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JOURNAL OF CHROMATOGRAPHY

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CONTRIBUTION TO THE STUDY OF MICROPACKED COLUMNS IN GAS CHROMATOGRAPHY

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(Received May 23rd, 1985)

SUMMARY

Several chromatographic columns made from glass tubes of I.D. 0.5–1 mm and with solid support particle sizes of 100–120 and 120–140 mesh were evaluated. The effect of liquid film thickness, column diameter, permeability and particle diameter to column diameter ratio on column efficiency is discussed. The pressure drop at the optimum gas velocity per unit column length and per theoretical plate is considered. A column is discussed with 6250 theoretical plates per metre, and others, less efficient per unit length, with a total of about 60 000 theoretical plates which can be run at about 7 kg/cm^2 if nitrogen is used.

INTRODUCTION

One of the major advances in the separation of substances after the onset of gas chromatography was the development in 1958 of capillary columns[1], which had such high separation powers that they seemed likely to bring to an end the use of traditional packed columns. Halász and Heine, however, warned in 1967[2] that the best results would be obtained with the type of column most suited to a particular problem. Nowadays chromatographers have various posibilities to choose from, with the following columns in order of increasing gas flow resistance: thin-layer capillary columns (1958); thick-layer capillary columns (1979); porous-layer capillary columns (1963); packed capillary columns (1963); packed columns of small diameter (1963); and conventional packed columns (1952).

Increasing resistance to the gas flow implies a parallel increase in the head gas pressure per unit length necessary to obtain the optimum flow-rate, and therefore the practical maximum length of a column diminished in the same order as above, with a concomitant decrease in the number of theoretical plates that can be obtained in the column. On the other hand, the load capacity of the different column types increases in the same order. Packed capillary columns and packed columns of small

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diameter represent the borderline between two ways in which the gas flows along the column in contact with the stationary phase: laminar flow parallel to the tube axis, with a considerable resistance to mass transfer in the gas phase (open-tubular columns), or passing through small multiple channels that facilitate the gas-phase mass transfer (classical packed columns). Packed capillary columns[2,3] and small-diameter packed columns[2,4,5] offer intermediate properties between the two flow regimes. The important difference between the two is not the diameter of the column, but rather the ratio of particle diameter to column inner diameter (d_p/d_c), which makes the chromatographic behaviour of the two types different. Packed capillary columns, with a d_p/d_c ratio between 0.2 and 0.5, have a loose packing, whereas small-diameter packed columns are tightly packed, with values of d_p/d_c of the order of 0.1 or lower[2]. Cramers and Rijks[6] proposed the name micropacked columns for packed columns of I.D. below 1 mm and d_p/d_c below 0.3.

Attempts to improve the efficiency of small-diameter packed columns have followed two different approaches: either using large particle diameters so that the special characteristics of the flow pattern found in packed capillary columns could be obtained or approximated[7–9], or seeking a flow pattern similar to that found in conventional packed columns[10,11] by using smaller particle diameters. The efficiencies obtained with the first procedure are lower, with reported values of the mean height equivalent to a theoretical plate ($\bar{H}$) varying from 0.26 to 0.6 mm. Packing the column with small-diameter particles produce values of $\bar{H}$ of the order of 0.1 mm. Jonker *et al.*[12] seem to have achieved the lowest value of $\bar{H}$ recorded with a value of 0.02 mm for tubes of 1.2 mm I.D. diameter and particles of 0.01 mm diameter, normally used in high-performance liquid-chromatography. This type of column needs a head pressure of about 200 atm/m, which imposes a limit on the length of the column. Following the same idea, Berezkin *et al.*[13] obtained a value of $\bar{H}$ of 0.09 mm for a head pressure of the order of 25 atm/m. Obviously, with this specific pressure drop columns cannot be very long.

THEORETICAL

The factors that affect the separation efficiency in packed column technology have been extensively studied by Giddings[14]. Optimization of resolution should be partially based on a consideration of the equation describing the height equivalent to a theoretical plate (H):

$$H = \frac{2vD_g}{u} + \frac{(1-R)2v_lD_l}{Ru} + \frac{w\,d_p^2}{D_g}\cdot u + \frac{q(1-R)d_l^2}{D_l}\cdot u + \frac{1}{\dfrac{1}{2\lambda d_p} + \dfrac{D_g}{wd_p^2\,u}} \qquad (1)$$

where v is the obstruction factor for the mobile phase, D_g and D_l are the solute diffusion coefficients in the gas and liquid phases, respectively, u is the linear gas velocity, R is the ratio between the velocities of the solute band and the gas in the column, v_l is the obstruction factor for the liquid phase, d_p is the particle diameter, d_l is the liquid phase film thickness, λ is a constant depending on the packing geometry, w is a parameter related to the gas velocity distribution inside the column

and q is the gas flow per unit area of column cross-section, proportional to pressure drop per unit length.

This equation is sometimes written as

$$H = \frac{B_g + B_l}{u} + (C_g + C_l)u + \frac{1}{\frac{1}{A} + \frac{1}{C_g u}} \quad (2)$$

In order to improve the packed column performance, the mass transfer terms (C_g and C_l) must be decreased. Therefore, attention should be paid to all parameters affecting them: uniformity of the packing may decrease the C_g term by a factor as high as ten[14]. A decrease in the particle diameter would increase the column efficiency but severe practical difficulties such as impossible pressure drops or even difficulties in packing the column with very small particles will place a limit on the improvements that can be obtained in this way.

On the other hand, considering the problem from a different point of view, Halász and Heine[2] decided that under certain circumstances it may be more practical to have a loose packing of the column with fewer theoretical plates per metre but with a much lower pressure drop per unit length, thus allowing the construction of much longer columns with more theoretical plates and the possibility of higher gas velocities, which would make analyses much faster. This is the goal with packed capillary columns. Therefore, there is another property of chromatographic columns that must be considered, *viz.*, permeability, the facility with which a column allows the gas to pass through it. This can be calculated using the Kozeny–Carman expression for spherical particles

$$K = \frac{d_p^2\, \varepsilon^3}{180(1-\varepsilon)^2} \quad (3)$$

According to this expression, permeability is independent of the column inner diameter as long as the particle diameter (d_p) and the porosity (ε) (the fraction of the cross-sectional area occupied by the gas) remain constant. The equation is valid for small values of d_p/d_c. Therefore, in order to prepare efficient packed columns, attention should be paid to factors that affect efficiency, such as support characteristics, inner diameter of the tube and uniformity and thickness of the liquid film, in addition to those which modify the permeability.

EXPERIMENTAL

Solid support

Volaspher A-2[15], 100–120 and 120–140 mesh (125–150 and 100–125 μm) was used throughout. Polar liquids were used on a non-silanized support, prepared by treating silanized Volaspher A-2 with concentrated hydrochloric acid at 70°C for 1 h followed by washing. Fine particles were eliminated in all instances by floatation in ethanol, and the powder was dryed for 1 h under a stream of nitrogen.

Preparation of the packing

The stationary phase was dissolved and mixed with the solid in a round-bottomed flask that had indentations to facilitate homogenization of the mixture while the flask turned. The operation was carried out at room temperature under vacuum. In this way air was eliminated from the solid[16] and the resultant liquid film was more uniform.

Filling the columns

All columns were made of glass tubing that was drawn and coiled while empty. The packing was introduced under pressure while ultrasonic vibration was applied. Short columns were filled at a constant pressure of about 8 kg/cm^2. Long columns were filled at low pressures for the first few turns, increasing the pressure gradually until about 8 kg/cm^2 at the end. In all instances the column was placed with the turns parallel to the table-top[6]. Columns were conditioned in the normal way.

The gas chromatograph

Experiments were carried out in a Hewlett-Packard 5830A apparatus. Split injection was employed and a make-up gas was added before gases leaving the column reached the flame-ionization detector.

RESULTS

Effect of liquid film thickness on column efficiency

The number of theoretical plates per metre depends on the liquid loading, as may be observed in Fig. 1, where two stationary phases of different polarity are shown. Column efficiency was measured on peaks corresponding to *n*-alkanes with a capacity factor of approximately 3. The highest efficiency was obtained with a liquid loading of *ca.* 4% in both instances, which corresponds to an average film thickness of about 0.04 μm. For higher loadings, the efficiency decreased quickly. Lower column efficiencies were found with polar liquid stationary phases, as may be

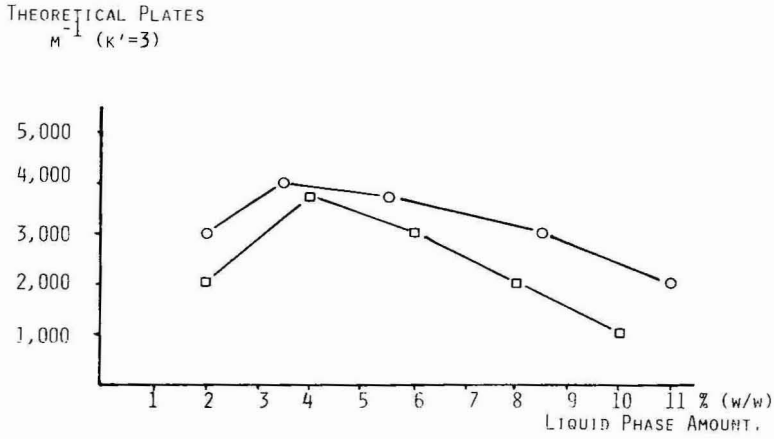

Fig. 1. Dependence of specific efficiency on liquid loading. d_c = 1.0 mm; L = 2 m; d_p = 0.113 mm; carrier gas, N_2. ○, non-polar phase (SE-30); □, polar phase (Carbowax 20M).

TABLE I

SPECIFIC EFFICIENCY OF SIMILAR COLUMNS WITH DIFFERENT STATIONARY PHASES

Common characteristics: d_p = 0.113 mm; d_c = 1.0 mm. Carrier gas, N_2. Column length, 2 m. $p_i/p_o(\bar{u}_{op})$ is the inlet to outlet pressure ratio at optimum gas velocity. N/m is the number of theoretical plates per metre, at $\bar{u}_{op}$.

Stationary phase	*Liquid load (%, w/w)*	p_i/p_o $(\bar{u}_{op})$	*N/m*
SE-30	3.5	1.7	4000
Carbowax 20M	4	1.8	3800
Fractonil III	5	2.2	3300
Triton X	5	2.0	3000
OV-225	5	1.9	2800
Fomblin Y	5	2.3	2400
Carbowax 300	5	1.8	2000
OV-275	5	1.7	2000

deduced from Table I. The effect has also been observed with mixed stationary phases.

Effect of column diameter on column efficiency

A number of columns of different diameter were prepared using a solid support of a size of 100–125 μm and a liquid loading of 4% of OV-1. The effect of column diameter on the number of theoretical plates per metre of column under these conditions is shown in Fig. 2. The lower limit of column diameter depends on the difficulty of filling a tube smaller than 0.5 mm with the support employed. The dependence of column efficiency on d_p/d_c is shown in Fig. 3, which seems to place a lower limit on the specific efficiency of large-diameter columns made with this packing of *ca.* 2000 theoretical plates per metre.

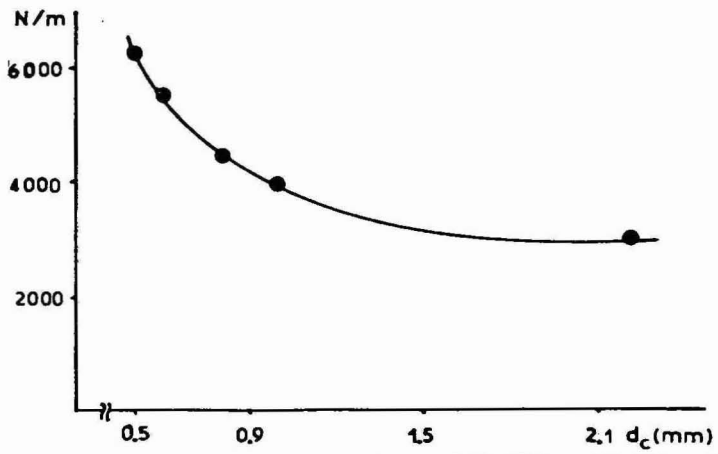

Fig. 2. Specific efficiency of different columns of 4% OV-1 on 120–140-mesh Volaspher A-2. L = 3 m; carrier gas, N_2.

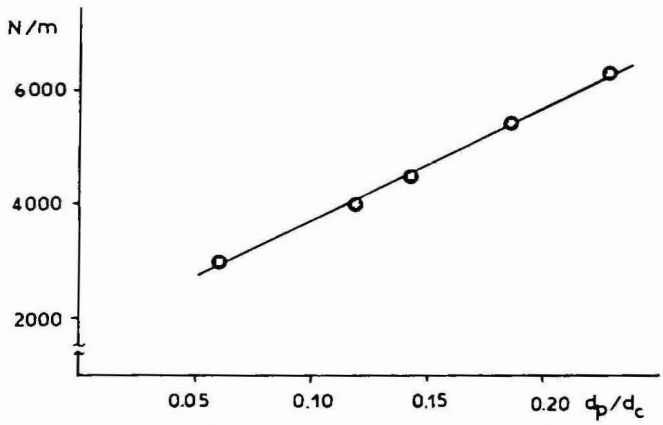

Fig. 3. Dependence of specific efficiency of the columns shown in Fig. 2 on d_p/d_c.

TABLE II

PERMEABILITY (K) OF SOME COLUMNS

Common characteristics: 4% OV-1 on 120–140-mesh Volaspher A-2. Column length: 3 m.

d_c	d_p/d_c	*Packing density* (g/cm^3)	ε	$K \cdot 10^7$	p_i/p_o (p_o = 1 atm)	$\bar{u}_{op}$ (cm/s)
0.5	0.23	0.411	0.211	10.56	2.5	4.4
0.6	0.19	0.460	0.107	1.10	2.3	3.8
0.8	0.14	0.475	0.087	0.56	2.3	3.4
1.0	0.11	0.478	0.047	0.08	2.5	3.3

Effect of permeability on various experimental parameters

Columns of different diameter, prepared with the same packing of 4% OV-1 on 120–140-mesh support, and of length 3 m allowed us to study the packing density, porosity, permeability and head pressures necessary to run the column at the optimum gas velocity. Table II gives a summary of the results.

DISCUSSION

From the point of view of the specific efficiency (plates/metre), the best column is one of I.D. 0.5 mm, whose characteristics are summarized in Table III. Despite the low packing density, an efficiency of 6250 plates/metre was measured at a capacity factor of *ca.* 3. This high efficiency was achieved by paying attention to the term C_g in eqn. 2. The procedure followed for filling the column limits the practical maximum column length to about 5 m. Therefore, about 30 000 theoretical plates could be achieved with a working head pressure of nitrogen of the order of 4 kg/cm^2, which is easily handled on any gas chromatograph and without special injection difficulties. Such numbers of theoretical plates are more than sufficient for many separation problems. Table IV compares various columns, cited in the literature, with special

TABLE III

CHARACTERISTICS OF THE MOST EFFICIENT COLUMN

Characteristic	*Value*
Packing	4% OV-1 on 120–140-mesh Volaspher A-2
I.D.	0.5 mm
Column length	3 m
d_p/d_c	0.23
Packing density	0.411 g/cm^3
Porosity, ε	0.21
Permeability, K	$10.56 \cdot 10^{-7}$ cm^2
$\bar{u}_{op}$ (N_2)	4.4 cm/s
p_i/p_o at $\bar{u}_{op}$	2.5
$\bar{H}$ at $\bar{u}_{op}$	0.16 mm
$C_s + C_l$	$4.17 \cdot 10^{-4}$ s

attention to the pressure needed, specific efficiency and length or pressure necessary to achieve 10^4 theoretical plates. Although the column shown in Table III has neither the highest efficiency nor the lowest pressure drop per metre, it shows a good equilibrium between performance and pressure drop, with a clear advantage if the cost of one theoretical plate in terms of head pressure is considered.

According to Cramers and Rijks[6], all columns shown in Figs. 2 and 3 (Table II) with I.D. between 0.5 and 1.0 mm may be considered as micropacked columns as they have an I.D. below 1.0 mm and d_p/d_c values below 0.3. In these, a gradual increase in the column efficiency may be observed as the column diameter decreases (Fig. 3) without much change in the column head pressure needed, as may be deduced from Table II. However, if other characteristics are considered, differences can be observed. The ratio d_p/d_c is close to 0.2 for the 0.5 and 0.6 mm I.D. columns, which would make them packed capillary columns[2], whereas the 0.8 and 1.0 mm I.D. columns should be considered as micropacked. An evaluation of the C terms in eqn. 2, as shown in Fig. 4 for the 0.5, 0.6 and 0.8 mm I.D. columns, again shows a clear

TABLE IV

CHARACTERISTICS OF SOME COLUMNS REPORTED BY DIFFERENT WORKERS

d_p *(mm)*	$\bar{H}_{min}$ *(mm)*	d_p/d_c	p/m *(atm)*	$N/atm\ m$	$L/10^4$ *T.P.** *(m)*	$p_o/10^4$ *T.P.** *(atm)*	*Ref.*
0.175	1.0	0.70	1	1000	10	10	17
0.150	0.26	0.17	3	1282	2.6	7.8	7
0.140	0.50	0.18	1	2000	5	5.0	9
0.125	0.60	0.39	0.5	3333	6	3.0	8
0.113	0.16	0.23	0.8	7813	1.6	1.3	This work
0.030	0.1	0.03	7	1430	1	7	11
0.030	0.1	0.03	8	1250	1	8	10
0.025	0.09	0.067	25	440	0.9	22.5	13
0.010	0.02	0.008	240	208	0.2	48.0	12

* T.P. = theoretical plates.

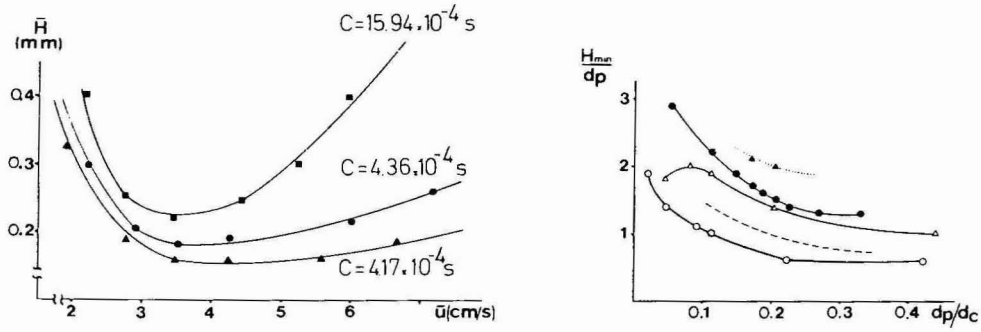

Fig. 4. Plot of $\bar{H}$ *versus* average linear gas velocity for the columns in Table II. ■, 0.8; ●, 0.6; ▲, 0.5 mm I.D. The C terms in eqn. 2 are shown.

Fig. 5. Dependence of $\bar{H}_{min}/d_p$ on d_p/d_c. ▲, Chromosorb P with OV-1 ($d_l \approx 0.04$ μm) (this work); ●, Volasphere A-2 with OV-1 ($d_l \approx 0.04$ μm) (this work); △, Chromosorb P without stationary phase[19]. ○, glass beads without stationary phase[19]. Broken line, calculated for Volaspher A-2 without stationary phase.

distinction between the two smaller columns and the 0.8 mm I.D. column, which has a C term about four times as large. A change in column I.D. from 0.8 to 0.5 mm brings about a decrease in the value of $\bar{H}$ at the optimum gas velocity from 0.22 to 0.16 mm while increasing the optimum gas velocity from 3.4 to 4.2 cm/s, thus decreasing analysis time by nearly 20%, as had been suggested by Kaiser[18].

The dependence of H on d_p/d_c was first considered by Sternberg and Poulson[19], who used solid particles without a liquid coating. Their findings are presented in Fig. 5 compared with our results. The curves corresponding to Chromosorb P and glass beads without any liquid coating show the dependence of the C_g term in eqns. 1 and 2 on d_p/d_c, and the curves corresponding to our results show the effect of d_p/d_c on both the C_g and C_l terms in these equations. If the ratio between the $\bar{H}/d_p$ values corresponding to coated Chromosorb P (our points) and uncoated Chromosorb P[19] is assumed to hold for coated and uncoated Volaspher A-2, the dotted line shown in the Fig. 5 may be drawn, corresponding to an uncoated porous spherical support, with a behaviour close to that of spherical glass beads and superior to what can be expected from a non-spherical support such as Chromosorb P.

TABLE V

CHARACTERISTICS OF LONG MICROPACKED COLUMNS

N/m = theoretical plates per metre.

Stationary phase	*Liquid load (%, w/w)*	d_p	d_c	p_i/p_o ($\bar{u}_{op}$)	*Carrier gas*	*L (m)*	*N/m*
OV-1	4	0.113	0.8	7.0	N_2	13	4600
Superox 20M	3	0.137	0.9	5.0	N_2	10	2900
Superox 20M + SE-30	4	0.137	0.9	5.0	H_2	11	3400
OV-101	15	0.137	0.9	5.0	H_2	10	2400
Superox 20M	10	0.137	0.9	4.0	H_2	10	2000
Superox 20M + SE-30	8	0.137	0.9	5.0	H_2	9	2900

Bearing in mind the relationship between efficiency, permeability, stationary phase load, particle and tube diameters, sample capacity, head pressure needed and construction facility, other columns could be used to advantage for particular problems, even if they may not be considered as the best overall micropacked columns possible. Table V lists several columns made from 0.8 and 0.9 mm I.D. tubing of length 9–13 m. The lower specific efficiencies may be compensated for with a greater length, resulting in columns with a sufficient number of theoretical plates and larger sample capacity.

Comparing the two OV-1 columns in Tables III and V, it can be seen that the latter has a lower specific efficiency but about twice as many theoretical plates as the longest column which could be made with the characteristics of the former, working at a head pressure of nitrogen of 7 kg/cm^2, which is not too high for commercial gas chromatographs but which could be made lower if hydrogen is used as the carrier gas, with the additional advantages of a higher analysis speed.

In some particular instances, a higher sample capacity must be achieved by increasing the stationary phase percentage, even if a lower specific column efficiency is obtained. As can be seen in Table V, an increase in Superox 20M from 3 to 10% lowers the efficiency from 2900 to 2000 theoretical plates per metre, but if this can

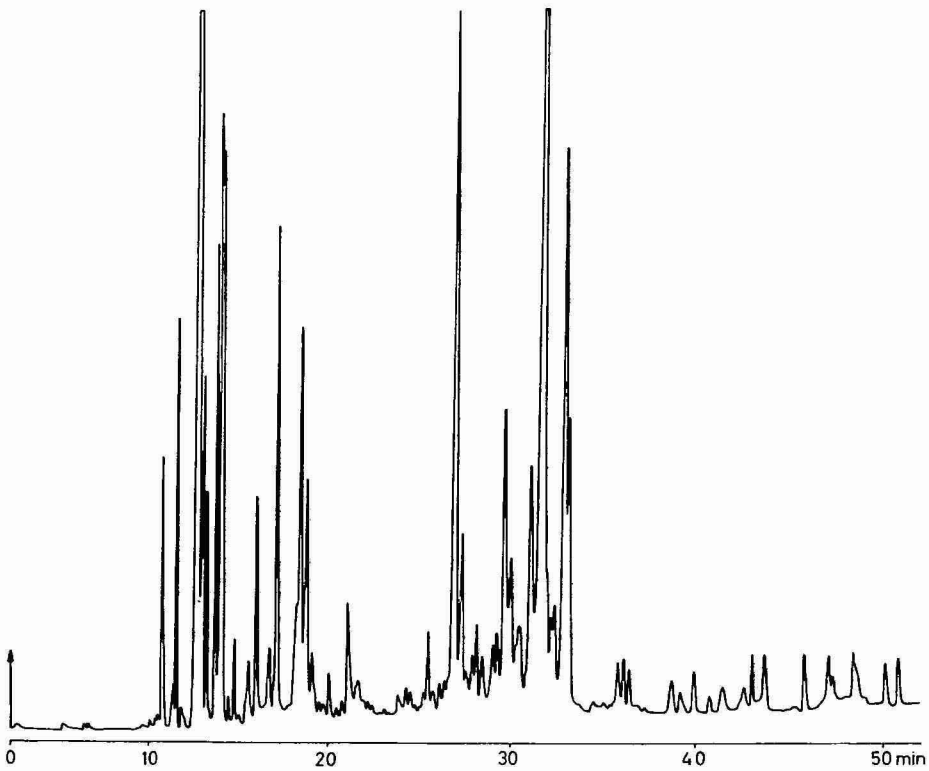

Fig. 6. Chromatogram of extract of *Santolina rosmarinifolia* obtained on a 4% SE-30 on Volaspher A-2 (120–140-mesh) column (5.5 m × 1.0 mm I.D.). Carrier gas, H_2 (8 cm/s). Temperature, programmed from 40°C at 5°C/min to 200°C.

be compensated for with a greater column length, the result may still be a very reasonable column. As already mentioned, these lower specific column efficiencies were the norm with polar liquid stationary phases (see Table I). Mixed stationary phase columns, made by mixing two liquids of different polarity and covering the solid support with the mixture (homogeneous mixed stationary phase), show specific efficiencies between the values corresponding to the individual stationary phase columns. Once again, the normal dependence of column efficiency on the amount of stationary phase on the support may be observed, with the lowest value of $\bar{H}$ at a *ca.* 4% loading.

Columns of the type shown here may be used to solve problems of some complexity. Figs. 6 and 7 show two examples of short columns with a non-polar liquid stationary phase applied to complex separations. Fig. 8 shows the separation of the components of a wine extract on an OV-275 column only 2 m long.

To summarize, if a good solid support is used and the coating procedure produces a homogeneous thin layer of stationary phase, then it is not necessary to have very low values of d_p/d_c, as values between 0.11 and 0.23 produce columns with 4000–6000 theoretical plates per metre with reasonable head pressures. All columns prepared in our laboratories and described in this paper have specific pressure drops below 1 atm/m.

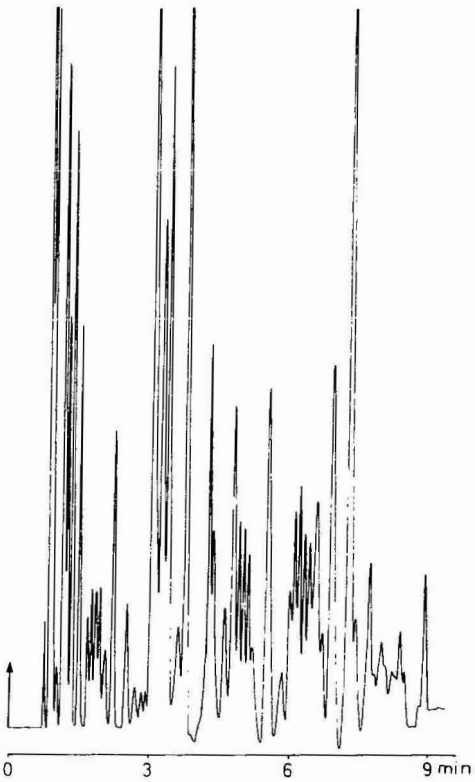

Fig. 7. Chromatogram for direct injection of naphtha into a 4% OV-1 on 120–140-mesh Volaspher A-2 column (3 m × 0.5 mm I.D.). Carrier gas, N_2 (4.5 cm/s). Temperature, programmed from 60°C at 10°C/min to 210°C.

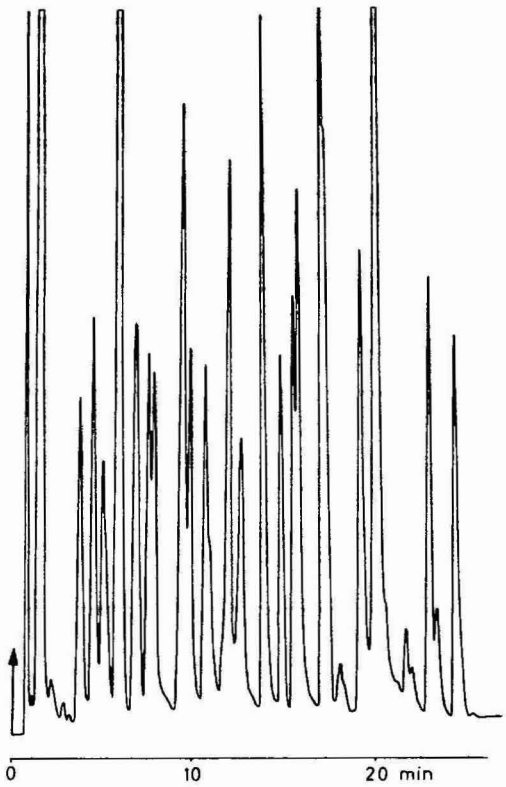

Fig. 8. Chromatogram of an organic extract of a wine obtained on a 5% OV-275 on Volaspher A-2 (120–140-mesh) column (2 m × 1 mm I.D.). Carrier gas, N_2 (4 cm/s). Temperature, programmed from 70°C at 5°C/min to 200°C.

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REVERSED-FLOW GAS CHROMATOGRAPHIC TECHNIQUE APPLIED TO MEASUREMENT OF MASS TRANSFER COEFFICIENTS OF *n*-HYDROCARBONS ON PORAPAK P

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SUMMARY

The reversed-flow gas chromatographic technique has been used to measure mass transfer coefficients of *n*-hexane, *n*-heptane, and *n*-octane on a Porapak P solid sorbent. The values found correspond not to infinite solute dilution but to finite solute concentrations (non-linear chromatography). The results show that mass transfer in the porous polymer is mainly governed by surface diffusion. The increase of the *C* coefficients for *n*-heptane with temperature is due to a decrease in the partition ratio *k* value. Some results for *n*-heptane and *n*-octane are in good agreement with literature values found by infinite dilution methods.

INTRODUCTION

Mass transfer phenomena play an important role in gas chromatography (GC), not only from the analytical point of view[1], but also with respect to physicochemical measurements[2]. Virtually all mass transfer studies in GC were made at infinite solute dilution by using conventional elution of an injected pulse. No mass transfer coefficient measurements at finite solute concentration (non-linear region of chromatography) have come to our attention. These would obviously require the use of some kind of frontal analysis, displacement development or special equipment for finite concentration work. However, a new technique, termed reversed-flow gas chromatography (RFGC), requires only a slight modification of a conventional gas chromatograph, and can lend itself to mass transfer measurements *at finite concentration*, by means of elution bands like those obtained with trace amounts of solute.

The RFGC technique has been successfully used to determine diffusion coefficients in binary and ternary gas mixtures[3,4], together with their temperature variation[5], adsorption equilibrium constants[6], rates of drying in solids[7], rate coefficients for evaporation of liquids[8], rate constants, activation parameters and catalytic conversions of surface-catalysed reactions[9–16], relative molar responses of the thermal conductivity detector and from these molecular diameters and critical volumes of gases[17], and finally Lennard-Jones parameters[18]. The method has recently been reviewed[19].

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For the present application of RFGC a porous polymer (Porapak P) was chosen, because such materials are widely used as sorbent phases in gas solid chromatography, and also because relatively few quantitative data on solute mass transfer in gas–solid systems are available. An additional reason for this study was that similar measurements on porous polymers at infinite dilution by Rakshieva *et al.*[20] and Guha *et al.*[21] revealed that adsorption–desorption kinetics make a negligible contribution to the C coefficients found, and that surface diffusion controls the mass transfer phenomenon in the solid state. The possible role of surface diffusion in chromatography and the lack of relevant experimental data were pointed out by Giddings a long time ago[22].

EXPERIMENTAL

Materials

Porapak P was a product of Waters Assoc., U.S.A., Batch No 1995, 80–100 mesh. The carrier gas (helium) was obtained from Linde (Greece) and had a purity of $\geqslant 99.99\%$. The solutes *n*-hexane and *n*-heptane were purchased from Merck (Uvasol, 99.9%), whereas *n*-octane was a product of Fluka (purum, 99.8%).

Apparatus

An experimental set-up similar to that used to measure gas diffusion coefficients[3] was used with a slight modification as shown in Fig. 1. An ordinary gas chromatograph (Pye Unicam 104) with a flame ionization detector contained in its oven two sections l and l' of a chromatographic column ($39+39$ cm $\times$ 4 mm I.D.) filled with Porapak P. The ends of this column D_1 and D_2 were connected to the carrier gas supply and the detector *via* a six-port valve S, so that when the valve was turned from one position to the other the direction of the carrier gas flow *was reversed.* A diffusion column L (80 cm $\times$ 4 mm I.D.), empty of any chromatographic material and with a U-shaped liquid reservoir at its upper end, was connected perpendicularly at its lower end to the middle of the filled column $l+l'$. The column L and the liquid reservoir were kept at a constant temperature in the range 48–62°C by means of water circulated around them from a thermostat.

Procedure

The conditioning of the chromatographic column $l+l'$ containing the Porapak P was carried out at 170°C for 24 h with a carrier gas flow-rate of 0.33 cm^3s^{-1}. After that, the column was brought to each working temperature, and while carrier gas was flowing in a certain direction through the column $l+l'$, a small amount of liquid solute (usually 0.5 cm^3) was introduced by injection into the heated upper reservoir of column L. After a certain time, during which no signal was noted, an ascending concentration–time curve for the solute was recorded. This reached a maximum plateau and remained there as long as there was still liquid in the reservoir (see Fig. 4 in ref. 8). Thus, a finite solute concentration in the Porapak column was established, the magnitude of which depended on the vapour pressure of the liquid solute at the temperature of the reservoir. When sufficient stability of the recorded signal was attained, valve S was switched to the other position (broken lines), thereby *reversing* the direction of the carrier gas flow. After a short time interval t' (20–60 s) of back-

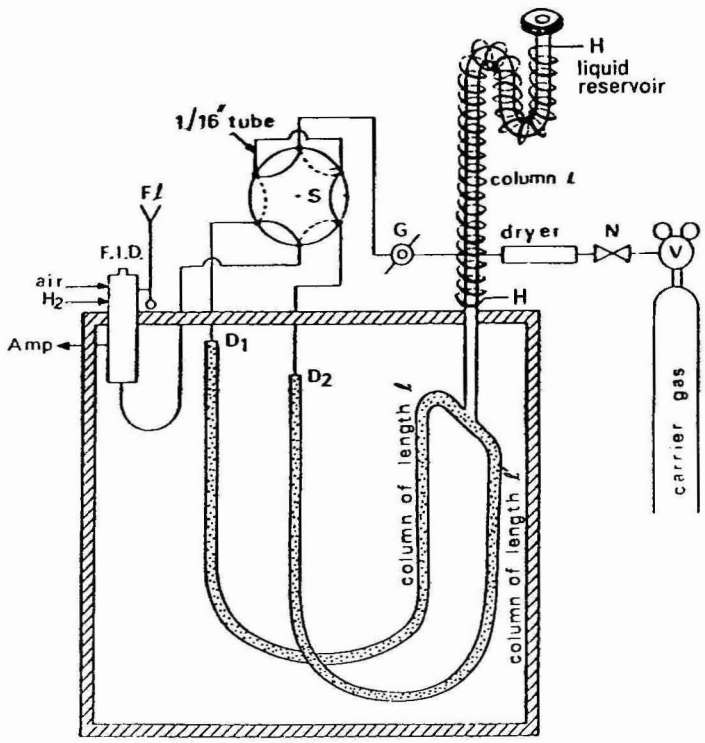

Fig. 1. Gas lines and connections for measuring mass transfer coefficients by RFGC: V = two-stage reducing valve and pressure regulator; N = needle valve; H = heating coil by water circulation; G = gas flow controller; S = six-port valve with a short 1/16-in. tube connecting two alternate ports; Fl = bubble flow-meter; Amp = signal to amplifier.

ward flow, the valve was turned back to its previous position, thus restoring the gas flow to its original direction.

Because the duration t' of backward flow was smaller than the retention time of the solute t_R and t'_R on column sections l and l', respectively, it created a perturbation in the concentration–time line with the form of a bell-shaped peak ("sample peak"). This emerged with a characteristic retention time after the restoration of the gas flow to its original direction, and "sited" on the otherwise finite concentration signal (*cf.* Fig. 2). The procedure was repeated several times, giving a series of sample peaks.

The pressure drop along column l or l' was found by measuring the pressure at the injection point of the solute with an open mercury manometer.

Experiments at 120, 135, 150 and 165°C were carried out with each solute, using time reversals t' of 20, 30, 40, 50 and 60 s.

Processing of the results

The mass transfer resistance coefficients C, together with the coefficients $\bar{B}$, were found by fitting the experimental data to the simple classical Van Deemter equation $\hat{H} = A + \bar{B}/\bar{v} + C\bar{v}$, using a least-squares program on a desk-top computer.

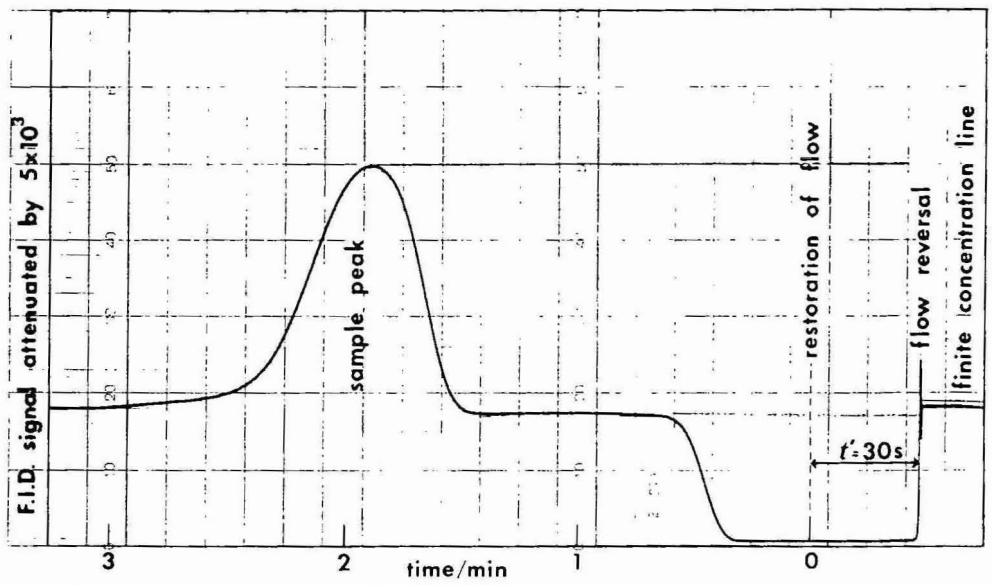

Fig. 2. A sample peak created by reversing the flow direction of carrier gas for 30 s while a finite concentration of *n*-heptane was passing through the column at 408 K, and a volumetric flow-rate of 1.07 cm^3s^{-1}.

The apparent (i.e. experimental) plate height $\hat{H}$ was calculated from the relation $\hat{H} = l/N$, and the number of theoretical plates N from the ratio t_R^2/σ_{net}^2, where t_R is the retention time of the sample peak, measured from the moment of the restoration of the flow to it original direction, and σ_{net}^2 is the net variance of the peak; these two parameters were computed as follows.

According to the theory of the RFGC method[5,19], the reversal of the flow for a time $t' < t_R$ creates a plug (*i.e.* a square function) on the continuous signal due to the diffusion of vapours from column L into column $l+l'$ (see Fig. 1). This square function has a theoretical width t', *i.e.* equal to the time of the backward flow, and its variance is therefore $t'^2/12$. When passed through the Porapak column section l or l', the sample peak will be given by the difference of two error functions[23]. Its variance σ_{exp}^2 and retention time t_R were calculated by the rectangular method of peak-area measurement introduced by Sternberg[24]. For each peak, 9–13 horizontal rectangles were used with 0.5–1-cm increments. In a few cases, where the peaks were of small height, the variance was calculated from the relation[2]

$$\sigma_{exp}^2 = \frac{1}{4}(t_+ - t_-)^2$$

where t_+ and t_- are the times corresponding to the inflection points of the curve. Finally, the net variance due to the chromatographic process was found from the difference

$$\sigma_{net}^2 = \sigma_{exp}^2 - \frac{t'^2}{12} \qquad (1)$$

RESULTS AND DISCUSSION

Table I summarizes the results obtained as outlined in the previous section. As was pointed out in the Introduction, the present study is a plate height measurement at finite concentration. Therefore, before discussing the mass transfer term C, it is worth commenting on the values of the coefficients A and $\bar{B}$. The particle diameter for a 80–100 mesh solid material, such as that used here, is 0.018–0.015 cm and most of the A values in Table I are of that order of magnitude, as in most infinite dilution studies. The negative values are probably due to experimental errors, since the term A is very sensitive to these errors.

The diffusion coefficients D_m of the solute vapours in the carrier gas helium were calculated, at each column temperature from the Fuller–Schettler–Giddings equation[25]. These values (given in Table I for 1 atm pressure) were used to find the obstruction factor γ from the well-known relation $\bar{B} = 2\gamma D_m$. In this calculation, each D_m value was first reduced to the mean column pressure, corresponding to the mean flow velocity of the carrier gas. The γ values found, listed in the last column of Table I, seem reasonable.

Coming now to the C coefficients, we first note that they do not include a mobile phase diffusion term C_m, since they are independent of pressure. There are three main points to be discussed: (1) the magnitude of C terms; (2) their variation with t', *i.e.* with the width of the square function created by the flow reversal; and (3) their variation with temperature.

(1) The C coefficients found are too large to be attributed to adsorption–desorption kinetics, *i.e.* being $C_k = 2k/(1+k)^2 k_d$, where k is the partition ratio and k_d is the desorption rate constant. The C_k terms are of the order of 10^{-8} to 10^{-6} s, and therefore their contribution to the magnitude of C found must be negligible. This has also been pointed out by Rakshieva *et al.*[20], who studied the mass transfer coefficients of *n*-heptane on several kinds of Porapak, at 170°C. Their value of C_s for Porapak P-411 is 0.076 s, and this agrees with our values at the same temperature ranging from 0.028 to 0.089 s (*cf.* Table I). Also our value of C (0.011 s) for *n*-octane at 170°C agrees with the C_s (0.008 s) found by Guha *et al.*[21] under similar conditions.

TABLE I

VALUES FOR THE COEFFICIENTS A, $\bar{B}$ AND C OF THE VAN DEEMTER EQUATION DETERMINED BY RFGC AT FINITE SOLUTE CONCENTRATION

Solute	*T(K)*	*t'(s)*	*A(cm)*	$\bar{B}(cm^2s^{-1})$	$10^2C(s)$	$D_m(cm^2s^{-1})$	γ
n-Heptane	393	30	0.029	0.271	1.34	0.443	0.39
	393	60	−0.004	0.289	3.40	0.443	0.39
	408	20	0.058	0.253	1.40	0.462	0.32
	408	30	0.079	0.225	1.85	0.462	0.29
	408	40	−0.015	0.376	2.98	0.462	0.51
	423	20	–	–	2.76	–	–
	423	30	0.041	0.294	4.95	0.503	0.33
	423	40	−0.179	0.544	8.90	0.503	0.64
	438	30	0.002	0.264	5.40	0.535	0.29
n-Hexane	423	30	−0.139	0.410	9.80	0.515	0.49
n-Octane	423	30	–	–	1.15	–	–

We can therefore adopt the explanation given by the above authors[20,21], that it is surface diffusion that controls the mass transfer of solute in the solid phase.

(2) Table I shows that there is a 2.1 to 3.2 fold increase of C when t' is doubled. This can be explained by referring to the finite solute concentration, on account of which the isotherm may not be linear. A decrease in the isotherm slope brought about by an increase in t' will cause a decrease in k. Since C is usually of the form[26]

$$C = N \frac{k}{(1+k)^2 D_{\text{eff}}} \tag{2}$$

where N is a constant and D_{eff} is an effective diffusion coefficient pertaining to the appropriate mass transfer mechanism, C will increase with decreasing k (provided that $k > 1$) and hence with increasing t'.

(3) The variation of C with temperature at constant t' (30 s) is shown in Fig. 3 in the form of a plot of ln C *versus* $1/T$. It shows an increase of C with temperature, and this is an unusual dependence, at least for infinite dilution studies. For instance, Guha *et al.*[21] found a small decrease of C_s with increasing T for *n*-octane on Porapak P, whereas for iso-octane the decrease was large, corresponding to an activation energy of 73.6 kJ mol^{-1} for diffusion into the micropores of the polymer. *n*-Octane, on the other hand, was assumed to have almost free access into the pores. If this argument applies also to *n*-heptane, the variation of D_{eff} of eqn. 2 with temperature cannot be responsible for our C dependence on T, not only because of the small magnitude of the activation energy expected, but also because it is in the opposite

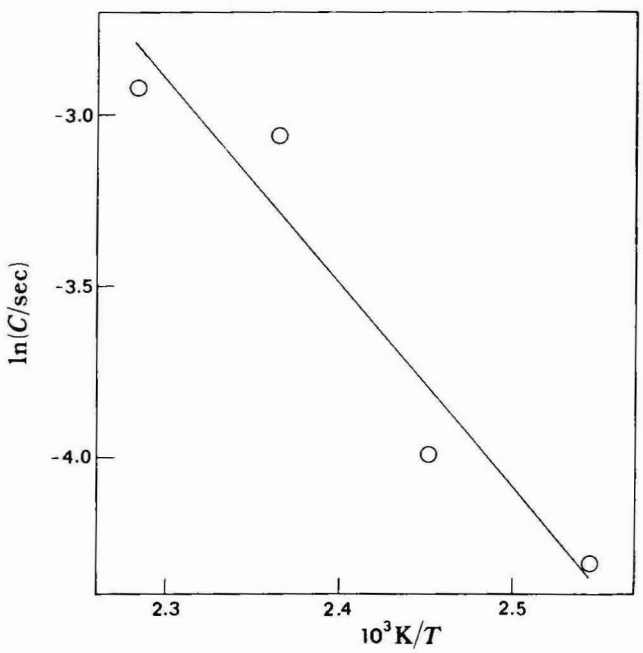

Fig. 3. Temperature dependence of C for *n*-heptane on a Porapak P column.

direction. An obvious explanation for the behaviour of C in the present case lies on the decrease of k with temperature. For $k \gg 1$, eqn. 2 becomes

$$C \simeq \frac{N}{kD_{\text{eff}}} = \frac{N'}{KD_{\text{eff}}} = N''\exp\left[(\Delta H_{\text{ad}} + E)/RT\right] \tag{3}$$

where N' and N'' are constants, k is the adsorption equilibrium constant, ΔH_{ad} the heat of adsorption and E the activation energy of D_{eff}. According to this equation, a plot of ln C *versus* $1/T$ should be linear with a slope equal to $(\Delta H_{\text{ad}} + E)/R$. From Fig. 3 we find a slope of $-5.931 \cdot 10^3$ and this gives $\Delta H_{\text{ad}} + E = -49.3$ kJ mol^{-1}. The heat of vaporization for *n*-heptane at its normal boiling point (98.4°C) is 31.7 kJ mol^{-1}, and if we take $E = 0$, because of the small activation energy expected (*cf.* above), the heat of adsorption is only 1.6 times bigger than the heat of vaporization. This places the adsorption of *n*-heptane on Porapak P in the domain of physical adsorption. For comparison we quote here the heat of adsorption of *n*-heptane on graphitized carbon black, equal to -52.3 kJ mol^{-1}.

CONCLUSION

The RFGC technique can be employed for mass transfer coefficient studies at finite solute concentrations, with only a slight modification of an ordinary gas chromatograph. In spite of finite concentrations (non-linear chromatography), the method is based on simple elution of extra peaks created on the existing finite solute concentration.

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INSULIN AND INSULIN DERIVATIVES

A COMPARATIVE STUDY*

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(First received February 19th, 1985; revised manuscript received August 14th, 1985)

SUMMARY

The reversed-phase separation of crystalline insulin (I) and monoiodoinsulins (II) has been investigated, with respect to the effects of buffer, substitution group, pore-size and column support backbone. The separations were performed either isocratically (for II) or by gradient elution with very narrow gradients (for I).

Fourteen reversed-phase columns, the majority being silica-based, were investigated, and three main results emerged.

(1) Trifluoroacetic acid is unsuitable as a buffer for this type of analysis, whereas trialkylammonium phosphates are very suitable.

(2) The separation between the major components in crystalline insulin was comparable in the main for all the columns tested except one. However, the ability to distinguish between the numerous minor components (co-extracted with insulin peptide) varied a great deal between the columns.

(3) In an optimized buffer system only three columns were able to separate insulin peptide and the four monoiodoinsulin isomers; all three were 80–100-Å silica-based C_{18} columns.

INTRODUCTION

In recent years a large number of reports have been published describing the application of insulin to a variety of reversed-phase high-performance liquid chromatographic (RP-HPLC) systems. In the majority of these reports, insulin has been used as one among several polypeptides and proteins in order to characterize the separation capacity of the system. The separation of insulin, insulin-related and non-insulin-related substances has also been studied in more detail[1–14].

* This paper has been presented at the *Fourth International Symposium on High-Performance Liquid Chromatography of Proteins, Peptides and Polynucleotides, Baltimore, MD, December 10–12, 1984*. The majority of the papers presented at this symposium have been published in *J. Chromatogr.*, Vols. 326 and 327 (1985).

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The separation of crystalline insulin, containing one major constituent (insulin peptide) and a large number of insulin-like contaminants (desamidoinsulin, arginine insulin, ethylesterinsulin, des-alanine insulin, des-pentapeptide and des-octapeptide insulin, insulin dimers, proinsulin and intermediary insulins) as well as non-insulin-related substances (glucagon, pancreatic polypeptide) represents a typical challenge to the separation capacity of RP-HPLC.

Three types of buffer system have commonly been used: ion-pairing substances (*e.g.* alkylammonium phosphates, trifluoroacetic acid); salts (*e.g.* acetates, sulphates, phosphates); and chaotropic compounds (*e.g.* perchlorate). In almost all insulin separations acetonitrile was used as organic modifier. No matter which buffer, organic modifier and column support were used, insulin peptide and insulin-related substances with similar molecular weights (*ca.* 6000) were eluted before insulin-like substances with higher molecular weight [proinsulin and intermediary insulin (mol.wt. 9000) and insulin dimers (mol.wt. 12 000)]. The individual elution order of proinsulin, intermediary insulin and insulin dimers depended upon the actual buffer system[5].

However, with one exception[5], all analyses of the reversed-phase separation of insulin and insulin-like components have been performed with a single column and a single buffer–modifier system. In this paper we report the influence of a number of parameters (column, buffer, pore-size, column packing material, substitution group and batch-to-batch variation) on the RP-HPLC separation of crystalline porcine insulin.

MATERIALS AND METHODS

An M6000 A/M660 solvent programmer (Waters) or a Spectra-Physics SP 8700 chromatograph was used, with a WISP 710B or U6K injector (Waters). A Waters M440 with an extended-wavelength module (214 nm) or a Pye Unicam variable-wavelength UV detector was used. Fractions (1 min) were collected in a Pharmacia FRAC 300 fraction collector and assayed for radioactivity in a Hydrogamma sixteen-channel gamma counter.

The following fourteen columns were tested: LiChrosorb RP-18, 5 μm, 250 × 4.0 mm I.D. (Merck); Vydac 218 TPB 5, 5 μm, 250 × 8.0 mm I.D. (Separation Group); Spherisorb ODS2, 3 μm, 150 × 4.0 mm I.D. (Phase Separation); Spherisorb ODS2, 3 μm, 250 × 4.0 mm I.D. (Phase Separation); TSK ODS-120T, 5 μm, 250 × 4.6 mm I.D. (Toyo Soda); Nova-Pak C_{18}, 5 μm, 150 × 3.9 mm I.D. (Waters); Techogel C_4, 5 μm, 250 × 4.0 mm I.D. (HPLC Technology); Techogel C_{18}, 5 μm, 250 × 4.0 mm I.D. (HPLC Technology); Protesil diphenyl, 10 μm, 250 × 4.6 mm I.D. (Whatman); Protesil octyl, 10 μm, 250 × 4.6 mm I.D. (Whatman); Chrompack C_{18}, 8 μm, 100 × 3.0 mm I.D. (Chrompack); PEP-RPC, 10 μm, 50 × 5.0 mm I.D. (Pharmacia); PRP-1, 10 μm, 150 × 4.0 mm I.D. (Hamilton); PLRP-S, 5 μm, 150 × 4.0 mm I.D. (Polymer Laboratories).

Three buffers were used: 0.25 *M* triethylammoniumphosphate, pH 3.00 (TEAP); 0.25 *M* triethylammoniumformate, pH 6.00 (TEAF); 0.1% trifluoroacetic acid (TFA). Acetonitrile and isopropanol (HPLC quality) were used as organic modifiers.

The gradients were prepared by mixing two solutions. The first was one of the three buffers, and the second was a mixture of equal amounts of the buffer and the organic modifier.

Except for Protesil diphenyl and Protesil octyl, each type of column was tested with three or more individual specimens.

All solvents were Millipore-filtered (0.45 μm) and degassed (vacuum/ultrasound) before use. During chromatography helium is bubbled continuously through the mobile phases.

The samples were crystalline porcine sodium insulinate, batch G-63 (Nordisk Gentofte), containing *ca.* 75% insulin constituents and 25% sodium chloride and water. The dilute iodination mixture was prepared as described previously[15], 50 μl containing *ca.* 100 ng of insulin, 1–5 ng of monoiodoinsulins and 50 pg of diiodoinsulins.

RESULTS

Figs. 1 and 2 show the isocratic elution pattern of insulin peptide and monodesamidoinsulin on two different silica-based C_{18} column supports: LiChrosorb RP-18 (Fig. 1) and Chrompack C_{18} (Fig. 2). Both columns were eluted with acetonitrile as organic modifier, with TFA (upper panel) or TEAP (lower panel) as buffer substance. For comparable capacity factors (k') notable differences were observed (with respect to peak shape and resolution) by the change from one buffer substance to the other. The peak shape obtained with TFA was non-ideal and considerably inferior to that achieved with TEAP [also for higher and lower k' values (data not shown)], the latter was used throughout the insulin analyses.

Figs. 3–10 show the resolution obtained using an acetonitrile gradient in TEAP

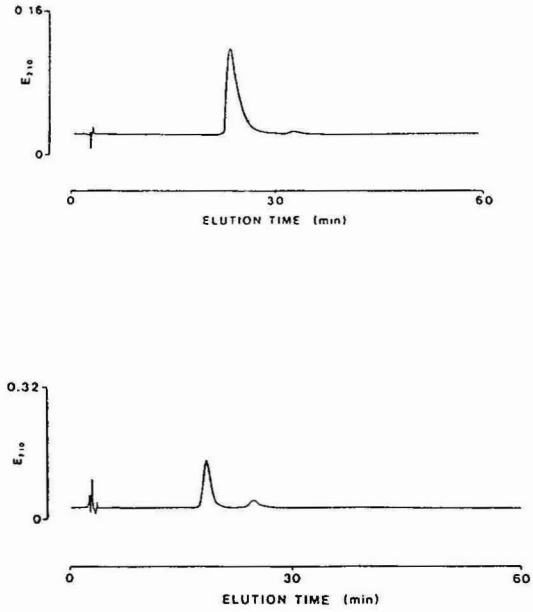

Fig. 1. Isocratic separation of 5 μg of crystalline porcine insulin on a 250 × 4.0 mm I.D. LiChrosorb RP-18 column eluted with 0.1% TFA–27.0% acetonitrile (upper curve) and 0.25 *M* TEAP–22.0% acetonitrile (lower curve), at 0.5 ml/min.

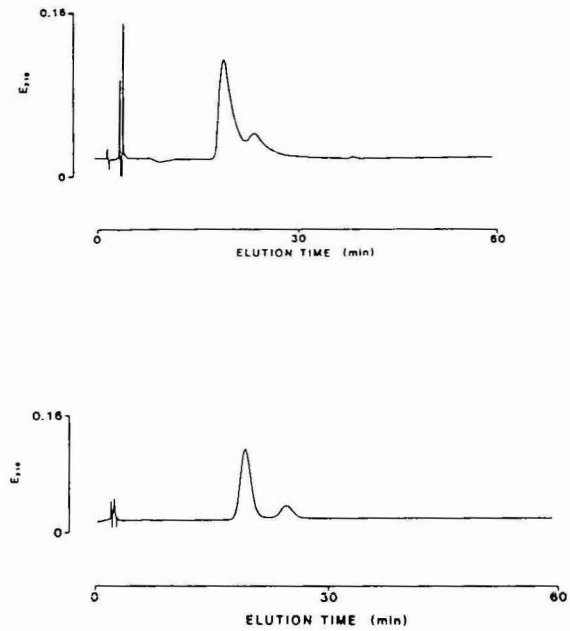

Fig. 2. Isocratic separation of 5 μg of crystalline porcine insulin on a 100 × 3.0 mm I.D. glass cartridge packed with Chrompack C_{18}. Elution was performed at 0.5 ml/min, with 0.1% TFA–23.5% acetonitrile (upper curve) and 0.25 *M* TEAP–22.5% acetonitrile (lower curve).

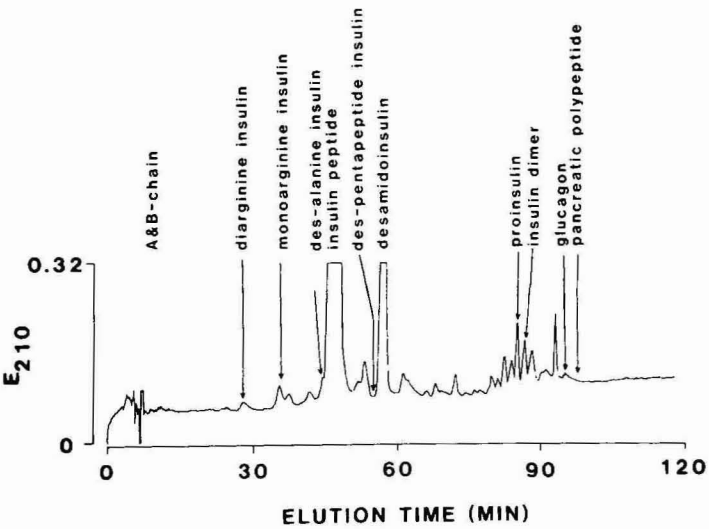

Fig. 3. Separation of 200 μg of crystalline porcine insulin on a 250 × 4.0 mm I.D. LiChrosorb RP-18 column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23–28% acetonitrile over 60 min (gradient No. 8), at 0.5 ml/min.

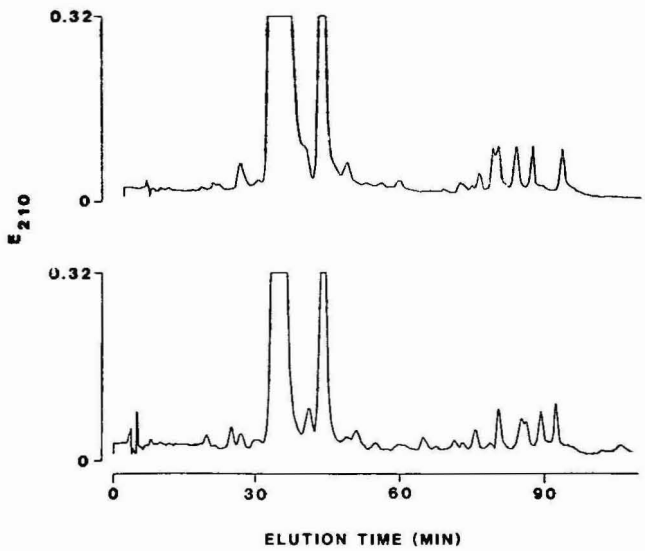

Fig. 4. Separation of 200 μg of crystalline porcine insulin on a 250 × 8.0 mm I.D. Vydac 218 TPB 5 column (top), or a 250 × 4.0 mm I.D. Spherisorb ODS2 column (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23–28% acetonitrile over 60 min (gradient No. 8). Flow-rate, 1.0 ml/min (top), 0.5 ml/min (bottom).

buffer for the elution of a number of reversed-phase column supports, silica-based (Figs. 3–9) as well as polymer-based (Fig. 10). Each column was evaluated in the following way. With a target k' value for insulin peptide between 15 and 25, the shape, duration and end-point of the gradient was varied in order to obtain the best possible resolution between insulin peptide and monodesamidoinsulin, as well as between proinsulin, insulin dimers and intermediary insulin in the last part of the chromatogram. A slightly concave acetonitrile gradient (gradient No. 8 in the Waters M660 solvent programmer) which increased the acetonitrile concentration 2.5–5.5%

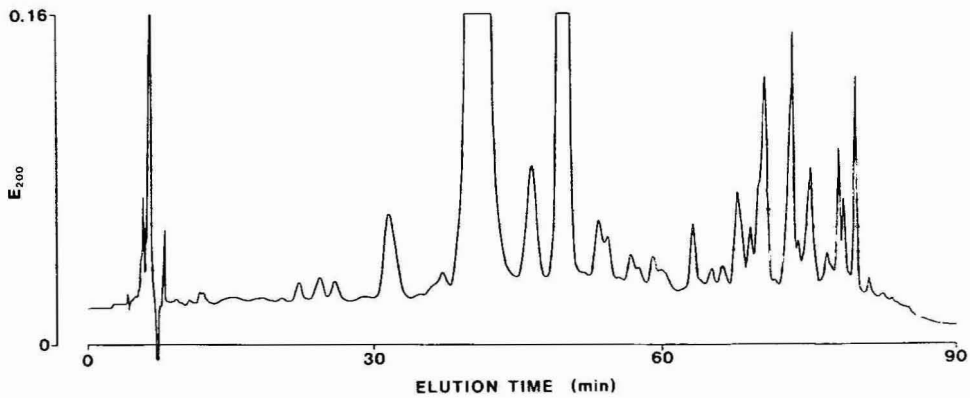

Fig. 5. Separation of 100 μg of crystalline porcine insulin on a 250 × 4.6 mm I.D. TSK ODS-120T column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 27.5–32.5% acetonitrile over 70 min (gradient No. 8) at 0.5 ml/min.

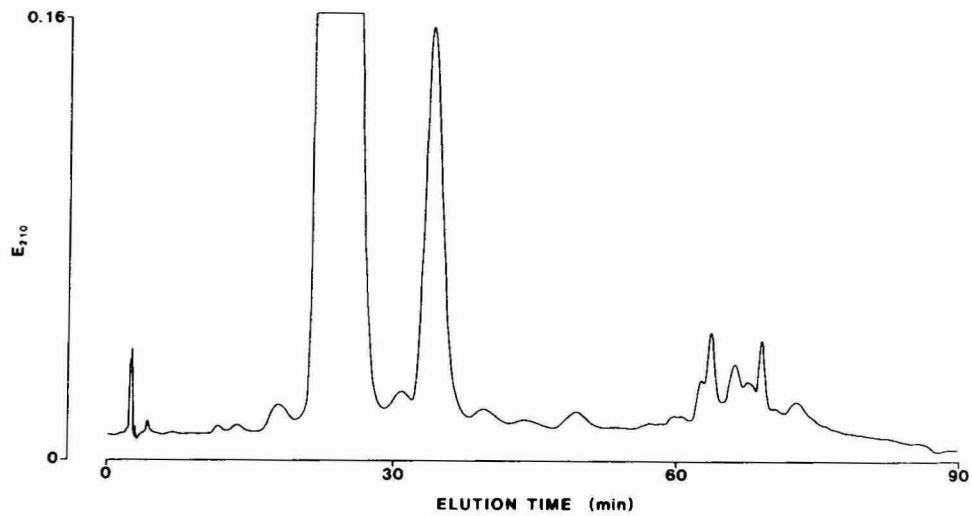

Fig. 6. Separation of 200 μg of crystalline porcine insulin on a 150 × 3.9 mm I.D. Nova-Pak C_{18} column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23.5–29% acetonitrile (gradient No. 8) for 60 min, at 0.5 ml/min.

during 50–70 min, was found to yield the best possible separation of crystalline insulin in all the columns tested except one. Except for the PEP-RPC column, these conditions allowed a more or less prominent separation between insulin peptide and desamidoinsulin, as well as between insulin-like components with a molecular weight higher than 6000. It was found impossible to separate insulin peptide and desamidoinsulin on the PEP-RPC column under these conditions.

Fig. 11 shows the isocratic elution of insulin peptide and the four monoiodoinsulins on three different silica-based C_{18} column supports with a pore-size of *ca.* 100 Å. The buffer system [0.25 *M* TEAF (pH 6.0)–isopropanol] has been optimized for this type of separation with respect to alkylammonium buffer, pH and organic modifier[16]. Although the three column supports separate the five constituents, it should be noted that the order of elution for the iodoinsulin isomers is different on the TSK ODS-120T from what it is on the LiChrosorb and the Spherisorb columns.

The Techogel C_4, Techogel C_{18} and PLRP-S columns were all incapable of separating the four monoiodoinsulins (Figs. 12 and 13). Either A14 and B16 monoiodoinsulin are eluted in the same position (Techogel C_{18}, PLRP-S) or B26 and A14 monoiodoinsulin are unresolved (Techogel C_4).

Batch-to-batch variation for one column, LiChrosorb RP-18, is shown in Fig. 14, where three different columns obtained from the local manufacturer over a period of 6 months were tested under similar conditions. For each column the organic modifier concentration was adjusted in order to achieve a k' value of *ca.* 15 for A19 monoiodoinsulin. Under these conditions two of the columns were unable to resolve A14 and B16 monoiodoinsulin.

DISCUSSION

Reversed-phase separation of numerous compounds with small differences in

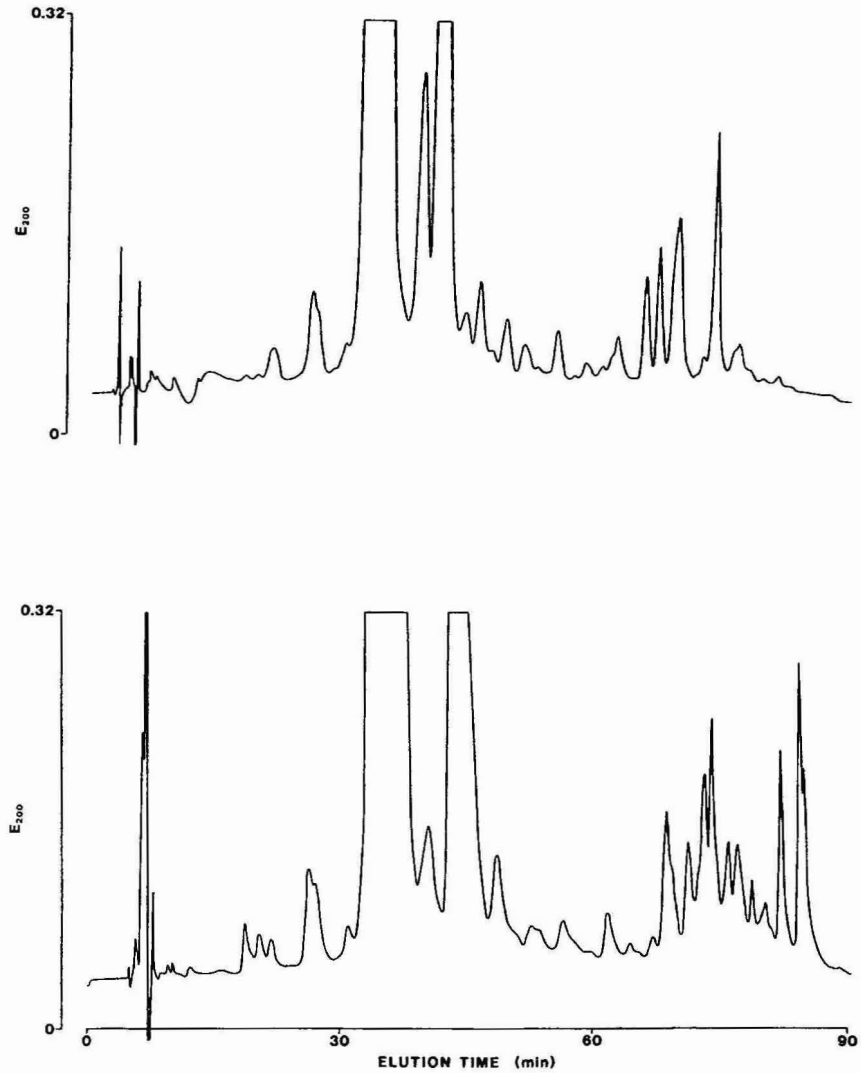

Fig. 7. Separation of 200 μg of crystalline porcine insulin on a 250 × 4.0 mm I.D. Techogel C_4 column (top) or a 250 × 4.0 mm I.D. Techogel C_{18} column (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 25–27.5% acetonitrile (gradient No. 8) for 60 min, at 0.5 ml/min.

hydrophobicity is generally performed using isocratic elution or a very narrow gradient of the organic modifier. In the case of crystalline insulin, the constituents can be divided in three groups (Table I). The main fraction contains insulin peptide (constituting 80–90% of the total amount of polypeptide) and a number of insulin derivatives with small structural differences from insulin peptide (loss of amide group(s), loss, addition or substitution of one or a few amino acid residues, etc.) and with a molecular weight similar to that of insulin peptide (5800). The second group

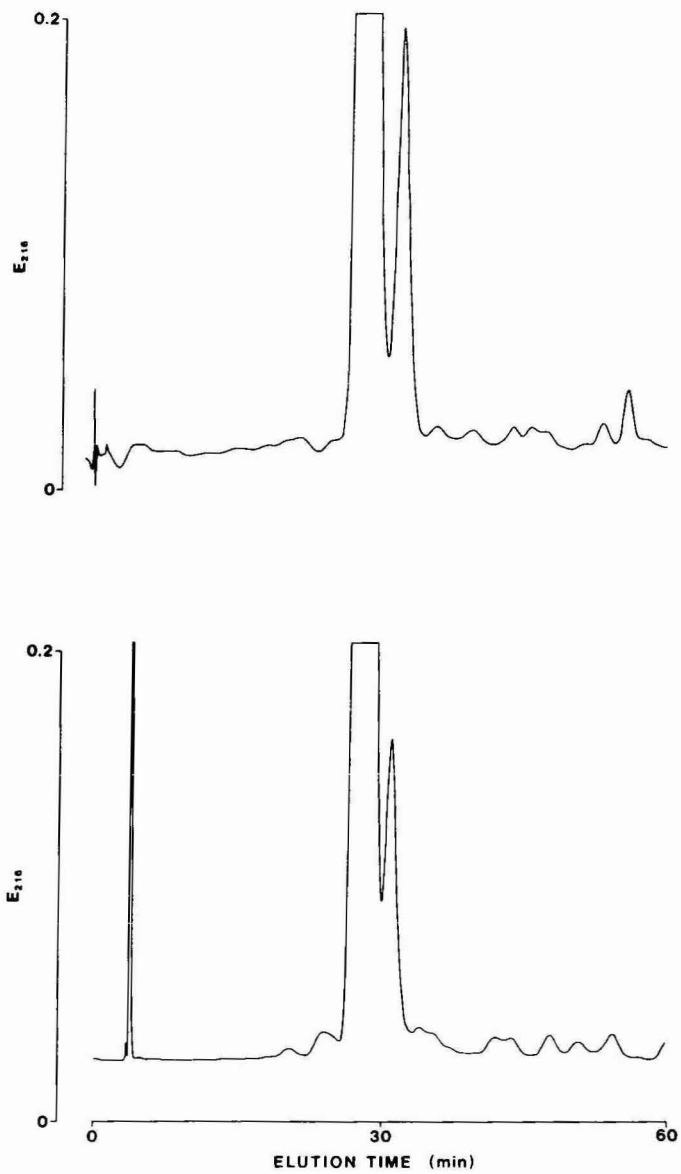

Fig. 8. Separation of 200 μg of crystalline porcine insulin on a 250 × 4.6 mm I.D. column packed with Protesil diphenyl (top) or Protesil octyl (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23–28% acetonitrile (gradient No. 8) over 50 min, at 0.5 ml/min.

contains proinsulin, covalently bound insulin dimers and intermediary insulin, compounds with higher molecular weights than insulin peptide (9000–12 000) but containing the insulin peptide as part of the molecule. The third group contains non-insulin-related polypeptides co-extracted with insulin during the processing of the pancreatic glands (glucagon, pancreatic polypeptide, etc.).

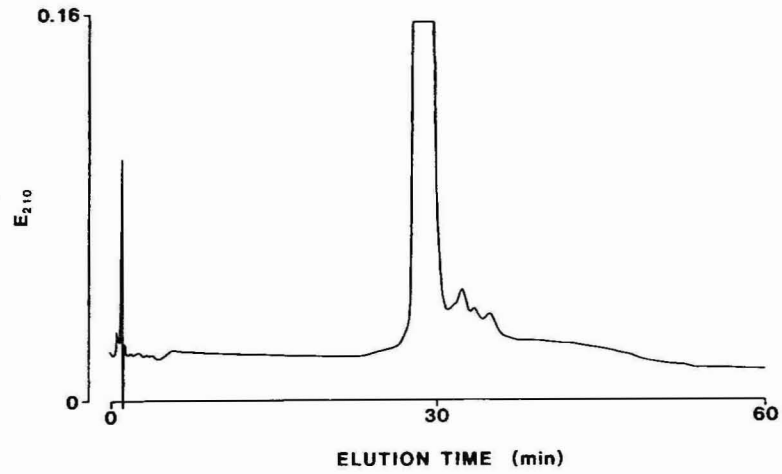

Fig. 9. Separation of 50 μg of crystalline porcine insulin on a 50 × 5.0 mm I.D. PEP-RPC column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 15–35% acetonitrile over 45 min (gradient No. 8), at 1.0 ml/min.

The members of the first group can be resolved by isocratic elution, but no published RP-HPLC separation of insulin peptide and proinsulin has been accomplished without the use of gradient elution.

In our studies we therefore used a very narrow, concave gradient, thereby operating close to isocratic conditions in the first part of the chromatogram where insulin and closely related substances are eluted, thereafter raising the organic modifier concentration to elute insulin-like components with higher molecular weights.

In isocratic elution —or close-to-isocratic elution— the choice of buffer is extremely important. As can be seen from Figs. 1 and 2, the peak shape of insulin peptide eluted isocratically is highly asymmetrical when TFA is used in combination with either C_{18} column support, whereas almost symmetrical peaks are obtained when the columns were eluted with TEAP buffer. This is probably because TEAP effectively masks residual silanol groups and thereby eliminates non-specific adsorption.

The isocratic elution pattern also reveals differences in selectivity, *i.e.* the ability to separate closely related substances. As can be seen from Figs. 1 and 2, there is a considerable difference in the abilities of the two C_{18} columns to distinguish between insulin peptide and monodesamidoinsulin (comparable k' values, identical mobile phase).

The separation pattern obtained for crystalline porcine insulin on a number of reversed-phase column supports, all eluted with an optimized TEAP–acetonitrile gradient, are shown in Figs. 3–10. The columns show considerable variation in their abilities to separate the numerous components in crystalline insulin, especially those with molecular weights higher than 6000. However, because many parameters are different (basic silica, substitution group, end-capping, pore size, particle size, etc.) from column to column, it seems too uncertain to correlate the fundamental characteristics —the hydrophobic interaction between the stationary phase and the sam-

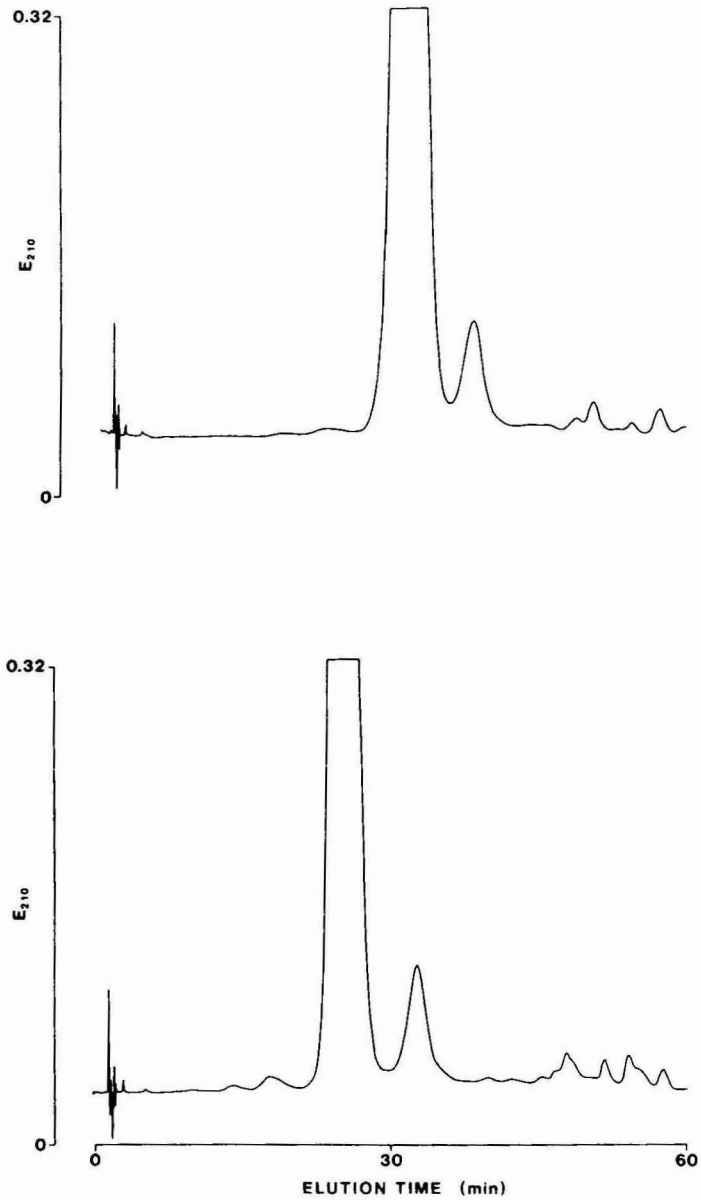

Fig. 10. Separation of 200 μg of crystalline porcine insulin on a 150 × 4.0 mm I.D. column packed with PRP-1 (top) or PLRP-S (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 22–25.5% acetonitrile (gradient No. 8), at 1.0 ml/min.

ple molecules— to one or more of the physicochemical parameters commonly used in column characterization.

The RP-HPLC separation of the four monoiodoinsulins (iodinated in Tyr A14, Tyr A19, Tyr B16 or B26) can be performed on LiChrosorb RP-18, TSK ODS-120T

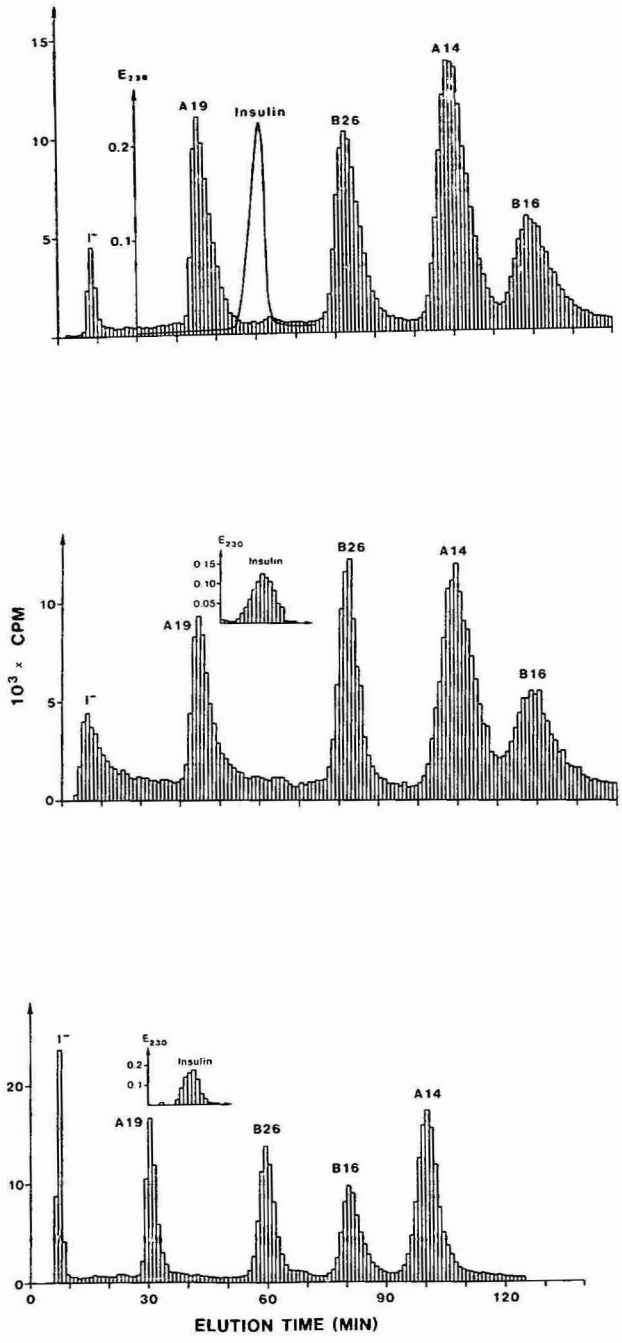

Fig. 11. Isocratic separation of 50 μl of diluted iodination mixture on a 250 × 4.6 mm I.D. TSK ODS-120T column (bottom), a 150 × 4.0 mm I.D. Spherisorb ODS2 column (middle) or a LiChrosorb RP-18 column (250 × 4.0 mm I.D.) (top), with 0.25 *M* TEAF (pH 6.0)–isopropanol concentrations of 21.5, 21.5 and 20.5% (from top to bottom). Flow-rate, 0.5 ml/min.

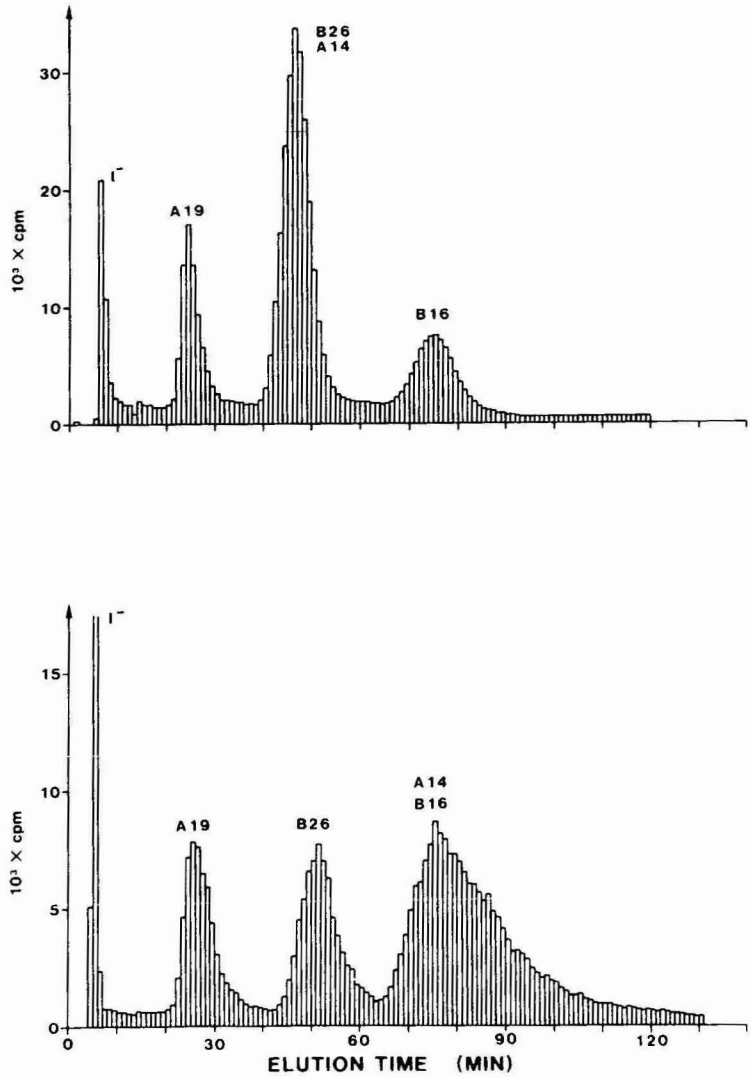

Fig. 12. Isocratic separation of 50 μl of diluted iodination mixture on a 250 × 4.0 mm I.D. column packed with Techogel C_4 (top) or Techogel C_{18} (bottom). The columns were eluted with 0.25 *M* TEAF (pH 6.0)–21.0% isopropanol, at 0.5 ml/min.

or Spherisorb ODS2 columns eluted isocratically with 0.25 *M* TEAF (pH 6.0)–isopropanol. This highly sensitive separation, which has been optimized with respect to alkylammonium buffer, pH, column and organic modifier[16], cannot be performed on the Techogel C_4 and C_{18} columns (Fig. 12), the resin-based PLRP-S column (Fig. 13), nor the PRP-1 column (data not shown). Furthermore, the Vydac 218 TPB 5 column was unable to separate the monoiodoinsulins[16].

Even though the separation can be performed on the LiChrosorb RP-18 col-

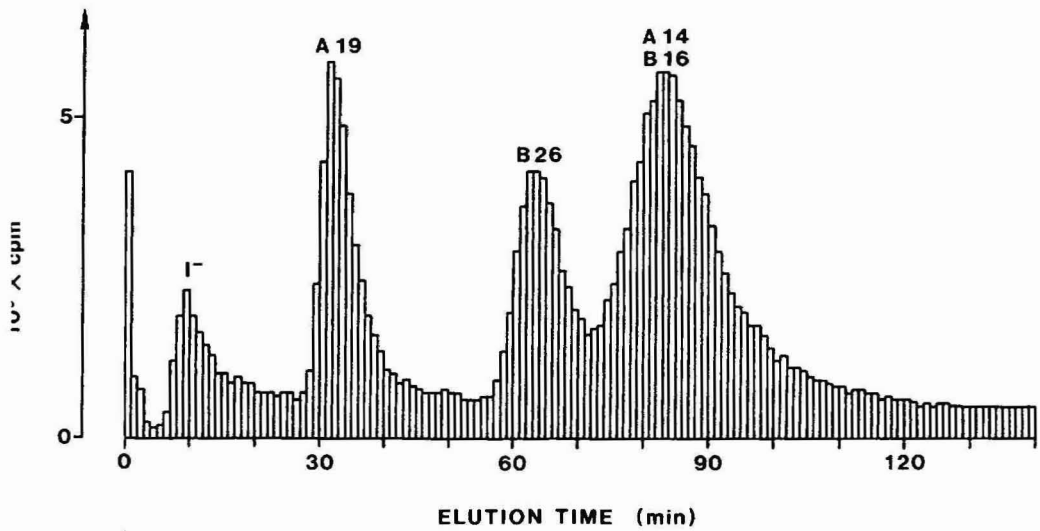

Fig. 13. Isocratic separation of 50 μl of diluted iodination mixture on a 150 × 4.0 mm I.D. PLRP-S column eluted with 0.25 *M* TEAF (pH 6.0)–22.5% isopropanol, at 0.5 ml/min.

umn, the task is only just within the capacity of this column. This is illustrated in Fig. 14, which shows the separation pattern obtained from three randomly selected LiChrosorb RP-18 columns. One column behaved well, but separation was not achieved on the two other columns, which suffered from lack of selectivity and theoretical plates and from too high an asymmetrical factor.

It has been claimed in the literature that reversed-phase columns with different chain lengths perform more or less identically. However, when the Techogel C_4 and C_{18} columns are compared (Fig. 12) it can be seen that they separate the four monoiodoinsulins in different ways: the C_4 column is unable to resolve the B26 and A14 monoiodoinsulin, whereas A14 and B16 are eluted in the same position from the C_{18} column.

It is worth emphasizing that the three columns that were able to separate the

TABLE I

INSULIN-RELATED AND NON-INSULIN-RELATED IMPURITIES COMMONLY FOUND IN CRYSTALLINE INSULIN

Insulin-related compounds, mol.wt. ca. 5800	*Insulin-related compounds, mol.wt. 9000–12 000*	*Non-insulin related compounds*
Insulin peptide	Proinsulin	Glucagon
Monoarginine insulin	Insulin dimer	Pancreatic polypeptide
Diarginine insulin	Intermediary insulin	
Desamidoinsulins		
Ethylesterinsulins		
Des-alanine insulin		
Des-pentapeptide insulin		
Des-octapeptide insulin		

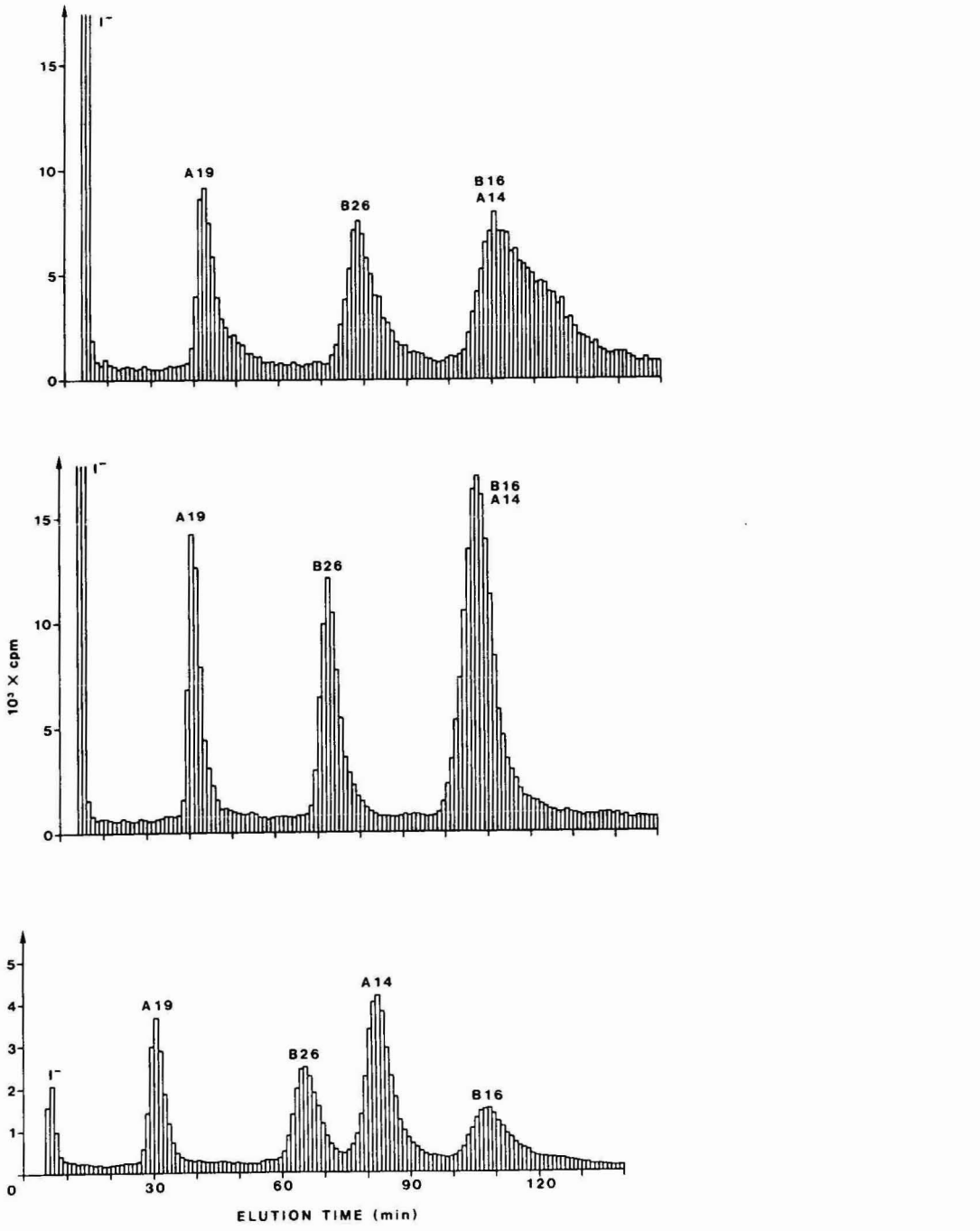

Fig. 14. Isocratic separation of 50 μl of diluted iodination mixture on three different LiChrosorb RP-18 columns (250 × 4.0 mm I.D.) eluted with 0.25 *M* TEAF (pH 6.0) containing (from top to bottom) 22.0, 21.0 and 21.0% isopropanol. Flow-rate 0.5 ml/min.

iodoinsulins were all silica-based C_{18} columns with 80–100 Å pore size. None of the wide-pore columns tested in this work could separate all four iodoinsulins, and, to the best of our knowledge, the successful use of wide-pore reversed-phase columns for complete separation of iodoinsulins has not been published. However, the three C_{18} columns found to be useful for this important separation differ with respect to other parameters, *i.e.* carbon load and end-capping, and it therefore seems premature to connect polypeptide selectivity with a single column parameter —or combination of parameters.

Although this investigation deals with only one type of buffer and one group of compounds (insulin and insulin derivatives), it may be concluded that, before a reversed-phase polypeptide separation is undertaken, some column research (bringing the actual sample into play) can be a very profitable investment.

ACKNOWLEDGEMENT

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PURIFICATION AND CHARACTERIZATION OF CAMP-FACTOR FROM *STREPTOCOCCUS AGALACTIAE* BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY AND CHROMATOFOCUSING

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(Received July 29th, 1985)

SUMMARY

CAMP-factor from *Streptococcus agalactiae* (group B streptococcus) was purified 60-fold from the culture supernatant to electrophoretic homogeneity in 57% yield. The purification procedure involved ammonium sulphate precipitation, ultrafiltration, hydrophobic interaction chromatography on Octyl-Sepharose and chromatofocusing on polybuffer exchanger PBE 94. The purified CAMP-factor consists of a single polypeptide chain with an apparent molecular weight of 25 kD and an isoelectric point of 8.9. The properties of the CAMP-factor demonstrated by charge-shift electrophoresis were consistent with those of an amphiphilic polypeptide.

INTRODUCTION

The CAMP-factor (CAMP-F) is produced by most strains of *S. agalactiae*[1] and was first described by Christie *et al.*[2]. The authors demonstrated that filtrates of *S. agalactiae* contained an agent (CAMP-F) that lysed sphingomyelinase-treated sheep and cow erythrocytes, whereas red blood cells (RBC) from human, horse, rabbit and guinea pig were not lysed[2]. Further studies by Esseveld *et al.*[3,4] and Bernheimer *et al.*[5] revealed that the agent was a polypeptide to which molecular weight values of 33 kD[6], 15 kD[4] and 23.5 kD[5] have been assigned. Part of the mechanism of the CAMP reaction was disclosed in the past by the work of Doery *et al.*[7], Bernheimer *et al.*[5] and Sterzik *et al.*[8]. These authors investigated the role of *S. aureus* sphingomyelinase in the sensitization process of either susceptible RBC or target liposomes.

Attempts to purify CAMP-F resulted in preparations of different grades of purity. This is obvious from the different purification protocols and the considerable differences in the reported amino acid composition[4,5,9]. Moreover, the isoelectric focusing (IEF) data of the purified CAMP-F reported by Bernheimer *et al.*[5] suggested considerable microheterogeneity of the purified CAMP-F. The present study was thus intended not only to clarify the reported discrepancies but also to obtain a homogeneous CAMP-F preparation that would permit further studies of the primary structure and chemical nature of this amphiphilic polypeptide.

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EXPERIMENTAL

Strain and culture conditions for CAMP-F production

S. agalactiae (T.C.C. strain No. 8181) was obtained from Central Public Health Laboratory, Colindale, London, U.K. Organisms were cultured on sheep blood agar plates and inoculated at 37°C for 12–24 h in a 5% carbon dioxide atmosphere.

Fermenter cultivation of *S. agalactiae* was performed as reported earlier[10].

Purification of CAMP-F

Ultrafiltration. The CAMP-F was concentrated in the supernatant by ultrafiltration (Amicon "hollow fiber" dialyser, H5 P2, Fa. Amicon, Witten, F.R.G.) at 4°C to one third of the original volume.

Ammonium sulphate precipitation. The concentrated supernatant was precipitated with solid ammonium sulphate at a saturation of 75%. The precipitation of CAMP-F at pH 8.5 and 8°C was terminated after 96 h. The precipitate was collected by centrifugation (10 000 *g*, 4°C, for 20 min) and dissolved in 0.05 *M* Tris–HCl buffer, 1 *M* potassium chloride (pH 8.0). Ammonium sulphate was removed by hollow fiber ultrafiltration using H1 P2 fibers.

Hydrophobic interaction chromatography (HIC). Binding of CAMP-F to Octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was performed in a batchwise procedure. The CAMP-F solution was diluted with 0.05 *M* Tris–HCl buffer (pH 8.0) containing 1 *M* potassium chloride to a protein concentration of 1 mg/ml. Octyl-Sepharose (75 g) equilibrated in the same buffer was used to bind CAMP-F (750 mg) at 4°C after 20 h with constant stirring.

The gel was then washed sequentially on a sintered glass funnel with 1 l of each of the following cold buffer solutions: (a) 0.05 *M* Tris–HCl, pH 8.0; (b) 0.01 *M* Tris–HCl, pH 8.0; (c) 0.001 *M* Tris–HCl, pH 8.0; (d) 0.05 *M* Tris–HCl, pH 9.0; (e) 0.01 *M* Tris–HCl, pH 9.0; (f) 0.001 *M* Tris–HCl, pH 9.0; (g) 0.05 *M* Tris–HCl, pH 8.5.

HIC was carried out by transferring the gel to a column (15 × 2.6 cm I.D.). A sodium desoxycholate gradient (SDOC: 0 → 1%) in 750 ml, 0.05 Tris–HCl (pH 8.5) was applied first to remove extraneous protein. Thereafter, CAMP-F was desorbed by a Triton X-100 gradient (750 ml; 0 → 1% in water). Fractions containing CAMP-F activity were pooled and lyophilized.

Chromatofocusing. The polybuffer exchanger PBE 94 (Pharmacia) was packed into a column (35 × 1 cm I.D.) and equilibrated with 0.025 *M* ethanolamine–hydrochloric acid (pH 9.4). The CAMP-F (3 mg protein) purified by HIC was dissolved in 2 ml of polybuffer PB 96–hydrochloric acid (pH 7.0), and applied to the column for chromatofocusing. CAMP-F was eluted with a 1:10 dilution of polybuffer PB 96–hydrochloric acid (pH 7.0). The elution volume was 250 ml and the flow-rate 12 ml/h. Fractions of 2.5 ml containing CAMP-F were pooled and lyophilized.

Removal of polybuffer PB 96. To remove polybuffer PB 96 from CAMP-F, the freeze-dried protein was dissolved in a minimal volume of 0.05 *M* ammonium bicarbonate (pH 8.2) and applied to a Sephadex G-75 column (50 × 1.6 cm I.D.). Gel filtration was performed in the same solution at a flow-rate of 12 ml/h. Fractions containing CAMP-F were collected, and the absence of polybuffer monitored by reading the absorption at $\lambda = 245$ nm.

Preparation of CAMP-F antibodies. To produce antibodies, 1 mg of purified CAMP-F (specific activity $1.3 \cdot 10^5$ U/mg) was injected in 1 ml of Freund's complete adjuvant (Difco Labs., Detroit, MI, U.S.A.) subcutaneously into a rabbit at multiple sites. A booster injection was given at weeks 5 and 6, and the production of antibodies was followed by the Ouchterlony technique. Blood was drawn from the rabbit's ear, usually 6 days after the last injection.

Determination of protein. Protein was measured according to Peterson[11] with BSA as a standard.

Determination of CAMP-F activity. CAMP-F activity was measured by the kinetic test developed by Sterzik[12].

Characterization of CAMP-F

Analytical polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulphate (SDS–PAGE in slab gels (80 × 120 × 1 mm, T = 12%, C = 2.6%) was performed according to Laemmli[13]. Phosphorylase E.C. 2.4.1.1. (M_r 95 000), bovine serum albumin (M_r 68 000), fumarase E.C. 4.1.2.2. (M_r 49 000), carboanhydrase E.C. 4.2.1.1. (M_r 30 000) and lysozyme E.C. 3.2.1.17. (M_r 14 500) were used as standard proteins and purchased from Boehringer (Mannheim, F.R.G.). The proteins were denaturated in the presence of 2.5% (v/v) 2-mercaptoethanol and 1% (w/v) SDS by boiling for 5 min; 5–30 μg of protein were applied and the protein stained with Coomassie brilliant blue.

PAGE of CAMP-F under non-denaturating conditions was carried out in tubes (140 × 5 mm I.D.) using polyacrylamide gels of T = 7.5% and, C = 2.6%. The buffer system of Reisfeld[14] and Laemmli[13] was used and the protein (40 μg/gel) was separated at 3.5 mA per tube.

Protein titration curve. The titration curves in slab gels (125 × 125 mm, T = 5%, C = 2.6%) were obtained according to Righetti *et al.*[15].

RESULTS

Purification of CAMP-F

Concentration of CAMP-F and ammonium sulphate precipitation. Fermenter cultivation of *S.* agalactiae[10] in Trypticase Peptone broth (Becton Dickinson, Heidelberg, F.R.G., No. 11 921) in the presence of glucose (2%) and carbon dioxide resulted in the production of CAMP-F in the supernatant with a specific activity (SA) of $3.9 \cdot 10^3$ (U/mg). After ultrafiltration, concentration, ammonium sulphate precipitation, the SA increased to $9 \cdot 10^3$ (U/mg). A further increase was achieved by a second ultrafiltration step, resulting in the CAMP-F preparation of SA = $2.2 \cdot 10^4$ (U/mg). The yield of CAMP-F at this stage of purification was still 94% of the starting material.

Hydrophobic interaction chromatography. The successful purification of *S. aureus* lipase by HIC[16,17] suggested that HIC could be useful in the purification of the amphiphilic CAMP-protein. Preliminary batch experiments with Octyl-Sepharose CL-4B confirmed the binding of CAMP-F to this matrix. The experiments also revealed that CAMP-F was separated from extraneous protein on Octyl-Sepharose by a combination of a batchwise and a column procedure. Quantitative binding of CAMP-F to Octyl-Sepharose was achieved in batch experiments after 20 h at 8°C in

0.05 *M* Tris–HCl buffer (pH 8.0), containing 1.0 *M* potassium chloride, at a gel/protein ratio of 1 (g):10 (mg). The washing procedure (steps a–g, *cf.* Experimental) with buffers of different pH and molarities removed contaminating material but not CAMP-F.

CAMP-F was then further purified by gradient elution chromatography after packing the gel into a column. When the SDOC gradient (0 → 1%) was applied in the first step, 20% of contaminating protein and a small amount of CAMP-F (5%) of total activity) were eluted (Fig. 1). CAMP-F activity was then removed from the gel with a Triton X-100 gradient (0 → 1%). After HIC, 89% of the CAMP-F activity (SA = $113.3 \cdot 10^3$, U/mg) was recovered.

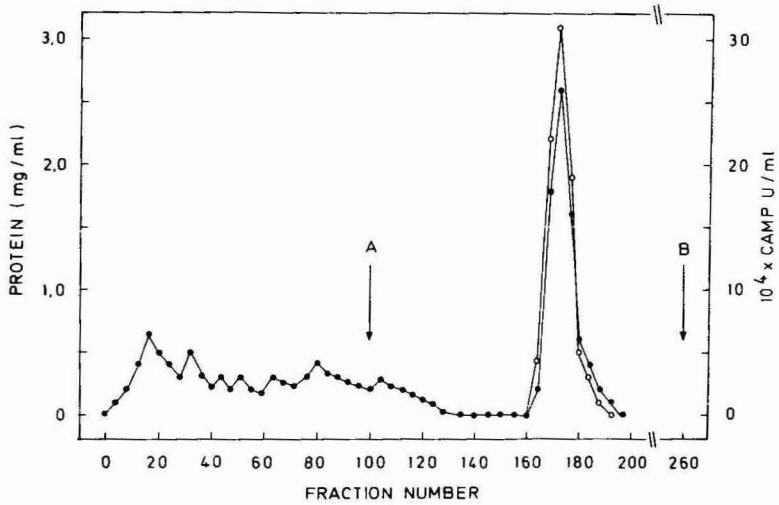

Fig. 1. Purification of CAMP-factor by hydrophobic interaction chromatography on Octyl-Sepharose CL-4B (column, 40 × 2.6 cm I.D.). Arrow indicates change of eluent. (A) Gradient elution of extraneous protein with sodium desoxycholate, 0 → 1% (w/v) in 0.05 *M* Tris–HCl buffer (pH 8.5) (fraction Nos. 1–100 ≙ 750 ml); flow-rate, 20 ml/h. (B) Gradient elution of CAMP-factor with Triton X-100, 0 → 1% (w/v) in water (fraction Nos. 101–260 ≙ 750 ml); flow-rate, 20 ml/h. Protein mg/ml (–●–●–); activity of CAMP-factor U/ml (–○–○–).

Subsequent analysis of CAMP-F by native electrophoresis at different pH values[13,14] exhibited two protein bands only as shown in Fig. 2A and B. In contrast, protein titration[15] revealed five polypeptides of very similar hydrophobicity (Fig. 2C), electrophoretic mobility and isoelectric points.

Chromatofocusing of CAMP-F. It was shown by Sluyterman *et al.*[18–20] and Kopetzki and Entian[21] that chromatofocusing is an excellent technique for the separation of polypeptides with similar isoelectric points. Thus, CAMP-F was separated from its "satellite proteins" by chromatofocusing using the polybuffer exchanger PBE 94 (pH 7–9.4). A typical elution profile is depicted in Fig. 3. CAMP-F was eluted with polybuffer 96–hydrochloric acid at pH 9 and thus successfully separated from its contaminants.

The separation steps are summarized in Table I.

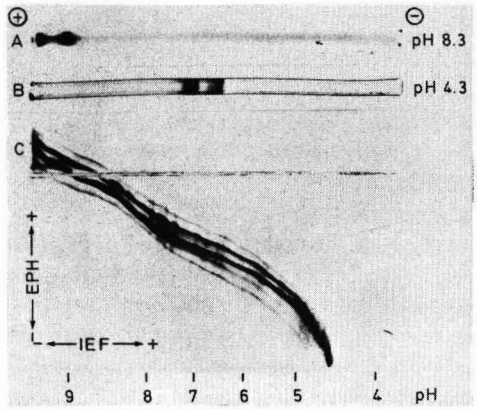

Fig. 2. Native PAGE of partially purified CAMP-factor was performed in tubes (T = 7.5%; C = 2.6%; 140 × 5 mm I.D.) at 3.5 mA per tube using the following buffer systems: (A) system of Laemmli[13]; (B) system of Reisfeld[14]. (C) Protein titration curves were obtained with the PAGE technique (slab gels; T = 5%; C = 3%; 125 × 125 mm) in the presence of 2% ampholine (pH 3.5–10). First dimension: isoelectric focusing (IEF), 50 min at 8°C; 10 W/gel; second dimension: electrophoresis (EPH) of 100 μg of CAMP-factor preparation, 10 min at 8°C; 700 V/gel.

Characterization of CAMP-F

Homogeneity and isoelectric point of CAMP-F. The technique to characterize proteins by "protein titration"[15] provides an excellent means to determine both the isoelectric point and the degree of homo- or heterogeneity of an individual polypeptide. "Protein titration" of the purified CAMP-F revealed an isoelectric point of pI = 8.9 ± 0.2. The titration curve demonstrated in addition the presence of a single

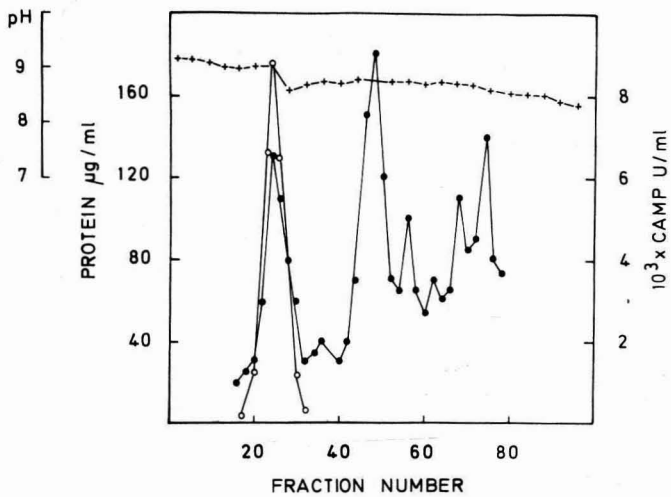

Fig. 3. Chromatofocusing of the CAMP-factor on polybuffer exchanger PBE 94 (column, 40 × 1 cm I.D.). Separation of CAMP-factor (fraction Nos. 18–32) from contaminating material (fraction Nos. 40–53) was done by elution with polybuffer 96–hydrochloric acid at pH 7.0. Starting buffer, 0.025 *M* ethanolamine–hydrochloric acid (pH 9.4); flow-rate, 12 ml/h. Protein μg/ml (–●–●–); activity of CAMP-factor U/ml (–O–O–).

TABLE I

SUMMARY OF PURIFICATION OF CAMP-FACTOR FROM *S. AGALACTIAE*

Purification step	*Total activity (units)*	*Specific activity (units/mg)*	*Recovery (%)*	*Purification ×-fold*
(1) Culture supernatant	$35.8 \cdot 10^6$	$3.9 \cdot 10^3$	100.0	1.0
(2) Ultrafiltration after ammonium sulphate precipitation	$33.6 \cdot 10^6$	$22.4 \cdot 10^3$	94.0	5.6
(3) Hydrophobic chromatography	$31.9 \cdot 10^6$	$113.0 \cdot 10^3$	89.0	29.1
(4) Chromatofocusing	$20.6 \cdot 10^6$	$236.9 \cdot 10^3$	57.6	60.8

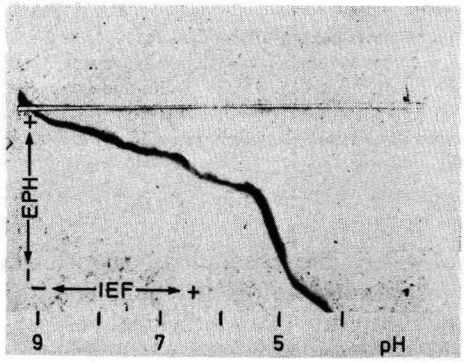

Fig. 4. Protein titration of the purified CAMP-factor (see legend of Fig. 2).

polypeptide band within a wide range of pH (Fig. 4). In addition, analytical SDS-PAGE confirmed that CAMP-F isolated by chromatofocusing appeared as a single protein band (Fig. 5A).

Molecular weight of purified CAMP-F. Estimation of the molecular weight of

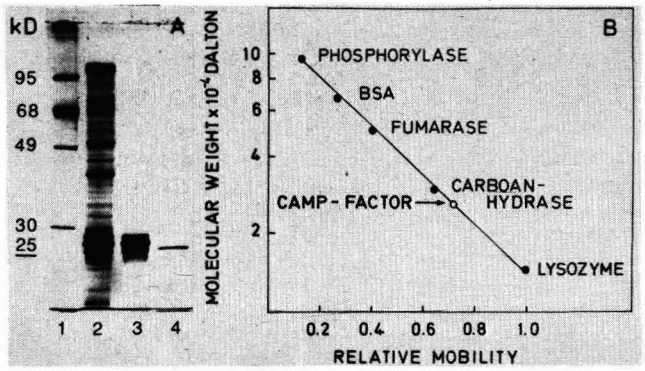

Fig. 5. (A) Analytical SDS–PAGE of CAMP-factor after different purification steps (T = 12%; C = 2.6%; 40 × 120 × 1 mm). Channels: 1 = marker proteins, phosphorylase (M_r 95 000), BSA (M_r 68 000), fumarase (M_r 49 000), carboanhydrase (M_r 30 000); 2 = ammonium sulphate precipitate of culture supernatant; 3 = CAMP-factor after hydrophobic interaction chromatography; 4 = CAMP-factor after chromatofocusing. (B) Determination of molecular weight of purified CAMP-factor by SDS–PAGE.

CAMP-F was performed by SDS–PAGE with the aid of appropriate calibration proteins. As can be seen from Fig. 5B, an apparent molecular weight of 25 kD ± 5% was found. The value was in good agreement with that of Bernheimer *et al.*[5] and corresponded well to the figure calculated from the amino acid analysis by Jürgens *et al.*[9]. It differed considerably, however, from the molecular weights reported earlier[4,6].

Antigenicity of CAMP-F and detergent binding. CAMP-F produced antibodies in the rabbit when used for immunization with complete Freund's adjuvant. When the purified CAMP-F was analysed in immunodiffusion, one precipitation line was observed. The antiserum was used in addition to detect CAMP-F in charge-shift electrophoresis according to Helenius and Simons[22]. Charge-shift electrophoresis revealed binding of cationic and anionic detergents resulting in a shift of the direction of migration and electrophoretic mobility of CAMP-F (Fig. 6). This type of immunoelectrophoresis indicated also that the purified CAMP-F, after detergent binding, migrated as a homogeneous protein fraction.

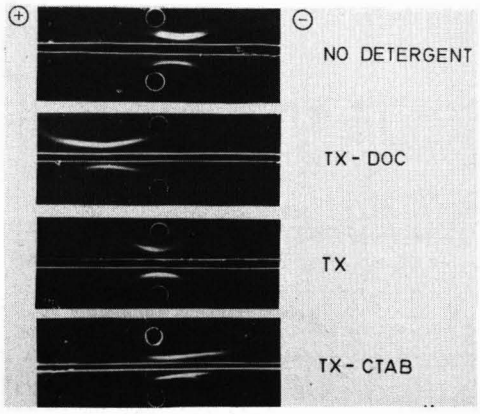

Fig. 6. Characterzation of CAMP-factor by charge-shift immunoelectrophoresis at pH 8.7[20]. Electrophoretic mobility of purified CAMP-factor in the presence of Triton X-100 and sodium desoxycholate (TX-SDOC), Triton X-100 (TX), Triton X-100 and cetyltrimethylammonium bromide (TX-CTAB), respectively.

DISCUSSION

Purification and characterization of CAMP-factor of group B streptococci by different groups in the past gave rise to discussions of the chemical nature of this extracellular polypeptide. Besides the molecular weights determined in different laboratories[4–6], the isoelectric point and particularly the amino acid composition[4,5,9] were at variance. These results are difficult to explain because the CAMP-F derived from different strains of group B streptococci may well represent a different gene product with differences in the amino acid composition. Thus, Bernheimer *et al.*[5] found 8 lysine, 18 alanine, 22 valine, and 4 methionine residues per mol, whereas 22, 31, 33, and 1 methionine residue(s) per mol, respectively, were found in our laboratory[9]. The existence of one methionine residue, however, is of considerable interest since BrCN cleavage should result in two peptide fragments. Recent experiments in

our laboratory[23] confirmed this view and demonstrated that, instead of the five fragments predicted from an earlier amino acid analysis[5], only two fragments are found.

Purification protocols suggested also that the CAMP-F preparation obtained by different groups[3–6] varied considerably in their degree of purification. Proofs of homogeneity by stringent methods were either lacking or indicated that the purified material still exhibited considerable microheterogeneity, as revealed by IEF analysis[5].

It is consistent with our findings that polypeptides with flat titration curves[15] are not readily separated and characterized by IEF. When the CAMP-F purified by gel filtration was subjected to isoelectric focusing, the active material was detected within a wide pH range (7–9.5) with maximum activity at pH 8.6 SDS–PAGE revealed, however, in contrast to earlier work[5] that the peak fraction still contained five polypeptides, visualized by silver staining[24], which have not been separated from CAMP-F. These contaminants could be separated successfully from CAMP-F by chromatofocusing[18–20] in polybuffer exchanger PBE 94. Thus the CAMP-F fraction eluted from the column at pH 9.0 was homogeneous as judged by SDS–PAGE, charge-shift electrophoresis[22] and protein titration[15]. The apparent molecular weight of 25 kD was in good agreement with that of 23.5 kD determined earlier by Bernheimer[5].

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NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NON-DERIVATIZED GANGLIOSIDE MIXTURES

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SUMMARY

A new analytical and semi-preparative high-performance liquid chromatographic method for the separation of a brain ganglioside mixture into individual components is described. Gangliosides were applied to a LiChrosorb-NH_2 column and eluted with the solvent system acetonitrile–phosphate buffer at different volume ratios and ionic strengths. The elution profile was monitored by flow-through detection of UV absorbance at 215 nm. The separation of mono- to polysialogangliosides was performed in one step in a total elution time lower than 90 min and with high reproducibility.

INTRODUCTION

Gangliosides, glycosphingolipids containing sialic acid, which are normally present in the cell plasma membranes[1,2], are extracted from tissues as mixtures of many species that differ in their oligosaccharide and/or ceramide portions. In recent years, high-performance liquid chromatography (HPLC) has been introduced as a methodological tool for the separation and quantification of glycosphingolipids[3–18]. The described procedures, with analytical aims, generally make use of a pre-column derivatization[3–6,9,12,13,15–18]. However, although these methods have the advantage of being acceptably sensitive, they do not seem to be easily applicable to preparative purposes. Besides, they generally require prewashing before injection to eliminate unspecific by-products that may interfere with gangliosides during UV detection. Recently, non-derivatized gangliosides have been separated on normal-phase silica gel columns with solvent systems containing *n*-hexane[7,14]. Under these conditions direct UV detection, in the wavelength range of ganglioside absorption (190–230 nm), is not convenient owing to solvent cut-off; therefore, tedious control thin-layer chromatographic (TLC) analyses are required.

Gangliosides have also been separated by reversed-phase HPLC, in solvent systems that permit direct UV detection[8,10,11]. In these cases, the analytical and preparative methods allow ganglioside molecular species to be separated because of both the oligosaccharide and ceramide portions, but when it is applied to the resolution of complex ganglioside mixtures, some overlap occurs[10].

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In the present paper, we report a HPLC methodology, that surmounts above the disadvantages and can separate and quantify non-derivatized ganglioside mixtures by direct flow-through reading at 215 nm. The system, which can also be used for preparative purposes, utilizes a LiChrosorb-NH_2 column and a gradient of acetonitrile–phosphate buffer as solvent system. The method has been standardized by the use of nine pure gangliosides and has been applied to the purification of a ganglioside mixture extracted from calf brain.

EXPERIMENTAL

Materials

Silica gel precoated thin-layer plates (HPTLC, Kieselgel 60, 250 μm thick, 20 × 10 cm) were purchased from Merck (Darmstadt, F.R.G.); the total ganglioside mixture was extracted from calf brain and partially purified according to Tettamanti *et al.*[19]. Traces of contaminants were eliminated from the ganglioside lipid extract as follows. The lyophilized crude ganglioside mixture was washed three times with cold acetone (1 ml per 5 mg crude mixture). The organic phase containing less than 0.4% of total ganglioside mixture, as sialic acid, was discarded and the ganglioside mixture was dissolved in doubly distilled water at a concentration of 200 mg of crude mixture per millilitre, sonicated for 2 min and centrifuged for 5 min at 8500 *g*, the clear supernatant containing gangliosides was carefully removed and lyophilized. TLC separation followed by densitometric quantitation[20] of the ganglioside mixture, carried out before and after the described purification steps, did not show any significant differences.

Standard gangliosides were extracted according to Tettamanti *et al.*[19]. GM1, GD1a, GD1b, GT1b and GQ1b were prepared from calf brain, Fuc-GM1 and Fuc-GD1b from pig brain, GM2 from a Tay-Sachs brain and GM3 from human spleen (the nomenclature of Svennerholm[21], for ganglioside designation, is used). All

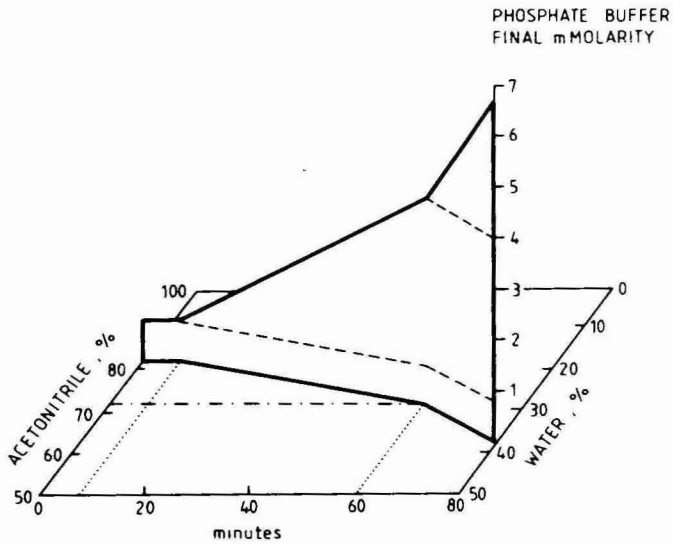

Fig. 1. Graphical representation of the elution gradient used in the HPLC separation of gangliosides.

gangliosides were more than 99.5% pure, as assessed by HPLC analysis following the suggestion of Gazzotti *et al.*[10].

HPLC separation of gangliosides

A 0.1–20-nmol portion of pure GM3, GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b, GQ1b gangliosides, or a 1–50 nmol portion of calf brain ganglioside mixture, as lipid-bound sialic acid, was dissolved in 5–10 μl of redistilled water in a microtube and introduced into a syringe-loading sample injector (Model 7125 Rheodyne) equipped with a 20-μl loop. The microtube was washed with 5–10 μl of redistilled water and the washing was added to the previous sample in order to minimized loss of material. Gangliosides were then chromatographed on a LiChrosorb-NH_2 column, 250 × 4 mm I.D., 7 μm average particle diameter (Merck, Darmstadt, F.R.G.) with a Gilson.HPLC apparatus, equipped with an Apple II computer system for the selection of elution gradient.

The separation was carried out at 20°C with a gradient of the following solvent mixtures: solvent A, acetonitrile–5 m*M* phosphate buffer, pH 5.6 (83:17); solvent B, acetonitrile–20 m*M* phosphate buffer, pH 5.6 (1:1). The gradient elution programme was as follows: 7 min with solvent A; 53 min with a linear gradient from solvent A to solvent A–solvent B (66:34); 20 min with a linear gradient from solvent A–solvent B (66:34) to solvent A–solvent B (36:64). The resulting gradient, expressed as acetonitrile and water percentages, and the final phosphate buffer molarity as a function of elution time, is represented in Fig. 1.

A complete analysis took 80 min. The flow-rate was 1 ml/min and the elution profile was monitored by flow-through detection of UV absorbance at 215 nm (Gilson UV detector model Holochrome).

Before a new analysis cycle, the column was washed with solvent B for 10 min and then equilibrated with solvent A for 15 min, in order to eliminate highly polar contaminants.

Preparative HPLC of the ganglioside mixture from calf brain was achieved using preparative LiChrosorb-NH_2, 250 × 25 mm I.D. (Merck), according to the above gradient programme. A 1–5-mg portion of ganglioside mixture (as sialic acid), was dissolved in 100 μl of redistilled water in a microtube, and introduced into the syringe-loading sample injector, equipped with a 200-μl loop. The microtube was washed with 100 μl of redistilled water and the washing was added to the previous sample. Gangliosides were chromatographed at a flow-rate of 39 ml/min. The elution profile was monitored by flow-through detection, as in the analytical procedure, and by TLC (see below). For this purpose 20-ml fractions were automatically collected and 0.1 ml of each fraction was dried, solubilized in 20 μl of chloroform–methanol (2:1) and spotted on a HPTLC plate. After separation, the combined fractions corresponding to each purified ganglioside were dried, dialysed, lyophilized and submitted to analytical HPLC as described.

Thin-layer chromatography (TLC)

TLC of gangliosides was carried out on HPTLC precoated plates under the following conditions: temperature, 18–20°C; solvent system, chloroform–methanol–0.2% aqueous calcium chloride (50:42:11); detection of the spots by spraying with an Erlich reagent and heating at 120°C[20].

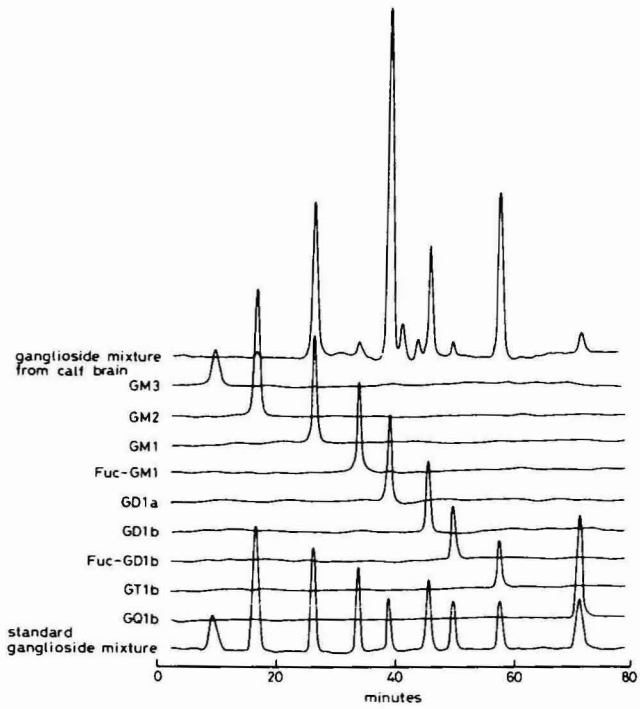

Fig. 2. Application of the HPLC analytical method to the separation of gangliosides present in a calf brain ganglioside mixture. The HPLC profile is compared with that of individual standard gangliosides and with that of a standard ganglioside mixture.

TABLE I

RELATIVE RETENTION TIMES (RRT) AND RELATIVE MOLAR RESPONSES (RMR) OF STANDARD GANGLIOSIDES

GD1a is taken as 1.00.

Ganglioside	*RRT**	*RMR ± S.D. (n = 6)*
GM3	0.202	0.536 ± 0.010
GM2	0.421	0.561 ± 0.006
GM1	0.656	0.607 ± 0.041
Fuc-GM1	0.900	0.621 ± 0.023
GD1a	1.000	1.000 ± 0.014
GD1b	1.184	1.045 ± 0.024
Fuc-GD1b	1.350	1.055 ± 0.041
GT1b	1.503	1.498 ± 0.088
GQ1b	1.869	1.901 ± 0.091

* $RRT = (RT_x - RT_m)/(RT_{GD1a} - RT_m)$, where RT_x is the retention time of the tested ganglioside, RT_{GD1a} is the retention time of GD1a, and RT_m is the dead retention time. S.D. (on six determinations) of RT_{GD1a} and of all RT_x values were lower than 1.2%.

Colorimetric methods

Ganglioside bound sialic acid was determined by the method of Warren[22] after acid hydrolysis of the sample in 0.05 *M* sulphuric acid (1 h at 80°C) and purification of liberated sialic acid by ion-exchange chromatography on a Dowex 2-X8 (CH_3COO^-) column[23]. Pure N-acetylneuraminic acid was used as the standard.

RESULTS AND DISCUSSION

Fig. 2 shows the separation of GM3, GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b and GQ1b standard gangliosides, obtained by analytical HPLC, according to the elution gradient programme in Fig. 1. Fig. 2 also illustrates the chromatographic behaviour of each individual ganglioside injected alone. Gangliosides were eluted from the column depending from their degree of polarity. GM3 and GQ1b gangliosides, which were the first and last to be eluted, displayed retention times of 572 and 4318 s (mean values), respectively. The relative retention times of each ganglioside, referred to GD1a, are listed in Table I.

Although gangliosides show an UV maximum absorption at 195 nm[10], column elution was followed at 215 nm, where the ganglioside absorption corresponded to 60% of that recorded at 195 nm. This was necessary in order to avoid zero-line variation caused by variations in the solvent absorption at 195 nm during the gradient

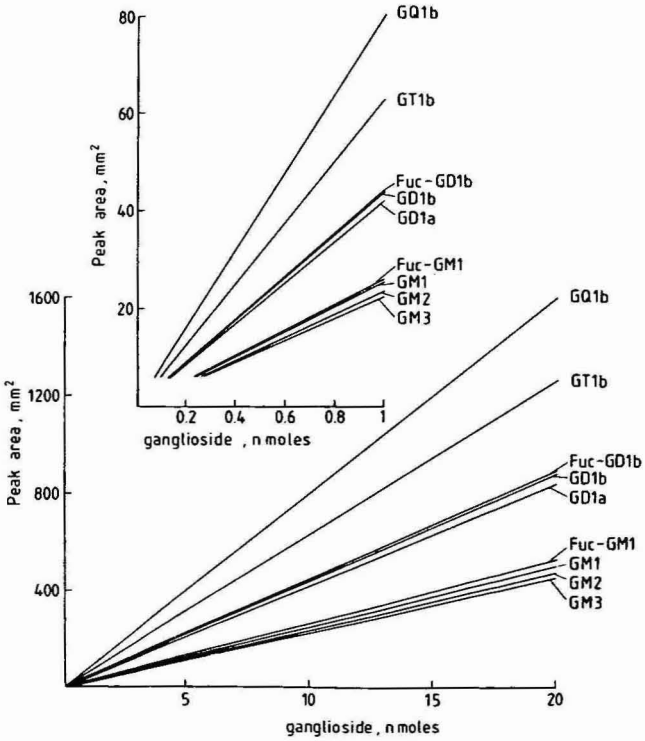

Fig. 3. Application of the HPLC analytical method to ganglioside quantification: relationship between peak area (mm^2 at 0.025 a.u.f.s.) and amount of injected gangliosides (nmol).

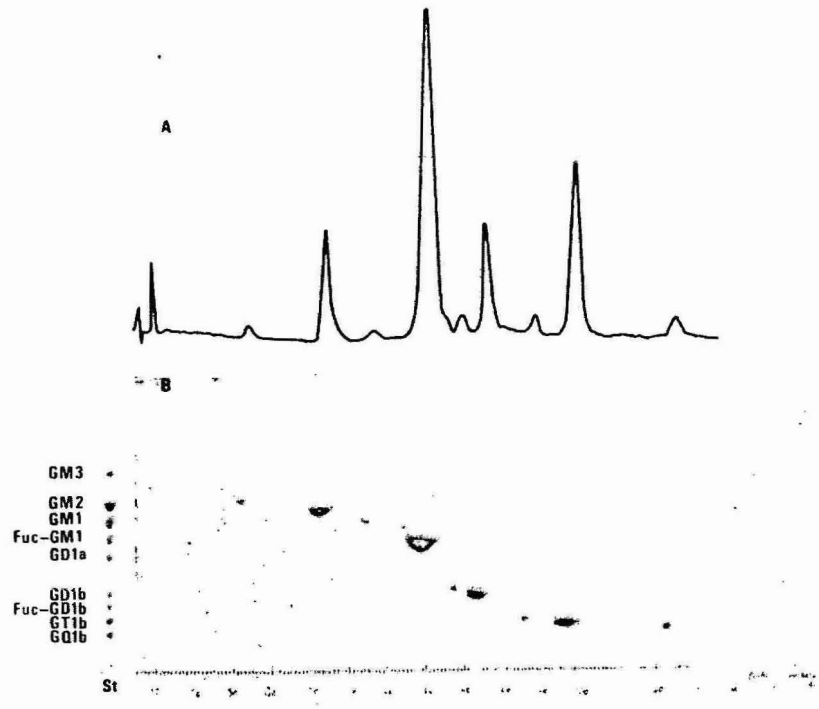

Fig. 4. Application of the HPLC preparative method to the isolation of individual gangliosides from a calf brain ganglioside mixture. (A) HPLC elution profile, recorded by UV absorbance at 215 nm. (B) HPLC elution profile monitored by HPTLC; 20-ml fractions were collected, and 0.1-ml aliquots were spotted after drying and redissolving in 20 μl of chloroform–methanol (2:1). St = Standard ganglioside mixture.

programme. The UV absorbance responses of equimolar amounts of different gangliosides increased with increasing sugar content, and in particular with an increase in the number of sialic acid residues. The response was linear with ganglioside content up to 20 nmol (Fig. 3): at this value the resolving power of the column is still adequate. The lowest amount of each ganglioside considered suitable for quantification is deemed to be that corresponding to an electrical impulse at the recorder of six-fold over the instrumental noise at 0.005 a.u.f.s.; this yields a peak area of *ca.* 30 mm^2. As indicated in Fig. 3, this limit corresponds to 0.26 nmol for GM3, 0.08 nmol for GQ1b ganglioside, and intermediate values for the other gangliosides used. Standard deviation values were less than 1% for ganglioside amounts between 1 and 20 nmol, less than 3% between 0.5 and 1 nmol, and still acceptably low ($\pm$ 5% of mean value) for ganglioside amounts less than 0.5 nmol. The relative molar responses (RMR) of the gangliosides analysed, referred to GD1a as reference standard, are listed in Table I.

The calf brain ganglioside mixture, known to contain four major gangliosides, GD1a, GT1b, GM1 and GD1b, which contain 38%, 16%, 13.5% and 9.4%, respectively[20], of the total brain sialic acid, and a number of minor species, was submitted to analytical (Fig. 2) and preparative (Fig. 4) HPLC separation, according to

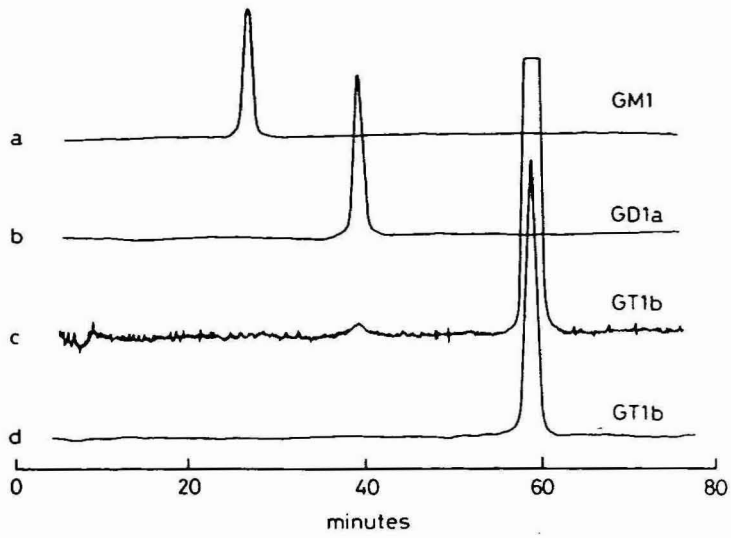

Fig. 5. Application of the HPLC analytical method to verification of the homogeneity of GT1b ganglioside purified by preparative HPLC, from a calf brain ganglioside mixture: (a) 10 nmol of standard GM1, range 0.025 a.u.f.s.; (b) 10 nmol of standard GD1a, range 0.025 a.u.f.s.; (c) 20 nmol of purified GT1b, range 0.05 a.u.f.s.; (d) 20 nmol of purified GT1b, range 0.005 a.u.f.s.

the presented method. Under preparative conditions, up to 5 mg of ganglioside mixture (as sialic acid) could be injected, producing an excellent peak resolution, with practically no overlap, as demonstrated by the TLC column monitoring (Fig. 4). If the injected amount was larger, the resolving power of the column decreased and some cross-contamination occurred.

The purity of each ganglioside separated by the analytical HPLC procedure described was at least 99%, with respect to other contaminant gangliosides. Fig. 5 shows the HPLC analysis of GT1b purified from the calf brain ganglioside mixture: 20 nmol of GT1b were injected and the elution was recorded at two different a.u.f.s. values, in order to detect very minor contaminants. In this particular case, the GT1b ganglioside was more than 99.5% pure.

CONCLUSION

The described procedure fulfills all the requirements of a method suitable for both quantification and preparation of pure ganglioside, starting from complex mixtures. It gives highly reproducible results (1–2% S.D. in retention times), even after hundreds of injections, because the solvent gradient programme is accurately computer-controlled.

The linearity range of the UV responses, and the low calculated standard deviations for RMR values, make this method reliable for accurate quantification of single gangliosides; in addition, the sensitivity of this method is much higher than that provided by conventional colorimetric procedures.

Finally, the ability to detect and quantify, in a precise way, ganglioside contaminants as minor as 0.1% of the total amount injected, makes this procedure very

useful in purity analysis. Adaptations of the method to the resolution of complex ganglioside mixtures extracted from extranervous tissues and body fluids, and from brains of non-mammalian species, are already in progress in our laboratory.

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STUDIES ON RESIDUAL ANTIBACTERIALS IN FOODS

IV*. SIMULTANEOUS DETERMINATION OF PENICILLIN G, PENICILLIN V AND AMPICILLIN IN MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and simple method for the simultaneous determination of penicillin G (PCG), penicillin V (PCV) and ampicillin (ABPC) in milk is described. The retention behaviour of these β-lactam antibiotics in reversed-phase liquid chromatography with mobile phases containing sodium alkylsulphonate was studied. Good separations were obtained with methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 11 m*M* sodium 1-heptanesulphonate and a LiChrosorb RP-18 column. The sample was pre-treated with a Sep-Pak C_{18} cartridge. The peaks corresponding to each β-lactam antibiotics can be confirmed with the treatment using penicillinase.

The recoveries from milk fortified with sodium PCG, potassium PCV and ABCP at levels of 0.5 and 0.1 μg/g each were generally better than 87% and the relative standard deviations were 1.17–4.98%. The detection limits corresponded to 0.03 μg/g of these β-lactam antibiotics in milk.

INTRODUCTION

β-Lactam antibiotics continue to be used in both human and veterinary medicine. In modern agricultural practice, however, frequent utilization of these antibiotics has led to problems with the spread of resistance factors and environmental pollution, and a simple, sensitive and selective method for the determination of residual β-lactam antibiotics in livestock products is therefore required.

Microbiological assays are mainly used for the determination of residual β-lactam antibiotics in foods as they are very sensitive. However, these methods require a long period of incubation, lack specificity and are difficult to quantify accurately.

Although numerous chemical methods[1–6] are available for the determination of β-lactam antibiotics, most of them are inadequate for determining trace levels in

* For Part III, see ref. 17.

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livestock products because they were developed for clinical applications. The detection of β-lactam antibiotics at residue levels requires much higher sensitivity and selectivity.

Recently, high-performance liquid chromatographic (HPLC) techniques for the analysis of β-lactam antibiotics have been developed[7–14] and applied also to the determination of residual antibiotics in foods[15–17]. In previous work, we established an HPLC method[17] for the determination of residual penicillin G (PCG) in animal tissues using an on-line concentration and purification system and successfully applied it to analyses of cattle liver, kidney and muscle. This method, however, is inapplicable to the simultaneous determination of other kinds of β-lactam antibiotics such as ampicillin (ABPC).

The purpose of this work was to investigate the retention behaviour of penicillin V (PCV), PCG and ABPC in a reversed-phase ion-pair HPLC system and to establish a rapid, sensitive and selective method for the simultaneous determination of these β-lactam antibiotics in milk.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of a Jasco (Tokyo, Japan) Uniflow 211 pump, a VL 611 variable-loop injector with a 100-μl sample loop, a Uvidec 100 II UV detector operating at 210 nm and a Nippon Denshi Kogaku (Kyoto, Japan) U-125M recorder.

Separations were carried out by using a stainless-steel column (15 cm × 4.3 m I.D.) (Umetani, Osaka, Japan) packed by the balanced slurry technique with LiChrosorb RP-18 (5 μm) (E. Merck, Darmstadt, F.R.G.). The column was encased in an acrylic jacket connected to a Yamato (Tokyo, Japan) BT-35 circulating waterbath to maintain the temperature at 45°C.

A 5 cm × 2.1 mm I.D. guard column was fitted in front of the analytical column and was tap-packed with Permaphase ETH (DuPont, Wilmington, DE, U.S.A.).

Reagents

Sodium PCG (1650 U/mg), potassium PCV (1560 U/mg) and ABPC were obtained from Sigma (St. Louis, MO, U.S.A.), penicilinase from Calbiochem (San Diego, CA, U.S.A.), sodium alkylsulphonates from Tokyo Kasei (Tokyo, Japan), 18-crown-6 ether from E. Merck and methanol (HPLC grade) from Wako (Osaka, Japan). A Sep-Pak C_{18} cartridge was purchased from Waters Assoc. (Milford, MA, U.S.A.).

The phosphate buffer was prepared from 0.2 *M* potassium dihydrogen phosphate by titration to the required pH with 0.2 *M* phosphoric acid or 0.2 *M* sodium monohydrogen phosphate.

All the water used was purified with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Chromatographic procedure

Mobile phases were prepared immediately before use by dissolving the calcu-

lated amount of sodium alkylsulphonate in methanol–water–0.2 *M* phosphate buffer. The operating conditions are given in the figure captions.

Sample preparation

A Sep-Pak C_{18} cartridge was attached to a 20-ml glass syringe and pre-conditioned with 20 ml of methanol, 20 ml of water and 2 ml of 2% sodium chloride solution prior to use.

A milk sample was filtered through a glass-wool plug, then *ca.* 30 g of it was weighed accurately in a 50 ml beaker and poured into the Sep-Pak C_{18} cartridge at a rate of 2 ml/min. After all of the sample had been added to the cartridge, the beaker was rinsed with 10 ml of water. The rinsing solution was poured into the cartridge at a similar rate to above, then the cartridge was washed with 5 ml of water and 10 ml of methanol–water–20% sodium chloride solution (1:8:1) containing 20 m*M* 18-crown-6 ether. The cartridge was then attached to another glass syringe and the β-lactam antibiotics were eluted with 10 ml of 15% (v/v) methanol. Aliquots (100 μl) of the eluate were subjected to HPLC.

Quantitation and confirmation

Quantitation was carried out using calibration graphs obtained from a standard solution containing 15% (v/v) of methanol.

When the peaks coinciding with PCG, PCV and ABPC appeared on the chromatogram, a confirmation test using penicillinase was carried out in the following manner. An aqueous solution of penicillinase (1000 U/ml, 0.2 ml) was added to 5 ml of eluate from a Sep-Pak C_{18} cartridge and the mixture allowed to stand at room temperature for at least 5 min. Then the solution (100 μl) was subjected to HPLC again and the disappearance of each peak on the chromatogram was confirmed.

RESULT AND DISCUSSION

For chromatographic separations of β-lactam antibiotics, ion-exchange or reversed-phase chromatography are usually chosen. However, ion-exchange chromatography provides separations of low efficiency and reversed-phase chromatography is unsuitable for monobasic penicillins and more polar amphoteric penicillins simultaneously. Recently, ion-pair reagents and crown ethers have been employed to increase the retention time of polar β-lactam antibiotics.

Fig. 1 shows the relationship between the capacity factor (k') of PCG, PCV and ABPC and carbon number of sodium alkylsulphonate added to the mobile phase as an ion-pair reagent, and Fig. 2 shows the effect of the concentration of sodium 1-heptanesulphonate added as an ion-pair reagent on the k' of PCG, PCV and ABPC. The k' of ABPC increased with increasing carbon number and/or concentration of ion-pair reagent, whereas the k' of PCG and PCV decreased. These results suggest that alkylsulphonates exert opposite effects on the retention of β-lactam antibiotics, giving an increasing and a decreasing effect on k'. The former effect predominates in the retention of ABPC by ion-pair formation by the amino group of ABPC and the alkylsulphonates. The latter effect is observed in the retention of PCG and PCV, which have no amino groups in the structures, owing to competition between the β-lactam antibiotics and the alkylsulphonates in binding to the stationary phase.

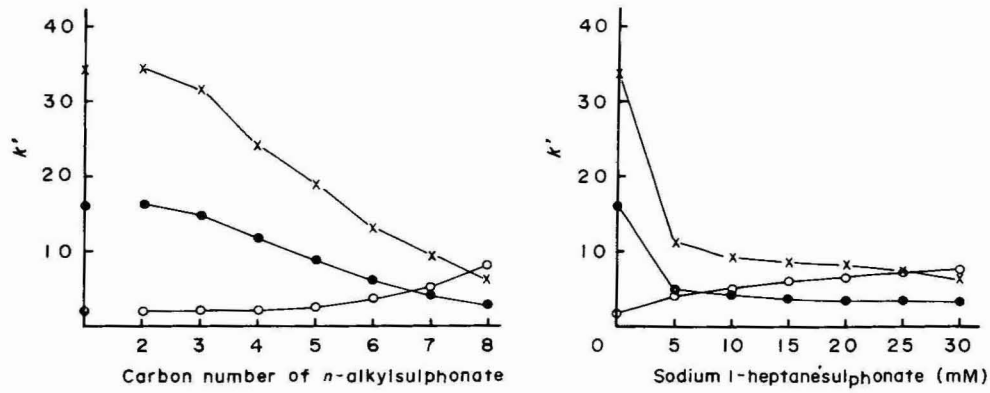


Fig. 1. Relationship between the k' of PCG, PCV and ABPC and carbon number of the sodium alkylsulphonate added to the mobile phase. Column, LiChrosorb RP-18 (5 μm) (15 cm × 4.3 mm I.D.); mobile phase, methanol–water–0.2 M phosphate buffer (pH 4.0) (5:13:2) containing 10 mM sodium alkylsulphonate; flow-rate, 1.0 ml/min; column temperature, 45°C; detection, UV (210 nm). ●, PCG; ○, ABPC; ×, PCV.

Fig. 2. Effect of the concentration of sodium 1-heptanesulphonate on the k' of PCG, PCV and ABPC. Mobile phase, methanol–water–0.2 M phosphate buffer (pH 4.0) (5:13:2) containing 0–30 mM sodium 1-heptanesulphonate. Other conditions as in Fig. 1.

Fig. 3 shows the effect of the pH of the phosphate buffer added to the mobile phase on the k' of PCG, PCV and ABPC. The k' of ABPC decreased with increasing pH, whereas at pH below 5 the k' of PCG and PCV decreased sharply with increasing the pH but at pH above 5 k' increased slightly, with a maximum at pH 7.

Fig. 4 shows the effect of the concentration of phosphate buffer (pH 4.0) added to the mobile phase on the k' of PCG, PCV and ABPC. The k' of PCG and PCV

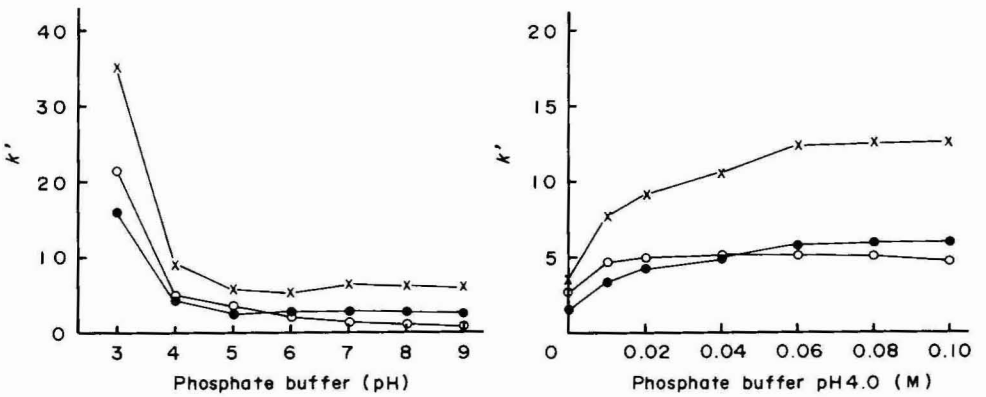
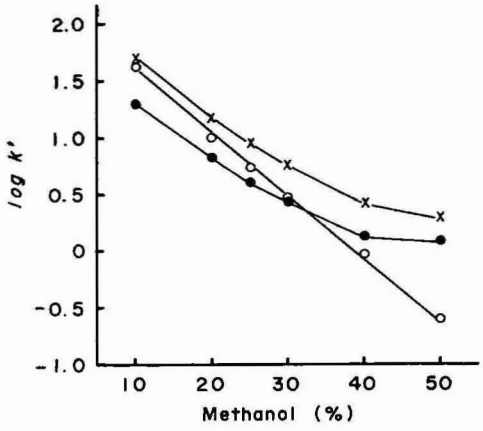


Fig. 3. Effect of the pH of the phosphate buffer added to the mobile phase on the k' of PCG, PCV and ABPC. Mobile phase, methanol–water–0.2 M phosphate buffer (pH 3.0–9.0) (5:13:2) containing 10 mM sodium 1-heptanesulfonate. Other conditions as in Fig. 1.

Fig. 4. Effect of the concentration of phosphate buffer (pH 4.0) on the k' of PCG, PCV and ABPC. Mobile phase, methanol (25%, v/v)–water–phosphate buffer (pH 4.0) containing 10 mM sodium 1-heptanesulphonate. Other conditions as in Fig. 1.



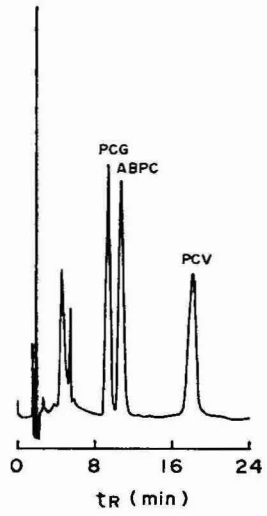
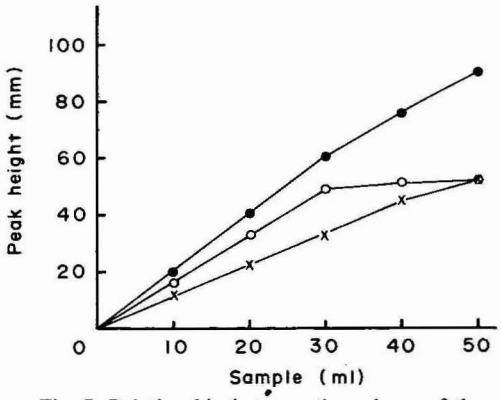


Fig. 5. Effect of the concentration of methanol on the k' of PCG, PCV and ABPC. Mobile phase, methanol–water containing 0.02 *M* phosphate buffer (pH 4.0) and 10 m*M* sodium 1-heptanesulphonate. Other conditions as in Fig. 1.

Fig. 6. Typical liquid chromatogram of PCG, PCV and ABPC. Mobile phase, methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 11 m*M* sodium 1-heptanesulphonate. Other conditions as in Fig. 1.

increased with increasing concentration, whereas the k' of amphoteric ABPC decreased slightly with increasing the concentration above 0.02 *M*.

Fig. 5 shows the effect of the concentration of methanol on the log k' of PCG, PCV and ABPC. Increasing methanol concentration caused them to elute earlier, but amphoteric ABPC was more affected than PCG and PCV and an approximately linear relationship existed between the log k' of ABPC and the methanol concentration.



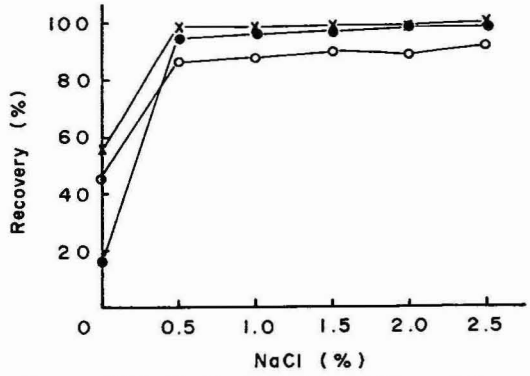


Fig. 7. Relationship between the volume of the sample poured into the Sep-Pak C_{18} cartridge and the peak height.

Fig. 8. Effect of sodium chloride concentration in the rinsing solution [containing 20 m*M* 18-crown-6 ether and 10% (v/v) methanol] for the Sep-Pak C_{18} cartridge on the recoveries of PCG, PCV and ABPC.

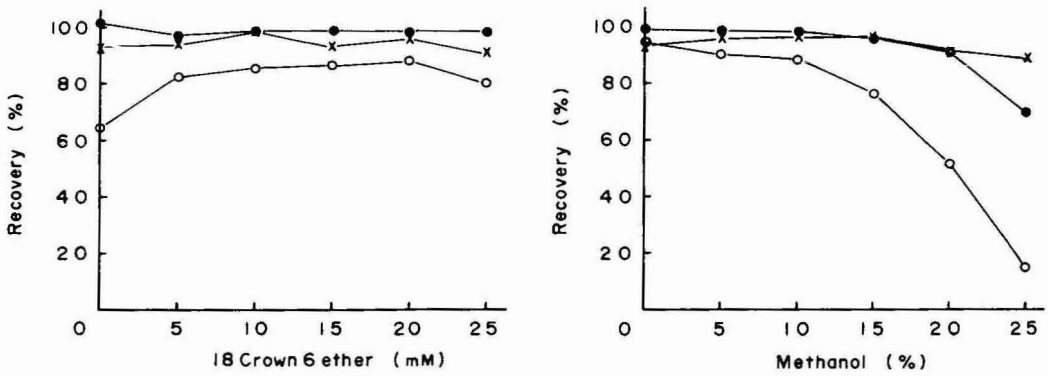


Fig. 9. Effect of 18-crown-6 ether concentration in the rinsing solution [containing 2% (w/v) sodium chloride and 10% (v/v) methanol] for the Sep-Pak C_{18} cartridge on the recoveries of PCG, PCV and ABPC.

Fig. 10. Effect of methanol concentration in the rinsing solution [containing 20 m*M* 18-crown-6 ether and 2% (w/v) sodium chloride] for the Sep-Pak C_{18} cartridge on the recoveries of PCG, PCV and ABPC.

On the basis of these results, methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 11 m*M* sodium 1-heptanesulphonate was chosen as the mobile phase. Fig. 6 shows the typical chromatogram obtained from a standard mixture. A good separation was attained in only 20 min, even without gradient elution.

The use of a Sep-Pak C_{18} cartridge for the pre-treatment led to a rapid and effective sample preparation. PCG, PCV and ABPC were absorbed in the cartridge from a milk sample injected directly. Fig. 7 shows the relationship between the volume of sample poured into the Sep-Pak C_{18} cartridge and the peak height obtained with the overall procedure. It indicates that the volume of sample should be less than 30 ml to ensure stable recoveries of PCG and ABPC. Consequently, 30-ml volumes (about 30 g) of sample were used.

Optimal clean up conditions for the Sep-Pak C_{18} cartridge could be chosen by reference to the investigation of chromatographic behaviour in HPLC, because the packing material of the Sep-Pak C_{18} cartridge had similar properties to those of the analytical column.

Figs. 8–10 show the effect of sodium chloride, 18-crown-6 ether and methanol concentration, respectively, in the rinsing solution for a Sep-Pak C_{18} cartridge on the recoveries of the β-lactam antibiotics. PCG and PCV showed good recoveries independent of the concentration of 18-crown-6 ether when the sodium chloride concentration was above 0.5%, but the recovery of ABPC fell to 64% when 18-crown-6 ether was not added to the rinsing solution. Nakagawa *et al.*[18] reported that the addition of crown ethers to the mobile phase in reversed-phase HPLC enhanced the k' of β-lactam antibiotics that had primary amino groups in the structure. Therefore, the absorption efficiency with respect to ABPC was enhanced by the addition of 18-crown-6 ether.

The background readings on the chromatogram decreased with increasing methanol content in the rinsing solution, but above 15% for ABPC and 20% for PCV the peak height decreased sharply. On the basis of these results, methanol–

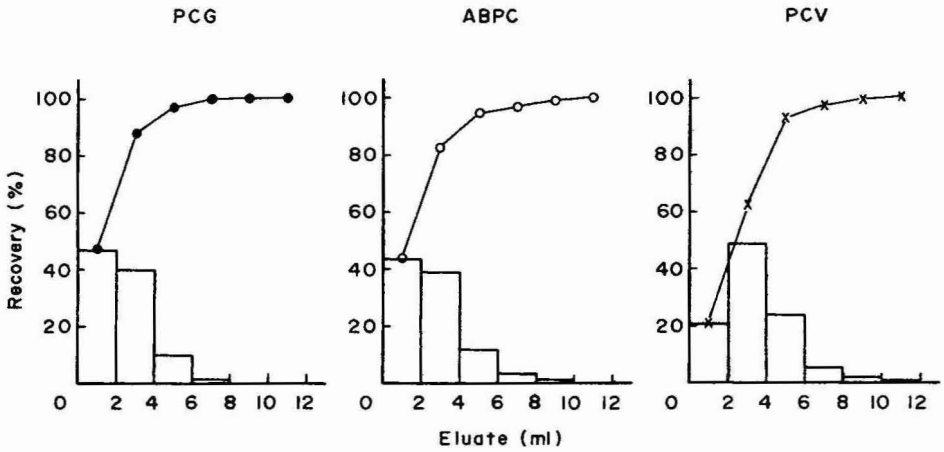


Fig. 11. Elution pattern of PCG, PCV and ABPC from the Sep-Pak C_{18} cartridge.

water–20% sodium chloride solution (1:8:1) containing 20 m*M* 18-crown-6 ether was used to rinse the Sep-Pak C_{18} cartridge.

Fig. 11 shows the elution pattern of the β-lactam antibiotics from a Sep-Pak C_{18} cartridge. Most part of the PCG, PCV and ABPC were eluted with 10 ml of 15% (v/v) methanol.

In a recovery test, the proposed method was applied to milk samples spiked with sodium PCG, potassium PCV and ABPC at levels of 0.5 and 0.1 μg/g. The reproducibility was determined by carrying out five identical analyses. The results are summarized in Table I.

TABLE I

RECOVERIES OF PCG, PCV AND ABPC IN MILK

Added (μg/g)	*PCG*		*PCV*		*ABPC*	
	Av. (%)*	*CV** (%)*	*Av.* (%)*	*CV** (%)*	*Av.* (%)*	*CV** (%)*
0.5	98.4	4.34	95.9	3.41	88.0	3.59
0.1	101.1	1.17	97.7	3.17	87.0	4.98

* Average of five determinations.
** Coefficient of variation.

Fig. 12 shows the chromatograms obtained in the recovery experiment (a) and from the sample solution treated with penicillinase (b). The peaks corresponding to PCG, PCV and ABPC disappeared. The detection limit of this method was 0.03 μg/g for each β-lactam antibiotic.

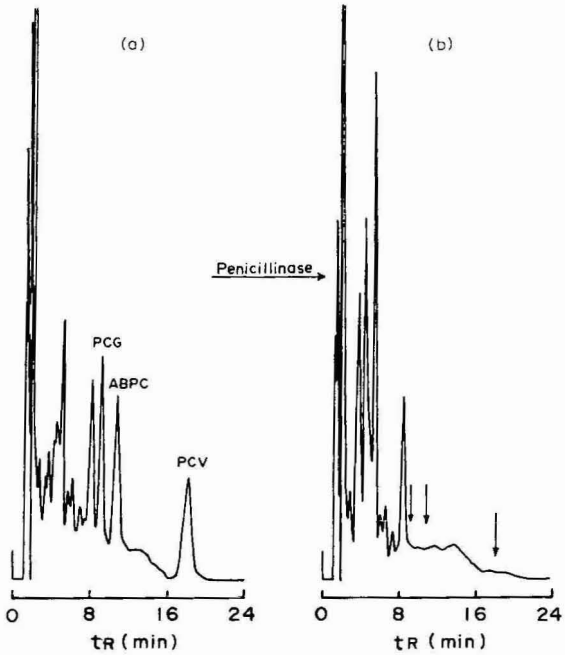


Fig. 12. Liquid chromatograms obtained from a milk sample by the overall procedure (a) and the sample solution treated with penicillinase (b). Sample spiked with 0.5 μg/g each of sodium PCG, potassium PCV and ABPC. Operating conditions as in Fig. 6.

CONCLUSION

A method for the HPLC determination of PCG, PCV and ABPC has been developed that has several advantages. The addition of sodium 1-heptanesulphonate to the mobile phase gives good separations, as PCG, PCV and ABPC can be completely separated in only 20 min without gradient elution. The use of a Sep-Pak C_{18} cartridge makes it possible to pre-treat the sample rapidly. It is applicable to unstable substances such as β-lactam antibiotics, which are liable to degrade on extraction and evaporation. The peaks corresponding to each β-lactam antibiotics are easily confirmed by treatment with penicillinase.

The proposed method, therefore, should be applicable to routine determinations of residues of β-lactam antibiotics in milk.

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REVERSED-PHASE ION-INTERACTION CHROMATOGRAPHY OF SODIUM DIALKYLDITHIOCARBAMATES IN THE PRESENCE OF TETRAALKYLAMMONIUM SALTS

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SUMMARY

The elution behaviour of sodium dialkyldithiocarbamates (R_2NCS_2Na: $R=CH_3$, C_2H_5, n-C_3H_7 and n-C_4H_9) was examined by high-performance liquid chromatography on C_1-, C_8- and C_{18}-bonded silica columns in the presence of tetraalkylammonium salts. The best separation of the carbamates was obtained from the C_8-bonded column, with a mobile phase of methanol–1/15 *M* phosphate buffer (60:40) at pH 6.8 containing tetra-*n*-butylammonium bromide (0.025 *M*). This method was applied to the analyses of commercial R_2NCS_2Na. The results are in good agreement with those obtained by potentiometric titration.

INTRODUCTION

Dithiocarbamate salts are widely used as colouring or extracting agents or titrants for metal ions and also as pesticides or vulcanization accelerators. Many methods have been reported for the determination of R_2NCS_2Na[1–6], but they are all specific only for the dithiocarbamate group and cannot simultaneously determine several R_2NCS_2Na salts with different alkyl substituents. The most suitable technique for their determination is high-performance liquid chromatography (HPLC).

In recent years, HPLC has been successfully applied to the separation of various dialkyldithiocarbamate–metal complexes in both normal-phase[7–9] and reversed-phase[10–12] modes for the simultaneous analysis of metals. On the other hand, Smith *et al.*[13] reported the determination of R_2NCS_2Na by HPLC on a C_{18}-bonded silica column using transition-metal salts as ion-pairing reagents. The injection of more than two kinds of carbamate brought about the complicated interpretation of the results, because of the formation of mixed complexes. Kirkbright and Mullins[14] used cetyltrimethylammonium bromide (CTAB) above its critical micelle concentration as

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the surfactant in the mobile phase to achieve the micellar HPLC separation of five dithiocarbamates on a CN-bonded silica column. It is assumed in micellar chromatography that if a solute partitions to the micelle in the mobile phase, then its retention decreases with an increase of the micelle concentration. This is true for the above-mentioned separation: the retention of the carbamates decreased as the CTAB concentration increased.

We tried to determine R_2NCS_2Na directly or after esterification by HPLC with a column packed with hydrophobic styrene–divinylbenzene porous polymer beads, but could not achieve satisfactory precision. However, the aplication of a hydrophilic porous polymer column, together with tetraalkylammonium salts as ion-interaction reagents, gave satisfactory separation and quantification of four R_2NCS_2Na by HPLC[15].

In this paper we describe the determination of R_2NCS_2Na by HPLC on a C_1-, C_8- or C_{18}-bonded silica column in the presence of tetraalkylammonium salts. This method was applied to the analysis of commercially available R_2NCS_2Na. The results indicate that the method is more precise than the previous one using the hydrophilic porous polymer column.

EXPERIMENTAL

Materials

The four kinds of R_2NCS_2Na ($R = CH_3$, C_2H_5, n-C_3H_7 and n-C_4H_9) used in this study were obtained from Ohuchi Shinko (Tokyo, Japan) and are denoted by Me_2NCS_2Na, Et_2NCS_2Na, Pr_2NCS_2Na and Bu_2NCS_2Na, respectively. These were purified twice by recrystallization from ethanol. Methanol and deionized water were distilled, and all other chemicals from Wako (Osaka, Japan) were of analytical reagent grade.

Apparatus

A Shimadzu LC-4A system (Kyoto, Japan) was used for HPLC. The eluates were detected with a SPD-2AS UV detector at 254 nm. The C_1-bonded silica column and the C_8- and C_{18}-bonded ones (particle size 5 μm, 150 × 4.6 mm I.D.) were obtained from Chemco (Osaka, Japan) and Gasukuro Kogyo (Tokyo, Japan), respectively. The column temperature was kept at 25°C, and the flow-rate of the eluents was 1.0 ml/min. All the eluents were filtered through a 0.45-μm membrane filter (Millipore) prior to use. A 10-μl portion of sample solutions was injected. The retention times and peak areas were measured with a Shimadzu C-R1A computer integrator.

A Kyoto Denshi AT-02 instrument (Kyoto, Japan) was used for potentiometric titration.

RESULTS AND DISCUSSION

Effects of eluent composition and tetraalkylammonium salts

First, the retention of the four R_2NCS_2Na salts was examined by changing the ratio of methanol to 1/15 *M* phosphate buffer (pH 6.8) in the eluent in the absence of tetraalkylammonium salts. Each R_2NCS_2Na was retained strongly on the C_1, C_8

and C_{18} stationary phases and could not be eluted, so the retention was reduced by the addition of tetramethyl- or tetrabutylammonium bromide (TMAB or TBAB, respectively) or CTAB to the eluents. At a constant concentration of the tetraalkylammonium salt, the retention and separation of all four R_2NCS_2Na salts increased as the methanol content in the eluent was decreased: a large decrease led to considerable peak tailing. The retention of each R_2NCS_2Na also varied with both the stationary phase and the tetraalkylammonium salt.

Optimum separation conditions

In order to obtain the optimum separation of the four R_2NCS_2Na salts, the effect of the methanol content in the eluent on the retention and on the peak shape was investigated for each of the nine combinations stationary phase and tetraalkylammonium salt. The concentration of the latter was kept constant at 0.025 *M*.

Fig. 1 shows the results for the C_{18} stationary phase, which is used widely in the reversed-phase mode. The optimum eluent compositions, chosen with regard to both the separation and the retention times of R_2NCS_2Na, are marked with arrows. The liquid chromatograms obtained in such optimum eluents are depicted in Fig. 2. It is apparent that the four peaks are well separated in the presence of TMAB, TBAB or CTAB. However, the considerable peak tailing is observed with both TMAB and TBAB.

The equivalent results for the C_1 stationary phase are shown in Figs. 3 and 4. The peak tailing is much less marked than with the C_{18} phase, but the separation of Me_2NCS_2Na and Et_2NCS_2Na is poor. The four solutes cannot be separated at all in the presence of TMAB.

As shown in Figs. 5 and 6, the C_8 stationary phase gives sharp, well separated peaks of the four R_2NCS_2Na. In particular, the addition of TBAB to the eluent of containing 60% methanol results in a complete separation within 10 min (Fig. 6b).

The effect of TBAB concentration on the retention was examined over a range

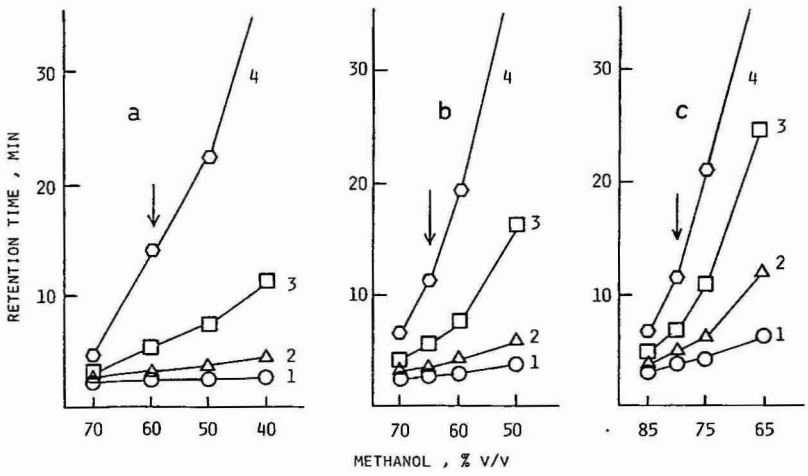

Fig. 1. Effect of methanol content in the eluent methanol–1/15 *M* phosphate buffer (pH 6.8) on the retention of R_2NCS_2Na on C_{18}-bonded silica in the presence of (a) TMAB, (b) TBAB and (c) CTAB. Carbamates: 1 = Me_2NCS_2Na; 2 = Et_2NCS_2Na; 3 = Pr_2NCS_2Na; 4 = Bu_2NCS_2Na.

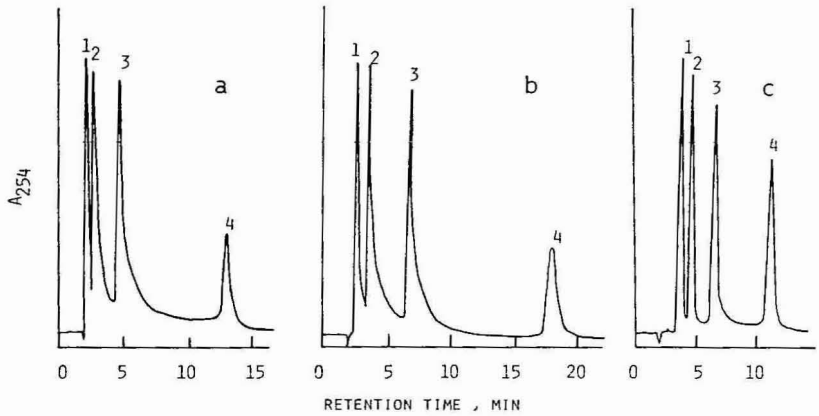

Fig. 2. Separation of R_2NCS_2Na in the eluents marked with arrows in Fig. 1. Other details as in Fig. 1.

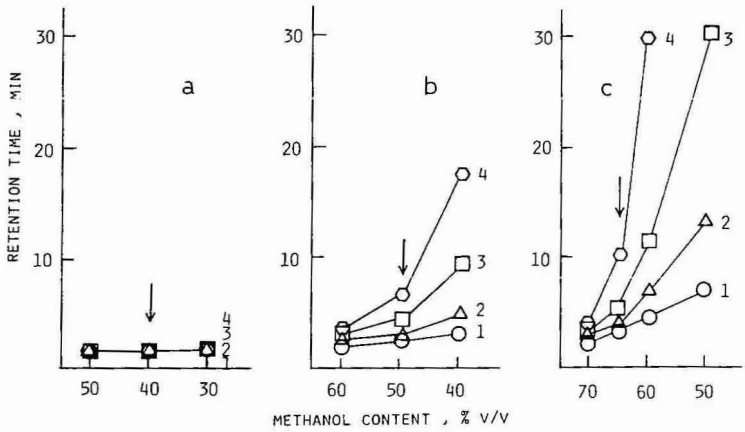

Fig. 3. Effect of methanol content in the eluent on the retention of R_2NCS_2Na on C_1-bonded silica. Other details as in Fig. 1.

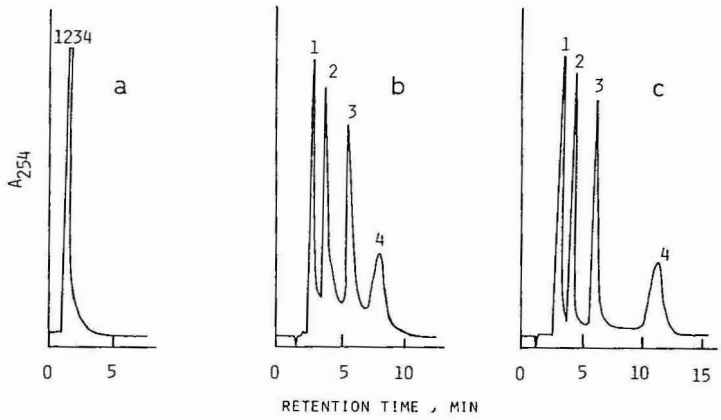

Fig. 4. Separation of R_2NCS_2Na in the eluents marked with arrows in Fig. 3. Other details as in Fig. 3.

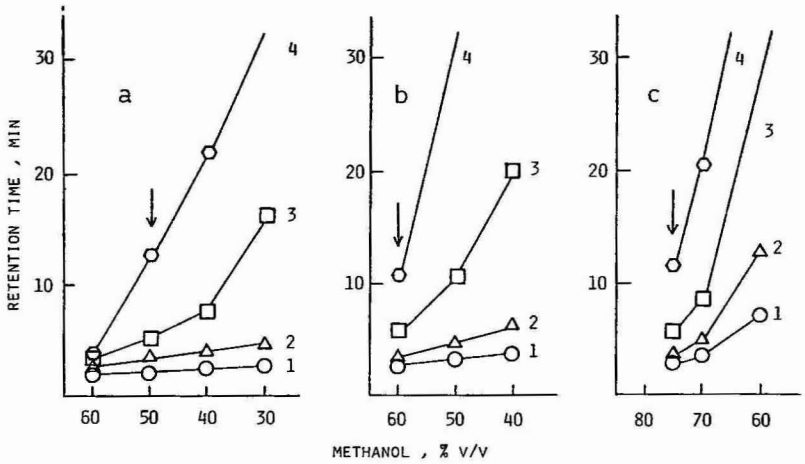

Fig. 5. Effect of methanol content in the eluent on the retention of R_2NCS_2Na on C_8-bonded silica. Other details as in Fig. 1.

of 0.001–0.1 *M*. A linear relationship was obtained between log (capacity factor) and log (TBAB concentration), as shown in Fig. 7. A decrease in the retention of R_2NCS_2Na is observed as the concentration of TBAB increases. This suggests an important role for micelles in this system, as previously reported for CTAB[14]. The optimum separation was obtained by eluting with 60:40 methanol–1/15 *M* phosphate buffer (pH 6.8) containing 0.025 *M* TBAB. Under these conditions, the retention times of the solutes are: Me_2NCS_2Na, 2.42 min; ET_2NCS_2Na, 3.00 min; Pr_2NCS_2Na 4.70 min and Bu_2NCS_2Na 9.84 min (see Fig. 6b).

Analytical calibration and application

To evaluate the quantitative applicability of the method, standard reference samples containing each R_2NCS_2Na over the concentration range 0.1–1.0 mg/ml were determined, and each calibration curve was constructed by plotting the peak

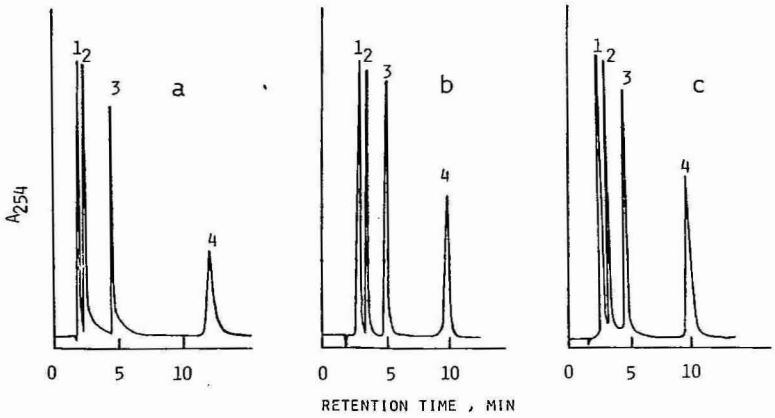

Fig. 6. Separation of R_2NCS_2Na in the eluents marked with arrows in Fig. 5. Other details as in Fig. 5.

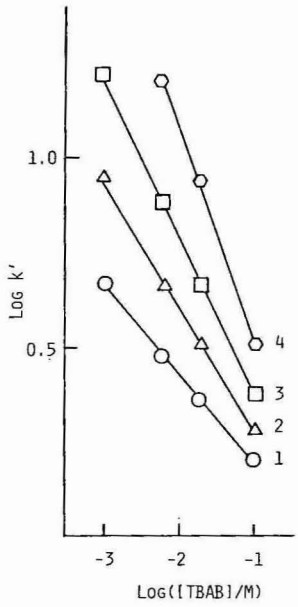

Fig. 7. Variation of log capacity factor (k') with log concentration of TBAB. Numbers as in Fig. 1.

TABLE I

REGRESSION ANALYSIS

Y = concentration of R_2NCS_2Na (10^{-6} M); X = peak area.

Sample	*Regression equation*	*Correlation coefficient*
Me_2NCS_2Na	$Y = 1.6489X + 0.0034$	0.999
Et_2NCS_2Na	$Y = 1.9061X - 0.0021$	1.000
Pr_2NCS_2Na	$Y = 2.3502X + 0.0069$	0.998
Bu_2NCS_2Na	$Y = 2.9426X - 0.0072$	0.998

area against the concentration of R_2NCS_2Na. Table I gives the results for these regression analyses and indicates good applicability of the method to the determination of R_2NCS_2Na.

The proposed HPLC method was applied to the analysis of commercial

TABLE II

COMPARISON STUDY OF THE ANALYSIS OF COMMERCIAL R_2NCS_2Na

Sample	*This method*		*Potentiometric method*		*G-320 method*	
	Purity (%)	*c.v.*	*Purity (%)*	*c.v.*	*Purity (%)*	*c.v.*
Me_2NCS_2Na	78.72	0.32	78.71	0.27	78.69	1.34
Et_2NCS_2Na	75.88	0.15	75.89	0.24	75.94	1.15
Bu_2NCS_2Na	51.20	0.87	51.22	0.95	51.43	1.87

R_2NCS_2Na, and the results obtained were compared with those determined by both the potentiometric method[16] and the G-320 method[15] using hydrophilic porous polymer beads (cross-linked poly(vinyl alcohol); Asahipack G-320 from Asahi Kasei, Tokyo, Japan) as column packing with methanol–water (30:70) containing TMAB (0.10 *M*) as eluent. As shown in Table II, the purities for each sample determined by the three methods are in good agreement. The coefficients of variation (C.V.) for this method are comparable with those for the potentiometric method and smaller than those for the G-320 method.

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LIQUID CHROMATOGRAPHY WITH VOLTAMMETRIC DETECTION FOR QUANTITATION OF PHENOLIC ACIDS

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SUMMARY

High-performance liquid chromatography with voltammetric detection was used to separate and quantitate a mixture of ten phenolic acids. Resolution from both the column and detector was necessary in order to achieve baseline separation of the mixture. The voltammetric detector yielded two-fold better precision than for amperometric detection of the same compounds, due to an inherent discrimination against electrode passivation. In addition, for all of the compounds studied, a significant improvement in precision was obtained when the difference in response at two separate potentials, was used for quantitation.

The best behavior was observed for 3,4-dihydroxycinnamic acid which exhibited a detection limit of 20 pmol. The detection limits for the other compounds were within a ten-fold range of this value.

INTRODUCTION

Within the past decade the popularity of electrochemical detectors has increased rapidly due to their high sensitivity and their increased selectivity within the voltage domain. However, a major limitation exists for quantitative analysis of species that are not chromatographically separable and/or where electroactive impurities are convoluted within the analytical peak. While it is possible to optimize the chromatographic conditions for a given sample, the same set of chromatographic conditions are often unacceptable for a different sample type.

Recently this problem has been addressed by using voltammetric detection where the potential domain was used successfully to resolve an overlapped chromatogram[1]. In this study, we present the results for various phenolic acids and their derivatives to further demonstrate the utility of voltammetric detectors.

Phenolic compounds are widely distributed in plants and animals. Phenolic acids are known for their hormonal activity in plant growth and their stimulatory or inhibitory activity (depending on the concentration) in biological tissue[2]. Various chromatographic procedures have been developed for identification of these compounds, including gas chromatography[3] and high-performance liquid chromato-

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graphy (HPLC)[4,5]. In contrast, quantitative analysis of these compounds has not been developed due to poor chromatographic separation and the absence of a selective detector. In most cases only an estimate of their concentration is reported[3–6].

Most phenolic compounds are electroactive and can be oxidized at a relatively low potential[7,8]. Those compounds with oxidation potentials 100–200 mV apart can be resolved by a voltammetric detector even if they are not separated by the chromatographic column.

EXPERIMENTAL SECTION

HPLC apparatus

Chromatography was carried out with a Model 8800 gradient liquid chromatographic system (DuPont Analytical Instrument Division, Wilmington, DE, U.S.A.). Sample was injected via a Micromeritics Model 725 automatic injector equipped with a 20-μl sample loop (Micromeritics, Norcross, GA, U.S.A.). Separations were achieved on a 250 × 4.6 mm I.D. DuPont Zorbax ODS reversed-phase column (5 μm porous support particles) or a 300 × 4.6 mm I.D. μBondapak C_{18} reversed-phase column (10 μm porous support particles, Waters Assoc.). The column effluent was monitored simultaneously with two detectors in series. The first detector was a Milton Roy variable-wavelength Spectromonitor D with a wavelength range of 190 to 700 nm (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). In addition to monitoring the chromatograms for any non-electroactive components which might be present, this detector provided a means to assess any band broadening caused by the second detector. The second detector was a coulostatic electrochemical detector equipped with a glassy carbon electrode. Technical details of this instrument have been reported elsewhere[9]. With this instrument, it is possible to scan the applied potential at a rate of 3 V/s, while recording up to 15 channels of chromatographic data, each corresponding to a different applied potential. This is analogous to scanning the wavelength and recording spectra with a diode array detector.

A microVAX (Digital Equipment) computer with one megabyte of memory was used to control the measurement process and record the data. The large memory was necessary in order to store the vast quantity of data generated by multiple-channel chromatograms.

Chemicals and reagents

Phenolic compounds were purchased from Aldrich, and from Sigma (4,8-dihydroxyquinoline-2-carboxylic acid and 1,3,5-trihydroxybenzol). All reagents were analytical reagent grade.

The mobile phase was methanol–acetate buffer (10:90, v/v), from which dissolved oxygen was removed by saturation with nitrogen. The acetate buffer was prepared by adjusting a 0.036 *M* ammonium acetate solution to pH 4.0 with 3 *M* acetic acid.

Standard samples were prepared by dissolving the appropriate amount of phenolic compounds in the mobile phase. The grape juice samples were diluted ten-fold prior to injection. Samples were filtered through Sep-Pak C_{18} cartridges (Waters Assoc.) before injection into the chromatograph.

RESULTS AND DISCUSSION

Table I lists the relative peak area as a function of applied potential for a series of phenolic compounds. These data were obtained from single component injections of each analyte. The detector was operated in an identical manner for all samples; a series of 50 mV steps was applied, and the response was recorded at each potential. In this manner, several parallel data channels were obtained for each chromatographic run. For any given compound, the chromatographic peak was integrated over the same time interval for each channel. Each value reported is the average of four identical samples.

The values listed for each compound in Table I represent points along the hydrodynamic voltammogram. Therefore, the table can be used to select the appropriate voltage for quantitation of each compound in addition to showing the relative responses. The table also indicates the degree of voltammetric resolution which can be attained between the various compounds.

The behavior observed in Table I for 4-hydroxybenzoic acid is unusual. The response initially increases rapidly to a plateau, then decreases severely before returning to a significant level, and eventually going off-scale as the solvent begins to oxidize. No satisfactory explanation for this behavior can be given at this time; however, it can not be attributed to instrumental artifact because the compound exhibits similar behavior when subjected to cyclic voltammetry.

Fig. 1 is a three dimensional chromatogram of a mixture of phenolic acids. The electrochemical response (y-axis) was plotted *versus* voltage (z-axis) and time (x-axis). Each horizontal trace represents a single channel separated by 50 mV from the adjacent channels, all of which were collected simultaneously from a single chromatographic injection. The point density in the figure has been reduced four-fold from that of the acquired data, in order to avoid overlap between the lines which run parallel to the z-axis. Although the column resolution is quite good for this complex mixture, compounds b, c, and d, and also f and g, are not completely resolved chromatographically. Voltammetric resolution of a pair of overlapped peaks is possible whenever their oxidation potentials differ by at least 120 mV/n; where n is the number of electrons transferred. For example, peaks c and d in the figure are resolved in the voltage domain, and peaks b and c are partially resolved, while no voltammetric resolution can be achieved for peaks f and g. Peak g actually represents two compounds which are partially voltammetrically resolved as evidenced by the shoulder which appears on the trailing edge of the peak at higher potentials. Compounds i and j are resolved in both the voltage domain and by the column under the current chromatographic conditions.

The peaks for compounds f, g and h in the figure are very broad. Comparison between the UV and voltammetric outputs indicated that the unusual peak shape was due to poor oxidation characteristics, rather than chromatographic behavior. With amperometric detection, the response for these compounds was found to be erratic and decreased rapidly indicating electrode passivation by the oxidation products.

The peak integrity of each component was determined using the peak ratio method[10]. Peak areas for the phenolic compounds were obtained at two different potentials; one corresponding to the mass transfer limited region (maximum re-

TABLE I

RELATIVE RESPONSES *VERSUS* VOLTAGE

Responses in percent, relative to 3,4-dihydroxybenzoic acid at 0.9 V.

Compound	*Voltage (V)*									
	0.60	*0.65*	*0.70*	*0.75*	*0.80*	*0.85*	*0.90*	*0.95*	*1.00*	*1.05*
2-Hydroxybenzoic acid	0.00	0.00	0.00	0.00	0.69	2.39	6.49	12.74	36.98	44.60
4-Hydroxybenzoic acid	3.86	15.47	53.38	23.59	0.84	7.57	5.64	9.67	42.55	–
4-Hydroxy-3-methoxybenzoic acid	0.00	0.00	0.00	0.00	0.70	8.51	10.87	11.60	10.05	35.57
2,3-Dihydroxybenzoic acid	34.04	34.16	34.23	69.70	77.75	80.84	87.07	81.62	81.54	80.31
2,6-Dihydroxybenzoic acid	0.00	0.00	0.00	0.00	0.00	0.00	1.35	2.20	3.98	14.93
3,5-Dihydroxybenzoic acid	0.00	0.00	0.00	0.00	0.00	5.92	8.92	7.67	20.54	66.92
3,4-Dihydroxybenzoic acid	33.76	24.37	49.90	65.37	76.59	85.14	100.00	71.18	60.34	49.36
3-Hydroxyphenylacetic acid	0.00	0.00	0.00	0.00	0.00	0.24	0.37	1.92	4.83	16.55
3,4-Dihydroxyphenylacetic acid	6.38	13.69	35.74	49.51	52.80	84.33	81.27	69.09	65.37	63.00
4,8-Dihydroxyquinolin-2-carboxylic acid	0.00	0.00	0.00	0.00	2.52	26.30	23.75	34.62	38.14	33.34
1,3,5-Trihydroxybenzol	0.00	0.00	0.00	0.00	0.00	2.63	5.02	6.96	18.56	40.61
3,4-Dihydroxycinnamic acid	32.00	38.92	62.00	75.00	80.50	85.06	76.20	68.10	65.31	53.20

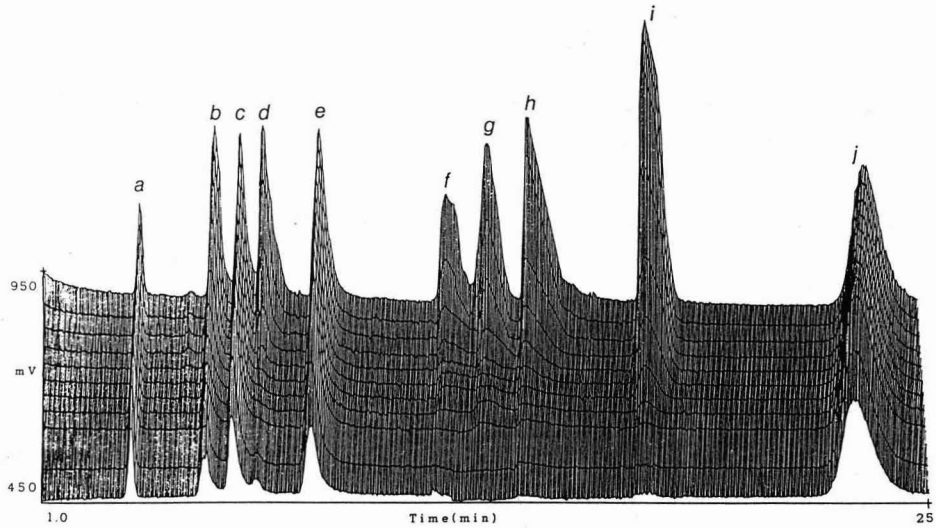

Fig. 1. Three dimensional chromatogram of phenolic acid mixture. Response *versus* voltage and time. a = 6 ng of ascorbic acid, b = 6 ng of trihydroxybenzoic acid, c = 6 ng of 2,3-dihydroxybenzoic acid, d = 6 ng of 3,5-dihydroxybenzoic acid, e = 4 ng of 3,4-dihydroxybenzoic acid, f = 10 ng of 2-hydroxybenzoic acid, g = 8 ng of 4,8-dihydroxyquinoline-2-carboxylic acid and 4 ng of 4-hydroxybenzoic acid, h = 12 ng of 2,6-dihydroxybenzoic acid, i = 15 ng of 4-hydroxy-3-methoxybenzoic acid, and j = 6 ng of 3,4-dihydroxycinnamic acid.

sponse) and the other at the lower Tafel region (slight response). Table II lists the characteristic peak area ratio for each compound. For most of the compounds the relative standard deviation (R.S.D.) of the ratio was less than 5% for five repetitive measurements; however, for 3,4-dihydroxybenzoic acid the coefficient of variation was 7%. In addition to testing for peak integrity, the peak–area ratio can also be used to identify an unknown phenolic acid. The retention time can be used to obtain

TABLE II

PEAK AREA RATIOS AND RETENTION TIMES

Compound	*V1 (V)**	*V2 (V)***	*Peak area ratio ± S.D.*	t_R *(min)*
1,3,5-Trihydroxybenzol	1.00	0.85	7.05 ± 0.31	5.21
2,3-Dihydroxybenzoic acid	0.85	0.60	2.37 ± 0.12	6.70
3,5-Dihydroxybenzoic acid	1.00	0.85	3.47 ± 0.02	7.25
3,4-Dihydroxybenzoic acid	0.85	0.65	3.49 ± 0.25	9.08
2-Hydroxybenzoic acid	1.05	0.85	18.66 ± 0.82	16.50
4-Hydroxybenzoic acid	1.00	0.85	5.38 ± 0.20	16.90
2,6-Dihydroxybenzoic acid	1.05	0.95	6.78 ± 0.32	17.50
3-Hydroxyphenylacetic acid	1.00	0.85	20.13 ± 0.53	18.12
4-Hydroxy-3-methoxybenzoic acid	1.05	0.85	4.18 ± 0.06	19.50
3,4-Dihydroxycinnamic acid	0.80	0.60	2.68 ± 0.07	22.00

* Mass transfer limited region (maximum response).
** Lower Tafel region (slight response).

a list of several possible candidates, and the peak–area ratio can be used to select the most likely possibility, or eliminate all of the candidates selected from the retention time alone. Of the compounds listed in Table II, only 3,4-dihydroxybenzoic acid and 3,5-dihydroxybenzoic acid have similar retention times *and* peak–area ratios which differ by less than their statistical variation.

Fig. 2 is a section of the chromatogram of the phenolic acids shown in Fig. 1. The upper trace corresponds to an applied potential of 900 mV. The large peak in the center represents 13 nmol of 3,5-dihydroxybenzoic acid and the smaller peak 0.47 nmol of 2,3-dihydroxybenzoic acid. The lower trace corresponds to 750 mV, at which 3,5-dihydroxybenzoic acid is not significantly oxidized. Therefore, the 2,3-dihydroxybenzoic acid can be quantitated at 750 mV without significant interference from the 3,5-dihydroxybenzoic acid. If the response at 750 mV is subtracted from that at 900 mV, one obtains the difference chromatogram shown in the center trace. This trace can be used to quantitate the 3,5-dihydroxybenzoic acid without significant interference from the 2,3-disubstituted acid. Although the column resolution for these compounds is about 0.64 (determined by UV absorption), the voltammetric detector provided complete resolution.

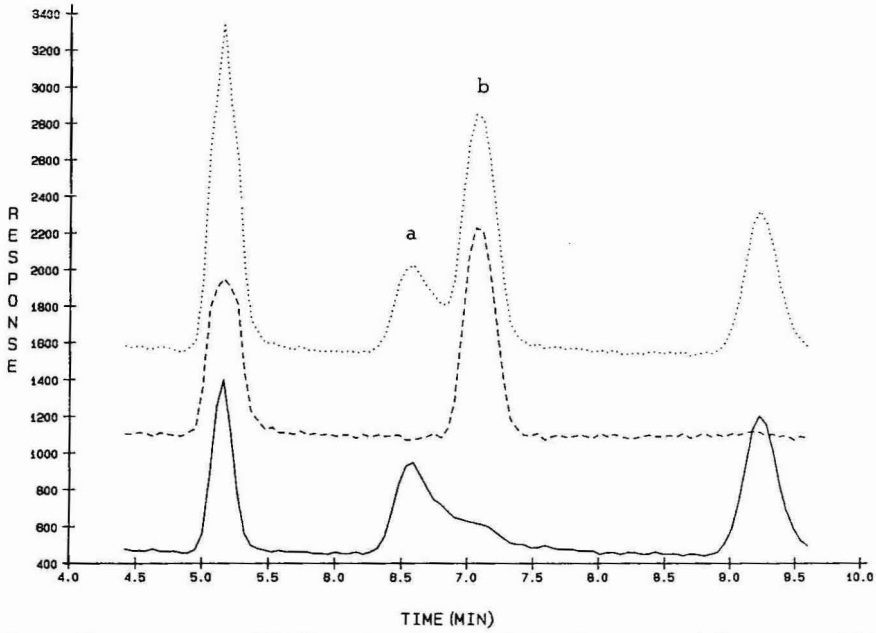

Fig. 2. Chromatograms which illustrate voltage resolution. Response at 900 mV potential (.....), response at 750 mV (———), difference chromatogram (upper trace – lower trace) (---------). a = 0.47 nmol of 2,3-dihydroxybenzoic acid, b = 13 nmol of 3,5-dihydroxybenzoic acid.

In order to test the precision of the detector for quantitative measurements, experiments were performed in which five measurements of 0.8 nmol of each sample, were made under identical conditions. The peak area was measured at two different potentials as in the previous experiment; however, in this case a peak–area-difference

TABLE III

PRECISION FOR VOLTAMMETRIC DIFFERENCE-PEAKS

Area values in percent, relative to 3,4-dihydroxybenzoic acid at 900 mV.

Compound	*V1 (V)*	*V2 (V)*	*Δ Peak area ± S.D.*	*R.S.D. (%)*
1,3,5-Trihydroxybenzol	1.00	0.85	15.93 ± 0.34	2.1
2,3-Dihydroxybenzoic acid	0.85	0.60	46.80 ± 0.13	0.3
3,5-Dihydroxybenzoic acid	1.00	0.85	14.62 ± 0.13	0.9
3,4-Dihydroxybenzoic acid	0.85	0.65	60.77 ± 0.26	0.4
2-Hydroxybenzoic acid	1.05	0.85	42.21 ± 0.62	1.5
4-Hydroxybenzoic acid	1.00	0.85	35.61 ± 0.38	0.1
2,6-Dihydroxybenzoic acid	1.05	0.85	14.93 ± 0.38	2.5
3-Hydroxyphenylacetic acid	1.00	0.85	4.59 ± 0.10	2.2
4-Hydroxy-3-methoxybenzoic acid	1.05	0.85	27.06 ± 0.34	1.3
3,4-Dihydroxycinnamic acid	0.85	0.60	42.42 ± 0.63	1.5

was obtained. The differences were used as a measure of response for each analyte. These results are shown in Table III, and the average peak areas for single potential measurements are shown in Table IV. For any given compound, the entries in both tables were obtained from the same set of data. In all cases the R.S.D. of the single-channel peak areas are considerably larger than those for difference peaks, even though the peak area is decreased in the latter case. This is a remarkable finding, since the precision of the voltammetric measurement is already two-fold better than for amperometric determinations (in our hands) of the same compounds (*e.g.*, for 2-hydroxybenzoic acid the R.S.D. was 3.0% and 5.6% for voltammetric and amperometric methods, respectively). The precise reason for this phenomenon is unknown; however, it is definitely associated with electrode ageing. For voltammetric, and more so amperometric measurements, the electrode response slowly decreases as oxidation products are deposited on the electrode surface. The problem is less severe for voltammetric measurements because the sawtooth waveform tends to either clean

TABLE IV

PRECISION FOR VOLTAMMETRIC SINGLE-PEAK MEASUREMENTS

Area value in percent, relative to 3,4-dihydroxybenzoic acid at 900 mV.

Compound	*Voltage (V)*	*Peak area ± S.D.*	*R.S.D. (%)*
1,3,5-Trihydroxybenzol	1.00	18.16 ± 0.50	2.8
2,3-Dihydroxybenzoic acid	0.85	80.84 ± 2.62	3.2
3,5-Dihydroxybenzoic acid	1.00	20.57 ± 1.13	5.5
3,4-Dihydroxybenzoic acid	0.85	85.14 ± 2.16	2.5
2-Hydroxybenzoic acid	1.05	44.60 ± 1.32	3.0
4-Hydroxybenzoic acid	1.00	43.75 ± 0.63	1.4
2,6-Dihydroxybenzoic acid	1.05	14.93 ± 0.42	2.8
3-Hydroxyphenylacetic acid	1.00	4.83 ± 0.21	4.3
4-Hydroxy-3-methoxybenzoic acid	1.05	35.57 ± 1.30	3.7
3,4-Dihydroxycinnamic acid	0.80	80.50 ± 1.96	2.4

the electrode, or reduce a large fraction of the oxidation products from the previous scan, before they become deposited (or polymerized) on the electrode surface. The voltammetric difference measurements apparently discriminate against the electrode ageing by removing a variable component of the oxidation current. The electrode ageing affects the oxidation current to the same extent for both channels involved in the difference-peak measurement. When the responses at the two different potentials are subtracted, the component of the analytical current affected by the electrode passivation is removed, leaving a second more stable component which is the value reported for the difference-peaks in Table II.

The ageing effect described above can not be due simply to a reduction in the active electrode surface area. If it were, the current decrease would be proportional to the actual current at any potential, and the peak-difference technique would yield no better precision than for single-potential measurements. A possible explanation, which is consistent with all of the observed facts, is that the oxidation current for the phenolic acids is composed of two components. One which is affected by the electrode passivation, and another which is unaffected or affected to a much smaller degree. Furthermore, one must conclude that the oxidation associated with the latter component occurs at a higher potential than for the component of the current affected by passivation, and that the variable component represents oxidation of an intermediate generated by the high-voltage process. In that case, the stable current component would be observed only at the larger of the two potentials involved in the peak-difference measurement, while the variable component would be observed at both potentials. Therefore, when the difference in the two responses was calculated, the variable part of the oxidation current would be removed.

Other researchers[11] have also observed complex voltammetric behavior for phenolic acids. This is particularly true for the monohydroxy phenolic acids which are often irreversible due to competing chemical reactions that consume electrochemical intermediates. A thorough study of the oxidation mechanism will be necessary before a definitive explanation can be given for the improvement in precision observed for difference-peaks.

If two channels are to be used for quantitation, it is necessary for the response to be linear in both channels. Therefore, the response for several phenolic acids was evaluated over a 0.1–1 nanomole range. In all cases examined, the response was linear with a correlation coefficient of at least 0.99. The best behavior was observed for 3,4-dihydroxycinnamic acid which exhibited a slope of 28 800 area units/nmol, an intercept of -1550 area units, and standard error of estimate of 200 area units. This leads to a detection limit of 20 pmol at the 95% confidence level. The detection limits for the other compounds tested were within a ten-fold range of this value.

Fig. 3 is a three dimensional chromatogram representing the first 20 min of elution for a sample of Welch's grape juice which was diluted ten-fold, spiked with 3,4-dihydroxybenzoic acid, and injected directly on the column after an initial pass through a Sep-Pak C_{18} column. Some components eluted up to 90 min after injection; however, several phenolic acids are eluted within the time span of the figure. By inspection along the voltage axis, it can be seen that the large unknown peak which elutes between ascorbic acid and trihydroxybenzoic acid is actually composed of at least three components. Many of the unknown compounds which coelute with trihydroxybenzoic acid are also resolved in the voltage domain.

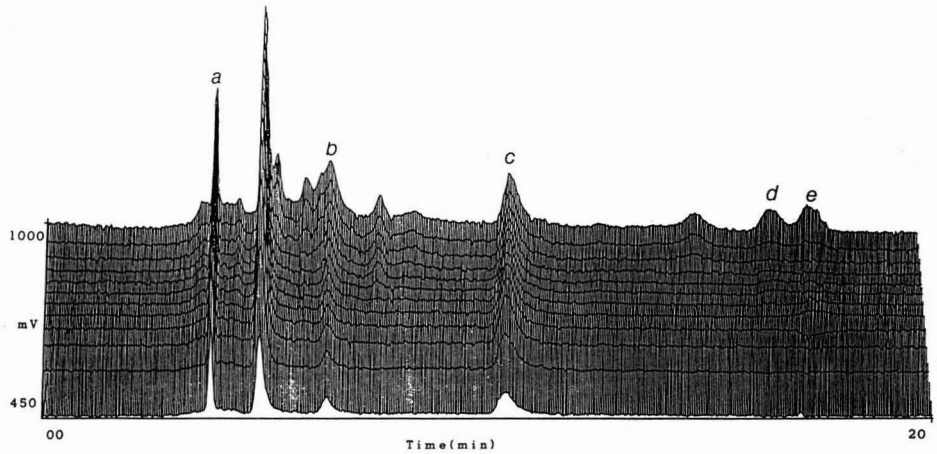

Fig. 3. Three dimensional chromatogram of first 20 min of elution for grape juice. a = Ascorbic acid, b = trihydroxybenzoic acid, c = 3,4-dihydroxybenzoic acid, d = 2-hydroxybenzoic acid, and e = 4-hydroxybenzoic acid.

CONCLUSIONS

The added resolution provided by voltammetric detection allows separation and quantitation of the phenolic acids at the sub-nmol level. Of the compounds investigated, only two (4-hydroxybenzoic acid and 4,8-dihydroxyquinoline-2-carboxylic acid) were not completely resolved by the column–detector pair in complex mixtures.

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DETERMINATION OF PHENYLETHANOLAMINE N-METHYLTRANSFERASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A highly sensitive assay method for phenylethanolamine N-methyltransferase in rat adrenal medulla and brain is described which employs high-performance liquid chromatography with fluorescence detection. Epinephrine formed enzymatically from the substrate norepinephrine and isoproterenol (internal standard), after chromatography on a small cartridge of a cation exchanger, Toyopak SP, are converted into the corresponding fluorescent compounds by reaction with 1,2-diphenylethylenediamine, a selective fluorescence derivatization reagent for catechol compounds. The derivatives are separated by reversed-phase chromatography on TSK gel ODS-120T. The detection limit for epinephrine formed enzymatically is 0.66 pmol per assay tube.

INTRODUCTION

Phenylethanolamine N-methyltransferase (PNMT; phenylethanolamine:S-adenosylmethionine N-methyltransferase, E.C. 2.1.1.28) catalyses the transfer of the methyl group of S-adenosylmethionine to the amino groups of norepinephrine or other phenylethanolamines. PNMT activity is high in the adrenal gland[1], where epinephrine is synthesised. As PNMT activity also occurs in mammalian brain[2] and the enzyme has been implicated in the pathogenesis of experimental hypertension in animals[3], the role of PNMT in brain has attracted great attention.

Many assay methods for PNMT in biological materials have been reported. They are classified into two types, radiochemical methods[1,2,4–10] and high-performance liquid chromatographic (HPLC) methods coupled with electrochemical[11–13] and post-column fluorescence detection[14]. The radiochemical methods are sensitive but require complicated procedures and expensive radioactive substrates, and the HPLC methods with electrochemical detection require careful manipulation to attain high sensitivity and reproducibility. The HPLC method with fluorescence detection uses

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the trihydroxyindole method and thus requires a post-column derivatization apparatus.

We have developed a highly sensitive assay method for PNMT in rat adrenal medulla and brain involving HPLC with fluorescence detection, based on the determination of epinephrine formed from substrate norepinephrine under the optimum conditions for the enzyme reaction. Epinephrine and isoproterenol as an internal standard, after chromatography on a small cartridge of cation exchanger, are converted into fluorescent compounds by reaction with 1,2-diphenylethylenediamine, a fluorogenic reagent for catechol compounds[15–17]. The fluorescent compounds from the amines are separated by reversed-phase HPLC on TSK gel ODS-120T.

EXPERIMENTAL

Reagents and materials

Norepinephrine hydrogen tartrate, dopamine hydrochloride and glutathione (reduced form) were purchased from Wako (Osaka, Japan). Epinephrine hydrogen tartrate and isoproterenol hydrochloride were obtained from Nakarai Chemicals (Kyoto, Japan). 1,2-Diphenylethylenediamine and Good's buffers (bicine, TAPS, tricine and glycinamide hydrochloride) were obtained from Dojindo Labs. (Kumamoto, Japan). All other chemicals were of analytical-reagent grade. Deionized, distilled water was used. 1,2-Diphenylethylenediamine solution (0.1 *M*, pH 6.7) and Toyopak SP (strong cation exchanger, sulphopropyl resin, sodium ion form; Toyo Soda, Tokyo, Japan) cartridge for sample clean-up were prepared as described previously[17]. The cartridge was washed successively with 2 ml of 2 *M* sodium hydroxide solution, 5 ml of water, 2 ml of 2 *M* hydrochloric acid and 10 ml of water. The used cartridge can be regenerated by washing in the same way and is usable for more than five times.

Enzyme preparations

Male Donryu rats (4 weeks old) were decapitated and the adrenal medullae and brains were immediately removed and chilled on ice. All further procedures were carried out at 0–5°C. Adrenal medulla (5 mg) was homogenized with 1.25 ml of isotonic potassium chloride and the homogenate was dialysed at 4°C for 12–15 h against 5 m*M* phosphate buffer (pH 6.8) containing 0.1 m*M* reduced glutathione. Brain (500 mg) was homogenized with 2.0 ml of isotonic potassium chloride. The homogenates were stored at −20°C until used, and the amounts of protein were measured by the method of Lowry *et al.*[18] using bovine serum albumin as a standard protein.

HPLC apparatus and conditions

An Eyela PLC-10 liquid chromatograph (Tokyo Rika Kikai, Tokyo, Japan) was used, equipped with a Rheodyne 7125 syrine-loading sample injector valve (100-μl loop) and a Shimadzu FLD-1 fluorescence detector fitted with a 14-μl flow cell and an EM-4 emission filter. The column was TSK gel ODS-120T (250 × 4.6 mm I.D.; Toyo Soda). The column temperature was ambient (20–25°C). This column can be used for more than 2000 injections when washed with acetonitrile–methanol–water (52:3:45, v/v) at a flow-rate of *ca.* 1 ml/min for 25 min each day.

The mobile phase was acetonitrile–methanol–50 m*M* Tris–HCl buffer (pH 7.0) (52:3:45, v/v) and the flow-rate was 1.0 ml/min. Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi 850 fluorescence spectrophotometer fitted with an 18-μl flow cell, setting the spectral bandwidths at 5 nm in both the excitation and emission monochromators.

Assay procedure

Adrenal medulla PNMT. To 100 μl of the enzyme preparation from rat adrenal medulla were added 150 μl of 0.1 *M* bicine buffer (pH 8.5) and 50 μl of 0.7 m*M* S-adenosylmethionine. The mixture was pre-incubated at 37°C for 10 min and again incubated for 30 min after the addition of 50 μl of 0.3 m*M* norepinephrine. At the end of the incubation, 50 μl each of 3.0 *M* trichloroacetic acid and 2.0 μ*M* isoproterenol as an internal standard were added. The mixture was centrifuged at 1000 *g* at 4°C for 10 min and the supernatant (300 μl) was poured on to a Toyopak SP (H^+) cartridge. The cartridge was washed successively with 10 ml of water, 3 ml of 0.2 *M* phosphate buffer (pH 5.5) and 10 ml of water. The adsorbed norepinephrine, epinephrine and isoproterenol were eluted with 2.0 ml of ethanol–1.0 *M* sodium chloride (7:3, v/v). To the eluate, 100 μl each of the 1,2-diphenylethylenediamine solution and 15 m*M* potassium hexacyanoferrate(III) were added and the mixture was allowed to stand at 37°C for 40 min to derivatize the amines to the fluorescent compounds. The resulting mixture (100 μl) was injected into the chromatograph. For the blank, the enzyme preparation was carried through the procedure except that the order of the addition of norepinephrine and trichloroacetic acid was reversed, incubation being omitted. Michaelis constants (K_m) for norepinephrine and S-adenosylmethionine were calculated from the Lineweaver-Burk plots.

Brain PNMT. To 100 μl of the enzyme preparation from rat brain were added 100 μl of 0.1 *M* bicine buffer (pH 8.5) and 50 μl each of 0.7 m*M* S-adenosylmethionine and 1.0 m*M* pargyline. The mixture was pre-incubated at 37°C for 10 min and again incubated at 37°C for 60 min after the addition of 50 μl of 0.3 m*M* norepinephrine. At the end of the incubation, 50 μl each of 3 *M* trichloroacetic acid and 0.5 μ*M* isoproterenol were added. The mixture was then treated in the same way as for the adrenal medulla preparation.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained with the adrenal medulla and brain preparations and those of the blanks. The fluorescent compounds for norepinephrine, epinephrine and isoproterenol (peaks 1, 2 and 3, respectively) can be well separated from the fluorescent components of the blanks within 14 min under the HPLC conditions used. The eluates from peaks 1 and 2 in Fig. 1 have fluorescence excitation (maxima 350 and 360 nm, respectively) and emission (maxima 470 and 480 nm, respectively) spectra that are identical with those for authentic norepinephrine and epinephrine, respectively. Peak 4 in Fig. 1c and d increases in height when authentic dopamine is added to the enzyme reaction mixtures for the test and blank, and the eluate from the peak shows fluorescence excitation (maximum 350 nm) and emission (maximum 480 nm) spectra identical with those for dopamine; the peak is ascribable to endogenous dopamine in brain. Peaks 5–8 in Fig. 1 increase in height

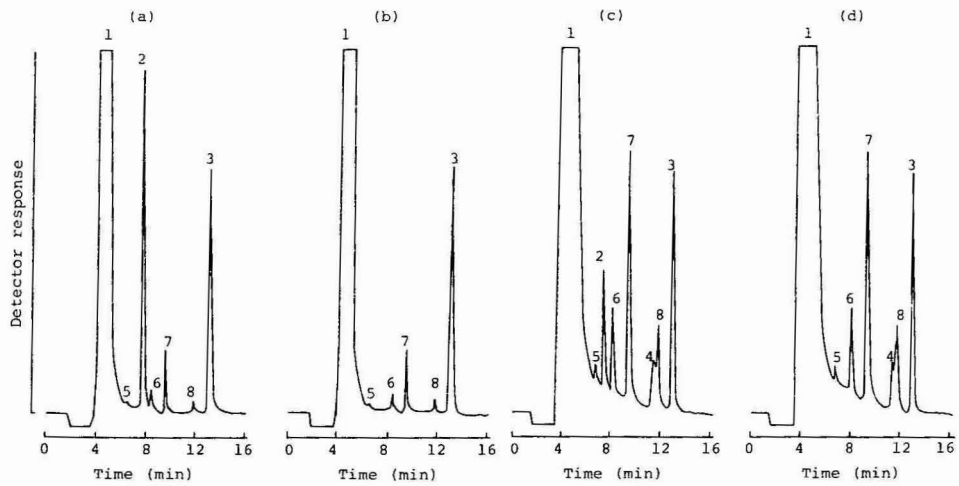

Fig. 1. Chromatograms obtained with the enzyme preparations from (a) adrenal medulla, (c) brain and (b) and (d) their blanks, respectively. Portions (100 μl) of the enzyme preparations were treated according to the procedure. Peaks: 1 = norepinephrine; 2 = epinephrine; 3 = isoproterenol; 4 = dopamine (endogenous); 5–8 = impurities in commercial norepinephrine. PNMT activity (pmol/min/mg protein): (a) 230; (c) 0.52. Detector sensitivity: (a) and (b) 2; (c) and (d) 8.

with increasing substrate concentration, and do not occur when the 1,2-diphenylethylenediamine solution or the norepinephrine solution is omitted from the procedure. The eluates from these peaks have fluorescence excitation (maxima around 350 nm) and emission (maxima around 460 nm) spectra that are fairly characteristic of 1,2-diphenylethylenediamine derivatives of catechol compounds[17]. These observations suggest that the peaks are due to some catechol compounds present as impurities in commercial norepinephrine. These peaks, however, do not interfere with the determination of epinephrine formed enzymatically and so further purification of norepinephrine is unnecessary.

The HPLC conditions are essentially the same as those described previously[17]. However, acetonitrile–methanol–50 m*M* Tris–HCl buffer (pH 7.0) was used as the mobile phase in order to obtain a complete seperation of the peaks for epinephrine and isoproterenol from those for dopamine and the impurities in norepinephrine.

Adrenal medulla contains a fairly large amount of epinephrine, which may interfere with the reproducible determination of the amine formed enzymatically; the endogenous epinephrine can be removed by dialysing the tissue homogenate at 4°C for 12 h or more against 5 m*M* phosphate buffer (pH 6.8) containing 0.1 m*M* reduced glutathione. PNMT in adrenal medulla is stable enough to be dialysed for at least 1 day.

PNMT in both the enzyme preparations is most active at pH 8.5–8.6 (Fig. 2) in bicine buffer and the bicine concentration at 0.1 *M* gives maximum activity. Although other buffers, *i.e.*, 0.1 *M* tricine, 0.1 *M* glycinamide, 0.1 *M* TAPS and 0.1 *M* Tris–HCl buffers, give maximum activities at pH 8.5, the activities did not exceed 90% of that given by the bicine buffer (pH 8.5–8.6) (Table I); 0.1 *M* bicine buffer of pH 8.5 was therefore used in the recommended procedure. Catecholamines are easily

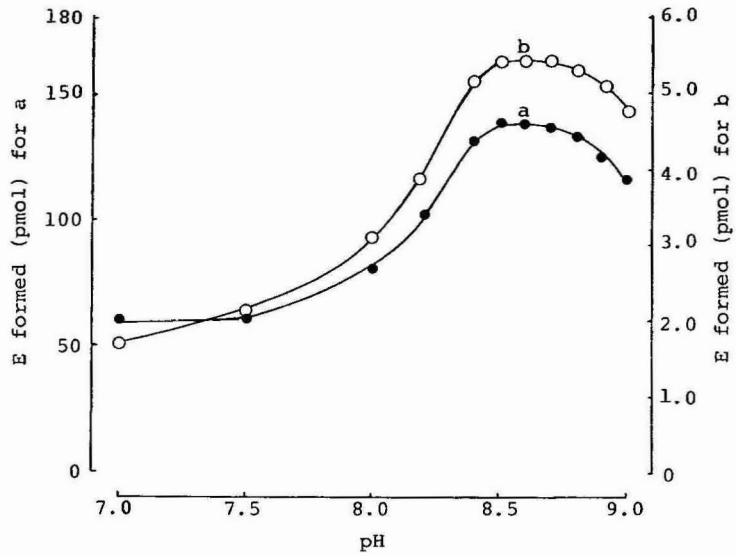

Fig. 2. Effect of pH of 0.1 *M* bicine buffer on the amount of epinephrine (E) formed in (a) adrenal medulla and (b) brain PNMT-catalysed reactions. Portions (100 μl) of the enzyme preparations were treated according to the procedure at various pHs. PNMT activity (pmol/min/mg protein): (a) 220; (b) 0.51.

oxidized in alkaline media[19]. However, epinephrine and norepinephrine dissolved in the bicine buffer, provided that the enzyme preparations are added, are stable at 37°C for more than 90 min; the enzyme preparations may act as antioxidant(s).

Norepinephrine in the enzyme reaction mixture gives maximum and constant activity in the concentration ranges 30–70 μ*M* for adrenal medulla PNMT and 30–50 μ*M* for brain PNMT (Fig. 3), with K_m values for norepinephrine of 10.3 ± 1.2 and 13.8 ± 1.5 μ*M* (mean ± S.D., $n=5$ in each instance), respectively; 43 μ*M* norepinephrine was used as a saturating concentration for the enzyme reactions.

S-Adenosylmethionine in the concentration range 30–150 μ*M* in the enzyme reaction mixture of the adrenal medulla preparation gives a maximum and constant

TABLE I

EFFECT OF BUFFERS ON PNMT ACTIVITY

Portions (100 μl) of the enzyme preparations were treated as in the procedure with various buffers.

Buffer (0.1 M, pH 8.5)	*PNMT activity (epinephrine formed, pmol/min/mg protein)*	
	Rat adrenal medulla	*Rat brain*
Bicine	220 (100)*	0.51 (100)*
Tricine	190 (86)	0.46 (90)
Glycinamide	170 (77)	0.42 (82)
TAPS	160 (72)	0.45 (88)
Tris–HCl	160 (72)	0.28 (55)

* Relative activity in parentheses. The activity obtained with the bicine buffer was taken as 100.

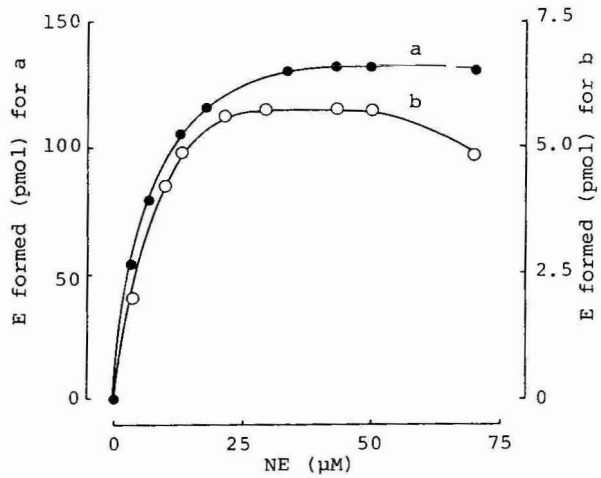

Fig. 3. Effect of the substrate concentration on the amount of epinephrine (E) in (a) adrenal medulla and (b) brain PNMT-catalysed reactions. Portions (100 μl) of the enzyme preparations were treated according to the procedure at various norepinephrine (NE) concentrations. PNMT activity (pmol/min/mg protein): (a) 220; (b) 0.54.

activity with an observed K_m value of 5.4 ± 0.8 μM (mean ± S.D., $n=5$). In the brain preparation, a weak PNMT activity, which corresponds to 10% of that given in the presence of 100 μM S-adenosylmethionine in the enzyme reaction mixture, arises even when S-adenosylmethionine is not added to the enzyme reaction mixture. This may be due to an endogenous methyl donor (probably S-adenosylmethionine), and can be completely eliminated by dialysing the enzyme preparation against 5 mM phosphate buffer (pH 6.8) containing 0.1 mM reduced glutathione at 4°C for 12 h or more. This dialysis does not cause a loss of the enzyme activity. S-Adenosylmethionine in the concentration range 50–120 μM gives maximum and constant activity in both the dialysed and non-dialysed brain preparations; dialysis was unnecessary for the assay. The K_m value for S-adenosylmethionine observed with the dialysed brain preparation is 9.1 ± 1.7 μM (mean ± S.D., $n=5$), which is higher than that obtained with the adrenal medulla preparation. This supports the view that PNMT in brain is slightly different from that in adrenal medulla in its electrophoretic and gel chromatographic behaviours[9]. Hence 100 μM S-adenosylmethionine was selected in the procedure.

As norepinephrine and epinephrine are deaminated and/or methylated *in vivo* by monoamine oxidase (MAO) and/or catechol O-methyltransferase (COMT) catalysed reactions[20,21], there is a possibility of deamination and/or O-methylation of epinephrine formed enzymatically and the substrate norepinephrine during the incubation period. The amount of epinephrine formed enzymatically is unaffected for the adrenal medulla preparation by the addition of the MAO inhibitor pargyline (0.05–0.5 mM in the enzyme reaction mixture). In the brain preparation, however, the PNMT activity in the absence of pargyline was only 28% of that obtained in its presence (0.05–0.5 mM in the enzyme reaction mixture); 0.14 mM pargyline was used in the assay for brain PNMT. The COMT inhibitor pyrogallol had no effect on

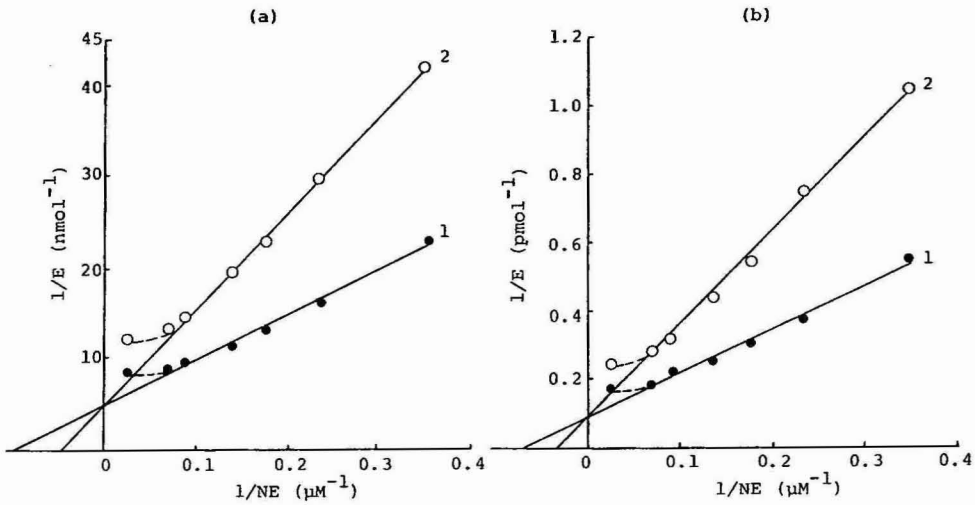

Fig. 4. Inhibition of (a) adrenal medulla and (b) brain PNMT by octopamine. Portions (100 μl) of the enzyme preparations were treated as in the procedure. Concentrations of octopamine in the enzyme reaction mixture: 1, nil; 2, 7.14 μM. The data were plotted by linear regression analysis.

PNMT activity in either of the enzyme preparations in the concentration range 10 μM–1.0 mM in the enzyme reaction mixture.

PNMT works on both octopamine and norepinephrine and these amines can be competitive inhibitors for each other[22]. Octopamine inhibited PNMT in adrenal medulla and brain in a competitive mode against norepinephrine (Fig. 4), with observed inhibitory constant (K_i) values of 6.3 and 6.2 μM, respectively, which were obtained according to the method of Dixon[23]. The enzyme activity is inhibited by thiol blocking agents[21,24]. *p*-Chloromercuribenzoic acid added as a thiol blocking agent at cocentrations of 0.125 and 0.4 mM in the enzyme reaction mixture inhibits 28 and 100% of PNMT activity in the adrenal medulla preparation, respectively, and at concentrations of 0.125, 0.4 and 1 mM inhibits 17, 63 and 100% of the enzyme activity in the brain preparation, respectively. All the above observations suggest that epinephrine formed under the enzyme reaction conditions of the present procedure can be ascribed to the enzymatic N-methylation of norepinephrine.

The amounts of epinephrine formed enzymatically are proportional to protein amounts (μg per tube) of up to 200 (adrenal medulla) and 450 (brain), respectively; amounts (μg per tube) of approximately 20 (adrenal medulla) and 180 (brain) were used in the procedure.

PNMT activity in the adrenal medulla preparation is proportional to incubation time at 37°C up to 60 min and in the brain preparation up to 90 min (Fig. 5); incubation times of 30 and 60 min, respectively, are recommended.

For clean-up of the enzyme reaction mixture, a strong cation exchanger, Toyopak SP (H^+) cartridge was used under conditions the same in principle as those described previously[25]. Recoveries (%; mean ± S.D.) of epinephrine and isoproterenol (0.1 nmol each) added to the incubated enzyme reaction mixtures for the blanks were 77.7 ± 1.8 and 84.7 ± 1.3 in adrenal medulla, and those of epinephrine (5.0

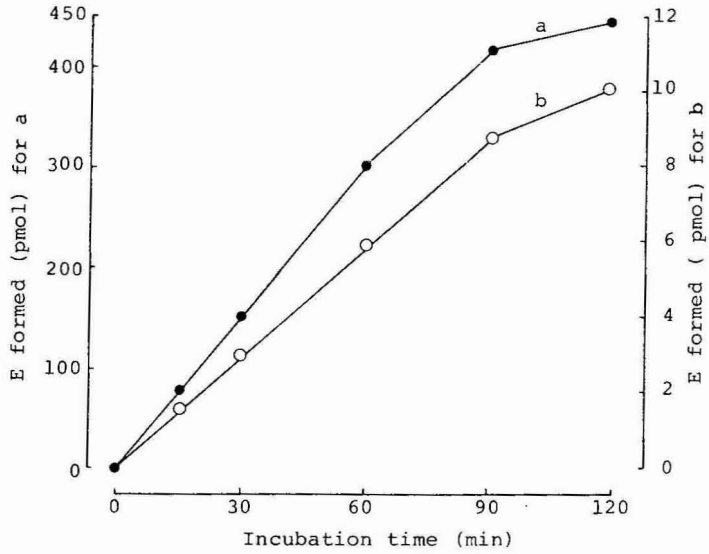

Fig. 5. Effect of the incubation time on the amount of epinephrine (E) formed in (a) adrenal medulla and (b) brain PNMT-catalysed reactions. Portion (100 μl) of the enzyme preparations were treated according to the procedure for various incubation times. PNMT activity (pmol/min/mg protein): (a) 240; (b) 0.58.

pmol) and isoproterenol (40 pmol) 71.3 ± 2.8 and 75.2 ± 1.6 in brain ($n=8$ in each instance), respectively.

A linear relationship was observed between the ratio of the peak height of epinephrine to that of isoproterenol and the amount of epinephrine added to the blank in each enzyme preparation over the range 1–100 pmol. The detection limit for epinephrine formed enzymatically was 0.66 pmol per tube (30 fmol per 100 μl injection volume) at a signal-to-noise ratio of 2. The precision was established with respect to repeatability. The coefficients of variations were 2.3 and 3.2% ($n=8$) for mean activities of 230 and 0.52 pmol/min/mg protein in the adrenal medulla and brain preparations, respectively.

PNMT activities in the adrenal medulla and brain preparations from rats (Donryu, male, 4 weeks old) were 233 ± 13 and 0.52 ± 0.06 pmol/min/mg protein (mean ± S.D., $n=5$), respectively. These values are in good agreement with reported data[14].

This method is sensitive enough to assay low PNMT activity in brain and precise, and should be useful for biological and biomedical investigations of catecholamines.

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CHROM. 18 125

Note

A problem of optimization in capillary gas chromatography

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The problem of optimizing the capacity ratio of a capillary column for a selected, presumably most critical component, is considered as a function of the diffusion time in the stationary phase, and the essential column parameters are derived for given inlet pressures, analysis times, etc. This study indictes that a double advantage can be derived from the use of high inlet pressures.

DISCUSSION

Consider a capillary column optimally designed for the separation of a given component at some predetermined temperature. At the outset the operating parameters are the inlet pressure, p_i, the analysis time, t_s, up to the elution of the particular component considered, the carrier gas viscosity μ, the product of the diffusion coefficient in the carrier gas times the gas pressure, $Dp = j$, which is invariant from injection to elution, and the pressure $p_e = bp_i$ at which the diffusion time in the gas phase is equal to the diffusion time in the stationary phase.

Two basic equations are used to solve the problem. The first is the Poiseuille flow equation

$$-\mathrm{d}p/\mathrm{d}x = Rv$$

where v is the carrier gas velocity and R is the resistance to flow:

$$R = 8\,\mu/r^2$$

As the product $vp = i$ is invariant throughout the column, the flow equation can be rewritten as

$$-\frac{\mathrm{d}p}{\mathrm{d}x} = \frac{8\mu}{r^2} \cdot \frac{i}{p}$$

whence, by integration

$$p^2 = 16\mu \cdot \frac{i}{r^2}(l - x) + p_e^2 \qquad (1)$$

where p_e designates the elution pressure at the column exit where $x = l$.

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The carrier gas velocity is given by

$$\frac{dx}{dt} = v = \frac{i}{p} = \frac{i}{\sqrt{16\mu \cdot \frac{i}{r^2}(l - x) + p_e^2}}$$

whence, by integration from $x = 0$ to $x = l$ we obtain the time of passage, t_c, of an element of the carrier gas:

$$t_c = \frac{1}{24} \cdot \frac{r^2}{\mu i^2}(p_i^3 - p_c^3)$$

The time of passage of the sample component of interest is given by

$$t_s = \frac{1}{24}(1 + k)\frac{r^2}{\mu i^2}(p_i^3 - p_e^3)$$

wherefrom:

$$r = \sqrt{\frac{24\mu t_s}{(1 + k)(p_i^3 - p_e^3)}} \cdot i \tag{2}$$

Likewise from eqns. 1 and 2 we obtain:

$$l = \frac{p_i^2 - p_e^2}{16\mu i} \cdot r^2 = \frac{3}{2}\frac{(p_i^2 - p_e^2)\, t_s i}{(1 + k)(p_i^3 - p_e^3)} \tag{3}$$

For the determination of i and k we introduce the second equation, which gives the height equivalent to a theoretical plate (HETP) of a capillary column

$$h = 2\frac{D}{v} + \frac{1 + 6k + 11k^2}{24(1 + k)^2} \cdot \frac{vr^2}{D} + \frac{k^3}{6(1 + k)^2} \cdot \frac{vr^2}{c^2 D_1}$$

where c designates the partition coefficient between the stationary and mobile phases and D_1 the diffusion coefficient of a sample molecule in the stationary phase, a quantity independent of pressure. For $c^2 D_1$ we may write

$$c^2 D_1 = \frac{j}{p_c} = \frac{j}{bp_i}$$

and for h

$$h = 2\frac{j}{i} + \frac{1}{24} \cdot f_k \cdot \frac{ir^2}{j} + \frac{k^3}{6(1 + k)^2} \cdot \frac{ir^2}{j} \cdot \frac{bp_i}{p}$$

where

$$f_k = \frac{1 + 6k + 11\,k^2}{(1 + k)^2}$$

The optimum condition sought here will be one in which the ratio $\Delta t_s/\Delta t_b$ of the difference, Δt_s, in the time of elution of the component of interest and that of its nearest neighbour over the elution bandwidth, Δt_b, will be maximal with a suitable choice of k. As $t_s = (1 + k)t_c$ for the component of interest, a neighbouring component with a k value differing by εk from the first will be eluted at a time differing from that of the first by

$$\Delta t_s = \varepsilon k t_c = \frac{\varepsilon k}{1 + k} \cdot t_s$$

The bandwidth of either component must be obtained by first calculating the second moment of mass, w, the differential of which, $dw = p^2 h dx$, remains invariant with pressure decrease as the component flows through the column. Thus:

$$w = \int_{x=0}^{l} p^2 h dx = \int_{x=0}^{l} p^2 \left[2\frac{j}{ir} + \frac{1}{24} \cdot f_k \cdot \frac{ir}{j} + \frac{k^3}{6\,(1 + k)^2} \cdot \frac{bp_i}{p} \cdot \frac{ir}{j} \right] r dx$$

Setting the dimensionless quantity $ir/j = y$, from eqns. 1–3 we obtain:

$$w = \left[\left(\frac{1}{y} + \frac{1}{48} \cdot f_k y \right) (p_i^2 + p_e^2) + \frac{k^3}{9\,(1 + k)^2} \cdot \frac{bp_i(p_i^3 - p_e^3)}{(p_i^2 - p_e^2)} \cdot y \right] lr$$

$$= \left[\left(\frac{1}{y} + \frac{1}{48} \cdot f_k y \right) (p_i^4 - p_e^4) + \frac{k^3}{9\,(1 + k)^2} \cdot bp_i\,(p_i^3 - p_e^3) y \right] \cdot i^2 \cdot$$

$$\cdot \sqrt{\frac{54 \mu t_s^3}{(1 + k)^3\,(p_i^3 - p_e^3)^3}}$$

Δt_b is obtained from w by use of the relationship:

$$\Delta t_b = (1 + k) \cdot \frac{\sqrt{w}}{i}$$

Maximizing the ratio $\Delta t_s/\Delta t_b$

$$\frac{\Delta t_s}{\Delta t_b} = \frac{\varepsilon k t_s i}{(1 + k)^2 \sqrt{w}} =$$

$$\frac{\varepsilon k t_s^{1/4} (p_i^3 - p_e^3)^{3/4}}{(54\mu)^{1/4} (1 + k)^{5/4} \left[\left(\frac{1}{y} + \frac{1}{48} \cdot f_k y\right)(p_i^4 - p_e^4) + \frac{k^3 y}{9 (1 + k)^2} \cdot b p_i (p_i^3 - p_e^3)\right]^{1/2}}$$

and writing $p_e = a p_i$ we obtain:

$$\frac{\Delta t_s}{\Delta t_b} = \varepsilon \left(\frac{p_i t_s}{\mu}\right)^{1/4} \cdot$$

$$\cdot \frac{k (1 - a^3)^{3/4}}{54^{1/4} \sqrt{\left(\frac{1}{y} + \frac{1}{48} \cdot f_k y\right)(1 - a^4) + \frac{k^3}{9 (1 + k)^2} \cdot b (1 - a^3) y} \cdot (1 + k)^{5/4}} \quad (4)$$

From the above equation it is seen that regardless of how high the value of b is, *i.e.*, of how slow the diffusion in the stationary phase, the ratio $\Delta t_s/\Delta t_b$ will be highest when a is negligibly small, a somewhat surprising result. As for y, the value of $\Delta t_s/\Delta t_b$ will be highest when the value of $1/y$ is equal to the sum of the terms in y under the square root. Eqn. 4 may therefore be rewritten as

$$\frac{\Delta t_s}{\Delta t_b} = \varepsilon \left(\frac{p_i t_s}{\mu}\right)^{1/4} Q(k, b) \quad (4a)$$

where

$$Q(k, b) = \frac{k}{216^{1/4} (1 + k)^{5/4} \left[\frac{1}{42} f_k + \frac{b k^3}{9 (1 + k)^2}\right]^{1/4}}$$

The term $p_i t_s/\mu$ in parentheses on the right-hand side of eqn. 4a is an important dimensionless and also nameless quantity, which could be considered a measure of the "effort" made, or of the "price" paid, to obtain a chromatogram, as its fourth root is directly related to the effective resolving power of the chromatograph. As for $Q(k, b)$, its optimum value is listed below for several values of b; also given are the values of k which maximize it, as well as the values of y which, by use of eqns. 2 and

3 give the radius, r, and length, l, of the column:

b =	0	0.1	0.3	1	3	10	30	100
k =	2.692	2.366	2.032	1.581	1.206	0.899	0.713	0.593
Q =	0.221	0.216	0.208	0.192	0.170	0.141	0.113	0.087
y =	2.599	2.547	2.475	2.327	2.113	1.790	1.430	1.009

These figures emphasize the double benefit to be derived from an increased inlet pressure: first, by increasing the measure of the "effort", and secondly by increasing the value of Q for correspondingly decreased values of b.

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Note

Investigation of the synthesis of tryptathionine using high-performance liquid chromatography

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The amino acid tryptathionine, which occurs in phalloidin and related toxic peptides of *Amanita phalloides*, provides a crosslink between a tryptophan and a cysteine residue[1]. The most successful synthetic route to this amino acid, the Savige–Fontana reaction[2,3] involves the reaction of cysteine (or other thiols in the case of related amino acids) with 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-[2,3-*b*]indole-2-carboxylic acid[4].

During a study of the kinetics of the reaction the need arose for a rapid method for the separation and estimation of a variety of tryptophan oxidation products. Existing methods used in this laboratory[3,5] although satisfactory with regard to separation, proved to be too slow for our purposes. In this communication we describe the results obtained using a high-performance liquid chromatography (HPLC) system in conjunction with a photodiode-array detector.

EXPERIMENTAL

Materials

Samples of a 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (Hpi)[4], dioxindolyl-3-alanine[6], and tryptathionine and some of its analogues[3], were prepared according to published methods. All other materials were obtained from normal commercial sources.

Method and instrumentation

HPLC analysis was performed using a Perkin-Elmer Series 4 solvent delivery system and a LCI-100 laboratory computing integrator. Sample injection was performed using a Rheodyne 7125-075 syringe-loading sample injector with a 6-μl loop.

Elutions were monitored at 289 nm using a Hewlett-Packard 1040A photodiode-array detector controlled by an 85B microcomputer. Chromatography was performed on a Vydac 218 TP (250 × 4.6 mm I.D.) 5 μm, C_{18} reversed-phase column at 60°C.

All samples were run at 1.0 ml/min with solvent A (1 g/l aqueous ammonium acetate) and solvent B (acetonitrile). The column was eluted for 4 min with solvent A and then for 11 min following a linear gradient to 60% B.

A mixture of L-cysteine hydrochloride monohydrate (18.5 mg, 0.105 mmole)

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and Hpi (29.9 mg, 0.126 mmole) in 25% trifluoroacetic acid (1.00 ml) was kept at room temperature. Aliquots (50 μl) were taken at recorded hourly intervals and added to water (950 μl). The diluted samples (2 μl) were then analysed by HPLC using tryptathionine and Hpi as controls.

RESULTS AND DISCUSSION

Table I summarizes the chromatography of tryptophan and the separation of the reactants and possible products of the Savige–Fontana reaction. These conditions were also found to be suitable for rapid quantitative assays for products resulting from various oxidative treatments of tryptophan, even though some derivatives were found to be eluted close together. The use of the photodiode-array detector enabled simultaneous confirmation of the identity of peaks by comparison of their ultraviolet absorption spectra with those of known standards. This feature made the procedure particularly suitable for materials with distinctive UV-absorbing characteristics, such as the tryptophan oxidation products.

TABLE I

SEPARATION OBTAINED BY HPLC OF TRYPTOPHAN, REACTANTS AND POSSIBLE PRODUCTS OF TRYPTATHIONINE SYNTHESIS, AND TRYPTOPHAN OXIDATION

Compound	*Retention time (min)*	*Colour factor 289 nm (Trp = 1)*
L-Tryptophan	8.80	1.00
Reactants		
L-Cysteine	2.68	–*
L-Hpi.	4.28	0.37
Products		
L-Tryptathionine	6.02	2.20
L-Cystine	2.61	–*
Dioxindolyl-3-alanine	4.12, 4.48	0.23**
L-Kynurenine	5.70	0.06
Formyl-DL-kynurenine	7.00	–*
L-Oxindolyl-3-alanine	7.38	0.24
Tryptamine	10.29	0.83

* 289 nm is unsuitable for detection of these compounds. Their retention times were established by detection at a wavelength suitable for their absorption maximum.

** Summation of two peaks.

When the technique was applied to the products obtained in the tryptathionine synthesis, separation of the reactants, tryptathionine and the byproducts was clearly obtained (Fig. 1). The method of Savige and Fontana[3] had specified a 48-h reaction time, but from our study (Fig. 2) any increase in time beyond 8 h is not necessary. The yield does increase slightly with longer times but so do the amounts of secondary products. A small quantity of oxindolyl-3-alanine was identified as a byproduct of the reaction, also reported by Savige and Fontana[3], but the major byproducts could not be identified. They did not correspond to any of a number of possible oxidation

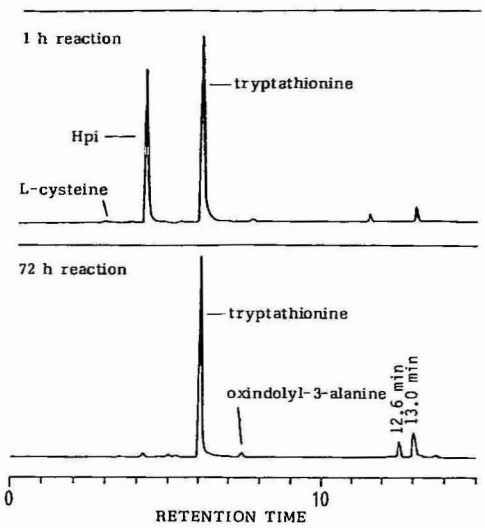


Fig. 1. HPLC trace of samples taken from the tryptathione reaction mixture after 1 h and 72 h at 289 nm. L-Cysteine does not absorb maximally at this wavelength.

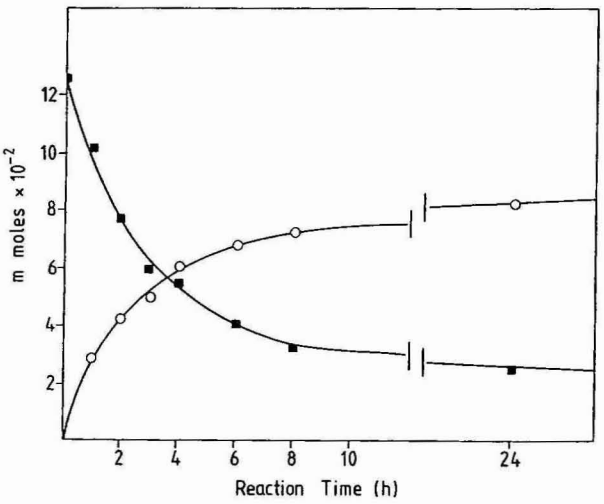


Fig. 2. The formation of tryptathionine (O) and the consumption of Hpi (■) plotted against time of reaction.

products of tryptophan reported in Table I and the UV-absorption spectrum of the major peak most closely resembled that of a tryptophan dimer (Fig. 3)[7].

The HPLC procedure described also proved to be suitable for the detection and analysis of a range of tryptathionine analogues (Table II), without modification to the chromatography. These compounds were eluted on the reversed-phase column in the manner consistent with their polarity and structure.

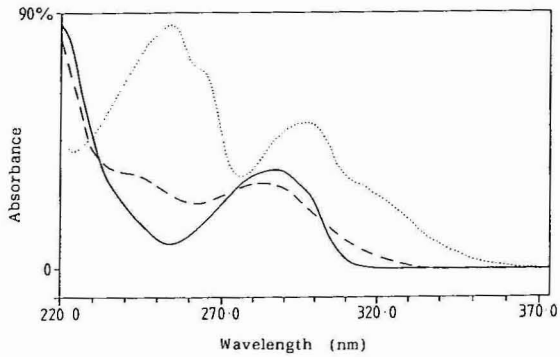
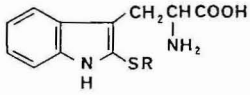


Fig. 3. The UV-absorption spectra of the byproducts (shown in Fig. 1) compared with that of tryptathionine obtained by the photodiode-array detector. ——, Tryptathionine; ...., compound eluted at 12.6 min; – – –, compound eluted at 13.0 min.

TABLE II

SEPARATION OBTAINED BY HPLC OF TRYPTATHIONINE AND ITS ANALOGUES

Basic formula	*R*	*Retention time (min)*
	$CH_2CH(NH_2)COOH$ (tryptathionine)	6.02
	CH_2COOH	8.65
	CH_2CH_2COOH	10.65
	CH_2CH_2OH	11.99
	CH_3	12.43
	CH_2CH_3	13.25

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Note

Fluorescent labelling of amino acids with 9-anthryldiazomethane and its applications to high-performance liquid chromatography

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We have recently developed 9-anthryldiazomethane (ADAM)[1] as a reagent for the fluorescent labelling of carboxylic acids. ADAM has widely been used[2–5] in high-performance liquid chromatography (HPLC) for the precolumn derivatization of biologically significant carboxylic acids such as fatty acids[1–3] and prostaglandins[5]. It can react with carboxylic acids at room temperature without the presence of a catalyst, and even in the presence of water. Accordingly, it was expected to be useful also for the derivatization of carboxylic acids carrying polar residues such as hydroxyl or amino groups. However, preliminary experiments gave only poor yields of amino acid esters. This may be due to the formation of zwitterions between the carboxylic and amino groups. Prevention of the zwitterion formation seemed to be necessary for the improvement of reaction yield. The present study deals with the use of additives, such as sodium dodecyl sulphate (SDS), which intercalate between the amino and carboxylic groups. The resulting improved method for the derivatization was then applied to the separation and determination of amino acids by HPLC.

EXPERIMENTAL

Reagents and chemicals

ADAM was purchased from Funakoshi Yakuhin (Tokyo, Japan), alanine, phenylalanine and SDS from Wako Pure Chemical Industries (Osaka, Japan). Other amino acids, organic amines and arene- and alkenesulphonates were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Non-ionic surfactants were purchased from Nikko Chemicals (Tokyo, Japan), acetonitrile and methanol (for HPLC) from Wako. Other organic solvents were obtained from Kokusan Kagaku (Tokyo, Japan). Water was purified by use of a MILLI-Q water purification system (Millipore, Bedford, MA, U.S.A.) before use.

Reagent solutions

A solution of ADAM (1 mg/ml) was prepared by dissolving the reagent in

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acetone. It was kept in a refrigerator and used within 2 days. Amino acid solutions were prepared by dissolving the acids in water (100 μg/ml), and diluting 2 ml of the solutions in isopropanol to 20 ml. Solutions of additives (0.02 *M*) were prepared by dissolving organic amines, non-ionic surfactants, hydrochloric acid and arene- and alkanesulphonates in isopropanol–water (19:1).

HPLC

The HPLC system consisted of a Model 638 liquid chromatograph (Hitachi, Tokyo, Japan) equipped with an autosampler Model 710B (Waters, Milford, MA, U.S.A.), a RF-530 spectrofluoromonitor (Shimadzu Seisakusho, Kyoto, Japan) and an integrator Model 7000B (System Instrument, Tokyo, Japan). Besides the spectrofluoromonitor, a Model 635M multi-wavelength UV-monitor (Hitachi, Tokyo, Japan) was also used for the detection of samples at higher concentrations.

The separation was performed with a C_{18} reversed-phase column (150 × 4.0 mm I.D.) packed with TSK gel LS-410 (Particle size 5 μm; Toyo Soda, Tokyo, Japan). The column temperature was kept at 50°C. All the chromatographic separations were carried out isocratically using acetonitrile–1% SDS–phosphoric acid (450:550:1) as the mobile phase at a flow-rate of 1.0 ml/min.

Derivatization of amino acids

To 1 ml of a sample solution were added 1 ml of an additive solution and 1 ml of ADAM solution. The mixture was allowed to stand at 50°C for 3 h and then cooled to room temperature. Isopropanol was then added to make exactly 5 ml. An aliquot of 5 μl of the resultant mixture was directly injected for HPLC.

Fluorescence spectra of the ADAM derivative of alanine

To 1 ml of alanine solution (1 mg/ml) was added 1 ml of ADAM solution and 1 ml of 0.02 *M* SDS solution and the resultant mixture was allowed to stand for 3 h at 50°C. An aliquot of 20 μl was injected for HPLC. The fraction containing the ADAM derivative of alanine was collected and extracted with diethyl ether. The whole procedure was repeated several times and the ether layers were combined. The combined ether fraction was separated and evaporated to dryness, and the residue was redissolved in 10 ml of an appropriate solvent. The excitation and emission spectra of the solution were measured with a Shimadzu Model RF-500 fluorescence spectrophotometer equipped with a xenon discharge lamp.

RESULTS AND DISCUSSION

Fluorescent labelling of the amino groups of amino acids for HPLC has extensively been studied using dansyl chloride (Dns-Cl)[6] or *o*-phthalaldehyde (OPTA)[7,8]. On the other hand, fluorescent labelling of the carboxyl groups of amino acids has been hampered because of the lack of suitable reagents. Halogenoalkyl[9–13] or halogenoacyl[14–16] labelling agents, such as 4-bromomethyl-7-methoxycoumarin[11] or 1-bromoacetylpyrene[16] have been used for the esterification of carboxylic acids. However, these reagents have seldom been employed for the derivatization of amino acids because they require anhydrous media and react also with the amino groups to give complex products.

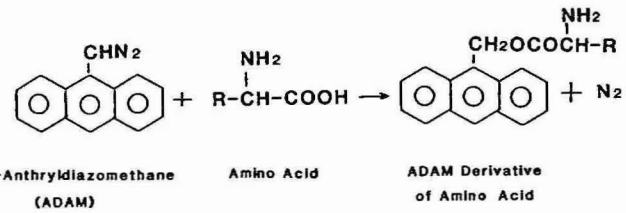

Fig. 1. Esterification of amino acids with ADAM.

ADAM has been proved to be highly reactive with carboxyl groups so that most carboxylic acids can be esterified at room temperature. Protection of the amino groups against alkylation seemed unnecessary under such mild reaction conditions. ADAM was therefore applied to the selective labelling of carboxyl groups in amino acids. Fig. 1 shows the proposed reaction course.

However, the yields of the ADAM derivatives of amino acids were unexpectedly poor. In order to improve the yields, the reaction conditions were reinvestigated using alanine. The effects of organic amines, hydrochloric acid, non-ionic surfactants, arene- and alkanesulphonates and SDS were tested. The amount of alanine derivative formed in each reaction was monitored by HPLC. Hydrochloric acid, alkane- and arenesulphonates and SDS improved the yields, whereas amines and non-ionic surfactants reduced the yields. However, hydrochloric acid was effective only in a very narrow range of concentration. The effects of the sulphonates and SDS were approximately the same. SDS was adopted as the additive as it is readily available.

The influence of the SDS concentration and organic solvents used as the reaction media on the yield of the alanine derivative were investigated. The peak height reached a plateau at SDS concentrations of more than 0.4%. The use of ethanol, isopropanol and acetone gave almost identical yields which were higher than those obtained by the use of other solvents. However, ethanol gave an unknown peak close to the peak of alanine, and acetone showed a large when the UV detector was used. Therefore, isopropanol was routinely used as the solvent for the reaction.

The optimum temperature for the reaction of ADAM with amino acids was found to be 50°C. The relative peak height at 60°C was lower than that at 50°C on account of the decomposition of ADAM. On the other hand, the reaction proceeds only slowly at room temperature and should be allowed to continue for more than 16 h.

Even under the improved reaction conditions, involving the addition of SDS and elevated temperature, amino groups were not affected by ADAM. Tests using

TABLE I

EFFECT OF SOLVENTS ON FLUORESCENCE INTENSITY

Solvent	*Fluorescence intensity*
Acetonitrile	1.0
Acetonitrile–phosphoric acid (1000:1)	2.5
Acetonitrile–water (9:11)	1.3
Acetonitrile–1% SDS–phosphoric acid (450:550:1)	1.7

TABLE II

HPLC CONDITIONS FOR SEPARATION OF AMINO ACIDS

Column	TSK gel LS-410, 150 × 4 mm I.D. ODS, particle size 5 μm
Column temperature	50°C
Mobile phase	Acetonitrile–1% SDS–phosphoric acid (450:550:1)
Flow-rate	1.0 ml/min
Detector	Fluorescence: excitation, 255 nm; emission, 412 nm. UV: 250 nm

ninhydrin and OPTA confirmed that the amino groups of the products remained intact.

The excitation and emission spectra of the ADAM derivative of alanine were measured in various HPLC mobile phases. The spectra were similar to those of other carboxylic acids[1–5] such as fatty acids.

Table I shows the relative fluorescence intensity of the ADAM derivative of alanine in various solvent systems compared with that in acetonitrile. The addition of phosphoric acid was found to enhance the fluorescence intensity. A comparatively high intensity was observed for alanine dissolved in the mobile phase, acetonitrile–1% SDS–phosphoric acid (450:550:1), and the highest intensity was observed in acetonitrile–phosphoric acid (1000:1).

The conditions for chromatographic separation of the ADAM derivatives of amino acids are summarized in Table II. A typical chromatogram of the ADAM derivatives of glycine, alanine and γ-amino-*n*-butyric acid is shown in Fig. 2. Since the ADAM derivatives are relatively polar owing to their free amino groups, their

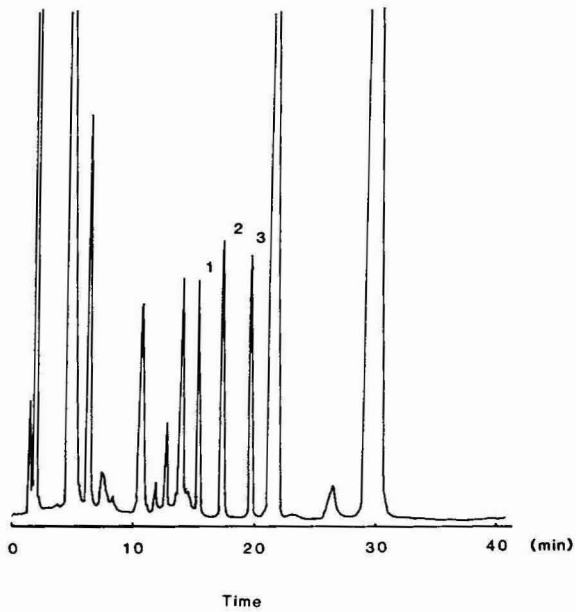

Fig. 2. Chromatogram of the ADAM derivatives of glycine (1), alanine (2) and γ-amino-*n*-butyric acid (3). Each sample solution contained 10 ng of amino acid.

retention times were very close to those of impurities and hydrolysed products of ADAM, and, therefore, they could hardly be separated. Addition of SDS into the mobile phase was found to be effective for the separation of the amino acid esters by increasing their retention times due to the formation of ion pairs. The retention times of impurities and hydrolysed products of ADAM were not so influenced.

A calibration curve for alanine using SDS as the additive was linear in the range 0.02–10 ng, and passed through the origin ($y = 2.539\ x - 0.109$, $r = 0.999$). The detection limit for alanine was about 20 pg per injection (signal-to-noise ratio = 4). The derivatization procedure described here was satisfactorily reproducible for the examined amino acids.

The present study provided a simple and reliable method for fluorescent labelling of the carboxyl groups of amino acids with ADAM. This result seems to extend the use of this reagent to the labelling of carboxylic acids having polar groups.

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Note

Analysis of *tert.*-butylated cresol mixtures by capillary gas chromatography and capillary gas chromatography–mass spectrometry

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The alkylation of cresol with isobutene is a method often used for the production of *tert.*-butylcresols. Mono- and dialkylated cresols as well as cresylic ether are the reaction products. The xylenols, contained in impure cresol, phenol and guaiacol react in the same way. By alkylation with a technical C_4 fraction, the appearance of disubstituted mixed *sec.*-butyl–*tert.*-butyl derivatives can also be expected.

Table I shows the number of isomers of some types of compounds which theoretically can be formed during the alkylation. Even, if the actual number of isomers produced is less, a multicomponent mixture is expected. The complete analysis of the *tert.*-butylation products of cresol has, to our knowledge, hitherto not been reported. Some workers described only the analysis of selected pairs of isomers on packed columns[2]. We decided to use capillary gas chromatography for the analysis of reaction mixtures.

The only way to identify the constituents was to use gas chromatography–mass spectrometry (GC–MS), since boiling point data are known only for a few constituents (Table II), structure–retention relationships are not available to the extent as, *e.g.*, in the case of alkyl aromatics and generally only a limited number of test substances was available. Thus, whereas the different types of compounds may

TABLE I

THEORETICALLY POSSIBLE ISOMERS OBTAINED UPON *tert.*-BUTYLATION OF TECHNICAL CRESOL MIXTURES

Type of compound	*Number of isomers*
tert.-Butyl-phenols	3
-guaiacols	4
-cresols	10
-xylenols	16
Di-*tert.*-butyl-phenols	6
-guaiacols	6
-cresols	14
-xylenols	16
sec.-Butyl–*tert.*-butyl-cresols	28
	113

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TABLE II

BOILING POINTS OF *tert.*-BUTYLATED PHENOLS, CRESOLS AND XYLENOLS[1]

tert.-bu = *tert.*-butyl; *sec.*-bu = *sec.*-butyl; phe = phenol; cre = cresol; xyl = xylenol.

No.	*Compound*	*Boiling point (°C)*
1	6-*tert.*-bu-*o*-cre	231
2	6-*tert.*-bu-*p*-cre	232.7
3	6-*sec.*-bu-*p*-cre	237
4	6-*tert.*-bu-*m*-cre	244
5	6-*sec.*-bu-*m*-cre	246–250
6	4-*tert.*-bu-*o*-cre	246.8
7	4-*tert.*-bu-2,6-xyl	248
8	6-*tert.*-bu-2,4-xyl	249
9	6-*tert.*-bu-2,3-xyl	252
10	2,6-di-*tert.*-bu-phe	253
11	6-*tert.*-bu-3,4-xyl	257
12	4-*tert.*-bu-2,3-xyl	259
13	2,4-di-*tert.*-bu-phe	264
14	4-*tert.*-bu-2,5-xyl	265
15	2,6-di-*tert.*-bu-*p*-cre	265
16	4,6-di-*tert.*-bu-*o*-cre	269
17	2,4,6-tri-*tert.*-bu-phe	278
18	4,6-di-*tert.*-bu-*m*-cre	282
19	4,6-di-*tert.*-bu-2,3-xyl	284

successfully be identified, in most cases their exact isomerism cannot be determined. GC–MS investigations on substituted phenols have been reported by a number of workers, *e.g.*, Hunt *et al.*[3], but *tert.*-butylated cresols were not included in their work.

EXPERIMENTAL

Capillaries of soft glass (60 m × 0.25 mm) were used, which had been pre-treated twice with Carbowax 20M according to Aue *et al.*[4] after treatment with hydrochloric acid. The coating with Carbowax 20M was also carried out according to the dynamic method[5]. A Varian 1800 instrument equipped with a glass evaporator and a flame ionization detector was employed. Hydrogen was the carrier gas, and a temperature of 300°C was maintained in the injection system. The temperature of the column was held at 90°C for 8 min, then raised to 200°C at 4°C/min; the analysis was completed isothermally. The constituents were identified with an Hewlett-Packard 5992 B system.

The following compounds were available as authentic samples: 2-*tert.*-butyl-*p*-cresol; 6-*tert.*-butyl-*m*-cresol; 6-*tert.*-butyl guaiacol; 2,6-di-*tert.*-butyl-*p*-cresol; 4,6-di-*tert.*-butyl-*o*-cresol; 4,6-di-*tert.*-butyl-*m*-cresol. Solid samples were dissolved in a small quantity of methanol. The quantitative evaluation was carried out with a computer via an LEDA interface[6].

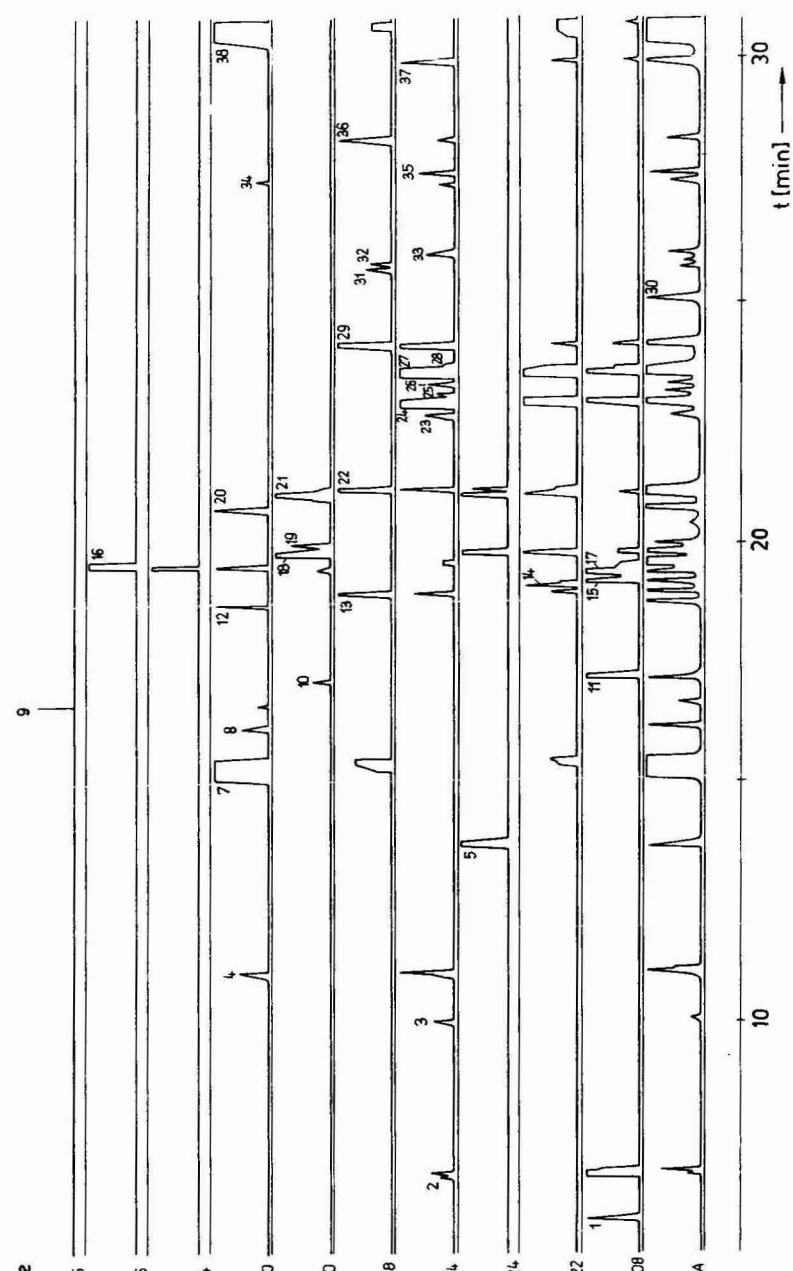

Fig. 1. Mass chromatogram (molecular ions) of a *tert.*-butylated cresol fraction. The peaks are designated according to Table III. The *m/e* values represent the molecular ions of the following compounds (TA = total ionization): 108, cresol; 122, xylenols; 124, methoxyphenols; 164, *tert.*-butylcresols; 178, *tert.*-butylxylenols; 180, methoxy-*tert.*-butylphenols; 220, di-*tert.*-butylcresols; 234, di-*tert.*-butylxylenols; 236, methoxy-di-*tert.*-butylphenols; 276, *tert.*-butoxy-di-*tert.*-butyltoluenes.

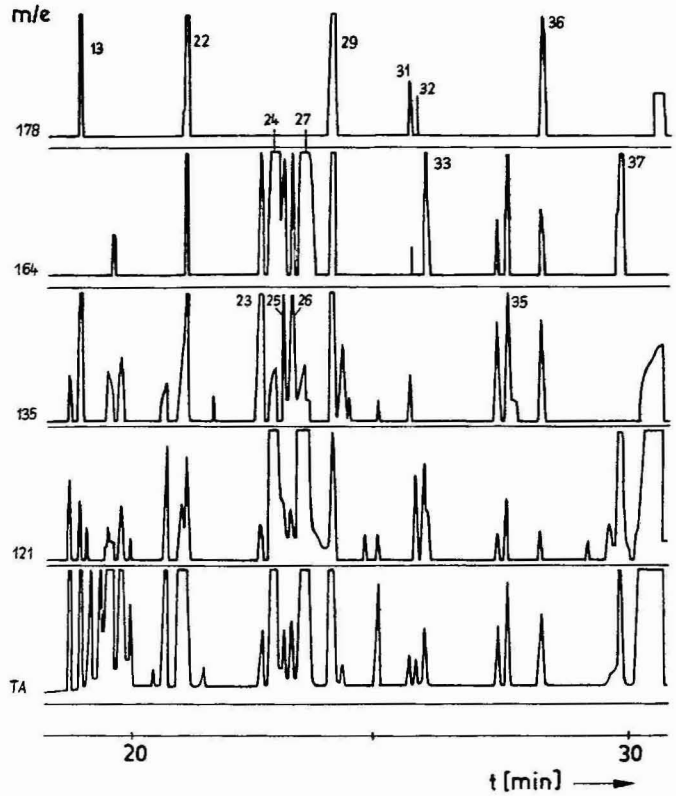

Fig. 2. Mass chromatogram (selected molecular and main fragment ions) of a *tert.*-butylated cresol fraction. The peaks are designated according to Table III (TA = total ionization): 121, main fragment of the *tert.*-butylcresols (M − 43); 135, main fragment of the *sec.*-butylcresols (M − 29); 164, molecular ion of the *tert.*-butylcresols; 178, molecular ion of the *sec.*-butylcresols.

RESULTS AND DISCUSSION

In addition to the molecular peak, the fragment ions M − 15 (basic peak) and M − 43 are significant for the identification of mono- and di-*tert.*-butylated compounds by means of mass spectrometry. In case of *sec.*-butyl–*tert.*-butyl substituted compounds, the fragment M − 29 occurs, whereas *tert.*-butyl–aryl ethers are characterized by the fragment M − 56.

Fig. 1 shows a mass chromatogram in playback representation. The occurrence of some peak overlappings can be seen. For instance, 2,6-di-*tert.*-butyl-*p*-cresol (*m/e* = 220) is eluted together with a *tert.*-butylxylenol (*m/e* = 178), and a di-*tert.*-butylxylenol (*m/e* = 234) and a di-*tert.*-butylmethoxyphenol (*m/e* = 236) are coeluted.

Fig. 2 shows the intensities of the key fragments for *tert.*-butylcresols (*m/e* = 121) and *sec.*-butylcresols (*m/e* = 135) as well as those of the molecular peaks. Both types of derivatives can be clearly differentiated.

Fig. 3. represents a capillary chromatogram of a sample. Table III contains the relative retentions. As expected, compounds with sterically hindered OH groups

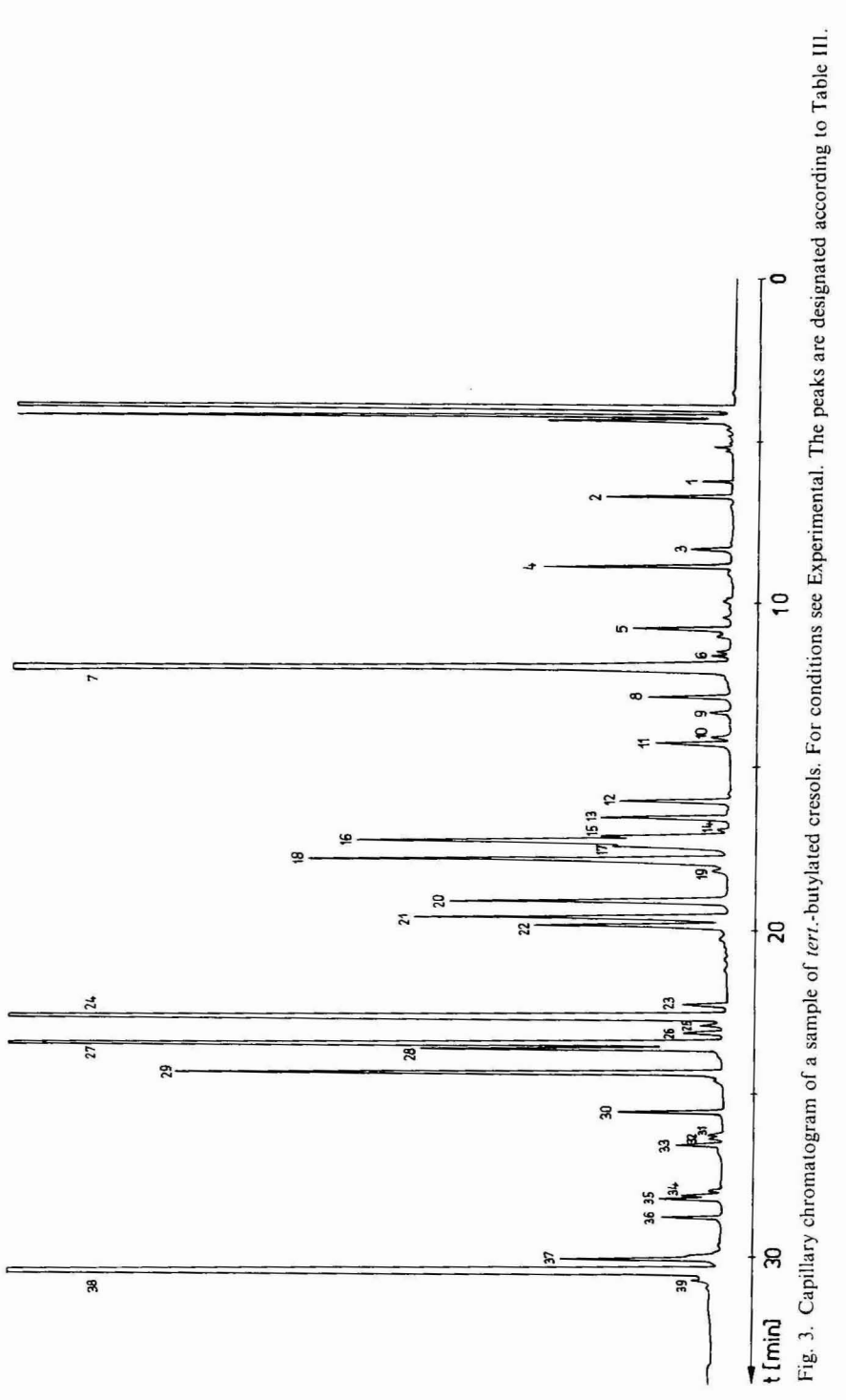

Fig. 3. Capillary chromatogram of a sample of *tert.*-butylated cresols. For conditions see Experimental. The peaks are designated according to Table III.

TABLE III

RELATIVE RETENTIONS

Values are relative to that of 2,6-di-*tert.*-butyl-*p*-cresol, 1.00; for conditions see Experimental. meo = Methoxy; buo = butoxy; bzn = benzene; tol = toluene; gua = guaiacol.

No.	*Substance*	*Relative retention*
1	meo-bzn	0.53
2	*tert.*-buo-tol	0.56
3	*tert.*-bu-cre	0.70
4	*tert.*-buo-*tert.*-bu-tol	0.74
5	Guaiacol	0.90
6	2,4,6-tri-bu-phe	0.92
7	2,6-di-*tert.*-bu-*p*-cre	1.00
8	*sec.*-bu-*tert.*-bu-cre	1.08
9	di-*tert.*-bu-*tert.*-buo-tol	1.13
10	6-*tert.*-bu-gua	1.18
11	*o*-Cresol + 6-*tert.*-bu-*o*-cre	1.19
12	*sec.*-bu-*tert.*-bu-cre	1.33
13	*tert.*-bu-xyl	1.36
14	Xylenol	1.40
15	*p*-Cresol	1.41
16	4,6-di-*tert.*-bu-gua + di-*tert.*-bu-xyl	1.42
17	*m*-Cresol	1.43
18	*tert.*-bu-gua	1.47
19	*tert.*-bu-gua	1.50
20	4,6-di-*tert.*-bu-*o*-cre	1.57
21	*tert.*-bu-gua	1.61
22	*tert.*-bu-xyl	1.63
23	*sec.*-bu-cre	1.83
24	2-*tert.*-bu-*p*-cre	1.85
25	*sec.*-bu-cre	1.87
26	*sec.*-bu-cre	1.89
27	*tert.*-bu-*m*-cre	1.91
28	*tert.*-bu-*o*-cre	1.92
29	*tert.*-bu-xyl	1.98
30	di-*tert.*-bu-phe	2.08
31	*tert.*-bu-cre	2.14
32	*sec.*-bu-xyl	2.15
33	*tert.*-bu-cre	2.17
34	di-*sec.*-bu-cre	2.28
35	*sec.*-bu-cre	2.29
36	*tert.*-bu-xyl	2.34
37	*tert.*-bu-cre	2.43
38	4,6-di-*tert.*-bu-*m*-cre	2.48
39	isooctyl-cre	2.50

(*e.g.*, 2,6-di-*tert.*-butyl-*p*-cresol and 2,4,6-tri-*tert.*-butylphenol) are eluted considerably earlier than would be expected from their boiling points.

For the investigation of such complex mixtures with different qualitative and quantitative compositions the use of the mass-selective detection proved indispensable in many cases in order to identify constituents from their retention data.

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Note

Gas–liquid chromatographic method for the determination of dodine in fruit

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Dodine (*n*-dodecylguanidine acetate, $CH_3(CH_2)_{10}CH_2NH–C(=NH)–NH_3^+CH_3COO^-$) is a fungicide widely used for the control of apple and pear scab, leaf spot in cherry, olive and other crops and many other diseases. Its residues decrease only slowly during storage of treated crops[1], therefore it is necessary to have a suitable method for their determination.

Several methods for dodine determination in biological materials have been developed. The spectrophotometric procedure of Steller *et al.*[2] is very laborious and its sensitivity is relatively low. Dodine, as with other monosubstituted guanidine-containing compounds[3], undergoes hydrolysis in alkaline media yielding the corresponding amine, *n*-dodecylamine[4], which is amenable to gas–liquid chromatography (GLC) after trifluoroacetylation. The disadvantage of this procedure is the long period necessary to complete hydrolysis. In the method developed by Newsome[5], dodine is determined as its volatile substituted pyrimidine, the product of reaction with hexafluoroacetylacetone.

In the procedure described in this paper, dodine is converted into a volatile compound suitable for determination by GLC via reaction with trifluoroacetic anhydride. In comparison with the method mentioned above[5], the derivatization step is very quick and no further clean-up of the reaction mixture is necessary.

EXPERIMENTAL

Chemicals

Dodine analytical standard was supplied by Serva (Feinbiochemica, Heidelberg, F.R.G.). All other chemicals were of reagent grade.

Procedure

A 50-g amount of a representative fruit sample was homogenized for 2 min with 200 ml of methanol–chloroform (2:1, v/v). The homogenate was then filtered through Whatman No. 1 paper on a Büchner funnel using a gentle vacuum. The filter was rinsed with 50 ml of the mixture used for extraction. The filtrate was acidified with 1 ml of concentrated hydrochloric acid and its volume was reduced to 30–40 ml using a vacuum evaporator (water-bath temperature 40°C). The pH of this

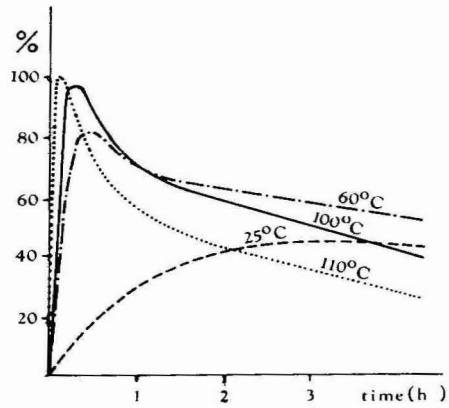

Fig. 1. The influence of the reaction temperature on the yield of dodine derivative.

concentrate was adjusted to 2 by means of hydrochloric acid, followed by extraction with two 25-ml portions of chloroform and one 25-ml portion of hexane. The organic layer was discarded and the aqueous phase was adjusted to pH 11 using sodium hydroxide (4 mol/l). Dodine was then extracted with three 25-ml portions of chloroform. The collected extracts were dried over anhydrous sodium sulphate and then evaporated to dryness under vacuum at 40°C. The residue was dissolved in 0.2 ml of hexane, 0.2 ml of trifluoroacetic anhydride were added and the flask was tightly stoppered. The reaction mixture was heated for 10 min in an oil-bath held at 100°C. After cooling, a solution of hexadecane (internal standard) in hexane was added.

GLC analyses were performed (using an Hewlett-Packard 5880 gas chromatograph) under the following conditions: glass column (1200 × 2 mm I.D.) packed with 3% OV-101 on Chromaton N-AW-DMCS (0.125–0.160 mm); column temperature programmed from 100 to 250°C at 10°C/min; injection port held at 250°C; carrier gas (nitrogen) flow-rate, 30 ml/min; flame ionization detector.

RESULTS AND DISCUSSION

The formation of a volatile derivative of dodine as the result of its reaction with trifluoroacetic anhydride was utilized as described. In order to obtain the max-

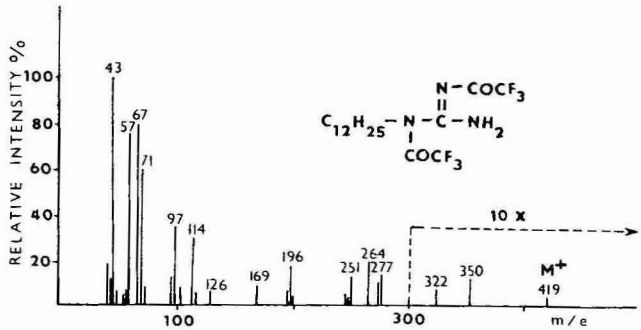

Fig. 2. Mass spectrum of 1-dodecyl-1,2-bis(trifluoroacetyl)guanidine (ionization voltage 70 eV).

imum yield of this substance, various conditions for the derivatization step were studied, see Fig. 1. It is obvious that the compound decomposed when heating (at 100°C) was prolonged, *i.e.*, greater than 10 min. The stability of the dodine derivative at room temperature was satisfactory; in the presence of an excess of trifluoroacetic anhydride, the decrease in the initial content of a model mixture (1 mg of dodine was used for derivatization) was found to be less than 1% after 1 h. Removal of the derivatizing agent resulted in relative rapid decomposition of the dodine derivative.

The mass spectrum of this derivative was obtained by means of gas chromatography–mass spectrometry of a standard solution and as expected, see Fig. 2, 1-dodecyl-1,2-bis(trifluoroacetyl)guanidine was identified. Trifluoroacetic anhydride reacts in a similar way with the guanidino group contained in arginine[6].

Typical gas chromatograms of dodine in apples and pears are shown in Fig. 3. Under the described conditions the time of dodine elution was 8.8–8.9 min; no interfering substances were present in the analysed samples. Efficient clean-up of raw extracts was achieved after their acidification by means of solvent extraction. After making basic the aqueous phase, dodine (in the form of its free base) could be immediately extracted; because of its limited stability under these conditions, pH values higher than 11 should be avoided.

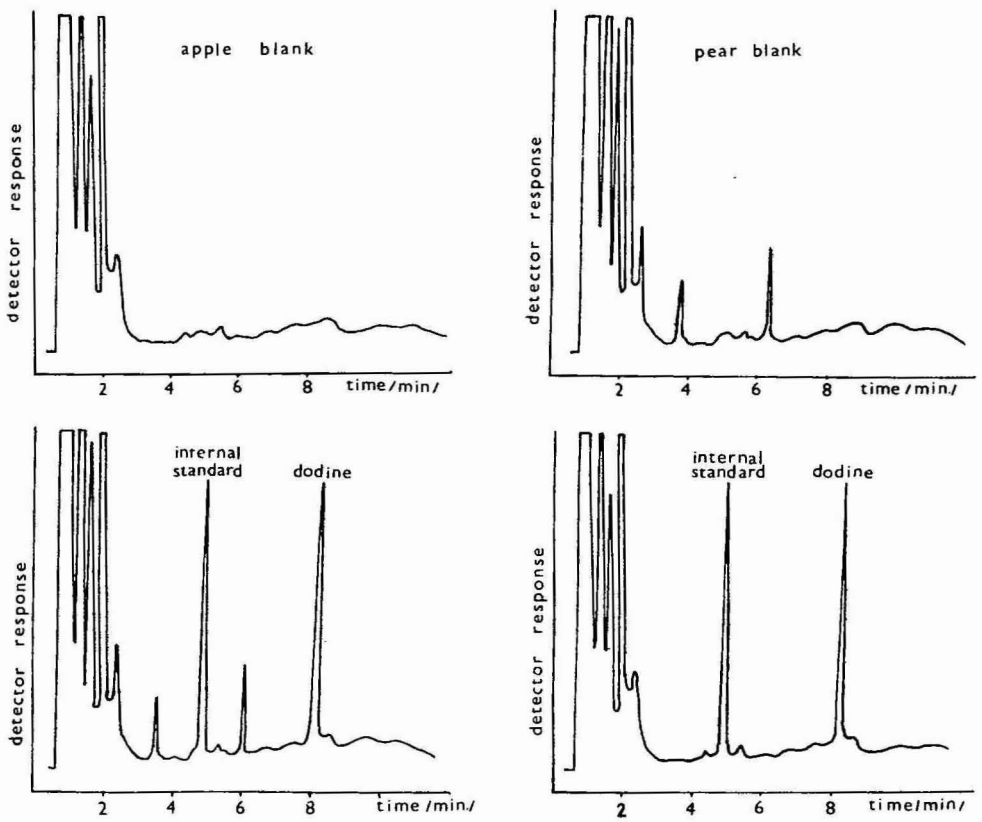

Fig. 3. Gas–liquid chromatograms of dodine in apples (left) and pears (right). Each injection represents the equivalent of 1 mg of sample (0.05 mg/kg of dodine were added).

TABLE I

RECOVERIES OF DODINE FROM APPLES AND PEARS

Apples		*Pears*	
Dodine added (mg/kg)	*Recovery* (%)*	*Dodine added (mg/kg)*	*Recovery* (%)*
0.01	96.5	0.01	94.4
0.05	97.0	0.05	97.6
0.10	99.0	0.10	95.0
0.50	98.6	0.50	98.0
Mean recovery (%)	97.8		96.3
Standard deviation	0.5		0.8

* Values calculated from five parallel determinations.

The average recoveries in the range 0.01–0.5 mg/kg are summarized in Table I. It is evident that a linear relationship exists between the amount of contaminant added to fruits and that determined.

The minimum detectable amount of dodine (signal to noise ratio 2:1) was found to be 0.005 mg/kg when using a flame ionization detector; comparable concentrations could be detected by means of a nitrogen–phosphorus detector. Because of the large amount of trifluoroacetic anhydride present, it was not possible to employ an electron-capture detector.

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Note

High-performance liquid chromatography of triglycerides of Flacourtiaceae seed oils containing cyclopentenyl fatty acids (chaulmoogric oils)

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The seeds of a great number of tropical shrubs and trees belonging to the plant family Flacourtiaceae contain oils that exhibit exceptional dextro-rotatory powers owing to their constituent cyclopentenyl fatty acids[1–4]. These unusual fatty acids are chiefly found as constituents of triacylglycerols, together with straight-chain fatty acids.

During the course of our studies directed towards the elucidation of the biosynthesis of cyclopentenyl fatty acids[5,6], we have developed methods for the identification of straight-chain fatty acids and cyclopentenyl fatty acids in a natural mixture[3,7,8].

Recently we have described a simple and direct high-performance liquid chromatographic (HPLC) method that provides a quick estimate of the triglyceride composition of different oils and fats[9–11]. This paper reports the application of HPLC to the analysis of triglycerides of Flacourtiaceae seed oils.

EXPERIMENTAL

Materials

Reference compounds were obtained from NU-CHEK-Prep (Elysian, MN, U.S.A.) and Larodan Chemicals (Malmø, Sweden).

Seeds of *Caloncoba echinata* and *Hydnocarpus anthelminthica* were obtained from Honolulu Botanical Gardens and from the Harold L. Lyon Arboretum of the University of Hawaii (Honolulu, HI, U.S.A.).

Oil extraction and the fractionation of triglycerides by thin-layer chromatography (TLC) were performed as reported earlier[3].

Analytical techniques

The HPLC system consisted of a Spectra Physics (SP 8000) chromatograph, coupled with a Pye Unicam LC-UV detector at 220 nm, and a Rheodyne loop (10 μl) injector (model 7120). A Knauer differential refractometer model 98,00 was used

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for refractive index measurements. An SP 8000 electronic integrator was used to obtain accurate retention times at a chart speed of 0.25 cm/min. The columns used for the separations consisted of two 150 × 4.5 mm I.D. Spherisorb S3 ODS 2 (Phase Sep UK) arranged in series and packed with 3 μm C_{18} bonded phase particles. The columns were maintained at 20°C by coupling the column oven with Hetofrig cooling system (type 03 PF 623 CB 11).

The mobile phase consisted of acetonitrile–tetrahydrofuran (68:32, v/v), both of "HPLC Grade" (Rathburn, Walkerburn, U.K.). Tetrahydrofuran was used without an inhibitor. The mobile phase was prepared by adding appropriate known volumes of each solvent to a flask and was degassed ultrasonically. The compositions reported are volume per cents. The two columns in series produced *ca.* $3.5 \cdot 10^3$ theoretical plates as measured for CCC triglyceride with a total system pressure of $2.5 \cdot 10^3$ p.s.i. at 1.5 ml/min, the usual flow-rate used. The column void volume was *ca.* 3.0 ml.

The sample size was 5–10 μl of *ca.* 10% solutions of triglycerides of *H. anthelminthica* and *C. echinata* in tetrahydrofuran.

Triglyceride peaks were collected from the HPLC effluent and analysed as their methyl esters by gas chromatography (GC). The methyl esters were prepared as described earlier[12]. Analysis of the methyl esters were carried out on a Chrompack fused-silica Silar 10 CP (0.2 μm) wall-coated open-tubular column (50 m × 0.22 mm I.D.). The instrument used was a Perkin-Elmer Sigma 2 gas chromatograph equipped with a flame ionisation detector. The data presented were recorded isothermally at 200°C with helium as carrier gas. The injector (split) and detector temperatures were maintained at 250°C. Quantitation was achieved by integration with a Hewlett-Packard Model 3390 A reporting integrator and comparison with standard mixtures. Triglyceride compositions of the eluted peaks were determined by evaluation of the

TABLE I

CONSTITUENT FATTY ACIDS OF TRIACYLGLYCEROLS FROM SEED OILS OF FLACOURTIACEAE

Analysed as methyl esters by capillary GC; traces below 0.1 wt% are omitted; "cy" denotes the cyclopentene structure of the acid.

Chain length: No. of double bonds	*Caloncoba echinata (weight %)*	*Hydnocarpus anthelminthica (weight %)*
14:1	0.2	0.2
14:1 cy	1.0	0.2
16:0	11.0	5.3
16:1	0.2	1.3
16:1 cy	1.4	57.1
18:0	0.4	0.5
18:1	2.5	3.5
18:2	1.4	0.9
18:1 cy	61.7	26.3
18:2 cy	19.2	3.6
20:1 cy	0.2	0.4
20:2 cy	0.4	0.3

methyl ester composition of each eluted peak. The triglyceride abbreviation CCG means that it is composed of two molecules of chaulmoogric acid (18:1 cy) and one molecule of gorlic acid (18:2 cy).

RESULTS AND DISCUSSION

The results from the fatty acid methyl esters of the two oils studied are presented in Table I. They show a slight deviation compared with the earlier publications[3,13]. This change in the fatty acid pattern is due to the different times of harvest and maturation. However, the characteristics of the oils *i.e.* rather high amounts of gorlic acid in *C. echinata* and high proportions of hydnocarpic acid in *H. anthelminthica* are clearly evident from the values shown in the Table I.

The complete separations of triglycerides of *C. echinata* and *H. anthelminthica* are shown in Figs. 1 and 2, respectively. The total analysis time until the elution of

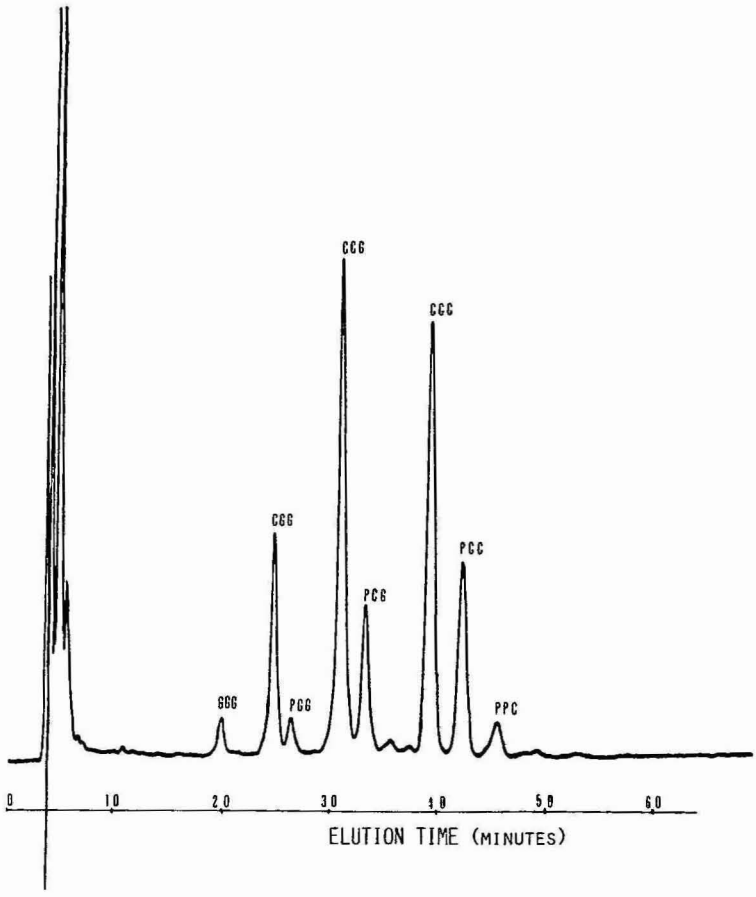

Fig. 1. Separation of triglycerides in *Caloncoba echinata* using two 150 × 4.5 mm I.D. Spherisorb S 3 ODS 2 columns and acetonitrile–tetrahydrofuran (68:32, v/v) as mobile phase at 1.5 ml/min; inlet pressure, 2500 p.s.i. at 20°C; UV detection at 220 nm. Abbreviations: P = palmitic acid; O = oleic acid; H = hydnocarpic acid 16:1 cy; C = chaulmoogric acid 18:1 cy; G = gorlic acid 18:2 cy.

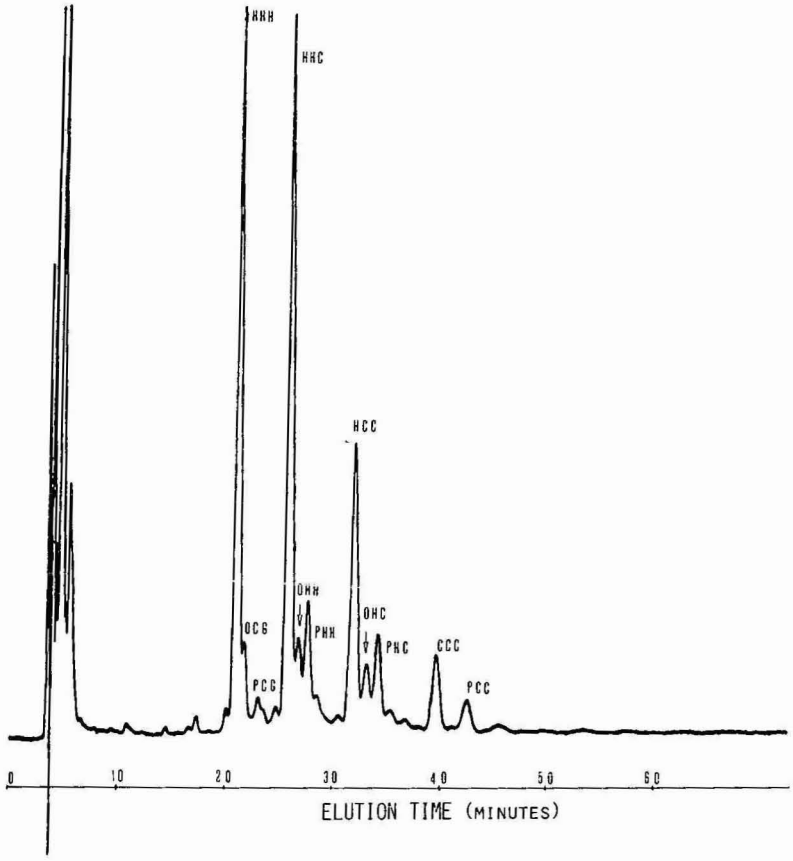

Fig. 2. Separation of triglycerides in *Hydnocarpus anthelminthica*; conditions and abbreviations as in Fig. 1.

PPC is *ca.* 45 min. The chromatograms of the purified triglycerides as presented here are much cleaner than those of the original oils owing to the removal of the early-appearing polar components with higher molar absorptivities. The structural elucidation of triglycerides was achieved by collecting each peak and analysing the respective methyl esters by GC.

In the case of *H. anthelminthica* the collection of sufficient material for analysis of OCG, PCG and OHH was not possible owing to the small amounts available; they were thus tentatively identified by linear logarithming.

The triglyceride compositions of the two oils are shown in Table II. Although Table I shows the presence of a total of 11 straight chain and cyclic fatty acids in each of the two oils only eight different triglyceride species were detected above a level of 1%. However, the present method thus far does not differentiate between different triglyceride isomers such as CCG, GCC and CGC: therefore individual peaks represent mixture of isomers.

The prevalence of C_{18} cyclic fatty acids in *C. echinata* is reflected by the exclusive contribution to the major triglyceride species CCG, CCC and CGG of this

TABLE II

MOLECULAR SPECIES OF TRIACYLGLYCEROLS FROM FLACOURTIACEAE SEED OILS

Traces (below 0.5%) are omitted; abbreviations as in Fig. 1.

Triacyl-glycerol (or their isomers)	*Relative retention time*	*Caloncoba echinata (mole %)*	*Hydnocarpus anthelminthica (mole %)*
GGG	0.97	2.9	
HHH	1.00		39.0
OCG	1.05		0.7
PCG	1.12		0.7
CGG	1.20	17.1	
HHC	1.24		27.1
PGG	1.29	1.4	
OHH	1.30		0.6
PHH	1.34		2.3
CCG	1.51	30.2	
HCC	1.55		15.1
OHC	1.62		3.2
PCG	1.64	7.2	
PHC	1.67		4.1
CCC	1.94	26.2	4.1
PCC	2.11	10.8	1.5
PPC	2.29	1.7	

oil. In *H. anthelminthica* the predominance of the C_{16} cyclic fatty acid is shown by its occurrence in the major triglycerides HHH, HHC and HCC. The triglycerides containing C_{20} cyclic fatty acids were not detected because of the presence of very low concentrations (Table I). The triglycerides containing only straight-chain fatty acids as well as those containing two straight-chain fatty acids were absent from both the oils.

A comparison of retention volumes of triglycerides containing cyclopentenyl fatty acids with those of straight-chain fatty acids show that CCC is equal to OOO, PCC is equal to POO and PPC is equal to PPO, respectively. Thus the chromatographic behaviour of triglycerides containing cyclopentenyl fatty acids and straight-chain fatty acids with the same number of double bonds is quite similar.

The triglyceride composition of *H. wightiana* oil, a plant related to *H. anthelminthica*, has been studied by lipolytic methods[14] and by GC of the hydrogenated oil[15]. Both methods show the presence of HHC and HHH as major triglycerides.

The major advantage of non-aqueous reversed-phase HPLC lies in the fact that it separates the triglyceride critical pairs that are not separated in TLC[16] and GLC[15] techniques. The one-step HPLC method offers simplicity, speed and ease of operation. Since HPLC is a non-destructive analytical technique it allows the collection of pure triglycerides containing cyclopentenyl structures to be used as standards for further identification purposes by complementary methods.

This appears to be the first report in the literature describing the separation of triglycerides of cyclopentenyl fatty acids by HPLC. An early appearance of these triglycerides will be of great help in identifying contaminations of edible oils with

chaulmoogric oils. The latter appear to be poisonous when ingested[1]; however, these oils and their constituent fatty acids have been used for the treatment of leprosy, tuberculosis and other skin diseases[17].

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Note

High-performance liquid chromatography of caffeoylquinic acid derivatives of *Cynara scolymus* L. leaves

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The choleraic and cholagogic action of *Cynara scolymus* L. extracts is already known[1]. Research is at present being carried out into the hepatotropic activity of such extracts, when faced with acute intoxication by carbon tetrachloride. This activity has always been attributed to the phenolic compounds present in the extract, and in particular to its caffeoylquinic derivatives[2,3]. These compounds are highly unstable[4,5] and during extraction may undergo isomerization through hydrolysis and posterior intramolecular transesterification[6–8]. This implies modifications in the composition of the extracts, depending on whether they are in aqueous or alcoholic solution, in which enzymatic activity is inhibited[9,10].

Many papers on the analysis of phenolic compounds have been published[11–15]. The aim of the present study was to develop a technique capable of analyzing all caffeoylquinic derivatives in any vegetable extract which contains phenol acids and flavonoids.

EXPERIMENTAL

Chromatography

A Perkin-Elmer Series 2 liquid chromatograph, equipped with a spectrophotometric detector LC-85 and a Sigma 15 chromatographic data station, was used. The column was a stainless-steel tube (15 cm × 4 mm I.D.) packed with Spherisorb C_{18} (Supelco, Bellefonte, PA, U.S.A.) having an average particle size of 5 μm. A short stainless-steel precolumn, packed with Spherisorb C_{18} Superguard (5 μm), was used. The UV detector was set at 325 nm.

Solvents were filtered using a 0.45-μm Millipore filter and degassed at room temperature under vacuum with magnetic stirring.

Two solvents were used: A, methanol–acetic acid (97.5:2.5); B, water–acetic acid (97.5:2.5). The linear gradient was from 13% A to 43% A in 30 min. The flow-rate was 1.6 ml/min, the temperature was 40°C and the column pressure was 1500 p.s.i.

Reagents

Phenols commercially available were obtained from Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland) and Sarsyntex (Merignac, France).

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Neochlorogenic and cryptochlorogenic acids were obtained through isomerization of chlorogenic acid according to procedure used by Walkowski[16].

1,3-Dicaffeoylquinic acid was isolated from the artichoke following a modified procedure reported by Michaud[9].

The isomers of isochlorogenic acid (3,4-, 3,5- and 4,5-dicaffeoylquinic acids) were separated from coffee by the method of Barnes *et al.*[17].

All the above-mentioned products were isolated by means of semipreparative high-performance liquid chromatography (HPLC)[18]. They were identified by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry[19,20].

Quantitative calibration

Each phenolic compound was examined by HPLC in order to determine its retention time, order of elution and capacity factor (Table I). α-Naphthol (Merck, Darmstadt, F.R.G.) was used as internal standard (retention time, t_R, 25.02).

A calibration graph of peak area against weight was obtained for each caffeoylquinic acid derivative and flavonoid, in the range of 1–200 μg. A linear relationship was obtained in each case.

Samples

Standards of caffeoylquinic acids and flavonoids were dissolved in methanol.

The purification method used for the artichoke extracts depended on whether aqueous (a) or alcoholic (b) solutions are used.

(a) A dried artichoke sample (10 g) was extracted with boiling water for 2 h, the process being repeated twice. The extract was filtered while warm, concentrated, diluted in a minimum of water and precipitated with methanol (10 volumes); this was repeated twice. The methanolic fractions were dried *in vacuo*.

(b) A similar sample was extracted warm (at 75°C) with 70% methanol for 2 h, three times. The extract was filtered while warm, concentrated and diluted in 50% methanol. It was subsequently extracted with light petroleum (b.p. 40–60°C)–diethyl ether (2:1). The aqueous alcoholic fraction was dried *in vacuo*.

It was shown that in both cases the purification processes do not modify the extract composition.

The dry residues were redissolved in 70% methanol, the volume was adjusted to 50 ml with methanol, and mixed with 10 ml of a 0.84 mg/ml solution of α-naphthol in methanol. After filtration (Millipore 0.45-μm filter), 3–5 μl of the solution were injected.

RESULTS AND DISCUSSION

The mobile phase was selected by examining different series of solvents. It was noted that the selectivity of methanol–water mixtures was hardly affected by the introduction of tetrahydrofuran or acetonitrile.

The simultaneous analysis of compounds with marked differences in polarity (mono- and dicaffeoylquinic derivatives, flavonic glycosides and aglycones) requires the use of a gradient programme. An identical concentration of acetic acid was maintained in the two pumps, to ensure a constant pH, independent of the gradient conditions. The reproducibility of the analysis was verified by use of 2.5% acetic acid[21].

Effect of structure on retention

Table I shows the retention times of the phenolic compounds. The values are averages from five analyses, the error being about 1.5%.

TABLE I

RETENTION TIMES, t_R, AND CAPACITY FACTORS, k', OF CAFFEOYLQUINIC ACID DERIVATIVES AND FLAVONOIDS

Compound	t_R(*min*)	k'
Neochlorogenic acid	2.11	0.81
Cryptochlorogenic acid	4.21	2.62
Chlorogenic acid	4.44	2.82
Caffeic acid	5.11	3.40
1,5-Dicaffeoylquinic acid	6.58	4.67
3,4-Dicaffeoylquinic acid	16.02	12.81
3,5-Dicaffeoylquinic acid	17.22	13.84
Luteolin-7-glucoside	17.50	14.08
1,3-Dicaffeoylquinic acid	18.18	14.67
4,5-Dicaffeoylquinic acid	21.35	17.40
Luteolin	28.57	23.62
α-Naphthol	25.02	20.56

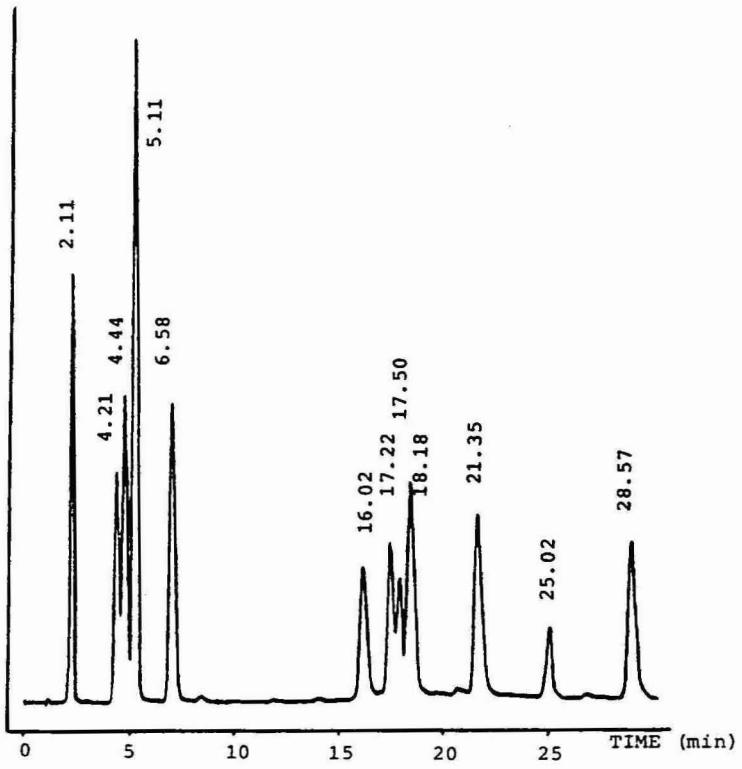
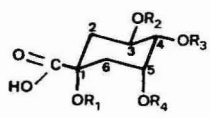
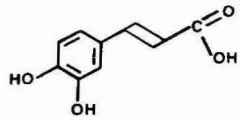


Fig. 1. The retention times of caffeoylquinic acid derivatives and flavonoids eluted using the linear gradient on a Spherisorb C_{18} column. A list of the compounds separated is given in Table I. For the elution system see Experimental.

The order of elution of the different groups is in accord with previous results[12]: monocaffeoylquinic acids, caffeic acid, dicaffeoylquinic acids, flavonic glycosides and flavones (Fig. 1). As verified by Court[22], the introduction of a caffeoyl group increases the retention of a molecule; however, the monocaffeoylquinic derivatives are eluted before the caffeic acid which may be considered to be more polar.

The chromatographic retention of the monocaffeoylquinic isomers is in agreement with the results obtained by Krause and Strack[23] and Nagels *et al.*[24], but not those described by Court[22]. The first to elute are the isomers that have axial caffeoyl substituent (pseudochlorogenic and neochlorogenic acids), followed by those with an equatorial caffeoyl moiety (criptochlorogenic and chlorogenic acids) (Fig. 2). The above effect was observed experimentally by Haslam *et al.*[7] for *p*-coumaroylquinic derivatives; compounds having the hydroxycinnamic moiety in positions 1 and 5 exhibit increased solubility in water.





Caffeic acid

	R_1	R_2	R_3	R_4
Quinic acid	H	H	H	H
1-O-Caffeoylquinic acid (pseudochlorogenic)	Caffeoyl	H	H	H
3-O-Caffeoylquinic acid (chlorogenic)	H	Caffeoyl	H	H
4-O-Caffeoylquinic acid	H	H	Caffeoyl	H
5-O-Caffeoylquinic acid (neochlorogenic)	H	H	H	Caffeoyl
1,5-O- Dicaffeoylquinic acid (cynarin)	Caffeoyl	H	H	Caffeoyl
1,3-O-Dicaffeoylquinic acid	Caffeoyl	Caffeoyl	H	H
3,4-O-Dicaffeoylquinic acid (isochlorogenic)	H	Caffeoyl	Caffeoyl	H
3,5-O-Dicaffeoylquinic acid (isochlorogenic)	H	Caffeoyl	H	Caffeoyl
4,5-O-Dicaffeoylquinic acid (isochlorogenic)	H	H	Caffeoyl	Caffeoyl

Fig. 2. Structures of caffeoylquinic acid derivatives.

In the case of dicaffeoylquinic derivatives, whereas cynarin (1,5-dicaffeoylquinic acid, axial-axial) is eluted faster than the rest of isomers, 3,4-dicaffeoylquinic acid (equatorial-equatorial) which should be the most strongly retained is eluted before 1,3-dicaffeoylquinic acid (axial-equatorial) and 3,5- and 4,5-dicaffeoylquinic acids (both equatorial-axial) (Fig. 2).

Quantitative analysis

The analysis of the alcoholic extracts, in which the initial composition of the

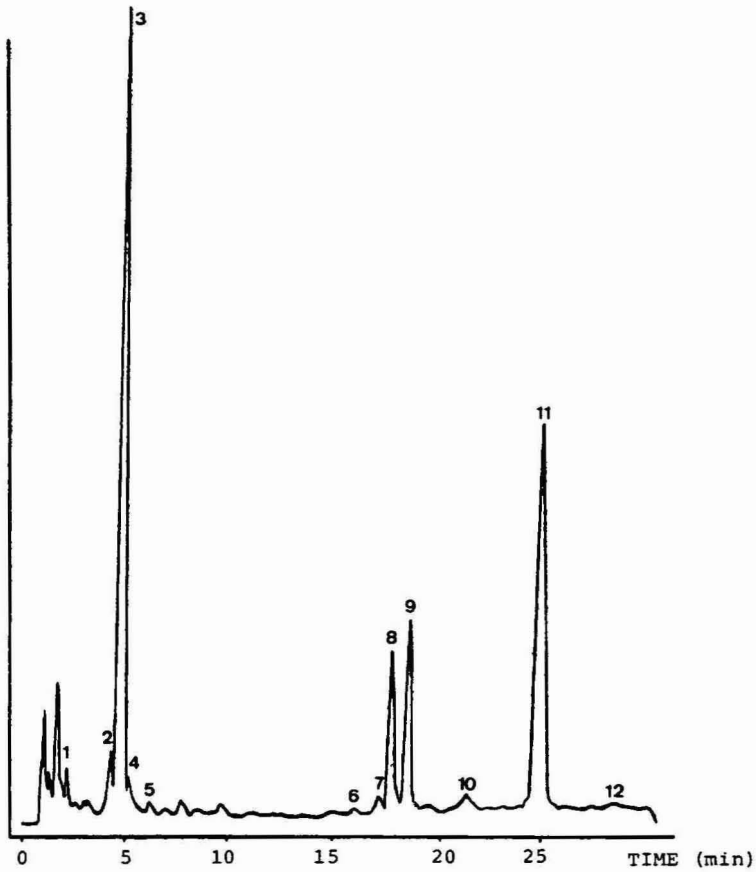


Fig. 3. The separation of caffeoylquinic acids and flavonoids in an alcoholic extract of artichoke using the linear gradient. Peaks: 1 = neochlorogenic acid; 2 = cryptochlorogenic acid; 3 = chlorogenic acid; 4 = caffeic acid; 5 = 1,5-dicaffeoylquinic acid; 6 = 3,4-dicaffeoylquinic acid; 7 = 3,5-dicaffeoylquinic acid; 8 = luteolin-7-glucoside; 9 = 1,3-dicaffeoylquinic acid; 10 = 4,5-dicaffeoylquinic acid; 11 = α-naphthol; 12 = luteolin. For the elution system see Experimental.

drug is maintained, shows the main components to be chlorogenic acid, 1,3-dicaffeoylquinic acid and luteolin-7-glucoside, with the other caffeoylquinic derivatives in much lower amounts (Fig. 3).

In a warm aqueous medium, isomerization phenomena occur, leading to a variation in the extract composition (Fig. 4). There is an increase in neochlorogenic and criptochlorogenic acids as a result of the decrease in chlorogenic acid[25]. On the other hand, the decrease in 1,3-dicaffeoylquinic acid results in an increase in 1,5-dicaffeoylquinic acid (cynarin) and the isomers of isochlorogenic acid (Table II).

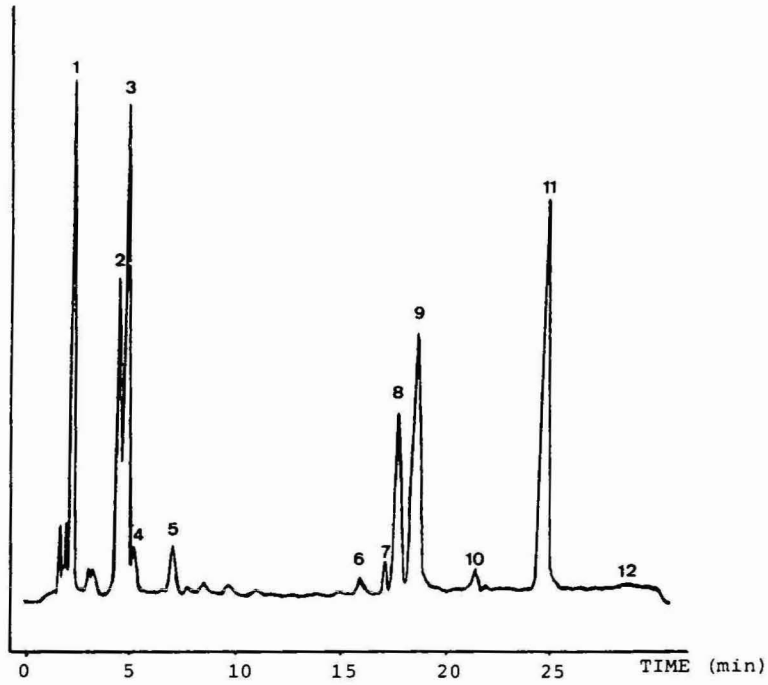


Fig. 4. The separation of caffeoylquinic acids and flavonoids in an aqueous extract of artichoke using the linear gradient. Details as in Fig. 3.

TABLE II

PERCENTAGES OF PHENOLIC COMPOUNDS IN AQUEOUS AND METHANOLIC EXTRACTS OF THE ARTICHOKE

Values are relative to total polyphenol content.

Compound	*Aqueous extract*	*Methanolic extract*
Neochlorogenic acid	19.50	1.30
Cryptochlorogenic acid	23.03	3.02
Chlorogenic acid	30.02	73.80
Caffeic acid	1.31	0.63
Cynarin	3.83	1.13
3,4-Dicaffeoylquinic acid	1.12	0.50
3,5-Dicaffeoylquinic acid	1.55	0.90
1,3-Dicaffeoylquinic acid	6.98	7.30
4,5-Dicaffeoylquinic acid	1.75	1.01
Luteolin-7-glucoside	10.98	9.83
Luteolin	0.18	0.15

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Addendum

In reference to the article "Less hazardous derivatization procedure for gas chromatography of plant hormone abscisic acid." published in the *Journal of Chromatography*, Vol. 325 (1985) p. 425; Dr. T. L. Davenport requests us to note that some of the work was funded by the Binational Agricultural Research and Development Fund (BARD) Research Project 1-44-81, during the 2.5 months that D. Tietz worked for the University of Florida, Institute of Food and Agricultural Sciences.

PUBLICATION SCHEDULE FOR 1986

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1985	N 1985	D 1985	J	F	
Journal of Chromatography	346 347/1	347/2 347/3 348/1	348/2 349/1 349/2 350/1 350/2	351/1 351/2 351/3	352 353 354	The publication schedule for further issues will be published later
Chromatographic Reviews						
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Biomedical Applications				374/1 374/2		

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 329, No. 3, pp. 449–452. A free reprint can be obtained by application to the publisher.)

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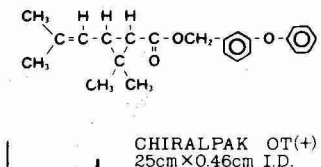

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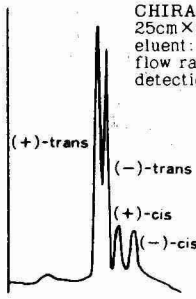
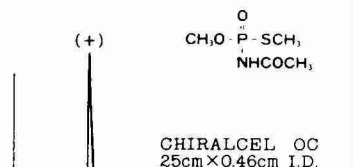


J. Am. Chem. Soc., 103, 6971 (1981)



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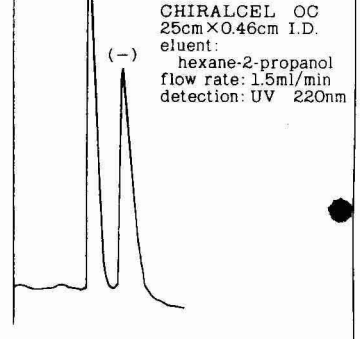

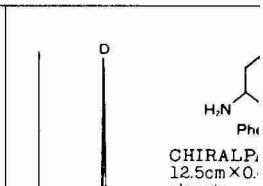

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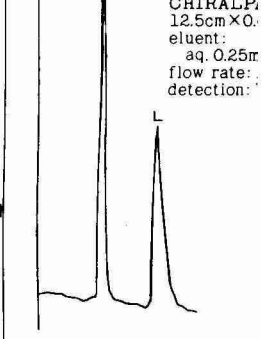
detection:



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