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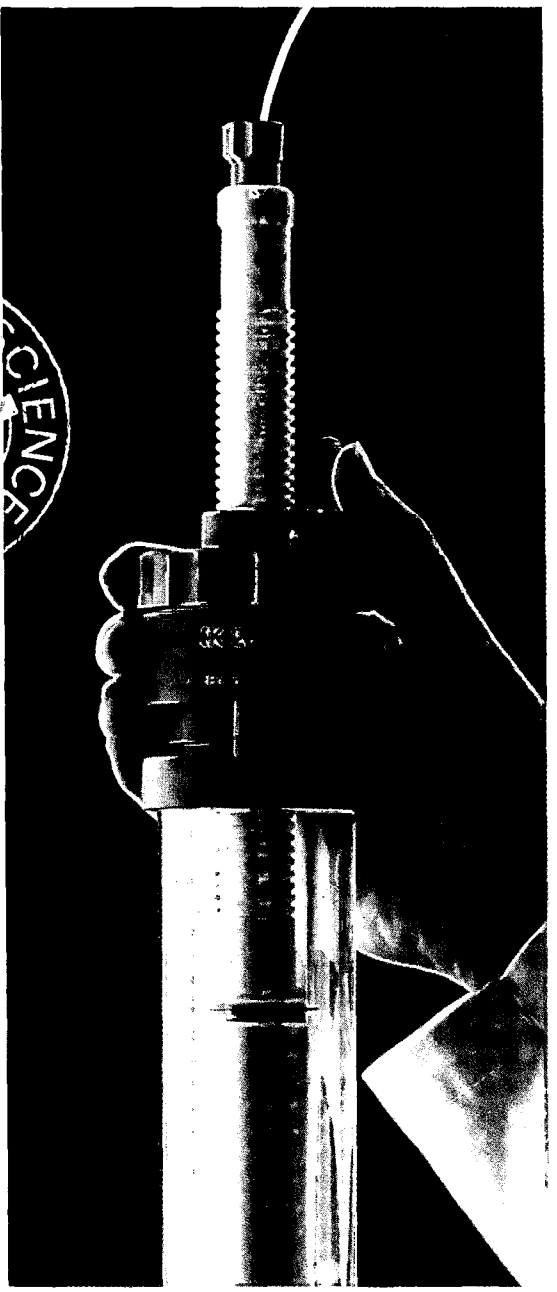

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Experimental Design: A Chemometric Approach

by **S.N. Deming,** *University of Houston, Houston, TX, USA*
and **S.L. Morgan,** *University of South Carolina, Columbia, SC, USA*

(Data Handling in Science and Technology, 3)

One of the greatest needs in all areas of competitive research and development is an efficient approach to experimentation. To be effective scientists, engineers or managers, individuals today require a clear understanding of the principles of experimental design. This book - unlike most current textbooks - approaches experimental design from the point of view of the experimenter, rather than that of the statistician. It provides a practical approach to experimental design and allows the reader to obtain the required information in the minimum number of experiments.

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OPTICAL ERRORS IN A LIQUID CHROMATOGRAPHY ABSORBANCE CELL

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(Received April 19th, 1988)

SUMMARY

The shadowgraph method is used to visualize mobile phase flow through a liquid chromatography absorbance cell with "Z" flow geometry. It is shown that mixing is incomplete in the cell. Composition gradients persist throughout the elution of any sample. The gradients systematically change as the sample flows through the cell. Additionally, window wedge angles are measured in several cells. The implications for low noise absorbance detectors are discussed.

INTRODUCTION

The detection method most widely used in liquid chromatography (LC)[1,2] is ultraviolet–visible (UV–VIS) absorption. The sensitivity of most absorption spectroscopic measurements is limited by intensity fluctuations caused by various experimental problems, and is usually well above the electronic shot noise limit. Modern mercury, zinc, or cadmium lamps are sufficiently intense to yield shot noise limits equivalent to 10^{-7} absorbance unit (a.u.). With modern deuterium lamps, the shot noise limit is about 10^{-6} a.u. But the current detection limits for absorbance measurements are in the order of 10^{-5} a.u. Therefore, it is important to identify and eliminate or compensate for excess noise sources.

One common noise source is mobile phase impurities which absorb at detection wavelengths. These impurities are present at relatively high levels in commercial solvents and in other chemicals used as components of the mobile phase. Therefore, careful purification of the solvents or the use of chromatographic-grade solvents are necessary to achieve good sensitivity.

Another noise source is fluctuation of mobile phase refractive index during measurement. Commonly, this arises from temperature and composition gradients in the mobile phase. The temperature gradient causes a refractive index variation, which in turn appears as noise in the absorption measurement. To achieve shot noise detection limit on the order of 10^{-7} a.u. requires controlling the temperature of the mobile phase to ± 0.004°C at 195 nm or ± 0.4°C at 254 nm (ref. 2). Such precise control would require much more sophisticated flow cell designs and thermostatting than are

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currently employed. In addition, a refractive index change can also be caused by variations in mobile phase flow-rate or pressure, and solvent gradients due to incompletely mixed solvents. The solvent gradient profile itself in gradient elution LC is another noise source[3].

The design of the flow cell and the properties of a photodiode contribute to excess noise. Any change in refractive index at the window of the flow cell or within the cell itself causes light rays in the cell to be refracted. Some of the light which would normally pass directly through the cell strikes the cell wall instead and is lost. The detector cannot distinguish light lost by sample absorption from light lost to cell walls.

To prevent the light from hitting the cell wall, Little and Fallick[4] designed a cell with diverging walls to accommodate much of the refraction. In principle, with this configuration all of the light which enters the cell should leave the cell, unless there is true absorption. More recent studies of chromatographic flow cells have been aimed at maximizing the light throughput in a small working volume[5,6].

But even the light rays that do not hit the cell wall are deflected from one point on the photodiode to another by refractive index gradients. Most photodiodes have inhomogeneity in spatial response inversely proportional to the illuminated area. For a 0.13-mm spot, the response inhomogeneity may be as much as 1% (ref. 7). To the extent that the mobile phase is uniform and at constant temperature and pressure, there are no refractive index gradients when pure mobile phase is flowing. But, in gradient elution measurements or in high-sensitivity isocratic measurements, deflection itself becomes another noise sources which may increase detection limits. While sample is flowing through the cell, deflection can generate small systematic errors in measured absorbance.

A typical chromatographic flow cell has a tapered design and a Z-shaped flow path. The Z-path is used to minimize stagnant regions in the cell and to reduce peak tailing[4]. Variants on this design have largely replaced straight-through capillaries or U shaped flow paths in commercial LC instruments. Typically, the flow is confined by two quartz windows held in place by caps screwed though or into the cell body. Mobile phase flows in from the bottom of the cell, hits the cell window and is directed through the body of the cell, which is aligned along the light path. The flow hits the window on the other side of the cell and then is forced out of the top of the cell.

The design minimizes stagnant regions, but introduces new problems. First, it is easy to assemble the cell so that the windows are wedged, deflecting the entire light beam. However, unless the screws used to hold the cell windows in place are carefully adjusted, the cell windows will usually be wedged with respect to the cell body. More importantly, the Z path itself leads to complicated time-dependent mixing patterns in the cell.

A wedged cell behaves like a prism[8], its influence can be seen from eqn. 1 and Fig. 1.

$$\delta = \theta_i + \sin^{-1}[(\sin \alpha)\,(n^2 - \sin^2 \theta_i)^{1/2} - \sin \theta_i \cos \alpha] - \alpha \qquad (1)$$

where n is the refractive index. Fig. 1 shows the deviation angle, δ, *versus* incident angle, θ_i, for sample cell wedged at two different angles, α. It is clear from the graphs that at different incident angles the curves have different slopes. The incident angle can vary by improper assembly or mounting of the cell or even by prolonged operation.

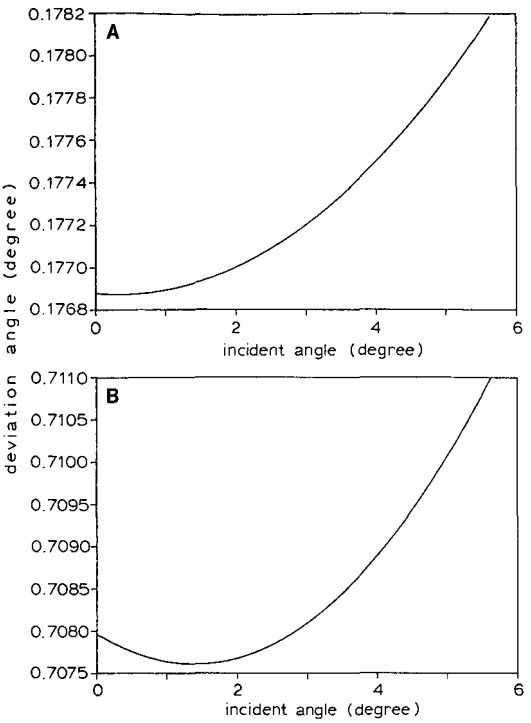

Fig. 1. Effect of cell angle on beam deviation in a wedged cell. (A) 9 mrad wedge angle; (B) lower graph 36 mrad wedge angle.

The temperature of the assembly may vary during operation because of heat dissipated from the electronic circuits or by the illumination with the light source. The temperature change can cause the beam to wander.

It is useful to observe these effects in a chromatographic flow cell in order to estimate their importance and to guide further designs. Flow visualization is an important experimental tool of fluid mechanics[9]. The range of flow visualization techniques is broad, but these techniques have been developed for observation of flow in or around large structures such as chemical reactor pipes or automobiles. At least three flow visualization techniques can be adapted to the scale of a LC flow cell. While the general optical principles remain unchanged, special techniques are required to observe refractive index gradients in a cell whose diameter is about 1 mm.

Mach–Zehnder interferometry is based upon the interference of object light passing through the flow cell and reference light in a stationary medium to create a visible intensity distribution. The Mach–Zehnder interferometer measures the change of refractive index ∂n or the distribution of refractive index directly. In principle, it produces a more direct measurement than the more common schlieren or shadow methods which measure $\partial n/\partial x$ and $\partial^2 n/\partial x^2$ respectively.

The schlieren method measures directly refractive index gradients, using a knife edge as a spatial filter. It is probably the most familiar technique for rendering weak phase objects visible. In a chromatographic cell, schlieren measurements are difficult

because the knife edge reduces an already low transmitted intensity to a level difficult to measure.

The shadow method is the simplest flow visualization procedure. The basic apparatus is just a point light source, a lens and a photographic plate or imaging detector. In the object under investigation, individual light rays are refracted from their original path. If the gradient of the refractive index, $\partial n/\partial y$, is constant along the length of the test section, l, then the deflection angle of all rays is the same, and the image plane or photographic plate is uniformly illuminated. Such a density distribution can be represented by a linear wedge of a uniform, transparent medium. However, if the contour of the wedge is curved, the photographic plate will still receive constant illumination corresponding to a density field of constant $\partial^2 n/\partial y^2$. Thus the shadow method visualizes those fields where the second $\partial^2 n/\partial y^2$ is not zero[9]. The relative intensity change recorded by the detector is given[10] by eqn. 2. Eqn. 2 assumes that the detector has a linear response. For photographic film, however, blackening is proportional to the logarithm of incident light intensity, I, and eqn. 2 is valid only over a limited range.

$$\frac{I - I'}{I'} = \frac{\Delta I}{I'} = l \int \left(\frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2} \right) (\ln n)\, dz \tag{2}$$

EXPERIMENTAL

Flow visualization measurements were carried out with an apparatus consisting of a pump system, the flow cell, and flow visualization optics. No column was used. The mobile phase was methanol–water (60:40). The samples were aqueous methyl orange or pure water. The flow cell was a Kratos SFA-234, 1 mm I.D., 1 mm path length. A single channel piston pump (LDC Milton Roy, Mini-Pump) was used at flow-rate about 2 ml/min. A flat coil pulse dampener (Handy and Harmon) was used to reduce flow fluctuations. Samples of 20 μl were injected into the system with a rotary injection valve (Rheodyne 7010).

The shadowgraph apparatus is shown in Fig. 2. A 75-mW Xe arc lamp was used

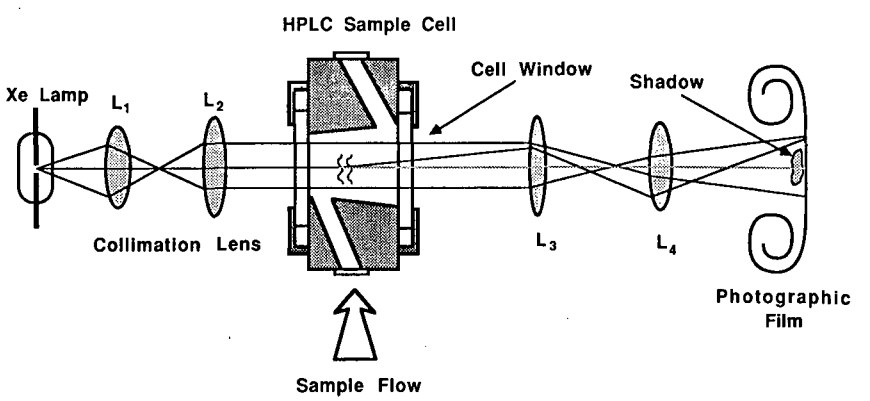

Fig. 2. Shadowgraph apparatus, L_1, f = 25 mm; L_2, f = 90 mm; L_3, f = 32 mm; L_4, f = 3 mm.

as the point light source. The output from the Xe arc lamp was collimated with lenses of 25 and 90 mm focal length and directed to the flow cell, which was mounted on translation stages for precise positioning. The images were recorded on Kodak Ektachrome 400 film or Kodak Panatomic X film mounted in a 35-mm SLR camera. In order to enlarge the image from the 1-mm diameter flow cell for photography, two microscope objective lenses of focal length, *f*, 32 mm and 3 mm respectively, were used. The 3 mm lens was mounted on a bellows attached to the camera for focusing.

To observe wedge angles among commercial flow cells, a He–Ne laser was directed onto the cell windows and the angle between reflections from entrance and exit windows was measured directly.

RESULTS AND DISCUSSION

To visualize flow phenomena in a chromatographic cell, the images must be greatly magnified. Preliminary experiments were made with a schlieren apparatus. However, the schlieren method requires blocking of the zero spatial frequency component. This requirement reduced the transmitted light intensity to a level which could not be photographed with apparatus available to us. Thus, the shadowgraph method was used for further experiments.

The shadowgraphs of Fig. 3 show the progress of an aqueous methyl orange sample flowing through the high-performance liquid chromatographic (HPLC) absorbance cell. With pure water as the sample, the progression of shadowgraph images is about the same. The pictures were taken with approximately 2-s intervals between each frame. These images are photographs of color transparencies.

Fig. 3.1 shows the cross section of a HPLC sample cell filled with only mobile phase. Although the mobile phase is moving, it is homogeneous and the flow is not visible.

In Fig. 3.2, methyl orange enters the cell from the bottom. The image shows a bright region in the center and two dark regions on either side. The dark spots are the area where light has been deflected by an inhomogeneous refractive index distribution. The light rays which encounter a small refractive index gradient are deflected but still reach the photographic film. The redistributed intensity is not lost but gives a brighter spot in the center of the image. But some light rays encounter a large refractive index gradient in the opposite direction. They are deflected at large angles and either hit the cell wall or escape detection by the high *f*-number photographic system. The result is that intensity is lost, creating the two dark lobes in the image.

The flow pattern in the cell which gives rise to Fig. 3.2 and the subsequent shadowgraph images, can be qualitatively described. The mobile phase is pumped against the cell window, causing the flow to bifurcate into two streams. The two streams are not exactly equal, so that the shadows are somewhat different on the two sides of the cell. As these two streams continue along the flow path, the shadowgraphs evolve, but always with the presence a two-lobe pattern.

In Fig. 3.3, the sample has flowed through the length of the cell and reached the exit window. At this point the flow has begun to swirl the walls of the cell. Even though the sample has hit the exit window, the upper part of the cell has not yet completely filled with methyl orange. This effect is even clearer in color images made with methyl orange. In these the upper part of the cell is only faintly orange while the bottom is brightly colored.

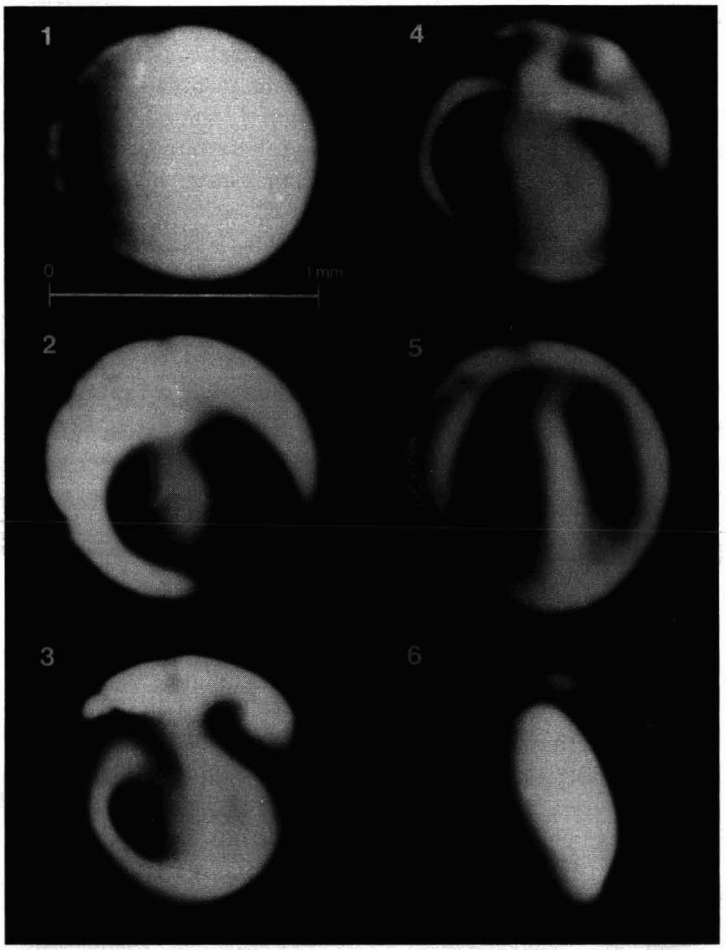

Fig. 3. Shadowgraph images of flow in a liquid chromatographic cell. The sample is aqueous methyl orange in a methanol–water (60:40) mobile phase. Flow-rate, 2 ml/min. (1) Mobile phase only; (2)–(6) sample through flow cell, at 2-s intervals.

In Fig. 3.4, the sample fills the cell. Part of the sample has already been washed from the cell. But as can be seen from the picture, the fluid in the cell is still not homogeneous. In Fig. 3.5, the tail of the sample flow has entered the cell. Again, the fluid is inhomogeneous. The last image shows the end of the sample flow. Here, mobile phase flows into the cell and generates the bright spot in the center. The remaining sample is distributed along the cell wall deflecting the light and creating shadows. After this the system returns to the uniform intensity distribution of Fig. 3.1.

At no time throughout the evolution of the band, can a homogeneous light intensity be seen. Inhomogeneity occurs because the cell transit time is only 25–30 ms. In the strongly directional flow there is no time for mixing. The constantly changing refractive index distribution becomes a source of detector excess noise. As the band evolves the transmitted intensity wanders around the detector surface, generating small changes in signal caused by local detector response inhomogeneities.

The shadowgraphs and the flow patterns observed for distilled water are the same as for methyl orange solutions. This observation confirms that the flow pattern is associated with the geometry of the absorbance cell rather than with the nature of the sample. Consequently, the details of the flow patterns will be strongly dependent on the design of the flow cell.

The shadowgraph method does not provide sufficient data to derive a complete description of refractive index distribution in an absorbance cell[11,12]. From eqn. 2, in order to evaluate the refractive index, n, we must perform a double integration of the data. Because the constants of integration are not known it is impossible to obtain the refractive index distribution.

A complete description of the index field is not necessary for evaluation of chromatographic cells. The shadowgraph technique provides information on the spatial redistribution of light at the exit window of the cell. With this information and the projected area of the beam at the detector surface distribution of light on the detector is readily calculated. A description of light intensity distribution on the photodetector surface, rather than of the spatial inhomogeneities within the cell itself, is needed for design of an improved absorbance detector.

The information in the shadowgraphs is sufficient to estimate the distances over which refractive index inhomogeneities extend at the exit window of the cell. Deionized water has a refractive index of 1.3325 at 20°C and water–methanol (40:60) mobile phase has a refractive index of 1.3302 at 20°C. From the small refractive index difference between the mobile phase and deionized water, 0.0023 at 20°C, a small angular deflection can be assumed. The aperture of the flow cell is 1 mm. Therefore we can estimate from the location of intensity peaks and saddles in the shadowgraphs that inhomogeneities of refractive index extend between 50–100 μm in this LC flow cell.

We measured wedge angles in four different LC cells, which were available in our laboratory. The measurements were made on cells to which no special adjustments had been made. We found that wedge angles varied from 0.5° to 2°. With careful adjustment, we could reduce the wedge angle to less than 0.06° in any of the cells. Although our measurements do not constitute a statistically valid study, they do suggest that the windows of a LC flow cell can not be assumed to be parallel unless care is taken in the assembly of the cell.

Even if the front window were aligned perfectly perpendicular to the light path, a wedged cell can cause the baseline to drift. Eqn. 1 shows that at normal incidence, $\theta_i = 0$, the deviation angle $\delta \approx \alpha(n - 1)$ and

$$\frac{d\delta}{dn} = \alpha \tag{3}$$

For a cell wedged at 0.5°, a refractive index change of 10^{-3} unit can cause the light to deviate 0.0005°. This effect can be important if the mobile phase is not mixed well or in gradient elution. Because photodiodes have different responses from point to point, this beam deviation is a further, if small, source of noise in absorbance detection.

We can estimate the temperature rise, ΔT, in the cell caused by light absorption. Using the equation, $\Delta T = (P\alpha l/J)/k2\pi wl$ where k is thermal conductivity, α is absorptivity, l, absorption length, w, beam radius, $J = 4.18$ J/cal is Joule's constant and assuming 0.1 mW power, P, delivered to the cell, and absorbance $1 \cdot 10^{-5}$, we

calculate the temperature rise in a flow cell to be on the order of 10^{-6}–10^{-8} degree. For most compounds dn/dT is approximately 10^{-4}, so that Δn is about 10^{-10}. The thermal lens effect is probably not a significant noise source.

Eliminating the noise sources associated with flow in a LC absorbance cell appears to be a formidable problem. There is no simple design which eliminates boundary layers and provides sufficient mixing to generate completely uniform flow. Because flow cell length and diameter are the same the mixing time must always be greater than the nominal transit time. To remove the problems associated with wedged cell windows, one must align cell windows carefully. To be certain that thermal effects are negligible, the cell and the detector must be designed to dissipate heat rapidly and uniformly. Compensation for these problems appears to be an attractive alternative to attempts at their complete elimination.

Pang and Morris[13] proposed the use of a retroreflective array as an approximate phase conjugator capable of compensating for the problems identified in this communication. They described an absorbance detector configured with a retroreflective array in the phase conjugation configuration. The system provided dynamic compensation of refractive artifacts, and, in addition, doubled the measured absorbances. The signal-to-noise ratio was increased overall by a factor of 4 to 6. A similar system was shown to provide at least a ten-fold reduction of noise in a flame atomic absorption system[14].

Pang and Morris[13] used a commercial array with elements spaced on 0.16 mm centers. Their work was carried out before detailed information about the actual non-idealities of a chromatographic flow cell, or even detailed knowledge of the optical properties of the array[15] was available. Our observations demonstrate that the Pang and Morris array is too coarse for optimum compensation for refractive index gradients in a chromatographic flow cell. Better results might be expected if arrays of 20–50 elements/mm are employed. Arrays with this element spacing are difficult to fabricate. Alternatively, beam-expanding optics might be used to magnify the exit at least five times and roughly collimate it for efficient operation of the array. Performance of detectors using this compensation scheme will be reported in a subsequent communication.

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USE OF RETENTION TEMPERATURES FOR THE IDENTIFICATION OF PHENOLS SEPARATED ON OV-1701 CAPILLARY COLUMNS USING LINEAR TEMPERATURE-PROGRAMMED GAS CHROMATOGRAPHY

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(First received April 7th, 1988; revised manuscript received May 17th, 1988)

SUMMARY

A procedure is described by means of which linear temperature-programmed gas chromatographic (LTPGC) retention data can be predicted from isothermal Kováts retention indices of substituted phenols separated on capillary columns coated with OV-1701 under LTPGC conditions. The identification algorithm included the comparison of retention temperatures predicted from isothermal data ($T_{R,P}$) with retention temperatures obtained experimentally under LTPGC conditions ($T_{R,E}$) in a window which was within 1.5°C. The identification of phenol sample constituents based on the comparison of retention data was checked using spectra obtained by gas chromatography–mass spectrometry because in the identification window several sample constituents overlapped.

INTRODUCTION

Substituted phenols are often used as disinfectants and gas chromatography (GC) is a very suitable method for their analysis. The separation of the constituents of a complex phenol sample requires capillary columns. As phenols containing electronegative substituents are strong acids, the inner surface of capillary columns for their analysis must be very inactive. In previous studies we have established conditions in which glass or fused-silica capillary columns coated with OV-1701 can be successfully used for the separation of phenols[1,2]. Further, we have shown that when separating phenol samples by linear temperature-programmed GC (LTPGC), the elution order of phenols depends on the programme mode, which complicates the qualitative analysis[2]. In subsequent studies we developed a procedure that allows the prediction of the retention temperatures ($T_{R,i}$) of solutes chromatographed by LTPGC from isothermal Kováts retention indices (I) and the retention temperatures of n-alkanes ($T_{R,z}$, $T_{R,z+1}$) found by LTPGC[3,4]. The proposed procedure was tested on hydrocarbons separated

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in capillary columns coated with polydimethylsiloxane (OV-101) stationary phase. The predicted retention temperatures allowed us to predict Van den Dool and Kratz indices (I_{progr})[4]. The comparison of predicted retention temperatures and/or retention indices with the data obtained experimentally allowed us to identify the hydrocarbons in complex mixtures separated by LTPGC[5].

The aim of this paper is to show the precision by means of which the LTPGC retention data can be predicted from isothermal Kováts retention indices of substituted phenols separated in capillary columns coated with polyphenylsilicone OV-1701 under LTPGC conditions.

EXPERIMENTAL

Two capillary columns used in this work were prepared by Grob[6]. Capillary tubes of length 25 m and I.D. 0.25 mm were drawn from Duran glass tubes. The inner surface of the capillary tubes was leached with 20% hydrochloric acid for 12 h (overnight) at 150°C, then rinsed with 1% hydrochloric acid. The inner surface was further dried for 2 h at 300°C using a water pump vacuum and then silanized using 1,3-diphenyl-1,1,3,3-tetramethyldisilazane overnight at 360°C. Free silanes were removed from the surface by rinsing the column with dichloromethane. The capillaries were then coated statically using a 0.5% solution of OV-1701 in dichloromethane, this stationary phase contained 1% (w/w) dicumyl peroxide. Two columns (1/1 and 1/2) were prepared. Further details on the preparation of the columns can be found in a booklet published on the occasion of a Summer School in Bratislava, Czechoslovakia[6].

A model sample of phenols containing 65 constituents was prepared by mixing the pure compounds. This mixture was analysed simultaneously with C_{12}–C_{20} *n*-alkanes by GC under isothermal conditions in an OV-1701 capillary column placed in a Fractovap 2350 gas chromatograph (Carlo Erba, Milan, Italy). The chromatograph was equipped with a flame ionization detector and an all-glass inlet splitter, operating with a splitting ratio of 1:100. Hydrogen was used as the carrier gas at a velocity of 30 cm/s in isothermal operations.

The LTPGC measurements on a model phenol sample were carried out in a Fractovap 4180 gas chromatograph (Carlo Erba) equipped with a flame ionization detector and a Grob on-column injector. Hydrogen with an inlet pressure of 250 kPa, corresponding to $\bar{u} \approx 30$ cm/s at 60°C, was used as the carrier gas. The temperature programme starting at 80°C with an increase of 1, 2, 3 and 4°C/min or at 70, 80, 90, 100, 110 and 120°C with an increase of 2°C/min.

The temperature in the thermostat oven was controlled by an LT 410 digital temperature programmer (Carlo Erba). Retention temperatures (T_{RE}) were calculated from the rates of temperature increase (r) and retention times (t_R) according to the equation

$$T_{RE} = T_0 + rt_R \tag{1}$$

where T_0 is the initial temperature of LTPGC. Retention times were measured with a Chromatopack C-R3A computing integrator (Shimadzu, Kyoto, Japan).

For identification of the individual peaks on a chromatogram, mass spectra obtained by combination of GC and mass spectrometry (GC–MS) were evaluated.

Mass spectra were measured in an HP 5995 GC–MS instrument (Hewlett Packard, Avondale, PA, U.S.A.) using the OV-1701 capillary column (column No. 1/1) under similar conditions to those used in GC.

The dead time was estimated both by analysing methane (t_{ME}) and calculation (t_{MC}) from the retention times (t_R) of *n*-alkanes:

$$t_{MC} = \frac{t_{R,z}^2 - t_{R,z+1} \cdot t_{R,z-1}}{2t_{R,z} - t_{R,z+1} - t_{R,z-1}} \quad (2)$$

where z denotes the number of carbon atoms in the *n*-alkane.

As the solubility of methane in the stationary phase varies with temperature, all adjusted relative retention data are based on the dead time calculated from retention times of dodecane (C_{12}), tridecane (C_{13}) and tetradecane (C_{14}).

RESULTS AND DISCUSSION

Isothermal gas chromatography

A model phenol sample was analysed by capillary GC on OV-1701 at temperatures from 70 to 170°C with a 5°C step (isothermal). From the retention data, Kováts retention indices were calculated for each experimental temperature [$I(T)$][7]. The values obtained were correlated with temperature and coefficients of the following linear equation were found:

$$I(T) = I(0°C) + \frac{dI}{dT} \cdot T \quad (3)$$

where $I(0°C)$ is the Kováts retention index of a given compound at 0°C and dI/dT is the temperature coefficient of the Kováts retention index.

In Table I values of $I(0°C)$ and dI/dT are given together with the correlation coefficients (r) and the temperature regions in which the data were measured (ΔT). It is surprising that the dI/dT values for most alkylphenols are negative. Relatively good correlation coefficients were obtained for compounds with relatively high dI/dT values (positive or negative) whereas compounds with very low dI/dT values were considerably influenced by the reproducibility of the measurements and therefore the correlation coefficients were poor. Deviations of the Kováts retention indices calculated for a given temperature on a given column ranged within 0.6 index unit. Deviations of the corresponding data obtained experimentally on the columns 1/1 and 1/2 (corresponding to the Kováts retention index reproducibility) were within 1.5 index unit.

Compounds corresponding to peaks on a chromatogram were identified via mass spectra obtained by GC–MS under similar conditions to those used during GC analysis. As the retention order of the peaks on a chromatogram changes dramatically with temperature, the mass spectra were compared by analysing a model phenol sample by GC/MS at 70, 90, 110, 130 and 170°C.

When the mass spectra did not give an unambiguous identification, the retention data were correlated with the proposed structure by considering chromatograms

VÁTS RETENTION INDICES (I) AND THEIR TEMPERATURE COEFFICIENTS (dI/dT) FOUND BY LINEAR REGRESSION ANALYSIS
ENOL SAMPLE CONSTITUENTS ANALYSED ISOTHERMALLY ON OV-1701

the correlation coefficient and ΔT is the temperature range in which the retention indices were measured.

k No.	Compound	I (0°C)	dI/dT	r	T (°C)
	2-Chlorophenol	1133.8	0.228	0.9857	90–150
	Phenol	1247.2	−0.162	−0.9705	90–170
	6-Chlorotrimethylphenol	1212.1	0.391	0.9854	90–170
	2,6-Dimethylphenol	1269.4	0.192	0.9701	90–170
	2-Methylphenol	1288.2	−0.088	−0.8912	90–170
	2-Nitrophenol	1197.9	0.730	0.9952	90–170
	4-Methylphenol	1329.1	−0.112	−0.9123	90–170
	3-Methylphenol	1333.5	−0.134	−0.9637	90–170
	2-Methylphenol	1380.8	−0.205	−0.9693	90–130
	2,5-Dimethylphenol	1378.6	−0.140	−0.9608	90–130
	2,4-Dichlorophenol	1324.2	0.425	0.9998	130–150
	2,3-Dichlorophenol	1319.1	0.505	0.9992	130–150
	2,5-Dichlorophenol	1327.9	0.420	0.9999	130–150
	2,6-Dichlorophenol	1308.3	0.610	0.9984	130–150
	2,4,6-Trimethylphenol	1361.4	0.154	0.9968	90–150
	2,3-Dimethylphenol	1412.0	−0.092	–	90–130
	3,5-Dimethylphenol	1440.9	−0.308	−0.9969	90–150
	4,6-Dichloro-2-methylphenol	1333.3	0.640	0.9996	130–150
	4-Ethylphenol	1415.5	−0.076	−0.9876	90–150
	3-Ethylphenol	1427.1	−0.142	−0.9507	90–150
	2,3,6-Trimethylphenol	1367.5	0.355	0.9997	130–150

28	2-Methylphenol	1518.1	−0.186	−0.972	90–150
29	4-Chlorophenol	1520.6	−0.068	−0.9780	90–150
30	3-Chlorophenol	1520.6	−0.068	−0.9780	90–150
31	2-Methyl-5-isopropylphenol	1522.6	−0.118	−0.9874	90–130
32	2,3,5,6-Tetramethylphenol	1485.2	0.365	0.9997	110–150
33	2,4,6-Trichlorophenol	1500.2	0.410	0.9980	110–150
34	4-Chloro-2-methylphenol	1545.1	0.085	0.9729	110–150
35	2-*tert.*-Butyl-4-methylphenol	1573.7	−0.182	−0.9676	110–150
36	2,3,5-Trichlorophenol	1507.1	0.485	0.9975	110–150
37	2-*tert.*-Butyl-5-methylphenol	1560.2	−0.017	−0.8478	110–170
38	4-*tert.*-Butyl-2-methylphenol	1571.3	−0.028	−0.4593	110–170
39	4-Chloro-3-methylphenol	1576.0	0.0925	0.9989	130–170
40	2,3,6-Trichlorophenol	1504.9	0.638	0.9993	130–170
41	2,3,4-Trichlorophenol	1529.7	0.585	0.9985	130–150
42	2,4,5-Trichlorophenol	1547.5	0.472	0.9930	130–170
43	4-Bromophenol	1592.8	0.162	0.9996	130–170
44	3-Bromophenol	1592.8	0.162	0.9960	130–170
45	4-Chloro-3,5-dimethylphenol	1651.2	0.125	0.9380	130–170
46	2,4,6-Trichlorophenol	1625.2	0.570	0.9944	130–170
47	2-Phenylphenol	1616.4	0.702	0.9996	130–170
49	4-Chloro-2-isopropyl-5-methylphenol	1736.8	0.128	0.9677	130–170
50	4-Bromo-3,5-dimethylphenol	1718.2	0.365	–	150–170
52	3,4-Dichlorophenol	1741.6	0.148	0.9996	150–170
53	2,3,4,5-Tetrachlorophenol	1726.2	0.680	–	170–190
54	Methyl 4-hydroxybenzoate	1823.6	0.060	–	170–190
55	Ethyl 4-hydroxybenzoate	1885.8	0.080	–	170–190
56	3-Nitrophenol	1887.3	0.350	–	170–190
57	2-Benzylphenol	1873.4	0.475	–	170–190
58	*n*-Propyl-4-hydroxybenzoate	1973.4	0.080	–	170–190
59	Pentachlorophenol	1936.4	0.335	–	170–190
60	4-Nitrophenol	1975.1	0.410	–	170–190
61	4-Phenylphenol	1996.0	0.300	–	170–190
62	3,4,5-Trichlorophenol	1999.2	0.370	–	170–190
63	4-Benzylphenol	1988.8	0.395	–	170–190
64	*n*-Butyl 4-hydroxybenzoate	2096.8	0.03	–	170–190
65	4-Hexylresorcinol*	–	–	–	–

* The retention index was not measured as C_{22} *n*-alkane was not present in the sample.

already published for this sample[2]. The compounds with peak numbers 11–15 and 16–22 were identified by analysing the individual compounds separately.

Temperature-programmed gas chromatography

As the retention order of phenols on a chromatogram also changes dramatically with changes in the LTPGC parameters (initial temperature T_0 and rate of temperature increase r), separations were performed gradually using different temperature rates, ranging from 0 to 4°C with a step of 0.5°C/min, and starting from various initial temperatures, from 70 to 120°C with a step of 10°C.

Fig. 1 shows a chromatogram obtained by analysing the model phenol sample with addition of C_{12}–C_{20} *n*-alkanes by LTPGC starting from 70°C at a rate of 2°C/min in the OV-1701 capillary column No. 1/1.

The peaks obtained on the chromatogram under LTPGC conditions were characterized by retention temperature (T_{RE}) calculated by employing eqn. 1. In the process of identification in LTPGC, experimental retention temperatures (T_{RE}) were compared with the predicted retention temperatures (T_{RP}) calculated from the following equation, published recently[3]:

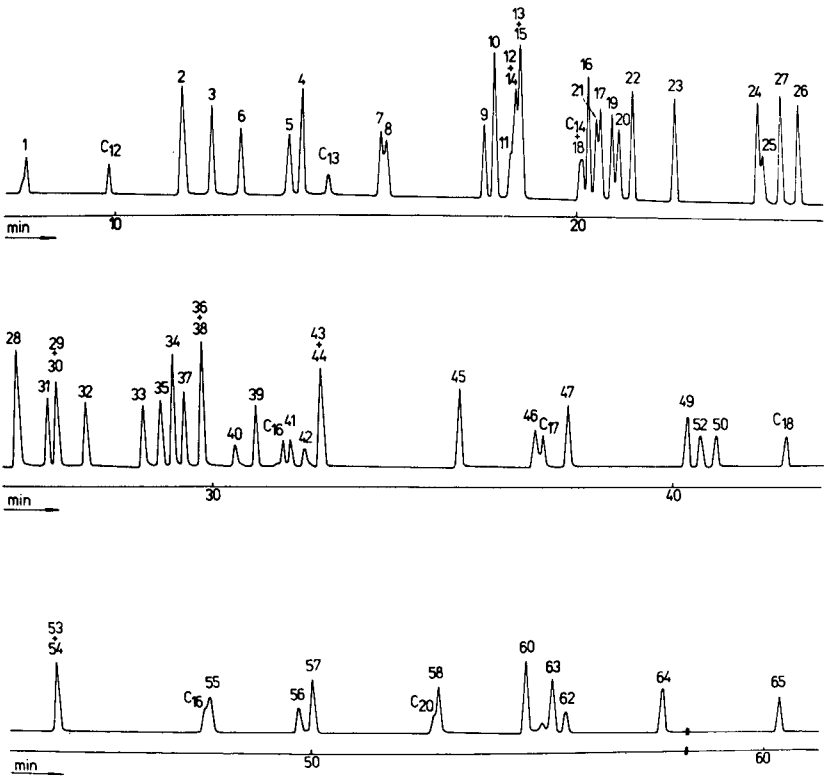

Fig. 1. Chromatogram obtained by analysing the model phenol sample with addition of C_{12}–C_{20} *n*-alkanes by LTPGC in an OV-1701 glass capillary column (column No. 1/1) starting from 70°C with a temperature rise of 2°C/min. The peak numbers correspond to those in Table I.

TABLE II

RETENTION TEMPERATURES OF *n*-ALKANES FOUND EXPERIMENTALLY ON AN OV-1701 CAPILLARY COLUMN BY LTPGC STARTING AT DIFFERENT TEMPERATURES WITH A TEMPERATURE RISE OF 2°C/min

n-Alkane	*Starting temperature (°C)*					
	70	*80*	*90*	*100*	*110*	*120*
C_{12}	89.67	95.13	101.48	108.79	116.81	125.4
C_{13}	99.20	102.91	107.57	113.3	120.09	127.73
C_{14}	110.23	112.61	115.69	119.69	124.9	131.34
C_{15}	121.45	123.03	125.16	127.7	131.33	136.35
C_{16}	132.75	133.97	135.44	136.87	139.23	142.87
C_{17}	144.12	144.91	146.00	146.6	148.14	150.6
C_{18}	154.81	155.53	156.45	156.66	157.54	159.09
C_{19}	165.40	165.87	166.61	166.53	167.10	168.15
C_{20}	175.33	175.85	176.52	176.29	176.63	177.17

$$T_{RP} = \frac{(T_{RE,z+1} - T_{RE,z})[I(T_1) - T_1(dI/dT) - 100z] + 100T_{RE,z}}{100 - (dI/dT)(T_{RE,z+1} - T_{R,z})} \quad (4)$$

where T_{RE} is the retention temperature found under given LTPGC conditions, $I(T_1)$ the Kováts retention index at temperature T_1, (dI/dT) the temperature coefficient of the Kováts retention index and z the number of carbon atoms in the *n*-alkane chain.

Experimental retention temperatures of *n*-alkanes obtained under the various LTPGC conditions are given in Tables II and III. In Tables IV and V experimental retention temperatures (T_{RE}) are compared with retention temperatures predicted from eqn. 4 by applying the data given in Tables I–III. The reproducibility of the T_{RE} measurements can be obtained by comparing the corresponding data (T_0 = 80°C and

TABLE III

RETENTION TEMPERATURES OF *n*-ALKANES FOUND EXPERIMENTALLY ON AN OV-1701 CAPILLARY COLUMN BY LTPGC STARTING AT 80°C WITH VARIOUS RATES OF TEMPERATURE INCREASE

n-Alkane	*Temperature gradient (°C/min)*			
	1	*2*	*3*	*4*
C_{12}	90.03	95.27	100.1	102.2
C_{13}	95.03	103.1	109.15	112.27
C_{14}	103.0	112.77	119.8	123.47
C_{15}	112.16	123.5	131.1	135.2
C_{16}	122.27	134.33	142.55	146.8
C_{17}	132.42	145.27	153.9	158.2
C_{18}	142.89	156.0	164.7	169.27
C_{19}	152.81	166.3	175.3	179.86
C_{20}	162.48	176.27	185.45	190.2

TABLE IV

RETENTION TEMPERATURES OF PHENOL SAMPLE CONSTITUENTS PREDICTED (T_{RP}) AND FOUND EXPERIMENTALLY (T_{RE}) ON AN OV-1701 CAPILLARY COLUMN BY LTPGC STARTING AT DIFFERENT TEMPERATURES WITH A TEMPERATURE RISE OF 2°C/min

Peak No.	*70°C*		*80°C*		*90°C*		*100°C*		*110°C*		*120°C*	
	T_{RE}	T_{RP}	T_{RE}	T_{RP}	T_{RE}	T_{RP}	T_{RE}	T_{RP}	T_{RE}	T_{RP}	T_{RE}	T_{RP}
1	86.0	84.8	92.3	91.5	99.5	97.7	107.4	106.9	115.9	115.5	124.8	124.5
2	92.8	92.8	97.4	97.6	103.1	103.3	109.8	110.2	117.5	117.8	125.8	126.0
3	94.1	94.4	98.7	99.1	104.1	104.7	111.0	111.3	118.5	118.8	126.7	126.8
4	98.0	98.1	101.9	102.0	106.8	107.6	112.7	113.3	119.7	119.9	127.5	127.6
5	97.5	97.3	101.2	101.3	106.1	106.3	112.1	112.4	119.2	119.4	127.0	127.2
6	95.4	96.2	100.0	100.7	105.6	106.1	112.1	112.4	119.4	119.7	127.5	127.5
7	101.5	101.2	104.6	104.6	108.8	109.0	114.2	114.4	120.7	120.9	128.1	128.2
8	101.7	101.4	104.8	104.8	109.0	109.2	114.3	114.5	120.7	121.0	128.2	128.3
9	106.0	105.7	108.5	108.6	112.1	112.2	116.7	117.1	122.6	122.8	129.5	129.6
10	106.4	106.2	108.9	109.1	112.5	112.7	117.0	117.4	122.8	123.0	129.8	129.8
11	107.2	106.9	109.7	109.8	113.1	113.5	117.9	118.3	123.6	123.8	130.5	130.6
12	107.3	107.3	109.9	110.1	113.6	113.8	118.1	118.6	123.8	124.0	130.6	130.8
13	107.5	107.3	109.9	110.1	113.6	113.7	118.1	118.5	123.8	123.9	130.5	130.7
14	107.3	107.3	110.1	110.3	113.6	113.9	118.2	118.7	124.0	124.1	130.8	130.9
15	107.5	107.8	110.1	110.5	113.6	113.9	118.1	118.6	123.8	123.9	130.5	130.6
16	110.5	110.4	112.6	112.8	115.7	115.8	119.7	120.0	124.9	124.9	131.3	131.3
17	111.0	111.0	113.0	113.2	116.0	116.2	119.9	120.3	125.0	125.0	131.3	131.3
18	110.2	110.6	112.6	113.2	116.0	116.4	120.3	120.8	125.7	125.8	132.1	132.2
19	111.5	111.0	113.5	113.3	116.4	116.3	120.3	120.5	125.3	125.2	131.6	131.6
20	111.8	111.4	113.7	113.7	116.6	116.6	120.4	120.7	125.5	125.4	131.7	131.7
21	110.8	111.0	113.0	113.4	116.3	116.5	120.3	120.8	125.5	125.6	131.9	132.0
22	112.4	112.0	114.2	114.2	117.1	117.0	120.7	121.0	125.7	125.7	131.9	131.9
23	114.3	114.0	116.0	116.1	118.7	118.8	122.1	122.5	126.8	127.0	132.8	132.9
24	118.0	117.6	119.4	119.4	121.2	121.8	124.7	124.9	128.8	129.0	134.3	134.5
25	118.2	117.8	119.6	119.6	121.9	122.0	124.8	125.0	128.9	129.1	134.3	134.6
26	119.8	119.6	121.2	121.2	123.4	123.5	126.2	126.4	130.1	130.2	135.3	135.4
27	119.0	120.4	120.8	122.1	123.4	124.5	126.6	127.3	130.8	131.2	136.2	136.4

28	121.5	121.0	122.7	122.5	124.7	124.6	127.2	127.2	130.9	130.9	135.9	136.0
29	123.2	122.8	124.3	124.3	126.3	126.4	128.6	128.8	132.1	132.2	137.0	137.1
30	123.2	122.8	124.3	124.3	126.3	126.4	128.6	128.8	132.1	132.2	137.0	137.1
31	122.8	122.4	124.0	123.8	126.0	125.9	128.4	128.4	131.9	131.8	136.8	136.7
32	124.5	124.9	125.8	126.4	127.8	128.4	130.2	130.7	133.6	134.0	138.4	138.7
33	127.0	127.4	128.1	128.8	130.0	130.6	132.1	132.7	135.3	135.8	139.8	140.1
34	128.3	127.7	129.2	129.1	130.8	130.9	132.7	132.9	135.6	135.8	139.8	140.1
35	127.8	127.1	128.7	128.4	130.3	130.3	132.1	132.2	135.1	135.2	139.4	139.5
36	129.6	129.3	130.4	130.7	132.0	132.5	134.0	134.4	136.8	137.2	141.0	141.3
37	128.8	128.0	129.7	129.3	131.3	131.1	133.0	133.0	135.8	135.9	139.9	140.1
38	129.6	129.1	130.4	130.4	132.0	132.1	133.7	133.9	136.4	136.7	140.5	140.7
39	131.5	131.4	132.6	132.7	134.1	134.3	135.7	135.9	138.1	138.4	142.0	142.2
40	130.7	131.4	131.9	132.8	133.6	134.5	135.4	136.2	138.1	138.7	142.2	142.6
41	133.0	133.6	134.2	134.9	135.8	136.4	137.3	137.9	139.7	140.3	143.5	144.0
42	134.0	134.0	134.7	135.2	136.2	136.7	137.7	138.2	140.1	140.5	143.8	144.1
43	134.3	134.4	135.3	135.6	136.7	137.0	138.1	138.4	140.3	140.7	143,8	144.2
44	134.3	134.4	135.3	135.6	136.7	137.0	138.1	138.4	140.3	140.7	143.8	144.2
45	140.5	140.5	141.2	141.4	142.3	142.7	143.3	143.8	144.7	145.4	147.9	148.2
46	144.0	145.0	144.5	145.8	145.7	147.0	146.6	147.5	148.1	149.0	150.9	151.7
47	145.4	146.1	145.9	146.9	147.0	148.2	147.8	148.7	149.3	150.2	151.9	152.6
49	150.7	150.1	151.0	150.8	151.9	151.9	145.3	152.3	153.4	153.4	155.3	155.5
50	151.7	151.9	152.2	152.7	153.2	153.8	153.6	154.2	154.7	155.2	156.6	157.0
52	151.1	150.9	151.5	151.7	152.5	152.8	152.9	153.2	153.9	154.2	155.9	156.2
53	158.9	158.4	159.1	159.1	159.9	160.0	160.0	160.1	160.8	161.0	162.1	162.4
54	158.9	158.4	159.1	159.0	159.9	159.9	160.0	160.0	160.8	160.7	162.1	162.1
55	165.5	165.2	165.9	165.7	166.6	166.4	166.5	166.4	167.1	167.0	168.2	167.9
56	169.1	170.0	169.8	170.6	170.5	171.2	170.4	171.2	170.9	171.6	171.8	172.4
57	170.1	170.8	170.4	171.4	171.2	172.0	171.0	172.0	171.5	172.4	172.4	173.1
58	175.6	174.6	175.9	175.2	176.5	175.7	176.3	175.7	176.6	176.0	177.2	176.5
59		174.8		175.4		175.9		175.9		176.2		176.8
60*	179.4	180.1	179.8	180.8	180.4	181.2	180.2	181.2	180.5	181.3	181.0	181.7
61*		180.2		180.9		181.3		181.3		181.4		181.8
62*	181.2	181.8	181.5	182.4	182.1	182.8	181.9	182.8	182.2	182.9	182.7	183.3
63*	180.6	181.2	181.0	181.8	181.6	182.2	181.3	182.3	181.7	182.4	182.1	182.7
64*	185.5	185.4	185.7	186.0	186.4	186.4	186.0	186.4	186.3	186.5	186.6	186.6
65	194.6		194.8		195.4		195.0		194.9		195.4	

* Data were predicted using retention temperatures for C_{20} and C_{21} *n*-alkanes.

TABLE V

RETENTION TEMPERATURES OF PHENOL SAMPLE CONSTITUENTS PREDICTED (T_{RP}) AND FOUND EXPERIMENTALLY (T_{RE}) ON AN OV-1701 CAPILLARY COLUMN BY LTPGC STARTING AT 80°C WITH VARIOUS RATES OF TEMPERATURE INCREASE

Peak No.	*1°C/min*		*2°C/min*		*3°C/min*		*4°C/min*	
	T_{RE}	T_{RP}	T_{RE}	T_{RP}	T_{RE}	T_{RP}	R_{RE}	T_{RP}
1	88.2	87.5	92.5	91.7	96.7	96.1	98.5	97.8
2	91.7	91.8	97.6	97.8	102.8	102.9	105.1	105.3
3	92.5	92.7	98.9	99.3	104.5	104.9	107.3	107.8
4	94.8	94.8	102.1	102.3	108.1	108.3	111.1	111.5
5	94.3	94.4	101.5	101.5	107.3	107.3	110.2	110.2
6	93.3	93.7	100.3	100.9	106.2	107.1	109.3	110.2
7	97.0	96.9	104.9	104.8	111.2	111.0	114.3	114.2
8	97.2	97.1	105.1	105.0	111.4	111.2	114.5	114.4
9	100.1	100.0	108.9	108.8	115.5	115.3	118.9	118.7
10	100.4	100.3	109.3	109.3	116.0	115.9	119.4	119.3
11	100.9	100.5	110.1	110.0	117.0	117.2	120.7	120.9
12	100.9	100.7	110.4	110.3	117.4	117.6	121.0	121.4
13	101.1	100.7	110.4	110.3	117.4	117.5	121.0	121.2
14	100.9	100.7	110.4	110.4	117.4	117.8	121.0	121.6
15	101.1	101.2	110.4	110.7	117.4	117.7	121.0	121.3
16	103.4	103.2	113.0	113.0	120.0	120.0	123.7	123.7
17	103.8	103.8	113.4	113.4	120.4	120.3	124.1	123.9
18	103.0	102.9	113.0	113.4	120.4	121.2	124.6	125.2
19	104.2	103.7	113.9	113.5	121.0	120.6	124.6	124.3
20	104.5	104.1	114.1	113.9	121.3	121.0	124.9	124.7
21	103.5	103.4	113.4	113.6	120.7	121.1	124.6	125.0
22	104.9	104.6	114.7	114.4	121.7	121.4	125.4	125.1
23	106.3	106.2	116.4	116.4	123.7	123.6	127.5	127.4
24	109.3	109.4	119.9	119.7	127.4	127.1	131.2	131.1
25	109.5	109.5	120.1	119.9	127.6	127.2	131.4	131.2
26	110.7	110.9	121.6	121.6	129.3	129.1	133.2	133.2
27	109.7	110.9	121.2	122.6	129.3	130.8	133.7	135.3
28	112.2	112.3	123.1	122.9	130.7	130.3	134.6	134.4
29	113.6	113.6	124.8	124.8	132.6	132.4	136.7	136.5

30	113.6	113.6	124.8	124.8	132.6	132.4	136.7	136.5
31	113.3	113.1	124.5	124.4	132.2	131.9	136.3	136.0
32	114.4	114.9	126.2	126.9	134.4	135.1	138.7	139.4
33	116.5	117.1	128.6	129.2	137.0	137.6	141.1	142.0
34	118.0	117.7	129.6	129.6	137.7	137.6	141.9	141.8
35	117.6	117.5	129.2	128.9	137.0	136.7	141.1	140.8
36	118.9	118.8	130.9	131.2	138.0	139.7	143.7	144.2
37	118.5	118.1	130.1	129.8	138.1	137.7	142.2	141.9
38	119.1	119.1	130.9	130.8	138.9	138.9	143.1	143.0
39	121.1	121.1	133.2	133.1	141.3	141.3	145.5	145.6
40	119.9	120.5	132.5	133.2	141.0	142.1	145.5	146.7
41	122.3	122.4	134.7	135.3	143.3	144.0	147.8	148.8
42	122.8	122.9	135.3	135.6	143.7	144.1	148.2	148.8
43	123.6	123.7	135.8	136.0	144.2	144.2	148.5	148.8
44	123.6	123.7	135.8	136.0	144.2	144.2	148.5	148.8
45	129.1	129.1	141.7	141.8	150.3	150.4	154.7	154.8
46	131.7	132.6	145.5	146.2	154.0	155.3	158.8	160.1
47	133.0	133.6	146.3	147.4	155.5	156.7	160.2	161.5
49	139.3	138.1	151.5	151.2	160.2	160.0	164.6	164.7
50	139.5	139.6	152.8	153.2	161.7	162.2	166.3	167.0
52	139.0	138.9	152.1	152.2	160.7	160.9	165.3	165.6
53	144.6	145.2	158.5	159.6	167.8	169.1	172.7	174.0
54	146.5	146.0	159.4	159.4	168.5	168.2	173.1	172.9
55	153.2	152.4	166.4	166.0	175.3	175.1	179.9	179.8
56	156.3	156.8	170.3	170.8	179.7	180.3	184.5	185.2
57	156.8	157.4	170.9	171.7	180.3	181.3	185.3	186.2
58	162.7	162.2	176.3	175.4	185.5	184.4	190.2	189.0
59		161.5		175.6		185.3		190.2
60*	165.9	166.6	180.2	181.0	189.8	190.9	194.8	196.0
61*		166.9		181.1		190.9		195.9
62*	167.6	168.3	181.9	182.7	191.5	192.6	196.5	197.7
63*	167.0	167.7	181.3	182.1	190.9	191.9	195.9	197.0
64*	172.2	172.3	186.2	186.2	195.5	195.9	200.3	200.8
65*	181.2		195.3		204.7		209.6	

* Data were predicted using retention temperatures for C_{20} and C_{21} *n*-alkanes.

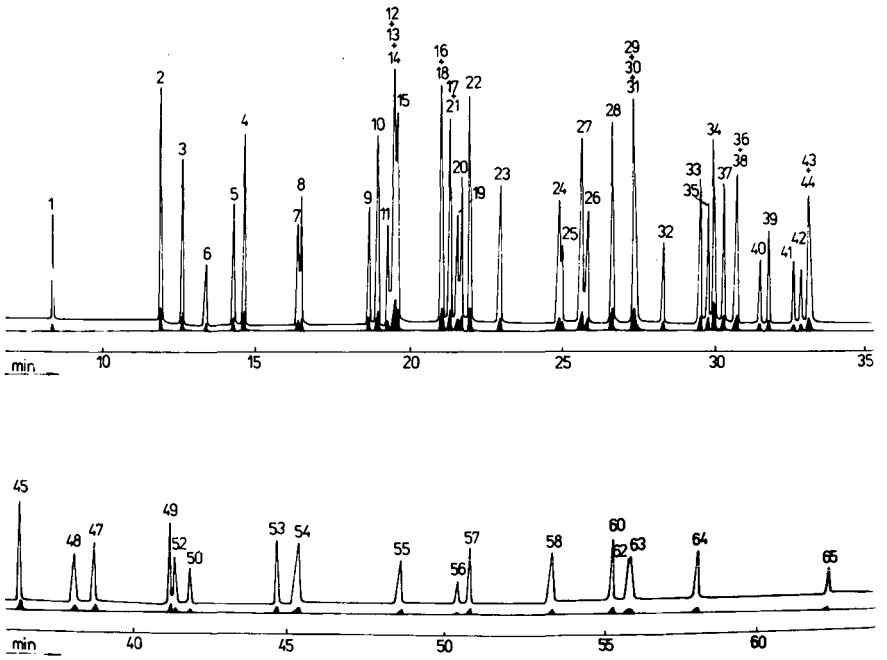

Fig. 2. Chromatogram obtained by analysing a model phenol sample by LTPGC in an OV-1701 glass capillary column (column No. 1/1) placed in the GC–MS instrument (HP 5995) starting from 70°C with a temperature rise of 2°C/min. The chromatogram with shaded peaks was recorded using a 10-fold higher attenuation.

r = 2°C/min obtained on columns 1/1 and 1/2, respectively) in Tables IV and V. Deviations of the corresponding T_{RE} values are in the range 0–0.5°C. Most of the corresponding differences, $T_{RE} - T_{RP}$, are also in this range. However, these differences are much higher for peak 6 (1.2°C in Table IV) and peak 27 (1.4°C in Table IV). The relatively good agreement between T_{RE} and T_{RP} allows us to predict the LTPGC retention of the constituents of the phenol sample from the data given in Tables I–III (excluding peaks 6 and 27). The correctness of the prediction was checked by mass spectra obtained by GC–MS. Fig. 2 shows a chromatogram obtained by analysing the model sample by LTPGC in the OV-1701 glass capillary column No. 1/1 placed in the HP 5995 GC–MS instrument starting from 70°C with a temperature rise of 2°C/min.

The retention temperature can be predicted graphically from the combination of the dependences of the Kováts retention indices of phenol sample constituents on temperature and of the temperature-programmed retention indices of *n*-alkanes on retention temperature (Figs. 3–8). For the construction of Figs. 3–5 data from Tables I and II were used and for the construction of Figs. 6–8 data from Tables I and III were used.

The reprodicibility of measurements of retention temperatures and their agreement with the predicted values are used as a basis for the window in which the identification is performed. However, from the data in Tables IV and V it can be

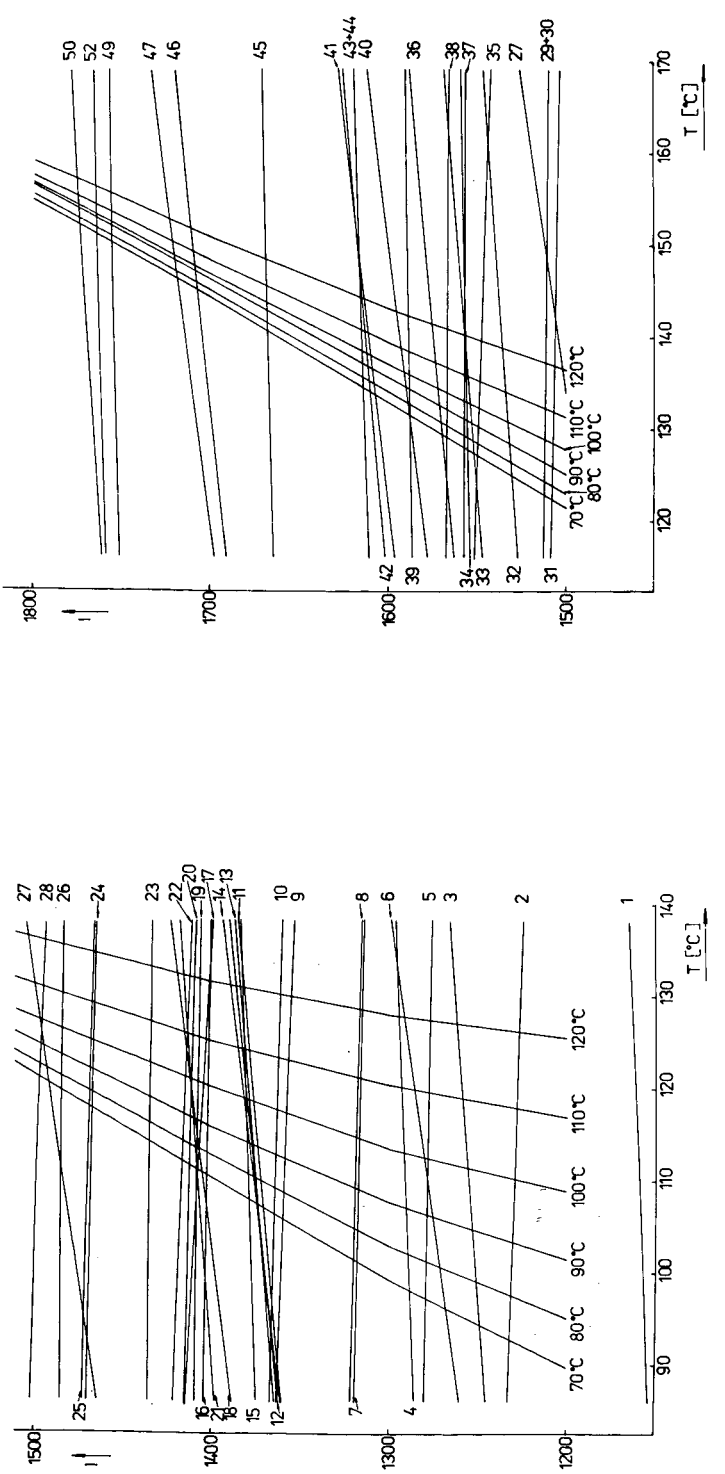

Fig. 3. Dependences of isothermal Kováts retention indices of phenol sample constituents eluted from OV-1701 between dodecane and pentadecane on temperature, combined with the dependences of the retention indices of *n*-alkanes on retention temperatures found under LTPGC conditions starting from different initial temperatures with a temperature rise of 2°C/min.

Fig. 4. Dependences of isothermal Kováts retention indices of phenol sample constituents eluted from OV-1701 between pentadecane and octadecane on temperature, combined with the dependences of the retention indices of *n*-alkanes on retention temperatures (details as in Fig. 3).

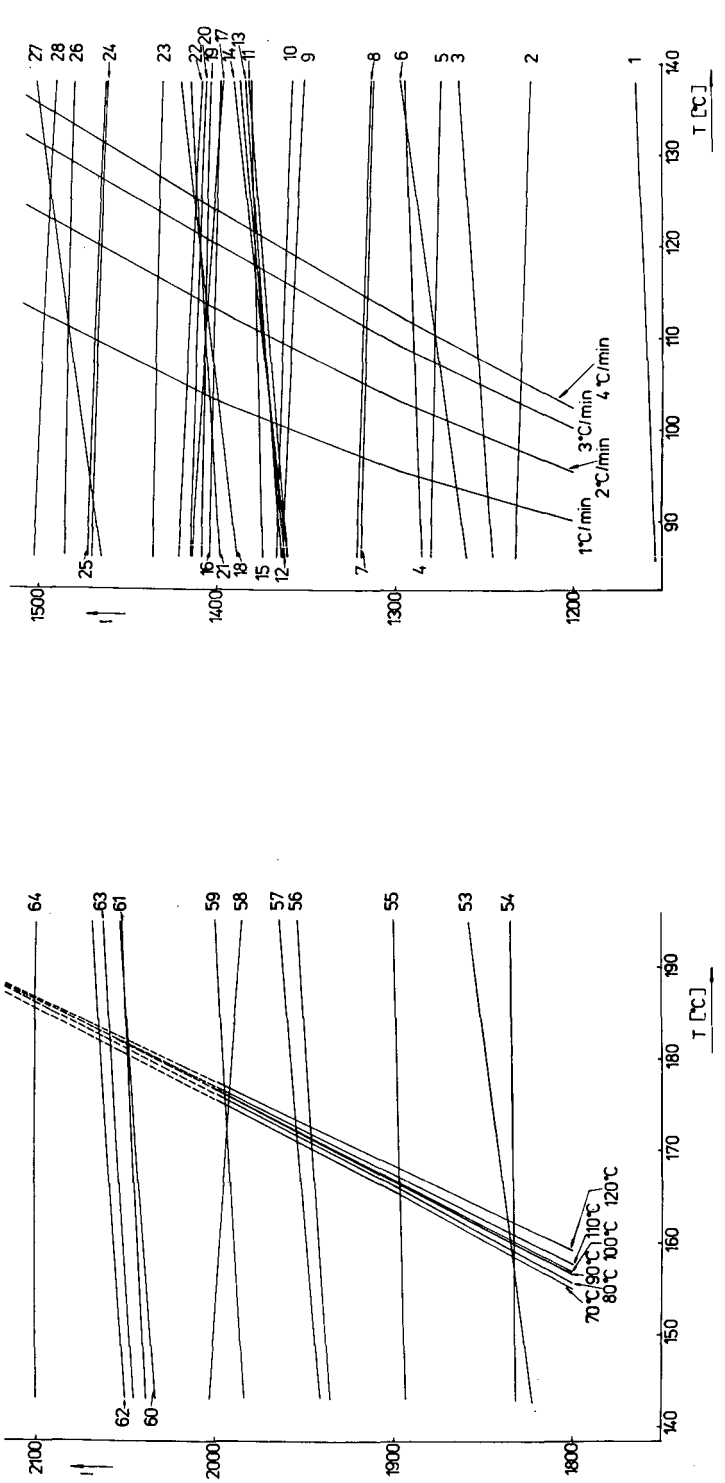

Fig. 5. Dependences of isothermal Kováts retention indices of phenol sample constituents eluted from OV-1701 between octadecane and heneicosane on temperature, combined with the dependences of the retention indices of *n*-alkanes on retention temperatures (details as in Fig. 3). Dashed lines represent extrapolation of dependences from nonadecane and eicosane.

Fig. 6. Dependences of isothermal Kováts retention indices of phenol sample constituents eluted from OV-1701 between dodecane and pentadecane on temperature, combined with the dependences of the retention indices of *n*-alkanes on retention temperatures found under LTPGC conditions starting from 80°C with various rates of temperature increase.

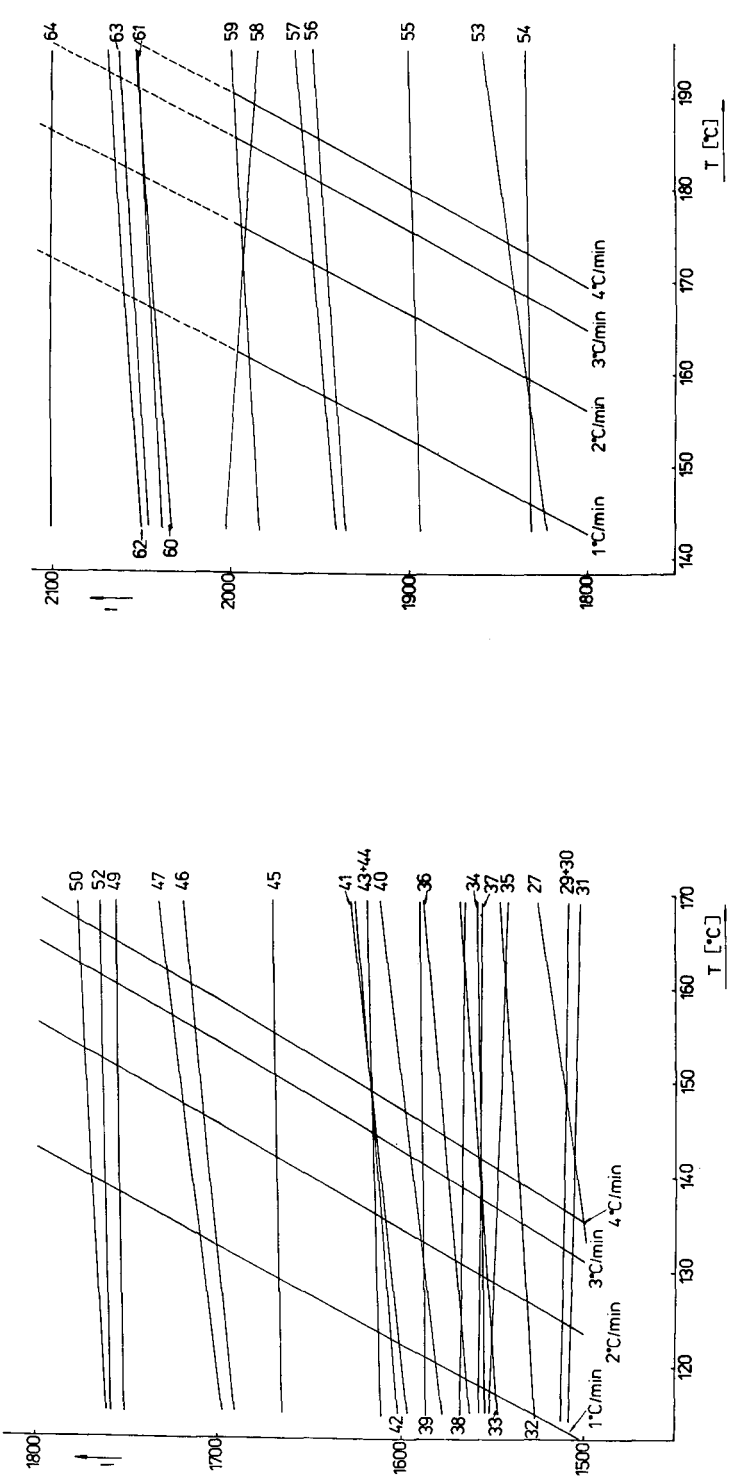

Fig. 7. Dependences of isothermal Kováts retention indices of phenol sample constituents eluted from OV-1701 between pentadecane and octadecane on temperature, combined with the dependences of the retention indices of *n*-alkanes on retention temperatures (details as in Fig. 6).

Fig. 8. Dependences of isothermal Kováts retention indices of phenol sample constituents eluted from OV-1701 between octadecane and heneicosane on temperature, combined with the dependences of the retention indices of *n*-alkanes on retention temperatures (details as in Fig. 6). Dashed lines represent extrapolation of dependences from nonadecane and eicosane.

concluded that in the window found in this work several peaks overlapped. The width of such a window is larger for phenols than for hydrocarbons, as the dI/dT values of phenols are much larger than those of hydrocarbons[3]. The correlation of retention data measurements of phenols with experimental conditions in both isothermal and LTPGC is currently being studied and will be published separately.

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POLARITY OF SOME INDIVIDUAL DERIVATIVES OF α,ω-DIAMINOOLIGOETHERS AS MEASURED BY GAS CHROMATOGRAPHY

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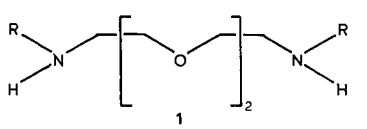
SUMMARY

The polarity of α,ω-diaminooligoether derivatives, as determined by inverse gas chromatography, was investigated. The effects of structures of the compounds on their polarities and on the hydrophobic effective lengths are discussed. Different relationships between polarity parameters are considered and appropriate increments of the polarity parameters were determined for characteristic groups.

INTRODUCTION

The polarities of several compounds containing one nitrogen atom were studied previously[1,2]. The relationships between different polarity parameters were discussed and the increments of the polarity parameters for different characteristic structural fragments were calculated.

The aim of this work was to study the relationships between the structures and polarities of some derivatives of α,ω-diaminooligoethers which contain two nitrogen atoms bridged by an oligooxyethylene chain, and each linked to combinations of hydrogen atoms and alkyl and acyl groups. Their structures are as follows:



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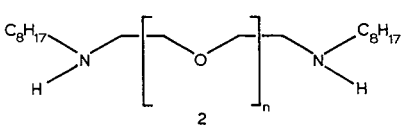

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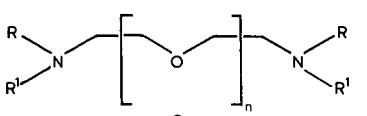

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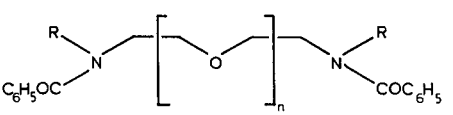

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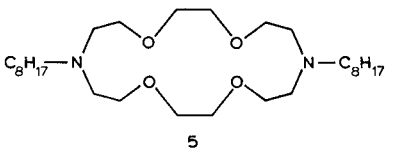

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where R and R^1 denote alkyl groups (CH_3 to $C_{12}H_{25}$), a cyclohexyl or a benzoyl group and $n = 2$, 3 and 4.

Some of these compounds were obtained previously and tested as drugs[3,4] and pesticides[5]. Some new compounds are described in this work. They were tested as crown ether analogues for the extraction of mercury(II) chloride[6], alkali metals and alkaline earth metals[7].

EXPERIMENTAL

Individual derivatives of α,ω-diaminooligoethers and their preparation

Nineteen pure model compounds (Table I) were used for polarity measurements.

TABLE I

STRUCTURES AND ANALYTICAL DATA FOR α,ω-DIAMINOOLIGOETHER DERIVATIVES

Compound	*R*	R^1	*n*	*B.p. (°C/mmHg)*	*M.p. (°C)*	n_D^{20}
1a	CH_3	–	2	85–88/0.3**	–	1.4453**
1b	C_2H_5	–	2	88–90/0.01***	–	1.4439***
1c	C_4H_9	–	2	124/0.01	–	1.4472
1d	C_6H_{13}	–	2	169–172/0.3	–	1.4514
1e	C_8H_{17}	–	2	167/0.01	–	1.4545
1f	$C_{10}H_{21}$	–	2	–	23	–
1g	$C_{12}H_{25}$	–	2	–	38–39§	–
1h	C_6H_{11}*	–	2	–	–	1.4829
1i	$C_6H_5CH_2$	–	2	198–200/0.01	–	1.5425
2a	C_8H_{17}	–	3	220/0.01	–	1.4553
2b	C_8H_{17}	–	4	226–281/0.1	–	1.4570
3a	C_4H_9	C_4H_9	2	139/0.01§§	–	1.4529
3b	C_4H_9	C_4H_9	4	206–208/0.08	–	1.4535
3c	CH_3	C_7H_{15}	4	197–206/0.02	–	1.4550
4a	H	–	2	–	120–122	–
4b	C_8H_{17}	–	2	Oil	–	–
4c	C_8H_{17}	–	3	Oil	–	–
4d	C_8H_{17}	–	4	Oil	–	–
5	C_8H_{17}	–	4	223–236/0.02	22	–

* Cyclohexyl.
** 115°C/12 mmHg: 1.4453[12].
*** 148°C/22 mmHg; 1.4420[12].
§ 39–40°C[8].
§§ 122–39/0.02 mmHg[8].

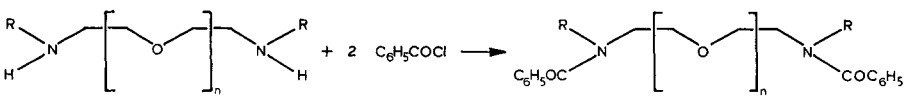
They were synthesized according to methods described previously[3,8,9]. All reagents used for syntheses were of analytical-reagent grade.

Compounds 1 and 2 were obtained as follows:

A 0.1-mol amount of the required α,ω-dichlorooligoether was added slowly with stirring at 70–120°C to the required primary amine (0.6–1.2 mol) and the reaction mixture was maintained at 70–120°C for 10–15 h. The cooled mixture was treated with aqueous sodium hydroxide (20 g in 40 cm^3) and the precipitated salt was filtered off. The organic mother liquor was then dried over potassium hydroxide and distilled two to four times under reduced pressure. The yield was 50–80%.

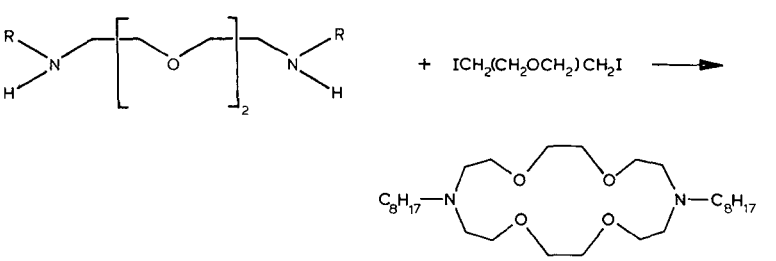
Compounds 3 were prepared by heating (100–150°C) a mixture of 0.1 mol of the required α,ω-dichlorooligoether, 0.3 mol of the required secondary amine and 0.3 mol of sodium carbonate with stirring for 20 h. After the precipitated salt had been filtered off, the mother liquor was distilled three to five times under reduced pressure. The yield was 40–60%.

The bisbenzamides were prepared as follows:



To a suspension of 0.05 mol of 1 in 30 cm^3 of 1,4-dioxane and aqueous potassium hydroxide solution (10 g in 10 cm^3) was added dropwise 0.11 mol of benzoyl chloride at 5–10°C. The mixture was stirred for 2 h at 50–70°C and, after cooling, diluted with 50 cm^3 water. The solid product was collected on a glass filter, washed with water and recrystallized from ethanol. The oil obtained was washed five times with water and five times with *n*-heptane. The yield was 70–95%.

The crown ether 5 was obtained according to the method of Kulstad and Malmsten[10]:



Treatment of 1e (0.05 mol) with 1,8-diiodo-3,6-dioxaoctane (0.05 mol) in the presence of sodium carbonate (0.1 mol) in acetonitrile afforded 5 in 28% yield. The product was purified by distillation and recrystallization.

The purities and structures of all the compounds were confirmed by elemental analyses, ^{1}H NMR spectra, and gas (GC) and thin-layer chromatography (TLC). Their structures and some analytical data are given in Table I. ^{1}H NMR spectra were recorded on a Tesla BS 487B 80-MHz instrument using C^2HCl_3 as solvent and hexamethyldisiloxane (HMDS) as internal standard. Compounds 1–3: 0.80–0.83 (t, CH_3), 1.22–1.32 (m, CH_2 in alkyls), 2.29–2.57 (t, CH_2 in alkyls bonded with hydrogen), 2.49–2.73 (4H, t, CH_2 in oligooxyethylene chain bonded with nitrogen), 3.47–3.54 (4H, t, CH_2 in oligooxyethylene chain in β-position), 3.52–3.60 ppm (s, CH_2 in oligooxyethylene chain).

The purity of α,ω-diaminooligoether derivatives was checked by GC using SE-30 as the stationary phase and by TLC on silica using toluene–acetone–methanol–25% ammonia solution (45:42:10:3) as the eluent and iodine vapour and cobalt(II) thiocyanate[11] for detection.

Chromatographic measurements

Chromatographic measurements were carried out using a gas chromatograph (Chrom 5, Kovo, Czechoslovakia) equipped with a flame ionization detector. The conditions were as follows: column, 1 m × 3 mm I.D.; column temperature, 70 and 90°C; column packing, 25% (w/w) of the compound on Porolith (mesh size 0.2–0.5 mm); carrier gas (helium) flow-rate, 40 ml/min; solutes, methanol, ethanol, 1-propanol, 1-butanol, 2-butanone, 2-pentanone, benzene, pyridine, 1-nitropropane and C_5–C_{12} alkanes; time for column stabilization, 10 h. The compounds examined were used as liquid stationary phases.

For each surfactant five different measurements were made and the average values of the polarity parameters were calculated. The following polarity parameters were considered; retention index of methanol and ethanol, I_R (ref. 13); polarity index of methanol or ethanol, $PI = 100 \log (C - 4.7) + 60$, where C is the apparent number of carbon atoms in a standard n-alkane having the same retention time as the alcohol[14]; coefficient ρ, defined as the ratio of the retention times of alcohol and n-hexane[15,16]; partial molal free energies of solution of hydroxyl group, ΔG_s^m(OH) and carbonyl group, ΔG_s^m(C=O); and McReynolds constants.

Partial molal Gibbs free energies of solution were calculated according to the procedure described by Risby and co-workers[17,18] and used in our previous work[2,19,20]. The sum of the first five McReynolds constants, $\sum_{i=1}^{5} \Delta I_i$, was calculated in the standard way using differences in retention indices, ΔI_i, values of benzene, 1-butanol, 2-pentanone, pyridine and 1-nitropropane, as determined on a considered compound and on squalane, respectively.

RESULTS AND DISCUSSION

The values of the polarity parameters obtained are given in Tables II–IV (the compounds are numbered as in Table I). The precision of the determination of the polarity parameters considered is good and similar to that in our previous work[1,2,19,20]. The relationships between the polarity parameters are also similar to those reported previously[2,19,20] and hence they are not discussed here.

TABLE II

EMPERICAL POLARITY PARAMETERS

Compound	*Temperature (°C)*	I_R		*PI*		ρ	
		CH_3OH	C_2H_5OH	CH_3OH	C_2H_5OH	CH_3OH	C_2H_5OH
1a	70	920	1047	114.0	116.0	4.91	9.76
	90	–	–	–	–	–	–
1b	70	837	868	106.6	110.0	2.89	6.30
	90	–	–	–	–	–	–
1c	70	712	742	98.8	103.6	2.40	3.29
	90	703	726	96.9	101.1	2.36	2.67
1d	70	693	727	95.0	101.1	2.19	2.91
	90	675	699	90.4	94.9	1.79	2.14
1e	70	660	691	91.0	96.9	1.68	2.54
	90	655	681	86.0	94.4	1.60	2.06
1f	70	653	689	87.0	95.0	1.60	2.22
	90	618	656	79.9	93.5	1.52	2.25
1g	70	647	672	84.9	91.1	1.51	1.89
	90	610	650	74.7	85.7	1.08	1.78
1h	70	698	726	96.0	101.0	2.35	2.98
	90	683	703	92.9	96.9	1.90	2.22
1i	70	685	724	94.0	95.5	1.78	2.62
	90	683	703	92.8	93.6	1.27	2.18
2a	70	681	701	92.5	97.4	1.90	2.61
	90	693	696	95.1	95.6	2.04	2.09
2b	70	683	711	92.9	98.3	2.18	2.75
	90	692	707	94.7	97.7	2.00	2.25
3a	70	758	780	106.1	109.3	3.73	4.48
	90	778	783	109.0	109.7	3.78	3.93
3b	70	829	849	115.8	118.2	5.87	6.86
	90	853	874	119.4	121.2	6.20	6.95
3c	70	756	773	105.8	108.4	3.85	4.19
	90	727	765	101.6	106.2	2.66	4.14
4a	70	805	824	112.8	115.5	4.78	5.62
	90	803	803	114.0	113.6	4.27	4.18
4b	70	708	728	100.2	103.2	2.53	3.16
	90	737	764	103.2	107.4	2.73	3.31
4c	70	729	751	101.6	105.1	2.80	3.33
	90	722	749	100.5	104.9	2.38	2.88
4d	70	740	755	103.4	105.9	3.08	3.47
	90	723	752	101.8	105.3	2.72	2.97
5	70	682	705	92.8	97.2	1.97	2.39
	90	699	703	96.0	96.8	2.10	2.17

The polarity of $RHNCH_2(CH_2OCH_2)_nCH_2NHR$ depends significantly on their structures, and increases as the lengths of the alkyl groups and of the oligooxyethylene chain decrease and increase, respectively. These effects are similar for the all polarity parameters and hence the relationships presented in Figs. 1 and 2 as examples have a general character. For the first homologues having short alkyl chains (methyl, ethyl

TABLE III

THERMODYNAMIC POLARITY PARAMETERS

Compound	$\Delta G_s^m(OH)$ *(kJ mol^{-1})*		$\Delta G_s^m(C=O)$ *(kJ mol^{-1})*	
	CH_3OH	C_2H_5OH	*2-Butanone*	*2-Pentanone*
1a	–	–	–	–
1b	–	–	–	–
1c	−11.3	− 9.7	− 9.1	− 8.8
1d	−11.2	− 9.6	− 8.8	− 8.5
1e	−10.3	− 9.1	− 8.7	− 8.4
1f	− 9.9	− 8.4	− 8.4	− 8.2
1g	− 9.4	− 7.6	− 8.0	− 7.8
1h	−11.1	− 9.6	− 8.8	− 8.5
1i	−10.7	− 9.1	−11.0	−10.8
2a	−10.4	− 8.2	− 8.3	− 7.9
2b	−10.7	− 9.0	− 8.7	− 8.4
3a	−12.4	−10.6	− 9.2	− 8.8
3b	−12.4	−10.7	− 9.7	− 9.3
3c	−11.4	− 9.7	− 9.2	− 8.9
4a	−13.0	−11.2	−11.6	−10.0
4b	−11.3	− 9.6	−10.7	−10.4
4c	−10.5	− 8.8	−10.1	− 9.8
4d	−10.8	− 8.9	−10.2	− 9.9
5	−10.5	− 8.7	− 8.8	− 8.5

and butyl) their polarity decreases rapidly as the alkyl chain length increases. However, a further increase in the alkyl length causes a much smaller decrease in polarity, and approximately linear relationships are observed for compounds having 4–12 carbon atoms (m) in each alkyl group. The gradients of these relationships, which are different for each polarity parameter, do not depend significantly on the type of polar solute and they are similar for methanol and ethanol and for 2-butanone and 2-pentanone, respectively. The effect of the number of oxyethylene groups (n) on the polarity is also approximately linear. The gradients calculated for the relationships $PP_i = A + Bm$ and $PP_i = A + Bn$, where PP_i denotes the polarity parameter considered, give appropriate increments of the polarity parameter, which are negative and positive for a methylene group and an oxyethylene group, respectively (Table V). These increments and/or the relationships obtained for compounds 1 can be further used to estimate the polarity of other homologues, to determine the so-called "effective length of the hydrophobe" for other compounds and to compare the effect of cyclohexyl, benzyl and benzoyl groups on the polarity of the compounds and the effective length of their hydrophobes.

The use of these increments to calculate the polarity for the other compounds considered which have different structures is limited, because polarity depends significantly on the distribution of carbon in the X and Y groups. Isomers having each nitrogen atom linked with two short alkyl chains are much more polar than isomers in

TABLE IV

SUM OF McREYNOLDS CONSTANTS AND CONTRIBUTIONS OF SUCCESSIVE TEST SOLUTES

Compound	*Benzene* (%)	*1-Butanol* (%)	*2-Pentanone* (%)	*Pyridine* (%)	*1-Nitropropane* (%)	$\sum_{i=1}^{5} \Delta I_i$
1a	–	–	–	–	–	–
1b	–	–	–	–	–	–
1c	12.2	34.3	13.9	18.5	21.9	1032
1d	8.3	30.5	22.1	14.5	24.6	972
1e	9.3	33.8	14.2	17.0	24.4	936
1f	6.8	39.1	10.6	13.5	29.8	912
1g	9.4	38.2	12.9	16.7	22.6	717
1h	10.1	37.2	13.4	17.3	22.0	885
1i	10.2	22.9	22.4	18.5	25.8	1053
2a	9.4	36.2	14.1	17.0	23.3	875
2b	10.2	33.9	14.3	18.0	23.5	970
3a	1.8	38.3	14.6	19.1	26.1	1031
3b	9.8	32.9	14.2	18.6	24.4	1529
3c	9.3	35.4	14.0	17.5	23.7	1041
4a	11.6	15.2	26.2	23.1	22.1	1952
4b	10.1	26.3	15.6	24.2	23.2	1357
4c	10.8	27.4	16.5	19.8	25.4	1391
4d	11.0	27.2	16.3	20.4	24.9	1409
5	9.6	33.2	14.2	18.2	24.8	967

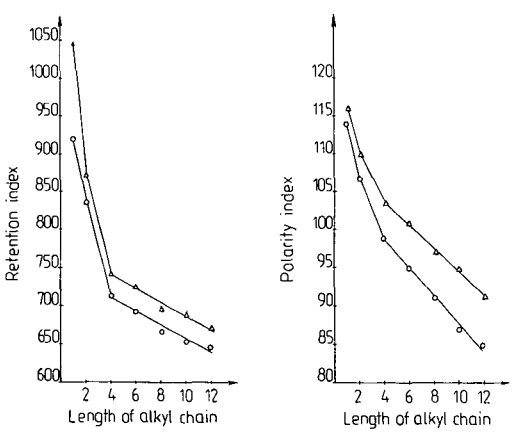


Fig. 1. Effect of the length of alkyl chain on the polarity of compounds 1. ○, Methanol; △, ethanol.

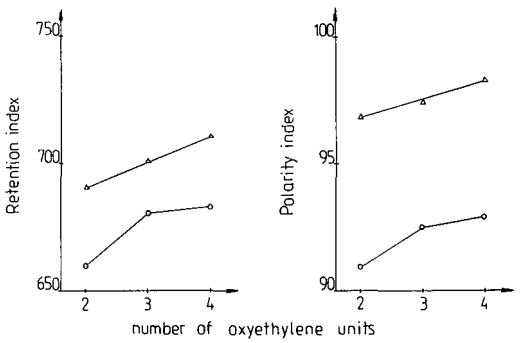
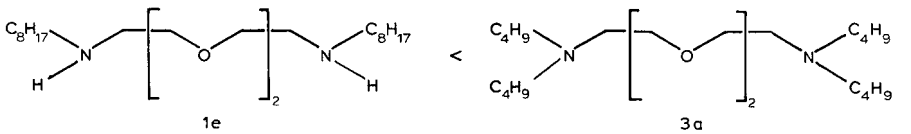


Fig. 2. Influence of the number of oxyethylene units on the polarity of compounds 2. ○, Methanol; △, ethanol.

which each nitrogen atom is linked with only one but long alkyl group, as in compounds 1e and 3a:



1e 3a

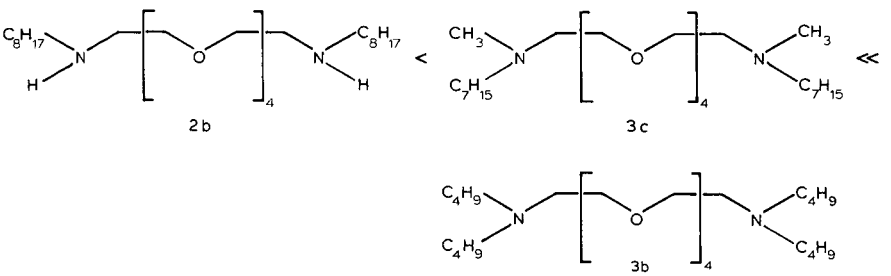
The polarity parameters for compound 3a are higher than those for compound 1e by about 100, 15, 2, 2 and 100 units for I_R, PI, ρ, $\Delta G_s^m(OH)$ and $\sum_{i=1}^{5} \Delta I_i$, respectively. Hence

TABLE V

INCREMENTS OF POLARITY PARAMETERS

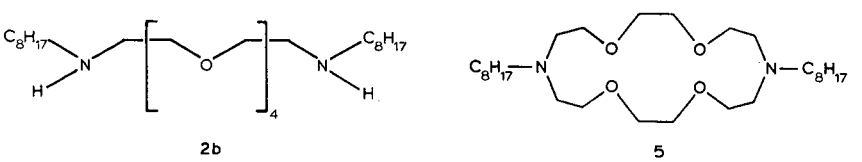
Polarity parameter	*Polar solute*	*Temperature (°C)*	*Increment*	
			–CH₂–	*–OCH₂CH₂–*
I_R	CH_3OH	70	−4.25	11.5
		90	−6.07	18.5
	C_2H_5OH	70	−4.45	10.0
		90	−4.87	13.0
PI	CH_3OH	70	−0.895	0.949
		90	−1.372	4.340
	C_2H_5OH	70	−0.777	0.700
		90	−0.905	1.650
ρ	CH_3OH	70	$-5.95 \cdot 10^{-2}$	0.250
		90	$-7.07 \cdot 10^{-2}$	0.200
	C_2H_5OH	70	$-8.67 \cdot 10^{-2}$	0.105
		90	$-7.92 \cdot 10^{-2}$	0.095

this effect is very strong and much more important than the effect of the lengths of the non-polar alkyl groups and of the polar oligooxyethylene chain. This effect becomes even stronger when the length of the polar oligooxyethylene chain increases. When compounds 2b and 3b are compared for which $n = 4$, the observed increase in the polarity parameters is about 50% higher than with compounds 1e and 3a. This increase is observed when the hydrogen atoms are replaced by methyl groups (compound 3c). This replacement causes a similar increase in the polarity parameters with a further change from methyl to butyl group (compounds 3c and 3b):

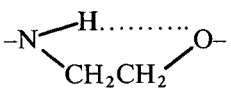


The effect of the oligooxyethylene chain length is also much stronger for compounds having two short alkyl chains linked with each nitrogen atom (for compounds 3 in comparison with compounds 1 and 2), *e.g.*, for compounds 3a and 3b in comparison with compounds 1e and 2b. In this instance, $PP_i(3b) - PP_i(3a) \gg PP_i(2b) - PP_i(1e)$.

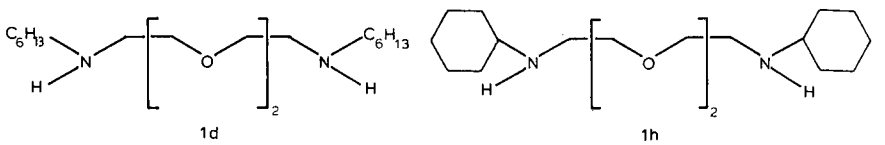
It is interesting that the linear compound 2b exhibits the same polarity as the cyclic compound 5. The same values of the polarity parameters, including the contributions for the McReynolds constants, were obtained for these two compounds:



Probably this is a result of a hydrogen bonding in compound 2b between hydrogen atoms bonded with nitrogen atoms and oxygen atoms present in the oxyethylene groups linked with nitrogen atoms:

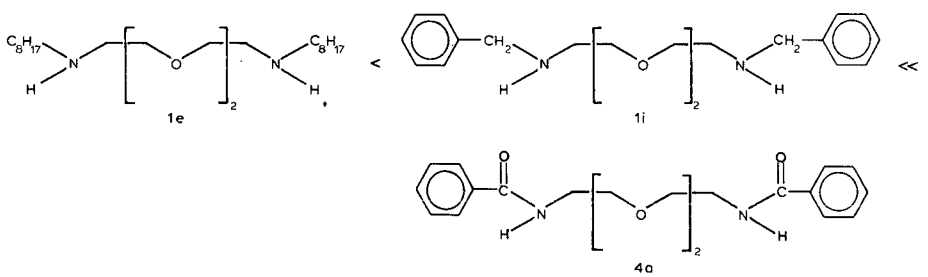


Comparison of the polarity parameters obtained for compounds 1d and 1h demonstrates that these compounds exhibit similar polarities as far as the retention times of alcohols and ketones are considered. The retention indices of alcohols, polarity indices, ρ values and partial molal Gibbs free energies of solution are almost the same for these two compounds. However, when more complex McReynolds constants are considered, a higher $\sum_{i=1}^{5} \Delta I_i$ is obtained for compound 1d.



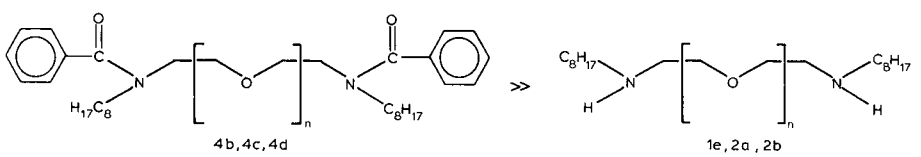
1d 1h

The replacement of the octyl group by a benzyl group results in only a small polarity increase (compounds 1e and 1i), which rapidly increases when the octyl group is replaced by a benzoyl group (compounds 1e and 4a). Thus,



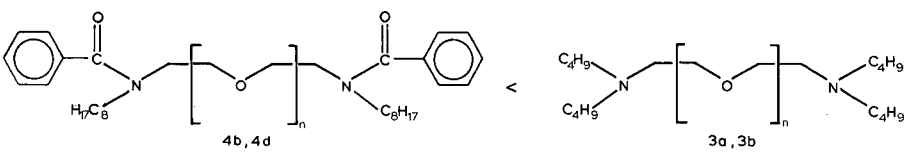
1e 1i 4a

This effect is so strong that compounds 4b, 4c and 4d are much more polar than compounds 1e, 2a and 2b, respectively, and they exhibit polarities near those of compounds 3a and 3b in which each nitrogen atom is bonded with two short butyl groups. Thus,



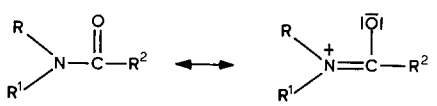
4b,4c,4d 1e,2a,2b

and



4b,4d 3a,3b

Compounds 4 have slightly acidic nitrogen atoms and they form two mesomeric structures:



As a result, they are more polar than appropriate compounds 1, 2, 3 and 5, in which the nitrogen atoms are strongly basic owing to the free electron pairs.

The effects of different groups on the polarities of the compounds can be quantitatively measured by means of the so-called "hydrophobe effective length". Compounds 1, $RNHCH_2(CH_2OCH_2)_2CH_2NHR$, were selected as the standard series and the hydrophobe effective length was obtained from the relationship of PP_i *vs.* $2m$, where m denotes the number of carbon atoms in each alkyl group and PP_i is the polarity parameter, valid for the considered standard series and from the polarity parameter determined experimentally for a compound considered. Thus the hydrophobe effective length equals the number of carbon atoms in both alkyl groups linked to the nitrogen atoms in a hypothetical $RNHCH_2(CH_2OCH_2)_2CH_2NHR$ which exhibits the same polarity as the compound considered.

The hydrophobe effective lengths obtained (Table VI) depend on the polarity parameter used for the calculations as a result of different interactions taken into consideration. They demonstrate that the polarities of cyclohexyl and benzyl groups are similar to those of the alkyl groups when only the interactions with the alcohol are considered. However, a much lower hydrophobe effective length was obtained for compound 1i, having two aromatic rings, when the McReynolds constants were considered. Compounds 3 have their lower hydrophobe effective lengths than the sum of the carbon atoms present in their alkyl groups. Thus, the second short alkyl group has only a small effect on the polarity. For compounds 4 negative values were obtained

TABLE VI

HYDROPHOBE EFFECTIVE LENGTHS

Compound	*Actual number of carbon atoms*	*Hydrophobe effective length*				
		I_R		PI		$\sum_{i=1}^{5} \Delta I_i$
		CH_3OH	C_2H_5OH	CH_3OH	C_2H_5OH	
1h	12 ($2 \times C_6H_{12}$)	12.5	13.2	9.9	10.3	17.7
1i	14 ($2 \times C_6H_5CH_2$)	14.2	13.3	13.0	18.2	7.9
2a	16	14.5	15.7	13.4	14.9	18.2
2b	16	14.2	14.6	12.9	13.6	12.7
3a	16	7.4	9.1	4.1	4.3	9.2
3b	16	4.0	5.6	1.8	1.7	5.7
3c	16	7.5	9.5	4.3	4.7	8.6
4a	12 ($2 \times C_6H_5CO$)	5.0	6.7	2.3	2.2	−44.2
4b	14 ($C_8 + C_6H_5CO$)	11.4	13.0	6.9	8.1	− 9.7
4c	14 ($C_8 + C_6H_5CO$)	9.5	11.1	6.1	6.7	−11.7
4d	14 ($C_8 + C_6H_5CO$)	8.6	10.8	5.2	6.1	−12.7
5	16	14.3	15.2	13.0	15.3	12.9

TABLE VII

REGRESSION AND CORRELATION COEFFICIENTS FOR THE RELATION

$$PP_i = a + b\sum_{i=1}^{5} \Delta I_i$$

PP	*Solute*	*a*	*b*	*r*
I_R	CH_3OH	393.7	0.308	0.8953
	C_2H_5OH	427.8	0.296	0.9185
PI	CH_3OH	42.004	0.0533	0.8526
	C_2H_5OH	55.700	0.0422	0.9244
ρ	CH_3OH	−4.18	$6.67 \cdot 10^{-3}$	0.9378
	C_2H_5OH	−4.70	$7.61 \cdot 10^{-3}$	0.9466
ΔG_s^m(OH)	CH_3OH	−7.316	$-3.64 \cdot 10^{-3}$	0.7688
	C_2H_5OH	−5.587	$-3.69 \cdot 10^{-3}$	0.7632
ΔG_s^m(C=O)	MEK*	−6.737	$-2.09 \cdot 10^{-3}$	0.8812
	MPK*	−6.629	$-1.89 \cdot 10^{-3}$	0.8651

* MEK = 2-butanone; MPK = 2-pentanone.

for the hydrophobe effective lengths as a result of the high polarity of the benzoyl group present in these amides.

As previously[2], approximately linear relationships exist between the considered polarity parameters and the sum of the first five McReynolds constants. However, the correlation coefficients are lower but still acceptable. The values of the regression coefficients presented in Table VII were obtained for compounds that do not contain an aryl group in their molecules. For this group of compounds the contribution of ΔI values of 1-butanol in $\sum_{i=1}^{5} \Delta I_i$ is much lower than for the other compounds with non-aromatic hydrophobes (Table IV), as the contribution of the respective McReynolds solutes to the $\sum_{i=1}^{5} \Delta I_i$ values depends significantly on the chemical nature of the stationary phase used.

Linear relationships of PP_i *vs.* $1/C_n$ where C_n is the number of carbon atoms in the alkyl group, were found for the homologous series 1. For the retention index and ρ (solute ethanol) (Fig. 3a) the correlation coefficients are high (0.9939 and 0.9889, respectively). For the other polarity parameters considered the correlation coefficients are low (<0.9) and the errors are high. The polarity index may be correlated with the reciprocal of the molecular weight (M) of the stationary phase (Figs. 3b): for methanol, $PI = 93.33 + 7790/M$, $r = 0.9950$, and for ethanol, $PI = 79.39 + 6408M$, $r = 0.9874$.

CONCLUSIONS

Relationships between the polarity parameters and the structures of α,ω-diaminooligoether derivatives are much more complex than those obtained previously

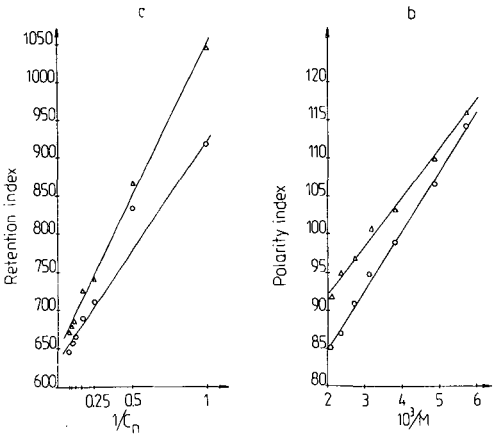


Fig. 3. (a) Retention index *vs.* reciprocal of the length of the alkyl chain; (b) polarity index *vs.* reciprocal of the molecular mass of the stationary phase. ○, Methanol; △, ethanol.

for linear compounds. Linear relationships between the polarity parameters and the numbers of oxyethylene groups and carbon atoms present in the alkyl groups are only observed for homologous series and are observed for compounds having enough long alkyl chains and/or oligooxyethylene chain. Two short alkyl chains have a much smaller effect on the polarity than one long alkyl chain.

A cyclohexyl group is equivalent to an *n*-hexyl group. Amides having benzoyl groups are much more polar than the appropriate intermediate α,ω-diaminooligoether derivatives.

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GAS CHROMATOGRAPHY ON POROUS POLYMERS

IV. INFLUENCE OF THE GEOMETRIC STRUCTURE OF POROUS COPOLYMERS OF 1,4-DI(METHACRYLOYLOXYMETHYL)NAPTHTHALENE WITH 1,4-DIVINYLBENZENE ON THEIR CHROMATOGRAPHIC BEHAVIOUR

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SUMMARY

Four copolymers of 1,4-di(methacryloyloxymethyl)naphthalene and 1,4-divinylbenzene were prepared with surface areas ranging from 184.5 to 342 m^2/g and porosities ranging from 1.06 to 1.36 cm^3/g. The chromatographic behaviour of homologous series of *n*-alkanes, *n*-alkan-1-ols, *n*-methyl ketones and *n*-alkanoic acids was similar on all four polymers.

INTRODUCTION

We recently discussed the behaviour of various aromatic compounds[1] and the peculiarities in the behaviour of *n*-alkanes[2] chromatographed on polymers of different polarities obtained by copolymerization of 1,4-di(methacryloyloxymethyl)naphthalene (DMN) and 1,4-divinylbenzene (DVB). An increase in the molar fraction of the polar monomer (DMN) increased the polarity of the sorbents and their selectivity towards different benzene derivatives. The relationships between the enthalpy change due to sorption of the molecules chromatographed and the corresponding entropy change for the homologous series studied indicated the existence of specific solute–sorbent interactions. We also observed linear relationships between the retention indices of benzene derivatives and the total selectivity of the sorbents, ΣI_{McR}[3], suggesting an unifiorm retention mechanism, irrespective of the sorbent polarity. For the solute–sorbent interactions we considered only the chemistry of the copolymers, as the physical properties, such as the specific surface areas and total porosity, were very similar for all sorbents.

The present work considers the effects of the geometric structure of porous polymer beds (PPBs) obtained by copolymerization of DMN and DVB on the chromatographic behaviour of various homologoues series of aliphatic compounds: *n*-alkanes, *n*-alkan-1-ols, *n*-methyl ketones and *n*-aliphatic acids. All the polymers studied have similar polarities, but different specific surface areas and total porosities.

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EXPERIMENTAL

Preparation of copolymers

Porous copolymers of 1,4-di(methacryloyloxymethyl)naphthalene with 1,4-divinylbenzene were obtained by suspension copolymerization in the presence of a mixture of toluene and *n*-decan-1-ol used as a diluent[4,5]. Polymers with different geometric structures were obtained by changing the proportion of toluene (v/v) to *n*-decan-1-ol. An increase in the proportion of toluene in the diluents led to an increase in specific surface areas, a decrease in the average pore radius and a modification of the pore-size distribution functions[5,6].

The proportion of toluene used in the syntheses of the copolymers together with some of their properties are presented in Table I.

Chromatographic measurements

Chromatography was carried out as previously[1]. The bridge current was 200 mA. Measurements were performed at 185, 190 and 195°C. Samples (0.2 μl) of *n*-alkanes, *n*-alkan-1-ols, *n*-methyl ketones and *n*-aliphatic acids were injected.

RESULTS AND DISCUSSION

The data in Table I show that changes in the composition of the diluent mixture lead to large changes in the geometric structure of the resulting copolymers. Similar effects were observed by Sederel and De Jong[7] for styrene–divinylbenzene copolymers. On the other hand, the results of the McReynolds test (Table I) suggest that changes in the geometric structures of our copolymers are not accompanied by any important changes in the chemical structure of their surfaces. From

$$\ln k' = -\Delta H/RT + A\Delta S \tag{1}$$

where

$$k' = (t_R - t_0)/t_0 \tag{2}$$

TABLE I

PROPERTIES OF COPOLYMERS

S = Specific surface area; ΣI_{McR} = total selectivity as determined by the McReynolds test. The specific surface areas were determined by using the standard nitrogen adsorption method with help of a Sorptomatic 1806 (Carlo Erba, Milan, Italy) apparatus and the porosity was determined with help of a mercury porosimeter (Carlo Erba).

Sample No.	*Toluene conc. (%, v/v)*	*S (m^2/g)*	*Porosity (cm^3/g)*	ΣI_{McR}
1	20	184.5	1.36	624.0
2	25	222.0	1.24	618.0
3	40	281.0	1.10	602.0
4	65	342.0	1.06	609.0

t_R being the retention time, t_0 the dead time, ΔH the change in enthalpy, ΔS the change in entropy, R the gas constant, T the temperature and A a temperature-independent constant, characteristic of the chromatographic system, we can determine ΔH and ΔS due to the sorption of solutes. A plot of ΔH *versus* $A\Delta S$ for all the solutes and all the sorbents studied (Fig. 1) shows a well defined linear relationship suggesting that (i) the interactions between solutes and sorbents are non-specific and (ii) the nature of these interactions does not depend on the sorbent used. However, we do find some differences in the magnitude of the molecular interactions for each homologous series on different sorbents. This is demonstrated in Fig. 2 which shows the relationships between the enthalpy change and the number or carbon atoms, n_c, in the chain of the solute molecule. There is a linear relationship between ΔH and n_c for all homologous series considered. Similar relationships are observed for the entropy changes. These linear relationships suggest that one can consider the total changes in the enthalpy and entropy as comprised of the contributions due to the functional group in the solute molecule (ΔH_0 and ΔS_0) and to the methylene groups of the carbon chain (ΔH_1 and ΔS_1). Thus we can write

$$\Delta H = \Delta H_0 + n_c \Delta H_1$$

and

$$\Delta S = \Delta S_0 + n_c \Delta S_1$$

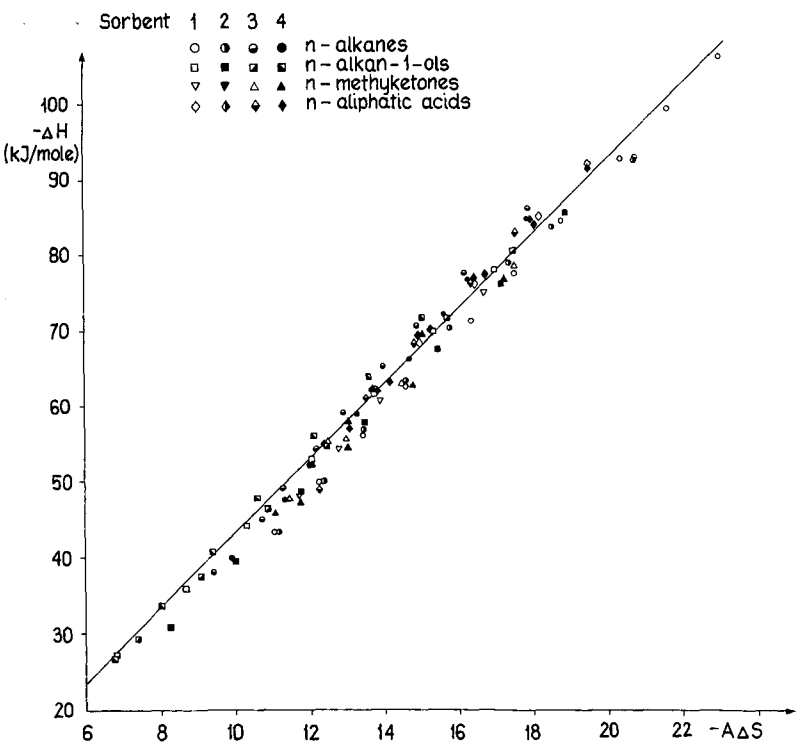
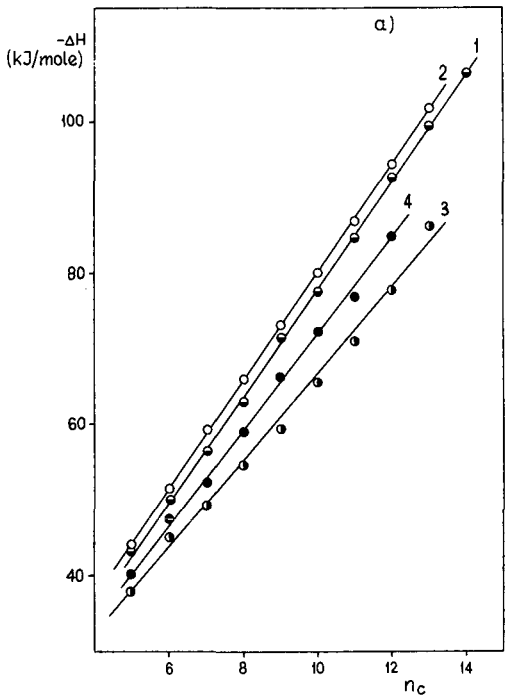


Fig. 1. Relationships between ΔH and $A\Delta S$ for various homologous series and the sorbents from Table I.


a)
-ΔH
(kJ/mole)
100
80
60
40
6
8
10
12
14
n_c
1
2
3
4

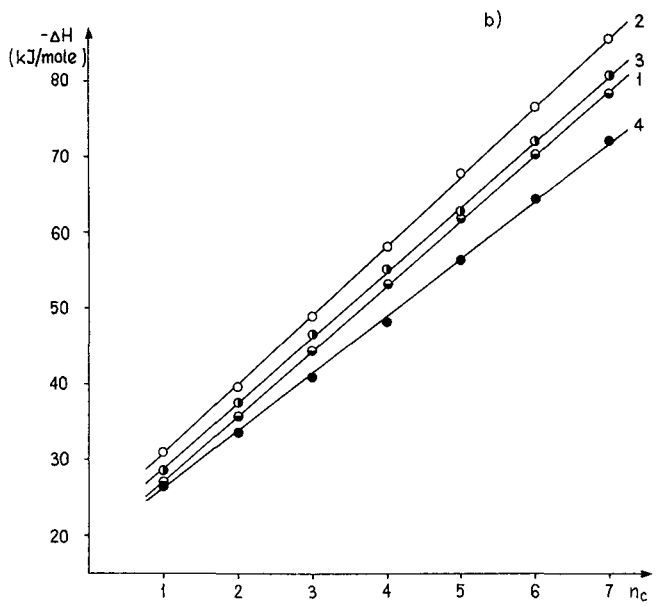

b)
-ΔH
(kJ/mole)
80
70
60
50
40
30
20
1
2
3
4
5
6
7
n_c
2
3
1
4

Fig. 2.

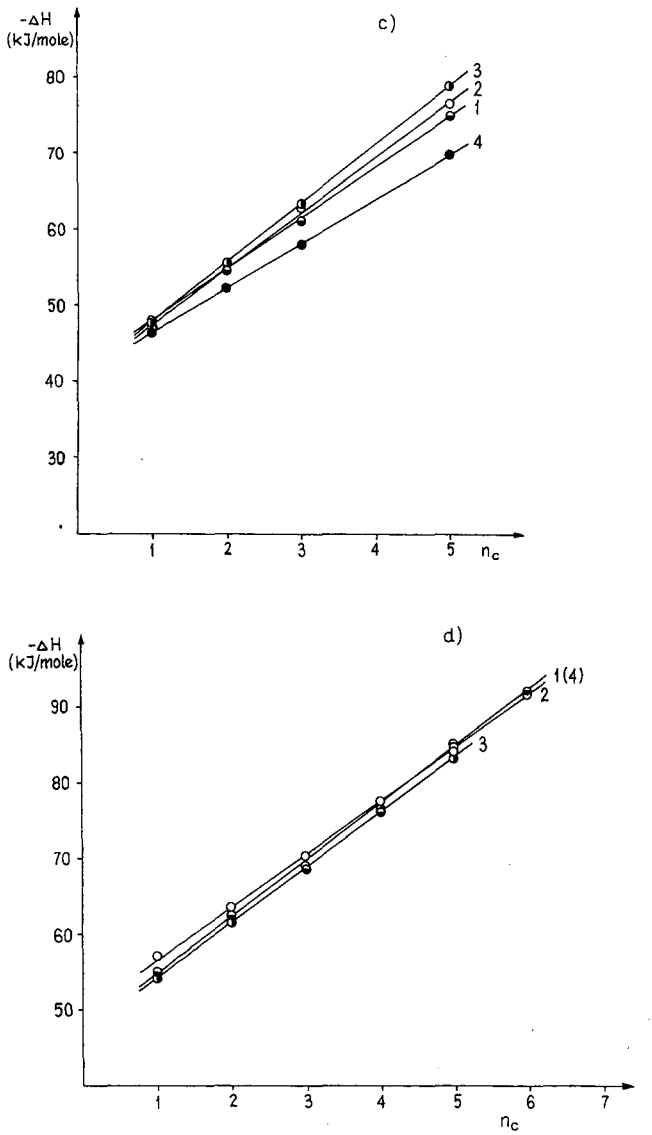


Fig. 2. Relationships between ΔH and the number of carbon atoms in the chain for various homologous series and the sorbents from Table I: (a) *n*-alkanes; (b) *n*-alkan-1-ols; (c) *n*-methyl ketones; (d) *n*-aliphatic acids.

The results in Table II demonstrate clearly that ΔH_1 and ΔS_1 remain practically constant for all homologous series considered here. On the other hand, the values of ΔH_0 and ΔS_0 increase with the polarity of the functional group in an homologous series of solute molecules, *i.e.*, these parameters characterize the chemical nature of the solute molecules. The sorbent structure seems to have little influence on the values of ΔH_0, $A\Delta S_0$, ΔH_1 and $A\Delta S_1$.

TABLE II

THE COEFFICIENTS ΔH_1, ΔH_0, $A\Delta S_1$ AND $A\Delta S_0$ FOR VARIOUS HOMOLOGOUS SERIES OBTAINED FOR THE COPOLYMERS STUDIED

Homologous series	*Copolymer No.*	$-\Delta H_1$ *(kJ/mol)*	$-\Delta H_0$ *(kJ/mol)*	$-A\Delta S_1$	$-A\Delta S_0$
n-Alkanes	1	7.07	7.32	1.34	4.15
	2	6.90	8.58	1.28	4.78
	3	5.58	10.11	0.94	4.79
	4	6.25	9.12	1.11	4.49
n-Alkan-1-ols	1	8.54	18.76	1.67	5.31
	2	9.16	21.44	1.78	6.44
	3	8.61	20.15	1.65	5.75
	4	7.58	18.41	1.40	5.20
n-Methyl ketones	1	6.76	34.29	1.25	9.07
	2	7.37	32.85	1.37	9.06
	3	7.77	40.00	1.51	9.96
	4	5.82	34.73	0.99	9.40
n-Aliphatic acids	1	7.46	47.17	1.41	10.94
	2	6.89	43.01	1.24	10.70
	3	7.31	46.83	1.34	10.85
	4	7.40	47.48	1.38	10.95
Average		7.28		1.35	

TABLE III

KOVÁTS RETENTION INDICES FOR VARIOUS HOMOLOGOUS SERIES AT 190°C OBTAINED ON THE COPOLYMERS LISTED IN TABLE I

Compound	I_R *on copolymer No.*			
	1	*2*	*3*	*4*
Methanol	490.7	439.7	413.2	422.4
Ethanol	587.0	546.6	527.3	536.6
Propan-1-ol	702.0	676.75	652.2	656.9
Butan-1-ol	816.0	796.3	777.0	772.8
Pentan-1-ol	922.8	910.3	889.1	883.2
Hexan-1-ol	1028.3	1016.3	997.7	987.4
Heptan-1-ol	1131.0	1122.9	1101.2	1092.7
Acetone	614.7	598.7	591.0	594.6
Methyl ethyl ketone	723.4	713.6	693.9	698.5
Methyl *n*-propyl ketone	818.4	809.5	784.0	791.6
Methyl *n*-pentyl ketone	1026.8	1023.1	985.5	995.3
Acetic acid	823.2	796.3	773.1	779.2
Propionic acid	927.1	906.8	875.4	883.0
Butyric acid	1036.0	1013.9	985.5	987.4
Valeric acid	1140.9	1121.0	1098.5	1101.7
Caproic acid	1245.6	1228.3	1203.3	1206.3
Heptanoic acid	1351.5	1336.3	–	–

TABLE IV

RETENTION TIMES FOR *n*-ALKANES AT 190°C OBTAINED ON THE COPOLYMERS LISTED IN TABLE I

n-Alkane (C_n)	t_R (s) on copolymer No.			
	1	*2*	*3*	*4*
C_5	19.9	20.2	20.5	22.3
C_6	27.0	27.0	29.1	31.8
C_7	38.7	38.5	43.0	47.7
C_8	58.0	58.5	66.2	73.9
C_9	89.6	90.6	105.8	119.0
C_{10}	141.0	144.0	172.5	194.0
C_{11}	225.0	236.0	277.0	323.0
C_{12}	366.0	380.0	460.0	537.0
C_{13}	595.0	–	–	–

In Table III we give the retention indices at 190°C for *n*-alkan-1-ols, *n*-methyl ketones and *n*-alkanoic acids determined on all the sorbents used. For any substance, the values of I_R decrease as the proportion of toluene in the diluent mixture increases to 40% (v/v). With a still higher proportion of toluene in the diluent mixture (sorbent 4) and equal to 65% (v/v), some of the retention indices increase again. There is a gradual decrease in the difference between the retention indices obtained for different sorbents as the number of carbon atoms in the solute molecule increases, confirming that the contribution of the functional group to the retention decreases with an increase in the number of carbon atoms in the aliphatic chain. All the sorbents considered exhibit very similar interactions with the methylene groups and, hence, the retention of long-chain molecules should become similar for all the sorbents. In Table IV we give the retention times for *n*-alkanes at 190°C. It is seen that these times increase with the polarity of the sorbent.

The results presented above show that the chromatographic properties of the polymeric column packings with porosities in the range of 1.06–1.36 cm^3/g are not sensitive to their geometric structure.

ACKNOWLEDGEMENT

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EFFECT OF THE CONTENT OF DIVINYLBENZENE IN ION-EXCHANGE RESINS ON THE CHROMATOGRAPHIC SEPARATION OF α-CYCLODEXTRIN AND GLUCOSE

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SUMMARY

In the chromatographic separation of α-cyclodextrin and glucose, the parameters relating to the adsorption equilibria and rate processes of the solutes were evaluated for the sodium forms of cation-exchange resins with contents of divinylbenzene (DVB) of 2, 4, 6 and 8%. By using these parameters, the chromatograms of the solutes were then calculated. It is concluded that the resin with 6% DVB is most suitable for the chromatographic separation of the solutes on a preparative scale.

INTRODUCTION

Cyclodextrins (CDs) are cyclic and non-reducing maltooligosaccharides produced from starch by cyclodextrin glucosyltransferases. They have an ability to form complexes with guest compounds, and have been utilized in food and pharmaceutical industries. The enzymes, regardless of their origins, produce a mixture of α-, β- and γ-CDs and maltooligosaccharides[1]. In order to produce a specific CD, a separation process is indispensable.

β-CD can easily be separated from other saccharides due to its low solubility. The co-operative action of taka-amylase and glucoamylase hydrolyzes γ-CD and maltooligosaccharides to glucose, but the enzymes do not affect α-CD[2]. Therefore, production of α-CD with high purity requires the separation of α-CD and glucose.

Among separation procedures, chromatography may be promising because of the non-use of additives. Lammers[3] demonstrated the separation by adsorption

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chromatography using gradient elution on charcoal, and Carter and Lee[4] reported the adsorption chromatography of CDs on Sephadex G-15. These methods are, however, tedious and time-consuming. Chromatography on polyacrylamide gels has been applied to the separation of CDs and maltooligosaccharides[5,6]. Since the gels are relatively expensive and give a large pressure drop in the packed bed because of their small diameter and softness, they are not suitable for industrial-scale application. Okada *et al.*[7] showed that the sodium or calcium form of strongly acidic cation-exchange resins, comprised of a co-polymer of styrene and divinylbenzene (DVB), could be used to separate α-CD and glucose. However, they did not present details of the adsorption isotherms and rate processes.

In this study we have evaluated the parameters relating to adsorption equilibria and rate processes of α-CD and glucose for the sodium forms of cation-exchange resins with various contents of DVB. Since the separability of the solutes is a complex fraction of the parameters, it is difficult to conclude which ion-exchange resin is most suitable for the chromatographic separation through a simple experiment. Therefore, the optimum DVB content of the ion-exchange resin was determined through simulation of the chromatograms of the solutes on columns packed with each ion-exchange resin, using the parameters estimated.

EXPERIMENTAL

Materials

Four kinds of strongly acidic cation-exchange resins, possessing sulphonate groups, and different DVB contents were used. The DVB contents were 2, 4, 6 and 8%, and the corresponding exchange capacities were 0.7, 1.2, 1.6 and 1.7 mequiv./ml bed, respectively, in the hydrogen forms. Each of the ion-exchange resins (Amberlites HFS-471X, Japan Organo) was conditioned by 1 *M* sodium chloride, and then washed with a large quantity of distilled water. α-Cyclodextrin was obtained from Ensuiko Seito (Japan), glucose from Kanto Chemicals (Japan).

Physical properties of ion-exchange resins

The apparent density, ρ_p, of each ion-exchange resin was measured pycnometrically. The water regain, W_r, which is defined as the weight of water held in the wet resin per unit weight of dry resin, was evaluated from the difference in weights between the wet and dry resins. The diameter was measured by a microphotograph for at least 250 particles. The mean value was calculated by the following equation[8]:

$$\overline{d_p} = (\Sigma d_p^5/\Sigma d_p^3)^{1/2} \tag{1}$$

Chromatography

A cation-exchange resin (Na^+) was carefully packed under vibration in a cylindrical glass column equipped with a water jacket. The inner diameter of the column was $1.5 \cdot 10^{-2}$ m and the bed length was *ca.* 0.45 m (measured exactly in each experiment). The bed was kept at 333 K by circulating thermostatted water through the jacket. A small amount (usually 0.5 ml) of a solute solution was carefully loaded on top of the bed, and the flow was started. When all the sample solution had been sucked into the bed, an eluent, degassed distilled water, was fed by use of a constant-delivery

pump (MP-101; Tokyo Rikakiki, Japan). At appropriate intervals, the effluent was collected at the bottom of the bed by a fraction collector (SF-100G; Toyo Roshi, Japan). The concentration of α-CD in the sample was in the range of 3–11% (w/v), while that of glucose was 10–30% (w/v). The bed voidage, ε_b, was estimated from the response curve of an impulse of 1% (w/v) soluble starch, whose molecular weight was more than $1 \cdot 10^4$.

Analysis

α-CD in the effluent was assayed by the following method. The effluent was appropriately diluted in distilled water. A 0.5-ml volume was mixed with 2.5 ml of iodine–potassium iodide solution ($1.25 \cdot 10^{-4}$ *M*), and then the increase in absorbance at 352.5 nm was recorded. The increase was proportional to the α-CD concentration below 0.02% (w/v), without any interference from glucose. The concentration of glucose in the effluent was determined by the glucose oxidase–peroxidase method (New Glucostat, Washington Comp.). A high-performance liquid chromatograph (L-5000; Yanagimoto Seisakusho, Japan) equipped with a separation column (TSKgel SCX; Tosoh, Japan) and a differential refractometer (SE-11; Showa Denko, Japan) was also used to determine the concentrations of α-CD and glucose.

The soluble starch in the effluent was completely hydrolyzed to glucose by addition of glucoamylase (pure grade, Seikagaku Kogyo, Japan) dissolved in a 0.05 *M* acetate buffer (pH 4.5). The glucose produced was analyzed by the glucose oxidase–peroxidase method.

Determination of distribution, intraparticle diffusion and axial dispersion coefficients

Preliminary experiments showed that the adsorption isotherms of α-CD and glucose on the ion-exchange resin, regardless of the DVB content, were linear and independent of each other. Therefore, the moment analysis of the response curve of an impulse of the solute was adopted to estimate the values of the distribution coefficient, m, the axial dispersion coefficient, D_z, and the intraparticle diffusion coefficient, D_s. The first-order normalized statistical moment, μ_1, and the second-order normalized central moment, μ_2, of a chromatogram are related to the values of m, D_z, D_s and the bed voidage, ε_b, by the following equations assuming that the film mass-transfer resistance is insignificant[9]

$$\mu_1 = \int_0^\infty tC(Z, t)\,\mathrm{d}t \Big/ \int_0^\infty C(Z, t)\,\mathrm{d}t$$

$$= (Z/u_0)[\varepsilon_b + (1 - \varepsilon_b)m] \tag{2}$$

$$\mu_2 = \int_0^\infty (t - \mu_1)^2 C(Z, t)\,\mathrm{d}t \Big/ \int_0^\infty C(Z, t)\,\mathrm{d}t \tag{3}$$

$$\mu_2/(2Z/u) = (D_z/u)\,(1 + Hm)^2\,(1/u) + HmR^2/(15D_s) \tag{4}$$

where C is the concentration of a solute, Z the height of the bed, u_0 the superficial velocity, u the interstitial velocity ($= u_0/\varepsilon_b$), R the particle radius and $H = (1 - \varepsilon_b)/\varepsilon_b$. Eqn. 4 was used in this study since the corresponding mass balance equations included all the parameters related to the spreading of the chromatogram. Eqns. 2 and 4 have been successfully utilized to estimate the parameters in gel chromatography[9]. A plot of $\mu_2/(2Z/u)$ *versus* $1/u$ gives a straight line since D_z/u is independent of the flow-rate in a range of Reynolds numbers[10] in these experiments. The value of the bed voidage was determined from a plot of μ_1 *versus* Z/u_0 for soluble starch, which is too large to penetrate the resins.

Calculation of elution curve

The elution curve, $C(Z, t)$, was calculated numerically through an inverse transformation of its solution in the Laplace domain[11].

RESULTS AND DISCUSSION

Table I lists the apparent densities, the water regains and the mean diameters of the ion-exchange resins. The water regain, which may reflect the porosity of the resins, changes considerably between the DVB contents of 4 and 6%. Since the physical properties of the resins are influenced by the DVB content, the equilibrium and mass transfer parameters of α-CD and glucose may depend largely on the DVB content of the resin.

The equilibrium and mass transfer parameters were evaluated from moment analysis of the elution curves for each component. Fig. 1a shows a plot of μ_1 *versus* Z/u_0 for the resin with 2% DVB. The slope of the line for soluble starch gives the bed voidage. The distribution coefficients of α-CD and glucose were also estimated from the slope of the line for each component. The values are listed in Table II. Fig. 1b shows a plot of $\mu_2/(2Z/u)$ *versus* $1/u$. The values of D_z/u and D_s were calculated from the slope and intercept of the line, respectively, according to eqn. 4. The results shown in the figure were also obtained for the resin with 2% DVB. The same kinds of plots were drawn for other resins with different DVB contents. The values of m, D_z/u and D_s of the components are listed in Table II together with the bed voidages. The m and $D_s$values for α-CD and glucose depend on the DVB content. Large changes in the values result upon varying the DVB content between 4 and 6%, as predicted from the water regains. It is known that D_z/u is one to two times the particle diameter[9,10]. The D_z/u values obtained in this study were also within this range.

TABLE I

PHYSICAL PROPERTIES OF THE SODIUM FORMS OF THE CATION EXCHANGERS USED (AMBERLITE HFS-471X)

	DVB content (%)			
	2	*4*	*6*	*8*
Mean diameter, $\bar{d}_p \cdot 10^4$ (m)	3.43	3.73	3.24	3.38
Water regain, W_r (kg/kg)	2.449	2.071	1.214	1.092
Apparent density, ρ_p (kg/m^3)	1139	1159	1229	1251

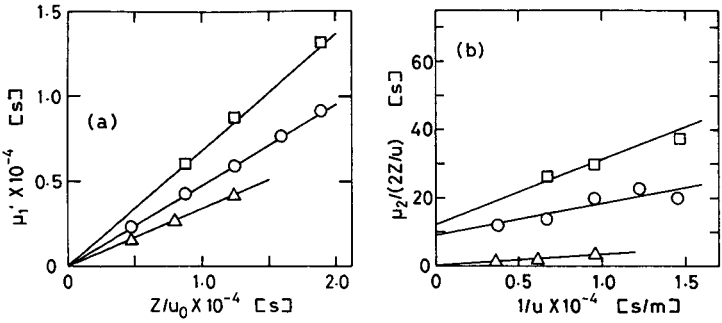

Fig. 1. Estimation of the bed voidage by soluble starch (△) and the distribution, the axial dispersion and the intraparticle diffusion coefficients for glucose (□) and α-CD (○) for the resin with 2% DVB.

The distribution coefficients of both α-CD and glucose become larger as the water regain increases. The difference between them, however, is not greatly dependent on the water regain. A lower water regain means that the resin is harder. The use of hard ion-exchange resins in chromatography has some advantages such as a small bed shrinkage at high concentrations of solutes and a low pressure drop.

The D_s values of α-CD and glucose also depend on the water regain. The D_s value of glucose is about one tenth of the molecular diffusivity, *ca.* $1.4 \cdot 10^{-9}$ m^2/s at 60°C, estimated from the data of Gladden and Dole[12]. The D_s values of α-CD for resins with DVB contents of 6 and 8% are less than those for resins with DVB contents of 2 and 4%. Although it is supposed that these lower values may result in spreading of the elution curve, this may not always be correct because the extent of spreading depends on the ratio of m to D_s.

From these results it may be supposed that the use of resins having a DVB content of 6 and 8% is advantageous in the chromatographic separation of α-CD and glucose. The selectivity, which is defined as the ratio of m_G to m_α, also supposes the predominance of the resins with DVB contents of 6 and 8%. This supposition was examined in detail.

By varying the sample volume loaded on the chromatographic column, the elution curves of α-CD and glucose were calculated under the same conditions for each of the resins. The column was 0.5 × 0.02 m I.D., the volumetric flow-rate was 0.25 ml/min and the concentrations of α-CD and glucose loaded were 10 and 30% (w/v), respectively. By using the values of the parameters listed in Table II, the elution curves of α-CD and glucose were calculated. Fig. 2 shows the elution curves calculated for the resin with 6% DVB; X_0 is the sample volume normalized by the void volume of the bed. Although the figure shows the curves in intervals of 0.1 in X_0, the actual calculations were carried out in more detail. The elution curves were also calculated for other resins with different DVB contents. From these elution curves the recovery, Y, the purity, P, and the average concentration, C_{av}, of α-CD in its fraction were calculated by

$$Y = Q \int_{t_{1\alpha}}^{t_{2\alpha}} C_\alpha \, dt/(C_{\alpha 0} V_0) \tag{5}$$

TABLE II

THE DISTRIBUTION, m, AXIAL DISPERSION, D_z, AND INTRAPARTICLE DIFFUSION, D_s, COEFFICIENTS OF α-CD AND GLUCOSE FOR THE SODIUM FORMS OF THE CATION EXCHANGER (AMBERLITE HFS-471X) WITH VARIOUS DVB CONTENTS, AND THE BED VOIDAGE, ε_b (60°C)

	2% DVB			*4% DVB*			*6% DVB*			*8% DVB*		
	Starch	*α-CD*	*Glucose*	*Starch*	*α-CD*	*Glucose*	*Starch*	*α-CD*	*Glucose*	*Starch*	*α-CD*	*Glucose*
ε_b	0.341	–	–	0.340	–	–	0.351	–	–	0.343	–	–
m	–	0.204	0.508	–	0.152	0.503	–	0.042	0.304	–	0.035	0.239
$D_z/u \cdot 10^4$ (m)	3.33	4.87	4.90	3.00	3.98	5.80	3.13	5.97	3.17	3.06	3.45	4.48
$D_s \cdot 10^{10}$ (m^2/s)	–	0.86	1.59	–	0.76	1.33	–	0.33	1.09	–	0.28	0.93

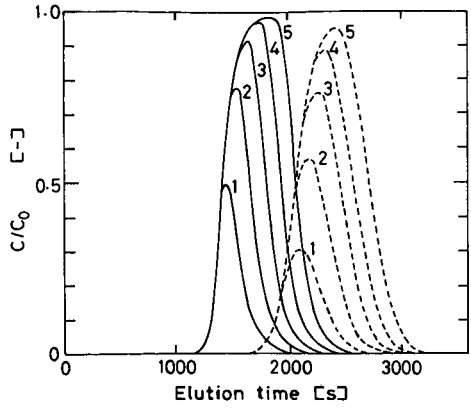

Fig. 2. Chromatograms of α-CD (——) and glucose (– – –) calculated for the column packed with the cation-exchange resin having 6% DVB at various volumes of sample solution loaded. X_0 = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4) and 0.5 (5). The details of the conditions are given in the text.

$$P = \int_{t_{1\alpha}}^{t_{2\alpha}} C_\alpha \, dt \Big/ \left(\int_{t_{1\alpha}}^{t_{2\alpha}} C_\alpha \, dt + \int_{t_{1\alpha}}^{t_{2\alpha}} C_G \, dt \right) \tag{6}$$

$$C_{av} = \int_{t_{1\alpha}}^{t_{2\alpha}} C_\alpha \, dt/(t_{2\alpha} - t_{1\alpha}) \tag{7}$$

where Q is the volumetric flow-rate, $C_{\alpha 0}$ the feed concentration of α-CD and V_0 the sample volume loaded. The subscripts α and G denote α-CD and glucose. t_{1i} and t_{2i} (i = α and G) are the times when C_i/C_{i0} = 0.1 in the leading and tailing parts of the chromatogram, respectively. When the chromatograms of α-CD and glucose cross

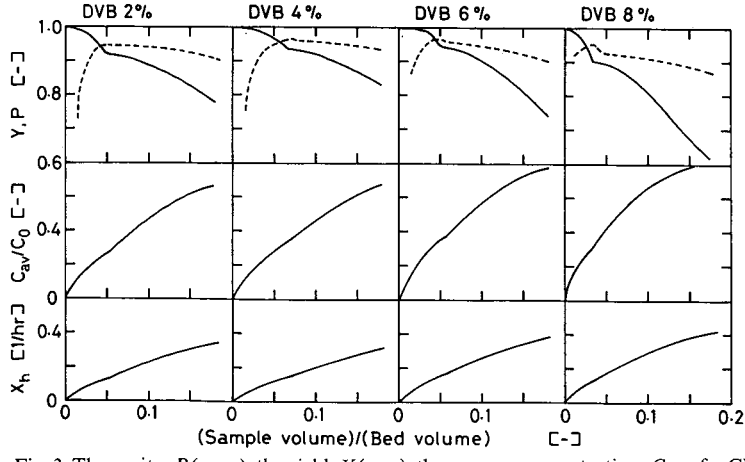

Fig. 3. The purity, P(——), the yield, Y(– – –), the average concentration, C_{av}, of α-CD in its fraction and the sample volume capable of being treated per hour, X_h, normalized by the whole volume of the bed volume, at various volumes of sample solution loaded.

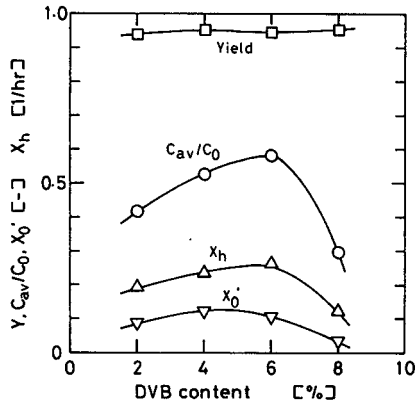

Fig. 4. Comparison of the efficiency of columns packed with cation-exchange resins with various DVB contents to obtain the α-CD solution with a purity of 90%. Y is the yield of α-CD; C_{av} is the average concentration of α-CD in its fraction and C_0 its concentration in the feed; X_h is the sample volume capable of being treated per hour, normalized by the bed volume; X_0 is the sample volume normalized by the bed volume.

over 10% of the feed concentrations, $t_{2\alpha}$ and t_{1G} are regarded as identical. In preparative chromatography the sample injection and the supply of an eluent are alternated. Since the time necessary for an elution is $t_{2G} - t_{1\alpha}$, the sample volume capable of being treated per hour, X_h, which is normalized by the whole volume of the bed, V_t, is given by

$$X_h = [60V_0/(t_{2G} - t_{1\alpha})]/V_t \tag{8}$$

where t_{2G} and $t_{1\alpha}$ are expressed in minutes.

Fig. 3 illustrates Y, P, C_{av} and X_h at various sample volumes for each resin with different DVB contents. The abscissa shows the sample volume normalized by the whole volume of the bed.

The yield, the average concentration and the sample volume at the purity = 0.9 are plotted against the DVB content of the resin in Fig. 4. This reveals that the resin with 6% DVB is most suitable for the preparative chromatographic separation of α-CD and glucose. The mechanical strength of the resin also seems to be good, judging from its water regain and apparent density.

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METHOD FOR EVALUATING THE BIOCONVERSION OF RADIOACTIVE POLYUNSATURATED FATTY ACIDS BY USE OF REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography on a thermostatted octadecylsilyl column was used to separate mixtures of labelled polyunsaturated fatty acids (as their methyl esters) formed by successive desaturations and elongations of labelled linoleic (18:2 $n - 6$) or linolenic (18:3 $n - 3$) acid by rat liver microsomes. Acetonitrile–water mixtures were used for elution of the esters. Unsaturated and saturated esters were detected by their refractive indices. The order of elution of fatty acid methyl esters in complex mixtures varies as a function of the chain length and unsaturation, analysis temperature, water concentration and solvent flow-rate. The peak areas vary as a function of the unsaturation. Specific radioactivities of ^{14}C-labelled fatty acids and the percentage distribution of radioactivity among fatty acids from complex mixtures can be efficiently determined by collection and direct measurement of the radioactivity in the solvent by liquid scintillation counting. The method can be applied to complete compositional analysis, but is especially useful for determination of specific radioactivities during studies on the metabolic conversion of labelled polyunsaturated fatty acids.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are significant constituents of biological membrane phospholipids[1]. They are also substrates for oxygen-containing compounds such as prostaglandins, leucotrienes, hydroxylated fatty acids and related substances[2].

In animal cells such as hepatocytes, the most important PUFAs are synthesized from linoleic acid (18:2 $n - 6$)* and linolenic acid (18:3 $n - 3$) of dietary origin according to Scheme 1.

* This symbolism means that the fatty acid comprises eighteen carbon atoms and two double bonds, the first one being at the sixth carbon atom when counted from the methyl end, the second one being three carbon atoms further on.

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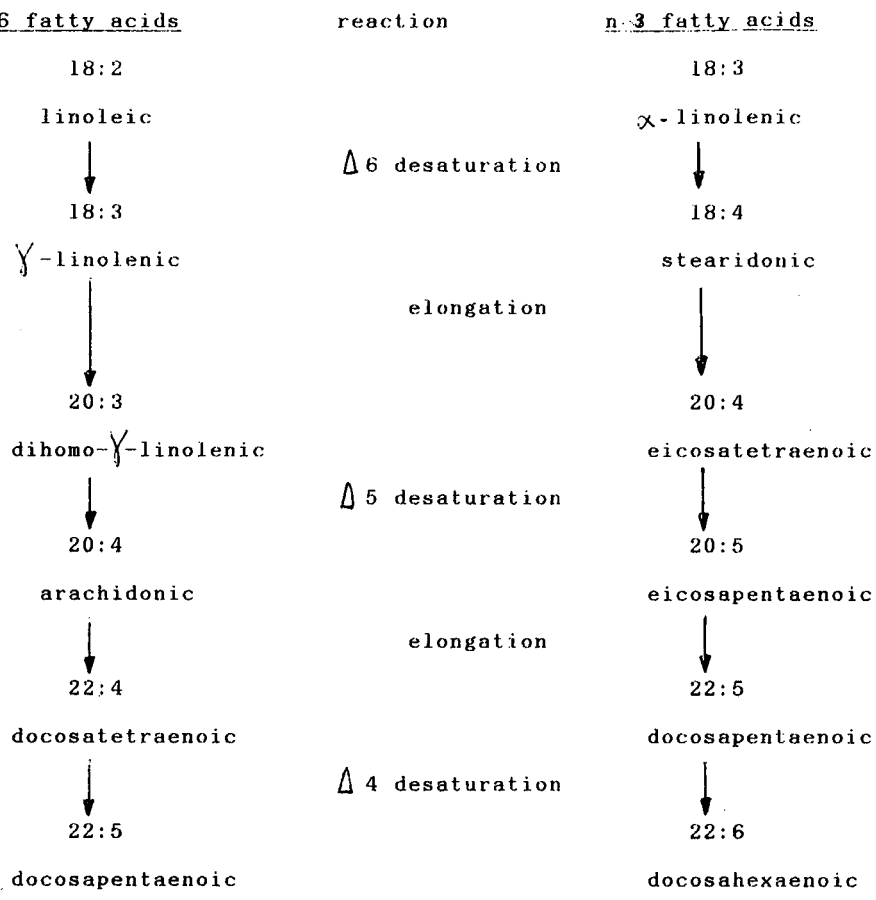

Scheme 1. The symbol Δ6, etc. means that the double bond is formed at the sixth carbon atom when counted from the carboxyl end.

In the $n - 6$ series, another pathway might start as follows:

18:2 ⟶ 20:2 ⟶ 20:3 as above
elongation Δ8 desaturation
eicosadienoic

The biosynthesis of PUFAs can be studied in animals by using ^{14}C-labelled fatty acids as precursors and by determining the radioactivity of the newly formed fatty acids after they have been separated.

Up to now this separation has usually been achieved by gas chromatography (GC) and the radioactivity measured either automatically at the column outlet for relatively high radioactivities[3] or after trapping on glass wool and counting by liquid scintillation for low radioactivities[4]. In both cases packed columns were used which

gave good separations. However, with this type of chromatography, only small quantities of fatty acids can be separated in one step. This represented a drawback when low specific radioactivities were encountered. Furthermore, the analysis was time-consuming since in the presence of polar phases permitting the best separations the most unsaturated fatty acids exhibited the longest retention times. Besides, they were eluted after the precursor, when biosynthesis experiments were performed. These newly formed fatty acids, generally of low radioactivity, may be affected by peak tailing of the precursor, usually highly radioactive. Finally, the method is still more time-consuming when the radioactive fatty acid has to be trapped before being counted.

It is now possible to separate fatty acids as esters by reversed-phase liquid chromatography (RPLC)[5–42], especially PUFAs, in less time than by GC[7,16,21,26,27,33,36,39] and in higher amounts when needed[11,41]. This prompted us to experiment with this type of chromatography to solve our analytical problems. The good results obtained with labelled trioleoylglycerol fractionated by RPLC[43] suggested that the same might be true with labelled polyunsaturated fatty acid esters, as demonstrated by others[21,27].

Accordingly we propose a method for separating and fractionating methyl esters of radioactive polyunsaturated fatty acids by RPLC and for measuring their radioactivity directly in the eluting solvent by liquid scintillation counting.

EXPERIMENTAL

Samples

[1-^{14}C]18:2 [Centre d'Etudes Nucléaires (CEA), Gif sur Yvette, France] was used in all the experiments carried out with a radioactive fatty acid. It was diluted with cold 18:2 (Sigma, St. Louis, MO, U.S.A.). Pure methyl linoleate was prepared by collecting the methyl ester separated by RPLC[43]. To study the separation of PUFAs by RPLC, two GC mixtures of methyl esters from Nu-Chek-Prep (Elysian, MN, U.S.A.) were used: the 1A mixture (16:0, 18:0, 18:1 $n-9$, 18:2 $n-6$, 18:3 $n-3$) and the 8A mixture (20:0, 20:1 $n-9$, 20:2 $n-6$, 18:3 $n-6$). The following fatty acid methyl esters were obtained from Sigma: 18:2 $n-6$, 20:4 $n-6$, 22:4 $n-6$, 20:5 $n-3$, 22:6 $n-3$. A distillate from blackcurrent seed oil (Nestlé, Vevey, Switzerland) provided 18:4 $n-3$. The methyl ester of 22:5 $n-6$ was isolated by RPLC from adipose tissue of trout deficient in 18:3 $n-3$ (ref. 44).

Liquid chromatography

A Model 6000 A solvent-delivery system and a R 401 differential refractometer (Waters Assoc., Milford, MA, U.S.A.) were used. The 250 mm × 4.0 mm I.D. Hibar Lichrocart, Superspher RP 18 (4 μm particles) column was obtained from Merck (Darmstadt, F.R.G.). It was maintained at a constant temperature in a jacket with circulating water. The water temperature was regulated by a thermostat (Model 33194 Polystat I, Bioblock, Strasbourg, France). The device, shown in Fig. 1, allows very reproducible analyses to be performed. The analyses were carried out isocratically at 30 or 40°C, using acetonitrile–water (95:5 or 93:7, v/v) as the mobile phase at a flow-rate of 1.2 or 1.0 ml min^{-1}. Acetonitrile was of analytical grade (SDS, Peypin, France) and water was twice-distilled. The mixture was filtered through a Millipore

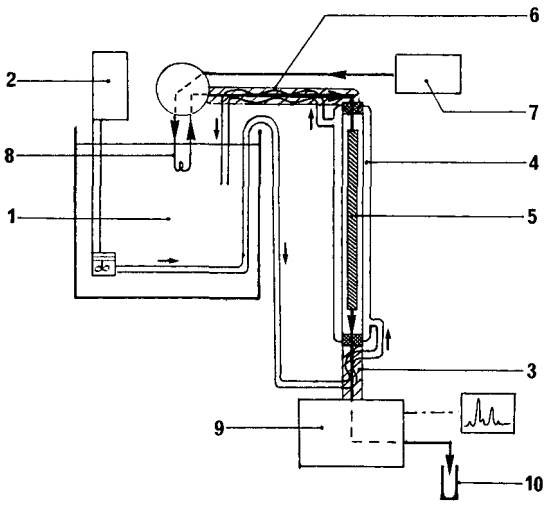

Fig. 1. Device for column thermostatting. Several litres of distilled water contained in a bath (1) are heated by a Polystat (2) which regulates the temperature and forces the water to circulate first close to the outlet column capillary into a glass wool, isolated muff (3), then through a jacket (4) containing the RPLC column (5) and lastly close to the column-inlet capillary into the same type of device as at the outlet (6), before returning to the water-bath. The solvent is delivered via a pump (7) from a reservoir at room temperature and is heated by circulating through a stainless-steel capillary loop (8) placed in the water-bath. Temperature decrease is avoided by the thermostatted devices (3, 6) before the solvent enters the column and at the outlet before it enters the refractive index detector (9). Samples are injected in the heated loop (8), avoiding possible precipitation of saturated long-chain compounds. A thermometer inside the water jacket (4) controls the column temperature. Solvent from the detector and containing an eluted labelled fatty acid methyl ester is collected in a 20-ml polyethylene vial (10) for radioactivity counting.

membrane (pore size 0.5 μm) and vacuum degassed for 1 min before use. The fatty acid methyl ester mixtures were dissolved in pure acetonitrile before injection. Peak areas were measured by means of an Enica 21 integrator-calculator (Delsi Instruments, Suresnes, France). Under the conditions used the detector response was found to be linearly related to the amount of the individual methyl ester detected.

To collect the radioactive methyl ester fractions, the solvent issuing from the detector through a 15 cm × 0.1 mm I.D. stainless-steel tube was collected into a polyethylene vial, routinely thirteen drops after the beginning of the peak registered on the chromatogram. This delay represents the void volume of the detector plus that of the collection tube. It was first determined after chromatography of [^{14}C]trioleoylglycerol as previously described[43] and verified with methyl-[1-^{14}C]linoleate in this work.

Gas chromatography

The fatty acid methyl esters were prepared from methanol–boron trifluoride[45]. The analyses were performed on a Becker-Packard Model 417 gas chromatograph, equipped with a 30 m × 0.4 mm I.D. glass capillary column coated in the laboratory with Carbowax 20M (Applied Science Labs., State College, PA, U.S.A.) at a constant temperature of 195°C and a nitrogen flow-rate of 3 ml min^{-1}. The column was equipped with a ROS injector[46] (Spiral, Dijon, France). Flame ionization detection

was employed. Peak areas were measured by means of an Enica 21 integrator-calculator (Delsi Instruments). Calibration factors for quantitations were calculated using standard mixtures of fatty acid methyl esters (Nu-Chek-Prep).

Radioactivity

Radioactivity was measured by liquid scintillation counting with Permafluor III or Picofluor 15 (Packard Instrument, Rungis, France) using a Packard Model A 300 CD spectrometer. The polyethylene vials (7 or 20 ml) were obtained from Kartell (Noviglio, Italy). Each sample was counted four times for 5 min. Counting efficiencies were estimated by external standardization (Packard).

RESULTS AND DISCUSSION

Separation of polyunsaturated fatty acid methyl esters

In experiments on PUFAs biosynthesis, the mixture to be analyzed generally comprises different labelled fatty acids, *i.e.*, the precursor and the derived fatty acids. It also contains different unlabelled fatty acids originating from the biological material used. The first problem encountered with such a mixture is to separate these different fatty acids with sufficient resolution in order to provide accurate radioactivity measurements. Also, accurate mass determinations from peak areas must be made in order to determine the fatty acid composition.

The best separation of PUFAs in preliminary experiments, and from data in the literature, can be achieved by using RPLC[21,26,27,33,36,39], with a mixture of acetonitrile and water as the mobile phase[14,18,19,25,28,36,40], applied to methyl esters[10,12,16,21,22,27,29,41,42], especially when a differential refractometer was used as the detector[10,12,22,42]. Several chromatographic conditions were tested for different mixtures of fatty acid methyl esters.

Simple mixtures

Two chromatograms (Fig. 2) were obtained by RPLC of two synthetic mixtures under the conditions reported. The first mixture (A) comprised six PUFAs of the $n - 6$ series, expected to be found after the bioconversion of 18:2 $n - 6$ (see Introduction). The fatty acid methyl esters were well separated within a relatively short time (20 min) under the experimental conditions. The low peak tailing does not lead to cross-contamination between adjacent radioactive peaks. As previously stated by others[8,9,13,14,18,20,23,28–30,37], the retention time is related to chain length and to degree of unsaturation of the fatty acids. The higher the chain length, the longer is the retention time (compare 18:3 and 20:3 or 18:2 and 20:2), and the greater the unsaturation, the lower is the retention time (compare 20:4, 20:3 and 20:2). This illustrates the characteristic of RPLC; compounds are eluted in order of decreasing polarity[9,13,16,18]. Moreover, when a new double bond is added to a given fatty acid, the more unsaturated this fatty acid, the smaller is the influence of the additional double bond on the retention (compare 20:4 to 20:3 and 20:3 to 20:2)[18]. The reason may be that the new double bond is nearer to the polar (carboxyl) end of the molecule. Consequently, the notion of partition number[47], the number of carbon atoms -2 $\times$ number of double bonds, cannot apply to the separation of these fatty acid methyl esters by RPLC, as with triacylglycerols[48] and as is apparent from the effect of the position of the double bond on the retention time[13,18,23,42].

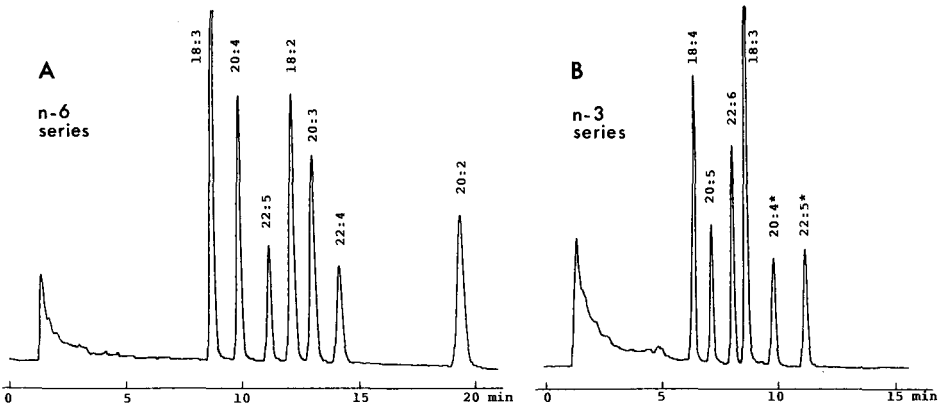

Fig. 2. Analysis by RPLC of a mixture of polyunsaturated fatty acid methyl esters of the $n - 6$ family (A) and of the $n - 3$ family (B). In the $n - 3$ family the last two fatty acids 20:4 $n - 6$ and 22.5 $n - 6$ replaced their unavailable $n - 3$ isomers. Stainless-steel column: 250 mm × 4 mm I.D. packed with 4 μm octadecylsilyl (C_{18}) reversed-phase materials. Eluent: acetonitrile–water (93:7, v/v) at 1 ml min^{-1}. Temperature: 30°C. Refractive index detector. Isocratic analysis.

In desaturation experiments, the elution order of PUFAs in RPLC is favourable for radioactivity measurement, since the newly formed fatty acids are clearly eluted before their less unsaturated precursors. This is the case for the pairs 18:3–18:2 ($\Delta 6$ desaturation) and 20:4–20:3 ($\Delta 5$ desaturation).

The second mixture (chromatogram B in Fig. 2) comprised four PUFAs of the $n - 3$ series. The other two fatty acids 20:4 $n - 3$ and 22:5 $n - 3$ necessary to complete the mixture (see Introduction) were not available. They were replaced by their respective isomers of the $n - 6$ series, whose retention volumes must not differ under our experimental conditions, since the retention volumes of 18:3 $n - 3$ and 18:3 $n - 6$ do not differ (see below). Similarly, the six fatty acid methyl esters were clearly separated, particularly the derivative–precursor pairs, 18:4–18:3 ($\Delta 6$ desaturation) and 20:5–20:4 ($\Delta 5$ desaturation) for which accurate radioactivity determinations can be predicted. The same remarks as made above apply to the influence of the chain length and the degree of unsaturation of fatty acids on their retention volumes in this type of chromatography.

Complex mixtures

When an *in vitro* experiment is undertaken to study the biosynthesis of polyunsaturated fatty acids, labelled 18:2 $n - 6$ or 18:3 $n - 3$ is incubated in an appropriate medium containing rat liver microsomes. At the end of the experiment, an analysis of the radioactive fatty acids is performed on the lipid extract of the incubation medium containing the labelled precursors, the labelled newly synthesized fatty acid and the unlabelled microsomal fatty acids. The last are comprised mainly of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 $n - 9$), linoleic acid (18:2 $n - 6$), arachidonic acid (20:4 $n - 6$) and docosahexaenoic acid (22:6 $n - 3$). Such a mixture was prepared qualitatively and analyzed by RPLC under the same conditions as above. The chromatogram thus obtained is reported in Fig. 3A. It shows that the different polyunsaturated fatty acids expected to be formed from the precursors can

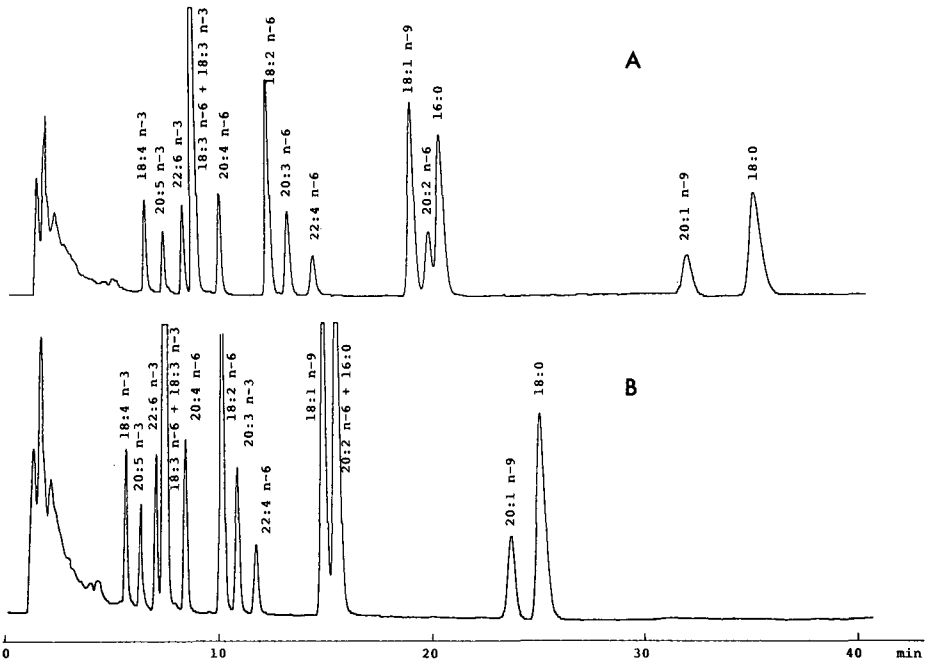

Fig. 3. Analysis by RPLC of a complex mixture of fatty acid methyl esters at 30 (A) or at 40°C (B). Other conditions as in Fig. 2.

easily be identified and thus their radioactivity measured after fractionation. Moreover, all the expected labelled and unlabelled fatty acids were sufficiently well separated to permit the fatty acid composition of the mixture to be determined precisely from the peak areas (see below). Under the conditions used, the analysis time was relatively short since 18:0 was eluted about 35 min after the injection. However, it can be shortened without decreasing the resolution too much, by modifying different chromatographic parameters, as will be shown below.

Influence of temperature. The preceding complex mixture of fatty acid methyl esters was analyzed at 40 instead of 30°C, the other conditions remaining the same. The chromatogram thus obtained (Fig. 3B) shows that the retention times were markedly reduced when the analysis temperature was increased, confirming the results reported by Tsuchiya *et al.*[28] for a wide variety of fatty acid 4-bromomethyl-7-acetoxycoumarin derivatives and by Ichinose *et al.*[26] for the 9-anthryldiazomethane derivative of eicosapentaenoic acid. In our experiment, the retention time of the stearic acid methyl ester was reduced from *ca.* 35 to 25 min for a 10°C increase, that is a 30% reduction in the analysis time of the mixture.

However, the time reduction was not the same for the different fatty acids. In Table I (first line) are reported the ratios of the retention times measured from the beginning of the solvent peak, at 40 and 30°C respectively for the different fatty acids listed according to the elution sequence. The ratios decreased when the retention times increased, which means that the fatty acids eluted last at 30°C had the most shortened retention times at 40°C or, in other words, that the retention time reduction affected the saturated or monounsaturated fatty acids much more than the polyunsaturated

TABLE I

EFFECT OF TEMPERATURE, FLOW-RATE AND NATURE OF SOLVENT ON RETENTION TIME FOR FATTY ACIDS OF DIFFERENT CHAIN LENGTHS AND DEGREES OF UNSATURATION

	18:4 n − 3	*20:5 n − 3*	*22:6 n − 3*	*18:3 n − 3, n − 6*	*20:4 n − 6*	*18:2 n − 6*	*20:3 n − 6*	*22:4 n − 6*	*18:1 n − 9*	*20:2 n − 6*	*16:0*	*20:1 n − 9*	*18:0*
40/30°C*	0.87	0.87	0.86	0.85	0.84	0.82	0.82	0.81	0.78	0.78	0.76	0.74	0.71
1.2/1.0 ml**				0.81	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.79	0.79
95:5/93.7***				0.75	0.73	0.72	0.71	0.69	0.69	0.67	0.70	0.65	0.66

* Ratios of the retention times, at 40 and 30°C respectively, with acetonitrile–water (93:7, v/v) at 1 ml min^{-1}.

** Ratios of the retention times, at 30°C with acetonitrile–water (93:7, v/v) at flow-rates of 1.2 and 1.0 ml min^{-1} respectively.

*** Ratios of the retention times at 30°C with acetonitrile–water, in the proportions 95:5 and 97:3 (v/v) respectively, at 1 ml min^{-1}. All the retention times were measured from the beginning of the solvent peak.

fatty acids. Consequently, for the latter, the loss of resolution was low, not impairing the accuracy of radioactivity determinations. In view of the data reported in Table I, it appears that the fatty acid chain length has a greater contribution to the reduction than the degree of unsaturation, when the analysis temperature is increased. This discriminative effect has been previously underlined by Ichinose *et al.*[26] with different fatty acid derivatives. This is illustrated in Fig. 3B by the cochromatography of 20:2 and 16:0 at 40°C, whereas these two fatty acids were separated at 30°C.

In conclusion, from a practical point of view, in order to reduce the duration of analysis, the temperature can be slightly increased since the resolution of the principal PUFAs is not greatly affected.

Influence of solvent flow-rate. Another mixture of fatty acids was analyzed at 30°C with the same mixture of acetonitrile–water (93:7, v/v) as the eluting solvent but at two different flow-rates, 1.0 and 1.2 ml min^{-1}. The ratios of the two retention times observed for the different fatty acids are reported in Table I (second line). The ratios were the same, *ca.* 0.80. Thus, whatever the type of fatty acid the retention time was reduced in the same proportion, *i.e.*, 20% for a 20% increase in the solvent flow-rate, indicating that the retention volume was not affected by the change in flow-rate. This implies that there is practically no loss of resolution between two close peaks. An increase in the solvent flow-rate thus appears to be a good means of reducing the analysis duration without modifying the elution pattern.

Influence of solvent nature. The same mixture as above was analyzed with the same acetonitrile–water mixture but at two different proportions of water, namely 93:7

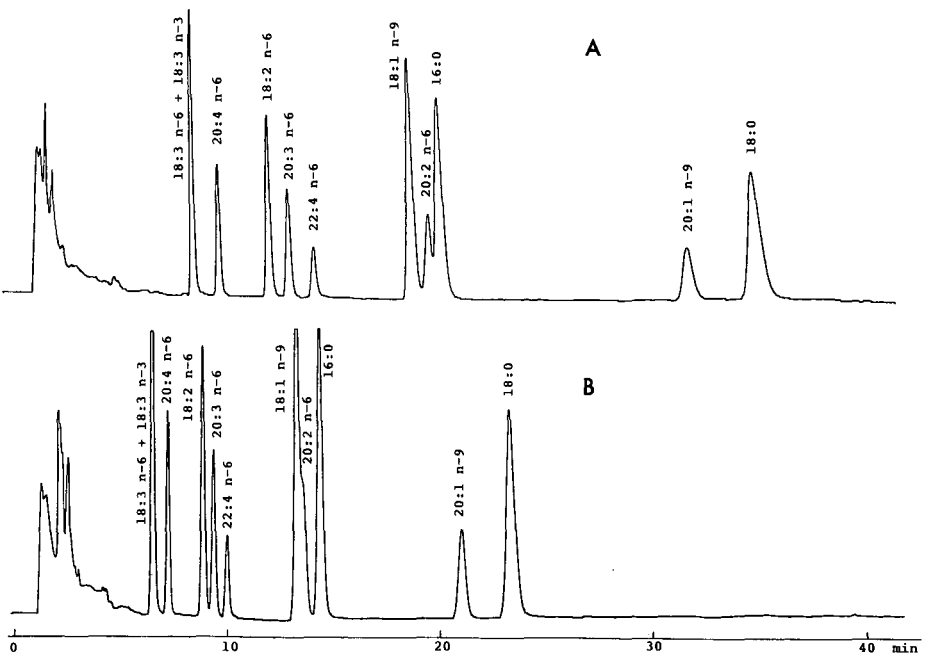

Fig. 4. Analysis by RPLC of a complex mixture of fatty acid methyl esters with acetonitrile–water at two different water concentrations: 93:7, v/v (A), 95:5, v/v (B). Other conditions as in Fig. 2.

TABLE II

RESPONSE OF THE REFRACTIVE INDEX DETECTOR TO FATTY ACID METHYL ESTERS ANALYZED BY RPLC

	18:4 n − 3	*20:5 n − 3*	*22:6 n − 3*	*18:3 n − 3, n − 6*	*20:4 n − 6*	*18:2 n − 6*	*20:3 n − 6*	*22:4 n − 6*	*18:1 n − 9*	*20:2 n − 6*	*16:0*	*20:1 n − 9*	*18:0*
GC/RPLC* (n = 5)	0.90 ±0.03	0.84 ±0.04	0.86 ±0.04	0.92 ±0.03	0.93 ±0.04	0.95 ±0.02	0.92 ±0.02	0.88 ±0.03	1.00 ±0.03	1.01 ±0.02	1.08 ±0.03	1.01 ±0.02	1.08 ±0.03

* Ratios of the percentages of the fatty acid methyl esters present in the complex mixture previously analyzed (Fig. 3A) as determined by GC after application of calibration factors, to the percentages of areas determined after analysis of the mixture by RPLC. Analytical conditions: temperature, 30°C; solvent, acetonitrile–water (93:7, v/v) at 1.0 ml min^{-1}. Values are means ± S.E. of five determinations.

TABLE III

RADIOACTIVITY MEASUREMENT BY LIQUID SCINTILLATION OF FATTY ACID METHYL ESTERS ACCORDING TO THE RELATIVE PROPORTION OF THE ELUTING SOLVENT AND SCINTILLATING LIQUID

Scintillating liquid*	*Permafluor III*		*Picofluor 15*		*Permafluor III*							
Quantity of elution solvent (ml)	0	1	0	1	0	1	2	3	0	1	2	3
Quantity of scintillating liquid (ml)	5	4	5	4	10	10	10	10	15	15	15	15
Counting efficiency (%)**	94.2	90.8	92.5	90.4	96.6	94.7	94.2	93.3	97.1	96.4	94.7	94.4

* Scintillating liquids from Packard (Rungis, France).

** Efficiencies (cpm/dpm · 100) were determined by external standardization (Packard).

and 95:5 (v/v). The two chromatograms are shown in Fig. 4 and the ratios of the two retention times determined from the solvent peak start are listed in Table I (third line). It is seen that for a low decrease in water proportion (from 7 to 5%), the retention times were considerably reduced (from 25 to 35% according to the fatty acids). The significant influence of water (or of the solvent strength) has been observed by several authors using different fatty acid derivatives and different reversed-phase columns[8,9,11,14,15,18,20,21,25–27,30,32,34,35,38]. The most extensive study on free fatty acids was done by Aveldano *et al.*[21] who made the same observations as can be made for the methyl esters from our results (Table I). That is, the higher the hydrophobicity (the longer the chain length or the lower the unsaturation) the larger is the decrease in retention time with decreasing water proportion. Thus, with regard to chain length, for 16:0 and 18:0 the decrease was 30 and 34%, for 18:1 and 20:1 31 and 35%, for 18:2 and 20:2 28 and 33% and for 18:3 and 20:3 25 and 29% respectively. With regard to unsaturation, for the series 20:4, 20:3, 20:2, 20:1 the decrease in retention time was 27, 29, 33 and 35% respectively.

Quantitative analysis of fatty acid methyl esters

At the end of *in vitro* desaturation experiments, in addition to determining the radioactivity of certain fatty acids present in the medium lipids, it can also be useful to calculate the fatty acid composition of the total lipids, starting from the peak areas, or to calculate a specific activity, assuming that a measured radioactivity can be ascribed to a known quantity of the labelled fatty acid. To determine the detector (differential refractometer) response to the fatty acid methyl esters according to chain length and unsaturation, the most complex synthetic mixture (Fig. 3) was analyzed both by liquid chromatography and gas chromatography (GC) under the conditions reported in the Experimental section. Calibration factors determined by use of standard mixtures were applied to peak areas in GC analysis. The peak area percentage calculated from the values thus obtained represented the exact weight percentages of the fatty acid methyl esters present in the mixture. The ratios of these figures to those of the peak area percentages calculated after RPLC analysis are reported in Table II. It is seen that PUFA methyl esters exhibited a ratio less than one, and the more unsaturated the fatty acid, the lower was the ratio. The lowest value was observed with 20:5 $n - 3$. Conversely, the saturated fatty acids showed the highest ratios. The question can be raised whether the ratios increase with retention time and not with decreasing unsaturation. Comparison of the ratios obtained with 22:4 (0.88) and 18:1 (1.00), which are not too distant on the chromatogram, or with 18:1 and 16:0 (1.08) which are very close to each other, shows that unsaturation is mostly responsible for the decrease in ratio.

The values reported in Table II indicate that for the same quantity of fatty acid methyl ester the area registered by using the differential refractometer in RPLC was higher with polyunsaturated than with saturated fatty acids. A similar result has been obtained previously with methyl esters[10,42] or with triacylglycerols[48].

Therefore, to calculate the composition of fatty acid mixtures analyzed by RPLC under the experimental conditions, it is necessary first to apply calibration factors to peak areas. These are the ratio values reported in Table II. Such factors can be determined directly using commercial standard mixtures. Analyses of fatty acid mixtures as methyl esters by RPLC under these conditions can replace GC analyses,

TABLE IV

RADIOACTIVITY RECOVERED AND AREA REGISTERED DURING PEAK COLLECTION

Results are expressed as means ± S.E. for *n* determinations.

							Mean
Injected radioactivity (dpm) $n = 4$	839 ±6	1626 ±16	3195 ±40	6317 ±12	12 635 ±88	24 715 ±132	
Recovered radioactivity (dpm) $n = 6$	820 ±10	1578 ±32	3110 ±44	6192 ±116	12 259 ±277	24 177 ±280	
$\frac{\text{Recovered}}{\text{Injected}}$	0.973 ±0.008	0.970 ±0.016	0.973 ±0.014	0.980 ±0.018	0.970 ±0.022	0.978 ±0.011	0.974* ±0.004
Peak area (arbitrary units) $n = 6$	3462 ±214	7109 ±347	13 420 ±337	26 840 ±338	53 173 ±1361	105 373 ±965	
$\frac{\text{Recovered radioactivity}}{\text{peak area}}$	0.233 ±0.008	0.222 ±0.002	0.232 ±0.004	0.231 ±0.002	0.231 ±0.001	0.229 ±0.001	0.229* ±0.004

* Mean ± S.E. of the six means.

when the amount of the available material is not too low. However, to separate positional isomers of PUFAs, capillary GC has to be used, in our case, whereas Pei *et al.*[13] succeeded in separating 18:3 $n-3$ and 18:3 $n-6$ *p*-bromophenacyl esters by RPLC. When labelled fatty acids are present in the mixture, mass determinations in addition to radioactivity measurement allow calculation of the specific radioactivity of these fatty acids.

Radioactivity measurements

Particular attention was paid to radioactivity measurements in the eluting solvent. In order to save time it was essential that radioactivity be measured directly after collection of a peak, in the presence of the eluting solvent. The latter contains water generally not miscible with scintillating liquids, which can impair radioactivity measurements. Accordingly, we have tested two scintillating liquids from Packard Instruments, namely Permafluor III in which water is insoluble and the more expensive Picofluor 15 in which water is soluble. When Permafluor III was added to the acetonitrile–water mixture used as the eluting solvent, water first appeared as an emulsion and then progressively precipitated leaving a clear supernatant. In contrast, when Picofluor 15 was added, water was maintained in solution and the mixture remained clear.

Tests of radioactivity counting were undertaken with both scintillating liquids. In a first set of experiments, vials of low capacity (7 ml) were chosen because of the high cost of the scintillating liquids. They contained 12.000 dpm of [1-^{14}C]18:2 and 1 ml of acetonitrile–water (93:7, v/v) corresponding to the elution of a peak at 20 min under the usual conditions of analysis. The vials were shaken, then placed in 20 ml polyethylene vials and the radioactivity was immediately counted (Table III). In the absence of eluting solvent (columns 1 and 3), the efficiency of counting was 94.2% with Permafluor III and less (92.5%) with Picofluor 15. In both cases, the presence of solvent decreased the efficiency to values too low for accurate counting. Apparently the presence of water droplets with Permafluor III did not hamper counting and this scintillating solution was preferred because of its lower cost, in the search for better conditions of counting. We therefore tested ethylene vials of higher capacity (20 ml) which can contain a higher amount of solvent, in the case of radioactive peaks eluted after 18:2 and a higher amount of scintillating liquid to improve the counting efficiency. Experiments were carried out in the presence of 0–3 ml of solvent added with a constant volume of 10 or 15 ml of scintillating liquid. Results reported in Table III (columns 5–12) show that the efficiency of counting was higher than before. It was also slightly higher with 15 ml than with 10 ml of scintillating liquid, but not sufficiently for 15 ml to be preferred.

Thus the use of 10 ml of Permafluor III in 20 ml polyethylene vials was adopted. The only problem was the formation of water droplets when the Permafluor solution was added to the elution solvent. Additional tests were carried out by counting for only 1 min the radioactivity 1, 5, 11, 16 and 60 min after the addition of Permafluor. At 60 min the water emulsion had completely disappeared, the supernatant over the precipitated water being quite clear. For each time tested, the efficiency of counting was exactly the same, showing that the presence of water in the elution solvent did not hamper radioactivity measurement.

Peak collection

Increasing amounts of pure methyl-[1-^{14}C]linoleate were injected onto the RPLC column. The peak was collected and its radioactivity was measured. Results in Table IV show that the radioactivity eluted with the peak increased in proportion to the radioactivity injected. The ratio was equal to 0.974 ($\pm$0.004). Thus 2.6% ($\pm$0.4%) of the injected radioactivity was not recovered. Experiments carried out under the same conditions but in the absence of the column showed that 1.6% ($\pm$0.1% for four determinations) was lost during injection. Peak tailing can most probably account for the rest (1.0%). Indeed, the radioactivity collected after elution of the labelled peak and during the same elution time amounted to 0.44% ($\pm$0.10% for six determinations). The radioactivity eluted just before was found to be 0.25% ($\pm$0.11%). It probably represented the beginning of the peak emergence.

The radioactivity following elution of the labelled peak is very low. However, it could cause relatively high contamination in biological experiments, when a newly synthesized PUFA of low radioactivity would be eluted just after the labelled precursor of high radioactivity. That is the reason why this method in which the more unsaturated fatty acids formed from the precursors are eluted first is preferred.

Table IV also shows that area of the labelled peak collected increased proportionally to the radioactivity collected. The ratio (radioactivity/peak area) was found to be 0.225 ($\pm$0.004). It is equivalent to a specific radioactivity. The lowest value was observed for the lowest amount of labelled material injected. The relative lack of precision is ascribable to the peak area determination (precision: $\pm$6%), not to the radioactivity measurement (precision: $\pm$1.2%). In consequence, the radioactivity distribution among fatty acids can be determined more precisely, even for low radioactivities, than the specific radioactivities of fatty acids.

CONCLUSIONS

In *in vivo* or *in vitro* experiments on the biosynthesis of polyunsaturated fatty acids, using radioactive precursors, one or several mixtures of fatty acids have eventually to be analyzed. The mixtures contain both labelled and unlabelled fatty acids. We have shown that it is possible by using RPLC both to measure the radioactivity of the newly formed fatty acids and to determine the fatty acid composition from the peak areas. From these two determinations, it is also possible to calculate the specific radioactivity of the labelled fatty acids. In RPLC, under the conditions used, the elution sequence of fatty acids is quite different from that observed in GC with polar phases. Polyunsaturated fatty acids are eluted very early, as narrow peaks, so that the solvent volume containing such a radioactive fatty acid is low. The subsequent measurement of radioactivity by liquid scintillation counting is thus accurate without high quenching which might be due to water in the elution solvent. Morever, the fatty acids synthesized by desaturation from the labelled precursors are more unsaturated than the precursors and thus are eluted earlier. This prevents them from being highly contaminated by peak tailing of the precursor which is generally highly labelled. The opposite is true in GC and in addition the method is much more time-consuming.

The RPLC separations of the different fatty acids in a mixture are generally of good quality. When the amount of material to be analyzed is not too low, the

sensitivity of the refractometer being relatively low, RPLC can replace GC advantageously. Different conditions can be used according to the nature of the fatty acids present in the mixture. If the mixture comprises long-chain monounsaturated and saturated fatty acids, the temperature can be increased and/or the water content of the solvent in acetonitrile can be decreased to shorten the analysis duration without loss of resolution. Conversely, if the mixture is rich in polyunsaturated fatty acids, as for example when PUFA biosynthesis is studied, it seems appropriate to work at a relatively low temperature (30°C) at a relatively high water content in acetonitrile (7%) and to increase the solvent flow-rate to decrease the duration. However, to achieve separation of isomers, capillary GC remains the method of choice.

To solve our problems linked to PUFA metabolism, RPLC presents the same accuracy as GC with, as additional qualities, simplicity and reduction in analysis time.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MOLLUSCICIDAL SAPONINS FROM *PHYTOLACCA DODECANDRA* (PHYTOLACCACEAE)

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of oleanane saponins in *Phytolacca dodecandra* (Phytolaccaceae), a plant with potent molluscicidal properties. The molluscicidal monodesmosidic saponins of the berries were determined at 254 nm as their 4-bromophenacyl derivatives, whereas the non-derivatized bidesmosidic saponins, lacking a free carboxyl group, were determined at 206 nm. A comparison of different extraction procedures showed that with cold water predominantly monodesmosidic saponins were obtained, whereas hot water gave mainly bidesmosidic (non-molluscicidal) saponins.

INTRODUCTION

Molluscicides of plant origin are currently of great interest for the potential focal control of schistosomiasis in endemic countries[1]. This widespread tropical disease, affecting over 200 million people, is caused by parasites which require aquatic snails as intermediate hosts for their transmission. Some saponin-containing plants are particularly toxic to these snails and fulfil the majority of criteria for an effective plant molluscicide. One of the most promising and intensively studied of these plants is *Phytolacca dodecandra* l'Hérit (Phytolaccaceae), from Ethiopia[2]. A number of molluscicidal monodesmosidic and non-molluscicidal bidesmosidic saponins have been isolated from the berries of *P. dodecandra*[3–5] and a high-performance liquid chromatographic (HPLC) method has been developed for their determination[6]. The aim of this work was to determine quantitatively the content of saponins (Table I) in *P. dodecandra*. This is necessary for several reasons, the most important being the investigation of plant material from different strains and geographical locations, as it is advisable to maximize the content of molluscicidal saponins for the most effective treatment of infected sites. Quantitative analysis is also required in order to evaluate

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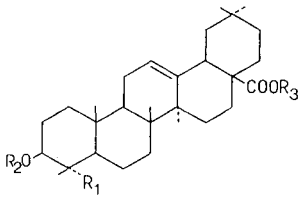
TABLE I

STRUCTURES OF SAPONINS



Compound	R_1	R_2*	R_3
1	CH_3–	Rha–^{2}Glc–^{2}Glc– $\vert_{4}$ Glc	H–
1a	CH_3–	Rha–Glc–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–
1b	CH_3–	Rha–Glc–Glc– | Glc	Glc–
2	CH_3–	Glc–^{2}Glc– $\vert_{4}$ Glc	H–
2a	CH_3–	Glc–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–
2b	CH_3–	Glc–Glc– | Glc	Glc–
3	CH_3–	Gal–^{3}Glc– $\vert_{4}$ Glc	H–
3a	CH_3–	Gal–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–
3b	CH_3–	Gal–Glc– | Glc	Glc–
4	$HOCH_2$–	Glc–^{2}Glc– $\vert_{4}$ Glc	H–
4a	$HOCH_2$–	Glc–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–

* Rha = α-L-rhamnopyranosyl; Glc = β-D-glucopyranosyl; Gal = β-D-galactopyranosyl.

the efficiency of different extraction methods, involving changes of solvent, temperature and time. Information on the content of active saponins is essential for biodegradation and toxicological studies.

EXPERIMENTAL

Materials

Acetonitrile and methanol were of LiChrosolv grade (Merck, Darmstadt, F.R.G.). Pure saponins were previously isolated from *P. dodecandra*[4,5].

Preparation of saponin derivatives

Saponin (*ca.* 5 mg) was dissolved in water (2 ml) containing 1 mg of potassium hydrogencarbonate. The solution was lyophilized and treated with 1 ml of a mixture of 4-bromophenacyl bromide (Fluka, Buchs, Switzerland) (3.5 g) and 18-crown-6 (Fluka) (680 mg) in acetonitrile (100 ml). After refluxing at 100°C for 90 min and subsequent cooling, by-products were removed by passing the reaction mixture through a short column (2 cm × 1 cm I.D.) of Kieselgel 60 (15–40 μm) (Merck, Darmstadt, F.R.G.) and eluting first with dichloromethane (10 ml), followed by chloroform–methanol (1:1) (10 ml)[7]. Final purification was achieved by low-pressure liquid chromatography on a Lobar LiChroprep Si 60 (40–63 μm) column (13 cm × 1 cm I.D.) (Merck) with chloroform–methanol (9:1) as eluent.

For plant material, the lyophilized extract (20 mg) was treated in a similar fashion to the pure saponins, without the final column chromatographic step.

Apparatus

HPLC–UV analyses were carried out on a system consisting of a Spectra-Physics (San Jose, CA, U.S.A.) 8700 pump, a Rheodyne injector, a Hewlett-Packard (Palo Alto, CA, U.S.A.) 1040A photodiode array detector, an HP-85 computer and an HP 7470A plotter. Quantitative analyses were performed with Waters Assoc. (Milford, MA, U.S.A.) 6000A pumps, a Waters Assoc. 480 UV spectrophotometer, a Waters Assoc. 720 system controller and a Waters Assoc. 730 data module. Separations were performed on 10-μm μBondapak C_{18} (30 cm × 3.9 mm I.D.), 4-μm NovaPak C_{18} (15 cm × 3.9 mm I.D.) and 5-μm RP-8 (25 cm × 4.6 mm I.D.) (Knauer, Bad Homburg, F.R.G.) columns.

Chromatographic conditions

For non-derivatized saponins and extracts, a Knauer 5-μm RP-8 column was used, with a linear gradient from 30% to 50% of acetonitrile in water over 60 min, an elution rate of 1.5 ml/min and detection at 206 nm. Saponins were dissolved in methanol at a concentration of 1 mg/ml and extracts at 2 mg/ml. Samples of 10 μl were injected.

For derivatized saponins and extracts, RP-18 NovaPak and μBondapak columns were used, with a linear gradient from 40% to 70% of acetonitrile in water, an elution rate of 1 ml/min and detection at 254 nm. For injection 10-μl samples of 1 mg/ml saponin solutions or 5 mg/ml extract solutions were employed.

Extractions

In each instance, 1 g of dried berries was treated with 100 ml of water. The extract (50 ml) was filtered, lyophilized and weighed.

Method 1. Ground berries were extracted at 20°C for 24 h.

Method 2. Whole berries were heated at 90°C for 24 h in a stoppered flask.

Method 3. Ground berries were extracted under reflux for 2 h.

Method 4. Ground berries were extracted under reflux for 12 h.

Quantitative determination

For quantification purposes, derivatized saponins 1a, 2a and 3a were used as standards, together with the bidesmosidic saponins 1b, 2b and 3b. After the establishment of calibration graphs (obtained from the respective peak areas at different injected concentrations; least-squares analysis gave correlation coefficients of 0.998 for 1a, 0.999 for 2a and 0.999 for 3a), the amounts of the individual saponins in the extracts were determined as percentages of the total extracts. The amounts of the other saponins were calculated by summing the areas of the remaining minor saponin peaks in the chromatogram, using 2a and 2b as standards. HPLC runs were carried out in triplicate to obtain average results.

RESULTS AND DISCUSSION

Separations of saponins on reversed-phase columns have previously been carried out with detection at 206 nm owing to their poor absorption at higher wavelengths[6]. Consequently, there are limitations concerning the solvents and gradients that can be used. HPLC of an aqueous *P. dodecandra* extract (90°C, 24 h) with an acetonitrile–water gradient demonstrates the problems of baseline drift at 206 nm (Fig. 1). The bidesmosidic saponins elute between 10 and 20 min but the monodesmosidic saponins elute later and are consequently much more difficult to quantify. A three-dimensional representation of this chromatogram (Fig. 2) shows the rapid decrease in UV absorption of the saponins when moving to higher wavelengths.

Refractive index detection is not practicable under these conditions, so an

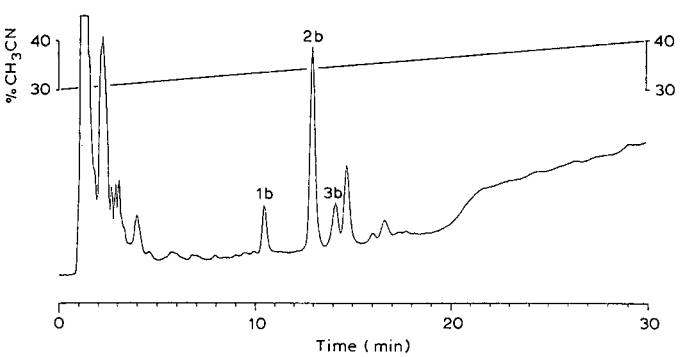


Fig. 1. HPLC analysis of an aqueous extract (method 2) of *Phytolacca dodecandra* berries. Column, Knauer RP-8, 5 μm (25 cm × 4.6 mm I.D.); 30–40% acetonitrile over 30 min; flow-rate, 1.5 ml/min; detection, 206 nm.

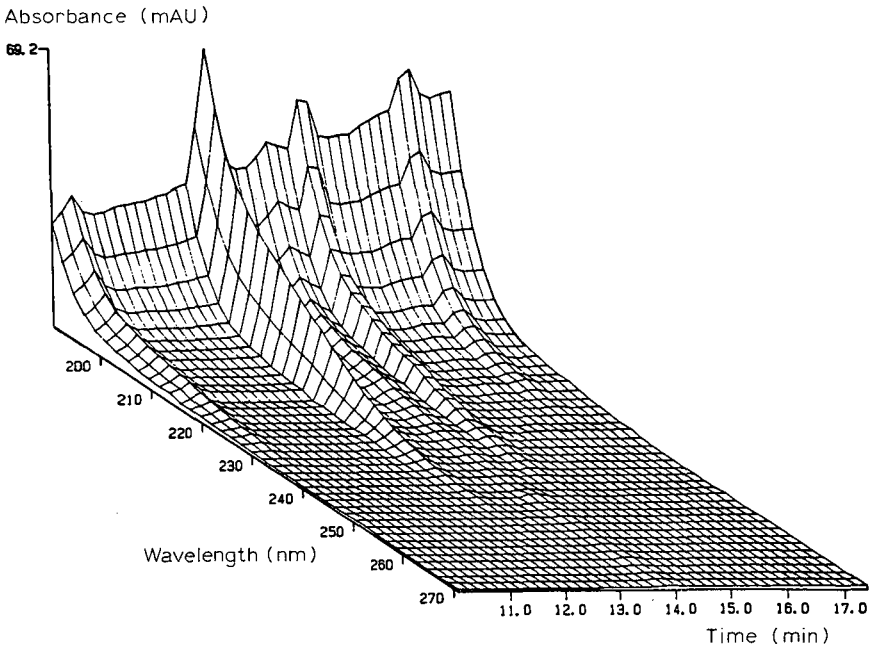


Fig. 2. Three-dimensional representation of a part of the chromatogram shown in Fig. 1.

alternative is to derivatize the saponins with a chromophore which facilitates UV detection at 254 nm. As the monodesmosidic saponins from *P. dodecandra* (which are responsible for the molluscicidal activity) possess a free carboxyl group at the C-28 position, derivatization can be carried out at this function. The approach used here is to introduce a 4-bromophenacyl chromophore by a reaction involving 4-bromophenacyl bromide and a crown ether (see Experimental). This method has previously been employed for the analysis of fatty acids[8] and prostaglandins[9]. The structures of four chosen derivatized pure saponins (1a, 2a, 3a, 4a) from *P. dodecandra* are shown in

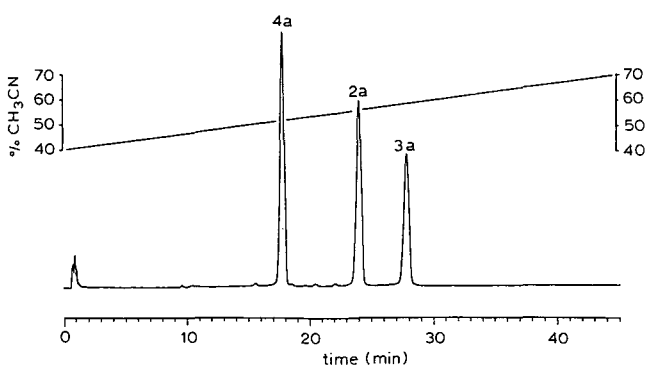


Fig. 3. Separation of derivatized saponins 2a, 3a and 4a. Column, NovaPak C_{18}, 4 μm (15 cm × 3.9 mm I.D.); 40–70% acetonitrile over 45 min; flow-rate, 1 ml/min; detection, 254 nm.

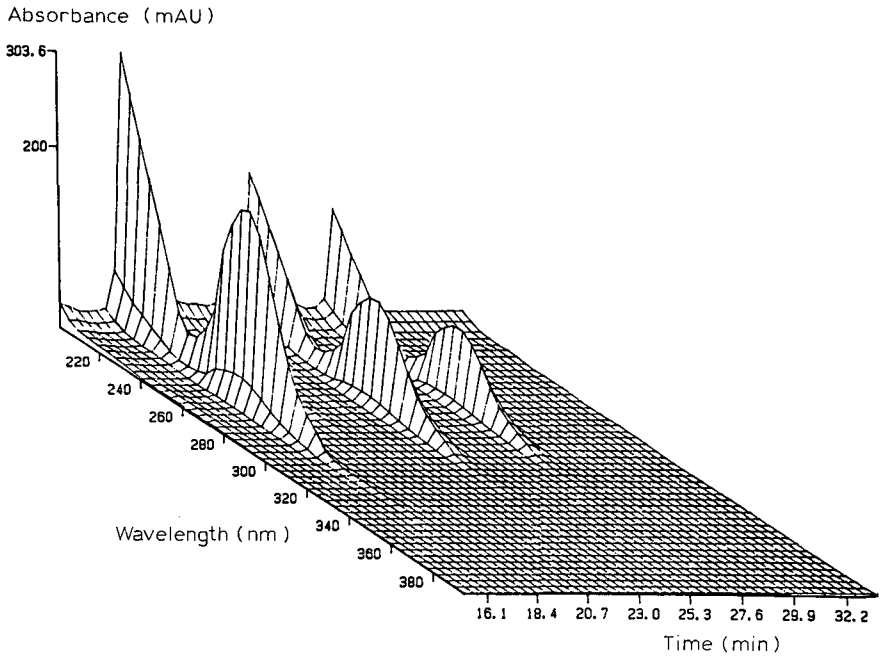


Fig. 4. Three-dimensional representation of the separation of derivatized saponins 2a, 3a and 4a shown in Fig. 3.

Table I. The four saponins selected for derivatization are found in appreciable amounts in *P. dodecandra* extracts and can be used for standardization and identification purposes. A mixture of three of these saponins was separated on an RP-18 column with detection at 254 nm, using a gradient from 40% to 70% of acetonitrile in water over 45 min without any baseline drift (Fig. 3). The three-dimensional chromatogram (Fig. 4) shows the excellent UV absorption of the derivatized saponins at 254 nm.

For quantification of the saponins in *P. dodecandra* extracts, two methods were considered: (a) use of the derivatives of the three isolated saponins as external standards; (b) use of naphthalene as internal standard. Comparison of the results showed virtually identical values for the two methods. Consequently, method (a) was employed throughout. Mixtures of the three derivatized saponins 1a, 2a and 3a were injected at different concentrations onto the HPLC column and the surface area under each peak was plotted against concentration to obtain the calibration graphs illustrated in Fig. 5.

Calculation of the percentages of saponins 1, 2 and 3 in any extract is therefore a relatively straightforward matter, after derivatizing the extract and performing a preliminary purification step before HPLC analysis.

The bidesmosidic saponins lack a free carboxyl functionality and therefore cannot be derivatized by the method employed for the monodesmosidic saponins. In order to quantify extracts containing bidesmosidic saponins, HPLC analyses were performed at 206 nm. By constructing calibration graphs with the pure glycosides 1b, 2b and 3b, the percentages of 1b, 2b and 3b in the extracts were ascertained.

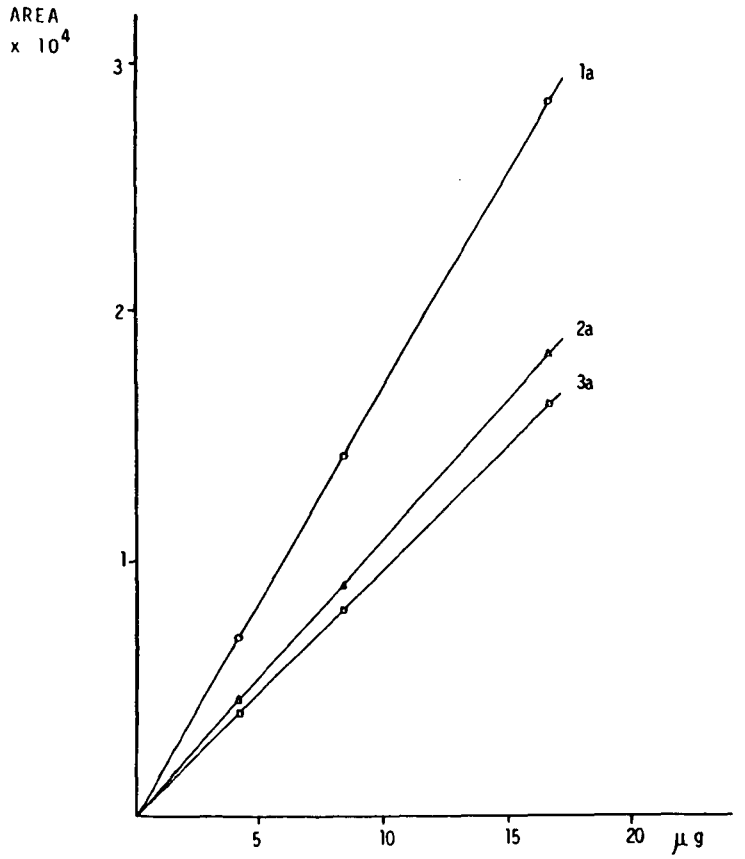


Fig. 5. Calibration graphs for derivatized saponins 1a, 2a and 3a. For conditions, see Fig. 3.

An aqueous extract of *P. dodecandra* berries (strain no. 17) was derivatized and subjected to preliminary purification (see Experimental) before analysis on a NovaPak C_{18} column (Fig. 6). The individual derivatized monodesmosidic saponins 1a–4a were clearly separated with a gradient of acetonitrile and water. Chromatography on a μBondapak C_{18} column gave a lower resolution of the peaks and a less satisfactory result. Analysis of the aqueous extract of another strain (no. 44) of *P. dodecandra* berries by the derivatization method is shown in Fig. 7. Although the modified saponins 1a, 2a and 3a are present, some of the other saponins found in strain 17 are missing, most notably the hederagenin glycoside 4a. Hence this analytical method is useful for both qualitative and quantitative purposes.

Different methods of extraction of *P. dodecandra* berries with water were investigated (it has already been established that extraction of the berries with methanol provides mainly bidesmosidic saponins[4,6]) in order to find both the weights of the extracts and the saponin compositions. The results are summarized in Table II.

The largest amount of extract was obtained by method 4, which involved refluxing ground berries with water for 12 h at 100°C (see Experimental). Extraction by method 1 gave both mono- and bidesmosidic saponins, whereas methods 2–4 gave

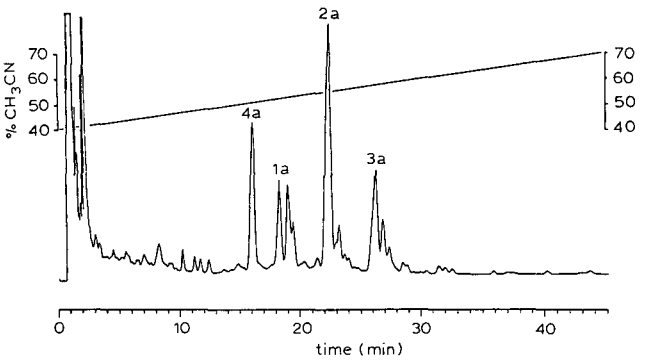


Fig. 6. HPLC analysis of the derivatized aqueous extract (method 1) from *Phytolacca dodecandra* berries (strain 17). Column, NovaPak C_{18}, 4 μm (15 cm × 3.9 mm I.D.); 40–70% acetonitrile over 45 min; flow-rate, 1 ml/min; detection, 254 nm.

only bidesmosidic saponins. Thus elevated temperatures increase the yield of extract but produce only bidesmosidic saponins. Kinetic experiments showed that the temperature limit for obtaining monodesmosidic saponins appeared to be *ca.* 60°C. A possible explanation for this phenomenon is that enzymes present in the berries cleave the glycosidic chain at position C-28 during extraction with cold water but that on heating the water the enzymes are deactivated and hydrolysis of the ester-linked sugars does not occur. Extraction of ground berries with cold water gave approximately 30% of total saponins by weight of extract. Saponin 2 constituted nearly 19% of the extract. All the hot water extraction methods gave a total of *ca.* 50% saponins as a percentage of the water extract. Bidesmosidic saponin 2b was the most important constituent, in amounts varying from 27 to 33% of the total extract. Extraction of non-ground berries (not shown) at 20°C gave 21% less extract than at 90°C. Thin-layer chromatography of the extracts obtained by method 1, 2 or 3 confirmed the presence or absence of saponins 1–3, with monodesmosidic saponins only detectable after method 1.

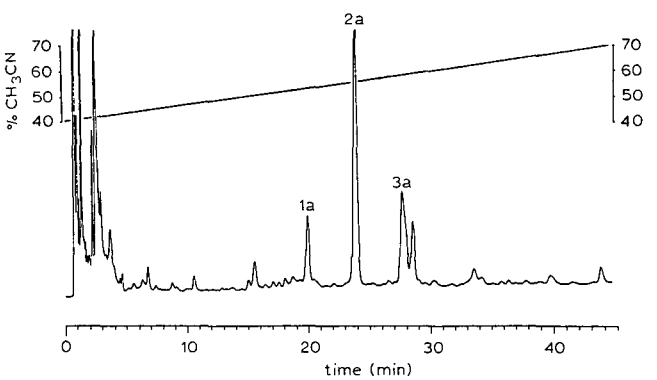


Fig. 7. HPLC analysis of the derivatized aqueous extract (method 1) from *Phytolacca dodecandra* berries (strain 44). Conditions as in Fig. 6.

TABLE II

PERCENTAGES OF SAPONINS IN *PHYTOLACCA DODECANDRA* AQUEOUS EXTRACTS

Method	*Total weight of extract (g)*	*Saponin in total extract (%, w/w)*						
		1a	*2a*	*3a*	*1b*	*2b*	*3b*	*Remaining saponins*
(1) 20°C, 24 h	0.413	3.5	18.9	3.6	*	*	*	5.4
(2) 90°C, 24 h	0.533	–	–	–	6.4	28.8	4.3	10.8
(3) 100°C, 2 h	0.500	–	–	–	8.3	32.9	4.5	9.9
(4) 100°C, 12 h	0.573	–	–	–	6.2	27.2	4.	9.8

* Bidesmosidic saponins not calculated by this procedure.

CONCLUSIONS

An HPLC method has been developed that permits the quantitative determination on reversed-phase supports of saponins in the molluscicidal plant *Phytolacca dodecandra* l'Hérit (Phytolaccaceae). The monodesmosidic saponins, which are also responsible for the molluscicidal activity of *P. dodecandra* berries, are quantified by means of their 4-bromophenacyl derivatives, permitting HPLC detection at 254 nm. Thus the concentrations of the most active saponins 2 and 3, with molluscicidal activities of 6 and 3 mg/ml, respectively[4], can be determined in crude plant extracts. The content of bidesmosidic saponins is calculated by HPLC analysis at 206 nm, without derivatization but using standard pure saponins.

This HPLC procedure involving derivatization with 4-bromophenacyl bromide can be extended to any saponins, provided either the aglycone or the sugar moiety contains a free carboxyl group.

A comparison of different aqueous extraction procedures for *P. dodecandra* berries showed that measureable amounts of monodesmosidic saponins were obtained uniquely at ambient temperatures. Extractions with hot water produced only bidesmosidic saponins, presumably owing to inactivation of the enzymes responsible for cleaving the glycosidic chain in position C-28 of the triterpene moiety. This important observation is relevant to the problem of schistosomiasis, as obviously only cold water extracts which contain monodesmosidic saponins will have any application as plant molluscicides. Thus, pounding the berries with cold water, the most practicable method of obtaining a vegetable molluscicide in endemic regions, conveniently provides the greatest concentration of saponins from *P. dodecandra* for application to sites of infestation by transmitter snails.

For toxicological and other investigations, it is therefore easy to reproduce field extraction conditions and then rapidly quantitate standard extracts for their respective active saponins by HPLC before submitting them to the tests required.

ACKNOWLEDGEMENTS

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ANALYSIS AND PURIFICATION OF TOXIC PEPTIDES FROM CYANOBACTERIA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received January 19th, 1988; revised manuscript received May 3rd, 1988)

SUMMARY

A simple, rapid and reliable chemical analysis method for microcystins (cyanoginosins) has been studied. Three different mobile phases for high-performance liquid chromatography were selected and optimized. Also the adsorptive powers of three commercially available C_{18} cartridges were compared and the results successfully applied to the clean up of three of the toxins. Finally a total system for the analysis and isolation of microcystins was established.

INTRODUCTION

Low-molecular-weight peptide toxins which affect the liver have been the predominant toxins involved in cases of animal poisonings due to cyanobacterial toxins. They are mainly produced by *Microcystis aeruginosa* and are called microcystins or cyanoginosins[1]. Several microcystins have been isolated and their structures determined to be cyclic heptapeptides[2,3] (Fig. 1). Although mouse bioassay provides a general assessment of the toxicity[4], it is necessary to determine the concentrations of the toxins more accurately by an appropriate chemical assay. The establishment of a suitable chemical assay method would also facilitate studies on toxin production and toxicokinetics.

Some chemical assay methods for microcystins using high-performance liquid chromatography (HPLC)[5–7] and thin-layer chromatography (TLC)[8] have been reported. However, these methods have been used mainly for the separation of

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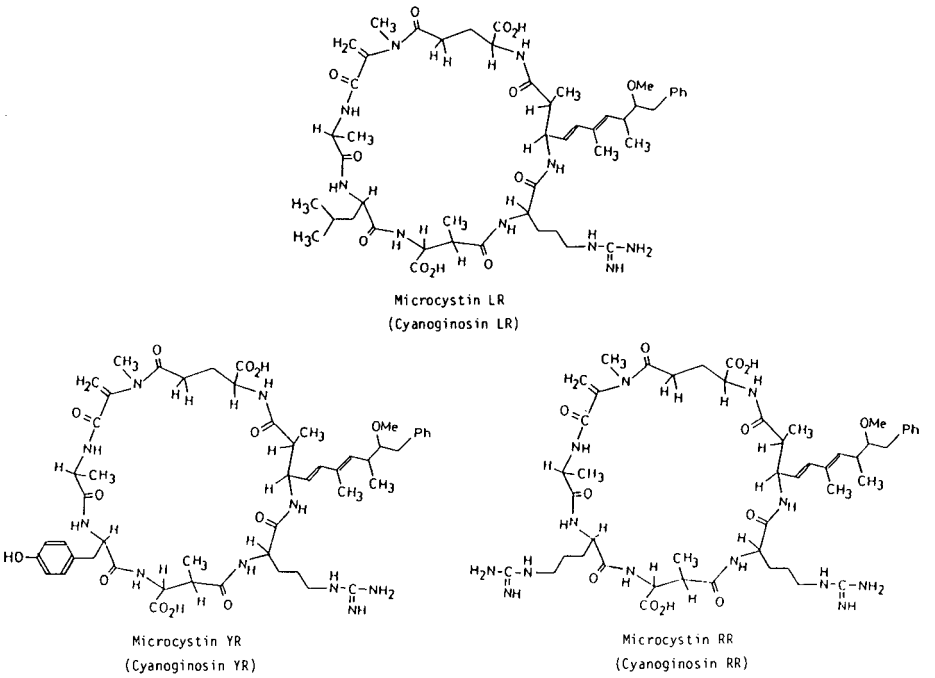


Fig. 1. Structures of microcystins LR, YR and RR (cyanoginosins LR, YR and RR).

microcystins LR (cyanoginosin LR). We have recently proposed an efficient purification method which consists mainly of extraction on ODS (octadecylsilanized)-silica gel, separation on silica gel and HPLC with ODS-silica gel, and which has been successfully applied to some *Microcystis aeruginosa* strains[9].

In this paper we describe a chemical analysis method using reversed-phase HPLC with modifications based on the above purification method. The method can now be used in the detection and determination of all microcystins we have investigated to date and was applied successfully to the analysis of the toxins from several *Microcystis* species. This work establishes a total system for both analysis and isolation of microcystins.

EXPERIMENTAL

Materials

Three *Microcystis* cell materials were used. The first was from culture strain *Microcystis aeruginosa*, M-228[10], the second from culture strain *Microcystis viridis*, TAC-44[11] and the third from a naturally occurring surface bloom, *Microcystis aeruginosa*, Monroe strain[12].

Baker 10 C_{18}, Bond Elut C_{18} and Sep-Pak C_{18} were obtained from J. T. Baker (Phillipsburgh, NJ, U.S.A.), Analytichem International (Harbour City, CA, U.S.A.) and Waters Assoc. (Milford, MA, U.S.A.), respectively. The HPLC packing materials were Nucleosil $3C_{18}$ and $5C_{18}$ (Chemco Scientific, Osaka, Japan) having particle sizes of 3 and 5 μm, respectively.

Methanol, chloroform, ethyl acetate, isopropanol, trifluoroacetic acid (TFA), acetic acid, sodium sulphate, disodium hydrogenphosphate, potassium dihydrogenphosphate and phosphoric acid were analytical grade.

Toxins

Microcystins LR, YR and RR (cyanoginosins LR, YR and RR) were isolated and purified from M-228 and TAC-44 according to the previously reported method[9].

Thin-layer chromatography

Samples applied to plates (Kieselgel $60F_{254}$; E. Merck, Darmstadt, F.R.G.) were developed with the following solvent systems; ethyl acetate–isopropanol–water (4:3:7, upper layer) and chloroform–methanol–water (65:35:10, lower layer). The developed plate was heated at 105°C for 5 min to evaporate remaining solvents. The plate was visualized under short-wavelength UV light and then placed in a chamber with iodine vapour, and after 5 min the positions of the toxins were evaluated. Toxin concentrations as low as 100 ng can be detected with this detection system.

High-performance liquid chromatography

An high-performance liquid chromatograph equipped with a constant-flow pump (LC-5A; Shimadzu, Kyoto, Japan) was used with a variable-wavelength UV detector (Shimadzu SPD-2AM) operated at 238 nm. The separation was performed on Nucleosil $3C_{18}$ (3 μm, 75 mm × 4.6 mm I.D.) and $5C_{18}$ (5 μm, 150 mm × 4.6 mm I.D.) columns with the following mobile phases; (A) methanol–0.05% (v/v) TFA (6:4), (B) methanol–0.05 *M* phosphate buffer (pH 3.0, 6:4) and (C) methanol–0.05 *M* sodium sulphate (1:1).

Analytical procedure

Lyophilized cells (500 mg) were extracted three times with 50 ml of 5% (v/v) acetic acid for 30 min while stirring. The extract was centrifuged at 9300 *g* and then the supernatant was applied to a C_{18} cartridge. The cartridge which contained microcystins was rinsed with 20 ml of water, followed by 20 ml of 10–25% methanol in water (10% for Sep-Pak C_{18}, 20% for Bond Elut C_{18} and 25% for Baker 10 C_{18}, respectively). Microcystins were finally eluted from the C_{18} cartridge with 20 ml of methanol. The eluate was evaporated under reduced pressure and then the residue was dissolved in 0.5 ml of methanol. The solution was subjected to TLC or HPLC analysis.

RESULTS AND DISCUSSION

In our previous study[9] we found that: (1) extraction with 5% aqueous acetic acid was very effective because excellent toxin recovery was obtained and pigment extraction was limited; (2) clean-up using ODS-silica gel efficiently eliminated inorganic materials and polar contaminants; (3) TLC with silica gel gave good separation and (4) HPLC with methanol–0.05% TFA as a mobile phase provided excellent resolution between microcystins LR and YR.

Separation of microcystins by HPLC

In several previous reports on HPLC methods for separation of microcystins,

acetonitrile–10 m*M* ammonium acetate (26:74) has often been used as a mobile phase on C_{18} columns[5–8]. We attempted to separate microcystins LR and YR under these HPLC conditions but they were coeluted. We were previously able to separate microcystins LR and YR using a mobile phase containing TFA[9]. However, more than five microcystins exist in this group[2,3], so the identification of the toxins should be achieved by the complementary usage of plural HPLC solvent systems. In addition, preparative HPLC is a suitable technique to obtain pure microcystins. Therefore three different HPLC conditions were compared for separation between microcystins LR and YR in view of the analysis and preparation and the results are discussed below.

As shown in Fig. 2, the three different mobile phases [(A) methanol–0.05% TFA (6:4), (B) methanol–0.05 *M* phosphate buffer (pH 3.0) (6:4) and (C) methanol–0.05 *M* sodium sulphate (1:1)] gave good separation between both toxins on a short column (Nucleosil $3C_{18}$, 3 μm, 75 mm × 4.6 mm I.D.). However, since microcystin LR is frequently accompanied by a small peak (arrow) in liquid chromatography, the separation between these compounds was also examined. The compound corresponding to the small peak has the same molecular weight as microcystin LR and its isolation and characterization will be discussed elsewhere[13]. With the use of mobile phase A, both toxins were separated within 5 min and this mobile phase was applicable to preparative HPLC because it is very easy to evaporate the mobile phase. Despite these advantages the separation between microcystin LR and the small peak was not sufficient. Although mobile phase B was not suitable for preparative HPLC because of

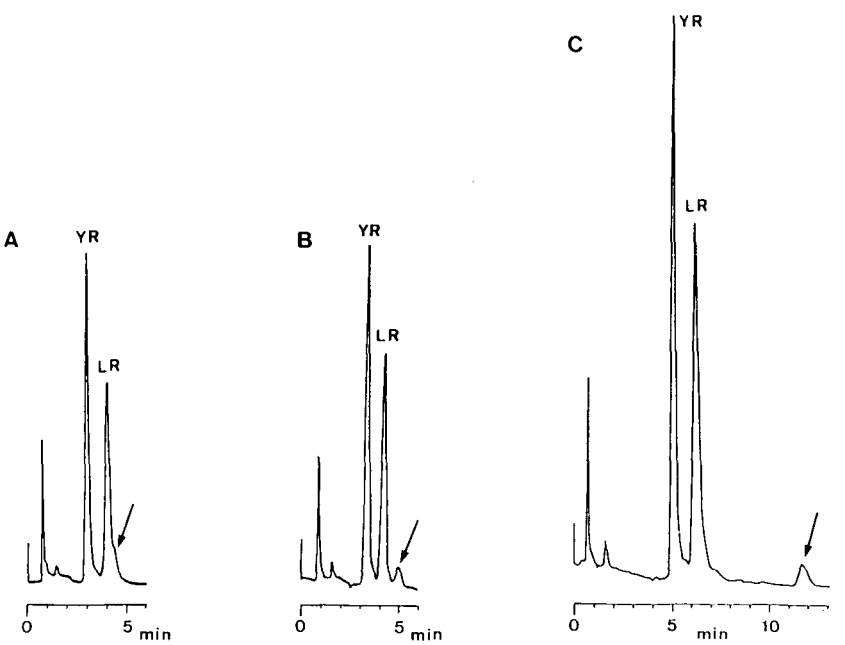


Fig. 2. HPLC separation between microcystins LR and YR. Column: Nucleosil $3C_{18}$ (3 μm, 75 mm × 4.6 mm I.D.). Mobile phases: (A) methanol–0.05% TFA (6:4); (B) methanol–0.05 *M* phosphate buffer (pH 3.0) (6:4); (C) methanol–0.05 *M* sodium sulphate (1:1). Flow-rate: 1 ml/min. Detection: 238 nm. Arrows indicate the small peak.

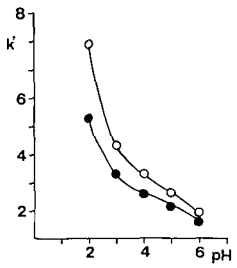


Fig. 3. Influence of the pH on the capacity factor, k', using phosphate buffer in the mobile phase for separation of microcystins LR (○) and YR (●).

the presence of non-volatile salts, all compounds including the small peak were separated within 5 min. Fig. 3 shows the influence of the pH of the phosphate buffer in the mobile phase on the capacity factors, k' (ref. 14), of both toxins. The k' of both toxins are strongly dependent upon the pH. When phosphate buffer with a pH of 3.0 was used, the most suitable k' values of these toxins were obtained. Therefore this mobile phase was adopted subsequently. With mobile phase C, it took 12 min to separate the above three compounds. This mobile phase is the most suitable for preparative HPLC because it was able to separate completely microcystin LR from the small peak and sodium sulphate is inert. We examined the effect of the concentration of sodium sulphate in the mobile phase on k' values of both toxins using methanol–aqueous sodium sulphate (1:1) solutions. The resolution was improved with increasing salt concentration, so that good resolution was obtained above 0.01 *M*. The most suitable retention time was obtained using 0.05 *M* sodium sulphate.

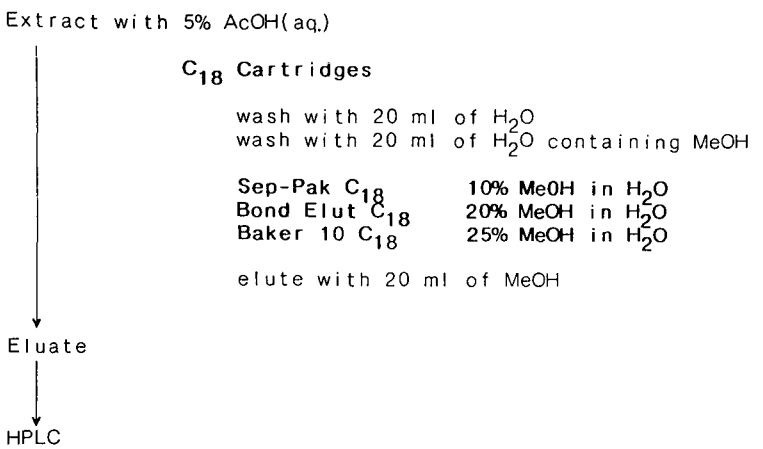
At this time we recommend the following mobile phases for identification and purification of microcystins: A, methanol–0.05% TFA (6:4), B, methanol–0.05 *M* phosphate buffer (pH 3.0) (6:4) and C, methanol–0.05 *M* sodium sulphate (1:1). Their complementary use would enable a ready identification of any microcystins investigated to date, and mobile phase C is most effective for preparative HPLC of the toxins.

Purification of microcystins LR with C_{18} cartridges

Recently, Poon *et al.*[8] and Brooks and Codd[5] extracted microcystins using Sep-Pak C_{18} cartridges. However the adsorbing power of the cartridge is relatively weak, so they were compelled to use two cartridges connected in series. Although Krishnamurthy *et al.*[6] efficiently used Bond Elut C_{18} cartridges, it has not always been possible to optimize the clean-up conditions. We have already shown the differences in the adsorption behaviour of cartridges from different suppliers[15]. Consequently, the clean-up conditions for microcystins were investigated using three commercially available cartridges (Baker 10 C_{18}, Bond Elut C_{18} and Sep-Pak C_{18}).

A lyophilized *Microcystins aeruginosa* (Monroe strain, dry weight 500 mg), which contained only microcystin LR, was extracted three times with 50 ml of 5% aqueous acetic acid solution and then the extract was passed through a C_{18} cartridge. The cartridge was washed with 20 ml of water and then with 20 ml of 5–40% methanol in water. Each eluate passed through the cartridge was subjected to HPLC to test for the elution of microcystin LR from the cartridge. In the case of the Baker 10 C_{18}

cartridge the toxin was completely retained using less than 25% methanol in water as a washing agent. When the cartridge was washed with 30% methanol in water, the toxin began to leak from the cartridge. It is desirable to wash the sample-containing cartridge with 20 ml of 25% methanol in water when the Baker 10 C_{18} cartridge is used at the clean-up step to separate impurities from the toxin. Using Bond Elut and Sep-Pak C_{18} cartridges, and respectively 25 and 15% methanol in water as the washing agents, the toxin began to elute. Twenty and ten percent methanol in water are suitable for Bond Elut and Sep-Pak C_{18} cartridges, respectively because no toxin elution can be detected. These results indicate the adsorptive power for microcystin LR is increasing in the order: Baker 10, Bond Elut and Sep-Pak C_{18}. The clean-up procedures for the toxin using the three different cartridges are shown in Scheme 1. The chromatograms before and after the clean up show the effect of eliminating the more polar contaminants (Fig. 4). The results should be applicable to microcystins other than microcystin LR.



Scheme 1. Clean-up procedure for microcystin LR with C_{18} cartridges. AcOH = Acetic acid; MeOH = methanol.

Finally, in order further to ensure the applicability of the methods mentioned above, mobile phase B and Baker 10 C_{18} were used for the analysis of microcystins from a *Microcystis viridis* (TAC 44) which was collected in Lake Kasumigaura in Japan[11]. In this case a normal type column (Nucleosil $5C_{18}$, 5 μm, 150 mm × 4.6 mm I.D.) and a slightly modified mobile phase [methanol–0.05 *M* phosphate buffer (58:42)] were employed. Fig. 5 shows the high-performance liquid chromatogram after the clean up, and the three toxins are successfully detected. Two of the three peaks were readily identified as microcystins LR and YR by comparison with standard samples. The remaining large peak which has a molecular weight of 1037 is thought to be microcystin RR containing two argines as the L amino acid variants. The toxin is also considered to be the same compound as cyanoviridin RR and cyanogenosin RR recently reported by Kusumi *et al.*[16] and Painuly *et al.*[17], respectively. The toxins from *Microcystins viridis* will be reported in detail elsewhere[18].

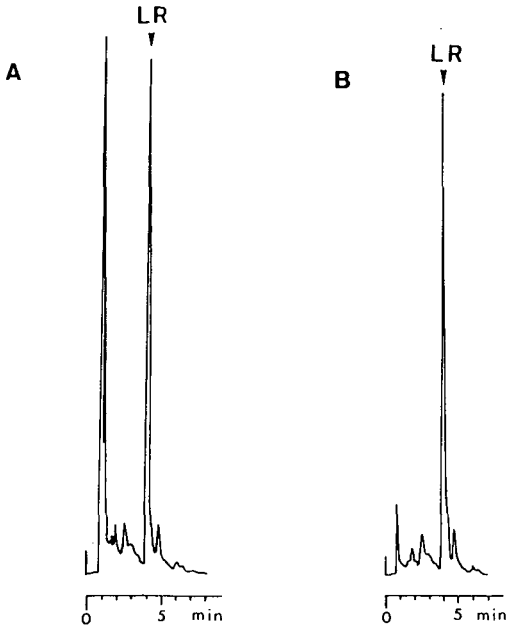


Fig. 4. High-performance liquid chromatograms of the methanol fraction from Monroe cells. (A) Before the clean up using Baker 10 C_{18}; (B) after the clean up using Baker 10 C_{18}. Conditions as in Fig. 2B.

A total system for analysis and isolation of microcystins

Herein we have established analytical methods for microcystins using three HPLC solvent systems and three commercially available C_{18} cartridges. In addition we have also described an isolation procedure for microcystins by using TLC, HPLC and silica gel and C_{18}-silica gel chromatographies[9]. A combination of these methods can therefore provide an isolation and analytical system for the toxins as shown in Scheme 2. Namely, after extraction with 5% aqueous acetic acid the sample is purified with

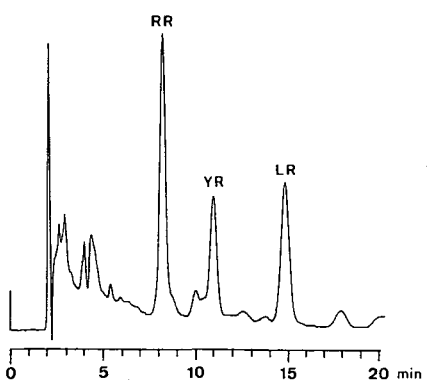
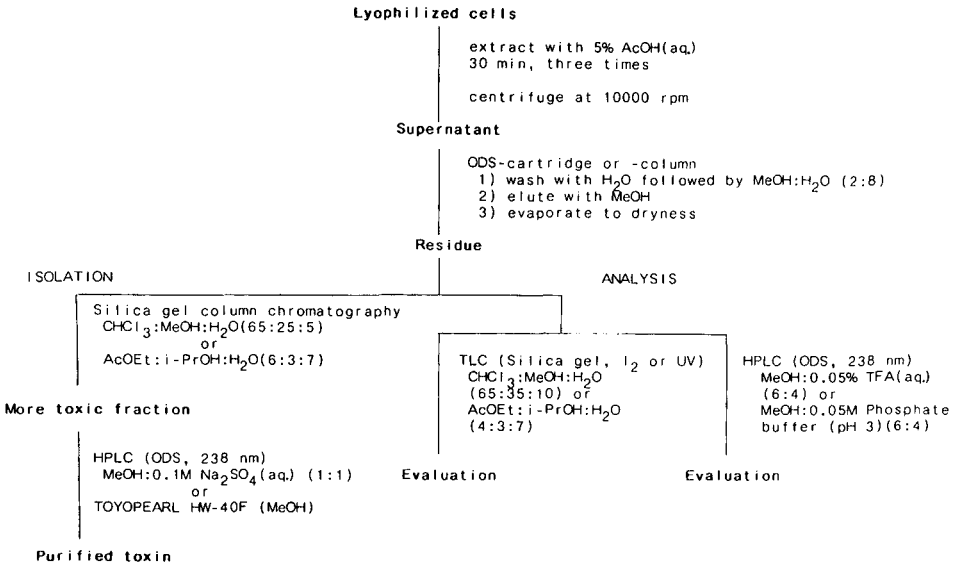


Fig. 5. High-performance liquid chromatogram of the methanol fraction from TAC-44. Column: Nucleosil $5C_{18}$ (5 μm, 150 mm × 4.6 mm I.D.). Mobile phase: methanol–0.05 *M* phosphate buffer (pH 3.0) (58:42). Flow-rate: 1 ml/min. Detection: 238 nm.



Scheme 2. Total system for analysis and isolation of microcystins. MeOH = Methanol; AcOH = acetic acid; AcOEt = ethyl acetate; i-PrOH = isopropanol.

a C_{18} cartridge and then the toxins are detected by HPLC with mobile phase A or B, and TLC using iodine and UV as detection systems. In this HPLC system, 1 ng of the toxin can be precisely detected, while the TLC method enables a ready detection of not only the toxins but also contaminants which are not detected by UV alone. When the toxins are detected at the analysis stage, the toxic fraction from a large amount of cells is applied successively to C_{18}-silica gel and silica gel column chromatographies. Finally the toxins are purified by preparative HPLC (mobile phase C) or Toyopearl HW-40 (methanol) and are checked by HPLC and TLC again. The systems developed are simple, rapid and reliable, and should contribute to progress in various studies on the microcystins.

ACKNOWLEDGEMENT

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Note

Novel isoferulate esters identified by gas chromatography–mass spectrometry in bud exudate of *Populus nigra*

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Previous investigations have shown that bud exudate of Populus spp. consists of a complex mixture of compounds including substituted benzoic acids and esters, substituted phenolic acids and esters and flavonoid aglycones[1–7]. Recent studies have identified 3-methyl-2-butenyl caffeate (prenyl caffeate) in bud exudate of *Populus nigra* L.[6] (black poplar) and a series of 3-methyl-2-butenyl- and 3-methyl-3-butenyl esters of caffeic, coumaric, ferulic and isoferulic acids in bud exudate of *P.* × *euramericana* (Dode) Guinier[2]. The prenyl esters are of particular interest because 3-methyl-2-butenyl caffeate has been found to be a major contact allergen in propolis[8], the "bee-glue" collected by bees from poplar buds[2–4,6,9], which finds wide use in homeopathic medical and cosmetic products[9,10].

We here report the identification from a fraction of *P. nigra* bud exudate (LB3)[6] of a series of esters of isoferulic acid with both aromatic and aliphatic alcohols. This series includes several novel esters of the prenyl type.

EXPERIMENTAL

Fractionation of poplar bud exudate

P. nigra bud exudate was extracted and fractionated as described previously[6].

Reagents and materials

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) was obtained from Sigma (Dorset, U.K.).

Esters of isoferulic acid were synthesised from the corresponding alcohol and *trans*-isoferulic acid as previously described[2]. The appropriate alcohols were purchased from Sigma, Aldrich (Dorset, U.K.), Lancaster Synthesis (Morecambe, U.K.), or provided as a gift by Shell Research (Sittingbourne, U.K.).

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Sample preparation

The samples (0.5–1 mg) were prepared for gas chromatography (GC) by heating for 30 min at 100°C with 50 μl pyridine and 100 μl BSTFA (including 1% TMCS) in a sealed glass tube to produce the trimethylsilyl (TMS) derivatives.

Gas chromatography–mass spectrometry (GC–MS)

The derivatized samples were separated and analysed in a Finnigan 1020 automated GC–MS system (incorporating a Data General Nova 3 computer); the GC system was fitted with a 30 m × 0.32 mm I.D. J & W Scientific silica column coated with 0.25 μm DB-1, and a splitless injector with a flush 30 s after sample injection to remove residual gases. The end of the column was introduced directly into the mass spectrometer analyser chamber. The system was operated under the following conditions: helium pressure 11 lbs/un.^{2}; injector temperature 300°C; GC temperature 75–300°C at 3°C min^{-1}. The mass spectrometer was set to scan 40–650 a.m.u. per nominal second with an ionizing voltage of 70 eV. The filament was switched on 250 s after injection of the sample into the gas chromatograph.

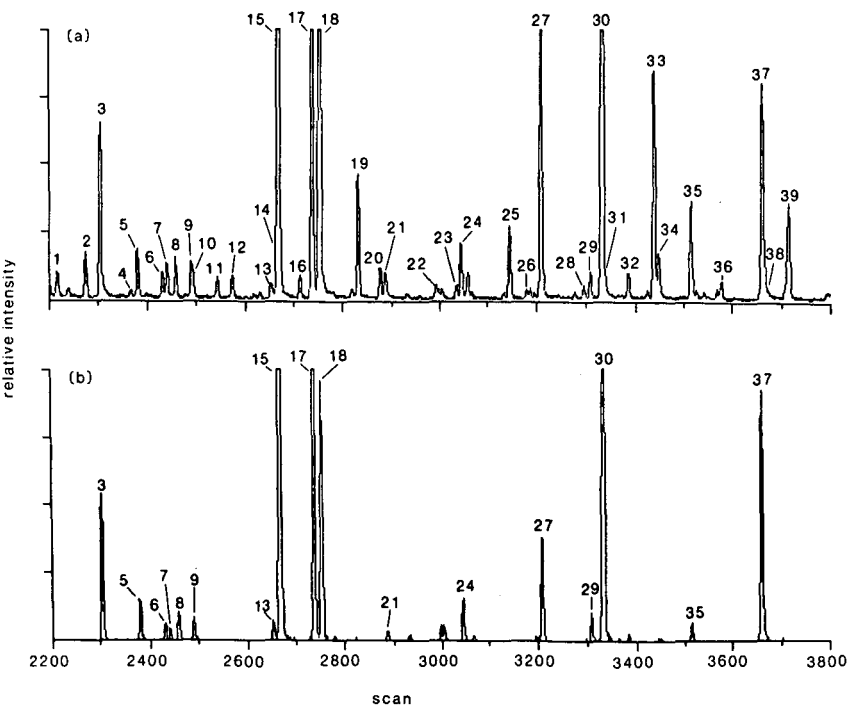
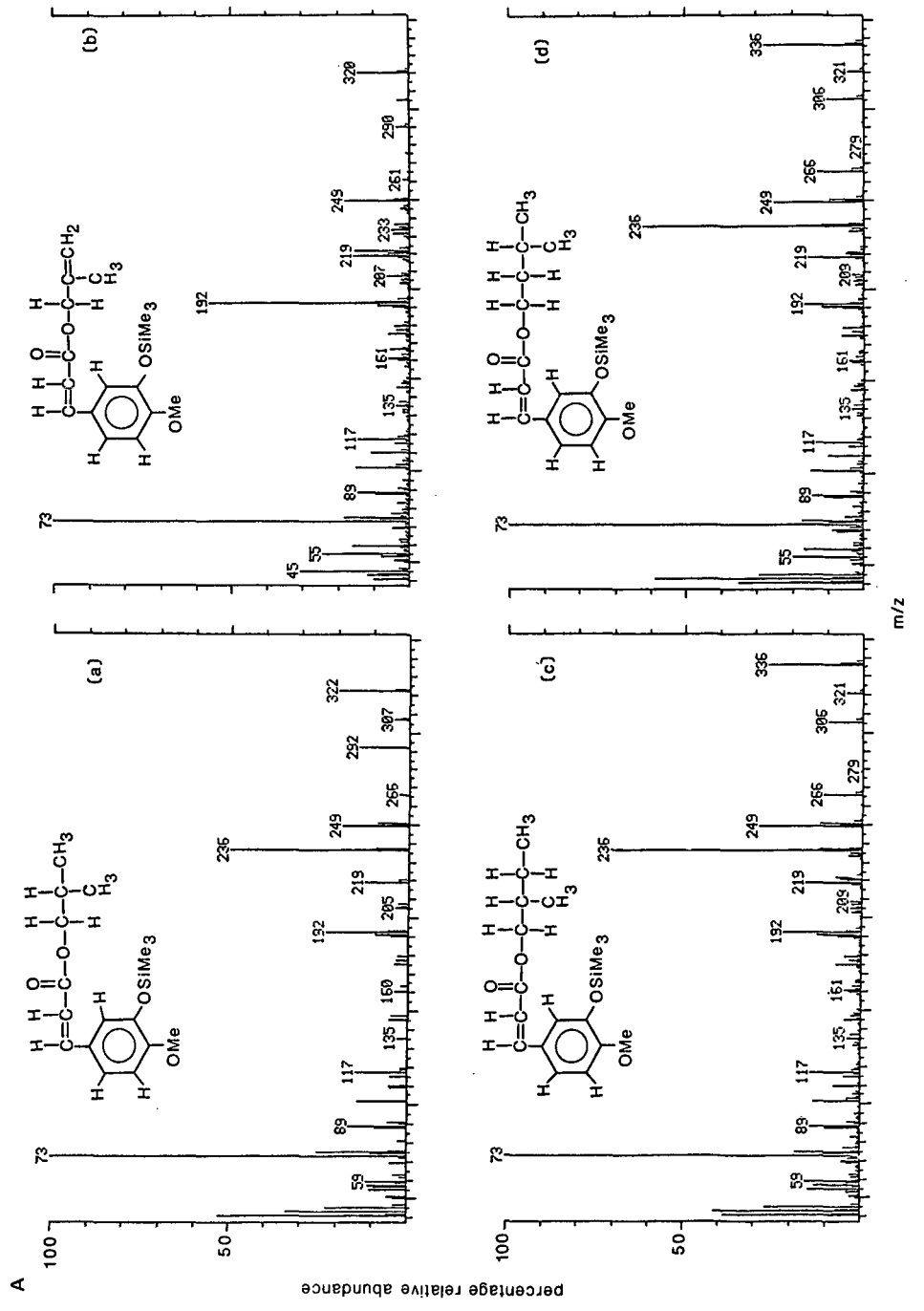


Fig. 1. (a) Reconstructed ion chromatogram of poplar bud exudate fraction LB3. The area shown [c MU 20–30; 175–260°C] contains all the isoferulic acid esters in the fraction. In addition to the isoferulic acid esters listed in Table I the following acids are identified: hexadecenoic (1); hexadecanoic (2); octadecenoic (12); phenylethyl *trans*-caffeate (34) and the following unbranched hydrocarbon alcohols are identified; C_{18}–C_{26} straight chain-1-ols (10, 14, 19, 23, 25, 28, 33, 36, 39), C_{18}–C_{24} straight chain-2-ols (4, 11, 16, 20, 23, 26, 31) and C_{24}–C_{26} iso branched chain-1-ols (32, 38). (b) Single-ion reconstruction of *m/z* 249 (for structure of *m/z* 249 see ref. 12) indicating positions of isoferulic acid and its esters.


A
percentage relative abundance
100
50
(a)
(b)
(c)
(d)
73
59
89
117
135
150
161
192
219
236
249
265
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OSiMe3
OMe
m/z

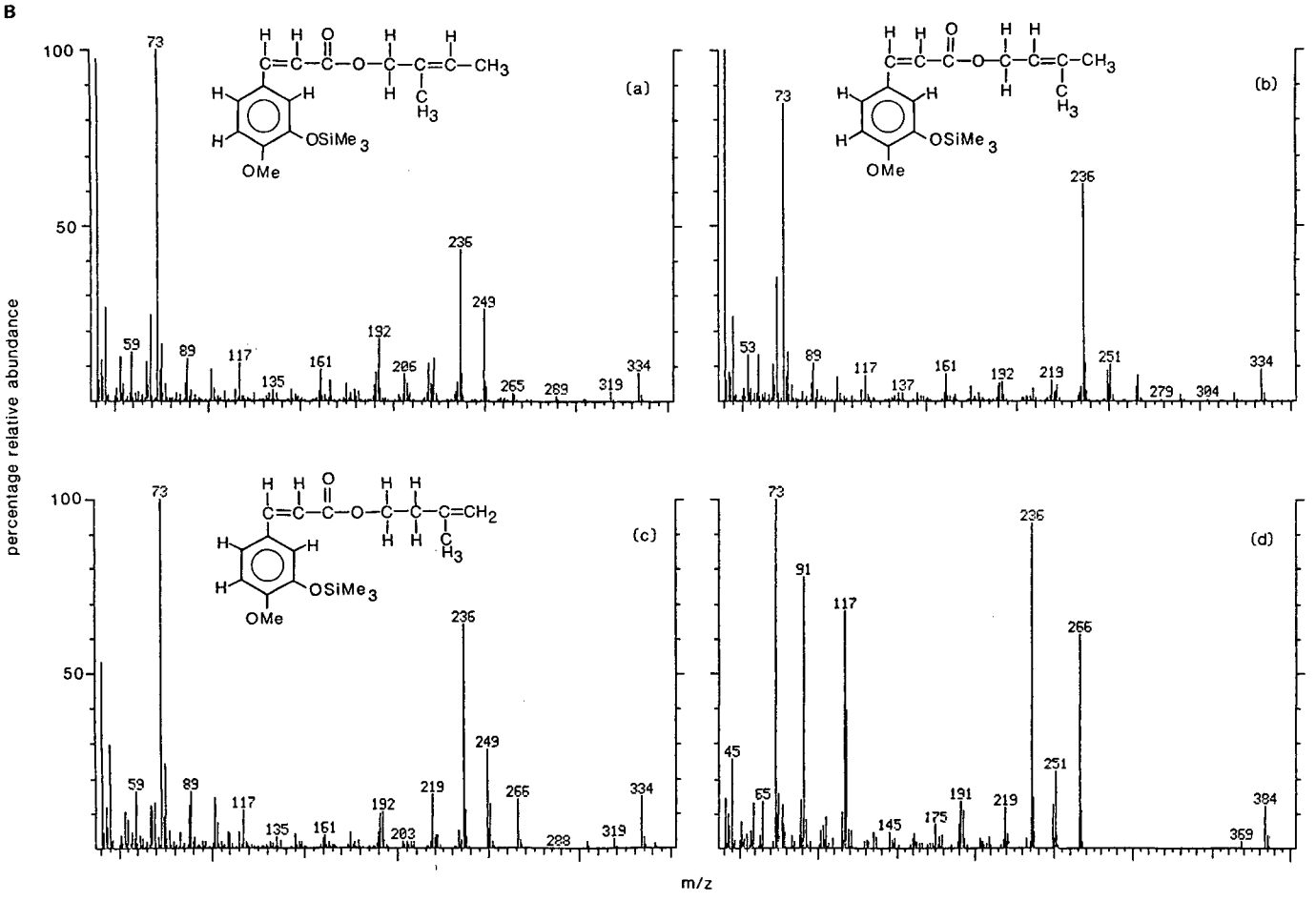

Fig. 2. (A) Mass spectra, recorded at 70 eV, of the 2-methylpropyl ester $[M]^+$ m/z = 322 (a), 2-methyl-2-propenyl ester $[M]^+$ m/z = 320 (b), 2-methylbutyl ester $[M]^+$ m/z = 336 (c), 3-methylbutyl ester $[M]^+$ m/z = 336 (d), of *trans*-isoferulic acid mono-TMS. Spectrum (a) is from poplar bud exudate; (b), (c) and (d) are reference standards, because (b) co-chromatographs with 1-octadecanol in bud exudate and (c) and (d) chromatograph together (see Results section). (B) Mass spectra, recorded at 70 eV, of the 2-methyl-2-butenyl ester $[M]^+$ m/z = 334 (a), 3-methyl-2-butenyl ester $[M]^+$ m/z = 334 (b), 3-methyl-3-butenyl ester $[M]^+$ m/z = 334 (c) and hydrocinnamyl ester $[M]^+$ m/z = 384 (d) of isoferulic acid mono-TMS. These spectra are from poplar bud exudate (Me = methyl).

Identification of compounds

Peaks were identified by computer search of user-generated reference libraries, incorporating GC retention times and mass spectra. Reference compounds were co-chromatographed with the experimental sample to confirm GC retention times and mass spectral patterns. Peaks were examined by single-ion chromatographic reconstructions to confirm their homogeneity; mixed peaks were resolved by a computer program aimed at resolving the mass spectral data of one compound from overlapping mass spectra of another.

RESULTS

Analysis by GC–MS and co-chromatography with appropriate reference compounds enabled separation and identification of *trans*-isoferulic acid (3-hydroxy-

TABLE I

ISOFERULIC ACID AND ISOFERULATE ESTER COMPONENTS OF FRACTION LB3 OF *P. NIGRA* BUD EXUDATE

GC retention times in methylene units (MU; defined by Dalgliesh *et al.*[11]) are given to two decimal places to indicate the elution sequence of peaks which chromatograph closely. Factors such as concentration of the compound concerned and concentration of adjacent compounds, together with the characteristics of a particular GC column are liable to affect the chromatography, and for general purposes the MU figures are probably reliable only to a single decimal place.

Peak No.	*Compound**	*Retention time (MU)*	*Percentage of total** isoferulate*
3	*trans*-Isoferulic acid	20.63	4.3
5***	3-Methyl-3-butenyl-*cis*-isoferulate	21.07	1.1
6***	2-Methyl-2-butenyl-*cis*-isoferulate	21.35	0.6
7***	3-Methyl-2-butenyl-*cis*-isoferulate	21.40	0.8
8	2-Methylpropyl-*trans*-isoferulate	21.49	0.9
9	2-Methyl-2-propenyl-*trans*-isoferulate	21.67	1.4
13§	2-Methylbutyl-*trans*-isoferulate 3-Methylbutyl-*trans*-isoferulate	22.50 22.55	0.5
15	3-Methyl-3-butenyl-*trans*-isoferulate	22.63	21.6
17	2-Methyl-2-butenyl-*trans*-isoferulate	23.04	10.9
18	3-Methyl-2-butenyl-*trans*-isoferulate	23.13	18.0
21***	Benzyl-*cis*-isoferulate	23.87	0.8
24***	Phenylethyl-*cis*-isoferulate	24.93	1.9
27	Benzyl-*trans*-isoferulate	26.00	8.0
29***	*trans*-Cinnamyl-*cis*-isoferulate	26.62	0.6
30	Phenylethyl-*trans*-isoferulate	26.79	19.7
35	Hydrocinnamyl-*trans*-isoferulate	28.13	2.9
37	*trans*-Cinnamyl-*trans*-isoferulate	29.13	6.0

* Isoferulic acid chromatographs as the bis-TMS derivative; all others listed chromatograph as mono-TMS derivatives.

** The total ion current generated depends on the characteristics of the compound concerned and is not a true quantitation (see ref. 2).

*** Identified on the basis of mass spectral and gas chromatographic characteristics. We have not confirmed our identification by co-chromatography with an authentic reference standard as we do not have *cis*-isoferulic acid available for syntheses of the *cis* derivatives.

§ The 2-methylbutyl- and 3-methylbutyl esters co-chromatograph and are not resolved (see Results).

4-methoxycinnamic acid) together with a series of *cis*- and *trans*-isoferulate esters and of aliphatic alcohols (Fig. 1a). A single-ion reconstruction of $m/z = 249$ enables the isoferulic esters to be identified (Fig. 1b). *Cis* and *trans* isomers of 2-methyl-2-butenyl-, 3-methyl-3-butenyl-, 3-methyl-2-butenyl-, benzyl-, phenylethyl- and *trans*-cinnamyl isoferulate were identified, as were the *trans* isomers of 2-methylpropyl-, 2-methyl-2-propenyl-, 2-methylbutyl-, 3-methylbutyl- and hydrocinnamyl isoferulate (Table I).

The 2-methylbutyl- and 3-methylbutyl esters have similar GC retention times (MU 22.50; 22.55) and mass spectra which are noticeably different only in some of the lower mass ions ($m/z = 40$–60, see Fig. 2A). Both esters chromatograph very close to the 3-methyl-3-butenyl ester (Fig. 1b), a major peak, and this causes some problems in attempting to exactly co-chromatograph reference standards to confirm positively the presence of both the 2-methylbutyl- and 3-methylbutyl esters. From our co-chromatography results and examination of the mass spectra we consider both to be present, although it is difficult to be certain of this.

Mass spectra of the esters of isoferulic acid with saturated and unsaturated aliphatic alcohols of the prenyl type and with hydrocinnamyl alcohol are shown in Fig. 2A and B.

DISCUSSION

Preliminary analysis of this fraction of poplar bud exudate (LB3)[6] indicated that it contained esters of ferulic and isoferulic acid. Our current, more detailed, analysis confirms the identification of isoferulic acid esters only. Although spectra of isoferulate (3-hydroxy-4-methoxycinnamate) and ferulate (4-hydroxy-3-methoxycinnamate) esters are virtually identical, esters of the former compound chromatograph about 0.1–>0.2 MU before those of the latter[2], and co-chromatography with appropriate reference compounds enables them to be clearly identified. Esters identified include the 2-methylpropyl-, 2-methyl-2-propenyl-, 2-methyl-2-butenyl-, 2-methylbutyl-, 3-methylbutyl- and hydrocinnamyl esters. Isofar as we are aware this is the first report of the natural occurrence of these esters.

There are traces of a further novel isoferulate ester at MU 24.45 and the $[M]^+$ ($m/z = 348$) suggest it to be a methylpentenyl ester.

We have identified from mass spectral patterns and GC retention times the *cis*-isoferulate esters of several compounds (Table I). We have consistently found that *cis* isomers are not formed from *trans* isomers of isoferulate esters during derivatization and chromatography. We conclude therefore that these occur naturally in poplar bud exudates, although we do not know whether the *cis* isomers are secreted as such, or whether they are subsequently formed from *trans* isomers due to environmental conditions.

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Note

Analysis of catechin and epicatechin by high-performance liquid chromatography after benzoylation

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(First received February 25th, 1988; revised manuscript received May 3rd, 1988)

The flavan-3-ols catechin and epicatechin are widespread in nature, for example, as naturally occurring monomers of tannins in wood plants[1,2]. The analysis of catechin and epicatechin from plant material has recently been carried out by means of reversed-phase high-performance liquid chromatography (HPLC) after extraction with methanol or methanol–water and purification on polyamide. The HPLC separation is effected by gradient elution with 2% acetic acid–methanol and detection at 280 nm[3–5]. The method of sample clean up on polyamide was optimal for catechins as well as for the ubiquitous hydroxycinnamic acid derivatives[5]. To confirm the results obtained, a second method of similar quality is required for the determination of catechins. To separate catechins quantitatively from aqueous solutions RP-18 cartridges have been tested as has the possibility of catechin detection as their benzoate derivatives. RP-18 cartridges are often used for rapid and selective clean up and/or accumulation procedures[3,6–12]. The isolation and detection of different polyhydroxy compounds from food after benzoylation have been described in earlier publications[9–12].

EXPERIMENTAL

Chemicals

Catechin and epicatechin are commercially available substances. The solvents used for HPLC were of analytical reagent grade purified with Extrelut® (Merck, Darmstadt, F.R.G.). All evaporations were performed in a rotary vacuum evaporator at a temperature not higher than 40°C.

Sample preparation

An 100-g amount of the fruit material without stones (cores) was cut into small pieces and frozen. The frozen or freeze-dried tissue was homogenized using an household blender and extracted with 400 ml of methanol. The residue was extracted twice more with 400 ml of 80% aqueous methanol. The pooled extracts were evaporated. The residual aqueous solution was made up to 100 ml with water. The resulting concentrations are 1.0 g fruit/ml solution. In the case of pome fruit, freeze drying helps to avoid enzymatic browning and allows more rapid extractions and separations of the pulp through a P-4 glass frit.

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Clean up on polyamide

A methanolic suspension of polyamide (MN-SC-6, 0.05–0.16 mm; Machery & Nagel, Düren, F.R.G., without traces of iron), was poured into glass columns (250 mm × 27 mm I.D.) used for separation. For preliminary treatment, the column was flushed with water. A 25-ml volume of the aqueous extract was applied to the column, washed with 150 ml of water to remove carbohydrates, acids and salts.

Catechins besides hydroxycinnamoylglucoses and glucosides were eluted from the column with 300 ml of methanol, concentrated and diluted to 25 ml of aqueous solution. These solutions (microfiltered, Millipore-Filter 0.2 μm; Sartorius, Göttingen, F.R.G.) were used for direct HPLC analysis or for separation and concentration with RP-18 cartridges. For quantitation of catechins it was necessary to insert the aqueous solutions immediately.

RP-18 cartridges

Extract solutions of pome and stone fruit in concentrations of g/ml were applied to Sep-Pak RP-18 cartridges (Waters Assoc., Milford, MA, U.S.A.). The cartridges were conditioned with 2 ml of methanol and 8 ml of water by removing them from each solvent by pressing with a syringe. The separation potential was not influenced by repeated use (tested five times). Plastic syringes with a capacity of 10 ml were used.

Benzoylation

The benzoylation procedure is described in several publications[9–12]. The reagents used were those for chemical reaction with 50 mg of solids or 30 μl of liquids. The eluate containing catechins obtained with or without cartridges was carefully evaporated to dryness before benzoylation. Reagents and conditions for benzoylation: dissolution in 4 ml of pyridine addition of 0.5 ml of benzoyl chloride, reaction in an ultrasonic apparatus at 60°C for 1 h; addition of 0.5 ml of methanol, precipitation of benzoates with 50 ml of water, separation with RP-18 cartridges; flushing with water in portions (4 × 5 ml); final elution of the benzoates with isooctane–diethyl ether–acetonitrile (150:80:20) made up to 50 ml in a graduated flask. This solution is used for HPLC.

Analytical HPLC

The HPLC analysis was carried out with an isocratic system. A Beckman pump 114 M or an LC-XPD-pump (Pye Unicam, Kassel, F.R.G.) was used with a flow-rate of 0.8 ml/min. The 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) was equipped with a 20-μl sample loop. Chromatograms, spectra and results were registered with a diode-array detector (Philips, Kassel, F.R.G.) consisting of a Pye Unicam PU 4021 multichannel detector and a PU 4850 video chromatography control center. Chromatograms were recorded at 231 nm, spectra from 190 to 390 nm. Three-dimensional presentations (chromascans) are possible. A stainless-steel column (125 mm × 5 mm I.D.) packed with SC-Hypersil 3 μm (Gynkotek, München, F.R.G.) was used for separations with the solvent isooctane–diethyl ether–acetonitrile (150:60:10).

RESULTS AND DISCUSSION

A second method of determination only for catechins was developed without the disadvantages of the method described in earlier publications[3–5]. The first method was harmonized for a simultaneous determination of catechins and hydroxycinnamic acid esters and therefore represents a compromise. In the process of separation and purification, catechins and some hydroxycinnamic acid derivatives are obtained by elution with methanol from polyamide. The previous flushing with water to remove sugars, salts and other undesired compounds is to limit so that the hydroxycinnamic acid derivatives are obtained quantitatively. Also separations by analytical HPLC with gradient elution take a longer time. The employment of aqueous extract solutions is required for HPLC on a RP-18 phase with the eluents used. Because of their lower stability in aqueous solutions, catechin should be detected immediately. These solutions cannot be stored for later quantitations. The change to benzoates as employed for the direct quantitation of several polyhydroxy compounds from food[9–12] makes it possible to keep the catechins for a longer time.

To compare the results obtained by direct HPLC, we used the same aqueous

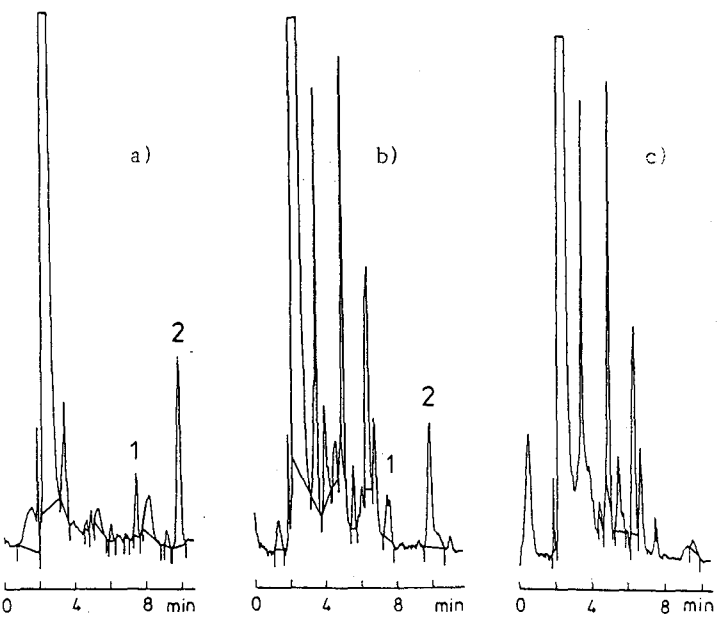

Fig. 1. Chromatograms of "Granny Smith" apple after benzoylation. HPLC separation of derivated catechin (1) and epicatechin (2) in the extracts without and with the use of RP-18 cartridges for clean up before benzoylation. A 2-ml volume of aqueous sample solution in concentrations of g/ml was used in each case. Column: SC-Hypersil 3 μm (125 mm × 5 mm I.D.). Detection: 231 nm. Flow-rate: 0.8 ml/min. Isocratic elution with isooctane–diethyl ether–acetonitrile (150:60:10). (a) Extract purified with a RP-18 cartridge before derivatization. Catechins were eluted with 5 ml of methanol after flushing with 2 ml of water. They can be detected without overlapping and measurable losses. (b) Extract benzoylated without use of RP-18 cartridge. Overlapping and losses can be seen. (c) Catechin and epicatechin retained on a RP-18 cartridge as shown by the absence in the chromatogram of the benzoylated extract and of the water used for flushing (2 ml) after separation of the catechins.

solutions (purified by polyamide) for tests of separation and benzoylation. Catechin and epicatechin exist only in small amounts compared with the naturally occurring sugars in fruit, which are also UV-detectable after benzoylation. To achieve good conditions for a subsequent benzoylation of the dry residues, we developed an additional clean-up procedure. The use of aqueous solutions (without cartridges) did not yield reproduciable results. Also, direct benzoylation of apple tissue fresh or freeze dried did not give an acceptable proof of catechins. On the other hand, RP-18 cartridges conditioned as shown retain catechin and epicatechin quantitatively from aqueous extracts and standard solutions. They are eluted with methanol which can be removed quickly and carefully without losses of catechins. Additional clean-up steps with water before the elution with methanol seemed to be necessary for quantitation of epicatechin from some extract solutions as well as to reduce or avoid overlapping of catechins with undesired compounds in the HPLC chromatograms of the benzoates, as shown by the example of an apple extract (Fig. 1).

Test series with different kinds of fruit (apple, pear, peach, apricot, plum, cherry) and tea as well as with standard solutions of catechins with contents comparable to those in the fruit extracts showed that catechin and epicatechin could be detected quantitatively after application of Sep-Pak RP-18 cartridges to 1–3 g (ml) fruit extract, flushing with 2 ml of water and elution with 5 ml of methanol. Clean up with more than 4 ml of water gave losses of catechin of about 5%. The results obtained are independent of the aqueous sample solutions investigated and reproducible and agree with those obtained by analytical HPLC of the underivatized catechins. The contents of catechins in 57 fruit samples examined by direct HPLC varied from less than 10 to 180 mg/kg[5]. Samples tested five times under the same conditions using the same aqueous solutions from the polyamide column showed standard deviations of the average contents of benzoylated catechins of not more than ±4%.

Optimum separation with isocratic HPLC was carried out on silica gel with isooctane–diethyl ether–acetonitrile (150:60:10). The wavelength of detection is 231 nm. Catechins are eluted before most of the sugars with short retention times of less than 10 min. They can also be distinguished from sugars and other benzoylated compounds by their UV spectra. Fig. 2 shows the normalized and superimposed UV spectra of derivatized catechins and rhamnose.

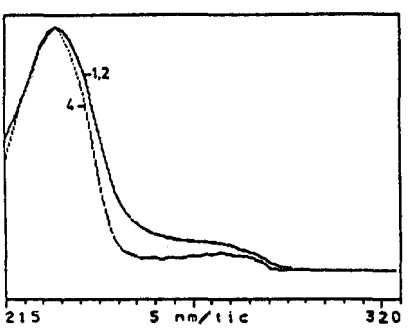

Fig. 2. Normalized and superimposed UV spectra of benzoylated catechin (1), epicatechin (2) and rhamnose (4).

Catechins were found with a reproducibility of 92 ± 3%. The detection limit of catechins as their benzoates increases by up to 10- or 20-fold compared with the underivatized compounds. Catechin and epicatechin in standard solutions of 0.2 mg/kg each can be detected after benzoylation. The linearity of detection was tested up to 100 mg/kg of derivatized catechins in the final solutions applied for HPLC. For samples with low contents of catechins it is also possible and useful to concentrate the final solutions of 50 ml as described here. The clean-up procedure allows the amount of sample used to be increased without changing the reagents used for benzoylation.

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Note

Identification of xanthene dyes in lipsticks by reversed-phase high-performance liquid chromatography

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(Received May 3rd, 1988)

In a previous paper[1] we described a method for the identification of 75 organic colours commonly used in the cosmetic industry, based on ion-pair reversed-phase high-performance liquid chromatography (HPLC), which can be used for the rapid screening of samples for non-permitted colours. The law 713|86 and subsequent adjournments which comprise the Italian legislation on cosmetics in response to the Directive 76|768 of the European Economic Community has stimulated us to study other methods of identification and quantitation of cosmetic dyes.

In particular, we have considered the series of acidic xanthene dyes. These compounds are widely employed in lipsticks in the form of aluminium lacquers or free acids, practically insoluble in the matrix used[2] and their analysis is generally performed by chromatographic techniques[3–8]. This paper reports the results of an analysis performed on 99 samples of commercial lipsticks. Since this kind of cosmetic product usually contains very complex mixtures of dyes, the samples were subjected to a preliminary extraction to isolate the class of xanthene dyes from the other colouring agents. The presence of this class of colourants in the extracts was tested by thin-layer chromatography (TLC) before performing the separation and identification of the dyes selected by reversed-phase HPLC. In particular, the dyes CI 45350 for which the law 713|86 establishes a maximum limit of 6% in the finished product and CI 45396 (maximum limit 1% as the free acid) were quantitated in order to test the compliance of the cosmetic product with the EEC legislation.

EXPERIMENTAL

Materials

All dyes, referred to by their CI reference numbers[1], were commercial samples used as received. Standard solutions of individual dyes and mixtures were prepared in methanol. For CI 45396 and CI 45350, serial dilutions were made to obtain a set of solutions with concentrations ranging from 0.01 to 0.8 mg/ml. The samples of

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lipsticks were freely available. All chemicals used were of analytical grade (Farmitalia-Carlo Erba, Milan, Italy). Water was deionized and doubly distilled from glass apparatus; acetonitrile was of HPLC grade. All solvents and solutions for HPLC analysis were filtered through a filter (Millipore, Bedford, MA, U.S.A.), pore size 0.5 μm, and vacuum degassed by sonication before use.

Apparatus

A model 5000 liquid chromatograph (Varian, Zug, Switzerland) equipped with a variable-wavelength UV–VIS detector (Varichrom UV 50), a Valco AH 60 injection valve and a Model 730 integrator recorder (Waters Assoc., Milford, MA, U.S.A.) was used. The analytical column was a 5-μm Altex C_{18} Ultrasphere (150 mm × 46 mm I.D., Beckman).

Chromatographic conditions

The HPLC conditions were as follows: mobile phase, acetonitrile–water (pH 3 adjusted with glacial acetic acid) with a linear gradient from 30 to 35% acetonitrile in 5 min and 75% acetonitrile in 30 min, and finally a purge with 100% acetonitrile for 12 min; flow-rate, 2.5 ml/min; column temperature, 25°C; injection volume, 10 μl; detection wavelengths, 400, 475 and 525 nm; detector sensitivity, 0.64 a.u.f.s.; chart speed, 0.5 cm/min.

Sample treatment

According to Lehmann's extraction[9], a 500-mg amount of lipstick was dissolved in 10 ml of a solution of orthophosphoric acid (5%, v/v) in dimethylformamide (DMF) by heating in a water-bath. After cooling, the mixture was filtered through cotton-wool into a separatory funnel and 5 ml of water and 5 ml of hexane were added. The organic phase, if coloured, was kept for the analysis of lipophilic dyes, whereas the aqueous phase was extracted with dichloromethane. The organic phase, containing xanthene dyes, was concentrated until a detectable absorbance was obtained. If that did not occur the initial amount of lipstick was doubled. An alternative extraction was the one proposed by Etournaud and Aubort[2], by which all acidic dyes, including xanthene dyes, were extracted into the ammonia–methanol fraction.

RESULTS AND DISCUSSION

The chromatographic properties of the xanthene dyes examined and their relative absorptions at the three detection wavelengths are reported in Table I. The retention times are reproducible under the experimental conditions used. The mobile phase employed enables good column performance for long periods of time. Chromatograms of most of the individual dye samples (not shown) clearly demonstrated the presence of coloured impurities whose nature was not investigated. In such cases we have put the symbols (I), (II), etc., next to the CI number for labelling the impurities. The separation obtained for a standard mixture of the seven xanthene dyes is illustrated in Fig. 1.

The extraction of the dyes from lipsticks has been performed both with the technique proposed by Etournaud and Aubort[2] and with that proposed by Lehmann[9]. Both techniques can be utilized since they are equally effective in extracting

TABLE I

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

Colour index No.	*Retention time (min)*	A_{400}	A_{475}	A_{525}
45350	6.2	15	100	5
45396	8.4	15	100	5
45370 (I)	9.4			
45425	10.3	45	60	100
45380 (I)	11.4			
45370	11.8	5	60	100
45430 (I)	12.0			
45425 (I)	13.0			
45380	13.1	5	15	100
45430 (II)	13.8			
45430	14.4	15	30	100
45425 (II)	18.2			
45410	23.6	5	20	100

xanthene dyes from the cosmetic samples. The applicability of this HPLC method has been demonstrated on commercial lipstick samples of unknown composition. Fig. 2 gives a typical chromatogram of a lipstick extract, recorded at 400, 475 and 525 nm. Two xanthene dyes must be present: CI 45410 and CI 45380. The minor peaks at about 11 and 14 min were assigned to impurities of CI 45380.

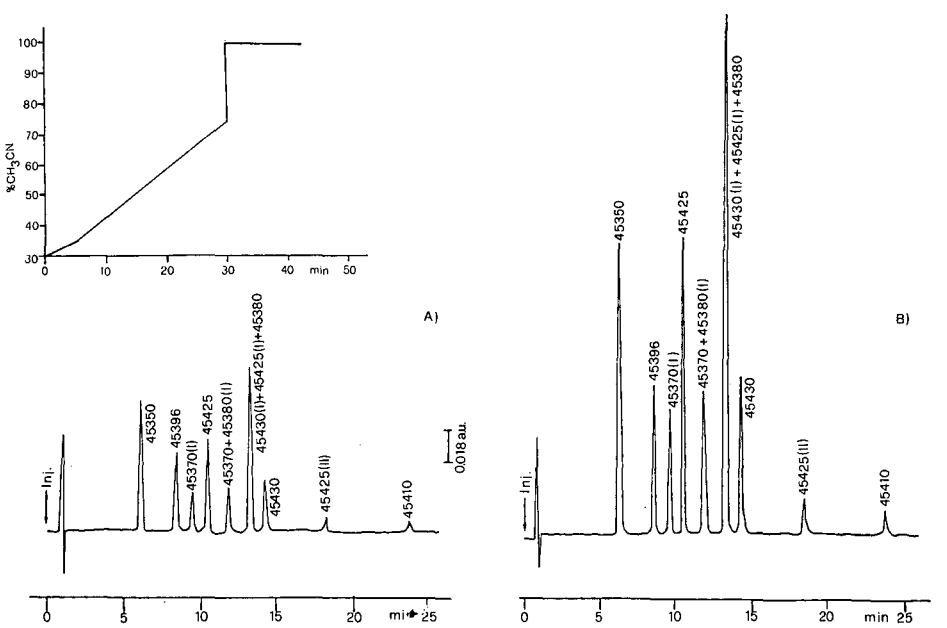

Fig. 1.

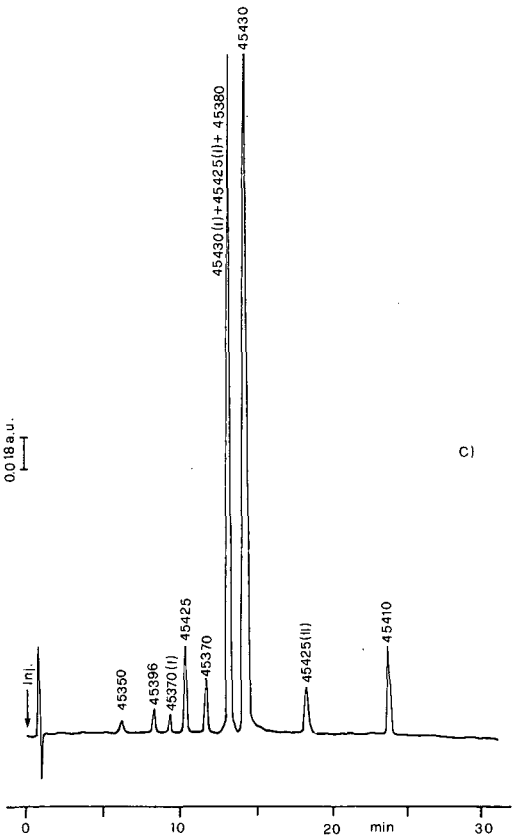

Fig. 1. Typical chromatogram of a standard mixture of the xanthene dyes. Detection at 400 (A), 475 (B) and 525 nm (C), respectively. In the upper part of (A) is reported the solvent programme for the chromatographic separation.

Ninety-nine samples of commercial lipsticks have been analyzed for the presence of the xanthene dyes and the results obtained are reported in Table II. A quantitation has been performed for the dyes CI 45430 and 45396. Standard solutions

TABLE II

FREQUENCY OF OCCURRENCE OF THE XANTHENE DYES IN THE 99 SAMPLES OF LIPSTICKS ANALYZED

Colour index No.	*Name*	*Frequency of occurrence*
45430	Erythrosin	21
45380	Eosin S-13	28
45410	Phloxin B	9
45370	Eosin S-10	23
45396	Dinitrofluorescein	1
45350	Fluorescein	0
45425	Diiodofluorescein	0

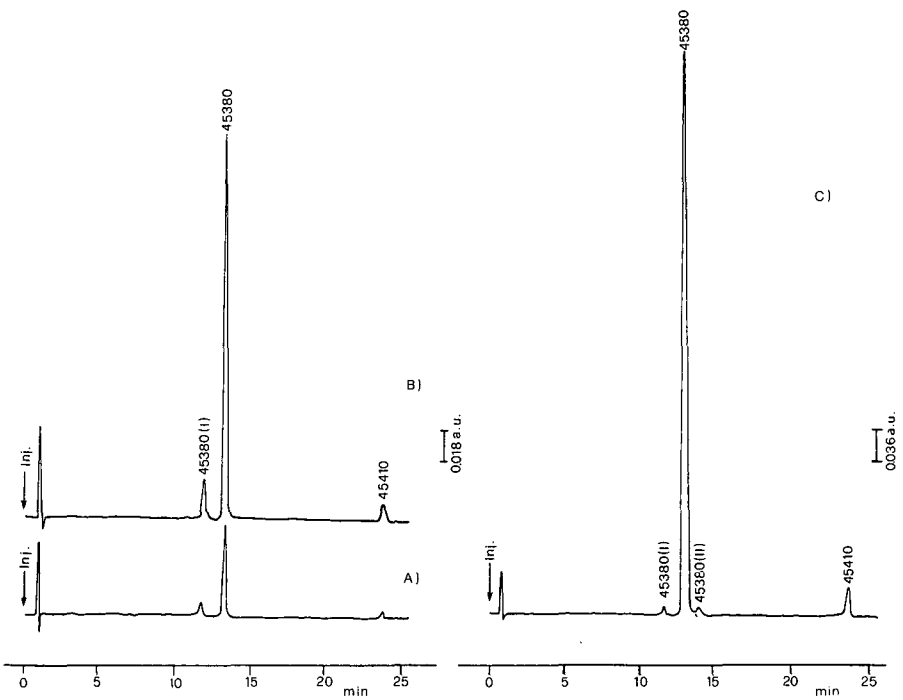

Fig. 2. Chromatogram of a lipstick extract recorded at 400 (A), 475 (B) and 525 nm (C).

were prepared by dissolving known amounts of the two standards in methanol and the solutions were processed using the HPLC conditions described above. Calibration graphs were constructed by use of the peak areas measured for the various amounts injected and were linear over the ranges of concentrations used, with regression coefficients of 0.9998. Their slopes were used in the quantitation of the two dyes in commercial lipsticks. No cosmetic sample contained the dye CI 45430 and only one contained the dye CI 45396 at a level lower than the limit imposed by the law 713 | 86.

In conclusion, the HPLC method described enables the identification, by a relatively simple procedure, of the xanthene dyes and can be used for determining those subject to legislation as well as the impurities associated with them.

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Note

Thin-layer chromatographic characterization of essential oils

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The use of thin-layer chromatography (TLC) for the analysis of essential oils has been very limited in the last 10 years[1,2], gas chromatography (GC) on packed and capillary columns and the head-space technique being the chromatographic methods most widely used[3,4]. The interpretation of GC data is quite difficult and, when possible, mass spectrometry is employed[3].

The characterization of these compounds is of importance in industry (production and quality control) and in physiologic and genetic studies of the plants from which they are extracted[5].

This paper deals with a rapid method which can differentiate and characterize the essential oils of different plants (pine and juniper), different species (*Pinus halepensis, Pinus cembra, Hybridus P. halepensis* × *P. brutia*) and different parts of the same plants (needles and small branches in the case of pine and needles and fruits in the case of *Juniperus phoenicia*).

EXPERIMENTAL

The chromatographic determinations were carried out on silica layers 60 F_{254} (Merck). There are no substantial differences between the data on these layers and those on high-performance ones, there being no problems of sensitivity or of elongated spots. The spots were visualized by spraying first with 5% sulphuric acid solution in ethanol and then with 10% vanillin solution in methanol[1]. The plates were then heated at 100°C for 10 min. The migration distance was 10 cm. Fresh standard solutions were prepared by dissolving the pure compounds (Aldrich, Fluka, Roth) or the essential oils in methanol. The amount deposited on the layer was between 0.1 and 2 μg in the case of the reference compounds and between 10 and 30 μg in the case of essential oils. All the measurements were carried out at 20°C.

The quantitations were effected with a Shimadzu LS 200 densitometer. The measurements were carried out at a wavelength of 540 nm, which gave the best results both in terms of the sensitivity and baseline constancy; in this way the separation of spots which differ very little in their R_F values is possible. Most spots were visualized even by fluorimetric measurements using an excitation beam wavelength of 365 nm and an emission filter of 400 nm. In this case, however, the densitometric peaks were less sharp and the baseline quite high and therefore the interpretation is rather dif-

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ficult. The layers were scanned with the densitometer 30 min after the end of heating: the intensity of the spots decreased considerably in the first 20 min, but after this time the colour intensity was constant for about 1 h.

The essential oils were obtained by steam distillation of minced needles small branches or fruits of *Pinus halepensis, Pinus cembra, Hybridus* (*P. halepensis* × *P. brutia*) and *Juniperus phoenicia.*

RESULTS AND DISCUSSION

Table I lists some constituents of essential oils, the colours observed upon spraying with the vanillin solution and the R_F values on silica gel layers in four eluents chosen among those which gave the best results[1,2]. Among the reference compounds studied were the most common consistuents of pine and juniper essential oils. It should be noted that, with the vanillin solution, only those compounds with a certain degree of double bond conjugation can be detected. The compounds are listed according to their increasing R_F values in chloroform–toluol (75:25) mixture. From the data, it is secn that quite good separations can be obtained even with a single eluent and that the identification of the spots may be improved by the use of two plates in two different eluents. For instance, with toluol–ethyl acetate (90:10),

TABLE I

R_F VALUES OF SOME COMPONENTS OF ESSENTIAL OILS ON SILICA GEL LAYERS AND COLOURS OF THE SPOTS WHEN SPRAYED WITH VANILLIN SOLUTION

Eluents: (a) chloroform–toluol (75:25); (b) toluol–ethyl acetate (90:10); (c) two developments in *n*-hexane–ethyl acetate (90:10); (d) first development in dichloromethane followed by a second one in *n*-hexane–ethyl acetate (90:10).

Compound	*Colour*	*Eluent*			
		a	*b*	*c*	*d*
Nerol	Blue	0.20	0.30	0.30	0.42
α-Terpineol	Blue	0.22	0.27	0.32	0.32
D-citronellol	Red	0.23	0.29	0.32	0.40
Borneol	Red	0.25	0.35	0.35	0.43
Geraniol	Red	0.26	0.27	0.30	0.38
Myrtenol	Violet	0.28	0.35	0.39	0.32
Terpinen-4-ol	Blue	0.31	0.43	0.47	0.51
Linalool	Blue	0.34	0.43	0.47	0.46
α-Phellandrene	Blue	0.35	0.59	0.37	0.32
Carvacrol	Pink	0.40	0.56	0.65	0.57
Eugenol	Yellow	0.42	0.55	0.68	0.44
Thymol	Red	0.43	0.62	0.70	0.55
1,8-Cineole	Blue	0.47	0.54	0.68	0.65
α-Terpinene	Brown	0.50	0.58	0.70	0.64
Carvone	Pink	0.50	0.61	0.65	0.73
Isobornyl acetate	Violet	0.67	0.66	0.82	0.85
Geranyl acetate	Blue	0.68	0.78	0.83	0.85
Methylchavicol	Pink	0.85	0.95	0.90	0.98
β-Caryopillene	Violet	0.97	0.96	0.98	0.98
β-Myrcene	Violet	0.98	0.97	0.95	0.98

linalool and α-phellandrene can be separated (R_F = 0.43 and 0.59, respectively), while this separation is not possible in chloroform–toluol (eluent a). In the latter eluent, terpinen-4-ol and linalool (R_F = 0.31 and 0.34) can be separated, in contrast to their behaviour in eluent b. The reference compounds listed in Table I can be detected if present at the level of 1 or 2 μg at most. The retention of the compounds is correlated to the polarity of their molecules. The higher the polarity of the compounds, the more they are retained by the silica gel layers. Terpenes with an alcoholic group are the most strongly retained, then the phenylpropane derivatives, the acetates and finally compounds such as β-myrcene and β-caryophillene which migrate with the solvent front (see Table I).

In Fig. 1 are shown the chromatograms of different essential oils obtained by steam distillation. Very good separations are achieved and some components can be identified (see Fig. 1). The data are in good agreement with these obtained by GC[6,7].

As regards the quantitation of the different components, some critical factors must be considered, that is the temperature and the time during which the layers are heated, and the time after which the densitometric measurements are made. Because of the influence of these parameters, besides those which are peculiar to TLC quantitations[8], a standard of known concentration must always be deposited on the layer.

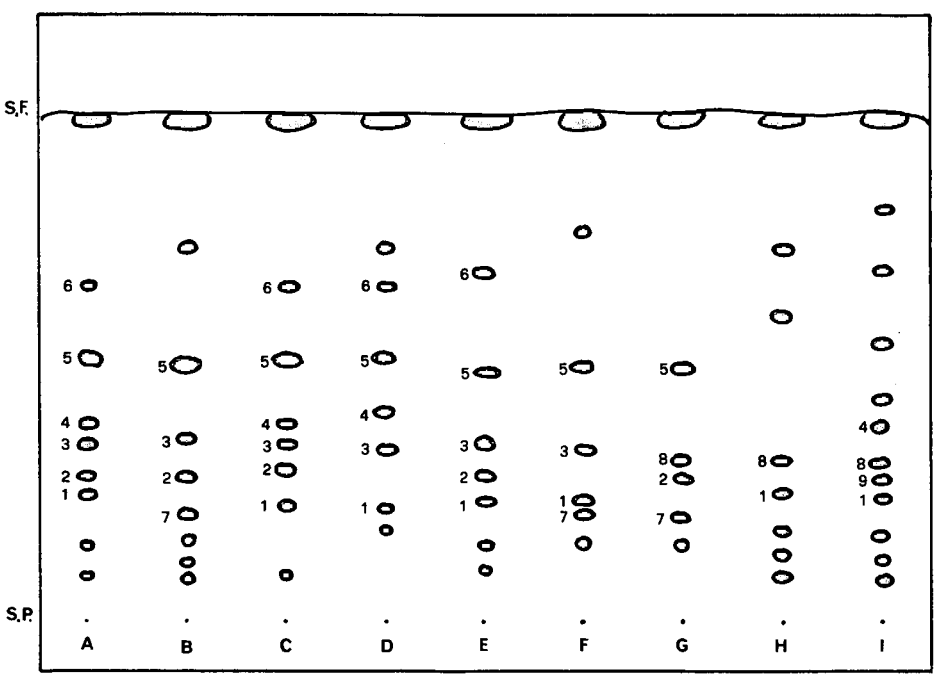

Fig. 1. Thin-layer chromatograms of essential oils. Eluent: chloroform–toluol (75:25). A = *Hybridus* (needles); B = *Hybridus* (branches); C = *Pinus halepensis* (needles); D = *Pinus halepensis* (branches); E = *Pinus cembra* (needles); F = *Pinus cembra* (branches); G = *Pinus cembra* (commercial oil); H = *Juniperus phoenicia* (fruits); I = *Juniperus phoenicia* (needles). 1 = D-Citronellol; 2 = myrtenol; 3 = linalool; 4 = carvacrol; 5 = 1,8-cineole; 6 = geranyl acetate; 7 = α-terpineol; 8 = terpinen-4-ol; 9 = geraniol.

TABLE II

RATIOS OF INTEGRATION UNITS FOR THE DIFFERENT COMPONENTS OF PINE ESSENTIAL OILS TO THOSE OF LINALOOL (SEE FIG. 2)

The ratios are the means for eight determinations. S.D. = Standard deviation.

Component	*Ratio*	*S.D.*	*Component*	*Ratio*	*S.D.*
P. halepensis (needles)			*P. halepensis (branches)*		
1	1.78	0.05	1	1.05	0.18
2	4.46	0.33	3	1	
3	1		4	0.90	0.14
4	3.51	0.33	5	6.68	1.61
5	10.38	1.47	6	0.61	0.02
6	0.84	0.06			
P. cembra (needles)			*P. cembra (branches)*		
1	2.89	0.08	1	1.08	0.16
2	2.88	0.12	3	1	
3	1		5	1.69	0.13
5	0.37	0.01	7	0.78	0.10
6	0.18	0.02			
Hybridus (needles)			*Hybridus (branches)*		
1	3.98	0.66	2	1.13	0.12
2	3.67	0.71	3	1	
3	1		5	10.22	1.33
4	2.40	0.52	7	1.95	0.07
5	16.14	1.67			
6	0.66	0.07			

Notwithstanding such difficulties, quite reproducible data are obtained; however the whole procedure is time-consuming, since fresh solutions of all the reference compounds must always be used.

In order to effect a quality control or to identify the origin of an essential oil (for instance from small branches, needles or from different species), the quantitative data may be used as ratios with respect to a given component which must always be present so that the above-mentioned critical factors can be neglected. In the case of the pine essential oils, we calculated the ratios of the integration units for the different components identified relative to those of linalool. We chose linalool as the reference since it is present in all samples, can easily be identified and its concentration is relatively low (its amount changes from 0.5 to 1.5% in the different essential oils studied). Table II lists some ratios for the compounds identified; the values are the averages from eight determinations; the standard deviations are also listed.

In Fig. 2 are reported maps of the different oils. The results are very interesting, since significant differences among them can be seen. These maps can also be used for the study of the essential oil composition as a function of the genetic origins of the plants[9,10].

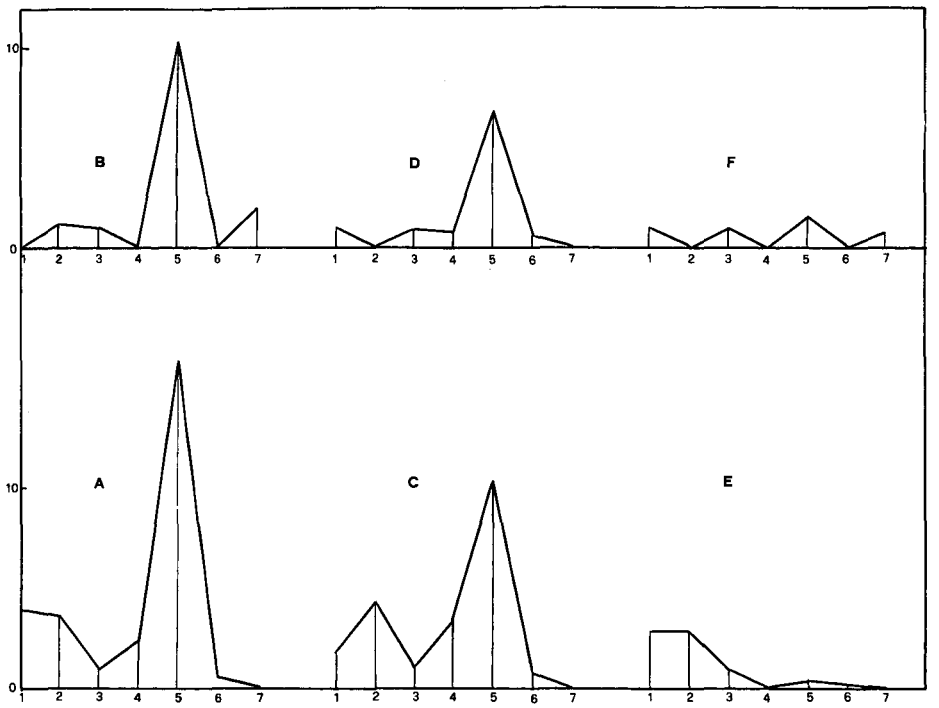

Fig. 2. Maps of essential oils. A = *Hybridus* (needles); B = *Hybridus* (branches); C = *Pinus halepensis* (needles); D = *Pinus halepensis* (branches); E = *Pinus cembra* (needles); F = *Pinus cembra* (branches). *X* axis, numbers as in Fig. 1; *Y* axis, ratios of integration units (see text).

ACKNOWLEDGEMENT

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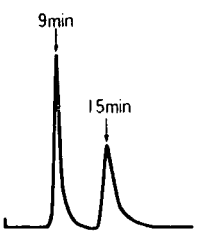
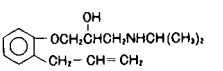


Column: 4.6mm ID × 250mm
Eluent: ethanol-H_2O (95:5)
Flow rate: 0.5ml/min
Detection: UV254nm

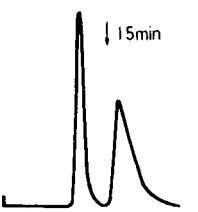
CHIRALCEL OD

Column: 4.6mm ID × 250mm
Eluent: hexane-2-propanol-diethylamine (80:20:0.1)
Flow rate: 0.5ml/min
Detection: UV254nm

CHIRALCEL OD



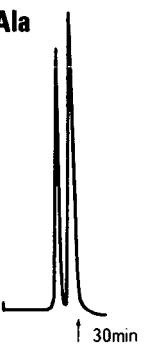
Sample: 150mg



Column: 20mm ID × 500mm
Eluent: hexane-2-propanol-diethylamine (80:20:0.1)
Flow rate: 12ml/min
Detection: UV254nm

CHIRALPAK WE

DL-Ala



Column: 4.6mm ID × 250mm
Eluent: aq 0.25mM $CuSO_4$
Flow rate: 1.0ml/min
Detection: UV230nm Temp. 35°C

Name	Separation
CHIRALPAK OT(+) CHIRALPAK OP(+)	Compounds possesing aromatic group (see: Technical Brochure No.1)
CHIRALCEL OA CHIRALCEL OB CHIRALCEL OC CHIRALCEL OD CHIRALCEL OK CHIRALCEL CA-1	(see: Technical Brochure No.1, No.3)
CHIRALPAK WH CHIRALPAK WM CHIRALPAK WE	DL-Amino acid or its derivative (see: Technical Brochure No.2, No.4)

■Size of column

Analytical Column	4.6mm (I.D.) × 250mm (L)
Precolumn	4.6mm (I.D.) × 50mm (L)
Semipreparative column	10mm (I.D.) × 250mm or (500mm)
	20mm (I.D.) × 250mm or (500mm)

■Preparation of pure enantiomer (~kg) with Chiral column are acceptable.

Please contact!

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