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Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2		The publication schedule for further issues will be published later.								
Chromatographic Reviews			184/1										
Biomedical Applications		181/1	181/2	181/3-4									

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For further information, see page 3 of cover.

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CONTENTS

(Continued overleaf)

Contents (continued)

Notes

Letter to the Editor

Journal of Chromatography, 187 (1980) 285–295

CHROM. 12,336

THEORETICAL CONSIDERATIONS OF MOLECULAR SIEVE EFFECTS

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(First received April 11th, 1979; revised manuscript received August 21st, 1979)

SUMMARY

The separation of molecules in neutral gel and controlled-pore glass columns is studied on the basis of a statistical model. The theoretical results are compared with experimental results obtained from Sephadex gel columns. The good agreement between theoretical and experimental results over the whole working range enables complete calibration of columns with just one reference molecule and a knowledge of the void volume, V_0. By means of the derived equation, the molecular weight can be calculated from the elution volume with greater accuracy. The equation also permits the calculation of the elution volume of aggregates of molecules which can assume different sterical conformations with high frictional ratios. An accurate functional correlation between elution volume and molecular weight applicable over the whole working range not only makes analytical steric chromatography less time-consuming but also renders it more amenable to automation. Providing active transport can be neglected, the derived equation may also be valid for filtration across membranes and synapses.

INTRODUCTION

Although the use of gel filtration to estimate the dimensions of macromolecules was suggested by Lathe and Ruthven[1] in 1956, three years elapsed before the work of Porath and Flodin[2] on crossed-linked dextrans and the introduction of Sephadex opened the way to further developments. Since then, gel chromatography, together with sucrose density gradient centrifugation and analytical ultracentrifugation, has become an established technique for the characterization of macromolecular systems.

As in other areas of applied science, progress on the theoretical side has not been as rapid as that on the practical. Many attempts have been made to correlate elution volume with molecular size and shape and there exist a number of equations based on empirically-fitted curves, various geometrical models or statistical assumptions[3–9]. Most of the derived equations are, however, impracticable for normal laboratory purposes and the implied structural features of the gels are arbitrary. One

empirically-based equation[10,11] has nevertheless found wide acceptance; it expresses a logarithmic relationship between the elution volume, V_e, and the molecular weight, M

$$V_e = c_1 - c_2 \log (M) \quad (1)$$

where c_1 and c_2 are empirical constants. Eqn. 1 is used for simplicity, although it cannot be applied accurately over the whole working range of dextran gels (see Fig. 6), an obvious drawback in establishing a valid calibration curve.

This paper presents an equation which describes the functional correlation over the whole range of filtration, and which enables the influence of axial ratios on the elution volume to be calculated. The equation is very similar to an expression derived by Hjertén[12]: neither formula makes any assumptions as to the structure of the gel or the accessible space within the gel, but whereas Hjertén's derivation rests on thermodynamic considerations, the present treatment is based on a statistical model.

MATERIALS AND METHODS

Preparation of gel columns

Columns (100 × 2 cm I.D.) were filled with Sephadex G-25, G-50, G-75, G-100 and G-200 according to the procedure described by the manufacturer and equilibrated with acetate buffer (10 mM CH_3COONa, 60 mM KCl, 10 mM $MgCl_2$; pH 5.5).

Calibration proteins

The calibration of Sephadex gel columns was performed with the following molecules: α-alanine, phenol red, ribonuclease T1, pancreatic ribonuclease, myoglobin, lysozyme, cytochrome *c*, chymotrypsinogen, crotoxin, phosphofructokinase, hexokinase, haemoglobin, ovalbumin, glucose-6-phosphate dehydrogenase, aldolase, pyruvate kinase, transferrin (all from Boehringer, Mannheim, G.F.R.) and dextran blue (Pharmacia, Uppsala, Sweden).

The extinction of the elution solvent was measured at 252 and 280 nm with a 8300 Uvicord II photometer, and the elution volume was determined by collecting 1 ml fractions.

The measurements were repeated at least five times for each reference molecule in order to calculate the respective standard errors.

THEORETICAL

Preliminaries

Electron micrographs of swollen, frozen-etched Sephadex reveal a network of crossed-linked dextrans (Fig. 1). Gel filtration can be described as a diffusional partitioning of solute molecules between the external liquid phase (V_0) around the porous, bead-like particles, and the internal solvent phase (V_i), within the particles themselves. Molecules larger than the largest pores of the swollen Sephadex particles can move only in the volume V_0. Smaller molecules, however, penetrate the particles to an extent dependent on their size and shape. A schematical representation of how

Fig. 1. Electron micrograph of frozen-etched swollen Sephadex G-75.

partitioning may occur is illustrated in Fig. 2: the smaller molecule R_1 penetrates the "gel matrix" by pushing the fibres aside, whereas the larger molecule R_2 is, at first, excluded and has to move horizontally until it, in turn, is able to penetrate the matrix.

The derivation of the function is based on the following assumptions: (1) the fibres in the porous regions of the gel are distributed randomly; (2) the size of the penetrable area, $A(r)$, is determined by the square of the longest axis of the largest molecule that can penetrate; (3) the probability, $w(r)$, of finding an area $A(r)$ at height h is normally distributed; and (4) the volume $(V_e - V_0)$ is proportional to the total area $A_t(r')$ which can be penetrated by a molecule with a radius r', *i.e.*, the distribution of $A(r)$ is the same along the whole length of the column.

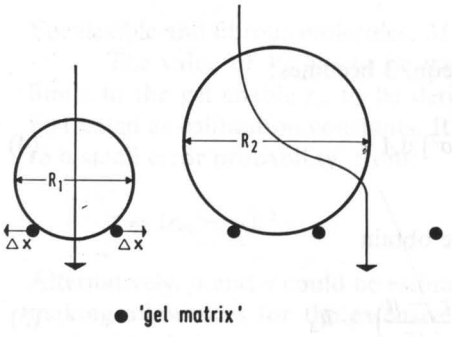

Fig. 2. Schematic representation of a gel filtration process.

An arbitrary Gaussian probability distribution of the penetrable areas $A(r)$ is shown in Fig. 3; the distribution of accessible areas for a molecule with a radius r' is indicated by hatching.

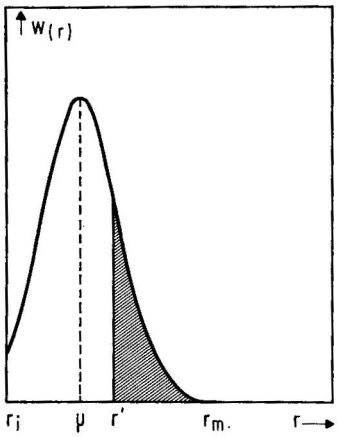

Fig. 3. Arbitrary Gaussian distribution of penetrable areas.

RESULTS

The total penetrable area, $A_t(r')$, within a small vertical distance, Δx, for a molecule with a radius r' is the sum over all areas $A(r)$ with $r' \leqslant r \leqslant r_m$, where r_m is the radius of the largest molecule that can penetrate the gel particles.
Thus:

$$A_t(r') = \sum_{r' \leqslant r \leqslant r_m} w(r)\, A(r) \tag{2}$$

Assuming a continuous distribution:

$$A_t(r') = \int_{r' \leqslant r \leqslant r_m} W(r)\, \mathrm{d}A \tag{3}$$

Inserting the Gaussian distribution function, eqn. 3 becomes:

$$A_t(r') = \int_{A(r')}^{A(r_m)} \exp\left[-(r - \langle r \rangle)^2/2\sigma^2\right] \mathrm{d}A \tag{4}$$

Substituting $r^2 = A/\pi$ and $(V_e - V_0) \propto A_t$, we obtain

$$V_e = k_1 \exp\left(-\frac{r^2 - 2\mu r}{2\sigma^2}\right) - k_2 \operatorname{erf}\left(\frac{r - \mu}{\sqrt{2}\sigma}\right) + k_3 \tag{5}$$

where

$$\mu = \langle r \rangle \tag{6}$$

$$k_1 = L\sqrt{2\pi}\sigma \exp\left(-\frac{\mu^2}{2\sigma^2}\right) \tag{7a}$$

$$k_2 = 2L\sqrt{\pi}\mu \tag{7b}$$

$$k_3 = L\sqrt{\pi}\left[2\mu \operatorname{erf}\left(\frac{r_m - \mu}{2\sigma}\right) - \sqrt{2}\sigma \exp\left(-\frac{(r_m - \mu)^2}{2\sigma^2}\right)\right] + V_0 \tag{7c}$$

$$\operatorname{erf}(x) = {}_0\int^x \exp(-t^2)\,dt, \tag{8}$$

and L is a constant.

In accordance with Oncley[13], the molecular weight, M, of globular particles can be expressed as

$$M = \alpha r^3, \quad \text{with} \quad \alpha = \frac{4\pi}{3}\frac{N}{(\bar{v} + \delta\bar{v}_s)} \tag{9}$$

where N is Avogadro's number, $\bar{v}$ is the partial specific volume, δ is the number of grams of solvent per gram of dry macromolecular material and $\bar{v}_s$ is the specific volume of pure solvent.

Eqn. 5 can now be written

$$V_e = k_1 \exp\left(-\frac{M^{2/3} - 2\tilde{\mu}M^{1/3}}{2\tilde{\sigma}^2}\right) - k_2 \operatorname{erf}\left(\frac{M^{1/3} - \tilde{\mu}}{\sqrt{2}}\right) + k_3 \tag{10}$$

where

$$\tilde{\mu} = \alpha^{1/3}\mu \tag{11a}$$

and

$$\tilde{\sigma} = \alpha^{1/3}\sigma \tag{11b}$$

For flexible and fibrous molecules, $M = \beta r^2$ can be used instead of $M = \alpha r^3$ (ref. 14).

The value of V_0 has to be measured directly. A knowledge of the exclusion limits to the gel enable r_m to be derived from eqn. 9. The parameters μ and σ can be treated as calibration constants. It is also possible, however, to estimate σ, subject to a small error probability, from:

$$\sigma \approx (r_m - \mu)/3 \tag{12}$$

Alternatively, μ and σ could be estimated from micrographs of Sephadex particles by making allowances for the extensive hydrophilic envelope surrounding each polysaccharide chain.

As can be seen from Fig. 4, the theoretical curve calculated from eqn. 10 agrees extremely well with the experimental results obtained from Sephadex G-75 gel filtration of globular proteins over the complete range of M from zero to the exclusion limits of the gel—in other words, even for small, totally non-excluded molecules.

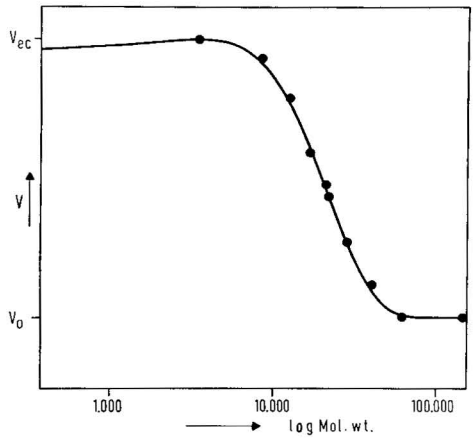

Fig. 4. Relationship between elution volume V_e and logarithm of molecular weight for Sephadex G-75 according to eqn. 10. The empirical data are indicated by closed circles.

An estimate of the sample mean μ of the distribution function for five different types of Sephadex is given in Table I. Calculations with various μ show that the value of μ has a significant influence on the theoretical results only in the range of lower M values. In the working range of the gel, therefore, $\mu = 0$ can be used for all gels. Thus, eqn. 10 reduces to

$$V_e = k_1 \exp\left(-\frac{M^{2/3}}{c}\right) + k_2 \tag{13}$$

where

$$k_1 = L\sqrt{2\pi}\,\sigma \text{ } (L \text{ is a constant}) \tag{14a}$$

$$c = 2\alpha^{2/3}\sigma^2 \tag{14b}$$

$$k_2 \approx V_0 \tag{14c}$$

TABLE I

SAMPLE MEAN OF THE DISTRIBUTION FUNCTION $w(r)$ FOR FIVE DIFFERENT TYPES OF SEPHADEX

Material	*Molecular range of* μ
G-25	0– 100
G-50	100–1000
G-75	1000–3000
G-100	100–1000
G-200	0

and the standard deviation σ can be estimated by:

$$\sigma \approx r_m/3 \tag{14d}$$

For different values of $c \propto \sigma^2$, the parameters k_1 and k_2 of eqn. 13 were determined for Sephadex G-75, G-100 and G-200 using the method of least squares. With the resultant values of k_1 and k_2, the theoretical elution volume V_e was calculated from eqn. 13. The averaged deviations between the theoretical and the empirical elution volumes, $f_w = [\Sigma(V_{\text{theoretical}} - V_{\text{empirical}})^2]^{1/2}/n$, were then plotted against the respective values of c (Fig. 5) in order to ascertain the magnitude of c which yields the smallest f_w value. The minima, thus determined (see Fig. 5), do not differ greatly from the corresponding values estimated from eqn. 14b: $c_{th}^{75} = 429.6$ ($M = 85{,}000$), $c_{th}^{100} = 882.0$ ($M = 250{,}000$) and $c_{th}^{200} = 2071.5$ ($M = 900{,}000$) (exclusion limits according to ref. 15).

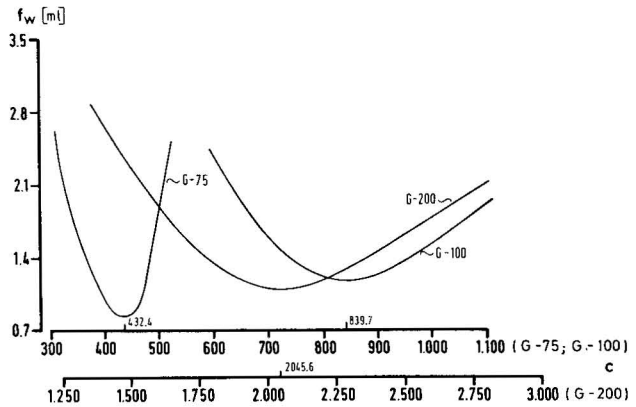

Fig. 5. Empirical approximation of the c calues for different types of Sephadex.

Empirical (V_{em}) and theoretical ($V_{ep}^{(13)}$; V_{st}; V_{1g}) values for the elution volumes of Sephadex G-75 and G-200 are compiled in Table II. In the case of $V_{ep}^{(13)}$, the optimal values for k_1 and k_2 of eqn. 13 were determined by the method of least squares; for V_{st}, k_1 was calculated from eqn. 13 using a single standard molecule ($M = 13{,}500$) and k_2 was evaluated from eqn. 14c. The values of V_{1g} were computed from the generally applied logarithmic relationship between V_e and M (eqn. 1), the parameters c_1 and c_2 being optimized by the method of least squares.

As can be seen from Table II, there is a close similarity between V_{em} and both $V_{ep}^{(13)}$ and V_{st}, but discrepancies exist between V_{em} and V_{1g}. Moreover, the good agreement between $V_{ep}^{(13)}$ and V_{st} demonstrates that a column could be calibrated accurately with only one, or maximally two (should a control be deemed necessary), reference molecules, providing, of course, the void volume had already been established. The conventional calibration procedure could thus be considerably shortened and the efficacy of the column correspondingly enhanced.

The calibration curves computed from a logarithmic and an exponential correlation between V_e and M, according to eqns. 1 and 13, respectively, are

TABLE II

ELUTION VOLUMES OF SEPHADEX G-75 AND G-200

For details see text.

M	V_{cm} (ml)	$V_{ep}^{(13)}$ (ml)	V_{st} (ml)	$V_{ep}^{(16)}$ (ml)	V_{lg} (ml)
Sephadex G-75: $V_0 = 67.2$ ml, $V_t = 254.5$ ml					
11,000	225.2	224.4	225.1	222.7	209.4
13,500	199.6	199.0	199.7	199.8	192.9
16,900	171.9	172.4	173.2	173.9	174.9
20,000	149.4	153.7	154.9	154.6	161.3
24,000	133.5	133.1	134.0	132.7	145.0
30,000	118.8	115.0	116.1	113.2	128.6
60,000	74.2	74.4	76.0	73.6	72.8
75,000	67.2	67.9	69.7	69.3	54.8
	f_w =	±0.7 ml	±0.9 ml	±1.1 ml	±3.6 ml
Sephadex G-200: $V_0 = 67.0$ ml, $V_t = 259.6$ ml					
25,000	190.0	188.5	190.1	185.1	178.1
37,500	173.2	170.6	173.9	170.9	164.3
52,500	153.0	154.0	158.9	156.3	152.8
58,000	150.0	149.6	154.3	151.5	149.4
67,000	137.0	142.5	147.5	144.3	144.5
123,000	112.5	113.6	118.6	112.4	123.8
158,000	101.0	103.1	107.4	100.4	115.2
237,000	98.6	89.0	91.3	85.7	101.4
1,000,000	67.0	70.8	65.9	75.1	52.3
	f_w =	±1.4 ml	±1.8 ml	±2.0 ml	±3.5 ml

illustrated in Fig. 6; the closed circles represent empirical values. In order to emphasize the differences between the curves, a linear scale is used for M instead of the customary logarithmic.

If calibration is conducted with just one or two reference molecules, it is advisable to select the molecules from the M ranges given in Table III. The table also contains a list of the most appropriate empirical c values (see also Fig. 5) for Sephadex G-75, G-100 and G-200. In the given deviation interval i, changes in

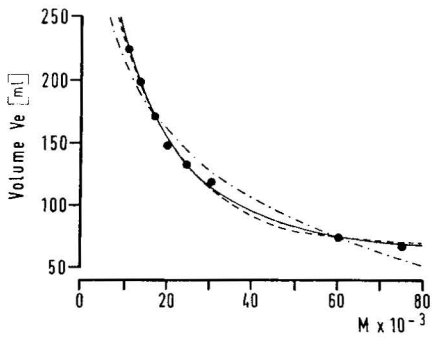

Fig. 6. Comparison of empirical and theoretical data for Sephadex G-75: —·—·—, according to the generally applied eqn. 1; ———, according to eqn. 13; — — —, according to eqn. 16a.

TABLE III

BEST VALUES FOR c AND M RANGE FOR CALIBRATION WITH STANDARD PROTEINS

In the given interval, i, changes in f_w are less than 0.2 ml.

Material	$c \pm i$ *(eqn. 13)*	$c_0 \pm i$ *(eqn. 16)*	*M range*
G-75	432 ± 15	15,700 ± 1300	4000– 50,000
G-100	840 ± 30	29,500 ± 5000	5000–100,000
G-200	2050 ± 60	90,500 ± 20,000	5000–200,000

f_w are less than 0.2 ml (indicated by the dashed lines in Fig. 7) and the parameter k_1 derived from eqn. 13 is more or less constant. The larger the mean size of the dextran pores, the smaller is the range over which k_1 can be varied without significantly affecting the f_w value; on the other hand, the more accurately can k_1 be derived.

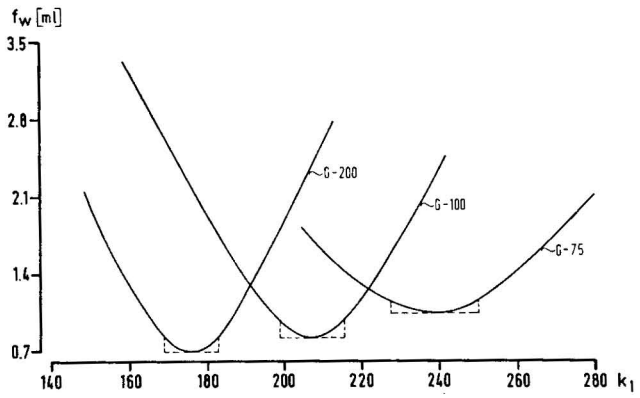

Fig. 7. Variation of the parameter k_1 of eqn. 13 within a given interval, according to Table III, for different types of Sephadex.

DISCUSSION

In the present paper, eqn. 13 is derived which permits a more accurate calibration of molecular sieve columns than that afforded by the conventional logarithmic relationship between V_e and M (eqn. 1). An optimal calibration can be achieved by employing the method of least squares to determine the unknown parameters; alternatively, the latter can be ascertained by adopting the less time-consuming, but slightly less exact, procedure of measuring the V_0 and V_e values of at least one reference molecule (the M value of which should lie within the range given in Table III).

The derived equation might also provide a better understanding of the theoretical implications underlying the processes of filtration across membranes and synapses, always assuming, of course, that active transport can be neglected. The parameter c is a constant and well-defined characteristic of the filtration medium and

the solute, the precision of its determination being such, for example, that small productivity changes in 1965, designed to improve the quality of Sephadex G-25, G-50 and G-75, were readily detectable. The next step would obviously seem to be the compilation of a table of c values covering all the different filtration media and isochemical substances: the c values could be calculated from a consideration of the exclusion limits of the molecular sieve material and the partial specific volume, $\bar{v}$, of the molecules, although a more accurate procedure would be to evaluate c using the method of least squares.

For globular molecules with low frictional ratios, the elution volume and molecular weight can be determined from

$$V_e - V_0 = k \exp(-M^{2/3}/c) \tag{15a}$$

whilst more flexible and elliptical molecules, with $M \propto r^2$ (ref. 14), will obey, according to eqn. 5:

$$V_e - V_0 = K \exp(-M/c_0) \tag{16a}$$

As can be seen from Table II, the elution volumes ($V_{ep}^{(16)}$) calculated from eqn. 16a are very similar to those obtained empirically (see also Fig. 6). The best c values for Sephadex G-75, G-100 and G-200 are listed in Table III.

Eqns. 15a and 16a can also be used to estimate M values directly from logarithmic calibration curves simply by rewriting them in the form

$$-\log(V_e - V_0) = A_1 M^{2/3} + B_1 \tag{15b}$$

and

$$-\log(V_e - V_0) = A_2 M + B_2 \tag{16b}$$

It is interesting to note that eqns. 15b and 16b, derived from a statistical model, are analogous to eqns. 20 and 19, respectively, derived by Hjertén[12] on the basis of thermodynamic considerations. The coefficients A_1 and A_2 in the present equations are essentially dependent on the partial specific volume $\bar{v}$ of the solute and the standard deviation σ of the penetrable areas $A(r)$; σ itself is determined by the distribution of the interfaces between the solute particles and the gel, and the pressure brought to bear on the system. Similarly, the corresponding coefficients C_2 and C_1 in Hjertén's equations are mainly dependent on the partial specific volume $\bar{v}$ of the solute, the interfacial tension, γ, and the pressure, p.

Finally, the molecular weights of rod-like molecules or aggregates of molecules with high frictional ratios can be calculated from the respective elution volumes with greater accuracy than that provided by conventional methods, by a statistical consideration of the orientation of the axes of symmetry of the molecular structures to the gel "network". The theoretical procedure can be simplified by assuming that molecular structures which attempt to penetrate the gel obliquely will orientate themselves either parallel or perpendicular to the "network" depending on the angle of approach. The total penetrable area accessible to such molecular structures can be expressed by

$$A_t(a,b) = \alpha_1 A_t(a) + \alpha_2 A_t(b) \tag{17}$$

where a,b = lengths of the two longest axes of symmetry, and α_i = the probability of axis a or b entering the gel parallel to the "network" ($\Sigma\alpha_i = 1$). The parameter α_i is known from investigations on the rotation of rigid rod-shaped particles in a solution and its treatment is therefore subject to probability theory. The basic equation describing the distribution of particulate orientations was derived by Peterlin and Stuart[16].

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Journal of Chromatography, 187 (1980) 297–305

CHROM. 12,331

GAS CHROMATOGRAPHY OF ESTERS

XIII*. INTERRELATIONSHIP OF EQUIVALENT CHAIN LENGTH (*ECL*) AND RETENTION INDEX VALUES OF FATTY ESTERS

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(Received August 23rd, 1979)

SUMMARY

The interrelationship of equivalent chain length and retention index values of straight- and branched-chain fatty esters is demonstrated on a typical polyester phase (DEGS), a phenyl-substituted polysiloxane (OV-17) and on two low-polarity, thermally stable hydrocarbons. Hydrogenated Apiezon M and the synthetic hydrocarbon $C_{87}H_{176}$ were used, and have lower polarities than the simple Apiezon greases that have been used with fatty esters. These stationary phases have polarities comparable to that of squalane and retention increments of fatty esters may be considered comparatively with those of simple esters which have been compared with squalane.

INTRODUCTION

A variety of relationships have been used to specify retention behaviour and the interrelation of nine of the commoner structure retention parameters was shown mathematically by Guerin and Banks[1]. The growing use of automated gas chromatographic equipment with accompanying computer processing of retention data will continue to eliminate tedious measurements from charts and provide data virtually simultaneously with the elution of compounds. The determination of mathematical dead-time has recently been reviewed[2] and this, together with the interpolation of

* Part XII: see J. K. Haken, A. Nguyen and M. S. Wainwright, *J. Chromatogr.*, 178 (1979) 471.
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the results for a compound on a graph prepared with homologous compounds, *e.g.*, *n*-alkanes, to provide retention indices are simple procedures that can be rapidly handled by on-line processors.

Retention indices[3] are established by the relationship

$$I = \frac{100(\log t_{RS} - \log t_{RN}) + 100N}{\log t_{R(N+1)} - \log t_{RN}} \tag{1}$$

where

t_{RS} = retention of unknown *n*-substrate;
t_{RN} = retention of *n*-alkane with N carbon atoms;
$t_{R(N+1)}$ = retention of *n*-alkane with $N + 1$ carbon atoms;
N = integer equal to carbon number of t_N.

The slope (b) of the semi-logarithmic plot of carbon number *versus* retention of *n*-alkanes is common to many of the schemes considered by Guerin and Banks[1] and is determined from two points on a graph prepared with *n*-alkanes, as shown in eqn. 2, although in a computer plot the slope is determined statistically by considering all of the *n*-alkanes used in the calibration:

$$b = \frac{\log t_{R(N+x)} - \log t_{R(N)}}{x} \tag{2}$$

where

$t_{R(N+x)}$ and $t_{R(N)}$ = retention of *n*-alkanes with N and $N + x$ carbon atoms, respectively

The retention index is therefore given by

$$I = \frac{100(\log t_{RN} + N)}{b} \tag{3}$$

The methylene unit (MU) nomenclature proposed by VandenHeuvel *et al.*[4] is essentially identical with the retention index system. A calibration using *n*-docosane and *n*-tetracosane was used and the methylene unit multiplied by 100 gives the retention index. The proposal based on a two-point calibration line is less satisfactory than the normally determined multi-point *n*-alkane calibration line.

The difficulty in selecting a reference series suitable for all polarities of solute and solvent is well known. Lorenz and Rogers[5] considered the effect of sorption phenomena on retention indices and found significant errors when the solute and solvent polarities were widely variant. These findings were similar to those of VandenHeuvel and Horning[6], who recommended that the polarities of the two species should be similar.

The use of secondary standards with *n*-alkanes has been suggested, and Hawkes[7] discussed the difficulty of using primary *n*-alkanols as proposed by Grobler[8], who had criticised the retention index system using polar stationary phases owing to the rapid elution of hydrocarbons and indicated that compounds with very different boiling points cannot be chromatographed under strictly identical conditions. *n*-Propyl ethers were recommended as standards by Hawkes[7], with ketones and

aldehydes as alternatives. 2-Alkanones had earlier been suggested as standards by Dymond and Kilburn[9] and the merits of using ketones as secondary reference standards were later detailed by Ackman[10].

The difficulty or impossibility of providing a universally acceptable reference series has been demonstrated by a study of the behaviour of compounds of moderately polar character, *e.g.*, *n*-alkyl esters. With dimethyl polysiloxane, the solvent of lowest polarity included, it is apparent that methyl acetate (b.p. 57.1°) has a retention index of 509, *i.e.*, identical with that of *n*-pentane (b.p. 36.2°), while *n*-hexyl acetate (b.p. 169.2°) has a retention index of 988, *i.e.*, almost identical with that of *n*-decane (b.p. 174.0°), so that for a given retention index the esters have higher boiling points than the comparable *n*-alkanes. With the introduction of two phenyl groups, *i.e.*, using DC-710, the situation is reversed, with methyl acetate ($I = 609$) being comparable to *n*-hexane (b.p. 69.0°) and *n*-hexyl acetate ($I = 1092$) being comparable to *n*-undecane (b.p. 196°). With weaker acceptor phases, *i.e.* XE-60, these retention indices are increased to 737 and 1221, respectively, the former being equivalent to an *n*-alkane with a boiling point of approximately 220°. With a more polar stationary phase (Silar 5CP), retention indices of 894 and 1313 are obtained, which are equivalent to *n*-nonane (b.p. 151°) and *n*-tridecane (b.p. 235°), respectively.

The great differences in boiling points at each end of the scale reflect the differences in the slopes for the *n*-alkanes and simple esters on the various stationary phases. As alternative secondary reference standards, acetate esters and symmetrical esters with equal acid and alcohol chain lengths have been suggested[11].

The use of esters as standards has found considerable acceptance with fatty esters in the form of the equivalent chain length (*ECL*) relationship developed by Woodford and Van Gent[12] and Miwa *et al.*[13]. The relationship is equivalent to the retention index system, results for an unknown ester being interpolated on graph prepared using homologous long-chain methyl esters.

The relationship is shown in eqns. 4 and 5 and is identical with eqns. 1 and 3 except for the reference series used.

$$ECL = \frac{\log t_{RS} - \log t_{RN} + N}{\log t_{R(N+2)} - \log t_{RN}} \tag{4}$$

$$ECL = \frac{(\log t_{RN} + N)}{b} \tag{5}$$

The quantities are as shown above but refer to methyl fatty esters, while the calibration line is usually constructed using even-carbon-number esters.

With computer processing of chromatographic data it is possible to provide retention data relative to multiple reference series and with the retention index and *ECL* representations a simple consideration of the slopes of plots for standard homologous series is necessary.

In this paper we consider the retention of straight- and branched-chain fatty esters with the data expressed in terms of both retention indices and *ECL* values. The data were determined on a typical polyester phase (DEGS) and on two low-polarity, thermally stable hydrocarbons. Hydrogenated Apiezon M and the synthetic hydrocarbon $C_{87}H_{176}$ were used, and have lower polarities than the simple Apiezon greases that have been used with fatty esters. These stationary phases are comparable

TABLE I

EQUIVALENT CHAIN LENGTHS (*ECL*), RELATIVE RETENTIONS (*RR*) AND RETENTION INDICES (*I*) OF METHYL ALKANOATES AND *n*-ALKANES

Compounds	*Carbon number*	*Hydrogenated Apiezon M (230°)*			$C_{87}H_{176}$ *(200°)*			*OV-17 (200°)*			*DEGS (200°)*		
		ECL	*RR*	*I*	*ECL*	*RR*	*I*	*ECL*	*RR*	*I*	*ECL*	*RR*	*I*
Methyl esters	C_{10}	10.00	0.104	1256	10.00	0.075	1266	10.00	0.091	1410	10.00	0.202	1794
	C_{12}	12.00	0.221	1457	12.00	0.177	1468	12.00	0.211	1613	12.00	0.347	2003
	C_{14}	14.00	0.471	1659	14.00	0.424	1659	14.00	0.465	1814	14.00	0.585	2201
	C_{16}	16.00	1.000	1859	16.00	1.000	1860	16.00	1.000	2015	16.00	1.000	2401
	C_{18}	18.00	2.120	2059	18.00	2.350	2061	18.00	2.170	2215	18.00	1.703	2599
	C_{20}	20.00	4.479	2257	20.00	5.530	2262	20.00	4.550	2414	20.00	2.907	2796
	C_{22}	22.00	9.499	2454	22.00	13.109	2460				22.00	4.963	2993
n-Alkanes	C_{10}	7.43	0.040	1000									
	C_{12}	9.40	0.083	1200				7.96	0.044	1200			
	C_{14}	11.39	0.176	1400				9.89	0.093	1400			
	C_{16}	13.37	0.371	1600	13.37	0.342	1600	11.87	0.201	1600			
	C_{18}	15.37	0.789	1800	15.36	0.762	1800	13.86	0.436	1800	10.05	0.205	1800
	C_{20}	17.36	1.670	2000	17.36	1.791	2000	15.85	0.943	2000	11.98	0.343	2000
	C_{22}	19.34	3.520	2200	19.35	4.184	2200				13.99	0.585	2200
	C_{24}										15.98	0.995	2400
											18.03	1.756	2600
											20.15	3.021	2800

in behaviour to squalane and retention increments of fatty esters may be considered comparatively with those of simple esters which have been compared with squalane.

EXPERIMENTAL

Gas chromatography

A Hewlett-Packard 5700A gas chromatograph, fitted with dual flame-ionization detectors, was used. Nitrogen (30 ml/min) was used as the carrier gas, and hydrogen (30 ml/min) and air (200 ml/min) were supplied to the detector. All results were determined isothermally at 200°. Four stationary phases were used, *viz.*, in order of increasing polarity, hydrogenated Apiezon M (refs. 14 and 15), $C_{87}H_{176}$ (ref. 16), OV-17 and DEGS, all coated to the extent of 10% on Chromosorb W AW DMCS (80–100 mesh) and packed into 2 m × 3 mm O.D. stainless-steel columns.

The gas chromatograph was connected to a Hewlett-Packard 3370B integrator with facilities for paper-tape output on a teletype (STC, ASR-33). The chromatograph was also fitted with a Hewlett-Packard 7671A auto-sampler.

Preparation of fatty acid methyl esters from wool wax

Wool from Merino sheep was extracted with light petroleum (b.p. 40–60°), using a Soxhlet apparatus. Small portions of wax were saponified with alcoholic sodium hydroxide solution. Unsaponifiable components were removed with light petroleum, the solution was then acidified and fatty acids together with hydroxy fatty acids were extracted with light petroleum. Fatty acids were separated from hydroxy fatty acids by thin-layer chromatography on silica gel G using *n*-hexane–diethyl ether–acetic acid (70:30:1). The bands were detected under UV light after spraying with 0.1% ethanolic 2,7-dichlorofluorescein and identified using standards (Applied Science Labs., State College, Pa., U.S.A.) run on the same plate. Fatty acid bands were scraped from the plate and extracted with diethyl ether. Methyl esters were prepared by adding diazomethane solution.

Calculations

The data were processed by means of a PDP 11/15 computer fitted with a high-speed paper-tape reader and disc pack (RK05), dead-times being calculated by the procedure of Guardino *et al.*[17].

ECL values were computed from the modified regression equation first obtained by the procedure of Guardino *et al.* from a homologous series of straight-chain fatty acid methyl esters. In order to allow for the normal variations that occur with net retention times with time (as up to 36 samples could be automatically processed), all experimental data were converted into retentions relative to methyl palmitate (C16). The net retention time of methyl palmitate was constantly re-measured in each sample, as it either occurred naturally or, if not, was added as an internal standard. Retention indices were similarly determined using homologous *n*-alkanes.

RESULTS AND DISCUSSION

Retention data for methyl alkanoates and *n*-alkanes on the four stationary phases are shown in Table I as retentions relative to methyl palmitate, *ECL* values

TABLE II

EQUIVALENT CHAIN LENGTHS (*ECL*), RELATIVE RETENTIONS (*RR*) AND RETENTION INDICES (*I*) OF WOOL WAX METHYL ESTERS

*Compound**	*Hydrogenated Apiezon M (230°)*			$C_{87}H_{176}$ *(200°)*			*OV-17 (200°)*			*DEGS (200°)*		
	ECL	*RR*	*I*	*ECL*	*RR*	*I*	*ECL*	*RR*	*I*	*ECL*	*RR*	*I*
a-C_9												
iso-C_{10}	9.63	0.091	1217				9.56	0.082	1365			
n-C_{10}	9.99	0.104	1252				9.99	0.096	1407			
a-C_{11}	10.72	0.137	1325	10.71		1336	10.69	0.127	1481	10.78	0.249	1883
U	11.35	0.174	1390									
iso-C_{12}	11.62	0.192	1417	11.61	0.153	1425	11.57	0.179	1571	11.55	0.306	1963
n-C_{12}	11.99	0.221	1454	11.96	0.177	1455	11.98	0.210	1612			
U	12.33		1487									
a-C_{13}	12.71	0.290	1527	12.69	0.243	1528	12.69	0.276	1683	12.75	0.421	2085
U	13.35	0.369	1590				12.99	0.310	1713			
iso-C_{14}	13.62	0.408	1618	13.61	0.360	1619	13.58	0.390	1772	13.52	0.516	2161
n-C_{14}	14.00	0.471	1656	13.99	0.424	1658	13.99	0.458	1814	14.00	0.587	2210
a-C_{15}	14.71	0.615	1727	14.69	0.572	1727	14.70	0.603	1885	14.71	0.709	2280
n-C_{15}	15.00	0.618	1756			1752	15.00	0.677				
U	15.33	0.777	1789									
iso-C_{16}	15.62	0.867	1818	15.62	0.849	1820	15.58	0.849	1973	15.48	0.871	2357
n-C_{16}	16.00	1.00	1856	16.00	1.000	1858	16.00	1.000	2015	16.00	1.000	2409
U	16.31	1.119	1886									
a-C_{17}	16.71	1.304	1927	16.69	1.345	1928	16.69	1.310	2085	16.70	1.206	2478
n-C_{17}	16.99	1.452	1955				17.00	1.476	2115			

U	17.26	1.605	1982	17.24		1978						
iso-C_{18}	17.60	1.823	2016	17.57	1.957	2016	17.57	1.840	2172	17.48	1.484	2555
n-C_{18}	18.00	2.120	2056	18.00	2.340	2059	17.98	2.158	2213	17.99	1.701	2605
a-C_{19}	18.70	2.763	2126	18.68	3.153	2128	18.68	2.835	2283	18.71	2.056	2676
n-C_{19}	19.01	3.101	2157									
U	19.19		2175	19.14	3.833	2175						
iso-C_{20}	19.60	3.880	2216	19.59	4.636	2220	19.55	3.969	2370	19.49	2.533	2753
n-C_{20}	19.98	4.479	2254							20.00	2.902	2803
U	20.26	4.962	2281									
a-C_{21}	20.68	5.821	2321						2481	20.71	3.511	2873
U	21.18	7.036	2374									
U	21.31		2386									
iso-C_{22}	21.61		2412							21.48	4.310	2948
n-C_{22}										22.02	4.976	3001
a-C_{23}										22.72	5.985	3069
iso-C_{24}										23.49	7.345	3144
n-C_{24}										24.02	8.476	3197
a-C_{25}										24.72	10.198	3265
iso-C_{26}										25.50	12.574	3342
a-C_{27}										26.93	17.441	3462

* U = Unknown.

and retention indices, and Table II gives similar data for a series of wool wax fatty esters on the same phases.

From Table II, it is evident that on the DEGS column the iso-esters (terminal isopropyl group) have shorter retention times than the anteiso-esters (terminal isobutyl group); the same elution pattern is, as expected, observed with the non-polar stationary phases as the three homologous series are all of the same functional class.

Semi-logarithmic plots for the different series are essentially parallel, and the separation factors are essentially identical, as indicated previously by Gerson[18] and Haken[19]. The separation factors are given in Table III; as expected, the separations are enhanced on the stationary phases of lower polarity.

TABLE III

SEPARATION FACTORS OF BRANCHED- AND STRAIGHT-CHAIN FATTY ESTER SERIES

Structure	*Hydrogenated Apiezon M*	$C_{87}H_{176}$	*OV-17*	*DEGS*
n-	2.11	2.35	2.14	1.76
Iso-	2.11	2.35	2.14	1.76
Anteiso-	2.11	2.35	2.14	1.76
U	2.11			

The polarity has a greater influence on the elution of iso-esters than on that of anteiso-esters; this is shown in Table IV, where variations in *ECL* values are given.

TABLE IV

REDUCTION IN RETENTION (*ECL* UNITS) OF ESTERS ON STATIONARY PHASES OF VARYING POLARITY

Structure	*Hydrogenated Apiezon M*	$C_{87}H_{176}$	*OV-17*	*DEGS*
Iso-	−0.37	−0.38	−0.43	−0.53
Anteiso-	−0.28	−0.31	−0.32	−0.30
n-	1.00	1.00	1.00	1.00
U	±0.65			

The improved separations on the non-polar columns are further evident by the appearance of a fourth series of minor components on hydrogenated Apiezon M column shown as U in Tables II–IV. The same separation factors are exhibited as for the other series on this stationary phase, and on a non-polar column separation of an unsaturated species would be very unlikely. The composition of wool wax fatty acids has recently been reviewed[20] and, in addition to the saturated esters described, series of normal, iso- and anteiso-hydroxy esters are present and the peaks observed are due to small amounts of the materials that remain after the separation reported.

Tables I and II show that the retention is essentially identical on both of the non-polar phases, with retention indices being approximately 250 units or 2.5 carbon numbers greater than the *ECL* values. With increased polarity of the stationary phase,

retention relative to the *n*-alkanes is increased by 410 units or 4.1 carbon numbers on the OV-17 column and 800 units or 8 carbon numbers on the DEGS column. These values show the same trends as in a previous study in which the retention of simple esters on polysiloxane stationary phases of increasing polar character was considered[21].

The results indicate that the interrelationship of retention indices and *ECL* values or other indices relative to some other homologous calibration line can be readily achieved by using simple data reduction units or by manual operation.

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FRACTIONATION OF FISH OIL FATTY ACID METHYL ESTERS BY MEANS OF ARGENTATION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, AND ITS UTILITY IN TOTAL FATTY ACID ANALYSIS

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SUMMARY

The utility of reversed-phase and argentation high-performance liquid chromatography (HPLC) as pre-fractionation methods in fatty acid analysis is discussed. Both HPLC modes were applied to cod liver oil fatty acid methyl esters. Apart from positional isomers, the fractions obtained by reversed-phase HPLC were analysed by gas–liquid chromatography and appear to be free of the usually occurring "critical pairs". The mechanism of retention of the fatty acid methyl esters on low-loaded silver nitrate-impregnated silicas is discussed. It is shown that argentation HPLC is a rapid semi-preparative pre-fractionation method for highly unsaturated fatty esters with 3–6 double bonds.

INTRODUCTION

Thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) are important analytical methods in lipid research. The latter has found widespread use in the analysis of apolar lipids such as fatty acid esters. It has become common practice to use very polar stationary phases in GLC in order to obtain a good separation of saturated and unsaturated esters. However, even for simple mixtures not all peaks in the chromatogram are completely resolved.

Usually, the position of a peak in a gas chromatogram is characterized by the equivalent chain length (*ECL*) of the fatty acid ester. On a polar GLC column

$$ECL = n + xn_{C=C}$$

where n and $n_{C=C}$ are the numbers of carbon atoms and double bonds, respectively, in

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the fatty acid. The magnitude of x depends on the number, position and the *cis/trans* geometry of the double bonds and on the polarity of the stationary phase. Generally, it decreases from 1 to about 0.5 as $n_{C=C}$ increases from 1 to 6. Fatty acid esters that have similar *ECL* values (critical pairs) cannot be separated completely. Some of these ester pairs can be resolved on apolar columns but then other peaks may overlap to various extents. Several workers have attempted to separate geometrical and positional isomers by means of GLC[1–9]. Generally, the selectivity towards positional isomers increases with the polarity of the stationary phase. However, as the polarity is increased, overlap with a preceding and/or next eluting ester species may occur. When also *cis/trans* isomers are present, the problems become difficult to solve, even with capillary columns[2,7].

In this paper the utility of high-performance liquid chromatography (HPLC) in fatty acid ester analysis is considered in connection with the problems mentioned above. The aim of the work was to examine the possibility of pre-fractionating the fatty acid ester mixture by HPLC in such a manner that the two esters of a critical pair would be isolated in different fractions. To that end, the utility of reversed-phase (RP-) and argentation (Ag-) HPLC is compared.

Argentation chromatography has been applied extensively on TLC plates[10]. It is based on the formation of a reversible charge-transfer complex involving the silver ion and an (olefinic) double bond[11,12]. Dudley and Anderson[13] showed that Ag-TLC can be used to fractionate fatty acid ester mixtures according to the number of double bonds. However, in order to obtain reproducible results the relative humidity in the TLC chamber must be kept within the narrow limits of 42–44%. The recovery of highly unsaturated esters ($n_{C=C} = 5$ and 6) was about 80%, presumably as a result of oxidation. This recovery can be improved by means of HPLC, as will be shown.

Heath *et al.*[14] showed that the geometrical isomers of 3,13-octadecadiene-1-ol acetate can be separated by Ag-HPLC. Similar results were obtained by Lam and Grushka[15] for some *p*-bromophenacyl fatty acid esters. The *trans*-isomers are eluted before the corresponding *cis*-isomers, owing the less pronounced influence of steric effects and to the release of intramolecular strain on the formation of the *cis* double bond–silver (I) complex[11]. Hence, a good resolution of *cis*- and *trans*-fatty acid methyl esters can be expected.

Positional isomers will be resolved in only a few instances, as follows from Ag-TLC work[8,16–18].

Reversed-phase chromatographic separations are largely based on the sorption of hydrophobic moieties of the solute molecules from a polar solvent to an apolar sorbent. Therefore, the order of elution of fatty acid esters will be in accord with their alkyl chain length, *i.e.*, be analogous to that in GLC columns. However, because of (polar) eluent–double bond interactions, the solute retention decreases with increasing number of double bonds[19] at constant chain length, *i.e.*, contrary to that on polar GLC columns. Therefore, *ECL* values of fatty acid methyl esters on RP columns can be presented by the equation

$$ECL = n - yn_{C=C}$$

From unresolved ester pairs in RP chromatograms given by Jordi[20], it can be deduced

that y decreases as $n_{C=C}$ increases. *Cis*- and *trans*-isomers have been separated successfully by RP-HPLC[20,21]. Owing to the influence of steric effects on the eluent–double bond interaction, the *cis*-isomer is eluted first. Although the C18:3ω3 and C18:3ω6 *p*-bromophenacyl esters have been separated by RP-HPLC[22], RP systems would hardly be expected to show good selectivity towards positional isomers in general because of the similar hydrophobicities of these isomers.

Summarizing, it can be concluded that critical pairs will occur in (capillary) GLC and Ag- and RP-HPLC, but that (apart from some positional isomers) the ester pairs involved are generally not the same.

Some investigators have combined the selectivities of both HPLC modes by using an RP system with a dilute aqueous silver salt solution as the eluent[23–27]. Excellent separations of the *cis–trans* isomers of 1,5,9-cyclododecatriene[26,27] and of the positional isomers of cyclooctadiene have been obtained in this way[27].

At present, the most suitable HPLC detectors for fatty acid methyl esters are the refractive index and the moving wire detector[28]. UV detection can be applied in the 203–214 nm range[29]. Unfortunately, the detection limits for these esters, in the microgram range, leave much to be desired. Therefore, in this work quantitation was performed in a final GLC analysis step by flame-ionization detection, the sensitivity and ease of which are unsurpassed.

The aim of this work was to examine the extent to which an HPLC pre-fractionation step can eliminate potential critical pair problems in GLC analysis. In order to compare the merits of Ag- and RP-HPLC in this respect, fatty acid ester mixtures from fish oils (*e.g.*, cod liver oil) were chosen because of their complex fatty acid profiles[9]. Further, some consideration is given to the mechanism of retention of unsaturated fatty acid esters in Ag-HPLC.

EXPERIMENTAL

Chemicals and sorbent specification

Even-carbon-numbered saturated fatty acid methyl esters (C14:0 up to C22:0), palmitoleate, palmitelaidate, oleate, elaidate, linoleate and linolenate were purchased from Applied Science Labs. (State College, Pa., U.S.A.). Their purities were at least 99%. Purified cod liver oil was a gift from the Institute for Fishery Products TNO (IJmuiden, The Netherlands). The oil was *trans*-esterified by the boron trifluoride–methanol method according to Morrison and Smith[30]. All ester samples were stored under nitrogen in a refrigerator.

LiChrosorb 10 RP-18 (E. Merck, Darmstadt, G.F.R.; particle diameter about 10 μm) was used as the RP sorbent. Elemental analysis gave 20.3% (w/w) of carbon.

Silver nitrate-impregnated silicas were prepared as follows. Partisil-10 (Reeve Angel Scientific, London, Great Britain; particle diameter 10 μm, specific surface area 400 m^2/g) was suspended in 15 ml of aqueous silver nitrate solutions, the concentrations of which corresponded to amounts of 5.0 and 7.5% (w/w) of silver (I) on to Partisil, respectively. The water was removed in a rotary vacuum evaporator. The residue was kept under purified nitrogen at 110° for 2 h. During the preparation, the adsorbent was protected from light.

The GLC packing was 12% (w/w) OV-275 on Chromosorb P AW DMCS (100–120 mesh).

Apparatus and procedure

The apparatus was a Pye LCM 2 chromatograph, equipped with a moving wire detector. The wire was coated with the sample components in the eluent by means of a stainless-steel spray nozzle (type V) as described by Van Dijk[31]. It was slightly modified by heating the spray nozzle and the spray nitrogen in order to promote evaporation of the eluent. The vapour was sucked away by pumping. Optimal detector performance was achieved with 0.5 atm of spray nitrogen at a nozzle tip-wire distance of 10 mm and maximum wire speed (13 cm/sec). The temperatures of the cleaner, evaporator, oxidizer oven, reduction chamber and flame-ionization detector were 900°, 150°, 700°, 390° and 175°, respectively. The nitrogen, air and hydrogen flow-rates were as recommended by the supplier. The samples were injected by means of a six-port Valco sample valve (7000 p.s.i., sample loop 50 μl). The detection limit for oleate was about 0.2 μg, *i.e.*, about a factor 25 better than that obtained with the conventional dip-coating device.

The GLC analyses were carried out with a Packard-Becker (Delft, The Netherlands) M417 chromatograph, equipped with a dual flame-ionization detector with quartz flame tips. The samples were injected directly on to the column packing.

The HPLC columns (polished precision-bore stainless steel, length 25 cm, I.D. 4.6 mm) were packed by the viscous slurry method[32]. The slurry [10% (w/w) of sorbent in *n*-hexane–squalane (1:3)] was degassed and homogenized by ultrasonic treatment and forced into the column at 300 atm. Finally, 200 ml of *n*-hexane were flushed through the column in order to settle the packed bed. The HETP of linoleate [$k' = 2.19$ on the 5% Ag(I) column, eluent 0.4% acetonitrile in *n*-hexane, flow-rate 0.20 cm/sec] was 0.14 mm. The HETP of stearate ($k' = 6.24$ on the LiChrosorb RP-18 column, eluent acetonitrile, flow-rate 0.16 cm/sec) was 0.09 mm.

The GLC columns (coiled glass, length 2 m, I.D. 2.7 mm) were filled according to the method described by Kuksis and Breckenridge[33]. The column packings were equilibrated at 250° for 24 h by flushing purified nitrogen through the columns. GLC analyses were carried out at 215°.

As eluents 0.3, 0.4 and 0.5% (v/v) of acetonitrile in *n*-hexane (Ag-HPLC) were used at ambient temperature. Both solvents were dried with molecular sieve 5A and carefully degassed by sonication. The eluent flow-rates were about 1.25 and 1.5 ml/min. In GLC, purified water- and oxygen-free nitrogen was used as the carrier gas (flow-rate 10 ml/min).

Retention times were corrected for the eluent hold-up in the column and the dead time of the transport detector by means of the retention time of squalane (Ag-HPLC) or approximately by that of sucrose (RP-HPLC). The measurements of the capacity ratios (k') were made in triplicate and were reproducible within 4%. In the GLC k' measurements, methane was used as the dead volume marker (precision better than 1%).

RESULTS AND DISCUSSION

Reversed-phase HPLC

Log k' values of some fatty acid ester standards (indicated in Table I) and those of palmitelaidate and elaidate are plotted against n in Fig. 1. It was assumed that the data points of homologous fatty acid esters lay on lines parallel to that for the

TABLE I

FATTY ACID ESTER CONTENTS OF THE RP-HPLC FRACTIONS OF COD LIVER OIL, THEIR LOG *k'* AND *ECL* VALUES ON OV-275, *ECL* VALUES ON SILAR 10C GIVEN BY HECKERS *et al.*[4] AND QUANTITATIVE RESULTS FROM THIS WORK AND THOSE BY WORKERS AT THE TNO[35]

Fatty ester	*RP fraction*			*OV-275*		*Silar 10C: ECL*[4]	*Concentration (%, w/w)*	
	I	*II*	*III*	*Log [k'/k' (C18:0)]***	*ECL*		*This work*	*TNO*[35]
14:0*		+		−0.343	14.01		4.6	4.2
15:0 (+ iso)			+	−0.265	14.92	—	0.5	0.7***
15:1 (ω5)	+			−0.174	15.98	16.04	<0.2	—
16:0*			+	−0.173	15.99		11.6	10.4
?		+		−0.130	16.49		<0.2	—
16:1ω7*		+		−0.086	17.00	16.89	10.4	11.7
17:1 (ω7)?		+		−0.012	17.87	17.89	<0.2	0.1
16:2	+			−0.002	17.98	—	0.7	1.0§
18:0*			+	0.000	18.01		2.3	2.5§§
18:1ω9*			+	0.075	18.88	18.77	25.5	26.0
16:3	+			0.098	19.15	—	1.0	—
18:2ω6*		+		0.165	19.93	19.71	1.7	1.5
20:0*				0.170	19.99		—	—
18:3 (ω6)	+			0.207	20.42	20.51	<0.2	0.5
18:3ω3*	+			0.274	21.20	20.91	0.7	
20:1 (ω7)			+	0.240	20.81	20.84	11.6	12.9
20:2 (ω6)			+	0.334	21.90	21.71	<0.2	—
22:0*				0.343	22.01		—	—
18:4	+			0.353	22.12	—	2.7	2.5
22:1 (ω7)			+	0.414	22.83	22.77	5.7	4.9
20:4 (ω6)	+			0.512	23.98	(22.92?)	0.6	—
20:5 (ω3)	+			0.571	24.67	(24.08?)	9.4	9.8
21:5	+			0.665	25.76	—	<0.2	—
22:5	+			0.741	26.65	—	0.6	0.5
22:6 (ω3)	+			0.786	27.17	27.16	10.4	10.8

* Also applied as standards.
** Log k' (C18:0) = 0.622.
*** C15:0 (0.3%) + iso-C15:0 (0.4%).
§ C16:2 + C17:0 (1.0%).
§§ C18:0 (2.3%) + iso-C18:0 (0.2%).

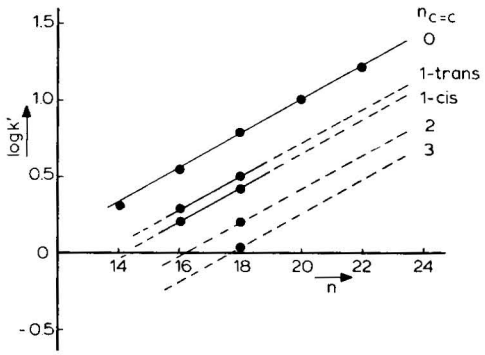

Fig. 1. Log *k'* values of some standard fatty acid methyl esters on LiChrosorb RP-18 *versus* the number of (chain) carbon atoms (*n*). Eluent: acetonitrile.

saturated esters. The *trans*-isomers are eluted after the corresponding *cis*-esters, as expected.

Fig. 2 shows the chromatogram of the cod liver oil esters. Geometrical isomers can be separated, provided that their contents are not too large. Otherwise, a longer column or a 5-μm sorbent has to be used. A 25-μl sample containing about 0.6 mg of fatty acid esters was injected and collected in three fractions as indicated in Fig. 2. The collected eluate was concentrated nearly to dryness by vacuum evaporation. The residue was dissolved in 100 μl of *n*-heptane and 0.5–2-μl samples were analysed by GLC. The chromatograms are given in Fig. 3. The small shaded peaks are traces

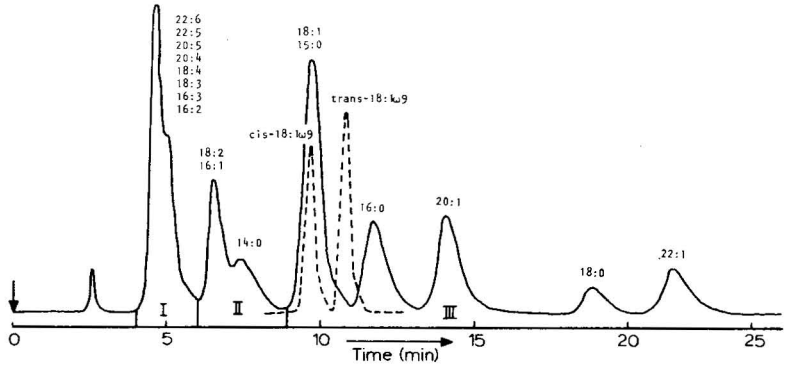

Fig. 2. Fractionation of cod liver oil fatty acid methyl esters on LiChrosorb 10 RP-18. Eluent: acetonitrile. Flow-rate: 1.27 ml/min. Sample size: 25 μl containing 0.6 mg of fatty acid esters. Broken lines: methyl oleate and elaidate standards.

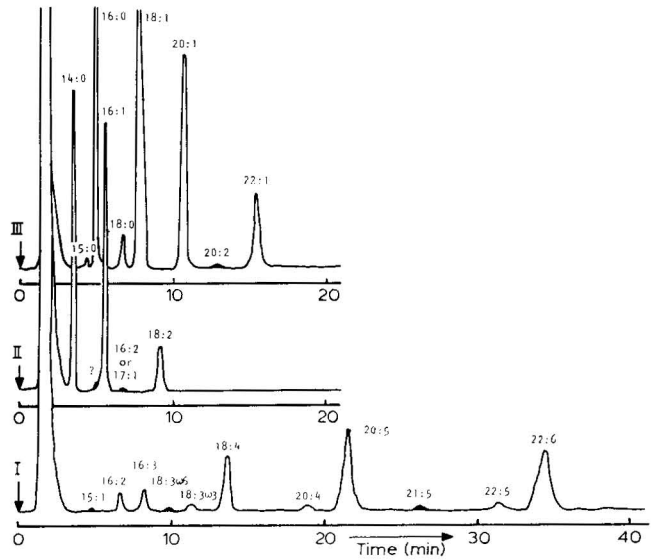

Fig. 3. Gas chromatograms of reversed-phase HPLC fractions I–III (see Fig. 2). Column: 2 m × 2.7 mm I.D. (glass), 12% (w/w) of OV-275 on Chromosorb P AW DMCS. Carrier gas: nitrogen (10 ml/min). Temperature: 215°.

(amounts $< 0.2\%$). The small peak in II, ascribed to C17:1, can be a trace of C16:2 from fraction I.

The log k' values (relative to that of C18:0) are given in Table I. The corresponding esters were identified from $\log[k'/k'(\text{C18:0})]$ *versus* n plots. Both the predictability of the esters potentially present in a certain fraction on the basis of Figs. 1 and 2, and the fact that esters with similar log k' values belong to different fractions (see Table I), greatly facilitates this procedure.

Using saturated fatty acid esters as standards, *ECL* values were calculated from the log k' values of all traced fatty esters by linear regression. As OV-275 and Silar 10C have similar chemical constitutions[4], the *ECL* values on the former can be compared with those on the latter given by Heckers *et al.*[4]. The agreement is good, except for C20:4 and C20:5. The large discrepancies in this instance can hardly be due to positional isomerism or different polarities of the two stationary phases (compare the *ECL* values for C22:6). The *ECL* values of these fatty acid esters on OV-275 are consistent with a contribution of 1 *ECL* unit per methylene group (compare the *ECL* values of C20:4 and C20:5 with those of C18:4 and C22:5, respectively).

The indication of double bond positions (ω notation in Table I) refers to *ECL* values given by Heckers *et al.*[4], and does not exclude the occurrence of other isomers in cod liver oil. Generally, they are hard to distinguish on packed GLC columns. Inspection of the *ECL* values in Table I shows that neighbouring esters which belong to the same fraction have *ECL* values, the difference of which is about 1 *ECL* unit in most instances. Hence a pre-fractionation by RP-HPLC can facilitate the search for positional isomers in complex mixtures by capillary GLC. *Cis–trans* separations of monoenoic esters by this HPLC method can also be useful in this respect, as follows from Fig. 2.

Quantitative results were obtained by triangulation of the peaks and normalization of the peak areas to constant sample size by means of resolved peaks in the chromatogram of the unfractionated cod liver oil esters. Response factors were estimated from the weight fractions of carbon in the esters (the carbonyl carbon atom was excluded because its contribution to the response of the flame-ionization detector can be neglected[34]). The weight percentages are given in Table I, together with GLC data* provided by TNO[35] for the same sample. The former are precise to about 0.5% and the latter (obtained with a digital integrator) to about 0.2%. The agreement is satisfactory in most instances.

Argentation HPLC

The log k' values of the fatty acid esters with 0–6 *cis* double bonds on both silver (I) columns are plotted *versus* $n_{C=C}$ in Fig. 4A. The data points for $n_{C=C} = 0$, 1, 2 and 3 belong to standard C18 esters and those for $n_{C=C} = 4$, 5 and 6 to C18:4, C20:5 and C22:6, respectively. The latter were obtained from the chromatograms of the cod liver oil fatty acid esters. The (smoothed) chromatogram on the 5% Ag(I)–0.4% acetonitrile–*n*-hexane column is given in Fig. 5.

* Analytical procedure according to the Dutch NEN-norm 3428 entitled "Gas chromatographic determination of fatty acid composition of vegetable and animal fats and oils, and of technical fatty acids". The hydrogenation step was excluded and replaced with analyses on EGA and Apiezon L columns.

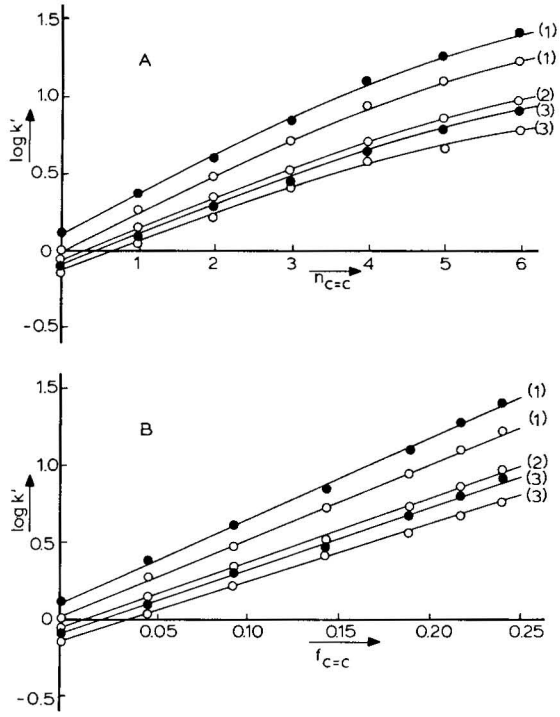

Fig. 4. Log k' values of fatty acid esters *versus* (A) the number of double bonds ($n_{C=C}$) and (B) their double bond fraction ($f_{C=C}$) on 5 and 7.5% (w/w) Ag(I) on Partisil 10 (data points indicated by open and full circles, respectively). Eluents: (1) 0.3, (2) 0.4 and (3) 0.5% (v/v) acetonitrile in *n*-hexane.

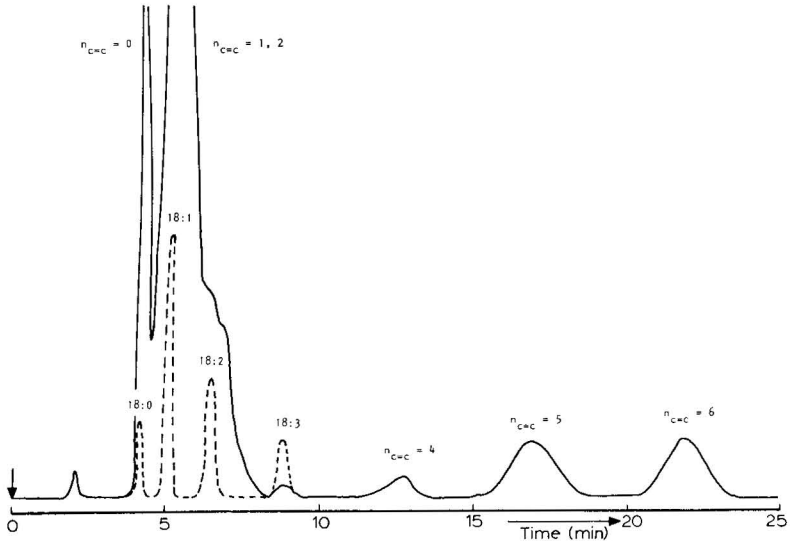

Fig. 5. Fractionation of cod liver oil fatty esters on 5% (w/w) Ag(I) on Partisil 10. Eluent: 0.4% (v/v) acetonitrile in *n*-hexane. Flow-rate: 1.54 ml/min. Sample size: 25 μl containing 0.6 mg of fatty esters. Broken lines: C18 fatty acid ester standards.

The log k' values increase with increasing silver(I) content, as expected. Further, the log k' values increase rapidly with decreasing acetonitrile content of the eluent. Finally, the log k' values are not linearly related to $n_{C=C}$ and hence solute retention is not merely related to the number of double bonds. As the retention mechanism in Ag-HPLC has not yet been described in detail, a few comments will be made.

It is assumed that the contribution of the adsorption of the ester group to log k' is similar for all fatty acid esters under examination. Hence, it cancels in the log α_i $\{\equiv \log[k'_i/k'(C18:0)]\}$ values. As the adsorption of an olefinic double bond to silanol sites will be weak because of the strong adsorption of the acetonitrile molecules to the silica surface[39], the log α_i values represent the contribution of double bond–silver(I) interactions. This contribution increases as the probability of double bond–silver(I) complexation increases, *i.e.*, with increasing silver(I) content.

The ratios of the α_i values obtained at 0.3 and 0.5% of acetonitrile in the eluent are equal, within the limits of experimental error, on both columns [5 and 7.5% Ag(I)]. This result would be expected if the silver(I) sites on both columns are roughly equivalent, *i.e.*, act as single sites towards all fatty esters under discussion and merely form 1:1 complexes. These sites are surrounded by acetonitrile molecules, the number of which is merely dependent on the acetonitrile concentration in the eluent. It can be estimated[36] that the influence of a 0.2% change in the acetonitrile concentration on log α_i would be negligible on silica. Hence, the relatively large eluent effect on silver nitrate-impregnated silica must be related to competitive complex formation between acetonitrile and unsaturated fatty acid ester molecules and silver(I) sites. This phenomenon has recently been described in detail[37].

The equivalence of the complexing sites can also be expected from a consideration of the site distribution. At the low surface concentrations of silver(I) in our columns (about 1.2 and 1.7 μmole/m^2), the mean distance between adjacent sites will be at least 13.5 and 11.4 Å, respectively. If part of the silver nitrate molecules form small clusters (which seems plausible), these distances are even larger and simultaneous complexation with two sites becomes even less probable. Hence, the most plausible explanation for the curved plots in Fig. 4A is that 1:1 complexes are formed to an extent that is simply proportional to the chance of complexation. A measure of this chance is the ratio of $n_{C=C}$ and the total number of carbon and oxygen atoms in the fatty acid ester, $f_{C=C}$. Indeed, plots of log k' against $f_{C=C}$ are linear (Fig. 4B). Obviously, the double bonds are equivalent with respect to complex formation. It is noteworthy that the influence of the chain length on the contribution of complexation to log k' values is also evident on 4% Ag aluminosilicate[15], where C16:1Δ^9 elutes *after* C18:1Δ^9 in 0.011% acetonitrile–chloroform+*n*-hexane (1:12).

This simple picture of the adsorption process does not apply to the R_F values of series of C18 positional isomers[8,16–18], which are a shallow sinusoidal function of the double bond position. For these isomers the strongest complexation is invariably found for the Δ^5 or Δ^6 isomer, irrespective of the silver content (10–30% of silver nitrate on silica gel G). Presumably, the surface concentration of silver(I) is so large here that the mean spacing between the ester group and the sixth carbon atom fits to the average distance between a silver site and its surrounding silanol groups[8]. Similar "localization" phenomena have been described by Snyder[38] for a series of dithiaalkanes on alumina.

Fig. 5 shows the chromatogram of the cod liver oil fatty acid esters together

with that of a synthetic mixture of C18 standards. The bad resolution of the saturated, mono- and dienoic esters can be expected for most vegetable and fish oils, in which C16:0, C18:0, C18:1, C18:2, C20:1 and C22:1 are generally abundantly present. The resolution can be improved by using a larger amount of silver(I) and/or a lower acetonitrile content. However, the analysis time will then be unacceptably long. Gradient elution is not suitable because the regeneration of these columns to their original condition with *n*-hexane is time consuming. Analysis of the combined fraction ($n_{C=C} = 0$, 1 and 2) is not advantageous in view of the large number of esters involved. Therefore, it is concluded that this type of argentation HPLC is not suitable for fractionation in total fatty acid analysis.

On the other hand, the resolution of the highly unsaturated esters is good and can be achieved within 25 min. Hence, Ag-HPLC is a promising fractionation technique (milligram scale) for highly unsaturated esters under favourable chromatographic conditions. The recovery of the esters from the column was examined by comparing the peak heights of unsaturated esters (relative to that of C22:1) in gas chromatograms of a sample that passed the silver(I) column and of a sample that was injected directly into the gas chromatograph. The recoveries of C22:6, C20:5 and C18:4 were 96 ± 4, 101 ± 2 and $101 \pm 2\%$, respectively. Previously, De Vries[39] reported a complete recovery of linolenate (C18:3). Obviously, oxidation of the esters by silver nitrate does not occur. A disadvantage of columns with silver nitrate-impregnated silicas is their restricted lifetime. Under the chromatographic conditions used here, the column properties were constant for only 2 months with daily use. This is probably due to the slight solubility of silver nitrate in the eluents used. This problem may be overcome by saturating the eluent with silver nitrate by means of a pre-column.

CONCLUSIONS

Reversed-phase HPLC on LiChrosorb RP-18 with acetonitrile as the eluent is a promising pre-fractionation method for complex fatty acid methyl ester mixtures. Apart from positional isomers, the collected fractions are free of most of the "critical pairs" which can occur in GLC analysis on polar columns. The isolation of *cis*- and *trans*-isomers in complex ester mixtures in different RP fractions (although restricted to monoenoic esters with chain lengths of at least 15 carbon atoms) further facilitates the analysis of the RP fractions by means of (capillary) GLC, including positional isomers.

Argentation HPLC appears to be less suitable as a pre-fractionation method in total fatty acid ester analysis. However, it seems to be a good method for the fractionation of unsaturated ester species with 3–6 double bonds (milligram scale). The mechanism of retention of fatty acid methyl esters on low-loaded silver nitrate-impregnated silicas [surface coverages up to 1.7 μmol/m^2 of Ag(I)] is controlled by their degree of unsaturation, *i.e.*, the ratio of the number of double bonds to the total number of carbon and oxygen atoms in the ester. The large influence of the content of acetonitrile in *n*-hexane on the retention of the unsaturated esters is due to competitive 1:1 complex formation with silver(I) sites.

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USE OF ISOTHERMAL GAS CHROMATOGRAPHY FOR THE DETERMINATION OF THE ADSORPTION ENTHALPIES AND ENTROPIES OF INORGANIC HALIDES AT HIGH TEMPERATURES

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SUMMARY

Gas chromatography was used to determine the adsorption enthalpies and entropies of inorganic chlorides on solid non-porous adsorbents (silica, alkali metal chlorides, graphite, etc.) at high temperatures (650–1100°K). The results demonstrate that gas chromatography is also a useful method for investigations in the field of inorganic chemistry. The adsorption enthalpies and entropies (or rather, chemisorption enthalpies and entropies) indicate that the adsorption (or chemisorption process) involves complex formation between the adsorbed molecules and the adsorbent (coordinative by bonded surface complex).

INTRODUCTION

In recent years various types of gas–solid chromatography for application to inorganic compounds have been developed. The techniques applied include isothermal and temperature-programmed gas chromatography[1], and thermochromatography[2] and on-line separations or continuous retention time measurements[3]. The aims of the investigation of the different methods of inorganic gas chromatography are different:

(1) the development of separation methods for trace elements;

(2) the determination of thermodynamic data, especially adsorption enthalpies and entropies[4,5];

(3) the separation of new artificial elements and the investigation of the chemical properties of these elements and their compounds[6].

In an extensive study, we have investigated the chromatographic behaviour of various metal chlorides and oxychlorides in columns with different solid inorganic stationary phases. An important part of this study was the determination of adsorption enthalpies and entropies by isothermal gas–solid chromatography. In this paper we present the arguments and theory for the calculation of these values from experimental data and the results of the investigations.

THEORETICAL

Most of the investigations were carried out at extremely low surface concentrations and with non-porous solid stationary phases. As such low concentrations were used, it is justified to assume that the adsorption isotherms are linear and therefore that the partition ratio is independent of concentration. For these nearly ideal conditions (non-porous solid stationary phase, linear adsorption isotherm), the migration velocity ($dx/dt = v$) of the first statistical moment of the chromatographic peaks (or the peak maximum of a symmetrical peak) can be expressed by

$$\frac{dx}{dt} = v = \frac{u}{1+k} \tag{1}$$

where u = linear velocity of the carrier gas.

The partition coefficient (or retardation ratio), k, can be expressed as

$$k = \frac{as}{c_g V_g} = K_a \cdot \frac{s}{V_g} \tag{2}$$

where $K_a = a/c_g$ (cm), a = surface concentration, s = surface area, V_g = free column volume and c_g = concentration in the gas phase.

To convert the gas-phase concentration into the partial pressure (p), as a first approximation an ideal gas is assumed:

$$k = \frac{asRT}{pV_g} = K_p \cdot \frac{sRT}{V_g} \tag{3}$$

where $K_p = a/p$ (mol N^{-1}).

In principle, the adsorption enthalpy can be determined from the temperature dependence of the partial pressure p in the gas phase at constant surface pressure π:

$$\Delta H_{ads} = -RT^2\left(\frac{\partial \ln p}{\partial T}\right)_\pi \tag{4}$$

Theoretically, the adsorption enthalpy can be determined from retention data by combining eqn. 1 (in the integrated form) for constant experimental conditions [$t_r^0 = t_0 \cdot (1 + k)$] with eqns. 3 and 4:

$$-\left\{\frac{\partial \ln\left[\frac{asRT}{V_g(t_r/t_0)}\right]}{\partial T}\right\}_\pi RT^2 = \Delta H_{ads} \tag{5}$$

where (t_r^0 = total retention time, t_0 = dead time and $t_r = t_r^0 - t_0$ = net retention time)

The correlation between $(\partial \ln p/\partial T)_\pi$ and $(\partial \ln p/\partial T)_a$ and the following expression, which was derived by Hill[7]:

$$\Delta H_{ads} = -RT^2\left(\frac{\partial \ln p}{\partial T}\right)_a + T\,a\left(\frac{\partial \pi}{\partial T}\right)_\theta \tag{6}$$

where θ = relative surface coverage, may be used. The term $RT^2\,(\partial \ln p/\partial T)_a = q_{is}$ is often called the isosteric heat of adsorption.

For the borderline cases of the adsorbed state (totally unhindered movement of the molecules along the surface and completely fixed molecules), the following two equations are obtained:

(1) for immobile adsorbed molecules at low surface concentration:

$$\Delta H^{f}_{ads} = -R\left[\frac{d\ln\left(\frac{t_r}{t_0 T}\right)}{d\left(\frac{1}{T}\right)}\right] = -R\left[\frac{d\ln\left(\frac{V_r}{TV_g}\right)}{d\left(\frac{1}{T}\right)}\right] \tag{7}$$

where V_r = net retention volume, and

(2) for an ideal two-dimensional gas:

$$\Delta H^{m}_{ads} = -R\left[\frac{d\ln\left(\frac{t_r}{t_0}\right)}{d\left(\frac{1}{T}\right)}\right] = -R\left[\frac{d\ln\left(\frac{V_r}{V_g}\right)}{d\left(\frac{1}{T}\right)}\right] \tag{8}$$

As the net retention time, t_r, and the net retention volume, V_r, are independent of the surface concentration provided that the adsorption isotherms are linear, the adsorption enthalpies do not depend on the surface concentration and in this instance it is no longer necessary to use partial differentials.

According to eqns. 7 and 8, the adsorption enthalpies can be determined from a graph of either the logarithms of the net retention volumes at the column temperature or the logarithms of the quotients of the net retention volumes at the column temperature and the absolute temperature *versus* the inverse of the absolute temperature. It should be noted that both equations were derived by assuming different states of the adsorbed molecules.

An example is presented in Fig. 1 for a range of temperatures. The retention volumes of tellurium tetrachloride were determined in a gas chromatographic column with yttrium trichloride (on quartz-glass particles as the support) as the stationary phase. Fig. 1a shows a plot according to eqn. 7 and Fig. 1b a plot according to eqn. 8. The two adsorption enthalpies determined from these data are $\Delta H^{f}_{ads} = -73.2 \pm 2.3$ kJ mole^{-1} and $\Delta H^{m}_{ads} = -66.0 \pm 2.3$ kJ mole^{-1}, respectively (data are given for a yttrium trichloride column). As $\theta \ll 1$ in all experiments, it is justified to assume that the adsorption isotherms will be linear. The difference between the above two values is 7.2 kJ mole^{-1}, which is as expected in accordance with RT at the average of the experimental temperatures (*ca.* 880°K).

The adsorption enthalpies listed in Table I are based on the assumption of immobile adsorbed molecules, as our results indicate chemisorption of the molecules rather than a purely physical adsorption process. The standard adsorption enthalpies are determined from the intercept of the curve with the $\ln V_r$ axis according to the following equation:

$$\Delta S^{\circ}_{ads} = \frac{\Delta H^{\circ}_{ads}}{T} - R\ln\left(\frac{a_s s RT}{V_r P_0}\right) \tag{9}$$

where a_s = standard surface concentration and P_0 = standard (gas phase) pressure.

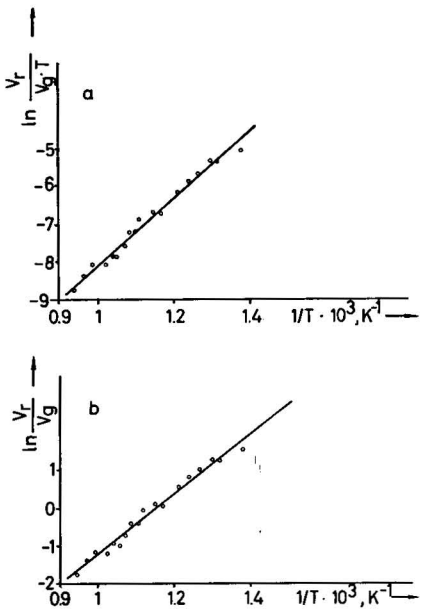

Fig. 1. Arrhenius-type plots for the determination of adsorption enthalpies. (a) Immobile adsorbed molecules; (b) ideal two-dimensional gas. Example: $TeCl_4$ on a YCl_3 surface.

It can be seen from this equation that the value obtained for the standard adsorption entropy depends on the values used for a_s and P_0. This is not a problem with P_0, as a standard pressure of 1 atm is generally used in thermodynamics, but to our knowledge there is no generally accepted standard state for the adsorbed phase. We adopted the standard state suggested by De Boer[8] for a temperature of 298° K (this gives a value of *ca.* $1.37 \cdot 10^{-11}$ mole cm^{-2}).

EXPERIMENTAL

The experimental procedure and equipment are described in detail elsewhere[9]. The chromatographic section of the column is 75 cm long with an I.D. of 8 mm; the column material was quartz. The columns were packed with quartz-glass or graphite particles or with coated quartz-glass particles of diameter *ca.* 0.25 mm (total surface area = $6 \cdot 10^3$ cm^2). For the investigation, radioactively labelled compounds of high specific activity (carrier free or practically carrier free) were used. Therefore, it was possible to carry out the experiments with extremely small amounts of adsorbate (*ca.* 10^3 molecules cm^{-2}) and the surface coverage, θ, is consequently extremely small ($\theta \ll 1$). The chlorides and oxychlorides were prepared *in situ*, in order to reduce their handling as far as possible. The different radionuclides (Tc-99m, Mo-99, Te-132, Zr-95, Nb-95 and Nb-97) were detected by gamma spectroscopy. The carrier gas was nitrogen with different carbon tetrachloride partial pressures (flow-rate = 1–10 cm^3 min^{-1}). Carbon tetrachloride was used as a reactive gas for the preparation of volatile chlorides and oxychlorides and was present in the carrier gas to remove oxygen and water impurities as far as possible in order to

prevent the formation of non-volatile oxides and to reduce the thermal dissociation of the investigated compounds into chlorine and compounds of lower oxidation states.

RESULTS AND DISCUSSION

The standard adsorption enthalpies and entropies that were determined by gas–solid chromatography are listed in Table I. Standard states are 1 atm for the gas phase and a surface concentration of $1.37 \cdot 10^{-11}$ mole cm^{-2} for the adsorbed phase. The stated errors are determined from the linear regression of the ln V_r/T *versus* T^{-1} plot. The results of identical measurement series are in agreement within the limits of experimental error. The results show that it is justified to neglect the temperature dependence of the adsorption enthalpies and entropies because it is small compared with the experimental errors.

The identification of the chemical compounds presents difficulties in several instances. The measurement method (gamma spectroscopy) permits an unambiguous identification of the element, whereas direct identification of the chemical compounds is not possible. Two sources of information can be used for the identification of the chemical compounds: (1) the known macroscopic properties (*e.g.*, sublimation or evaporation temperatures, enthalpies or entropies); and (2) the chromatographic behaviour and the data derived from the chromatographic properties of the investigated compounds.

The compounds of elements that form only one or two volatile chlorides or oxychlorides can easily be identified from their known macroscopic properties, whereas the compounds of elements that form several different volatile chlorides or oxychlorides (*e.g.*, molybdenum and technetium) can seldom be easily identified. To obtain further evidence about the identity of an unknown compound, it is necessary to compare the observed chromatographic properties with the properties of already identified compounds. By this means it is possible to identify a considerable proportion of the various compounds of different elements, although in some instances the identification is still ambiguous. Several difficulties arise if the macroscopic properties of the chlorides or oxychlorides are not known or are unreliable (*e.g.*, for technetium chlorides or oxychlorides) or if there are no experimental data on the chromatographic properties of similar, already identified compounds.

The high adsorption enthalpies and entropies and the strong dependence of these values on the type of the surface on which the compounds are adsorbed suggest a chemisorption rather then a physisorption process. Both results indicate a selective mutual interaction between adsorbent and adsorbate. The most probable description of this interaction is obtained if coordinative chemical bonding (complex formation) between the surface (electron-pair donor, Lewis base) and the adsorbed molecules (electron-pair acceptor, Lewis acid) is assumed. The experimental data are insufficient in both number and accuracy for any reliable assumptions to be made about the degree of ionic or covalent bonding. For the same reason, it is not possible to make any statements about the contribution of physical forces (repulsion or attraction) to the adsorption enthalpies and entropies. The assumption of the formation of a "surface complex" is supported by the following arguments:

(1) the chlorides and oxychlorides of tellurium, zirconium, niobium, molyb-

TABLE I

ADSORPTION ENTHALPIES AND ENTROPIES

*Compound**	*Surface*	*Partial pressure of reactive gas (mmHg)*	ΔH_{ads} *(kJ mole^{-1})*	ΔS_{ads} *(J mole^{-1} °K^{-1})*	*Regression coefficient*	*Number of points*
$NbCl_5$	SiO_2, 1st series	90 (CCl_4)	− 69 ± 5	− 20 ± 6	0.9806	9
$NbCl_5$	SiO_2, 2nd series	90 (CCl_4)	− 65 ± 5	− 11 ± 5	0.9757	12
$NbCl_5$	SiO_2, carbon modified	90 (CCl_4)	− 60 ± 2	− 8 ± 3	0.9912	15
$NbCl_5$	Graphite	90 (CCl_4)	−109 ± 11	− 41 ± 11	0.9581	13
$NbCl_5$	Graphite, $RuCl_3$ treated	90 (CCl_4)	−121 ± 11	− 53 ± 12	0.9502	14
$NbCl_5$	SiO_2	55 (CCl_4)	− 72 ± 7	− 20 ± 8	0.9710	8
$NbCl_5$	SiO_2	25 (CCl_4)	− 64 ± 5	− 10 ± 5	0.9783	10
$NbCl_5$	SiO_2	15 (CCl_4)	− 69 ± 4	− 15 ± 5	0.9825	11
$NbOCl_3$	SiO_2	25 (CCl_4)/ 15 (H_2O)	− 94 ± 13	− 34 ± 14	0.8814	17
$NbCl_5$	NaCl	90 (CCl_4)	− 76 ± 11	− 22 ± 11	0.9372	9
$NbCl_5$	$MgCl_2$	90 (CCl_4)	− 76 ± 4	− 23 ± 5	0.9939	7
$NbCl_5$	$CaCl_2$	90 (CCl_4)	− 82 ± 3	− 30 ± 4	0.9891	16
$NbCl_5$	YCl_3	90 (CCl_4)	− 78 ± 4	− 20 ± 5	0.9814	16
$NbCl_5$	CsCl heated at 950 °K	90 (CCl_4)	− 62 ± 3	− 12 ± 4	0.9967	5
$NbCl_5$	CsCl heated at 870 °K	90 (CCl_4)	− 86 ± 4	− 44 ± 6	0.9963	5
$NbCl_5$	KCl, 1st series	90 (CCl_4)	−101 ± 4	− 52 ± 5	0.9968	6
$NbCl_5$	KCl, 2nd series	90 (CCl_4)	−102 ± 4	− 57 ± 5	0.9974	5
$NbCl_5$	KCl, $HfCl_4$ heated	90 (CCl_4)	− 84 ± 8	− 34 ± 10	0.9779	6
$NbCl_5$	KCl, $RuCl_3$ heated	90 (CCl_4)	− 81 ± 8	− 32 ± 9	0.9871	5
Mo	SiO_2, 1st series	90 (CCl_4)	− 75 ± 3	− 30 ± 3	0.9975	5
Mo	SiO_2, 1st series	90 (CCl_4)	−119 ± 10	− 56 ± 21	0.9933	4
($MoOCl_3$)	SiO_2, 1st series	90 (CCl_4)	− 94 ± 8	− 46 ± 9	0.9939	4
$MoCl_5$	SiO_2, 2nd series	90 (CCl_4)	− 57 ± 2	− 10 ± 3	0.9952	7
($MoOCl_3$)	SiO_2, 2nd series	90 (CCl_4)	− 81 ± 6	− 31 ± 7	0.9767	10
Mo	SiO_2, 2nd series	90 (CCl_4)	−111 ± 20	− 46 ± 21	0.9696	4
$MoCl_5$	SiO_2, carbon modified	90 (CCl_4)	− 46 ± 4	1 ± 4	0.9797	9
($MoOCl_3$)	SiO_2, carbon modified	90 (CCl_4)	− 71 ± 6	− 19 ± 7	0.9868	6
($MoOCl_3$)	Graphite	90 (CCl_4)	− 95 ± 11	− 27 ± 11	0.9809	4
$MoCl_5$	SiO_2	55 (CCl_4)	− 60 ± 5	− 13 ± 7	0.9830	5
Mo	SiO_2	55 (CCl_4)	− 77 ± 5	− 26 ± 6	0.9978	3
Mo	SiO_2	25 (CCl_4)	− 65 ± 4	− 21 ± 5	0.9909	6
$MoCl_5$	SiO_2	25 (CCl_4)	− 66 ± 11	− 12 ± 13	0.9444	6
$MoCl_5$	SiO_2	15 (CCl_4)	− 56 ± 3	− 10 ± 3	0.9935	7
Mo	SiO_2	15 (CCl_4)	−115 ± 19	− 66 ± 22	0.9858	3
($MoOCl_3$)	SiO_2	25 (CCl_4)/ 15 (H_2O)	− 91 ± 5	− 26 ± 6	0.9914	7
($MoOCl_4$)	NaCl	90 (CCl_4)	− 43 ± 3	17 ± 12	0.9865	5
$MoCl_5$	NaCl	90 (CCl_4)	− 72 ± 8	− 12 ± 7	0.9790	6
Mo	NaCl	90 (CCl_4)	−136 ± 6	− 65 ± 6	0.9990	3
$MoCl_5$	$MgCl_2$	90 (CCl_4)	− 68 ± 9	− 35 ± 12	0.9752	5
Mo	$MgCl_2$	90 (CCl_4)	−112 ± 8	− 62 ± 9	0.9916	5

TABLE I *(continued)*

*Compound**	*Surface*	*Partial pressure of reactive gas (mmHg)*	ΔH_{ads} *(kJ mole^{-1})*	ΔS_{ads} *(J mole^{-1} °K^{-1})*	*Regression coefficient*	*Number of points*
$MoCl_5$	$CaCl_2$	90 (CCl_4)	− 73 ± 3	− 21 ± 3	0.9942	13
$MoCl_3$	$CaCl_2$	90 (CCl_4)	−100 ± 5	− 34 ± 5	0.9970	4
$MoOCl_4$	YCl_3	90 (CCl_4)	− 48 ± 5	15 ± 6	0.9601	11
$MoCl_5$	YCl_3	90 (CCl_4)	− 76 ± 3	− 28 ± 3	0.9925	16
$MoOCl_3$	CsCl heated at 950 °K	90 (CCl_4)	− 78 ± 6	− 30 ± 8	0.9928	5
$MoCl_5$	CsCl heated at 870 °K	90 (CCl_4)	− 76 ± 10	− 27 ± 12	0.9672	5
$MoCl_5$	KCl, 1st series	90 (CCl_4)	− 84 ± 4	− 40 ± 4	0.9955	3
Mo	KCl, 1st series	90 (CCl_4)	−136 ± 25	− 81 ± 30	0.9376	6
($MoOCl_4$)	KCl, 2nd series	90 (CCl_4)	− 41 ± 1	2 ± 1	0.9995	4
$MoCl_5$	KCl, 2nd series	90 (CCl_4)	− 83 ± 6	− 40 ± 3	0.9865	7
($MoOCl_4$)	KCl, $HfCl_4$ coated	90 (CCl_4)	− 42 ± 3	2 ± 4	0.9939	4
$MoCl_5$	KCl, $HfCl_4$ coated	90 (CCl_4)	− 83 ± 7	− 38 ± 8	0.9815	5
Mo	KCl, $HfCl_4$ coated	90 (CCl_4)	−135 ± 42	− 78 ± 46	0.9085	3
$MoCl_5$	KCl, $RuCl_3$ treated	90 (CCl_4)	− 78 ± 5	− 33 ± 7	0.9975	3
$MoCl_5$	KCl (with KCl packed column)	90 (CCl_4)	− 85 ± 8	− 41 ± 9	0.9886	5
Mo	KCl (with KCl packed column)	90 (CCl_4)	−129 ± 39	− 71 ± 23	0.9773	4
($TcOCl_3$)	SiO_2, 1st series	90 (CCl_4)	− 94 ± 9	− 29 ± 10	0.9678	8
$TcCl_4$	SiO_2, 1st series	90 (CCl_4)	− 94 ± 2	− 24 ± 2	0.9992	7
$TcOCl_4$	SiO_2, 1st series	90 (CCl_4)	− 50 ± 1	− 3 ± 1	0.9999	3
$TcCl_5$**	SiO_2, 1st series	90 (CCl_4)	− 65 ± 5	− 13 ± 5	0.9924	7
$TcCl_4$	SiO_2, 2nd series	90 (CCl_4)	− 85 ± 5	− 15 ± 5	0.9961	4
$TcOCl_4$	SiO_2, 2nd series	90 (CCl_4)	− 52 ± 4	− 6 ± 4	0.9891	6
$TcCl_5$	SiO_2, 2nd series	90 (CCl_4)	− 67 ± 3	− 15 ± 4	0.9935	5
$TcOCl_4$	SiO_2, carbon modified	90 (CCl_4)	− 44 ± 2	2 ± 2	0.9925	11
$TcCl_5$	SiO_2, carbon modified	90 (CCl_4)	− 62 ± 3	− 10 ± 4	0.9956	5
$TcOCl_4$	SiO_2	Together 15, 25, 55, (CCl_4)	− 52 ± 2	− 5 ± 2	0.9907	15
$TcCl_5$	SiO_2	Together 15, 25, 55 (CCl_4)	− 69 ± 2	− 17 ± 2	0.9954	16
($TcOCl_3$)	SiO_2	25 (CCl_4) 15 (H_2O)	−101 ± 7	− 37 ± 7	0.9934	5
$TcCl_4$	SiO_2	25 (CCl_4) 15 (H_2O)	− 87 ± 15	− 16 ± 17	0.9709	4
$TcOCl_4$	NaCl	90 (CCl_4)	− 50 ± 1	2 ± 2	0.9997	6
$TcCl_5$	NaCl	90 (CCl_4)	− 69 ± 1	− 9 ± 1	0.9997	5
$TcCl_4$	$MgCl_2$	90 (CCl_4)	− 62 ± 6	− 26 ± 8	0.9821	7
$TcCl_5$	$MgCl_2$	90 (CCl_4)	− 67 ± 12	− 12 ± 14	0.9222	5
$TcCl_5$	$CaCl_2$	90 (CCl_4)	− 79 ± 1	− 28 ± 2	0.9985	11
$TcCl_4$	$CaCl_2$	90 (CCl_4)	− 80 ± 6	− 21 ± 7	0.9845	6
$TcOCl_4$	$CaCl_2$	90 (CCl_4)	− 70 ± 5	2 ± 5	0.9862	7

(Continued on p. 326)

TABLE I *(continued)*

*Compound**	*Surface*	*Partial pressure of reactive gas (mmHg)*	ΔH_{ads} *(kJ mole^{-1})*	ΔS_{ads} *(J mole^{-1} °K^{-1})*	*Regression coefficient*	*Number of points*
$TcCl_4$	YCl_3	90 (CCl_4)	− 66 ± 3	− 17 ± 3	0.9877	16
$TcCl_5$	YCl_3	90 (CCl_4)	− 75 ± 6	− 17 ± 7	0.9785	8
$TcOCl_4$	YCl_3	90 (CCl_4)	− 68 ± 3	− 1 ± 2	0.9953	8
$TcCl_5$	KCl, 1st series	90 (CCl_4)	− 80 ± 4	− 33 ± 6	0.9933	5
$TcCl_4$	KCl, 1st series	90 (CCl_4)	− 85 ± 10	− 20 ± 12	0.9789	6
$TcCl_5$	KCl, 2nd series	90 (CCl_4)	− 79 ± 14	− 36 ± 17	0.9285	7
($TcOCl_3$)	KCl, 2nd series	90 (CCl_4)	−102 ± 20	− 43 ± 20	0.9819	3
$TcCl_5$	KCl (with KCl packed column)	90 (CCl_4)	− 74 ± 4	− 26 ± 5	0.9951	5
$TcCl_5$	KCl, $HfCl_4$ treated	90 (CCl_4)	− 79 ± 12	− 36 ± 14	0.9301	3
$TcCl_4$	KCl, $HfCl_4$ treated	90 (CCl_4)	− 81 ± 12	− 22 ± 13	0.9775	4
($TcOCl_3$)	CsCl heated at 950 °K	90 (CCl_4)	−100 ± 5	− 44 ± 5	0.9977	4
$TcCl_4$	CsCl heated at 950 °K	90 (CCl_4)	− 75 ± 7	− 27 ± 8	0.9918	4
$ZrCl_4$	SiO_2, 1st series	90 (CCl_4)	− 97 ± 12	− 54 ± 8	0.9465	9
$ZrCl_4$	SiO_2, carbon modified	90 (CCl_4)	− 81 ± 4	− 18 ± 5	0.9955	5
$ZrCl_4$	YCl_3	90 (CCl_4)	− 90 ± 6	− 32 ± 6	0.9892	7
$TeCl_4$	SiO_2, 1st series	90 (CCl_4)	− 54 ± 2	− 6 ± 2	0.9946	11
$TeCl_4$	SiO_2, 2nd series	90 (CCl_4)	− 57 ± 5	− 7 ± 6	0.9669	11
$TeCl_4$	SiO_2, carbon modified	90 (CCl_4)	− 43 ± 3	− 8 ± 3	0.9689	16
$TeCl_4$	Graphite	90 (CCl_4)	− 83 ± 8	− 17 ± 5	0.9462	13
$TeCl_4$	Graphite, $RuCl_3$ treated	90 (CCl_4)	− 74 ± 14	− 15 ± 14	0.8347	15
$TeCl_4$	SiO_2	55 (CCl_4)	− 57 ± 3	− 5 ± 3	0.9943	7
$TeCl_4$	SiO_2	25 (CCl_4)	− 51 ± 3	5 ± 4	0.9803	11
$TeCl_4$	SiO_2	15 (CCl_4)	− 41 ± 3	14 ± 4	0.9751	11
Te	SiO_2	25 (CCl_4)/ 15 (H_2O)	− 81 ± 13	− 27 ± 15	0.9209	9
$TeCl_4$	NaCl	90 (CCl_4)	− 70 ± 15	− 18 ± 17	0.9029	6
$TeCl_4$	$MgCl_2$	90 (CCl_4)	− 70 ± 3	− 17 ± 3	0.9946	9
$TeCl_4$	$CaCl_2$	90 (CCl_4)	− 75 ± 4	− 26 ± 5	0.9820	12
$TeCl_4$	YCl_3	90 (CCl_4)	− 73 ± 3	− 18 ± 3	0.9917	18
$TeCl_4$	CsCl heated at 950 °K	90 (CCl_4)	− 42 ± 3	10 ± 3	0.9940	5
$TeCl_4$	CsCl heated at 870 °K	90 (CCl_4)	− 90 ± 4	− 54 ± 6	0.9960	5
$TeCl_4$	KCl, 1st series	90 (CCl_4)	− 85 ± 8	− 38 ± 10	0.9785	6
$TeCl_4$	KCl, 2nd series	90 (CCl_4)	− 79 ± 10	− 36 ± 12	0.9703	6
$TeCl_4$	KCl, $HfCl_4$ treated	90 (CCl_4)	− 77 ± 2	− 32 ± 2	0.9985	5
$TeCl_4$	KCl, $RuCl_3$ treated	90 (CCl_4)	− 72 ± 19	− 24 ± 22	0.9108	5

* The compounds listed are the most probably formed species: where we consider that the assignment might not be completely justified, the formular is enclosed in parentheses. If there is insufficient evidence of the identity of the compound, only the element is stated.

** Although $TcCl_5$ has not yet been positively identified, we think that the pentachloride is probably formed under the experimental conditions and the observed chromatographic properties indicate that this compound is a pentachloride; therefore, we are convinced that the observed compound is $TcCl_5$.

denum and technetium have higher coordination numbers in the condensed phases than in the gas phase;

(2) there is a correlation between the complexing abilities of the solid phase and the experimental adsorption enthalpies (*e.g.*, potassium chloride forms stronger complexes with niobium pentachloride than does sodium chloride and the adsorption enthalpy for niobium pentachloride is -75 kJ mole^{-1} on sodium chloride and -101 kJ mole^{-1} on potassium chloride);

(3) there is a correlation between adsorption enthalpies and entropies (see Fig. 2); this is to be expected as stronger chemical bonding causes a higher loss of enthalpy and a higher loss of entropy.

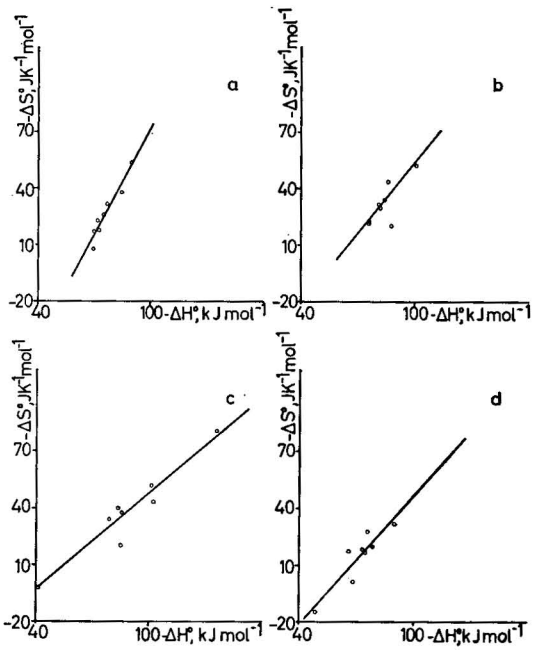

Fig. 2. Correlations between adsorption enthalpies and entropies. (a) $TeCl_4$ on different surfaces; (b) $NbCl_5$ on different surfaces; (c) different compounds on KCl; (d) different compounds on YCl_3.

Although a general trend for all adsorbent–adsorbate pairs can be recognized, the correlation between adsorption enthalpies and entropies is better for a given adsorbate on different adsorbents or, *vice versa*, for different adsorbates on a given adsorbent. This is demonstrated by the data in Fig. 2 and the values obtained by linear regression of these data (Table II). This may be explained by the effect of the individual geometry of the different surface complexes. As both the adsorbate and adsorbent have considerable influence on the geometry of the surface complex, better correlations can be expected when only one of them is varied than for a variation of both adsorbent and adsorbate. Consequently, the best correlations are obtained if adsorbents and adsorbates with similar properties and structures are

TABLE II

CORRELATIONS BETWEEN ADSORPTION ENTHALPIES AND ENTROPIES ($\Delta S_{ads} = a\Delta H_{ads} + b$)

Compound	*a* (10^{-3} °K^{-1})	*b* (*J* °$K^{-1}mole^{-1}$)	*Regression coefficient*	*Number of points*
$TeCl_4$ on different surfaces	1.86 ± 0.24	115 ± 19	0.9521	8
$NbCl_5$ on different surfaces	1.28 ± 0.20	74 ± 17	0.9317	8
Different compounds on KCl	0.85 ± 0.11	38 ± 10	0.9532	8
Different compounds on YCl_3	1.14 ± 0.21	67 ± 15	0.9126	8

compared (*eg.*, the adsorption of a given compound on different ionic chlorides)*.

Further information can be obtained from a simplified statistical thermodynamic calculation. The adsorbed molecule has lost three degrees translationel of freedom and gained three degrees of vibrational freedom and an amount of configurational entropy due to the many possibilities of distribution of the adsorbed molecules on the surface. A comparison of calculated and experimental entropies indicates that the surface complex has gained one or more degrees of easily excitable vibrational freedom with frequencies in the range 10^{11}–10^{12} sec^{-1}. This is a striking analogy to the well known gas-phase complexes, where the experimentally determined entropies of formation also indicate easily excitable vibrations. From this point of view, it is obvious that in addition to the strength of the bonding between chemisorbed molecules and the surface, the geometry and properties of both the surface and the chemisorbed molecule have a considerable influence on the various possible movements (vibration, rotations) of the chemisorbed molecules. Unfortunately, the experimental results do not allow a detailed interpretation of the surface complexes, as the errors in the experimental data obscure the fine detail required. Nevertheless, we think that it is reasonable to assume that the surface complexes have structures similar to those of their known complexes in bulk phases, *e.g.*, niobium pentachloride has a six-fold chloride coordination in the surface complex with one of the chloride ligands supplied by the solid phase, and zirconium tetrachloride had a six-fold coordination with two of the chloride ligands supplied by the solid phase.

CONCLUSIONS

The inorganic gas chromatographic method described is promising for the investigation of gas–solid interactions at high temperatures with solid phases with very small surface areas and at extremely low surface coverages. It is possible to determine adsorption (chemisorption) enthalpies and entropies for the formation of inorganic surface complexes, which supplies information that gives a deeper insight into the mechanisms of the chemisorption of metal halides (or oxides, etc.).

The results suggest parallels between these "surface complexes" and the well known gas-phase complexes. It is to be hoped that further investigations of these

* Nevertheless, it should be mentioned that the general similarity of the enthalpy–entropy correlations points towards the uniformity of the chemisorption mechanismn, that is, surface complex formation.

chemisorption phenomena will present results that are as interesting as the similar gas-phase complexes. An understanding of the chemisorption of volatile inorganic halides, oxides and similar compounds is the basis for many applications of the gas–solid chromatography of these compounds. We think that the method presented for the determination of adsorption data provides a useful means of collecting valuable information about the chemisorbed state, but additional methods can be applied in order to obtain further complementary information about the interesting field of "surface complexes" of halides and similar compounds.

ACKNOWLEDGEMENTS

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CHROM. 12,334

tert.-BUTYLPENTAFLUOROPHENYLMETHYLCHLOROSILANE AS A REAGENT FOR THE FORMATION OF HYDROLYTICALLY STABLE ALKYLSILYL DERIVATIVES WITH ELECTRON-CAPTURING PROPERTIES

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SUMMARY

The synthesis of a new silylating reagent, *tert.*-butylpentafluorophenylmethylchlorosilane (*tert.*-buflophemesyl chloride) is described. The reagent forms volatile derivatives of alcohols, phenols, carboxylic acids, thiols and amines which are suitable for gas chromatography with electron-capture detection. The *tert.*-buflophemesyl derivatives are many-fold more stable towards hydrolysis than the flophemesyl derivatives to conditions such as partitioning between organic solvents and acid or base, thin-layer chromatography and column chromatography. The mass spectra of the derivatives show characteristics of both the flophemesyl and *tert.*-butyldimethylsilyl derivatives and are suitable for compound identification.

INTRODUCTION

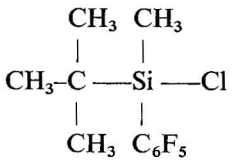
Trimethylsilyl reagents are the most versatile of all derivatization reagents, reacting with a wide range of protonic functional groups and some enolizable ketone groups[1]. Their derivatives are volatile and generally thermally stable, with good separation characteristics, which has led to their widespread use in gas chromatography (GC). However, for use in trace analysis two problems remain. The trimethylsilyl derivatives are not very stable towards hydrolysis, which makes the manipulation of trace amounts in preliminary purification or sample clean-up operations [*e.g.*, thin-layer chromatography (TLC), column chromatography, partition with protonic solvents] likely to result in large and variable losses of derivatives through hydrolysis. Secondly, the search for a silicon-selective and specific detector for trace analysis has not been successful[2].

Owing to their poor stability towards hydrolysis, trimethylsilyl ethers have made little impact as a protecting group in organic synthesis. Recently, higher alkyl homologues than the trimethylsilyl ethers have been used in the synthesis of prostaglandins and nucleosides and shown to be stable to a wide range of chemical reagents (for a review, see ref. 2). Corey and Venkateswarlu[3] found that *tert.*-butyldimethylsilyl ethers were 10^3–10^4 times more stable towards hydrolysis than trimethylsilyl ethers. This

observation has not gone unnoticed by analytical chemists and the last few years have seen an increasing use of reagents of this type in gas chromatography[2].

To improve the detection limit of the silyl ethers, reagents have been devised that contain halomethyl or pentafluorophenyl groups, which can be used with the selective and sensitive electron-capture detector. Halomethylsilanes are vulnerable to nucleophilic attack, resulting in displacement of the halomethyl group and thus limiting their general usefulness[2]. Pentafluorophenyldimethylsilyl reagents (abbreviated to flophemesyl for convenience) have been shown to form derivatives with steroids[4–6], alcohols[7–10] and phenols, amines and carboxylic acids[9,10]. These derivatives can be detected with the electron-capture detector at the picogram to femtogram (10^{-12}–10^{-15} g) level and have similar stabilities towards hydrolysis to trimethylsilyl derivatives.

In an attempt to improve the stability towards hydrolysis of flophemesyl derivatives while maintaining the high response to the electron-capture detector, the new reagent *tert.*-butylpentafluorophenylmethylchlorosilane was synthesized and evaluated. For convenience this reagent has been given the name *tert.*-buflophemesyl chloride:



EXPERIMENTAL

Flophemesyl chloride and *tert.*-buflophemesyl chloride are available from Lancaster Synthesis (St. Leonard Gate, Lancaster, Great Britain) and Alfa Products Division, Ventron Corp. (Danvers, Mass., U.S.A.).

tert.-Buflophemesyl chloride was prepared for the first time in this laboratory by a three-step reaction sequence as described below.

All reactions were carried out under a nitrogen atmosphere in oven-dried glassware and using anhydrous solvents. Methyldichlorosilane was obtained from Sigma (St. Louis, Mo., U.S.A.), *tert.*-butyllithium from Tridom Chemical (Hauppauge, New York, N.Y., U.S.A.), *n*-butyllithium from Aldrich Chemical (Milwaukee, Wisc., U.S.A.) and pentafluorobenzene from PCR Research Chemicals (Gainesville, Fla., U.S.A.).

tert.-Butylmethylchlorosilane

To a solution of methyldichlorosilane (1.0 *M*) in approximately 250 ml of *n*-pentane was added slowly over about 2 h a solution of *tert.*-butyllithium (500 ml, 2 *M*) in *n*-pentane. The mixture was allowed to reflux spontaneously for about 1 h and was then heated to reflux for a further 1 h. The solution was cooled, the precipitate of lithium chloride filtered off and the solvent removed *in vacuo*. The residue was fractionally distilled at atmospheric pressure to give *tert.*-butylmethylchlorosilane, b.p. 89–91 °C, in 56% yield. IR: ν (Si–H) 2160 cm^{-1} (s). NMR: CH_3–Si 0.38 ppm (d), $(CH_3)_3$ C–Si 0.95 ppm (s), Si–H 4.50 ppm (q). MS: *m/e* 136/138 (16.4/5.9) M^+, 93/95 (31.8/15.6), 79/81 (46.2/17.1) [M–*tert.*-Bu]$^+$, 57 (100).

tert.-Butylpentafluorophenylmethylsilane

A solution of *n*-butyllithium (0.5 moles of a 1.6 *M* solution in *n*-hexane) was added slowly with stirring to pentafluorobenzene (0.5 *M*) in diethyl ether (160 ml) at −70 °C. The mixture was allowed to warm up to −20 °C, stirred for 0.5 h and cooled again to −70 °C. To this was added slowly *tert.*-butylmethylchlorosilane (0.5 *M*) in diethyl ether (50 ml) over about 1 h and the mixture allowed to warm up to −20 °C for 1 h and finally to reach room temperature. The precipitate of lithium chloride was filtered off and the solvent removed *in vacuo*. The remaining liquid was distilled to give *tert.*-butylpentafluorophenylmethylsilane, b.p. 105–107 °C at 152 mmHg, in 61% yield. IR: ν (Si–H) 2160 cm^{-1} (s). NMR: CH_3–Si 0.40 ppm (d), $(CH_3)_3C$–Si 0.93 ppm (s), Si–H 4.35 ppm (q). MS: *m/e* 268 (15.1) M^+, 129 (10), 125 (11.5), 81 (21.8) 77 (15.8), 75 (10.2), 63 (22.2), 57 (100) 47 (11.9), 41 (25.3).

tert.-Butylpentafluorophenylmethylchlorosilane

Chlorine was bubbled through a solution of *tert.*-butylpentafluorophenylsilane (0.5 *M*) in cabon tetrachloride (250 ml) in a reaction vessel covered with aluminium foil to exclude light and arranged so that it could be intermittently immersed in an *ice*–salt bath to maintain the temperature below 25 °C. A buffer volume in the gas line prior to the reaction vessel and an auxilliary supply of nitrogen connected to the chlorine were used in order to prevent loss of material due to suck-back. The reaction was rapid and when complete (approximately 1 h, monitored by GC), excess of chlorine was purged with nitrogen, the solvent removed *in vacuo* and the remaining liquid distilled to give *tert*-butylpentafluorophenylmethylchlorosilane, b.p. 99–100 °C at 12 mmHg, in 77% yield. NMR: Si–CH_3 0.73 ppm, Si–$C(CH_3)_3$ 0.97 ppm (s). MS: *m/e* 302/304 (24.9/8.7) M^+, 245/247 (7.9/4.0) [M—*tert.*-Bu]$^+$, 129 (10.2) 125 (9.5), 99 (11.5), 97 (22.9), 81 (13.6), 57 (100).

Preparation of derivatives

For the preparation of derivatives, 20 μl of reagent (flophemesyl chloride or *tert.*-buflophemesyl chloride) and 20 μl of triethylamine were added to 20 μl of ethanol (or other substrate) in 100 μl of acetronitrile or *n*-hexane in a 1.0 ml Reacti-Vial. The mixture was heated at 60 °C until the reaction was complete. For studies on reaction rate and stability towards hydrolysis using *n*-octanol as a representative alcohol, *n*-tridecane for the flophenesyl derivative and *n*-tetradecane for the *tert.*-buflophemesyl derivative were used as internal standards.

Gas chromatography

For gas chromatography, a Perkin-Elmer Sigma 2 gas chromatograph with a flame-ionization and a nickel-63 constant-current electron-capture detector was used. Retention times were determined on a 3 ft. × 1/8 in. I.D. nickel column packed with 5% SE-30 on Gas-Chrom Q (100–120 mesh) with a nitrogen flow-rate of 40 ml min^{-1}. For gas chromatography–mass spectrometry, a Hewlett-Packard 5992A mass spectrometer equipped with a single-stage glass-jet separator and a 6 ft. × 0.4 cm I.D. glass column packed with 3% OV-1 on Gas-Chrom Q (100–120 mesh) and operated with a helium flow-rate of 30 ml min^{-1} was used. Electron-impact mass spectra were recorded at an ionization potential of 70 eV.

RESULTS AND DISCUSSION

Flophemesyl and *tert.*-buflophemesyl chlorides in the presence of an acid acceptor catalyst such as triethylamine show a similar range of application as far as the alcohols, amines, thiols, carboxylic acids and phenols used in this study are concerned. Although we were primarily interested in the determination of alcohols, for which retention time data are given in Table I, derivatives of phenol, 1-propionic acid, 1-butenoic acid, benzoic acid, *n*-butylamine, *sec.*-butylamine, *tert.*-butylamine, aniline, *n*-butanethiol and *n*-dodecanethiol were also easily formed with *tert.*-buflophemesyl chloride. For some derivatives *tert.*-buflophemesyl chloride reacted more slowly than the flophemesyl chloride; whereas the latter gave a complete reaction within 0.25 h at room temperature, the former occassionally required heating at 60° for 0.5–1.0 h. This slower rate of reaction is probably a consequence of the greater steric bulk of the reagent due to the presence of the *tert.*-butyl group. All derivatives showed good separation properties and peak shapes in gas chromatography. For the alcohols listed in Table I, the *tert.*-buflophemesyl derivatives were approximately 2–3 times less volatile than the equivalent flophemesyl derivatives on the non-polar SE-30 column.

TABLE I

RELATIVE VOLATILITIES OF FLOPHEMESYL AND *tert.*-BUTYLPENTAFLUOROPHENYLMETHYLSILYL ALCOHOL DERIVATIVES

Alcohol	*Flophemesyl derivative (R_1) (min)*	*tert.-Butylpentafluorophenylmethylsilyl derivative (R_2) (min)*	R_2/R_1	*Column temperature (°C)*
Methanol	1.2	3.2	2.67	120
Ethanol	1.6	3.2	2.00	120
1-Propanol	2.0	5.8	2.90	120
2-Propanol	1.8	4.8	2.67	120
1-Butanol	3.8	10.1	2.66	120
2-Butanol	3.3	8.5	2.58	120
tert.-Butanol	—	—	—	—
2-Pentanol	1.5	3.7	2.43	150
1-Hexanol	2.9	6.5	2.24	150
1-Heptanol	5.1	10.3	2.00	150
1-Octanol	7.4	16.4	2.22	150
Benzyl alcohol	6.3	13.6	2.16	150
Cyclohexanol	3.4	8.3	2.44	150
Phenol	3.8	9.0	2.37	150
1-Decanol	2.6	5.0	1.92	180
1-Dodecanol	4.9	9.7	1.98	180

Stability towards hydrolysis

The limited stability towards hydrolysis of the trimethylsilyl and flophemesyl derivatives is a disadvantage for some applications in which more than the minimum of sample manipulation or preliminary chromatography of the derivatives by thin-layer or column chromatography, etc., is required. The stabilities of the flophemesyl and *tert.*-buflophemesyl derivatives of *n*-octanol under a variety of hydrolytic conditions are compare in Table II. The *tert.*-buflophemesyl derivative is many times more

TABLE II

CONDITIONS FOR HYDROLYSIS OF *n*-OCTANOL DERIVATIVES

Hydrolysis medium	*Hydrolysis (%)* *	
	Flophemesyl derivative	*tert.-Butylpentafluorophenylmethylsilyl derivative*
Acetonitrile–water (4:1)	70% in 5 min 95% in 30 min	5% in 5 min 5% in 6 h
Acetonitrile–acetic acid (4:1)	78% in 5 min 95% in 2 h 100% in 24 h	5% in 5 min 5% in 2 h 12% in 24 h
Toluene–acetic acid (4:1)	50% in 5 min 95% in 2 h	5% in 5 min 8% in 24 h
Toluene–6 *N* HCl (4:1)	10% in 5 min 17% in 30 min	No change over 24 h
Acetonitrile–6 *N* HCl (2:1)	90% in 5 min 100% in 30 min	5% in 5 min 5% in 24 h
Acetonitrile–6 *N* NaOH (2:1)	95% in 5 min 100% in 30 min	No change over 24 h

* ±5% is approximately the reproducibility of the analytical data.

stable towards hydrolysis than the flophemesyl derivative and is not affected by partitioning an organic solution of the derivative against 6 *M* sodium hydroxide or hydrochloric acid. The *tert.*-buflophemesyl derivative is also remarkably stable towards dissolution in acidic or basic media under conditions in which the flophemesyl derivative is extensively hydrolysed. The *tert.*-buflophemesyl alcohol derivatives have also been submitted to column and thin-layer chromatography without noticeable hydrolysis. The *tert.*-buflophemesyl derivatives can be detected by their absorption of UV light on fluorescent TLC plates.

Sensitivity to the electron-capture detector

A general feature of the operation of the electron-capture detector is the dependence of its sensitivity on temperature[11]. This feature arises as a consequence of the mechanism of the interaction between thermal electrons and the compound being determined[12]. The mechanism can be evaluated by a plot of $\ln AT^{3/2}$ *versus* $1/T$ (A = peak area for a fixed mass of derivative and T = absolute detector temperature). For the flophemesyl derivatives, the mechanism is dissociative and the optimal detector temperature for their determination is the highest that can practically be used[7]. For the *tert.*-buflophemesyl derivative of *n*-octanol, a plot of $\ln AT^{3/2}$ *versus* $1/T$ shows regions of both dissociative and non-dissociative electron-capture (Fig. 1).

For convenience a high detector temperature is preferred as this allows the downtime due to contamination to be minimized as well as providing the highest detector response for the derivatives over the temperature range likely to be used for the gas chromatographic separations. With a detector temperature of 350 °C, the detection limit for the *n*-octanol derivative was $12 \cdot 10^{-12}$ g. This response is of the same order as that obtained with the flophemesyl derivative previously discussed[7,9].

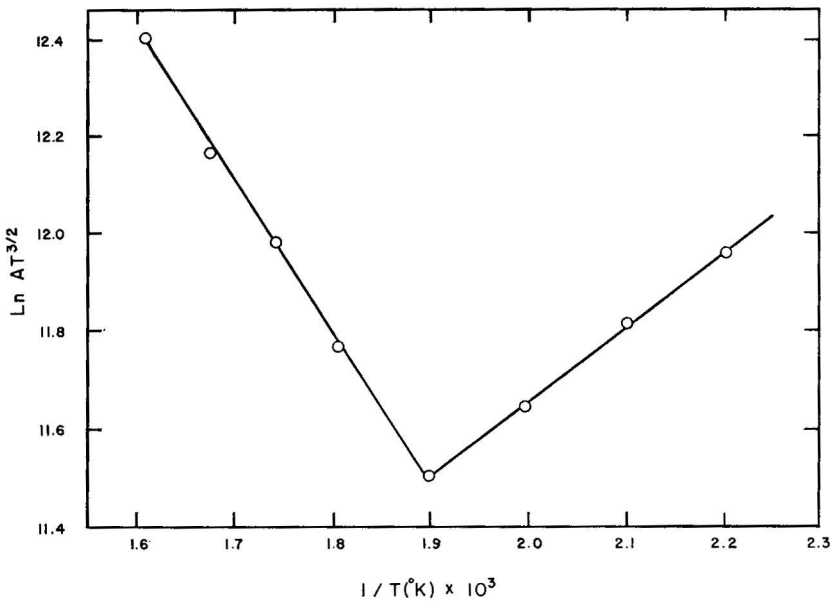


Fig. 1. Temperature dependence of electron-capture detector response to the *tert.*-buflophemesyl derivative of *n*-octanol.

Mass spectra of tert.-buflophemesyl derivatives

The mass spectra of the *tert.*-buflophemesyl derivatives show the combined features of the prominent modes of fragmentation observed with the *tert.*-butyldimethylsilyl[2] and the flophemesyl derivatives[6,10]. The molecular ion is generally weak or absent but the molecular weight can be established by the abundant ion $[M-57]^+$ due to facile loss of the *tert.*-butyl radical (phenols show a loss of 56 rather than 57 a.m.u.). An abundant *tert.*-butyl (*m/e* 57) and allylic ion (*m/e* 41) are also characteristic of the mass spectra. The allylic ion is usually very abundant, in some instances the base peak, and is presumably derived directly from the *tert.*-butyl radical or by exchange of a methyl group in *tert.*-butyl with fluorine on the pentafluorobenzene ring with involvement of the silicon centre and elimination as indicated in Fig. 2 (ref. 13). An exchange of this type is the probable origin of the ion at *m/e* 225 $[C_6F_5Si(CH_3)_2]^+$ observed in some mass spectra. Phillipou[14] has discussed the origin of ions characteristic of the trimethylsilyl group in the mass spectra of *tert.*-butyldimethylsilyl derivatives. Unlike the flophemesyl derivatives, silicon-containing fragments are more prominent although relatively few in number. The mass spectra of the derivatives of *n*-propanol (Fig. 3), 3-butenoic acid (Fig. 4), aniline (Fig. 5), *n*-butanethiol (Fig. 6) and phenol (Fig. 7) are typical of those studied. The phenol and aniline *tert.*-buflophemesyl derivatives, analogous to the flophemesyl derivatives[10], have some characteristic differences in the mass spectra from those of the alcohols, carboxylic acids and thiols. The presence of a prominent silatropylium ion (*m/e* 175) is observed and is derived from the aromatic portion of the derivative by an as yet unknown rearrangement process. Cyclic fluorohydrocarbon ions are not important in the mass spectrum of the phenol derivative. Stable ions of this type (Fig. 2) derived from methyl and fluorine

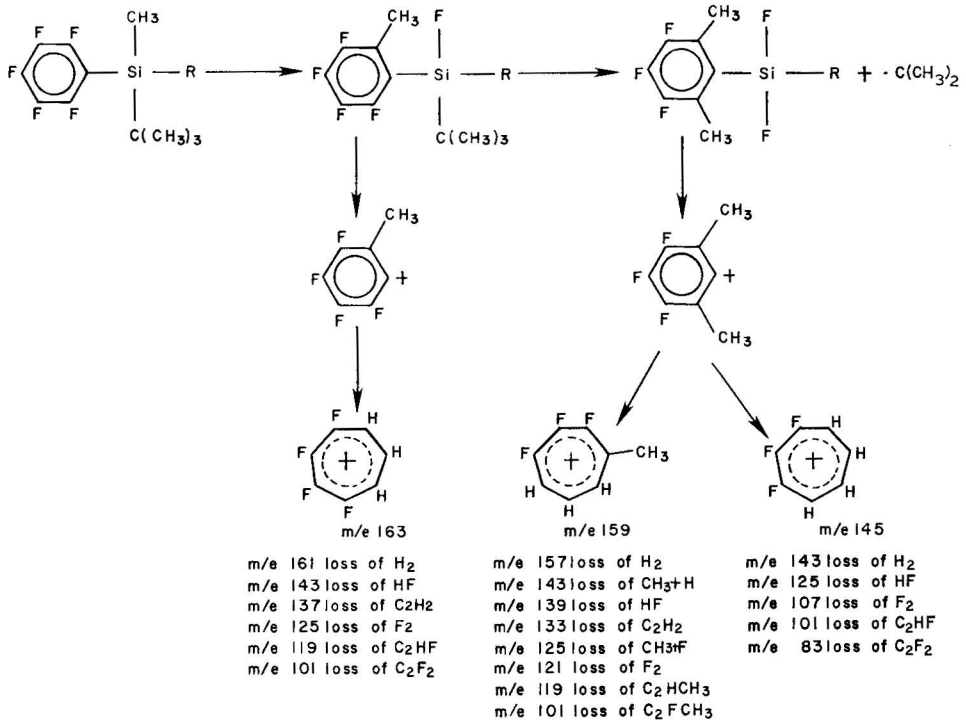


Fig. 2. Schematic representation of the origin of fluorohydrocarbon tropylium ions in the mass spectra of *tert.*-buflophemesyl derivatives.

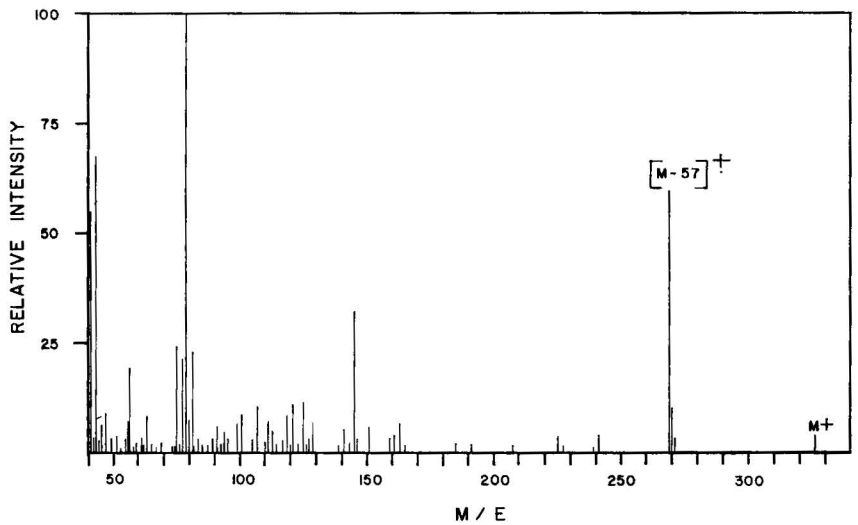


Fig. 3. Electron-impact mass spectra of the *tert.*-buflophemesyl derivative of *n*-propanol.

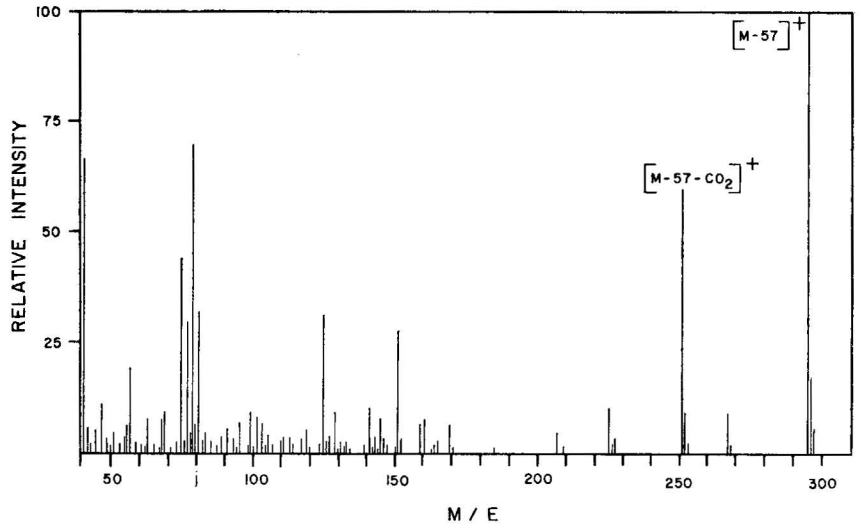


Fig. 4. Electron-impact mass spectra of the *tert.*-buflophemesyl derivative of 3-butenoic acid.

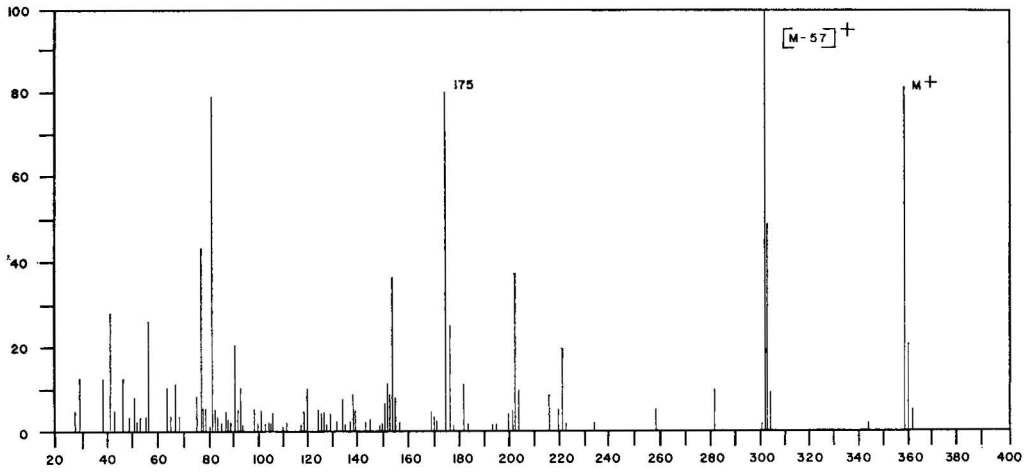
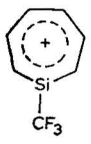


Fig. 5. Electron-impact mass spectra of the *tert.*-buflophemesyl derivative of aniline.

interchange and elimination dominate the remaining features of the alcohol, carboxylic acid, thiol and amine mass spectra. The ions at *m/e* 163, 159 and 145 (formulated as tropylium ions) and their further decomposition by elimination of neutral fragments are of moderate intensity[13].



Silatropylium ion (*m/e* 175)

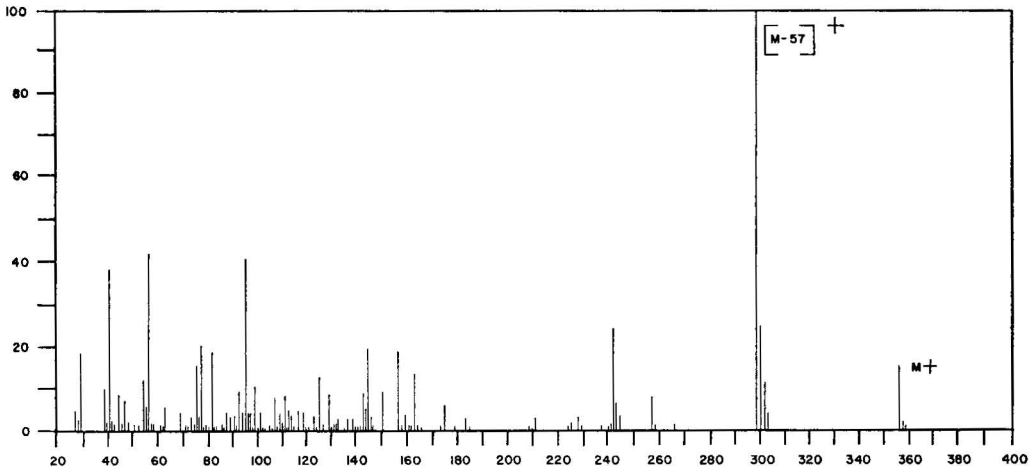


Fig. 6. Electron-impact mass spectra of the *tert.*-buflophemesyl derivative of *n*-butanethiol.

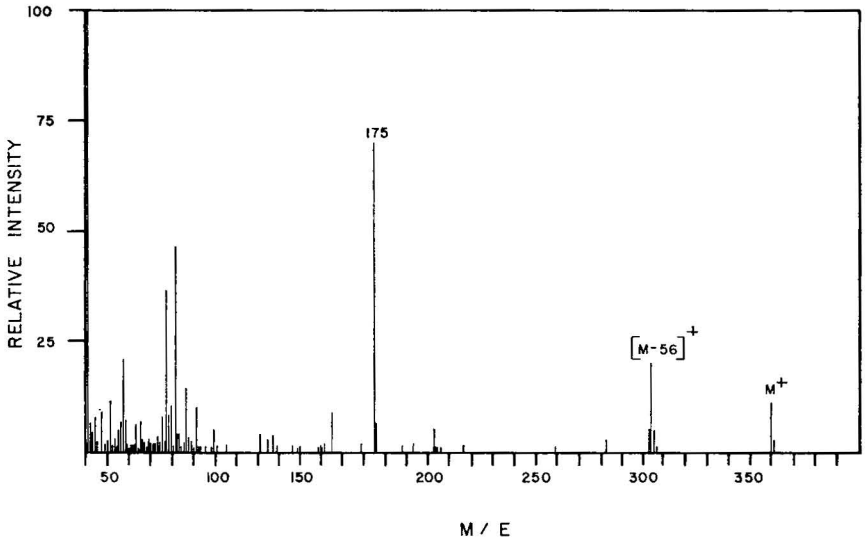


Fig. 7. Electron-impact mass spectra of the *tert.*-buflophemesyl derivative of phenol.

CONCLUSIONS

In comparison with flophemesyl chloride, *tert.*-buflophemesyl chloride reacts less rapidly but to completion under mild conditions, forms derivatives with alcohols that have retention times longer by a factor of 2–3 on GC, has similar sensitivity to the electron-capture detector and forms derivatives that are many times more stable towards hydrolysis. The mass spectra of these derivatives show characteristic features with fragmentation being directed by both the *tert.*-butyl and the pentafluorophenyl group. The greater stability towares hydrolysis of the *tert.*-buflophemesyl derivatives

and their high response to the electron-capture detector should make them very useful for trace level analyses of complex mixtures for which derivative stables to a wide range of chemical conditions are required.

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Journal of Chromatography, 187 (1980) 341–349

CHROM. 12,314

COMPARISON OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOUR OF *s*-TRIAZINE DERIVATIVES ON VARIOUS STATIONARY PHASES

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SUMMARY

The high-performance liquid chromatographic behaviour of 23 *s*-triazine derivatives was studied using LiChrosorb SI-5 as the stationary phase and 2–4% isopropanol in *n*-pentane as the mobile phase. The chromatographic data obtained were correlated with both the structures of the stationary phases and the polarities of the mobile phases that have been used so far for the analysis of *s*-triazines. Some of these relationships can be useful for the identification of *s*-triazines.

INTRODUCTION

The extensive use of *s*-triazine herbicides has stimulated a great interest in rapid and sensitive methods for their analysis. In addition to non-specific spectrophotometric methods[1], paper chromatographic[2], thin-layer chromatographic[3,4] and especially gas chromatographic (GC)[5–12] methods have been successfully applied in this field. GC plays a predominant role in the analysis of *s*-triazine residues. Using specific detectors[9,10], subnanogram amounts of *s*-triazines can be determined in water, soil and grain by GC without prior purification of the extracts. The procedure has been fully automated[11,12].

However, GC fails in some instances: some substances, *e.g.*, cyanatryne, decompose under the conditions used in GC and others, *e.g.*, 2-hydroxy derivatives, are so polar and therefore non-volatile that they cannot be analyzed by GC without prior derivatization. High-performance liquid chromatography (HPLC) seems an attractive alternative to GC as *s*-triazines absorb strongly in the UV region (the molar absorp-

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tivity is $\varepsilon = 3 \cdot 10^4$–$4 \cdot 10^4$ l mol^{-1} cm^{-1} at $\lambda_{max} = 220$–240 nm) and the detection is sufficiently sensitive for the determination of residues over the required concentration range (0.005–0.1 ppm).

HPLC has not been used extensively for the analysis of *s*-triazine derivatives. Published work[13–18] deals chiefly with practical applications, always using the reversed-phase technique. Permaphase ETH (siloxane) has been used as the stationary phase for the analysis of terbutryne[13] and cyanatryne[14,15] with water–methanol as the mobile phase. Vitali *et al.*[16] separated 13 *s*-triazines on chemically bonded Zipax-ODS stationary phase with 5% aqueous methanol as the mobile phase. A reversed-phase system was used by Roth[17] and Jork and Roth[18], who studied the effect of the mobile phase composition (methanol–water) on the separation of *s*-triazines on μBondapak C_{18} stationary phase. They compared various chromatographic methods and critically evalueted their advantages and disadvantages from the point of view of the practical determination of *s*-triazines in plant extracts.

In view of the importance of *s*-triazines in the environment, *s*-triazine herbicides have been studied systematically in our laboratories by various methods, namely spectrophotometry, electrochemistry, GC and GC combined with mass spectrometry[5–8]. The acid–base behaviour and the dissociation constants of a series of *s*-triazines were also studied[10]. The determination of *s*-triazine residues in soil has also been investigated[6].

As some *s*-triazines could not be analysed by GC, HPLC was also used[19,20]. A number of *s*-triazines were not separated satisfactorily uxing the reversed-phase technique and therefore chemically bonded CN[19] and NH_2[20] stationary phases were employed in our earlier work. In this paper, the data obtained on LiChrosorb SI-5 stationary phase are given and the relationship between the structure of *s*-triazines, the structure of the stationary phase and the mobile phase composition is discussed.

EXPERIMENTAL

Materials

The *s*-triazine derivatives were obtained from Ciba-Geigy (Basle, Switzerland). Isopropanol (UV grade) was purchased from Lachema (Brno, Czechoslovakia) and *n*-pentane (p.a. grade) from Merck (Darmstadt, G.F.R.).

Method

The HPLC measurements were carried out on a Varian Model 4100 liquid chromatograph equipped with a Variscan 635 UV detector. The wavelength selected for all measurements was 235 nm. A stainless-steel column (25 cm × 2.2 mm I.D.) was packed with LiChrosorb SI-5, particle size 5 μm. *n*-Pentane containing 2–4% of isopropanol was used as the mobile phase, at a flow-rate of 30 ml/h for a 2% mobile phase and 18 ml/h for 3 and 4% mobile phases. The column was pre-tested by the manufacturer, showing 7800 theoretical plates for *m*-nitraniline at a flow-rate of 60 ml/h with *n*-hexane–dichloromethane–isopropanol (90:10:0.5) as the mobile phase.

The dead retention volume was determined in the same manner as in our previous paper[19] and a value of $V_M = 0.72$ ml was obtained.

RESULTS AND DISCUSSION

s-Triazines have similar chemical structures and offer wide possibilities for correlation of their structural characteristics with chromatographic data. A list of *s*-triazines studied and some of their physico-chemical characteristics are given in Table I.

The adjusted retention volumes, V'_R, and the capacity factors, k', are given in Table II for the three compositions of the mobile phase. Optimal separation was achieved using 2% isopropanol in *n*-pentane. An example of the separation of a mixture of *s*-triazines with this mobile phase is shown in Fig. 1.

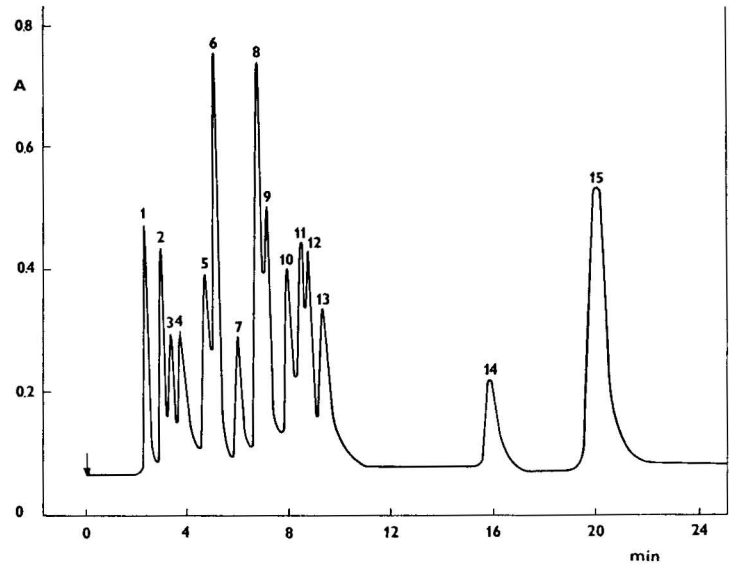

Fig. 1. Separation of a mixture of *s*-triazines on LiChrosorb SI-5 with 2% isopropanol in *n*-pentane as the mobile phase. Flow-rate, 30 ml/h; UV detection at 235 nm; 0–1 a.u.f.s.; 1 cm/min; 1050 p.s.i. Peaks: 1 = chlorazine; 2 = ipazine; 3 = aziprotryne; 4 = prometryne; 5 = terbutylazine; 6 = ametryne; 7 = atrazine; 8 = prometone; 9 = simetryne; 10 = metoprotryne; 11 = simazine; 12 = norazine; 13 = atratone; 14 = noretone; 15 = cyanazine.

To compare the retention behaviour of *s*-triazines on various stationary phases, ratios of the capacity factors (*i.e.*, the relative retentions) were calculated for substances that differ in only a single substituent, either in the alkyl group bound to the amino groups in positions 4 and 6, or in the substituent in position 2. These values are analogous to the functional group retention indices used in GC for identification purposes. The average k'_1/k'_2 values corresponding to the replacement of one substituent in the molecule are given in Table III for all stationary phases so far studied, together with the standard deviations.

On the μBondapak C_{18} reversed phase[17,18] with methanol–water (60:40) as the mobile phase, thiomethyl derivatives are the most strongly retained. Methoxy derivatives cannot be separated from chloro derivatives on this stationary phase (see Table III). *s*-Triazines containing the same number of carbon atoms in the alkyl groups

TABLE I

STRUCTURE AND PROPERTIES OF *s*-TRIAZINES

ε_1, ε_2 = molar absorptivities at absorption maxima (1/mol · cm); $E_{1/2}$ = anodic half-wave potentials (V *vs.* S.C.E.); I_A = retention index on XE-60 at 195°; I_B = retention index on SE-30 + Reoplex 400 at 195°.

Common name	*Substituent*			*Mol.wt.*	ε_1^5	ε_2^5	$E_{1/2}^5$	pK_a^{21-23}	I_A^{24}	I_B^{24}
	2—	*4—*	*6—*							
Chlorazine	Cl	$N(C_2H_5)_2$	$N(C_2H_5)_2$	257.8	—	—	—	1.74	2286	1791
Ipazine	Cl	$NHCH(CH_3)_2$	$N(C_2H_5)_2$	243.6	43,100	4300	1.77	1.99	2378	1907
—	Cl	$NHC(CH_3)_3$	$NHC(CH_3)_3$	257.8	36,000	3300	2.07	—	2418	1938
Trietazine	Cl	NHC_2H_5	$N(C_2H_5)_2$	229.7	44,300	4300	1.77	1.88	2415	1932
Propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	229.7	32,000	3100	1.95	1.85	2462	1973
Terbutylazine	Cl	NHC_2H_5	$NHC(CH_3)_3$	229.7	19,500	1800	2.01	1.94	2504	1999
Atrazine	Cl	NHC_2H_5	$NHCH(CH_3)_2$	215.7	41,000	3900	2.02	1.68	2509	2023
Simazine	Cl	NHC_2H_5	NHC_2H_5	201.5	36,000	3100	1.97	1.65	2553	2078
Norazine	Cl	$NHCH_3$	$NHCH(CH_3)_2$	201.5	—	—	—	1.88	2518	2029
Cyanazine	Cl	NHC_2H_5	$NHC(CH_3)_2CN$	240.7	—	—	—	1.30	—	—
Aziprotryne	SCH_3	$NHCH(CH_3)_2$	N_3	224.3	—	—	—	—	—	—
Prometryne	SCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	241.3	42,000	—	1.83	4.05	2558	2099
Terbutryne	SCH_3	NHC_2H_5	$NHC(CH_3)_3$	241.3	21,200	—	1.78	4.38	2608	2122
Ametryne	SCH_3	NHC_2H_5	$NHCH(CH_3)_2$	227.3	40,000	—	1.77	4.00	2610	2139
Simetryne	SCH_3	NHC_2H_5	NHC_2H_5	213.3	44,400	—	1.79	4.00	2656	2185
Desmetryne	SCH_3	$NHCH_3$	$NHCH(CH_3)_2$	213.3	33,700	—	1.78	3.93	2622	2141
Metoprotryne	SCH_3	$NHCH(CH_3)_2$	$NH(CH_2)_3OCH_3$	271.4	—	—	—	3.98	2983	2457
Prometone	OCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	225.3	40,200	—	1.77	4.28	2350	1916
Terbutone	OCH_3	NHC_2H_5	$NHC(CH_3)_3$	225.3	33,600	—	1.79	4.46	2396	1938
sec.-Bumetone	OCH_3	NHC_2H_5	$NHCH(CH_3)C_2H_5$	225.3	—	—	—	4.23	2470	2015
Atratone	OCH_3	NHC_2H_5	$NHCH(CH_3)_2$	211.3	—	—	—	4.20	2418	1972
Simetone	OCH_3	NHC_2H_2	NHC_2H_5	197.2	38,300	—	1.79	4.17	2435	1990
Noretone	OCH_3	$NHCH_3$	$NHCH(CH_3)_2$	197.2	—	—	—	4.15	2411	1966

TABLE II

RETENTION DATA FOR *s*-TRIAZINES ON LiCHROSORB SI-5 STATIONARY PHASE

Mobile phase: *n*-pentane with 2, 3 or 4% of isopropanol (IPA).

s-Triazine	*2% IPA*		*3% IPA*		*4% IPA*	
	V_R	k'	V_R	k'	V_R	k'
Chlorazine	0.06	0.08	0.02	0.04	0.02	0.04
Ipazine	0.26	0.36	0.20	0.28	0.15	0.20
2-Chloro-4,6-di-*tert.*-butyl-*s*-triazine	0.37	0.50	0.27	0.38	0.24	0.32
Trietazine	0.38	0.50	0.29	0.41	0.26	0.36
Aziprotryne	0.41	0.57	0.32	0.46	0.26	0.35
Prometryne	0.57	0.79	0.47	0.65	0.37	0.50
Terbutryne	0.61	0.85	0.55	0.76	0.40	0.54
Propazine	0.68	0.95	0.67	0.80	0.49	0.68
Terbutylazine	0.86	1.19	0.72	1.00	0.57	0.78
Ametryne	1.02	1.42	0.86	1.21	0.64	0.87
Atrazine	1.38	1.91	1.12	1.57	0.83	1.14
Prometone	1.75	2.43	1.36	1.92	0.98	1.34
Terbutone	1.86	2.58	1.53	2.15	1.07	1.47
Simetryne	1.86	2.58	1.47	2.07	1.08	1.48
Desmetryne	1.95	2.71	1.51	2.13	1.19	1.65
Metoprotryne	2.30	3.19	1.70	2.40	1.27	1.75
Simazine	2.40	3.33	1.86	2.62	1.37	1.88
sec.-Bumetone	2.53	3.51	1.92	2.70	1.49	2.04
Norazine	2.62	3.64	1.95	2.75	1.47	2.01
Atratone	2.88	4.00	2.15	3.02	1.61	2.21
Simetone	4.81	6.68	4.06	5.72	2.69	3.68
Noretone	5.43	7.54	4.09	5.76	3.02	4.14
Cyanazine	7.58	10.53	5.36	7.56	3.91	5.35

bound to the amino groups in positions 4 and 6 are also poorly separated. For example, simetone–simazine–norazine, prometone–propazine and atratone–atrazine–desmetryne–simetryne systems are not separated.

s-Triazines were better separated using a chemically bonded CN phase[19] in combination with both a non-polar and a polar mobile phase. Methoxy derivatives were well separated from chloro and thiomethyl derivatives using *n*-heptane–isopropanol. Whereas at a low isopropanol concentration the retention order is $OCH_3 \gg Cl > SCH_3$, an increase in the retention times of thiomethyl derivatives occurs with increasing polarity of the mobile phase and the retention order changes at a 15% isopropanol concentration ($k'_{Cl}/k'_{SCH_3} < 1$; see Table III). The retention order in the *s*-triazine series with the same substituent in position 2 is unaffected by an increase in the polarity of the mobile phase over the range of isopropanol concentrations studied and depends solely on the spacial shielding of the amino groups by alkyl groups, analogous to the situation in GC[5].

s-Triazines differing in the substituent in position 2 were best separated on a chemically bonded NH_2 phase[20]. *s*-Triazines can form hydrogen bonds of the phase–N–H$\cdots$N–triazine or phase–N$\cdots$H–N–triazine type with the NH_2 phase, which are responsible for an increased selectivity of the stationary phase. The strength of the hydrogen bonds depends on the mutual steric accessibility of the sites.

TABLE III

DEPENDENCE OF k_1/k_2 RATIOS ON VARIOUS STATIONARY PHASES ON THE MOBILE PHASE POLARITY

Mobile phases: A = 60% methanol in water; B_1, B_2, B_3 = 2, 3, 4% isopropanol in *n*-pentane; C_1, C_2, C_3 = 1.5, 5, 15% isopropanol in *n*-heptane; D_1, D_2, D_3, D_4 = 0.25 *M* methanol, 0.25 *M* ethanol, 0.25 *M* isopropanol, 0.25 *M tert.*-butanol in *n*-heptane; E_1, E_2, E_3, E_4, E_5 = 0.131, 0.196, 0.25, 0.261, 0.323 *M* isopropanol in *n*-pentane.

R_1/R_2	*Parameter**	C_{18}[17,18]	*SI-5*			*CN*[19]							NH_2[20]				
		A	*B1*	*B2*	*B3*	*C1*	*C2*	*C3*	*D1*	*D2*	*D3*	*D4*	*E1*	*E2*	*E3*	*E4*	*E5*
Et/H	$\bar{x}$	3.96	0.17	0.14	0.16	0.19	0.22	0.26	0.21	0.20	0.18	0.17	0.15	0.17	0.18	0.19	0.21
	s	0.39	0.02	0.04	0.04	0.01	0.02	0.02	0.01	0.01	—	—	0.02	0.01	0.01	0.01	0.01
Et/Me	$\bar{x}$	1.57	0.53	0.55	0.54	0.62	0.74	0.80	0.61	0.58	0.56	0.58	0.53	0.55	0.56	0.56	0.56
	s	0.01	0.01	0.03	0.02	—	—	—	0.01	0.01	0.01	0.05	0.01	0.01	0.01	0.01	0.03
Et/iPr	$\bar{x}$	0.64	1.73	1.73	1.70	1.60	1.46	1.51	1.52	1.56	1.61	1.62	1.68	1.66	1.65	1.65	1.64
	s	0.01	0.19	0.18	0.05	0.09	0.26	0.09	0.12	0.10	0.07	0.07	0.16	0.12	0.10	0.10	0.11
Et/tBu	$\bar{x}$	—	2.70	2.69	2.52	2.39	1.90	1.98	2.16	2.27	2.36	2.42	2.61	2.51	2.41	2.39	2.34
	s	—	0.28	0.16	0.15	0.07	0.42	0.10	0.17	0.13	0.04	0.01	0.11	0.01	0.06	0.11	0.11
iPr/Me	$\bar{x}$	2.46	0.29	0.31	0.32	0.41	0.59	0.70	0.41	0.38	0.36	0.35	0.32	0.33	0.34	0.34	0.36
	s	0.04	0.03	0.02	0.03	—	—	—	0.06	0.04	0.02	0.03	0.02	0.03	0.03	0.03	0.02
OCH_3/Cl	$\bar{x}$	0.98	2.18	2.14	1.96	2.84	2.73	2.61	1.29	1.32	1.39	1.42	1.40	1.39	1.38	1.37	1.38
	s	0.02	0.22	0.17	0.06	0.16	0.09	0.12	0.05	0.05	0.05	0.14	0.05	0.05	0.06	0.05	0.05
OCH_3/SCH_3	$\bar{x}$	0.58	2.86	2.75	2.57	3.57	2.86	2.32	1.91	1.99	2.06	2.14	2.18	2.15	2.08	2.05	2.00
	s	0.01	0.20	0.17	0.09	0.12	0.06	0.02	0.17	0.16	0.08	0.11	0.18	0.18	0.15	0.15	0.06
Cl/SCH_3	$\bar{x}$	0.59	1.32	1.33	1.32	1.29	1.29	0.89	1.42	1.46	1.46	1.47	1.56	1.53	1.49	1.48	1.44
	s	0.01	0.08	0.09	0.08	0.10	0.22	0.04	0.13	0.09	0.06	0.08	0.08	0.08	0.06	0.08	0.06

* $\bar{x}$ = mean k_1/k_2 value; *s* = standard deviation.

Compared with the NH_2 phase, methoxy derivatives are more strongly retained on silica gel. The bonds of the –Si–OH groups to the π-electrons of the *s*-triazine ring or to the electron pairs on the nitrogen atoms in the substituent groups can play a role with silica gel. As the dependence on the ring electron density is not obeyed (the retention volume order is $OCH_3 > Cl > SCH_3$, whereas the total group electronegativity order, expressed in terms of pK_a or σ_m, is $Cl > SCH_3 \geqslant OCH_3$), a predominant effect of silica gel on the ring π-electrons can be excluded, otherwise the ring would not be sterically affected by substitution and would exhibit only an induction effect. As far as the bonds of the silica gel OH groups to the electron pairs on the nitrogen atoms are concerned, the opposite effects of the substituent donor ability (*tert.*-butyl > isopropyl > ethyl > methyl) and steric accessibility (an increase in the entropic factor in the equilibrium) play a role, the latter predominating. Hence it can be assumed for the Si–OH···N–triazine bond that the equilibrium constant for the complex formation will decrease with increased branching of the alkyl group, which has been confirmed experimentally.

From the point of view of the effect of substituents of types other than amine (with the possibility of hydrogen bonding), an increase in the retention volumes in the order $OCH_3 \gg SCH_3 \approx Cl$ can be expected. However, these groups affect the electron density in the whole conjugated system in the order $Cl > SCH_3 \gg OCH_3$. The resultant retention order for substances differing only in the substituent in position 2 is $OCH_3 \gg Cl > SCH_3$.

s-Triazines with the same number of carbon atoms in the amino alkyl groups in positions 4 and 6, *e.g.*, the pairs norazine–simazine and noretone–simetone, are best

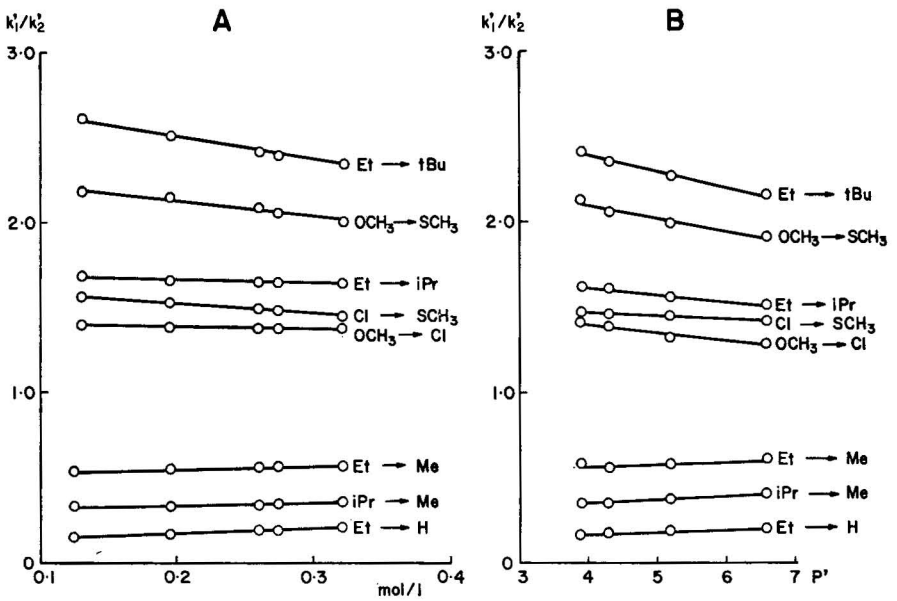

Fig. 2. Dependence of the capacity factors, k'_1/k'_2, on the isopropanol concentration (A) and the alcohol polarity, P', (B) for LiChrosorb NH_2 stationary phase. $P' = 6.6$ for methanol, 5.2 for ethanol, 4.3 for isopropanol and 3.9 for *tert.*-butanol).

separated on silica gel, whereas their separation on the NH_2 phase is difficult and on μBondapak C_{18} impossible.

As can be seen from Table III, the k_1'/k_2' values depend both on the stationary phase and on the polarity of the mobile phase. With increasing isopropanol concentration (Fig. 2A) or with increasing alcohol polarity (expressed in terms of the polarity index, P', see Fig. 2B)[25], the k_1'/k_2' ratios decrease (on substitution of ethyl for isopropyl, ethyl for *tert.*-butyl, OCH_3 for Cl, Cl for SCH_3 and OCH_3 for SCH_3), or increase (on substitution of ethyl for methyl, ethyl for hydrogen and isopropyl for ethyl) and approach unity, which means that separation is not achieved at a certain iopropanol concentration. The dependence shown in Fig. 2 for the LiChrosorb NH_2 phase also hold for the CN phase and silica gel (see Table III).

The k_1'/k_2' ratios given in Table III can be used to predict the retention behaviour of *s*-triazines that were not available. For example, the capacity ratios were calculated for ipatone (0.78), ipatryne (0.25), trietatone (1.09) and trietatryne (0.35) on LiChrosorb SI-5 and with 2% isopropanol in *n*-pentane as the mobile phase.

The constancy of the k_1'/k_2' ratio can be utilized in correlations of the retention data with the number of carbon atoms in the alkyl groups bound to the amino groups in positions 4 and 6. In the series of *s*-triazines obtained by substitution of a single kind of substituent, linear dependences of log k' on the number of carbon atoms in the alkyl groups were obtained, and some of them are depicted in Fig. 3. The log k' dependences on the number of carbon atoms are non-linear in series of *s*-triazines that differ only in the substituent in position 6, because of different spacial shielding of the alkyl groups. Dependence similar to those given in Fig. 3 could also be employed for the identification of *s*-triazines.

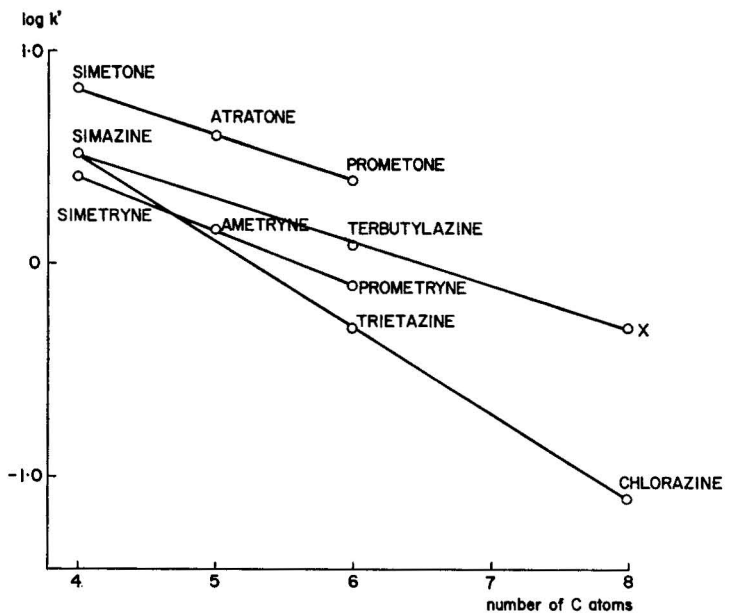

Fig. 3. Dependence of log k' on the number of carbon atoms in the alkyl groups bound to *s*-triazine amino groups in positions 4 and 6. X = 2-chloro-4,6-di-*tert.*-butyl-*s*-triazine.

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SURFACE AFFINITY CHROMATOGRAPHIC SEPARATION OF BLOOD CELLS

I. SEPARATION OF HUMAN AND RABBIT PERIPHERAL GRANULOCYTES, LYMPHOCYTES AND ERYTHROCYTES USING POLYETHYLENE GLYCOL-BONDED COLUMN PACKINGS

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SUMMARY

Two different types of column packings with bonded polyethylene glycol (PEG) as the stationary phase, PEG 20M-bonded Porasil AX and bisoxirane-coupled PEG 20M–Sepharose 6B, were prepared for chromatographing human and rabbit peripheral blood cells. The best separation, especially of granulocytes and lymphocytes (separation factor 2.65), was obtained with the latter packing and a phosphate-buffered solution (pH 7.5) of 8% (w/w) dextran as the mobile phase.

The selectivity of the column for blood cells depended on the concentration and molecular weight of dextran and on the ionic composition of the electrolytes in the mobile phase. The recoveries of human granulocytes, lymphocytes and erythrocytes by this chromatographic system were about 67, 82 and 50%, respectively.

INTRODUCTION

Improvements in fractionation methods used in the investigation of cells have long been required. In column chromatographic methods, which seem to have potential for cell fractionation, many practical difficulties are experienced, such as the adsorption of a large number of cells on the column. The subsequent separation of blood cells according to their adherence characteristics is exemplified by the technique of adherence chromatography, which uses a column of siliconized glass beads about 200 μm in diameter[1]. The glass bead column does not separate human lymphocytes from erythrocytes. The recovery from the column is about 60–70% for small lymphocytes and 35–45% for polymorphonuclear leukocytes[1]. Several reports have described the chromatographic behaviour of certain types of mammalian cells on columns filled with agarose beads[2], poly(methyl methacrylate) beads[3], cross-linked dextran beads[3,4], etc. However, only a few papers have described the adherence of cells to packing materials[3,5].

On the other hand, the use of immiscible aqueous two-phase systems for the fractionation of cell particles and biological macromolecules was established by Albertsson[6]. Of various aqueous polymeric two-phase (APTP) systems, partition in aqueous dextran–polyethylene glycol (PEG) two-phase systems is useful in many instances, these containing a PEG-rich top and a dextran-rich bottom phase.

The manual operation of a counter-current device is tedious; separation with immiscible aqueous systems is particularly slow as the solutions used are viscous. Such systems can be rendered isotonic by incorporation of inorganic salts and are suitable for partition and separation by a counter-current distribution apparatus of a special design for the partitioning of cell particles and cell organelles.

It is clear that partition in APTP systems could be carried out in a column if one of the polymer phases were held immobile. By binding one of the polymers to the support material in liquid chromatography, it should be possible to achieve partition of cell particles between the bonded stationary phase and the mobile phase in a manner similar to the APTP systems. In this work, we have attempted to prepare column packing materials with chemically bonded PEG as the stationary phase. By the use of such a packing, fractionation of peripheral blood cells from human and rabbit could be carried out by means of column chromatography, based on the partition between chemically bonded PEG and dextran constituting the aqueous mobile phase.

EXPERIMENTAL

Materials

Porasil AX (particle size 75–125 μm) and Corasil II (particle size 37–75 μm) (Waters Assoc., Milford, Mass., U.S.A.), epoxy-activated Sepharose 6B, dextran T40 (weight-average molecular weight $M_w = 40{,}000$) and dextran T500 ($M_w = 500{,}000$) (Pharmacia, Uppsala, Sweden) were commercial products. Polyethylene glycol, number-average molecular weight $M_n = 6000$-7500 and 15,000-20,000, was purchased as PEG 6000 and 20M, respectively. Other reagents were of analytical reagent grade.

Instruments

A Hitachi Model 034 liquid chromatograph, equipped with a Model 0037 multi-wavelength effluent monitor (Hitachi, Tokyo, Japan), was used for chromatography. An LKB 2112 RediRac fraction collector (LKB, Bromma, Sweden) was used for fractionation of eluates. A Coulter Model D counter (Coulter Electronics, Harpenden, Great Britain) was used for counting the number of blood cells.

Preparation of PEG-bonded column packings

PEG-bonded silica beads. Porasil AX and Corasil II were used as supports for the stationary phases. Both types of silica bead were dried for 12 h at 120° before use. Replacement of the surface hydroxyl groups with chloro groups was carried out with titanium tetrachloride or silicon tetrachloride, according to the procedure of Locke *et al.*[7]. The chlorinated silica beads were dried over phosphorus pentoxide under reduced pressure at room temperature. A mixture of 15 g of melted PEG 6000 or 20M and 0.3 g of metallic sodium was heated for 2–3 h at 140° until the sodium had dissolved. To this melt, 2.5 g of chlorinated silica beads were added and the mixture was heated for about 15 h at 180° until the aqueous solution of an aliquot of this reaction mixture did not show a red colouration with phenolphthalein. The product

was washed repeatedly with tetrahydrofuran until the washings no longer had the blue colour due to peroxochromic acid [oxodiperoxochromium(VI)] and PEG[8] *. Either PEG 6000- or 20M-bonded Corasil II and/or Porasil AX was dried under vacuum at 130° for 48 h, and the carbon and hydrogen contents were determined by elemental analysis. The X-ray diffraction patterns showed characteristic diffractions due to PEG at $\theta = 25$, 37.7 and 47.8.

Bisoxirane-coupled PEG-Sepharose 6B. One gram of epoxy-activated Sepharose 6B was washed on a sintered-glass filter funnel (G3) with *ca.* 100 ml of water and then mixed with PEG solution. The solution was prepared by dissolving 10 g of either PEG 6000 or 20M in 20 ml of a solution adjusted to a pre-determined pH of 9.0, 10.0 or 11.0 with sodium hydrogen carbonate–sodium carbonate buffer, and pH 12.0 and 13.0 with sodium hydroxide solution. The reaction mixture was shaken for 16 h at 32.5°, 40° and 45° in a thermostat. The coupled product was washed on a sintered-glass filter funnel successively with water, 0.1 *M* sodium tetraborate–boric acid–0.5 *M* sodium chloride buffer (pH 8.0), and 0.1 *M* sodium acetate–acetic acid–0.5 *M* sodium chloride buffer (pH 4.0) until PEG was no longer detected in the filtrate[8]. The product, suspended in 25 ml of 1 *M* 2-aminoethanol, was shaken for 12 h at room temperature, thoroughly washed with water and dried under vacuum at 105° for 48 h. The amount of PEG bonded to epoxy-activated Sepharose 6B was determined by spectrophotometry[9] from the amount of unchanged PEG in the combined solution of the filtrate of the reaction mixture and the washings.

Collection of blood cells

Human blood was drawn from normal male adult donors by venepuncture and heparin (Shimizu Seiyaku, Shizuoka, Japan) added (0.05 ml of a 1000 U/ml solution per 10 ml of the blood). Rabbit defibrinated blood was purchased from Nippon Seibutsu Zairyo Centre, Tokyo, Japan.

Isolation of blood cells. Siliconized glassware was used in all procedures.

For erythrocytes, blood was centrifuged at 500 *g* for 10 min, and the supernatant and buffy coat layer were removed. The cells were washed three times with saline and packed by centrifugation.

For granulocytes, sodium metrizoate–dextran sedimentation technique of Bøyum[10] was used. A mixture of 10 ml of blood and 2 ml of 0.9% physiological saline was carefully layered on 10 ml of a mixed solution of 32.8% (w/w) sodium metrizoate (N-methyl-3,5-diacetamido-2,4,6-triiodobenzoate)–6% (w/w) dextran T500 (1:2). The system was allowed to stand for 1–2 h until the sedimentation of erythrocytes was complete. The supernatant plasma was pipetted off. The supernatant was centrifuged at 130 *g* for 10 min and the precipitated cells were then washed three times with 5-ml volumes of saline and centrifuged. The granulocyte preparation contained a variable amount of contaminating erythrocytes.

* Polyethylene glycols are high-molecular-weight ethers and are able to stabilize CrO_5 [oxodiperoxochromium(VI)] through addition. This stabilization permits the detection of polyethylene glycols. *Procedure.* To one drop of polyethylene glycol solution, one drop of 5% K_2CrO_4, followed by a drop of 4% H_2SO_4 containing 3% H_2O_2 is added. A blank test without polyethylene glycol is also carried out. In the blank the blue colour initially obtained changes to green within a few minutes. The possitive response is indicated through the persistence of the blue colour during 15–20 min.

For lymphocytes, a sodium metrizoate–Ficoll sedimentation technique based on that of Thorsby and Bratlie[11] was used. A 5-ml volume of blood was defibrinated by rotating the test-tube vertically for 10 min with five glass spheres of 5 mm diameter, and 5 ml of the defibrinated blood were mixed with an equal volume of saline in a centrifuge tube and carefully layered on 7.5 ml of Lymphoprep [a mixture of 9.6% (w/v) sodium metrizoate and 5.6% (w/v) Ficoll; Nyegaard, Oslo, Norway]. The tube was centrifuged at exactly 400 *g* for 30–40 min. The lymphocyte layer was separated and the cells were diluted with about 2 ml of saline and washed and centrifuged at 500 *g* for 10 min. The precipitated cells were washed and centrifuged three times with 5-ml volumes of saline. The contamination in the lymphocyte suspension of erythrocytes was usually between 1 and 5% of the total number of cells.

Chromatography

Four eluents were used, as follows: 0.03 *M* sodium dihydrogen orthophosphate–0.03 *M* disodium hydrogen orthophosphate buffer (pH 7.5) containing 4.5% (w/w) of dextran T500 and 0.1 *M* sodium chloride (eluent I); 0.045 *M* sodium dihydrogen orthophosphate–0.045 *M* disodium hydrogen orthophosphate buffer (pH 7.5) containing 4.5% (w/w) of either dextran T500 (eluent II) or T40 (eluent III); and 0.045 *M* sodium dihydrogen orthophosphate–0.045 *M* disodium hydrogen orthophosphate buffer (pH 7.5) containing 8.0% (w/w) of dextran T40 (eluent IV).

A jacketed glass column (10.5 × 0.9 cm I.D.), filled with PEG 20M-bonded Porasil AX, and a column (25 × 0.9 cm I.D.) filled with bisoxirane-coupled PEG 20M–Sepharose 6B were used. The packing materials were suspended in each of the above eluents, and the columns were filled with the slurried packings. The columns were thoroughly washed with the eluent to equilibrate the chemically bonded phases by the use of a reciprocating or a peristaltic pump.

The total amount of granulocytes, lymphocytes and erythrocytes prepared as above was suspended in 0.6 ml of the eluent used, 0.5 ml of the cell suspension containing $13.9 \cdot 10^4$–$19.8 \cdot 10^4$ of granulocytes, $9.9 \cdot 10^4$–$15.8 \cdot 10^4$ of lymphocytes or $2.4 \cdot 10^4$–$3.2 \cdot 10^4$ of erythrocytes was loaded in the column, and the column was eluted with each of the eluents. These operations were performed at 4° by circulation of cold water through the column jacket. A flow-rate of 1.5–12 ml/h was maintained by the use of a pump. The absorbance of the eluate at 230, 260 and 570 nm was monitored continuously with a multi-wavelength effluent monitor. The fractions were collected in test-tubes every 15 min (for PEG 20M-bonded Porasil AX) or 30 min (for bisoxirane-coupled PEG 20M-Sepharose 6B); in the former instance the volume was about 2 ml and in the latter about 1 ml. An aliquot of each fraction was diluted with 5 ml of Isoton (aqueous electrolyte diluent for blood cell counting; Coulter Diagnostics, Hialeah, Fla., U.S.A.) and the number of granulocytes, lymphocytes and erythrocytes was counted with a Coulter counter. The recovery of the eluted cells was calculated from the combined number in each fraction against the cells loaded on the column.

RESULTS

PEG bonded-phase column packings

Chlorination of the surface silanol hydroxyl groups of the silica support material Porasil C was investigated by Locke *et al.*[7]. In this study, chlorination of the surface silanol hydroxyl groups according to the method of Locke *et al.*[7] was compared

for the two types of spherical siliceous supports: deactivated Porasil AX, with totally porous silica particles, and Corasil II, with spherical glass particles that have a double layer of porous silica. Chlorination of silanol hydroxyl groups was carried out with both titanium tetrachloride and silicon tetrachloride in *n*-pentane as the solvent. After pyrolytic decomposition of the reaction products with sodamide, the chlorine content of the products was determined by argentimetric titration. As shown in Table I, the chlorine content of chlorinated Porasil AX was higher than that of Corasil II with both chlorination reagents. When these chlorinated siliceous supports were allowed to react with PEG 6000 at 180° for 15 h, the carbon content of the PEG-bonded Porasil AX was also higher than that of Corasil II.

TABLE I

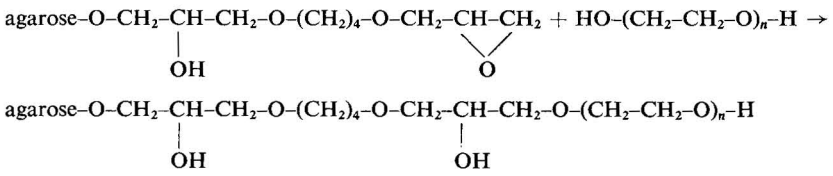
CHLORINE AND CARBON CONTENTS OF CHLORINATED AND PEG-BONDED SPHERICAL SILICA BEADS

Samples: silica beads chlorinated with (A) $TiCl_4$ or (B) $SiCl_4$; PEG-bonded silica beads with (C) PEG 6000 or (D) PEG 20M.

Starting material	*Chlorine content (mequiv. per 100 mg)*		*Carbon content (%)*	
	Sample A	*Sample B*	*Sample C*	*Sample D*
Porasil AX	26.89	12.86	3.17	3.59
Corasil II	0.91	0.61	0.61	

On the basis of this result, Porasil AX was condensed with PEG of two different molecular weights, PEG 6000 and 20M, at 180° for 15 h. The carbon contents are shown in Table I for the products from two types of silica beads: PEG 6000 or 20M bonded on porous beads (Porasil AX), and PEG 6000 bonded on solid core beads (Corasil II). These results also demonstrate the greater capacity of totally porous beads for binding with PEG. Because the carbon content of the former was five times greater than that of the latter, PEG 20M-bonded Porasil AX (PEG-bonded Porasil) was used as the column packing for subsequent liquid chromatography of blood cells.

As a soft gel type of packing material, PEG 20M was coupled to bead-shaped agarose through the mediation of bisoxirane [1,4-bis(2,3-epoxypropoxy)butane]. A terminal hydroxyl group in PEG 20M was directly coupled to an active oxirane group of epoxy-activated Sepharose 6B, and a stable hydrophilic uncharged ether linkage was formed between PEG and Sepharose 6B. The reaction is as follows:



As the coupling conditions depend greatly on the properties of the ligand and the functional group to be coupled[12], suitable conditions for the oxirane coupling of PEG 20M with epoxy-activated Sepharose 6B were investigated at 32.5°, 40.0° and 45.0° for 16-h periods in aqueous solution adjusted to pH 9.0, 10.0, 11.0, 12.0 or 13.0. The amount of PEG 20M coupled was determined from the recovered amount of unchanged PEG in the reaction mixture and washings of the products. As shown in

TABLE II

RETENTION VOLUMES AND SEPARATION FACTORS OF GRANULOCYTES, LYMPHOCYTES AND ERYTHROCYTES

Column: PEG 20M-bonded Porasil AX (10.5 × 0.9 cm I.D.)

Blood cells	*Mobile phase**							
	I		*II*		*III*		*IV*	
	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*
Human:								
Erythrocytes	18.0	1.50	42.0	3.50	14.0	(1.56)**	14.0	1.33
Granulocytes	12.0	1.20	14.0	1.17	7.0	1.29	10.5	1.67
Lymphocytes	10.0		12.0		9.0		17.5	
Rabbit:								
Erythrocytes			44.0	(3.57)**	33.0	5.50	14.3	(1.14)**
Granulocytes	14.3	1.05	10.5	1.17	6.0	1.00	12.3	1.04
Lymphocytes	15.0		12.3		6.0		12.5	

* Mobile phase I contained 4.5% (w/w) of dextran T500, 0.03 *M* NaH_2PO_4, 0.03 *M* Na_2HPO_4 and 0.1 *M* NaCl (pH 7.5); mobile phase II contained 4.5% (w/w) of dextran T500, 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5); mobile phase III contained 4.5% (w/w) of dextran T40, 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5); mobile phase IV contained 8.0% (w/w) of dextran T40, 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5).

** Separation factor against lymphocytes.

Fig. 1, the optimum coupling condition for PEG 20M was a 16-h period at 40.0° in solution of pH 12.0. The content of PEG coupled under these optimal condition as the bonded stationary phase was 15.7 μmol per gram of dry powder (314 mg per gram of dry powder). In addition, the amount of PEG 20M bound was also expressed by means of the carbon and nitrogen content of the products, the latter obtained from the hydroxyethylamino substituent which was introduced by treatment with 1 *M* 2-aminoethanol after the coupling, for the purpose of blocking residual free epoxy groups. The analysis of the dried product prepared under optimal conditions was C 47.09, H 6.64 and N 0.30%. The carbon content was the highest and the nitrogen content the lowest compared with the contents in the products prepared according to the bisoxirane coupling conditions shown in Fig. 1. On the other hand, epoxy-activated Sepharose 6B hydrolysed under optimal coupling conditions gave values of C 45.08, H 6.69 and N 0.46% as a control.

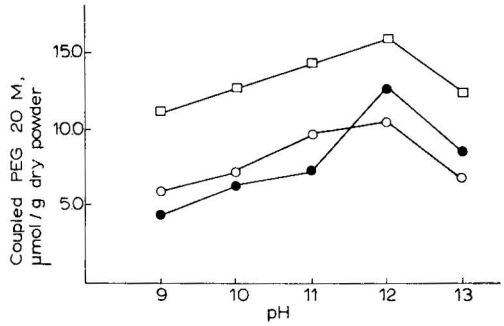


Fig. 1. Dependence of coupling of PEG 20M to epoxy-activated Sepharose 6B on pH and temperature. Carbonate–hydrogen carbonate buffers were used in the pH range 9–11 and sodium hydroxide solution in the range pH 12–13. PEG 20M concentration, 500 mg/ml. Temperature: ○, 45°; □, 40°; ●, 32.5°.

Chromatography of peripheral blood cells

Each suspension of granulocytes, lymphocytes and erythrocytes from human and rabbit peripheral blood was chromatographed on chemically bonded PEG stationary phases. Four kinds of mobile phases containing either dextran T40 orT500 were used, with isotonic phosphate buffers containing either sodium chloride or no salt except as a buffer. One of the principal advantages of these mobile phases was that the fundamental behaviour of some of the blood cells in APTP systems is known[13,14].

Table II shows retention volumes and separation factors for human and rabbit granulocytes, lymphocytes and erythrocytes eluted independently from PEG-bonded Porasil column. It can be readily seen that the order of elution of human granulocytes and lymphocytes was reversed by the use of dextran T500 and T40 as the mobile-phase. Decreasing the molecular weight of dextran would reverse the elution order with both kinds of human cells, whereas it gave similar retention volumes for rabbit cells.

The increased retention volume of human lymphocytes was observed from the increase in dextran concentration in the mobile phase from 4.5 to 8.0% (w/w). The delayed elution of human lymphocytes resulted in an improved separation from granulocytes. Of the four mobile phases used, eluent IV, containing 8% (w/w) of dextran T40, produced the highest separation factor for human granulocytes and

TABLE III

RETENTION VOLUMES AND SEPARATION FACTORS OF GRANULOCYTES, LYMPHOCYTES AND ERYTHROCYTES

Column: bisoxirane-coupled PEG 20M–Sepharose 6B (25 × 0.9 cm I.D.)

Blood cells	*Mobile phase**							
	I		*II*		*III*		*IV*	
	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*
Human:								
Erythrocytes					5.4	1.66	3.8	2.18
Granulocytes	12.0	1.25	12.1	1.49	9.0	2.33	8.3	2.65
Lymphocytes	15.0		18.0		21.0		22.0	
Rabbit:								
Erythrocytes	22.0	1.24	17.0	1.24	5.0	4.20	4.5	(2.33)**
Granulocytes	31.0	2.17	21.0	3.56	21.0	2.24	22.0	2.10
Lymphocytes	14.3		5.6		47.0		10.5	

* Mobile phases I–IV as in Table II.

** Separation factor against lymphocytes.

lymphocytes. The separation factor for both kinds of rabbit blood cells was lower than that for human cells, and the highest value was obtained by the use of eluent II. The separation factor for human erythrocytes and lymphocytes was 1.56 with eluent III and 3.57 for those of the rabbit with eluent II. Because of irreversible adsorption on the column, the best recoveries of human granulocytes and lymphocytes were not more than 24% and 16%, respectively, of the number of these cells applied, when using eluent III. Similarly, 23% of rabbit granulocytes and 14% of lymphocytes were recovered by using eluent IV.

Table III shows the retention and separation of human and rabbit granulocytes, lymphocytes and erythorcytes chromatographed independently on bisoxirane-coupled PEG–Sepharose 6B (PEG-bonded Sepharose) column using the same eluents. In eluent I the affinity of lymphocytes for the mobile phase increased and the separation from granulocytes was not good. Eluent II, with a similar composition to eluent I but without sodium chloride, gave a better separation. Human granulocytes and lymphocytes were eluted from the column with increasing retention volumes with every eluent. A decrease in the molecular weight of dextran in the mobile phase from T500 to T40 appreciably increased the retention of human lymphocytes. The separation factor against granulocytes was improved by the delayed elution of lymphocytes. When the concentration of dextran T40 was increased from 4.5 to 8.0% (w/w), the elution of lymphocytes was retarded and the separation factor against granulocytes became 2.65. The separation factor of human erythrocytes against granulocytes was 1.66 with eluent III and 2.18 against granulocytes with eluent IV. With eluents I and II the erythrocytes aggregated and were adsorbed on the column.

On the other hand, eluents I, II and IV reversed the order of elution of rabbit granulocytes and lymphocytes in contrast to that of human cells. A reversal of values of the retention volume, similar to human cells in Table II, was also obtained when dextran T500 was replaced with 4.5% (w/w) of T40 (eluent III). An increase in the concentration of dextran T40 resulted in lower separation factors for both cells, similar to that observed in Table II. The highest separation factor for rabbit granulocytes and lymphocytes was obtained with eluent II, and this result was comparable to that obtained on a PEG-bonded Porasil column. By taking advantage of the anomalous behaviour of rabbit lymphocytes in eluent II, an almost complete separation from granulocytes was accomplished by the use of either eluent II or III. Rabbit erythrocytes gave a separation factor of 4.20 against granulocytes with eluent III and 2.33 against lymphocytes with eluent IV. It was considered that the anomalous behaviour of rabbit lymphocytes may be species-dependent owing to the difference in the surface properties compared with human cells.

A good selectivity of the PEG-bonded Sepharose column for human and rabbit blood cells was shown. High separation factors for these cells were observed compared with the PEG-bonded Porasil. The selectivity of the column depended greatly on the molecular weight and concentration of dextran in the mobile phases used.

In order to demonstrate the capability of the PEG-bonded Sepharose column, a mixture of human granulocytes and lymphocytes was chromatographed, using eluent IV on the basis of the results described above. As shown in Fig. 2, the two kinds of cells were completely separated and eluted within 12 h. The recoveries of granulocytes and lymphocytes from the column were 67 and 82%, respectively, of the number of cells applied.

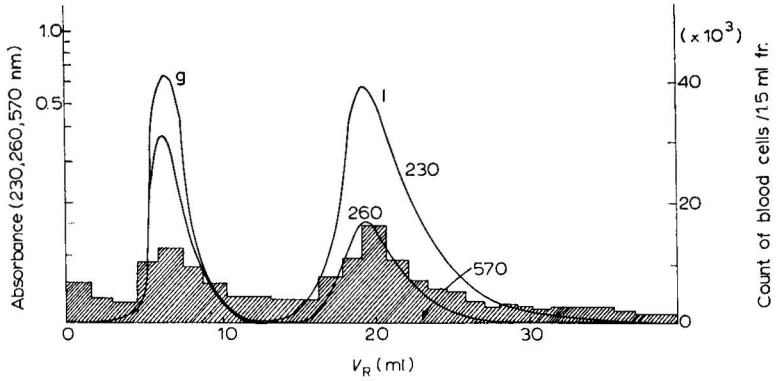


Fig. 2. Elution profile of human granulocytes (g) and lymphocytes (l). Column: 25 × 0.9 cm I.D., bisoxirane-coupled PEG 20M–Sepharose 6B. Eluent: IV, 8.0% (w/w) of dextran T40–0.045 *M* NaH_2PO_4–0.045 *M* Na_2HPO_4, pH 7.5; flow-rate, 3.0 ml/h. A mixture of $2.4 \cdot 10^4$ cells (g) and $3.9 \cdot 10^4$ cells (l) in 0.5 ml of the eluent was loaded on the column.

Fig. 3 shows a chromatographic pattern for a synthetic mixture of human erythrocytes and lymphocytes obtained by using the same column and eluent. The two kinds of cells were fairly well separated and eluted within about 5.8 h. The recovery of the erythrocytes from the column was 50%.

The selectivity of the chemically bonded PEG stationary phase for chromatographic separations is further illustrated in Fig. 4, which shows an elution profile for a synthetic mixture of human erythrocytes, granulocytes and lymphocytes with eluent IV. Three kinds of blood cells were well separated and eluted from the column within 15 h. A similar chromatogram was obtained at a higher flow-rate of 3.0 ml/h. The separation was attained without a serious decrease in column efficiency.

The viability of the blood cells recovered by these chromatographic separations was evaluated by means of phase-contrast microscopy and by respiratory and glycolytic activity.

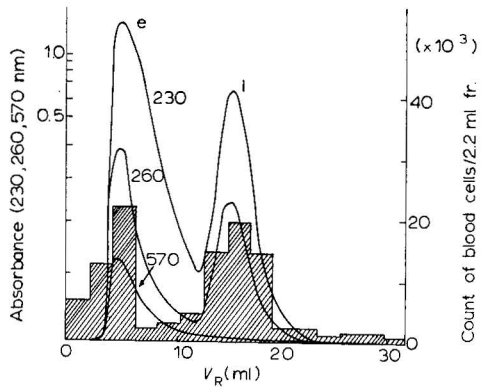


Fig. 3. Elution profile of human erythrocytes (e) and lymphocytes (l). Column and conditions as in Fig. 2, except the flow-rate was 4.4 ml/h. A mixture of $2.3 \cdot 10^4$ cells (e) and $5.8 \cdot 10^4$ cells (l) in 0.5 ml of the eluent was loaded on the column.

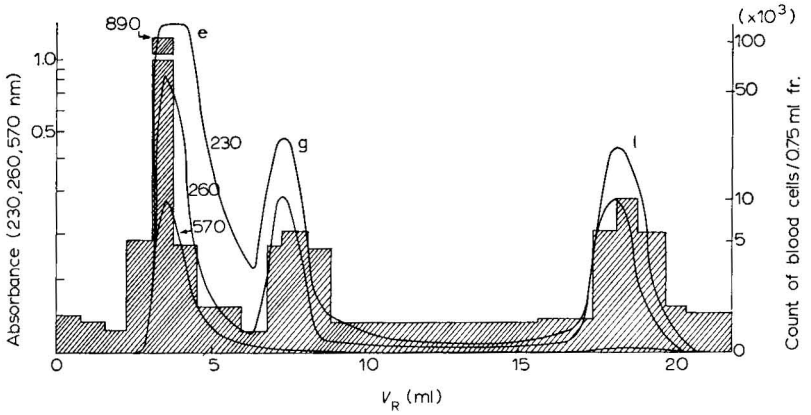


Fig. 4. Elution profile of human erythrocytes (e), granulocytes (g) and lymphocytes (l). Column and conditions as in Fig. 2, except the flow-rate was 1.5 ml/h. A mixture of $1.9 \cdot 10^6$ cells (e), 2.6×10^4 cells (g) and $2.7 \cdot 10^4$ cells (l) in 0.5 ml of the eluent was loaded on the column.

DISCUSSION

The usefulness of APTP systems for fractionation by partition and counter-current distribution of blood cells is illustrated by the successful resolution of erythrocytes of different ages[15,16], the separation of erythrocytes from leukocytes[14], etc. With blood cells, partition in APTP systems depends considerably on their surface properties, on the polymer composition and concentration, on the ionic composition and concentration (including the ratio between the ions present, pH), etc. In general, it has been found that a phase system composed of 5% (w/w) of dextran T500 and 4% (w/w) of PEG 6000 is suitable for the partition of most blood cells. Inorganic salts show characteristic partition behaviour in aqueous two-polymer phases[17]. Sodium and phosphate ions distribute unequally between the phases, giving rise to an electrostatic interfacial potential difference between them[6,18]. This phenomenon explains why the surface charge of suspended cells is involved in determining their partition and why the partition of such materials is sensitive to the ionic composition of the phases.

In order to carry out the separation of peripheral blood cells by means of column chromatography, we prepared two types of packing materials with chemically bonded PEG as the stationary phase. Dextran was chosen as the polymer constituent of the mobile phase because its viscosity must be decreased by the use of a branched polymer. The PEG-bonded Sepharose packing performs excellent separations of human erythrocytes, granulocytes and lymphocytes, as described above. This chromatographic method eliminates many of the problems usually associated with the operation of counter-current distribution in APTP systems. It was also found that the capability of the PEG-bonded Sepharose column depended greatly on the concentration and molecular size of dextran and the ionic composition of coexisting inorganic electrolytes in the mobile phase. The results indicate that the selectivity of the column decreased on the addition of sodium chloride. As the salt partitions equally between the phases and there is virtually no electrical interfacial potential[17], the negatively charged blood cells should have a preferential affinity for the mobile phase and the cells should be eluted more rapidly from the column.

PEG-bonded Sepharose shows a much lower adsorption for blood cells than

PEG-bonded Porasil. It should be pointed out that Sepharose, the support material for the stationary phase, has a hydrophilic surface, and the soft and bulky bead-shaped agarose gel particles do not substantially adsorb proteins. In contrast, Porasil forms fine and rigid particles with a large surface area, and it is known to adsorb proteins spontaneously so that irreversible adsorption can take place between such a surface and proteins of the cell membrane. PEG-bonded Sepharose is easily prepared from epoxy-activated Sepharose 6B and PEG 20M, and the column can be used repeatedly. Scaling up of this technique can readily be achieved by use of a larger bore and/or a longer column.

It is known that lamb leukocytes show heterogeneity of population in their distribution curve by means of an APTP system composed of 5% (w/w) of dextran T500, 4% (w/w) of PEG 6000 and phosphate buffer, but lymphocytes and granulocytes were not separated sufficiently well[14]. Preliminary studies of the counter-current distribution of human leukocytes showed only a single peak[14] or partial resolution[19]. Similarly, separation of rabbit erythrocytes and leukocytes is feasible in similar phase systems[20].

The versatility and usefulness of PEG-bonded Sepharose for the separation and subfractionation of blood cells and cell populations have been demonstrated. The proposed method makes it possible to separate leukocyte populations without difficulty, but the chromatographic separation of lymphocyte populations has not been accomplished.

Mobile phase systems in this method can often be modified so as to solve a particular problem. Once a suitable mobile phase has been found, this technique should prove useful for a wide variety of fractionations and characterizations of blood cells that differ in surface properties, size, specific gravity of the cells and hydrophobicity of the cell surface. This aspect and some applications to the chromatographic separation of platelets from other blood cells will be reported in a subsequent paper.

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CHROM. 12,315

QUANTITATIVE ANALYSIS OF ZINC, COPPER AND NICKEL DIETHYLDITHIOCARBAMATES BY GAS–LIQUID CHROMATOGRAPHY

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(First received April 9th, 1979; revised manuscript received August 13th, 1979)

SUMMARY

A column packed with a 1:1 mixture of 5% OV-101 and 5% QF-1 on Gas-Chrom Q was used for the gas–liquid chromatographic assay of zinc, copper and nickel diethyldithiocarbamates in artificial mixtures and in samples of natural origin. The quantitative determination of complexes in artificial mixtures may be performed at concentration ratios of metal to *n*-dotriacontan (internal standard) of 0.11–5.57. The precision obtained for the determination of zinc, copper and nickel was *ca.* 11% in sea sand and 10% in sea mud.

INTRODUCTION

In an earlier paper[1], the possibility of the gas–liquid chromatographic (GLC) determination of zinc, copper and nickel in the form of their diethyldithiocarbamate (DEDTC) complexes in marine bottom sediments was demonstrated. The most suitable stationary phases were 5% OV-101 or 5% QF-1 on Gas-Chrom Q. As described in another paper[2], a mixed column packing consisting of a 1:1 mitxure of 5% OV-101 and 5% QF-1 on Gas-Chrom Q is particularly useful for the GLC assay of the complexes. This packing has now been used for assaying the complexes in standard solutions and in samples of natural origin.

EXPERIMENTAL

Preparation of metal chelates

Pure DEDTC complexes of zinc, copper and nickel, synthesized by known procedures[3,4], were used for preliminary investigations.

Gas–liquid chromatography

A Pye Unicam series 104 gas chromatograph with a flame-ionization detector (FID) was used. A glass column (1.5 m × 2 mm I.D.) was packed with a 1:1 (w/w) mixture of 5% OV-101 and 5% QF-1 coated on Gas-Chrom Q (100–120 mesh).

Preparative GLC and identification of the DEDTC complexes of zinc, copper and nickel

The DEDTC complexes of the metals were isolated from the column by using

a 1:25 splitting ratio. Solutions of pure zinc, copper or nickel complexes, solutions containing artificial mixtures of the complexes and solutions containing a mixture of the complexes obtained from the digestion of a sample of bottom sediment were applied on to the column.

The identities of the complexes in the eluate were determined by comparing their UV spectra with those of authentic samples.

Sample preparation and procedure for assaying zinc, copper and nickel in marine bottom sediments

A study of the effect of pH, addition of the carrier, extent of extraction at different pHs and the ratio of phases on the results of analyses by GLC[5] indicated that it was necessary to modify the original procedure slightly, as follows. A ground sample (0.3 g) was placed in a Perkin-Elmer Model 2 autoclave, wetted with doubly distilled water; 2 ml of concentrated nitric acid were added, followed by 10 ml of 40% hydrofluoric acid. The contents were mixed and autoclaved for 45 min at 150°. After cooling, the contents were transferred into a 100-ml PTFE dish, 10 ml of 40% hydrofluoric acid and 1 ml of concentrated nitric acid were added and the solution was evaporated to dryness on a sand-bath. After adding to the residue 1 ml of concentrated nitric acid and 10 ml of 40% hydrofluoric acid, the evaporation was repeated. Then the residue was dissolved in 10 ml of 8 *M* hydrochloric acid at elevated temperature. The solution was transferred quantitatively into a 25-ml volumetric flask and made up to the mark with doubly distilled water. This solution was used in the following analyses.

(i) Aliquots of 7 ml were withdrawn, placed in a 25-ml volumetric flask, treated with 0.5 ml of lanthanum(III) solution (1000 μg/ml) and made up to the mark with doubly distilled water. The contents of zinc, copper and nickel were determined by atomic-absorption spectrometry with an air–acetylene flame.

(ii) Aliquots of 7 ml were withdrawn, placed in a 250-ml beaker, treated with 5 ml of lanthanum(III) solution (1000 μg/ml) and diluted with doubly distilled water to *ca.* 100 ml. Then the beaker was placed on a boiling water-bath. After 2 min, 20 ml of 25% ammonia solution followed by 1 ml 30% hydrogen peroxide were added, mixed and heated for 10 min. The solution was filtered through a small-pore filter-paper and the precipitate was washed twice with 10-ml portions of warm ammonia solution. The filtrate was transferred into a 250-ml separating funnel and extracted with three 5-ml portions of methyl isobutyl ketone (MIBK). After extraction, 10 ml of 3% NaDEDTC solution, previously freed from metal impurities by extraction with MIBK, was added to the solution in the separating funnel. The complexes were extracted after 10 min with one 10-ml portion and two 5-ml portions of MIBK. The combined extracts were placed in a 25-ml volumetric flask and made up to the mark with MIBK. The contents of zinc, copper and nickel were determined by atomic-absorption spectrometry with an air–acetylene flame (lean fuel).

(iii) In the GLC assay, 10-ml aliquots were withdrawn and placed in a 250-ml beaker. The following analytical operations were the same as in (ii). However, for the extraction of the DEDTC complexes chloroform was used (three 5-ml volumes). The combined extracts were evaporated to dryness on a water-bath at 80°. To the dry residue a 717.6 μg/ml solution of *n*-dotriacontan as internal standard was added.

Preparation of standard solutions for assaying zinc, copper and nickel in marine bottom sediments

The concentrations of the aqueous solutions of zinc, copper and nickel were 5, 10, 15, 20 and 50 μg per 150 ml. After complexing the metals with ammoniacal NaDEDTC solution, the complexes were extracted with chloroform as described. After evaporation of chloroform from the combined extracts, a 717.6 μg/ml solution of *n*-dotriacontan was added with a microsyringe to the residue and the mixture was diluted with chloroform to a pre-determined volume.

The same procedure was employed for the preparation of a solution of extracts from marine bottom sediments, using the same volume of the internal standard solution and the same final dilution as for the standard solutions.

RESULTS AND DISCUSSION

The separation of metal complexes and internal standard is shown in Fig. 1.

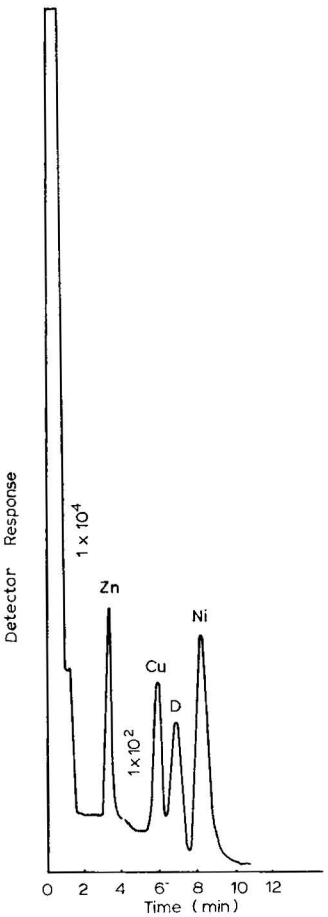

Fig. 1. GLC of $Zn(DEDTC)_2$, $Cu(DEDTC)_2$ and $Ni(DEDTC)_2$ and *n*-dotriacontane on a 1.5 m × 2 mm I.D. glass column packed with a 1:1 (w/w) mixture of 5% OV-101 and 5% QF-1 on Gas-Chrom Q. Column temperature, 240°; detector temperature, 250°; argon flow-rate, 17.6 ml/min. D = *n*-dotriacontan.

The peaks are symmetrical and well separated. To identify the DEDTC complexe of metals, they are isolated by preparative GLC.

As can be seen in Fig. 2, the retention times of the metal complexes obtained from a sample of the sediment were identical with those of each standard used. This was also confirmed by the UV spectra shown in Fig. 3. The similarity of the spectra indicates that the complexes are eluted without decomposition.

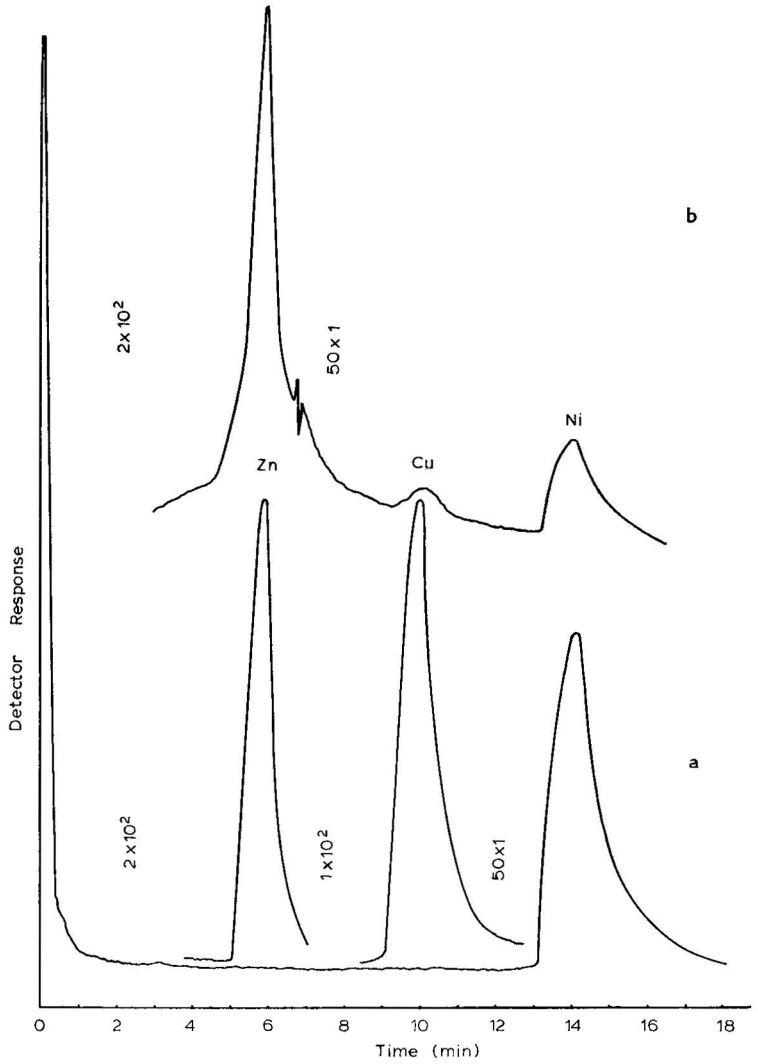

Fig. 2. Preparative GLC of $Zn(DEDTC)_2$, $Cu(DEDTC)_2$ and $Ni(DEDTC)_2$ on a 1.5 m × 2 mm I.D. glass column packed with a 1:1 (w/w) mixture of 5% QF-1 and 5% OV-101 on Gas-Chrom Q. Column temperature, 240°; detector temperature, 250°; splitting ratio, 1:25; argon flow-rate at outlet of the preparative section, 21.4 ml/min. (a) Separation of a simple complex; (b) separation of a mixture of complexes obtained from a marine bottom sediment.

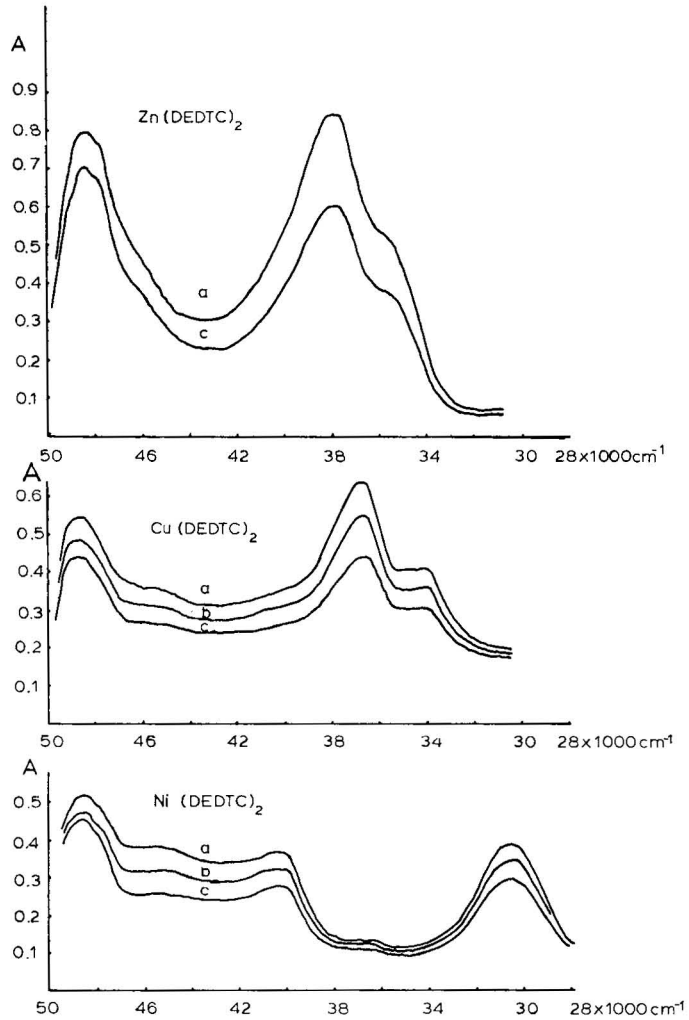

Fig. 3. UV spectra of $Zn(DEDTC)_2$, $Cu(DEDTC)_2$ and $Ni(DEDTC)_2$. (a) Pure complex; (b) complex obtained from the preparative column; (c) complex obtained from a bottom sediment sample after GLC separation.

The analytical usefulness of the column packing employed was further demonstrated by the unaltered peak shapes obtained after repeated injections of a solution consisting of a mixture of the complexes and *n*-dotriacontan (*cf.*, Figs. 4–6). A suitable ratio of the concentration of the complexes (based on the metal content) to the concentration of *n*-dotriacontan is 1.55. The values of the ratio of the peak area of a given complex to that of *n*-dotriacontan (D) were as follows: for nickel, $D = 5.40 \pm 0.22$ (coefficient of variation, C.V. = 6.5%); for copper, $D = 2.38 \pm 0.13$ (C.V. = 9.2%); and for zinc, $D = 7.16 \pm 0.63$ (C.V. = 13.1%).

For quantitation, calibration graphs were constructed (Fig. 7) for each complex based on the chromatogram of an appropriate standard mixture of known concentration of the complex and *n*-dotriacontan. The ratio of the peak area of a given

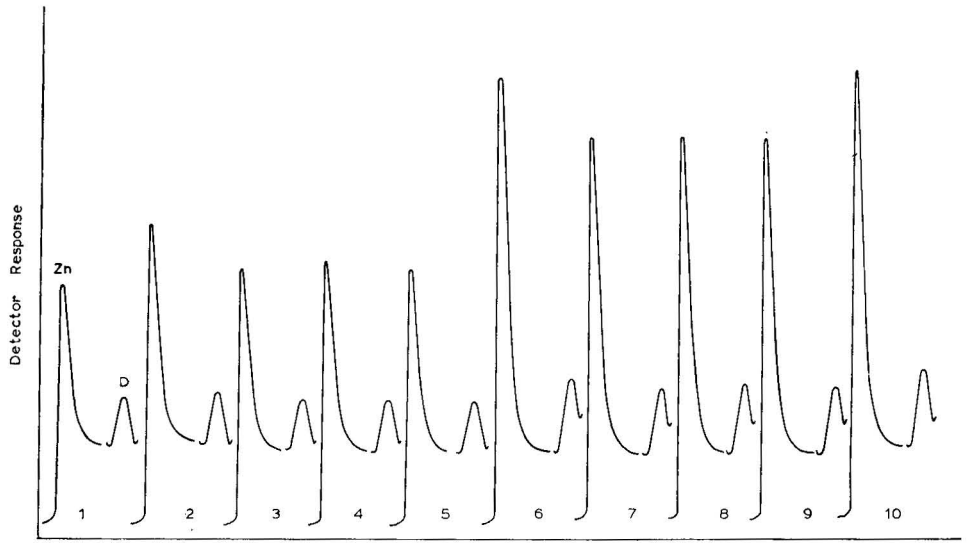

Fig. 4. Gas chromatogram of extracted mixture of $Zn(DEDTC)_2$, illustrating sample stability under chromatographic conditions. Glass column, 1.5 m × 2 mm I.D., packed with a 1:1 (w/w) mixture of 5% OV-101 and 5% QF-1 on Gas-Chrom Q. The column was operated isothermally at 210° for 3 min, than programmed at 15°/min and held at 240°; argon flow-rate, 35.7 ml/min. D = *n*-dotriacontan.

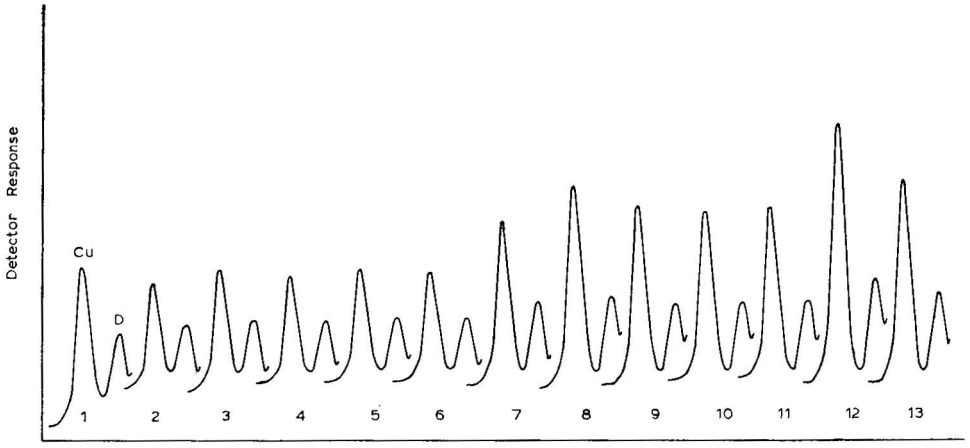

Fig. 5. As Fig. 4, for $Cu(DEDTC)_2$.

complex to that of *n*-dotriacontan was plotted against the ratio of the concentration of the complex (based on its metal content) to the concentration of *n*-dotriacontan. As the response of the detector to small amounts of complex is poor[2], the calibration graphs do not pass through the origin. Corrections to the graphs were effected by the least-squares method. The concentration ranges studies for the zinc and nickel complexes and for *n*-dotriacontan were 44.4–114.3 and 76.4–416.0 ng/μl, respectively;

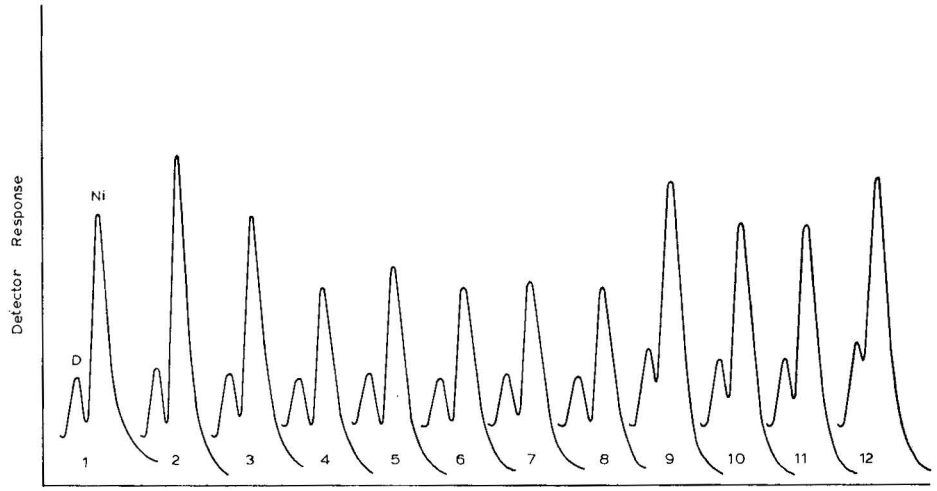

Fig. 6. As Fig. 4, for $Ni(DEDTC)_2$.

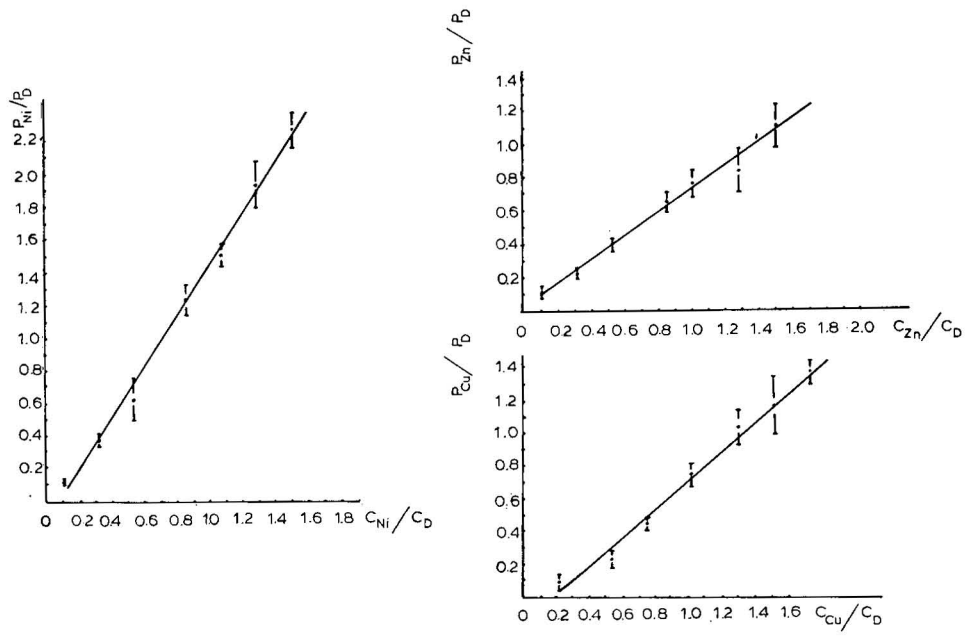

Fig. 7. Calibration graphs of peak area ratio ($P_{complex}/P_D$) *versus* concentration ratio (C_{metal}/C_D).

for copper the concentration range was 88.8–143.5 ng/μl and for *n*-dotriacontan 96.0–416 ng/μl.

Fig. 8 shows chromatograms in which the ratios of the concentrations of the complexed metal to that of *n*-dotriacontan and the peak areas differ widely.

Typical chromatograms obtained from a sample of sea sand and from a sample of sea mud are shown in Figs. 9 and 10, respectively. It is worth noting that larger

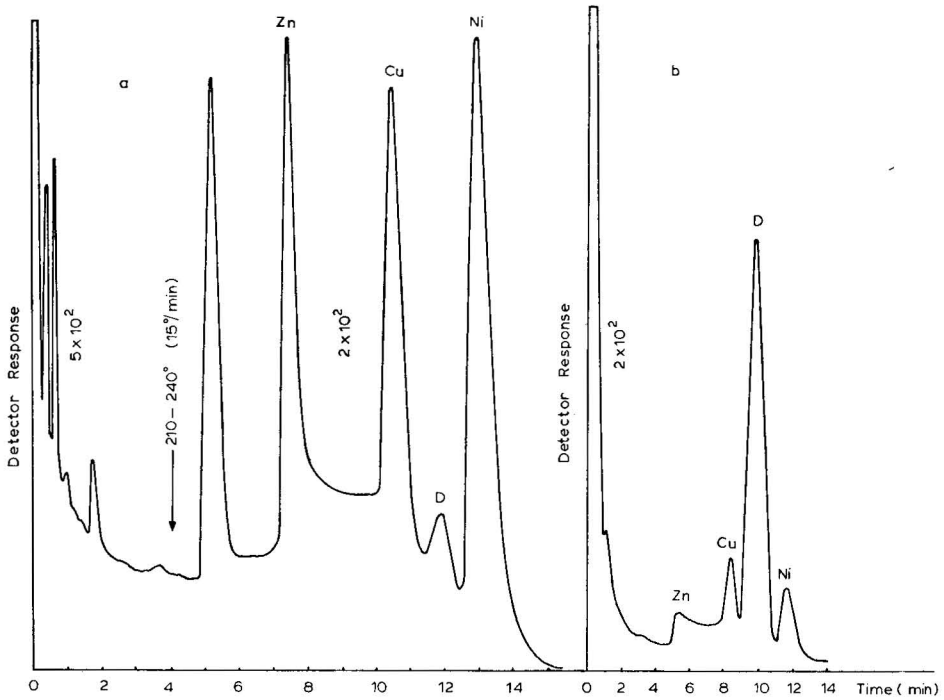

Fig. 8. (a) GLC of $Zn(DEDTC)_2$, $Cu(DEDTC)_2$ and $Ni(DEDTC)_2$ with *n*-dotriacontan (D) at a C_{metal}/C_D ratio of 5.57. This corresponds to 200 ng of metal and 35.88 ng of *n*-dotriacontan. (b) GLC of DEDTC complexes of Zn, Cu and Ni with *n*-dotriacontan. Concentration ratio (C_{metal}/C_D) for Zn and Ni is 0.11 and for Cu 0.21. This corresponds to 44.4 ng of Zn and Ni, 88.8 ng of Cu and 416.0 ng of *n*-dotriacontan.

TABLE I

RESULTS OF ANALYSIS OF MARINE SEDIMENTS

Metal	*Method**	*No. of replicates*	*Sea sand*		*Sea mud*	
			*Found** (μg/g dry weight)*	*C.V.*** (%)*	*Found** (μg/g dry weight)*	*C.V.*** (%)*
Zinc	(i)	6	42.72 ± 0.46	1.01	99.21 ± 1.27	1.78
	(ii)	6	40.73 ± 1.48	4.9	97.45 ± 2.08	2.8
	(iii)	10	37.98 ± 3.97	9.87	92.35 ± 3.75	5.70
Copper	(i)	6	11.03 ± 0.28	2.44	27.96 ± 1.96	5.94
	(ii)	6	9.26 ± 0.39	4.1	26.11 ± 1.73	9.26
	(iii)	10	9.40 ± 0.84	8.5	25.87 ± 1.83	9.86
Nickel	(i)	6	12.93 ± 0.22	1.62	30.40 ± 0.96	4.6
	(ii)	6	11.46 ± 0.52	4.36	27.14 ± 1.37	7.07
	(iii)	10	10.01 ± 1.19	11.3	27.05 ± 1.44	7.43

* See text.

** Found value = mean at 95% probability level.

*** C.V. = coefficient of variation

$$= \left(\frac{\text{standard deviation of single measurement}}{\text{mean value}} \right) \times 100\%.$$

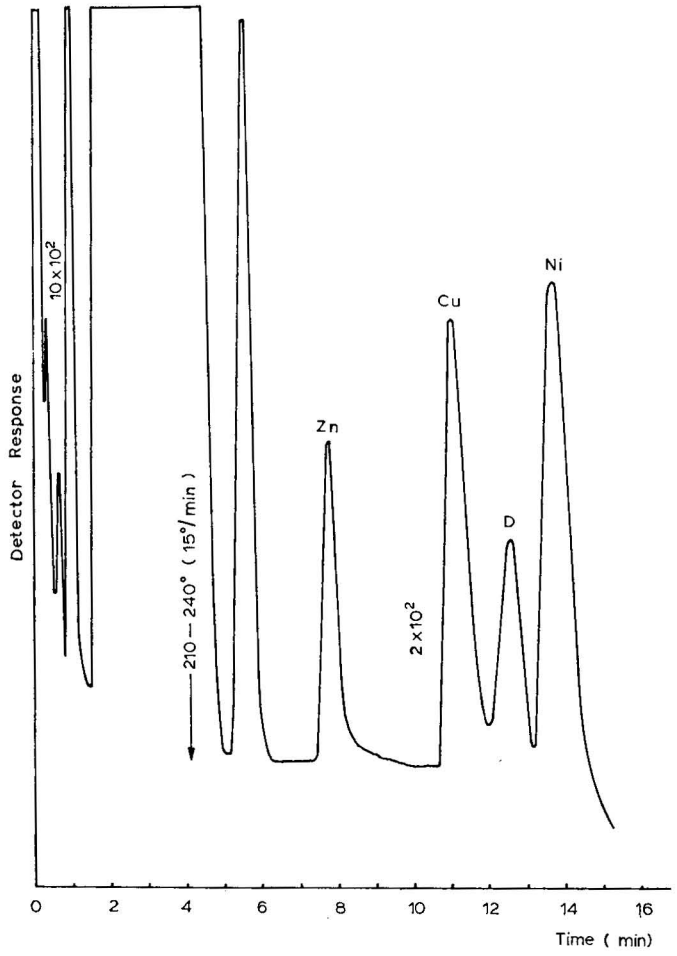

Fig. 9. Typical chromatogram obtained with the solution of extracted complexes from the sample of sea sand. The column was operated isothermally at 210° for 4 min, then programmed at 15°/min and held at 240°. Argon flow-rate, 19.74 ml/min; addition of 5 μl of *n*-dotriacontan (D) solution (717.6 μg/ml). Final volume, 100 μl; injection volume, 0.8 μl.

zinc levels than those of copper and nickel can be quantified by suitable attenuation of the signal if it falls within the range of the linear response of the detector.

The GLC measurements of zinc, copper and nickel in samples of sediments carried out according to method (iii) are shown in Table I together with the results obtained by methods (i) and (ii).

The zinc, copper and nickel contents calculated from chromatograms shown in Figs. 9 and 10 are averages of five runs. Each extract of the complexes was applied on to the column three times.

The results are encouraging for the use of an FID in the GLC assay of the DEDTC complexes of zinc, copper and nickel. Further, they show the usefulness of the procedure for processing maring bottom sediments for the simultaneous assay of

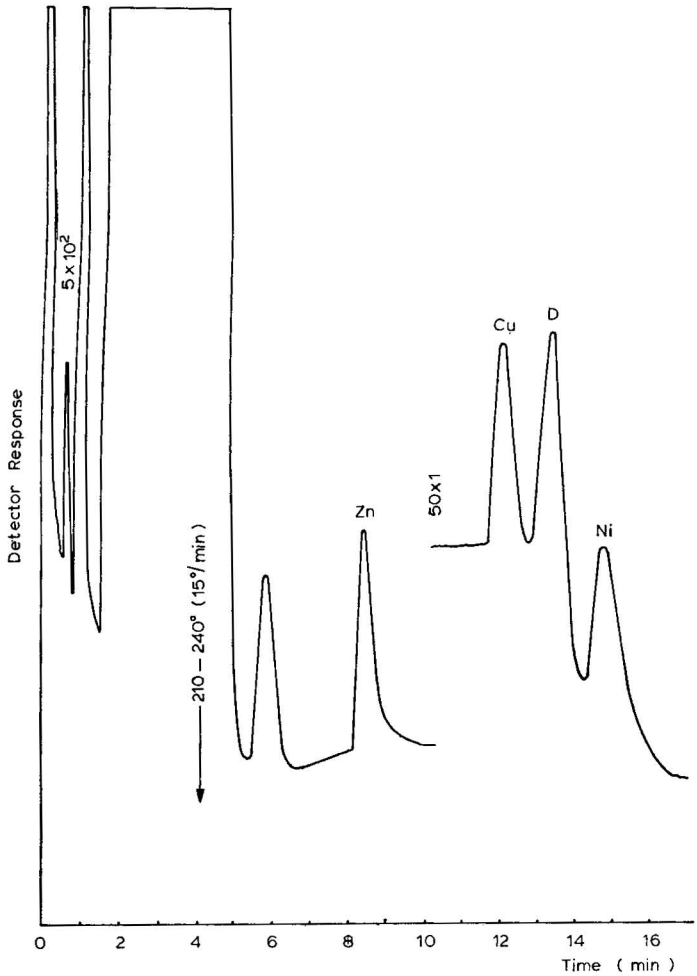

Fig. 10. Typical chromatogram obtained for a solution of a sample of mud. Column temperature, 240°; detector temperature, 250°; argon flow-rate, 18.75 ml/min. Addition of 10 μl of *n*-dotriacontan (D) solution (717.6 μg/ml). Final volume, 100 μl; injection volume, 0.8 μl.

zinc, copper and nickel without the risk of thermal decomposition of the metal complexes in the column or interferences by other metals that form complexes with DEDTC.

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Journal of Chromatography, 187 (1980) 373–379

CHROM. 12,341

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HYALURONIC ACID AND OLIGOSACCHARIDES PRODUCED BY BOVINE TESTES HYALURONIDASE

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SUMMARY

A high-performance liquid chromatographic method of analysing hyaluronic acid and oligosaccharides produced by bovine testes hyaluronidase is presented. Using silica gel packed columns and an aqueous mobile phase, it is possible to separate the mono-, di-, tetra- and hexasaccharides from higher oligosaccharides and hyaluronic acid and to follow the enzymatic conversion of polysaccharides to oligosaccharides. The analysis is performed within 30 min and only 20 μl of sample (containing 20–60 μg of saccharides) are needed.

INTRODUCTION

The glycosaminoglycan hyaluronic acid (HA) is a linear naturally occurring polysaccharide having the repeating structure -O-β-D-glycopyranuronosyl-(1 $\rightarrow$ 3)-O-(2-acetamido-2-desoxy-β-D-glycopyranosyl)-(1 $\rightarrow$ 4)-, known as N-acetylhyalobiuronic acid.

Enzymatic digestion of HA can be performed by *exo*-glycanohydrolases such as β-N-acetylglucosaminidase (E.C. 3.2.1.30) and β-gluruconidase (E.C. 3.2.1.31), cutting off monomer units from the end of the HA molecule, or by *endo*-glycanohydrolases (E.C. 3.2.35), cleaving the 1 $\rightarrow$ 3 bond leaving a reducing glucuronic acid end group, or cleaving the 1 $\rightarrow$ 4 bond leaving a reducing glucosamine end group. Bovine testes hyaluronidase is of the latter type, being an *endo*-β-N-acetylglucosaminidase.

For investigating the action of different hydrolases on HA, several techniques have been used *e.g.*, end group analysis, gel filtration and electrophoresis, paper chromatography, radial diffusion, viscometric and turbidimetric measurements.

In this paper we describe the application of high-performance liquid chromatography (HPLC) to the well known enzymatic digestion of HA with bovine testes hyaluronidase.

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MATERIALS AND METHODS

High-performance liquid chromatography

A 1010B liquid chromatograph from Hewlett-Packard (Böblingen, G.F.R.) was used with a differential refractometer (LD Refracto Monitor; Laboratory Data Control, Riviera Beach, Fla., U.S.A.) as detector. The detector response was recorded on a Hewlett-Packard 3380A integrator. Injections were made with a Rheodyne Model 7210 injector (Rheodyne, Berkeley, Calif., U.S.A.) with a 20-μl loop.

Separation was performed on a μBondagel E linear column, followed in series by two μPorasil GPC 60 Å columns (Waters Assoc., Milford, Mass., U.S.A.). Each had dimensions of column 300 × 4 mm I.D. The μBondagel column had a nominal molecular weight separation range of 2000–2,000,000, the μPorasil column a range of 100–10,000. The mobile phase was 20 m*M* sodium acetate buffer (pH 4.0) containing 1.5 mg/l of HA. The column compartment was heated to 40°, and a flow-rate of 0.40 ml/min was used. The pressure drop over the three columns was about 65 kp/cm^2. Injections were made with a 100-μl SGE syringe (SGE, North Melbourne, Australia).

Enzymatic digestion

HA as the potassium salt obtained from human umbilical cord (Grade I; Sigma, St. Louis, Mo., U.S.A.) was dissolved in 20 m*M* sodium acetate buffer (pH 4.0) at concentrations of 1.5–4.0 mg/ml, and this solution was used as a substrate for the digestion with chromatographically purified bovine testes hyaluronidase (Sigma). The enzyme was supplied in vials containing 15,000 NF units, which was dissolved in the acetate buffer to give an activity of 4000 NF units/ml. Digestion was started by the addition of 25 NF units of enzyme per milligram of HA. Similar additions of enzyme were repeated after 24, 48 and 72 h to give a total of 100 NF units added per milligram of HA.

Samples from the incubation mixture were drawn at different times, the enzyme activity being stopped by boiling in a water bath for 5 min, and stored at 4°.

Preparation of low-molecular-weight oligosaccharides

The 120-h digestion product was separated on a Sephadex G-25 Superfine (Pharmacia, Uppsala, Sweden) column (935 × 26 mm I.D.), equilibrated and eluted with 10% (v/v) of ethanol in 0.2 *M* sodium chloride solution according to Flodin *et al.*[1]. A constant flow of 10 ml/h was maintained with a peristaltic pump (P3; Pharmacia). The effluent was continuously monitored with a differential refractometer (LDC), and 5-ml fractions were collected.

The fractions containing oligosaccharides were pooled and desalted on Sephadex G-25 Superfine columns (80 × 30 mm I.D.) with 10% (v/v) of ethanol in water as the eluent. The desalted fractions were lyophilized and redissolved in water.

Determination of reducing N-acetylglucosamine

During digestion with bovine testes hyaluronidase, reducing N-acetylglucosamine end groups are liberated, and their analysis was performed according to Reissig *et al.*[2] with modifications. To 250 μl of sample, 50 μl of 1 *M* sodium hydroxide solution and 60 μl of 0.8 *M* dipotassium tetraborate solution were added in screw-

capped tubes to give a final pH of 9.1. The capped tubes were heated for 3 min at 100° in a heating block (BT 3; Grant, Cambridge, Great Britain). After cooling, 1.8 ml of DMAB reagent (5 g of *p*-dimethylaminobenzaldehyde dissolved in 5 ml of concentrated hydrochloric acid and 50 ml of glacial acetic acid, and diluted with 9 volumes of glacial acetic acid just before use) was added. After incubation for 20 min in a water-bath at 37°, the extinctions at 543 nm were measured in an LKB 2074 spectrophotometer (LKB, Bromma, Sweden), 0.250 m*M* N-acetylglucosamine being used as a standard.

Determination of total glucuronic acid

The analysis was performed according to Bitter and Muir[3]. To 250 μl of sample was added 1.5 ml of a 0.05 *M* solution of dipotassium tetraborate in concentrated sulphuric acid, carefully avoiding an increase in temperature above ambient. The capped tubes were heated for 10 min at 100° in a heating block (Grant). After cooling, 50 μl of a 0.25% (w/v) solution of carbazole in ethanol were added, followed by heating for 15 min at 100°. The tubes were cooled to ambient temperature and the extinctions at 514 nm were measured in the LKB 2074 spectrophotometer. A mixture of 0.100 m*M* glucuronic acid and 0.100 m*M* N-acetylglucosamine was used as a standard.

Determination of total N-acetylglucosamine

The analysis was performed according to Blix[4] with modifications. The sample (750 μl) was hydrolysed with 750 μl of concentrated hydrochloric acid in sealed glass ampoules for 3 h at 100° in a heating block (Grant). The mixture was cooled and transferred into 15-ml vials with about 0.5 ml of water, quickly frozen in acetone–carbon dioxide and lyophilized (Hetosicc; Heto, Birkerød, Denmark). The residue was dissolved in 1500 μl of water and 500 μl of this solution were added to 500 μl of acetylacetone reagent [10 ml of 0.2 *M* dipotassium tetraborate (pH 9.1), 220 μl of 5 *M* sodium hydroxide solution and 200 μl of acetylacetone] in a capped tube. After 30 min at 100° in a heating block (Grant), the tubes were cooled with ice and 200 μl were added to 1000 μl of DMAB reagent (see *Determination of reducing N-acetylglucosamine*) in LKB cuvettes. Colour development took place at 50° for 45 min in an LKB incubator, followed by measurement of extinctions in the LKB 2074 spectrophotometer at 543 nm. As a standard, a mixture of 0.5 m*M* N-acetylglucosamine and 0.5 m*M* glucuronic acid, treated in the same way as the samples, was used.

Paper chromatography

Descending paper chromatography was performed according to Partridge[5]. The solvent used was 1-butanol–acetic acid–water (44:16:40). Volumes of 20 μl of sample containing 10–40 μg of oligosaccharides were applied on Whatman No.1 paper. The strips (180 × 420 mm) were irrigated for 40 h at room temperature. The formation of chromogen took place in a box containing saturated water vapour at 105° for 10 min. After spraying with DMAB reagent, the reducing N-acetylglucosamine-containing compounds gave a red colour.

Determination of exo-glycanohydrolases

β-N-Acetylglucosaminidase and β-glucuronidase activity was assayed accord-

ing to Khar and Anand[6] and Brot *et al.*[7]. The assays were modified for performance on an ABA-100 analyser (Abbott, Pasadena, Calif., U.S.A.) using a 415/450 nm filter set at 37° and *p*-nitrophenol substrates (Koch-Light, Colnbrook, Great Britain).

RESULTS

A 4 mg/ml solution of umbilical cord HA was incubated with bovine testes hyaluronidase as described under Materials and Methods. No β-glucuronidase or N-acetylglucosaminidase activity could be measured in the enzyme preparation. After incubation for 120 h the mixture was chromatographed on the Sephadex column. Part of the elution profile is shown in Fig. 1. The effluent was continuously monitored by the refractometer and 5-ml fractions were analysed for reducing N-acetylglucosamine content.

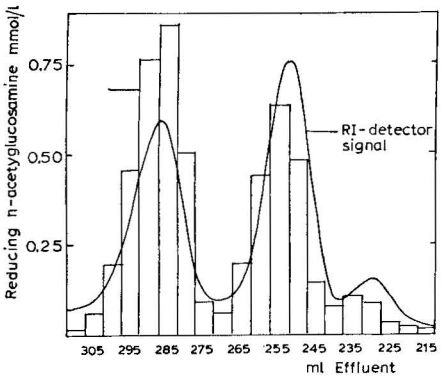

Fig. 1. Separation of oligosaccharides on Sephadex G-25 Superfine from the enzymatic digestion of HA. The peak with a maximum at about 250 ml of effluent was shown to contain hexasaccharide and the peak at 285 ml was shown to contain mainly tetrasaccharide.

The fractions eluted from 245 to 265 ml were pooled [pool (a)], and also the fractions eluted from 280 to 300 ml [pool (b)]. Each of the two pools was desalted, lyophilized and redissolved in 2 ml of water.

Pool (a) was found to give a total N-acetylglucosamine to glucuronic acid ratio of 1.04 and a total N-acetylglucosamine to reducing N-acetylglucosamine ratio of 2.95. Paperchromatography revealed a single spot with a mobility relative to N-acetylglucosamine of 0.41.

Pool (b) gave a total N-acetylglucosamine to glucuronic acid ratio of 1.00 and a total N-acetylglucosamine to reducing N-acetylglucosamine ratio of 1.77. Paper chromatography showed a heavily coloured spot with a relative mobility of 0.58, and a less coloured spot with a mobility of 0.77 relative to N-acetylglucosamine.

We conclude that pool (a) consisted of hexasaccharide and pool (b) of a mixture of tetrasaccharide and a small amount of disaccharide.

Fig. 2 shows the HPLC separation of a mixture of standards of approximately 1.5 mg/ml of each of the constituents of umbilical cord HA (elution time 13.79 min), hexasaccharide from pool (a) (17.84 min), tetrasaccharide from pool (b) (18.95 min), disaccharide from pool (b) (20.52 min), glucuronic acid (21.90 min) and N-acetyl-

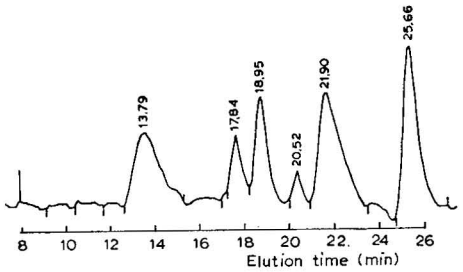

Fig. 2. HPLC of a mixture of six compounds. The refractometer response shows that HA is eluted at 13.79, hexasaccharide at 17.84, tetrasaccharide at 18.95, disaccharide at 20.52, glucuronic acid at 21.90 and N-acetylglucosamine at 25.66 min.

glucosamine (25.66 min). The void volume of the column system was about 3 × 1.8 ml, corresponding to an elution time of 13.5 min (0.40 ml/min) and a total volume of 3 × 3.4 ml, corresponding to 25.5 min, according to specifications from Waters Assoc.

In another experiment, a 1.6 mg/ml solution of HA was digested with bovine testes hyaluronidase. The HA had been desiccated over silica gel, and when analysed an N-acetylglucosamine to glucuronic acid ratio of 1.02 and a glucuronic acid content of 3.0 m*M* (theoretical value of pure potassium salt of HA, 3.8 m*M*) were found. Fig. 3 shows the recorder traces obtained in the HPLC analysis of the digest. After 120 h HA is degraded mainly to hexasaccharides and tetrasaccharides with only minor amounts of higher-molecular-weight oligosaccharides. Paper chromatography of the 120-h digestion product showed the same result.

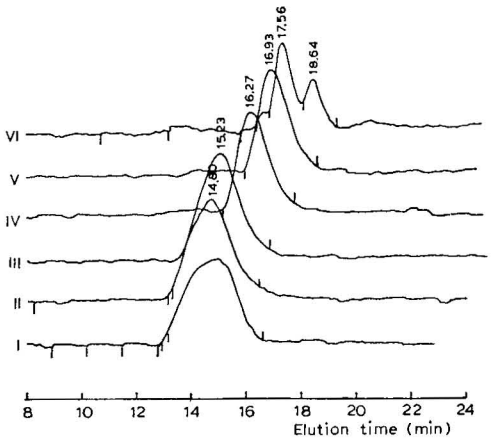

Fig. 3. HPLC of six samples (I–VI) drawn at different times from enzymatic digestion of HA. Data on the samples are given in Table I.

Table I shows the time course of the same digestion. Samples drawn from the incubation mixture at the times indicated were analysed for released end groups, and from these results the average molecular chain length could be calculated as the ratio of end groups to total monosaccharides. Using a molecular weight of 379 for the

TABLE I

DATA ON THE SIX SAMPLES FROM THE EXPERIMENT IN FIG. 3

Samples I–VI were drawn at times shown in the second column. The third column gives the concentration of liberated end groups, and the fourth and fifth columns show calculated values of the average number of disaccharides per chain and the number-average molecular weight.

Sample No.	*Time of digestion (h)*	*Reducing N-acetyl-glucosamine (mmol/l)*	*Average number of disaccharides per chain*	*Number-average molecular weight*
I	0	0	—	—
II	4	0.046	65.2	24700
III	12	0.101	29.7	11250
IV	48	0.405	7.4	2800
V	96	0.750	4.0	1520
VI	120	1.110	2.7	1050

repeating structure (N-acetylhyalobiuronic acid minus one molecule of water), the number-average molecular weights could be calculated.

DISCUSSION

Separation of the hyaluronic acid compounds in the system described is achieved by size exclusion in the three silica gel packed columns. Addition of trace amounts of HA to the mobile phase gave a better recovery and prevented adsorption to the columns. The μBondagel column has a packing material of varying porosity (125–1000 Å) with an ether-modified hydrophilic surface of the silica gel. The μPorasil columns have a narrow pore size range around 60 Å and a non-bonded surface. The packing material is not completely inert, however. The negatively charged HA and oligosaccharides seem to be partly excluded from the pores, *e.g.*, N-acetylglucosamine is eluted significantly later than glucuronic acid. Similarly, the Dextran T series (Pharmacia) is eluted much later than the glucuronic acid containing anions of the same molecular weight: Dextran T 10 (mol. wt. 10,000) at 18.7 min, Dextran T 20 (mol. wt. 20,000) at 17.8 min. Consequently, for our purpose the dextrans cannot be used as standards for molecular weight determinations.

The chosen column combination favours the separation of lower oligosaccharides. Fractionation must occur with elution times in the range 13.50 min (void volume) to 25.50 min (total volume) at a flow rate of 0.40 ml/min, but in this range the hexasaccharide already appears at 17.80 min, leaving the range 13.50–17.80 min to fractionation of higher oligosaccharides and polysaccharides. Umbilical cord HA is considered to have a very high molecular weight. Assuming (1) a molecular weight of several millions, corresponding to the peak of sample I in Fig. 3, and a molecular weight of 1150 corresponding to hexasaccharide in sample VI (17.56 min), and (2) gel filtration with a linear relationship between elution time and log (molecular weight), we would find weight-average molecular weights of the peaks in samples II–V up to ten times higher than the calculated number-average molecular weights shown in Table I. This means that the HA is degraded into a very polydisperse mixture by the bovine testes hyaluronidase, which fits well with the broad peaks in Fig. 3.

HA of other origin, such as pig skin (Seikagaku, Tokyo, Japan), vitreous

humour (EGA-Chemie, Steinheim/Albuch, G.F.R.) and bacteria (a gift from Dr. E. Kjems, Statens Seruminstitut, Copenhagen, Denmark), were also chromatographed; they were all eluted slightly later than the umbilical cord HA, and the peaks were narrower. Other charged glycosaminoglycanes such as the chondroitin sulphates and their disaccharide constituents (Seikagaku) were readily chromatographed under the same conditions.

The columns were stable for several months, but could be regenerated if necessary with 3% (w/v) of urea in the mobile phase.

We conclude that the proposed HPLC method for the characterization of HA and the enzymatic digestion products is advantageous because of the small sample volume, the speed of separation and the possibility of using a mobile phase with the same composition as the medium used for the enzymatic reaction.

ACKNOWLEDGEMENT

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A RAPID AND HIGH-RESOLUTION METHOD TO DETERMINE THE COMPOSITION OF CORN SYRUPS BY LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure was developed that provides saccharide distributions up to the pentasaccharides. The method requires less than 20 min and has been employed in research and in industrial laboratories. The precision and accuracy of results compare favorably with accepted but more time-consuming techniques.

INTRODUCTION

The high-performance liquid chromatographic (HPLC) separation of saccharides in sucrose, glucose and fructose mixtures is well known. Brobst *et al.*[1] and others[2–4] used cation-exchange resins with metallic counterions (Ca^{2+} and others) to separate the mono- and oligosaccharides in several carbohydrate sweeteners. Kesler[5] and Lee[6] used anion resins and a borate buffer eluent for their work. More recently, silica columns with specific functionalities have been used by Conrad and Fallick[7], Cegla and Bell[8] and Richter and Woelk[3] for sugar mixtures.

Several of the above procedures are excellent for separations up to the octasaccharides, but are either time-consuming[1,4] or require the use of undesirable organic solvents[3,7,8]. An alternative method[2,4] based on an aqueous eluent is rapid, but is limited to a molecular weight distribution up to the trisaccharides. Some procedures[5,6] provide excellent separations among the mono-, di-, and trisaccharides but are time-consuming and use systems of buffers as eluents. Fig. 1A, B and C are examples of excellent but time-consuming chromatograms; Fig. 1D is an example of a chromatogram obtained rapidly at the expense of the degree of separation.

In the work of Brobst and co-workers[1,4] with cation-exchange resins, many data were presented on the relative value of resin cross-linking but the discussion was limited to 4 and 8% cross-linked products. Resin particle size was also examined, but only when more resolution was needed among the tri- and lower saccharides to enable shorter analysis time with 8% cross-linked resin. Although not specifically mentioned, a separation based on an aqueous mobile phase was advantageous from economic, health hazard and waste disposal standpoints. It was clear that the benefits of this chromatographic approach—employing calcium counter-

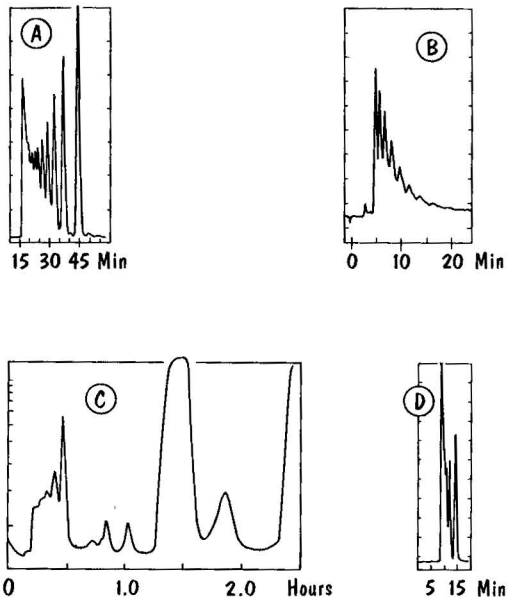

Fig. 1. A, Chromatogram of 42 D.E.* acid-hydrolyzed corn syrup, using a stainless-steel column (61 cm × 7 mm I.D.) packed with Aminex 50W-X4 (Ca^{2+}) and operated at 80°. The eluent was pumped at 0.5 ml/min, and a differential refractometer was used at ambient temperature. B, as in A, but using a column (30 cm × 3 mm I.D.) packed with chemically modified silica (μBondapak/CHO), and acetonitrile–water (60:40) as eluent at 1.0 ml/min. C, Partial chromatogram of a high-fructose corn syrup using a glass column (35 cm × 6.3 mm I.D.) filled with Durrum DA-X4 anion resin (borate) and operated at 55°. Borate buffer gradient (70 ml of 0.4% borate, pH 8.0, and 70 ml of 0.4% borate, pH 10.0) was pumped at 0.75 ml/min. An anthrone–sulfuric acid AutoAnalyzer detector was used and absorbance was plotted. D, Chromatogram of a 42 D.E. acid-hydrolyzed corn syrup using a stainless-steel column (61 cm × 7 mm I.D.) filled with Aminex Q-15S (8% cross-linked, Ca^{2+}) and operated at 80°. A differential refractometer was used at ambient temperature.

ions and aqueous eluents— could be further exploited by investigating the effect of resin cross-linking on both separation and speed of analysis. Perhaps a 4, 6 or 7% cross-linked cation-exchange resin of small particle size and with an appropriate metallic ion would provide a rapid oligosaccharide separation suitable for routine laboratory use. The objective was to develop a method with an analysis time under 20 min and providing separation up to the pentasaccharides. The result was, indeed, an HPLC determination of oligosaccharides up to the pentasaccharides that utilizes a controlled particle size, 4% cross-linked cation-exchange resin (Ca^{2+}). Approximately 17 min are needed for sample elution through glucose using water as eluent. An automatic sampler and integrator permit rapid processing of a large number of samples.

EXPERIMENTAL

Materials

A piece (30.5 cm × 7 mm I.D.) of chromatography grade stainless-steel tubing

* Throughout this article, D.E. (dextrose equivalent) is defined as a measure of reducing power using a modified Lane and Eynon procedure[12].

(Anspec., Ann Arbor, Mich., U.S.A.) was fitted with two 3/8-in. end fittings (Waters Assoc., Milford, Mass., U.S.A.), nuts and ferrules. Fittings with a 10-μm frit were selected for compatability with resin particle sizes. Strong cation resin was evaluated from three sources: A, Aminex 50W-X4, particle size 20–30 μm (Cat. No. 147-4208; Bio-Rad Labs., Richmond, Calif., U.S.A.), B, HC-X4.00 and HC-X6.00, particle size 10–15 μm (Cat. Nos. 77761 and 77768; Hamilton, Reno, Nev., U.S.A.) and C, BC-X4.00, particle size 10–15 μm (Cat. No. 605; Benson, Reno, Nev., U.S.A.).

Distilled deionized water from a Millipore Milli-Q water purification system (Millipore, Bedford, Mass., U.S.A.) was used in the preparation of aqueous solutions and as the mobile phase during the evaluation and analyses.

Apparatus

A Waters Model ALC 201 liquid chromatograph equipped with a Model 401 differential refractometer (capable of measuring 10^{-7} refractive index units) and a Model U6K universal injector was used throughout this study (Waters Assoc.). During the latter part of this investigation, a DuPont Model 834 (DuPont, Wilmington, Del., U.S.A.) automatic sampler was employed to provide 24-h operation during routine laboratory tests. A Spectra-Physics System I electronic integrator (Spectra-Physics, Santa Clara, Calif., U.S.A.) was used for peak integration and the calculations of results. Chromatograms were recorded on a Houston Omniscribe™ strip chart recorder (Houston Instruments, Austin, Texas, U.S.A.). Finally, an electrically heated column block of our own design (D. E. Just, CPC International) was used to maintain column temperature during the packing and evaluation of the analytical columns. It should be noted that stability of the detector is excellent but is dependent on ambient temperature if the temperature is not controlled with an external water-bath.

Resin preparation

The resin preparation method described by Brobst *et al.*[1] was used to clean the resin and convert it into the appropriate metallic form (*i.e.*, Ca^{2+}). Special attention, in the form of a double treatment, was given to the resin during the conversion step to insure complete metallic ion saturation and prevent sucrose inversion after the resin was packed. Resin fines, a detrimental fraction in some resin products, was not found to any appreciable amounts in the resin products tested.

Column packing

Analytical columns were packed using conventional slurry packing techniques. Briefly, two columns of the same length were connected using a tubing union. A column end fitting was attached to the bottom of the assembly and the lower section was inserted into a constant temperature block previously set to 80°. After securing the assembly into a vertical position, the column was filled with resin slurry, capped with an end fitting and pumped for several hours at *ca.* 0.1 ml/min above the final operating flow-rate. Then the flow was stopped, the top end fitting was removed and the resin bed depth was checked with a small probe. When more resin was required, the supernatant liquid was removed with a syringe attached to a narrow bore Teflon™ tube, more resin slurry was added, the end fitting was replaced and the pumping continued for another hour.

The resin bed was again checked for height and the above procedure was repeated as required. When the resin bed extended into the tubing union or above, the column was capped. Capping was accomplished by first removing the upper half of the column assembly and tubing union and immediately attaching a column end fitting, previously packed with *ca.* 3 mm of resin bed. In principle, the column could be used in either direction but the end capped last was usually designated as the inlet.

Sample preparation

Samples were adjusted to a *ca.* 5–10% solids basis. Particulates were removed by filtration through a 0.45-μm membrane filter. Soluble protein and inorganic salts were removed by ion-exchange. Samples with low pH can be quite harmful to the resin since localized concentration of hydrogen ions will displace the calcium ions and decrease the efficiency of the analytical column. The ease of sample preparation is one of the strengths of this and other similar procedures[4].

New column evaluation

A corn syrup conventionally prepared by acid hydrolysis (so-called acid-converted corn syrup) is a sample type well suited for column evaluation. Twenty microliters of a 10% solution offers the chromatographer a dual benefit. First, the number of theoretical plates can be calculated from the glucose peak, which is last to emerge. It is important that chart speed is adjusted to obtain desirable chromatograms so that small errors in measurement do not result in a large error in plate number. Second, the oligosaccharides possessing similar linkages and derived from a common monomeric form (*i.e.*, glucose) can be counted upward from glucose to determine the highest saccharide resolved. If there is doubt as to a particular saccharide assignment, assistance can be provided by constructing a plot of log molecular weight of saccharide *versus* elution time or volume as in Fig. 2. It is worthy of note that a plot of the saccharide molecular weights *versus* time or volume conforms to the

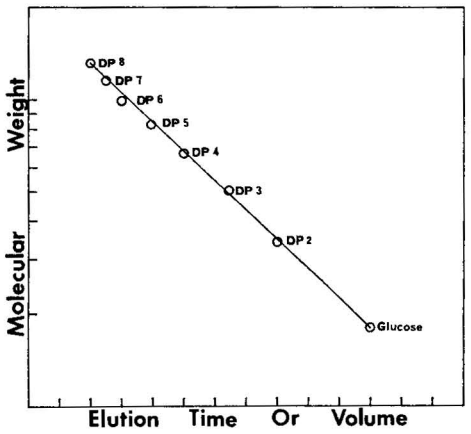

Fig. 2. Semi-log plot of molecular weight *versus* elution time or volume of the glucose homologous series. Saccharides were separated using a column (61 cm × 7 mm I.D.) of Aminex 50W-X4 ion-exchange resin (Ca^{2+}) operated at 80°. Water was pumped at 0.5 ml/min for elution and a differential refractometer used for the detector.

principle set forth by molecular exclusion chromatography. A periodic check of calcium saturation in the column can be accomplished using a dilute sucrose solution, since inversion of sucrose occurs when calcium is replaced by hydrogen ions on the resin[9].

RESULTS AND DISCUSSION

It is well known that small particles contribute to resolution within a given type of column packing, and that with the additional resolution comes an increase of pressure drop across the column. With the more highly cross-linked resins (6–8%) a high-pressure drop is not as damaging to resin bead integrity as with 4% cross-linked resins. With this in mind, 6 and 7% cross-linked resins (Ca^{2+}) of particle size 10–15 μm were tested in a (61 cm) column at 1.0 ml/min using water as eluent. It was hoped that, under these conditions, the 6 or 7% resin would combine the best

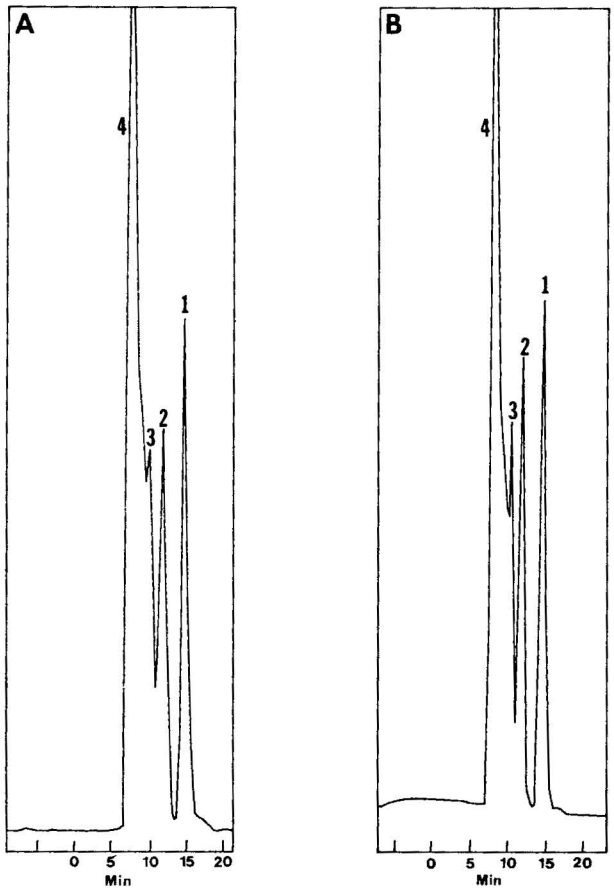

Fig. 3. Chromatograms of 42 D.E. acid-hydrolyzed corn syrup. Experimental parameters: column (61 cm × 7 mm I.D.) temperature, 80°; counterion, Ca^{2+}; flow-rate, 1.0 ml/min (water); column loading held essentially constant. Resin cross-linking: A, 8%; B, 6%. Peaks: 1 = glucose; 2 = maltose; 3 = maltotriose; and 4 = tetra- and higher saccharides.

features of the 4 and 8% cation resins. However, Fig. 3 shows a high degree of similarity between the 6 and 8% resin chromatograms: whereas a small particle size (7–10 μm), 6% cross-linked resin provided an acceptable value for the trisaccharides, it was not substantially better than an 8% resin and was not adequate for corn syrup characterization. In summary, the 6, 7 and 8% cross-linked ion-exchange resins (Ca^{2+}) were nearly equivalent in saccharide resolution, and the 4% crosslinked resin (Ca^{2+}) was still the best resolving medium.

The HC-X4.00 resin was available in a variety of particle sizes that might provide a better opportunity to optimize a separation, but primary emphasis was given to the particle sizes of 10–15 μm. All of the 4% resin tested was initially evaluated in a 61-cm column even though it was known that the chromatogram would be too time-consuming. The information obtained from the 61-cm column was used as a basis of comparison for the resins and also a means to relate to the prior work. Column pressure drop was found to be comparable for the resins tested. Resolution, as defined by the depth of the valleys between the peaks (peak sharpness) and the number of oligosaccharides resolved, was slightly better for the HC-X4.00 (particle size 10–15 μm) than the Aminex 50W-X4 (20–30 μm). The HC-X4.00 (particle size 7–10 μm) was examined briefly and gave a dramatic improvement to the overall resolution but it is not known how long the resin would perform at the higher pressure drops found. Because the 61-cm column procedures were too lengthy, mainly on account of the low flow-rates needed to maintain resin integrity, it was felt that a column [30.5 cm (1 ft.) $\times$ 7 mm I.D. (3/8 in. O.D.)] filled with a typical 4% resin and operated at 0.7 ml/min and at 80° could provide a desired analysis of corn syrup in the short time required.

Syrups examined

Fig. 4A, B, C and D show a variety of common corn syrups chosen because of the wide economic importance and for the differences in the saccharide distributions. Saccharide data from each of the various syrup types shown were quantitated on an area percent basis (component area expressed as a percent of the total area). Two reference procedures were used to analyze the syrups so that the results from the rapid method (described here) could be compared to the results from accepted methods. The reference methods were liquid chromatography[10] on high-resolution HC-X4.00 resin in a 2-ft. column pumped at 0.6 ml/min using saccharide calibration up to the trisaccharides, and classical quantitative paper chromatography[11]. Tables I–IV show the data comparison.

By virtue of the excellent agreement among the methods, the utility of the 30.5 cm column procedure was demonstrated. It was felt, however, that precision was equally important. Precision, expressed as the coefficient of variation, CV, can be demonstrated in several different ways. The same day and day-to-day variations were calculated for a typical syrup on a research basis during the method development. Because the procedure is rapid (less than 20 min), the approach lends itself to the needs of routine laboratory use. To test the utility of the procedure in a laboratory environment, an automatic sampler was added for 24-h operation. As a part of the normal operation, a daily control sample analyzed with other samples served two important purposes: (1) to monitor daily the quality of performance of the system by observing separation and quantitation; (2) to observe the long term

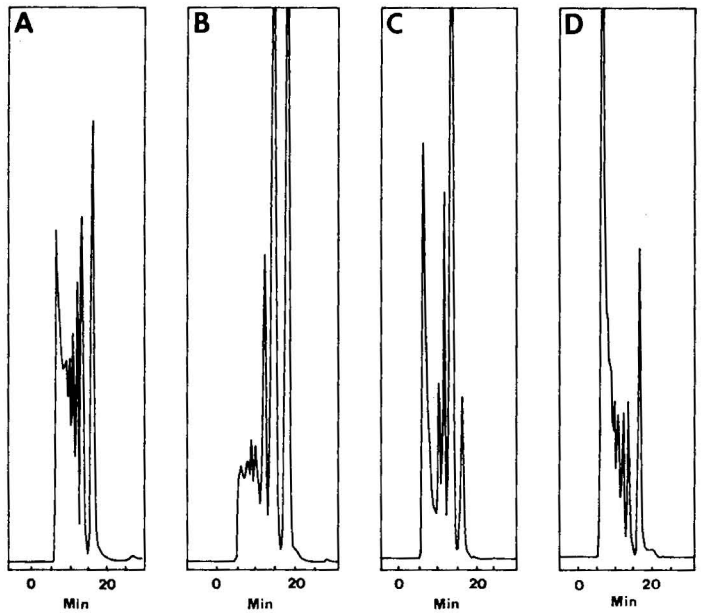

Fig. 4. Chromatograms of a 42 D.E. acid-hydrolyzed corn syrup (A), a 62 D.E. enzyme-converted corn syrup (B), a 42 D.E. high-maltose syrup (C) and a 30 D.E. acid-hydrolyzed corn syrup (D). Chromatographic conditions: stainless-steel column (30.5 cm × 7 mm I.D.) packed with Aminex 50W-X4 (20–30 μm) operated at 80° and pumped with water using a flow of 0.6 ml/min.

TABLE I

COMPARISON OF COMPOSITION BY LIQUID AND PAPER CHROMATOGRAPHY FOR 42 D.E. ACID-CONVERTED CORN SYRUP

*Component identification**	*% by liquid chromatography*		*% by paper chromatography*
	*1-ft.***	*2-ft.****	
DP 1	19.3	19.2	19.3
DP 2	14.1	13.8	14.4
DP 3	11.7	11.3	12.0
DP 4	9.3	9.0	9.9
DP 5	7.8	7.6	8.0
DP 6+	37.6	39.0	35.8

* Oligosaccharide degree of polymerization.

** Aminex 50W-X4 resin (20–30 μm) operated in a 30.5-cm (1-ft.) column at 80° using water at a flow-rate of 0.6 ml/min.

*** Aminex 50W-X4 resin operated in a 61-cm column at 80° using water at a flow-rate of 0.6 ml/min.

performance during the life of the analytical column. Table V shows typical values for precision.

The first column evaluated operated for about 2 months during which time about 1400 samples were analyzed. During this period, values for monosaccharides up to the pentasaccharide were provided although slight corrections were applied to the

area percent calculations near the end of the life of the column. A 2-month column-life is considered average for our application. This value could vary widely depending on the quality of samples injected, quality of eluent and general operating procedures.

TABLE II

COMPARISON OF COMPOSITION BY LIQUID AND PAPER CHROMATOGRAPHY FOR A 62 D.E. ENZYME-CONVERTED CORN SYRUP

Details as in Table I.

Component identification	*% by liquid chromatography*		*% by paper chromatography*
	1-ft.	*2-ft.*	
DP 1	46.7	46.2	47.4
DP 2	27.1	27.2	26.8
DP 3	6.2	6.1	6.1
DP 4	5.3	5.1	4.5
DP 5	3.8	3.6	3.6
DP 6^+	10.9	11.8	11.2

TABLE III

COMPARISON OF COMPOSITION BY LIQUID CHROMATOGRAPHY AND PAPER CHROMATOGRAPHY FOR A HIGH MALTOSE SYRUP

Because of the saccharide distribution, only DP 5^+ is reported instead of DP 6^+. Other details as in Table I.

Component identification	*% by liquid chromatography*		*% by paper chromatography*
	2-ft.	*1-ft.*	
DP 1	7.1	7.5	7.5
DP 2	39.3	39.8	40.1
DP 3	15.4	15.1	15.3
DP 4	7.3	7.1	7.8
DP 5^+	30.9	30.5	29.3

TABLE IV

COMPARISON OF COMPOSITION DATA FROM TWO METHODS OF LIQUID CHROMATOGRAPHY FOR A 30 D.E. ACID-CONVERTED CORN SYRUP

Details as in Table I.

Component identification	*% by liquid chromatography*	
	2-ft.	*1-ft.*
DP 1	14.4	14.4
DP 2	7.1	7.0
DP 3	6.3	6.4
DP 4	5.9	6.1
DP 5	6.0	6.1
DP 6^+	60.1	60.0

TABLE V

ANALYTICAL PRECISION FOR CORN SYRUPS USING THE PROPOSED RAPID PROCEDURE

Component	*Coefficient of variation*			
	Research data (short term) *		*Routine lab. data (long term)* **	
	Product A	*Product B*	*Product A*	*Product B*
DP 1	0.41	0.20	1.55	1.06
DP 2	0.21	0.33	2.03	1.25
DP 3	0.60	0.97	2.18	2.44
DP 4	0.22	1.13	3.80	5.14
DP 5	0.77	0.79	6.46	6.28
DP 6^+	0.48	1.01	2.25	2.49

* Measured over the span of several days.
** Measured over the span of 6–8 weeks.

CONCLUSION

We conclude that a quality 4% resin packed in a column (30.5 cm × 7 mm I.D.) operated at 80° using water as the eluent pumped at 0.6 ml/min offers a rapid (less than 20 min), accurate means for saccharide characterization of corn syrup. With peripheral equipment, such as an automatic sampler and computing integrator, this system is a powerful tool for routine laboratory analysis.

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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF AMIODARONE IN BLOOD PLASMA OR SERUM AT THE CONCENTRATIONS ATTAINED DURING THERAPY

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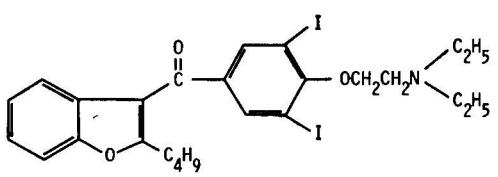
SUMMARY

A simple high-performance liquid chromatographic method has been developed for the measurement of the antiarrhythmic drug amiodarone in small (200 μl) volumes of plasma or serum. After addition of 2 mole/l phosphate solution, pH 4.5 (20 μl), containing the internal standard, the sample is vortex-mixed with diisopropyl ether (200 μl) for 30 sec. A portion (100 μl) of the resulting extract is analysed on a microparticulate (5 μm) silica column using methanol–diethyl ether (85:15) containing perchloric acid (0.02% v/v) as the mobile phase, and the absorption of the column effluent is monitored at 240 nm. No endogenous sources of interference have been observed, and interference from other drugs is minimal. The procedure is rapid, an analysis in duplicate taking less than 15 min to complete. The limit of sensitivity of the assay is 0.05 mg/l, and the concentrations of amiodarone measured in plasma samples from patients under treatment with this compound ranged from 0.15 to 4.5 mg/l.

INTRODUCTION

Amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)benzofuran; Fig. 1] is an orally effective antiarrhythmic agent. It is thought that this compound may have a relatively long half-life in man since optimal therapeutic effects may only be seen some days after the commencement of therapy[1], and may persist for some time after withdrawal of the drug[2]. However, the pharmacokinetics of amiodarone have received only scant attention using radio-labelled drug[3].



Amiodarone

Fig. 1. Structural formula of amiodarone.

The method described here for the measurement of plasma amiodarone concentrations is based upon the principle of solvent extraction of a relatively small plasma volume, followed by the direct chromatographic analysis of a portion of the resulting extract[4]. In this case, the primary chromatographic system chosen was a microparticulate (5 μm) silica column using methanol–diethyl ether containing 0.02% (v/v) perchloric acid as the mobile phase. However, in the absence of an established reference technique the results of sample analyses were compared to those obtained on a nitrile-bonded microparticulate silica column.

EXPERIMENTAL

Materials and reagents

Amiodarone and the internal standard, fenethazine [10-(2-dimethylaminoethyl)phenothiazine hydrochloride], were obtained from Labaz (Brussels, Belgium) and Rhône-Poulenc (Paris, France), respectively. The internal standard was used as a 12.5 mg/l solution in 2 mole/l aqueous potassium dihydrogen orthophosphate (analytical reagent grade), pH 4.5. Methanol and diethyl ether were both HPLC Grade (Rathburn Chemicals, Walkerburn, Great Britain), and diisopropyl ether (laboratory reagent grade) and perchloric acid (70%) (analytical reagent grade) were obtained from BDH (Poole, Great Britain).

High-performance liquid chromatography (HPLC)

The solvent delivery system was a constant-flow reciprocating pump (Applied Chromatography Systems, Model 750/03) and sample injection was performed using a Rheodyne Model 7120 syringe-loading valve fitted with a 100-μl sample loop. Stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet port of the valve to the analytical column, a stainless-steel tube 125 × 5 mm I.D. packed with Spherisorb 5 silica (Hichrom, Woodley, Great Britain), which was used at ambient temperature (normally 22°). The column effluent was monitored at 240 nm (Applied Chromatography Systems, Model 750/11) and integration of peak areas was performed using a Hewlett-Packard 3352 data system. The mobile phase was methanol–diethyl ether (85:15) containing 0.02% (v/v) perchloric acid, and this was helium-degassed before use. The flow-rate was 2.0 ml/min, maintained by a pressure of *ca.* 40 bar.

The chromatography on this system of an extract from a plasma standard containing amiodarone and fenethazine is illustrated in Fig. 2. The retention times of these compounds and some additional drugs on this system are given in Table I.

Sample preparation

Plasma or serum (200 μl) was pipetted into a small (Dreyer) test-tube (Poulten, Selfe and Lee, Wickford, Great Britain). Internal standard solution (20 μl) and diisopropyl ether (200 μl) were added using Hamilton repeating mechanisms fitted with 1.0- and 5.0-ml Hamilton gas-tight luer-fitting glass syringes (Field, Richmond, Great Britain), respectively. Everett stainless-steel needles (No. II serum) were fitted to these syringes. The contents of the tube were vortex-mixed for 30 sec and the tube was centrifuged at 9950 *g* for 2 min in an Eppendorf 5412 centrifuge (Anderman, East Molesey, Great Britain) which was modified to accept Dreyer tubes

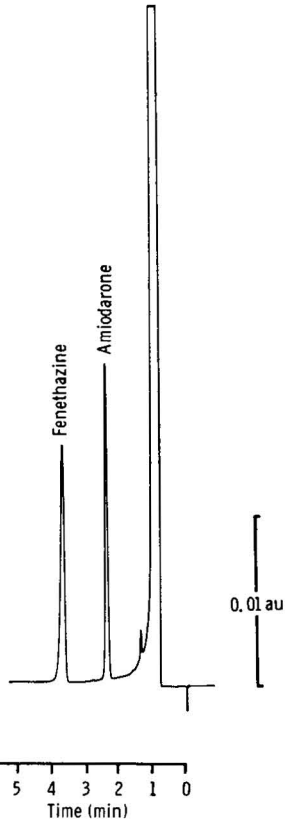


Fig. 2. Chromatogram obtained on analysis of a diisopropyl ether extract of heparinised bovine plasma containing amiodarone (1.0 mg/l) on the 5-μm silica column; 100 μl injection. See text for chromatographic conditions.

by slightly drilling-out the 0.4-ml test-tube centrifuge adapters. Subsequently, *ca.* 110 μl of the extract were taken and used to fill the sample loop of the injection valve. Analyses were performed in duplicate and the mean result taken.

Instrument calibration

Standard solutions containing amiodarone at concentrations of 0.10, 0.20, 0.50, 1.00, 2.00 and 5.00 mg/l were prepared in heparinised bovine plasma by dilution of a 1 g/l solution of this compound in methanol. The plasma and methanolic solutions were stable for at least 1 and 3 months, respectively, if stored at 4° and in the absence of light.

On analysis of these solutions, the ratio of the peak area of drug to the peak area of the internal standard, when plotted against drug concentration, was linear and passed through the origin of the graph. The calibration gradient normally obtained was 1.12 l/mg.

TABLE I

RETENTION TIMES RELATIVE TO FENETHAZINE OF AMIODARONE AND OTHER COMPOUNDS ON (i) THE MICROPARTICULATE SILICA AND (ii) THE NITRILE-BONDED COLUMNS

Compound	*Relative retention times*	
	(i) 5 μm Silica column	*(ii) 5 μm Nitrile column*
Glutethimide	0.20	0.23
Butobarbitone	0.21	0.24
Quinalbarbitone	0.21	0.24
Barbitone	0.22	0.23
Phenytoin	0.22	0.24
Cyclobarbitone	0.23	0.25
Ethotoin	0.23	0.27
Phenylbutazone	0.23	0.25
Amylobarbitone	0.24	0.24
Chlorpropamide	0.24	0.24
Ethosuximide	0.24	0.23
Feprazone	0.24	0.28
Heptabarbitone	0.24	0.23
Methoin	0.24	0.25
Methosuximide	0.24	0.27
Pentobarbitone	0.24	0.24
Pheneturide	0.24	0.23
Phenobarbitone	0.24	0.25
Phenylethylmalondiamide	0.24	0.25
Thiopentone	0.24	0.27
Azapropazone	0.26	0.25
Phenacetin	0.26	0.25
Primidone	0.26	0.23
Caffeine	0.27	0.31
Carbamazepine	0.27	0.29
Lorazepam	0.29	0.33
Oxazepam	0.36	0.55
Temazepam	0.37	0.49
Desalkylflurazepam	0.38	0.72
Nitrazepam	0.44	0.81
Nordiazepam	0.44	0.85
"Amiodarone metabolite"	0.45	0.78
Lignocaine	0.53	0.81
Carbamazepine-10,11-epoxide	0.54	0.82
Chlordiazepoxide	0.55	0.90
Diazepam	0.56	1.00
Propylphenazone	0.60	0.73
Lorcainide	0.64	1.03
Methaqualone	0.62	0.80
Amiodarone	0.68	0.90
Chlormethiazole	0.76	0.75
Chlorpromazine	0.81	0.96
Promethazine	0.83	1.00
Medazepam	0.84	1.00
Phenazone	0.85	0.95
Prochlorperazine	0.85	0.97
Fenethazine	1.00	1.00
Promazine	1.06	1.00
Flurazepam	1.75–1.90*	1.55

* Tailing peak; retention times measured at 1 g/l and 10 mg/l, respectively.

RESULTS AND DISCUSSION

Choice of extraction and chromatographic conditions

Under the extraction conditions chosen (pH 6), the recovery of amiodarone from heparinised bovine plasma was 98.6% ± 3.3 (S.D.) over the range 0.5, 1.0, 2.0 and 5.0 mg/l ($n = 3$ at each concentration). In contrast, the recovery was only 21.0% ± 3.9 (S.D.) at pH 9. Solutions prepared in heparinised human plasma gave analogous results. These findings were obtained by direct comparison of the peak areas obtained on analysis of 100 μl portions of methanolic solutions of amiodarone to those obtained from freshly-prepared sample extracts. However, an internal standard was incorporated in the final analytical procedure in order to minimize possible errors due to partial evaporation of the extract prior to analysis, and also to facilitate the use of injection volumes of less than 100 μl should this prove necessary. For example, duplicate results can be obtained where only 100 μl of sample is available for each extract by using the other reagents in proportion.

Diisopropyl ether was found to be a suitable extraction solvent since it is considerably less volatile than solvents such as diethyl ether and, thus, evaporation of the extract prior to analysis was minimised. On the other hand, diethyl ether was used as the lipophilic constituent of the mobile phase since commercially available diisopropyl ether contains 0.01% (w/v) hydroquinone resulting in a very high absorbtion at 240 nm, a convenient $\lambda_{max.}$ of amiodarone. The presence of hydroquinone in the extracts did not interfere in the analysis since it gave rise in part to the "solvent front" observed (Figs. 2–4).

It was necessary to add perchloric acid in low concentrations (0.02%, v/v) to the mobile phase in order to promote elution of the compounds under study. Under these conditions, the relative retention times of these compounds (Table I) varied by ±0.01, although during routine use the absolute retention times did vary by up to 15%. A higher perchlorate concentration than that indicated gave very rapid elution but with some loss of resolution, whilst lower concentrations gave longer retention times and permitted some peak tailing. Acetic acid, at concentrations up to 1% (v/v), was ineffective in promoting the elution of these compounds; higher concentrations than 1% could not be used since the absorption of the solvent at 240 nm became limiting.

Specificity

No endogenous sources of interference have been observed. The chromatogram obtained on analysis of a specimen of drug-free human plasma is illustrated in Fig. 3, and the chromatogram obtained from a plasma specimen from a patient treated chronically with amiodarone is given in Fig. 4. In both cases, analyses of these and other specimens performed without the addition of fenethazine have not revealed the presence of compounds which could co-elute with this standard.

The compound which eluted with a retention time of 0.45 relative to fenethazine (Fig. 4) has only been observed in extracts from patients treated chronically with amiodarone, and increased in concentration with time in samples obtained from a patient soon after the start of amiodarone therapy. This presumed metabolite of amiodarone appears to have a similar UV absorption spectrum to the parent compound, but is extracted more efficiently at pH 7.4 than under the conditions used in the assay.

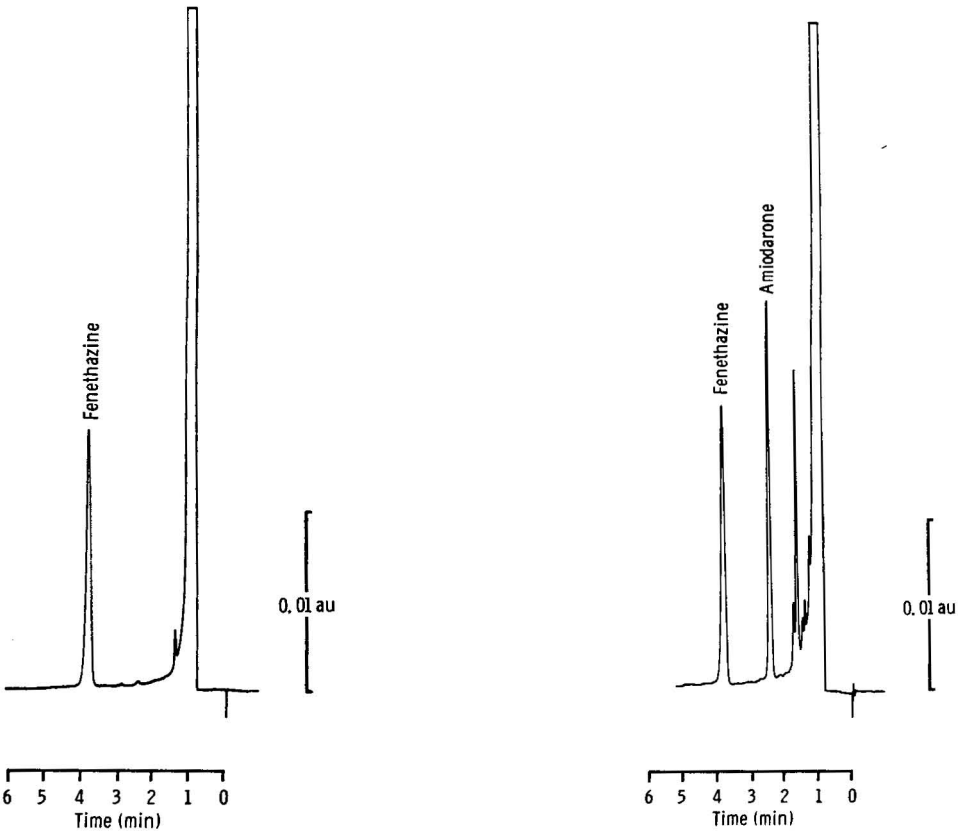


Fig. 3. Chromatogram obtained on analysis of an extract of drug-free human plasma on the 5 μm silica column; 100 μl injection.

Fig. 4. Chromatogram obtained on analysis of an extract of plasma from an amiodarone-treated patient on the 5 μm silica column; 100 μl injection. The amiodarone dose was 200 mg/day, and the plasma amiodarone concentration was found to be 1.1 mg/l.

Neither amiodarone nor this presumed metabolite have been detected in urine specimens from two of the amiodarone-treated patients studied.

Additional cardio-active drugs which might have interfered (disopyramide, mexiletine, procainamide, quinidine and tocainide) were not extracted under the conditions of the assay. The potential interference from a number of other drugs which are extracted under these conditions and have significant absorption at 240 nm has been studied (Table I). The concentrations of lorcainide attained during oral therapy are normally below 0.4 mg/l (ref. 5) and since this compound is poorly extracted at an acidic pH, such interference as may occur is unlikely to prove serious. Of the remaining compounds, only methaqualone and promazine eluted with amiodarone and fenethazine, respectively. Should interference from promazine occur, quantitative analyses can be performed by direct comparison of sample and standard peak areas of amiodarone from freshly-prepared extracts as discussed previously. Interference from methaqualone may be countered by using an alternative chromatographic system, such as that discussed in the following paragraph.

Comparison with results obtained on a second chromatographic system

The results of sample analyses were compared to those obtained on a second chromatographic system in order to provide additional evidence as to the specificity of the assay. The extraction and instrument calibration were identical to those described previously. The column used was a stainless-steel tube (250 × 5 mm I.D., packed with Spherisorb 5 nitrile (Hichrom), and the mobile phase was methanol–propan-2-ol (AR Reagent grade), 75:25, containing 0.02% (v/v) perchloric acid. The other chromatographic conditions were stated previously for the Spherisorb 5 silica column, except that a pressure of 120 bar was required to give a flow-rate of 2.0 ml/min.

The retention times of amiodarone and its presumed metabolite together with those of some other compounds on this nitrile-bonded column are given in Table I. The retention time of fenethazine under these conditions was 6.9 min. No compounds in addition to amiodarone and this presumed metabolite were observed in sample extracts from patients treated with amiodarone. Of the commonly-used drugs, only diazepam interfered in the assay on this column since it was not resolved from the internal standard (Table I). Thus, for the purposes of the comparison specimens which contained diazepam were assayed by direct measurement of amiodarone peak areas from freshly-prepared sample and standard extracts.

Twenty-eight plasma specimens (five containing diazepam) were analysed using both the nitrile and silica columns and the results of this comparison are presented in Fig. 5. The mean results obtained on each system were almost identical [silica: 1.32 mg/l ± 1.03 (S.D.); nitrile: 1.30 mg/l ± 1.02 (S.D.)] and the regression coefficient (using the data from the silica column as the independent variable) was 0.994 with an intercept of −0.011 mg/l.

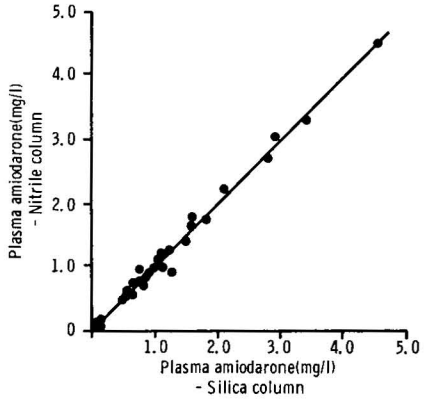


Fig. 5. Results of amiodarone assays performed independently on two different column systems. (The regression line shown is that of x on y.)

Reproducibility

The intra-assay coefficients of variation (C.V.) measured from replicate analyses ($n = 10$) of standard solutions prepared in heparinised bovine plasma containing amiodarone at concentrations of 0.2, 1.0 and 2.0 mg/l were 3.8%, 2.5% and 2.2%, respectively. The inter-assay C.V. at 1.0 mg/l was 6.9% ($n = 10$).

Limit of sensitivity

The limit of accurate measurement of the assay was 0.05 mg/l; the intra-assay C.V. at this concentration was 7.1% ($n = 10$). The plasma amiodarone concentrations measured in specimens obtained from patients receiving from 200 to 600 mg/day of this compound ranged from 0.15 to 4.5 mg/l (Fig. 5); no amiodarone was detected in a blood sample from one patient prescribed 200 mg/day, non-compliance with therapy being the most likely explanation.

CONCLUSIONS

The method described here has been found to be suitable for the measurement of the plasma amiodarone concentrations attained during therapy and may prove useful in single-dose pharmacokinetic studies. Only 400 μl of specimen are required for a duplicate analysis, which can be completed within 15 min, and sources of interference are minimal. In addition, although no amiodarone was detected in two urine specimens from patients treated with this compound, the method may prove useful in the analysis of amiodarone in other biological fluids such as saliva. Finally, the nature of the presumed metabolite of amiodarone observed on sample extract chromatograms is the subject of active investigation.

ACKNOWLEDGEMENTS

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Journal of Chromatography, 187 (1980) 399–408

CHROM. 12,330

SEPARATION OF FLUORESCENT COMPOUNDS IN MEMBERS OF THE GENUS *RHIZOPUS* BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Numerous fluorescent compounds were detected and separated by thin-layer chromatography in the chloroform extracts of species of *Rhizopus*. Qualitative similarities and differences existed among the species with respect to specific fluorescent compounds.

INTRODUCTION

The presence of secondary metabolites from mycelium of fungi has been well documented by numerous investigators[1–5]. These secondary metabolites, which serve no known function in the life cycle of many fungi[6], include antibiotics, pigments, mycotoxins and others. Chromatographic methods, which include gas–liquid chromatography[7], high-performance liquid chromatography[8], thin-layer chromatography (TLC)[9–13], and paper chromatography[14–20] have proved very useful in their separation.

This paper describes the use of TLC for the detection of UV-absorbing substances in *Rhizopus* species.

METHODS AND MATERIALS

Species of *Rhizopus* used in this work were obtained from the American Type Culture Collection (Rockville, Md., U.S.A.) and the Department of National Health and Welfare (Ottawa, Canada). Stock cultures were maintained at 5° on slants of potato dextrose agar (PDA) enriched with 0.2% yeast extract. Spores from heavily sporulated cultures, which had been previously grown on slants of PDA enriched with 0.2% yeast extract (PDAYE) for 7 days at 27°, were washed off the PDAYE slant with sterile distilled water. The spore suspension was adjusted to an optical density of 10.0 Klett Units at 450 nm with a blue filter (photoelectric colorimeter, Klett Sumnerson, New York, N.Y., U.S.A.) before being used to inoculate the medium. 50 ml of mycological broth enriched with 0.5% yeast extract (MYE) and 15% sucrose plus 2% yeast extract (YES) were inoculated with 1.0 ml of spore suspension and allowed to incubate for 7 and 14 days respectively at 27° (ref. 21).

The procedure for extraction of secondary metabolites was based on that of

Armbrecht *et al.*[22]. The mycelial mats were extracted with two volumes of warm (60°) chloroform in a Waring Blendor and then concentrated to dryness in a flash evaporator.

For the separation of the secondary metabolites, the chloroform extracts were dissolved in 0.2 ml of chloroform and 15 μl of each extract were spotted on 0.500 mm TLC plates (Redi-Coat 5-8172, Aflasil, Supelco, Bellefonte, Pa., U.S.A.). The plates were developed in two solvent systems (chloroform–methanol (99:1, v/v) (CM) and benzene–methanol–acetic acid (24:4:1, v/v) (BMA). After development, the plates were examined under longwave UV light. Fluorescent materials from the extracts and separated on TLC plates were compared with authentic mycotoxins and aflatoxins. To determine whether non-fluorescent materials were also present in the extracts, the plates were (1) sprayed with a freshly prepared mixture of 0.5 ml of *p*-anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulfuric acid and then heated at 210° for 8–20 min[23] and (2) sprayed with 1% ethanolic ferric chloride[24].

RESULTS AND DISCUSSION

Seven members of the *Rhizopus* group were evaluated for their ability to produce metabolites in a chemically undefined culture medium and a semi-chemically defined culture medium. Visual examination of the TLC plates accomplished in the dark, with the aid of UV light, revealed numerous fluorescent compounds, as shown in Tables I–IV. The number of fluorescent compounds separated and detected in the two solvent systems CM and BMA, respectively, are as follows: (YES medium for 7 days) *R. arrhizus*, 6 and 5; *R. chinensis*, 8 and 8; *R. circinans*, 8 and 6; *R. kazanensis*, 4 and 6; *R. oryzae*, 6 and 6; *R. stolonifer*, 8 and 7; *R.* 66-81-2, 4 and 6; (YES medium for 14 days) *R. arrhizus*, 5 and 6; *R. chinensis*, 5 and 8; *R. circinans*, 7 and 6; *R. kazanensis*, 10 and 7; *R. oryzae*, 6 and 6; *R. stolonifer*, 6 and 5; *R.* 66-81-2, 5 and 7; (MYE medium for 7 days) *R. arrhizus*, 5 and 2; *R. chinensis*, 4 and 3; *R. circinans*, 2 and 1; *R. kazanensis*, 4 and 3; *R. oryzae*, 3 and 4; *R. stolonifer*, 4 and 4; *R.* 66-81-2; (MYE medium for 14 days) *R. arrhizus*, 8 and 4; *R. chinensis*, 6 and 4; *R. circinans*, 6 and 5; *R. kazanensis*, 7 and 6; *R. oryzae*, 7 and 5; *R. stolonifer*, 6 and 4; and *R.* 66-81-2, 4 and 5.

The data indicate that the seven fungi examined produced a number of fluorescent substances when incubated in chemically undefined and semi-chemically defined media. The fluorescent compounds were analyzed by their chromatographic behavior, reaction to spray reagents, and spectra. The data obtained from these analyses were then compared with those of known mycotoxins. The data on the fluorescent compounds did not reveal the presence of any of the known mycotoxins[25]. Qualitative differences were apparent in the number of detectable fluorescent substances with respect to (1) separation in the two solvent systems, (2) the media, and (3) period of incubation. It was of interest, however, to note the presence of a common fluorescent compound in all seven of the fungi which appeared green before spray treatment and purple after spray treatment with *p*-anisaldehyde in visible light. Caution must be exercised, however, in the interpretation of the significance of the differences and similarities of fluorescent compounds which were found in the fungi examined during this investigation. Studies on the structural elucidation and identi-

TABLE I

TLC OF THE CHLOROFORM EXTRACT OF *RHIZOPUS* SPECIES GROWN IN YES MEDIUM FOR 7 DAYS

Organism	*Spot*	R_F	*Solvent system*	*Detection*					
				Before spray treatment		*After spray treatment*			
				Visible light	*Ultra-violet*	*p-Anisaldehyde*		*Iron chloride*	
						Visible light	*Ultra-violet*	*Visible light*	*Ultra-violet*
R. arrhizus	1	0.23	CM	—	Blue	—	—		
	1b	0.38	CM	—	Green			—	Green
	2	0.48	CM	—	—			—	Pale Blue
	3	0.53	CM	—	Green	Purple	—		
	4	0.76	CM	—	Pale Blue	Green	—	—	Pale Blue
	5	0.97	CM	—	Bright Blue	Green	—		
	1	0.09	BMA	—	—	Green	—		
	2	0.31	BMA	—	Blue	—	—		
	2b	0.38	BMA	—	Green			—	Green
	3	0.48	BMA	—	Pale Blue			—	Pale Blue
	4	0.72	BMA	—	Pale Blue	Green	—	—	Pale Blue
R. chinensis	1	0.14	CM	—	Blue	—	—		
	2	0.29	CM	—	Blue	—	—		
	3	0.41	CM	—	Green	—		—	Green
	3b	0.48	CM	—	Bright Blue			—	Green
	4	0.64	CM	—	Green	Purple	—		
	5	0.73	CM	—	Dull Blue	—	—		
	6	0.85	CM	—	Blue	Green	—	—	Blue
	7	0.94	CM	—	Pale Blue	—	—	—	Pale Blue
	1	0.07	BMA	—	Blue	—	—	—	Blue
	2	0.15	BMA	—	Blue			—	Blue
	3	0.28	BMA	—	Blue			—	Blue
	4	0.34	BMA	—	Blue			—	Blue
	5	0.44	BMA	—	Blue	—	—		
	6	0.73	BMA	—	Green	Purple	—	—	Green
	7	0.84	BMA	—	Blue	Green	—	—	Blue
	8	0.91	BMA	—	Dull Blue	Green	—		
R.circinans	1	0.12	CM	—	Blue	—	—		
	2	0.28	CM	—	Blue	—	—		
	3	0.41	CM	—	Green			—	Green
	4	0.50	CM	—	Bright Blue			—	Bright Blue
	5	0.63	CM	—	Green	Purple	—		
	6	0.75	CM	—	Blue	Green	—		
	7	0.83	CM	—	Dull Blue	Green	—	—	Dull Blue
	8	0.97	CM	—	Bright Blue	—	—		
	1	0.09	BMA	—	—	Green	—		
	2	0.16	BMA	—	Blue			—	Blue
	3	0.333	BMA	—	Blue	—	—	—	Blue
	4	0.44	BMA	—	Blue	—	—		
	5	0.71	BMA	—	Green	Purple	—	—	Green
	6	0.82	BMA	—	Dull Blue	Green	—	—	Dull Blue

(Continued on p. 402)

TABLE I *(continued)*

Organism	*Spot*	R_F	*Solvent system*	*Detection*					
				Before spray treatment		*After spray treatment*			
						p-Anisaldehyde		*Iron chloride*	
				Visible light	*Ultra-violet*	*Visible light*	*Ultra-violet*	*Visible light*	*Ultra-violet*
R. kazanensis	1	0.12	CM	—	Blue	—	—		
	2	0.20	CM	—	Blue	—	—		
	3	0.50	CM	—	Green	Purple	—	—	Green
	4	0.82	CM	—	Pale Blue	Green	—	—	Pale Blue
	1	0.06	BMA	—	Blue			—	Blue
	2	0.14	BMA	—	Blue	Green	—	—	Blue
	3	0.31	BMA	—	Blue			—	Blue
	4	0.40	BMA	—	Blue	—	—	—	Blue
	5	0.74	BMA	—	Green	Purple	—	—	Green
	6	0.94	BMA	—	Pale Blue	—	—		
R. oryzae	1	0.09	CM	—	Blue	—	—		
	2	0.22	CM	—	Blue	—	—		
	3	0.43	CM	—	Green	—	—	—	Green
	4	0.53	CM	—	Bright Blue	Purple	—	—	Bright Blue
	5	0.66	CM	—	Blue	Green	—		
	6	0.80	CM	—	Pale Blue			—	Pale Blue
	1	0.05	BMA	—	Blue			—	Blue
	2	0.14	BMA	—	Blue	—	—	—	Blue
	3	0.28	BMA	—	Blue	—	—	—	Blue
	4	0.40	BMA	—	Blue	—	—	—	Blue
	5	0.68	BMA	—	Green	Purple	—	—	Green
	6	0.78	BMA	—	Blue			—	Blue
R. stolonifer	1	0.09	CM	—	Blue	—	—		
	2	0.22	CM	—	Blue	—	—		
	3	0.41	CM	—	Green			—	Green
	4	0.53	CM	—	Bright Blue			—	Bright Blue
	4b	0.56	CM	—	Green	Purple	—		
	5	0.69	CM	—	Blue	Green	—		
	6	0.81	CM	—	Dull Blue	Green	—		
	7	0.90	CM	—	Pale Blue			—	Pale Blue
	1	0.06	BMA	—	Blue			—	Blue
	2	0.14	BMA	—	Blue	Green	—	—	Blue
	3	0.28	BMA	—	Blue			—	Blue
	4	0.37	BMA	—	Blue			—	Blue
	5	0.42	BMA	—	Blue	—	—		
	6	0.68	BMA	—	Green	Purple	—	—	Green
	7	0.75	BMA	—	Blue			—	Blue
R. 66-81-2	1	0.21	CM	—	Blue	—	—		
	2	0.40	CM	—	Green			—	Green
	3	0.56	CM	—	Green	Purple	—		
	4	0.75	CM	—	Blue	Green	—	—	Blue
	1	0.06	BMA	—	Blue			—	Blue
	2	0.14	BMA	—	Blue	—	—	—	Blue
	3	0.26	BMA	—	Blue			—	Blue
	4	0.35	BMA	—	Blue	—	—	—	Blue
	5	0.65	BMA	—	Green	Purple	—	—	Green
	6	0.81	BMA	—	Pale Blue	Green	—	—	Pale Blue

TABLE II

TLC OF THE CHLOROFORM EXTRACT OF *RHIZOPUS* SPECIES GROWN IN YES MEDIUM FOR 14 DAYS

Organism	*Spot*	R_F	*Solvent system*	*Detection*					
				Before spray treatment		*After spray treatment*			
				Visible light	*Ultra-violet*	*p-Anisaldehyde*		*Iron chloride*	
						Visible light	*Ultra-violet*	*Visible light*	*Ultra-violet*
R. arrhizus	1	0.08	CM	—	Bright Blue	—	—		
	2	0.41	CM	—	Green	Purple	—	—	Green
	3	0.72	CM	—	Blue			—	Blue
	4	0.76	CM	—	Dull Blue	Green	—	—	Dull Blue
	5	0.97	CM	—	Bright Blue	Green	—		
	1	0.09	BMA	—	Blue			—	Blue
	2	0.16	BMA	—	Blue			—	Blue
	3	0.41	BMA	—	Blue	—	—		
	4	0.63	BMA	—	Green	Purple	—	—	Green
	5	0.76	BMA	—	Blue	Green	—	—	Blue
	6	0.91	BMA	—	Bright Blue	—	—		
R. chinensis	1	0.12	CM	—	Blue			—	Blue
	2	0.25	CM	—	Blue			—	Blue
	3	0.46	CM	—	Green	Purple	—	—	Green
	4	0.76	CM	—	Blue	—	—	—	Blue
	5	0.94	CM	—	Dull Blue	Green	—		
	1	0.06	BMA	—	Blue			—	Blue
	2	0.13	BMA	—	Blue			—	Blue
	3	0.18	BMA	—	Blue			—	Blue
	4	0.41	BMA	—	Blue	—	—		
	5	0.65	BMA	—	Green	Purple	—	—	Green
	6	0.75	BMA	—	Blue			—	Blue
	7	0.88	BMA	—	Blue	—	—		
	8	0.95	BMA	—	Dull Blue	—	—	—	Dull Blue
R. circinans	1	0.08	CM	—	Blue	—	—		
	2	0.23	CM	—	Blue	—	—		
	3	0.45	CM	—	Green	Purple	—	—	Green
	4	0.67	CM	—	Blue			—	Blue
	5	0.76	CM	—	Blue			—	Blue
	6	0.84	CM	—	Dull Blue	Green	—		
	7	0.97	CM	—	Bright Blue	Green	—		
	1	0.07	BMA	—	Blue			—	Blue
	2	0.16	BMA	—	Blue			—	Blue
	3	0.28	BMA	—	Blue	—	—	—	Blue
	4	0.51	BMA	—	Blue	—	—	—	Blue
	5	0.81	BMA	—	Green	Purple	—		
	6	0.91	BMA	—	Dull Blue	Green	—	—	Dull Blue
R. kazanensis	1	0.03	CM	—	Blue			—	Blue
	2	0.06	CM	—	Blue	—	—	—	Blue
	3	0.19	CM	—	Blue	—	—		

(Continued on p. 404)

TABLE II *(continued)*

Organism	*Spot*	R_F	*Solvent system*	*Detection*					
				Before spray treatment		*After spray treatment*			
				Visible light	*Ultra-violet*	*p-Anisaldehyde*		*Iron chloride*	
						Visible light	*Ultra-violet*	*Visible light*	*Ultra-violet*
	4	0.26	CM	—	Green			—	Green
	5	0.38	CM	—	Dull Blue	—	—		
	6	0.50	CM	—	Blue	—	—		
	7	0.63	CM	—	Green	Purple	—		
	8	0.66	CM	—	Blue			—	Blue
	9	0.75	CM	—	Blue			—	Blue
	10	0.87	CM	—	Blue	Green	—	—	Blue
	1	0.15	BMA	—	Blue	—	—	—	Blue
	2	0.23	BMA	—	Green	—	—		
	3	0.35	BMA	—	Blue	—	—	—	Blue
	4	0.47	BMA	—	Blue	—	—	—	Blue
	5	0.61	BMA	—	Green	—	—	—	Green
	6	0.75	BMA	—	Green	Purple	—	—	Green
	7	0.90	BMA	—	Blue	Green	—	—	Blue
R. oryzae	1	0.07	CM	—	Blue	—	—	—	Blue
	2	0.19	CM	—	Blue	—	—		
	3	0.41	CM	—	Green	Purple	—	—	Green
	4	0.71	CM	—	Blue			—	Blue
	5	0.81	CM	—	Blue			—	Blue
	6	0.88	CM	—	Blue	Green	—	—	Blue
	1	0.01	BMA	—	—	Green	—		
	2	0.17	BMA	—	Blue	—	—	—	Blue
	3	0.31	BMA	—	Blue	—	—	—	Blue
	4	0.47	BMA	—	Dull Blue	—	—		
	5	0.64	BMA	—	Green	Purple	—	—	Green
	6	0.84	BMA	—	Dull Blue	Green	—	—	Dull Blue
R. stolonifer	1	0.08	CM	—	Blue	—	—		
	2	0.22	CM	—	Blue	—			
	3	0.45	CM	—	Green	Purple	—	—	Green
	4	0.68	CM	—	Blue			—	Blue
	5	0.78	CM	—	Blue			—	Blue
	6	0.93	CM	—	Blue	Green	—	—	Green
	1	0.15	BMA	—	Blue	—	—	—	Blue
	2	0.25	BMA	—	Blue	—	—	—	Blue
	3	0.34	BMA	—	Blue	—	—	—	Blue
	4	0.66	BMA	—	Green	Purple	—	—	Green
	5	0.94	BMA	—	Blue	Green	—	—	Blue
R. 66-81-2	1	0.12	CM	—	Blue	—	—	—	Blue
	2	0.39	CM	—	Green	Purple	—	—	Green
	3	0.56	CM	—	Blue			—	Blue
	4	0.65	CM	—	Blue			—	Blue
	5	0.86	CM	—	Blue	Green	—	—	Green
	1	0.10	BMA	—	—	Green	—		
	2	0.15	BMA	—	Blue	Green	—	—	Blue
	3	0.25	BMA	—	Blue	—	—	—	Blue
	4	0.33	BMA	—	Blue	—	—		
	5	0.43	BMA	—	Dull Blue	—	—		
	6	0.57	BMA	—	Green	Purple	—	—	Green
	7	0.86	BMA	—	Blue	Green	—	—	Blue

TABLE III

TLC OF THE CHLOROFORM EXTRACT OF *RHIZOPUS* SPECIES GROWN IN MYE MEDIUM FOR 7 DAYS

Organism	*Spot*	R_F	*Solvent system*	*Detection*					
				Before spray treatment		*After spray treatment*			
						p-Anisaldehyde		*Iron chloride*	
				Visible light	*Ultra-violet violet*	*Visible light*	*Ultra-violet*	*Visible light*	*Ultra-violet*
R. arrhizus	1	0.50	CM	—	Green			—	Green
	2	0.65	CM	—	Blue			—	Blue
	3	0.69	CM	—	Green	Pink	—		
	4	0.73	CM	—	Blue	Green	—		
	5	0.80	CM	—	Green	—			
	1	0.62	BMA	—	Green	Pink	—		
	2	0.68	BMA	—	Green			—	Green
R. chinenensis	1	0.20	CM	—	Blue	—	—	—	Blue
	2	0.60	CM	—	Green	Pink	—	—	Green
	3	0.76	CM	—	Blue	—	—	—	Blue
	4	0.91	CM	—	Blue	Green	—	—	Blue
	1	0.53	BMA	—	Blue	—	—		
	2	0.80	BMA	—	Green	—	—		
	3	0.89	BMA	—	Blue	Green	—	—	Blue
R. circinans	1	0.53	CM	—	Green	Pink	—	—	Green
	2	0.77	CM	—	Blue	Green	—	—	Blue
	1	0.61	BMA	—	Green	Pink	—	—	Green
R. kazanensis	1	0.05	CM	—	Blue			—	Blue
	2	0.58	CM	—	Green	Pink	—	—	Green
	3	0.73	CM	—	Blue	Green	—	—	Blue
	4	0.87	CM	—	Blue	—	—	—	Blue
	1	0.25	BMA	—	Blue	—	—		Blue
	2	0.68	BMA	—	Green	Pink	—		Green
	3	0.84	BMA	—	Blue	Green	—	—	Blue
R. oryzae	1	0.04	CM	—	Blue			—	Blue
	2	0.50	CM	—	Green	Pink	—	—	Green
	3	0.77	CM	—	Blue	Green	—	—	Blue
	1	0.09	BMA	—	Blue				Blue
	2	0.34	BMA	—	Blue	Pink	—	—	Blue
	3	0.62	BMA	—	Green	Pink	—	—	Blue
	4	0.85	BMA	—	Blue	Green	—		
R. stolonifer	1	0.20	CM	—	Blue	—	—	—	Blue
	2	0.55	CM	—	Green	Pink	—	—	Green
	3	0.78	CM	—	Pale Blue	Green	—	—	Pale Blue
	4	0.80	CM	—	Blue	Green	—		
	1	0.13	BMA	—	Blue			—	Blue
	2	0.40	BMA	—	Blue	Green	—	—	Blue
	3	0.67	BMA	—	Green	Pink	—	—	Green
	4	0.81	BMA	—	Blue	Green	—	—	Blue
R. 66-81-2	1	0.28	CM	—	Blue	—	—		
	2	0.59	CM	—	Green	Pink	—	—	Green
	3	0.87	CM	—	Blue	Green	—	—	Blue
	1	0.20	BMA	—	Blue	—	—	—	Blue
	2	0.47	BMA	—	Blue	—	—	—	Blue
	3	0.68	BMA	—	Green	Pink	—	—	Green
	4	0.83	BMA	—	Blue	—	—	—	Blue

TABLE IV

TLC OF THE CHLOROFORM EXTRACT OF *RHIZOPUS* SPECIES GROWN IN MYE MEDIUM FOR 14 DAYS

Organism	*Spot*	R_F	*Solvent system*	*Detection*					
				Before spray treatment		*After spray treatment*			
				Visible light	*Ultra-violet*	*p-Anisaldehyde*		*Iron chloride*	
						Visible light	*Ultra-violet*	*Visible light*	*Ultra-violet*
R. arrhizus	1	0.04	CM	—	Blue			—	Blue
	2	0.11	CM	—	Blue	—	—	—	Blue
	3	0.29	CM	—	Blue	—	—	—	Blue
	4	0.49	CM	—	Green			—	Green
	5	0.69	CM	—	Blue			—	Blue
	6	0.75	CM	Yellow	—	—	—	—	—
	7	0.83	CM	—	Green	Pink	—		
	8	0.98	CM	—	Blue	Green	—		
	1	0.25	BMA	—	Blue	—	—	—	Blue
	2	0.47	BMA	—	Blue	—	—	—	Blue
	3	0.74	BMA	—	Green	Pink	—	—	Green
	4	0.87	BMA	—	Blue	Green	—	—	Blue
R. chinensis	1	0.10	CM	—	Blue	—	—	—	Blue
	2	0.25	CM	—	Blue	—	—		
	3	0.45	CM	—	Green			—	Green
	4	0.62	CM	—	Blue			—	Blue
	5	0.77	CM	—	Green	Pink	—		
	6	0.92	CM	—	Blue	Green	—	—	Blue
	1	0.29	BMA	—	Blue	—	—		
	2	0.45	BMA	—	Blue	—	—	—	Blue
	3	0.70	BMA	—	Green	Pink	—	—	Green
	4	0.86	BMA	—	Blue	Green	—		
R. circinans	1	0.09	CM	—	Blue	—	—	—	Blue
	2	0.25	CM	—	Blue	—	—		
	3	0.50	CM	—	Green			—	Green
	4	0.67	CM	—	Blue			—	Blue
	5	0.78	CM	—	Green	Pink	—		
	6	0.89	CM	—	Blue	Green	—	—	Blue
	1	0.27	BMA	—	Blue	—	—	—	Blue
	2	0.37	BMA	—	Blue			—	Blue
	3	0.44	BMA	—	Blue	—	—	—	Blue
	4	0.68	BMA	—	Green	Pink	—	—	Green
	5	0.82	BMA	—	Blue	Green	—	—	Blue
R. kazanensis	1	0.08	CM	—	Blue			—	Blue
	2	0.16	CM	Yellow	Dull Blue			—	Dull Blue
	3	0.26	CM	—	Blue	—	—	—	Blue
	4	0.60	CM	—	Green	Pink	—	—	Green
	5	0.71	CM	—	Blue			—	Blue
	6	0.78	CM	Orange	Dull Blue			—	Dull Blue
	7	0.90	CM	—	Blue	Green	—	—	Blue

TABLE IV *(continued)*

Organism	*Spot*	R_F	*Solvent system*	*Detection*					
				Before spray trearment		*After spray treatment*			
						p-Anisaldehyde		*Iron chloride*	
				Visible light	*Ultra-violet*	*Visible light*	*Ultra-violet*	*Visible light*	*Ultra-violet*
	1	0.25	BMA	—	Blue			—	Blue
	2	0.35	BMA	—	Blue			—	Blue
	3	0.49	BMA	—	Blue	—	—	—	Blue
	4	0.76	BMA	—	Green	Pink	—	—	Green
	5	0.87	BMA	—	Blue	—	—		Blue
	6	0.95	BMA	—	Blue	Green	—	—	Blue
R. oryzae	1	0.03	CM	—	Blue			—	Blue
	2	0.08	CM	—	Blue			—	Blue
	3	0.15	CM	—	Blue			—	Blue
	4	0.53	CM	—	Green	Pink	—		
	5	0.72	CM	—	Blue	Green	—	—	Blue
	6	0.90	CM	—	Blue			—	Blue
	7	0.95	CM	—	Blue	Green	—	—	Blue
	1	0.23	BMA	—	Blue			—	Blue
	2	0.45	BMA	—	Blue	—	—	—	Blue
	3	0.70	BMA	—	Green	Pink	—	—	Green
	4	0.82	BMA	—	Blue	Green	—	—	Blue
	5	0.95	BMA	—	Blue	Green	—	—	Blue
R. stolonifer	1	0.08	CM	—	Blue			—	Blue
	2	0.20	CM	—	Blue	—	—		
	3	0.56	CM	—	Green	Pink	—	—	Green
	4	0.66	CM	—	Blue			—	Blue
	5	0.84	CM	—	Blue	Green	—	—	Blue
	6	0.94	CM	—	Blue	Green	—	—	Blue
	1	0.46	BMA	—	Blue	—	—	—	Blue
	2	0.73	BMA	—	Green	Pink	—	—	Green
	3	0.84	BMA	—	Blue	—	—	—	—
	4	0.94	BMA	—	Blue	Green	—		
R. 66-81-2	1	0.14	CM	—	Blue	—	—	—	Blue
	2	0.60	CM	—	Green	Pink	—	—	Green
	3	0.77	CM	—	Blue	Green	—	—	Blue
	4	0.94	CM	—	Blue	Green	—	—	Blue
	1	0.43	BMA	—	Blue			—	Blue
	2	0.49	BMA	—	Blue	—	—	—	Blue
	3	0.75	BMA	—	Green	Pink	—	—	Green
	4	0.87	BMA	—	Blue	Green	—	—	Green
	5	0.93	BMA	—	Blue	Green	—	—	Green

fication of the metabolites are presently under way and will be reported on in the near future.

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CHROM. 12,327

Note

Covalent coupling of cholic acid to aminohexylamino-Sepharose 4B and its use in affinity chromatography of serum albumin

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Conventional methods for removal or purification of albumin from serum have been generally disappointing. In more recent methods advantage has been taken of the ability of albumin to bind reversibly a variety of biological substances. The high affinity that albumin shows for bilirubin, fatty acids and certain organic dyes has led to the use of these compounds as ligands coupled to Sepharose for the separation of albumin from serum[1–3]. Bile acids are also bound by albumin[4] although use of this ligand has not yet been reported. This paper describes the synthesis of a cholic acid–aminohexylamino-Sepharose 4B gel, its serum albumin binding properties and its advantages over other affinity chromatography methods.

MATERIALS AND METHODS

Coupling of cholic acid to aminohexylamino-Sepharose 4B

^{14}C-Labelled cholic acid (40 mg; 1 μCi) was dissolved in 5 ml of absolute ethanol and added to 20 ml of 50% (v/v) aqueous ethanol containing 160 mg of 1 ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl. The pH of the solution was adjusted as required. The coupling solution was then slowly added to 2 ml of pre-swollen aminohexylamino(AH)-Sepharose 4B (Pharmacia, Uppsala, Sweden) and gently stirred at 24° for 16 h.

The substituted Sepharose was washed extensively on a Buchner funnel with 50% aqueous ethanol (pH 7.4) and 0.5 *M* NaCl (pH 7.4) until ^{14}C-labelled cholic acid was free from the effluent. Since the capacity of the column to bind albumin did not alter with the use of the column, it appears that washing was complete and that there was no leakage of coupled bile acid. The cholic acid coupled Sepharose was stored in buffer (20 m*M* phosphate; 0.5 *M* NaCl pH 7.4) at 4° in the presence of 0.1% sodium azide and was stable for periods greater than 3 months.

The bile acid content of the various gel preparations was estimated by counting an aliquot of gel slurry dispersed in 10 ml of Triton X-100–toluene scintillant in a Packard Tri-Carb scintillation counter (Model B2450).

Affinity chromatography

Bile acid–Sepharose preparations were packed in 5-ml polypropylene syringes,

plugged with glass wool (packed gel volume 2 ml). Solutions of bovine serum albumin (BSA) were passed through these columns at room temperature in buffer (20 m*M* phosphate, 0.14 *M* NaCl, pH 7.4) at a flow-rate of 30 ml/h, and further washed with 40 ml of the same buffer before elution of bound protein. Protein was measured by absorbance at 280 nm using BSA as standard. For re-use the columns were washed and equilibrated with phosphate–saline buffer.

The maximal capacity for uptake of BSA was measured by saturation of the column with a solution of the protein, washing with 40 ml of buffer and subtraction of the amounts of albumin in the effluent from the amount added.

Affinity chromatography of albumin from human serum was studied by passing 4 ml of 1:20 diluted serum sample through the column and washing as above.

Electrophoresis

Sodium dodecyl sulphate (SDS) gel electrophoresis was carried out in 7% polyacrylamide gels[5]. Quantitation of protein was done using a gel scanner fitted to a Beckman Spectrophotometer Model 25 at 280 nm and after staining with Coomassie Blue at 620 nm. Immunoelectrophoresis was carried out on 1% agarose gel plates before addition of whole human antisera (Dakopatts, Copenhagen, Denmark) to troughs running parallel to the samples. Precipitation lines were stained with Coomassie Blue.

RESULTS AND DISCUSSION

Coupling reaction

Cholic acid was coupled to AH-Sepharose 4B using ethyl(dimethylaminopropyl)carbodiimide, at various pH values (Table I). Maximal coupling of 3.3 μmol/ml swollen gel was obtained at pH 7.0.

TABLE I

EFFECT OF pH ON COUPLING OF CHOLIC ACID TO AH-SEPHAROSE 4B

pH	*Cholic acid coupled (μmol/ml packed gel)*
4	0.0
5	1.2
6	2.8
7	3.3
8	0.3

Binding and elution of albumin

Columns containing substituted or native AH-Sepharose 4B preparations were loaded with BSA and washed with buffer. Retention of albumin was specific to cholic acid coupled Sepharose. No BSA was retained by the native unsubstituted Sepharose. The maximal capacity of the gel for BSA was 0.044 μmol/ml of gel. Elution of BSA bound to Sepharose could be accomplished at pH 7.4 by 8 *M* urea, or under non-denaturing conditions by competitively eluting with 5 m*M* cholic acid or 5 m*M* taurocholic acid.

Purification of albumin from human serum

When diluted human serum was added to the affinity column, immunoelectrophoretic studies of the eluent showed that nearly all of the albumin had been bound on the gel. There was some minor binding of other serum proteins but these also bound to the unsubstituted control Sepharose. These non-specifically bound proteins could be removed almost entirely by elution with 0.5 *M* NaCl, but this also resulted in a greater than 50% loss of bound albumin. The remaining bound albumin could be eluted by 8 *M* urea or 5 m*M* bile acid (Fig. 1B).

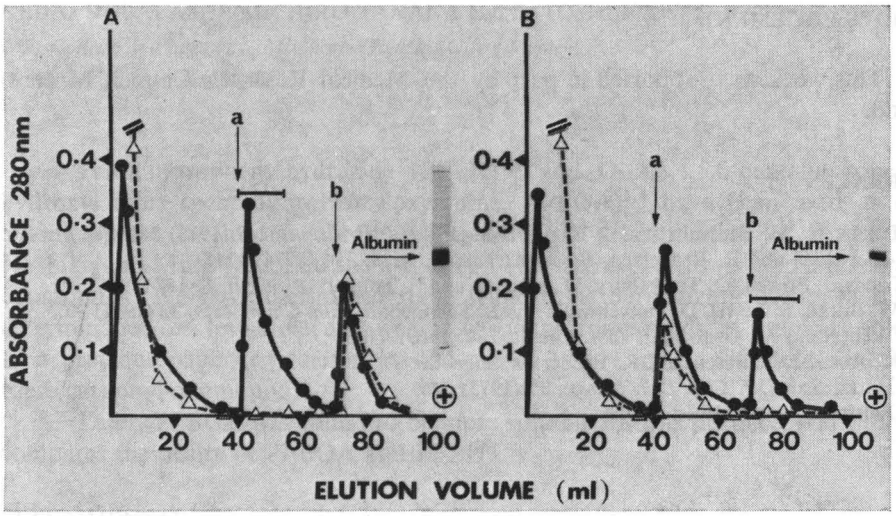

Fig. 1. Affinity chromatography of albumin from human serum. 4 ml of dilute (1:20) serum was applied to both substituted (●—●) and native (△---△) columns (packed gel volume 2 ml) and washed immediately with phosphate–saline buffer (pH 7.4). (A) Bound albumin was specifically eluted with (a) 5.5 m*M* cholic acid (pH 7.4). Non-specifically bound serum protein which remained on the column was removed by (b) either 0.5 *M* NaCl (pH 7.4) or 8 *M* urea (pH 7.4). (B) Non-specifically bound protein together with 40–60% albumin was eluted with (a) 0.5 *M* NaCl (pH 7.4). The remaining albumin bound with higher affinity was subsequently removed by (b) 8 *M* urea or 5 m*M* cholic acid (pH 7.4). The albumin peak indicated by the horizontal bar was pooled, concentrated and analysed for purity by SDS–polyacrylamide electrophoresis, run in the presence of mercaptoethanol.

In contrast, specific elution with 5 m*M* cholic acid gave complete recovery of albumin uncontaminated with other serum proteins (Fig. 1A). Non-specifically bound protein remained bound to the gel. Purity of the eluted albumin was assessed by SDS–polyacrylamide electrophoresis. Scanning of the SDS gels at 280 nm and at 620 nm after staining with Coomassie Blue showed the purity of the eluted albumin to be greater than 99%. The ability of the bile acids (cholic acid and taurocholic acid) to elute albumin indicates that the binding of albumin to the gel is through a specific bile acid binding site. Elution of albumin by bile acids is not due to their general detergent properties since non-specifically bound serum protein on both substituted and native Sepharose remained bound to the gel in the presence of 5 m*M* cholic acid.

The cholic acid–Sepharose gel has proved useful for the purification of albumin from serum. Although the capacity of the gel for albumin (3 mg/ml swollen gel) is not

as high as that reported for other affinity methods[1–3], it does have a number of advantages. The technique is simple and allows elution of albumin (>99% pure) in one step, in a non-denatured form. The matrix is very stable and the ligand readily coupled. None of the other methods reported has all of these advantages. Since the various ligands appear to take advantage of different binding sites[6,7], cholic acid coupled Sepharose may prove a useful tool in studying binding sites on albumin. The gel has already proved useful in the isolation of receptor/carrier proteins involved in the enterohepatic transport of bile acids[8].

ACKNOWLEDGEMENT

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CHROM. 12,353

Note

Gas chromatographic determination of low-molecular-weight carbonyl compounds in aqueous solution as their O-(2,3,4,5,6-pentafluorobenzyl) oximes

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Pentafluorophenylhydrazine (PFPH)[1,2] and O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (pentafluorobenzyloxyamine, PFBOA)[3,4] have been used as derivatizing agents in the gas chromatographic (GC) determination of keto-steroids. Hoshika and Muto[5] reported the use of PFPH in the GC of lower aliphatic carbonyl compounds. We have considered these reagents as derivatizing agents in the GC determination of low-molecular-weight carbonyl compounds in aqueous solution. In a previous paper[6], we described the use of PFPH in the GC determination of these carbonyl compounds.

Later, PFBOA was found to be more suitable for this purpose, and this paper compares the utility of PFBOA and PFPH.

EXPERIMENTAL

Reagents

PFPH was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). PFBOA hydrochloride was synthesized from pentafluorobenzyl bromide (Aldrich) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan) according to the procedure of Youngdale[7]. White plates (m.p. 115°) were obtained. Elemental analysis: calculated for $C_7H_5NOF_5Cl$, C 33.69, H 2.02, N 5.61%; found, C 33.69, H 1.97, N 5.52%.

The internal standard (IS) solution was a 0.025% solution of *p*-chlorobenzyl chloride in ethyl acetate.

Apparatus and conditions

A Shimadzu Model GC-4APF gas chromatograph equipped with a flame-ionization detector(FID) was used. The GC conditions were as follows: 2-m glass column packed with 3% XE-60 on 80–100-mesh Celite 545 (AW DMCS); column temperature, 90°; detector temperature, 120°; injection temperature, 120°; chart speed, 0.25 cm/min.

Standard procedure

To a 0.5-ml aliquot of sample solution in a 10-ml centrifuge tube was added 0.5 ml of PFBOA solution (0.2 mg/ml; *ca.* $8.0 \cdot 10^{-4}$ *M*). The mixture was shaken well and allowed to stand for 40 min at room temperature (overnight if the sample

solution contained ketones). To the reaction mixtures, saturated with sodium chloride, was added 1 drop of 18 *N* sulphuric acid and the mixture was extracted with 0.2 ml of ethyl acetate containing 50 μg of *p*-chlorobenzyl chloride as internal standard. Excess of sodium chloride and the aqueous layer were removed with the aid of a syringe with a long needle, a few grains of anhydrous sodium sulphate were added to dry the ethyl acetate extract and an aliquot was injected on to the GC column.

RESULTS AND DISCUSSION

The retention times of thirteen O-pentafluorobenzyloximes (O-PFBO) relative to the internal standard obtained on different columns are given in Table I.

TABLE I

RELATIVE RETENTION TIMES OF THE O-PENTAFLUOROBENZYLOXIMES OF CARBONYL COMPOUNDS

Parent compound	*Stationary phase and column temperature*			
	3% XE-60 (90°)	*3% XF-1105 (100°)*	*3% SE-30 (80°)*	*2% OV-17 (70°)*
PFBOA	0.62	0.69	0.88	0.65
HCHO	0.19	0.27	0.29	0.19
CH_3CHO	0.32, 0.34*	0.48, 0.51*	0.57, 0.59*	0.43
C_2H_5CHO	0.47, 0.51*	0.80	1.05	0.72
n-C_3H_7CHO	0.80	1.41	1.83, 1.92*	1.30, 1.38*
iso-C_3H_7CHO	0.57	1.01	1.32	0.88
n-C_4H_9CHO	1.35, 1.44*	2.68	3.33, 3.52*	2.52, 2.68*
iso-C_4H_9CHO	2.72	1.91, 1.99*	2.55, 2.72*	1.76, 1.94*
CH_3COCH_3	0.40	0.70	0.88	0.65
$CH_3COC_2H_5$	0.61	1.13	1.50	1.04
CH_3CO-*iso*-C_3H_7	0.73	1.48	2.08	1.24, 1.43*
CH_3CO-*iso*-C_4H_9	1.16	2.36	3.38	2.08
$C_2H_5COC_2H_5$	0.85	1.74	2.43	1.61
C_2H_5CO-*n*-C_3H_7	1.27	2.70	3.95	2.61
$ClC_6H_4CH_2Cl$**	1.00	1.00	1.00	1.00

* Double peaks.
** Internal standard.

A typical GC separation of some carbonyl compounds as their O-PFBO derivatives is illustrated in Fig. 1; *p*-chlorobenzyl chloride was used as the internal standard. The procedure was essentially the same as that described previously[6].

A calibration graph was constructed by plotting the ratio of the peak height of a carbonyl compound to that of the internal standard, and a straight line passing through the origin was obtained for formaldehyde, acetaldehyde, isobutyraldehyde and diethyl ketone in the range 1–50 μg in 0.5 ml of aqueous solution. The reproducibility of the method for five replicate determinations on an identical sample solution containing 30 μg of isobutyraldehyde was examined; the standard deviation was 1.48%, which was less than that obtained with PFPH (1.84%).

When some carbonyl compounds reacted with PFBOA (marked with asterisks in Table I), the two peaks in each instance can be considered to correspond to *syn*-

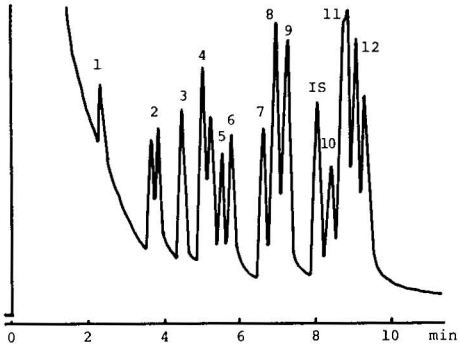

Fig. 1. Gas chromatogram of some carbonyl compounds as their pentafluorobenzyloximes. Conditions: 3% XE-60, 2.0-m glass column, temperature programmed from 80° to 120° at 4°/min, FID. Peaks: 1 = formaldehyde; 2 = acetaldehyde; 3 = acetone; 4 = propionaldehyde; 5 = isobutyraldehyde; 6 = methyl ethyl ketone; 7 = methyl isopropyl ketone; 8 = *n*-butyraldehyde; 9 = diethyl ketone; 10 = methyl isobutyl ketone; 11 = ethyl *n*-propyl ketone; 12 = *n*-valeraldehyde; IS = *p*-chlorobenzyl chloride.

and *anti*-isomers resulting from condensation reactions with PFBOA. A similar phenomenon was observed with PFPH.

The utility of PFBOA was compared with that of PFPH. As shown in Fig. 2, the PFPH concentration required was about ten times greater than those of the aldehydes being determined, in order to complete the reaction, whereas the formation of the O-PFBO derivatives was easily achieved with a much lower concentration of PFBOA. As can be seen in Table II, the condensation reaction was complete in 20 min at room temperature, after which the measured values were constant. Using isobutyraldehyde, the peak-area ratios of both O-PFBO and hydrazone derivatives to the same internal standard (*p*-chlorobenzyl chloride) were measured at column temperatures of 90° (Fig. 2) or 120°. The values obtained were O-PFBO 0.58 and hydrazone 0.28 at 90°, and O-PFBO 0.62 and hydrazone 0.28 at 120°. Hence the value for the O-PFBO derivative was approximately double that of the hydrazone at each temperature. The condensation reaction of PFBOA with ketones also proceeded

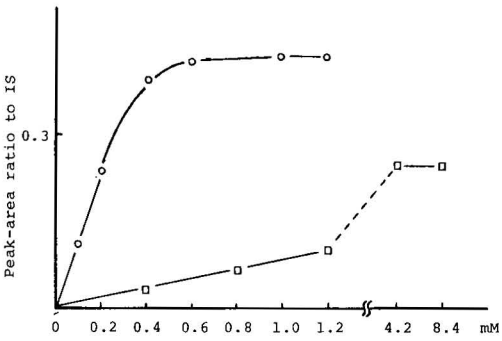

Fig. 2. Effect of PFBOA (○) and PFPH (□) concentration on reaction with *iso*-C_3H_7CHO (0.7 mM). The measurements were carried out according to the procedure described under Experimental and that in the previous paper[6]. IS: *p*-chlorobenzyl chloride for the determination of both derivatives produced. Column temperature: 90°.

TABLE II

EFFECT OF REACTION PERIOD ON EXTENT OF CONDENSATION REACTION WITH PFBOA

Reaction at room temperature. The values given for extent of reaction are the peak-area ratios of the compound peaks to that of the internal standard (*p*-chlorobenzyl chloride).

Compound	*Reaction period*				
	20 min	*40 min*	*1 h*	*2 h*	*24 h*
HCHO	1.78	1.88	1.82	1.82	1.63
CH_3CHO	0.85	0.88	0.82	0.85	0.71
C_2H_5CHO	0.92	0.82	0.88	0.85	0.75
n-C_3H_7CHO	0.81	0.79	0.82	0.77	0.71
iso-C_3H_7CHO	1.01	1.00	1.09	1.00	0.95
n-C_4H_9CHO	0.68	0.66	0.68	0.68	0.68
iso-C_4H_9CHO	0.56	0.52	0.56	0.50	0.78
CH_3COCH_3	0.44	0.60	0.86	1.06	1.23
$CH_3COC_2H_5$	0.18	0.31	0.38	0.58	1.18
CH_3CO-*iso*-C_3H_7	0.07	0.12	0.15	0.25	1.11
CH_3CO-*iso*-C_4H_9	0.05	0.07	0.09	0.14	0.60
$C_2H_5COC_2H_5$	0.07	0.12	0.17	0.21	0.98
C_2H_5CO-*n*-C_3H_7	0.03	0.04	0.06	0.11	0.97

slowly, being analogous to the reaction of PFPH but proceeding further to completion (Table II, Fig. 3), a measurable yield obtained within 24 h.

Nambara *et al.*[3] heated the reaction mixture in pyridine at 60° for 1 h in order to accelerate the condensation between PFBOA and keto-steroids. Koshy *et al.*[4] heated the mixture in benzene and pyridine at 65° for 30 min to prepare the O-PFBO derivatives of steroids with keto groups. In our experiment, the condensation reaction was carried out in aqueous solution. It can be seen in Fig. 3 that heating improved the yield, but the yield decreased gradually with an increase in the heating period, giving a value after 60 min that was considerably lower than that obtained after 24 h at room temperature.

With aldehydes, heating of the reaction mixture also did not give very good

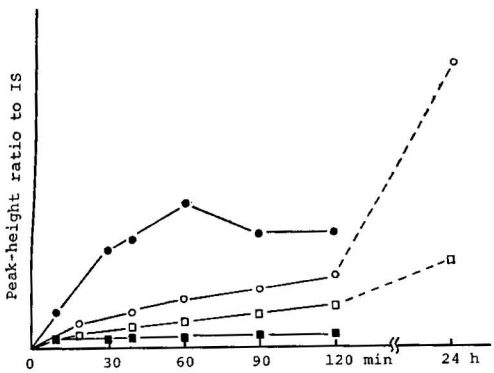

Fig. 3. Effect of reaction temperature and reaction period on the condensation reactions of diethyl ketone with PFBOA or PFPH. Reaction: ○, with PFBOA at room temperature; ●, with PFBOA at 70°; □, with PFPH at room temperature; ■, with PFPH at 70°.

results, and the procedure at room temperature was much more satisfactory with regard to the yield of the reaction and deviation of the values obtained from repeated determinations on an identical sample solution. A decrease in the reagent concentration in the reaction mixture led to an additional advantage with PFBOA that the removal of the unreacted reagent was almost complete. PFBOA in aqueous solution was slowly hydrolyzed on storage for more than a week and the pentafluorobenzyl alcohol liberated was extracted, giving an undesirable peak that overlapped that of the O-PFBO derivative of acetone on the gas chromatogram. The O-PFBO derivatives were much more volatile than the corresponding pentafluorophenylhydrazones, and therefore the GC separation could be carried out at lower temperatures. In this work, the column temperature was maintained at 90° for all separations, which was 30° lower than that used with the pentafluorophenylhydrazones.

The extent of the condensation reaction with PFPH was greater in neutral media, whereas the reaction with PFBOA proceeded readily in weakly acidic media (pH 4–6), so that the use of a buffer solution was unnecessary.

The stability of the O-PFBO derivatives was studied using isobutyraldehyde, and it appeared that the derivative was stable in ethyl acetate at room temperature for at least a few days. Because of the ease of preparation of the derivatives, the complete removal of unreacted reagent and the increase in volatility of O-PFBO, the reagent would be more suitable for the GC determination of carbonyl compounds and the derivatives would be extremely sensitive to electron-capture detection.

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CHROM. 12,319

Note

Detection of thiols on thin-layer chromatograms with 3,5-di-tert.-butyl-1,2-benzoquinone–iron(III) chloride

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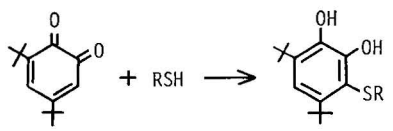
5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) has been used for selective detection of thiols on chromatograms as yellow spots[1]. The colour is that of the thiophenolate anion of 2-nitro-5-mercaptobenzoate formed by a disulphide exchange reaction[1] and therefore the reagent gives the same colour with different thiols.

o-Quinones are known to react readily with thiols to give catechol thioethers[2]. Thus, reaction of 3,5-di-*tert.*-butyl-1,2-benzoquinone (I) affords 6-thioether derivatives of 3,5-di-*tert.*-butylcatechol (II)*. Catechols can form coloured chelates with iron(III) ion. We report here that thiols can be detected with high sensitivity and specificity by spraying chromatograms with quinone I followed by iron(III) chloride solution and that cysteine and glutathione (reduced) can be differentiated from each other by their characteristic colours.



I II

EXPERIMENTAL

Reagents

Quinone solution. A 0.1% solution of 3,5-di-*tert.*-butyl-1,2-benzoquinone (Aldrich, Milwaukee, Wisc., U.S.A.) in 2-butanone was prepared.

Iron(III) chloride solution. A 1.6% solution of iron(III) chloride hexahydrate in ethanol was prepared.

Procedure

Appropriate amounts of compounds were spotted on pre-coated cellulose thin-layer plates (E. Merck, Darmstadt, G.F.R.) of length 10 cm and the chro-

* The reaction of cysteine with quinone I gave 3-*S*-cysteinyl-4,6-di-*tert.*-butylcatechol, for which correct elemental analyses were obtained.

matogram was developed in 1-butanol–acetic acid–water (12:3:5) until the solvent front had migrated 7–8 cm past the origin. After being dried, the chromatogram was sprayed with the quinone solution and subsequently after 1 min at room temperature, with the iron(III) chloride solution.

RESULTS AND DISCUSSION

Table I summarizes the results. Most of the thiols tested gave a positive reaction to the reagent, giving different colours. Amounts of cysteine, cysteamine, glutathione and 2-mercaptopurine of 2 nmole were easily detected on a yellow background, which gradually darkened.

TABLE I

SENSITIVITIES AND COLOURS OF THIOLS AND OTHER COMPOUNDS ON THIN-LAYER CHROMATOGRAMS OBTAINED WITH QUINONE–IRON(III) CHLORIDE SPRAY REAGENT

Compound	*Sensitivity**	*Colour*
Cysteine	2:+, 5:++	Dark green
Cysteamine	2:+, 5:++	Dark green
Glutathione (reduced)	2:+, 5:++	Pinkish orange
Ergothioneine	5:±, 10:+	Dark green
2-Mercaptopurine	2:+, 5:++	Grey
6-Mercaptopurine	20:−, 50:±/+	
Cysteinesulphinic acid	20:+, 50:++	Orange**
Cysteic acid	100:−	
Cystine	100:−	
Methionine	20:−, 50:±	
3,4-Dihydroxyphenylalanine	2:+, 5:++	Dark green**
Tryptophan	20:−/±, 50:±	Orange
Histidine	50:−, 100:−/±	
Tyrosine	100:−	
Lysine	50:−, 100:−/±	
Arginine	50:−, 100:−/±	

* Numbers are amounts (nanomoles) of compounds spotted; −, not detected; ±, faint; +, weak but easily detectable; ++, clear.

** The same colours were also produced with iron(III) chloride only.

Catechols such as 3,4-dihydroxyphenylalanine can be easily distinguished from thiols by their positive test with iron(III) chloride. Although compounds with nucleophilic functional groups such as indoles and thioethers were detectable at levels of 50–100 nmole, their spots were dull and faint, and were thereby distinguishable from those of thiols. However, if these compounds were allowed to react with the quinone for 10 min, their sensitivities to iron(III) chloride increased several-fold. Therefore, in order to achieve a higher specificity for thiols, it is essential to spray the chromatograms with iron(III) chloride exactly 1 min after spraying with the quinone.

Under similar conditions, the DTNB reagent detected cysteine and glutathione with sensitivities comparable to those obtained in the present method, but it did not react with ergothioneine and 2-mercaptopurine, probably owing to their olefinic

character[1]. The marked difference between the reactivities of 2-mercaptopurine and 6-mercaptopurine indicates that the sensitivity of the quinine–iron(III) chloride reagent to heterocyclic thiols is also dependent on the thiol–thione tautomerism.

In conclusion, the quinone–iron(III) chloride reagent is a selective and sensitive spray reagent for detection of thiols on thin-layer chromatograms. An advantage of this reagent over other reagents for thiols and other sulphur-containing compounds such as DTNB, platinum(IV) iodide[3] and sodium nitroprusside[3] is that it can differentiate between cysteine and glutathione, which are difficult to separate from each other on thin-layer chromatograms.

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CHROM. 12,367

Note

High-performance liquid chromatographic determination of L-ascorbate-2-phosphate in phosphorylation reactions*

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(Received September 4th, 1979)

Several of us recently reported[1] the synthesis of L-ascorbic acid 2-phosphate (A-2-P, Fig. 1). In that work we used a rapid, high-performance chromatographic method to assay the products of reaction between 5,6-O-isopropylidene-L-ascorbic acid (IAA) and phosphorus oxychloride. A number of reactions were done under a variety of conditions to maximize the yield of A-2-P. Previous workers[2] used gravity column chromatography to separate the phosphorylation products. In this paper we present the details of the high-performance liquid chromatographic (HPLC) procedure used in the synthesis work.

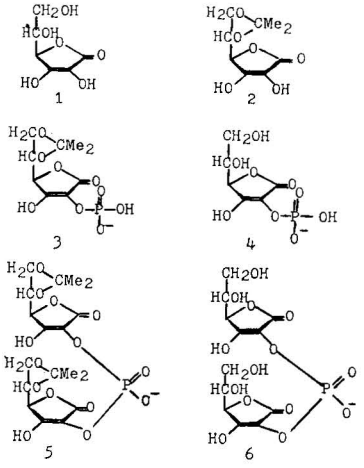

Fig. 1. Compounds involved in this work: 1 = AA; 2 = IAA; 3 = IA-2-P; 4 = A-2-P; 5 = BIA-2-P; 6 = BA-2-P.

* Contribution No. 79-395-J, Department of Grain Science and Industry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, Kan. 66506, U.S.A.

EXPERIMENTAL

Reagents

The following compounds were used in analytically pure form: IAA[3], m.p. 217–223°; tricyclohexylammonium A-2-P[1], m.p. 178–182° (decomposition); barium bis(L-ascorbate)-2,2′-phosphate (BA-2-P)[1], m.p. 250° (decomposition), dipotassium L-ascorbate-2-sulfate[4] (A-2-S), m.p. 96–97°; and L-ascorbic acid (AA), m.p. 192° (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.).

Standard solutions (0.05–1.0 m*M*) of the water-soluble salts were prepared in water. In the case of the sparingly soluble phosphodiester, barium BA-2-P (50 mg, 0.081 mmole) was added to water (100 ml) containing sodium sulfate (20 mg, 0.141 mmole). After barium sulfate had been removed by gravity filtration, standard solutions were prepared.

Phosphate buffers (0.1–0.5 *M*) of pH 3.5–5.0 were prepared by mixing aqueous potassium hydrogen phosphate (0.1–0.5 *M*) with aqueous phosphoric acid (0.1–0.5 *M*). The buffers were degassed by boiling momentarily.

Apparatus

Separations were carried out on a Waters Model ALC/GPC 201 high-performance liquid chromatograph fitted with a septum injector and a UV detector (Waters Assoc., Milford, Mass., U.S.A.). The detector operated at 254 nm with a cylindrical flow cell of 1 mm diameter and 10 mm length (8 μl cell volume). The detector, which was equipped with a binary attenuator (2× to 64×), produced a linear output over $A = 0.02$–0.64. The stainless-steel column (1.22 m × 3.17 mm) was packed with a pellicular, strongly basic anion-exchange resin (Bondapak AX/Corasil, 37–50 μm, Waters Assoc.). The chromatography was done under ambient temperature conditions. Samples (10 μl) were injected (Series "B-110" syringe, Precision Sampling, Baton Rouge, La., U.S.A.) and the components were eluted with potassium dihydrogen phosphate buffer (0.1 *M*) at a flow-rate of 0.5 ml/min. Peaks were recorded on a 0.1 mV recorder at a chart speed of 0.5 cm/min. The retention times are given with reference to the mobility of AA (Table I). When retention times changed gradually with sustained use of the column, spiking was done to verify peak assignments.

Analysis of phosphorylation reactions

To a mixture of IAA (6.15 g, 28.5 mmoles) in water (50 ml) with or without pyridine (10 ml, 124 mmoles; or 12 ml, 155 mmoles), was quickly added 10 *M* aqueous potassium hydroxide to a final pH of 12 or 13. Each reaction mixture was kept at about 0–5° under an atmosphere of nitrogen while phosphorus oxychloride (3.65 ml, 39.9 mmoles) was added dropwise and the pH of the reaction mixture was maintained constant by periodic addition of 10 *M* aqueous potassium hydroxide. The reaction mixture was allowed to warm to 25° and was made to volume (250.0 ml). Each reaction mixture was examined by HPLC before hydrolytic removal of the isopropylidene group. For that purpose, an aliquot (1.0 ml) of the diluted reaction mixture was added to dipotassium A-2-S (0.1 mmole), and the resulting mixture was diluted again (250 ml). For quantitative analysis, the isopropylidene group was hydrolyzed and the pyridine removed by treatment with a cation-exchange resin at 25°. An aliquot (1.0 ml)

TABLE I

RELATIVE MOBILITIES (R_A) OF DERIVATIVES OF AA

Relative mobilities (R_A) = (distance migrated by derivative)/(distance migrated by L-ascorbic acid). HPLC; stationary phase, pellicular anion-exchange resin; mobile phase 0.1 *M* KH_2PO_4, pH 4.4, at 0.5 ml/min and 25°. The retention time of AA was 4.5–6.0 min depending on the age of the column. The figures in the table were observed using fresh resin. Paper chromatography (PC): development solvent, *n*-propanol–water–trichloroacetic acid (15:4:1, v/v/w).

Compound	R_A	
	HPLC	*PC*
AA	1.0	1.0
IAA	1.3	1.6
A-2-S	1.8	0.5
A-2-P	2.8	0.6
BA-2-P	2.3	0.4
IA-2-P*	3.4	—
BIA-2-P*	6.0	—
Inorganic phosphate	—	1.2

* Compounds not isolated; structures assigned based on products identified after mild acid hydrolysis.

of the once diluted reaction mixture was passed through Amberlite IR-120 (H^+) (15 ml) which had been previously washed free of UV-absorbing material. The reaction mixture became strongly acidic (pH 0.5–1.0) after passing through the exchange resin, because of the exchange of sodium ions. The column was washed generously with water (200 ml), the effluent was made to volume (250 ml), and an aliquot (10 μl) was injected in triplicate into the liquid chromatograph. Standard curves were prepared from analytically pure samples of known tricyclohexylammonium A-2-P. The precision of the assay was $\pm 3\%$.

Paper chromatography was also used to examine the reaction mixtures. The products of a reaction mixture were converted to sodium salts by a cation-exchange resin, concentrated to a small volume, and spotted on Whatman No. 1. The papers were developed in a descending manner at 25° using *n*-propanol–water–trichloroacetic acid (15:4:1, v/v/w). Components were visualized with three spray reagents. Strongly reducing compounds such as IAA reacted with silver nitrate spray[5] (1 ml of saturated aqueous silver nitrate in 100 ml of acetone). Ferric chloride spray[6,7] (1%, w/w, $FeCl_3$ in ethanol–water, 95:5) gave a permanent intense red color on a yellow background for the 2-substituted esters of AA and permanent white spots for strongly reducing ascorbic acids. Phosphate containing spots were detected as blue spots using an acid–molybdate spray[8] followed by treatment of the paper with hydrogen sulfide.

RESULTS AND DISCUSSION

Fig. 2 shows the HPLC separation of the products when IAA was reacted at a constant pH of 12 with 1.4 equivalents of phosphorus oxychloride in alkali. The peak with the shortest retention time is the internal standard A-2-S. The other two prominent peaks represent the principal products of the phosphorylation reaction, namely 5,6-O-isopropylidene-L-ascorbate 2-phosphate (IA-2-P) (51%) and bis(5,6-O-

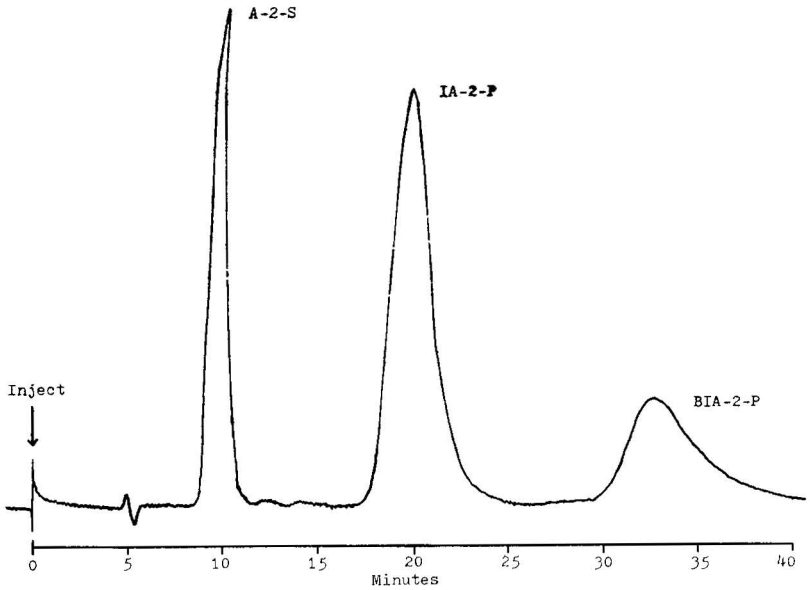

Fig. 2. HPLC separation of reaction products between IAA and phosphorus oxychloride at pH 12 and 2°. An internal reference standard was added, A-2-S. Other abbreviations identified in Fig. 1.

isopropylidene-L-ascorbate)-2,2′-phosphate (BIA-2-P) (22%). Those two products were not isolated, but upon hydrolytic removal of the acetal group two new peaks appeared with a ratio of intensities equal to that of the acetonated products, and with mobilities identical to those of analytically pure samples of A-2-P and BA-2-P. The chromatographic mobilities of various AA compounds are given in Table I. The mobilities of the compounds varied somewhat as the use of the column increased. However, the relative positions of the peaks did not change.

Fig. 3 shows the effect of adding pyridine (initially 1.9 *M*) to the phosphorylation reactions mixture at pH 12. The chromatogram shows that the quantity of phospho-diester (retention time, $t_R = 37$ min) is greatly reduced compared to that seen in Fig. 2. In Fig. 3 the first peak to elute is pyridine followed by the internal standard A-2-S ($t_R = 14.5$ min).

The optimum reaction medium for the conversion of IAA to IA-2-P was found to be a mixture of potassium hydroxide at pH 13 and pyridine at an initial concentration of 2.3–2.8 *M*. Under those conditions, as seen in Fig. 4, IA-2-P ($t_R =$ 24.5 min) was formed in 97% yield and only a trace (<1%) of BIA-2-P was observed. When the optimum reaction mixture was treated at 25° with a strongly acidic cation-exchange resin in the H^+ form, pyridine was removed and the 5,6-acetal was quantitatively cleaved (Fig. 5). The response area of the major peak ($t_R = 14.5$ min) compared to that of the pure standard tricyclohexylammonium A-2-P showed a 97% conversion of IAA to the monophosphate ester. Prior studies[9] on the hydrolysis of A-2-P have indicated that the phosphate ester would probably be stable in acid at pH 1.0 and 25°. Fig. 5 shows a slight shoulder on the left side of the major peak due to traces of BA-2-P ($t_R \approx 12$ min) in the reaction mixture. It should be noted that

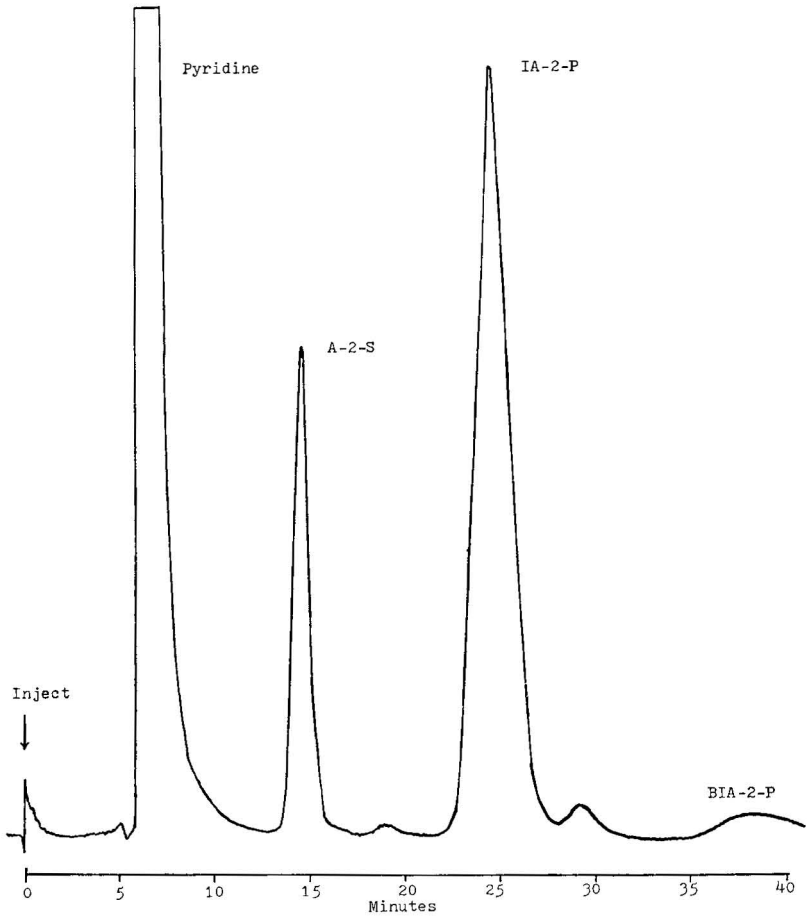

Fig. 3. HPLC separation of reaction products between IAA and phosphorus oxychloride at pH 12 and 2° in presence of pyridine (1.9 *M*). See Figs. 1 and 2 to identify abbreviations.

BIA-2-P has a slower mobility on the analytical column than IA-2-P, but the reverse is true of BA-2-P and A-2-P.

In another phase of this work, several phosphorylation reactions were done directly on AA in strong alkali (pH 13–13.5) without pyridine. In one example, we repeated the conditions of a reaction described in a German patent[10]. AA (8.3 g) was dissolved in a mixture of water (30 ml) and calcium hydroxide (11.5 g), and phosphorus oxychloride (7.5 g) was added at 0°. After 90 min the reaction mixture was diluted and examined by HPLC (Fig. 6). At pH 13–13.5 with no pyridine, the reaction mixture contained mainly BA-2-P (37%) along with A-2-P (24%) and unreacted AA (24%). The barium salt of BA-2-P, which crystallizes rather easily from water, was readily isolated[1] in pure form from that reaction mixture.

The HPLC separation of A-2-P and BA-2-P depended on the ionic strength and pH of the eluting buffer (Table II). The best resolution was found with a mobile

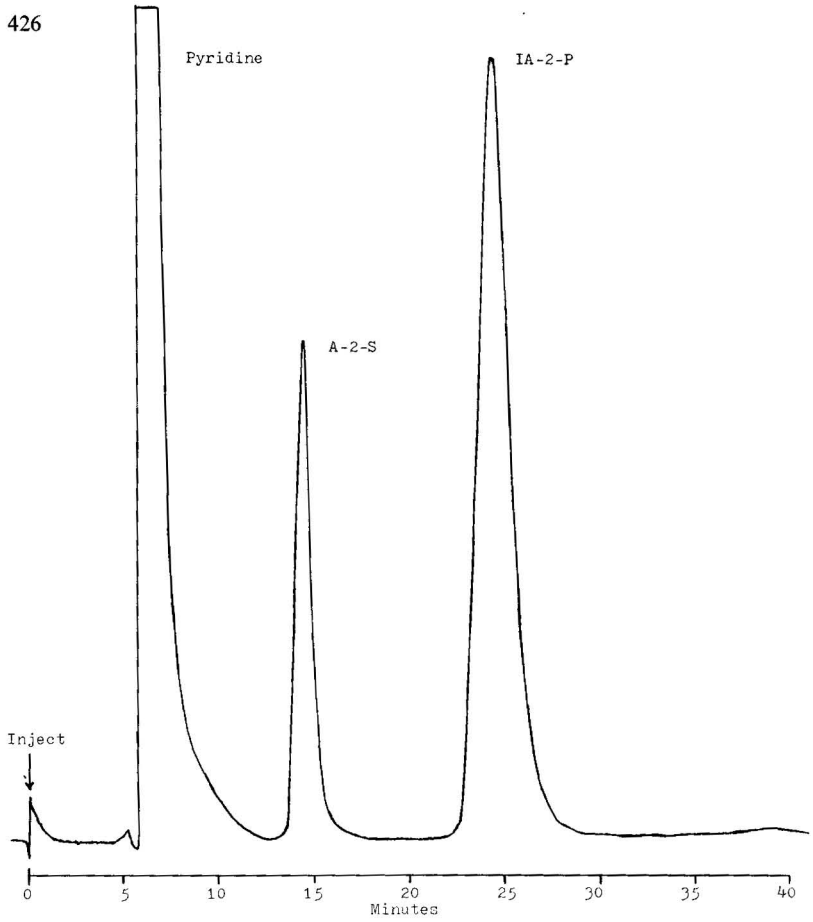

Fig. 4. HPLC separation of reaction products between IAA and phosphorus oxychloride at pH 13 and 2° in the presence of pyridine (2.3 *M*). See Figs. 1 and 2 to identify abbreviations.

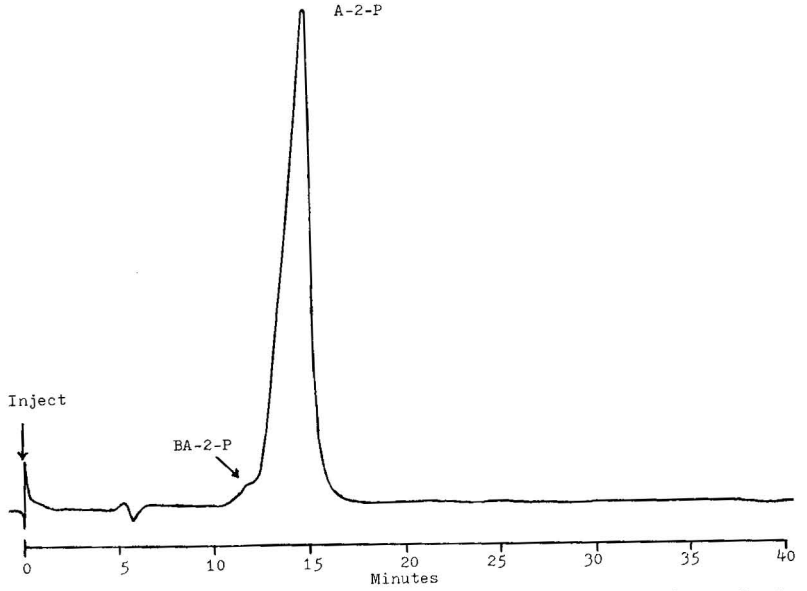

Fig. 5. HPLC separation of de-acetonated reaction products obtained at optimal conditions (pH 13, 2°, and pyridine at 2.3 *M*) for formation of A-2-P.

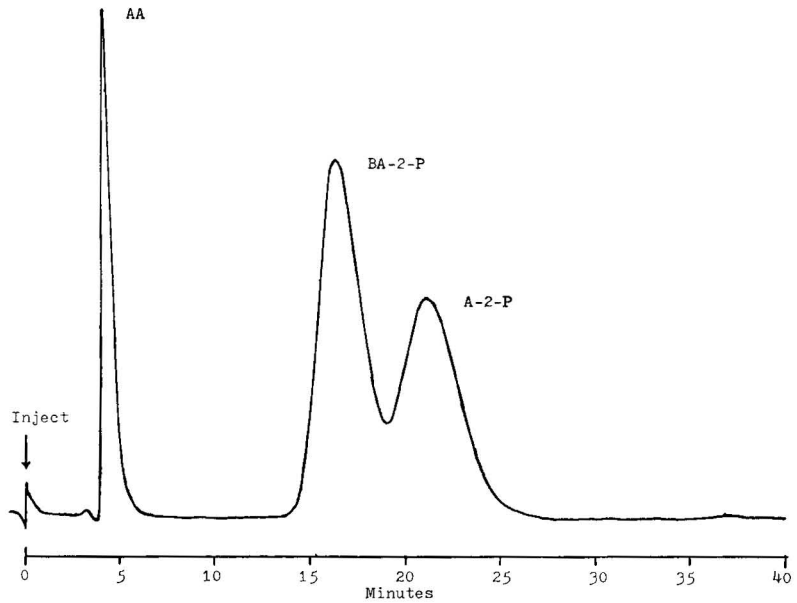

Fig. 6. HPLC separation of reaction products formed when phosphorus oxychloride is added to AA in aqueous calcium hydroxide (pH 13.1) at 2°.

TABLE II

MOBILE PHASE FOR SEPARATION OF PHOSPHO-MONOESTER AND PHOSPHO-DIESTER OF AA

Eluent flow-rate, 0.5 ml/min.

Potassium dihydrogen phosphate (M)	*pH*	*Retention time (min)*	
		Phospho-diester	*Phospho-monoester*
0.5	4.4	5.8	6.2
0.3	4.4	6.6	7.2
0.1	4.4	12.8	15.4
0.1	5.0	12.4	13.6
0.1	4.7	12.6	14.8
0.1	4.0	14.0	16.4
0.1	3.5	17.2	17.2

phase of 0.1 *M* potassium dihydrogen phosphate at pH 4.4. That same eluent gave baseline resolution of the 5,6-acetal derivatives of those two phosphate esters (Fig. 2).

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Note

High-pressure liquid chromatography of caprolactam and its metabolites in urine and plasma

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Caprolactam (Fig. 1) is a cyclic amide chemically synthesized in a multi-step process from phenol. The last stage of the synthesis usually involves the Beckmann rearrangement of cyclohexanone oxime to the seven-membered ring. In the synthesis of nylon 6, caprolactam is hydrolyzed to the straight-chain ε-aminocaproic acid which then spontaneously polymerizes via amide linkages into the linear polymer. U.S. production of nylon 6 is estimated at over $0.5 \cdot 10^9$ kg per year, the applications of which include tire cord, apparel, carpeting, plastics and packaging films.

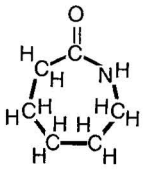

Fig. 1. Structure of caprolactam, $C_6H_{11}ON$.

A moderate order of toxicity has been reported for caprolactam, with acute oral lethalities of >1 g/kg body weight in the rat[1–3] and rabbit[2]. Other effects attributed to caprolactam include pulmonary[4] and skin[5,6] irritation in humans and sensitization in guinea pigs[7,8]. After administration to the rat of a lethal dose of caprolactam (>900 mg/kg, intraperitoneal) toxic effects include stupor and bleeding from the nostrils, followed by clonic convulsions which progress to tonic convulsions and death[11]. Chronic and subchronic administration of caprolactam to rodents produced no pathological changes[9–11]. Greene *et al.*[12], using a variety of bacterial and mammalian cell screens, have recently reported that caprolactam shows no mutagenic activity.

Several gas[13–15], liquid[16] and gel permeation[17–20] chromatographic procedures exist for the determination of caprolactam, but most were developed primarily for the determination of caprolactam in mixtures of oligomers from extracts of the polymer. The present study, therefore, was undertaken to develop a sensitive and reliable high-pressure liquid chromatographic (HPLC) procedure for the determination of caprolactam in biological samples, adaptable to pharmacokinetic and metabolic studies.

EXPERIMENTAL

Standards

Commercial grade caprolactam (purity >99%) was obtained from the Fibers Division of Allied Chemical Corp. (Hopewell, Va., U.S.A.). Caprolactam was dissolved in distilled water to yield a stock standard containing 1 mg/ml. Aliquots of the stock standard were dissolved in the appropriate volume of distilled water to yield caprolactam standards containing 0.1, 0.01 and 0.001 mg/ml.

Equipment

Reversed-phase chromatography was performed using an LDC Constametric IIG HPLC system including an LDC Spectromonitor III variable-wavelength absorbance detector (Laboratory Data Control, Riviera Beach, Fla., U.S.A.). Samples were injected onto the column using a Rheodyne model 7120 sample injector (Rheodyne, Berkeley, Calif., U.S.A.). Separations were achieved with two Lion Technology (Dover, N.J., U.S.A.) FNP 018 reversed-phase columns (particle size, 10 μm; column dimensions, 25 cm $\times$ 4.6 mm I.D.) connected in series. A guard column (Whatman, Clifton, N.J., U.S.A.) packed with pellicular octadecylsilane (particle size, 25–37 μm) was attached preceeding the analytical columns. The elution rate was 2 ml/min, and caprolactam was detected at a wavelength of 210 nm, with the absorbance detector at sensitivities of 0.005–1.0 absorbance units full scale (AUFS). Solvent programming (Gradient Master, LDC) was used to establish optimum solvent ratios.

Elution solvent

The elution solvent consisted of glass-distilled acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) and micro-filtered distilled water (Milli-RO and Milli-Q Water Purification System, Bedford, Mass., U.S.A.). Acetonitrile–water (11:89) was found to give adequate resolution of caprolactam from interferring peaks in urine and plasma samples. The elution solvent was degassed under vacuum before use, and kept under nitrogen during chromatography.

RESULTS AND DISCUSSION

Retention time

At the flow-rate of 2 ml/min, using acetonitrile–water (11:89) as the elution solvent, caprolactam eluted as a sharp, symmetrical peak (Fig. 2). The peaks appearing between three and four minutes on the chromatograms were found to be due to the distilled water in which the standards were dissolved. The retention time was highly reproducible, 20 injections of caprolactam standards of varying concentrations over a 5-day period gave a mean retention time (t_R) of 576 sec, with a coefficient of variation of 1% (Table I).

Precision and sensitivity

Precision was evaluated by injecting, over a 3-day period, ten 9.5-μl aliquots of standard solution containing 95 ng caprolactam. Reproducibility of peak height was good, with a coefficient of variation of 1.03%, representing the combined errors

of injection, detection and flow-rate fluctuation (Table I). Mean sensitivity of detection, mm peak height per ng of caprolactam (Table I) and the chromatograms shown in Fig. 2 indicate that 5–10 ng of caprolactam can easily be detected and quantitated.

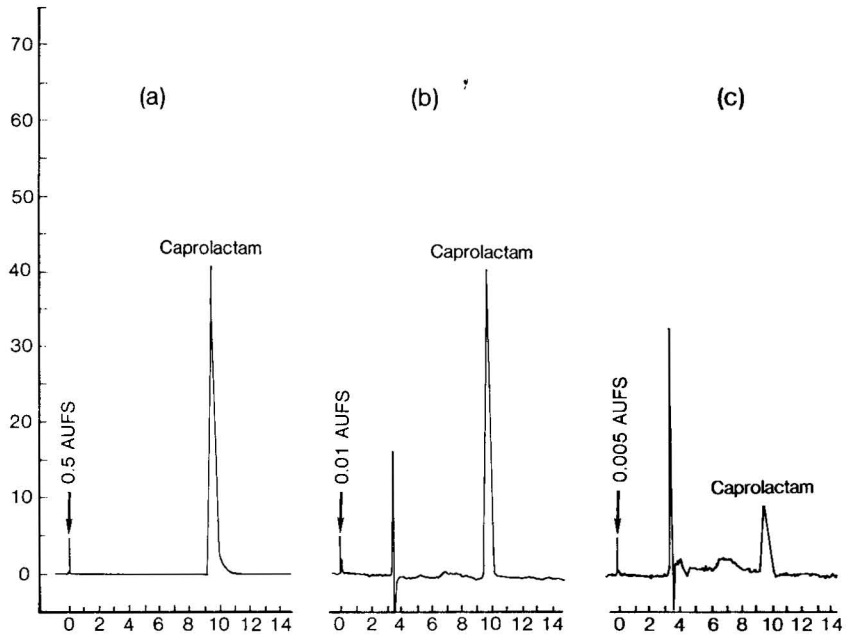

Fig. 2. HPLC chromatogram tracings of caprolactam standards; (a) 0.95 μg, (b) 95 ng, (c) 9.5 ng. Elution solvent, acetonitrile–water (11:89); flow-rate, 2 ml/min.

TABLE I

REPRODUCIBILITY OF RETENTION TIME AND PEAK HEIGHT FOR CAPROLACTAM BY HPLC

Retention time	
Injections, N*	20
Range (sec)	564–582
Mean	576
Standard deviation (sec)	5.78
Coefficient of variation (%)	1.00
Peak height	
Injections, N**	10
Range (mm)	95.5–98.5
Mean	96.6
Standard deviation (mm)	0.994
Coefficient of variation (%)	1.029
Sensitivity (mm peak height/ng)***	2.034

* Successive injections of caprolactam standards (9.5 ng–0.95 μg) over a 5-day period, using several separately-prepared batches of the elution solvent.

** Successive injections of caprolactam standard (95 ng) over a 3-day period.

*** Calculated to maximum sensitivity, 0.005 AUFS.

Linearity

The relationship between peak height and amount of caprolactam injected was linear over a range of 9.5 ng–9.5 μg (Fig. 3). Fig. 3 contains a logarithmic graph of peak heights (converted to a common sensitivity) plotted against the quantity of caprolactam injected. Injections (5–9.5 μl) of each caprolactam standard solution were made over a two-day period using microliter syringes (Hamilton, Reno, Nev., U.S.A.).

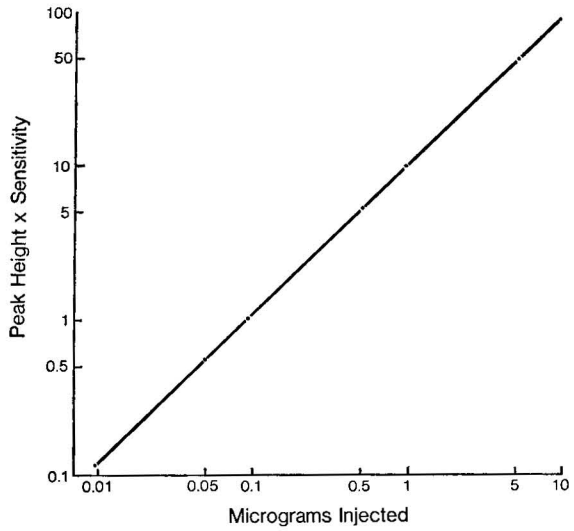

Fig. 3. Linearity, peak height *vs.* amount of caprolactam injected (9.5 ng–9.5 μg).

BIOLOGICAL APPLICATIONS

Due to the nature of mobile and stationary phases used in reversed-phase chromatography, aqueous (such as biological) samples may be loaded directly onto the column, provided that adequate measures are taken to protect the column from particulate matter and components in the samples which might bind irreversibly to the packing material. If a chromatographic procedure is to be adapted to biomonitoring and metabolic studies, the advantage of direct analysis becomes important in terms of convenience and speed. Furthermore, in the analysis of biological fluids for metabolites, reversed-phase chromatography allows a view of the total metabolic profile, rather than the selective glimpses provided by extraction with organic solvents followed by normal-phase chromatography.

Determination of caprolactam in rat urine and plasma

For the determination of caprolactam in urine, caprolactam (dissolved in distilled water) was added to aliquots of untreated rat urine to yield concentrations of 0.05, 0.1, 0.5 and 1 mg/ml. The spiked urine samples were then injected directly onto the HPLC for caprolactam determination.

For the determination of caprolactam in plasma, caprolactam (dissolved in

distilled water) was added to aliquots of untreated rat plasma to yield concentrations of 0.05, 0.1, 0.5 and 1.0 mg/ml. An equal volume of acetonitrile was added to each spiked sample to precipitate to plasma proteins. Following centrifugation (5 min at *ca.* 1000 *g*), the supernatants were carefully removed using pasteur pipettes. The supernatants were then injected onto the HPLC for determination of caprolactam.

As indicated in Table II, determination of caprolactam in both urine and plasma was quantitative over the concentration range investigated. Chromatogram tracings of spiked urine and plasma samples are shown in Fig. 4a and 4b, respectively.

TABLE II

RECOVERY OF CAPROLACTAM FROM SPIKED URINE AND PLASMA SAMPLES

Caprolactam added (mg/ml)	*Recovery (%)**	
	Urine	*Plasma*
0	—	—
0.05	95.56 ± 1.44	98.72 ± 3.80
0.1	100.08 ± 1.99	97.30 ± 1.56
0.5	96.47 ± 5.06	98.5 ± 1.35
1.0	93.57 ± 5.65	99.60 ± 2.98

* Recovery given as mean ± standard deviation.

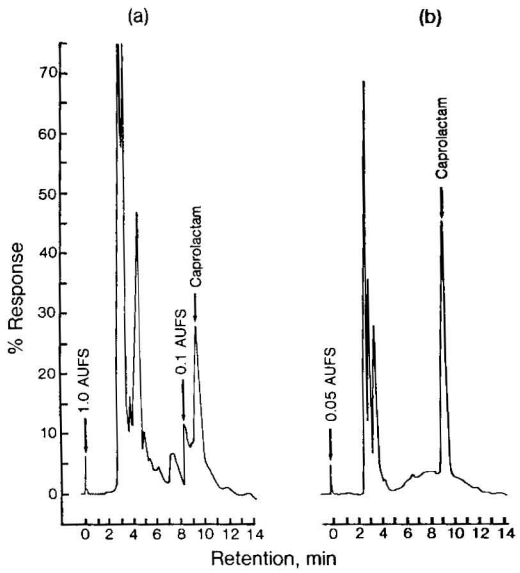

Fig. 4. Chromatogram tracings of urine spiked to 50 μg/ml (a), and plasma spiked to 100 μg/ml (b). Spiked plasma samples were diluted 1:1 with acetonitrile to precipitate the proteins. The large peak (t_R, 7.5 min) immediately preceeding caprolactam was not observed in all urine samples.

Determination of urinary metabolites

For the determination of urinary metabolites of caprolactam, male Fischer 344 rats were dosed (by oral intubation) with 1 μCi [carbonyl-^{14}C]caprolactam

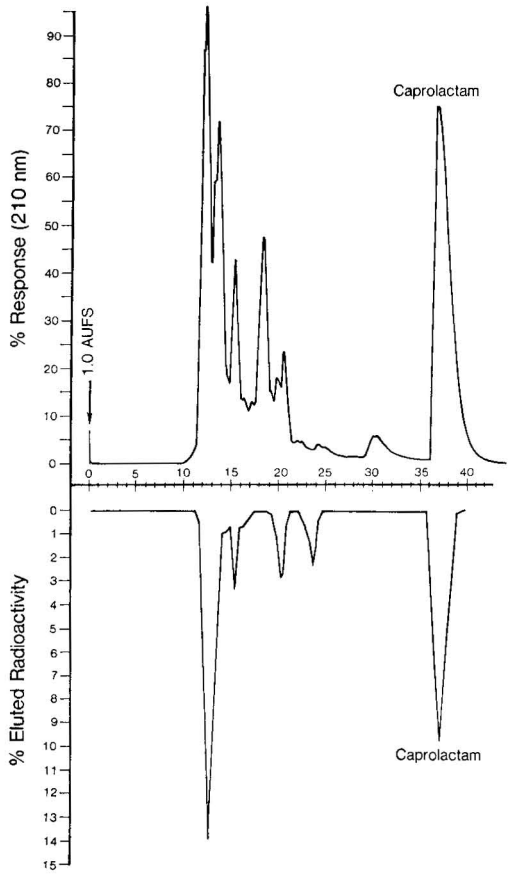

Fig. 5. Chromatogram tracing of 6-h urine of rat orally dosed with 1.5 g/kg of [carbonyl-^{14}C]caprolactam (a), and elution profile of radioactivity (b). Flow-rate, 0.5 ml/min.

(1.5 g/kg body weight). Six hours after dosing, the urine was collected, centrifuged to remove particulate matter, and analyzed by HPLC. The flow-rate was reduced to 0.5 ml/min to allow better resolution of the early-eluting metabolites. The eluate was collected at 24-sec intervals and radioassayed. Fig. 5a shows an HPLC chromatogram of the urine and the radioactivity contained in each of the corresponding vials of the eluate (Fig. 5b). As shown in this figure, the radioactivity administered as [^{14}C]caprolactam was excreted primarily as a major metabolite (t_R, 12.5 min) and as the parent compound. Three lesser metabolites were also observed, with t_R values of approximately 15.5, 20.5 and 23.5 min.

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CHROM. 12,332

Note

High-performance liquid chromatographic determination of tert.-butylhydroquinone in vegetable oils

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tert.-Butylhydroquinone (TBHQ) is used as an anti-oxidant in vegetable oils. It can be used alone or in combination with other synthetic anti-oxidants[1]. For the quantitative determination of TBHQ a spectrophotometric[2] or a gas chromatographic (GC) method[3] can be applied. Although the GC method is more specific it is time- and labour-consuming as it is necessary to extract and derivatize the TBHQ. Recently, a gel permeation chromatographic method for the analysis of TBHQ and other anti-oxidants was reported[4]. Analysis time for this method is *ca.* 30 min and the detection limit for TBHQ is 500 ng. We report here a rapid and specific high-performance liquid chromatographic (HPLC) method for the quantitative determination of TBHQ in oils.

EXPERIMENTAL

Reagents

Dioxane containing 0.1 g water/100 g, *n*-hexane (spectroscopic grade) containing 0.004 g water/100 g and Oxinex 2378 were obtained from Merck (Darmstadt, G.F.R.). TBHQ was obtained from Eastman (Kingsport, Tenn., U.S.A.), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and 2,4,5-trihydroxybutyrophenone (TBP) from BDH (Poole, Great Britain) and dodecyl gallate (DG) from Naarden (Wormerveer, The Netherlands).

Equipment

The HPLC equipment consisted of a Varian 5000 pump, Valco inlet valve and a Farrand Mk 1 spectrofluorometer, equipped with a 7-μl flow cell. A primary wavelength of 309 nm, a secondary wavelength of 340 nm and a slit width of 10 nm were used. Peak retention times and areas were determined with a Hewlett-Packard 3352B laboratory data system. The stainless steel column (25 $\times$ 0.4 cm I.D.) was slurry-packed[5] with 5 μm LiChrosorb SI 60 (Merck).

Method

The mobile phase consisted of dioxane and *n*-hexane (24:76, v/v), pumped at a flow-rate of 3 ml/min. TBHQ standard solutions containing 1, 2, 2.8, 4 and 10

mg/100 ml in *n*-hexane were prepared. A calibration curve (peak areas against concentration) was obtained by injecting 13.9 μl of each standard solution in duplicate. TBHQ in oil samples was determined by direct injection of 13.9 μl oil. Recovery determinations were conducted by adding 20 mg TBHQ to 100 g maize oil (oil density 0.918 g/cm^3 at 20°).

RESULTS AND DISCUSSION

A typical chromatogram of TBHQ dissolved in oil is reproduced in Fig. 1. TBHQ eluted with a capacity factor of $k' = 2.1$ from a column giving 4600 theoretical plates. The maize oil tocopherols, which are also detectable under the prescribed conditions of analysis, eluted with a capacity factor close to zero. An oil sample without TBHQ did not show any peaks at or near $k' = 2.1$.

Solutions of Oxinex 2378, BHT, BHA, DG and TBP in *n*-hexane were prepared and injected. Only BHA gave a detectable peak which eluted with a k' value similar to that of the tocopherols. Subsequently a mobile phase of 4% dioxane in

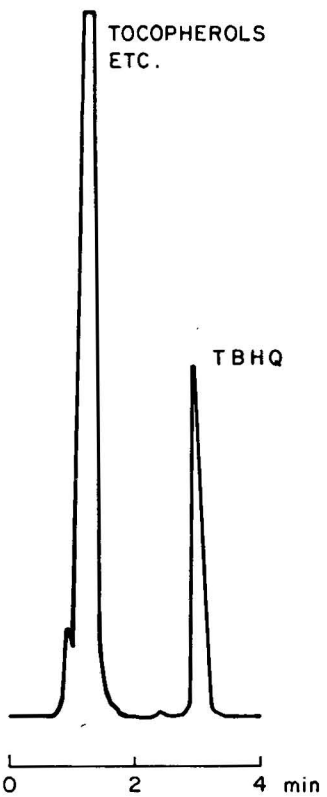

Fig. 1. Chromatogram of 20 mg TBHQ/100 g maize oil. Steel column (25 × 0.4 cm I.D.) slurry-packed with LiChrosorb SI 60 (5 μm), with dioxane and *n*-hexane (24:76) as mobile phase (3 ml/min) and a fluorometric detector.

n-hexane, which was more suitable for the separation of the tocopherols[6], was used and we found that BHA eluted with γ-tocotrienol.

A linear calibration curve for TBHQ was obtained and the least-squares method was applied to calculate the line of best fit for the data ($y = 48.3x - 2.2$, $r = 0.9998$). A recovery figure of 98.5% for TBHQ from maize oil was obtained with a coefficient of variation of 2.4% for eight determinations. The detection limit was 6 ng, and the average analysis time per sample was 5 min. The described method is therefore fast, sensitive and convenient.

ACKNOWLEDGEMENTS

We wish to thank Mrs. S. C. C. Smit for assistance with analyses, Mr. M. Steyn (Simba-Quix) and Dr. P. van Twisk (Federal Foods) for supplying literature on TBHQ and for helpful discussions.

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Note

Rapid analysis of doxycycline from biological samples by high-performance liquid chromatography

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A high-performance liquid chromatographic (HPLC) method for the determination of tetracyclines, except for the more lipophilic compound doxycycline, in urine, plasma and animal tissues has been described by Sharma and co-workers[1,2]. Maximum resolution on reversed phase columns can be achieved with acidic eluents when their pH is below the isoelectric point of the tetracyclines (*ca.* pH 5)[3].

As the analysis of tetracyclines in biological samples, according to Sharma and co-workers[1,2], requires extraction into an organic solvent and reextraction into aqueous acids, I have developed a single-step extraction method with subsequent chromatographic analysis on RP-8 material that is applicable for tetracycline and pyrrolidinomethyltetracycline[4,5]. In principle, this method is also applicable to doxycycline since at pH 2.4 tetracycline and pyrrolidinomethyltetracycline are eluted prior to the more lipophilic doxycycline, as is also described by others[3]. However, the rate of recovery of doxycycline varies when different batches of column packing material are used. This difficulty can be overcome and the reproducibility of the results can be sufficiently enhanced by: (1) preequilibration of the columns with a compound more lipophilic than doxycycline or any other tetracycline to be analyzed; and (2) use of a final eluent less apolar than the equilibration eluent.

The improved procedure is described in this paper.

MATERIALS AND METHODS

All chemicals used were of analytical grade. Anhydrotetracycline was kindly provided by Hoechst (Frankfurt/M, G.F.R.). Doxycycline was a gift from Pfizer (Karlsruhe, G.F.R.).

The HPLC apparatus used in this study was essentially that described previously[4]. Columns (25 × 0.4 cm) packed with Nucleosil® 10 C_8 (Macherey, Nagel & Co., Düren, G.F.R.) were conditioned for 20 min with eluent I (0.01 *M* NaH_2PO_4 in water–acetonitrile, 70:30; adjusted to pH 2.4 with 1 *M* HNO_3) containing 0.1 mg anhydrotetracycline/100 ml, at a flow-rate of 1.9 ml/min. Complete equilibration was checked by UV analysis of the eluent before and after passing the column. The UV absorption was measured at 270 nm, *i.e.*, at the absorption maximum of anhydrotetracycline.

The chromatographic analysis of doxycycline was performed with eluent II (0.01 *M* NaH_2PO_4 in water–acetonitrile, 73:27; pH 2.4), the UV absorption being measured at 357 nm.

For the extraction of doxycycline from biological samples the procedure described for tetracycline and pyrrolidinomethyltetracycline[4] was slightly modified.

(a) Extraction from blood or serum. All steps were performed in the cold (*ca.* 0°). A 50- or 100-μl volume of blood or serum was mixed with 150 μl 0.03 *M* H_3PO_4; after 15 min 1 ml acetonitrile–buffer (0.01 *M* NaH_2PO_4, pH 2.4) (50:50) was added. 5 min later most of the precipitated proteins were spun down (2000 *g* for 5 min) and the supernatant taken for the HPLC analysis.

(b) Extraction from tissue. Organs were resected from the animals, rinsed in ice-cold 0.9% NaCl, dried on filter-paper and homogenized in ice-cold 0.03 *M* NaH_2PO_4 (1 part organ + 4 parts buffer) with an Ultra Turrax (Janke & Kunkel, Staufen, G.F.R.) at 15,000 rpm for 30 sec. After 15 min, 200 μl of the homogenate were mixed with 1 ml acetonitrile–buffer (0.01 *M* NaH_2PO_4, pH 2.4) (50:50) and centrifuged. For the chromatographic analysis, 50- or 100-μl aliquots of the supernatant were applied to the column.

For recovery experiments, doxycycline dissolved in 1.5 m*M* $MgSO_4$ was added to serum or organ homogenates (20% in 0.9% NaCl) to give a final concentra-

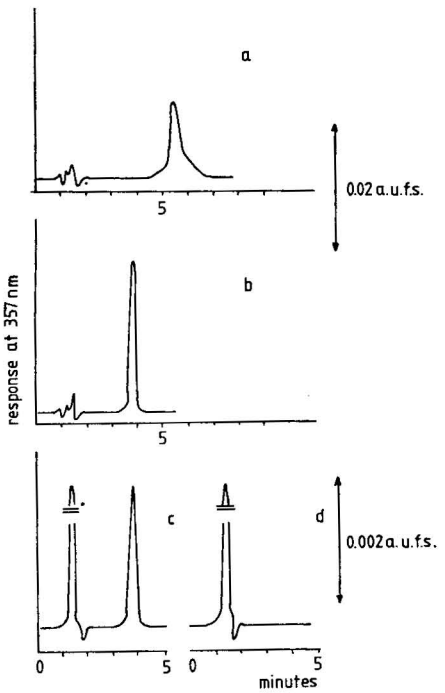

Fig. 1. Chromatographic analysis of doxycycline on RP C_8. Elution patterns: a, 208 ng doxycycline on an unconditioned column (tailing); b, 208 ng doxycycline on a column preequilibrated with anhydrotetracycline (no tailing, symmetric peak); c, 21.9 ng doxycycline, extracted from serum (10.5 μg/ml); doxycycline from tissue homogenates gave the same result; d, a control serum. The sensitivity of the photometer in c and d was ten times that in a and b.

tion of 0.5, 1, 5, 10, 50, 100 or 200 μg/ml. After incubation at 37° for 30 min, doxycycline was extracted as described above.

RESULTS AND DISCUSSION

High ionic strength of the eluent or an unconditioned column result in tailing of the eluted compound (Fig. 1)[3,4]. When the column is conditioned with the lipophilic anhydrotetracycline, an eluent with relatively low content of acetonitrile (27%) elutes doxycycline within 5 min (Fig. 1b). The anhydrotetracycline adsorbed to the column is not eluted as can be proven by UV analysis at 270 nm.

Doxycycline extracted from biological samples has the same retention time of 3.9 min as doxycycline standards dissolved either in eluent or in 1.5 m*M* $MgSO_4$.

The recovery of doxycycline from serum and organ homogenates is complete when the doxycycline concentrations are not higher than 100 μg/ml. At a concentration of 200 μg/ml the recovery from liver homogenates and from serum is only 80–85%. This may be due to a partial precipitation of doxycycline together with some proteins, as described for tetracycline[5]. In repeated experiments the standard deviation of the extraction and the chromatographic analysis was between 4 and 5%.

Pilot studies showed that in mice treated with doxycycline (50 μg/g body weight), i.v. doxycycline could be found in serum and liver 60 min after the injection of the drug. The serum concentration was 5.5 μg/ml; the liver contained 63 μg/g.

In conclusion, the main advantages of the method described are: (1) complete and simple extraction from serum and tissue samples; (2) rapid and highly reproducible analysis on different batches of the column packing material.

ACKNOWLEDGEMENTS

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Note

Automated ion-exchange chromatographic analysis of usual and unusual natural polyamines

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(Received September 3rd, 1979)

For the study of polyamine content and metabolism in *Euglena gracilis* Z[1–3], it was necessary to develop a convenient and rapid method for the determination of usual and unusual polyamines such as *sym*-nor-spermidine, *sym*-nor-spermine, *sym*-homospermidine and aminopropylcadaverine, most of which are present in this algal flagellate[1–5]. The method described here allows the determination of histidine, lysine, ethanolamine, carbamylputrescine, arginine, 1,3-diaminopropane, putrescine, histamine, cadaverine, *sym*-nor-spermidine, spermidine, *sym*-homospermidine, agmatine, aminopropyl cadaverine, *sym*-nor-spermine, spermine and 1,7-diaminoheptane in a single sample and without prior purification of the sample at the picomole level in less than 2 h.

MATERIALS AND METHODS

Sample preparation

Aliquots of 50 ml ($1 \cdot 10^5$–$10 \cdot 10^5$ cells) of *Euglena gracilis* (strain Z), grown as described elsewhere[6], were harvested by centrifugation at 5000 *g* for 15 min and the pellet was re-suspended in 0.5 ml of 5% trichloroacetic acid (TCA) in 0.05 *M* hydrochloric acid. The cells were broken by sonication for 20 sec with an MES ultrasonic cell disruptor. The homogenate was centrifuged at 5000 *g* for 15 min and the pellet was then re-extracted with a further 250 μl of 5% TCA in 0.05 *M* hydrochloric acid and re-centrifuged. The two supernatants were then pooled and a 50-μl sample was used directly for the determination of polyamines.

Chemicals

Amino acids and the usual polyamines used were obtained from Sigma (St. Louis, Mo., U.S.A.). *Sym*-nor-spermidine and *sym*-nor-spermine were purchased from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.). N-3-Aminopropylcadaverine and *sym*-homospermidine were gifts from Professor S. S. Cohen (Stony Brook, N.Y., U.S.A.), Dr. D. E. Worth (Parke, Davis & Co., Ann Arbor, Mich., U.S.A.) and Prof. A. N. Radhakrishnan (Hyderabad, India). *o*-Phthalaldehyde was

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obtained from Fluka (Buchs, Switzerland), and all other chemicals used for the preparation of buffers and reagents were obtained as the highest purity grade from Merck (Darmstadt, G.F.R.). All of the amino acid and polyamine solutions were prepared at a concentration of $6 \cdot 10^{-6}$ *M* in 0.1 *M* hydrochloric acid.

Instrumentation and chromatographic conditions

An amino acid analyser (Liquimat-Labotron; Kontron, Vélizy-Villacoublay, France) equipped with a fluorimeter (Labotron FFM-31) using a 50-μl flow cell was employed. An integrator (ICAP-10; LTT, Saint-Honorine, France) was coupled to the fluorimeter for quantification of the amines by the internal standard method. 1,7-Diaminoheptane was used as the internal standard. The recorder (W+W 600-Tarkan; Kontron) was set at 100 mV for 100% relative fluorescence. Separation of amines was carried out on a 11.5 × 0.45 cm I.D. column of DC-4A cation-exchange resin (Durrum, Palo Alto, Calif., U.S.A.). The composition of the two buffers used was as follows: first buffer (pH 5.60), 0.20 *N* sodium citrate dihydrate–0.30 *N* sodium chloride–4% ethanol; second buffer (pH 5.65), 0.20 *N* sodium citrate dihydrate–2.50 *N* sodium chloride–6% ethanol. The buffers and reagent were prepared as described earlier[7–9]. Two temperatures were used: 61° during the first 48 min and thereafter 78° until the end of the chromatographic run. Flow-rates of 26 ml/h for the buffer and 20 ml/h for the reagent were maintained; the first buffer was run for 44 min, then the

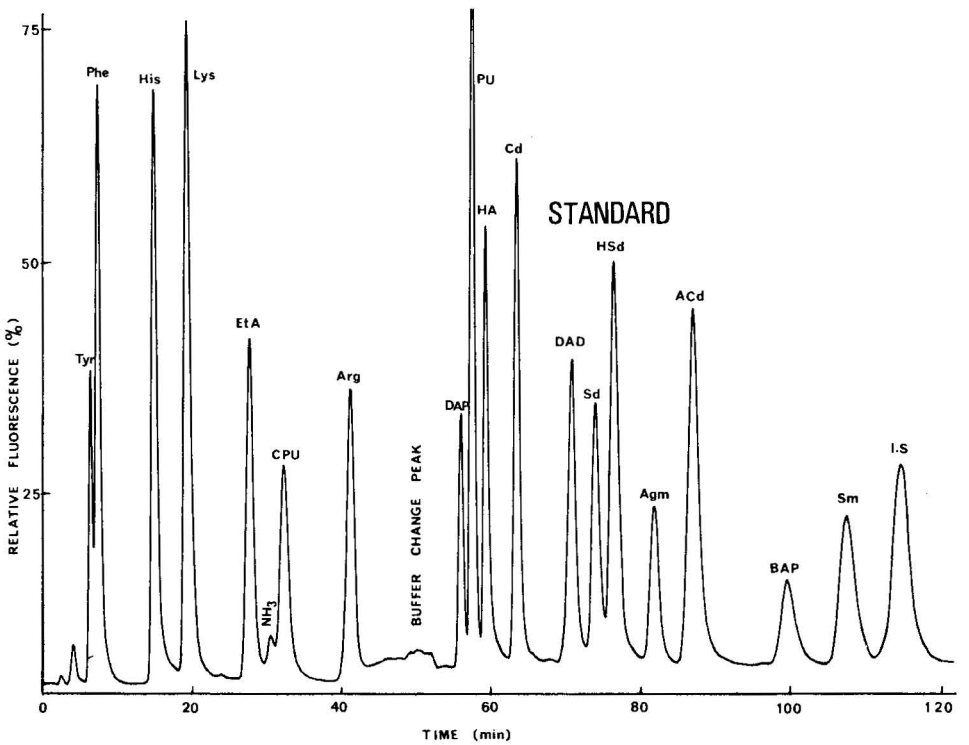

Fig. 1. Chromatographic resolution of a standard mixture of 300 pmole of each of the common basic amino acids and usual and unusual polyamines. Abbreviations as in Table I.

second buffer was run for 68 min. The column was regenerated with 0.2 *M* sodium hydroxide solution containing 250 mg/l of EDTA for 8 min and equilibrated with the first buffer for 30 min.

RESULTS

Fig. 1 shows the elution pattern of a standard mixture (300 pmole of each) of common basic amino acids and usual and unusual polyamines. The components of the mixture, retention times, relative fluorescence peak areas (in arbitrary units) and the coefficient values used for the concentration calculations (KC), using 1,7-diaminoheptane as the internal standard, are presented in Table I. Acidic and neutral amino acids (aspartic acid to phenylalanine) were eluted in a group with the first buffer, and subsequently histidine, lysine, ethanolamine, ammonia, carbamylputrescine and arginine were eluted with good resolution. The second buffer eluted and resolved well the usual and unusual polyamines. The given concentrations of salt and ethanol gave optimal chromatographic resolution. It must be emphasized that the time at which the temperature is changed as well as salt and ethanol concentrations are critical for obtaining good separations of these compounds. Complete analysis was effected in 118 min.

TABLE I

PRECISION AND ACCURACY OF THE METHOD

Compound	*Abbreviation*	*Retention time* (min)*	*Surface area of peaks*	*Constant KC**
Histidine	His	14.7 ± 0.2	86,949	1086
Lysine	Lys	19.2 ± 0.2	103,900	909
Ethanolamine	EtA	27.7 ± 0.1	67,741	1395
Ammonia	NH_3	30.5 ± 0.2	—	—
Carbamylputrescine	CPu	32.1 ± 0.1	67,021	1410
Arginine	Arg	41.1 ± 0.2	64,467	1465
1,3-Diaminopropane	DAP	55.8 ± 0.1	28,465	3320
Putrescine	PU	57.4 ± 0.2	81,197	1182
Histamine	HA	59.3 ± 0.2	72,283	1347
Cadaverine	Cd	63.5 ± 0.1	82,202	1149
3,3′-Diaminodipropylamine (*sym*-nor-spermidine)	DAD	70.7 ± 0.1	72,590	1329
Spermidine	Sd	73.8 ± 0.3	53,158	1777
Sym-homospermidine	HSd	76.3 ± 0.1	91,092	1037
Agmatine	Agm	81.6 ± 0.2	41,229	2472
Aminopropylcadaverine	ACd	86.8 ± 0.1	123,453	833
N,N′-Bisaminopropyl-1,3-propanediamine (*sym*-nor-spermine)	BAP	99.8 ± 0.1	40,014	2546
Spermine	Sm	107.7 ± 0.2	72,596	1329
1,7-Diaminoheptane	I.S.**	114.8 ± 0.2	94,508	

* Average of ten determinations on a mixture containing 300 pmole of each of the products. Quantification was effected with an ICAP 10 integrator coupled to the fluorimeter.

** Internal standard.

Fig. 2 shows the chromatogram obtained from a crude sample of *Euglena gracilis* Z grown in presence of continuous light. Putrescine, *sym*-nor-spermidine, spermidine and *sym*-nor-spermine were found to be the major polyamines present.

The concentrations [expressed as picomoles per cellular volume (μl)] obtained were 257 for putrescine, 179 for *sym*-nor-spermidine, 1160 for spermidine and 1526 for *sym*-nor-spermine. In addition, small amounts of carbamylputrescine, *sym*-homospermidine, 1,3-diaminopropane and spermine were also found.

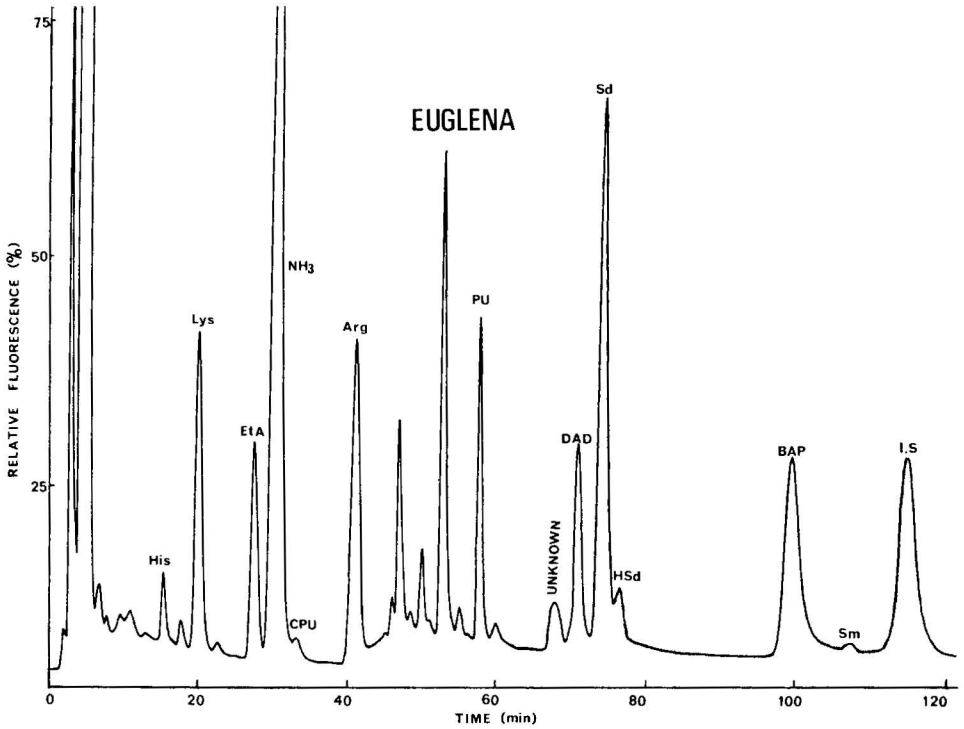

Fig. 2. Chromatogram of a 50-μl crude sample of *Euglena gracilis* Z grown in the presence of continuous light. Abbreviations as in Table I.

DISCUSSION

In this work, the previously described method for the determinations of common basic amino acids, mono-, di- and polyamines and phenolic and indole amines[9] has been further modified so that it could be adapted to the separation of unusual polyamines. The method has been applied to the analysis of these polyamines in crude extracts of *Euglena*. The sensitivity of the method is 1–300 pmole. The presence of large amounts of amino acids in *Euglena* did not interfere with the determination of the amines. The method has proved to be very reliable and the use of an integrator and an internal standard contributed to easier and reproducible quantification. The results presented are averages of ten determinations and the difference between them was never greater than 3%. This method, together with our previously described methods[7–9], would allow the detection of all presently known polyamines, in any kind of crude biological sample.

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Note

Thin-layer chromatography of phosphonic acids

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(First received November 23rd, 1978; revised manuscript received September 4th, 1979)

The phosphonic acids described here are essentially hydrolysis residues of intermediate synthesis compounds or in some cases from their highly toxic anticholinesterase products. Although the original esters are readily determinable in trace quantities by gas chromatography[1] or chemical ionization mass spectrometry[2], the acids must be extracted and derivatized to be effectively determined by these methods.

We have developed a thin-layer chromatography (TLC) method that allows the detection and identification of a variety of phosphonic acids on the basis of R_F and relatively specific visualization tests.

The method development discussed here evolved from a required capability for additional confirmation in the detection and identification of various labile diester and haloester phosphonates. In principle, the residual phosphonic acids could be evidence of pre-existing diester or haloester compounds that had been present in environmental or biological media. For best separation of our phosphonic acids a cellulose chromatoplate and a developing solvent of 1 *M* ammonium hydroxide–*n*-butanol–*n*-propanol (1:3:1) were found to be most favorable. A variety of detection (or visualization) systems, all for the microgram and lower levels, are reported. The phosphonic acids included in this study are itemized in Table I.

EXPERIMENTAL

Apparatus and reagents

Glass plates (50 × 200 mm or 200 × 200 mm) were coated with a 0.5 mm thick layer of cellulose MN 300, and a Desaga thin-layer applicator (Brinkmann Westbury, N.Y., U.S.A.). The plates were allowed to air dry and then were stored over silica gel until used. No activation of adsorbent was necessary. Thin-layer chromatograms were developed with a solvent system of 1 *M* aqueous ammonium hydroxide–*n*-butanol–*n*-propanol (1:3:1). The solvents and other compounds were of reagent grade and required no further purification.

Reagents for visualization of developed chromatograms included a modified Hanes and Isherwood reagent for phosphorus[3], a horse serum cholinesterase preparation and indoxyl acetate substrate[4,5] for phosphonic acids, as well as for true inhibitors; a modified Dragendorff procedure for amines; and a nitropyridine disulfide method for thiols[6]. The phosphorus reagent was prepared by dissolving 1 g of am-

TABLE I

R_F VALUES OF SELECTED PHOSPHONIC ACIDS

Acid	*Abbreviation*	R_F	*Sensitivity threshold (μg)*			
			Molybdate reagent (blue spots)	*Cholinesterase reagent (white-on-blue spots)*	*Dragendorff reagent (orange-red/ orange spots)*	*Nitropyridine disulfide (yellow spots)*
Pinacolyl methylphosphonic	PMPA	0.75	1	5	$>10^3$	$>10^3$
S-2-Diisopropylaminoethyl methylphosphonothioic	DPAESMPA	0.72	1	0.05	6.5	10^3
Cyclohexyl methylphosphonic	CHMPA	0.67	0.5	5	$>10^3$	$>10^3$
Ethyl methylphosphonothiolic	EMPSA	0.57	0.5	5	$>10^3$	0.5
Cyclopentyl methylphosphonic	CPMPA	0.55	0.5	5	$>10^3$	$>10^3$
Isopropyl methylphosphonic	IMPA	0.44	0.5	5	$>10^3$	$>10^3$
Methylphosphonofluoridic	MPFA	0.31	0.5	1	$>10^3$	$>10^3$
Methylphosphonic	MPA	0.0	0.5	5	$>10^3$	$>10^3$

monium molybdate in 40 ml of water, followed by 3 ml of concentrated hydrochloric acid, 5 ml of 70% perchloric acid and diluted under chilled conditions to 100 ml with acetone. The light-yellow colored reagent was stable for 1 to 2 weeks when stored in the dark. The enzyme solution was Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) horse serum cholinesterase made up as 1 mg/ml in 0.05 *M* tris-(hydroxymethyl)aminomethane(Tris buffer); and the substrate, 15 mg of indoxyl acetate (Mann Labs., New York, N.Y., U.S.A.) in 5 ml of absolute ethanol. The indoxyl acetate solution was mixed just prior to use with a solution of 4 ml of 0.05 *M* Tris buffer, 5 ml of 2 *M* sodium chloride, 0.2 ml of 1 *M* calcium chloride and 2.8 ml of distilled water. The Dragendorff reagent was composed of 1.7 g of bismuth subnitrate, 20 g of tartaric acid and 80 ml of distilled water, to which was added a solution of 16 g of potassium iodide in 40 ml of water. The mixture was heated carefully until solution was complete. On cooling, some crystals were formed. This solution (reagent A) was stable indefinitely when kept in the dark. As a working solution for spraying, a 5 ml aliquot of the clear mixture of stock solution was dissolved daily with 10 g of tartaric acid in 50 ml of distilled water (reagent B). Nitropyridine disulfide, the reagent for thiols, was procured as a 0.03% in acetone aerosol spray from Newcell Biochemicals, Berkeley, Calif., U.S.A. For the thiol test a chromatographic development tank served as a chamber containing just enough concentrated hydrochloric acid to maintain a saturated acid atmosphere.

All of the phosphonic acids reported here with one exception, were synthesized by the Chemical Branch, Research Division, Chemical Systems Laboratory. S-2-Diisopropylaminoethyl methylphosphonothioic acid (DPAESMPA) was prepared by the authors through the hydrolysis of a prepared sample of bis(S-2-diisopropylaminoethyl) methylphosphonothioate and subsequent clean-up.

Procedures

The 50 × 200 mm cellulose chromatoplate was scribed 100 mm above the point of sample application as a horizontal break. Vertical scribing allowed up to five separate chromatography channels separated by parallel breaks in the adsorbent. This vertical discontinuity prevented overlapping of developing samples and allowed selective masking of plate areas for separate sprays of different detector solutions on the same plate. To determine the amount of unknown liquid (aqueous or other) sample to apply to the chromatoplate, aliquots of 1 μl, 5 μl, and 10 μl were applied to a test plate.

The 50 × 200 mm plate developing chamber was maintained at 23° to 25° and contained 20 ml of the 1 *M* ammonium hydroxide–*n*-butanol–*n*-propanol solution. This amount of developing solvent produced a desired depth of 10 mm. The chamber was lined with filter paper or filter paper wick (50 × 150 mm) to aid in saturating the chamber atmosphere. Samples were applied as water, methanol or chloroform solutions of the acids or esters and by means of micropipets or drawn out tubes. If particular acids were suspected, known samples of these were pipetted as potential references on a parallel channel. Development in this system required approximately 90 min for a 100 mm frontal movement.

Detection of the acids was performed as follows. The chromatoplate was air dried in a fume hood until no trace of an *n*-butanol odor was detected (approximately 30 min). An exposed portion of the plate was sprayed with the modified molybdate

reagent and while still wet was irradiated using a 254 nm ultraviolet source for 3 to 5 min. The plate channel was then further exposed to air and light for 1 to 2 h to assure complete color development. The phosphonic acids appear as blue spots on a white background.

Detection of the acids with the cholinesterase reagent was performed as follows. The previously masked channels, or preferably those on a fresh chromatoplate, were then sprayed with the enzyme solution in Tris buffer and the plate was incubated at room temperature for 20 min. The incubated plate was sprayed immediately after dilution of the indoxyl acetate reagent. The phosphonic acids appear as white spots on a blue background in approximately 5 min with DPAESMPA being the most sensitive.

DPAESMPA could also be detected by use of the Dragendorff reagent via the amine moiety of the compound. The unmasked plate was sprayed with reagent B (diluted Dragendorff) and a pink to red color at R_F 0.72 indicated the presence of DPAESMPA as opposed to pinacolyl methylphosphonic acid (PMPA) (R_F 0.75 by molybdate).

To detect as well as distinguish between phosphonic and phosphonothiolic acids, the nitropyridine disulfide reagent was sprayed on another unexposed channel and the chromatoplate was placed into the hydrochloric acid vapor chamber for several minutes for color development. This allowed the discrimination between cyclopentyl methylphosphonic acid (CPMPA) (R_F 0.55) and ethyl methylphosphonothiolic acid (EMPSA) (R_F 0.57). The thiolate appears as a stable yellow spot on a white background and is detectable down to 0.5 μg.

RESULTS AND DISCUSSION

Initial attempts to effectively chromatograph the various acids were made on silica gel plates and with various solvents and mixtures thereof. Solvents used included methanol, chloroform, 1 *M* ammonium hydroxide, *n*-propanol, ethanol, 1,4-dioxane, acetone, 2-propanol, and *n*-butanol. At best, only partial resolution was obtained with even the best of combinations. The work of Plapp and Casida[7] on the paper chromatography of hydrolysates from organophosphate pesticides appeared to most closely approximate our type of problem.

The cellulose chromatoplate and the 1 *M* NH_4OH–*n*-butanol–*n*-propanol (1:3:1) combination was selected after having tested also methanol, ethanol, dioxane and 2-propanol separately, as mixtures with one another, and with NH_4OH.

Samples of the acids up to 10 mg/ml in water were applied to the chromatoplates as 1 μl spots and developed separately to obtain their average R_F under the procedural conditions. Ten such spots were used for each R_F determination. The standard deviation was found to be less than 0.05 R_F units under the conditions of minimum spot blossoming. The average R_F values found for the acids, the visualization methods, and the sensitivity thresholds are shown in Table I.

Mixtures were made of equal volumes of the acid solutions. Aliquots in the range of 1 to 4 μl were applied to cellulose plates and the development allowed to proceed. After air drying, the molybdate reagent was sprayed onto the plates leaving some of the channels masked. With the exception of the compounds that were within 0.03 R_F units of one another, definitive spots were apparent. Even those that showed

overlapping still indicated the presence of at least two acids. Another developed channel was unmasked and sprayed with the cholinesterase reagent and subsequently, after incubation, with the indoxyl acetate substrate. At a level of 5 μg or higher per spot the acids showed the positive test of white on blue, but more as enzyme denaturants. DPAESMPA, the only true anticholinesterase among the compounds, was detectable at a level of 50 ng or higher. Similar observations had been made in our laboratory for parathion *versus* some of its acid decomposition products. Our experience over the years has been to distinguish between denaturation and inhibition by sensitivity of the test on the plate and the relative degree of reversibility.

The nitropyridine disulfide spray was applied to another previously masked channel and the plate placed into the hydrochloric acid vapor chamber. This produced a definitive yellow, EMPSA spot (R_F 0.57) which by molybdate had been detected as the upper of two overlapping spots, the other being CPMPA (R_F 0.55).

With the employment of TLC and the visualization methods, sufficient definition was obtained on the chromatoplates to give reasonable certainty of the presence of all of the acids in the mixture. Where fusion of one spot into another was evident, the selective detection systems aided in proving the presence of both compounds.

Attempts to extract the alkali salts of the phosphonic acids into chloroform prior to chromatography failed because their partition coefficients were highly favorable to the water. When a very minimal amount of hydrochloric acid was added to the sample solution with adjustment to approximate pH 4, extraction into chloroform of all acids with the exception of methylphosphonic acid (MPA) was quantitative. The addition of sodium chloride solution aided also, as a salting out process. Concentration of MPA in water through heating presented no problem since it is the most stable of the phosphonic acids.

Another chromatographic system tested here (also on a cellulose chromatoplate) was based on a paper chromatographic study made by the authors as an in-house report[8]. In this research, a butanol–acetic acid–water system was found to be very effective for separating phosphonic acids as well as basic (amino compounds) components related to some intact phosphonates. When checked against the cellulose TLC plate, blossoming of the phosphonic acid spots was significantly more evident thus providing poorer separations. The solvents were also more persistent thus affecting some of the visualization reactions, especially the enzyme method. Similarly, the same solvents when used on a silica gel plate produced sluggish separation, and poor visualization tests.

In one attempt at a reversed-phase type system using a C_{18} silica gel, we were less than successful mainly because the aqueous reagents did not effectively wet or penetrate the non-polar adsorbent.

High-performance liquid chromatography employing a cellulose column and an eluent system of *tert.*-amyl alcohol–1 *M* NH_4OH (7:1) had proved to be effective for separating a variety of phosphonic acids[9]. However, sensitivity was limited to that of the existing refractive index detectors (> 20 μg of compound). The TLC visualization systems described here give not only better sensitivity but also greater specificity of detection.

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CHROM. 12,321

Note

Separation of halogenated uracil derivatives from nucleobases and nucleosides by thin-layer chromatography on silica gel

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During studies on pyrimidine analogues in biological systems, a rapid and simple method was required for the detection of 5-fluorouracil, 5-chlorouracil, 5-bromouracil, 5-iodouracil, 5-fluorodeoxyuridine, 5-chlorodeoxyuridine, 5-bromodeoxyridine and 5-iododeoxyuridine in biological specimens and in mixtures of bases and nucleosides.

Nucleic acid constituents and their analogues have been extensively characterized by paper chromatography[1,2] and thin-layer chromatography[3,4]; the clear identification of the 5-halogenated uracil derivatives, however, remains a difficult problem.

In this paper, thin-layer chromatographic systems are described that allow the rapid separation of 5-halouracils and 5-halodeoxyuridines from the common nucleic acid constituents and the differentiation of these halogenated compounds.

EXPERIMENTAL

Analytical-reagent grade substances were purchased from Serva (Heidelberg, G.F.R.), Merck (Darmstadt, G.F.R.) or Calbiochem (San Diego, Calif., U.S.A.). Each substance (10–50 nmol) was applied to aluminium sheets (20 × 20 cm, not pre-treated) coated with a 0.2-mm layer of silica gel 60 with fluorescence indicator F_{254} obtained from Merck. The chromatograms were developed in standard chambers from Desaga (Heidelberg, G.F.R.) with the solvents given in Table I for 45–90 min at room temperature. When silica gel 60 HPTLC aluminium sheets (5.0 × 7.5 cm) were used, 2 nmol of each compound were sufficient and the time to develop the chromatograms was reduced to 10–15 min. Equilibration was not necessary. After air-drying of the developed chromatograms, the spots were detected under UV light (254 nm). When radioactive substances were employed, the spots were eluted or scraped off for scintillation counting.

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TABLE I

R_F VALUES FOR BASES, NUCLEOSIDES AND 5-HALOGENATED URACIL DERIVATIVES

Solvents: I, ethyl acetate–methanol (5:1); II, ethyl acetate–ethanol–water (32:4:1); III, diethyl ether–acetone–water (20:20:1); IV, methyl acetate; V, chloroform–ethanol–water (20:14:1); VI, chloroform–ethyl acetate–ethanol (5:24:6).

Compound	*Solvent*					
	I	*II*	*III*	*IV*	*V*	*VI*
Adenine	0.15	0.17	0.16	0.00	0.48	0.05
Guanine	0.05	0.06	0.09	0.00	0.26	0.00
Cytosine	0.00	0.03	0.02	0.00	0.20	0.00
Thymine	0.69	0.62	0.74	0.46	0.85	0.54
Uracil	0.57	0.54	0.62	0.29	0.75	0.39
5-Fluorouracil	0.82	0.72	0.87	0.71	0.85	0.63
5-Chlorouracil	0.86	0.78	0.90	0.78	0.89	0.72
5-Bromouracil	0.87	0.85	0.90	0.79	0.90	0.74
5-Iodouracil	0.89	0.85	0.91	0.82	0.93	0.77
Adenosine	0.16	0.18	0.17	0.00	0.51	0.08
Guanosine	0.00	0.02	0.02	0.00	0.27	0.00
Cytidine	0.00	0.02	0.03	0.00	0.18	0.00
Uridine	0.30	0.33	0.38	0.00	0.61	0.10
Deoxyadenosine	0.21	0.19	0.21	0.02	0.60	0.07
Deoxyguanosine	0.02	0.06	0.06	0.00	0.44	0.00
Deoxycytidine	0.04	0.05	0.05	0.00	0.35	0.00
Thymidine	0.59	0.48	0.65	0.20	0.82	0.41
5-Fluorodeoxyuridine	0.76	0.70	0.82	0.51	0.83	0.57
5-Chlorodeoxyuridine	0.80	0.68	0.85	0.58	0.90	0.60
5-Bromodeoxyuridine	0.79	0.75	0.86	0.61	0.88	0.63
5-Iododeoxyuridine	0.84	0.72	0.87	0.65	0.91	0.63

RESULTS

The R_F values of the compounds tested in six solvent systems are given in Table I; these are average values determined from 3–8 independent chromatographic runs. The results demonstrate that an efficient and rapid separation of the 5-halogenated uracil derivatives from the naturally occurring bases and nucleobases was achieved. This improvement over other solvent systems is due to the higher R_F values of the 5-halogenated uracil derivatives. To the best of the author's knowledge, these solvent systems provide much more rapid separations than comparable systems. Table I also shows that the different 5-halogenated base derivatives have distinct R_F values. In order to separate these closely related compounds completely, a combination of solvent systems is recommended.

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Note

Qualitative thin-layer chromatographic separation of 1,5-anhydroglucitol in the presence of other carbohydrates on silica gel impregnated with borate buffer

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Considerable attention has recently been focused on carbohydrates in body fluids[1,2]. Changes in the monosaccharide content are frequently associated with pathological conditions. The presence of 1,5-anhydroglucitol in cerebrospinal fluid (CSF) was established by Pitkänen[3] and confirmed by Smith *et al.*[4]. The separation of 1,5-anhydroglucitol from other polyols and sugars is desirable in an investigation of its role in diseases. Most common carbohydrates can be separated easily using thin-layer chromatography (TLC). We found that the separation of 1,5-anhydroglucitol can be performed on plates impregnated with borate buffer. We used nine specially selected reference polyols and sugars, and the separation of 1,5-anhydroglucitol from them was satisfactory under the conditions suggested.

EXPERIMENTAL

Materials and methods

All solvents were analytical-reagent grade reagents and were used without further purification. Borate buffer (0.1 *M*) was prepared from boric acid and sodium tetraborate.

Spray reagents

The stock solution contained 4.2 g of sodium metaperiodate in 75 ml of water. The spray reagent was prepared from the stock solution by dilution with acetone (1:100) just before use. Five minutes after the application of metaperiodate reagent the chromatograms were sprayed with *o*-tolidine solution containing 184 mg of *o*-tolidine, 95 ml of acetone and 0.6 ml of glacial acetic acid.

Chromatographic plates

The commercial plates used were DC-Karten SI, 10 × 20 cm (Riedel-De Häen, Hannover, G.F.R.), and high-performance thin-layer chromatographic (HPTLC) plates (silica gel 60 RP-2; E. Merck, Darmstadt, G.F.R.). Our coated plates were prepared from Kieselgel G nach Stahl (E. Merck), making the slurry in a borate-buffered solution (methanol–water, 1:1). Glass plates (10 × 20 cm) were

then coated with this slurry to a thickness of 0.25 mm. The plates were allowed to dry at room temperature for 24 h and stored at the humidity and temperature of the laboratory air.

The commercial plates were impregnated by soaking them in buffered methanol solution and allowing them to dry in air.

Application of sugar solutions

A micropipette (Drummond, Broomall, Pa., U.S.A.) was used to spot the samples. The sugar solutions were prepared at a concentration of 0.5% in water and spots of 1–2 μl were applied.

Development

The development solvent was 1-butanol–acetone–water (5:4:1). The plates were allowed to develop by ascending chromatography to a height of about 10 cm in closed glass tanks at room temperature. The average development time was 30–45 min. The plates were dried in air.

RESULTS AND DISCUSSION

The identification of 1,5-anhydroglucitol in the presence of other polyols and sugars is not possible on TLC plates using normal methods[5]. It has been reported[6] that 1,5-anhydroglucitol does not form a chelate with boric acid. We therefore decided to compare the influence of borate impregnation on the R_F values of 1,5-anhydroglucitol and other sugars and to find the optimal concentration of boric acid and pH of the impregnating solution. Boric acid solution (0.1 *M*) was chosen for the impregnation of the plates as it gave the best separation and did not produce diffused or tailed spots, and a good separation of 1,5-anhydroglucitol from other sugars was obtained. The optimal pH of the impregnating solution was *ca.* 8.4. If the pH was greater than 9 the migration of 1,5-anhydroglucitol was too slow, and if it was less than 8 the separation of 1,5-anhydroglucitol from other carbohydrates was poor. We tested several mixtures of the following eluates on borate-impregnated plates: methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, ethyl acetate, acetone, acetic acid, methyl ethyl ketone and water.

A good separation was obtained with the solvent system 1-butanol–acetone–water (5:4:1), which favoured the migration of carbohydrates with a wide range of R_F values.

Symmetrical spots were obtained both on HPTLC plates and on the home-made plates. The good separation of 1,5-anhydroglucitol from the other compounds is evidently due to the poor chelate-forming capacity of 1,5-anhydroglucitol with boric acid. The other sugars and polyols seem to be easily chelated on plates. The chelation is clearly dependent on the boric acid concentration and on the pH of the impregnating solution (Fig. 1). In order to achieve a good separation we used impregnation with 0.1 *M* borate buffer. This buffer was more concentrated than those commonly used for carbohydrate separations[7].

Table I and Fig. 1 summarize the data on the migration and the R_F values of the ten carbohydrates run on TLC plates.

The method permits a rapid, one-dimensional and reproducible separation of 1,5-anhydroglucitol from other closely migrating reference carbohydrates.

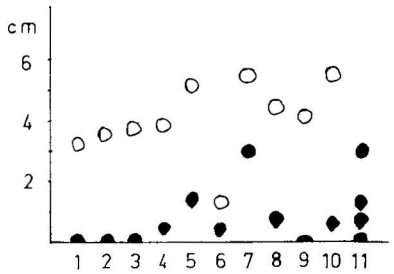

Fig. 1. Influence of borate impregnation on the TLC migration of the following carbohydrates: 1 = sorbitol; 2 = mannitol; 3 = xylitol; 4 = arabinitol; 5 = erythritol; 6 = inositol; 7 = 1,5-anhydroglucitol; 8 = glucose; 9 = fructose; 10 = xylose; 11 = mixture of 1, 5, 7 and 8. Open spots, methanol-impregnated plates; closed spots, borate buffer-impregnated plate, pH 8.5.

TABLE I

R_F VALUES OF CARBOHYDRATES RUN ON HPTLC PLATES AND ON HOME-MADE PLATES

The plates were impregnated either non-buffered or with borate buffer. 1-Butanol–acetone–water (5:4:1) was used as the eluent.

Carbohydrate	*HPTLC plate*			*Home-made plate*		
	Non-buffered	*Buffered*		*Non-buffered*	*Buffered*	
		pH 8.25	*pH 8.5*		*pH 8.25*	*pH 8.5*
Sorbitol	0.20	0.05	0	0.41	0.01	0.01
Mannitol	0.26	0.09	~0	0.49	0.04	0.02
Xylitol	0.32	0.10	0.02	0.45	0.02	0.01
Arabinitol	0.40	0.16	0.05	0.55	0.07	0.03
Erythritol	0.57	0.36	0.17	0.64	0.17	0.12
Inositol	0.03	0.03	0.02	0.34	0.16	0.08
1,5-Anhydro-glucitol	0.59	0.53	0.37	0.70	0.44	0.36
Glucose	0.35	0.25	0.13	0.65	0.20	0.13
Fructose	0.35	0.14	0.04	0.58	0.05	0.03
Xylose	0.59	0.35	0.12	0.72	0.16	0.11

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Note

Gel filtration of soluble coloured metal hexacyanoferrate(II) compounds

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The use of soluble Prussian blue for measuring the void volume in gel filtration was recently advocated by Saito and Matsumoto[1], which is of interest in our studies on the gel filtration behaviour of inorganic polymers. Chromium hexacyanoferrate(II) and mixed chromium–iron(III) hexacyanoferrate(II) have also been studied by gel filtration by Matsumoto *et al.*[2].

We report here results obtained with coloured hexacyanoferrate(II) compounds of UO_2^{2+} and Cu^{2+}.

EXPERIMENTAL AND RESULTS

Soluble hexacyanoferrate(II) compounds were prepared by mixing equal volumes of 0.1 *M* solutions of potassium hexacyanoferrate(II) [$K_4Fe(CN)_6$] and of the metal salts, *viz.*, $Fe(NO_3)_3 \cdot 6H_2O$, $CuCl_2 \cdot H_2O$, $CrCl_3 \cdot 6H_2O$ (violet) and $UO_2(NO_3)_2$. Precipitates often form if other concentrations are used.

Gel filtration

Thin-layer gel filtration was carried out using a Pharmacia TLG chamber and the conditions described previously[3]. Dextran Blue 2000 and $Co(en)_3^{3+}$ were used as void volume indicator and small ion reference, respectively.

Fig. 1 shows the movement of the various hexacyanoferrate(II) compounds on Sephadex G-75, G-150 and G-200 gel. Except for chromium(III) hexacyanoferrate(II) all of the soluble compounds are completely excluded on all gels when eluted with distilled water. On some gels, *e.g.*, Sephadex G-150, Dextran Blue 2000 is retarded in comparison with the hexacyanoferrate(II) compounds. This phenomenon is well known in thin-layer gel filtration and seems to be due to the high viscosity of the concentrated Dextran Blue 2000 solutions employed in thin-layer work.

In the presence of electrolytes in the eluent the gel chromatographic behaviour of the soluble hexacyanoferrate(II) compounds is very different, as shown in Fig. 2. Soluble Prussian blue is retarded and can form long comets; copper and uranyl hexacyanoferrate(II) seem to be precipitated at the point of application. None moves with the speed of Dextran Blue 2000. As we were more interested in the polymeric nature of these compounds than in their utility as void volume markers, we tried to characterize the compounds by a variety of techniques.

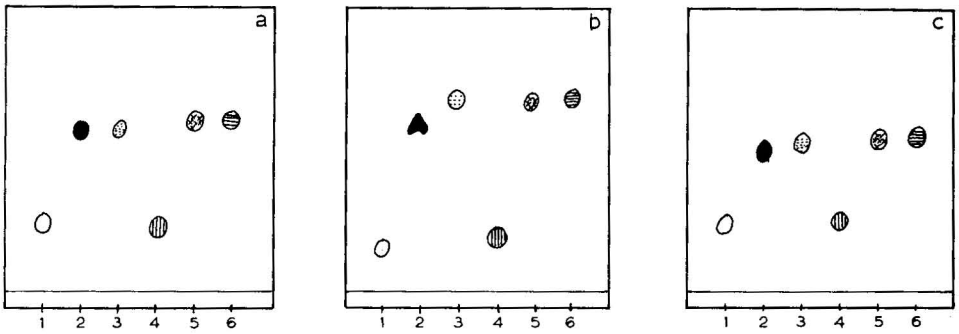

Fig. 1. Thin-layer gel chromatograms of soluble metal hexacyanoferrate(II) compounds. The solutions (chromatographed side-by-side) were as follows: 1 = $Co(en)_3Cl_3$; 2 = Dextran Blue 2000; 3 = uranyl hexacyanoferrate(II) prepared by mixing 0.1 *M* solutions of $UO_2(NO_3)_2$ and $K_4Fe(CN)_6$; 4 = chromium(III) hexacyanoferrate(II) prepared by mixing 0.1 *M* solutions of chromium chloride (violet) and $K_4Fe(CN)_6$; 5 = copper hexacyanoferrate(II) prepared by mixing 0.1 *M* solutions of $CuCl_2$ and $K_4Fe(CN)_6$; 6 = iron(III) hexacyanoferrate(II) (Prussian blue) prepared by mixing 0.1 *M* solutions of iron(III) nitrate and $K_4Fe(CN)_6$. (a) On Sephadex G-75; (b) on Sephadex G-150; (c) on Sephadex G-200; development with distilled water in each instance. Visible spots were recorded except for that with $Co(en)_3^{3+}$, which was detected by spraying with ammonium sulphide solution, and that of uranyl hexacyanoferrate(II), which was sprayed with Na_3PO_4, CH_3COONa and $Zr(CH_3COO)_2$ aqueous solutions and viewed under a UV lamp.

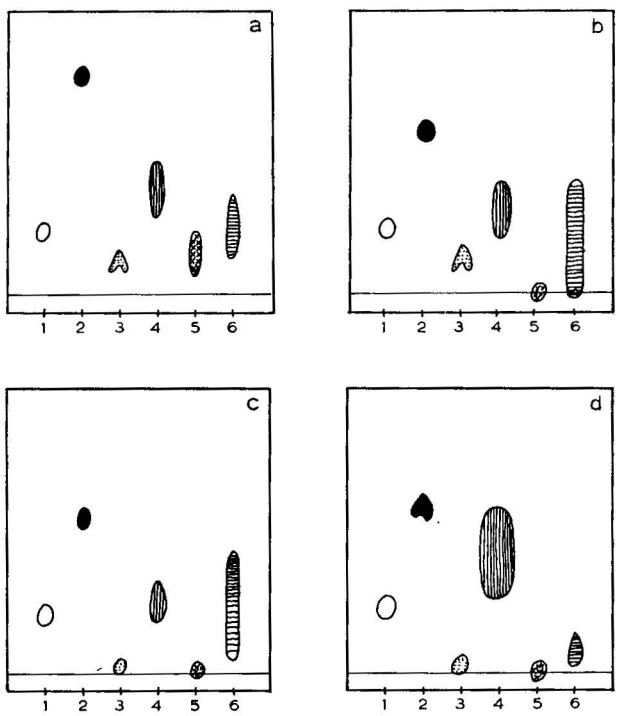

Fig. 2. Thin-layer gel chromatograms of soluble metal hexacyanoferrate(II) compounds obtained using salt solutions as eluents. The solutions chromatographed are the same as in Fig. 1. (a) On Sephadex G-200 developed with 0.1 *M* LiCl; (b) on Sephadex G-75 developed with 0.1 *M* $NaClO_4$; (c) on Sephadex G-200 developed with 0.1 *M* $NaClO_4$; (d) on Sephadex G-75 developed with 0.1 *M* Na_2SO_4.

Chromatography on cellulose paper and thin layers

Chromatograms obtained on acid-washed paper strips and on Polygram Cel 300 layers are shown in Fig. 3. It is remarkable that Prussian blue yields long trails on cellulose whereas it is completely excluded from all Sephadex thin layers when eluted with distilled water. The only compound that seem only slightly adsorbed on cellulose is chromium hexacyanoferrate(II), which is found near the liquid front even in the presence of electrolytes.

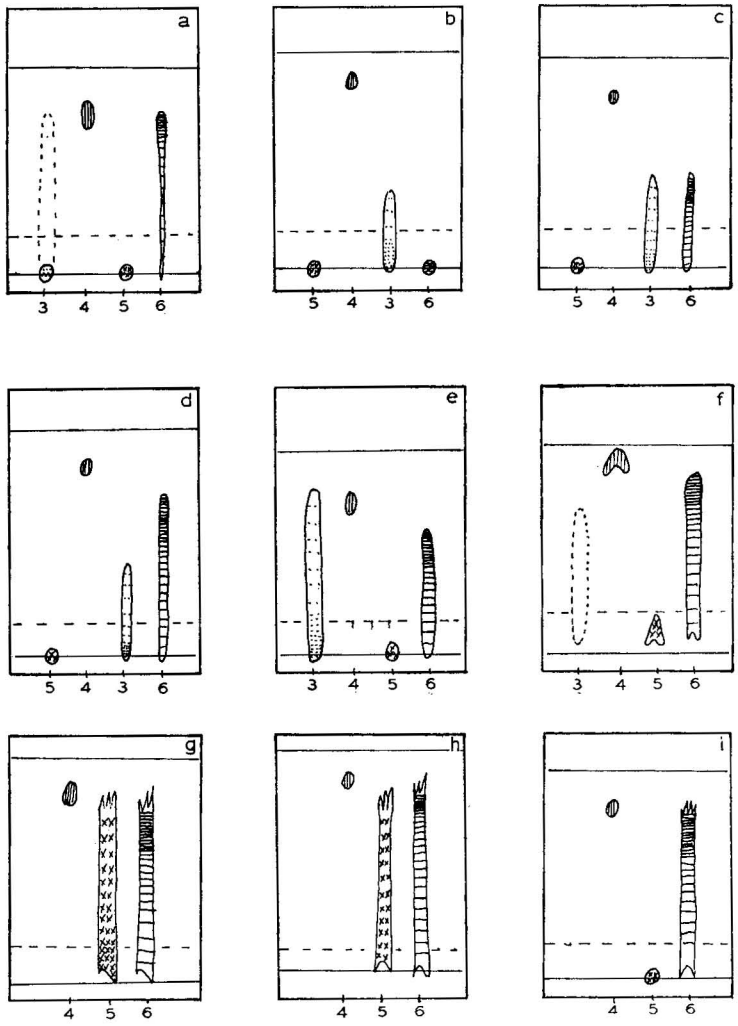

Fig. 3. Chromatograms of soluble hexacyanoferrate(II) compounds on cellulose paper and thin layers. Samples were spotted behind the solvent front (broken line). The solutions chromatographed are numbered as in Fig. 1. (a) Whatman No. 1 paper developed with distilled water; (b) Whatman No. 1 paper developed with 1 *M* acetic acid; (c) Whatman No. 1 paper developed with 0.1 *M* acetic acid; (d) Whatman No. 1 paper developed with 0.005 *M* acetic acid; (e) Whatman No. 1 paper developed with 0.1 *M* LiCl; (f) Polygram Cel 300 developed with distilled water; (g) Polygram Cel 300 developed with 0.1 *M* LiCl; (h) Polygram Cel 300 developed with 0.1 *M* Na_2SO_4; (i) Polygram Cel 300 developed with 0.1 *M* $NaClO_4$.

Filtration through Millipore filters

The solutions of the coloured hexacyanoferrate(II) compounds passed readily through a Millipore membrane with pores between 0.45 and 0.25 μm. However, the membrane became intensely coloured.

DISCUSSION

It had already been found that Prussian blue[1] and chromium(III) hexacyanoferrate(II)[2] are excluded by Sephadex gels. We have now observed that copper and uranyl hexacyanoferrate(II) are excluded from Sephadex gels when eluted with distilled water but are adsorbed or precipitated when eluted with electrolytes. Copper, uranyl and iron(III) hexacyanoferrate(II) are also adsorbed or precipitated when chromatographed on cellulose paper or thin layers. They pass through Millipore filters with pores between 0.45 and 0.25 μm.

Unfortunately, our experiments do not permit even an approximate estimate of the molecular size of these soluble species, because recently Kirkland[4] has shown that even large colloidal silica sols can be excluded in gel filtration. All we can say is that evidently polymeric species are formed but these may be either true solutions or already in the colloidal range.

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Journal of Chromatography, 187 (1980) 462–465

CHROM. 12,329

Note

Determination of some anions separated by thin-layer chromatography

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(First received June 15th, 1979; revised manuscript received August 22nd, 1979)

Procedures for determining cations have received great attention in analytical chemistry. Reported methods include atomic absorption or emission spectroscopy, polarography and others.

On the other hand, the situation is different where the determination of small amounts of anions is concerned. There exist various polarographic methods or methods using ion-selective electrodes, but these fail when the anions are to be determined in mixtures with various organic compounds. In such a case only separation methods, especially those based on chromatographic principles, may be of help.

Chromatographic separation of anions has received great attention as evidenced by a review by Brinkman *et al.*[1], which covers the papers published to 1972, and by further papers[2–4]. Similar problems were solved by electrophoresis[5,6] and by isotachophoresis[7] and even by gel chromatography on Sephadex[8]. All these papers discuss only the separation and identification of anions, not their determination. Only a few papers dealing with phosphates also cover their determination[9–11]. The work reported here was aimed at solving the problem of determining nitrates in various organic materials (*e.g.*, molasses) where the hitherto known methods failed owing to the presence of interfering organic compounds and to low concentration of nitrates.

A recent paper reported the determination of nitrates by gas chromatography[12], in which aqueous nitrate ion is converted into nitrobenzene by reaction with benzene in the presence of a catalyst. The nitrobenzene is then quantitated by gas chromatography. This method would be adequate for our purposes, although it determines only nitrates and no other oxidizing anions.

We used thin-layer chromatography (TLC) to separate the nitrates and also performed the separation of some other oxidizing anions.

The determination was done by using diphenylamine, which reacts with these anions to give blue stains on the chromatograms. The stains may be evaluated densitometrically.

EXPERIMENTAL

The anions were separated on commercial plates of the Silufol 254 type using two solvent systems, *n*-propanol–conc. ammonia (2:1) and *n*-butanol–pyridine–water (5:3:3).

The anions were detected using several agents: (a) diphenylamine (0.2% soln. in conc. sulphuric acid); (b) 1% aqueous soln. of KI in 1.0 *M* HCl; (c) 1% aqueous soln. of $FeCl_3$; (d) aqueous 2 *M* $AgNO_3$ soln. with several drops of conc. ammonia added; (e) aqueous soln. of 2,6-dichlorophenolindophenol (a freshly prepared saturated soln.).

The model compounds were potassium salts, except the NO_3^-, SeO_3^{2-}, and SeO_4^{2-} anions (sodium salts), the $Mo_7O_{24}^{6-}$ and VO_3^- anions (ammonium salts) and the ClO_4^- anion (Mg^{2+} salt) because of the low solubilities of the corresponding potassium salts.

In addition to TLC the separations were done by paper electrophoresis under following conditions: (a) 3*M* NH_4OH for 1.5 h at 220 V, paper Whatman 1 (6 V/cm); (b) 1 *M* CH_3CO_2H for 1.5 h at 220 V, paper Whatman 1 (6 V/cm).

The R_F values, the mobilities observed on electrophoresis, and the stain colors are listed in Table I.

TABLE I

THE R_F VALUES, MOBILITIES, AND STAIN-COLOR INTENSITIES OF ANIONS

S_1 = *n*-propanol–conc. ammonia (2:1). S_2 = *n*-butanol–pyridine–water (5:3:3). S_3 = 3 *M* NH_4OH. S_4 = 1 *M* CH_3CO_2H. D_1 = diphenylamine; D_2 = KI; D_3 = $FeCl_3$; D_4 = $AgNO_3$; D_5 = 2,6-dichlorophenolindophenol. X = tailing. Key to the detection: b = blue; sl = slight; y = yellow; g = green; bl = black; w = white; r = red; br = brown; rs = rose; v = violet; l = light.

Anion	R_F		U^*		*Detection*				
	S_1	S_2	S_3	S_4	D_1	D_2	D_3	D_4	D_5
NO_2^-	0.43	0.23	20.8	0.0	b	v	y	—	b
NO_3^-	0.44	0.19	22.4	0.0	b	slv	—	—	rs
ClO^-	0.29	0.25	10.3	0.0	b	v	—	bl	rs
ClO_3^-	0.46	0.43	20.9	3.2	b	—	—	—	rs
ClO_4^-	0.50	0.72	3.7	0.0	—	—	—	w	rs
IO_4^-	0.26	0.28	11.3	7.7	b	v	b	w	—
BrO_3^-	0.61	X	22.1	2.9	b	v	—	w	slrs
CrO_4^{2-}	0.12	X	22.0	13.4	b	v	y	yrbl	—
$Cr_2O_7^{2-}$	0.17	0.40	23.0	13.1	b	v	—	ybr	—
$[Fe(CN)_6]^{3-}$	0.14	0.20	25.4	16.2	b	v	bg	y	yg
$[Fe(CN)_6]^{4-}$	0.00	0.14	21.4	16.2	—	—	b	w	—
$S_2O_8^{2-}$	0.46	X	23.3	5.1	b	v	—	br	rs
$S_2O_5^{2-}$	0.00	0.11	19.2	4.5	—	—	—	w	—
MnO_4^-	0.00	0.00	0.0	0.0	b	b	—	br	—
$Mo_7O_{24}^{6-}$	0.20	0.13	20.5	2.9	slb	slb	ly	w	—
WO_4^{2-}	0.07	0.06	15.2	1.3	b**	slv	—	ly	—
SeO_3^{2-}	0.13	0.15	14.6	0.0	slb	v	ly	w	rs
SeO_4^{2-}	0.08	0.12	—	—	slb	v	ly	w	rs
VO_3^-	0.00	0.00	13.4	8.0	b	y	y	y	rs

* Mobility U ($cm^2\ s^{-1}\ V^{-1}$) $\times\ 10^{-5}$.

** After a prolonged period.

Determination

A sample containing 1–5 μg of anion was applied to a Silufol 254 plate. After the chromatogram was developed, preferably in *n*-propanol–ammonia, it was allowed to dry, and then sprinkled with a 0.2% soln. of diphenylamine in conc. sulphuric acid.

The blue colour obtained was evaluated by means of a ERI-10 densitometer (Carl Zeiss Jena, Jena, G.D.R.) with a yellow filter (max. 550 nm) and a slit of 18 mm.

Because the colour intensity varies with time, it was necessary to carry out the densitometric measurements within 2–4 min after the sprinkling with diphenylamine (*cf.* Fig. 1). The chromatogram was cut into strips, each strip being sprinkled with the detection agent immediately prior to the measurement.

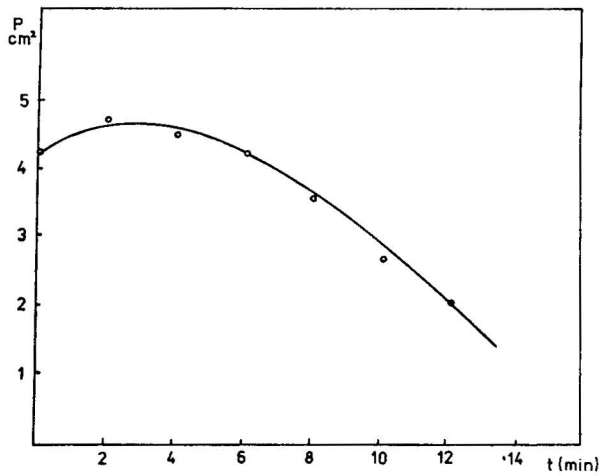

Fig. 1. The time dependence of intensity of the color stain formed by reaction of an oxidizing anion with diphenylamine (Silufol 254 plates).

The chromatogram was always fitted with a standard scale showing, preferably, a concentration of 1, 3, and 5 μg of the anion concerned. It is necessary to allow for a certain error in the results, in accordance with the time dependence of the colour intensity. In an interval of 2–4 min following the sprinkling the error corresponds to *ca.* 10%. As in most cases low concentrations of anions are involved the error does not appear considerable. The detection limit for readily reacting anions (such as NO_3^-) is 0.1 μg.

For the determination of NO_3^- and $[Fe(CN)_6]^{3-}$ in molasses with the contents 1.0 and 0.1%, respectively, the standard deviation of the method was found to be $\sigma = \pm 6\%$ and $\sigma = \pm 8\%$, respectively.

RESULTS AND DISCUSSION

The method described can be used for determining the anions listed when present in concentrations down to hundredths of one per cent in various organic mixtures. The method is applicable to all anions that give a blue colour with diphenylamine. The stability of the colour could be improved by working with plates on a polyester substrate, as the aluminium substrate results in a reaction of aluminium with the sulphuric acid. The relationship of extinction to the concentration of anions shows a straight-line dependence in the range 0–5 μg.

The method has proved applicable to determining the concentration of anions

passing from fertilizers into soil and various plants and from these into further products. The method could also be used to follow the residual concentration in various oxidation processes run with nitric acid in chemical industry. Unfortunately, the separation of nitrates and nitrites is poorer than with other chromatographic methods.

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Journal of Chromatography, 187 (1980) 466–467

CHROM. 12,322

Letter to the Editor

Two-dimensional

Sir,

At a recent symposium devoted to progress in chromatography, the term "multi-dimensional chromatography" was used to signify the combination of several chromatographic systems or techniques. In the subsequent discussion attention was drawn to the well established usage of the term "two-dimensional chromatography" in paper (PC) and thin-layer chromatography (TLC). This can be defined as the use of a flat bed in such a way that migration in one direction is followed by migration at 90° to the first, usually under different solvent conditions[1,2]. In comparison with the use of a single solvent system, the separation achieved is better if the separation principle in the two systems differs. This improvement may be evaluated visually on the chromatogram, and it is negligible if the spots of most substances are near the diagonal line of the resulting quadrangle.

The same principle is applicable to flat-bed zone electrophoresis and its combination with chromatography. The technique of column chromatography, where the effluent is applied to a moving sheet, which then serves for chromatography in the second dimension, is also possible[3–5].

During informal discussion afterwards, the lecturer stressed that in mathematics and physics the term "dimension" is not necessarily limited to path length and is not measured only in metres. This would suggest that the term "two-dimensional", as traditionally used in PC and TLC, is incorrect and should be discontinued.

If this is so, one would have to look for an alternative term for use with flat-bed techniques. The terms "bi-directional" or, as used by Smith[6], a "two-way procedure", could be possibilities.

However, before we decide to reject the traditional meaning of "two-dimensional" in PC and TLC, it would be advisable to examine whether the intrinsic meaning of "multi-dimensional chromatography" as used in the lecture referred to (*cf.*, ref. 7), corresponds to general usage. Various principles have been used in succession or even in parallel for analytical, preparative and other, not necessarily chemical, purposes for many years, without calling their combination a "multi-dimensional" procedure. The term "multi-dimensional chromatography", rather than referring to a combination of chromatographic procedures, was obviously selected with a view to modelling and treating the procedures mathematically by applying information theory in a virtual multi-dimensional space. (Incidentally, one of the earliest papers suggesting the application of information theory in systematic analysis by chromatography is that by Drozen[8].)

Such an *n*-dimensional model may, of course, be applied more generally and

often one cannot include all the individual procedures or "dimensions" under a common designation, as one can do if one limits oneself to a single area, such as chromatography, psychological tests or medical symptomatology.

The use of identical terms with different connotations in different fields (information theory and chromatography in the case under discussion) is, of course, common. But why not speak about "multi-dimensional treatment of" or "approach to" chromatographic data and thereby avoid misunderstanding? If we were to agree that it is not necessary to redesignate a combination of n chromatographic procedures as "n-dimensional chromatography", it would cease to be necessary to abandon the established meaning of the term "two-dimensional chromatography" in flat-bed techniques.

I would welcome readers' opinions on this terminological question.

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Author Index

Errata

J. Chromatogr., 175 (1979) 243–260

Page 245, section *Optimal sequence for use of systems*, line 8, "$S_3\sqrt{1 - r_{1,2}^2} \cdot \sqrt{1 - r_{2,3}^2}$" should read "$S_3\sqrt{1 - r_{1,3}^2} \cdot \sqrt{1 - r_{2,3}^2}$".

Page 250, compound 109, "3β,17α-ol-5αP-11,20-ome" should read "3β,17α-ol-5αP-11, 20-one".

Page 250, compound 139, "3β-ol-P^4-20-one" should read "3β-ol-P^5-20-one".

J. Chromatogr., 178 (1979) 411–417

Page 417, ref. 15 should read "C. H. Lochmüller and R. W. Souter, *J. Phys. Chem.*, 77 (1973) 3016".

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
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The Dutch guilder price is definitive. US $ prices are subject to exchange rate fluctuations.