroid of unidentified structure, $C_{24}H_{34}O_4$	0.18	386	280	0.70
3	Ethynylestradiol 17-methyl ether	0.20	310	264	2.00
4	16-Methoxyethynylestradiol	0.23	310	260	2.00
5	Estradiol	0.29	272	280	0.80
6	Steroid of unidentified structure, $C_{21}H_{26}O_2$	0.30	310	266	0.10
7	Estrone	0.34	270	280	1.00
8	Estradiol 3-methyl ether	0.39	286	278	0.80
9	Mestranol	0.41	310	280	0.57
10	Estrone 3-methyl ether	0.53	284	278	1.00

RESULTS AND DISCUSSION

We found ten minor compounds (with steroidal structures) in ethynylestradiol (Table I). The presence of the same minor components in all the samples of drugs analysed (commercial samples from different firms) showed that they had been prepared in a similar manner.

Compounds 2 and 6 were analysed by mass and NMR spectrometry and were proved to have a steroidal skeleton. In order to verify the hypothesis of a steroidal structure, quantitative reactions of steroid estrogen were conducted in the usual way.

Typical chromatograms are shown in Figs. 2–4.

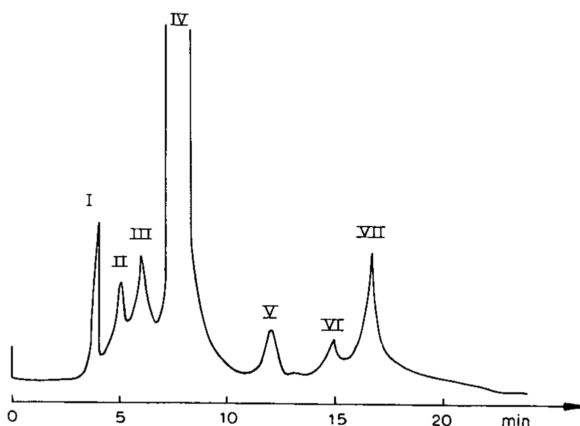

Fig. 2. Semi-preparative separation of the third fraction (see Fig. 1) on a DuPont 830 chromatograph with a LiChrosorb Si 60 column (250 × 22.7 mm I.D.) with ethyl acetate–*n*-hexane (5:95) at a flow-rate of 13 ml min and UV detection at 254 nm.

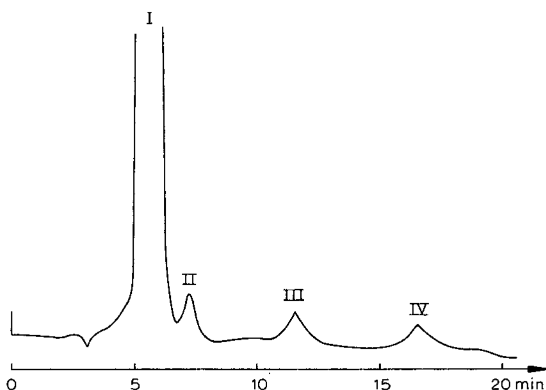

Fig. 3. HPLC trace of 17α-hydroxyprogesterone capronate obtained on a DuPont 830 chromatograph with a Silasorb 600 (250 × 4.6 mm I.D.) with ethyl acetate–*n*-hexane (20:80) at a flow-rate of 1.2 ml min and UV detection at 254 nm.

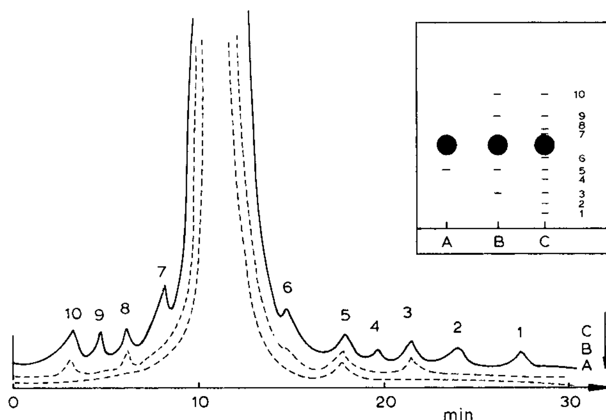

Fig. 4. Detection of impurities in different samples of ethynylestradiol (using markers isolated from the mother liquor product of ethynylestradiol) by means of TLC and HPLC. (A) Farmacon product; (B) Akrichin product; (C) mother liquor product. 1–10 = Compounds identified (see Table I).

CONCLUSIONS

It has been shown that ethynylestradiol (and also mestranol) contain more than ten, 17α-hydroxyprogesterone acetate more the six and progesterone and 17α-hydroxyprogesterone caproate three minor steroidal compounds[5,6].

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CHROM. 20 995

Note

Liquid chromatographic resolution of hypoglycin A from leucine

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Hypoglycin A (HG-A) or 2-amino-4,5-methylenehex-5-enoic acid is the natural toxic compound found in ackee fruit. Because of the toxicity of HG-A, there is great concern about the existence and content levels of HG-A in canned ackee fruit if immature fruit is packed. Since current liquid chromatographic methods do not give baseline resolution of HG-A from leucine, methodology is needed to accurately resolve and measure HG-A in ackee fruit products.

Early work on HG-A quantitation relied upon paper chromatography. Patrick[1] tried 17 different paper chromatographic systems and failed to separate HG-A from leucine. Other paper chromatographic methods[2,3] which appear to work are lengthy and unreliable.

Published procedures utilizing ion-exchange amino acid analyzer (IE-AAA) systems are not adequate for HG-A quantitation. Scott *et al.*[4] developed a method for the determination of HG-A using a Beckman IE-AAA system and post-column ninhydrin reaction. However, HG-A and leucine were not baseline resolved. Fincham[5] used an indirect AAA method to quantitate HG-A in ackee fruit with HG-A and leucine coeluting as one peak. The peak was measured before and after destroying the HG-A by bromination. The resulting peak was leucine with the difference between the nonbrominated and brominated samples being HG-A. The author indicated that the method is not precise because of the differential method of analysis[5]. Attempts to use the bromination procedure in our laboratory were unsuccessful. This paper describes an IE-AAA system which gives baseline resolution of HG-A from leucine and 16 other amino acids.

EXPERIMENTAL

Reagents

Buffers. The IE-AAA buffers were pre-prepared (Pickering Labs., Mountain View, CA, U.S.A.): (1) buffer A-Na 315 (0.2 *M* sodium citrate, pH 3.15); (2) buffer B-Na 740 (0.9 *M* sodium chloride–sodium acetate, pH 7.40).

Ninhydrin. Trione ninhydrin reagent (Pickering Labs).

HG-A standard solution. Dissolve HG-A in buffer A (72.6 μg/ml). Make further dilutions with buffer A to give 18.15, 12.10 and 4.84 μg/ml. Note: Hypoglycin A standard is not available commercially. The standard used for this study was provided by Dr. Tanaka, Yale University School of Medicine.

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Amino acid standard mixture. Pre-prepare a mixture of 17 amino acids and ammonia to give 0.5 μmol amino acid/ml (No. 20079, Pierce, Rockford, IL, U.S.A.).

Amino acid standard mixture/HG-A. Combine equal volumes of the HG-A standard and amino acid standard mixture (0.25 μmol amino acid/ml).

Method development standard mixtures. Make up a 0.5 μmol/ml solution of L-methionine, L-isoleucine, L-leucine and L-tyrosine (No. 185009, 185006, 185007, and 185015, respectively, Pierce) in buffer A. Combine equal volumes of this mixture with HG-A standard.

Apparatus

As amino acid analyzer a Waters Assoc. ion-exchange ninhydrin detection system was used. It consisted of a 25 cm × 4.6 mm amino acid column (Waters No. 80002), two Model 510 pumps, a temperature control module (TCM) system, a Model A-30-SW ninhydrin pump, a WISP Model 710B auto sample injection system, a Model 440 detector, and an interface module system. The system was operated by a Digital Pro 350 computer with a Digital LA50 printer.

Chromatographic conditions

Instrument parameters. Injection volume, 15 μl; flow-rate, 0.4 ml/min; detector wavelength, 436 and 546 nm (additive); column temperature, 62°C; reaction oven temperature, 120°C; ninhydrin flow-rate, 0.4 ml/min.

Mobile phase. Buffer A–buffer B (30:70), set pump A (buffer A) at 30% and pump B (buffer B) at 70% of the total flow-rate of 0.4 ml/min for baseline reolution of HG-A and leucine.

System calibration

Inject the high standard solution (72.6 μg/ml) to calibrate the system and establish baseline, peak response and retention time parameters. These parameters were entered into the computer program. Linearity and detection limits were established by running a series of four standards in duplicate ranging from 4.84 to 72.6 μg/ml.

RESULTS AND DISCUSSION

The binary buffer gradient systems recommended by the manufacturer for separation of 17 amino acids and ammonia in 110 min failed to resolve HG-A and leucine. A method development standard mixture which incorporated those amino acids immediately preceding and following HG-A elution (methione, isoleucine, leucine, and tyrosine) was used. Various modifications in the binary gradient failed to resolve HG-A and leucine; however, a small distortion in the HG-A/leucine peak was observed during an isocratic segment in one of the gradient program modifications, where the ratio of buffers A and B was 80:20. This small distortion of the HG-A/leucine peak was believed to be a crude initial separation between HG-A and leucine.

This was further investigated by trying different isocratic buffer systems. Fig. 1 shows the progressive improvement in the resolution of HG-A and leucine by using isocratic buffer systems consisting of 50:50, 40:60 and 30:70 mixtures of buffers A and

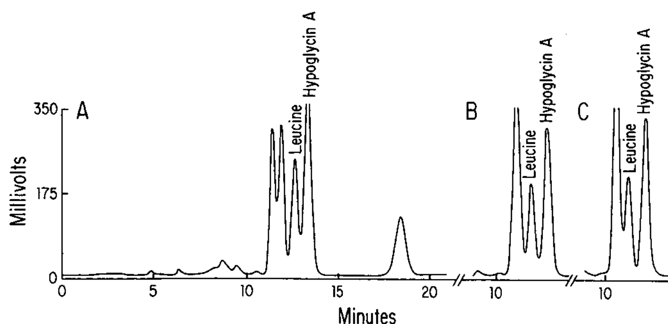

Fig. 1. Chromatogram of method development standard mixture of methionine, isoleucine, leucine, HG-A and tyrosine with a mobile phase of varying buffer A–buffer B ratios at a flow-rate of 0.4 ml/min. Elution order: Methionine, isoleucine, leucine, HG-A and tyrosine. (A) 50:50; (B) 40:60; (C) 30:70.

B. The 50:50 mixture of buffers A and B resolved all four amino acids of the method development standard with partial coelution of HG-A and leucine (Fig. 1A). As the amount of buffer A decreased, the methionine and isoleucine peaks coeluted and HG-A and leucine resolution increased (Fig. 1B). Optimal baseline resolution of HG-A and leucine was obtained with the isocratic buffer system made of a 30:70 mixture of buffers A and B (Fig. 1C). This buffer system was further evaluated using a standard mixture of the 17 naturally occurring amino acids. Fig. 2 is the chromatogram of the 17 amino acid mixture with HG-A. In a separate chromatogram not shown here, the 17 amino acid mixture without HG-A was run and showed that there were no coeluting amino acids with HG-A.

Evaluation of the method showed a linear peak response to increasing HG-A concentrations from 4.84 to 72.6 μg/ml (72.6–1089 ng/15 μl injection volume) with a correlation coefficient of 0.999. The precision of the system was evaluated by analyzing the high standard (72.6 μg/ml) five times and gave a peak area of 7.920 $\pm$ 0.0147 (0.19% C.V.) and indicated good reproducibility.

This method presents a rapid, reliable procedure for the baseline resolution of HG-A and leucine and will serve as a basis for the study of HG-A content in ackee fruit.

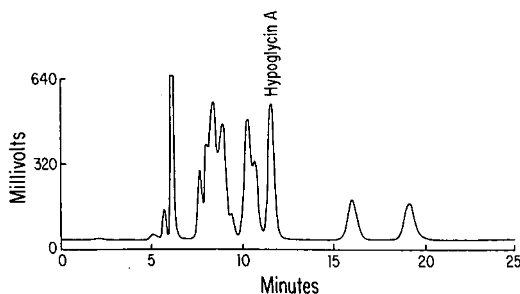

Fig. 2. Chromatogram of 17 naturally occurring amino acids with HG-A. Mobile phase, buffer A–buffer B (30:70) at a flow-rate of 0.4 ml/min.

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Note

Determination of chlorthalidone and its impurities in bulk and in dosage forms by high-performance thin-layer chromatographic densitometry

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Chlorthalidone (I), 2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl)benzenesulphonamide, is a diuretic, antihypertensive agent. Chlorthalidone has been quantitated in various media using a variety of methodologies. The major techniques for biological materials involves extractive alkylation and gas chromatography[1–3] or deamination followed by UV spectrophotometric quantitation[4,5]. For the determination of chlorthalidone in pharmaceutical dosage forms, a normal-phase chromatographic method has been reported[6], which separated the hydrolysis product (II), 4′-chloro-3′-sulphamoyl-2-benzophenonecarboxylic acid, on a polyamide column. A reversed-phase liquid chromatographic (LC) system had been used for the determination of I in tablets also containing clonidine hydrochloride[7]. The original USP procedure was a spectrophotometric determination[8], subsequently updated to an LC method[9]. In the Italian Pharmacopeia[10], chlorthalidone is determined by potentiometric titration. A study of the degradation products of chlorthalidone, II and III [2-chloro-5-(1-methoxy-3-oxo-1-isoindolinyl)benzenesulphonamide], was made using an LC system[11] to separate these products from chlorthalidone. A stability-indicating reversed-phase LC method was developed and validated for the assay of I and II in tablet formulation[12].

This paper describes a simple and rapid method for determining the chlorthalidone and its impurities II and III by scanning quenched zones on high-performance silica gel F_{254}.

EXPERIMENTAL

Reagents and materials

Chlorthalidone bulk material and related impurities were kindly supplied by pharmaceutical manufacturers. Pharmaceutical dosage forms were obtained commercially. All reagents used were of analytical-reagent grade.

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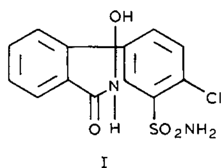

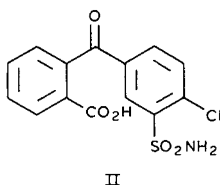

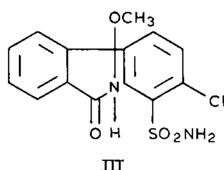

Standard and sample solutions were separated on pre-coated silica gel F_{254} high-performance thin-layer chromatographic (HPTLC) plates (Merck, Darmstadt, F.R.G.).

Equipment

Quantitative analysis was performed with Sigma FTR-20 TLC scanner (Biochem) in the reflectance mode using a 5-mm light beam and a shortwave (254 nm) UV source, equipped with a CR-3-A integrator printer–plotter (Shimadzu). Standards and samples were spotted on plates using a Camag Nanomat II device in order to increase the precision and reproducibility of the analysis.

Standards for calibration

The standard solutions for establish calibration graphs for compounds I, II and III were prepared in absolute methanol from concentrated solutions to give the following final concentrations: I, 5, 10, 15, 20 and 25 μg/μl; II, 0.0015, 0.0030, 0.0050, 0.0070 and 0.0090 μg/μl; and III, 0.050, 0.070, 0.090, 0.110 and 0.130 μg/μl.

In addition, five different methanol solutions with the same amount of chlorthalidone and different concentrations of impurities II and III were prepared as standard mixtures.

Sample preparation

Raw materials. Accurately weigh *ca.* 200 mg of chlorthalidone and transfer it into a 100-ml volumetric flask. Dissolve and dilute to volume with methanol.

Tablets. Weigh and finely powder not less than 20 tablets. Accurately weigh a portion of powder, equivalent to *ca.* 200 mg of I, transfer it into a 100-ml volumetric flask, dilute to volume with absolute methanol and mix well in an ultrasonic bath.

High-performance thin-layer chromatography

Aliquots of 1 μl of each standard and sample solution were transferred with the Camag Nanomat II onto HPTLC plates (10 × 20 cm) with alternating depositions of samples, standards and standard mixtures. The solvent used for development was dioxane–isopropyl alcohol–25% ammonia solution–toluene–xylene (30:30:20:10:10). All chromatograms were developed to about 8 cm from baseline (requiring about 10

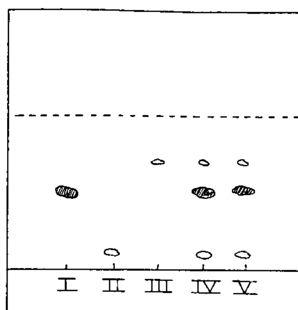

Fig. 1. Chromatogram of I (20 μg/ml), II (0.1 μg/μl), III (0.09 μg/μl), IV (standard mixture) and V (tablet extract).

min) in a chromatographic tank, previously saturated with eluent mixture, at room temperature. The layers were dried with a forced current of cool air from a hair dryer and measured at 254 nm.

The analysis conditions were as follows: width, 5 mm; attenuation, 5; slit, 5.8 × 0.4 mm. The scanning was performed at 40 mm/min with a time constant of 200 ms. The areas of peaks I, II and III were measured directly by the integrator printer–plotter. The amounts of chlorthalidone and its impurities in a spotted sample were calculated using the equation

$$\frac{\text{average area of standard peaks}}{\mu\text{g of standard spotted}} = \frac{\text{average area of sample peaks}}{\mu\text{g of I (or II or III) in a 1-}\mu\text{l aliquot}}$$

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram for a single standard, standard mixture and tablet extract. The R_F values allow quantitative densitometric analysis (Fig. 2). Fig. 3 shows the linearity for standard solutions that was established by spotting different concentrations. The linearity correlation coefficient was between 0.97 and 0.98 for II and III but always above 0.99 for I.

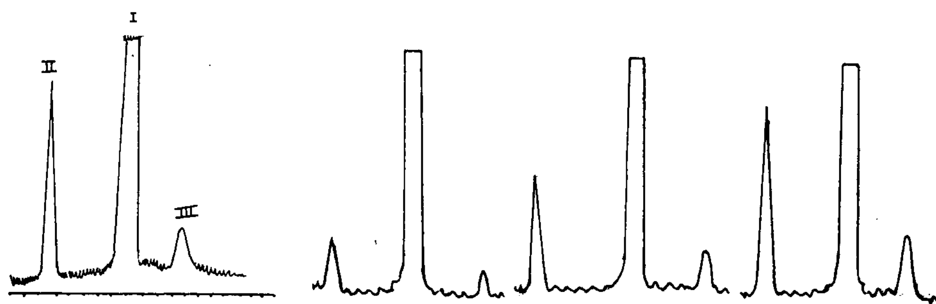

Fig. 2. Scanning of a chromatogram with I (20 μg/μl), II (0.1 μg/μl) and III (0.09 μg/μl).

Fig. 3. Linearity for standard solutions.

TABLE I

RECOVERY OF CHLORTHALIDONE AND IMPURITIES II AND III FROM STANDARD MIXTURES

a = Added; f = found; R = recovery.

I			*II*			*III*		
a (mg/ml)	*f (mg/ml)*	*R (%)*	*a (mg/ml)*	*f (mg/ml)*	*R (%)*	*a (mg/ml)*	*f (mg/ml)*	*R (%)*
5	4.97	99.4	0.0015	0.0012	–	0.050	0.051	102.0
10	9.87	98.7	0.0030	0.0028	93.3	0.070	0.069	98.6
15	15.10	100.7	0.0050	0.0047	94.0	0.090	0.090	100.0
20	19.91	99.6	0.0070	0.0071	101.4	0.110	0.106	96.4
25	25.12	100.5	0.0090	0.0089	98.9	0.130	0.129	99.2
		S.D. 0.82			S.D. 3.9			S.D. 2.04

TABLE II

DETERMINATION OF I, II AND III IN BULK MATERIAL AND TABLETS

Sample No.	*Bulk materials*			*Tablets*		
	I	*II*	*III*	*I*	*II*	*III*
1	98.7	0.030	0.11	99.1	–	0.13
2	98.9	0.027	0.09	97.9	0.040	0.10
3	98.0	0.032	0.12	98.4	0.035	0.097

The recovery of chlorthalidone and the two impurities from artificial mixtures prepared in order to validate the method are summarized in Table I. The results obtained from the analysis of samples are reported in Table II.

A study was made of the reproducibility of the HPTLC procedure by spotting eight times the same amounts and volumes of I, II and III standards on a single plate. The zones were scanned after development and the relative standard deviation of the peak areas was 1.9%.

The proposed quantitative HPTLC method proved to be accurate, reproducible and selective for the determination of chlorthalidone and its two impurities. Impurity II below 0.0015 μg is not observed, but the densitometric analysis of this concentration is possible. The ability to spot multiple samples together with a standard of each product and standard mixtures of three compounds on the same plate allows a high sample throughput to be achieved.

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Note

Hot thin-layer chromatographic fractionation of polyethylene

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(Received September 16th, 1988)

High-performance size-exclusion chromatography (HPSEC) is the predominant chromatographic method used to analyze polymers. Recently there has been an increased interest in the use of non-aqueous high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) methods for the analysis of polymers[1–5]. As a point of departure, this work built upon earlier normal-phase chromatographic results obtained by Inagaki[6], Belenkii and Gankina[7,8], and Otocka[9]. By varying the experimental conditions one can do either qualitative analysis of polymers or separate them by molecular weight[4]. These approaches were shown to have over an order of magnitude more resolving power than conventional size-exclusion methods[10]. In addition, the theory and mechanism of the separation process was elucidated[2,11,12]. It was demonstrated via calculations and experiment that conventional LC partition and kinetic theory for small molecules cannot be applied generally to polymer separations. For binary mobile phases, the concept of a critical mobile phase composition was introduced[2,11,12]. Below this critical composition, no elution occurs while at concentrations above it, there is rapid elution. Factors such as polymer molecular weight and chemical make-up can affect its critical solvent composition. By engineering a system in which strong stationary phase adsorption is minimized one can fractionate polymers by molecular weight[2,4,12]. In the case of silica gel based stationary phases this is done by silanizing all available silanol groups (as in typical reversed-phase media) or by using mobile phases or additives that deactivate the silica gel. Both LC and TLC can be used to fractionate polymers according to molecular weight provided a proper binary solvent combination is used (consisting of a good and poor solvent for the polymer). In addition, polymer fractionation requires gradient elution. In TLC, a gradient can form spontaneously via demixing during development when the stationary phase preferentially adsorbs one component, thereby enriching the mobile phase in the other[1,2].

Crystalline polymers (such as polyethylene and polypropylene) are difficult to analyze by any chromatographic method because they cannot be dissolved easily except at elevated temperatures in certain solvents. Given the problems and cost involved in doing HPSEC it seemed reasonable to examine simpler alternatives. Carrying out TLC separations at elevated temperatures (100–140°C) is one possible alternative that is analyzed in this communication. To our knowledge, there have been no prior reports on using "hot" TLC to fractionate crystalline polymers. When doing experiments of this type, close attention must be paid to safety factors (see cautionary note in the experimental section).

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EXPERIMENTAL

Materials

Silica gel TLC plates (KGF, 5 × 20 cm) were obtained from Whatman. Dodecane, hexadecane, diethylmalonate, 1,2,4-trichlorobenzene and polyethylene glycol (MW ≈ 400) were obtained from Aldrich. Phenyl ether was obtained from Fisher Scientific and benzyl alcohol from MCB Manufacturing. Polyethylene of molecular weight 2000 was obtained from Polysciences. Polyethylene standards of molecular weight 13 600; 32 000; 52 000 and 119 600 were obtained from the U.S. National Bureau of Standards. All standards were of a narrow molecular weight distribution except for the 52 000 MW standard which had a M_w/M_n of 2.9. All developments were done in 15 cm high × 8.5 cm diameter cylindrical glass jars.

Methods

Each 5 × 20 cm plate was cut in half (to make two 5 × 10 plates). The polyethylene standards were dissolved in a hot solvent such as dodecane and individually spotted one cm from the bottom of the plate. A covered jar containing the binary solvent mobile phase was placed in a heated, insulated chamber at the desired temperature between 110°C and 125°C (to ± 1°C). The spotted TLC plate was placed along side the developing chamber and both were allowed to reach the higher chamber temperature (≈ 30 min). Subsequently, the "hot" TLC plate was placed into the "hot" developing jar. The chromatogram was allowed to develop (30–120 min depending on the mobile phase composition) at the elevated temperature. After development, the solvent was evaporated from the plate in a vacuum oven and the polyethylene spots were visualized using sulfuric acid charring (yellow-brown spots on a white background).

In general, the best binary mobile phase for polyethylene seemed to be 1,2,4-trichlorobenzene and benzyl alcohol. Exact conditions are given in the figure legends and the Results and Discussion section.

Cautionary note

The TLC developing chamber should not be tightly sealed or it could explode upon heating. The TLC development should not be done in an oven with exposed heating coils or any surface that could cause catalytic decomposition of the organic vapors, otherwise ignition could occur. All vapors from the experiment should be properly ventilated. When using high-temperature TLC, it is advisable to use solvents with low vapor pressures, high boiling points and high flash points.

RESULTS AND DISCUSSION

The TLC fractionation of polymers by molecular weight is most effective on reversed-phase plates[1,4]. This is because silanization eliminates most of the "strong adsorption sites" and strong adsorption tends to accentuate separations based on differences in chemical composition and structure rather than molecular weight. Unfortunately, it was difficult to visualize polyethylene on reversed-phase TLC plates. For example, sulfuric acid charring methods affected the hydrocarbon C_{18} stationary phase as much as the hydrocarbon polymer. Hence, it was necessary to use normal-

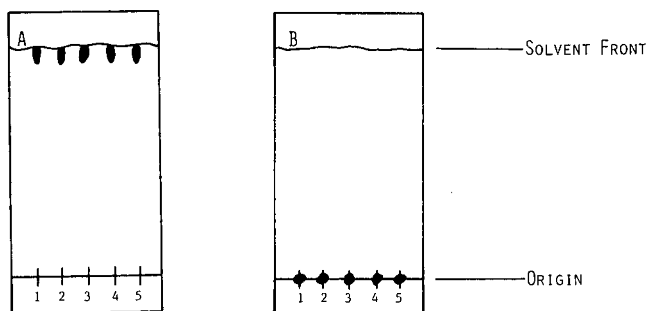

Fig. 1. (A) Schematic of TLC plate after development at 125°C with a pure good solvent (*i.e.*, 1,2,4-trichlorobenzene, diethyl malonate or hexadecane). All polyethylene standards travel with the solvent front (R_F = 1). (B) An analogous chromatogram in which neat, poor solvents were used as mobile phases (*i.e.*, benzyl alcohol, polyethylene glycol, or phenyl ether). In this case there is no movement of any polymer (R_F = 0). The molecular weight of the polyethylene standards are: (1) 2 000; (2) 13 600; (3) 32 100; (4) 52 000 and (5) 119 600.

phase silica gel TLC plates. Previously, it was demonstrated that a rough molecular weight dependence for polystyrene could be seen on silica gel if the appropriate mobile phase was chosen[6,9]. In general, this means that a component of the mobile

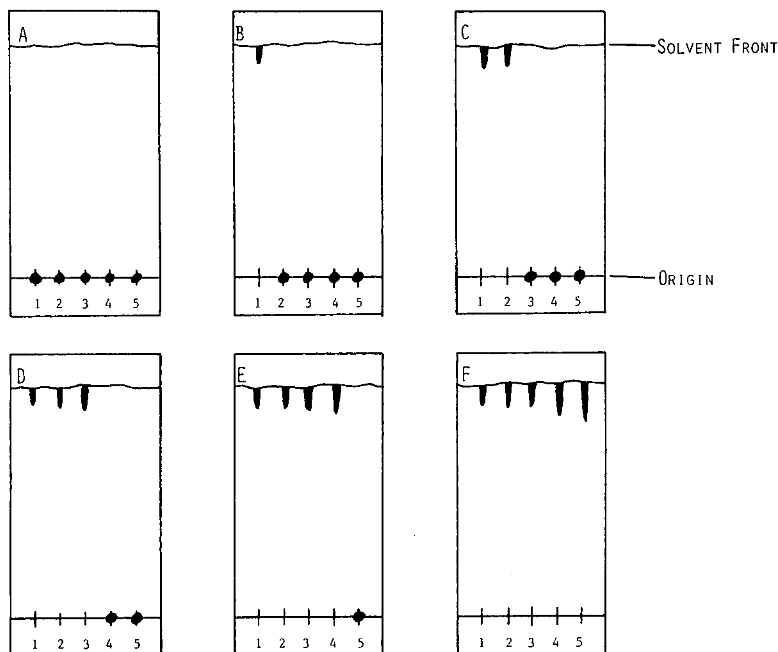

Fig. 2. Schematic showing the effect of mobile phase composition on elution of different molecular weight polyethylenes on silica gel TLC plates. The mobile phase consisted of various mixtures of the good–poor solvent pair, 1,2,4-trichlorobenzene–benzyl alcohol. The molecular weight of each polymer (1–5) is the same as indicated in Fig. 1. The volume percent of 1,2,4-trichlorobenzene in each chromatogram was: (A) 1%, (B) 25%, (C) 45%, (D) 52%, (E) 60% and (F) 75%.

phase must be more polar (and therefore more strongly adsorbed) than any segment of the polymer.

As in previously reported TLC polymer fractionation techniques, a binary good–poor solvent pair is used as the mobile phase. When using the pure good solvent as the mobile phase at 125°C, polyethylenes of all molecular weights move with the solvent front (Fig. 1A). Conversely, when neat poor solvents are used, under identical conditions, there is no movement from the origin (Fig. 1B). By mixing the good and poor solvents in various proportions, polymers of different molecular weight can be moved. For each molecular weight polymer, there is a critical mobile phase composition. This molecular weight-critical composition dependence arises from the flexibility of the polymer which enables it to change configuration in response to its environment[2,4,11,12]. Fig. 2 shows the effect of mobile phase composition on the movement of polyethylene polymers of different molecular weight. As the volume percent of the good solvent (1,2,4-trichlorobenzene) increases, higher-molecular-weight polymers elute with the solvent front (Fig. 2). In the case of polymers with a wider molecular weight range (such as the 52 000 MW polyethylene standard) a mobile phase can be used which causes the lower MW species to move while leaving the higher MW species at the origin.

There are two ways to obtain a chromatogram in which each molecular weight polymer has a slightly different R_F (as has been done for non-crystalline polymers[1,2]). The first way is to use gradient-elution TLC[9]. The second is to use a reversed-phase plate that forms its own gradient during development[1,4]. The former technique has already been described in the literature. The latter approach requires the use of reversed-phase plates and therefore awaits a solution to the visualization problem (*vide supra*).

ACKNOWLEDGEMENT

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CHROM. 21 027

Letter

Adsorption of Co(III) complexes on cellulose

In a recent note Ray and Kauffman[1] reported R_F values of Co(III) complexes on Whatman No. 1 paper developed with various aqueous solutions of inorganic salts.

We were very interested in their reporting rather strong adsorption for $[Co(NH_3)_6]Cl_3$ and $[Co(en)_3]Cl_3$ (where en = ethylenediamine) when developed with 0.1 M potassium iodide (R_F values 0.40 and 0.30, respectively). The adsorption of these complexes had been examined in our laboratory by M. Casillo with very similar electrolyte solutions (in connection with a paper electrophoretic study of ion-pair formation[2]) and no such adsorption was noted.

We thus repeated the chromatographic runs of $[Co(en)_3]Cl_3$ with 0.01 M potassium iodide on Whatman No. 1 paper as well as on Whatman No. 3MM paper and on cellulose thin layers and in all experiments we obtained a single fast-moving spot near the liquid front with an R_F value of *ca.* 0.9.

These results were communicated to Professor Kauffman on April 25th, 1988. In his letter of May 23rd, 1988, he informed me that he had forwarded my letter to Dr. Ray with the request to reply to me directly. Unfortunately, to date I have not yet received the authors' comments on the above discrepancy.

We thus feel that we should publish our observations that the work of Ray and Kauffman[1] contains results which we could not repeat.

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M. LEDERER

1 R. K. Ray and G. B. Kauffman, *J. Chromatogr.*, 442 (1988) 381.
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CHROM. 20 989

Book Review

Countercurrent chromatography —Theory and practice (*Chromatographic Science Series*, Vol. 44), edited by N. B. Mandava and Y. Ito; Marcel Dekker, New York, 1988, X + 841 pp., price US$ 115.00 (U.S.A. and Canada), US$ 138.00 (rest of world). ISBN 0-8247-7815-4.

This is a book which fills a gap in the literature on chromatography, the "big book" on liquid–liquid extraction methods. Further, it is edited and largely written by one of the modern exponents of the field.

The first chapter by Mandava and Ruth is an "Introduction to chromatography". It suffers from quoting largely from other "histories" of chromatography rather than consulting the original literature and from vague statements such as, "It is said that when one of the great discoveries in chromatographic science was submitted for publication, the journal of a renowned chemical society rejected it. The importance of the work was eventually emphasized by a Nobel Prize. Such instances are not uncommon in the history of scientific society publications. The paper on brassinolide [25] was rejected by the journals of two learned societies. After it was published in *Nature* and its impact was realized, the journals of those societies carried news stories." The reader would have liked to know the author and the journal and the year of the cited reference 25!

Then comes a chapter on "The origins of countercurrent chromatography" by the same authors as the Introduction. It covers the principles of the work of Craig on the Craig machine fairly well. However, this technique is not sufficiently mentioned in later chapters, hence a good survey of the applications of the Craig machine is sadly lacking.

Chapter 3 by Y. Ito deals with "Principles and instrumentation of countercurrent chromatography". This chapter, consisting of 363 pages, is an excellent treatise on Ito's methods and on its own would already be an excellent book and reference work. It is followed by a short chapter on "Theoretical aspects of countercurrent chromatography" by W. D. Conway.

The reader will be astonished by the following statement:

I. FUNDAMENTAL PARAMETERS

A. The Capacity Factor

Perhaps the most fundamental parameter in chromatography is the capacity factor k', which is defined as

$$k' = \frac{Q_s}{Q_m} = \frac{\text{quantity of solute in stationary phase}}{\text{quantity of solute in mobile phase}} \qquad (1)$$

as the term "capacity factor" is an misnomer confined to high-performance liquid chromatographic techniques.

The second half of the volume deals with various applications (312 pages). It is impressive that a chapter on the combination of countercurrent chromatography and mass spectrometry has as many as 194 references. Then there are several appendices: the first has a table of applications of high-speed countercurrent chromatography, another those of centrifugal countercurrent chromatography other than high-speed countercurrent chromatography and a further table on compounds separated by droplet countercurrent chromatography and one on non-aqueous solvent systems for countercurrent chromatography.

The second appendix gives lists of terms used and the third appendix the symbols used. Appendix four lists manufacturers of instruments with some good but badly reproduced figures.

The book can be recommended equally as a laboratory handbook and as a reading list for students in separation science.

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CHROM. 21 034

Book Review

Chromatographic enantioseparation —Methods and applications, by S. G. Allenmark, Ellis Horwood, Chichester/Halsted (Wiley), New York, 1988, 224 pp., price £ 32.50, ISBN 0-84312-988-6 (Ellis Horwood), 0-470-21080-X (Halsted).

This is easily the best introduction to chromatographic enantioseparation which is available today. It was written by one of the pioneers in the field and as the entire book comes from the pen of one author it has a homogeneity which is so often lacking in multi-author volumes.

There is an adequate introduction with a coverage of the historical background and an explanation of stereochemical concepts. This is followed by a survey of techniques used for measuring optical rotation and techniques of chromatography.

The main part of the book comprises three chapters dealing with the theory of chiral chromatography for direct optical resolutions, chiral gas chromatography and chiral liquid chromatography, respectively. All of it is eminently readable and contains a wealth of data in the form of tables.

The last chapters concern analytical applications in academic research and industry, preparative methods and future trends.

This reviewer enjoyed it all and found the text very easy to follow. As such it can be recommended for the reading list of students.

Now to come to some regrettable omissions: there is no mention of the separation of enantiomeric metal complexes, such as the Co(III) complexes. This is the more a pity as a number of principles were first tried with such complexes and that it forms an important chapter of chemistry, with at least one Nobel prize, that of A. Werner.

There is also no discussion of the related technique of electrophoresis, which has yielded interesting separations as well as shedding light on the interactions of optically active ions in solution. In the section on applications the work on age determination by the measurement of the racemization of aspartic acid in bones and teeth could also usefully have found a place.

And finally, a British publisher really should have avoided such awkward phrases as "...and was awarded a Docent..." or "To fully comprehend the modern chromatographic methods..."; on the other hand there are very few printer's errors and excellent figures with adequate legends.

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CHROM. 21 041

Book Review

Neuromethods, Vol. 8, Imaging and correlative physicochemical techniques, edited by A. A. Boulton, G. B. Baker and D. P. J. Boisvert, Humana Press, Clifton, NJ, 1988, 460 pp., price US$ 69.50, US$ 79.50 (export), ISBN 0-89603-116-0.

This book is part of a series, the previous volumes of which were either concerned with chromatographic methods or based on them*. The book presently under review deals, however, with other topics. The first chapter is on "Measurement of cerebral blood flow using diffusible gases" and the arguments used remind one of capillary liquid chromatography. The reviewer would have been pleased to see also a reference to the separation of noble gases by high-performance liquid chromatography. (Yes, high-performance liquid chromatography as all of them have different hydrophobic properties when dissolved in the eluent.) The following chapter deals with measurement of pial vessel hydrodynamics, and here again the principles are the same as in hydrophobic capillary chromatography and this is also mentioned in the discussion.

The next four chapters are entitled: Autography and cerebral function, Measurement of regional cerebral hemodynamics and metabolism by positron emission tomography, NMR spectroscopy of brain metabolism *in vivo*, and NMR imaging of the central nervous system.

With the very sensitive and elegant chromatographic techniques used in neurochemistry there was always the question in how far the results obtained from killed cells are also valid for living cells, as in the tale of the drunk found on all fours under a street lamp by the police, whose explanation was that he was looking for his car keys not where he had lost them but where there was light enough to look for them...

The final chapter on the measurement of cerebral ions poses the same question as the fine chromatographic analysis; by inserting an ion-selective electrode into a live cell do you alter the cell by this, or are we again looking under the street lamp?

This book will make interesting reading not only for the specialist but also for any chromatographer with some knowledge of physiology.

* Reviews of the first five volumes in the series were published in *J. Chromatogr.*, 403 (1987) 396–397, 404 (1987) 296 and 407 (1987) 415.

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CHROM. 20 926

Book Review

Analysis —What analytical chemists do, by J. Tyson, Royal Society of Chemistry, London, 1988, XIV + 186 pp., price £ 9.95, US$ 19.00, ISBN 0-85186-463-5.

This reviewer must first acknowledge a certain prejudice towards books which tend to talk down to the reader and hence his judgement may be felt by others to be on the harsh side.

Nevertheless, the Preface states "This book is for students, but it is not meant to be a textbook used in conjunction with a formal teaching programme", but on the back cover the publisher states that "The book is a must for chemistry students and will be a boon to any scientist wishing to be better informed about analytical chemistry and what it can and cannot do".

Some unintentional humour throughout reminds one somewhat of Joyce Grenfell's "George, don't do that", but some of Jaroslav Hašek's "Good Soldier Schweijk" is also represented, as in the statement on page 67: "Two types of stripping voltammetry are known, anodic and cathodic". Some of the other explanations offered are in the style of a book on driving a car which starts with: "Of course everybody today knows how a car works...".

In the chapter on chromatography, and only there, are the names of some investigators mentioned: "and the originators of modern instrumental chromatography A. J. P. Martin and R. L. M. Synge were rightly awarded their Nobel prize" (sic). In polarography Heyrovsky is not mentioned and the reason becomes clear in the following statement on page 65: "The full equation (known as the Ilkovič —pronounced "Ilkovitch"— equation) involves a number...".

May we still cite two further highlights? On page 138: Principles of Immunoassay. "When a sufficiently large molecule (relative molecular mass of 6000 or more) is injected into an animal, it triggers the animal's immune response system...".

On page 159: Electrophoresis. "Electrophoresis is a technique in which a potential gradient is produced along a gel bed...".

The book calls to mind G. B. Shaw's dictum on he who teaches... The author teaches!

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CHROM. 20 937

Book Review

Aqueous size-exclusion chromatography (*Journal of Chromatography Library*, Vol. 40), edited by P. L. Dubin, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, XVIII + 454 pp., price Dfl. 275.00/US$ 144.75, ISBN 0-444-42957-3.

This book attempts to cover the most fundamental aspects of aqueous size-exclusion chromatography (SEC) and also several areas of technological progress. However, as the Editor states in the Preface, "The reader will find a strong bias towards the elucidation of principles with a correspondingly reduced emphasis on instrumentation *per se*". The volume is divided into five sections: Separation Mechanisms (SEC parameters, hydrophobic interactions, electrostatic effects and SEC of inorganic compounds), Characterization of Stationary Phases (pore size distribution, structural analysis of porous materials using SEC and column efficiency), New Packings (native and bonded silica and rigid polymer gels), Biopolymers (protein separations in denaturing and non-denaturing solvents, serum lipoproteins and application of SEC–low-angle laser-light scattering) and Associating Systems (measurement of equilibration constants by SEC, frontal analysis in SEC and SEC of micelles).

As one might expect for a multi-author volume, the quality of the different chapters varies, but in this particular book the quality differences are especially great. There is also considerable overlap between some of the chapters. The different authors use different nomenclature and definitions, which could be confusing or even misleading. For example, some authors mix up the definitions for K_D and K_{av}.

In a volume entitled *Aqueous Size-Exclusion Chromatography* one would not expect that, in a chapter dealing with column efficiency, several of the examples discussed would be taken from experiments performed in organic solvents. The same chapter gives advice regarding the sample volume to be used for the determination of the theoretical plate number for a SEC column; altough it states that the sample volume should be 0.1–0.2 ml, it does not give the column volume. In another chapter (Rigid polymer gels for SEC and their application to biopolymers) the Superose gels are presented as dextran-based, whereas it is well known that they are based on an agarose matrix.

Unfortunately, a few more examples of this type can be found in the book. The volume would have been improved if some of the chapters had been revised more carefully.

Despite these shortcomings, most of the chapters are well written and informative, describing fundamental areas in aqueous SEC in a comprehensive way. The book was produced from camera-ready manuscripts, all having figures and graphs of good quality.

Uppsala (Sweden) KJELL-OVE ERIKSSON

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CHROM. 20 994

Book Review

Supercritical fluid chromatography, edited by R. M. Smith, Royal Society of Chemistry, London, 1988, 238 pp., price £ 27.50, US$ 59.00, ISBN 0-85186-577-1.

The book was written in conjunction with a course in supercritical fluid chromatography (SFC), which was organized by the Editor in July 1986 at the Loughborough University of Technology in England, and it is the first book in the series *RSC Chromatography Monographs*. The eight chapters by authors from Europe and Japan were written in the summer of 1987. It is, moreover, one of the first four books on SFC, which appeared in 1987 and 1988, and which, by reason of timeliness alone, definitely respond to a need. As is to be expected for a multi-authored book, not all important aspects of the subject could be touched upon, let alone covered in depth. The aspects which are covered in the individual chapters, however, are useful for informing the reader about current practical aspects, theory and results in SFC.

The first chapter, by Bartle, gives an introduction to SFC, emphasizing basic principles, and the second chapter, by Sanagi and Smith, presents first a brief historical account of SFC with an overview of the number of publications that have appeared and their coverage. The larger part of the chapter is then devoted to instrumentation, placing particular emphasis on the mobile phase delivery system and cooling devices.

Chapter 3, by Leyendecker, is the largest in the book and gives guidance for selecting proper separation conditions in SFC. The effect of changing one parameter at a time, *i.e.*, density, pressure, temperature, mobile phase or mobile phase composition, is discussed, followed by a treatment of the effect of varying two parameters simultaneously, *i.e.*, density and temperature, pressure and temperature, and composition and temperature. The chapter also deals with programming the parameters singly or simultaneously, stressing the importance of gradients for SFC.

In Chapter 4, by Schoenmakers, a comparison is made between open and packed columns. At the outset it is rightly pointed out that there are no sharp borderlines between SFC and gas (GC) or high-performance liquid chromatography (HPLC). The advantage of open-tubular columns is seen primarily in the higher plate number attainable when using long columns. The important advantage of packed columns is their higher speed of analysis. The role of the pressure drop is discussed, emphasizing that efficiency deteriorates if the pressure at the column exit is too low. The author also points out the importance of adjustable resistors (valves) at the column exit, without which pressure and linear velocity cannot be changed independently of each other. Chapter 5, by Sandra, compares capillary SFC with capillary high-temperature GC. Not surprisingly, the high-temperature version of capillary GC leads to higher efficiencies than capillary SFC, but suffers the drawback of only a few stationary phases and not all substrates being able to withstand temperatures of 400°C and higher.

The next two chapters, 6 and 7, deal with SFC–mass spectrometry (MS). Games *et al.* describe MS interfaces for both capillary and packed columns, prominently

among them a moving belt with a thermospray deposition device. Both chemical ionization (CI) and electron impact (EI) mass spectra can be obtained when the proper conditions are attainable, *e.g.*, a high vacuum for EI. SFC–MS in the pharmaceutical industry is the topic of the chapter by Lance, who uses a packed column and a similar SFC–MS interface as before, and in addition an UV detector in tandem. A large number of total ion current (TIC) and selected ion monitoring (SIM) chromatograms are presented, together with spectra of some of the individual peaks, to document the capabilities of the method.

The last chapter, 8, deals with fractionation by supercritical fluid extraction (SFE) and the on-line combination of SFE with SFC. After a discussion of principles, attention is directed to a new type of back-pressure regulator useful for SFE and SFC. The regulator consists of a flow-switching valve which has the advantage of reducing clogging of the extremely small passageway between stem and seat. Two- and three-dimensional profiles are utilized to show the extraction kinetics for the whole extract, coming from the SFE part, and also for the separation of the extract on the SFC column.

The book is general enough and treats enough practical aspects to be of value for the analyst starting out with SFC. At the same time, it treats enough of more specialized topics to be of use to the chromatographer already working with SFC.

Aachen (F.R.G.) E. KLESPER

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CHROM. 20 947

Book Review

Analytical applications of spectroscopy, edited by C. S. Creaser and A. M. C. Davies, Royal Society of Chemistry, London, 1988, XIII + 488 pp., price £47.50, US$ 99.00, ISBN 0-85186-383-3.

This volume is a collection of camera-ready printed papers presented at the international conference *Spectroscopy Across the Spectrum*, incorporating the proceedings of the *First International Near-Infrared Spectroscopy Conference*, held on July 12–15th, 1987, in Norwich, U.K.

The book contains six sections dealing with near-infrared spectroscopy (22 papers), infrared spectroscopy (14 papers), mass spectrometry (6 papers), nuclear magnetic resonance spectroscopy (6 papers), atomic and UV–VIS spectroscopy (8 papers) and chemometrics and data analysis (13 papers, some of which belong thematically to the above-mentioned spectral methods). The Preface, subject and author indexes complete the book.

As in many conference collections, the individual papers differ in concept and form, covering more deeply or less well the theoretical basis, instrumentation and/or practical applications of the spectral methods considered.

Although the practical applications of near-IR are discussed most often, the advantage of the presented collection of papers is that the Fourier transform (FT) approach and data-handling techniques have diversified almost all the spectral methods concerned. For readers who specialize in separation methods a few papers on "hyphenated" techniques may be of considerable interest, *viz.*, on chromatography–FT-IR (P.R. Griffiths), supercritical fluid chromatography–FT-IR (M.W. Raymor, I.L. Davies, K.D. Bartle, A. A. Clifford, A. Williams, J.M. Chalmers and B.W. Cook), liquid chromatography–mass spectrometry (F.A. Mellon, G.R. Fenwick, J.A. Lewis and E.A. Spinks), high-performance liquid chromatography–atomic absorption spectrometry or –atomic emission spectrometry (L. Ebdon and S. Hill), capillary gas chromatography–fluorescence detection (H. Babhai and C.S. Creaser) and high-performance liquid chromatography–fluorescence detection (M.A.J. Bayliss, R.B. Homer and M.J. Shepherd).

The book can be recommended as a source of useful information from very different fields of application (foodstuffs, leather, agrochemicals, orthopeadic bone cement, trace analysis of metals, etc.).

Brno (Czechoslovakia) JAROSLAV JANÁK

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Erratum

J. Chromatogr., 452 (1988) 265–281
Page 275, last line above the equation, "see page 3" should read "see page 267".

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NEW BOOKS

Size-exclusion chromatography, edited by B.J. Hunt and S.R. Holding, Blackie Publishers, Glasgow, 1988, 292 pp., price £ 45.00.

Countercurrent chromatography: theory and practice, edited by N. Bushan Mandova and Y. Ito, Marcel Dekker, New York, 1988, X + 842 pp., price US$ 115.00 (U.S.A. and Canada), US$ 138.00 (rest of world), ISBN 0-8247-7815-4.

Electrophoresis '88, 6th Meeting of the International Electrophoresis Society, Copenhagen, July 4–7, 1988, edited by C. Schafer-Nielsen, VCH Verlagsgesellschaft, Weinheim, Basel, Cambridge, New York, 1988, 502 pp., price DM 148.00, £ 51.00.

Analysis — What analytical chemists do, by J. Tyson, Royal Society of Chemistry, London, 1988, XIV + 186 pp., price £9.95, US$ 19.00, ISBN 0-85186-463-5.

The chemical physics of solvation. Part C: Solvation phenomena in specific physical, chemical, and biological systems, edited by R.R. Dogonadze, E. Kálmán, A.A. Kornyshev and J. Ulstrup, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, XXXII + 788 pp., price US$ 250.00, Dfl. 475.00, ISBN 0-444-42984-0. (Set price, Parts A–C: US$ 508.00, Dfl. 965.00.)

Silylating agents, by G. van Look, Fluka, Buchs, 1988, 154 pp., price US$ 14.40, S.Fr. 16.00, ISBN 90561702-1.

Petroanalysis 87, Analytical development in the petroleum industry (*Proceedings of the 3rd Petro Analysis Symposium, Lancaster*), edited by G.B. Crump, Wiley, Chichester, New York, 1988, *ca.* 500 pp., price *ca.* US$ 115.00, ISBN 0471-91946-2.

Flavors and flagrances: A world perspective, edited by B.M. Lawrence, B.D. Mookherjee and B.J. Willis, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, XIV + 1108 pp., price US$ 302.75, Dfl. 575.00, ISBN 0-444-42964-6.

FELLOWSHIP

ANNIVERSARY FELLOWSHIP FOR ANALYTICAL CHEMISTRY

Council of the Analytical Division of The Royal Society of Chemistry has approved proposals for a Robert Boyle Fellowship in Analytical Chemistry to mark the 150th Anniversary in 1991 of the founding of The Chemical Society. The Fellowship will be awarded by the Trustees of the Analytical Chemistry Trust Fund.

It is intended that the Fellowship be awarded to an applicant making the most prestigious proposal within the realm of and of direct benefit to the advancement of analytical chemistry in the modern world.

Although the initiative for the Fellowship arises from the desire to mark the 150th Anniversary of The Chemical Society, founded in 1841, it is noted that 1991 marks the tercentenary of the death of Robert Boyle (1627–1691) in whose work were to be seen the beginnings of modern chemical analysis. Therefore, the Fellowship is also dedicated to him.

It is expected that the Fellow appointed will be at work at the time of the 150th Anniversary Celebrations of the Royal Society of Chemistry (within which are amalgamated The Chemical Society, The Royal Institute of Chemistry, The Society for Analytical Chemistry and The Faraday Society) to be held at Imperial College, University of London between April 9 and 12, 1991.

The Fellowship will be tenable at a British university, polytechnic, or research establishment having facilities to meet the approval of the Trustees. Prospective applicants should register their interest with the Secretary of the Analytical Division at the following address: Burlington House, London W1V 0BN, U.K. Tel.: (01) 4378656.

ANNOUNCEMENTS OF MEETINGS

WORKSHOP ON ION CHROMATOGRAPHY, SILS-MARIA, SWITZERLAND, APRIL 13–14, 1989

The workshop will follow the three-day symposium in Sils-Maria, Switzerland, and will be coordinated by Professor R.W. Frei (Free University Amsterdam, The Netherlands). Workshop leader will be Professor P.R. Haddad (University of New South Wales, Australia). Participation of other speakers from the preceding symposium in Sils-Maria is envisaged.

Topics covered will include: sample handling, enrichment, clean-up, automation; dual and single column concepts; theory and practice of ion separations (isocratic and gradients); detection principles; applications in the pharmaceutical, environmental, biomedical, alimentation fields etc.

The registration fee (DM 690) will include luncheons, refreshments, reception, manual and company information. Upon registration you will receive further information on the course.

For further details contact: Workshop Office, IAEAC, M. Frei-Häusler, Postfach 46, CH-4123 Allschwill 2, Switzerland.

11th ANNUAL SPRING SYMPOSIUM OF THE MINNESOTA CHROMATOGRAPHY FORUM, MINNEAPOLIS, MN, U.S.A., MAY 8–10, 1989

The Minnesota Chromatography Forum (a non-profit organization) is presenting the 11th Annual Spring Symposium, May 8–10, 1989. The symposium will be held at the Thunderbird Hotel and Convention Center, Minneapolis, MN, U.S.A.

The keynote speaker will be Dr. Lloyd Snyder of LC Resources Incorporated. The topic of his presentation will be *Methods Development*. The symposium will also focus on applications and developments in all aspects of chromatography.

For further information, please contact: Kowler Associates, 4948 Lyndale Avenue South, Minneapolis, MN 55409, U.S.A. Tel.: (612) 823-6034.

8th INTERNATIONAL SYMPOSIUM ON ADVANCES AND APPLICATION OF CHROMATOGRAPHY IN INDUSTRY, BRATISLAVA, CZECHOSLOVAKIA, JULY 2–7, 1989

The 8th International Symposium on Advances and Application of Chromatography will be held in the House of the Trade Union, František Zupka square, Bratislava, Czechoslovakia, July 2–7, 1989. An exhibition of chromatographic and ancillary equipment will be held at the same place. The exhi-

bition will be organized by INCHEBA, Foreign Trade Company.

The scientific programme will comprise plenary lectures, as well as lectures and poster sessions, dealing with the theory and practice of: gas chromatography, liquid chromatography, supercritical-fluid chromatography, electromigration and other methods (advances, new separation systems, identification of substances, optimization, detection systems); and their application in: industry, biochemistry, clinical chemistry, biotechnology, environmental analysis, trace analysis. The conference language will be English.

An abstract of between 200–300 words in English will be required from prospective authors by December 31, 1988. Abstracts of all papers and poster presentations will be distributed in book form to all participants at the symposium. The full papers of the presentations will be published in a special issue of the *Journal of Chromatography.*

These interested in attending the Symposium should write to the address given below before December 31, 1988. The registration fee will be SF 290.

Correspondence concerning organizational features of the symposium as well as registration of attendance including paper registration should be addressed as follows: Department of Analytical Chemistry, Symposium on Chromatography; Faculty of Chemical Technology, Radlinského 9, 812 37 Bratislava, Czechoslovakia. Tel.: 560 43.

11th INTERNATIONAL SYMPOSIUM ON MICROCHEMICAL TECHNIQUES, WIESBADEN, F.R.G., AUGUST 28–SEPTEMBER 1, 1989

The 11th International Symposium on Microchemical Techniques, organized by the Fachgruppe Analytische Chemie of the Gesellschaft Deutscher Chemiker, will be held in Wiesbaden, F.R.G., August 28–September 1, 1989.

The topics that will be covered at the symposium include: pure and applied aspects of analytical chemistry concerning inorganic and organic trace, micro and surface analysis in natural sciences, medicine, technology and environment. The scientific programme will comprise plenary lectures, main lectures and poster presentations of original contributions.

Authors wishing to submit contributions should write to the address given below. Contributions can be given only as posters. The scientific committee will decide on the acceptance of the contributions. The discussion of the posters should represent an essential feature of the symposium.

Abstracts of all papers and poster presentations will be distributed to delegates at the commencement of the meeting. The full text of the lectures will be published in a special issue of *Fresenius Zeitschrift für Analytische Chemie.* Authors are, therefore, requested to hand their manuscripts to the editor at the meeting. An exhibition of modern analytical equipment and new literature concerning the methods discussed will be held adjacent to the meeting.

For further details contact: Gesellschaft Deutscher Chemiker, Abteilung Tagungen, Varrentrappstrasse 40–42, P.O. Box 900440, D-6000 Frankfurt am Main 90, F.R.G. Tel.: (069) 7917-360/366, Fax: (069) 7917-322, Telex: 4170 497 gdch d.

CIS '89, 10th INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY, TOKYO, JAPAN, OCTOBER 17–20, 1989

CIS '89, the 10th International Symposium on Chromatography, will be held at the Japan City Center in Tokyo, Japan, October 17–20, 1989. The symposium will be sponsored by The Division of Liquid Chromatography of The Japan Society for Analytical Chemistry and will commemorate the 20th anniversary of this division.

The main theme of the symposium will be chromatography in life science. The scientific programme will include numerous oral and poster papers on subjects discussing recent advances in chromatography and related separation techniques such as FFF, SFC and column electrophoresis. The symposium will cover the latest topics on separation methods ranging from theory and fundamental aspects to instrumental development and applications. Poster sessions are planned as a major part of

the sessions. Discussion sessions will also be organized to deal with recent advances and prospectives of wide chromatographic techniques and applied topics. The symposium language will be English.

Those intending to present an oral discussion paper or a poster communication should submit a one-page 300-word summary on plain white paper to Dr. T.Hoshino, MD., the Secretary General of CIS'89 Tokyo. Title, author(s), affiliation, and mailing address should be placed at the top of the page. At the bottom of the page in the lower right hand corner, please note whether a poster or oral presentation is desired. The summary should arrive before June 17, 1989. The review procedure will be completed and notification of acceptance and details of the abstract form mailed to authors by early July, 1989. The abstracts will be produced in a booklet and distributed at the meeting. The authors should submit their abstracts completed in the abstract form no later than August 19, 1989.

Papers presented at the symposium will be refereed for publication in a special issue of the *Journal of Chromatography*. Authors wishing to submit their papers should bring three copies of the manuscript and one set of reproducible figures with them to the conference.

The tentative registration fee will be Y 15 000. A reduced registration fee will be offered for students.

In conjuction with the symposium, an exhibition of the latest chromatographic instrumentation, columns and accessories is planned. Companies interested in participating in this exhibition should direct their inquiries to the Exhibition Office, Ad-point, Ltd., 1-9-21 Fujimi-cho, Chiyoda-ku, Tokyo 102, Japan, FAX 81-03-261-5345.

An attractive social programme is planned including "Welcome to CIS '89/Tokyo", a reception and an "Enkai", a traditional Japanese style banquet in the Tatami-room.

For further details, contact: Dr. T. Hoshino, Secretary General of CIS '89/Tokyo, c/o Pharmaceutical Institute, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160, Japan. Tel.: (81) 03-353-1211, ext 2737; FAX 81-03-225-1479.

6th SYMPOSIUM ON SEPARATION SCIENCE AND TECHNOLOGY FOR ENERGY APPLICATIONS, KNOXVILLE, TN, U.S.A., OCTOBER 22–27, 1989

The United States Department of Energy, Oak Ridge National Laboratory, the Subdivision of Separation Science and Technology of the American Chemical Society, and the American Institute of Chemical Engineers are sponsoring the 6th of a series of symposia entitled, Separation Science and Technology for Energy Applications. This symposium will be held October 22–27, 1989 in Knoxville, TN, U.S.A. This will be a most beautiful time in East Tennessee, and the symposium will be scheduled so that participants can experience the impact of the Great Smoky Mountains and the Cumberland Mountains. Both are convenient areas near Knoxville.

A variety of separation science topics will be covered in the symposium such as: solvent extraction, adsorption and ion exchange, membrane separations, absorption, distillation, physical separations (solid–liquid, solid–gas, and solid–solid), photochemical separations, supercritical fluid-based separations, chromatography, field flow fractionation, separations by leaching processes, electrochemical separations, biochemical separations, and novel or new separation techniques.

If you are interested in presenting a paper at one of the sessions, please submit a 200- to 250-word abstract, no later that April 1, 1989, to the address given below. Since we are planning to have the proceedings published in a refereed journal in a timely manner, you will be required to submit the paper in a form suitable for submission to the journal at the time of the symposium.

As with previous symposia, a special poster session is being scheduled for the presentation of selected papers on the session topics indicated, as well as for the description of significant advances in other areas of separation science. Although all papers will be considered for presentation in the poster session, priority will be given to those papers which the author indicate to be particularly appropriate for this mode of presentation.

For further details, contact: Dr. J.T. Bell, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6268, U.S.A. Tel.: (615) 574-4934 or 574-6795.

3rd EUROPEAN MEETING ON BIO-CHROMATOGRAPHY AND MOLECULAR AFFINITY, DIJON, FRANCE, MAY 22–25, 1990

The 3rd European Meeting of "Groupe Français de Bio-Chromatographie" will be held at the Congress Center in Dijon, France, from May 22–25, 1990.

The meeting is entitled "Bio Chromatography and Molecular Affinity" and offers scientists, whose investigations involve chromatographic techniques in the field of biological molecules, a forum to discuss their results as well as the problems they encounter in their work.

For further details contact: Dr. J.-P. Dandeu, Institut Pasteur, Unité d'Immuno-Allergie, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (1) 45 68 80 00.

COURSES

BRADFORD ANALYTICAL COURSE ON CHIRAL SEPARATIONS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, BRADFORD, U.K., MARCH 20–23, 1989

Chiral separations in liquid chromatography are of key strategic importance in the pharmaceutical and related industries, due to legislative concern for stereoselective consequences in drug therapy and drug metabolism. Chiral separations are also being vigorously developed for a wide range of applications in the life sciences. This intensive course is designed to operate at two levels: to introduce the novice to the fundamental principles and applications of chiral separations; to enable the more experienced user to extend and strengthen their expertise in state-of-the-art chiral technology for practical problem solving.

This four-day Bradford Analytical Course will run from 10 a.m. on Monday 20 March to 2 p.m. on Thursday 23 March 1989. The course is designed to give a thorough training in the principles and practical applications of chiral separations in HPLC. Participants will have extensive opportunities to familiarise themselves with state-of-the-art chiral technology from many of the leading manufacturers, in practical demonstrations and hands-on workshop sessions. The range of topics covered in lectures and tutorial discussion sessions will include: principles and implications of chirality in drugs and metabolites; design and utilisation of chiral column technology; development of chiral eluents; principles of stereoselective method optimisation; semi-prep and prep-scale chromatography; chiroptical techniques for method validation (ORD, CD, FT-NMR); applications in chiral quality control, chiral metabolism, therapeutic drug monitoring, clinical biochemistry; trends in legislative implications for stereoselective drug design and control. The course is presented by Professor Anthony F. Fell and Dr. Brian J. Clark.

The course fee (£650) includes full documentation, hotel accommodation, all meals and the course dinner.

Applicants wishing to register for the course should return the attached registration form with their remittance to: Miss Susan O'Brien, Continuing Education Unit, University of Bradford, Bradford, BD7 1DP, West Yorkshire, UK.

ANALYTICAL CHEMISTRY SHORT COURSES, LOUGHBOROUGH, U.K.

The following short courses will be held in The Department of Chemistry, Loughborough University of Technology, Loughborough, U.K., in the spring of 1989:

- Intracellular calcium — new fluorescence methods; February 22, 1989; fee £75 including coffee, lunch, tea.
- Separations for biotechnology and biochemistry; April 10–14, 1989; fee £480 including residence and all meals (£450 if paid with booking form), non-residents £405 (£375 if paid with booking form).

— Basic microbiological methods for the analytical chemist; April 17–21, 1989; fee £510 including residence and all meals, non-residents £435.
— Gas–liquid chromatography; April 17–21, 1989; fee £480 including residence and all meals (£450 if paid with booking form), non-residents £405 (£375 if paid with booking form).

Further details may be obtained from: Mrs. J.E. Stirling, Department of Chemistry, Loughborough University of Technology, Loughborough, Leics. LE11 3TU, U.K. Tel.: (0509) 222549.

CALENDAR OF FORTHCOMING MEETINGS

Jan. 5–7, 1989 Orlando, FL, U.S.A.	**Winter Conference on Flow Injection Analysis** Contact: G.D. Christian or J. Ruzicka, Department of Chemistry, BG-10, University of Washington, Seattle, WA 98195, U.S.A. (Further details published in Vol. 450, No. 3.)
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. (Further details published in Vol. 448, No. 3.)
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 6–9, 1989 San Francisco, CA, U.S.A.	**1989 Joint Symposium on Stationary Combustion NO_x Control** Contact: Claudia Runge, Electric Power Research Institute, 3412 Hillview Avenue, Palo Alto, CA 94303, U.S.A.
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.)
March 20–23, 1989 Bradford, U.K.	**Analytical Course on Chiral Separations in High-Performance Liquid Chromatography** Contact: Dr. B.J. Clark, Pharmaceutical Chemistry School of Chemistry, University of Bradford, BD7 1DP, U.K. Tel.: (0274) 733466, ext. 585

April 9–12, 1989 Sils-Maria, Switzerland	**6th Symposium on Ion 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.)
April 11, 1989 Washington, DC, U.S.A.	**Chromexpo 1989, Chromatography Exhibition and Poster Session** Contact: Janet E. Cunningham, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.
April 13–14. 1989 Freiburg i. Br., F.R.G.	**Workshop on Ion Chromatography** Contact: Workshop Office, IAEAC, M. Frei-Häusler, Postfach 46, CH-4123 Allschwill, Switzerland.
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 8–10, 1989 Minneapolis, MN, U.S.A.	**11th Annual Spring Symposium of the Minnesota Chromatography Forum** Contact: Kowler Associates, 4948 Lyndale Avenue South, Minneapolis, MN 55409, U.S.A. Tel.: (612) 823-6034.
May 8–12, 1989 Colorado Springs, CO, U.S.A.	**11th Symposium on Biotechnology for Fuels and Chemicals** Contact: Elias Greenbaum, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6194, U.S.A.
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. (Further details published in Vol. 450, No. 3.)

May 22–25, 1989
Riva del Garda,
Italy

10th International Symposium on Capillary Chromatography
Contact: Dr. P. Sandra, Laboratory for Organic Chemistry, University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent, Belgium.

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. and Vol. 448, No. 3.)

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. (Further details published in Vol. 450, No. 3.)
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. (Further details published in Vol. 448, No. 3.)
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. (Further details published in Vol. 448, No. 3.)
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.
Sept. 27–29, 1989 Paris, France	**Euro Food Chem V, 5th European Conference on Food Chemistry** Contact: G.A.M.S., 88 Boulevard Malesherbes, 75008 Paris, France. Tel.: (161) 45639304.
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. 17–20, 1989 Tokyo, Japan	**10th International Symposium on Chromatography, CIS '89** Contact: Tadao Hoshino, Pharmaceutical Institute, School of Medicine, Keio University, 35-Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.
Oct. 22–27, 1989 Knoxville, TN, U.S.A.	**6th Symposium on Separation Science and Technology for Energy Applications** Contact: Dr. J.T. Bell, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6268, U.S.A. Tel.: (615) 574-4934 or 574-6795.
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. (Further details published in Vol. 448, No. 3.)
Nov. 5–8, 1989 Philadelphia, PA, U.S.A.	**9th International Symposium for High-Performance Liquid Chromatographic Separation of Proteins, Peptides and Polynucleotides** Contact: Janet E. Cunningham, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.

May 22–25, 1990 Dijon, France	**3rd European Meeting on Bio-Chromatography and Molecular Affinity** Contact: J.-P. Dandeu, Groupe Français de Bio-Chromatographie, Institut Pasteur, Unité d'Immuno-Allergie, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (1) 45688000.
Aug. 5–10, 1990 Hamburg, F.R.G.	**7th IUPAC Congress on Pesticide Chemistry** Contact: GDCH-Geschaeftstelle, Abteilung Tagungen, Postfach 900440, D-6000 Frankfurt 90, F.R.G.
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. (Further details published in Vol. 445, No. 1.)
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. (Further details published in Vol. 448, No. 3.)

PUBLICATION SCHEDULE FOR 1988

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	435/1 435/2 435/3 436/1	436/2 436/3	437/1 437/2	438/1 438/2	439/1 439/2 440 441/1	441/2 442 443	444 445/1 445/2 446	447/1 447/2 448/1	448/2 448/3 449/1	449/2 450/1 450/2 450/3 452	453 454 455	456/1 456/2 457 458 459
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Cumulative Indexes, Vols. 401–450												451
Biomedical Applications	424/1	424/2	425/1 425/2	426/1 426/2	427/1	427/2 428/1	428/2 429	430/1	430/2 431/1	431/2	432	433 434/1 434/2

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.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

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

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

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

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

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

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

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Selected references:
D.C. Turnell and J.D.H. Cooper, J. Chromatogr., 395 (1987) 613-621.
J.D.H. Cooper, D.C. Turnell, B. Green and F. Vérillon, J. Chromatogr., 456 (1988) 53-69.

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