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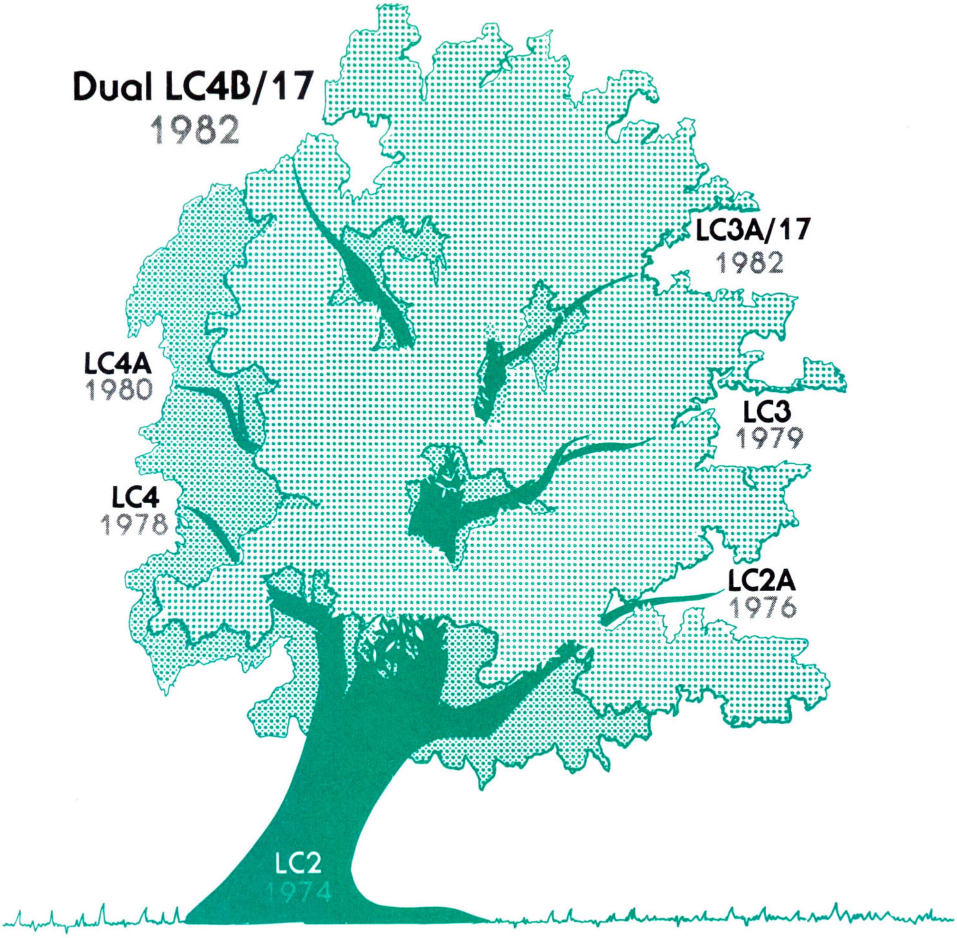

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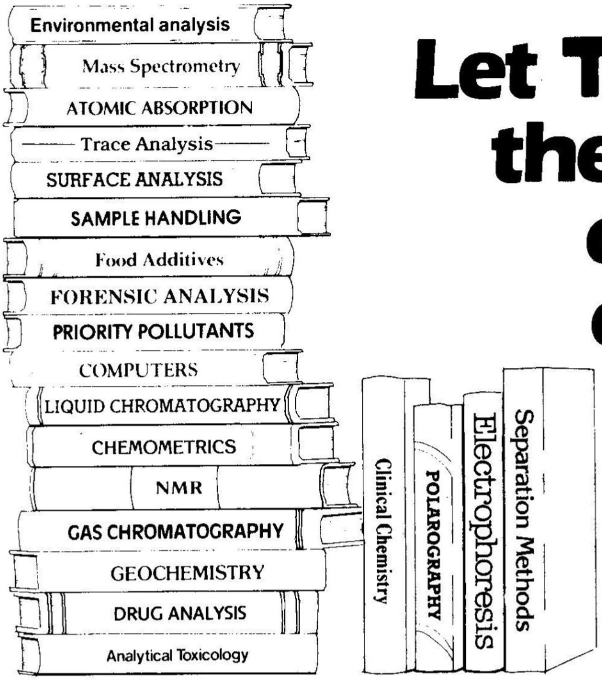
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CORRECTION OF GAS CHROMATOGRAPHIC RETENTION VOLUMES FOR SMALL TEMPERATURE CHANGES

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(Received April 25th, 1982)

SUMMARY

Two simple equations are proposed of sufficient accuracy for correcting retention volumes for small changes in column temperature. One equation (eqn. 1) is suitable for use when column temperature is sufficiently close to the boiling point; otherwise eqn. 2 is more accurate. The equations are derived and shown to be valid for most solutes over a wide range of gas–liquid chromatographic conditions.

INTRODUCTION

Since retention varies with temperature, retention volumes often have to be corrected for small changes in column temperature, *e.g.* when oven temperature drifts with change in ambient temperature. The need for a suitable correction equation arises in two applications of gas chromatography. In analytical work, identification and accurate quantitation of peaks depends on accurate determination of the retention volume: in particular, automatic data processing requires a simple means of correcting for variations in column temperature. Secondly, in applications to physicochemical measurement[1], retentions often need to be measured with great accuracy. The significance of the problem is seen in the fact that a temperature change of only 0.3°K is sufficient to change the retention by 1% or so, an amount which may be enough to vitiate measurement of a physicochemical property or cause wrong identification of a peak. Even with use of an internal standard, peaks can be misidentified because, as a second order effect, the relative retention may also change with temperature[2].

We will show that the correction can be made with the aid of the simple equation

$$\frac{\delta V_N}{V_N} = -9.7 \frac{\delta T_c}{T_c} \tag{1}$$

when column temperature is sufficiently close to the boiling point of the solute concerned, and otherwise with the equation

$$\frac{\delta V_N}{V_N} = -\frac{\delta T_c}{T_c}\left[\frac{17.1\ T_b}{T_c} - 7.4\right] \tag{2}$$

In these equations, V_N is the net retention volume, T_c and T_b are respectively the temperature of the column and the normal boiling point of the solute, in °K, and δV_N is the correction to be made to the retention volume for a change δT_c in column temperature. The negative sign arises because retention falls as temperature rises. Eqn. 2 reduces to eqn. 1 as $T_c \rightarrow T_b$. Eqn. 1 is independent of solute parameters.

We consider, first, the derivation of these equations, and then their accuracy and applicability.

DERIVATION OF EQUATIONS

Combining the Clausius–Clapeyron equation

$$\frac{1}{p^0}\frac{dp^0}{dT_c} = \frac{\lambda_c}{RT_c^2}$$

and Trouton's rule,

$$\tau = \lambda_b/T_b = 85 \text{ J mol}^{-1} \text{ °K}^{-1}$$

we obtain

$$\frac{\delta p^0}{p^0} = \frac{\tau}{R}\frac{\lambda_c T_b}{\lambda_b T_c}\frac{\delta T_c}{T_c} = 10.2\frac{\lambda_c T_b}{\lambda_b T_c}\frac{\delta T_c}{T_c} \tag{3}$$

where p^0 is the saturation vapour pressure of pure solute at column temperature T_c, λ its latent heat, τ is Trouton's constant and $R = 8.314$ J mol^{-1} °K^{-1}, the gas constant. Therefore, if the column temperature is close to the boiling point,

$$\frac{\delta p^0}{p^0} = 10.2\frac{\delta T_b}{T_b} \tag{4}$$

whence we have the useful rule of thumb that a temperature rise of 1°K raises the vapour pressure by *ca.* 3% for a liquid boiling in the 310–380°K region, or 2.5% for a liquid boiling in the range 380–450°K.

The net retention volume is given by

$$V_N = \frac{R\ T_c W_L}{\gamma\ p^0 M_L}$$

where W_L and M_L are the mass and molecular weight of liquid stationary phase in the column[3], and γ is the activity coefficient of solute at infinite dilution in the liquid at temperature T_c. Differentiating, we obtain

$$\frac{\delta V_N}{V_N} = \frac{\delta T_c}{T_c} - \frac{\delta p^0}{p^0} - \frac{\delta \gamma}{\gamma} \tag{5}$$

In many cases the third term, $\delta\gamma/\gamma$, is the smallest of the three on the right-hand side and a mean value of minus half the first term is assumed, as explained below. If, in addition, $T_c \approx T_b$, substitution of eqn. 4 in eqn. 5 yields eqn. 1, which is thereby proved.

If these assumptions are not satisfied, we obtain, instead of eqn. 1,

$$\frac{\delta V_N}{V_N} = -\frac{\delta T_c}{T_c}\left[\frac{\tau}{R}\frac{\lambda_c T_b}{\lambda_b T_c} - 1 - \frac{T_c}{\gamma}\frac{d\gamma}{dT_c}\right]$$

$$= -\frac{\delta T_c}{T_c}\left[\frac{\tau}{R}\left\{1 + \frac{1}{\lambda_b}\frac{d\lambda}{dT}(T_c - T_b)\right\}\frac{T_b}{T_c} - 1 - \frac{T_c}{\gamma}\frac{d\gamma}{dT_c}\right] \tag{6}$$

The normal boiling point of a pure liquid is *ca.* two-thirds of its critical temperature[4]. Hence, from the universal normalised plot of latent heat of vaporisation against reduced temperature[5], we find

$$\frac{1}{\lambda_b}\cdot\frac{d\lambda}{dT}\bigg|_{T_b} = -\frac{1}{1.5\,T_b} \tag{7}$$

In eqn. 6, $d\lambda/dt$ is evaluated at a mean temperature between T_b and T_c. Accordingly we write

$$k = \left(\frac{d\lambda}{dT}\bigg|_{(T_b+T_c)/2} \bigg/ \frac{d\lambda}{dT}\bigg|_{T_b}\right) - 1 \tag{8}$$

noting that $k \ll 1$ since λ falls by only 0.15–0.3% for each 1°K rise in temperature around the normal boiling point. Combination of the last three equations and some manipulation then gives

$$\frac{\delta V_N}{V_N} = -\frac{\delta T_c}{T_c}\left[(1.67 + 0.67k)\frac{\tau\,T_b}{R\,T_c} - 0.67\frac{\tau}{R} - 0.67\frac{\tau k}{R} - 1 - \frac{T_c}{\gamma}\frac{d\gamma}{dT_c}\right] \tag{9}$$

This equation is simplified by assigning, as before, an average value of -0.5 to the final term within the square brackets and setting $k \approx 0$, giving

$$\frac{\delta V_N}{V_N} = -\frac{\delta T_c}{T_c}\left[\left(1.67\frac{\tau}{R}\right)\frac{T_b}{T_c} - \left(0.67\frac{\tau}{R} + 0.5\right)\right] \tag{10}$$

which yields eqn. 2 when $\tau = 85$ J mol^{-1} °K^{-1} and $R = 8.314$ J mol^{-1} °K^{-1}.

APPLICABILITY OF EQUATIONS

Because of the assumptions and approximations made in the derivation, the accuracy of eqns. 1 and 2 is affected by five considerations.

(1) Variation of activity coefficient with temperature

Inspection of published chromatographic data for a variety of solute–liquid phase systems shows that the last term within the square brackets in eqn. 9, (T_c/γ) $(d\gamma/dT_c)$, usually lies between 0 and -1, so contributing less than 10% to the square-bracketed summation in eqn. 9. Assigning the mean value -0.5 in deriving eqns. 1 and 2 means that the error in the equations from this source is usually less than 5%. However, charge transfer or other complexing interactions between solute and stationary phase (or occasionally pronounced steric effects) can give values of $(T_c/\gamma) \times (d\gamma/dT_c)$ as large as $+2$ or -5 or occasionally larger. Even larger variations in activity coefficient may be observed over narrow ranges of temperature in liquid crystalline transitions[6–8], in cases where a low-loaded liquid (usually a long straight-chain methylenic molecule) forms an oriented monolayer[9,10], or in other types of phase transition[11]. In these circumstances, both eqns. 1 and 2 break down.

In gas–solid chromatography, the analogue of the (T_c/γ) $(d\gamma/dT_c)$ term may be expected to be small for physically adsorbed solutes having heats of adsorption similar to their heats of liquefaction. However, in many cases the difference in the heats is too large[12] for this term to be neglected.

(2) Trouton's rule

Trouton's constant, τ, is 85 ± 6 J mol^{-1} °K^{-1} for most organic and inorganic liquids, solids and vapours, giving the constants shown in eqns. 1 and 2. The main exceptions are: liquids, such as water, alcohols and amines, which associate through hydrogen bonding in the liquid phase, giving high values of τ; carboxylic acids in which the hydrogen-bonding persists into the vapour phase, giving low values of τ; and some permanent gases and inorganic solids[13–15]. Examples of these solutes are given in Table I, together with corresponding revised values of the constants in eqns. 1 and 2.

TABLE I

REVISED CONSTANTS IN EQNS. 1 AND 2 FOR SOME SOLUTES WHICH DO NOT OBEY TROUTON'S RULE

Solute	τ *(J mol^{-1} °K^{-1})*	*Constant in eqn. 1*	*Constants in eqn. 2 from eqn. 10*	
		$\left(\frac{\tau}{R} - 0.5\right)$	$1.67\,\frac{\tau}{R}$	$0.67\,\frac{\tau}{R} + 0.5$
Most solutes (obeying Trouton's rule)	85 ± 6	9.7 ± 0.7	17.1 ± 1.2	7.4 ± 0.5
Water	109	12.6	21.9	9.3
Methanol	104	12.0	20.9	8.9
Ethanol	110	12.7	22.1	9.4
Methylamine	97	11.1	19.4	8.3
Dimethylamine	94	10.8	19.0	8.1
Formic acid	64	7.2	12.9	5.7
Acetic acid	62	7.0	12.5	5.5
Potassium chloride	100	11.5	20.1	8.6

(3) Relation between boiling and critical temperatures

In deriving eqns. 9 and 2 it is assumed that the ratio of the normal boiling point to the critical temperature is *ca.* two-thirds. Values of this ratio tabulated by Moelwyn-Hughes[4] range between extremes of 0.58, for certain small molecules containing less than five atoms, and 0.74 for long, unbranched hydrocarbons (*n*-decane). At these extremes, eqn. 2 is 5% in error, which is quite acceptable as a "correction to a correction".

(4) Closeness of column temperature to boiling point

When the boiling point of a solute is equal to column temperature, eqn. 2 reduces to eqn. 1. The effect of using a column temperature different from the boiling point may be examined by comparing eqns. 1, 2 and 9, which are successively more accurate in this order when $T_b \neq T_c$, and taking τ as 85 J mol^{-1} °K^{-1} and the final term in square brackets in eqn. 9 as -0.5. Values of k were obtained from eqn. 8 and the universal normalised plot of latent heats of evaporation against reduced temperature. The calculated errors due to difference between T_b and T_c are shown in Table II. The error in using eqn. 2 is *ca.* a quarter of that in eqn. 1. If a 10% error in δV_N is acceptable, eqn. 1 is useable up to values of $|T_b - T_c|$ of 12 and 25°K for solutes boiling at 230 and 470°K, respectively. With the same error and solutes eqn. 2 is useable up to *ca.* 45 and 100°K, respectively. The boiling points 230 and 470°K are quoted as representative of the more extreme values encountered in most gas–liquid chromatographic work, even though the technique is available for solutes boiling well outside this range.

TABLE II

ERROR IN $\delta V_N/V_N$ PREDICTED BY EQNS 1 AND 2 AS A FUNCTION OF DEVIATION OF T_b/T_c FROM UNITY

$\left\lvert \frac{T_c - T_b}{T_c} \right\rvert$	*Percentage error in*	
	Eqn. 1	*Eqn. 2*
0.05	9	2.5
0.1	*ca.* 20	5
0.2	>40	10

(5) Size of δT_c

The substitution of finite differences for differentials restricts the validity of eqns. 1 and 2 to small temperature corrections, δT_c. The error in both equations reaches *ca.* 2.7% when δT_c reaches 2°K and 5.5% at 4°K.

CONCLUSIONS

For making retention volume corrections to an accuracy of 10% in δV_N, eqn. 1 is useable at column temperatures within 12–25°K of the boiling point of the solute concerned. Eqn. 2, which is *ca.* four times as accurate, is useable up to temperatures differing by much more from the boiling point, *i.e.* by *ca.* 45–100°K for 10% accuracy. These ranges cover the great majority of column temperatures normally used: keeping the temperature in the general region of the boiling points of the solutes chromatographed not only ensures a satisfactory compromise between resolution and analysis time but also gives peaks whose symmetry is least sensitive to large samples. The simple form of these equations, particularly eqn. 1, which is solute-independent with few exceptions, makes them quick to apply and suitable for automatic data processing.

Since the equations are intended for obtaining small corrections, 10 % accuracy in the correction term itself is usually sufficient. Both equations are accurate to this level or better for most combinations of organic or inorganic solutes with a liquid stationary phase, with the few exceptions listed under headings 1 and 2 above. The equations will cope with temperature changes δT_c up to at least 0.5°K for 0.15 % accuracy in the corrected V_N, or 3°K for 1 %.

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GAS–SOLID CHROMATOGRAPHY OF PROPANE ON CROSS-LINKED POLYSTYRENE AT 25°C

JAN-CHAN HUANG*, BAO-GANG WU, DANIEL ROTHSTEIN and RICHARD MADEY*
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(First received April 23rd, 1982; revised manuscript received June 7th, 1982)

SUMMARY

Breakthrough curves of propane in a chromatographic column packed with cross-linked polystyrene were measured for different concentrations and flow-rates. The adsorption isotherm, as determined from a mass-balance equation, was found to be a Freundlich-type isotherm. A theoretical model for the asymptotic concentration profile is discussed in order to examine the longitudinal diffusion coefficient and the lumped mass-transfer coefficient. Slopes of breakthrough curves at the inflection points were applied to calculate these two parameters for different input concentrations. The imperfection of the model is disclosed in this result because the data deviate from the theoretical prediction at high flow-rates, and the longitudinal diffusion coefficient and the lumped mass-transfer coefficient depend on the input concentration which contradicts the assumption of the model.

INTRODUCTION

In a recent paper[1], we studied the asymptotic concentration profile (ACP) of ethane under an equilibrium assumption for a column packed with activated carbon. The ACP, sometimes called a self-sharpening boundary, occurs for a step-increase in the concentration for an adsorbate that exhibits a convex isotherm and also for a step-decrease in the concentration for an adsorbate with a concave isotherm[2]. Mathematically, the ACP occurs as a result of cancellation between the curvature of the isotherm and the effect of diffusion. Thus, when an observer moves with the average speed of the adsorbate, the observer will see a time-independent profile in his coordinate system. Because of the existence of this moving coordinate system, it is possible to describe the adsorbate concentration by an ordinary differential equation instead of a partial differential equation.

Since the asymptotic concentration profile depends on the balance between the curvature of the isotherm and the diffusion processes in the column, study of the profile provides information about diffusion processes. There are three diffusion pro-

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cesses that are important in gas–solid chromatography (GSC): longitudinal diffusion in the gas-phase, interfacial diffusion resistance, and solid-phase diffusion. Longitudinal diffusion in a packed column is a function of flow-rate; it depends also on the size and geometry of the adsorbent. For a spherical adsorbent, it can be correlated with the Reynolds number and the Schmidt number of the fluid phase[3]. Interfacial diffusion resistance was correlated[4] also as a function of the Reynolds number and the Schmidt number; however, in most GSC, the last effect can be neglected[5]. Solid-phase diffusion depends on pore structure and adsorbent molecular properties. A wide range of values has been reported[6] for the solid-phase diffusion coefficient. When the solid-phase diffusion rate is small, the speed of equilibrium is slow and the ACP is no longer valid.

Solid-phase diffusion incurs mathematical difficulties also, because the concentration depends on the radial coordinate of the adsorbent pellet. In an early study of chromatography theory, Glueckauf and Coates[7] proposed to describe the time-dependent concentration of the adsorbate in the solid phase by a lumped first-order ordinary differential equation. Their suggestion is followed in the literature. In this paper, we use a model that involves Glueckauf and Coates diffusion term and calculate the longitudinal diffusion coefficient and the rate coefficient of Glueckauf and Coates expression from data on the ACP.

THEORY

Consider a mixture of trace components and the carrier gas passing with a constant interstitial flow-rate u through a packed column with a void fraction ε. The relation between the gas-phase concentration C and solid-phase concentration q is given as

$$\frac{\partial C}{\partial t} = D_{\mathrm{L}} \frac{\partial^2 C}{\partial z^2} - u \frac{\partial C}{\partial z} - \frac{1 - \varepsilon}{\varepsilon} \frac{\partial q}{\partial t} \tag{1}$$

$$\frac{\partial q}{\partial t} = k(q^* - q) \equiv k[f(C) - q] \tag{2}$$

The initial and boundary conditions for an initially desorbed column are

$$C(z,0) = 0;\ q(z,0) = 0 \tag{3}$$

$$C(0,t) = C_0;\ q(0,t) = 0 \tag{4}$$

$$C(\infty,t) = 0;\ q(\infty,t) = 0 \tag{5}$$

The following dimensionless variables are defined

$$\xi \equiv \frac{z}{L} - \frac{\lambda u t}{L} \tag{6}$$

$$m \equiv \frac{1-\varepsilon}{\varepsilon} \tag{7}$$

$$\lambda \equiv \left[1 + m\frac{f(C_0)}{C_0}\right]^{-1} \tag{8}$$

$$Pe \equiv \frac{uL}{D_L} = \text{axial Peclet number} \tag{9}$$

$$St \equiv \frac{kL}{u} = \text{Stanton number} \tag{10}$$

When the ACP is reached, both the gas-phase and the solid-phase concentration are functions of ξ only. But the gas-phase concentration and the solid-phase concentration do not equilibrate for the same ξ; instead, they are governed by the following simultaneous equations

$$\frac{1}{Pe}\frac{d^2C}{d\xi^2} - (1-\lambda)\frac{dC}{d\xi} + \lambda m\frac{dq}{d\xi} = 0 \tag{11}$$

$$-\lambda\frac{dq}{d\xi} = St(q^* - q) \tag{12}$$

Eqns. 11 and 12 are transformations of eqns. 1 and 2 under the ACP assumption. By differentiating eqn. 12 with respect to ξ and combining with eqn. 11, the solid-phase concentration q can be eliminated to give

$$\frac{\lambda}{PeSt}\frac{d^3C}{d\xi^3} - \left\{\frac{1}{Pe} + \frac{\lambda(1-\lambda)}{St}\right\}\frac{d^2C}{d\xi^2} + (1-\lambda)\frac{dC}{d\xi} - \lambda m\frac{dq^*}{d\xi} = 0 \tag{13}$$

An integration of eqn. 13 from $-\infty$ to ξ gives

$$-\frac{\lambda}{PeSt}\frac{d^2C}{d\xi^2} + \left\{\frac{1}{Pe} + \frac{\lambda(1-\lambda)}{St}\right\}\frac{dC}{d\xi} = (1-\lambda)C - \lambda m q^* \tag{14}$$

Here we used the fact that C, q^*, and all derivatives are zero at $\xi = -\infty$. Eqn. 14 was derived by Rhee and Amundson[9] in their theoretical discussion of the asymptotic profile for a Langmuir system. They used a numerical method to calculate concentration profiles based on assumed parameters. Because the adsorption isotherm and the parameters Pe and St are to be measured in our study, an alternative method is applied in the discussion later.

EXPERIMENTAL

The flow system and experimental procedure were described earlier[5]. The chromatographic column is a stainless-steel cylinder, 10 × 1.08 cm I.D., which contains 3.23 g of (20–40 mesh) Chromosorb 106 (Johns-Manville, Denver, CO, U.S.A.). This adsorbent is a cross-linked polystyrene with true density 1.12 g/cm^3 and pore volume 0.98 cm^3/g. The chromatographic column was immersed in a water bath which controlled the temperature at 25°C to within ±0.02°C. The composition of the inlet gas was controlled by two valves which separately adjusted the flow-rates of the pure helium and the calibrated propane–helium mixture. The calibrated propane–helium mixture was prepared by Matheson Gas Products (East Rutherford, NJ, U.S.A.) with a nominal propane concentration of 10,000 ppm. The concentration of propane at the outlet of the chromatographic column was measured at regular time intervals (of 50 sec in most runs) by a Varian 3700 gas chromatograph with a column packed with Chromosorb 102. At a column temperature of 135°C, the elution time of propane is 30 sec. The area of each sample peak is integrated by a Spectra-Physics Minigrator. The resolution of successive peaks is good. The Minigrator did not produce any event marker which would indicate overlapping signals.

RESULTS AND DISCUSSION

Determination of the isotherm

The time-dependent concentration of propane at the column exit was measured for several concentrations and for several flow-rates at 25°C. The first series of runs was conducted to measure the adsorption isotherm. The equilibrium solid-phase concentration q_0, corresponding to the gas-phase concentration C_0, was calculated from the mass-balance equation[5]:

$$q_0(1 - \varepsilon)L + C_0\varepsilon L = uC_0\varepsilon \int^{\infty} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t \equiv uC_0\varepsilon t_\mathrm{p} \tag{15}$$

The propagation time t_p was calculated from transmission data by numerical integration. The propagation time represents the average retention time of the sample. Mathematically, it is the zeroth-order moment of the breakthrough curve relative to the time origin. Eqn. 15 gives a relation between the solid-phase concentration and the gas-phase concentration without any assumption on diffusion processes.

Fig. 1 shows the relation between the solid-phase concentration and the gas-phase concentration. These results follow the Freundlich isotherm very well; therefore, in this study we have

$$f(C) \equiv q^* = AC^n \tag{16}$$

A linear least-squares calculation based on the logarithms of the gas- and solid-phase concentrations gives $n = 0.783$ and $A = 5.60$ when both q^* and C are expressed as mole/cm^3. The fact that propane follows the Freundlich isotherm indicates that the surface-energy distribution of polystyrene is more homogeneous than that of carbon[10]. It has been pointed out by Tompkins[11] that the Freundlich isotherm can be derived from the Langmuir isotherm with an assumption of an exponential distribution of surface energy. According to the equation of Tompkins, the exponent n of eqn. 16 varies inversely with the absolute temperature. A detailed discussion on

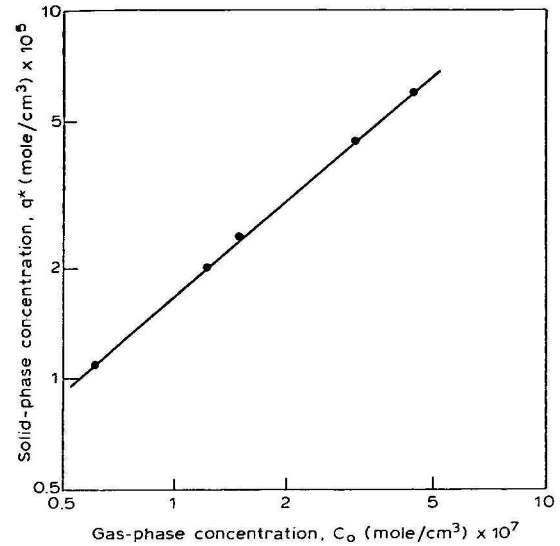

Fig. 1. Adsorption isotherm of propane on cross-linked polystyrene at 25°C.

physicochemical properties of adsorption on polystyrene will be the subject of a forthcoming article.

Determination of diffusion coefficients

A simple method is suggested here to evaluate D_L and k. Since the breakthrough curve is an S-type curve, an inflection point exists where the second derivative of the breakthrough curve *versus* time or coordinate is zero. From eqn. 16, we have

$$\left\{\frac{1}{Pe} + \frac{\lambda(1 - \lambda)}{St}\right\}\left(\frac{\mathrm{d}C}{\mathrm{d}\xi}\right)_i = (1 - \lambda)C_i - \lambda m q^* \tag{17}$$

or

$$\frac{D_L}{\lambda u^2} + \frac{(1 - \lambda)}{k} = -\left[(1 - \lambda)T_i - \lambda m \frac{f(C_i)}{C_0}\right]\left(\frac{\mathrm{d}t}{\mathrm{d}T}\right)_i \tag{18}$$

where

$$T = \frac{C}{C_0} \tag{19}$$

The subscript i in eqns. 17 and 18 denotes the condition at the inflection point. Eqn. 18 indicates that a plot of the right-hand-side *versus* $1/\lambda u^2$ will be a straight line. Both D_L and k can be calculated from the slope and the intercept of this line.

The second series of runs was made for three input concentrations. The input concentration, flow-rate, transmission at the inflection point, and slope of the

TABLE I

THE INPUT CONCENTRATION, THE TRANSMISSION AND THE SLOPE AT THE INFLECTION POINT AND THE INTERSTITIAL FLOW-RATE OF PROPANE

Run	*Input propane concentration* $C_0 \times 10^7$ *(mole/cm³)*	*Transmission at inflection point* T_i	*Slope at inflection point* $\left(\frac{dT}{dt}\right) \times 10^3$ *(sec⁻¹)*	*Interstitial flow-rate m(cm/sec)*
1	1.51	0.466	1.277	2.54
2	1.46	0.437	1.094	1.72
3	1.42	0.439	0.847	1.33
4	1.43	0.438	0.715	1.16
5	3.01	0.491	1.820	2.53
6	2.91	0.463	1.292	1.70
7	2.89	0.449	1.042	1.31
8	2.86	0.433	0.878	1.13
9	4.38	0.516	1.970	2.51
10	4.28	0.472	1.431	1.73
11	4.30	0.447	1.145	1.31
12	4.21	0.425	1.020	1.14

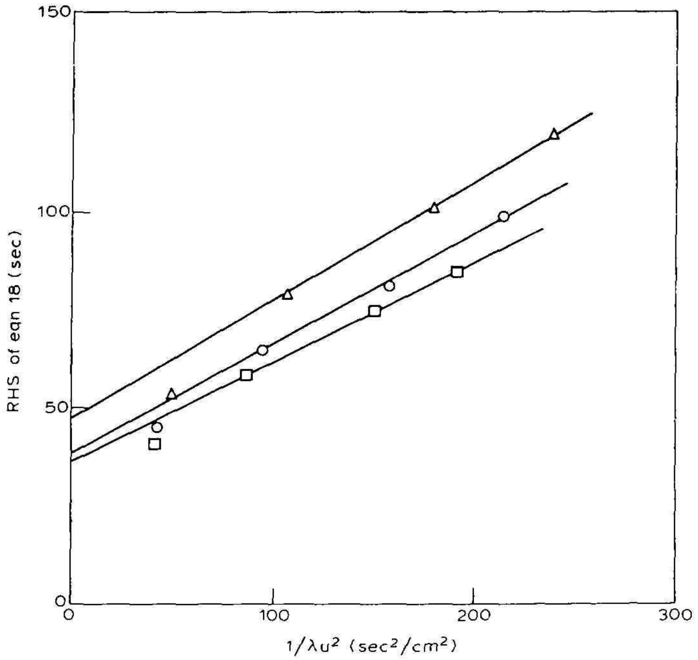

Fig. 2. Plot of the right-hand-side of eqn. 18 *versus* $1/\lambda u^2$ for various input concentrations. The symbols △, ○, and □ represent average input concentrations of $1.46 \cdot 10^{-7}$, $2.92 \cdot 10^{-7}$, and $4.37 \cdot 10^{-7}$ moles/cm³, respectively.

breakthrough curve at the inflection point are given in Table I. The inflection point is determined by interpolating the second-order finite difference of the transmission *versus* time. The right-hand-side of eqn. 18 is plotted in Fig. 2. It can be seen that there is a systematic deviation in the data at the highest flow-rate. This discrepancy can be attributed to the inadequacy of the model as discussed later. Three straight lines were obtained from low flow-rate data, indicating that both k and D_L are functions of the input concentration. The value of these parameters are listed in Table II.

TABLE II

THE LONGITUDINAL DIFFUSION COEFFICIENT, THE LUMPED MASS-TRANSFER COEFFICIENT, AND THE ESTIMATED SOLID-PHASE DIFFUSION COEFFICIENT CALCULATED AT DIFFERENT PROPANE INLET CONCENTRATIONS

Average input concentration $C_0 \times 10^7$ *(mole/cm³)*	*Longitudinal diffusion coefficient* D_L *(cm²/sec)*	*Lumped mass-transfer coefficient* $k \times 10^2$ *(sec⁻¹)*	*Estimated solid-phase coefficient* $D_s \times 10^5$ *(cm²/sec)*
1.46	0.300	2.12	4.3
2.92	0.278	2.60	5.3
4.37	0.255	2.73	5.5

Glueckauf and Coates[7] pointed out that the solid-phase diffusion coefficient can be related to the rate coefficient of eqn. 2 by the following relation

$$k = \eta \frac{D_s}{R^2} \tag{20}$$

where η is a constant on the order of unity, and R is the radius of the adsorbent granules. For polystyrene beads with a 20–40 mesh, R is *ca.* 0.45 mm. Values of D_s are estimated by assuming $\eta = 1$ in Table II. We can see that D_L is *ca.* 0.28 and D_s is on the order of $5 \cdot 10^{-4}$ cm²/sec. The value of D_s is in the middle of values reported for various porous solids.

Because of the concentration dependence of D_L and k, it is interesting to reexamine the approximation in eqn. 2. The coefficients D_L and k are generally assumed to be independent of concentration. This assumption is not only for convenience, but also is essential for the differentiation to be carried out in eqn. 13. If either D_L or k depends on concentration, they will depend also on ξ because the concentration is a function of ξ. Our result is not a surprise. It can be explained in terms of the following three reasons: (1) the solid-phase diffusion coefficient of propane is a function of concentration, which in turn makes k a function of concentration; (2) the ACP is not reached under the conditions of this study; or (3) the solid-phase diffusion coefficient is constant, but eqn. 2 is not a good approximation.

The argument 1 is not a strong reason because the diffusion process in a polystyrene bead, which is attributed to diffusion in the pore space, is constant at low partial pressure. For a system with linear isotherm, the solid-phase diffusion coefficient can be determined precisely through moment analysis[12]. Our study[13] using 1 % ethane in a helium carrier gas indicates that the solid-phase concentration is essentially constant up to 1 %. The argument 2 is not easy to verify in this study; however, an order-of-magnitude analysis can rule out this possibility. From eqn. 2, we note that $1/k$ is effectively a time constant that represents the time for solid particles to reach equilibrium. The dimensionless product $t_p k$ gives the ratio of the elution time to the time required to reach equilibrium. The value of t_p in this study ranges from 1200 to 2700 sec, which is large enough to reach equilibrium. It is likely that the concentration dependence of the parameters originate from the approximation in eqn. 2. Further work should be undertaken to look for an approximation method for the solid-phase concentration in systems with non-linear isotherms.

CONCLUSION

We attempted to measure the longitudinal diffusion coefficient and the rate coefficient of Glueckauf and Coates from the breakthrough curves of propane. Theoretical argument indicates that the measurement of the inflection point of the ACP at different flow-rates can give the two coefficients. The experimental results at different input concentrations show different rate coefficients and different longitudinal coefficients. We concluded that the lumped first-order expression is not adequate when the adsorption is non-linear and that a new approximation method is needed.

SYMBOLS

A	constant
C	gas-phase concentration
C_0	$C(0,t)$
D_L	longitudinal diffusion coefficient
D_s	solid-phase diffusion coefficient
$f(C)$	solid-phase concentration equilibrated with C
i	condition at the inflection point
k	lumped mass-transfer coefficient
L	length of adsorber bed
q	solid-phase concentration
q^*	solid-phase concentration equilibrated with C
R	radius of adsorbent granules
t	time
t_p	propagation time
u	flow-rate
z	distance from column entrance
ε	void fraction
ξ, m, λ, Pe, St, T	see eqns. 6, 7, 8, 9, 10, and 19, respectively
η	constant

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FREE RADICAL CROSS-LINKING IN THE PREPARATION OF NON-EXTRACTABLE STATIONARY PHASES FOR CAPILLARY GAS CHROMATOGRAPHY*

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SUMMARY

Free radical cross-linking of methylpolysiloxane stationary phases to form insoluble rubbers recently became of widespread interest in capillary column gas chromatography. The use of various peroxides as free radical generators to form carbon–carbon cross-links in conventional stationary phases has been reported. In this study the applicability of various peroxides and azo compounds for free radical cross-linking and the effects that these free radical generators have on commercially available stationary phases are described. Characteristics of the stationary phases such as chain length and functional groups and their roles in the cross-linking mechanism are discussed. The properties of benzoyl peroxide, dichlorobenzoyl peroxide, dicumyl peroxide, *tert.*-butylperoxide, and azo-*tert.*-butane as free radical generators were evaluated. Stationary-phase polarity change, column activity change and loss of stationary phase were all considered in the overall evaluation of the effectiveness of these free radical generators. Azo-*tert.*-butane showed minimal effect on phase polarity and column activity while benzoyl peroxide and dicumyl peroxide were the most effective in forming insoluble stationary phases.

INTRODUCTION

Although there has been some controversy in the past about the definition of a "bonded phase", the generally accepted characteristic of primary importance is the non-extractability or insolubility of the stationary phase. The advantages resulting from the production of efficient insoluble stationary phases have been described by Grob and Grob[1]. Some of the more important advantages include the ability to wash

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columns of non-volatile compound deposits, minimal phase stripping from injection solvents and enhanced film stability after cross-linking.

Recently, the approach of free radical cross-linking of polysiloxane stationary phases with peroxides to form insoluble rubbers has been reported by several workers[1–5]. This type of cross-linking results in carbon–carbon bonds between methyl groups attached to silicon atoms (Si–C–C–Si). The applicability of this method to several conventional stationary phases as well as some of the aspects of the free radical generator and its use have been examined. In addition to peroxides, certain azo compounds and radiation[6] can also be used as free radical generators. Very little cross-linking (0.1–1 %) is necessary to change long polymeric chains to insoluble rubbers. Consequently, only low levels of cross-linking agent are needed. Adsorption and other undesirable properties resulting from the decomposition of cross-linking agents are minimized since most of the decomposition products are volatile or can be removed from the column with a solvent wash.

The free radical cross-linking of silicone polymers to form insoluble rubbers is well documented and has been performed industrially for many years[7,8]. Unfortunately, this procedure has not been used for the preparation of immobilized stationary phases for capillary chromatography until recently[1]. Free radical cross-linking of stationary phases has been reported earlier, however. One of the earliest examples was reported by Sinclair *et al.*[9] some ten years ago in which the preparation of thermally stable silicone liquid phases by the oxidation of SE-52 on Gas Chrom Q was described. Evans *et al.*[10] have recently examined the deleterious effects of excessive peroxide cross-linking on the efficiency of squalene on Chromosorb G. The approach for capillary columns has previously been the bonding of the phase directly to the column surface, probably due to the influence of high-performance liquid chromatography bonding technology. Grob[11] first attempted this approach using organolithium compounds. Later, Madani *et al.*[12–15] and Blomberg *et al.*[16–20] developed methods in which the condensation of hydroxy and alkoxy groups to split out water, alcohols or ethers were used to form Si–O–Si cross-links in the preparation of non-extractable stationary phases. Problems associated with this approach included the observation of higher activity and lower column efficiencies than were obtained from columns coated with commercial stationary phases. These problems were caused by (a) residual silanol or alkoxy groups left in the phase after cross-linking that cannot be chemically deactivated or thermally removed because of steric problems, and (b) sample molecules being less soluble in the stationary phase as a result of the cross-linking levels (10–50 %, depending on prepolymer chain length) required by this approach. The thermal stabilities of these phases, however, were extremely good owing to the stability of the Si–O–Si bond.

This paper reports the results of an investigation of suitable stationary phases for free radical cross-linking, further studies on the applicability of various free radical producers to this process and the effect of different free radical producers on the final column performance. Various characteristics of the stationary phases such as chain length and functional groups and their roles in the cross-linking mechanism are discussed. The properties of several aroyl and alkyl peroxides and azo compounds as free radical generators are described. The influence of these compounds on column performance was carefully quantified utilizing retention indices to monitor polarity changes, peak asymmetry factors and peak area ratios to assess activity changes and capacity factor (k') measurements to determine the loss of stationary phase after cross-linking and solvent washing.

EXPERIMENTAL

The following free radical generators were obtained from commercial sources and used as supplied: *tert.*-butyl peroxide (TBP) (Pfaltz and Bauer, Stamford, CT, U.S.A.), azo-*tert.*-butane (ATB) and 2,2′-azobis-(2-methylpropionitrile) (AIBN) (Alfa Products, Danvers, MA, U.S.A.), and dicumyl peroxide (DCP) (Lucidol Pennwalt, Buffalo, NY, U.S.A.). The benzoyl peroxide (BP) (Fischer Scientific, Pittsburgh, PA, U.S.A. or Tridom/Fluka, Hauppauge, NY, U.S.A.) was dried prior to use by dissolving it in benzene followed by separation of the benzene from the water layer and removal of the benzene under reduced pressure. 2,4-Dichlorobenzoyl peroxide (DCBP) was synthesized from 2,4-dichlorobenzoyl chloride (Aldrich, Milwaukee, WI, U.S.A.) following published procedures[21]. Polysiloxane stationary phases were chromatographic grade and were obtained from Applied Science (State College, PA, U.S.A.) or Ohio Valley Specialty Chemicals (Marietta, OH, U.S.A.).

Preliminary testing of various stationary phases with BP, DCBP and AIBN was done by preparing 4% (v/v) solutions in methylene chloride of OV-101, OV-3, OV-17, OV-22, SP-2340, Silar-10C, SE-52, SE-54 and SE-30. These solutions were doped with the desired free radical producer by adding measured volumes of 1% (w/v) solutions of the desired peroxide or azo compound in methylene chloride. These polymer solutions were then put in 0.5-ml Pyrex test tubes and the methylene chloride evaporated under reduced pressure. In all cases the cross-linking was performed by heating the open test tubes in an oven at 150°C. The solubilities of the polymers were then tested by quickly washing with methylene chloride and hexane. If the polymers survived this initial wash they were allowed to sit for 2 h in methylene chloride as a more rigorous test of solubility.

Capillary columns were prepared from uncoated and untreated fused silica capillary tubing (0.20 mm or 0.31 mm I.D.; Hewlett-Packard, Avondale, PA, U.S.A.). Generally, 50-m column lengths were deactivated[22] and then broken into four equal lengths for subsequent coating and cross-linking. Deactivation consisted (in most cases) of first rinsing the column with 5–10 ml of methanol at room temperature. Afterwards, nitrogen was passed through the column for several hours to evaporate any traces of methanol. Next, octamethylcyclotetrasiloxane (D_4) (Ohio Valley Specialty Chemicals) was dynamically coated on the column by filling *ca.* 20% of the column and then rapidly pushing the D_4 plug through the column with nitrogen pressure. After the D_4 plug was expelled from the column, both ends were sealed. Finally, the column was heated for 2 h at 420°C to effect the deactivation. To protect the polyimide outer coating from oxidation during heating, the column was placed in a nitrogen-purged oven or wrapped in aluminum foil and the interior chamber which was formed was purged with nitrogen. After this heat treatment, the column ends were broken and the column was purged with nitrogen for about 30 min at 350°C to remove any residual D_4.

Depending on the type of free radical generator used, the coating and cross-linking procedures differed. For the free radical generators which are solids at room temperature (BP, DCBP, DCP), the peroxide was doped directly into the stationary phase solution and the column coated normally. However, for the free radical generators which are liquids at room temperature (TBP, ATB), it was necessary to coat the columns first and then saturate the stationary phase with the vapors of the free radical generator. This was done by bubbling nitrogen through the free radical generator and purging the coated columns at 40°C for 2 h.

All columns were statically coated. Fresh coating solutions were prepared daily using purified pentane. The concentration of the stationary phase was selected to give film thicknesses of 0.10 to 0.50 μm. Solutions (0.5–4.0%) of DCP, BP, and DCBP in methylene chloride were also prepared daily and used to spike the coating solutions 30 min prior to coating.

Cross-linking was done by both static and dynamic curing methods. For the static method, the coated columns already containing peroxide were purged with nitrogen and sealed. The columns purged with free radical generator were sealed immediately after disconnecting from the bubbler. The columns were then temperature-programmed from 40°C to the curing temperature at 4°C/min and held for a specified time. The temperature was raised slowly to prevent any stationary phase disruption from the decomposing peroxide. After cross-linking, the columns were washed for 30–60 min with 5–10 ml of methylene chloride. The columns were conditioned with a slow carrier gas flow for 1 h at 40°C to desorb any residual solvent from the stationary phase left from the washing procedure and then temperature programmed to 260°C at 0.5°C/min and held for 8 h. The columns that were coated prior to purging with peroxide or azo compound were conditioned and evaluated prior to and after cross-linking for comparison.

For dynamic curing, columns were attached to an argon manifold in an oven and heated at 5–10°C/min to the curing temperature. Typical conditions for curing were an argon linear velocity of 10 cm/sec and a hold at 250°C for 15–30 min. The columns were rinsed with 10–25 column volumes of methylene chloride–acetone (50:50, v/v) and then conditioned with rapid carrier flow for 30–60 min at ambient temperature. After being reconnected to the argon manifold, the columns were heated at 5°C/min to 350°C and held for 4 h with an argon linear velocity of 25 30 cm/sec.

Columns were evaluated using either a Carlo Erba 4160 gas chromatograph equipped with an on-column injector and a flame-ionization detector (FID) or a Hewlett-Packard 5880A gas chromatograph equipped with a split injector and a FID. Two test mixtures, one containing acidic components and the other containing basic components, were used. Hydrogen carrier gas was adjusted to a linear velocity of 30–45 cm/sec for each column. The oven was temperature-programmed from 40°C after a 2-min isothermal period at 4°C/min to *ca.* 160°C. The sensitivity was adjusted to give full-scale peak height for approximately 1 ng of dodecane. The FID signal from the Carlo Erba gas chromatograph was also connected to the external analog imput of a HP 5880A gas chromatograph and the data processed to give accurate retention times and cardinal point data for peak asymmetry calculations.

RESULTS AND DISCUSSION

Of the polysiloxane stationary phases tested in the 0.5-ml test tubes, only SE-30, SE-52 and SE-54 gave insoluble coatings with 2% or less of the free radical generators. Satisfactory insoluble films were formed with benzoyl peroxide loadings of 0.2%, 0.5% and 1.0% (w/w), respectively, in SE-30, SE-54 and SE-52. Loadings of 0.2% and 0.5% benzoyl peroxide in SE-54 and SE-52, respectively, washed out slightly with the methylene chloride rinses, but were almost entirely dissolved on sitting in methylene chloride for more than 1 h. SE-54 and SE-30 each required at least 2% AIBN to give insoluble polymers and OV-101 was still soluble with an 8% loading. Furthermore, the use of AIBN resulted in the discoloration of the polymer. These unsatisfactory results for AIBN discouraged any further testing with this azo compound.

Two other stationary phases were also rendered insoluble with peroxides. OV-101 required at least 4% (w/w) benzoyl peroxide and OV-3 required 10% (w/w) benzoyl peroxide to form insoluble rubbers. No other polysiloxane materials tested resulted in non-extractable materials. Higher than 10% (w/w) loadings of peroxides were not tried. The amount of 2,4-dichlorobenzoyl peroxide required was found to be roughly 1.5 times that of the benzoyl peroxide in all cases. This is reasonable since the molecular weight of the dichloroperoxide is about 1.5 times that of benzoyl peroxide.

The influence of the initial polymer chain length on the level of peroxide or azo compound needed is clearly shown in a comparison of the percentages required to yield sufficiently insoluble rubbers from SE-30 and OV-101. OV-101 has a polymer chain length that is *ca.* 0.04 times that of SE-30, and it required roughly 25 times as much peroxide to make it insoluble. SE-30 consists of extremely long chains and therefore requires very few cross-links to render it insoluble. Clearly, long-chain polymeric materials are to be preferred when low levels of peroxide additives are sought.

The functional groups present in the siloxane polymer also have a definite effect on the polymerization reactions and on the nature of the cross-linked product obtained. An understanding of the chemical processes leading to cross-links between polysiloxane chains helps to explain this. Cross-linking of polysiloxanes with free radicals occurs in mechanisms[23,24] similar to that shown in Fig. 1. In the case of polysiloxanes containing vinyl groups the principle cross-linking reaction is not methyl-to-methyl cross-linking as shown, but rather methyl-to-vinyl cross-linking[25,26]. The increased tendency of vinyl groups to cross-link over other functional groups is advantageous since lower levels of peroxides are necessary to achieve similar or higher levels of cross-linking[27]. This can be illustrated by a comparison between SE-52 and SE-54. Both polysiloxanes contain 5% phenyl, but the SE-54 also contains 1% vinyl. The SE-54 required 2.5 times less benzoyl peroxide than the SE-52 to achieve the same level of insolubility. Another example of the tendency for vinyl groups to cross-link is the spontaneous cross-linking of SE-54 when conditioned at 400°C for a

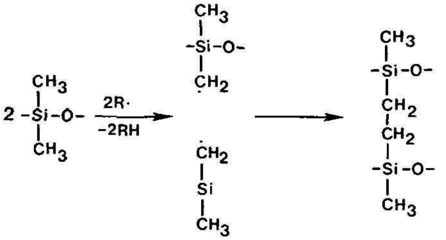

Fig. 1. Typical free radical cross-linking mechanism of polysiloxanes.

few hours. When an SE-54 column was conditioned in this way, only 50% of the phase was extractable, whereas an OV-1 column similarly treated was totally extractable.

In the absence of vinyl groups the cross-linking efficiency of the more reactive aroyl peroxides is low with only 10–30% of the peroxide molecules giving rise to ethylene bridges between siloxane chains[25,28]. Less reactive free radicals formed from alkyl peroxides and azo compounds tend to be vinyl specific, forming free radicals primarily from vinyl groups. The vinyl-specific free radicals will cross-link polysiloxanes containing no vinyl groups, but higher levels of the azo or peroxide free radical producer are required[28]. The higher reactivity of peroxides in the presence of vinyl groups can be attributed to more than one cross-link being formed per molecule of decomposing peroxide[28,29].

Results from experiments using vinyl-specific peroxides[3] showed that 36% of SE-52 (no vinyl) stationary phase was extractable after cross-linking with the vinyl-specific dicumyl peroxide, whereas only 14% of the stationary phase in a similarly cross-linked SE-54 (vinyl-containing) column was extractable. This same point was illustrated by the *tert.*-butyl peroxide cross-linking of a SE-52 column. By measuring k' before cross-linking, and again after cross-linking and solvent washing it was determined that 23% was extracted, compared to less than 5% when SE-54 was similarly cross-linked.

Phenyl groups in the polysiloxane framework markedly hinder free radical cross-linking[8]. This is exemplified by a comparison between the peroxide levels required for SE-30 and SE-52. Both of these polysiloxanes are similar in chain length, but SE-52 with 5% phenyl groups on the siloxane chain required between three and five times more peroxide than SE-30 to give an insoluble polymer. As the phenyl content increases, cross-linking becomes more difficult simply because the phenyl groups are non-reactive. OV-3 (10% phenyl) required 10% benzoyl peroxide to render it insoluble. Grob and Grob[1] have reported the upper phenyl limit for feasible cross-linking to be 33% (OV-61). This conclusion was based on experiments using commercially available phenyl siloxanes which are composed of relatively short-chain polymers. Recent work in this laboratory[30] has shown that methylphenylpolysiloxane gum phases containing greater polymer chain lengths and containing up to 70% phenyl groups can be easily cross-linked, although peroxide levels greater than required for methylpolysiloxanes are necessary.

Typical data illustrating the stability of variously prepared cross-linked columns as assessed by the change in k' before and after solvent washing are given in Table I. It appears that after a minimum threshold amount of peroxide was reached, additional peroxide did not significantly decrease the amount of phase that was extractable. Generally, additional solvent washes did not significantly remove any additional stationary phase.

The effects of vinyl substitution, phenyl substitution, chain length and peroxide specificity were investigated simultaneously with OV-1 and SE-54 cross-linked with BP or DCP. When SE-54 and OV-1 were cross-linked with BP, similar amounts were extracted. Cross-linking with DCP gave 5% extraction for SE-54 and 15% extraction for OV-1. Although the absolute amounts extracted were different, the ratio of amount extracted between OV-1 and SE-54 did not change as the BP was varied between 0.1 and 2.0% and the DCP between 0.5 and 3.0%. The vinyl groups in the

TABLE I

LOSS OF STATIONARY PHASE AFTER CROSS-LINKING AND SOLVENT RINSING

Stationary phase	*Column I.D. (µm)*	*Film-thickness (µm)*	*Crosslinking agent*	*Cure conditions**		*Loss of stationary phase (%)*
				Temperature (°C)	*Time (min)*	
OV-1	310	0.5	0.1% BP	Dynamic		8
OV-1	310	0.5	0.05% BP	Dynamic		22
SE-54	310	0.5	0.1% BP	Dynamic		6
SE-54	310	0.5	0.05% BP	Dynamic		8
SE-54	200	0.1	2% BP	Dynamic		4
SE-54	200	0.1	2% DCP	Dynamic		6
SE-54	310	0.25	0.25% BP	150	120	<1
SE-54	310	0.25	0.25% BP	135	15	6
SE-54	310	0.25	ATB	220	15	15
SE-54	310	0.25	TBP	165	120	5
SE-54	310	0.25	TBP	220	15	5
SE-54	310	0.25	0.28% DCP	175	15	<1
SE-54	310	0.25	0.38% DCBP	115	15	39
SE-54	310	0.25	0.38% DCBP	120	30	33
SE-54	310	0.25	0.38% DCBP	150	120	13

* Static cross-linking unless specified differently.

SE-54 easily compensated for the 5% phenyl content and an initial chain length of about one-half that of OV-1. The vinyl preference of DCP is again quite clear.

Various free radical generators react with and modify to different degrees the polysiloxane being cross-linked. This is largely accounted for by the reactivity of the free radicals and by the nature of the decomposition products which are formed. Ideally, low decomposition temperatures and non-polar and unreactive decomposition products are desired. It is possible that the decomposition products can be incorporated into the polysiloxane chain. Therefore, any polar products would tend to make the phase active and also alter its polarity. Higher decomposition temperatures enhance this possibility. High cross-linking temperatures can also be a serious problem for the more volatile free radical generators if dynamically cured (loss of generator by evaporation). However, this problem can be completely eliminated by static curing (ends sealed).

Data which indicate the reactivity of the free radical generators used in this study are contained in Table II. The decomposition temperature which is listed is the temperature at which 50% of the cross-linking agent decomposes in 15 min. The aroyl peroxides tend to be the most reactive, thus allowing cross-linking to be done at the lowest temperatures. However, they also form the most polar decomposition products. The major decomposition products of DCBP are *m*-dichlorobenzene and 2,4-dichlorobenzoic acid, and those of BP are benzene and benzoic acid. Although, a portion of these products can be removed by solvent washing or mild conditioning, residual amounts of these acids can cause degradation of the polysiloxane framework with exposure at elevated temperatures[27,31]. Such decomposition reactions produce silanol and silicon ester groups, both of which are capable of adsorptive interactions with acids, alcohols and amines. The major decomposition products of the alkyl

TABLE II

REACTIVITY DATA OF VARIOUS FREE RADICAL GENERATORS

Free radical generator	*Activation energy (kcal/mole)*	*Decomposition temperature* (°C)*	*Ref.*
Bis-2,4-dichlorobenzoyl peroxide	28.1	87	35
Dibenzoyl peroxide	29.9	109	36
Di-*tert.*-butyl peroxide	37.5	160	36
Dicumyl peroxide	38.0	142	36
2′,2′-Azobutane	43.0	187	37

* Temperature for $t_{1/2}$ = 15 min.

peroxides are less polar. The by-products of TBP are acetone and methane and those for DCP are acetophenone and 2-hydroxy-2-phenylpropane[25,27]. These products are not extremely polar or acidic and would not be expected to cause polysiloxane degradation under normal conditions. These peroxides decompose at higher temperatures than the aroyl peroxides, however. The major decomposition products of ATB are nitrogen, isobutane and isobutene. These products are completely non-polar and very unreactive with the polysiloxane chain. No acidic compounds are formed which could react to cause activity problems. ATB is, unfortunately, the least reactive of the free radical producers and requires high temperatures to obtain acceptable free radical generation rates.

Another disadvantage associated with the aroyl peroxides is their low solubility in the methylpolysiloxane stationary phases. In the preliminary test-tube tests in which the methylene chloride was evaporated, crystals of the doped peroxide could be detected at 0.5% levels in the polymer and were abundant in 1.0% and higher levels. After cross-linking, irregularities in the polymer coating on the insides of the test tubes could be seen where crystals previously existed. This was particularly evident for BP. Another indication of problems associated with the aroyl peroxides was the presence of a light brown discoloration of the polymer[27]. With BP this discoloration could be observed at all levels tested, but with DCBP this was only observed at levels greater than 8%. Part of this discoloration was removed with the methylene chloride washes, but even on prolonged exposure (overnight), the intensity was only reduced by about half of the original. This residue is likely a source of undesireable activity. The poor solubility of BP in the non-polar polysiloxanes probably leads to its reacting with itself and, thereby accounting for the discoloration problem.

In addition to the slight increases in acidity resulting from the decomposition products of the free radical generators, the surface of fused silica itself is slightly acidic. This has been experimentally deduced from the tailing peak shapes of the basic alkyl amines and the reduced peak height of the methylated aniline using the classic acid–base test mixture on columns prepared with undeactivated fused silica. Consequently, it was necessary to develop a deactivation procedure that gave an essentially inert surface before the effects of various free radical generators and curing conditions could be properly assessed. Part of the residual acidic surface activity of fused silica is due to deposits of nitrates and nitrites formed during the high-temperature drawing process from the pyrolysis of atmospheric oxygen and nitrogen[32]. Removal of these deposits with a mild water rinse followed by D_4 deactivation to block the silanol groups gave significant improvement to column inertness, but was still incomplete[33]. Rinsing with methanol instead of water, however, gave better results. Not only were the inorganic ions removed, but the surface was not hydroxylated to the same extent as with water rinsing, and the surface-adsorbed methanol probably catalyzed the D_4 reaction.

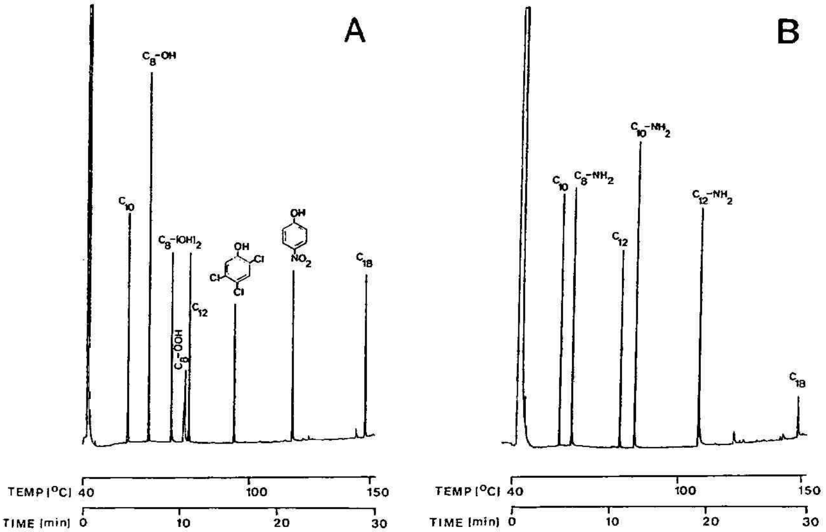

Fig. 2. Capillary gas chromatograms of (A) an acidic test mixture and (B) a basic test mixture obtained on a methanol-washed and D_4-deactivated fused-silica column (11 m × 0.31 mm I.D.) coated with an uncross-linked 0.25-μm film-thickness of SE-54. Chromatographic conditions: temperature programmed at 4°C/min from 40°C after a 2-min isothermal period after injection. Hydrogen carrier gas at 45 cm/sec linear velocity. Sensitivity set for full-scale response for 1 ng of dodecane.

Chromatograms of an acidic and a basic test mixture obtained on a methanol-rinsed and D_4-deactivated fused-silica column coated with SE-54 are shown in Fig. 2. The components in the acidic test mixture (chromatogram 2A) including a highly acidic free fatty acid and a very polar diol all have very symmetrical peaks, and the nitrophenol and C_8-diol have essentially the same peak heights as the dodecane reference, all three of which were in essentially the same stoichiometric concentration (see Tables IV and V for a quantitative description of the column inertness). Likewise, the components in the basic test mixture (chromatogram 2B) have very symmetric peaks shapes and the peak heights of the basic amines compared to the dodecane reference coincide with their stoichiometric concentrations. These results indicate that the finished column had a very inert surface with little residual acidic surface activity.

Several SE-54 columns were cross-linked with the various free radical generators and carefully evaluated to compare their effectiveness. Care was taken to ensure that all the column parameters (except for the different cross-linking procedures) and operating conditions were standardized. The concentrations of the free radical generators were chosen to be slightly above the minimum threshold values to maximize phase stability and to minimize the deleterious effects of the decomposition products. The concentrations of the solid peroxides were adjusted to give equivalent mole percentages (*e.g.* BP = 0.25; DCBP = 0.38; DCP = 0.28 %). The vapors of ATB and TBP were purged through the columns for a sufficiently long time to ensure maximum saturation of the SE-54 stationary phase. It appears that this method provided an evenly treated surface and a homogeneously cured insoluble rubber. Typical chromatograms obtained on columns cross-linked with the five different free radical generators of the same acidic and basic test mixtures as shown in Fig. 2 are shown in Figs. 3–7.

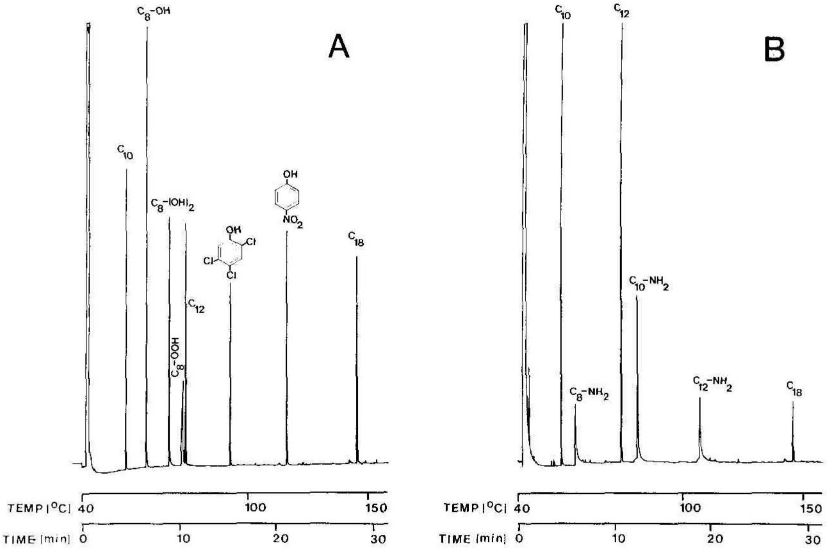

Fig. 3. Capillary gas chromatograms of (A) an acidic text mixture and (B) a basic test mixture obtained on a methanol-washed and D_4-deactivated fused-silica column (11 m × 0.31 mm I.D.) coated with a 0.25-μm film-thickness of SE-54, cross-linked with 0.25 % (w/w) BP, and cured at 135°C for 15 min followed by a methylene chloride wash. Chromatographic conditions as in Fig. 2.

The cross-linking efficiency of the five free radical generators and the effectiveness of different static-curing conditions were evaluated by measuring the loss of stationary phase after rinsing with methylene chloride (See Table I). For those columns treated with TBP and ATB, k' measurements for C_{10}, C_{11} and C_{12} at 60°C were made before and after cross-linking and solvent washing. For columns which were cross-linked and washed prior to evaluation, predicted k' values obtained from the average of several identical uncross-linked and non-extracted columns were used to obtain these data. It is clear that some free radical generators are more efficient than others, and that the proper curing conditions are necessary. From the decomposition properties of the free radical generator, reasonable curing conditions were calculated. To prevent any thermal rearrangements and possible adverse activity increases, the most mild curing conditions possible were preferred. However, under-

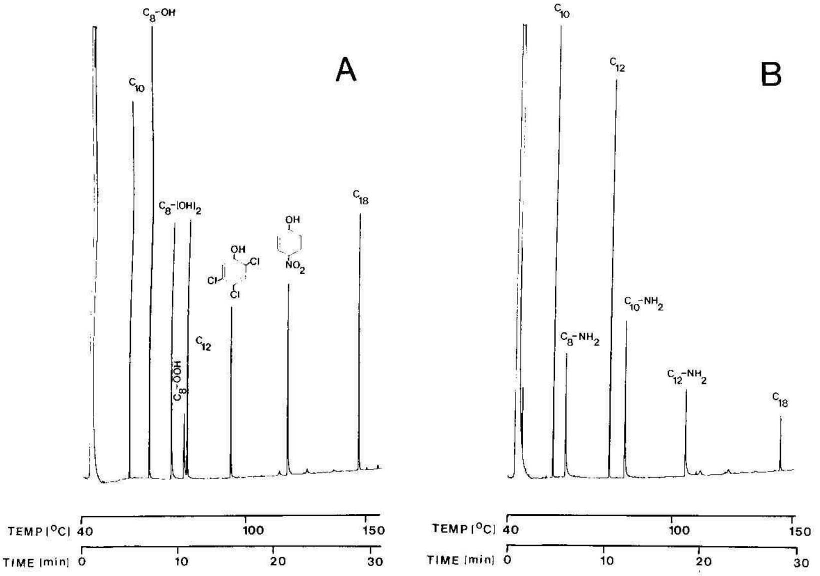

Fig. 4. Capillary gas chromatograms of (A) an acidic test mixture and (B) a basic test mixture obtained on a methanol-washed and D_4-deactivated fused-silica column (11 m × 0.31 mm I.D.) coated with a 0.25-μm film-thickness of SE-54, cross-linked with 0.38 % (w/w) DCBP, and cured at 150°C for 2 h followed by a methylene chloride wash. Chromatographic conditions as in Fig. 2.

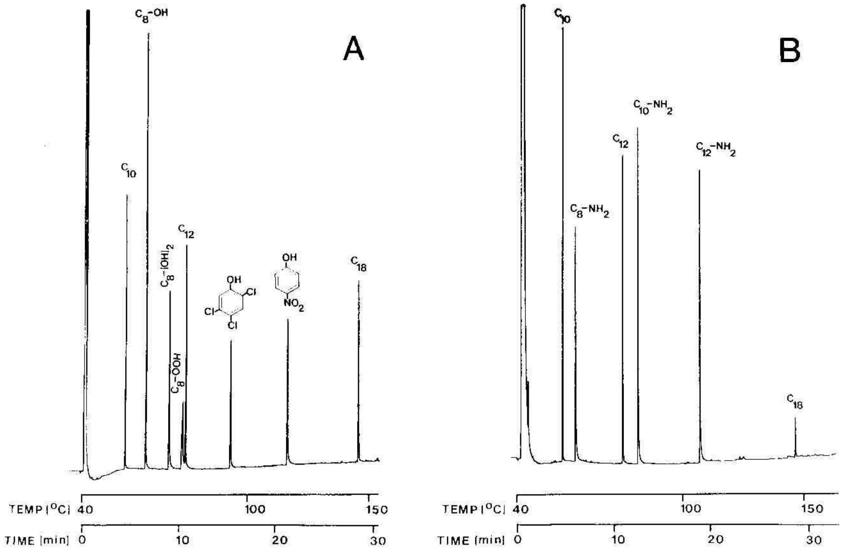

Fig. 5. Capillary gas chromatograms of (A) an acidic test mixture and (B) a basic test mixture obtained on a methanol-washed and D_4-deactivated fused-silica column (11 m × 0.31 mm I.D.) coated with a 0.25-μm film-thickness of SE-54, cross-linked with 0.28 % (w/w) DCP, and cured at 175°C for 15 min followed by a methylene chloride wash. Chromatographic conditions as in Fig. 2.

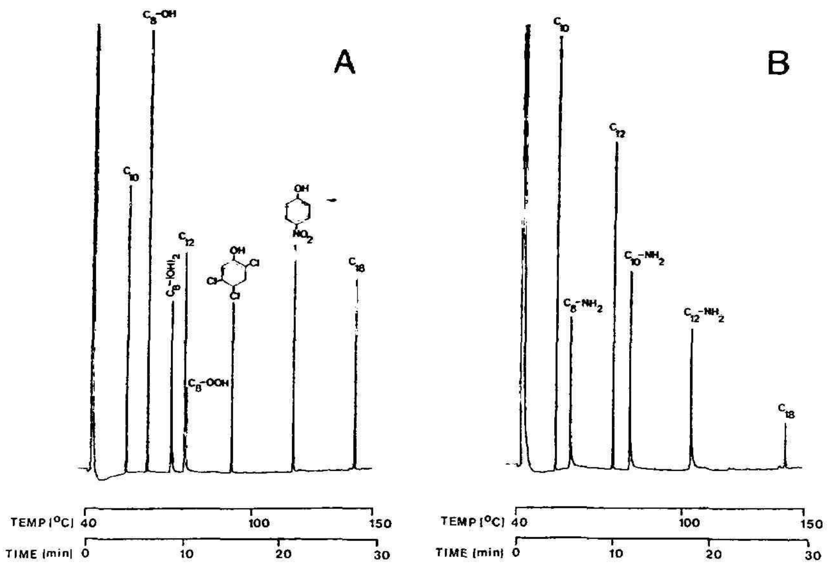

Fig. 6. Capillary gas chromatograms of (A) an acidic test mixture and (B) a basic test mixture obtained on a methanol-washed and D_4-deactivated fused-silica column (11 m × 0.31 mm I.D.) coated with a 0.25-μm film-thickness of SE-54 cross-linked with TBP, and cured at 220°C for 15 min followed by a methylene chloride wash. Chromatographic conditions as in Fig. 2.

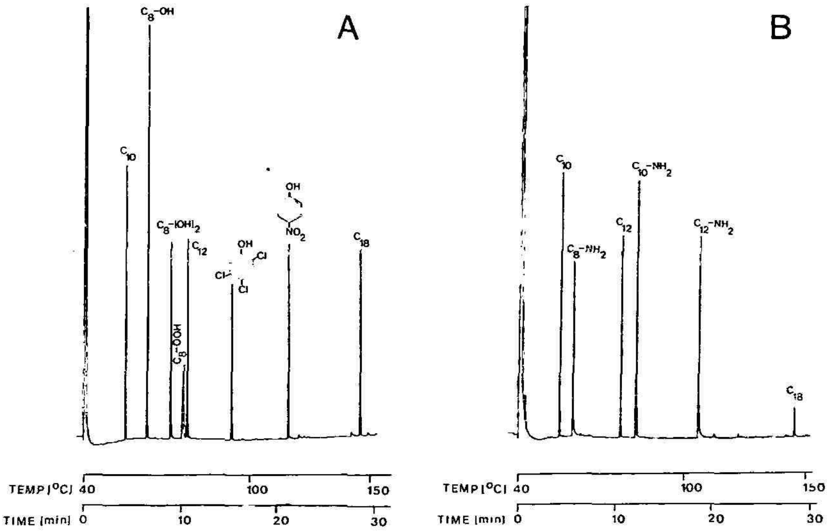

Fig. 7. Capillary gas chromatograms of (A) an acidic test mixture and (B) a basic test mixture obtained on a methanol-washed and D_4-deactivated fused-silica column (11 m × 0.31 mm I.D.) coated with a 0.25-μm film-thickness of SE-54, cross-linked with ATB, and cured at 220°C for 15 min followed by a methylene chloride wash. Chromatographic conditions as in Fig. 2.

curing led to the loss of significant stationary phase which is also undesireable (*e.g.* DCBP, 115°C, 15 min).

To assess any changes in polarity of the cross-linked SE-54 induced by the incorporation of decomposition products into the phase, careful retention measurements were made on each cross-linked column and compared to retention measurements on uncross-linked SE-54. A retention system was defined in which all of the acidic or basic test components were bracketed between decane and octadecane. The relative position of the test compounds between these standards could then be determined. Consequently, it was possible to calculate retention changes between cross-linked and uncross-linked SE-54, and thus, detect subtle changes in phase polarity. These retention change data are tabulated in Table III. Several identical columns and several chromatographic runs on the same column were used to obtain these data. A positive shift in retention indicates that the components were being eluted earlier which suggests that the phase was becoming less polar. On the other hand, a negative retention shift indicates that the components were being retained longer which suggests that the phase was becoming more polar. This is exemplified by a comparison of ATB and BP. The decomposition products of ATB are non-polar, and if incorporated into the phase, would be expected to make it less polar. The decomposition products of BP are polar and should increase the polarity of the SE-54. These examples are verified by the data in Table III. The greatest polarity changes were surprisingly observed when TBP was used as the cross-linking agent. The alkoxy-type decomposition products were evidently incorporated into the SE-54 phase to a significant extent. The free fatty acid actually elutes after the dodecane (see Fig. 6A) rather than before it as usual. The more harsh curing conditions for TBP (220°C, 30 min *vs.* 165°C, 120 min) also tended to increase the polarity of the cross-linked phase.

Probably the most serious problem encountered in free radical cross-linking has been the increased column activity from the free-radical-generator decomposition products. The inertness of the finished columns was evaluated in two ways. Both reversible and irreversible adsorption mechanisms were monitored. Reversible adsorption characterized by peak tailing was quantified with peak asymmetry factors[34]. Using this system, a perfectly symmetrical peak has an asymmetry factor of 1.00. Peaks tailing on the trailing edge (adsorption) have asymmetry factors greater than 1.00, and peaks with the leading edge tailing (overloaded) have asymmetry factors less than 1.00. Peak asymmetry factors for the acidic and basic test components calculated from the chromatographic runs in which the chromatograms shown in Figs. 2–7 were obtained are given in Table IV. Except for the alkyl amines, the peaks exhibited excellent symmetry. The BP caused the greatest increase in peak asymmetry while the DCBP, TBP, DCP and ATB had minimal influences. In most cases, the shapes of the alcohol, diol and acidic phenol peaks were nearly as sharp as the alkane peaks.

In addition to reversible adsorption, irreversible adsorption is an important factor in column inertness. Compounds undergoing this type of adsorption are characterized by symmetric peak shapes, but reduced chromatographic peak areas or heights. This phenomenon was evaluated by comparing the ratio of the peak areas of the test compounds to the area of the dodecane peak contained in the test mixture. The matching peak area ratio data for the chromatographic runs listed in Table IV and shown in Figs. 2–7 are given in Table V. Once again, the most significant effects

TABLE III

RETENTION CHANGES* FOR VARIOUSLY CROSS-LINKED SE-54 COLUMNS

Test compound	*Retention number***	*Cross-linking procedures*						
		BP (0.25%) 135°C, 15 min	*BP (0.25%) 150°C, 120 min*	*DCBP (0.38%) 150°C, 120 min*	*DCP (0.28%) 175°C, 15 min*	*TBP 165°C, 120 min*	*TBP 220°C, 20 min*	*ATB 220°C, 15 min*
C_8-OH	11.33 ± 0.11	−0.08 ± 0.06	−0.17 ± 0.08	−0.24 ± 0.14	−0.17 ± 0.09	−0.04 ± 0.12	−0.49 ± 0.17	+0.21 ± 0.04
C_8-diol	5.37 ± 0.02	−0.05 ± 0.02	−0.08 ± 0.01	−0.08 ± 0.03	−0.07 ± 0.03	−0.23 ± 0.03	−0.26 ± 0.04	+0.06 ± 0.02
C_8-FFA	4.09 ± 0.00	−0.03 ± 0.01	−0.05 ± 0.01	−0.02 ± 0.03	−0.10 ± 0.03	−0.26 ± 0.04	−0.30 ± 0.03	+0.04 ± 0.04
C_{12}	3.86 ± 0.03	+0.01 ± 0.02	−0.01 ± 0.02	+0.06 ± 0.04	−0.01 ± 0.02	+0.07 ± 0.03	+0.10 ± 0.02	+0.06 ± 0.01
Trichloro-phenol	2.23 ± 0.01	0.00	0.00	+0.02 ± 0.01	−0.01 ± 0.01	−0.07 ± 0.01	−0.07 ± 0.01	+0.02 ± 0.01
Nitro-phenol	1.45 ± 0.00	−0.01 ± 0.00	−0.01 ± 0.00	0.00	−0.01 ± 0.00	−0.08 ± 0.00	−0.09 ± 0.00	0.00
C_8-NH_2	19.65 ± 0.79	−1.60 ± 0.76	−5.49 ± 1.04	−0.90 ± 0.59	−1.65 ± 0.43	−3.63 ± 0.16	−2.69 ± 0.37	−0.38 ± 0.20
C_{12}	3.87 ± 0.04	+0.01 ± 0.02	−0.01 ± 0.02	+0.08 ± 0.05	−0.02 ± 0.03	+0.08 ± 0.02	+0.12 ± 0.02	+0.06 ± 0.01
C_{10}-NH_2	3.11 ± 0.03	−0.02 ± 0.03	−0.11 ± 0.03	−0.03 ± 0.03	−0.03 ± 0.02	−0.04 ± 0.02	−0.02 ± 0.01	−0.01 ± 0.01
C_{12}-NH_2	1.70 ± 0.01	−0.01 ± 0.01	−0.02 ± 0.01	0.00	−0.01 ± 0.01	−0.01 ± 0.00	0.00	0.00

* "+" Change indicates earlier elution and "−" change indicates retarded elution.

** Retention number = $\frac{R_{C_{18}} - R_{C_{10}}}{R_{\text{Test compound}} - R_{C_{10}}}$, where R is the retention time.

TABLE IV

PEAK ASYMMETRY FACTORS* (A_s) FOR VARIOUSLY CROSS-LINKED SE-54 COLUMNS

Test compound	*Cross-linking procedures*						
	Uncross-linked	*BP (0.25%)* *135°C, 15 min*	*DCBP (0.38%)* *150°C, 120 min*	*DCP (0.28%)* *175°C, 15 min*	*TBP* *165°C, 120 min*	*TBP* *220°C, 20 min*	*ATB* *220°C, 15 min*
C_{10}	1.19	1.20	1.03	1.08	1.13	1.16	1.08
C_8-OH	1.25	1.74	1.32	1.42	1.09	1.14	1.17
C_8-diol	1.36	1.95	1.73	1.51	2.20	1.17	1.48
C_8-FFA	0.78	1.26	0.83	1.30	1.22	0.80	0.76
C_{12}	1.06	1.10	1.15	1.05	0.86	1.00	1.07
Trichlorophenol	1.14	1.11	1.41	1.61	1.25	1.29	1.12
Nitrophenol	1.25	0.95	1.28	1.94	1.46	1.50	1.33
C_{18}	1.08	1.20	1.18	1.20	1.21	1.02	1.16
C_{10}	1.10	1.03	1.08	1.05	1.05	1.08	0.91
C_8-NH_2	2.36	7.56	2.00	3.97	3.67	1.79	3.43
C_{12}	1.14	1.08	0.93	1.08	1.07	0.93	1.13
C_{10}-NH_2	1.31	5.23	1.81	2.53	1.53	1.54	1.83
C_{12}-NH_2	1.52	6.73	1.67	1.69	2.24	1.40	1.47
C_{18}	0.88	1.16	0.97	1.05	1.07	0.76	1.08

* $A_s = \frac{a + b}{(a + b) - (b - a)}$, where a is the front half and b the back half of the peak, measured from the perpendicular drawn through the peak maximum[34].

TABLE V

PEAK AREA RATIOS* FOR VARIOUSLY CROSS-LINKED SE-54 COLUMNS

Test compound	*Cross-linking procedures*						
	Uncross-linked	*BP (0.25%)* *135°C, 15 min*	*DCBP (0.38%)* *150°C, 120 min*	*DCP (0.28%)* *175°C, 15 min*	*TBP* *165°C, 120 min*	*TBP* *220°C, 20 min*	*ATB* *220°C, 15 min*
C_{10}	0.98	0.94	0.96	0.86	0.89	0.95	0.94
C_8-OH	1.74	1.72	1.74	1.57	1.65	1.71	1.75
C_8-diol	0.96	1.00	0.88	0.84	0.94	0.84	0.94
C_8-FFA	0.40	0.56	0.39	0.58	0.55	0.39	0.69
C_{12}	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Trichlorophenol	0.73	0.75	0.71	0.63	0.71	0.65	0.76
Nitrophenol	0.96	1.04	0.80	0.82	0.94	0.65	0.98
C_{18}	0.97	0.95	0.93	0.89	0.91	0.97	0.94
C_{10}	0.97	0.97	0.98	0.99	0.96	0.99	0.99
C_8-NH_2	1.07	0.20	0.30	0.78	0.51	0.77	0.87
C_{12}	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C_{10}-NH_2	1.54	0.36	0.39	1.15	0.65	0.97	1.30
C_{12}-NH_2	1.39	0.28	0.26	1.09	0.49	0.47	1.05
C_{18}	0.26	0.15	0.16	0.14	0.42	0.11	0.22

* Peak areas normalized to the dodecane peak area in each of the test mixtures.

were detected with the alkyl amines. For the BP column, 80% of the octylamine was completely and irreversibly adsorbed. Similarly, although the peak symmetry for octylamine on the DCBP column was excellent (2.00), 70% of the compound was totally adsorbed. Consequently, it is necessary to consider both factors when evaluating column inertness. Although the effects of each free radical generator were slightly different for the individual test components, it appears that ATB had overall fewer deleterious effects on the activity and polarity of cross-linked SE-54 stationary phase.

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COMPORTEMENT CHROMATOGRAPHIQUE DE PHASES STATIONNAIRES

II. APPLICATION DE L'ANALYSE FACTORIELLE

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SUMMARY

Chromatographic behaviour of stationary phases. II. Application of factor analysis

A "principal component analysis" applied to a set of retention times of 17 benzenic monosubstituted compounds on a collection of 21 stationary phases, leads to the following relationship:

$$x_{\mathrm{P},\varphi_i} = a_\varphi \cdot x_{\mathrm{P},\varphi_1} + b_\varphi \cdot x_{\mathrm{P},\varphi_2} + c_\varphi \cdot x_{\mathrm{P},\varphi_3} + d_\varphi$$

Knowing the experimental data x_{P,φ_1}, x_{P,φ_2} and x_{P,φ_3} of any substance P, measured on three phases φ_1, φ_2 and φ_3 (respectively non-polar, polyfluorinated and polar) one can estimate the gas–liquid chromatographic retention data x_{P,φ_i} of the same substance P on any stationary phase φ_i with the aid of the above relationship. (a_φ, b_φ, c_φ and d_φ are the coefficients of the non-restricted multiparametric relationship.)

INTRODUCTION

La prévision des grandeurs de rétention en chromatographie gaz–liquide a été abordée sous des aspects divers.

Une première série de travaux est basée sur la recherche de relations empiriques entre les grandeurs de rétention et des variables explicatives[1], telles que le point d'ébullition; la masse moléculaire, le nombre d'atomes de carbone d'une molécule ou encore des constantes physicochimiques issues de traitements particuliers, tels que la chaleur de vaporisation[1], les coefficients de partage[2] ou encore les coefficients d'activi-

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té[3]. On est alors rapidement confronté avec l'interprétation de ces relations et leur limite d'application pour l'analyse des interactions soluté–solvant.

En retenant comme hypothèse fondamentale que chaque élément de structure contribue pour sa part à la rétention et que cette contribution n'est pas affectée par l'environnement, il devient possible, à partir de ces relations empiriques, d'établir des règles d'additivité[4–14]. On assiste ainsi à la naissance de relations de "Topologie-Information"[15] telles qu'on peut les dégager par exemple à l'aide du logiciel DARC[16,17]. Ces relations permettent de déterminer la contribution à la rétention de chacun des sites d'une molécule et de chiffrer ainsi la participation de chacun de ses éléments structuraux à cette rétention.

Une deuxième approche fait appel aux méthodes de "l'analyse des données" qui permettent entre autres une étude prévisionnelle des grandeurs de rétention, ainsi qu'une analyse globale des interactions soluté–solvant, c'est à dire une analyse qui ne préjuge pas de la nature des solutés et des solvants étudiés.

Parmi les applications de l'analyse des données à la chromatographie gaz–liquide, signalons en particulier "l'analyse en composantes principales" ou "A.C.P." utilisée surtout par Weiner et Howery[18–21].

Cette méthode a permis à Malinowski *et al.*[22] de prévoir les valeurs des coefficients d'activité, à certains auteurs d'identifier les "facteurs principaux" qui influencent d'une manière prépondérante le phénomène complexe de la rétention[20,23–27] et enfin à d'autres d'établir des classements des solutés[28] et/ou des solvants[28–31]. Un tel classement des solvants a été abordé d'autre part à l'aide des méthodes taxonomiques d'agrégation[32,33] et de la théorie de l'information[33,34]. Pratiquement, toutes ces méthodes ont été testées à partir des lots de mesures réalisées soit par Rohrschneider[35], soit par McReynolds[36].

Dans le cadre de nos propres études chromatographiques[37] nous avons retenu un autre lot de substances; celles-ci possèdent toutes un même squelette fixe, le noyau benzénique, et des fonctions chimiques diverses. Les données relatives à ces substances nous ont déjà permis, à l'aide d'une étude taxonomique et sur la base du critère du coefficient de corrélation, de regrouper les phases stationnaires en 3 classes et de voir apparaître, à côté des phases dites "polaires" et "apolaires", la famille des phases "fluorées", indépendante des précédentes de par son comportement.

Le but de ce travail est d'étudier les différentes variantes d'A.C.P. et d'envisager un modèle multiparamétrique de prévision de comportement de phases stationnaires pour l'évaluation des grandeurs de rétention.

PARTIE EXPÉRIMENTALE

Les logarithmes de temps réduits qui nous permettent de réaliser cette étude sont issus de mesures que nous avons effectuées, dans des conditions isothermes et pour un ensemble de phases stationnaires, sur une série de dérivés mono- et polysubstitués du benzène et pour lesquelles nous avons déjà défini le détail des conditions expérimentales[37]. Le Tableau I regroupe les logarithmes de temps réduit (log t') du benzène sur les différentes phases et permet de déterminer les données de rétention réduites des différents composés, à partir des données publiées antérieurement[37].

TABLEAU I

LOGARITHMES DE TEMPS RÉDUIT DU BENZÈNE

Phase	*log t′ (benzène)*	*Phase*	*log t′ (benzène)*
Apiezon L	0.9906	Carbowax 20M	0.0523
SE-30	0.6180	XF-1150	0.7380
DC-200	0.8170	FFAP	0.9857
PMPE 5R	0.6218	PDEAS	0.6876
Apiezon H	0.9476	DEGA	0.9360
OV-17	0.8100	DEGS	0.4771
UCON 50 HB 2000	0.9868	EGS	0.5211
ULB 550X	0.7482	DEGSeb	1.1009
OV-210	0.4298	QF-1	0.7251
OV-225	0.6907	DCL 5X	−0.2565
NPGS	0.6207		

RÉSULTATS

L'application de l'A.C.P. par diagonalisation de la matrice de covariance, aux logarithmes de temps réduits, montre que trois variables factorielles suffisent à cerner les tendances du phénomène examiné et rendent compte de 99.8 % de la variance totale (la variance liée à la première composante principale représente 96.3 % de la variance totale, celle de la seconde 2.8 % et celle de la troisième 0.7 %). Dans ce type d'A.C.P., les variables initiales qui ont une forte variance ont une importance accrue.

Dans les plans factoriels b_1,b_2 et b_2,b_3 nous avons représenté les projections des vecteurs unitaires portés par les axes des variables initiales (Figs. 1 et 2). Dans chacun des deux plans factoriels les vecteurs de directions voisines correspondent à des phases stationnaires fortement corrélées entre-elles.

En examinant la Fig. 1 on observe un regroupement des phases en deux faisceaux très proches et situés de part et d'autre de l'axe b_1. Ce regroupement et la proximité au cercle de corrélation de l'ensemble des vecteurs "phases" dénote à la fois l'absence d'un pouvoir discriminant et la forte "représentativité", c'est à dire le poids très élevé du plan factoriel b_1,b_2. Par contre, le plan factoriel b_2,b_3 (Fig. 2) met en évidence la distribution des phases en trois classes: les phases polaires (groupe C), apolaires (groupe A) et fluorées (groupe B). Nous avons déjà abouti à une distribution semblable à l'aide de la classification ascendante hiérarchique[37].

Pour les trois premiers facteurs principaux b_1, b_2 et b_3 les valeurs des coefficients de corrélation f_1,f_2,f_3, issus de la régression entre les variables factorielles et les variables initiales, ainsi que les variances liées à chacun de ces axes, sont rassemblées dans le Tableau II.

Rappelons que les variables factorielles ne peuvent être rattachées à l'avance à tel ou tel facteur physicochimique. L'étude du Tableau II fait ressortir les faits suivants:

(1) Les variables "phases" sont toutes étroitement corrélées avec le facteur b_1, les deux faisceaux de vecteurs qui en résultent sont fortement pincés et proches de l'axe b_1. D'autre part, des valeurs voisines des coefficients de corrélation f_1 expriment le fait que le canevas des grandeurs de rétention d'une population de produits divers

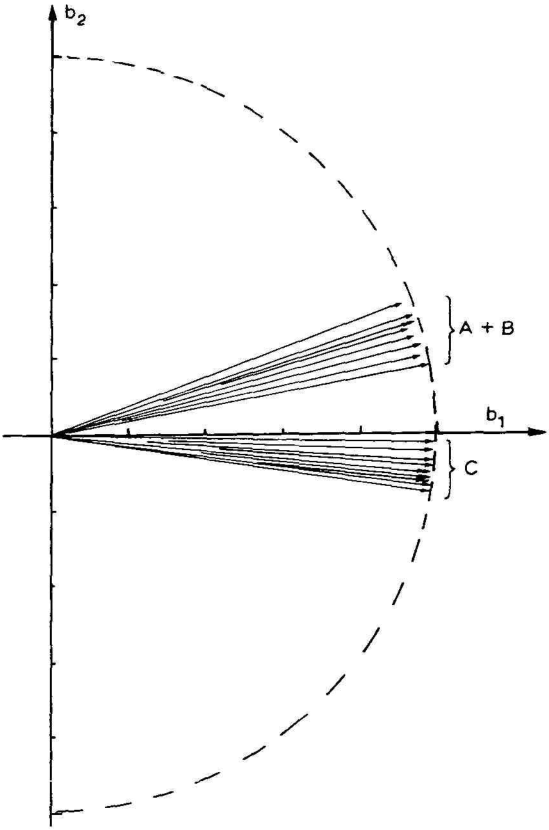

Fig. 1. Projection des vecteurs unitaires portés par les axes des variables dans le plan factoriel b_1,b_2.

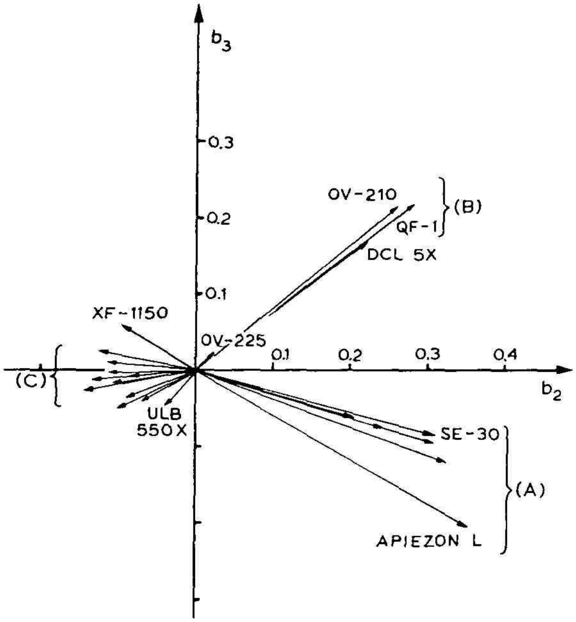

Fig. 2. Projection des vecteurs unitaires portés par les axes des variables dans le plan factoriel b_2,b_3.

TABLEAU II

COEFFICIENTS DE CORRÉLATION DES TROIS PREMIERS FACTEURS PRINCIPAUX b_1, b_2, b_3 AVEC LES VARIABLES INITIALES

φ_i	f_1	f_2	f_3
Apiezon L	0.9119	0.3521	−0.2060
SE-30	0.9471	0.3014	−0.0823
DC-200	0.9427	0.3064	−0.0971
PMPE 5R	0.9771	0.1980	−0.0581
Apiezon H	0.9397	0.3185	−0.1221
OV-17	0.9672	0.2370	−0.0762
UCON 50 HB 2000	0.9922	−0.1037	−0.0535
ULB 550X	0.9959	−0.0369	−0.0454
OV-225	0.9988	0.0177	0.0280
NPGS	0.9960	−0.0779	0.0064
Carbowax 20M	0.9897	−0.1325	−0.0124
XF-1150	0.9931	−0.0907	0.0661
FFAP	0.9876	−0.1472	−0.0248
PDEAS	0.9932	−0.1072	−0.0166
DEGA	0.9938	−0.1097	−0.0054
DEGS	0.9913	−0.1175	0.0090
EGS	0.9907	−0.1224	0.0284
DEGSeb	0.9961	−0.0694	−0.0358
OV-210	0.9418	0.2603	0.2075
DCL 5X	0.9596	0.2141	0.1654
QF-1	0.9322	0.2834	0.2182
Variance liée à chacun des axes (%)	96.3	2.8	0.7

est le même pour l'ensemble des variables "phases", indépendamment des plages des valeurs des grandeurs numériques.

En clair, si sur une phase φ_1 le temps de rétention du corps A est "court" et celui du corps B "long", la situation sera qualitativement analogue pour les temps de rétention sur une phase φ_2. Les raisons qui font que A "sorte" plus rapidement que B sont liées à la nature des facteurs b_2, b_3, $b_4 \ldots b_n$.

(2) Les variables "phases" se répartissent en deux groupes, en fonction de la valeur du facteur b_2; celles pour lesquelles f_2 est négative présentent un caractère "polaire" et la capacité de s'engager dans des liaisons hydrogène.

On peut mettre en évidence des relations linéaires entre les valeurs de f_2 et les "échelles de polarité" P de McReynolds[39], Z_1 de Wold[29] et X de Lowry[31]. On aboutit aux relations

$$f_2 = -0.161\, P \cdot 10^{-3} + 0.373 \qquad r = 0.901 \quad p = 18 \qquad (1)$$

$$f_2 = -2.452\, Z_1 + 0.083 \qquad r = 0.935 \quad p = 14 \qquad (2)$$

$$f_2 = -0.056\, X + 0.120 \qquad r = 0.944 \quad p = 14 \qquad (3)$$

Ces résultats montrent bien les parallélismes existant entre les "échelles de polarité" et permettent de préciser le rôle de l'axe b_2 que l'on peut considérer comme discriminant vis à vis de la "polarité" d'une phase.

(3) Le facteur b_3 permet d'approfondir l'analyse des propriétés des phases dites "non polaires" qui se scindent en deux groupes distincts dont l'un rassemble les phases fluorées. Celles-ci constituent donc bien un groupe à part entière.

En définitive, la variance absorbée par les trois axes ($b_1 + b_2 + b_3$) est de 99.8 %; aussi un espace à trois dimensions suffit pour décrire l'information significative de nos données, comme le confirme l'application de la méthode Varimax[40]. Cette dernière consiste en une analyse en composantes principales suivie d'une rotation orthogonale des axes factoriels. On remarque, après cette rotation, que les valeurs les plus élevées de f_1' concernent la classe des variables "phases" la plus fortement représentée (groupe C), et que celles de f_2 sont les plus élevées pour les phases du groupe A. Quant aux valeurs de f_3, elles ne sont importantes que pour les phases fluorées. Pour les axes au-delà de b_3, les composantes principales sont très faibles et dispersées positivement et négativement, ce qui montre bien qu'il s'agit d'un "facteur de bruit" non significatif.

Les résultats de l'A.C.P. par diagonalisation de la matrice de covariance sont certainement fortement influencés par la représentativité inégale des trois classes de phases mises en évidence. En particulier, le fait que les phases polaires soient mieux représentées par l'axe b_1 que les autres phases pourrait être dû, soit à une cause intrinsèque restant à expliquer, soit à leur nombre élevé dans l'échantillon des phases retenues. Aussi, pour confirmer la représentativité du groupe des phases fluorées, nous avons procédé à une A.C.P. sur la matrice de corrélation avec un jeu de pondération des variables[41] qui donne une importance égale à chacun des trois groupes de phases considérées: tout se passe comme si chaque groupe comportait le même nombre de représentants. Dans notre cas, où le groupe le plus important rassemble 12 termes, on fait intervenir chaque phase apolaire deux fois et chaque phase fluorée quatre fois. Les résultats de cette A.C.P. permettent de constater: (1) que l'axe b_1 n'est pas plus représentatif des phases polaires que des autres phases; (2) que la distinction première entre phases apolaires/fluorées et polaires est maintenue; et (3) que l'axe b_3 rend compte d'une rôle particulier des phases fluorées.

Il en résulte qu'un modèle prévisionnel des grandeurs de rétention, réalisé uniquement à l'aide des données relevées sur seulement des phases polaires et apolaires ne peut pas rendre compte avec précision des grandeurs de rétention sur l'ensemble des phases. La déviation devient importante dans le cas de phases fluorées. Ce phénomène avait été déjà constaté par Rohrschneider[42].

L'ensemble de nos analyses en composantes principales nous amène à proposer un modèle de prévision des données de rétention linéaire par régression faisant intervenir trois variables "phases" correspondant à trois phases stationnaires prises chacune dans l'un des trois groupes.

Modèle de régression à trois variables explicatives

Plusieurs critères permettent la sélection des trois phases, destinées chacune à représenter son groupe. Parmi ces critères, nous avons envisagé:

(1) *la régression multiple*[37]; elle conduit à retenir les phases SE-30 (groupe A), OV-210 (groupe B) et NPGS (groupe C);

(2) *l'espace factoriel* issu de l'analyse en composantes principales. Dans notre cas cet espace est tridimensionnel et amène à retenir comme meilleur choix les phases Apiezon H (groupe A), OV-210 (groupe B) et DEGA (groupe C);

(3) *l'analyse discriminante*[40] dans laquelle on recherche parmi un ensemble de variables "phases" celles qui conduisent à la meilleure discrimination des produits. Contrairement aux critères précédents, l'analyse discriminante établit un ordre indépendant de la classification en groupes et basé sur l'efficacité de la discrimination. Les phases placées en tête sont cette fois NPGS (groupe C), DC-200 (groupe A) et QF-1 (groupe B).

En effectuant des régressions affines du type

$$x_{\mathrm{P},\varphi_i} = a_\varphi \cdot x_{\mathrm{P},\varphi_1} + b_\varphi \cdot x_{\mathrm{P},\varphi_2} + c_\varphi \cdot x_{\mathrm{P},\varphi_3} + d_\varphi \qquad (4)$$

où x_{P,φ_i} représente la grandeur de rétention d'un produit P sur une phase φ_i.

Dans chacun des cas ci-dessus, on observe que les écarts entre les grandeurs de rétention expérimentales et les grandeurs recalculées sont très voisins ($\sigma_{e,\varphi} \approx 0.04$). En réalisant par ailleurs des régressions avec les données relatives aux phases les plus usuelles (SE-30, OV-210, DEGS), la valeur $\sigma_{e,\varphi}$ obtenue dans la reconstitution des données ne diffère guère de la précédente. Cette concordance provient de la bonne corrélation observée entre phases d'un même groupe taxonomique.

Avec ces dernières phases, l'application de la relation 4 aux dérivés monofonctionnels du benzène conduit aux résultats du Tableau III. Si, pour la plupart des phases, le coefficient b_φ est peu différent de zéro, il faut noter qu'il devient réelle-

TABLEAU III

APPLICATION DE LA RELATION 4 AUX DÉRIVÉS MONOFONCTIONNELS DU BENZÈNE

$\log t'_{\phi\mathrm{Z},\varphi} = \mathrm{a}_\varphi \cdot \log t'_{\phi\mathrm{Z,SE-30}} + \mathrm{b}_\varphi \cdot \log t'_{\phi\mathrm{Z,OV-210}} + \mathrm{c}_\varphi \cdot \log t'_{\phi\mathrm{Z,DEGS}} + \mathrm{d}_\varphi$
r_φ = coefficient de corrélation; $\sigma_{\mathrm{e},\varphi}$ = écart type moyen de l'erreur d'estimation.

φ_i	a_φ	b_φ	c_φ	d_φ	r_φ	$\sigma_{e,\varphi}$
Apiezon L	1.753	−0.268*	−0.047*	−0.012	0.9916	0.05
DC-200	1.112	−0.025*	−0.005*	0.149	0.9993	0.01
PMPE 5R	1.158	0.092*	0.229	−0.334	0.9953	0.05
Apiezon H	1.472	−0.029*	−0.007*	−0.040	0.9926	0.05
OV-17	1.141	0.067*	0.127*	−0.061	0.9940	0.05
UCON 50 HB 2000	0.410	−0.233*	0.808	0.428	0.9967	0.04
ULB 550X	0.536	−0.124*	0.614	0.149	0.9968	0.04
OV-225	0.256	0.252	0.541	0.116	0.9988	0.03
NPGS	0.160*	0.043*	0.782	0.137	0.9984	0.03
Carbowax 20M	0.046*	−0.057*	0.934	−0.472	0.9960	0.05
XF-1150	−0.315	0.342	0.832	0.336	0.9991	0.02
FFAP	0.122*	−0.174*	0.980	0.511	0.9979	0.04
PDEAS	0.125*	−0.049*	0.913	0.123	0.9975	0.04
DEGA	0.085*	−0.016*	0.901	0.380	0.9982	0.04
EGS	−0.149*	0.105*	0.975	0.060	0.9994	0.02
DEGSeb	0.375	−0.099*	0.754	0.472	0.9975	0.04
QF-1	−0.019*	0.935	−0.030*	0.339	0.9966	0.03
DCL 5X	0.156*	0.879	0.103	−0.712	0.9975	0.03

* Valeur des coefficients de la régression pour laquelle le test de Fischer est supérieur à 2%

TABLEAU IV

APPLICATION DE LA RELATION 4 AUX DÉRIVÉS POLYFONCTIONNELS DU BENZÈNE

Benzènes disubstitués	φ_i	a_φ	b_φ	c_φ	d_φ	r_φ	$\sigma_{e,\varphi}$
ortho	Carbowax 20M	−0.045*	0.029*	0.972	−0.016	0.979	0.08
	ULB 550X	0.482	−0.278	0.682	0.363	0.993	0.04
	OV-225	0.221	0.290	0.510	0.062	0.997	0.02
	PDEAS	0.247*	0.017*	0.813	0.058	0.994	0.04
	Apiezon L	1.385	−0.139*	−0.094*	0.221	0.996	0.03
	PMPE 5R	1.066	0.108	0.140	−0.181	0.999	0.02
meta	Carbowax 20M	0.142*	−0.056*	1.030	−0.421	0.995	0.04
	ULB 550X	0.811	−0.170*	0.510	−0.072	0.995	0.03
	OV-225	0.256	0.269	0.543	−0.056	0.999	0.01
	PDEAS	0.245*	0.059*	0.851	−0.111	0.998	0.03
	Apiezon L	1.393	−0.185*	−0.039*	0.170	0.997	0.02
	PMPE 5R	0.760	0.327*	0.253*	−0.210	0.976	0.07
para	Carbowax 20M	−0.026*	−0.115*	1.117	−0.210	0.995	0.04
	ULB 550X	0.157*	−0.085*	0.768	0.431	0.985	0.06
	OV-225	0.210	0.244	0.574	0.005	0.999	0.02
	PDEAS	0.263	−0.006*	0.872	−0.059	0.996	0.03
	Apiezon L	1.530	−0.178	−0.120	0.096	0.995	0.03
	PMPE 5R	1.294	0.002*	0.119*	−0.376	0.995	0.04
Benzènes tri-substitués	Carbowax 20M	−1.460*	−1.080	2.832	0.511	0.992	0.06
	ULB 550X	0.987	−0.002*	0.247	0.178	0.999	0.01
	OV-225	0.391*	0.443	0.293	0.105	0.999	0.02
	PDEAS	0.421	0.178	0.707	−0.084	0.999	0.01
	Apiezon L	1.631	−0.213	−0.053*	0.031	0.998	0.01
	PMPE 5R	0.912	0.036*	0.342	−0.080	0.999	0.01

ment significatif non seulement dans le cas des phases fluorées (QF-1 et DCL 5X), mais aussi dans celui des phases cyanées (OV-225 et XF-1150).

Les coefficients a_φ, b_φ et c_φ de la relation 4 ont été déterminés à partir d'une famille de dérivés monofonctionnels du benzène. Il restait à vérifier si la relation 4 pouvait constituer un modèle de portée plus générale. A cet effet, nous l'avons appliquée aux populations suivantes: benzènes disubstitués en *o*, benzènes disubstitués en *m*, benzènes disubstitués en *p*, benzènes trisubstitués[37]. Les résultats, regroupés dans le Tableau IV, montrent que le modèle représenté par la relation 4 permet une bonne reconstitution de l'ensemble des données de rétention.

Enfin, en appliquant (Tableau V) la relation 4 à une population de 27 composés RZ (où R correspond à une chaine aliphatique et Z à des fonctions diverses) pour laquelle nous disposions des indices de rétention déterminés par McReynolds[36] sur les phases SE-30, DC-1265 F (très voisine de la phase OV-210) et DEGS, nous obtenons des valeurs recalculées dont l'écart $\sigma_{e,\varphi}$ est inférieur à 16 unités d'indice de rétention (sauf en ce qui concerne la phase Apiezon L). La bonne concordance entre indices de rétention expérimentaux et recalculés, atteinte à partir d'une population restreinte, nous a semblé assez prometteuse pour tenter d'appliquer la relation 4 à l'ensemble des données de rétention déterminées par Rohrschneider[35] (Tableau VI) pour la mise au point de son propre système de prévision. Ce dernier est basé sur cinq substances judicieusement choisies et censées représenter les différents types d'interactions pouvant coexister dans la chromatographie en phase gazeuse. Compte-tenu de la variété des produits examinés et des phases, l'écart $\sigma_{e,\varphi}$ qui se situe presque toujours en-dessous de 20 pèse peu sur les valeurs recalculées. Cette valeur de $\sigma_{e,\varphi}$ est du même ordre de grandeur que celle que l'on observe dans le système de prévision établit par Rohrschneider.

Une analyse en composantes principales par diagonalisation de la matrice de covariance que nous avons réalisée par ailleurs à partir des indices de rétention de Rohrschneider confirme d'une part la qualité de notre relation 4 et d'autre part la légitimité de notre choix d'un espace factoriel à trois dimensions.

DISCUSSION

L'A.C.P. est une méthode d'analyse de données qui, à travers ses différentes variantes, répond à de nombreuses approches visant toutes à condenser des informations groupées généralement dans un tableau. Celui-ci représente la "matrice des données de rétention étudiées", composée de n lignes "produits" —indicées par P— et de m colonnes "phases" —indicées par φ. Un élément de cette matrice, situé dans une colonne φ et dans une ligne P, est noté $x_{\mathrm{P},\varphi}$. Il représente la grandeur de rétention Gr(P,φ) du produit P sur la phase φ. Gr(P,φ) peut exprimer, selon le cas, soit un logarithme de temps réduit (log t'), soit un logarithme de temps réduit relatif (log t'_H), soit un logarithme de volume de rétention (log V_g, log V_N), soit enfin un indice de rétention (I) ou encore une différence d'indice (δI), considérée comme un incrément de fonction.

Le tableau, constitué d'observations interdépendantes, permet d'analyser des relations entre "produits" —les lignes— ou entre "phases" —les colonnes—. L'objectif consiste alors à condenser l'essentiel des informations résultant de l'observation en un nombre plus restreint de nouvelles informations, non observables directement et appelées "variables factorielles" ou "composantes principales".

Ces variables factorielles, notées $b_1, \ldots, b_q$ permettent, par des régressions multiples, de reconstituer de façon approchée la matrice des données initiales. Le choix de $b_1, \ldots, b_q$ est déterminé par l'optimisation d'un critère mesurant la qualité de cette reconstitution. Le pourcentage d'inertie associé aux q premières variables factorielles $b_1, \ldots, b_q$, constitue un indice de qualité de la reconstitution de la matrice. Si cet indice est proche de 100%, cela signifie généralement que (1) les variables factorielles situées au-delà de b_q décrivent en fait un "bruit" sans signification, issue en général des erreurs de mesures, et (2) les variables initiales x_φ, aux erreurs de mesures près, sont situées dans un espace de dimension réduite, cette situation étant due à leurs interdépendances.

On peut donc, à l'aide de l'A.C.P., déterminer un nombre minimal de variables observées qui permettent une reconstitution du tableau de données sans perte sensible d'information. Par ailleurs, la représentation des variables initiales dans l'espace de dimension réduite engendré par les q variables factorielles retenues permet de mettre en évidence les similitudes et les différences de comportement des phases étudiées.

Les différentes variantes de l'A.C.P. classiquement utilisées dans "l'Analyse de Données" sont au nombre de 4 et se distinguent entre-elles par:

le type de matrice à diagonaliser (produits scalaires, produits scalaires normalisés, covariances, corrélations);

le type de régression (affine ou linéaire) intervenant dans la reconstitution des données;

les paramètres dont les valeurs sont à estimer;

le critère d'optimisation (somme des carrés des écarts entre grandeurs mesurées et valeurs recalculées avec ou sans normalisation).

En chimiométrie il convient de déterminer les relations qui existent entre ces différentes variantes et de choisir le (ou les) modèle(s) le(s) mieux adapté(s).

Dans le cas de la chromatographie en phase gazeuse, nous avons mis en évidence[38] les résultats suivants:

l'A.C.P. par diagonalisation de la matrice de corrélation présente l'avantage de donner des résultats identiques, quelle que soit la grandeur de rétention utilisée. Par ailleurs, pour la détermination des axes factoriels, cette A.C.P. attribue la même importance aux variables à fortes ou faibles variances, c'est-à-dire aux phases stationnaires qui retiennent fortement ou peu les produits étudiés;

selon les grandeurs de rétention choisies, les A.C.P. par diagonalisation de la matrice de covariance donnent des résultats différents; cependant, ce type d'A.C.P. a l'avantage de présenter un critère d'optimisation identique à celui utilisé en régression multiple. Ce dernier type d'A.C.P. appliqué aux indices de rétention (I) (respectivement au logarithme de temps réduit, log t') donne des résultats pratiquement identiques à ceux d'une A.C.P. par diagonalisation des produits scalaires sur des différences d'indices δI (ou sur des logarithmes de temps réduits relatifs log t'_H). L'identité serait totale si le composé de référence (le benzène dans notre cas) était parfaitement recalculé dans l'A.C.P. initiale.

Dans la pratique, le temps de rétention réduit du benzène ne peut être déterminé avec une très grande précision, compte-tenu des caractéristiques techniques de notre appareillage et des températures de travail qui sont liées aux propriétés des substances analysées.

Cette imprécision se répercutant sur les valeurs de rétention réduites relatives des composés étudiés, il nous a semblé préférable d'appliquer l'A.C.P. par diagonalisation de la matrice de covariance aux logarithmes de temps réduit (log t'); on observe alors que la précision des valeurs recalculées reste compatible avec la précision expérimentale.

CONCLUSION

Les méthodes de l'analyse des données permettent d'aborder avec fruit le problème de la prévision des grandeurs de rétention en chromatographie en phase gazeuse. Wold[43] a réalisé des A.C.P. à l'aide des indices de Kovats par diagonalisation de la matrice de corrélation; les régressions résultant de ce traitement sont en général affines. Nous-mêmes avons pu montrer que l'analyse en composantes principales (A.C.P.), et plus particulièrement sa variante issue de la diagonalisation de la matrice de covariance, conduit à un espace tridimensionnel. Nous avons ainsi été amenés à proposer le modèle

$$x_{P,\varphi_i} = a_{\varphi} \cdot x_{P,\varphi_1} + b_{\varphi} \cdot x_{P,\varphi_2} + c_{\varphi} \cdot x_{P,\varphi_3} + d_{\varphi}$$

TABLEAU V

APPLICATION DE LA RELATION 4 AUX DONNÉES DE McREYNOLDS

$I_{RZ,\varphi} = a_{1,\varphi} \cdot I_{RZ,SE-30} + b_{1,\varphi} \cdot I_{RZ,DC1265F} + c_{1,\varphi} \cdot I_{RZ,DEGS} + d_{1,\varphi}$

R = *tert.*-C_4H_9, iso-C_4H_9, *sec.*-C_4H_9, *n*-C_4H_9, *n*-C_5H_{11}, *n*-C_6H_{13} et Z = OH, CHO, $COCH_3$, $COOCH_3$, OCH_3

φ_i	$a_{1,\varphi}$	$b_{1,\varphi}$	$c_{1,\varphi}$	$d_{1,\varphi}$	r_φ	$\sigma_{e,\varphi}$
Apiezon L	1.255	-0.010*	-0.119*	-57.63	0.9814	29.0
Carbowax 20M	0.518	-0.324	0.856	-141.23	0.9986	9.2
DEGA	0.277	-0.178	0.917	-95.89	0.9993	6.7
DEGSeb	0.565	-0.163	0.618	-78.11	0.9988	7.7
Diéthylhexylsébacate	0.773	-0.061*	0.224	-13.70	0.9986	6.9
Didecyl phtalate	0.711	0.058*	0.198	-22.38	0.9982	8.5
EGS	0.636	-0.186	0.581	-84.18	0.9986	8.4
NPGS	0.372	-0.054*	0.679	-81.54	0.9990	7.3
PMPE 5R	0.693	0.199	0.144	-15.41	0.9989	7.2
UCON 50 HB 2000	0.688	-0.292	0.640	-124.63	0.9991	6.6
XF-1150	-0.117*	0.359	0.738	-108.30	0.9966	15.5
Tricrésyl phosphate	0.589	-0.011*	0.423	-54.97	0.9980	9.8

TABLEAU VI

APPLICATION DE LA RELATION 4 AUX DONNÉES DE ROHRSCHNEIDER

$I_{RZ,\varphi} = a_{1,\varphi} \cdot I_{RZ,DC-200} + b_{1,\varphi} \cdot I_{RZ,QF-1} + c_{1,\varphi} \cdot I_{RZ,DEGS} + d_{1,\varphi}$

φ_i	$a_{1,\varphi}$	$b_{1,\varphi}$	$c_{1,\varphi}$	$d_{1,\varphi}$	r_φ	$\sigma_{e,\varphi}$
Squalane	1.144	-0.071*	-0.059	-4.013	0.9962	13.4
Apiezon L	1.110	-0.109*	0.012*	-5.080	0.9927	18.1
Diéthylhexylsébacate	0.866	-0.100*	0.185	21.228	0.9957	12.4
Célanèse ester NR9	0.842	-0.081*	0.202	11.886	0.9965	11.3
Diisodécylphtalate	0.736	0.074*	0.160	10.033	0.9965	11.1
DC-710	0.705	0.201	0.121	-11.308	0.9949	14.1
Polypropylèneglycol	0.815	-0.225	0.345	22.350	0.9922	17.3
Acétyltributylcitrate	0.612	0.065*	0.297	3.807	0.9947	14.3
Tricrésylphosphate	0.562	0.040*	0.371	8.969	0.9959	13.1
PMPE	0.639	0.206	0.229	-36.985	0.9911	19.9
Marlophène 87	0.603	-0.140*	0.472	19.780	0.9931	17.6
Polypropylènesébacate	0.630	-0.081*	0.434	-2.142	0.9973	11.1
Marlophène 814	0.552	-0.174	0.560	19.480	0.9938	17.9
NPGS	0.321	0.040*	0.605	5.300	0.9957	16.3
XF-1150	-0.015*	0.433	0.543	4.811	0.9961	15.9
Carbowax 20M	0.442	-0.210	0.755	-13.486	0.9923	24.0
Carbowax 4000	0.433	-0.222	0.775	-14.974	0.9931	23.1
Réoplex 400	0.334	-0.106	0.785	-22.634	0.9981	12.5
Éthylèneglycolcyanoéthyléther	-0.368	0.492	0.953	-64.168	0.9988	13.1
Tris1,2,3cyano-2-éthoxypropane	-0.510	0.462	1.096	-35.029	0.9988	14.3

comme outil de prévision des grandeurs de rétention. Ce modèle, mis au point à partir des données relatives à des benzènes monosubstitués, présente un caractère plus général; en effet, son domaine de validité peut être étendu non seulement à des benzènes polysubstitués, mais même à des populations de structure quelconque.

Par ailleurs, les résultats obtenus montrent très nettement que les phases polyfluorées se distinguent dans leur comportement chromatographique tant des phases dites "polaires" que de celles qui sont "apolaires". Elles constituent par conséquent une classe particulière.

RÉSUMÉ

L'analyse en composantes principales appliquée à un ensemble de données chromatographiques recueillies sur 21 phases stationnaires et relatives à une population de 17 benzènes monosubstitués permet de déduire un modèle multiparamétrique simple. La relation qui en découle

$$x_{P,\varphi_i} = a_\varphi \cdot x_{P,\varphi_1} + b_\varphi \cdot x_{P,\varphi_2} + c_\varphi \cdot x_{P,\varphi_3} + d_\varphi$$

présente un caractère de généralité. Elle permet d'estimer la grandeur de rétention x_{P,φ_i} d'un produit P sur une phase φ_i à partir des valeurs expérimentales des grandeurs de rétention x_{P,φ_1}, x_{P,φ_2} et x_{P,φ_3} de ce même produit P, relevées sur trois phases stationnaires φ_1, φ_2 et φ_3, respectivement apolaire, polyfluorée et polaire.

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CORRELATION BETWEEN THE RETENTION OF CARDIAC GLYCOSIDES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A DIPHENYLSILYL STATIONARY PHASE, THE STRUCTURE OF THEIR MOLECULES AND THEIR BIOLOGICAL ACTIVITY

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SUMMARY

The separation of mixtures of cardiac glycosides by reversed-phase high-performance liquid chromatography on silica gel with chemically grafted diphenylsilyl groups using water–ethanol as the eluent was carried out. It is shown that the configuration and conformation of the glycoside molecules, and the hydrophilic properties of their aglycones and glycones, influence the separation. The hydrophilic properties of the aglycones are more important than those of the glycones. The glycosides with more hydrophilic aglycones have higher biological activity. This is probably related to the easier transport of these glycosides to the receptor.

INTRODUCTION

The investigation of the correlation between molecular structure and chromatographic retention is of great interest, as it permits the mechanisms of intermolecular interactions in adsorption from solution to be established and the reverse chromatographic problem, *i.e.*, to obtain information about molecular structure from chromatographic data, to be solved. Such problems have been solved in simpler cases in gas chromatography[1–5]. For the development of such an approach using liquid chromatography, it is first necessary to obtain reproducible experimental results, for which we need to take into account more complicated intermolecular interactions in the system adsorbent–compound under investigation–eluent (often multi-component).

In previous papers[6–9] we attempted to find a correlation between the structure of cardiac glycoside molecules and their retention on hydrophobic silica gel when eluting with water–ethanol solutions. It was found that the retention volume does not depend directly on the molecular weight of the glycosides, but on the hydrophilic properties of their molecules. It was also found that the retention volume of glycosides depends on the configuration of their molecules.

Cardiac glycosides, mainly digitalis glycosides, have been studied by high-performance liquid chromatography (HPLC) by many workers[10–26]. We have considered the HPLC not only of digitalis glycosides but also of other types of cardiac glycosides without derivatization on the silica gel surface with attached rigid diphenylsilyl groups, while silica gels with octadecylsilyl (ODS) groups having conformational mobility were usually used.

The correlation between the chemical structure and chromatographic behaviour of cardiac glycosides was investigated earlier by paper (PC) and thin-layer chromatography (TLC) (see, for example, refs. 27–33). This correlation depends on the type of adsorbent and on the composition of the eluent. In PC and TLC, hydrophilic adsorbents and multi-component solvents were usually used.

The correlation between biological activity and chromatographic behaviour (QSAR studies) on the basis of hydrophobic, electronic and structural properties of biologically active molecules is important[34,35]. However, these properties are difficult to determine for such complicated molecules as glycosides. The retention volume depends on many types of intermolecular interactions and on the properties of adsorbent, eluent and solute. Therefore, the correlation between the retention volume itself and the biological activity of cardiac glycosides is useful.

In order to find a more general relationship between the retention and molecular structure of glycosides, in this work the retention volumes of different glycosides on silica gel with chemically grafted diphenylsilyl groups have been determined and the separation of glycoside molecules, which differ only in the molecular configuration, has been investigated. We have also attempted to find a correlation between the chromatographic properties of glycosides and their biological activity.

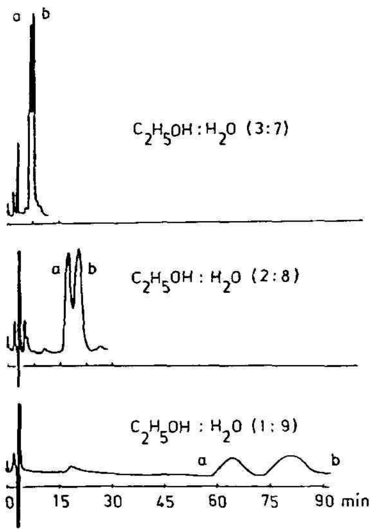
EXPERIMENTAL

The cardiac glycosides investigated are listed in Table I. The biological activities were taken from ref. 36.

A Spectra-Physics 3500B liquid chromatograph was used with a UV–VIS 700 detector at 220 nm. Stainless-steel columns (125 mm × 4.8 mm I.D.) were packed with silica gel modified with diphenyldichlorosilane[8,9], LiChrosorb Si 60 or LiChrosorb Si 100 of particle size 5 and 7 μm, respectively. In contrast to *n*-alkylsilyl groups, the attached diphenylsilyl groups do not exhibit conformational transformations in polar eluents (phenyl groups are rigid and can undergo only restricted rotation around the silicon–carbon bonds, producing an adsorbent with a considerably more uniform surface).

RESULTS AND DISCUSSION

The isomeric cardiac glycosides convallatoxin and desglucocheirotoxin have the same aglycone, strophanthidin, and the monosaccharide glycones have L-rhamnose and D-gulomethylose, respectively. They possess an identical number of hydroxyl groups. The structural difference between these isomers is only the different saccharide conformation and their bonds with the aglycone, which produces a relatively small difference in the molecular configuration. However, this difference is enough for the separation of these isomers on the silica surface modified with diphenylsilyl groups. Fig. 1 shows the separation of convallatoxin and desglucocheirotoxin on LiChrosorb Si 100 (specific surface area, s = 285 m^2/g) with chemically grafted diphenylsilyl groups using water–ethanol as the eluent. The increase in the ethanol content of



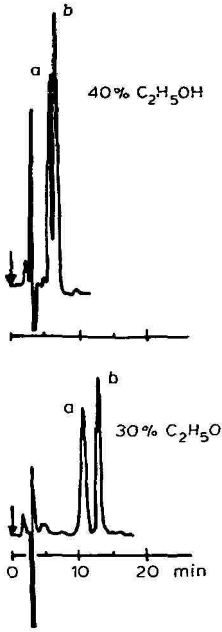


Fig. 1. Separation of a mixture of convallatoxin (a) and desglucocheirotoxin (b) on LiChrosorb Si 100 (7 μm) with diphenylsilyl groups grafted to the surface. Water–ethanol eluent composition as indicated. Column, 12.5 cm × 4.8 mm I.D.; flow-rate, 0.7 cm^3/min; temperature, 30°C.

Fig. 2. Separation of a mixture of convallatoxin (a) and desglucocheirotoxin (b) on LiChrosorb Si 60 (5 μm) with attached diphenylsilyl groups from water–ethanol eluent of different compositions as indicated. Column as in Fig. 1. Flow-rate, 0.65 cm^3/min; temperature, 40°C.

the eluent improves the separation, but does not lead to complete separation. The use of LiChrosorb Si 60, which has a larger specific surface area (s = 500 m^2/g), with attached diphenylsilyl groups made possible their complete separation (Fig. 2). It can be clearly seen that owing to the bond in strophanthidin–3β-O-α-L-rhamnose and the C1 conformation of L-rhamnose, the convallatoxin molecule as a whole is more curved than the desglucocheirotoxin molecule, for which the bond in strophanthidin–3β-O-β-D-gulomethylose and the 1C conformation of the D-gulomethylose form a flatter molecule.

Fig. 3 shows the dependence of the retention volumes of these two isomers on the water–ethanol eluent composition. With a decrease in the ethanol content the retention volume for both glycosides sharply increases, but the difference in the specific retention volumes, $V_{m,1}$, *i.e.*, in the adsorption equilibrium constant (Henry's constant)[7], increases. It was interesting to determine the dependence of other thermodynamic characteristics of adsorption of convallatoxin and desglucocheirotoxin from water–ethanol on silica gel with attached diphenylsilyl groups on the eluent composition. For this purpose the retention volumes of these glycosides at different temperatures were measured. Fig. 4 shows the dependence of ln $V_{m,1}$ on reciprocal temperature for different compositions of the eluent. From these plots the changes in the differential enthalpy and entropy of adsorption from dilute solutions were evaluated.

Fig. 5 shows the dependence of the retention volume (Henry's constant), the

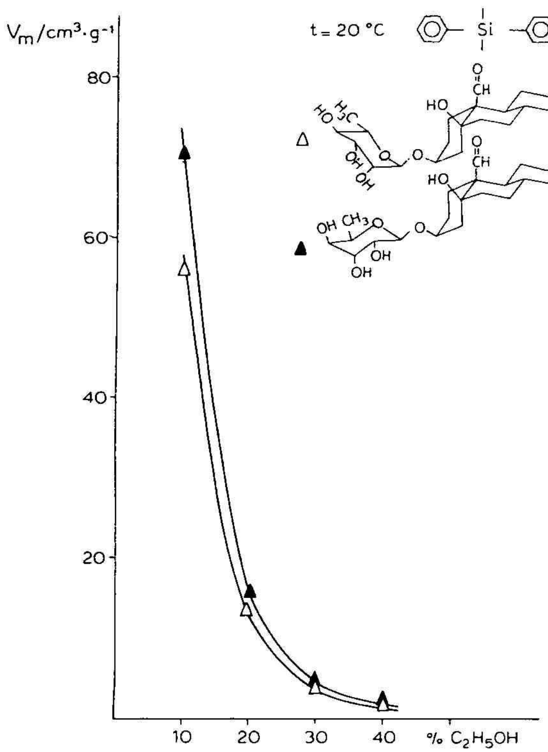


Fig. 3. Change in the retention volume of convallatoxin (a) and desglucocheirotoxin (b) on LiChrosorb Si 100 with attached diphenylsilyl groups on the ethanol concentration in the eluent. Temperature, 20°C.

differential enthalpy, $-\overline{\Delta H}_1$, and the standard differential entropy, $\overline{\Delta S}_1^\circ$, changes for the adsorption of these isomeric glycosides from water–ethanol on the eluent composition. The general character of the dependence of the thermodynamic characteristics of adsorption on the eluent composition is the same for these glycosides when adsorbed on the hydrophobic surface of modified silica gel from water–ethanol solution: a sharp decrease in the adsorption equilibrium constant occurs with increase in ethanol concentration in the eluent and a slow change in $-\overline{\Delta H}_1$ on increasing the ethanol concentration up to 20 %, in comparison with the sharp decrease in $-\overline{\Delta H}_1$ at higher ethanol concentrations. A large increase in the adsorption equilibrium constant at lower ethanol concentrations is connected with the higher influence of the entropic factor, $-\overline{\Delta S^\circ}/R$.

For the elucidation of the relationship between the structure of the cardiac glycosides molecules and their retention, the seprations of mixtures of cardiac glycosides containing mono-, di-, tri- and tetrasaccharide glycones was carried out. Fig. 6 shows the separation of a mixture of glycosides containing the monosaccharide glycones G-strophanthin, convallatoxin, desglucocheirotoxin, erysimin, cymarin and oleandrin. The elution sequence of these glycosides from a column filled with silica gel with attached diphenylsilyl groups is determined by the number of hydrophilic groups in the glycoside molecule, as can be seen from the structural formulae. The largest

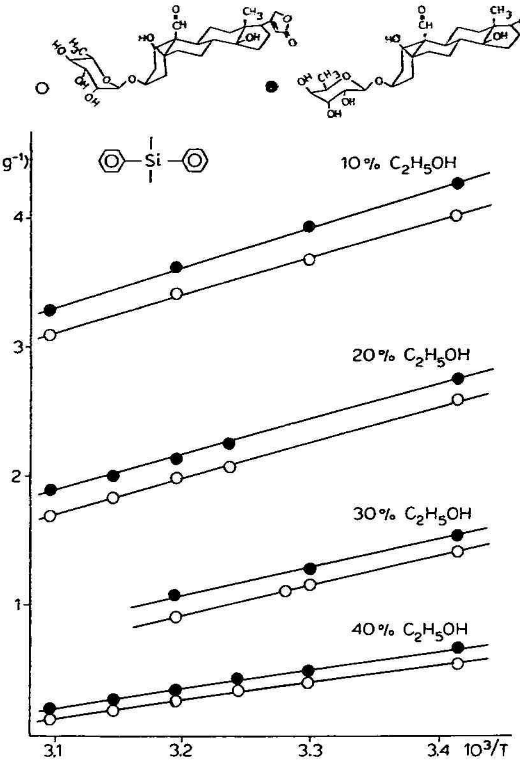


Fig. 4. Dependence of the logarithm of the retention volume on reciprocal temperature for convallatoxin and desglucocheirotoxin at different water–ethanol eluent compositions. Column filled with silica gel with diphenylsilyl groups grafted to the surface.

number of hydroxyl groups are present in the G-strophanthin molecule; there are five hydroxyl groups in the aglycone (oubagenin) and three in the glycone (L-rhamnose) of this molecule. As it is the most hydrophilic molecule in this mixture, G-strophanthin produces the strongest hydrogen bond type interaction with the water–ethanol eluent and the weakest interaction with the hydrophobic adsorbent surface. Therefore, G-strophanthin is eluted first. The convallatoxin molecule has the same glycone as G-strophantin (L-rhamnose) and a different aglycone, strophanthidin, which possesses only two hydroxyl groups. Thus convallatoxin is eluted second from this column. Third to be eluted is desglucocheirotoxin, which does not differ from its isomer convallatoxin in the number of hydroxyl groups, but has a flatter molecule (Fig. 1). The fourth glycoside, erysimin, differs from desglucocheirotoxin in the number of hydroxyl groups: its glycone (digitoxose) has only two hydroxyl groups. Then follows cymarine, whose glycone (cymarose) contains only one hydroxyl group. Last to be eluted is oleandrin, which also contains one hydroxyl group in its glycone (oleandrose), but in the aglycone (oleandrigenin) it has only one hydroxyl group.

Hence the larger the number of hydroxyl groups in the molecules of the glycosides in this mixture, the weaker is their retention on the silica gel with chemically grafted diphenylsilyl groups on the surface from water–ethanol solution.

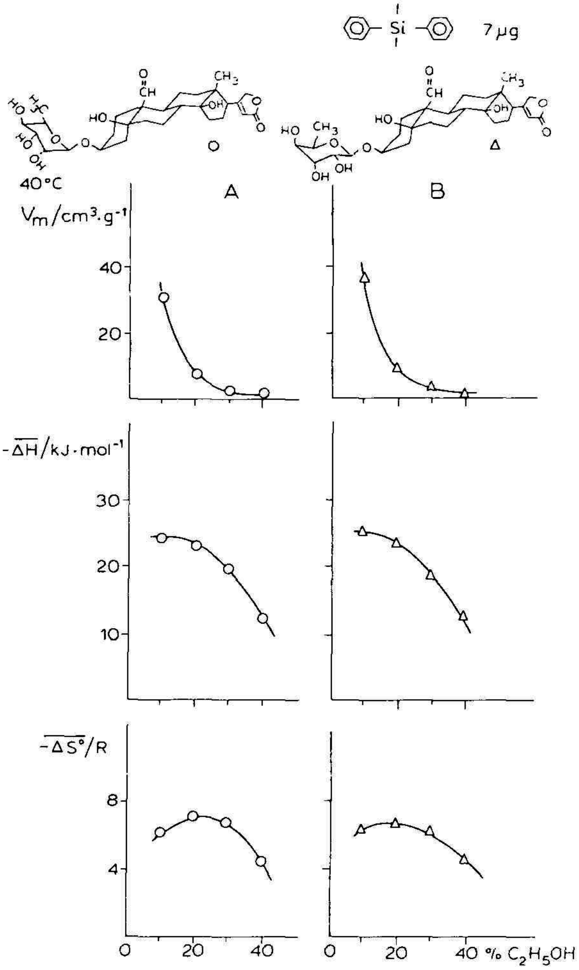


Fig. 5. Dependence of the specific retention volume, $V_{m,1}$, and the differential changes of enthalpy, $-\overline{\Delta H}_1$, and entropy, $-\overline{\Delta S}_1^\circ/R$, for the adsorption of convallatoxin (A) and desglucocheirotoxin (B) on LiChrosorb Si 100 with attached diphenylsilyl groups on composition of the water–ethanol eluent.

An analogous dependence of the retention of glycosides on the number of hydroxyl groups in the molecule is observed for glycosides with disaccharide glycones, such as for the mixture of corelborin-π, olitoriside and K-strophanthin-β. The aglycones of these three glycosides have an identical number of hydroxyl groups (two), and therefore a different number of hydroxyl groups in the glycone mainly determines the retention difference (Fig. 7). First to be eluted is corelborin-π (six hydroxyl groups in the glycone), second is olitoriside (five hydroxyl groups) and third is K-strophanthin-β (four hydroxyl groups).

By separating a mixture of glycosides containing a trisaccharide glycone, the same regularity is observed (Fig. 8): first to be eluted is the more hydrophilic glycoside K-strophanthoside (seven hydroxyl groups in the glycone and two in the aglycone),

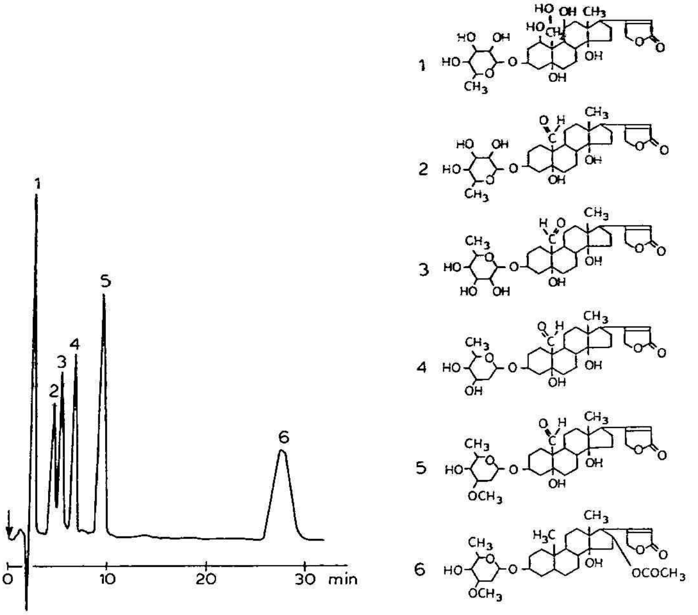


Fig. 6. Chromatogram of a mixture of cardiac glycosides on LiChrosorb Si 60 (5 μm) with attached diphenylsilyl groups from water–ethanol (65:35) eluent. Column, 12.5 cm × 4.8 mm I.D.; flow-rate, 0.9 cm^3/min; temperature, 50°C. Peaks: 1 = G-strophanthin; 2 = convallatoxin; 3 = desglucocheirotoxin; 4 = erysimin; 5 = cymarine; 6 = oleandrin.

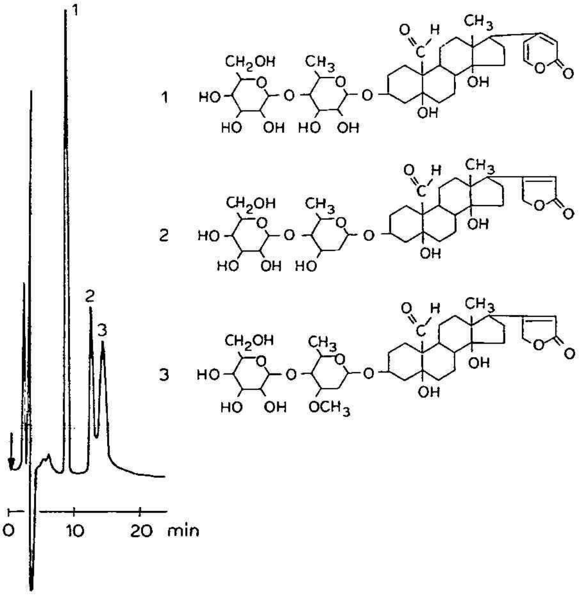


Fig. 7. Chromatogram of a mixture of cardiac glycosides on LiChrosorb Si 60 (5 μm) with attached diphenylsilyl groups from water–ethanol (70:30) eluent. Column as in Fig. 6. Flow-rate, 0.65 cm^3/min; temperature, 40°C. Peaks: 1 = Corelborin-π; 2 = olitoriside; 3 = K-strophanthin-β.

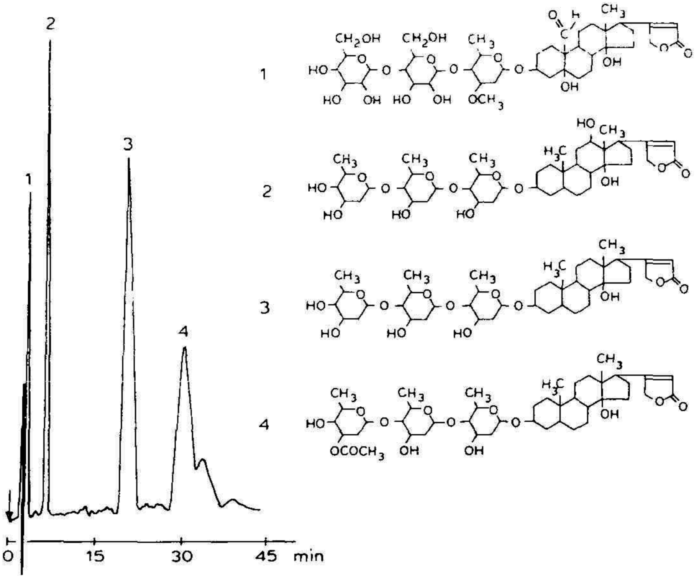


Fig. 8. Chromatogram of a mixture of cardiac glycosides on LiChrosorb Si 60 (5 μm) with attached diphenylsilyl groups from water–ethanol (60:40) eluent. Column as in Fig. 6. Flow-rate, 0.9 cm^3/min; *temperature*, 60°C. Peaks: 1 = K-strophanthoside; 2 = digoxin; 3 = digitoxin; 4 = acetyldigitoxin.

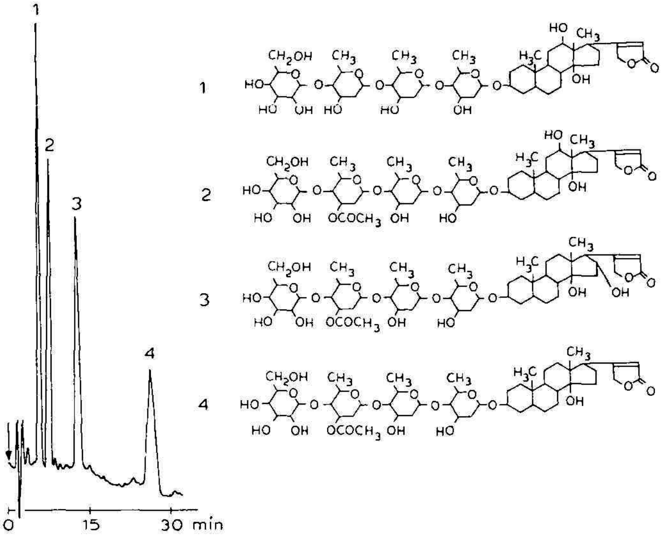


Fig. 9. Chromatogram of a mixture of cardiac glycosides on LiChrosorb Si 60 (5 μm) with attached diphenylsilyl groups from water–ethanol (60:40) eluent. Column as in Fig. 6. Flow-rate, 0.7 cm^3/min; temperature, 50°C. Peaks: 1 = Desacetyl-lanatoside C; 2 = lanatoside C; 3 = lanatoside B; 4 = lanatoside A.

second is digoxin (four and two hydroxyl groups, respectively), third is digitoxin (four and one hydroxyl groups, respectively) and last is acetyldigitoxin (three and one hydroxyl groups, respectively).

Fig. 9 shows the separation of a mixture of lanatosides having tetrasaccharide glycones: first to be eluted is desacetyl-lanatoside C (seven hydroxyl groups in the glycone and two in the aglycone), second is lanatoside C (six and two hydroxyl groups, respectively) and third is lanatoside B (also six and two hydroxyl groups, respectively). With lanatoside B, the hydroxyl group of the aglycone (gitoxigenine) in position C_{16} is screened, which results in a decrease in the hydrophilicity of the molecule[37], and therefore in the molecule of lanatoside B only seven hydroxyl groups remain "free". Last to be eluted is lanatoside A, which contains the aglycone digitoxigenin (six hydroxyl groups in the aglycone and one in the glycone).

The retention volumes of the 17 glycosides investigated were measured under identical conditions and are presented in Table I. Fig. 10 shows plots of ln $V_{m,1}$ against the number of hydroxyl groups, n_{OH}, in the whole cardiac glycoside molecules for each of these four mixtures containing mono-, di-, tri- and tetrasaccharide glycones. Fig. 10 shows that an increase in the number of hydroxyl groups in the molecules of cardiac glycosides produces a decrease in the retention volume but in a different manner for each mixture.

By grouping the glycosides with identical aglycones into families and arranging the aglycones so that their hydrophilic properties increase, *i.e.*, the number of all hydrophilic groups, it is possible to arrange the cardiac glycosides within each family according to the increase in the number of hydroxyl groups in the glycone. This permits a comparison of the change in the ln $V_{m,1}$ values with changes in the hydrophilic characteristics of the aglycone and of the whole glycoside molecule, although there is some uncertainty in the determination of the hydrophilic properties of parts of the molecule and the hydrophilic properties of the molecule as a whole. Fig. 11 shows this comparison. The glycosides are arranged in sequence according to the increase in the hydrophilic properties of the aglycone (by considering the increment in

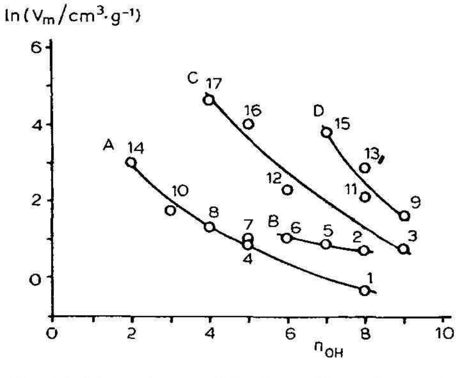
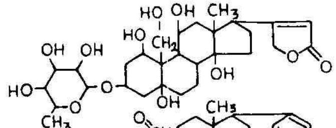
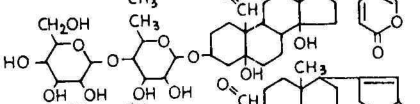
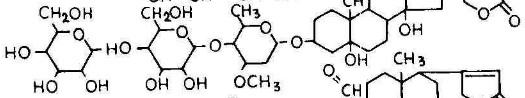
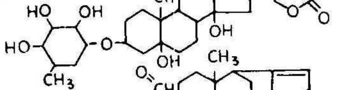
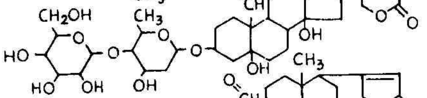
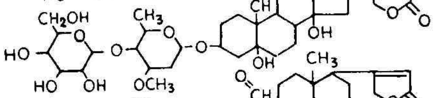
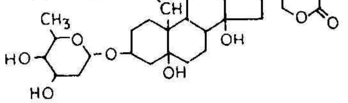
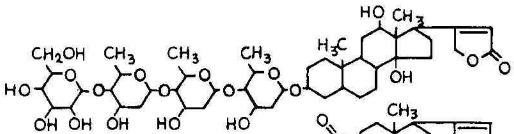
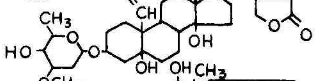
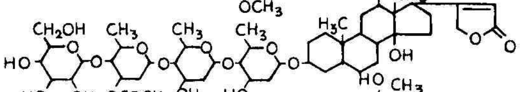
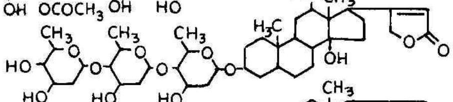
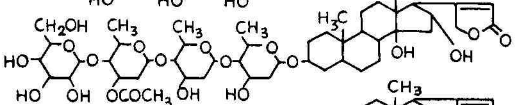
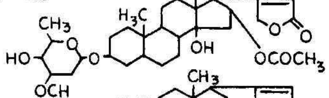
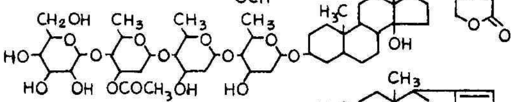
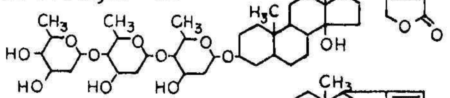
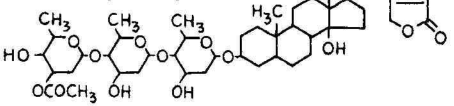


Fig. 10. Dependence of the logarithm of the retention volume on the number of hydroxyl groups in the glycoside molecule for the families of cardiac glycosides containing (A) mono-, (B) di-, (C) tri- and (D) tetrasaccharide glycones. The values of $V_{m,1}$ were determined on LiChrosorb Si 60 (5 μm) with attached diphenylsilyl groups from water–ethanol (65:35) eluent at 50°C. Numbers indicate the corresponding glycosides in Table I.

TABLE I

STRUCTURAL FORMULAE OF CARDIAC GLYCOSIDES, MOLECULAR WEIGHTS, VALUES OF HENRY'S CONSTANT, $K_1 = V_{m,1}$, FOR THE ADSORPTION OF GLYCOSIDES ON SILICA GEL (LICHROSORB Si 60, 5 μm) MODIFIED WITH DIPHENYLDICHLOROSILANE FROM WATER–ETHANOL (65:35) ELUENT AT 50°C, AND VALUES OF BIOLOGICAL ACTIVITY, LD (REF. 36)

No.	*Cardiac glycoside*	*Structural formula*	*Molecular weight*	$V_{m,1}^{50°C}$ *(cm^3/g)*	*LD (mg/kg)*
1	G-strophanthin		584	0.72 ± 0.02	0.116
2	Corelborin-π		696	2.09 ± 0.09	0.104
3	K-strophanthoside		872	2.18 ± 0.09	0.126
4	Convallatoxin		550	2.36 ± 0.05	0.079
5	Olitoriside		680	2.44 ± 0.02	0.103
6	K-strophanthin-β		694	2.83 ± 0.03	0.120
7	Desglucocheirotoxin		550	2.87 ± 0.03	0.096
8	Erysimin		534	3.90 ± 0.08	0.087

9	Desacetyl-lanatoside C		942	5.3 ± 0.2	0.228
10	Cymarin		548	6.0 ± 0.1	0.111
11	Lanatoside C		984	8.6 ± 0.4	0.280
12	Digoxin		780	10.4 ± 0.6	0.280
13	Lanatoside B		984	18.6 ± 0.9	0.403
14	Oleandrin		576	20.4 ± 0.1	0.197
15	Lanatoside A		967	46.5 ± 0.5	0.380
16	Digitoxin		764	58.3 ± 3.5	0.370
17	Acetyldigitoxin		806	107.2 ± 7.5	0.447

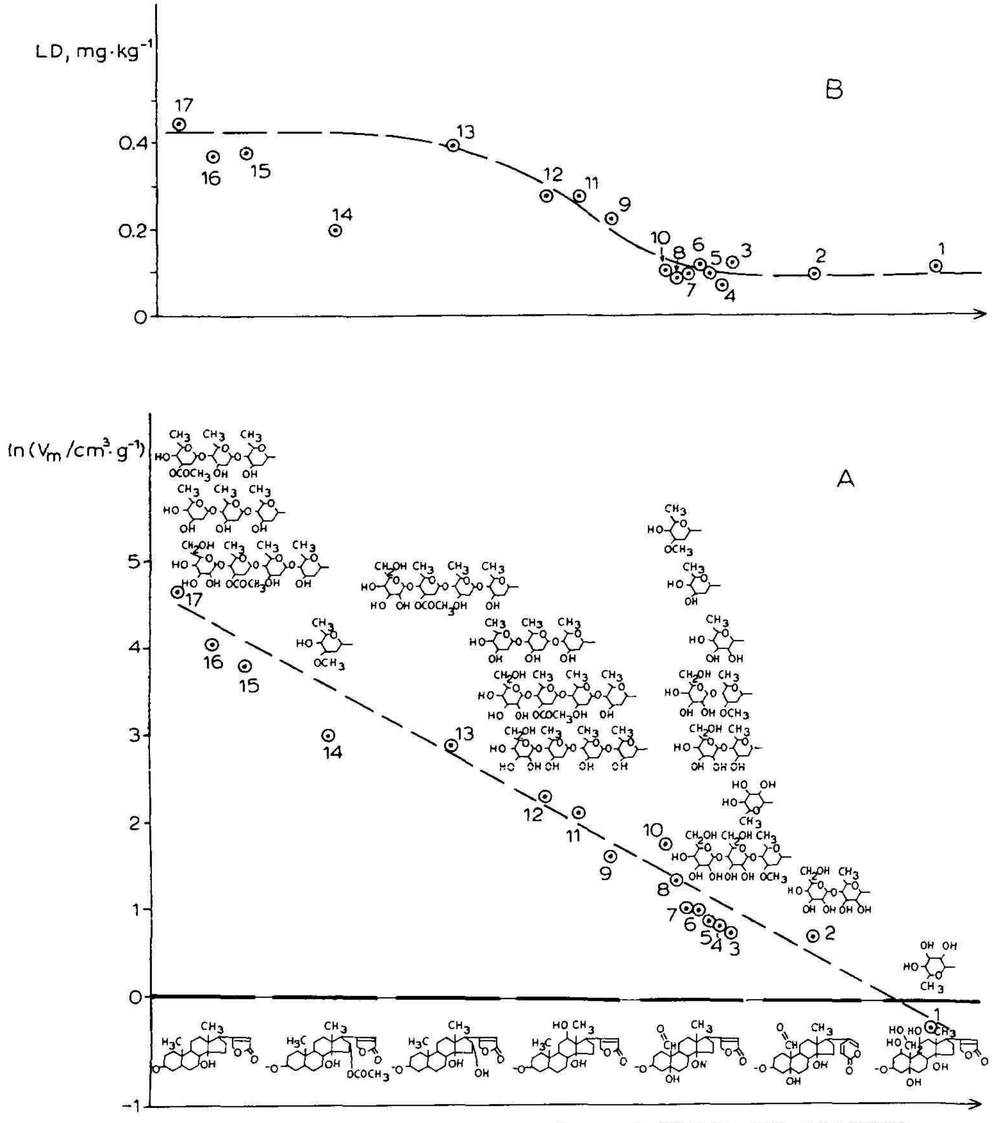


Fig. 11. Correlation of (A) ln $V_{m,1}$ and (B) biological activity, LD, of cardiac glycosides with the hydrophilic properties of their glycones and aglycones. Numbers indicate the corresponding glycosides in Table I.

the number of hydroxyl groups, their position in the steroid part and the presence of other groups such as aldehyde and acetoxy) and, for a given aglycone, in sequence according to the increase in the number of hydroxyl groups in the glycone. The $V_{m,1}$ values for the adsorption of cardiac glycosides on silica gel with attached diphenylsilyl groups from water–ethanol solution depend mainly on the hydrophilicity of the aglycones and to a smaller extent on the hydrophilicity of the glycones. Fig. 11 also

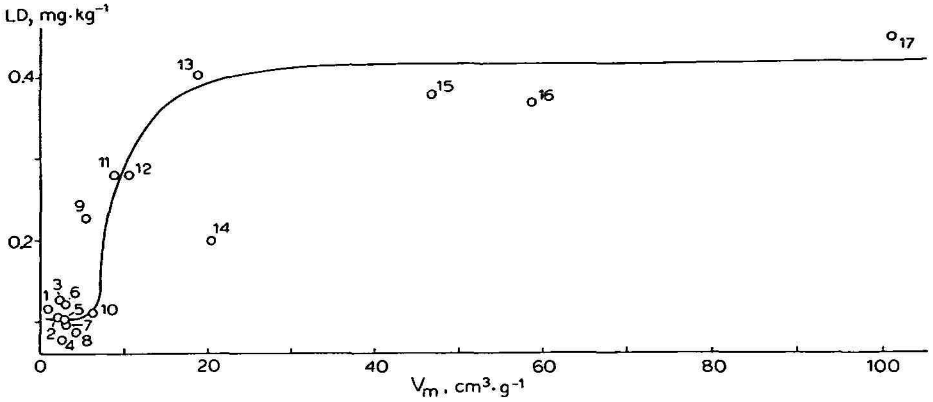


Fig. 12. Correlation between the biological-activity, LD, of cardiac glycosides and their retention volumes, $V_{m,1}$, on the hydrophobic silica gel surface from water–ethanol eluent. Numbers indicate the corresponding glycosides in Table I.

shows the values of biological activity LD (ref. 36), expressed in milligrams of glycoside per kilogram of cat body-weight.

Fig. 12 shows the relationship between the biological activity of the cardiac glycosides and the retention volumes on adsorption on the hydrophobic silica gel surface containing diphenylsilyl groups. At values of $V_{m,1}$ up to *ca.* 6 cm^3/g, the biological activities of different glycosides are approximately the same and maximal (0.1 mg/kg). For $V_{m,1} > 18$ cm^3/g the cardiac glycosides manifest nearly identical biological activities but four times lower in comparison than those of the glycosides in the first group. Glycosides with $V_{m,1}$ between 6 and 18 cm^3/g have moderate biological activity.

The comparison of the biological activities of cardiac glycosides with their retention volumes on the hydrophobic surface of silica gel may demonstrate that the biological activity is connected with the transport of the glycosides to the corresponding receptor. The difference in the biological activities of different cardiac glycosides is also connected with the existence of the lactone ring in the steroid part of their molecules. The destruction of the lactone ring under the action of ultraviolet light, for example, results in the loss of biological activity[38].

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AROMATIC COMPOUNDS ON POLYCHLORAL

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SUMMARY

Chloral was polymerized by the lithium alkoxide of cholesterol and the polymer obtained was used as a packing material for high-performance liquid chromatography. Various types of aromatic compounds were chromatographed on the polychloral column mostly with methanol as carrier solvent, and correlations between the relative retention times of the solutes and their ^{13}C nuclear magnetic resonance chemical shifts were found. Three isomers of xylene were separated with this column at lower temperatures with a methanol–water mixture as carrier.

INTRODUCTION

Chloral can be polymerized to an isotactic, helical polymer with anionic initiators such as lithium alkoxides[1–4]. The polychloral obtained is insoluble in all solvents and can be successfully used as a column packing material for liquid chromatography. In a previous paper[5] we reported that polychloral, initiated by the lithium alkoxide of cholesterol, is capable of partially resolving isotactic polymers of *R*-(+)- and *S*-(−)-α-methylbenzyl methacrylates. We suggested that the resolution resulted from the specific interaction between the optically active polychoral helix of one screw-sense and the two poly(α-methylbenzyl methacrylate)s.

In this work, various types of aromatic compounds were chromatographed on polychloral and correlations between the relative retention times of these solutes and their ^{13}C nuclear magnetic resonance (NMR) chemical shifts were found.

EXPERIMENTAL

Chloral was purified by refluxing over phosphorus pentoxide for 1 day followed by fractional distillation in a nitrogen atmosphere[6,7].

The polymerization of chloral was carried out with the lithium alkoxide of cholesterol at 0°C in hexane according to a method previously described[1,2,5]. The mole percentage (mol %) of the initiator used relative to chloral was 0.2. The polymer plug obtained was grated and the powder stabilized by successive treatments with 10% hydrochloric acid in methanol and a 0.5 *M* solution of phosphorus pentachloride in carbon tetrachloride[8,9]. The polymer was then extracted with acetone for 3 days in a Soxhlet apparatus and dried at 0.1 mmHg. The polychloral particles of diameter 44–105 μm were prepared by grinding the stabilized polymer powder in a small ball-mill and subsequent sifting through a sieve. The particles were suspended in a carrier solvent and packed into a stainless-steel column (50 × 0.72 cm) with the pressure increased to 100 kg/cm^2.

A JASCO Tri Rotar-II liquid chromatograph was used with either a JASCO UV-254-II UV detector operating at 254 nm or with a Shodex RI SE-11 refractive index (RI) detector. The temperature of the column was controlled by jacketing the column and pumping water or ethanol through the jacket from a thermostat bath. The samples were injected as 1–5-μl aliquots from 1–5% solutions in the carrier solvent. The number of theoretical plates for the column was determined from the benzene peak except for the experiment with toluene carrier where pentane was used for the determination.

The ^{13}C NMR spectra were measured on a JEOL JNM FX-100 Fourier transform NMR spectrometer at room temperature and at 25 MHz. Tetramethylsilane was used as an internal standard. The ^{13}C chemical shifts of polycyclic aromatic hydrocarbons and of substituted benzenes were determined in 2 and 5% (w/v) solutions, respectively, in [^{2}H]chloroform; the spectra of dihydroxybenzenes were measured in [^{2}H]methanol.

RESULTS AND DISCUSSION

A mixture of several aromatic hydrocarbons was chromatographed on polychloral prepared with the lithium alkoxide of cholesterol in hexane. Table I shows the relative retention times of these hydrocarbons with toluene, hexane and methanol as carrier solvents. When the polychloral was used with toluene, short retention times and poor separations resulted. Some improvements were achieved with hexane but, with methanol as the carrier, the retention times were even longer and good separation was obtained. Fig. 1 shows the chromatograms obtained with methanol as a carrier at different temperatures. The resolution was improved with an increase in the temperature and complete separation was observed at 50 and 60°C.

Vogl and Hatada[10] measured the ^{1}H NMR line-widths of aromatic solvents including toluene and xylenes in a polychloral matrix and found that the line-broadening of the signal of the entrapped solvent molecule was greater for the aromatic protons than for those of the aliphatic substituents. They suggested a charge-transfer-type interaction between the aromatic ring and the trichloromethyl group as the latter has an electron-withdrawing ability. The higher affinity of toluene for polychloral may cause "squeezing-out" of the solute molecules from the polychloral stationary phase and thus result in poor resolution. However, when methanol is used as carrier, elution of the aromatic solute molecules is delayed and separation is improved because of the decreased affinity of the methanol carrier for the polychloral.

A correlation between ^{13}C NMR chemical shifts of polycyclic aromatic hydrocarbons and their relative retention times in high-performance liquid chromatogra-

TABLE I

RELATIVE RETENTION TIMES OF SEVERAL AROMATIC HYDROCARBONS ON POLYCHLORAL AT 24°C

Column: Polychloral (44–105 μm) in a 50 × 0.72 cm I.D. stainless-steel column; flow-rate, 1.0 ml/min.; pressure, 20–50 kg/cm^2 depending on the mobile phase.

Hydrocarbon	*Mobile phase**				
	Toluene (2640)	*Hexane (3067)*	*Methanol (2300)*		
			24°C	*50°C*	*60°C*
Benzene	1.00	1.00	1.00	1.00	1.00
Toluene	–	1.02	1.14	–	–
p-Xylene	1.01	1.04	1.54	1.34	1.28
Naphthalene	1.02	1.14	1.39	1.35	1.33
Biphenyl	–	1.13	1.76	1.73	1.64
Phenanthrene	1.03	1.42	2.41	2.38	2.27
Anthracene	1.04	1.49	3.58	2.97	2.74
Pyrene	1.05	1.62	4.05	3.61	3.47
1,3,5-Triphenylbenzene	–	1.43	8.95	7.46	6.52

* Values in parentheses represent the number of theoretical plates determined at 24°C.

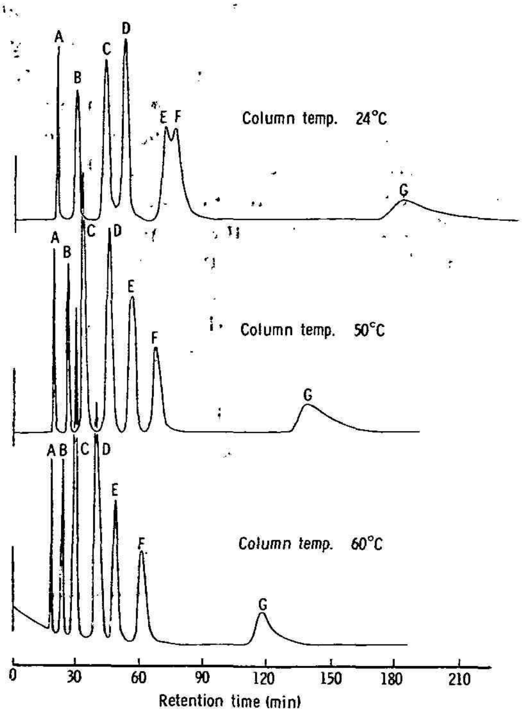


Fig. 1. HPLC separation of aromatic hydrocarbons on polychloral at different temperatures with methanol as carrier. Peaks: A = benzene; B = naphthalene; C = biphenyl; D = phenanthrene; E = anthracene; F = pyrene; G = 1,3,5-triphenylbenzene. Column, polychloral (44–105 μm) in a 50 × 0.72 cm I.D. stainless-steel column; flow-rate, 1.0 ml/min; pressure, 50 kg/cm^2.

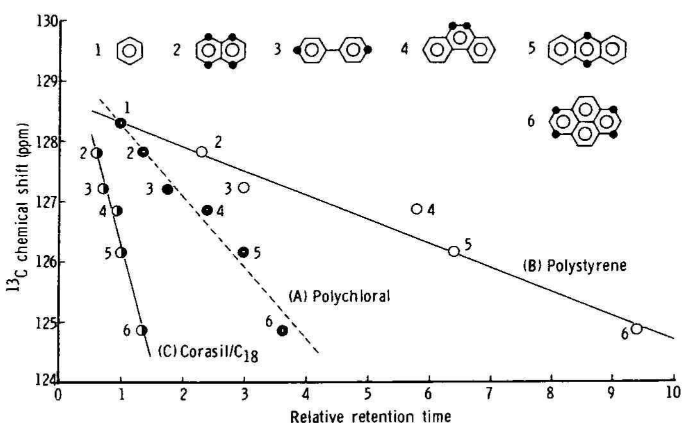
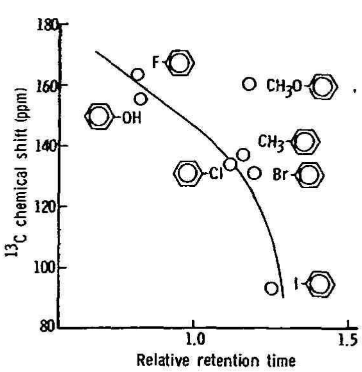


Fig. 2. Correlation between ^{13}C chemical shifts of aromatic hydrocarbons and their relative retention times in HPLC on (A) polychloral (44–105 μm) in a 50 × 0.72 cm I.D. stainless-steel column; solvent, methanol; flow-rate, 1.0 ml/min; pressure, 50 kg/cm^2; temperature, 50°C; number of theoretical plates, 2300; (B) polystyrene gel (7 μm) in a 25 × 0.72 cm I.D. stainless-steel column; solvent, methanol; flow-rate, 1.0 ml/min; temperature, 21°C; number of theoretical plates, 4800; (C) Corasil/C_{18} (37–50 μm) in a 105 × 0.22 cm I.D. stainless-steel column; solvent, methanol–water (3:1); flow-rate, 0.5 ml/min; pressure, 650 p.s.i.; temperature, 25°C (data for Corasil/C_{18} taken from ref. 13).

phy (HPLC) on polychloral was found and is shown in Fig. 2. The ^{13}C chemical shift values are for those carbon atoms at which the frontier electron density is highest[11,12]. The points marked in the structural formulae in Fig. 2 indicate the position of the highest density of the frontier electrons. The ^{13}C chemical shift for the aromatic-ring carbons is considered to be controlled mainly by the π-electron density and shows an upfield shift when the density on the carbon atom increases. The results in Fig. 2 indicate that the frontier electrons of these hydrocarbons play an orienting or guiding role in the retention process and that the charge-transfer-type interaction between the aromatic hydrocarbons and polychloral takes place at the position of the highest frontier-electron density. Linear relationships between the ^{13}C NMR chemical shifts and the relative retention times were also observed in HPLC on polystyrene gel with methanol as carrier or on Corasil/C_{18} with methanol–water (3:1) as carrier (Fig. 2). The separation may be caused by π-electron interactions with the polystyrene gel and by Van der Waals interactions with the Corasil/C_{18}–methanol–water system. All the results mentioned here indicate that the frontier electrons play an important role in the retention processes for aromatic hydrocarbons.

1,3,5-Triphenylbenzene had a very long retention time, as shown in Table I and Fig. 1, and *p*-diphenylbenzene was not eluted from the column. The significance of these phenomena is not clear at present. Perhaps the terminal phenyl rings of these compounds geometrically fit the neighbouring trichloromethyl groups of polychloral with the molecules being tightly adsorbed on the polychloral surface.

In Fig. 3 are plotted the ^{13}C chemical shift values for the substituted carbon (*ipso*-carbon) atoms of various monosubstituted benzenes against their relative retention times. The retention times increase as the chemical shift values of the *ipso*-carbons shift upfield, *i.e.* as the electron densities of the carbons increase. In disubstituted benzenes similar correlations were observed between the average chemical-



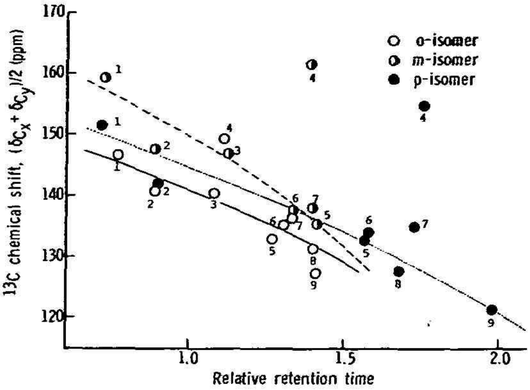
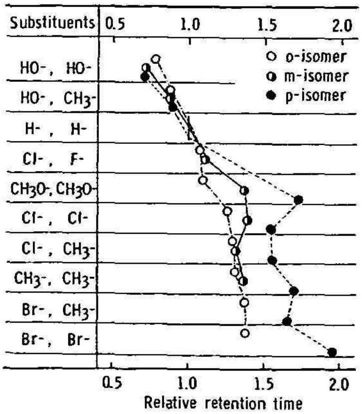


Fig. 3. Correlation between ^{13}C chemical shifts of *ipso*-carbons in monosubstituted benzenes and their relative retention times in HPLC on polychloral at 24°C with methanol as carrier.

Fig. 4. Correlation between the average chemical shift of substituted carbons in disubstituted benzenes and their relative retention times in HPLC on polychloral at 24°C with methanol as carrier. Compounds: 1 = dihydroxybenzene; 2 = hydroxymethylbenzene; 3 = chlorofluorobenzene; 4 = dimethoxybenzene; 5 = dichlorobenzene; 6 = chloromethylbenzene; 7 = dimethylbenzene; 8 = bromomethylbenzene; 9 = dibromobenzene.



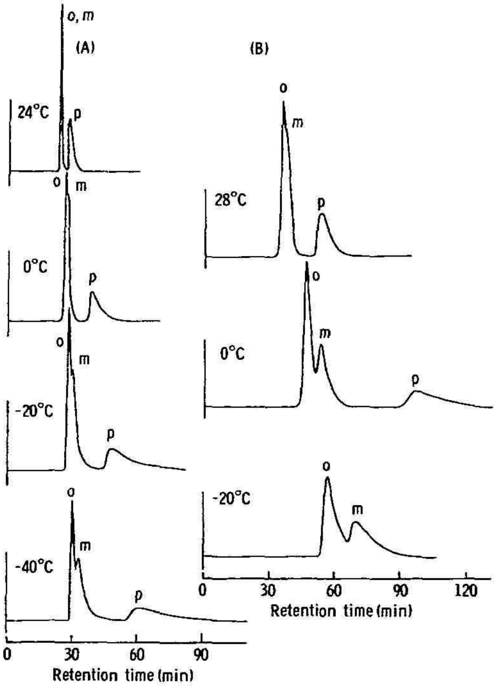


Fig. 5. Relative retention times of disubstituted benzenes in HPLC separation on polychloral at 24°C with methanol as carrier.

Fig. 6. HPLC separation of xylene isomers on polychloral at various temperatures with (A) methanol and (B) methanol–water (9:1) as a carrier solvent. Column, polychloral (44–105 μm) in a 50 × 0.72 cm I.D. stainless-steel column; flow-rate, 1.0 ml/min; pressure, 30–80 kg/cm^2 depending on the temperature and the mobile phase.

shift values of the two *ipso*-carbon atoms and their relative retention times, as shown in Fig. 4. These results indicate that the π-electron densities at the *ipso*-carbons are of significance in the retention process. However, the effect of interactions between the dipole of the substituent–*ipso*-carbon bond in the solute and the dipole of a trichloromethyl group in the polychloral matrix cannot be ruled out, since changes in the ^{13}C chemical shift values could be attributed to the difference in the electronegativities of the substituents. The deviation of the plots for methoxy-substituted benzenes from the curves may suggest an additional interaction between the lone-pair electrons of the oxygen atom and the polychloral. On the other hand, the plots for the hydroxy-substituted benzenes fit the curves, owing to the strong affinity of the solute for methanol. This would compensate for the interaction between the electron pair of the oxygen atom and the polychloral.

Fig. 5 shows the relative retention times of various disubstituted benzenes on polychloral. The order of elution was *o*-, *m*- and *p*-isomers for most of the disubstituted benzenes. The ^1H NMR line-broadening of the phenyl proton signal of xylenes in the polychloral matrix increased in the order of *o*- and *p*-isomers, indicating a stronger interaction of *p*-xylene with polychloral than that of *o*-xylene[10]. The two *ipso*-carbons are further apart from each other in *p*- than in *o*-isomers. Therefore the *p*-isomer interacts with more adsorption sites on the polychloral matrix than the *o*-isomer. This may be one of the reasons for the elution order of the three isomers, but it is not completely understood at present. For dihydroxybenzenes and hydroxytoluene the elution order was reversed. In these hydroxy-substituted benzenes the interaction between the solute and solvent overcomes the solute–polymer interaction and the order of elution is controlled predominantly by the solvent–solute interaction.

It was difficult to separate the three positional isomers of xylene by liquid chromatography. Fig. 6 shows the chromatographic separation of xylenes using polychloral and methanol or a methanol–water (9:1) mixture. The bands of *o*- and *m*-xylenes overlapped notably when xylenes were chromatographed at room temperature; however the separation improved when the temperature was lowered. Addition of water to the solvent methanol also improved the separation because it decreased the affinity of the carrier for the polychloral matrix. A fairly good chromatographic separation was achieved at −20°C by using a methanol–water mixture as a carrier.

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GAS–LIQUID CHROMATOGRAPHIC ANALYSES

IX*. GLASS CAPILLARY GAS CHROMATOGRAPHY OF ALIPHATIC C_1–C_8 *n*-ALKYL MONOCHLORO ESTERS OF ACETIC, CHLOROACETIC, DICHLOROACETIC AND TRICHLOROACETIC ACIDS

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SUMMARY

The gas chromatography (GC) of *n*-alkyl acetates (CH_3–COOR), chloroacetates (CH_2Cl–COOR), dichloroacetates ($CHCl_2$–COOR) and trichloroacetates (CCl_3–COOR), where the alcohol chain length (R) varied between 1 and 8, and certain of their monochlorinated derivatives, 176 compounds altogether, has been studied on SE-30 and OV-351 glass capillary columns under the same operating conditions. The isomeric monochlorinated esters are eluted in direct order from the 1-chloro to the ω-chloro isomer, the separation of the isomers being complete on OV-351. On SE-30, however, the peaks of the 6- and 7-chlorooctyl esters are partly overlapped. The separation of the mixtures of odd- and even-carbon-number esters was better on a non-polar column.

The GC analysis of the combined mixtures of all isomers resulted in several overlapping peaks, particularly with the chloroacetates. The isomeric chloro esters are eluted on SE-30 in the order mono-, di- and trichloro isomers, whereas on OV-351 the di- and trichloro esters are eluted in the reverse order while the 1-chloroalkyl C_2–C_8 trichloroacetates are eluted before the corresponding monochloro isomers. A different elution order of the compounds is observed on a polar column, 1-chloro and ω-chloro isomers giving rise to the greatest difference in elution order. The gas chromatograms of the mixtures and the relative retention times of the compounds are given.

INTRODUCTION

Recently, the gas chromatographic (GC) separation of mixtures with a wide range of chain lengths of aliphatic *n*-alkyl acetates and their monochlorinated derivatives on SE-30 and Carbowax 20M glass capillary columns has been reported[1]. Also, the GC of a series of *n*-alkyl esters of acetic acid and its chlorinated derivatives has

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[*] For Part VIII, see ref. 2.

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been studied on polar and non-polar columns[2], as have halogenated esters[3].

This paper describes a GC study of homologous series of aliphatic *n*-alkyl acetates, chloroacetates and certain of their monochlorinated derivatives. The parent esters are CH_3COOR, $CH_2ClCOOR$, $CHCl_2COOR$ and CCl_3COOR, where the carbon number of the alcohol chain (R) varied between 1 and 8. The separations of mixtures of the parent esters and their isomeric chloroalkyl derivatives were carried out on a non-polar SE-30 and a highly polar OV-351 glass capillary column under the same operating conditions. The relative retention times for all 176 compounds are given and the retention order on both columns is discussed.

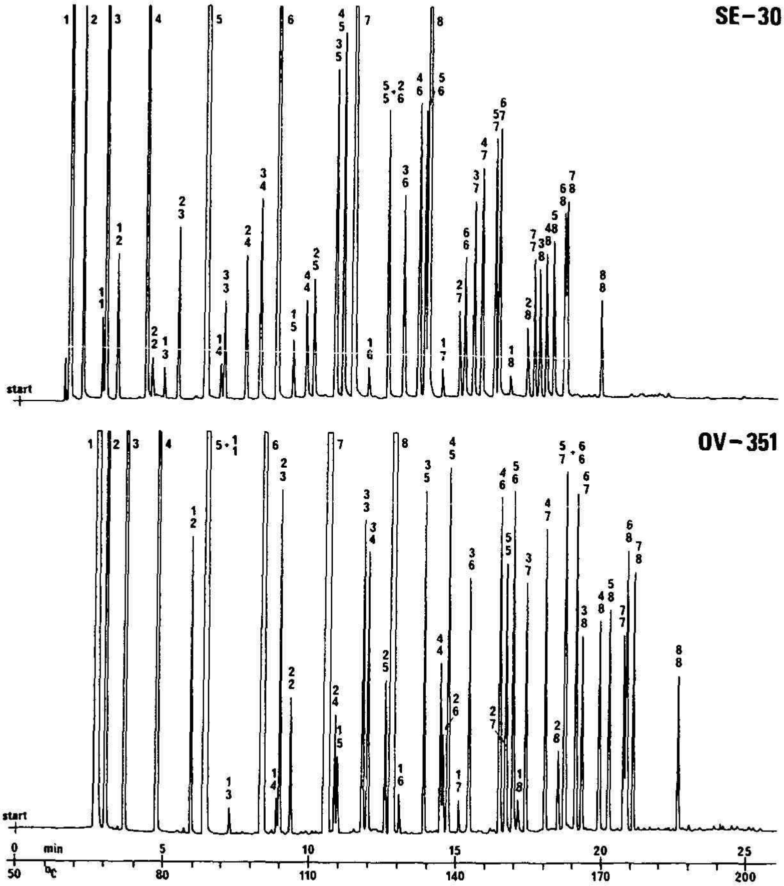

Fig. 1. Chromatograms of the mixture of aliphatic C_1–C_8 *n*-alkyl acetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. Methyl acetate (1) = solvent. Notation of compounds: 1–8 are the parent esters from methyl acetate (1) to *n*-octyl acetate (8); the upper number indicates the position of the Cl-substituent, the lower number the alcohol chain length (*e.g.*, $^{3}_{6}$ denotes 3-chlorohexyl acetate). The additional peaks are unidentified polychloro isomers.

TABLE I

RELATIVE RETENTION TIMES (RRT) OF ALIPHATIC C_1–C_8 *n*-ALKYL ACETATES AND THEIR MONOCHLORINATED DERIVATIVES ANALYSED ON SE-30 AND OV-351 GLASS CAPILLARY COLUMNS

Conditions as shown in Fig. 1.

n-Alkyl acetate, CH_3*–COOR, R=*	*Column*						
	SE-30			*OV-351*			
	*Time (min)**	*RRT***	*RRT****	*Time (min)**	*RRT***	*RRT****	*RRT*§
Methyl	1.65	1.00	0.19	2.65	1.00	0.32	1.61
Chloromethyl	2.80	1.70	0.32	6.34	2.39	0.76	2.26
Ethyl	2.10	1.00	0.24	3.01	1.00	0.36	1.43
1-Chloroethyl	3.30	1.57	0.38	5.94	1.97	0.72	1.80
2-Chloroethyl	4.48	2.13	0.52	9.36	3.11	1.13	2.09
Propyl	2.86	1.00	0.33	3.61	1.00	0.44	1.26
1-Chloropropyl	4.87	1.70	0.56	7.27	2.01	0.88	1.49
2-Chloropropyl	5.39	1.88	0.62	8.99	2.49	1.08	1.67
3-Chloropropyl	6.95	2.43	0.80	11.83	3.28	1.43	1.70
Butyl	4.21	1.00	0.49	4.71	1.00	0.57	1.12
1-Chlorobutyl	6.81	1.62	0.79	8.88	1.89	1.07	1.30
2-Chlorobutyl	7.69	1.83	0.89	10.86	2.31	1.31	1.41
3-Chlorobutyl	8.15	1.94	0.94	11.99	2.55	1.45	1.47
4-Chlorobutyl	9.74	2.31	1.12	14.46	3.07	1.74	1.48
Pentyl	6.21	1.00	0.72	6.34	1.00	0.76	1.02
1-Chloropentyl	9.30	1.50	1.07	10.95	1.73	1.32	1.18
2-Chloropentyl	10.00	1.61	1.15	12.58	1.98	1.52	1.26
3-Chloropentyl	10.75	1.73	1.24	13.92	2.20	1.68	1.30
4-Chloropentyl	11.02	1.77	1.27	14.73	2.32	1.78	1.34
5-Chloropentyl	12.51	2.01	1.44	16.70	2.63	2.01	1.33
Hexyl	8.67	1.00	1.00	8.29	1.00	1.00	0.96
1-Chlorohexyl	11.87	1.37	1.37	13.05	1.57	1.57	1.10
2-Chlorohexyl	12.51	1.44	1.44	14.53	1.75	1.75	1.16
3-Chlorohexyl	13.05	1.51	1.51	15.46	1.86	1.86	1.18
4-Chlorohexyl	13.57	1.57	1.57	16.50	1.99	1.99	1.22
5-Chlorohexyl	13.79	1.59	1.59	16.97	2.05	2.05	1.23
6-Chlorohexyl	15.13	1.75	1.75	18.76	2.26	2.26	1.24
Heptyl	11.26	1.00	1.30	10.45	1.00	1.26	0.93
1-Chloroheptyl	14.39	1.28	1.66	15.11	1.45	1.82	1.05
2-Chloroheptyl	14.98	1.33	1.73	16.70	1.60	2.01	1.11
3-Chloroheptyl	15.45	1.37	1.78	17.42	1.67	2.10	1.13
4-Chloroheptyl	15.72	1.40	1.81	18.08	1.73	2.18	1.15
5-Chloroheptyl	16.18	1.44	1.87	18.76	1.80	2.26	1.16
6-Chloroheptyl	16.33	1.45	1.88	19.11	1.83	2.31	1.17
7-Chloroheptyl	17.55	1.56	2.02	20.78	1.99	2.51	1.18

(Continued on p. 72)

TABLE I *(continued)*

n-Alkyl acetate, CH_3-COOR, R=	*Column*						
	SE-30			*OV-351*			
	*Time (min)**	*RRT***	*RRT****	*Time (min)**	*RRT***	*RRT****	*RRT§*
Octyl	13.80	1.00	1.59	12.70	1.00	1.53	0.92
1-Chlorooctyl	16.75	1.21	1.93	17.14	1.35	2.07	1.02
2-Chlorooctyl	17.31	1.25	2.00	18.51	1.46	2.23	1.07
3-Chlorooctyl	17.74	1.29	2.05	19.32	1.52	2.33	1.09
4-Chlorooctyl	17.97	1.30	2.07	19.92	1.57	2.40	1.11
5-Chlorooctyl	18.20	1.32	2.10	20.27	1.60	2.45	1.11
6-Chlorooctyl	18.60	1.34	2.15	20.85	1.64	2.52	1.12
7-Chlorooctyl	18.66	1.35	2.15	21.10	1.66	2.55	1.13
8-Chlorooctyl	19.85	1.44	2.29	22.64	1.78	2.73	1.14

* Absolute retention times (min) measured from Fig. 1.

** Relative retention times for the parent esters taken as 1.00.

*** Relative retention time for *n*-hexyl acetate taken as 1.00.

§ Relative retention times for compounds on SE-30 taken as 1.00.

EXPERIMENTAL

Gas chromatography

GC analyses were carried out on a Perkin-Elmer Model Sigma 3 gas chromatograph with the following operating conditions: injector temperature, 250°C; flame-ionization detector temperature, 270°C; nitrogen carrier gas flow-rate, 0.9 ml/min; splitting ratio, 1:20; chart speed, 10 mm/min. The two columns used were a vitreous silica SE-30 wall-coated open-tubular (WCOT) column (25 m × 0.22 mm I.D.), supplied by Scientific Glass (North Melbourne, Australia), and a fused silica OV-351 WCOT column (25 m × 0.32 mm I.D.), supplied by Orion Analytica (Espoo, Finland). The column temperature was programmed from 50°C at 6°C/min until elution of peaks had ceased.

Samples

Aliphatic C_1–C_8 *n*-alkyl acetates, chloroacetates, dichloroacetates and trichloroacetates were prepared in our laboratory as described earlier[1,2]. The corresponding chloroalkyl esters were obtained by chlorination of the parent esters with chlorine in the liquid phase[4]. The products were identified by gas chromatography–mass spectrometry (GC–MS). The crude chlorination mixtures were used for GC analyses. The additional peaks illustrated in Figs. 1, 3, 4 and 5 are unidentified higher chlorinated isomers formed in the chlorinations.

RESULTS AND DISCUSSION

Recently, the GC separation of mixtures of aliphatic C_1–C_8 *n*-alkyl acetates and their monochlorinated derivatives on SE-30 and Carbowax 20M glass capillary

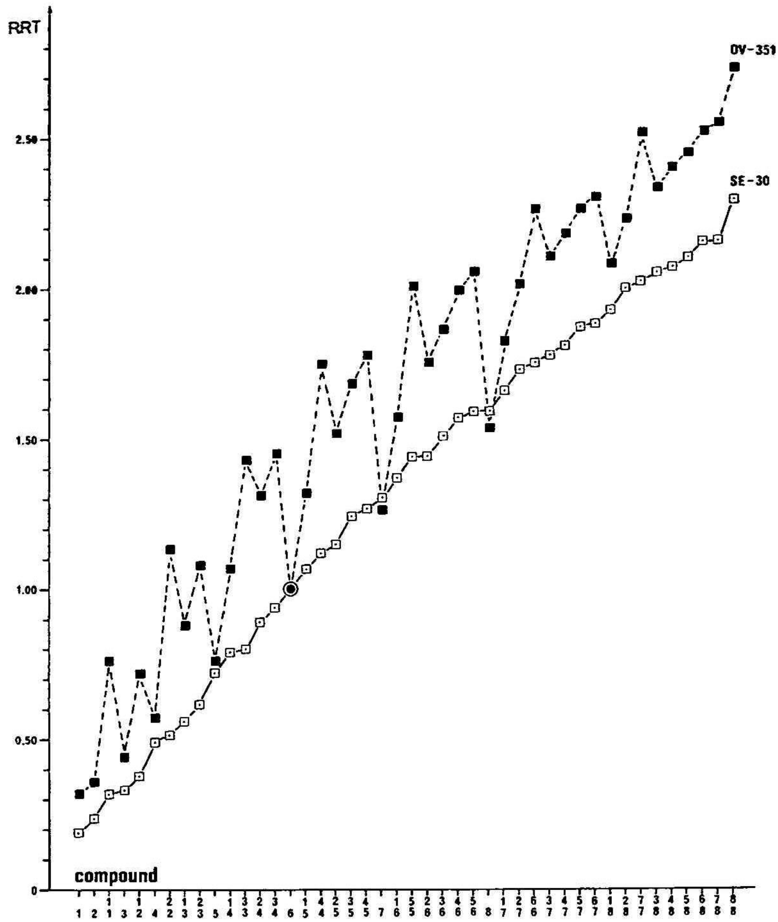

Fig. 2. Relative retention times (RRT) of aliphatic C_1–C_8 *n*-alkyl acetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. Relative retention time for *n*-hexyl acetate (6) taken as 1.00 (see Table I). Notation of compounds as in Fig. 1.

columns has been reported[1]. To compare the GC behaviour, the separation of these acetates has now been studied under the same experimental conditions with their chlorinated derivatives.

The gas chromatograms of the mixture of all C_1–C_8 *n*-alkyl acetates are illustrated in Fig. 1 for both SE-30 and OV-351 columns. Table I gives the absolute and relative retention times of the compounds. All retention times were measured from sample injection and are tabulated relative to the parent esters and relative to *n*-hexyl acetate = 1.00 (Fig. 2). The retentions are also expressed as the ratios of the retention times of the compounds on OV-351 divided by those on SE-30.

The results show that the separations of the esters are similar on a highly polar OV-351 column and, as previously reported, on Carbowax 20M[1]. Methyl and ethyl acetates are completely separated, but 2-chloroheptyl and 5-chloropentyl isomers overlap. *n*-Pentyl and chloromethyl acetates and 5-chloroheptyl and 6-chlorohexyl isomers completely overlap, as on Carbowax 20M.

Figs. 3–5 illustrate the GC separations of the mixtures of odd- and even-carbon-number C_1–C_8 *n*-alkyl chloro-, dichloro- and trichloroacetates and their monochlorinated derivatives, respectively. However, the GC analysis of the combined mixtures of all isomeric esters resulted in several overlapping peaks, making the chromatograms complicated. Tables II–IV give the retention data for the compounds, the retention also being expressed relative to the corresponding *n*-alkyl acetates = 1.00, given in Table I. The retention times relative to *n*-hexyl esters = 1.00 are illustrated in Figs. 6–8.

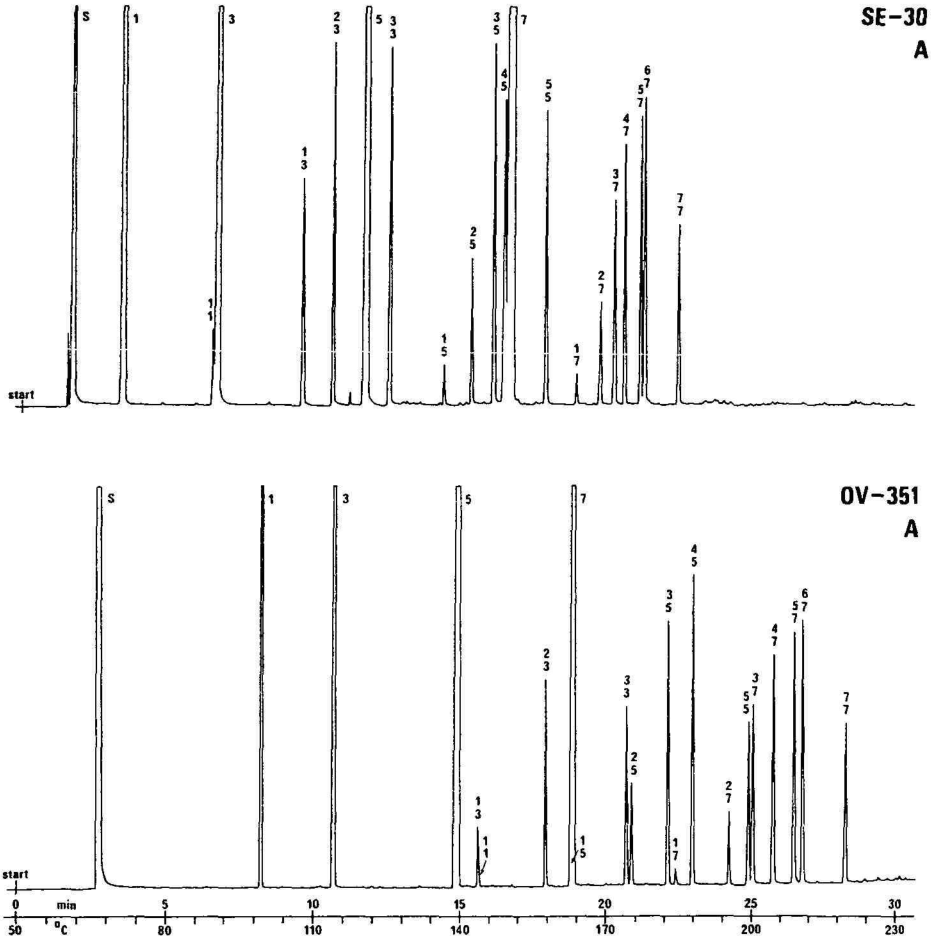

Fig. 3.

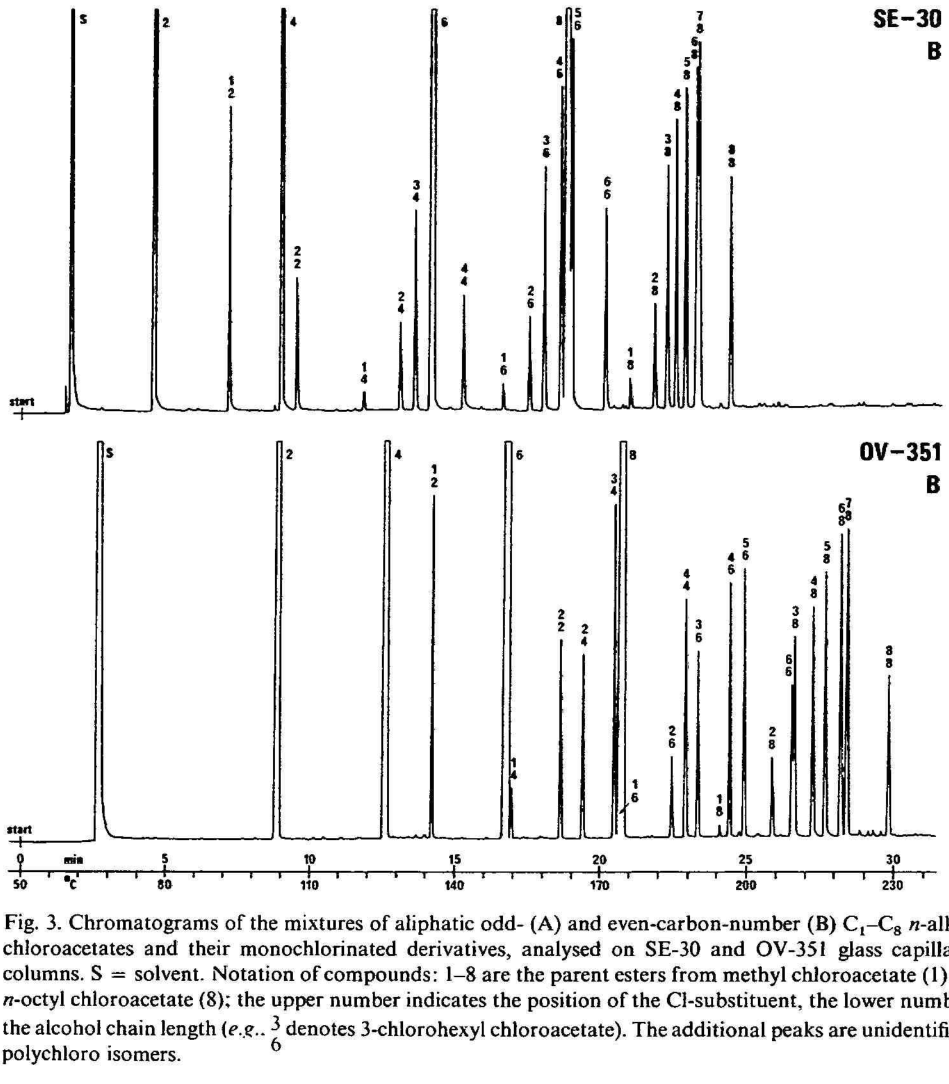

Fig. 3. Chromatograms of the mixtures of aliphatic odd- (A) and even-carbon-number (B) C_1–C_8 *n*-alkyl chloroacetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. S = solvent. Notation of compounds: 1–8 are the parent esters from methyl chloroacetate (1) to *n*-octyl chloroacetate (8); the upper number indicates the position of the Cl-substituent, the lower number the alcohol chain length (*e.g.*, $\genfrac{}{}{0pt}{}{3}{6}$ denotes 3-chlorohexyl chloroacetate). The additional peaks are unidentified polychloro isomers.

Figs. 3–5 show that the separations of the mixtures are better on SE-30 than on OV-351. The isomeric monochlorinated esters are eluted in direct order from the 1-chloro to the ω-chloro compound, their separation being complete on a polar column[1]. On SE-30, however, the peaks of 6- and 7-chlorooctyl esters partly overlap. As can be seen from Fig. 3, the following chloroacetates are poorly separated on SE-30: chloromethyl and *n*-propyl, 4-chloropentyl and *n*-heptyl and *n*-octyl and 5-chlorohexyl esters. Figs. 4 and 5 show that the corresponding di- and trichloroacetates are separated from each other.

TABLE II

RELATIVE RETENTION TIMES (RRT) OF ALIPHATIC C_1–C_8 *n*-ALKYL CHLOROACETATES AND THEIR MONOCHLORINATED DERIVATIVES ANALYSED ON SE-30 AND OV-351 GLASS CAPILLARY COLUMNS

Conditions as shown in Fig. 3.

n-Alkyl chloroacetate, CH_2Cl–COOR, R=	*Column*								
	SE-30				*OV-351*				
	*Time (min)**	*RRT***	*RRT****	*RRT§*	*Time (min)**	*RRT***	*RRT****	*RRT§*	*RRT§§*
Methyl	3.26	1.00	0.23	1.98	8.19	1.00	0.49	3.09	2.51
Chloromethyl	6.41	1.97	0.46	2.29	15.65	1.91	0.94	2.47	2.44
Ethyl	4.49	1.00	0.32	2.14	8.77	1.00	0.53	2.91	1.95
1-Chloroethyl	7.17	1.60	0.51	2.17	14.27	1.63	0.86	2.40	1.99
2-Chloroethyl	9.50	2.12	0.68	2.12	18.62	2.12	1.12	1.99	1.96
Propyl	6.43	1.00	0.46	2.25	10.63	1.00	0.64	2.94	1.65
1-Chloropropyl	9.46	1.47	0.67	1.94	15.65	1.47	0.94	2.15	1.65
2-Chloropropyl	10.52	1.64	0.75	1.95	17.96	1.69	1.08	2.00	1.71
3-Chloropropyl	12.40	1.93	0.88	1.78	20.73	1.95	1.25	1.75	1.67
Butyl	8.90	1.00	0.63	2.11	12.49	1.00	0.75	2.65	1.40
1-Chlorobutyl	11.84	1.33	0.84	1.74	16.94	1.36	1.02	1.91	1.43
2-Chlorobutyl	13.08	1.47	0.93	1.70	19.39	1.55	1.17	1.79	1.48
3-Chlorobutyl	13.58	1.53	0.97	1.67	20.47	1.64	1.23	1.71	1.51
4-Chlorobutyl	15.23	1.71	1.08	1.56	22.93	1.84	1.38	1.59	1.51
Pentyl	11.49	1.00	0.82	1.85	14.74	1.00	0.89	2.32	1.28
1-Chloropentyl	14.25	1.24	1.01	1.53	18.86	1.28	1.14	1.72	1.32
2-Chloropentyl	15.20	1.32	1.08	1.52	20.90	1.42	1.26	1.66	1.38
3-Chloropentyl	15.95	1.39	1.14	1.48	22.16	1.50	1.34	1.59	1.39
4-Chloropentyl	16.35	1.42	1.16	1.48	23.03	1.56	1.39	1.56	1.41
5-Chloropentyl	17.75	1.54	1.26	1.42	24.93	1.69	1.50	1.49	1.40

Hexyl	14.05	1.00	1.00	1.62	16.59	1.00	1.00	2.00	1.18
1-Chlorohexyl	16.61	1.18	1.18	1.40	20.50	1.24	1.24	1.57	1.23
2-Chlorohexyl	17.50	1.25	1.25	1.40	22.45	1.35	1.35	1.55	1.28
3-Chlorohexyl	18.01	1.28	1.28	1.38	23.34	1.41	1.41	1.51	1.30
4-Chlorohexyl	18.57	1.32	1.32	1.37	24.40	1.47	1.47	1.48	1.31
5-Chlorohexyl	18.91	1.35	1.35	1.37	24.91	1.50	1.50	1.47	1.32
6-Chlorohexyl	20.12	1.43	1.43	1.33	26.54	1.60	1.60	1.41	1.32
Heptyl	16.37	1.00	1.17	1.45	18.75	1.00	1.13	1.79	1.15
1-Chloroheptyl	18.82	1.15	1.34	1.31	22.43	1.20	1.35	1.48	1.19
2-Chloroheptyl	19.66	1.20	1.40	1.31	24.25	1.29	1.46	1.45	1.23
3-Chloroheptyl	20.12	1.23	1.43	1.30	25.08	1.34	1.51	1.44	1.25
4-Chloroheptyl	20.49	1.25	1.46	1.30	25.79	1.38	1.55	1.43	1.26
5-Chloroheptyl	21.01	1.28	1.50	1.30	26.51	1.41	1.60	1.41	1.26
6-Chloroheptyl	21.16	1.29	1.51	1.30	26.79	1.43	1.61	1.40	1.27
7-Chloroheptyl	22.31	1.36	1.59	1.27	28.30	1.51	1.71	1.36	1.27
Octyl	18.70	1.00	1.33	1.36	20.55	1.00	1.24	1.62	1.10
1-Chlorooctyl	21.00	1.12	1.49	1.25	24.09	1.17	1.45	1.41	1.15
2-Chlorooctyl	21.81	1.17	1.55	1.26	25.88	1.26	1.56	1.40	1.19
3-Chlorooctyl	22.25	1.19	1.58	1.25	26.61	1.29	1.60	1.38	1.20
4-Chlorooctyl	22.56	1.21	1.61	1.26	27.26	1.33	1.64	1.37	1.21
5-Chlorooctyl	22.89	1.22	1.63	1.26	27.70	1.35	1.67	1.37	1.21
6-Chlorooctyl	23.25	1.24	1.65	1.25	28.20	1.37	1.70	1.35	1.21
7-Chlorooctyl	23.32	1.25	1.66	1.25	28.40	1.38	1.71	1.35	1.22
8-Chlorooctyl	24.41	1.31	1.74	1.23	29.82	1.45	1.80	1.32	1.22

* Absolute retention times (min) measured from Fig. 3.

** Relative retention times for the parent esters taken as 1.00.

*** Relative retention time for *n*-hexyl chloroacetate taken as 1.00.

§ Relative retention times for the corresponding *n*-alkyl acetates (Table I) taken as 1.00.

§§ Relative retention times for compounds on SE-30 taken as 1.00.

TABLE III

RELATIVE RETENTION TIMES (RRT) OF ALIPHATIC C_1–C_8 *n*-ALKYL DICHLOROACETATES AND THEIR MONOCHLORINATED DERIVATIVES ANALYSED ON SE-30 AND OV-351 GLASS CAPILLARY COLUMNS

Conditions as shown in Fig. 4.

n-Alkyl dichloroacetate, $CHCl_2$ COOR, R=	*Column*								
	SE-30				*OV-351*				
	*Time (min)**	*RRT***	*RRT****	*RRT§*	*Time (min)**	*RRT***	*RRT****	*RRT§*	*RRT§§*
Methyl	4.49	1.00	0.29	2.72	10.22	1.00	0.57	3.86	2.28
Chloromethyl	8.06	1.80	0.51	2.88	17.29	1.69	0.96	2.73	2.15
Ethyl	5.97	1.00	0.38	2.84	10.62	1.00	0.59	3.53	1.78
1-Chloroethyl	8.65	1.45	0.55	2.62	15.42	1.45	0.85	2.60	1.78
2-Chloroethyl	11.32	1.90	0.72	2.53	20.45	1.93	1.13	2.18	1.81
Propyl	8.18	1.00	0.52	2.86	12.19	1.00	0.68	3.38	1.49
1-Chloropropyl	11.02	1.35	0.70	2.26	16.45	1.35	0.91	2.26	1.49
2-Chloropropyl	12.21	1.49	0.78	2.27	19.25	1.58	1.07	2.14	1.58
3-Chloropropyl	14.12	1.73	0.90	2.03	22.01	1.81	1.22	1.86	1.56
Butyl	10.65	1.00	0.68	2.53	14.14	1.00	0.78	3.00	1.33
1-Chlorobutyl	13.28	1.25	0.85	1.95	17.78	1.26	0.99	2.00	1.34
2-Chlorobutyl	14.67	1.38	0.94	1.91	20.70	1.46	1.15	1.91	1.41
3-Chlorobutyl	15.16	1.42	0.97	1.86	21.69	1.53	1.20	1.81	1.43
4-Chlorobutyl	16.91	1.59	1.08	1.74	24.30	1.72	1.35	1.68	1.44
Pentyl	13.21	1.00	0.84	2.13	16.01	1.00	0.89	2.53	1.21
1-Chloropentyl	15.60	1.18	1.00	1.68	19.32	1.21	1.07	1.76	1.24
2-Chloropentyl	16.72	1.27	1.07	1.67	21.93	1.37	1.22	1.74	1.31
3-Chloropentyl	17.45	1.32	1.12	1.62	23.12	1.44	1.28	1.66	1.32
4-Chloropentyl	17.89	1.35	1.14	1.62	24.15	1.51	1.34	1.64	1.35
5-Chloropentyl	19.43	1.47	1.24	1.55	26.20	1.64	1.45	1.57	1.35

Hexyl	15.65	1.00	1.00	1.81	18.04	1.00	1.00	2.18	1.15
1-Chlorohexyl	17.92	1.15	1.15	1.51	21.11	1.17	1.17	1.62	1.18
2-Chlorohexyl	18.95	1.21	1.21	1.51	23.52	1.30	1.30	1.62	1.24
3-Chlorohexyl	19.48	1.24	1.24	1.49	24.38	1.35	1.35	1.58	1.25
4-Chlorohexyl	20.12	1.29	1.29	1.48	25.59	1.42	1.42	1.55	1.27
5-Chlorohexyl	20.54	1.31	1.31	1.49	26.24	1.45	1.45	1.55	1.28
6-Chlorohexyl	21.78	1.39	1.39	1.44	27.85	1.54	1.54	1.48	1.28
Heptyl	18.00	1.00	1.15	1.60	19.88	1.00	1.10	1.90	1.10
1-Chloroheptyl	20.14	1.12	1.29	1.40	22.85	1.15	1.27	1.51	1.13
2-Chloroheptyl	21.11	1.17	1.35	1.41	25.17	1.27	1.40	1.51	1.19
3-Chloroheptyl	21.60	1.20	1.38	1.40	25.97	1.31	1.44	1.49	1.20
4-Chloroheptyl	22.04	1.22	1.41	1.40	26.79	1.35	1.49	1.48	1.22
5-Chloroheptyl	22.65	1.26	1.45	1.40	27.64	1.39	1.53	1.47	1.22
6-Chloroheptyl	22.78	1.27	1.46	1.39	27.93	1.40	1.55	1.46	1.23
7-Chloroheptyl	23.95	1.33	1.53	1.36	29.43	1.48	1.63	1.42	1.23
Octyl	20.25	1.00	1.29	1.47	21.79	1.00	1.21	1.72	1.08
1-Chlorooctyl	22.31	1.10	1.43	1.33	24.62	1.13	1.36	1.44	1.10
2-Chlorooctyl	23.21	1.15	1.48	1.34	26.81	1.23	1.49	1.45	1.15
3-Chlorooctyl	23.67	1.17	1.51	1.33	27.57	1.27	1.53	1.43	1.16
4-Chlorooctyl	24.05	1.19	1.54	1.33	28.31	1.30	1.57	1.42	1.18
5-Chlorooctyl	24.44	1.21	1.56	1.34	28.81	1.32	1.60	1.42	1.18
6-Chlorooctyl	24.79	1.22	1.58	1.33	29.35	1.35	1.63	1.41	1.18
7-Chlorooctyl	24.88	1.23	1.59	1.33	29.56	1.36	1.64	1.40	1.19
8-Chlorooctyl	25.96	1.28	1.66	1.31	30.94	1.42	1.72	1.37	1.19

* Absolute retention times (min) measured from Fig. 4.

** Relative retention times for the parent esters taken as 1.00.

*** Relative retention time for *n*-hexyl dichloroacetate taken as 1.00.

§ Relative retention times for the corresponding *n*-alkyl acetates (Table I) taken as 1.00.

§§ Relative retention times for compounds on SE-30 taken as 1.00.

TABLE IV

RELATIVE RETENTION TIMES (RRT) FOR ALIPHATIC C_1–C_8 *n*-ALKYL TRICHLOROACETATES AND THEIR MONOCHLORINATED DERIVATIVES ANALYSED ON SE-30 AND OV-351 GLASS CAPILLARY COLUMNS

Conditions as shown in Fig. 5.

n-Alkyl trichloroacetate, *CCl_3-COOR*, *R* =	*Column*								
	SE-30				*OV-351*				
	*Time**	*RRT***	*RRT****	*RRT§*	*Time**	*RRT***	*RRT****	*RRT§*	*RRT§§*
Methyl	6.29	1.00	0.36	3.81	9.86	1.00	0.57	3.72	1.57
Chloromethyl	9.98	1.59	0.57	3.56	16.19	1.64	0.93	2.55	1.62
Ethyl	7.83	1.00	0.45	3.73	10.09	1.00	0.58	3.35	1.29
1-Chloroethyl	10.38	1.33	0.59	3.15	13.73	1.36	0.79	2.31	1.32
2-Chloroethyl	13.36	1.71	0.76	2.98	20.10	1.99	1.16	2.15	1.50
Propyl	10.18	1.00	0.58	3.56	11.59	1.00	0.67	3.21	1.14
1-Chloropropyl	12.70	1.25	0.73	2.61	14.79	1.28	0.85	2.03	1.16
2-Chloropropyl	14.08	1.38	0.81	2.61	18.62	1.61	1.07	2.07	1.32
3-Chloropropyl	15.98	1.57	0.91	2.30	21.40	1.85	1.23	1.81	1.34
Butyl	12.71	1.00	0.73	3.02	13.42	1.00	0.77	2.85	1.06
1-Chlorobutyl	14.94	1.18	0.85	2.19	16.03	1.19	0.92	1.81	1.07
2-Chlorobutyl	16.40	1.29	0.94	2.13	19.93	1.49	1.15	1.84	1.22
3-Chlorobutyl	16.97	1.34	0.97	2.08	21.00	1.56	1.21	1.75	1.24
4-Chlorobutyl	18.76	1.48	1.07	1.93	23.92	1.78	1.37	1.65	1.28
Pentyl	15.10	1.00	0.86	2.43	15.40	1.00	0.89	2.43	1.02
1-Chloropentyl	17.15	1.14	0.98	1.84	17.71	1.15	1.02	1.62	1.03
2-Chloropentyl	18.35	1.22	1.05	1.84	21.12	1.37	1.21	1.68	1.15
3-Chloropentyl	19.15	1.27	1.10	1.78	22.32	1.45	1.28	1.60	1.17
4-Chloropentyl	19.61	1.30	1.12	1.78	23.60	1.53	1.36	1.60	1.20
5-Chloropentyl	21.24	1.41	1.22	1.70	25.90	1.68	1.49	1.55	1.22

Hexyl	17.48	1.00	1.00	2.02	17.40	1.00	1.00	2.10	1.00
1-Chlorohexyl	19.40	1.11	1.11	1.63	19.50	1.12	1.12	1.49	1.01
2-Chlorohexyl	20.54	1.18	1.18	1.64	22.72	1.31	1.31	1.56	1.11
3-Chlorohexyl	21.11	1.21	1.21	1.62	23.62	1.36	1.36	1.53	1.12
4-Chlorohexyl	21.80	1.25	1.25	1.61	25.06	1.44	1.44	1.52	1.15
5-Chlorohexyl	22.21	1.27	1.27	1.61	25.85	1.49	1.49	1.52	1.16
6-Chlorohexyl	23.47	1.34	1.34	1.55	27.59	1.59	1.59	1.47	1.18
Heptyl	19.70	1.00	1.13	1.75	19.35	1.00	1.11	1.85	0.98
1-Chloroheptyl	21.55	1.09	1.23	1.50	21.32	1.10	1.23	1.41	0.99
2-Chloroheptyl	22.60	1.15	1.29	1.51	24.38	1.26	1.40	1.46	1.08
3-Chloroheptyl	23.13	1.17	1.32	1.50	25.21	1.30	1.45	1.45	1.09
4-Chloroheptyl	23.60	1.20	1.35	1.50	26.29	1.36	1.51	1.45	1.11
5-Chloroheptyl	24.24	1.23	1.39	1.50	27.31	1.41	1.57	1.46	1.13
6-Chloroheptyl	24.39	1.24	1.40	1.49	27.61	1.43	1.59	1.44	1.13
7-Chloroheptyl	25.52	1.30	1.46	1.45	29.19	1.51	1.68	1.40	1.14
Octyl	21.91	1.00	1.25	1.59	21.27	1.00	1.22	1.67	0.97
1-Chlorooctyl	23.61	1.08	1.35	1.41	23.20	1.09	1.33	1.35	0.98
2-Chlorooctyl	24.62	1.12	1.41	1.42	26.06	1.23	1.50	1.41	1.06
3-Chlorooctyl	25.09	1.15	1.44	1.41	26.85	1.26	1.54	1.39	1.07
4-Chlorooctyl	25.50	1.16	1.46	1.42	27.83	1.31	1.60	1.40	1.09
5-Chlorooctyl	25.95	1.18	1.48	1.43	28.53	1.34	1.64	1.41	1.10
6-Chlorooctyl	26.30	1.20	1.50	1.41	29.08	1.37	1.67	1.39	1.11
7-Chlorooctyl	26.39	1.20	1.51	1.41	29.30	1.38	1.68	1.39	1.11
8-Chlorooctyl	27.49	1.25	1.57	1.38	30.80	1.45	1.77	1.36	1.12

* Absolute retention times (min) measured from Fig. 5.

** Relative retention times for the parent esters taken as 1.00.

*** Relative retention time for *n*-hexyl trichloroacetate taken as 1.00.

§ Relative retention times for the corresponding *n*-alkyl acetates (Table I) taken as 1.00.

§§ Relative retention times for compounds on SE-30 taken as 1.00.

The use of a highly polar OV-351 column resulted in more overlapping peaks, particularly with the chloroacetates (Fig. 3), chloromethyl and 1-chloropropyl, *n*-heptyl and 1-chloropentyl and 3-chlorobutyl and 1-chlorohexyl esters being completely overlapped. Also, a poor separation of 6-chlorohexyl and 3-chlorooctyl chloroacetates is observed. As can be seen from Figs. 4 and 5, however, 1-chloroalkyl di- and trichloroacetates are separated better owing to their relatively short retention times in comparison with the other isomers.

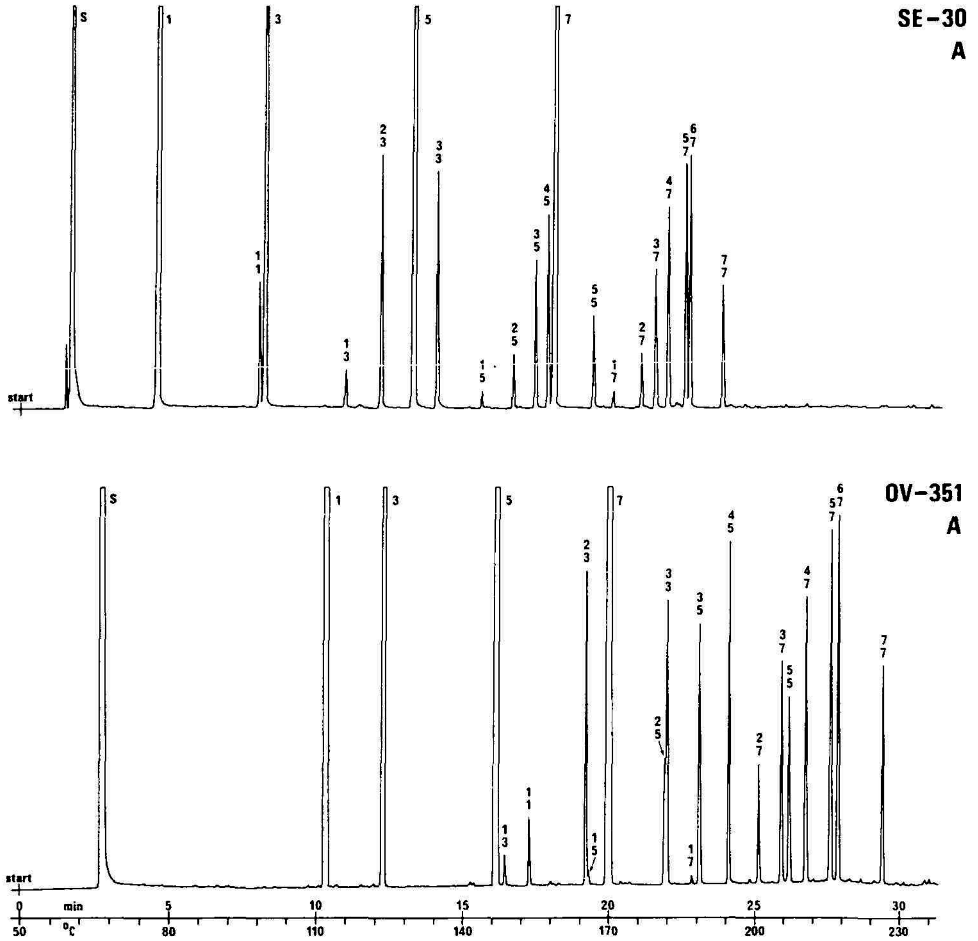

Fig. 4

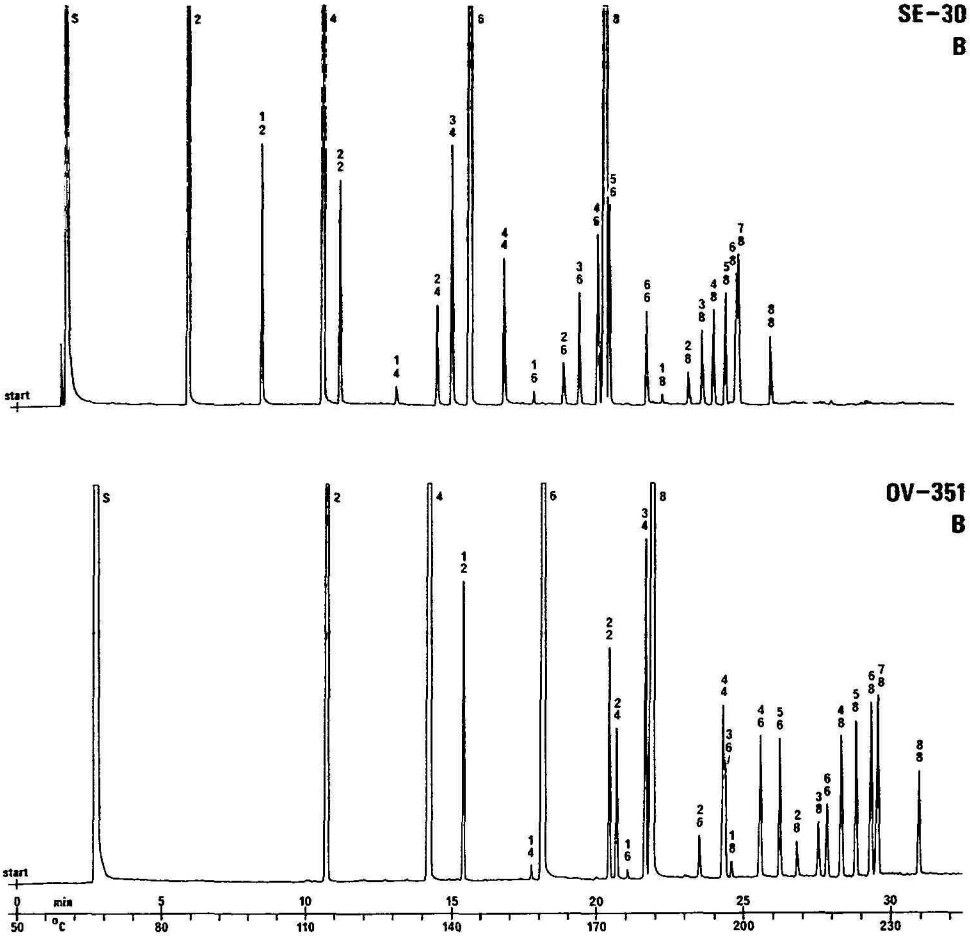

Fig. 4. Chromatograms of the mixtures of aliphatic odd- (A) and even-carbon-number (B) C_1–C_8 *n*-alkyl dichloroacetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. S = solvent. Notation of compounds: 1–8 are the parent esters from methyl dichloroacetate (1) to *n*-octyl dichloroacetate (8); the upper number indicates the position of the Cl-substituent, the lower number the alcohol chain length (*e.g.*, $^{3}_{6}$ denotes 3-chlorohexyl dichloroacetate). The additional peaks are unidentified polychloro isomers.

Tables II–IV show that the chloroacetates are always eluted on SE-30 in the order mono-, di- and trichloro esters. On a polar OV-351 column the elution order is generally mono-, tri- and dichloro esters. With the 1-chloroalkyl esters, except for the chloromethyl isomers, however, trichloroacetates are eluted before monochloro isomers.

The last column of Tables I–IV shows that the retention times of the compounds are generally longer on OV-351 than on SE-30. As can be seen from Figs. 2, 6, 7 and 8, relatively short retention times for the parent esters on a polar column are observed, giving rise to lower values for *n*-hexyl, *n*-heptyl and *n*-octyl acetates (Table I). As with chlorinated chloromethyl esters[5,6], the chlorine substituent at a carbon atom adjacent (*i.e.* at C-1) to the ether oxygen has a stronger effect on the polarity of the compound than a substituent further away. This leads to relatively short retention times of the 1-chloro isomers on a polar column, 1-chloroheptyl and 1-chlorooctyl trichloroacetates being eluted on OV-351 before than on SE-30 (Table IV).

The ω-chloroalkyl esters have longer retention times on a polar column than the other isomers, as have also the ω-chloromethyl[7] and chloromethyl esters[5]. The values for the ω-chloroalkyl acetates (relative to the parent esters = 1.00) varied on OV-351 between 3.28 and 1.78 and on SE-30 between 2.43 and 1.44 (Table I), *n*-propyl acetate giving the highest and *n*-octyl acetate the lowest value. Tables II–IV show that the lower relative retention times for the ω-chloro isomers of chlorinated acetic acids are observed. On OV-351 the values vary from 2.12 to 1.45 for mono-,

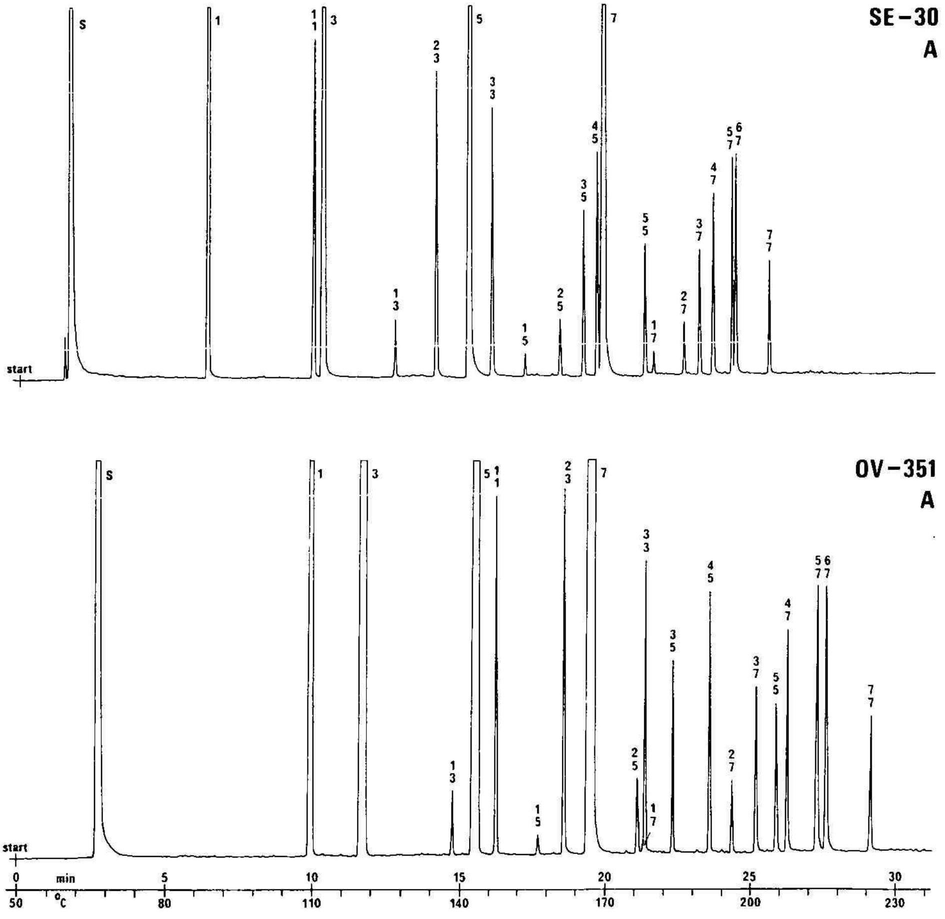

Fig. 5

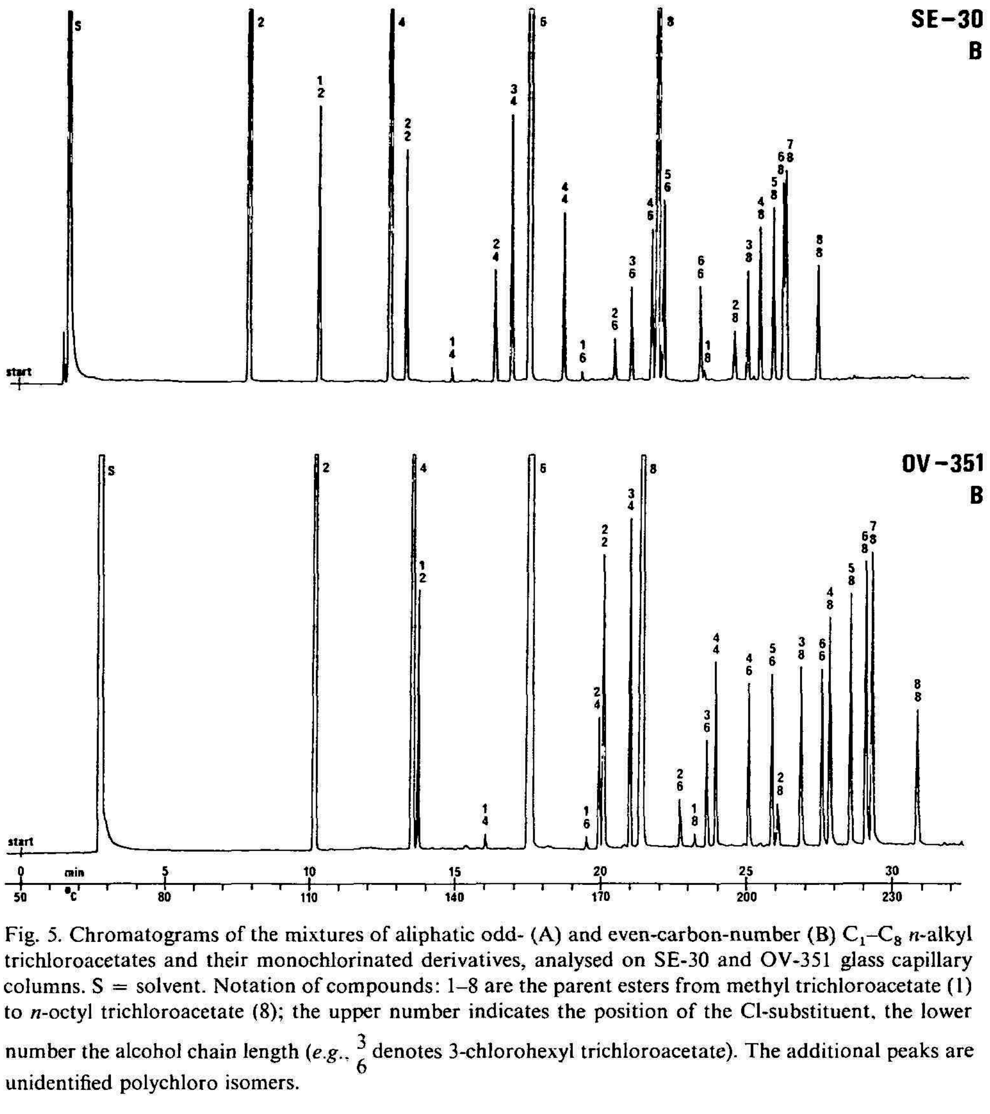

Fig. 5. Chromatograms of the mixtures of aliphatic odd- (A) and even-carbon-number (B) C_1–C_8 *n*-alkyl trichloroacetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. S = solvent. Notation of compounds: 1–8 are the parent esters from methyl trichloroacetate (1) to *n*-octyl trichloroacetate (8); the upper number indicates the position of the Cl-substituent, the lower number the alcohol chain length (*e.g.*, $\overset{3}{6}$ denotes 3-chlorohexyl trichloroacetate). The additional peaks are unidentified polychloro isomers.

from 1.93 to 1.42 for di- and from 1.99 to 1.45 for trichloroacetates. The corresponding values on SE-30 are 2.12–1.31, 1.90–1.28 and 1.71–1.25, respectively. The highest relative retention times for the ω-chloroethyl and the lowest values for the ω-chlorooctyl esters are observed under the operating conditions used.

As can be seen from Figs. 1–8, different elution orders of the esters are observed on OV-351 and on SE-30. By separating the mixtures of closely related compounds, the order of elution on a non-polar column is largely determined by the

TABLE V

ELUTION ORDER OF ALIPHATIC C_1–C_8 *n*-ALKYL ACETATES, CHLOROACETATES, DICHLOROACETATES AND TRICHLOROACETATES AND THEIR MONOCHLORINATED DERIVATIVES ON SE-30 AND OV-351 GLASS CAPILLARY COLUMNS

Compound *R = n-C_1–C_8* *and n-C_1(Cl)–C_8(Cl)*	*Column*	*Elution order of compounds**
CH_3-COOR	SE-30	1 1 2 1 2 1 3 2 3 1 4 2 3 4 1 5 2 3 4 5 1 2 6 3 4 5 6 1 2 7 3 4 5 6 7 8 1 2 1 3 2 4 2 3 3 5 4 3 4 4 6 5 4 5 5 5 7 6 5 + 6 6 6 6 8 7 7 6 7 7 7 7 8 8 7 8 8 8 8 8 8
CH_2Cl-COOR		1 1 1 2 2 1 3 2 3 1 2 4 3 4 1 2 5 3 4 1 5 2 6 3 4 1 5 6 2 3 7 4 5 6 7 8 1 2 1 3 2 4 3 2 3 5 4 3 4 4 6 5 5 4 5 5 7 6 6 5 6 6 8 7 6 7 6 + 7 7 8 7 7 8 8 7 8 8 8 8 8
$CHCl_2$ COOR		1 1 1 2 2 1 3 2 3 1 2 4 3 4 1 2 5 3 4 1 5 2 3 6 4 1 5 6 2 3 7 4 5 6 7 8 1 2 1 3 2 4 3 2 3 5 4 3 4 4 5 6 5 4 5 5 6 7 6 5 6 6 7 8 6 7 7 6 7 8 7 7 8 8 7 8 8 8 8 8
CCl_3-COOR		1 1 1 2 2 1 3 2 3 1 2 4 3 1 4 2 3 5 1 4 5 2 3 6 4 1 5 6 2 3 4 7 5 6 7 8 1 2 1 3 2 3 4 2 3 4 5 3 4 4 5 6 5 4 5 6 5 7 6 6 5 7 6 8 6 7 7 6 7 8 7 7 8 8 8 7 8 8 8 8
CH_3-COOR	OV-351	1 1 1 1 2 2 2 1 3 3 2 1 3 4 2 4 1 3 4 2 5 5 1 3 4 2 5 6 6 3 4 5 7 6 7 8 1 2 3 4 2 5+1 3 6 4 3 2 7 4 5 3 4 5 8 6 5 4 6 5 7 6 6 7 + 5 6 8 7 7 8 7 + 6 7 8 8 8 7 8 8 8
CH_2Cl-COOR		1 1 1 1 2 2 1 2 3 1 3 2 3 1 2 4 4 3 1 2 4 5 5 3 4 2 5 6 3 6 4 5 6 7 7 8 1 2 3 4 2 5 1 + 3 6 4 3 2 7 5 4 4 6 8 3 5 5 7 6 4 5 6 8 7 6 6 5 7 7 8 7 6 8 7 8 8 8 7 8 8
$CHCl_2$-COOR		1 1 1 1 2 1 7 2 2 1 3 2 3 1 3 2 4 4 3 1 2 4 3 5 5 4 2 3 5 6 6 4 5 6 7 7 8 1 2 3 4 2 5 3 1 4 6 3 5 7 2 4 6 4 8 5 3 7 5 6 5 4 6 8 7 6 7 5 6 7 8 8 7 6 7 8 8 8 7 8 8
CCl_3-COOR		1 1 1 1 1 2 1 2 2 3 2 1 3 3 2 1 4 3 4 2 4 3 5 5 2 4 3 5 6 6 4 5 6 7 7 8 1 2 3 4 2 3 5 4 1 6 5 3 7 6 4 2 4 5 8 7 3 5 6 8 5 6 4 7 6 7 6 5 8 7 8 7 6 7 8 8 8 7 8 8

* Notation of compounds: 1–8 are the parent esters from the methyl ester (1) to the *n*-octyl ester (8); the upper number indicates the position of the Cl-substituent, the lower number the alcohol chain length.

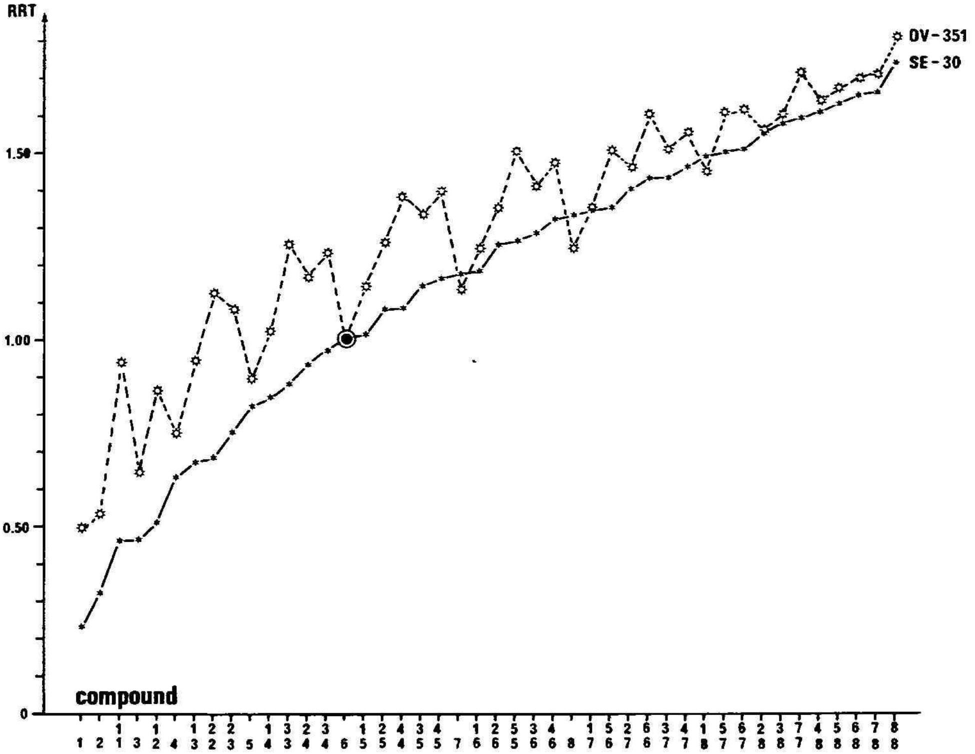

Fig. 6. Relative retention times (RRT) of aliphatic C_1–C_8 *n*-alkyl chloroacetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. Relative retention time for *n*-hexyl chloroacetate (6) taken as 1.00 (see Table II). Notation of compounds as in Fig. 3.

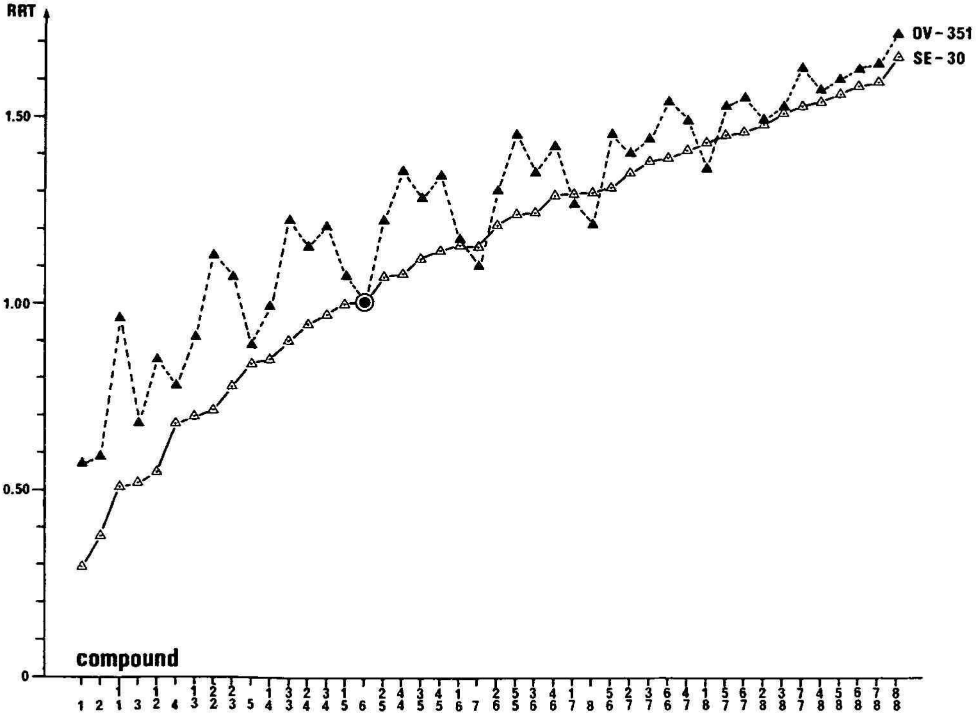

Fig. 7. Relative retention times (RRT) of aliphatic C_1–C_8 *n*-alkyl dichloroacetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. Relative retention time for *n*-hexyl dichloroacetate (6) taken as 1.00 (see Table III). Notation of compounds as in Fig. 4.

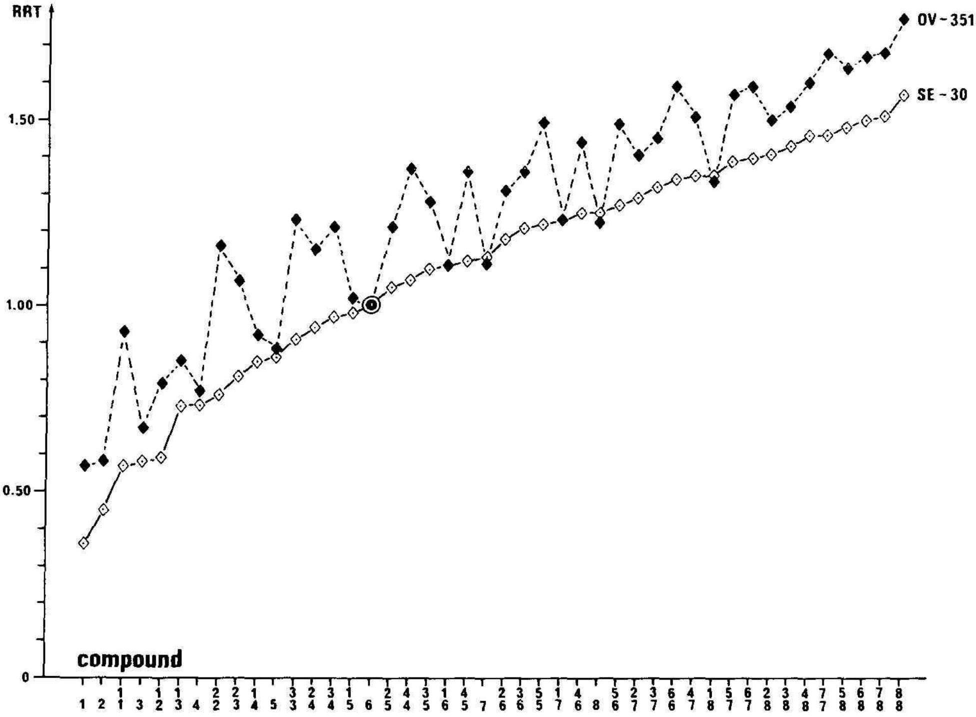

Fig. 8. Relative retention times (RRT) of aliphatic C_1–C_8 *n*-alkyl trichloroacetates and their monochlorinated derivatives, analysed on SE-30 and OV-351 glass capillary columns. Relative retention time for *n*-hexyl trichloroacetate (6) taken as 1.00 (see Table IV). Notation of compounds as in Fig. 5.

boiling point of the compounds. On a polar column, however, the order in which the compounds appear is greatly influenced by their structure[6]. Table V gives the retention order of the compounds. It can be seen that with increasing degree of chlorination 1-chloro and ω-chloro isomers give rise to the greatest difference in elution orders, the former being eluted before and the latter after the other isomers. As expected, this tendency is stronger on OV-351 than on SE-30, particularly with the less polar 1-chloroalkyl trichloroacetates. The decrease in the polarity of the 2- and 3-chloro isomers on increasing the degree of chlorination seems to be smaller.

ACKNOWLEDGEMENT

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QUALITATIVE AND QUANTITATIVE ANALYSIS OF DITERPENE RESIN ACIDS BY GLASS CAPILLARY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

Wall-coated open-tubular glass capillary columns are useful for the qualitative gas–liquid chromatographic analysis of diterpene resin acid methyl ester mixtures. The elution characteristics of 77 compounds on six liquid stationary phases (Silar 10C, BDS, SP-2330, SP-1000, SE-54 and SE-30) are given. For most applications, two stationary phases, one polar and the other non-polar, will suffice. The reproducibility of quantitative data was found to be unacceptable when typical size samples were injected into an inlet splitter by an automatic injector. However, by injecting large samples (over 20 ng per component on to the column) reliable quantitative data are obtainable.

INTRODUCTION

The gas–liquid chromatography (GLC) of diterpene resin acid methyl esters is an important procedure in the study of extractives, oleoresin, rosin and tall oil. Although the esters can range in molecular weight from about 300 to 375 daltons, most of those found in pines and other conifers are related isomers within a molecular weight range of 10 daltons. Separation of many of the isomers by packed column GLC has been achieved with polar liquid stationary phases. Retention data for a large number of diterpene resin acid methyl esters on DEGS and SE-30/EGiP packed columns have been reported as part of a compilation of spectral data[1]. More recent developments in GLC of resin acid esters with packed columns have been reviewed by Zinkel and Engler[2].

The use of wall-coated open-tubular (WCOT) capillary columns for the analysis of resin acids has been attempted by several workers. In 1965, Sandermann and Weissman[3] used stainless-steel WCOT columns coated with BDS and with SE-30 for the GLC of resin acid methyl esters. Although no chromatographic conditions were

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** Maintained at Madison, WI, U.S.A., in cooperation with the University of Wisconsin.

given, one illustration (apparently for a BDS column) showed a resolution that was no better than for a 6-ft. packed column. Partial resolution of the difficult palustrate–levopimarate pair was demonstrated by Weissmann and Simatupang[4] in an analysis of an Indonesian rosin on a 50-m stainless-steel ethylene glycol phthalate WCOT column. From their published chromatogram it can be estimated that the column gave about 20,000 theoretical plates. Hasson and Kulkarni[5] used stainless-steel WCOT columns coated with Apiezon N and with Versamid 930 for analysis of rosin fluxes. Palustrate and isopimarate were resolved on Versamid 930 but not on Apiezon N. The palustrate–levopimarate separation was not considered because levopimarate is not a component of rosin flux; however, levopimarate is extensively decomposed on packed Versamid columns. Claeys[6] used stainless-steel Silar 10C WCOT columns for the analysis of resin acids in kraft mill effluent but did not address the problem of on-column isomerization of abietadienoic acid esters such as methyl palustrate and methyl levopimarate.

Retention data of several resin acids from tall oil have been reported by Holmbom *et al.*[7] for BDS and Apiezon L coated stainless-steel WCOT columns and for OV-101 (a methyl silicone) and SP-1000 (a substituted polyethylene glycol terephthalate) on glass WCOT columns. Holmbom[8] later included glass WCOT columns coated with BDS, Apiezon L, SE-30 and Silar 10C. Complete palustrate–levopimarate resolution was not achieved even with the Silar 10C column, and high on-column losses of levopimarate are evident in the data. Recently, Mayr *et al.*[9] described the chromatography of mixtures of resin acids on glass WCOT columns by temperature programming. FFAP (free fatty acid phase) was shown to separate methyl palustrate and methyl levopimarate; however, methyl isopimarate was not well resolved from methyl palustrate.

All of these attempts to use WCOT capillary columns for the analysis of resin acids have had limited objectives. This work has determined the retention characteristics of a large number of resin acid methyl esters for several columns coated with stationary phases spanning the range of available polarities. This will facilitate selection of columns appropriate for various applications. The quantitative reproducibility of WCOT glass capillary column GLC was evaluated using the response factors for the resin acids common in pines.

EXPERIMENTAL

A Hewlett-Packard Model 5840A gas chromatograph equipped with an 18835B inlet splitter and a flame-ionization detector was used. Retention times and areas of peaks were measured through the data system of the gas chromatograph. WCOT glass capillary columns were obtained from the following sources: SE-30, SE-54 and SP-1000 (a Carbowax derivative similar to FFAP) from J&W Scientific. (Orangevale, CA, U.S.A.), BDS (butane-1,4-diol succinate) and Silar 10C from Altech Assoc. (Deerfield, IL, U.S.A.) and SP-2330 (68 % cyanopropyl silicone) from Supelco (Bellefonte, PA, U.S.A.). The helium carrier gas flow-rate was set at *ca.* 30 cm/sec. Column temperature (see footnotes in Table I) was optimized to resolve the commonly found pine resin acid methyl esters (resolution of the palustrate–levopimarate pair was the criterion) while maintaining reasonable analysis times (elution of methyl pimarate in less than 20 min). For the determination of response factors, resin acid methyl esters were purified by the rigorous methods required in preparing reference standards[1].

RESULTS AND DISCUSSION

Qualitative GLC with WCOT glass capillary columns

Nearly all of the resin acids derived from pine and other conifers belong to four basic diterpene skeletal classes: abietane, pimarane, isopimarane and labdane (Fig. 1). This classification by skeletal types has been used to organize the retention characteristics of 77 resin acid esters as obtained with six stationary phases (Silar 10C, BDS, SP-2330, SP-1000, SE-54 and SE-30; see Table I). These stationary phases were chosen to span the range of liquid phase polarity available. In practice we find that a polar and a non-polar stationary phase are adequate for most applications. Either stationary phase can resolve all the components commonly found in pines, but the two phases are complementary. The polar stationary phase provides good resolution of the common resin acids, but the analysis time may extend to several hours when oxygenated derivatives are present. When oxygenated components are encountered, the non-polar stationary phase reduces analysis times to within reasonable limits. As discussed below, the non-polar SE-30 will resolve all the common resin acids if the

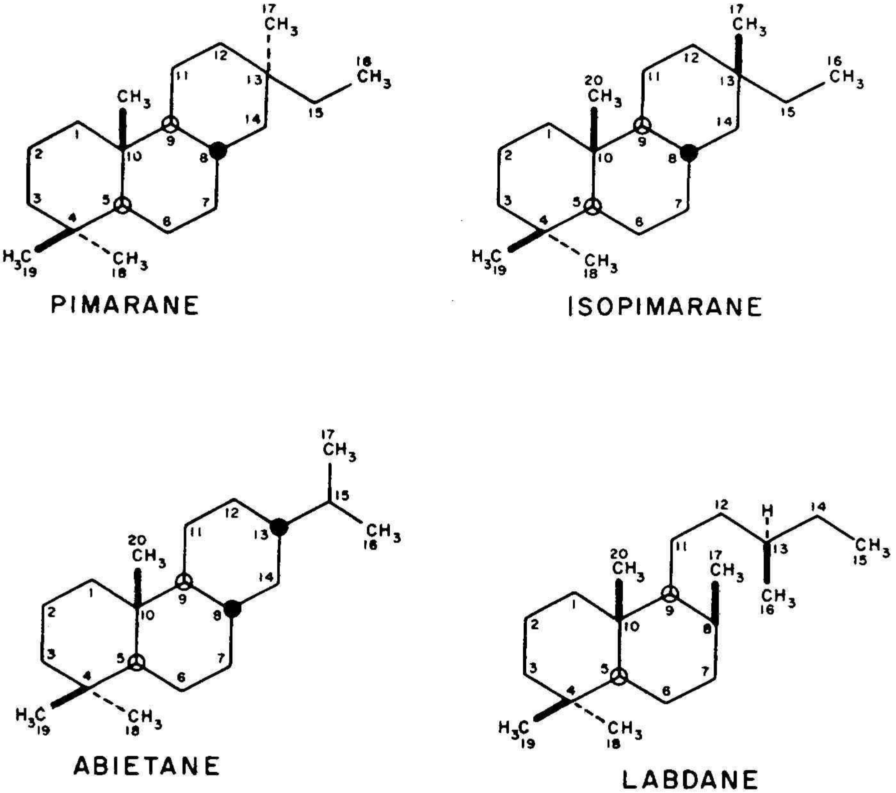

Fig. 1. Parent hydrocarbon structures for common diterpene resin acids (for nomenclature, see refs. 10 and 11).

TABLE I

RETENTION CHARACTERISTICS OF DITERPENE RESIN ACID METHYL ESTERS ON GLASS CAPILLARY GLC COLUMNS

Retentions relative to methyl pimarate.

*Systematic name**	*Common name*	*Silar 10C*** *(230°C)*	*BDS**** *(190°C)*	*SP2330*§ *(190°C)*	*SP1000*§§ *(200°C)*	*SE-54*§§§ *(190°C)*	*SE-30*† *(190°C)*	*SE-30*†† *(170°C)*
Abietane skeleton:								
18-Abietanoate		1.041	1.111	1.098	1.119	1.309	1.319	1.373
13β-Abietan-18-oate		1.012	0.996	1.039	0.986	1.183	1.195	1.212
8α,13β-Abietan-18-oate		1.096	1.202	1.240	1.206	1.379	1.377	1.418
9β,13β-Abietan-18-oate		1.044	1.081	1.087	1.090	1.264	1.281	1.313
7-Abieten-18-oate		1.387	1.551	1.528	1.520	1.508	1.477	1.544
13β-Abiet-7-en-18-oate		1.329	1.389	1.409	1.362	1.381	1.349	1.392
8-Abieten-18-oate		1.026	1.133	1.181	1.141	1.249	1.242	1.287
13β-Abiet-8-en-18-oate		1.082	1.220	1.188	1.229	1.334	1.323	1.371
8(14)-Abieten-18-oate		1.094	1.162	1.158	1.180	1.297	1.300	1.335
13β-Abiet-8(14)-en-18-oate		1.172	1.187	1.217	1.190	1.292	1.287	1.316
13-Abieten-18-oate		1.031	1.123	1.090	1.134	1.211	1.211	1.234
13(15)-Abieten-18-oate		1.431	1.565	1.583	1.560	1.556	1.504	1.570
7,13,15-Abietatrien-18-oate	Abietate	1.897	2.182	2.155	2.071	1.697	1.603	1.703
8,12-Abietadien-18-oate		1.506	1.792	1.768	1.779	1.564	1.476	1.581
8,13-Abietadien-18-oate	Palustrate	1.252	1.396	1.397	1.379	1.287	1.247	1.301
8,13(15)-Abietadien-18-oate		1.597	1.938	1.870	1.932	1.719	1.638	1.760
8(14),13(15)-Abietadien-18-oate	Neoabietate	2.225	2.505	2.571	2.469	2.036	1.892	2.032
13β-Abieta-7,9(11)-dien-18-oate		1.450	1.654	1.637	1.622	1.432	1.368	1.438
8(14),12-Abietadien-18-oate	Levopimarate	1.320	1.354	1.421	1.352	1.282	1.242	1.279
8,11,13-Abietatrien-18-oate	Dehydroabietate	2.117	2.274	2.447	2.098	1.506	1.362	1.439
8,11,13-Abietatrien-19-oate	Callitrisate	1.734	1.808	1.944	1.680	1.303	1.216	1.260
5β-Abieta-8,11,13-trien-18-oate		1.321	1.158	1.358	1.080	0.923	0.885	0.882
5β-Abieta-8,11,13-trien-19-oate		1.539	1.605	1.740	1.511	1.195	1.119	1.164
7,13,15-Abietatrien-18-oate		3.840	4.308	4.829	3.864	2.150	1.866	2.047
6,8,11,13-Abietatetraen-18-oate		2.499	2.662	2.959	2.421	1.501	1.326	1.414
8,11,13,15-Abietatetraen-18-oate		3.884	4.299	4.842	3.863	2.152	1.889	2.045
20-*nor*-Abieta-5,7,9-trien-18-oate		2.566	2.754	3.139	2.531	1.570	1.358	1.445
12α-Methoxy-7,13-abietadien-18-oate	12-Methoxyabietate	3.414	4.108	4.398	3.689	2.323	2.342	2.660
12α-Acetoxy-7,13-abietadiene-18-oate		—	7.91	8.85	6.98	3.950	3.370	3.957
7-Oxo-8,11,13-abietatrien-18-oate		12.91	12.20	17.86	9.36	3.726	2.829	3.217

Pimarane skeleton:								
18-Pimaranoate		0.930	0.912	0.944	0.903	1.087	1.109	1.117
8α-Pimaran-18-oate		1.183	1.171	1.224	1.156	1.309	1.317	1.344
8-Pimaren-18-oate		0.893	0.900	0.929	0.902	1.039	1.045	1.061
8(14)-Pimaren-18-oate	Dihydropimarate	0.946	0.953	0.959	0.962	1.097	1.115	1.160
8,15-Pimaradien-18-oate		0.990	0.981	1.016	0.968	0.966	0.953	0.961
8(14),15-Pimaradien-18-oate	Pimarate	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Isopimarane skeleton:								
18-Isopimaranoate		0.998	1.012	1.024	1.000	1.179	1.322	1.308
8α-Isopimaran-18-oate		1.154	1.130	1.177	1.115	1.275	1.286	1.223
7-Isopimaren-18-oate	Dihydroisopimarate	1.293	1.350	1.370	1.327	1.341	1.319	1.366
8-Isopimaren-18-oate		0.879	0.874	0.906	0.891	1.013	1.015	1.032
8(14)-Isopimaren-18-oate		0.966	0.988	0.988	1.004	1.129	1.145	1.159
7,15-Isopimaradien-18-oate	Isopimarate	1.426	1.470	1.500	1.406	1.241	1.201	1.230
8,15-Isopimaradien-18-oate		0.913	0.889	0.922	0.873	0.879	0.875	0.868
8(14),15-Isopimaradien-18-oate	Sandaracopimarate	1.121	1.122	1.142	1.099	1.065	1.057	1.065
ent-7,15-Isopimaradien-19-oate	Oblongifolate	1.058	1.098	1.065	1.073	1.026	1.019	1.022
Labdane skeleton:								
8(17)-Labdene-15,18-dioate	Pinifolate	3.590	4.353	4.837	3.665	2.625	2.358	2.730
8(17)-*E*-13-Labdene-15,18-dioate	Dehydropinifolate	5.80	7.60	8.20	6.02	3.558	3.073	3.603
8(17),*E*-13-Labdadiene-15,19-dioate	Agathate	4.94	6.23	6.79	5.10	3.158	2.766	3.214
8(17)-Labdene-15,19-dioate	Dihydroagathate	3.073	3.637	4.090	3.101	2.308	2.129	2.407
15-Hydroxy-8(17)-labden-19-oate	Imbricatoloate	–	–	–	–	2.480	2.090	2.405
15-Oxo-8(17)-labden-19-oate	Imbricataloate	4.002	3.325	4.753	2.675	1.698	1.539	1.639
15-Methyl-15-oxo-8(17)-labden-19-oate		4.639	4.025	5.57	3.219	2.132	1.932	2.144
15-Oxo-8(17)-labden-18-oate	(Epiimbricataloate)	4.741	4.128	5.82	3.220	1.950	1.737	1.895
15-Acetoxy-8(17),*E*-13-labdadien-19-oate	(Acetoxyisocupressate)	4.575	5.85	6.55	5.29	3.431	3.040	3.521
8(17),*E*-12,14-Labdatrien-19-oate	Communate	1.242	1.287	1.358	1.196	1.082	1.061	1.100
8(17),*E*-12,14-Labdatrien-18-oate	(Epicommunate)	1.451	1.559	1.645	1.438	1.239	1.228	1.267
8(17),13(16),14-Labdatrien-19-oate	Myrcecommunate	0.889	0.866	0.909	0.835	0.811	0.815	0.816
8,13β-Epoxy-14-labden-19-oate	Manoyl oxide ester	1.302	1.186	1.339	1.079	0.987	0.969	0.974
8,13β-Epoxylabdan-19-oate		1.124	1.074	1.154	1.010	1.091	1.095	1.104
8,15-Epoxy-14-labden-19-oate	Epimanoyl oxide ester	1.276	1.214	1.300	1.132	1.065	1.052	1.062
8(17),13-Labdadien-16,15-olid-19-oate	Pinusolid	–	–	–	–	7.99	4.970	6.22
15,16-Epoxy-8(17),13(16),14-labdatrien-19-oate	Lambertianate	2.463	2.511	2.767	2.240	1.438	1.313	1.397
8(17),*E*-13-Labdadien-15-oate	Anticopalate	1.346	1.482	1.472	1.323	1.286	1.278	1.319
3-Oxo-8(17),*E*-13-labdadien-15-oate	Ketoanticopalate	10.81	9.77	14.28	6.59	3.041	2.474	2.807
3-Acetyl-8(17),*E*-13-labdadien-15-oate	Acetylanticopalate	8.03	10.77	11.83	7.90	4.693	4.037	4.919

(Continued on p. 94)

TABLE I (*continued*)

*Systematic name**	*Common name*	*Silar 10C*** *(230°C)*	*BDS**** *(190°C)*	*SP2330*[§] *(190°C)*	*SP1000*[§§] *(200°C)*	*SE-54*[§§§] *(190°C)*	*SE-30*[†] *(190°C)*	*SE-30*[††] *(170°C)*
Others:								
14S,17-Cyclolabda-8(17),12-dien-18-oate	Strobate	1.600	1.697	1.728	1.673	1.453	1.399	1.446
14S,17-Cyclolabd-8(17)-en-18-oate		1.392	1.423	1.466	1.388	1.355	1.306	1.358
14S,17-Cyclolabd-13-en-18-oate		1.738	1.934	1.:47	1.935	1.804	1.828	1.828
14S,17-Cyclolabdan-18-oate		1.092	1.109	1.130	1.093	1.244	1.254	1.277
8βH,14S,17-Cyclolabdan-18-oate		1.455	1.508	1.551	1.496	1.588	1.568	1.621
2α-[2′-(*m*-Isopropylphenyl)ethyl]-1β,3α-dimethylcyclohexanecarboxylate	Secodehydroabietate	0.881	0.811	0.947	0.772	0.794	0.792	0.802
15,16-Epoxy-3,13(16),14-clerodatrien-18-oate	Hardwickate	4.370	4.657	5.28	3.836	2.116	1.885	2.068
ent-13α,16-Cycloatisan-19-oate		1.032	0.947	0.984	0.945	0.989	0.993	0.981
12-Methoxy-8,11,13-podocarpatrien-19-oate	Podocarpate (methyl ether)	3.863	3.388	4.304	2.900	1.397	1.195	1.250
ent-5-Epicleroda-3,13*E*-dien-15-oate	Happlopappate	7.19	8.80	10.12	6.78	3.823	3.231	3.837
13-Ketopodocarp-8(14)-en-18-oate		18.36	10.49	20.56	7.43	2.406	1.761	1.882
ent-16-Kauren-19-oate		1.499	1.353	1.449	1.306	1.191	1.162	1.160

* See refs. 10 and 11.

** 10-m Silar 10C column, 230°C, u = 30.8 cm/sec, t'_{pim} = 4.16 min.

*** 10-m BDS column, 190°C, u = 30.3 cm/sec, t'_{pim} = 14.03 min.

[§] 10-m SP2330 column, 190°C, u = 30.3 cm/sec, t'_{pim} = 2.52 min.

[§§] 10-m SP1000 column, 200°C, u = 33.3 cm/sec, t'_{pim} = 8.37 min.

[§§§] 10-m SE-54 column, 190°C, u = 30.3 cm/sec, t'_{pim} = 8.55 min.

[†] 10-m SE-30 column, 190°C, u = 30.3 cm/sec, t'_{pim} = 7.82 min.

[††] 10-m SE-30 column, 170°C, u = 28.7 cm/sec, t'_{pim} = 18.12 min.

column temperature is reduced from 190 to 170°C; this, of course, increases the analysis time, and it is more practical to carry out the analysis on the two stationary phases.

The retention data (for BDS and SE-30) from Table I for pimarate, sandaracopimarate, levopimarate, palustrate, isopimarate, abietate, dehydroabietate and neoabietate were compared with those reported by Holmbom *et al.*[7]. The relative retention data for BDS agreed to within 1% even though the column temperatures differed by 5°C. Good agreement was also found for the SE-30 data when we extrapolated the 170 and 190°C retention data to 200°C and compared these data with those reported for 200°C by Holmbom *et al.* Holmbom *et al.* reported that palustrate and levopimarate are not resolved at 200°C on SE-30. Indeed, our data show that while this pair is completely resolved at 170°C, the retention times are predicted to converge at 200°C.

Although data for comparison are limited[2,13], capillary retention characteristics of diterpene resin acid methyl esters generally parallel those from packed columns containing the same stationary phases. For the polar stationary phases (Silar 10C and BDS) the order of elution from the capillary columns remains the same as from the packed column, but the separation factors from the capillary columns increase progressively with retention time.

Zinkel *et al.* have discussed the considerable difficulty in the separation of methyl isopimarate and methyl anticopalate, which have nearly identical retention characteristics on three stationary phases (packed columns) of different polarities. However, these two compounds were resolved on all six of the capillary columns tested.

In packed column GLC analysis of the methylated acidic fraction[14] of oleoresin or extractives, the most perplexing problem has been the resolution of the palustrate–levopimarate pair. This resolution was accomplished only on the polar

TABLE II

RESPONSE CHARACTERISTICS OF DITERPENE RESIN ACIDS ON GLASS CAPILLARY COLUMNS COATED WITH SE-30 AT LOW LOADING

Sample range 3–10 ng applied to the column (*i.e.*, injection of 0.3–1.0 μg in 1 μl of *n*-hexane with a 100:1 split).

Resin acid methyl ester	*$F_c^\star$ for SE-30*			
	Average	*No. of data points*	*Range*	*Standard deviation (σ)*
Abietate	0.986	23	0.835–1.147	0.056
Dehydroabietate	0.965	9	0.927–1.008	0.024
Isopimarate	0.996	16	0.948–1.038	0.018
Neoabietate	0.993	10	0.904–1.153	0.083
Levopimarate	0.989	17	0.951–1.058	0.024

[★] F_c = correction factor $= \frac{A_s W_x}{W_s A_x} = 1/F$ (where F = response factor); when the response of a component X is multiplied by F_c it is corrected to equivalence with the reference component S (in this instance S is pimarate).

stationary phase Silar 10C[2]. However, concurrent with the resolution of palustrate–levopimarate on Silar 10C, the resolution of neoabietate and dehydroabietate was lost. Hence the analysis of a typical mixture of resin acid methyl esters requires GLC using at least two packed columns. On the 10-m glass capillary column coated with SE-30, palustrate and levopimarate can be resolved by lowering the column temperature from 190 to 170°C. Resolution can be effected on the polar stationary phases (BDS and Silar 10C). At 230°C neoabietate and dehydroabietate do not converge on Silar 10C WCOT glass columns.

Caution must be used in applying the data in Table I for the identification of compounds. For example, a peak found on SE-30 at 190°C at $r_{\mathrm{pim}} = 1.323$ should not necessarily be interpreted as resulting from 13β-abiet-8-en-18-oate. There are four other compounds (18-abietanoate, 6,8,11,13-abietatetraen-18-oate, 8α-isopimaran-18-oate and 7-isopimaren-18-oate) that elute within the range of uncertainty of the retention data ($= r_{\mathrm{pim}} \pm 0.005$). In this example, one could apply BDS at 190°C for a clear resolution of identity.

Quantitation by glass capillary GLC

Capillary GLC will provide useful retention data at very low sample levels. However, obtaining reliable quantitative data with less than 10–20 ng applied to the column (*i.e.*, injection of 1–2 μg in 1 μl of *n*-hexane with a 100:1 split) is difficult. For example, the response data in Table II were obtained by introducing 3–10 ng of each component on to the column using an automatic sampling device injecting into an inlet splitter. At the 3σ confidence level, the data in Table II indicate that the error may range from 6 to 25%. A similar lack of reproducibility was recently reported by Lanza *et al.*[15] for the analysis of fatty acid methyl esters on a cyanosilicone stationary phase. Grob and Neukom[16] have also experienced a lack of precision with vaporizing injectors and recommend a cold on-column injection technique as the solution to the problem. Later, Grob and Rennhard[17] recommended a manual injection technique wherein the syringe needle is pre-heated for several seconds before the sample is injected from the syringe barrel. This technique was applied to the analysis of diterpene resin acid esters, and the precision of response data was maintained at levels

TABLE III

COMPARISON OF DETECTOR RESPONSE RELATED TO SAMPLE SIZE

The sample consisted of methyl pimarate and dehydroabietate in approximately a 1:1 mixture.

Sample size (ng)	*Average* $F_c^{\star}$	*Standard deviation* (σ)
500	0.975	0.009
350	0.982	0.011
175	0.966	0.004
85	0.973	0.007
45	0.978	0.004
20	0.983	0.006
10	1.021	0.090

[★] F_c is the correction factor, which is multiplied by the peak area of dehydroabietate to give an equal area response per unit weight of both resin acid esters.

TABLE IV

QUANTITATIVE CORRECTION FACTORS FOR METHYL ESTERS OF RESIN ACIDS ON A 10-m GLASS CAPILLARY COATED WITH SE-30 AT HIGH SAMPLE LOADING

*Ester**	F_c	*Standard deviation* (σ)
Isopimarate	0.999	0.011
Levopimarate	1.025	0.007
Palustrate	1.062	0.004
Dehydroabietate	1.001	0.007
Abietate	0.997	0.010
Neoabietate	1.018	0.010

* The reference compound is pimarate.

down to less than 10 ng applied to the column. Unfortunately, this injection technique cannot be reproduced by most autosamplers.

When using an autosampler with the inlet splitter, accurate results may be obtained by introducing samples of greater than 20 ng per component on to the column (Tables III and IV). It is clear from Table III that the relative response by this method is linear for amounts of sample from 20 to 500 ng. The reproducibility of the response for several resin acid methyl esters is shown in Table IV. This method of quantitation is much easier than the on-column method described by Grob and Neukom[16], and the standard deviations show that the reproducibility is better.

Obviously, for meaningful quantitative results the components must endure the chromatographic process without isomerization or loss. A useful test of packed column activity is the isomerization of certain abietadienoic acid esters (levopimarate and palustrate). On-column transformation is recognized by the elevation of the baseline following the levopimarate and palustrate peaks, the elevation extending through the elution of neoabietate[13]. With capillary columns it is more difficult to observe this transformation because the peaks are usually displayed on the chart at greater attenuation and more closely grouped than with packed columns. Consequently, the baseline deviation is not so apparent. Therefore, it is imperative that response factors are regularly monitored.

Table IV lists the correction factors (reciprocal of response factors) of several of the more common pine resin acid methyl esters, relative to pimarate as determined on the SE-30 column at 190°C. Because the correction factors fall within the range of experimental error of the method, normalization of peak areas will suffice for most analyses.

CONCLUSION

WCOT glass capillary GLC is superior to packed column GLC for the qualitative analysis of diterpene resin acid mixtures. Tentative identification of components may be accomplished more rapidly and with greater certainty than with packed columns.

When samples of less than 10–20 ng per component are introduced on to the capillary column from an inlet splitter, specific injection techniques are necessary for reliable quantitation. Most automatic injectors cannot produce these techniques.

However, it is possible to achieve accurate quantitation by glass capillary GLC using an automatic injector and inlet splitter if samples of adequate size (greater than about 20 ng per component) are used. When the samples involved have widely varying component concentrations, some components will overload the column. As a result of overloading, the analysis must often be repeated at different sampling levels in order to obtain complete retention data and quantitative analysis. However, this usually takes less time than carrying out several analyses on two or more packed columns.

ACKNOWLEDGEMENT

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NOTE ADDED IN PROOF

Mediantsev *et al.*[18] recently claimed to have optimized the resolution of a mixture of common resin acid methyl esters chromatographed on a 50 m × 0.3 mm glass column coated with a 1:1 mixture of EGS–DEGS. However, they did not resolve the levopimarate–palustrate pair as would be expected considering our success with the slightly less polar BDS liquid phase on a 10-m column.

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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF AROMATIC HYDROCARBON METABOLITES FROM LIVERS OF FISH EXPOSED TO FUEL OIL

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SUMMARY

Metabolites of several two- and three-ring aromatic hydrocarbons (AHs) have been found in livers of English sole exposed to No. 2 fuel oil. Four metabolites of the C_2H_5-naphthalenes, six of the C_3H_7-naphthalenes and one each of fluorene, phenanthrene and anthracene have been partially characterized and their concentrations, which ranged from 50 to 1100 ng/g, were determined. Metabolites were separated from the liver matrix using an automated extractor/concentrator. The resulting extract was then purified by high-performance liquid chromatography, and the metabolites were characterized and quantitated by gas chromatography–mass spectrometry.

INTRODUCTION

In many areas, marine waters and sediments contain hundreds of chemicals[1–3], many of which are petroleum-related aromatic hydrocarbons (AHs). Marine organisms accumulate AHs and metabolize them[4–7] to products which may be more deleterious than the parent compounds[8,9]. In order to assess the effects of exposing organisms to a multitude of xenobiotics, techniques are needed for determining the complex mixtures of these chemicals and their metabolites in animal tissues.

Although analyses for parent AHs in tissue samples from aquatic organisms are performed routinely[10–12], the determination of AH metabolites is a more difficult task. The polar metabolites often are non-volatile or labile and, thus, must be determined with special gas chromatographic (GC) columns or procedures. High-performance liquid chromatography (HPLC) can be used to analyze for individual compounds in simple mixtures of polar compounds; however, the resolution is usually insufficient to separate a mixture of metabolites as complex as those resulting from the exposure of organisms to petroleum.

Nevertheless, progress has been made in analyzing for some mixtures of metabolic products and other polar compounds. For example, 2,6-dimethylnaphthalene metabolites can be separated from biological matrices using an automated extractor/concentrator[13], and metabolites in the extracts can be determined by reversed-phase

HPLC with fluorescence detection[14,15]. Alternatively, a method combining silica gel column chromatography, preparative normal-phase HPLC and gas chromatography–mass spectrometry (GC–MS) has been used to analyze for polar compounds in tissue extracts from marine organisms[16].

In this study, we have combined several of these techniques to determine metabolic products resulting from the exposure of English sole (*Parophyrs vetulus*) to No. 2 fuel oil. We used an extractor/concentrator to isolate, HPLC to purify and GC–MS to identify and quantitate AH metabolites from livers of fish exposed to the oil.

EXPERIMENTAL

*Chemicals**

2-Methyl-1,4-naphthoquinone, 9-phenanthrol, α-methyl-2-naphthalenemethanol and 9-hydroxyfluorene were obtained from Aldrich (Milwaukee, WI, U.S.A.), and 4-methyl-1-naphthol, 1-naphthaldehyde and 2,2-dihydroxybiphenyl from K & K Labs (Plainview, NY, U.S.A.). 1-Naphthol was purchased from Sigma (St. Louis, MO, U.S.A.) and 2,6-dimethyl-3-naphthol, 6-methyl-2-naphthalenemethanol and *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene were prepared in our laboratories[7]. Ultrex-grade acetic acid and HPLC-grade water were purchased from J. T. Baker Chemical (Phillipsburg, NJ, U.S.A.). Distilled-in-glass methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

GC–MS standard

A GC–MS standard of the following reference compounds (ng/μl), dissolved in methanol, was prepared: 1-naphthaldehyde (30.8), 2,2′-dihydroxybiphenyl (26.5), α-methyl-2-naphthalenemethanol (26.5), 9-hydroxyfluorene (26.2), 4-methyl-1-naphthol (25.0), 9-phenanthrol (24.2), 2-methyl-1,4-naphthoquinone (26.2), 1-naphthol (34.8), 2,6-dimethyl-3-naphthol (14.9), 6-methyl-2-naphthalenemethanol (7.88), *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (8.65) and hexamethylbenzene (4.10) (internal standard).

Fish exposures

English sole were caught near Meadow Point, WA, and maintained in flow-through seawater aquaria. The water temperature was 9.5 ± 1°C and the salinity was 24 ± 2‰. Fish were exposed to polychlorinated biphenyls (PCBs, Aroclor 1254) and to fuel oil in an LC_{50} study[17]. Eight days prior to fuel oil exposure, fish (13.8 ± 0.6 cm, 21.5 ± 2.3 g) were injected intraperitoneally with 25 μg/g of Aroclor 1254 in *ca.* 100 μl of salmon oil. Control fish received a single i.p. injection of 100 μl of salmon oil carrier. Then, fish in flow-through aquaria were exposed to 50 mg/l of fuel oil for 5 days. At the end of the LC_{50} study, the fish were killed and their livers were removed and frozen until needed for analysis. A water sample (1 l) was collected from the exposure tank at the end of the experiment, extracted, concentrated to 1 ml and analyzed by GC for total hydrocarbon content by comparing the total peak areas of hydrocarbons from the water extract with those from a No. 2 fuel oil standard.

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* Reference to a company or product does not imply endorsement by the U.S. Department of Commerce to the exclusion of others that may be suitable.

Metabolite extraction

Sample preparation. Livers were thawed (seven experimental, four unexposed controls), pooled within groups and then homogenized in 4 parts of 1.15% potassium chloride solution (described previously[13]).

Extraction. Homogenized liver samples were extracted with a Prep I automated extractor/concentrator (DuPont, Wilmington, DE, U.S.A.), which used glass microbeads and Type W extraction cartridges packed with a styrene–divinylbenzene copolymer resin (DuPont)[13,18]. Aliquots (500 and 750 μl, respectively) of liver homogenates (5.0 ml experimental, 1.5 ml control) were into cleaned cartridges, diluted with 1 ml of buffer (1.0 *M* citric acid, 0.2 *M* disodium hydrogen orthophosphate heptahydrate, pH 2.5), and loaded into the extractor/concentrator. The samples were washed with water (10 ml) and extracted twice with 10 ml of acetone–methanol (1:1). The extraction sequence was repeated twice more with 10 ml of a second solvent [methylene chloride–2-propanol–water (75:25:2)]. After the extraction steps had been completed, liver extracts from the experimental fish (1.0 g of liver) were combined and the volume was reduced, at 30°C under a flow of nitrogen, to 1.0 ml. Similarly, liver extracts from control fish (0.3 g of liver) were combined and reduced to a volume of 0.2 ml.

Reversed-phase HPLC

Instrumentation. Separations were performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 8000B high-performance liquid chromatograph. A Perkin-Elmer (Norwalk, CT, U.S.A.) Model MPF-44A fluorescence spectrometer was used for detection (excitation at 290 nm and measurement at 335 nm). Samples were injected using a Waters (Milford, MA, U.S.A.) Model 710A variable-volume automatic sampler.

Columns. The analytical column was a 25 × 0.26 cm I.D. reversed-phase HC-ODS column (Perkin-Elmer). A 5 × 0.21 cm I.D. stainless-steel guard column dry-packed with Vydak 37-μm reversed-phase packing (Separations Group, Hesperia, CA, U.S.A.) was also used.

Gradient elution conditions and fraction collection. Acetic acid–water (0.5:100; solvent A) and methanol (solvent B) were used to separate non-conjugated metabolites from conjugated metabolites and parent compounds in a linear gradient as follows: 100% solvent A to 100% solvent B in 5 min; 10 min at 100% B; 2 min to return to 100% A; and 8 min equilibration at 100%. The flow-rate was 1.5 ml/min, except during equilibration, when it was increased to 2.5 ml/min. The oven temperature was 50°C. Liver extract (200 μl) ($n = 3$ for exposed organisms, $n = 1$ for control) was injected into the HPLC system, and the non-conjugated fraction was collected during an elution interval of 8.7–11.2 min. The conjugated fraction was not collected because low volatilities of conjugated metabolites precluded their determination by GC.

Concentration of HPLC fraction. Metabolites in fractions collected by HPLC were extracted from the HPLC eluate using the extractor/concentrator. The HPLC fraction was first concentrated under a stream of nitrogen at room temperature to remove most of the methanol. The volume was reduced to *ca.* 1 ml, and 2 ml of water and 1 ml of the pH 2.5 citrate–phosphate buffer were added. Two extractor/concentrator extractions (10 ml of acetone, then 10 ml of methanol) were then carried out[13].

The combined extracts were reduced to *ca.* 200 μl in the instrument, then further evaporated under a flow of nitrogen to *ca.* 20 μl. (Note: concentrating samples to dryness should be avoided because this can result in the loss of some analytes[14].) Hexamethylbenzene (the GC internal standard) was added to each sample.

Recoveries of reference standards

Recoveries of AHs from liver homogenate by automated extraction were generally $>90\%$[13]. Compounds in the GC–MS reference standard were processed by the same techniques used to recover metabolites from the liver extract. The GC–MS standard (200 μl, $n = 3$) was injected into the HPLC system and fractions were collected and concentrated. Recoveries were greater than 90% except for three compounds: 1-naphthaldehyde (23%), α-methyl-2-naphthalenemethanol (59%) and 4-methyl-1-naphthol (82%). These compounds have the lowest melting points of the reference standards, so losses may have resulted owing to their relatively high volatilities.

Gas chromatography and mass spectrometry

Instrumentation and chromatographic conditions. Portions (2 μl) of the concentrated fractions containing the metabolites (samples from HPLC) were each injected, without splitting, into a Hewlett-Packard Model 5840A gas chromatograph equipped with a flame-ionization detector. GC–MS was performed using an identical

TABLE I

AMOUNTS OF AROMATIC HYDROCARBONS IN NO. 2 FUEL OIL AND IN LIVERS FROM ENGLISH SOLE EXPOSED TO THE OIL

Compound	*Fuel oil (µg/g)**	*English sole livers (ng/g wet wt.)***
Naphthalene	760	320
1-Methylnaphthalene	1100	1800
2-Methylnaphthalene	1800	3600
2,6-Dimethylnaphthalene	1100	3500
Other C_2H_5-naphthalenes (7 isomers)***	4800	N §
Diphenyl	300	850
Acenaphthene	34	62
2,3,5-Trimethylnaphthalene	580	1400
Other C_3H_7-napthalenes (16 isomers)***	3100	N
Fluorene	120	830
Phenanthrene	220	810
1-Methylphenanthrene	135	94
Other methylphenanthrenes (3 isomers)***	400	N
3,6-Dimethylphenanthrene	200	<30
Other C_2H_5-phenanthrenes (9 isomers)***	1300	N

* GC–MS analysis of No. 2 fuel oil.

** GC analysis of livers from English sole exposed to No. 2 fuel oil. Preparation and analysis of sample by method described previously[10].

*** Amounts determined by GC–MS using response factors from immediately preceding isomeric compound.

§ N = Not determined.

GC system interfaced with a Finnigan 3200 mass spectrometer (Finnigan MAT, Sunnyvale, CA, U.S.A.) and an Incos 2300 data system (Finnigan MAT). The fused silica capillary column was coated with DB-5 (30 m × 0.25 mm I.D.; J&W Scientific, Rancho Cordova, CA, U.S.A.). The flow-rate of the carrier gas (helium) was adjusted to a linear velocity of *ca.* 33 cm/sec at 150°C. The column temperature was programmed from 90 to 300°C at 4°C/min. Injections made at 90°C were best for methanolic solutions.

GC–MS identifications and quantitations. Metabolites were identified by comparison with reference compounds or with spectra in the expanded National Bureau of Standards/National Institutes of Health Mass Spectral Library (Finnigan Library).

Commercially available reference compounds were not, in general, the same isomers present in the experimental samples. Only three of the reference compounds were found in our liver sample. Therefore, many compounds were quantitated using the response factor for an isomer (*e.g.*, the C_2H_5-naphthols were quantitated using the response factor for 2,6-dimethyl-3-naphthol). For some metabolites, no isomers were available: amounts of these compounds were determined using a response factor for the most chemically similar reference standard (*e.g.*, 2,6-dimethyl-3-naphthol was used to quantitate the C_3H_7-naphthols).

RESULTS

No. 2 fuel oil and livers of English sole exposed to the oil were analyzed for AHs by conventional analytical methods[10], and a partial listing of the compounds found is given in Table I. Existing procedures were modified to enable us to quantitate metabolites. Thirteen AH metabolites were found*, in amounts of 50–1100 ng/g wet wt. in livers of fish exposed to oil; none were found in control livers (Fig. 1 and Table II). Five other polar compounds, dibenzothiophene, fluorenemethanol, C_2H_5-fluorenone, ethoxybiphenyl and C_5H_{11}-quinoline, were tentatively identified through the Finnigan Library. Many of the metabolites in Table II were present in amounts near the lower limit of detection and quantitation, and relative standard deviations ranged from 6 to 41%.

Estimated minimum detectable amounts of the reference compounds in GC–MS analysis are 1-naphthaldehyde 30, 2,2′-dihydroxybiphenyl 70, α-methyl-2-naphthalenemethanol 20, 9-hydroxyfluorene 15, 4-methyl-1-naphthol 50, 9-phenanthrol 150, 2-methyl-1,4-naphthoquinone 20, 1-naphthol 20, 2,6-dimethyl-3-naphthol 20, 6-methyl-2-naphthalenemethanol 50 and *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene 100 ng/g.

It was possible that the identified polar compounds were actually acquired by accumulating oxidized hydrocarbons from the fish-exposure tanks. To rule out this

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* In a preliminary study[22], analysis of a single liver sample from English sole exposed to oil showed an even wider variety of metabolites including [approximate amounts (ng/g)]: 1-naphthol (750), 1-naphthaldehyde (170), 4-methyl-1-naphthol (150), 2,6-dimethyl-3-naphthol (500), 9-hydroxyfluorene (130), 9-phenanthrol (1500), five other methylnaphthols (150–500), six other C_2H_5-naphthols (80–2000), another hydroxyfluorene (60), biphenol, naphthoic acid and five isomeric C_3H_7-naphthols.

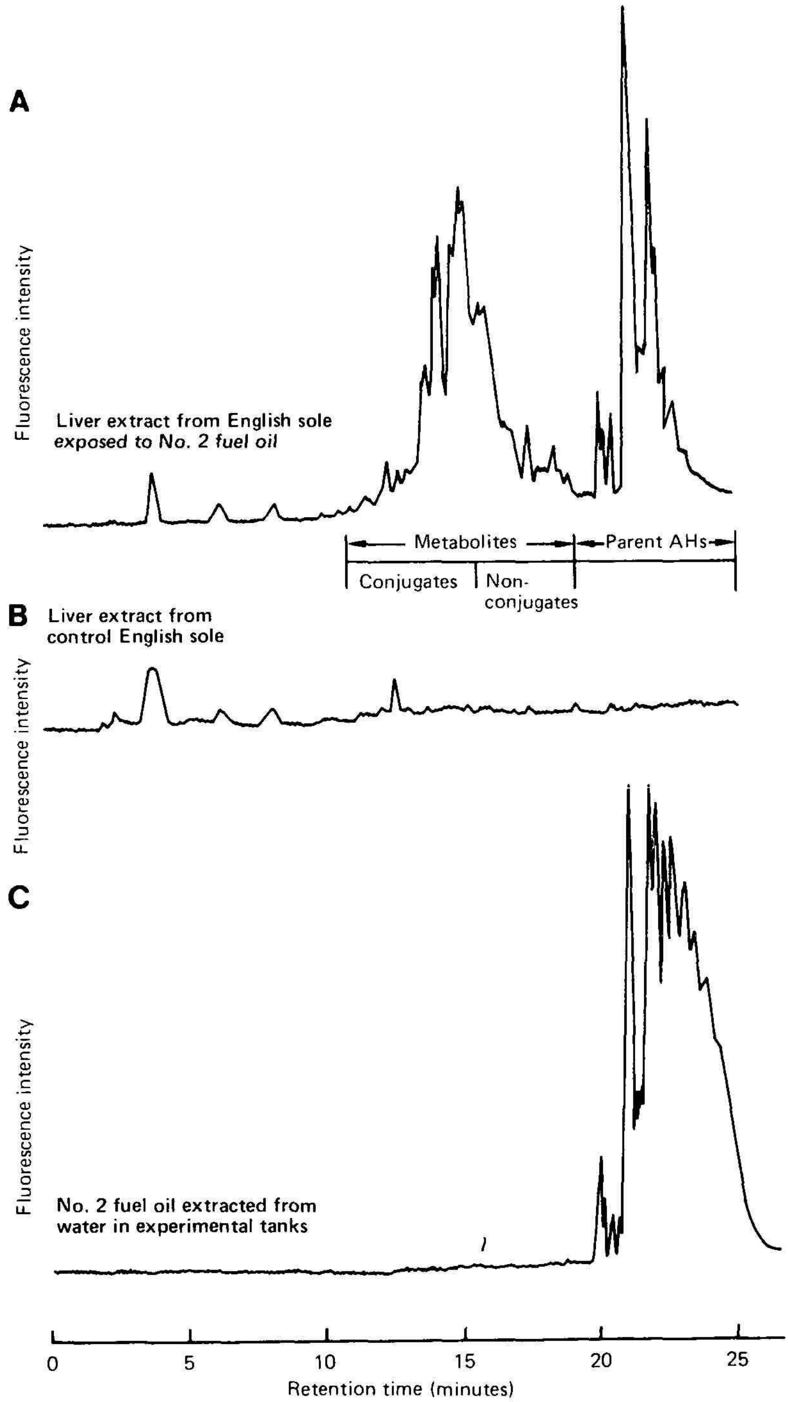

Fig. 1. Reversed-phase HPLC/fluorescence chromatograms (excitation at 290 nm, measurement at 335 nm, the optimum wavelengths for the naphthalene compounds). Gradient elution conditions have been reported previously[13]. (A) Liver extracts from fish exposed to 50 ppm of No. 2 fuel oil for 5 days. Retention times at which the naphthalene family of parent hydrocarbons and their metabolites elute are indicated. (B) Liver extracts from control fish. (C) Fuel oil was extracted from water in the experimental tank. Concentration of the oil is 10 times that of the experimental tank.

TABLE II

GC–MS QUANTITATION OF AROMATIC HYDROCARBON METABOLITES IN LIVERS OF FISH EXPOSED TO NO. 2 FUEL OIL

Scan No.	*Compound**	*Amount (ng/g wet wt)*	*R.S.D. (%)*	*Quantitation method***
1625	C_2H_5-Naphthol	160	38	2
1650	Methylnaphthalenemethanol	460	41	2
1659	6-Methyl-2-naphthalenemethanol	1000	6	1
1741	C_2H_5-Naphthol	180	28	2
1749	9-Hydroxyfluorene	49	39	1
1844	C_3H_7-Naphthol	720	19	3
1849	C_3H_7-Naphthol	440	23	3
1882	C_3H_7-Naphthol	120	12	3
1889	C_3H_7-Naphthol	750	17	3
1914	C_3H_7-Naphthol	160	22	3
1924	C_3H_7-Naphthol	150	33	3
2397	9-Phenanthrol	1100	30	1
2412	Phenanthrol or anthrone	920	25	2

* Compounds present, but not quantitated: C_5H_{11}-quinoline, dibenzothiophene, ethoxybiphenyl, fluorenemethanol, C_2H_5-fluorenone. Control livers contained no metabolites.

** GC–MS determination (or estimation) of amounts using response factor of (1) compound; (2) isomer (estimated); (3) most chemically similar compound, 2,6-dimethyl-3-naphthol (estimated).

route of accumulation, tank water was analyzed by HPLC; only parent hydrocarbons were shown to be present in concentrations above the minimum detectable amount (Fig. 1C).

DISCUSSION

The variety of AHs found in livers of fish exposed to fuel oil resembled that found in the fuel oil itself (see list of selected AHs in Table I). However, each of these AHs is rapidly converted to a series of metabolic products, most often phenols, alcohols and diols[4–8,19,20]. Conjugating enzymes can then transform these metabolites into the corresponding glucuronides, sulfates or other products.

Previous studies of fish exposed to radioactive AHs have demonstrated that metabolites account for a large fraction of the total radioactivity in livers after short periods of exposure (1 day to 1 week)[4–7,19,20]. Our studies of fish exposed to fuel oil substantiate this; HPLC results of liver extracts showed that significant portions of the AHs were converted to metabolites after 5 days (Fig. 1). However, HPLC did not resolve individual metabolites in our complex mixture, so GC–MS was used to separate, identify and quantitate the metabolites.

Several comments about the methods chosen to analyze for metabolites are necessary. Metabolites were extracted from the liver matrix using an automated extractor/concentrator because that method was rapid and recoveries were good (generally >90%). Then, HPLC was used to separate the non-conjugated naphthalene-type metabolites from the conjugates and naturally occurring compounds (*e.g.*, lipids) to prepare the sample for GC–MS analysis. No attempt was made to determine metabolites of all AHs found in oil; we limited the study to non-conjugated metabolites of two- and three-ring AHs. A bonded-phase GC column and methanol as solvent functioned well for the GC analysis of our polar compounds. However, two problems were encountered. Residues tended to build up in the GC injection system because many polar compounds were non-volatile, so the GC liner had to be cleaned frequently. In addition, GC columns appeared to degrade more rapidly, as measured by peak broadening and tailing, with polar samples than with samples containing predominately parent AHs.

Two points of interest were apparent from our interpretation of the mass spectral data (Table II). First, parent AHs were oxidized to the expected types of metabolites. The metabolic products detected (Table II) were primarily alcohols and phenols. Second, several metabolites were present in livers in substantial amounts (as high as 1100 ng/g). Analyses conducted previously on non-radiolabeled environmental samples had not been able to determine these xenobiotics.

Certain AH metabolites may have serious consequences to the health or behavior of organisms. For example, Varanasi *et al.*[8] have shown that the extent of binding of benzo[*a*]pyrene intermediates to hepatic DNA was comparable to values reported for mammalian tissues susceptible to AH-induced neoplasms[8]. Also, in a study on the effect of petroleum exposure on the predatory behavior of coho salmon (*Oncorhynchus kisutch*), Folmar *et al.*[21] postulated that the non-eating behavior of one group of experimental animals was probably a result of the accumulation of metabolites in the animals. However, because no method was available to determine metabolites, the postulate could not be tested. These observations, in conjunction with the results of our study, illustrate the necessity for determining both parent hydrocarbons and their metabolites in order to draw valid conclusions about the effects of xenobiotics on marine organisms and on the consumer of fishery products.

CONCLUSIONS

Tissues of fish exposed to the complex mixture of chemicals in fuel oil can now be analyzed for both parent hydrocarbons and metabolites of two- and three-ring AHs. Analyses have shown that several metabolites are present in livers of English sole in amounts from 50 to 1100 ng/g. Although metabolites may be more harmful to the organism than the parent hydrocarbons, they have previously gone undetected in environmental samples. Thus, our procedures for determining metabolites are an important first step toward routinely determining polar compounds in aquatic organisms exposed to xenobiotics in their natural environment.

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DETERMINATION OF TRI-*n*-OCTYLPHOSPHINE OXIDE BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

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SUMMARY

The determination of tri-*n*-octylphosphine oxide by gas chromatography has been studied. Packed columns gave irreproducible results, probably owing to the thermolability of the compound. Moreover, tailing was observed in most systems, which might be due to adsorption. Good results have been obtained with a glass capillary column (7 m × 0.26 mm I.D.) coated with Carbowax 20M and with temperature programming starting at 140°C until 1 min after injection, then increased at 30°C/min to 240°C. Tri-*n*-nonylphosphine oxide was used as an internal standard. A coefficient of variation of 1.8% was obtained (flame-ionization detector). Less than 1 ng/ml can be determined by the use of a thermionic detector.

INTRODUCTION

Tri-*n*-octylphosphine oxide (TOPO) is a strong electron donor and proton acceptor and is therefore used as a complex-forming agent for metals and carboxylic acids, in batch extraction[1,2] and in liquid chromatography[3,4]. TOPO, dissolved in an alkane, has been applied as a liquid stationary phase in reversed-phase liquid–liquid chromatography[4]. The retention behaviour of organic acids in these systems can be described qualitatively by their complex formation with TOPO[4]. Strong indications were found, however, that the concentration of TOPO in the chromatographic system is much lower than was assumed[5]. The quantitation of TOPO was therefore of interest.

TOPO has been determined by non-aqueous titrimetry[6] and by spectrophotometry of the complex with titanium(IV) and thiocyanate after extraction into an organic phase[7]. The molar ratio between TOPO and bis(2-ethylhexyl)phosphoric acid has been estimated by electron spectroscopy (ESCA), nuclear magnetic resonance spectroscopy and mass spectrometry[8]. For the determination of TOPO in aqueous and organic solutions none of these methods is suitable. Some gas chromatographic investigations have been reported[9-13], mostly of arylphosphine oxides. Organophosphorus compounds can also be determined as phosphine by flame photometry after reduction with hydrogen and gas chromatographic separation from by-products[14].

TOPO has no chromophoric groups and is not electrochemically active. It contains, however, a phosphorus atom, which could be suitable for phosphorus-sensitive detection, *e.g.*, in combination with gas chromatography. Preliminary experiments with packed columns and various stationary phases gave unsatisfactory results, however. We found a spread of up to 10% between consecutive injections of microgram amounts of TOPO (9,10-dibromoanthracene as internal standard and flame-ionization detection). Non-polar, medium polar and many polar stationary phases gave tailing peaks. Almost symmetrical peaks were obtained only with 5% Carbowax 20M–2% potassium hydroxide. The column temperature, however, had to be kept at 250°C, owing to the high molecular weight (386) and polarity of TOPO, and the column packing rapidly deteriorated. The better chromatographic behaviour on Carbowax–potassium hydroxide might be explained by the slight basicity of the oxygen atom of TOPO.

Other experiments showed that TOPO decomposed before its boiling point was reached. The colourless liquid (melting point *ca.* 52°C) turned light yellow after 6 min at 250°C. The bad reproducibility in our preliminary experiments might be due to uncontrollable decomposition of TOPO in the gas chromatographic system.

In this paper we present conditions for the determination of TOPO by gas chromatography, using a Carbowax 20M glass capillary column and flame-ionization or thermionic detection. The use of tri-*n*-nonylphosphine oxide (TNPO) as an internal standard greatly improved the quantitation.

EXPERIMENTAL

Gas chromatographic conditions

A Varian 3700 gas chromatograph with flame-ionization and thermionic (nitrogen–phosphorus) detectors was used. It was modified with adaptors for capillary column gas chromatography[15] for split flow and make-up connections. The inlet pressure of the carrier gas (helium) was 150 kPa, giving a column flow-rate of 7 ml/min at 150°C. The make-up gas (helium) flow-rate was 20 ml/min. The detector and the injector were kept at 300 and 240°C, respectively. Air and hydrogen flow-rates for the thermionic detector were 175 and 5 ml/min, respectively.

The capillary column (soda glass) had an inner diameter of 0.26 mm. A 7 m long piece was etched twice with hydrogen chloride gas at 350°C for 3 h and was then filled with a 1% solution of Carbowax 20M in methylene chloride and left overnight. The column was subsequently rinsed with methylene chloride and coated dynamically with the same solution as above. The column ends were closed after drying and the column was baked at 250°C[16] and finally rinsed with methylene chloride.

Reagents and chemicals

Tri-*n*-octylphosphine oxide (TOPO) of zur Extraktionsanalyse grade, *n*-decane of zur Synthese grade and ethyl acetate of analytical-reagent grade were obtained from E. Merck (Darmstadt, G.F.R.), *n*-Hexane of HPLC grade from Rathburn Chemicals (Walkerburn, Great Britain) and *n*-dotriacontane from Fluka (Buchs, Switzerland).

The internal standard tri-*n*-nonylphosphine oxide (TNPO) was synthesized from *n*-nonylmagnesium bromide and phosphorus oxychloride[17]. The structure and purity were confirmed by ^{13}C NMR spectroscopy. The melting point was 41.5–42.5°C, as determined with a Büchi melting point apparatus and a temperature gradient of 2°C/min.

Determination of TOPO content of liquid chromatographic columns

The liquid stationary phases, containing TOPO, were stripped from the columns with ethanol[5] and 1 ml of the eluate was diluted with ethanol (1–5 ml) to give an expected concentration of TOPO in the range 55–175 μg/ml. A 1-ml volume of this solution was mixed with 200 μl of TNPO in ethanol (1 mg/ml) and analysed by capillary column gas chromatography with flame-ionization detection. A volume of 1 μl was injected with the split valve closed. The valve was opened after 0.8 min. The column temperature was held at 140°C for 1 min after the injection, then increased to 240°C at a rate of 30°C/min. The peak heights of the phosphine oxides were measured. The amount of TOPO in the sample was calculated from the peak-height ratio and a calibration graph. Standards were prepared from solutions of known amounts of TOPO in ethanol: 1 ml was mixed with 200 μl of the internal standard solution. They were then gas chromatographed as above.

Determination of TOPO in aqueous solutions

An aqueous phosphate buffer solution of pH 6.4 ($\mu = 0.1$) was equilibrated with a solution of TOPO in *n*-decane. After centrifugation the organic phase was discarded. A 20-ml volume of the aqueous phase was extracted with 2 ml of *n*-hexane and 100 μl of the internal standard TNPO in ethanol (2.09 μg/ml) for 30 min. A volume of 1 ml of the organic phase was evaporated to dryness using a stream of nitrogen at room temperature. The residue was dissolved in 20 μl of ethyl acetate, 1 μl of which was analysed by gas chromatography with phosphorus-selective detection. Standard samples were prepared by spiking 20-ml aliquots of phosphate buffer solution with 0–100 μl of TOPO in ethanol (2.09 μg/ml) and then treated as above.

RESULTS AND DISCUSSION

Choice of chromatographic method

Determination of TOPO using packed columns was not successful. A system with Carbowax–potassium hydroxide gave symmetrical peaks (see above), but had a short lifetime. A 30-cm short glass column with only 5 cm of chromatographic packing and a high carrier gas flow-rate gave no improvement.

The use of a short capillary column with a polar character was then investigated. A relatively high temperature and a high carrier gas flow-rate were required to elute TOPO within 10 min. The samples were introduced by splitless injections at a low column temperature, although this was only required for the determination of small amounts of TOPO, *e.g.*, in aqueous samples.

As the preliminary studies showed that TOPO might decompose at high temperatures, a solution of TOPO and *n*-dotriacontane was injected at different column temperatures. The hold-up time of TOPO was kept constant by adjusting the inlet pressure. The area ratios are presented in Table I and indicate that TOPO does not

TABLE I

INFLUENCE OF COLUMN TEMPERATURE ON PEAK-AREA RATIO

Sample: 177 μg/ml of TOPO and 117 μg/ml of *n*-dotriacontane in ethyl acetate. Volume injected: 1 μl, 3–4 times at each temperature. Splitting ratio: *ca.* 1:10.

Column temperature (°C)	*Peak-area ratio** (± S.D.)	t_R *(TOPO)*** (min)	*Inlet pressure* (kPa)
200	0.97 ± 0.07	2.8	210
210	1.20 ± 0.05	2.6	135
220	1.03 ± 0.01	2.4	100
230	1.16 ± 0.06	2.3	65

* Peak-area ratio TOPO/*n*-dotriacontane.

** Relative retention of *n*-dotriacontane/TOPO = 0.53.

decompose under these conditions. Temperature-related decomposition would have given a lower peak-area ratio of TOPO to the hydrocarbon at 230°C compared with 200°.

Determination of TOPO content in liquid chromatographic columns

The calibration graph was linear with a small intercept (Fig. 1), which may be due to the presence of a small amount of TOPO in the internal standard. Each point

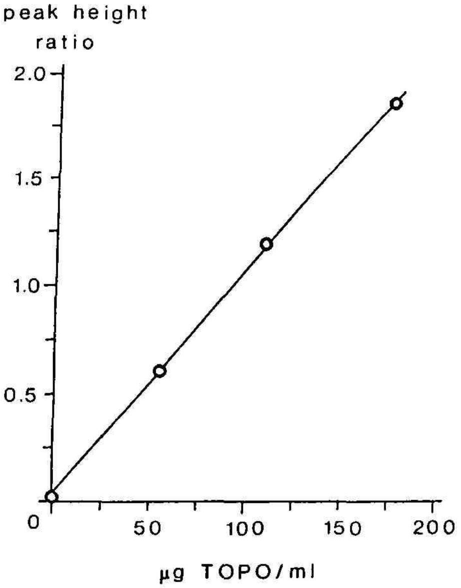

Fig. 1. Calibration graph for the determination of TOPO. Internal standard: TNPO. Column: 7 m × 0.26 mm I.D. glass capillary, coated with Carbowax 20M. Carrier gas (helium) flow-rate: 7 ml/min (at 150°C). Temperature: 140°C for 1 min after injection, then increased at 30°C/min to 240°C. Intercept: 0.039 ± 0.009, slope 0.01042 ± $8 \cdot 10^{-5}$.

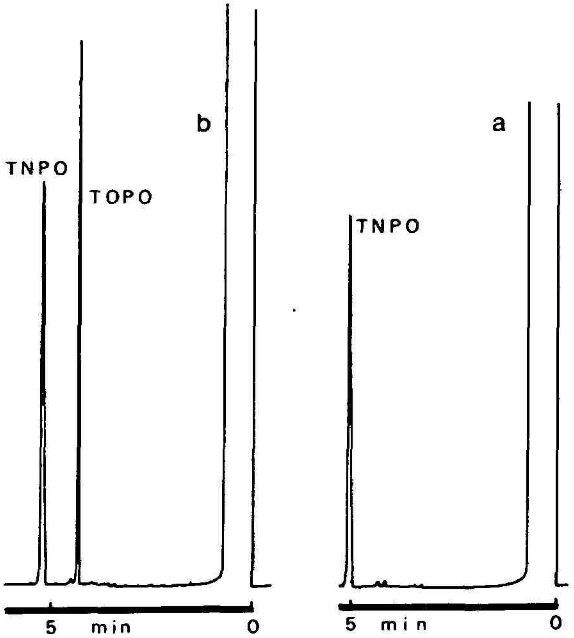

Fig. 2. Capillary column gas chromatograms of TOPO and TNPO (internal standard). (a) Blank; (b) eluate from liquid chromatographic column. Conditions as in Fig. 1.

on the graph was the mean value of eight or nine injections, except at [TOPO] = 0 (three injections). The regression coefficient was 0.999.

A sample containing approximately 25 mg of TOPO per 100 ml of ethanol was analysed five times. The coefficient of variation, including an initial 1 + 2 dilution, was 1.8 %. The use of TNPO as an internal standard probably contributes to this low value. The two chromatograms in Fig. 2 illustrate a blank with only TNPO added (a) and a sample found to contain 127 μg/ml of TOPO (b). The chromatograms and the data above show that this system is well suited for the chromatographic determination of TOPO and TNPO. About 20 samples have been analysed by this method[5].

Determination of TOPO in aqueous solutions

The detection limit could be lowered by the use of a thermionic detector. Less than 1 ng/ml of TOPO could be determined in aqueous solutions after extraction into *n*-hexane and evaporation of the organic solvent. A new injection could be made every 8 min. It has not yet been possible to use this method for the determination of the partition of TOPO between an alkane, such as *n*-decane, and an aqueous buffer solution. High and varying concentrations of TOPO were measured in the aqueous phase, which might be due to the presence of small droplets of the organic phase in the aqueous solution, as a cause of difficulties in separating the phases.

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GAS CHROMATOGRAPHIC IDENTIFICATION OF COMMON DRUGS BY THEIR MULTIPLE PEAKS AND THOSE OF THEIR TRIMETHYLSILYL DERIVATIVES

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SUMMARY

The extent to which common drugs produce multiple peaks in gas chromatography on an OV-17 column at an injection port temperature of 300°C and temperature programme from 120 to 270°C or at an isothermal temperature of 300°C was studied. Forty-six out of 116 drugs tested produced more than one peak either as the parent compounds or as their trimethylsilyl derivatives. The retention times provide a useful means of identification.

INTRODUCTION

In gas chromatography (GC) dual columns[1,2] or even three columns[3] have sometimes been utilized although, for basic drugs, the use of more than one stationary phase is reported to be of very limited value for identification purposes[4]. Many compounds, moreover, may have the same retention times for a given set of conditions and need the use of gas chromatography–mass spectrometry (GC–MS) for more reliable identification[2,5].

Generally, published GC retention data record only single values for each compound[1–3,6] and only in rare instances is more than one retention value given for a few odd compounds[4–5,7–10].

GC of derivatized compounds, principally by silylation to give trimethylsilyl derivatives, has been used for identification purposes[6,11] and, although the possibility of more than one derivative being formed has been suggested[12], only a few compounds have been reported to produce more than one peak[13].

This study was undertaken to explore the extent to which common drugs produce more than peak and the feasibility of using their retention times and those of their silylated derivatives for identification.

EXPERIMENTAL

The instrument used was a Perkin-Elmer F17 gas chromatograph with a 3% OV-17 Gas-Chrom Q (100–120 mesh) glass column (2 m × $\frac{1}{4}$ in. × 3 mm I.D.) and a flame-ionization detector (FID). The carrier gas was nitrogen at a flow-rate of 30 ml/min and the hydrogen and air flow-rates were 30 and 450 ml/min, respectively. The injection port and detector temperatures were 300°C and the oven temperature was programmed from 120 to 270°C at 10°C/min and held at 270°C for 16 min. For isothermal work on compounds not eluted by temperature programming, the injection port, detector and oven temperatures were 300°C. A linear recorder with a chart speed of 30 cm/h and an input voltage of 5 mV was used. Retention times were read manually.

Pure samples of the drugs were obtained from the Singapore Pharmaceutical Department, manufacturers, the British Pharmacopoeia Commission (Reference Substances), the United States Pharmacopoeia (Reference Standards) and the United Nations Narcotics Division of the World Health Organization (International Chemical Reference Substances).

Approximately 1% solutions were prepared fresh in either analytical-reagent grade chloroform, ethanol or 50% ethanol. For silylation, about 0.5 mg of the compound was mixed with about 0.1 ml of chloroform and 0.1 ml of bis(trimethylsilyl)trifluoracetamide (Merck, Darmstadt, G.F.R.) in a 60 × 7 mm tapered-rimmed test-tube, in duplicate, and heated in an air-oven at 80°C for about 1.5 h. Silylated products should not be diluted as precipitation may occur. If the solution is found to be too strong the reaction should be repeated with a smaller amount of the compound.

Volumes of 0.5–2 *µl* of the standard solution and the silylated mixture (or its supernatant liquid if undissolved solids remained) were each injected on to the column. If the height of the main peak was outside the range of 4–7 in. the run was repeated with an adjusted volume of solution and, if necessary, adjustment of the attenuation.

As the silylated derivatives could undergo hydrolysis on standing[8], they were injected within 1–2 h of their formation.

RESULTS AND DISCUSSION

The retention times of 116 compounds, with temperature programming, of the parent compounds and their silylated derivatives are listed in Table I. Table II lists similar data obtained under isothermal conditions for compounds that are not eluting with temperature programming. A plus or a minus sign denotes the presence of absence of a peak, indicated by the retention time in the neighbouring column. Peaks with heights below 10% of that of the highest peak have been omitted to allow for impurities and artifacts.

An analysis of the data in Tables I and II is given in Table III. By defining that two peaks are distinct when their retention times differ by more than 1.5 min, 35 compounds and the stilylated derivatives of 11 others have more than one distinct peak, making a total of 46 (39.6%) of the 116 compounds tested. This does not take into account another 19 compounds that gave single distinct peaks of their silylated derivatives with retention times different from those of their parent compounds.

Hence, under selected conditions the gas chromatograph can play a useful role in identifying a large number of compounds by multiple peak formation as the parent compounds and/or as their silylated derivatives. The phenomenon of multiple peak

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES OBTAINED WITH TEMPERATURE PROGRAMMING

Compound	*Retention time (min)**	
	Parent compound	*Silylated derivative*
Adephenine hydrochloride	18.9	+
Allobarbital	10.8	+
Amethocaine hydrochloride	19.0	+
Aminoglutethimide	22.0	+
Amitriptyline hydrochloride	17.2	+
Amphetamine sulphate	3.6	+
Antazoline hydrochloride	4.0	–
	13.3	+
	14.0	+
	–	17.5
Atropine sulphate	15.4	–
	–	16.0
	–	16.7
	–	18.0
Benzhexol hydrochloride	–	16.4
	17.1	+
Buclizine hydrochloride	2.8	+
	6.1	+
	10.4	+
	11.8	+
	12.9	+
	13.4	+
	14.4	–
	19.2	+
	28.2	–
Butobarbitone	11.2	+
Caffeine	14.5	+
Carbemazepin	15.2	+
	22.6	+
Chlordiazepoxide	–	2.0
	–	14.2
	–	16.6
	–	24.6
	–	27.3
Chloroquin sulphate	14.2	+
	16.1	+
	17.6	+
Chlorpheniramine maleate	2.3	–
	–	4.8
	14.9	+
Chlorphentermine hydrochloride	6.6	+
Chlorpromazine hydrochloride	26.2	+
Clomipramine	16.8	+
Cocaine hydrochloride	18.2	+
Codeine phosphate	–	4.4
	20.6	–
	–	21.5
	22.8	–

(Continued on p. 118)

TABLE I *(continued)*

Compound	*Retention time (min)**	
	Parent compound	*Silylated derivative*
Cyclizine hydrochloride	15.8	+
Dextroamphetamine sulphate	3.8	+
	–	5.0
Dextromethoprhan hydrobromide	17.5	+
Diazepam	25.4	+
Diethylcarbamazine citrate	2.4	–
	8.2	+
	–	10.0
Diphenhydramine hydrochloride	13.6	+
Diphenidol hydrochloride	19.3	+
	23.0	+
Diphenoxylate hydrochloride	16.2	+
Disopyramide	14.5	+
	–	15.4
	18.0	+
	–	25.0
Ephedrine hydrochloride	2.6	–
	–	5.8
	6.7	–
	7.0	–
Ergometrine maleate	–	5.3
Ergotarmine tartrate	–	8.4
Emetine hydrochloride	–	12.8
	14.3	+
	19.0	+
Guanethidine sulphate	–	2.0
Homatropine hydrobromide	–	15.6
	16.8	–
Hydralazine hydrochloride	10.4	+
	–	13.8
	–	14.0
	–	14.3
	17.7	–
Hydroxyamphetamine hydrochloride	8.4	+
	12.0	+
	–	13.2
Hydroxyzine hydrochloride	10.8	+
	13.2	+
	19.8	+
Hyoscine N-butyl bromide	17.5	+
	–	19.2
	20.8	+
	–	22.6
Hyoscine hydrobromide	17.8	–
	–	18.8
	21.0	–
Imipramine hydrochloride	15.0	+
	18.0	+

TABLE I *(continued)*

Compound	*Retention time (min)**	
	Parent compound	*Silylated derivative*
Iproniazid phosphate	–	4.5
	–	5.0
	10.8	+
Isoniazid	11.2	+
	12.0	–
Isopropamide iodide	–	15.6
	16.8	+
	18.6	+
	19.2	+
	23.2	+
	23.8	+
Isopropylhexidine hydrochloride	3.9	+
	–	4.9
	–	8.7
Lignocaine hydrochloride	13.6	+
Lorazepam	24.6	+
Maprotiline hydrochloride	20.8	+
	–	22.2
Medazepam	19.8	+
Meprobamate	–	3.2
	–	7.7
	–	8.2
	13.7	+
Mepyramine maleate	1.7	–
	–	5.2
	18.6	+
Mepyrylcaine hydrochloride	–	4.8
	10.8	+
Methadone hydrochloride	16.8	+
Methaqualone	18.6	+
Methimazole	12.0	+
Methoxyphenamine hydrochloride	9.2	+
Methylampheatamine hydrochloride	4.1	+
Metronidazole	–	10.8
	12.4	–
Morphine hydrochloride	–	21.7
Naphazoline nitrate	17.2	+
	–	18.8
	–	19.6
Narcotine	13.5	+
Nalorphine hydrobromide	24.2	+
Neostigmine bromide	13.6	+
Nortriptyline hydrochloride	18.3	+
	–	19.6
Oxazepam	21.8	+
Oxyphenbutazone	2.3	–
	2.8	–
	11.6	–
	–	29.4

(Continued on p. 120)

TABLE I *(continued)*

Compound	*Retention time (min)**	
	Parent compound	*Silylated derivative*
Oxyphencyclimine hydrochloride	10.2	+
	14.1	–
	26.6	–
Oxyphenonium bromide	10.2	+
	16.8	–
	17.6	–
Paracetamol	–	12.0
	12.8	–
Pethidine hydrochloride	12.0	+
Phenacetin	12.1	+
Phenalzine sulphate	1.6	+
	1.8	+
	–	3.8
	–	4.6
	–	5.5
	7.6	–
	–	8.9
	–	10.0
	16.4	–
	–	16.8
Phendimetrazine tartrate	8.0	+
Phenformin	–	3.5
	–	3.9
	4.4**	–
	–	8.0
	11.7	+
	–	15.4
	–	16.0
	–	17.4
	22.8	–
Phenoxybenzamine hydrochloride	14.4	+
	17.4	+
	18.0	+
Pentazocine hydrochloride	–	16.6
	18.4	–
Phenylephrine hydrochloride	–	9.4
	13.0	–
	13.4	–
	13.8	–
Phenylpropanolamine hydrochloride	7.3	–
	–	6.0
	–	6.6
Phenytoin sodium	24.6	+
Physostigmine salicylate	8.0	+
	–	12.6
	14.0	+
Piperazine citrate	–	1.8
	–	10.3

TABLE I *(continued)*

Compound	*Retention time (min)**	
	Parent compound	*Silylated derivative*
Prilocaine	15.4	+
Procaine hydrochloride	16.0	+
Prochlorperazine dimaleate	–	5.2
Promethazine hydrochloride	16.6	+
	19.0	+
Propantheline bromide	10.8	+
	–	14.1
	–	19.0
	19.6	+
Propranolol hydrochloride	–	15.7
	17.0	–
Propylhexidrine	3.6	+
	+	8.9
Propylthiouracil	15.6	+
Quinidine sulphate	–	25.1
Quinine sulphate	–	26.1
Thebaine	28.2	+
Theophylline	16.9	+
Tolbutamide	12.6	+
Tranylcypromine sulphate	–	5.5
	–	7.6
	–	8.2
	–	18.4
Tripelennamine hydrochloride	14.9	+
Triprolidine hydrochloride	15.4	+
	18.8	+
	22.6	+
	26.5	+
Trifluoperazine tartrate	15.8	+
	30.0	+
Xylocaine hydrichloride	13.8	+

* A + or – sign indicates the presence or absence of a peak at the corresponding retention time.
** Does not always appear.

formation should, or course, be avoided as much as possible, especially in trace analysis work, in order to achieve optimum detection and quantitative results of drugs, but when they do occur it would be useful to know their number.

The retention times listed in Tables I and II should be used only as a guide as it may be difficult to reproduce exactly the chromatographic conditions of another laboratory[3]. Also, the data in these tables are limited to specific instrument parameters and operating conditions. Most important, the number of decomposition peaks formed will depend on the type of support used, its activity and age and perhaps also on the size of column. It is therefore important that selected standards should always be chromatographed when confirming the identity of an unknown compound.

The results also emphasize the dearth of multiple peaks in published data. The highest number of compounds with more than one peak that has been reported is only fourteen[7], and this was in a list of 570 retention data. Twelve compounds out of the fourteen had not even been tested in this study.

TABLE II

GAS CHROMATOGRAPHIC RETENTION TIMES OBTAINED UNDER ISOTHERMAL CONDITIONS

*Compound**	*Retention time (min)***	
	Parent compound	*Silylated derivative*
Bromazepam	7.0	+
	9.3	+
Brucine	Nil	Nil
Cinchocaine hydrochloride	6.0	+
Chlordiazepoxide	2.0	+
	6.1	+
Clonazepam	3.6	+
	11.4	–
	13.0	–
	14.9	+
Ergometrine maleate	–	6.0
Ergotarmine tartrate	3.4	–
	4.0	–
Flurazepam	8.0	+
Flunitrazepam	7.2	+
Guanethidine sulphate	Nil	Nil
Heroin hydrochloride	7.0	+
	8.8	+
Morphine hydrochloride	–	3.1
	–	3.5
Narceine hydrochloride	Nil	Nil
Nitrazepam	2.8	+
	11.7	+
Papaverine hydrochloride	12.4	+
Pholcodine	–	3.2
	–	3.7
Pilocarpine nitrate	2.4	+
Piperazine citrate	Nil	Nil
Prochlorperazine dimaleate	3.2	+
	–	13.0
Quinidine sulphate	–	4.8
Quinine sulphate	–	4.8
Reserpine	Nil	Nil
Strychnine hydrochloride	–	25.0
	–	28.4
Tranylcypromine sulphate	–	2.0
	–	2.4
	–	2.5
	2.8	+
Tubocurarine hydrochloride	Nil	Nil
Yohimbine hydrochloride	Nil	Nil

* Compounds that do not elute with temperature programming.

** A + or – sign indicates the presence or absence of a peak at the corresponding retention time.

To confirm that the phenomenon of multiple peaks was not due to sample inpurity, international standards were tested under similar GC conditions and, except for minor peaks for two compounds, there was corroboration of the data in all instances.

Combined GC–MS was carried out on hyoscine hydrobromide and oxyphenbutazone using a Hewlett-Packard HP 5985B instrument, in order to investigate the

TABLE III

ANALYSIS OF DATA IN TABLES I AND II

	No. of compounds					
	Parent compound			*Silylated derivative*		
	Temperature programming	*Isothermal*	*Total*	*Temperature programming*	*Isothermal*	*Total*
Single peak	59	6	65	70	6	76
Multiple peaks	32	4	36	29	6	35
No peaks			15			5
Distinct* multiple peaks	31	4	35	1	–	1**
Parent compound single peak; silylated derivative:						
Single peak with distinot retention time	–	–	–	12	1	13
Multiple distinct peaks	–	–	–	6	–	6
Parent compound no peaks; silylated derivatives:						
Single peak with distinct retention time	–	–	–	6	–	6
Multiple distinct peaks	–	–	–	3	2	5
Parent compound single peak; silylated derivative identical peak***	40	5	45	–	–	–

* Retention time difference between two peaks is more than 1.5 min.

** Excludes compounds with the same retention time as the parent compound and 11 others with distinct retention times which have been included elsewhere in the table.

*** Presumably no reaction had taken place.

nature of the products formed. The ionization voltage was 70 eV and the ion source temperature was 200°C. The chromatograph was fitted with a 6 ft. × 2 mm I.D. glass column packed with 3% OV-1 on Chromosorb W HP (100–200 mesh) and interfaced to the mass spectrometer by a jet separator maintained at 275°C. The injection temperature was 275°C and the oven temperature was programmed from 120 to 270°C at 10°C/min and held at 270°C for 10 min. The carrier gas was helium at a flow-rate of 30 ml/min. The EI mode was used for both the compounds. The results obtained are given in Table IV.

It can be seen that although oxyphenbutazone remains intact with the direct insertion probe, it undergoes complete decomposition in the gas chromatograph, forming at least three products which bear hardly any resemblance to the parent compound. With hyoscine the two peaks correspond to unchanged hyoscine and hyoscine with the loss of a molecule of water, probably between the α-hydroxy and β-hydrogen atoms. No doubt for other compounds other reactions could take place, but their elucidation is beyond the scope of this study.

Higher injection port temperatures are expected to produce greater decomposition and when lower temperatures, for example 200°C[3], are used the absence of multiple retention times can be expected. However, at 300°C, their absence or almost complete absence[2,4,5], except in a few instances[12,14], remains unexplained. Due importance to subsidiary peaks had probably not been accorded, although this study has demonstrated their usefulness in confirming the identity of compounds.

TABLE IV

RESULTS OBTAINED BY GC–MS

Compound	*Mol.wt.*	*DIP*/GC*	*Retention time (min)*	*m/e*
Oxyphenbutazone	324.37	DIP	–	55 65 77 93 107 121 135 199 268 324
		GC	0.97**	51 64 74 91 119
			8.07***	55 64 80 93 106 119 165 217
			11.40***	52 65 80 93 107 119 135 148 161 177 187 204 231
Hyoscine	303.35	GC	11.18***	51 68 77 94 103 120 138 154 285
			12.70**	55 65 77 94 108 120 138 154 303

* Direct insertion probe.
** Major peak.
*** Minor peak.

CONCLUSION

With injection port and detector temperatures of 300°C and an OV-17 column, a large number of common drugs produce characteristic peaks as the parent compounds or as their trimethylsilylated derivatives, and thereby afford a means of identification.

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DETERMINATION OF IMPURITIES IN LEVODOPA AND CARBIDOPA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

The detection of 3-(3,4,6-trihydroxyphenyl)alanine and 3-methoxytyrosine in levodopa and methyldopa and 3-O-methylcarbidopa in carbidopa by high-performance liquid chromatography (HPLC) with electrochemical detection is described. An octyl-bonded reversed-phase column is employed with a buffered aqueous methanol mobile phase containing an "ion-pairing" reagent. All components are well resolved and sensitively detected by amperometric oxidation at a glassy carbon electrode maintained at +0.90 V *versus* Ag/AgCl. The impurities can be qualitatively characterized for identification purposes by hydrodynamic voltammetry, in which the peak height is observed for a range of oxidation voltages. The analysis of levodopa–carbidopa combination tablets is discussed.

INTRODUCTION

Levodopa (*l*-dopa, 3-hydroxy-L-tyrosine) and carbidopa (*S*-α-hydrazino-3,4-dihydroxy-α-methylbenzenepropanoic acid) are used in combination to treat Parkinson's disease[1]. The current *United States Pharmacopeia* (USP XX[2]) specifies maximum acceptable limits of two impurities in each of the two separate bulk powders. For levodopa, the USP XX requires that the major component be assayed by non-aqueous titration. The hydroxylated impurity 3-(3,4,6-trihydroxyphenyl)alanine and the O-methylated impurity 3-methoxytyrosine are measured by thin-layer chromatography (TLC). For carbidopa, the assay is also by non-aqueous titration. The non-hydrazine impurity methyldopa is measured by TLC and the O-methylated impurity, 3-O-methylcarbidopa, is determined by high-performance liquid chromatography (HPLC) on a 1-m pellicular cation-exchange column.

Each of these compounds contains a phenol or catechol functionality and thus should be amenable to sensitive and selective detection by amperometric oxidation[3].

We chose to develop an HPLC method with electrochemical detection (ElCD) which would be applicable to the determination of components in the bulk powders and in the combination dosage form. In combination with simultaneous ultraviolet absorption detection, both high and low levels of many compounds can be monitored.

Chromatographic separation and electrochemical detection of catecholamines in biological samples is a well developed technique with picogram detection limits for suitable compounds[4]. Few detection methods can compare for simplicity and performance[3]. With current improvements in chromatographic resolution[5] and detection sensitivity, standard methods of pharmaceutical analysis need to be re-examined and updated to insure acceptable drug quality and to maximize analytical laboratory efficiency.

In this paper we present a single HPLC–ElCD method which can detect and quantitate two drug substances and four specified USP impurities and which also indicates the presence of additional impurities. This approach is seen to be much more sensitive, simple and efficient than existing methodology.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a pump (Altex 110A single piston with pulse dampener, or Waters Assoc. 6000 dual piston), a valve injector with a 10-μl sample loop (Rheodyne 7125), a variable-wavelength ultraviolet absorption detector operated at 283 nm (DuPont Model 837) and an amperometric detector (Bioanalytical Systems TL-8A cell with an LC-3 controller). The electrochemical detector consists of a thin-layer flow cell with 3 mm diameter glassy carbon working and auxiliary electrodes and an Ag/AgCl reference electrode (4 *M* potassium chloride). For this study the working electrode was maintained in the oxidation mode at +0.90 V *versus* Ag/AgCl. Samples under 25 mg were weighed on an automatic electrobalance (Cahn Model 27). Chromatograms were recorded on a Linear Instruments dual-pen strip-chart recorder.

Materials

The water was deionized, distilled, suitable for HPLC. The methanol was HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), the sodium heptanesulfonate (Eastman Chemical, Rochester, NY, U.S.A.) was used as received and all other chemicals were ACS Reagent Grade. The drug substances and reference compounds were either from a commercial source or were purchased from USP. All sample solutions were prepared by dissolving the weighed solute in mobile phase with extra 1 *M* orthophosphoric acid added if needed to effect dissolution.

Chromatographic conditions

The HPLC column was a 12.5 cm × 4.0 mm I.D. 5-μm octyl-bonded reversed-phase column (LiChrosorb RP-8 Hibar) or a 25.0 cm × 4.0 mm I.D. 5-μm reversed-phase column (Ultrasphere). Both columns were commercially prepared. The mobile phase consisted of 16.5 g of sodium dihydrogen orthophosphate, 980 ml of water, 1.0 ml of 0.10 *M* disodium ethylenediaminetetraacetic acid, 20 ml of methanol and 1.2 ml of 0.5 m*M* sodium heptanesulfonate with the pH adjusted to 3.4 with 1 *M* orthophosphoric acid. The mobile phase was filtered through a 0.45-μm cellulose membrane filter, vacuum degassed and used at ambient temperature with a flow-rate generally of 0.8 ml/min.

RESULTS AND DISCUSSION

Limits test of USP impurities

A reference chromatogram of all six known components is shown in Fig. 1 with corresponding retention information in Table I. The retention order for this reversed-phase separation can be explained on the basis of polarity. The primary amine levodopa elutes well before the less polar hydrazine-containing carbidopa. The more polar trihydroxyphenylalanine elutes before the dihydroxy levodopa and the extra methyl group in methyldopa causes it to be more retained relative to levodopa. The O-methylated levodopa derivative is more retained than levodopa and also elutes later than the dihydroxy methyldopa. Finally, the O-methylated carbidopa is most highly retained.

The mobile phase used is the same as that specified for the HPLC–ElCD analysis of epinephrine in combination with lidocaine which is described in USP XX Supplement 2[2], and is similar to another levodopa–carbidopa separation[6] as well as to that proposed for the separation of other catecholamines[7]. The EDTA is used to sequester any oxidizable metal ions. The effect on the separation of the "ion-pair" reagent concentration, pH and methanol for related compounds has been discussed elsewhere[7].

A dual detector chromatogram at 0.8 ml/min of a 10-μl injection of a 2.0 mg/ml sample of levodopa USP standard material is shown in Fig. 2. This would correspond to the analysis of a 100 mg levodopa–10 mg carbidopa tablet dissolved in 50 ml of solution. The UV detector is at its highest sensitivity (0.01 a.u.f.s.) while the electrochemical detector is at its lowest sensitivity (100 nA f.s.). Both impurities can be easily seen, but ElCD is much more sensitive.

TABLE I

CHROMATOGRAPHIC RETENTION TIMES

Compound	*Retention time (min)*	
	*Column 1**	*Column 2***
3-(3,4,6-Trihydroxyphenyl)alanine	5.5	2.9
Levodopa	6.9	3.5
Unknown impurity 1	9.1***	
Methyldopa	16.5	8.5
3-Methoxytrosine	19.3	9.8
Carbidopa	21.7	11.0
Unknown impurity 2	25.4***	
Unknown impurity 3	35.5***	
Unknown impurity 4	37.5***	
3-O-Methylcarbidopa	52.5	39.5

* 25-cm Ultrasphere column at 0.4 ml/min, increased to 1.0 ml/min after 30 min.

** 12.5-cm RP-8 column at 0.8 ml/min.

*** These impurities did not appear in fresh solutions.

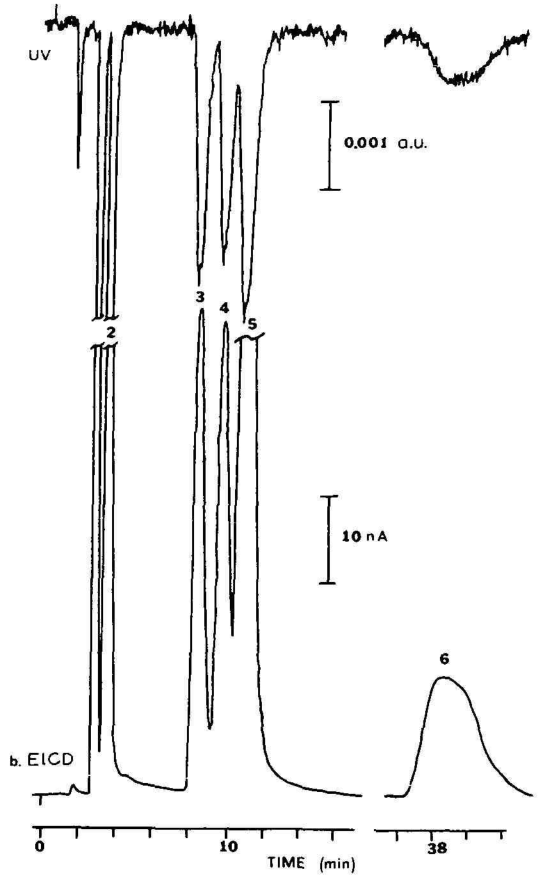

Fig. 1. Dual detector chromatogram of the six known components. (a) UV detection at 0.01 a.u.f.s.; (b) electrochemical detection of 100 nA f.s. Peaks: (1) trihydroxyphenylalanine; (2) levopoda; (3) methyldopa; (4) methoxytyrosine; (5) carbidopa; (6) methylcarbidopa.

Fig. 3 shows a chromatogram of USP standard samples of the two specified impurities at concentrations that are at the limit of USP acceptability for levodopa bulk powder. This corresponds to 2.0 μg/ml for trihydroxyphenylalanine and 10.0 μg/ml for methoxytyrosine. The ElCD limit, estimated judiciously as 5 % of the 5 nA f.s. sensitivity, is seen to be about 20 ng/ml for trihydroxyphenylalanine and 40 ng/ml for methoxytyrosine. This is a factor of 100–250 lower than the current USP limits, demonstrating the much higher sensitivities obtained with HPLC–ElCD compared with conventional TLC.

A dual detector chromatogram at 0.8 ml/min of a 10-μl injection of a 0.20 mg/ml solution of USP primary standard carbidopa is shown in Fig. 4. This would correspond to the analysis of a 100 mg levodopa–10 mg carbidopa tablet dissolved in 50 ml of solution. The retention times of the two USP specified impurities are indicated. Fig. 5 shows a chromatogram of USP standard samples of the two impurities at the concentrations that are at the limit of USP acceptability for carbidopa bulk powder. This corresponds to about 1 μg/ml for both methyldopa and 3-O-methylcarbidopa.

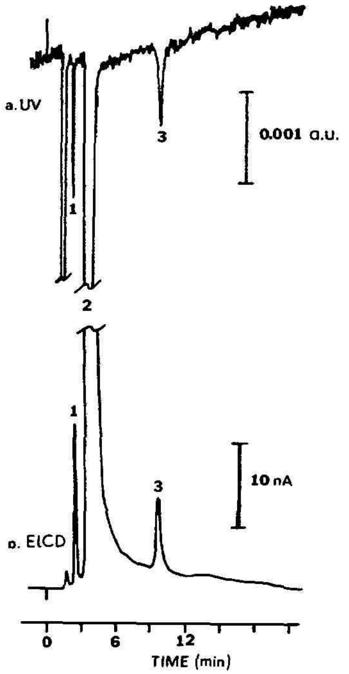

Fig. 2. Dual detector chromatogram of 2.0 mg/ml levodopa USP Standard. Peaks: (1) trihydroxyphenylalanine; (2) levodopa; (3) methoxytyrosine.

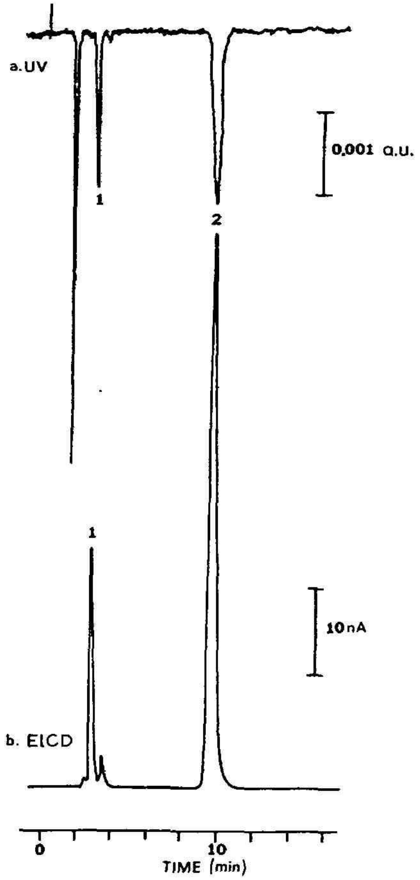

Fig. 3. Dual detector chromatogram of (1) 2.0 μg/ml of trihydroxyphenylalanine and (2) 10.0 μg/ml of methoxytyrosine.

Detection limits of about 80 ng for methyldopa and 60 ng for 3-O-methylcarbidopa can be obtained as 5% of the 5nA f.s. sensitivity. This is a factor of 10–20 lower than the current USP limits and could be further lowered by using a sample solution as concentrated as that used here for levodopa.

For comparison, Fig. 6 shows a dual detector chromatogram from a 0.20 mg/ml solution of a commercial source of carbidopa. In neither the USP reference sample nor the commercial material was the last impurity, 3-O-methylcarbidopa, observed.

Reproducibility

The reproducibility of each detector based on peak-height precision was evaluated by replicate injection of 100-ng amounts of 3-methoxytyrosine. For five replicates the UV absorption detector exhibited a coefficient of variation (relative standard deviation) of 0.96%. For the same injections the electrochemical detector

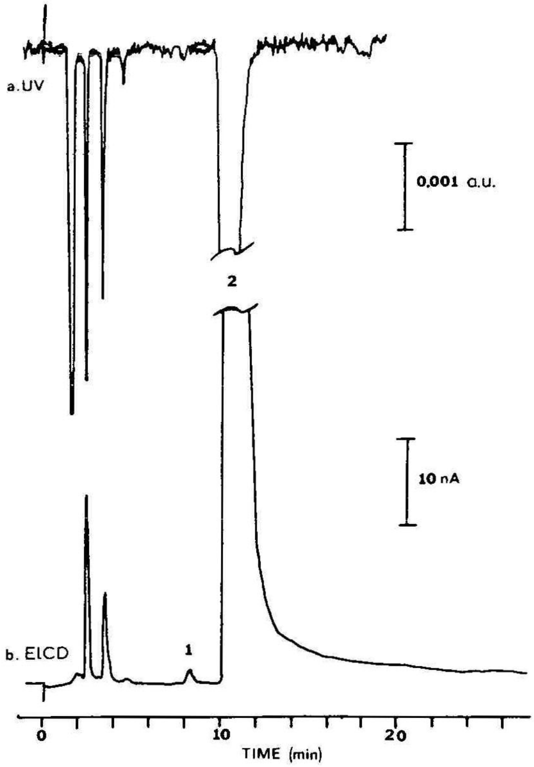

Fig. 4. Dual detector chromatogram of 0.20 mg/ml of carbidopa USP Standard. Peaks: (1) methyldopa; (2) carbidopa.

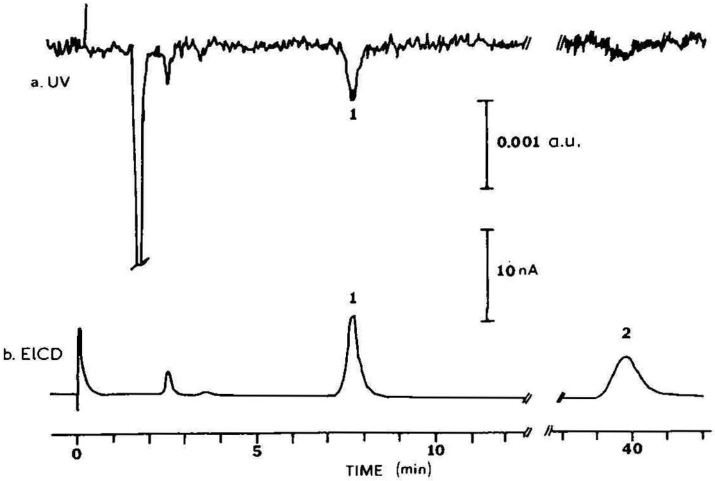

Fig. 5. Dual detector chromatogram of (1) 1.4 μg/ml of methyldopa and (2) 1.4 μg/ml of methylcarbidopa.

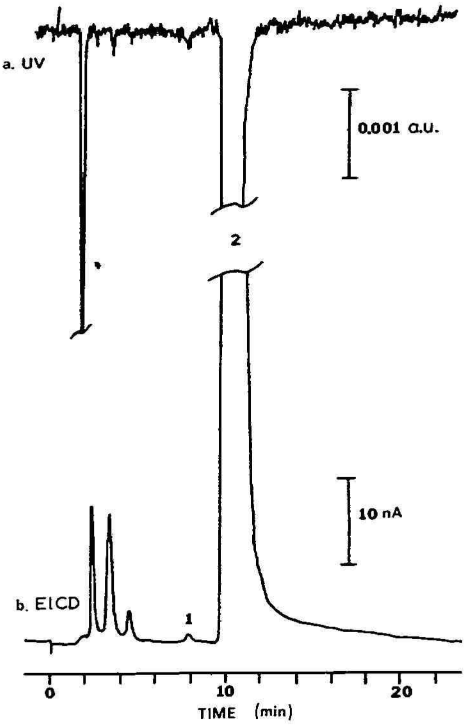

Fig. 6. Dual detector chromatogram of a 0.20 mg/ml solution of commercial carbidopa. Peaks: (1) methyldopa; (2) methylcarbidopa.

yielded a coefficient of variation of 0.28 %. This difference is mainly due to the fact the ElCD trace was virtually noise-free whereas the UV peak had a signal-to-noise ratio of only about ten.

Qualitative identification

There is no guarantee in chromatography that two peaks from different samples that elute at the same time arise from the same substance. As the chromatographic efficiency is increased, such "coincidental coelution" becomes increasingly unlikely. However, it is advisable, when possible, to use some means of qualitative identification to insure that measured concentrations of impurity bear a high degree of validity. This identification generally becomes increasingly difficult as the level of the impurity decreases.

ElCD for HPLC can provide such qualitative identification capability by observing the change in peak height *versus* applied oxidation potential. This is termed "hydrodynamic voltammetry"[3].

In this study a standard solution of each of the six compendial substances was repetitively injected while the detector operating voltage was increased from roughly 0.0 to 1.0 V in 25-mV increments. The resulting data are presented in Fig. 7 in which the peaks heights have been normalized with respect to the largest response observed for each compound. From these pseudo-polarograms characteristic half-wave potentials ($E_{1/2}$) have been extracted and are reported in Table II. The correlation between

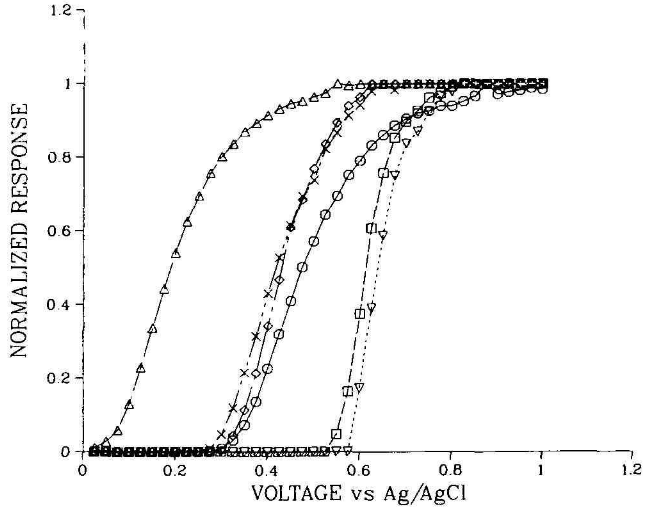

Fig. 7. Hydrodynamic voltammograms of the six compounds. ○, Levodopa; △, 2,4,6-trihydroxyphenylalanine; ×, carbidopa; ◇, methyldopa; ▽, 3-O-methylcarbidopa; □, 3-methoxytyrosine.

chemical structure and use of oxidation is clearly seen: The trihydroxy compound has the lowest $E_{1/2}$, the three dihydroxy species have intermediate $E_{1/2}$ and the two monohydroxy substances have the largest $E_{1/2}$. This information can be used to provide qualitative information in support of the assignment of identity of impurity peaks.

Degradation study

It was our observation that additional impurity peaks developed in the HPLC–ElCD trace for standard solutions which had been allowed to stand for more than a day (Table I). Although we have not pursued this to the point of identifying these impurities, we have monitored the behavior of these components for a week using a solution containing all six target compounds at the 1–2 mg/ml level.

Impurities 1, 2 and 3 appear in solutions that are a day old. The response due to impurity 1 became > 100 nA after 6 days and remained high, while impurity peak 2 reached a maximum of 16 nA on day 3 and fell off thereafter. Impurity 3 reached a level of 10 nA on day 8. Impurity 4 appeared on day 3 and reached 1.5 nA on day 8.

TABLE II

HYDRODYNAMIC VOLTAMMETRIC HALF-WAVE POTENTIALS

Compound	$E_{1/2}$ *(V vs Ag/AgCl)*
3-(3,4,6-Trihydroxyphenyl)alanine	0.19
Carbidopa	0.42
Methyldopa	0.43
Levodopa	0.48
3-Methoxytrosine	0.61
3-O-Methylcarbidopa	0.64

The only significantly noticeable change in peak height for the six compounds was for 3,4,6-trihydroxyphenylalanine, which initially had a peak height >100 nA but which had fallen to 12 nA after 8 days. A solution containing only 3,4,6-trihydroxyphenylalanine was prepared. This solution developed impurity peaks upon standing which appear to correspond to impurity peaks 2 and 3 in Table I. This initial degradation study demonstrates the potential of HPLC–ElCD to monitor the storage and breakdown pathways of oxidizable organic compounds.

CONCLUSIONS

The combination of the high sensitivity of amperometric detection with the superb resolving power of liquid chromatography has been seen to be used advantageously to provide a simple and effective means by which to determine the amount of impurities in commercial samples of levodopa and carbidopa as specified by the USP[2]. The method can supersede four existing methods for the specified impurities and is sensitive enough for single tablet analysis.

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SEPARATION AND QUANTIFICATION OF VARIOUS PHOSPHORUS OXOACIDS CONTAINING TWO PHOSPHORUS ATOMS BY ISOTACHOPHORESIS

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SUMMARY

The separation and determination of various phosphorus oxoacids containing two phosphorus atoms, such as $\overset{5}{\mathrm{P}}$–O–$\overset{5}{\mathrm{P}}$, $\overset{3}{\mathrm{P}}$–O–$\overset{5}{\mathrm{P}}$ and $\overset{3}{\mathrm{P}}$–O–$\overset{3}{\mathrm{P}}$ salts, by capillary isotachophoresis was investigated. The potential unit (PU) values, which are indicators in quantitative analysis, increased in the order $\overset{3}{\mathrm{P}}$–O–$\overset{3}{\mathrm{P}}$ < $\overset{3}{\mathrm{P}}$–O–$\overset{5}{\mathrm{P}}$ < $\overset{5}{\mathrm{P}}$–O–$\overset{5}{\mathrm{P}}$. The calibration graphs for these oxoacids were linear in the range 0–10^{-7} mole as phosphorus oxoacids. Separation times were approximately 20 min.

The procedure was applied to the analysis of some crude products and to the study of the hydrolytic degradation of some condensed phosphates. The amount of each phosphorus oxoacid could be determined rapidly and easily, and the results were in good agreement with those obtained by ion-exchange chromatography.

INTRODUCTION

Phosphorus oxoacids and their salts are widely used in food products and processing, detergents and cleaning compounds, etc. For their separation and determination, paper, gel and ion-exchange chromatography have been used. These methods lack speed and simplicity.

In this work, the separation and determination of some phosphorus oxoacids containing two phosphorus atoms, such as $\overset{5}{\mathrm{P}}$–O–$\overset{5}{\mathrm{P}}$, $\overset{3}{\mathrm{P}}$–O–$\overset{5}{\mathrm{P}}$ and $\overset{3}{\mathrm{P}}$–O–$\overset{3}{\mathrm{P}}$, by isotachophoresis was investigated and was applied to the analysis of some crude products and hydrolytic degradation products of $\overset{3}{\mathrm{P}}$–O–$\overset{5}{\mathrm{P}}$ and $\overset{3}{\mathrm{P}}$–O–$\overset{3}{\mathrm{P}}$.

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EXPERIMENTAL

Reagents

Unless otherwise stated, guaranteed reagents from Wako (Osaka, Japan) were used without further purification.

Disodium phosphonate pentahydrate ($Na_2PHO_3 \cdot 5H_2O$; $\overset{3}{P}$), sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$; $\overset{5}{P}$) and tetrasodium diphosphate decahydrate ($Na_4P_2O_7 \cdot 10H_2O$; $\overset{5}{P}$–O–$\overset{5}{P}$) were purified by repeated recrystallization. Disodium diphosphonate ($Na_2P_2H_2O_5$; $\overset{3}{P}$–O–$\overset{3}{P}$) and trisodium isohypophosphate tetrahydrate ($Na_3P_2HO_6 \cdot 4H_2O$; $\overset{3}{P}$–O–$\overset{5}{P}$) were prepared according to the method given in the literature[1–5].

Isotachophoresis

A Shimadzu, IP-2A Capillary Tube Isotachophoretic Analyzer was used. The isotachophoretic tubes for the separation consisted of a main capillary column (100 mm × 0.5 mm I.D.) and a pre-column (100 mm × 1.0 mm I.D.).

The operational system of the isotachophoretic analysis is given in Table I. The analytical procedure used was as described previously[6].

TABLE I

OPERATIONAL SYSTEM FOR SEPARATION OF PHOSPHORUS OXOACIDS

Parameter	*Electrolyte*	
	Leading	*Terminating*
Anion	Cl^-	Hexanoate$^-$
Concentration	0.01 *M*	0.01 *M*
Counter ion	Histidine$^+$	H^+
pH	5.5	3.4
Additive	0.1 % Triton X-100	
Solvent	Water	Water
Capillary tube	100 × 1.0 mm I.D. + 100 × 0.5 mm I.D.	
Current	200 μA (12 min) → 100 μA	
Detector	Potential gradient detector	
Temperature	20°C	
Chart speed	20 or 40 mm min^{-1}	

Anion-exchange chromatography

The gradient elution technique was employed to separate phosphorus oxoacids by anion-exchange chromatography. The elution conditions were as follows: column, 65 × 1.5 cm I.D., Bio-Rad AG 1-X8 (100–200 mesh); mixing bottle, 0.08 *M* sodium chloride–0.005 *M* EDTA-Na$_2$ mixed solution, 750 ml; reservoir, 0.2 *M* sodium chloride–0.005 *M* EDTA-Na$_2$ mixed solution; fraction size, 10 g; sample volume, 1 ml.

The pH of the eluting solution was adjusted to 7.0 with dilute sodium hydroxide solution.

Colorimetric determination and identification of phosphorus oxoacids

The colorimetric determination was carried out according to the orthophosphoric heteropoly-blue method, by means of which phosphorus as oxidation states 3 and 5 can be determined simultaneously$^{7-9}$.

Hydrolytic degradation of $\overset{3}{P}$*–O–*$\overset{5}{P}$ *and* $\overset{3}{P}$*–O–*$\overset{3}{P}$

Hydrolytic degradation of $\overset{3}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{3}{P}$ was started with the addition of hydrochloric acid. The pH of reaction mixtures was 2.0. After fixed time intervals, samples were removed with a 2-ml pipette into a test-tube, instantly neutralized with sodium hydroxide solution and cooled in an ice-bath to stop hydrolytic degradation.

RESULTS AND DISCUSSION

The separation of the phosphorus oxoacids is affected by the pH of the leading solution. Figs. 1 and 2 show the relationship between the pH of the leading solution and the PU values of five phosphorus oxoacids. The decreasing order of PU★ values is $\overset{5}{P} > \overset{3}{P} > \overset{5}{P}\text{–O–}\overset{5}{P} > \overset{3}{P}\text{–O–}\overset{5}{P} > \overset{3}{P}\text{–O–}\overset{3}{P}$. With L-glutamic acid as the terminating electrolyte, the PU values of the phosphorus oxoacids differ sufficiently except at pH

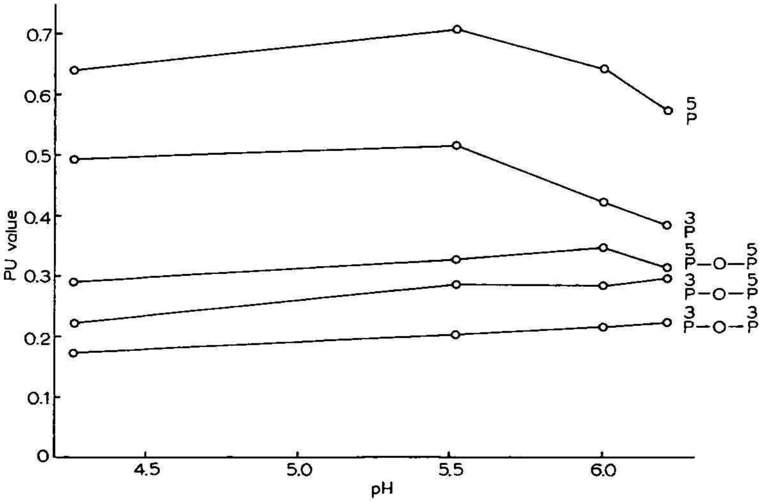

Fig. 1. Effect of pH of leading electrolyte on PU values. Leading electrolyte: L-histidine. Terminating electrolyte: L-glutamic acid.

★ The PU (potential unit value) represents the ratio of the potential gradient difference of the sample ion to that of the leading ion and of the terminating ion to that of the leading ion $(P_S - P_L/P_T - P_L)$.

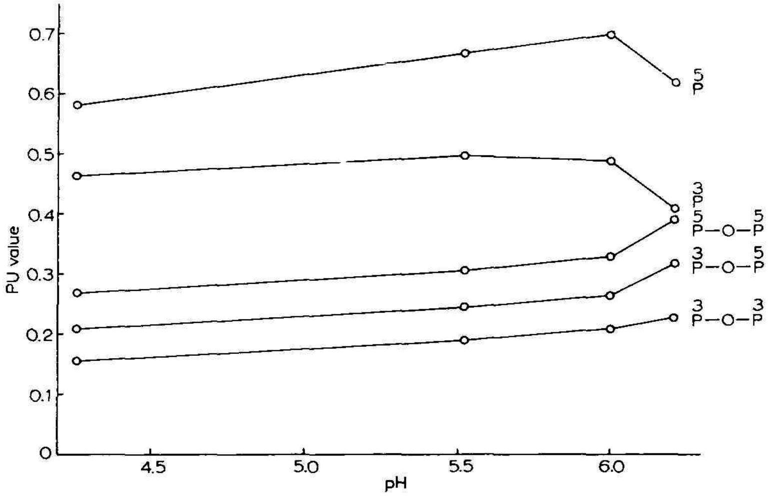

Fig. 2. Effect of pH of leading electrolyte on PU values. Leading electrolyte: L-histidine. Terminating electrolyte: hexanoic acid.

6.2, where the values for $\overset{5}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{5}{P}$ are so close that their separation is difficult.

With hexanoic acid as the terminating electrolyte, the PU values also differ sufficiently, except at pH 6.2, where $\overset{3}{P}$ and $\overset{5}{P}$–O–$\overset{5}{P}$ are too close to separate. Hence good separations are achieved at pH values below 6.0 with both the terminating electrolytes. In this work, a pH of 5.5 was chosen with histidine as the counter ion and hexanoic acid as the terminating electrolyte.

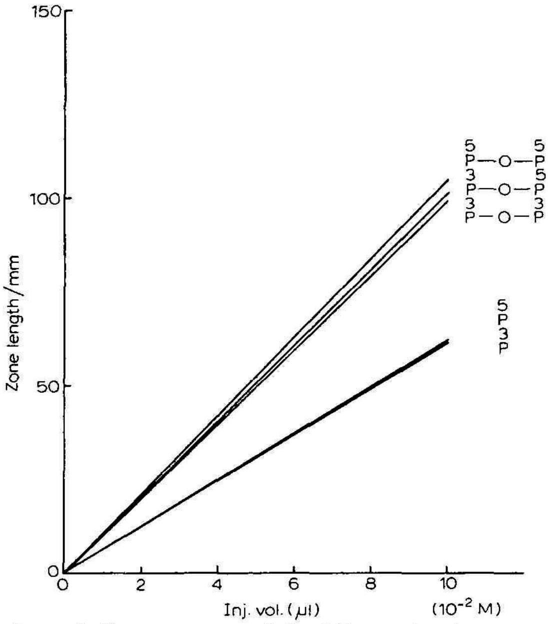

Fig. 3. Calibration graphs of the different phosphates.

Fig. 3 shows the calibration graphs for five varieties of phosphorus oxoacids. Linear relationships were obtained over the range of 0–10^{-7} mole of phosphorus oxoacids.

The slopes of the calibration graphs for $\overset{5}{P}-O-\overset{5}{P}$, $\overset{3}{P}-O-\overset{5}{P}$ and $\overset{3}{P}-O-\overset{3}{P}$, and for $\overset{5}{P}$ and $\overset{3}{P}$, are approximately equal.

From the relationship between zone length and the amount of the phosphorus oxoacid species, the zone length per phosphorus atom is almost the same for each. This finding shows that the volumes of $\overset{5}{P}-O-\overset{5}{P}$, $\overset{3}{P}-O-\overset{5}{P}$ and $\overset{3}{P}-O-\overset{3}{P}$, and those of $\overset{5}{P}$ and $\overset{3}{P}$, in the capillary tube during electrophoresis are almost equal.

Fig. 4 shows a typical isotachopherogram of a mixed sample of $\overset{5}{P}-O-\overset{5}{P}$, $\overset{3}{P}-O-\overset{5}{P}$ and $\overset{3}{P}-O-\overset{3}{P}$. The three phosphorus oxoacids were successfully separated, their PU values increasing in the order $\overset{3}{P}-O-\overset{3}{P} < \overset{3}{P}-O-\overset{5}{P} < \overset{5}{P}-O-\overset{5}{P}$. Although $\overset{3}{P}$ and $\overset{5}{P}$ were included as contaminants, they did not interfere in the separation of the three phosphorus oxoacids because their PU values were greater. The injection volume of the samples (10^{-2} *M*) had to be less than 2 μl because with more than 4 μl a mixed zone was formed.

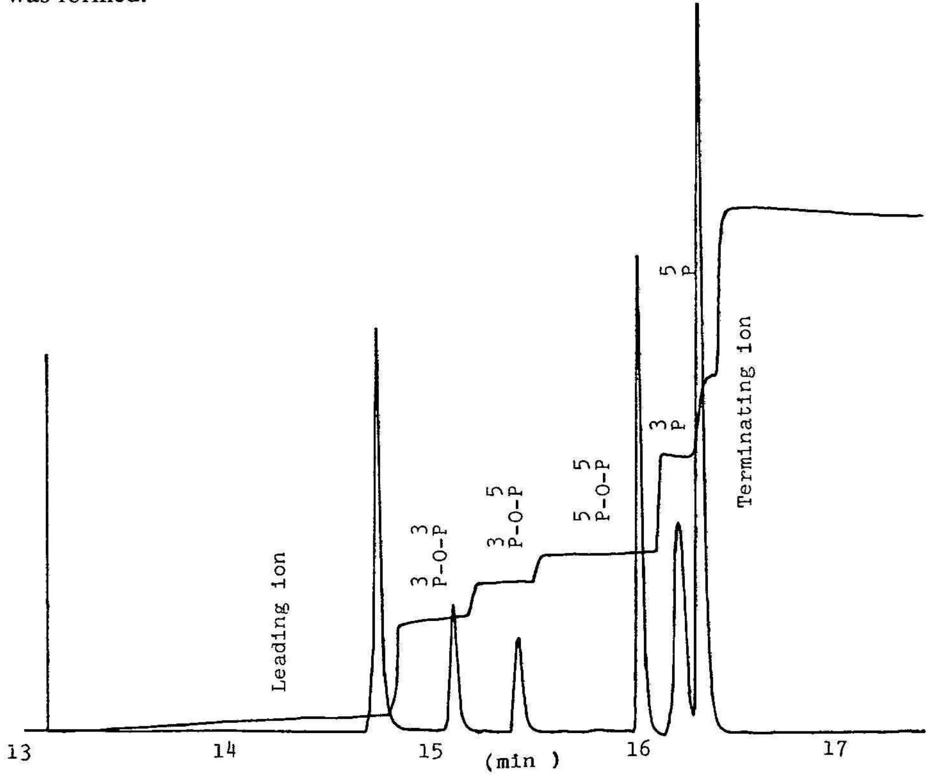

Fig. 4. Isotachopherogram of mixed sample of $\overset{5}{P}-O-\overset{5}{P}$, $\overset{5}{P}-O-\overset{3}{P}$ and $\overset{3}{P}-O-\overset{3}{P}$.

Application

The above procedure was applied to the analysis of some crude products and to the study of the hydrolysis of some condensed phosphates, *e.g.*, $\overset{3}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{3}{P}$.

Analysis of crude products. As the salts of $\overset{3}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{3}{P}$ were synthesized, they are always contaminated by some impurities. Figs. 5 and 6 show typical iso-

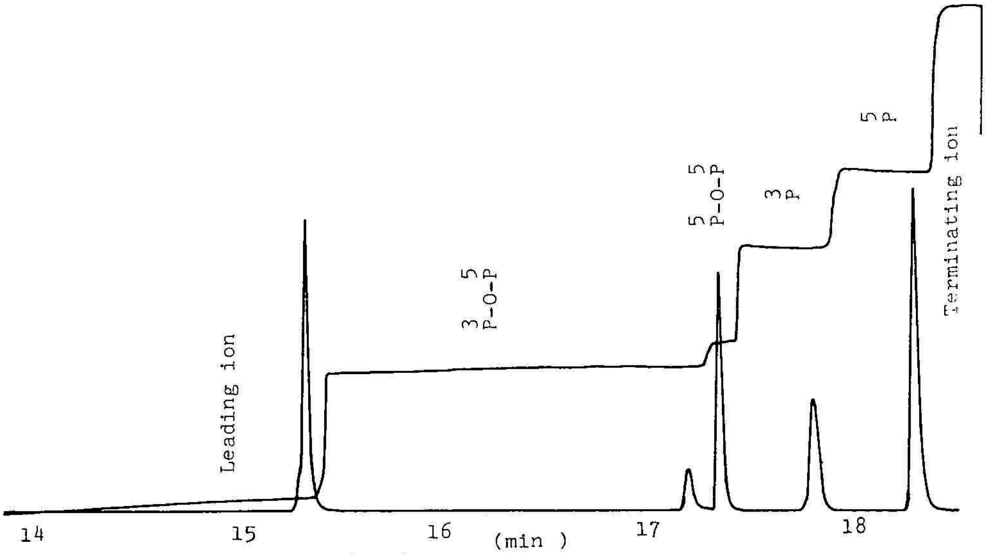

Fig. 5. Isotachopherogram of crude $\overset{3}{P}$–O–$\overset{5}{P}$.

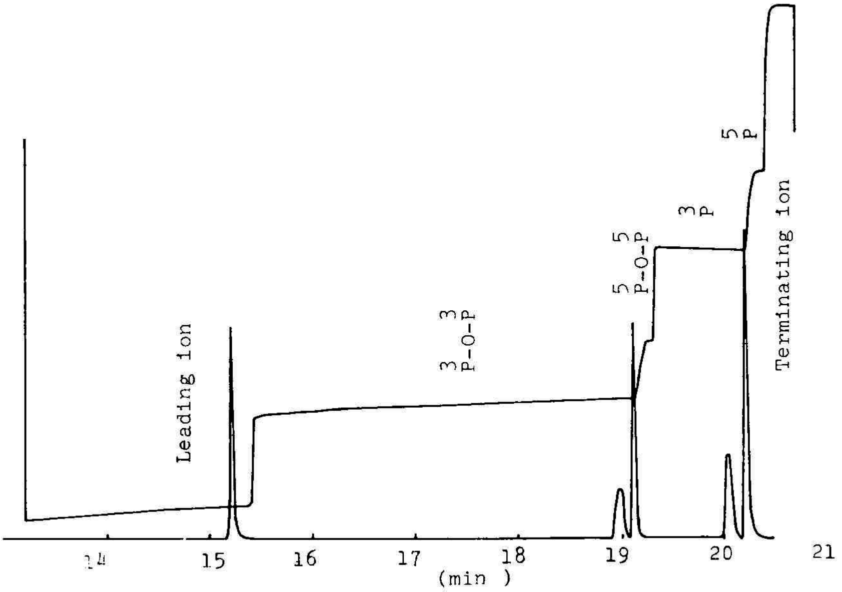

Fig. 6. Isotachopherogram of crude $\overset{3}{P}$–O–$\overset{3}{P}$.

tachopherograms of the crude $\overset{3}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{3}{P}$. Table II compares the results for some crude products obtained by isotachophoresis (IP) and by ion-exchange chromatography (IEC). The amounts of each phosphorus oxoanion were determined from the zone length. IP and IEC gave comparable results, but the former method was far superior in speed of analysis and ease of handling.

TABLE II

COMPARISON OF IP AND IEC RESULTS

Sample	*$\overset{5}{P}$ (%)*		*$\overset{3}{P}$ (%)*		*$\overset{5}{P}$–O–$\overset{5}{P}$ (%)*		*$\overset{3}{P}$–O–$\overset{5}{P}$ (%)*		*$\overset{3}{P}$–O–$\overset{3}{P}$ (%)*	
	IP	*IEC*	*IP*	*IEC*	*IP*	*IEC*	*IP*	*IEC*	*IP*	*IEC*
$\overset{3}{P}$–O–$\overset{3}{P}$	2.4	3.1	15.9	16.6	2.2	1.7			79.5	78.6
$\overset{3}{P}$–O–$\overset{3}{P}$	1.1	0.5	12.4	14.8			6.6	4.8	79.8	79.8
$\overset{3}{P}$–O–$\overset{5}{P}$	14.6	14.7	13.7	13.8	3.1	2.7	68.7	68.8		
$\overset{3}{P}$–O–$\overset{5}{P}$	3.8	3.3	3.9	3.6			92.3	93.1		

Hydrolysis of $\overset{3}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{3}{P}$. Hydrolytic degradation products were analysed by IP, and the results were in good agreement with those obtained by IEC. The plots of log (zone length) *versus* time for $\overset{3}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{3}{P}$ were straight lines, indicating that the hydrolytic degradation of these phosphorus oxoanions obeys first-order kinetics. The first-order rate constants were determined from the values of the half-life period: for $\overset{3}{P}$–O–$\overset{5}{P}$, $k_{20} = 0.30 \cdot 10^{-2}$, $k_{30} = 0.75 \cdot 10^{-2}$ and $k_{40} = 1.78 \cdot 10^{-2}$ min^{-1}; for $\overset{3}{P}$–O–$\overset{3}{P}$, $k'_{20} = 0.54 \cdot 10^{-2}$, $k'_{30} = 1.54 \cdot 10^{-2}$ and $k'_{40} = 3.37 \cdot 10^{-2}$ min^{-1}. The hydrolysis of $\overset{3}{P}$–O–$\overset{3}{P}$ proceeded more rapidly than that of $\overset{3}{P}$–O–$\overset{5}{P}$. Values of the activation energies (E_a) of 16 kcal mol^{-1} for both $\overset{3}{P}$–O–$\overset{5}{P}$ and $\overset{3}{P}$–O–$\overset{3}{P}$ were determined from Arrhenius plots.

The values of k, k' and E_a agreed well with the literature values[10,11], and these quantities can therefore be determined rapidly and easily by IP.

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Note

Liquid chromatographic determination and resolution of the enantiomers of the acid moieties of pyrethroid insecticides as their (–)-1-(1-phenyl)ethylamide derivatives

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(Received June 9th, 1982)

Most synthetic pyrethroid insecticides have from one to three chiral centers. Typical commercial samples are unresolved and therefore consist of mixtures of from two to eight optical and geometrical isomers, the biological activities of which range from highly toxic to virtually non-toxic[1,2]. Pyrethroid isomers also differ considerably in their rates and preferred pathways of biodegradation[3–5]. Thus, the study of the biological activity and the biological and environmental fate of pyrethroid isomer mixtures may not adequately describe the behavior of the toxic components, and methods for both the determination of pyrethroid isomer composition and the preparation of optically pure samples are clearly needed.

Several gas–liquid chromatographic (GLC) procedures for the determination of the optical isomers of chiral pyrethroid acid moieties have been reported. Most of these involve cleavage of the pyrethroid ester and derivatization of the acid moiety with optically active alcohols[6–9] or amines[10], followed by GLC separation of the resulting diastereomeric esters or amides. A recent method[11,12] involves the separation of amides prepared from optically inactive amines on optically active stationary phases. Although high-resolution liquid chromatographic (HRLC) methods have been reported for the analysis of pyrethroids[13–16], no HRLC method has been described for the determination of the optical isomers of pyrethroid acids.

Methods are also required for the optical resolution of pyrethroid acids as intermediates in the synthesis of optically pure esters. Pyrethroid acids are generally resolved on a preparative scale by fractional crystallization of diastereomeric salts prepared from racemic acids and optically active amines[17–19]. Although these methods are satisfactory for large-scale preparations, fractional crystallization is not suited to situations where only small amounts of racemic acid (*e.g.* radiolabeled preparations) are available. The HRLC separation of acid derivatives that could be readily converted into the optically pure free acids would provide a useful micro-scale alternative to conventional optical resolution procedures.

HRLC analysis of optically active arylethylamides has been used to determine the optical purity of several chiral terpenoid acids[20–22]. In this paper we report the

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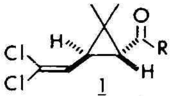

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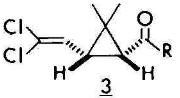

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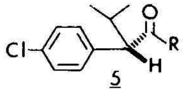

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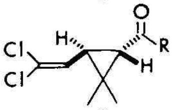

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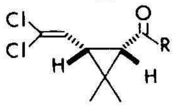

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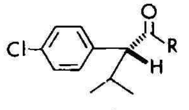

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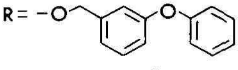
R = -OH

A



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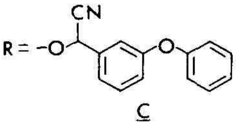

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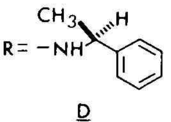

D

HRLC determination and small-scale preparative resolution of the four isomers of 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (DCVA; 1A–4A), the acid moiety of the pyrethroid insecticides permethrin (1B–4B) and cypermethrin (1C–4C), and the enantiomers of 2-(4-chlorophenyl)-3-methylbutyric acid (CMBA; 5A–6A), the acid moiety of the pyrethroid insecticide fenvalerate (5C–6C), as their (−)-1-(1-phenyl)ethylamide derivatives (1D–6D).

EXPERIMENTAL

Acid moieties of pyrethroids

Samples of a mixture of all four DCVA isomers and of the separated 1*RS*,*trans* (1A + 2A) and 1*RS*,*cis* (3A + 4A) racemates were provided by R. A. Robinson, FMC Corporation, Middleport, NY, U.S.A. Samples of optically pure 1*S* enantiomers (2A and 4A) prepared by fractional crystallization[18], were provided by M. Elliott, Roth-

TABLE I

HRLC AND TLC PARAMETERS FOR THE SEPARATION OF THE DIASTEREOMERIC (−)-1-(1-PHENYL)ETHYLAMIDES OF DCVA AND CMBA

Acid moiety	*Absolute configuration*	*HRLC parameters**,**					*TLC* R_F***	
		V'_1	k'	N	α	R_s	*A*	*B*
DCVA	1*S*,*cis*	26	1.51	3810			0.27	0.56
					1.56	4.24		
	1*R*,*cis*	40.4	2.35	3320			0.22	0.51
	1*S*,*trans*	53.2	3.09	2203			0.23	0.45
					1.44	2.58		
	1*R*,*trans*	76.4	4.44	973			0.19	0.40
CMBA	*R*	35.2	2.05	2750			0.38	0.35
					1.64	4.07		
	S	58.0	3.37	1745			0.32	0.31

* See Experimental section for analytical conditions.

** V'_1 = Corrected retention volume (ml); k' = capacity ratio; N = number of theoretical plates; α = selectivity ratio; R_s = resolution factor.

*** TLC solvent systems: A = carbon tetrachloride–diethyl ether (9:1); B = hexane–diethyl ether (1:1).

amsted Experimental Station, Harpenden, Great Britain. Racemic CMBA (5A +6A) was purchased from Frinton Labs., Vineland, NJ, U.S.A., and was recrystallized three times from hexane–benzene (10:1, v/v) prior to use. Optically pure 5A and 6A were obtained by fractional crystallization[19].

Preparation of diastereomeric derivatives

DCVA and CMBA samples (10–50 mg) were converted into their acid chlorides using an excess of $SOCl_2$ (Aldrich, Milwaukee, WI, U.S.A.) at 50–55°C for 2 h. Unchanged $SOCl_2$ was removed by rotary evaporation *in vacuo*. Derivatives were prepared by treating the crude acid chlorides with excesses of (–)menthol, (–)-2-octanol, or (–)-1-(1-phenyl)ethylamine (all from Aldrich) in 1–2 ml of dry benzene containing 30 μl of dry pyridine at 70–80°C for 0.5 h and then at room temperature for 16 h. The resulting esters or amides were purified by preparative thin-layer chromatography (TLC) on silica (Merck silica gel 60 F_{254} chromatoplates, 0.5 mm gel thickness, E. M. Labs., Elmsford, NY, U.S.A.) prior to HRLC analysis. Appropriate TLC solvent systems and R_F values for the amide derivatives are given in Table I.

Liquid chromatography

The liquid chromatograph consisted of an Altex Model 110A pump, a Valco CV-UHP loop injector and an LDC Model 1202 variable-wavelength UV detector operated at 235–240 nm. Separations were performed on a 25 × 1.02 cm I.D. column slurry-packed with LiChrosorb SI-60 (E. M. Labs.) at 3500 p.s.i. and eluted with various mixtures of hexane and diethyl ether, depending on the derivative analyzed, at a flow-rate of 4 ml/min.

Regeneration of free acids from derivatives

The (–)-1-(1-phenyl)ethylamides of the resolved acids (*ca.* 20 mg) recovered from HRLC were hydrolyzed in 6 *M* hydrochloric acid (5 ml) at 90–100°C for 6 h (DCVA) or in 50% aqueous sulfuric acid (5 ml) at 50°C for 6 h (CMBA). The reaction mixture was extracted with diethyl ether (3 × 2 ml), and the organic fractions combined and concentrated *in vacuo*. The residue was dissolved in dilute aqueous sodium hydroxide (pH 10), extracted with diethyl ether (2 × 2 ml), acidified to pH 2 (hydrochloric acid), and re-extracted with diethyl ether (2 × 3 ml) to recover the resolved acid free of any amine impurity (yield from amide, 75–90%). The resolved acids were converted into their acid chlorides as described above for preparation of (–)-1-(1-phenyl)ethylamides to confirm their optical purity.

RESULTS

HRLC separation of derivatized DCVA isomers

The (–)-1-(1-phenyl)ethylamides of the four DCVA isomers were resolved completely and rapidly on silica upon elution with 25% ether–hexane (Fig. 1a). This small-scale preparative column exhibited adequate efficiency and excellent resolution for this separation (Table I). Separate experiments with the *trans* (1A+2A) and *cis* (3A+4A) enantiomer pairs showed that the amides of *cis*-substituted acids eluted first. Analysis of the amides of authentic samples of 2A and 4A established that the 1*S* enantiomer of each pair eluted first, thus allowing the unambiguous assignment of

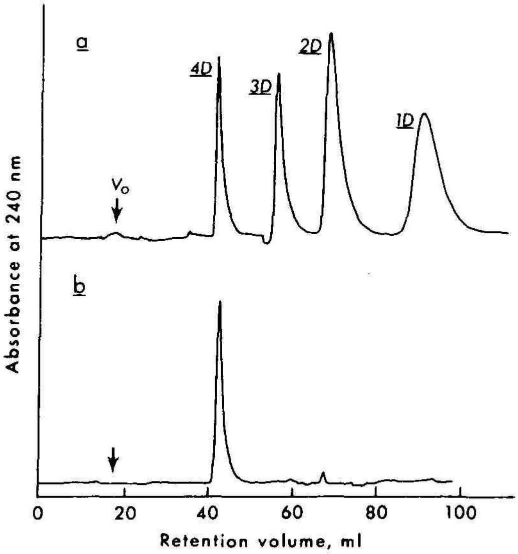

Fig. 1. (a) HRLC separation of diastereomeric amides 1D–4D; see Experimental section for conditions; (b) HRLC analysis of amide 4D after recovery from the amide mixture, acid hydrolysis to give free DCVA (4A) and re-preparation of the amide.

absolute configuration to each separated derivative. Minimum detectable levels at optimum detector sensitivity (A_{240}, 0.01 a.u.f.s.) were 0.1 and 0.5 μg for the amides of 2A and 4A, respectively.

In preliminary studies, we also explored the use of menthyl and 2-octyl ester derivatives for the resolution of the DCVA isomers by HRLC. These derivatives proved much more difficult to separate than the amides, requiring low solvent strengths ($\leqslant 1\,\%$ diethyl ether in hexane) and long analysis times for the achievement of partial resolution.

HRLC separation of enantiomeric CMBA amides

We extended the use of (−)-1-(1-phenyl)ethylamide derivatives to explore the resolution of the enantiomers of CMBA, a pyrethroid acid moiety lacking the cyclopropane ring and having only one chiral center. The diastereomeric amides were well resolved in the same chromatographic system used for the DCVA amides (Fig. 2a). Analysis of the amide prepared from the authentic *R* enantiomer (6A) established that this enantiomer eluted first. The efficiency and degree of resolution for this separation were similar to those observed for the amides of 3A and 4A (Table I). The minimum detectable of the amide of 5A at optimum detector sensitivity (A_{237}, 0.01 a.u.f.s.) was approximately 0.5 μg.

TLC separation of amide derivatives

We routinely used TLC for preliminary clean-up of amide derivatives and for the selection of candidate HRLC mobile phases. These procedures identified two solvent systems capable of separating the amides of *cis*-DCVA, *trans*-DCVA and

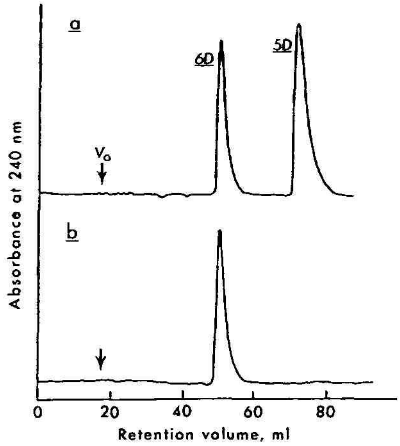

Fig. 2. (a) HRLC separation of diastereomeric amides 5D and 6D; see Experimental section for conditions; (b) HRLC analysis of amide 6D after recovery from the amide mixture, acid hydrolysis to give free CMBA (6A) and re-preparation of the amide.

CMBA on both analytical and preparative layers (Table I). The degree of resolution observed for TLC was considerably lower than that obtained by HRLC, so that rechromatography of each initial fraction was required to obtain optically pure material.

Regeneration of optically pure acids from amide derivatives

The chromatographically resolved DCVA amides were hydrolyzed in 6 *M* hydrochloric acid, and the free acids were recovered directly from the reaction mixtures by organic extraction. Re-preparation of the amide derivatives of the resolved acids, followed by HRLC analysis, demonstrated that neither chiral center underwent significant epimerization under the conditions required for hydrolysis (Fig. 1b). Attempts to hydrolyze the DCVA amides under basic conditions were unsuccessful, resulting in all cases in complex product mixtures and low yields of the recovered optically pure acids.

The CMBA amides were not hydrolyzed under the same conditions used for the DCVA derivatives, but the free CMBA enantiomers were obtained from hydrolysis in 50% aqueous sulfuric acid. Sulfuric acid-catalyzed hydrolysis at 50°C gave the optically pure acid without epimerization, as determined by re-preparation and chromatographic analysis of the amide derivative (Fig. 2b). However, higher temperatures (60–80°C) resulted in 15–30% epimerization as determined by analysis of the re-prepared amide derivatives.

DISCUSSION

Our results extend the usefulness of optically active arylethylamines as derivatizing agents for the HRLC separation of enantiomeric carboxylic acids to the optical and geometrical isomers of the acid moieties of pyrethroid insecticides. We found (−)-1-(1-phenyl)ethylamine, readily available from commercial sources at a high degree of optical purity, to be a suitable derivatizing agent for the resolution of pyrethroid acids. In particular, these amide derivatives proved to be more readily separable than the menthyl and 2-octyl derivatives under normal-phase HRLC conditions.

We determined that optically pure pyrethroid acids could be regenerated by acid hydrolysis from the chromatographically isolated amide derivatives in satisfactory yields without epimerization. This finding suggests that the HRLC or TLC separation of amide derivatives may be an efficient method for the micro-scale preparative resolution of pyrethroid acid moieties. Chromatographic resolution may be particularly applicable for radiolabeled preparations necessary for metabolism, mode of action and environmental degradation studies. Previous approaches have required optical resolution of labeled pyrethroid acid moieties by conventional techniques[23,24] or the radiosynthesis of a single isomer from an unlabeled chiral intermediate[25]. Chromatographic micro-scale resolution may offer access to a wide variety of acid-labeled optically pure pyrethroids presently available only as racemates. This technique may also prove useful in the preparation of unlabeled optically pure pyrethroids in instances where the availability of racemic acid is limited.

Because of our primary interest in preparative resolution, we did not optimize chromatographic conditions for enantiomer composition determinations at high sensitivity. Nevertheless, we were able to detect sub-microgram quantities of the amides using essentially preparative chromatographic conditions. It is likely that the use of more efficient, smaller-diameter analytical columns and an amide derivative having a higher molecular extinction coefficient[22] would result in a several-fold increase in the sensitivity of this method. With analytical scale optimization, this HRLC method for the determination of pyrethroid isomer composition may prove to be a useful alternative to the GLC methods[6–12] available.

ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatography of the janthitrems: fluorescent tremorgenic mycotoxins produced by *Penicillium janthinellum*

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Tremorgenic mycotoxins from *Penicillium* species and other fungi found in the pasture environment are being examined in studies on the neurological disease of sheep and cattle known as ryegrass staggers[1,2]. Recently, three tremorgenic toxins named janthitrem A, B and C (Jan A, Jan B, Jan C) were isolated from *Penicillium janthinellum* cultures, and their behaviour on silica gel thin-layer chromatography (TLC), molecular weights and molecular formulae reported[3]. The janthitrems were found to have strong UV absorption spectra and strong fluorescent spectra[3]. Methanolic solutions of Jan B, one of the major janthitrems produced, had absorption maxima at 228, 258, 265 (major) and 329 nm and a fluorescence emission maximum at 385 nm. The tremorgenic activity of cultures and TLC fractions were determined by a mouse bioassay[3].

In order to screen a range of fungal culture variants for the occurrence of these new tremorgens, and to relate the activity in mouse bioassay to the presence of these or other known tremorgens, a rapid method of identification and quantitation was required. We have developed a method of analysis for the janthitrems using reversed-phase high-performance liquid chromatography (HPLC). The use of both UV and fluorescence detectors allows differentiation of the janthitrems from other compounds of similar retention times. A fourth compound, first seen on HPLC, has been separated and isolated by preparative TLC, and is tentatively named janthitrem D (Jan D).

EXPERIMENTAL

Apparatus

The liquid chromatograph has been described[4]. The fluorescence detector was an LDC Fluoromonitor III fitted with either 370-nm bandpass excitation and a 400-nm cutoff emission filter or 254-nm line excitation and a 370-nm cutoff emission filter. It was placed in-line after the variable-wavelength UV detector.

Chromatographic conditions

The columns used were Zorbax C_8 (25 cm × 4.6 mm I.D.) (Dupont, Wilmington, DE, U.S.A.), μPorasil (30 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA,

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U.S.A.) and a column (25 cm × 3.1 mm I.D.) slurry-packed with Spherisorb S5 CN (Phase Separations, Queensferry, Great Britain). The solvent systems were water–methanol, hexane–ethyl acetate–methanol and hexane–isopropanol for respective columns; relevant conditions are listed in the text. The analyses were performed at room temperature, 19–22°C. Solvents were analytical grade and were purified as previously described[5].

Janthitrem samples and culture extracts

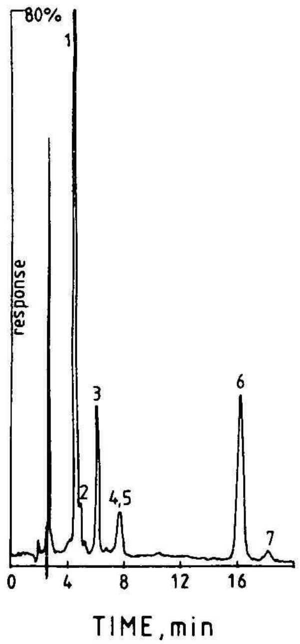
Dried mycelial mats of *P. janthinellum* cultures[2] grown on Czapek-Dox yeast extract broth[6] or potato–milk–sucrose broth[7], were mixed with chloroform–methanol (2:1) in a blender for 3 min. The resultant slurries were filtered and the filtrates dried over anhydrous sodium sulphate, filtered, and evaporated to dryness under vacuum on a rotary evaporator. The crude extracts were stored in the dark at −5°C. Pure janthitrems were separated by TLC[3] and stored dry in a similar manner. Standard solutions (10 μg/ml) of Jan B were made in methanol and ethyl acetate, and of Jan C in methanol. The vials containing these solutions were held in cardboard cylinders, and kept at 2°C between use. The Jan C standard was found to be stable for at least six months. Jan B was stable in methanol for four months, but breakdown was apparent after seven months. In ethyl acetate, considerable degradation had occurred within three months.

RESULTS AND DISCUSSION

Standard solutions of Jan B and Jan C were used to test the suitability of different chromatographic conditions. The C_8 column was found to have far greater selectivity than either the silica or the CN column. Both the silica column, using hexane–ethyl acetate–methanol (85:14.7:0.3), and the CN column, using hexane–isopropanol (90:10), showed little separation of Jan B and Jan C. The compounds could be well resolved on the C_8 column, however. An additional advantage of the C_8 column was that the janthitrems exhibited much stronger fluorescence in water–methanol than in either of the other two solvent systems.

In order to test for the presence of janthitrems in fungal cultures, extracts were analysed on the C_8 column using water–methanol (20:80). Under these conditions, the major janthitrems, Jan B and Jan C, were well separated from each other and from other common tremorgens such as penitrem A, verruculogen and fumitremorgen B (Fig. 1). To verify further the presence of the janthitrems, and to enhance sensitivity, a fluorescence detector could be used in conjunction with the UV absorbance detector. The janthitrems are the only tremorgens of those mentioned above to exhibit significant fluorescence.

In the isocratic system used to screen fungal culture extracts for janthitrems, Jan A and Jan B were eluted very close together and were only partially resolved (see Fig. 1). In addition, once several extracts had been analysed, the presence of strongly retained co-extractives on the column degraded its efficiency and Jan A merged into the tail of Jan B. This effect was aggravated by the fact that Jan B generally occurred in much greater concentrations than Jan A. Increasing the water content of the mobile phase allowed better resolution of the two peaks, and at water–methanol (36:64), near baseline separation of Jan A from Jan B was achieved at the relative



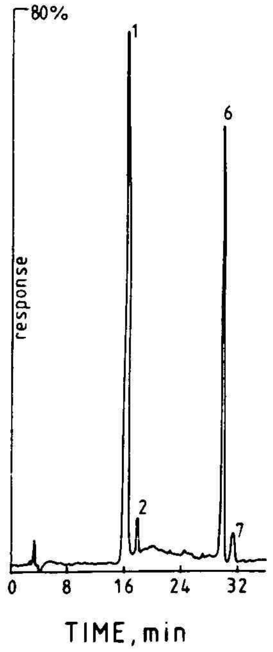


Fig. 1. HPLC of janthitrems Jan A, Jan B, Jan C and Jan D in combination with verruculogen, penitrem A and fumitremorgen B. Conditions: column, Zorbax C_8; eluent, water–methanol (20:80); flow-rate, 1 ml/min; UV detector wavelength, 265 nm; detector sensitivity, 0.04 a.u.f.s.; sample size, 20 μl. The sample was a composite mixture made by spiking a janthitrem-containing culture extract with three other tremorgens. Sample dissolved in eluent. Peaks, with approximate concentrations (μg/ml): 1 = Jan B (12); 2 = Jan A (estimate 0.5); 3 = verruculogen (24); 4 = penitrem A (20); 5 = fumitremorgen B (12); 6 = Jan C (17); 7 = Jan D (estimate 1).

Fig. 2. HPLC conditions for quantitation of janthitrems A, B, C and D in fungal culture extracts. Conditions as in Fig. 1 except for eluent, water–methanol (36:64) for 10 min followed by linear gradient over 5 min to water–methanol (20:80); UV detector wavelength, 330 nm; detector sensitivity, 0.16 a.u.f.s.; sample size, 50 μl. The sample was the extract used for Fig. 1 dissolved in water–methanol (50:50). Peaks as in Fig. 1.

concentrations commonly found in fungal culture extracts. Jan C and Jan D were retained indefinitely with this solvent, and a step gradient to water–methanol (20:80) was required to elute them in reasonable time. Quantitation of the four janthitrems in fungal culture extracts could be achieved using the conditions shown in Fig. 2.

The extract shown in Fig, 2 was used for preparative TLC[3] to isolate samples of each of the four janthitrems and to confirm their HPLC designations. The samples were recovered from TLC in order of decreasing R_F, as Jan D, Jan C, Jan A, Jan B. The isolation of Jan D has not been reported before. No molecular formula has yet been obtained for this compound, but it has been tentatively named as a janthitrem because of the characteristic UV and fluorescent properties, and because of its behaviour on HPLC and TLC. Attempts to isolate the individual janthitrems by preparative HPLC were not successful due to the low solubility of the compounds in methanol containing significant amounts of water. Further investigation of the straight-phase systems may be necessary to make preparative HPLC a viable alternative to TLC.

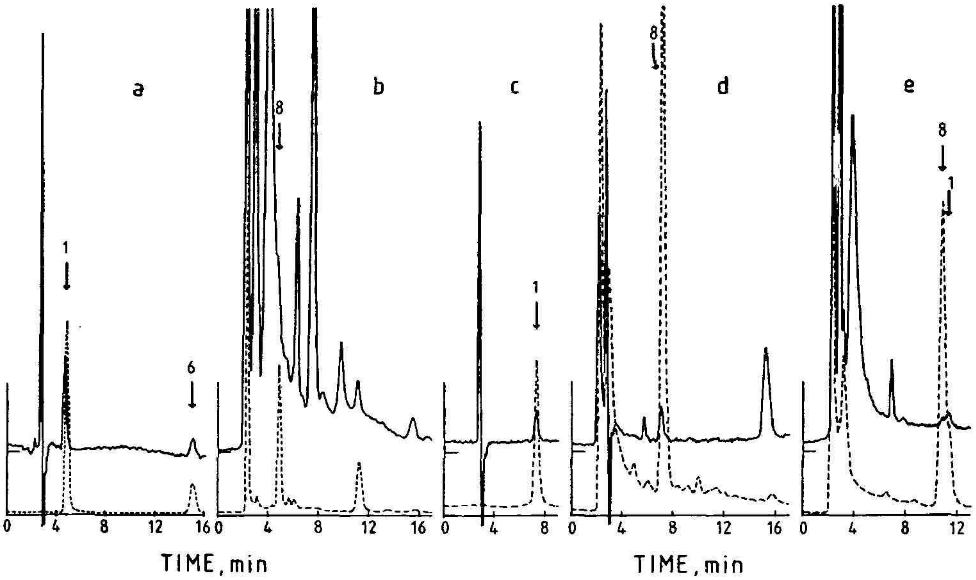


Fig. 3. Combined UV (upper trace) and fluorescent (lower trace) estimation of janthitrems in a fungal culture extract. Conditions as in Fig. 1 except for eluent, c, d, water–methanol (28:72); e, water–methanol (32:68); UV detector wavelength, a, b, e, 265 nm; c, d, 330 nm; detector sensitivity, 0.04; fluorescence excitation/emission wavelength combinations, a, b, 254/370 cutoff; c, d, e, 370/400 cutoff; fluorescence range, a, b, 500; c, d, e, 10. Samples, in methanol, were: a, 1 μg/ml Jan B plus Jan C; b, tremorgenic fungal culture extract; c, 1 μg/ml Jan B; d, as in b (first part of trace); e, 1:1 mixture of b and c (first part of trace). Peaks 1–7 as in Fig. 1; 8 = unknown fluorescent compound.

For UV detection of the janthitrems the wavelength was set at either 265 nm or 330 nm. At 330 nm the detector was more selective while at 265 nm a better indication of the presence of any co-extracted impurities could be obtained. Some strongly retained peaks, and much of the early eluting co-extractives seen at 265 nm, are not seen at 330 nm. The janthitrems also show an enhanced response at 265 nm compared to 330 nm; by 30–40% for Jan B and by 10–20% for Jan C and Jan D. On our system run at 265 nm and with water–methanol (20:80), the minimum detectable amount of Jan B was 1 ng, and of Jan C, 5 ng.

The use of a fluorescence detector allowed smaller amounts of the janthitrems to be detected. In our system, with the fluorescence detector connected in line after the UV detector, a twenty-fold increase in sensitivity over UV determination was obtained using the standard 370-nm bandpass excitation lamp and filter combination in conjunction with a 400-nm cutoff emission filter. A further 50-fold increase in sensitivity could be obtained by using a 254-nm line excitation and a 370-nm cutoff emission filter. The ratio of the absorbance response to the fluorescence response was similar for each of the four janthitrems. An added advantage of using a combination of UV and fluorescence detection was that the janthitrems could be differentiated from compounds with identical retention times but with different ratios of absorbance to fluorescence response. For example, when run in water–methanol (20:80) the culture extract shown in Fig. 3b was originally thought to contain Jan B. However, when run in water–methanol (28:72) with the UV wavelength set at 330 nm (more specific for janthitrems) and the fluorescent excitation wavelength set at 370 nm (less sensitive for janthitrems), a simplified chromatogram results, as shown in Fig. 3d. In Fig. 3c, Jan B occurs at retention time 443 sec and has an absorbance/fluorescence ratio of *ca.* 1:4. In Fig. 3d, the extract peak occurs at retention time 428 sec and has an absorbance/fluorescence ratio of 1:16, strongly suggesting a compound other than Jan B. As Fig. 3e shows, these two compounds are partly resolved in water–methanol (32:68). Single injections show the peaks for Jan B and the unknown extract component at 678 sec and 648 sec respectively.

The monitoring system for janthitrems in fungal culture extracts described here has been used on fourteen *P. janthinellum* extracts previously tested for tremorgenic activity by mouse bioassay. Janthitrems were found in eight samples, with concentrations in the order Jan B, Jan C ⩾ Jan A, Jan D. Only four of these samples, with very high levels of janthitrems, showed activity in the bioassay. The screening of fungal culture extracts for janthitrems is rapid and accurate using isocratic reversed-phase HPLC with combined variable UV–fluorescence detection. Quantitation of the individual janthitrems is possible with a gradient system or with an isocratic system set up to allow a simple step gradient.

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We thank Dr. M. E. di Menna for preparation of the fungal cultures, and Mr. P. A. Wyatt for the mouse bioassay results.

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Note

Rapid high-performance liquid chromatographic method for the analysis of benzene in gasoline

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The authorities of many countries have decided to reduce the lead content of automobile gasoline. This has forced the producers to look for other ways of maintaining a high octane number. One possibility is to increase the content of benzene and alkylbenzenes in gasoline. Because of the toxic properties of benzene, Norwegian authorities have set a maximum limit for this compound of 5% (v/v) in automobile gasoline. Different techniques such as gas chromatography[1] nuclear magnetic resonance spectroscopy[2] and infrared spectrometry[3] have been used for the determination of benzene in gasoline. In addition the retention behaviour of benzene and some alkylbenzenes using high-performance liquid chromatography (HPLC) with different reversed-phase columns has been studied[4].

Both high-resolution capillary gas chromatography (HRGC) and HPLC have been investigated. A simple method based on a single dilution step and quantification by HPLC has been developed for benzene. The results were controlled by HRGC which was used as an independent method. Except for one sample the agreement of the measuring values was better than ±4%. This special gasoline sample contained an unknown compound which interfered strongly with the gas chromatographic determination. Using HRGC–mass spectrometry this compound was tentatively identified as an isomer of hexadiyne.

EXPERIMENTAL

Standards and reagents

All reagents used were at least of analytical-reagent grade. Benzene standard solutions were prepared from analytical-reagent grade benzene (E. Merck, Darmstadt, G.F.R.; No. 1783) which was diluted in isooctane for HPLC determination or in light petroleum (b.p. 40–60°C) for quantification by HRGC. 2-Butanone (E. Merck, No. 9708) was used as an internal standard for the HRGC method.

Apparatus

The HPLC system consisted of a CCM control module, a Constametric III and I pump and a UV III monitor Model 1203 with a fixed wavelength of 254 nm (all from Laboratory Data Control, Division of Milton Roy Co., FL, U.S.A.). The injector was a Rheodyne 7125 with 20-μl loop which was completely filled. A Supelcosil LC-8

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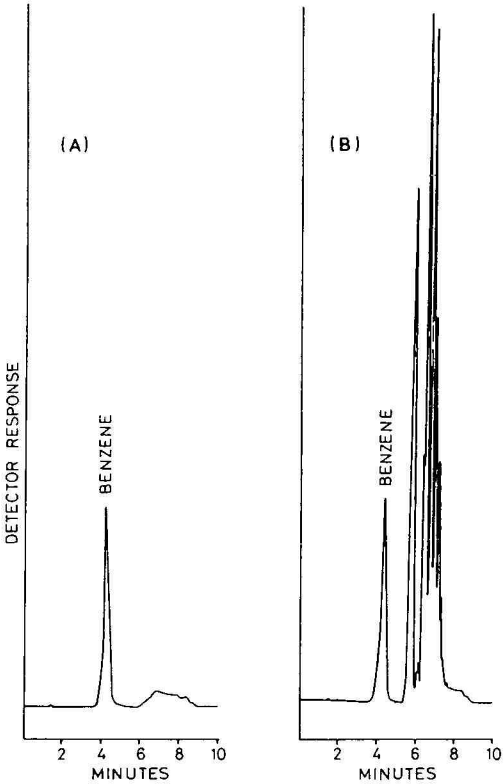

Fig. 1. Chromatograms from HPLC analysis showing (A) a standard of 0.3% benzene and (B) a gasoline sample.

column (150 × 4.6 mm; Supelco, Bellefonte, PA, U.S.A.) was used. The gas chromatographic analysis was carried out on a Carlo Erba Fractovap 2150 equipped with an electrometer type Type 180, a LT programmer 232, a flame-ionization detector, a Grob-type split/splitless injector and a Spectra-Physics system I computing integrator. A glass capillary column (25 m × 0.3 mm I.D.) coated with OV-225 (film thickness 0.2 μm) was used.

Analytical procedure

HPLC method. Prior to the HPLC analysis the gasoline samples were diluted (1:9) in methanol. Base standards of 1, 3 and 5% benzene content were diluted further with methanol to working standards of 0.1, 0.3 and 0.5% respectively. After injecting a 20-μl sample a solvent mixture of methanol–water (70:30) was used to elute benzene at a flow-rate of 1 ml/min. Afterwards all other compounds were washed out using pure methanol at a flow-rate of 2.5 ml/min. A new sample could be injected after 11 min. The benzene concentration was calculated from the peak area using the external-standard method. The relationship between area and concentration was linear in the actual measuring range. A 0.3% benzene standard was injected after every five samples for control and correction of the response factor. Fig. 1 shows a chromatogram of a standard and a gasoline sample.

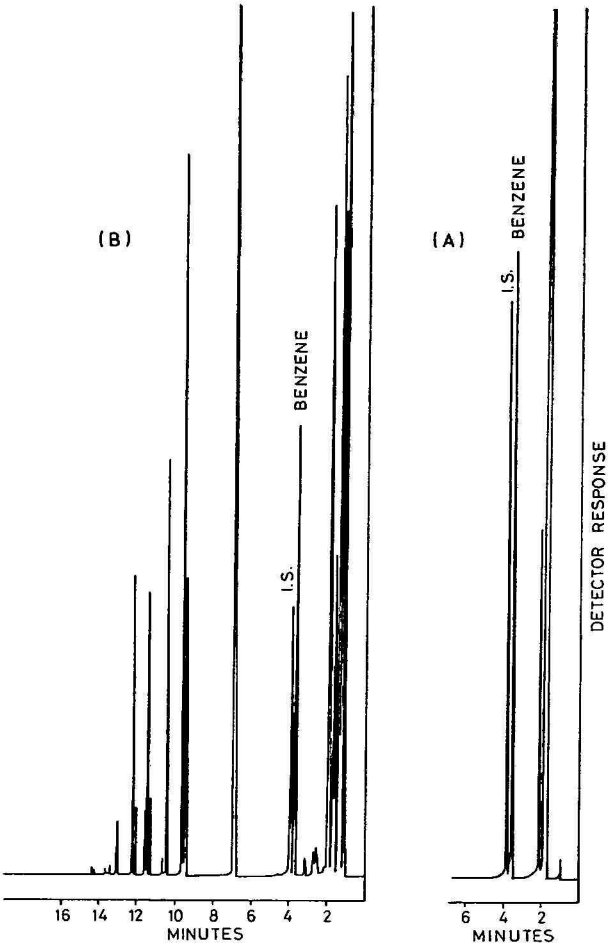

Fig. 2. Chromatograms from HRGC analysis showing (A) a standard of 3.0% benzene and (B) a gasoline sample.

HRGC method. A 4% sample of the internal standard (I.S.) was added to the undiluted gasoline sample and to standard solutions containing 2 or 4% benzene in light petroleum. A 0.1-μl aliquot was injected with a split ratio of 1:100. The oven was kept at 30°C for 5 min, then the temperature was raised at a rate of 15°C/min to 150°C. At least two standard solutions were injected each day for calculation of the relative response factor. The resin OV-225 is a polar stationary phase which gives benzene a relatively long retention time. This results in a better separation from other less polar compounds in the same range of boiling point than with less polar stationary phases.

Fig. 2 shows a chromatogram of a standard and a gasoline sample.

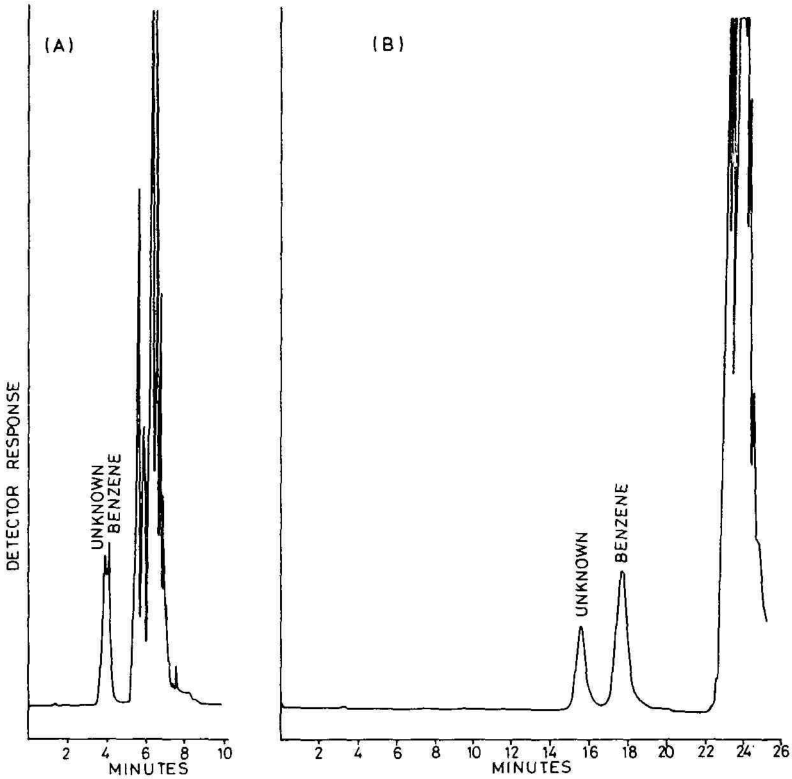

Fig. 3. HPLC chromatograms of a gasoline sample containing an interfering compound eluted at (A) 70 % and (B) 50 % methanol.

RESULTS AND DISCUSSION

Table I compares the results from both HRGC and HPLC quantification of thirteen samples from six oil companies. The results of both methods are the average of two analyses each. Except for sample no. 8, the results from the HRGC and the HPLC method are in good agreement and no systematic deviation is observed.

Sample No. 8 showed a considerably higher value when analysed by HRGC. This also was the only sample showing an unknown component as an unresolved peak in front of the benzene peak when analysed by HPLC (see Fig. 3). A better separation was obtained by reducing the methanol concentration in the eluent from 70 to 50 %, and the benzene content could be measured without any interference from the first peak.

The undiluted sample No. 8 was injected on to the HPLC column and both the unknown and the benzene was collected separately. The two solutions were extracted with hexane and the extracts were analysed by HRGC. This revealed that the two compounds had the same retention on the OV-225 column. HRGC–mass spectrometric analysis of the two extracts using the same HRGC column showed that the two compounds had identical mass spectra using electron-impact ionization. A reasonable proposal for the structure of the unknown is 1,4-hexadiyne.

TABLE I

COMPARISON OF THE RESULTS FROM HGRC AND HPLC ANALYSIS OF THIRTEEN GASOLINE SAMPLES

Sample No.	*Oil company*	*Octane number*	*Concentration (%, v/v)*		*Difference (%)*
			GC	*HPLC*	
1	A	98	4.33	4.29	+ 0.9
2	A	93	3.67	3.64	+ 0.8
3	B	98	3.37	3.35	+ 0.6
4	B	93	3.22	3.14	+ 2.5
5	C	98	3.17	3.25	− 2.5
6	C	93	3.27	3.21	+ 1.9
7	D	99	5.10	5.00	+ 2.0
8	D	99	2.80	2.20	+27.3
9	D	93	4.45	4.29	+ 3.7
10	E	98	1.78	1.76	+ 1.1
11	E	93	1.55	1.59	− 2.5
12	F	98	4.44	4.40	+ 0.9
13	F	93	4.35	4.33	+ 0.5

Until now, 42 gasoline samples have been analysed by the HPLC method and only two samples, both coming from the same supplier, showed this interfering peak. This study indicates that apart from being faster and demanding less pretreatment, the HPLC method is less exposed to interferences than the HRGC method.

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Note

Rapid high-performance liquid chromatographic determination of acetaminophen in dosage form using a totally aqueous mobile phase

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(Received June 9th, 1982)

Over the last decade, acetaminophen has become a widely used substitute for aspirin and aspirin-type analogues. Since it contains no carboxylic acid functionality, it is promoted as an effective analgesic and antipyretic with gentler gastrointestinal properties. As a result of its rapid success and popularity as an alternative medication, the analysis of acetaminophen has received considerable attention.

In just the last half dozen years, numerous techniques have been employed for the analysis of acetaminophen in biological samples. Various chromatographic[1–3], spectrophotometric[4], and electrochemical[5] methods have been reported. Of the liquid chromatographic procedures, reversed-phase separations employing an octadecyl column often are utilized[6,7].

The quantitation of acetaminophen in various dosage forms also has received considerable attention. Titration with tetraalkylammonium hydroxides[8,9], colorimetric methods[10–12] and an oxidative electrochemical reaction using a carbon electrode[13] have been reported. Gas and liquid chromatographic[14,15] assays for acetaminophen in dosage forms also have been devised.

Recently published high-performance liquid chromatographic (HPLC) methods have employed both silica and alkyl phases as packings. Many of these separations require moderately long analysis times. For example, the use of a silica column and a mobile phase consisting of butyl chloride, tetrahydrofuran, methanol and glacial acetic acid has been reported to require *ca.* 30 min per sample[15].

The current HPLC procedure is a rapid simple method for the analysis of acetaminophen in tablets and capsules either as single or composite samples. It is designed as an internal standard method for process control. The procedure consists of a simple dissolution step followed by a single dilution. After sample preparation, the complete chromatographic analysis takes *ca.* 2 min per sample injection, using only water as the mobile phase.

EXPERIMENTAL

Reagents

All separations were carried out on 5 cm × 4.5 mm I.D. octyl columns obtained from IBM Instruments (Danbury, CT, U.S.A.). Prior to use, the column was phase-rearranged by initially conditioning with at least 50 ml of acetonitrile followed

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by an equivalent volume of water. The flow was turned off for an additional 20 min. This conditioning was carried out at 55°C. The column was then brought to thermal equilibrium at the analysis temperature of 40°C.

The acetonitrile was distilled-in-glass UV grade obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All water was obtained from a Barnstead (Boston, MA, U.S.A.) 4-module NANO pure purification system.

Equipment

Column temperature was controlled to 0.1°C using a Neslab (Portsmouth, NH, U.S.A.) Model EX-300 temperature and Model DCR-1 digital controller. All detection was at 254 nm.

Procedure

A 0.5 mg/ml internal standard solution was prepared by dissolving 50 mg of methyl *p*-hydroxybenzoate in 100 ml of water. Calibration curve standards were prepared by quantitatively adding respectively 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of 1.30 mg/ml acetaminophen standard to 10-ml volumetric flasks. All flasks were volumetrically filled following the addition of 2.0 ml of internal standard solution to each.

Individual tablet, capsule or equivalent composite samples were placed in 250-ml volumetric flasks to which *ca.* 100 ml of water were added. The mixtures were shaken for 20–30 min after which the content of each flask was diluted to volume. After allowing a solution to settle for at least 15 min, 0.5 ml of it was transferred to a 10-ml volumetric flask. Then 2 ml of internal standard were added and the contents were diluted to volume with water.

Aliquots of 5 μl of the above samples and standards were chromatographed on a 5-cm octyl column using 100% water as the mobile phase. In all cases, the flow-rate was maintained at 2.0 ml/min at a column temperature of 40°C.

RESULTS AND DISCUSSION

Shown in Fig. 1 are representative chromatograms of acetaminophen and the internal standard methyl *p*-hydroxybenzoate. These data were obtained from a series of calibration standards. All separations were carried out, using a totally aqueous mobile phase, on a 5-cm octyl column in the phase-rearranged orientation.

The concept of phase rearrangement has been discussed previously[16,17]. Briefly, surfaces with chemically attached hydrocarbon moieties of appropriate chain length and silane-backbone structures can be oriented in one of two configurations in a totally aqueous environment. These two arrangements have been termed the folded or down-state and the extended or up-state. The down-state is obtained after an initial conditioning procedure with an organic water-miscible solvent, usually methanol or acetonitrile, followed by treatment with water. The up-state is formed by thermal rearrangement at elevated temperatures. For the particular surface used, treatment at 55°C was sufficient for formation of the up-state configuration.

As the result of phase rearrangement, solutes may be made to elute rapidly or to be retained for greater lengths of time, depending on the nature of the bonded alkyl chains. Shorter retentions are obtained when the phase is wetted or in the up-state

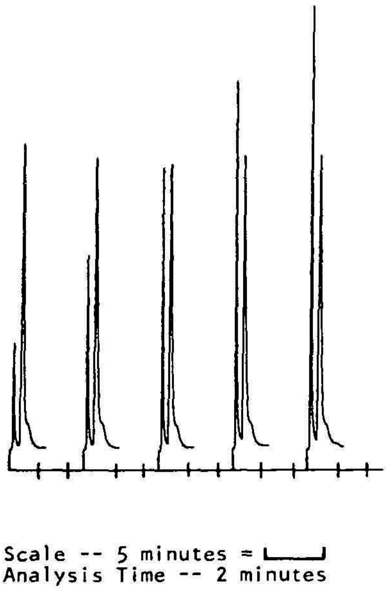

Fig. 1. Representative chromatograms of acetaminophen standard solutions. Peaks: first peak, acetaminophen; second peak; methyl *p*-hydroxybenzoate. Conditions: mobile phase, 100% water; flow-rate, 2.0 ml/min; column temperature, 40°C.

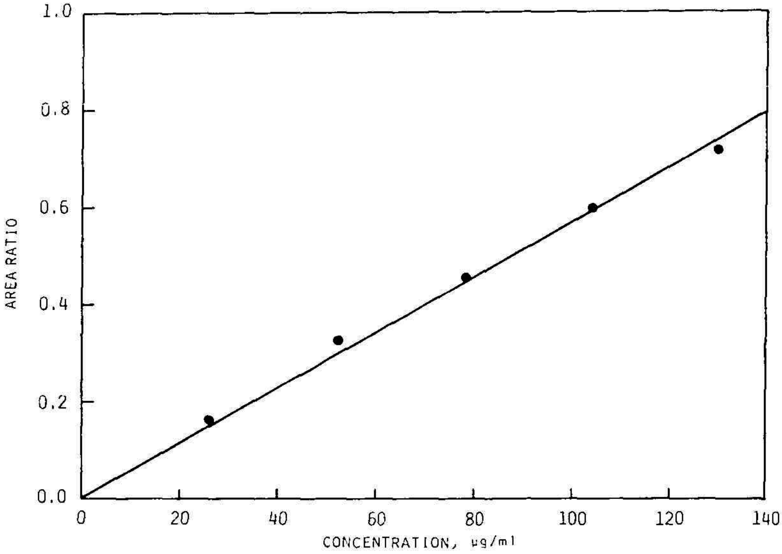

Fig. 2. Calibration curve for the area ratio of acetaminophen to internal standard (*i.e.*, methyl *p*-hydroxybenzoate) *vs.* concentration of acetaminophen in μg/ml.

orientation. This arrangement greatly reduces retentivity of the surface, permitting solutes which would ordinarily be retained for relatively long periods of time in a totally aqueous mobile phase to be analyzed more rapidly. In some cases, changes in retention of almost two orders of magnitude have been produced[18].

Shown in Fig. 2 is a plot of area ratio of acetaminophen to internal standard *vs.* acetaminophen concentration. The plotted points represent the means of at least five replicate injections per standard. The plot showed good linearity over the desired concentration range (*i.e.*, up to 130 μg/ml).

Summarized in Tables I and II are results from single 325-mg tablet and composite analyses. These values represent the mean of at least five replicate injections per sample. The overall single tablet variation was from 98.6% to 102.4% acetaminophen with an average value of 100.2% for the ten trials. This is in excellent agreement with an overall average value for the composite samples of 99.8% and a variation of 98.7% to 101.4% for the individual trials.

Summarized in Table III are the analysis results from single 500-mg capsules. The precision of these data is in good agreement with that obtained on the single

TABLE I

SINGLE 325-mg ACETAMINOPHEN TABLET RESULTS

Sample	*Percentage**
Tablet 1	100.1
Tablet 2	99.4
Tablet 3	101.3
Tablet 4	99.2
Tablet 5	98.6
Tablet 6	98.8
Tablet 7	100.1
Tablet 8	100.6
Tablet 9	102.4
Tablet 10	101.7
Average value	100.2 ± 1.3%

* Mean of at least five replicate injections per sample.

TABLE II

COMPOSITE 325-mg ACETAMINOPHEN RESULTS

Sample	*Percentage**
Weighing 1	99.2
Weighing 2	99.4
Weighing 3	101.4
Weighing 4	98.7
Weighing 5	100.2
Average value	99.8 ± 1.1%

* Mean of at least five replicate injections per sample.

TABLE III

SINGLE 500-mg ACETAMINOPHEN CAPSULE RESULTS

Sample	*Percentage**
Capsule 1	99.4
Capsule 2	101.4
Capsule 3	102.6
Capsule 4	98.3
Capsule 5	99.0
Capsule 6	99.8
Capsule 7	99.6
Capsule 8	101.1
Capsule 9	98.5
Capsule 10	98.4
Average value	99.8 ± 1.4%

* Mean of at least five replicate injections per sample.

tablet and composite tablet samples. A mean value of 99.8% with a standard deviation of ±1.4% was obtained. To verify the absence of interference from the capsule blank, empty capsules were carried through the procedure. Upon chromatographic examination, no peaks were observed.

CONCLUSION

The method described represents a quick and simple means of determining acetaminophen in tablets and capsules using only water as the mobile phase. Totally aqueous chromatography offers further advantages in terms of solvent safety and disposal.

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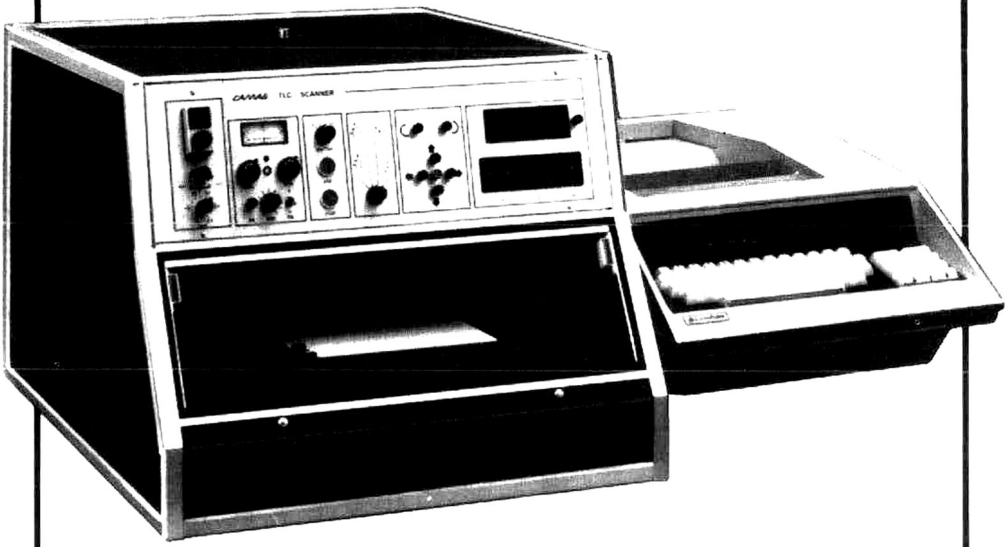
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